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European Journal of Pharmacology 351 (1998) 253–260

ejp

Factors contributing to differences in the regulation of cGMP in isolated porcine pulmonary vessels

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Received 22 January 1998; revised 9 April 1998; accepted 15 April 1998

Abstract

Guanosine 3′,5′-cyclic monophosphate (cGMP) is an important second messenger in many biological systems including vascular smooth muscle where it mediates relaxation. Cellular levels of cGMP are regulated primarily by three enzymes; nitric oxide (NO) synthase, soluble guanylate cyclase, and cGMP-phosphodiesterase. Basal cGMP levels of isolated endothelium intact porcine pulmonary vein are five fold higher than in pulmonary artery. The objective of this study was to investigate possible reasons for this difference. Therefore, we compared NO synthase activity of pulmonary vein with artery and used pharmacologic approaches to compare soluble guanylate cyclase and phosphodiesterase activities in these vessels. NO synthase activities of pulmonary vein and artery were measured by monitoring the conversion of exogenous L-[^{14}C]arginine to L-[^{14}C]citrulline and by quantifying NO formation from endogenous L-arginine. Rates (pM/min per mg protein) of basal L-citrulline and NO formation from endothelium intact pulmonary vein (29.0 ± 4.8 and 44 ± 7.1, respectively) were significantly higher than from artery (8.3 ± 2.2 and 17.1 ± 3.3). Western blot analysis indicated higher constitutive NO synthase protein in the vein than in artery. *N*-nitro-L-arginine (0–100 μM), a potent inhibitor of NO synthase, induced contractions of the pulmonary vein which were significantly higher than those of the artery. *N*-nitro-L-arginine (5 and 20 μM) in the presence of phosphodiesterase inhibitors, decreased basal cGMP levels of endothelium intact blood vessels. In endothelium denuded pulmonary vein and artery, basal cGMP levels were not different from each other, but increased significantly following stimulation of soluble guanylate cyclase with exogenous NO. In the presence of both non-specific and specific cGMP phosphodiesterase inhibitors, exogenous NO-induced cGMP levels of endothelium denuded tissues were not significantly different from each other. However, in the absence of the phosphodiesterase inhibitors, exogenous NO-induced cGMP was significantly less in the artery than in the vein. These results suggest that (I) the intact porcine pulmonary vein contains higher levels of NO synthase activity than pulmonary artery, and that (II) the soluble guanylate cyclase activities in pulmonary vein and artery are equally responsive to NO, and finally (III) pulmonary artery expresses greater phosphodiesterase activity than vein. Higher NO synthase and lower phosphodiesterase activity may explain the greater accumulation of cGMP in the pulmonary vein compared to the artery.

Keywords: Nitric oxide (NO); Nitric oxide (NO) synthase; Phosphodiesterase; cGMP; Porcine pulmonary vessel; Guanylate cyclase, soluble

1. Introduction

The endothelium has been shown to play an essential role in the regulation of basal and active vascular contraction both in vivo and in isolated vessel preparations (Cherry and Gillis, 1987; Furchgott and Vanhoutte, 1989). Endothelial cells produce multiple vasoactive substances, including endothelium-derived relaxing factor (EDRF). Recent studies have reported evidence that EDRF is identical to, or shares many properties with, nitric oxide (NO) (Ignarro et al., 1987). NO, a relatively unstable, free radical plays a role as a biological messenger in a wide range of physiological processes in humans and animals (Moncada et al., 1991). NO synthase catalyzes the stepwise five electron oxidation of one of the equivalent guanidino nitrogens of the amino acid L-arginine to L-citrulline and NO which diffuses out of the endothelial cell in all directions (Sakuma et al., 1988). Production of NO from L-arginine is inhibited by a number of drugs including N^G-substituted L-arginine analogs (Gross et al., 1990; Ghislaine et al., 1994). When NO reaches the underlying vascular smooth muscle, it binds to the ferrous moiety of the heme group of guanylate cyclase, causing activation of

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0014-2999/98/$19.00

the soluble form of guanylate cyclase. Activated soluble guanylate cyclase converts guanosine 5′-triphosphate to the cellular second messenger molecule guanosine 3′,5′-cyclic monophosphate (cGMP) which ultimately causes relaxation of blood vessels. Cyclic GMP is the intracellular messenger for NO and its levels in tissues are dependent on a number of factors such as activity levels of NO synthase and thus amount of NO, soluble guanylate cyclase, and cGMP-phosphodiesterase, the intracellular enzyme responsible for the hydrolysis of cGMP.

