Cyclooxygenase-2 and an early stage of chronic hypoxia-induced pulmonary hypertension in newborn pigs

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Fike, Candice D., Mark R. Kaplowitz, Yongmei Zhang, and Sandra L. Pfister. Cyclooxygenase-2 and an early stage of chronic hypoxia-induced pulmonary hypertension in newborn pigs. J Appl Physiol 98: 1111–1118, 2005. First published October 29, 2004; doi:10.1152/japplphysiol.00810.2004.—Our objective was to determine whether cyclooxygenase (COX)-2-dependent metabolites contribute to the altered pulmonary vascular responses that manifest in piglets with chronic hypoxia-induced pulmonary hypertension. Piglets were raised in either room air (control) or hypoxia for 3 days. The effect of the COX-2 selective inhibitor NS-398 on responses to arachidonic acid or acetylcholine (ACH) was measured in endothelium-intact and denuded pulmonary arteries (100- to 400-μm diameter). Pulmonary arterial production of the stable metabolites of thromboxane and prostacyclin was assessed in the presence and absence of NS-398. Dilution to arachidonic acid was greater for intact control than for intact hypoxic arteries, was unchanged by NS-398 in intact arteries of either group, and was augmented by NS-398 in denuded hypoxic arteries. ACh responses, which were dilatation in intact control arteries but constriction in intact and denuded hypoxic arteries, were diminished by NS-398 treatment of all arteries. NS-398 reduced prostacyclin production by control pulmonary arteries and reduced thromboxane production by hypoxic pulmonary arteries. COX-2-dependent contracting factors, such as thromboxane, contribute to aberrant pulmonary arterial responses in piglets exposed to 3 days of hypoxia.

arachidonic acid metabolites; prostacyclin; thromboxane; NS-398

It has been known for many years that prostanoids play an important role in regulation of neonatal pulmonary vascular tone. It is also well known that one of the critical, rate-limiting biosynthetic steps in prostanoid production is the conversion of arachidonic acid by cyclooxygenase (COX) to prostaglandin (PG) G2 (PGG2) and subsequent peroxidation of PGG2 to PGH2 by the same enzyme (Fig. 1A) (32). To date, two COX isoforms, COX-1 and COX-2, have been shown to be constitutively expressed in adult lung tissue, including pulmonary vascular endothelium and smooth muscle (11, 13). Evaluation of COX-1 and COX-2 distribution in the neonatal lung has been limited to one report in human lungs during the perinatal period (23) and to a few studies with ovine lungs (5, 6, 26). Notably, although both COX isoforms have been identified in neonatal and adult pulmonary vasculature of some species, there is a paucity of physiological information regarding the contribution of COX-2 to regulation of pulmonary vascular tone (12). Furthermore, studies evaluating the potential contribution of COX-2 to altered regulation of pulmonary vascular tone in lungs of animals with pulmonary hypertension have received almost no attention (9, 18).

Because of their critical role in regulation of pulmonary vascular tone, elucidation of derangements in arachidonic acid pathways in resistance-level pulmonary arteries is of particular importance. Changes that occur with short exposure to hypoxia are critical to delineate because of the potential significance for developing therapies to intervene with the onset and progression of pulmonary hypertension. Our laboratory previously showed that resistance-level pulmonary arterial responses to arachidonic acid and acetylcholine are altered in newborn piglets with pulmonary hypertension induced by 3 days of exposure to hypoxia (18, 19). The major purpose of this study was to test the hypothesis that metabolites of the COX-2 pathway of arachidonic acid metabolism are involved with the aberrant pulmonary vascular responses that develop at this early stage of pulmonary hypertension in newborn piglets.

