UNIVERSITE DE STRASBOURG

ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

Mitochondries, Stress Oxydant et Protection Musculaire – EA 3072

Année universitaire 2018/2019

HABILITATION A DIRIGER LES RECHERCHES

Discipline : Sciences du Vivant
Domaine : Physiologie et Biologie des Organismes

ISCHEMIE-REPERFUSION DU MUSCLE SQUELETTIQUE : IMPLICATION MITOCHONDRIALE ET ETUDE DE L'EFFET DU CONDITIONNEMENT DANS LA MODULATION DU STRESS OXYDANT

Conseil national des Universités : Section 51-04, Chirurgie Vasculaire

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Le 21 Novembre 2018

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Docteur Christian BOISSIER	Saint-Etienne, France	Rapporteur externe
Professeur Bernard GENY	Strasbourg, France	Examinateur interne
Professeur Fabien THAVEAU	Strasbourg, France	Examinateur interne
Professeur Nabil CHAKFE	Strasbourg, France	Garant de recherche

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I. ETAT CIVIL

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II. DIPLOMES

1. Formation médicale

- Doctorat en Médecine, Strasbourg, 2009
- Diplôme d'Etude Spécialisée, Chirurgie générale, Strasbourg, 2010
- Diplôme d'Etude Spécialisée Complémentaire, Chirurgie vasculaire, Strasbourg, 2012
- Collège Français de Chirurgie Vasculaire, Lyon, 2016
- European Board of Vascular Surgery, Maastricht, 2017

2. Diplômes universitaires et interuniversitaires

- Diplôme interuniversitaire – Leçons de pratiques chirurgicales, Nancy, 2007
- Diplôme universitaire – Chirurgie laparoscopique, Strasbourg, 2007
- Diplôme interuniversitaire – Chirurgie des abords vasculaires pour hémodialyse, Reims, 2008
- Diplôme interuniversitaire – Chirurgie endovasculaire, Reims, 2010
- Diplôme interuniversitaire – Pédagogie médicale, Strasbourg, 2014

3. Parcours scientifique

- Master 1 – Physiopathologie cellulaire et moléculaire, Strasbourg, 2010
- Master 2 – Sciences chirurgicales, Paris 2011
- Doctorat d'Université, EA3072, Strasbourg, 2014
- Post-doctorat, Institute of Medical Sciences, Toronto, 2014-2015
- Membre de l'EA 3072 Mitochondries, stress oxydant et protection musculaire

4. Bourses et distinctions

- 1er prix du concours de communications, Collège de Chirurgie Vasculaire, 2005
- 1er prix du concours de communications, Congrès de Chirurgie Thoracique, 2009
- Bourse de 1 000 euros allouée par la Société de Chirurgie Thoracique, 2010
- Bourse de 10 000 euros allouée par la Société française de Chirurgie Vasculaire, 2010
- Bourse de 15 000 euros allouée par la Société française de Chirurgie Vasculaire, 2014
- Protocole Hospitalier de Recherche Clinique interrégional, PHRC HUS 5831, subventionné à hauteur de 204 228 euros, 2015

5. Sociétés savantes

- Membre de la Société française de Chirurgie Vasculaire et Endovasculaire
- Membre de l'European Society of Vascular and Endovascular Surgery

III. FONCTIONS HOSPITALO-UNIVERSITAIRES

1. Parcours hospitalo-universitaire

- 2005 à 2010 : Interne de chirurgie des Hôpitaux Universitaires de Strasbourg
- 2011 à 2014 : Chef de Clinique Universitaire – Assistant des Hôpitaux, Service de Chirurgie Vasculaire et Transplantation Rénale, Hôpitaux Universitaires de Strasbourg
- Depuis 2015 : Maître de Conférences – Praticien Hospitalier, Service de Chirurgie Vasculaire et Transplantation Rénale, Service de Physiologie et Explorations Fonctionnelles, Hôpitaux Universitaires de Strasbourg
- Participation à la permanence des soins à hauteur de 8 à 10 astreintes par mois

2. Responsabilités hospitalières

- Responsable du versant chirurgical de l'activité de transplantation rénale
- Membre permanent du COPIL (Comité Pilote pour le prélèvement d'organes Maastricht 3 aux Hôpitaux Universitaires de Strasbourg)
- Membre du Conseil d'Administration de la Société de Physiologie

3. Responsabilités universitaires

- Reviewer pour l'European Journal of Vascular and Endovascular Surgery depuis 2017
- Reviewer pour Annals of Vascular Surgery depuis 2014
- Expert désigné par l'Agence de Biomédecine pour le reviewing des appels à projets 2018
- Participation au Writing Committee des Guidelines de l'European Society of Vascular and Endovascular Surgery sur le traitement des infections de prothèses

4. Perspectives

Depuis Novembre 2011, l'activité de greffe rénale est assurée par la collaboration entre l'équipe de Chirurgie Vasculaire et l'équipe de Néphrologie des Hôpitaux Universitaires de Strasbourg. Cette collaboration a permis la réalisation d'un nombre croissant de greffes rénales : 90 greffes rénales en 2012 versus 113 greffes rénales en 2017, pour un nombre moyen de 104 greffes rénales par an.

Etant responsable du versant chirurgical de cette activité, j'ai collaboré à la mise en place de plusieurs axes de développement, dans le but de répondre au plan greffe 2017-2021, nous demandant un accroissement important du nombre de greffes rénales.

a. Greffe à donneur vivant

Le nombre de greffes à donneur vivant est en permanente augmentation (11 greffes en 2012, 25 greffes en 2017). Il est ainsi estimé que 50 greffes à donneur vivant par an seront réalisées dans les prochaines années.

De plus, suite à l'obtention du nouveau robot Da Vinci Xi par le CHU de Strasbourg, notre objectif est de réaliser les néphrectomies pour greffe à donneur vivant en bénéficiant de

l'assistance robotique. L'utilisation de cette nouvelle technologie permet d'offrir une récupération plus rapide au patient mais surtout plus grande précision du geste opératoire pour l'équipe chirurgicale, afin de réaliser une intervention qui se doit d'être sans complication aux vues du contexte. Nous avons ainsi réalisé avec le Professeur Fabien Thaveau la première néphrectomie robotisée dans le cadre d'une greffe à donneur vivant le 15 Janvier 2018, et souhaitons pérenniser l'utilisation du robot Da Vinci Xi de manière systématique à l'ensemble des néphrectomies pour greffe à donneur vivant.

Dans le cadre de la greffe à donneur vivant, la greffe ABO incompatible est également développée au CHU de Strasbourg par l'équipe de Néphrologie et Transplantation Rénale (Professeur Bruno Moulin, Professeur Sophie Caillard). Le receveur est préparé à la greffe de manière à obtenir une diminution prolongée et significative du taux d'anticorps circulants par des techniques d'aphérèse spécifiques, et par la perfusion d'anticorps monoclonaux. Le Centre Hospitalier Universitaire de Strasbourg est le seul centre de la région Grand Est à proposer ces techniques.

b. Bigreffe

Le programme de bigreffe est proposé aux Hôpitaux Universitaires de Strasbourg depuis 2016, dans le but d'augmenter le taux d'accès à la greffe des patients âgés, via l'implantation de deux greffons dans le même temps, qui n'auraient pas été utilisés en monogreffe. Les résultats de cette technique sont encourageants à l'échelon national. Les patients de plus de 70 ans, après avoir été informés de cette possibilité, sont inscrits à la fois sur liste conventionnelle de greffe rénale, ainsi que sur liste de bigreffe. Depuis 2016, 5 bigreffes ont été réalisées. Il est estimé qu'environ 10 bigreffes par an pourront être réalisées dans les prochaines années.

c. Greffe à donneur à cœur arrêté (Maastricht 3)

Un Comité Pilote dirigé par le Professeur Philippe Wolf, concernant le prélèvement d'organes sur donneur à cœur arrêté (Maastricht 3) se réunit depuis Juin 2017. Le prélèvement d'organes M3 se fait sur des patients à cœur arrêté, après limitation des thérapeutiques et mise en place d'une circulation régionale normothermique.

Après dépôt du dossier de candidature, le Centre Hospitalier Universitaire a été accrédité depuis Juin 2018 pour le prélèvement Maastricht 3. Depuis, deux prélèvements Maastricht 3 ont été réalisés. Le nombre de prélèvements Maastricht 3 est estimé à 10 à 20 prélèvements par an dans les prochaines années. Ceci permettra d'augmenter le pool de greffons, puisqu'il sera possible selon les règles de répartition de greffer un greffon M3 prélevé par notre centre, mais également de recevoir un greffon M3 prélevé dans d'autres centres, selon les règles de répartition nationale.

d. Greffe des sujets obèses

Un BMI supérieur à 35 kg/m2 est considéré comme une contre-indication à l'inscription sur liste d'attente de greffe rénale dans de nombreux centres, du fait de la complexité opératoire et du risque de retard de cicatrisation post-opératoire inhérents au tablier abdominal.

Dans notre centre, nous ne donnons pas de limite de BMI pour l'inscription sur liste. Ainsi, au cours de la dernière année, 15 patients dont le BMI était supérieur à 35 ont été greffés et 12 patients dont le BMI est supérieur à 40 kg/m2 ont été inscrits sur liste.

e. Greffe des patients porteurs de lésions aorto-iliaques sévères

Nous mettons à profit notre expertise vasculaire pour proposer la greffe rénale à des patient porteurs de lésions aorto-iliaques sévères, en proposant une revascularisation préalable ou concomittante à la greffe.

IV. ACTIVITE D'ENSEIGNEMENT

1. Enseignements en 1er cycle des études médicales

a. PACES

- Séméiologie vasculaire (cours magistral, 1 heure)
- Equilibre acido-basique (enseignement dirigé, 6 heures)
- Bioénergétique (enseignement dirigé, 6 heures)
- Transferts membranaires (enseignement dirigé, 6 heures)
- Physiologie de l'exercice (enseignement pratique, 8 heures)
- Physiologie respiratoire (enseignement pratique, 8 heures)

b. DFGSM2

- Physiologie rénale (cours magistral, 8 heures)

c. DFGSM3

- Physiologie cardio-vasculaire (cours magistral, 6 heures)
- Pathologie anévrismale et artériopathie (enseignement dirigé, 4 heures)

2. Enseignements en 2ème cycle et 3ème cycle des études médicales

a. Centre de simulation

- Simulation endovasculaire et sutures (enseignement pratique, 30 heures)

b. Diplômes interuniversitaires

- DIU Pied diabétique (cours magistral, 2 heures)
- DIU Podologie (cours magistral, 2 heures)

c. Accueil des internes de chirurgie

- Voies d'abord chirurgicales (cours magistral, 1 heure)
- Bases chirurgicales (enseignement pratique, 8 heures)

d. Vascular summer schools

- Mitochondrial disease in peripheral arterial disease (cours magistral, 2 heures)
- Mitochondriopathy in peripheral arterial disease (enseignement dirigé, 2 heures)

e. eVASC

- Principes de la greffe rénale (powerpoint sonorisé)
- Technique chirurgicale de la greffe rénale (powerpoint sonorisé)
- Conduite à tenir devant une ischémie aigue de membre (powerpoint sonorisé)
- Les prothèses vasculaires (powerpoint sonorisé)
- Recherche sur pubmed (powerpoint sonorisé)

3. Enseignements en écoles paramédicales

a. Faculté de chirurgie dentaire

- Physiologie rénale (cours magistral, 6 heures)

b. Institut de formation en masso-kinésithérapie

- Physiologie cardiaque (cours magistral, 2 heures)
- Physiologie rénale (cours magistral, 2 heures)
- Physiologie vasculaire (cours magistral, 2 heures)

c. Ecole de sages-femmes

- Physiologie rénale (cours magistral, 6 heures)

d. AURAL

- Chirurgie des accès vasculaires pour hémodialyse (cours magistral, 8 heures)
- Principes de revascularisation chez le patient diabétique (cours magistral, 8 heures)

5. Encadrement des étudiants

a. Direction de thèse de médecine

Charline Delay : Traitement des anévrismes de l'aorte abdominale : évolution en 10 ans des stratégies thérapeutiques. 2014.
Thèse ayant permis la publication de trois manuscrits :
- Delay C, Deglise S, **Lejay A**, Georg Y, Roussin M, Schaeffer M, et al. Zenith bifurcated iliac side branch device: mid-term results and assessment of risk factors for intraoperative thrombosis. *Ann Vasc Surg 2017;41:141-50.*
- Deglise S, Delay C, Saucy F, **Lejay A**, Dubuis C, Briner L, et al. Endovascular versus open abdominal aortic aneurysm: best decision. *Curr Pharm Des 2015;21:4076-4083.*
- Delay C, **Lejay A**, Deglise S, Mantz F, Schwein A, Gaertner S, et al. Should we treat abdominal aortic aneurysms from 5.0 cm in France while the cut-off is 5.5 cm in English-speaking countries? *J Mal Vasc 2016;41:1-3.*

Benjamin Del Tatto : Maladie anévrismale de l'artère poplitée : étude comparative entre la chirurgie conventionnelle et la chirurgie endovasculaire sur 153 cas. 2017.
Manuscrit publié dans Annals of Vascular Surgery :
- Del Tatto B, **Lejay A**, Meteyer V, Roussin M, Georg Y, Thaveau F, et al. Open and endovascular repair of popliteal artery aneurysms. *Ann Vasc Surg 2018;50:119-27.*

Anne-Florence Rouby : Evolution volumétrique au long cours des artères iliaques de plus de 20 mm de diamètre traitées par chirurgie endovasculaire. Soutenance prévue en Avril 2019. Manuscrit en cours de préparation pour l'European Journal of Vascular and Endovascular Surgery.

b. Direction de mémoire de diplômes d'études spécialisées

Charline Delay : Evolution iliaque après traitement endovasculaire pour pathologie anévrismale iliaque ou aorto-iliaque. 2017.

Bettina Chenesseau : Comparaison de la chirurgie robotique et de la chirurgie ouverte dans la prise en charge des anévrismes de l'aorte abdominale sous-rénale. 2018

c. Direction de master 2

Charline Delay : Ischémie-reperfusion du muscle squelettique et préconditionnement ischémique, implication des voies RISK et SAFE. 2016.
Manuscrit publié dans le Journal des Maladies Vasculaires :
- Delay C, Paradis S, Charles AL, Chakfe N, Geny B, **Lejay A**. Skeletal muscle ischemia-reperfusion and ischemic conditioning pathophysiology – clinical applications for the vascular surgeon. *J Mal Vasc 2017;42:29-38.*

d. Encadrement de thèse d'université

Adeline Schwein : Développement d'un modèle porcin de thrombose veineuse profonde et développement d'un stent veineux dédié. Soutenance prévue en 2019.

6. Synopsis et score SIAPS

<table>
<tr><th>Charge annuelle d'enseignement
134 heures</th><th>Formation pédagogique</th><th>Activités pédagogiques</th><th>Score SIAPS</th></tr>
<tr><td>Cours magistraux :
56 heures</td><td rowspan="2">DIU de pédagogie médicale
(2014)</td><td>Productions pédagogiques numérisées :
5 chapitres eVASC</td><td rowspan="3">554</td></tr>
<tr><td>Enseignements dirigés :
24 heures</td><td>Participation à la rédaction d'un polycopié national</td></tr>
<tr><td>Enseignements pratiques : 54 heures</td><td>Certification SIDES
(2011)</td><td>Rédaction de 6 dossiers progressifs et 15 QCM de fin d'année</td></tr>
</table>

V. ACTIVITE DE RECHERCHE

1. Présentation générale

Mon activité de recherche prend place dans le cadre de l'Equipe d'Accueil 3072 Mitochondries, stress oxydant et protection musculaire (Professeur Bernard Geny) au sein de la Fédération de Médecine Translationnelle de Strasbourg, et dans le cadre du Service de Chirurgie Vasculaire et Transplantation Rénale (Professeur Nabil Chakfé).

Cette activité de recherche, qu'elle soit fondamentale ou clinique, a été façonnée, au cours des dix années passées, par les Maîtres qui m'ont encadrée, les équipes de recherche qui m'ont accueillie, et par l'orientation clinique et scientifique du service dans lequel j'ai la chance de travailler. Ainsi, à mi-parcours de mon internat de chirurgie vasculaire, le Professeur Fabien Thaveau m'a fait part de ses travaux sur l'ischémie-reperfusion (*Thaveau F, et al. J Vasc Surg 2007 ; Thaveau F, et al. J Surg Res 2009 ; Thaveau F, et al. Fundam Clin Pharmacol 2010*). Mon premier contact avec la démarche expérimentale s'est donc fait en l'accompagnant au sein de l'EA 3072. J'ai ainsi découvert que l'ischémie-reperfusion était un sujet d'importance majeure en chirurgie vasculaire.

Néanmoins, il m'est clairement apparu que les phénomènes d'ischémie-reperfusion ont été largement étudiés dans le cadre de l'ischémie aiguë, mais qu'il existait peu de données étudiant les lésions liées à une ischémie chronique. Il m'a ainsi semblé indispensable de développer la compréhension des phénomènes d'ischémie-reperfusion dans le cadre de l'ischémie critique chronique, dont la prévalence allait augmenter dans les années à venir du fait de ses facteurs de risque majeurs (diabète, tabagisme, vieillissement de la population). Ma préoccupation principale était cependant que cette activité de recherche soit translationnelle, basée sur la compréhension de la physiologie cellulaire et notamment de la fonction mitochondriale, pour pouvoir l'appliquer ensuite à un modèle animal et ensuite au patient. Il était donc important de l'intégrer dans un schéma directeur qui fasse sens, pour que mon cheminement soit source d'ouverture et d'enrichissement.

2. Rationnel du projet de recherche

L'ischémie critique chronique définit un degré d'insuffisance artérielle chronique tel que la pression distale résiduelle compromet sévèrement la fonction microcirculatoire et le débit nutritif du membre inférieur, mettant en jeu le pronostic fonctionnel et le pronostic vital du patient. En effet, l'ischémie critique chronique des membres inférieurs expose le muscle squelettique à des cycles répétés d'ischémie-reperfusion : la marche ou le décubitus correspondant à une situation d'ischémie, et le repos ou la station debout à une situation de reperfusion. L'augmentation de la longévité et la prévalence du diabète conduisent à une augmentation de l'incidence et de la prévalence de l'ischémie critique chronique, faisant de cette pathologie un véritable problème de santé publique.

Le traitement de l'ischémie critique chronique repose sur la revascularisation chirurgicale, lorsque celle-ci est possible. En effet, un geste de revascularisation n'est possible que pour 50% des patients, car 25% des patients nécessiteront une amputation majeure d'emblée, et 25% des patients ne pourront bénéficier que d'un traitement médical du fait d'une atteinte

trop importante contre-indiquant tout geste de revascularisation (*Norgren L et al. Eur J Vasc Endovasc Surg 2018*). Ce constat explique l'intérêt de développer des alternatives médicamenteuses à la chirurgie, ayant pour but de limiter les lésions d'ischémie-reperfusion chroniques.

3. Objectifs

Dans ce contexte, nos objectifs étaient les suivants :

- Réaliser une mise au point des mécanismes mitochondriaux impliqués dans l'ischémie-reperfusion
- Mettre au point un modèle murin d'ischémie critique chronique
- Déterminer sur ce modèle les effets de différents protocoles susceptibles de moduler le stress oxydant
- Envisager *in fine* l'application d'un protocole de modulation du stress oxydant à l'Homme

4. Résultats obtenus

a. Mécanismes mitochondriaux impliqués dans l'ischémie-reperfusion

Dans l'ischémie critique chronique, le muscle squelettique est soumis à des cycles répétés d'ischémie-reperfusion. En effet, lors des efforts de marche ou lors du décubitus, les besoins en oxygène et donc en substrats énergétiques du muscle squelettique vont dépasser les apports du fait de l'altération du lit vasculaire, générant une situation d'ischémie ; suivie d'une situation de reperfusion lorsque le patient sera debout et au repos. Ces cycles répétés d'ischémie-reperfusion sont extrêmement délétères pour le muscle squelettique, conduisant à une véritable myopathie liée au stress oxydant et à l'inflammation, mais à terme à une atteinte des organes à distance (*Pipinos I, et al. Free Radic Biol Med 2006 ; Zorov DB, et al. Biochim Biophys Acta 2006*).

L'énergie nécessaire au bon fonctionnement de la cellule est fournie par la mitochondrie, à partir de l'oxydation des nutriments, et stockée sous forme d'ATP. L'ensemble des réactions conduisant à la formation de l'ATP est assimilé à une « respiration mitochondriale », qui consomme plus de 90% de l'oxygène que nous consommons. De façon plus précise, l'oxydation des nutriments va fournir par l'intermédiaire du cycle de Krebs des coenzymes réduits (NADH et à un moindre degré $FADH_2$), qui sont des donneurs d'électrons. Ce flux d'électrons est pris en charge par différentes réactions d'oxydoréduction assurées par les 4 complexes de la chaîne respiratoire mitochondriale jusqu'à la réduction de l'oxygène moléculaire en eau. Les complexes respiratoires utilisent l'énergie générée par ce transfert d'électrons pour permettre une translocation active de protons depuis la matrice vers l'espace inter-membranaire mitochondrial. Cette expulsion de protons va avoir comme conséquence la création d'un gradient de concentration de protons et d'un potentiel de membrane mitochondrial à travers la membrane interne. Les protons ainsi expulsés de la matrice vont du fait d'un gradient électrochimique favorable, retourner dans la matrice en empruntant un canal, ce qui active ATP synthase qui transforme alors l'ADP en ATP. Le maintien de ce gradient électrochimique, encore appelé force protomotrice, est un élément indispensable au rôle énergétique de la mitochondrie.

La respiration mitochondriale génère toutefois dans le même temps la formation de radicaux libres dérivés de l'oxygène. Un radical libre est une espèce chimique contenant un électron non apparié. Extrêmement instable, ce composé peut réagir avec les molécules plus stables pour apparier son électron. Il peut alors soit arracher un électron (se comportant alors comme un oxydant), soit en céder un (et agir comme un réducteur). Si le radical libre se comporte comme un oxydant, ceci conduit généralement à la formation en chaîne de nouveaux radicaux, et explique que la production d'un premier radical libre puisse causer d'importantes lésions dans une cellule. Les principaux radicaux libres dérivés de l'oxygène sont l'anion superoxyde et le radical hydroxyle. La production de radicaux libres dérivés de l'oxygène est normale pour tous les organismes vivant en aérobie. Il existe en effet des systèmes de détoxification, qu'ils soient enzymatiques (superoxide dismutase, catalase, glutathion peroxydase) ou non (vitamines et oligoéléments).

En situation d'ischémie, l'ATP est générée par la glycolyse anaérobie. Il s'en suit une déplétion des réserves en glycogène, un métabolisme anaérobie et une acidose lactique locale. La déplétion en ATP qui en résulte réduit la fonction des pompes membranaires et provoque un œdème cellulaire. En effet, la cellule va tendre à corriger l'acidose en expulsant les ions H^+ par l'intermédiaire de l'échangeur Na^+/H^+, le cytoplasme va donc être saturé en ions Na^+, provoquant un appel osmotique vers le cytoplasme. L'œdème cellulaire est aggravé par la dysfonction de l'échangeur Na^+/K^+ ATP-dépendant du fait du manque d'ATP, qui conduit également à l'accumulation de Na^+ dans le cytoplasme. L'acidose va activer également des médiateurs comme la phospholipase A2 qui va métaboliser les phospholipides membranaires en acide arachidonique, un précurseur des médiateurs de l'inflammation, comme les leucotriènes et les prostaglandines. L'ischémie va également initier la conversion de la xanthine déshydrogénase en xanthine oxydase (*Makris KI, et al. Vascular 2007)*.

Seule la reperfusion est capable d'empêcher les lésions irréversibles de l'ischémie. Néanmoins, la reperfusion génère également des lésions propres qui aggravent les lésions tissulaires dues à la seule ischémie. Au niveau cellulaire, la réoxygénation va interrompre les lésions induites par l'ischémie, mais elle va provoquer une série de lésions propres, dites lésions de reperfusion. Au cours des premières minutes de reperfusion, la correction rapide de l'acidose par l'échangeur Na^+/H^+ et le co-transporteur $Na^+/HCO3^-$, ainsi que l'épuration de l'acide lactique, vont provoquer une activation inverse de l'échangeur Na^+/Ca^{2+} et donc augmenter le Ca^{2+} cytosolique. Cette accumulation de Ca^{2+} cytosolique va être responsable de l'ouverture du pore de transition de perméabilité mitochondrial (*Bernardi P, et al. Front Physiol 2013*). L'ouverture du pore de transition de perméabilité va provoquer un changement brusque de la perméabilité membranaire mitochondriale, entraînant un collapsus énergétique incompatible avec la survie cellulaire et induit la libération de facteurs pro-apoptotiques de l'espace inter-membranaire mitochondrial vers le cytosol, conduisant à la mort cellulaire. En parallèle, la reperfusion va générer un stress oxydant massif puisque la xanthine oxydase, produite pendant l'ischémie, va catalyser la formation d'acide urique à partir de l'hypoxanthine, accompagnée par la formation de grandes quantités de radicaux libres. Les radicaux libres ainsi produits vont dépasser les défenses anti-oxydantes cellulaires. Il se crée alors un cercle vicieux, car la production de radicaux libres va entraîner une dysfonction de la chaîne respiratoire mitochondriale, mais la dysfonction de la chaîne respiratoire mitochondriale va à son tour générer davantage de radicaux libres. La

surproduction de radicaux libres va alors avoir plusieurs effets délétères : la peroxydation des lipides, l'oxydation des protéines, des mutations de l'ADN, mais également l'ouverture du pore de transition de perméabilité mitochondrial.

Néanmoins, lorsque la production radicalaire reste contenue en deçà d'un certain seuil, les radicaux libres exercent un effet de signalisation moléculaire prompt à développer des mécanismes de défense cellulaire et d'activation de la biogenèse mitochondriale (Figure 1). Regroupés sous le nom de mitohormèse, ces mécanismes constituent l'une des cibles thérapeutiques à atteindre pour limiter les lésions liées aux cycles répétés d'ischémie-reperfusion dans l'ischémie critique chronique (*Lejay A, et al. Int J Bioch Cell Biol 2014*).

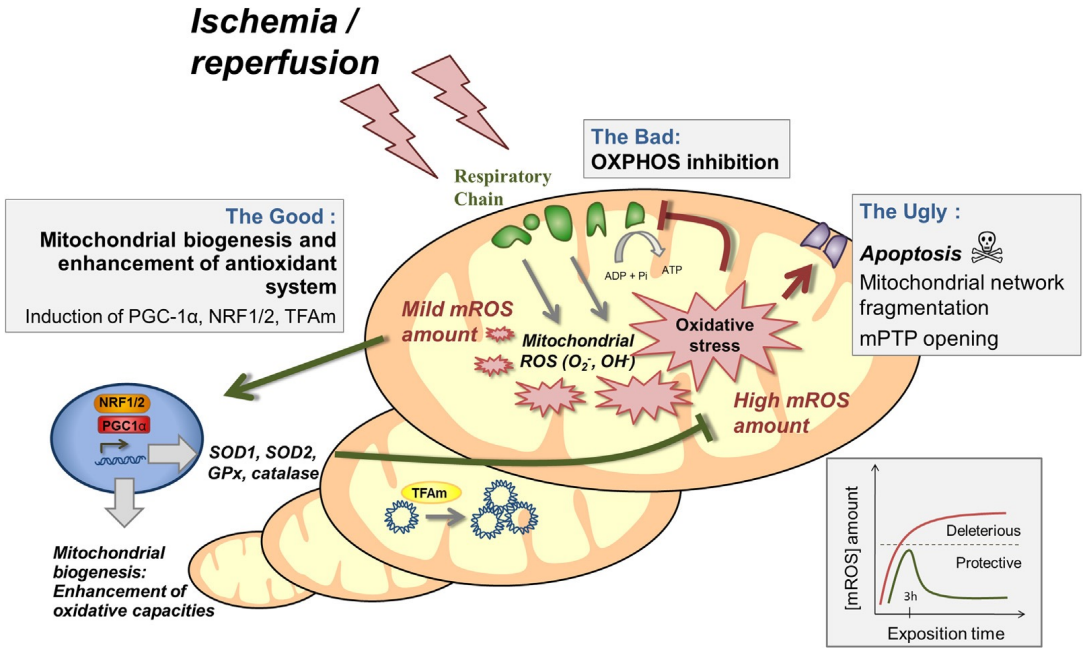

Figure 1. Variabilité des effets mitochondriaux de l'ischémie-reperfusion en fonction du niveau de stress oxydant (*d'après Lejay A, et al. Int J Biochem Cell Biol 2014*).

En résumé, l'ischémie-reperfusion va engendrer des lésions cellulaires si la production de radicaux libres générée est importante. Néanmoins, si la production de radicalaire reste contenue en deçà d'un certain seuil, la mitohormèse et la stimulation des défenses anti-oxydantes va avoir un effet protecteur pour la cellule. Ainsi, la modulation du stress oxydant peut être considérée comme une cible thérapeutique pour limiter les lésions d'ischémie-reperfusion.

Ce constat a fait l'objet d'une première publication présentée en Annexes (Annexe 1):
Lejay A, Meyer A, Schlagowski AI, Charles AL, Singh F, Bouitbir J, et al. Mitochondria: Mitochondrial participation to ischemia-reperfusion injury in skeletal muscle. *Int J Biochem Cell Biol 2014;50:101-5.*

b. Mise au point d'un modèle murin d'ischémie critique chronique

Faisant suite à ces différents constats sur l'ischémie-reperfusion, nous avons voulu étudier de façon plus approfondie les effets de l'ischémie critique chronique, et donc des cycles répétés d'ischémie-reperfusion au niveau du muscle squelettique. Il nous a donc semblé indispensable de mettre au point un modèle animal d'ischémie critique chronique. Notre choix s'est porté sur la mise au point d'un modèle d'ischémie critique chronique chez la souris, de façon à avoir accès, une fois le modèle animal validé, aux souris transgéniques.

Pour poser le diagnostic d'ischémie critique chez l'Homme, les trois critères suivants sont nécessaires : 1 / des signes cliniques (douleurs de décubitus ou troubles trophiques), 2 / une mesure objective témoignant de l'hypoperfusion du membre ischémique, 3 / une durée des symptômes supérieure à trois semaines (*Norgren L et al. Eur J Vasc Endovasc Surg 2018).* Nous avons donc voulu développer un modèle animal qui répondait à ces trois critères, à savoir obtenir des signes physiques d'ischémie critique chronique chez l'animal, confirmer l'hypoperfusion par une imagerie dédiée, et obtenir une stabilité des lésions au cours du temps.

Il existe de nombreux modèles animaux d'ischémie aiguë, notamment chez la souris (*Bonheur JA, et al. J Surg Res 2004 ; Crawford RD, et al. Am J Physiol Heart Circ Physiol 2007; Tran TP, et al. Eur J Pharmacol 2011*), mais pas de modèle valide d'ischémie critique chronique (*Lofti S, et al. Atherosclerosis 2013)*. L'absence de modèle murin d'ischémie critique chronique est liée à deux particularités propres à la souris, d'une part une collatéralité artérielle importante, et d'autre part une néoangiogenèse rapide. L'importante collatéralité artérielle explique qu'une ligature fémorale isolée ne permet pas d'obtenir une ischémie aiguë du membre postérieur chez la souris car les très nombreuses collatérales permettent d'assurer un flux sanguin suffisant à la perfusion du membre postérieur ; alors qu'une oblitération aiguë de l'artère fémorale commune entraîne aussitôt une ischémie aiguë du membre inférieur chez l'homme. Cette collatéralité artérielle explique qu'un des modèles d'ischémie aiguë le plus utilisé chez la souris soit la méthode du tourniquet : ce modèle permet, grâce à un garrot appliqué au niveau de la racine du membre, de bloquer le flux sanguin au niveau de l'artère fémorale, mais également au niveau de l'ensemble de ses collatérales (*Tran TP, et al. Eur J Pharmacol 2011*). Il a également été démontré qu'il existe chez la souris des mécanismes de néoangiogenèse importants, stimulés à la fois par l'hypoxémie et par l'inflammation liées à l'ischémie (*Shweiki D, et al. Science 1992*).

Cette importante collatéralité artérielle associée à l'activation rapide de la néoangiogenèse explique la difficulté d'obtenir chez la souris un modèle d'ischémie critique chronique, c'est à dire un modèle d'hypoperfusion progressive, mais surtout prolongée chez l'animal. Dans l'objectif d'obtenir une atteinte progressive et chronique, nous avons donc développé un modèle basé sur des ligatures artérielles séquentielles. Le membre postérieur droit était soumis au protocole, tandis que le membre postérieur gauche servait de contrôle. Une première incision était réalisée au niveau fémoral, sous anesthésie générale par isoflurane, et permettait de ligaturer sélectivement l'artère fémorale, ainsi que ses trois premières collatérales. Une deuxième intervention était réalisée 4 jours plus tard, permettant par une courte laparotomie médiane de ligaturer l'artère iliaque commune, 0,5 cm après son origine.

La symptomatologie ischémique a été évaluée quotidiennement à partir de scores cliniques permettant d'objectiver un score d'atteinte tissulaire et un score d'atteinte fonctionnelle. Ainsi, à compter du 5ème jour suivant la première intervention, les souris développaient une cyanose du membre soumis aux ligatures séquentielles, puis des nécroses d'orteil. D'un point de vue fonctionnel, les souris présentaient également des déficits de flexion au niveau du membre ischémique.

Des scintigraphies de perfusion au Technetium 99m ont été réalisées pendant 30 jours pour objectiver l'hypoperfusion du membre soumis aux ligatures artérielles. La perfusion du membre ischémique a été mesurée, de même que celle du membre contrôle, permettant d'obtenir un rapport de perfusion membre ischémique/membre contrôle. Durant toute la durée du protocole, le rapport de perfusion était négatif, ce qui témoignait de l'hypoperfusion durable et stable du membre soumis aux ligatures séquentielles, et ce jusqu'au 30ème jour après la première ligature, date du sacrifice des animaux.
Ainsi, nous avons obtenu dans notre modèle animal les critères permettant de poser le diagnostic d'ischémie critique chronique : en associant des signes cliniques (nécrose d'orteils), des mesures de perfusion artérielle et une durée des symptômes supérieure à 21 jours, nous avons pu valider notre modèle animal d'ischémie critique chronique : 1 / des signes cliniques d'ischémie, 2 / une mesure objective de l'hypoperfusion du membre inférieur, 3 / une durée des symptômes supérieure à trois semaines.

Nous nous sommes ensuite intéressés à l'atteinte mitochondriale spécifique de l'ischémie critique chronique, en réalisant l'analyse de la production de radicaux libres, de la respiration mitochondriale, de la capacité de rétention calcique au niveau du muscle squelettique, et l'analyse moléculaire des transcrits codant pour les systèmes anti-oxydants et la biogenèse mitochondriale. Au niveau des membres ischémiques, il existait un stress oxydant du fait d'une production de radicaux libres augmentée et d'une diminution des systèmes anti-oxydants et de la biogenèse mitochondriale. Il existait également une altération de la respiration mitochondriale, de même que de la capacité de rétention calcique, témoignant d'une plus grande susceptibilité de la cellule à l'apoptose. L'analyse histologique des muscles a mis en évidence un aspect myopathique du muscle ischémique, avec une diminution de la taille des myofibrilles, un aspect central des noyaux, et des zones de fibrose.

En résumé, notre modèle animal nous a permis de confirmer le rôle majeur du stress oxydant dans l'atteinte mitochondriale liée à l'ischémie critique chronique.

La mise au point du modèle animal a fait l'objet d'une deuxième publication présentée en Annexes (Annexe 2) :
Lejay A, Choquet P, Thaveau F, Singh F, Schlagowski AI, Charles AL, et al. A new murine model of sustainable and durable chronic ischemia fairly mimicking human pathology. *Eur J Vasc Endovasc Surg 2015;49:205-12.*

c. Modulation du stress oxydant par l'exercice

Notre modèle d'ischémie critique chronique validé, notre objectif suivant a été de mettre au point des stratégies qui pourraient protéger le muscle squelettique en limitant la dysfonction mitochondriale. Aux vues de nos précédents travaux, notre idée était de mettre en place différentes stratégies qui engendreraient une production radicalaire limitée, de façon à stimuler la mitohormèse et les défenses anti-oxydantes, afin de contrebalancer le stress oxydant induit par l'ischémie critique chronique.

Notre équipe avait déjà travaillé sur les effets de l'exercice, tant sur l'animal que sur l'Homme, et avait mis en évidence l'effet protecteur de l'exercice sur le muscle squelettique (*Bouitbir J, et al. Muscle Nerve 2012 ; Isner-Horobeti ME, et al. Muscle Nerve 2014 ; Schlagowski AI, et al. J Appl Physiol 2014 ; Bouaziz W, et al. Arch Gerontol Geriatr 2017*).

Sur la base de ces travaux, nous avons donc développé un protocole d'exercice d'intensité et de durée faibles, qui puisse d'une part être adapté à une situation d'ischémie critique chronique et qui engendre d'autre part une production radicalaire limitée. Notre hypothèse était que l'activation des systèmes anti-oxydants et la stimulation de la biogenèse mitochondriale par cette production radicalaire limitée pourraient contrebalancer le stress oxydant engendré par l'ischémie critique chronique.
Nous avons donc testé les effets d'un exercice sur tapis roulant sur notre modèle animal d'ischémie critique chronique. Les animaux ont été soumis à notre protocole de ligatures séquentielles du membre postérieur droit, et au 6ème jour le protocole d'exercice a été mis en place. L'exercice a été conduit sur une durée de 3 semaines, 5 jours par semaine. Une phase d'échauffement de 2 minutes (10° d'inclinaison, 15 cm/s) était réalisée, puis ensuite la phase d'exercice de durée et d'intensité croissantes au cours des semaines (10° d'inclinaison à 25 cm/s pendant 45 minutes la première semaine, 10° d'inclinaison à 30 cm/s pendant 60 minutes la deuxième semaine, et 10° d'inclinaison à 30 cm/s pendant 90 minutes la troisième semaine) a été réalisée. Les animaux ont été sacrifiés au 30ème jour.

L'effet protecteur de l'exercice, même d'intensité et de durée faibles a été mis en évidence au niveau du muscle squelettique. En effet, nous avons observé une restauration de la respiration mitochondriale et de la capacité de rétention calcique au niveau du muscle en ischémie critique chronique, du fait d'une activation des capacités anti-oxydantes (super-oxyde dismutases 1 et 2, catalase) et de la biogenèse mitochondriale (PGCα et β).

