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Effects of Chronic Nitric Oxide Synthase Inhibition on Endothelium-Dependent and -Independent Relaxation in Arteries that Perfuse Skeletal Muscle of Swine

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Abstract

The purpose of this investigation was to test the hypothesis that chronic L-NAME treatment produces differential effects on conduit artery and resistance arteriole relaxation responses to endothelium-dependent and -independent vasodilators in arteries that perfuse skeletal muscle of swine. To test this hypothesis conduit skeletal muscle arteries and second order skeletal muscle arterioles were harvested from 14 Yucatan swine that were chronically administered L-NAME and 16 controls. In vitro assessments of vasorelaxation to increasing doses of acetylcholine (ACH), bradykinin (BK), and sodium nitroprusside (SNP) were performed in both conduit and 2A arterioles. L-NAME treatment produced a significant reduction in both BK and ACH relaxation responses in the conduit arteries. In contrast, the relaxation response and/or sensitivity to SNP were significantly greater in the intact, but not denuded, conduit arterial rings from chronically L-NAME treated swine. There were no significant effects of chronic L-NAME treatment on vasodilation of skeletal muscle arterioles. These findings suggest: (1) that unlike arterioles, skeletal muscle conduit arteries do not functionally compensate for a lack of NO through the upregulation of alternative vasodilator pathways. (2) that the greater relaxation response in conduit arteries of chronically L-NAME treated swine to SNP can be explained by alterations to the endothelium.

Keywords

Nitric oxide synthase; conduit arteries; arterioles; skeletal muscle

Introduction

Chronic inhibition of nitric oxide synthase (NOS) is commonly utilized to estimate the role of nitric oxide (NO) in cardiovascular function. Interestingly, endothelium-dependent relaxation in vitro to either acetylcholine (ACh) or flow has been reported to be maintained in skeletal muscle and mesenteric arterioles of rats that were chronically administered N^G-nitro-L-arginine methyl ester (L-NAME) (Dowell *et al.*, 1996; Wu *et al.*, 2001). This finding is in agreement with data collected from various arterioles of eNOS gene-disrupted mice (Meng *et al.*, 1996; Godecke *et al.*, 1998; Sun *et al.*, 1999). The maintained dilatory response of arterioles lacking the ability to synthesize NO with eNOS has been attributed to the upregulation of prostacyclin (PGI₂) bioavailability (Godecke *et al.*, 1998; Sun *et al.*, 1999; Wu *et al.*, 2001). These findings are consistent with the finding that production of NO within the endothelial cell is known to inhibit the synthesis of PGI₂ and endothelium-

dependent hyperpolarizing factor (EDHF) (Doni *et al.*, 1988; Bauersachs *et al.*, 1996; Bauersachs *et al.*, 1997; Nishikawa *et al.*, 2000), a paracrine effect that would not occur in the absence/inhibition of eNOS. Similar in vitro experiments utilizing conduit arteries from L-NAME treated and eNOS knockout mice suggest, however, that the upregulation of alternative vasodilator pathways in the absence of NO is not consistently observed since ACh-induced relaxation of the aorta, basilar and carotid arteries has been reported to be attenuated in chronically L-NAME treated and eNOS knockout mice when compared to controls (Moreau *et al.*, 1995; Faraci *et al.*, 1998; Kojda *et al.*, 1999; Lake-Bruse *et al.*, 1999; Linder *et al.*, 2005).

Relaxation responses of vascular smooth muscle (endothelium-independent relaxation), such as responses to sodium nitroprusside (SNP), have been reported to be enhanced in the conduit arteries of chronically L-NAME treated animals (Linder *et al.*, 2005). Consistent with this finding, SNP-induced relaxation has been reported to be enhanced in the carotid arteries of eNOS gene disrupted mice (Faraci *et al.*, 1998). Interestingly, enhanced SNP-induced relaxation has not been reported in the pial arterioles of eNOS gene disrupted mice (Meng *et al.*, 1996) or in mesenteric resistance arteries of animals chronically treated with L-arginine analogs (Dowell *et al.*, 1996). Considering these results in light of the effects of chronic NOS inhibition on endothelium-dependent relaxation discussed above suggests that smooth muscle of conduit arteries and arterioles also exhibits differential adaptations to chronic NOS inhibition in that altered smooth muscle responsiveness is seen in conduit arteries but not in arterioles. Unfortunately, the experiments on conduit arteries and arterioles have generally not been conducted in the same animal.

