Arachidonic acid metabolites and an early stage of pulmonary hypertension in chronically hypoxic newborn pigs

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Fike, Candice D., Mark R. Kaplowitz, and Sandra L. Pfister. Arachidonic acid metabolites and an early stage of pulmonary hypertension in chronically hypoxic newborn pigs. Am J Physiol Lung Cell Mol Physiol 284: L316–L323, 2003. First published September 27, 2002; 10.1152/ajplung.00228.2002.—Our purpose was to determine whether production of arachidonic acid metabolites, particularly cyclooxygenase (COX) metabolites, is altered in 100–400-μm-diameter pulmonary arteries from piglets at an early stage of pulmonary hypertension. Piglets were raised in either room air (control) or hypoxia for 3 days. A cannulated artery technique was used to measure responses of 100–400-μm-diameter pulmonary arteries to arachidonic acid, a prostacyclin analog, or the thromboxane mimetic U46619. Radiomunonassay was used to determine pulmonary artery production of thromboxane B2 (TxB2) and 6-keto-prostaglandin F1α, (6-keto-PGF1α), the stable metabolites of thromboxane and prostacyclin, respectively. Assessment of abundances of COX pathway enzymes in pulmonary arteries was determined by immunoblot technique. Arachidonic acid induced less dilation in pulmonary arteries from hypoxic than in pulmonary arteries from control piglets. Pulmonary artery responses to prostacyclin and U46619 were similar for both groups. 6-Keto-PGF1α production was reduced, whereas TxB2 production was increased in pulmonary arteries from hypoxic piglets. Abundances of both COX-1 and prostacyclin synthase were reduced, whereas abundances of both COX-2 and thromboxane synthase were unaltered in pulmonary arteries from hypoxic piglets. At least partly due to altered abundances of COX pathway enzymes, a shift in production of arachidonic acid metabolites, away from dilators toward constrictors, may contribute to the early phase of chronic hypoxia-induced pulmonary hypertension in newborn piglets.

cyclooxygenase-1; cyclooxygenase-2; prostacyclin; thromboxane

ARACHIDONIC ACID IS METABOLIZED by a number of pathways to a variety of vasoactive agents known to contribute to regulation of pulmonary vascular tone in lungs of both newborns and adults (4, 19). It follows that perturbations in one or more arachidonic acid metabolic pathways could disrupt regulation of pulmonary vascular tone and lead to the development of pulmonary hypertension. Along these lines, altered production of both vasodilator and vasoconstrictor arachidonic acid metabolites has been implicated in the pathogenesis of pulmonary hypertension in newborns and adults of a number of species (1, 2, 5–7, 13, 17, 18).

To date, almost all studies evaluating the role of arachidonic acid metabolites in pulmonary hypertension have been performed with whole lungs, with large conduit level pulmonary arteries, or by determination of serum or urine levels of arachidonic acid metabolites (1, 2, 5–7, 13, 17, 18). Because of their critical role in regulation of pulmonary vascular tone, elucidation of derangements in arachidonic acid pathways in resistance level pulmonary arteries are of particular importance. We previously showed that pulmonary hypertension develops when newborn pigs are exposed to chronic hypoxia for time periods of either 3 days (referred to as short hypoxia) or 10 days (referred to as long hypoxia) (10). The major purpose of this study was to test the hypothesis that a shift in the production of arachidonic acid metabolites from dilators to constrictors occurs in resistance-level pulmonary arteries of newborn piglets during in vivo exposure to hypoxia. We evaluated changes that occur with short exposure to hypoxia because of the potential significance for developing therapies to intervene with the onset and progression of pulmonary hypertension at an early stage. Moreover, we performed studies specifically evaluating the cyclooxygenase (COX) pathway of arachidonic acid metabolism based on our previous evidence suggesting that thromboxane, a vasoconstrictor COX metabolite, is involved with the pulmonary hypertension resultant from short hypoxia in newborn piglets (12).

METHODS

Animals. A total of 19 hypoxic piglets and a total of 22 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 (Therma-care). Oxygen content was regulated at 8–10% O2 (P02 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. We have 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 (11, 12). Therefore, for this study, many (n = 11) of the control piglets were studied on the day of arrival from the farm at 5–8 days of age.