En résumé, un protocole d'exercice de durée et d'intensité faibles a permis de stimuler la biogenèse mitochondriale et les systèmes anti-oxydants, réduisant ainsi l'atteinte mitochondriale liée à l'ischémie critique chronique.

Ce constat a fait l'objet d'une publication présentée en Annexes (Annexe 3) :
Lejay A, Laverny G, Paradis S, Schlagowski AI, Charles AL, Singh F, et al. Moderate exercise allows for shorter recovery time in critical limb ischemia. *Frontiers Physiol 2017;8:523.*

d. Modulation pharmacologique du stress oxydant

Après avoir démontré que la modulation du stress oxydant par l'exercice pouvait stimuler les défenses anti-oxydantes et la biogenèse mitochondriale, et ainsi limiter les effets délétères liés à l'ischémie critique chronique, nous avons voulu mettre en place un protocole pharmacologique. Notre objectif était d'obtenir une diminution du stress oxydant et donc une diminution de la dysfonction mitochondriale liée à l'ischémie critique chronique, mais de manière moins contraignante que par l'intermédiaire d'un protocole d'exercice.

Notre équipe avait déjà étudié les effets d'un anti-oxydant, la N-acétyl-cystéine, sur la cellule, mettant en évidence la stimulation de la mitohormèse lorsque les cellules étaient exposées de manière chronique à un traitement par N-acétyl-cystéine (*Singh F, et al. Biochim Biophys Acta 2015*). La N-acétyl-cystéine, est une molécule utilisée de longue date, notamment comme agent mucolytique ou comme antidote après une intoxication au paracétamol. Néanmoins, il existe un intérêt croissant pour cette molécule, en raison de ces propriétés anti-oxydantes (*Elbini Dhouib I, et al. Life Sci 2016*).

Sur la base de ces travaux, nous avons donc testé les effets d'un conditionnement pharmacologique par N-acétyl-cystéine sur notre modèle d'ischémie critique chronique. Notre hypothèse était qu'un traitement quotidien par N-acétyl-cysteine pourrait restaurer la fonction mitochondriale et réduire la myopathie ischémique, en contrecarrant le stress oxydant lié à l'ischémie critique chronique.

Nous avons donc soumis des animaux en ischémie critique chronique selon notre modèle de ligatures séquentielles à un traitement par N-acétyl-cystéine, à la dose d'1,5 g/kg/j dans l'eau de boisson, pour une durée de 30 jours. Les animaux ont été sacrifiés au 30ème jour.

En effet, nous avons observé une restauration de la respiration mitochondriale et de la capacité de rétention calcique au niveau du muscle en ischémie critique chronique, de même qu'une réduction du taux de radicaux libres. D'un point de vue histologique, le muscle squelettique ne présentait plus de caractéristique myopathique.

En résumé, l'administration chronique de N-acétyl-cystéine dans l'eau de boisson dans le cadre de l'ischémie critique chronique a permis de restaurer la fonction mitochondriale, et de réduire la myopathie qui en découlait. La modulation pharmacologique du stress oxydant peut donc avoir un effet protecteur au niveau du muscle squelettique en ischémie critique chronique.

Ce constat a fait l'objet d'une publication présentée en Annexes (Annexe 4) :
Lejay A, Paradis S, Lambert A, Charles AL, Talha S, Enache I, et al. N-Acetyl Cysteine restores limb function, improves mitochondrial respiration and reduces oxidative stress in a murine model of critical limb ischemia. *Eur J Vasc Endovasc Surg 2018 (epub ahead of print).*

e. Applications à l'Homme

En parallèle de ces travaux expérimentaux, et dans une optique plus transversale, nous avons voulu dégager des pistes pour améliorer la prise en charge des patients en chirurgie vasculaire. En effet, même si la chirurgie de revascularisation est nécessaire, elle nécessite un clampage vasculaire, et induit donc une souffrance ischémique. La restauration de la perfusion liée au déclampage va paradoxalement provoquer des lésions supplémentaires, dites lésions de reperfusion. Les lésions d'ischémie-reperfusion peuvent donc devenir un facteur limitant le succès des interventions chirurgicales nécessitant un clampage artériel, que la pathologie soit occlusive ou anévrismale.

Dans ces conditions, nous nous sommes intéressés au conditionnement ischémique. Le conditionnement ischémique a été développé pour prévenir les lésions d'ischémie-reperfusion. L'idée est de faire subir à l'organisme de brèves séquences répétées d'ischémie-reperfusion, de façon à stimuler les défenses anti-oxydantes de l'organisme pour lutter contre une ischémie-reperfusion plus conséquente (*Hausenloy DJ, et al. Nat Rev Cardiol 2011*). Le concept de préconditionnement ischémique a été décrit initialement au niveau du muscle cardiaque (*Murry CE, et al. Circulation 1986*).

Les mécanismes cellulaires mis en jeu dans le conditionnement ischémique sont complexes et incomplètement élucidés à ce jour. Le préconditionnement ischémique est un processus multifactoriel activant de nombreuses cascades de signalisation interagissant les unes sur les autres. Des signaux inducteurs sont libérés pendant le préconditionnement, ils vont activer des seconds messagers, qui à leur tour vont transmettre le signal à des effecteurs pendant la période d'ischémie prolongée, afin d'atténuer les lésions liées à l'ischémie-reperfusion (*Heusch G. Circ Res 2015*). Ces mécanismes ont cependant été essentiellement étudiés au niveau cardiaque.

De façon simplifiée, le préconditionnement ischémique génère la production de radicaux libres, mais en faible quantité. Cette faible production de radicaux libres protège l'organisme contre une production ultérieure plus importante, puisque cette production de radicaux libres en deçà du seuil critique stimule les défenses anti-oxydantes de la cellule. L'organe venant de subir ce préconditionnement garde en quelque sorte en mémoire l'agression subie, et met en jeu des mécanismes endogènes lui permettant de mieux tolérer une prochaine ischémie (*Kalogeris T, et al. Int Rev Cell Mol Biol 2012*).

On distingue le préconditionnement ischémique local, où le protocole de préconditionnement ischémique est réalisé au niveau de l'organe cible ; et le préconditionnement ischémique à distance, où le protocole de préconditionnement ischémique est réalisé à distance de l'organe cible, c'est à dire au niveau d'un autre organe. Les mécanismes cellulaires mis en jeu dans le conditionnement à distance sont encore très incertains, mais les études expérimentales suggèrent que les mécanismes cellulaires et les voies de signalisation au sein de l'organe protégé sont les mêmes que lors du conditionnement local. Cependant, les métabolites de transmission du message de l'organe préconditionné à l'organe cible ne sont pas clairement identifiés. Plusieurs hypothèses suggèrent une implication des facteurs humoraux, une implication neuronale et un effet anti-inflammatoire et anti-apoptotique systémique (*Hausenloy DJ, et al. Heart Fail Rev 2007; Lim SY, et al. Basic Res Cardiol 2010*).

Notre équipe a mis en évidence les effets bénéfiques du préconditionnement ischémique local et à distance chez l'animal (*Thaveau F, et al. J Vasc Surg 2007; Mansour Z, et al. J Vasc Surg 2012*). Le préconditionnement ischémique, local ou à distance, avait des effets protecteurs en diminuant l'atteinte mitochondriale liée à l'ischémie-reperfusion.

Ainsi, sur la base de ces travaux, nous avons voulu développer un protocole clinique de préconditionnement ischémique chez l'Homme, pour pallier aux effets délétères de l'ischémie-reperfusion induite par le clampage-déclampage nécessaire à toute intervention vasculaire. La chirurgie ouverte des anévrismes de l'aorte abdominale correspond à une de ces situations, puisqu'elle nécessite un clampage aortique et donc une situation d'ischémie-reperfusion induisant une souffrance des organes situés en dessous du niveau du clampage artériel, mais également une souffrance des organes à distance, tels que le myocarde, les reins, les poumons et le muscle squelettique. Cette souffrance peut s'expliquer par plusieurs mécanismes physiopathologiques, dont les perturbations hémodynamiques majeures, chez des patients souvent fragiles et présentant d'autres comorbidités (*Greenhaigh RM, et al. Lancet 2004*). Cette chirurgie est donc grevée d'une morbi-mortalité postopératoire importante. L'idée d'un protocole de préconditionnement ischémique était donc de protéger les organes situés en dessous du niveau de clampage mais également les organes à distance.

Une première étude clinique avait été effectuée chez des patients porteurs d'un anévrisme de l'aorte abdominale avec mise à plat chirurgicale. La réalisation d'un protocole de préconditionnement ischémique à distance a permis d'obtenir une protection cardiaque avec une réduction significative de l'incidence du nombre d'infarctus du myocarde (*Ali ZA, et al. Circulation 2007*). Le protocole de préconditionnement était réalisé par deux cycles d'ischémie-reperfusion par clampage de l'artère iliaque commune. Notre objectif était donc de développer un protocole moins invasif, ne nécessitant pas la réalisation de clampages itératifs d'artères déjà pathologiques.

Nous avons ainsi obtenu le financement d'un **Protocole Hospitalier de Recherche Clinique (PHRC HUS 5831)**, qui a pour objectif de mettre en évidence les effets protecteurs du préconditionnement ischémique à distance sur les fonctions cardiaque, rénale, pulmonaire et musculaire lors de la chirurgie ouverte des anévrismes de l'aorte abdominale. Il s'agit d'une étude multicentrique devant inclure 200 patients. Les patients sont randomisés en deux groupes : un groupe contrôle et un groupe préconditionnement ischémique. Le protocole de préconditionnement ischémique à distance est réalisé avec un brassard huméral : 3 cycles d'ischémie-reperfusion (10 minutes d'ischémie suivies de 10 minutes de reperfusion) sont réalisés juste avant le clampage aortique dans le groupe préconditionnement. Le but de ce protocole de préconditionnement ischémique est de diminuer la morbidité du clampage aortique. L'objectif principal est d'obtenir une diminution de la survenue d'une atteinte myocardique dans le groupe de patients ayant bénéficié du préconditionnement ischémique. Les objectifs secondaires sont la diminution de la survenue d'un infarctus du myocarde, la diminution de la survenue d'une atteinte rénale, la diminution de la survenue d'une atteinte musculaire, et la diminution du nombre de décès sous 30 jours. Le protocole a été approuvé par le Comité de Protection des Personnes le 27 Juin 2018, et est ouvert aux inclusions depuis cette date.

En résumé, le conditionnement ischémique permet de lutter contre les effets délétères des lésions liées à l'ischémie-reperfusion. L'idée est de faire subir à l'organisme de brèves séquences répétées d'ischémie-reperfusion, de façon à stimuler les défenses anti-oxydantes pour lutter contre une ischémie-reperfusion ultérieure plus conséquente.

Il en découle des applications immédiates en chirurgie vasculaire, puisque le clampage-déclampage nécessaire à toute intervention vasculaire induit une situation d'ischémie-reperfusion programmée. Notre protocole hospitalier de recherche clinique a pour objectif de mettre en évidence les effets protecteurs du préconditionnement ischémique à distance sur les fonctions cardiaque, rénale et musculaire lors de la chirurgie ouverte des anévrismes de l'aorte abdominale.

Une mise au point concernant le conditionnement ischémique et ses applications en chirurgie vasculaire est présentée en Annexes (Annexe 5) :
Delay C, Paradis S, Charles AL, Chakfe N, Geny B, **Lejay A**. Skeletal muscle ischemia-reperfusion and ischemic conditioning pathophysiology – clinical applications for the vascular surgeon. *J Mal Vasc 2017;42:29-38.*

f. Limites

A l'heure actuelle, de nombreux protocoles de conditionnement ischémique sont à l'étude. Néanmoins, bien qu'offrant des résultats prometteurs en expérimentation animale, le conditionnement ischémique conduit à des résultats plus mitigés au niveau clinique. En effet, il existe une grande disparité concernant les résultats des différentes études réalisées. Au niveau cardiaque, de nombreuses études concluent à l'efficacité du préconditionnement ischémique local ou à distance, mais de nombreuses études ne retrouvent aucun effet cardioprotecteur (*Ferdinandy P, et al. Pharmal Rev 2014; Hausenloy DJ, et al. N Eng J Med 2015; Meybohm P, et al. N Engl J Med 2015*).

Il en est de même au niveau vasculaire. Si certaines études mettent en évidence un effet protecteur du préconditionnement ischémique, d'autres ne retrouvent aucune protection (*Walsh SR, et al. Vasc Endovascular Surg 2010; Li C, et al. Anesthesiology 2013; Murphy N, et al. J Cardiothorac Vasc Anesth 2014*).

Ces résultats variables nous ont conduit a étudié plus précisément les voies de signalisation intracellulaires impliquées dans la transmission des signaux du conditionnement ischémique. La séquence moléculaire lors du conditionnement ischémique inclut des signaux inducteurs, activant ensuite des seconds messagers qui à leur tour vont transmettre le signal à des effecteurs pendant la période d'ischémie prolongée, afin d'atténuer les lésions d'ischémie-reperfusion. Les signaux inducteurs sont essentiellement les radicaux libres en petites quantités, la bradykinine, l'adénosine, le TNFalpha, ou certaines interleukines (*Xuan YT, et al. Circulation 2005*). Les seconds messagers intracellulaires impliquent principalement la protéine kinase C (PKC), l'oxyde nitrique, la protéine kinase G (PHG) et le GMP cyclique. Ces seconds messagers vont agir sur le principal effecteur qui est le pore de transition de perméabilité mitochondrial. Le signal transmis inhibe l'ouverture du pore de transition de perméabilité mitochondrial, et donc l'apoptose (*Costa AD, et al. Am J Physiol Heart Circ Physiol 2008*; *Oldenburg O, et al. Am J Physiol Heart Circ Physiol 2004*). Cette distinction entre signaux

inducteurs-seconds messagers-effecteurs est cependant complexe car il existe de nombreuses interactions, et une même molécule peut interagir à plusieurs niveaux de la cascade de signalisation. De ce fait les mécanismes protecteurs liés au conditionnement ischémique ont été décrits d'une autre façon, en les regroupant sous deux voies principales : la voie Reperfusion Injury Salvage Kinase (RISK) et la voie Survivor Activating Factor Enhancement (SAFE) (*Hausenloy DJ, et al. Trends Cardiovasc Med 2005; Lecour S. J Mol Cell Cardiol 2009*).

Ainsi, certains facteurs, tels que l'âge, l'hypertension artérielle, et surtout le diabète peuvent limiter l'efficacité du préconditionnement. Nous avons ainsi mis en évidence une diminution de l'effet protecteur du conditionnement ischémique liée au diabète. La diminution de l'effet protecteur du conditionnement ischémique est liée à deux éléments principaux : un niveau de stress oxydant basal plus élevé, et une altération des voies de signalisation RISK et SAFE (Figure 2).

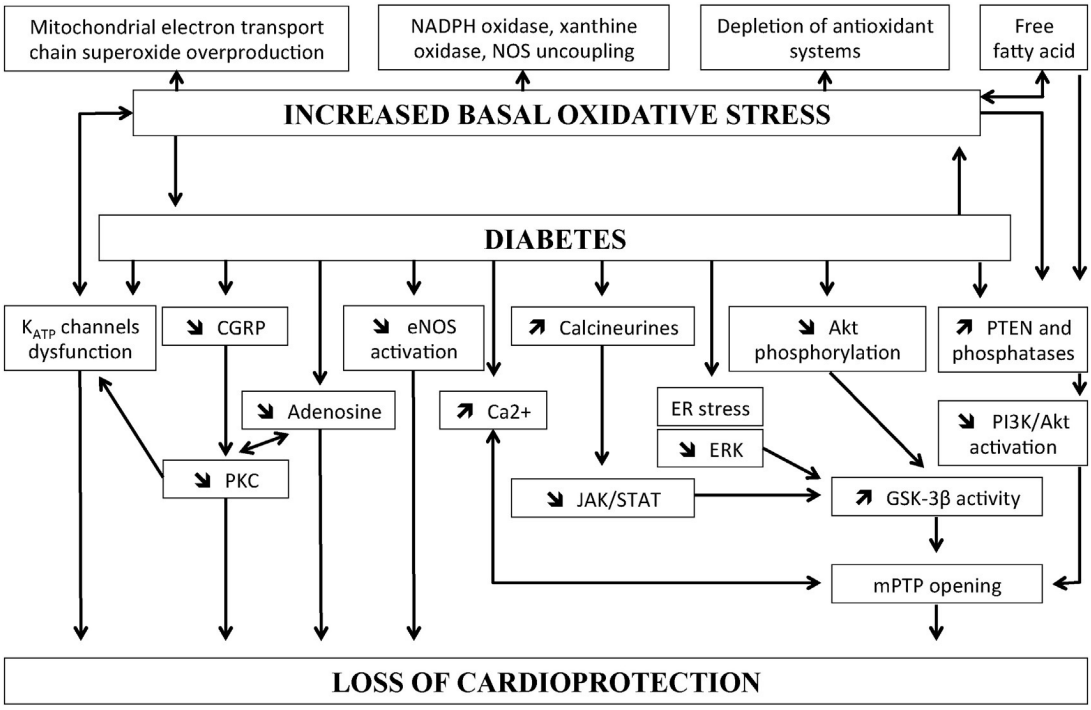

Figure 2. Diminution de l'effet protecteur du conditionnement ischémique due au diabète (*d'après Lejay A, et al. J Mol Cell Cardiol Biol 2016*).

Le niveau basal de stress oxydant plus élevé est lié à deux mécanismes : une production basale de radicaux libres plus élevée, et une altération des systèmes anti-oxydants. Lors d'une hyperglycémie, de nombreuses protéines, principalement plasmatiques, vont subir le phénomène de glycation, qui consiste à la fixation de sucres réducteurs sur les protéines. La glycation des protéines aboutit à la formation des produits avancés de glycation (AGE). Les AGE sont directement impliqués dans la genèse du stress oxydant, car ils peuvent se fixer sur des récepteurs membranaires spécifiques (RAGE). Cette fixation aux récepteurs génère des radicaux libres. De plus, les protéines glyquées peuvent induire un stress oxydant en

réagissant directement avec les radicaux libres. Certains produits d'oxydation peuvent se lier à des protéines et amplifier les lésions générées par la glycation. Il s'agit donc d'un mécanisme délétère et ininterrompu qui s'auto-entretient. En parallèle, les activités anti-oxydantes de la cellule sont réduites. Au total, le niveau élevé de radicaux libres et les capacités anti-oxydantes réduites conduisent à un stress oxydant basal important, qui dépasse les capacités de défense de la cellule (*Heusch G. Lancet 2013*).

En parallèle, les voies RISK et SAFE sont altérées dans le diabète. L'hyperglycémie bloque l'activation des différents composants des voies de signalisation, qui ne peuvent alors plus inhiber l'ouverture du pore de transition de perméabilité mitochondrial et donc l'apoptose.

En résumé, certains facteurs peuvent limiter l'effet protecteur du conditionnement ischémique. Ainsi le diabète, par le stress oxydant basal élevé et l'altération des voies de protection intracellulaires est un des facteurs limitants majeurs.

Nos travaux expliquant l'inefficacité du conditionnement ischémique sont présentés en Annexes (Annexe 6 et Annexe 7) :
Lejay A, Fang F, John R, Van JA, Barr M, Thaveau F, et al. Ischemia reperfusion injury, ischemic conditioning and diabetes mellitus. *J Mol Cell Cardiol 2016;91:11-22.*
Pottecher J, Adamopoulos C, **Lejay A**, Bouitbir J, Charles AL, Meyer A, et al. Diabetes worsens skeletal muscle mitochondrial function, oxidative stress and apoptosis after lower-limb ischemia-reperfusion: implication of the RISK and SAFE pathways? *Frontiers Physiol 2018;9:579.*

5. Perspectives

Nos travaux ont mis en évidence l'atteinte mitochondriale associée à l'ischémie critique chronique des membres inférieurs, et la diminution des lésions observées par une modulation du stress oxydant.

Nous avons également mis en évidence l'intérêt du conditionnement ischémique pour pallier aux effets délétères de l'ischémie-reperfusion, malgré certains facteurs limitants tels que le diabète. Néanmoins, avec un protocole bien conduit, dans des situations cliniques adaptées, le conditionnement ischémique peut avoir une place majeure dans la pratique clinique courante.

Etant responsable du versant chirurgical du programme de transplantation rénale, mes perspectives sont d'appliquer nos travaux sur l'ischémie-reperfusion et sur la modulation du stress oxydant au domaine de l'ischémie-reperfusion rénale. Lors de mon post-doctorat à l'institute of Medical Sciences au Canada, je me suis formée à un modèle murin d'ischémie-reperfusion rénale. D'un point de vue expérimental, il convient d'étudier si un protocole de préconditionnement ischémique peut diminuer l'atteinte mitochondriale rénale ; et si tel est le cas appliquer un tel protocole à l'Homme lors des transplantations rénales.

VI. COLLABORATIONS

1. Collaborations locales

a. Service de Néphrologie et Transplantation Rénale

La collaboration médico-chirurgicale entre le service de Chirurgie Vasculaire et Transplantation Rénale et celui de Néphrologie et Transplantation Rénale (Professeur Bruno Moulin, Professeur Sophie Caillard) a pour objectif d'améliorer la prise en charge pré-opératoire et per-opératoire des patients sur liste d'attente de greffe rénale en mettant à profit notre expertise vasculaire, et d'optimiser le suivi des patients greffés. Cette collaboration a permis la publication de quatre manuscrits :

- **Lejay A**, Caillard S, Thaveau F, Chakfé N. Response to Letter to the Editor: 'Why should vascular surgeons be more involved in kidney transplantation?' *Eur J Vasc Endovasc Surg 2018 (Epub ahead of print).*
- **Lejay A**, Caillard S, Thaveau F, Chakfé N. Why should vascular surgeons be more involved in kidney transplantation? *Eur J Vasc Endovasc Surg 2018;55:455-6*.
- **Lejay A**, Thaveau F, Caillard S, Georg Y, Moulin B, Wolf P, et al. How can a vascular surgeon help in kidney transplantation. *J Cardiovasc Surg 2017;58:351-9.*
- Collange O, Jazaerli L, **Lejay A**, Biermann C, Caillard S, Moulin B, et al. Intraoperative pleth variability index is linked to delayed graft function after kidney transplantation. *Transplant Proc 2016;48:2615-21.*

b. Service de Radiologie Vasculaire

Notre service travaille en étroite collaboration avec le service de Radiologie Vasculaire (Professeur Catherine Roy, Docteur Mickaël Ohana). L'essor de l'endovasculaire a conduit à une augmentation importante du nombre d'examens radiologiques, programmés ou urgents, à visée diagnostique ou thérapeutique. Ceci a conduit à des procédures plus longues, plus complexes, parfois itératives. La collaboration avec le service de Radiologie Vasculaire prend ainsi place dans une démarche d'amélioration de l'analyse systématisée de l'image et d'optimisation dosimétrique. Cette collaboration a permis la publication de neuf manuscrits :

- Girsowicz E, Georg Y, Lefebvre F, **Lejay A**, Thaveau F, Roy C, et al. Anatomical study of healthy aortic arches. *Ann Vasc Surg 2017;44:179-89.*
- **Lejay A**, Delay C, Georg Y, Gaertner S, Ohana M, Lee JT, et al. Five-years outcomes of surgical treatment for popliteal artery entrapment syndrome. *Eur J Vasc Endovasc Surg 2016;51:557-564.*
- **Lejay A**, Caspar T, Ohana M, Delay C, Girsowicz E, Ohlmann P, et al. Vascular access complications in endovascular procedures with large sheaths. *J Cardiovasc Surg 2016;57:311-21.*
- **Lejay A**, Ohana M, Delay C, Georg Y, Girsowicz E, Scholey JW, et al. Cystic adventitial pathology as an entity in peripheral arterial disease. *J Cardiovasc Surg 2016;57:282-91.*
- Schwein A, Georg Y, Ohana M, Delay C, **Lejay A**, Thaveau F, et al. Treatment of aneurysmal aberrant right subclavian artery with trop-barrel stentgraft. *Ann Vasc Surg 2015;29:595.*

- Ohana M, Georg Y, **Lejay A**, Girsowicz E, Gaertner S, Labani A, et al. Current optimal morphological evaluation of peripheral arterial disease. *J Cardiovasc Surg 2015;56:287-297.*
- Ohana M, El Ghannudi S, Girsowicz E, **Lejay A**, Georg Y, Thaveau F, et al. Detailed cross-sectional study of 60 superficial femoral artery occlusions: morphological quantitative analysis can lead to a new classification. *Cardiovasc Diagn Ther 2014;4:71-9.*
- **Lejay A**, Ohana M, Lee JT, Georg Y, Delay C, Lucereau B, et al. Popliteal artery entrapment syndrome. *J Cardiovasc Surg 2014;55:225-37.*
- Girsowicz E, Georg Y, **Lejay A**, Ohana M, Delay C, Bouamaied N, et al. Mid-term failure after endovascular treatment of a persistent sciatic artery aneurysm. *Ann Vasc Surg 2014;28:7-12.*

c. Service d'Hypertension et Maladies Vasculaires

La collaboration médico-chirurgicale entre le service de Chirurgie Vasculaire et Transplantation Rénale et celui d'Hypertension et Maladies Vasculaires (Professeur Dominique Stephan, Docteur Sébastien Gaertner) a pour objectif d'améliorer le parcours de soins des patients présentant une artériopathie des membres inférieurs. Cette collaboration a permis la publication de quatre manuscrits :

- Stephan D, Cordeanu M, Mirea C, Faller A, **Lejay A**, Gaertner S. Place of non-vitamin K antagonist oral anticoagulant-antiplatelet combinations in peripheral arterial disease. *Arch Cardiovasc Dis 2016;109:634-40.*
- Lejay A, Delay C, Georg Y, Gaertner S, Ohana M, Thaveau F, et al. Five-years outcomes of surgical treatment for popliteal artery entrapment syndrome. *Eur J Vasc Endovasc Surg 2016;51:557-564.*
- **Lejay A**, Delay C, Georg Y, Schwein A, Gaertner S, Thaveau F, et al. Endovascular surgery, open surgery and primary amputation in nonagenarians presenting with critical limb ischemia. *Ann Vasc Surg 2016;32:25-33*.
- Delay C, Schwein A, **Lejay A**, Gaertner S, Aleil B, Thaveau F, et al. Aortitis and aortic occlusion in Crohn's disease. *Ann Vasc Surg 2015;29:365-369.*

d. Institut de Génétique et Biologie Moléculaire et Cellulaire

D'un point de vue fondamental, la collaboration entre l'Equipe d'Accueil 3072 et l'Institut de Génétique et Biologie Moléculaire et Cellulaire (Docteur Daniel Metzger, Dr Gilles Laverny) a permis d'explorer les voies moléculaires impliquées dans nos travaux de modulation du stress oxydant dans l'ischémie reperfusion, et d'approfondir ainsi notre connaissance de la physiopathologie de l'ischémie des membres inférieurs. Cette collaboration a permis la publication de deux manuscrits :

- **Lejay A**, Laverny G, Paradis S, Schlagowski AI, Charles AL, Singh F, et al. Moderate exercise allows for shorter recovery time in critical limb ischemia. *Front Physiol 2017;8:253*.
- **Lejay A**, Choquet P, Thaveau F, Singh F, Laverny G, Metzger D, et al. A new murine model of sustainable and durable chronic critical limb ischemia fairly mimicking human pathology. *Eur J Vasc Endovasc Surg 2015;49:205-212.*

e. Centre Européen d'Etude du Diabète

Nos travaux sur le stress oxydant dans les situations d'ischémie-reperfusion et sur la transplantation d'organes offrent des perspectives de recherche, notamment sur la préservation des greffes d'îlots pancréatiques, avec la publication récente d'une étude pilote :

- Schaschlow A, Sigrist S, Mura C, Dissaux C, Bouzakri L, **Lejay A**, et al. Extra-hepatic islet transplantation: validation of the h-omental matrix islet filing (HOMING) technique on a rodent model using an alginate carrier. *Cell Transplant 2018;27:1289-93.*

2. Collaborations internationales

a. Université de Bâle, Suisse

Nos travaux de recherche sur l'ischémie reperfusion et le stress oxydant sont basés sur une collaboration avec le Service de Pharmacologie et Toxicologie de l'Université de Bâle (Docteur Jamal Bouitbir, Docteur François Singh). Celle collaboration a donné lieu à la publication de deux manuscrits :

- Pottecher J, Adamopoulos C, **Lejay A**, Bouitbir J, Charles AL, Meyer A, et al. Diabetes worsens skeletal muscle mitochondrial function, oxidative stress and apoptosis after lower-limb ischemia-reperfusion: implication of the RISK and SAFE pathways? *Frontiers Physiol 2018;9:579.*
- **Lejay A**, Meyer A, Schlagowski AI, Charles AL, Singh F, Bouitbir J, et al. Mitochondria: Mitochondrial participation to ischemia-reperfusion injury in skeletal muscle. *Int J Biochem Cell Biol 2014;50:101-5.*

b. Université de Novossibirsk, Russie

Nous venons de débuter une collaboration avec l'Institut de Recherche sur la Pathologie Circulatoire de l'Université de Novossibirsk (Docteur Rabtsum, Docteur Karpenko), basée sur la biomécanique de l'artère fémorale superficielle. Cette collaboration a permis la publication récente d'un premier manuscrit :

- Rabtsun A, Karpenko A, Zoloev DG, Starodubtsev V, **Lejay A**, Chakfe N. Remote endarterectomy and lamina vastoadductoria dissection improves superficial femoral artery biomechanical behaviour during limb flexion. *Ann Vasc Surg 2018;50:112-8.*

c. Université de Toronto, Canada

Mon année de mobilité en post-doctorat à l'Institut de Sciences Médicales de l'Université de Toronto (Professeur James Scholey, Docteur Fang, Docteur John) m'a permis d'étudier les voies de signalisation moléculaires impliquées dans les mécanismes de conditionnement ischémique, ainsi que les facteurs limitant l'efficacité des protocoles de conditionnement ischémique.

D'un point de vue expérimental, cette collaboration m'a permis de me former à un modèle murin d'ischémie-reperfusion rénale et d'étudier les effets mitochondriaux de l'ischémie-reperfusion à ce niveau.
La collaboration entre l'équipe de Toronto et l'Equipe d'Accueil EA3072 a ainsi permis d'aboutir à la publication de deux revues sur la physiopathologie de l'ischémie-reperfusion et du préconditionnement ischémique :

- **Lejay A**, Fang F, John R, Van JA, Barr M, Thaveau F, et al. Ischemia reperfusion injury, ischemic conditioning and diabetes mellitus. *J Mol Cell Cardiol 2016;91:11-22.*
- Paradis S, Charles AL, Meyer A, **Lejay A**, Scholey J, Chakfe N, et al. Chronology of mitochondrial and cellular events during skeletal muscle ischemia-reperfusion. *Am J Physiol Cell Physiol 2016;310:968-82*.

d. Université de Stanford, Etats Unis

Nous travaillons en étroite collaboration avec le Service de Chirurgie Vasculaire de l'Université de Standford (Professeur Jason Lee, Docteur Benjamin Colvard). Nos travaux communs concernent la pathologie de l'artère poplitée piégée ainsi que l'analyse des explants. Cette collaboration a donné lieu à quatre publications :

- **Lejay A**, Colvard B, Magnus L, Dion D, Georg Y, Papillon Y, et al. Explanted vascular and endovascular devices: where do we stand and what should we do? *Eur J Vasc Endovasc Surg 2018;55:567-76.*
- **Lejay A**, Monnot A, Georg Y, Colvard B, Thaveau F, Geny B, et al. Pathology of graft and stent-graft infections: lessons learned from examination of explant materials. *Semin Vasc Surg 2017;30:70-4.*
- **Lejay A**, Delay C, Georg Y, Gaertner S, Ohana M, Lee J, et al. Five-years outcomes of surgical treatment for popliteal artery entrapment syndrome. *Eur J Vasc Endovasc Surg 2016;51:557-564.*
- **Lejay A**, Ohana M, Lee JT, Georg Y, Delay C, Lucereau B, et al. Popliteal artery entrapment syndrome. *J Cardiovasc Surg 2014;55:225-37.*

e. Working group : Allemagne, Espagne et Serbie

Depuis Juin 2017, dans le cadre de la rédaction des Guidelines de l'European Society for Vascular and Endovascular Surgery, nous travaillons en collaboration avec le Service de Chirurgie Vasculaire et Endovasculaire de l'Université de Belgrade (Professeur Igor Koncar), le Service de Médecine Vasculaire de l'Université d'Hambourg (Professeur Holger Diener) et le Service d'Angiologie et Chirurgie Vasculaire de l'Université de Bizkaia (Docteur Melina Vega de Ceniga). Nous avons ainsi pu établir une revue systématique concernant l'infection de matériel implanté au niveau des troncs supra-aortiques :

- **Lejay A**, Koncar I, Diener H, Vega De Ceniga M, Chakfé. Postoperative infection of prosthetic materials or stents involving the supra-aortic trunks: a comprehensive review. *Eur J Vasc Endovasc Surg 2018* (*Epub ahead of print*).

VII. PUBLICATIONS ET COMMUNICATIONS

1. Publications internationales dans des revues à comité de lecture

(1) **Lejay A**, Paradis S, Lambert A, Charles AL, Talha S, Enache I, et al. N-acetyl cysteine restores limb function, improves mitochondrial respiration and reduces oxidative stress in critical limb ischemia. *Eur J Vasc Endovasc Surg 2018 (Epub ahead of print).*
(2) **Lejay A**, Chakfé N. Commentary on 'In vitro evaluation of aortic stent graft deployment accuracy in the distal landing zone'. *Eur J Vasc Endovasc Surg 2018 (Epub ahead of print).*
(3) **Lejay A**, Koncar I, Diener H, Vega De Ceniga M, Chakfé. Postoperative infection of prosthetic materials or stents involving the supra-aortic trunks: a comprehensive review. *Eur J Vasc Endovasc Surg 2018* (*Epub ahead of print*).
(4) **Lejay A,** Caillard S, Thaveau F, Chakfé N. Response to Letter to the Editor: 'Why should vascular surgeons be more involved in kidney transplantation?' *Eur J Vasc Endovasc Surg 2018 (Epub ahead of print).*
(5) **Lejay A**, Koelbel T, Chakfé N. When surgeons create their own tools. *Eur J Vasc Endovasc Surg 2018 (Epub ahead of print).*
(6) Schaschlow A, Sigrist S, Mura C, Dissaux C, Bouzakri L, **Lejay A**, et al. Extra-hepatic islet transplantation: validation of the h-omental matrix islet filing (HOMING) technique on a rodent model using an alginate carrier. *Cell Transplant 2018;27:1289-93.*
(7) **Lejay A,** Chakfé N. Keep in mind an endograft is a spring! Aorto-enteric fistula after endovascular repair for Behcet's disease patient: a case report. *EJVES Short Rep 2018;39:61.*
(8) Pottecher J, Adamopoulos C, **Lejay A**, Bouitbir J, Charles AL, Meyer A, et al. Diabetes worsens skeletal muscle mitochondrial function, oxidative stress and apoptosis after lower-limb ischemia-reperfusion: implication of the RISK and SAFE pathways? *Frontiers Physiol 2018;9:579.*
(9) **Lejay A**, Chakfé N. Do many hands make outcomes better? Commentary on 'Use of an assistant surgeon does not mitigate the effects of lead surgeon volume on outcomes following open repair of intact abdominal aneurysms' *Eur J Vasc Endovasc Surg 2018;55:720*.
(10) **Lejay A,** Caillard S, Thaveau F, Chakfé N. Why should vascular surgeons be more involved in kidney transplantation? *Eur J Vasc Endovasc Surg 2018;55:455-6*.
(11) Del Tatto B, **Lejay A,** Meteyer V, Roussin M, Georg Y, Thaveau F, et al. Open and endovascular repair of popliteal artery aneurysms. *Ann Vasc Surg 2018;50:119-27.*
(12) Rabtsun A, Karpenko A, Zoloev DG, Starodubtsev V, **Lejay A**, Chakfé N. Remote endarterectomy and lamina vastoadductoria dissection improves superficial femoral artery biomechanical behaviour during limb flexion. *Ann Vasc Surg 2018;50:112-8.*
(13) Chenesseau B, Heim F, Pidancier C, **Lejay A**, Thaveau F, Georg Y, Chakfé N. How compression inside a delivery system can degrade the cover of aortic endografts. *Ann Vasc Surg 2018;50:1-10.*
(14) **Lejay A,** Colvard B, Magnus L, Dion D, Georg Y, Papillon Y, et al. Explanted vascular and endovascular devices: where do we stand and what should we do? *Eur J Vasc Endovasc Surg 2018;55:567-76.*
(15) **Lejay A,** Chakfé N. Commentary on 'A prospective study to evaluate complete wound healing and limb salvage rates after angiosome targeted infrapopliteal balloon angioplasty in critical limb ischemia patients'. *Eur J Vasc Endovasc Surg 2018;55:398.*

(16) **Lejay A,** Monnot A, Georg Y, Colvard B, Thaveau F, Geny B, et al. Pathology of graft and stent-graft infections: lessons learned from examination of explant materials. *Semin Vasc Surg 2017;30:70-4.*
(17) **Lejay A,** Chakfé N. A huge thoracic aneurysm. *Eur J Vasc Endovasc Surg 2018;55:248.*
(18) **Lejay A,** Kuntz S, Rouby AF, Georg Y, Thaveau F, Geny B, et al. Late peroneal neuropathy after open surgical treatment of popliteal artery aneurysm. *Ann Vasc Surg 2018;47:283-4.*
(19) **Lejay A,** Delay C, Girsowicz E, Chenesseau B, Bonnin E, Ghariani MZ, et al. Cryopreserved cadaveric arterial allograft for arterial reconstruction in patients with prosthetic infection. *Eur J Vasc Endovasc Surg 2017;54:636-44.*
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(7) **Lejay A,** Delay C, Charles AL, Thaveau F, Zoll J, Chakfe N, et al. Effet bénéfique expérimental de l'exercice sur le muscle quelettique soumis à une ischémie critique chronique par restauration de la fonction mitochondriale. Société Française de Chirurgie Vasculaire et Endovasculaire, *Grenoble 2016*.
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(10) **Lejay A,** Thaveau F, Delay C, Charles AL, Zoll J, Chakfé N, et al. Effets des statines sur le muscle squelettique dans un modèle murin d'ischémie critique des membres inférieurs. Société Française de Chirurgie Vasculaire et Endovasculaire, *Bordeaux 2014*.
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4. Ouvrages à caractère didactique

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5. Bibliométrie

Nombre de publications par catégorie et par position							
Position	A	B	C	D	E	NC	**Total**
1er auteur	0	23	4	6	6	1	**40**
2ème auteur	0	1	0	0	2	1	**4**
3ème auteur	0	1	1	5	4	0	**11**
Autre position	0	5	5	5	3	2	**20**
Avant-dernier auteur	0	0	1	1	1	0	**3**
Dernier auteur	0	1	0	0	0	1	**2**
Total	**0**	**31**	**11**	**17**	**16**	**5**	**80**

Nombre de publications par catégorie et par année								
Année	A	B	C	D	E	NC	**Total**	**SIGAPS**
2008	0	0	0	0	1	0	**1**	**8**
2009	0	0	0	0	1	0	**1**	**8**
2011	0	0	0	1	2	0	**3**	**22**
2012	0	1	0	1	0	0	**2**	**36**
2013	0	2	0	3	0	0	**5**	**60**
2014	0	1	3	4	0	1	**9**	**64**
2015	0	7	1	5	0	0	**13**	**193**
2016	0	3	5	2	6	2	**18**	**166**
2017	0	6	1	1	2	1	**11**	**128**
2018	0	11	1	0	4	1	**17**	**282**
Total	**0**	**31**	**11**	**17**	**16**	**5**	**80**	**967**

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IX. ANNEXES

Annexe 1 : Lejay A, Meyer A, Schlagowski AI, Charles AL, Singh F, Bouitbir J, et al. Mitochondria: Mitochondrial participation to ischemia-reperfusion injury in skeletal muscle. *Int J Biochem Cell Biol 2014;50:101-5.*

Annexe 2 : Lejay A, Choquet P, Thaveau F, Singh F, Schlagowski AI, Charles AL, et al. A new murine model of sustainable and durable chronic ischemia fairly mimicking human pathology. *Eur J Vasc Endovasc Surg 2015;49:205-12.*

Annexe 3 : Lejay A, Laverny G, Paradis S, Schlagowski AI, Charles AL, Singh F, et al. Moderate exercise allows for shorter recovery time in critical limb ischemia. *Frontiers Physiol 2017;8:523.*

Annexe 4 : Lejay A, Paradis S, Lambert A, Charles AL, Talha S, Enache I, et al. N-Acetyl Cysteine restores limb function, improves mitochondrial respiration and reduces oxidative stress in a murine model of critical limb ischemia. *Eur J Vasc Endovasc Surg 2018 (epub ahead of print).*

Annexe 5 : Delay C, Paradis S, Charles AL, Chakfe N, Geny B, Lejay A. Skeletal muscle ischemia-reperfusion and ischemic conditioning pathophysiology – clinical applications for the vascular surgeon. *J Mal Vasc 2017;42:29-38.*

Annexe 6 : Lejay A, Fang F, John R, Van JA, Barr M, Thaveau F, et al. Ischemia reperfusion injury, ischemic conditioning and diabetes mellitus. *J Mol Cell Cardiol 2016;91:11-22.*

Annexe 7 : Pottecher J, Adamopoulos C, Lejay A, Bouitbir J, Charles AL, Meyer A, et al. Diabetes worsens skeletal muscle mitochondrial function, oxidative stress and apoptosis after lower-limb ischemia-reperfusion: implication of the RISK and SAFE pathways? *Frontiers Physiol 2018;9:579.*

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Organelles in focus

Mitochondria: Mitochondrial participation in ischemia–reperfusion injury in skeletal muscle

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ABSTRACT

Irrespective of the organ involved, restoration of blood flow to ischemic tissue is vital, although reperfusion *per se* is deleterious. In the setting of vascular surgery, even subtle skeletal muscle ischemia contributes to remote organ injuries and perioperative and long-term morbidities. Reperfusion-induced injury is thought to participate in up to 40% of muscle damage.