Methods

Animals. A total of 12 hypoxic piglets and a total of 15 control piglets were studied. For the hypoxic piglets, newborn pigs (2–3 days old) were placed in a hypoxic normobaric chamber for 3–4 days. Normobaric hypoxia was produced by delivering compressed air and N2 to an incubator (Thermocare). O2 content was regulated at 8–10% (PO2 60–72 Torr), and CO2 was maintained at 3–6 Torr by absorption with soda lime. The chamber was opened two times per day for cleaning and to weigh the piglets. The animals were fed ad libitum with an artificial sow milk replacer from a feeding device attached to the chamber. Our laboratory has previously found no differences in vascular responses between piglets raised in a room-air environment for 3–5 days and piglets raised on a farm (16, 19). Therefore, for this study, most (n = 12) of the control piglets were studied on the day of arrival from the farm at 5–8 days of age, i.e., the same postnatal age as the hypoxic piglets on the day of study.

Cannulated artery preparation. On the day of study the piglets were preanesthetized with ketamine (30 mg/kg im) and then anesthetized with pentobarbital sodium (10 mg/kg iv). All animals were given heparin (1,000 IU/kg iv) and then exsanguinated. The thorax was opened, and the lungs were removed and placed in cold (4°C) physiological saline solution (PSS) until use. The PSS had the following composition (in mM): 141 Na+ 4.7 K+, 125 Cl−, 2.5 Ca2+, 0.72 Mg2+, 1.7 H2PO4−, 25 HCO3−, and 11 glucose. Immediately before use, segments of 100- to 400-μm-diameter pulmonary arteries were dissected from a lung lobe.

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The system used to study cannulated arteries has been described in detail previously (19). Briefly, it consists of a water-jacketed plastic chamber in which proximal (inflow) and distal (outflow) cannulas were mounted. An arterial segment was threaded onto the proximal cannula and tied in place with a 22-μm nylon suture. The distal end of the artery was then tied onto the distal cannula, and the artery was filled with PSS, and all side branches were tied off. The distance between the cannula tips was adjusted with a micrometer connected to the proximal cannula so that the slack was taken out of the artery. The exterior of the artery was suffused with PSS from a reservoir at 37°C and aerated with a gas mixture containing O2, CO2, and N2, giving a PO2 of 140 Torr, a PCO2 of 38 Torr, and a pH of 7.37. The arterial lumen was filled from a syringe containing PSS, aerated with the same gas mixture as the reservoir, and connected to the cannula with polyethylene tubing. Gas concentrations and pH were monitored in all solutions (reservoir, vessel chamber, and infusion syringes) by using a blood-gas analyzer.

Inflow pressure was adjusted by changing the height of the infusion syringe. Pressure transducers were placed both on the inflow side between the syringe and the artery and at the outflow end of the system. Both inflow and outflow pressures were monitored continuously on a recorder, and the artery was discarded if the pressures were not equal (indicates leak in vessel). The external diameter of the artery was observed continuously with a video system containing a color camera (model 5000, Panasonic) and television monitor. Vessel diameters were measured with a video scaler (FORA IV). The video scaler was calibrated with a micrometer scale. Measurements with the video scaler are repeatable within ±1 μm.

Cannulated artery protocols. Each artery was allowed to equilibrate for 60–90 min to establish basal tone. The control arteries were equilibrated at a transmural pressure of 15 cmH2O, and the hypoxic arteries were equilibrated at a transmural pressure of 25 cmH2O. These pressures were chosen as they represent in vivo pressures (15, 16). Our laboratory has previously shown no effect from these transmural pressures on pulmonary arterial responses to ACh (19). After establishment of basal tone, all arteries were tested for viability by contraction to the thromboxane (Tx) A2-mimetic U-46619 (10^{-8} M). To check for a functional endothelium in control arteries, responses to ACh (10^{-6} M) were evaluated. Our laboratory previously found that hypoxic arteries constricted to ACh but dilated to another endothelium-dependent agent, the calcium ionophore A-23187 (19). Therefore, responses to A-23187 were used to check for a functional endothelium in hypoxic arteries.

