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RESEARCH PAPER

Alteration of vascular reactivity in heart failure: role of phosphodiesterases 3 and 4

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BACKGROUND AND PURPOSE

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

EXPERIMENTAL APPROACH

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

KEY RESULTS

HF rat aortas exhibited signs of endothelial dysfunction, with alterations of the NO pathway, and alteration of PDE3 and PDE4 subtype expression, without changing total aortic cAMP-hydrolytic activity and PDE1, PDE3 and PDE4 activities. Vascular reactivity experiments using PDE inhibitors showed that PDE3 and PDE4 controlled the level of $PGF_{2\alpha}$-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 and restored by PDE3 inhibition. In $PGF_{2\alpha}$-precontracted arteries, β-adrenoceptor stimulation-induced relaxation in SHAM aorta, which was abolished in the absence of functional endothelium, as well as in HF aortas, but 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 had a major effect on PDE3 function and PDE3 inhibition restored a functional relaxation to β-adrenoceptor stimulation.

Abbreviations

BAY, BAY-60-7550; cAMP-PDE, cAMP-hydrolysing PDE; CRC, concentration–response curve; HF, heart failure; MIMX, 8-methoxymethyl-3-isobutyl-1-methylxanthine; PSS, physiological salt solution; Ro, Ro-20-1724; SMC, smooth muscle cell; SNP, sodium nitroprusside

Tables of Links

TARGETS	LIGANDS
β-Adrenoceptors	BAY-60-7550
PDE3A	Cilostamide
PDE3B	IBMX
PDE4B	Isoprenaline
PDE4D	Ro-20-1724

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b)

Introduction

In the vascular system, cAMP is a key physiological second messenger that inhibits contraction, proliferation and migration of the smooth muscle cells (SMCs). The intracellular concentration of cAMP is determined by the balance between its production by adenylyl cyclase (AC) and degradation by phosphodiesterases hydrolysing cAMP (cAMP-PDEs).

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

The cAMP-mediated relaxation can be decreased by the degradation of cAMP through the action of PDEs. PDEs comprise a large group of more than 50 isoenzymes that are classified into 11 families. 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 and Strada, 1996; Zhai *et al.*, 2012). In rat aorta, both PDE3 and PDE4 inhibitors induce a vasorelaxation and potentiate the relaxation to β-adrenoceptor agonists (Komas *et al.*, 1991; Lugnier and Komas, 1993; Delpy *et al.*, 1996). These cAMP-mediated responses may be modulated by the endothelium and the NO/cGMP pathway, because cGMP inhibits PDE3 activity by competition with cAMP on its catalytic site (Lugnier and Komas, 1993; Delpy *et al.*, 1996).

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-adrenoceptor signalling 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 (Yanaka *et al.*, 2003; Abi-Gerges *et al.*, 2009; Mokni *et al.*, 2010) 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 endothelium-independent responses (Francis and Cohn, 1990; Negrao *et al.*, 2000; Nakamura *et al.*, 2001). 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 to the effects of HF on the vascular β-adrenoceptor/cAMP/PDE pathway. Most studies reported a decrease in the β-adrenoceptor-mediated vasorelaxation in systemic and/or pulmonary arteries isolated from different models of HF animals (Mathew *et al.*, 1993; Nasa *et al.*, 1996; McGoldrick *et al.*, 2007), which was in some cases related to a decrease in β-adrenoceptor density (Kiuchi *et al.*, 1993; Gaballa *et al.*, 2001). 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 effects of HF 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-adrenoceptor stimulation and to evaluate the effects of HF on these functions, and on the expression profile of the cAMP-PDEs.

Methods

Animals

All animal care and experimental procedures conformed to the European Community guiding principles in the care and use of animals (Directive 2010/63/EU of the European Parliament) and authorizations to perform animal experiments according to this decree were obtained from the French Ministry of Agriculture, Fisheries and Food (No. D-92-283, 13 December 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). A total of 106 animals were used in the experiments described here.

Detailed methods are included in Supporting Information Appendix S1.

Surgical procedure

Aortic stenosis was induced in male Wistar rats (60–70 g; Elevage Janvier, Le Genest St Isle, France) by placing a stainless steel haemoclip on the ascending aorta to induce HF, as previously described (Joubert *et al.*, 2008). SHAM-operated animals were used as controls.

Echocardiography and blood pressure measurement

Two-dimensional-guided M-mode echocardiography was performed at 22 weeks after surgery on 10 SHAM-operated and 9 HF rats, using a 12 MHz transducer (Vivid 7; General Electric Healthcare, Vélizy Villacoublay, France) under isoflurane gas anaesthesia. Arterial blood pressure was measured in 9 SHAM-operated and 6 HF conscious rats using a tail-cuff system (CODA™, Kent Scientific, Torrington, CT, USA).

Rat aorta sampling

Twenty-two weeks after surgery, rats were killed and the descending thoracic aorta excised and cut into rings, 2-mm long. 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), as previously reported (Zhai *et al.*, 2012). The radioenzymatic assay was performed in the absence or presence of selective PDE inhibitors: 10 μM 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 μM cilostamide for PDE3 (Sudo *et al.*, 2000), 10 μM Ro-20-1724 (Ro) for PDE4 or 1 mM 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, CA, USA) and β-actin (sc-47778; Santa Cruz Biotechnology, Dallas, TX, USA) were used. The PDE signal was normalized to the β-actin signal.

Quantitative RT-PCR analysis

mRNAs encoding four PDE subtypes (PDE3A, PDE3B, PDE4B and PDE4D) and two housekeeping genes [TBP (TATA box-binding protein) and Ywhaz: 14-3-3 protein zeta/delta] were analysed, as previously described (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 $PGF_{2\alpha}$ were obtained in arteries pretreated with or without a selective PDE inhibitor (1 μM cilostamide for PDE3 or 10 μM Ro for PDE4). In a second set of experiments, aortic rings were submaximally precontracted with $PGF_{2\alpha}$ and CRCs were conducted using increasing concentrations of either the muscarinic agonist carbachol, the selective PDE inhibitors, cilostamide or Ro, or the β-adrenoceptor agonist isoprenaline, in the presence of an α-adrenoceptor antagonist (10 μM phentolamine) (Leblais *et al.*, 2008). In some cases, these experiments were performed in arteries pretreated in the presence of the following agents: the PDE3 inhibitor cilostamide (1 μM), the PDE4 inhibitor Ro (10 μM) or the NOS inhibitor L-NAME (300 μM).