Recently, we reported that chronic L-NAME treatment altered relaxation responses of conduit coronary arteries but not coronary arterioles from the same swine (Ingram *et al.*, 2007). It is unknown whether these findings from the coronary circulation are unique to this vascular bed or if these effects of chronic L-NAME treatment are generalized. This question is of particular importance for the skeletal muscle circulation because it plays a greater role in the regulation of blood pressure than does the coronary circulation. In our view at least three factors indicate that it is reasonable to expect that chronic NOS inhibition has relatively greater effects on skeletal muscle arterioles/resistance arteries than on conduit arteries of skeletal muscle vascular beds. First, skeletal muscle tissue represents approximately 40% of total body mass in most mammals (Rowell, 1986). Second, with constant cardiac output, blood pressure is directly related to vascular resistance determined by the caliber of resistance arterioles in the periphery (Rowell, 1986). And third, chronic NOS inhibition consistently has been reported to increase mean arterial pressure in vivo (Moreau *et al.*, 1995; Dowell *et al.*, 1996; Puybasset *et al.*, 1996; Henrion *et al.*, 1997; Wu *et al.*, 2001; Linder *et al.*, 2005). Thus we conceived that, because chronic NOS inhibition causes increased blood pressure, it must produce a greater effect on skeletal muscle arterioles which are a major determinant of vascular resistance. Based on this rationale, one would expect that chronic NOS inhibition would have greater effects on endothelium-dependent dilation of skeletal muscle arterioles than of conduit arteries. Therefore, the purpose of this investigation was to test the hypothesis that chronic L-NAME treatment would produce differential effects on skeletal muscle conduit artery and resistance arteriole relaxation responses to endothelium-dependent and -independent vasorelaxation. We designed our experiment to compare and contrast the effects of L-NAME treatment on conduit arteries and skeletal muscle arterioles from the forelimb and hindlimb of the same swine.

Methods

Experimental Animals

Thirty female Yucatan miniature swine, ranging in ages between 11 and 13 months, were utilized for this experiment. L-NAME was chronically administered to 14 of these animals at an average dose of 8.3 ± 0.6 mg/kg/d in their drinking water for ≥ 4 weeks. The additional 16 animals were used as controls. Effectiveness of the L-NAME treatment was assessed by measuring resting mean arterial pressure (MAP) via an externalized catheter, which was surgically implanted in the aorta (via the internal thoracic artery) of control (n=5) and L-NAME (n=7) swine. The catheter was connected to a pressure transducer that was interfaced with a digital pressure monitoring system (MicroMed BP Analyzer). In addition, Plasma NO_x concentration was determined via VCl_3 -induced reduction of NO_x to NO. NO, in turn, was reacted with O_3 to produce chemiluminescence that was detected by a photomultiplier tube (Sievers NOA 280i analyzer). Lastly, NOS activity was determined in the brachial and femoral arteries of control (n=4) and L-NAME (n=5) swine as previously described in detail (McAllister *et al.*, 2005).

Conduit Arteries

Pharmacological studies—Brachial and femoral arteries were harvested, cleaned, measured and set to the optimal point in the length tension relationship ($L_{\max}$) as previously described (Newcomer *et al.*, 2007). Contractile responses of the brachial and femoral rings to KCl were performed prior to vasorelaxation studies. KCl (80mM) was administered twice to all of the arterial rings until the increase in tension had reached a plateau (~ 10 min). Krebs bicarbonate solution was replaced to wash out KCl and arterial rings were allowed to regain prior resting tensions between the first and second administrations of KCl. Following the KCl studies, Krebs bicarbonate solution was replaced every 20 minutes until resting tension was achieved.

Endothelium-dependent, dose-dependent vasorelaxation was assessed in all rings using cumulative addition of bradykinin (BK; 10^{-11} - 10^{-6} M) and ACh (10^{-10} - 10^{-4} M). The assessment of endothelium-independent vasorelaxation utilized increasing doses of SNP (10^{-10} - 10^{-4} M). All rings were precontracted with $\text{PGF}_{2\alpha}$ (30 μ M) and allowed to achieve a plateau in tension development before the addition of either endothelium-dependent or –independent vasodilators. The role of the nitric oxide synthase (NOS) pathway in the relaxation responses to endothelium-dependent and –independent vasodilators was assessed through the addition of L-NAME (300 μ M) to the Krebs bicarbonate solution of one ring from the brachial and femoral artery 30 minutes before precontraction with $\text{PGF}_{2\alpha}$. Similarly, a ring from the brachial and femoral arteries was pretreated with indomethacin (INDO; 5 μ M) to investigate the role of the cyclooxygenase (COX) pathway in relaxation responses to endothelium-dependent and –independent vasodilators. NOS and COX independent pathways to endothelium-dependent and –independent relaxation were assessed by pretreating a ring from the brachial and femoral arteries with a combination of L-NAME and INDO. Lastly, rings from the brachial and femoral arteries of 10 swine (5 control and 5 L-NAME treated) were denuded, by gently rubbing the lumen of the ring with forceps, to determine the role of the endothelium in BK and SNP induced vasorelaxation. The order of agonists throughout the entire study was BK, ACh, and SNP. Following each agonist induced dose response, Krebs bicarbonate solution was replaced at 20 minute intervals until resting tension of all arterial rings was reached (~ 60 min).

Immunoblot analysis—To elucidate potential mechanisms underlying the results from the functional experiments guanylate cyclase (GC; α -subunit) protein content, and eNOS protein content were assessed with immunoblot analysis in the brachial and femoral

samples. The portion of the right brachial and femoral artery that was not utilized for vasomotor function experiments was cut into two ~1–2cm length segments. One arterial segment was placed in Laemmli buffer solution in a microcentrifuge tube and stored until immunoblot analysis at -80°C . This segment was later used to assess eNOS and GC α protein content in both the endothelium and vascular smooth muscle. The other segment was cut longitudinally and pinned to expose the lumen of the arterial segment. The endothelium was then scraped after application of 200 μL of Laemmli buffer; this buffer was then transferred from the luminal surface into a microcentrifuge tube for storage at -80°C . This segment was utilized to assess eNOS protein content in the endothelium alone.