In one series of studies, we evaluated the contribution of COX-2-dependent metabolites to arachidonic acid responses in control (n = 12 arteries from 12 control piglets) and hypoxic (n = 9 arteries from 9 piglets) arteries. For these studies, changes in vessel diameter to arachidonic acid (10^{-8} to 10^{-5} M) were measured before and after addition of the COX-2-selective inhibitor NS-398 (10^{-6} M). Because we have previously shown that the change in diameter to arachidonic acid in vessels at basal tone is minimal (18), these studies were performed in vessels with elevated tone. After being assessed for viability and a functional endothelium, vessel tone was elevated by addition of endothelin to the reservoir in increasing doses until the arterial diameter had decreased by 40–50%. Changes in vessel diameter were then measured in response to cumulative doses of arachidonic acid (10^{-8} to 10^{-5} M). Next, the vessels were washed with PSS, and the COX-2-selective inhibitor NS-398 (10^{-6} M) was added to the reservoir. Twenty minutes after addition of the NS-398, vessel tone was again elevated by 40–50% with endothelin, and dose responses to arachidonic acid (10^{-8} to 10^{-5} M) were repeated.

To determine the influence of the endothelium, air was infused into arteries of control (n = 11 arteries from 11 piglets) and hypoxic (n = 6 arteries from 6 piglets) piglets (22). Functional disruption of the endothelium was verified by loss of dilation to ACh and/or A-23187 in the control arteries and to A-23187 in the hypoxic arteries. Reactivity to U-46619 was used to confirm viability of the arteries. Then, responses to arachidonic acid (10^{-8} to 10^{-5} M) were measured in vessels with elevated tone before and after addition of the COX-2-selective inhibitor NS-398 (10^{-6} M).

In another series of studies, we evaluated the contribution from COX-2-dependent metabolites to changes in vessel diameter in re-
response to ACh, an agonist known to stimulate release of endogenous arachidonic acid (14). Some of these studies were performed with endothelium-intact vessels, and others were performed with vessels in which the endothelium was disrupted by air infusion, as described in the preceding paragraph. On the basis of our laboratory’s previous use of vessels at basal tone to evaluate ACh responses (17), these studies were performed with vessels at basal tone. For all of these studies, changes in vessel diameter (for intact control arteries, n = 9 arteries from 9 piglets; for endothelium-denuded control arteries, n = 10 arteries from 10 piglets; for intact hypoxic arteries, n = 9 arteries from 9 piglets; for endothelium-denuded hypoxic arteries, n = 7 arteries from 7 piglets) were continuously monitored while cumulative doses of ACh were added (10⁻⁸ M to 10⁻⁵ M) before and then 20 min after the addition of the COX-2-selective inhibitor NS-398 (10⁻⁶ M).

For all of the above studies, vessel viability was tested at the completion of the study by addition of U-46619. In addition, in some studies, vessel responses to DMSO, the vehicle used for solubilization of NS-398, were evaluated.

Radioimmunoassay of 6-keto-PGF₁α, the stable metabolite of thromboxane, TxB₂, and enzyme-immunoassay of the stable metabolite of prostacyclin, 6-keto-PGF₁α. This series of studies was performed to assess whether production of either of the COX metabolites, thromboxane (TxA₂) or prostacyclin (PGI₂), are COX-2 dependent in control and hypoxic arteries. To do this, pulmonary arteries (20- to 600-μm diameter) were dissected; weighed; immediately placed in HEPES buffer of the following composition (in mM): 10 HEPES, 150 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, and 11 glucose, pH 7.4; and then incubated to assess production of the stable metabolites of TxA₂, TxB₂, and PGI₂, 6-keto-PGF₁α. A total of 9 control and 10 hypoxic piglets were used for these determinations. Note that the same piglet’s lung was often used to obtain pulmonary arteries for cannulated artery studies and the radioimmunoassay and enzyme immunoassay determinations. As described above, all piglets were preanesthetized with ketamine (30 mg/kg im), anesthetized with pentobarbital sodium (10 mg/kg iv), given heparin (1,000 IU/kg iv), and then exsanguinated. Next, the lungs of the piglets were excised and vessels dissected. Vessels were first incubated for 15 min at 37°C in HEPES buffer containing the vehicle, DMSO, or NS-398 (10⁻⁶ M). Then, the supernatant was discarded, and the vessels were incubated for an additional 15 min at 37°C in HEPES buffer containing arachidonic acid (10⁻⁵ M). After the second 15-min incubation, the supernatant was collected and stored at −20°C until the time of assay for specific metabolites. The vessels were dried at 60°C for at least 72 h. Synthesis of TxB₂ was measured by specific radioimmunoassay by use of the method of Campbell and Ojeda (7). The antibody for TxB₂ was from Dr. Pfister’s laboratory. Sensitivity of the assay is 1 pg/ml for TxB₂. Synthesis of 6-keto-PGF₁α was measured by enzyme immunoassay following standard methods and using kits from Cayman Laboratories. Sensitivity of the assay is 11 pg/ml. Radioimmunoassay determinations of TxB₂ and enzyme immunoassay determinations of 6-keto-PGF₁α were normalized to vessel dry weight.