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 contents were determined by an enzyme immunoassay (monoclonal anti-cAMP and anti-cGMP EIA kits; NewEast Biosciences, King of Prussia, PA, USA) on lysates obtained from rings incubated with the selective PDE inhibitors (1 μM cilostamide for PDE3 or 10 μM Ro for PDE4) or the vehicle. As positive controls, some rings were incubated with L-858051 (10 μM) or sodium nitroprusside (SNP; 1 μM) in the presence of 100 μM IBMX. Results are expressed in pmol of cAMP or cGMP per ring.

Data and statistical analysis

All data are expressed as mean ± 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 ANOVA. Different parameters were compared using Student's t-test. Values of $P < 0.05$ were considered to show statistical significance.

Materials

$PGF_{2\alpha}$ (Dinoprost tromethamine, Dinoprost®) was obtained from Pfizer Animal Health (Paris, France). Carbamylcholine chloride (carbachol), IBMX, (-)-isoprenaline hydrochloride, L-NAME, phentolamine hydrochloride and SNP were purchased from Sigma Aldrich (St Quentin, Fallavier, France). BAY-60-7550 was from Cayman Chemical (Bertin Pharma, Montigny-le-Bretonneux, France), Cilostamide from Tocris Bioscience (Bristol, UK), MIMX and Ro-20-1724 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-aqueous vehicle, control experiments were performed in the presence of equivalent concentration of vehicle (DMSO).

Results

Anatomical, echocardiographic and blood pressure parameters

Twenty-two weeks after surgery, rats with aortic stenosis exhibited decreased body weight and increased heart and

Table 1

Anatomical, echocardiographic and blood pressure parameters from SHAM and HF rats

Anatomical 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)	90 ± 2.7	77 ± 3.9	<0.05
Systolic blood pressure (mmHg)	132 ± 3.1	108 ± 4.4	<0.001
Mean blood pressure (mmHg)	103 ± 2.7	87 ± 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).

lung weight normalized to the tibia length, compared with SHAM animals. Signs of congestive HF, such as ascites, pleural effusions and oedema, were also observed in these rats. Echocardiographic analysis showed an increase in left ventricular mass by 80% ($P < 0.01$) and a twofold decrease in fractional shortening ($P < 0.001$) after stenosis (Table 1). Overall, these observations provide evidence for the presence of left ventricular hypertrophy associated with major cardiac dysfunction in rats with aortic stenosis, confirming the occurrence of severe HF. Furthermore, these rats displayed a decrease in blood pressure parameters by about 14–18% compared with SHAM animals (Table 1).

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 μM), but not modified by the PDE2 inhibitor, BAY (100 nM), suggesting the absence of PDE2 activity (Figure 1B). Both PDE3 (cilostamide, 1 μM) and PDE4 (Ro, 10 μM) 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 was PDE3 > PDE4 >> PDE1. Removal of endothelium from SHAM aortas did not affect this pattern of cAMP-hydrolysing activities (Figure 1B). Aortas isolated from HF rats exhibited similar cAMP-PDE family activities compared with aortas isolated from SHAM rats. Overall, endothelium removal had no 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 of PDE3 and PDE4 families in aorta from SHAM and HF rats

We then evaluated the effects 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 previous reports in vascular cells (Zhao *et al.*, 2008) and myocardium (Abi-Gerges *et al.*, 2009). Expression of the 98 kDa isoform was significantly increased in aorta isolated from HF compared with 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) that was lost in aorta isolated from *Pde4b*$^{-/-}$ mice compared with 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 with that from 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 with SHAM rats (Figure 2E). PDE3A mRNA was slightly although not significantly higher in HF aortas (40% increase compared with

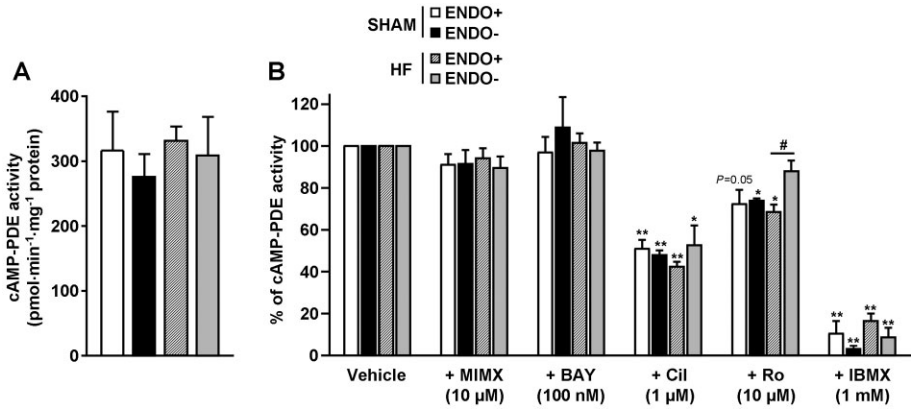

Figure 1

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 µM [^{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 µM MIMX; PDE2: 100 nM BAY; PDE3: 1 µM cilostamide (Cil); PDE4: 10 µM 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 ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, significantly different from vehicle group. $^{\#}P < 0.05$, significant effect of endothelial removal.

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 $PGF_{2\alpha}$, a vasoconstrictor agent acting through stimulation of prostanoid receptors, was significantly shifted to the left in intact aorta from HF rats compared with SHAM rats (Figure 3B). Endothelium removal caused a significant leftward shift of the CRC to $PGF_{2\alpha}$ 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 slightly increased after endothelium removal (Figure 3B). In $PGF_{2\alpha}$-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 $PGF_{2\alpha}$ in aortas isolated from SHAM and HF rats

In intact arteries isolated from SHAM and HF rats, pretreatment with the PDE3 inhibitor cilostamide (1 µM) similarly shifted to the right the contractile response curves to $PGF_{2\alpha}$ compared with their respective controls, without modification of the maximum contraction (Figure 4A and B). The inhibitory effect of cilostamide on the $PGF_{2\alpha}$-induced contraction was preserved in endothelium-denuded rings from both groups (Figure 4A and B). These data indicate that PDE3 inhibition similarly decreases the sensitivity to $PGF_{2\alpha}$ 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 $PGF_{2\alpha}$. However, in endothelium-denuded arteries, the effect of Ro was abolished (Figure 4C). In HF aorta, Ro treatment did not alter the contractile response to $PGF_{2\alpha}$, 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 cilostamide and Ro were obtained in rat aorta submaximally precontracted with $PGF_{2\alpha}$. In aortas isolated from SHAM rats, cilostamide 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 indicated that in SHAM aortas, removing endothelial function or reducing NO production facilitated the relaxant response to the PDE3 inhibitor, suggesting that endothelial NO exerted an inhibitory control on PDE3 activity. In HF aortas, cilostamide 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 significant], which was markedly potentiated after endothelium removal and to a lower extent in the presence of L-NAME (Figure 5B). This indicated that in HF aortas, the endothelium-dependent inhibitory control of PDE3 activity was maintained but only partly mediated by NOS activity.