We have previously reported a five fold difference in the basal cGMP levels between pulmonary vein and artery of the pig (Freas et al., 1995). The aim of this study was to explore the basis for this difference. In endothelium intact vessels the role of NO synthase was compared by quantifying NO and L-citrulline accumulation under basal conditions. The effects of *N*-nitro-L-arginine a potent inhibitor of constitutive endothelial NO synthase, on basal tension and cGMP levels of pulmonary vein and artery were examined (Gross et al., 1990). Activity of soluble guanylate cyclase in endothelium denuded vessels was compared by examining the effects of increasing concentrations of exogenous NO in the presence and absence of 3-isobutyl-1-methylxanthine (IBMX), a nonspecific inhibitor of phosphodiesterase (Beavo et al., 1970; Kramer and Wells, 1979), and zaprinast, a specific cGMP phosphodiesterase inhibitor (Burns et al., 1992).

2. Materials and methods

2.1. Preparation of pulmonary arteries and veins

This study was approved by the Laboratory Animal Care Committee of the Uniformed Services University of the Health Sciences (Bethesda, MD, USA). Lungs were removed from anesthetized (sodium pentobarbital, 30 mg/kg, i.v.) pigs (male or female, 15–25 kg) and placed in Krebs–Ringer solution of the following composition (mM): 118.2 NaCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 4.7 KCl, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, and 5.6 glucose (pH 7.4). Second generation intrapulmonary blood vessels were carefully removed and following removal of adhering fat and connective tissues, arterial and venous segments, were either cut into rings (3–4 mm in length and 3–5 mm in diameter) for the tension studies and cGMP levels measurement or were sliced into pieces (3–4 mm^2) for homogenization. In some experiments, endothelium was removed from the vessels by rotating rings around a small metal blade. The presence or absence of endothelium was verified with acetylcholine (1 μM) as described in Section 2.5.

2.2. Assay of NO synthase activity by L-[^{14}C]citrulline formation

NO synthase activity was measured by monitoring the conversion of exogenous L-[^{14}C]arginine to L-[^{14}C]citrulline, as described previously (Bredt and Snyder, 1990). Endothelium intact pulmonary vein (270 ± 18 mg) and artery (286 ± 21 mg, wet weight) were homogenized in ice-cold buffer (1 ml) containing Tris–HCl (25 μM/ml, pH 7.4), ethylenediaminetetraacetic acid (EDTA, 1 μM/ml), EGTA (1 μM/ml), antipain (10 μg/ml), leupeptin (10 μg/ml), and phenylmethylsulfonyl fluoride (100 μg/ml). NO synthase in the soluble and membrane-associated fractions was separated by centrifuging the homogenized tissues at $25\,000 \times g$ for 60 min at 4°C. Homogenate or supernatant (10 μl) was added to the reaction mixture (40 μl) containing Tris–HCl (25 mM, pH 7.4), tetrahydrobiopterin (5 nM/ml), flavin adenine dinucleotide (1 nM/ml), flavin adenine mononucleotide (1 nM/ml), nicotinamide adenine dinucleotide phosphate, reduced form (10 nM/ml), calcium chloride (50 nM/ml), and L-[^{14}C]arginine (0.812 nM/ml = 250 nCi/ml). After incubation of samples for 30 min at 37°C, assays were terminated by addition of 0.4 ml HEPES buffer (50 mM, pH 5.4) containing EDTA (5 μM/ml). Both the incubation time (30 min) and substrate concentration in the assay medium were chosen in order to achieve about 20% conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline by NO synthase in the pulmonary vein. Then Bio-Rad AG50WX-8 resin, suspended in water (0.2 ml, 1:1), was added to the reaction mixture to remove endogenous bivalent cations and arginine. After mixing, samples were transferred into micro-spin cups and holders and were centrifuged for 30 s. The radioactivity of the eluate was measured in a liquid scintillation counter (Packard). NO synthase activity was expressed as rate of L-citrulline formation in pM/min/mg protein. The ratio of unreacted L-[^{14}C]arginine to L-[^{14}C]citrulline was measured by placing the spin cups containing resin in fresh microcentrifuge tubes, and adding 0.4 ml of elution buffer (0.5 M NH_4Cl), and then spin cups were centrifuged (30 s). The eluate (containing L-[^{14}C]arginine) was quantified by liquid scintillation counting. Protein content was measured by dissolving homogenate (0.1 ml) in NaOH (0.9 ml, 2 M) and analyzing it for total protein content using a Bio-Rad protein assay kit.