Recently, the pathophysiology of lower limb ischemia–reperfusion (IR) has been largely improved, acknowledging a key role for mitochondrial dysfunction mainly characterized by impaired mitochondrial oxidative capacity and premature mitochondrial permeability transition pore opening. Increased oxidative stress triggered by an imbalance between reactive oxygen species (ROS) production and clearance, and facilitated by enhanced inflammation, appears to be both followed and instigated by mitochondrial dysfunction.

Mitochondria are both actors and target of IR and therapeutic strategies modulating degree of ROS production could enhance protective signals and allow for mitochondrial protection through a mitohormesis mechanism.

1. Organelle facts

- Mitochondria produce ATP, the main energy source of cells and generate ROS, acting either as causes of cellular injuries or as second messengers allowing mitohormesis.
- Mitohormesis is a phenomenon triggered by moderate oxidative stress activating mitochondrial biogenesis and therefore improving cellular and mitochondrial antioxidant capacities.
- Ischemia–reperfusion increases oxidative stress, inflammation and triggers oxidative damage in tissues.
- Lower limb ischemia–reperfusion initiates muscle mitochondrial dysfunctions including reduced oxidative capacity and mitochondrial pore transition opening.
- Skeletal muscle injuries aggravate the prognosis of patients suffering from peripheral arterial disease.
- Ischemic conditioning generally protects skeletal muscle, reducing ROS production, inflammation and mitochondrial dysfunctions.

2. Introduction

Life requires energy, and this energy is stored in adenosine triphosphate (ATP) molecules that are produced in the mitochondria by oxidative phosphorylation. The roles of mitochondria extend far beyond energy production, as they are important generators of reactive oxygen species (ROS), which can either act as second messengers or as a source of cellular damage, depending on the amount produced.

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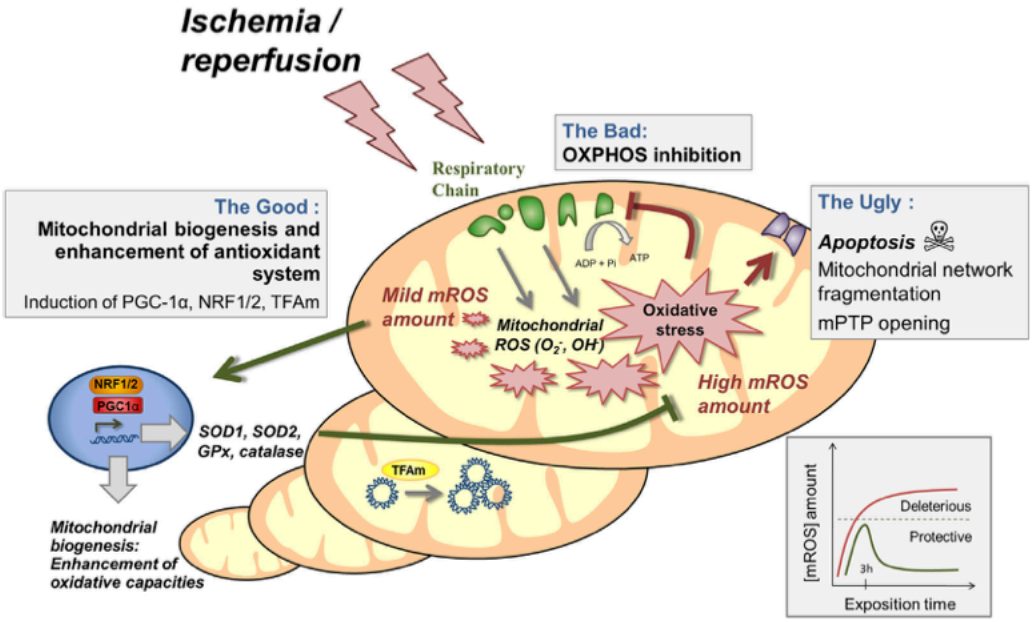

Fig. 1. Mitochondria and reactive oxygen species interactions: the good, the bad and the ugly. ADP, adenosine diphosphate; ATP, adenosine triphosphate; GPx, glutathione peroxidase; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; NRF: nuclear respiratory factor; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; Pi, inorganic phosphate; SOD, superoxide dismutase; TFAm, transcription factor A, mitochondrial.

Peripheral arterial disease (PAD) is a very common manifestation of atherosclerosis related to lower limb arterial stenosis or occlusions. The resulting ischemia leads to exercise or resting pain and, ultimately, to tissue necrosis resulting in leg amputation.

Insufficient oxygen supply was long presumed to be the main and sole cause for the manifestations of PAD; however, reperfusion-related impairment in skeletal muscle mitochondria associated with oxidative stress now appear as key mechanisms.

Such recent advances in mitochondrial participation to IR injury in skeletal muscle support new therapeutic approaches targeting mitochondria. Reducing the amount of ROS perceived by the cells might allow for a shift from a vicious (increased ROS, increased mitochondrial dysfunction, further ROS increase and oxidative damage) to a virtuous cycle (ROS signaling, mitochondrial protection and antioxidative system stimulation) (Fig. 1).

2.1. Organelle function and cell physiology

The physiological functions of mitochondria include ATP production, ROS generation and detoxification, apoptosis involvement, regulation of cytoplasmic and mitochondrial matrix calcium, metabolite synthesis and catabolism. An abnormality in any of these processes can be termed as mitochondrial dysfunction and can impair cell physiology which, when considering skeletal muscle cells, includes contractility and participation in glycemic control.

2.1.1. Oxidative phosphorylation

This process allows the production by the mitochondrial respiratory chain complexes of cellular free energy in the form of ATP and is one of the most prominent functions of mitochondria. Maximal oxidative capacity varies widely depending on the prominence of muscle fiber types (Meyer et al., 2014) and exercise capacity appears linked to skeletal muscle mitochondrial oxidative capacities and coupling. Interestingly, type I muscle fibers (slow-twitch oxidative fibers with high mitochondrial content) participate in euglycemia maintenance, as opposed to the more glycolytic type II fibers (Dela and Helge, 2013).

2.1.2. Mitochondrial permeability transition pore (mPTP)

An increase in calcium concentration in the mitochondrial matrix triggers this high-conductance inner membrane channel opening. While transient openings may serve the purpose of providing a fast Ca^{2+} release mechanism, persistent mPTP opening is followed by a deregulated release of matrix Ca^{2+}, termination of oxidative phosphorylation, matrix swelling with inner membrane unfolding and eventually outer membrane rupture with release of apoptotic proteins and cell death. Pore opening can also cause production of reactive oxygen species, as shown by the occurrence of "superoxide flashes" triggered by transient openings of the mPTP in cardiomyocytes (Wang et al., 2008). The molecular nature of mPTP remains under debate. The long-standing notion that mPTP formation occurs at contact sites of the inner and outer membranes through voltage-dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT) is unlikely since VDAC- and ANT-null mitochondria still display a cyclosporin A permeability transition. Interestingly, reconstituted dimers of F_0F_1 ATP synthase form a channel with properties identical to those of the mPTP, leading to the hypothesis that complex V dimers may actually form the pore (Bernardi, 2013).

2.1.3. Mitochondrial dynamics

Mitochondria are highly dynamic organelles that undergo fission (division) and fusion (joining). Mitochondrial fission and fusion play critical roles in maintaining functional mitochondria in stress conditions. Fusion helps mitigate stress by mixing the contents of partially damaged mitochondria. Fission enables the removal of damaged mitochondria and is necessary to create new mitochondria, but can also facilitate apoptosis during high levels of cellular stress (Youle and van der Bliek, 2012). These processes are regulated by GTPases including optic atrophy protein and mitofusin 1 and 2 for fusion, and dynamin-related protein 1 (Drp1) and the Drp1 targeting molecule fission 1 (Fis1) for fission.

2.1.4. Reactive oxygen species (ROS)

Under resting conditions, over 90% of cellular ROS is produced in the mitochondria. The major sites for ROS generation are electron transport chain complexes I and III. Interestingly, ROS are a

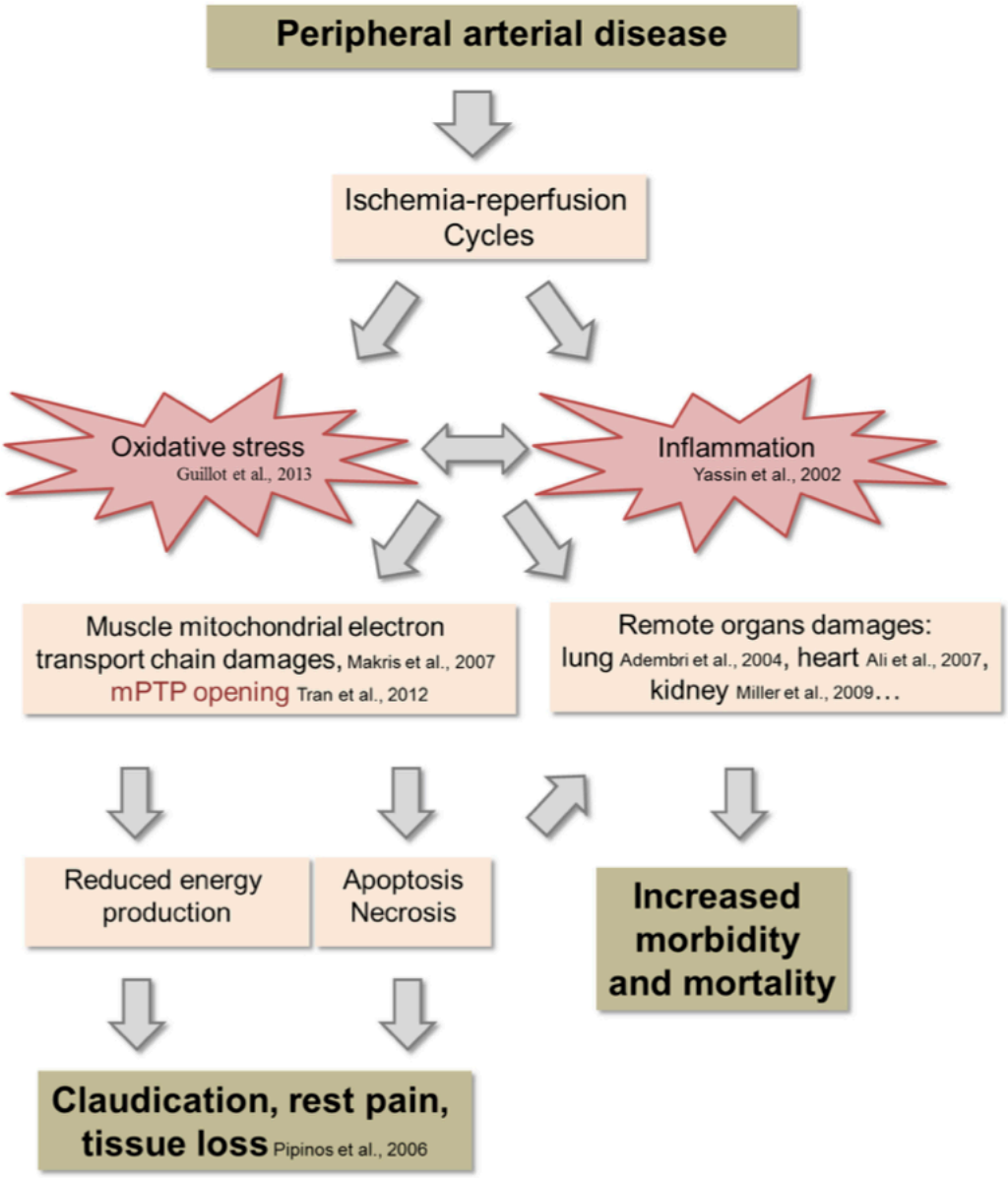

Fig. 2. Implication of mitochondria in the pathophysiology of peripheral arterial disease. Modified from Pipinos et al. (2006).

double-edged sword. They are beneficial *via* cell signaling involved in the antioxidant defense network, but can be harmful by inducing oxidative stress (Zorov et al., 2006). Superoxide anion ($O_2^{\bullet -}$) appears to be a highly important ROS, its toxicity being related to the generation of further reactive species able to attack intracellular biomolecules and resulting in protein carboxylation, lipid peroxidation and DNA damage.

Defense systems can reduce ROS-induced damage. Briefly, $O_2^{\bullet -}$ in the matrix is converted to H_2O_2 by matrix MnSOD (SOD2), while $O_2^{\bullet -}$ released in the intermembrane space is partly dismuted by intermembrane space CuZnSOD (SOD_1). Any residual $O_2^{\bullet -}$ that diffuses into the cytosol is converted by cytosolic CuZnSOD. If mitochondrial $O_2^{\bullet -}$ reaches the extracellular space, it is detoxified by extracellular CuZnSOD (SOD_3). Glutathione-based systems constitute the major redox buffer in the cytosol and H_2O_2 can also be reduced to water by catalase or glutathione peroxidase.

3. Organelle pathology

There are a number of muscular pathologies associated with mitochondrial dysfunction due to either a primitive defect in mitochondrial protein or to secondary injury in the setting of general disease such as diabetes, cardiac or pulmonary failure, cancer and/or inflammatory diseases (Moylan and Reid, 2007). With particular focus oriented toward cardiovascular diseases, data indeed demonstrate an impairment of skeletal muscle mitochondria with decreased mitochondrial oxidative capacities in patients suffering from peripheral arterial disease (PAD) (Brass and Hiatt, 2000; Brass et al., 2001; Pipinos et al., 2000, 2006; Makris et al., 2007). This dependence on oxygen and ATP is critical in skeletal muscle. In PAD muscle, suboptimal energy production from defective mitochondria participates in pathogenesis in addition to reduced oxygen supply (Pipinos et al., 2006) while damage to the mitochondria enhances the production of ROS. Furthermore, MnSOD (the initial

line of ROS defense in mitochondria) has been found to be deficient in PAD muscles (Pipinos et al., 2006; Makris et al., 2007). These findings reflect an impaired mitochondrial antioxidant defense system which is unable to respond to abnormally elevated ROS production, leading to significant oxidative damage to muscle proteins and lipids. Of note, IR-induced injury differs according to the skeletal muscle phenotype and thus, type II fibers probably sustain more damage than type I muscle fibers.

Although not fully elucidated in skeletal muscle, the origin of mitochondrial dysfunction related to ischemia–reperfusion has been studied in the myocardium and can occur at several levels. A decrease in electron transport chain (ECT) protein may result from increased autophagy and/or from decreases in PGC1-α protein content (Lee et al., 2012). Posttranslational alterations of ETC in the ischemic myocardium are also involved, including increased protein tyrosine nitration of complex I and complex II, decreased protein S-glutathionylation of complex II, inactivation of Fe–S protein of complex III and increased hyperphosphorylation of complex IV. Finally, during ischemia and/or reperfusion phases, a loss of mitochondrial cardiolipin (a mitochondrial membrane phospholipid) has also been proposed to explain the decline in complex I (Paradies et al., 2004), III and complex IV activities.

Accordingly, ischemia–reperfusion of the lower limb, largely studied in experimental models using either aortic cross-clamping or leg tourniquet, has confirmed the reduction in mitochondrial oxidative capacity associated with increased ROS production (Brandão et al., 2003; Charles et al., 2011). Although endogenous up-regulation of mitochondrial biogenesis and enhancement of the antioxidant defense may delay muscle injury, IR also increases the Bax/Bcl2 ratio and reduces the capacity for mitochondrial calcium retention thus favoring early mPTP opening and apoptosis (Mansour et al., 2012a; Tran et al., 2012). Interestingly, reduced CyP-D expression in gastrocnemius muscle likely prevented cyclosporin A from blocking mPTP opening during lower-limb IR (Pottecher et al., 2013).

Taken together, both apoptosis and necrosis can lead to tissue loss and ultimately to leg amputation. In addition, nerve, skin and subcutaneous tissue damages also occur, leading to the characteristic "trophic legs" of patients with advanced PAD having thin and brittle muscles, and thin hairless skin with impaired sensorimotor function. Interestingly, surgical by-pass or endovascular therapies do not always relieve patient symptoms. Leg pain has consequently been related to metabolic alterations rather than to reduced blood flow. Furthermore, even subtle skeletal muscle alterations participate in inflammation and remote organ injuries aggravating patient morbidity and mortality rates through multi-organ failure (Yassin et al., 2002; Adembri et al., 2004; Fowkes et al., 2006; Ali et al., 2007; Miller et al., 2009) (Fig. 2).

Importantly, a better understanding of PAD pathophysiology and particularly in view of the fact that defective mitochondria and oxidative stress are central to this myopathy allows for new therapeutic approaches. Increased ROS production occurs before mitochondrial dysfunction (Guillot et al., 2013) suggesting that strategies aiming to reduce ROS production might be successful.

Accordingly, ischemic preconditioning (i.e. repeated short lasting ischemia–reperfusion cycles applied before sustained ischemia) has been shown to protect both ischemic muscles and remote organs in experimental animals and humans (Eberlin et al., 2008; Mansour et al., 2012a; Ali et al., 2007). Controlled reperfusion and ischemic post-conditioning (applied at the onset of reperfusion) was also shown to protect skeletal muscle from IR injuries (Beyersdorf and Schlensak, 2009; McAllister et al., 2008). This is clinically relevant since improved skeletal muscle mitochondrial function is associated with enhanced walking capacities, both in healthy subjects and in patients suffering from PAD.

Similarly, pharmacological approaches using a mimetic of the antioxidant system also decrease ROS production, protect muscle mitochondrial function and reduce infarct size (Tran et al., 2012).

During IR, mitochondria undergo fission that is dependent on Drp1 activation. Inhibition of Drp1 activation by Mdivi-1 (mitochondrial division inhibitor 1) was indeed shown to preserve mitochondrial morphology, lower mitochondrial reactive oxygen species, reduce cytosolic calcium (Sharp et al., 2014), prevent mPTP opening as well as reduce infarct size. Mdivi-1 was conversely protective if administered prior to or following myocardial ischemia, thereby opening promising therapeutic strategies in other organs submitted to IR (Sharp et al., 2014).

4. Future outlook

Controversial data have nevertheless been reported. Local and remote ischemic post-conditioning was found to decrease muscle mitochondrial function and trigger ROS production and inflammation (Mansour et al., 2012b). The temporal relationship between inflammation, oxidative stress and mitochondrial function hence deserves further investigation. There is also a need to investigate the main source of ROS arising in skeletal muscle during IR and, in particular, the specific extent of mitochondrial, xantine and NAPDH oxidase involvement. Further studies with regard to molecular pathways (SAFE, RISK, etc.) ultimately acting on the mPTP are also warranted in order to determine whether these endogenous protective pathways are impaired by IR.

Finally, defining the threshold level of ROS that will induce mitohormesis rather than oxidative stress is of key importance. This represents an obvious challenge knowing that such a threshold may vary according to the metabolic phenotype of the organ involved in IR and to comorbidity factors (hypertension, diabetes, old age, etc.) often present in patients suffering from PAD.

In summary, mitochondrial dysfunction resulting from- and enhancing ROS production is clearly a key mechanism involved in the deleterious effects of IR on skeletal muscles. While accessible to therapy, further knowledge is nonetheless needed to allow a broader translation of ischemic conditioning into clinical practice.

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A New Murine Model of Sustainable and Durable Chronic Critical Limb Ischemia Fairly Mimicking Human Pathology

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WHAT THIS PAPER ADDS

Critical limb ischemia (CLI) is frequent and associated with a very poor local and general prognosis. An experimental model fulfilling all CLI characteristics is lacking. This is the first study to analyze not only clinical and perfusion parameters, but also cellular and molecular mechanisms involved in CLI up to 30 days postoperatively. Clinical and scintigraphic scores confirmed CLI, together with impaired mitochondrial respiration, calcium retention capacity, and biogenesis. Ischemic muscles also demonstrated increased production of reactive oxygen species, decreased antioxidant enzymes, and myopathic features. Sequential femoral and iliac arteries ligations closely mimic human functional, clinical, scintigraphic, and skeletal muscle mitochondrial impairments, and could allow testing of therapeutic approaches needed in view of the gravity of CLI in humans.

Objective: To establish a chronic mouse model of critical limb ischemia (CLI) with *in vivo* and *ex vivo* validation, closely mimicking human pathology.
Methods: Swiss mice ($n = 28$) were submitted to sequential unilateral femoral (day 0) and iliac (day 4) ligatures. Ischemia was confirmed by clinical scores (tissue and functional damages) and methoxyisobutylisonitrile (MIBI) scintigraphies at days 0, 4, 6, 10, 20, and 30. At days 10, 20, and 30, muscle mitochondrial respiration, calcium retention capacity (CRC), and production of reactive oxygen species (ROS) were investigated, together with transcripts of mitochondrial biogenesis and antioxidant enzymes. Histological analysis was also performed.
Results: Clinical and functional damage confirmed CLI. MIBI scintigraphies showed hypoperfusion of the ischemic limb, which remained stable until day 30. Mitochondrial respiration was impaired in ischemic muscles compared with controls ($V_{max} = 7.93 \pm 0.99$ vs. 10.09 ± 2.87 mmol/L O2/minute/mg dry weight [dw]; $p = .01$), together with impaired CRC (7.4 ± 1.6 mmol/L minute/mg dw vs. 11.9 ± 0.9 mmol/L minute/mg dw; $p < .001$) and biogenesis (41% decrease in peroxisome proliferator-activated receptor gamma coactivator [PGC]-1α, 49% decrease in PGC-1β, and 41% decrease in nuclear respiratory factor-1). Ischemic muscles also demonstrated increased production of ROS under electron paramagnetic resonance (0.084 ± 0.029 vs. 0.051 ± 0.031 mmol/L minute/mg dw; $p = .03$) and with dihydroethidium staining (3622 ± 604 arbitrary units of fluorescence vs. 1224 ± 324; $p < .01$), decreased antioxidant enzymes (32% decrease in superoxide dismutase [SOD]1, 41% decrease in SOD2, and 49% decrease in catalase), and myopathic features (wider range in fiber size, rounded shape, centrally located nuclei, and smaller cross-sectional areas). All defects were stable over time.
Conclusion: Sequential femoral and iliac ligatures closely mimic human functional, clinical, scintigraphic, and skeletal muscle mitochondrial characteristics, and could prove useful for testing therapeutic approaches.

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INTRODUCTION

Critical limb ischemia (CLI) defines an advanced stage of chronic arterial insufficiency describing patients with typical ischemic rest pain, or patients with ischemic skin lesions, either ulcers or gangrene.[1–4] The term CLI should only be

used in relation to patients with chronic ischemic disease, defined as the presence of symptoms for >2 weeks.[4] Diagnosis of CLI should also only be made in patients with symptoms attributable to objectively proven arterial occlusive disease, and verified by ankle or toe pressure. The diagnosis of CLI is thus a clinical diagnosis of chronic arterial disease, but, inevitably, has to be supported by objective tests, and must have a duration of >2 weeks.[4] However, despite recent advances in surgical and endovascular techniques, a large number of patients with CLI is not eligible for these revascularization procedures because of the anatomic location of the lesions, the extent of the disease, or extensive comorbidity, largely supporting the need for adequate experimental models allowing the testing of new therapeutic approaches.[1,4,5]

Understanding of the pathophysiology of lower-limb ischemia–reperfusion has been greatly improved with both skeletal muscle dysfunction and increased oxidative stress appearing as major components.[6–10] All of these alterations should be observed in relevant experimental models of CLI. Furthermore, notwithstanding that ischemia induces neoangiogenesis and formation of collateral vessels—showing that even after femoral artery excision the perfusion of the limb returns to normal within a few days—stability of the lesions over time should be obtained.[11] Models already exist, but result in progressive blood flow restoration, with complete restoration 7 days after surgery. Further, these models have not always been fully characterized on a clinical, functional, and pathophysiological basis, particularly when considering oxidative stress and mitochondrial functions that are now considered as key factors in CLI, including in human pathology.[12–14]

The main objective of the present study was to develop and comprehensively characterize, both *in vivo* and *ex vivo*, a chronic, stable, and long-lasting established CLI experimental model, closely mimicking human functional, clinical, and skeletal muscle mitochondrial pathology. Indeed, such a durable model is currently lacking and may prove useful for testing therapeutic approaches.

MATERIALS AND METHODS

Animals

Male Swiss mice ($n = 28$) weighing 30–35 g were handled according to French laws for animal use and care, and in accordance with the guidelines of the European Community Council.

CLI model

Surgery was performed under general anesthesia. Induction was conducted in an airtight ventilated chamber with a mixture of 3% isoflurane (Aerrane; Baxter Healthcare, Maurepas, France) and air. Maintenance of anesthesia was ensured by spontaneous ventilation through a mask delivering a mixture of 2% isoflurane and air.

Ligation of the right femoral artery was performed midway between the superficial epigastric artery and the bifurcation of the popliteal and saphenous arteries under microscope. Three collateral vessels were also ligatured. Four days later, ligation of the right common iliac artery 0.5 cm distal to its origin, after visualization of the origin of internal iliac artery, was performed by laparotomy. In this unilateral ischemia model, the contralateral limb can be considered as a control.[15] The duration of ischemia was counted from the first operation onwards.

Animals were divided into three groups: one group to be sacrificed 10 days after surgery (group 1; $n = 10$); one group to be sacrificed 20 days after surgery (group 2; $n = 8$), and one group to be sacrificed 30 days after surgery (group 3; $n = 10$) in order to study *in vivo* perfusion, as well as *ex vivo* muscle damage over time. Of these 28 mice, one from group 1 died at day 1 and one from group 3 died at day 4. Nine mice were then allocated to *ex vivo* tissue harvest at day 10, eight at day 20, and nine at day 30.

Chosen outcomes

In order to mimic as closely as possible human pathology and according to Trans-Atlantic InterSociety Consensus (TASC II) guidelines, *in vivo* chosen outcomes were functional signs objectified by clinical scores—scanographic arguments proving the hypoperfusion of the ischemic limb, for >2 weeks. *Ex vivo* outcomes were alteration of mitochondrial function, by decreased mitochondrial respiratory chain complex activities and calcium retention capacity (CRC), increased production of reactive oxygen species (ROS), and alteration of biogenesis and antioxidant system.

In vivo *follow-up of clinical and functional damages and of limb perfusion*

Using already established clinical scores, clinical tissue damage was graded as follows: normal or white aspect of the limb, toe cyanosis or necrosis, and spontaneous amputation of a toe (attributed 1, 2, 3, 4, or 5 points, respectively).[16]

Functional damage was graded as follows: normal function of the limb, plantar flexion without toe flexion, no plantar flexion, and dragging the limb (attributed 0, 1, 2 or 3 points, respectively).[16] Clinical scores were assessed at days 0, 4, 6, 10 ($n = 26$), 20 ($n = 17$), and 30 ($n = 9$).

Tissue perfusion was assessed with gamma camera scans (Gaede MedizinSysteme GmbH, Freiburg im Breisgau, Germany) under general anesthesia, the animals being placed in a dedicated heating cell (Minerva, Esternay, France). Scans lasting 15 minutes were performed 30 minutes after injection of the tracer (methoxyisobutylisonitrile [MIBI]) at the tail vein, at day 0 (before femoral ligation), day 4 (before iliac ligation), and at days 6, 10 ($n = 26$), 20 ($n = 17$), and 30 ($n = 9$).

Ex vivo *muscle analysis*

Nine mice were allocated to *ex vivo* tissue harvest at day 10, eight at day 20, and nine mice at day 30. Ischemic and contralateral gastrocnemius tibialis muscles were collected. Gastrocnemius muscles were harvested and immediately

used for measurement of mitochondrial respiration, CRC, and production of free radicals. Tibialis muscles were frozen in order to perform histological sections and transcripts analyses.

Mitochondrial respiratory chain complex activities. The mitochondrial respiratory chain is made of four complexes generating electron transfers in order to produce energy. This activity requires oxygen. The study of mitochondrial respiratory chain complex activities is a technique based on the measurement of oxygen consumption in skinned fibers in order to determine the functional oxidative capacity of the skeletal muscle in its cellular environment. Oxygen concentration is measured with a Clark electrode, and substrates are then added in order to activate or inhibit the different complexes of the respiratory chain. Adenosine diphosphate (2 mM) was added in order to study complex I, III, and IV activity. Succinate (25 mM) was then added to study complex I, II, III, and IV activity, which determines the maximal oxidative capacity (V_{max}). The addition of amytal (0.02 mM) subsequently inhibited complex I, allowing determining vamytal (complex II, III, and IV activities). The addition of N, N, N′, N′-tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.5 mM) and ascorbate (0.5 mM) specifically activated complex IV (V_{tmpd}).

CRC. The CRC of gastrocnemius muscle fibers measured by spectrofluorometry is the amount of calcium required to enable the opening of the mitochondrial transition pore, leading to apoptosis.[17] Calcium pulses (20 mmol/L) were applied to the skinned gastrocnemius muscle fibers until calcium release. The number of calcium additions needed to trigger mitochondrial permeability transition provided the CRC.

Production of ROS using electron paramagnetic resonance. Gastrocnemius muscles (1-mm^3 fragments) were incubated with a 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine HCl (CMH) molecular probe, which is oxidized in the presence of unpaired electrons of ROS. The amount of oxidized CMH, and thus the amount of free radicals produced, was measured by the intensity of the resonance signal.

Histological analysis: muscle structure and ROS production. Tibialis muscles were immersed in liquid nitrogen and stored at −80 °C. Muscles were then embedded in paraffin, and 10-mm-thick sections were prepared using a cryostat microtome, and mounted onto glass slides. Two types of analyses were subsequently performed: images of slide specimens stained with hematoxylin and eosin were acquired under bright-field microscopy, while other slides were incubated with 2.5 mM dihydroethidium (DHE). DHE produces red fluorescence when oxidized to ethidium bromide, mainly by superoxide anion. After staining, sections were examined under epifluorescence microscope (Nikon Eclipse E800; Nikon, Tokyo, Japan) and emission signals recorded.

Protein transcripts encoding for mitochondrial biogenesis and antioxidant defense. The main protein-encoding transcripts involved in mitochondrial biogenesis (peroxisome proliferator-activated receptor gamma coactivator [PGC]-1α, PGC-1β, and nuclear respiratory factor [NRF]1) and antioxidative enzymes (superoxide dismutase [SOD]1, SOD2, catalase) were analyzed. RNA (2 µg), isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), was converted to cDNA with SuperScript II reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA) and hexamer primers according to the manufacturer's instructions. Quantitative reverse transcription polymerase chain reaction was performed using the QuantiTectTM SYBR Green PCR kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Results are expressed as mean ± SD. Comparison were assessed using the nonparametric Mann—Whitney test. p-Values <0.05 were considered as indicative of statistical significance.

RESULTS

Follow-up of clinical and functional parameters and of limb perfusion

Clinical and functional damages. Clinical examination showed tissue damage: toe necrosis in 22 (85%) mice and cyanosis in 4 (15%) at day 10; toe necrosis in 15 (88%) mice and cyanosis in 2 (12%) at day 20; and autoamputation in 2 (22%) mice, toe necrosis in five (56%), and cyanosis in 2 (22%) at day 30. Clinical examination also showed functional damage: 20 (77%) mice dragged the ischemic limb and 6 (23%) mice could not achieve plantar flexion at day 10, day 20, or day 30 (Fig. 1A, B).

Limb perfusion. MIBI scans showed hypoperfusion of the ischemic limb, in comparison with control limb and such hypoperfusion remained stable until day 30 after surgery (Fig. 1C).

Ex vivo muscle analysis

Mitochondrial respiratory chain complex activities. Mitochondrial respiration was significantly impaired in ischemic muscles compared with control muscles at days 10, 20, and 30 after surgery. This was also true for all mitochondrial respiratory chain complexes.

At day 10 ($n = 9$), V_0 was 2.05 ± 1.01 versus 3.57 ± 1.02 mmol/L O_2/minute/g dry weight (dw) ($p < .01$) in ischemic and control legs, respectively. V_{max} was 6.97 ± 2.41 versus 10.29 ± 2.02 mmol O_2/minute/g dw ($p < .01$). Complex II, III, and IV activity (V_{amytal}) was 4.32 ± 1.84 versus 6.87 ± 1.46 mmol/L O_2/minute/g dw ($p < .01$). Finally, complex IV activity (V_{tmpd}) was 8.27 ± 2.47 versus 11.01 ± 2.60 mmol/L O_2/minute/g dw ($p < .01$) in ischemic and control legs, respectively.

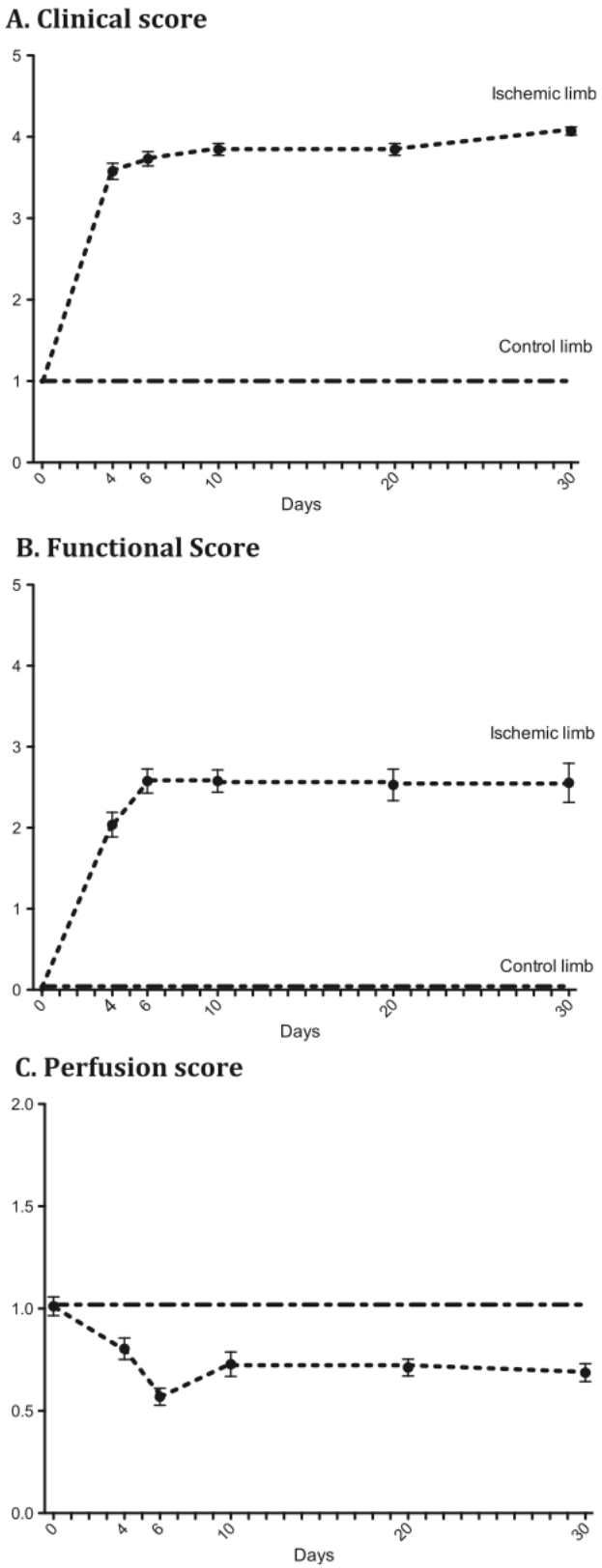


Figure 1. (A) Clinical, (B) functional and (C) perfusion scores are impaired after induction of ischemia. Day 10, $n = 26$; day 20; $n = 17$, day 30, $n = 9$. Mean ± SD at days 0, 4, 6, 10, 20, and 30.

