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IMPLICATION DES PHOSPHODIESTERASES SPECIFIQUES DES NUCLEOTIDES CYCLIQUES DANS LE RELACHEMENT VASCULAIRE ET LA FONCTION RENALE CHEZ LE RAT CIRRHOTIQUE

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Review of the literature

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Rima TAHSELDAR-ROUMIEH

Review of the literature

I- Introduction

II- Part 1: Specific cyclic nucleotide phosphodiesterase Families

III- Part 2: Introduction to liver cirrhosis

I- Introduction

The second intracellular messengers, 3',5'-cyclic AMP (cAMP) and 3',5'-cyclic GMP (cGMP), play pivotal regulatory roles in a wide variety of signal transduction pathways in various tissues, and mediate many physiological processes (Beavo., 1995). In particular, in the vascular system, it has been clearly shown that the cyclic nucleotides induce vasorelaxation. (Ignarro & Kadowitz, 1985; Murad, 1986; Waldman & Murad, 1987; Schoeffter et al., 1987). In fact, the intracellular levels of cAMP and cGMP are controlled by their rate of synthesis by adenylyl and guanylyl cyclase respectively and by their rate of hydrolysis by the cyclic nucleotides phosphodiesterases (PDEs). Therefore, the PDEs play an important role in the regulation of vascular tone. In our laboratory it has been demonstrated that the PDEs are differently distributed in the vascular system: PDE1, PDE3, PDE4 and PDE5 are present in the smooth muscle cells (Lugnier et al., 1986; Komas et al., 1991), whereas in the endothelium the PDE2 and PDE4 are majority (Lugnier & Schini, 1990).

The PDEs form a large family of intracellular enzymes and the only cellular mechanism that catalyse the hydrolysis of 3', 5' cyclic nucleotides to the corresponding non-active nucleotide 5'-monophosphate. Currently, 11 PDE families (PDE1-11) have been identified, based on their substrate specificities, tissue distribution, amino acid sequence, and inhibitors sensitivities (Beavo, 1995; Conti & Jin, 1999; Soderling & Beavo, 2000; Francis et al., 2001). Each family displays distinct tissue, cell, and subcellular expression patterns, which give them the opportunity to participate in physiological signal transduction pathways such as vision process, cardiac contractility, aldosterone synthesis, platelet aggregation, and smooth muscle relaxation, as well as pathophysiological processes, male impotence (PDE5), pulmonary hypertension, atherosclerosis and diabetes (Juilfs et al., 1999; Rickards et al., 2003). Hence, PDEs may provide fundamental and

therapeutical interest, especially in the cardiovascular system (Lugnier & Komas., 1993; Stoclet et al., 1995; Lugnier et al., 1999b).

Furthermore, recent studies have showed an alteration of the expression and activity of the PDE2, PDE3 and PDE4 in the endothelial cell proliferation (Keravis et al., 2000; Favot et al., 2004; Netherton & Maurice, 2005). Moreover, previous studies have showed increased activity and expression of the PDE5 in the pathogenesis of renal resistance to endogenous natriuretic peptides, and on the other hand an overproduction of NO in the vascular system is well demonstrated in advanced liver cirrhosis (Lee & Humphreys, 1996; Angeli et al., 2000; Ni et al., 2001).

The clinical course of liver cirrhosis is complicated by a number of important physiological perturbations as consequences of the disease, including portal hypertension (PHT), a common syndrome associated with chronic liver cirrhosis (Cahill et al., 2001). A consequence of the chronic PHT is a hyperdynamic circulatory state, characterized by peripheral vasodilation, decreased vascular resistance and increased cardiac out put, in association with renal function abnormalities such as sodium and water retention, and the consequent ascites formation (Groszmann, 1994; Blendis & Wong, 2001) Arterial vasodilation of the splanchnic area is considered the key factor for the altered hemodynamic profiles in PHT. Moreover, in order to maintain a physiological homeostasis, this arterial vasodilation stimulates a compensatory mechanism by a baroreceptor-mediated activation of vasoconstrictor sytems, renin-angiotensin-aldosterone, sympathetic nervous system and the arginine-vasopressin, which in turn induce renal vasoconstriction accompanied by sodium and water retention and the consequent ascites formation (Lopez et al., 1991; Ginès & Arroyo, 1993).

Finally, PHT promotes the development of vasculopathy, with intrahepatic and renal vasoconstriction, and a splanchnic and pulmonary vasodilation.

Currently, it is believed that the overproduction of endothelial derived relaxing factor, NO (Palmer et al., 1988; Moncada et al., 1988), plays a major role in the pathogenesis of arterial vasodilation that occurrs in cirrhosis (Shreir et al., 1988; Vallance & Moncada, 1991; Claria et al., 1992; Van Obergh et al., 1995; Niederberger et al; 1995, 1996; Martin et al., 1998a; Wiest et al., 1999; Wiest & Grozmann, 2002). Hence, in the vascular system it is clearly established that the NO induces vasorelaxation by increasing the cGMP level in smooth muscle cells through activation of the soluble guanylyl cyclase. In the vascular smooth muscle cells the cGMP is hydrolysed specifically by the PDE1 and PDE5, and cGMP also regulates the PDE3 activity present in this tissue by inhibiting its hydrolyzing activity for cAMP (Komas et al., 1989). Therefore, the NO participates obviously through cGMP in the pathogenesis of arterial vasodilation in cirrhosis, and thus alterations in the PDEs function may arise to regulate the vascular tone in this disease.

The aims of the study

The aims of this study are to examine first: 1) the participation of PDE5 with its specific and potent inhibitor (DMPPO), in the changes in the vascular reactivity of the aorta (*in vitro* study) in liver cirrhosis four weeks after bile duct ligation (BDL); 2) the participation of PDE5 in the hemodynamics changes and renal dysfunction (*in vivo* study after acute i.v administration of DMPPO 5 mg/kg) in BDL rats or sham-operated rats (SO).

Second, this study emerges to examine the effects of chronic administration of sildenafil Viagra® (a selective inhibitor of PDE5 used in treatment in human impotence) (20 mg/kg subcutaneously 3 times/day) on the renal sodium retention and hemodynamic disturbances associated with liver cirrhosis in rats.

Third, in order to define the relation between the hemodynamic changes in liver cirrhosis and the findings in the effects of PDE5 inhibitors in cirrhosis, a biochemical study was done to evaluate the variations of the activity and expression of the different PDE isozymes in the various altered tissues (renal cortex, kidney, aorta, mesenteric arteries) to put in evidence the PDE targets.

II- Specific cyclic nucleotide phosphodiesterase Families

A- General properties

Cyclic nucleotide phosphodiesterases (PDEs) are a large family of enzymes that specifically hydrolyses the second messengers cyclic-3', 5'-adenosine monophosphate (cAMP) and/or cyclic-3', 5'-guanosine monophosphate (cGMP) to their inactivated-noncyclic nucleotides 5'-AMP and 5'GMP. PDEs, which are the only cellular mechanism for degrading cAMP and cGMP, play a major role in regulating the intracellular levels of these second messengers and, subsequently, control functional responses of cells.

Once primary protein sequence and initial cDNA isolation and sequencing had been accomplished for the several different PDEs, it became clear that a number of different PDEs gene families exist, and the first five PDE families were designated by roman numerals as PDE-I through PDE-V (Beavo & Reifsnyder, 1990).

The application of cloning, polymerase chain reaction (PCR) and techniques of molecular biology to the problems of PDE resulted in a more specific classification. Currently, they have been classified into eleven families (PDEs 1-11) according to their structure, their primary sequence, their substrate specificity, and their pharmacological properties.

Most PDE families comprise more than one gene (~20 PDE genes), which generate multiple protein products (> 50 PDE proteins) (Manganiello & Degerman., 1999; Soderling and Beavo, 2000). Furthermore, PDE genes have one or more mRNA splice variants transcribed from them, which appears to be highly tissue specific. Recently, the international nomenclature of PDE designated the gene families (types) of PDE by Arabic numerals followed by a capital letter to designate the product of a single PDE isogene also called "PDE subtype". PDE isoforms or "variants", which confer an additional layer of diversity and of specificity to the PDE family system, are expressed by an Arabic number. For example for PDE1A2: gene family 1 (type), gene A (subtype),

and variant 2 (isoform). This nomenclature is also preceded by a capital letter to show the species (figure. 1).

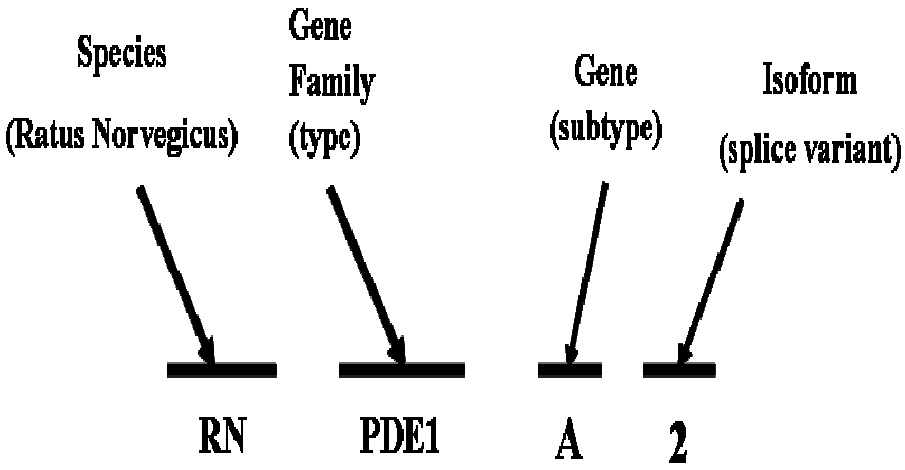


Figure1. Current nomenclature of PDE isozymes (Dousa, 1999). The nomenclature follows criteria for naming PDE isozymes according to the entries into LOCUS field of Gene Bank (Beavo et al., 1994). The first two letters at the beginning denote the species by the initials of taxonomic classification, the following three letter (PDE) plus arabic numeral designate gene family (type), the next letter denote isogene product (subfamily or subtype), last numeral denotes isoform (splice variant). Current nomenclature often does not specify the first two letters (species).

In spite of the large number of PDE isoforms, they share some structural features (figure. 2). These include a central catalytic domain that is highly conserved in all mammalian PDEs consisting of approximately 270 amino acids and positioned close to the COOH terminus. This region is much more similar in terms of amino acid identity within one individual PDE gene family (more than 80 %) rather than between two distinct PDE gene

families (~25 % to 40 %). Further more, this catalytic domain contains signature consensus sequences and motifs that are common for all PDEs and two consensus Zn^{2+}-binding sites (~3 moles of Zn^{2+} to 1 mol of PDE5 monomer) (Francis et al., 1994). In addition, the catalytic domain contains family specific sequences, responsible for substrate affinities, catalytic activities, and sensitivities to specific inhibitors.

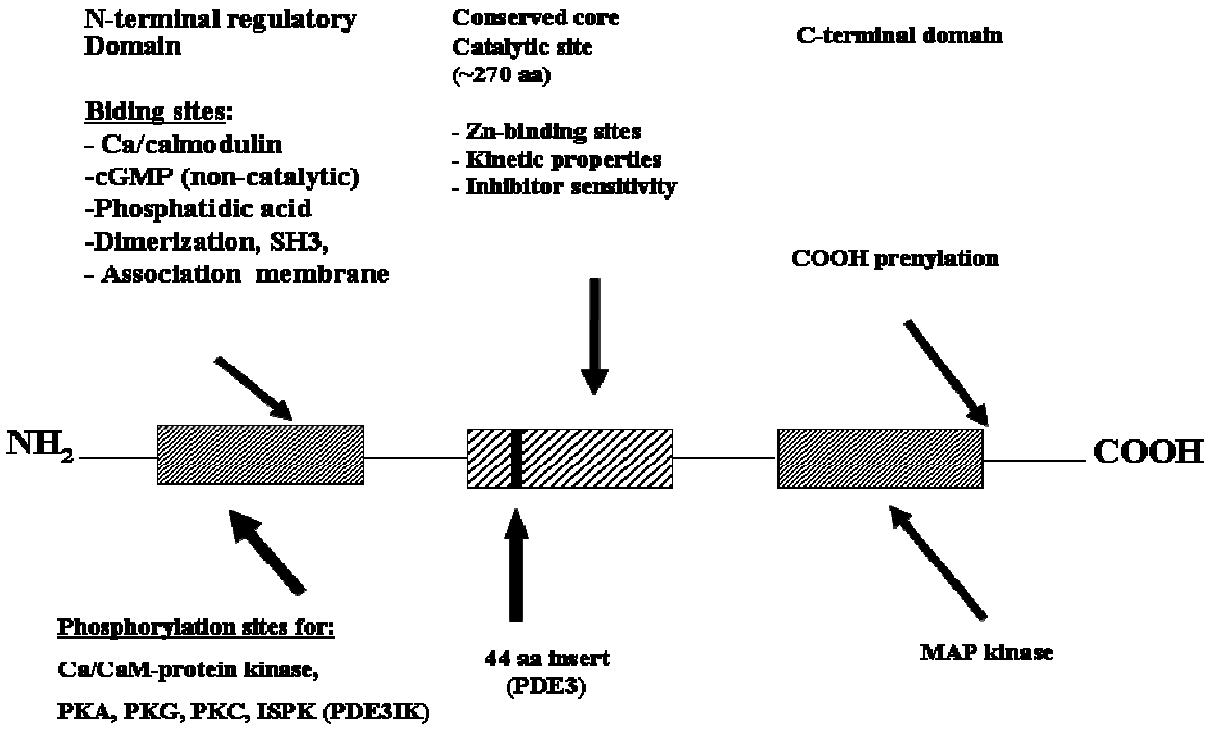


Figure 2. Schematic representation of the general structure of the PDEs (Dousa, 1999). It consists of an N-terminal domain, central catalytic core, and C-terminal domain, connected hinge regions. Abbreviations are: PKA, protein kinase A; PKG, protein kinase G; PKC, protein kinase C; ISPK (PDE3IK), insulin-dependent PDE3B protein kinase.

In contrast to the central catalytic domain, the NH_2-terminal "regulatory domain" of PDE diverges widely among PDEs in structure and size and contains sequences that are target sites for various modes of regulation (figure. 3) (Rybalkin et al., 2003). These include binding sites for Ca^{2+}-CaM (calmodulin), non-catalytic sites for cGMP or phosphatidic acid, phosphorylation site for protein kinases (Ca^{2+}-CaM PKII, PKA, PKG, PKC and

insulin stimulated PK "PDE3IK"), sites for dimerization and membrane association sites. Experimental manoeuvres that delete the N-terminal regulatory domain from the rest of the molecule result in many-fold increase of PDE hydrolytic activity, supporting the hypothesis that the N-terminal regulatory domain of PDE exerts an auto inhibitory effect on the enzymatic activity (Dousa, 1999).

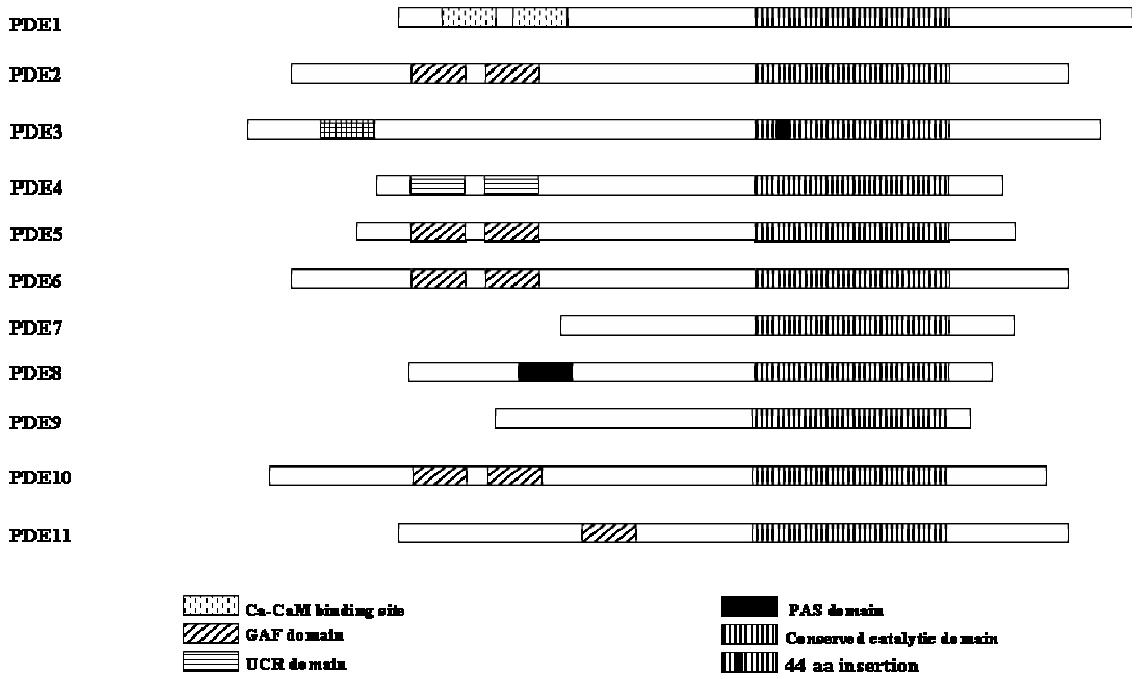


Figure 3. Domain organization of 11 PDEs gene families (Francis et al., 2001; Soderling & Beavo, 2000)**.** All PDEs share significant homology in the catalytic domain but differ greatly in the N-terminal part, which contains different regulatory domain. UCR domain is an upstream conserved found in PDE4. GAF domain for binding cGMP found in PDE2, PDE5 and PDE6. PAS found only in PDE8. ~44 aa insertion in the catalytic domain of PDE3.

The functional small C-terminal domain of PDEs is largely unknown. According to recent report, this domain may apply some regulatory functions: It can be phosphorylated by MAPK (mitogen-activated protein kinase) on the Ser^{487} of the PDE4B2 isoform, and it

contains consensus sequences for prenylation representing a mechanism for anchoring PDEs to membranes.

The intracellular localization of the PDEs is based on the distribution of PDE activity in tissues or cells preparations. The PDEs are not intrinsic membrane proteins but are often associated tightly with membranes or are present in the cytosol compartments (soluble) (Shakur et al., 1994, 1995). Within the cell, recent studies showed precise interactions between the PDEs and intracellular structures and that the N-terminal domain has the potential to interact with plasma membrane and Golgi apparatus as well as with cytoskeletal proteins and proteins involved in cell signaling (Shakur et al., 1995; Houslay, 1996). The deletion of this N-terminal allows enzyme diffusion in the cytoplasm. Furthermore, PDE isozymes and isoforms are differentially expressed in the cell types and tissues, which constituted a determining factor to the great fundamental diversity of the cell-specific PDE isozyme systems.

In principle, signal transduction via cyclic nucleotides (cAMP or cGMP) in a cell type is controlled by: 1) the rate of adenylyl cyclase or guanylyl cyclase to synthesize the nucleotides in response to extracellular signals, and 2) the rate of the PDE isozymes to hydrolyse the nucleotides. Therefore, the PDEs participate discretely in signal transduction pathways and thus play a fine tunning role in the physiological and pathophysiological processes that is now widely demonstrated in the literature such as penile erection, asthma, pulmonory hypertension, atherosclerosis, heart failure, and diabetes.

Here, I will cite the recent information relating to the structure, localization, regulation, for the expanding PDE families and their physiological role in the control of the cell signaling pathways via cAMP and cGMP.

B- Different PDE gene families

PDE1 family

The PDEs of the PDE1 gene family are called calcium/calmodulin-dependent phosphodiesterases (CaM-PDEs) because they are activated by Ca^{2+}/CaM complex. Three different gene products have been cloned: PDE1A, PDE1B and PDE1C (Sonnenburg et al., 1998). PDE1A and PDE1B have higher affinity for cGMP more than cAMP, whereas, PDE1C hydrolyses cGMP and cAMP with equal efficiency. (Beavo., 1995). The PDE1 isozyme family is diverse and includes numerous splice-variants PDE isoforms. At least seven splice-variants of the PDE1A gene have been reported, among them PDE1A1 and PDE1A2 have been isolated from bovine heart and brain with molecular weight 59 and 61 KDa respectively (Sonnenburg et al., 1993, 1995).

For PDE1B, only one gene product has identified the PDE1B1, which encodes the bovine brain 63 KDa (Bentley et al., 1992; Repaske et al., 1992). It is of interest that in the brain PDE1B is particulary high in the striatum whereas the PDE1A is highly expressed in the cortex and the hippocampus (Beavo, 1995).

Five PDE1C splice variants, PDE1C1 to 5, have been identified in human in and mouse tissues (brain, olfactory neurons, testis) and the cDNA for these PDEs have predicted molecular masses of ~70-80KDa (Borisy et al., 1992; Loughney et al., 1996; Yan et al., 1995, 1994). At least two of the PDE1C variants have high affinity for the cAMP and one of these is highly expressed in olfactory receptor neurons, which mediate some aspects of odorant-induced signal transduction (Yan et al., 1995).

In the vascular system, the PDE1 family is widely expressed in the vascular smooth muscle of the coronary artery, the aorta, the mesenteric artery (Keravis et al., 1980; Lugnier et al., 1986; Komas et al., 1991) The Ca^{2+}-CaM complex increases the PDE1 activity to at least 10 fold. The binding site for the CaM/Ca^{2+} is positioned on the N-terminal domain of the enzyme (Beavo, 1995), which also can be phosphorylated on two

sites of serine and therefore modulates negatively the enzyme activity. The PDE1A1 from heart and PDE1A2 from brain are phosphorylated by cAMP protein kinase (PKA), whereas PDE1B is phosphorylated by CaM-dependent protein kinase II (Hashimoto et al., 1989; Sharma & Wang, 1986). These phosphorylations decreased the affinity of the enzyme towards CaM-Ca^{2+}, and can be reversed by calcineurin, a Ca^{2+}/CaM dependent-phosphatase (Sharma & Wang, 1985). The PDE1C is expressed in the proliferating smooth muscle cells (SMCs) but not in the quiescent human aorta (Rybalkin et al., 1997). The inhibition of the PDE1C isozyme from atherosclerotic lesions, with specific PDE1 inhibitor, result in suppression of SMC proliferation (Rybalkin et al., 2002a, 2003b). Thus, PDE1 isoforms may constitute therapeutic targets in atherosclerosis and cancer. Moreover, Jiang et al. (1996) have also shown that the PDE1B is expressed in human lymphocytes of leukemic cells and that inhibition of PDE1B induced apoptosis of these cells. Moreover, Lefièvre et al. (2002) have demonstrated that calmodulin is tightly bound to PDE1A in human spermatozoa, which lead them to suggest that PDE1A is permanently activated in this tissue, raising the question about its potentiality in human fertility.

The compounds that chelate Ca^{2+} act as effective inhibitors of PDE1 but not selective, and the 8-MeoM-IBMX (xanthine) and vinpocetine are described as selective inhibitors of the basal PDE1 activity, acting directly on the catalytic domain of the enzyme (Nicholson et al., 1991; Beavo & Reifsnyder, 1990). However, they have poor selectivity since vinpocetine inhibits K^+ channel at a concentration of 30 microM (Bukanova and Solntseva, 1998).

PDE2 Family

The PDE2 family also known as cGMP-stimulated PDE (cGs-PDE) because it is specifically stimulated by cGMP that binds to the cGMP binding sites (GAF A and GAF

B) located in the N-terminal domain (Beavo et al., 1995; Manganiello et al., 1995a). To date, only one gene has been identified PDE2A, and three splice variant encoded by this gene have been described, PDE2A1, A2 and A3, which differ in their N-terminal domain (Yang et al., 1994; Rosman et al., 1995). The two cGMP-binding sites localized on the N-terminal domain regulated positively the PDE2 activity. Furthermore, in response to hormonal stimulations PKC activates PDE2 (Geoffroy et al., 1999).

PDE2 isoforms can be cytosolic and particulate. The cytosolic PDE2A1 is characterized in the adrenal cortex (MacFarland et al., 1991). The PDE2A2 isozyme is identified as membrane-bound from brain and cardiac tissue (Yang et al., 1994) and the N-terminal domain of this isozyme has a hydrophobic region that accounts for membrane targeting.

Both cAMP and cGMP are substrates for PDE2 with positive cooperative kinetic effects (Beavo et al., 1995).

The PDE2 is found in high concentration in the olfactory neurons, the brain and the adrenal cortex, where it may contribute to regulate physiological processes (Juilfs et al., 1997; Beavo et al., 1995).

In the adrenal cortex, it mediates the inhibitory effect of ANP on aldosterone synthesis. Moreover, in frog cardiac myocytes, it has been shown that the PDE2 activity is physiologically coupled to decrease the Ca^{2+} channel activity (Fischmeister & Hartzell, 1987). In vascular tissue, PDE2 is present in the endothelial cells (Lugnier et Schini, 1990) and recently it has been shown that PDE2 is implicated in endothelial cell proliferation and migration, and that PDE2 inhibitor had an anti-angiogenic effect (Keravis et al., 2000; Favot et al., 2003, 2004).

Table 1. Characteristics of different PDE families

Isoenzyme Family	Characteristics	cAMP Km (μM)	cGMP Km (μM)	Inhibitor	Sensitivity to IBMX
PDE1	Ca^{2+}-calmodulin dependent	1-30	3	Vinpocetine 8-methoxy-IBMX	+
PDE2	activity stimulated by cGMP	30-300	10-30	EHNA	+
PDE3	activity inhibited by cGMP	0.1-0.5	0.01-0.5	Cilostamide, milrinone Zardaverine	+
PDE4	cAMP specific	0.5-4	>50	Rolipram	+
PDE5	cGMP specific	>40	1.5	Zaprinast sildenafil DMPPO	+
PDE6	Photoreceptor cGMP specific	2000	60	Sildenafil Zaprinast DMPPO	+
PDE7	High specificity for cAMP, rolipram insensitive	0.2	>1000	None reported	+
PDE8	Specific for cAMP hydrolysis, IBMX insensitive	0.7	>100	Dipyridamole	-
PDE9	cGMP specific	>100	0.07	Zaprinast	-
PDE10 PDE11	cAMP /cGMP cAMP/cGMP	0.5 1	3 0.5		+ +

The only known selective inhibitor of PDE2 is erythro-9-(2-hydroxyl-3-nonyl-)-adenine (EHNA), which is also a potent inhibitor of adenosine deaminase (Schaeffer & Schwender, 1974).

PDE3 family

The PDE3s, purified from a variety of tissues, can be distinguished from different PDEs by their high affinities for both cAMP and cGMP, with K_m values in the range of 0.1-0.8 μM respectively and V_{max} for cAMP 4-10 times higher than that for cGMP, which allows this later to act as a competitive inhibitor for cAMP hydrolysis by this enzyme (Lugnier & Komas, 1993; Beavo et al., 1995; Degerman et al., 1997). Thus, PDE3 is referred as the cGMP-inhibited PDE.

There are two different gene products identified as being part of PDE3 family (Beavo, 1995; Degerman et al., 1997; Matsumoto et al., 2003). The PDE3A exist in three isoforms (PDE3A1-A3) and is identified and more abundant in the heart, vascular smooth muscle, and platelets, whereas the PDE3B is prominent in the adipocytes, and hepatocytes (Reinhart et al., 1995; Manganiello et al., 1995a, 1995b; Manganiello & Degerman, 1999).

The structural organization of PDE3A and 3B protein is identical to all PDEs, with specificity in the conserved catalytic domain, an insert of 44 amino acids, which distinguishes the PDE3 family from other PDE families. This insertion within the catalytic domain may be involved in the interaction of PDE3 with substrates and inhibitors. The widely divergent and determinant regulatory N-terminal domain of the PDE3 is regulated positively by PKA via its phosphorylation on the serine 302 (Degerman et al., 1997). In addition, PDE3B is activated by phosphorylation on the same site Ser^{302}, by the PDE3 protein kinase (PDE3IK) activation via the insulin receptor-

activated cascade (Degerman et al., 1997; Manganiello & Degerman, 1999). Moreover, the N-terminal hydrophobic region is involved in membrane association, whether the cytosolic PDE3s isoforms are generated by proteolytic removal of the membrane association region and this later is not required for PDE3 catalytic activity or sensitivity to specific PDE3 inhibitors. Thus, the PDE3 isoforms could be cytosolic or membrane-associated: related to their cytosolic localization the PDE3A have shorter forms, whereas the PDE3B are membrane-associated (Liu & Maurice, 1998; Choi et al., 2001).

Because of tissue-specific distributions of the PDE3 isoforms, specific inhibitors have been used to promote clinical practice. Therefore, PDE3 inhibitors such as cilostamide, cilostazol and milrinone induce smooth muscle relaxation, platelet anti-aggregation, stimulate myocardial contractility and block the antilipolytic action of insulin (Eriksson et al 1995; Degerman et al., 1996).

PDE4 family

The PDE4 family referred as "cAMP-specific PDE" is the most diverse family of PDEs and hydrolyses selectively and with high affinity only cAMP (Conti & Jin., 1999; Houslay & Adams., 2003). Four PDE4 genes have been identified (A, B, C, D) in most mammalians cells and encode more than 16 different isoforms (Beavo, 1995; Manganiello et al., 1995a). Analysis of the primary sequence of the different PDE4 isoforms showed two highly conserved regions found only in PDE4 within the N-regulatory domain, UCR1 and UCR2 (upstream conserved region 1and 2). Two regions named LR1 and LR2 (linker region 1and 2) separated UCR1 from UCR2 and UCR2 from the catalytic domain respectively. These linker regions represented a variability of sequence that confers the specificity to the different isoforms (Bolger et al., 1994).

Moreover, alternative mRNA splicing generates “long” PDE4 isoforms (85 to 110 kDa) having UCR1 and UCR2, and “short” PDE4 isoforms (68 to75 kDa) having only UCR2 and super-short isoforms that have a truncated UCR2 (Johnston et al., 2004).

The N-terminal domain plays a major role in regulating PDE4 isoforms activity as well as cellular and subcellular localization. This domain has the potential to interact with plasma membrane and cell organelle as well as with cytoskeletal proteins involved in cell signaling. Thus, the PDE4 isozymes can be cytosolic or associated to membranes. The PDE4A1 is a membrane associated isoform and the deletion of the first 67 amino acids residues of the N-terminal domain lead to its cytsolic form (Shakur et al., 1995). The “short” PDE4D isoforms are cytosolic whereas the “long” can be membrane-associated or cytosolic (Liu & Maurice, 1999a). Similarly, two PDE4B isoforms have been identified in rat brain tissue with both subcellular localization: membrane and cytosolic (Lobban et al., 1994). Furthermore, the proline residues of the N-terminal for PDE4A5 are implicated in the interaction with SH3 domains of protein of other signaling pathway such as tyrosine protein kinases of the Src family (O’Connell et al., 1996).

On the other hand, the N-terminal regulates the PDE4 activity consequently to its phosphorylation. For instance, phosphorylation of PDE4D “long isoforms” on Ser^{54} of the UCR1 by PKA causes a rapid several-fold increase in catalytic activity of PDE4D3, and also greatly increases its sensitivity to the selective inhibitor rolipram (Mackenzie et al., 2002). Another bioregulator is phosphatidic acid, which activates PDE4D3 probably via binding on the N-terminal domain (Nemoz et al., 1997). Furthermore, the MAP kinases phosphorylate the PDE4 on the C-terminal domain and can regulate positively the “short form”, whereas it regulates negatively “long form” PDE4 activity (Mackenzie et al., 2000; Houslay, 2001).

A selective inhibitor of PDE4 first described is rolipram (K_i=0.8μM) (Lugnier et al., 1986). Rolipram binds with a very high affinity (1 nM) to PDE4 isozymes at a site that is probably within the core catalytic domain but not clearly defined. Furthermore PDE4 may exist in various conformers (low and high affinities) which can be regulated by physiological states (Méhat et al., 2000). Several PDE4 inhibitors with low affinity (1 μM) have been described Ro-20-1724, denbufylline, and with high affinity (1 nM) CDP 840, RP73401. All these inhibitors have experimental and potential therapeutic applications (Dousa, 1999).

PDE5 family

a) PDE5 localization, molecular organization and splice variants

This enzyme is highly identified in lung, vascular and tracheal smooth muscles, platelets and it is also found in the kidney (Francis et al., 1980; Lugnier et al., 1986; Hamet and Tremblay, 1988; Thomas et al., 1990). PDE5 isozyme is cytosolic, and lately it was identified in the heart where preparations from whole left ventricule and isolated myocytes expressed PDE5A isozyme (Senzaki et al., 2001). The PDE5 isozyme known as cGMP-specific PDE or cGMP-binding PDE, is highly specific for cGMP hydrolysis and binds cGMP to allosteric cGMP-binding sites. The enzyme is a dimer with two regulatory, putative GAF domains that bind cGMP at the N-terminus, and one catalytic domain at the C-terminal site (Corbin & Francis, 1999; Kameni-Tcheudji et al., 2001; Rybalkin et al., 2003a) (Figure. 6). The regions mediating dimerization of the enzyme have been suggested to be present in the allosteric cGMP-binding domain (Martinez et al., 2002; Muradov et al., 2003). Recently, the GAF regulatory domains are defined as GAF A and GAF B, based on their sequence homology with similar regulatory motifs of a large group of proteins, including the cGMP-regulated PDEs (PDE2, PDE5 and PDE6), several adenylyl cyclase (A) and a bacterial transcription factor called FhlA. The GAF

nomenclature is derived from the first letters of these groups. The GAF A and B of PDE5 are homologous; however GAF A of this enzyme is most homologous to GAF B of the PDE2 that also binds cGMP with high specificity.

Only one gene has been identified PDE5A, which encodes three different isoforms, PDE5A1, PDE5A2 and PDE5A3 that differ only in the N-terminal domain. The first PDE5 purified from bovine lung was soluble and was identified as PDE5A1 isoform (Thomas et al., 1990). Later, a human PDE5A1 was identified and it is very similar to bovine PDE5A1, except an additional 10 amino acid insert at the N-terminal end (Loughney et al., 1998). Another variant, PDE5A2 contains a significantly shorter N-terminal domain, and PDE5A3 is identified only in human tissues (Kotera et al., 1999; Lin et al., 2000).

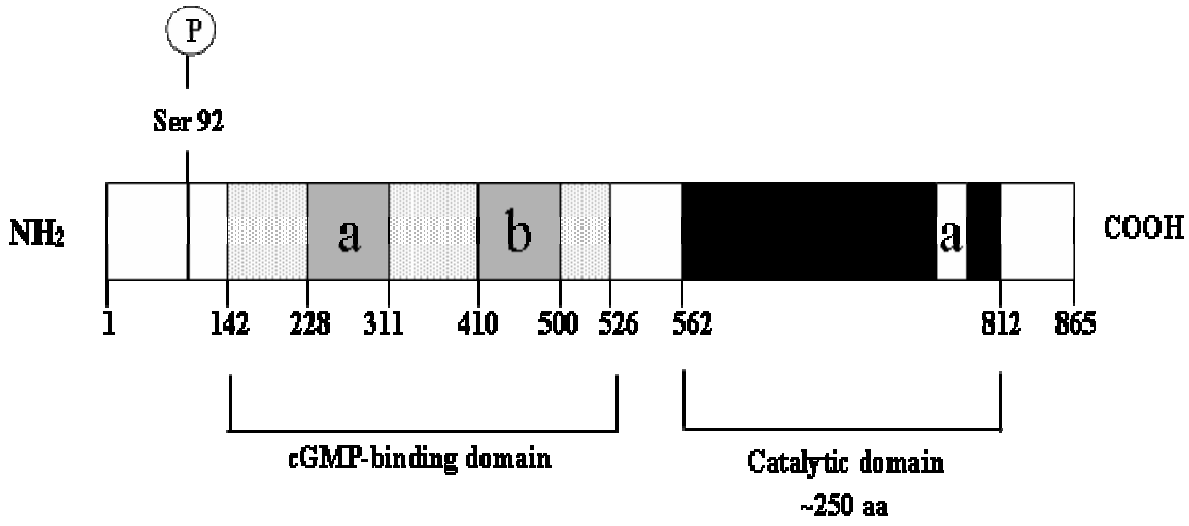


Figure 4. Linear structural model of PDE5 (Fink et al., 1999). PDE5 monomeric fragment contains two allosteric cGMP-binding sites **a** and **b** within the N-terminal domain that is required for phosphorylation of serine 92 by PKG. The catalytic domain is located in the center of the C-terminal portion and contains the cGMP-binding substrate site **a**.

b) Mechanisms for regulation of PDE5 activity

Like all PDEs the N-terminal domain regulated the PDE activity via its phosphorylation. For the PDE5, the N-terminal domain is phosphorylated on the Ser^{92} and the binding of cGMP to the non-catalytic domain of the N-terminal portion (GAF domain) is necessary for enhancing phosphorylation by protein kinase A (PKA) or protein kinase G (PKG) (Turko et al., 1998) (Figure. 4). Recently, an *in vitro* study showed that a 50 to 70% increase in the activity of the partially purified recombinant bovine PDE5 was achieved after its phosphorylation (Corbin et al., 2000). Furthermore, more experiments provide evidence that PDE5 phosphorylation *in vivo* is predominantly mediated through the cGMP/PKG I and not PKA. A study done on transgenic mice that lack in the PKG I gene showed the inability of aortic smooth muscle cells from these mice to produce any phosphorylation of PDE5 (Rybalkin et al., 2002). However, in platelets from PKG I-deficient mice PDE5 phosphorylation still occurred but was found to be reduced to approximately 75% as evaluated by densitometric analysis. This phosphorylation of PDE5 was accounted for by the activation of PKA in response to NO/cGMP (Mullershaussen et al., 2003). The phosphorylation status of PDE5 after cGKI activation by *in vivo* injection of sodium nitroprusside has also been demonstrated in the cerebral Purkinje neurons and the phospho-PDE5 may then serve as an indicator of *in vivo* PKG activation (Shimizu-Albergine et al., 2003).

Mullershaussen and his colleagues (2003) also demonstrated that cGMP could directly induce activation of PDE5 independent of phosphorylation, most likely via binding to GAF domain. In this study, preparations from PKG I and ATP-depleted platelet were incubated with cGMP, and PDE activity was measured. Surprisingly, PDE5 activity was enhanced 2.3-fold suggesting that cGMP by itself is able to activate PDE5.

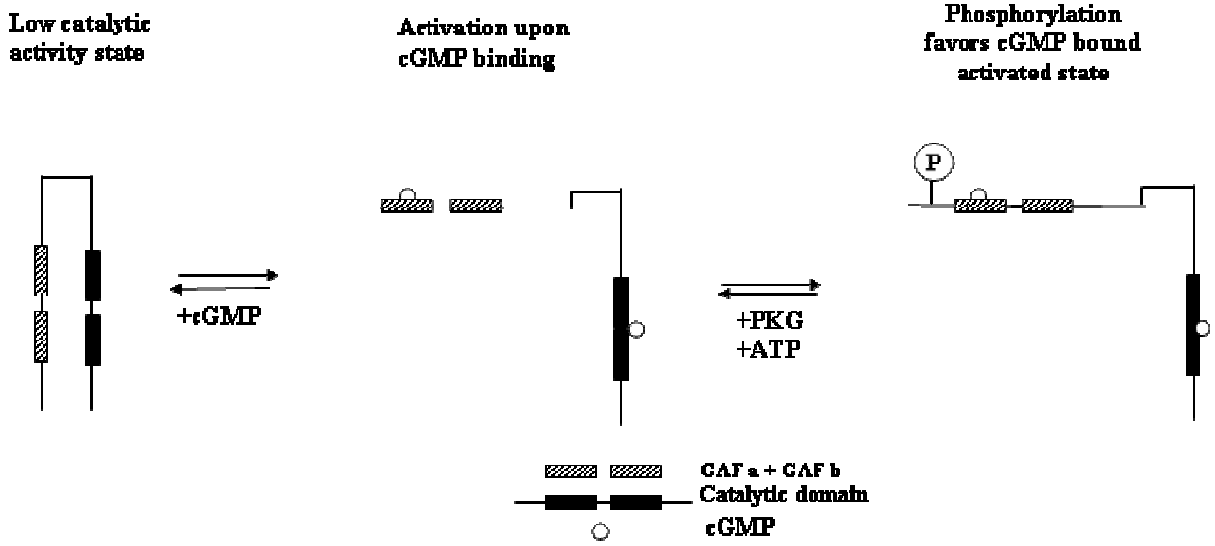


Figure 5. Conformational change of PDE5 via cGMP-binding to non-catalytic binding site (Rybalkin et al., 2003a). PDE5 is directly activated upon cGMP-binding to its GAF a domain; cGMP bound converted non–activated PDE5 into an activated state. Only this activated PDE5 is phosphorylated by PKG.

Moreover, it has been demonstrated that the recombinant PDE5 is activated upon cGMP binding to the GAF A domain (Rybalkin et al., 2003a). The authors showed in their study that the activation of PDE5 was not a result of phosphorylation because the mutation of the phosphorylation site (Ser^{92}) to alanine did not change the pattern of PDE5 activation by cGMP. In addition, to verify their concept a monoclonal antibody generated against the GAF domain of PDE5 was applied before preincubation with cGMP. This prevented the PDE5 activation and greatly reduced the hydrolytic activity of PDE5, which strongly ensured their concept. Interestingly, these authors have found that the ability of PDE5 to be directly activated by cGMP was limited to fresh preparations.

Therefore, it is reported (Rybalkin et al., 2003a) that PDE5 can exist in at least two different conformational states *in vivo*: non-activated and activated upon cGMP-binding (figure. 5). The non-activated PDE5 is in a state with low intrinsic catalytic activity that can be converted reversibly to an activated state upon cGMP binding. Very recently, it has been shown that cGMP binding to the cGMP-binding sites increase ^{3}H-sildenafil binding to the catalytic site. The results suggested that two conformations states of PDE5 catalytic sites existed with Kd's of 12 and 0.83 nmol/L (Corbin et al., 2003).

Finally, binding of cGMP proved to be necessary and sufficient to achieve full activation of PDE5, and to enhance its phosphorylation by PKG (Turko et al., 1998), which increases the hydrolytic activity of PDE5. Thus, elevation of intracellular cGMP provides negative feedback control and enhances its own destruction via direct, cGMP-binding allosteric activation of PDE5 as well as activation caused by phosphorylation of PDE5 by PKG (Francis et al., 2001; Mullerhausen et al., 2003; Okada and Asakawa, 2002; Rybalkin et al, 2002b, 2003a).

c) Regulation of PDE5 activity by Zn^{2+}

It has been suggested that PDE5 isozyme contain binding site for Zn^{2+} (Francis et al., 1994; Beavo, 1995) (Figure. 6), since Zn^{2+} could be detected in the pure enzyme by using radioactive Zn^{2+}. Moreover, the PDE5 isozyme is also purified by binding to a Zn^{2+} chelate affinity column and PDE5 is activated by Zn^{2+} ions binding to the catalytic core domain.

d) PDE5 and vascular relaxation

The PDE5 isozyme is the most highly expressed and a major cGMP-hydrolyzing PDE in smooth muscle cells, and thus an effective controller of the NO/cGMP-PKG signaling pathway. Thus, the specific inhibition of the PDE5 plays a major role in the regulation of vascular tone. In the vascular smooth muscle cells, four types of PDEs have been

described (Lugnier et al., 1986; Komas et al., 1991): PDE1 (Ca^{2+}-calmodulin-dependent PDE), PDE3 (cyclic GMP-inhibited PDE), PDE4 (cyclic AMP-specific PDE) and PDE5 (cyclic GMP-specific PDE). In vascular endothelial cells, PDE2 (cyclic AMP-stimulated PDE) and PDE4 represent the main isoforms (Lugnier & Schini, 1990). However, it is well established that cyclic guanosine 3'-5'-monophosphate (cyclic GMP) is a second messenger implicated in signal transduction mechanisms in eukaryotic cells. The role of cyclic GMP has been extensively studied in the cardiovascular system. In vascular smooth muscle cells, it has been clearly shown that an increase in cGMP levels induces vasorelaxation (Ignarro & Kadowitz, 1985; Murad, 1986; Waldman & Murad, 1987; Schoeffter et al., 1987). The intracellular elevation of cyclic GMP is regulated by the activity of guanylyl cyclase, which catalyzes GTP to form cGMP. Moreover, it is well established that in smooth muscle nitric oxide (NO) and atrial natriuretic peptide (ANP) regulate the vascular tone through activation of soluble and particulate guanylyl cyclases respectively, elevation of cGMP and activation of cGMP dependent protein kinase (PKG) (Ignarro, 1992; Garbers et al., 1988; Chinkers et al., 1989). There are several specific physiological substrates for PKG to induce vasorelaxation. The key event in the mechanism of vasorelaxation appears to be Ca^{2+}-mediated (Walsh, 1994). An increase of $[Ca^{2+}]_i$ results in the production of the complexe Ca^{2+}/calmodulin, which binds with the enzyme myosin light chain kinase (MLCK), reduces its kinase activity and allows phosphorylation of myosin light chain subunit. Myosin cross-bridges are then able to cycle along actin filaments, which results in the interaction between actin-myosin, which results in shortening of the muscle and the development of a contractile force. PKG-activated is able to phosphorylate the MLCK, myosin phosphatases, as well as the modulators of the intracellular Ca^{2+}expressed in the smooth muscle cells (calcium-

activated maxi K^+ (BK_{Ca}); IP_3 receptor associated cGMP kinase substrate) (Surks et al., 1999; Fukao et al., 1999; Schlossmann et al., 2000).

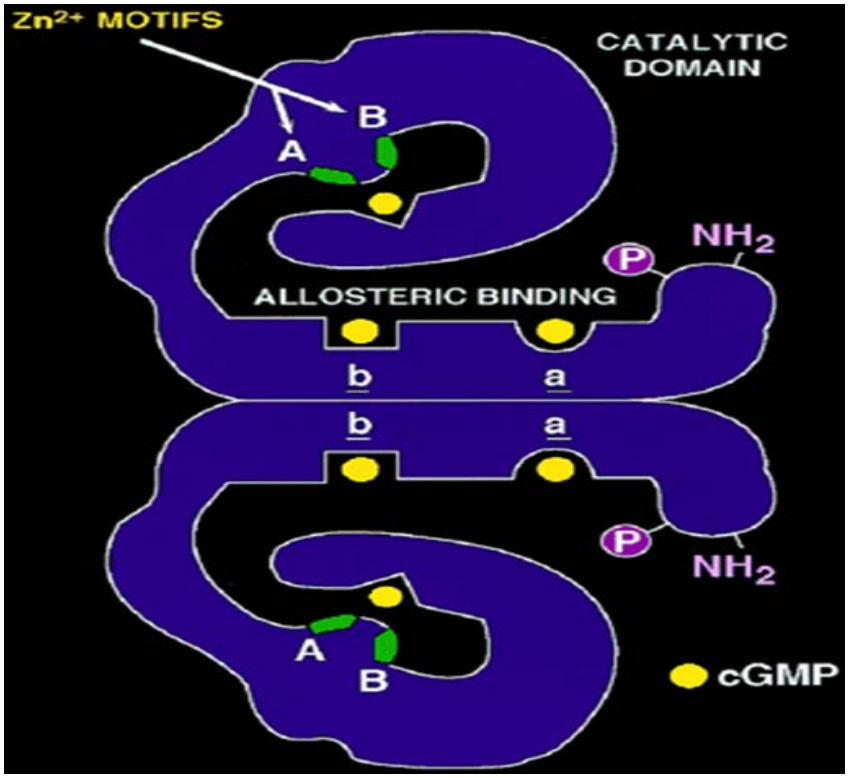


Figure 6. Dimer structure of PDE5 (Corbin and Francis., 1999). The regulatory domain in the N-terminal domain of PDE5 contains the phosphorylation site (Ser-92), the two allosteric cGMP-binding sites *a* and *b*, and at least a portion of the dimerization domain. The catalytic domain in the C-terminal domain of the protein contains the two Zn2+-binding motifs A and B and a cGMP-binding substrate site. Amino acids that are potentially involved in the catalytic mechanism are indicated in *green* and those involved in binding cGMP in an allosteric or catalytic site are indicated in *yellow*.