Cannulated artery preparation. On the day of study, the piglets were preanesthetized with ketamine (30 mg/kg im) and then anesthetized with pentobarbital (10 mg/kg iv). All animals were given heparin (1,000 IU/kg iv) and were 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 Ca++, 0.72 Mg++, 1.7 H2PO4−, 25 HCO3−, and 11 glucose. Immediately before use, segments of 100–400 μm-diameter pulmonary arteries were dissected from a lung lobe.

The system used to study cannulated arteries has been described previously (12). Briefly, it consisted of a water-jacketed plastic chamber in which proximal (inflow) and distal (outflow) cannulas with equally matched tip diameters 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, 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) with a blood gas analyzer.

Inflow pressure was adjusted by changing the height of the infusion syringe. Pressure transducers were placed on both 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 exterior diameter of the artery was observed continuously with a video system containing a color camera (Panasonic 5000) and a 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 because they represent in vivo pressures (10, 11). We have previously shown no effect from these transmural pressures on pulmonary arterial responses to acetylcholine (ACh) (12). After establishment of basal tone, all arteries were tested for viability by contraction to U46619 (10−7 M). To check for a functional endothelium in control arteries, responses to ACh (10−6 M) were evaluated. We previously found that hypoxia-constricted ACh but dilated to another endothelium-dependent agent, the calcium ionophore A-23187 (12). Therefore, responses to A-23187 were used to check for a functional endothelium in hypoxic arteries.

To evaluate whether the production of arachidonic acid metabolites shifts from a predominance of dilators to contractors with short hypoxia, for one series of studies, we evaluated responses to arachidonic acid in control and hypoxic arteries. Some of these studies were performed with vessels at basal tone (n = 17 arteries from 11 control piglets and 19 arteries from 8 hypoxic piglets), while other studies were performed with vessels at elevated tone (n = 28 arteries from 12 control piglets and 23 arteries from 11 hypoxic piglets). For studies at basal tone, tests for viability and a functional endothelium were performed as described above, after which the vessels were washed with fresh PSS and allowed to return to basal tone. The vessel diameter was then continuously monitored while cumulative doses of arachidonic acid (10−8 to 10−5 M) were added to the reservoir. For studies at elevated tone, after checking for viability and a functional endothelium, endothelin was added to the reservoir in increasing doses until the arterial diameter had decreased by 40–50%, following which cumulative doses of arachidonic acid (10−8 to 10−5 M) were added. Next, in some of these studies, reproducibility of repeated dose responses to arachidonic acid was assessed. To do this, some of the hypoxic and some of the control vessels were washed with fresh PSS reconstricted with endothelin, and a second dose response to arachidonic acid was performed. For all the preceding studies, 15 min were allowed between each dose of arachidonic acid.

Because of the minimal change in diameter to arachidonic acid in vessels at basal tone, the following series of studies were performed in vessels with elevated tone.

In the first series of studies, the influence of the endothelium on arachidonic acid responses in control (n = 11 arteries from 7 piglets) and hypoxic (n = 11 arteries from 6 piglets) arteries was assessed. For these studies, air was infused into arteries from control and hypoxic piglets (14). Effective functional disruption of the endothelium was verified by loss of relaxation to ACh and/or A-23187 for the control arteries and by loss of relaxation to A-23187 for the hypoxic arteries. Reactivity to U46619 was used to confirm viability of the artery. Endothelin was then added to the reservoir in increasing doses until the arterial diameter had decreased by 40–50%, after which the diameter of control and hypoxic arteries was continuously monitored while cumulative doses of arachidonic acid (10−8 to 10−5 M) were added to the reservoir.