Immunohistochemical localization of COX-2. Control (n = 3) and hypoxic (n = 3) piglets were preanesthetized with ketamine (30 mg/kg im), anesthetized with pentobarbital sodium (10 mg/kg iv), given heparin (1,000 IU/kg iv), and then exsanguinated. Cannulas were placed into the trachea, pulmonary artery, and left atrium of each piglet. After perfusion with normal saline to remove all blood from the pulmonary circulation, the lungs were perfused for 5 min with 10% neutral-buffered formalin (4°C). The lungs were fixed by instillation of the formalin into the airway, pulmonary artery, and left atrium. After 24 h of fixation, pieces of lung were embedded in paraffin and sectioned. The tissue sections were deparaffinized, rehydrated, and then microwaved in citrate buffer (Bio-Genex, San Ramon, CA) to improve antigen detection (33). Next, the tissue sections were treated with blocking serum and incubated with a COX-2 primary antibody (1:500; antibody kindly provided by Dr. William Campbell, Medical College of Wisconsin). Methanol-H₂O₂ was used to block endogenous peroxidase activity, and a standard peroxidase method was used for antigen detection (Elite ABC kit, Vector Laboratories, Burlingame, CA). The tissue sections were counterstained with hematoxylin. Immunohistochemical staining controls included omission of the primary antibody and omission of the secondary antibody.

Materials. Concentrations for each drug listed in cannulated artery protocols were expressed as final molar concentrations in the vessel bath. ACh, A-23187, and NS-398 were obtained from Sigma Chemical. Arachidonic acid was from Nu-Chek Prep. Arachidonic acid was prepared in ethanol previously sparged with nitrogen. Endothelin was from Calbiochem and was solubilized in ethanol. ACh was solubilized in saline. NS-398 was solubilized in DMSO. [3H]TxB₂ was from Amershams.

Statistics. Data are presented as means ± SE. To compare changes in vessel diameters between endothelium-intact and endothelium-disrupted vessels, both with and without treatment with NS-398, a one-way ANOVA with post hoc multiple-comparison test was used. To compare TxB₂ or 6-keto-PGF₁α production with and without NS-398 for control or hypoxic arteries, a paired t-test was used. To compare NS-398-induced changes in TxB₂ or 6-keto-PGF₁α production between control and hypoxic arteries, an unpaired t-test was used. P < 0.05 was considered significant.

RESULTS

For the cannulated artery studies, the mean diameters of vessels used for all studies were 216 ± 8 μm for control arteries and 225 ± 6 μm for hypoxic arteries. None of the vehicles significantly changed arterial diameter in the concentrations used for solubilization of any of the agents.