At day 20, V_0 ($n = 8$) was 1.90 ± 0.70 versus 3.26 ± 0.41 mmol/L O_2/minute/g dw ($p < .01$); V_{max} was 7.30 ± 1.59 versus 9.86 ± 1.44 mmol/L O_2/minute/g dw ($p < .01$); V_{amytal} was 4.64 ± 1.23 versus 6.03 ± 1.05 mm/L O2/min/g dw ($p < .01$) and V_{tmpd} was 8.07 ± 1.25 versus 10.25 ± 0.93 mmol/L O_2/minute/g dw ($p < .01$) in ischemic and control legs, respectively.

At day 30 ($n = 9$), V_0 was 2.33 ± 0.70 versus 3.34 ± 0.84 mmol/L O_2/minute/g dw ($p = .01$) in ischemic and control legs, respectively. V_{max} was 7.93 ± 0.99 versus 10.09 ± 2.87 mmol O_2/minute/g dw ($p = .01$). Complex II, III, and IV activity (V_{amytal}) was 4.99 ± 0.58 versus 6.45 ± 2.39 mmol/L O_2/minute/g dw ($p = .06$). Finally, complex IV activity (V_{tmpd}) was 8.68 ± 1.18 versus 10.36 ± 2.02 mmol/L O_2/min/g dw ($p = .04$) in ischemic and control legs, respectively (Fig. 2A).

CRC. CRC was also impaired in ischemic muscles compared with control muscles: 7.3 ± 1.2 versus 10.6 ± 1.2 µM/mg dw for the nine mice sacrificed at day 10 ($p < .01$).

At day 20 ($n = 8$), CRC was 20 6.8 ± 0.9 mmol/L mg dw versus 11.4 ± 0.7 mmol/L mg dw ($p < .01$).

At day 30 ($n = 9$), CRC was 7.4 ± 1.6 mmol/L mg dw versus 11.9 ± 0.9 mmol/L mg dw ($p < .01$) in ischemic and control legs, respectively (Fig. 2B).

Production of ROS using electron paramagnetic resonance and DHE staining. Production of free radicals was increased in ischemic gastrocnemius compared with control muscles: 0.089 ± 0.033 versus 0.080 ± 0.022 mmol/L min/mg dw for the nine mice sacrificed at day 10 ($p = .46$); 0.102 ± 0.024 versus 0.083 ± 0.034 mmol/L min/mg dw for the eight mice sacrificed at day 20 ($p = .01$); and 0.084 ± 0.029 versus 0.051 ± 0.031 mmol/L min/mg dw for the nine mice sacrificed at day 30 ($p = .03$). Fluorescence after DHE staining was also higher in ischemic tibialis fibers: 3622 ± 604 arbitrary units of fluorescence (AUFs) versus 1224 ± 324 AUFs ($p < .01$) (Fig. 3A, B).

Histological analysis: muscle structure. Chronically ischemic tibialis muscle exhibited myopathic features, as established by hemotoxylin and eosin coloration with a wider range in fiber size, a more rounded shape, centrally located nuclei, and smaller cross-sectional areas than control fibers (Fig. 3C).

Stability of the model over time. As shown in Fig. 1, clinical, functional, and scintigraphic parameters were similar in the control leg at days 10, 20, and 30 after surgery. Importantly, impairments in ischemic legs were also stable during the study follow-up.

Mitochondrial respiration, CRC, and production of free radicals showed a similar evolution, demonstrating the stability of the model (Fig. S1; see Supplementary Information).

Transcripts encoding proteins involved in mitochondrial biogenesis and antioxidant defense. RNA analysis revealed a decrease in biogenesis, with a decrease in PGC-1α of 63% ($p = .01$), 44% ($p = .01$), and 41% ($p = .04$) at days 10, 20, and 30, respectively; a decrease in PGC-1β of 60% ($p = .01$), 36%

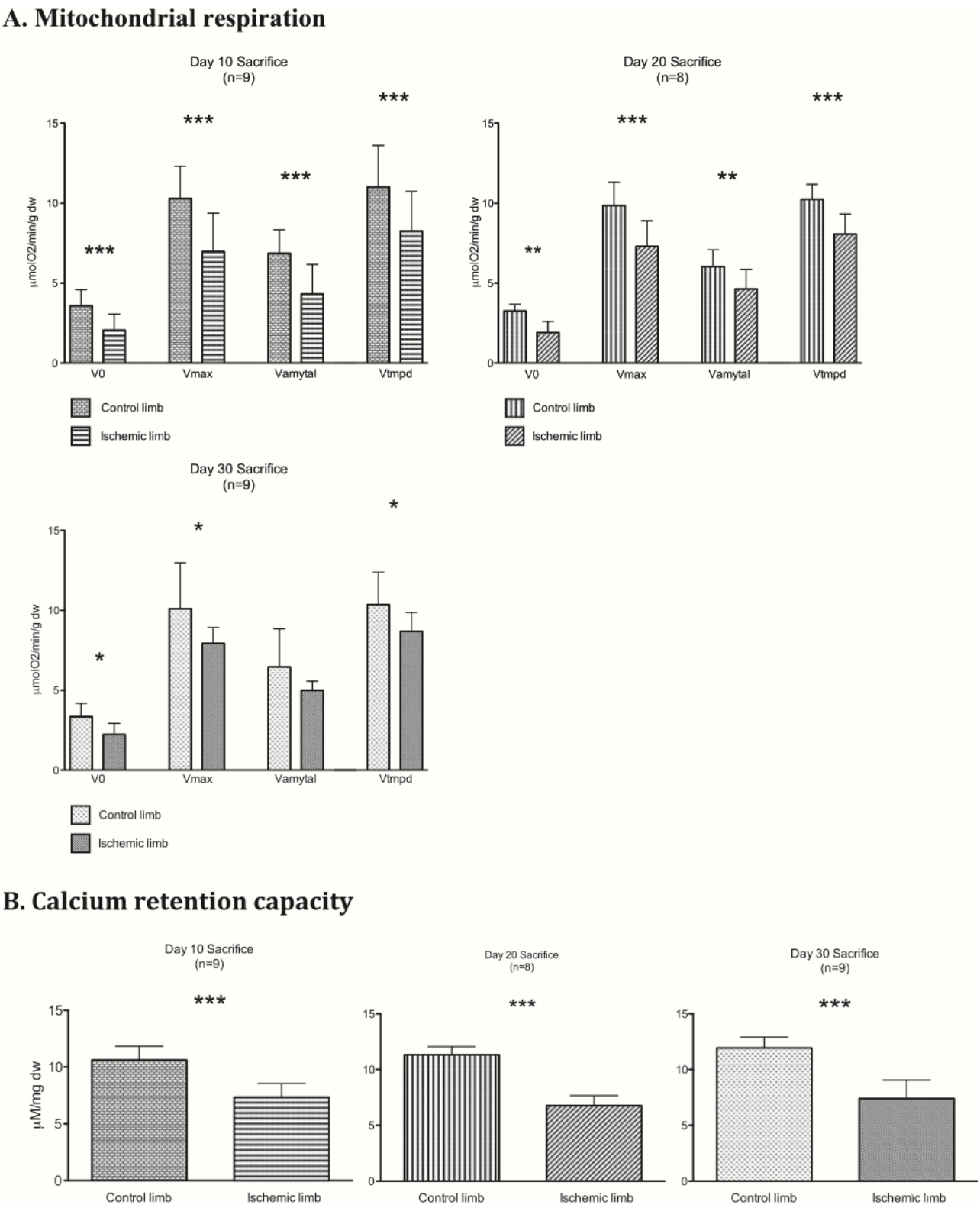
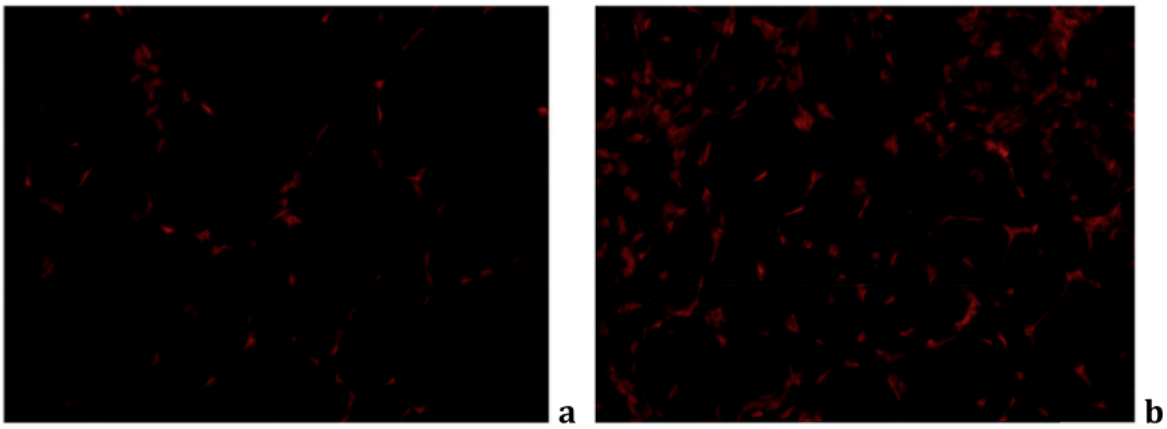


Figure 2. (A) Mitochondrial respiration and (B) calcium retention capacity are decreased after induction of ischemia. *Note.* V_0 = basal mitochondrial oxydative capacity; V_{max} = maximal mitochondrial oxidative capacity; V_{amytal} = complex II, III, and IV activity; V_{tmpd} = complex IV activity. *$p < .05$; **$p < .01$; ***$p < .001$.

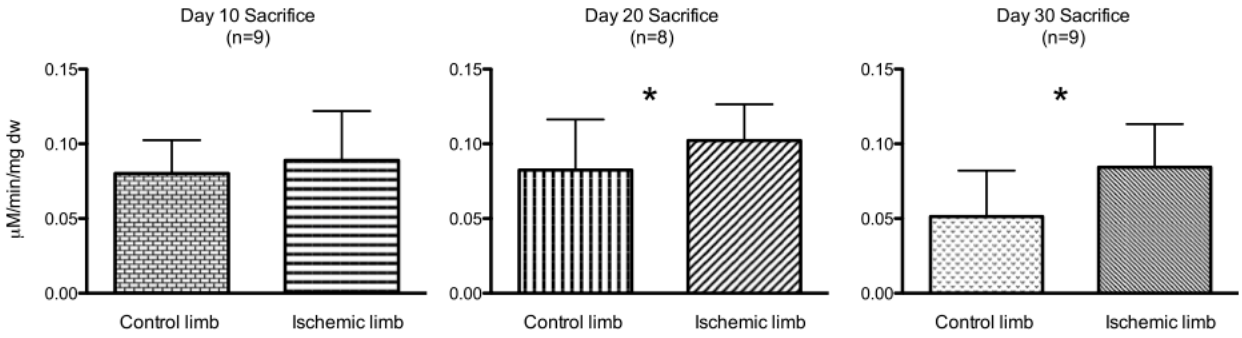
($p = .03$), and 49% ($p = .01$) at days 10, 20, and 30, respectively; and a decrease in NRF1 of 19% ($p = .04$), 35% ($p = .02$) and −41% ($p = .02$) at days 10, 20, and 30, respectively.

The antioxidant system was impaired at days 10, 20, and 30: SOD1 was decreased by 39% ($p = .01$), 44% ($p = .01$), and 32% ($p = .04$), respectively; SOD2 by 59% ($p = .01$), 38% ($p = .04$), and 41% ($p = .02$), respectively; and catalase by 60% ($p = .01$), 36% ($p = .04$), and 49% ($p = .03$), respectively (Fig. S2; see Supplementary Information).

A. Dihydroethidium stain (**a** Control limb, **b** Ischemic limb)



B. Production of free radicals



C. Hematoxylin-eosin stain (**a** Control limb, **b** Ischemic limb, x40)

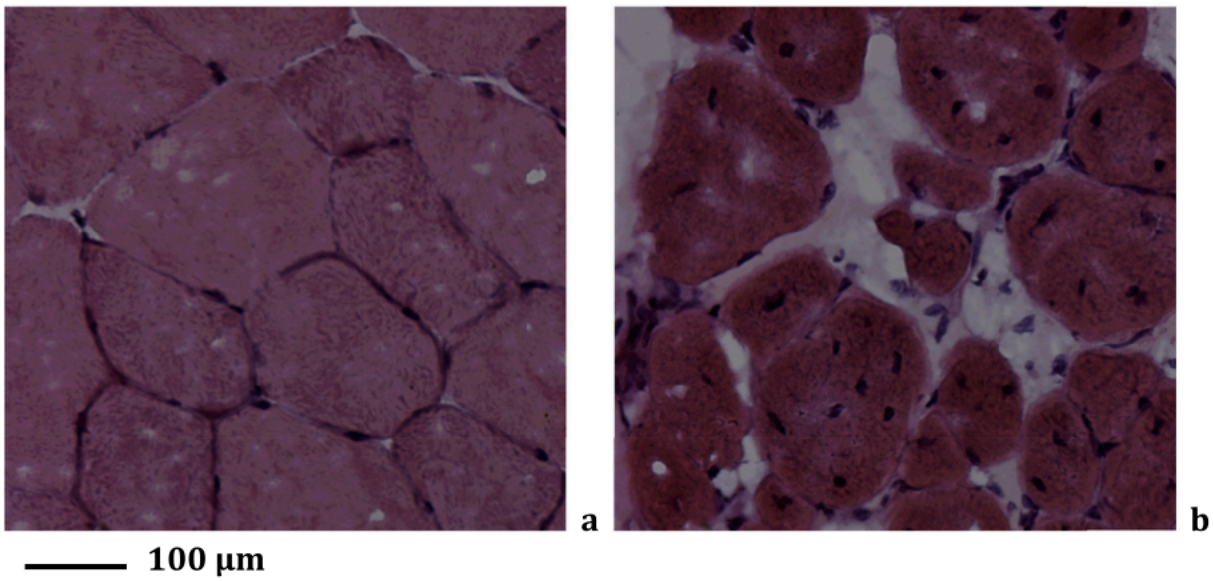


Figure 3. Oxidative stress is increased after induction of ischemia and chronic limb ischemia leads to myopathic features. (A) Dihydroethidium stain. (B) Production of free radicals. (C) Hematoxylin and eosin stain.

DISCUSSION

A sustainable and stable-over-time model of CLI, according to TASC II guidelines, has been developed with clinical signs, confirmation of hypoperfusion by objective measures, lasting for more than 2 weeks, and using sequential artery ligations. Models already used in order to induce ischemia

are based on a single arterial ligation. In rodents, ligation of the femoral artery just distal to the origin of the profunda femoris is most commonly used.[11–14,18,19] However, this method leaves most of the collateral circulation to the lower limb intact and, consequently, blood flow to the limb is fully restored within 7 days.[19] Another method consists of total excision of the femoral artery, removing the collateral bed. However, blood flow is restored progressively, with a third of the original blood flow restored only 7 days after excision of the femoral artery. In fact, collateral vessels in the mice arise mostly from the internal iliac artery.[20] By performing sequential ligations in the CLI model presented herein, a sustainable ischemia was obtained, likely because the second ligation performed on the common iliac artery reduces the collateral perfusion provided by the internal iliac artery.

Herein, a new CLI model is proposed, which fulfills all criteria defining CLI. In order to diagnose CLI, current recommendations require that both clinical signs and symptoms are present for >2 weeks along with objective measurements of arterial perfusion.[4] Using already-established clinical scores and MIBI scintigraphies, it has been demonstrated that the present model fully complies with these recommendations. While clinical scores are not routinely used when characterizing animal models, they can, nevertheless, be easily transposed as animals also present with several degrees of lower limb impotency and, moreover, the observed tissue lesions follow the same pattern as in humans, ranging from a cyanotic aspect of the limb to necrosis. Also, rather than using invasive arteriography, scintigraphy was preferentially used, as it has already been proven to be a good approach for cardiac or skeletal muscle perfusion studies.[21–23] Indeed, such a noninvasive technique enables potential perfusion changes to be followed over time. Furthermore, Tc-99m MIBI scintigraphy can detect hypoperfusion earlier than with laser Doppler imaging, as the uptake of this radiopharmaceutical compound is dependent on the distribution of regional blood flow, an early indicator of hypoperfusion.[22] Currently, MIBI scintigraphies are under consideration for the study of peripheral arterial disease (PAD).[23] Finally, in the present study, particular care was taken to follow the animals until 30 days after the first surgical ligation, thus allowing assessment of the observed symptoms and hypoperfusion for a period of >2 weeks.[4]

The pathophysiology of lower limb ischemia–reperfusion has been largely improved in recent years and, as a result, key mechanisms, such as muscle mitochondrial function and oxidative stress, were further investigated. Given that mitochondrial dysfunction and increased oxidative stress are largely involved in acute lower limb ischemia, whether these elements are also key factors in CLI was investigated.[24–26] Accordingly, in our CLI model, ischemic murine muscles displayed impaired mitochondrial respiration involving all mitochondrial respiratory chain complexes. Furthermore, early Ca^{++} release in the ischemic muscle was also observed herein. These data support that chronically ischemic muscles are prompt to apoptose. Finally, mitochondrial dysfunctions were associated with a reduced mitochondrial biogenesis, which also probably participated in the chronic alteration of ischemic muscles. Mitochondria may potentially be both actors and targets in chronic ischemia–reperfusion-related injuries as they both enhance and are impaired by ROS production. A reduction in antioxidant capacities was also observed, as mRNA encoding SOD1 and SOD2, and catalase, the main enzymes involved in ROS detoxification, were significantly decreased in ischemic muscles. Pipinos et al.[8] observed that ischemic human muscles presented mitochondrial alterations and exhibited myopathic features, with ischemic myofibers demonstrating a broader range in terms of size, a more rounded shape, centrally located nuclei, and smaller cross-sectional area than control fibers. In the CLI model presented herein, mitochondrial alterations and myopathic features of ischemic myofibers also mimic human pathology.

However, despite its wide similitude with human pathology, this model has limitations. First, human CLI generally occurs on already pathological arteries linked to cardiovascular risk factors and comorbidities associated to PAD. Thus, CLI is a result of a chronic process associated with the build-up of atherosclerotic plaque over many years leading to arterial stenosis. Here, sequential ligations are performed on healthy arteries. Further, CLI pathogenesis in humans develops gradually at multiple and different locations (iliac arteries, superficial femoral arteries). These obliterations are progressive, and not acute, as were the ligations done in the model. However, as in PAD, sequential ligations likely allowed neoangiogenesis and collaterality, leading mainly to hypoperfusion rather than to complete acute ischemia of the limb. Thus, sequential ligations fairly mimic the progressivity of PAD, until a threshold of hypoperfusion causing CLI.[18]

In conclusion, the present study shows that sequential artery ligations lead to a valid CLI model based on both *in vivo* (clinical, functional and perfusion scores) and *ex vivo* (mitochondrial respiration, biogenesis, CRC and oxidative stress) impaired functions. Although needing to be tested in other mice strands demonstrating, for instance, cardiovascular risk factors, this experimental model would likely help to better investigate efficient therapeutic strategies to be later proposed in patients with CLI.

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CONFLICTS OF INTEREST

None.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejvs.2014.12.010.

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N-Acetyl Cysteine Restores Limb Function, Improves Mitochondrial Respiration, and Reduces Oxidative Stress in a Murine Model of Critical Limb Ischaemia

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WHAT THIS PAPER ADDS

This study shows that targeting inhibition of oxidative stress confers muscle protection in a murine model of critical limb ischaemia. In this study, *N*-acetyl cysteine allowed muscle protection and might be considered as potential adjunctive therapy to revascularisation procedures.

Objective/background: The aim of this study was to investigate whether antioxidant therapy might decrease oxidative stress related deleterious effects in the setting of critical limb ischaemia (CLI).
Methods: Twenty Swiss mice were submitted to sequential right femoral and iliac ligatures; the left limb served as control. The mice were assigned to two groups: in the first group (no-treatment group, $n = 10$) no treatment was administered; in the second group (*N*-acetyl cysteine [NAC] group, $n = 10$) NAC was administered by dissolution in drinking water for 4 weeks, starting on day 7, when CLI was effective. Clinical and functional scores were assessed by two blinded investigators. Mice were killed on day 40 and mitochondrial respiratory chain complex activities, calcium retention capacity, oxidative stress, and histological analysis were analysed.
Results: Ischaemic muscles in the no-treatment group showed significantly impaired mitochondrial respiration and calcium retention capacity, with increased production of reactive oxygen species; but no statistical difference was noticed when comparing ischaemic muscles in the NAC group ($n = 10$) to contralateral muscles ($n = 10$) and to control muscles in the no-treatment group ($n = 10$). Ischaemic muscles in the no-treatment group exhibited myopathic features such as wider range in fibre size, rounded shape, centrally located nuclei, and smaller cross sectional areas, but none of these features were observed in contralateral muscles or in NAC-group muscles (ischaemic or controls).
Conclusion: Targeting inhibition of oxidative stress may be a potential therapeutic strategy for muscle protection in CLI and might be considered as potential adjunctive therapy to revascularisation procedures.

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INTRODUCTION

In a previous study, a stable and long lasting critical limb ischaemia (CLI) murine model was developed, closely mimicking human clinical and functional pathology.[1] This allowed assessment of the key role of mitochondrial dysfunction in CLI, characterised mainly by impaired mitochondrial respiratory chain complex activities and premature mitochondrial pore transition permeability opening, leading to apoptosis.[1] Additionally, increased oxidative stress secondary to reactive oxygen species (ROS) production and clearance imbalance appeared both to precede and be enhanced by mitochondrial dysfunction,[1] suggesting a therapeutic approach focused on ROS modulation.[2–8]

However, experimental data assessing the potential usefulness of modulating ROS using a pharmacological approach are still lacking in the setting of CLI. The main objective of the present study was therefore to investigate whether a pharmacological approach with antioxidants would allow decreasing oxidative stress and therefore CLI deleterious effects. Glutathione is currently one of the most

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studied antioxidants. However, supplementation with glutathione encountered little success as glutathione bioavailability is very poor, owing to the hydrolytic enzymes breaking down glutathione on ingestion.[9] Relative to glutathione availability, maintaining the availability of cysteine in the blood is mandatory, as it is the rate limiting substrate for glutathione resynthesis.[9,10] Among the most widely used agents to maintain the cysteine pool, *N*-acetylcysteine (NAC) is the most commonly used and is considered as a direct scavenger of free radicals, especially in mitochondria.[9,10] Thus, the aim was to investigate whether NAC supplementation could have beneficial effects in the setting of CLI mitochondriopathy.

As NAC has been shown to reduce ROS overproduction and reduce ischaemia reperfusion injury,[11–16] it was hypothesised that NAC treatment could restore skeletal muscle function in the setting of chronic ischaemic situations, such as CLI.

MATERIALS AND METHODS

Animals

Twenty Swiss male mice weighing 30–35 g were handled in accordance with the principles of French laws for animal use and care. Research was approved by the ethical committee (reference number AL/70/77/02/13).

CLI was induced in all animals in the right limb, according to an established model, whereas the left limb served as the control.[1] It has previously been shown that the animal can be considered as its own control.[17] Surgery was performed by the same surgeon for all animals: ligation of the right femoral artery was performed between the superficial epigastric artery and the bifurcation of the popliteal and saphenous arteries under microscope and the first three collateral vessels were also ligatured. Four days later, ligation of the right common iliac artery 0.5 cm from its origin, after visualisation of the origin of the internal iliac artery, was performed by laparotomy.[1] It has previously been shown that ischaemia is effective from day 6 and is sustainable.[1] As a consequence, at day 6, mice were assigned to two groups by a single animal technician: in the first group (no-treatment group, $n = 10$) no treatment was administered; in the second group (NAC group, $n = 10$) NAC treatment was administered by dissolution in drinking water for 4 weeks, starting on day 7. The rest of the team did not know into which group mice were assigned. A dose of 1.5 g/kg/day NAC was chosen as it has previously been shown that this dose reduced oxidative stress and triggered mitochondrial biogenesis.[18] Daily, 1.5 mg buprenorphine was administered subcutaneously to all animals by an animal technician, until the animal was killed.

All animals were killed on day 40. Gastrocnemius and tibialis muscles were collected and then brought from the animal laboratory to the experimental laboratory. Samples were analysed by the experimental team, who were blinded to the experimental condition.

Clinical and functional scores

Clinical tissue damage was graded as follows: normal aspect of the limb, white aspect, toe cyanosis, necrosis, and spontaneous amputation of a toe were attributed 1, 2, 3, 4, or 5 points, respectively.[1,19] Functional damage was graded as follows: normal function of the limb, plantar flexion without toe flexion, no plantar flexion, and dragging the limb were attributed 0, 1, 2, or 3 points, respectively.[1,19] Clinical and functional scores were assessed daily by both an animal technician and a research student.

Mitochondrial respiratory chain complex activities

The mitochondrial respiratory chain is made of four complexes generating electron transfers in order to produce energy. This activity requires oxygen. The study of mitochondrial respiratory chain complex activities is a technique based on the measurement of oxygen consumption in skinned fibres in order to determine the functional oxidative capacity of the skeletal muscle in its cellular environment. Substrates are then added in order to activate or inhibit the different complexes of the respiratory chain. Adenosine diphosphate (2 mM) and succinate (25 mM) were added in order to activate all complexes and determine maximum oxidative capacity (Vmax). Addition of amytal (0.02 mM) inhibited complex I, determining Vamytal (complexes II, III, and IV activities). Addition of tetramethyl-p-phenylenediamine dihydrochloride (TMPD 0.5 mM) and ascorbate (0.5 mM) activated complex IV, determining Vtmpd.[1,2,20]

Calcium retention capacity

The calcium retention capacity (CRC) was measured by spectrofluorometry. It represents the amount of calcium required to enable the opening of the mitochondrial transition pore, leading to apoptosis. Calcium pulses were applied to skinned gastrocnemius muscle fibres, until calcium release. The number of calcium additions needed to trigger mitochondrial permeability transition pore opening provided the CRC.[1,2,20,21]

Production of ROS

Production of ROS was assessed with electron paramagnetic resonance. Gastrocnemius muscles were incubated with a 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) molecular probe, which is oxidised in the presence of unpaired electrons of ROS. The amount of oxidised CMH, and thus the production of free radicals, was measured through the intensity of the resonance signal.

Despite the great potential of electron paramagnetic resonance, the high reactivity and low steady state concentration of ROS generate high variations, which is why ROS production was also assessed by dihydroethidium staining.[21]

Tibialis muscles were immersed in liquid nitrogen and stored at −80 °C. Muscles were then embedded in paraffin, and sections were prepared using a cryostat microtome,

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and mounted onto glass slides. Slides were incubated with 2.5 mM dihydroethidium, which produces red fluorescence when oxidised to ethidium bromide, mainly by superoxide anion. After staining, sections were examined under epi-fluorescence microscope and signals were recorded.

Histological analysis

After immersion in liquid nitrogen, tibialis muscles were embedded in paraffin and cryostat sections were mounted onto glass slides. Slide specimens were stained with hematoxylin and eosin and acquired under bright field microscopy.

Data analysis

Power calculation was made by the statistician, on the basis that a minimum of eight mice were needed in order to obtain a statistically significant decrease of at least 20% for mitochondrial respiration, CRC and production of free radicals in an established chronic ischaemia protocol.[1] Consequently, 20 mice were ordered and assigned in both groups, in order to investigate whether NAC administration could restore mitochondrial parameters. Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Normal distribution of the variables was confirmed by Shapiro–Wilk test. Intra-individual comparison was assessed using the paired *t* test. Inter-individual comparison was assessed with a one way ANOVA. Results are expressed as median and interquartile range (IQR). *p* values $< .05$ were considered to be statistically significant.

RESULTS

Clinical and functional parameters

No animal died. No disagreement was noted concerning clinical and functional assessment between the animal technician and the research student. In the no-treatment group, ischaemic limbs showed auto-amputation of a toe in three mice and toe necrosis in seven mice at day 40. The tissue damage score increased progressively from day 4 to day 40 (Fig. 1A), the functional damage score also increased as nine mice dragged the limb and one could not achieve plantar flexion at day 40 (Fig. 1B).

In the NAC group, the ischaemic limb appeared white in seven mice and was normal in three mice; four mice could not achieve plantar flexion and six mice could achieve plantar but no toe flexion at day 40. Tissue damage and functional scores decreased after NAC administration (Fig. 1A, B).

Mitochondrial respiratory chain complex activities

Mitochondrial respiration was significantly impaired in ischaemic muscles ($n = 10$) in the no-treatment group compared with contralateral muscles ($n = 10$), as well as control muscles ($n = 10$) in the NAC group: V0 was 2.30 mmol O_2/min/g dry weight (dw); (IQR 0.28) vs. 3.58 mmol O_2/min/g dw (IQR 0.49) and 3.43 mmol O_2/min/g dw (IQR 0.24) ($p < .001$). The same applied to Vmax: Vmax was 7.13 mmol O_2/min/g dw (IQR 1.63) vs. 10.25 mmol O_2/min/g dw (IQR 0.98) and 9.77 mmol O_2/min/g dw (IQR 1.62) ($p < .001$). This also applied to Vamytal: Vamytal was 4.38 mmol O_2/min/g dw (IQR 1.07) vs. 6.63 mmol O_2/min/g dw (IQR 1.43) and 6.37 mmol O_2/min/g dw (IQR 1.11) ($p < .001$). Finally, this again applied to Vtmpd: Vtmpd was 7.97 mmol O_2/min/g dw (IQR 2.61) vs. 10.48 mmol O_2/min/g dw (IQR 0.73) and 9.99 mmol O_2/min/g dw (IQR 1.37) ($p < .001$) (Fig. 2).

No statistical difference was noted when comparing ischaemic NAC treated muscles ($n = 10$) with contralateral muscles ($n = 10$) or to untreated control muscles ($n = 10$): V0 was 3.15 mmol O_2/min/g dw (IQR 0.71) vs. 3.43 mmol O_2/min/g dw (IQR 0.24) and 0.58 mmol O_2/min/g dw (IQR 0.49); Vmax was 9.89 mmol O_2/min/g dw (IQR 2.37) vs. 9.77 mmol O_2/min/g dw (IQR 1.62) and 10.25 mmol O_2/min/g dw (IQR 0.98); Vamytal was 6.01 mmol O_2/min/g dw (IQR 1.36) vs. 6.37 mmol O_2/min/g dw (IQR 1.11) and 6.63 mmol O_2/min/g dw (IQR 1.43); and Vtmpd was 9.52 mmol O_2/min/g dw (IQR 1.1) vs. 9.99 mmol O_2/min/g dw (IQR 1.37) and 10.48 mmol O_2/min/g dw (IQR 0.73) (Fig. 2).

CRC

CRC was impaired in ischaemic muscles in the no-treatment group (7.40 mmol/mg dw; IQR 1.62; $n = 10$) vs. contralateral muscles (11.60 mmol/mg dw; IQR 1.83; $n = 10$), as well as control muscles in the NAC group (11.30 mmol/mg dw; IQR 1.57; $n = 10$) ($p < .001$) (Fig. 3).

No statistical difference was noted when comparing ischaemic NAC treated muscles ($n = 10$) with contralateral muscles ($n = 10$) or with untreated control muscles ($n = 10$): CRC was 11.05 mmol/mg dw (IQR 1.23) vs. 11.30 mmol/mg dw (IQR 1.57) and 11.60 mmol/mg dw; (IQR 1.83).

Production of ROS using electron paramagnetic resonance and dihydroethidium (DHE) staining

Production of ROS was increased in ischaemic muscles in the no-treatment group (0.104 mmol/min/mg dw; IQR 0.026; $n = 10$) vs. contralateral muscles (0.073 mmol/min/mg dw; IQR 0.043; $n = 10$), as well as control muscles in the NAC group (0.047 mmol/min/mg dw; IQR 0.049; $n = 10$) ($p < .001$) (Fig. 4A).

No statistical difference was noted when comparing ischaemic NAC treated muscles ($n = 10$) with contralateral muscles ($n = 10$) or with untreated control muscles ($n = 10$): CRC was 0.038 mmol/min/mg dw (IQR 0.03) vs. 0.047 mmol/min/mg dw (IQR 0.049) and 0.073 mmol/min/mg dw (IQR 0.043) (Fig. 4A).

Fluorescence after DHE staining was higher in ischaemic muscles in the no-treatment group (3143 $\pm$ 312 arbitrary units of fluorescence; $n = 4$) than in contralateral muscles (1034 $\pm$ 425 arbitrary units of fluorescence; $n = 4$), as well as control muscles in the NAC group (725 $\pm$ 112 arbitrary units of fluorescence; $n = 4$) ($p < .01$) (Fig. 4B).

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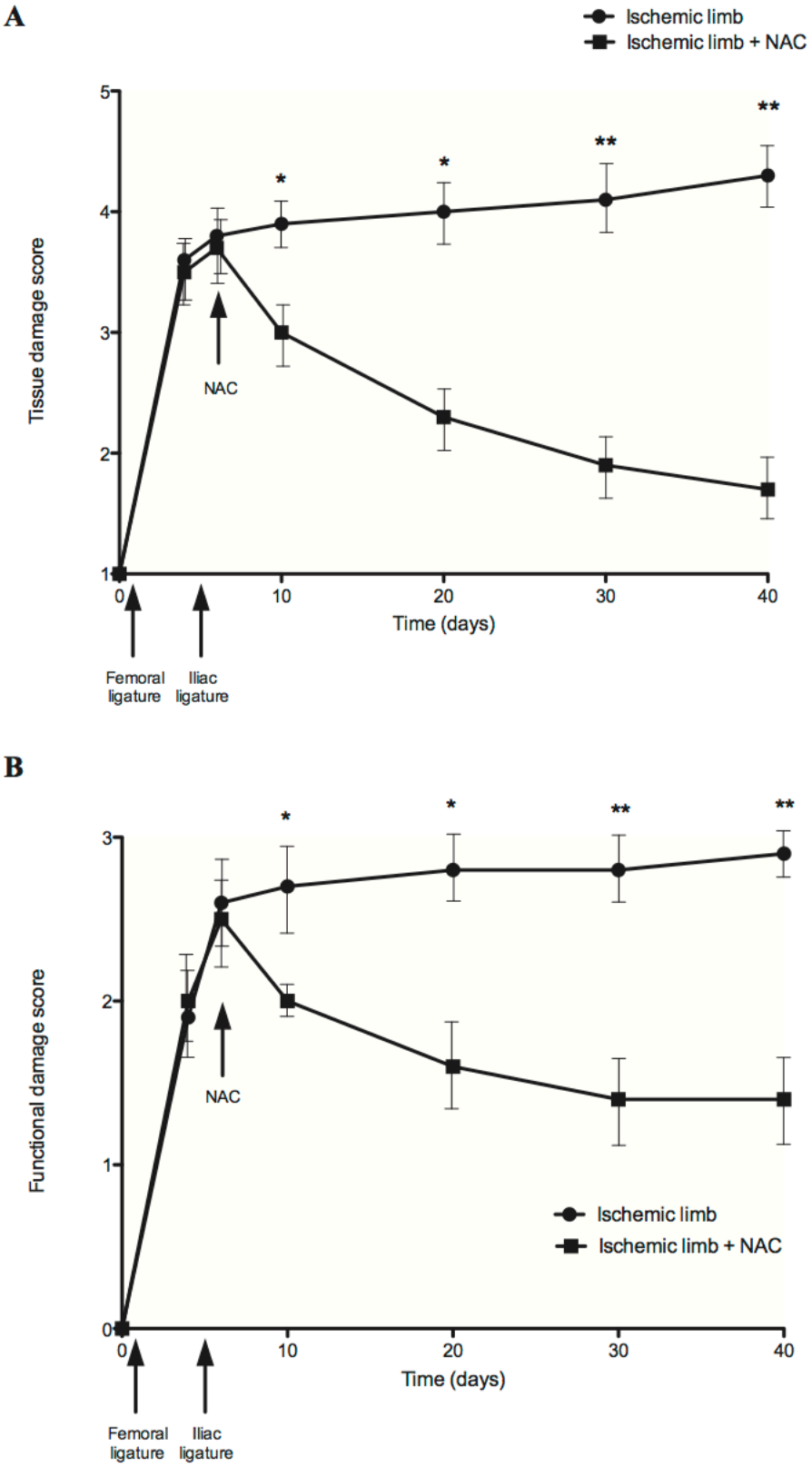

Figure 1. *N*-acetylcysteine (NAC) treatment has beneficial effects on tissue damage and functional scores. (A) Tissue damage and (B) functional scores were evaluated in the critical limb ischaemia (CLI) group ($n = 10$) and CLI—NAC group ($n = 10$). Results are expressed as mean ± SD at days 0, 4, 6, 10, 20, 30, and 40. *$p < .05$; **$p < .01$.

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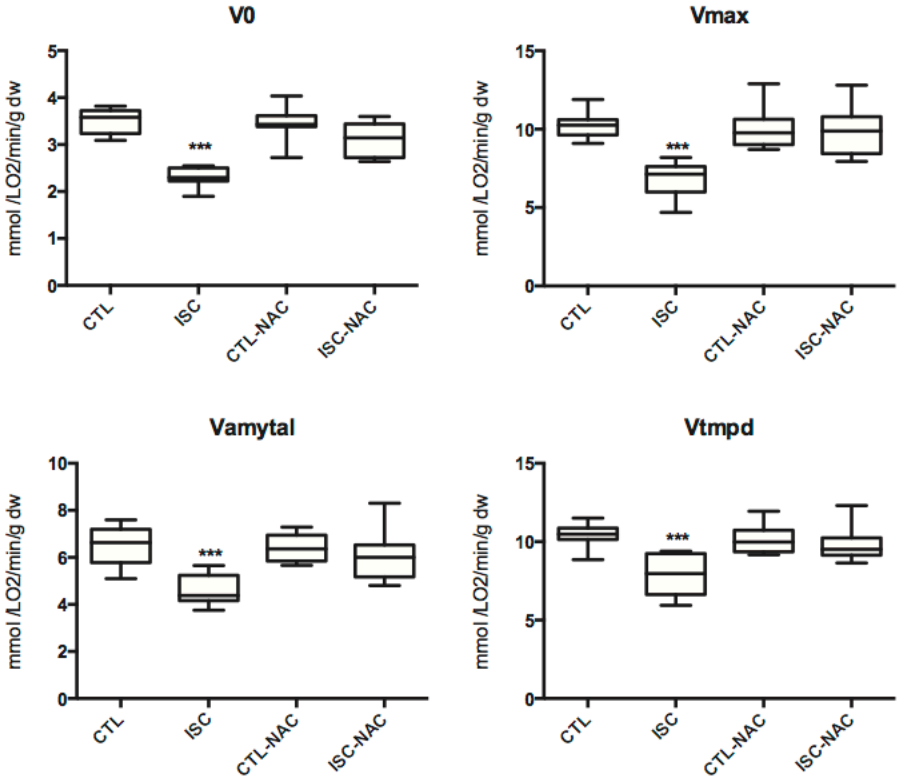

Figure 2. Mitochondrial respiration is restored in ischaemic muscles after *N*-acetylcysteine (NAC) treatment. Mitochondrial respiration was evaluated in the no-treatment group (control [CTL] limbs $n = 10$, ischaemic [ISC] limbs $n = 10$) and NAC treatment group (CLT—NAC limbs $n = 10$, ISC—NAC limbs $n = 10$). Box plot showing quantification of basal mitochondrial oxidative capacity (V0), maximal oxidative capacity (Vmax), complex II, III, and IV activity (Vamytal), and complex IV activity (Vtmpd). ***$p < .001$ (one way ANOVA).

