Thèse

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Characterisation of triterpenoids as TGR5 agonists and their effects on metabolism

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Je finirai par cette citation : « Plus on partage, plus on possède, voilà le miracle », Léonard Nimoy.

ABBREVIATIONS

AC	adenylate cyclase
ADAM	α disintegrin and metalloproteinase
ADP	adenosine-5'-diphosphate
AGE	advanced glycation end-products
α-MEM	alpha-minimum essential medium
AMP	adenosine-5'-monophosphate
AMPK	AMP-dependent kinase
APCI	atmospheric pressure chemical ionization
ATP	adenosine-5'-triphosphate
BA	bile acid
BAT	brown adipose tissue
bp	base pair
CA	cholic acid
cAMP	cyclic AMP
CD26	DPP4
CDCA	chenodeoxycholic
cDNAs	complementary DNA
CHO	chinese hamster ovary
coA	coenzyme A
COS-1	cell line derived from the CV-1 (simian cells containing PAPOVAVIRUS DNA)
CRE	cAMP response element
CREB	CRE binding protein
CYP7A	cholesterol 7α-Hydroxylase
CYP8B1	12-alpha-hydroxylase
D2	type 2 iodothyronine deiodinase
DAD	diode array detector
DCA	deoxycholic acid
DIO2	D2 gene
DNA	deoxyribonucleic acid
DPP4	dipeptidyl peptidase 4
DREAM	diabetes reduction assessment with ramipril and rosiglitazone medication
e.g.	exempli gratia
EC_{50}	half maximal effective concentration
EDG	endothelial differentiation, lysophosphatidic acid GPCR
EDTA	ethylene-diamine-tetraacetique acid
EGCG	epigallocatechin-3-gallate
EGF	endothelial growth factor
EGFR	EGF receptor
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxid synthase
ERK	extracellular signal-regulated kinase mitogen-activated protein kinase
ESI	electrospray ionization
EtOH	ethanol
$FAD^+/FADH_2$	flavin adenine dinucleotide couple redox
FAS	fatty acid synthase
FBS	foetal bovine serum
FCS	foetal calf serum
FFA	free fatty acid
Foxo1	forkhead box, sub-group O1
FXR	farnesoid X receptor
GCN5	histone acetyltransferase

GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide-1
GLUTag	stable immortalized murine enteroendocrine cell line
Gpbar1	G-protein coupled bile acid receptor
GPCR	G-protein coupled receptor
GSH	glutathione
GTP	guanine triphosphate
HbA_{1c}	glycosylated haemoglobin
hBG37	human G-protein coupled bile acid receptor BG37
HDL	high density lipoprotein
HEK293 cells	human embryonic kidney 293 cells
HPLC	high- pressure liquid chromatography
HS	horse serum
IBMX	isobutylmethylxanthine
IL-1	interleukin-1
IL-6	interleukin-6
IR	insulin receptor
IRS	insulin receptor substrate
Kcal	kilocalory
kDa	kilodalton
LCA	lithocholic acid
LC–MS	liquid chromatography-mass spectrometry
LPS	lipopolysaccharide
LRH-1	liver receptor homolog 1
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
NaAc	sodium acetate
NADH, H^+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NEAA	non essential amino acid
NMR	nuclear magnetic resonance
NO	nitric oxide
NOS	nitric oxide synthase
NPH insulin	neutral protamine haegdorn insulin
NRF1	nuclear respiratory factor 1
OA	oleanolic acid
OE	*Olea Europaea*
p38MAPK	p38mitogen-activated protein kinase
PCR	polymerase chain reaction
PGC-1α	PPARγ coactivator 1 alpha
P_i	pyrophosphate
PKA	cAMP protein kinase
PKC	protein kinase C
PIPOD	pioglitazone in the prevention of diabetes
PPARγ	peroxisome proliferator-activated receptor-gamma
PPARα	peroxisome proliferator-activated receptor-alpha
PROactive	prospective pioglitazone clinical trial in macrovascular events
PTP	protein tyrosine phosphatase
PXR	pregnane X receptor
Q-PCR	quantitative PCR
ROS	reactive oxygen species
Rp-cAMPS	Rp-3',5'-cyclic adenosine monophosphorothioate
rRNA	Ribosomal RNA
RXRα	retinoid X receptor alpha
SAR	structure activity relationship

SDS	sodium dodecyl sulfate
SEC	sinusoidal endothelial cells
SHP	small heterodimer partner
shRNA	short hairpin RNA
SIRT1	sirtuin 1
SOD	superoxide dismutase
SRC-1	steroid receptor coactivator-1
STC-1	intestinal enteroendocrine cell line
SU	sulfonylureas
SXR	steroid and xenobiotic receptor
T_3	3,5,3'-tri-iodothyronine
T_4	thyroxine
TCA	tetracarboxylic acid
TCDCA	taurochenodeoxycholic acid
TDCA	taurodeoxycholic acid
TFAM	transcription factor A, mitochondrial
TGR5	transcription growth factor beta receptor, gene name is Gpbar-1
TLCA	taurolithocholic acid
TNFα	tumor necrosis factor α
TRIPOD	troglitazone in the prevention of diabetes
TRβ	thyroid receptor beta
TZD	thiazolidinedione
UCP	uncoupling protein
UPS	ubiquitin proteasome system
UQ	ubiquinol
UQH_2	ubiquinone
VDR	vitamin D receptor
VLDL	very low density lipoprotein
WAT	white adipose tissue
3D-QSAR	three-dimensional-quantitative SAR

ABSTRACT

La balance énergétique résulte de l'équilibre entre la quantité de calories ingérées par l'organisme (lipides, glucides) et l'énergie dépensée. Les calories absorbées sont métabolisées et transformées par les mitochondries en énergie utilisable, c'est la dépense énergétique. Lorsque l'apport calorique est supérieur à la dépense énergétique, les maladies métaboliques apparaissent, avec l'obésité en premier lieu. La perturbation du métabolisme lipidique entraîne à terme celle du métabolisme glucidique et le développement du diabète de type 2. Ces maladies sont donc des pathologies ayant comme point commun une perturbation du métabolisme et notamment au niveau mitochondrial, siège de la production/dépense énergétique. Les traitements des maladies métaboliques atteignent leurs limites en raison d'effets secondaires, de fait la prévention devient un élément clé pour la prise en charge de ces pathologies. L'identification de molécules agissant aux stades précoces de ces maladies, et en particulier sur les mitochondries, pourrait donc ouvrir de nouvelles voies de traitement en « brûlant » les graisses stockées et donc en évitant la glucolipotoxicité et l'ultérieure aggravation des maladies métaboliques.

Il a été récemment démontré au sein du laboratoire que les mitochondries peuvent être activées par un récepteur membranaire appartenant à la famille des récepteurs couplés aux protéines G et activé par les acides biliaires, le TGR5. L'activation de ce récepteur a des effets positifs sur l'expression de gènes impliqués dans les fonctions mitochondriales, sur la dépense énergétique, sur le poids corporel et sur de multiples aspects des métabolismes lipidiques et glucidiques. Dans les cellules intestinales, l'activation du TGR5 entraine la sécrétion de l'incrétine glucagon like peptide-1 (GLP-1). Ce double effet sur les mitochondries et sur la sécrétion de GLP-1 fait de TGR5 une nouvelle cible d'intérêt pour prévenir, voire traiter, les désordres métaboliques. Les acides biliaires, seuls agonistes TGR5 connus à ce jour, ont des effets pléïotropiques en stimulant d'autres récepteurs, en particulier le récepteur nucléaire FXR. Trouver de nouveaux agonistes de ce récepteur, plus puissants et plus sélectifs que les acides biliaires est donc essentiel pour comprendre sa

physiologie, pour confirmer son potentiel en tant que cible thérapeutique et pour découvrir de potentiels agents thérapeutiques pour les maladies métaboliques.

Le premier objectif de ce travail était donc de trouver des agonistes TGR5 originaux. A cet effet, une librairie d'extraits de plantes a été criblée sur un test d'activité TGR5. Parmi les extraits de plantes testés, celui de feuilles d'olivier *Olea Europaea* a montré une activité agoniste significative. Grâce à un fractionnement bioguidé, nous avons purifié la molécule portant l'activité TGR5, et l'avons identifiée comme étant l'acide oléanolique, un triterpène naturel. *In vivo*, l'acide oléanolique diminue les taux plasmatiques de glucose et d'insuline, améliore la tolérance au glucose et diminue la prise de poids. Il est à noter que la perte de poids semble être due à une perte de la masse graisseuse. Ces résultats décrivent pour la première fois, que l'acide oléanolique contenu dans les feuilles d'olivier possède une activité hypoglycémiante, et ces données ont fait l'objet d'une publication (Manuscrit 1). L'autre aspect intéressant de ces travaux est que TGR5 est activé par un triterpène, une molécule dont la structure, bien que proche, est différente de celle des acides biliaires. Les effets métaboliques provoqués par l'acide oléanolique, et qui semblent être TGR5-dépendants, soulignent l'intérêt thérapeutique de TGR5 et des triterpènes.

Nous avons montré au sein du laboratoire que TGR5 activé par les acides biliaires stimule la dépense énergétique dans le muscle et le tissu adipeux. Plus précisément, l'activation du TGR5 conduit à l'augmentation de l'expression de gènes impliqués dans les fonctions mitochondriales. En outre, dans le muscle, l'acide oléanolique augmente l'expression de gènes impliqués dans la biogenèse et dans l'activité mitochondriales. Ce sont ces deux aspects de la fonction mitochondriale qui ont ensuite été particulièrement explorés. Le nombre de mitochondries (biogénèse) a été calculé via la mesure de la quantité d'ADN mitochondrial par rapport à la quantité d'ADN nucléaire, alors que la cytochrome c oxydase a été utilisée pour mesurer l'effet sur l'activité. Ce complexe enzymatique est le complexe IV de la chaine de transport des électrons et mesurer son activité nous renseigne sur l'activité de toute la chaine. Ces expériences nous ont permis de montrer que l'acide

oléanolique augmente la biogénèse et l'activité des mitochondries de muscle des souris. De plus, l'acide oléanolique augmente l'activité mitochondriale dans des cellules de muscle, *in vitro*.

Dans un deuxième temps nous avons cherché à identifier un dérivé de triterpène qui soit un agoniste de TGR5 plus puissant en conduisant une étude de relation structure-activité à partir de triterpènes naturels. Cela nous a permis de synthétiser et de caractériser le composé RG239 (18 dia 2), un agoniste TGR5 plus puissant que l'acide oléanolique avec une EC_{50} de l'ordre du nM alors que l'EC_{50} de l'acide oléanolique est de l'ordre du µM. Ces résultats sont actuellement soumis à publication (Manuscrit 2). Ces travaux ont donné des éléments nécessaires à la modélisation de la poche de liaison du TGR5 : un étroit site accepteur d'électrons qui lie le groupe hydroxyle (ou tout autre groupement donneur d'électrons) en C-3 ; une poche hydrophobe qui lie l'acide carboxylique en C-17 ; et une poche latérale neutre qui accueille le groupe alkène. Ces hypothèses sont en adéquation avec celles émises par différentes études décrivant la poche de liaison du TGR5 aux acides biliaires, ce qui suggère que les triterpènes et les acides biliaires lient TGR5 de la même façon.

En ce qui concerne les effets biologiques de RG239, nous n'avons pas pu mettre en évidence d'effets significatifs de ce composé *in vivo*, probablement du fait d'un problème de biodisponibilité. En effet, les triterpènes sont des molécules hydrophobes dont les problèmes de solubilité qui ont des effets négatifs sur leur absorption intestinale sont connus. Il est à noter que des modifications chimiques, telles que l'ajout d'agents solubilisant, pourraient aider à améliorer les caractéristiques physicochimiques de RG239. Cependant, même si RG239 n'est pas efficace *in vivo*, c'est un excellent outil *in vitro*, permettant d'avancer dans la compréhension de la biologie du récepteur TGR5.

La troisième partie de ce travail est basée sur la description cellulaire et moléculaire des effets de RG239 sur les fonctions mitochondriales. Dans le but de déterminer comment le récepteur TGR5 activé par les triterpènes stimule les mitochondries dans le muscle et le

tissu adipeux (tissus de la dépense énergétique) ainsi que dans l'intestin (tissu exprimant fortement TGR5), nous avons utilisé RG239 comme molécule outil. Nous avons mis en évidence que RG239 stimule l'activité et la biogénèse mitochondriales dans les cellules musculaires et intestinales, et ce de façon TGR5-dépendante. De plus, une approche pharmacologique nous a permis de montrer que l'activation des mitochondries semble emprunter la voie de signalisation TGR5-adénylate cyclase-AMPc-protéine kinase AMPc-dépendante (PKA). En parallèle, RG239 stimule la sécrétion de GLP-1 dans les cellules intestinales, également de façon TGR5-dépendante. Ce résultat permet de poser la potentialité des triterpènes comme sécrétagogues d'incrétines, et donc comme agents antidiabétiques.

Ces derniers travaux, soumis à publication (Manuscrit 3), permettent de confirmer le potentiel de TGR5. En effet, son double effet sur les mitochondries et sur la sécrétion de GLP-1 argumente pour une double utilisation préventive (mitochondries) et thérapeutique (GLP-1) des maladies métaboliques. L'action sur les mitochondries permettrait d'agir sur les stades précoces de ces maladies puisque l'affaiblissement de la dépense énergétique est une des causes les plus en amont de cette pathologie. En parallèle, maintenir la sécrétion de GLP-1 permettrait de soutenir son effet, et donc d'améliorer la sensibilité à l'insuline.

Pour conclure, nous avons décrit pour la première fois que les triterpènes, en particulier l'acide oléanolique, sont des agonistes de TGR5. Ces molécules ont une activité hypoglycémiante, amaigrissante et stimulent les mitochondries dans le muscle. RG239, un dérivé triterpénique plus puissant sur TGR5, stimule l'activité mitochondriale dans le muscle, le tissu adipeux et l'intestin et induit la sécrétion de GLP-1 dans les cellules entéroendocrines de l'intestin. L'ensemble de ce travail a permis de confirmer la voie de signalisation impliquant TGR5 dans l'activation des mitochondries, et ainsi l'intérêt thérapeutique de ce récepteur, de part son double effet sur les mitochondries et sur la sécrétion de GLP-1. De plus, par leur activité agoniste TGR5, nous avons mis en évidence que les triterpènes pourraient être utilisés comme agents de prévention de l'obésité et du

diabète de type 2. Au-delà de ces conclusions, les tests d'activité TGR5 et mitochondriaux utilisés ici pourront être employés pour développer de nouveaux composés ciblant les phases précoces du diabète de type 2.

L'intérêt du récepteur TGR5 en tant que cible thérapeutique est supporté par les entreprises pharmaceutiques qui progressent actuellement sur des projets de recherche d'agonistes TGR5. D'autre part, les triterpènes naturels sont extraits de plantes utilisées depuis des temps anciens comme traitement de différentes maladies. Ils sont donc facilement accessibles et surtout apparemment sans danger, en tous les cas en combinaison avec les autres composants de ces plantes. Le problème de leur biodisponibilité reste le prochain challenge à surmonter afin de disposer de molécules thérapeutiques originales.

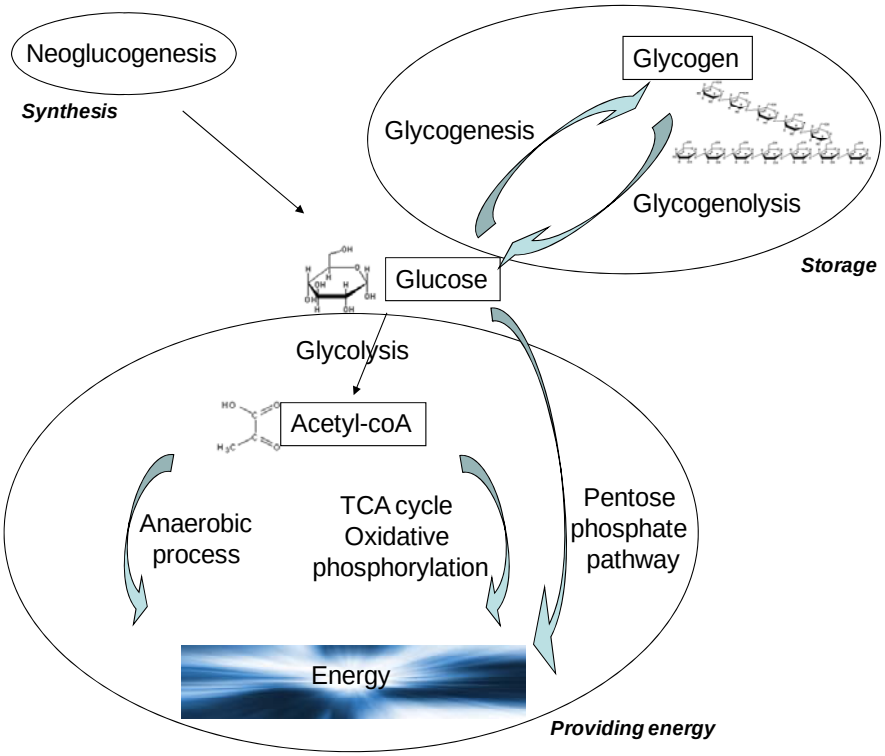


Figure 1: Glucose homeostasis

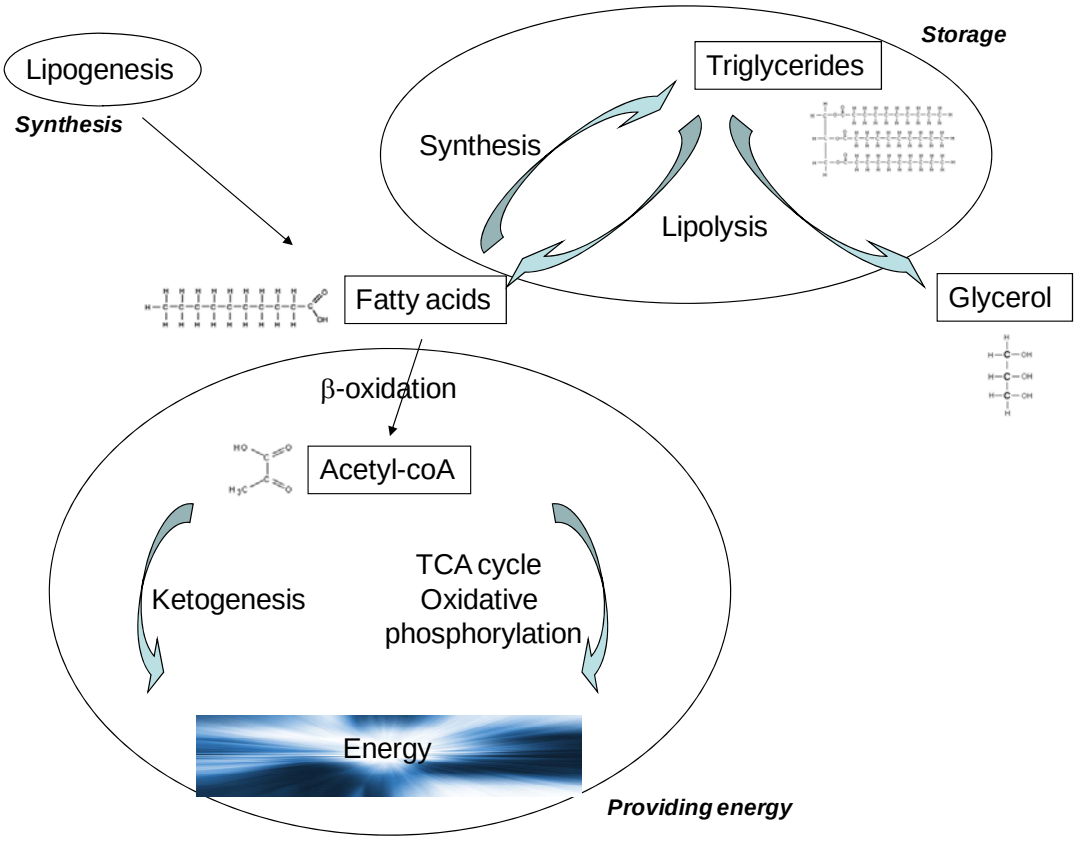


Figure 2: Lipid homeostasis

I. Mitochondria in energy homeostasis and in metabolic disorders

A. Energy homeostasis

1. Introduction

Under normal conditions, metabolic homeostasis is maintained in the body, balancing energy intake and energy expenditure. Food intake supplies the carbohydrates and fats necessary for the production of energy required for physiological functions. Carbohydrate metabolism is described in Figure 1 and lipid metabolism in Figure 2. The final stage of their catabolism, glycolysis and beta-oxidation respectively, is the production of acetyl-coA. Mitochondria are the central sites of energy production, where the last reactions are performed which convert acetyl-coA to ATP. Numerous metabolic activities such as fatty acid beta-oxidation, the tetracarboxylic acid (TCA) cycle, oxidative phosphorylation, synthesis and degradation of amino acids (urea cycle), and the generation of heat occur in this organelle. Mitochondria play a dominant role not only in the oxidation of fuel molecules but also in heat production and the generation of oxidative stress. Heat and ATP production are the two primary processes of energy expenditure with mitochondria as the *in situ* organelles housing this energy expenditure. The mechanisms of the TCA cycle and of cellular respiration, including oxidative phosphorylation, reactive oxygen species (ROS) generation and heat production, will be described.

2. TCA cycle: the intersection of metabolisms

Acetyl-coA, the end product of carbohydrate and lipid metabolism (Figure 3), enters the mitochondria where it is oxidized in the TCA cycle, a series of eight reactions that oxidize acetyl-coA into CO_2, producing ATP, ($NADH, H^+$) and $FADH_2$:

$$1 \text{ pyruvate} + 1 \text{ ADP} + 1 \text{ } P_i + 3 \text{ } NAD^+ + 1 \text{ } FAD^+ \rightarrow 2 \text{ } CO_2 + 1 \text{ ATP} + 3 \text{ } (NADH, H^+) + 1 \text{ } FADH_2$$

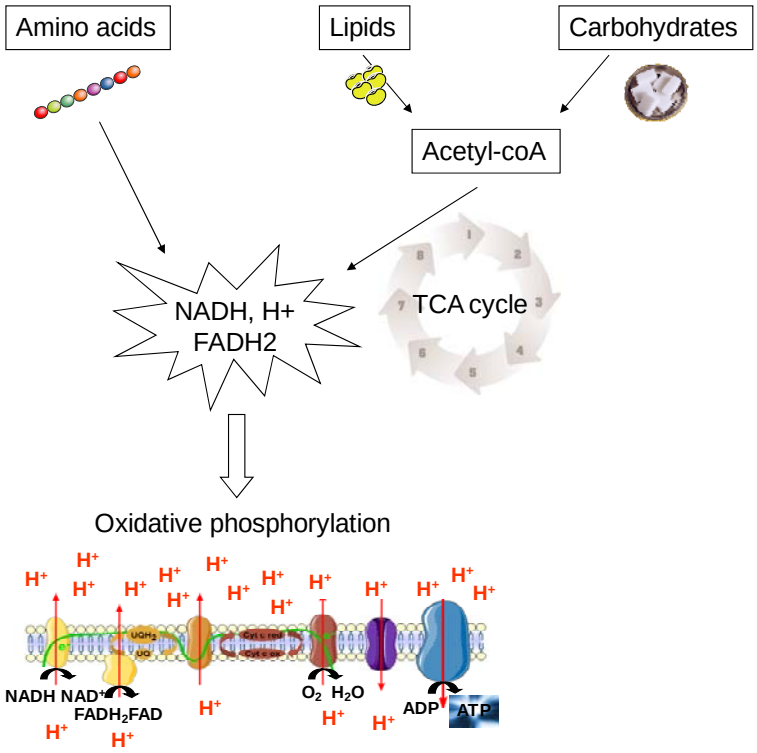
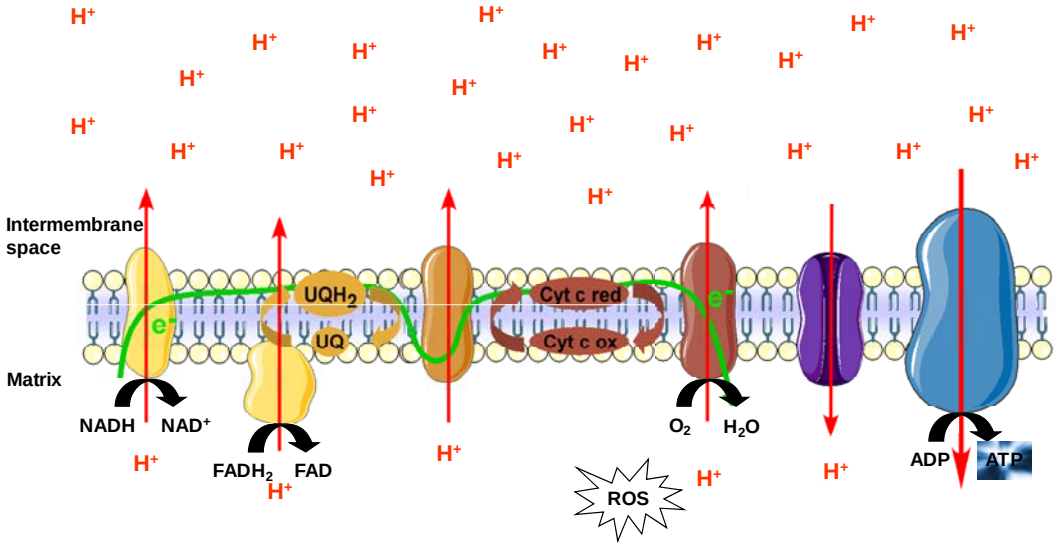


Figure 3: Energetic sources



Complex I NADH-ubiquinone reductase	Complex II Succinate-ubiquinone reductase	Complex III Ubiquinol-cytochrome c reductase	Complex IV Cytochrome c oxidase	Uncoupling protein	ATP synthase

Figure 4: Electron transport chain

Given that the reoxidation of NADH and $FADH_2$ by oxidative phosphorylation generates 3 and 2 ATP molecules respectively, the TCA cycle produces 12 ATP per acetyl-coA degraded. The intermediates of the TCA cycle are used by many essential energy consuming biosynthetic pathways which emphasizes the important role of the catabolic pathways in providing enough intermediates for the flow of the cycle to produce enough energy/ATP.

3. Cellular respiration

Cellular respiration allows the reoxidation of the reduced agents (NADH, H^+) and $FADH_2$ provided by different catabolism pathways (glycolysis, TCA cycle, etc). This reoxidation is performed by the electron transport chain itself coupled to ATP synthesis, the final step of energy production.

a. Oxidative phosphorylation (Figure 4)

The electron transport chain is localised in the inner-membrane of mitochondria and comprises enzyme complexes I, II, III and IV plus the electron carriers cytochrome c and ubiquinone. The first electron donor NADH donates electrons to NADH:ubiquinone reductase complex (complex I) which transfers them to ubiquinone. Ubiquinone (UQH_2) is also reduced to ubiquinol (UQ) by electrons from $FADH_2$, which is performed by the succinate:ubiquinone reductase complex (complex II). Ubiquinol transports electrons to the ubiquinol:cytochrome c reductase complex (complex III), where electrons are carried by cytochrome c to the cytochrome c oxidase complex (complex IV) which uses them to reduce molecular oxygen O_2 to H_2O. In summary, electrons derived from different metabolic pathways are funnelled through the redox carriers of the respiratory chain to the final electron acceptor, molecular oxygen.

The free energy of transferred electrons generates a proton gradient with high proton levels in the intermembrane space. The ATP synthase machinery (complex V) uses the

energy released when protons return to the mitochondrial matrix to perform phosphorylation of ADP into ATP in a process that links the electron transport chain to ATP production by the proton gradient. The activity of the respiratory chain complexes is inherently governed by the transmembrane proton gradient and the membrane potential, and more generally by the ratio of [NADH]/[NAD^+]. However, a proton leak can be induced by uncoupling protein, as discussed later.

Considering the whole set of catabolic processes, including oxidative phosphorylation, the energetic productivity per carbon atom is 8 ATPs for fatty acid oxidation and 6 ATPs for glucose oxidation. Thus, fatty acids constitute a greater energetic source per unit weight than carbohydrates.

b. Reactive oxygen species production

During normal metabolism, incompletely reduced reactive forms of oxygen superoxide (ROS) are produced. Normally, only 0.1% of total oxygen consumption leaks from the respiratory chain to generate ROS. The variety of sources of ROS includes several enzymatic and nonenzymatic systems. Enzymatic sources include NADPH oxidases (1), cytochrome P450-dependant oxygenases (2) and xanthine oxidase (3). The non-enzymatic production of ROS takes place in the mitochondrial respiratory chain, which contains several redox centers (flavine, iron-sulfur clusters, ubisemiquinone, etc) able to transfer one electron to oxygen to form O_2^- (4). The two major sites of superoxide production are complexes I and III and in small part, complex IV (5). The primary factor governing mitochondrial ROS generation is therefore the redox state of the respiratory chain, i.e. the mitochondrial metabolic state. For example, when oxygen consumption is low and proton motive force is high, the complexes of the electron transport chain are in their reduced states and ROS production is highest. This is the case when ATP demand is low (6). There is a strong positive correlation between ROS production and mitochondrial membrance potential since

an increase/decrease in potential results in an increase/decrease in ROS production, respectively (7).

ROS exist in all aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted due to an excess of ROS, antioxidant depletion, or both. Antioxidants can be exogenous or endogenous, such as cellular non-protein thiols and antioxidant enzymes. The enzymes, that neutralize ROS include superoxide dismutase (SOD) (8), catalase, and peroxidase. The low molecular weight antioxidants comprise ascorbate, glutathione (GSH), phenolic compounds, and tocopherols. Another example is coenzyme Q which is a source of O_2^-. when partially reduced (ubisemiquinone form) and an antioxidant when fully reduced (ubiquinol) (9).

c. Uncoupling/Thermogenesis

As described earlier, the process of degrading fuel molecules is normally coupled to ATP synthesis via the energetic proton gradient. The establishment of this gradient is only possible if the mitochondrial inner membrane is totally impermeable to protons. Apart of being the driving force of ATP synthesis, the energy of the proton gradient is dissipated by proton leak reactions, whereby protons pass back into the mitochondrial matrix, but not through ATP synthase (10). This leakage reduces the number of protons flowing through the ATP synthase and uncouples the electron transport chain from phosphorylation of ADP to ATP. Thus the energy derived from the oxidation of metabolic fuels is dissipated/“wasted” and released as heat. These pathways are physiologically important and account for 20–25% of basal metabolic rate. Their suggested functions include thermogenesis, regulation of energy metabolism, control of body mass, and attenuation of ROS production. Indeed, this proton leak is considered as an effective and naturally safe mechanism since it keeps the cell NAD^+/NADH ratio sufficiently high to allow substrate oxidation to continue. In addition, the proton leakage causes partial depolarization of the mitochondrial transmembrane potential. As mentioned in the previous chapter, the membrane potential is positively correlated with

ROS production. So uncoupling, through its effects on membrance potential, has a natural antioxidant effect (11, 12). Whatever its consequences, the process of uncoupling is achieved by the uncoupling proteins (UCPs) (UCP1, UCP2, UCP3, UCP4, UCP5), which are members of the anion carrier protein family located in the inner mitochondrial membrane.

UCP1 was the first member of the UCP family to be described and is specifically expressed in the mitochondria of brown adipose tissue (BAT). In mammals, two kinds of adipose tissues exist: white adipose tissue (WAT) which is responsible for energy storage and brown adipose tissue (BAT), which is the site of heat production (non-shivering thermogenesis rather than shivering thermogenesis that occurs in muscle tissues) (13). This non-shivering thermogenesis is highly important in neonates and small rodents for maintaining body temperature under cold conditions as muscle tissues are not well developed (14). Furthermore, functional BAT has recently been described in adult humans (15). This result has proved highly controversial, however, a more recent and precise examination determined unequivocally that thermogenesis occurs in these tissues (16). To accomplish this thermogenic function, BAT expresses high concentrations of UCP1 (up to about 10% of the membrane proteins) and low level of ATPsynthase. UCP1 is greatly induced by fatty acids (via PPARγ) and ROS while strongly inhibited by nucleotides (17). The free energy conserved in the proton gradient is dissipated through UCP1, producing heat, indispensable for thermogenesis. When animals are exposed to cold, the expression of UCP1 is increased, potentiating thermogenesis. In addition, other genes implicated in energy homeosatsis such as CREB, PPARγ, PGC-1α and TRβ (thyroid receptor) are also upregulated (18, 19), suggesting that an enhancement of energy metabolism is required to activate thermogenesis in BAT. Mice with a disruption in the UCP1 gene are known to be cold sensitive (20).

Transcription factors such as CREB and PGC-1α are significantly expressed in muscle tissues, another important organ of the energy expenditure (19). Indeed, muscle needs ATP

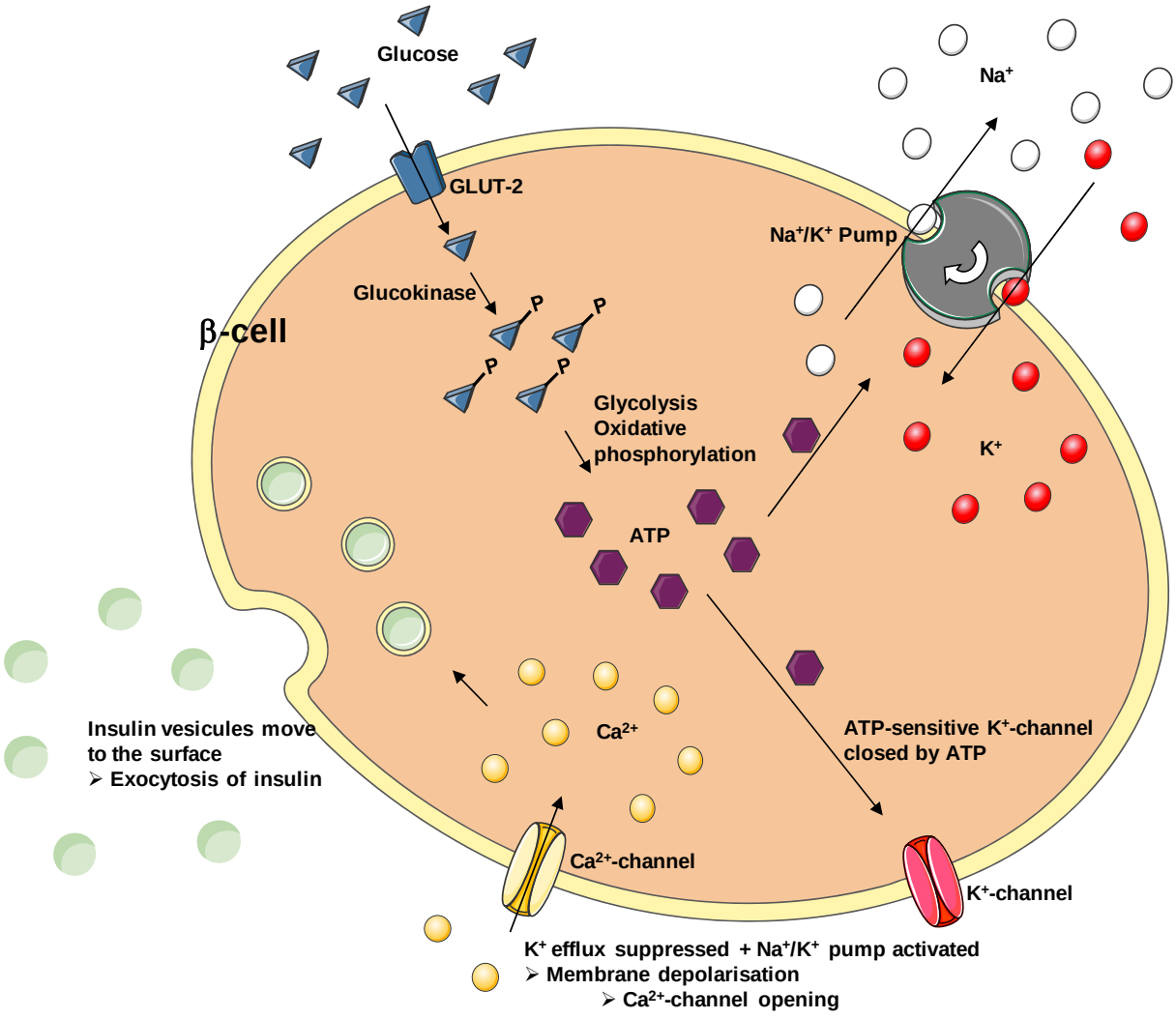


Figure 5: Glucose-stimulated insulin secretion

to contract. This highlights the key role of mitochondria in the organs of the energy expenditure, BAT and muscle.

Due to their homology with UCP1, both UCP2 and UCP3 were first postulated to be thermogenic and involved in regulation of energy expenditure and body weight. Mice genetically deficient in UCP2 and UCP3 are normally resistant on exposure to cold and are protected against obesity, indicating that these proteins are implicated in energy expenditure but not in thermogenesis (21). This might be explained by the fact that UCP2 and UCP3 are expressed at lower levels than UCP1 and their rate of proton conductance is lower. UCP3 favors thermogenesis in muscle though experiments performed on knock-out mice did not reveal any evidence for the influence of this protein on organism thermogenesis (21). Considered together, these data lead us to anticipate that UCPs could have more general functions.

UCP2 is expressed ubiquitously and seems to play a role in limiting ROS production and may be implicated in insulin secretion (12, 22). This is supported by the fact that UCP2 is well expressed in beta cells (23) and reduces the ATP/ADP ratio. In fact, beta-cells sense glucose by its catabolism: glycolysis results in increasing the ATP/ADP ratio, leading to the closure of the ATP-sensitive K^+ channel and causing plasma membrane depolarization. The subsequent opening of voltage sensitive Ca^{2+}-channels produces an influx of Ca^{2+} that triggers insulin secretion (Figure 5). UCP2, by virtue of its proton-leak activity, reduces the generation of ATP and consequently impairs glucose-stimulated insulin secretion (24, 25). In the same way, by decreasing the mitochondrial membrane potential, UCP2 provokes reduced ROS production. As impaired insulin action and secretion, perturbed fatty acid and glucose metabolisms and ROS production are all involved in the pathophysiology of type 2 diabetes, UCPs are possible therapeutic targets for the treatment of obesity and type 2 diabetes.

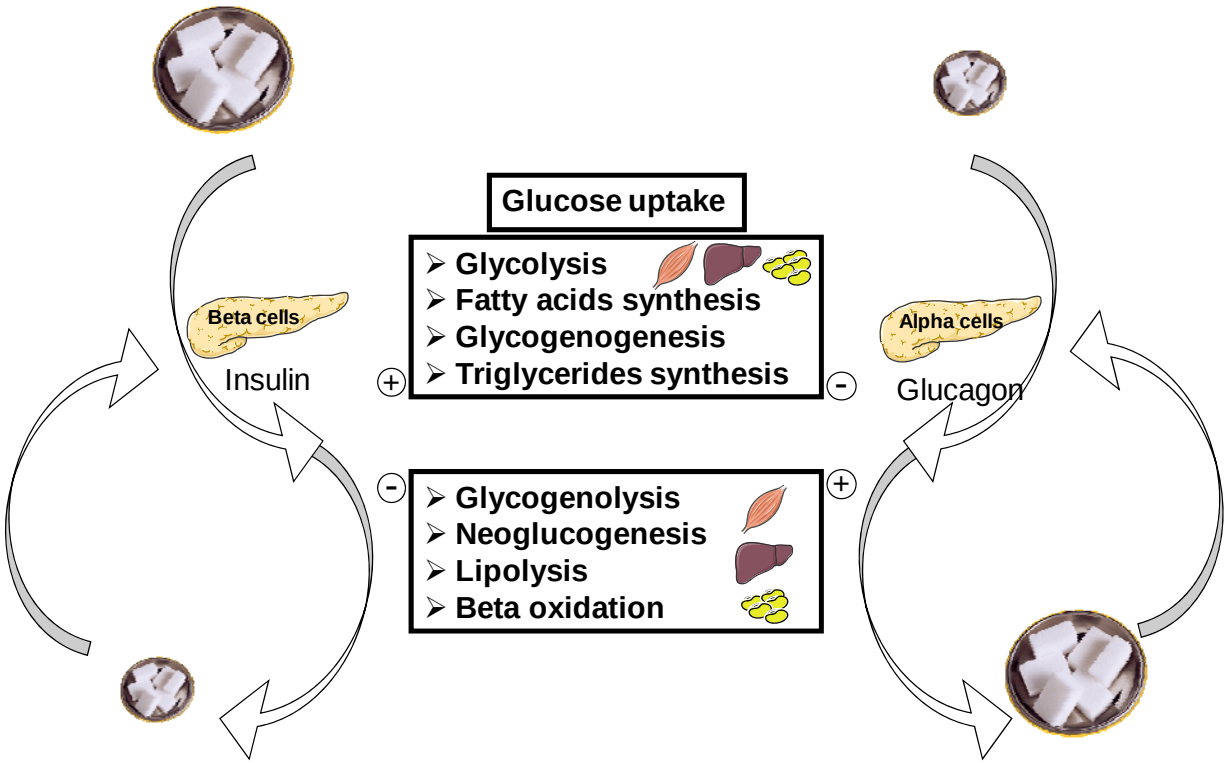


Figure 6: Roles of insulin and glucagon: balance in energy homeostasis

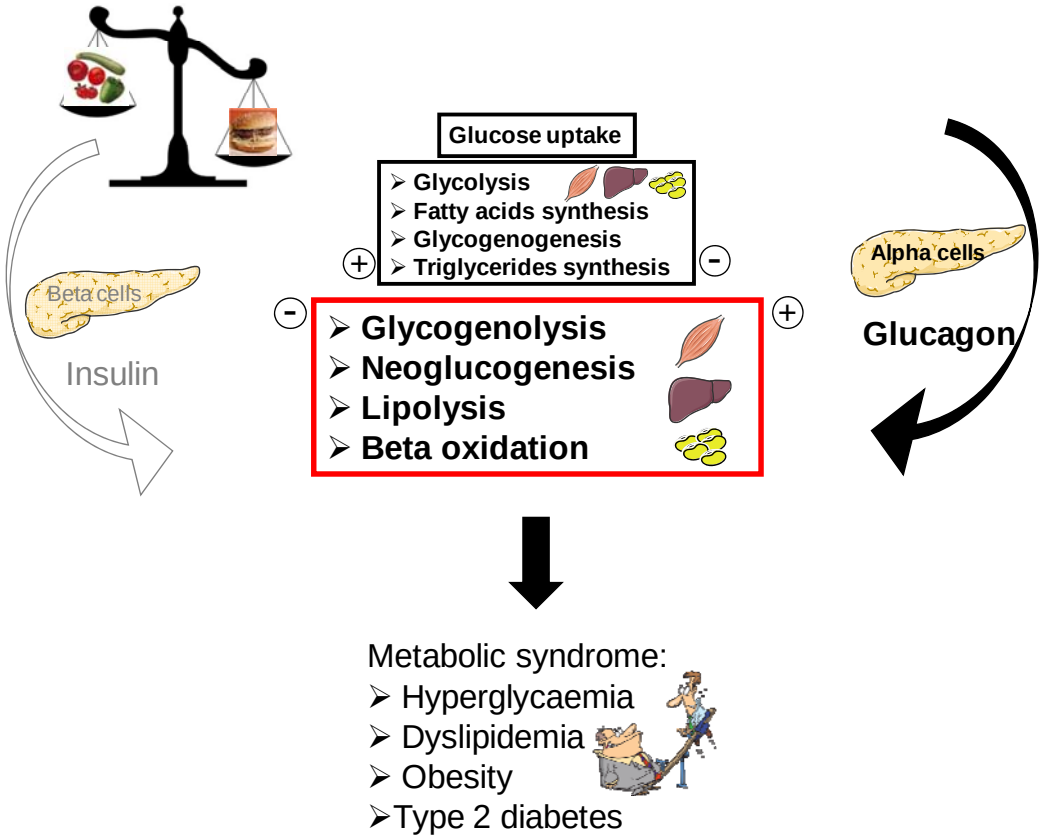


Figure 7: Imbalance in energy homeostasis

UCP3, mainly expressed in BAT and muscle, transports fatty acid anions and superoxide out of mitochondria, thus protecting this key organelle from lipotoxicity (26, 27). Furthermore, UCP3 seems to play a role in glucose homeostasis (27). UCP4 and UCP5, although thought to be implicated in metabolic rate and thermoregulation, are mainly expressed in the brain where their physiological role remains unclear (28).

B. Mitochondria in metabolic disorders

1. Introduction

Perturbation of energy homeostasis results in metabolic diseases. Among them, obesity and type 2 diabetes are prevalent, posing a worldwide health problem. In 2000, 171 million people were suffering from diabetes and this number is projected to rise to 366 million by 2030 (29). Energy homeostasis is under the control of the balance between the actions of insulin and glucagon (Figure 6); hormones which play a key role in glucose and lipid homeostasis. Insulin is produced in response to hyperglycaemia, stimulates glucose consumption in different tissues (storage, ATP production), and exerts profound effects on fat metabolism. Glucagon exerts the opposite effects. The balance between insulin and glucagon is defined by the availability of glucose and directly controls both metabolic features and the flux of lipids and carbohydrates.

An excess of food intake, rich in saturated fatty acids and carbohydrates, generates a positive energetic balance, and results in the intake of potential energy becoming greater than energy expenditure. This situation is associated with an imbalance in the actions of insulin and glucagon, leading to impaired glucose and lipid metabolism (30) and resulting in a number of consequences associated with the metabolic syndrome such as obesity and type 2 diabetes. The mechanisms underlying this positive energetic balance include physical inactivity, genetics, stress, smoking and excessive alcohol consumption, frequent features of modern, urbanized societies (Figure 7).

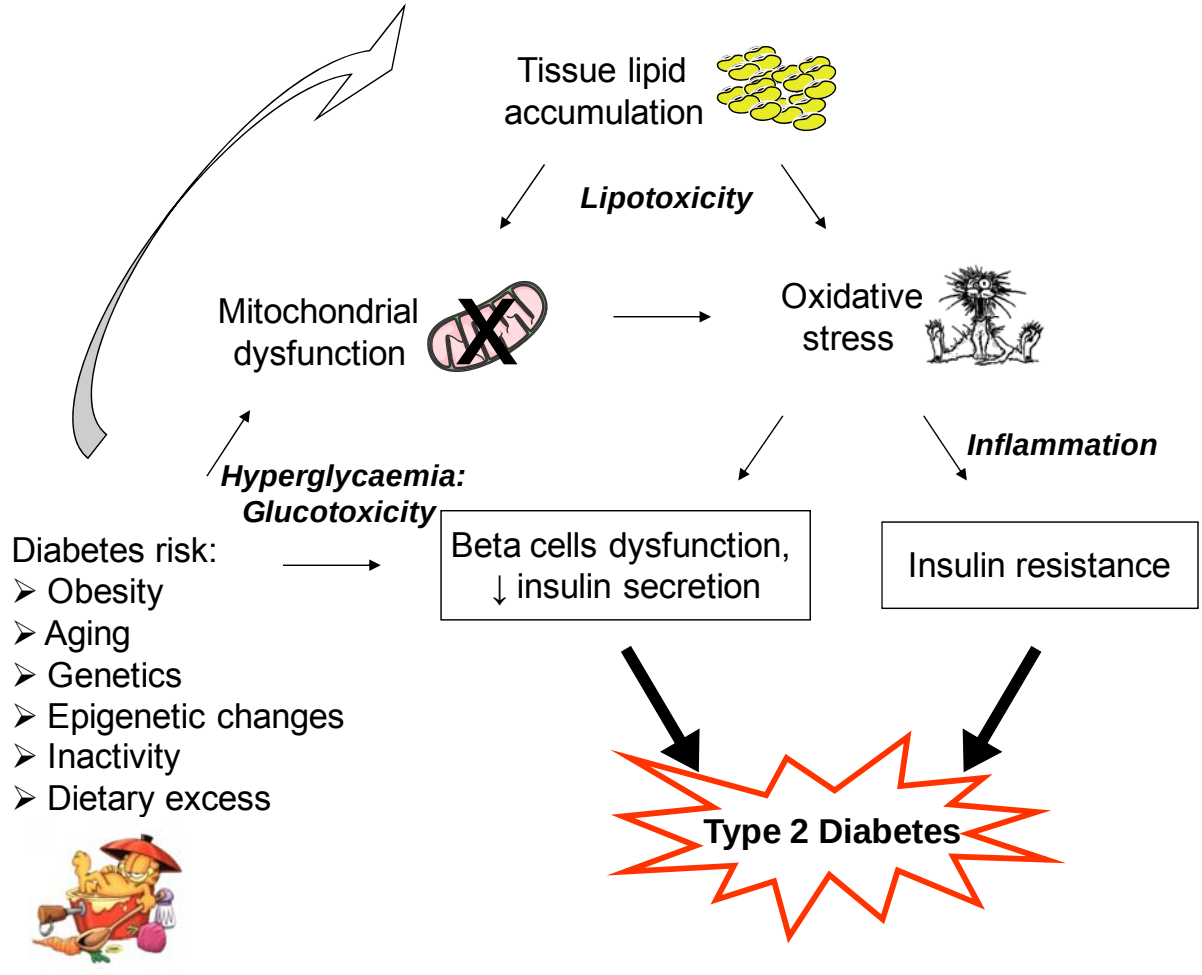


Figure 8: Type 2 diabetes pathophysiology

In genetically predisposed subjects, the combination of excess caloric intake and relatively reduced physical activity/energy expenditure, with the likely consequence of obesity, can induce a state of hyperglycaemia/insulin resistance (Figure 8) (31). When beta cells are no longer able to compensate for insulin resistance by adequately increasing insulin production, impaired glucose tolerance appears, which may evolve into overt diabetes.

2. Correlation with mitochondria

We have been seeing that mitochondria play a key role in energy expenditure and that perturbation of energy homeostasis is well correlated to mitochondrial defects, notably in muscle, adipose and beta cells, at the early stages of metabolic syndrome. Indeed, mitochondrial impairments may constitute a central factor in the development of insulin resistance and β-cell dysfunction. This new and very interesting hypothesis still requires the elucidation of a number of questions, such as the exact nature of the mitochondrial dysfunctions: the role of ROS, uncoupling proteins and the cofactor PGC-1α and its target genes in the events leading to type 2 diabetes (32)(33).

a. Importance of oxidative stress

Oxidative stress is widely accepted as playing a key role in the development and progression of type 2 diabetes and its complications: hyperglycaemia and induced cellular dysfunction are interrelated and augmented by increased oxidative stress/ increased ROS production which is associated with insulin resistance, impaired glucose tolerance, overt diabetes and the development of cardiovascular disease (31, 34-39). In fact, hyperglycaemia leads to an increased production of electron donors (NADH and $FADH_2$) entering the mitochondrial electron transport chain (36-38). A high mitochondrial membrane potential is generated followed by a subsequent increase in ROS production. Cell damage results from the proinflammatory and the mutagenic properties of superoxide since the close proximity of mtDNA and ROS-generating sites renders mtDNA vulnerable to oxidative damage (40).

Normally, ROS-induced cell damage is prevented by enzymes which convert superoxide to H_2O_2: superoxide dismutase, catalase, glutathion peroxidase and glutathion reductase. When this enzymatic detoxification system is overwhelmed or dysfunctional, oxidative stress occurs. Inhibition of ROS production by overexpression of these enzymes or UCP (which collapses the proton electrochemical gradients) prevents hyperglycaemia-induced superoxide overproduction and increased intracellular AGE formation, PKC activation, and hexosamine formation (35, 37).

b. Impaired mitochondrial functions in metabolic disease

More generally, a growing body of evidence has demonstrated a link between various disturbances in mitochondrial function and type 2 diabetes. For example, mutations in mtDNA, or a decrease in mtDNA copy number can be observed in overt diabetes.

The majority of mitochondrial proteins are synthesized in the nucleus and shuttled to the mitochondria while only 13 proteins involved in oxidative phosphorylation are exclusively encoded by mtDNA. Since mitochondrial number and function require both nuclear and mitochondrial-encoded genes, coordinated mechanisms exist to regulate the two genomes and to determine the overall oxidative capacity (41). PGC-1α is an integrator of the molecular regulatory circuit involved in the transcriptional control of cellular energy metabolism, including mitochondrial biogenesis, hepatic gluconeogenesis and fatty acid beta-oxidation (42, 43). For example, PGC-1α regulates PPARγ and its target NRF1, which controls the expression of respiratory chain complexes, and the mitochondrial activator of replication and transcription TFAM (43). In type 2 diabetes, expression of genes involved in fatty acid oxidation and of PGC-1α are reduced (44, 45).

Another event that results from mitochondrial dysfunction is an increase in intramyocellular lipid content, associated with insulin resistance in muscle, as observed by Petersen *et al.* (46). This deregulation of intramyocellular fatty acid metabolism is probably due to an inherited defect in mitochondrial oxidative phosphorylation (46). Different studies

have shown an increased intramyocellular lipid content and decreased mitochondrial ATP synthesis, density and substrate oxidation rates, in the muscle of lean insulin-resistant offspring of type 2 diabetic patients (46-49). This observation indicates the significance of mitochondrial dysfunction in diabetes progression.

As previously described, mitochondria play an important role in the two principal aspects of energy expenditure: ATP production and thermogenesis. The capacity to activate heat production in response to diet contributes significantly to the ability of some individuals to resist to obesity (fast-burners) while others become readily obese (slow-burners) (50). This correlates with the fact that obese people store fat instead of oxidizing it. Conversely, negative energetic balance leads to the suppression of thermogenesis (51). In other words, the induction of thermogenesis is an interesting way to fight lipid accumulation in the organism, and thus prevent lipotoxicity and its consequences. Indeed, a reduced UCP3 content is associated with insulin resistance in muscle of prediabetic and diabetic individuals (27).

The more general roles of UCP include the control of ROS production, of lipid fluxes and of insulin secretion. An assessment of the implication of these proteins in the development of type 2 diabetes should reflect upon these three activities as they are important during the early stages of the disease (26). For example, decreased UCP3 content is associated with insulin resistance in muscle of prediabetic and diabetic individuals (27). Another example is the implication of UCP2 in the inhibition of glucose-stimulated insulin secretion (24, 25) (Figure 5).

Thus, the decrease in mtDNA content and the inhibition of mitochondrial function may be considered as pathogenic hallmarks in the altered metabolic state associated with diabetes (52).

C. Metabolic diseases: therapeutic strategies

1. Prevention

Obesity and type 2 diabetes result from an excessive energy intake in the context of low energy expenditure. A method of prevention would then be action upon the lifestyle with calorie restriction (low saturated fat and increase of fibre intake) and increased physical activity (at least 150 minutes each week of brisk walking, cycling or jogging). Diet, exercise or both reduces the incidence of type 2 diabetes in high risk groups by 31% to 46% and has favorable effects on body weight, waist circumference, blood pressure and blood glucose levels (53-57). An energy-controlled diet with regular aerobic exercise is central to type 2 diabetes prevention and therapy, alone or in combination with drugs (58-60).