In another series of studies, the contribution of COX metabolites to arachidonic acid responses in both intact and endothelium-disrupted control arteries (for intact arteries n = 18 arteries from 10 control piglets; for endothelium-disrupted arteries n = 11 arteries from 7 control piglets) and hypoxic (for intact arteries n = 9 arteries from 7 hypoxic piglets; for endothelium-disrupted arteries n = 11 arteries from 6 hypoxic piglets) was determined. That is, 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 above. For all of these studies, responses to arachidonic acid (10−8 to 10−5 M) were measured in vessels with elevated tone both before and after adding the COX inhibitor indomethacin (10−5 M). After assessing for viability and for either a functional or disrupted endothelium as appropriate, vessel tone was elevated by adding endothelin to the reservoir in increasing doses until the arterial diameter had decreased by 40–50%, and changes in vessel diameter were then measured in response to cumulative doses of arachidonic acid (10−8 to 10−5 M).
10⁻⁵ M). Next, the vessels were washed with PSS, and the COX inhibitor indomethacin (10⁻⁵ M) was added to the reservoir. Twenty minutes after the indomethacin was added, vessel tone was again elevated by 40–50% with endothelin, and dose responses to arachidonic acid (10⁻⁸ to 10⁻⁵ M) were repeated.

A further series of studies was performed to determine whether differences in responses to arachidonic acid between arteries from control and hypoxic piglets involve altered sensitivity to the specific COX metabolites prostacyclin or thromboxane. Vessels were assessed for viability and a functional endothelium. They were then washed with PSS and allowed to return to basal diameter, after which changes in vessel diameter were measured in response to adding cumulative doses of either prostacyclin (PGI₂ sodium salt, 10⁻⁹ to 10⁻⁶ M; n = 9 arteries from 6 control piglets and n = 8 arteries from 5 hypoxic piglets) or the thromboxane mimetic U46619 (10⁻⁹ to 10⁻⁶ M; n = 17 arteries from 9 control piglets and n = 10 arteries from 5 hypoxic piglets) to the reservoir. In some additional vessels from both control (n = 10 arteries from 8 control piglets) and hypoxic piglets (n = 8 arteries from 5 hypoxic piglets), responses to prostacyclin (10⁻⁹ to 10⁻⁶ M) were assessed in vessels after elevating tone 40–50% with endothelin.

For all of the above studies, vessel viability was tested at the completion of the study by addition of U46619. In addition, in some studies, vessel responses to the vehicle used for solubilization of each agent were evaluated.

**Pulmonary artery preparation for radioimmunoassay and immunoblot analyses.** Control (n = 9) and hypoxic (n = 8) piglets were preanesthetized with ketamine (30 mg/kg im), anesthetized with pentobarbital (10 mg/kg iv), given heparin (1,000 I.U/kg iv), and then exsanguinated. Next, the lungs of the piglets were excised, and pulmonary arteries (20–600-μm diameter) were dissected. Some pulmonary arteries were frozen in liquid nitrogen and stored at −80°C for immunoblot analysis. Other pulmonary arteries were used for radioimmunoassay determinations (see below).

**Radioimmunoassay of TxB₂ and 6-keto-PGF₁α.** This series of studies was performed to assess whether a difference in production of either of the COX metabolites, thromboxane or prostacyclin, might contribute to altered responses to arachidonic acid in control and hypoxic arteries. For these studies, pulmonary arteries dissected from control (n = 4) and hypoxic (n = 4) piglets were weighed and then 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. Vessels were incubated in HEPES buffer containing arachidonic acid (10⁻⁵ M) for 15 min. After being incubated, the media were collected and stored at −20°C until the time of assay for specific metabolites. Synthesis of TxB₂ and of 6-keto-PGF₁α was measured by specific radioimmunoassays by use of the method of Campbell and Ojeda (3). The antibodies for TxB₂ and 6-keto-PGF₁α were kindly provided by Dr. William B. Campbell (Medical College of Wisconsin). Sensitivity of the assay is 1 pg/0.3 ml for TxB₂ and 5 pg/0.3 ml for 6-keto-PGF₁α. The cross-reactivity of the antisera with known arachidonic acid metabolites is <0.1%.

**Immunoblot analysis for COX-1, COX-2, prostacyclin synthase, and thromboxane synthase.** This series of studies assessed whether a change in abundance of either COX-1, COX-2, prostacyclin synthase, or thromboxane synthase might contribute to altered responses to arachidonic acid in control and hypoxic arteries.