2.3. Assay of NO synthase activity by nitrite anion formation

NO accumulation from endothelium intact blood vessels was measured as its breakdown product, nitrite anion. Endothelium intact pulmonary vein and artery were placed in two small vials containing 1 ml Krebs–Ringer solution (pH 7.4). An additional vial containing Krebs–Ringer solution was used as a blank. The wet weights of the pulmonary vein (24 ± 8 mg) and artery (28 ± 9 mg) rings were not significantly different from each other. Vials were incubated at 37°C while continuously aerated with 95% O_2–5% CO_2. After 15, 30, and 120 min incubation, two sets of aliquots (20 μl) were removed. Nitrate in one set was reduced to nitrite by a 15 ± 5 min incubation with

nitrate reductase (0.1 unit). A standard curve was plotted before each experiment by using standard sodium nitrite solutions (1–200 pM). Standards and samples were analyzed for NO by chemiluminescence (NO-analyzer, Sievers 270 B, Boulder, CO) (Palmer et al., 1987; Brien et al., 1991). Briefly, 5 μl of standards or samples were injected into the gas purged vessel containing (10 ml) KI (30 mM) in 20% acetic acid to reduce nitrite anion to NO. This technique allowed transfer of NO from the aqueous phase into the gas phase for chemiluminescence detection. Nitrogen gas was used to purge NO from liquid phase to gas phase. At the end of the experiment, protein content was measured by dissolving rings in NaOH (1 ml, 2 M) and analyzing for total protein content as described previously.

2.4. Western blot analysis

Tissues (100 ± 20 mg) were homogenized in disposable Dounce homogenizers in buffer (400 μl) containing Tris–HCl (50 μM/ml, pH 7.5), NaCl (100 μM/ml), Nonidet P-40 (1%), aprotinin (10 μg/ml), leupeptin (10 μg/ml), *p*-nitrophenylguanidinobenzoate (25 μg/ml), EDTA (2 μM/ml), and sodium orthovanadate (1 μM/ml) and incubated for 20 min at 4°C. After incubation, homogenates were centrifuged ($14\,000 \times g$, 30 min). The supernatants were analyzed for total protein content, then protein contents were adjusted to equal amounts and samples applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% Tris–glycine gel. Human endothelium lysate (1 mg/ml) derived from an aortic endothelium cell line cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum was used as a positive control. After separation, the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and then probed with polyclonal rabbit anti-mouse endothelium NO synthase or inducible NO synthase antibodies. Alkaline phosphatase-conjugated monoclonal anti-rabbit immunoglobulin G (IgG) antibody was used to detect primary rabbit antibodies. Chemiluminescence detection was performed using alkaline phosphatase substrate CSPD (Tropix). The optical density of the endothelium NO synthase band in the film was measured by densitometer (Image 1/AT, Universal Imaging, Brandywine, PA) using calibrated optical density standard.

2.5. Vessel tension studies

Vessel rings (wet weight, mg: vein 12.0 ± 2.4, artery 16.2 ± 2.4) were suspended in water-jacketed organ chambers (25 ml) filled with Krebs–Ringer solution, aerated with 95% O_2 and 5% CO_2 at 37°C. Isometric tension was measured with Grass (Quincy, MA) force transducers (FT03) and recorded on a Gould (Valley View, OH) recorder (Model 3400). Two pulmonary vein and two pulmonary artery rings from the same pig were studied simultaneously. The rings were stretched to the optimum point on their length–tension curve (vein and artery 1.8 ± 0.2, and 2.5 ± 0.3 g respectively) and were equilibrated at this optimal tension for 60 ± 10 min during which time the Krebs solutions were changed at 15 min intervals to remove vasoactive substances. The pulmonary vein rings were contracted with 5-hydroxytryptamine (0.1 μM) and pulmonary artery rings with L-phenylephrine (1 μM). The absence or presence of endothelium was verified with acetylcholine (1 μM) by either lack of relaxation in endothelium denuded rings or at least 75% relaxation in endothelium intact rings. Then a series of cumulative concentrations of *N*-nitro-L-arginine (1–100 μM) were added. Preliminary studies indicated that the use of indomethacin (10 μM in Krebs), to prevent release of cyclooxygenase products from vessels, did not significantly affect either induced contractions or cGMP levels of the tissues.

