Prostacyclin-induced relaxations of small porcine pulmonary arteries are enhanced by the basal release of endothelium-derived nitric oxide through an effect on cyclic GMP-inhibited-cyclic AMP phosphodiesterase

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ABSTRACT

AIM To study the interactions between prostacyclin and endothelium-derived nitric oxide in porcine pulmonary arteries. METHODS Rings of 5th order of porcine pulmonary arteries were studied in vitro for the measurement of tension and the content in cyclic nucleotides. RESULTS Prostacyclin given exogenously caused endothelium-potentiated relaxations  inhibition of phenylephrine contraction that were inhibited by the inhibitors of the L-arginine nitric oxide pathway  oxyhemoglobin and N\textsuperscript{-}nitro-L-arginine. These inhibitors did not affect the tension in rings without endothelium. Cyclic GMP-concentrations were not increased above basal concentrations in the presence of prostacyclin. Increases were seen with acetylcholine and sodium nitroprusside. Prostacyclin-stimulated cyclic AMP concentrations did not reach statistical significance compared to controls. The addition of 8-bromo-cyclic GMP to prostacyclin  however increased the cyclic AMP content. The nitric oxide synthase inhibitor nitro-L-arginine NLA reduced the prostacyclin-stimulated cyclic AMP content to basal level. Inhibition of cyclic GMP-inhibited cyclic AMP phosphodiesterase by 8-bromo-cyclic GMP or amrinone a specific inhibitor of this enzyme potentiates the prostacyclin-induced relaxations in rings without endothelium to a magnitude similar to that observed in rings with endothelium. CONCLUSION These data suggest that the augmentation by the endothelium of the prostacyclin-induced relaxation of porcine pulmonary arteries is secondary to the inhibition of cyclic GMP-inhibited cyclic AMP phosphodiesterase by basally released endothelium-derived nitric oxide.

INTRODUCTION

The endothelium synthesizes and releases relaxing and contracting factors that are responsible for vascular tone under normal and pathologic conditions. Prostacyclin and endothelium-derived nitric oxide  EDNO are synthesized and released by pulmonary endothelial cells both factors cause vasodilatation and inhibit platelet aggregation. Prostacyclin mediates these actions through the accumulation of cyclic AMP while nitric oxide mediates vasodilation through the accumulation of cyclic GMP. In porcine systemic vessels prostacyclin stimulates the release of EDNO the quantity of which varies with the type of blood vessel. Shimokawa et al concluded that the vasodilator effects of endothelium-derived relaxing factors  EDRF  nitric oxide and prostacyclin are synergistic in the porcine coronary artery. They interpreted their data to suggest that the synergism is secondary to stimulated release of EDRF/nitric oxide by the prostanoid. Another synergism exists between agonists that stimulate cyclic AMP and those that stimulate cyclic GMP. A cyclic GMP-inhibited cyclic AMP phosphodiesterase cG1-PDE was first isolated and characterized in bovine cardiac muscle

The enzyme is also present in platelets and vascular smooth muscle. Inhibition of this enzyme results in elevated platelet cyclic AMP concentrations and is responsible for the synergistic antiaggregatory actions of sodium nitroprusside and prostaglandin E\textsubscript{1} and likely EDRF/nitric oxide and prostacyclin on platelets.
Dilatation of vascular smooth muscle also results from the inhibition of the enzyme \[ \text{IP} \overset{1,14}{\rightarrow} \text{cGMP} \]. The cardiotonic drugs \[ \text{amrinone and milrinone} \] inhibit cG-IP \[ \overset{1,14,37}{\rightarrow} \text{cGMP} \] and increasing cardiac myocyte cyclic AMP concentrations \[ \overset{130}{\rightarrow} \text{cAMP} \] and causing peripheral vasodilatation \[ \overset{19-21}{\rightarrow} \text{cGMP} \]. Presumably secondary to an increase in cyclic AMP concentration in the vascular smooth muscle. Inhibition of the enzyme by nitrovasodilators appears responsible for the enhanced relaxations to \( \beta \)-adrenergic agonists \[ \overset{161}{\rightarrow} \text{cGMP} \]. Indeed, the presence of the endothelium enhances relaxations of pulmonary arterial smooth muscle to the \( \beta \)-adrenergic agonist isoproterenol \[ \overset{47}{\rightarrow} \text{cGMP} \].