Calorie restriction is beneficial in rodents and primates to slow aging and maintain health and vitality. This strategy results in: lower plasma insulin levels and greater sensitivity, lower body temperatures, reduced cholesterol, triglycerides, blood pressure and arterial stiffness, elevated HDL, and slower age-related decline in circulating levels of DHEAS. Collectively, these biomarkers offer a protection through diet against type 2 diabetes (61, 62). Application of long-term 30% calorie restriction offer its own constraints and mimetics were researched to ascertain the positive effects of restriction without necessarily reducing caloric intake (61). In addition to this caloric restriction mimetic, a wealth of literature (both scientific and popular) exists in the area of compounds that might elicit some of the same beneficial effects as caloric restriction.

Medicinal plants are often used to target the early stages of the obesity and type 2 diabetes and in particular, to avoid the development of oxidative stress (31, 63). As explained earlier, this is the central factor of insulin resistance, impaired insulin secretion and glucose tolerance processes. This is the reason why in traditional medicines, numerous plants are used in a preventive manner for their antioxidant properties. Observational epidemiologic studies have revealed the protective association of dietary or plasma antioxidants against the

Class			Examples	Roles
Monophenol			hydroquinone (from Busserole) Carvacrol (From Thym)	
Polyphenols/Flavonoids	flavonols		resveratrol kaempferol quercetol rutin	anti-cancer, anti-inflammatory, blood-sugar-lowering and other beneficial cardiovascular effects of resveratrol pigment, antioxydant, anti-inflammatory antioxidant, anti-inflammatory antioxidant, angiogenesis inhibitor
	flavanones		naringenin	antioxidant, anti-inflammatory, carbohydrate metabolism promoter, immune system modulator
	flavones		apigenin luteolin	inhibitor of CYP2C9 antioxidant, anti-inflammatory, carbohydrate metabolism promoter, immune system modulator
	flavanols		catechines (EGCG)	powerful antioxidant
	anthocyanins		cyanidin	antioxidant and radical-scavenging effects
	isoflavones		soy seeds like genistein	antioxidants
	chalcones			antibacterial, antifungal, antitumor and anti-inflammatory properties
Phenolic acid			vanillin salicylic acid gallic acid	flavoring agent skin-care anti-fungal and anti-viral properties
Hydroxycinnamic acid	Coumarines	furanocoumarine	psoralene	perfumery, aroma, antioedema nut hepatotoxic
		pyranocoumarines		
			Cafeic acid	carcinogenic inhibitor, antioxidant
Lignanes				antioxidant, phytooestrogen, antimitotic
Tyrosol esters			Hydroxytyrosol Oleuropein	antioxidant antioxidant
Tannins				antioxidant, precipitation of proteins

Table1: Classification of phenols

Number of isoprene units	Class			Examples	Roles
n = 1	Hemiterpene				
n = 2	Monoterpene	Monocyclic		limonene menthol thymol carvacrol	solvent, perfumery, antitumoral, insecticide anti-inflammatory, antiviral, anesthesic antibacteria, antifongic, antiseptic antibacteria, antiproliferation
		Bicyclic		α-/β-pinene camphor carene eucalyptol azulene naphtalene	antiseptic antiseptic, anesthesic aroma synthesis analgesic, antitumoral, aroma antiinflammatory insecticid, cancerigen
n = 3	Sesquiterpene	Linear		farnesol	perfumery, antimites, insect pheromon
		Monocyclic			
		Bicyclic			
		Tricyclic		cubelol dihydrohelenaline viridoflorol	aroma anti-inflammatory, analgesic phlebotonic, oestrogen mimetic
n = 4	Diterpene			cafestol phytol	hypercholesterolemiant chlorophyll composant, liposoluble
n = 6	Triterpene	Steroids	sterols and derivatives	cholesterol	membrane structure
			steroid hormones		signalling molecules
			secosteroid	vitamine D	
			bile acids		detergents and signalling molecules
		Others		oleanolic acid ursolic acid betulinic acid	antitumor, and hepatoprotective anticancer anticancer, anti-retroviral, anti-malarial, and anti-inflammatory
n ≥ 8	Polyterpene	Carotenoids	Carotenes	lycopene beta-carotene =vitamin A dimer	essential nutrient, anticancer antioxydant, antitumoral, dye
			Xanthophylles	luteine others pigments	antioxydant, eye and skin protector from UV

Table 2: Classification of terpenes

development of type 2 diabetes (63, 64). However, this has not yet been assessed by clinical trials(65-69) and worth remembering is that some powerful antioxidant phytochemicals such as melatonin are correlated with increasing type 2 diabetes risk (70). Examples of antioxidant phytochemicals such as phenols and terpenes are shown in Table 1 and Table 2 respectively.

2. Classical treatment

Since metabolic diseases are slowly progressing diseases, prevention by itself is often insufficient and the disease will ultimately evolve, underlining the requirement for new therapies. Among these, we can distinguish classical approaches that are currently used in clinical practices, and novel therapies that are under development (see below). Classical approaches to the treatment of obesity and type 2 diabetes consist of counteracting dyslipidemia and hyperglycaemia through lifestyle intervention alone or combined with administration of antiobesity and antidiabetic molecules.

a. Anti-obesity (Table 3)

Whatever treatment used, forced exercise and diet intervention are often an essential part of the treatment of obesity.

Appetite suppressors are a way to decrease food intake i.e. energy input, in the same idea than calorie restriction.

Intestinal lipase inhibitors inhibit fat absorption and that results in reduction of plasma FFA levels, improvement in insulin sensitivity and subsequent reduction of type 2 diabetes incidence (71, 72). Fatty acid synthase (FAS) inhibitors decrease plasma lipid levels and weight (73). Energy expenditure enhancers increase the metabolic rate, allowing the rebalance of the metabolic disequilibrium. This last strategy is of particular interest, since increasing energy expenditure will allow to burn fat storages and thus body weight loss. As

Classification			Examples
Non-pharmacological therapy: Lifestyle intervention			Diet and calorie restriction Physical activity
Pharmacological therapy: Anti-obesity	Appetite suppressors		Sibutramine (Meridia®) Rimonabant *Hoodia gordonii*
	Others	Lipase inhibitor	Orlistat (Alli®, Xenical®) *Panax ginseng*
		FAS inhibitor	Orlistat (Alli®, Xenical®) Polyphenols Genistein
		Energy expenditure enhancers	Caffeine Polyphenols Others antidiabetics

Table 3: Classification of antiobesity

Classification				Examples
Non-pharmacological therapy:				Diet and calorie restriction Physical activity
Pharmacological therapy: Antidiabetics	Insulin and analogues			NPH insulin Glargine Detemir
	Insulin secretagogues	K+/ATP	Sulfonylurea (SU)	Glyburide/Glybenclamide Glipizide Glimepiride
			Non-SU/Glinides	Repaglinide Nateglinide
		Incretin modulators	GLP-1 analogues	Liraglutide Exenatide/exendin 4
			DPP4 inhibitors	Sitagliptin (Januvia®) Vildagliptin (Galvus®)
	Insulin sentitizers	Biguanides		Metformin
		PPARs agonists	PPARγ agonists/TZD	Troglitazone Pioglitazone Rosiglitazone
			Dual agonists	Chiglitazar Netoglitazone ONO-5129
		Amylin analogue		Pramlintide
		PTP1B inhibitors Glycogen phosphorylase inhibitors UPS inhibitors AMPK activators		
	α-glucosidase inhibitors			Acarbose Miglitol Voglibose
	Intervention on Mitochondria	Antioxydants		Vitamines, natural molecules....
	Preserving beta cell function	AMPK activators		

Table 4 : Classification of antidiabetics (classical approaches are in black and novel therapies in blue)

example, caffeine, shown to increase metabolic rate by 12% (74), is used in nutraceutic and cosmetic as antiobesity.

In addition to the described targeted treatment, some natural medicines act simultaneously at different levels of the pathophysiology of obesity. For example the ayurvedic folk medicine Brindall berry *Garcinia cambogia* contains hydroxycitrate which increases lipolysis, decreases lipogenesis and suppresses appetite (75).

b. Antidiabetics

Antidiabetic strategies, which are described in Figure 9 and classified in Table 4, target different levels of pathophysiological states of type 2 diabetes. The pathophysiology of type 2 diabetes is characterized by a lack of insulin action and/or secretion that is the basis of the therapeutic strategies which directly bring insulin to the organism, enhance insulin secretion, or improve insulin sensitization. The fourth currently used therapy prevents carbohydrates absorption.

i. Insulin and analogs

Insulin and analogs were among the first used therapy. The new long-acting basal insulin analogs such as glargine and detemir offer improved pharmacokinetic and pharmacodynamic profiles compared with NPH insulin (76, 77) and exhibit ameliorated efficacy and safety by improving glycemic control, lowering risk of hypoglycaemia, and reducing weight gain better when compared to NPH insulin (78, 79).

ii. Insulin secretagogues

Sulfonylurea (SU) and non-SU drugs are insulin secretagogues. They bind to the SU receptor found on the surface of pancreatic beta cells, leading to the following signalling cascade: closure of voltage-dependent ATP potassium (K_{ATP}) channels, cell membrane depolarization, calcium entry into the cell, and finally insulin secretion (80, 81) (Figure 5). As

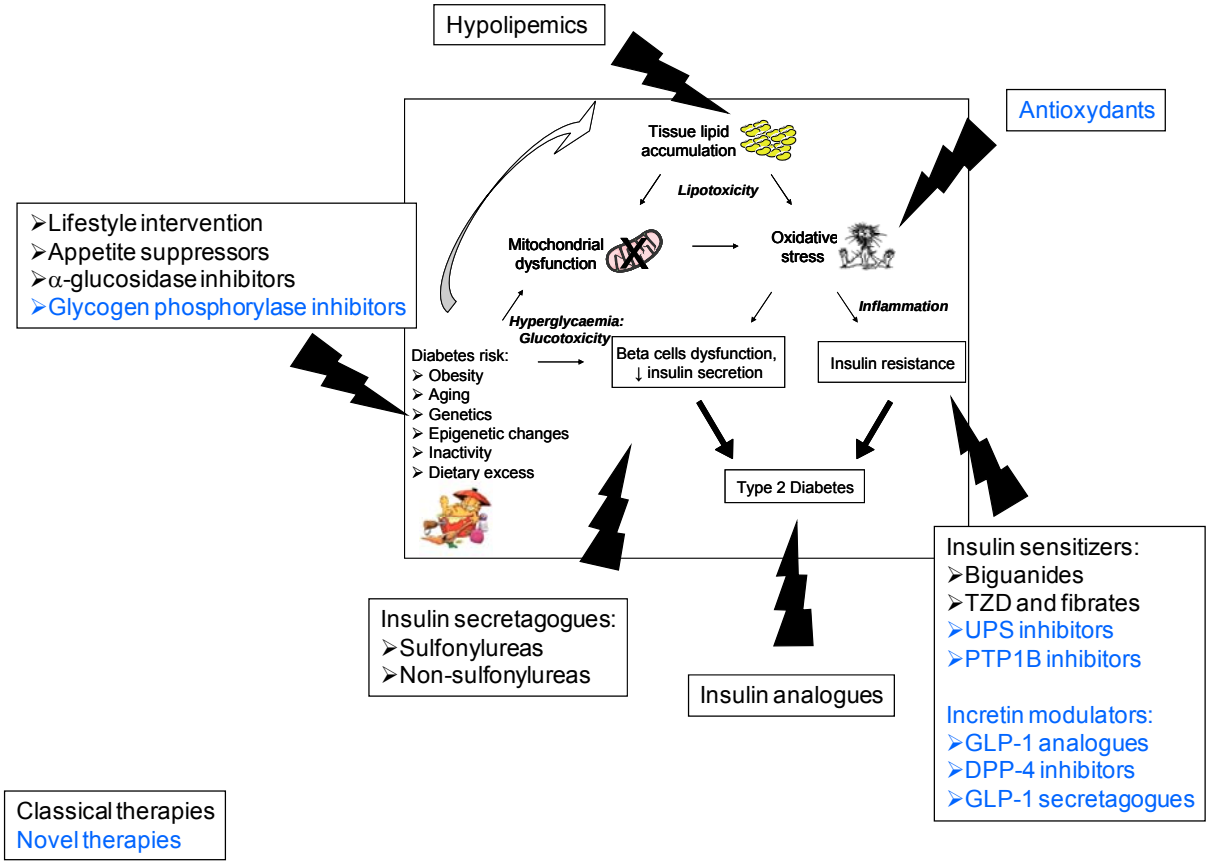


Figure 9: Antidiabetic strategies

seen with insulin analogs therapy, SU therapy is associated with weight gain and hypoglycaemia (80, 82) but to a lesser extent (208). The non-SU drugs are distinguished from the SU by their short metabolic half-lives, which results in brief episodic stimulation of insulin secretion (83) but which requires frequent dosing schedule with meals. The short-term insulin secretion allow the decrease of adverse hypoglycaemia events (84).

iii. Insulin sensitizers

Insulin sensitizers may act on neoglucogenesis (biguanides) or on lipid homeostasis (PPARs agonists). Biguanides reduces neoglucogenesis, which results in a decreased glycaemia and lower blood lipids. The more commonly employed in clinic is metformin, extracted from Galega officinalis (Bailey, cj, 2004; 21(3):115-7). Metformin treatment associated with lifestyle intervention reduces type 2 diabetes incidence by 31% (85, 86). Moreover, metformin's glucose-lowering effects are equivalent to those obtained with SU (87). In addition, metformin treatment causes weight loss (or no weight gain) and much less hypoglycaemia than SU therapy (88, 89).

The agonists of the transcription factors PPARs act on lipid homeostasis. The fibrates are PPARα agonists. They are classified as hypolipidemic agents, like statins, which are used in the treatment of hyperlipidemia, in the context of cardiovascular diseases principally (90-92). Their lipid lowering properties allow us to consider them in the treatment of metabolic diseases. Indeed, fibrates lower plasma lipid levels by stimulating fatty acids oxidation (93-95). Through these lowering effects on FFA, fibrates contribute to the reduction of insulin resistance and restoration of insulin secretion. .

Compounds of the class of the thiazolidinediones (TZD) are PPARγ agonists. They exert a beneficial effect on the lipid profile since they promote fat storage in the adipose tissue thus contributing to decreased insulin resistance (96-98), andimprove muscle insulin-stimulated glucose uptake (99)(100, 101). That results in stabilized glucose levels and protected beta-cell function, effects which together decrase the risk of type 2 diabetes and

death by 60% (102-107). The described metabolic effects of TZD were clearly demonstrated in different clinical studies, such as TRIPOD(106), PIPOD (108), DPP (85), PROactive and DREAM (102). It has to be noted that administration of TZD is associated with side effects such as weight gain and oedema (109-112).

iv. α-glucosidase inhibitors

α-glucosidase is an enzyme present in the brush border of the small intestine responsible of the degradation of carbohydrates in sugar. The α-glucosidase inhibitors delay the breakdown of complex carbohydrates, that slows down their absorption, and consequently lowers postprandial glucose peaks and insulin levels (113, 114). They eventually reduce type 2 diabetes development risk by 25% (115). The efficacy of α-glucosidase inhibitors is considerably less than that of either SU or metformin (116) but they remain attractive because unassociated with hypoglycaemia and weight gain, thus used in combination with SU. Side effects are mostly of gastro-intestinal origin (flatulence, diarrhoea) but there is no evidence for long-term detrimental effects (113). It has to be noted that numerous alpha-glucosidase inhibitors come from natural world and in particular triterpene acids isolated from Lagerstroemia speciosa (117).

v. Antidiabetic plants

In addition to all these therapies, few traditional antidiabetic plants have received scientific or medical scrutiny, and for the most of them, they act simultaneously at different levels of type 2 diabetes pathophysiology. The study of such medicines offer a natural key to unlock the diabetologist's pharmacy. Considering the numerous plants which are used to treat diabetes (118), only some well-known examples will be detailed.

Gymnema Sylvestre leaves act on intestinal glucose absorption (119). Some botanics such as Artemisia dracunculus L (120), shilianhua (120), polyphenols contained in green tea (121), wild ginseng (122) enhance insulin action. Olive leaves (*Olea europaea* L., Oleaceae)

exert hypoglycaemic, antiproliferative and apoptotic, hypotensive, anticancer, antimicrobial and antioxidant properties (212, 216-218). Concerning the hypoglycaemic and antioxidant effect of this plant, oleuropein seems to be the active constituent (123).

3. Novel therapies

a. Alternative therapies

The new therapeutic strategies are based on developing drugs against new targets, which have been identified through the increased knowledge in the ethiology and the pathophysiology of diabetes.

The protein tyrosine phosphatase (PTP) PTP1B dephosphorylates the primary substrates (IRS) of the tyrosine kinase insulin receptor (IR). Inhibition of PTP1B could be effective as a treatment of type 2 diabetes by improving insulin-mediated events such as glucose uptake (124), without the adverse weight gain effect of numerous antidiabetics. The search for effective and safe orally available compounds is ongoing.

Glycogen phosphorylase is an enzyme responsible of the glycogenolysis and its inhibition mimics one effect of insulin, that is thus an interesting strategy for the treatment of type 2 diabetes (125). Some glycogen phosphorylase inhibitors previously discovered, emphasize the benefits of this strategy: carbohydrate derivatives and pentacyclic triterpenes, the triterpenoid maslinic acid isolated from *Olea Europaea* (126).

The ubiquitin proteasome system (UPS) is implicated in the degradation of the IR and IRS (127-129), leading to insulin resistance. Moreover, increased expression of components of the UPS has been observed in the muscles of patients in catabolic states (130). Reversal of UPS effects appears as a novel approach in the treatment of type 2 diabetes. The recent development of large scale screening technologies will accelerate the pace of such discoveries. For example, shRNA libraries against UPS genes have been constructed (131) and could be used to characterize alterations in insulin action in cultured cells.

AMP-activated kinase (AMPK) was revealed to be implicated in numerous processes which correlate with type 2 diabetes: adiponectin action (132), regulation of genes linked to oxidative metabolism (133), hepatic glucose output (134), neoglucogenesis, glucose transport in muscle, prevention of hyperglycaemia, inhibition of insulin release in beta cell (135), cardiovascular disease (136) and increased food intake (137). Considered together, these data emphasize the importance of AMPK in the regulation of cellular energy homeostasis since they result in improvements of peripheral insulin sensitivity in muscle (138) and delayed beta cell dysfunction associated with type 2 diabetes (139). Furthermore, it was recently shown in our laboratory that AMPK is implicated in regulation of energy expenditure by SIRT1/ PGC-1α activation (140). AMPK is therefore a potential therapeutic target in the prevention and the treatment of type 2 diabetes (141). Pharmacological (e.g., A-769662) and nutritional (e.g., polyphenols) AMPK activators have been employed with promising results (139). Interestingly, metformin treatment of subjects with type 2 diabetes significantly increases AMPK activity in skeletal muscle (142, 143). Moreover, the natural polyphenols resveratrol and epigallocatechin-3-gallate were recently identified as potent AMPK activators *in vitro* and *in vivo* (144, 145).

As explained earlier, fatty acids are in part responsible for the lipotoxicity that leads to beta cell dysfunction and apoptosis. Preservation of beta cell function would seem relevant to a decrease in the development of type 2 diabetes. Recently, the reduction of Foxo1 activity has been highlighted as a potential strategy to protect beta cells (146). Indeed, Foxo1 seems to play a major role in fatty acid-induced beta cell apoptosis and expression of a dominant-negative allele of Foxo1 reduces the expression of apoptotic and ER stress markers and promotes beta cell survival (146).

Finally, an interesting strategy is to combine different strategies. In all the cases, combinations of drugs and lifestyle intervention proved beneficial. Combining different drugs could also be attractive. For example, the combined treatment with PPARγ and PPARα agonists (rosiglitazone and fenofibrate respectively) results in a normalization of triglyceride

and total cholesterol levels with no increased body mass index in type 2 diabetic patients (147-149). Alternatively, PPARγ/δ dual agonists deliver optimal glycemic control with minimized adverse effects, as demonstrated by a new compound **23**, which represents superior profiles and weight gain reduction compared with the currently marketed thizolidinediones (TZD) (150). A final example is given by the positive effects of the combination of statins with bile acids sequestrant, ezetimibe, niacin, fibrates, omega 3 fatty acid (151-154).

b. Intervention on mitochondria

As described above, the early stages of metabolic diseases is linked to mitochondrial dysfunction in two principal respects: excessive ROS generation and defective energy expenditure system (UCP is an example).

Indeed endogenous (overexpression of manganese dismutase) and exogenous (vitamin E, C, carotenoids, polyphenols, etc) antioxidants have been proposed as preventive agents in metabolic diseases. This strategy concomitantly prevents increased intracellular AGE formation, PKC activation, and increased hexosamine formation (35, 37). Similarly, UCPs, through their proton-leak activity, could decrease ROS generation and avoid oxidative stress (26, 27). This method must be used with caution in beta cells, as this property leads to a decreased generation of ATP and consequently will impair glucose-stimulated insulin secretion (24, 25). Antioxidants are currently widely used and new therapeutics targeting energy expenditure could improve the existing medications.

For example, natural compounds such as polyphenols (EGCG) have been shown to target mitochondria by enhancing energy expenditure but not lipid oxidation (155, 156). Similarly, bitter melon (*Momordica charantia*) reduces adiposity by increasing lipid oxidation and mitochondrial uncoupling (157). Others medicinals such as the medinical plant *Undaria pinnatifida* upregulate UCP1 expression in WAT, leading to enhanced energy expenditure and reduced WAT weight (158). The active component is fucoxanthin, a carotenoid

contained in this plant. This last interesting therapeutic strategy requires further study before it can be used in clinic to prevent and treat metabolic disorders.

c. Incretin modulators

i. Introduction

Eating provokes the secretion of multiple gastrointestinal hormones or incretins such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which are synthesized in enteroendocrine cells and the levels of which increase within minutes of eating. A combination of endocrine and neural signals probably promotes their rapid secretion, but their circulating levels decrease rapidly through enzymatic inactivation by dipeptidyl peptidase-4 (DPP4) and through renal clearance (159). Indeed, both GIP and GLP-1 contain alanine at position 2, and hence are excellent substrates for DPP4 (160).

Both GIP and GLP-1 act on GPCRs expressed in beta cells, in peripheral tissues and, in the case of GIP also in the central nervous system. Activation of both incretin receptors on beta cells leads to insulin biosynthesis, insulin exocytosis in a glucose-dependent manner (161), and stimulation of beta cell proliferation (162), thus promoting resistance to apoptosis and enhanced beta cell survival (163). GLP-1 also inhibits glucagon secretion, gastric emptying, food intake and appetite, and promotes enhanced glucose disposal through neural mechanisms (164), actions that also contribute to the control of glucose homeostasis. In fact, GLP-1 and GIP inhibition (by antagonists or mutations) lowers insulin secretion, increases plasma glucose and glucagon levels, impairs glucose tolerance, reduces glucose clearance, and leads to quicker gastric emptying (165-167); the same pattern is observed in type 2 diabetes patients, confirming the therapeutic potential of these hormones (168). Indeed, GLP-1 injection lowers blood glucose levels in patients with type 2 diabetes by improving insulin secretion, suppressing glucagon secretion and gastric emptying, improving insulin sensitivity, and reducing HbA_{1c} (169, 170). Intravenous or subcutaneous GLP-1 infusions could be useful for the short-term control of hyperglycaemia whereas long-term treatment of

type 2 diabetes requires a more feasible approach such as injectable GLP-1 receptor agonists and/or oral DPP-4 inhibitors.

Two new classes of drug based on the action of the incretin hormones have recently been approved for type 2 diabetes therapy: incretin mimetics such as injectable long-acting stable analogs of GLP-1 and incretin enhancers such as orally available inhibitors of DPP4 (171).

ii. GLP-1 analogs

The stabilization of GLP-1 molecules against DPP4, by substituting alanine in position 2 with valine, for example, does not affect the biological activity of the peptide (172). However, renal clearance eliminates this stabilized molecule extremely rapidly (with a half life of 4–5 min). Another incretin mimetic under clinical development is liraglutide (NovoNordisk), which is based on the structure of native human GLP-1, but modified to bind to albumin, thereby preventing renal elimination and DPP4 degradation (half-life of 11–13 h) (173, 174). Liraglutide is slowly absorbed and its half-life renders it suitable for once-daily injection. Clinically, the molecule has similar actions to continuously infused GLP-1 and appears to have a similar clinical potential to exendin 4 (see below) (175). Indeed, liraglutide alone is capable of decreasing fasting plasma glucose levels, HbA_{1c} and weight with transient and mild nausea as side effects (175).

Another strategy involves the isolation of exendin 4, a peptide with about 50 % sequence homology to GLP-1, from the saliva of the Gila Monster (*Heloderma suspectum*). This molecule appears to be a full agonist of the GLP-1 receptor and stable against DPP4-mediated degradation, with a plasma half-life of 30 minutes (176, 177). Exenatide, a synthetic replica of exendin 4, was developed by Amylin Corporation and Lilly for type 2 diabetes treatment (Byetta®). In the clinic, exenatide induced weight loss, lowered HbA1c and improved beta cell function ; indicating that exenatide is effective in a primary care setting (178). Observed side effects were primarily dose-dependent nausea and vomiting. Treatment for 3 years or more was associated with significant improvements in

cardiovascular risk factors and hepatic biomarkers (179). New drug status were obtained from the authorities in both the USA and EU in May 2008.

iii. DPP4 inhibitors

DPP4, also known as the T-cell antigen CD26, is a serine peptidase present in kidney, intestinal brush-border membranes, hepatocytes and vascular endothelium, as well as in a soluble form in plasma (180). It cleaves the N-terminal dipeptide from substrate peptides such as GLP-1 and GIP (181). DPP4 is thought to contribute to T-cell activation and proliferation, but its role in the immune system is independent of its enzymatic action and its absence can be compensated for.

Since degradation of GLP-1 by DPP4 is extremely rapid and extensive, inhibitors of the enzyme could be used as a therapy for type 2 diabetes by protecting and thereby enhancing circulating levels of GLP-1 (182). Indeed, mice with targeted inactivation of the DPP4 gene present raised levels of plasma GIP and GLP-1, increased insulin secretion, and reduced glucose excursion (160). DPP4 inhibition completely protects both endogenous and exogenous incretins, which furthermore greatly enhance insulin responses to glucose (183, 184). Numerous studies focused on the development of DPP4 inhibitors for clinical use and long-term studies have proven their safety, their good tolerance, and their absence of adverse immune effects (185). Moreover, these inhibitors exhibit good oral bioavailability and a relatively long duration of action, allowing once-daily dosing (186, 187). As opposed to the incretin mimetics, DPP4 inhibitors appear weight neutral, which could be explained by the fact that attained GLP-1 concentrations are limited compared to these attained with the incretin mimetics.

The first DPP4 inhibitors on the market were sitagliptin by Merck & Co., Inc. (Januvia®) and vildagliptin by Novartis AG, Inc. (Galvus®) (188, 189). Both agents used in monotherapy significantly reduce fasting blood glucose and HbA1c in patients with type 2 diabetes (190). This glycemic control is further improved when these inhibitors are given in combination with

other antidiabetic agents including metformin, sulfonylurea and thiazolidinediones (186, 191). Since recent studies have indicated that metformin may increase GLP-1 biosynthesis and secretion, combination of DPP4 inhibitors with metformin may result in a greater increase in the concentrations of active GLP-1 (192). If this combination is demonstrated to prevent deterioration of beta cell function better than metformin alone, it may be recommended as the initial treatment of type 2 diabetes patients. DPP4 inhibition may also be combined with insulin treatment leading to improved glycemic control with lower significant hypoglycaemic events (193).

iv. GLP-1 secretagogues

A third strategy concerning incretin-based therapies could be to enhance GLP-1 secretion. This route has not been exploited greatly to date but could prove a promising strategy to improve therapy with DPP4 inhibitors. As an example, a plant decoction was recently shown to exert antidiabetic action by increasing GLP-1 secretion (194), and berberine extracted from *Berberis vulgaris* showed similar effects (195).

II. TGR5

A. Introduction

1. Discovery and molecular characteristics

TGR5 is a G protein coupled receptor (GPCR), first identified as an orphan membrane receptor and then classified as the first BAs-responding GPCR. Indeed, a search of the GenBank DNA database based on amino acid sequences of known GPCR lead to a new DNA coding sequence named the human BG37 (hBG37). The putative coding sequence was independently cloned by PCR by Kawamata *et al.* (196) and Maruyama *et al.* (197). TGR5 is encoded by a two exons which sizes a 993 bp mRNA (accession number of human and mouse mRNA in DDBJ/EMBL/GenBank™ AB089307 ans AB089308) and maps to chromosome position 2q35 in humans and chromosome 1c3 in mouse. This sequence encodes a 330 amino acid protein (35kDa) constituted of seven transmembrane domains, characteristic of GPCR. It revealed 28% identity to human EDG-1 (sphingosine-1-phosphate receptor), a member of the class 1 (rhodopsin-like) GPCR superfamily. hBG37/TGR5/Gpbar1 cDNAs was also identified among species showing high conservation with mouse (83%), rat (82%), dog (85%), cow (86%) and chimpanzee (99%) (Genaltas HGNCid 19680, Pubmed NCBI Entrez gene AB086170.1).

In TGR5-expressing HEK293 cells, some compounds related to cyclopentanoperhydro-phenanthrene, which present a similar structure to steroid hormones, bile acids (BAs) and sterols, induced an increase of cAMP levels in a TGR5-dependent manner. After screening of BAs, the rank order of potency was determined: lithocholic acid (LCA) > deoxycholic acid (DCA) > chenodeoxycholic acid (CDCA) > cholic acid (CA) with EC_{50} values of 35±5, 575±75, 4004±662nM, >10µM respectively. However, steroid hormones proved not substantially active at physiological concentrations (197).

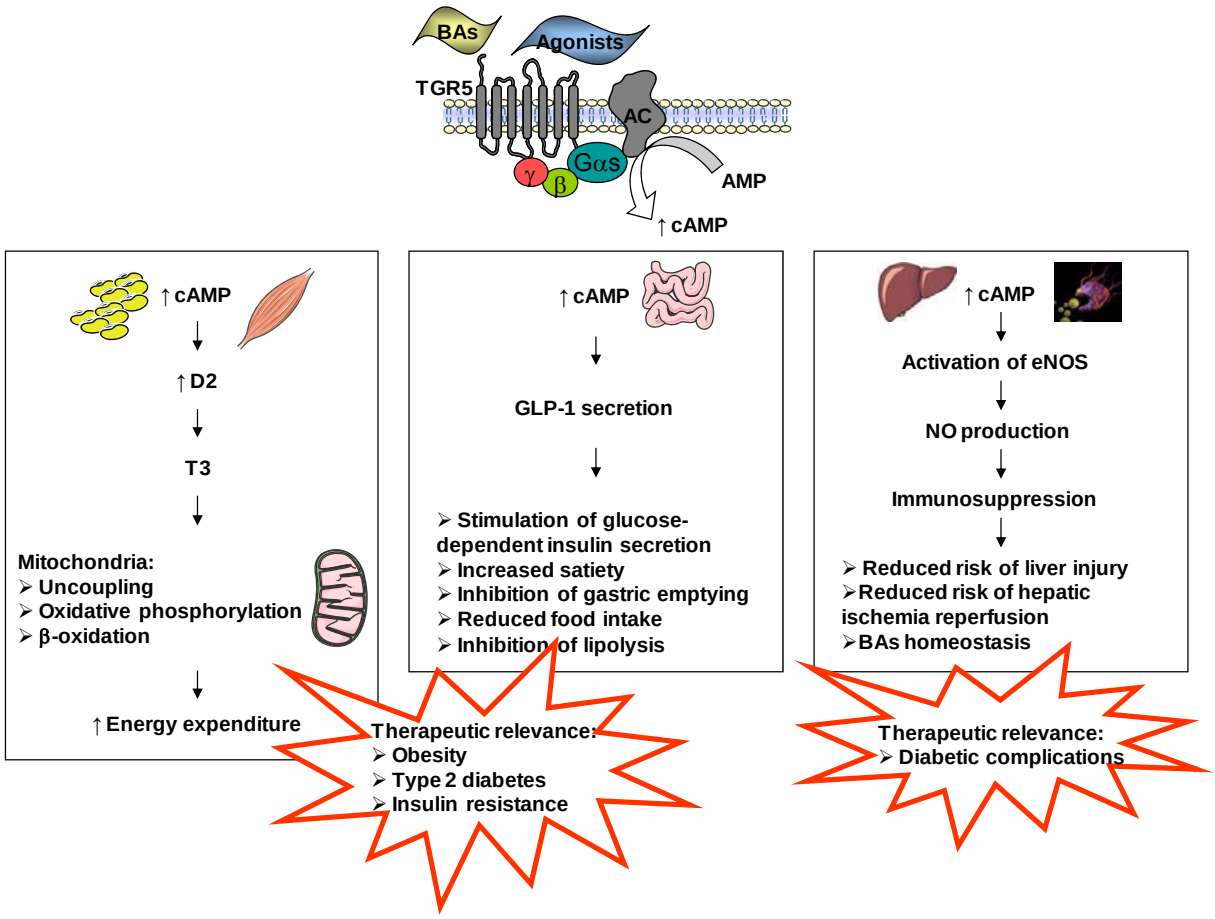


Figure 10: TGR5 actions

2. Tissue distribution

TGR5 is ubiquitously expressed in human tissues: high levels were detected in placenta and spleen, whereas moderated levels were found in other tissues including lung, liver, stomach, small intestine, adipose tissue, heart, skeletal muscle, uterus mammary gland, bone marrow, liver, kidney and leukocytes/monocytes/macrophages. However no expression is observed in brain and thymus (197-199). Among rabbit tissues, the highest level was detected in the spleen (196). In vitro, TGR5 is expressed in enteroendocrine cell lines such as NCI-H716, STC-1 and GLUTag cells but not in epithelial cells (196, 197). In addition, TGR5 is expressed in Kupffer cells (liver macrophages) and liver sinusoidal endothelial cells (SEC) (200).

3. Roles

BAs-activated TGR5 induces intracellular cAMP production in TGR5-expressing cells leading to subsequent GLP-1 release in intestine cells (196, 197), inhibition of inflammation in macrophages and associated cells and metabolic effects in adipose tissue and muscle (Figure 10).

4. KO/Transgenic mice

To study the implication of TGR5 in physiology, TGR5 deficient transgenic mice were generated. In 2006, Maruyama *et al.* and Vassileva *et al.* independently published the effect of targeted deletion of TGR5 on mice physiology (198, 201).

The team of Vassileva *et al.* observed that TGR5-/- mice are viable, develop normally and have no physiological abnormalities when compared to TGR5+/+ mice. They did not observe significant differences in the content in cholesterol, BAs and phospholipids of bile. However, when challenged with a high fat diet containing cholic acid, TGR5-/- mice were protected from cholelithiasis with gallstones of cholesterol type. Indeed, TGR5-deleted mice produced an excess of phospholipids in their gall-bladders, resulting in a low bile salt/phospholipids

ratio, unfavourable for gallstones formation. Furthermore the loss of TGR5 impairs the negative feedback regulation of BAs synthesis, resulting in the failure to repress Cyp7a1 mRNA in response to cholic acid feeding. This study did not reveal significant differences in weight gain although TGR5-/- mice were slightly lighter than the wild-type (198).

Maruyama *et al.* demonstrated that the total BAs pool size was significantly decreased in homozygous mice. Independent of the gender, TGR5-/- mice were shown to gain more weight than the wild-type mice under high fat diet conditions (significantly for females, as a tendency for males). Furthermore, locomotor activity and food intake were not changed, suggesting that weight gain is due to decreased energy expenditure in homozygous mice (201). Indeed, the lack of TGR5-cAMP-D2 pathway may reduce energy expenditure and elicit adiposity, in accordance with the hypothesis developed by Watanabe *et al.* (199). Moreover, lipolysis is regulated by intracellular cAMP levels (202). As TGR5 stimulates cAMP production and as it is expressed in WAT, it is possible that TGR5 may play a role in the regulation of the lipolysis in WAT, however further investigations are required to assess this hypothesis.

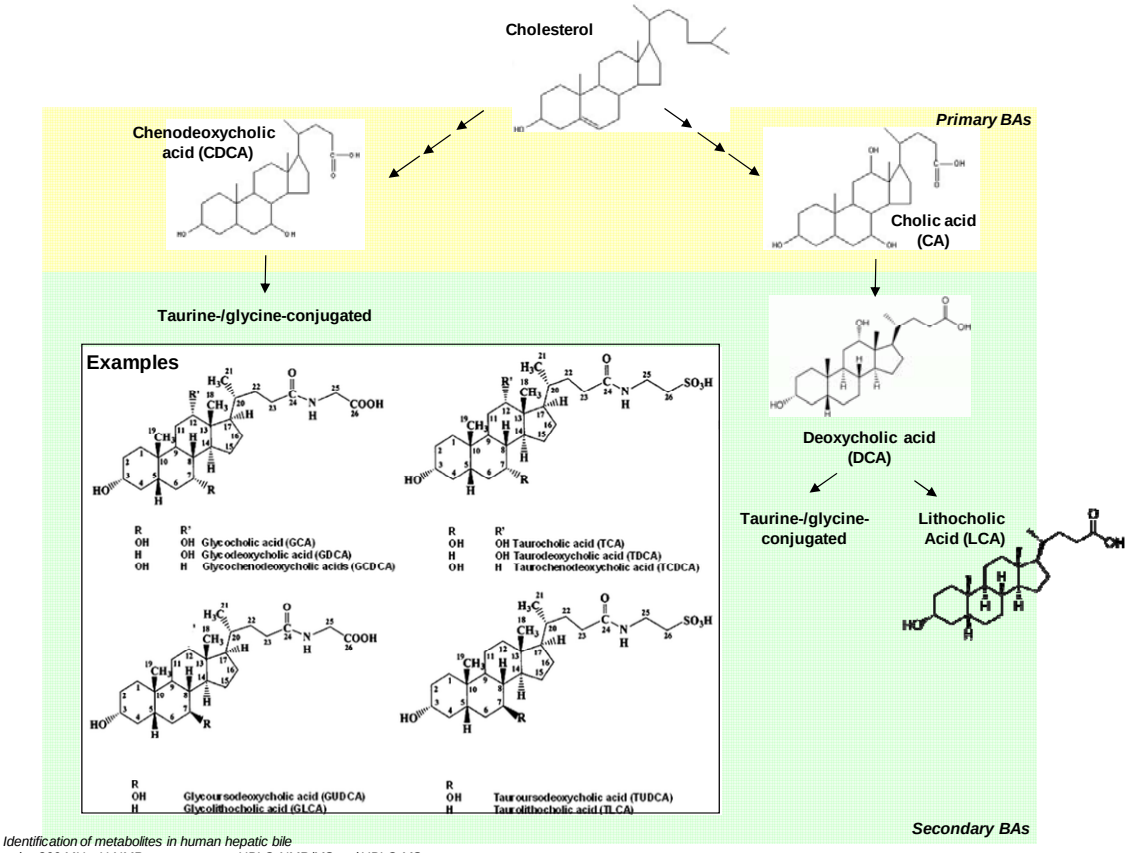
In both studies, no difference in FXR expression was observed; plasma levels of total BAs and triglycerides were also similar between wild-type and homozygous mice. Altogether, these data suggest that TGR5 contributes to BAs homeostasis. A recent study from our laboratory demonstrated, using mice that overexpress TGR5, that TGR5 signalling induces GLP-1 release, leading to improved liver and pancreatic functions (Annexe 5).

B. Agonists

1. Bile acids

a. Introduction

Bile is secreted by the liver and plays an important part in fat digestion and absorption since digesting enzymes and bile salts emulsify and transport digested fat. The bile is



Identification of metabolites in human hepatic bile using 800 MHz ^{1}H NMR spectroscopy , HPLC-NMR/MS and UPLC-MS. Duarte IFet al. Mol. BioSyst., 2009, 5, 180-190

Figure 11: Bile acid synthesis and structures

synthesized, secreted by the liver and then stored by the gall-bladder until needed in the duodenum. There, bile is concentrated because of absorption of water and nutrients by the gall-bladder mucosa. The most abundant substances composing bile are bile salts (about half of total solutes). The synthesis of BAs involves cholesterol as precursor and leads to the synthesis of primary and secondary BAs (Figure 11). After a meal, when food begins to be digested in the upper gastrointestinal tract, the gall-bladder begins to empty until excretion of bile into the intestine occurs, where BAs serve as detergents to facilitate lipid absorption. Upon completion of their function, BAs are recycled (203).

BAs are unique amphipathic steroids that make up the major organic component of secreted bile. The distinct hydrophilic and hydrophobic faces of these steroids are vital for their ability to aid in the absorption and the digestion of fats and fat soluble vitamin. BAs are produced in the liver, secreted into the bile, stored in gall-bladder and after use in intestine, reabsorbed from the intestine by both active and passive mechanisms, and returned to the liver via the portal blood. BAs are cholesterol derivatives essential for the absorption of dietary lipids and fat-soluble vitamins.

b. Roles

BAs were considered for many years as the final products of cholesterol catabolism involved in dietary lipids and vitamins absorption. Now, they are also recognized as key elements of paracrine and endocrine functions related to the homeostasis of cholesterol levels and regulation of the immune system. BAs exert their biological functions through two independent pathways, nuclear receptors and GPCR (Figure 12).

i. Role in lipid homeostasis

The amphipathic BAs can serve as powerful physiological detergents for absorption and transport of nutrients, fats and vitamins. First, BAs emulsify fat particles from food, which decreases the surface tension of the particles and allows the agitation in the intestinal tract to

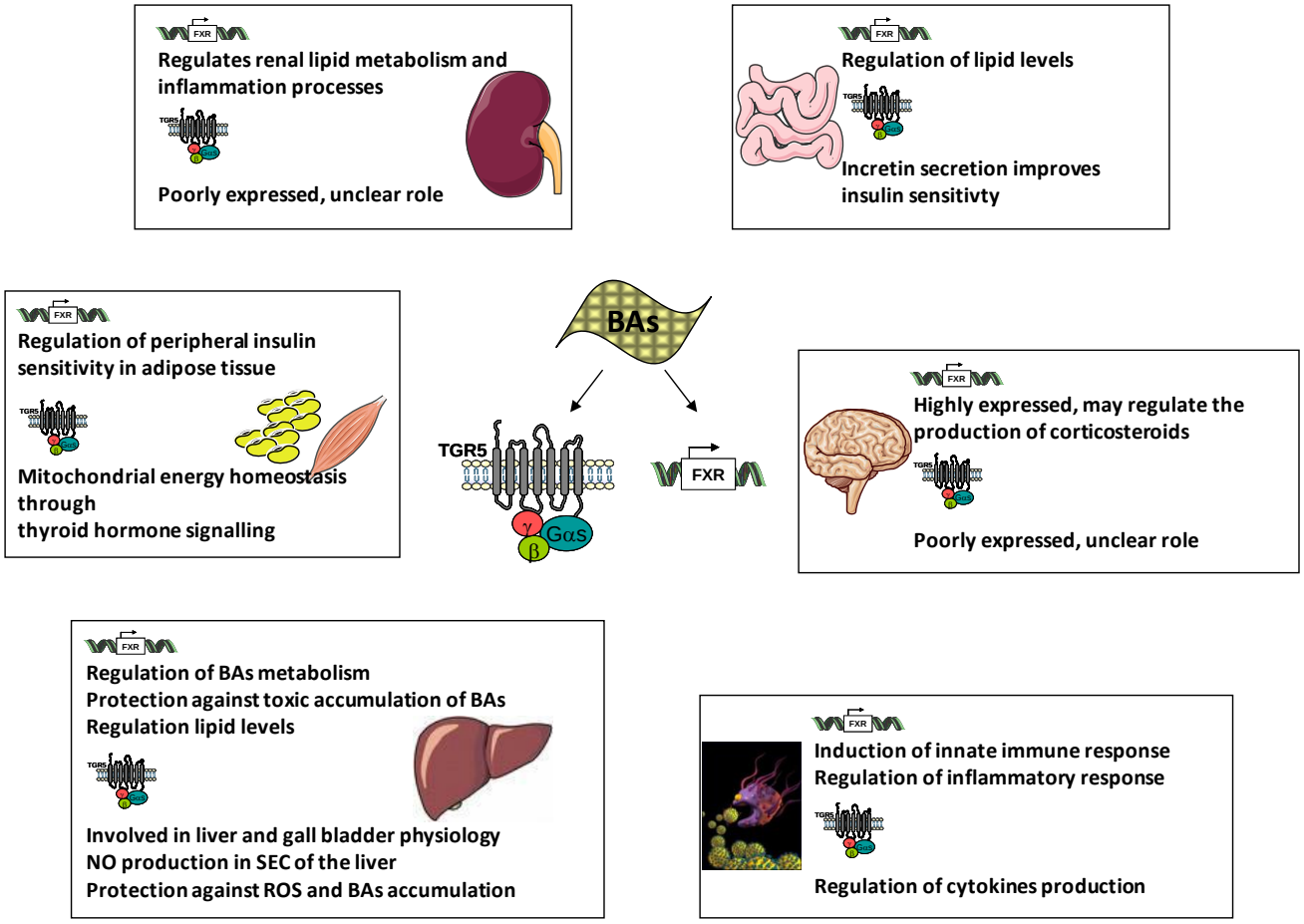


Figure 12: BAs actions

break fat globules into minute sizes. Second, BAs help the absorption of fatty acids, monoglycerides, cholesterol and other lipids from the intestinal tract by forming micelles, which are highly soluble complexes due to the electrical charges of BAs (203).

BAs are also signalling molecules and inflammatory agents that rapidly activate nuclear receptors and cell signalling pathways that regulate lipid, glucose and energy metabolism.

ii. Role in immunologic and inflammatory mechanisms

At high concentrations, BAs are known to exhibit immunosuppressive effects on cell-mediated immunity and macrophages functions (204). Indeed, BAs increase the GTP binding in membrane fractions and the intracellular cAMP production, leading to the activation of extracellular signal-regulated kinase mitogen-activated protein kinase (ERK) and the final suppression of LPS-stimulated cytokine production in macrophages (196)(152). Kupffer cells are macrophages residing in liver, which represent the major source of inflammatory cytokines in this organ (205). In these cells, BAs that activate TGR5 once again inhibit LPS-stimulated cytokine production via TGR5-cAMP dependent pathways and thus protect the liver by preventing excessive cytokine production (206).

In sinusoidal endothelial cells, BAs instantaneously activate eNOS (whose expression is under CRE control, CRE present in the eNOS promoter; activity regulated by phosphorylation of eNOS) by activating the TGR5-cAMP signaling pathway (200). This leads to NO production in the liver and thus vasodilatation. An excess of NO is correlated with the observed increase in BAs, both implicated in cirrhosis. In addition to its role in vascular resistance, NO may protect from oxidative stress generated by BAs themselves (207, 208) (Figure 12).

iii. Role in apoptosis and cell proliferation

At high concentrations, bile salts are toxic by altering membrane fluidity (209) and by enhancing apoptosis (207, 210). Otherwise, BAs were shown to prevent apoptosis through a

TGR5-ADAM-HB-EGF-EGFR signalling pathway (211) since TGR5 mediates the BAs activation of epidermal growth factor receptor (EGFR), leading to cell proliferation and inhibition of apoptosis, in gastrointestinal-tract cell carcinogenesis (211).

c. Bile acids receptors

i. FXR

❖ Introduction

FXR belongs to the superfamily of nuclear receptors which contains 48 different members in humans, many of which are orphan receptors that have no known ligands. Both free and conjugated-BAs are the primary endogenous ligands of FXR by binding its ligand-binding domain. FXR forms a heterodimer with retinoid X receptor (RXR).

❖ Roles

Activation of FXR by BAs prevents the accumulation of toxic concentrations of these steroids in cells by decreasing their synthesis and transport, increasing their excretion from cells and inducing the expression of proteins that sequester them (212). Indeed, FXR is the BAs nuclear receptor which regulates the transcription of genes encoding pivotal BAs biosynthetic enzymes and transport proteins, since FXR represses the expression of CYP7A, thereby repressing the conversion of cholesterol to BAs. This repression has been shown to be mediated by liver receptor homolog-1 (LRH-1) and small heterodimer partner (SHP). The target genes of FXR are CYP7A and CYP8B1, ABCB11, ABCC2STD (213, 214).

❖ Agonists and natural agonists

The rank of order of potency for natural BAs to induce FXR activation is CDCA>LCA=DCA (197). The potent synthetic FXR agonist 3-[2-[2-chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]-methoxy]phenyl]ethenyl]benzoic acid (GW4064) could be used to prevent liver damage caused by cholestatic disease and prevent cholesterol gallstone

disease (215). This hepatoprotective effects is thought to be accomplished by promoting BAs and phospholipids excretion while repressing BAs synthesis. 6alpha-ethyl-chenodeoxycholic acid (6α-ECDCA) is the most potent FXR agonist reported (216) and it is actually in phase II clinical trials for the treatment of cholestasis in subjects with primary biliary cirrhosis and for the management of nonalcoholic steatohepatitis in patients with the metabolic syndrome.

Alternatively, FXR antagonists may be useful therapeutically to increase the conversion of cholesterol to BAs resulting in lower LDL levels in hyperlipidemic patients. The plant sterol and FXR antagonist guggulsterone (pregna-4,17(20)-diene-3,-16-dione) was tested for this indication but failed to show any improvement in LDL cholesterol levels. This was explained by the lack of specificity of this molecule which is also an activator of PXR (217, 218).

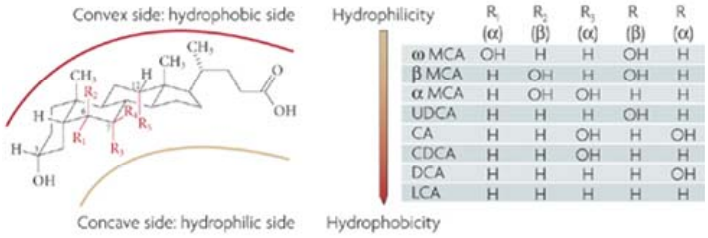
ii. TGR5 (Figure 10)

The TGR5 agonist activity of the molecules seems to increase in accordance with their hydrophobicity. Free and taurine and glycine conjugated forms are active. The rank or order of potency is:

LCA > DCA > CDCA > CA = TLCA ≥ TDCA > TCDCA > TCA (196, 197) (Figure 11).

LCA is the most potent BA-derived TGR5-agonist since its EC_{50} values in HEK239-TGR5 cells and NCI-H716 cells are 35nM and 3µM, respectively, which is much more potent than other BAs (197, 219). These concentrations of LCA are within the physiological range reported *in vivo*. DCA was used to prove the implication of TGR5 in promoting cell proliferation and inhibiting apoptosis (211, 219). In Kupffer cells, TLCA and TCA activation of TGR5 inhibits the LPS-stimulated cytokine production and inflammation (196, 206). TLCA, TCDCA and TCA-activated TGR5 induce mRNA expression of eNOS in liver sinusoidal endothelial cells (200). In addition, they activate eNOS by phosphorylation. TLCA induces the internalization of TGR5 and the subsequent ERK activation (196).

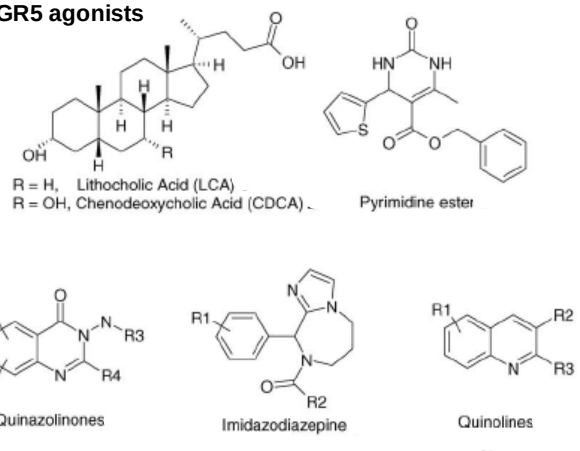
A. Chemical characteristic of BAs



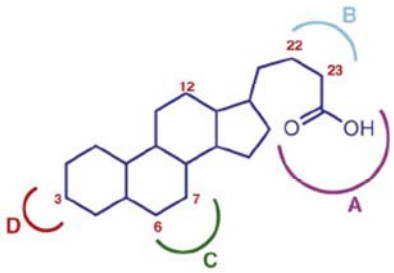
	R_1 (α)	R_2 (β)	R_3 (α)	R (β)	R (α)
ω MCA	OH	H	H	OH	H
β MCA	H	OH	H	OH	H
α MCA	H	OH	OH	H	H
UDCA	H	H	H	OH	H
CA	H	H	OH	H	OH
CDCA	H	H	OH	H	H
DCA	H	H	H	H	OH
LCA	H	H	H	H	H

Targeting BAs signalling for metabolic diseases. Thomas C et al. Nature Reviews Drug Discovery 7, 678-693 (2008)

B. TGR5 agonists



C. TGR5 interaction sites for binding BAs



TGR5: an emerging BAs GPCR target for the potential treatment of metabolic dissorders. Tiwari, A et al. Drug Discovery Today 14,523-530 (2009)

Figure 13: TGR5 agonists and binding site

Ursodeoxycholic acid and cholesterol are only slightly active on TGR5 but pregnandione shows significant activity. These results suggest that hydrophobic groups as well as the 5b-cholanic acid structure are important for the ligands to exhibit agonistic activity on TGR5. TLCA induces a response to TGR5 but not to homolog receptors such as EDG6, EDG7 or EDG8. Alternatively, absence of hydroxylation on position 6, 7, 12 of the steroid nucleus of LCA further increase TGR5 potency (220).

iii. Others

BAs activate different nuclear hormone receptors. For example, LCA activates FXR, SXR/PXR (human steroid and xenobiotic receptor (SXR) and its rodent homolog PXR) and VDR (vitamin D receptor). Furthermore, LCA-activated SXR/PXR inhibits BAs synthesis (CYP7A) and enhances BAs transport and metabolism (Oatp2) (221).

However, unlike FXR, BAs are not the only known endogenous ligands of these two receptors. SXR/PXR can be activated by (5β)-pregnane-3,20-dione, a progesterone reduction product, as well as a large and diverse class of xenobiotics. In addition, the PXR agonist rifampicin, in addition to having antimicrobial properties, has been shown to alleviate cholestasis and pruritis resulting from cholestatic disease (222). Concerning VDR, 1,25-$(OH)_2$-vitamin D_3 is the well known agonist, but novel ligands have also been investigated, which could be interesting because of their potential anti-inflammatory, anti-cancer, and anti-microbial properties.

2. Others agonists and binding pocket of TGR5 (Figure 13)

a. Agonists

The search of new TGR5 agonists, in order to characterize pharmacologically and pathophysiologically this receptor, as well as to validate it as a clinically useful target, requires the availability of potent and selective ligands.

LCA is the more potent agonist of natural BAs on TGR5 and research continues to elaborate on CDCA and CA derivatives which are also expected to have a high potency while keeping a reasonable hydrophobic/hydrophilic balance.

