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Alteration of vascular reactivity in heart failure:

Role of phosphodiesterases type 3 and 4

Short running title: Vascular reactivity and cAMP-PDE in heart failure

Authors: F Hubert^{1,2,*}, M Belacel-Ouari^{1,2}, B Manoury^{1,2}, K Zhai^{1,2,*}, V Domergue-Dupont^{2,3}, P Mateo^{1,2}, F Joubert^{1,2}, R Fischmeister^{1,2}, V Leblais^{1,2}.

¹*Inserm UMR-S 769, LabEx LERMIT, DHU TORINO, F-92296 Châtenay-Malabry, France,*

²*Université Paris-Sud, Faculté de Pharmacie, F-92296 Châtenay-Malabry, France, and*

³*IPSIT/IFR141, Plateforme « Animalerie et exploration fonctionnelle », F-92296 Châtenay-Malabry, France.*

* Current address for: F H, Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Canada; and K Z, National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China.

Correspondence to: V Leblais, UMR-S 769, LabEx LERMIT, DHU TORINO, Faculté de Pharmacie, Université Paris-Sud, 5 rue J.-B. Clément 92296 Châtenay-Malabry, France.

E-mail: veronique.leblais@u-psud.fr; Tel: +33-1.46.83.59.06; Fax: +33-1.46.83.54.75.

Summary

BACKGROUND AND PURPOSE

This study examined the role of the main vascular cAMP-phosphodiesterases (cAMP-PDE) in the regulation of basal vascular tone and β -adrenergic (β -AR) relaxation of rat aorta following heart failure (HF).

EXPERIMENTAL APPROACH

Twenty-two weeks after proximal aortic stenosis (HF) or SHAM-surgery in rats, we evaluated the cAMP-PDE activity, expression and function in the descending thoracic aorta.

KEY RESULTS

HF rat aortas exhibited signs of endothelial dysfunction linked to an alteration of the nitric oxide (NO) pathway. Expression pattern of PDE3 and PDE4 subtypes was altered, although total cAMP hydrolytic activity in aorta, as well as PDE1, PDE3 and PDE4 activities, were unchanged. Vascular reactivity experiments using PDE inhibitors showed that PDE3 and PDE4 controlled the level of $\text{PGF}_{2\alpha}$ -stimulated contraction in SHAM aorta. PDE3 function was partially inhibited by endothelial NO, whereas PDE4 function required a functional endothelium and was under the negative control of PDE3. In HF, PDE3 function was preserved, but its regulation by endothelial NO was altered. PDE4 function was abolished, although it could be restored by PDE3 inhibition. In $\text{PGF}_{2\alpha}$ -precontracted arteries, β -AR stimulation induced a relaxation of SHAM aorta. Such a response was abolished in the absence of functional endothelium, as well as in HF aortas, but it was restored after PDE3 inhibition in all unresponsive arteries.

CONCLUSIONS AND IMPLICATIONS

Our study underlines the key role of the endothelium in controlling the contribution of smooth muscle PDE to contractile function. In HF, endothelial dysfunction has a major impact on PDE3 function and PDE3 inhibition is able to restore a functional β -AR relaxation.

Keywords

Heart Failure; Vascular smooth muscle; Endothelial dysfunction; Vasorelaxation; Phosphodiesterase; Adrenergic receptor

Abbreviations

AC, adenylyl cyclase; β -ARs, β -adrenoceptors; BAY, BAY-60-7550; cAMP-PDE, cAMP-hydrolysing PDE; Cil, cilostamide; CRC, concentration-response curve; HF, heart failure; IBMX, 3-isobutyl-1-methylxanthine; MIMX, 8-methoxymethyl-3-isobutyl-1-methylxanthine; L-NAME, N ω -nitro-L-arginine-methyl ester; NO, nitric oxide; NOS, NO synthase; PDEs, 3',5'-cyclic nucleotide phosphodiesterases; PGF_{2 α} , prostaglandin F_{2 α} ; PSS, physiological salt solution; Ro, Ro-20-1724; SMCs, smooth muscle cells; SNP, sodium nitroprusside.

Introduction

In the vascular system, 3'-5'-cyclic adenosine monophosphate (cAMP) is a key physiological second messenger that inhibits contraction, proliferation and migration of the smooth muscle cells (SMCs). Intracellular concentration of cAMP is determined by the balance between its production and degradation by adenylyl cyclases (AC) and 3',5'-cyclic nucleotide phosphodiesterases (PDEs), respectively.

Stimulation of β -adrenoceptors (β -ARs), which are typically coupled to the AC/cAMP pathway, causes vasodilation through protein kinase-dependent mechanisms (Eckly-Michel *et al.*, 1997). β -ARs may be located on endothelial cells, on SMCs or both depending on the vascular bed and the β -AR subtype (Flacco *et al.*, 2013). In rat aorta, the endothelium appears not to be necessary for β -AR relaxation but it exerts a regulatory role by controlling the SMC precontraction level (Eckly *et al.*, 1994b) and the SMC concentration of cGMP through nitric oxide (NO) release (Eckly *et al.*, 1994a; Lugnier *et al.*, 1993).

The cAMP-mediated relaxation can be blunted by its degradation under the action of PDEs. PDEs comprise a large group of more than 50 isoenzymes that are classified into 11 families. It is usually admitted that blood vessels express four dominant cAMP-hydrolysing PDE (cAMP-PDE) families: the Ca^{2+} /calmodulin-stimulated PDE1, the cGMP-stimulated PDE2, the cGMP-inhibited PDE3 and the cAMP-specific PDE4, with PDE3 and PDE4 providing the main cAMP-hydrolysing activity (Komas *et al.*, 1991; Polson *et al.*, 1996; Zhai *et al.*, 2012). In rat aorta, both PDE3 and PDE4 inhibitors induce a vasorelaxation and potentiate the β -AR relaxation (Delpy *et al.*, 1996; Komas *et al.*, 1991; Lugnier *et al.*, 1993). These cAMP-mediated responses may be modulated by the endothelium and the NO/cGMP pathway as cGMP inhibits PDE3 activity by competition with cAMP on its catalytic site (Delpy *et al.*, 1996; Lugnier *et al.*, 1993).

Heart failure (HF) is a clinical syndrome related to a decreased ability of the heart to

provide sufficient cardiac output and resulting in inadequate tissue perfusion. Numerous studies have reported down-regulation of the cardiac β -AR signaling pathway in HF (Lohse *et al.*, 2003). More recently, alterations of the expression, distribution or activity of cardiac PDEs were also shown to be involved in cardiac hypertrophy (Abi-Gerges *et al.*, 2009; Mokni *et al.*, 2010; Yanaka *et al.*, 2003) and HF (Ding *et al.*, 2005; Lehnart *et al.*, 2005; Pokreisz *et al.*, 2009). HF is also characterized by vascular morphological and functional alterations, in particular an increase in vessel wall thickness, an increase in the vasomotor tone at rest and a decrease in vasodilator endothelium-dependent and -independent responses (Francis *et al.*, 1990; Nakamura *et al.*, 2001; Negrao *et al.*, 2000). This endothelial dysfunction may result from impaired release of endothelium-derived relaxing factors such as NO or increased release of endothelium-derived contracting factors (Kaiser *et al.*, 1989; Katz *et al.*, 1993). Less attention was paid on the consequence of HF on the vascular β -AR/cAMP/PDE pathway. Most studies reported a decrease in the β -AR-mediated vasorelaxation in systemic and/or pulmonary arteries isolated from different models of HF animals (Mathew *et al.*, 1993; McGoldrick *et al.*, 2007; Nasa *et al.*, 1996), which was in some cases related to a decrease in β -AR density (Gaballa *et al.*, 2001; Kiuchi *et al.*, 1993). One study also reported an enhanced PDE3 activity in rat aorta isolated from a model of salt-induced hypertension and HF (Takahashi *et al.*, 2002). However, the impact of HF disease on the functional role of vascular PDEs has never been evaluated.

This study was thus designed to characterize the role of the main vascular cAMP-PDE families in the regulation of the basal vascular tone and the relaxant response to β -AR stimulation and to evaluate the impact of HF on these functions, as well as on cAMP-PDE expression profile.

characterize the impact of HF on the contributions of the main vascular cAMP-PDE families to the regulation of basal vascular tone and the relaxant response to β -AR stimulation.

Methods

All experiments performed conform to the European Community guiding principles in the care and use of animals (Directive 2010/63/EU of the European Parliament). Authorizations to perform animal experiments according to this decree were obtained from the French Ministry of Agriculture, Fisheries and Food (no. D- 92-283, December 13, 2012). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Detailed methods are included in the online supplementary information.

Surgical procedure

Aortic stenosis was induced in male Wistar rats (Elevage Janvier, Le Genest St Isle, France) by placing a stainless steel hemoclip on the ascending aorta, as previously described (Joubert *et al.*, 2008). SHAM-operated animals were realized as controls.

Echocardiography and blood pressure measurement

Two-dimensional-guided (2D) M-mode echocardiography was performed at 22 weeks after surgery on 10 SHAM-operated and 9 stenosed rats using a 12 MHz transducer (Vivid 7, General Electric Healthcare) under isoflurane gas anesthesia.

Arterial blood pressure was measured in 9 SHAM-operated and 6 stenosed conscious rats using a tail-cuff system (CODA™, Kent Scientific).

Rat aorta sampling

Twenty-two weeks after surgery, rats were sacrificed to isolate the descending thoracic aorta and cut it into 2 mm length rings. In some preparations, the endothelium was removed. For biochemical studies, rings were frozen in liquid nitrogen.

Cyclic AMP-PDE activity assay

Cyclic AMP-PDE activity was measured according to the method described by Thompson and Appleman (1971) (Thompson *et al.*, 1971), as previously reported (Zhai *et al.*, 2012). The radioassay was performed in the absence or presence of selective PDE inhibitors: 10 μ M 8-methoxymethyl-3-isobutyl-1-methylxanthine (MIMX) for PDE1 (Rich *et al.*, 2001), 100 nM BAY-60-7550 (BAY) for PDE2 (Boess *et al.*, 2004), 1 μ M Cilostamide (Cil) for PDE3 (Sudo *et al.*, 2000), 10 μ M Ro-20-1724 (Ro) for PDE4 or 1 mM 3-isobutyl-1-methylxanthine (IBMX) as a non-selective PDE inhibitor (Rich *et al.*, 2001). The residual hydrolytic activity observed in the presence of PDE inhibitors was expressed as a percentage of the total cAMP-PDE activity, corresponding to the cAMP-PDE activity in the absence of inhibitor (vehicle).