Arteries from both control (Fig. 2A; n = 12 arteries from 12 piglets) and hypoxic (Fig. 2B; n = 9 arteries from 9 piglets) piglets dilated to all doses of arachidonic acid, but at the higher doses of arachidonic acid the dilation was less in the hypoxic arteries than in the control arteries. NS-398 treatment did not alter the degree of dilation to arachidonic acid in endothelium-intact arteries of either type (Fig. 2A control and Fig. 2B hypoxic). After air infusion, both control (Fig. 2A, n = 11 arteries from 11 control piglets) and hypoxic (Fig. 2B, n = 6 arteries from 6 hypoxic piglets) arteries dilated to all doses of arachidonic acid. The magnitude of dilation to arachidonic acid was less at the higher doses of arachidonic acid for air-infused, henceforth referred to as endothelium-denuded, than for endothelium-intact arteries from control piglets (Fig. 2A). Responses to arachidonic acid were similar at all doses of arachidonic acid for endothelium-denuded and endothelium-intact arteries of hypoxic piglets (Fig. 2B). NS-398 treatment did not alter the degree of dilation to arachidonic acid in endothelium-intact arteries of either type (Fig. 2A control and Fig. 2B hypoxic). Air-infused control arteries (Fig. 2A, n = 11 arteries from 11 control piglets) and hypoxic (Fig. 2B, n = 6 arteries from 6 hypoxic piglets) arteries dilated to all doses of arachidonic acid. The magnitude of dilation to arachidonic acid was less at the higher doses of arachidonic acid for air-infused, henceforth referred to as endothelium-denuded, than for endothelium-intact arteries from control piglets (Fig. 2A). Notably, the magnitude of dilation to arachidonic acid was augmented after NS-398 treatment in endothelium-denuded arteries of hypoxic piglets (Fig. 2B).

The diameter of endothelium-intact control arteries (n = 9 arteries from 9 piglets) increased with ACh (Fig. 3A), whereas the diameter of endothelium-intact arteries of hypoxic piglets (n = 9 arteries from 9 piglets) decreased with ACh (Fig. 3B). NS-398 treatment reduced the dilation to the higher concentrations of ACh in endothelium-intact control arteries and abolished the constriction to all concentrations of ACh in endothelium-intact hypoxic arteries.

Endothelium-denuded vessels of both control (n = 10 arteries from 10 piglets) (Fig. 3A) and hypoxic (n = 7 arteries from 7 piglets) piglets (Fig. 3B) constricted to all doses of ACh. In
hypoxic arteries, the magnitude of constriction to the higher doses of ACh was augmented in endothelium-denuded vessels. For both types of endothelium-denuded arteries, treatment with NS-398 almost completely abolished constriction to ACh (Fig. 3, A and B).

Enzyme immunoassay determinations of the supernatant from incubated pulmonary arteries showed that values for production of the stable metabolite of PGI₂, 6-keto-PGF₁α, were less for NS-398-treated control arteries (12,333 ± 3,055 pg/mg) than for DMSO-treated control arteries (17,444 ± 5,000 pg/mg; P = 0.04, paired t-test). By comparison, NS-398 had little influence on production of 6-keto-PGF₁α in vessels from hypoxic piglets: values of 6-keto-PGF₁α were 9,860 ± 2,700 pg/mg for NS-398-treated arteries from hypoxic piglets compared with 9,360 ± 2,220 pg/mg for DMSO-treated hypoxic vessels (P = 0.6, paired t-test). That is, NS-398 had different effects on production of the stable metabolite of PGI₂, 6-keto-PGF₁α in control than in hypoxic arteries. As shown in Fig. 4A, 6-keto-PGF₁α was decreased by NS-398 treatment of control vessels but was minimally affected by NS-398 treatment of hypoxic vessels (P = 0.027 unpaired t-test).

Production of the stable metabolite of TxA₂, TxB₂, as assessed by radioimmunoassay, tended to be less (P = 0.08) in the supernatant of control vessels treated with NS-398 (6.5 ± 2.9 pg/mg) than in the supernatant of control vessels treated with DMSO (9.5 ± 3.5 pg/mg). For hypoxic vessels, production of TxB₂ was less (P = 0.049) in the supernatant of vessels treated with NS-398 (4.1 ± 2.6 pg/mg) compared with the supernatant of vessels treated with DMSO (7.0 ± 2.9 pg/mg). That is, Fig. 4B shows that, by comparison with the disparate effect on 6-keto-PGF₁α production, TxB₂ production was affected similarly by NS-398 treatment of control and hypoxic arteries.

