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Induction of PDE5 and de-sensitization to endogenous NO signaling in a systemic resistance artery under altered blood flow

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Abstract

In the classical pathway, the opposing activities of guanylyl cyclases (GC) and phosphodiesterases (PDE), and the effect of the cGMP-dependent protein kinase (cGK) on its targets, determine the biological responses to NO signaling. Here we tested the hypothesis that vascular dysfunction may be due to altered expression and activity of these effectors of NO signaling. Every other set of rat second order mesenteric resistance arteries (MA) were ligated, resulting in chronic low flow (LF) in the upstream MA1 and high flow (HF) in the adjacent MA1 without tissue ischemia. eNOS and iNOS were up-regulated in HF and LF MA1, respectively, in the sub-acute phase (four days) of vascular remodeling. The Day4 HF/LF MA1s were under increased control of NO as indicated by reduced sensitivity to the vasoconstrictor phenylephrine and its normalization with the NOS antagonist L-NAME. PDE5 mRNA and protein were also significantly up-regulated in the HF/LF MA1 with no change in sGC or PKG1, an effect that was dependent upon NO synthesis. The PDE5 inhibitor Sildenafil was several-fold more powerful in relaxing the HF/LF MA1s, and pre-treatment with Sildenafil uncovered an increased responsiveness of HF/LF MA1s to the NO donor DEA/NO. We conclude that induction of PDE5 de-sensitizes this systemic resistance artery to sustained NO signaling under chronic HF/LF. Treatment with PDE5 antagonists, in contrast to NO donors, may more specifically and effectively increase blood flow to chronically hypo-perfused tissues.

Keywords

nitric oxide; cGMP; Sildenafil; vasorelaxation

INTRODUCTION

The endothelial derived relaxing factor nitric oxide (NO) plays a key role in the regulation of vascular tone. Vascular resistance and vascular function are determined by the state of contraction of the smooth muscle of the small arteries and arterioles according to the Law of Poiseuille. In the classical pathway NO activation of soluble guanylyl cyclase in smooth muscle leads to the generation of the second messenger cGMP. The subsequent activation of the cGMP-dependent protein kinase 1 (PKG1) causes smooth muscle relaxation through the inhibition of calcium flux via an effect on the IP3 receptor complex, and activation of myosin phosphatase, thereby reducing the sensitivity of the myofilaments to calcium.[20;31] This

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signaling system is regulated by phosphodiesterases (PDE), which constitute a large and diverse family of proteins. [2] In smooth muscle PDEs1 and 5 degrade cGMP to GMP and thereby terminate cGMP signaling. In contrast to the well studied role of reduced bioavailability of NO in vascular disease, termed endothelial dysfunction [5] the contribution of the phenotypic state of the smooth muscle to vascular dysfunction has not received much attention. Are there conditions in which systemic resistance arteries are less (or more) sensitive to NO signaling, so-called "NO resistance"? [10] This question may be critical for understanding vascular dysfunction and its medical treatment. Many of the drugs approved or in clinical trials to treat vascular dysfunction target this pathway in smooth muscle, including NO donor drugs, PDE5 inhibitors such as Sildenafil, Atrial Natriuretic Protein, and direct activators of guanylyl cyclase (reviewed in [32]). Drugs that target this pathway, e.g. PDE5 inhibitors, may also have direct effects on muscle in conditions such as cardiac hypertrophy [14] and ischemia-reperfusion injury. [6]

In order to study the function of NO/cGMP signaling in resistance artery smooth muscle under altered flow, we implemented a model in which alternating pairs of rat second order mesenteric arteries are ligated. This results in very low flow (LF) in the upstream first order mesenteric artery (MA1), and a corresponding increase in blood flow (high flow, HF) in the adjacent MA1 [26;37] which supplies the territory of the occluded MA2s through the mesenteric collateral arcade. In a prior study [40] we observed dynamic switching of myosin phosphatase isoforms, an end target of NO/cGMP signaling [35;15;13], in the MA1s under HF and LF. After 28 days of sustained LF there was a complete shift in myosin phosphatase isoforms associated with the predicted increase in sensitivity to NO/cGMP-mediated vasorelaxation. Here we tested the hypothesis that sustained LF/HF may alter the expression and activity of the more proximal components of the NO/cGMP signaling pathway in the resistance artery smooth muscle.