2.6. Measurement of cGMP levels

Preliminary experiments indicated that basal cGMP levels of stretched and unstretched tissues were not significantly different from each other. Therefore, unstretched rings were used. Blood vessels (wet weight of pulmonary vein and artery were 4.1 ± 0.5 and 5.2 ± 0.7 mg, respectively) with or without endothelium were placed in 25 ml water-jacketed organ baths filled with Krebs–Ringer solution (37°C) which was continuously aerated with 95% O_2–5% CO_2 for about 60 min. For concentration response experiments, one set of rings was removed and frozen in liquid nitrogen at the end of the 60 min equilibration period and served as the untreated controls. The second set of rings was incubated for 10 min with either *N*-nitro-L-arginine, IBMX or zaprinast. Zaprinast and IBMX were dissolved in dimethyl sulfoxide (0.1% final concentration in organ bath). Preliminary experiments indicated that dimethyl sulfoxide at the concentration used did not significantly affect cGMP levels of the tissues. After the incubation period, tissues were treated with NO (0.1–10 μM) and incubated for an additional 30 ± 5 s. Tissues were then frozen in liquid nitrogen, homogenized and extracted twice in 1 ml of 80% ethanol. Levels of the cGMP were measured by an Amersham cGMP radioimmunoassay kit and results were expressed as pM/mg protein. Protein content was measured by dissolving the rings in 2 N sodium hydroxide (1 ml) and analyzing the total dissolved protein using a Bio-Rad protein assay kit. Concentrations of NO, *N*-nitro-L-arginine, IBMX, and zaprinast are final bath concentrations. Each determination (n) was performed on a ring from a different animal.

2.7. Preparation of nitric oxide

NO gas was generated daily prior to use as previously described (Bina et al., 1995): briefly, in a 100 ml deoxygenated flask with a septum inlet, potassium iodide (20

mM) was dissolved in deoxygenated hydrochloric acid solution (20 ml, 3 M), followed by addition of sodium nitrite (10 ml, 0.3 mM/ml), at a rate of 30 drops/min. The generated NO gas was passed over solid potassium hydroxide to scavenge traces of higher oxidation states of nitrogen oxides (Feelisch, 1991). Using a gas-tight syringe, NO gas (10 ml) was injected into a gas-tight round bottom flask with a septum inlet, filled with deoxygenated water (100 ml, deoxygenated with nitrogen or argon for 30 min) at room temperature. The concentration of NO in saturated solutions was measured by employing the chemiluminescence procedure (Palmer et al., 1987; Brien et al., 1991). The measured concentration of saturated NO solution was 1.6 ± 0.2 mM ($n = 5$). Different volumes of saturated NO solution, without further dilution, were used to achieve the desired final bath concentrations.

2.8. Reagents

N-nitro-L-arginine, IBMX, Zaprinast, sodium nitrite, dimethyl sulfoxide, potassium iodide, protease inhibitors, nitrate reductase, and other common laboratory chemicals were obtained from Sigma, St. Louis, MO. [^{14}C]arginine (307.3 mCi/mM) was purchased from DuPont, Boston, MA. Human endothelial lysate (control endothelium NO synthase protein) and endothelium NO synthase/inducible NO synthase antibodies were obtained from Transduction Laboratories, Lexington, KY. Cyclic GMP radioimmunoassay kits were obtained from Amersham, Arlington Heights, IL. AG50W-X8 cation exchange resin was obtained from Bio-Rad, Richmond, CA. Protein assay kits were from Bio-Rad except for those used in Wester blot analysis which were from Pierce, Rockford, IL.

2.9. Statistical analysis

Data were expressed as means ± S.E.M. Comparisons for repeated measures between groups were made using one-way analysis of variance (ANOVA). Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. No synthase activity by L-[^{14}C]citrulline formation

The NO synthase activity of the supernatant (soluble NO synthase) was significantly less than homogenate (soluble plus membrane-associated NO synthase, data not shown). This indicated that NO synthase was predominantly associated with membrane fractions. Therefore, to compare total NO synthase activities in pulmonary vein with those in pulmonary artery, data obtained from homogenate (soluble plus membrane-associated NO synthase) samples were used. The rate of conversion of [^{14}C]arginine to [^{14}C]citrulline in endothelium intact pulmonary vein was significantly (3.5 fold) higher than in pulmonary artery (Table 1, column a).