On the day of immunoblot analysis whole brachial and femoral arterial segments were solubilized in Laemmli buffer (0.01 $\mu\text{g}/100\text{uL}$), vortexed, centrifuged at 9000rpm for 30 seconds, boiled for 2 minutes, and sonicated for 10 seconds. This series of steps was repeated 5 times to adequately solubilize the arterial segment. Endothelial scrapes were diluted 1:2 (15 μL :15 μL) in Laemmli buffer and vortexed. Total protein content of brachial and femoral whole vessel segments and endothelial scrapes was measured utilizing the NanoOrange protein assay. Protein samples from the whole vessel segment (5 $\mu\text{g}/\text{lane}$) and endothelial scrapes (2.5 $\mu\text{g}/\text{lane}$) were loaded on separate 12 lane 5–12% NuPage Bis Tris gradient gels, electrophoresed at 200 volts for 50 minutes, and transferred at 34V for 60 minutes to a polyvinylidene difluoride membrane (Hybond-ECL, Amersham). The membranes were blocked in 5% nonfat milk and TBST (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20) at room temperature for 1 hour. Overnight the membrane was incubated with the primary antibody against eNOS (1:1000; Transduction Laboratories). This was followed by one hour incubation with a secondary antibody (1:2,500; horseradish peroxidase-conjugated anti-mouse; Sigma Chemical). Analysis of eNOS protein was performed with chemiluminescence and quantified by densitometry through the use of NIH Image software (National Institute of Health, Bethesda, MD). Whole vessel segment blots were reblocked for 1 hour at room temperature and incubated overnight in an antibody against GC α (1:5000; Cayman Chemical). Blots were then incubated for 1 hour in a secondary antibody (1:3333; horseradish peroxidase-conjugated anti-rabbit; Sigma Chemical). Equal loading was confirmed with Coomassie staining.

Resistance Arterioles

Second order arterioles (2A arterioles) were selected from the extensor carpi ulnaris (ECU) and gracilis, muscles located in the forelimb and hindlimb, respectively. The harvesting, preparation and mounting of these arterioles for in vitro vasomotor function experiments has been previously described in detail (Newcomer *et al.*, 2007). Isolated arterioles were allowed to warm-up to 37°C and equilibrate at 60 cmH_2O intraluminal pressure. At the end of 1 hour of warm-up the vessels were challenged with 80mM KCl. If the vessels did not constrict to 80mM KCl then they were considered dead and were not included in the experiment. Following the 80 mM KCl challenge, arterioles that did not develop spontaneous tone were stimulated to constrict with phenylephrine (PE) until 30% constriction based on the measured baseline was achieved. This preconstruction protocol was repeated prior to each vasodilatory curve as necessary. To assess endothelium-dependent and independent dilation arterioles were exposed to three vasodilators; BK (10^{-12} - 10^{-6} M), ACh (10^{-9} to 10^{-4} M), and SNP (10^{-9} to 10^{-4} M) added in whole log increments to determine the dose-response relationship for each vessel to the various dilators. Vessels that did not exhibit a response to two or more dilators were not included in the following data. At the end of the experiment the PSS was removed from the bath and replaced with calcium free PSS. The vessel was allowed to remain in calcium free PSS for 30 to 45 minutes while the solution was changed every 15 minutes. To confirm that maximal calcium free diameter had been achieved the bathing solution was removed and replaced

with 10mM caffeine dissolved in calcium free PSS and thapsigargin, a SERCA inhibitor. The vessel was then allowed to bathe in this solution for an additional 15 minutes.

Solutions and drugs

Krebs bicarbonate buffer solution was comprised of (in mM) 131.5 NaCl, 5.0 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.5 CaCl₂, 11.2 glucose, 20.8 NaHCO₃, 0.003 propranolol, and 0.025 EDTA. Krebs bicarbonate solution pH was adjusted to 7.4. The PSS used for isolated arterioles contained the following (in mM): 145.0 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.17 MgSO₄, 3.0 3-(N-morpholino) propanesulfonic acid (MOPS), 1.2 NaH₂PO₄, 5.0 glucose, 2.0 pyruvate, and 0.02 EDTA. Calcium free PSS was made with the reagents above except that 2.0 mM CaCl₂ was omitted and 2mM EDTA was added. Caffeine was diluted in calcium free PSS. Thapsigargin was diluted in ethanol such that in the bath the final concentration of ethanol was not more than 1% of the total bath volume. All chemicals were acquired from Sigma Chemical.

Statistical analysis

Repeated measures two-way ANOVA models were applied to determine the effects of chronic L-NAME treatment on forelimb and hindlimb vascular responses to bradykinin, acetylcholine, and sodium nitroprusside. An unpaired Student's t test was utilized to compare blood pressure, vessel characteristics, IC₅₀, maximal relaxation, eNOS protein expression, and GCα protein expression in the brachial and femoral arteries of chronically L-NAME treated and control swine. Values for IC₅₀ were calculated using a nonlinear regression analysis (SigmaPlot 8.0 & Prism). Data for eNOS and GCα protein expression were normalized such that control values were set to 1 and L-NAME values were expressed as a fold value relative to control (Woodman *et al.*, 2003). Statistical significance was set at P<0.05. All data are presented as means ± se.