For each protein, we performed preliminary studies with different amounts of total protein to determine the dynamic range of the immunoblot analysis. An amount of protein within the dynamic range of the immunoblot analysis for that protein was then used to compare protein abundance between homogenates of small pulmonary arteries from control and short hypoxic piglets as follows. Specifically, based on preliminary studies used to determine the dynamic range for immunoblot analysis for each protein, for determinations of COX-1, prostacyclin synthase, and thromboxane synthase, we used 15-μg protein samples. For COX-2 analysis, we used 40-μg protein samples.

Frozen samples of pulmonary arteries (20–600-μm diameter) from control (n = 5) and hypoxic (n = 4) piglets were homogenized in 10 mM HEPES buffer containing 250 mM sucrose, 3 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4, on ice using three 15-s pulses of a Polytron blender, taking care to avoid foaming of the homogenate. Protein concentration of the vessel homogenate was determined by the Bio-Rad protein assay. Each vessel homogenate was diluted with phosphate-buffered saline (PBS) to obtain a protein concentration of 1 mg/ml. Aliquots of the protein concentrations were solubilized in equal volume of denaturing, reducing sample buffer [Novex; 0.25 M Tris⋅HCl, 5% sucrose, 3 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 6.8, heated to 80°C for 15 min and centrifuged for 3 min at 5,800 g in a microfuge. Equal volumes of these supernatants were then applied to Tris-glycine precast 8% polyacrylamide gels (Novex) so that equal amounts of protein were loaded. Electrophoresis was carried out in 25 mM Tris, 192 mM glycine, and 0.1% SDS (pH 8.3) at 125 V for 1.7 h. The proteins were transferred from the gel to a nitrocellulose membrane (Novex) at 100 V for 1 h in 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). The membrane was incubated overnight at 4°C in PBS containing 10% nonfat dried milk and 0.1% Tween 20 to block nonspecific protein binding. To detect the protein of interest (COX-1, COX-2, thromboxane synthase, or prostacyclin synthase), the nitrocellulose membrane was incubated for 1 h at room temperature with the primary antibody diluted in PBS containing 0.1% Tween 20 and 1% nonfat dried milk (carrier buffer), followed by incubation for 30 min at room temperature with a biotinylated secondary antibody (Vector Elite, ABC kit, Vector Laboratories) diluted in the carrier buffer, followed by incubation for 30 min at room temperature with streptavidin-horseradish peroxidase conjugate (Amersham) diluted in PBS containing 0.1% Tween 20. The nitrocellulose membrane was washed three times between the first two incubations with the carrier buffer and three times with the carrier buffer plus one time with PBS containing 0.1% Tween 20 after the final incubation. To visualize the biotinylated antibody, the membranes were developed with enhanced chemiluminescence reagents (ECL, Amersham), and the chemiluminescent signal was captured on X-ray film (ECL Hyperfilm, Kodak). The bands for each protein were quantified using densitometry.

Similar procedures were followed using primary antibodies for COX-1 (Cayman), COX-2 (Oxford), prostacyclin synthase (Cayman), thromboxane synthase (from S. L. Pfister’s laboratory), and appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma or Zymed).

**Statistics.** Data are presented as means ± SE. To compare data between control and hypoxic animals, an unpaired t-test was used. To compare changes in vessel diameters between endothelium-intact and endothelium-disrupted vessels both with and without treatment with indomethacin, a one-way ANOVA with post hoc multiple comparison test was used. P < 0.05 was considered significant.