Fig. 3. A: acetylcholine-induced changes in diameter for endothelium-intact (n = 9 arteries from 9 piglets) and endothelium-denuded (n = 10 arteries from 10 piglets) control arteries before and after NS-398. Data are expressed as percent dilation of contraction elicited by endothelin. Values are means ± SE. *Different from intact, P < 0.05. B: acetylcholine-induced changes in diameter for endothelium-intact (n = 9 arteries from 9 piglets) and endothelium-denuded (n = 6 arteries from 6 piglets) hypoxic arteries before and after NS-398. Data are expressed as percent dilation of contraction elicited by endothelin. Values are means ± SE. *Different from values for all other conditions, P < 0.05.

Fig. 4. A: arachidonic acid-induced changes in diameter for endothelium-intact (n = 12 arteries from 12 piglets) and endothelium-denuded (n = 11 arteries from 11 piglets) control arteries before and after NS-398. Data are expressed as percent dilation of contraction elicited by endothelin. Values are means ± SE. *Different from intact, P < 0.05. B: arachidonic acid-induced changes in diameter for endothelium-intact (n = 9 arteries from 9 piglets) and endothelium-denuded (n = 6 arteries from 6 piglets) hypoxic arteries before and after NS-398. Data are expressed as percent dilation of contraction elicited by endothelin. Values are means ± SE. *Different from intact, P < 0.05.
Taken altogether, the effect of NS-398 on the ratio of production of the stable metabolites of PGI2 and TxA2 differed between control and hypoxic arteries. Specifically, the ratio of production of the stable metabolites of PGI2 and TxA2 was similar for DMSO-treated (PGI2/TxA2 \(1,836:1\)) and NS-398-treated (PGI2/TxA2 \(1,897:1\)) arteries of control piglets. In contrast, the ratio of production of the stable metabolites of PGI2 and TxA2 was greater for hypoxic vessels treated with NS-398 (PGI2/TxA2 \(2,283:1\)) than for hypoxic vessels treated with DMSO (PGI2/TxA2 \(1,418:1\)).

For both control (Fig. 5A) and hypoxic (Fig. 5B) piglets, immunohistochemistry revealed the presence of COX-2 in the endothelium and smooth muscle cells of arteries within the size range used for cannulated artery studies.

**DISCUSSION**

Our laboratory has previously shown that newborn piglets exposed to 3 days of chronic hypoxia develop pulmonary hypertension (15, 16). Specifically, they have elevated pulmonary vascular resistances and exhibit pulmonary arterial morphometric changes that are consistent with pulmonary hypertension (15, 16). Our laboratory has also shown that responses to some agonists are altered in resistance-level pulmonary arteries from newborn piglets exposed to 3 days of chronic hypoxia (18, 19). Consistent with our laboratory’s previous findings (19), in this study we show that responses to ACh, an agonist known to stimulate release of arachidonic acid (14), are altered in resistance-level pulmonary arteries of piglets exposed to 3 days of hypoxia. By use of nonspecific COX inhibition, our laboratory previously showed that COX pathways contribute to the aberrant ACh responses in pulmonary arteries from hypoxic piglets (19). An important new finding in this study is that the impaired ACh responses are due, at least in part, to COX-2-dependent contracting factors.

Physiological studies evaluating the contribution of the COX-2 pathway of arachidonic acid metabolism to regulation of pulmonary vascular tone are scarce. In studies with lungs of normal rats, arachidonic acid has been shown to be converted into vasoactive prostanoids by way of the COX-2 pathway (2, 3, 12). Findings in our study support the possibility that metabolites of the COX-2 pathway contribute to regulation of vascular tone in the normal neonatal piglet pulmonary circulation. In particular, we show that COX-2 may modulate agonist-induced responses, as suggested by our finding that ACh responses in pulmonary arteries from control piglets were diminished by a COX-2 inhibitor. In addition to the pulmonary circulation, there are physiological studies showing that COX-2 is important in regulation of tone, such as modulation of agonist-induced responses, in other vascular beds of normal animals, including the systemic peripheral vasculature (2, 3, 30), thoracic aorta (1), renal medulla (30), and the ductus arteriosus (10, 24). As with other signaling pathways, the role of COX-2 in regulation of normal vascular tone is likely to
differ between vascular beds (25), with postnatal age (25), and between species (20).