Results

Treatment efficacy

Resting blood pressures in the chronically treated L-NAME swine were significantly higher (P<0.05) than those measured in the control swine (Fig 1A), while, plasma NO_x concentrations were significantly lower (P<0.05) in the chronically L-NAME treated compared to control swine (Fig 1B). In addition, NOS activity was significantly higher (P<0.05) in the brachial arteries (Fig 1C) and tended (P=0.1) to be higher in the femoral arteries (Data not shown) of control swine.

Conduit artery characteristics

Table 1 presents structural characteristics of rings cut from the brachial and femoral arteries and used for functional experiments. There were no significant differences in axial length, outer diameter, inner diameter, wall thickness, resting tension, resting 2A arteriole diameter, maximal 2A arteriole diameter and constriction to both 80mM KCl and 30μM PGF_{2α} between rings from control and L-NAME treated animals (Table 1). Similarly, no significant differences in these same variables were observed in rings treated with INDO, L-NAME, or L-NAME + INDO from control and L-NAME treated animals (data not shown).

Conduit artery pharmacological experiments

Bradykinin responses—Bradykinin produced a concentration dependent relaxation in all brachial (Fig. 2A,B) and femoral rings (Fig. 2C,D). Compared to controls, chronic L-NAME treatment was associated with a blunted overall relaxation response to bradykinin in both the intact and INDO treated brachial and femoral rings (P=0.0001). Similarly, maximal

relaxation to bradykinin was significantly attenuated in the intact (Brachial, $P=0.0002$; Femoral, $P=8\times 10^{-8}$) and INDO (Brachial, $P=0.001$; Femoral, $P=0.00002$) treated brachial and femoral rings of swine chronically administered L-NAME compared to controls (Tables 2&3). Chronic treatment with L-NAME also produced a rightward shift in the dose response curve, as indicated by greater IC_{50} values, of the intact (Brachial, $P=0.0001$; Femoral, $P=0.0003$) and INDO (Brachial, $P=0.0001$; Femoral, $P=0.0008$) treated brachial and femoral rings when compared to the corresponding rings from control swine (Tables 2&3). However, brachial and femoral rings from the control swine that were incubated in L-NAME or L-NAME+INDO had similar bradykinin-induced relaxation responses, maximal relaxation, and IC_{50} as the corresponding rings from chronically treated L-NAME swine.

Similar effects [as those described above] of chronic L-NAME treatment on bradykinin-induced dose response, maximum relaxation, and IC_{50} were observed in the intact brachial and femoral rings of ten additional swine. These additional experiments were done to establish that there was no significant bradykinin-induced relaxation of denuded brachial and femoral rings from either L-NAME treated or control swine (Data not shown).

Acetylcholine responses—Acetylcholine produced a concentration dependent relaxation in all brachial rings from control and chronically L-NAME treated swine (Fig. 3A,B). The relaxation response to acetylcholine was significantly attenuated ($P<0.02$) in all brachial rings of chronically L-NAME treated swine when compared to the corresponding rings of control swine (Fig 3A,B). In addition, the maximal relaxation to acetylcholine was significantly less in the intact ($P=0.0001$), INDO ($P=0.00009$), and L-NAME ($P=0.03$) brachial rings of the chronically L-NAME treated compared to control swine (Table 2). Chronic treatment with L-NAME also produced a rightward shift in the dose response curve, as indicated by IC_{50} , of the intact ($P=0.007$) and INDO ($P=0.001$) treated brachial rings when compared to the corresponding rings from control swine (Table 2).

Similar to the brachial rings, acetylcholine produced a concentration dependent relaxation in all femoral rings from control and chronically L-NAME treated swine (Fig. 3C,D). The relaxation response to acetylcholine was significantly attenuated ($P<0.05$) and shifted to the right ($P=0.04$) in the femoral rings of chronically L-NAME treated swine compared to corresponding rings of control swine (Table 3). Acetylcholine also produced a significantly greater maximal relaxation in the femoral intact ($P=0.0007$) and INDO ($P=0.0001$) treated rings of control compared to the chronically L-NAME treated swine (Table 3).

Sodium nitroprusside responses—Sodium nitroprusside produced a concentration dependent relaxation in the brachial rings from control and chronically treated L-NAME swine (Fig. 4). The relaxation response to sodium nitroprusside was significantly greater ($P=0.02$) in the brachial INDO ring of the chronically L-NAME treated compared to control swine (Fig. 4B). The sensitivity to sodium nitroprusside was significantly shifted to the left in the chronically L-NAME treated swine's intact ($P=0.03$) and INDO ($P=0.04$) rings as compared to the corresponding rings from the control swine (IC_{50} ; Table 2). However, there were no significant differences in maximal relaxation of any of the brachial rings from control and L-NAME treated swine (Table 2).