Materials and Methods

Animal model

Seventy-one adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used for the study. Chronic high flow (HF)/ low flow (LF) in the rat MA1 was induced as previously described.[26;37;40] In brief, after general anesthesia a mid-line abdominal incision was made and 6-0 silk suture was used to ligate paired 2nd order mesenteric arteries originating from alternating 1st order small mesenteric arteries (MA1). Animals were sacrificed and MA1s harvested 1 to 7 days after surgery. Control MA1s were taken from the same operated rats. Two separate time course series (*Days 1, 4, 7*) were performed for analyses of RNA. Forty seven additional rats at *Day4* were sacrificed for analyses of RNA, protein, cGMP level and functional studies of MA1s. In a separate experiment L-NAME was placed in the drinking water of rats (0.6g/L, ~60mg/day/kg body weight) each day to block NO synthesis, as previously described,[21] from 3 days prior to surgery until the rats were sacrificed 4 days after surgery. Animal experiments were conducted in accordance with and approved by the Animal Care and Use Committee of Case Western Reserve University.

Real-time PCR analyses

The isolation of total RNA and reverse transcription were performed as previously described. [40] Primers to amplify eNOS, iNOS, sGC, PKG1, PDE5, PDE1A, MYPT1, PP1c δ and cyclophilin in real-time PCR (Table 1 and [40]) were designed using Primer3 online software. Cyclophilin abundance was measured in separate reactions as an internal control for normalization, and as previously reported was invariant. All reactions were run in duplicate and averaged. Fold change of target gene transcripts of HF/LF MA1 to that of control MA1 were calculated as $2^{-\Delta\Delta C_t}$. The specificity of the PCR reactions were confirmed by melting

curve analysis and the absence of PCR products when reverse transcriptase was not included in the reaction mix (data not shown).

Western blots

Abundance of PDE5 and NOS proteins were examined by Western blot. Twenty five μ g of total protein isolated from pooled MA1s from each rat was separated by electrophoresis through 3-8% NuPage Gel (Invitrogen, Carlsbad, CA), transferred to PVDF membrane (Millipore, Bedford, MA) or nitrocellulose membrane (Micron Separations Inc., Westborough, MA), treated with Mizer™ antibody extender solution (Pierce, Rockford, IL) and probed with the following primary antibodies 1) PDE5[33] 2) eNOS (1185-1205, Sigma, St. Louis, MO) 3) iNOS (Affinity BioReagents, Golden, CO) 4) smooth muscle α -actin (clone 1A4, Sigma, St. Louis, MO). Signals were detected with horseradish peroxidase conjugated secondary antibodies followed by ECL. Band intensities were quantified by densitometry using NIH image software and normalized to that of smooth muscle α -actin.

Vascular function studies

Segments of MA1 2mm in length were dissected free of connective tissue, mounted in a four chamber wire myograph (Model 610 M, Danish Myo Technology, Aarhus, Denmark), and normalization, internal diameter measurement and priming performed as previously described. [40] All chemicals were purchased from Sigma (St. Louis, MO) unless specified otherwise. Vascular reactivity was assessed simultaneously in isolated Day4 HF, LF and control MA1s from a single rat. Force was continuously recorded as vessels were exposed to the following agents singly or in combination: 1) Phenylephrine (PE), an α -adrenergic vasoconstrictor 2) U46619 (Cayman Chemicals), a thromboxane analogue and vasoconstrictor 3) Sildenafil, (Pfizer Inc., New York, NY), a selective PDE5 antagonist 4) Vinpocetine, a selective PDE1 antagonist 5) IBMX, a general PDE antagonist 6) The direct NO donor DEA/NO 7) L-NAME, a competitive antagonist of L-arginine that inhibits endogenous NO production 8) L-NIL (Cayman chemical), a competitive antagonist of L-arginine with selectivity towards iNOS. Dose response studies were performed with single agents. When two agents were used, the concentration of one agent was fixed to achieve sub-maximal effect and dose-response with the second agent tested. Treatment regimens are described in each figure legend. Data are presented as tension (mN/mm length vessel wall) for PE-induced contraction, and percentage of maximum tension for DEA/NO, Sildenafil and vinpocetine induced relaxation.

cGMP assays

Three control, HF and LF MA1s were collected from each rat four days after surgery, pooled, and placed in wells with PSS at 37°C. All samples were pre-treated with L-NAME (0.1 mM, 30 min) to block endogenous NO synthesis and in addition were treated with either 1) Sildenafil (1 μ M, 30 min) 2) IBMX (0.5mM, 60 min) 3) no additional drug. DEA/NO was then added to a final concentration of 0.1 μ M. After five minutes the vessels were snap frozen in liquid nitrogen. MA1s were homogenized in cold 10% trichloroacetic acid. After centrifugation, the supernatant was extracted with water saturated ether and the ether was removed. A cGMP EIA kit (Biomedical Technologies Inc., Stoughton, MA) was used to measure cGMP concentrations per the manufacturer's instruction. Each sample was run in duplicate with replicate variation of less than 10%. A standard curve was generated with each assay and was linear over a range of 0.5-200 pmol/ml of cGMP. OD405nm readings were converted to pmol/ml and then normalized to total protein measured in the TCA precipitate. The data is presented as pmol/mg protein.