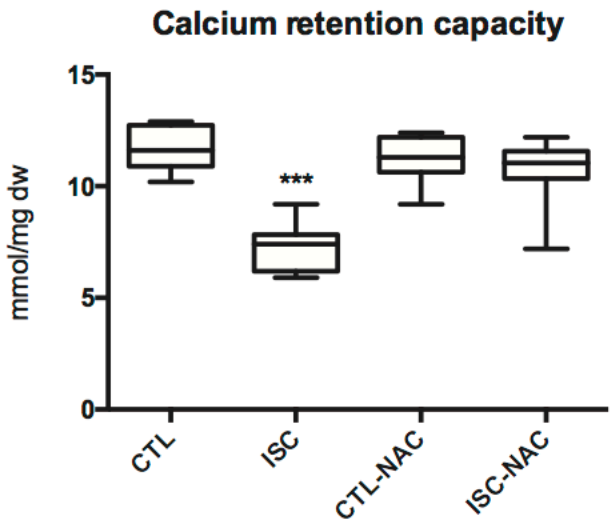

Figure 3. Calcium retention capacity is restored in ischaemic limbs after *N*-acetylcysteine (NAC) treatment. Calcium retention capacity was evaluated in the no-treatment group (control [CTL] limbs $n = 10$, ischaemic [ISC] limbs $n = 10$) and the NAC group (CTL—NAC limbs $n = 10$, ISC—NAC limbs $n = 10$). Box plot showing quantification of calcium retention capacity. ***$p < .001$ (one way ANOVA).

No statistical difference was noticed when comparing ischaemic NAC treated muscles ($n = 4$) with contralateral muscles ($n = 10$) or with untreated control muscles ($n = 10$): fluorescence was 826 ± 152 arbitrary units of fluorescence vs. 725 ± 112 arbitrary units of fluorescence and 1034 ± 425 arbitrary units of fluorescence (Fig. 4B).

Histological analysis: muscle structure

In the no-treatment group, chronically ischaemic tibialis muscle ($n = 3$) exhibited myopathic features, as established by hematoxylin-eosin coloration with a wider range in fibre size, a more rounded shape, centrally located nuclei, and smaller cross sectional areas than control fibres ($n = 3$). NAC treatment allowed recovery of muscle structure in ischaemic muscles ($n = 3$) (Fig. 5). As the number of samples was low for histological analysis, results were left at a descriptive stage.

DISCUSSION

The main finding of this study is that NAC treatment allowed functional improvement and recovery of mitochondrial function. Consequently, modulating oxidative stress with antioxidant therapy could be considered as an adjunctive therapy in ongoing CLI.

It has long been thought that reduced blood flow and impaired oxygen delivery were the only factors limiting function in patients with CLI. However, significant advances have been made and there is now clear evidence that metabolic defect in oxygen utilisation exists in ischaemic muscle.[21–23] In physiological conditions, skeletal muscles continually produce moderate levels of ROS, because of

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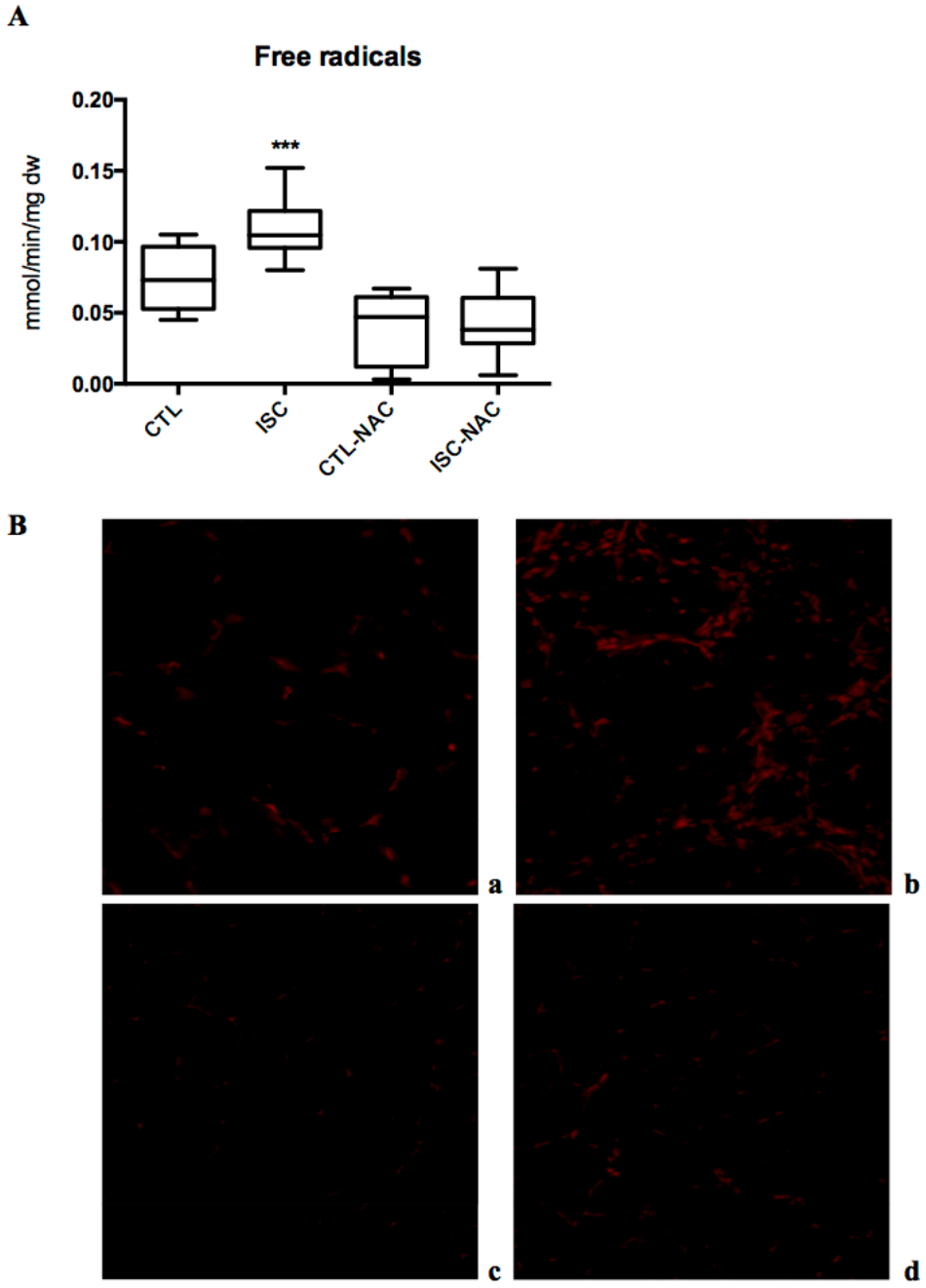

Figure 4. Oxidative stress is reduced after *N*-acetylcysteine (NAC) treatment. (A) Production of free radicals was evaluated in the no-treatment group (control [CTL] limbs $n = 10$, ischaemic [ISC] limbs $n = 10$) and NAC group (CTL—NAC limbs $n = 10$, ISC—NAC limbs $n = 10$). Box plot showing quantification of calcium retention capacity. ***$p < .001$ (one way ANOVA). (B) Fluorescence in dihydroethidium stain in the no-treatment group ([a] CTL limb, [b] ISC limb) and in the NAC group ([c] CTL—NAC limb, [d] ISC—NAC limb).

their contractile activity and their high oxygen consumption and metabolic rate. These ROS are buffered by multiple antioxidant systems in order to maintain redox homeostasis. Maintenance of muscle architecture, which is highly organised to optimise contraction and force generation, also requires basal energy and high amounts of oxygen. However, skeletal muscle also has the capacity to regenerate after injury by recapitulating the myogenesis process, which depends on the activation of muscle stem cells that can differentiate into myotubes and ultimately muscle fibres. In the setting of CLI, the imbalance between ROS production and the biological ability to readily detoxify the reactive intermediates or to repair the resulting damage results in oxidative stress, whereas impaired basal energy mediates a large number of structural and metabolic changes in skeletal muscle, resulting in reduced strength and function, and decreased capacity to regenerate.[2] Histopathological and imaging studies in patients with peripheral arterial disease have consistently documented a number of adverse adaptations in the leg muscles, such as atrophy, connective tissue proliferation, and higher muscle percent fat.[22–25] Moreover, it has been shown that the

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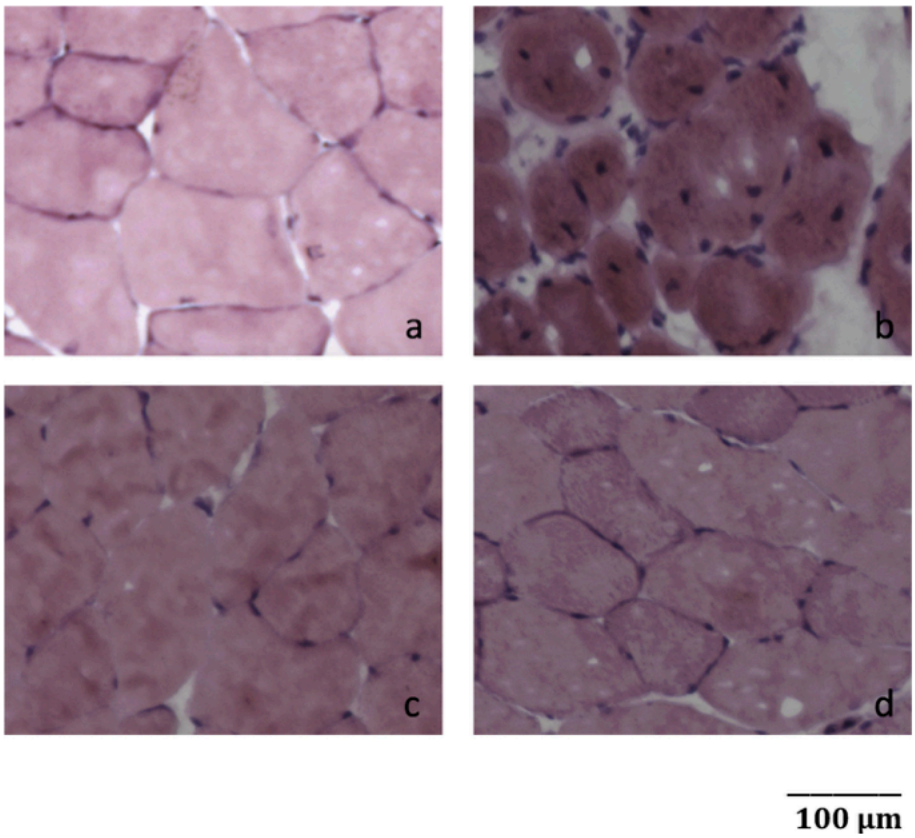

Figure 5. *N*-acetylcysteine (NAC) treatment allows the recovery of myopathic features. Tibialis muscle specimens (n = 3) were examined under bright field microscopy after hematoxylin and eosin (H&E) stain (×40). H&E staining showed myopathic features of ischaemic muscles in the no-treatment group ([a] control [CTL], [b] ischaemic [ISC] limb) but not in the NAC group ([c] CTL—NAC limb, [d] ISC—NAC limb).

pathophysiological changes in calf muscle predict mobility loss at two year follow up in patients with peripheral arterial disease.[23]

Several pharmacological approaches have been proposed to reduce mitochondrial and muscle dysfunction in the setting of ischaemia—reperfusion,[18,20,26] but in the present study, the key players in CLI pathophysiology appeared to be defective mitochondria associated with oxidative stress. CLI generates a vicious circle: skeletal muscles receive a decreased supply of nutrients and oxygen as a result of occluded arteries, but mitochondrial respiratory defect amplifies injury through the inefficient use of available nutrients and oxygen for adenosine triphosphate production, resulting in high oxidative stress.[2,3] In such a context, it appears mandatory to counteract these physio-pathological mechanisms using antioxidants in order to maintain an optimal redox state in the cell.

In this study, NAC treatment decreased oxidative stress and restored mitochondrial respiratory chain complex activities and CRC. Moreover, no difference was noticed between NAC treated ischaemic muscles and contralateral and even untreated control limbs. These results also appeared clinically significant with an improved functional score in treated ischaemic limbs, even if recovery was not complete. The duration of treatment may have been too short to obtain complete recovery. However, it has been shown that NAC treatment had meaningful effects on skeletal muscle in peripheral arterial insufficiency, as NAC supplementation could delay fatigue and/or improve exercise performance.[9,10]

Importantly, despite the increasing burden of CLI and its detrimental consequences for patients, there is to date, no specific pharmacological therapy for CLI skeletal muscle alterations, highlighting that identifying physio-pathological mechanisms involved in these alterations is important. Besides revascularisation and exercise, developing pharmacological strategies to prevent and treat muscle deterioration might be an approach, as redox balance is drug targetable.[27–29] The present results show that antioxidant treatment with NAC could attenuate mitochondrial dysfunction leading to CLI myopathy, supporting the idea that impaired arterial perfusion is not the only factor determining leg function in CLI. As a consequence, NAC could be considered as a potential adjunctive therapeutic option during revascularisation procedures. For example, when performing a femoropopliteal bypass for CLI due to superficial femoral artery occlusion, common femoral artery clamping is necessary, meaning that the patient will suffer from an additional level of ischaemia (over and above the pre-existing CLI) through profunda femoris artery clamping. This will be followed by reperfusion injury when the bypass is performed and clamps removed. Using NAC perfusion

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before reperfusion could modulate oxidative stress and decrease skeletal muscle injury in the setting of CLI. However, mode of administration, as well as dosage, in humans should be determined.

Although the present findings confirm that oxidative stress plays a central role in CLI and that targeting oxidative stress with a pharmacological approach can be an option in order to protect skeletal muscle, the study suffers from several limitations and it is complicated to extrapolate these results and put them into practice in humans in the current state. First, a critical aspect in the preclinical search for adjunctive novel therapy for CLI is that the animal model should exhibit physio-pathological features found in patients. Despite the wide similarity between the murine model and human pathology, cardiovascular risk factors and comorbidities are not present. Moreover, CLI in humans is the result of a chronic process associated with a progressive build up of atherosclerotic plaque, which develops gradually at multiple and different locations over many years, leading to arterial stenosis or occlusions. In the present model, sequential ligations were performed on healthy arteries and this could be considered an "acute" development of CLI, while CLI development is staged in patients. One might consider that NAC was administered in developing CLI, although, in clinical reality, patients with CLI are usually not treated during the development of CLI but rather when CLI is well established. Further, although this study aimed to investigate functional and morphological status after the administration of NAC, this could be at least indirectly associated with improved microvascularisation, which was not investigated, neither was NAC concentration in the ischaemic limbs.

In summary, targeting inhibition of oxidative stress using pharmacological agents may be a potential therapeutic strategy for muscle protection in CLI and might be considered as potential adjunctive therapy to revascularisation procedures, further allowing functional improvement in the setting of CLI.

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MISE AU POINT

Physiopathologie de l'ischémie-reperfusion et du conditionnement ischémique du muscle squelettique — applications cliniques pour le chirurgien vasculaire

Skeletal muscle ischemia-reperfusion and ischemic conditioning pathophysiology—clinical applications for the vascular surgeon

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MOTS CLÉS
Ischémie-reperfusion ;
Muscle squelettique ;
Chirurgie vasculaire ;
Conditionnement ischémique

Résumé Le chirurgien vasculaire est confronté quotidiennement au phénomène d'ischémie-reperfusion. En effet, toute intervention chirurgicale nécessitant un clampage artériel induit une situation d'ischémie, qui est ensuite suivie d'une reperfusion au moment du déclampage. L'objectif de ce travail est de faire une mise au point sur la physiopathologie de l'ischémie-reperfusion au niveau du muscle squelettique et de dégager des pistes pour prévenir les lésions d'ischémie-reperfusion dans la pratique clinique courante du chirurgien vasculaire. Les mécanismes contribuant aux lésions d'ischémie-reperfusion sont multifactoriels et complexes. La surcharge calcique et le stress oxydant jouent un rôle majeur, conduisant à l'ouverture du pore de transition de perméabilité mitochondrial. Les mitochondries sont donc une cible privilégiée pour lutter contre les lésions d'ischémie-reperfusion. Le conditionnement ischémique est un processus qui tend à lutter contre les effets délétères de l'ischémie-reperfusion. L'idée est en fait de faire subir à l'organisme de brèves séquences répétées d'ischémie-reperfusion, de façon à stimuler les défenses antioxydantes pour lutter contre une ischémie-reperfusion

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plus conséquente. Bien qu'offrant des résultats prometteurs en expérimentation animale, le conditionnement ischémique a des résultats plus mitigés au niveau clinique. Néanmoins, avec un protocole bien conduit, dans des situations cliniques adaptées, le conditionnement ischémique pourrait avoir une place majeure. Ainsi, s'il s'avère efficace, une vaste application du conditionnement ischémique s'offrirait aux chirurgiens vasculaires.

KEYWORDS
Ischemia-reperfusion;
Skeletal muscle;
Vascular surgery;
Ischemic conditioning

Summary Ischemia-reperfusion, which is characterized by deficient oxygen supply and subsequent restoration of blood flow, can cause irreversible damage to tissue. The vascular surgeon is daily faced with ischemia-reperfusion situations. Indeed, arterial clamping induces ischemia, followed by reperfusion when declamping. Mechanisms underlying ischemia-reperfusion injury are complex and multifactorial. Increases in cellular calcium and reactive oxygen species, initiated during ischemia and then amplified upon reperfusion are thought to be the main mediators of reperfusion injury. Mitochondrial dysfunction also plays an important role. Extensive research has focused on increasing skeletal muscle tolerance to ischemia-reperfusion injury, especially through the use of ischemic conditioning strategies. The purpose of this review is to focus on the cellular responses associated with ischemia-reperfusion, as well as to discuss the effects of ischemic conditioning strategies. This would help the vascular surgeon in daily practice, in order to try to improve surgical outcome in the setting of ischemia-reperfusion.

Introduction

L'ischémie est définie par la diminution (liée à une sténose significative) ou l'arrêt de l'apport sanguin artériel dans un territoire donné, ce qui entraîne une inadéquation entre les apports et les besoins de la cellule pour son fonctionnement et sa survie, provoquant des lésions locales dont l'importance dépend du temps écoulé avant la reperfusion et du caractère aigu ou chronique de l'ischémie (l'ischémie chronique étant mieux tolérée du fait du réseau de collatéralité). La reperfusion correspond à la restauration de l'apport sanguin artériel nécessaire aux besoins de la cellule. Le chirurgien vasculaire est confronté quotidiennement au phénomène d'ischémie-reperfusion. En effet, toute intervention chirurgicale nécessitant un clampage artériel induit une situation d'ischémie, qui est ensuite suivie d'une reperfusion au moment du déclampage. La chirurgie conventionnelle aorto-iliaque ou la chirurgie conventionnelle des artères des membres inférieurs soumet donc particulièrement le muscle squelettique au phénomène d'ischémie-reperfusion.

La restauration de la perfusion avec réoxygénation des tissus doit être la plus précoce possible pour limiter les lésions liées à l'ischémie mais la reperfusion va paradoxalement provoquer des lésions supplémentaires avec une aggravation de l'état initial [1,2]. Cet effet paradoxal lié à la perfusion, induit une agression tissulaire et constitue le syndrome de reperfusion [3]. La dénomination « Ischémie-Reperfusion » (IR) rappelle ainsi que les dégâts tissulaires liés à une interruption ou une réduction du flux sanguin sont le résultat de deux phénomènes : l'effet de l'ischémie, mais également l'effet de la reperfusion. Les lésions d'IR peuvent donc devenir un facteur limitant le succès des interventions chirurgicales nécessitant un clampage artériel. Ces lésions sont liées à des mécanismes cellulaires complexes, notamment au niveau mitochondrial, avec atteinte de la chaîne respiratoire mitochondriale et production de radicaux libres dérivés de l'oxygène (ROS).

L'objectif de ce travail est de faire une mise au point sur la physiopathologie de l'IR au niveau du muscle squelettique et de dégager des pistes pour prévenir les lésions d'IR dans la pratique clinique courante du chirurgien vasculaire.

Physiopathologie de l'IR au niveau du muscle squelettique

Les mécanismes contribuant aux lésions d'IR sont multifactoriels et complexes. La surcharge calcique et le stress oxydant jouent un rôle majeur, conduisant à l'ouverture du pore de transition de perméabilité mitochondrial (mPTP) [4—7].

La surcharge calcique

La surcharge calcique intracellulaire survient en phase d'ischémie et sera accentuée lors de la reperfusion avec des conséquences délétères pour la cellule. Pendant l'ischémie, la production d'ATP est réduite et provient essentiellement de la glycolyse anaérobie. La conversion du pyruvate en lactate lié à la glycolyse anaérobie entraîne une surcharge intracellulaire en ions H^+ et diminue donc le pH intracellulaire. L'acidose ainsi générée active l'échangeur Na^+/H^+ afin de restaurer le pH intracellulaire par la sortie d'ions H^+ contre des ions Na^+. L'entrée d'ions Na^+ dans la cellule active à son tour l'échangeur Na^+/Ca^{2+} permettant la sortie des ions Na^+ contre l'entrée d'ions Ca^{2+}, aboutissant à une surcharge calcique intracellulaire [5]. Cette surcharge est également liée au fait que la réentrée de calcium dans le réticulum endoplasmique est inhibée, puisque cette réentrée est dépendante de la Ca^{2+}ATPase (SERCA) qui ne peut plus fonctionner en l'absence d'ATP [6—8].

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Pendant les premières minutes de la reperfusion, la correction de l'acidose par l'échangeur Na^+/H^+ et le cotransporteur $Na^+/HCO3^-$, ainsi que l'épuration de l'acide lactique, vont provoquer une activation inverse de l'échangeur Na^+/Ca^{2+} et donc augmenter le Ca^{2+} cytosolique [9]. Par la suite, le Na^+ va être épuré par des canaux Na^+ utilisant l'ATP de nouveau disponible. Ce n'est qu'à ce moment-là que l'échangeur Na^+/Ca^{2+} va refonctionner selon son mode normal pour épurer le Ca^{2+} cytosolique vers le milieu extra-cellulaire. Ainsi la surcharge calcique intracellulaire est aggravée lors des premières minutes de la reperfusion.

Les lésions d'ischémie-reperfusion sont ainsi, en partie, secondaires à l'accumulation accrue des ions Ca^{2+} au niveau du cytosol, provoquant l'activation de médiateurs chimiques et enzymatiques contribuant aux lésions cellulaires. Tout d'abord l'excès de calcium va atteindre directement la mitochondrie par ouverture des mPTP causant la mort cellulaire par apoptose [10]. Une deuxième cible de l'hypercalcémie intracytosolique est l'activation des calpaïnes. Ces enzymes provoquent une protéolyse intracellulaire conduisant à la dégradation du cytosquelette, du réticulum endoplasmique et des protéines mitochondriales aboutissant à la mort cellulaire par nécrose ou apoptose. La surcharge calcique intracellulaire active également des précurseurs de l'inflammation, cytokines et chémokines, aggravant encore les lésions d'ischémie-reperfusion [11—14].

Le stress oxydant

Le stress oxydant joue aussi un rôle majeur dans les lésions d'IR, avec la production de ROS par la mitochondrie, aussi bien lors de l'ischémie que lors de la période de reperfusion, aboutissant également à des lésions délétères pour la cellule.

Pendant l'ischémie, le fonctionnement des complexes I et III de la chaîne respiratoire est réduit, aboutissant à la formation de ROS [15—17]. Plus la durée de l'ischémie est longue, plus la production de ROS est importante. D'autre part, pendant l'ischémie, le déficit énergétique conduit à la formation de xanthine oxydase à partir des stocks intracellulaires de xanthine déshydrogénase. La xanthine oxydase est une oxydoréductase qui catalyse l'oxydation de l'hypoxanthine en xanthine, puis l'oxydation de la xanthine en acide urique. Ces deux réactions s'accompagnent de la formation de ROS [11].

$$\text{Hypoxanthine} + O_2 + H_2O \xrightarrow{\text{Xanthine oxydase}} \text{Xanthine} + \text{ROS}$$

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{\text{Xanthine oxydase}} \text{acide urique} + \text{ROS}$$

Ainsi pendant la reperfusion, du fait de l'apport d'oxygène, de grandes quantités de ROS sont produites.

Les ROS ainsi produits vont dépasser les capacités de défense antioxydante de la cellule, entraînant la peroxydation des acides gras polyinsaturés de la membrane cellulaire, la carboxylation des protéines et des dommages au niveau de l'ADN.

Le mPTP

La surproduction de ROS et la surcharge calcique vont agir directement au niveau du mPTP. Le mPTP est un pore large, normalement fermé. Lorsque ce pore est ouvert, sous l'effet de la surcharge calcique intracellulaire et de la surproduction de ROS, la perméabilité de la membrane interne de la mitochondrie aux ions produit une dissipation du gradient électrochimique des protons, ainsi qu'une perte de l'homéostasie ionique. Diverses susbstances apoptogènes sont alors libérées dans le cytosol, comme le cytochrome C ou encore l'*apoptosis-inducing factor* (AIF), ce qui engendre la mort cellulaire [18—20] (Fig. 1).

Les mitochondries jouent donc un rôle clé dans les altérations liées à l'IR principalement par la production de ROS et l'ouverture du mPTP [21]. Les mitochondries sont donc une cible privilégiée pour lutter contre les lésions d'IR.

Prévention des lésions d'IR par le conditionnement : principes

Le conditionnement ischémique est un processus qui tend à lutter contre les effets délétères de l'IR [22—25]. L'idée est en fait de faire subir à l'organisme de brèves séquences répétées d'IR, de façon à stimuler les défenses antioxydantes pour lutter contre une IR plus conséquente [24]. En fonction du moment où la séquence de conditionnement est pratiquée, on distingue le pré-, per-, et postconditionnement (Fig. 2). Le conditionnement est également divisé en deux sous-groupes selon la localisation du conditionnement par rapport à l'organe cible à protéger. On distingue ainsi le conditionnement local où le protocole de conditionnement est réalisé au niveau de l'organe cible ; et le conditionnement à distance, où le protocole de conditionnement est réalisé à distance de l'organe cible c'est-à-dire au niveau d'un autre organe (ou d'un autre territoire vasculaire d'un même organe). Par analogie avec le conditionnement ischémique, il existe également le conditionnement pharmacologique, qui consiste en l'administration d'une molécule protectrice avant (préconditionnement pharmacologique) ou après (postconditionnement pharmacologique) un évènement ischémique prolongé. Ce type de conditionnement ne sera pas abordé ici.

Le préconditionnement ischémique

Le préconditionnement ischémique est un processus réalisé avant la période d'IR.

Le concept de préconditionnement a été décrit pour la première fois par Murry et al. en 1986 au niveau du muscle cardiaque [26]. Cette étude réalisée sur le myocarde de chien consistait en la réalisation d'un préconditionnement ischémique par clampage de l'artère circonflexe avec 4 cycles d'IR (soit 5 minutes d'ischémie par clampage de l'artère circonflexe, puis 5 minutes de reperfusion), suivi d'une ischémie prolongée et d'une reperfusion. Il a ainsi été mis en évidence que le préconditionnement ischémique local protégeait le myocarde des lésions d'IR ultérieures avec diminution de la taille de l'infarctus myocardique. Przyklenk et al. ont, quant à eux, démontré la protection

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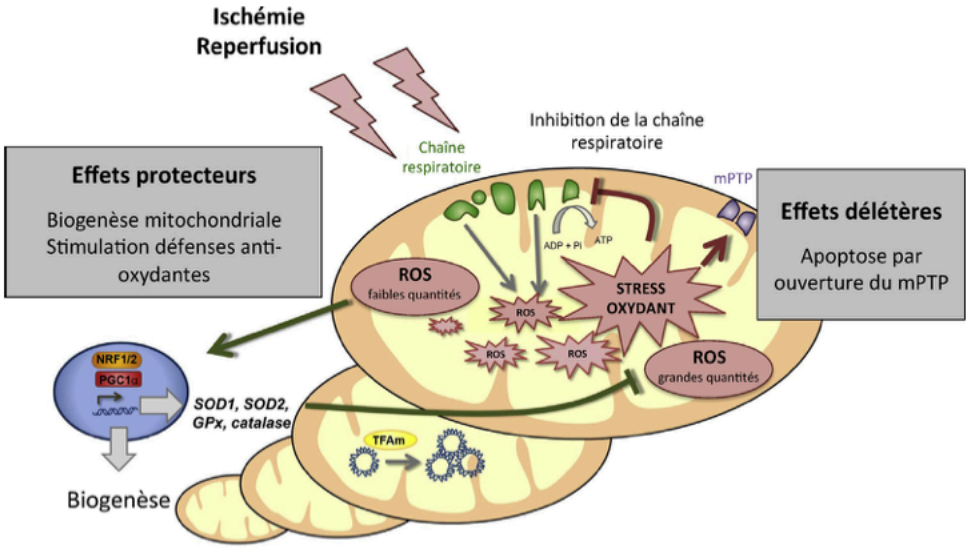

Figure 1 Effets protecteurs et délétères des radicaux libres. ADP : adénosine diphosphate ; ATP, adénosine triphosphate ; GPx, glutathion peroxidase ; mPTP, pore de transition de perméabilité mitochondriale ; ROS, radicaux libres dérivés de l'oxygène ; NRF : facteur respiratoire nucléaire ; PGC-1 : *peroxisome proliferator-activated receptor gamma coactivator 1 alpha* ; Pi : phosphate inorganique ; SOD : superoxide dismutase ; TFAm : facteur de transcription mitochondrial A.
Protective and deleterious effects of reactive oxygen species.
Modifié d'après Lejay A. et al., Int J Biochem Cell Biol (2014) [21].

du myocarde par le préconditionnement à distance [27]. Le préconditionnement ischémique à distance a été réalisé au niveau de l'artère circonflexe par 4 cycles d'IR (soit 5 minutes d'ischémie, puis 5 minutes de reperfusion). Ce préconditionnement a été suivi d'une ischémie prolongée par clampage de l'artère interventriculaire antérieure. Il a été montré que le préconditionnement à distance par clampage de l'artère circonflexe a protégé le territoire myocardique de l'artère interventriculaire antérieure avec une diminution de la taille de l'infarctus.

Au niveau du muscle squelettique, c'est Pang et al. en 1995, qui ont mis en évidence un effet protecteur du préconditionnement local [28]. Leurs expérimentations portaient sur la réalisation d'une ischémie prolongée des muscles grand dorsal et gracile du cochon. Le préconditionnement local a été effectué par 3 cycles d'IR (10 minutes d'ischémie par clampage des différents pédicules musculaires, puis 10 minutes de reperfusion). Ils ont conclu à un effet protecteur du fait d'une réduction de la taille de la nécrose musculaire de 44 et 62 % pour les muscles grand dorsal et gracile respectivement. Addison et al., en 2003, ont démontré l'effet protecteur du préconditionnement à distance chez le cochon [29]. En effet, les muscles grand dorsal, gracile et droit de l'abdomen ont été protégés des lésions d'IR par un préconditionnement à distance effectué au niveau du membre postérieur du cochon par 3 cycles d'IR (5 minutes d'ischémie, puis 5 minutes de reperfusion) avec une diminution de la taille de la nécrose musculaire de 55, 60 et

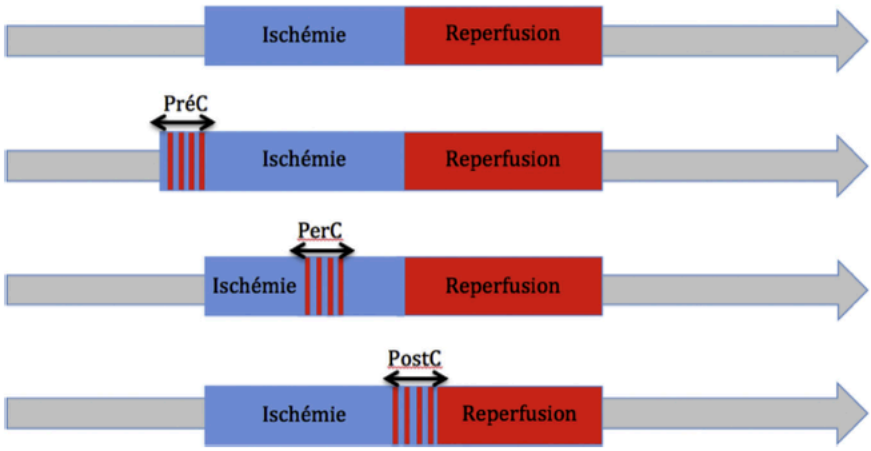

Figure 2 Le préconditionnement (PréC), le perconditionnement (PerC) et le postconditionnement ischémique (PostC).
Ischemic preconditioning (PréC), perconditioning (PerC) and postconditioning (PostC).

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55 % au niveau des muscles grand dorsal, gracile et droit de l'abdomen respectivement. De nombreuses études précliniques menées sur différentes espèces animales ont suivi, confirmant l'effet protecteur du préconditionnement sur le myocarde principalement, ainsi que sur le muscle squelettique.

Le perconditionnement ischémique

Le perconditionnement ischémique consiste en l'alternance de périodes d'ischémie et de reperfusion au décours même de l'évènement ischémique. Cette méthode est difficilement applicable en pratique clinique, raison pour laquelle elle ne sera pas détaillée ici.

Le postconditionnement ischémique

Enfin, le postconditionnement ischémique consiste à effectuer, après un évènement ischémique durable, une série de brèves périodes d'IR avant de permettre une reperfusion finale [30].

Le concept du postconditionnement ischémique a été décrit pour la première fois par Zhao et al. en 2003 au niveau du myocarde de chien, en postconditionnant le territoire myocardique irrigué par l'artère interventriculaire antérieure ayant subi une ischémie prolongée par 3 cycles d'IR (30 secondes d'ischémie, puis de 30 secondes de reperfusion) avant la reperfusion totale [31]. Il a ainsi été mis en évidence que le postconditionnement ischémique local protégeait le myocarde des lésions d'IR avec une réduction de 44 % de la taille de l'infarctus. Il en est de même pour le postconditionnement à distance, en obtenant un effet cardioprotecteur par le postconditionnement ischémique du rein. Cette expérimentation a été réalisée par Kerendi et al. en 2005, et consistait à réaliser chez le rat, un cycle d'IR de l'artère rénale (5 minutes d'ischémie, puis de 1 minute de reperfusion) à la fin de l'ischémie myocardique [32]. La taille de l'infarctus du myocarde était réduite de 50 % par le postconditionnement à distance.

Au niveau musculaire squelettique, c'est McAllister et al. en 2008 qui ont mis en évidence un effet protecteur du postconditionnement ischémique local au niveau du muscle grand dorsal chez le cochon [33]. Le protocole de postconditionnement consistait en 3 cycles d'IR (5 minutes d'ischémie, puis 5 minutes de reperfusion) à la fin de l'ischémie, c'est-à-dire juste avant la reperfusion définitive. Ils ont constaté une réduction de la taille de la nécrose musculaire de 44 à 22 % par le postconditionnement local. Tsubota et al. en 2010, ont quant à eux mis en évidence, un effet protecteur du postconditionnement à distance chez la souris [34]. En effet, le postconditionnement du membre postérieur gauche par 1 cycle d'IR (5 minutes d'ischémie, puis 5 minutes de reperfusion) a permis une protection des lésions d'IR du muscle squelettique du membre postérieur droit qui venait de subir une IR.

Les différents travaux précliniques montrent cependant que le postconditionnement doit être initié dans les plus brefs délais ; l'effet du postconditionnement est perdu s'il est débuté tardivement après la fin de l'ischémie [30]. Par ailleurs, Mansour et al., précisent que le postconditionnement, qu'il soit local ou à distance, peut être délétère, si le protocole de postconditionnement n'est pas adapté. Celui-ci doit, en effet, être adapté à chaque espèce [35].

Mécanismes cellulaires mis en jeu lors du conditionnement ischémique

Les mécanismes cellulaires mis en jeu dans le conditionnement ischémique sont complexes et incomplètement élucidés à ce jour. Il s'agit d'un processus multifactoriel activant de nombreuses cascades de signalisation interagissant les unes sur les autres, nécessitant l'interaction de nombreux signaux inducteurs, de seconds messagers (ou médiateurs) et des effecteurs [22]. Il semble que l'effecteur final soit la mitochondrie [18].

Inducteurs/seconds messagers/effecteurs

Les mécanismes du conditionnement ont cependant été principalement étudiés au niveau du myocarde. De façon simplifiée, la séquence moléculaire lors du conditionnement ischémique est la suivante : des signaux inducteurs sont libérés pendant le conditionnement, ils vont activer des seconds messagers, qui à leur tour vont transmettre le signal à des effecteurs pendant la période d'ischémie prolongée afin d'atténuer les lésions liées à l'IR [22].

Les signaux inducteurs sont essentiellement les ROS en petites quantités, la bradykinine, l'adénosine, le TNFalpha, l'interleukine 6, l'interleukine 10 et le Calcitonin Gene-Related Peptide (CGRP) [36—40].

Les seconds messagers intracellulaires impliquent quant à eux, essentiellement la protéine kinase C (PKC) [41]. La principale cible de la PKC sont les canaux mitochondriaux K_{ATP} dont l'ouverture permet de réduire la surcharge calcique [42]. L'oxyde nitrique, la protéine kinase G (PKG) et le cGMP sont également des seconds messagers qui régulent également l'activité des canaux mitochondriaux K_{ATP} [43].