Sodium nitroprusside also produced a concentration dependent increase in relaxation of the femoral rings of control and chronically treated L-NAME swine (Fig. 5). There was a significantly greater ($P<0.04$) relaxation response to sodium nitroprusside in all the femoral rings from the chronically L-NAME treated swine except for the ring incubated in L-NAME + INDO. This greater relaxation response to sodium nitroprusside in the intact, INDO, and L-NAME femoral rings of chronically treated L-NAME swine (Fig. 5A,B,C) was accompanied by a significant leftward shift ($P<0.04$) compared to controls in the dose

response curves of these rings (Table 3). Also, similar to brachial rings, there were no significant differences in the maximal relaxation of any of the femoral rings from control and L-NAME treated swine (Table 3).

In a set of ten additional swine, chronic L-NAME treatment also produced a significantly greater sodium nitroprusside-induced dose response and leftward shift in the intact brachial and femoral rings (Fig 6A,C). There were no significant differences in either the concentration dependent relaxation to sodium nitroprusside or IC_{50} of denuded brachial and femoral rings from chronically L-NAME treated and control swine (Fig 6B,D).

Resistance arteriole pharmacological experiments

Bradykinin, acetylcholine, and sodium nitroprusside all produced concentration dependent relaxations in arterioles harvested from the forelimbs and hindlimbs of chronically L-NAME treated and control swine (Fig 7 and 8). However, unlike the conduit arteries, forelimb and hindlimb arteriole responses to bradykinin, acetylcholine, and sodium nitroprusside were not significantly different between chronically L-NAME treated and control swine (Fig 7 and 8).

Immunoblots

Chronic L-NAME treatment produced a significant ($P<0.05$) increase in eNOS protein content in femoral artery endothelial cell scrapes (Fig. 9). These differences in eNOS protein content were not observed in the brachial artery endothelial scrapes. There were no significant differences in GC α protein content in either the brachial or femoral arteries of chronically L-NAME treated and control swine (Fig 10).

Discussion and Conclusions

The purpose of this investigation was to test the hypothesis that chronic L-NAME treatment would elicit differential effects on conduit artery and resistance arteriole relaxation responses to endothelium-dependent and -independent vasodilators in arteries perfusing limb skeletal muscles of swine. Results indicate that chronic inhibition of NOS is associated with decreased endothelium-dependent relaxation in conduit arteries and maintained endothelium-dependent relaxation in resistance arterioles. An important strength of our study is that it is the first to demonstrate these differential effects in skeletal muscle conduit arteries versus skeletal muscle arterioles in the same experimental animals. Our results also indicate that chronic L-NAME treatment resulted in an enhanced response and/or sensitivity of the femoral and brachial arteries to SNP, an endothelium-independent vasodilator. Finally, our results demonstrate that the endothelium plays an apparent role in the enhanced response of chronically L-NAME treated brachial and femoral artery rings to SNP since endothelial removal abolished the differences between L-NAME treated and control responses.

Effects of Chronic inhibition of NOS on endothelium-dependent relaxation in skeletal muscle conduit arteries and resistance arterioles of swine

Relaxation mediated by both bradykinin and acetylcholine was attenuated in the brachial and femoral arteries of chronically L-NAME treated Yucatan miniature swine. These findings are in agreement with previous published data indicating that chronic inhibition of NOS reduces endothelium-dependent relaxation in several conduit arteries (Moreau *et al.*, 1995; Puybasset *et al.*, 1996; Linder *et al.*, 2005; Ingram *et al.*, 2007). In the current study, differences in endothelium-dependent relaxation between the control and chronically L-NAME treated swine can be attributed to the successful inhibition of NOS as evidenced by increased MAP and decreased plasma NO x concentrations. This interpretation is supported by the finding that acute incubation of control brachial and femoral rings in either L-NAME

or L-NAME+INDO ameliorated differences in the bradykinin responses between the control and chronically L-NAME swine, whereas the differences in the bradykinin responses were maintained in rings pretreated with INDU alone (Figure 2). Similar findings have also been reported in the femoral arteries of dogs infused with N^ω-nitro-L-arginine (L-NNA) for 7 days (Puybasset *et al.*, 1996).

It is surprising that the femoral and brachial arteries of chronically L-NAME treated swine did not maintain endothelium-dependent dilation through the upregulation of alternative vasodilator pathways in light of data suggesting that nitric oxide normally inhibits the synthesis of PGI₂ and EDHF (Doni *et al.*, 1988; Bauersachs *et al.*, 1996; Bauersachs *et al.*, 1997; Nishikawa *et al.*, 2000). Specifically, findings from experiments using cultured aortic endothelial cells, coronary arterioles, and conduit arteries suggest that chronic inhibition of NOS enhances the release of both PGI₂ and EDHF by the endothelium. Consistent with the data suggesting an inhibitory effect of NO on alternative vasodilator pathways are the facts that mesenteric resistance arterioles from chronically eNOS inhibited rodents have maintained endothelium-dependent (Dowell *et al.*, 1996) and that skeletal muscle arterioles have maintained shear stress induced dilation (Wu *et al.*, 2001) that are mediated through the upregulation of the PGI₂ pathway. This is also consistent with our previous finding of a trend toward an increased reliance on PGI₂ and/or EDHF in the coronary arterioles of chronically L-NAME treated swine (Ingram *et al.*, 2007). Our current observation of maintained endothelium-dependent relaxation in skeletal muscle arterioles from the forelimb and hindlimb of swine is consistent with these previous reports. Maintained agonist and shear stress dependent relaxation through the compensatory upregulation of PGI₂ have also been reported in coronary, pial, and skeletal muscle arterioles from eNOS gene-disrupted mice (Bauersachs *et al.*, 1996; Meng *et al.*, 1996; Godecke *et al.*, 1998; Sun *et al.*, 1999). We believe that careful review of the literature in combination with the present results indicate that chronic inhibition of NOS produces greater effects on vasomotor function of conduit arteries than arterioles, with compensation for the loss of NO by PGI₂ and/or EDHF in resistance arterioles but not conduit arteries.