Table 1
Nitric oxide synthase activity of isolated porcine pulmonary vein (PV) and artery (PA)

	(a) L-[^{14}C]citrulline (pM/min/mg protein)	(b) NO (pM/min/mg protein)
PV	29.4 ± 4.8 *	44.1 ± 7.1 *
PA	8.3 ± 2.2	17.1 ± 3.3

Nitric oxide synthase activities were measured by two different protocols: (a) by monitoring the conversion of exogenous L-[^{14}C]arginine to L-[^{14}C]citrulline and (b) by measuring accumulation of NO formation from endogenous L-arginine by chemiluminescence.
Data are expressed as the means ± S.E.M., $n = 5$.
* Significantly different from corresponding pulmonary artery values, $P < 0.01$.

3.2. No synthase activity by nitrite anion formation

Incubation of samples with nitrate reductase did not significantly increase nitrite anion levels of the samples (data not shown), indicating that the major product of oxidation of NO was nitrite and not nitrate anion. Basal accumulation of NO from endothelium intact pulmonary vein was significantly higher than in artery. Rate of NO formation obtained from samples that were incubated for 30 min at 37°C are presented in Table 1, column b.

3.3. Western blot analysis of NO synthase

Both pulmonary vein and artery expressed distinct bands equal to the control value at about 140 kDa. The optical density analysis of the endothelium NO synthase in the

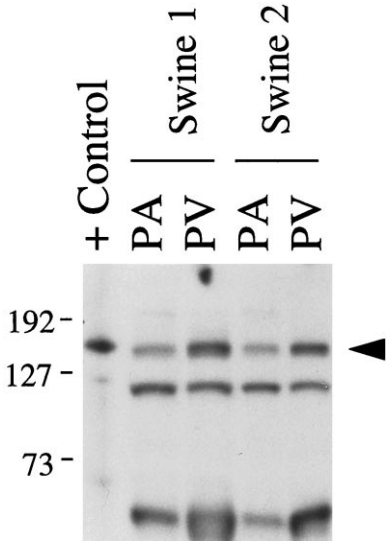

Fig. 1. Typical Western blot analysis of endothelium NO synthase protein in pulmonary artery (PA) and vein (PV) obtained from two different pigs. Arrow shows endothelium NO synthase band with molecular weight of 140 kDa. Control endothelium NO synthase protein derived from human aortic endothelium cell line cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Optical density analysis of the endothelium NO synthase bands indicated greater quantities of endothelium NO synthase protein in pulmonary vein than in artery, $n = 6$.

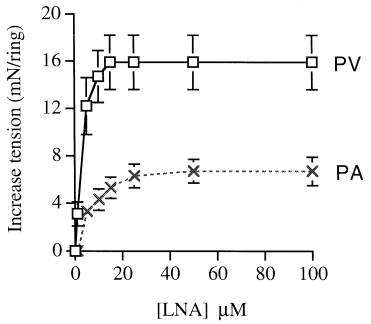

Fig. 2. Effect of increasing *N*-nitro-L-arginine (LNA) concentrations on basal tension of endothelium intact porcine pulmonary vein (PV) and artery (PA). Values are expressed as means ± S.E.M., $n = 5$. At all concentrations, LNA induced significantly greater tensions in pulmonary vein than in artery.

film for pulmonary vein (0.607 ± 0.072 o.d., $n = 6$) was significantly more intense than pulmonary artery (0.289 ± 0.036 o.d., $n = 6$) (Fig. 1). There was no sign of inducible NO synthase band in any of these blood vessels (figure not shown).

3.4. Effect of N-nitro-L-arginine on basal tension

N-nitro-L-arginine (1–100 μM) did not increase basal tension of endothelium denuded blood vessels but it induced contractions of endothelium intact blood vessels, and reached a plateau at 15 μM. *N*-nitro-L-arginine induced contractions of pulmonary vein were significantly higher than artery (Fig. 2).