Statistical analysis

Data are expressed as mean $\pm$ SEM. All statistics were done with Prism software (GraphPad software Inc., San Diego, CA). One-way analysis of variance (ANOVA), Newman-Keuls post test were used for comparison among experimental groups. Students' t-test was used for comparison of phenylephrine dose-response with or without sildenafil (or L-NAME) pre-treatment. $P<0.05$ was considered statistically significant.

Results

Induction of NOS and PDE5 in HF/LF MA1

We first examined the time course of changes in the expression of PDE5 by real-time PCR in the HF/LF model. PDE5 mRNA was increased as early as 1 day after the induction of HF and LF in the MA1s, peaked at 4 days and returned to baseline by 7 days (Fig. 1A). We therefore focused our subsequent studies on the Day 4 time point. eNOS mRNA was increased by 4.1-fold in Day4 HF MA1 while iNOS mRNA was unchanged (Fig. 1B,C). iNOS mRNA was increased by 15.7-fold in Day4 LF MA1 while eNOS mRNA was unchanged. There was no significant change in the expression of sGC α 1 or β 1 or PKG1 in the HF and LF MA1s. PDE5 mRNA was increased 8.7-fold in the Day4 HF and 4.9-fold in the LF MA1. There was no significant change in the PDE1A mRNA in the Day4 HF and LF MA1, indicating the specificity of the up-regulation of PDE5. In contrast to the up-regulation of NOS isozymes and PDE5, the expression of the myosin phosphatase subunits MYPT1 and PP1c δ , an end-target of NO/cGMP signaling, were decreased to ~25% and ~50% of their control values, respectively, in Day4 LF and HF MA1, consistent with our previous report.[40] Of these transcripts only iNOS was 1) not detected in the un-operated rat MA1 and 2) slightly induced (above background) in control MA1s from operated (HF/LF) rats. To determine if the induction of PDE5 was related to the induction of NOS in the HF/LF MA1s, the MA2 ligation procedure was performed in rats that were treated with L-NAME to block NO synthesis (0.6g/L each day in the drinking water, ~60mg/day/kg body weight) for 3 days prior to surgery and until the time of sacrifice four days after surgery. In these rats there was no induction of PDE5 mRNA in the Day4 HF/ LF MA1 as compared to the control MA1s from the same rats (Fig. 1D). PDE5 mRNA was modestly increased in the L-NAME treated control MA1 as compared to untreated control MA1 (HF 1.5 $\pm$ 0.3 fold, LF 1.5 $\pm$ 0.4 fold, control 2.0 $\pm$ 0.4 fold vs. untreated rat control MA1, n=4, $P>0.05$).

PDE5 protein abundance as measured by Western blot followed the trends identified by real-time PCR, though the magnitude of the changes was less. PDE5 protein was increased by 1.5-fold and 1.9-fold in the HF and LF MA1, respectively ($P<0.05$ vs. control, Fig. 2). eNOS protein was increased 2.8-fold in the HF MA1 ($P<0.05$ vs. control), consistent with a previous report [7], and was not significantly increased in the LF MA1 (Fig. 2). iNOS protein was not detectable in the control and HF MA1 and was detected at low levels in the D4 LF MA1 (not shown). All signals were normalized to smooth muscle α -actin, which was not different among groups.

Vascular Function

We studied the Day4 HF/LF and control MA1s in a wire myograph system to determine how the altered expression of the genes in the NO/cGMP signaling pathway may affect resistance artery function under sustained high and low flow conditions. At this time point there was already evidence of outward and inward remodeling of HF and LF MA1s, respectively (internal diameters: control 306 $\pm$ 7 μ m, n=43 ; HF 330 $\pm$ 8 μ m, n=34 $P<0.05$; LF 256 $\pm$ 6 μ m, n=34, $P<0.01$). The HF and LF Day4 MA1s were less sensitive than control MA1 to α -adrenergic agonist PE-induced force production (Fig. 3A; EC50: HF 2.15 $\pm$ 0.49 μ M, n=5; LF 2.05 $\pm$ 0.23 μ M, n=6 vs. control 1.12 $\pm$ 0.23 μ M, n=6; $P<0.01$). Control and HF MA1 produced equal amounts of maximum force at the highest concentration of PE, while maximum force in the LF MA1 was reduced by ~15%, likely due to the inward remodeling of the vessel and loss of