Although endothelium-dependent relaxation was blunted in brachial and femoral arteries of chronically NOS inhibited pigs, we found that eNOS protein content was increased in the endothelium of femoral arteries of these swine (Fig 8). Interestingly, previous studies utilizing similar models of chronic eNOS inhibition have not reported a compensatory upregulation of eNOS protein in conduit arteries (Kobayashi *et al.*, 2000; De Gennaro Colonna *et al.*, 2002; Husain, 2004). Moreover, eNOS protein expression and activity have been reported to be significantly reduced in the cardiac tissue of rats chronically administered L-NAME for 8 weeks (Husain, 2004). In addition, chronic inhibition of eNOS has also been associated with a significant reduction of eNOS mRNA in rodent aorta and left ventricular tissue (Kobayashi *et al.*, 2000; De Gennaro Colonna *et al.*, 2002). Thus current literature does not present a clear consensus about the effects of chronic NOS inhibition on eNOS expression. One potential explanation for this controversy is differences in the tissues utilized for eNOS protein measurements. Previous studies measured eNOS protein or mRNA in either whole vessel or cardiac tissue samples (Kobayashi *et al.*, 2000; De Gennaro Colonna *et al.*, 2002; Husain, 2004), whereas we measured eNOS protein content in the endothelial cells from the brachial and femoral arteries. We did this because our histological data demonstrate that most eNOS is located in the endothelial cells. Interestingly, when we measured eNOS protein with immunoblots on whole brachial and femoral artery samples from the same animals, no significant differences were found between the chronically treated L-NAME arteries and controls (data not shown). Thus analysis of whole tissue eNOS content may mask changes in endothelial cell protein content. Nonetheless, this increase in eNOS protein content of femoral arteries was not associated with improved endothelium-dependent relaxation.

Effects of chronic inhibition of NOS on endothelium-independent relaxation in the brachial and femoral arteries of swine

Linder et al. reported that chronic NOS inhibition was associated with increased sensitivity to sodium nitroprusside in rodent aorta (Linder *et al.*, 2005). Similarly, we recently reported that chronic inhibition of NOS is associated with an increased sensitivity to sodium nitroprusside in the left anterior descending coronary artery of swine (Ingram *et al.*, 2007). Current results confirm these observations as sensitivity to SNP was increased in intact brachial and femoral arteries of chronically L-NAME treated swine (Tables 2,3) while maximal dilation to SNP was similar between NOS inhibited animals and controls (Moreau *et al.*, 1995; Puybasset *et al.*, 1996; De Gennaro Colonna *et al.*, 2002; Linder *et al.*, 2005). Results from carotid arteries of eNOS gene disrupted mice also exhibit enhanced sensitivity to sodium nitroprusside (Faraci *et al.*, 1998). In contrast, data collected from aortic rings of eNOS gene disrupted rodents suggest that sodium nitroprusside responses are either maintained (Huang *et al.*, 1995; Brandes *et al.*, 2000) or decreased (Lake-Bruse *et al.*, 1999). It seems plausible that vascular smooth muscle of murine aorta may respond differently to a lack of NO than does the carotid artery and differently from the conduit arteries of swine.

An attractive hypothesis concerning the underlying mechanism involved in an accentuated relaxation to SNP in the conduit arteries of NOS inhibited swine is that chronic NOS inhibition and the subsequent reduction in endogenous nitric oxide results in a compensatory upregulation of the GC pathway in vascular smooth muscle. We tested this hypothesis by measuring GC α protein content in the brachial and femoral arteries of chronically L-NAME treated swine. Results demonstrate that GC α protein content was similar, compared to controls (Fig 9). Based on these results we conclude that increased relaxation and/or sensitivity of the brachial and femoral arteries to sodium nitroprusside of L-NAME treated swine are not a consequence of increased vascular smooth muscle GC α protein. This finding is consistent with previous results showing maintained GC activity in lung tissue of eNOS knockout mice (Kojda *et al.*, 1999) and similar GC protein content in aortic tissue of eNOS knockout mice (Brandes *et al.*, 2000). Future investigations will need to elucidate whether alternative regulatory proteins within the GC/cGMP signaling pathway distal to GC are altered with chronic L-NAME administration.

Also consistent with the conclusion that the vascular smooth muscle GC pathway may not be the underlying mechanism responsible for accentuated sodium nitroprusside responses following chronic L-NAME treatment in swine is our observation that sodium nitroprusside-induced relaxation responses in denuded brachial and femoral (Fig. 5B,D) rings of chronically L-NAME treated pigs were similar to those of control swine. These results, from the denuded brachial and femoral rings, suggest that interactions between the endothelium and sodium nitroprusside account for an altered sodium nitroprusside response of the brachial and femoral arteries of chronic L-NAME treated swine. Future investigations will need to elucidate how chronic L-NAME treatment alters the endothelium to enhance sodium nitroprusside responses in conduit arteries.