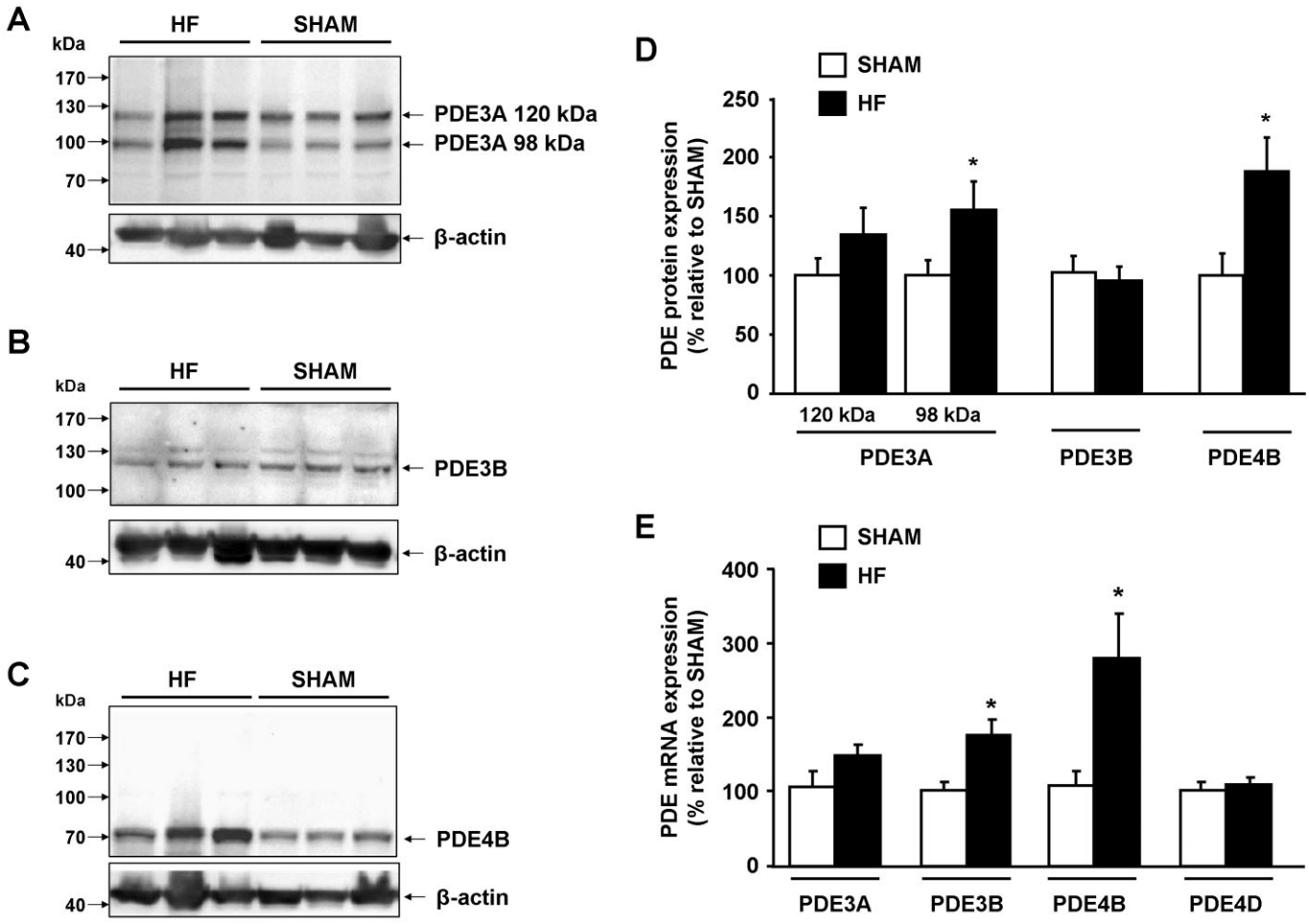

Figure 2

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 and PDE4B proteins. Results are expressed in % of the mean expression level in SHAM group. Data are means ± SEM of 9–12 SHAM and 9–11 HF rats, detected in three to four 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 ± SEM of six SHAM and six HF rats. *$P < 0.05$, significant effect of HF.

In $PGF_{2\alpha}$-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 precontractile tone, either in control conditions or 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 cilostamide (1 μM for 10 min prior to $PGF_{2\alpha}$ application) on the relaxant response to Ro. Pretreatment with cilostamide 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 5E). In HF rats, cilostamide pretreatment restored a strong relaxant response to Ro in both endothelium-intact and endothelium-denuded aortas (Figure 5F). These results indicated that PDE4 function was under the negative control of PDE3.

Relaxations to β-adrenoceptor stimulation in aortas isolated from SHAM and HF rats

In endothelium-intact rings of SHAM aortas precontracted with $PGF_{2\alpha}$, the non-selective β-adrenoceptor agonist isoprenaline produced a concentration-dependent relaxation [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 endothelium-intact arteries (Figure 6B). Thus, aortic relaxation to β-adrenoceptor stimulation requires a functional endothelial NO pathway which is lost in HF aortas due to endothelial dysfunction.