**Materials.** Concentrations for each drug listed in cannulated artery protocols were expressed as final molar concen-
trations in the vessel bath. ACh, A-23187, and indomethacin were obtained from Sigma Chemical. Arachidonic acid was from Nu-Chek Prep and was prepared in ethanol previously sparged with nitrogen. Prostaglandin E_2 sodium salt and 3H-6-ethanol. ACh was solubilized in saline. Indomethacin was solubilized in saline. 10% ethanol (vol/vol) with ethanol (10% vol/vol) or 8% NaHCO_3. 3H-6-ethanol. ACh was solubilized in saline. Indomethacin was solubilized in saline. Notably, arachidonic acid treatment for endothelium-disrupted control arteries diminished the responses measured before and after indomethacin treatment (Fig. 2B). After air infusion, both control (Fig. 2A, n = 11 arteries from 7 control piglets) and hypoxic (Fig. 2B, n = 11 arteries from 6 hypoxic piglets) arteries dilated to all doses of arachidonic acid, but the magnitude of dilation to arachidonic acid was less at the higher doses of arachidonic acid for air-infused than for endothelium-intact arteries of both types. The effect of indomethacin treatment was not the same on endothelium-disrupted arteries as on endothelium-intact arteries of both types. Specifically, in contrast to endothelium-intact control arteries (Fig. 2A), the magnitude of dilation to all doses of arachidonic acid was similar before and after indomethacin treatment for endothelium-disrupted control arteries (Fig. 2A). Moreover, different from endothelium-intact hypoxic arteries (Fig. 2B), the magnitude of dilation to the highest doses of arachidonic acid was augmented after indomethacin treatment for endothelium-disrupted hypoxic arteries (Fig. 2B).

Table 1. Change in vessel diameter to two consecutive dose responses to arachidonic acid in control and hypoxic vessels preconstricted with endothelin

<table>
<thead>
<tr>
<th>Group</th>
<th>10^{-8} M</th>
<th>10^{-7} M</th>
<th>10^{-6} M</th>
<th>10^{-5} M</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
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<tr>
<td>First dose response</td>
<td>10 ± 2</td>
<td>25 ± 4</td>
<td>50 ± 6</td>
<td>80 ± 8</td>
</tr>
<tr>
<td>Second dose response</td>
<td>20 ± 4</td>
<td>40 ± 6</td>
<td>60 ± 9</td>
<td>86 ± 9</td>
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<tr>
<td>Hypoxic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First dose response</td>
<td>14 ± 4</td>
<td>28 ± 9</td>
<td>42 ± 12</td>
<td>54 ± 14</td>
</tr>
<tr>
<td>Second dose response</td>
<td>20 ± 6</td>
<td>36 ± 10</td>
<td>47 ± 9</td>
<td>60 ± 9</td>
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All data are means ± SE. For control, n = 9; for hypoxic, n = 5.

RESULTS

For the cannulated artery studies, the mean diameter of vessels used for all studies was 230 ± 7 μm for control arteries and 249 ± 6 μm for hypoxic arteries. None of the vehicles significantly changed arterial diameter in the concentrations used for solubilization of any of the agents.

At basal tone (Fig. 1A), the diameter of control arteries (n = 17 arteries from 11 piglets) increased with arachidonic acid, whereas the diameter of arteries of hypoxic piglets (n = 19 arteries from 8 piglets) decreased with arachidonic acid. When tone was elevated with endothelin (Fig. 1B), arteries from both hypoxic (n = 23 arteries from 11 piglets) and control (n = 28 arteries from 12 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.

Table 1 summarizes the changes in pulmonary arterial diameter for those control and hypoxic vessels in which two sequential dose responses to arachidonic acid were performed. For both control and hypoxic arteries, the magnitude of the arachidonic acid-induced dilation at each dose was similar in both trials. Therefore, differences in responses to arachidonic acid measured before and after addition of indomethacin cannot be attributed to tachyphylaxis.

After indomethacin treatment, endothelium-intact arteries from both control (Fig. 2A, n = 18 arteries from 10 control piglets) and hypoxic (Fig. 2B, n = 9 arteries from 7 hypoxic) piglets dilated to arachidonic acid. Notably, indomethacin treatment diminished the magnitude of dilation to the highest doses of arachidonic acid in endothelium-intact control arteries (Fig. 2A). By comparison, for endothelium-intact hypoxic arteries, the magnitude of dilation to all doses of arachidonic acid after indomethacin treatment was similar to the responses measured before indomethacin treatment (Fig. 2B). After air infusion, both control (Fig. 2A, n = 11 arteries from 7 control piglets) and hypoxic (Fig. 2B, n = 11 arteries from 6 hypoxic piglets) arteries dilated to all doses of arachidonic acid, but the magnitude of dilation to arachidonic acid was less at the higher doses of arachidonic acid for air-infused than for endothelium-intact arteries of both types. The effect of indomethacin treatment was not the same on endothelium-disrupted arteries as on endothelium-intact arteries of both types. Specifically, in contrast to endothelium-intact control arteries (Fig. 2A), the magnitude of dilation to all doses of arachidonic acid was similar before and after indomethacin treatment for endothelium-disrupted control arteries (Fig. 2A). Moreover, different from endothelium-intact hypoxic arteries (Fig. 2B), the magnitude of dilation to the highest doses of arachidonic acid was augmented after indomethacin treatment for endothelium-disrupted hypoxic arteries (Fig. 2B).