23(S)-methyl-CDCA and 6α-ethyl-23(S)-methyl-3α,7α-dihydroxy-5β-cholan-24-oic acid have been identified as potent and selective TGR5 agonists (223). In particular, methylation at the C-23(S) position of natural BAs confers a marked selectivity for TGR5 over FXR, while the 6α-alkyl substitution increases the potency of both receptors (220). These findings allow for the first time the pharmacological differentiation of genomic versus nongenomic effects produced by BAs and derivatives. Indeed, the binding pocket for BAs seems to be not entirely conserved between TGR5 and FXR, allowing pharmacological differentiation between genomic and nongenomic effects.

The screening of libraries of nonsteroidal compounds has led to the disclosure of 6-methyl-2-oxo-4-thiophen-2-yl-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid (WO2004067008, Takeda chemical industries LTD, Japan, 2004) (224), a pyrimidine ester, as TGR5 agonist.

Another strategy was to research BAs enantiomers. Enantiomeric compounds are non-superimposable mirror images of one other. Complementary pairs of enantiomers have identical physical properties with the exception of their optical rotations, which are equal in magnitude and opposite in direction leading to different three-dimensional structures. A pair of enantiomers possessing a single different chiral centre, one (R), the other (S), could interact, or not, with a protein (or other molecule), in a unique manner because of their spatially distinct three-dimensional ligand binding pockets. This suggests that whenever a BA causes strong activation of a receptor, its enantiomer does not. This is true for LCA and CDCA whose enantiomers did not activate TGR5 (225).

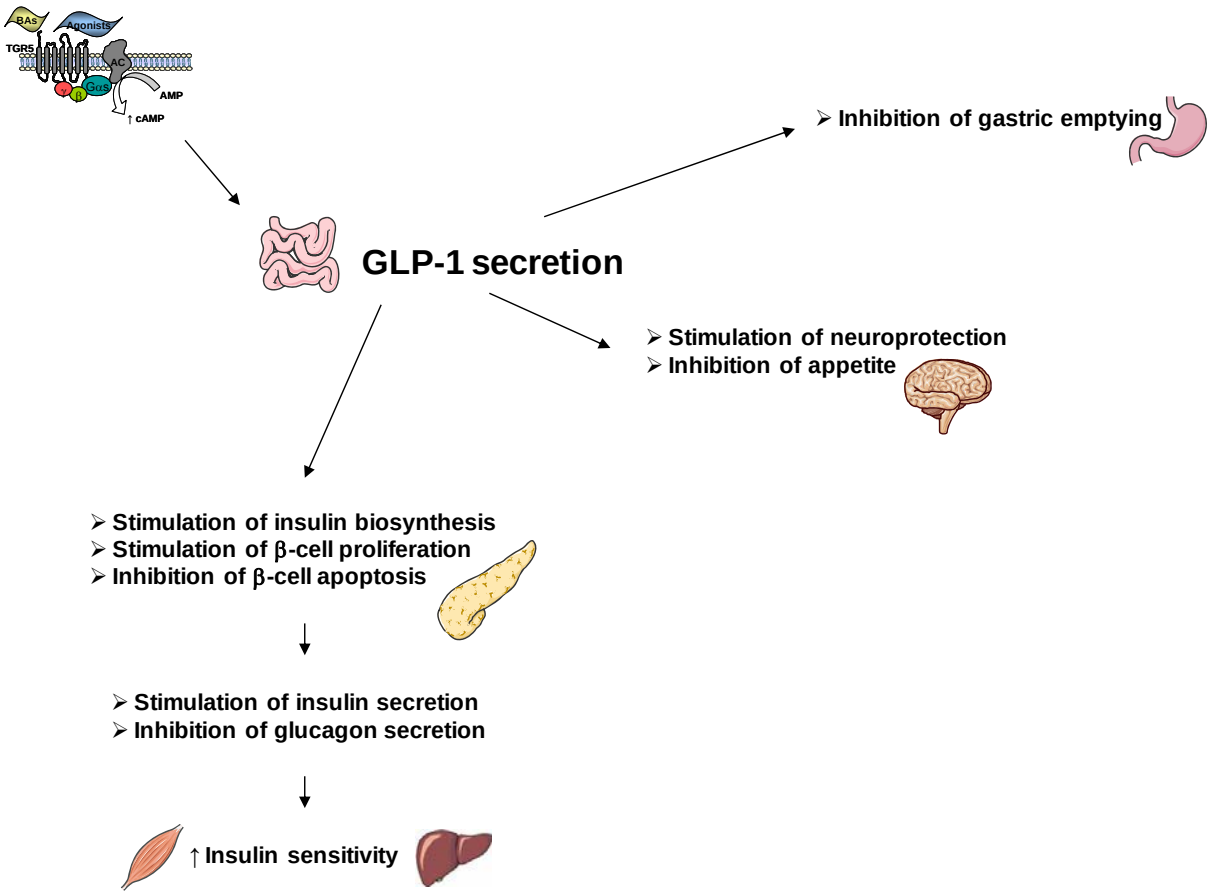


Figure 14: GLP-1 actions

b. Consequences on knowledge of binding pocket

Information yielded by two studies allows the prediction of some of the TGR5 binding site properties. The first, mentioned above, described a recognition method (linear discriminant analysis), providing a classification model of agonists. Furthermore, the screening of BAs, BA derivatives and some steroid hormones, permitted the disclosure of 7ξ-Me-LCA, 7a-F-LCA and CDC-Sul as some of the most potent and selective TGR5 agonists currently available (220). The second was an interesting 3D-QSAR study (226).

Combined, they permit the suggestion that the TGR5 binding site hosting BAs and derivatives comprises: a selectivity pocket lining the C-23(S) position of natural BAs (region A), a large polar site recognizing the acidic side chain of BAs (region B), a hydrophobic pocket hosting the C6 and C7 positions of the BAs steroid nucleus (region C), and a narrow hydrogen bond donor site binding the 3-hydroxyl group of BAs (region D) (Figure 13).

C. Biological effects on diabetic parameters

1. GLP-1

As explained earlier, GLP-1 is implicated in the control of glucose homeostasis by protecting beta cells and improving insulin sensitivity (161-164, 169, 170) (Figure 14). These two roles highlight the therapeutic potential of GLP-1 in type 2 diabetes treatment (168). This gastrointestinal hormone is synthesized in enteroendocrine cells and increases within minutes of eating until it is secreted. GLP-1 secretion is under the control of different mechanisms of neural and hormonal nature. Indeed, cholinergic muscarinic, β-adrenergic and peptidergic fibres enhance GLP-1 secretion, whereas α-adrenergic and somatostatinergic fibres restrain it. GIP stimulates this excretion, while somatostatin inhibits it locally (227).

Another regulatory pathway of GLP-1 release involves TGR5, since the major role of this GPCR in endocrine cells is to enhance GLP-1 excretion through a cAMP-dependent mechanism. This accords well with the fact that GLP-1 secretion depends on cAMP production (228).

In addition to the two new therapeutic strategies, incretin mimetics and DPP4 inhibitors, potentiation of incretin secretion by agonist activity on TGR5 could also be of particular interest in type 2 diabetes therapy.

2. D2

The type 2 iodothyronine deiodinase (D2) is a widely expressed enzyme which converts T4 into T3. Thyroid hormones influence heart rate, serum lipids, metabolic rate, body weight and multiple aspects of lipid, carbohydrate, protein and mineral metabolism (229). D2 expression is controlled by BAs-activated TGR5 which triggers the cAMP pathway to activate the D2 promoter and finally leads to increased T3 and heat production in BAT (199).

3. PGC-1α

The cofactor PGC-1α is an integrator of the molecular regulatory circuit involved in the transcriptional control of cellular energy metabolism, including mitochondrial biogenesis, hepatic neoglucogenesis and fatty acid beta-oxidation (42, 43). PGC-1α controls adaptative thermogenesis in adipose tissue and skeletal muscle by stimulating the generation of mitochondria and the oxidative phosphorylation (43, 230). In addition, PGC-1α protects against ROS-induced cell damage (231). To potentiate transcriptional activity, PGC-1α recruits proteins that display histone acetyltransferase (HAT) activity such as SRC-1 and CBP (cAMP response element (CRE) binding (CREB) protein) (42). The importance of PGC-1α in type 2 diabetes pathophysiology is testified by the fact that expression of genes involved in fatty acid oxidation and of PGC-1α is reduced in this disease (232).

PGC-1α is regulated by acetylation/deacetylation processes, involving GCN5 as acetyltransferase and the SIRT1 deacetylase (233, 234), and by cytokine-activated p38MAPK phosphorylation. As an example, SIRT-1 activation, by either resveratrol or SRT1720, improves mitochondrial function and protects against metabolic diseases through its activation of PGC-1α (235, 236).

BAs appear to enhance energy expenditure via a PGC-1α-dependent mechanism (199, 237). Despite the fact that PGC-1α action is regulated by cAMP/CREB/CBP and that TGR5 stimulates cAMP production, whether BAs-activated TGR5 regulate PGC-1α via the same signalling pathway remains unknown for the present.

I. Oleanolic acid as TGR5 agonist

A. Introduction

TGR5 is a potential target for a new therapy aimed to prevent and treat obesity or type 2 diabetes. Indeed, activation of this GPCR by BAs has positive effects on metabolic rate, body weight and multiple aspects of lipid and glucose metabolisms (229). Asides from its effect on TGR5, BAs activate other receptors such as FXR suggesting that finding novel TGR5 agonists, more selective and potent than BAs, is of particular interest. This would lead to a deeper understanding of TGR5 signalling, enable an assessment of the potential of this receptor as a therapeutic target for the treatment of diabetes, and allow the discovery of new antidiabetic agents with limited secondary effects. Since innumerable medicines are derived from the plant world, it was interesting to look for new TGR5 agonists in plants, as it has already been attempted successfully, as exemplified by the identification of the FXR agonist guggulsterone contained in *Commiphora wightii* (238). Our first task was to find new selective and original TGR5 agonists from vegetal sources.

B. Results and discussion

We first screened a library of plant extracts on TGR5 activity. Among the plants tested, the *Olea Europaea* (OE) leaves extract revealed a significant TGR5 agonistic activity (Manuscript 1). The hydroalcoholic extract of OE was purified and the TGR5 activity was identified as the triterpenoid oleanolic acid (OA). Interestingly, this activity was selective for TGR5 but not for FXR, demonstrating enhanced potential over BAs. These data were surprising because it has been reported that the antioxidant and hypoglycaemic effects of OE were carried by the iridoid oleuropein (123); but this potent antioxidant exhibited no any TGR5 activity. As ethnopharmacological studies showed that OE was used to treat type 2 diabetes in south-eastern Morocco (118), we wanted to examine to which extent OA extracted from OE exerted an hypoglycaemic effect. Treatment of high fat diet-fed mice with

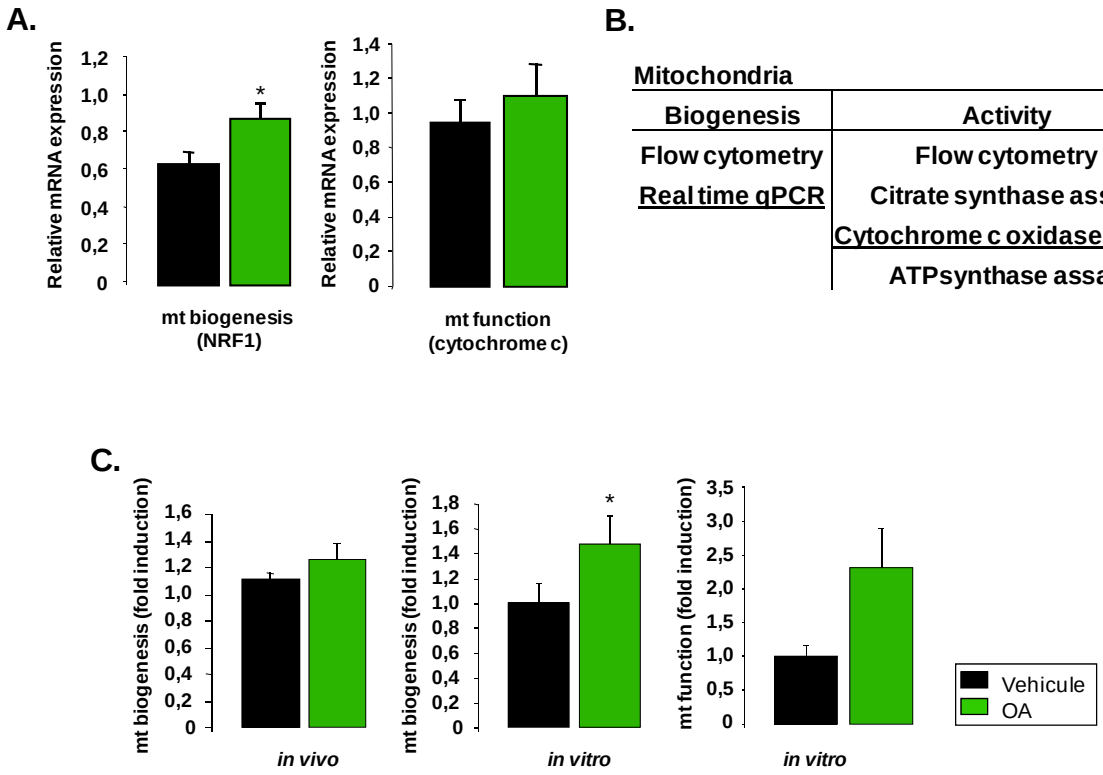


Figure 15: Mitochondrial effects of OA. A. Relative mRNA expression levels of genes related to mitochondrial biogenesis and function as measured by real time-quantitative PCR in the gastrocnemius muscle of HFD or HFD+OA mice (n=8 animals/group). Data represent mRNA level relative to 36B4 and are given as means ± SEM. * = p<0,05. **B.** Table of mitochondrial tests. Those underlined are used in the next experiments. **C.** Mitochondrial biogenesis as measured by Q-PCR *in vivo* in the gastrocnemius muscle of HFD (vehicle) or HFD+OA (OA) mice (n=8 animals/group) and *in vitro* in C2C12 myotubes treated 48h with vehicle or OA 10 µM. Mitochondrial function as measured by cytochrome c oxidase assay *in vitro* in C2C12 myotubes treated 3h with vehicule or OA 10 µM. * = p<0,05.

OA led to a lowering of serum glucose and insulin levels and to a reduced weight gain. Moreover, OA improved glucose tolerance. It should be noted that weight loss seems to be due to a decrease in adipose tissue.

This study demonstrated for the first time that TGR5 is selectively activated by triterpenoids such as OA and that OA-activated TGR5 exhibits antidiabetic effects, emphasizing the potential therapeutic role of this receptor and of triterpenoids, in general, to improve metabolic disorders.

As detailed earlier, BAs-activated TGR5 was shown, in our laboratory, to activate energy expenditure in muscle and BAT by stimulating gene expression involved in mitochondrial activity (199). Similarly, the new TGR5 agonist OA stimulates expression of genes implicated in mitochondrial biogenesis (NRF1) and in mitochondrial activity (cytochrome c) in muscle (Figure 15-A). We wanted to further evaluate these mitochondrial effects in a functional context. The effects on mitochondria are generally modest and it is difficult to find an assay which presents a reasonable working window to detect statistical changes. The increase in energy expenditure is characterized by the fact that more energy is produced and this can be achieved either by an increase in mitochondrial activity or by an increase in mitochondrial number (biogenesis) (Figure 15-B).

The biogenesis was evaluated by estimating the number of mitochondria per cell through the assessment of the number of the mitochondrial genome copies corrected to nuclear genome by quantitative PCR (qPCR). In parallel, we tried to use a flow cytometry method, with a mitochondrial membrane-inserted dye (MitoTracker^{TM} green FM, Invitrogen). In spite of the fact that this dye is membrane potential-independent, we met some technical problems and the molecular analysis by PCR revealed to be more reproducible. Concerning the mitochondrial activity, we chose to perform the cytochrome c oxidase assay (CYTOCOX1, Sigma-Aldrich): this enzyme complex represents the last of the electron transport chain and measuring its activity gives information on the total activity of the electron transport chain and

therefore oxidative phosphorylation. The citrate synthase and ATP synthase assays were also tested. The first represents the activity of TCA cycle while the activity of the second depends on the coupling between the electron transport chain and ATP production. Finally, we tried to measure mitochondrial activity by using a membrane potential-dependent dye in flow cytometry (MitoProbe^{TM} JC-1, Molecular Probes), but we met some technical problems and we therefore choosed to evaluate mitochondrial activity by using the cytochrome c oxidase assay.

The techniques chosen (mtDNA quantification and cytochome c oxidase assay) allowed us to show that mitochondrial biogenesis was increased in muscles from OA-treated mice (*in vivo*) and in OA-treated myotubes (*in vitro*). Moreover, OA improves mitochondrial activity in a muscle cell line. The mitochondrial tests developed here could be further improved for high-speed screening of potential compounds enhancing mitochondrial activity, serving as a basis for potential antidiabetic drugs.

II. SAR study around triterpenoids: discovery of RG239

A. Introduction

The triterpenoids belong to the broad family of terpenes classified in Table 2. In nature, they exert a broad variety of roles, notably in ecology since they ensure communication between organisms, such as antagonistic and mutualistic interactions (anti-bacteria, anti-insect, anti-fungi). They are currently studied for the properties which permit them to undertake roles ranging from antioxidants (239), antimicrobial (240), anti-tumoral (241), anti-inflammatory (242) and detergent (243) activities.

Among the terpenes, carotenoids and triterpenes are well known for their antioxidant properties. Indeed, numerous antioxidant effects of plants were shown to be exhibited by

triterpenes, and more particularly, to improve metabolic disorders. For example, the gymnosides and gymnemic acids (dymnosied-W1 and W2) extracted from *Gymnema Sylvestre* act on intestinal glucose absorption to finally reduce blood sugar concentration (119, 244). The triterpenoids oleanolic acid, ursolic acid, erithrodiol extracted from *Olea Europaea* leaves exhibit antioxidant properties (245-251). In addition, oleanolic and ursolic acids have cardiotonic and antidysrhythmic effects (250).

The properties that TGR5 agonists must exhibit were defined by the first structure-activity relationship (SAR) study of a potential TGR5 agonist, based on natural occurring BAs, semisynthetic BA derivatives and some steroid hormones (220). This study indicated that the binding pocket of TGR5 is endowed with narrow hydrogen binding donor sites which recognizes the 3-hydroxyl group of BAs, a hydrophobic pocket lining to C_6 and C_7 position of BAs and a large and neutrally formal charged pocket that anchors the acidic side chain. Furthermore, chemical elaboration around the steroid nucleus of BAs revealed that methylation at the C23-(*S*) position of natural BAs confers a marked selectivity to TGR5 while the 6α-alkyl substitution increases the potency (223). These informations are useful for the design of novel selective and potent TGR5 agonists.

B. Results and discussion

We observed previously that the triterpenoid OA is a TGR5 agonist. This is not surprising since triterpenoid and BAs exhibit similar hydrophobic structures and moreover, Sato *et al.* demonstrated the requirement of a hydrophobic skeleton for a relevant TGR5 agonist (220).

Based on the discovery that the triterpenoid OA was a selective TGR5 agonist, we screened a collection of naturally occuring triterpenoids on TGR5 activity in order to generate more potent TGR5 agonists. The results revealed that, in addition to OA, ursolic acid and betulinic acid are also selective TGR5 agonists (Manuscript 2). The comparison between natural triterpenoids led us to consider the importance of the hydroxyl (or either hydrogen donor-acceptor) at C-3 and of the carboxylic acid at C-17. Additional hydroxyl groups may

A.

Body weight parameters		
	High fat diet	
	Vehicle	RG239
Body weight (g)		
J0	32,3 ± 1,3	35,5 ± 1,3
J14	33,4 ± 1,6	35,5 ± 1,3
Epididymal WAT (%BW)	4,1 ± 0,52	5,1 ± 0,56

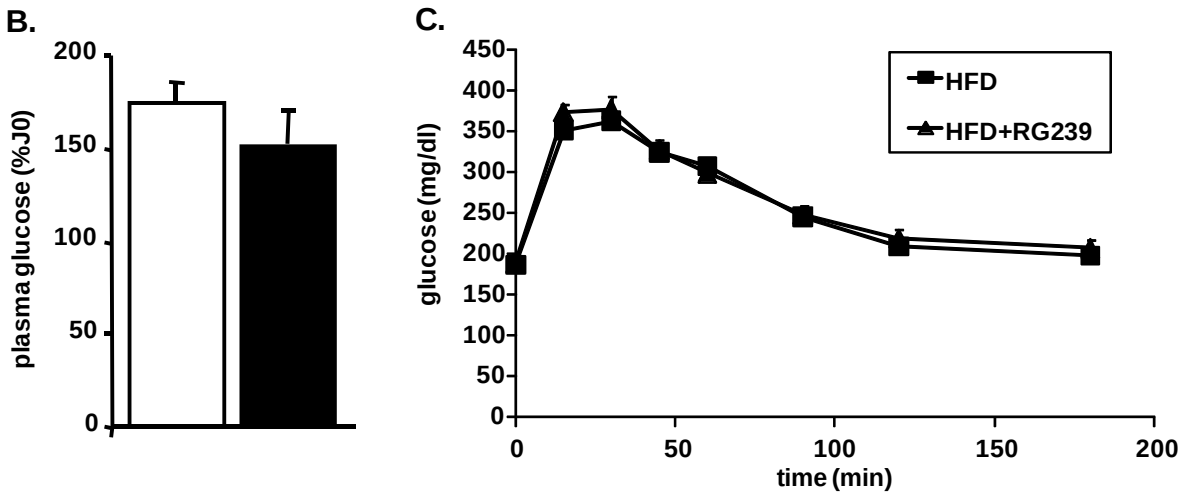


Figure 16: *in vivo* effects of RG239. A. Body weight parameters. **B.** Plasma glucose levels of mice fed for 2 weeks whith high fat diet (white bars) or high fat diet (black bars) supplemented with 30 mg/kg RG239. **C.** Intraperitoneal glucose tolerance test performed after 2 weeks of RG239 treatment at 30 mg/kg. Values are mean ± SEM, n=10 animals/group.

disrupt hydrophobic interactions or generate unfavourable steric constraints. These considerations are in agreement with Sato *et al.* Who reported a narrow pocket with hydrogen bond acceptor groups in this section of the TGR5 binding site and emphasized the similar location of BAs and triterpenoids within the receptor (220).

Betulinic acid is the most potent natural triterpenoid acting as TGR5 agonist. The SAR study based on this lead compound drove us to determine that structural variations around C-3 position (maintaining the hydrogen donor character), could improve the potency of the compound and particularly by alkylation. The fact that this position maintains its hydrogen donor properties is in agreement with Macchiarulo et al. who predicted by molecular field analysis and 3D-quantitative, the structure of a narrow bond donor at the C-3 position (226). Moreover, for a good TGR5 binding, the chiral configuration of C-3 needs to be (*R*). Following the same idea, modifications to the carboxylic acid at the C-17 position led us to suggest that the hydrogen donor character of COOH was essential supposing binding to a small polar site in the TGR5 binding site. This is in contradiction with the hypothesis of Sato *et al.* who proposed a large binding pocket in this region of the receptor binding to BAs, probably explaining the selectivity of triterpenoids on TGR5 over FXR, unlike BAs. Interestingly, simple modifications of the alkene did not change TGR5 activity. The RG239 compound (18 dia 2) meets all of these property requirements and is a better TGR5 agonist *in vitro* than the other triterpenoids tested. EC_{50} (OA) is in µM range while EC_{50} (RG239) is in nM range.

We demonstrated that OA exhibits anti-hyperglycaemic effects and since RG239 is a more potent TGR5 agonist *in vitro*, we evaluated its biological effects, in particular on metabolic parameters. RG239 showed no significant activity since it does not decrease glucose or lipid levels, neither weight nor fat distribution (Figure 16), whereas OA did. This lack of action *in vivo* could be explained by a weak bioavailability of this hydrophobic molecule. Indeed, the physicochemical properties of RG239 do not respect one of the four

predicting Lipinsky rules: the partition coefficient logP is out of range, leading to high hydrophobicity and most likely bioavailability problems.

Chemical modifications could help to improve the bioavalability of this kind of molecule. For example, a possible strategy is to modify the alkene by adding solubilising agents. Indeed, the alkene moiety seems not to be involved in ligand-receptor interactions since its modifications did not affect TGR5 activity. Triterpenoids are known to be poor water soluble molecules, because of their hydrophobic structure, however recently, considering their therapeutic potential, some strategies have been developed to improve their dissolution and oral bioavailability, permitting their therapeutic use (252).

Here, we discovered a new TGR5 agonist, the most potent amongst the triterpenoids and derivatives. Yet despite this, a biological effect *in vivo* could not be established. The molecule remains interesting to use *in vitro*, as a tool compound to elucidate the TGR5 pharmacology and thus assess the potential therapeutic role of TGR5.

III. RG239 enhances mitochondrial functions

A. Introduction

TGR5 activated by BAs enhances energy expenditure in muscle and adipose tissue by stimulating mitochondria, as demonstrated by our laboratory (199) and stimulates GLP-1 secretion in enteroendocrine cells (219). Alternatively, we showed that the natural triterpenoid OA enhances energy expenditure by stimulating mitochondria in muscle and a more potent and selective TGR5 agonist RG239 was synthesized. The question now is to determine how TGR5 agonists triterpenoids exert positive effects on energy expenditure.

B. Results and discussion

To describe the TGR5 signalling pathways, we used the triterpenoid RG239 and performed a pharmacological study in different cell lines. RG239 enhances mitochondrial activity and biogenesis in muscle, adipose tissue and intestine cell lines (Manuscript 2 and Manuscript 3). More precisely, this process occurs in a TGR5-dependent manner in muscle and intestine cell lines, implicating adenylate cyclase and cAMP-dependent protein kinase activation. Furthermore, in enteroendocrine cell line, RG239-activated TGR5 results in GLP-1 secretion, arguing in favour of triterpenoid as GLP-1 secretagogues and thus as antidiabetic agent. Indeed, the enteric hormone GLP-1 is known to stimulate insulin secretion and improve glycaemia in type 2 diabetes. Altogether, these data allow us to establish triterpenoids as antidiabetic agents and TGR5 as an antidiabetic target. Particularly, its dual actions on mitochondria and GLP-1 could argue for a dual therapeutic use of TGR5: both for prevention (mitochondria) and treatment (GLP-1 secretion). However, some points need to be further discussed: how, for example, does PKA activation lead to mitochondria activation in the context of triterpenoid-activated TGR5-stimulated mitochondria? Is there a link between GLP-1 secretion and mitochondria activation? Which are the consequences of the activation of mitochondria in the different tissues, and in terms of oxidative stress?

1. How does PKA activation lead to mitochondria activation?

Watanabe *et al*. described that BAs-activated TGR5 increases D2 and PGC1-α expression, leading to mitochondria activation in BAT and muscle. Indeed, this transmembrane receptor is coupled to a Gs protein, and its activation induces cAMP levels. This second messenger regulates numerous cellular pathways by activating enzymes such as the protein kinase cAMP-dependent (PKA) or by activating the functional cAMP-responsive element (CRE) binding protein (CREB). The transcription factor P-CREB could transactivate its target genes by binding to CRE contained in their promoter. In metabolic tissues such as liver, BAT and muscle, CRE was identified in D2 and PGC-1α promoters, regulating their expression (253-255). The cofactor PGC1-α is a key regulator of energy

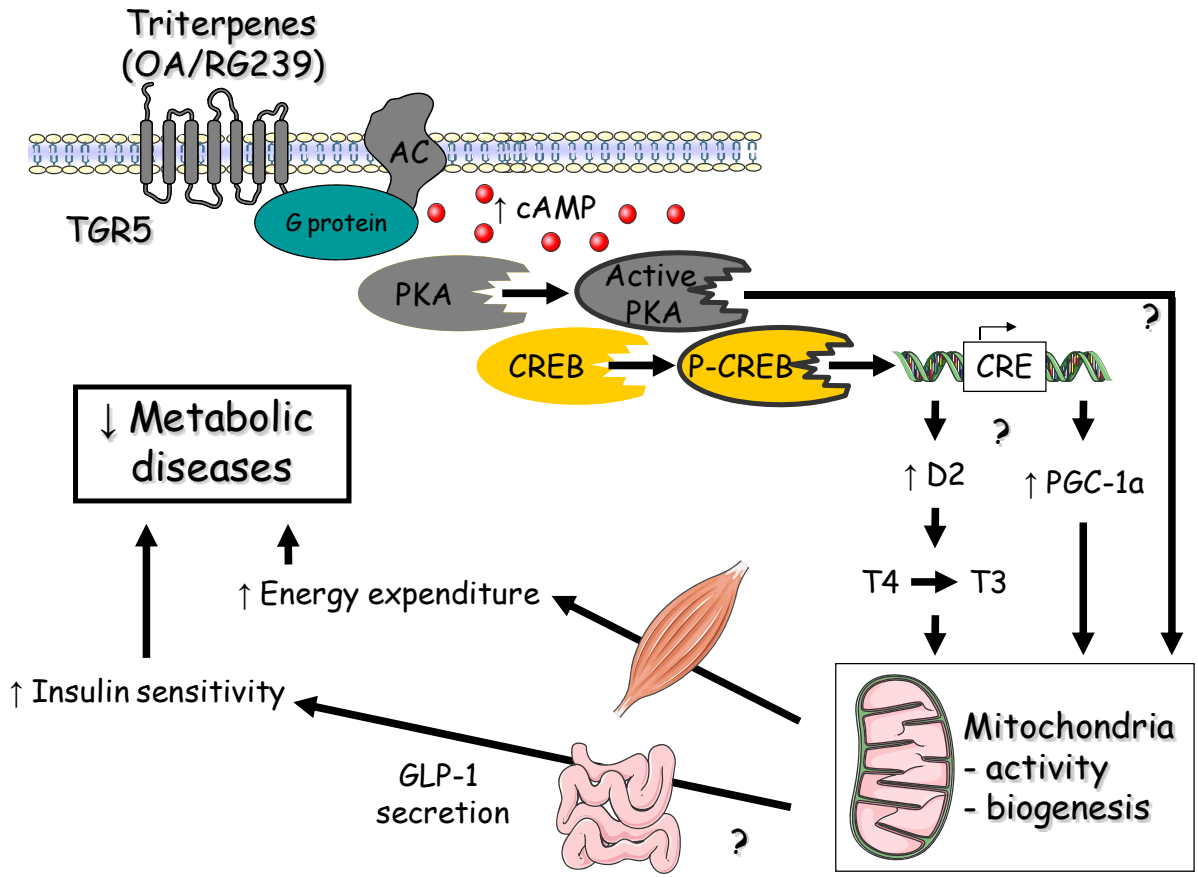


Figure 17: Triterpenoid-activated TGR5 signalling pathway

homeostasis, inducing and coordinating gene expression that stimulates mitochondrial pathways, from β-oxidation of fatty acids to oxidative phosphorylation, to uncoupling in BAT and muscle. PGC1-α plays a key role in energy expenditure and is regulated by different pathways. Among them, the deacetylase SIRT1 whose activation induces PGC1-α, enhances fat oxidation and thus protect against metabolic disorders as demonstrated in our laboratory (Annexe 2).

On the other hand, D2 converts inactive thyroxine (T_4) into active 3,5,3'-tri-iodothyronine (T_3)(253), emphasizing its major role in energy homeostasis. Indeed, activation of the thyroid receptor increases mitochondrial oxidative phosphorylation in muscle and uncoupling in BAT, resulting in enhanced energy expenditure, i.e. body fat loss and basal metabolic rate (199, 229, 255-257). Furthermore, a functionally significant polymorphism in D2 gene is strongly associated with decreased whole-body glucose disposal rate, which is determined by muscle glucose uptake. In patients with type 2 diabetes, this polymorphism is associated with increased insulin resistance (258).

Altogether, our data and the data in the literature allow us to hypothesize the existence of an intracellular triterpene-stimulated TGR5 signalling, schematized in Figure 17. Our findings demonstrated that the triterpenoid derivative RG239 activates selectively TGR5, leading to adenylate cylclase activation, subsequent cAMP production, PKA activation and finally, mitochondria activation in both muscle and intestine cell lines. These results seem rational since evidence has emerged that cAMP-dependent phosphorylation of mitochondrial enzymes playing a role in the electron transport chain regulation (259, 260). Moreover, this is supported by a recent work, which depicts the existence of a mitochondrial AC-PKA pathway of cAMP production, bypassing the fact that the inner mitochondrial membrane is impermeable to cAMP (261).

These results corroborate the study of Watanabe *et al*., which demonstrated the implication of TGR5-stimulated cAMP production in mitochondria activation (199). Based on

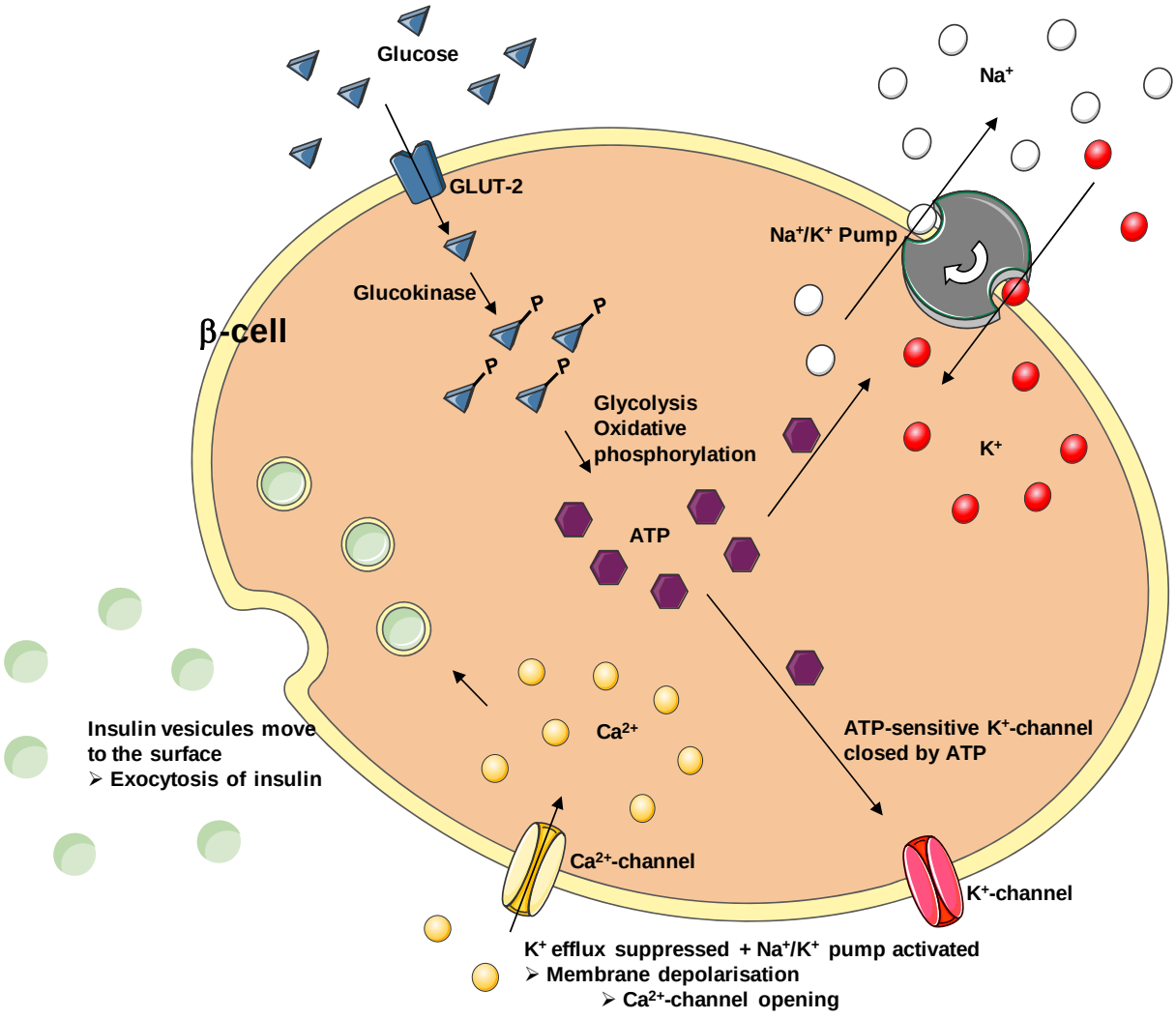


Figure 5: Glucose-stimulated insulin secretion

these results, we can hypothesize that triterpenoid-activated TGR5 activates CREB, leading to increased PGC-1α and D2 expression and subsequent mitochondrial activation. This hypothesis must be further validated. It is plausible that such a mechanism is active in muscle where PGC-1α and D2 are expressed, but not in intestine, since no link between PGC-1α and intestinal cells has been reported until now. Concerning D2, in spite of its presence already shown in hagfish intestine, no proof exits for its expression in mouse or human intestine (199, 262). The pathway by which mitochondria are activated in intestine could be different than that proposed in muscle, but this question remains to be answered.

2. Are GLP-1 secretion and mitochondria activation linked?

The putative enhancement of GLP-1 secretion by TGR5-activated mitochondria should be elucidated. However, this hypothesis is based on analogy with the process established in pancreatic β cells to secrete insulin (Figure 5). Normally, elevated cytosolic ATP production results in plasma membrane K_{ATP} channels closure, cellular depolarization and subsequent voltage-gated Ca^{2+} influx, which leads to insulin granule exocytosis (263, 264). This links nutrient metabolism (glucose metabolism results in ATP production) to insulin secretion. For example, the A3243G mutation in mitochondrial DNA affects insulin secretion by attenuation of ATP production upon aging (40). As enteroendocrine STC-1 are secretary cells as well, we can suppose that RG239-activated TGR5 promotes GLP-1 exocytosis by stimulating mitochondria. Further experiments however are needed to fully explore this eventuality.

3. What are the consequences of mitochondria activation?

The RG239-activated TGR5-stimulation of mitochondria in muscle and in adipose tissue could result in enhanced energy expenditure i.e. body weight loss, as already shown with BAs-activated TGR5 (199). In intestine cells, we supposed the involvement of mitochondrial activation in GLP-1 secretion, a major feature of these cells. Altogether, both effects could

result in improving energy homeostasis, consolidate the metabolic importance of TGR5 and underline the potential role of triterpenoids as metabolic homeostasis stabilisers.

However, attention is needed because activation of mitochondria leads simultaneously to enhanced production of ROS. Caution has to be exerted as the therapeutic target of TGR5 at this point is not clear and further investigation of the oxidative stress generated by TGR5 activation has to be performed. However, we can expect that the consequences to oxidative stress are not dramatic, since BAs-activated TGR5 enhances PGC-1α and UCP expression, which are known to stimulate endogenous antioxidative systems (265). Moreover, numerous triterpenoids are known for their antioxidant properties (245-251) and RG239, as triterpenoid, has to be tested for this.

TGR5

We described here for the first time that triterpenoids, the natural OA and the hemisynthetic RG239, are TGR5 agonists, in addition to the well-known BAs and derivatives. From a biological point of view, OA improves diabetic parameters in mice as it induces weight loss due to reduced fat, is antihyperglycemic and enhances insulin sensitivity. This action occurs in a mitochondria-dependent manner in muscle. RG239 furthermore stimulates mitochondria in adipose, muscle and intestine cell lines and enhances GLP-1 secretion in intestine cells.

These results, in agreement with the literature, emphasize the dual action of TGR5, on mitochondria and on GLP-1 secretion, and position this receptor as a promising therapeutic target. Indeed, mitochondria play a central role in energy expenditure and its dysfunctions are involved in the early stages of obesity and type 2 diabetes. Moreover, activating energy expenditure i.e. mitochondria could improve obesity and metabolic parameters in diabetic parameters, thanks to the burning of stored fats, thus avoiding glucolipotoxicity and subsequent worsening of these diseases. In summary, stimulating mitochondrial action of TGR5 is an interesting preventive and therapeutic strategy for these metabolic diseases.

Secondly, activated TGR5 stimulates GLP-1 secretion. We mentionned earlier that developing incretin secretagogues is a new, interesting therapeutic strategy in the context of obesity and type 2 diabetes. This will improve the amount of GLP-1 available and through effects on insulin secretion, will sustain its positive actions on glucose metabolism. This second biological effect of TGR5 activation by BAs and triterpenoids further underscores the value of this receptor as a therapeutic target to treat type 2 diabetes.

Furthermore, the therapeutic interest of TGR5 is supported by the fact that TGR5 agonists research projects are in progress in pharmaceutical companies. For example, Intercept pharmaceutical (www.**interceptpharma**.com) performed studies and has identified a novel

TGR5 agonist which is currently in preclinical testing, to treat metabolic disorders. Moreover, Exelixis (www.**exelixis**.com) company's current drug development programs are focused on modulating bile acid receptors with an initial focus on the improvement of metabolic and hepatic function in chronic liver diseases.

Triterpenoids

In the present work, we described for the first time triterpenoids as a new class of selective TGR5 agonists, allowing us to consider triterpenoids as interesting pharmaceutical agents. Indeed, triterpenoids-activated TGR5 mediates the same effects that BAs-activated TGR5 on mitochondria and GLP-1 secretion.

First we described OA, a natural triterpenoid extracted from OE leaves. Triterpenoids are ubiquitous secondary metabolites of plants, apparently used safely. Our results indicate that these molecules could be used directly as drugs, but the problems of bioavailability *in vivo* due to their structure and the inherent problem to cross the physico-chemical barriers have to be considered. For example, despite a better activity *in vitro* compared to OA, RG239 did not improve diabetic parameters *in vivo*, unlike OA. However, adding functional groups on these molecules could ameliorate their Lipinsky parameters. We described earlier that chemical modifications around the alkene of RG239 may enhance solubility without consequences on the binding properties of the molecule but pending this improvement in structure, preparation enriched with plants containing relevant triterpenoids could be useful in nutraceutical prevention of metabolic disorders.

Alternatively, triterpenoids exhibit numerous biological effects: antioxidant properties (245-251), cardiotonic and antidysrhythmic effects (250), etc. Caution has to be exerted before therapeutic use: complementary tests have to be performed to evaluate the potentiality of RG239 or other more bioavailable molecules on known adverse activities. These activities could reinforce the benefits of these molecules in context of metabolic diseases but could also lead to unwanted side effects. For example, the triterpenoid betulinic acid presents pro-

apoptotic properties (266), inferring antitumoral characteristics. This is not necessarily interesting for metabolic diseases since some cellular dysfunction (adipocyte, beta cell) are at the origin of these diseases and could be worsened by a molecule with apoptotic properties.

To conclude, this work presented for the first time triterpenoids as novel TGR5 agonists able to enhance energy expenditure and mitochondria in muscle and BAT, while stimulating GLP-1 secretion in intestine. These data position TGR5 as a promising therapeutic target and triterpenoids as potential agents to prevent (by mitochondira activation) and treat (by GLP-1 secretion) obesity and type 2 diabetes. Future work will have to focus on physico-chemical properties of triterpenoids to avoid the classical problem of bioavailability encountered with these promising molecules.

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Anti-hyperglycemic activity of a TGR5 agonist isolated from *Olea europaea*

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Abstract

Olive tree (*Olea europeaea*) leaves are well known for their effect on metabolism in particular as a traditional anti-diabetic and anti-hypertensive herbal drug. These properties are until now only attributed to oleuropein, the major secoiridoid of olive leaves. Here we describe the isolation and the identification of another constituent implicated in the anti-diabetic effect of this plant, i.e. oleanolic acid. We show that this triterpene is an agonist for TGR5, a member of G-protein coupled receptor activated by bile acids and which mediates some of their various cellular and physiological effect. Oleanolic acid lowers serum glucose and insulin levels in mice fed with a high fat diet and it enhances glucose tolerance. Our data suggest that both oleuropein and oleanolic acid are involved in the anti-diabetic effect of olive leaves and further emphasize the potential role of TGR5 agonists to improve metabolic disorders.

Keywords: Triterpenoid; TGR5; Insulin sensitization; Metabolic diseases

The incidence of metabolic diseases and in particular of type 2 diabetes is growing rapidly worldwide [1]. Despite the number of therapies available, there remains a large unmet medical need for the treatment of those diseases. Identification of new therapies for type 2 diabetes retains the attention of the scientific community, especially discovering ways to act early in the development of diabetes, particularly on the development of insulin resistance since it almost always precedes type 2 diabetes onset. One possibility to combat insulin resistance arises from the recent observation that it is invariably associated with mitochondrial dysfunction in skeletal muscle [2] suggesting that a reduction in mitochondrial activity is one of the early hallmarks of type 2 diabetes [2,3]. Identification of new biological targets that could interfere with mitochondrial function could hence be of great therapeutical relevance for the treatment of type 2 diabetes.

A recently identified member of the G-protein coupled receptor (GPCR) family i.e. TGR5 [4] could be such a drugable diabetes target. TGR5 is identified as the first cell surface receptor activated by bile acids and this receptor is reported to mediate some of the endocrine functions of bile acids [4]. This observation is in line with the fact that besides their role in dietary lipid absorption and cholesterol homeostasis, bile acids are also emerging as important and metabolic signalling molecules [for review, 5]. Most relevant in that context is that bile acids have been shown to increase energy expenditure in part through activation of mitochondrial function, hence preventing the development of obesity and insulin resistance in mice fed a high fat diet. This metabolic effect was shown to be mediated by TGR5

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whose activation results in an increased intracellular activation of thyroid hormone, subsequently leading to an increase in energy expenditure [6]. Based on these data, it is clear that TGR5 has the potential of becoming a good candidate for the development of new anti-diabetic drugs to be used in early stages of the treatment of metabolic diseases.

Numerous plants have been reported to possess anti-hyperglycemic or anti-diabetic activity [for review 7]. Indeed their use as complementary and alternative medicine (CAM) has increased among the general public in the past years. It is in fact estimated that about one third of the US diabetic population actually uses CAM to treat their condition [8]. Plants are particularly interesting since they not only can be used as CAM to prevent metabolic diseases, but also serve as an interesting source of drug candidates for the pharmaceutical industrial research. Noteworthy, about 45% of the drugs on the market are from vegetal origin [9]. Different mechanisms are used by plants to reduce blood sugar levels. In fact, plants which exhibit properties similar to those of known classes of anti-diabetic drugs such as sulfonylureas, inhibitor of hepatic neoglucogenesis, like metformin, or inhibitors of α-glucosidase, amongst others, have been identified [7,10]. It is therefore conceivable that plants which possess TGR5 agonist or antagonist activities can be identified, facilitating a better understanding of the biology of this receptor. The goals of the present study were hence: (1) to screen plants in order to identify TGR5 agonists and (2) to test the potential of a TGR5 agonists identified as such to act as anti-hyperglycemic agents.

Materials and methods

Materials. Oleanolic acid was purchased from Extrasynthèse (Genay, France). Oleuropein, Lithocholic acid, and Chenodeoxycholic acid were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France).

Plant extraction. For ethanol extraction, 10 g of the air-dried powdered olive leaves (*Olea europaea* L. Oleaceae, PhytoEst, France) were exhaustively extracted with 80% aqueous ethanol by maceration at room temperature for 3 h. The mixture was then filtered on a suction pump and the filtrate evaporated under reduced pressure. For dichloromethane and methanol extraction, the powdered plant material (10–20 g) was successively extracted with cyclohexane (100 mL), dichloromethane (100 mL), and methanol (100 mL), using an automatic soxhlet apparatus (2055 Aventi Soxtex, Foss Tecator). This procedure generates three types of extracts which concentrate respectively the very lipophilic, the nonpolar and the polar constituents of the plant. The solvent was evaporated under reduced pressure and the corresponding residues were weighted and then stored in the freeze until use.

HPLC analysis. For the analytical profiles of each extract, a methanolic solution of the residue (5 mg/mL) was subjected to analytical HPLC (Varian Pro Star) coupled with a DAD detector (Varian), using a reversed phase column (Nucleodur 100-10-C18, 250/4.6, Macherey-Nagel). A mixture of acetonitrile (CH_3CN) and acidified water (0.1% trifluoroacetic acid) was used for elution. In analytical conditions, the flow rate was 1 mL/min with the following gradient: H_2O/CH_3CN (20:80) for 30 min, CH_3CN 100% for 5 min, and H_2O/CH_3CN (20:80) for 5 min. For LC–MS (Agilent 1200SL), the analyses were performed with APCI coupled with an electrospray ionization (ESI) interface in negative mode.

NMR analysis. A DPX300 NMR spectrometer operating at 300 MHz for ^1H was used for NMR experiments. Chemicals shifts are expressed in δ (parts per million) referring to the solvent peak 7.58 for pyridine-d5 C5D5N.

TGR5 luciferase assay. Chinese hamster ovary (CHO) cells were obtained from ATCC (Manassas, VA) and were maintained in minimum essential medium alpha (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 μM nonessential amino acids (NEAA), 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. For the TGR5 assay, a stable cell line was obtained by transfection of CHO cells with 3.8 μg of TGR5 expression plasmid (pCMVSPORT6/TGR5), 3.8 μg of CRE-driven luciferase reporter plasmid (pCRE-Luc), and 0.4 μg of neomycin-resistant gene expression plasmid (pcDNA3.1(+)) using Lipofectamine 2000 (Invitrogen, Cergy Pontoise, France). The transfected cells were selected with 400 μg/mL G418 sulfate and single clones were grown in 96-well plate, independently. TGR5-expressing CHO cells were treated with 10 μM lithocholic acid (LCA) or plant extracts, followed by luciferase assays [11]. Luminescence was determined with CentroXS3 LB960 (Berthold Technologies, Bad Wildbad, Germany).

FXR luciferase assay. To evaluate FXR activity of compounds, COS1 (ATCC) cells were transfected with 25 ng of hFXR expression plasmid (pCMX-hFXR), 25 ng of mouse (m) retinoid X receptor α (RXRα) expression plasmid (pCMX-mRXRα), 50 ng of reporter plasmid (pEcREx7-Luc) and 50 ng of pCMVβ as internal control in each well, using the Lipofectamine 2000 reagent. About 18 h after transfections, cells were incubated for 24 h with different concentrations of each compound in fresh MEM (or DMEM). After this treatment, the cells were lyzed and normalized luciferase activity was determined [11].

Animals and diet. Six week old male C57BL/6 J mice were purchased from Charles River. The chow diet (SAFE, DO4) and the high fat diet (60% kcal Fat, D12492) were purchased from Research Diets (New Brunswick, NJ). The mice were housed by 4 or 5 under controlled temperature and 12 h light–dark cycle and had free access to food and water. Animals were kept for 10 weeks either on chow diet ($n = 5$) or on high fat diet ($n = 16$). After 10 weeks on high fat, the animals were divided in two groups of 8 animals: one group was kept on high fat diet only, the other received the same food supplemented with oleanolic acid at a 100 mg/kg/day dose (food intake was measured throughout the study and the amount of drug was adjusted to keep the dosing stable). Body weight and food intake was determined every other day. The experiment was carried out according to the ethical guidelines.

Glucose tolerance test. Glucose tolerance test was performed on animals that were fasted 10 h. Blood glucose levels were monitored using a handheld glucometer (Maxi Kit Glucometer 4, Bayer Diagnostic). For the test, a sterile solution containing 2 g of glucose/kg body weight was injected intraperitoneally. Blood glucose levels were sampled from tail vein blood at 0, 15, 30, 45, 60, 90, 120, 150, and 180 min. Fasting blood glucose and insulin levels were measured after 7 days of treatment. Mice were deprived of food for 10 h and the blood levels were quantified with Glucose RTU (BioMérieux Inc.) for glucose and by ELISA (Mercodia, Uppsala, Sweden) for insulin.

Results and discussion

A triterpene i.e. oleanolic acid contained in olive leaves is a TGR5 agonist

In an effort to isolate new agonists for TGR5 that are structurally different from bile acids, a library of plant extracts was screened for activity on the CHO-TGR5 stable TGR5 reporter cell line. Numerous total extracts (ethanolic extracts) were tested and among them an extract obtained from *O. europaea* leaves showed an activation of TGR5 similar to the one observed with the positive control lithocholic acid (LCA, Fig. 1A). The isolation of the active

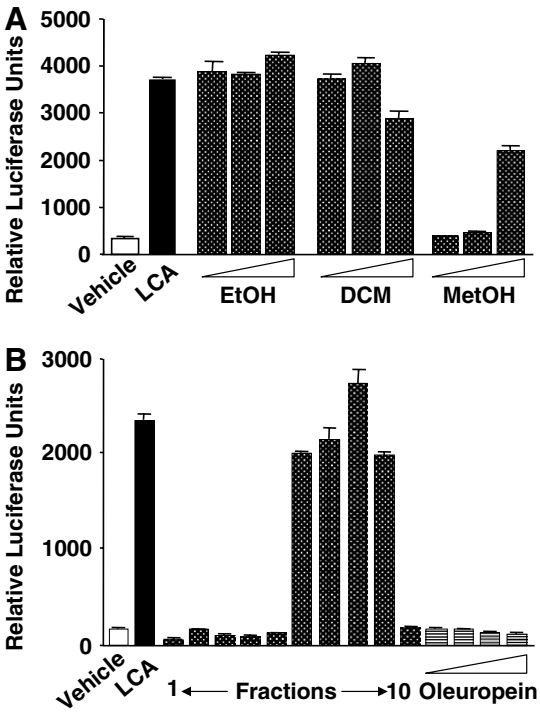

Fig. 1. (A) *Olea europaea* leaf extracts induces TGR5 activity. CHO cells were incubated for 5 h with increasing doses (5, 15, and 50 μg/mL in DMSO) of various extracts and luciferase activity measured thereafter. Ethanol (EtOH), dichloromethane (DCM), methanol (MetOH) extracts. (B) CHO cells were incubated for 5 h with 50 μg/mL of each of the 10 *O. europaea* fractions or with oleuropein at doses of 1.5, 5, 15, and 50 μg/mL, and luciferase activity measured thereafter.

component was then pursued by testing extracts enriched either in hydrophobic molecules (DCM) or hydrophilic molecules (MeOH). Data in Fig. 1A show that most of the activity is present in the hydrophobic extract. The hydrophobic fraction of the extract of *O. europaea* was then further fractioned by preparative RP-HPLC. The DCM extract was hence subjected to silica gel chromatography and 10 fractions numbered 1–10 were obtained and tested in the TGR5 luciferase assay. As shown in Fig. 1B, activity was present only in fraction 6–9. Interestingly, when oleuropein, a well characterized active component of olive leaves [12], was tested at doses ranging from 1.5 to 50 μg/mL, no agonist effect on TGR5 was observed (Fig. 1B). These data indicate that the active molecule contained in DCM *Olea* extract was distinct from oleuropein.