Ces seconds messagers vont agir sur le principal effecteur qui est le mPTP, en inhibant son ouverture, et donc l'apoptose (par diminution de la surcharge calcique) [44,45].

Voies RISK et SAFE

Cette distinction entre signaux inducteurs/seconds messagers/effecteurs est cependant complexe car il existe de nombreuses interactions, et il est difficile de « classer » une molécule dans une position donnée de la cascade. De ce fait, les mécanismes protecteurs liés au conditionnement ischémique ont été décrits d'une autre façon, via les voies Reperfusion Injury Salvage Kinase (RISK) et Survivor Activating Factor Enhancement (SAFE) [37,46—50]. L'étude de l'effet protecteur des voies RISK et SAFE a été menée essentiellement au niveau du muscle cardiaque, et il existe à l'heure actuelle très peu de données concernant le muscle squelettique.

Mécanismes d'action

Pour résumer, le conditionnement ischémique génère la production de radicaux libres, mais en faible quantité. Cette production de ROS à un niveau sublétal protège contre une

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augmentation successive ultérieure à de plus fortes doses puisqu'elle stimule les défenses antioxydantes de la cellule. Tout se passe comme si l'organe venant de subir ce préconditionnement gardait en mémoire cette agression et mettait en jeu des mécanismes endogènes lui permettant de mieux tolérer une prochaine ischémie [40]. Par ailleurs, cette production de ROS en petites quantités va jouer le rôle d'inducteur, puisqu'elle va permettre de diminuer la surcharge calcique par l'activation des canaux K_{ATP} et donc l'ouverture du mPTP et l'apoptose qui s'ensuit.

Les mécanismes cellulaires mis en jeu dans le conditionnement à distance sont encore très incertains. Les études expérimentales suggèrent que les mécanismes cellulaires et les voies de signalisations au sein de l'organe protégé serait les mêmes que lors du conditionnement local décrit plus haut [47]. Cependant, les métabolites de transmission du message de l'organe préconditionné à l'organe cible ne sont pas identifiés. Plusieurs hypothèses suggèrent une implication des facteurs humoraux, une implication neuronale et un effet anti-inflammatoire et anti-apoptotique systémique [47,51].

Applications cliniques

Les lésions d'IR peuvent être observées dans plusieurs spécialités chirurgicales impliquant un clampage, principalement en chirurgie cardiaque et en chirurgie vasculaire [52].

Le préconditionnement ischémique en chirurgie cardiaque

Les premières études cliniques sur le préconditionnement ischémique ont d'abord été effectuées au niveau cardiaque. Yellon et al., ont publié en 1993 la première série de patients ayant bénéficié d'un préconditionnement ischémique local avant pontage aorto-coronarien [53]. Le préconditionnement local a été effectué par 2 cycles de 6 minutes d'IR (3 minutes de clampage aortique, puis 3 minutes de reperfusion). Ils ont démontré un effet cardioprotecteur du préconditionnement avec une préservation du niveau d'ATP dans les cellules musculaires dans le groupe préconditionnement par rapport au groupe contrôle. Depuis 1993, de nombreuses études sur le préconditionnement ischémique local ont été publiées, avec confirmation de cet effet cardioprotecteur. Le principal inconvénient de cette technique est son caractère invasif nécessitant des clampages artériels itératifs, induisant donc potentiellement un risque emboligène chez des patients souvent polyathéromateux.

Cheung et al., en 2006, ont démontré l'effet cardioprotecteur du préconditionnement à distance [54]. Ce préconditionnement à distance a été effectué par 4 cycles de 10 minutes d'IR (5 minutes d'ischémie, puis 5 minutes de reperfusion) grâce un garrot au niveau d'un des membres inférieurs chez des enfants devant se faire opérer de cardiopathies congénitales. Ce préconditionnement à distance a réduit significativement les taux postopératoires de Troponine I et réduit les doses nécessaires de drogues inotropes. Depuis 2006, du fait de sa faible invasivité et de sa facilité de réalisation, une multitude d'études sur le préconditionnement ischémique à distance ont été réalisées. Cependant, bien que de nombreuses études concluent à l'efficacité de ce conditionnement à distance, un nombre non négligeable d'études ne retrouvent aucun effet cardioprotecteur [55—57]. Les raisons pour lesquelles le conditionnement à distance n'aurait pas d'effet cardioprotecteur sont multifactorielles et seront détaillées plus bas.

Le préconditionnement ischémique en chirurgie vasculaire

En chirurgie vasculaire, le préconditionnement a été étudié dans la pathologie anévrismale de l'aorte abdominale. En effet, cette chirurgie nécessite un clampage aortique et induit une souffrance ischémique des organes situés en dessous du niveau du clampage mais également une souffrance des organes à distance, tels que le myocarde, les reins, les poumons et le système digestif. Cette souffrance peut s'expliquer par plusieurs mécanismes physiopathologiques, dont les perturbations hémodynamiques majeures, chez ces patients souvent fragiles et présentant d'autres comorbidités. Cette chirurgie est donc grevée d'une morbi-mortalité postopératoire importante [58]. L'idée est donc de protéger l'organisme des lésions d'IR, que ce soit les organes situés en dessous du niveau de clampage, mais également les organes situés à distance.

La première étude clinique a été effectuée par Ali et al. en 2007 [59]. Cet essai prospectif, randomisé, a inclus 82 patients bénéficiant de la mise à plat d'un anévrisme de l'aorte abdominale (AAA) par chirurgie ouverte avec clampage aortique sous-rénal. Le préconditionnement à distance a été réalisé au niveau des muscles squelettiques du membre inférieur par 2 cycles d'IR (10 minutes d'ischémie par clampage de l'artère iliaque commune, puis 10 minutes de reperfusion). Ce préconditionnement à distance a permis d'obtenir une protection au niveau cardiaque avec une réduction significative de l'incidence de l'élévation des marqueurs myocardiques de 27 % et une réduction du nombre d'infarctus du myocarde de 22 %. Ce préconditionnement a également permis d'obtenir une protection rénale avec une diminution du nombre de patients présentant une insuffisance rénale postopératoire de 23 % (augmentation de plus de 20 % du taux de créatininémie par rapport à la valeur préopératoire). Le préconditionnement s'est donc avéré être un facteur indépendant de protection contre la survenue postopératoire de lésions myocardiques et de dysfonction rénale après clampage aortique.

Le même protocole de préconditionnement a été réalisé par Walsh et al., chez 40 patients, mais aucune différence entre les 2 groupes n'a été mise en évidence en ce qui concerne la protection rénale [60]. Cette même équipe a également étudié la protection cérébrale et cardiaque d'un préconditionnement à distance chez les patients bénéficiant d'une endartériectomie de la carotide [61]. Aucune différence entre les deux groupes de patients n'a été mise en évidence.

Quelques années plus tard, en 2013, une autre étude randomisée concernant la protection du préconditionnement à distance avant clampage aortique sur les fonctions pulmonaires et digestives a été réalisé par Li et al. Soixante-deux

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Tableau 1 Études cliniques sur le préconditionnement ischémique en chirurgie vasculaire.
Clinical studies on ischemic conditioning in vascular surgery.

Auteurs Année de publication	Pathologies	PréC à distance	Résultats
Ali et al. 2007	AAA	Clampage de l'artère iliaque commune	Protection cardiaque et rénale
Walsch et al. 2010	AAA	Clampage de l'artère iliaque commune	Aucune protection rénale
Walsch et al. 2010	TEA carotide	Brassard à tension au membre inférieur	Aucune protection cérébrale ou cardiaque
Li et al. 2013	AAA	Brassard à tension au membre supérieur	Protection digestive et pulmonaire
Murphy et al. 2014	AAA	Brassard à tension au membre supérieur	Aucune protection cardiaque ou rénale

PréC : préconditionnement ; TEA : thromboendartériectomie de l'artère carotide.

patients ont été inclus [62]. Le préconditionnement a cette fois-ci été réalisé par 3 cycles d'IR au niveau du membre supérieur (5 minutes d'ischémie par mise en place d'un brassard à tension au niveau du bras gauche du patient, puis 5 minutes de reperfusion). Cet essai a conclu à une réduction significative des lésions digestives et pulmonaires par le préconditionnement.

Un dernier essai randomisé concernant la pathologie anévrismale aortique a été publié en 2014 par Murphy et al. et incluait 62 patients [63]. Le préconditionnement ischémique à distance a été effectué par 3 cycles d'IR (5 minutes d'ischémie par un brassard à tension au niveau du membre supérieur, puis 5 minutes de reperfusion). Cette équipe n'a pas mis en évidence d'effet protecteur du préconditionnement sur la fonction rénale et cardiaque (Tableau 1).

Le postconditionnement ischémique

Le postconditionnement a principalement été étudié au niveau cardiaque, notamment en cas de prise en charge endovasculaire de l'infarctus du myocarde. Staat et al., en 2005 ont publié la première étude clinique concernant 30 patients présentant un infarctus du myocarde avec prise en charge en urgence en salle de coronarographie [64]. Le postconditionnement a été effectué par 4 cycles d'IR (1 minute d'ischémie grâce à l'inflation d'un ballon d'angioplastie en amont du stent mis en place, puis 1 minute de reperfusion par déflation du ballon). Cette équipe a montré que cette procédure a pu réduire de 36 % les marqueurs de souffrance myocardique, témoignant d'un effet cardioprotecteur du postconditionnement. Une multitude d'études concernant le postconditionnement a ensuite été réalisée avec des résultats variables [65—67].

Quelques études ont également été effectuées lors de pontages aorto-coronariens avec des résultats en faveur de l'effet cardioprotecteur du postconditionnement ischémique [68,69]. Toutefois, à l'instar du préconditionnement, le caractère invasif lié aux clampages artériels itératifs chez des patients athéromateux induit un risque embolique, raison pour laquelle peu d'études sur ce protocole ont été réalisées.

Limites du conditionnement ischémique

Ainsi, que ce soit en chirurgie cardiaque ou vasculaire, les résultats des études portant sur le conditionnement ischémique sont variables d'une étude à l'autre. Plusieurs arguments peuvent être apportés quant à la disparité de ces résultats. Tout d'abord, il existe une grande variabilité dans le type de préconditionnement ischémique utilisé dans ces différentes études, et aucun marqueur fiable de l'efficacité de la réalisation de l'ischémie. En effet, certaines études réalisent un clampage artériel direct, tandis que d'autres utilisent un brassard à tension appliqué au niveau des membres pour créer l'ischémie. Ces deux techniques ne présentent pas la même fiabilité et aucun marqueur standardisé d'ischémie n'est utilisé pour démontrer l'efficacité des cycles d'IR. De la même façon, la durée des cycles d'IR est également variable d'une étude à l'autre, sans consensus. Il est également à noter que le muscle squelettique peut varier au niveau de son volume d'un individu à l'autre, et est aussi plus volumineux au niveau du membre inférieur que supérieur. Nous pouvons alors nous demander si le préconditionnement des muscles des membres inférieurs serait plus efficace que celui des membres supérieurs, d'où la variabilité des résultats. Aucune étude n'a à l'heure actuelle démontré si le conditionnement ischémique est plus ou moins efficace en fonction du volume du muscle squelettique.

Une autre donnée importante à prendre en compte quant à l'analyse des résultats est l'interaction d'autres facteurs, tels que les comorbidités du patient, à savoir le diabète, l'âge, l'hypertension. Ces facteurs peuvent en effet limiter l'efficacité du conditionnement. Par exemple, plusieurs études ont mis en évidence une diminution de l'effet protecteur du conditionnement ischémique chez les patients diabétiques [40,70]. D'autre part, pendant une chirurgie cardiaque ou vasculaire, le patient reçoit de nombreux traitements pouvant interférer avec le conditionnement ischémique tels que les drogues anesthésiques (propofol) ou les morphiniques, pouvant ainsi réduire l'efficacité du conditionnement [55,71,72]. De plus, étant donné que d'autres stratégies de protection myocardique sont déjà utilisées pendant la chirurgie cardiaque ou vasculaire, les lésions du

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myocarde périopératoire sont relativement faibles, ce qui fait qu'il devient difficile de mettre en évidence un effet cardioprotecteur surajouté.

Enfin, les critères de jugement sont variables en fonction des études ainsi que les définitions et les taux cibles des biomarqueurs qui définissent la souffrance myocardique ou rénale [63]. Ainsi d'autres études de plus fortes puissances seraient nécessaires à l'avenir, ainsi que des consensus quant à la réalisation du protocole de conditionnement ischémique. D'autre part, la mise en évidence de tous les facteurs confondants, cliniques, anesthésiques ou chirurgicaux, qui peuvent interférer avec l'efficacité du conditionnement est également nécessaire.

Perspectives

En chirurgie vasculaire, d'autres applications du conditionnement ischémique pourraient être envisagées.

L'étude expérimentale du préconditionnement ischémique se limite actuellement au traitement des anévrismes de l'aorte abdominale par chirurgie ouverte nécessitant un clampage aortique. Mais nous pourrions envisager d'étudier le préconditionnement lors des pontages artériels des membres inférieurs. Par exemple, lors de la réalisation d'un pontage fémoropoplité, le clampage, puis déclampage de l'artère fémorale commune induit des lésions d'IR des muscles squelettiques de la cuisse et de la jambe. En effet, dans ce type de chirurgie, les patients présentent dans la majorité des cas un tableau d'oblitération fémorale superficielle, mais gardent le plus souvent une artère fémorale profonde perméable. Le clampage fémoral commun aura alors des conséquences sur le lit d'aval de par ce clampage de l'artère fémorale profonde, seul axe perméable. Nous pourrions alors effectuer un préconditionnement local ou à distance avant la réalisation du pontage. Le préconditionnement local pourrait s'effectuer par plusieurs cycles d'IR par clampages itératifs de l'artère fémorale commune avant le clampage prolongé. Le préconditionnement à distance pourrait quant à lui, être réalisé au niveau du membre supérieur ou du membre inférieur controlatéral avec plusieurs cycles d'IR par l'application d'un garrot à la racine du membre, avec une pression contrôlée.

Il serait également envisageable de tester le postconditionnement ischémique en chirurgie vasculaire. Ce postconditionnement pourrait être étudié tout d'abord en chirurgie programmée, après les périodes de clampages prolongés lors de la réalisation de pontages aortiques ou périphériques. Nous devrions pratiquer plusieurs cycles d'IR avant le déclampage définitif. Une autre application du postconditionnement en chirurgie vasculaire concerne la pathologie ischémique aiguë des membres. En effet, l'avantage du postconditionnement est qu'il peut, contrairement au préconditionnement, s'appliquer à la chirurgie en urgence. Nous pourrions alors facilement effectuer un postconditionnement en cas d'ischémie aiguë d'étiologie embolique sur artères « saines ». Ce postconditionnement consisterait en une revascularisation progressive du membre ischémique après avoir effectué l'embolectomie à la sonde de Fogarty par plusieurs cycles d'IR avant le déclampage définitif. Le postconditionnement en chirurgie vasculaire nous permettrait d'espérer une meilleure récupération postopératoire par diminution des lésions d'IR des cellules musculaires avec une diminution de la rhabdomyolyse et une meilleure tolérance de l'organisme.

Conclusion

Les mécanismes contribuant aux lésions d'IR du muscle squelettique sont multifactoriels et complexes. Le conditionnement ischémique permet de lutter contre les effets délétères de ces lésions d'IR. L'idée est en fait de faire subir à l'organisme de brèves séquences répétées d'IR, de façon à stimuler les défenses antioxydantes pour lutter contre une IR plus conséquente. Bien qu'offrant des résultats prometteurs en expérimentation animale, le conditionnement ischémique a des résultats plus mitigés au niveau clinique. Des consensus pour harmoniser les prises en charge seraient nécessaires. En chirurgie vasculaire, les perspectives seraient d'utiliser le pré- et postconditionnement ischémique pour protéger les organes et muscles squelettiques lors de clampages prolongés. Ainsi, s'il s'avère efficace, une vaste application du conditionnement ischémique s'offrirait aux chirurgiens vasculaires.

Déclaration de liens d'intérêts

Les auteurs déclarent ne pas avoir de liens d'intérêts.

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Review article

Ischemia reperfusion injury, ischemic conditioning and diabetes mellitus

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ABSTRACT

Ischemia/reperfusion, which is characterized by deficient oxygen supply and subsequent restoration of blood flow, can cause irreversible damages to tissue. Mechanisms contributing to the pathogenesis of ischemia reperfusion injury are complex, multifactorial and highly integrated. Extensive research has focused on increasing organ tolerance to ischemia reperfusion injury, especially through the use of ischemic conditioning strategies. Of morbidities that potentially compromise the protective mechanisms of the heart, diabetes mellitus appears primarily important to study. Diabetes mellitus increases myocardial susceptibility to ischemia reperfusion injury and also modifies myocardial responses to ischemic conditioning strategies by disruption of intracellular signaling responsible for enhancement of resistance to cell death.
The purpose of this review is twofold: first, to summarize mechanisms underlying ischemia reperfusion injury and the signal transduction pathways underlying ischemic conditioning cardioprotection; and second, to focus on diabetes mellitus and mechanisms that may be responsible for the lack of effect of ischemic conditioning strategies in diabetes.

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1. Introduction

The heart is one of the most energy demanding tissues in the body and is totally dependent upon oxidative phosphorylation to supply the large amount of ATP required for continuous contraction and relaxation. Ischemia, caused by disruption in blood flow leads to deficient oxygen supply to tissue. This results in cessation of oxidative phosphorylation, causing decrease in tissue ATP and creatine phosphate, and concomitant rise in ADP, AMP and Pi concentrations. Glycolysis is activated, but is unable to produce the required level of ATP. The heart rapidly ceases to beat as the contractile machinery is inhibited by elevated Pi and ADP, combined with the decreasing pH from the accumulation of glycolytic lactic acid [1]. Short duration ischemia is associated with full recovery of the tissue after reperfusion, while prolonged ischemia can lead to cell death. The extent of tissue injury is influenced by both the magnitude and duration of ischemia. Immediate restoration of blood flow remains the mainstream treatment. Reperfusion delivers oxygen and nutrients to support aerobic metabolism and ATP generation, and normalizes extracellular pH by washing out accumulated H^+ ions. However, reperfusion may paradoxically exacerbate tissue injury, and this additional damage is called reperfusion injury.

Mechanisms contributing to the pathogenesis of ischemia reperfusion injury are multifactorial and complex but also highly integrated. Increases in cellular calcium and reactive oxygen species (ROS), initiated during ischemia and then amplified upon reperfusion are thought to be the main mediators of reperfusion injury. Mitochondrial dysfunction also plays an important role, both in the production of ROS and as a target for downstream effects of both ROS and calcium overload [1–5].

Extensive research has focused on increasing heart tolerance to ischemia reperfusion injury using conditioning strategies. Brief episodes of coronary ischemia reperfusion preceding (ischemic preconditioning) or following (ischemic postconditioning) sustained myocardial ischemia reperfusion reduce infarct size. Even ischemia reperfusion in organs remote from the heart provides cardioprotection (remote ischemic conditioning) [6–10]. Diabetes is a common comorbidity in patients with cardiovascular disease [11]. Despite progress in coronary intervention strategies, diabetes mellitus is still associated with higher mortality after acute myocardial infarction. Diabetes mellitus increases myocardial susceptibility to ischemia reperfusion injury and also modifies myocardial responses to ischemic conditioning strategies by disruption of intracellular signaling responsible for conditioning-induced enhancement of resistance to cell death [12–16]. These alterations in the diabetic heart appear to underlie the poor prognosis of diabetic patients after acute myocardial infarction.

The purpose of this review is twofold. First, to summarize mechanisms underlying ischemia reperfusion injury and signal transduction pathways underlying ischemic conditioning-related cardioprotection. Second, to focus on diabetes mellitus and mechanisms that may be responsible for the lack of effect of ischemic conditioning strategies in the diabetic heart.

2. Part I: ischemia–reperfusion injury and ischemic conditioning

2.1. A. Mechanisms of ischemia reperfusion injury

The mechanisms contributing to the pathogenesis of ischemia reperfusion injury are multifactorial, complex, and moreover highly integrated. Processes as varied as calcium overload, oxidative stress, endoplasmic reticulum stress, mitochondrial dysfunction, apoptosis, protein kinases activation, and inflammation all play an important role and are also inter-related [2–5].

2.1.1. Calcium overload

When oxygen delivery to tissue is impaired, cells undergo a transformation from aerobic to anaerobic metabolism, switching to glycolysis for ATP generation. This leads to an accumulation of lactates, protons, and NAD^+, and decreases cell pH. Cells restore pH by extruding H^+ ions for Na^+ through the Na^+/H^+ exchanger. Na^+ ions are then exchanged for Ca^{2+} by the Na^+/Ca^{2+} exchanger[3]. The increase in cytosolic Ca^{2+} is exacerbated upon reperfusion, because the washout of accumulated extracellular H^+ ions further increases the proton gradient across the cell membrane, thereby accelerating Na^+/H^+ exchanger function[3]. In addition to changes in transmembrane Ca^{2+} transport during ischemia–reperfusion, Ca^{2+} transport across the sarcoplasmic reticulum is also affected. Ca^{2+} reuptake into the sarcoplasmic reticulum by the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) is impaired, while Ca^{2+} release through the ryanodine receptor is enhanced (Fig. 1) [4–17]. (See Fig. 2.)

Calcium overload activates a variety of systems, all of which can contribute to cell injury following ischemia reperfusion. Excess Ca^{2+} is taken into the mitochondria via the mitochondrial Ca^{2+} uniporter, a protein that uses the negative mitochondrial transmembrane potential to drive uptake of the positively charged Ca^{2+} ions into the matrix. However, when Ca^{2+} levels in the mitochondria become excessive, Ca^{2+} binds and activates the Ca^{2+} binding domains of the mitochondrial permeability transition pore (mPTP), leading directly to mPTP opening and causing cell death (Fig. 1) [18]. The calpains are another target for calcium overload. This family of cysteine proteases is activated by increased Ca^{2+} and degrades intracellular proteins such as cytoskeletal, endoplasmic reticulum and mitochondrial proteins. On the other hand, the endogenous inhibitor of calpains, calpastatin, is often degraded during ischemia reperfusion, which further enhances calpain availability and ischemia reperfusion injury [1]. Finally, calcium overload can lead to the generation of calcium pyrophosphate complexes and the formation of uric acid, both having the possibility to bind to

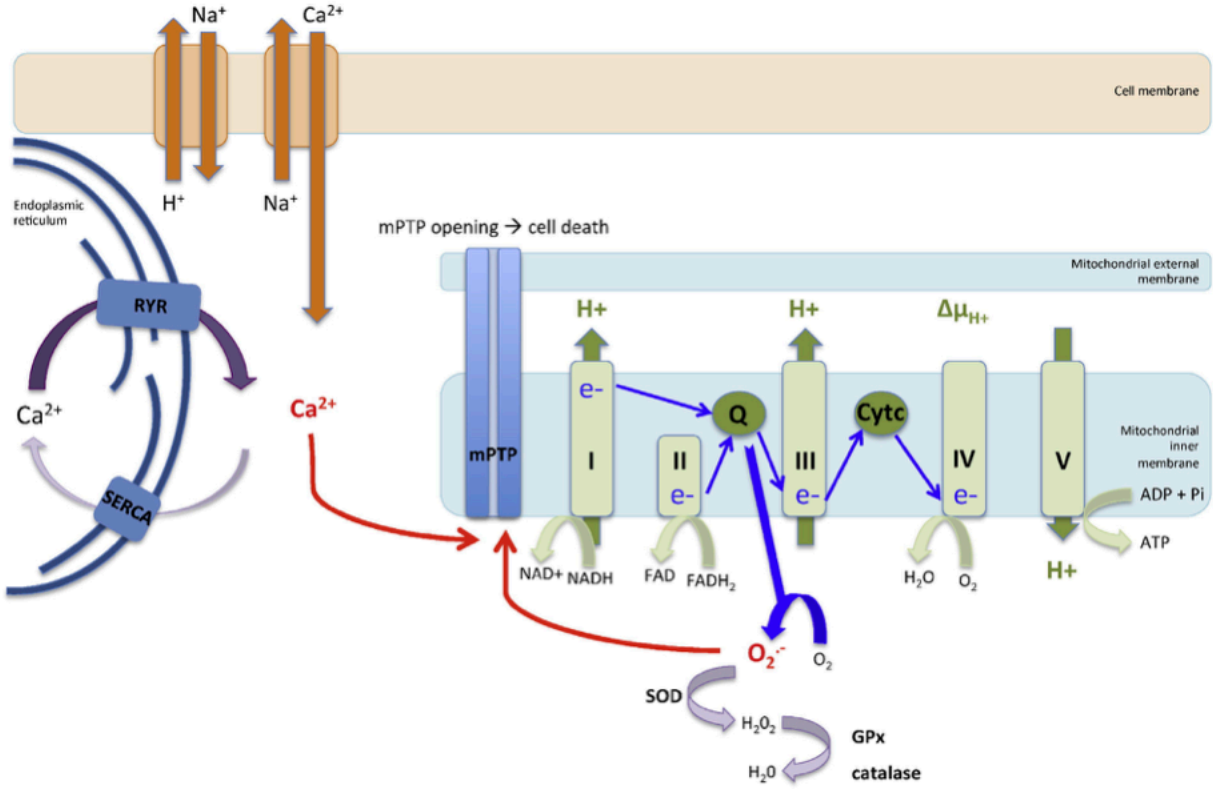

Fig. 1. Ischemia–reperfusion: role of oxidative stress, calcium overload, and mPTP.

inflammasomes, thereby increasing the expression of cytokines and chemokines, and also contributing to ischemia reperfusion injury [1].

2.1.2. Oxidative stress

Free radicals are molecules or atoms with unpaired electrons in their outer shell and are highly reactive. Free radicals produced from oxygen are called ROS, and include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) [19,20]. Under physiologic conditions, the generation of ROS is low, and is mainly due to electron leakage from the mitochondrial electron transport chain. Low levels of ROS contribute to normal cellular function, including signal transduction [20].

During ischemia, complexes I and III of the mitochondrial electron transport chain are kept in their reduced state, thereby increasing ROS production to the point that the cellular antioxidant systems are

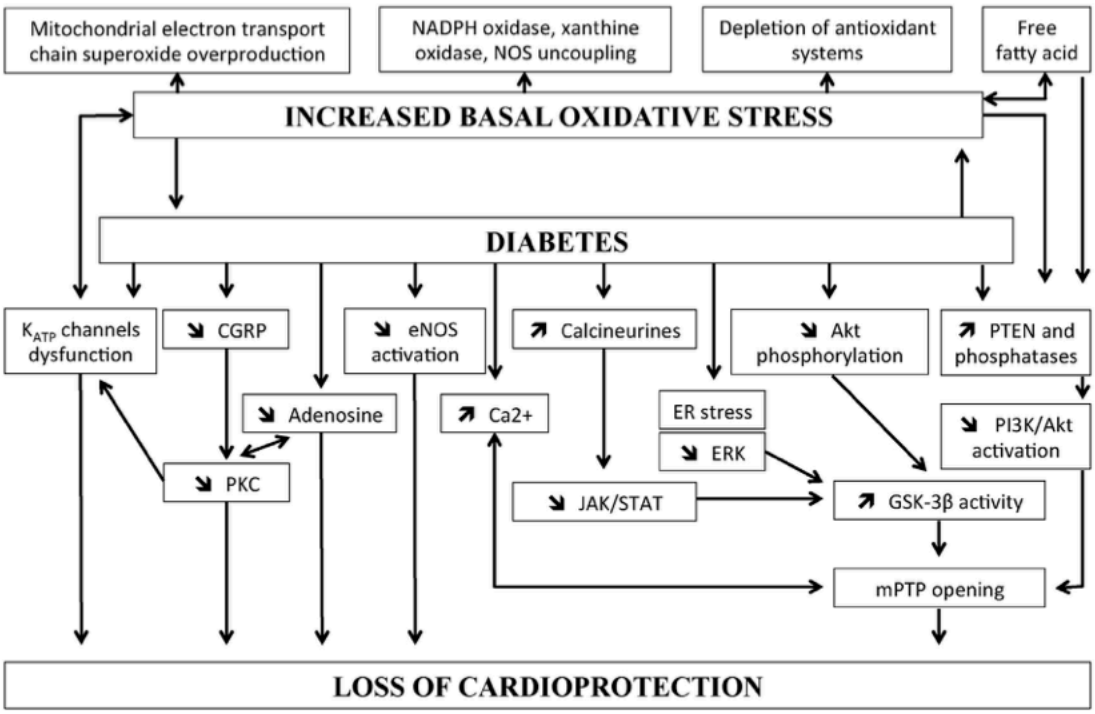

Fig. 2. Increased oxidative stress and impaired ischemic conditioning cardioprotection in the setting of diabetes mellitus.

overwhelmed, resulting in oxidative stress. Restoration of oxygen upon reperfusion exacerbates this pathogenic mechanism. High amounts of ROS cause cell toxicity, including from direct protein carboxylation, lipid peroxidation, and DNA damage.

The primary ROS initially produced during ischemia reperfusion is the superoxide anion radical (O_2^-) from the univalent reduction of molecular oxygen as part of the mitochondrial electron transport chain. Superoxide anion serves as a precursor to the other ROS. Superoxide anion can also be produced by a number of cytosolic and membrane enzyme. Superoxide anion is generally thought not to be toxic mainly because of rapid and spontaneous dismutation to the less cytotoxic hydrogen peroxide (H_2O_2), a conversion accelerated about 10^4-fold by the action of superoxide dismutase enzyme (SOD). SOD1 is located in the cytosol, and SOD2 is present in mitochondria. Hydrogen peroxide is subsequently converted to water by either catalase or by glutathione peroxidase (GPx), which use reduced gluthatione (GSH) as a substrate [9]. The antioxidant effect of GPx requires sufficient levels of reduced GSH, and the latter can be generated by glutathione reductase (GR) (Fig. 1) [16].

However, under conditions of low pH, such as might be expected in ischemic tissue, superoxide anion radical can be rapidly and spontaneously converted to its conjugate acid, the more highly potent oxidant, hydroperoxyl radical (HOO). Hydrogen peroxide is less reactive than superoxide anion radical, and can diffuse across cell membranes and act as second messenger. Hydrogen peroxide also participates in the generation of highly reactive radicals such as hydroxyl (OH) via the Fenton reaction, or can react with hemoglobin and myoglobin to form damaging ferryl derivatives of these hemoproteins. Finally, superoxide anion radical can react with nitric oxide to form peroxynitrite anion (ONOO), which can be protonated to the highly cytotoxic peroxynitrous acid (ONOOH), a strong oxidant [1].

2.1.3. Endoplasmic reticulum stress

The endoplasmic reticulum is a complex membranous network found in all cells where it plays an important role in calcium homeostasis, proteins folding, and lipid biosynthesis. A wide variety of stressors, including oxidative stress and ischemia, disrupt endoplasmic reticulum function, which leads to protein misfolding and unfolding. The accumulation of misfolded and unfolded proteins in the endoplasmic reticulum, a state referred to as endoplasmic reticulum stress, elicits the unfolded protein response [21]. Through a variety of mechanisms, including chaperone expression and protein degradation, the unfolded protein response acts to ameliorate endoplasmic reticulum stress, but can also direct the cell to apoptosis. Excess stress, as part of ischemia reperfusion, can thus exacerbate cell death [21].

2.1.4. Mitochondrial dysfunction

Mitochondria are the major source of cell energy by producing ATP through the electron transport chain. The electron transport chain comprises an enzymatic series of electron donors and acceptors. Each donor passes electrons to a more electronegative acceptor, which in turn donates these electrons to another acceptor; a process that continues until electrons are passed to oxygen, the most electronegative and terminal electron acceptor in the chain. Passage of electrons between donor and acceptor releases energy, which is used to generate a proton gradient across the mitochondrial membrane by actively pumping protons into the intermembrane space, and producing a thermodynamic state that has the potential to do work. The entire process is called oxidative phosphorylation, since ADP is phosphorylated to ATP using the energy of hydrogen oxidation in many steps.

Mitochondria play a critical role in the progression of ischemia reperfusion injury, through various related processes such as mitochondrial ROS production, opening of the mPTP, and mitochondrial fission.

During ischemia, superoxide anion is produced by respiratory complexes I and III, which leads to increased ROS production. Due to the lack of oxygen during ischemia, electron flow through the respiratory chain is inhibited. As a consequence, the ATP synthase can no longer phosphorylate ADP to generate ATP [1]. Moreover, in an attempt to maintain the mitochondrial transmembrane potential, ATP synthase actually functions in reverse, hydrolyzing what ATP remains, leading to the formation of adenosine. Adenosine is then converted to inosine, which is in turn converted into hypoxanthine and finally xanthine. During reperfusion, as a result of greater availability of oxygen, the enzyme xanthine oxidase is activated. This oxidation results in molecular oxygen being converted into highly reactive superoxide and hydroxyl radicals. Oxygen restoration is necessary to restore ATP production, but paradoxically induces cell dysfunction by ROS overproduction, the so-called oxygen paradox [22].

Opening of the mPTP is believed to be the final common pathway leading to cell death by necrosis and apoptosis during reperfusion following prolonged ischemia [23–27]. Inhibited by low pH, the mPTP is closed during ischemia. However, during reperfusion, high intracellular calcium levels and increased ROS production induce opening of the mPTP. When the mPTP is open, K+ ions can pass back into the matrix, thereby dissipating the mitochondrial transmembrane potential, uncoupling the electron transport chain, and inhibiting ATP synthesis [18]. In addition, water enters the mitochondria through its osmotic gradient causing the mitochondria to swell and even rupture [1].

By blocking oxidative phosphorylation, ischemia reperfusion also inhibits the breakdown of fatty acids, leading to an accumulation of toxic acids within the cell and the production of inflammatory metabolites through the arachidonic acid pathway, as well as opening of the mPTP [28].

Mitochondria are dynamic organelles in that they can undergo fission and fusion. Alterations in mitochondrial morphology, and hence function, occur when these two processes become unbalanced. Low ATP levels and increased mitochondrial ROS production due to ischemia reperfusion lead to excessive mitochondria fission, thereby resulting in the fragmentation of the mitochondria and apoptosis.

2.1.5. Apoptosis

Besides necrotic death, ischemia reperfusion injury also causes apoptotic cell death. Activation of pro-apoptotic Bcl2 proteins such as Bax, Bak, Bid or PUMA, and their upregulation, translocation and integration into mitochondrial membranes have been reported in the setting of ischemia reperfusion [1]. These proteins, by a mechanism that still remains controversial, permeabilize the outer membrane, thereby enabling the release of proapoptotic proteins, such as cytochrome c, Smac/DIABLO, and endonuclease G from the intermembrane space. Cytochrome c binds to the cytosolic protein apaf1 and the resultant apoptosome activates the caspase-9 and -3 protease system. Smac/DIABLO activates caspases by sequestering caspase-inhibitory proteins. Endonuclease G mediates DNA fragmentation. However, ischemia alone is not sufficient for pro-apoptotic Bcl2 proteins activation since reperfusion is required, consistent with the fact that many of the pro-apoptotic Bcl2 proteins are redox sensitive [1].

2.1.6. Protein kinase activation

The activation of intracellular signal transduction pathways plays an important role in ischemia reperfusion injury pathogenesis. The protein kinases that are considered as mediators of ischemia reperfusion injury are the mitogen-activated protein kinases (c-Jun. N-terminal kinases and p38 MAPK), the protein kinase Cδ, the Ca^{2+}/calmodulin-dependent protein kinase (CaMK), and the receptor-interacting protein kinases (RIP kinases). These pro-death kinases have numerous targets that can contribute to ischemia reperfusion injury. JNK and p38 lead to upregulation of inflammatory cytokines such as TNFα and IL1; JNK, p38, and protein kinase Cδ lead to the activation of mitochondrial-dependent death pathways or activation of several pro-death Bcl2 proteins [1]. CaMK can lead to calcium overload, by activating Ca^{2+} and Na^{2+} channels and facilitating Ca^{2+} release from sarcoplasmic reticulum, while RIP kinases induce ROS production [29].

2.1.7. Inflammation

Ischemia reperfusion induces inflammation, so-called sterile inflammation due to the absence of pathogens. Ischemia reperfusion-induced inflammation is characterized by the recruitment of neutrophils and the production of cytokines and chemokines [30]. The sequestration of innate immune cells occurs primarily during reperfusion, which restores the delivery of oxygen but also neutrophils to the tissues. This fuels the formation of ROS by xanthine oxidase or NADPH oxidase present in immune cells. Neutrophil infiltration is a result of inflammatory responses to necrotic cells and formation of mediators, some of which depend on the generation of ROS, which exacerbates ischemia reperfusion injury [30].

2.2. B. Ischemic conditioning

To date, extensive research has focused on increasing organ tolerance to IRI through the use of ischemic conditioning strategies [6–8,31].

There are three approaches to ischemic conditioning that differs in terms of the site and timing of a brief ischemic conditioning stimulus: ischemic preconditioning, ischemic postconditioning and remote ischemic conditioning.

2.2.1. Ischemic conditioning approaches and mechanisms

Classic ischemic preconditioning, as first described by Murry et al., consists of 2 to 4 repeated episodes of 2 to 5 min ischemia, interrupted by intervening 5 min periods of reperfusion, before the onset of the sustained ischemic injury [32]. Ischemic preconditioning is a well-described adaptive response by which brief exposure to ischemia/reperfusion before sustained ischemia markedly enhances the ability of the organ to withstand a subsequent ischemic insult [7]. Ischemic preconditioning elicits a bi-phasic pattern of cardioprotection. The first phase manifests almost immediately following the stimulus and lasts for 1 to 2 h. The second phase of cardioprotection appears 12 to 24 h later, lasts for 48 to 72 h. This second phase, termed the Second Window of Protection (SWOP) or delayed ischemic preconditioning, lasts longer but is less protective in strength [33]. The first phase of ischemic preconditioning relies on the recruitment of acutely available signaling modules, whereas the delayed form involves increased synthesis and expression of protective proteins in response to an acute signal [33].

In contrast, postconditioning, as first described by Zhao et al., is performed near the end of the sustained ischemic period, before complete reperfusion. It is characterized by a progressive restoration of blood flow in a staggered manner, consisting of 3 to 6 cycles of 10–30 s of reperfusion, interspersed with 10 to 30 s periods of re-occlusion, before establishing complete reperfusion [34,35].

In remote conditioning, brief periods of ischemia are applied to a site that is distant to the organ of interest. With regard to timing, protection can be achieved when the stimulus is applied before (remote preconditioning), during sustained myocardial ischemia (remote perconditioning), or at the time of reperfusion (remote postconditioning) [36].