Role of PDE3 and PDE4 in the relaxation to β-adrenoceptor stimulation in aortas isolated from SHAM and HF rats

As shown in Figure 7A, cilostamide pretreatment increased the maximum relaxation to isoprenaline by 16% ($P < 0.05$) in

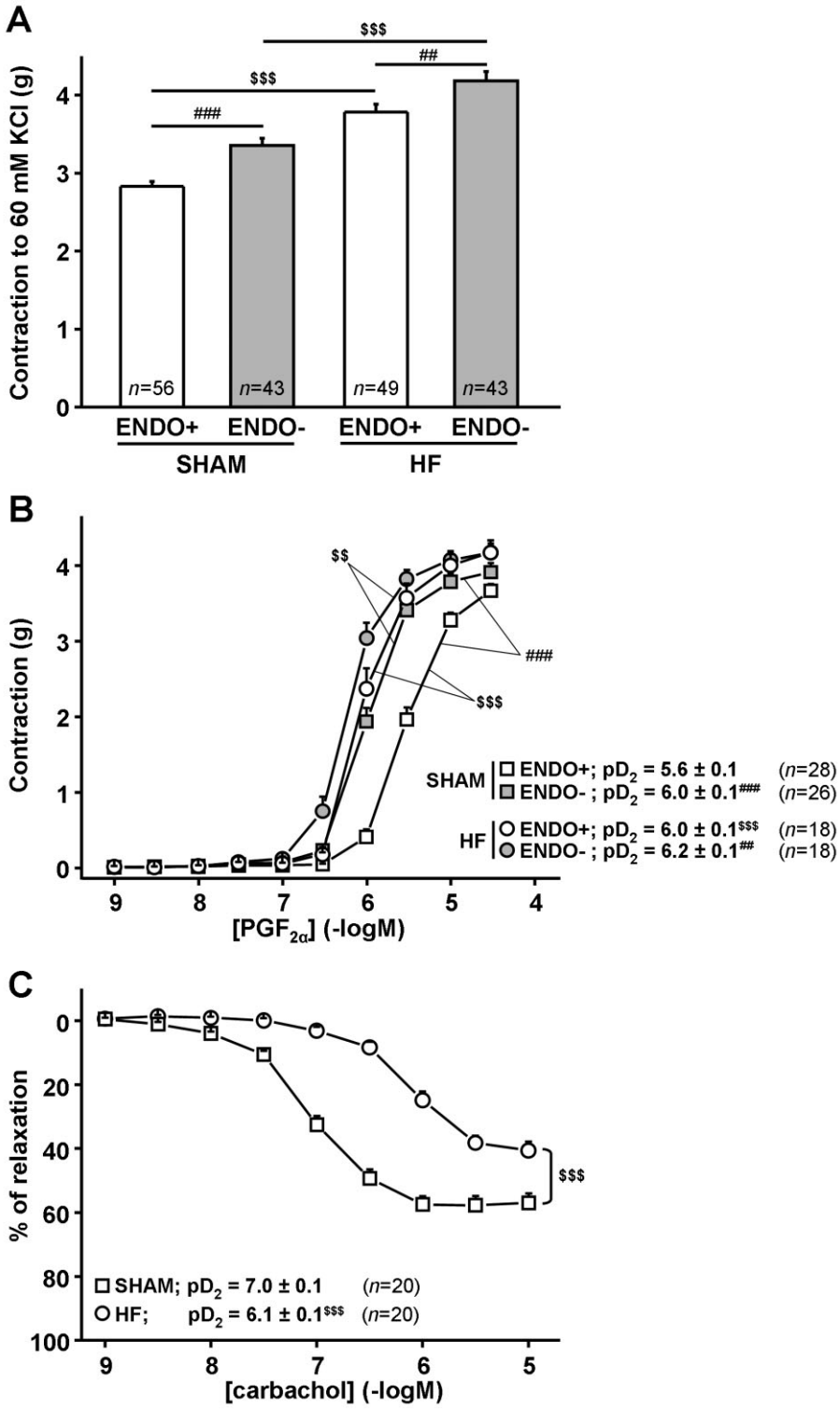

Figure 3

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 endothelium-intact (*ENDO+*) or endothelium-denuded (*ENDO–*) rat aortas. (B) CRCs to $PGF_{2\alpha}$ (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 $PGF_{2\alpha}$-precontracted ENDO+ aortas isolated from SHAM and HF rats. Data are means ± SEM. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, significant effect of endothelial removal; $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$, significant effect of HF.