muscle mass. Pre-treatment of the MA1s with 0.1 mM L-NAME to block NO synthesis increased sensitivity to PE in all three groups (Fig. 3B-D). The magnitude of the shift was larger in the HF/LF MA1s, such that under conditions of NO blockade the PE dose-response between groups was not different (EC50: HF $0.75 \pm 0.27 \mu\text{M}$, $n=5$; LF $0.77 \pm 0.12 \mu\text{M}$, $n=6$ vs. control $0.56 \pm 0.09 \mu\text{M}$, $n=6$; $P>0.05$). This indicates that endogenous NO signaling exerted a greater inhibition of α -agonist induced force in Day4 HF/LFMA1 as compared to controls, consistent with the induction of NOS isozymes as described above. We were not able to functionally define the contribution of the different NOS isozymes to this effect, as the semi-selective iNOS inhibitor L-NIL at $10 \mu\text{M}$ had effects similar to that of L-NAME in all three groups (data not shown). L-NAME also caused a greater potentiation of force production in HF and LF as compared to control MA1s after force activation with the thromboxane analogue U46619. Developed tensions at half-maximal concentrations of U46619 (40 nM) before and after L-NAME were in mN/mm CON: $1.46 \pm .53$, $3.17 \pm .15$; LF: $1.15 \pm .40$, $3.53 \pm .37$; HF $1.5 \pm .62$, $4.3 \pm .36$; $n=3$ each, $p<0.05$ for Δ L-NAME CON vs HF and LF.

We next used Sildenafil, a potent and selective inhibitor of PDE5, to examine the effect of PDE5 induction on NO control of resistance artery tone under chronic high and low flow. Pre-treatment with Sildenafil ($1 \mu\text{M}$) caused a greater reduction in sensitivity to PE in the Day4 HF/LF MA1 as compared to control MA1 (Fig. 4A-C; PE EC50 with Sildenafil: HF $8.56 \pm 1.81 \mu\text{M}$, $n=4$; LF $9.93 \pm 1.75 \mu\text{M}$, $n=4$ vs. control $3.10 \pm 0.77 \mu\text{M}$, $n=7$; $P<0.01$). Sildenafil reduced maximum force in all groups, and while this effect tended to be greater in the HF and LF MA1 as compared to control, these differences were not statistically significant. We next tested the dose-response to Sildenafil after activation of force in the MA1s with a sub-maximal concentration of PE ($10 \mu\text{M}$). At all concentrations the effect of Sildenafil in promoting vascular relaxation tended to be greater in the HF and LF MA1 as compared to control MA1 (Fig. 5). At the highest concentration tested ($1 \mu\text{M}$), Sildenafil produced more powerful relaxation of the HF and LF MA1 as compared to control MA1 (% maximum tension: HF 23.3 ± 8.0 , $n=6$ vs. CON 53.7 ± 11.1 , $n=3$, $P<0.05$; LF 9.7 ± 4.7 , $n=6$ vs. CON, $P<0.01$). Pre-incubation of the MA1s with L-NAME completely abrogated the effect of Sildenafil excepting a very modest relaxation at the highest concentration. This demonstrates the expected requirement of endogenous NO signaling for Sildenafil's vaso-relaxant activity. Relaxation to Sildenafil was also abolished by the guanylate cyclase inhibitor ODQ, indicating cGMP/PKG-dependence. The increased efficacy of Sildenafil was not observed in D7 HF and LF MA1 (data not shown).

The increased efficacy of Sildenafil in the HF/LF MA1 could reflect the increased activation of this pathway due to induction of NOS and/or increased activity of PDE5 in these arteries. To distinguish these possibilities, the vasorelaxation studies were repeated with the inclusion of L-NAME to inhibit endogenous NO synthesis followed by incubation of HF, LF and control MA1s with equivalent concentrations of the NO donor DEA/NO. There was no significant difference between control, HF and LF MA1 in the dose-responses to DEA/NO in the presence of L-NAME (Fig. 6A; EC50: HF $0.22 \pm 0.06 \mu\text{M}$; LF $0.20 \pm 0.03 \mu\text{M}$ vs. control $0.28 \pm 0.03 \mu\text{M}$, $P>0.05$, $n=4$ each), though the HF/LF MA1s tended to be slightly more sensitive at the intermediate concentrations. Pre-treatment of the MA1s with the PDE5 inhibitor Sildenafil ($0.1 \mu\text{M}$) increased the sensitivity of all 3 groups to DEA/NO (Fig. 6B; EC50: HF $0.030 \pm 0.004 \mu\text{M}$, $n=5$; LF $0.020 \pm 0.003 \mu\text{M}$, $n=6$ and CON $0.052 \pm 0.015 \mu\text{M}$, $n=5$; $P>0.05$). In the presence of Sildenafil the HF/LF MA1 were significantly more responsive to sub-maximal concentrations of the NO donor (Fig. 6B; % of maximal tension at $10^{-7.5} \text{M}$ DEA/NO: HF $34.7 \pm 9.9\%$, $n=5$ vs. CON $66.5 \pm 11.0\%$, $n=5$, $P<0.05$; LF $19.5 \pm 4.7\%$, $n=6$, $P<0.01$ vs. CON). Thus PDE5 inhibition had a greater effect in the HF and LF MA1 as compared to the control MA1 at sub-maximal concentrations of DEA/NO. The NO donor at the highest concentrations caused complete relaxation of the MA1s in the presence or absence of Sildenafil.