The present experiments were designed to determine whether or not \[ \overset{-}{\text{cGMP}} \] in isolated porcine pulmonary arteries \[ \overset{-}{\text{cGMP}} \] the potentiation by the presence of the endothelium of the response to prostacyclin is secondary to the basal or the stimulated release of nitric oxide \[ \overset{-}{\text{cGMP}} \] and if it depends upon a cyclic GMP-inhibited cyclic AMP phosphodiesterase.

**MATERIALS AND METHODS**

**Drugs** The following drugs were used in the experiments \[ \overset{\rightarrow}{\text{acetylcholine chloride}}, \overset{\rightarrow}{\text{amrinone}}, \overset{\rightarrow}{\text{bradykinin}}, \overset{\rightarrow}{\text{8-bromo-cyclic GMP}}, \overset{\rightarrow}{\text{hemoglobin}}, \overset{\rightarrow}{\text{bovine}}, \overset{\rightarrow}{\text{indomethacin}}, \overset{\rightarrow}{\text{methylene blue}}, \overset{\rightarrow}{\text{N}}^\text{\textsuperscript{-}} \overset{\rightarrow}{\text{nitro-L-arginine}}, \overset{\rightarrow}{\text{NLA}} \overset{\rightarrow}{\text{potassium chloride}}, \overset{\rightarrow}{\text{and prostacyclin}} \] all from Sigma Chemical Co \[ \overset{\rightarrow}{\text{St. Louis}}, \overset{\rightarrow}{\text{Mo.}} \]. The drugs were made fresh daily using distilled water. Indomethacin was first dissolved in \( \text{NaCl} \) \[ 10 \mu \text{mole L}^{-1} \] and then distilled water. NLA was first dissolved in \( \text{HCl} \) \[ 1 \text{mole L}^{-1} 100 \mu \text{L} \] and then 10 mL of distilled water. Prostacyclin was first dissolved in \( \text{NaHCO}_3 \) and then \( \text{NaOH} \) \[ 100 - 200 \mu \text{L} \] was added to bring the pH of the solution to 9.0. The drugs were kept on ice during the experiments.

Bovine hemoglobin \[ \overset{\rightarrow}{\text{type I}} \] was obtained from Sigma Chemical Co. It contains a mixture of oxyhemoglobin and methemoglobin. The oxyhemoglobin solution was prepared by adding 600 mg of bovine hemoglobin to 10 mL of distilled water containing 70 mg of sodium dithionite \[ \text{Na}_2\text{S}_2\text{O}_4 \]. The sodium dithionite was then removed by dialyzing the solution against 15 liters of water containing 0.001 % edetic acid, which was maintained at room temperature and bubbled with nitrogen. Following two hours of dialysis \[ \overset{-}{\text{dialysis}} \] the percent of oxyhemoglobin was determined spectrophotometrically \[ \overset{-}{\text{spectrophotometrically}} \].

Animal research was performed using an Institutional Review Board approved protocol and was carried out according to guidelines set forth by the National Institutes of Health for animal research.