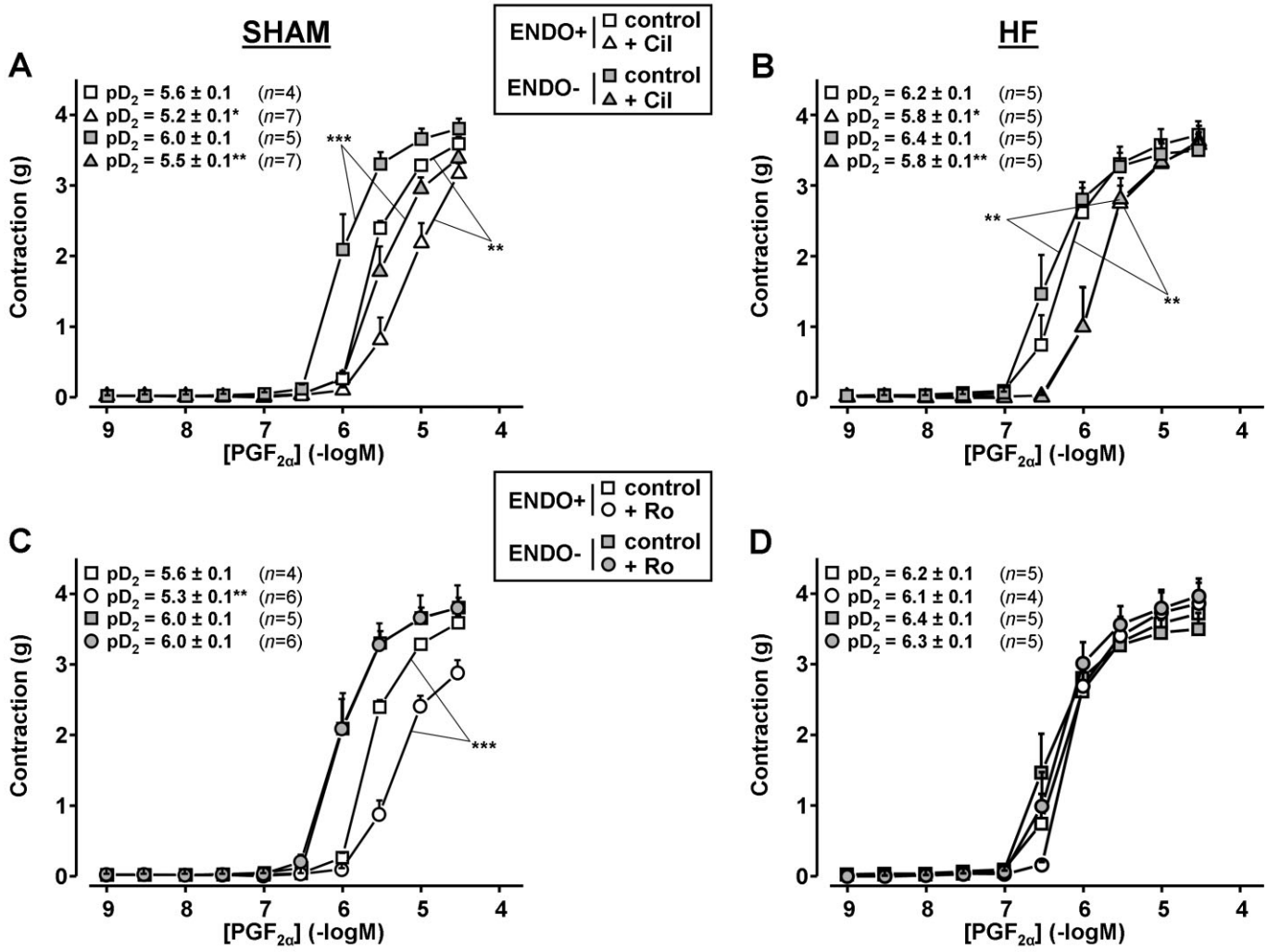

Figure 4

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

endothelium-intact SHAM aortas. In HF aortas, cilostamide treatment unmasked a marked relaxation to isoprenaline (Figure 7B), which was similar to that observed in SHAM rat arteries in the presence of cilostamide In endothelium-denuded aortas (Figure 7C and D) or under NOS inhibition by L-NAME (Figure 7E and F), incubation with cilostamide restored a relaxation to isoprenaline in both SHAM and HF groups. These results indicate that the relaxation of aorta mediated by β-adrenoceptor stimulation was negatively controlled by PDE3 activity. In HF aortas, the loss of this relaxation was unmasked when PDE3 was 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 indicated that the aortic relaxation mediated by β-adrenoceptor stimulation was also negatively controlled by PDE4 activity which was largely dependent on the endothelium functionality. In HF aortas, the loss of relaxation to β-adrenoceptor stimulation was 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 pmol per ring ($n = 6$) vs. 2.8 ± 0.6 pmol per ring ($n = 6$) respectively]. Direct stimulation of AC with L-858051, a forskolin analogue, in the presence of IBMX strongly raised cAMP levels by 25- and 32-fold in SHAM and HF aortas respectively (data not shown, $n = 5$).

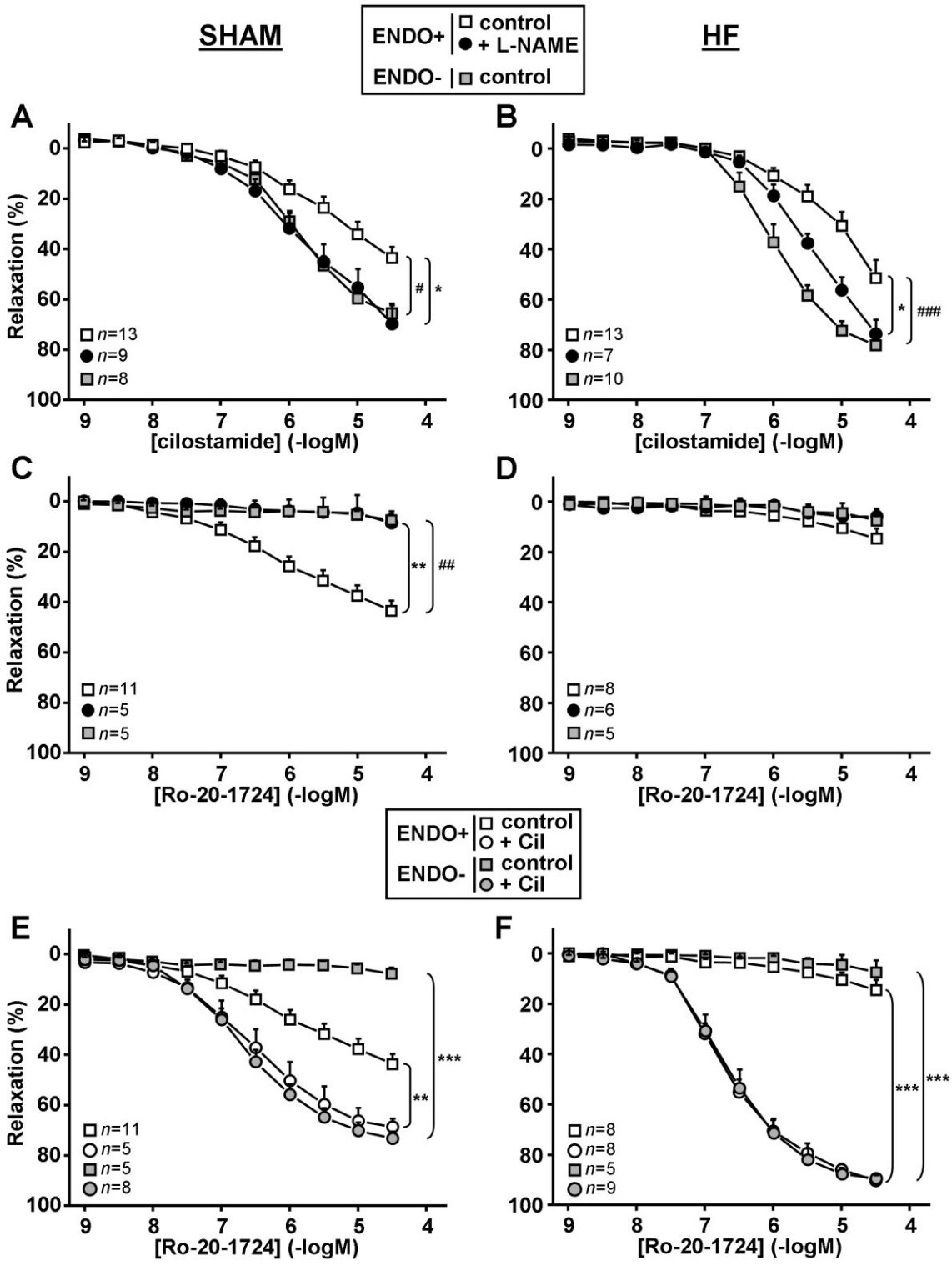

Figure 5

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 (*ENDO+*) pretreated in the absence (*control*) or presence of the NOS inhibitor (300 μM L-NAME) and in endothelium-denuded arteries (*ENDO–/control*) isolated from SHAM (A and C) and HF (B and D) rats and precontracted with $PGF_{2\alpha}$. (E–F) CRCs to Ro (1 nM to 30 μM) were performed either in endothelium-intact (*ENDO+*) or endothelium-denuded (*ENDO–*) arteries isolated from SHAM (E) and HF (F) rats, and pretreated in the absence (*control*) or presence of 1 μM Cil. Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significant effect of L-NAME or PDE inhibitor; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, significant effect of endothelial removal.