Western blot analysis

Primary antibodies directed against PDE3A (gift from Dr. Chen Yan, Columbia University, NY, USA), PDE3B (gift from Dr. Emilio Hirsch, University of Torino, Italy), PDE4B (gift from Dr. Marco Conti, University of California, San Francisco, USA) and β -actin (sc-47778, Santa Cruz Biotechnology) were used. PDE signal was normalized to β -actin signal.

Quantitative RT-PCR analysis

mRNAs encoding 4 PDE subtypes (PDE3A, PDE3B, PDE4B and PDE4D) and 2 housekeeping genes (TBP: Tata Box Binding Protein, and Ywhaz: 14-3-3 protein zeta/delta) were analyzed according to the procedure previously described by Zhai *et al.* (Zhai *et al.*, 2012). PDE gene expression level was calculated using the comparative threshold (Ct) method ($2^{\Delta Ct}$).

Vascular reactivity measurement

Aortic rings were mounted in standard organ bath chambers. In a first set of experiments, concentration-response curves (CRCs) to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were obtained in arteries pretreated in the absence or presence of a selective PDE inhibitor (1 μ M Cil for PDE3 or 10 μ M Ro for PDE4). In a second set of experiments, aortic rings were submaximally precontracted with $PGF_{2\alpha}$ and CRCs were performed using increasing concentrations of either the muscarinic agonist carbachol, the selective PDE inhibitors [Cil or Ro] or the β -AR agonist isoprenaline in the presence of an α -AR antagonist (phentolamine, 10 μ M) (Leblais *et al.*, 2008). In some cases, these experiments were performed in arteries pretreated in the presence of the following agents: the PDE3 inhibitor Cil (1 μ M), the PDE4 inhibitor Ro (10 μ M) or the NOS inhibitor N ω -nitro-L-arginine-methyl ester (L-NAME; 300 μ M).

Contractile responses were expressed in grams as a difference from baseline tone. Vasorelaxant responses were expressed as the percentage of the precontraction evoked by $PGF_{2\alpha}$.

Cyclic nucleotide measurements

Cyclic AMP and cGMP content were determined by an enzyme immunoassay (monoclonal anti-cAMP and anti-cGMP EIA kits, NewEast Biosciences) on lysates obtained from rings incubated with the selective PDE inhibitors (1 μ M Cil for PDE3 or 10 μ M Ro for PDE4) or the vehicle. As positive controls, some rings were incubated with L-858051 (10 μ M) or sodium nitroprusside (SNP; 1 μ M) in the presence of 100 μ M IBMX. Results are expressed in pmole of cAMP or cGMP per ring.

Data and statistical analysis

All data are expressed as mean \pm SEM, where “n” represents the number of rats, except for Figure 3 and Figure 8 where “n” represents the number of vessels. Differences between CRCs

were analysed using a two-way repeated-measures analysis of variance (ANOVA). Pharmacological parameters were compared using Student's *t*-test. Values of $P < 0.05$ were considered for statistical significance.

Results

Anatomic, echocardiographic and blood pressure parameters

Twenty-two weeks after surgery, stenosed rats exhibited a decreased body weight and an increase in heart and lungs weight normalized to the tibia length, compared to SHAM animals. Signs of congestive HF (i.e. ascites, pleural effusions and oedema) were also observed in these stenosed rats. Echocardiographic analysis showed an increase in left ventricular mass by 80% ($P<0.01$) and a 2-fold decrease in fractional shortening ($P<0.001$) after stenosis (Francis *et al.*, 2003). Overall, these observations provide evidence for the presence of left ventricular hypertrophy associated with major cardiac dysfunction in stenosed rats confirming the occurrence of severe HF. Furthermore, stenosed rats displayed a decrease in blood pressure parameters by about 14 to 18% compared to SHAM animals (Francis *et al.*, 2003).

cAMP-PDE activity in aorta from SHAM and HF rats

Total cAMP-PDE activity was similar in intact or endothelium-denuded aorta isolated from SHAM and HF rats (Figure 1A). In intact aorta isolated from SHAM rats, the total cAMP-PDE activity was barely decreased by the PDE1 inhibitor, MIMX (10 μ M), but not modified by the PDE2 inhibitor, BAY (100 nM), suggesting the absence of PDE2 activity (Figure 1B). Both PDE3 (Cil, 1 μ M) and PDE4 (Ro, 10 μ M) inhibitors reduced the cAMP hydrolytic activity by 48% ($P<0.01$) and 28% ($P=0.05$), respectively. Finally, the broad-spectrum PDE inhibitor, IBMX (1 mM), almost completely abolished the total cAMP-PDE activity ($P<0.01$). This suggests that the rank order of PDE families contributing to the global cAMP-PDE activity in SHAM aortas is PDE3>PDE4>>PDE1. Endothelium removal of SHAM aortas did not affect this pattern of cAMP hydrolyzing activities (Figure 1B). Aortas isolated from HF rats exhibited similar cAMP-PDE family activities compared to aortas isolated from SHAM rats. Overall, endothelium removal had not effect in HF aortas, except that it significantly lowered the

decrease in cAMP-PDE activity elicited by the addition of Ro ($P<0.05$) (Figure 1B).

Expression PDE3 and PDE4 families in aorta from SHAM and HF rats

We then evaluated the impact of HF on the vascular expression of the two main PDE families, PDE3 and PDE4, by the Western blot technique. Two PDE3A isoforms of 98 and 120 kDa were detected (Figure 2A), in accordance with literature (Abi-Gerges *et al.*, 2009), especially in vascular cells (Zhao *et al.*, 2008). Expression of the 98 kDa isoform was significantly increased in aorta isolated from HF compared to SHAM rats, whereas the expression of the 120 kDa isoform tended to increase although the difference did not achieve significance (Figure 2D). Using a PDE3B polyclonal antibody, we detected a main band around 125 kDa (Figure 2B). We confirmed the identity of this band as PDE3B protein, by using HEK cells overexpressing PDE3B (data not shown). Expression of aortic PDE3B protein was similar in SHAM and HF animals (Figure 2D). Using a PDE4B polyclonal antibody, we detected a band of 68 kDa (Figure 2C) which was lost in aorta isolated from *Pde4b*^{-/-} mice compared to their wild-type littermates (data not shown). A 68 kDa PDE4B isoform has been previously reported in vascular cells (Zhao *et al.*, 2008). PDE4B expression was significantly raised in aorta isolated from HF compared to SHAM rats (Figure 2D).

We also evaluated the pattern of PDE gene expression by RT-PCR. PDE3B and PDE4B mRNAs were significantly increased in aortas isolated from HF compared to SHAM rats (Figure 2E). PDE3A mRNA was slightly although not significantly higher in HF aortas (40% increase compared to SHAM aortas, n=6), whereas PDE4D mRNA expression was similar in both groups (Figure 2E).

Vascular function in aortas isolated from SHAM and HF rats

The contractile response induced by the 60 mM KCl depolarizing solution in intact aortic rings

was significantly higher in HF than SHAM animals ($P<0.001$) (Figure 3A). Endothelium removal increased this response in both groups, by 18.5% in SHAM ($P<0.001$) and 10.5% in HF ($P<0.01$). The CRC to $\text{PGF}_{2\alpha}$, a vasoconstrictor agent acting through stimulation of prostanoid receptors, was significantly shifted to the left in intact aorta from HF rats compared to SHAM rats (Figure 3B). Endothelium removal caused a significant leftward shift of the CRC to $\text{PGF}_{2\alpha}$ in aortas from SHAM rats without any change in the maximum response (Figure 3B). In HF aortas, the curves were not significantly different in the presence or absence of endothelium, although the pD_2 value was weakly increased after endothelium removal (Figure 3B). In $\text{PGF}_{2\alpha}$ -precontracted aortic rings, the endothelial NO-dependent relaxation elicited by the muscarinic agonist carbachol was severely impaired in aortas from HF rats (Figure 3C). These results provide evidence that aortas isolated from HF rats exhibit signs of vascular dysfunction, with hyper-reactivity to contractile agents and endothelial dysfunction.

Effect of pretreatment with PDE3 and PDE4 inhibitors on the contractile response to $\text{PGF}_{2\alpha}$ in aortas isolated from SHAM and HF rats

In intact arteries isolated from SHAM and HF rats, pretreatment with the PDE3 inhibitor Cil (1 μM) similarly shifted to the right the contractile response curves to $\text{PGF}_{2\alpha}$ compared to their respective controls, without modification of the maximum contraction (Figures 4A and B). The inhibitory effect of Cil on the $\text{PGF}_{2\alpha}$ -induced contraction was preserved in endothelium-denuded rings from both groups (Figures 4A and B). These data indicate that PDE3 inhibition similarly decreases the sensitivity to $\text{PGF}_{2\alpha}$ in aortas from SHAM and HF rats, independently of the presence of a functional endothelium.

In intact aortas from SHAM rats, the PDE4 inhibitor Ro (10 μM) decreased the contractile response to $\text{PGF}_{2\alpha}$. In endothelium-denuded arteries, however, the effect of Ro was abolished (Figure 4C). In HF aorta, Ro treatment did not alter the contractile response to $\text{PGF}_{2\alpha}$.

either in the presence or absence of endothelium (Figure 4D). Thus, to counter aortic contraction, PDE4 inhibition requires an intact and functional endothelium.

Effect of PDE3 and PDE4 inhibitors in precontracted aortas isolated from SHAM and HF rats

To further elucidate the role of PDE3 and PDE4, CRCs to Cil and Ro were obtained in rat aorta submaximally pre-contracted with PGF_{2α}. In aortas isolated from SHAM rats, Cil induced a concentration-dependent relaxation, which was increased to a similar extent after endothelium removal or pretreatment with 300 μM L-NAME (Figure 5A). This indicates that in SHAM aortas, removing endothelial function or reducing NO production facilitates the relaxant response to the PDE3 inhibitor, suggesting that endothelial NO exerts an inhibitory control on PDE3 activity. In HF aortas, Cil induced a similar relaxation to that observed in SHAM aortas [relaxation at the maximum tested concentration of Cil (30 μM): 54.6±7.2% (n=13) and 46.7±4.9% (n=13), respectively; not significantly different], which was markedly potentiated after endothelium removal and to a lower extent in the presence of L-NAME (Figure 5B). This indicates that in HF aortas, the endothelium-dependent inhibitory control of PDE3 activity is maintained, but only partly mediated by NOS activity.

In PGF_{2α}-precontracted SHAM aorta, increasing concentrations of Ro produced a concentration-dependent relaxation, which was abolished by L-NAME or removal of the endothelium (Figure 5C), indicating that the relaxant effect induced by PDE4 inhibition requires a functional endothelium and NO. In HF aortas, Ro had no effect on the pre-contractile tone, neither in control conditions nor after incubation with L-NAME or endothelium removal (Figure 5D).