3.5. Effect of N-nitro-L-arginine on cGMP levels of endothelium intact pulmonary vein and artery in presence of IBMX

In the presence of IBMX (1 mM), basal cGMP levels (pM/mg protein) of endothelium intact pulmonary vein

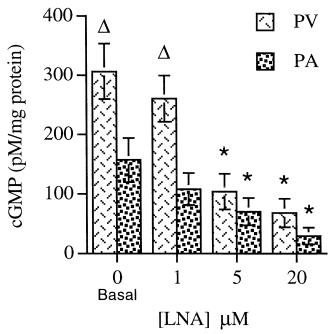

Fig. 3. Effect of increasing *N*-nitro-L-arginine (LNA) concentrations on basal cGMP levels in IBMX (1 mM) treated, endothelium intact pulmonary vein (PV) and artery (PA). Values are expressed as mean ± S.E.M., $n = 7$. At 5 and 20 μM *N*-nitro-L-arginine, cGMP levels of pulmonary vein were not significantly different from corresponding pulmonary artery. (*) Levels of cGMP in both tissues were significantly less than corresponding basal values. (△) At basal and 1 μM *N*-nitro-L-arginine, cGMP levels of pulmonary vein were significantly greater than corresponding pulmonary artery.

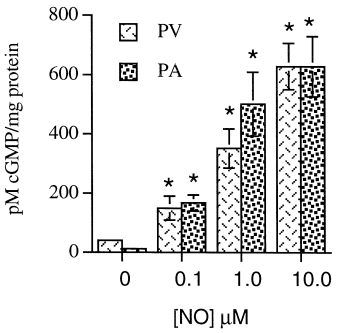

Fig. 4. Effect of increasing NO concentrations on cGMP levels in IBMX (1 mM) treated, endothelium denuded pulmonary vein (PV) and artery (PA). Values are expressed as means ± S.E.M., $n = 7$. Levels of cGMP of pulmonary vein were not significantly different from artery at any NO concentrations. (*) Levels of cGMP of both tissues were significantly increased above basal at all NO concentrations.

(306 ± 47) were significantly higher than in the endothelium intact pulmonary artery (157 ± 37). *N*-nitro-L-arginine at 1 μM had no significant effect on cGMP levels of both tissues but at 5 and 20 μM it significantly decreased basal cGMP levels of pulmonary vein (104 ± 30 and 68 ± 24, respectively) and artery (70 ± 23 and 39 ± 8, respectively) (Fig. 3).

3.6. Effect of IBMX and zaprinast on cGMP accumulation in response to NO

Endothelium denuded rings and IBMX or zaprinast were used to eliminate the influence of endothelium derived relaxing factor and phosphodiesterase on cGMP levels, thus permitting comparisons of soluble guanylate cyclase activities between pulmonary vein and artery. Basal cGMP levels (pM/mg protein) of endothelium intact pulmonary vein (19.1 ± 1.9) were about five fold higher than in intact pulmonary artery (3.6 ± 0.4). Removal of the endothelium significantly decreased cGMP from basal levels to 0.6 ± 0.3 in pulmonary vein and 0.7 ± 0.5 in artery which were not significantly different from each other. In

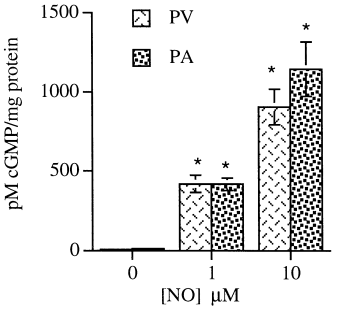

Fig. 5. Effect of NO (1 and 10 μM) on cGMP levels in zaprinast (0.5 mM) treated, endothelium denuded pulmonary vein (PV) and artery (PA). Values are expressed as means ± S.E.M., $n = 7$. Levels of cGMP of pulmonary vein were not significantly different from artery at either NO concentration. (*) Both concentrations of NO significantly increased basal cGMP levels of both vessels.

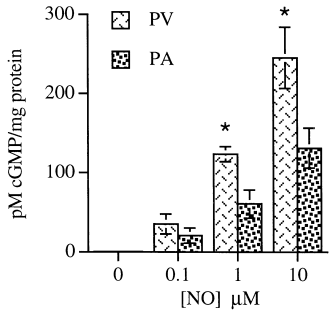

Fig. 6. Effect of increasing exogenous NO concentrations, on cGMP levels of endothelium denuded porcine pulmonary vein (PV) and artery (PA), in the absence of phosphodiesterase inhibitors. Values are expressed as means ± S.E.M., $n = 6$. At 0.1 μM NO, cGMP levels of pulmonary vein and artery were not significantly different from each other. (*) cGMP levels of pulmonary vein were significantly higher than corresponding levels of artery at 1 and 10 μM NO.