Olea europaea has been known for long to improve metabolic disorders and to carry anti-diabetic properties [13,14]. In vivo, olive leaves extracts and oleuropein are known to act on diabetes by at least two mechanisms: increased peripheral glucose utilization and improved glucose stimulated insulin release [12]. In addition, part of the effects of oleuropein on diabetes and its complications derives from its antioxidant activity [15]. According to the same authors, this effect on oxidative stress may contribute to the increased glucose utilization observed after treatment with oleuropein [15]. Our hypothesis was therefore that there was another active constituent contained in olive leaves which contributes to its anti-diabetic effect. We therefore attempted to identify the active molecule using a TGR5 luciferase assay. Fraction 6, 7, 8, and 9 were subjected to analytical HPLC. Fraction 7 exhibited a high degree of purity and indeed the UV detection showed only one major peak with a retention time at 21.9 min (Fig. 2A). Fraction 7 was hence subjected to LC/MS analysis which identified the molecular weight of the molecule at 455.3 g/mol (Fig. 2B). The analysis was done in the negative scan mode which means that the molecule lacks one hydrogen proton. The real molecular weight of the major detected peak was therefore estimated around 456–457 g/mol. The identification was completed by analysis of fraction 7 by NMR and the obtained spectrum was characteristic of a triterpenoid molecule (Fig. 2C). By comparison of the HPLC, LC/MS, and NMR analytical data with triterpenes already described in olive leaves, we postulated the presence of oleanolic acid (Fig. 2C). Its identity was confirmed by performing HPLC co-injection with a commercial standard (Extrasynthèse, Genay, France) and comparison of the NMR spectrum since the oleanolic acid spectrum was known [16]. In a final step, agonist activity on TGR5 for oleanolic acid was confirmed: the EC_{50} obtained for oleanolic acid in the human TGR5 luciferase assay was 1.42 μM (Fig. 2D), which was comparable to 0.89 μM, the EC_{50} for the potent natural TGR5 ligand LCA.

Biologically, bile acids act through at least two receptors, TGR5 and the nuclear receptor Farnesoid X Receptor (FXR) whose activation is responsible for many of the known effects of bile acids on metabolism [5]. To rule out a potential implication of the nuclear bile acid receptor in explaining the biological activity of oleanolic acid, we next tested whether oleanolic acid activates FXR in a FXR-dependent luciferase reporter assay. Interestingly, oleanolic acid did not activate FXR (Fig. 2E) which indicates that this triterpene is a selective TGR5 agonist, clearly distinguishing it from bile acids, which activate both TGR5 and FXR.

The TGR5 agonist oleanolic acid promotes insulin sensitization in mice fed a high fat diet

Oleanolic acid belongs to the oleanane-type triterpenoids which have been extensively studied in the oncology field because of their anti-tumoral properties [for review, 17]. No effects of oleanolic acid on diabetes have been reported, although this triterpene has been shown to inhibit, in enzymatic studies, the activity of α-glucosidase, an enzyme involved in intestinal glucose absorption [18]. Since oleanolic acid has been successfully administrated to rodents [19], we decided to evaluate the effect of oleanolic acid in a rodent model of metabolic syndrome, the high fat fed mouse. C57BL/6J mice were fed with either chow or a high fat diet for 10 weeks prior to the start of the treatment. During this period, the high fat diet mice exhibited a

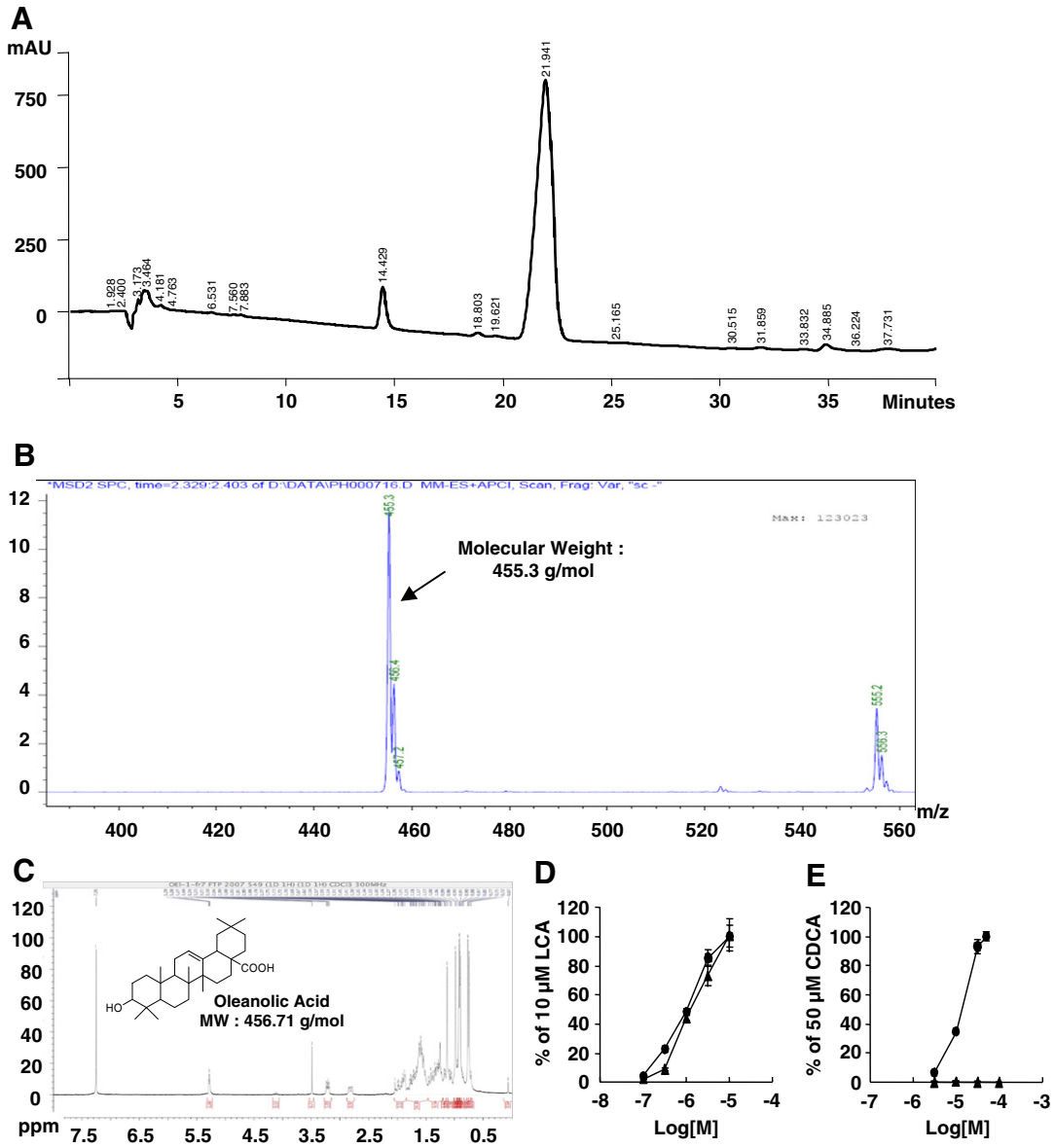

Fig. 2. Analysis and identification of compound contained in fraction 7 of *Olea europaea* leaves DCM extract: (A) Analytical RP-HPLC profile at 254 nm. (B) MS analytical data. (C) NMR spectrum. (D) Oleanolic acid (triangles) and LCA (circles) effect on TGR5. (E) Oleanolic acid (triangles) and CDCA (circles) effect on FXR.

50% increase in body weight accompanied by an increase in caloric intake (Table 1). Even though there was only a tendency towards an effect on body weight, a significant decrease of epididymal fat pad mass was observed (Table 1), which indicates that oleanolic acid affects energy metabolism, potentially through a mechanism involving mitochondrial activation.

The results on energy metabolism obtained with oleanolic acid, a non bile acid related TGR5 agonist, are consistent with the data previously obtained with bile acids, which also improved energy homeostasis and protected against weight gain induced by a high fat diet [6]. Together with these data [6], our study further emphasizes the role of TGR5 agonists in preventing body weight increase. These

Table 1
Body weight parameter

	Chow diet	High fat diet	
		Vehicle	Oleanolic acid 100 mg/kg
Body weight (g)			
J0	24.3 ± 0.5	36.1 ± 1	34.5 ± 0.7
J14	26.3 ± 0.4	38.03 ± 1.1	30.4 ± 1.4
Average food intake (Kcal/animal/day)	7.20 ± 0.29	14.79 ± 0.88	11.43 ± 0.98
Epididymal WAT (% BW)	1.73 ± 0.09	5.77 ± 0.27	3.7 ± 0.7*

Data represent mean values ± SEM, chow diet $n = 5$, vehicle $n = 8$, oleanolic acid $n = 8$ (*$p < 0.001$).

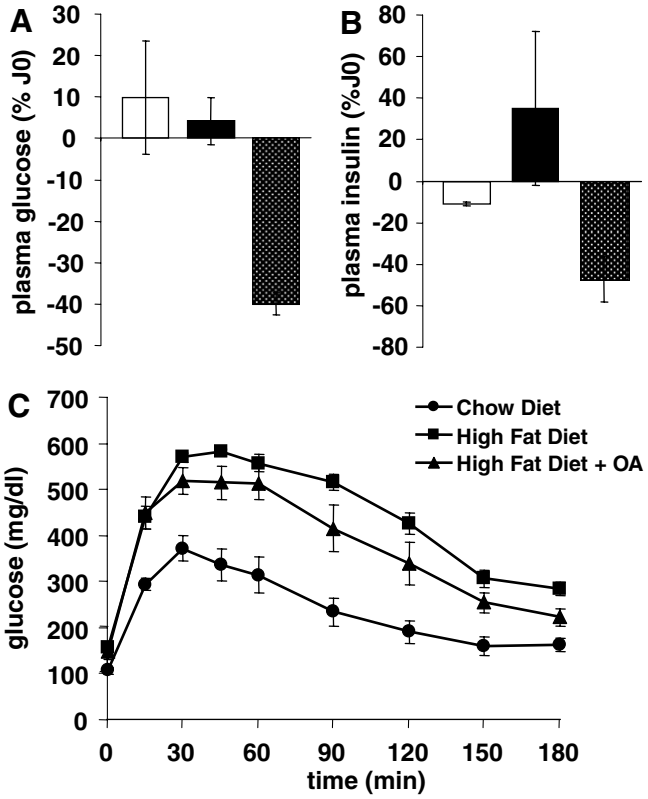

Fig. 3. Oleanolic acid decreases plasma glucose (A) and insulin (B) levels in mice treated for 7 days. Chow diet (□), high fat diet (■), high fat diet + 100 mg/kg oleanolic acid (▩). (C) Oleanolic acid improved the glucose tolerance in high fat diet mice. An intraperitoneal glucose tolerance test was performed after 14 days of treatment on C57BL/6J mice fed with chow diet (●), high fat diet (■), and high fat diet + oleanolic acid 100 mg/kg (▲). Values are means ± SEM, $n = 5$ for chow diet and $n = 8$ for high fat diet and high fat diet + oleanolic acid 100 mg/kg.

data are also consistent with those observed in another study where another triterpenoid i.e. ursolic acid was tested in the same model i.e. high fat fed mice [20]. In this study, the authors tested ursolic acid (50 mg/kg) in a prevention mode, with the treatment initiated from the beginning of the high fat diet, which resulted in a significant inhibition of body weight increase. In contrast in the current experiment, the high fat diet was maintained for 10 weeks before treatment was actually initiated. Our study therefore looks at the ability to correct, rather than prevent, the onset of insulin resistance. Even though the design of our study tested a more ambitious objective, it achieved the same conclusions.

Seven days of treatment with oleanolic acid furthermore clearly decreased plasma glucose ($-40 \pm 3\%$ vs. $4 \pm 6\%$) and plasma insulin levels ($-47 \pm 11\%$ vs. $35 \pm 37\%$), which indicates that oleanolic acid was able to improve metabolic homeostasis in high fat fed mice (Fig. 3A and B). To further ascertain this effect, an intraperitoneal glucose tolerance test (IPGTT) was performed after 14 days of treatment. The average blood glucose levels prior to the IPGTT were 103 ± 7, 154 ± 5, and 144 ± 18 mg/dL respectively for the chow diet, the high fat diet and the high fat diet + oleanolic acid groups. IPGTT curves clearly indicated that feeding a high fat diet induces glucose intolerance and insulin resistance (Fig. 3C). This glucose tolerance was partially corrected when mice were treated with oleanolic acid.

Oleanolic acid at 100 mg/kg/day showed some toxicity, since compared to non treated high fat diet mice, the transaminases (GOT, GPT), as well as phosphatase alkaline ALP were increased at 360 ± 108 vs. 76 ± 5 U/L, 655 ± 224 vs. 55 ± 13 U/L, and 154 ± 14 vs. 56 ± 1 U/L, respectively. The increase in liver enzymes was probably indicative of cholestasis, since plasma total bilirubin levels were also increased (13.6 ± 5.6 vs. 2.4 ± 0.05 μmol/L). This toxic effect was mainly attributed to 2 individual animals out of the experimental group of 8 mice. The 6 remaining animals showed only a very mild increase in these liver enzymes, which indicates that the 100 mg/kg/day dose was at the upper limit of the pharmacological dose.

Although oleanolic acid shows some promising features to treat diabetes, some challenges remains ahead. Future work will be needed to identify the molecular mechanism involved in mediating the effects of oleanolic acid on metabolism. Since TGR5 activation is both reported to enhance mitochondrial energy expenditure in muscle and brown adipose tissue in vivo [6] and to induce GLP1 release in cultured intestinal cells [21], future studies should explore whether the effect of oleanolic acid on glucose tolerance, that we observe in this study, derive from effects on mitochondrial function and/or insulin release from the pancreas. In addition, detailed pharmacological dose finding studies and chemical optimization of oleanolic acid could help to ameliorate safety and to improve efficacy of the molecule.

In conclusion, we have shown that oleanolic acid, a triterpenoid from olive leaves is a highly specific and potent TGR5 agonist. We have furthermore established that this molecule slows weight increase induced by high fat and has a potent anti-hyperglycemic activity, which contributes to the anti-diabetic effect of *O. europaea* leaves, previously thought to be mainly explained by the activity of oleuropein.

Acknowledgments

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References

[1] P. Zimmet, K.G. Alberti, J. Shaw, Global and societal implications of the diabetes epidemic, Nature 414 (2001) 782–787.
[2] K.F. Petersen, S. Dufour, D. Befroy, R. Garcia, G.I. Shulman, Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes, N. Engl. J. Med. 350 (2004) 664–671.
[3] J. Auwerx, Improving metabolism by increasing energy expenditure, Nature 12 (2006) 44–45.

[4] Y. Kawamata, R. Fujii, M. Hosoya, M. Harada, H. Yoshida, M. Miwa, S. Fukusumi, Y. Habata, T. Itoh, Y. Shintani, S. Hinuma, Y. Fujisawa, M. Fujino, A G protein-coupled receptor responsive to bile acids, J. Biol. Chem. 278 (2003) 9435–9440.
[5] S.M. Houten, M. Watanabe, J. Auwerx, Endocrine functions of bile acids, EMBO J. 25 (2006) 1419–1425.
[6] M. Watanabe, S.M. Houten, C. Mataki, M.A. Christoffolete, B.W. Kim, H. Sato, N. Messaddeq, J.W. Harney, O. Ezaki, T. Kodama, K. Schoonjans, A.C. Bianco, J. Auwerx, Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation, Nature 439 (2006) 484–489.
[7] G.Y. Yeh, T.K. Kaptchuk, D.M. Eisenberg, R.S. Phillips, Systematic review of herbs and dietary supplements for glycemic control in diabetes, Diabetes Care 26 (2003) 1277–1294.
[8] G.Y. Yeh, D.M. Eisenberg, R.B. Davis, R.S. Phillips, Complementary and alternative medicine use among patients with diabetes mellitus: result of a national survey, Am. J. Pub. Health 92 (2002) 1648–1652.
[9] D.J. Newman, G.M. Cragg, Natural products as sources of new drugs over the last 25 years, J. Nat. Prod. 70 (2007) 461–477.
[10] M. Bnouham, A. Zyyat, H. Mekhfi, A. Tahri, A. Legssyer, Medicinal plants with potential antidiabetic activity—a review of ten years of herbal medicine research (1990–2000), Int. J. Diabetes Metab. 14 (2006) 1–25.
[11] F. Picard, M. Gehin, M.C. Annicotte, S. Rocchi, M.F. Champy, B.W. O'Malley, P. Chambon, J. Auwerx, SRC-1 and TIF2 control energy balance between white and brown adipose tissues, Cell 111 (2002) 931–941.
[12] M. Gonzalez, A. Zarzuelo, M.J. Gamez, M.P. Utrilla, J. Jimenez, J. Osuna, Hypoglycemic activity of olive leaf, Planta Med. 58 (1992) 513–515.
[13] N. Bennabi-Kabchi, H. Fdhil, Y. Cherrah, F. El Bouayadi, L. Kehel, G. Marquie, Therapeutic effect of *Olea europaea* var. *oleaster* leaves on lipidic and carbohydrate metabolism in obese and prediabetic sand rats (*Psammomys obesus*), Ann. Pharm. Fr. 58 (2000) 271–277.
[14] A. Keys, Mediterranean diet and public health: personal reflections, Am. J. Clin. Nutr. 61 (1995) 1321S–1323S.
[15] H.F. Al-Azzawie, M.S. Saeed Alhamdani, Hypoglycemic and anti-oxidant effect of oleuropein in alloxan-diabetic rabbits, Life Sci. 78 (2006) 1371–1377.
[16] W. Seebacher, N. Simic, R. Weiss, R. Saf, O. Kunert, Magn. Reson. Chem. 41 (2003) 636–638.
[17] K.T. Liby, M.M. Yore, M.B. Sporn, Triterpenoids and rexinoids as multifunctional for the prevention and treatment of cancer, Nat. Rev. Cancer 7 (2007) 357–369.
[18] M.S. Ali, M. Jahangir, S.S. ul Hussan, M.I. Choudhary, Inhibition of α-glucosidase by oleanolic acid and its synthetic derivatives, Phytochemistry 60 (2002) 295–299.
[19] D.W. Jeong, Y.H. Kim, H.H. Kim, H.Y. Ji, S.D. Yoo, W.R. Choi, S.M. Lee, C.K. Han, H.S. Lee, Dose linear pharmacokinetics of oleanolic acid after intravenous and oral administration in rats, Biopharm. Drug Dispos. 28 (2007) 51–57.
[20] B. Jayaprakasam, L.K. Olson, R.E. Schutzki, M.H. Tai, M.G. Nair, Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in cornelian cherry (*Cornus mas*), J. Agric. Food Chem. 54 (2006) 243–248.
[21] S. Katsuma, A. Hirasawa, G. Tsujimoto, Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC1, Biochem. Biophys. Res. Commun. 329 (2005) 386–390.

Characterization of the Effect of a TGR5 Agonist Isolated from *Olea Europea* on Metabolic Disorders

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Introduction: Metabolic disorders have been increasing at a fast pace over the past decades and intense drug discovery efforts in the metabolic field have focused on enhancing energy expenditure. Mitochondrial dysfunction in skeletal muscle is invariably associated with the early stages of this diseases[1,2]. In this context, the G-protein coupled receptor TGR5 is an interesting target since its activation by bile acids results in increased energy expenditure through activation of mitochondrial function[3]. Numerous plants have been reported to possess anti-hyperglycemic and anti-diabetic activity. Olive tree (*Olea Europaea*) leaves are well known for their effect on metabolism and are used as a traditional anti-diabetic and anti-hypertensive herbal drug[4,5]. In the present study, we describe the identification in this plant of a highly specific and potent TGR5 agonist, the terpenoid oleanolic acid (OA). Our data suggest that OA has antidiabetic and antiobesity properties through increasing mitochondrial function, that was confirmed *in vivo* and *in vitro*.

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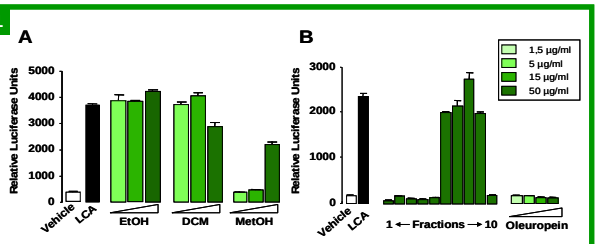

Fig 1 : TGR5 acivity of *Olea Europaea* extracts
(A) *Olea Europaea* leaf extracts induces TGR5 activity. TGR5-expressing CHO cells were treated 5h with increasing doses (5, 15 and 50 µg/ml in DMSO) of ethanol (EtOH), dichloromethane (DCM) or methanol (MetOH) extracts. Luciferase activity was measured thereafter. (B) CHO cells were treated 5h with 50µg/ml of each of the 10 *O. Europaea* fractions or with 1,5, 5, 15, or 50 µg/ml of oleuropein. Luciferase activity was measured thereafter.

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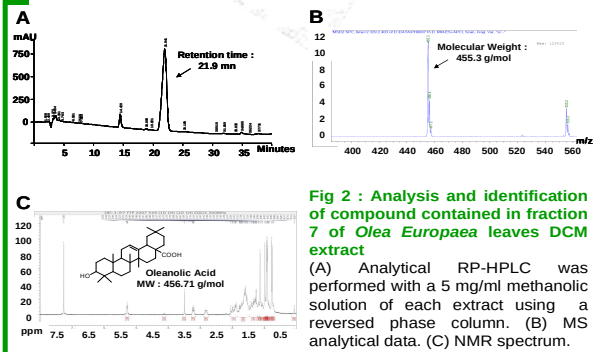

Fig 2 : Analysis and identification of compound contained in fraction 7 of *Olea Europaea* leaves DCM extract
(A) Analytical RP-HPLC was performed with a 5 mg/ml methanolic solution of each extract using a reversed phase column. (B) MS analytical data. (C) NMR spectrum.

3

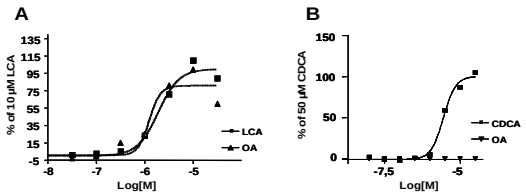

Fig 3 : TGR5 or FXR activity of *Olea Europaea*
(A) TGR5-expressing CHO cells were treated with LCA (circles) or oleanolic acid OA (triangles) and luciferase assay was performed. EC_{50} (OA) = 1,42 µM. EC50 (LCA) = 0,89 µM. (B) FXR-expressing COS cells were treated with CDCA (circles) or OA (triangles) and luciferase assay was performed.

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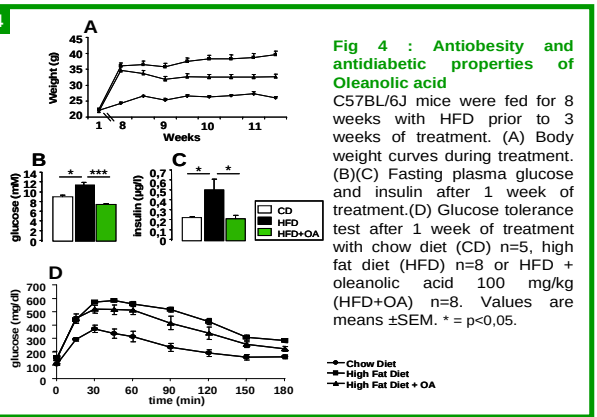

Fig 4 : Antiobesity and antidiabetic properties of Oleanolic acid
C57BL/6J mice were fed for 8 weeks with HFD prior to 3 weeks of treatment. (A) Body weight curves during treatment. (B)(C) Fasting plasma glucose and insulin after 1 week of treatment.(D) Glucose tolerance test after 1 week of treatment with chow diet (CD) n=5, high fat diet (HFD) n=8 or HFD + oleanolic acid 100 mg/kg (HFD+OA) n=8. Values are means ±SEM. * = p<0,05.

5

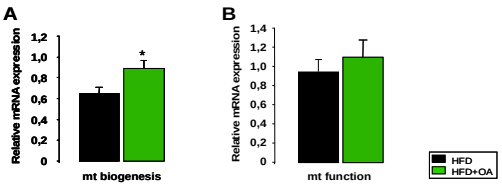

Fig 5 : Oleanolic acid improves the expression of genes implicated in mitochondrial biogenesis and function in skeletal muscle
Relative mRNA expression levels of genes related to mitochondrial biogenesis (A) and function (B) as measured by Q-RT-PCR in the gastrocnemius muscle of HFD or HFD+OA mice (n=8 animals/group). Data represent mRNA level relative to 36B4 and are given as means ± SEM. * = p<0,05.

6

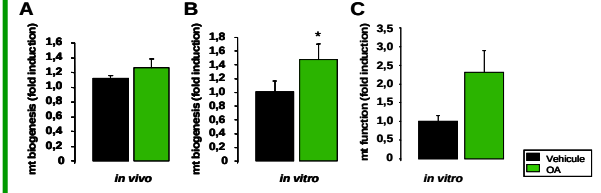

Fig 6 : Oleanolic acid improves the mitochondrial biogenesis and function in vivo and in vitro
(A) Mitochondrial biogenesis as measured by Q-PCR in the gastrocnemius muscle of HFD (vehicule) or HFD+OA (OA) mice (n=8 animals/group). (B) mtDNA content as measured by Q-PCR in C2C12 myotubes treated 48h with vehicule or OA 10µM. (C) Mitochondrial function as measured by enzymatic assay in C2C12 myotubes treated 3h with vehicule or OA 10µM. * = p<0,05.

Conclusions:

- the triterpenoid OA contained in *olive leaves* is a TGR5 agonist
- anti-diabetic effect of olive leaves is at least partly dependent of oleanolic acid
- OA improves diabetic parameters in vivo through increasing mitochondrial biogenesis and function.

This work emphasizes the potential role of TGR5 agonists to improve metabolic disorders.

References:

[1] Petersen K.F. *et al*, *N.Eng.J.Med.*: 350(664-671), 2004
[2] Auwerx J., *Nature*: 12(44-45), 2006
[3] Watanabe M. *et al*, *Nature*: 439 (484-489), 2006
[4] Gonzalez M. *et al*, *Planta Med.*: 58 (513-515), 1992
[5] Bennabi-Kabchi N. *et al*, *Ann. Pharm. Fr.*: 58(271-277), 2000

Structure−Activity Relationship Study of Betulinic Acid, A Novel and Selective TGR5 Agonist, and Its Synthetic Derivatives: Potential Impact in Diabete

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TGR5-dependant activation of mitochondrial function by terpenoid derivatives

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Running title: Triterpernoid-mediated TGR5 activation enhances mitochondrial function

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Abstract

We recently identified the natural triterpenoid oleanolic acid (OA) as a selective agonist of TGR5, a G protein-coupled receptor which mediates the increase in energy expenditure induced by bile acids (BAs). This triterpenoid TGR5 agonist was shown to enhance glucose homeostasis through a stimulation of mitochondria *in vivo* and *in vitro* in muscle. A novel triterpenoid derivative, RG239, endowed with selective and more potent TGR5 agonist activity than OA, was synthesized through hemisynthesis. In order to characterize the biological effects of this newly identified TGR5 agonist, we assessed the impact of RG239 on mitochondrial function in muscle and in intestinal enteroendorine cell lines. In the muscle cell line C2C12 and in the intestinal enteroendocrine cell line STC-1, RG239 stimulates mitochondrial biogenesis and activity as assessed by quantification of mitochondrial DNA content and cytochrome c oxidase assay, respectively. These effects were blunted by specific TGR5 antibodies and by inhibiting the activity of adenylate cyclase or cAMP-dependent protein kinase. Interestingly, in STC-1 cells, these events were correlated with a TGR5-dependent increase in the release of glucagon-like peptide 1 (GLP-1). Altogether, our findings further validate TGR5 as an interesting target for the improvement of metabolic homeostasis and indicate that triterpenoids derivatives are promising pharmacological compounds, with effects on mitochondrial function in muscle and enteroendocrine cell lines, that could find applications in the treatment of metabolic disorders.

Introduction

Diabetes is a major worldwide health problem. In 2000, 171 million people were living with diabetes and this number is projected to rise to 366 million in 2030 (1). The huge human and economic costs of diabetes and associated complications incited an intensive research to identify novel preventive and therapeutic strategies. To date, the therapies available target the consequences of diabetes and it is therefore of particular interest to find ways to tackle early stages of diabetes. Obesity and insulin resistance, which generally precede type 2 diabetes, are correlated with mitochondrial disorders in skeletal muscle (2). Correlatively, hyperglycaemia was shown to decrease mitochondrial function (3). For example, in muscle of diabetic subjects, a defect in energy expenditure is observed and acting on mitochondria could be helpful to optimize energy expenditure in this organ (4). Targeting and improving mitochondria could hence be relevant to prevent and treat Type 2 diabetes.

In addition to their well-established roles in dietary lipids absorption and cholesterol metabolism (5), bile acids (BAs) have been shown to activate mitogen-activated protein (6) and nuclear hormone receptors such as the farnesoid X receptor α (FXR-α) (7). Through activation of these two pathways, BAs control cholesterol and their own metabolism. In addition, BAs act as signalling molecules through TGR5 (GPR131, M-BAR, GPBAR-1), a Gs protein-coupled receptor activated by BAs (5, 8). This receptor is broadly expressed but is relatively enriched in metabolic tissues (8-10). In this context, it was recently described that TGR5 activation in brown adipocytes results in the activation of the type 2 iodothyronine deiodinase (D2), followed by a local production of thyroid hormones which eventually increases mitochondrial activity, an effect instrumental in preventing obesity and insulin resistance in DIO mice fed with BAs (11). These findings suggest that TGR5 activation enhances mitochondrial function. Consistent with the role of TGR5 in the control of energy metabolism, female TGR5 knockout mice gain less weight when challenged with a high fat diet, indicating that the lack of TGR5 decreases energy expenditure and promote obesity (10). These findings link BAs through TGR5 and mitochondria with energy expenditure in BAT and muscle.

Likewise and in line with the involvement of TGR5 in metabolic homeostasis, BAs activation of TGR5 has also been reported to promote glucagon-like peptide 1 (GLP-1) secretion in murine enteroendocrine cell lines, as a consequence of an induction of cyclic adenosine monophosphate

(cAMP) accumulation (12). GLP-1 is an enteric hormone that stimulates insulin secretion in pancreatic β cells and improves glycaemia in Type 2 diabetes (13, 14). The link between TGR5 and GLP-1-mediated metabolic homeostasis underlines that TGR5 could be a good candidate target for the development of new anti-diabetic drugs.

Recently, and in contrast to BAs, which activate both TGR5 and FXR-α, a number of novel and selective TGR5 agonists, that do not activate FXR-α have been described. A biological screening of a collection of natural occurring BAs, BA derivatives and some steroid hormones has resulted in the discovery of potent and selective TGR5 ligands such as 6α-ethyl-23(S)methyl-chenodeoxycholic acid and derivatives (15, 16). This compound was described to induce cAMP production in CHO-TGR5 cells and in human intestinal NCI-H716, suggesting that it could induce GLP-1 secretion(16) as was proposed by Katsuma *et al.*(12). We previously identified oleanolic acid (OA), a triterpenoid extracted from *Olea Europaea* leaves, as a selective and potent TGR5 agonist which enhances mitochondrial functions and improves glucose tolerance (17, 18). A structure-activity relationship (SAR) approach led us to the synthesis of a novel more selective and potent TGR5 agonist, 18 dia 2 also named RG239 (19).

Since TGR5 was shown to improve BAT and muscle energy expenditure through the activation of mitochondria, we hypothesized that TGR5 enhances also mitochondrial functions in other cell types. We hence evaluated the impact of RG239 relative to BAs, on mitochondrial function in two cell lines of muscle and intestinal origin, whose biological functions are closely related to the maintenance of metabolic homeostasis.

Materials and Methods

Materials. Synthesis of RG239 / 18 dia 2 was described elsewhere (19). Rp-3',5'-cyclic Adenosine MonoPhosphorothioate (Rp-cAMPS) and *cis*-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine (MDL-12,330A) were purchased by Sigma-Aldrich (Saint-Quentin Fallavier, France). Oleanolic acid was purchased from Extrasynthèse (Genay, France). An antibody directed against TGR5 was produced by intradermally injection of the peptide N-KRDNPGPSTAYHTSSQC-C in a rabbit. The production of the peptide and the immunization of the rabbit were performed by the IGBMC core facilities.

Cells. Chinese hamster ovary (CHO) cells, COS-1 cells and enteroendocrine L-cells (STC-1) were obtained from ATCC (MANASSAS, VA). Mouse myoblast (C2C12) cells were purchased by ECACC (Salisbury, UK). CHO cells were maintained in minimum essential medium-alpha (α-MEM) supplemented with 10%C (v/v) foetal calf serum (FCS), 100µM non essential amino acid (NEAA), 100u/ml penicillin and 100µg/ml streptomycin-sulphate. They were used for the TGR5 luciferase assay (see below). COS-1 cells were maintained in DMEM (1g/l glucose) supplemented with 10% (v/v) fetal bovine serum (FCS) and 100µg/ml gentamycine. They were used for the FXR luciferase assay (see below). C2C12 mouse myoblast cell line was maintained in DMEM (4,5g/l glucose) supplemented with 10% FCS. Differentiation of myoblasts in myotubes was induced with media containing 2% horse serum (HS) during four days. STC-1 cells were maintained DMEM (1g/l glucose) supplemented with 15% HS, 10 % FCS and 100µg/ml gentamycin. C2C12 myotubes and STC-1 were treated for cytochrome c oxidase assay and mitochondrial DNA quantification (see below). For the need of the experiment, STC-1 cells were transfected using JETPEI (PolyPlus Transfection, Illkirch, France).

TGR5 luciferase assay. A stable cell line was obtained by transfection of CHO cells with 3,8µg of human TGR5 expression plasmid (pCMVSPOR6/hTGR5) or mouse TGR5 expression plasmid (pCMVSPORT6/mTGR5), 3,8µg of CRE-driven luciferase reporter plasmid (pCRE-Luc), and 0,4µg of neomycin-resistant gene expression plasmid (pcDNA3,1 (+)) using lipofectamine 2000 (Invitrogen, Cergy-Pontoise, France). The transfected cells were selected with 400µg/ml G418 sulphate and single clones were grown in 96-well plate, independently. TGR5-expressing CHO cells were treated with

10µM Lithocholic acid (LCA) or RG239 followed by luciferase assay (20). Luminescence was determined with CentroXS3 LB960 (Berthold Technologies, Bad Wildbad, Germany).

FXR luciferase assay. To evaluate FXR activity of compounds, COS-1 cells were transfected with 25ng of hFXR expression plasmid (pCMX-hFXR), 25ng of mouse retinoid X receptor α (mRXRα) expression plasmid (pCMX- mRXRα), 50ng of reporter plasmid (pEcREx7-Luc) and 50ng of pCMVβ as internal control in each well, using the Lipofectamine 2000 reagent. About 18h after transfection, cells were incubated for 24h with different concentrations of each compound in fresh MEM (or DMEM). After this treatment, the cells were lysed and normalized luciferase activity was determined (20).

Mitochondrial DNA quantification. STC-1 cells or C2C12 myotubes were treated with RG239 ± compounds. The cells were then lysed with a lysis buffer allowing DNA extraction by conventional phenol-chloroform method (10mM Tris, 1mM EDTA, 0.3M NaAc, 1% SDS). DNA was quantified by Q-PCR on sixplicate using 10µM each primer (mitochondrial DNA specific PCR (16S rRNA), forward 5'-CCGCAAGGGAAAGATGAAAGAC-3', reverse 5'- TCGTTTGGTTTCGGGGTTTC-3' ; and nuclear specific PCR (UCP2), forward 5'-CTACAGATGTGGTAAAGGTCCGC-3', reverse 5'-GCAATGGTCTTGTAGGCTTCG-3') and QuantiTect SYBR Green PCR Kit (Sigma-Aldrich, Saint-Quentin Fallavier, France), in a LightCycler 480 (Roche diagnostics, Mannheim, Germany) with a program of 20 minutes at 95°C, followed by 45 to 50 cycles of 15 seconds at 95°C, 20 seconds at 56°C and 20 seconds at 72°C. Single-product amplification was verified by an integrated post-run melting curve analysis. Exponential amplification efficiency was verified during each PCR run using a standard dilution series made from pooled samples.

Cytochrome c oxidase assay. STC-1 cells or C2C12 myotubes were treated with RG239 ± compounds and then lysed (20mM HEPES, 0.1%Triton, 1mM EDTA). Cell lysate was incubated with reduced cytochrome c (from equine heart, Sigma). Disappearance of reduced cytochrome c was followed spectrophotometrically at 550nM (according to the manufacturer's instruction, Sigma).

GLP-1 release. STC-1 cells were treated with RG239 ± compounds. The incubated medium was collected and the concentration of GLP-1 was determined by enzyme immunoassay with a specific GLP-1 amide Enzyme Immunoassay Kit (Millipore).

Results and Discussion

The activity of the synthetic triterpenoid RG239 on TGR5 was compared to that of OA by luminescence assay in stable cotransfection of an hTGR5 expression vector and a Cre-luciferase reporter gene in CHO cells. In these assays, RG239 is a more potent hTGR5-agonist than OA (Fig. 1A: EC_{50} of 82 nM and 1.3 µM respectively). As opposed to OA that exhibited partial agonist activity on mTGR5 (data not shown), RG239 is also a potent agonist on mTGR5, with an EC50 of 0.5 µM (Fig. 1B). Furthermore, RG239 was devoid of activity on FXR as evaluated in transiently cotransfected COS cells with FXR and RXR expression vectors and an FXR-dependent Cre-luciferase reporter (Fig. 1C). These data suggest that triterpenoids, such as OA or RG239, have improved selectivity over BAs, which activate both TGR5 and FXR, making triterpenoids interesting tools to further characterize TGR5 physiology.

In a previous study, we demonstrated that OA, a triterpenoid extrated from *Olea Europaea* leaves, increases mitochondrial biogenesis and activity of muscle *in vivo* and *in vitro* (18). Here, we first explored the effects of RG239 on mitochondrial functions (biogenesis and activity) in the muscle cell line C2C12. To assess mitochondrial biogenesis, the ratio of mitochondrial DNA over nuclear DNA from treated C2C12 cells was evaluated by Q-PCR using mitochondrial and nuclear specific primers. Mitochondrial activity of treated C2C12 cells was analyzed by measuring cytochrome c oxidase activity spectrophotometrically. Cytochrome c oxidase is a mitochondrial enzyme (complex IV of the electron transport chain) which provides energy for the cells by coupling of the electron transport chain with the process of oxidative phosphorylation. In these tests, RG239 induced mitochondrial biogenesis (Fig. 2A) and activity (Fig. 2B) in C2C12 cells. Secondly, we wanted to examine RG239 effects on mitochondria of another cell type, the intestinal enteroendocrine L-type cells (STC-1), which secrete GLP-1 and express TGR5 (8, 11). Also in STC-1 cells, RG239-treatment induced mitochondrial biogenesis (Fig. 3A) and activity (Fig. 3B), similarly as observed in C2C12 cells (Fig. 2). These findings demonstrate that the TGR5-agonist RG239 activates mitochondrial functions both in muscle and intestinal L cell lines.

We then set out to determine whether the enhanced mitochondrial functions induced by the triterpenoid RG239 are TGR5-dependent. To assess this question, we used a blocking polyclonal

antibody directed against TGR5. RG239-stimulation of mitochondria was abolished in presence of the blocking antibody in STC-1 cells (Fig. 3). The TGR5 dependence of the effects of RG239 on mitochondria biogenesis was corroborated using mouse embryonic fibroblasts (MEF) isolated from mice in which the TGR5 gene was flanked by LoxP sites and that were infected with an adenovirus expressing the CRE recombinase. The RG239-mediated increase in the number of mitochondria was inhibited in these TGR5 deficient MEFs in a similar fashion as in our studies that used a blocking antibody (data not shown). These data indicate that mitochondrial effects of the triterpenoid RG239 are TGR5-dependent.

The activation of the Gs coupled receptor, TGR5, by BAs and BA derivatives lead to cAMP production in the STC-1 cell line and in primary brown adipocytes (8, 11, 12). In fact, activation of receptors coupled to Gs protein leads to stimulate the adenylate cyclase (AC), responsible from the cAMP production. To demonstrate the implication of AC in RG239-induced mitochondrial effects, we pretreated both intestine and muscle cells with the AC inhibitor MDL-12,330A, before analyzing the effects of RG239 on mitochondrial biogenesis. As expected, MDL-12,330A-pretreatment blocked RG239-stimulated mitochondrial biogenesis both in STC-1 cells (Fig. 4A) and in C2C12 cells (Fig. 4B). The increase in the second messenger cAMP could act on several targets, one of which is the protein kinase A (PKA). We therefore explored the role of PKA in the RG239 induced phenotype, using the specific membrane-permeable inhibitor Rp-cAMPS, a cAMP analogue. In the presence of Rp-cAMPS, RG239 was unable to further enhance mitochondrial biogenesis in STC-1 cells (Fig. 4C) and in C2C12 cells (Fig. 4D). We hence conclude that the triterpenoid RG239 induces mitochondrial functions by a pathway implicating TGR5, AC and PKA in muscle and intestinal cell lines.

The RG239-activated TGR5-stimulation of mitochondria in muscle could result in enhancing energy expenditure, as already shown with BAs-activated TGR5 (11) and as discussed below. Concerning the consequence of mitochondria activation in intestinal L cells, we hypothesized a link with GLP-1 secretion, a major role of these cells. To begin to investigate this hypothesis, STC-1 cells were treated with RG239 and GLP-1 secretion was measured with an enzyme immunoassay. Addition of RG239 to STC-1 cells was shown to stimulate GLP-1 secretion. The stimulation of GLP-1 release is prevented with the blocking antibody directed against TGR5 (Fig. 5). These data lead us to conclude that

triterpenoids induce GLP-1 secretion from enteroendocrine L cells through their effects on TGR5 arguing that triterpenoids could have antidiabetic effects. Indeed, the enteric hormone GLP-1 is known to stimulate insulin secretion and improve glycaemia in type 2 diabetes. The putative enhancement of GLP-1 secretion subsequent to TGR5-dependent mitochondria activation should hence be further explored as a potential way to increase insulin secretion. However, this hypothesis is plausible and shows many analogies with insulin secretion in pancreatic β cells. Normally, elevated cytosolic ATP production results in plasma membrane K_{ATP} channels closure, cellular depolarization and subsequent voltage-gated Ca^{2+} influx, whose lead to insulin granule exocytosis (21, 22). That link nutrient metabolism (glucose metabolism results in ATP production) to insulin secretion. As example, the A3243G mutation in mitochondrial DNA will impact on insulin secretion by attenuation of ATP production upon aging (23). STC-1 being also secretary cells, we can hypothesize that RG239-induced TGR5 activation promotes GLP-1 exocytosis by stimulating mitochondria. Further experiments are needed to explore this eventuality.

Altogether, these data and literature allow us to hypothesize the existence of an intracellular triterpenoid-stimulated TGR5 signalling pathway schematized in Figure 6. In the present paper, we demonstrated that the triterpenoid RG239 activates selectively TGR5. Ligand binding is followed by release of guanine-dependent stimulatory protein $G\alpha_s$ subunit and thus activation of AC. The increase in cAMP levels activates PKA, leading to mitochondria activation in both muscle and intestine cell lines. The link between cAMP, PKA and mitochondria make sense since evidence has emerged that cAMP-mediated phosphorylation of mitochondrial enzymes plays a role in OXPHOS regulation (24, 25). More recently, a mitochondrial AC-PKA pathway of cAMP production has been described, corroborating with our results and bypassing the fact that the inner mitochondrial membrane is impermeable to cAMP (26). Furthermore, transcriptional pathways mediated by the PKA-induced activation of the cAMP response element (CRE) binding protein (CREB) and the subsequent induction of the activity of CREB target genes, such as dioiodinase 2 (D2), which activates thyroid signalling, and proliferator-activated receptors γ (PPARγ) coactivator-1α (PGC-1α), the master regulator of

mitochondrial biogenesis and function, can contribute to explain the dramatic biology of RG239 activation of TGR5(11, 27).

The work presented here described triterpenoids as potent and selective TGR5-agonists. Research on terpenes provide evidences of their large scale of activities, including antimicrobial (28), antioxidant (29), anti-tumoral (30) and anti-inflammatory (31) actions. Although triterpenoids were considered for those properties, we demonstrated that OA is a highly selective and potent TGR5 agonist and possesses anti-hyperglycaemic effects (17) through enhanced mitochondrial functions (18). Here, we characterized RG239 as a more potent TGR5-agonist activating mitochondria in muscle and intestine. These findings allow us to consider triterpenoids as interesting molecules to prevent and treat Type 2 diabetes and associated metabolic disorders. An advantage of triterpenoids is that they are natural products since they are present in numerous plants, and hence are widely available. Furthermore, different triterpenoids are already on the market to treat other pathologies. Nevertheless, because of the structure of triterpenoids, the problems of bioavailability *in vivo* must be considered. By example, despite a better activity *in vitro* compared to OA, RG239 did not improve diabetic parameters *in vivo*, unlike OA. Even though RG239 levels were not measured in the plasma of the treated animals, the fact that RG239 does not seem to respect the five Lipinsky rules argues for a poor bioavailability (19, 32). This characteristic has to be kept in mind in the perspective of therapeutic use of triterpenoids. The addition of decorations on the molecule through hemisynthesis could be envisaged in order to ameliorate Lipinsky parameters of those molecules.

Altogether, our results confirm TGR5 as a promising therapeutical target for the treatment of the early stages of Type 2 diabetes through the action on mitochondria. Furthermore, they allow us to consider triterpenoids as interesting pharmaceutical agents in type 2 diabetes and metabolic diseases.

Acknowledgments

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Figure legends

Figure 1: TGR5 and FXR activity of RG239. (A) Stable hTGR5-expressing CHO cells were treated with RG239 (▼), OA (▲) or LCA (■) and luciferase assay was performed. (B) Transient mTGR5-expressing CHO cells were treated with RG239 (▼) or LCA (■) and luciferase assay was performed. (C) Transient FXR-expressing COS cells were treated with RG239 (▼), LCA (■) or UPF747 (▲) and a luciferase assay was performed.

Figure 2: Mitochondrial effects of RG239 in C2C12 cells. (A) Mitochondrial biogenesis as measured by mitochondrial DNA quantification using Q-PCR and (B) mitochondrial activity as measured spectrophotometrically by cytochrome c oxidase assay in C2C12 cells treated with RG239. Data represent means ± SEM, *n=3* for mitochondrial biogenesis (confirmed below) and *n=8* for mitochondrial function. Student unpaired t-test. * ($p<0.05$) compared to vehicle condition.

Figure 3: Mitochondrial effects of RG239 are TGR5-dependent. (A) Mitochondrial biogenesis as measured by mitochondrial DNA quantification using Q-PCR and (B) mitochondrial activity as measured spectrophotometrically by cytochrome c oxidase assay in TGR5-overexpressed STC-1 cells treated with RG239 ± antibody. Data represent means ± SEM, *n=4* for mitochondrial biogenesis and *n=4* for mitochondrial function. Student unpaired t-test. § ($p<0.05$) compared to RG239 treated condition.

Figure 4: Mitochondrial effects of RG239 are dependent of AC signalling pathway. Mitochondrial biogenesis as measured by mitochondrial DNA quantification using Q-PCR in TGR5-overexpressed STC-1 cells (A) and in C2C12 cells (B) treated with RG239 ± MDL-12,330A. *n=3* for STC-1 cells and *n=3* for C2C12 cells. Mitochondrial biogenesis as measured by mitochondrial DNA quantification using Q-PCR in TGR5-overexpressed STC-1 cells (C) and in C2C12 cells (D) treated with RG239 ± Rp-cAMPS. Data represent means ± SEM, *n=3* for STC-1 cells and *n=3* for C2C12 cells. Student

unpaired t-test. * ($p<0.05$) compared to vehicle condition. § ($p<0.05$) or §§ ($p<0.005$) compared to RG239 treated condition.

Figure 5: RG239-stimulated GLP-1 release is TGR5-dependent. GLP-1 release as measured by Elisa test in TGR5-overexpressed STC-1 cells treated with RG239 ± antibody. Data represent means ± SEM.

Figure 6: Triterpenoids activated TGR5 act on metabolic function in a mitochondria-dependant way. According to the data obtained in this paper, we conclude that triterpenoid-enhanced TGR5 act on mitochondria and on GLP-1secretion through activation of AC and PKA. Supported by literature, we propose a hypothesis by which PKA phosphorylates CREB, increasing D2 and PGC-1α expression, depending of the cell type. These events lead to mitochondria activation, and subsequently to the improvement of metabolic parameters.