We then evaluated the effect of a pretreatment of the arteries with the PDE3 inhibitor Cil (1 μM for 10 min prior to PGF_{2α} application) on the relaxant response to Ro. The

pretreatment with Cil significantly enhanced the Ro-induced relaxation in SHAM aorta, and restored a relaxation in endothelium-denuded arteries to a similar extent as observed in intact arteries (Figure 4E). In HF rats, Cil pretreatment restored a strong relaxant response to Ro in both intact and -denuded aortas (Figure 5F). These results indicate that PDE4 function is under the negative control of PDE3.

β-adrenergic vasorelaxant response in aortas isolated from SHAM and HF rats

In endothelium-intact SHAM aortas precontracted with $\text{PGF}_{2\alpha}$, the non-selective β -AR agonist isoprenaline produced a concentration-dependent vasorelaxation [pD_2 : 7.5 ± 0.2 (n=13)] which was abolished in the presence of L-NAME or after endothelium removal (Figure 6A). In HF aortas, the relaxant effect of isoprenaline was absent even in arteries with intact endothelium (Figure 6B). Thus, aortic β -AR relaxation requires a functional endothelial NO pathway which is lost in HF aortas due to endothelial dysfunction.

Role of PDE3 and PDE4 in the β-adrenergic vasorelaxant response in aortas isolated from SHAM and HF rats

As shown in Figure 7A, Cil pretreatment increased the maximum relaxation to isoprenaline by 16% ($P < 0.05$) in endothelium-intact SHAM aortas. In HF aortas, Cil treatment unveiled a strong relaxation to isoprenaline (Figure 7B), which was similar to that observed in SHAM rats arteries in the presence of Cil. In endothelium-denuded aortas (Figure 7C and D) or under NOS inhibition by L-NAME (Figure 7E and F), incubation with Cil restored a relaxation to isoprenaline in both SHAM and HF groups. These results indicate that the aortic β -AR relaxation is negatively controlled by PDE3 activity. In HF aortas, the loss of β -AR relaxation is unmasked when PDE3 is inhibited.

As shown in Figure 7A, Ro treatment significantly increased the isoprenaline relaxation

in endothelium-intact SHAM aortas. In intact HF aortas, Ro restored a small relaxant response to isoprenaline (Figure 7B). By contrast, Ro treatment did not restore the isoprenaline-induced relaxation that was impaired by endothelium removal (Figure 7C and D) or NOS inhibition (Figure 7E and F). These results indicate that the aortic β -AR relaxation is also negatively controlled by PDE4 activity which is largely dependent on the endothelium functionality. In HF aortas, the loss of β -AR relaxation is unmasked by PDE4 inhibition albeit to a lower extent than with PDE3 inhibition.

Effect of PDE3 and PDE4 inhibitors on cAMP and cGMP levels in aortas isolated from SHAM and HF rats

Basal cAMP levels were similar in intact aorta rings isolated from SHAM and HF rats (2.2 ± 0.4 pmole/ring, n=6, *versus* 2.8 ± 0.6 pmole/ring, n=6, respectively). Direct stimulation of adenylyl cyclase with L-858051 in the presence of IBMX strongly raised cAMP levels by 25 and 32 fold in SHAM and HF aortas, respectively (data not shown, n=5). Ro treatment but not Cil treatment induced a significant 2.5 fold increase in cAMP levels in SHAM intact aortas (Figure 8A). This effect was absent in intact HF aortas, and deeply reduced in endothelium-denuded SHAM aortas (Figure 8A).

In HF aortas, basal cGMP levels were slightly reduced by 25% compared to SHAM aortas (0.8 ± 0.2 pmole/ring, n=6, *versus* 1.1 ± 0.3 pmole/ring, n=5, respectively). Endothelium denudation decreased cGMP levels by 38% and 58% in HF and SHAM aortas, respectively (0.5 ± 0.2 pmole/ring, n=5, and 0.4 ± 0.1 pmole/ring, n=3, respectively). Treatment with the NO donor SNP in the presence of IBMX strongly enhanced cGMP levels by 28 and 17 fold in SHAM and HF aortas, respectively (data not shown, n=5). By contrast, Cil and Ro treatments had no significant effect on cGMP levels in any groups of SHAM and HF aortas (Figure 8B).

Discussion

In this study, we characterized the activity, expression and functional role of the cAMP-PDE isoforms in aortas isolated from SHAM and HF rats. Our main results can be summarized as follows: 1) PDE3 and PDE4 are the main PDE families responsible for cAMP hydrolysis in SHAM and HF aortas; 2) HF alters aortic PDE3 and PDE4 family expression; 3) PDE3 inhibition with Cil induces a vasorelaxation in pre-contracted SHAM and HF aortas whether the endothelium is present or not; 4) moreover, the relaxant effect induced by Cil is potentiated when the endothelium is removed or upon NOS inhibition; 5) PDE4 inhibition with Ro induces an increase in cAMP level and a vasorelaxation in SHAM but has no effect in HF aorta; 6) these effects of Ro are absent when the endothelium is removed or upon NOS inhibition; 7) however, a large vasorelaxant effect of Ro is revealed in endothelium-denuded SHAM aorta as well as in HF aorta when PDE3 is inhibited; 8) β -AR stimulation induces an endothelium- and NOS-dependent vasorelaxation in SHAM aorta but has no effect in HF; 9) the β -AR vasorelaxant response is increased in SHAM and unveiled in HF aorta by PDE3 or PDE4 inhibition, but only by PDE3 inhibition in denuded aortas. We conclude that cAMP metabolism plays an important role in the endothelial regulation of vascular tone. Endothelial-derived NO, by increasing cGMP level in SMCs, limits the cAMP hydrolytic activity of PDE3 and contributes to maintaining a fine tuning between the activities of PDE3 and PDE4. This mechanism also determines the amplitude of the β -AR vasorelaxant response. In HF, this modulation is lost due to endothelial dysfunction: thus, PDE3 activity in SMCs is increased which makes the contribution of PDE4 negligible, blocks PKA-mediated relaxation and abolishes the β -AR vasorelaxant response. Inhibition of vascular PDE3 may thus represent an attractive approach to restore a proper vasorelaxation in HF.

We used a rat model of cardiac chronic pressure overload which undergoes with time the transition from cardiac hypertrophy to HF (Abi-Gerges *et al.*, 2009; Joubert *et al.*, 2008).

This animal model mimics HF in patients with stenosis of the aortic valve (Muders *et al.*, 2000). To our knowledge, systemic blood pressure has never been reported in this rat model of HF. Here, we observed that HF rats exhibited a decrease in blood pressure, which might be correlated to the decompensated stage of HF. The aortas isolated at the HF stage exhibited clear signs of vascular dysfunction, with hyper-reactivity to contractile agents and endothelial dysfunction linked to an inhibition of the NOS/NO pathway. This is a hallmark of cardiovascular diseases including HF (Francis *et al.*, 1990; Nakamura *et al.*, 2001; Negrao *et al.*, 2000), although the mechanisms leading to this dysfunction are unclear, including neurohumoral activation, oxidative stress, haemodynamics alterations and vascular remodeling (Indik *et al.*, 2001; McGoldrick *et al.*, 2007; Muders *et al.*, 2000). By using an *in vitro* biochemical assay, we found that the rank order of cAMP hydrolyzing activity in rat aorta was PDE3>PDE4>>PDE1, whereas PDE2 activity was not detected. This pattern is in agreement with the literature (Maurice *et al.*, 2003; Polson *et al.*, 1996). Aortas isolated from HF rats exhibited a similar total cAMP hydrolyzing activity and PDE isoform contribution. Endothelium removal did not affect cAMP-PDE activities in SHAM aortas, suggesting either that the contribution of endothelium to the vascular PDE activity is minor compared to that of SMCs, or that the sensitivity of the assay is insufficient to detect this endothelial contribution. However, in HF aortas, PDE4 activity was found to be decreased in the absence of endothelium. We also observed that HF stage is associated with alterations of vascular PDE3 and PDE4 family expression, essentially an increase in PDE3A and PDE4B proteins. The apparent discrepancy between biochemical activity and protein expression might be explained either by a too small increase in protein expression to be detectable in activity or by a local confinement of these new PDE proteins dedicated to one particular function. Takahashi *et al.* (2002) observed an increase in PDE3 activity without modification of PDE4 activity in the intact aorta from Dahl salt-sensitive rat model of HF. Thus, the alteration in vascular PDE activity might

depend on the HF model used.

Both PDE3 and PDE4 were found to control the vascular tone in precontracted SHAM aorta but their contribution differed in several ways. PDE3 inhibition with Cil induced a relaxant effect which did not require a functional endothelium. However, this effect was potentiated by NOS inhibition or when the endothelium was removed. The most likely explanation for this is that NO released from intact endothelium elevates cGMP in SMCs and this leads to a partial inhibition of PDE3 which minimizes the effect of Cil. By contrast, the vasorelaxant effect of a PDE4 inhibition with Ro was abolished when the endothelium was removed (when PDE3 was uninhibited) and was increased in the presence of Cil (when PDE3 was inhibited). This suggests that, when fully active, PDE3 largely dominates over PDE4. A somewhat similar conclusion was reached by Komasa *et al.* (1991) in noradrenaline- and PGF_{2α}-precontracted arteries. Surprisingly, PDE3 inhibition was not associated with an increase in intracellular level of cAMP or cGMP levels, raising the question of its vasorelaxant mechanism. However, this is in agreement with literature showing an increase in cAMP level only after PDE4 inhibition (Eckly *et al.*, 1994a). This supports the idea that functional responses elicited by cyclic nucleotides require local instead of diffused changes in their concentrations.

Another difference between the functional role of PDE3 and PDE4 was observed in HF. While the relaxant effect of a PDE3 inhibition was similar in aortas from SHAM and HF rats, PDE4 inhibition did not relax aortas from HF rats. This loss of function for PDE4 in HF aortas was similar to that observed in endothelium-denuded SHAM aortas and was therefore likely due to the endothelial dysfunction in HF. Interestingly, PDE3 inhibition restored a significant relaxant response to PDE4 inhibition in both intact and endothelium-denuded HF aortas. This supports the hypothesis that PDE4 function is under the negative control of PDE3 and that in HF aortas, PDE3 activity masks that of PDE4. This is not in contradiction with our finding that global PDE3 cAMP-hydrolytic activity was similar in SHAM and HF aortas, because the *in*

vitro assay in lysates and controlled buffer solution cannot reveal a change in cellular PDE3 activity by a soluble factor such as cGMP. Alternatively, changes in PDE3 activity may occur in a subcellular compartment, e.g. in the vicinity of PDE4, which would not be detectable in biochemical assays.