**Organ chamber experiments** Lungs were obtained from mature pigs \( n = 40 \) three months old \[ \overset{-}{\text{following}} \] ketamin \[ 300 \text{ mg/kg intramuscularly} \] and pentobarbital \[ 12.5 \text{ mg/kg intravenously} \] anesthesia and euthanasia \[ \overset{-}{\text{or}} \] from a local slaughterhouse. The lungs were immersed in cold modified Krebs-Ringers bicarbonate solution \[ \overset{-}{\text{control solution}} \] of the following composition \[ \overset{-}{\text{composition}} \] in mmol L\(^{-1}\) \[ \text{NaCl} 118 \text{ KCl 4.7 CaCl}_2 2.5 \text{ MgSO}_4 1.2 \text{ KH}_2\text{PO}_4 \text{ NaHCO}_3 25 \text{ edetic acid-Ca 0.026 glucose} 11.1 \text{ and transported to the laboratory.} \] The small pulmonary arteries \[ \overset{-}{\text{5}} \text{th order} \overset{-}{\text{2 - 3 mm diameter}} \overset{-}{\text{were dissected free from the parenchyma and loose connective tissue was removed taking care not to touch and damage the endoluminal surface.}} \overset{-}{\text{In some rings}} \overset{-}{\text{the endothelium was removed by passing a thin stainless steel wire 0.25 mm into the lumen and gently rolling the vessels back and forth on tissue soaked with control solution. This method has proven effective in removing the endothelium without damaging the underlying vascular smooth muscle}} \overset{-}{\text{[45,50]}} \]. The cleaned vessels were cut into rings and placed in organ chambers filled with control solution \[ 25 \text{ mL} \overset{-}{\text{that was maintained at constant temperature 37 °C and pH}} 7.4 \text{ and bubbled with a 95 % O}}_2 - 5 \% \text{ CO}_2 \text{ gas mixture. The rings were suspended between a fixed stirrup within the organ chamber} \overset{-}{\text{and a force transducer}} \overset{-}{\text{grass UTC3 Grass Instruments Quincy MA. Isometric tension was recorded Gould 8000S Gould Electronics Cleveland OH}} \overset{-}{\text{The rings were stretched gradually to the optimal point on the length-tension curve as determined by a maximal contraction to either phenylephrine 50 μmole L}^{-1} \text{ or histamine 3 μmole L}^{-1}} \overset{-}{\text{The vessels were washed and allowed to equilibrate for 30 minutes in the presence of indomethacin 10 μmole L}^{-1} \text{ an inhibitor of cyclooxygenase}} \overset{-}{\text{Experimental protocols were then performed. Rings with and without endothelium were studied in parallel. The integrity of the endothelium was verified by the response to bradykinin 300 nmole L}^{-1}} \overset{-}{\text{Endothelium-dependent responses}} \overset{-}{\text{Following the equilibration period rings with and without endothelium were contracted with a submaximal dose of phenylephrine 1 μmole L}^{-1} \text{ to 50 μmole L}^{-1} \text{ or KC} \overset{20}{\text{mmole L}^{-1}} \text{ These concentrations cause a contraction that approximates 50 % ED}_{50} \text{ of the maximal contraction to 10 mmole L}^{-1} \text{ norepinephrine ED}_{50} =}} \text{ED}_{50} =
1.0–1.5 grams of tension[]. Increasing cumulative concentrations of prostacyclin[] 100 fmol·L⁻¹ to 1 µmol·L⁻¹[] or acetylcholine[] 1 µmol·L⁻¹ to 1 µmol·L⁻¹[] were added to organ chambers containing rings with and without endothelium[] to determine the effect of the endothelium on the agonist-induced relaxations. All of these experiments were performed in the presence of indomethacin[] 10 µmol·L⁻¹[]. Vehicle controls for prostacyclin[] using NaHCO₃ and NaOH[] were performed. The pH of the bath following vehicle and prostacyclin addition was measured. To determine the contribution of nitric oxide to these relaxations[] the following antagonists were added to the organ chamber[] 5–30 min[] prior to the addition of prostacyclin or acetylcholine[] a scavenger of EDRF/nitric oxide[] methylene blue[] 10 µmol·L⁻¹ a nonspecific inhibitor of soluble guanylate cyclase[] N⁰-nitro-L-arginine[] NLA[] 10 µmol·L⁻¹ a stereoselective antagonist of nitric oxide synthases[]. Concentrations of cyclic GMP and cyclic AMP in the vascular smooth muscle were measured by radioimmunoassay to determine if increased nitric oxide release and the subsequent accumulation of cyclic GMP and AMP were responsible for the endothelium-potentiated relaxations observed to prostacyclin.