There has also been interest in the potential contribution of COX-2 to the disrupted regulation of tone found in a number of vascular diseases. For example, COX-2 inhibitors have been shown to increase flow-mediated dilation in brachial arteries of adult humans with atherosclerotic vascular disease (8, 34), to ameliorate the augmented responses to angiotensin II found in the aorta of spontaneously hypertensive rats (35), and to diminish the vasoconstrictor effects of arachidonic acid in renal arteries of diabetic rats (31). Moreover, limited evidence has been provided showing that COX-2 may be involved in the physiological changes, including changes in blood flow distribution, accompanying acute lung injury associated with endotoxemia (21). Despite the long-standing interest in the role of COX metabolites and pulmonary hypertension, to our knowledge, our finding that ACh constriction was abolished by a COX-2 inhibitor is the first physiological evidence that COX-2-dependent contracting factors are involved with the aberrant agonist-induced responses that develop in pulmonary arteries of animals during exposure to chronic hypoxia.

Other findings in our study also indicate that COX-2 functions as an important pathway for arachidonic acid metabolism in resistance pulmonary arteries of both control and hypoxic piglets. Our finding that COX-2 inhibition did not alter the degree of dilation to arachidonic acid in either endothelium-intact or denuded arteries of control piglets need not be interpreted to indicate a lack of COX-2 metabolite production. Rather, a more likely explanation for these findings is that COX-2 inhibition altered the production of downstream COX-2-dependent dilators and constrictors by similar amounts in arteries of control piglets. Indeed, this explanation is supported by our findings with control pulmonary arteries showing that COX-2 inhibition reduced production of the downstream dilator, PGI₂, and the downstream constrictor, TxA₂, such that the ratio of production of these metabolites remained the same.

Unlike control arteries, we found that COX-2 inhibition reduced production of the constrictor, TxA₂, without altering production of the dilator, PGI₂, such that the ratio of dilator production increased. In view of this, it might seem that COX-2 inhibition should augment arachidonic acid dilation in arteries from hypoxic piglets. Instead, we found that arachidonic acid dilation was augmented by COX-2 inhibition in denuded arteries but was unchanged by COX-2 inhibition in endothelium-intact arteries of hypoxic piglets.

To understand the disparity in findings between endothelium-intact and denuded arteries, it should be kept in mind that some pathways of arachidonic acid metabolism, such as the lipoxygenase pathway and/or some of the cytochrome P-450 pathways (Fig. 1A and B), might be present in one cell type but not the other (e.g., endothelial cells but not in smooth muscle cells). In addition, it is possible that, after 3 days of hypoxia, arachidonic acid metabolism is shunted toward these non-COX-dependent pathways (Fig. 1B), potentially leading to augmented production of non-COX-dependent metabolites, including products of the lipoxygenase pathway that act as constrictors, such as some of the leukotrienes and some of the lipoxygenase-dependent hydroxyeicosatetraenoic acids (HETEs; Fig. 1B). Indeed there is evidence that chronic hypoxia activates lung 15-lipoxygenase in newborn rabbits and enhances production of the pulmonary vasoconstrictor, 15-HETE (37). Moreover, we previously found that COX-1 and PGI₂ synthase levels were reduced but that COX-2 and TxA₂ synthase levels were not altered in resistance pulmonary arteries of newborn piglets exposed to 3 days of hypoxia (Fig. 1B; Ref. 18). Therefore, it could be that, after 3 days of in vivo hypoxia, the COX-2 pathway becomes a more prominent pathway for arachidonic acid metabolism in piglet resistance pulmonary arteries because of the reduced availability of COX-1 (Fig. 1B). Furthermore, because PGI₂, the enzymatic product of COX-2, will be preferentially metabolized by the relatively abundant downstream enzyme(s), in this case TxA₂ synthase (Fig. 1B), the balance shifts toward constriction.