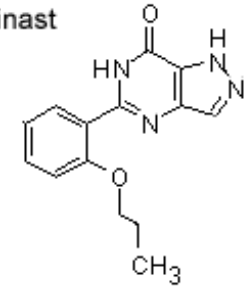
Phosphorylation of these regulatory targets by PKG contributes to a reduction in sensitivity to Ca^{2+} or a reduction in intracellular Ca^{2+} concentration and thereby decreased smooth muscle tone.

e) PDE5-specfic inhibitors as therapeutic targets

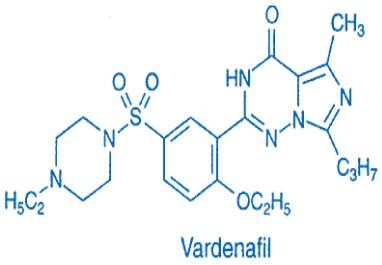
The physiological importance of the PDE5 in the regulation of smooth muscle tone has been most effectively demonstrated by the successful clinical use of its specific inhibitors. Sildenafil (Viagra^{TM}), a potent and selective PDE5 inhibitor has been used clinically in the treatment of human impotence (Corbin & Francis, 2002). PDE5 is abundant in the corpus cavernosum and relatively controls the cGMP pool that mediates relaxation of smooth muscle of the corpus cavernosum (Francis et al., 2001; Corbin, 2004). Relaxation of the corpus cavernosum is induced by NO release from endothelial cells and noncholinergic nonadrenergic neurons that surrounds the arteries and sinusoids in this tissue. Specific inhibition of PDE5 with sildenafil causes accumulation of cGMP in response to NO, thus enhancing erectile response (Ballard et al., 1998; Corbin et al., 2002; Corbin et al., 2004).

Currently, two other specific PDE5 inhibitors targeting erectile dysfunction, vardenafil and tadalafil are also in the market (Corbin et al., 2004). Vardenafil has been shown in a rabbit model to increase intracavernosal pressure more quickly and for a longer period than sildenafil (Saenz et al., 2001).

Zaprinast

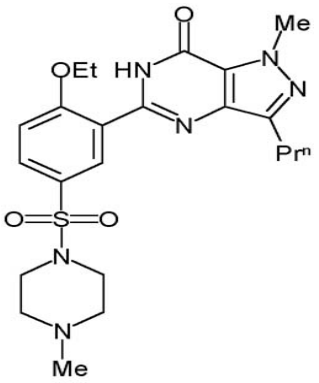


Vardenafil

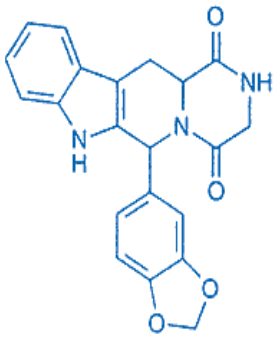


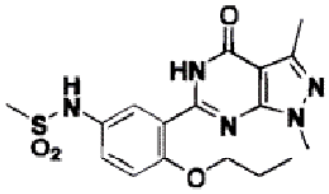
Vardenafil

Sildenafil



Tadalafil





DMPPO

Figure 8. Structures of the different PDE5 inhibitors: Zaprinast, sildanafil, vardenafil, tadalafil and DMPPO.

Tadalafil has been shown in clinical trials to have a long half-life of 17 hours and no visual side effect (Eardley and Cartledge, 2002). Among all PDE inhibitors only sildenafil (Viagra^{TM}) has first fulfilled the promise of PDEs as therapeutic targets. However, the PDE4 inhibitors such as cilomilast and rofumilast, potent anti-inflammatory agents, remain in phase III clinical trials as potential therapeutic agents for asthma and chronic pulmonary disease, although PDE3 inhibitors, which enhance myocardial contractility and anti-aggregation of platelet, failed in clinical trials of long term of cardiac failure, but are currently used for acute therapy and cilostazol for intermittent claudication.

Sildenafil exertes the inhibition of PDE5 via binding to the catalytic domain, and cGMP binding to the cGMP-binding sites increases this binding (Corbin et al., 2003). Sildenafil is a potent inhibitor that can be administered orally and acts pharmacologically in the presence of local production of cGMP via NO release, which constitutes the one major contra-indication of sidenafil therapy because concurrent treatment with nitroglycerine or nitrates have been demonstrated to cause a severe decrease in systemic arterial pressure (Kloner et al., 2000; Corbin et al., 2002; Corbin & Francis, 2002; Manganiello, 2003).

In addition, the pulmonary hypertention characterized by high pulmonary arterial pressure and vascular resistance may constitute a clinical targert for the PDE5 inhibitors. Recently, the treatment of acute pulmonary hypertension with oral or intravenous sildenafil has shown positive results in humans superior to inhaled NO, by decreasing pulmonary arterial pressure and vascular resistance (Weimann et al, 2000; Michelakis et al., 2002; Schulze-Neick et al., 2003). However, in early clinical investigations sildenafil interestingly has modest effect on reduction of systemic blood pressure and no effect on heart rate has been observed in non-pathological state (Kloner, 2004). Chiu & Reid

(2002) have shown that oral administration of sildenafil exerts a stimulatory action on renin secretion, which could explain why it has a minor effect on blood pressure.

On the other hand, Zaprinast was the first selective PDE5 inhibitor described in the literature which was able to increase cGMP content and vasodilate smooth muscle (Lugnier et al., 1986, Schoeffter et al., 1987). This compound was therefore used as selective PDE5 inhibitor and presently used as a scaffold to conceive new PDE5 inhibitor. Coste and Grondin (1995) described a highly selective PDE5 inhibitor active in the nanomolar range concentration named DMPPO that is 66-fold more potent than Zaprinast. These authors showed that this enzyme controlled the intracellular levels of cGMP and induced vasorelaxation (Delpy et al., 1996). Furthermore, DMPPO decreased blood pressure when given i.v. or orally without modifying heart rate and dilatated the pulmonary circulation during chronic hypoxia (Delpy & le Monnier de Gouville, 1996; Eddahabi et al., 1998). The prolonged hypotensive effect of orally administered DMPPO indicated that this compound possessed good bioavailibility and stability over time. Therefore, it is possible that PDE5 inhibitor may represent a new therapeutic agent in the treatment of cardiovascular disorders.

Finally, the selectivity profiles of most PDE inhibitors are binding to the catalytic domain because all PDEs share a high degree of sequence similarity in their catalytic domains. The fact that binding of cGMP to the GAF domain can cause activation of PDE5 suggests that the antagonists and agonists targeting this domain might be developed and might have a different selectivity than achieved with the catalytic site antagonists.

PDE6 family

PDE6 family also called photoreceptor cGMP phosphodiesterase are highly expressed in the outer the retinal rod and cones, and function as effector proteins in visual transduction (Beavo, 1995). Recently, the PDE6 isozymes have been also identified in the chicken

pineal gland (Morin et al., 2001). The PDE6 isozymes are heterotetramers or homotetramers composed of variations of catalytic subunits (α and β for the rods; two α' subunits for the cones) and inhibitory subunits (γ and γ'). The key role of γ subunit is to inhibit cGMP hydrolysis by the catalytic subunits of the enzyme in the dark (Hamilton and Hurley, 1990) and the role of δ is less clear but it appears only in the soluble form of PDE6, which may serve to modulate binding of the PDE6 to the membrane (Beavo, 1995). Moreover, the mutation of the gene encoding for the β subunit induces visual pathologies such as *retinis pigmentosa* (Gal et al., 1994).

PDE6 isozyme activation is initiated by photons that interact with rhodopsin, which is coupled via the heteromeric G-protein "transducin" to membrane-associated PDE6, and results in increased PDE6 activity by dissociating the inhibitory γ subunit from the catalytic core of the enzyme. Then this activation of PDE6 results in a rapid drop in cGMP levels and opening cGMP-gated channel (Beavo, 1995).

The photoreceptor enzymes could be soluble or membrane-associated and display a high homology with the PDE5 isozyme (45-48 % identity) in the catalytic domain, a strong substrate preference for cGMP, and show similar patterns for PDE5 inhibitors such as zaprinast, dipyridamole and sildenafil (Gillepsie & Beavo, 1989; Turko et al., 1999). The clinical use of sildenafil in the human impotence has been reported to show visual side effects, which can inhibit the photoreceptor PDE6 in the nmol/L range of concentration (Ballard et al., 1998). For this reason, new PDE5 inhibitors, such as vardenafil and tadalafil, less effective on PDE6 are developed.

PDE7 family

This PDE was recently discovered, cloned expressed and identified as a new family of PDE (Michaeli et al., 1993). The PDE7 is highly specific for cAMP as a substrate similar to PDE4, but it is not sensitive neither to rolipram nor to the other PDEs inhibitors. PDE7

has an amino acid sequence distinct from those of cAMP PDEs, and no specific regulatory domain that characterized its N-terminal parts has been cited (Michaeli et al., 1993; Bloom & Beavo, 1996; Han et al., 1997).

Two genes have been identified to encode the PDE7 (PDE7A and B) (Gardner et al., 2000; Hetman et al., 2000b). Three isozymes derived from the A gene by alternative mRNA splicing: PDE7A1, PDE7A2, PDE7A3. The PDE7 isoforms are differently distributed in the various tissues. PDE7A2 mRNA is expressed abundantly in heart, kidney and skeletal muscle, whereas the testis and the immune system (thymus, spleen, lymph node, blood leukocytes) are enriched of PDE7A1 (Bloom & Beavo., 1996; Han et al., 1997; Wang et al., 2000; Smith et al., 2004). Moreover, the mRNAs for PDE7A1 and PDE7A2 and also PDE7A1 protein, have been found to be expressed in vascular smooth muscle cells derived from lung pulmonary artery (Smith et al., 2004), and the mRNAs for PDE7A1 and PDE7A2 are expressed in vascular endothelial cells (Miro et al., 2000).

PDE8 family

PDE8 isozyme family is a specific for the hydrolysis of cAMP with a K_m of approximately 70 nM (Soderling et al., 1998a; Fisher et al., 1998a). Interestingy, PDE8 is not sensitive to IBMX but it is inhibited by dipyridamole, which inhibits PDE5 and PDE2 (Lugnier and Komas, 1993). Analysis of mRNA PDE8 indicates that this isozyme is prominently abundant in the testis, ovary and the intestin (Soderling et al., 1998a; Fisher et al., 1998a). On the basis of sequence homology with a domain found in proteins from bacteria to eucaryotes, a PAS (Period, Arnt, Sim) domain has been identified in PDE8 (Soderling et al., 1998a; Wang et al., 2001). The function of PAS domain in PDE8 is not clear and it may be important in protein-protein interaction or for sensing the concentration of a small ligand suggesting a novel mode of regulation for the PDEs

(Soderling et al., 2000). To date, it is not known whether the PDE8 expression and activities are present within the vascular system.

PDE9 family

The PDE9 isozyme family specifically hydrolyses cGMP, with a K_m of 70 nM (Fisher et al., 1998b; Soderling et al., 1998b). PDE8, PDE9 are not inhibited by IBMX (van Staveren et al., 2002) and are also resistant to the inhibitors of PDEs instead of the PDE5 inhibitor, Zaprinast (IC_{50} 30 μM) (Soderling et al., 1998b; Fisher et al., 1998b). Compared with cGMP-specific PDEs sequences, PDE9 lacks non-catalytic cGMP-binding domain or GAF domain at the N-terminal part, which is present in the PDE2, PDE5 and PDE6 (Sonnenburg & Beavo, 1994; Beavo, 1995; Manganiello et al., 1995a). PDE9 mRNA is widely distributed in the various tissue including spleen, small intestine and brain. PDE9 is widely distributed throughout the brain, and its expression pattern closely ressembles that of soluble guanylyl cyclase in the rat brain, suggesting a possible functional association or coupling of these two enzymes in the regulation of cGMP levels (Andreeva et al., 2001). To date, four variants have been identified for PDE9, however, the functional implications of these variants remain yet unknown (Guipponi et al., 1998).

PDE10 and PDE11 families

The PDE10 and PDE11 isozymes families can hydrolyse both cAMP and cGMP and contains GAF domain (Hetman et al., 2000; Fujishige et al., 1999; Loughney et al., 1999; Soderling et al., 1999). The primary sequence of PDE10 showed similar structure to those cGMP-binding PDEs (PDE2, PDE5and PDE6): It represents a carboxy-terminal region with homology to the catalytic regions of mammalian PDEs, and contains also two cGMP-binding domains (GAF domains), within the N-terminal core. One gene encoded two PDE10 variants, PDE10A1 and PDE10A2 (Loughney et al., 2005). PDE10A1 encodes a protein that is 779 amino acids in length, and an incomplete cDNA for

PDE10A2 was isolated. Inhibitors that are selective for other PDE families are poor inhibitors of PDE10A; however, PDE10A is inhibited by the non-specific PDE inhibitor, IBMX (Loughney et al., 2005).

PDE11 family is the most recently discovered (Loughney et al., 2005). This family contains one gene, PDE11A, with four splice variants (PDE11A1-PDE11A4). PDE11A exhibits 50% amino acid identity with the catalytic domains of all other PDEs, being most similar to PDE5, and has distinct biochemical properties (Fawcett et al., 2000). At the N terminus PDE11A1 has a single GAF domain homologous to that found in other signaling molecules, including PDE2, PDE5, PDE6, and PDE10. The physiological role of PDE11A has not been determined. The PDE11A protein is strongly expressed prostate, kidney, liver, pituitary, and salivary glands and testis glandular epithelium of the prostate, and no PDE11A protein is detected in blood vessels or cardiac myocytes (Loughney et al., 2005**).** The PDE5 inhibitors, such as tadalafil, vardenafil, and sildenafil inhibit the PDE11 with selectivities 40-, 9300-, and 1000-fold for PDE5A than PDE11A respectively (Weeks et al., 2005). Tadalafil, PDE5 inhibitor, used for treatment of male erectile dysfunction, partially inhibit PDE11. PDE11A is sensitive to the nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) as well as zaprinast and dipyridamole, inhibitors that are generally considered relatively specific for the cGMP-selective PDEs, with IC(50) values of 49.8 microM, 12.0 microM, and 0.37 microM, respectively (Fawcett et al., 2000).

C- Role of PDEs in the cardiovascular system

In the cardiovascular system, it is well established that: i) an increase in cAMP level induces positive inotropic effect in heart, and vasorelaxation in the vessel; ii) an increase in cGMP level decreases cardiac contraction and induces vasorelaxation. Due to their

cyclic nucleotide-inactivating role, PDEs play a major role in the fine regulation of these functions.

a) Role of PDEs in cardiac contraction

In cardiac muscle, cAMP is the intracellular second messenger mediating the positive inotropic effects of β-agonists (Tsien, 1977). PDE3 appeared to be the molecular target for PDE inhibitors described as new cardiotonic drugs, such as amrinone, milrinone, piroximone, SK&F 94120, imazodan or CI 930 (Harrison et al., 1986; Weishaar et al., 1987; Komas et al., 1989). Implication of cAMP in the positive inotropic effect of PDE3 was shown on guinea pig isolated left atria, since PDE3 inhibitors decrease the EC_{50} value of isoproterenol (β-adrenergic agonist) and their inotropic effects were enhanced by forskolin (adenylyl cyclase activator) (Muller et al., 1990a). Furthermore, this study also clearly showed that cGMP acts in an opposite manner on cardiac contraction, since 8-Br-cGMP increases the EC50 value of isoproterenol and decreases PDE3 inhibitor inotropic effects. This is in agreement with the opposite effects of cAMP and cGMP reported on Ca^{2+} current in single heart cells (Hartzell and Fischmeister, 1986). Further characterization of PDE4 and PDE2 in guinea pig and dog-cardiac ventricles, as well as in frog atrial fibers and ventricles (Komas et al., 1989; Lugnier et al., 1992) raised the question about their functional roles in heart, in respect with to PDE3. Study performed with rolipram, denbufylline and Ro 20-1724 as PDE4 inhibitors, showed that in contrast to PDE3 inhibitors, these PDE4 inhibitors did not produce any positive inotropic in normal or elevated external calcium concentration. However, in presence of low concentrations of forskolin or a PDE3 inhibitor, all exerted concentration-dependent positive inotropic effects suggesting that PDE4 may play a role in regulation of cardiac contraction in physiological conditions, in which the sympathetic outflow produces a stimulation of adenylyl cyclase in cardiac cells (Muller et al., 1990b). Interestingly, (-)

rolipram was 10-fold more potent than the (+) enantiomer as positive inotropic agent, in agreement with its 20-fold higher affinity for specific binding to rat brain cAMP-PDE (Schneider et al., 1986). Biochemical characterization of cardiac isolated subcellular fractions revealed that PDE4 is preferentially associated to sarcolemma, whereas PDE3 is enriched in sarcoplasmic reticulum and that PDE2 is mainly associated to sarcolemma (Lugnier et al., 1993; Okruhlicova et al., 1997). These differential distributions of PDE4, PDE3 and PDE2 suggest a possible role of PDEs in the reported control of various cAMP compartments having a subcellular basis within the cardiomyocyte (Buxton and Brunton, 1983). The participation of PDEs in compartmentation was therefore shown in frog cardiac myoctes by using IBMX, a non selective PDE inhibitor (Jurevicius and Fischmeister, 1996). Recent studies, performed by using a transfected cyclic nucleotide gated channel sensitive to cAMP, demonstrated that PDE4, preferentially to PDE3, controls subsarcolemmal cAMP level (Rochais et al., 2004) and that its activity was increased consequently to β-stimulation. Furthermore, in transgenic mice, cardiac expression of AC8 is accompanied by a rearrangement of PDE isozymes (increase in PDE4 and decrease in PDE2), leading to a strong compartmentation of the cAMP signal that shields L-type Ca^{2+} channels and protects the cardiomyocytes from Ca^{2+} overload (Georget et al., 2003). Altogether, data concerning the localized role of PDE4 in the control of cAMP level and calcium regulation are in accordance with the positive inotropic effects reported for PDE4 inhibitors consequently to adenylyl cyclase stimulation (Muller et al., 1990b). In addition, the use of EHNA (PDE2 inhibitor) reveals the regulation of basal calcium current by PDE2 in cardiac myocytes (Rivet-bastide et al., 1997). Furthermore, in cardiac myocytes cGMP like NO was shown to act in an opposite manner on Ca^{2+} level. At low concentration cGMP by inhibiting PDE3 increases Ca^{2+}

level, whereas at higher concentration cGMP by stimulating PDE2 decreases Ca^{2+} level, indicating a concerted regulation of PDE2 and PDE3 (Vandecasteele et al., 2001). However, PDEs inhibitors were shown to have been linked to heart failure and cardiac arrhythmias, although the mechanisms are not understood. Recently, in cardiac myocytes PDE4 cAMP hydrolyzing activity has been shown to be localized to the transverse (T) tubule/sarcoplasmic reticulum (SR) junctional space that is involved in excitation-contraction coupling (Mongillo et al., 2004), and lately, in human heart failure a study showed that the PDE4D3 levels were reduced in the the cardiac ryanodine receptor (RyR2)/calcium-release-channel complex, which may contribute to heart failure and arrhythmias that prevents PKA phosphorylation of the RyR2 channel promoting defective regulation of these channels (Stephan et al., 2005).

2) Role of PDEs in vascular contraction (figure.7)

The role of PDE in vascular contraction was firstly reported when Kukovetz and Pöch (1970) showed that the vasodilator papaverine inhibits cAMP-PDE in vessel. This study was extent to papaverine analogs, showing a correlation between potencies of their vasodilatatory effects and their inhibitory effects toward vascular cAMP-PDE (Lugnier et al., 1972). Separations of PDE isoforms from human, bovine and rat aorta allowed to characterize PDE1, PDE4 and PDE5 as well as the specific inhibitors rolipram (PDE4) and zaprinast (PDE5) (Lugnier et al, 1986). Characterization of PDE3 and PDE4 in the aorta (Komas et al., 1991) questioned about their respective role in vascular contraction. Relaxation study performed on precontracted aorta, with and without endothelium, with specific PDE3 and PDE4 inhibitors in combination with NO modulator demonstrates that PDE3 inhibitors are endothelium independent vasorelaxant, whereas PDE4 inhibitors are endothelium dependent. Furthermore, NO donor, functional endothelium or PDE3

inhibitor reveals the vasorelaxing effect of PDE4 inhibitor, indicating that cGMP elevation, consequently to NO-induced guanylyl activation, inhibits PDE3 and therefore increases cAMP level allowing PDE4 to control cAMP level (Komas et al., 1991; Eckly et Lugnier, 1994). This firstly showed a cross talk between PDE3 and PDE4 mediated by cGMP**.** To go further, study was performed to investigate the participation of protein

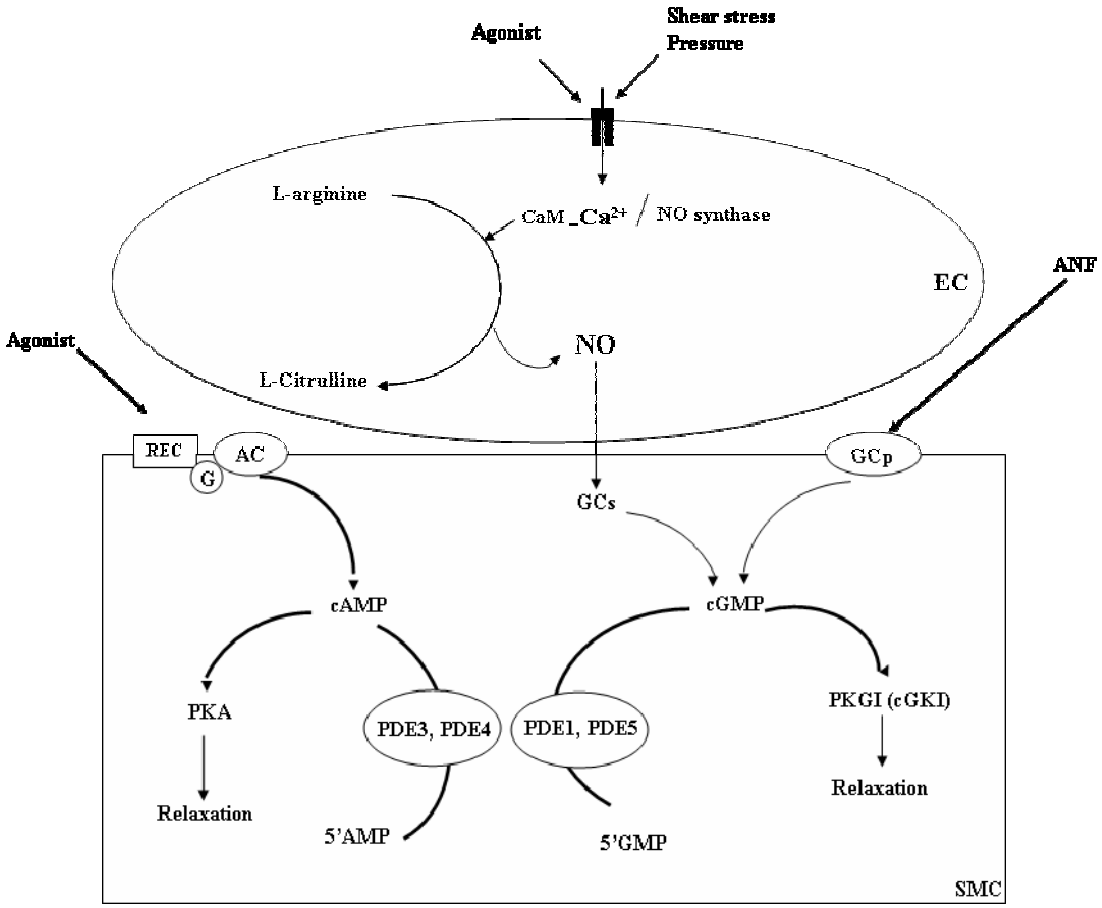


Figure 7. PDEs regulating vascular smooth muscle tone. Current concepts of cGMP and cAMP induces vasorelaxation of smooth muscle cells (SMC) through PKG and PKA respectively, and regulation of vascular tone by PDEs , enzymatic system responsible for degradation of cAMP or cGMP to their non-cyclic non-activated forms, 5'AMP and 5'GMP.

kinases in vascular contraction. Contrary to isoprenaline-induced relaxation mediated by PKA, the results obtained with PDE3 and PDE4 inhibitors showed that: i) relaxation induced either by PDE3- or PDE4-inhibitor alone is mediated by protein kinase G (PKG) despite that they only increase cAMP level; ii) contrary to isoprenaline, rolipram inhibits the ATP-induced transient increase in $[Ca^{2+}]_i$; iii) this inhibition is only mediated by PKG (Eckly-Michel et al., 1997). This inhibitory effect of rolipram on $[Ca^{2+}]_i$ is in agreement with PDE4 localization on sarcolemma. This reveals a cross-activation of PKG by cAMP in rat aorta, due to a localized increase in cAMP, as much as the relaxing effect of isoprenaline (0.01 to 0.3 μM) is only mediated by PKA. Furthermore, the combination of PDE3 and PDE4 inhibitors, which induces a higher increase in cAMP level in rat aorta (Eckly et al., 1994), is mediated by PKA (shown by an increased PKA activity ratio and by PKA inhibitor effect). The differences in the participation of PKA vs. PKG observed when the inhibitors of PDE3 and PDE4 were used alone or together could be due to differences in the degree of accumulation of cyclic AMP, resulting in the activation of PKA or PKG which are differently localized in cell and differently associated to PDE3 and PDE4. These data argue for a compartmentation of cAMP and cGMP regulated by PDE4 and PDE3 in rat aorta, the participation of PKA or PKG depending on the cellular pathway used to increase the cAMP level (Eckly et Lugnier, 1994; Lugnier et al., 1999a). Moreover, in rat aorta without endothelium and in absence of extracellular calcium, PDE1 and PDE3 seem to play an important role as modulators of rat aortic smooth muscle contractility (Noguera et al., 2001).

PDE5, which is mainly present in vascular smooth muscle, participates to vascular contraction since zaprinast increases cGMP level and relaxes rat aorta (Schoeffter et al., 1987), this effect being endothelium-dependent (Martin et al., 1986; Komas et al., 1991).

The recent discovery of sildenafil, a very potent and selective inhibitor of PDE5, allowed going further in the investigation of PDE5

participation in vascular contraction (Pauvert et al., 2003). Sildenafil inhibits potently vascular smooth muscle PDE5, which is mainly cytosolic, with an IC_{50} of 3.4 nM and induces vasorelaxation with an EC_{50} of 11 nM; this effect in pulmonary rat artery is not altered by endothelium removal or in the presence of potent inhibitors of PKG and PKA. Moreover, in isolated arterial myocytes, sildenafil (10-100 nM) antagonized ATP- and endothelin-1-induced calcium oscillations but had no effect on the transient caffeine-induced $[Ca^{2+}]_i$ response, indicating that PDE5 may be involved in the inositol triphosphate-mediated calcium signaling pathway (Pauvert et al., 2003).

D- Role of PDEs in the kidney (figure.10)

In the kidney cAMP and cGMP system are known to regulate the renal function in response to hormone action: i) arginin-vasopressin (AVP) increases cAMP levels, which makes the renal tubules and collecting ducts more permeable to water, so that more water is reabsorbed into the bloodstream; ii) atrial natriuretic factor (ANF) increases cGMP levels in the renal collecting duct, which produces sodium and water excretion.

Subsequently, the existence of PDE isozymes in the kidney that hydrolyze both cyclic nucleotides may play a role in the regulation of these functions.

However, the studies devoted to PDE activities were viewed of advances in renal pathophysiology. It became obvious to investigate the expression and the significance of PDE isozymes in nephron segments, the glomeruli and the tubules.

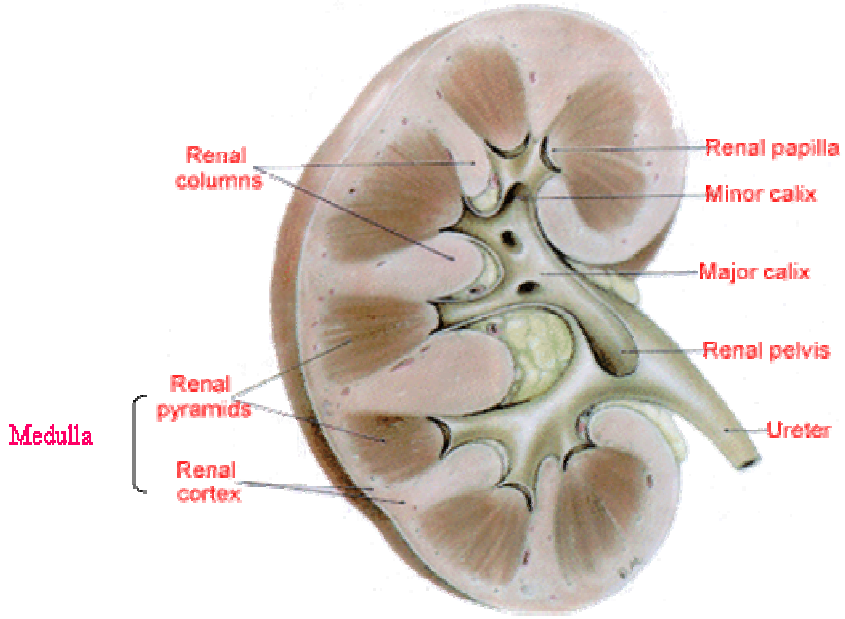
Based on PDEs activity, initial studies showed that the specific activities and regulatory properties of cyclic nucleotide phosphodiesterase in glomeruli differ markedly from tubules or renal cortical tissue.The specific cGMP-PDE activity was higher than cAMP-PDE activity in rat glomeruli isolated from rat renal cortex (Torres et al., 1978). The use

of PDE inhibitors indicates that this cGMP-PDE activity is due effectively to PDE5 and to a lesser extent to PDE1 and PDE2 activity is not detectable (Matousovic et al., 1997; Dousa, 1999). In extracts from suspensions of renal cortical tubules, cAMP was metabolized predominantly by isozyme PDE4; activity of PDE3 was about three times lower (Matousovic et al., 1997). Recent study also showed that glomeruli had the capacity to hydrolyze cAMP by isozymes PDE2, PDE3 and PDE4, whereas cGMP is hydrolyzed by PDE1 and PDE5. In this study, they suggested that selective inhibitor of PDE-IV rolipram via the cAMP-signaling pathway suppressed generation of reactive oxygen metabolites (ROM) possibly via phosphorylating ras-type GTP-binding protein component of NADPH oxidase, and thereby blocking assembly of functional NADPH oxidase complex (Chini et al., 1994).

In the tubules homogenates cAMP-PDE activity is attributed to PDE4, which shows highest activity of this isozyme, while PDE3 and PDE1 are much less active. In contrast to glomeruli, in the tubule cGMP is equally hydrolyzed by PDE1 and PDE5. More studies done in the tubule segments from kidneys of different animal species (rat, rabbit, mouse; Kusano, 2001) showed a tendency toward higher cAMP-PDE activity.

Moreover, most studies done on segments of the collecting duct (inner medullary collecting duct (IMCD); outer medullary collecting duct (OMCD)) showed that cGMP-PDE activity was higher than the cAMP-PDE activity (Jackson et al., 1980). The use of specific PDE inhibitors indicates the presence of PDE4 and PDE3 in IMCD (Dousa et al., 1998), and activation of cAMP-PDE by Ca^{2+} in IMCD and in medullary thick ascending limb (MTAL) improve the presence of PDE1 (Kusano et al., 1985). On the other hand, studies were also conducted on renal cells, which could come from cells grown in primary culture or cells from fresh isolated tissue and grown in culture, by which the implication of PDEs in the renal functional could be referred.

Renal morphology



Nephron

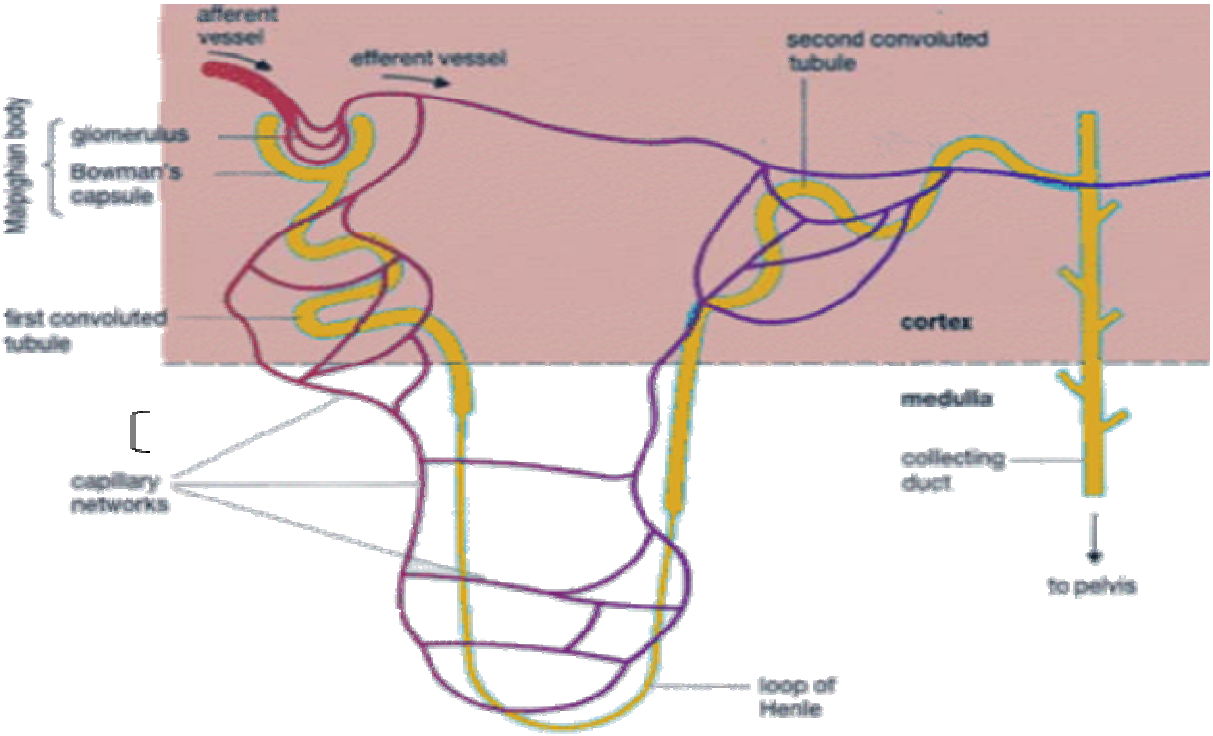


Figure10. Renal morphology. The kidney has a pale outer region- the **cortex**- and a darker inner region- the **medulla**.The medulla is divided into 8-18 conical regions, called the renal pyramids. The task of cleaning, or filtering, the blood is performed by millions of **nephrons**, remarkable structures that extend between the cortex and the medulla.

a) PDEs in the cell biology of the kidney

The $LLCPK_1$ cells (line of renal tubular cells with AVP-sensitive adenylyl cyclase) are used as model for studies of AVP-dependent cAMP signaling and PDEs isozymes in the kidney. In these cells the five PDEs, PDE1 to PDE5 are present (Rassier et al., 1992), PDE7 is not excluded, and there is evidence for a marginal presence of PDE3 based on minor effects of PDE3 inhibitors in cell extracts. In these cells, the PDE1 and PDE4 are the isozymes that play a role in metabolism of the cAMP pool generated in response to AVP (Rassier et al., 1992; Yamaki et al., 1992).

Furthermore, in the IMCD cells isolated from rat kidney and grown in culture (rIMCD), which preserved biological properties of the wild IMCD, expressed PDE1, PDE3 and PDE4 isozymes, but PDE2 and PDE5 were not detected (Yamaki et al., 1992; Dousa, 1994). In these cells the PDE4 inhibitor rolipram increased accumulation of cAMP both basal and stimulated by AVP, whereas AVP-dependent cAMP pool was metabolized primarily by PDE1 (Yamaki et al., 1992; Dousa, 1994). Besides, the levels of cGMP in rIMCD cells, both basal and stimulated with ANP were enhanced by inhibitors of PDE1 (Yamaki et al., 1992). However, in $LLCPK_1$-S#16 transfected with PDE4, only rolipram, PDE4 inhibitor, but not 8-Meo-IBMX (PDE1 inhibitor) enhanced AVP-stimulated accumulation of cAMP. This result suggests that the PDE4 controls AVP-dependent cAMP pool in the $LLCPK_1$ cells (Yamaki et al., 1993).

In the rat mesangial cells (MCs) (that constitutes the major cells of the glomerular) grown in primary culture, three PDE4 isoforms were found in these cells: PDE4A5, PDE4D3 and PDE4D5 (Walker et al., 1998). PDE3 was found with a lesser degree and PDE7 and PDE8 account for "residual" cAMP PDE activity. The cGMP activity was equally attributed to PDE1 and PDE5, and a portion of PDE5 is likely due to PDE9. Mesangial

cells are often the target of pathologic processes involved in the glomerulonephritis that is marked by a rapid proliferation of mesangial cells (Tsuboi et al., 1996).

b) Implication of PDEs in renal pathology

The major disorder of the kidney is the inability to excrete salt, which is known as renal sodium retention. Various mechanisms are involved in this renal disorder, including abnormal function of glomeruli with insufficient release of ultrafiltrate to the tubules. In the nephron, response to ANP is mediated through glomeruli-cGMP, which may be impaired in experimental nephrotic syndrome marked by renal sodium retention (Lee and Humphreys, 1996). However, resistance to the natriuretic action of ANP is a hallmark of states of pathological sodium retention including congestive heart failure, cirrhosis of the liver, nephrotic syndrome and hyperglycemic diabetics. Therefore, the blunted response to ANP was corrected by the PDE5 inhibitor, zaprinast, in these pathologies accounting for the increase in PDE5 activity in the glomeruli (Valentin et al., 1992; Supaporn et al., 1996; Angeli et al., 1994; Ni et al., 2001). But in hyperglycemic diabetic rats, ANP-stimulated cGMP in glomeruli is reduced but cannot be corrected by inhibiting PDE5, due to a decrease in the ANP receptor density (Sechi et al., 1995).

Recent studies have showed the involvement of PDEs in the control of mesangial cells proliferation and reactive oxygen metabolite (ROM) generation in glomeruli, widely involved in the renal diseases, particularly glomerulonephritis (Tsuboi et al., 1996). It has been shown that increases in cAMP enhance cell proliferation. However, incubation of mesangial cells with PDE inhibitors without stimulation of adenylyl cyclase, showed that PDE3 inhibitors cause a profound decrease of mitogenesis comparable to to that elicited by forskolin (Matousovic et al., 1995). PDE4 inhibitors at high concentrations caused a minor inhibition of MC proliferation, and at low concentration PDE4 inhibitors had non antiproliferative effect (Dousa, 1998). PDE1 and PDE5 inhibitors were without effect on

mitogenesis in these cells but in another study increase in cGMP in response to ANP had antimitogenic effect in MCs. Also, dual PDE3/PDE4 inhibitors showed a strong antiproliferative effect (Matousovic et al., 1995). However, PDE4 inhibitor (rolipram) suppressed ROM generation and PDE3 inhibitor (cilostamide) had much less suppressive effect on ROM generation, and PDE1 and PDE5 had no effect of all (Chini et al., 1994).

III- Introduction to liver cirrhosis

The liver, the largest organ of the body, performs different functions. These functions include, storing energy in form of glycogen, serving as a bacterial filter of the portal circulation, producing the bile, albumin, globin, fibrinogen, prothrombin and cholesterol. Hence, a functional liver is indispensable to life.

Cirrhosis of the liver is the twelfth leading causes of death by disease in the western world killing about 26,000 people each year (Tierney et al, 2002), and it is largely the result of alcohol abuse; other major contributors include chronic viral hepatitis, primary biliary cirrhosis (PBC), and iron accumulation in the liver (Cotran et al, 1999).

Liver cirrhosis is characterized by the destruction of the liver parenchyma, thus leading to a hepatic insufficiency. The natural history of this disease is associated with circulatory complications, which are independent of the etiology of the underlying liver cirrhosis. Portal hypertension (PHT) is the major and common syndrome associated with liver disease. It is characterized by a pathophysiological increase in portal pressure, due to an elevated portal venous inflow and an increased hepatic vascular resistance (Benoit & Granger, 1986; Sikuler & Groszmann, 1986; Lebrec & Moreau, 1999). Important sequels of liver cirrhosis (Bratovic and Jazic, 2003) are: esophageal and gastric varices (Park et al., 2004), portal hypertensive gastropathy and colonopathy (Kitano & Dolgor, 2000) and splenomegaly, in addition to ascites formation, spontaneous bacterial peritonitis (Garcia & Sanyal., 2001; Ferretto et al., 2004), hepatic encephalopathy, hepatocellular carcinoma (Lesi et al., 2004) and cardiomyopathy (Moller & Henriksen, 2002).

In addition, a hyperdynamic circulation (HC) begins in the portal venous bed as the consequence of portal hypertension due to the increased resistance to flow from altered hepatic vasculature. This was first described many years ago by Kowalski and Abelman

in 1953 and was defined as an increase in heart rate and cardiac output and a decrease in systemic vascular resistance. The initiating mechanism of this appears to be an arterial vasodilation that occurs in the splanchnic circulation due to portal hypertension.

As the liver disease progresses and liver function deteriorates the systemic hyperdynamic circulation becomes more manifest with activation of the vasoconstrictor renin-angiotensin-aldosterone system. Renal failure (hepatorenal syndrome) occurs by intensive vasoconstriction of renal arteries, which causes a decrease in renal perfusion and glomerular filtration rate (GFR). These complications are referred to as the hepatorenal syndrome (HRS).

Therefore, in liver cirrhosis, portal hypertension induces a hyperdynamic circulation, with high cardiac output, reduced systemic vascular resistance and resulting arterial vasodilation mainly in the splanchnic vasculature, associated with renal water and sodium retention and consequent ascites formation.

Several studies have been conducted to clarify the mechanisms by which these hemodynamic complications are established, including evaluation of the role of many vasoactive agents that play a major role in regulation of vascular tone. In addition, the pathogenesis of sodium and water retention and ascites formation has been largely a subject of debate and experimentation.

A- Animal models of liver cirrhosis

Extensive studies have been performed to elucidate the mechanism of the hyperdynamic circulation, hepatorenal syndrome and sodium retention. Human studies have yielded important and clinically relevant data. Ethical considerations, which limit the extent of investigations in patients with liver disease especially when invasive techniques and

repetitive measurements are needed, led to the development of several animal models of cirrhosis and portal hypertension that mimic the human disease. There are many advantages of using animal models to undertake research including, the reproducibility of the results, the rapid onset, and the ability to address mechanisms using interventions and experimental tools that may be difficult in humans. In general, there are two approaches to induce cirrhosis in animals: surgical and drug-induced.

a) Surgically induced-cirrhosis

- Bile duct ligation (BDL) (figure. 11)

Ligation of the common bile duct known as bile duct ligation (BDL) is a widely used experimental method to reproduce human cirrhosis. Animals undergoing only bile duct ligation develop a moderate hyperbilirubinemia and hypotension, which disappear by the end of the 2nd week, and the animal appears normal. On the other hand, the ligation and resection of the common bile duct induces more severe hyperbilirubinemia and results in secondary biliary cirrhosis with accompanying jaundice by the end of fourth week. The evolution of changes in systemic haemodynamics was characterized in a model of liver cirrhosis induced by chronic bile duct ligation (BDL) (Martinez et al., 2000). BDL rat model shows early portal hypertension, peripheral vasodilation and arterial hypotension, several days before sodium retention is detectable, and in the absence of changes in plasma levels of renin and aldosterone. Overall, these data suggest that, in the BDL rat model, sodium retention is secondary to portal hypertension and peripheral vasodilation. However, the disadvantage of this model is elevated mortality rate.

-Partial ligation of portal vein (PVL)

Stenosis of portal vein is a model of portal hypertension without liver cirrhosis. Stenosis of portal vein is a surgical method achieved by the partial ligation of the portal vein

(PVL). It is characterised by an acute marked increase of portal pressure but no liver pathology. In this model, the hyperdynamic changes are fully developed by 4 days, and at 8 days, maximal portosystemic shunting is achieved (Sikuler et al., 1985).

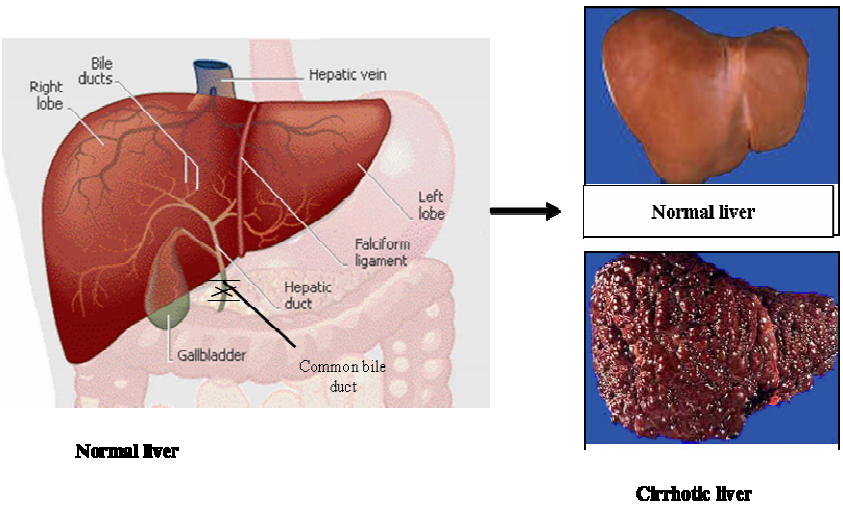


Figure 11. Liver cirrhosis is induced experimentally by common bile duct ligation; a double suture placed around the common bile duct and the duct is excised between the sutures (Wensing et al., 1990 a, b). Cirrhotic liver is marked by the presence of nodules on the surface of the liver.