Several studies in systemic and/or pulmonary arteries isolated from different models of HF animals have reported a decrease in the β -AR-mediated vasorelaxation (Mathew *et al.*, 1993; McGoldrick *et al.*, 2007; Nasa *et al.*, 1996). In our model of HF, we also observed a loss of aortic β -AR relaxation. This loss of response was likely a consequence of the endothelial dysfunction since in SHAM aorta the β -AR response was abolished when the endothelium was removed or upon NOS inhibition. A similar loss of the β -AR response in the absence of a functional endothelium was reported in earlier studies and this led the authors to conclude that the response involved β -ARs located on the endothelial cell surface (Gray *et al.*, 1992; Kamata *et al.*, 1989). However, our results contradict this conclusion. Indeed, we found that the β -AR vasorelaxation was totally rescued in the absence of endothelium, both in SHAM and HF aortas, when PDE3 was inhibited. Therefore, we propose a different mechanism by which NO released from the endothelium leads to SMC PDE3 inhibition by cGMP, and this acts as a brake on cAMP degradation to allow SMC β -AR/cAMP/~~PKA~~ pathway to convey relaxation. In HF, because of endothelial dysfunction, the brake is released and PDE3 becomes fully active and serves as a sink for cAMP to prevent it from activating ~~PKA and~~ relaxation. Thus, only a pharmacological inhibition of PDE3 can restore β -AR vasorelaxation, as also observed in a different pathological situation such as restenosis after balloon angioplasty (Zhao *et al.*, 2007).

Conclusions

Our study underlines a key role of the vascular endothelium on smooth muscle PDEs and contractile function. Endothelial dysfunction in HF exacerbates smooth muscle PDE3 activity

and this prevents β -AR relaxation. Inhibition of vascular PDE3 may thus represent an attractive therapeutic approach to restore a proper vasorelaxation in HF. A limit of our study is that aorta is a conductance artery which is not critically involved in the regulation of blood pressure. Thus, further studies would be required to investigate PDE contractile function in HF resistance arteries. However, our study clearly shows that in HF, endothelial dysfunction leads to alteration of smooth muscle PDE function. This phenomenon could be a common feature of cardiovascular diseases associated with endothelial dysfunction.

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Conflict of interest

None declared.

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Table 1

Anatomic, echocardiographic and blood pressure parameters from SHAM and HF rats.

Anatomic parameters	SHAM (n=48)	HF (n=43)	P
Body weight (g)	601 ± 10	566 ± 11	<0.05
Heart weight (g)	1.81 ± 0.03	3.85 ± 0.11	<0.001
Tibia length (cm)	4.47 ± 0.02	4.44 ± 0.02	NS
Heart weight/tibia length (mg.cm⁻¹)	404 ± 6	859 ± 25	<0.001
Lung weight/tibia length (mg.cm⁻¹)	397 ± 6	908 ± 37	<0.001
Liver weight/tibia length (g.cm⁻¹)	3.82 ± 0.08	3.93 ± 0.11	NS
Echocardiographic parameters	SHAM (n=10)	HF (n=9)	P
Left ventricular mass (mg)	1104 ± 73	1993 ± 238	<0.01
Fractional shortening (%)	48.5 ± 2.3	24.4 ± 2.7	<0.001
Blood pressure	SHAM (n=9)	HF (n=6)	P
Diastolic blood pressure (mmHg)	89.5 ± 2.7	77.1 ± 3.9	<0.05
Systolic blood pressure (mmHg)	131.6 ± 3.1	107.6 ± 4.4	<0.001
Mean blood pressure (mmHg)	103.2 ± 2.7	86.9 ± 4	<0.01

Values are mean ± SEM. n = number of animals. *P*: statistical differences between SHAM and HF groups, NS: non significant (Student's *t*-test).

Figure legends

Figure 1

cAMP-PDE activity in aorta with or without functional endothelium, isolated from SHAM and HF rats. (A): Total cAMP-PDE activity was determined in the presence of 1 μ M [3 H]-cAMP in lysates of SHAM and HF endothelium-intact (*ENDO+*) or endothelium-denuded (*ENDO-*) aortas. (B): cAMP-PDE activity pattern was determined in the absence (*vehicle*) or presence of a selective PDE family inhibitor (PDE1: 10 μ M MIMX; PDE2: 100 nM BAY; PDE3: 1 μ M Cil; PDE4: 10 μ M Ro) or a non-selective PDE inhibitor (IBMX: 1 mM). Results are expressed in % of cAMP-PDE activity measured in the absence of inhibitors. Data are means \pm SEM of 3 independent experiments. * P <0.05, ** P <0.01 *versus* corresponding vehicle group. # P <0.05 between *ENDO+* and corresponding *ENDO-* group.

Figure 2

Expression of PDE3 and PDE4 proteins (A-D) and mRNAs (E) in aorta isolated from SHAM and HF rats. (A-C): Representative Western blot images showing PDE3A, PDE3B, PDE4B and β -actin expression in aorta from SHAM and HF rats. (D): Corresponding graph showing relative expression level of PDE3A, PDE3B, PDE4B proteins. Results are expressed in % of the mean expression level in SHAM group. Data are means \pm SEM of 9-12 SHAM and 9-11 HF rats, detected in 3-4 independent immunoblots. (E): PDE3A, PDE3B, PDE4B and PDE4D mRNA expression in aorta from SHAM and HF rats. Results are expressed in % of the mean expression level in SHAM group. Data are means \pm SEM of 6 SHAM and 6 HF rats. * P <0.05 between SHAM and HF.

Figure 3

Evaluation of vascular reactivity in aorta isolated from SHAM and HF rats with or without

functional endothelium. (A): Contractile response to a depolarizing solution of 60 mM KCl in SHAM and HF rat aortas, either with intact endothelium (*ENDO+*) or endothelium-denuded (*ENDO-*). (B): CRCs to $\text{PGF}_{2\alpha}$ (1 nM to 30 μM) in SHAM and HF *ENDO+* and *ENDO-* aortas. (C): Relaxant-response curves to carbachol (1 nM to 10 μM) on $\text{PGF}_{2\alpha}$ -precontracted *ENDO+* aortas isolated from SHAM and HF rats. Data are means \pm SEM. $^{##}P<0.01$, $^{###}P<0.001$ between *ENDO+* and corresponding *ENDO-* group; $^{$$}P<0.01$, $^{$$$}P<0.001$ between SHAM and corresponding HF group.

Figure 4

Effects of PDE3 or PDE4 inhibition on $\text{PGF}_{2\alpha}$ -induced contraction in aorta isolated from SHAM and HF rats with or without functional endothelium. CRCs to $\text{PGF}_{2\alpha}$ (1 nM to 30 μM) were performed in SHAM (A and C) and HF (B and D) aortas with intact endothelium (*ENDO+*) or endothelium-denuded (*ENDO-*), in the absence (*control*) or presence of selective PDE inhibitors: (A-B): 1 μM Cil for PDE3; (C-D): 10 μM Ro for PDE4. Data are means \pm SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ between control and PDE inhibitor-treated in each group.

Figure 5

Effect of L-NAME, Cil and endothelium removal on PDE3 or PDE4 inhibition-induced relaxant response in precontracted aorta isolated from SHAM and HF rats. (A-D): CRCs to Cil (1 nM to 30 μM ; A and B) or Ro (1 nM to 30 μM ; C and D) were performed in endothelium-intact arteries pretreated in the absence (*ENDO+/control*) or presence of the NOS inhibitor (300 μM L-NAME; *ENDO+/+ L-NAME*) and in endothelium-denuded arteries (*ENDO-/control*) isolated from SHAM (A and C) and HF (B and D) rats and precontracted with $\text{PGF}_{2\alpha}$. (E-F): CRCs to Ro (1 nM to 30 μM) were performed either in arteries with intact endothelium

(*ENDO+*) or endothelium-denuded (*ENDO-*) arteries isolated from SHAM (E) and HF (F) rats, and pretreated in the absence (*control*) or presence of 1 μ M Cil. Data are means \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 between control and L-NAME- or PDE inhibitor-treated in each group. # P <0.05, ## P <0.01, ### P <0.001 between *ENDO+* and corresponding *ENDO-* group.

Figure 6

Effect of L-NAME and endothelium removal on β -adrenergic-induced relaxant response in precontracted aorta isolated from SHAM and HF rats. CRCs to isoprenaline (0.01 nM to 30 μ M) were performed in arteries with intact endothelium pretreated in the absence (*ENDO+/control*) or presence of the NOS inhibitor (300 μ M L-NAME), and in endothelium-denuded arteries (*ENDO-/control*), isolated from SHAM (A) and HF (B) rats and precontracted with $\text{PGF}_{2\alpha}$. Data are means \pm SEM. *** P <0.001 between control and L-NAME. ### P <0.001 between *ENDO+* and *ENDO-*.

Figure 7

Effect of PDE3 or PDE4 inhibition on β -adrenergic-induced relaxant response in precontracted aorta isolated from SHAM and HF rats. CRCs to isoprenaline (0.01 nM to 30 μ M) were performed in intact (A-B), in endothelium-denuded (C-D) or in L-NAME pretreated (E-F) aortas isolated from SHAM (A, C, E) or HF (B, D, F) rats, pretreated in the absence (*control*) or presence of the PDE3 inhibitor (1 μ M Cil), or the PDE4 inhibitor (10 μ M Ro). Data are means \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 between control and PDE inhibitor-treated in each group.

Figure 8

Effect of PDE3 or PDE4 inhibition on cAMP and cGMP levels in aorta isolated from SHAM

and HF rats. Cyclic AMP (A) and cGMP (B) levels were determined in lysates of SHAM and HF intact (*ENDO+*) or endothelium-denuded (*ENDO-*) rings pretreated in the absence (*vehicle*) or presence of the PDE3 inhibitor (1 μ M Cil) or the PDE4 inhibitor (10 μ M Ro). Results are expressed in % of mean cyclic nucleotides levels measured in the absence of inhibitors. Data are means \pm SEM of n rings from different animals. ** $P < 0.01$ versus corresponding vehicle group. # $P < 0.05$ between *ENDO+* and corresponding *ENDO-* group.