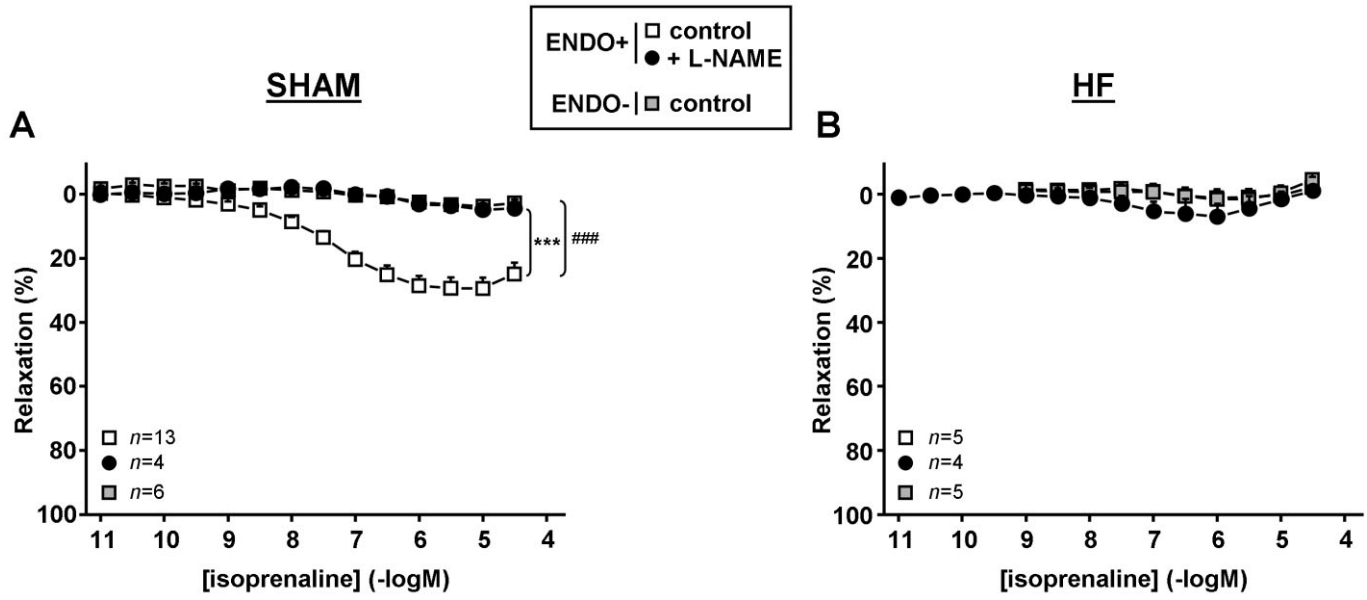

Figure 6

Effect of L-NAME and endothelium removal on relaxation to β-adrenoceptor stimulation in precontracted aorta isolated from SHAM and HF rats. CRCs to isoprenaline (0.01 nM to 30 μM) were performed in endothelium-intact arteries (*ENDO+*) pretreated in the absence (*control*) or presence of the NOS inhibitor (300 μM L-NAME) and in endothelium-denuded arteries (*ENDO–/control*) isolated from SHAM (A) and HF (B) rats and precontracted with $PGF_{2\alpha}$. Data are means ± SEM. ***$P < 0.001$, significant effect of L-NAME. $^{###}P < 0.001$, significant effect of endothelial removal.

Treatment with Ro but not with cilostamide 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 markedly reduced in endothelium-denuded SHAM aortas (Figure 8A).

In HF aortas, basal cGMP levels were slightly reduced by 25% compared with SHAM aortas [0.8 ± 0.2 pmol per ring ($n = 6$) vs. 1.1 ± 0.3 pmol per ring ($n = 5$) respectively]. Endothelium denudation decreased cGMP levels by 38 and 58% in HF and SHAM aortas respectively [0.5 ± 0.2 pmol per ring ($n = 5$) and 0.4 ± 0.1 pmol per 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, treatment with cilostamide or Ro had no significant effect on cGMP level in any groups of SHAM and HF aortas (Figure 8B).

Discussion

In this study, we characterized the expression, activity and functional role of the cAMP-PDE subtypes in aortas isolated from SHAM and HF rats. Our main results can be summarized as follows: (i) PDE3 and PDE4 are the main PDE families responsible for cAMP hydrolysis in SHAM and HF aortas; (ii) PDE3 and PDE4 family expression is altered in HF; (iii) PDE3 inhibition with cilostamide induces a vasorelaxation in precontracted SHAM and HF aortas whether the endothelium is present or not; (iv) moreover, the relaxant effect induced by cilostamide is potentiated when the endothelium is removed or upon NOS inhibition; (v) PDE4 inhibition with Ro induces an increase in cAMP level and a vasorelaxation in SHAM but has no effect in HF aorta; (vi) these effects of Ro are absent when the endothelium is removed or upon NOS inhibition; (vii) however, a large vasorelaxant effect of Ro is revealed in endothelium-denuded SHAM aorta as well as in HF aorta when PDE3 is inhibited; (viii) β-adrenoceptor stimulation induces an endothelium- and NOS-dependent vasorelaxation in SHAM aorta but has no effect in HF; and (ix) the relaxation mediated by β-adrenoceptor stimulation is increased in SHAM and unmasked 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 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-adrenoceptor 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 and abolishes the relaxation mediated by β-adrenoceptor stimulation. Inhibition of vascular PDE3 may thus represent an attractive approach to restore a normal vasorelaxation in HF.