A study done by Niederberger et al. (1996) on PVL and cirrhotic rats with ascites showed a typical hypercirculatory state compared to control. Both models had increased cardiac index, decreased systemic vascular resistance and hypotension. The differences between the two models are the absence of jaundice and ascites (Bomzon & Blendis, 1987) and the more rapid onset of portal hypertension in the PVL model. Thus the importance of the PVL model is that it reproduces all hemodynamic disturbances without liver damage and a hepatic deficiency (Ohno et al., 1991, 1993)..

b) Drug induced cirrhosis

- Carbon tetrachloride-induced cirrhosis in the rat

An alternate model of inducing cirrhosis in experimental animals is by the administration of hepatotoxins. Two hepatotoxic compounds have been successfully used to induce cirrhosis: carbon tetrachloride (CCl_4) in the rat (Jimenez et al., 1992) and dimethylnitrosamine in the dog. The first objective of using this model was to evaluate sodium retention and ascites formation in cirrhosis. The evaluation of cardiovascular changes was of secondary importance.

In 1969, McLean and colleagues reported that the rats treated with CCl_4 for 8 to 12 weeks developed liver cirrhosis. In this model, all the features of hyperdynamic circulations appear after 8 to 12 weeks of treatment. It should be noted that the cessation of administration results in spontaneous reversal of the hepatocellular damage and sodium retention by the kidney. This model has been used to clarify the relation between liver dysfunction and sodium retention and ascites formation (Mc Lean et al., 1969).

- Dimethylnitrosamine-induced cirrhosis in the dog

In 1970, Madden and his colleagues showed that intermittent oral administration of dimethylnitrosamine for 4 weeks produced hepatic cirrhosis which was progressive for at least 5 months after discontinuing drug. Many investigators also used this model to study the relationships between sodium retention, ascites formation and systemic abnormalities (Levy & Allotey, 1978). Using dimethylnitrosamine to induce cirrhosis in the dog, four arbitrary phases would be described:

1- During the treatment phase (8 weeks), portal hypertension develops soon after commencement of treatment.

2- Preascitic phase (8 to 12 weeks) in which systemic hymodynamics appear to be normal but sodium retention is occurring.

3- An ascitic phase (12 to 20 weeeks), in which all the features of the hyperdynamic circulation are present.

4- The fatal phase (20 to 22 weeks), in which the animal usually dies following gastrointestinal hemorrhage.

However, the disadvantage with this model is the fact that the dimethylnitrosamine is a human carcinogen; hence its use has not been widespread. In conclusion, all the features of portal hypertension are observed in all these animal models.

c) Comparison between the animal models

Irrespective of the method used, all the animal models develop the overall hyperdynamic circulation, i.e, systemic hypotension, a decrease in total peripheral ressistance, an elevated cardiac output, portal hypertension, portosystemic shunting and an apparent hypervolemia. However, the sequence or the order of onset of the elements that contribute to the hyperdynamic circulation differs. In the carbon tetrachloride-induced cirrhosis in the rat and in dimethylnitrosamine-induced cirrhosis in the dog, the altered portal and splanchnic hemodynamics precede the systemic changes. This sequence is also observed in the rat which undergone partial ligation of the portal vein. However, in the animal models of cirrhosis induced by ligation of the bile duct, the systemic changes occur before the onset of the portal hypertension and changes in splanchnic hemodynamics.

It appears that the hepatocellular damage that was induced by hepatotoxins or surgically obstruction of biliary outflow is not critical, since the portal vein stenotic rat developed all features of the hyperdynamic circulation. Furthermore, in the bile-duct ligated animals the complications become sever with the development of hyperbilirubinemia, which diminishes the ability of the cardiovascular system to respond.

B- Pathophysiological Complications associated to liver cirrhosis

a) Sodium and water retention and ascites formation: "Underfilling"hypothesis. (Figure. 11)

Patients with advanced cirrhosis show abnormal regulation of extracellular fluid volume resulting in accumulation of fluids as ascites. The mechanism by which ascites develops in cirrhosis is multifactorial. Severe sinusoidal portal hypertension and hepatic insufficiency are the initial factors. The main consequence of this hepatic pathology is a hyperdynamic state, which is characterised by increased cardiac output, decrease in peripheral vascular resistance and evidence of arterial vasdilation in the splanchnic area. The rapid and high inflow of arterial blood into the splanchnic microcirculation is the main factor increasing hydrostatic pressure in the splanchnic capillaries leading to an excessive production of lymph. Leakage of lymph from liver and splanchnic organs is the mechanism of fluid accumulation in the abdominal cavity.

Ascites occurs in more than half of all patients with cirrhosis and indicates progression of the disease. Many theories have been proposed to clarify mechanisms of ascites formation. The traditional theory (Witte et al., 1971) considers that the hepatic pathology constitutes a mechanical block to portal blood flow. This backward flow results in an increased hydrostatic pressure in the hepatic sinusoids and the splanchnic capillaries resulting in a disruption of the Starling forces equilibrium, secondary to increased hydrostatic pressure and decreased oncotic pressure, the latter from impaired serum protein synthesis (albumin) by the liver. This later leads to filtration of the fluids into the peritoneal space. The hyperfiltration is initially compensated for by an increase of the lymphatic flow, which returns the fluid to the systemic circulation via the thoracic duct. As the portal hypertention worsens, however, the lymphatic system fails to drain the interstitial fluid, which accumulates in the peritoneal cavity as ascites.

Loss of fluids from the intravascular compartment results in a decrease in plasma volume that leads through activation of vasoconstrictor mechanisms to the activation of the sympathetic and the renin-angiotensin-aldosterone system (RAAS) leading to sodium and water retention by the kidney (Bichet et al, 1982; Bernadi et al, 1993). The retained fluid tends to refill the intravascular compartment, but fluids continue to leak into the peritoneal cavity such that the kidney continues to retain sodium and water, thus creating a vicious cycle.

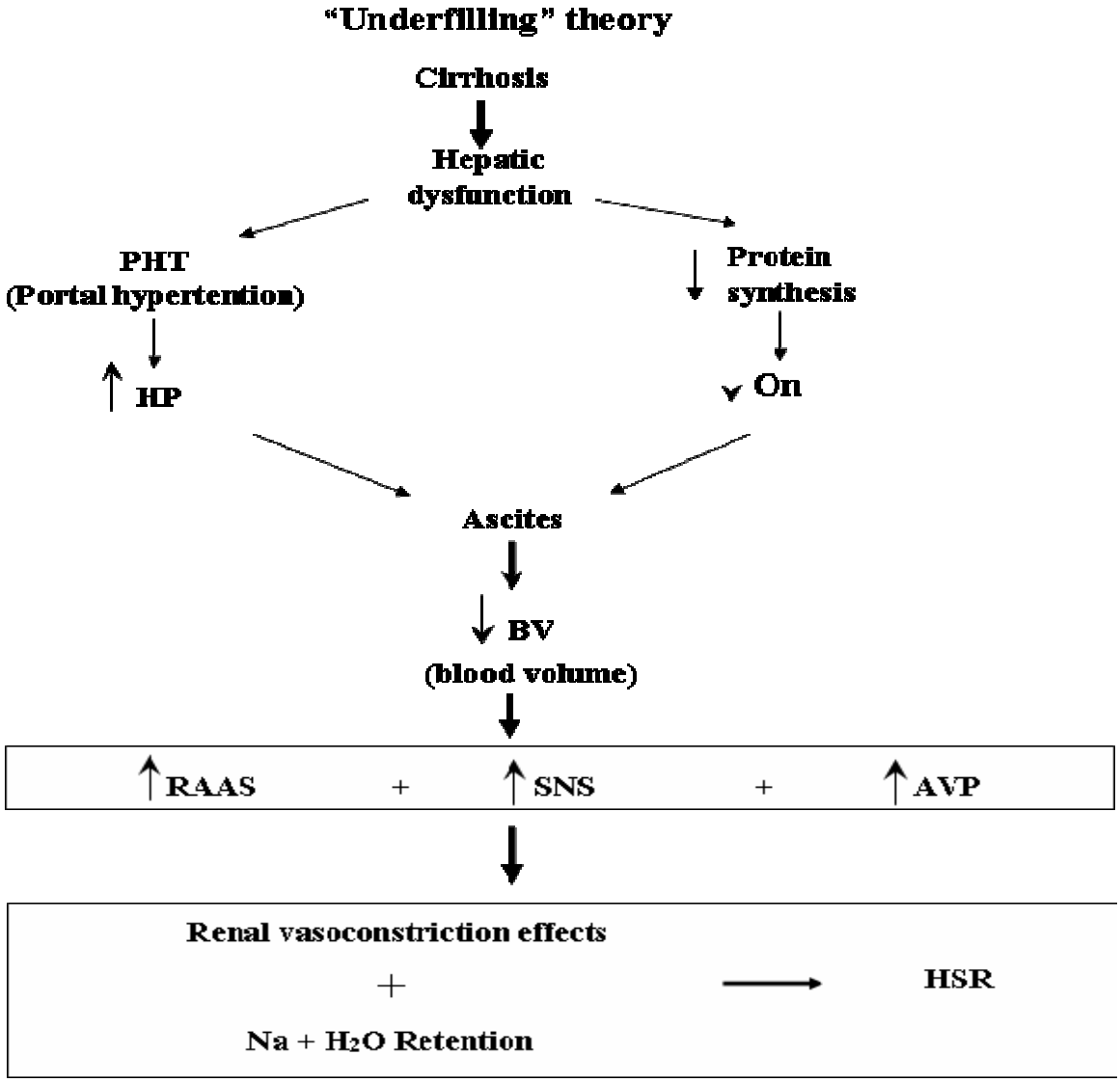


Figure 11. Underfilling theory of Na retention (Witte et al., 1971; Bichet et al., 1982). Liver cirrhosis forms a mechanical block to portal blood flow. Increased hepatic hydrostatic pressure (HP) and decreased oncotic pressure (On) in the splanchnic capillaries leads to ascites formation through the interstitial space that results in a decrease in BV. Increased activity of renin-angiotensin-aldosterone system (RAAS), sympathetic nervous system (SNS), and arginine-vasopressin secretion (AVP). Excessive renal vasoconstriction and sodium retention lead to the hepatorenal syndrome (HRS).

b) "Overflow" hypothesis for sodium retention. (Figure. 12)

The underfilling hypothesis therefore suggests that sodium and water retention develops as consequence of ascites formation, and strongly supports the concepts that a diminished intravascular volume is the major determinant of the enhanced tubular reabsorption of sodium in patients with established cirrhosis as a result of the RAA and sympathetic nervous system activation. Liebermann and his colleagues (1967) criticized this theory because they found that the plasma volume was elevated in the presense of ascites and not reduced as suggested by the underfilling hypothesis.

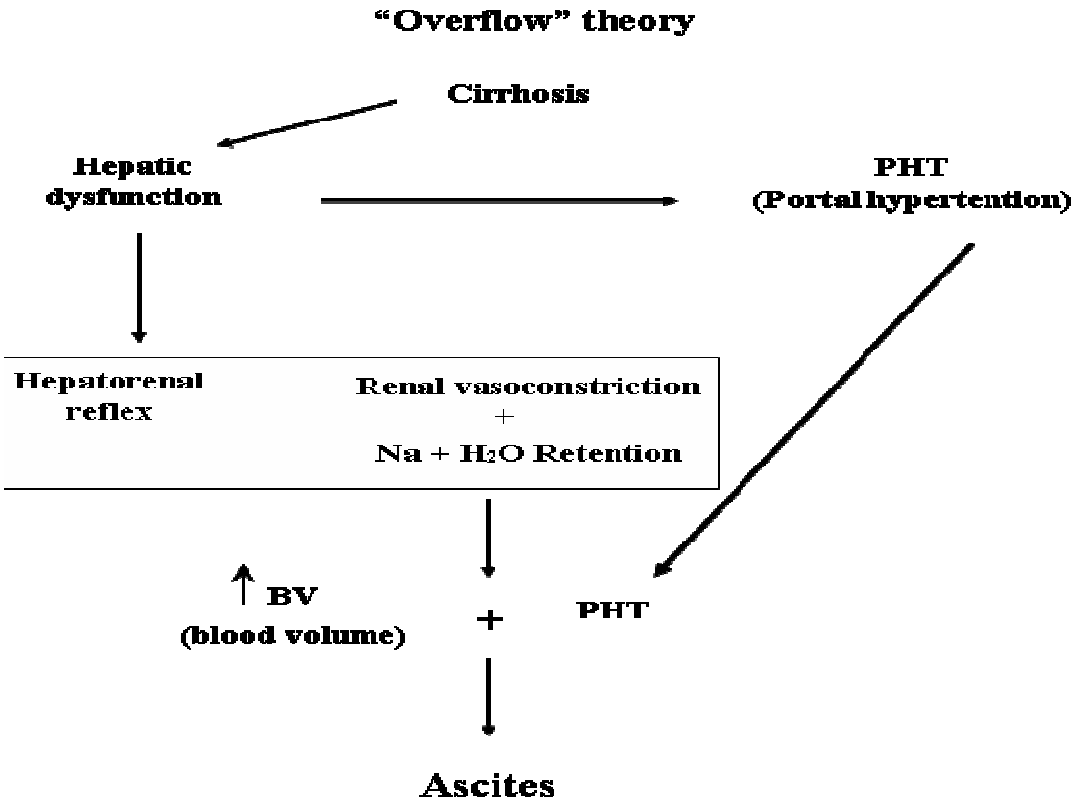


Figure12. "Overflow" theory of Na retention (Lieberman et al., 1967, 1970)**.** Hepatic dysfunction in cirrhosis increases renal vasoconstriction through a hepatorenal reflex leading to sodium and water retention. The increase in blood volume (BV) is corrected by the formation of ascite. No changes occur in the activity of renin-angiotensin-aldosterone system (RAAS), sympathetic nervous system (SNS), and arginine-vasopressin secretion (AVP).

Moreover, two additional observations were made by these authors in 1970: (1) the rate of ascites formation did not increase following paracentesis, nor did plasma volume fall, both of which should occur if the traditional concept of ascites formation is correct; and (2) ascites could be made to reform in patient with cirrhosis by administering a sodium retaining hormone (Florinef). Such a drug expands plasma volume and extracellular fluid in normal people so that before oedema forms, sodium-losing factors are activated to prevent further blood volume expansion. Sodium losing increases in the urine, this response is known as renal "sodium escape" (Reilman et al., 1958). Cirrhotic patients, however, failed to escape the sodium–retaining effect of this drug indicating that ascites probably formed as a way of preventing further plasma-volume expansion.

These results lead the authors to propose that a disturbance of the kidney causes water and salt retention prior to ascites. Liebermann & collegues (1970) proposed the theory of "overflow": Primary renal sodium retention occurred and led to increase blood volume and consequently increase cardiac index and reduce vascular resistance as adaptive circulatory mechanisms to the excess of the intravascular volume. The abnormal Starling forces of the hepatic and splanchnic vascular beds and the circulating hypervolemia induced by portal hypertension would lead to "overflow" ascites formation. The mechanism by which the liver cirrhosis leads to renal sodium retention was not identified, however.

A hepathorenal reflex mediated by the intrahepatic mechanoreceptors was one of the mechanisms later proposed for primary sodium retention, as described by Kostreva et al. (1980). They suggest that there is a hepatorenal reflex which is activated during cirrhosis causing increase in the renal sympathetic efferent nerve activity after the hepatic venous system is congested, and this renal efferent nerve activity is associated with increased

renal vasoconstriction (Block et al., 1952) and tubular sodium and water retention (Slik et al., 1974). Recently Ming et al. (2002) proposed a mechanism for this hepatorenal reflex. In this study, they show that the water and sodium retention commonly seen in the hepatorenal syndrome is related to intraportal adenosine accumulation due to the decrease in intraportal flow.

Moreover, in a series of studies, Levy et al., (1977, 1978) provided support to the overflow hypothesis. They suggest the existence of a hepatorenal reflex resulting from increased sinusoidal pressure in cirrhosis that contributed to the sodium retention by the kidney (Unikowsky et al., 1983). Furthermore, it was shown that elevation of portal and sinusoidal pressure by occlusion of the thoracic vena cava resulted in increased activity in hepatic afferent nerves and in cardiopulmonary and renal sympathetic efferent nerves; section of the anterior hepatic nerves eliminated the increase in renal sympathetic activity (Kostreva et al., 1980). Later studies showed that infusion of glutamine into the superior mesenteric vein at a rate known to induce liver cell swelling led to marked decreases in renal glomerular filtration rate, renal plasma flow and urinary flow rate, while the same dose administered intravenously did not induce any effect (Lang et al., 1991). Further evidence for a hepatorenal reflex was provided by Morita and his colleagues (1993) who demonstrated decreased renal nerve activity following a high salt diet associated with increased natriuresis. Both responses were abolished or attenuated by hepatic denervation. Similar results were obtained by by Meredith et al (1997). Dibona and Sawin (1995) demonstrated that the sensitivity of the hepatic baroreflex was not altered in rats with cirrhosis due to bile duct ligation, thus raising the possibility that its activation may indeed play a role in the sodium retention observed in cirrhosis. More recently, it was susuggested that the water and sodium retention observed in the

hepatorenal syndrome may be related to intraportal adenosine accumulation due to the decrease in portal blood flow (Ming et al., 2001, 2001).

A hepatorenal link to sodium and water retention has also been proposed which is not dependent on hepatic or renal nerve activity but is humoraly mediated: the role of cAMP released by the liver (and or other organs) in the regulation of renal hemodynamics and function. Bankir et al., (1997) proposed that the increased level of cAMP in the plasma could result from action of hormones such as glucagon on the liver, and these high levels of cAMP could lead to an increase in GFR, urine flow and renal sodium excretion.

These results collectively give evidence that sodium and water retention that occurs in cirrhosis is associated with a decrease in hepatic function, which causes sodium and water reabsorption by the kidney. Neverless, a hepatorenal role of cAMP not dependent on nerve activity is also interesting to note, and may be a subject of debate and experimentation.

c) Peripheral arterial vasodilation (PAV), a hypothesis of Na retaining. (Figure. 13)

Schrier et al. (1988) proposed that patients with ascites behaved as if their arterial vascular compartment was actually underfilled rather than overfilled. This led them to propose a third hypothesis: the peripheral vasodilation is the event that initiates sodium and water retention in cirrhosis. They considered that the portal hypertension is the initial event to happen, with resultant splanchnic arteriolar vasodilation causing relative underfilling of the vascular compartment. This would decrease effective arterial blood volume in the central arterial tree. This reduction in arterial filling stimulates the neurohormonal mediators such as the renin-angiotensin-aldosterone system and sympathetic nervous system and vasopressin release (antidiuretic hormone) and promotes

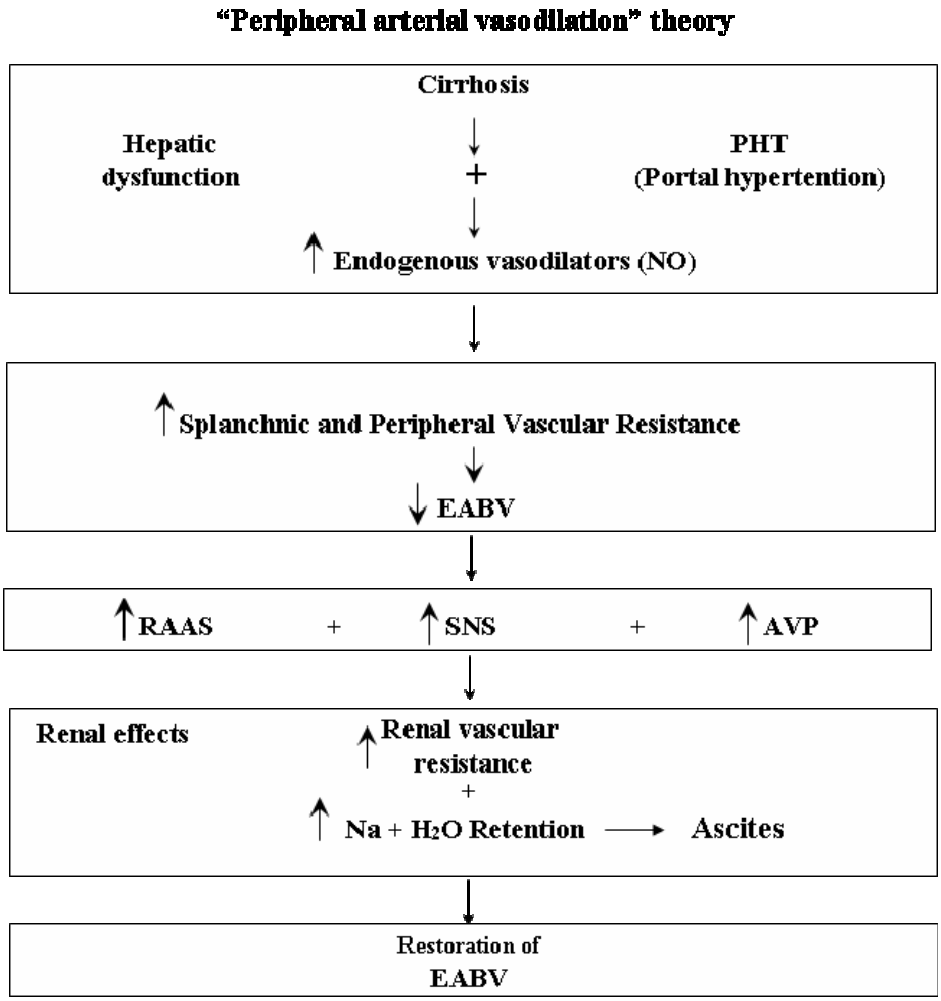


Figure 13. "Peripheral arterial vasodilation" PAV hypothesis of sodium and water retention and ascites formation in liver cirrhosis (Francis and Dudley, 1992). This hypothesis has both characteristics of both classical "underfilling" and "overflow" theories. Decreased effective blood volume (EABV) relative to the enlargement of the arterial compartment. Increased activity of renin-angiotensin-aldosterone system (RAAS), sympathetic nervous system (SNS), and arginine-vasopressin secretion (AVP).

sodium and water retention. According to this theory, the splanchnic arterial vasodilation causes a forward increase in splanchnic capillary pressure that enhances the effects of portal hypertension on the filtration coefficient in splanchnic capillaries, which facilitates the formation of ascites (Korthuis et al., 1988).

Moreover in human and experimental cirrhosis with ascites, hemodynamic studies showed low arterial pressure despite very high levels of angiotensin II, noradrenaline, and vasopressin (antidiuertic hormone), reflecting a great disparity between the capacity of arterial vascular bed and the arterial blood volume (Fernadez-Seara et al., 1989). The increased cardiac index and decrease in peripheral vascular resistance were greater in patients with ascites and functional renal failure compared to patients with ascites but preserved renal function, and this exaggerated reduction in peripheral resistance activated the pressor system more intensely in these patients suggesting that an intense splanchnic vasodilation may play a crucial role in the development of hepatorenal syndrome.

In addition, studies using head out water immersion (HWI) of cirrhotic patients supported this theory. These studies showed that ascitic patients that underwent HWI, a manoeuvre that redistributes the blood volume into the central compartment, demonstrated striking normalization of renal sodium handling, and experienced marked natriuresis and diuresis in association with reduction in levels of norepinephrine, vasopressin, and aldosterone (Bichet et al., 1983; Gerbes et al., 1993). These results suggested that the primary abnormality in these patients was a diminution in the central or effective blood volume resulting in activation of the compensatory mechanisms.

On the other hand, the greatest attention has focused on nitric oxide release as the cause of splanchnic peripheral vasodilation and numerous studies have provided evidence to support such a role in liver cirrhosis (see review of Wiest and Grozann, 2002). Similarly, recent studies(Garcia-Estan et al., 2002) have shown that acute inhibition of nitric oxide (NO) synthesis improves sodium and water excretion and increases blood pressure in cirrhotic rats with ascites, thus suggesting that NO is an important factor contributing to the arterial hypotension and sodium retention of liver cirrhosis. Garcia-Estan et al. (2002) shows that chronic NO synthesis inhibition with L-NAME (NO synthase inhibitor)

improves renal perfusion pressure and increases the lower sodium and water excretion of cirrhotic rats with ascites. Thus, an enhanced production of NO is an important factor contributing to the renal sodium and water retention characteristic of liver cirrhosis.

C- Neurohormonal agents mediating regulation of renal vasculature function

Even though the PAV hypothesis is widely accepted, some studies in ascitic patients with avid sodium retention showed normal neurohormonal levels (Salo et al., 1995). Furthermore, evidence in pre-ascitic cirrhosis has shown the presence of subclinical sodium retention and adequately filled effective arterial blood volume in the absence of the neurohormonal systems activation (Wong et al., 1996). These finding suggested that the peripheral arterial vasodilation does not fully explain all the alterations that occurred in cirrhosis at all stages of the disease. It is clear that evidence in the litterature exists to support both the overflow and the PAV of ascites formation. It is possible that the "overflow" hypothesis may apply at early phases of the disease and PAV become predominent at late stages of liver cirrhosis.

However, according to the PAV hypothesis, renal arterial vasoconstriction is the cause of renal failure in patients with advanced liver cirrhosis. Many studies have showed elevated plasma levels of vasoconstrictors during progression of cirrhosis (Figure. 14), such as angiotensin II (Laragh et al., 1963) and norepinephrine (Nicholls et al., 1985; Blendis et al., 1987), vasopressin (Akriviadis et al., 1997), which correlate with severity of the cirrhosis and targeted the renal vasculature leading to the sodium and water retention by the kidney (figure. 14).

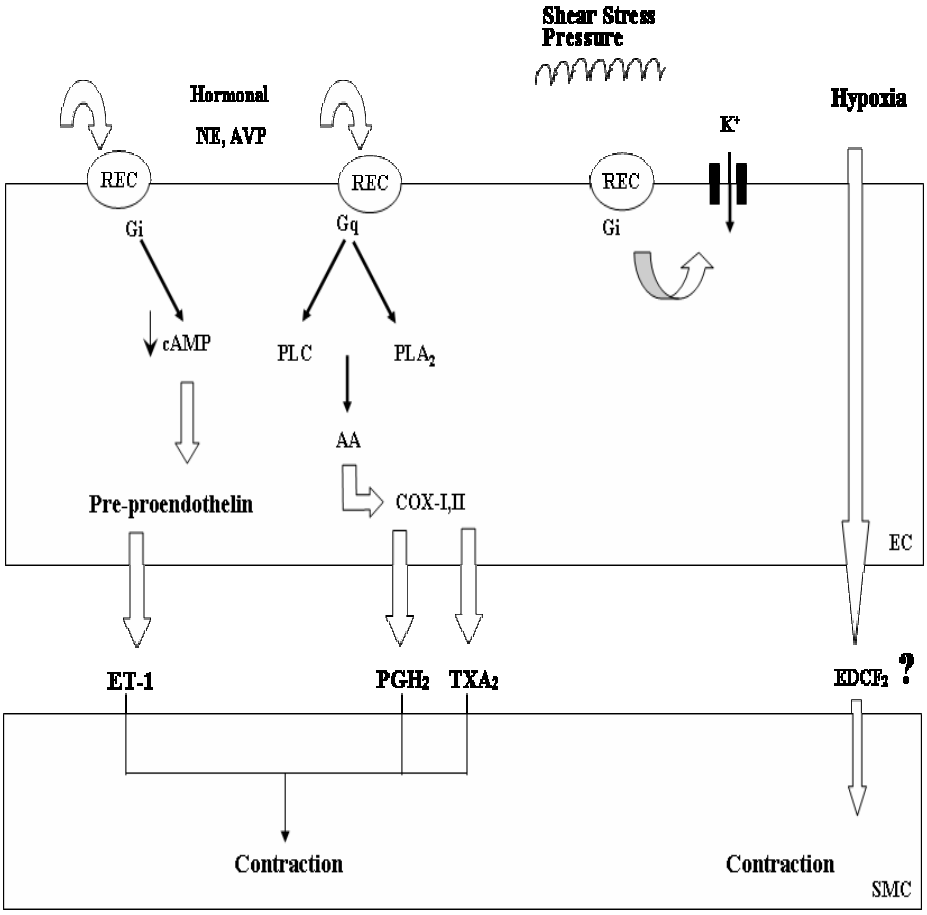


Figure 14. Agonist-stimulation of endothelium derived contracting factors (EDCFs) (Cahill et al., 2001). Endothelium initiate vasoconstriction through the release of diffusible vasoconstrictor substances: endothelin (ET), thromboxane (TXA2), and endothelium derived contracting factor (EDCF2), the nature of which is unknown.

Angiotensin II is an important end product of the renin-angiotensin axis that is activated in advanced stages of liver cirrhosis (Wilkinson & Williams, 1980). In addition, there is evidence of the intrarenal role of this antidiuretic factor in the proximal tubule cells (Ingelfinger et al., 1990). Wong and coworkers (1998) used the angiotensin II receptor antagonist losartan, in human preascitic cirrhosis, at a dose that did not disturb renal or systemic hemodynamic. Lower doses of losartan induced but not fully sodium excretion in these patients. This observation raised the suggestion that other factors may also contribute to the increase sodium and water retention in cirrhosis, such as endothelin (ET). On one hand, there is evidence for increase plasma level of ET in cirrhotic patients compared to normal people (Bernardi et al., 1996; Trevisani et al., 1997). In addition, the ET receptor antagonist bosentan does not induce changes in the arterial pressure or systemic hemodynamic in advanced cirrhotic rats (Sogni et al, 1998), nor does it ameliorate the sodium retention (Asbert et al, 1993).

More studies have shown other factors within the kidney which could contribute to renal dysfunction such as eicosanoids with anti-natriuretic or vasoconstrictors activities. It was suggested that the hepatorenal syndrome (HRS) could be the consequence of imbalance between the renal synthesis of the prostaglandin vasodilator (PGE_2 and PGI_2) and vasoconstrictor eicosanoids such as thromboxanes (TXA2). A study done by Laffi and coworkers in 1986 in cirrhotic patients with HRS showed a decrease in urinary TXB2. In contrast, inhibition of thromboxane synthesis did not improve renal function in these patients (Zipser et al., 1984). Moreover, cirrhotic patients with HRS had increased urinary excretion of other eicosanoids like leukotriene E4, and N acetyl-leukotriene E4 as compared to healthy people and patients without ascites (Huber et al., 1989). Thus, leukotrienes could participate in the pathogenesis of renal dysfunction in cirrhosis.

Altogether these results raised the possibility of a role for these vasoconstrictor agents in the renal vasoconstriction occurring in association with the liver cirrhosis.

In addition, a decrease in vasodilating agents (Figure. 15) such as prostaglandins might play a role in the renal vasoconstriction leading to sodium and water excretion by the kidney. Accordingly, patients with HRS have shown lower urinary excretion of PGE2 and 6-Keto-PGF1α than patients with ascites and without renal failure (Laffi et al., 1986). Moreover, cirrhotic patients with ascites without renal failure showed increase urinary levels of vasodilating prostaglandins PGE2 and PGI2 compared to healthy people (Laffi et al., 1986), and the use of a cyclooxygenase inhibitor induced a significant decrease in renal blood flow and GFR in patients with cirrhosis and ascites (Quintero et al., 1986). Garcia-Estan et al., (1996) also revealed a compensatory increase in renal prostaglandin activity in response to phenylephrine in BDL rats. These results suggested that increased renal synthesis of vasodilating prostaglandins contributed to maintaining sodium excretion and blood flow in cirrhosis, and the decreased synthesis of these prostaglandins contributed to renal vasoconstriction and sodium retention.

Moreover, there was an evidence for a role of the natriuretic hormones like atrial natriuretic peptide (ANP) in liver disease. Such hormones play an important role by contributing to sodium and water excretion by the kidney.

The role of ANP and potential defects of ANP in liver disease were widely reviewed. Patients with cirrhosis of the liver showed no decrease of ANP plasma concentrations (Gerbes, 1993), in contrast, patients showed progressively elevated plasma ANP to maintain a sodium balance (Trevisani et al., 1995) Recent studies have shown increased plama levels of ANP in patients with liver cirrhosis (Montoliu et al., 2005). In this study the authors found that both increased ANP and increased activation of soluble guanylyl cyclase by nitric oxide contribute to increased plasma cGMP in patients. The

concentrations of ANP and cGMP in plasma increase with the degree of disease and are higher in patients with ascites.

Additionnaly to the elevated plasma level of ANP, previous studies found a resistance to ANP in liver disease (Ni et al., 1996). The authors showed that a blunted response to ANP in cirrhotic rats devoid of intrarenal disease was related to increased activity of cGMP specific phosphodiesterase (PDE5) in renal target cells for ANP as well as to heightened renal nerve activity. Zaprinast (a selective PDED5 inhibitor) restored ANP responsiveness in both innervated and denervated kidneys from common bile-duct ligation rats.

Ni et al., (2001) also showed that DMPPO a more potent and selective PDE5 inhibitor (DMPPO exhibited very poor inhibitory activity against the calcium-calmodulin-dependent PDE1 in renal homogenates cells) normalized the blunted ANP-dependent cGMP accumulation by renal cells from CBDL rats in vitro and in vivo. Indeed, immunoprecipitation studies indicated an increase in PDE5 protein level. These results demonstrate that renal resistance to ANP in CBDL rats is accompanied by heightened activity of PDE5, which is due largely to an increase in PDE5 protein, and contributed to the observed increase in cGMP hydrolysis. Finally, this PDE5-dependent ANP resistance might represent an important contributor to the sodium retention of liver disease.

The effects of adenosine in the kidney have been extensively studied. Adenosine induces vasodilation of most vascular beds except the lungs and the kidney, where it causes vasoconstriction through activation of A1 receptors. Therefore, administration of dipyridamole (drug that increases level of adenosine in the extracellular fluid by blocking its reuptake) has been shown to result in renal vasoconstriction and sodium and water retention, an effect which was enhanced in cirrhotic patients with increase RAAS activity

(Llach et al, 1993). Thus adenosine by potentiating the effect of angiotensin II may play a role in the renal vasoconstriction in cirrhosis.

Therefore, it is believed that in compensated liver cirrhosis, sodium retention and eventually renal vasoconstriction and the hepatorenal syndrome are consequences of intense activation of the SNS, RAAS and vasopressin (ADH) as well as the renal resistance to the vasodilation effect of prostaglandin and ANP.

D- Vascular effectors of splanchnic vasodilation (Figure. 15)

The hyperdynamic circulation associated with decreased systemic and splanchnic vascular resistance and increased cardiac output are features of advanced liver cirrhosis. The initial mechanism of the circulatory abnormalities is an arterial vasodilation that occurs especially in the splanchnic area. The vasodilation was widely attributed to the increased circulating levels of endogenous vasodilating factors such as substance P, PGI2 and PGE2, calcitonin gene related peptide (CGRP), glucagons, adrenomedullin, carbon monoxide and vasoactive intestinal peptide (Hadoke, 2001).

Glucagon is a prime gut peptide mediating vasodilation especially in the splanchnic vasculature (Nakahara et al., 1997). Increased pancreatic production of glucagons was observed in cirrhotic patients compared to normal subjects (Silva et al., 1990; Lin et al., 1996). The infusion of a glucagon antiserum induced significant reduction in splanchnic blood flow, but did not ameliorate the systemic vasodilation (Benoit et al., 1986). Thus, despite the increase circulating level of glucagons in cirrhosis, it seemed that its contribution to the hyperdynamic circulation appeared to be limited.

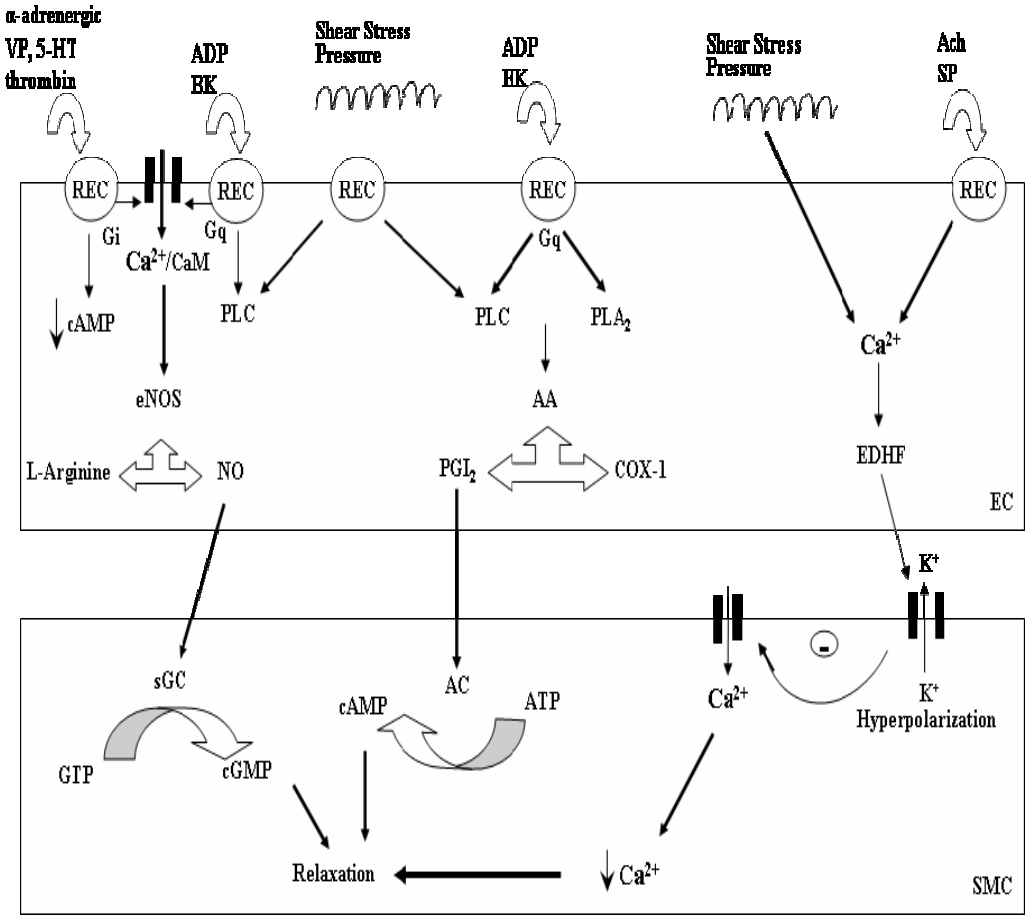


Figure 15. Endothelial NO, PGI2 and EDHF production induce vasorelxation (Cahill et al., 2001). Release of the endothelial vasorelaxant factors may be mediated through endothelium agonists activated-receptors coupled to G proteins, Gi or Gq (vasopressin, bradikinin, 5-HT, ADP and thrombin), or stress induced-EDHF release.

Circulating concentrations of substance P are elevated in patients with liver cirrhosis (Uemura et al., 1998). Substance P is a potent vasodilator, stimulates NO release from endothelium through activation of the endothelial L-arginine-NO pathway (Whittle et al., 1989). It role has been investigated by Chu and his colleagues (1997), who showed higher plasma level of substance P in cirrhotic rats with ascites than cirrhotic rats without ascites, and control rats. The difference in plasma substance P values however was not significant between cirrhotic rats and control rats, but plasma levels of nitrate and nitrite progressively increased in relation with the severity of the disease. However, no correlation was found between the substance P levels and those of nitrate/nitrite. The evidence for substance P as cause of vasodilation is still minimal in liver cirrhosis.

Plasma levels of vasoactive intestinal peptide (VIP) plasma levels were also shown to be elevated in cirrhotic patients (Henriksen et al., 1986). Lee and colleagues (1996) showed that plasma VIP concentrations were significantly higher in cirrhotic rats than in control rats. Hemodynamic parameters like cardiac index, systemic resistance, portal blood flow, and splanchnic blood flow were measured in both groups before and after infusion of VIP. This infusion did not induce any hemodynamic changes in bile duct ligated rats, whereas in the sham-operated rats VIP infusion increased mesenteric blood flow without affecting any other parameter. This finding suggests that the VIP does not contribute as a major mediator of the hyperdynamic circulation.

Cirrhotic patients and control people underwent an investigation with determination of systemic hemodynamic and circulating calcitonin gene related peptide levels (CGRP) (Moller et al., 1996). The circulating CGRP was significantly elevated in cirrhotic patients compared to controls and it increases with the severity of the disease. The increased CGRP level covaried negatively with the reduced vascular resistance, reduced central blood volume, and reduced central circulation time, and positively with the non-

central blood volume. Moreover, Hori et al. (1997) have shown reduced vascular response in cirrhotic rats after infusion of human α-CGRP (inhibitor of CGRP) as compared to controls, and the administration of high dose human α-CGRP significantly increased the mean arterial blood pressure and total vascular resistance in cirrhotic rats compared to controls, which remained less than controls. These authors suggested that the CGRP contributed to the systemic hyperdynamic circulation occurred in cirrhosis.

Recent studies have suggested that carbon monoxide, a product of the heme oxygenase (HO) reaction, may be involved in the splanchnic circulation in portal hypertensive rats (PVL). Fernandez et al. (2001) found increased expression of the heme oxygenase (HO-1) in splanchnic tissues from portal hypertensive rats compared to sham-operated rats (SO). In this study, the perfusion pressure in the mesenteric vessels in response to KCl and methoxamine (α-adrenergic agonist) was determined in both groups. The perfusion pressure in response to these vasoconstrictor agents was significantly lower in the PVL rats than in sham-operated rats (SO). After perfusion of ZnMP, a heme-oxygenase inhibitor, the pressure response to KCl increases slightly. In addition, the simultaneous perfusion with ZnMP and L-NAME (NO synthase imhibitor) significantly potentiated the increase pressure response to KCl in PVL rats, but no significant difference was obtained between vessels from PVL and SO rats. Thus, carbon monoxide appears to contribute to splanchnic vasodilation but its exact role is still unclear.

A variety of other ciculating vasodilators have been evaluated, such as bile acid. Thomas et al. (1991) showed that the bile duct cannulation effectively depleted both normal and portal hypertension animals of their circulating bile acid pool and significantly reduced portal venous inflow in portal hypertensive but not in control rats. Moreover, bile acid depletion did not completely abolish gastrointestinal hyperemia. Thus bile acids showed a partial role in the splanchnic hyperemia of portal hypertension.

Evidence from several studies supported the role of prostaglandin in the hyperdynamic circulation of portal hypertension. PGI_2 levels in portal hypertension were significantly increased over the normal in humans (Sitzmann et al., 1993). Briux et al. (1985) showed that indomethacin administration (50 mg/kg for 24h) to cirrhotic patients significantly reduced cardiac output and increased peripheral vascular resistance. These changes in the hemodynamics were associated with a significant reduction in hepatic blood flow and a slight decrease in portal pressure.

Recently, a newly potent vasodilating peptide, adrenomedullin has been described (Tahan et al., 2003).In this study, adrenomedullin levels were found high in non-cirrhotic and cirrhotic portal hypertension patients, increased proportionally with the severity of cirrhosis, and were significantly higher than those with non-cirrhotic portal hypertension patients. Portal hypertension plays an important role in the increase of ADM and NO. Parenchymal damage in cirrhosis may contribute to the increase in these parameters. No study examined the importance of the role of adrenomedullin in the hemodynamic abnormalities occurring in cirrhosis.

The role of ATP-sensitive potassium channels (K_{ATP}) in cirrhosis has been raised (figure. 15). Moreau et al. (1994) examined the role of K_{ATP} in vasodilation in cirrhosis. In cirrhotic rat glibenclamide, a K_{ATP} inhibitor, at high dose 20 and 30 mg/kg significantly increased vascular resistance in systemic and portal territories compared to baseline in cirrhotic rats. Administration of aprikalim, K_{ATP} channel opener, induced arterial hypotention, which was less marked in cirrhosis than in control. In the same study another investigation was done; following administration of glibenclamide, aprikalim-induced arterial hypotension was significantly less marked in cirrhotic than in normal rat. Thus, the authors concluded that cirrhosis contributed to an alteration of the vascular tone by altering the activity of K_{ATP} channels.

Because the major site of the arterial vasodilation is the splanchnic circulation, a local mechanism is more likely possible. Most available data, obtained primarily from arterial vessels of cirrhosis indicate that nitric oxide (NO) is the main mediator of the arterial vasodilation in liver cirrhosis (Martin et al., 1998a, b), and that endothelial NO production is markedly increased in the arterial vessels of cirrhotic rats compared to controls (Martin et al., 1998a, b), and predominant in the splanchnic vasculature (Wiest et al., 1999). Saphr et al. (2002) examined the acute effects of L-NMMA, a NOS inhibitor, on the systemic, hepatic, and renal hemodynamic in cirrhotic patients. After L-NMMA infusion, cirrhotic patients showed a decrease in their cardiac output, hepatic blood flow and plasma renin activity, and an increase in the systemic resistance. Thus, these results confirmed that NO depletion improved clinical status of the patients.

Because the direct measurement of NO levels is technically difficult, the production of NO has been indirectly assessed in rats with cirrhosis by investigating the effect of NO synthesis inhibition on the systemic hemodynamic.

The main source of vascular NO production is the endothelium constitutive NO synthase (eNOS-3), as suggested by Theodorakis.et al. (2003). The authors used portal ligated mice of the wild type, and eNOS and iNOS-knock out mice (inducible NOS) to determine the implication of the different NOS to hemodynamic changes, in portal venous pressure, abdominal aortic blood flow, and portosystemic shunt 2 weeks postoperatively. iNOS-knockout mice developed increases in portal venous pressure, abdominal aortic blood flow and shunt after PVL compared to sham-operated mice; in contrast, eNOS knockout mice had no significant changes in portal venous pressure, and abdominal aortic blood flow but developed shunts compared to sham-operated mice. This study showed that the presence of eNOS contributes to the hemodynamic changes associated to portal hypertension.

Moreover, Niederberg et al. (1996) demonstrated a marked increase of eNOS expression in the mesenteric arteries of PVL rats and cirrhotic rats with ascites as compared to sham-operated rats (SO). Another finding by Cahill et al. (1996) was that eNOS activity was elevated in the superior mesenteric artery of portal hypertensive rats.

Moreover, in order to investigate the contribution of NO in the hemodynamic abnormalities of liver cirrhosis, studies were conducted to estimate the concentration of cyclic guanosine monophosphate (cGMP), a second messenger of NO. Hence, Ros et al. (1995) estimated the vascular NO production in the arterials vessels of the cirrhotic and controls rats, using a co-incubation assay. Measurement of reporter monolayer cell-associated to cGMP levels allows evaluating the influence of nitric oxide release by arterial segments. Rat foetal lung fibroblast cells (RFL) served as reporter cells. Basal and bradykinin-stimulated cGMP was significantly higher in RFL-6 cells coincubated mesenteric arterial segments of cirrhotic rats as compared to control mesenteric arterial segments.

However, in the intrahepatic vasculature of cirrhotic rats a paradoxial vasoconstriction in response to acetylcholine, was observed suggesting diminished NO production (Gupta et al., 1998). This reduction of endothelial NO release also led to a greater increase in perfusion pressure in response to incremental increase in flow and subsequent shear stress (Wiest and Grozmann, 2002). Since, shear stress is a potent physiological regulator of eNOS (Ranjan et al., 1995), NO production in cirrhotic vasculature did not increase in response to increased shear stress.