Figure 1

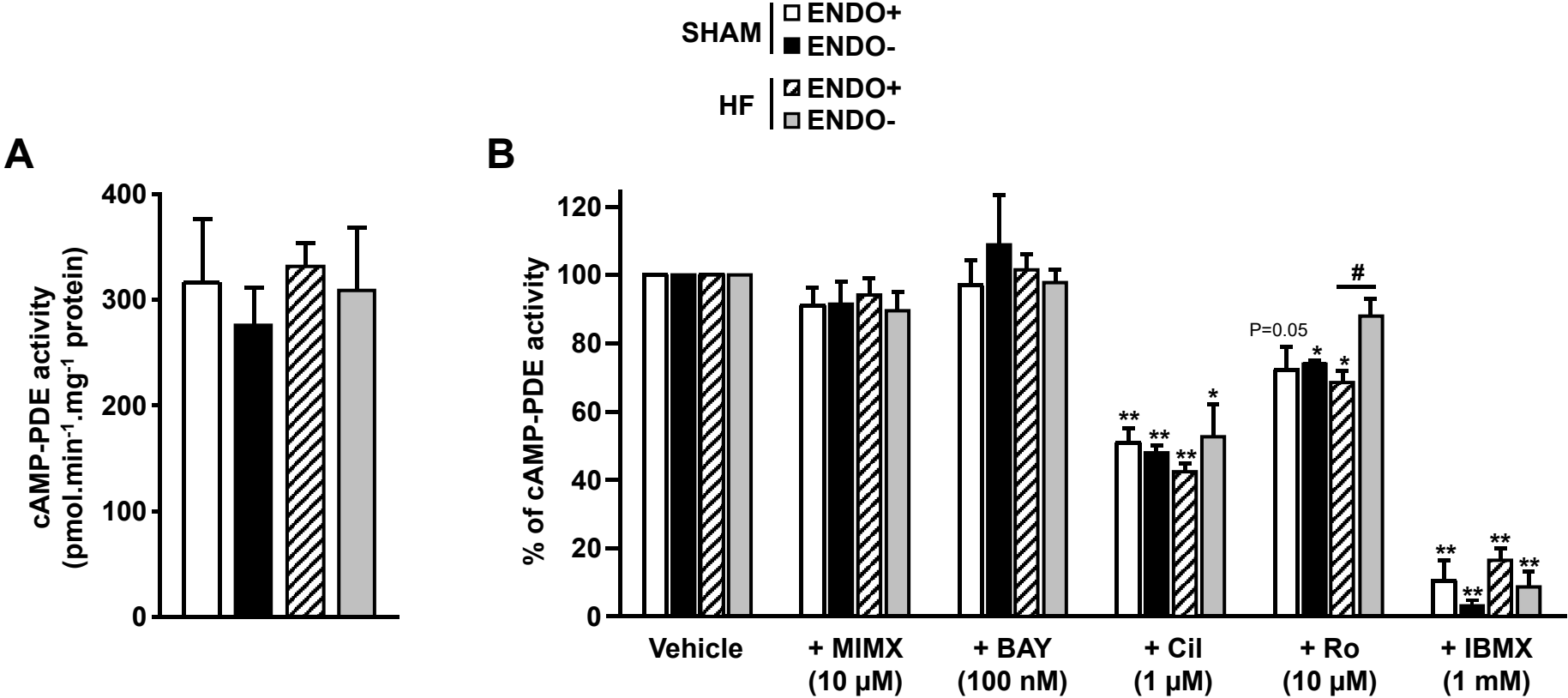


Figure 2

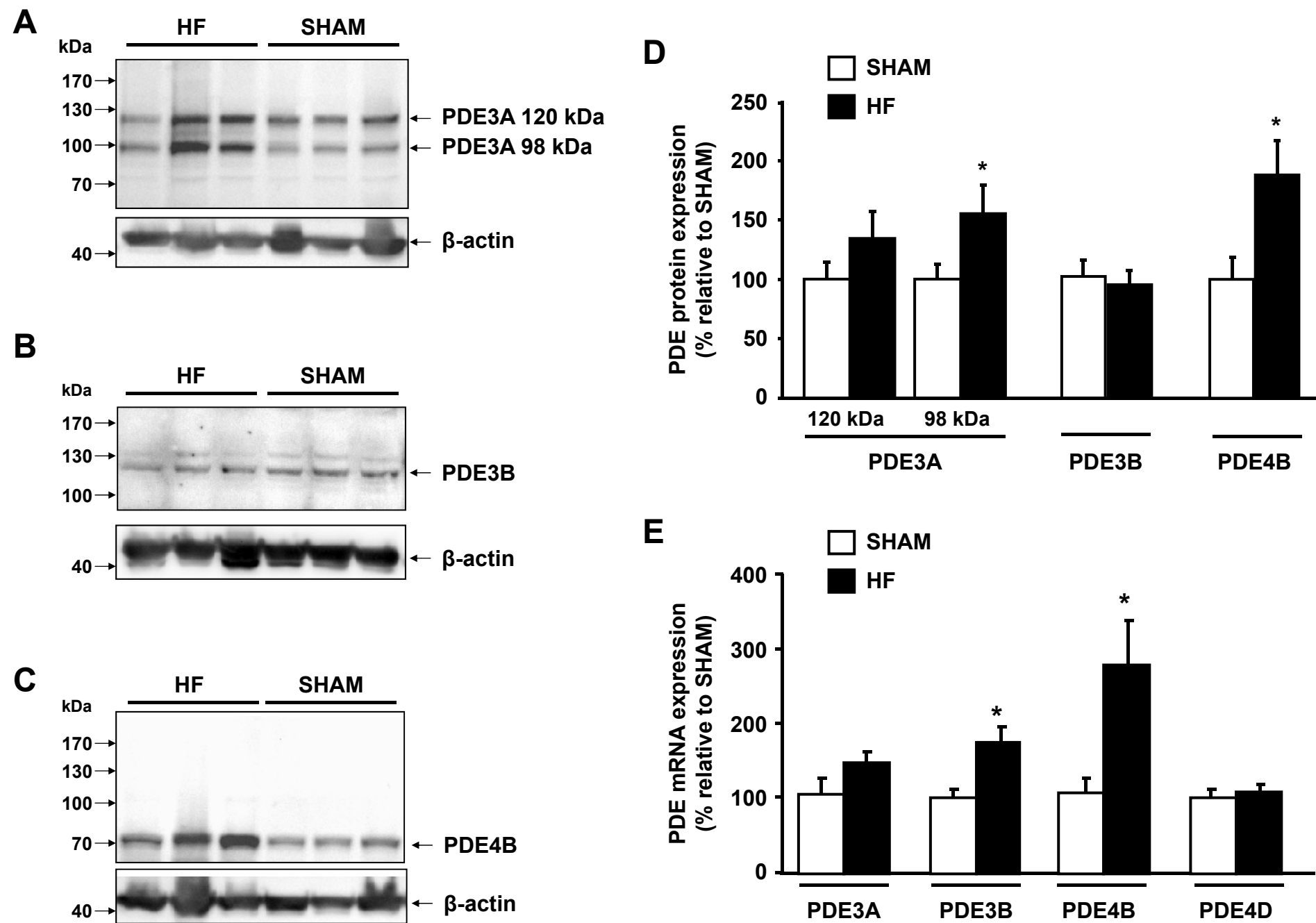


Figure 3

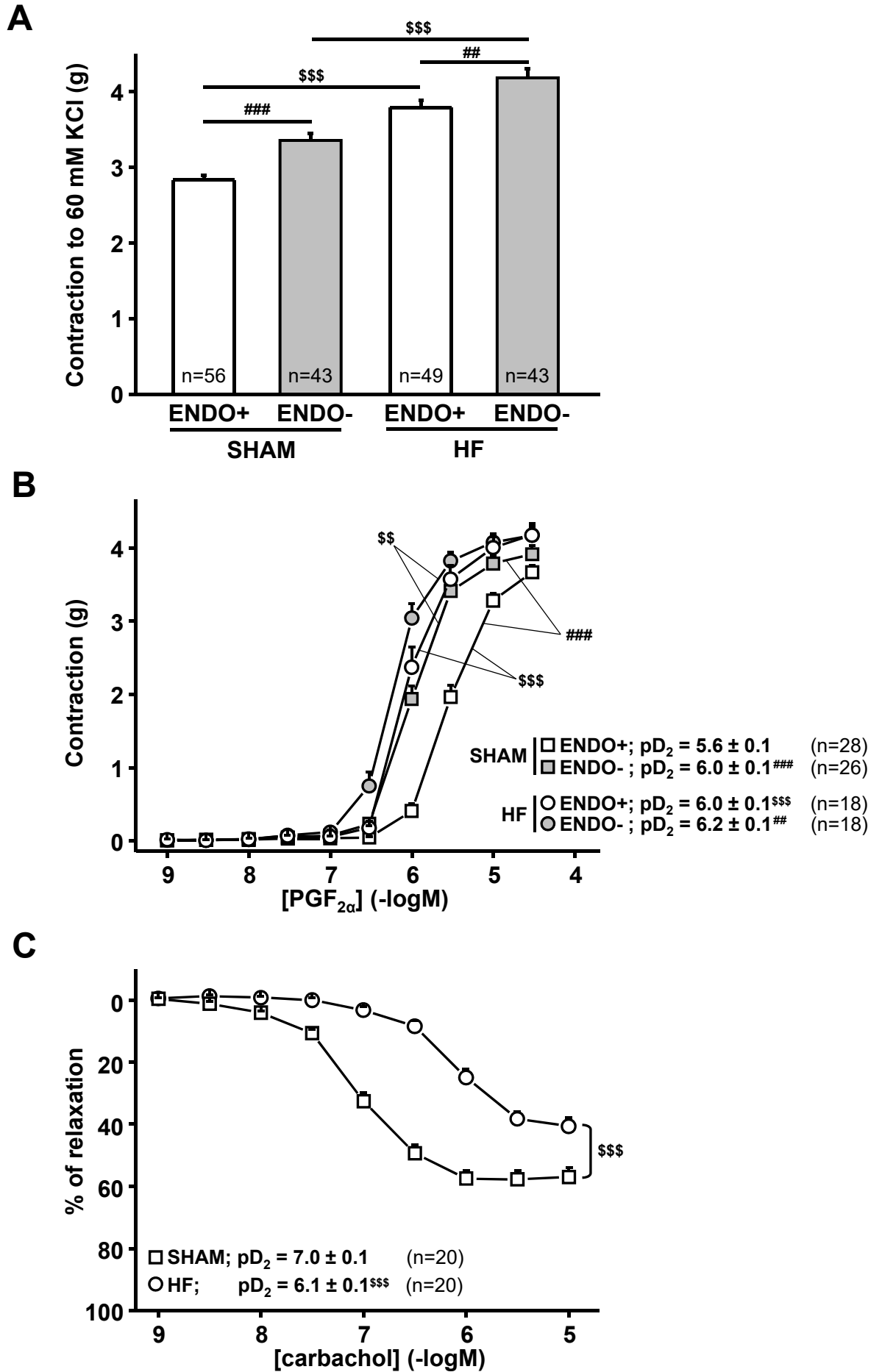


Figure 4

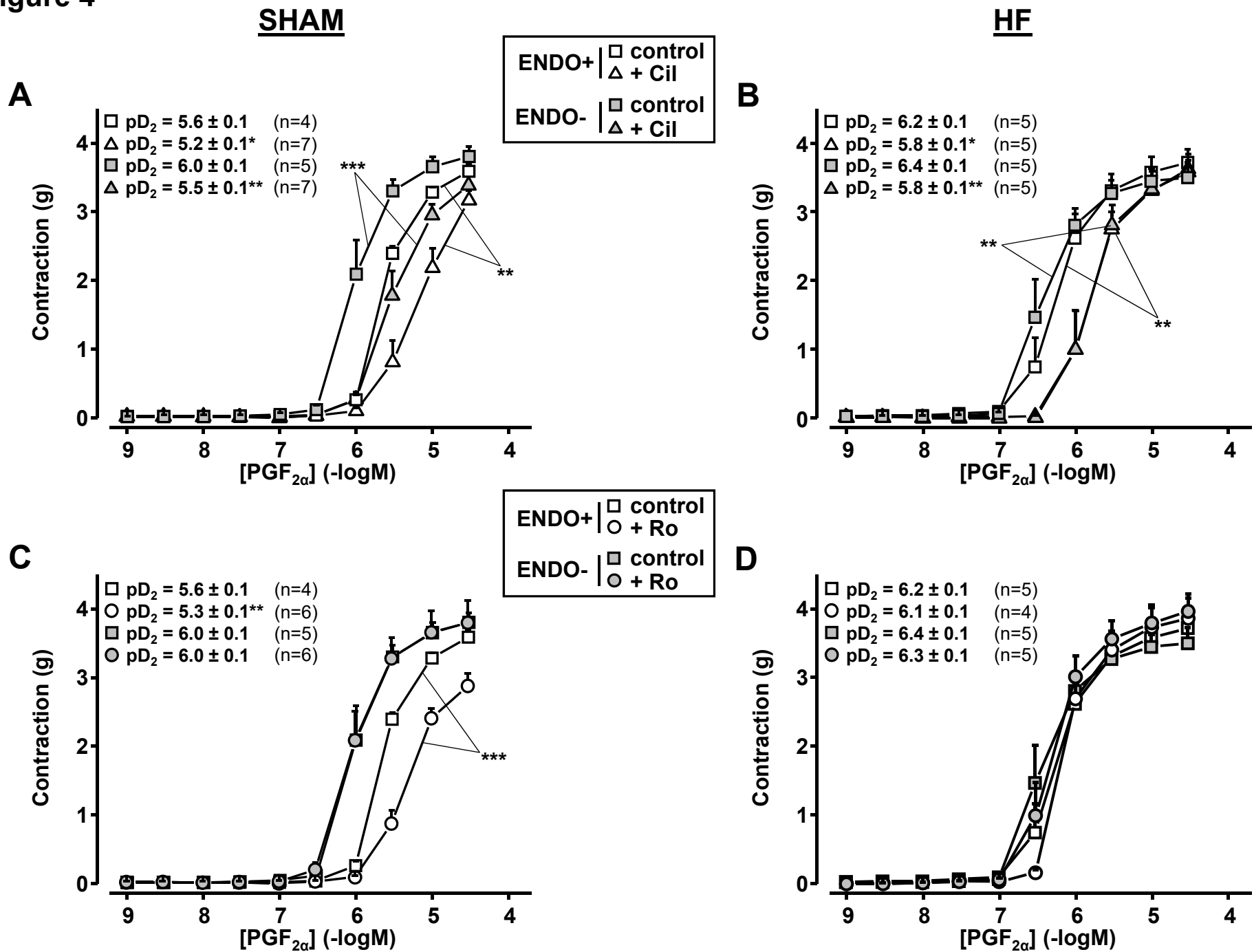


Figure 5

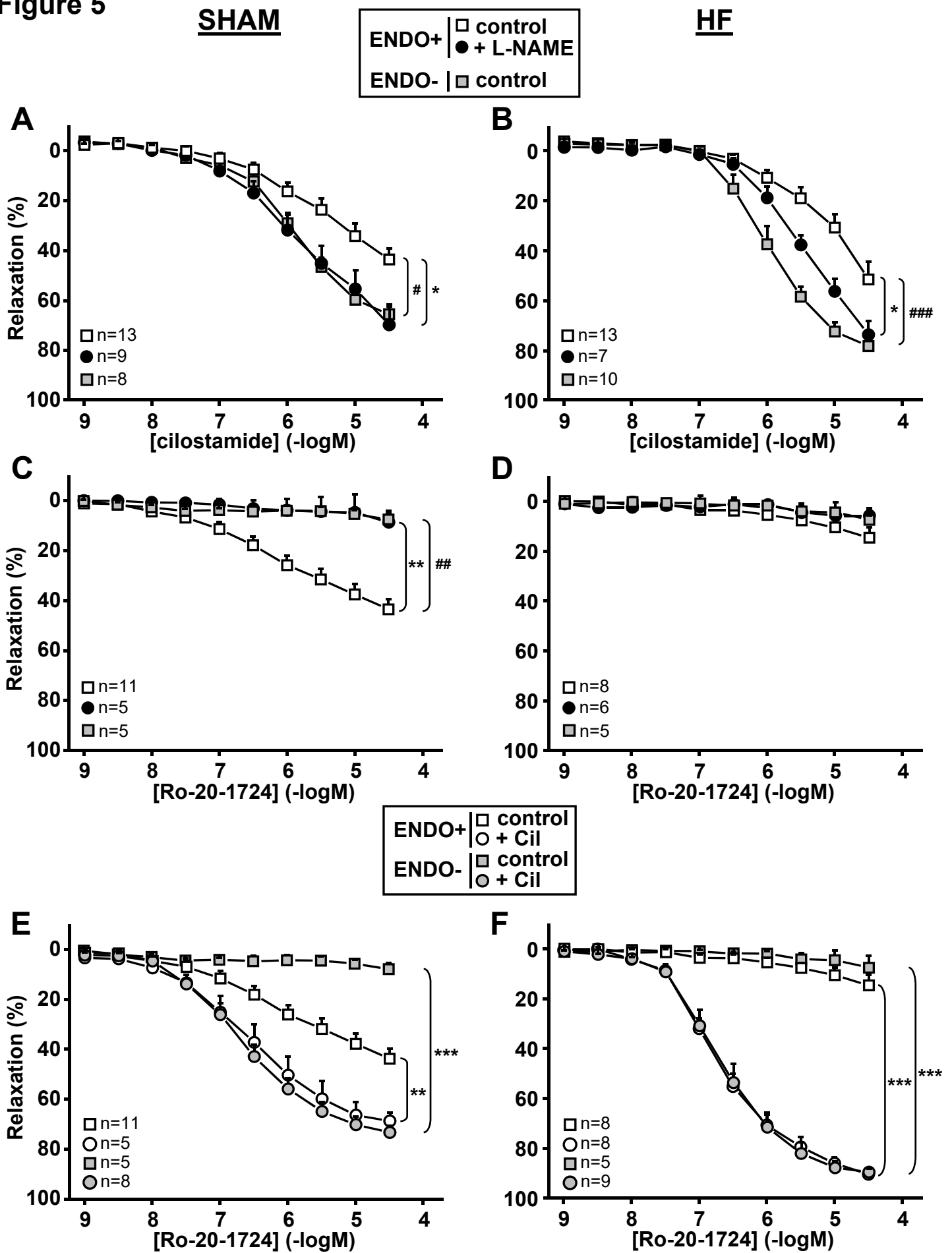


Figure 6

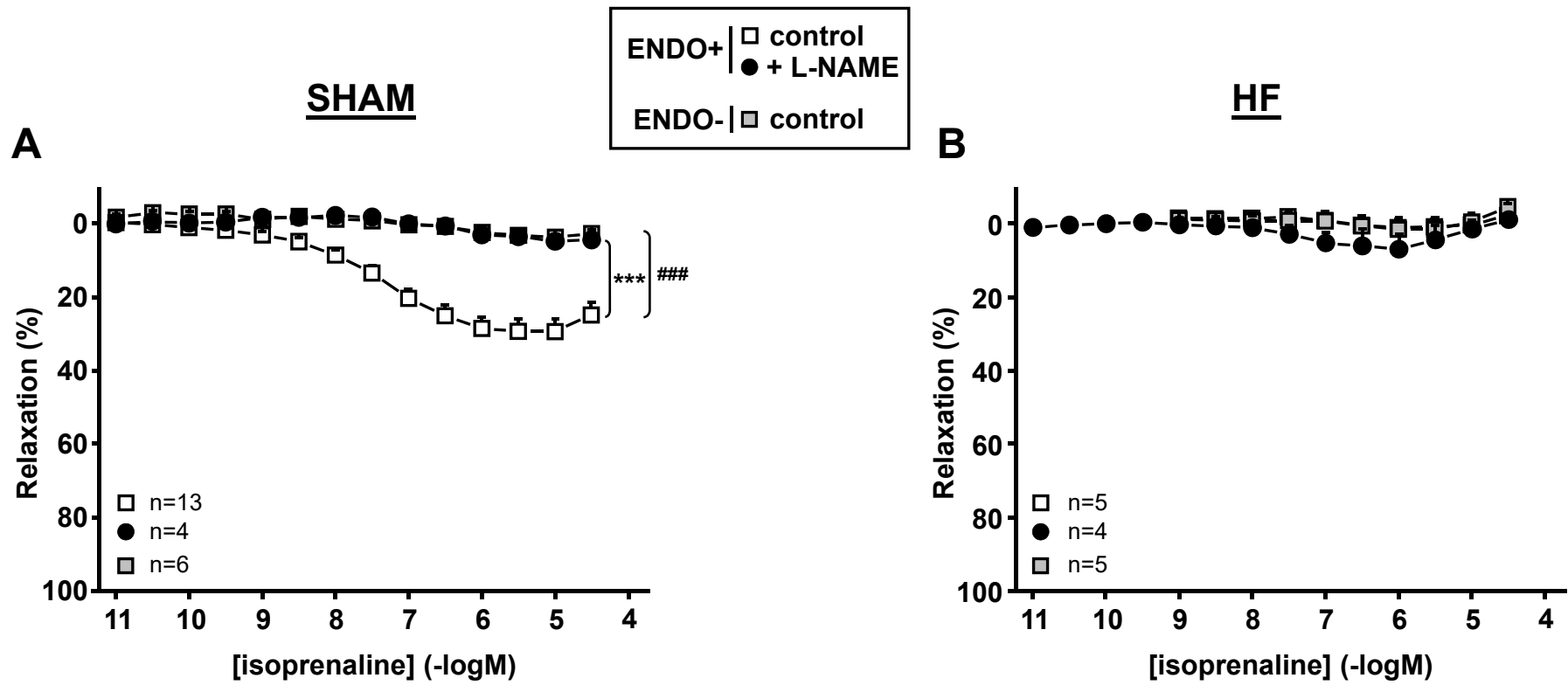


Figure 7

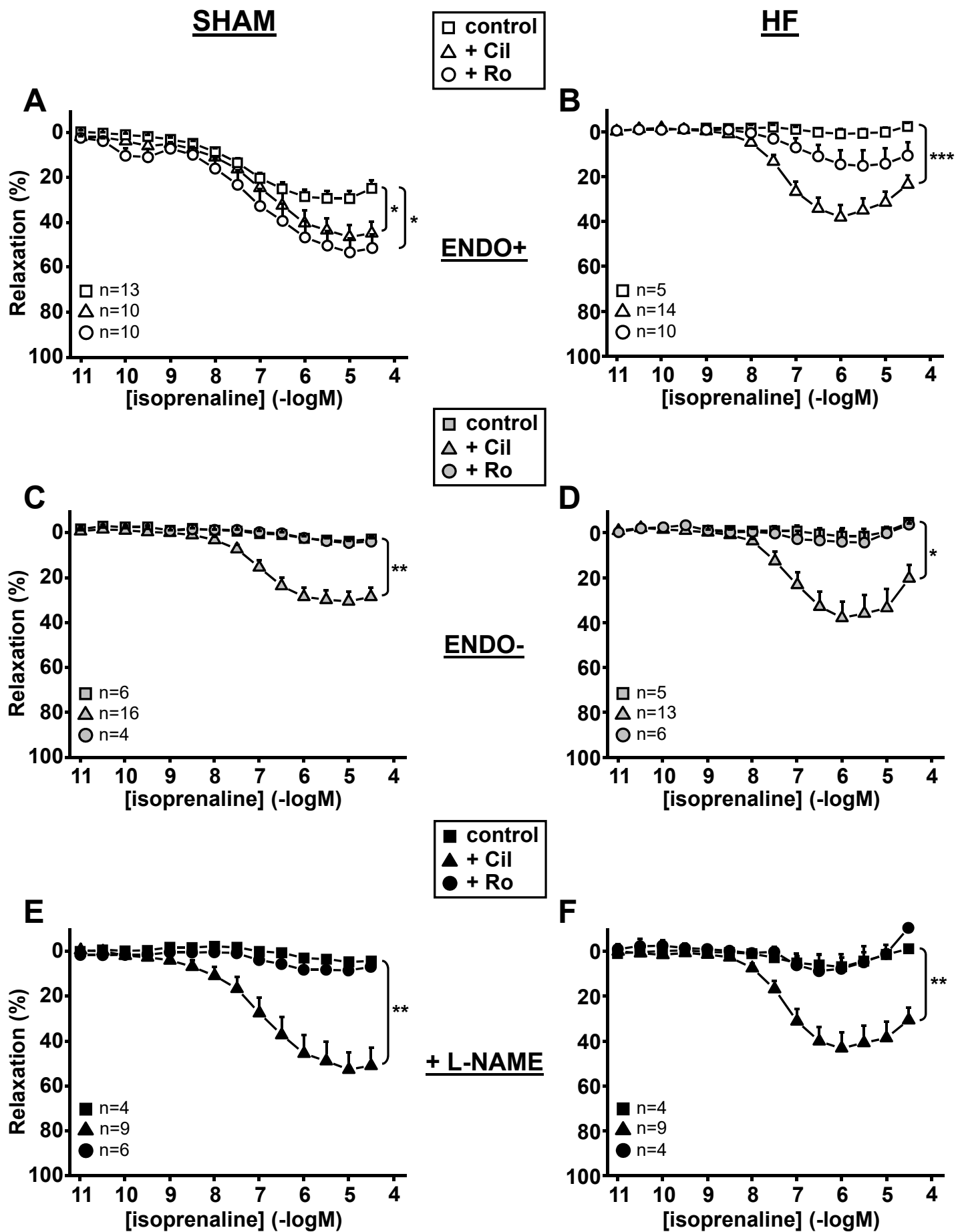
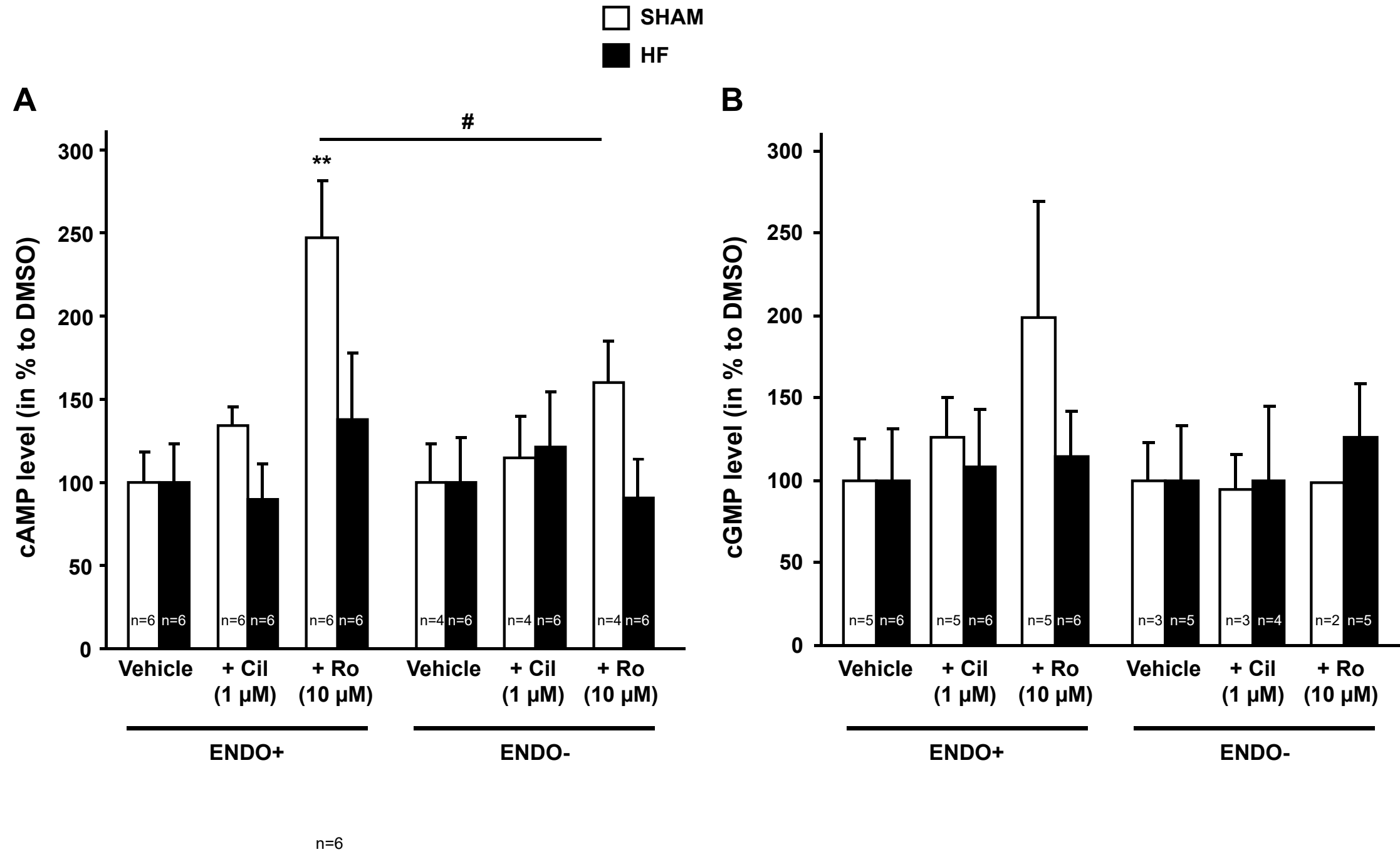


Figure 8



SUPPLEMENTARY MATERIAL

Alteration of vascular reactivity in heart failure:

Role of phosphodiesterases type 3 and 4

Short running title: Vascular reactivity and cAMP-PDE in heart failure

Authors: F Hubert^{1,2,*}, M Belacel-Ouari^{1,2}, B Manoury^{1,2}, K Zhai^{1,2,*}, V Domergue-Dupont^{2,3}, P Mateo^{1,2}, F Joubert^{1,2}, R Fischmeister^{1,2}, V Leblais^{1,2}.

¹*Inserm UMR-S 769, LabEx LERMIT, DHU TORINO, F-92296 Châtenay-Malabry, France,*