![Graph A](image1.png)

**Fig. 1.** A: arachidonic acid-induced changes in diameter for control (n = 17 arteries from 11 piglets) and hypoxic (n = 19 arteries from 8 piglets) arteries at basal tone. All values are means ± SE, *different from control, P < 0.05. B: arachidonic acid-induced changes in diameter for control (n = 28 arteries from 12 piglets) and hypoxic (n = 23 arteries from 11 piglets) arteries with elevated tone. Data are expressed as % dilation of contraction elicited by endothelin. All values are means ± SE, *different from control, P < 0.05.
At both basal and elevated tone, dilation to all doses of prostacyclin was similar for control and hypoxic arteries (Fig. 3A, basal tone: \( n = 9 \) arteries from 6 piglets and hypoxic \( n = 8 \) arteries from 5 piglets) arteries at basal tone. All values are means ± SE. B: change in diameter to cumulative doses of prostacyclin for control and hypoxic arteries at elevated tone \( (n = 10 \) arteries from 8 control piglets; \( n = 14 \) arteries from 9 hypoxic piglets). Data are expressed as % dilation of contraction elicited by endothelin. All values are means ± SE.

Radioimmunoassay determinations of the media from incubated pulmonary arteries (20–600-μm diameter) showed that production of the stable metabolite of 6-keto-PGF\(_{1\alpha}\) was less \( (P = 0.04) \) for hypoxic \( (45 ± 6 \) pg/mg, 12 samples from 4 piglets) than for control \( (103 ± 27 \) pg/mg, 12 samples from 4 piglets) arteries, whereas production of TxB\(_2\), was greater \( (P = 0.03) \) for hypoxic \( (2.3 ± 0.5 \) pg/mg, 12 samples from 4 piglets).

Fig. 2. A: arachidonic acid-induced changes in diameter for endothelium-intact \( (n = 18 \) arteries from 10 piglets) and air-infused \( (n = 11 \) arteries from 7 piglets) control arteries before and after indomethacin. Data are expressed as % dilation of contraction elicited by endothelin. All values are means ± SE. *different between control intact and control air infusion, \( P < 0.05 \). **different between control intact and intact + indomethacin, \( P < 0.05 \). †different between control intact and air infusion + indomethacin, \( P < 0.05 \). B: arachidonic acid-induced changes in diameter for endothelium-intact \( (n = 9 \) arteries from 7 piglets) and air-infused \( (n = 11 \) arteries from 6 piglets) hypoxic arteries before and after indomethacin. Data are expressed as % dilation of contraction elicited by endothelin. All values are means ± SE. *different between hypoxic intact and hypoxic air infusion, \( P < 0.05 \). †different between hypoxic air infusion and air infusion + indomethacin, \( P < 0.05 \).

Fig. 3. A: change in diameter to cumulative doses of prostacyclin for control \( (n = 9 \) arteries from 6 piglets) and hypoxic \( (n = 8 \) arteries from 5 piglets) arteries at basal tone. All values are means ± SE. B: change in diameter to cumulative doses of prostacyclin for control and hypoxic arteries at elevated tone \( (n = 10 \) arteries from 8 control piglets; \( n = 14 \) arteries from 9 hypoxic piglets). Data are expressed as % dilation of contraction elicited by endothelin. All values are means ± SE.

Fig. 4. Change in diameter to cumulative doses of the thromboxane mimetic U46619 for control \( (n = 17 \) arteries from 9 piglets) and hypoxic \( (n = 10 \) arteries from 5 piglets) arteries. All values are means ± SE.
than for control (0.8 ± 0.2 pg/mg, 12 samples from 4 piglets) arteries. Therefore, the ratio of production of the stable metabolites of prostacyclin to thromboxane was decreased almost 10-fold in pulmonary arteries of hypoxic compared with control piglets (Fig. 5).