Therefore, a large body of evidence suggests that increased NO production, particularly in the splanchnic vascular bed, which causes of the arterial vasodilation in liver cirrhosis and the hyperdynamic circulation. In addition to the emphasize findings of enhanced

activity of vasoconstrictive systems and marked increase in circulating level of endogenous vasoconstrictors in liver cirrhosis, impaired contractile hyporesponsiveness was detected.

E- Vascular reactivity in cirrhosis and pre-hepatic portal hypertension models

Portal hypertension is a common feature of cirrhotic patients; it is characterized by pronounced arterial vasodilation in the splanchnic bed and contributes to an increase in the portal pressure despite the development of portosystemic collateral shunt formation. Many mechanisms were proposed to demonstrate the induced splanchnic vasodilation, including opening of aterio-venous fistula, reduced sensitivity to vasoconstrictors, dysfunction of the autonomic nervous system, and increased circulating levels of vasodilators. Blood vessels isolated from cirrhotic and or hypertensive models have been used to identify the underlying mechanisms. Knowledge of intracellular signaling processes that mediates contraction and relaxation of the vessel has been exploited to understand vascular function in cirrhosis.

a) Vasoactive factors regulated-vascular tone in cirrhosis. (Figure. 15)

The development of the hyperdynamic circulation in cirrhosis is associated with activation of many endogenous pressor and dilator systems mentioned previously that are shown to be elevated in the plasma of cirrhotic patients. Most of contractile agents induced vascular response through receptors present on either smooth muscles cells or endothelium. This later could modulate the contractile response by release of relaxing factors (such as NO, prostacyclin and endothelial hyperpolarizing factor). The stimulation of endothelial receptors results in release of vasorelaxant factors that could counterbalance the contractile response. Functional responses of these agents were assessed on isolated human or animal vessels in order to clarify their potential role in

both development of the hemodynamic abnormalities and implication of the endothelium (NO) of the impaired response to vasoconstrictors.

b) *In vivo* vascular reactivity to vasoconstricors

Clinical evidence suggests that the development of a hyperdynamic circulation in cirrhosis is associated with impaired contractile responsiveness to vasoconstrictors.

α-adrenoreceptor agonists are the most widely used compounds in investigations of contractile hyporesponsiveness in cirrhosis. Clinical investigations have shown increased plasma levels of pressor factors renin, aldosterone and noradrenaline (Pinzani et al., 1991).

Moreover, pharmacological investigations of the whole body are difficult to interpret, as infusion of agonists may cause central effects, hormonal responses, and changes in the sympathetic output. In order to avoid these factors, venous occlusion of plethysmography has been used to assess vascular reactivity to α-adrenoreceptor in the isolated forearm of cirrhotic patients. Infusion of noradrenaline decreases forearm blood flow in cirrhotic and in control people and its response in cirrhotic forearm vessels was significantly lower than in control group (Ryan et al., 1996). Recently many studies in cirrhotic animal models have postulated that humoral mediators cause the release of NO through activation of specific endothelial receptors. Circulating catecholamines are among these endogenous factors stimulating release of NO. Liao & Homey (1993) showed that the release of NO is mediated through a α_2-adrenoreceptor activation.

Cirrhosis induced arterial vasodilation in cirrhotic patients, despite increased plasma levels of the potent vasoconstrictor endothelin 1 (ET-1) in cirrhotic patients (Uchihara et al., 1992). Therefore, Hartleb and coworkers (1994) examined the effect of NO synthesis inhibitor (L-NAME) on vascular response to ET-1 in normal and cirrhotic rats. Increased doses of ET-1 were given intravenously to rats pretreated with L-NAME or with its

vehicle. The dose-response curve of ET-1 revealed a significant decrease in the maximal vasopressor responses (Rmax) compared to the response in the control rats, without a change in the ED_{50} value. L-NAME induced a more pronounced contractile response to ET-1 in both groups control and cirrhotic rats but the maximal response was only increased in the cirrhotic rats. The pressor response to low doses of ET-1 in the presence of L-NAME remained lower in cirrhotic than in control rats, thus, L-NAME did not normalize the response to low doses of ET-1 in cirrhotic rats. Moreover, indomethacin (COX inhibitor) did not normalize the pressor response to ET-1 either. These results lead the authors to suggest that NO is responsible for the hyporesponsiveness of ET-1 in cirrhosis only for the high doses, and an NO-independent mechanism is implicated for low doses of ET-1.

Recently, the contribution of NO and prostacyclin to the development of vascular hyporesponsiveness for phenylephrine was examined in the mesenteric circulation of cirrhotic and PVL and SO rats (Colle et al., 2004). Blood flow was measured during intra-mesenteric infusion of phenylephrine alone or after incubation with combined L-NAME and indomethacin. The mesenteric blood flow to phenylephrine was lower in cirrhotic and PVL than in SO and was maintained after incubation with L-NAME and indomethacin. Thus, these results led the authors to suggest that there was an abnormality at the vascular smooth cell level rather than at the endothelial level leading to an overproduction of NO or prostacyclin.

c) *In vitro* splanchnic vascular reactivity to vasoconstrictors

Isolated vessels have also been used to investigate the vascular hyporesponsiveness in cirrhosis. The effects of the circulating vasopressor agents or their pharmacological analogues were studied in the mesenteric arteries and the aorta. The role of NO in the modulation of the vascular hyporeactivity response to these factors was also investigated

in both cirrhotic and PVL rats. As shown in the whole animal studies, the contribution of NO to altered contractile function is still unclear. In vitro methodology, using isolated vessels must allow for the possibility for more understanding the altered responsiveness of the vasopressor factors.

Several studies have shown a hyporeactivity to the exogenous vasopressor agent, phenylephrine. Lee et al. (1995) showed a hyporeactivity to phenylephrine in arterial mesenteric rings from cirrhotic rats compared to control rats. Furthermore, a significant hyporesponsiveness to phenylephrine in the superior mesenteric artery of cirrhotic rats was observed as compared to normal rats (Pateron et al., 1999). This hyporesponsiveness was abolished by pretreatment with N(omega)-nitro-L-arginine, an NO synthase inhibitor.

Moreover, a study done by Sieber et al. (1993) investigated the vascular response to KCl of Krebs-perfused superior mesenteric artery rings. The contractile response to KCl perfused-Krebs in cirrhotic rats was significantly lower than control. This hyporeactivity was overcome by incubating the same vessels with the NO synthase inhibitor, N^{ω}-nitro-L-arginine (NNA); even the vascular response to KCl in cirrhotic rats was also higher than in control. This result indicated a significantly more pronounced role of NO production in vessels preparations of cirrhotic rats.

In further studies, the contractile response of methoxamine an α–adrenoreceptor agonist was elucidated in cirrhotic models. In a study done by Atucha et al. (1996), *in vitro* perfused mesenteric arterial beds showed a hyporesponsiveness to methoxamine in cirrhotic rats compared to control rats. An interesting finding in this study was that cirrhotic rats with ascites showed a larger reduction in the vascular response than in non-ascitic cirrhotic rats, which suggested that, the severity of the disease, as indicated by ascites formation, more profoundly alters the response to this vasoconstrictor. Mechanical

removed of the endothelium potentiated the pressor response to methoxamine in cirrhotic rats and completely eliminated the vascular hyporesponsiveness.

In addition Atucha et al. (2000) introduced pharmacological tools to investigate the mechanisms by which NO contributes to the hyporesposivness to methoxamine in the isolated mesenteric vessels of control and cirrhotic rats. Pressor responses to methoxamine were analysed under different conditions: after inhibition of the soluble guanylyl cyclase with methylene blue (MB), which is considered as the receptor of NO; after inhibition of NO production with NNA and after blockade of potassium channels with tetraethylammonium (TEA), that are considered as a target of the EDHF (endothelium derived hyperpolarization factor) -induced hyperpolarization of smooth muscle cell and produced vasorelaxation. In this study, mesenteric preparations from cirrhotic rats showed a lower pressor response to methoxamine compared to controls. Pretreatment with MB or NNA increased the contractile response to methoxamine in both groups, but the response in cirrhosis remained lower than in control rats. This result suggested that the hyporesponsivenes to methoxamine was not exclusively due to NO overproduction and that another mechanism might be involved. Pretreatment with TEA plus MB or TEA plus NNA potentiated the vasopressor response to methoxamine to a greater extent than that induced by pretreatment with NNA or MB, but the responses were not significantly different from the responses observed with MB or NNA. Indeed, TEA alone did not increase the response to methoxamine in any group, and the pressor response to methoxamine was greater with combination of TEA and L-NNA than TEA with MB, suggesting that NO acted through a cGMP-independent mechanism. Although pretreament with TEA and NNA increased the vascular reactivity to methoxamine in cirrhosis, it remained less than in the control group. Actually no treatment could completely normalize the lower response to vasopressor factors in the mesenteric arteries

of cirrhotic rats, which suggested that mesenteric hyporesponse to vasoconstrictors was not solely due to NO overproduction or to EHDF-induced vasorelaxation, and that another mechanism might be involved.

In addition to models of cirrhosis, several investigations used the PVL model to study vascular reactivity. The PVL model produces portal hypertention and a hyperdynamic circulation similar to what is observed in liver cirrhosis, but it shows mild and transient sodium retention with less marked activation of vasoconstrictor systems, and no hepatic damage.

The effects of two major endogenous vasopressors, norepinephrine and arginine-vasopressin (AVP) and a non-receptor vasoconstrictor agent KCl were tested on the vascular reactivity of the mesenteric arterial beds. Sieber and Grosmann (1992) have conducted *in vitro* study to demonstrate the contribution of NO in the perfused superior mesenteric arterial beds. They showed that portal hypertension in PVL rats model is accompanied by a hyporeactivity of the vasopressor methoxamine, which was overcome by blockade of NO synthesis with NNA, a stereospecific NO formation inhibitor. These results lead the authors to suggest that locally released NO in this preparation is responsible for the decreased vasoconstriction. They demonstrated also in a previous study (Sieber & Grozmann, 1992) that a hyporeactivity to norepinephrine, AVP and KCl, exists *in vitro* perfused mesenteric vessels of PVL rats, and that NO is involved in this hyporesponse.

Moreover, a study done by Atucha et al (1996), in the *in vitro* perfused mesenteric arterial bed showed a hyporesponsiveness to methoxamine, in PVL rats compared to controls. Mechanical removal of the endothelium potentiated the vasopressor effect of methoxamine in PVL model and totally eliminated the hyporesponsiveness.

In addition, another study was performed by Atucha et al., (1998) to investigate the role of the potassium channels (K_{ATP} channels) and NO in the hyporeactivity response to methoxamine in the perfused mesenteric vascular bed of PVL rats. In this study, they found that blockade of NO did not completely correct the difference between PVL and control rats in response to methoxamine. This finding was different from the results of Sieber & Grozmann (1992), and led them to propose another mechanism of action for the decrease response to methoxamine. Pretreatment of the vessels with inhibitors of potassium channels (TEA, glibenclamide or charybdotoxin) did not improve the reduced pressor response to methoxamine in PVL rats. However, when preparations were simultaneously pretreated with MB (guanylate cyclase inhibitor) and TEA or with L-NNA (inhibitor of NO synthesis) and TEA, they completely corrected the hyporesponsiveness to methoxamine but not with MB or L-NNA alone. Thus, they suggested that NO blunted the vascular pressor response to methoxamine of PVL vessels by three mechanisms of action: 1) NO-dependent vasodilation mediated by cGMP, 2) NO-dependent activation of potassium channels, 3) and activation of potassium channels independently of NO.

This study was unlike the authors' results (Atucha et al., 2000) obtained in mesenteric vessels from cirrhotic rats, in which the hyporesponsiveness to methoxamine was not corrected after preincubation MB plus L-NNA or TEA plus L-NNA. The authors suggested that this discrepancy in the results between the PVL and cirrhotic model was due to the fact that the two models were different in spite of overexpression of eNOS in the mesenteric arteries in both models (Niederberger et al., 1996), and that other endothelial or smooth muscle factors are involved in cirrhosis.

d) *In vitro* aortic vascular reactivity to vasoconstrictors

In attempt to evaluate the role of NO in vascular reactivity to vasoactive drugs, many studies determined the function and expression of NOS and NO production by estimating its messenger cGMP in PVL and cirrhotic models.

In the study of Niederberg et al. (1996), Western blotting analysis showed that expression of endothelial nitric oxide synthase (eNOS) was increased in the aorta of cirrhotic rats compared to control rats. In addition, aortic cGMP was markedly increased in cirrhotic rats with ascites (Niederberger et al., 1995). This increase was abolished by chronic administration of N^G-nitro-L-arginine methyl ester (L-NAME, NOS inhibitor). In contrast, in PVL rats the aortic eNOS expression did not differ from controls, and cGMP concentration was also similar to controls and significantly lower than that found in cirrhotic rats with ascites (Niederberg et al., 1995). These results lead the authors to suggest that in cirrhosis there is an overproduction of aortic vascular NO that might play an important role in the arterial vasodilation

Moreover, Cahill et al. (1996) showed an increase in basal activity of a Ca^{2+}dependent endothelial nitric oxide synthase (eNOS) that was significantly elevated in thoracic aorta of PVL compared to controls.

On one hand, it is well established according to these results that the vascular NO production is higher in cirrhotic models than in PVL. This is consistent with the possible stimulus of iNOS (NOS2) induction in cirrhosis with ascites by endotoxin via cytokine cascade (Chu et al, 1999), which might play an important role in cirrhosis. Therefore, Liu et al. (1999b) have conducted a study to evaluate the entire pathway from cytokines to nitrate/nitrite final metabolites in bile duct and sham-operated rats. The plasma TNF-α and NOx levels were measured and iNOS and eNOS aortic content were determined. Compared to control rats, TNF-α levels were significantly elevated in cirrhotic rats,

iNOS mRNA was detectable in the aorta of BDL rats whereas absent in controls, and eNOS mRNA levels were significantly higher in the aorta of cirrhotic rats. Aortic eNOS protein content was significantly higher in cirrhotic rats, but iNOS protein was undetectable. Plasma NOx concentrations were significantly higher in cirrhosis than those in controls. Moreover, the failure to detect iNOS protein expression in cirrhotic animals and the failure of polymixin B, an endotoxin antagonist, and aminoguanidine, a preferential inducible nitric oxide synthase inhibitor, to ameliorate the hyperdynamic circulation of portal hypertensive rats suggests that iNOS may not play a role in the hymodynamic abnormalities (Chu et al., 1998, 1999). Overall, these findings do not support the contribution of iNOS the role of endotoxin in the hyperdynamic circulation of PVL rats. These results suggest that NO activity is increased in cirrhosis and it is predominantly due to eNOS, which might play an important role in the systemic hemodynamic changes occurring in cirrhosis.

Consequently, the enhanced production of NO was assessed in the aortic vessels of rats with cirrhosis and ascites (Claria et al., 1994). In this study, vascular response to endothelium-derived, nitric oxide-dependent vasodilators, acetylcholine- and ADP, enhanced the relaxation in isolated aortic rings of cirrhotic rats, and differences between cirrhotic and control rings disappeared after nitric oxide synthesis inhibition with N omega-nitro-L-arginine. No difference in the relaxing effect of sodium nitrite, endothelium-independent vasodilator, was observed between cirrhotic and control rings. These results therefore support to the concept that nitric oxide activity is increased in cirrhosis.

On the other hand, the effect of vasopressor agents and the role of NO have been largely identified in aortic isolated preparations. A study one by Van Obbergh and his colleagues (1995) was to investigate the response of isolated aortic rings from BDL and SO rats to

two vasoconstrictors, phenylephrine and a thromboxane analogue (U46619). They found that the vasoconstriction produced by phenylephrine was decreased in cirrhotic vessels both with and without endothelium, but the response to U46619 was not modified by cirrhosis. In addition, nitric oxide inhibition with L-NMMA restored normal contractility of the rings with and without endothelium. This beneficial effect was not observed when cyclooxygenase activity was blocked with indomethacin. This study suggests that cirrhotic aortic vessel is hyporeactive to phenylephrine and this effect is mediated through increased nitric oxide production. The improvement observed after inhibition of NO pathway in denuded rings led the authors supposed that cirrhosis also may induce NO production in both endothelial and smooth muscle cells.

Bomzon et al. (1991) have performed a study, in which the *in vitro* vascular responsiveness to norepinephrine was measured in aortic rings from CCl_4 cirrhotic rats and five-week BDL rats. They found that vascular reactivity of aortic rings to norepinephrine was decrease in both cirrhotic models.

An impaired response to norepinephrine was observed in aortas from BDL rats compared to sham-operated (SO) rats and was associated with increased levels of nitrate in the blood taken from the aortic circulation (Gadano et al., 1999). This hyporeactivity was improved by eNOS inhibitors, but the authors did not show if this incubation eliminated the difference between BDL and SO.

Similarly, previous studies have shown a reduced response to angiotensin II in aortic rings of cirrhotic rats with ascites as compared to controls (Castro et al., 1993). This hyporesponse to angiotensin II was reversed when aortic rings were denuded of endothelium or after inhibition of nitric oxide synthase.

Unlike this study, Leehey (1993) showed that there is no significant difference in the vascular reactivity to angiotensin II in aortic rings from cirrhotic rats compared to

controls. Therefore, it is unclear whether the contraction response mediated by angiotensin II is impaired or unchanged in arteries from cirrhotic animals.

In conclusion, there are contradictory results regarding the vascular reactivity of the aorta to vasopressors in cirrhosis, with most studies showing hyporesponsiveness that appears to be related to overproduction of NO, and few studies showing hyperresponse to α_1-adrenoreceptor with the presence of high levels of circulating norepinephrine (Pinzani et al., 1991). The explanation of this discrepancy remains unclear. In contrast, a consistent hyporesponse to vasopressor agents in the mesenteric vascular bed of cirrhotic models was established, which might contribute the splanchnic vasodilation, primary trigger for the hyperdynamic circulation syndrome.

F- Role of PDE5 in complications of liver cirrhosis

ANP is a hormone involved in the regulation of body fluid and electrolyte balance (Gerbes et al., 1990). ANP induces natriuresis and diuresis by interaction with specific renal receptors. Following receptor stimulation a membrane-bound guanylyl cyclase is activated to generate cGMP, which in turn activates the protein kinase G (PKG). This later is able to phosphorylate diverse intracellular proteins and induces the various physiological response of ANP (vascular relaxation, natriuresis and diuresis). The intracellular cGMP level is controlled by the hydrolytic activity of the PDEs. The renal tissue contains multiple isozymes of PDEs, which have a high affinity for cGMP. These include PDE5 and PDE1 (Dousa, 1999), and recently described PDE9 (Soderling et al., 1998), PDE10 (Soderling et al., 1999), and PDE11 (Fawcett et al., 2000). In vascular smooth mucles, pharmacological studies have suggested that both PDE1 and PDE5 mediate catabolism of cGMP stimulated by ANP (Weishaar et al., 1990; Dousa, 1999), whereas in the inner medullary collecting duct (ICMD) cells, PDE1 appeared responsible for hydrolysis of ANP-stimulated cGMP (Yamaki et al., 1992).

Renal function abnormalities, such as sodium and water retention, are common findings in advanced cirrhosis, nephrotic syndrome and congestive heart failure. This renal dysfunction is manifested by resistance to the natriuretic action of the atrial natriuretic peptide (ANP) (Levy, 1997). Previous studies examined the role of ANP in experimental nephrotic rats with administration of adriamycin for 3 weeks (Lee & Humphreys, 1996). The ANP-induced cGMP accumulation in isolated glomeruli and dispersed inner medullary collecting duct cells (IMCD) of normal rats were markedly blunted in nephrotic rats. The responsiveness of nephrotic cells was normalized in the presence of the PDE5 inhibitor, zaprinast (10^{-3} M). These results lead the authors to suggest that increased cGMP phosphodiesterase activity is responsible for the renal resistance to ANP in nephrotic syndrome.

On the other hand, recent studies in liver cirrhosis resulting from bile common duct ligation, have found also a resistance to ANP correlated to an increase in the PDE5 activity in the renal cells. Ni and his colleagues (1996) conducted a study on normal sham-operated rats (SO) and rats with common bile-duct ligation (BDL), in which the rats received intravenous infusion of normal saline (2 %) in order to produce volume expansion (VE). Acute VE promotes increase ANP plasma levels in SO associated with an increase in urine flow rate, urinary Na and cGMP excretion, in contrast, BDL showed impaired natriuresis and urinary cGMP excretion in response to VE, despite high ANP plasma levels. Previously, it had been suggested that increased renal sympathetic activity mediated the blunted natriuresis response to ANP in cirrhotic rats (Koepke et al., 1987). Renal denervation, however, was not able to fully normalize the VE-induced natriuresis in BDL. In correlation, cGMP accumulation in isolated IMCD cells was examined in both groups. This accumulation was higher in SO compared to BDL rats either from innervated or denervated kidneys. The ANP analogue failed to induce cGMP

accumulation in BDL rats. Density of ANP receptors as well as affinity of ANP to its binding sites did not differ between the two groups. Zaprinast, a specific PDE5 inhibitor, restored ANP responsiveness in both innervated and denervated kidneys from BDL rats, and intrarenal infusion of zaprinast (10 μg/min) corrected both the blunted Na and cGMP excretion in response to VE in BDL rats. These results demonstrate that renal resistance to ANP in BDL rats is accompanied with increase activity of PDE5 rather than the increased renal sympathetic nerve activity mediating the blunted response to ANP (Koepke et al., 1987), or an alteration of ANP receptors (Gerbes et al., 1991).

Ni et al. (2001) conducted another study to confirm these findings. They measured cGMP hydrolysis in IMCD cell homogenates of BDL and SO rats, and quantitied the amount of PDE5 by Western blotting and immunoprecipitation in IMCD andrenal cortical cell homogenates of both groups. They also characterized ANP responsiveness *in vivo* of kidneys of anesthetized SO and BDL rats before and after VE.

The kinetic analysis of PDE5 activity from IMCD cells indicates a significant increase in V_{max} of BDL rats compared to SO without change the affinity (K_m). The PDE5 activity was inhibited with potent and specific PDE5 inhibitor 1,3-dimethyl-6-(2-propoxy-5-methanesulfonylamidophenyl) pyrazol [3,4d]-pyrimidin-4-(5H)-one (DMPPO), with no change in K_i or IC_{50} between the two groups.

Moreover, Western blot analysis showed no change in the protein expression in the cortex of both groups, whereas a two-fold increase in PDE5 protein in IMCD of BDL rats was observed that accounted for the increase in cGMP hydrolysis.

In addition, VE was not able to induce natriuresis in the BDL rats, whereas VE induces natriuresis from the kidney ofsham-operated (SO) rats. The infusion of DMPPO in BDL rats corrected the blunted natriuresis response to VE and was not statistically different from the SO ratstreated with DMPPO Differences were also observed in the urinary

cGMP excretion during DMPPO and zaprinast infusion before and after VE in BDL and SO rats. The cGMP excretion was significantly less from non-infused kidney of BDL rats compared to SO rats, and intrarenal infusion of either DMPPO or zaprinast normalized the blunted VE-induced increase in cGMP in infused BDL kidney.

On the other hand, ANP plasma level was not different between BDL and SO rats, but it rose equally in both groups after VE or infusion of either DMPPO or zaprinast.

Thus these data demonstrated that an increase in cGMP-PDE activity mediates renal resistance to ANP in rats with common bile duct ligation.

In support of these findings, Angeli & his colleagues (2000) tested the hypothesis of increased PDE5 activity correlated to a decrease in cGMP accumulation in renal tissue *in vitro* rats with CCl_4 induced liver cirrhosis. The PDE5 activity and cGMP concentration were evaluated in both cirrhotic and control rats before and after i.v administration of zaprinast (15 and 30 μg/kg/min). The renal content of cGMP was lower in renal tissue of cirrhotic rats because of increased PDE5 activity. The i.v infusion of zaprinast (60 μg/kg/min) over 60 min decreased PDE5 activity and increased cGMP level in the kidneys of cirrhotic rats.

In this study, cirrhotic rats compared to control rats showed a hyperdynamic circulation profile with reduced baseline in mean arterial pressure (MAP), total peripheral resistance (TPR), and increased cardiac out put (CO) and cardiac index (CI), and heart rate was similar in both groups. Administration of the highest doses of zaprinast (60 and 120 μg/kg/min) accentuated the hyperdynamic circulation, resulting in significant decrease in MAP and TPR, accompanied with an increase in CO and CI in both groups. However lower doses of zaprinast were ineffective.

As for renal function, cirrhotic rats showed a significantly decreased baseline of glomerular filtration rate (GFR), urine flow rate (V), urinary sodium excretion (UNaV)

and a very high level of ANP. Administration of 15 and 30 µg/kg/min of zaprinast increased all these parameters in cirrhotic rats, and only higher doses of zaprinast were effective in controls.

Plasma renin activity and aldosterone were also significantly higher in cirrhosis than in control rats. The administration of zaprinast resulted in a marked reduction of PRA without a change in the plasma aldosterone concentration in cirrhosis. No change was observed in controls. In different protocols a maintained hypotension marked the cirrhotic rats, and remained stable over all protocols in both animal groups.

Finally, administration of an ANP-receptor inhibitor (HS-142-1) prevented any renal effect of zaprinast in cirrhotic rats, confirming that the effect of zaprinast was due to enhancement of the effects of ANP.

In conclusion, altogether these results demonstrated an increased PDE5 activity in renal tissue, which contributes to the renal resistance to ANP in liver cirrhosis. The PDE5 inhibitor zaprinast and DMPPO corrected the blunted response to ANP both in vivo and *in vitro* in cirrhotic rats.

To date, no data are reported about cardiovascular alterations-induced in cirrhosis especially in cyclic nucleotide phosphodiesterases (PDEs) field, since cGMP mediates cross talk between various PDE isozymes in cardiovascular system. Hence, this work has targeted the various PDE isozymes as a key factor to understand the cardiovascular and renal complications associated to liver cirrhosis.

The above findings provide evidence supporting the following conclusions: 1) NO pathway is involved in generation of the splanchnic vasodilation of cirrhosis. 2) Vascular reactivity to vasopressor is reduced in bile duct ligation-induced cirrhosis, which is reversed by inhibition of NO synthase or by endothelial removal. 3) Increased PDE5

activity and expression in the kidney contributing to renal resistance to the natriuretic effect of ANP in cirrhosis.

Therefore, the purpose of this study base on the above findings, and focuse on: 1) the role of PDE5 with PDE5 inhibitor, DMPPO, in the vascular reactivity of the aorta to vasodilators and vasoconstrictors in bile duct ligated rat model. 2) The acute and chronic influence of PDE5 inhibitor (DMPPO and sildenafil) on hypodynamic changes and sodium retention by the kidney. 3) Influence of cirrhosis on the PDEs activity and expression in the various altered tissues (aorta, mesenteric arteries, and the kidney).

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PUBLICATIONS SOUMIS OU EN PREPARATION

Rana Ghali-Ghoul, Rima Tahseldar-Roumieh, Ramzi Sabra. Effect of chronic administration of sildenafil on sodium retention and on the hemodynamic complications associated with liver cirrhosis in the rat.

Tahseldar-Roumié R, Keravis T, Justiniano H , Sabra R, Lugnier C. Alteration of cyclic nucleotide phosphodiesterase (PDE) activities and expressions in rats with liver cirrhosis

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Résumés publiés

Sabra R, Tahseldar-Roumié R, Ghali R, Tumeh Y, El-Hajj I & Lugnier C. Role of phosphodiesterase-5 (PDE5) in altered reactivity in cirrhotic rats. FASEB J (2004), 18, abstrac#2767. Experimental Biology 2004, 17-20 April 2004, Washington DC

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Results

Article I

Effect of a Phosphodiesterase 5 Inhibitor on the Alteration in Vascular Smooth Muscle Sensitivity and Renal Function in Rats with Liver Cirrhosis

Article II

Effect of chronic administration of sildenafil on sodium retention and on the hemodynamic complications associated with liver cirrhosis in the rat

Article III

Assessment of Phosphodiesterase Isozyme Contribution in Cell and Tissue Extracts

Article IV

Alteration of cyclic nucleotide phosphodiesterase (PDE) activities and expressions in rats with liver cirrhosis

Effect of a Phosphodiesterase 5 Inhibitor on the Alteration in Vascular Smooth Muscle Sensitivity and Renal Function in Rats with Liver Cirrhosis

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Plusieurs études ont mis en évidence le rôle du monoxide d'azote (NO) dans la vasodilatation splanchnique liée à la cirrhose. Une production excessive du NO entraîne l'activation des mécanismes de compensation qui produisent une vasoconstriction excessive des artères rénales, entraînant la rétension de sodium par les reins. D'autre part, des études récentes ont montré, une augmentation de l'activité PDE5 rénale associée à une rétention sodée et à une diminution de l'effet vasodilatateur du peptide auriculaire natriurétique (ANP). Ces résultats mettent en évidence une implication de la PDE5 au niveau de la rétention rénale du sodium. Si une telle régulation de la PDE5 s'établit dans l'artère mésentérique, elle pourrait antagoniser l'effet du NO et améliorer les altérations hémodynamiques liées a la cirrhose.

Cependant, cette étude pour la première fois examine l'effet du DMPPO, inhibiteur puissant et spécifique de la PDE 5, et de l'endothélium (producteur de NO) sur la réactivité vasculaire *in vitro* et *in vivo* sur les réponses hémodynamiques et la fonction rénale chez le rat cirrhotique.

Etant donne que l'aorte joue un rôle dans la regulation de la pression arterielle, cette étude a été menée sur des anneaux d'aorte de rat, pour lesquels la cirrhose est induite par ligature du canal biliaire (BDL) selon la méthode de Wensing et Branch.

Les études menées *in vitro* chez les rats cirrhotiques (BDL) en comparaison avec des rats contrôles, sur le relâchement des artères dépourvues d'endothélium, mettent en évidence une diminution de sensibilité de l'aorte aux donneurs de NO (NG et SNAP) chez les rats BDL. L'incubation avec le DMPPO potentialise l'effet vasodilatateur des donneurs de NO (NG et SNAP) et élimine la différence de sensibilité existante entre les rats contrôles et les rats cirrhotiques. Ceci suggère qu'au niveau de l'aorte dépourvue d'endothélium, il y a une modification de la réponse NO/cGMP. Le fait que le SNAP, qui active directement la guanylyl cyclase soluble, ne différencie pas les réponses à la phényléphrine dans les deux groupes, s'oppose à l'hypothèse de l'implication de la

guanylyl cyclase et plaide en faveur du rôle de l'augmentation de l'activité de la phosphodiestérase dans cette diminution de sensibilité aux vasodilatateurs observée au niveau de l'aorte. En accord avec cette hypothèse, lorsque l'aorte est pourvue d'endothélium, les aortes BDL sont significativement moins sensibles à la vasodilatation induite par le DMPPO, suggérant qu'il y a une augmentation de l'activité phosphodiestérasique.

L'étude *in vivo* montre, que les rats BDL présentent tous les paramètres de la circulation hyper dynamique. L'administration aiguë du DMPPO (5 mg/kg, 5 min i.v.) diminue de façon significative la pression artérielle et la résistance vasculaire périphérique dans les deux groupes. De façon intéressante, l'administration du DMPPO induit un doublement significatif de l'excrétion sodique et de l'excrétion fractionnelle du sodium chez les BDL, sans agir chez les contrôles.

L'ensemble des résultats *in vitro* met en évidence une diminution de la réponse à un inhibiteur de PDE5, suggérant une augmentation de l'activité PDE5 chez les rats cirrhotiques.

Effect of a Phosphodiesterase 5 Inhibitor on the Alteration in Vascular Smooth Muscle Sensitivity and Renal Function in Rats with Liver Cirrhosis

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Abbreviated Title

Phophodiesterase 5 and cirrhosis

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Title

Effect of chronic administration of sildenafil on sodium retention and on the hemodynamic complications associated with liver cirrhosis in the rat

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BDL: bile duct ligation and excision; GFR: glomerular filtration rate, MAP: mean arterial pressure, cGMP: cyclic guanosine monophosphate, NO: nitric oxide, CI: cardiac index, CO: cardiac output, FE_{Na}: fractional sodium excretion, DMPPO: 1,3 dimethyl-6-(2-propoxy-5-methanesulphonylamidophenyl)-pyrazolol[2,4-d]pyrimidin-4-(5H)-one, ANF: atrial natriuretic factor.

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Abstract

Previous studies demonstrated increased PDE5 activity and expression in the kidneys of rats with liver cirrhosis. Acute intravenous administration of PDE5 inhibitors enhanced sodium excretion in these rats. The aim of the present study was to examine the effects of chronic administration of sildenafil (a selective inhibitor of PDE5) on the renal sodium retention and hemodynamic disturbances associated with liver cirrhosis in rats. Male Sprague-dawley rats underwent bile-duct-ligation and excision (BDL) or sham operation and were housed in metabolic cages throughout the study. Body weight, food intake, water intake and urine volume were measured daily, and plasma samples were obtained twice weekly. Fourteen days following surgery sildenafil or its vehicle (DMSO) were administered (20mg/kg subcutaneously 3 times/day). Two weeks later, systemic hemodynamics were measured under general anesthesia. The results revealed that sildenafil enhanced both the systemic vasodilatation and the fall in arterial pressure associated with liver cirrhosis. There was no further reduction in GFR, however. Despite these hemodynamic changes, sildenafil prevented the decrease in Na excretion observed in the BDL group receiving vehicle and markedly increased fractional sodium excretion relative to the other groups. These results are consistent with previous findings of increased activity of PDE5 in the kidney of rats with liver cirrhosis that contribute to Na retention. Conclusion: Despite aggravating the vasodilatation and hypotension, chronic administration of sildenafil in rats with liver cirrhosis improves sodium excretion while maintaining glomerular filtration rate.

The major cardiovascular complication of liver cirrhosis is a hyperdynamic circulation with peripheral and splanchnic vasodilatation, increased cardiac output and varying degrees of hypotension. The peripheral arterial vasodilation hypothesis suggests that primary peripheral (mainly splanchnic) vasodilation induces activation of compensatory mechanisms such as the renin-angiotensin-aldosterone system, antidiuretic hormone and the sympathetic nervous system to result in sodium and water retention in order to restore effective arterial volume and maintain arterial pressure [Schreier et al, 1988]. The primary mesenteric vasodilatation leading to the hyperdynamic circulation has been attributed to increased production of various vasodilator substances such as nitric oxide (NO) [Wiest and Groszman 1999, Vallance and Moncada, 1991], prostacyclin [Oberti et al, 1993], endothelium derived hyperpolarizing factor [Barriere et al 2000], glucagon [Lin et al, 1996], substance P and calcitonin-gene-related peptide [Gines et al, 1997]. Among all, nitric oxide has gained the most attention as the major cause of the decreased vascular resistance in cirrhosis [Martin et al, 19988]. Nitric oxide, formed by endothelial cells from its precursor L-arginine raises intracellular cGMP levels, its second messenger, leading to vasodilatation [Wiest and Groszman 2002].

Another hemodynamic abnormality associated with cirrhosis is a decreased responsiveness of the cirrhotic kidney to the natriuretic effect of the atrial natriuretic factor (ANF), a key component to blood pressure regulation and homeostasis. ANF, like nitric oxide, acts by increasing intracellular levels of cGMP which can be hydrolyzed by numerous phosphodiesterase isoforms [Beavo, 1995]. Among those isoforms, which are specific to cGMP hydrolysis, is phosphodiesterase type 5 (PDE5), located in the smooth muscle cells of blood vessels. PDE5 was found to be present in the mesenteric artery

[Sampson et al, 2001, Wallis et al, 1999] in pulmonary arteries [Ahn et al 1991] and in other vascular beds [Medina et al 2000]. It has been shown that the blunted renal response to ANF in cirrhosis is due to upregulation of PDE5 and that inhibition of PDE5 in the kidney can enhance the responsiveness to ANF by restoring intracellular levels of cGMP producing an increase in renal plasma flow, in glomerular filtration rate (GFR) and in sodium excretion [Ni et al 2001, Shah et al., 1999]. These results suggest that inhibition of PDE-5 may be a therapeutic strategy in patients with liver cirrhosis and ascites. However, it should be noted that inhibition of PDE5 in the splanchnic area may increase the vasodilator effect associated with nitric oxide by raising intracellular levels of cGMP. Inhibition of PDE 5, therefore, may have a beneficial effect in the kidney and on the response to ANF, but may worsen the hemodynamic picture in cirrhosis. In preliminary studies we have demonstrated that acute administration of a PDE-5 selective inbhibitor, DMPPO, led to worsening in the vasodilation and the decrease in arterial pressure; despite this, however, it induced an increase in sodium excretion by the kidney (Sabra et al, 2004). Others have shown that the acute administration of sildenafil in the splanchnic bed increased mesenteric blood flow and portal venous pressure and decreased mean arterial pressure [Colle et al, 2004]. While results of acute drug administration may be interesting, they do not reflect the possible clinical relevance of such an intervention. The hemodynamic disturbances induced by sildenafil may in the long term worsen the sodium handling by the kidney as a result of more severe activation of the compensatory mechanisms mentioned above. Therefore, this study was designed to explore the effect of chronic administration of a PDE5 inhibitor, sildenafil, on the hemodynamic and renal functional consequences of liver cirrhosis in rats, specifically to examine whether the beneficial effect on sodium excretion, observed in acute studies, will also be observed after chronic administration.

Methods

The animals used in the present study were reared, treated and euthanized in accordance with the provisions for animal welfare of the Institutional Animal Care and Use Committee of the American University of Beirut. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, National Academy of Sciences. Adult male Sprague-Dawley rats weighing between 230-290 g were randomized to undergo either sham operation (Sham) or bile duct ligation and excision (BDL). Two days prior to surgery, rats were housed in metabolic cages for adaptation and allowed access to normal rat chow and water. For surgery, rats were anesthetized with pentobarbital (40 mg/kg body weight ip) and their abdomen was opened through a midline incision. The common hepatic bile duct was isolated and transected between two ligatures in BDL animals. Sham rats underwent the same procedure but without ligation and excision of the bile duct. Following surgery, rats were housed individually in metabolic cages. Several parameters were measured on a 24-hour basis including: body weight, food intake, water intake and urine volume. Sodium excretion was calculated after measurement of Na concentrations in the urine over 24 h. During the first 2 weeks, blood was withdrawn from the rats' tail veins, under light ether anesthesia, once weekly then twice weekly for the remaining 2 weeks. Plasma and urine were used to measure sodium and creatinine levels from which sodium excretion rate, creatinine clearance, and fractional sodium excretion were calculated. Two weeks post-surgery, rats were started on either sildenafil (Pfizer Ltd, Sandwich, Kent, UK) (30mg/ml in DMSO) or its vehicle (DMSO) at a dose of 20 mg/kg subcutaneously 3 times daily. Four weeks following surgery, animals were anesthetized with pentobarbital (25 mg/kg for BDL and 50 mg/kg for Sham) and their tracheas were cannulated with PE-205 tubing. A PE-50 catheter was introduced into the right carotid artery and connected to a blood

pressure transducer (TDX-310) for measurement of arterial pressure using a BPA-100 blood pressure analyzer (Micro-Med Louisville, KY, USA), while another PE50 catheter was inserted in the right jugular vein and advanced to the right atrium. A thermistor probe was advanced to the aortic arch through a left carotid approach to monitor the cardiac output (CO) by the thermodilution technique using a cardiac output computer (Cardiotherm® Columbus Instruments, Columbus, OH). Three consecutive measurements of CO were obtained at 5 minute intervals and the peripheral vascular resistance (PVR) was calculated as MAP/CI where CI is the cardiac index (CO per /100g body weight). The bladder was then exposed through a supra-pubic incision and the urethra catheterized using PE 190 tubing for urine collection. ^{3}H-Inulin was then administered (0.75μCi loading dose followed by a continuous infusion of 0.05μCi/min). After 30-min of stabilization, a urine sample was collected over a 30-min period and a blood sample withdrawn at the midpoint of the collection. The urine and plasma samples were used to measure ^{3}H counts and the glomerular filtration rate (GFR) was estimated from the clearance of inulin.

Statistical Analysis

Data are presented as mean ± standard error of the mean. Comparison of values between two groups was conducted using the Students' unpaired t-test; comparisons among more than two groups was performed by ANOVA (analysis of variance) followed by the Newman-Keul test for individual comparisons. Comparison of mortality figures among groups was conducted using the z-test for proportions. All analyses were performed using the SigmaStat statistical Software (Jandel Corporation, USA). Differences in values were considered statistically significant at a P level of < 0.05.

Results

Survival following BDL surgery was determined over the 4 weeks period. Of those randomized to the sildenafil group, 5 of 17 rats died during the first 2 weeks of the study, before sildenafil or its vehicle were administered. However, none of the rats in those randomized to the vehicle group (n=9) died during the first 14 days. Analysis of mortality in BDL rats during the last 14 days of the study (after the start of sildenafil or vehicle treatment) revealed that none of the remaining 12 rats which received sildenafil died, compared with 3 of 9 (33%) in those receiving its vehicle. This difference was not statistically significant. None of the rats in the two sham-operated groups (n=11 for vehicle and n=10 for sildenafil treatment) died during the study period. .

Body Weight

All rats sustained a reduction in body weight on the first few days after surgery. After that, rats started to regain weight; however, the rate of weight gain was slower in the BDL groups compared with the sham-operated groups, irrespective of the treatment received, such that it remained lower in the BDL relative to the sham-operated group of rats especially during the last 2 weeks (Fig 1(a) and (b)). There was no difference, however, between the two BDL groups or the two sham-operated groups.

Food Intake

Food Intake was decreased early after surgery, and late during the study in the BDL relative to the sham-operated groups in both the sildenafil and vehicle treated rats (Fig.2). However, there were no differences between the BDL groups treated with sildenafil or vehicle or between the sham-operated groups similarly treated.

Sodium Handling by the Kidney:

Average urinary sodium excretion rate was analyzed over 3 days during the last week of the study (days 24-26). There was a significant reduction in the sodium excretion rate in the BDL rats treated with vehicle compared with the sham-operated rats treated with

vehicle (Fig. 3). However, BDL rats treated with sildenafil had a sodium excretion similar to that in sham-operated rats treated with sildenafil and significantly higher than that in BDL rats treated with vehicle. These findings were particularly significant considering the fact that food intake and, consequently, sodium intake, were not significantly different between the BDL group treated with sildenafil and the BDL group treated with vehicle, during the same 3 days (Fig. 4), both of which were lower than the values in the corresponding sham-operated groups. Neither Na excretion nor food intake was different between the 2 sham-operated groups..

Creatinine clearance

During the last week of the study there were 3 measurements of creatinine clearance. The average of the 3 values was significantly lower in both BDL groups relative to their sham-operated controls, irrespective of treatment with sildenafil or its vehicle. Furthermore, and there was no significant difference in creatinine clearance between the 2 BDL groups, nor between the 2 sham-operated groups (Fig. 5).

Fractional sodium excretion (FE_{Na})

In the BDL group treated with vehicle, FE_{Na} was similar to the one obtained in the corresponding sham-operated group. Treatment of sham-operated rats with sildenafil did not alter this parameter. However in the BDL group treated with sildenafil there was a significantly higher FE_{Na} compared with all the other groups (Fig. 6).

Systemic Hemodynamics:

At the end of the study, BDL rats treated with vehicle had a lower peripheral vascular resistance than their sham-operated controls. Treatment with sildenafil per se reduced the vascular resistance significantly in the sham operated group (fig 7). BDL rats receiving sildenafil sustained further vasodilation relative to their sham-operated sildenafil treated

controls, and relative to the BDL group treated with vehicle. Cardiac index was elevated in the BDL rats receiving vehicle such that mean arterial pressure was unchanged relative to their sham-operated control (figs. 8 and 9). In contrast, despite the enhancement of the vasodilation in the BDL group receiving sildenafil, there was no further increase in cardiac index relative to the BDL group receiving vehicle, such that mean arterial pressure was significantly reduced compared with that in both the sham-operated, sildenafil treated group and the BDL group receiving vehicle.

Glomerular filtration rate:

Measurement of GFR using inulin clearance revealed the same trends as those that were observed using creatinine clearance, with a decrease in both BDL groups relative to their controls. However, the differences did not reach significant levels (Fig. 10). It should be noted, however, that a number of rats died during the course of the hemodynamic studies, after measurement of cardiac output had been finished, and hence there were no values for GFR in these rats (2 in the sham-vehicle, 2 in the BDL-sildenafil).

Discussion

The present study is the first to investigate the hemodynamic and renal functional alterations associated with liver cirrhosis following chronic administration of sildenafil. Administration of sildenafil to rats with liver cirrhosis caused further reduction in arterial pressure by enhancing the peripheral vasodilation associated with this condition. Despite these unfavorable hemodynamic effects, sildenafil increased total and fractional sodium excretion and did not worsen renal function (GFR).

According to the peripheral vasodilatation theory of ascites formation in liver cirrhosis, vasodilation, mainly splanchnic in origin, initiates a sequence of events in order to compensate for the reduction in effective blood volume. This sequence includes activation of the renin-angiotensin-aldosterone system and the sympathetic nervous system, as well as ADH release, to retain sodium and water by the kidney [Schreier et al, 1988]. In the present study, the BDL group receiving vehicle reproduced the predictable hemodynamic and renal changes, with a decrease in vascular resistance, an increase in cardiac output, and a reduction in creatinine clearance and sodium excretion by the kidney. The blood pressure was unchanged since the increase in cardiac output compensated for the vascular dilatation.

Much evidence supports a role for increased NO production as the cause of the splanchnic and peripheral vasodilation in cirrhosis [Wiest and Groszman 1999, 2002]. NO, by activating the production of cGMP, decreases intracellular calcium levels and leads to relaxation of the smooth muscle cells [Moreau and Lebrec 1995]. cGMP is hydrolyzed by phosphodiesterases found in the vascular smooth muscle cells, mainly PDE-1 and PDE-5 [Beavo 1995]. Recently, studies have shown that kidneys of rats with liver cirrhosis express increased levels and activity of PDE5 and that acute administration of iInhibitors of PDE5 increases renal plasma flow and sodium excretion and improves the blunted response to a salt load or to infusion of atrial natriuretic peptide in rats with liver cirrhosis [Ni et al 1996, 2001, Angeli et al 2000]. These results suggest that the PDE-5 inhibitors may be beneficial in the management of patients with liver cirrhosis suffering from sodium retention and ascites. No studies were undertaken to examine the effect of chronic administration of PDE 5 inhibitors, however. This is especially

important because these agents may worsen the peripheral vasodilation attributed to NO due to inhibition of cGMP hydrolysis. Such enhancement of vasodilation may increase sodium retention by the kidney and annul the direct effect of the PDE-5 inhibitor on the kidney, by causing more severe activation of the sodium retaining mechanisms over time, an effect that may not be seen after acute administration only. This study was conducted to address that question.