A prior report has suggested that aortic smooth muscle becomes resistant to chronic exogenous NO donors through the induction of PDE1A expression and activity as assessed by the effect of the PDE1A inhibitor Vinpocetine.[16] We therefore tested the effect of Vinpocetine in this model. Pre-treatment with Vinpocetine (1 μ M) caused a similar reduction in sensitivity to PE and maximum tension in control, HF and LF MA1 (Fig. 7A-C; EC50: HF 16.31 $\pm$ 3.17 μ M, n=3; LF 16.88 $\pm$ 0.89 μ M, n=4 vs. CON 20.32 $\pm$ 2.77 μ M, n=4; P>0.05). After sub-maximal force activation with PE (10 μ M), the dose-response curves to Vinpocetine were not different between the groups, though the HF MA1 tended to be slightly less sensitive to Vinpocetine (Fig. 7D). This functional data is consistent with the lack of induction of PDE1A in the HF/LF MA1. Pre-treatment with IBMX (0.5 mM), a general antagonist of all PDEs, almost completely and equally blocked the ability of PE to contract the control, HF and LF MA1 (data not shown).

Measurements of cGMP in MA1

cGMP levels in MA1s were measured after blockade of endogenous NO production with L-NAME and exposure to equivalent concentrations of the NO donor DEA/NO (0.1 μ M) as another gauge of the relative activities of guanylate cyclase, which generates cGMP, and PDE, which degrades it. Control and Day4 HF/LF MA1 generated similar levels of cGMP when pre-treated with IBMX (0.5 mM) to inhibit all PDE activity (pmol cGMP/mg protein: HF 31.2 $\pm$ 10.9, LF 32.0 $\pm$ 10.2 vs. CON 29.6 $\pm$ 8.7, P>0.05, n=4 each). This is consistent with the gene expression data and suggests no change in the activity of guanylate cyclase in HF/LF MA1. When exposed to 0.1 μ M DEA/NO without PDE inhibition, the cGMP levels in the HF/LF MA1 were modestly less than the control MA1, a difference that was not statistically significant (Fig. 8). When exposed to 0.1 μ M DEA/NO with Sildenafil (1 μ M) pre-treatment, the cGMP levels were modestly higher in the HF/LF MA1s as compared to the control MA1, a difference that again was not statistically significant. Subtracting the differences in cGMP levels with DEA/NO exposure with and without Sildenafil is an indicator of the PDE5 activity in the different groups. The difference in cGMP levels after PDE5 inhibition with Sildenafil was 36% greater in the HF MA1 and 49% greater in the LF MA1 as compared to control MA1 (pmol cGMP/mg protein: HF 3.5 $\pm$ 0.3, LF 3.9 $\pm$ 0.3 vs. CON 2.6 $\pm$ 0.1, P<0.05, n=4 each). This is consistent with the gene expression and vascular reactivity which in toto indicate increased PDE5 activity in the HF and LF MA1s.

Discussion

In this study we have observed induction of NOS and PDE5 after four days of high and low flow in a mesenteric resistance artery. That the Day4 HF and LF MA1s were under increased control of endogenous NO is suggested by their reduced sensitivity to the vasoconstrictor PE and the near normalization of the PE dose-response in the HF/LF MA1 with the NOS inhibitor L-NAME. This reduced sensitivity to PE is similar to that observed in other high flow models, for example a rat portal vein stenosis model of portal hypertension.[11] The residual difference in the PE dose-response with L-NAME in the bath could reflect differences in the expression of the α -adrenergic receptors or proteins that regulate its signaling, e.g. RGS proteins. [38; 36] The induction of eNOS in the HF MA1 is consistent with prior studies which showed its induction in the HF MA1 associated with increased flow-mediated dilation.[7;39] In the current study we also observed induction of iNOS in both MA1s, though it was only statistically significant in the Day4 LF MA1. As iNOS is a constitutively active and high capacity enzyme, [24] its induction would contribute to a basal level of dilation to increase flow in the MA1. The low level of expression of iNOS in this model is consistent with low flow models of myocardial ischemia, where it has been suggested that iNOS plays a beneficial role (reviewed in [4]. Induction of iNOS may also reflect recruitment of inflammatory cells, as reported by Bakker and colleagues in the LF MA1 at Day7 in a slightly different version of this model in the mouse.

[1] We did not observe inflammatory cells in the HF/LF Day4 MA1s (data not shown), suggesting that the induction of iNOS in these arteries at this and earlier time points was not due to the recruitment of these cells.