Immunoblot analyses for COX-1, COX-2, prostacyclin synthase, and thromboxane synthase in pulmonary homogenates from control and hypoxic piglets are shown in Fig. 6, A–D. As determined by densitometry (Table 2), the mean data for the absorbance of COX-1 bands and prostacyclin synthase bands were decreased for homogenates of pulmonary arteries from hypoxic compared with control piglets. By comparison, the mean data for the absorbance of COX-2 bands and thromboxane synthase bands did not differ for homogenates of pulmonary arteries of both groups.

**DISCUSSION**

An important new finding in this study is that dilation to arachidonic acid is diminished in 100–400-μm-diameter pulmonary arteries from newborn piglets exposed to short hypoxia. We previously showed that dilation to both a nitric oxide donor and the calcium ionophore A-23187 was preserved in 100–400-μm diameter pulmonary arteries from piglets with pulmonary hypertension resultant from short hypoxia (12). Thus impaired smooth muscle dilation does not explain the reduced ability to dilate to arachidonic acid exhibited by the pulmonary arteries from piglets exposed to short hypoxia. Instead, it is likely that after 3 days of hypoxia, the metabolites produced from arachidonic acid pathways by resistance level pulmonary arteries shift away from dilators toward constrictors. Although it was not feasible to measure all vasoactive products of the multiple arachidonic acid metabolic pathways that function in the pulmonary vasculature, we did measure pulmonary artery production of the COX dilator prostacyclin and the COX constrictor thromboxane. Supportive of the likelihood that the ratio of arachidonic acid metabolites shifts toward constrictors during exposure to short hypoxia, we found that pulmonary arteries from piglets exposed to short hypoxia produced less prostacyclin and more thromboxane than did pulmonary arteries from comparable age control piglets.

Our specific interest in the COX pathway of arachidonic acid metabolism is based, in part, on findings from our previous study showing that COX contracting factors, such as thromboxane, are involved with the altered responses to ACh that develop in piglets with hypoxia-induced pulmonary hypertension (12). In fact,
a number of findings in this study suggest a role for the COX pathway and the impaired responses to arachidonic acid exhibited by pulmonary arteries from piglets exposed to short hypoxia. For example, we found that COX inhibition reduced arachidonic acid-induced dilation in endothelium-intact pulmonary arteries from control piglets but had no effect on arachidonic acid responses in endothelium-intact arteries from hypoxic piglets. In other words, the disparate effect of COX inhibition on endothelium-intact arteries suggests that during short hypoxic exposure, the balance of COX-derived metabolites changes.

Our findings with air-infused arteries also provide evidence that COX metabolites contribute to altered arachidonic acid responses and that the balance of COX-derived metabolites changes with short hypoxia. In contrast to findings with endothelium-intact arteries noted above, arachidonic acid responses in air-infused arteries from control piglets were unaltered by COX inhibition, whereas arachidonic acid-induced dilation was augmented by COX inhibition in air-infused arteries from hypoxic piglets. Air infusion unmasks the contribution of arachidonic acid metabolites derived from the vascular wall. Therefore, the disparity in findings between air-infused arteries from control and hypoxic piglets raises the possibility that the vascular wall is one source of the change in balance of COX-derived archidonic acid metabolites that occurs with short hypoxia.

Our findings regarding the enzyme proteins underlying COX metabolite production provide some information regarding possible mechanisms for the altered responses to arachidonic acid and the changed balance of COX metabolites resultant from exposure to short hypoxia. The decreased abundance of both COX-1 and prostacyclin synthase found in pulmonary arteries of piglets raised in hypoxia could underlie decreased production of the COX-derived dilator prostacyclin. Moreover, our additional finding that COX-2 and thromboxane synthase were preserved, and not affected in the same fashion as COX-1 and prostacyclin, could underlie the relative increase in thromboxane production that occurs during short hypoxia. Regardless of any change in abundance of the upstream enzymes COX-1 and COX-2, a change in abundance of one downstream enzyme, prostacyclin synthase, but not the other, thromboxane synthase, could alter the ratio of prostacyclin and thromboxane production. This is because the enzymatic product of the COX isoforms PGH2 would be preferentially metabolized by the relatively more abundant downstream enzyme.