As the results show, at the time the BDL group treated with vehicle was showing sodium retention as evidenced by a reduction in sodium excretion, the BDL group treated with sildenafil had a sodium excretion similar to the sham–operated groups, and hence, did not exhibit sodium retention. This occurred despite the fact that food intake (and consequently sodium intake) and GFR in this group were reduced to levels similar to that in the BDL group treated with vehicle. Consequently, the sildenafil treated BDL group had a significantly and markedly higher fractional sodium excretion relative to all the other groups. It is noteworthy that sildenafil, per se, did not alter renal handling of sodium (compare Sham-V with Sham-S); it was only in the context of liver cirrhosis that its effect on sodium excretion was evident. This is likely due to the fact that in liver cirrhosis, PDE-5 activity is stimulated, as previously shown [Angeli et al 2000, Ni et al, 2001], thus the effect of PDE-5 inhibition becomes more evident.

It is important to note that this salutary effect on sodium handling occurred despite worsening of the hemodynamic picture. It was clear that sildenafil treatment per se induced hemodynamic changes including a decrease in peripheral vascular resistance and an increase in cardiac output, with no change in arterial pressure, as evident by comparing the Sham-V and Sham-S groups. In the BDL group treated with sildenafil

there was a further vascular dilation (reflecting the effect of BDL). However, this was not met by an increase in cardiac output, likely due to the lack of significant sodium and water retention, thus resulting in a reduction in mean arterial pressure.

The results of this study are consistent with previous studies that showed an increase in sodium excretion following administration of zaprinast or DMPPO (both PDE-5 selective inhibitors), as noted above [Ni et al, 1996, 2001, Angeli 2000]. However, all these studies were acute interventional studies and none of them evaluated the systemic hemodynamic effects of PDE-5 inhibition. A more recent study by Colle et al. showed that sildenafil administration intravenously or into the mesenteric artery of rats with BDL increased mesenteric blood flow and portal venous pressure, but decreased arterial pressure [Colle et al, 2004]. The authors suggested that the rise in portal pressure may pause a risk for hemorrhagic complications and cautioned against prescription of sildenafil to patients with cirrhosis before further studies were conducted. Thiesson et al. administered a single oral dose of sildenafil to patients with liver cirrhosis [Thiesson et al, 2004]. The patients sustained an elevation in plasma renin activity, angiotensin II and aldosterone concentrations in the plasma at 60 minutes and a fall in sodium excretion at 120 and 180 minutes after the dose. Thus, in contrast to animal studies, single administration of sildenafil in humans did not induce natriuresis but worsened sodium retention. This appeared to be related to the activation of the renin-angiotensin aldosterone system as a result of the hypotension.

Our present study is the first (in humans or animals) to study the effect of chronic administration of sildenafil, or any PDE-5 inihibitor, in liver cirrhosis. It suggests that under these circumstances, the direct effect of sildenafil in the kidney overcomes the

indirect effects of activating sodium retaining compensatory mechanisms resulting from vasodilation; however, this occurs at the expense of further hypotension. The clinical significance of these findings cannot be ascertained from this study, which used a single dose regimen of the drug. It is possible that using a lower dose may induce a selective effect on the kidney without much worsening of the hemodynamic picture, and that requires specific studies in the future. Furthermore, the possible hemorrhagic complications need to be considered as suggested by Colle et al. [2004]. It is encouraging, however, that in the present study, the mortality rate during the second two weeks of the study, i.e., following the start of treatment, tended to be lower in the sildenafil treated group, although the study was not specifically designed, and was not powerful enough, to examine that parameter. In conclusion, the present study offers a possibility for a novel therapeutic approach to sodium retention and ascites in liver cirrhosis using sildenafil specifically, and PDE-5 inhibitors in general, that should be further tested in animal and human studies.

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Footnotes

a) Supported by a grant from the Pfizer Pharmaceuticals Group, Pfizer, Inc..

b) Send reprint requests to:

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Figure Legends

Figure 1 (a) and (b): Body weight over 28 days in BDL (n=9) and sham-operated (n=11) rats treated with vehicle (V)(Fig.1a) and in sham-operated (n=10) and BDL (n=12) rats treated with sildenafil (S) (Fig.1b). Values are Means±SE of mean. x:P<0.05 compared with the sham-operated group receiving the same treatment.

Figure 2 (a) and (b): Food intake over 28 days in BDL (n=9) and sham-operated (n=11) groups treated with vehicle (V) (Fig.2a) or in BDL (n=12) and sham-operated (n=10) groups treated with sildenafil (S) (Fig.2b). x:P<0.05 compared with the sham-operated group receiving the same treatment.

Figure 3: Average urinary Na excretion over days 24-26 of the study in the BDL-V (n=9) Sham-V (n=11), BDL-S (n=12) Sham-S (n=10) groups. Values represent the mean±SE of the mean x: P<0.05 vs. sham-operated group receiving the same treatment. *: P<0.05 vs. BDL-V group.

Figure 4: Average food Intake over days 24-26 of the study in the BDL-V (n=8) Sham-V (n=11), BDL-S (n=12) and Sham-S (n=10) groups. x: P<0.05 vs sham-operated group receiving the same treatment. The values represent the mean±SE of the mean.

Figure 5: Average creatinine clearance during the last week of the study in the BDL-V (n=8) Sham-V (n=11), BDL-S (n=12) Sham-S (n=10). The values represent the mean±SE of the mean.

x: P<0.05 compared with sham-operated group receiving the same treatment.

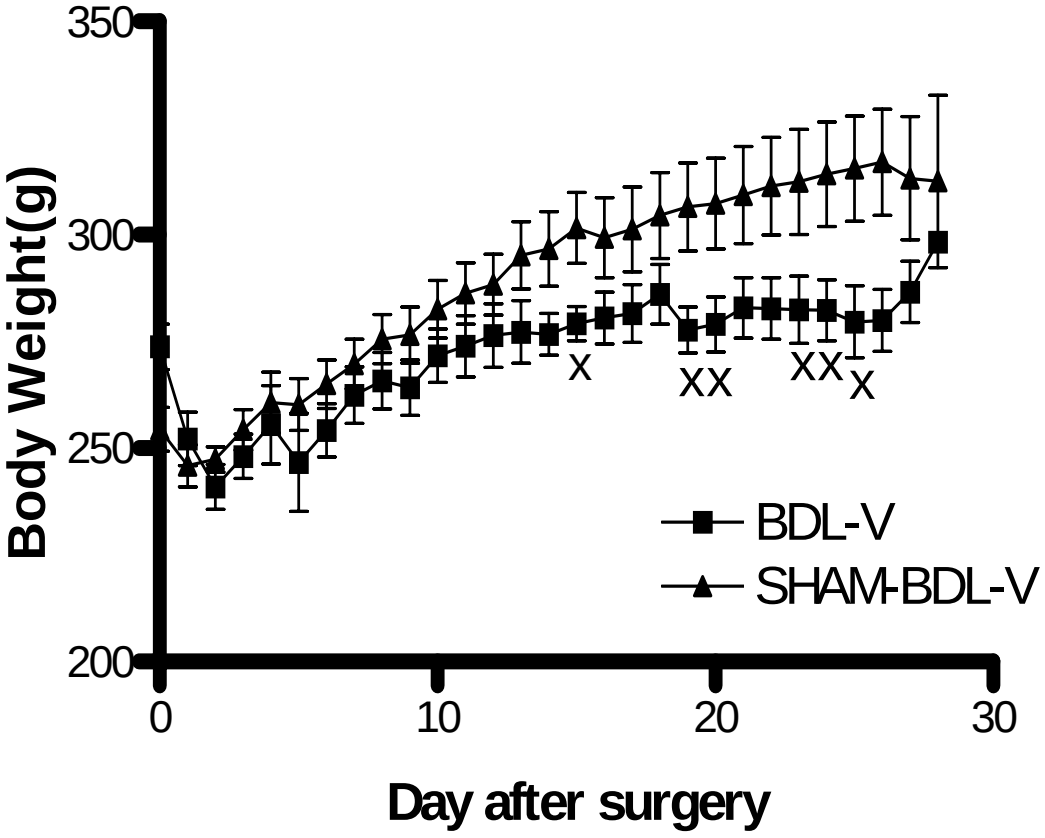
Figure 6: Average FE_{Na} over days 24-26 of the study in the BDL-V (n=8) Sham-V (n=11), BDL-S (n=12) Sham-S (n=10) groups. The values represent the mean±SE of the mean. #: P<0.05 compared with all al treated groups.

Figure 7: Peripheral vascular resistance in rats at the end of the study. BDL-V (n=6) Sham-V (n=9), BDL-S (n=10) Sham-S (n=10). The values represent the mean±SE of the mean. x: P<0.05 compared with sham-operated group receiving the same treatment.+: P<0.05 compared with sham-vehicle. *: P<0.05 compared with BDL-V.

Figure 8: Cardiac Index in rats at the end of the study. BDL-V (n=6) Sham-V (n=9), BDL-S (n=10) Sham-S (n=10). The values represent the mean±SE of the mean. x: P<0.05 compared with sham-operated group receiving the same treatment. +: P<0.05 compared with sham-vehicle.

Figure 9: Mean arterial pressure in rats at the end of the study. BDL-V (n=6) Sham-V (n=9), BDL-S (n=10) Sham-S (n=10). The values represent the mean±SE of the mean. *: P<0.05 compared with BDL-vehicle.

Figure 10: Glomerular filtration rate in the 4 experimental groups at the end of the study. The values represent the mean±SE of the mean. BDL-V (n=6) Sham-V (n=9), BDL-S (n=10) Sham-S (n=10).


350
300
250
200
Body Weight(g)
0
10
20
30
Day after surgery
BDL-V
SHAM-BDL-V
x
xx
xx
x

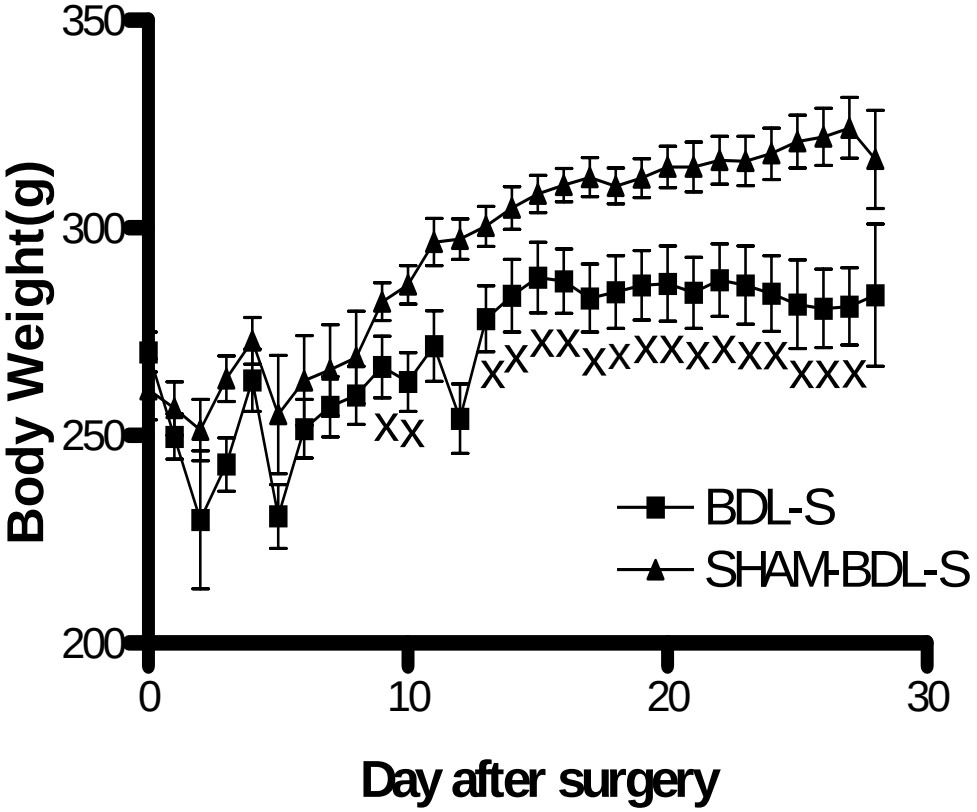
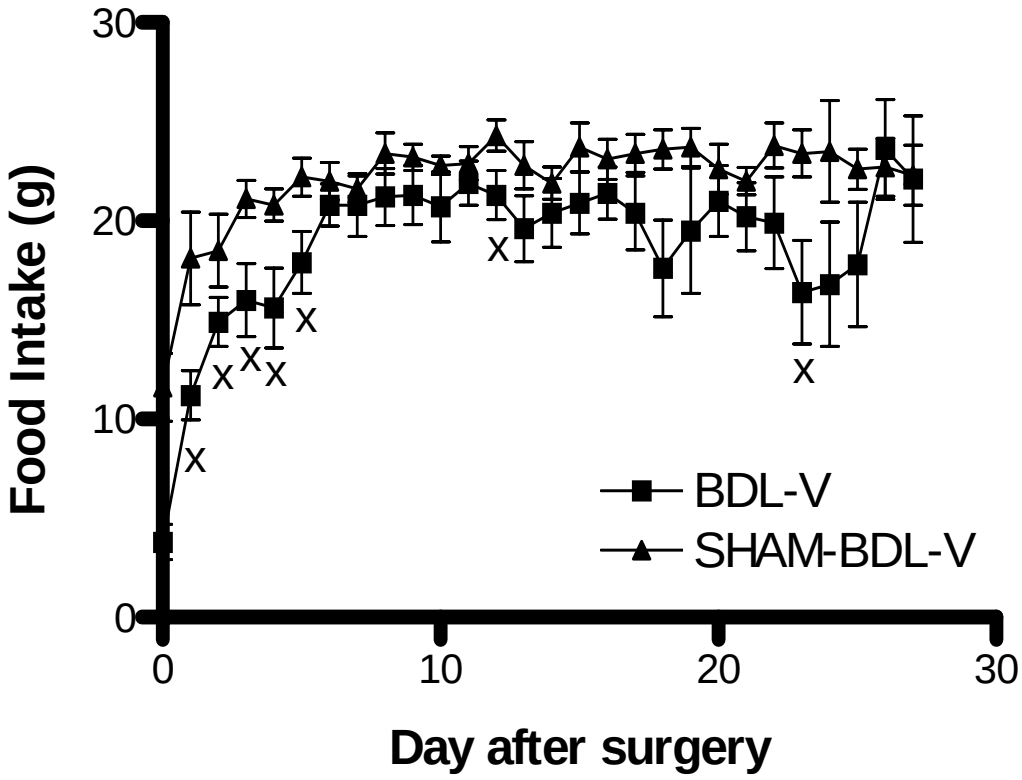

350
300
250
200
Body Weight(g)
0
10
20
30
Day after surgery
BDL-S
SHAM-BDL-S
xx
x
xx
xxxxxxxxx

Figure 1 (a) and (b)



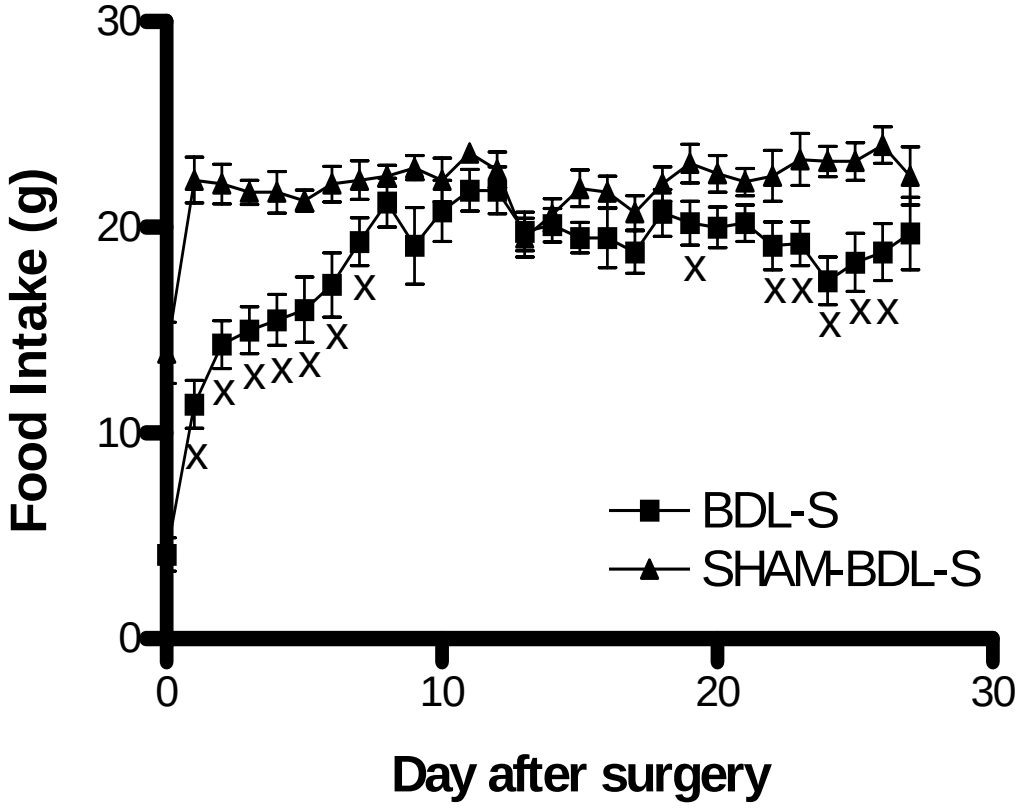


Figure 2 (a) and (b)

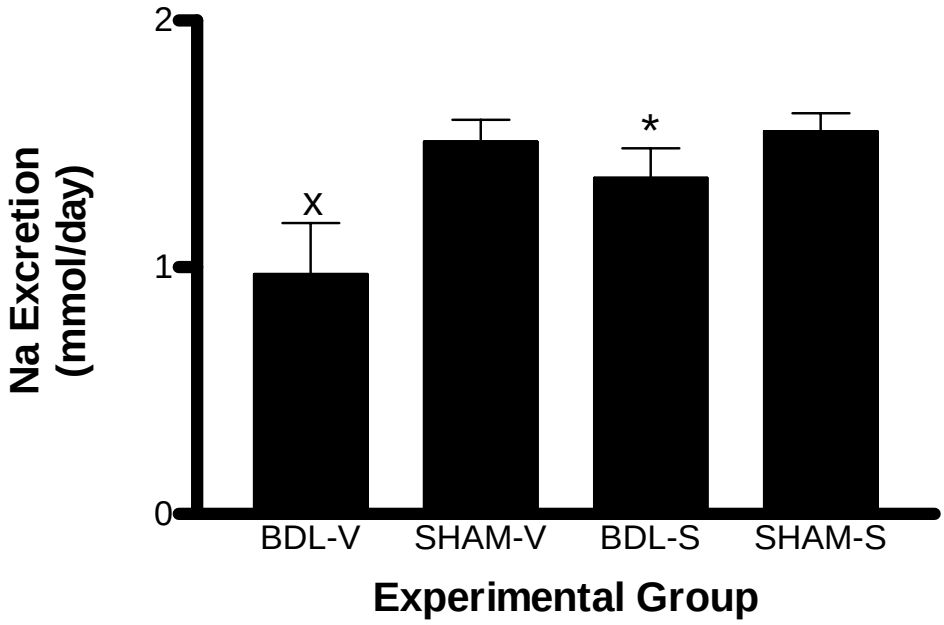

Na Excretion
(mmol/day)
2
1
0
X
*
BDL-V
SHAM-V
BDL-S
SHAM-S
Experimental Group

Figure 3

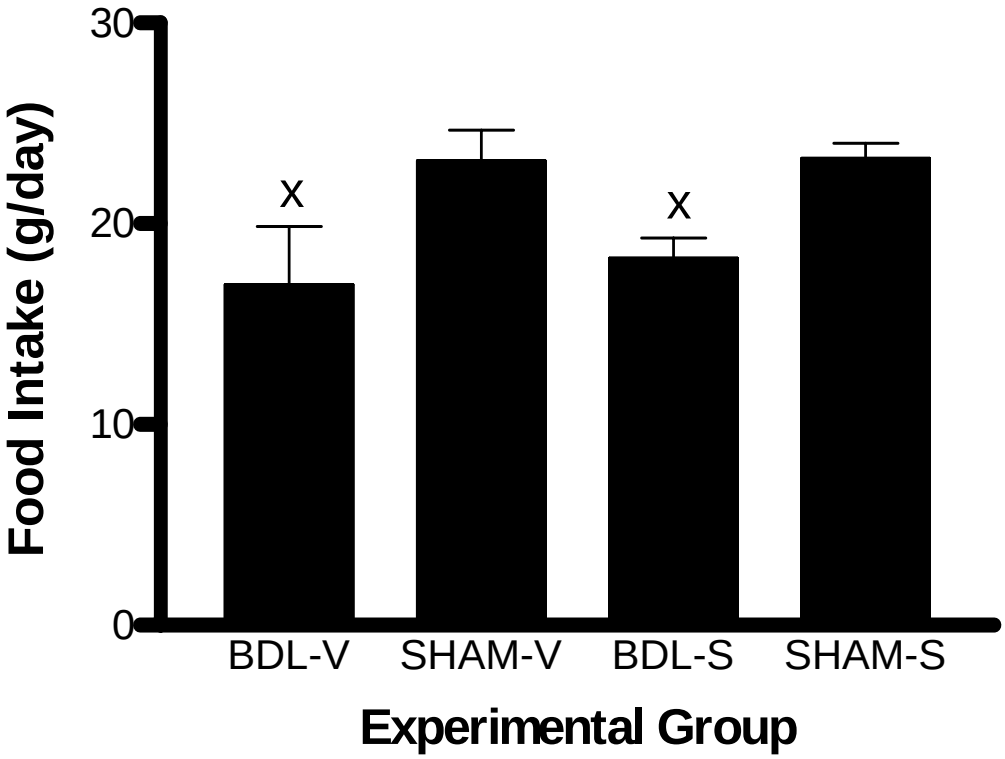

30
20
10
0
Food Intake (g/day)
X
X
BDL-V
SHAM-V
BDL-S
SHAM-S
Experimental Group

Figure 4

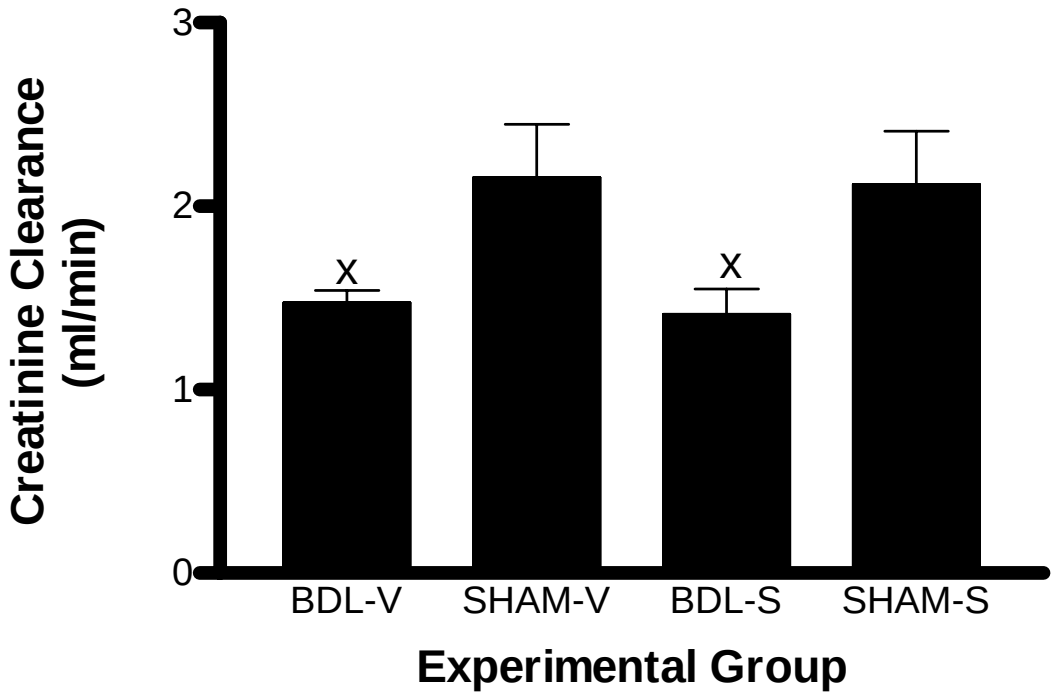

Creatinine Clearance
(ml/min)
3
2
1
0
X
X
BDL-V
SHAM-V
BDL-S
SHAM-S
Experimental Group

Figure 5.

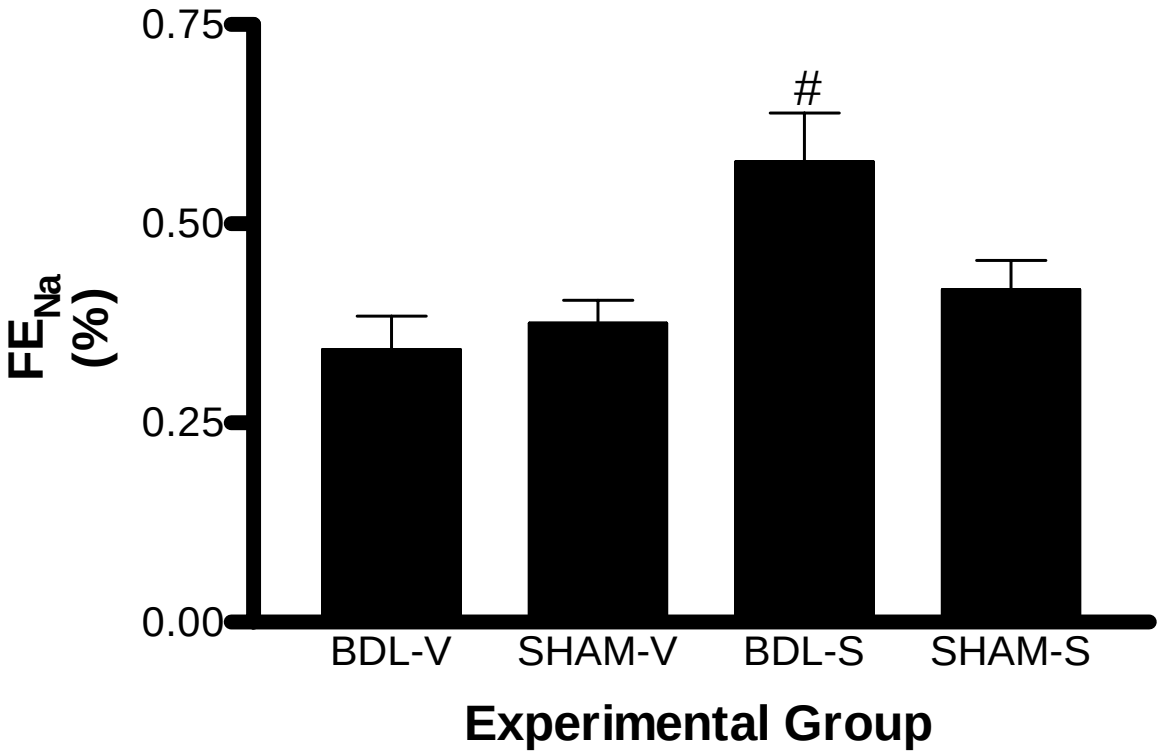

0.75
0.50
0.25
0.00
FE Na (%)
#
BDL-V
SHAM-V
BDL-S
SHAM-S
Experimental Group

Figure 6

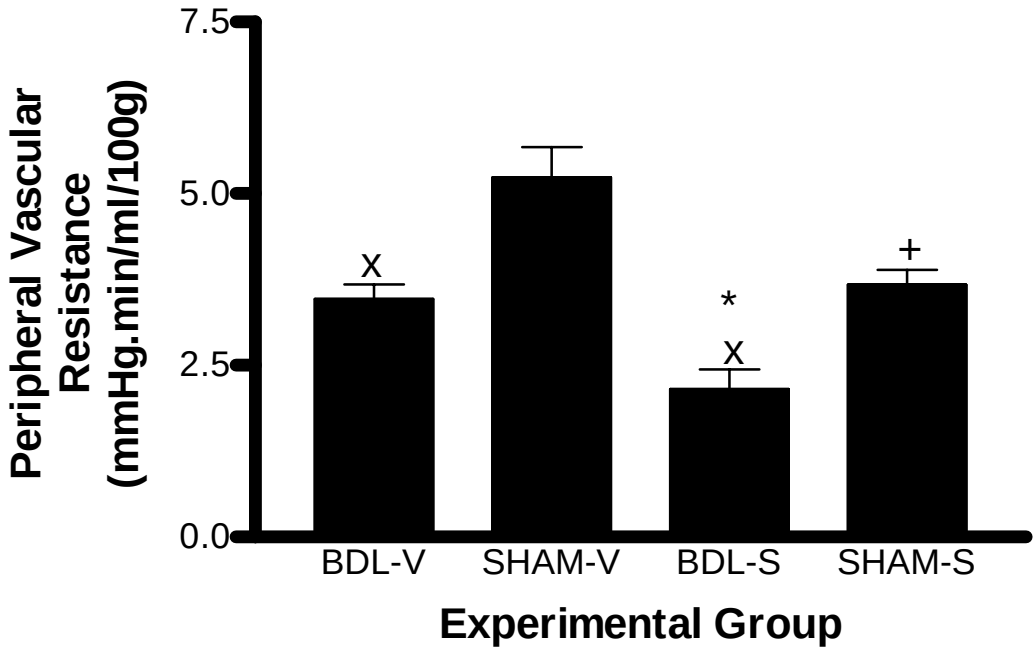

Peripheral Vascular Resistance (mmHg.min/ml/100g)
7.5
5.0
2.5
0.0
X
*
X
+
BDL-V
SHAM-V
BDL-S
SHAM-S
Experimental Group

Figure 7

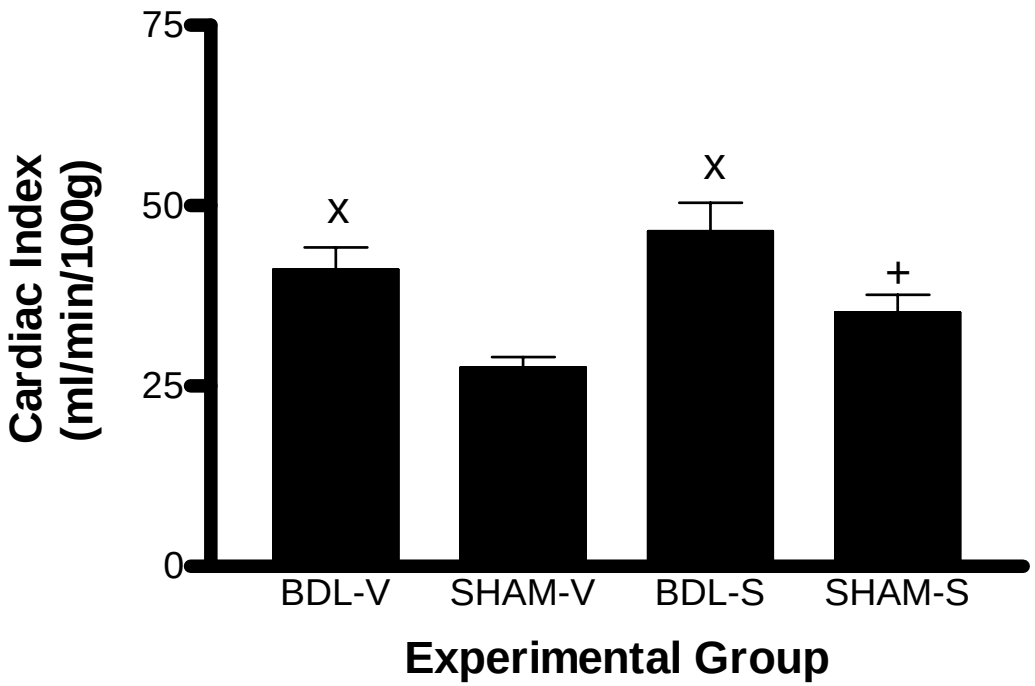

75
50
25
0
Cardiac Index
(ml/min/100g)
X
X
+
BDL-V
SHAM-V
BDL-S
SHAM-S
Experimental Group

Figure 8

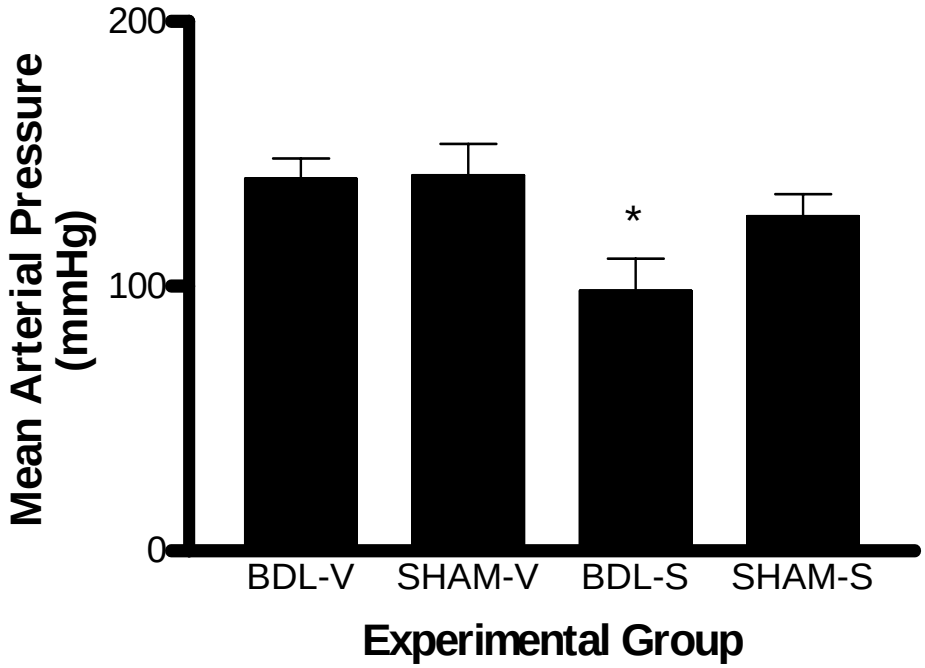

200
100
0
Mean Arterial Pressure (mmHg)
*
BDL-V
SHAM-V
BDL-S
SHAM-S
Experimental Group

Figure 9

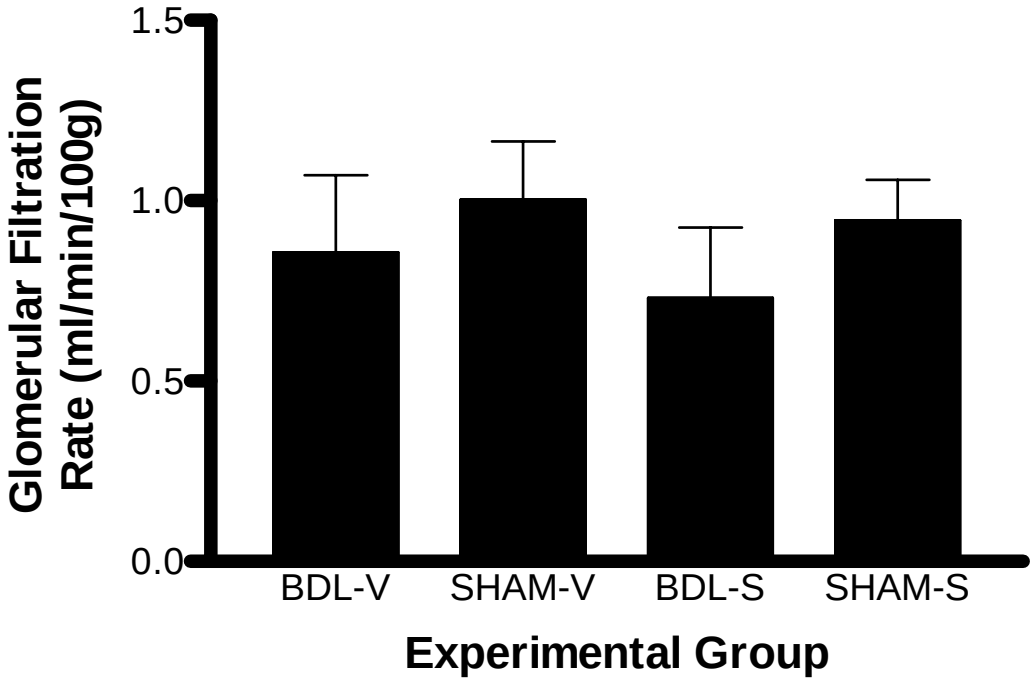

Glomerular Filtration Rate (ml/min/100g)
1.5
1.0
0.5
0.0
BDL-V
SHAM-V
BDL-S
SHAM-S
Experimental Group

Figure 10

Article II

Effect of chronic administration of sildenafil on sodium retention and on the hemodynamic complications associated with liver cirrhosis in the rat

Les résultats obtenus dans l'article I ont montré un effet bénéfique de l'inhibiteur de PDE5, dans la mesure où l'administration aiguë du DMPPO (5mg/kg i.v) induit une excrétion rénale du sodium chez les rats cirrhotiques, et restaure par la suite la fonction rénale. Ce dysfonctionnement rénal associé á la cirrhose est la conséquence d'une vasodilatation des artères de la surface splanchnique, qui afin de maintenir la pression artérielle entraîne l'activation des mécanismes de compensation au niveau du système rénine-angiotensine-aldostérone, du système nerveux parasympathique et du système sécrétoire de l'arginine-vasopressine qui, à leur tour produisent une vasoconstriction excessive des artères rénales. En plus, l'augmentation de l'activite rénale de la PDE5 s'oppose a l'effet natriuretique de l'ANF, une hormone régulatrice de la pression artérielle et de l'homéostasie, et induit la rétention rénale du sodium. Ces résultats suggèrent que l'inhibition de la PDE5 pourrait être une cible thérapeutique de la rétenion du sodium liée á la cirrhose.

Des études récentes ont montré que l 'administration aiguë du Sildenafil (Viagra^{TM}), inhibiteur puissant et spécifique de la PDE5 (médicament contre la dysfonction érectile) diminue la pression arterielle dans le lit mésenterique. Cependant, cet effet hémodynamique néfaste similaire à celui du DMPPO pourrait à long terme aggraver la rétention rénale du sodium suite a l'activation des systèmes de vasoconstrictions. l'objectif de ce travail est de montrer la reproductivité de l'effet bénéfique d'un inhibiteur de PDE5 au niveau de l'excrétion rénale du sodium, et son devenir après une administration chronique du Sildenafil.

Cette étude examine l'effet chronique du Sildenafil sur la rétention sodique et les altérations hémodynamiques associées à la cirrhose. Deux semaines après la chirurgie, les rats BDL et contrôles subissent ou non un traitemennt sous cutanée de 20 mg/kg 3 fois par jour ou du vehicule du Sildenafil, le DMSO, pendant 15 jours. Ces travaux montrent que le Sildenafil améliore la rétention rénale du sodium, mais potentialise cependant la vasodilatation péripherique et diminue la pression arterielle.

En conclusion, cette étude a montré que l'administration chronique du Sildenafil améliore l'excrétion de sodium dans les urines mais est néanmoins toujours néfaste envers la vasodilatation périphérique qu'elle aggrave.

Effect of chronic administration of sildenafil on sodium retention and on the hemodynamic complications associated with liver cirrhosis in the rat

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Short Title

Effect of sildenafil in cirrhosis

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Summary

Background: Previous studies demonstrated increased PDE5 activity and expression in the kidneys of rats with liver cirrhosis. Acute intravenous administration of PDE5 inhibitors enhanced sodium excretion in these rats. The aim of the present study was to examine the effects of chronic administration of sildenafil on renal sodium handling and hemodynamics in rats with liver cirrhosis. Methods: Male Sprague-dawley rats underwent bile-duct-ligation and excision (BDL) or sham operation and were housed in metabolic cages throughout the study. Body weight, food intake, water intake and urine volume were measured daily, and plasma samples were obtained twice weekly. Fourteen days following surgery sildenafil or its vehicle (Dimethylsulfoxide-DMSO) were administered (20 mg/kg subcutaneously 3 times/day). Two weeks later, systemic hemodynamics were measured under general anesthesia. Results: Sildenafil enhanced the systemic vasodilatation associated with liver cirrhosis and reduced the arterial pressure. There was no reduction in GFR, however. Despite these hemodynamic changes, sildenafil prevented the decrease in Na excretion observed in the BDL group receiving vehicle and markedly increased fractional sodium excretion relative to the other groups. Conclusions: These results suggest that chronic sildenafil administration may help prevent or ameliorate Na retention in cirrhosis, but that hemodynamic adverse effects may ensue.

Keywords
cirrhosis, hemodynamics, vasodilation, phosphodiesterase 5, sildenafil, cGMP, sodium excretion

Introduction

The major cardiovascular complication of liver cirrhosis is a hyperdynamic circulation with peripheral and splanchnic vasodilatation, increased cardiac output and varying degrees of hypotension. The peripheral arterial vasodilation hypothesis suggests that primary peripheral (mainly splanchnic) vasodilation induces activation of compensatory mechanisms such as the renin-angiotensin-aldosterone system, antidiuretic hormone and the sympathetic nervous system to result in sodium and water retention in order to restore effective arterial volume and maintain arterial pressure [1]. The primary mesenteric vasodilatation leading to the hyperdynamic circulation has been attributed to increased production of various vasodilator substances such as nitric oxide (NO) [2,3], prostacyclin [4], endothelium derived hyperpolarizing factor [5], glucagon [6], substance P and calcitonin-gene-related peptide [7]. Among all, nitric oxide has gained the most attention as the major cause of the decreased vascular resistance in cirrhosis [8]. Nitric oxide, formed by endothelial cells from its precursor L-arginine raises intracellular cGMP levels, its second messenger, leading to vasodilatation [9].

Another hemodynamic abnormality associated with cirrhosis is a decreased responsiveness of the kidney to the natriuretic effect of the atrial natriuretic factor, a key component to blood pressure regulation and homeostasis. Atrial natriuretic factor, like nitric oxide, acts by increasing intracellular levels of cGMP which can be hydrolyzed by numerous phosphodiesterase isoforms [10]. Among those isoforms which are specific to cGMP hydrolysis is phosphodiesterase type 5 (PDE5), located in the smooth muscle cells of blood vessels. PDE5 was found to be present in the mesenteric artery [11,12] in pulmonary arteries [13] and in other vascular beds [14]. It has been shown that the blunted renal response to atrial natriuretic factor in cirrhosis is due, at least partially, to

upregulation of PDE5, and that inhibition of PDE5 in the kidney can enhance the responsiveness to atrial natriuretic factor by restoring intracellular levels of cGMP producing an increase in renal plasma flow, in glomerular filtration rate (GFR) and in sodium excretion [15,16]. These results suggest that inhibition of PDE-5 may be a therapeutic strategy in patients with liver cirrhosis and ascites. However, it should be noted that inhibition of PDE5 in the splanchnic area may increase the vasodilator effect associated with nitric oxide by raising intracellular levels of cGMP. Inhibition of PDE 5, therefore, may have a beneficial effect in the kidney and on the response to atrial natriuretic factor, but may worsen the hemodynamic picture in cirrhosis. In preliminary studies we have demonstrated that acute administration of a PDE-5 selective inhibitor, DMPPO, led to worsening in the vasodilation and the decrease in arterial pressure; despite this, however, it induced an increase in sodium excretion by the kidney [17]. Others have shown that the acute administration of sildenafil in the splanchnic bed increased mesenteric blood flow and portal venous pressure and decreased mean arterial pressure [18]. While results of acute drug administration may be interesting, they do not reflect the possible clinical relevance of such an intervention. The hemodynamic disturbances induced by sildenafil may in the long term worsen the sodium handling by the kidney as a result of more severe activation of the compensatory mechanisms mentioned above. Therefore, this study was designed to explore the effect of chronic administration of a PDE5 inhibitor, sildenafil, on the hemodynamic and renal functional consequences of liver cirrhosis in rats, specifically to examine whether the beneficial effect on sodium excretion, observed in acute studies, will also be observed after chronic administration.

Materials and Methods

The animals used in the present study were reared, treated and euthanized in accordance with the provisions for animal welfare of the Institutional Animal Care and Use Committee of the American University of Beirut. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, National Academy of Sciences. Adult male Sprague-Dawley rats weighing between 230-290 g were randomized to undergo either sham operation (Sham) or bile duct ligation and excision (BDL). Two days prior to surgery, rats were housed in metabolic cages for adaptation and allowed access to normal rat chow and water. For surgery, rats were anesthetized with pentobarbital (40 mg/kg body weight ip) and their abdomen was opened through a midline incision. The common hepatic bile duct was isolated and transected between two ligatures in BDL animals. Sham rats underwent the same procedure but without ligation and excision of the bile duct. Following surgery, rats were housed individually in metabolic cages. Several parameters were measured on a 24-hour basis including: body weight, food intake, water intake and urine volume. Sodium excretion was calculated after measurement of Na concentrations in the urine over 24 h. During the first 2 weeks, blood was withdrawn from the rats' tail veins, under light ether anesthesia, once weekly then twice weekly for the remaining 2 weeks. Plasma and urine were used to measure sodium and creatinine levels from which sodium excretion rate, creatinine clearance, and fractional sodium excretion were calculated. Two weeks post-surgery, rats were started on either sildenafil (Pfizer Ltd, Sandwich, Kent, UK) (30 mg/ml in DMSO) or its vehicle (DMSO) at a dose of 20 mg/kg subcutaneously 3 times daily. Thus 4 groups resulted: sham-operated with vehicle (Sham-V) or sildenafil (Sham-S) treatment, or BDL with similar treatment (BDL-V and BDL-S, respectively). Four weeks following surgery, animals were anesthetized with pentobarbital (25 mg/kg for BDL and 50 mg/kg for Sham) and their tracheas were cannulated with PE-205 tubing. A

PE-50 catheter was introduced into the right carotid artery and connected to a blood pressure transducer (TDX-310) for measurement of arterial pressure using a BPA-100 blood pressure analyzer (Micro-Med Louisville, KY, USA), while another PE50 catheter was inserted in the right jugular vein and advanced to the right atrium. A thermistor probe was advanced to the aortic arch through a left carotid approach to monitor the cardiac output by the thermodilution technique using a cardiac output computer (Cardiotherm® Columbus Instruments, Columbus, OH). Three consecutive measurements of cardiac output were obtained at 5 minute intervals and the peripheral vascular resistance was calculated as the ratio of mean arterial pressure to cardiac index, which is the cardiac output expressed per 100g body weight. The bladder was then exposed through a supra-pubic incision and the urethra catheterized using PE 190 tubing for urine collection. ^{3}H-Inulin was then administered (0.75μCi loading dose followed by a continuous infusion of 0.05 μCi/min at 50 μL/min). After 30-min of stabilization, a urine sample was collected over a 30-min period and a blood sample withdrawn at the midpoint of the collection. The urine and plasma samples were used to measure ^{3}H counts and the glomerular filtration rate (GFR) was estimated from the clearance of inulin.