We used real-time PCR analysis of gene expression in these small arteries to identify candidate genes that may affect how the artery responds to sustained NO/cGMP signaling in this sub-acute phase of altered blood flow. Of the genes assayed that are in the pathway of NO signaling, PDE5 was specifically up-regulated in this HF/LF model. Of note, the level of induction of the protein was less than that of the mRNA, perhaps relating to the proteasomal activation observed in this model of vascular remodeling.[40] The selective PDE5 inhibitor Sildenafil caused a several-fold greater maximal relaxation of the Day4 HF and LF MA1 as compared to control MA1 under endogenous NO signaling, and resulted in greater relaxation of the HF/LF MA1 at fixed sub-maximal concentrations of the exogenous NO donor DEA/NO, consistent with increased expression and activity of PDE5 in the HF/LF MA1. Consistent with this Sildenafil resulted in a greater potentiation of cGMP levels after sub-maximal DEA/NO treatment in the HF/LF as compared to control MA1. Of note the dose-response curves to Sildenafil were not identical in the HF and LF MA1. PDE5 activity may be affected by the expression of splice variant isoforms or acute phosphorylation with de-sensitization to NO signaling [2;23] issues that require further study in this model.

DEA/NO at maximal concentrations caused equivalent and complete relaxation of all MA1s, and DEA/NO stimulated cGMP levels with all PDEs inhibited with IBMX was equivalent between groups. These data suggest that increased PDE5 activity can be overcome at higher concentrations of NO, and that guanylate cyclase activity does not limit the response to NO in this model of altered flow. We cannot exclude small differences in sGC activity between groups, though there was no significant change in mRNA levels. Other studies have suggested that oxidation or S-nitrosylation of sGC, and resultant reduced sGC activity, may play a role in vascular dysfunction.[29;34] The study by Stasch et al. examined the effects of NO donors and GC activators in models of disease such as diabetes and hyperlipidemia that are associated with increased vascular oxidative stress, while the current study used normal male rats. It will be of interest to determine how these components of the NO signaling pathway are affected in this MA1 HF/LF model in the presence of pre-existing vascular disease.

PDE5 induction was abrogated by pre-treatment of the animals with the NOS inhibitor L-NAME. This suggests that sustained endogenous NO signaling induces PDE5, serving to desensitize the signaling pathway, a common occurrence in receptor signaling. The induction of PDE5 could be a direct result of NO induced elevation of cGMP, as a prior study has shown that the transcription of PDE5 is cGMP and cAMP responsive *in vitro*, [19] and PKG1 is known to regulate transcription in vascular smooth muscle (reviewed in [20]). Alternatively, it is possible that the inhibition of NO synthesis had an effect on blood flow/pressure in the HF/LF MA1s and thereby indirectly blocked the induction of PDE5. Given that PDE5 is induced under both high and low flow conditions, it would seem unlikely that its induction is a direct result of the altered flow. In contrast a study using an exogenous NO donor observed no increase in PDE5 expression in the systemic artery (rat aorta) but increased expression and activity of PDE1A,[16] while a more recent study observed increased expression and activity of PDE5 in the glyceryl trinitrate tolerant rat femoral vein but not the femoral artery.[22] We did not observe induction of PDE1A in the Day4 HF/LF MA1, and the PDE1 selective inhibitor Vinpocetine produced equivalent reductions in sensitivity to PE in the different groups, suggesting that induction of PDE1A is not responsible for tolerance to sustained endogenous NO signaling. It is interesting to note that the PDE1A inhibitor Vinpocetine produced a larger shift in the EC50 for PE in the MA1s than did the PDE5 inhibitor Sildenafil, suggesting that PE induced force in the intact MA1 is greatly potentiated by calcium activation of PDE1A, providing another example of cross-talk between constrictor and dilator signaling pathways.

PDE5 is up-regulated in other circulations, e.g. in animal models of shunt-induced pulmonary hypertension[3;25] and in humans with pulmonary hypertension (along with PDE1 family members).[30] PDE5 is also induced in the placental vessels in an ovine model of fetal placental bypass resulting in a low flow and high NO state.[18] The current study establishes that PDE5 is induced in a systemic resistance artery under chronically altered flow with increased endogenous NO signaling. Identification of the control mechanisms regulating the transcription and processing of PDE 5 is required to further our understanding of the vessel and stimulus-specific induction of PDE5.