Bibliography

1. **Wild S, Roglic G, Green A, Sicree R, King H** 2004 Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27:1047-1053
2. **Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI** 2004 Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med 350:664-671
3. **Palmeira CM, Rolo AP, Berthiaume J, Bjork JA, Wallace KB** 2007 Hyperglycemia decreases mitochondrial function: the regulatory role of mitochondrial biogenesis. Toxicol Appl Pharmacol 225:214-220
4. **Befroy DE, Petersen KF, Dufour S, Mason GF, de Graaf RA, Rothman DL, Shulman GI** 2007 Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. Diabetes 56:1376-1381
5. **Houten SM, Watanabe M, Auwerx J** 2006 Endocrine functions of bile acids. Embo J 25:1419-1425
6. **Qiao L, Han SI, Fang Y, Park JS, Gupta S, Gilfor D, Amorino G, Valerie K, Sealy L, Engelhardt JF, Grant S, Hylemon PB, Dent P** 2003 Bile acid regulation of C/EBPbeta, CREB, and c-Jun function, via the extracellular signal-regulated kinase and c-Jun NH2-terminal kinase pathways, modulates the apoptotic response of hepatocytes. Mol Cell Biol 23:3052-3066
7. **Wang H, Chen J, Hollister K, Sowers LC, Forman BM** 1999 Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. Mol Cell 3:543-553
8. **Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M** 2003 A G protein-coupled receptor responsive to bile acids. J Biol Chem 278:9435-9440
9. **Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Nakamura T, Itadani H, Tanaka K** 2002 Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun 298:714-719
10. **Maruyama T, Tanaka K, Suzuki J, Miyoshi H, Harada N, Nakamura T, Miyamoto Y, Kanatani A, Tamai Y** 2006 Targeted disruption of G protein-coupled bile acid receptor 1 (Gpbar1/M-Bar) in mice. J Endocrinol 191:197-205
11. **Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC, Auwerx J** 2006 Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature 439:484-489
12. **Katsuma S, Hirasawa A, Tsujimoto G** 2005 Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. Biochem Biophys Res Commun 329:386-390
13. **Holst JJ** 2007 The physiology of glucagon-like peptide 1. Physiol Rev 87:1409-1439
14. **Reimann F, Habib AM, Tolhurst G, Parker HE, Rogers GJ, Gribble FM** 2008 Glucose sensing in L cells: a primary cell study. Cell Metab 8:532-539
15. **Pellicciari R, Sato H, Gioiello A, Costantino G, Macchiarulo A, Sadeghpour BM, Giorgi G, Schoonjans K, Auwerx J** 2007 Nongenomic actions of bile acids. Synthesis and preliminary characterization of 23- and 6,23-alkyl-substituted bile acid derivatives as selective modulators for the G-protein coupled receptor TGR5. J Med Chem 50:4265-4268
16. **Sato H, Macchiarulo A, Thomas C, Gioiello A, Une M, Hofmann AF, Saladin R, Schoonjans K, Pellicciari R, Auwerx J** 2008 Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. J Med Chem 51:1831-1841
17. **Sato H, Genet C, Strehle A, Thomas C, Lobstein A, Wagner A, Mioskowski C, Auwerx J, Saladin R** 2007 Anti-hyperglycemic activity of a TGR5 agonist isolated from Olea europaea. Biochem Biophys Res Commun 362:793-798
18. **Strehle A, Sato H, Genet C, Thomas C, Lobstein A, Wagner A, Auwerx J, Saladin R** 2008 Characterization of the effect of a TGR5 agonist isolated from *Olea Europaea* on metaolic disorders. Molecules et ingrédients Santé 2008, 3e colloque européen Rennes, 2008

19. **Genet C, Strehle, A., Boudjelal, G., Schmidt, C., Lobstein, A. Schoonjans, K. Souchet, M., Auwerx, J., Saladin, R. Wagner, A.** SAR study around betulinic acid a novel and selective TGR5 agonist. J. Med Chem, submitted
20. **Picard F, Gehin M, Annicotte J, Rocchi S, Champy MF, O'Malley BW, Chambon P, Auwerx J** 2002 SRC-1 and TIF2 control energy balance between white and brown adipose tissues. Cell 111:931-941
21. **Luciani DS, Ao P, Hu X, Warnock GL, Johnson JD** 2007 Voltage-gated Ca(2+) influx and insulin secretion in human and mouse beta-cells are impaired by the mitochondrial Na(+)/Ca(2+) exchange inhibitor CGP-37157. Eur J Pharmacol 576:18-25
22. **Wiederkehr A, Wollheim CB** 2008 Impact of mitochondrial calcium on the coupling of metabolism to insulin secretion in the pancreatic beta-cell. Cell Calcium 44:64-76
23. **Maassen JA, LM TH, Van Essen E, Heine RJ, Nijpels G, Jahangir Tafrechi RS, Raap AK, Janssen GM, Lemkes HH** 2004 Mitochondrial diabetes: molecular mechanisms and clinical presentation. Diabetes 53 Suppl 1:S103-109
24. **Bender E, Kadenbach B** 2000 The allosteric ATP-inhibition of cytochrome c oxidase activity is reversibly switched on by cAMP-dependent phosphorylation. FEBS Lett 466:130-134
25. **Pagliarini DJ, Dixon JE** 2006 Mitochondrial modulation: reversible phosphorylation takes center stage? Trends Biochem Sci 31:26-34
26. **Acin-Perez R, Salazar E, Kamenetsky M, Buck J, Levin LR, Manfredi G** 2009 Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation. Cell Metab 9:265-276
27. **Puigserver P, Spiegelman BM** 2003 Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. Endocr Rev 24:78-90
28. **Cantrell CL, Franzblau SG, Fischer NH** 2001 Antimycobacterial plant terpenoids. Planta Med 67:685-694
29. **Montilla MP, Agil A, Navarro MC, Jimenez MI, Garcia-Granados A, Parra A, Cabo MM** 2003 Antioxidant activity of maslinic acid, a triterpene derivative obtained from Olea europaea. Planta Med 69:472-474
30. **Rocha Gda G, Simoes M, Lucio KA, Oliveira RR, Coelho Kaplan MA, Gattass CR** 2007 Natural triterpenoids from Cecropia lyratiloba are cytotoxic to both sensitive and multidrug resistant leukemia cell lines. Bioorg Med Chem 15:7355-7360
31. **Medeiros R, Otuki MF, Avellar MC, Calixto JB** 2007 Mechanisms underlying the inhibitory actions of the pentacyclic triterpene alpha-amyrin in the mouse skin inflammation induced by phorbol ester 12-O-tetradecanoylphorbol-13-acetate. Eur J Pharmacol 559:227-235
32. **Lipinski CA, Lombardo F, Dominy BW, Feeney PJ** 2001 Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 46:3-26

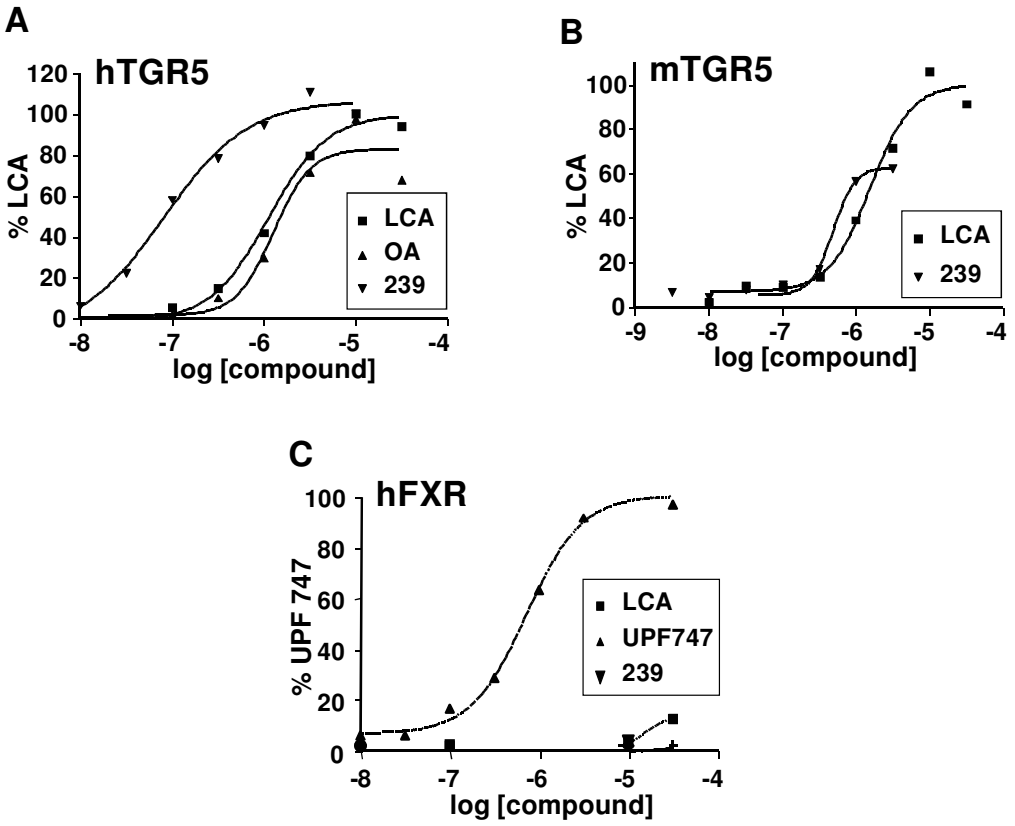
A
hTGR5
% LCA
log [compound]
LCA
OA
239
B
mTGR5
% LCA
log [compound]
LCA
239
C
hFXR
% UPF 747
log [compound]
LCA
UPF747
239

Figure 1

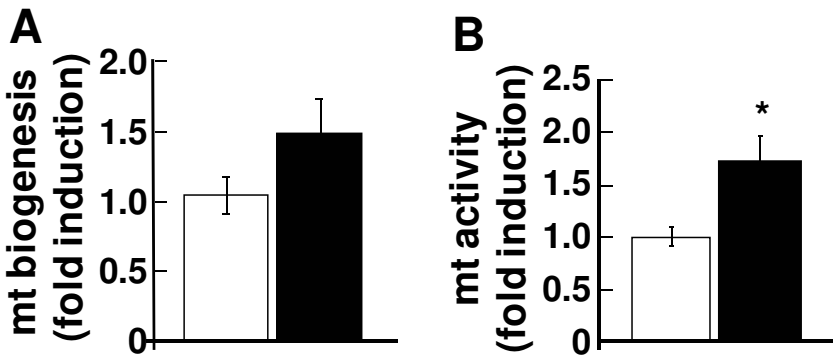
A
mt biogenesis
(fold induction)
2.0
1.5
1.0
0.5
0
B
mt activity
(fold induction)
2.5
2.0
1.5
1.0
0.5
0
*

Figure 2

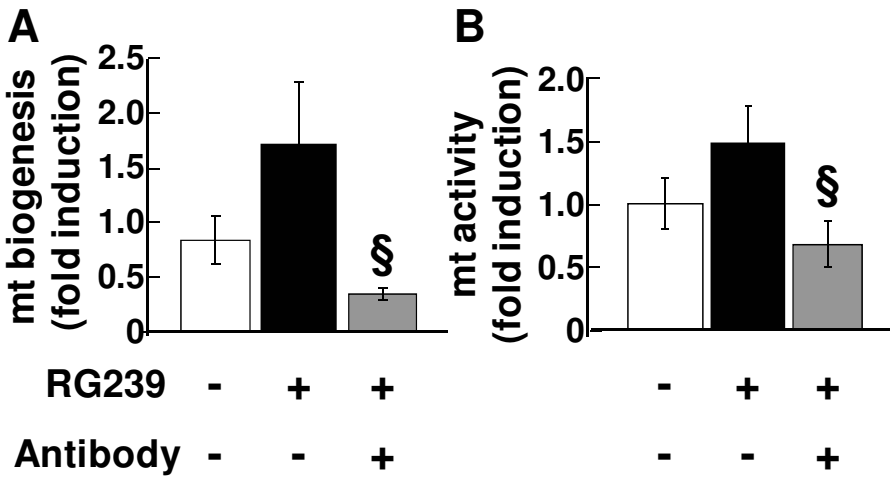
A
mt biogenesis
(fold induction)
2.5
2.0
1.5
1.0
0.5
0
§
RG239 - + +
Antibody - - +
B
mt activity
(fold induction)
2.0
1.5
1.0
0.5
0
§
- + +
- - +

Figure 3

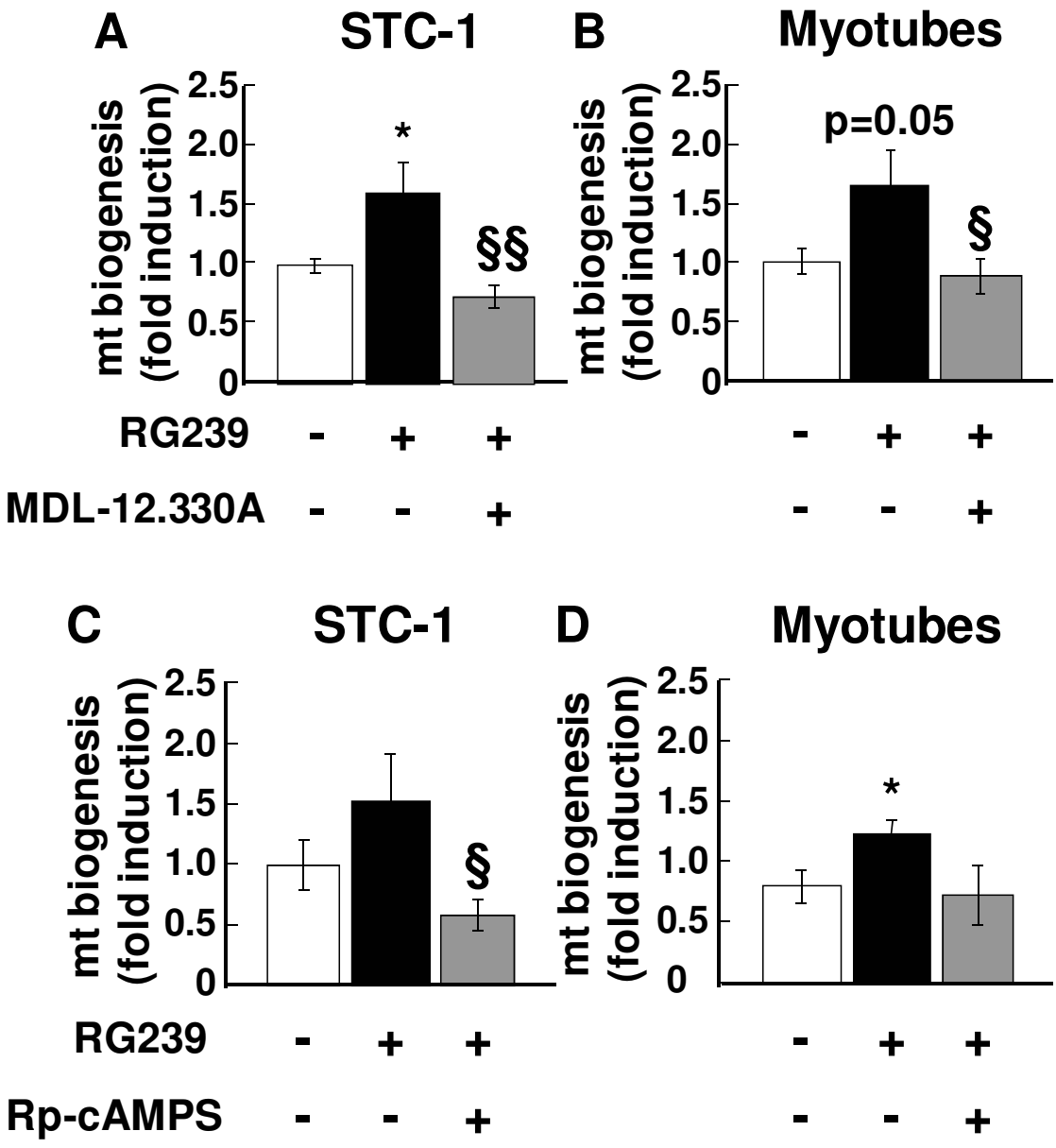
A
STC-1
B
Myotubes
mt biogenesis (fold induction)
2.5
2.0
1.5
1.0
0.5
0
*
§§
p=0.05
§
RG239
MDL-12.330A
C
STC-1
D
Myotubes
Rp-cAMPS

Figure 4

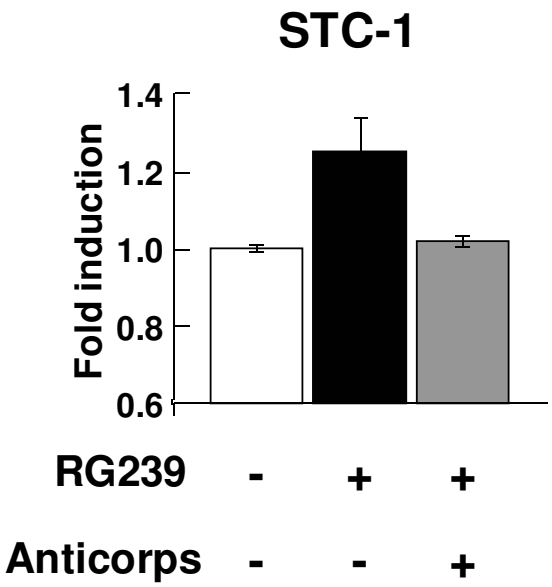

Figure 5

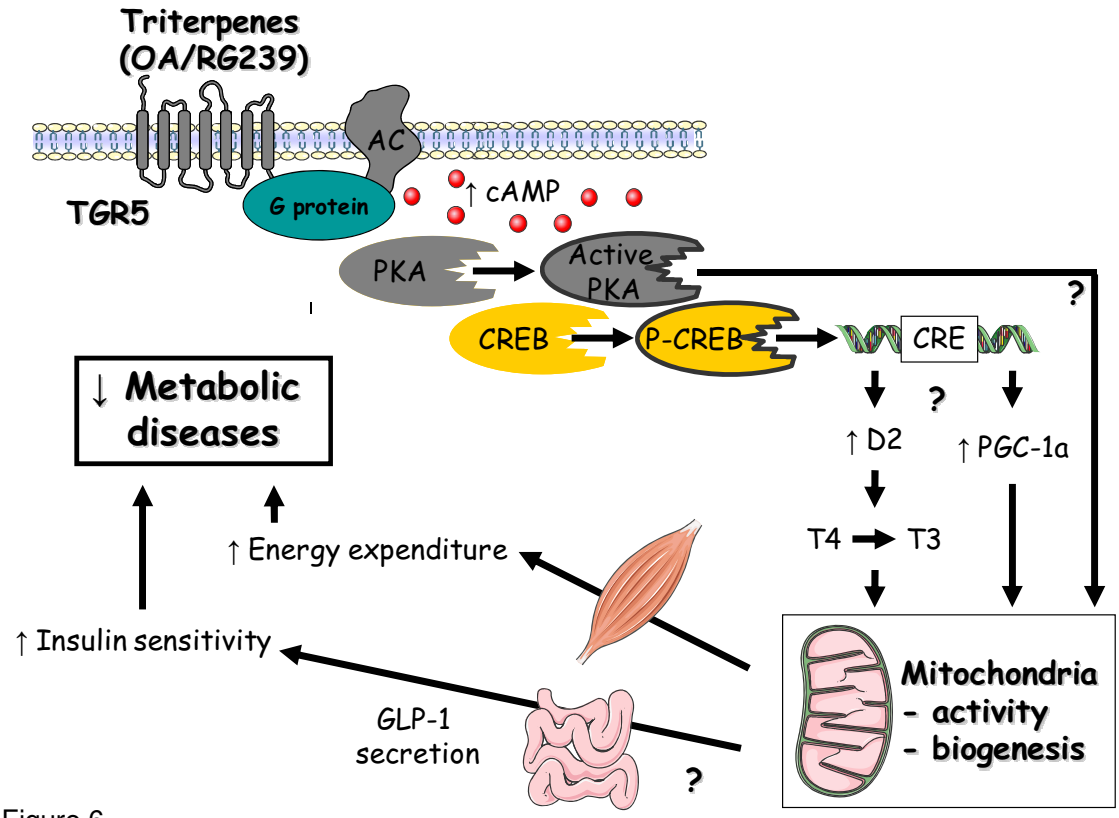
Triterpenes
(OA/RG239)
TGR5
G protein
AC
↑ cAMP
PKA
Active
PKA
?
CREB
P-CREB
CRE
↓ Metabolic
diseases
?
↑ D2
↑ PGC-1a
T4 → T3
↑ Energy expenditure
↑ Insulin sensitivity
GLP-1
secretion
?
Mitochondria
- activity
- biogenesis

Figure 6

I. Materials

Oleanolic acid was purchased from Extrasynthèse (Genay, France). Oleuropein, Lithocholic acid, and Chenodeoxycholic acid were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France).

Synthesis of RG239 was described elsewhere (267). Rp-3',5'-cyclic Adenosine MonoPhosphorothioate (Rp-cAMPS) and *cis*-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine (MDL-12,330A) were purchased by Sigma-Aldrich (Saint-Quentin Fallavier, France). An antibody directed against TGR5 was produced by intradermally injection of the peptide N-KRDNPGPSTAYHTSSQC-C in a rabbit. The production of the peptide and the immunization of the rabbit were performed by the IGBMC core facilities.

II. Cells

Chinese hamster ovary (CHO) cells, COS-1 cells, mouse 3T3L1 cells and enteroendocrine L-cells (STC-1) were obtained from ATCC (MANASSAS, VA). Mouse myoblast (C2C12) cells were purchased by ECACC (Salisbury, UK).

CHO cells were maintained in minimum essential medium-alpha (α-MEM) supplemented with 10%C (v/v) foetal calf serum (FCS), 100µM non essential amino acid (NEAA), 100u/ml penicillin and 100µg/ml streptomycin-sulphate. They were used for the TGR5 luciferase assay. COS-1 cells were maintained in DMEM (1g/l glucose) supplemented with 10% (v/v) fetal bovine serum (FCS) and 100µg/ml gentamycine. They were used for the FXR luciferase assay.

Mouse 3T3L1 cells were maintained in DMEM supplemented with 10% (V/V) foetal bovine serum (FBS). Two days post confluency, adipocyte differentiation was induced with a mixture of IBMX 500 µM, dexamethasone 1 µM and insulin 10 µg/ml. After 7 days of differentiation, cells were used. C2C12 mouse myoblast cell line was maintained in DMEM (4,5g/l glucose)

supplemented with 10% FCS. Differentiation of myoblasts in myotubes was induced with media containing 2% horse serum (HS) during four days. STC-1 cells were maintained in DMEM (1g/l glucose) supplemented with 15% HS, 10 % FCS and 100µg/ml gentamycin. 3T3L1, C2C12 myotubes and STC-1 were treated for cytochrome c oxidase assay and mitochondrial DNA quantification. For the need of the experiment, STC-1 cells were transfected using JETPEI (PolyPlus Transfection, Illkirch, France).

III. Plant extraction

For ethanol extraction, 10 g of the air-dried powdered olive leaves (*Olea europaea* L. Oleaceae, PhytoEst, France) were exhaustively extracted with 80% aqueous ethanol by maceration at room temperature for 3 h. The mixture was then filtered on a suction pump and the filtrate evaporated under reduced pressure. For dichloromethane and methanol extraction, the powdered plant material (10–20 g) was successively extracted with cyclohexane (100 mL), dichloromethane (100 mL), and methanol (100 mL), using an automatic soxhlet apparatus (2055 Aventi Soxtex, Foss Tecator). This procedure generates three types of extracts which concentrate respectively the very lipophilic, the nonpolar and the polar constituents of the plant. The solvent was evaporated under reduced pressure and the corresponding residues were weighted and then stored in the freeze until use.

IV. HPLC and NMR analysis

For the analytical profiles of each extract, a methanolic solution of the residue (5 mg/mL) was subjected to analytical HPLC (Varian Pro Star) coupled with a DAD detector (Varian), using a reversed phase column (Nucleodur 100-10-C18, 250/4.6, Macherey-Nagel). A mixture of acetonitrile (CH_3CN) and acidified water (0.1% trifluoroacetic acid) was used for elution. In analytical conditions, the flow rate was 1 mL/min with the following gradient: H_2O/CH_3CN (20:80) for 30 min, CH_3CN 100% for 5 min, and H_2O/CH_3CN (20:80) for 5 min.

For LC–MS (Agilent 1200SL), the analyses were performed with APCI coupled with an electrospray ionization (ESI) interface in negative mode.

A DPX300 NMR spectrometer operating at 300 MHz for 1H was used for NMR experiments. Chemicals shifts are expressed in δ (parts per million) referring to the solvent peak 7.58 for pyridine-d5 C5D5N.

V. TGR5 luciferase assay.

Chinese hamster ovary (CHO) cells were obtained from ATCC (Manassas, VA) and were maintained in minimum essential medium alpha (α-MEM) supplemented with 10% (v/v) foetal bovine serum (FBS), 100 IM nonessential amino acids (NEAA), 100 U/ ml penicillin, and 100 µg/ml streptomycin sulphate. For the TGR5 assay, a stable cell line was obtained by transfection of CHO cells with 3.8 µg of human TGR5 expression plasmid (pCMVSPOR6/hTGR5) or mouse TGR5 expression plasmid (pCMVSPORT6/mTGR5), 3.8 µg of CRE-driven luciferase reporter plasmid (pCRE-Luc), and 0.4 µg of neomycin-resistant gene expression plasmid (pcDNA3.1(+)) using Lipofectamine 2000 reagent (Invitrogen, Cergy Pontoise, France). The transfected cells were selected with 400 µg/ml G418 sulphate and single clones were grown in 96-well plate, independently. TGR5-expressing CHO cells were treated with 10 µM lithocholic acid (LCA) or plant extracts or triterpenoids or triterpenoid derivatives, followed by luciferase assays [11]. Luminescence was determined with CentroXS3 LB960 (Berthold Technologies, Bad Wildbad, Germany).

VI. FXR luciferase assay.

To evaluate FXR activity of compounds, COS-1 cells were transfected with 25 ng of hFXR expression plasmid (pCMX-hFXR), 25 ng of mouse (m) retinoid X receptor α (RXRα) expression plasmid (pCMX-mRXRα), 50 ng of reporter plasmid (pEcRE·7-Luc) and 50 ng of pCMVβ as internal control in each well, using the Lipofectamine 2000 reagent. About 18h

after transfections, cells were incubated for 24 h with different concentrations of each compound in fresh DMEM. After this treatment, the cells were lyzed and normalized luciferase activity was determined [11].

VII. Animals and diet

The first experiment on animals was performed to test OA ±high fat diet. Six week old male C57BL/6 J mice were purchased from Charles River. The chow diet (SAFE, DO4) and the high fat diet (60% kcal Fat, D12492) were purchased from Research Diets (New Brunswick, NJ). The mice were housed by 4 or 5 under controlled temperature and 12 h light–dark cycle and had free access to food and water. Animals were kept for 10 weeks either on chow diet (n = 5) or on high fat diet (n = 16). After 10 weeks on high fat, the animals were divided in two groups of 8 animals: one group was kept on high fat diet only, the other received the same food supplemented with oleanolic acid at a 100 mg/kg/ day dose (food intake was measured throughout the study and the amount of drug was adjusted to keep the dosing stable). Body weight and food intake was determined every other day. The experiment was carried out according to the ethical guidelines.

The second experiment on animals was realized to test RG239 ±high fat diet. Seven weeks old male C57BL/6J mice were purchased from Charles River. The high fat diet (60% kcal Fat, D12492) was purchased from Research Diet –New Brunnswick, NJ). The mice were housed by 5 under controlled temperature and 12h light-dark cycle and have free access to food and water. Animals were kept for 10 weeks on high fat diet (n=30). After 10 weeks on high fat, the animal were divided in three groups of 10 animals: one group was kept on high fat diet only, the second received the same food supplemented with RG239 compound at a 30 mg/kg/day dose and the third received the same food supplemented with oleanolic acid at a 50 mg/kg/day dose. The food intake was measured throughout the study and the amount of drug was adjusted to keep the dosing stable. Body weight and food intake

was determined every week. The experiment was carried out according to the ethical guidelines.

VIII. Glucose tolerance test

Glucose tolerance test was performed on animals that were fasted 10 h. Blood glucose levels were monitored using a handheld glucometer (Maxi Kit Glucometer 4, Bayer Diagnostic). For the test, a sterile solution containing 2 g of glucose/kg body weight was injected intraperitoneally. Blood glucose levels were sampled from tail vein blood at 0, 15, 30, 45, 60, 90, 120, 150, and 180 min. Fasting blood glucose and insulin levels were measured after 7 days of treatment. Mice were deprived of food for 10 h and the blood levels were quantified with Glucose RTU (BioMerieux Inc.) for glucose and by ELISA (Mercodia, Uppsala, Sweden) for insulin.

IX. Cytochrome c oxidase assay

Differenciated 3T3-L1 cells, STC-1 cells or C2C12 myotubes were treated with compounds and then lysed (20mM HEPES, 0.1%Triton, 1mM EDTA). Cell lysate was incubated with reduced cytochrome c (from equine heart, Sigma). Disappearance of reduced cytochrome c was followed spectrophotometrically at 550nM (according to the manufacturer's instruction, Sigma).

X. Mitochondrial DNA quantification.

STC-1 cells or C2C12 myotubes were treated with RG239 ± compounds. The cells were then lysed with a lysis buffer allowing DNA extraction by conventional phenol-chloroform method (10mM Tris, 1mM EDTA, 0.3M NaAc, 1% SDS). DNA was quantified by Q-PCR on sixplicate using 10µM each primer (mitochondrial DNA specific PCR (16S rRNA), forward 5'-CCGCAAGGGAAAGATGAAAGAC-3', reverse 5'- TCGTTTGGTTTCGGGGTTTC-3' ; and

nuclear specific PCR (UCP2), forward 5'-CTACAGATGTGGTAAAGGTCCGC-3', reverse 5'-GCAATGGTCTTGTAGGCTTCG-3') and QuantiTect SYBR Green PCR Kit (Sigma-Aldrich, Saint-Quentin Fallavier, France), in a LightCycler 480 (Roche diagnostics, Mannheim, Germany) with a program of 20 minutes at 95°C, followed by 45 to 50 cycles of 15 seconds at 95°C, 20 seconds at 56°C and 20 seconds at 72°C. Single-product amplification was verified by an integrated post-run melting curve analysis. Exponential amplification efficiency was verified during each PCR run using a standard dilution series made from pooled samples.

XI. GLP-1 release.

STC-1 cells were treated with RG239 ± compounds. The incubated medium was collected and the concentration of GLP-1 was determined by enzyme immunoassay with a specific GLP-1 amide Enzyme Immunoassay Kit (Millipore).

1. **Vignais PV** 2002 The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cell Mol Life Sci 59:1428-1459
2. **Coon MJ, Ding XX, Pernecky SJ, Vaz AD** 1992 Cytochrome P450: progress and predictions. Faseb J 6:669-673
3. **Yokoyama Y, Beckman JS, Beckman TK, Wheat JK, Cash TG, Freeman BA, Parks DA** 1990 Circulating xanthine oxidase: potential mediator of ischemic injury. Am J Physiol 258:G564-570
4. **Turrens JF** 2003 Mitochondrial formation of reactive oxygen species. J Physiol 552:335-344
5. **St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD** 2002 Topology of superoxide production from different sites in the mitochondrial electron transport chain. J Biol Chem 277:44784-44790
6. **Boveris A, Chance B** 1973 The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 134:707-716
7. **Votyakova TV, Reynolds IJ** 2001 DeltaPsi(m)-Dependent and -independent production of reactive oxygen species by rat brain mitochondria. J Neurochem 79:266-277
8. **Costa NJ, Dahm CC, Hurrell F, Taylor ER, Murphy MP** 2003 Interactions of mitochondrial thiols with nitric oxide. Antioxid Redox Signal 5:291-305
9. **Beyer RE** 1990 The participation of coenzyme Q in free radical production and antioxidation. Free Radic Biol Med 8:545-565
10. **Brand MD, Brindle KM, Buckingham JA, Harper JA, Rolfe DF, Stuart JA** 1999 The significance and mechanism of mitochondrial proton conductance. Int J Obes Relat Metab Disord 23 Suppl 6:S4-11
11. **Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC, Brand MD** 2002 Superoxide activates mitochondrial uncoupling proteins. Nature 415:96-99
12. **Skulachev VP** 1997 Membrane-linked systems preventing superoxide formation. Biosci Rep 17:347-366
13. **Nicholls DG, Locke RM** 1984 Thermogenic mechanisms in brown fat. Physiol Rev 64:1-64
14. **Cannon B, Nedergaard J** 2004 Brown adipose tissue: function and physiological significance. Physiol Rev 84:277-359
15. **Nedergaard J, Bengtsson T, Cannon B** 2007 Unexpected evidence for active brown adipose tissue in adult humans. Am J Physiol Endocrinol Metab 293:E444-452
16. **Seale P, Kajimura S, Spiegelman BM** 2009 Transcriptional control of brown adipocyte development and physiological function--of mice and men. Genes Dev 23:788-797
17. **Klingenberg M, Echtay KS** 2001 Uncoupling proteins: the issues from a biochemist point of view. Biochim Biophys Acta 1504:128-143
18. **Watanabe M, Yamamoto T, Mori C, Okada N, Yamazaki N, Kajimoto K, Kataoka M, Shinohara Y** 2008 Cold-induced changes in gene expression in brown adipose tissue: implications for the activation of thermogenesis. Biol Pharm Bull 31:775-784
19. **Unami A, Shinohara Y, Kajimoto K, Baba Y** 2004 Comparison of gene expression profiles between white and brown adipose tissues of rat by microarray analysis. Biochem Pharmacol 67:555-564

20. **Enerback S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, Kozak LP** 1997 Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. Nature 387:90-94
21. **Gong DW, Monemdjou S, Gavrilova O, Leon LR, Marcus-Samuels B, Chou CJ, Everett C, Kozak LP, Li C, Deng C, Harper ME, Reitman ML** 2000 Lack of obesity and normal response to fasting and thyroid hormone in mice lacking uncoupling protein-3. J Biol Chem 275:16251-16257
22. **Echtay KS** 2007 Mitochondrial uncoupling proteins--what is their physiological role? Free Radic Biol Med 43:1351-1371
23. **Zhou YT, Shimabukuro M, Koyama K, Lee Y, Wang MY, Trieu F, Newgard CB, Unger RH** 1997 Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation. Proc Natl Acad Sci U S A 94:6386-6390
24. **Zhang CY, Baffy G, Perret P, Krauss S, Peroni O, Grujic D, Hagen T, Vidal-Puig AJ, Boss O, Kim YB, Zheng XX, Wheeler MB, Shulman GI, Chan CB, Lowell BB** 2001 Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. Cell 105:745-755
25. **Chan CB, De Leo D, Joseph JW, McQuaid TS, Ha XF, Xu F, Tsushima RG, Pennefather PS, Salapatek AM, Wheeler MB** 2001 Increased uncoupling protein-2 levels in beta-cells are associated with impaired glucose-stimulated insulin secretion: mechanism of action. Diabetes 50:1302-1310
26. **Dulloo AG, Samec S** 2001 Uncoupling proteins: their roles in adaptive thermogenesis and substrate metabolism reconsidered. Br J Nutr 86:123-139
27. **Schrauwen P, Hesselink MK, Blaak EE, Borghouts LB, Schaart G, Saris WH, Keizer HA** 2001 Uncoupling protein 3 content is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes 50:2870-2873
28. **Andrews ZB, Diano S, Horvath TL** 2005 Mitochondrial uncoupling proteins in the CNS: in support of function and survival. Nat Rev Neurosci 6:829-840
29. **Wild S, Roglic G, Green A, Sicree R, King H** 2004 Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27:1047-1053
30. **Morgan NG** 2009 Fatty acids and beta-cell toxicity. Curr Opin Clin Nutr Metab Care 12:117-122
31. **Ceriello A, Motz E** 2004 Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. Arterioscler Thromb Vasc Biol 24:816-823
32. **Lowell BB, Shulman GI** 2005 Mitochondrial dysfunction and type 2 diabetes. Science 307:384-387
33. **Houstis N, Rosen ED, Lander ES** 2006 Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 440:944-948
34. **Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M** 2000 Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. Proc Natl Acad Sci U S A 97:12222-12226
35. **Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M** 2000 Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404:787-790
36. **Robertson RP** 2004 Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. J Biol Chem 279:42351-42354

37. **Brownlee M** 2001 Biochemistry and molecular cell biology of diabetic complications. Nature 414:813-820
38. **Rolo AP, Palmeira CM** 2006 Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. Toxicol Appl Pharmacol 212:167-178
39. **Yu T, Robotham JL, Yoon Y** 2006 Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci U S A 103:2653-2658
40. **Maassen JA, LM TH, Van Essen E, Heine RJ, Nijpels G, Jahangir Tafrechi RS, Raap AK, Janssen GM, Lemkes HH** 2004 Mitochondrial diabetes: molecular mechanisms and clinical presentation. Diabetes 53 Suppl 1:S103-109
41. **Kelly DP, Scarpulla RC** 2004 Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev 18:357-368
42. **Puigserver P, Spiegelman BM** 2003 Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. Endocr Rev 24:78-90
43. **Benton CR, Wright DC, Bonen A** 2008 PGC-1alpha-mediated regulation of gene expression and metabolism: implications for nutrition and exercise prescriptions. Appl Physiol Nutr Metab 33:843-862
44. **Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ** 2003 Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci U S A 100:8466-8471
45. **Jove M, Salla J, Planavila A, Cabrero A, Michalik L, Wahli W, Laguna JC, Vazquez-Carrera M** 2004 Impaired expression of NADH dehydrogenase subunit 1 and PPARgamma coactivator-1 in skeletal muscle of ZDF rats: restoration by troglitazone. J Lipid Res 45:113-123
46. **Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI** 2004 Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med 350:664-671
47. **Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, Neschen S, White MF, Bilz S, Sono S, Pypaert M, Shulman GI** 2005 Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. J Clin Invest 115:3587-3593
48. **Befroy DE, Petersen KF, Dufour S, Mason GF, de Graaf RA, Rothman DL, Shulman GI** 2007 Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. Diabetes 56:1376-1381
49. **Kahn SE** 2003 The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. Diabetologia 46:3-19
50. **Levine JA, Eberhardt NL, Jensen MD** 1999 Role of nonexercise activity thermogenesis in resistance to fat gain in humans. Science 283:212-214
51. **Weyer C, Walford RL, Harper IT, Milner M, MacCallum T, Tataranni PA, Ravussin E** 2000 Energy metabolism after 2 y of energy restriction: the biosphere 2 experiment. Am J Clin Nutr 72:946-953
52. **Palmeira CM, Rolo AP, Berthiaume J, Bjork JA, Wallace KB** 2007 Hyperglycemia decreases mitochondrial function: the regulatory role of mitochondrial biogenesis. Toxicol Appl Pharmacol 225:214-220
53. **Li G, Hu Y, Yang W, Jiang Y, Wang J, Xiao J, Hu Z, Pan X, Howard BV, Bennett PH** 2002 Effects of insulin resistance and insulin secretion on the efficacy of

interventions to retard development of type 2 diabetes mellitus: the DA Qing IGT and Diabetes Study. Diabetes Res Clin Pract 58:193-200
54. **Norris SL, Zhang X, Avenell A, Gregg E, Schmid CH, Lau J** 2005 Long-term non-pharmacological weight loss interventions for adults with prediabetes. Cochrane Database Syst Rev:CD005270
55. **Yamaoka K, Tango T** 2005 Efficacy of lifestyle education to prevent type 2 diabetes: a meta-analysis of randomized controlled trials. Diabetes Care 28:2780-2786
56. **Gillies CL, Abrams KR, Lambert PC, Cooper NJ, Sutton AJ, Hsu RT, Khunti K** 2007 Pharmacological and lifestyle interventions to prevent or delay type 2 diabetes in people with impaired glucose tolerance: systematic review and meta-analysis. Bmj 334:299
57. **Brynes AE, Lee JL, Brighton RE, Leeds AR, Dornhorst A, Frost GS** 2003 A low glycemic diet significantly improves the 24-h blood glucose profile in people with type 2 diabetes, as assessed using the continuous glucose MiniMed monitor. Diabetes Care 26:548-549
58. **Lindgren P, Lindstrom J, Tuomilehto J, Uusitupa M, Peltonen M, Jonsson B, de Faire U, Hellenius ML** 2007 Lifestyle intervention to prevent diabetes in men and women with impaired glucose tolerance is cost-effective. Int J Technol Assess Health Care 23:177-183
59. 1997 Translation of the diabetes nutrition recommendations for health care institutions. American Diabetes Association. Diabetes Care 20:106-108
60. **Garg A** 1998 High-monounsaturated-fat diets for patients with diabetes mellitus: a meta-analysis. Am J Clin Nutr 67:577S-582S
61. **Roth GS, Ingram DK, Lane MA** 2001 Caloric restriction in primates and relevance to humans. Ann N Y Acad Sci 928:305-315
62. **Lane MA, Ingram DK, Roth GS** 1999 Calorie restriction in nonhuman primates: effects on diabetes and cardiovascular disease risk. Toxicol Sci 52:41-48
63. **Mayer-Davis EJ, Costacou T, King I, Zaccaro DJ, Bell RA** 2002 Plasma and dietary vitamin E in relation to incidence of type 2 diabetes: The Insulin Resistance and Atherosclerosis Study (IRAS). Diabetes Care 25:2172-2177
64. **Montonen J, Knekt P, Jarvinen R, Reunanen A** 2004 Dietary antioxidant intake and risk of type 2 diabetes. Diabetes Care 27:362-366
65. **Liu S, Ajani U, Chae C, Hennekens C, Buring JE, Manson JE** 1999 Long-term beta-carotene supplementation and risk of type 2 diabetes mellitus: a randomized controlled trial. Jama 282:1073-1075
66. **Lonn E, Yusuf S, Hoogwerf B, Pogue J, Yi Q, Zinman B, Bosch J, Dagenais G, Mann JF, Gerstein HC** 2002 Effects of vitamin E on cardiovascular and microvascular outcomes in high-risk patients with diabetes: results of the HOPE study and MICRO-HOPE substudy. Diabetes Care 25:1919-1927
67. **Sacco M, Pellegrini F, Roncaglioni MC, Avanzini F, Tognoni G, Nicolucci A** 2003 Primary prevention of cardiovascular events with low-dose aspirin and vitamin E in type 2 diabetic patients: results of the Primary Prevention Project (PPP) trial. Diabetes Care 26:3264-3272
68. **Stranges S, Marshall JR, Natarajan R, Donahue RP, Trevisan M, Combs GF, Cappuccio FP, Ceriello A, Reid ME** 2007 Effects of long-term selenium supplementation on the incidence of type 2 diabetes: a randomized trial. Ann Intern Med 147:217-223
69. **Czernichow S, Couthouis A, Bertrais S, Vergnaud AC, Dauchet L, Galan P, Hercberg S** 2006 Antioxidant supplementation does not affect fasting plasma glucose in the Supplementation with Antioxidant Vitamins and Minerals (SU.VI.MAX) study

in France: association with dietary intake and plasma concentrations. Am J Clin Nutr 84:395-399

70. **Bouatia-Naji N, Bonnefond A, Cavalcanti-Proenca C, Sparso T, Holmkvist J, Marchand M, Delplanque J, Lobbens S, Rocheleau G, Durand E, De Graeve F, Chevre JC, Borch-Johnsen K, Hartikainen AL, Ruokonen A, Tichet J, Marre M, Weill J, Heude B, Tauber M, Lemaire K, Schuit F, Elliott P, Jorgensen T, Charpentier G, Hadjadj S, Cauchi S, Vaxillaire M, Sladek R, Visvikis-Siest S, Balkau B, Levy-Marchal C, Pattou F, Meyre D, Blakemore AI, Jarvelin MR, Walley AJ, Hansen T, Dina C, Pedersen O, Froguel P** 2009 A variant near MTNR1B is associated with increased fasting plasma glucose levels and type 2 diabetes risk. Nat Genet 41:89-94
71. **Kelley DE, Kuller LH, McKolanis TM, Harper P, Mancino J, Kalhan S** 2004 Effects of moderate weight loss and orlistat on insulin resistance, regional adiposity, and fatty acids in type 2 diabetes. Diabetes Care 27:33-40
72. **Torgerson JS, Hauptman J, Boldrin MN, Sjostrom L** 2004 XENical in the prevention of diabetes in obese subjects (XENDOS) study: a randomized study of orlistat as an adjunct to lifestyle changes for the prevention of type 2 diabetes in obese patients. Diabetes Care 27:155-161
73. **Tian WX, Li LC, Wu XD, Chen CC** 2004 Weight reduction by Chinese medicinal herbs may be related to inhibition of fatty acid synthase. Life Sci 74:2389-2399
74. **Astrup A, Toubro S, Cannon S, Hein P, Breum L, Madsen J** 1990 Caffeine: a double-blind, placebo-controlled study of its thermogenic, metabolic, and cardiovascular effects in healthy volunteers. Am J Clin Nutr 51:759-767
75. **Sullivan AC, Hamilton JG, Miller ON, Wheatley VR** 1972 Inhibition of lipogenesis in rat liver by (-)-hydroxycitrate. Arch Biochem Biophys 150:183-190
76. **Lepore M, Pampanelli S, Fanelli C, Porcellati F, Bartocci L, Di Vincenzo A, Cordoni C, Costa E, Brunetti P, Bolli GB** 2000 Pharmacokinetics and pharmacodynamics of subcutaneous injection of long-acting human insulin analog glargine, NPH insulin, and ultralente human insulin and continuous subcutaneous infusion of insulin lispro. Diabetes 49:2142-2148
77. **Haak T, Tiengo A, Draeger E, Suntum M, Waldhausl W** 2005 Lower within-subject variability of fasting blood glucose and reduced weight gain with insulin detemir compared to NPH insulin in patients with type 2 diabetes. Diabetes Obes Metab 7:56-64
78. **Hermansen K, Davies M, Derezinski T, Martinez Ravn G, Clauson P, Home P** 2006 A 26-week, randomized, parallel, treat-to-target trial comparing insulin detemir with NPH insulin as add-on therapy to oral glucose-lowering drugs in insulin-naive people with type 2 diabetes. Diabetes Care 29:1269-1274
79. **Rosenstock J, Schwartz SL, Clark CM, Jr., Park GD, Donley DW, Edwards MB** 2001 Basal insulin therapy in type 2 diabetes: 28-week comparison of insulin glargine (HOE 901) and NPH insulin. Diabetes Care 24:631-636
80. **Zimmerman BR** 1997 Sulfonylureas. Endocrinol Metab Clin North Am 26:511-522
81. **Doar JW, Thompson ME, Wilde CE, Sewell PF** 1976 Diet and oral antidiabetic drugs and plasma sugar and insulin levels in patients with maturity-onset diabetes mellitus. Br Med J 1:498-500
82. 1998 Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet 352:837-853
83. **Perfetti R, Ahmad A** 2000 Novel sulfonylurea and non-sulfonylurea drugs to promote the secretion of insulin. Trends Endocrinol Metab 11:218-223

84. **Nattrass M, Lauritzen T** 2000 Review of prandial glucose regulation with repaglinide: a solution to the problem of hypoglycaemia in the treatment of Type 2 diabetes? Int J Obes Relat Metab Disord 24 Suppl 3:S21-31
85. **Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM** 2002 Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med 346:393-403
86. **Orchard TJ, Temprosa M, Goldberg R, Haffner S, Ratner R, Marcovina S, Fowler S** 2005 The effect of metformin and intensive lifestyle intervention on the metabolic syndrome: the Diabetes Prevention Program randomized trial. Ann Intern Med 142:611-619
87. **Tessier D, Maheux P, Khalil A, Fulop T** 1999 Effects of gliclazide versus metformin on the clinical profile and lipid peroxidation markers in type 2 diabetes. Metabolism 48:897-903
88. **Bailey CJ, Turner RC** 1996 Metformin. N Engl J Med 334:574-579
89. **Inzucchi SE, Maggs DG, Spollett GR, Page SL, Rife FS, Walton V, Shulman GI** 1998 Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. N Engl J Med 338:867-872
90. **Collins R, Armitage J, Parish S, Sleigh P, Peto R** 2003 MRC/BHF Heart Protection Study of cholesterol-lowering with simvastatin in 5963 people with diabetes: a randomised placebo-controlled trial. Lancet 361:2005-2016
91. **Colhoun HM, Betteridge DJ, Durrington PN, Hitman GA, Neil HA, Livingstone SJ, Thomason MJ, Mackness MI, Charlton-Menys V, Fuller JH** 2004 Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial. Lancet 364:685-696
92. **Kearney PM, Blackwell L, Collins R, Keech A, Simes J, Peto R, Armitage J, Baigent C** 2008 Efficacy of cholesterol-lowering therapy in 18,686 people with diabetes in 14 randomised trials of statins: a meta-analysis. Lancet 371:117-125
93. **Forcheron F, Cachefo A, Thevenon S, Pinteur C, Beylot M** 2002 Mechanisms of the triglyceride- and cholesterol-lowering effect of fenofibrate in hyperlipidemic type 2 diabetic patients. Diabetes 51:3486-3491
94. **Jonkers IJ, Smelt AH, Hattori H, Scheek LM, van Gent T, de Man FH, van der Laarse A, van Tol A** 2003 Decreased PLTP mass but elevated PLTP activity linked to insulin resistance in HTG: effects of bezafibrate therapy. J Lipid Res 44:1462-1469
95. **Willson TM, Brown PJ, Sternbach DD, Henke BR** 2000 The PPARs: from orphan receptors to drug discovery. J Med Chem 43:527-550
96. **Lehrke M, Lazar MA** 2005 The many faces of PPARgamma. Cell 123:993-999
97. **Mayerson AB, Hundal RS, Dufour S, Lebon V, Befroy D, Cline GW, Enocksson S, Inzucchi SE, Shulman GI, Petersen KF** 2002 The effects of rosiglitazone on insulin sensitivity, lipolysis, and hepatic and skeletal muscle triglyceride content in patients with type 2 diabetes. Diabetes 51:797-802
98. **Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y** 2001 PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. Diabetes 50:2094-2099
99. **Tontonoz P, Spiegelman BM** 2008 Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem 77:289-312
100. **Campbell IW** 2004 Long-term glycaemic control with pioglitazone in patients with type 2 diabetes. Int J Clin Pract 58:192-200

101. **Smith SA, Porter LE, Biswas N, Freed MI** 2004 Rosiglitazone, but not glyburide, reduces circulating proinsulin and the proinsulin:insulin ratio in type 2 diabetes. J Clin Endocrinol Metab 89:6048-6053
102. **Gerstein HC, Yusuf S, Bosch J, Pogue J, Sheridan P, Dinccag N, Hanefeld M, Hoogwerf B, Laakso M, Mohan V, Shaw J, Zinman B, Holman RR** 2006 Effect of rosiglitazone on the frequency of diabetes in patients with impaired glucose tolerance or impaired fasting glucose: a randomised controlled trial. Lancet 368:1096-1105
103. **Dagenais GR, Gerstein HC, Holman R, Budaj A, Escalante A, Hedner T, Keltai M, Lonn E, McFarlane S, McQueen M, Teo K, Sheridan P, Bosch J, Pogue J, Yusuf S** 2008 Effects of ramipril and rosiglitazone on cardiovascular and renal outcomes in people with impaired glucose tolerance or impaired fasting glucose: results of the Diabetes REduction Assessment with ramipril and rosiglitazone Medication (DREAM) trial. Diabetes Care 31:1007-1014
104. **Dormandy JA, Charbonnel B, Eckland DJ, Erdmann E, Massi-Benedetti M, Moules IK, Skene AM, Tan MH, Lefebvre PJ, Murray GD, Standl E, Wilcox RG, Wilhelmsen L, Betteridge J, Birkeland K, Golay A, Heine RJ, Koranyi L, Laakso M, Mokan M, Norkus A, Pirags V, Podar T, Scheen A, Scherbaum W, Schernthaner G, Schmitz O, Skrha J, Smith U, Taton J** 2005 Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. Lancet 366:1279-1289
105. **Hartung DM, Touchette DR, Bultemeier NC, Haxby DG** 2005 Risk of hospitalization for heart failure associated with thiazolidinedione therapy: a medicaid claims-based case-control study. Pharmacotherapy 25:1329-1336
106. **Buchanan TA, Xiang AH, Peters RK, Kjos SL, Marroquin A, Goico J, Ochoa C, Tan S, Berkowitz K, Hodis HN, Azen SP** 2002 Preservation of pancreatic beta-cell function and prevention of type 2 diabetes by pharmacological treatment of insulin resistance in high-risk hispanic women. Diabetes 51:2796-2803
107. **Finegood DT, McArthur MD, Kojwang D, Thomas MJ, Topp BG, Leonard T, Buckingham RE** 2001 Beta-cell mass dynamics in Zucker diabetic fatty rats. Rosiglitazone prevents the rise in net cell death. Diabetes 50:1021-1029
108. **Xiang AH, Peters RK, Kjos SL, Marroquin A, Goico J, Ochoa C, Kawakubo M, Buchanan TA** 2006 Effect of pioglitazone on pancreatic beta-cell function and diabetes risk in Hispanic women with prior gestational diabetes. Diabetes 55:517-522
109. **Staels B, Fruchart JC** 2005 Therapeutic roles of peroxisome proliferator-activated receptor agonists. Diabetes 54:2460-2470
110. **Guan Y, Hao C, Cha DR, Rao R, Lu W, Kohan DE, Magnuson MA, Redha R, Zhang Y, Breyer MD** 2005 Thiazolidinediones expand body fluid volume through PPARgamma stimulation of ENaC-mediated renal salt absorption. Nat Med 11:861-866
111. **Takeda Pharmaceuticals America I** 2002 Actos® (pioglitazone hydrochloride) tablets.
112. **Smith-Kline-Beecham-Pharmaceuticals** 2001 Avandia® (rosiglitazone maleate) tablets.
113. **Van de Laar FA, Lucassen PL, Akkermans RP, Van de Lisdonk EH, Rutten GE, Van Weel C** 2005 Alpha-glucosidase inhibitors for type 2 diabetes mellitus. Cochrane Database Syst Rev:CD003639
114. **Goke B, Herrmann-Rinke C** 1998 The evolving role of alpha-glucosidase inhibitors. Diabetes Metab Rev 14 Suppl 1:S31-38

115. **Chiasson JL, Josse RG, Gomis R, Hanefeld M, Karasik A, Laakso M** 2002 Acarbose for prevention of type 2 diabetes mellitus: the STOP-NIDDM randomised trial. Lancet 359:2072-2077
116. **Hoffmann J, Spengler M** 1997 Efficacy of 24-week monotherapy with acarbose, metformin, or placebo in dietary-treated NIDDM patients: the Essen-II Study. Am J Med 103:483-490
117. **Hou W, Li Y, Zhang Q, Wei X, Peng A, Chen L, Wei Y** 2009 Triterpene acids isolated from Lagerstroemia speciosa leaves as alpha-glucosidase inhibitors. Phytother Res 23:614-618
118. **Tahraoui A, El-Hilaly J, Israili ZH, Lyoussi B** 2007 Ethnopharmacological survey of plants used in the traditional treatment of hypertension and diabetes in south-eastern Morocco (Errachidia province). J Ethnopharmacol 110:105-117
119. **Zhu XM, Xie P, Di YT, Peng SL, Ding LS, Wang MK** 2008 Two new triterpenoid saponins from Gymnema sylvestre. J Integr Plant Biol 50:589-592
120. **Cefalu WT, Ye J, Zuberi A, Ribnicky DM, Raskin I, Liu Z, Wang ZQ, Brantley PJ, Howard L, Lefevre M** 2008 Botanicals and the metabolic syndrome. Am J Clin Nutr 87:481S-487S
121. **Cao H, Hininger-Favier I, Kelly MA, Benaraba R, Dawson HD, Coves S, Roussel AM, Anderson RA** 2007 Green tea polyphenol extract regulates the expression of genes involved in glucose uptake and insulin signaling in rats fed a high fructose diet. J Agric Food Chem 55:6372-6378
122. **Mollah ML, Kim GS, Moon HK, Chung SK, Cheon YP, Kim JK, Kim KS** 2009 Antiobesity effects of wild ginseng (Panax ginseng C.A. Meyer) mediated by PPAR-gamma, GLUT4 and LPL in ob/ob mice. Phytother Res 23:220-225
123. **Al-Azzawie HF, Alhamdani MS** 2006 Hypoglycemic and antioxidant effect of oleuropein in alloxan-diabetic rabbits. Life Sci 78:1371-1377
124. **Egawa K, Maegawa H, Shimizu S, Morino K, Nishio Y, Bryer-Ash M, Cheung AT, Kolls JK, Kikkawa R, Kashiwagi A** 2001 Protein-tyrosine phosphatase-1B negatively regulates insulin signaling in l6 myocytes and Fao hepatoma cells. J Biol Chem 276:10207-10211
125. **Agius L** 2008 Glucokinase and molecular aspects of liver glycogen metabolism. Biochem J 414:1-18
126. **Guan T, Li Y, Sun H, Tang X, Qian Y** 2009 Effects of Maslinic Acid, a Natural Triterpene, on Glycogen Metabolism in Cultured Cortical Astrocytes. Planta Med
127. **Rui L, Fisher TL, Thomas J, White MF** 2001 Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2. J Biol Chem 276:40362-40367
128. **Rui L, Yuan M, Frantz D, Shoelson S, White MF** 2002 SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem 277:42394-42398
129. **Hicke L, Dunn R** 2003 Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu Rev Cell Dev Biol 19:141-172
130. **Khal J, Hine AV, Fearon KC, Dejong CH, Tisdale MJ** 2005 Increased expression of proteasome subunits in skeletal muscle of cancer patients with weight loss. Int J Biochem Cell Biol 37:2196-2206
131. **Stegmeier F, Rape M, Draviam VM, Nalepa G, Sowa ME, Ang XL, McDonald ER, 3rd, Li MZ, Hannon GJ, Sorger PK, Kirschner MW, Harper JW, Elledge SJ** 2007 Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities. Nature 446:876-881

132. **Nawrocki AR, Rajala MW, Tomas E, Pajvani UB, Saha AK, Trumbauer ME, Pang Z, Chen AS, Ruderman NB, Chen H, Rossetti L, Scherer PE** 2006 Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor gamma agonists. J Biol Chem 281:2654-2660
133. **Guigas B, Taleux N, Foretz M, Detaille D, Andreelli F, Viollet B, Hue L** 2007 AMP-activated protein kinase-independent inhibition of hepatic mitochondrial oxidative phosphorylation by AICA riboside. Biochem J 404:499-507
134. **Boon H, Bosselaar M, Praet SF, Blaak EE, Saris WH, Wagenmakers AJ, McGee SL, Tack CJ, Smits P, Hargreaves M, van Loon LJ** 2008 Intravenous AICAR administration reduces hepatic glucose output and inhibits whole body lipolysis in type 2 diabetic patients. Diabetologia 51:1893-1900
135. **da Silva Xavier G, Leclerc I, Varadi A, Tsuboi T, Moule SK, Rutter GA** 2003 Role for AMP-activated protein kinase in glucose-stimulated insulin secretion and preproinsulin gene expression. Biochem J 371:761-774
136. **Nishino Y, Miura T, Miki T, Sakamoto J, Nakamura Y, Ikeda Y, Kobayashi H, Shimamoto K** 2004 Ischemic preconditioning activates AMPK in a PKC-dependent manner and induces GLUT4 up-regulation in the late phase of cardioprotection. Cardiovasc Res 61:610-619
137. **Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, Mu J, Foufelle F, Ferre P, Birnbaum MJ, Stuck BJ, Kahn BB** 2004 AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428:569-574
138. **Smith JL, Patil PB, Fisher JS** 2005 AICAR and hyperosmotic stress increase insulin-stimulated glucose transport. J Appl Physiol 99:877-883
139. **Pold R, Jensen LS, Jessen N, Buhl ES, Schmitz O, Flyvbjerg A, Fujii N, Goodyear LJ, Gotfredsen CF, Brand CL, Lund S** 2005 Long-term AICAR administration and exercise prevents diabetes in ZDF rats. Diabetes 54:928-934
140. **Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, Auwerx J** 2009 AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 458:1056-1060
141. **Bertrand L, Ginion A, Beauloye C, Hebert AD, Guigas B, Hue L, Vanoverschelde JL** 2006 AMPK activation restores the stimulation of glucose uptake in an in vitro model of insulin-resistant cardiomyocytes via the activation of protein kinase B. Am J Physiol Heart Circ Physiol 291:H239-250
142. **Hojlund K, Mustard KJ, Staehr P, Hardie DG, Beck-Nielsen H, Richter EA, Wojtaszewski JF** 2004 AMPK activity and isoform protein expression are similar in muscle of obese subjects with and without type 2 diabetes. Am J Physiol Endocrinol Metab 286:E239-244
143. **Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE** 2001 Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 108:1167-1174
144. **Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R, Sinclair DA** 2006 Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444:337-342