Statistical Analysis

Data are presented as mean ± standard error of the mean. Comparison of values between two groups was conducted using the Students' unpaired t-test; comparisons among more than two groups was performed by one way analysis of variance (ANOVA) followed by the Newman-Keul test for individual comparisons. The responses of variables over time in one group were compared to other groups by 2-way ANOVA with repeated measures and followed by Bonferroni post-test for multiple comparisons. Comparison of mortality figures among groups was conducted using the z-test for proportions. Analyses were

performed using the SigmaStat statistical Software (Jandel Corporation, San Rafael, California, U.S.A.), or the GraphPad Prism software (GraphPad Software, Inc., San Diego, California, U.S.A.). Differences in values were considered statistically significant at a P level of < 0.05.

Results

Survival following BDL surgery was determined over the 4 weeks period. Of those randomized to the sildenafil group, 5 of 17 rats died during the first 2 weeks of the study, before sildenafil or its vehicle were administered. However, none of the rats in those randomized to the vehicle group (n=9) died during the first 14 days. Analysis of mortality in BDL rats during the last 14 days of the study (after the start of sildenafil or vehicle treatment) revealed that none of the remaining 12 rats which received sildenafil died, compared with 3 of 9 (33%) in those receiving its vehicle. This difference was not statistically significant. None of the rats in the two sham-operated groups (n=11 for vehicle and n=10 for sildenafil treatment) died during the study period. .

Body Weight

All rats sustained a reduction in body weight on the first few days after surgery. After that, rats started to regain weight; however, the rate of weight gain was slower in the BDL groups compared with the sham-operated groups, irrespective of the treatment received, such that it remained lower in the BDL relative to the sham-operated group of rats especially during the last 2 weeks (figures 1a and 1b). There was no difference, however, between the two BDL groups or the two sham-operated groups.

Urine volume and sodium excretion:

Urine volume increased in all groups over time (figure 2). Both BDL groups had significantly higher urine volumes than their sham-operated counterparts ($P<0.0001$). Furthermore, the BDL group treated with sildenafil had significantly higher values than the vehicle treated BDL groups especially during the last two weeks of the study ($P=0.0039$).

Urinary sodium excretion during the last week of the study is presented in figure 3. Previous studies have shown that at this time Na retention is manifested in rats with BDL [19,20]. Statistical analysis showed that the vehicle-treated BDL group had lower values than the vehicle treated sham-operated group ($P=0.0066$) and the sildenafil treated BDL group ($P=0.04$) during this period. These findings were particularly significant considering the fact that food intake and, consequently, sodium intake, were not significantly different between the BDL group treated with sildenafil and the BDL group treated with vehicle, during the same periods of time (figure 4), both of which were lower than the values in the corresponding sham-operated groups. Neither Na excretion nor food intake was different between the 2 sham-operated groups.

Calculation of Na balance showed that the sildenafil treated BDL group had a significantly lower sodium balance than both the sildenafil treated sham operated group and the vehicle treated BDL group (figure 5).

Creatinine clearance

Figure 6 represents the creatinine clearance during the last week of the study. The values were not difference among the 4 groups of rats. The mean creatinine clearance during the last week (average of the 3 values in each rat) was slightly but not significantly lower in both BDL groups relative to their sham-operated controls (Sham-V:0.73±0.09; BDL-V:

0.52±0.02; Sham-S: 0.65±0.09; BDL-S: 0.43±0.02 ml/min/100g). Furthermore, there was no significant difference in creatinine clearance between the 2 BDL groups, nor between the 2 sham-operated groups.

Fractional sodium excretion (FE_{Na})

In the BDL group treated with vehicle, FE_{Na} was similar to the one obtained in the corresponding sham-operated group. Treatment of sham-operated rats with sildenafil did not alter this parameter. In the BDL group treated with sildenafil, however, there was a significantly higher FE_{Na} compared with all the other groups (figure 7). The average FENa during the last week of the study provided similar results with an overall higher value in the sildenafil treated BDL group that all the other groups (Sham-V: 0.37±0.03 %; BDL-V: 0.30±0.05 %; Sham-S: 0.42±0.03 %; BDL-S: 0.53±0.04 %, P=0.002 by ANOVA, and $P<0.05$ comparing BDL-S with each of the other groups).

Systemic Hemodynamics and GFR:

At the end of the study, BDL rats treated with vehicle had a lower peripheral vascular resistance than their sham-operated controls. Treatment with sildenafil per se reduced the vascular resistance significantly in the sham operated group (Table 1). BDL rats receiving sildenafil sustained further vasodilation relative to their sham-operated sildenafil treated controls, and relative to the BDL group treated with vehicle. Cardiac index was elevated in the BDL rats receiving vehicle such that mean arterial pressure was unchanged relative to their sham-operated control. In contrast, despite the enhancement of the vasodilation in the BDL group receiving sildenafil, there was no further increase in cardiac index relative to the BDL group receiving vehicle, such that mean arterial pressure was significantly reduced compared with that in both the sham-operated, sildenafil treated group and the BDL group receiving vehicle.

Measurement of GFR using inulin clearance revealed the same trends as those that were observed using creatinine clearance, with a decrease in both BDL groups relative to their controls. However, the differences did not reach significant levels (Table 1).

Discussion

The present study is the first to investigate the chronic effects of sildenafil on the hemodynamic and renal functional alterations associated with liver cirrhosis. Administration of sildenafil to rats with liver cirrhosis caused reduction in arterial pressure by enhancing the peripheral vasodilation associated with this condition. Despite these unfavorable hemodynamic effects, sildenafil increased total and fractional sodium excretion and did not worsen GFR.

According to the peripheral vasodilatation theory of ascites formation in liver cirrhosis, vasodilation, mainly splanchnic in origin, initiates a sequence of events in order to compensate for the reduction in effective blood volume. This sequence includes activation of the renin-angiotensin-aldosterone system and the sympathetic nervous system, as well as ADH release, to retain sodium and water by the kidney [1]. In the present study, the BDL group receiving vehicle reproduced the predictable hemodynamic and renal changes, with a decrease in vascular resistance, an increase in cardiac output, and a reduction in creatinine clearance and sodium excretion by the kidney. The blood pressure was unchanged since the increase in cardiac output compensated for the vascular dilatation.

Much evidence supports a role for increased NO production as the cause of the splanchnic and peripheral vasodilation in cirrhosis [2, 9]. NO, by activating the

production of cGMP, decreases intracellular calcium levels and leads to relaxation of the smooth muscle cells [21]. cGMP is hydrolyzed by phosphodiesterases found in the vascular smooth muscle cells, mainly PDE-1 and PDE-5 [10]. Recently, studies have shown that kidneys of rats with liver cirrhosis express increased levels and activity of PDE5 and that acute administration of inhibitors of PDE5 increases renal plasma flow and sodium excretion and improves the blunted response to a salt load or to infusion of atrial natriuretic peptide in rats with liver cirrhosis [15, 22, 23]. These results suggest that the PDE-5 inhibitors may be beneficial in the management of patients with liver cirrhosis suffering from sodium retention and ascites. No studies were undertaken to examine the effect of chronic administration of PDE 5 inhibitors, however. This is especially important because these agents may worsen the peripheral vasodilation attributed to NO due to inhibition of cGMP hydrolysis. Such enhancement of vasodilation may increase sodium retention by the kidney and annul the direct effect of the PDE-5 inhibitor on the kidney, by causing more severe activation of the sodium retaining mechanisms over time, an effect that may not be seen after acute administration only. This study was conducted to address that question.

As the results show, at the time the BDL group treated with vehicle was showing sodium retention as evidenced by a reduction in sodium excretion, the BDL group treated with sildenafil had a sodium excretion similar to the sham–operated groups, and hence, did not exhibit sodium retention. This occurred despite the fact that food intake (and consequently sodium intake) and GFR in this group were reduced to levels similar to that in the BDL group treated with vehicle. Consequently, the sildenafil treated BDL group had a significantly and markedly higher fractional sodium excretion relative to all the other groups. It is noteworthy that sildenafil, per se, did not alter renal handling of

sodium (compare Sham-V with Sham-S); it was only in the context of liver cirrhosis that its effect on sodium excretion was evident. This is likely due to the fact that in liver cirrhosis, PDE-5 activity is stimulated, as previously shown [15, 23], thus the effect of PDE-5 inhibition becomes more evident.

It is important to note that this salutary effect on sodium handling occurred despite worsening of the hemodynamic picture. It was clear that sildenafil treatment per se induced hemodynamic changes including a decrease in peripheral vascular resistance and an increase in cardiac output, with no change in arterial pressure, as evident by comparing the Sham-V and Sham-S groups. In the BDL group treated with sildenafil there was a further vascular dilation (reflecting the effect of BDL). However, this was not met by a further increase in cardiac output to that observed in the BDL-V group, likely due to the lack of significant sodium and water retention, thus resulting in a reduction in mean arterial pressure.

The results of this study are consistent with previous studies that showed an increase in sodium excretion following administration of zaprinast or DMPPO (both PDE-5 selective inhibitors), as noted above [15, 22, 23]; however, all these studies were acute interventional studies. A more recent study by Colle et al. showed that sildenafil administration intravenously or into the mesenteric artery of rats with BDL increased mesenteric blood flow and portal venous pressure, but decreased arterial pressure [18]. The authors suggested that the rise in portal pressure may pause a risk for hemorrhagic complications and cautioned against prescription of sildenafil to patients with cirrhosis before further studies were conducted. Thiesson et al. administered a single oral dose of sildenafil to patients with liver cirrhosis [24]. The patients sustained an elevation in

plasma renin activity, angiotensin II and aldosterone concentrations in the plasma at 60 minutes and a fall in sodium excretion at 120 and 180 minutes after the dose. Thus, in contrast to animal studies, single administration of sildenafil in humans did not induce natriuresis but worsened sodium retention. This appeared to be related to the activation of the renin-angiotensin aldosterone system as a result of the hypotension. In that study, however, the authors failed to detect any change in urinary cGMP levels, which suggests that renal PDE5 activity was not effectively inhibited by that single dose of sildenafil.

Our present study is the first (in humans or animals) to study the effect of chronic administration of sildenafil, or any PDE-5 inihibitor, in liver cirrhosis. It suggests that under these circumstances, the direct effect of sildenafil in the kidney overcomes the indirect effects of activating sodium retaining compensatory mechanisms resulting from vasodilation which are the proposed mechanism of sodium retention in cirrhosis; however, this occurs at the expense of further vasodilation and hypotension. The clinical significance of these findings cannot be ascertained from this study, which used a single dose regimen of the drug. It is possible that using a lower dose may induce a selective effect on the kidney without much worsening of the hemodynamic picture, and that requires specific studies in the future. Furthermore, the possible hemorrhagic complications need to be considered as suggested by Colle et al. [18]. It is encouraging, however, that in the present study, the mortality rate during the second two weeks of the study, i.e., following the start of treatment, tended to be lower in the sildenafil treated group, although the study was not specifically designed, and was not powerful enough, to examine that parameter. In conclusion, the present study offers a possibility for a novel therapeutic approach to sodium retention and ascites in liver cirrhosis using sildenafil

specifically, and PDE-5 inhibitors in general, that should be further tested in animal and human studies.

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Table 1. Hemodynamic parameters 4 weeks after BDL or sham operation in vehicle or sildenafil treated rats. Sildenafil or its vehicle was started on day 14 after surgery.

	Vehicle		**Sildenafil**		**ANOVA**
	Sham (n=9)	BDL (n=6)	Sham (n=10)	BDL (n=10)	
MAP (mmHg)	142 ± 11	141 ± 7	126 ± 8	111 ± 11 *	P=0.0001
Cardiac Index (ml/min/100g)	27.6 ± 1.4	41.1 ± 3.1*	35.2 ± 2.5	46.5 ± 3.9*	P=0.0001
PVR (mmHg.min.100g/ml)	5.2 ± 0.5	3.5 ± 0.2*	3.7 ± 0.2+	2.2 ± 0.3*#	P<0.0001
GFR (ml/min/100g)	1.00 ± 0.16	0.86 ± 0.22	0.95 ± 0.11	0.73 ± 0.19	NSD

Values represent the mean ± the standard error of the mean.

*: P<0.05 compared with the sham-operated group receiving the same treatment

+: P<0.05 compared with the Sham-Vehicle group.

#: P<0.05 compared with the BDL-Vehicle group.

Table 2: Results of Two way Analysis of Variance for figures 1-7. Only values for P<0.1 were included; all others are P>0.1.

Parameter	Comparisons	Time factor	Group factor	Interaction of time and Group
Body weight	Sham-V v BDL-V	<0.0001	0.076	<0.0001
	Sham-S v BDL-S	<0.0001	0.028	<0.0001
	Sham-V v Sham-S	<0.0001	-	-
	BDL-V v BDL-S	<0.0001	-	-
Urine Volume	Sham-V v BDL-V	<0.0001	0.0001	0.019
	Sham-S v BDL-S	<0.0001	<0.0001	<0.0001
	Sham-V v Sham-S	<0.0001	-	<0.0001
	BDL-V v BDL-S	<0.0001	0.004	<0.0001
Na excretion	Sham-V v BDL-V	-	0.007	-
	Sham-S v BDL-S	0.027	-	-
	Sham-V v Sham-S	0.014	-	-
	BDL-V v BDL-S	-	0.041	-
Food Intake	Sham-V v BDL-V	-	0.065	0.033
	Sham-S v BDL-S	-	0.002	0.083
	Sham-V v Sham-S	-	-	-
	BDL-V v BDL-S	0.002	-	-
Na Balance	Sham-V v BDL-V	-	-	0.065
	Sham-S v BDL-S	0.054	0.004	0.094
	Sham-V v Sham-S	-	-	-
	BDL-V v BDL-S	0.004	0.054	-
Creatinine CL	Sham-V v BDL-V	-	-	-
	Sham-S v BDL-S	0.010	0.074	0.010
	Sham-V v Sham-S	0.083	-	-
	BDL-V v BDL-S	-	-	-
FE Na	Sham-V v BDL-V	-	-	0.031
	Sham-S v BDL-S	0.085	0.053	-
	Sham-V v Sham-S	-	-	0.066
	BDL-V v BDL-S	-	0.015	-

Figure Legends

Figure 1 (a) and (b): Body weight over 28 days in BDL (n=8) and sham-operated (n=11) rats treated with vehicle (V)(Fig.1a) and in sham-operated (n=10) and BDL (n=12) rats treated with sildenafil (S) (Fig.1b). S or V were started on day 14 after surgery. Values are Means±SE of mean. x:$P<0.05$ compared with the sham-operated group receiving the same treatment.

Figure 2: Urine volume over 28 of the study in the BDL-V (n=8) Sham-V (n=11), BDL-S (n=12) Sham-S (n=10) groups. The values represent the mean±SE of the mean x: $P<0.05$ vs. sham-operated group receiving the same treatment. Results of 2-way ANOVA with repeated measures are shown in table 2.

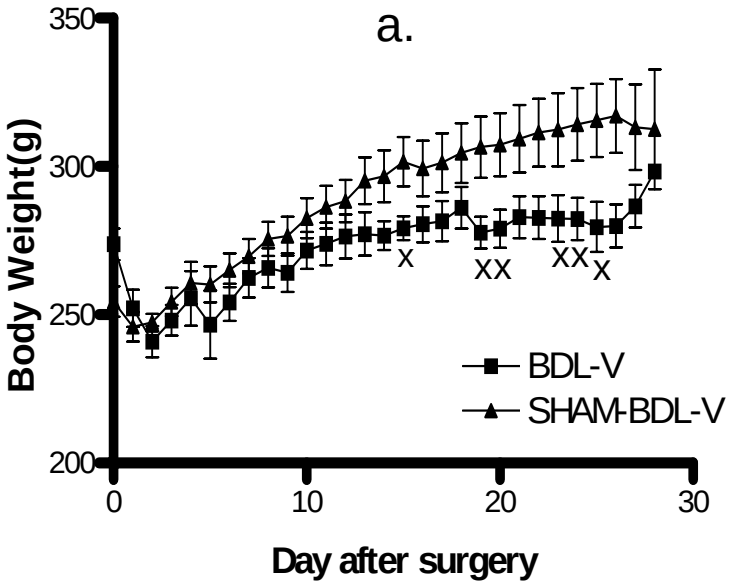
Figure 3: Sodium excretion during the last week of the study in the BDL-V (n=8)ham-V (n=11), BDL-S (n=12) and Sham-S (n=10) groups. The values represent the mean±SE of the mean. Results of 2-way ANOVA with repeated measures are shown in table 2.

Figure 4: Food intake during the last week of the study in the BDL-V (n=8) Sham-V (n=11), BDL-S (n=12) Sham-S (n=10). The values represent the mean±SE of the mean. Results of 2-way ANOVA with repeated measures are shown in table 2.

Figure 5: Na balance during the last week of the study in the BDL-V (n=8) Sham-V (n=11), BDL-S (n=12) Sham-S (n=10). The values represent the mean±SE of the mean. Results of 2-way ANOVA with repeated measures are shown in table 2.

Figure 6: Creatinine clearance on three days during the last week of the study in the BDL-V (n=8) Sham-V (n=11), BDL-S (n=12) Sham-S (n=10). The values represent the mean±SE of the mean. Results of 2-way ANOVA with repeated measures are shown in table 2.

Figure 7: Fractional excretion of Na on three days during the last week of the study in the BDL-V (n=8) Sham-V (n=11), BDL-S (n=12) Sham-S (n=10) groups. The values represent the mean±SE of the mean. Results of 2-way ANOVA with repeated measures are shown in table 2.


a.
350
300
250
200
Body Weight(g)
0
10
20
30
Day after surgery
BDL-V
SHAM-BDL-V

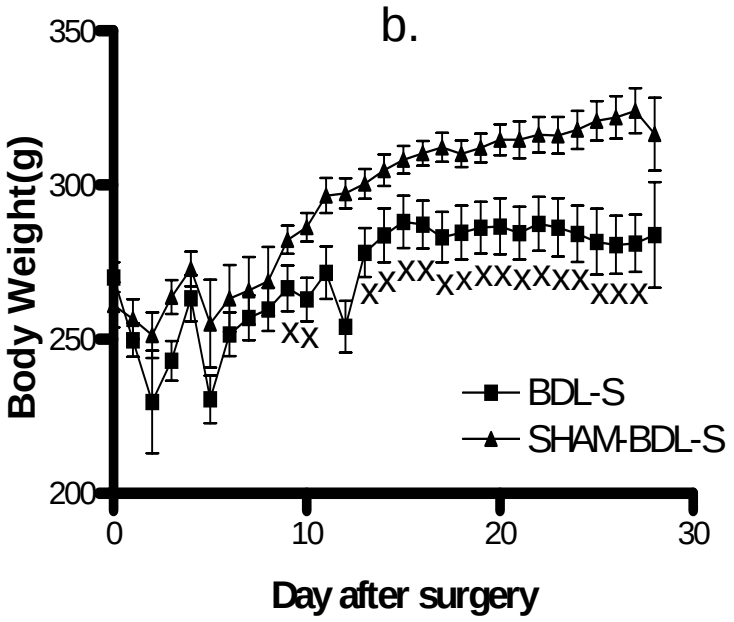

b.
350
300
250
200
Body Weight(g)
0
10
20
30
Day after surgery
BDL-S
SHAM-BDL-S

Figure 1

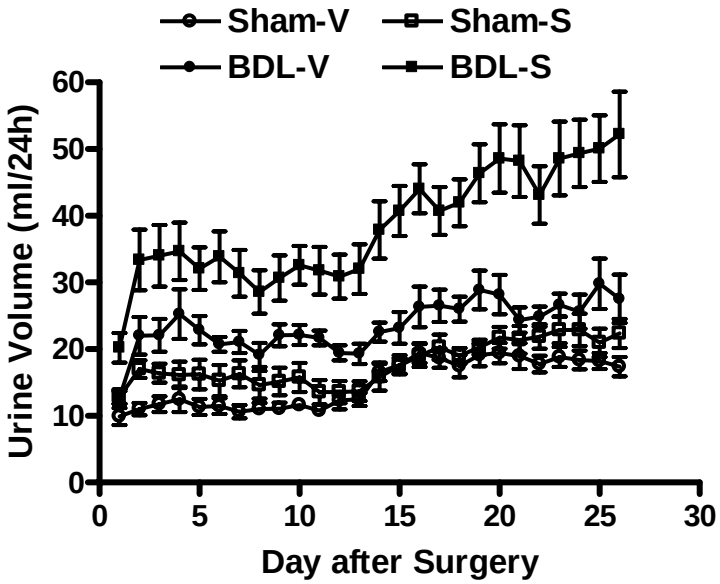

Sham-V
Sham-S
BDL-V
BDL-S
Urine Volume (ml/24h)
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Day after Surgery

Figure 2

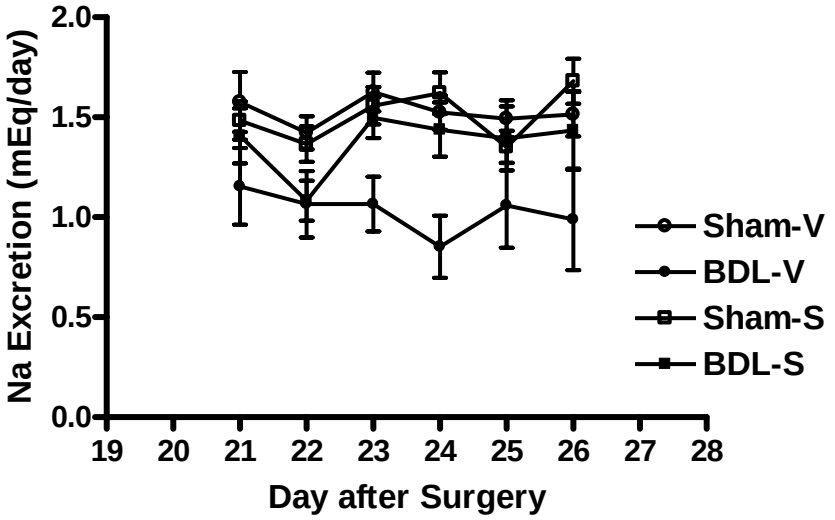

Na Excretion (mEq/day)
2.0
1.5
1.0
0.5
0.0
19
20
21
22
23
24
25
26
27
28
Day after Surgery
Sham-V
BDL-V
Sham-S
BDL-S

Figure 3

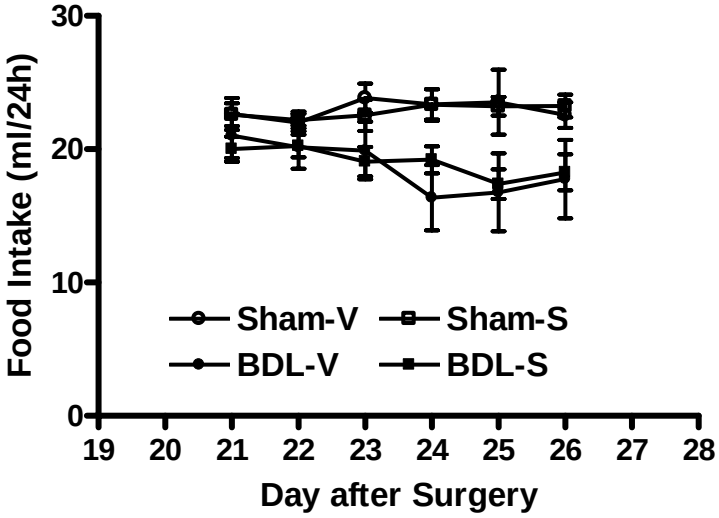

30
20
10
0
Food Intake (ml/24h)
Sham-V
Sham-S
BDL-V
BDL-S
19
20
21
22
23
24
25
26
27
28
Day after Surgery

Figure 4

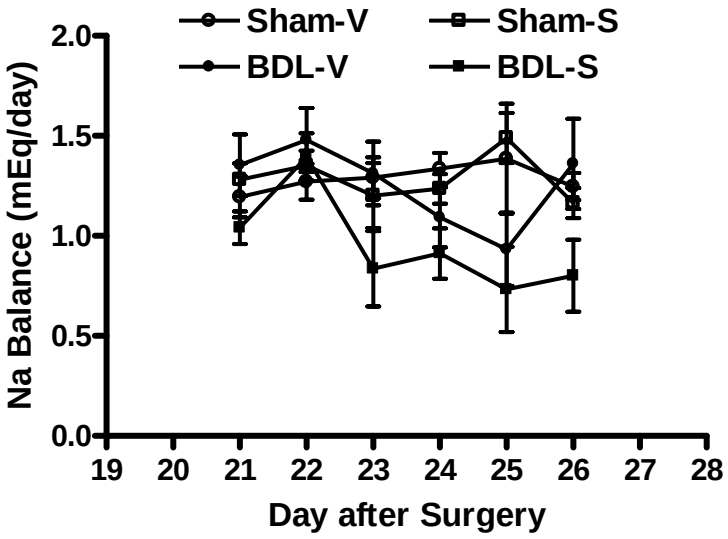

Sham-V
Sham-S
BDL-V
BDL-S
Na Balance (mEq/day)
2.0
1.5
1.0
0.5
0.0
19
20
21
22
23
24
25
26
27
28
Day after Surgery

Figure 5

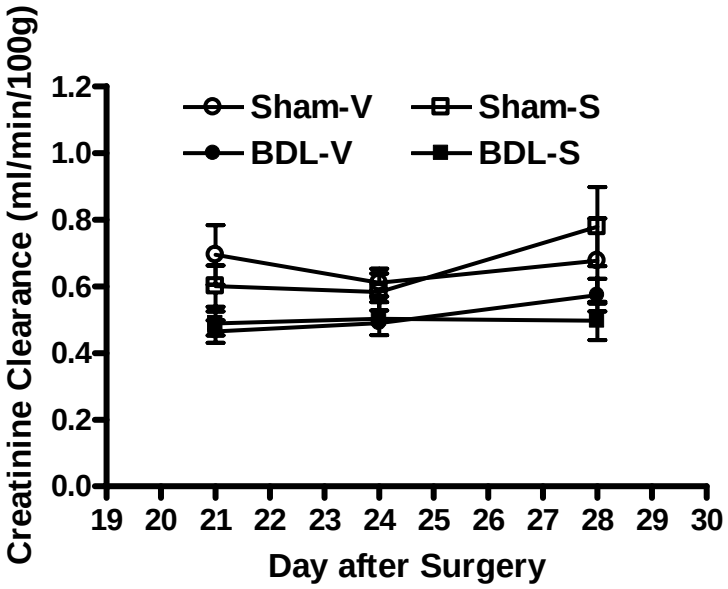

Creatinine Clearance (ml/min/100g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham-V
Sham-S
BDL-V
BDL-S
19
20
21
22
23
24
25
26
27
28
29
30
Day after Surgery

Figure 6

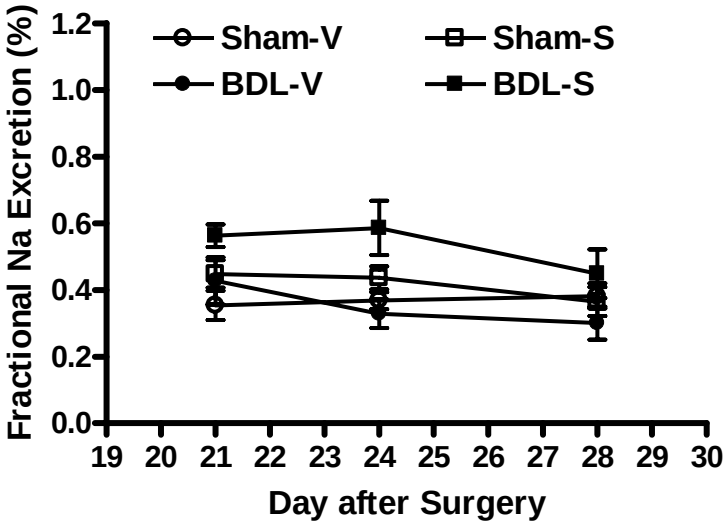

Fractional Na Excretion (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham-V
Sham-S
BDL-V
BDL-S
19
20
21
22
23
24
25
26
27
28
29
30
Day after Surgery

Figure 7

Article III

Assessment of Phosphodiesterase Isozyme Contribution in Cell and Tissue Extracts

(Methods in Molecular Biology, vol. 307: Phosphodiesterase Methods and Protocols)

Thérèse Keravis, Rima Thaseldar-Roumié, and Claire Lugnier

Les phosphodiesterases des nucléotides cycliques (cAMP et cGMP) jouent un role important dans les voies de signlisation cellulaire, comme étant le seul système enzymatique capable d'hydrolyser les seconds messager intracellulaire en leur forme inactive. Récemment, les PDEs de mamifères ont été classées en 11 familles qui sont différement distribuées dans la cellule : cytosolique ou associée a la membrane cellulaire. la méthode décrite permet de doser l'activité des différents PDEs à l'aide d' inhibiteurs spécifiques, en vue de determiner l'implication specifique et fonctionnelle des PDEs dans diverses conditions physiopathologiques.

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Assesment of PDE isozyme contribution in cell and tissue extracts.

Thérèse Keravis, Rima Thaseldar-Roumié and Claire Lugnier

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Keywords: cyclic nucleotide phosphodiesterase activity, PDE1, PDE2, PDE3, PDE4, PDE5, tissues, PDE inhibitor.

Abstract – Cyclic nucleotide phosphodiesterases (PDEs), which are ubiquitously distributed in mammalian tissues, play a major role in cell signaling by hydrolyzing cAMP and cGMP. Due to their diversity, which allows specific distribution at cellular and subcellular level, PDEs can selectively regulate various cellular functions. We present here a convenient and sensitive radioenzymatic assay for characterizing and determining the contribution of the various PDE families in cell and tissue extracts. This assay is based on the knowledge and the use of chosen PDE family-specific inhibitors in order to determine the distinct PDE isozyme contribution in the overall cyclic nucleotide hydrolyzing activity. It can be used to characterize total, cytosolic and membrane-associated PDE activities, as well as PDEs associated with purified membrane structures. This approach is useful for comparing data of control and treated extracts and is therefore quite valuable for viewing the PDE status in different physiopathological conditions.

1. Introduction

Cyclic nucleotide phosphodiesterases (PDEs) which inactivate cAMP and cGMP, play a major contribution in the control of cell signaling. They have been classified into eleven families (PDE1 to PDE11) according to their gene, substrate specificity, regulation, and pharmacology ***(***for review ***1-3).*** PDE1, PDE2, PDE3, PDE4, PDE7, PDE8, PDE10 and PDE11 hydrolyze cAMP. PDE1, PDE2, PDE3, PDE5, PDE6, PDE9, PDE10 and PDE11 hydrolyze cGMP. Their diversity and their specific distribution at cellular and subcellular levels allow a fine regulation of different cellular functions. Among these families, PDE1 to PDE5 families are the most representative and the best characterized isozymes present in cells and tissues. As shown in **Table 1**, PDE1 family is activated by Ca^{2+}-calmodulin (Ca^{2+}-CaM). PDE2 is allosterically activated by cGMP. PDE3, which mainly hydrolyzes cAMP, is competitively inhibited by cGMP, since it hydrolyzes cGMP at a lower rate than cAMP. PDE4 family specifically hydrolyzes cAMP and is insensitive to cGMP. PDE5 specifically hyrolyzes cGMP ***(4-6).*** The knowledge of the participation of these various PDE isozymes in cyclic nucleotide phosphodiesterase activity will help in understanding the regulation of cAMP and cGMP levels in tissues or cells in response to hormonal stimulation or in physiopathological states. Herein, we describe a convenient radioenzymatic approach, which determines the nature and the proportion of a given PDE isozyme activity by using its specific inhibitor. This approach can be used to evaluate PDE activity in cytosolic and membrane fractions as well as purified subcellular structures of normal and pathological tissues, thus contributing to ascertain the functional role of PDEs in various physiopathological states.

2. Materials

1. [2,8-^{3}H] adenosine 3',5'-cyclic phosphate, ammonium salt, 30-50 Ci/mmol; Amersham Biosciences, # TRK498.
2. [8-^{3}H] guanosine 3',5'-cyclic phosphate, ammonium salt, 5-25 Ci/mmol; Amersham Biosciences, # TRK392.

3. TLC plates 20x20 cm, Silicagel 60 F_{254}; Merck, # 1.05715.
4. TLC chromatography solvent: V/V/V: 70 isopropanol/15 NH_4OH/15 H_2O.
5. QAE-Sephadex^{TM}A-25; Amersham Biosciences, # 17-0190-02.
6. Econo-column, polypropylene , 0.8x4 cm; Bio-Rad, # 7311550.
7. **Buffer A**: 20 mM Tris-HCl pH 7.5, 2.0 mM Mg acetate, 5.0 mM EGTA, 1.0 mM dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml soya trypsin inhibitor, 2000 U/ml aprotinin and 0.33 mM Pefabloc.
8. **Formate solution**: 0.4 M $HCOONH_4$ to be diluted 20 fold and adjusted to pH 7.4 just before use.
9. **Tris-BSA**: 20 mM Tris-HCl pH7.5, 2.5 mg bovine serum albumine (BSA)/ml.
10. **Tris-Mg-Ca**: 400 mM Tris-HCl pH 7.5, 20 mM Mg acetate, 0.1 mM $CaCl_2$.
11. **EGTA-Mg**: 10 mM EGTA (ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'- tetraacetic acid), 10 mM Mg acetate, to be adjusted to pH 7.5.
12. **Mg-CaM**: 10 mM Mg acetate, 200 nM purified calmodulin (CaM), ***(7)***.
13. **SV**: 5 mg Crotalus Atrox snake venom /ml; Sigma-Aldrich, # V700-1G.
14. **PDE-stop**: 100 mM Tris-HCl pH 7.5, 50 mM EDTA, 30 mM theophylline, 5 mM cAMP, 5mM cGMP.
15. **SV-stop** : 15 mM EDTA, 0.1 mM adenosine, 0.1mM guanosine, to be adjusted to pH 6.5.
16. PDE inhibitors: cilostamide, nimodipine, EHNA (erythro-9-(2-hydroxy-3-nonyl) adenine), rolipram, zaprinast and IBMX (isobutyl-methyl xanthine) from Sigma-Aldrich.
17. Ultra-Turrax homogenizer.
18. Mortar and pestle.
19. Glass-glass potter homogenizer.
20. Well regulated 30°C-water bath.
21. β liquid scintillation counter.
22. Scintillation fluid: Ultima Gold^{TM}XR; Perkin Elmer®, # 6013119.

23. Millipore Water, 18 Mohm.

3. Methods

3.1. Tissue homogenization

1. Fresh or frozen (stored at –80° C) weighted tissues are hand-powdered in a mortar with a pestle under liquid nitrogen. The resulting powder is collected onto aluminum paper.
2. The powdered tissue is homogenized in buffer A (5 vol/weight; see **Note 1**) first with an ultra-turrax (6 times for10 s at 2000 rpm) and thereafter with a glass-glass potter homogenizer.
3. Homogenate is aliquoted (100-500 µl) in Eppendorf tubes and stored at –80°C for protein determination according to Lowry et al ***(8***) and for PDE assay (**Subheading 3.2**.).

3.2. PDE assay

PDE activities are measured by a two-step radioenzymatic assay as previously described ***(9-11)*** at a substrate concentration of 1 µM cyclic AMP or cyclic GMP in presence of 15,000 cpm [^{3}H]-cAMP or [^{3}H]-cGMP as a tracer, respectively (**Fig. 1**). During the first incubation (30°C for 30 min) performed in a final volume of 250 µl, cyclic nucleotides are hydrolyzed into their respective nucleotide monophosphates. This incubation is stopped by addition of an excess of cAMP and cGMP in presence of EDTA and a non-selective PDE inhibitor (theophylline) which inactivates PDE activity. The second incubation performed in the presence of an excess of 5'-nucleotidase (snake venom) results in the formation of adenosine or guanosine. This second incubation is stopped by addition of an excess of adenosine and guanosine in presence of EDTA. The enzymatic reaction products are separated by anion-exchange column chromatography on QAE-sephadex A-25 formate ***(12).*** The amount of resulting [^{3}H]-nucleoside is determined by liquid scintillation.

3.2.1. Purification of radiolabeled cAMP or cGMP

[3H]-cAMP and [3H]-cGMP are purified by thin layer chromatography (TLC) on Silicagel 60 F_{254} TLC plates.

1 Along an horizontal line drawn 2 cm from the bottom of a TLC plate, 2 µl of 10 mM unlabelled cyclic nucleotides are applied as migration markers.

2 80 µl of [3H]-cAMP and [3H]-cGMP are separetely spotted by successive additions of 10 µl under drying air.

3 The TLC plate is then placed in a vertical developing tank containing the TLC chromatography solvent and developed for approximately 4 h, until the solvent front is 4 cm beneath the top of the plate. The plate are then dried.

4 Under an ultra violet light (254 nm), the positions of the cyclic nucleotide standards, as well as those of the labeled cyclic nucleotides, are located and surrounded with pencil. Using a razor blade, the corresponding region for each labeled cyclic nucleotide is scraped off onto aluminum paper and the silicagel powder is transferred into a 1.5 ml Eppendorf tube.

5 To extract the cyclic nucleotide, 1ml of 50% ethanol is added to the tube, which is thoroughly mixed and centrifuged to settle down the silicagel; this extraction is repeated a second time with 0.4 ml. The supernatants are then combined.

A sample (10 µl) of the purified labeled cyclic nucleotide is counted. The supernatant is adjusted with 50% ethanol to give 15,000 cpm/µl and stored at -20°C (see **Note 2**).

*3.2.2. QAE Sephadex A-25 formate anion-exchange resin **(12**)*

The use of anion-exchange resin to recover [^{3}H]-adenosine or [^{3}H]-guanosine is critical for the accuracy of the assay. This method contrary to that described by Thompson et al. ***(9),*** allows recovering and regenerating the resin that can then be used over a long period of time. QAE-Sephadex A-25 being expensive, the recycling is quite valuable.

3.2.2.1 Resin preparation and equilibration

1 250 g of QAE-Sephadex A-25 (in the chloride form) is allowed to swell in 5 l of Millipore water, overnight at 4°C.

2 Resin is converted through the OH^- form to the formate form, through steps 3 to 8 of the procedure described for resin regeneration.

3 Resin is stored at +4°C as a 50% slurry.

3.2.2.2. Resin regeneration

Used resin collected from the assay columns is stored at + 4°C and regenerated in a large glass column (35x8cm) with sintered-glass bottom by successive treatments:

1 0.1 N HCl until the pH of the effluent is 1-2;

2 water until the pH of the effluent is 5-6;

3 0.2 N NaOH until the effluent is Cl^- free, as checked with $AgNO_3$ under acid conditions;

4 water until the pH of the effluent is 6-7;

5 0.2 N formic acid until the pH of the effluent is about 2.5;

6 2 M ammonium formate;

7 water until the pH of the effluent is about 4;

8 suspend the resin in 100 mM ammonium formate and store at 4°C.

3.2.3. Procedure for the two-step PDE assay

3.2.3.1 Enzymatic reactions

Adding reactive constituents sequentially by 25 or 50 µl fractions constitutes the 250 µl final assay medium, allowing maximum flexibility and convenience for several assay purposes. During this procedure test tubes are kept in iced-water bath.

1 25 µl of Millipore water, solvent or drug (made up soluble with solvent such as DMSO. or ethanol) at 10X concentration to be tested.

2 25 µl of Millipore water or additional drug.

3 25 µl of Tris-Mg-Ca.

4 25 µl Mg-EGTA or Mg-CaM.

5 50 µl Tris-BSA.

6 50 µl tissue homogenate (PDE extract) diluted with Tris-BSA to give around 15% of the substrate hydrolyzed in control conditions **(**see **Note 3).**

7 Vortex the test tubes.

8 Enzymatic reaction is initiated by addition of 50 µl substrate solution (25 µl of 10 µM cAMP or cGMP + 24 µl Millipore water + 1 µl of purified [^{3}H]-cAMP or [^{3}H]-cGMP, 15 000 cpm), gently vortexed and incubated at 30°C.

9 The reaction is stopped by adding 25 µl of PDE-stop and after being vortexed test tubes are returned to the iced-water bath.

10 The nucleotidase reaction is initiated by adding 20 µl of SV; tubes are vortexed and incubated for 20 min at 30°C.

11 This reaction is stopped by adding 300 µl of SV-stop.

3.2.3.2. QAE-Sephadex formate anion-exchange column chromatography

This step allows the separation of the dephosphorylated products (adenosine or guanosine) from the non-hydrolyzed cyclic nucleotide ***(12)***.

1 For each test tube, a chromatography column is filled out with 2 ml of 50% resin slurry (3.2.2) in order to get 1ml of settled resin.

2 The columns are then equilibrated by ading 3 ml of formate solution.

3 By using a homemade rack (**Fig. 2**), each column is placed above a scintillation vial.

4 Test tubes are poured onto their corresponding columns.

5 Test tubes are rinsed with 1.5 ml formate solution and poured again onto columns.

6 7 ml of scintillation fluid are added in each vial; after having been capped, vials are vigorously vortexed, and then placed in β liquid scintillation counter.

7 Resin is recovered from columns and stored at +4°C until regeneration; columns are washed exhaustively with tap water.

3.2.4. Data expression

To determine PDE activity, 4 test tubes without PDE extract (50 µl Tris-BSA) are run in each assay procedure until step **3.2.3.1.11.** Then the 2 first tubes, used to determine the total radioactivity (R_t), are directly poured into scintillation vials and added with 1.5 ml formate; the 2 other tubes, used to determine blank assay (R_o), are treated similarly to the other test tubes.

The percentage of cyclic nucleotide phosphate (CNP) hydrolyzed is calculated as follows:

$$\% \text{ CNP hydrolyzed} = R_x\text{-}R_o / R_T\text{-}R_o$$

R_x = cpm of sample

R_o = cpm of blank assay

R_T = cpm of total radioactivity

The PDE activity of the sample is calculated as follows:

$$\text{PDE activity } (\text{pmol.min}^{-1}.\text{mg}^{-1}) = \frac{(\% \text{ CNP})(0.25\text{x}10^{-3})(1\text{x}10^{-6}\text{M})}{\text{tx}(0.050\ \text{C})}$$

t = incubation time of the first enzymatic reaction in min

C = protein concentration in mg/ml

3.3. Characterization of PDE family contribution

To determine the contribution of one PDE isozyme in total cAMP or cGMP hydrolytic activity in tissue, both total PDE hydrolytic activity and residual PDE activities in presence of specific inhibitors are determined in the same assay. A PDE isozyme family activity is obtained by determining the activity which is sensitive to its corresponding specific inhibitor (see **Table 1**).

Cyclic AMP-hydrolyzing PDE isozymes are determined with 1 µM cyclic AMP as substrate in the presence of 1 mM EGTA by using 10 µM rolipram for PDE4, 1 µM cilostamide for PDE3 (see **Note 4)**. The proportion of cyclic GMP-hydrolyzing PDE isozymes is determined with 1 µM cyclic GMP as substrate in presence of 1 mM EGTA by using 20 µM EHNA for PDE2, 10 µM nimodipine for PDE1, 1 µM cilostamide for PDE3 , 10 µM zaprinast (or 0.1 µM DMPPO or sildenafil) for PDE5 (see **Note 5**).

1 In the same assay, the solvent (for total PDE activity) and the various inhibitors are added in the assay as previously indicated (see step **3.2.3.1.1**).

2 The whole assay procedure must be run as stated in **heading 3**.

3 The PDE activity of one isozyme family (PDEx) is expressed as follows:

PDEx ($pmol.min^{-1}.mg^{-1}$) = PDE_T – PDEn; (see **Note 6**)

PDE_T = total PDE activity

PDEn = activity assessed in the presence of specific PDEx inhibitor

4. Notes

1. To study subcellular distribution of PDE isozymes (cytosolic and membrane-bound PDEs or associated to isolated subcellular structures), tissue powder must be homogenized in an isotonic buffer, i.e. Buffer A with 0.25 M sucrose. To study PDE isozymes in cells, fresh or frozen (stored at -80°C), cell pellets are homogenized in a minimal volume of 500 µl and sonicated 6x10 sec at +4°C.
2. Purified tritium labeled cAMP and cGMP can be stored for 2 months at -20°C. When blank values represent more than 1% of total radioactivity, labeled cyclic nucleotides must be purified again.

3. Product accumulation should be determined to be linear with time and with enzyme dilution under all conditions studied.

4. For technical convenience, in this protocol we have chosen to determine PDE2 participation by evaluating the effect of EHNA towards its cGMP activity. This may directly reflect PDE2 activity in its stimulated state. It should also be possible to determine the participation of PDE2 in total cAMP hydrolytic activity. In this case, since EHNA is only effective on cGMP-stimulated PDE2, it is necessary to perform the assay in presence of cilostamide (1 μM) + cGMP (5 μM) + EHNA (20 μM). This must be done under conditions where the final concentration of DMSO will not exceed 1%. The difference between the hydrolytic activity in presence of 1 μM cilostamide and the activity obtained in presence of the PDE inhibitor cocktail will give the cAMP-PDE activity for PDE2 in its activated state. Usually, PDE3 and PDE4 activities represent more than 80% of the total cAMP PDE activity. Residual cAMP-PDE activity could be assigned to PDE1, PDE2, PDE7, PDE8, PDE10 and PDE11. The participation of PDE1 activity in cAMP hydrolysis could be evaluated by using 10 μM nimodipine as previously reported (***24***), keeping in mind that 10 μM nimodipine is able to inhibit by 25% the purified PDE4 activity. Characterization and participation of PDE8 (an IBMX-insensitive isoform) can be evaluated by using 100 μM IBMX, the residual cAMP-PDE activity representing PDE8, since all other cAMP-hydrolyzing isozymes are fully inhibited by 100 μM IBMX .

5. Nimodipine (10 μM) is a dihydropyridine compound, well known as a calcium antagonist by its inhibitory effect on L-type calcium channel. This compound can be used on tissue and cell homogenates to determine PDE1 participation with no information relative to the basal or calmodulin-activated states, since it acts on both states with the same potency (***5, 13***). Vinpocetine, referred to as a specific PDE1 inhibitor, is unable to inhibit bovine vascular and rat cardiac PDE1 (***5***). Its inhibitory effect depends on the PDE1 subtype splice variant studied (IC_{50} from 10 to 100 μM; ***25***). Vinpocetine is also able to inhibit PDE7B with an IC_{50} of 60 μM (***26***). It was also shown

to be a direct activator of BK(Ca) channels (***27***). Another compound used as PDE1 inhibitor, 8-methoxymethyl IBMX, is a poor selective PDE1 inhibitor, since it inhibits in the same range of concentrations PDE1 and PDE5 (IC_{50} of 8 and 10 µM, respectively; ***28)***.