²*Université Paris-Sud, Faculté de Pharmacie, F-92296 Châtenay-Malabry, France, and*

³*IPSIT/IFR141, Plateforme « Animalerie et exploration fonctionnelle », F-92296 Châtenay-Malabry, France.*

* *Current address for:* F H, Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Canada; and K Z, National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China.

Correspondence to: V Leblais, UMR-S 769, LabEx LERMIT, DHU TORINO, Faculté de Pharmacie, Université Paris-Sud, 5 rue J.-B. Clément 92296 Châtenay-Malabry, France.

E-mail: veronique.leblais@u-psud.fr; Tel: +33-1.46.83.59.06; Fax: +33-1.46.83.54.75.

SUPPLEMENTARY METHODS

Methods

All experiments performed conform to the European Community guiding principles in the care and use of animals (Directive 2010/63/EU of the European Parliament). Authorizations to perform animal experiments according to this decree were obtained from the French Ministry of Agriculture, Fisheries and Food (no. D- 92-283, December 13, 2012). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Surgical procedure

Experiments were performed on a total of 91 male Wistar rats (Elevage Janvier, Le Genest St Isle, France). The surgical procedure was carried out on 3-week-old rats, under anaesthesia with pentobarbital sodium (200 mg.kg⁻¹, i.p.). The depth of anaesthesia was checked by toe pinch. Aortic stenosis was induced by placing a stainless steel hemoclip (0.6 mm-internal diameter) on the ascending aorta via thoracic incision, as previously described {Joubert, Wilding, et al. 2008 #27424}. Age-matched control animals (SHAM-operated) underwent the same procedure without placement of the clip.