145. **Collins QF, Liu HY, Pi J, Liu Z, Quon MJ, Cao W** 2007 Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5'-AMP-activated protein kinase. J Biol Chem 282:30143-30149
146. **Martinez SC, Tanabe K, Cras-Meneur C, Abumrad NA, Bernal-Mizrachi E, Permutt MA** 2008 Inhibition of Foxo1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. Diabetes 57:846-859
147. **Boden G, Homko C, Mozzoli M, Showe LC, Nichols C, Cheung P** 2005 Thiazolidinediones upregulate fatty acid uptake and oxidation in adipose tissue of diabetic patients. Diabetes 54:880-885
148. **Seber S, Ucak S, Basat O, Altuntas Y** 2006 The effect of dual PPAR alpha/gamma stimulation with combination of rosiglitazone and fenofibrate on metabolic parameters in type 2 diabetic patients. Diabetes Res Clin Pract 71:52-58
149. **Martin G, Schoonjans K, Lefebvre AM, Staels B, Auwerx J** 1997 Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators. J Biol Chem 272:28210-28217
150. **Liu KG, Lambert MH, Leesnitzer LM, Oliver W, Jr., Ott RJ, Plunket KD, Stuart LW, Brown PJ, Willson TM, Sternbach DD** 2001 Identification of a series of PPAR gamma/delta dual agonists via solid-phase parallel synthesis. Bioorg Med Chem Lett 11:2959-2962
151. **Barter P, Ginsberg HN** 2008 Effectiveness of combined statin plus omega-3 fatty acid therapy for mixed dyslipidemia. Am J Cardiol 102:1040-1045
152. **Karalis DG** 2008 The role of lipid-lowering therapy in preventing coronary heart disease in patients with type 2 diabetes. Clin Cardiol 31:241-248
153. **Taylor AJ, Lee HJ, Sullenberger LE** 2006 The effect of 24 months of combination statin and extended-release niacin on carotid intima-media thickness: ARBITER 3. Curr Med Res Opin 22:2243-2250
154. **Wanner C, Krane V, Marz W, Olschewski M, Mann JF, Ruf G, Ritz E** 2005 Atorvastatin in patients with type 2 diabetes mellitus undergoing hemodialysis. N Engl J Med 353:238-248
155. **Lin JK, Lin-Shiau SY** 2006 Mechanisms of hypolipidemic and anti-obesity effects of tea and tea polyphenols. Mol Nutr Food Res 50:211-217
156. **Kim HJ, Jeon SM, Lee MK, Jung UJ, Shin SK, Choi MS** 2009 Antilipogenic effect of green tea extract in C57BL/6J-Lep ob/ob mice. Phytother Res 23:467-471
157. **Chan LL, Chen Q, Go AG, Lam EK, Li ET** 2005 Reduced adiposity in bitter melon (Momordica charantia)-fed rats is associated with increased lipid oxidative enzyme activities and uncoupling protein expression. J Nutr 135:2517-2523
158. **Maeda H, Hosokawa M, Sashima T, Funayama K, Miyashita K** 2005 Fucoxanthin from edible seaweed, Undaria pinnatifida, shows antiobesity effect through UCP1 expression in white adipose tissues. Biochem Biophys Res Commun 332:392-397
159. **Orskov C, Wettergren A, Holst JJ** 1993 Biological effects and metabolic rates of glucagonlike peptide-1 7-36 amide and glucagonlike peptide-1 7-37 in healthy subjects are indistinguishable. Diabetes 42:658-661
160. **Marguet D, Baggio L, Kobayashi T, Bernard AM, Pierres M, Nielsen PF, Ribel U, Watanabe T, Drucker DJ, Wagtmann N** 2000 Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26. Proc Natl Acad Sci U S A 97:6874-6879
161. **Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF** 1987 Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. Proc Natl Acad Sci U S A 84:3434-3438

162. **Drucker DJ** 2006 The biology of incretin hormones. Cell Metab 3:153-165
163. **Farilla L, Bulotta A, Hirshberg B, Li Calzi S, Khoury N, Noushmehr H, Bertolotto C, Di Mario U, Harlan DM, Perfetti R** 2003 Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. Endocrinology 144:5149-5158
164. **Burcelin R, Da Costa A, Drucker D, Thorens B** 2001 Glucose competence of the hepatoportal vein sensor requires the presence of an activated glucagon-like peptide-1 receptor. Diabetes 50:1720-1728
165. **Miyawaki K, Yamada Y, Yano H, Niwa H, Ban N, Ihara Y, Kubota A, Fujimoto S, Kajikawa M, Kuroe A, Tsuda K, Hashimoto H, Yamashita T, Jomori T, Tashiro F, Miyazaki J, Seino Y** 1999 Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. Proc Natl Acad Sci U S A 96:14843-14847
166. **Scrocchi LA, Brown TJ, MaClusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ** 1996 Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. Nat Med 2:1254-1258
167. **Schirra J, Sturm K, Leicht P, Arnold R, Goke B, Katschinski M** 1998 Exendin(9-39)amide is an antagonist of glucagon-like peptide-1(7-36)amide in humans. J Clin Invest 101:1421-1430
168. **Nauck M, Stockmann F, Ebert R, Creutzfeldt W** 1986 Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. Diabetologia 29:46-52
169. **Toft-Nielsen MB, Madsbad S, Holst JJ** 2001 Determinants of the effectiveness of glucagon-like peptide-1 in type 2 diabetes. J Clin Endocrinol Metab 86:3853-3860
170. **Zander M, Madsbad S, Madsen JL, Holst JJ** 2002 Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. Lancet 359:824-830
171. **Penfornis A, Borot S, Raccah D** 2008 Therapeutic approach of type 2 diabetes mellitus with GLP-1 based therapies. Diabetes Metab 34 Suppl 2:S78-90
172. **Deacon CF, Knudsen LB, Madsen K, Wiberg FC, Jacobsen O, Holst JJ** 1998 Dipeptidyl peptidase IV resistant analogs of glucagon-like peptide-1 which have extended metabolic stability and improved biological activity. Diabetologia 41:271-278
173. **Knudsen LB, Nielsen PF, Huusfeldt PO, Johansen NL, Madsen K, Pedersen FZ, Thogersen H, Wilken M, Agerso H** 2000 Potent derivatives of glucagon-like peptide-1 with pharmacokinetic properties suitable for once daily administration. J Med Chem 43:1664-1669
174. **Agerso H, Jensen LB, Elbrond B, Rolan P, Zdravkovic M** 2002 The pharmacokinetics, pharmacodynamics, safety and tolerability of NN2211, a new long-acting GLP-1 derivative, in healthy men. Diabetologia 45:195-202
175. **Vilsboll T, Zdravkovic M, Le-Thi T, Krarup T, Schmitz O, Courreges JP, Verhoeven R, Buganova I, Madsbad S** 2007 Liraglutide, a long-acting human glucagon-like peptide-1 analog, given as monotherapy significantly improves glycemic control and lowers body weight without risk of hypoglycemia in patients with type 2 diabetes. Diabetes Care 30:1608-1610
176. **Simonsen L, Holst JJ, Deacon CF** 2006 Exendin-4, but not glucagon-like peptide-1, is cleared exclusively by glomerular filtration in anaesthetised pigs. Diabetologia 49:706-712
177. **Edwards CM, Stanley SA, Davis R, Brynes AE, Frost GS, Seal LJ, Ghatei MA, Bloom SR** 2001 Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers. Am J Physiol Endocrinol Metab 281:E155-161

178. **Amori RE, Lau J, Pittas AG** 2007 Efficacy and safety of incretin therapy in type 2 diabetes: systematic review and meta-analysis. Jama 298:194-206
179. **Klonoff DC, Buse JB, Nielsen LL, Guan X, Bowlus CL, Holcombe JH, Wintle ME, Maggs DG** 2008 Exenatide effects on diabetes, obesity, cardiovascular risk factors and hepatic biomarkers in patients with type 2 diabetes treated for at least 3 years. Curr Med Res Opin 24:275-286
180. **Lambeir AM, Durinx C, Scharpe S, De Meester I** 2003 Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci 40:209-294
181. **Mentlein R, Gallwitz B, Schmidt WE** 1993 Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. Eur J Biochem 214:829-835
182. **Deacon CF, Nauck MA, Toft-Nielsen M, Pridal L, Willms B, Holst JJ** 1995 Both subcutaneously and intravenously administered glucagon-like peptide I are rapidly degraded from the NH2-terminus in type II diabetic patients and in healthy subjects. Diabetes 44:1126-1131
183. **Deacon CF, Hughes TE, Holst JJ** 1998 Dipeptidyl peptidase IV inhibition potentiates the insulinotropic effect of glucagon-like peptide 1 in the anesthetized pig. Diabetes 47:764-769
184. **Deacon CF, Danielsen P, Klarskov L, Olesen M, Holst JJ** 2001 Dipeptidyl peptidase IV inhibition reduces the degradation and clearance of GIP and potentiates its insulinotropic and antihyperglycemic effects in anesthetized pigs. Diabetes 50:1588-1597
185. **Williams-Herman D, Round E, Swern AS, Musser B, Davies MJ, Stein PP, Kaufman KD, Amatruda JM** 2008 Safety and tolerability of sitagliptin in patients with type 2 diabetes: a pooled analysis. BMC Endocr Disord 8:14
186. **Deacon CF, Holst JJ** 2006 Dipeptidyl peptidase IV inhibitors: a promising new therapeutic approach for the management of type 2 diabetes. Int J Biochem Cell Biol 38:831-844
187. **Richter B, Bandeira-Echtler E, Bergerhoff K, Lerch CL** 2008 Dipeptidyl peptidase-4 (DPP-4) inhibitors for type 2 diabetes mellitus. Cochrane Database Syst Rev:CD006739
188. **Kim D, Kowalchick JE, Edmondson SD, Mastracchio A, Xu J, Eiermann GJ, Leiting B, Wu JK, Pryor KD, Patel RA, He H, Lyons KA, Thornberry NA, Weber AE** 2007 Triazolopiperazine-amides as dipeptidyl peptidase IV inhibitors: close analogs of JANUVIA (sitagliptin phosphate). Bioorg Med Chem Lett 17:3373-3377
189. **Villhauer EB, Brinkman JA, Naderi GB, Burkey BF, Dunning BE, Prasad K, Mangold BL, Russell ME, Hughes TE** 2003 1-[[(3-hydroxy-1-adamantyl)amino]acetyl]-2-cyano-(S)-pyrrolidine: a potent, selective, and orally bioavailable dipeptidyl peptidase IV inhibitor with antihyperglycemic properties. J Med Chem 46:2774-2789
190. **Ahren B, Simonsson E, Larsson H, Landin-Olsson M, Torgeirsson H, Jansson PA, Sandqvist M, Bavenholm P, Efendic S, Eriksson JW, Dickinson S, Holmes D** 2002 Inhibition of dipeptidyl peptidase IV improves metabolic control over a 4-week study period in type 2 diabetes. Diabetes Care 25:869-875
191. **Ahren B, Gomis R, Standl E, Mills D, Schweizer A** 2004 Twelve- and 52-week efficacy of the dipeptidyl peptidase IV inhibitor LAF237 in metformin-treated patients with type 2 diabetes. Diabetes Care 27:2874-2880

192. **Green J, Feinglos M** 2008 New combination treatments in the management of diabetes: focus on sitagliptin-metformin. Vasc Health Risk Manag 4:743-751
193. **Fonseca V, Schweizer A, Albrecht D, Baron MA, Chang I, Dejager S** 2007 Addition of vildagliptin to insulin improves glycaemic control in type 2 diabetes. Diabetologia 50:1148-1155
194. **Yu YL, Lu SS, Yu S, Liu YC, Wang P, Xie L, Wang GJ, Liu XD** 2009 Huang-Lian-Jie-Du-Decoction modulates glucagon-like peptide-1 secretion in diabetic rats. J Ethnopharmacol
195. **Lu SS, Yu YL, Zhu HJ, Liu XD, Liu L, Liu YW, Wang P, Xie L, Wang GJ** 2009 Berberine promotes glucagon-like peptide-1 (7-36) amide secretion in streptozotocin-induced diabetic rats. J Endocrinol 200:159-165
196. **Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M** 2003 A G protein-coupled receptor responsive to bile acids. J Biol Chem 278:9435-9440
197. **Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Nakamura T, Itadani H, Tanaka K** 2002 Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun 298:714-719
198. **Vassileva G, Golovko A, Markowitz L, Abbondanzo SJ, Zeng M, Yang S, Hoos L, Tetzloff G, Levitan D, Murgolo NJ, Keane K, Davis HR, Jr., Hedrick J, Gustafson EL** 2006 Targeted deletion of Gpbar1 protects mice from cholesterol gallstone formation. Biochem J 398:423-430
199. **Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC, Auwerx J** 2006 Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature 439:484-489
200. **Keitel V, Reinehr R, Gatsios P, Rupprecht C, Gorg B, Selbach O, Haussinger D, Kubitz R** 2007 The G-protein coupled bile salt receptor TGR5 is expressed in liver sinusoidal endothelial cells. Hepatology 45:695-704
201. **Maruyama T, Tanaka K, Suzuki J, Miyoshi H, Harada N, Nakamura T, Miyamoto Y, Kanatani A, Tamai Y** 2006 Targeted disruption of G protein-coupled bile acid receptor 1 (Gpbar1/M-Bar) in mice. J Endocrinol 191:197-205
202. **Fruhbeck G, Gomez-Ambrosi J, Salvador J** 2001 Leptin-induced lipolysis opposes the tonic inhibition of endogenous adenosine in white adipocytes. Faseb J 15:333-340
203. **Arthur C, Guyton, M.D.** 1991 Textbook of Medical physiology.
204. **Keane RM, Gadacz TR, Munster AM, Birmingham W, Winchurch RA** 1984 Impairment of human lymphocyte function by bile salts. Surgery 95:439-443
205. **Ramadori G, Armbrust T** 2001 Cytokines in the liver. Eur J Gastroenterol Hepatol 13:777-784
206. **Keitel V, Donner M, Winandy S, Kubitz R, Haussinger D** 2008 Expression and function of the bile acid receptor TGR5 in Kupffer cells. Biochem Biophys Res Commun 372:78-84
207. **Reinehr R, Becker S, Keitel V, Eberle A, Grether-Beck S, Haussinger D** 2005 Bile salt-induced apoptosis involves NADPH oxidase isoform activation. Gastroenterology 129:2009-2031
208. **Rubbo H, Radi R, Trujillo M, Telleri R, Kalyanaraman B, Barnes S, Kirk M, Freeman BA** 1994 Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. J Biol Chem 269:26066-26075
209. **Asamoto Y, Tazuma S, Ochi H, Chayama K, Suzuki H** 2001 Bile-salt hydrophobicity is a key factor regulating rat liver plasma-membrane communication:

relation to bilayer structure, fluidity and transporter expression and function. Biochem J 359:605-610
210. **Higuchi H, Gores GJ** 2003 Bile acid regulation of hepatic physiology: IV. Bile acids and death receptors. Am J Physiol Gastrointest Liver Physiol 284:G734-738
211. **Yasuda H, Hirata S, Inoue K, Mashima H, Ohnishi H, Yoshiba M** 2007 Involvement of membrane-type bile acid receptor M-BAR/TGR5 in bile acid-induced activation of epidermal growth factor receptor and mitogen-activated protein kinases in gastric carcinoma cells. Biochem Biophys Res Commun 354:154-159
212. **Makishima M** 2005 Nuclear receptors as targets for drug development: regulation of cholesterol and bile acid metabolism by nuclear receptors. J Pharmacol Sci 97:177-183
213. **Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ, Evans RM** 2001 An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. Proc Natl Acad Sci U S A 98:3375-3380
214. **Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ** 2000 Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Mol Cell 6:507-515
215. **Liu Y, Binz J, Numerick MJ, Dennis S, Luo G, Desai B, MacKenzie KI, Mansfield TA, Kliewer SA, Goodwin B, Jones SA** 2003 Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis. J Clin Invest 112:1678-1687
216. **Pellicciari R, Fiorucci S, Camaioni E, Clerici C, Costantino G, Maloney PR, Morelli A, Parks DJ, Willson TM** 2002 6alpha-ethyl-chenodeoxycholic acid (6-ECDCA), a potent and selective FXR agonist endowed with anticholestatic activity. J Med Chem 45:3569-3572
217. **Szapary PO, Wolfe ML, Bloedon LT, Cucchiara AJ, DerMarderosian AH, Cirigliano MD, Rader DJ** 2003 Guggulipid for the treatment of hypercholesterolemia: a randomized controlled trial. Jama 290:765-772
218. **Owsley E, Chiang JY** 2003 Guggulsterone antagonizes farnesoid X receptor induction of bile salt export pump but activates pregnane X receptor to inhibit cholesterol 7alpha-hydroxylase gene. Biochem Biophys Res Commun 304:191-195
219. **Katsuma S, Hirasawa A, Tsujimoto G** 2005 Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. Biochem Biophys Res Commun 329:386-390
220. **Sato H, Macchiarulo A, Thomas C, Gioiello A, Une M, Hofmann AF, Saladin R, Schoonjans K, Pellicciari R, Auwerx J** 2008 Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. J Med Chem 51:1831-1841
221. **Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, Kliewer SA** 2001 The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc Natl Acad Sci U S A 98:3369-3374
222. **Kliewer SA, Willson TM** 2002 Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. J Lipid Res 43:359-364
223. **Pellicciari R, Sato H, Gioiello A, Costantino G, Macchiarulo A, Sadeghpour BM, Giorgi G, Schoonjans K, Auwerx J** 2007 Nongenomic actions of bile acids. Synthesis and preliminary characterization of 23- and 6,23-alkyl-substituted bile acid derivatives as selective modulators for the G-protein coupled receptor TGR5. J Med Chem 50:4265-4268

224. **Ito F, Hinuma, K., Kanzaki, N., Miki, T., Kawamata, Y., Oi, S., Tawaeaishi, T., Ishichi, Y., Hirohashi, M.** 2004 Preparation of aromatic Ring-fused cyclic compounds as TGR5 receptor agonists. .
225. **Katona BW, Cummins CL, Ferguson AD, Li T, Schmidt DR, Mangelsdorf DJ, Covey DF** 2007 Synthesis, characterization, and receptor interaction profiles of enantiomeric bile acids. J Med Chem 50:6048-6058
226. **Macchiarulo A, Gioiello A, Thomas C, Massarotti A, Nuti R, Rosatelli E, Sabbatini P, Schoonjans K, Auwerx J, Pellicciari R** 2008 Molecular field analysis and 3D-quantitative structure-activity relationship study (MFA 3D-QSAR) unveil novel features of bile acid recognition at TGR5. J Chem Inf Model 48:1792-1801
227. **Deacon CF** 2005 What do we know about the secretion and degradation of incretin hormones? Regul Pept 128:117-124
228. **Brubaker PL, Schloos J, Drucker DJ** 1998 Regulation of glucagon-like peptide-1 synthesis and secretion in the GLUTag enteroendocrine cell line. Endocrinology 139:4108-4114
229. **Baxter JD, Webb P** 2009 Thyroid hormone mimetics: potential applications in atherosclerosis, obesity and type 2 diabetes. Nat Rev Drug Discov 8:308-320
230. **Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM** 1998 A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 92:829-839
231. **St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, Spiegelman BM** 2006 Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell 127:397-408
232. **Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC** 2003 PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 34:267-273
233. **Lerin C, Rodgers JT, Kalume DE, Kim SH, Pandey A, Puigserver P** 2006 GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1alpha. Cell Metab 3:429-438
234. **Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P** 2005 Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature 434:113-118
235. **Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P, Auwerx J** 2006 Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell 127:1109-1122
236. **Feige JN, Lagouge M, Canto C, Strehle A, Houten SM, Milne JC, Lambert PD, Mataki C, Elliott PJ, Auwerx J** 2008 Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. Cell Metab 8:347-358
237. **Houten SM, Watanabe M, Auwerx J** 2006 Endocrine functions of bile acids. Embo J 25:1419-1425
238. **Shishodia S, Harikumar KB, Dass S, Ramawat KG, Aggarwal BB** 2008 The guggul for chronic diseases: ancient medicine, modern targets. Anticancer Res 28:3647-3664

239. **Montilla MP, Agil A, Navarro MC, Jimenez MI, Garcia-Granados A, Parra A, Cabo MM** 2003 Antioxidant activity of maslinic acid, a triterpene derivative obtained from Olea europaea. Planta Med 69:472-474
240. **Cantrell CL, Franzblau SG, Fischer NH** 2001 Antimycobacterial plant terpenoids. Planta Med 67:685-694
241. **Rocha Gda G, Simoes M, Lucio KA, Oliveira RR, Coelho Kaplan MA, Gattass CR** 2007 Natural triterpenoids from Cecropia lyratiloba are cytotoxic to both sensitive and multidrug resistant leukemia cell lines. Bioorg Med Chem 15:7355-7360
242. **Medeiros R, Otuki MF, Avellar MC, Calixto JB** 2007 Mechanisms underlying the inhibitory actions of the pentacyclic triterpene alpha-amyrin in the mouse skin inflammation induced by phorbol ester 12-O-tetradecanoylphorbol-13-acetate. Eur J Pharmacol 559:227-235
243. **Gershenzon J, Dudareva N** 2007 The function of terpene natural products in the natural world. Nat Chem Biol 3:408-414
244. **Yoshikawa M, Murakami T, Kadoya M, Li Y, Murakami N, Yamahara J, Matsuda H** 1997 Medicinal foodstuffs. IX. The inhibitors of glucose absorption from the leaves of Gymnema sylvestre R. BR. (Asclepiadaceae): structures of gymnemosides a and b. Chem Pharm Bull (Tokyo) 45:1671-1676
245. **Fito M, de la Torre R, Farre-Albaladejo M, Khymenetz O, Marrugat J, Covas MI** 2007 Bioavailability and antioxidant effects of olive oil phenolic compounds in humans: a review. Ann Ist Super Sanita 43:375-381
246. **Japon-Lujan R, Luque-Rodriguez JM, Luque de Castro MD** 2006 Dynamic ultrasound-assisted extraction of oleuropein and related biophenols from olive leaves. J Chromatogr A 1108:76-82
247. **Sudjana AN, D'Orazio C, Ryan V, Rasool N, Ng J, Islam N, Riley TV, Hammer KA** 2009 Antimicrobial activity of commercial Olea europaea (olive) leaf extract. Int J Antimicrob Agents 33:461-463
248. **Bisignano G, Tomaino A, Lo Cascio R, Crisafi G, Uccella N, Saija A** 1999 On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. J Pharm Pharmacol 51:971-974
249. **Juan ME, Wenzel U, Daniel H, Planas JM** 2008 Erythrodiol, a natural triterpenoid from olives, has antiproliferative and apoptotic activity in HT-29 human adenocarcinoma cells. Mol Nutr Food Res 52:595-599
250. **Somova LI, Shode FO, Ramnanan P, Nadar A** 2003 Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from Olea europaea, subspecies africana leaves. J Ethnopharmacol 84:299-305
251. **Briante R, Patumi M, Terenziani S, Bismuto E, Febbraio F, Nucci R** 2002 Olea europaea L. leaf extract and derivatives: antioxidant properties. J Agric Food Chem 50:4934-4940
252. **Xi J, Chang Q, Chan CK, Meng ZY, Wang GN, Sun JB, Wang YT, Tong HH, Zheng Y** 2009 Formulation development and bioavailability evaluation of a self-nanoemulsified drug delivery system of oleanolic acid. AAPS PharmSciTech 10:172-182
253. **Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR** 2002 Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocr Rev 23:38-89
254. **Puigserver P** 2005 Tissue-specific regulation of metabolic pathways through the transcriptional coactivator PGC1-alpha. Int J Obes (Lond) 29 Suppl 1:S5-9

255. **Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, Montminy M** 2001 CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature 413:179-183
256. **Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K** 2008 Targeting bile-acid signalling for metabolic diseases. Nat Rev Drug Discov 7:678-693
257. **Videla LA, Fernandez V, Tapia G, Varela P** 2007 Thyroid hormone calorigenesis and mitochondrial redox signaling: upregulation of gene expression. Front Biosci 12:1220-1228
258. **Canani LH, Capp C, Dora JM, Meyer EL, Wagner MS, Harney JW, Larsen PR, Gross JL, Bianco AC, Maia AL** 2005 The type 2 deiodinase A/G (Thr92Ala) polymorphism is associated with decreased enzyme velocity and increased insulin resistance in patients with type 2 diabetes mellitus. J Clin Endocrinol Metab 90:3472-3478
259. **Bender E, Kadenbach B** 2000 The allosteric ATP-inhibition of cytochrome c oxidase activity is reversibly switched on by cAMP-dependent phosphorylation. FEBS Lett 466:130-134
260. **Pagliarini DJ, Dixon JE** 2006 Mitochondrial modulation: reversible phosphorylation takes center stage? Trends Biochem Sci 31:26-34
261. **Acin-Perez R, Salazar E, Kamenetsky M, Buck J, Levin LR, Manfredi G** 2009 Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation. Cell Metab 9:265-276
262. **Sutija M, Joss JM** 2006 Thyroid hormone deiodinases revisited: insights from lungfish: a review. J Comp Physiol [B] 176:87-92
263. **Luciani DS, Ao P, Hu X, Warnock GL, Johnson JD** 2007 Voltage-gated Ca(2+) influx and insulin secretion in human and mouse beta-cells are impaired by the mitochondrial Na(+)/Ca(2+) exchange inhibitor CGP-37157. Eur J Pharmacol 576:18-25
264. **Wiederkehr A, Wollheim CB** 2008 Impact of mitochondrial calcium on the coupling of metabolism to insulin secretion in the pancreatic beta-cell. Cell Calcium 44:64-76
265. **Spiegelman BM** 2007 Transcriptional control of mitochondrial energy metabolism through the PGC1 coactivators. Novartis Found Symp 287:60-63; discussion 63-69
266. **Fulda S** 2009 Betulinic acid: a natural product with anticancer activity. Mol Nutr Food Res 53:140-146
267. **Genet C, Strehle, A., Schmidt, C., Boudjelal, G.,Lobstein, A., Schoonjans, K., Souchet, M., Auwerx, J., Saladin, R, Wagner, A..** 2009 Structure activity relationship around betulinic acid a novel and selective TGR5 agonist.

Cell Metabolism
Article

TGR5-Mediated Bile Acid Sensing Controls Glucose Homeostasis

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SUMMARY

TGR5 is a G protein-coupled receptor expressed in brown adipose tissue and muscle, where its activation by bile acids triggers an increase in energy expenditure and attenuates diet-induced obesity. Using a combination of pharmacological and genetic gain- and loss-of-function studies in vivo, we show here that TGR5 signaling induces intestinal glucagon-like peptide-1 (GLP-1) release, leading to improved liver and pancreatic function and enhanced glucose tolerance in obese mice. In addition, we show that the induction of GLP-1 release in enteroendocrine cells by 6α-ethyl-23(*S*)-methyl-cholic acid (EMCA, INT-777), a specific TGR5 agonist, is linked to an increase of the intracellular ATP/ADP ratio and a subsequent rise in intracellular calcium mobilization. Altogether, these data show that the TGR5 signaling pathway is critical in regulating intestinal GLP-1 secretion in vivo, and suggest that pharmacological targeting of TGR5 may constitute a promising incretin-based strategy for the treatment of diabesity and associated metabolic disorders.

INTRODUCTION

Bile acids (BAs) have evolved over the past few years from being considered as simple lipid solubilizers to complex metabolic integrators (Houten et al., 2006; Thomas et al., 2008; Zhang and Edwards, 2008). Beyond the orchestration of BA, lipid, and glucose metabolism by the nuclear receptor farnesoid X receptor (FXR), BAs also act as signaling molecules through the BA-dedicated G protein-coupled receptor (GPCR) TGR5 (GPR131) (Kawamata et al., 2003; Maruyama et al., 2002). Stimulation of the TGR5 signaling pathway confers to BAs the ability to modulate energy expenditure by controlling the activity of type 2 deiodinase and the subsequent activation of thyroid hormone in brown adipose tissue (BAT) and muscle. In accordance with this, pharmacological intervention with a diet containing 0.5% of cholic acid (CA) efficiently attenuates diet-induced obesity (DIO) in mice (Watanabe et al., 2006). These results have indicated that TGR5 could be a promising target for the treatment of metabolic disorders associated with weight gain. In addition, expression of TGR5 has recently been described in enteroendocrine L cells (Reimann et al., 2008) and enteroendocrine cell lines, such as the STC-1 cells (Katsuma et al., 2005), which secrete the incretin glucagon-like peptide-1 (GLP-1) upon nutrient intake. The therapeutic relevance of GLP-1 is currently well established, since enhancement of the half-life of GLP-1 upon treatment with dipeptidyl peptidase 4 inhibitors (DPP4i) or activation of the GLP-1 receptor by exendin-4 derivatives (Ex-4) have been proven efficacious in the treatment of type 2 diabetes (Drucker, 2006; Drucker and Nauck, 2006). Therefore, the direct stimulation of intestinal GLP-1 release could constitute another promising GLP-1-based therapeutic approach for the treatment of metabolic diseases.

In this context, we hypothesized that TGR5 could play a critical in vivo role in the control of intestinal GLP-1 release and in the maintenance of glucose homeostasis. This assumption was investigated in vivo using a combination of gain- and loss-of-function genetic approaches. Additionally, we identified and explored the therapeutic properties of the semisynthetic CA derivative, 6α-ethyl-23(*S*)-methyl-cholic acid (EMCA, INT-777), a potent TGR5 agonist with no FXR activity, which could hold promise for the treatment of diabesity.

RESULTS

TGR5 Activation Stimulates GLP-1 Release from Enteroendocrine L Cells by Enhancing Mitochondrial Oxidative Phosphorylation and Calcium Influx

We report here a semisynthetic CA derivative, 6α-ethyl-23(*S*)-methyl-CA (EMCA, INT-777), as a selective and potent TGR5 agonist (Figures 1A and 1B). The rational for selecting this compound was based on previous in vivo work showing that CA induces energy expenditure via activation of TGR5 (Watanabe et al., 2006), and on structure-activity studies showing that alkyl substitutions at positions 6α and 23(*S*) improve the potency and selectivity of chenodeoxycholic acid (CDCA)

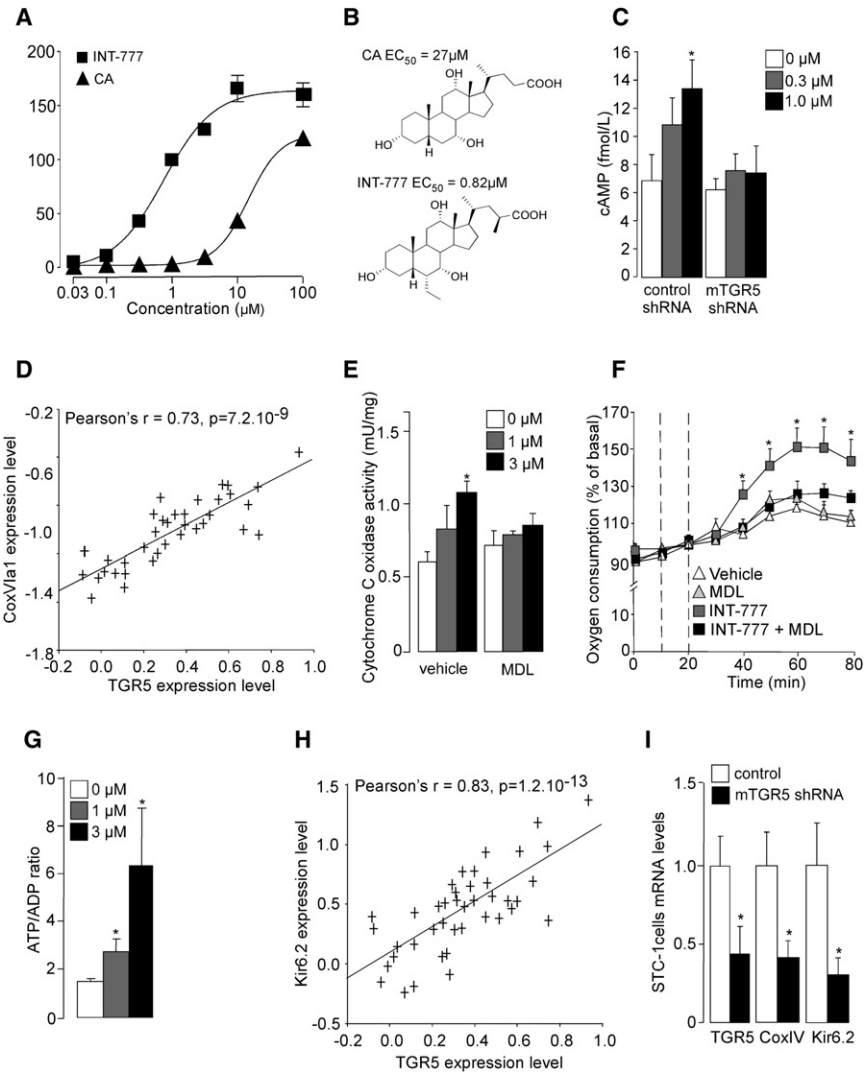

Figure 1. Activation of TGR5 Signaling Pathway Activates Mitochondrial Oxidative Phosphorylation and Increases ATP/ADP Ratio in Enteroendocrine L Cells

(A) Activity of 6α-ethyl-23(*S*)-methyl-cholic acid (INT-777) and CA on TGR5 in CHO cells transiently transfected with human TGR5 expression vector and a Cre-Luc reporter vector. EC_{50} values are expressed as percent of the activity of 10 μM of LCA (n = 3).

(B) Chemical structure of CA and INT-777 and respective TGR5 EC_{50}.

(C) Intracellular cAMP levels in STC-1 cells transfected with control or mTGR5 shRNA for 36 hr and treated for 1 hr with INT-777 at the concentrations indicated (n = 3).

(D) Correlation plots for liver mRNA expression of TGR5 and CoxVI1a in the mouse BxD genetic reference population (n = 41) as found in http://www.genenetwork.org/ and described in the Supplemental Experimental Procedures.

(E) Cox activity in STC-1 cells treated for 1 hr with INT-777 at the concentration indicated. Vehicle or adenylate cyclase inhibitor MDL-12330-A (MDL) (1 μM) was added 15 min prior to treatment (n = 3).

(F) Oxygen consumption in STC-1 cells was measured by using the XF24 extracellular flux analyzer (Seahorse Bioscience). The first vertical dotted line indicates the addition of vehicle or MDL-12330-A (MDL) to culture medium, and the second dotted line depicts the treatment with INT-777 at 1 μM (n = 10).

(G) ATP/ADP ratio in STC-1 cells treated as in (E) (n = 3).

(H) Correlation plots for liver mRNA expression of TGR5 and Kir6.2 in the mouse BxD genetic reference population according to a similar strategy as described (D).

(I) mRNA expression levels of TGR5, CoxIV, and Kir6.2 in STC-1 cells transfected for 36 hr with control or mTGR5 shRNA was measured by real-time quantitative PCR. Target mRNA levels were normalized to 36B4 mRNA levels (n = 3). The data are represented as mean ± SE; Student's unpaired t test; *p < 0.05.

toward TGR5 (Pellicciari et al., 2007; Sato et al., 2008). The addition of these alkyl substitutions to CA improved the resulting compound's EC_{50} on TGR5 by 30-fold (Figure 1B), while critically reducing its activity on FXR (see Figure S1A available online). In accordance with previous studies, activation of TGR5 (Katsuma et al., 2005; Kawamata et al., 2003; Maruyama et al., 2002) by INT-777 triggered a dose-dependent increase in intracellular cAMP levels in enteroendocrine STC-1 cells (Figure 1C). This induction was abrogated upon reduction of TGR5 expression by a specific TGR5 shRNA (Figure 1C), illustrating the specificity of the compound. The specificity of INT-777 for TGR5 was further confirmed by its inability to activate a subset of nuclear receptors involved in lipid and xenobiotic metabolism (Figure S1A), as well as other GPCRs that are phylogenetically related to TGR5 (Figures S1B and S1C).

Since we previously established a link between BAs and energy expenditure in vivo (Watanabe et al., 2006), we speculated that activation of TGR5 signaling could impact mitochondrial activity in a more general fashion. To find initial support for this hypothesis, we analyzed TGR5 mRNA expression via the GeneNetwork liver mRNA database in the BxD genetic reference population (http://www.genenetwork.org/). A wide range of variation in TGR5 mRNA expression was evident among the different BxD mouse strains (Figure S2A). Interestingly, TGR5 mRNA expression was highly significantly correlated with the expression of several genes encoding for subunits of complexes involved in oxidative phosphorylation, such as cytochrome *c* oxidase (Cox) (e.g., CoxVI1a; Figure 1D) and ATP synthase (Atp6v0b, ATPase H^+ transporting V0 subunit B; Atpaf2, ATP synthase mitochondrial F1 complex assembly factor 2; Atp1a3, ATPase $Na^+/K+$ transporting alpha 3 polypeptide; Atp6v1b2, ATPase H^+ transporting V1 subunit B isoform 2; Figure S2B). Consistent with this observation, treatment of STC-1 cells with INT-777 resulted in a cAMP-dependent increase in Cox activity (Figure 1E), which was associated with an increase in cellular oxygen consumption (Figure 1F) and a rise in the ATP/ADP ratio (Figure 1G). This result was confirmed in the human enteroendocrine cell line NCI-H716, in which INT-777 treatment increased

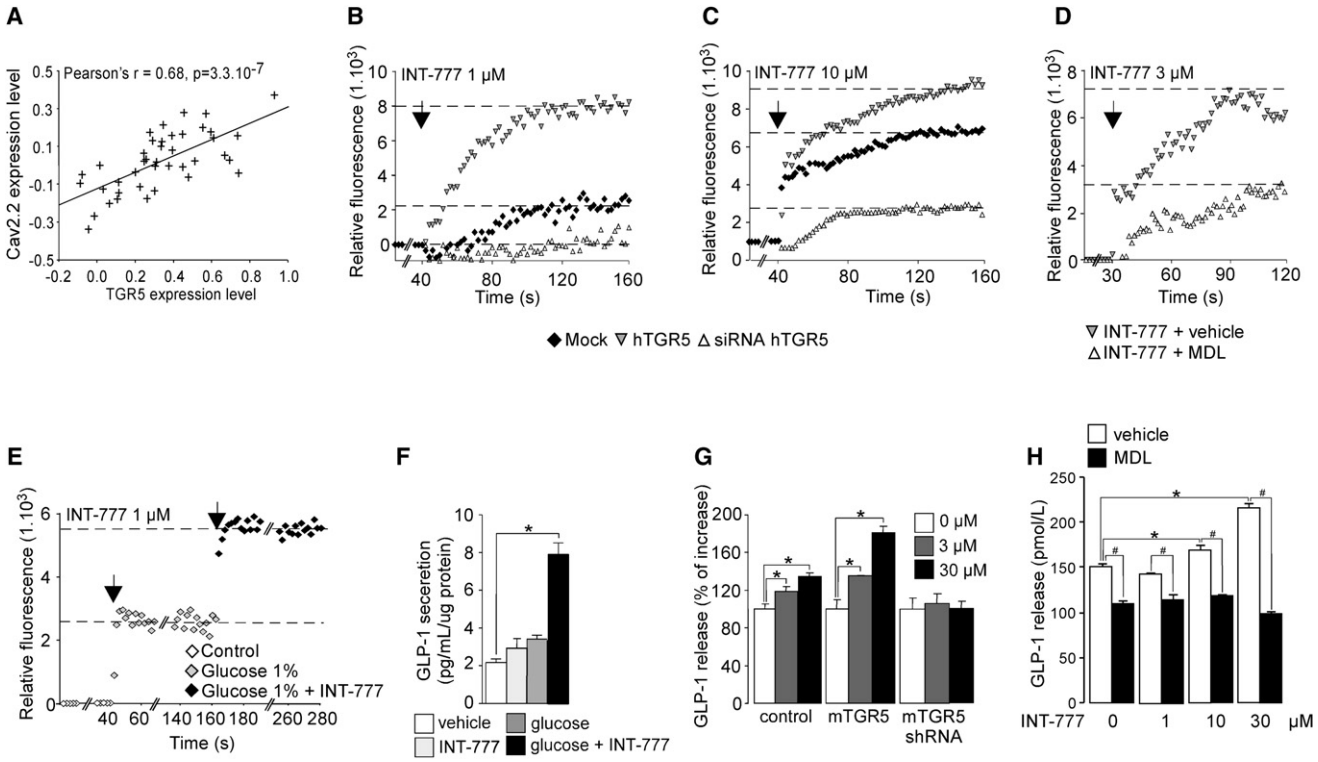

Figure 2. Activation of TGR5 Signaling Pathway Increases Intracellular Calcium Level and Stimulates GLP-1 Release in Enteroendocrine L Cells

(A) Correlation plots for liver mRNA expression of TGR5 and Cav2.2 in the mouse BxD genetic reference population (n = 41) as found at http://www.genenetwork.org/ and as described in the Supplemental Experimental Procedures.

(B and C) Intracellular calcium level in NCI-H716 cells transfected with mock vector, hTGR5 expression vector, or hTGR5 siRNA for 36 hr and treated with 1 (B) or 10 μM (C) of INT-777. The arrow represents INT-777 treatment (n = 3).

(D) Intracellular calcium level in NCI-H716 cells treated with 3 μM of INT-777 (indicated by the arrow) in the presence of vehicle or adenylate cyclase inhibitor MDL-12330-A (MDL) (10 μM). MDL or vehicle was added 15 min prior to INT-777 treatment (n = 3).

(E) Intracellular calcium level in NCI-H716 cells treated with 1% glucose and then with 1 μM of INT-777 (n = 3).

(F) GLP-1 release in NCI-H716 cells treated with 1% glucose or 1 μM INT-777, or a combination of both agents (n = 3).

(G) GLP-1 release in STC-1 cells transfected for 36 hr with control, mTGR5 expression vector, or mTGR5 shRNA and then exposed 30 min to INT-777 at the indicated concentration. A DPP4 inhibitor (Millipore) was added into culture medium at 0.1% (n = 3).

(H) Impact of 30 min of INT-777 treatment on GLP-1 release in STC-1 cells transfected with mTGR5 expression vector in the presence of vehicle or adenylate cyclase inhibitor MDL-12330-A (10 μM). MDL or vehicle was added 15 min prior to INT-777 treatment. A DPP4 inhibitor (Millipore) was added into culture medium at 0.1% (n = 3). The data are represented as mean ± SE. Student's unpaired t test; *$p < 0.05$ vehicle versus INT-777 treatment; #$p < 0.05$ vehicle versus MDL-12330-A treatment.

ATP production in a cAMP-dependent manner (Figure S3A). Interestingly, TGR5 expression was also strongly correlated with that of Kir6.2, a component of the ATP-dependent potassium channel (K_{ATP}) (Figure 1H). These correlations were further corroborated by TGR5 RNA interference in STC-1 cells, which resulted in a concomitant drop in the expression of CoxIV and Kir6.2 mRNAs (Figure 1I).

In pancreatic β cells, it is well established that an increase in the ATP/ADP ratio derived from glucose metabolism closes the K_{ATP} channels, resulting in depolarization of the plasma membrane. This membrane depolarization in turn opens calcium-gated voltage channels (Ca_v), causing calcium influx. The resultant increase in intracellular calcium then triggers the direct interaction between exocytotic proteins situated in the insulin-containing granule membrane and those located in the plasma membrane (Yang and Berggren, 2006), leading to the subsequent release of insulin (Nichols, 2006). Recent findings support the hypothesis that K_{ATP} and Ca_v channels also play a pivotal role in GLP-1 release from enteroendocrine L cells (Reimann and Gribble, 2002; Reimann et al., 2008). Fascinatingly, in the BxD reference population, we also found that TGR5 expression correlated with the expression of $Ca_v2.2$ (Figure 2A), whose expression was previously described in enteroendocrine cells (Reimann et al., 2005) and which participates in calcium-stimulated insulin release in pancreatic β cells (Yang and Berggren, 2006). Along with this, INT-777 robustly increased calcium influx in the human enteroendocrine cell line NCI-H716, an effect that was potentiated by TGR5 overexpression and, by contrast, blunted by TGR5 RNA interference (Figures 2B and 2C) or by the addition of the adenylate cyclase inhibitor MDL-12330A (MDL) (Figure 2D). In addition, the presence of glucose enhanced the TGR5-dependent increase in intracellular calcium (Figure 2E). This effect was correlated with a rise in GLP-1 release from the NCI-H716 cells (Figure 2F), which was inhibited by MDL-12-330A (Figure S3B).

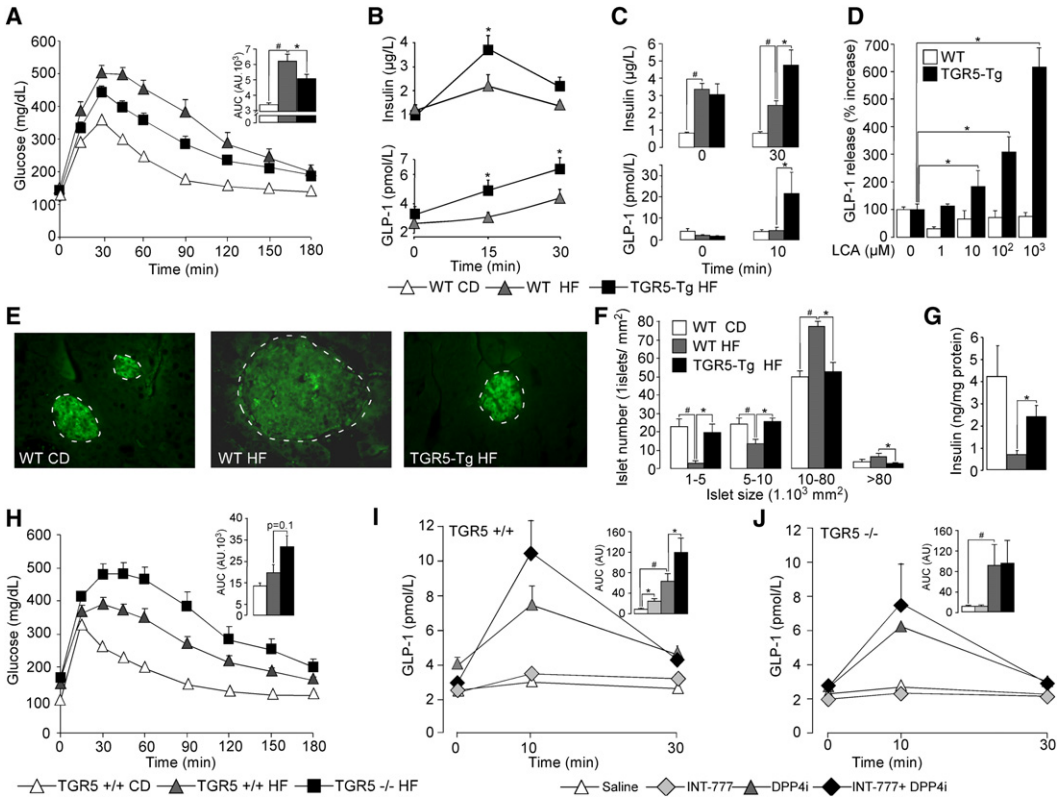

Figure 3. TGR5 Signaling Pathway Modulates GLP-1 Secretion In Vivo

(A) Oral glucose tolerance test (OGTT) in male TGR5-Tg mice fed for 10 weeks with HF diet and in age-matched male littermates fed with a CD or a HF diet for the same duration. All mice were 8 weeks old at the initiation of the HF diet. Body weight of TGR5-Tg and control littermates was 37.9 ± 1.7 g and 37.0 ± 1.8 g, respectively (n = 8; not statistically different). The adjacent bar graph represents the average area under the curve (AUC) (n = 8).

(B and C) Plasma levels of insulin (top panel) and GLP-1 (bottom panel) during OGTT (B) or before and after a test meal challenge (C) (n = 8).

(D) GLP-1 release from ileal explants isolated from control and TGR5-Tg male mice fed for 18 weeks with HF diet and exposed for 1 hr to the indicated concentrations of LCA (n = 4).

(E) Representative immunofluorescent insulin-stained pancreatic sections from TGR5-Tg male mice fed with a HF diet for 20 weeks or from male age-matched littermates fed with a CD or a HF diet for the same duration.

(F) Distribution profile of pancreatic islets from male TGR5-Tg mice and control littermates fed with a CD or a HF diet as described in (E). Islets were counted and sized by the ImageJ analysis software on four H&E-stained alternated pancreatic sections spaced each by 150 µM (n = 5).

(G) Insulin content in collagenase-isolated pancreatic islets from male TGR5-Tg mice and control littermates fed with a CD or a HF diet as described in (E).

(H) OGTT in *TGR5*$^{-/-}$ and *TGR5*$^{+/+}$ male mice fed with a HF diet for 8 weeks. The inset represents the average AUC. Body weight of *TGR5*$^{+/+}$ and *TGR5*$^{-/-}$ male mice at time of analysis was 46.3 ± 3.9 g and 51.9 ± 2.0 g, respectively (n = 8; not statistically different).

(I and J) Plasma GLP-1 levels in CD-fed *TGR5*$^{+/+}$ (I) and *TGR5*$^{-/-}$ mice (J) after an oral glucose challenge, preceded 30 min before by the oral administration of saline or INT-777 (30 mg/kg), alone or in combination with a dipeptidyl-peptidase-4 inhibitor (DPP4i, 3 mg/kg) (n = 6). The data are represented as mean ± SE. Student's unpaired t test; *p < 0.05, HF-fed compared to HF-fed INT-777-treated mice; and #p < 0.05, HF-fed versus CD-fed mice except for (I) and (J), where * assessed saline- or DPP4i-treated mice versus INT-777 or INT-777 + DPP4i-treated mice, and # saline- versus DPP4i-treated mice.

The TGR5-mediated GLP-1 release triggered by INT-777 was further confirmed in the mouse enteroendocrine STC-1 cells in which the impact of INT-777 on GLP-1 release was enhanced by TGR5 overexpression, while being prevented either by RNA interference (Figure 2G) or by MDL-12-330A, further underscoring the cAMP dependence of TGR5-mediated GLP-1 release (Figure 2H). Taken together, these data demonstrate that TGR5 regulates a key pathway governing the release of GLP-1 from enteroendocrine L cells.

TGR5 Overexpression Modulates GLP-1 Secretion In Vivo

To further evaluate the metabolic role of enhanced TGR5 signaling, we assessed the impact of transgenic overexpression of TGR5 in vivo in the context of DIO in mice. TGR5 transgenic mice (TGR5-Tg) were generated by oocyte injection of the bacterial artificial chromosome (BAC) RP23-278N11 (Figure S4A). By quantitative real-time PCR, TGR5-Tg mice were shown to have integrated six copies of the RP23-278N11 BAC clone, leading to a robust TGR5 mRNA expression, restricted to most tissues that express TGR5 normally (Figure S4B). Glucose tolerance was markedly improved in TGR5-Tg mice challenged for 10 weeks with a high-fat (HF) diet compared to control HF-fed littermates (Figure 3A), whereas no difference was noticed in mice on chow diet (CD) (data not shown). In contrast to our expectations, no differences were observed in body weight gain between wild-type and TGR5-Tg mice on CD or HF diet (Figures S5A and S5B), demonstrating that improvement of glucose tolerance in

TGR5-Tg mice could not be attributed to the confounding effects of weight loss. The absence of weight gain in TGR5-Tg mice, in the wake of an increase in energy expenditure (Figure S5C), was explained by a reduction of locomotor activity (Figure S5D). Since GLP-1 receptor knockout mice display a marked hyperactivity (Hansotia et al., 2007), we administered the GLP-1 receptor agonist Ex-4 to wild-type mice in order to assess whether the decrease in locomotor activity in TGR5-Tg mice could be linked to GLP-1 secretion. Ex-4 efficiently and dose-dependently reduced locomotor activity in mice. Interestingly, at 1 nmol/Kg, we noticed a significant decrease in locomotor activity while the mice were still eating properly (Figures S5E and S5F).

Interestingly, and according to our expectations, glucose tolerance in TGR5-Tg mice was associated with a robust GLP-1 secretion and insulin release in response to an oral glucose load (Figure 3B). The significance of the enhanced GLP-1 secretion was underscored by the fact that measurements of plasma GLP-1 levels were performed without preliminary oral administration of a dipeptidyl peptidase-4 (DPP4) inhibitor to the mice. This enhanced GLP-1 release in TGR5-Tg mice helps to explain the decreased locomotor activity in these mice. To further investigate the impact of TGR5 overexpression on GLP-1 secretion, the HF-fed mice were subsequently challenged with a test meal to stimulate BA release from the gallbladder. Interestingly, the impact of TGR5 overexpression on insulin and GLP-1 secretion was more pronounced postprandially than after simple glucose challenge (Figure 3C). We speculate that these effects are due to the increased BA flux triggered by the test meal as compared to the glucose challenge. In line with this hypothesis, the treatment of ileal explants from TGR5-Tg and control mice with lithocholic acid (LCA) confirmed that BAs provide an excellent signal to induce GLP-1 release in the context of high TGR5 expression (Figure 3D). These data are furthermore in accordance with results obtained in mTGR5-transfected STC-1 cells in which GLP-1 release was also boosted by increased expression of TGR5 (Figure 2G). We speculate that in the context of wild-type ileal explants, the quick degradation of GLP-1 by DPP4 enzyme might mask the moderate increase in GLP-1 release triggered by LCA.

The impact of GLP-1 on pancreatic function has been extensively documented during the last decade and ranges from insulin-secretagogue effects to the promotion of pancreatic islet survival and proliferation (Drucker, 2006). In this context, immunofluorescent staining of insulin on pancreatic sections revealed that, in contrast to hypertrophic islets with low insulin content, as observed in HF-fed control mice, islets of HF-fed TGR5-Tg mice were not hypertrophic and stained more intensively for insulin (Figure 3E). In line with these data, counting and sizing of pancreatic islets confirmed that TGR5 expression results in the maintenance of a normal islet distribution profile (Figure 3F), likely due to enhanced plasma GLP-1 levels. In addition, the insulin content of isolated pancreatic islets was significantly higher in HF-fed TGR5-Tg mice than in controls (Figure 3G).

To further establish a role of TGR5 signaling in the maintenance of glucose homeostasis, we assessed the glucose tolerance of germline TGR5-deficient mice (*TGR5*$^{-/-}$), generated by breeding mice in which the TGR5 allele was floxed with CMV-Cre transgenic mice (Figures S4C–S4F). In direct contrast with what was observed in TGR5-Tg mice, glucose tolerance was impaired in *TGR5*$^{-/-}$ mice challenged with a HF-diet for 8 weeks (Figure 3H), whereas no difference was observed in CD-fed mice (data not shown). GLP-1 secretion was then tested by challenging *TGR5*$^{+/+}$ and *TGR5*$^{-/-}$ mice with an oral glucose load 30 min after the administration of saline or INT-777 alone, or in combination with the DPP4 inhibitor (DPP4i), sitagliptin. Preadministration of INT-777 moderately increased GLP-1 release after a glucose challenge in *TGR5*$^{+/+}$ mice (Figure 3I). This effect was, however, markedly more pronounced when DPP4i was coadministered as a consequence of its ability to prolong the half-life of plasma GLP-1 (Drucker and Nauck, 2006) (Figure 3I). In contrast, the effects of INT-777 on plasma GLP-1 levels were blunted in *TGR5*$^{-/-}$ mice (Figure 3J). Together, these data underscore the critical role of TGR5 signaling in the control of GLP-1 release and further demonstrate the specificity of the semisynthetic agonist INT-777 in vivo.

The TGR5 Agonist INT-777 Increases Energy Expenditure and Reduces Hepatic Steatosis and Obesity upon High-Fat Feeding

In view of the improved glucose and insulin profile in TGR5-Tg mice, we next assessed the therapeutic potential of INT-777 admixed at a dose of 30 mg/kg/day (mkd) with the diet in an intervention study in C57BL/6J mice in which diabesity was induced by HF feeding for 14 weeks. As expected, the HPLC profile of plasma BAs confirmed the presence of INT-777 in the treated mice only (Figure 4A). The plasma levels of INT-777 were within the range of those of CA and β-muricholic acid (Figure S6A). It is noteworthy that INT-777 treatment affected neither plasma BA composition nor the expression profile of the enzymes involved in BA synthesis, whose expression is mainly under the control of nuclear receptors (Figures S6A and S6B). The complete absence of changes in the expression level of classical target genes of FXR in the liver, such as cholesterol 7α hydroxylase (CYP7A1) and bile salt export pump (BSEP) (Thomas et al., 2008), further confirmed the specificity of INT-777 toward TGR5 (Figure S6B).