As we have previously reported, zaprinast (M&B 22,948), which was first described as a CaM-PDE inhibitor ***(5)***, is in fact more potent as a PDE5 inhibitor (cGMP-PDE; ***17,19)***. Zaprinast, which is commercially available, should be used at 10 µM to minimize its effect on PDE1. More specific and potent inhibitors such as DMPPO ***(20)*** and sildenafil ***(21)*** can now be obtained from pharmaceutical companies. They are more convenient and should be used at 0.1 µM to evaluate PDE5. Nevertheless, it must be noted that zaprinast, DMPPO and sildenafil potently inhibit PDE6, which is known to be mainly present in retina (***1-6***) and pineal gland (***29***). They also inhibit PDE9, PDE10 and PDE11 to a lesser degree (***6***). Development of specific inhibitors for PDE6 to PDE11, will allow further characterization, thus enhancing the knowledge of their functional role, as recently shown for a PDE7 inhibitor conceived by Bristol-Meyer-Squibb Company ***(22)***.

6. For additional characterization of PDE isozymes, cAMP-PDE and cGMP-PDE activities could be determined in presence of calcium/calmodulin to evaluate the contribution of PDE isozymes to CaM-activated PDE activities. In that case, 25 µl of Mg-CaM is added instead of Mg-EGTA in step **3.2.3.1.4.** Furthermore, the comparison of the whole PDE activity with and without calmodulin gives an index of PDE1 contribution.

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Table 1: Classification of PDE family.

PDE family	Substrate	Property	Specific inhibitors
PDE1	cAMP, cGMP	Ca-calmodulin-activated	Nimodipine ***(13, 14)***
PDE2	cAMP, cGMP	cGMP-activated,	EHNA ***(15)***
PDE3	cAMP, cGMP	cGMP-inhibited	Cilostamide (***16, 17***), milrinone ***(17, 18)***
PDE4	cAMP	cGMP-insensitive	Rolipram ***(16, 19)***, R0 20-1724 ***(16, 19)***
PDE5	cGMP	PKA/PKG-phosphorylated	Zaprinast ***(16, 19)***, DMPPO ***(20)*** Sildenafil ***(21)***
PDE6	cGMP	Transducin-activated	Zaprinast ***(6)***, DMPPO ***(20)*** Sildenafil ***(21)***
PDE7	cAMP	Rolipram-insensitive	BMS-586353 ***(22)***
PDE8	cAMP	IBMX-insensitive	
PDE9	cGMP	IBMX-insensitive	
PDE10	cAMP, cGMP		Papaverine ***(23)***
PDE11	cAMP, cGMP		

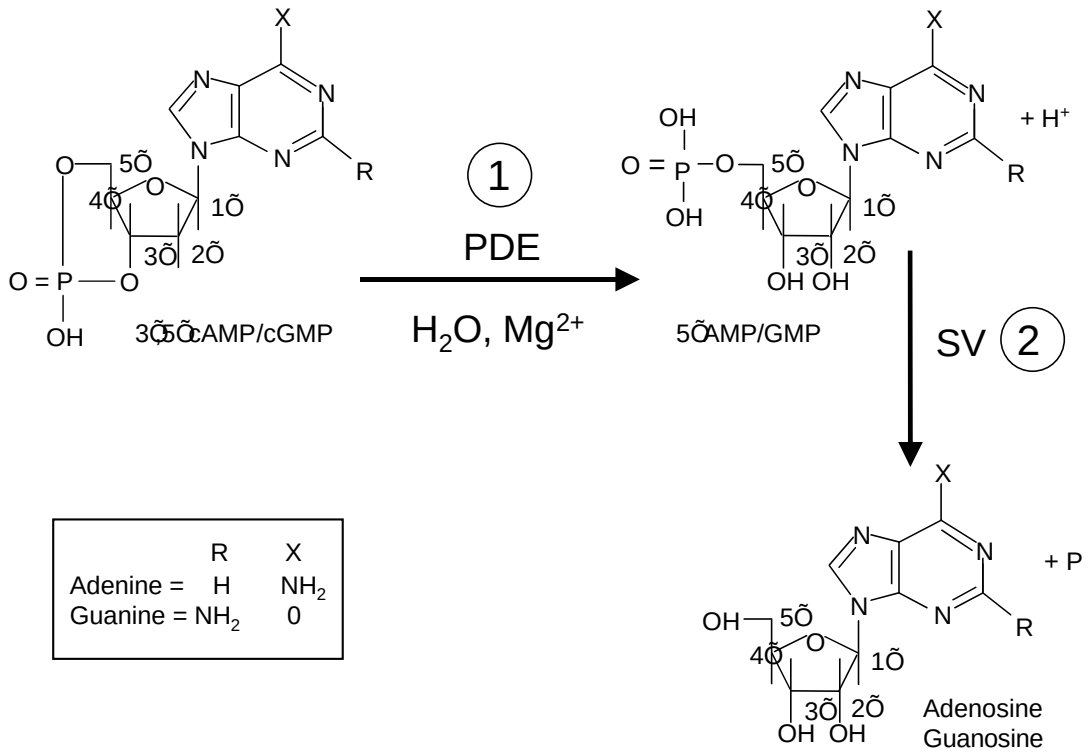

Fig. 1: Principle of the two-step PDE assay. In the first step, cyclic nucleotides are converted to 5' nucleotides by PDE. In the second step, an excess of 5' nucleotidase (snake venom) produces the corresponding nucleosides that are recovered by QAE-Sephadex A-25 column chromatography.

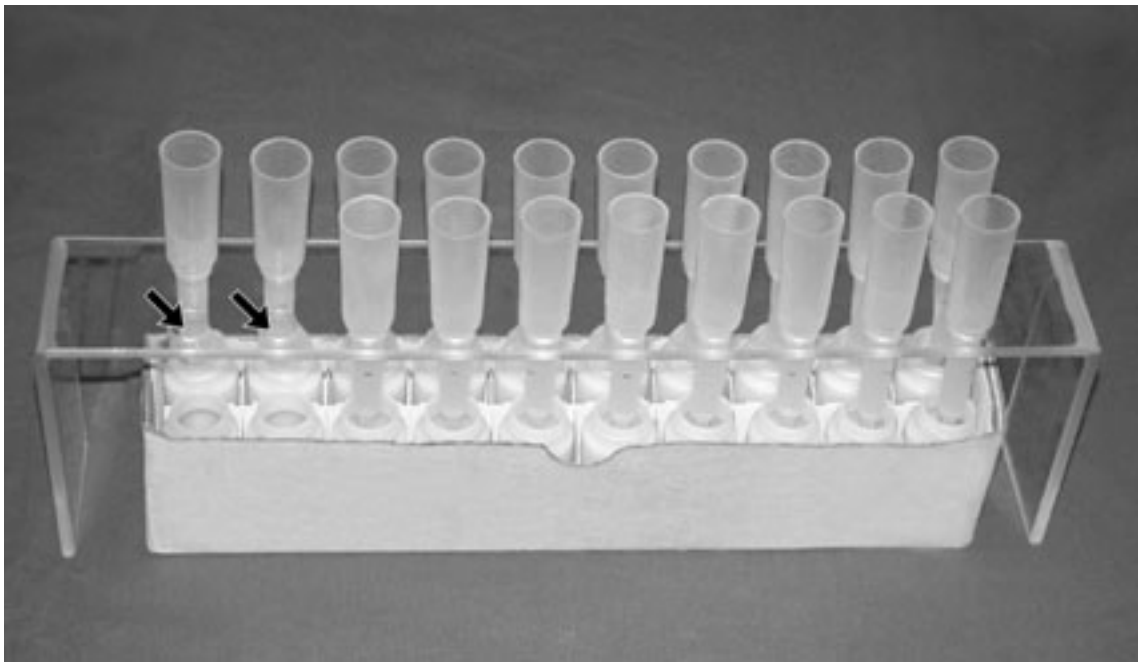

Fig. 2: Column chromatography with direct elution of each tube assay medium in scintillation vials. Note the absence of columns in the two first holes of the first raw of the column rack; the corresponding scintillation vials are used for the determination of total radioactivity.

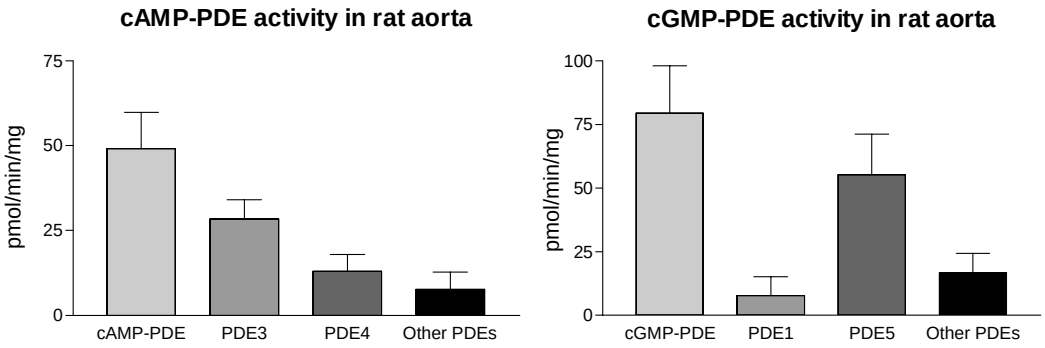

Fig. 3: Characterization of PDE isozyme activities in presence of EGTA in whole rat aorta (unpublished data). PDE1 and PDE5 activities were determined as cGMP-PDE activities sensitive to 10 µM nimodipine and 0.1 µM DMPPO, respectively. PDE3 and PDE4 activities were determined as cAMP-PDE activities sensitive to 1 µM cilostamide and 10 µM rolipram, respectively. The remaining cAMP-PDE activity (Other PDEs) is mainly due to PDE1 activity, which hydrolyses similarly cAMP and cGMP in its basal state (***13).*** The remaining cGMP-PDE activity (Other PDEs) is mainly due to PDE3.

Article IV

Alteration of cyclic nucleotide phosphodiesterase (PDE) activities and expressions in rats with liver cirrhosis

L'ensemble des résultats des deux articles (I et II) suggère une augmentation de l'activité PDE5 au niveau de l'aorte et du rein des rats cirrhotiques qui a pour conséquence une diminution de la réponse aux donneurs de NO et une rétention renale de sodium. En outre, des études récentes ont montré une augmentation de l'activite de la PDE4, et l'amélioration de l'excrétion rénale du sodium par l'inhibition spécifique de la PDE4 par le rolipram. L'ensemble de ces résultats suggère la présence de modifications au niveau de l'expression et de l'activite de la PDE5 dans la cirrhose aussi bien pour PDE4. L'objet de ce travail est d'étudier la relation entre les variations hémodynamiques liés à la cirrhose (suggérées dans les articles I, II) et les alterations d'activités enzymatiques et d'expression des différentes PDEs dans les différents tissus altérés au cours de la cirrhose: l'aorte, l'artère mésentérique, le rein et le cortex rénal. Pour cette étude La cirrhose est induite chez le rat par ligature du canal biliaire et excision, les dosages d'activite de PDEs à partir des homogénats tissulaires sont mesurés par méthode radioenzymatiques (article III) et la quantification des extraits de protéines est faites par électrophorèse et par immuno empreintes.

Notre étude contrairement aux résultats décrits dans la littérature, montre qu'il n'y a pas de modification significative de l'activité et de l'expression de la PDE5 rénale, mais par contre il y a une augmentation de l'activité et de l'expression de la PDE1 qui contribue 3 fois plus que la PDE5 à l'hydrolyse du GMPc dans le rein. Cependant, la cirrhose étant une maladie progressive, une implication de la PDE5 pourrait être impliquée aux premiers stades, alors que la PDE1 pourrait intervenir aux stades plus avancés.

Au niveau de l'aorte, on a suggère une diminution de sensibilité à l'inhibiteur de PDE5 due a une augmentation de son activité chez le rat cirrhotique. Cette diminution de sensibilité ne peut être attribuée à une augmentation d'expression de la PDE5, mais par contre peut s'expliquer par l'augmentation de l'activité de PDE3 associée à une augmentation de son expression démontrée dans cette étude. En effet, le DMPPO en inhibant la PDE5, qui représente plus de 50% de l'activité hydrolytique du GMPc dans l'aorte, pourrait augmenter le GMPc intracellulaire et de ce fait inhiber la PDE3. Dans la mesure où l'activité de la PDE3 est fortement augmentée dans l'aorte des rats

cirrhotiques, l'effet inhibiteur du GMPc, résultant de l'inhibition de la PDE5 inchangée, est moindre sur l'activité de la PDE3 engendrant un relâchement moindre. De plus cette étude, qui met en évidence d'autres modifications enzymatiques chez le rat cirrhotique au niveau du rein (augmentation de l'activité de la PDE1 hydrolysant le GMPc et de la PDE4 hydrolysant l'AMPc) et de l'artère mésentérique (augmentation de l'activité des PDE2 et PDE5 pour l'hydrolyse du GMPc, augmentation de l'activité des PDE3 et PDE4 pour l'hydrolyse de l'AMPc), fait ressortir des régulations complexes au niveau des PDEs qui peuvent impliquer des modifications transcriptionnelles et/ou post-transcriptionnelles. Ces nouvelles régulations nécessitent des recherches permettant de préciser l'implication des différents territoires dans la cirrhose, afin de préciser la nature des PDEs pouvant être des cibles potentielles dans la cirrhose. L'augmentation de PDE3 au niveau de l'aorte chez le rat cirrhotique indique qu'il serait plus judicieux d'inhiber directement la PDE3 surexprimée dans l'aorte. Ces résultats sont innovants puisqu'ils mettent à jour des implications de différentes PDEs dans la cirrhose et permettent d'envisager certaines PDEs comme nouvelles cibles thérapeutiques envers la cirrhose.

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Title

Alteration of cyclic nucleotide phosphodiesterase (PDE) activities and expressions in rats with liver cirrhosis

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Abbreviated Title

Alteration of PDE isozymes in the pathology of liver cirrhosis

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List of abbreviations:

PDE: cyclic nucleotide phosphodiesterase; BDL: bile duct ligation; ANP: atrial natriuretic peptide; IMCD: inner medullary collecting duct; VSMC: vascular smooth muscle cell;

ABSTRACT

Background. Liver cirrhosis is complicated by splanchnic vasodilation that contributes to sodium and water retention by the kidney. This renal dysfunction is manifested by the resistance to the natriuretic action of atrial natriuretic peptide (ANP). It was reported that an increase in the activity of cyclic nucleotide phosphodiesterase (PDE) specific of cGMP (PDE5) as well as an increase in cAMP-PDE contribute to the renal resistance to ANP in the rat liver cirrhosis model induced by common bile duct ligation (BDL). Therefore, this study examines the alteration of the different PDE families in kidney and vascular tissues (aorta and mesenteric arteries) in BDL model. Identifying the expression of the different PDE isozymes and the changes in PDE activity and expression in these tissues in liver cirrhosis, will increase our understanding of the pathological processes.

Methods. Rats underwent common bile duct ligation and excision (BDL) and sham operation (SO). Tissues were removed at 4 weeks after bile duct ligation, a delay necessary for the development of secondary biliary cirrhosis. PDE activities from tissue homogenates were measured by radioenzymatic assay and PDE amounts were quantified by Western blotting analysis.

Results. Analysis of PDE activities and PDE expressions in homogenates from the different tissues of SO and BDL rats shows: i) an increase in total cGMP-PDE specific activity in BDL rat kidney which is largely due to an increase (+ 83%) in PDE1 isozyme activity ; this increase is specifically accounted by the increased level of the 72 kDa-PDE1A; ii) in aorta, there is evidence for an increase in total cAMP-PDE specific activity in BDL rats, this increase is largely due to an increase (+93%) in PDE3 activity and it correlates with an increase in protein level of the two PDE3 variants, the 110 kDa-PDE3A and the 72 kDa-PDE3B; iii) in mesenteric arteries, PDE3 and PDE4 represent respectively 68.9% and 21% of the total cAMP-PDE activity, PDE1 and PDE5 represent respectively 35% and 54% of the total cGMP-PDE activity ; an increase in PDE3 (+23%) and PDE4 (96%) activities is observed in the mesenteric arteries of BDL rats, and is accompanied with the induction of PDE3A (84 kDa) and an increase in the PDE4D expression (71kDa); PDE2 and PDE5 activities are increased by 50% but no modification in the protein level of cGMP-PDE isozymes is observed.

Conclusion. This study demonstrates an increase in PDE1 activity and PDEA1 expression in kidneys from BDL rats which mainly participate in the increase of cGMP-PDE activity, but no modification of PDE5 as previously shown (Angeli et al., 2000; Ni et al., 2001) and a significant increase of cAMP-PDE related to PDE4. Furthermore no significant changes was observed in renal cortex indicating that major changes in PDE activity could be localized in medulla. In the aorta of

BDL rats, this study shows an increase in PDE3 activity and PDE3A and PDE3B expressions in this tissue, which may explain our previous finding showing an altered response to PDE5 inhibitor (Tahseldar-Romié et al., 2005) and suggests an important role of PDE3 isozymes in the regulation of smooth muscle tone. In conclusion, this study shows different regulation of PDEs in a specific tissue, which can involve transcriptional and/or post-transcriptional modifications. In the mesenteric arteries PDE2 to PDE5 activities are mainly increased in cirrhotic rats in order to overcome splanchnic vasodilation. Taken together, these results demonstrate that PDEs are widely and differently implicated in liver cirrhosis and thus specific PDE inhibitors may be useful for precising new potential targets for liver cirrhosis.

INTRODUCTION

Cyclic nucleotides (cAMP and cGMP) are important second messengers of intracellular signaling pathways by regulating many physiological processes (Francis et al., 2001, Mehats et al., 2001; Soderling et al., 2000). The cellular content and biological effects of cAMP and cGMP are regulated by their catabolyzing enzymes, the cyclic nucleotide phosphodiesterases (PDEs) (Beavo, 1995). PDEs are responsible for degrading cAMP and cGMP to their non cyclic nucleotides, 5'-AMP and 5'-GMP respectively. In mammals, eleven PDEs families are currently known (PDE1-PDE11); most of them consist of several isozymes and numerous PDE isozyme-splice variants. A comparison of sequences shows that all PDEs share a structural feature of a catalytic core, and they differ in substrate affinity (cAMP and/or cGMP), kinetic properties, specific inhibitor sensitivity and allosteric regulators, as well as differential expression in various cell types (Beavo & Reifsnyder., 1990; Conti and Jin., 1999; Francis et al., 2001; Lugnier, 2005).

Current evidence indicates that PDEs play diverse roles in many pathobiologic processes, especially in cardiovascular diseases (Stoclet et al., 1995; Takimoto et al., 2005), erectile dysfunction (Corbin, 2004), and lately in angiogenesis (Favot et al., 2003, 2004; Netherton & Maurice., 2005). Furthermore, the pathology of liver cirrhosis that is characterized by a hyperdynamic circulation with peripheral and splanchnic vasodilation (Shrier et al., 1988) is associated with the development of sodium retention by the kidney. Since the kidney is a key component of body fluid homeostasis, altered hormone action of pathological states accounts for numerous aspects of renal function abnormalities. Renal dysfunction, such as water and sodium retention leading to ascites formation, is a critical finding in advanced liver cirrhosis, and is manifested by the renal resistance to ANP (Levy et al., 1997). In liver cirrhosis produced by bile duct ligation (BDL), increased of cGMP-PDE activity is an important mediator of renal resistance to ANP (Lee & Humphreys., 1996). Such increased enzyme activity rapidly hydrolyses the second messenger cGMP, normally formed when ANP binds to its active receptors, and thereby leading to blunted ANP responsiveness. Zaprinast (1mM), a PDE5 inhibitor (K_i= 0.3 µM), which also inhibits PDE1 at higher concentration (K_i= 11 µM, Lugnier et al., 1986, Lugnier et Komas, 1993) was found to increase the ability of sodium excretion in response to ANP both *in vivo* and *in vitro* (Valentin et al., 1996; Ni et al., 1996 and 2001). Recently, we showed that DMPPO, a more potent and selective PDE5 inhibitor (PDE5 K_i = 3 nM, PDE1 IC_{50} = 1 µM ; Coste & Grondin, 1995) improves sodium excretion by the kidney in BDL and not in SO rats suggesting a possible alteration of PDE5 activity in vascular tissue related to alteration of vascular smooth muscle sensitivity (Tahseldar-Roumié et al., 2005). Furthermore,

after 7 to 10 days of BDL, there was an evidence for an increase in PDE5 activity in rat renal inner medullary collecting duct (IMCD) cells and not in renal cortex, which mediated renal resistance to ANP. This increase in PDE5 activity in ICMD was related directly to an increase in the enzyme protein level (Ni et al., 2001).

On the other hand, the pathogenesis contributor for sodium and water retention by kidney is an arterial vasodilation especially in the splanchnic vasculature (Shrier et al., 1988). Much evidence has been provided to support a role of an overproduction of nitric oxide (NO) in the genesis of splanchnic vasodilation (Wiest et al., 1999; Wiest & Groszmann, 2002). Lately, in the aorta as a model of the vascular system, functional studies suggested a possible increase in PDE5 activity, which may be a primary event of liver cirrhosis and results in a reduced response to NO donors (Tahseldar-Roumié et al., 2005). Furthermore, in BDL rats it was shown an increase in renal cAMP-PDE (Brønd et al., 2003; Ahloulay et al., 2005) and an improvement of sodium excretion by rolipram (a specific PDE4 inhibitor, Lugnier et al., 1986) treatment in BDL with cirrhosis and ascites (Ahloulay et al., 2005).

The finding of the alteration of activity and protein expression of PDE5 (Ni et al., 2001) as well as the change in cAMP-PDE activity (Brønd et al., 2003; Ahloulay et al., 2005) in the kidney of liver cirrhosis, lead us to investigate: 1) the relative contribution of PDE isozymes in total PDE activity toward cGMP as well as cAMP in normal tissues and 2) the eventual modifications in activity and protein expression that could occur to the major different PDE isozymes in kidney, renal cortex, aorta and mesenteric arteries of cirrhotic rats four weeks after BDL. It is important to define which PDE isozymes are altered and whether such alteration could result from either a greater amount of PDE protein (transcriptional) or a change in PDE activity, or a combination of these two. This report will present how targeting PDE expression is of importance for a better understanding of the altered physiology in cirrhosis.

MATERIALS AND METHODS

Drugs and Chemicals

PDE inhibitors were obtained as follows: nimodipine (specific for PDE1) was from Bayer, Berlin, Germany; erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA; specific for PDE2) was from Sigma; cilostamide (specific for PDE3) was synthetized by Dr C. Lugnier (1985). Rolipram was from Schering, Berlin, Germany; DMPPO (specific for PDE5) was a kind gift from Dr P. Grondin, GlaxoSmithKline, France). For PDE activity, all these compounds were dissolved in DMSO, and the final DMSO concentration in assay (1%) did not affect significantly PDE activity. All the other reagents for PDE assay were from Sigma.

Primary polyclonal antibodies were obtained as follows: anti-PDE1 from Chemicon (AB1653) anti-PDE1s subfamilies from FabGenix (PDE1A, #PD1A-101AP; PDE1B, #PD1B-201AP; PDE1C, #PD1C-301AP); Fabgenix anti-PDE2 (C-terminal); anti-PDE3A (targeting the regulatory domain 424-460) and anti-PDE3B (targeting the N-terminal domain 2-20) were a kind gift from Dr. V. Manganiello (NIH, USA); anti-PDE4s from FabGennix (4A, 4C and 4D); anti-PDE4B (K118) was a kind gift of Dr. M. Conti's laboratory (Standford University, Standford, USA); anti-PDE5 from Cell Signaling (#4072); anti-PDE5 from Fabgenix (PD5A-101AP) ; anti-PDE5 from Corbin, kind gift from Dr. J. Corbin (Vanderbilt University, USA).

Primary monoclonal antibodies were obtained as follows: anti β-actin (#A-5441) from Sigma and anti-GAPDH (#MAB374) from Chemicon. Anti-rabbit (#W4011) and anti-mouse (#W4021) HRP-conjugates secondaries antibodies were from Promega. The molecular weight markers were from Bio-Rad: Prestained, broad range Precision Protein Standards. The ECL assay kit was from Amersham. Autoradiographic films (Kodak Biomax light-1) were obtained from Sigma.

Preparation of animals

Sprague Dawley rats weighing 250-300 g underwent either sham operation (SO) or bile duct ligation (BDL), as described (Tahseldar-Roumié et al, 2005). BDL is achieved after a midline incision through whereby the common bile duct is identified, a double suture is placed around it and the duct is excised between the sutures. This procedure results in cirrhosis within 4-6 weeks. SO involves the identification of the common bile duct but without application of excision. The abdomen is double sutured at the end of the procedures. BDL and SO rats were sacrificed at 4

weeks. Whole aorta, whole mesenteric arteries, renal cortex and kidney were isolated, cleaned off adjacent tissues, immediately frozen in liquid N_2 and stored at –80 ^{o}C.

PDE activity assay

Frozen tissues were ground in liquid N_2. The resulting tissue-powders were homogenized with a glass-glass potter for 3x10 s at 4 ^{o}C, as 100 mg/ml of the following buffer: 2 mM Mg acetate, 5 mM EGTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soya trypsin inhibitor, 2000 U/ml aprotinin, 0.33 mM Pefabloc, 20 mM Tris, pH 7.5. The homogenates were divided into small aliquots and stored at –80 ^{o}C until used. Protein concentration was determined following Lowry (Lowry et al., 1951).

Total PDE activity was determined at 1 μM cAMP or cGMP in presence of 1 mM EGTA by a radioenzymatic assay, as previously described (Keravis et al., 1980). To determine the distinct PDE isozyme contribution in the overall cyclic nucleotide hydrolyzing activity, PDE family-specific inhibitors were included in the assay medium, as described elsewhere (Keravis et al, 2005). The proportion of cGMP-hydrolyzing PDE isozymes in the homogenates was assessed at 1 μM cGMP in presence of 10 μM nimodipine for PDE1, in the presence of 0.1 μM DMPPO for PDE5, and 20 μM EHNA for PDE2. The proportion of cAMP-hydrolyzing PDE isozymes was assessed at 1 μM cAMP in presence of 1 mM EGTA, including 1 μM cilostamide for PDE3 and 10 μM rolipram for PDE4.

Western blot anlysis

Frozen tissues were ground in liquid N_2. The resulting tissue-powders were homogenized with a glass-glass potter for 3x10 s at 4 ^{o}C in the following buffer: 250 mM NaCl, 5 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatinA, 1mM Pefabloc, 1% SDS, 25 mM Tris, pH 7.5. The homogenate was centrifuged at 12,000 *g* for 10 min at 4^{o}C and aliquots of supernatants were stored at – 80^{o}C until used. Protein concentration was determined following Lowry (Lowry et al., 1951).

Protein samples (30 μg for cortex and kidney and 50 μg for aorta and mesenteric arteries) were denatured and solubilized by heating for 5 min at 95 ^{o}C in Laemmli buffer, electrophoresed on SDS-8% polyacrylamide gel and electro-transferred onto PVDF membranes, as described previously (ref: Lugnier et al, BBA 1999). Membranes were processed for immunoblotting with the primaries antibodies anti-PDE1 (Fabgennix 1A, 1B, 1C; 1/1000 dilution), anti-PDE2 C-terminal (1/1000), anti-PDE3A (1/400), anti-PDE3B (1/400), anti-PDE4A (Fabgennix;1/2500), anti-PDE4B

(Conti-K118; 1/2000), anti-PDE4C (FabGennix; 1/2500), anti-PDE4D (FabGennix; 1/2500), and anti-PDE5 (Cell Signaling; 1/2500). The immobilized antigens were detected by chemiluminescence using HRP-conjugates as secondary antibodies (1/60,000), the ECL assay kit and autoradiography films.

The amounts of protein in each lane were checked by re-probing the membranes with an antibody directed against a house-keeping protein, shown not to be affected by the BDL treatment: β-actin for aortic and mesenteric artery samples and GAPDH for kidney and renal cortex samples.

Densitometric analysis

Autoradiography signals were captured on a GeneGenius Bio Imaging System (Syngene) using the GeneSnap soft wear and analyzed using the GeneTool soft wear. Relative values for PDE signals in BDL rats were calculated as a percentage of SO rats, corrected for the density of the β-actin or GAPDH bands.

Statistical analysis

Results are expressed as mean ± SEM of n experiments. Student's *t* test for unpaired data was used for statistical analysis, with $P < 0.05$ being considered significant.

RESULTS

Renal cortex

Analysis of homogenate PDE activity in the renal cortex of SO rats (Fig. 1A and 1B) shows that: (i) the total cAMP-PDE activity (Fig. 1B) is about 3-fold higher than the total cGMP-PDE activity (Fig. 1A), with values of 316 and 123 pmol/mg/min, respectively; (ii) the total cAMP-PDE specific activity is mainly due to PDE4 (62.2 %) and to a lesser extend to PDE3 (24 %) and to other PDEs (13.8 %), which might represent essentially PDE2 since it hydrolyses in the same range both cAMP and cGMP, nevertheless it might also include to a lesser extend PDE1, PDE7 and PDE8 as well as PDE10 and PDE11; (iii) the cGMP-PDE specific activity is mainly due to PDE2 (51.5 %) and PDE1 (32.1 %), and to a lesser extend to PDE5 (10.6 %) and others PDEs (5.8 %) which could include PDE9-PDE11.

The investigation of the expression of the different PDE families in the renal cortex of SO reveals also some subfamilies (Table 1). PDE1 was not done. PDE2 is expressed as a 74 kDa protein which could be a PDE2A1. The two subfamilies of PDE3 are expressed: PDE3A (~106 kDa) and PDE3B (~161 and 62 kDa). The four subtypes of PDE4 are expressed: PDE4A (~130 and ~47 kDa), PDE4B (~82 kDa), PDE4C (~75 kDa), and PDE4D (~74 kDa). PDE5 is expressed as a ~98 kDa protein (Table 1).

Cirrhosis does not induce any significant changes in the total and specific cGMP- (Fig. 1C) or cAMP- (Fig.1D) PDE activities. Except for PDE3 specific activity (-52 %; P = 0.06), the changes induced were small and not significant decreases. However, cirrhosis induces a significant decrease in PDE3 expression (Fig. 2): the 106 kDa-PDE3A and the 62 kDa-PDE3B were respectively reduced by 41 and 38 %, the 161 kDa-PDE3B disappears, and the expression of other studied PDEs was not modified.

Renal cortex is characterized by a higher cAMP-PDE than cGMP-PDE activity, with a predominance of PDE4 and PDE2 to hydrolyse cAMP and cGMP, respectively.

Although cirrhosis does not modify significantly any PDE activity, it induces a significant reduced expression of PDE3, which might correlate with the decrease in PDE3 activity.

Kidney

Analysis of homogenate PDE activities in the kidney of SO shows that : (i) the total cAMP-PDE activity is not significantly different from the total cGMP PDE activity, (Fig. 3A and 3B) ; (ii) the total cAMP-PDE specific activity is mainly due to PDE4 (51.5 %) and also to PDE3 (20.9 %) and other PDEs (27.6 %), which might essentially represent PDE2; (iii) the cGMP-PDE specific activity is mainly and similarly due to PDE1 (37 %) and PDE2 (43 %), and to a lesser extend to PDE5 (12.2 %) and others PDEs (7.8 %) which could include PDE9-PDE11.

Table 1 shows that the first five PDEs and their subtypes are expressed in the kidney of SO rats, PDE4C excepted.

Liver cirrhosis induces a significant increase in the total cGMP-PDE activity (+26 %) (Fig. 3C) and cAMP-PDE activities (+47 %) (Fig.3D).The increase in cGMP activity is reflected by a significant increase of PDE1 activity (+83 %). The increase in cAMP-PDE activity is credited to a significant increase of PDE4 activity (+76 %). While PDE1A (~78 kDa), PDE1B (~74 kDa) and PDE1C (~72 kDa) subtypes were expressed in SO, an induction of PDE1A 72k DA was seen in the kidney of BDL with no change for PDE1A, PDE1B and PDE1C.

Kidney is characterized by similar levels of cAMP-PDE and cGMP-PDE activities, and a predominance of PDE4 and PDE1 to hydrolyse cAMP and cGMP, respectively. PDE2, which hydrolyses both cAMP and cGMP, is as effective as PDE1 in hydrolyzing cGMP but is not affected in BDL rats.

Cirrhosis increases significantly both cAMP-PDE activity through the increase in PDE4 activity and cGMP-PDE activity through the increase in PDE1 activity, and it induces the expression of a 72 kDa-PDE1A.

Aorta

Analysis of homogenate PDE activity in the whole aorta of SO shows that: (i) the total cGMP-PDE activity is 1.6-fold higher ($P = 0.002$) than the total cAMP PDE activity (Fig. 5A and 5B); (ii) the total cAMP-PDE specific activity is mainly due to PDE3 (58 %) and to a lesser extend to PDE4 (26.5 %) and other PDEs (15.5 %), which might represent PDE2, PDE7, PDE8 PDE10 and PDE11; (iii) the cGMP-PDE specific activity is mainly due to PDE5 (69.6%) and to a lesser extend to PDE1 (9.6 %), PDE2 (14 %), and others PDEs (6.8 %) which could include PDE7 and PDE8 as well as PDE10 and PDE11.

Table 1 shows that PDE3A and PDE3B, many variants of PDE4 subtypes, and 2 variant of PDE5 are expressed in the whole aorta of SO rats, furthermore phosphorylated 104 kDa PDE4A was characterized.

While cirrhosis does not induce any significant changes in cGMP-PDE activity (Fig. 5C) with the exception of PDE2 activity (significant decrease of –30 %, but PDE2 represents only 14% of the total cGMP-PDE activity), it increases significantly the total cAMP-PDE activity (+71%) reflected by an increase of PDE3 (+93 %) and PDE4 (+73 %) activities (Fig. 5D). This increase in PDE3 activity is accompanied with an increased expression of the 110 kDa-PDE3A (Fig. 6A) and the 72 kDa-PDE3B (Fig. 6B), respectively +200 % and +300 %.

Aorta is characterized by a higher level of cGMP-PDE activity and a predominance of PDE3 and PDE5 to hydrolyse cAMP and cGMP respectively.
Cirrhosis increases significantly only the cAMP-PDE activity through the increase in PDE3 and PDE4 activities, and it increases the expression of a 110 kDa-PD3A and a 72 kDa-PDE3B.

Mesenteric arteries

Analysis of homogenate PDE activities in the whole mesenteric arteries of SO shows that : (i) the total cAMP- (Fig ; 7B) and cGMP- (fig. 7A) PDE activities are similar ; (ii) the total cAMP-PDE specific activity is mainly due to PDE3 (68.9 %) and to a lesser extend to PDE 4 (21.1 %) and other PDEs (10 %), which might represent essentially PDE2; (iii) the cGMP-PDE specific activity is mainly due to PDE5 (54.1 %) and PDE1 (35.4 %), and to a lesser extend to PDE2 (16.9 %).
Table 1 shows that the PDE3 to PDE5 s are expressed in the whole mesenteric arteries of SO rats, that all subtypes of PDE4 are present and that phosphorylated 105 kDa-PDE4A is also characterized.

Cirrhosis shows a significant increase in the total cAMP-PDE specific activity (+29.1 %), reflected by an increase of PDE3 (+23 %) and PDE4 (+96 %) activities (Fig. 7D). While cirrhosis does not show any significant modification in the total cGMP-PDE activity, PDE2 and PDE5 are nevertheless significantly increased by 50 % (Fig. 7C). The increase in PDE5 activity was not accompanied by an increased expression of the 101 kDa-PDE5 (not shown). While the expression of the 111 kDa-PDE3A and 96 kDa-PDE3B were not modified in BDL rats, cirrhosis induces an 84

kDa-PDE3B (Fig. 8A). The increase in PDE4 activity in cirrhosis is accompanied with an increased expression (+68 %) of the 71 kDa-PDE4D (Fig. 8B).

Mesenteric arteries are characterized by similar levels of cAMP-PDE and cGMP-PDE activities and a predominance of PDE3 and PDE5 to hydrolyse cAMP and cGMP, respectively.
Cirrhosis increases significantly the cAMP-PDE activity through the increase in PDE3 and PDE4 activities, and it induces the expression of an 84 kDa-PDE3B and it increases a 71 kDa-PDE4D. Cirrhosis increases significantly PDE2 and PDE5 activities, with no change in the PDE5 expression level and no significant change in the overall cGMP-PDE activity.

Discussion

cGMP - phosphodiesterase had been investigated to be a potential target for the blunted renal response to atrial natriuretic peptide (ANP) in experimental liver cirrhosis and nephrotic syndrome by using zaprinast (Lee & Humphreys.,1996; Angeli et al., 2000; Valentin et al., 1992). Increased PDE5 activity and expression in renal inner medullary collecting duct (IMCD) cells contributes to resistance to atrial natriuretic peptide (ANP), which lead to the excessive sodium retention seen in experimental liver cirrhosis, in nephrotic syndrome, and in pregnancy (Ni et al., 2001, Ni et al., 2004). Moreover, we have found in previous studies that DMPPO, a selective and potent PDE5 inhibitor, increased Na excretion in the kidney of BDL and not in SO rats, and restore vascular sensitivity to NO donor (Tahseldar-Roumié et al., 2005). Sildenafil a similarly potent PDE5 inhibitor accounted for this investigation by preventing the decrease in renal Na excretion (Ghali et al., 2005 submitted). In another way, an increase in cAMP-PDE was also implicated in BLD cirrhotic rats (Brønd et al., 2003, Ahloulay et al., 2005) questioning about the idendity and the participation of the various PDE subtypes in cirrhosis.

Therefore for the first time, we analyzed globally in different tissues the changes induced by cirrhosis of various PDEs isozymes. In this study, after characterizing activity, proportions and protein expresions of PDE subtypes in renal cortex, whole kidney, whole aorta and whole meseteric arteries, we characterize first the PDE alterations at two levels, isozyme activities and isozyme expressions, in these tissues of liver cirrhosis, four weeks after bile duct ligation.

In the SO rats, our investigations showed that the activities of cAMP-PDE are higher than of cGMP-PDE in the kidney and renal cortex, similar in rat mesenteric artery and lower in rat aorta.

Most information about renal cortex PDEs have showed higher cAMP-PDE activity in the cortical tubular segments (Torres. et al., 1978; Jackson et al., 1980; Kusano et al., 1982), whereas cGMP-PDE activity is higher than cAMP-PDE activity in the cortical medullary collecting ducts and in glomeruli (Jackson et al., 1980; Torres. et al., 1978). In the renal vasculature, type 4 phosphodiesterase has been shown to be the predominant cAMP-phosphodiesterase isozyme (Jackson et al., 1997), and another study has demonstrated that in all nephron segments cAMP-PDE activities were detected and PDE4 was responsible for cAMP control in the proximal tubule as well as in the medullary collecting duct (Yoshida et al., 1996). Our findings using the renal cortex and the whole kidney homogenates clearly show that cAMP-PDE activity is higher than cGMP-PDE activity in rat renal cortex (2.5 fold) and similar in the whole kidney, furthermore in both tissues cAMP-PDE activity is mainly attributed to PDE4 activity (more than 50 %). Thus, our findings

strenghten suggestions that the rat kidney exhibits higher cAMP-PDE activity than cGMP-PDE activity (Yoshida et al., 1996; Jackson et al., 1997). We also show that cGMP-PDE activity, mainly related to PDE1 and PDE2 is higher (1.6 fold) in whole kidney than in renal cortex suggesting a specific highest level of cGMP-PDE activity in medulla, in agreement with the higher cGMP-PDE activity reported in cortical medullary collecting ducts and in glomeruli (Jackson et al., 1980; Torres. et al., 1978). The higher level of PDE5 activity encountered in whole kidney (2 fold) in comparison with renal cortex might result from a highest PDE5 activity in the medulla. Nevertheless, the first published studies (Valentin et al., 1996; Ni et al., 1996) using zaprinast at high concentration (1 mM) to estimate PDE5 activities in renal tissues were unable to discriminate PDE5 from PDE1 activities since at the used concentration both PDEs are fully inactivated by zaprinast (Lugnier and Komas, 1993). The analysis of possible contribution of PDE1 and PDE2 activities relatively to PDE5 activities in kidney cGMP-PDE activity clearly demonstrates that PDE1 and PDE2 activities represent at least 80% of cGMP-PDE activity, revealing a low PDE5 contribution to cGMP-PDE activity.

In the pathology of liver cirrhosis induced by BDL cirrhosis, our results revealed in the whole kidney that 4 weeks cirrhosis induced at the transcriptional level the expression of PDE1A isoform, associated with an increase of PDE1 activity. This increase in PDE1 is in accordance with the increase of PDE1 in inner medullary collecting duct cells (Yamaki et al., 1992). The present increases in PDE1 activity and expression apparently did not match with previous investigations focused on PDE5 implication (Ni et al., 2001) using either DMPPO or zaprinast and PDE5 antibody showing changes in the PDE5 isozyme activity and expression in the IMCD 7 days after bile duct ligation. According to our data, PDE5 activity is far less representative than PDE1 and PDE2 contributions to total cGMP-PDE in kidney, suggesting that PDE1 upregulation could play a greater role than PDE5 in cGMP regulation. Furthermore, since the liver cirrhosis pathology progresses, the pathophysiogical complications become more manifest with activation of the vasoconstrictor renin-angiotensin-aldosterone system, which induced the renal sodium and water retaining. Thus, these results could enhance an implication of PDE5 at earlier stages of the disease whereas at the later stages the PDE1 could be upregulated. Moreover, studies of cultured rat mesangial cells (MC) suggested that the angiotensin II, which is increased in sodium retaining states, may activate PDE1 by increasing intracellular Ca^{2+} and may contribute to increased hydrolysis of ANP-stimulated cGMP pool in situ (Haneda et al., 1991). These finding accounted our results, since it is established that the plasma level of angiotensin II is increased in liver cirrhosis (Schroeder et al., 1970; Laffi et al., 1984), which may activate the PDE1A1 isozyme consequently to the increase in intracellular

Ca^{2+} and that PDE1A1 expression is associated with the development of nitrate tolerance. (Kim et al., 2001).

In the renal cortex, at a transcriptional level, our results showed decreases in the expression of 106 kDa-PDE3A and 161- and 62 kDa-PDE3B isozymes, and at a post-transcriptional level, there was a decrease in the PDE3 specific activity (-52%) that was not significant, however a two fold decrease in its relative contribution was significant. Information on PDEs in tubules from cortex showed low PDE3 activity (Dousa, 1999) and no previous study showed an implication of PDE3 in cirrhosis. This decrease in PDE3 in the renal cortex might allow more cAMP accumulation as a compensatory mechanism for the increase vasoconstrictor system of renin–angiotensin-aldosterone associated to liver cirrhosis resulting in the renal vasoconstriction (Bernardi et al., 1993).

In the whole aorta, the five PDE isozymes (PDE1 to PDE5) are differently distributed in the vascular tissues. PDE3 and PDE4 isozymes, which have been characterized in vascular smooth muscle cells (VSMC), play a major role in vasoconstriction (Lugnier & coll, 1986; Komas et al, 1991; Lugnier et Komas 1993,). PDE2 which is absent in VSMC from aorta (Lugnier et al , 1986, Maurice et al., 2003, Orallo et al., 2004) is present in endothelial cells (Lugnier and Schini, 1990, Favot et al., 2003 and 2004), regulates with PDE4 endothelial NO production (Kessler & Lugnier, 1995) and controles vasodilatation (Eckly-Michel et al., 1997). The role of PDE5 in regulating vascular tone has been extensively studied showing that the PDE5 inhibitors induced vasorelaxation and increases cGMP level (Lugnier et al.; 1986, Schoeffter et 1987, Komas et al., 1991, Pauvert et al., 2003). Western blots reveal the expression of 110 kDa-PDE3A in accordance with Liu and Maurice (1998) as well as 72 kDa-PDE3B, PDE4A expression of 131, 97, 82 and 67 kDa.

In cirrhotic rat aorta, surprisingly no change in cGMP-PDE activity could be detected. However a significant increase in PDE3 activity, associated with an increase in PDE3A 110 kDa and PDE3B 72 kDa is observed. This increase in PDE3 may explain our previous apparent contradictory result (Tahseldar-Roumié et al., 2005) showing that DMPPO (0.1 μM) restore the vasodilator responses to NO donors that were attenuated in BDL compared with SO and suggesting a possible increase in PDE5 activity. This restoration might be related to an indirect inhibition of upregulated PDE3 mediated by cGMP, consequently to PDE5 inhibition, since it was demonstrated in rat aorta that cGMP induced vasorelaxation by inhibiting PDE3 (Komas et al., 1991, Lugnier 2005). While previous studies have showed an increase in the plasma level of prostacyclin that increases cAMP in the vascular tissue (Laffi et al., 1986), an increase in PDE3 activity could develop a compensatory mechanism to reduce cAMP accumulation, which can worsen the vasodilation and the hypotension related to the hyperdynamic circulation syndrome of liver cirrhosis (Blendis &

Wong, 2001). Interestingly a significant decrease of PDE2 was observed in aorta, related to endothelial cells underlining smooth muscle cells and controling NO production, which could participate to an increase in NO production and/or in vasodilatation previously reported in cirrhotic rats (Atucha et al., 2005).

In the whole mesenteric arteries, cAMP-PDE and cGMP-PDE specific activities are similar, cAMP-PDE specific activity being 2 fold higher than in whole rat aorta. cAMP-PDE activity is mainly related to PDE3 (69 %), whereas cGMP-PDE is mainly related to PDE5 (54 %). Our results extend previous results characterizing the activity of four PDE isozymes (PDE1, 3, 4, and 5) in rat mesenteric without endothelium (Komas et al., 1991a) by the characterization of PDE2 related to endothelial cells that is widely described in the literature (Komas et al., 1991b, Wallis et al., 1999). Western blotting allows characterization of 101 kDa-PDE5, 111 kDa-PDE3A and 118 kDa- and 96 kDa-PDE3B, phosphorylated 105 kDa-PDE4A, 66 kDa-PDE4B, 71 kDa-PDE4D and 131 kDa-PDE4C.

The results about PDEs alteration in the mesenteric arteries, demonstrated significant increases in specific cAMP-PDE activities: PDE3 (+23 %) and PDE4 (+96 %) associated with an induction of 84 kDa-PDE3B and ans increase in the 71 kDa-PDE4D isozyme. No increase in total cGMP-PDE was observed despite significant increases in PDE2 (+50%) and PDE5 (+50%) activities which are rised by a decrease in PDE1 (-22%) activity. No modification in the PDE5 protein level of is observed, suggesting that other new cGMP-PDE enzymes may be altered during cirrhosis

It seemed that the excessive generation of vasodilating substances from the endothelium, nitric oxide and prostacylin (Atucha et al., 2005; Angeli et al., 2005), which contributed to the abnormal splanchnic vasodilation, lead to the activation of cyclic nucleotides PDEs as an up-regulation mechanism to decrease cyclic nucleotides level, by which it could regulate the vascular tone.