IBMX (1 mM) or zaprinast (0.5 mM) treated tissues, all exogenous NO concentrations tested significantly increased cGMP levels and the values were not different between pulmonary vein and artery (Figs. 4 and 5). In the absence of phosphodiesterase inhibitors, exogenous NO (0.1–10 μM) induced a concentration dependent increase in cGMP content of the denuded tissues. However, in contrast to studies with phosphodiesterase inhibitors, the cGMP levels of pulmonary vein (at 1 and 10 μM NO) were significantly higher than corresponding values for artery (Fig. 6).

4. Discussion

The basal levels of the important vasodilator second messenger cGMP was five fold greater in the isolated porcine pulmonary vein than in the pulmonary artery. The current results suggest that this difference in basal cGMP depends on differences between these vessels in the activities of two of the three enzymes (NO synthase, soluble guanylate cyclase, and phosphodiesterase) known to play critical roles in the regulation of cGMP in vascular smooth muscle. Basal endothelium NO synthase activity of the endothelium of the pulmonary vein was significantly higher and phosphodiesterase activity lower than that of the pulmonary artery. However, the activity of soluble guanylate cyclase, as determined by responsiveness to exogenous NO in the presence of phosphodiesterase inhibitors, was the same in pulmonary vein and artery. These are the first observations that provide information about the mechanisms of heterogeneity in regulation of vascular smooth muscle cGMP.

The enzymes responsible for the synthesis of NO from L-arginine in mammalian tissues are known as nitric oxide synthases. There are three known major isoforms of NO synthase. Two are constitutive enzymes, one normally expressed in the endothelium and one in neurons. A third isoform, inducible NO synthase, can be produced after induction by endotoxin in a variety of cells, including smooth muscle cells and macrophages. The subcellular distribution of NOS is not the same in all tissues. In adult rat brain NO synthase is primarily soluble, while in skeletal muscle endothelium NO synthase is largely associated with the membrane fraction as a result of N-terminal myristoylation (Busconi and Michel, 1993; Kobzik et al., 1994). In cultured bovine aortic endothelial cells about 5% of the total NO synthase activity is found in the soluble and 95% in the membrane fraction (Pollock et al., 1991). Our observations show that in porcine pulmonary vessels, NO synthase is also predominantly associated with the membrane fraction.

Different approaches were used to assess the NO synthase activity/levels in pulmonary vessels. All showed higher activity/levels in the pulmonary vein than in the artery. Two direct methods for analysis of NO synthase activity were used. In the first method, accumulation of L-[^{14}C]citrulline was measured. L-citrulline concentration may be taken as a index of NO synthase activity because it converts L-arginine to equimolar amounts of L-citrulline and NO. Results of this analysis indicated that the pulmonary vein produced approximately three times more L-[^{14}C]citrulline than the artery. The second method measured the amount of nitrite anion, the oxidation product of the free radical NO in aqueous solutions. In vitro, nitrite anion concentration is an index of NO release and thus NO synthase activity (Feelisch, 1991; Ford et al., 1993). This was confirmed by comparison of the nitrite anion accumulation in presence and absence of nitrate reductase which indicated that nitrate anion formation is insignificant. This method showed three fold greater nitrite anion accumulation in the pulmonary vein than in artery, which supports the higher endothelium NO synthase activity in the artery. The rates of L-[^{14}C]citrulline and nitrite anion formation in these two methods were not the same, probably as a result of the different methods used. In the first method L-[^{14}C]citrulline generated from exogenous L-[^{14}C]arginine was measured, while in the second method NO generated from endogenous L-arginine was measured.

The pulmonary vein bands for endothelium NO synthase were significantly more intense than those of artery, indicating greater endothelium NO synthase protein levels in the pulmonary vein than in artery, which also supports the greater activity of endothelium NO synthase in the vein.

The final method used to compare NO synthase activity involved the NO synthase inhibitor *N*-nitro-L-arginine. When *N*-nitro-L-arginine was present, the basal levels of cGMP were significantly reduced in both endothelium intact pulmonary vein and artery. Vessel tension changes were consistent with cGMP measurements. In the presence of this NO synthase inhibitor, the basal tensions of both pulmonary vein and artery increased in a concentration-dependent manner, with pulmonary vein tension about 2.5

times greater than tension of the artery. These findings are in agreement with results obtained in newborn and adult sheep where it has been reported that endothelium-derived nitric oxide activity is more pronounced in intact pulmonary veins than in arteries (Bansal et al., 1993; Gao et al., 1995; Steinhorn et al., 1995).