After 10 weeks of treatment with INT-777, a significant attenuation of body weight gain of about 15%, in association with a sharp reduction in fat mass, was observed in HF-fed INT-777-treated mice relative to HF-fed controls (Figures 4B and 4C). The increase in liver and fat pad mass was also attenuated in HF-fed INT-777-treated mice (Figure 4D). As noticed in our previous study with CA (Watanabe et al., 2006), the decrease in BAT mass was related to a diminution of white adipose tissue (WAT) in the interscapular region (Figure 4D and data not shown). The metabolic changes between control HF-fed and INT-777-treated HF-fed mice were not caused by a reduced calorie intake (Figure 4E) or fecal energy loss (Figure S6C), but rather were the consequence of enhanced energy expenditure, as indicated by the measurement of O_2 consumption and CO_2 production during indirect calorimetry (Figure 4F). During the dark period, the respiratory quotient of INT-777-treated mice was significantly reduced, consistent with increased fat burning (Figure 4F). Gene expression profiling of BAT confirmed that activation of the TGR5 signaling pathway triggers the increase of several mitochondrial genes involved in energy expenditure along with an induction of type 2 deiodinase gene expression (Figure 4G).

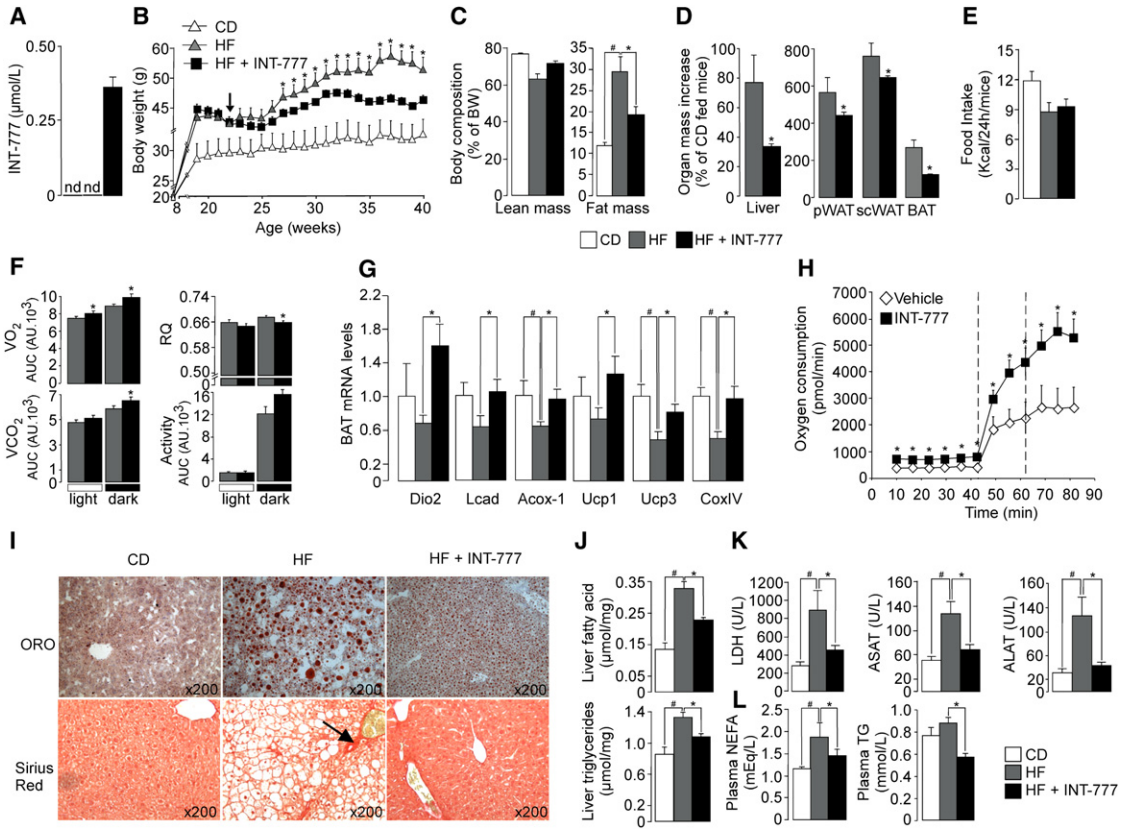

Figure 4. The TGR5 Agonist INT-777 Increases Energy Expenditure and Reduces Hepatic Steatosis and Adiposity in DIO Mice

(A) Measurement by HPLC of plasma INT-777 levels in CD-, HF-, and HF-fed INT-777-treated male C57BL6/J mice.

(B) Dietary intervention with INT-777 (30 mg/kg/d) was started after a 14-week period of HF feeding at the time indicated by the arrow. Body weight evolution in all groups was followed throughout the study (n = 8).

(C) Body composition was assessed by qNMR after 8 weeks of dietary intervention (n = 8).

(D) Organ mass was expressed as percent of the weight of CD-fed control mice.

(E) Food intake (n = 8).

(F) Spontaneous horizontal activity and energy expenditure, evaluated by the measurement of oxygen consumption (VO_2) and carbon dioxide release (VCO_2), were monitored over a 18 hr period 6 weeks after the initiation of the dietary intervention. The respiratory quotient (RQ) was calculated as the ratio VCO_2/VO_2. Bar graphs represent the average AUC. For the RQ, bar graphs represent the average (n = 8).

(G) Gene expression in BAT by real-time quantitative PCR after 18 weeks of dietary intervention. Target mRNA levels were normalized to 36B4 mRNA levels (n = 8).

(H) Primary brown adipocytes isolated from CD-fed C57BL/6J male mice were cultured for 12 hr with vehicle or 3 µM INT-777, and O_2 consumption was measured by using the XF24 extracellular flux analyzer (Seahorse Bioscience) (n = 5). The dotted lines illustrate the addition of the uncoupling agent FCCP at successive doses of 250 and 500 nM.

(I) Representative pictures of oil red O (ORO) staining of cryosections (top panel) and Sirius red staining of paraffin-embedded sections (bottom panel) of the liver at the end of the dietary intervention. Fibrosis is indicated by the arrow.

(J) Lipid content in liver samples extracted according to Folch's method (n = 8).

(K and L) Plasma levels of liver enzymes (K) and lipids (L) at the end of the dietary intervention (n = 8). The data are represented as the mean ± SE. Student's unpaired t test; *p < 0.05, HF-fed compared to HF-fed INT-777-treated mice; and #p < 0.05, HF-fed versus CD-fed mice.

The activation of the mitochondrial respiratory chain by INT-777 was further evidenced by measuring O_2 consumption in primary brown adipocytes isolated from C57BL/6J mice treated for 12 hr with INT-777. Addition of the uncoupling agent, carbonylcyanide-ptrifluoromethoxyphenylhydrazone (FCCP), boosted basal O_2 consumption in all conditions but was significantly more pronounced in those treated with INT-777 (Figure 4H). In addition to the enhanced energy expenditure, liver function was also improved, as evidenced by the reduction in liver steatosis, which was assessed by oil red O staining (Figure 4I) and biochemical quantification of liver lipid content (Figure 4J). Moreover, plasma levels of liver enzymes were markedly reduced compared to HF-fed controls, correlating with the absence of liver fibrosis in liver sections of INT-777-treated mice stained with Sirius red (Figures 4I and 4K). The improvement in liver function was also reflected by the significant drop in plasma triglycerides and nonesterified fatty acids (NEFAs) in HF-fed mice treated with INT-777 (Figure 4L).

The TGR5 Agonist INT-777 Improves Insulin Sensitivity in Obese Mice

We also ascertained the ability of INT-777 to improve glucose homeostasis. In both DIO and db/db mice, an environmental and genetic model of diabesity, respectively, treatment with

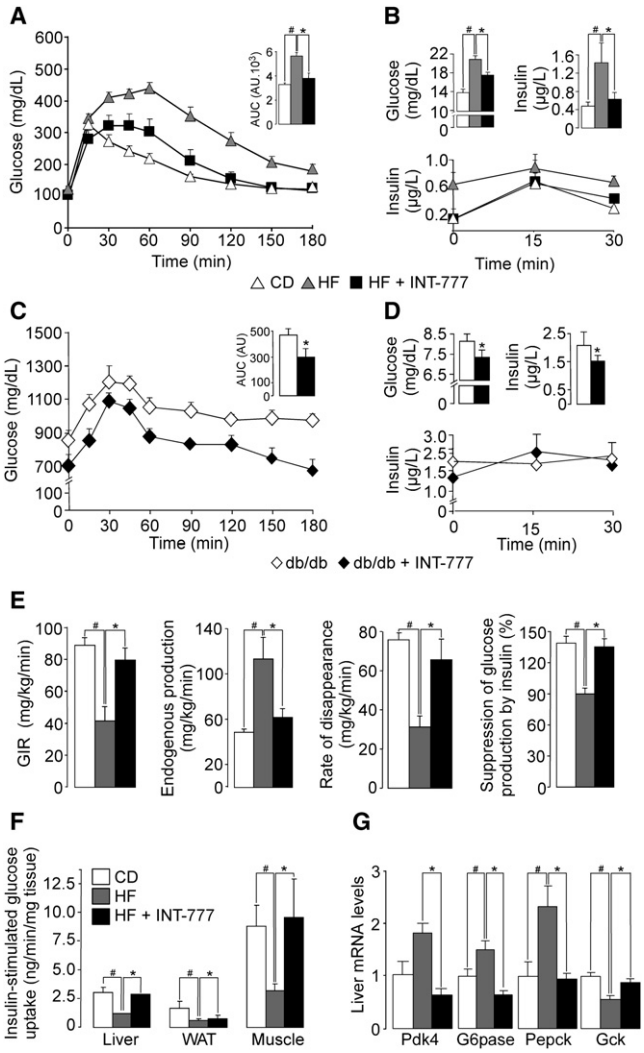

Figure 5. The TGR5 Agonist INT-777 Improves Insulin Sensitivity in Obese Mice

(A) OGTT in CD- and HF-fed male C57BL6/J mice supplemented with 30 mg/kg/d INT-777 for 8 weeks following the onset of obesity induced by feeding a HF diet during 10 weeks. The inset represents the average AUC. Body weight of vehicle and INT-777 treated mice was 38.08 ± 1.83 g and 32.26 ± 0.95 g, respectively ($n = 8$; $p < 0.05$).

(B) Fasting glycemia and insulinemia (4 hr fasting) in DIO mice after 3 weeks of dietary intervention with INT-777 (top panel). Plasma insulin levels during OGTT in DIO mice (bottom panel).

(C) OGTT in 14-week-old CD-fed db/db male mice treated with 30 mg/kg/d INT-777 for 6 weeks. The inset shows the average AUC ($n = 8$).

(D) Fasting (4 hr) glycemia and insulinemia in db/db mice after 6 weeks of treatment with INT-777 (top panel). Plasma insulin levels during OGTT in DIO mice (bottom panel).

(E) Insulin sensitivity evaluated through the average glucose infusion rate at equilibrium (euglycemia) in a hyperinsulinemic euglycemic clamp (10 mU insulin/min/kg) in DIO mice (following the onset of obesity induced by feeding a HF diet during 10 weeks) after 10 weeks of dietary intervention with INT-777 (30 mg/kg/d) ($n = 5$). The evaluation of liver glucose production and its suppression by insulin, as well as the rate of glucose disappearance, was assessed at equilibrium using ^{3}H-glucose ($n = 5$).

(F) Insulin-stimulated glucose uptake in the indicated tissues was measured by using ^{14}C-2-deoxyglucose tracers ($n = 5$).

(G) Gene expression profiling in liver was performed by real-time quantitative PCR. Target mRNA levels were normalized to 36B4 levels ($n = 8$). The data are represented as mean ± SE. Student's unpaired t test; *$p < 0.05$, HF-fed compared to HF-fed INT-777 treated mice; and #$p < 0.05$, HF-fed versus CD-fed mice.

INT-777 (30 mkd) admixed with the diet robustly improved glucose tolerance after an oral challenge with glucose (Figures 5A and 5C), along with an improvement of the glucose-stimulated insulin secretion profile (Figures 5B and 5D, lower panel). This feature is consistent with a GLP-1-mediated improvement in pancreatic function. Furthermore, fasting glucose and insulin levels were decreased in both DIO and db/db mice that were treated with INT-777 (Figures 5B and 5D, top panel). To further characterize the impact of INT-777 on glucose homeostasis and insulin sensitivity, a hyperinsulinemic euglycemic glucose clamp was performed on these DIO mice. Consistent with the improved glucose tolerance, the glucose infusion rate required to maintain euglycemia in DIO mice treated with INT-777 was virtually identical to that observed in CD-fed control mice (Figure 5E). While insulin-resistant HF-fed mice showed an increased endogenous production of hepatic glucose, together with a reduction of both glucose disposal rate and the suppression of glucose production by insulin, INT-777 treatment of HF-fed mice normalized these parameters to the values observed in CD-fed mice (Figure 5E). Measurement of insulin-stimulated ^{14}C-deoxyglucose uptake during the hyperinsulinemic euglycemic glucose clamp indicated that the improvement in glucose homeostasis by INT-777 could be mainly attributed to reduced insulin resistance in liver and muscle (Figure 5F). These effects correlated with normalization in the expression of key genes involved in hepatic glucose homeostasis (Figure 5G).

To address the specificity of INT-777 with regard to TGR5 in vivo, the impact of 4 weeks' treatment with INT-777 at 30 mkd on glucose tolerance was compared in *TGR5*$^{+/+}$ and *TGR5*$^{-/-}$ mice, primed by HF feeding for 9 weeks. Even over this short time period, INT-777 significantly improved glucose tolerance in *TGR5*$^{+/+}$ fed a HF diet (Figure 6A), along with a normalization of insulin secretion during oral glucose challenge (Figure 6B). These effects were blunted in *TGR5*$^{-/-}$ mice, thereby providing further arguments to support the specificity of INT-777 for TGR5 (Figures 6A and 6B).

DISCUSSION

Following our previous demonstration that TGR5 activation governs energy metabolism in BAT and muscle (Watanabe et al., 2006), and inspired by the fact that TGR5 is expressed in enteroendocrine cells (Katsuma et al., 2005; Reimann et al., 2008), we explored in this study whether TGR5 activation could alter glucose homeostasis. Our data demonstrate that GLP-1-producing enteroendocrine cells are sensitive not only to nutrients present in the intestinal lumen, such as glucose and lipids (Edfalk et al., 2008; Hirasawa et al., 2005; Overton et al., 2006; Reimann et al., 2008), but also to BAs, that activate the TGR5 signaling pathway. Along with the lipid-sensing GPCRs, GPR40, GPR119 and GPR120 (Edfalk et al., 2008; Hirasawa et al., 2005; Overton et al., 2006; Reimann et al., 2008), TGR5 represents a key pathway for the regulation of intestinal GLP-1 release. TGR5-mediated GLP-1 release in enteroendocrine L cells involves the closure/opening of the K_{ATP}/Ca_v channels

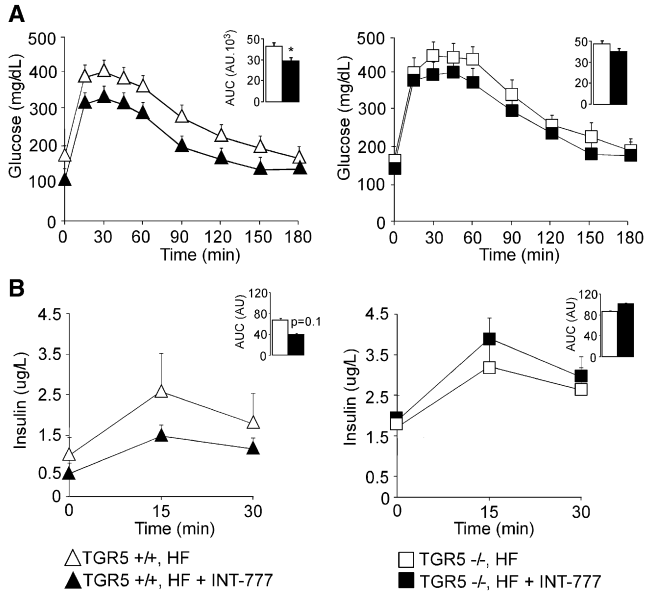

Figure 6. INT-777-Mediated Improvement of Glucose Tolerance Is TGR5 Dependent

(A) *TGR5*$^{+/+}$ and *TGR5*$^{-/-}$ male mice were fed a HF diet for 9 weeks, and a first OGTT was performed thereafter. HF was then supplemented with INT-777 at 30 mg/kg/d. A second OGTT was performed 4 weeks after treatment with INT-777 was initiated. Curves represent glucose tolerance before and after 4 weeks' treatment with INT-777 in *TGR5*$^{+/+}$ (left panel) and *TGR5*$^{-/-}$ (right panel) mice. The inset represents the average AUC. In *TGR5*$^{+/+}$ mice, body weight before and after INT-777 treatment was 46.86 ± 3.54 g and 43.50 ± 3.47 g, respectively (n = 8; not statistically different). In *TGR5*$^{-/-}$ mice, body weight before and after INT-777 treatment was 54.34 ± 2.23 g and 52.30 ± 2.72 g, respectively (n = 8; not statistically different).

(B) Plasma insulin levels were concurrently measured during the OGTT in DIO in *TGR5*$^{+/+}$ (left panel) and *TGR5*$^{-/-}$ (right panel) mice before and after 4 weeks' treatment with INT-777. The inset represents the average AUC (n = 8). The data are represented as mean ± SE. Student's unpaired t test; *p < 0.05, vehicle compared to INT-777-treated mice.

through a modulation of mitochondrial oxidative phosphorylation and the subsequent change in ATP/ADP ratio. Modulating this TGR5-driven pathway is of therapeutic potential, given that incretin-based therapies have emerged as potent antidiabetic strategies (Drucker, 2006; Drucker and Nauck, 2006).

In addition to our observations on GLP-1 release in vitro, we demonstrated through a gain- and loss-of-function genetic approach that the TGR5 pathway is a crucial determinant of glucose homeostasis in vivo. In fact, the activation of TGR5 by the selective and potent TGR5 agonist INT-777 may constitute a way to stimulate the release of incretins, such as GLP-1, translating into beneficial metabolic effects that could be of use in the treatment of diabesity and associated metabolic disorders. Although the receptor is expressed in many tissues (Kawamata et al., 2003; Maruyama et al., 2002), activation of TGR5 with the CA-derived INT-777 is particularly attractive, given the increased exposure of enteroendocrine cells in the gut due to the extensive enterohepatic recirculation of INT-777 with other BAs in the bile pool (data not shown). In this manner, the efficiency of intestinal BA reabsorption considerably increases the effective dose to which enteroendocrine cells are exposed. Despite these "gut-centered" properties of BAs and their derivatives, we cannot exclude the possibility that some of the beneficial metabolic actions of INT-777 may be unrelated to its modulation of GLP-1 levels. It is interesting to note within this context that other GPCRs expressed in enteroendocrine cells, such as GPR119 (Chu et al., 2007), are also expressed in pancreatic β cells (Chu et al., 2007; Sakamoto et al., 2006; Soga et al., 2005). We can therefore at present not ascertain that some TGR5-mediated effect on insulin release and glucose tolerance could involve direct pancreatic effects that are partially independent of GLP-1. Further investigations, using a β cell-specific TGR5-deficient mouse model, are required to test this assumption.

It should also be noted that, given the systemic bioavailability of INT-777 as evidenced by the plasma concentrations achieved, direct activation of TGR5 in other tissues is also a significant factor in the compound's effects in vivo. Indeed, in line with our previous experience with natural BAs (Watanabe et al., 2006), INT-777 also increased energy expenditure and attenuated weight gain upon high-fat feeding through TGR5-mediated effects in BAT and muscle. The more-limited impact of INT-777 on energy expenditure relative to our previous study using natural BAs can be attributed to the substantially lower doses of INT-777 that were administered in this study. Since the TGR5 signaling pathway has also been shown to be active in macrophages, further investigation is also required to evaluate the potential ameliorative effect of INT-777 on inflammatory processes, which also contribute to the metabolic syndrome (Despres and Lemieux, 2006).

Our current work also underscores the complexity of the endocrine activities of BAs, including TGR5- and FXR-mediated actions, which all integrate to maintain glucose homeostasis. TGR5-mediated effects of BAs appear to center on the stimulation of GLP-1 release and the subsequent improvement in insulin secretion and pancreatic function (Drucker, 2006; Drucker and Nauck, 2006). BAs that escape the first-pass hepatic clearance and end up in the systemic circulation (Angelin et al., 1982) then fine-tune energy expenditure through activation of TGR5 in BAT and muscle with a resulting attenuation of weight gain (Watanabe et al., 2004). By contrast, so far no significant weight loss has been reported in the context of FXR activation in mice (Duran-Sandoval et al., 2005; Ma et al., 2006; Watanabe et al., 2006; Zhang et al., 2006). Instead, FXR-mediated actions of BAs appear mainly to affect liver metabolism by increasing glycogen production (Duran-Sandoval et al., 2005; Ma et al., 2006; Zhang et al., 2006) and decreasing lipogenesis and VLDL production (Watanabe et al., 2004), thereby reducing hepatic glucose and fatty acid output, which in turn contributes to improved insulin sensitivity (Duran-Sandoval et al., 2005; Ma et al., 2006; Zhang et al., 2006).

The link between BAs and glucose homeostasis may be relevant not only in mice but also in humans. In support of this hypothesis, a correlation between BA levels and insulin sensitivity has already been evidenced in humans (Shaham et al., 2008). Interestingly, plasma BA levels were also found to be significantly higher in subjects after bariatric surgery than in matched obese patients, suggesting that BAs may contribute to the metabolic improvements of weight loss surgery (Patti et al., 2009). The inverse relationship between total BA

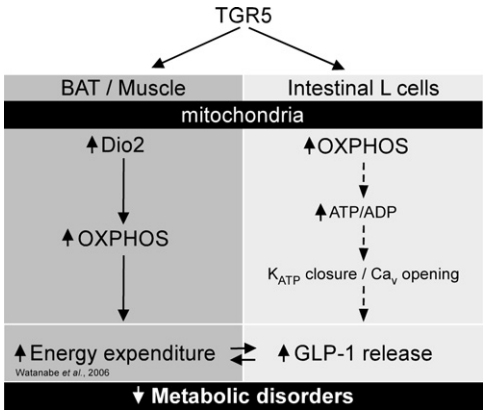

Figure 7. TGR5-Mediated BA Sensing Maintains Metabolic Function
In BAT (mouse) and in skeletal muscle (human), TGR5 activation triggers an increase in mitochondrial oxidative phosphorylation (OXPHOS), which results in energy expenditure and helps prevent obesity in mice treated with TGR5 agonists. Here we demonstrated that in enteroendocrine L cells, TGR5 activation also triggers an increase in mitochondrial OXPHOS, which is associated with a rise in the ATP/ADP ratio and a subsequent closure of the ATP-dependent potassium channel (K_{ATP}) and calcium mobilization (Ca_v). As a consequence, release of the incretin glucagon-like peptide-1 (GLP-1) is increased, which helps explain the improvement of glucose homeostasis in obese mice treated with a TGR5 agonist.

concentration and 2 hr postmeal glucose levels, as well as the positive correlation between BAs and peak GLP-1 in that study, further indicates that also in humans BAs may be key regulators of GLP-1 release and glucose homeostasis (Patti et al., 2009).

In conclusion, by employing a combination of pharmacological and genetic gain- and loss-of-function genetic approaches, our studies show that the activation of the TGR5 signaling pathway counteracts the metabolic dysfunction associated with diabesity. TGR5 activation results in a range of beneficial metabolic effects that include resistance to weight gain and hepatic steatosis, preservation of liver and pancreatic function, and the maintenance of glucose homeostasis and insulin sensitivity. These effects are due to enhanced mitochondrial function in muscle, BAT, and enteroendocrine cells, resulting in an increase in energy expenditure and incretin secretion (Figure 7). This leads us to conclude that TGR5 agonists could represent potential promising agents for the management of diabesity, along with associated disorders such as nonalcoholic steatohepatitis (NASH).

EXPERIMENTAL PROCEDURES

Chemicals and Reagents
All biochemical reagents were purchased from Sigma-Aldrich unless indicated. The DPP4 inhibitor (DPP4i) sitagliptin was a kind gift from Dr. C. Ullmer (Hoffmann-La Roche). INT-777 was synthesized as previously described (Macchiarulo et al., 2008; Pellicciari et al., 2007).

Cell Culture
In vitro experiments were carried out in STC-1 or NCI-H716 cells treated with vehicle (DMSO) or INT-777. INT-777 was assessed for its agonistic activity on TGR5 as previously described (Macchiarulo et al., 2008; Pellicciari et al., 2007). cAMP production was performed as described (Sato et al., 2008; Watanabe et al., 2006). Cox activity was evaluated by following the oxidation of fully reduced cytochrome *c* (Sigma) at 550 nm (Feige et al., 2008b). ATP/ADP ratio and GLP-1 release were measured according to the manufacturers' instructions (Biovision and Millipore, respectively). Primary brown adipocytes were prepared as previously described (Watanabe et al., 2006), and ileal explants were prepared according to an established method (Cima et al., 2004).

Intracellular Calcium Quantification
NCI-H716 (40,000 cells) was seeded in 96-well black plates coated with Matrigel (BD Biosciences). Seventy-two hours after transfection, cells were washed twice in assay buffer (HBSS1x, 20 mM HEPES [pH 7.4]) and assayed for intracellular calcium with Fluo-4 AM according to the manufacturer's protocol (Invitrogen).

Biochemistry and Histochemistry
Plasma parameters and hepatic and fecal lipid content were measured as described (Mataki et al., 2007). Hematoxylin and eosin (H&E), Sirius red, and oil red O staining were performed as described (Mark et al., 2007), and micrographs were taken on wide-field microscopes (Leica) with a CCD camera. For pancreatic sections, islets were sized and counted from four HE-stained alternated sections spaced of 150 μM using ImageJ software (five animals per group). Immunofluorescent staining of insulin was performed as described (Fajas et al., 2004). Additionally, pancreatic islets were isolated by collagenase digestion of pancreas from HF-fed TGR5-Tg mice according to online-available procedures (http://www.jove.com/index/Details.stp?ID=255). Insulin was extracted after O/N incubation at −20°C in acid ethanol and measured by ELISA on PBS-diluted samples according to the manufacturer's instructions (Mercodia). GLP-1 release was measured in vitro, ex vivo, and in vivo as described in the Supplemental Experimental Procedures.

Oxygen Consumption Measurement
Cellular oxygen consumption was measured using a Seahorse Bioscience XF24 analyzer with ten biological replicates per condition (Feige et al., 2008b).

Animal Experiments
Animals were housed and bred according to standardized procedures (Argmann and Auwerx, 2006b). Age-matched male mice were used for all experiments. Genetically engineered mouse models (GEMMs), i.e., TGR5-Tg and *TGR5*$^{-/-}$ mice, were generated as described in the Supplemental Data. DIO in the GEMMs or C57BL/6J mice (Charles River) was induced by feeding 8-week-old mice with a HF diet (60% cal/fat, D12492; Research Diets) for at least 8 weeks, as mentioned in the text and figure legends. In the dietary intervention experiments, INT-777 was mixed with diet (Feige et al., 2008a) at the dose sufficient to reach an in vivo dose of 30 mg/kg/d. Mouse phenotyping experiments were performed according to EMPRESS protocols (http://empress.har.mrc.ac.uk) and were aimed to assess food and water intake, body composition (Argmann et al., 2006a), energy expenditure (Argmann et al., 2006a), glucose and lipid homeostasis (Argmann et al., 2006b; Heikkinen et al., 2007; Mataki et al., 2007), and plasma biochemistry (Argmann and Auwerx, 2006a). Tissues and blood were collected and processed for histopathology, blood chemistry, and gene expression according to standardized procedures (Argmann and Auwerx, 2006a; Feige et al., 2008b; Mark et al., 2007; Watanabe et al., 2006). Hyperinsulinemic euglycemic clamp studies were performed as described (Feige et al., 2008b), with minor modifications including a change in the initial insulin bolus (30 mU/kg) and insulin infusion rate (10 mU/min/kg). Plasma GLP-1 levels were measured by ELISA (Millipore) on blood collected by retro-orbital puncture. Experiments with db/db mice (Charles River) were performed in 14-week-old animals fed a CD without or with INT-777 (30 mg/kg/d) for 6 weeks (Feige et al., 2008a).

Gene Expression Profiling
Gene expression profiling was performed by real-time quantitative PCR (Feige et al., 2008b; Watanabe et al., 2006). Primer sequences used have been previously published, except those used for the Kir6.2 gene: R-5′ AGATGCTAAACTTGGGCTTG, F-5′ TAAAGTGCCCACACCACTC.

Statistics
Statistical analyses were performed by using the unpaired Student's t test. Data are expressed as mean ± SEM, and P values smaller than 0.05 were considered statistically significant.

SUPPLEMENTAL DATA

Supplemental Data include six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cell-metabolism/supplemental/S1550-4131(09)00230-7.

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REFERENCES

Angelin, B., Bjorkhem, I., Einarsson, K., and Ewerth, S. (1982). Hepatic uptake of bile acids in man. Fasting and postprandial concentrations of individual bile acids in portal venous and systemic blood serum. J. Clin. Invest. *70*, 724–731.

Argmann, C.A., and Auwerx, J. (2006a). Collection of blood and plasma from the mouse. Curr. Protoc. Mol. Biol. Chapter 29, Unit 29A, 23.

Argmann, C.A., and Auwerx, J. (2006b). Minimizing variation due to genotype and environment. Curr. Protoc. Mol. Biol. Chapter 29, Unit 29A, 22.

Argmann, C.A., Champy, M.F., and Auwerx, J. (2006a). Evaluation of energy homeostasis. Curr. Protoc. Mol. Biol. Chapter 29, Unit 29B, 21.

Argmann, C.A., Houten, S.M., Champy, M.F., and Auwerx, J. (2006b). Lipid and bile acid analysis. Curr. Protoc. Mol. Biol. Chapter 29, Unit 29B, 22.

Chu, Z.L., Jones, R.M., He, H., Carroll, C., Gutierrez, V., Lucman, A., Moloney, M., Gao, H., Mondala, H., Bagnol, D., et al. (2007). A role for beta-cell-expressed G protein-coupled receptor 119 in glycemic control by enhancing glucose-dependent insulin release. Endocrinology *148*, 2601–2609.

Cima, I., Corazza, N., Dick, B., Fuhrer, A., Herren, S., Jakob, S., Ayuni, E., Mueller, C., and Brunner, T. (2004). Intestinal epithelial cells synthesize glucocorticoids and regulate T cell activation. J. Exp. Med. *200*, 1635–1646.

Despres, J.P., and Lemieux, I. (2006). Abdominal obesity and metabolic syndrome. Nature *444*, 881–887.

Drucker, D.J. (2006). The biology of incretin hormones. Cell Metab. *3*, 153–165.

Drucker, D.J., and Nauck, M.A. (2006). The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet *368*, 1696–1705.

Duran-Sandoval, D., Cariou, B., Percevault, F., Hennuyer, N., Grefhorst, A., van Dijk, T.H., Gonzalez, F.J., Fruchart, J.C., Kuipers, F., and Staels, B. (2005). The farnesoid X receptor modulates hepatic carbohydrate metabolism during the fasting-refeeding transition. J. Biol. Chem. *280*, 29971–29979.

Edfalk, S., Steneberg, P., and Edlund, H. (2008). Gpr40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion. Diabetes *57*, 2280–2287.

Fajas, L., Annicotte, J.S., Miard, S., Sarruf, D., Watanabe, M., and Auwerx, J. (2004). Impaired pancreatic growth, beta cell mass, and beta cell function in E2F1(−/−) mice. J. Clin. Invest. *113*, 1288–1295.

Feige, J.N., Lagouge, M., and Auwerx, J. (2008a). Dietary manipulation of mouse metabolism. Curr. Protoc. Mol. Biol. Chapter 29, Unit 29B, 25.

Feige, J.N., Lagouge, M., Canto, C., Strehle, A., Houten, S.M., Milne, J.C., Lambert, P.D., Mataki, C., Elliott, P.J., and Auwerx, J. (2008b). Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. Cell Metab. *8*, 347–358.

Hansotia, T., Maida, A., Flock, G., Yamada, Y., Tsukiyama, K., Seino, Y., and Drucker, D.J. (2007). Extrapancreatic incretin receptors modulate glucose homeostasis, body weight, and energy expenditure. J. Clin. Invest. *117*, 143–152.

Heikkinen, S., Argmann, C.A., Champy, M.F., and Auwerx, J. (2007). Evaluation of glucose homeostasis. Curr. Protoc. Mol. Biol. Chapter 29, Unit 29B, 23.

Hirasawa, A., Tsumaya, K., Awaji, T., Katsuma, S., Adachi, T., Yamada, M., Sugimoto, Y., Miyazaki, S., and Tsujimoto, G. (2005). Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat. Med. *11*, 90–94.

Houten, S.M., Watanabe, M., and Auwerx, J. (2006). Endocrine functions of bile acids. EMBO J. *25*, 1419–1425.

Katsuma, S., Hirasawa, A., and Tsujimoto, G. (2005). Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. Biochem. Biophys. Res. Commun. *329*, 386–390.

Kawamata, Y., Fujii, R., Hosoya, M., Harada, M., Yoshida, H., Miwa, M., Fukusumi, S., Habata, Y., Itoh, T., Shintani, Y., et al. (2003). A G protein-coupled receptor responsive to bile acids. J. Biol. Chem. *278*, 9435–9440.

Ma, K., Saha, P.K., Chan, L., and Moore, D.D. (2006). Farnesoid X receptor is essential for normal glucose homeostasis. J. Clin. Invest. *116*, 1102–1109.

Macchiarulo, A., Gioiello, A., Thomas, C., Massarotti, A., Nuti, R., Rosatelli, E., Sabbatini, P., Schoonjans, K., Auwerx, J., and Pellicciari, R. (2008). Molecular field analysis and 3D-quantitative structure-activity relationship study (MFA 3D-QSAR) unveil novel features of bile acid recognition at TGR5. J. Chem. Inf. Model. *48*, 1792–1801.

Mark, M., Teletin, M., Antal, C., Wendling, O., Auwerx, J., Heikkinen, S., Khetchoumian, K., Argmann, C.A., and Dgheem, M. (2007). Histopathology in mouse metabolic investigations. Curr. Protoc. Mol. Biol. Chapter 29, Unit 29B, 24.

Maruyama, T., Miyamoto, Y., Nakamura, T., Tamai, Y., Okada, H., Sugiyama, E., Itadani, H., and Tanaka, K. (2002). Identification of membrane-type receptor for bile acids (M-BAR). Biochem. Biophys. Res. Commun. *298*, 714–719.

Mataki, C., Magnier, B.C., Houten, S.M., Annicotte, J.S., Argmann, C., Thomas, C., Overmars, H., Kulik, W., Metzger, D., Auwerx, J., and Schoonjans, K. (2007). Compromised intestinal lipid absorption in mice with a liver-specific deficiency of liver receptor homolog 1. Mol. Cell. Biol. *27*, 8330–8339.

Nichols, C.G. (2006). KATP channels as molecular sensors of cellular metabolism. Nature *440*, 470–476.

Overton, H.A., Babbs, A.J., Doel, S.M., Fyfe, M.C., Gardner, L.S., Griffin, G., Jackson, H.C., Procter, M.J., Rasamison, C.M., Tang-Christensen, M., et al. (2006). Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. Cell Metab. *3*, 167–175.

Patti, M.E., Houten, S.M., Bianco, A.C., Bernier, R., Larsen, P.R., Holst, J.J., Badman, M.K., Maratos-Flier, E., Mun, E.C., Pihlajamaki, J., et al. (2009). Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism. Obesity. Published online April 9, 2009. 10.1038/oby.2009.102.

Pellicciari, R., Sato, H., Gioiello, A., Costantino, G., Macchiarulo, A., Sadeghpour, B.M., Giorgi, G., Schoonjans, K., and Auwerx, J. (2007). Nongenomic actions of bile acids. Synthesis and preliminary characterization of 23- and 6,23-alkyl-substituted bile acid derivatives as selective modulators for the G-protein coupled receptor TGR5. J. Med. Chem. *50*, 4265–4268.

Reimann, F., and Gribble, F.M. (2002). Glucose-sensing in glucagon-like peptide-1-secreting cells. Diabetes *51*, 2757–2763.

Reimann, F., Maziarz, M., Flock, G., Habib, A.M., Drucker, D.J., and Gribble, F.M. (2005). Characterization and functional role of voltage gated cation

conductances in the glucagon-like peptide-1 secreting GLUTag cell line. J. Physiol. *563*, 161–175.

Reimann, F., Habib, A.M., Tolhurst, G., Parker, H.E., Rogers, G.J., and Gribble, F.M. (2008). Glucose sensing in L cells: a primary cell study. Cell Metab. *8*, 532–539.

Sakamoto, Y., Inoue, H., Kawakami, S., Miyawaki, K., Miyamoto, T., Mizuta, K., and Itakura, M. (2006). Expression and distribution of Gpr119 in the pancreatic islets of mice and rats: predominant localization in pancreatic polypeptide-secreting PP-cells. Biochem. Biophys. Res. Commun. *351*, 474–480.

Sato, H., Macchiarulo, A., Thomas, C., Gioiello, A., Une, M., Hofmann, A.F., Saladin, R., Schoonjans, K., Pellicciari, R., and Auwerx, J. (2008). Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. J. Med. Chem. *51*, 1831–1841.

Shaham, O., Wei, R., Wang, T.J., Ricciardi, C., Lewis, G.D., Vasan, R.S., Carr, S.A., Thadhani, R., Gerszten, R.E., and Mootha, V.K. (2008). Metabolic profiling of the human response to a glucose challenge reveals distinct axes of insulin sensitivity. Mol. Syst. Biol. *4*, 214.

Soga, T., Ohishi, T., Matsui, T., Saito, T., Matsumoto, M., Takasaki, J., Matsumoto, S., Kamohara, M., Hiyama, H., Yoshida, S., et al. (2005). Lysophosphatidylcholine enhances glucose-dependent insulin secretion via an orphan G-protein-coupled receptor. Biochem. Biophys. Res. Commun. *326*, 744–751.

Thomas, C., Pellicciari, R., Pruzanski, M., Auwerx, J., and Schoonjans, K. (2008). Targeting bile-acid signalling for metabolic diseases. Nat. Rev. Drug Discov. *7*, 678–693.

Watanabe, M., Houten, S.M., Wang, L., Moschetta, A., Mangelsdorf, D.J., Heyman, R.A., Moore, D.D., and Auwerx, J. (2004). Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. J. Clin. Invest. *113*, 1408–1418.

Watanabe, M., Houten, S.M., Mataki, C., Christoffolete, M.A., Kim, B.W., Sato, H., Messaddeq, N., Harney, J.W., Ezaki, O., Kodama, T., et al. (2006). Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature *439*, 484–489.

Yang, S.N., and Berggren, P.O. (2006). The role of voltage-gated calcium channels in pancreatic beta-cell physiology and pathophysiology. Endocr. Rev. *27*, 621–676.

Zhang, Y., and Edwards, P.A. (2008). FXR signaling in metabolic disease. FEBS Lett. *582*, 10–18.

Zhang, Y., Lee, F.Y., Barrera, G., Lee, H., Vales, C., Gonzalez, F.J., Willson, T.M., and Edwards, P.A. (2006). Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. Proc. Natl. Acad. Sci. USA *103*, 1006–1011.

Cell Metabolism
Article

Specific SIRT1 Activation Mimics Low Energy Levels and Protects against Diet-Induced Metabolic Disorders by Enhancing Fat Oxidation

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SUMMARY

The NAD$^+$-dependent deacetylase SIRT1 controls metabolic processes in response to low nutrient availability. We report the metabolic phenotype of mice treated with SRT1720, a specific and potent synthetic activator of SIRT1 that is devoid of direct action on AMPK. SRT1720 administration robustly enhances endurance running performance and strongly protects from diet-induced obesity and insulin resistance by enhancing oxidative metabolism in skeletal muscle, liver, and brown adipose tissue. These metabolic effects of SRT1720 are mediated by the induction of a genetic network controlling fatty acid oxidation through a multifaceted mechanism that involves the direct deacetylation of PGC-1α, FOXO1, and p53 and the indirect stimulation of AMPK signaling through a global metabolic adaptation mimicking low energy levels. Combined with our previous work on resveratrol, the current study further validates SIRT1 as a target for the treatment of metabolic disorders and characterizes the mechanisms underlying the therapeutic potential of SIRT1 activation.

INTRODUCTION

The prevalence of metabolic disorders has been increasing over the past decades with the adoption of a sedentary lifestyle combined with excessive caloric intake. Increased physical activity and better feeding habits are clearly a requisite to limit or reverse weight excess and its deleterious metabolic consequences. However, dietary management and exercise are not usually successful as an intervention, underscoring the need for more efficient medication to treat metabolic disorders. Intense drug discovery efforts in the metabolic field currently focus on enhancing energy expenditure in organs specialized in energy consumption.

Integrated metabolic networks, which are governed at the transcriptional level by transcription factors and coregulators, enable the organism to adapt the metabolic state of different organs to nutrient availability (Desvergne et al., 2006; Feige and Auwerx, 2007; Spiegelman and Heinrich, 2004). Sirtuins, a family of NAD$^+$-dependent deacetylases, have recently emerged as integral components of these metabolic networks, which are particularly important for energy homeostasis (Guarente, 2006; Michan and Sinclair, 2007; Yamamoto et al., 2007). Sirtuins modulate gene expression according to the energetic state of the cell, which they sense through NAD$^+$ levels, by deacetylating histones as well as transcription factors and coregulators (Feige and Auwerx, 2008). The founding member of the family, SIRT1, promotes longevity in response to caloric restriction in species ranging from yeast to mammals, and it is believed that these protective actions may result, at least in part, from the regulation of energy homeostasis (Guarente, 2006). Consistently, SIRT1 is an important regulator of metabolic processes such as lipolysis, fatty acid oxidation (FAO), mitochondrial activity, and gluconeogenesis (Baur et al., 2006; Gerhart-Hines et al., 2007; Lagouge et al., 2006; Picard et al., 2004; Rodgers et al., 2005; Rodgers and Puigserver, 2007), which occur in response to an intracellular rise in the NAD$^+$/NADH ratio when energy supply is low. As SIRT1 is activated by caloric restriction, the ability to allosterically induce its activity opens the possibility to pharmacologically mimic low energetic levels and, thereby, stimulate fat utilization to prevent diet-induced obesity and its associated disorders. This concept has been validated in mice treated with the natural polyphenol resveratrol, which activates SIRT1 and protects from obesity by inducing oxidative mitochondrial metabolism through deacetylation of the PPARγ coactivator 1α (PGC-1α) (Baur et al., 2006; Lagouge et al., 2006). The observation that resveratrol can activate both SIRT1 and the AMP-activated protein kinase (AMPK) leaves open the question of what degree of efficacy is due to the direct activation of SIRT1 (Baur et al., 2006). To address this issue, we have used a selective synthetic SIRT1 activator with improved on-target selectivity, potency, and efficacy, and we have tested its metabolic actions for protection against diet-induced obesity and metabolic disorders in mouse models. Our results demonstrate that SRT1720 administration protects from diet-induced obesity and its negative consequences on glucose homeostasis by primarily promoting fat consumption in skeletal muscle, liver, and brown adipose tissue (BAT).

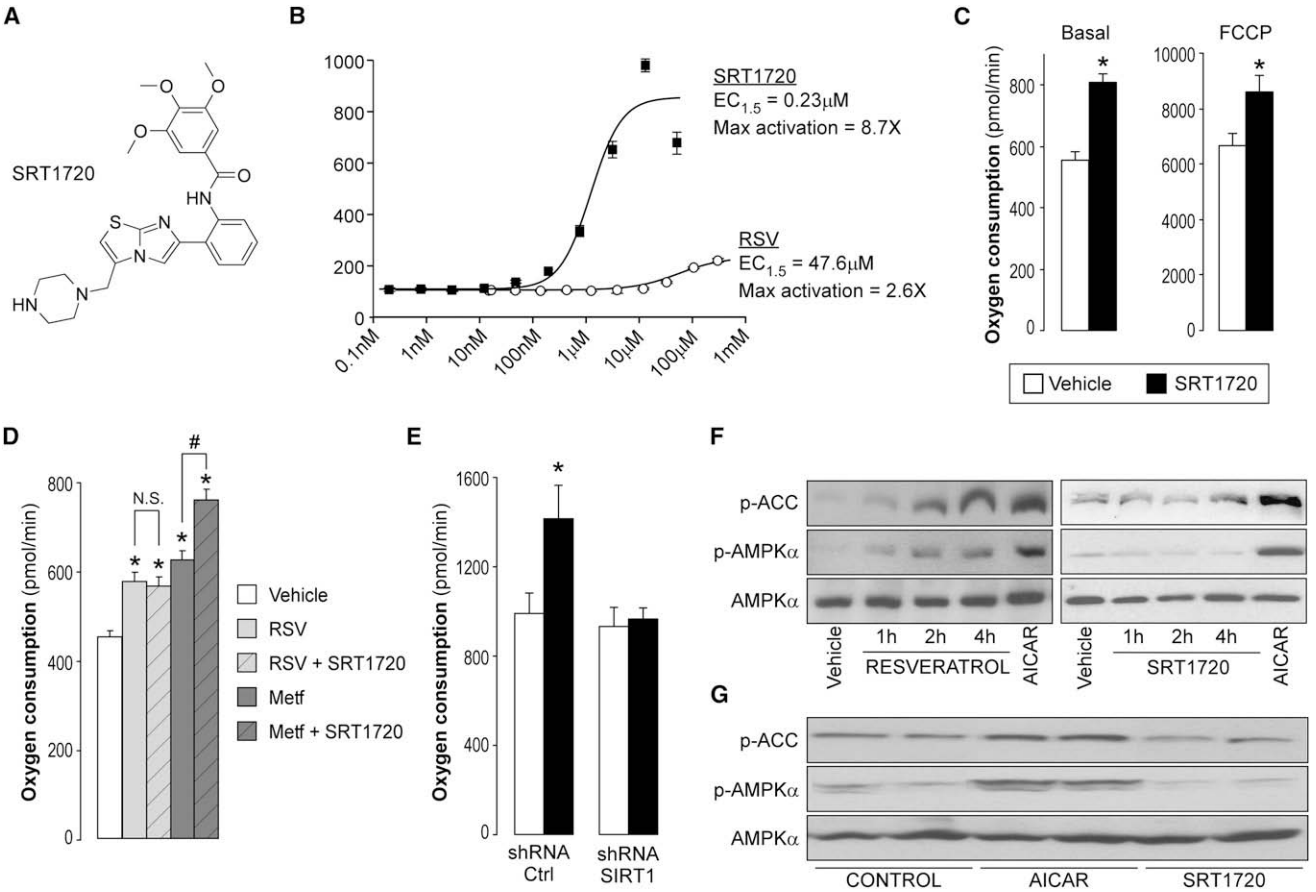

Figure 1. SRT1720 Is a High-Affinity Specific Activator of SIRT1

(A) Structure of the SRT1720 compound.

(B) The action of resveratrol (RSV) and SRT1720 on SIRT1 activity was assessed in a mass spectrometry-based deacetylation assay of a 20 residue peptide.

(C–E) Oxygen consumption was measured in C2C12 myotubes treated for 24 hr with 10 nM SRT1720 before or after a 1 hr treatment with the uncoupler FCCP at 500 nM (C), in combination with 5 μM resveratrol (RSV) or 2 mM metformin (Metf) (D), or 48 hr following an adenoviral infection with control or SIRT1 shRNAs (E).

(F) The activation of the AMP-activated protein kinase (AMPK) was assessed in C2C12 myotubes treated with vehicle, vehicle and 500 μM AICAR (15 min), 5 μM resveratrol, or 50 nM SRT1720 by evaluating the phosphorylation state of AMPKα and its target acetylCoA carboxylase (ACC) by western blotting.

(G) The acute action of SRT1720 on AMPK activation was evaluated after a single administration at 500 mg/kg in wild-type male C57BL/6J 4 hr prior to sacrifice. AICAR was administered as a positive control 4 hr prior to sacrifice at 500 mg/kg. AMPKα and ACC phosphorylation was analyzed by western blot in gastrocnemius muscle.

Error bars represent SEM, and significant differences to untreated controls ($p < 0.05$) are indicated by an asterisk. Other significant differences are indicated by a pound symbol.

RESULTS

SRT1720 Is a Potent and Specific Activator of SIRT1

Given the beneficial metabolic actions of the naturally occurring SIRT1 activator resveratrol, we selected a more potent and efficacious SIRT1 activator identified in a small molecule screen for SIRT1 agonists (Milne et al., 2007; Figure 1A). In a mass spectrometry deacetylation assay, SRT1720 induced the activity of SIRT1 by 8.7-fold, whereas the activation by resveratrol was limited to a 2.6-fold increase (Figure 1B). Importantly, this increased efficacy was also associated with enhanced potency, as the $EC_{1.5}$ for SIRT1, which represents the concentration required to reach a 50% increase over basal, was 0.23 μM for SRT1720 but 47.6 μM for resveratrol.

Since resveratrol protects against metabolic disorders by inducing energy expenditure, the action of SRT1720 was validated in a cellular model utilizing oxygen consumption as a readout of energy expenditure. In C2C12 myotubes, SRT1720 significantly stimulated oxygen consumption both in basal conditions and after stimulation with the chemical uncoupler FCCP (Figure 1C). Interestingly, this effect was observed after 24 hr of treatment, but not after a short 1 hr treatment (data not shown), a timing suggesting that the actions of SRT1720 on oxygen consumption are mediated by transcriptional mechanisms. In addition, the SRT1720-mediated stimulation of oxygen consumption was totally blocked by the ATP synthase inhibitor oligomycin (Figure S1A available online). To evaluate the specificity of SRT1720, we first analyzed whether it could synergize with other stimulators of cellular respiration in C2C12 cells (Figure 1D). The actions of SRT1720 were additive to those of the AMPK activator metformin, but not to those of resveratrol, suggesting that SRT1720 induces oxygen consumption via SIRT1. The direct involvement of SIRT1 in the

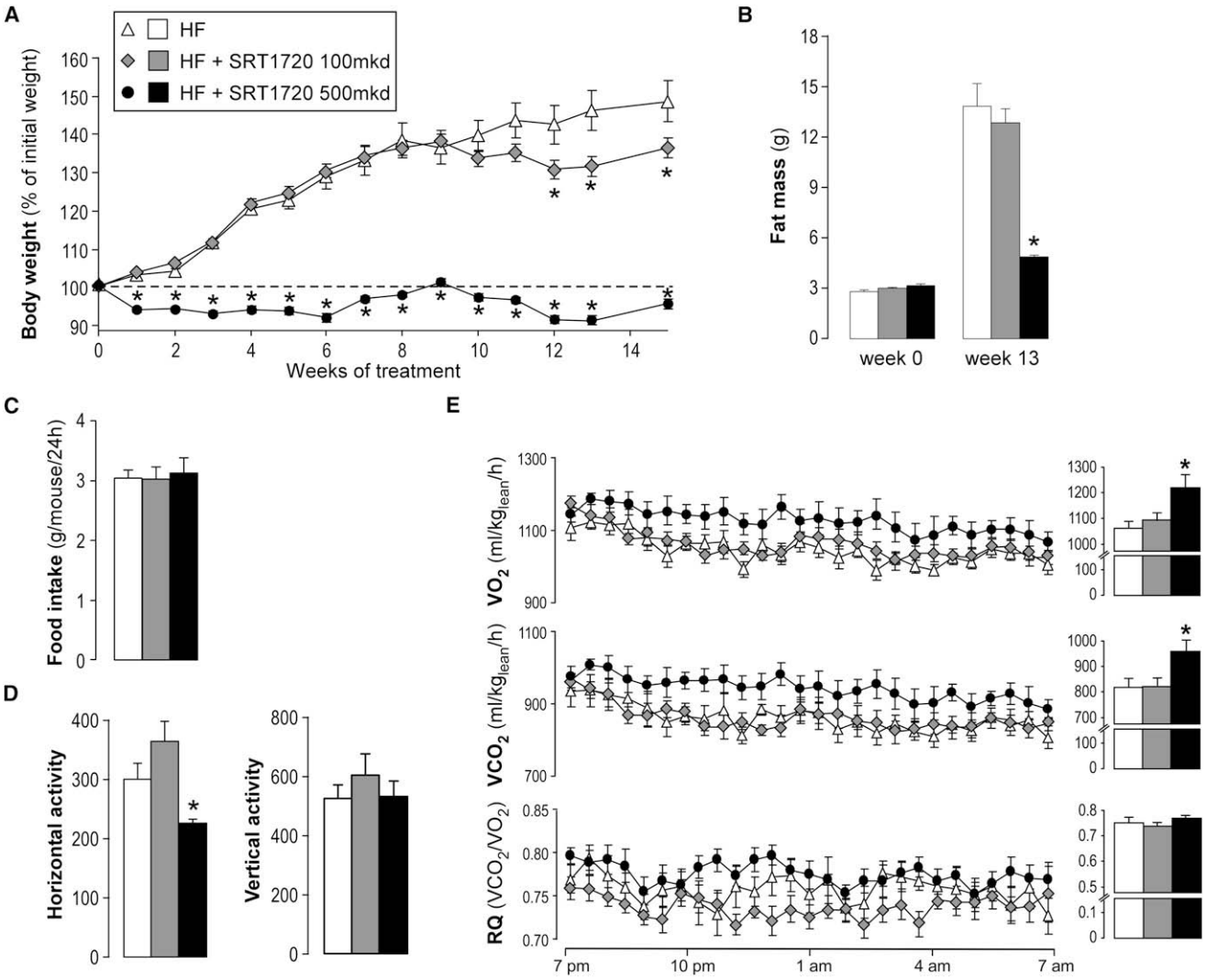

Figure 2. SRT1720 Prevents Diet-Induced Obesity in Male C57BL/6J Mice

(A) Body weight evolution of animals fed with a HF diet alone or supplemented with SRT1720 at 100 or 500 mg per kg of body weight per day (mkd) (n = 10).

(B) Fat mass was measured by dexascan before and after 13 weeks of treatment (n = 10).

(C) Average daily food intake (n = 10).

(D) Spontaneous activity of mice represented by the average horizontal and vertical activities monitored over a 24 hr period after 13 weeks of treatment (n = 8).

(E) Energy expenditure was evaluated by the measurement of oxygen consumption (VO_2), of carbon dioxide release (VCO_2), and by the calculation of the respiratory quotient (RQ) over a 12 hr period after 10 weeks of treatment. The adjacent bar graphs represent the average for each group (n = 8).