Many cellular functions are regulated by mechanisms that control the levels of cyclic nucleotides. In liver cirrhosis, the PDEs (PDE5 and PDE4) (Ni et al., 1996, 2001; Ahloulay et al., 2005) have been shown to be an important role in the renal resistance to the natriuretic effects of cyclic nucleotides. Our present study demonstrated that the pathology of liver cirrhosis induced alteration in various PDE activities, which can result from a transcriptional modification as well as from post-transcriptional level, especially in mesenteric artery for PDE5 and PDE4 whose activities are increased with increased expression of PDE4D. Furthermore, this study showed the presence of the five PDE families in the described tissues with different cellular patterns, and the pathology of liver cirrhosis provides a role of a specific PDE in a specific tissue.

In conclusion, our results showed that the PDE1 isozyme preferentially to PDE5 could play an important role in the pathogenesis of sodium retaining associated to liver cirrhosis, by hydrolyzing cGMP or formed in response to hormones acting on the kidney. In the vasculature system (aorta and mesenteric arteries), an upregulation in the PDE activities mainly related to cAMP hydrolysis could occur to decrease the effect of the vasodilating agent (NO), which provided the initial mechanism of splanchnic vasodilation abnormality.

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Table 1. PDE protein expression in rat tissues.

	PDE1			PDE2	PDE3		PDE4				PDE 5
PDE subfamily	**1A**	**1B**	**1C**		**3A**	**3B**	**4A**	**4B**	**4C**	**4D**	
Renal cortex				- 74	- 106	- 161 - 62	- 130 - 47	-82	- 75	- 74	- 98
Kidney	- 78	- 74	-72	-74	-66	-167 -84 -63	-83 -47	-87 -80		-71	-92
Aorta					- 110	- 72	-131 -97 -104 Phosphorylated	-87 -64F -99 -68C	- 138 - 117 -64	-87	-97 -92
Mesenteric arteries					- 111	- 96	-50 -105 4A phosphorylated	-66	- 131	- 71	- 101

PDE expression was determined by Western blot analysis as described in Material and Methods. Sizes of proteins are in kDa.

Table 2: Distributions and variations with cirrhosis (%) of PDE activities in rat tissues ($pmol.min^{-1}.mg^{-1}$).

PDE activity	*Kidney*	Renal cortex	Aorta	Mesenteric artery
cAMP-PDE	254±29 /+47%*	316±43	49±3 /+71%*	95±9 /+29%*
cGMP-PDE	201±21 /+26%*	124±8	79±6	92±11
PDE1	74±16 /+83%**	40 ± 3	8±2	33±8
PDE2	87±7	64 ± 5	11±0.8 /-30%*	16±1/+50%*
PDE3	53±7	76± 19 /-52%	28±2 /+93%*	65±6/+23%*
PDE4	131±28 /+76%**	197 ± 29	13±2 /+78%*	20±4 /+96%*
PDE5	25±9	13± 3	55±5	50±5 /+50%**

Figure legends

Figure 1. PDE activity pattern in the renal cortex of sham-operated rat. cGMP- **(**A**)** and cAMP- (B) PDE specific activities (pmol/mg/min) were assessed as described in Material and Methods. Results are expressed as the mean±SEM of four independent experiments. The proportion of the different isozymes is expressed as % of total activity.

Effect of cirrhosis on PDE activity in renal cortex. cGMP- (C) and cAMP- **(**D) PDE specific activities were assessed in sham-operated (□) and cirrhotic (■) rats. Results are expressed as the mean±SEM of four independent experiments. The effect of cirrhosis is expressed as % of total- and specific isozyme- activities in sham-operated rats. * P< 0.05; ns: not significant.

Figure 2. Effect of cirrhosis on PDE3 expression in renal cortex. PDE3A (A) and PDE3B (B) expressions in renal cortex are shown for sham-operated (□) and cirrhotic (■) rats. Histograms showing densitometric analysis are expressed as the mean±SEM of four independent experiments. ***P< 0.001. Underneath is shown a representative Western blot trace.

Figure 3. PDE activity pattern in the kidney of sham-operated rat. cGMP- (A) and cAMP- (B) PDE specific activities were assessed as described in Material and Methods. Results are expressed as the mean±SEM of four independent experiments. The proportion of the different isozymes is expressed as % of total activity.

Effect of cirrhosis on PDE activity in kidney. cGMP- (C) and cAMP- (D) PDE specific activities were assessed in sham-operated (□) and cirrhotic (■) rats. Results are expressed as the mean±SEM of four independent experiments. The effect of cirrhosis is expressed as % of total- and specific isozyme- activities in sham-operated rats. *P< 0.05; **P<0.01; ns: not significant.

Figure 4. Effect of cirrhosis on PDE1 expression in kidney. PDE1A expression in kidney is shown for sham-operated (□) and cirrhotic (■) rats. Histograms showing densitometric analysis are expressed as the mean±SEM of four independent experiments. ***P< 0.001. Underneath is shown a representative Western blot trace.

Figure 5. PDE activity pattern in the aorta of sham-operated rat. cGMP- (A) and cAMP- (B) PDE specific activities were assessed as described in Material and Methods. Results are expressed as the mean±SEM of four independent experiments. The proportion of the different isozymes is expressed as % of total activity.

Effect of cirrhosis on PDE activity in aorta. cGMP- (C) and cAMP- (D) PDE specific activities were assessed in sham-operated (□) and cirrhotic (■) rats. Results are expressed as the mean±SEM of four independent experiments. The effect of cirrhosis is expressed as % of total- and specific isozyme- activities in sham-operated rats. * P< 0.05; ns: not significant.

Figure 6. Effect of cirrhosis on PDE3 expression in aorta. PDE3A- (A) and PDE3B- (B) expressions in aorta are shown for sham-operated (□) and cirrhotic (■) rats. Histograms showing densitometric analysis are expressed as the mean±SEM of four independent experiments.*P<0.05; ***P< 0.001. Underneath is shown a representative Western blot trace.

Figure 7. PDE activity pattern in the mesenteric arteries of sham-operated rat. cGMP- (A) and cAMP- (B) PDE specific activities were assessed as described in Material and Methods. Results are

expressed as the mean±SEM of four independent experiments. The proportion of the different isozymes is expressed as % of total activity.

Effect of cirrhosis on PDE activity in mesenteric arteries. cGMP- (C) and cAMP- (D) PDE specific activities were assessed in sham-operated (□) and cirrhotic (■) rats. Results are expressed as the mean±SEM of four independent experiments. The effect of cirrhosis is expressed as % of total- and specific isozyme- activities in sham-operated rats. * P< 0.05; ** P<0.01; ns: not significant.

Figure 8. Effect of cirrhosis on PDE3 and PDE4 expression in mesenteric arteries. PDE3B (A) and PDE4D **(**B) expression in mesenteric arteries is shown for sham-operated (□) and cirrhotic (■) rats. Histograms showing densitometric analysis are expressed as the mean±SEM of four indepedent experiments. ***P< 0.001. Underneath is shown a representative Western blot trace.

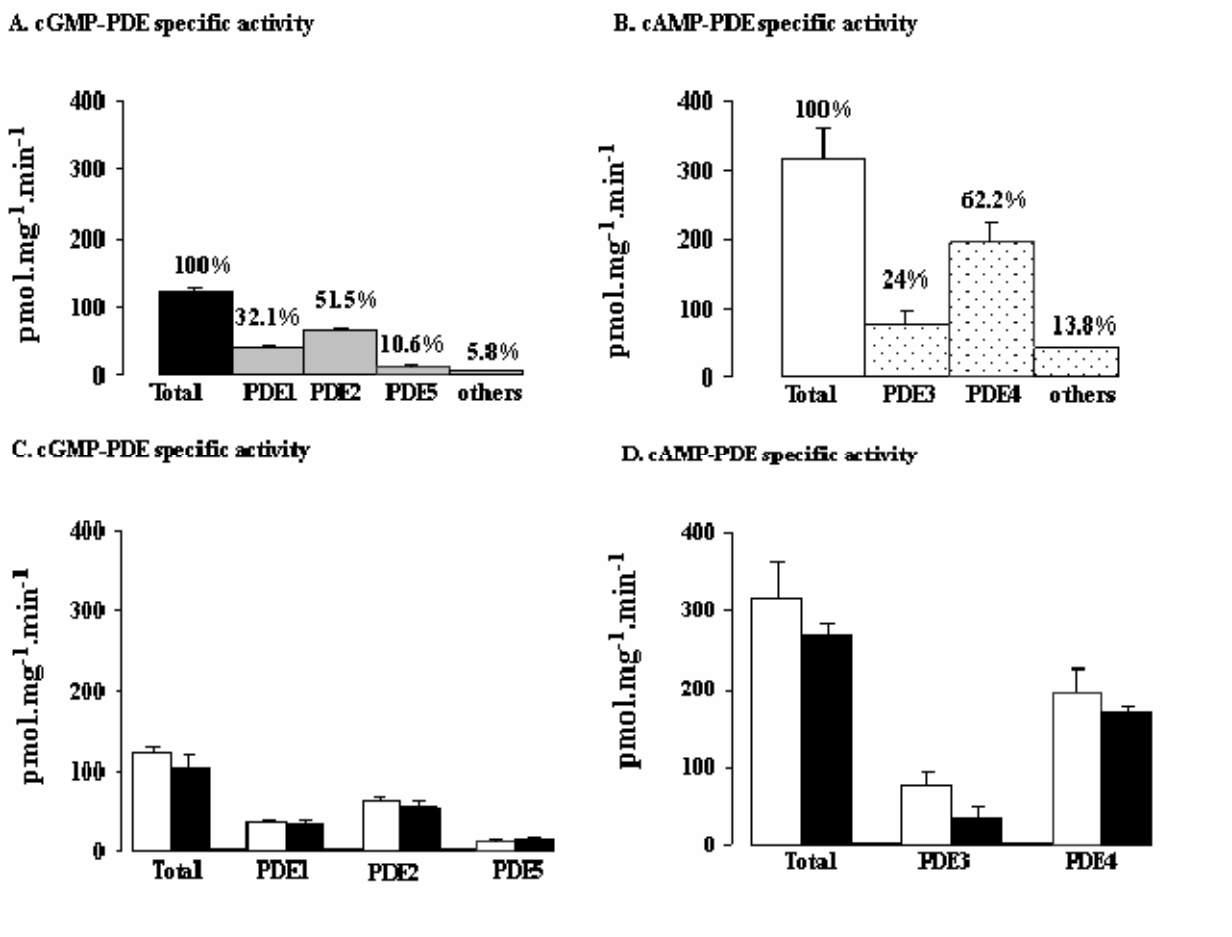

A. cGMP-PDE specific activity
pmol.mg-1.min-1
400
300
200
100
0
100%
32.1%
51.5%
10.6%
5.8%
Total
PDE1
PDE2
PDE5
others
B. cAMP-PDE specific activity
pmol.mg-1.min-1
400
300
200
100
0
100%
24%
62.2%
13.8%
Total
PDE3
PDE4
others
C. cGMP-PDE specific activity
pmol.mg-1.min-1
400
300
200
100
0
Total
PDE1
PDE2
PDE5
D. cAMP-PDE specific activity
pmol.mg-1.min-1
400
300
200
100
0
Total
PDE3
PDE4

Figure 1

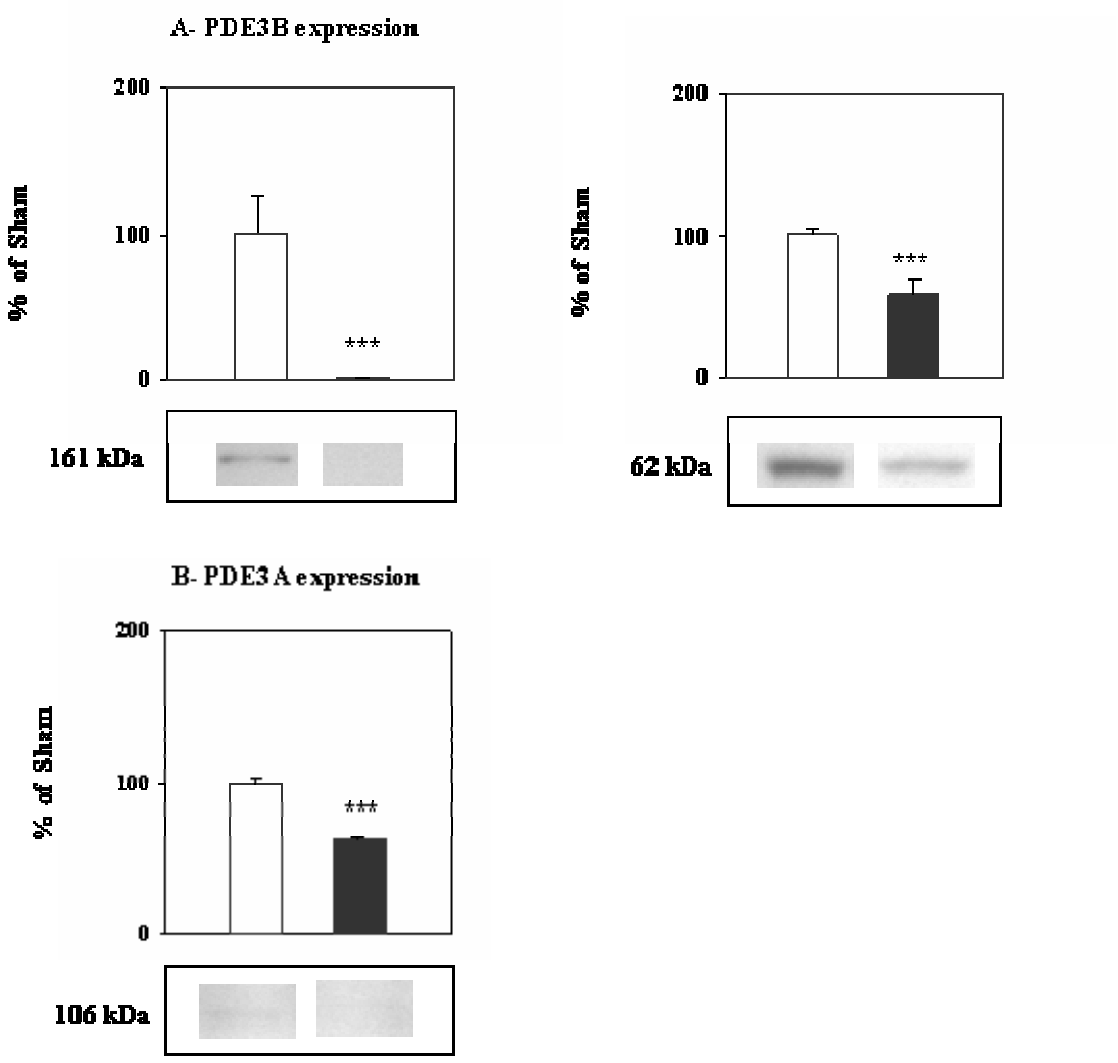

A- PDE3B expression
% of Sham
200
100
0
***
161 kDa
% of Sham
200
100
0
***
62 kDa
B- PDE3A expression
% of Sham
200
100
0
***
106 kDa

Figure 2

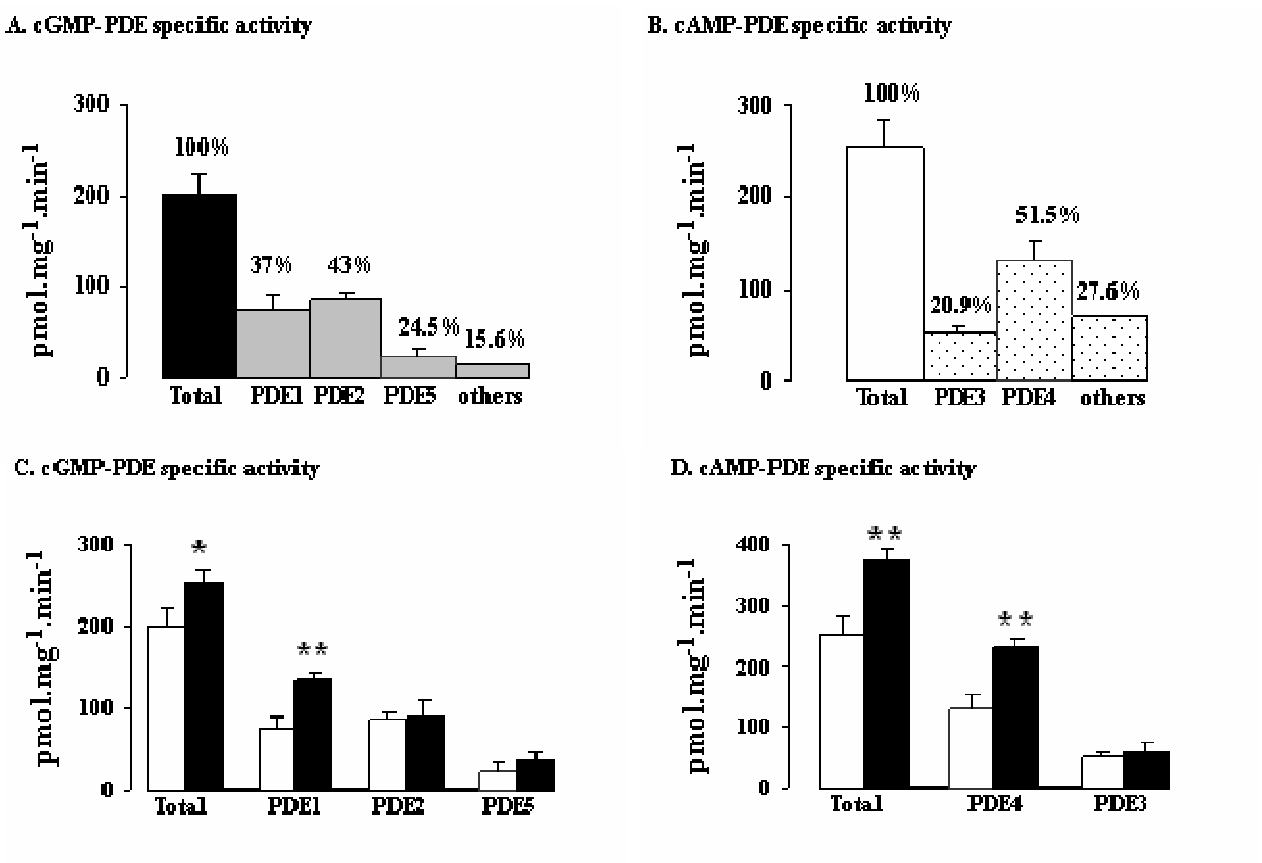

A. cGMP-PDE specific activity
pmol.mg-1.min-1
300
200
100
0
100%
37%
43%
24.5%
15.6%
Total
PDE1
PDE2
PDE5
others
B. cAMP-PDE specific activity
pmol.mg-1.min-1
300
200
100
0
100%
20.9%
51.5%
27.6%
Total
PDE3
PDE4
others
C. cGMP-PDE specific activity
pmol.mg-1.min-1
300
200
100
0
*
**
Total
PDE1
PDE2
PDE5
D. cAMP-PDE specific activity
pmol.mg-1.min-1
400
300
200
100
0
**
**
Total
PDE4
PDE3

Figure 3

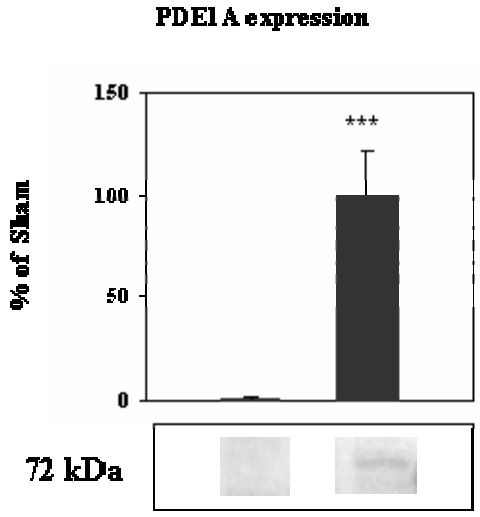


Figure 4

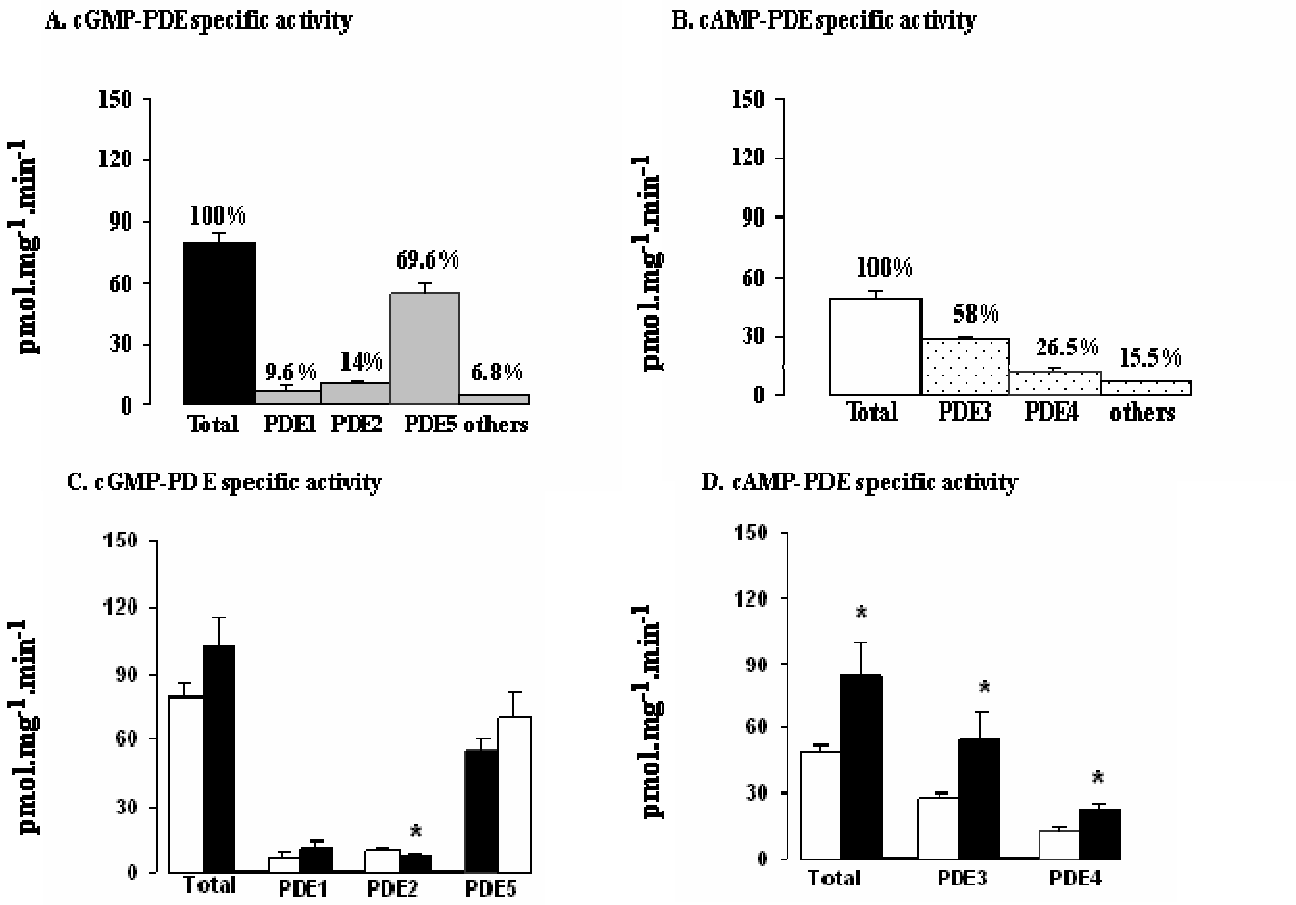

A. cGMP-PDE specific activity
pmol.mg-1.min-1
150
120
90
60
30
0
100%
9.6%
14%
69.6%
6.8%
Total
PDE1
PDE2
PDE5
others
B. cAMP-PDE specific activity
pmol.mg-1.min-1
150
120
90
60
30
0
100%
58%
26.5%
15.5%
Total
PDE3
PDE4
others
C. cGMP-PDE specific activity
pmol.mg-1.min-1
150
120
90
60
30
0
*
Total
PDE1
PDE2
PDE5
D. cAMP-PDE specific activity
pmol.mg-1.min-1
150
120
90
60
30
0
*
*
*
Total
PDE3
PDE4

Figure 5

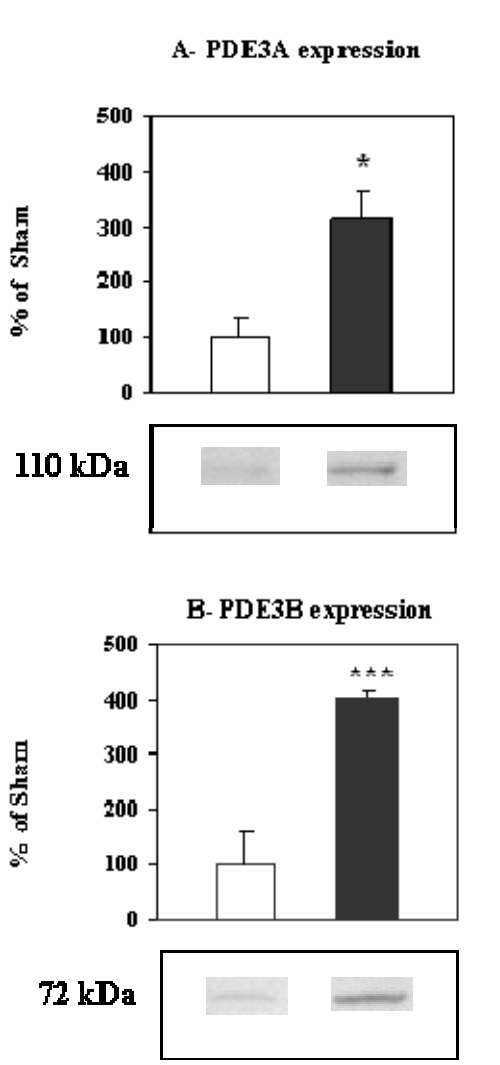

A- PDE3A expression
% of Sham
500
400
300
200
100
0
*
110 kDa
B- PDE3B expression
% of Sham
500
400
300
200
100
0
***
72 kDa

Figure 6

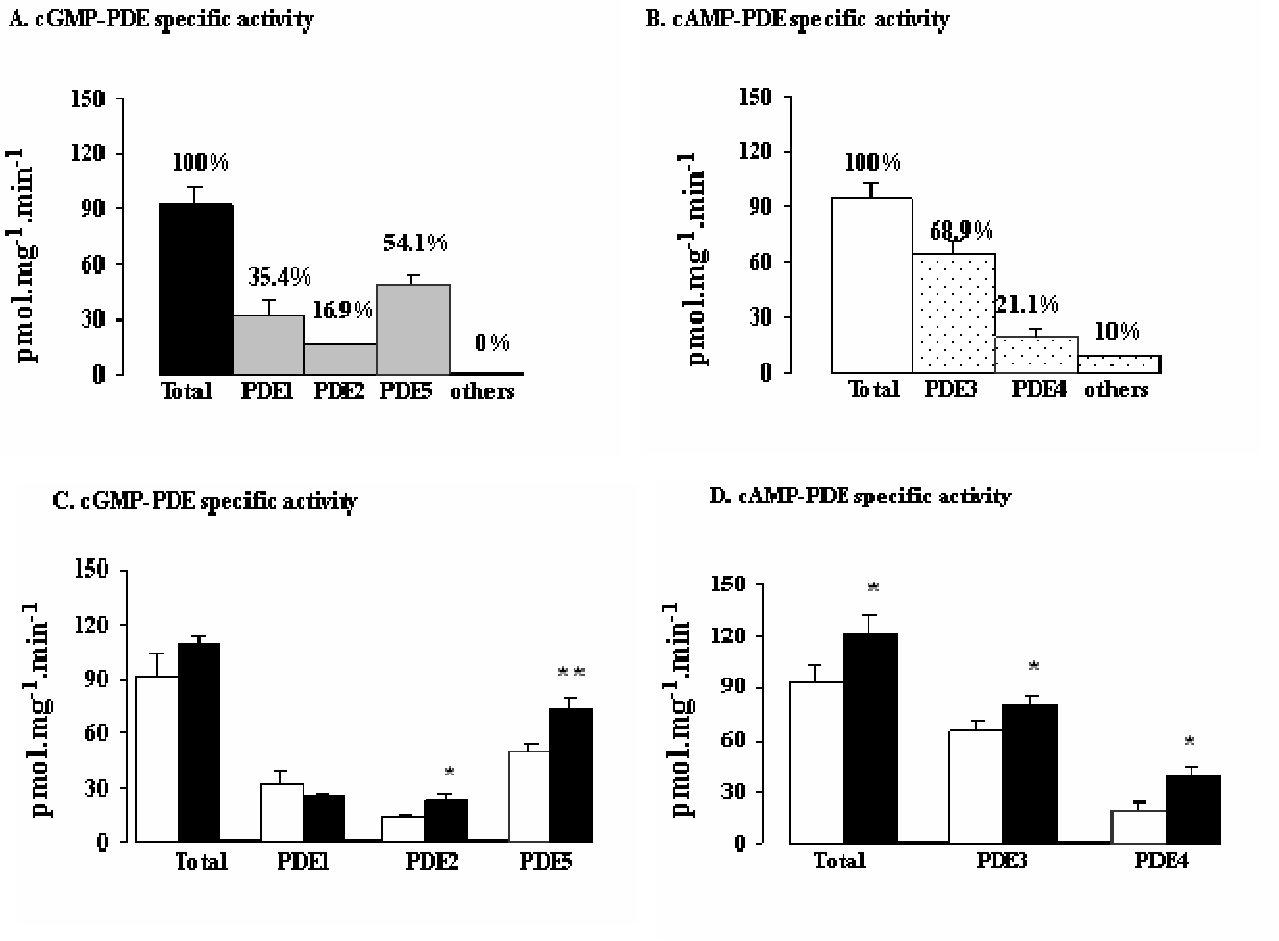
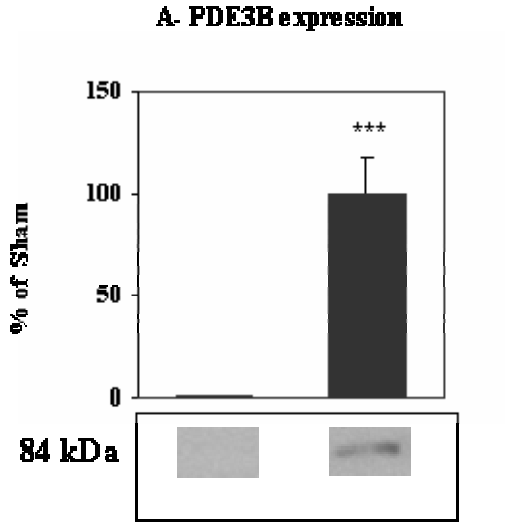

A. cGMP-PDE specific activity
pmol.mg-1.min-1
150
120
90
60
30
0
100%
35.4%
16.9%
54.1%
0%
Total
PDE1
PDE2
PDE5
others
B. cAMP-PDE specific activity
pmol.mg-1.min-1
150
120
90
60
30
0
100%
68.9%
21.1%
10%
Total
PDE3
PDE4
others
C. cGMP-PDE specific activity
pmol.mg-1.min-1
150
120
90
60
30
0
*
**
Total
PDE1
PDE2
PDE5
D. cAMP-PDE specific activity
pmol.mg-1.min-1
150
120
90
60
30
0
*
*
*
Total
PDE3
PDE4

Figure7


A- PDE3B expression
150
100
50
0
% of Sham
***
84 kDa

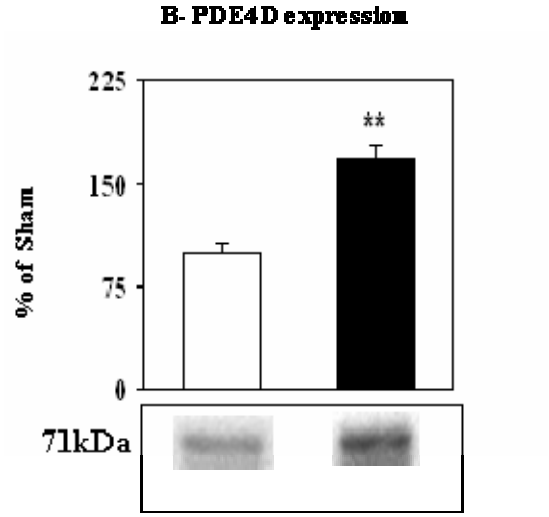

B- PDE4D expression
225
150
75
0
% of Sham
**
71kDa

Figure 8

Conclusion and Perspective

Our study for the first time has shown an implication of the PDEs in the hemodynamic and renal complications associated to advanced liver cirrhosis *in vitro* and *in vivo*.

Taking the thoracic aorta as a model, we have shown that there is a decreased sensitivity to the vasodilator effects of nitroglycerine in BDL compared to control rats, which was extended to SNAP, another NO donor. This difference was eliminated by pre-treatment of endothelium-denuded aortic rings with DMPPO. Vasodilation of DMPPO itself was also less in aortic rings of BDL rats. Since the response to diazoxide was the same in aortic rings from BDL and control rats (Tahseldar-Roumieh et al., 2005), these findings suggested that the specific decrease in vascular sensitivity account for an alteration in the NO-cGMP pathway and not to dysfunction at the level of vascular smooth muscle cells.Thus, we have provided evidence to suggest increased activity of PDE5, which manifested functionally as a reduced responsiveness to NO donors.

The studies involving phenylephrine were particularly revealing the decreased sensitivity to vasoconstrictors, attributed to increase NO production in liver cirrhosis as established in the literature. Therfore, pre-treatment with DMPPO enhanced hyporesponsiveness to phenylephrine, which was decreased in BDL in comparison with SO. This result accounted for the proposed increase in PDE5 activity that attenuated the effects of DMPPO in cirrhosis despite NO overproduction.

We also examined the effects of DMPPO on hemodynamic and renal parameters in cirrhotic and control rats. The results demonstrated that DMPPO enhanced the hemodynamic disturbance by causing worsening of the vasodilation (decrease PVR) and hypotension (decrease in MAP), which occurred equally in both sham operated and BDL rats and was of equal magnitude. However, despite the decrease in arterial pressure, there was an increase in Na excretion in cirrhotic rats that was not consistently seen in the control animals. This emphasizes the role of increased PDE5 activity locally in the kidney, and agrees with previous findings (Wensing and Branch, 1990; Moreau and LeBrec, 1995).

In conclusion, this first study revealed novel findings suggesting a role for PDE5 in regulating vascular reactivity, hemodynamics and renal function in cirrhotic rats. In at least two tissues (the aorta and kidney), there is evidence to suggest an increase in activity of PDE5 that has important functional consequences. Further studies are necessary in order to examine the usefulness of chronic administration of PDE5 inhibitors in ameliorating the renal complications of cirrhosis, especially sodium retention.

Therefore, the second part of the studies was to investigate the hemodynamic and renal functional alterations associated with liver cirrhosis following chronic administration of sildenafil (a selective inhibitor of PDE5). Administration of sildenafil to rats with liver cirrhosis caused further reduction in arterial pressure by enhancing the peripheral vasodilation associated with this condition. However, this is especially important because the worsened peripheral vasodilation attributed to NO due to inhibition of cGMP hydrolysis after administration of sildenafil may increase sodium retention by the kidney and annul the direct effect of the PDE-5 inhibitor on the kidney, by causing more severe activation of the sodium retaining mechanisms (the renin-angiotensin-aldosterone system and the sympathetic nervous system). Despite these unfavorable hemodynamic effects including a decrease in peripheral vascular resistance and in arterial pressure, sildenafil increased total and fractional sodium excretion and did not worsen renal function (GFR).

However, in contrast to our results, a single oral dose of sildenafil administred to patients with liver cirrhosis did not induce natriuresis but worsened sodium retention by the sustained elevation in plasma renin activity, angiotensin II and aldosterone concentrations.

Our present study is the first (in humans or animals) to investigate the effect of chronic administration of sildenafil, or any PDE-5 inihibitor, in liver cirrhosis. It suggests that under these circumstances, the direct effect of sildenafil in the kidney overcomes the indirect effects of activating sodium retaining compensatory mechanisms resulting from vasodilation; however, this occurs at the expense of further hypotension. The clinical significance of these findings cannot be

ascertained from this study, which used a single dose regimen of the drug. It is possible that using a lower dose may induce a selective effect on the kidney without much worsening of the hemodynamic picture and that requires specific studies in the future. Furthermore, the possible hemorrhagic complications need to be considered as suggested by Colle et al., (2004). In conclusion, the present study offers a possibility for a novel therapeutic approach to sodium retention and ascites in liver cirrhosis using sildenafil specifically, and PDE-5 inhibitors in general, that should be further tested in animal and human studies.

In the aorta as a model of vascular system, we suggested an increase PDE5 activity, which may be a primary event of liver cirrhosis and results in reduced the response to NO donors. Adding previous findings showing an increase in PDE5 activity and expression in the kidney of liver cirrhosis (Ni et al., 1996, 2001; Angeli et al., 2000) lead us to investigate the modifications that could occur to the different PDE isoforms at two levels, protein activity and protein expression in cirrhotic rats four weeks after bile duct ligation in the altered tissues; renal cortex, kidney, aorta and the mesenteric arteries. It is important to define if such vascular and hemodynamic alterations could result from a greater amount of the protein, from a change in the activity, or from combination of the two. This study reported how important is to target PDE expression and activity for understanding the physiology of the cirrhosis.

Therefore, analysis of PDE activities in homogenates and PDE expression in kidney of SO and BDL rats showed an increase in total cGMP-specific activity, which was due largely to an increase in PDE1 isozyme activity. This increase is specifically due to the increase in PDE1A isozyme protein level. This correlation in the increase in PDE1 activity and expression did not match with previous investigations of Ni and his collegues (2001) showing similar changes but in the PDE5 isozyme in the IMCD 7 days after bile duct ligation. Since the liver cirrhosis pathology progresses, the physiological complications become more manifest with activation of the vasoconstrictor renin-angiotensin-aldosterone system, which induced the renal sodium and water retaining. Thus, these

contradictory results could be explained by enhancing an implication of PDE5 at early stages of the disease where as the late stages the PDE1 could interfere. Moreover, studies of cultured rat mesangial cells (MC) suggested that the angiotensin II, which is increased in sodium retaining states, may activate PDE1 by increasing intracellular Ca^{2+} and may contribute to increased hydrolysis of ANP-stimulated cGMP pool in situ (Haneda et al., 1991). These findings may explain our results, since it is established that the plasma level of angiotensin II is increased in liver cirrhosis (Schroeder et al., 1970; Laffi et al., 1984), which may activate the PDE1 isozyme. It is possible that at the concentration of 5 mg/kg, the acute administration of DMPPO could enhance sodium excretion by inhibiting PDE1, leading to accumulation of cGMP via ANP pool.

On the other hand, the study of the PDE activity and expression in the vascular systems, aorta and mesenteric arteies of BDL rats, have shown for an increase in the total cAMP-specific PDE activity in BDL rats. In the aorta, this increase was due largely to the increase in PDE3 activity in the aorta from BDL that correlates with an increase in the protein level of the two PDE3 variants: PDE3A and PDE3B. Therefore, how do the results suggesting increased PDE3 activity in the aorta explain the effects of DMPPO?. DMPPO by inhibiting PDE5 in aortic smooth muscle, increases cGMP levels, which in turn could induce inhibition of PDE3 isozyme present in smooth musle cells. Since our data reveal an increase in PDE3 activity and expression, without significant increase in PDE5 activity, one can hypothezise that the decrease in DMPPO hyporesponsiveness to phenylephrine reported for BDL aorta in our first study could be attributed to PDE3. Moreover, most evidence accounted for a role of NO and prostacyclin in peripheral vasodilation in liver cirrhosis. Therefore, PDE3 inhibition may decrease hydrolysis of prostacyclin-stimulated cAMP leading to vasodilation.

In the whole mesenteric arteries, liver cirrhosis induced despite no significant change in total cGMP-PDE activity an increase in both cGMP- PDEs activity, PDE5 and PDE2, suggesting that other cGMP-PDEs might participatee in cGMP-PDE activity. Nevertheless, the fact that PDE2 is not present in vascular smooth muscle from rat mesenteric artery (Komas etal., 1991) and that

PDE5 activity is 3-fold greater than PDE2 activity, give evidence for PDE5 to control vascular tone. Therefore, increased PDE5 activity in the mesenteric arteries of rats with liver cirrhosis may be developed as a mechanism of down regulation as a response to the increase NO production contributing to peripheral vasodilatation abnormality and ascites formation, major complications of liver cirrhosis.

Moreover, increase in PDE3 and PDE4 activities and expression was enhanced in mesenteric arteries in liver cirrhosis. It seemed that the excessive generation of vasodilating substances from the endothelium, nitric oxide and prostacylin, which contributed to the abnormal splanchnic vasodilatation, stimulated activation of cyclic nucleotides PDEs as an up-regulation mechanism to decrease cyclic nucleotides level, by which it could regulate the vascular tone.

In conclusion, this work revealed novel findings suggesting the role for PDEs in regulating vascular reactivity, which was different in both vessels studied and pointed out that cAMP-PDE up regulation mediate decrease in DMPPO hyporesponsiveness in rat aorta revealing a possible cross-talk between cGMP and cAMP intracellular pools. Further studies are needed to explain the role of PDE3 in the rat aorta; whereas, in the mesenteric arteries, there is evidence for PDE5, as well as, a PDE3 and PDE4 up-regulations in the mesenteric arteries and newly a PDE1 up-regulation in the kidney. Our studies reveal complex regulations of various PDEs in various organs during liver cirrhosis and open a new research field. Therefore, many studies are needed to identify the various molecular mechanism participating in PDEs activity and expression alterations in cirrhosis (at early and late stage of the disease), which are manifested by a change in the vascular reactivity, the hemodynamic and the renal function.

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Abstract
Increased cGMP-specific PDE (PDE5) activity and expression in the kidneys of cirrhotic rats contributes to renal sodium retention. PDE5 hydrolyses cGMP and leads to a resistance of the kidney to the natriuretic effects of ANP. Liver cirrhosis is also complicated by peripheral vasodilation especially in the splanchnic vasculature, providing an evidence for a role of NO. These findings suggested that if such up-regulation of PDE5 occurs in cirrhosis, it would antagonize the effects of NO on the vasculature, and may lead to development of newer treatment modalities targeting inhibition of PDE5.
The aim of this study is to examine the effect of PDE5 inhibitors on the vascular reactivity of rat aorta to NO donors and phenylephrine, and on renal function and on systemic hemodynamics disturbances of liver cirrhosis induced by bile duct ligation (BDL). The vasodilator responses to nitroglycerine and S-nitroso-N-acetylpenicillamine (SNAP) were attenuated in the aortic rings without endothelium of BDL rats. DMPPO enhanced these responses and eliminated the differences between BDL and SO (sham-operated) rats. Vasodilation to DMPPO itself was also less in BDL. In vivo, acute administration of DMPPO (5 mg/kg i.v.) worsen vasodilation (decreased the arterial pressure and vascular resistance) in both groups equally, and caused a significant increase in Na excretion in BDL rats only. However, examining the chronic effect of a more potent PDE5 inhibitor and a clinical tool for human impotence, sildenafil, will be more revealing for the benefit effect of DMMPO on renal sodium excretion. Similarly, chronic sildenafil administration ameliorates Na retention in cirrhosis, but worsens the systemic hemodynamics disturbances of liver cirrhosis.
Therefore, identifying the alteration in PDEs activity and expression in liver cirrhosis will increase our understanding of the pathological processes. This study provides evidence for an increase PDE1 activity and expression in the kidney of cirrhosis. In the aorta, PDE3 activity correlates with an increase in protein level. This study may explain an implication of PDE5 at early stage of cirrhosis, whereas, at late stage a PDE1 isozyme is involved.
Taken together, these results demonstrate that PDEs are widely and differently implicated in liver cirrhosis and thus specific PDE inhibitors may be useful for precising new potential targets for liver cirrhosis.

Key words: PDEs, expression, activity, PDE5 inhibitor, rat, aorta, mesenteric artery, kidney, cirrhosis, Na retention.

Résumé
Au cours de l'installation de la cirrhose chez le rat, une augmentation de l'activité et de l'expression de la PDE5 rénale. Cet effet se manifeste par une rétention rénale du sodium conséquente d'une diminution de l'effet vasodilatateur du peptide auriculaire natriurétique (ANP), suite a l'hydrolyse du GMPc, substrat spécifique de la PDE5. De plus, étant donné que le NO est impliqué dans la vasodilatation splanchnique de la cirrhose, il est probable que la PDE5 puisse jouer un rôle dans la régulation du tonus vasculaire au cours de la cirrhose. Ces premiers résultats obtenus ont conduit à entreprendre une étude approfondie sur l'implication des PDEs, plus particulièrement de la PDE5, dans la réactivité vasculaire, et les altérations hémodynamiques lorsque la cirrhose est installée (4 semaines). Dans cette étude la cirrhose est induite chez le rat par ligature du canal biliaire (BDL).
Les études menées *in vitro* sur l'aorte de rat met en évidence une diminution de sensibilité aux donneurs de NO, nitroglycérine (NG) et S-nitroso-N-acetylpenicillamine (SNAP) chez les rats BDL. Le DMPPO, inhibiteur spécifique de la PDE5, potentialise l'effet vasodilatateur de NG et du SNAP et élimine la différence de sensibilité existante entre les BDL et les contrôles. L'administration aiguë du DMPPO (5 mg/kg, 5 min i.v.) diminue de façon significative la pression artérielle et la résistance vasculaire périphérique dans les deux groupes, et induit un doublement significatif de l'excrétion sodique et de l'excrétion fractionnelle du sodium chez les BDL, mais pas chez les contrôles. Nos premiers résultats suggèrent un effet bénéfique de l'inhibition de la PDE5 dans la rétention du sodium liée à la cirrhose. Cependant, le sildénafil administré chroniquement, inhibiteur puissant et spécifique de la PDE5, et outil thérapeutique de l'impuissance masculine, restaure le fonctionnement rénal, mais aggrave la vasodilatation périphérique.
L'étude approfondie menée sur l'activité et l'expression des diverses PDEs, montre une augmentation de l'activité et de l'expression de la PDE1 contribuant 3 fois plus à l'hydrolyse du GMPc dans le rein. La diminution de sensibilité de l'aorte de rat cirrhotique à l'inhibiteur de PDE5, DMPPO, est attribuée à une augmentation de l'activité de PDE3 sensible au GMPc associée à une augmentation de son expression. Cependant, la cirrhose étant une maladie progressive, une intervention de la PDE5 pourrait être impliquée aux premiers stades de la maladie, alors que la PDE1 pourrait intervenir aux stades plus avancés, induisant son effet néfaste sur les reins.
Ces résultats sont innovants puisqu'ils mettent à jour des implications de différentes PDEs dans la cirrhose et permettent d'envisager certaines PDEs comme nouvelles cibles thérapeutiques envers la cirrhose.

Mots clés : PDEs, expression, activité, inhibiteur de PDE5, rat, cirrhose, rétention du sodium, aorte, artère mésentérique, rein