Physiological perspective

The findings of the current study add insight into the role that NO produced by NOS plays in the regulation of blood pressure and blood flow. Our current data suggests that the elevated resting blood pressure observed in our chronically L-NAME administered swine is not mediated through decreased endothelium-dependent vasodilator function in skeletal muscle arterioles as we hypothesized. These findings support previously published data which suggests that the effects of chronic L-NAME administration on blood pressure regulation are mediated through the sympathoinhibitory effects of NO within the central nervous system (Patel *et al.*, 2001). In addition, our observation of maintained endothelium-dependent

relaxation in the skeletal muscle arterioles of swine that were chronically administered L-NAME is consistent with our recently published data demonstrating preserved resting and exercising skeletal muscle blood flow in chronically L-NAME treated swine (McAllister *et al.*, 2007).

In conclusion, the results of the current study indicate: (1) that chronic inhibition of NOS results in a diminished endothelium-dependent relaxation in the brachial and femoral arteries harvested from Yucatan miniature swine and (2) that the greater relaxation response and/or sensitivity in the brachial and femoral arteries of the L-NAME swine to SNP can be explained by alterations to the endothelium and not a higher guanylate cyclase α protein content. Therefore these results indicate that unlike arterioles, brachial and femoral arteries failed to compensate for a lack of nitric oxide by upregulating alternative vasodilator pathways. These heterogeneous responses of conduit arteries and arterioles to reduced nitric oxide synthesis may play a role in the preferential localization of atherosclerotic lesions to large vessels, given that NO inhibits several steps of the atherosclerotic process.

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Figure 1. Blood pressure (A), plasma NOx concentrations (B) and NOS activity (C) in control (■) and chronically L-NAME treated (□) swine
Values are means $\pm$ SE. * different than control (P<0.05).

Figure 2. Bradykinin-induced relaxation of the (A) control brachial (n=11), (B) chronically L-NAME treated brachial (n=9), (C) control femoral (n=11), and (D) chronically L-NAME treated femoral (n=9) arteries of swine
 Values are means $\pm$ SE. Data expressed as a percent relaxation in force from PGF_{2 α} (30 μ M)-induced tension. (X) Significantly greater than chronically treated L-NAME swine (P<0.05).

Figure 3. Acetylcholine-induced relaxation of (A) control brachial (n=11), (B) chronically L-NAME treated brachial (n=9), (C) control femoral (n=11), and (D) chronically L-NAME treated femoral (n=9) arteries of swine. Values are means $\pm$ SE. Data expressed as a percent relaxation in force from $\text{PGF}_{2\alpha}$ (30 μM)-induced tension. (X) Significantly greater than chronically treated L-NAME swine (P<0.05).

Figure 4. Sodium nitroprusside-induced relaxation of chronically L-NAME (n=9) treated and control (n=11) brachial arterial rings acutely treated with (A) INTACT, (B) INDO, (C) L-NAME, and (D) L-NAME + INDO
Values are means $\pm$ SE. Data expressed as a percent relaxation in force from $\text{PGF}_{2\alpha}$ ($30\mu\text{M}$)-induced tension. (†) Significantly less than chronically treated L-NAME swine ($P < 0.05$).

Figure 5. Sodium nitroprusside-induced relaxation of chronically L-NAME (n=9) treated and control (n=11) femoral arterial rings acutely treated with (A) INTACT, (B) INDO, (C) L-NAME, and (D) L-NAME + INDO

Values are means $\pm$ SE. Data expressed as a percent relaxation in force from $\text{PGF}_{2\alpha}$ (30 μM)-induced tension. (†) Significantly less than chronically treated L-NAME swine ($P < 0.05$).

Figure 6. Sodium nitroprusside-induced relaxation of chronically L-NAME (n=5) treated and control (n=5) (A) Brachial Intact, (B) Brachial Denuded, (C) Femoral Intact, and (D) Femoral Denuded arterial rings

Values are means $\pm$ SE. Data expressed as a percent relaxation in force from $\text{PGF}_{2\alpha}$ (30 μM)-induced tension. (†) Significantly less than chronically treated L-NAME swine ($P < 0.05$). (*) Significantly different IC_{50} than chronically treated L-NAME swine ($P < 0.05$).

Figure 7. Forelimb skeletal muscle arteriole relaxation responses to (A) bradykinin, (B) acetylcholine, and (C) sodium nitroprusside in chronically L-NAME treated (n=7) and control (n=10) swine

Values are means $\pm$ SE. Data expressed as a percent dilation of PE-induced constriction.

Figure 8. Hindlimb skeletal muscle arteriole relaxation responses to (A) bradykinin, (B) acetylcholine, and (C) sodium nitroprusside in chronically L-NAME treated (n=7) and control (n=10) swine

Values are means $\pm$ SE. Data expressed as a percent dilation of PE-induced constriction.