Although it has long been hypothesized that perturbations in arachidonic acid pathways could contribute to pulmonary hypertension, the influence from enzymes underlying the production of arachidonic acid metabolites has received little attention (5, 18). Consistent with our findings in piglets exposed to short hypoxia, prostacyclin synthase was decreased in the endothelium of pulmonary arteries of adult humans with pulmonary hypertension (18). By comparison, we previously found no change in amounts of prostacyclin synthase, thromboxane synthase, COX-1, or COX-2 in lung tissues, including pulmonary arteries, of piglets with high flow-induced pulmonary hypertension studied 5–6 wk after placement of aortopulmonary shunts (9). The difference between our findings in this study with piglets exposed to short hypoxia and those in our previous study with piglets after aortopulmonary shunts points out the likelihood that the mechanisms underlying pulmonary hypertension will differ with a multitude of factors, including duration of and postnatal age at exposure to the stimulus. In addition, species differences must be considered when evaluating mechanisms underlying pulmonary hypertension, since porcine pulmonary arteries may be more prone to production of constrictor prostaglandins than those of other species (15).

It is notable that in this study we found that COX-1 and COX-2 levels were affected differently by in vivo exposure to short hypoxia. A differential regulation of COX-1 and COX-2 by physiological stimuli, such as shear stress, has been previously described (16). It could be that the change in shear stress that occurs in the pulmonary circulation with in vivo hypoxia, and not decreased oxygen tension per se, underlies the differential effect on abundances of the two COX isoforms, and perhaps on the two downstream synthases, that we found in the resistance-level pulmonary arteries of newborn piglets.

Altered sensitivity to arachidonic acid metabolites could also contribute to altered responses to arachidonic acid. Our findings suggest that rather than changes in sensitivity to prostacyclin or thromboxane, altered production of these COX metabolites contributes to the impaired arachidonic acid responses that develop with short hypoxia.

Similar to ours, other studies have found evidence for altered production of COX metabolites and pulmonary hypertension. Findings from some studies are consistent with ours (2, 6), whereas findings from others are not (17). Maturation and species differences are possible sources for differences between findings. It also merits comment that the effect on COX metabolite production has been shown to vary with both the degree and duration of hypoxic exposure (8).

It is important to note that non-COX pathways of arachidonic acid metabolism might also be altered in pulmonary hypertension. Our findings that COX inhibition did not completely abolish arachidonic acid-induced responses in either control or hypoxic arteries suggest that other arachidonic acid pathways, such as hydroxyeicosatetraenoic acids (20), may contribute to regulation of pulmonary vascular tone in the newborn. Further studies will be required to evaluate the potential role of the non-COX pathways in the pathogenesis of neonatal chronic hypoxia-induced pulmonary hypertension.

It should also be considered that methodology might have influenced our results. For example, to assess dilation to arachidonic acid in vessels with elevated tone, we used concentrations of endothelin that decreased vessel diameter by 40–50%. It is possible that
endothelin concentrations differed between vessels from control and short hypoxic groups of piglets and that the use of higher endothelin concentrations might have interfered with the ability of one vessel group to dilate. However, our additional findings, i.e., differences in responses to arachidonic acid at basal tone combined with differences in COX pathway proteins and altered prostacyclin and thromboxane production, make it likely that dilatation of hypoxic vessels is impaired and involves arachidonic acid pathways.

To summarize, our findings indicate that a shift in production of arachidonic acid metabolites, away from dilators toward constrictors, occurs during exposure to short hypoxia. In particular, our findings suggest that decreased production of COX-dependent dilators in combination with increased production of COX-dependent constrictors alters the balance of vasoactive arachidonic acid metabolites produced by resistance-level pulmonary arteries. Because these changes occur at an early stage of pulmonary hypertension, future studies should be designed to evaluate whether early manipulation of arachidonic acid pathways will ameliorate or prevent the development and progression of neonatal pulmonary hypertension associated with chronic hypoxia.

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