There are now many studies reporting signaling molecules and mechanisms mediating conditioning in a wide range of experimental preparations. A classification of signaling using a causal sequence of events has been proposed [31]. A trigger is released during the preconditioning ischemia reperfusion cycles and acts as the stimulus to activate a mediator which transmits the signal during the sustained ischemia onto an effector, which then attenuates injury during early reperfusion. Such a causal sequence of events is also operative in postconditioning but is more difficult to define, as trigger, mediator and effector must all become immediately and almost simultaneously active during early reperfusion. Moreover, this classification into triggers/mediators/effectors is complex, because some of the components overlap in one or more categories.

2.2.2. Triggers of cardioprotection

Endogenously released chemical stimuli eliciting cardioprotection include ROS and reactive nitrogen species. ROS have an ambivalent role in the conditioning phenomena: as mentioned before, excess formation of ROS contributes to irreversible injury but small amounts of ROS contribute to cardioprotection, through oxidation of protective cytosolic kinases [31]. Reactive nitrogen species, notably nitric oxide, share this dose-dependent paradox: small concentrations of nitric oxide improve ventricular function while high concentrations depress contractile function [37]. Exogenous nitric oxide triggers acute ischemic preconditioning while endogenous nitric oxide triggers delayed ischemic preconditioning [31].

Autacoids, such as bradykinin but mostly adenosine, play a central role in cardioprotection. These can be released from cardiomyocytes, endothelium and interstitial cells during the cycles of ischemia reperfusion. Bradykinin activates the bradykinin receptor 2 subtype on cardiomyocytes, which couples to G proteins and activates the downstream reperfusion injury salvage kinase (RISK) and endothelial NOS/protein kinase G pathways. Bradykinin also activates cyclo-oxygenase (COX) and prostacyclin synthesis [31]. Adenosine exerts its effects by enrolling a family of four G-protein coupled receptors defined A_1, A_{2A}, A_{2B} and A_3 receptors expressed on cardiomyocyte sarcolemma [38,39]. These receptors differ in their affinity for adenosine and in the type of G proteins that they recruit [39]. Adenosine also activates protein kinase C directly, leading to downstream RISK and endothelial NOS/protein kinase G pathways activation [11,38–40]. The main effect of adenosine occurs at reperfusion, because adenosine is released in huge amounts immediately during the early phase of reperfusion [8]. Postconditioning produces cytoprotection by retaining adenosine in the coronary circulation in the early minutes after reperfusion, allowing the agonist enough time to activate adenosine receptors and trigger protective signal transduction pathways [41].

Other molecules, such as cytokines and chemokines also have a causal role in ischemic preconditioning and postconditioning. In particular, TNFα mediates protection through activation of TNF receptor, STAT3 and mitochondrial K_{ATP} channels, as part of the Survivor Activating Factor Enhancement (SAFE) pathway [42]. Interleukin 6 with subsequent STAT 3 signaling is important for ischemic preconditioning, as is interleukin 10 in remote ischemic conditioning [31]. Calcitonin gene-related peptide (CGRP) is also involved in the conditioning phenomenon, although there is limited data on its role [43].

2.2.3. Intracellular mediators of conditioning

Protein kinase C (PKC) plays a major role in conditioning. However, different isoforms of PKC serve contrasting functions, and intracellular translocation/colocalization of different isoforms is important [44]. The PKCε isoform confers protection by translocation to the mitochondria through a heat shock protein-dependent import, where it activates mitochondrial K_{ATP} channels during ischemic preconditioning to increase ROS formation, which then further activate PKCε in a positive feedback loop [45]. PKCε is also translocated to the sarcoplasmic reticulum where it reduces calcium content. PKC activation, mainly through the PKCε isoform, is also involved in ischemic postconditioning protection, through similar mechanisms (K_{ATP} channels and ROS formation). There is a positive feedback loop between PKC activation and adenosine: adenosine can directly activate PKC, while PKC sensitizes the A_{2B} adenosine receptor during early reperfusion and thereby contributes to ischemic preconditioning [46]. PKC is also a target of CGRP [43].

Nitric oxide, Protein kinase G (PKG) and cGMP are also intracellular mediators of conditioning. Endothelial nitric oxide synthase is activated after G protein-coupled receptor (GPCR) activation through a sequence of signals involving PI3K and Akt. The resulting nitric oxide activates soluble guanylate cyclase to form cGMP, which then activates PKG. PKG contributes to ischemic preconditioning by influencing K_{ATP} channel activity and subsequent ROS formation [47]. PKG is also involved in

ischemic postconditioning through the Na^+/K^+ exchanger, which delays the normalization of acidosis [48].

Hausenloy introduced the concept of a Reperfusion Injury Salvage Kinase (RISK) pathway, which consists of PI3K, Akt, ERK and GSK-3β components [40]. Activation of PI3K and its downstream target Akt and GSK-3β, and activation of ERK by the trigger adenosine are involved in ischemic preconditioning and postconditioning cardioprotection [40]. GSK-3β is proposed to be the downstream point of convergence of the RISK pathway, and when phosphorylated (and thus inhibited) acts to inhibit mPTP opening [31,40,42,49–53]. Akt activation can lead to morphological protection of mitochondria [31,54].

Lecours introduced the concept of a SAFE pathway, consisting of JAK and STAT-3 components [42]. Both TNFα and TNFα receptors are upregulated in myocardial cells after ischemic stimuli, exerting protective effects on cardiac cells during reperfusion via the SAFE pathway, by activating JAK and STAT-3 kinases [42]. Like the RISK pathway, the SAFE pathway is involved in both ischemic pre- and postconditioning [55]. Moreover, the SAFE pathway might be working parallel to or upstream of the RISK pathway and the RISK pathway must be functional for the SAFE pathway to work fully, highlighting the cross-talk between both pathways [7,23,31].

Upregulated proteins that act as mediators for delayed cardioprotection (SWOP) are inducible NOS, COX2, superoxide dismutase, aldose reductase, and heme oxygenase [31,33]. During preconditioning cycles, the generation of trigger molecules such as ROS, nitric oxide, and adenosine initiate a complex signal transduction cascade, including activation of GPCRs, tyrosine kinases and Janus kinases, as well as transcription factors such as nuclear factor B, STAT 1 and 3, ultimately inducing gene transcription and increasing the expression of cardioprotective proteins that play a role in the SWOP [8,34].

2.2.4. Effectors of cardioprotective conditioning

Mitochondria are the most important effectors of ischemic conditioning cardioprotection, via the mPTP, K_{ATP} channels, and STAT3. Mitochondria provide ATP for the maintenance of ionic gradients, thereby maintaining cell integrity. Ischemia with its inherent lack of oxygen supply inhibits the flow of electrons along the respiratory chain, inducing mitochondrial membrane depolarization and limiting the formation of ATP. Inner membrane depolarization, high concentrations of inorganic phosphate and ROS during ischemia, and more importantly during reperfusion, favor mPTP opening. Preventing mPTP opening is the point of convergence of cardioprotection.

The K_{ATP} channels are also important effectors of cardioprotective conditioning. It was initially thought that sarcolemmal K_{ATP} channels, which contribute to action potential duration shortening during ischemia, and hence alleviating calcium overload, were the main effector. However, it is now realized that the predominant site of cardioprotection signaling involving K_{ATP} channels is in the mitochondria, notably in the inner mitochondrial membrane [56]. The mitochondrial K_{ATP} channel is target of nitric oxide, PKC and PKG. When activated, it releases ROS, which in turn, activates PKCε in a positive feedback loop [31]. STAT 3, as part of the SAFE pathway, is a transcription factor involved in ischemic pre- and postconditioning cardioprotection. A mitochondrial location and function has been identified: STAT 3 is located in the mitochondrial matrix and serves to increase complex I respiration and attenuate mPTP opening and ROS formation [57].

The sarcoplasmic reticulum is also an effector of cardioprotective conditioning. Excessive calcium oscillations during early reperfusion, when sarcoplasmic reticulum and contractile machinery have been reenergized in the presence of cytosolic calcium overload contribute to uncoordinated contracture and eventual cellular disruption, rendering the sarcoplasmic reticulum a potential target of protection during early reperfusion. There is also close interaction between sarcoplasmic reticulum and mPTP during reperfusion, since calcium oscillations contribute to mPTP opening [58,59]. The sarcoplasmic reticulum is a target of PKCε and PKG, which lead to increased phosphorylation of the ryanodine receptor and improved function of the sarcoplasmic reticulum ATPase, preventing cytosolic calcium overload and oscillations [31,58,59].

The cytoskeleton may also serve as an end effector of ischemic conditioning. Decreased cytoskeletal stability and increased cell volume contribute to membrane disruption and cellular fragility. Ischemic conditioning attenuates osmotic fragility through stabilization of the actin cytoskeleton, via activation of PKCε [60].

2.2.5. Mechanisms involved in remote ischemic conditioning

The transfer of the cardioprotective signal from remote organs to the heart is the result of a complex neurohumoral interaction. Ischemia reperfusion induces activation of sensory fibers in the remote organ involving the PKCγ isoform. This activates the autonomic cardiac innervation through the central nervous system and releases an as yet unidentified humoral factor [31].

3. Part II: impact of diabetes mellitus

Of morbidities that potentially compromise the protective mechanisms of the heart, diabetes mellitus appears primarily important to study. Diabetes, from a variety of complications including cardiovascular disease, is an important cause of mortality and morbidity worldwide [11]. Susceptibility to ischemia reperfusion injury is increased in diabetes [12,13]. The mechanisms responsible for the exacerbation of ischemia reperfusion injury in diabetes have not been fully elucidated, but increased basal oxidative stress (excessive ROS production and/or endogenous antioxidant defense system depletion) likely plays an important role.

It is somewhat paradoxical that increased basal oxidative stress contributes to the increased susceptibility to ischemia reperfusion injury in the setting of diabetes mellitus, since ROS generation is an important component of cardioprotective conditioning maneuvers. The reasons for this discrepancy are not clear, but at least two possibilities can be proposed. First, that the time course of induction of ROS may be different in the two settings. Diabetes is associated with sustained oxidative stress, while conditioning maneuvers induce 'pulsed' and transient production of ROS. Second, that the threshold level of ROS (below which a protective effect is obtained, but above which a deleterious effect is observed) is surpassed with diabetes. In other words, conditioning mediates protection through low levels of ROS whereas diabetes is associated with high levels of ROS.

Additionally, ischemic conditioning strategies seem to be ineffective in diabetes, and this appears to be because of an impairment of prosurvival transduction pathways [14–16]. Diabetes leads to alterations in many of the different components that constitute the trigger, mediator and effector phases of cardioprotection.

3.1. A. Increased basal oxidative stress in diabetes

Previous studies have shown that diabetes mellitus is associated with increased susceptibility to ischemia reperfusion injury [12,13,61–66]. The underlying mechanisms responsible for this exacerbation are not clearly understood, but a higher basal level of oxidative stress secondary to hyperglycemia may play an important role [67–69]. Increased oxidative stress in diabetes is primarily due to an imbalance between ROS production and clearance by endogenous antioxidant defense systems. The magnitude of this imbalance likely contributes to the severity of ischemia reperfusion injury in the context of diabetes mellitus [14,16,70,71]. Both mitochondrial (hyperglycemia-induced superoxide overproduction and depletion of antioxidant defense systems) and non-mitochondrial (activation of NADPH oxidase and xanthine oxidase, and uncoupling of NOS) sources contribute to enhanced oxidative stress

in diabetes mellitus. Oxidative stress is a key player in diabetic cellular injury [14–16,69–84].

3.1.1. Mitochondrial sources of oxidative stress

Mitochondria are a major intracellular source of ROS. Approximately 4% of oxygen consumed by mitochondria is converted to ROS [85]. Diabetes mellitus has been considered to be a mitochondrial disorder, at least in part, because the mechanisms of hyperglycemia-induced injury, such as increased polyol pathway flux, increased advanced glycation end-product (AGE) formation, activation of protein kinase C (PKC) isoforms, and increased hexosamine pathway flux, reflect a single hyperglycemia-induced process: overproduction of superoxide by the mitochondrial electron transport chain [86,87].

Intracellular glucose oxidation begins with glycolysis in the cytoplasm, generating NADH and pyruvate. Cytoplasmic NADH may donate reducing equivalents to the mitochondrial electron-transport chain via two shuttle systems, or it can reduce pyruvate to lactate, which then exits the cell to provide a substrate for hepatic gluconeogenesis. Pyruvate may also be transported into the mitochondria, where it is oxidized by the tricarboxylic acid cycle to produce CO_2, H_2O, four molecules of NADH and one molecule of $FADH_2$. Mitochondrial NADH and $FADH_2$ enable the production of ATP through oxidative phosphorylation by providing electrons to the mitochondrial respiratory chain that is made up of four complexes, cytochrome c and the mobile electron carrier, ubiquinone. Regardless of whether NADH is derived from cytosolic glycolysis or mitochondrial activity, NADH donates electrons to Complex I, which ultimately transfers electrons to ubiquinone. Electrons donated from Complex II can also reduce ubiquinone. Electrons from reduced ubiquinone are then transferred to Complex III and proceed through cytochrome c, Complex IV and finally O_2. Electron transfer through Complexes I, III and IV generates a proton gradient that drives ATP synthase, otherwise known as Complex V (Fig. 1).

In diabetes, elevated levels of hyperglycemia-derived electron donors, from the tricarboxylic acid cycle (NADH and $FADH_2$), generate a high mitochondrial membrane potential by pumping protons across the inner mitochondrial membrane. When the electrochemical potential difference across the inner mitochondrial membrane is high, the half-life of superoxide-generating electron-transport intermediates, such as ubisemiquinone, is prolonged. As a result, superoxide production is markedly increased (Fig. 1) [72,73,88,89].

Excess superoxide partially inhibits the glycolytic enzyme GAPDH, thereby diverting upstream metabolites from glycolysis into glucose overutilization pathways. This results in an increased flux of dihydroxyacetone phosphate to diacylglycerol, an activator of PKC, and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Increased flux of fructose-6-phosphate to UDP-N-acetylglucosamine increases modification of proteins by O-linked N-acetylglucosamine. Furthermore, increased glucose through the polyol pathway consumes NADPH and depletes GSH [87]. These changes in intracellular glucose metabolism contribute to progressive organ injury. Furthermore, chronic elevated oxidative stress in diabetes may impair mitochondrial energy metabolism, leading to mitochondrial dysfunction and contributing to a vicious cycle of cell damage [1,81].

3.1.2. Non-mitochondrial sources of oxidative stress

The three predominant extra-mitochondrial systems that produce ROS are NADPH oxidase, xanthine oxidase, and uncoupled nitric oxide [90].

NADPH oxidase, a transmembrane enzyme, is located in intracellular organelles and contributes to the generation of superoxide. It mediates the penetration of electrons from NADPH across membranes of intracellular organelles and produces superoxide in the cytosol, by catalyzing the transfer of electrons from NADPH to molecular oxygen [91]. Several NADPH oxidase isoforms have been identified: Nox1-5, Duox 1, Duox 2. Activation of the membrane-bound Nox isoforms, Nox1 and Nox2, is dependent on recruitment and phosphorylation of several cytosolic subunits, including p47phox, which has been implicated in the generation of superoxide under high glucose conditions [91]. Elevated NADPH oxidase activity has been observed in diabetic animal models [68,92]. In diabetic patients, the NADPH oxidase system and the NADPH oxidase protein subunit expression levels were significantly increased [67]. NADPH oxidase inhibitors were also shown to significantly inhibit the elevated free radical formation in diabetic animals, suggesting that NADPH oxidases and ROS could be targeted to maintain glucose balance in the diabetic state [16,70,93,94].

Xanthine oxidase is involved in catalyzing the oxidation of hypoxanthine to xanthine, and the latter is further converted to uric acid. Plasma activity of xanthine oxidase is increased in diabetic mice, which was associated with an increased level of superoxide in blood [90,95].

Finally, uncoupling of nitric oxide synthase in diabetes leads to excessive superoxide anion production and diminishes nitric oxide availability [67]. In fact, superoxide anion rapidly reacts with nitric oxide, reducing nitric oxide bioactivity and producing oxidative peroxynitrite radicals. Nitric oxide confers antioxidant effects through direct scavenging of superoxide. However, nitric oxide synthase may be a source of superoxide production in diabetes because of enzymatic "uncoupling". High glucose enhances nitric oxide synthase expression and superoxide anion generation [96,97].

3.1.3. Impairment of antioxidant capacities

Clearance of ROS is decreased in diabetes, which also contributes to increased oxidative stress. Hyperglycemia can decrease antioxidant capacity by reducing SOD2, GPx and catalase activities [98–102]. Both SOD1, which is located in the cytoplasm, and SOD2, which is located in the mitochondria, catalyze the reaction that converts superoxide anion to hydrogen peroxide. Hydrogen peroxide is then converted to water by either catalase in peroxisomes or by GPx in the cytoplasm. Catalase and GPx are far more effective than SOD1 for oxidative cell protection [102].

In diabetes, the decrease in SOD 2 causes a relative increase in SOD1. Cells with increased levels of SOD1 are hypersensitive to oxidative stress rather than protected from it [103]. This is because SOD1 increases the formation of hydrogen peroxide, which is not efficiently converted to water when catalase and GPx levels are reduced. Normally, an increase in SOD1 is accompanied by a concomitant increase in catalase and GPx. However, this adaptive response does not occur in diabetes, making cells more susceptible to the detrimental effects of oxidative stress [102].

3.2. B. Impaired ischemic conditioning in diabetes

3.2.1. Inefficiency of ischemic conditioning in diabetes

While several studies have focused on ischemic pre-, post- and remote conditioning, only a few studies have investigated the effect of diabetes mellitus on ischemic conditioning [12,104–106].

The majority of preclinical experimental studies indicate that the presence of diabetes renders the heart more resistant to the infarct size-limiting effects of preconditioning, while considering differences in injury model and study design. Preconditioning either fails to reduce myocardial infarct size, or the efficacy of conditioning is attenuated since an amplified stimulus with increased numbers of episodes is required to evoke protection [107–118]. A similar lack of effect also applies to postconditioning studies [118–125].

Taken together, these findings suggest that the myocardium is resistant to ischemic conditioning stimuli in diabetes. Ischemic conditioning has been described as a 'healthy heart phenomenon', because aging and obesity also impair conditioning. The refractoriness of the diabetic heart to ischemic conditioning is largely related to changes in protective signal transduction pathways.

3.2.2. Adenosine signaling alterations

As described before, the release of adenosine is known to play a key role in mediating the cardioprotective effect of ischemic conditioning. Adenosine is produced in the extracellular space, first by hydrolysis of ATP or ADP to AMP through apyrase (CD39) and subsequently by conversion of AMP into adenosine through ecto 5′nucleotidase (CD73) [126]. The extracellular concentration of adenosine is controlled by reuptake processes triggered by nucleoside transporters (equilibrative and concentrative nucleoside transporters) or adenosine kinase. Adenosine deaminase is the enzyme responsible for adenosine breakdown, converting adenosine to the nucleoside inosine.

The suppressed effect of ischemic conditioning in the diabetic myocardium may be at least in part related to reduced adenosine availability, both due to decreased release and increased intracellular uptake. It has been reported that diabetes mellitus is associated with a reduced ability of cardiac fibroblasts to release adenosine during ATP deprivation [127]. Moreover, the expression of CD73 is decreased in the diabetic state, leading to impaired adenosine release [128]. Adenosine must be released at two important times for protection to occur. Following onset of the preconditioning ischemia, adenosine is released and serves as trigger for protection. During the subsequent prolonged ischemia, adenosine again must be released to mediate the protection. However, during the second period of ischemia, tissue pH remains less acidic. This relatively elevated pH causes increased adenosine kinase activity, which in turn results in less adenosine accumulation. Activation of CD73 opposes this effect and guarantees that a sufficient amount of adenosine will be present during the subsequent occlusion. Activation of CD73 serves as a positive feedback system to reinforce protection. This protective feedback is lost in the diabetic state, due to the decreased expression of CD73 [128]. On the other hand, adenosine reuptake is increased in the diabetic heart, due to an increased expression of nucleoside transporters and adenosine deaminase [127]. Overall, these findings suggest that a reduced availability of adenosine contribute to the loss of effect of preconditioning in the diabetic state.

3.2.3. RISK pathway alterations

The complete or partial failure of conditioning to limit myocardial infarct size in models of type 2 and type 1 diabetes mellitus has been importantly attributed to defects in the RISK pathway, and this occurs through many mechanisms.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is considered to be a key negative regulator of the PI3K/Akt pathway. The persistent hyperglycemia of diabetes has been shown to increase the expression and activity of PTEN [129,130]. Oxidative stress is involved in the increased activity of PTEN in the diabetic state. Hyperglycemia induces superoxide anion production and thereby peroxynitrite anion overproduction. This peroxynitrite anion generated by hyperglycemia blocks PI3K/Akt activation by up-regulating PTEN [129,130]. Moreover, a positive correlation between blood glucose levels, oxidative stress and PTEN levels have been detected in human diabetic myocardium [131]. In addition, high levels of circulating free fatty acids also impair insulin-stimulated activation of PI3K/Akt because free fatty acids have been shown to up-regulate PTEN expression [131]. Other phosphatases, such as MAPK phosphatases or protein phosphatase 2C are also implicated in blocking PI3K/Akt activation [105].

Insulin deficiency or resistance is also involved in RISK pathway alterations. Insulin normally leads to Akt activation by phosphorylation at Thr308 and Ser473. Activated Akt phosphorylates GSK-3β, inhibiting mPTP opening [12,83,115,116]. Therefore, insulin deficiency or resistance impacts this signal transduction pathway [132–134].

Diabetes-induced impairment of ERK1/2 activities has also been demonstrated [118,119,135,136]. Miki et al. reported that the presence of augmented endoplasmic reticulum stress in the diabetic heart blocked ERK1/2-mediated phosphorylation of GSK-3β, leading to increased susceptibility to mPTP opening and calcium overload [137].

3.2.4. SAFE pathway alterations

Loss of cardioprotection in the diabetic state may be due to an upregulation of calcineurin activity through angiotensin II-independent activation of angiotensin-II type 1 receptors, leading to direct impairment of phosphorylation of Janus Kinase 2, which is an upstream signal to STAT3 signaling [138,139].

3.2.5. Endothelial NOS signaling alterations

The diabetic myocardium is associated with a marked reduction in endothelial NOS activation, which is one of the mechanisms contributing to the inefficiency of ischemic conditioning strategies [106,135]. This reduction in endothelial NOS activation may involve caveolin, which is increased in the diabetic heart and interferes with endothelial NOS activity [140].

3.2.6. Calcitonin gene-related peptide signaling alterations

As reviewed before, CGRP release is involved in mediating cardioprotection. CGRP levels are decreased in the diabetic myocardium and may also play a role in the impaired response to conditioning [141].

3.2.7. Cardioprotective effector alterations

Finally, downstream targets and proposed end-effectors of ischemic- conditioning-induced cardioprotection may also be modified in the setting of diabetes mellitus. These include alterations in expression and activity of K_{ATP} channels (leading to increased ROS production) and increased propensity of mPTP opening in response to increased intracellular Ca^{2+} concentrations in the diabetic myocardium [142,143].

3.3. C. Conditioning and diabetes in the clinical settings

3.3.1. Cardiac consequences of diabetes in patients

The successful clinical translation of conditioning strategies to attenuate myocardial ischemia reperfusion injury has been identified as a major unmet need. This issue is of particular relevance and importance to diabetic patients as highlighted by the more than 2 fold greater incidence of cardiovascular disease and acute coronary syndromes, evidence of larger infarct sizes, and 2- to 4- fold greater incidence of cardiovascular disease-related mortality in diabetic versus non diabetic patients [144–148].

The wealth of preclinical evidence documenting reduction of infarct size with pre-, post- and remote conditioning has provided the groundwork and rationale for ongoing efforts to translate the concept of endogenous conditioning-induced cardioprotection for the clinical treatment of myocardial injury.

Ischemic preconditioning has been successfully translated to patients, but since it is by definition a pretreatment, clinical use is limited to planned ischemic events such as cardiac surgery and percutaneous coronary interventions. Thus, current attention is focused largely on postconditioning and remote conditioning strategies to alleviate ischemia reperfusion injury in the emergency unplanned situation. Both ischemic preconditioning and postconditioning involve manipulation of the atherosclerotic culprit lesion with the risk of coronary microembolization. This is particularly true in ischemic postconditioning, but studies of patient outcomes are absent. Remote ischemic conditioning has gained interest because it is the most attractive method of inducing cardioprotection, as it is both safe and easily feasible.

To date, results from about 40 phase II clinical trials including all forms of ischemic conditioning have been reported and reviewed [36,149]. All conditioning strategies seem to attenuate reperfusion injury; whereas ischemic preconditioning delays infarct development, ischemic postconditioning actually decreases infarct size. However, two recent meta-analyses underscored the variability among studies and concluded that there is reasonable evidence for cardioprotection with ischemic preconditioning, but borderline evidence for cardioprotection with either postconditioning or remote conditioning [150,151]. In addition to differences in patient demographics and enrollment criteria,

protocol logistics and choice of endpoints, the differing proportions of diabetic patients in the studies may contribute to the variability in outcomes.

3.3.2. Ischemic pre-, post- and remote conditioning in diabetic patients

Initial evidence appears to support the concept that preconditioning-induced cardioprotection is impaired or lost in diabetic patients [130,152,154]. In clinical trials in which prospective subset analyses were performed and cardiac enzyme release served as the surrogate for infarct size, preconditioning had no beneficial effect in diabetic patients [155].

There have been no clinical trials specifically investigating the effect of postconditioning on ischemia reperfusion injury in diabetes mellitus. Indirect evidence for the failure of ischemic postconditioning in diabetes comes from a large retrospective study of patients who underwent a postconditioning protocol for acute myocardial infarction, in whom multiple balloon inflations served as the postconditioning stimulus. Diabetes abolished the effects of postconditioning in a subgroup analysis [104,155]. Moreover, it was reported that postconditioning tended to exacerbate myocardial injury in the diabetic cohort [155].

Concerning remote ischemic conditioning, a clinical study showed that there was no reduction in the incidence or magnitude of myocardial injury in older patients with diabetes [156]. However, it is difficult to distinguish the effect of diabetes from the effects of age in this study. Jensen et al. investigated the role of neural and circulating humoral components, reporting that plasma from non-diabetic or diabetic patients exposed to remote ischemic conditioning protocol mediated cardioprotection in rabbit hearts subjected to ischemia–reperfusion [157]. In this regard, plasma from diabetic patients with peripheral neuropathy failed to produce this cardioprotective response in rabbit hearts [158]. This finding supports the notion that an intact neural pathway from the conditioned organ or tissue to the target organ is required to elicit the protective humoral factor. Similar findings were published using ex vivo human atrial tissue, which was only protected by a humoral factor from control patients, but not from diabetic patients, supporting the notion that humoral factors may also play an important role [159].

3.4. D. Restoring myocardial sensitivity to ischemic conditioning in the setting of diabetes

Since the defects that contribute to impaired cardioprotection in the diabetic myocardium involve many signal transduction pathways, it is reasonable to ask whether a therapy that targets one pathway will lead to a clinical benefit or be circumvented by the other defects in signaling. In this regard, interventions have focused on points of convergence in the various signal transduction pathways and targeted the final effectors of cardioprotection (either GSK3-β of mPTP) in order to overcome the resistance of the diabetic heart to ischemic conditioning [114].

Yadav et al. *showed that pharmacologic inhibition of GSK3-β by lithium chloride, indirubin-3 monooxime, or SB216763 was able to reduce infarct size in the diabetic heart* [116]. *Moreover, Jamwal* et al. *reported that pre- and post-ischemic treatment with the GSK3-β inhibitors, zinc chloride and zinc ionophore pyrithione, restored the cardioprotective potential of ischemic preconditioning in the diabetic heart* [160]. *This approach has been confirmed in other studies. Badalzadeh* et al. *demonstrated that the isolated diabetic rat heart was resistant to cardioprotection by either ischemic postconditioning or cyclosporine-A (mPTP opening inhibitor) alone, but that a combination of interventions led to a significant reduction in infarct size* [161]. *Interestingly, Najafi* et al. *reported that the inhibition of mPTP opening was sufficient to restore cardioprotection by postconditioning in diabetic hearts* [162].

The underlying mechanisms responsible for the effect of diabetes on conditioning strategies have not been fully elucidated, but higher basal levels of oxidative stress secondary to hyperglycemia may play an important role [67–69]. *In this regard, the effects of treatment strategies for diabetes on conditioning responses have been studied by several investigators. Gu* et al. *found that simvastatin treatment restored cardioprotection elicited by ischemic preconditioning in the presence of hyperglycemia* [163]. *Hausenloy* et al. *reported that treatment with the sulfonylurea, glimepiride, to lower blood glucose levels restored the sensitivity of the myocardium to ischemic preconditioning. They reported that one cycle instead of three cycles of ischemic preconditioning was sufficient to limit infarct size* [164]. *They also showed that administration of dipeptidyl peptidase-4 inhibitors (which lower blood glucose by augmenting endogenous levels of glucagon-like peptide-1) conferred cardioprotection in diabetic rats* [165]. *Przyklenk* et al. *reported that the transplantation of pancreatic islet tissue reversed the effect of diabetes on the myocardial response to postconditioning and conferred cardioprotection* [119–165].

Since diabetes is associated with increased basal oxidative stress, and since the imbalance between ROS production and endogenous antioxidant defense systems contributes to the severity of ischemia reperfusion injury in diabetes, we believe that newer pharmacologic approaches to cardioprotection will also focus on reducing oxidative stress by enhancing antioxidant systems. Ultimately, a combination of approaches that target both the oxidative response to hyperglycemia as well as the final common signaling molecules and effectors would seem to be the best strategy for restoring the cardioprotective response to conditioning strategies in diabetes.

4. Conclusion

Ischemia reperfusion injury remains an important clinical problem and its pathogenesis is complex and multifactorial. Although ischemic conditioning is a well-established cardioprotective strategy against ischemia reperfusion injury, growing evidence clearly suggests that the benefit of ischemic conditioning is abrogated in the diabetic state. Impaired activation of the PI3K/Akt/GSK3-β signaling pathway, impaired phosphorylation of ERK1/2, decreased generation and release of nitric oxide and CGRP, dysfunction of K_{ATP} channels and elevated oxidative stress including from mitochondrial dysfunction are highly integrated in the diabetic heart and may account for the lack of benefit of conditioning strategies in diabetes. *A better understanding of mechanisms underlying conditioning-associated cardioprotection should help to better utilize protective mechanisms of conditioning to lessen the inevitable injury associated with ischemia reperfusion. Furthermore, this would also help to tailor conditioning strategies so that cardioprotection can also be achieved in patients with diabetes.*

Disclosure

None of the authors have any disclosure with respect to the content of the review.

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Diabetes Worsens Skeletal Muscle Mitochondrial Function, Oxidative Stress, and Apoptosis After Lower-Limb Ischemia-Reperfusion: Implication of the RISK and SAFE Pathways?

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Objectives: Diabetic patients respond poorly to revascularization for peripheral arterial disease (PAD) but the underlying mechanisms are not well understood. We aimed to determine whether diabetes worsens ischemia-reperfusion (IR)-induced muscle dysfunction and the involvement of endogenous protective kinases in this process.

Materials and Methods: Streptozotocin-induced diabetic and non-diabetic rats were randomized to control or to IR injury (3 h of aortic cross-clamping and 2 h of reperfusion). Mitochondrial respiration, reactive oxygen species (ROS) production, protein levels of superoxide dismutase (SOD2) and endogenous protective kinases (RISK and SAFE pathways) were investigated in rat gastrocnemius, together with upstream (GSK-3β) and downstream (cleaved caspase-3) effectors of apoptosis.

Results: Although already impaired when compared to non-diabetic controls at baseline, the decline in mitochondrial respiration after IR was more severe in diabetic rats. In diabetic animals, IR-triggered oxidative stress (increased ROS production and reduced SOD2 levels) and effectors of apoptosis (reduced GSK-3β inactivation and higher cleaved caspase-3 levels) were increased to a higher level than in the non-diabetics. IR had no effect on the RISK pathway in non-diabetics and diabetic rats, but increased STAT 3 only in the latter.

Conclusion: Type 1 diabetes worsens IR-induced skeletal muscle injury, endogenous protective pathways not being efficiently stimulated.

Keywords: diabetes, ischemia-reperfusion, peripheral arterial disease, protective kinases, muscles, mitochondria

INTRODUCTION

Diabetes is a major risk factor for peripheral arterial disease (PAD) and in patients with diabetes, PAD is more severe and has a poorer response to revascularization (Jude et al., 2001; DeRubertis et al., 2008; Malmstedt et al., 2008). Yet, the mechanisms linking diabetes to worse outcomes in PAD are not well understood. Diabetes *per se* impairs skeletal muscle mitochondrial function (Kelley et al., 2002; Bonnard et al., 2008; Anderson et al., 2009). In type 1 diabetic patients the skeletal muscles present reduced mitochondrial oxidative phosphorylation, even without obvious vascular abnormalities (Karakelides et al., 2007) and the mitochondrial impairment may even precede hyperglycemia in type 2 diabetes (Petersen et al., 2004). These data suggest that mitochondrial dysfunction may be a common pathway of diabetes and PAD severity.

When PAD ischemia becomes critical, blood flow has to be reestablished by revascularization. However, the return in blood flow causes additional muscle damage. This paradoxical and detrimental effect is known as ischemia-reperfusion injury (IRI). Although the cause of PAD is occlusive arterial disease, muscle mitochondrial dysfunction is also critical for the severity of PAD. These abnormalities include impaired mitochondrial respiration and increased oxidative stress, and are present in both chronic PAD and after ischemia-reperfusion (IR) (Pipinos et al., 2006; Makris et al., 2007; Tran et al., 2012; Guillot et al., 2014; Lejay et al., 2014, 2017; Paradis et al., 2016). While it is generally assumed that muscle mitochondria are more susceptible to IRI in diabetic subjects, data supporting this assertion are lacking. Very little is known regarding the magnitude of the mitochondrial dysfunction and the mechanisms involved in this process.

It is well established that myocardial IR induces the reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) protective pathways (Lecour, 2009; Rossello and Yellon, 2017). Decreased myocardial activation of RISK and SAFE increases the susceptibility of the diabetic heart to IRI (Tsang et al., 2005; Drenger et al., 2011). Acute activation of RISK and SAFE effectors appear to converge on the mitochondria and avert cell damage. In brief, RISK signaling involves protein kinase B (Akt) phosphorylation which, in turn, phosphorylates and inactivates glycogen synthase kinase 3β (GSK-3β). Inactivated GSK-3β cannot induce mitochondrial permeability transition pore (mPTP) opening anymore, thus preventing mitochondrial dysfunction and apoptosis (Juhaszova et al., 2004). SAFE activation involves phosphorylation and hence, activation of signal transducer and activator of transcription 3 (STAT3). Activated STAT3 stimulates mitochondrial respiration, inhibits mPTP opening and attenuates apoptosis. Yet, the involvement of these pathways in skeletal muscle IR is unknown.

The purpose of this study was to investigate the impact of type 1 diabetes on the skeletal muscle IRI and examine the activation of the RISK and SAFE pathways. We assessed mitochondrial respiration, oxidative stress, and effectors of apoptosis and of RISK and SAFE pathways, in streptozotocin-treated, type 1 diabetic rats, in comparison to non-diabetic controls.

Abbreviations: Akt, protein kinase B; DHE, dihydroethidium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK-3β, glycogen synthase kinase 3β; IR, ischemia-reperfusion; IRI, ischemia-reperfusion injury; mPTP, mitochondrial permeability transition pore; PAD, peripheral arterial disease; RISK, reperfusion injury salvage kinases; ROS, reactive oxygen species; SAFE, survivor activating factor enhancement; SOD2, mitochondrial manganese superoxide dismutase; STAT3, signal transducer and activator of transcription 3; TMPD, N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride.

MATERIALS AND METHODS

Experimental Animals

Experiments were performed on 8-week-old male Wistar rats (Depré, Saint-Doulchard, France), either vehicle-treated or streptozotocin-treated to induce insulin-dependent type 1 diabetes. The study conformed to the "Principles of laboratory animal care" (NIH publication 85–23, revised 1985) and was approved by the Institutional Animal Care Committee (CREMEAS AL/02/10/06/2009).

Induction of Diabetes

Type 1 diabetes was induced in male rats by a single 65 mg/kg streptozotocin injection in the penile vein. Animals were considered diabetic when blood glucose was above 16.7 mmol/L, 8 days after induction of diabetes. Non-diabetic vehicle-treated animals received intravenous saline injection at the same time. Six weeks after diabetes induction, streptozotocin-treated and vehicle-treated rats were individually housed in metabolic cages for 2 days. After the first day (considered an acclimation period), blood glucose concentrations, food and water intake and urine output were recorded over a 24-h period.

Thirty days after diabetes induction, an oral glucose-tolerance test was performed in diabetic rats and non-diabetic animals to confirm the diabetic status of the formers. The test consisted in a 2 g/kg glucose loading given by oral gavage after a 12-h fasting period. Glucose concentrations were determined on tail vein blood at 20, 40, 60, 120, and 180 min after gavage.

Animal Surgical Preparation and Procedure

As previously described (Mansour et al., 2012), after performing a midline laparotomy under isoflurane anesthesia, the infra-renal abdominal aorta was dissected and freed from adjacent adhesions. All arterial collaterals located between the renal arteries and the aortic bifurcation, were coagulated and sectioned using electrocautery (Geiger®, thermal cautery unit).

Experimental Design

Seven weeks after vehicle or streptozotocin injection, rats (referred to as non-diabetic "n," and diabetic "d," respectively) were randomly assigned to the control (CON) or IR group. The control groups (nCON, dCON, 8 rats per group) underwent 5 h of isoflurane anesthesia and similar surgical manipulation to the IR groups, except for hindlimb ischemia (sham-operated). The ischemia–reperfusion groups (nIR and dIR, 10 rats per group) underwent 3 h of ischemia induced by infra-renal aortic occlusion and collateral vessel ligation, followed by 2 h of reperfusion. Ischemia was clinically characterized by cyanosis

and lack of an arterial pulse distal to the clamp, and biochemically by an increase in capillary blood lactate measured in the right hindlimb (Lactate Pro device, LT1710; Arkray, KGK, Japan).

After reperfusion, gastrocnemius muscles, that are considered more sensitive to IR (Charles et al., 2017), were harvested and either analyzed immediately (mitochondrial respiration) or kept in ice or in liquid nitrogen-cooled isopentane. Animals were sacrificed by heart retrieval under deep anesthesia (5% isoflurane).