It has been reported that when superoxide anions are generated from mammalian cells they react with nitric oxide to form nitrite/nitrate anions (Fridovich, 1975; Rubanyi and Vanhoutte, 1986). This raises the possibility that the differences between basal cGMP levels of porcine pulmonary vein and artery may be due to the greater production of superoxide anions in pulmonary artery, preventing NO from reaching its heme site in guanylate cyclase. In aqueous solutions NO reacts with superoxide anion to give peroxynitrite ($ONOO^-$), a powerful oxidant that further decomposes to hydroxyl radical and nitrogen dioxide (Beckman et al., 1990; Wing et al., 1993). Nitrogen dioxide reacts with water to give a mixture of nitrite and nitrate anions (Feelisch, 1991). If generation of different amounts of superoxide anions were responsible for the differences between basal cGMP levels of pulmonary vein and artery, assuming that NO release was the same in both tissues, then equal amounts of nitrite/nitrate anion accumulation in the pulmonary artery and vein would be expected. However, we found that nitrite anions were the predominant product of oxidation (nitrate anions level were insignificant) and their formation in pulmonary vein was three times greater that in artery. This result suggests that superoxide anion does not account for the differences observed in the cGMP levels of porcine pulmonary vessels.

Structural differences between the pulmonary arteries and veins may be a factor in the NOS differences observed. The veins, with a lower wall/lumen ratio than arteries, may have more endothelial cells/gram of protein than the arteries. However, the methods used here cannot provide information about the specific structural components of the vessels.

A pharmacologic approach was used to compare sGC activity in pulmonary vein and artery. Blood vessels without endothelium were treated with identical concentrations of exogenous NO. IBMX and zaprinast were used to eliminate the effect of phosphodiesterase on the hydrolysis of cGMP (Kramer and Wells, 1979; Burns et al., 1992; Waldman and Murad, 1987; Papappetropoulos et al., 1996). In the presence of both non-specific and specific phosphodiesterase inhibitors, exogenous NO-induced increases in the cGMP levels of the tissues were not significantly different from each other (Figs. 4 and 5), suggesting that the soluble guanylate cyclase activities in the pulmonary vein and artery are equally responsive to NO.

In the presence of phosphodiesterase inhibitors, exogenous NO-induced cGMP was not significantly different in the endothelium denuded pulmonary vein and artery (Figs. 4 and 5). However, in the absence of phosphodiesterase inhibitors, exogenous NO-induced cGMP was lower in the endothelium denuded artery than in the vein (Fig. 6). These observations suggest that the phosphodiesterase activity in the pulmonary artery is greater than in the vein, and that greater phosphodiesterase activity of the artery may contribute to the lower basal cGMP levels in the pulmonary artery. As previously indicated, basal cGMP levels of pulmonary vein were five fold greater than in the artery, while in the vein, basal NO release and rate of L-[^{14}C]citrulline formation were only three times greater than in the artery. Since our data indicated that soluble guanylate cyclase was equally active in both pulmonary artery and vein, it was expected that cGMP levels of pulmonary vein would also be three fold higher than in artery. We suggest that this discrepancy may be due to a greater phosphodiesterase activity in pulmonary artery than in vein. Similar observations have been reported in vessels from the adult sheep where the rate of hydrolysis of cGMP by phosphodiesterase isolated from pulmonary vein was slower than the rate of hydrolysis of cGMP by phosphodiesterase isolated from pulmonary artery (Okogbule-Wonodi et al., 1996). Thus it appears that in these two species the role of phosphodiesterase in regulation of the cGMP levels varies by vessel.

5. Conclusion

In conclusion, direct and indirect analysis of NO synthase activity demonstrated that intact porcine pulmonary vein contains significantly higher NO synthase levels/activity than artery. Pharmacologic analysis of phosphodiesterase and soluble guanylate cyclase indicated that phosphodiesterase activity in pulmonary vein was less than in artery and that the soluble guanylate cyclase activity levels were not significantly different from each other. This indicates that both endothelium NO synthase and phosphodiesterase play crucial roles in the regulation of cGMP levels of both isolated porcine pulmonary vein and artery and probably in the regulation of in vitro pulmonary vascular tone. However, the relative importance of these two enzymes is different in these vessels. Further direct evaluation of phosphodiesterase activity as it contributes to pulmonary tone, may lead to better therapeutic strategies in treating pulmonary vascular diseases.

Acknowledgements

We thank Dr. Leslie McKinney for thoughtful comments and Kathleen Gallagher for technical assistance. This work was supported by Uniformed Services University of the Health Sciences, research grant No. R08070.

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