Taking the above into account, one possible explanation as to why COX-2 inhibition did not alter arachidonic acid dilation in endothelium-intact arteries of hypoxic piglets could be that the inhibitory effect on production of COX-2-dependent contracting factors was offset by unmasking, and potentially enhancing, the influence from non-COX-2-dependent constrictors. Moreover, if the non-COX-2-dependent constrictor pathways are present in endothelial cells but not in smooth muscle cells, the effect of COX-2 inhibition would not be the same in endothelium-intact and denuded arteries. This is because the influence from the non-COX-2-dependent constrictors, perhaps leukotrienes and lipoxygenase-dependent HETEs (37), would be removed by endothelial denudation, such that the contribution from other cells in the vascular wall, including smooth muscle cells, would predominate. In other words, in endothelial-denuded arteries, an inhibitory effect on the production of COX-2-dependent contracting factors, such as TxA₂, by smooth muscle cells would lead to the augmented dilation to arachidonic acid that we found in denuded hypoxic arteries.

Other findings in this study also implicate a nonendothelial cellular source of COX-2-dependent contracting factors. Specifically, we found that COX-2 inhibition abolished the constrictor response to ACh in denuded arteries of both control and hypoxic piglets. Furthermore, immunohistochemistry revealed that COX-2 is found in smooth muscle cells of both control and hypoxic arteries. Taken together, our findings suggest that, in control arteries, the influence from COX-2-dependent dilators and constrictors is fairly well balanced. After 3 days of hypoxia, the influence from COX-2-dependent contracting factor(s), derived in part from smooth muscle cells, predominates over COX-2-dependent dilators.

It merits comment that nonphysiological evidence that COX-2 may be involved with pulmonary hypertension has been suggested by results of a few other studies. COX-2 protein levels were increased in whole lung homogenates from adult rats exposed to 2 wk of chronic hypoxia (9) and in proximal pulmonary arterial smooth muscle cells from adult humans cultured under hypoxic conditions for at least 48 h (4). Differences between species, postnatal ages, and length of hypoxic exposure might explain why our previous finding of no change in COX-2 amounts (18) differs from findings in these other studies (4, 9).

Limitations of our methodology should be discussed. Many of our conclusions are based on results of pharmacological inhibition. NS-398 (10⁻⁵ to 10⁻⁴ M) has been shown to effectively inhibit COX activity in COX-2-expressing cells, including newborn piglet endothelial and smooth muscle cells in primary culture, with no effect in COX-1 expressing cells, such as Swiss 3T3 fibroblasts and human umbilical vein...
endothelial cells (27–29). We chose to use an even lower concentration of NS-398, 10−6 M, to further minimize any potential influence on COX-1. Regardless of COX isoenzyme affected, use of any COX inhibitor can result in shunting of the precursor arachidonic acid to non-COX-mediated pathways, which would influence the results. In addition to a potential influence on arachidonic acid responses as noted above, other responses, such as the blunted response to ACh in control arteries after NS-398 treatment, could be partly attributed to shunting of arachidonic acid to the lipoxygenase pathway. Moreover, it should also be considered that non-COX pathways of arachidonic metabolism could be involved with pulmonary hypertension. Findings in this and our laboratory’s previous study show that neither COX-2-selective nor non-selective COX inhibition (19) abolishes arachidonic acid-induced responses in either control or hypoxic arteries. The possible contribution from non-COX pathways of arachidonic metabolism, such as leukotrienes and HETEs (36, 37), remains to be elucidated.

To summarize, our findings indicate that COX-2-dependent contracting factors contribute to aberrant responses that develop in resistance pulmonary arteries of newborn piglets exposed to 3 days of hypoxia. In addition, our findings suggest that use of a COX-2 inhibitor might diminish production of downstream COX-2-dependent constrictors, such as TxA2, without adversely affecting production of downstream COX-2-dependent dilators, such as PGI2, in resistance pulmonary arteries of piglets with pulmonary hypertension resulting from 3 days of exposure to hypoxia. Because the influence from COX-2-dependent constrictor production manifests at an early stage of pulmonary hypertension, determination of whether early manipulation of this pathway will ameliorate or prevent the development and progression of neonatal pulmonary hypertension associated with chronic hypoxia merits consideration.

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