A limitation of the current and prior studies is the reliance on pharmacological inhibition of PDE5 to test its function. Sildenafil is a ~5-fold less effective inhibitor of PDE6 (IC₅₀ 50nM), but this is only expressed in the rods and cones of the retina, accounting for the visual disturbances in patients who take Sildenafil. Sildenafil is a substantially weaker inhibitor of PDE1 (IC₅₀ 350 nM; reviewed in [2]). Given the equivalent sensitivity of the HF, LF and control MA1 to the PDE1 inhibitor Vinpocetine, and the efficacy of Sildenafil at concentrations below the IC₅₀ for PDE1, it is unlikely that the increased efficacy of Sildenafil in HF/LF MA1 is due to an effect on PDE1. The relative roles of PDE5 vs PDE1 in regulating vascular tone in the resistance arteries under normal and pathological conditions can be further tested through loss-of-function studies in the mouse. A second limitation of this study is the use of L-NAME to block NO synthesis in vivo in the HF/LF model. In this model there is a modest up-regulation of PDE5 in the control MA1, but no change under HF/LF. An alternative approach would be to use mouse models in which NOS isozymes are genetically inactivated. Our preliminary analyses of this model in wild type B16 mice suggest that the vascular remodeling and changes in gene expression are not as robust as in the rat.

It is only recently that the pharmacological targeting of the NO pathway for the treatment of vascular disease has changed from the centuries old practice of using NO donor drugs (reviewed in [8]). The PDE5 inhibitors were originally intended as a new treatment for patients with angina. However they were not effective in patients with stable anginal symptoms, and were found to have modest effects on systemic vascular resistance.[12] In contrast PDE5 inhibitors were found to be remarkably effective in increasing corpus cavernosal blood flow during sexual arousal, and more recently in the treatment of pulmonary hypertension.[9;27] Why the PDE5 inhibitors are effective in these conditions but not in the treatment of systemic vascular diseases has not been conclusively demonstrated, but likely reflects the relative contributions of NOS and PDE5 activities. Interestingly a recent study showed that the PDE5A inhibitors are effective in normalizing muscle blood flow in a mouse model of muscular dystrophy in which there is functional NOS deficiency and impaired exercise-induced augmentation of blood flow. [17] Other studies have found a beneficial effect of Sildenafil in the setting of ischemia (and reperfusion) by reducing tissue injury through PKG signaling. [28;6] The current study demonstrates induction of PDE5 in the setting of increased NO activity in the sub-acute phase of high and low-flow induced resistance artery remodeling and more powerful relaxation of these arteries to Sildenafil. By re-sensitizing vascular beds to the elevated levels of endogenous NO under sustained alterations in blood flow, PDE5 inhibitors may more effectively enhance blood flow to the target vascular bed than is achieved with the systemic administration of an NO donor drug.

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Figure 1.

Induction of PDE5 and NOS in HF and LF MA1. A, Time course of PDE5 mRNA induction in HF and LF MA1 as measured by real-time PCR (n=2 at each time point). B,C)Relative expression levels of genes in the NO/cGMP signaling pathway in Day4 B) HF and C) LF MA1 as measured by real-time PCR. Values shown are relative to control MA1s from the same rats and normalized to cyclophilin mRNA levels, which were not different between groups. n=4-6; this represents the number of rats. Five HF and five LF MA1s were pooled from each rat at 4 days after the induction of HF/ LF. * P<0.05, ** P<0.01. D)There was no induction of PDE5 mRNA in the Day4 HF/ LF MA1 as compared to the control MA1s from the same L-NAME fed rats (n=4, P>0.05). Values are normalized to control MA1 from rats not treated with L-NAME. HF, High flow MA1; LF, Low Flow MA1. sGC, soluble guanylyl cyclase; cGK, cGMP dependent kinase; PDE, phosphodiesterase; MYPT1, myosin phosphatase targeting subunit; PP1c, myosin phosphatase catalyzing subunit.

Figure 2.

Abundance of PDE5 and eNOS protein in Day4 HF and LF MA1 as measured by Western blot. Twenty five μ g of total protein were separated through 3-8% NuPAGE gels and blots probed with antibodies against PDE5, eNOS and smooth muscle α -actin as a loading control, as described in Methods. Five HF and five LF MA1s were pooled from each rat at 4 days after the induction of HF/LF. Control n=4, HF and LF n=6. Shown is a representative blot containing two different control and 3 different HF/LF samples. Bands were quantified by densitometry and normalized to α -actin. Positive controls: rat lung homogenates (25 μ g protein) for PDE5, eNOS. CON, control, kDa, kilo Daltons.

Figure 3.

Phenylephrine (PE) dose-response relationship of force production in CON and Day4 HF and LF MA1. In this and the following figures force was measured in vessels studied in parallel under isometric conditions in a four chamber wire myograph. Developed tension is normalized to vessel wall length as described in methods, and plotted against the log of the PE concentration. A) HF and LF vessels were less sensitive to PE than control MA1s ($n=5-6$, $P<0.01$ for EC_{50}). PE dose responses were examined in B) CON C) HF and D) LF MA1 before and after pre-treatment with the arginine analogue L-NAME (0.1 mM, 15 min) to block endogenous NO production. With L-NAME pre-treatment, the sensitivities of HF and LF Day4 MA1 to PE were not different from control ($n=5-6$, $P>0.05$ for EC_{50}).