We used a rat model of cardiac chronic pressure overload which changes over time from cardiac hypertrophy to HF (Joubert *et al.*, 2008; Abi-Gerges *et al.*, 2009). This animal model mimics HF in patients with stenosis of the aortic valve (Muders and Elsner, 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 with the decompensated stage of HF. The aortas isolated at the HF stage exhibited clear

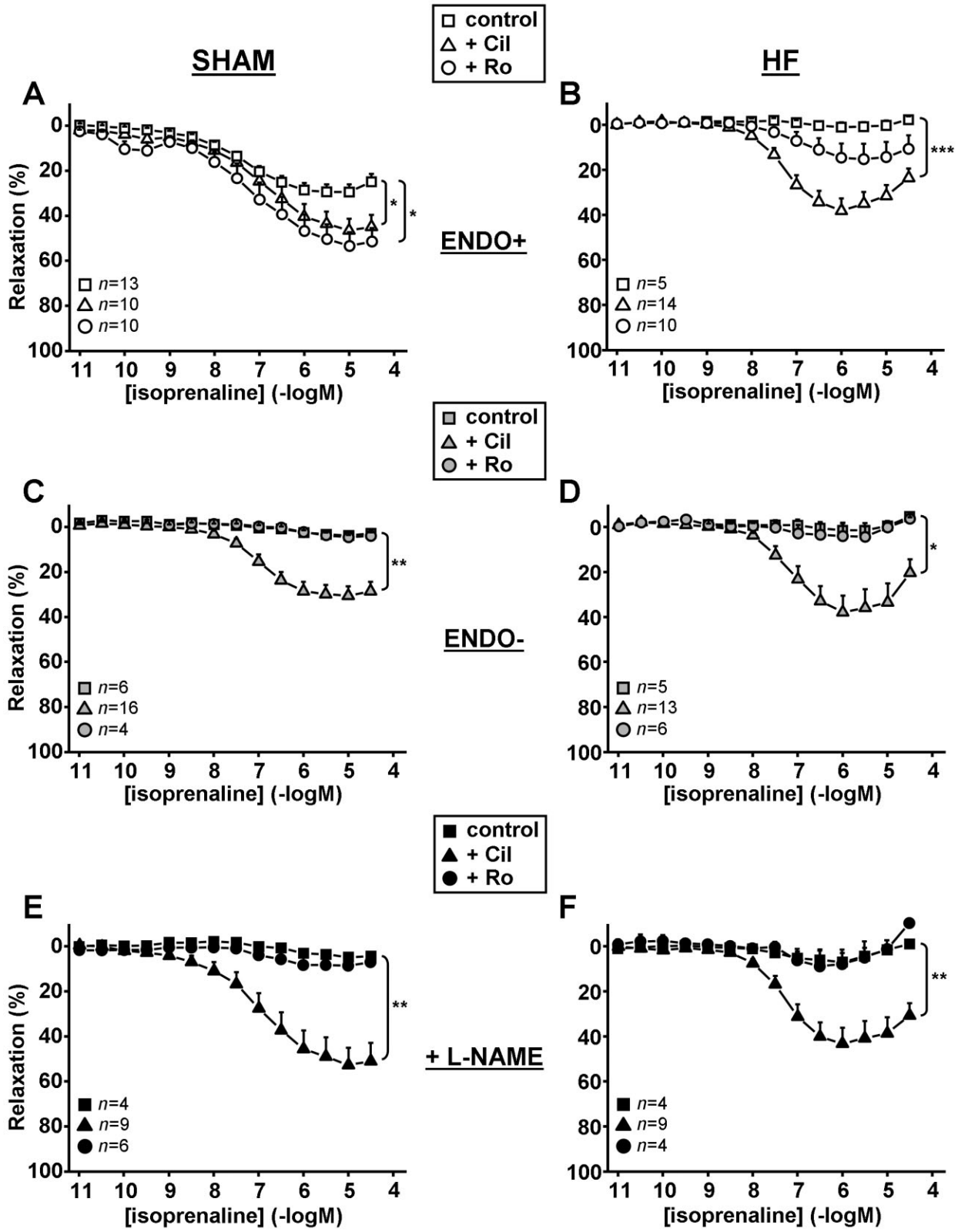

Figure 7

Effect of PDE3 or PDE4 inhibition on relaxant response to β-adrenoceptor stimulation in precontracted aorta isolated from SHAM and HF rats. CRCs to isoprenaline (0.01 nM to 30 μM) were performed in endothelium-intact (A and B), in endothelium-denuded (C and D) or in L-NAME-pretreated (E and 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 μM Cil), or the PDE4 inhibitor (10 μM Ro). Data are means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant effect of PDE inhibitor.