Figure 9. Immunoblot analysis of eNOS in the brachial (□) and femoral (■) arteries of chronically L-NAME treated (n=9) and control (n=10) swine
 Values are means ± SE. Data normalized such that control values were set to 1 and L-NAME values were expressed as a fold value relative to control. *P<0.05 vs. control

Figure 10. Immunoblot analysis of GCα in the brachial (□) and femoral (■) arteries of chronically L-NAME treated (n=5) and control (n=6) swine
 Values are means ± SE. Data normalized such that control values were set to 1 and L-NAME values were expressed as a fold value relative to control.

Table 1**Skeletal Muscle Conduit Artery Characteristics**

Variable	Brachial		Femoral	
	Control	L-NAME	Control	L-NAME
Axial Length (mm)	3.25±0.11	3.23±0.17	3.47±0.15	3.16±0.18
Outer Diameter (mm)	2.62±0.18	2.40±0.19	2.81±0.09	2.73±0.07
Inner Diameter (mm)	1.76±0.14	1.59±0.17	1.83±0.09	1.87±0.11
Wall Thickness (mm)	0.43±0.03	0.41±0.02	0.49±0.03	0.48±0.04
Resting Tension (g wt)	8.63±0.91	8.46±0.95	10.17±1.07	9.99±1.10
Specific Tension (mN/mm ²)	31.14±3.45	32.40±3.25	31.54±4.15	32.33±3.84
80mM KCl Tension (g)	29.0±2.3	26.0±1.7	27.9±2.3	24.9±2.9
PGF _{2α} Tension (g)	27.7±2.3	27.4±1.5	35.7±2.6	38.1±3.7
Resting 2A Arteriole Diameter (μm)	298±33	241±33	468±75	424±89
Maximal 2A Arteriole Diameter (μm)	321±37	287±33	523±75	439±91

Values are means ± SE. Resting tension, tension produced at optimal stretch; 80μM KCl tension, tension produced by adding 80mM KCl; PGF_{2α} tension, tension produced by PGF_{2α} prior to sodium nitroprusside dose response; mm, millimeters; g, grams.

Table 2

Maximal relaxation and IC₅₀ values for brachial artery rings

Variable	INTACT RING		INDO RING		L-NAME RING		L-NAME + INDO RING	
	Control	L-NAME	Control	L-NAME	Control	L-NAME	Control	L-NAME
BK								
Max relaxation, %	95.7±1.3*	79.2±4.0	94.6±1.7*	79.1±4.4	71.5±3.8	63.8±4.4	67.2±6.2	65.5±6.7
IC ₅₀ , -log M	-8.92±0.05*	-8.49±0.09	-8.99±0.05*	-8.47±0.11	-8.47±0.12	-8.44±0.08	-8.52±0.07	-8.37±0.10
ACh								
Max relaxation, %	97.8±2.1*	77.3±4.3	92.9±0.8*	71.7±4.9	73.5±2.5*	59.4±7.0	63.8±5.0	55.4±7.1
IC ₅₀ , -log M	-7.70±0.12*	-7.16±0.16	-7.83±0.09*	-7.31±0.13	-7.22±0.15	-6.99±0.25	-7.20±0.13	-7.06±0.18
SNP								
Max relaxation, %	106.4±4.4	108.9±6.5	95.0±1.4	96.8±1.8	110.6±5.9	104.1±3.8	102.4±2.4	99.8±1.6
IC ₅₀ , -log M	-6.33±0.12*	-6.63±0.08	-6.06±0.12*	-6.44±0.16	-6.58±0.12	-6.63±0.11	-6.56±0.11	-6.57±0.15

Values are means ± SE.

* P<0.05 vs. chronically treated L-NAME swine.

Table 3Maximal relaxation and IC₅₀ values for femoral artery rings

Variable	INTACT RING		INDO RING		L-NAME RING		L-NAME + INDO RING	
	Control	L-NAME	Control	L-NAME	Control	L-NAME	Control	L-NAME
BK								
Max relaxation, %	103.0±0.9*	85.7±2.1	100.3±0.5*	91.6±1.7	89.4±0.9*	85.2±2.4	88.4±1.1	86.0±3.6
IC ₅₀ , -log M	-8.97±0.04*	-8.64±0.07	-8.99±0.04*	-8.71±0.06	-8.71±0.05	-8.62±0.06	-8.78±0.07	-8.73±0.05
ACh								
Max relaxation, %	104.2±2.4*	89.6±3.2	98.4±0.9*	90.3±1.2	92.6±3.6	86.0±1.9	87.2±1.5	83.4±2.4
IC ₅₀ , -log M	-7.61±0.13*	-7.12±0.16	-7.74±0.10*	-7.36±0.12	-7.47±0.19*	-7.01±0.15	-7.59±0.08*	-7.31±0.11
SNP								
Max relaxation, %	103.6±3.0	109.3±3.8	99.0±1.2	101.2±0.6	110.8±4.5	114.9±4.7	106.3±1.6	103.9±1.0
IC ₅₀ , -log M	-5.70±0.15*	-6.17±0.17	-5.81±0.14*	-6.27±0.21	-5.71±0.15*	-6.17±0.12	-6.04±0.10	-6.17±0.14

Values are means ± SE.

* P<0.05 vs. chronically treated L-NAME swine.