**Cyclic GMP-inhibited cyclic AMP phosphodiesterase**  
To determine the contribution of nitric oxide[] with subsequent accumulation of cyclic GMP in the vascular smooth muscle[] leading to inhibition of cG1-PDE[] to the prostacyclin-induced relaxation[] anaminor[] 10 µmol·L⁻¹[] a specific cG1-PDE inhibitor[] and 8-bromo-cyclic GMP[] 10 µmol·L⁻¹[] the soluble cyclic GMP analog[] were added to the organ chambers containing rings without endothelium. Cumulative increases in the concentration of the prostacyclin were then added to the organ baths[] using rings with and without endothelium from the same lungs as parallel controls. All of these experiments were performed in the presence of indomethacin[] 10 µmol·L⁻¹[].

**Cyclic nucleotides**  
Rings with and without endothelium were placed in test tubes containing 2 ml of control solution. The rings were allowed to equilibrate for 30 minutes at 37 °C and then changed to solution containing isobutylmethylxanthine[] IBMX[] 100 µmol·L⁻¹ a phosphodiesterase inhibitor. The rings were exposed for two minutes to agonists. The rings were then removed from the tube[] frozen in liquid nitrogen and the reaction was stopped by the addition of 21% trichloroacetic acid to a final concentra-

**Statistics**  
Results are expressed as means ± SEM. Values from rings with and without endothelium were compared using Student’s t-test for paired or unpaired observations. When multiple means were compared[] an analysis of variance[] ANOVA[] was employed. For the isolated ring-tension experiments[] n” equals the number of animals and rings used for each experiment. For cyclic nucleotide experiments[] n” equals the number of animals used for each experiment[] two rings from contiguous sites were used for each drug exposure and the values were averaged. Values were considered statistically different when P was less than 0.05.

**RESULTS**  
**Endothelium-dependent relaxations**  
In rings contracted with phenylephrine[] concentrations of prostacyclin[] 0.1 µmol to 1 µmol·L⁻¹[] caused concentration-dependent relaxations in rings with and without endothelium[] that were larger in rings with endothelium[] n = 11[] Fig 1[]. The endothelium-dependent potentiation was abolished by oxyhemoglobin[] 10 µmol·L⁻¹ n = 6[] and NLA[] 10 µmol·L⁻¹ n = 7[]. Fig 1[]. The small relaxations to prostacyclin observed in control rings without endothelium were not altered when exposed to these inhibitors of the L-arginine-nitric oxide pathway. Vehicle controls using NaHCO₃ and NaOH did not alter the tension of the contracted rings[] n = 8 paired rings[] Fig 2[] nor did it alter the pH of the solution more than 0.04 unit[] 7.40 vs 7.44[] n = 12[]. Acetylcholine[] 1 µmol to 1 µmol·L⁻¹[] caused endothelium-dependent relaxations[] n = 6[] that were abolished by oxyhemoglobin[] n = 3[] or methylene blue[] 10 mmol·L⁻¹ n = 3[] and reduced by NLA[] n = 6[] P<0.05[] Fig 1[].

**Cyclic GMP content**  
Rings with and without
more cyclic GMP compared to rings without endothelium \[ 117.2 \pm 24.5 \text{ vs } 11.05 \pm 3.4 \text{ pmol/g protein} \] under basal conditions indicating the presence of basal release of nitric oxide. The cyclic GMP content was increased over basal production in the presence of acetylcholine \( 1 \mu \text{mol L}^{-1} \) rings with endothelium \( 339 \pm 91 \text{ pmol/g protein} \) and sodium nitroprusside \( 10 \mu \text{mol L}^{-1} \) rings with \( 227.3 \pm 44.5 \text{ pmol/g protein} \) and without endothelium \( 96.8 \pm 50.3 \text{ pmol/g protein} \) vs basal levels without endothelium \( 11.05 \pm 3.4 \text{ pmol/g protein} \) \( P < 0.05 \) for all comparisons. However, no significant increase over basal production was seen with exposure to prostaclin \( 1 \mu \text{mol L}^{-1} \) in rings with endothelium \( 160.2 \pm 40 \text{ pmol/g protein} \). Fig 3.