Error bars represent SEM, and significant differences compared to untreated controls ($p < 0.05$) are indicated by an asterisk.

response to SRT1720 was formally demonstrated using a shRNA knockdown of SIRT1, which ablated the SRT1720-mediated increase of oxygen consumption (Figure 1E). In addition, we evaluated the action of SRT1720 on AMPK, which has been demonstrated to be activated by resveratrol independently of SIRT1 (Baur et al., 2006; Dasgupta and Milbrandt, 2007). Unlike resveratrol and the synthetic AMPK activator AICAR, SRT1720 did not induce a significant level of phosphorylation of the α subunit of AMPK and of its downstream target acetylCoA carboxylase (ACC) in C2C12 myotubes at various time points and concentrations (Figures 1F and S1B). Furthermore, and perhaps most importantly, acute SRT1720 administration in vivo did not enhance AMPK signaling either (Figure 1G). Altogether, these results demonstrate that SRT1720 is a potent and specific SIRT1 activator that does not directly stimulate AMPK.

SRT1720 Protects from Diet-Induced Diabesity

The metabolic actions of SRT1720 were then evaluated by administering the compound by food admixture to wild-type C57BL/6J male mice challenged with a high-fat (HF) diet. The incorporation of the compound to the diet was adjusted weekly to food intake and body weight in order to achieve average exposures of 100 and 500 mg/kg/day (mkd). While the low dose only partially protected from weight gain after ~10 weeks of treatment, the high dose totally prevented diet-induced obesity by inhibiting fat accumulation (Figures 2A and 2B). Importantly,

this effect on body weight did not result from altered feeding behavior or increased locomotor activity (Figures 2C, 2D, and S2A). Enzymes associated with hepatic injury or tissue breakdown were normal during the study, indicating that SRT1720 was well tolerated and that the efficacy observed was not through a toxic response (Figure S2B). In addition, despite slightly elevated fecal lipid content, the histology of the gut was not altered by the treatment (Figures S2C and S2D), suggesting that SRT1720 did not significantly alter intestinal nutrient uptake.

The lean phenotype of SRT1720-treated mice fed HF diet correlated with enhanced energy expenditure, as both oxygen consumption and carbon dioxide release were increased by the treatment (Figure 2E). This stimulation of the metabolic rate occurred without changes in the respiratory quotient (RQ), which reflects the relative use of carbohydrates versus lipids as a source of energy. Since mice on HF diet use fatty acids as the main source of energy, the RQ of untreated animals was close to 0.7, the theoretical lower limit of this parameter corresponding to lipid utilization solely. To evaluate the action of SRT1720 on fuel preference in a setting where RQ variations were not masked by HF content in the diet, we treated mice on regular chow diet and repeated indirect calorimetry either in fed animals or in animals where endogenous fat utilization was stimulated by short-term fasting (Figure S3B). The RQ was higher on chow than on HF diet (0.95 versus 0.75, respectively) and was only slightly lowered by SRT1720 in fed conditions. However, the RQ of chow-fed mice decreased robustly upon fasting, demonstrating that SRT1720 can shift the fuel preference toward fatty acids.

Since diet-induced obesity exerts detrimental actions on plasma lipid profiles and glucose homeostasis, we analyzed whether SRT1720 could normalize these parameters. Lipid profiles in the plasma of SRT1720-treated mice were improved as both triglyceride and cholesterol levels were reduced on HF diet (Figure 3A). Reduced cholesterol concentrations resulted mainly from a decrease in LDL cholesterol (Figure 3A). On chow diet, cholesterol levels were unaffected by SRT1720 (data not shown), but triglyceride and VLDL levels were lowered by SRT1720 in chow-fed mice (Figure S3C). SRT1720 also prevented the negative impact of HF feeding on glucose homeostasis as fasting blood glucose and insulin were both reduced (Figure 3B). In addition, mice treated with SRT1720 had a better tolerance to a glucose load, which was particularly prominent in the late time points of an intraperitoneal glucose tolerance test (IP-GTT) (Figure 3C). Together with the reduced fasting insulin levels, this observation suggested that SRT1720 improves glucose homeostasis by enhancing insulin sensitivity. This hypothesis was confirmed in a hyperinsulinemic-euglycemic clamp in which the glucose infusion rate, which reflects the sensitivity of peripheral tissues to insulin, was significantly enhanced by SRT1720 treatment (Figure 3D). Since obesity and insulin resistance are intricately linked, we demonstrated that improved glucose tolerance at 500 mkd was not an indirect consequence of reduced adiposity since glucose tolerance was also improved in an IP-GTT in chow-fed mice treated with SRT1720 for 3 weeks, in which body weight was not substantially affected (Figure S3E). In addition, the direct action of SRT1720 on insulin sensitivity was confirmed by the increased glucose infusion rate in a hyperinsulinemic-euglycemic clamp in mice treated for 3 weeks on HF diet, for which the average body weight difference between groups does not exceed 3 g (Figure 3E). In this experiment, tritiated glucose was used as a tracer to evaluate whole-body glucose turnover before and after insulin perfusion (Figure 3F). Although the glucose turnover only tended to increase in SRT1720-treated mice, the ability of insulin to suppress endogenous glucose production was significantly enhanced upon SRT1720 administration, demonstrating that SRT1720 improves hepatic insulin sensitivity. Furthermore, insulin sensitivity was also enhanced in the skeletal muscle, as insulin-stimulated glucose uptake, measured through ^{14}C-2-deoxy-glucose uptake, was higher in the gastrocnemius muscle of SRT1720-treated mice (Figure 3G).

Altogether, these results demonstrate that activating SIRT1 by SRT1720 in a context of caloric excess strongly protects from fat accretion. Furthermore, our data show that SRT1720 improves glucose tolerance and insulin sensitivity both in the context of HF and chow feeding.

SRT1720 Promotes Energy Expenditure in Metabolic Tissues

A key factor controlling energy homeostasis is the balance between caloric intake and expenditure, which modulates fat accumulation and peripheral insulin sensitivity. Therefore, we analyzed the actions of SRT1720 on lipid accumulation and energy expenditure in organs that integrate the metabolic response of the entire body. The mass of epididymal white adipose tissue (WAT) from mice, where HF feeding was thwarted by 500 mkd SRT1720, was three times lower than that of control mice on HF diet only (Figure S4A). In addition, this reduced fat accumulation was associated with smaller adipocyte size (Figure S4B), a feature that correlates with leanness and insulin sensitivity. Since smaller adipose tissue could be linked to altered adipogenesis or enhanced lipolysis, we verified the expression of well-established markers of these processes in WAT. SRT1720 did not induce major impairments in the ability of adipocytes to differentiate and store lipids, as despite a slight induction of peroxisome proliferator-activated receptor (PPAR) γ expression, the levels of its downstream targets C/EBPα, aP2, and CD36 were not affected (Figure S4C). Consistent with the previous demonstration of a positive action of SIRT1 on lipolysis (Picard et al., 2004), the RNA levels of the hormone-sensitive lipase were enhanced in SRT1720-treated mice. The enhanced expression of oxidative markers, such as the PGC-1 coactivators and the nuclear receptor PPARβ/δ, suggests that SRT1720 could promote energy expenditure in WAT. Nevertheless, the minor contribution of WAT to whole-body energy consumption suggests that this regulation is not the primary cause of the lean phenotype, which most likely results from the reduced availability of lipids for storage.

Since the absence of fat accumulation in SRT1720-treated mice suggested that energy expenditure could be stimulated, we analyzed the metabolic capacities of energy dissipating organs. Skeletal muscle function was examined utilizing endurance and locomotor tests (Figure 4A). Mice treated with SRT1720 on HF diet ran approximately twice the distance as control animals in an endurance exercise test. To rule out an indirect effect of weight difference on running capacities, we exercised chow-fed mice treated for 6 weeks with SRT1720 and

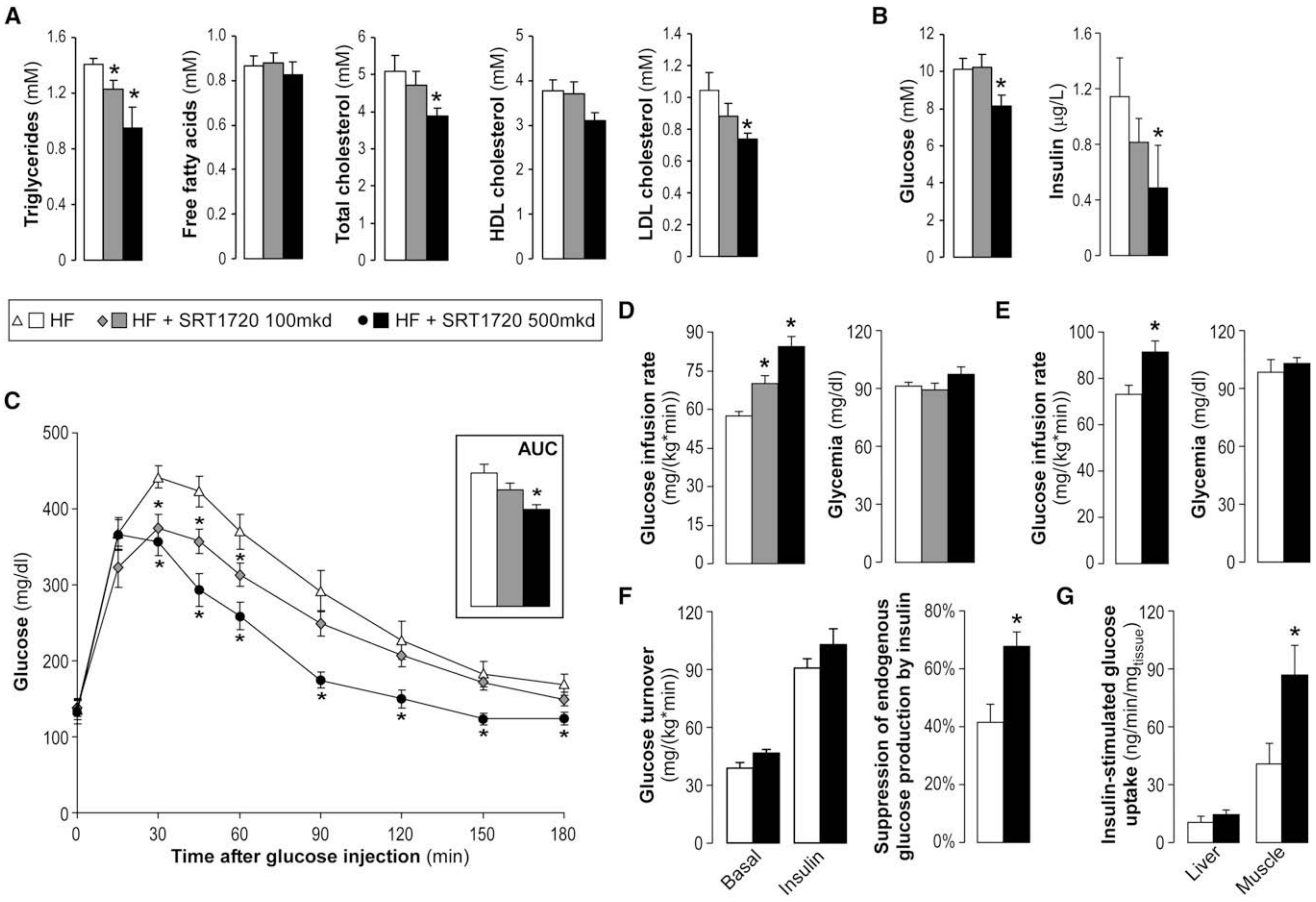

Figure 3. SRT1720 Improves Glucose and Cholesterol Homeostasis

(A and B) Lipid (A) and glucose and insulin (B) levels were measured after 16 hr of fasting in the plasma of mice treated with HF diet alone or supplemented with SRT1720 at 100 or 500 mkd for 6 weeks (n = 8).

(C) Intraperitoneal glucose tolerance test on mice treated with SRT1720 as in (A) for 12 weeks and injected with 2 g glucose/kg after 12 hr of fasting. The adjacent bar graph represents the average area under the curve (n = 10).

(D) Insulin sensitivity evaluated through the average glucose infusion rate at equilibrium in a hyperinsulinemic-euglycemic clamp (18 mU insulin/min/kg) in mice treated with SRT1720 as in (A) for 20 weeks. The equilibrium at clamp is reflected by the euglycemia shown in the adjacent graph (n = 5).

(E–G) A hyperinsulinemic-euglycemic clamp (18 mU insulin/min/kg) was performed using radioactive tracers in mice treated with SRT1720 as in (A) for 3 weeks. The evaluation of whole-body glucose turnover (F) and glucose uptake in tissues (G) was performed using ^{3}H-glucose and ^{14}C-2-deoxy-glucose tracers, respectively.

Error bars represent SEM, and significant differences compared to untreated controls (p value < 0.05) are indicated by an asterisk.

demonstrated that the animals on SRT1720 also became much better endurance runners (Figure S3D). Increased muscle strength in the grip strength test, as well as better locomotor behavior, as concluded from the increased latency to fall in a rotarod test and the reduced time to hind paw equilibration in a string test, all provide additional support to the improved muscle function upon SRT1720 administration. Since improved endurance can result from variations in the proportion of glycolytic and oxidative muscle fibers, we evaluated whether SRT1720 can promote fiber type switching through a succinate dehydrogenase (SDH) staining of muscle fibers (Figure 4B). The proportion of blue-stained oxidative fibers was higher in the gastrocnemius of SRT1720-treated mice, whereas it could not be further enhanced by the treatment in the purely oxidative soleus muscle. The shift toward more oxidative fibers occurred without changes in mitochondrial density (Figures S5B and S5C). In addition, citrate synthase (Figure S5D) and cytochrome C oxidase (Figure S5E) activities were not altered in SRT1720-treated mice, suggesting that SRT1720 enhances oxidative metabolism through other mitochondrial mechanisms.

Therefore, to understand the molecular basis of the improved endurance and fiber type switch, we analyzed gene expression from gastrocnemius skeletal muscle (Figure 4D). Despite an increased mRNA level of cytochrome C oxidase (Cox IV), the expression of most of the genes controlling mitochondrial function and oxidative phosphorylation (OxPhos) was only minimally affected by SRT1720 treatment. We observed, however, a switch in the contractile phenotype of the muscle fibers, as SRT1720-treated mice expressed higher mRNA levels of both troponin I slow (Tropol$_{slow}$) and type I and IIa myosin heavy chains (MyHC), three markers of slow-twitch oxidative fibers, but had reduced expression of the mRNA encoding the fast-twitch

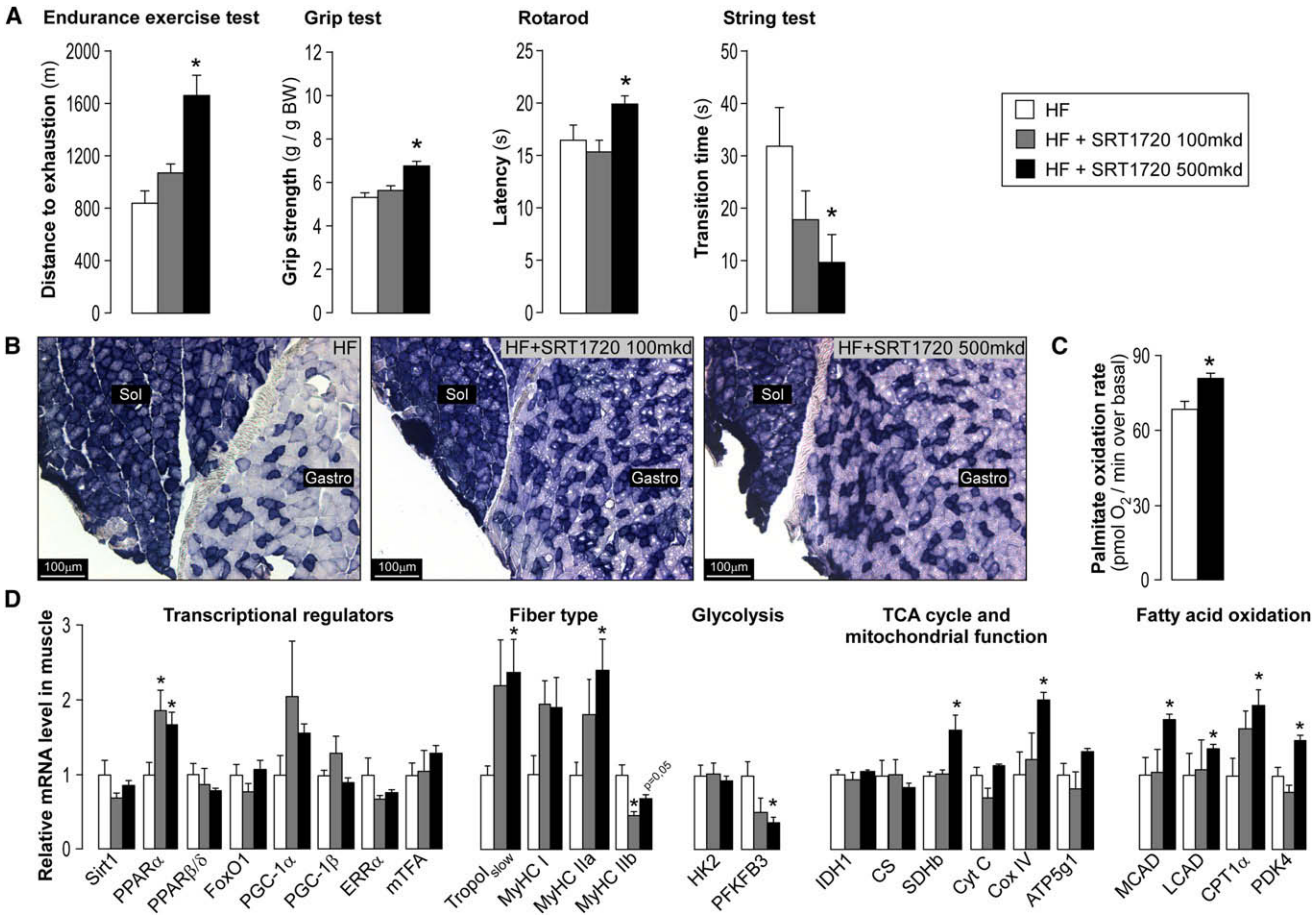

Figure 4. SRT1720 Improves Endurance and Locomotor Functions

(A) Endurance, evaluated by the average distance run until exhaustion on a treadmill, and locomotor functions, evaluated through the string, the grip strength, and the rotarod tests, were performed in mice treated for 15 weeks with HF diet alone or supplemented with either 100 or 500 mkd SRT1720 (n = 10).

(B) Representative succinate dehydrogenase staining on gastrocnemius (gastro) and soleus (sol) muscle sections.

(C) Fatty acid oxidation rates were measured in C2C12 cells treated for 24 hr with vehicle (white bars) or 10 nM SRT1720 (black bars) using oxygen consumption as a readout (n = 10).

(D) Gene expression in gastrocnemius skeletal muscle expressed relative to β2-microglobulin in the same mice as in (C). Abbreviations not used previously: HK, hexokinase; IDH, isocitrate dehydrogenase; Cyt C, cytochrome C; ATP5g1, ATP synthase subunit 5g1; CPT-1, carnitine palmitoyl transferase 1.

Error bars represent SEM, and significant differences compared to untreated controls ($p < 0.05$) are indicated by an asterisk.

glycolytic marker MyHC type IIb. Interestingly, this switch correlated with a lower expression of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and an increased expression of the pyruvate dehydrogenase kinase 4 (PDK4) mRNA, which favors the utilization of fatty acids over glucose by lowering the utilization of pyruvate through inhibition of the pyruvate dehydrogenase complex. Moreover, SRT1720 also stimulated the expression of genes promoting FAO, such as PPARα and the medium- and long-chain acyl-CoA dehydrogenases (MCAD and LCAD), to concomitantly increase the flux of fatty acid utilization in the muscle. In line with these observations, the genes upregulated by SRT1720 at the genome-wide level were enriched in genes from the mitochondrial β-oxidation pathway (Figure S5A). Moreover, these gene expression changes correlated functionally with enhanced FAO as SRT1720 increased palmitate oxidation rates in muscle cells (Figure 4C). Therefore, SRT1720 induces a switch toward more oxidative muscle fiber types by primarily stimulating FAO rather than by improving mitochondrial function per se.

Given the important contribution of hepatic FAO to whole-body energy expenditure, we evaluated whether the beneficial effects of SRT1720 on muscle oxidative functions could also be observed in the liver. Reduced staining with oil red O confirmed that hepatic fat storage in lipid droplets was significantly reduced in SRT1720-treated mice (Figure 5A), predominantly because the accumulation of triglycerides was inhibited (Figure 5B). In contrast, hepatic free fatty acid levels were not altered. We then tested the different metabolic pathways that SRT1720 could regulate to promote energy expenditure. The expression of genes controlling glycolysis was not altered in SRT1720-treated mice (Figure 5C), and gluconeogenesis was not consistently changed, as the expression of the phosphoenolpyruvate carboxykinase (PEPCK) was modestly enhanced, while that of glucose-6-phosphatase (G6Pase) remained unchanged. SRT1720, however, had

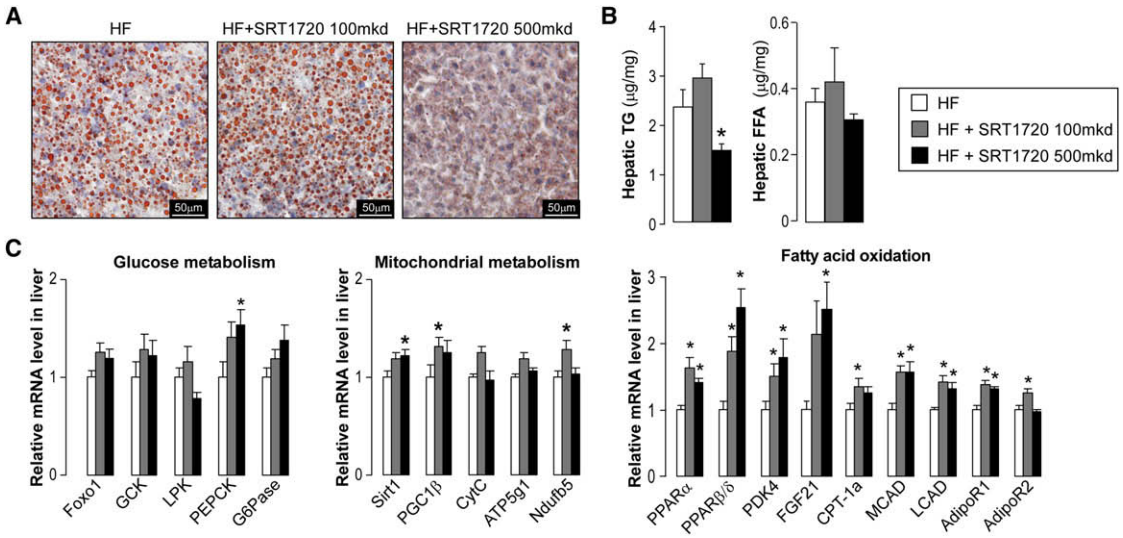

Figure 5. SRT1720 Promotes Hepatic Oxidative Functions

(A) Representative oil-red-O staining of liver sections from mice fed for 20 weeks with HF diet alone or supplemented with SRT1720 at 100 or 500 mkd.

(B) Hepatic lipid content was measured following Folch's extraction (n = 10).

(C) Gene expression in liver expressed relative to β-actin and 36B4 (n = 10). Abbreviations not used previously: GCK, glucocarboxykinase; LPK, L-type pyruvate kinase; Ndufb5, NADH dehydrogenase 1β5; AdipoR, adiponectin receptors.

Error bars represent SEM, and significant differences compared to untreated controls ($p < 0.05$) are indicated by an asterisk.

a strong impact on the expression of enzymes and regulators controlling FAO (Figures 5C and S5A). The expression of the two nuclear receptors PPARα and β/δ was robustly induced in the liver of SRT1720-treated mice. The functional relevance of this induction was validated by an increased expression of direct PPAR target genes. Among these, SRT1720 exerted particularly prominent actions on the mRNA expression of PDK4 and the fibroblast growth factor 21 (FGF21), an antiobesity signaling molecule secreted by the liver in response to PPARα activation (Kharitonenkov and Shanafelt, 2008). Consistent with what was observed in the muscle, the actions of SRT1720 on hepatic oxidative metabolism occurred with rather discrete changes in mitochondrial function and OxPhos (Figure 5C), suggesting that this SIRT1 activator stimulates hepatic energy expenditure by primarily acting on the degradation of fatty acids.

BAT is another major tissue where energy, coming predominantly from fat, is dissipated to maintain body temperature. Consistent with the lean phenotype induced by the treatment, mice on SRT1720 had smaller brown adipocytes, in which the size of lipid droplets was reduced (Figures 6A and 6B). Similar to what was observed in skeletal muscle, the number of mitochondria did not seem to be affected by SRT1720 despite a lower density caused by reduced lipid droplet size (Figure 6B). Low fat accumulation in BAT correlated with the global activation of a network of genes controlling energy expenditure (Figure 6C). The expression of several transcription factors, including PPARα and γ, thyroid hormone receptors (TR) α and β, and PGC-1α and β, was strongly activated in the BAT of SRT1720-treated animals. In addition, the upregulation of type 2 deiodinase (DIO2), an enzyme that converts inactive thyroid hormone T4 into T3, most likely synergizes with the elevated TR levels to promote thyroid hormone signaling. Several direct targets of PGC-1, such as the estrogen-related receptor α (ERRα), the mitochondrial transcription factor A (mTFA), and the superoxide dismutases (SOD) 1 and 2, were also significantly induced. Surprisingly, the expression of the uncoupling protein (UCP) 1, which uncouples mitochondrial electron transport from ATP synthesis to dissipate energy as heat, or that of its homolog UCP2 were not altered. In contrast, the expression of UCP3 was robustly increased by SRT1720, contributing potentially to energy dissipation by short-circuiting OxPhos. SRT1720 also potently stimulated the expression of genes controlling FAO, whereas its action on components of the mitochondrial electron transport chain was fairly limited. In SRT1720-treated mice, the excess of calories from the diet is most likely transformed to NADH and FADH2 by enhanced fat oxidation and generates a proton gradient in mitochondria, which can subsequently be eliminated through UCP3-mediated uncoupling. In line with these observations, SRT1720 stimulated oxygen consumption in primary brown adipocytes (Figure 6D). SRT1720 treatment did not change the body temperature at night. The physiological circadian temperature drop during daytime, because of inactivity and spontaneous fasting as normally observed in control mice, was, however, amplified by the treatment, which lowered body temperature by ~1°C (Figures 6E and 6F). Since this temperature phenotype could not be recapitulated in a short-term experiment where SRT1720 was administered by daily gavage, it is likely that the temperature phenotype from Figure 6F results from low fat accumulation in SRT1720-treated mice, which does not allow sufficient insulation and lipolysis to generate heat during resting periods.

SRT1720 Administration Mechanistically Mimics Low Energy Levels

SIRT1 has emerged as a pleiotropic modulator of transcription factor and coregulator activity, which it regulates through

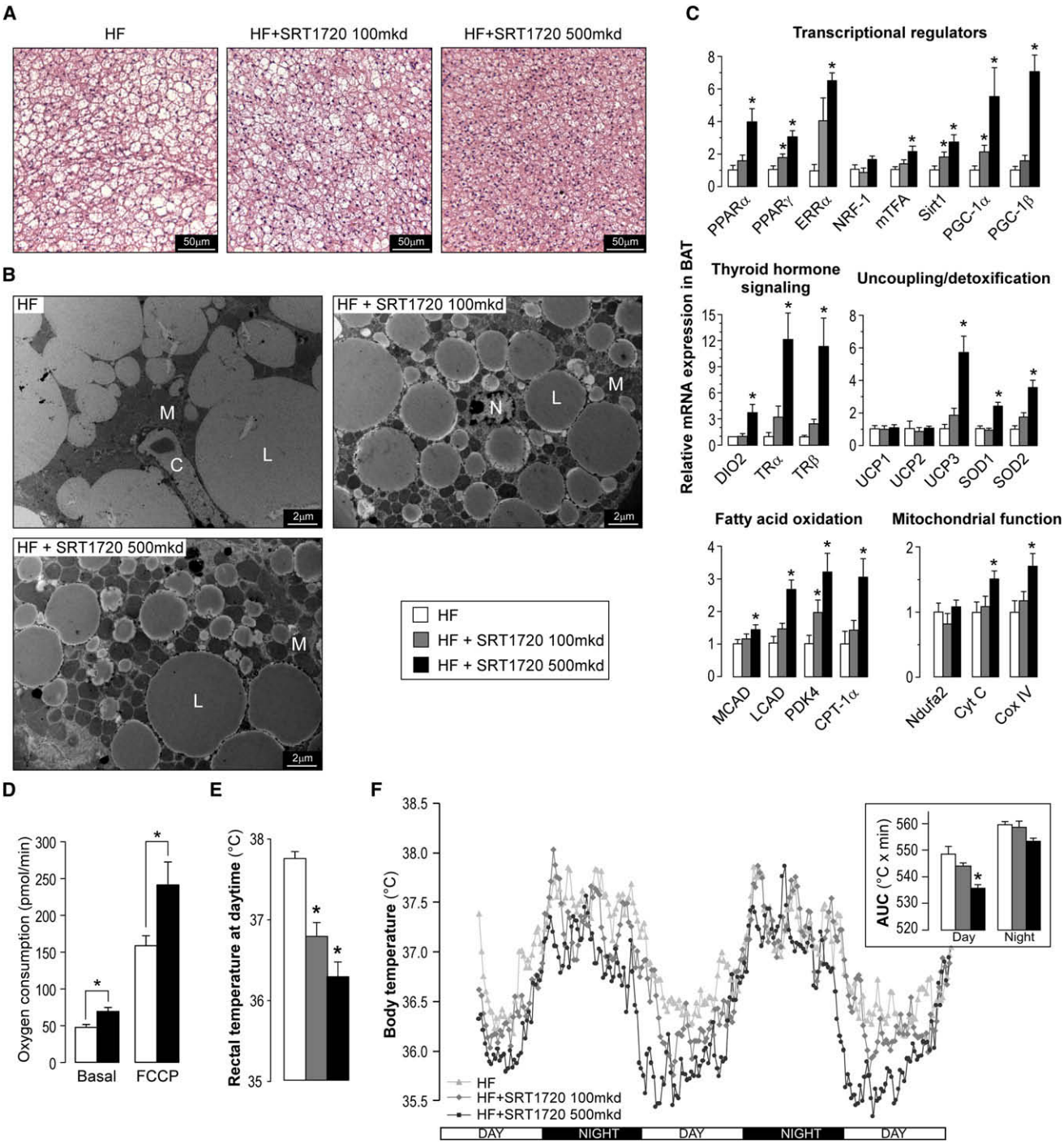

Figure 6. SRT1720 Stimulates Energy Expenditure from the Brown Adipose Tissue

(A and B) Representative hematoxylin and eosin staining (A) and electronic micrographs (B) from BAT sections of mice fed for 20 weeks with HF diet alone or supplemented with SRT1720 at 100 or 500 mkd (n = 10). Abbreviations not used previously: L, lipid droplet; M, mitochondrium; N, nucleus; C, capillary.

(C) Gene expression in BAT expressed relative β2-microglobulin (n = 10). Abbreviation not used previously: NRF1, nuclear respiratory factor 1.

(D) Oxygen consumption was measured in primary brown adipocytes treated for 24 hr with vehicle (white bars) or 10 nM SRT1720 (black bars) before or after a 1 hr treatment with the uncoupler FCCP at 500 nM.

(E) Rectal temperature measured at 11 a.m. (n = 10).

(F) Body temperature monitored by telemetry over 72 hr. The adjacent bar graph represents the average area under the curve over the diurnal and nocturnal phases, respectively (n = 5).

Error bars represent SEM, and significant differences compared to untreated controls ($p < 0.05$) are indicated by an asterisk.

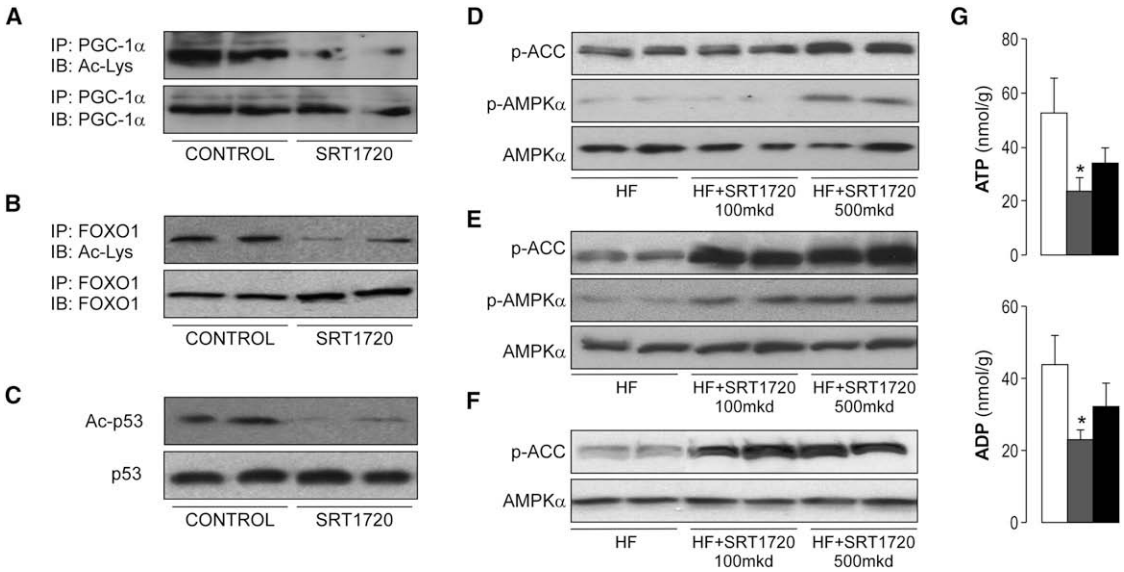

Figure 7. SRT1720 Promotes the Deacetylation of SIRT1 Targets and Indirectly Activates AMPK

(A–C) Acetylation levels of SIRT1 targets in response to chronic SRT1720 administration over 20 weeks. The acetylation of PGC-1α (A) and FOXO1 (B) was analyzed by immunoprecipitation from nuclear extracts from the gastrocnemius muscle of control mice or mice treated with SRT1720 at 500 mg per kg of body weight per day (mkd), followed by an immunoblot against acetylated lysine residues (top panel) or PGC-1α/FOXO1 (lower panel). P53 acetylation levels (C) were determined by direct immunoblotting against acetylated (top panel) or total (lower panel) p53 from hepatic nuclear extracts of the same animals as in (A) and (B).

(D–F) AMPK activation in mice treated for over 20 weeks with SRT1720 was evaluated in the gastrocnemius muscle (D), the liver (E), and BAT (F) by analyzing AMPKα and ACC phosphorylation levels by western blot.

(G) ATP and ADP concentrations were measured using a luciferase-based assay from acid-extracted BAT samples from mice described in (D)–(F) (n = 8). Error bars represent SEM, and significant differences compared to untreated controls ($p < 0.05$) are indicated by an asterisk.

deacetylation (Feige and Auwerx, 2008). Since our gene profiling experiments suggested that the metabolic effects of SRT1720 rely at least in part on transcriptional mechanisms, we analyzed the acetylation levels of metabolically relevant transcriptional targets of SIRT1 in response to SRT1720. One of the best characterized metabolic targets of SIRT1 is PGC-1α, which is activated by SIRT1-mediated deacetylation (Gerhart-Hines et al., 2007; Lagouge et al., 2006; Rodgers et al., 2005, 2008). Endogenous PGC-1α immunoprecipitated from the gastrocnemius muscle of mice treated with SRT1720 was robustly deacetylated (Figure 7A). In addition, SRT1720 also promoted the deacetylation of the Forkhead transcription factor family O1 (FOXO1) in skeletal muscle and of p53 in liver (Figures 7B and 7C), two other SIRT1 targets implicated in the coordination of metabolic homeostasis (Bensaad and Vousden, 2007; Feige and Auwerx, 2008; Frescas et al., 2005; Gross et al., 2008; Luo et al., 2001; Qiao and Shao, 2006; Vaziri et al., 2001). The ability of SRT1720 to induce the deacetylation of three established SIRT1 targets in different tissues, therefore, unequivocally demonstrates that it is a potent activator of SIRT1 in vivo.

SIRT1 is a well-recognized effector of the beneficial effects of calorie restriction on homeostasis (Guarente, 2006), and it has been speculated that treatment with SIRT1 activators might trigger metabolic pathways activated by low energetic levels (Chen and Guarente, 2007). Given that the systemic response to caloric deprivation is a metabolic switch toward the oxidation of lipids released from adipose tissue stores, SRT1720 seems to induce, at least in part, metabolic pathways activated by low energetic levels. Since AMPK is another key activator of fatty acid catabolism that senses low energetic levels, we tested whether prolonged SRT1720 administration could modulate AMPK activity. Although AMPK was not activated by SRT1720 treatment in cellular models or upon acute exposure in vivo (Figures 1F and 1G), the phosphorylation of AMPKα and its downstream target ACC was increased in the muscle, liver, and BAT of mice treated chronically with 500 mkd SRT1720 (Figures 7D, 7E, and 7F). Given that ATP and ADP levels were reduced in BAT of SRT1720-treated mice (Figure 7G), it is likely that SRT1720 targets AMPK indirectly through the metabolic status.

Altogether, these observations, therefore, support the concept that the SIRT1 activator SRT1720 acts as a calorie restriction mimetic that favors fat utilization by promoting the direct deacetylation of multiple SIRT1 targets and by inducing chronic metabolic adaptations that involve the indirect activation of AMPK.

DISCUSSION

Pharmacologically targeting transcriptional networks to regulate global gene expression programs favoring energy expenditure represents an attractive concept to combat metabolic diseases. In this context, SIRT1 has emerged as an interesting target with the demonstration that the naturally occurring SIRT1 activator resveratrol protects from diet-induced metabolic disorders (Baur et al., 2006; Lagouge et al., 2006). At present, it is, however, difficult to distinguish how many of the effects of resveratrol are specifically mediated by SIRT1 versus other resveratrol targets such as AMPK (Baur et al., 2006; Dasgupta and Milbrandt,

2007). A new generation of selective synthetic SIRT1 activators structurally unrelated to resveratrol and with improved potency, efficacy, and specificity have recently been developed (Milne et al., 2007). In the present study, we have analyzed the global metabolic actions of SRT1720, a member of this new generation of agonists that is more potent and efficacious than resveratrol and exhibits higher specificity with respect to off-target activation of other sirtuin homologs or AMPK (Milne et al., 2007; Figure 1). When incorporated in the diet at a dose of 100 mkd, SRT1720 improves glucose homeostasis by enhancing insulin sensitivity in mice where insulin resistance was induced by HF feeding, as also reported in other rodent models of type 2 diabetes using daily oral administration (Milne et al., 2007). These antidiabetic actions are even more pronounced at a dose of 500 mkd, which also protects from the weight gain induced by HF feeding. Importantly, the antidiabetic actions of SRT1720 are not the indirect consequences of reduced fat accretion, as they can be recapitulated after short-term treatment or under chow diet, where weight differences between groups are minimal. By selectively activating SIRT1, SRT1720 treatment, therefore, mimics the phenotype that is induced by resveratrol administration, as oxidative metabolism and energy expenditure are enhanced in mice treated with both compounds, leading to protection from diet-induced obesity and to improved muscle performance. Although it can be surprising to observe that high doses of SRT1720 are required to induce physiological effects in vivo despite a high affinity for SIRT1, this apparent discrepancy relates to the pharmacokinetics of the compound. The plasma concentrations of SRT1720 only reach nanomolar concentrations at both doses administered, presumably because of rapid metabolism and/or distribution, and further chemical refinement of the structure of SRT1720 will be required to enhance bioavailability. In addition, the observation that SRT1720 transiently affects core body temperature and spontaneous locomotor activity could constitute potential side effects for the treatment of metabolic disorders but could also be explored for other therapeutic interventions.

Despite a high correlation of the phenotypic outputs induced by resveratrol and SRT1720, which involve in both cases enhanced energy expenditure in muscle, liver, and BAT, the physiological mechanisms through which these compounds exert their actions only partially overlap. The most prominent difference relates to the way through which both compounds induce oxidative metabolism. Resveratrol acts primarily on mitochondrial biogenesis and function by activating PGC-1α and, subsequently, inducing the expression of regulators of mitochondrial metabolism such as NRF1, ERRα, and mTFA (Lagouge et al., 2006). In contrast, SRT1720 seems to have a more limited activity on mitochondrial density. It is, therefore, plausible that off-target effects, such as the direct activation of AMPK, are required for the actions of resveratrol on mitochondrial biogenesis. It seems that the activation of SIRT1 by SRT1720 acts upstream on oxidative metabolism by activating the pathways controlling the oxidation of fatty acids. Consistently, SRT1720 promotes palmitate oxidation in cellular models, and the nuclear receptors PPARα and PPARβ/δ, two major regulators of FAO (Evans et al., 2004; Feige et al., 2006), are upregulated by SRT1720 in several tissues with high rates of fatty acid utilization. Moreover, many oxidative PPAR target genes are also induced, suggesting that SIRT1 activation by SRT1720 drives a global oxidative program by stimulating fatty acid utilization. In this context, the strong enhancement of endurance and running capacities observed in SRT1720-treated mice most likely results both from a switch in the contractile phenotype of muscle fibers and from fast-twitch glycolytic fibers acquiring more resistance to fatigue by switching their substrate preference toward fatty acids. At the molecular level, the deacetylation of PGC-1α and FOXO1 by SRT1720 most probably plays a prominent role in this oxidative switch of skeletal muscle fibers, as the metabolic actions of these transcriptional regulators are stimulated by SIRT1-mediated deacetylation, leading to the promotion of oxidative muscle function (Frescas et al., 2005; Gross et al., 2008; Handschin et al., 2007; Lin et al., 2002; Rodgers et al., 2008). The SRT1720-mediated induction of PPARα expression could be linked to the coactivation of the PPARα promoter by PGC-1α (Huss et al., 2004). In addition, activation of PGC-1α signaling most likely synergizes with increased PPAR expression, as PGC-1α is a coactivator of PPARα- and PPARβ/δ-mediated FAO (Vega et al., 2000; Wang et al., 2003). Despite the induction of PGC-1α expression in various metabolic tissues of SRT1720-treated mice and the ability of SRT1720 to promote PGC-1α deacetylation, it is interesting to observe that the activation of PGC-1α signaling by SRT1720 seems limited to its action on FAO but, unlike resveratrol, does not extend to other PGC-1α-dependent actions. Several hypotheses could explain this more restricted activity. The bioavailability of SRT1720 in metabolic tissues could be distinct from that of resveratrol and dictate tissue-specific regulations. It is also possible that the interplay with other metabolically relevant SIRT1 targets such as FOXO1 or p53 (Bensaad and Vousden, 2007; Feige and Auwerx, 2008; Frescas et al., 2005; Gross et al., 2008; Luo et al., 2001; Qiao and Shao, 2006; Vaziri et al., 2001), which are deacetylated by SRT1720, also modulates the systemic metabolic response. Alternately, the observation that specific SIRT1 agonists modulate only a subset of PGC-1α target promoters in cellular models (Figure S6) suggests that SRT1720 can drive a selective activation of PGC-1α signaling.

It is well established that SIRT1 is activated by low energetic levels such as those occurring during fasting or calorie restriction (Michan and Sinclair, 2007; Rodgers et al., 2005), and it has been suggested that SIRT1 activators act, at least in part, by stimulating physiological pathways activated by low energetic levels (Barger et al., 2008; Pearson et al., 2008). Consistently, SRT1720 activates a global network enhancing fat oxidation, a process also stimulated upon fasting in mammals when fatty acids coming from adipose tissue lipolysis become the prominent energetic substrate. In this context, it is highly interesting to observe that prolonged SIRT1 activation by SRT1720 can activate AMPK, a sensor of low energetic levels, through indirect mechanisms. It is possible that the recently described SIRT1-dependent regulation of LKB1 plays a role in this regulation (Hou et al., 2008). However, since SRT1720 does not directly activate AMPK in cellular models or in vivo and energetic levels are reduced in certain tissues of SRT1720-treated mice, we believe that SRT1720 could act as a calorie restriction mimetic, which would induce a global metabolic adaptation similar to what occurs under low energetic levels. Given the important role of AMPK in inducing oxidative metabolism in response to low energetic levels, the indirect activation of this kinase and of its downstream effectors by SRT1720

most likely allows the amplification of increased fat oxidation. Since it has been recently suggested that the response to calorie restriction varies greatly between tissues (Chen et al., 2008), it will, therefore, be of importance to precisely characterize how the tissue-specific response to SIRT1 activators relates to energetic levels of individual organs.

Altogether, our results further validate SIRT1 as a bona fide target to combat metabolic disorders and establish SRT1720 as a prime candidate to explore the potential of SIRT1 as a therapeutic target.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

5-aminoimidazole-4-carboxamide riboside (AICAR; Toronto Research Chemicals) was dissolved in 0.9% NaCl, and metformin (Sigma), resveratrol (Orchid Chemicals), SRT1720, and SRT2183 (Sirtris) were dissolved in DMSO. Viral constructs for control and SIRT1 shRNAs, as well as PGC-1α WT and R13, were previously described (Rodgers et al., 2005). CytC and PDK4 reporter constructs were kind gifts of P. Puigserver and D. Kelly, respectively.

Animal Experiments

Male 7-week-old C57BL/6J mice (Charles River) were housed with a 12 hr light-dark cycle and fed a standard or HF diet containing 60% energy as fat (D12492; Research diet), supplemented or not with SRT1720, as described in Feige et al. (2008). Phenotyping tests and histology were performed according to EMPRESS standardized protocols as described in Argmann et al. (2006), Heikkinen et al. (2007), and Lagouge et al. (2006). For acute experiments, SRT1720 and AICAR were injected intraperitoneally at 500 mg/kg 4 hr before the sacrifice. Radiolabeled hyperinsulinemic-euglycemic clamps were performed as described in the Supplemental Experimental Procedures, using ^{3}H-glucose and ^{14}C-2-deoxy-glucose to evaluate glucose turnover and organ-specific glucose uptake, respectively.

Biochemistry and Immunoblotting

Plasmatic parameters and hepatic and fecal lipid content were measured as previously described (Mataki et al., 2007). Cytochrome C oxidase activity was evaluated by following the oxidation of fully reduced cytochrome C (Sigma) at 550 nm, and citrate synthase activity was measured as previously described (Lagouge et al., 2006). ATP and ADP concentrations were measured using a luciferase-based assay (Biovision) after acid extraction in 5% perchloric acid followed by neutralization with potassium carbonate. Western blots were performed on 0.5–2 mg of total proteins using antibodies directed against the total or phosphorylated subunit α of AMPK, phosphorylated ACC, p53, and acetylated p53 and diluted at 1/1000. PGC-1α and FOXO1 acetylation was evaluated by immunoprecipitating 1000 μg of total proteins from skeletal muscle with PGC-1α or FOXO1 antibodies, followed by immunoblotting against acetylated lysine or against PGC-1α or FOXO1. All antibodies were from Cell Signaling, and detection was performed using ultrasensitive horseradish peroxidase chemiluminescence (Pierce).

Gene Expression Profiling

SYBR-green qPCR and Affymetrix expression arrays were performed as described in the Supplemental Experimental Procedures on cDNAs from trizol-extracted (Invitrogen) RNA samples. Microarrays were analyzed using gene set enrichment analysis as previously described (Lagouge et al., 2006).

Oxygen Consumption Measurements

Cellular oxygen consumption and palmitate oxidation was measured using a Seahorse bioscience XF24 analyzer with ten biological replicates per condition, as described in the Supplemental Experimental Procedures.

Statistics

Statistical analyses were performed with a Student's t test for independent samples. Data are expressed as mean ± SEM, and p values smaller than 0.05 were considered as statistically significant.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cellmetabolism.org/supplemental/S1550-4131(08)00284-2.

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REFERENCES

Argmann, C.A., Champy, M.F., and Auwerx, J. (2006). Evaluation of energy homeostasis. In Current Protocols in Molecular Biology, F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds. (New York: John Wiley & Sons).

Barger, J.L., Kayo, T., Vann, J.M., Arias, E.B., Wang, J., Hacker, T.A., Wang, Y., Raederstorff, D., Morrow, J.D., Leeuwenburgh, C., et al. (2008). A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. PLoS ONE *3*, e2264.

Baur, J.A., Pearson, K.J., Price, N.L., Jamieson, H.A., Lerin, C., Kalra, A., Prabhu, V.V., Allard, J.S., Lopez-Lluch, G., Lewis, K., et al. (2006). Resveratrol improves health and survival of mice on a high-calorie diet. Nature *444*, 337–342.

Bensaad, K., and Vousden, K.H. (2007). p53: new roles in metabolism. Trends Cell Biol. *17*, 286–291.

Chen, D., and Guarente, L. (2007). SIR2: a potential target for calorie restriction mimetics. Trends Mol. Med. *13*, 64–71.

Chen, D., Bruno, J., Easlon, E., Lin, S.J., Cheng, H.L., Alt, F.W., and Guarente, L. (2008). Tissue-specific regulation of SIRT1 by calorie restriction. Genes Dev. *22*, 1753–1757.

Dasgupta, B., and Milbrandt, J. (2007). Resveratrol stimulates AMP kinase activity in neurons. Proc. Natl. Acad. Sci. USA *104*, 7217–7222.

Desvergne, B., Michalik, L., and Wahli, W. (2006). Transcriptional regulation of metabolism. Physiol. Rev. *86*, 465–514.

Evans, R.M., Barish, G.D., and Wang, Y.X. (2004). PPARs and the complex journey to obesity. Nat. Med. *10*, 355–361.

Feige, J.N., and Auwerx, J. (2007). Transcriptional coregulators in the control of energy homeostasis. Trends Cell Biol. *17*, 292–301.

Feige, J.N., and Auwerx, J. (2008). Transcriptional targets of sirtuins in the coordination of mammalian physiology. Curr. Opin. Cell Biol. *20*, 303–309.

Feige, J.N., Gelman, L., Michalik, L., Desvergne, B., and Wahli, W. (2006). From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. Prog. Lipid Res. *45*, 120–159.

Feige, J.N., Lagouge, M., and Auwerx, J. (2008). Dietary manipulations. In Current Protocols in Molecular Biology, F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds. (New York: John Wiley & Sons).

Frescas, D., Valenti, L., and Accili, D. (2005). Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. J. Biol. Chem. *280*, 20589–20595.

Gerhart-Hines, Z., Rodgers, J.T., Bare, O., Lerin, C., Kim, S.H., Mostoslavsky, R., Alt, F.W., Wu, Z., and Puigserver, P. (2007). Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. EMBO J. *26*, 1913–1923.

Gross, D.N., van den Heuvel, A.P., and Birnbaum, M.J. (2008). The role of FoxO in the regulation of metabolism. Oncogene *27*, 2320–2336.

Guarente, L. (2006). Sirtuins as potential targets for metabolic syndrome. Nature *444*, 868–874.

Handschin, C., Chin, S., Li, P., Liu, F., Maratos-Flier, E., Lebrasseur, N.K., Yan, Z., and Spiegelman, B.M. (2007). Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1alpha muscle-specific knock-out animals. J. Biol. Chem. *282*, 30014–30021.

Heikkinen, S., Argmann, C.A., Champy, M.F., and Auwerx, J. (2007). Evaluation of glucose homeostasis. In Current Protocols in Molecular Biology, F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds. (New York: John Wiley & Sons).

Hou, X., Xu, S., Maitland-Toolan, K.A., Sato, K., Jiang, B., Ido, Y., Lan, F., Walsh, K., Wierzbicki, M., Verbeuren, T.J., et al. (2008). SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. J. Biol. Chem. *283*, 20015–20026.

Huss, J.M., Torra, I.P., Staels, B., Giguere, V., and Kelly, D.P. (2004). Estrogen-related receptor alpha directs peroxisome proliferator-activated receptor alpha signaling in the transcriptional control of energy metabolism in cardiac and skeletal muscle. Mol. Cell. Biol. *24*, 9079–9091.

Kharitonenkov, A., and Shanafelt, A.B. (2008). Fibroblast growth factor-21 as a therapeutic agent for metabolic diseases. BioDrugs *22*, 37–44.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., et al. (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell *127*, 1109–1122.

Lin, J., Wu, H., Tarr, P.T., Zhang, C.Y., Wu, Z., Boss, O., Michael, L.F., Puigserver, P., Isotani, E., Olson, E.N., et al. (2002). Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature *418*, 797–801.

Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell *107*, 137–148.

Mataki, C., Magnier, B.C., Houten, S.M., Annicotte, J.S., Argmann, C., Thomas, C., Overmars, H., Kulik, W., Metzger, D., Auwerx, J., and Schoonjans, K. (2007). Compromised intestinal lipid absorption in mice with a liver-specific deficiency of liver receptor homolog 1. Mol. Cell. Biol. *27*, 8330–8339.

Michan, S., and Sinclair, D. (2007). Sirtuins in mammals: insights into their biological function. Biochem. J. *404*, 1–13.

Milne, J.C., Lambert, P.D., Schenk, S., Carney, D.P., Smith, J.J., Gagne, D.J., Jin, L., Boss, O., Perni, R.B., Vu, C.B., et al. (2007). Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. Nature *450*, 712–716.

Pearson, K.J., Baur, J.A., Lewis, K.N., Peshkin, L., Price, N.L., Labinskyy, N., Swindell, W.R., Kamara, D., Minor, R.K., Perez, E., et al. (2008). Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span. Cell Metab. *8*, 157–168.

Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., Leid, M., McBurney, M.W., and Guarente, L. (2004). Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature *429*, 771–776.

Qiao, L., and Shao, J. (2006). SIRT1 regulates adiponectin gene expression through Foxo1-C/enhancer-binding protein alpha transcriptional complex. J. Biol. Chem. *281*, 39915–39924.

Rodgers, J.T., and Puigserver, P. (2007). Fasting-dependent glucose and lipid metabolic response through hepatic sirtuin 1. Proc. Natl. Acad. Sci. USA *104*, 12861–12866.

Rodgers, J.T., Lerin, C., Haas, W., Gygi, S.P., Spiegelman, B.M., and Puigserver, P. (2005). Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature *434*, 113–118.

Rodgers, J.T., Lerin, C., Gerhart-Hines, Z., and Puigserver, P. (2008). Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. FEBS Lett. *582*, 46–53.

Spiegelman, B.M., and Heinrich, R. (2004). Biological control through regulated transcriptional coactivators. Cell *119*, 157–167.

Vaziri, H., Dessain, S.K., Ng Eaton, E., Imai, S.I., Frye, R.A., Pandita, T.K., Guarente, L., and Weinberg, R.A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell *107*, 149–159.

Vega, R.B., Huss, J.M., and Kelly, D.P. (2000). The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. Mol. Cell. Biol. *20*, 1868–1876.

Wang, Y.X., Lee, C.H., Tiep, S., Yu, R.T., Ham, J., Kang, H., and Evans, R.M. (2003). Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. Cell *113*, 159–170.

Yamamoto, H., Schoonjans, K., and Auwerx, J. (2007). Sirtuin functions in health and disease. Mol. Endocrinol. *21*, 1745–1755.