Figure 4.

Effect of PDE5 inhibitor Sildenafil on PE dose-response relationships. Dose-response to PE alone was measured followed by dose-response to PE after pre-treatment with sildenafil (1 μ M, 15 min) in CON (A) and Day4 HF (B) and LF (C) MA1. Sildenafil reduced the sensitivity to PE and reduced maximum force in all three groups. The shift in EC₅₀ with Sildenafil was significantly greater in the HF and LF MA1 as compared to CON (n=4-7, P<0.01 for EC₅₀s with sildenafil). * P<0.05, ** P<0.01. vs. no sildenafil pre-treatment.

Figure 5.

Sildenafil dose-response relationships after activation with PE. CON (A) and Day4 HF (B) and LF (C) MA1s were pre-constricted with 10 μ M PE with or without L-NAME (0.1 mM) pre-treatment. Force was then continuously monitored with cumulative addition of sildenafil. The data is plotted as percentage of maximum tension vs. log of sildenafil concentration. The HF and LF MA1s tended to be more sensitive to Sildenafil than the CON MA1. The highest concentration of sildenafil (1 μ M) caused a more powerful relaxation in HF and LF MA1 as compared to CON. (HF, $n=6$ vs. CON, $n=3$, $P<0.05$; LF, $n=6$ vs. CON, $n=3$, $P<0.01$). Inhibition of NO synthesis with L-NAME almost completely blocked the effect of Sildenafil in all three groups.

Figure 6.

DEA/NO dose-response relationships after activation with PE. A) CON and Day4 HF and LF MA1 were pre-treated with L-NAME (0.1 mM) to block endogenous NO synthesis, activated with PE (10 μ M), and force continuously recorded with cumulative addition of DEA/NO. Data is plotted as the percentage of maximal tension vs log of DEA/NO concentration. There was no difference in the sensitivities to DEA/NO between groups ($n=4$, $P>0.05$). B) Vessels were treated as in (A) and in addition pre-incubated with sildenafil (0.1 μ M). DEA/NO at intermediate concentrations ($10^{-7.5}$ M) in the presence of the PDE5 inhibitor produced more relaxation of the HF and LF vs CON MA1 ($n=5-6$. * $P<0.05$, ** $P<0.01$).

Figure 7.

Effect of PDE1A inhibitor Vinpocetine on PE induced force. Dose-response to PE alone followed by dose-response to PE after pre-treatment with Vinpocetine (0.1 mM, 15 min) in CON (A) and Day4 HF (B) and LF (C) MA1. Vinpocetine caused a similar shift in sensitivity to PE in all three groups (n=3-4). (D) Vinpocetine dose-response after activation of force with PE 10 μ M. There was no difference in the Vinpocetine EC50 among CON, HF and LF MA1 (n=4, P>0.05).

Figure 8.

cGMP levels in CON and Day4 HF and LF MA1. Three CON, HF and LF MA1s were pooled from each rat and treated in separate wells with L-NAME (0.1 mM, 30 min) to block endogenous NO synthesis. Another group of MA1s were also pre-treated with Sildenafil (1 μ M, 30 min). DEA/NO was then added to a final concentration of 0.1 μ M. After five minutes the vessels were snap frozen in liquid nitrogen. cGMP levels were measured in duplicate using an ELISA method as described in Methods and normalized to mg protein. cGMP levels were slightly lower in LF and HF MA1 with DEA/NO, and modestly higher with DEA/NO and Sildenafil. Sildenafil had a significantly larger effect on cGMP levels in the HF and LF MA1 as compared to CON (n=4). * P<0.05.

Table 1

Primers for real-time PCR

	GenBank Accession No.	primer (sense)	primer (antisense)	product size
eNOS	AF085195	GCAAGACCGATTACACGACA	CCGGTAGAGATGGTCCAGTT	100bp
iNOS	D12520	AGTGAGGAGCAGGTTGAGGA	AAAAGACCGCACCGAAGATA	82bp
sGCα1	U60835	TCATACGGGTGAGGAGATGG	ATGGCACTGGATTCAACGAT	97bp
sGCβ1	M22562	TTTCCACGGGATTCTTTCAC	CTCTGCCCCAGTCAGTTCAT	109bp
cGK1	(NM_011160) BLAT	TTACCTGCTTCTGCCTTGCT	TGACCCCGAGCACTaATCTT	94bp
PDE5A	D89093	AGTTTGCTTTGCACGCCTAT	TTGCCAGTCTTCTCCTCCAT	92bp
PDE1A	NM_030871	GCAGAACCAAGGAGAGGTGGA	TGTGTGTCAGCATGCTTTTCT	96bp
PP1cδ	D90164	GATGAGAAAATCTTCTGCTGTCA	TCAGGTACGTCAGTGGGTCTC	95bp