Cyclic AMP content Rings with and without endothelium \( n = 5 \sim 6 \) were studied under basal conditions and during stimulation with prostaclin. The cyclic AMP content was increased by prostaclin \( 1 \mu \text{mol L}^{-1} \) compared to basal levels but this did not reach statistical significance \( 3.99 \pm 0.97 \text{ vs } 1.86 \pm 0.52 \text{ nmol/g protein} \) \( P = 0.07 \). The addition of the cyclic GMP analog \( 8 \)-bromo-cyclic GMP \( 10 \mu \text{mol L}^{-1} \) to prostaclin increased the cyclic AMP levels \( 4.14 \pm 0.53 \text{ nmol/g protein} \) \( P = 0.019 \) compared to basal levels. The increase in cyclic AMP caused by prostaclin was reduced when NLA \( 10 \mu \text{mol L}^{-1} \) was added \( 1.93 \pm 0.24 \text{ pmol/mg protein} \) \( P = 0.005 \). The addition of \( 8 \)-bromo-cyclic GMP to rings in which nitric oxide production was inhibited by NLA restored the cyclic AMP levels toward the prostaclin-induced levels \( 3.22 \pm 0.35 \text{ nmol/g protein} \). Results with \( 8 \)-bromo-cyclic GMP alone were similar \( 3.26 \pm 0.61 \text{ nmol/g protein} \). Fig 4.

Cyclic GMP-inhibited cyclic AMP phosphodiesterase Rings with endothelium exhibited endothelium-potentiated relaxations to prostaclin under control conditions \( n = 10 \). Rings without endothelium \( n = 10 \) and with \( 8 \)-bromo-cyclic GMP \( n = 10 \) exhibited relaxations to prostaclin that were statistically increased compared to control rings without endothelium. They were similar to those observed in control rings with endothelium. Fig 5.


discussion

Prostaclin is an endothelium-derived vasodilator
prostaglandin that dilates several vascular beds and inhibits platelet aggregation. This process occurs through the activation of adenylate cyclase with subsequent formation and accumulation of cyclic AMP. In 1980 Furchgott and Zawadzki demonstrated the existence of EDRF subsequently identified as EDNO or a nitric oxide-liberating nitrosodihydroxyefrin. It causes relaxation of vascular smooth muscle and inhibits platelet aggregation by activating soluble guanylate cyclase with the subsequent accumulation of cyclic GMP. Several agonists and stimuli can cause the concomitant release of nitric oxide and prostacyclin; these include bradykinin, the calcium ionophore A23187, thrombin, flow and shear stress. In porcine coronary vessels, prostacyclin and EDNO appear synergistic in causing relaxation of vascular smooth muscle with prostacyclin stimulating the release of EDNO at least in vitro.

In the present studies, prostacyclin-induced relaxations were potentiated in the porcine pulmonary artery.
by the presence of an intact endothelium. These observations could be explained by prostacyclin stimulating the release of nitric oxide from the pulmonary arterial endothelium. This would be concordant with the observations that oxyhemoglobin, a scavenger of EDRF/nitric oxide,

and Nω-nitro-L-arginine, a stereospecific inhibitor of nitric oxide synthase, both inhibited the endothelium-potentiated relaxations induced by prostacyclin. Confirmation that the inhibition of the L-arginine nitric oxide pathways occurs in isolated porcine pulmonary artery rings with oxyhemoglobin and Nω-nitro-L-arginine is provided by the antagonists’ effects upon the acetylcholine-induced endothelium-dependent relaxation. Methylene blue, which nonspecifically inhibits soluble guanylate cyclase, the target of nitric oxide, also inhibited the acetylcholine-induced relaxation, further strengthening the evidence that this factor is released from the rings studied. However, although the cyclic GMP content measured in isolated rings of pulmonary artery is increased above basal by acetylcholine and sodium nitroprusside this is not the case with prostacyclin. These data indicate that in the porcine pulmonary artery prostacyclin does not stimulate the release of a significant amount of additional nitric oxide above the basal levels but potentiates the relaxation by another mechanism.