Echocardiography and blood pressure measurement

Echocardiography was performed at 22 weeks after surgery on 10 SHAM-operated and 9 stenosed rats using a 12 MHz transducer (Vivid 7, General Electric Healthcare) under 2.5% isoflurane gas anesthesia. Two-dimensional-guided (2D) M-mode echocardiography was used to determine wall thickness and left ventricular chamber diameter at systole and diastole, and contractile parameters such as fractional shortening. Left ventricular mass (LVM) was

calculated according to the Penn formula home-adapted for the rat heart: $(LVM = 1.04 \times [(Dtd + SIV + PP)^3 - (Dtd)^3])$ {Prunier, Gaertner, et al. 2002 #27476}.

Arterial blood pressure was measured in 9 SHAM-operated and 6 stenosed conscious rats using a tail-cuff system (CODA™, Kent Scientific) in a dark temperature-controlled room (22°C). Animals were trained for 3 days. They were placed in individual holders. An occlusion cuff and a volume pressure-recording cuff were applied close to the base of the tail. Each day, 20 sequential readings were obtained and the last 10 readings were averaged. Volume pressure recording allowed the noninvasive measurement of 3 blood pressure parameters simultaneously: systolic, diastolic and mean blood pressures.

Rat aorta sampling

Twenty-two weeks after surgery, rats were anesthetized with pentobarbital sodium (200 mg.kg⁻¹, i.p.). The descending thoracic aorta was isolated, placed in ice-cold physiological salt solution [PSS in mM: NaCl 119, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.17, KH₂PO₄ 1.18, glucose 11, NaHCO₃ 25], cleaned of adventitial tissue and cut into 2 mm length rings. In some preparations, the endothelium was removed by gently rubbing the intimal surface with fine forceps. Heart, lungs and liver were also removed and weighed. The right tibia was isolated to measure its length using a vernier calliper. For biochemical studies, aortic rings were frozen in liquid nitrogen.

Cyclic AMP-PDE activity assay

Cyclic AMP-PDE activity was measured according to the method described by Thompson and Appleman (1971), as previously reported {Zhai, Hubert, et al. 2012 #27203}. Frozen aortic rings were homogenized using a tissue homogenizer (Precellys®24, Bertin Technologies) in ice-cold lysis buffer [NaCl 150 mM, HEPES 20 mM, EDTA 2 mM, glycerol

10%, NP40 0.5% and microcystin 1 μ M]. Samples (4 μ g proteins) were assayed in a reaction mixture of 200 μ L containing 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM β -mercaptoethanol, 1 μ M cAMP, and 10^5 cpm of [3H]-cAMP for 25 min at 33°C. To evaluate cAMP-PDE family-specific activities, the assay was performed in the absence or presence of selective PDE inhibitors: 10 μ M 8-methoxymethyl-3-isobutyl-1-methylxanthine (MIMX) for PDE1 {Rich, Tse, et al. 2001 #20397}, 100 nM BAY-60-7550 (BAY) for PDE2 {Boess, Hendrix, et al. 2004 #24503}, 1 μ M Cilostamide (Cil) for PDE3 {Sudo, Tachibana, et al. 2000 #27462}, 10 μ M Ro-20-1724 (Ro) for PDE4 or 1 mM 3-isobutyl-1-methylxanthine (IBMX) as a non-selective PDE inhibitor {Rich, Tse, et al. 2001 #20397}. The residual hydrolytic activity observed in the presence of PDE inhibitors was expressed as a percentage of the total cAMP-PDE activity, corresponding to the cAMP-PDE activity in the absence of inhibitor (vehicle: 0.02% DMSO).

Western blot analysis

Frozen aortic rings were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors (Complete Protease Inhibitor Cocktail, Roche) using the tissue homogenizer and centrifuged at 3,000 g and 4°C for 5 min. Supernatants were heated at 95°C for 5 min in the presence of Laemmli buffer and β -mercaptoethanol. Proteins were resolved by electrophoresis on a 8% SDS-PAGE gel and transferred to a PVDF membrane (Millipore). The membranes were blocked for 1 h with Tris-buffered saline-Tween 20 (TBST) containing 5% skim milk at room temperature and then were incubated overnight at 4°C with antibodies directed against PDEs [PDE3A (1:10000; gift from Dr. Chen Yan, Columbia University, NY, USA), PDE3B (1:1000; gift from Dr. Emilio Hirsch, University of Torino, Italy), PDE4B (1:10000; gift from Dr. Marco Conti, University of California, San Francisco, USA)] or 2

hours at room temperature with antibody directed against β -actin (1:20000; sc-47778, Santa Cruz Biotechnology). After washing and 1 hour incubation at room temperature with the goat anti-rabbit IgG-HRP (1:10000; sc-2004, Santa Cruz Biotechnology), final detection was performed using chemiluminescence (Pierce ECL Western blotting substrate, Thermo Scientific). PDE signal was normalized to β -actin signal.

Quantitative RT-PCR analysis

Frozen aortic rings were homogenized in ice-cold TRIZOL Reagent (Gibco). Then total RNA extraction, cDNA synthesis and quantitative PCR were performed according to the procedure previously described by Zhai *et al.* {Zhai, Hubert, et al. 2012 #27203}. mRNAs encoding 4 PDE subtypes (PDE3A, PDE3B, PDE4B and PDE4D) and 2 housekeeping genes (TBP: Tata Box Binding Protein, and Ywhaz: 14-3-3 protein zeta/delta) were analyzed. PDE gene expression level was calculated using the comparative threshold (Ct) method ($2^{\Delta Ct}$).

Vascular reactivity measurement

Aorta rings were mounted in standard organ bath chambers filled with PSS maintained at 37°C and aerated with 95% O₂ and 5% CO₂ (pH 7.4). Vessels were passively stretched to their optimal resting force (2 g) and equilibrated for 60 min. Vessel integrity was evaluated by its contractile response to a 60 mM KCl depolarizing solution (PSS containing 60 mM KCl by equimolar substitution with NaCl). Rings developing a contraction below 1.5 g were discarded. Endothelial functionality was assessed by the relaxant response to carbachol (10 μ M) on 60 mM KCl-precontacted rings. The amplitude of relaxation was >20% and 10% in intact arteries isolated from SHAM and HF rats, respectively, and abolished in endothelium-denuded rings. Rings were then equilibrated for 60 min in PSS.

In a first set of experiments, concentration-response curves (CRCs) to increasing concentrations of the vasoconstrictor agent prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 1 nM to 30 μ M) were obtained in arteries pretreated in the absence or presence of a selective PDE inhibitor (1 μ M Cil for PDE3 or 10 μ M Ro for PDE4) for 10 min.

In a second set of experiments, aortic rings were submaximally precontracted with a concentration of $PGF_{2\alpha}$ inducing about 80% of their own contractile response to the 60 mM KCl depolarizing solution. The mean $PGF_{2\alpha}$ concentrations used in the different groups of aortas are 2.9 ± 0.1 μ M (n=37) and 2.0 ± 0.0 μ M (n=26) in intact aortas isolated from SHAM and HF rats, respectively, and 1.5 ± 0.3 μ M (n=19) and 1.2 ± 0.2 μ M (n=20) in endothelium-denuded aortas isolated from SHAM and HF rats, respectively. When the contractile response was stable, CRCs to increasing concentrations of a vasorelaxant drug were established with the muscarinic agonist carbachol (1 nM to 10 μ M), the selective PDE inhibitors [Cil (1 nM to 30 μ M) or Ro (1 nM to 30 μ M)] or the β -AR agonist isoprenaline (10 pM to 30 μ M) in the presence of the α -AR antagonist (10 μ M phentolamine) to avoid possible influence of α -ARs {Leblais, Delannoy, et al. 2008 #25385}. In some cases, these experiments were performed in arteries pretreated in the presence of the following agents: the PDE3 inhibitor Cil (1 μ M for 10 min), the PDE4 inhibitor Ro (10 μ M for 10 min) or the NOS inhibitor N ω -nitro-L-arginine-methyl ester (L-NAME; 300 μ M for 15 min).

Contractile responses were expressed in grams as a difference from baseline tone. Vasorelaxant responses were expressed as the percentage of the precontraction evoked by $PGF_{2\alpha}$.

Cyclic nucleotide measurements

Rat aortic rings were equilibrated in PSS at 37°C for 1 hour. Since an effective removal of endothelium could not be functionally tested, the NOS inhibitor L-NAME (300 μ M) was

added to endothelium-denuded rings. Rings were incubated for 30 min with the selective PDE inhibitors (1 μ M Cil for PDE3 or 10 μ M Ro for PDE4) or the vehicle (0.03% DMSO). As positive controls, some rings were incubated with the hydrophylic forskolin analog L-858051 (10 μ M for 15 min) or sodium nitroprusside (SNP; 1 μ M for 10 min) in the presence of 100 μ M IBMX. Rings were rapidly collected in the lysis buffer (0.1 M HCl and 1% Triton X-100) and frozen in liquid nitrogen. Each ring was homogenized using the tissue homogenizer and the homogenate centrifuged at 12,000 g for 10 min at 4°C. Cyclic AMP and cGMP content were determined on the supernatant by an enzyme immunoassay (monoclonal anti-cAMP and anti-cGMP EIA kits, NewEast Biosciences). Absorbance was read at 450 nm on a spectrophotometer (Infinite® 200 PRO, TECAN). Results are expressed in pmole of cAMP or cGMP per ring.

Data and statistical analysis

EC₅₀ value (concentration that produces 50% of the maximum response) was estimated, when relevant, in each individual CRC using the Boltzman equation fit (Origin 6 software) and converted to negative logarithm value (pD₂). All data are expressed as mean \pm SEM, where “n” represents the number of rats, except for Figure 3 where “n” represents the number of vessels. Differences between CRCs were analysed using a two-way repeated-measures analysis of variance (ANOVA). Different parameters were compared using Student’s *t*-test. Values of $P < 0.05$ were considered for statistical significance.

Pharmacological agents

PGF_{2 α} (Dinoprost tromethamine, Dinoprost®) was obtained from Pfizer Animal Health (Paris, France). Carbamylcholine chloride (carbachol), IBMX, (-)-isoproterenol hydrochloride (isoprenaline), L-NAME, phentolamine hydrochloride and SNP were purchased from Sigma

Aldrich (St Quentin, Fallavier, France). BAY was from Cayman Chemical (Bertin Pharma, Montigny-le-Bretonneux, France), Cil from Tocris Bioscience (Bristol, UK), MIMX and Ro from Calbiochem (Merck Chemicals Ltd, Nottingham, UK), and L-858051 from Biomol International (Enzo Life Sciences, Villeurbanne, France). When pharmacological inhibitors were dissolved in non water vehicle, control experiments were performed in the presence of equivalent concentration of vehicle.