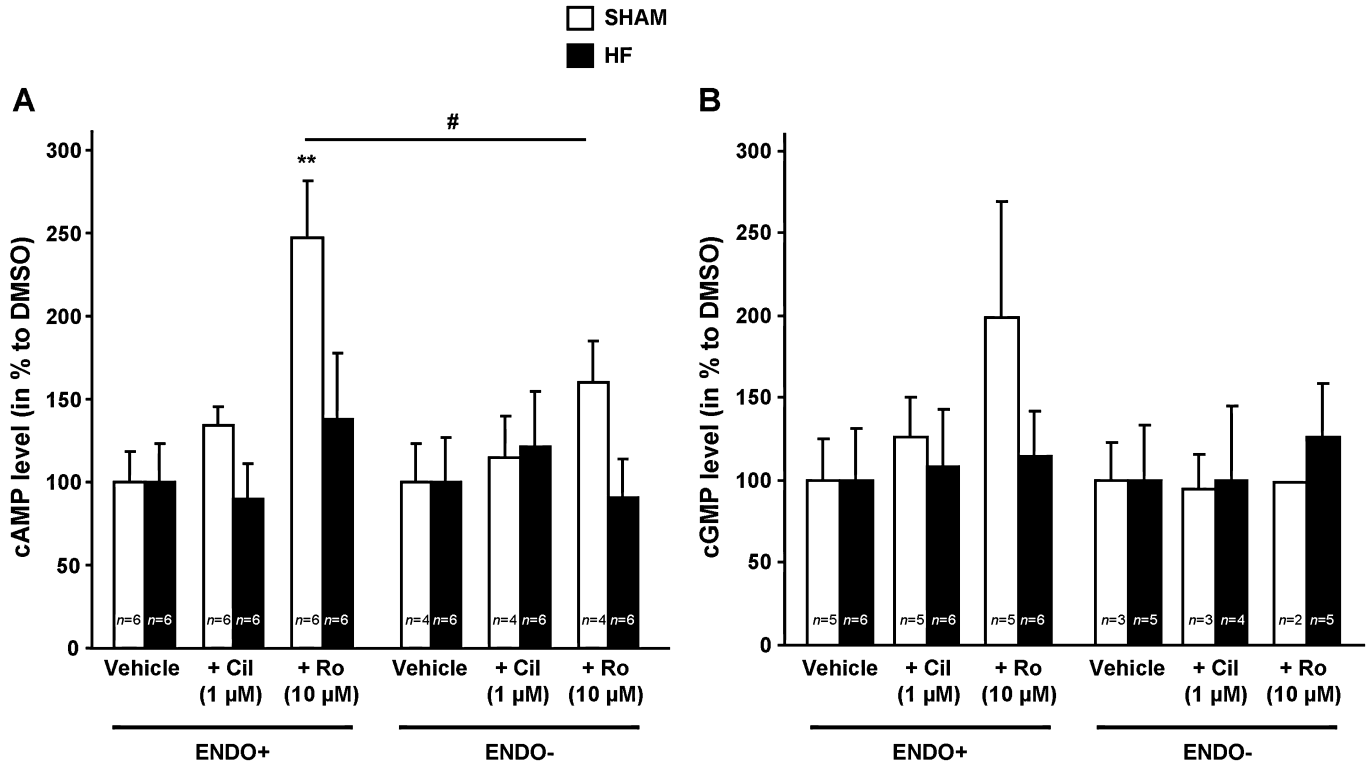

Figure 8

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 endothelium-intact (*ENDO+*) or endothelium-denuded (*ENDO–*) rings pretreated in the absence (*vehicle*) or presence of the PDE3 inhibitor (1 µM Cil) or the PDE4 inhibitor (10 µM Ro). Results are expressed in % of mean cyclic nucleotide levels measured in the absence of inhibitors. Data are means ± SEM of *n* rings from different animals. $^{**}P < 0.01$, significantly different from vehicle group. $^{\#}P < 0.05$, significant effect of endothelial removal.

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 characteristic of cardiovascular diseases, including HF (Francis and Cohn, 1990; Negrao *et al.*, 2000; Nakamura *et al.*, 2001), although the mechanisms leading to this dysfunction are unclear and include neurohumoral activation, oxidative stress, haemodynamic alterations and vascular remodelling (Muders and Elsner, 2000; Indik *et al.*, 2001; McGoldrick *et al.*, 2007). By using an *in vitro* biochemical assay, we found that the rank order of cAMP-hydrolysing activity in rat aorta was PDE3 > PDE4 >> PDE1, whereas PDE2 activity was not detected. This pattern is in agreement with previous reports (Polson and Strada, 1996; Maurice *et al.*, 2003). Aortas isolated from HF rats exhibited a similar total cAMP-hydrolysing activity and PDE family 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 with that of SMCs, or that the sensitivity of the assay is insufficient to detect this endothelial contribution. However, in HF aortas, PDE4 activity was decreased in the absence of endothelium. We also observed that the HF stage was 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 the Dahl salt-sensitive rat model of HF. Thus, alterations 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 cilostamide 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 cilostamide. By contrast, the vasorelaxant effect elicited by PDE4 inhibition with Ro was abolished when the endothelium was removed (when PDE3 was uninhibited) and was increased in the presence of cilostamide (when PDE3 was inhibited). This suggests that, when fully active, PDE3 largely dominates over PDE4. A somewhat similar conclusion was reached by Komas *et al.* (1991) in noradrenaline- and $PGF_{2\alpha}$-precontracted arteries. Surprisingly, PDE3 inhibition was not associated with an increase in global intracellular level of

cAMP or cGMP, as found previously (Eckly and Lugnier, 1994), which supports the hypothesis that functional responses elicited by cyclic nucleotides require local rather than global changes in their concentration.

Another difference between the functional role of PDE3 and PDE4 was observed in HF. While the relaxant effect induced by 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, for example, in the vicinity of PDE4, which would not been 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-adrenoceptor-mediated vasorelaxation (Mathew *et al.*, 1993; Nasa *et al.*, 1996; McGoldrick *et al.*, 2007). In our model of HF, we also observed a loss of aortic relaxation to β-adrenoceptor stimulation. This loss of response was likely a consequence of the endothelial dysfunction, because in SHAM aorta, the response to β-adrenoceptor stimulation was abolished when the endothelium was removed or upon NOS inhibition. A similar loss of the response to β-adrenoceptor stimulation in the absence of a functional endothelium was reported in earlier studies and this led the authors to conclude that the response involved β-adrenoceptors located on the endothelial cell surface (Kamata *et al.*, 1989; Gray and Marshall, 1992). However, our results contradict this conclusion. Indeed, we found that the relaxation to β-adrenoceptor stimulation 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-adrenoceptor/cAMP 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 relaxation. Thus, only a pharmacological inhibition of PDE3 can restore the vasorelaxation mediated by β-adrenoceptor stimulation, as also observed in another pathological situation, the restenosis after balloon angioplasty (Zhao *et al.*, 2007).

In conclusion, 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 relaxation to β-adrenoceptor stimulation. Inhibition of vascular PDE3 may thus represent an attractive therapeutic approach to restore a normal vasorelaxation in HF. A limitation of our study is that the aorta is a conductance artery which is not critically involved in the regulation of blood pressure. Thus, further studies would be required to assess the role of PDEs in contractile function in resistance arteries from HF models. However, our study clearly shows tha,t in HF, endothelial dysfunction leads to an altered function of PDEs in the smooth muscle. This phenomenon could be a common feature of cardiovascular diseases associated with endothelial dysfunction.

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Author contributions

F. H., M. B-O., B. M., P. M., F. J., R. F. and V. L. conceived and designed the experiments. F. H., M. B-O., B. M., Z. K., V. D-D. and P. M. performed the experiments. F. H., M. B-O., B. M., P. M. and V. L. analysed the data. F. H., M. B-O., R. F. and V. L. wrote the paper.

Conflict of interest

None declared.

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Supporting information

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http://dx.doi.org/10.1111/bph.12853

Appendix S1 Supplementary methods.