Cyclic GMP reduces the hydrolysis of cyclic AMP through inhibition of cyclic AMP phosphodiesterases. These enzymes have been found in cardiac muscle platelets and vascular smooth muscle. In the rat aorta cyclic GMP even in low concentrations increases the content of cyclic AMP. Inhibition of the cyclic GMP-inhibited cyclic AMP phosphodiesterases causes relaxation of vascular smooth muscle. This conclusion has been reached when the selective inhibitors of the enzyme milrinone and amrinone were given to rat aortic and guinea pig pulmonary artery rings in vitro. In vivo peripheral vasodilatation has also been observed with those drugs in humans. Additionally, agonists that stimulate cyclic GMP accumulation act synergistically to enhance β-adrenergic agonist-induced relaxation of isolated rat aortic rings as well as to enhance the antiaggregatory action of prostaglandin E1 and prostacyclin on platelets. In the present study, the endothelium potentiated the relaxations to prostacyclin a cyclic AMP-mediated agonist. The basal release of nitric oxide and the resulting accumulation of cyclic GMP could potentiate the relaxations to prostacyclin by inhibiting the cG1-PDE. This is supported by the documented accumulation of cyclic GMP under basal conditions which is not P > 0.05 changed by exposure to prostacyclin but which is accompanied by enhanced relaxations. Inhibition of basal release of nitric oxide using hemoglobin and NLA also inhibited the potentiation of the relaxation to prostacyclin. When simulating the presence of basally released nitric oxide in rings without endothelium by using 8-bromo-cyclic GMP prostacyclin-induced relaxations were enhanced to the level observed in rings without endothelium. In addition, amrinone, the specific inhibitor of cG1-PDE similarly enhanced the prostacyclin-induced relaxations in rings without endothelium mimicking relaxations observed in control rings. These observations strongly suggest that indeed the basal release of nitric oxide results in inhibition of cG1-PDE. The measurements of cyclic AMP levels offers further evidence that this is the case. The cyclic AMP-concentration is increased by the addition of 8-bromo-cyclic GMP.

Fig. 5. Cumulative concentration-response curves to prostacyclin in isolated rings of porcine pulmonary artery in rings with closed symbols and without endothelium open symbols. The effects of 8-bromo-cGMP and amrinone were tested in rings without endothelium. Data are expressed as means ± SEM n = 10. Indomethacin was present in all experiments. *P < 0.05 between control rings with endothelium as well as amrinone and 8-bromo-cGMP treated rings without endothelium compared to control rings without endothelium.
to prostacyclin. Inhibition of the cGI-PDE with subsequent increase in cyclic AMP levels would be expected with 8-bromo-cyclic GMP if this mechanism was active in the studied preparation. The addition of the nitric oxide inhibitor NLA decreased the cyclic AMP content toward basal levels. The most likely explanation for this observation is that the reduction in basal nitric oxide-induced cyclic GMP accumulation prevents the inhibition of cGI-PDE and the subsequent accumulation of cyclic AMP. A similar phenomenon has been observed in cerebral vessels and labeled cyclic nucleotide crosstalk [33].

In conclusion these present data confirm that the endothelium potentiates the relaxation to prostacyclin in the porcine pulmonary artery. This potentiation can be attributed to the basal release of nitric oxide which stimulates the accumulation of cyclic GMP on the vascular smooth muscle cells. Cyclic GMP in turn inhibits the cGI-PDE in these cells inhibiting the hydrolysis of cyclic AMP and increasing its concentration. This is turn enhances relaxation of the vascular smooth muscle by the cyclic nucleotides. Physiologically the potentiated relaxations by the basal release of nitric oxide may represent an important synergy to perpetuate vasodilatation in vivo.

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