Study of Muscle Mitochondrial Respiration in Skinned Fibers

Mitochondrial respiration was studied in saponin-skinned fibers of white gastrocnemius muscle (glycolytic muscle), as previously described (Talha et al., 2013). Fibers were separated and subsequently permeabilized in a bath of solution S containing 50 μg/ml saponin for 30 min at 4°C, under shaking. Permeabilized fibers were washed for 10 min under shaking, to remove the saponin, and placed in a bath with the respiratory solution for 5 min twice, in order to remove any phosphates. Finally, oxygen consumption was measured polarographically with a Clark-type electrode in a 3 ml oxygraphic cell (Strathkelvin Instruments, Glasgow, Scotland) at 22.1°C in incubation buffer using 5 mM glutamate and 2.5 mM malate as substrates for complex I, or 25 mM succinate (in combination with 0.02 mM amytal to inhibit complex I) as substrate for complex II.

After recording of basal oxygen consumption (V_0), maximal fiber respiration (V_{Max}) rate was measured under continuous stirring in the presence of a saturating amount of ADP (2 mM) as a phosphate acceptor. Relative contributions of the respiratory chain complexes I, III and IV to the global mitochondrial respiratory rates were also determined. When V_{Max} was recorded, the electron flow went through complexes I, III, and IV. For determining V_{Succ}, complex I was blocked with amytal (0.02 mM) and complex II was stimulated with succinate (25 mM). Mitochondrial respiration in these conditions allowed to determine the contribution of complexes II, III, IV activities. Thereafter, N, N, N′, N′-tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5 mM) and ascorbate (0.5 mM) were added as an artificial electron donor to cytochrome c. In these conditions, the activity of cytochrome c oxidase (complex IV) was determined as an isolated step of the respiratory chain (V_{TMPD}). In all cases, mitochondrial respiration assays in skinned fibers were performed immediately after harvesting. Fibers were then dried for 15 min at 150°C and respiration rates were expressed as μM O_2/min/g dry weight.

Assessment of Oxidative Stress in Skeletal Muscles

Assessment of Reactive Oxygen Species Production in Skeletal Muscle by Dihydroethidium Staining

As described previously (Pottecher et al., 2013), 10 μm-thick serial sections of white gastrocnemius muscle were prepared using a cryostat microtome and incubated with dihydroethidium (DHE) that produces red fluorescence when oxidized to ethidium bromide (EtBr) by ROS, including superoxide anion (Li and Jackson, 2002; Sheetz and King, 2002; Charles et al., 2011). To assess ROS production in each skeletal muscle section, mean fluorescence intensity (arbitrary units) was determined in 25 regions of interest under 20× epifluorescence magnification.

As ROS production by diabetic skeletal muscle mitochondria may be confounded by concurrent changes in respiration rates, this may leave raw, unadjusted ROS levels unchanged, while specific ROS production may increase, when considered relative to electron transport (Herlein et al., 2011). Consequently, the mean DHE fluorescence was divided by the mitochondrial respiration rate from skinned fibers.

Protein Levels of the Antioxidant Mitochondrial Manganese Superoxide Dismutase in Skeletal Muscles by Western Blot

This assessment is detailed below in the following paragraph.

Protein Extraction and Analysis

Gastrocnemius muscles were grounded in a mortar at 4°C in RIPA buffer [50 mM Tris pH 7.5, 1% Nonident P40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF and phosphatase and protease inhibitor cocktails according to the manufacturer's protocol (PhosphoStop and Complete-Mini EDTA free, Roche)]. Homogenates (50 μg of protein, $n = 3$–6 per group) were electrophoresed on polyacrylamide gels. Proteins were electroblotted onto nitrocellulose membranes using a Trans-blot turbo transfer system (Biorad) and immunodetected using primary antibodies directed against SOD2 (SOD-110, StressGen), Akt (4691, Cell Signaling), phospho-Akt Ser473 (4060, Cell Signaling), phospho-Akt Thr308 (4056, Cell Signaling), GSK-3β (610201, BD Biosciences), phospho-GSK-3β Ser9 (5558, Cell Signaling), STAT3 (9132, Cell Signaling), phospho-STAT3 Tyr705 (9131, Cell Signaling), cleaved caspase-3 (9661, Cell Signaling), tubulin and GAPDH (MAB374, Millipore Upstate Chemicon), according the manufacturer's instructions. Proteins were revealed with secondary antibodies conjugated to horseradish peroxidase (Amersham Biosciences) using an enhanced chemiluminescence detection system (ECLplus, GE Healthcare) and an ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare). Immunodetected proteins were quantified with the FIJI software and normalized to GAPDH levels (except cleaved caspase-3, which was normalized to tubulin levels).

Statistics

All data are expressed as mean ± standard error of the mean (SEM), and were analyzed using Prism software (GraphPad Prism 5, Graph Pad Software, San Diego, USA). Comparisons between two groups were performed by Student two-tailed *t*-test. Two-way analysis of variance (ANOVA) was applied to test simultaneously the main effects and interaction for diabetes status and ischemia. Comparisons between more than two groups were performed using one-way ANOVA with Newman-Keuls *post-hoc* correction for multiple comparisons. Repeated-measures ANOVA was conducted when appropriate. In all cases, a p-value < 0.05 was considered statistically significant.

RESULTS

Hemodynamic and Metabolic Changes in Diabetic and Non-diabetic Animals

Compared to vehicle-treated animals, streptozotocin-treated rats displayed typical features of type 1 diabetes with hyperglycemia, polyuria, polydipsia, polyphagia and decreased body weight. Baseline heart rate was similar in both groups although streptozotocin-treated rats exhibited mild arterial hypertension (164 ± 5, mmHg vs. 140 ± 3; $p < 0.001$). The absolute and relative maximal increases in blood glucose were higher in streptozotocin-treated than in the non-diabetic rats (+319 ± 9 mg/dL vs. +73 ± 4 mg/dL and 309 ± 24% vs. 200 ± 4%, respectively; $p < 0.001$) (**Figure 1**). Hind limb capillary blood lactate levels pre-IR were similar between streptozotocin- and vehicle-treated animals (2.8 ± 0.3 and 2.6 ± 0.4 mmol/L respectively, p = NS), and were similarly increased at the end of the 3 h ischemia (17.7 ± 0.5 vs. 18.9 ± 0.9 mmol/L respectively, p = NS).

Heart rate changes were also not statistically different between diabetic and non- diabetic animals at baseline, at the end of ischemia and after 2 h of reperfusion (data not shown). These results suggest that streptozotocin- and vehicle-treated animals were subjected to a similar ischemic insult.

Mitochondrial Respiratory Chain Complex Activities Are More Impaired After IR in Diabetic Animals Than in Controls

Among controls, oxygen consumption was halved in streptozotocin-treated rats as compared to non-diabetic rats (**Figure 2A**). Ischemia-reperfusion decreased V_{Max} in both diabetic and non-diabetic animals (**Figure 2A**). However, V_{Max} was significantly more reduced after IR in type 1 diabetic rats compared to the non-diabetics (−57 ± 14% vs. −23 ± 7%; $p < 0.05$, **Figure 2B**). While IR did not alter V_{Succ} in non-diabetic animals (−11 ± 6%; p = NS), it induced a significant decrease in V_{Succ} in diabetic rats (−60 ± 7%; $p < 0.001$ compared to non-diabetic animals). IR did neither alter $V_{TMPD/Asc}$ in streptozotocin- nor in non-diabetic animals (**Figure 2B**). These results show that, after IR, mitochondrial respiration is more impaired in diabetic than in non-diabetic animals.

Larger Increase in Oxidative Stress in Diabetic Animals

After IR, we observed a 2-fold increase in raw DHE fluorescence in vehicle-treated rats ($p < 0.001$). In contrast, DHE staining,

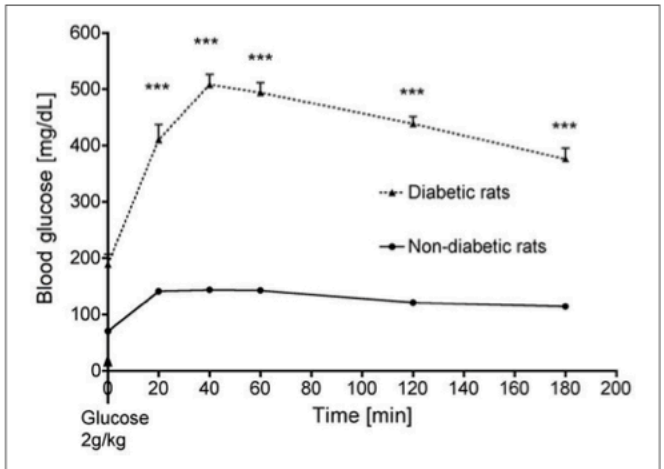

FIGURE 1 | Time course of blood glucose in streptozotocin-treated (diabetic) and non-diabetic rats during an oral glucose tolerance test. ***$p < 0.001$.

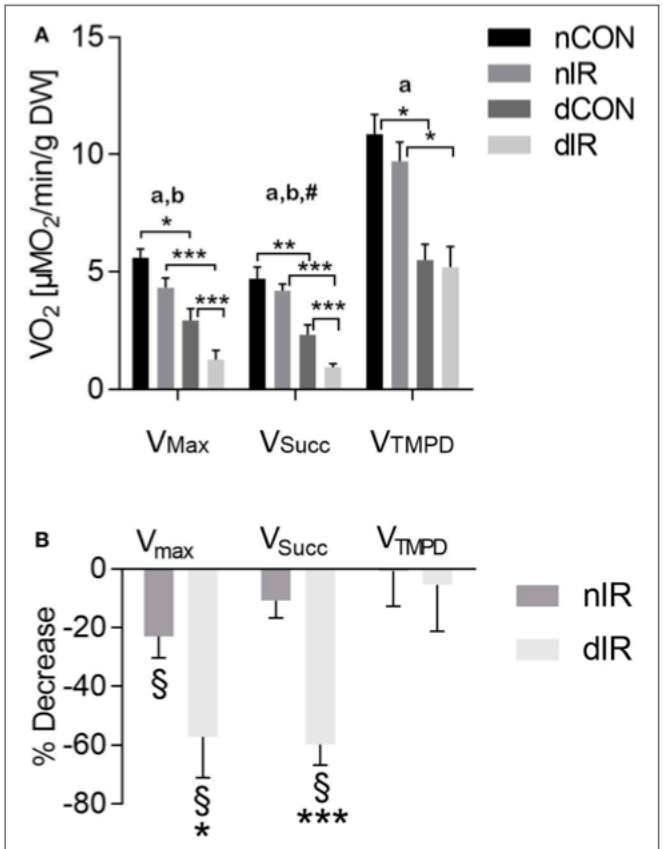

FIGURE 2 | Mitochondrial respiration in gastrocnemius muscles from vehicle-treated and streptozotocin-treated rats with and without hindlimb ischemia-reperfusion. **(A)** Mitochondrial respiration was determined in gastrocnemius muscles from sham-operated, vehicle-treated (nCON) or streptozotocin-treated rats (dCON), and after ischemia reperfusion in vehicle-treated (nIR) and streptozotocin-treated rats (dIR). Maximal fiber respiration (V_{Max}) rate, combined complexes II, III, IV activities (V_{Succ}) and isolated complex IV activity (VTMPD) were measured. Results are expressed as mean ± SEM [µM O_2/min/g dry weight tissue]. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, $^{a}p < 0.05$ for diabetes effect; $^{b}p < 0.05$ for IR effect; $^{\#}p < 0.05$ for interaction. **(B)** Variations in mitochondrial respiration in gastrocnemius muscles obtained after ischemia-reperfusion vs. sham operation in vehicle-treated ("nIR") and streptozotocin- treated rats ("dIR"). Results show a variation in oxygen consumption after ischemia- reperfusion vs. sham operation, expressed as percent decrease ± SEM. §indicates that the variation is significantly different from zero, while *$p < 0.05$ and ***$p < 0.001$ indicate that the variation is significantly different between diabetic and non-diabetic rats.

which was already enhanced in the diabetic rats, was not further increased after IR in this group (**Figures 3A,B**). However, ROS levels normalized either to V_{Max} (DHE/V_{Max}) or V_{Succ} (DHE/V_{Succ}) were significantly increased after IR in both diabetic and non-diabetic rats, but to a larger extent in diabetic animals (**Figure 3B**).

The antioxidant SOD2 level was conversely 3-fold lower in diabetic rats after IR (**Figure 3C**). Thus, diabetic muscles produced more ROS and had reduced antioxidant defenses after IR.

Safe and Risk Pathways Implications in Diabetic Rats After IR

To test whether the alterations on mitochondrial function and oxidative stress after IR were associated with activation of the endogenous protective kinases, we investigated the effector proteins of RISK (Akt) and SAFE (STAT3) pathways.

Among controls (no IR), the activated Akt (phosphorylated at the threonine 308 and the serine 473 residues) was higher in the non-diabetic rats ($p < 0.01$). After IR, the phosphorylated Akt levels did neither change in diabetic, nor in non-diabetic rats, indicating no RISK activation. However, both diabetic groups had consistently lower levels of activated Akt than their non-diabetic counterparts ($p < 0.01$; **Figures 4A,B**).

Regarding SAFE pathway, the active, phosphorylated form of STAT3 was not different between diabetic and non-diabetic rats who did not undergo IR. After IR, the phosphorylated STAT3 levels showed a significant 2-fold increase in the diabetic animals only ($p < 0.01$) (**Figure 4C**). Therefore, one effector of the SAFE pathway is activated by IR in diabetic but not in in the non-diabetic rats.

Decreased GSK-3β Inhibition and Increased Cleaved caspase-3 and Cell Damage in Diabetic Rats After IR

While the phosphorylated GSK-3β levels were not altered after IR in non-diabetic animals, they dropped markedly in diabetic rats ($p < 0.01$), resulting in a 2.5-fold decrease in phospho-GSK-3β/GSK-3β ratio (**Figure 5A**). Thus, after IR, the protective, phosphorylated GSK-3β was markedly lower in the diabetic than in the non-diabetic animals and was even lower compared to the diabetic rats that did not undergo IR ($p < 0.05$ and $p < 0.01$ respectively). Cleaved caspase-3 is a strong downstream effector of mitochondrial-mediated apoptosis and characterized as the "key executioner" of apoptosis.

IR was followed by an increased cleaved caspase-3 expression in gastrocnemius, which was non-significant in non-diabetic animals and highly significant in diabetic rats. Moreover, the increased expression of cleaved caspase 3 was 6-fold larger in the muscles of diabetic animals than in the muscles of their non-diabetic counterparts ($p < 0.0001$) (**Figure 5B**).

Taken together, the decreased levels of inactivated GSK-3β and the increased levels of cleaved caspase-3 in the diabetic animals after IR, denote a reduced protection against IRI and a strong trend for apoptosis (presumably through mPTP opening) and cell damage. Representative Western blots of the effector proteins

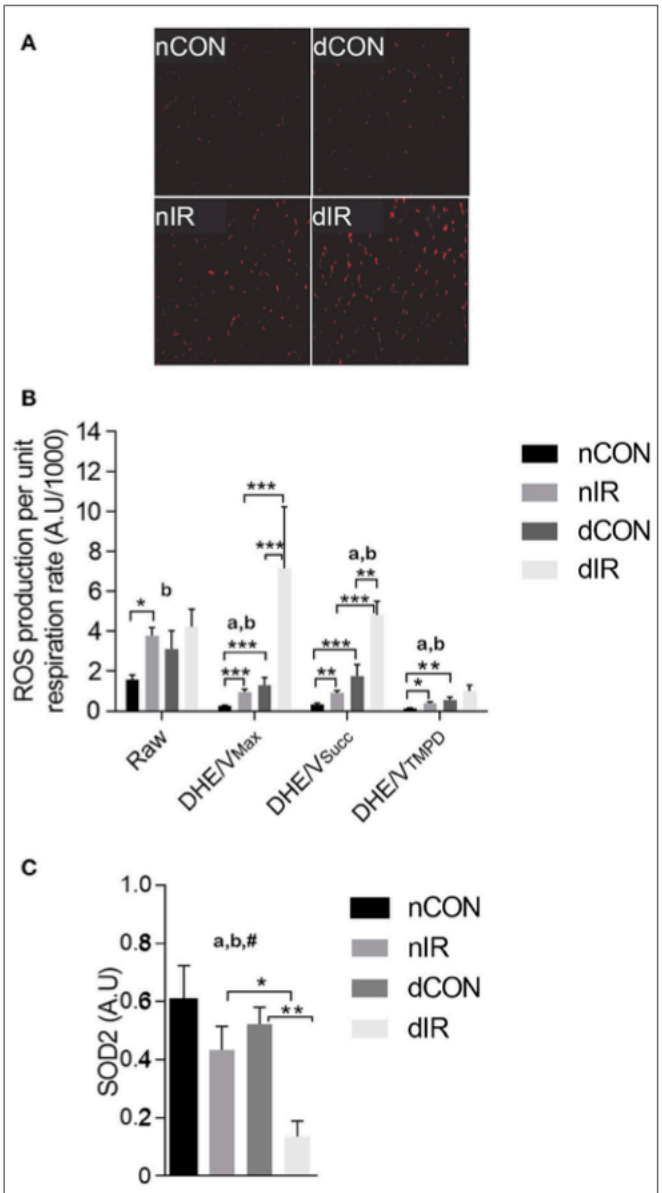

FIGURE 3 | Reactive oxygen species production and antioxidant protein level. **(A)** Representative histological sections after dihydroethidium (DHE) staining in sham- operated, vehicle-treated (nCON) or streptozotocin-treated rats (dCON), and after ischemia reperfusion in vehicle-treated (nIR) and streptozotocin-treated rats (dIR). **(B)** Raw and specific (normalized to respiration rate) reactive oxygen species (ROS) production in gastrocnemius muscles from sham-operated, vehicle-treated (nCON) or streptozotocin-treated rats (dCON), and after ischemia reperfusion in vehicle-treated (nIR) and streptozotocin-treated rats (dIR). Data are expressed as mean ± SEM [Arbitrary Unit/μM O_2/min/g dry weight]. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, $^{a}p < 0.05$ for diabetes effect; $^{b}p < 0.05$ for IR effect. **(C)** Quantification of mitochondrial superoxide dismutase (SOD2) protein level by Western blotting in gastrocnemius muscles from sham-operated, vehicle-treated (nCON) or streptozotocin-treated rats (dCON), and after ischemia reperfusion in vehicle-treated (nIR) and streptozotocin-treated rats (dIR). Data are expressed as the mean ± SEM of the intensity of the SOD2 bands reported to the intensity of the internal control (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). $^{*}p < 0.05$; $^{**}p < 0.01$, $^{a}p < 0.05$ for diabetes effect; $^{b}p < 0.05$ for IR effect; $^{\#}p < 0.05$ for interaction.

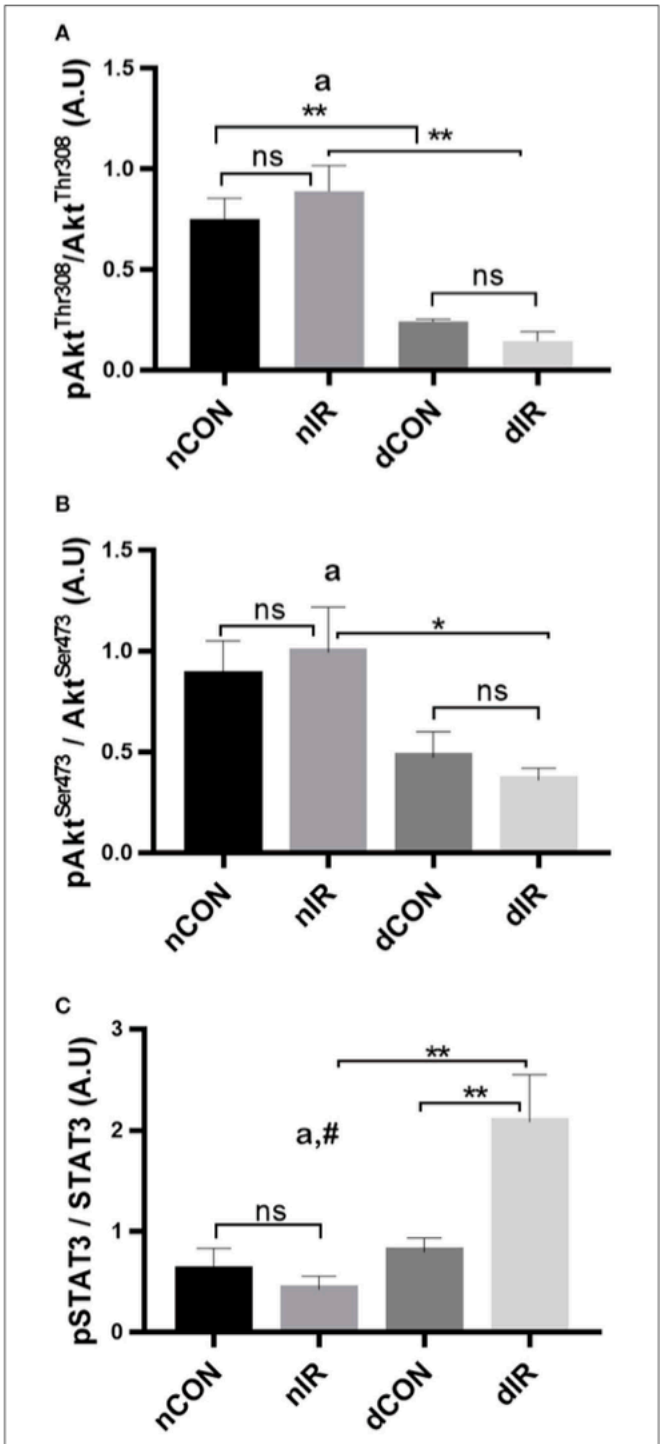

FIGURE 4 | Protein levels of key components of the endogenous protective RISK and SAFE pathways. Quantification of the levels of effector proteins from the RISK pathway [Akt, phospho-Akt/Total Akt Thr308 **(A)**, phospho-Akt/Total Akt Ser473 **(B)**] and the SAFE pathway [STAT3, phospho-STAT3/Total STAT3 tyr705 **(C)**] in gastrocnemius muscle from sham-operated, vehicle-treated (nCON) or streptozotocin-treated rats (dCON), and after ischemia reperfusion in vehicle-treated (nIR) and streptozotocin-treated rats (dIR). GAPDH was used as an internal control for all proteins. Data are expressed as the mean ± SEM of the intensity of the bands reported to the intensity of the internal control. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{a}p < 0.05$ for diabetes effect; $^{\#}p < 0.05$ for interaction.

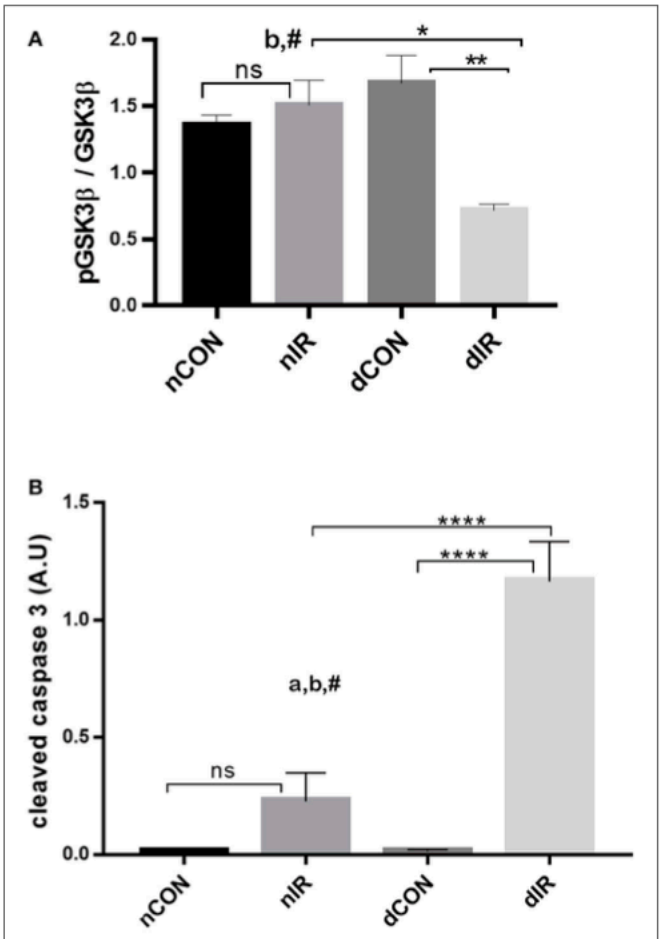

FIGURE 5 | Protein levels of GSK-3β and cleaved caspase-3. Quantification of the levels of **(A)** GSK-3β, (GSK-3β, phospho-GSK-3β/Total GSK-3β Ser9) and **(B)** caspase-3 effector of apoptosis in gastrocnemius muscle from sham-operated, vehicle-treated (nCON) or streptozotocin-treated rats (dCON), and after ischemia reperfusion in vehicle-treated (nIR) and streptozotocin-treated rats (dIR). GAPDH was used as an internal control except cleaved caspase-3 for which tubulin was used. Data are expressed as the mean ± SEM of the intensity of the bands reported to the intensity of the internal control. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{****}p < 0.0001$, $^{a}p < 0.05$ for diabetes effect; $^{b}p < 0.05$ for IR effect; $^{\#}p < 0.05$ for interaction.

(RISK and SAFE pathways, together with cleaved caspase 3) are provided in **Figure 6**.

Histological gastrocnemius samples from the diabetic-IR rats invariably showed totally distorted anatomy with extensive necrosis and cell lysis, compared to the non-diabetic-IR animals that presented much less severe cell injury (**Figure 7**).

DISCUSSION

The main finding of this study is that the impact of skeletal muscle IRI is worse in subjects with type 1 diabetes phenotype. Before ischemia, mitochondrial respiration and oxidative stress were more impaired in animals with diabetes than in those without, and these differences became more prominent after IR.

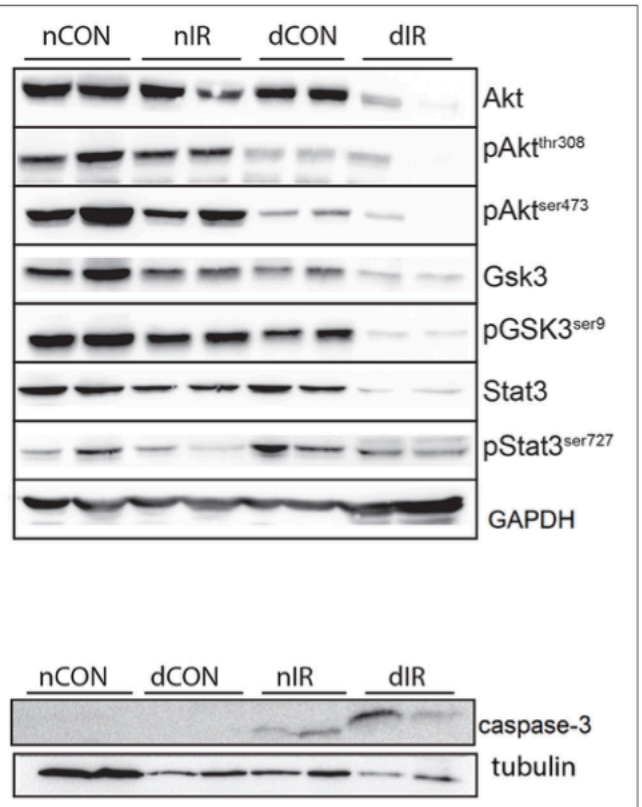

FIGURE 6 | Representative Western blots of the effector proteins. **(Upper panel)** Effector proteins of RISK and SAFE pathways. **(Lower panel)** Cleaved caspase-3. GAPDH was used as an internal control except cleaved caspase-3 for which tubulin was used.

Despite activation of one effector of the SAFE pathway, apoptosis and cell damage also appear to be more severe in diabetic subjects after IR. To the best of our knowledge, this is the first study that examined the combined effect of IR and diabetes on skeletal muscle.

Impaired skeletal muscle mitochondrial function and increased oxidative stress are the key elements of PAD-related IR injury (Tran et al., 2011; Guillot et al., 2014). Increased ROS production appears to precede (Guillot et al., 2014) and mediate mitochondrial dysfunction in skeletal muscles. In turn, dysfunctional mitochondria increase ROS production in a vicious cycle (Zorov et al., 2006; Chouchani et al., 2014). Type 1 and type 2 diabetes are also independently associated with increased oxidative stress and defective mitochondrial function, in both humans and experimental models (Kelley et al., 2002; Petersen et al., 2004; Karakelides et al., 2007; Bonnard et al., 2008; Anderson et al., 2009). Put in context, our results indicate that, after IR, diabetes further compromises mitochondrial function and oxidative stress in skeletal muscles, and suggest a cumulative or synergistic deleterious effect of both pathologies. A plausible mechanistic explanation could be that the diabetes-induced compromised mitochondrial function may be predisposing the limb muscles to respond poorly to IRI.

In the same line of evidence, Ryan et al., investigating the impact of type 2 diabetes on mice with acute limb ischemia, also reported exacerbation of skeletal muscle mitochondriopathy, oxidative stress and necrosis in diabetic animals. Although they did not extend their observations to the reperfusion period, they reported a reversal of these disturbances, by overexpression of catalase-mediated antioxidant defenses (Ryan et al., 2016). Bonnard et al. observed mitochondrial alterations that were similar in both type-1 and type-2 diabetic mice and associated with ROS production. Interestingly, the authors found that normalization of glycemia (by insulin) or antioxidant treatment restored mitochondrial integrity and reversed the proapoptotic process in the type-1 diabetic animals (Bonnard et al., 2008). Other studies, focusing on diabetic myocardium, have also shown that diabetes was associated with increased susceptibility to IRI (Whittington et al., 2012). Most of these studies suggested that the hyperglycemia-induced elevated levels of ROS and depletion of antioxidants were the key players in diabetic IRI (Ceriello et al., 2000; Nishikawa et al., 2000; Song et al., 2007).

Although present in skeletal muscles (Sandri, 2008), RISK and SAFE pathways activation has not been previously examined before in skeletal muscle IR. In this study, reduced Akt activation in diabetic animals indicates that the RISK pathway is not activated, and is consistent with the low inhibition of GSK-3β. GSK-3β is the downstream point of convergence of the RISK pathway and of several other prosurvival pathways and when phosphorylated (and thus inhibited) prevents mPTP opening (Juhaszova et al., 2004). Therefore, the lower levels of inactive GSK-3β in diabetic rats after IR may suggest failure of RISK and other protective pathways, ultimately resulting in mPTP opening, apoptosis and cell death. Consistently, downstream to GSK-3β, the proapoptotic cleaved caspase-3 levels were several times higher in diabetic rats. Several cardiac studies in diabetes also reported that hyperglycemia, insulin deficiency and insulin resistance were associated with loss of the conditioning-mediated cardioprotection and that this effect was driven by impaired signaling to Akt and GSK3β of the RISK pathway (Tsang et al., 2005; Gross et al., 2007; Song et al., 2007). In cardiomyocytes, upon IR, Akt activation appeared to be maximal after 10 min, remained elevated until 60 min of reperfusion, and returned toward basal levels by 2 h (Mockridge et al., 2000). Notwithstanding the lack of RISK activation at 2 h of reperfusion in our experiment, activation of RISK at an earlier timepoint cannot be excluded.

In contrast to RISK, SAFE pathway appeared to be activated in our diabetic animals after IR, as we observed an increased activation of STAT3. As a part of the SAFE pathway, STAT3 is involved in ischemic conditioning. Through its localization in the mitochondria of several organs, STAT3 modulates mitochondrial respiration and attenuates ROS production, mPTP opening and apoptosis (Wegrzyn et al., 2009). SAFE activation in diabetic animals should have been protective and yet, we found much higher cleaved caspase-3 levels in this group. These seemingly contradictory results may have several explanations. SAFE and RISK activation protects ischemic myocardium when triggered acutely, at the very first minutes of reperfusion, by specific conditioning maneuvers or pharmacological agents (Rossello and

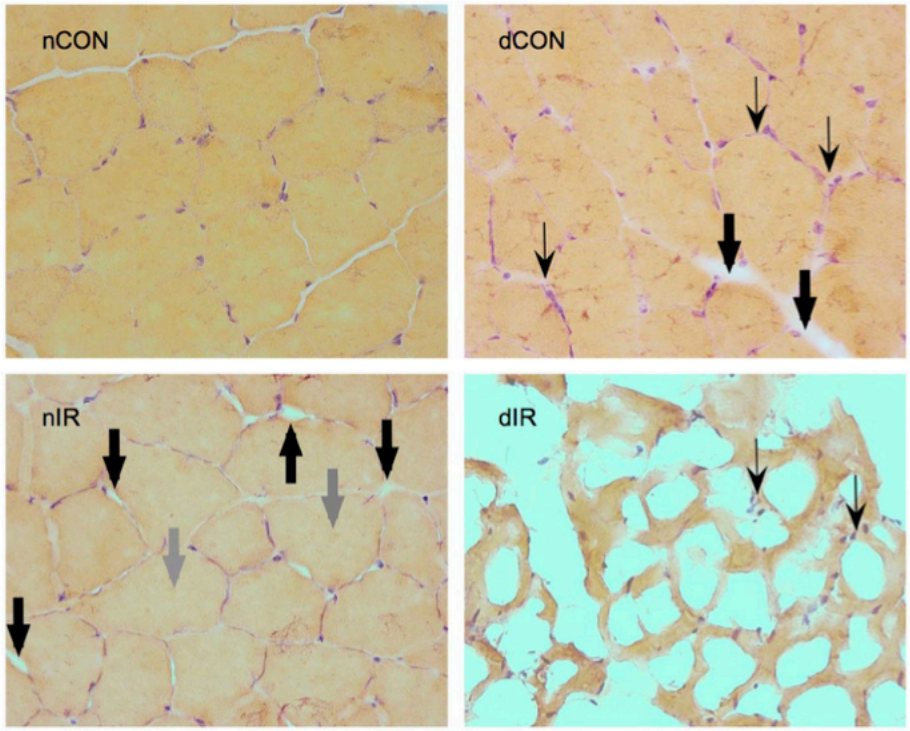

FIGURE 7 | Hematoxylin-eosin staining of gastrocnemius muscles from sham-operated, vehicle-treated (nCON) and streptozotocin-treated rats (dCON), and after ischemia reperfusion in vehicle-treated (nIR) and streptozotocin-treated rats (dIR). Magnification ×400. Compared to nCON, gastrocnemius muscles from dCON rats showed reduced muscle fiber diameter, increased number of inflammatory cells invading the intercellular space (thin black, swept arrowheads) and intercellular edema (large black, solid arrowheads). After IR, gastrocnemius muscles from the nIR group displayed both increased intercellular and intracellular edema (large gray, solid arrowheads). After IR, gastrocnemius from the dIR group showed totally distorted anatomy with extensive necrosis and cell lysis surrounded by clusters of inflammatory cells (thin black, swept arrowheads).

Yellon, 2017). In our study, STAT3 activation was spontaneous and hence, may not be sufficient or not activated early enough, to completely counteract the brunt of the increased oxidative stress. While spontaneous STAT3 activation has been shown to occur after myocardial ischemia and was further increased by reperfusion, it failed to reduce the infarct size (Hausenloy et al., 2011). Interestingly, STAT3 activation, despite its acute protective myocardial effects after IR, has also been strongly implied in atrophying skeletal muscle in experimental models of cancer cachexia or renal failure (Piccirillo and Giavazzi, 2015). Contrary to many chronic conditions, STAT3 activation usually triggers protective mechanisms in acute situations (like IR), probably accounting for the widespread "SAFE" label. In some situations, like in our study, one should probably not use the "SAFE" acronym, because STAT3 is not protective.

In our study, activated cleaved caspase-3 was several times higher in diabetic than in the non-diabetic subjects after IR, suggesting a strong trend toward apoptosis and cell death. Our histological findings, although not formally quantified, strongly corroborated this assertion. Chowdhry et al. reported comparable results in diabetic human myocardium. They demonstrated that diabetes increased both necrosis and apoptosis after IR, and that this effect was mediated by caspase-3 (Chowdhry et al., 2007). Other studies have also shown that increased activation of caspase-3 increased myocardial infarct size and that inhibiting caspases at the time of reperfusion, limited the infarct size (Mocanu et al., 2000; Condorelli et al., 2001). In skeletal muscles, caspases also mediate apoptosis after IR and trigger accelerated proteolysis in catabolic conditions (Du et al., 2004; Tran et al., 2012).

Several limitations of this study need to be acknowledged. Although the general limitations of the preclinical models may also apply to our study, we used a well-characterized pharmacological model of type 1 diabetes. Our data are descriptive and do not test specific mechanisms. That these pathways and mitochondrial function are altered in specific ways, simply make a series of observations and causality cannot be established. RISK and SAFE in skeletal muscle are investigated for the first time in this IR study and the analyses were performed at a single timepoint, after 3 h of ischemia and 2 h of reperfusion. In this regard, further studies are needed to determine the kinetics and the mechanisms of the different effectors. Executioner caspase-3 alone, although strongly inferring apoptosis and cell damage, cannot formally be interpreted as evidence of ultimate cell death. However, the histological findings seem to confirm a more extensive cell damage in the diabetic group.

In conclusion, our experimental data demonstrate that mitochondrial dysfunction, oxidative stress and apoptosis are

enhanced in type 1 diabetes, after lower-limb IR. The so-called RISK and SAFE protective pathways appeared not to be activated in diabetic animals after IR, despite STAT 3 enhancement, potentially explaining the increased susceptibility to apoptosis and cell damage. These disturbances may contribute toward the high failure rate of revascularization in diabetic subjects with PAD. More studies are needed to further elucidate the mechanisms of IR mitochondriopathy in diabetes, determine the impact of specific anti-diabetic treatments on mitochondrial IR dysfunction and clarify the kinetics and the role of the protective pathways. Therapeutic approaches targeting mitochondria may reduce lower-limb IR damage and improve local and general prognosis in patients with PAD.

AUTHOR CONTRIBUTIONS

Conception or design of the work JP, AL, JB, A-LC, GL, BG. Acquisition, analysis JP, CA, AL, JB, A-LC, AM, MS, VW, PD, GL, DM, BG. Interpretation of data for the work JP, CA, AL, JB, A-LC, AM, MS, VW, PD, GL, DM, BG. Drafting or revising the work: JP, CA, AL, AM, GL, BG. Final approval JP, CA, AL, JB, A-LC, AM, MS, VW, PD, GL, DM, BG. Agreement to be accountable of all aspects of the work JP, CA, AL, JB, A-LC, AM, MS, VW, PD, GL, DM, BG.

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