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Pulmonary Arterial Responses to Reactive Oxygen Species Are Altered in Newborn Piglets With Chronic Hypoxia-Induced Pulmonary Hypertension

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ABSTRACT: Reactive oxygen species (ROS) have been implicated in the pathogenesis of pulmonary hypertension. ROS might mediate vascular responses, at least in part, by stimulating prostanoid production. Our goals were to determine whether the effect of ROS on vascular tone is altered in resistance pulmonary arteries (PRAs) of newborn piglets with chronic hypoxia-induced pulmonary hypertension and the role, if any, of prostanoids in ROS-mediated responses. In cannulated, pressurized PRA, ROS generated by xanthine (X) plus xanthine oxidase (XO) had minimal effect on vascular tone in control piglets but caused significant vasoconstriction in hypoxic piglets. Both cyclooxygenase inhibition with indomethacin and thromboxane synthase inhibition with dazoxiben significantly blunted constriction to X+XO in hypoxic PRA. X+XO increased prostacyclin production (70 ± 8%) by a greater degree than thromboxane production (50 ± 6%) in control PRA; this was not the case in hypoxic PRA where the increases in prostacyclin and thromboxane production were not statistically different (78 ± 13% *versus* 216 ± 93%, respectively). Thromboxane synthase expression was increased in PRA from hypoxic piglets, whereas prostacyclin synthase expression was similar in PRA from hypoxic and control piglets. Under conditions of chronic hypoxia, altered vascular responses to ROS may contribute to pulmonary hypertension by a mechanism that involves the prostanoid vasoconstrictor, thromboxane. **(*Pediatr Res* 70: 136–141, 2011)**

Reactive oxygen species (ROS) have been implicated in the pathogenesis of a number of vascular diseases (1–6). We and others have provided evidence that ROS are involved in the development of pulmonary hypertension in both neonates and adults (7–14). However, whether ROS mediate different responses in the hypertensive *versus* normotensive pulmonary circulation is unknown. Aberrant responses to ROS could underlie abnormal pulmonary vascular tone and reactivity and thereby contribute to the progressive development of chronic pulmonary hypertension. Furthermore, the mechanisms by which ROS alter pulmonary vascular tone are not yet clear. This knowledge could have important clinical implications. Sudden elevations in ROS occur with sepsis (15,16) and abrupt increases in oxygen administration (17) in critically ill patients.

The purpose of this study was to evaluate whether responses to ROS were altered in resistance level pulmonary arteries (PRAs) of piglets with chronic hypoxia-induced pulmonary hypertension. ROS can stimulate release of arachidonic acid (18,19), the substrate for production of the prostanoid metabolites, including the potent dilator, prostacyclin (PGI_2), and the potent constrictor, thromboxane (TXA_2). Hence, an additional goal was to determine whether prostanoids are involved in the responses of normoxic, normotensive and hypoxic, hypertensive PRA to ROS.

METHODS

Animals: Chronic hypoxia model of pulmonary hypertension. Newborn pigs (2 d old) were placed in a hypoxic normobaric environment for 10 d. Oxygen content was regulated at 10–12% O_2 (inspired PO_2 64–78 Torr). CO_2 was absorbed with soda lime and inspired PCO_2 was maintained at 3–6 Torr. Healthy, normoxic piglets were used as controls on the day of arrival from the vendor, at postnatal age of 12 d, *i.e.* comparable postnatal age to the hypoxic piglets on the day of study. We have previously found no differences in vascular responses between piglets raised in a room-air environment and piglets raised by the vendor (20,21). At the time of study, all piglets were preanesthetized with ketamine (30 mg/kg i.m.) and acepromazine (2 mg/kg i.m.) and then anesthetized with pentobarbital (10 mg/kg i.v.). All animals were given heparin (1000 IU/kg i.v.) and then exsanguinated. The thorax was opened, and the lungs were removed and placed in cold (4°C) Krebs until use. All experimental protocols were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals and approved by the Animal Care and Use Committee of Vanderbilt University Medical Center. This animal resource facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Use.

Cannulated artery preparation. Using our previously published methods (9,22), piglet resistance level PRAs (80–200 μm diameter) were isolated, cannulated, and pressurized for continuous measurement of diameter.

Cannulated artery protocols. Each artery was equilibrated for 30 min to establish basal tone. Control and hypoxic arteries were equilibrated at transmural pressures that represent *in vivo* pressures (20,21): the control arteries at a transmural pressure of 15 cm H_2O and the hypoxic arteries at a transmural pressure of 25 cm H_2O. We have previously shown no effect between these transmural pressures on pulmonary arterial responses to acetylcholine (ACh) (23). After establishment of basal tone, all arteries were tested for viability by contraction to the thromboxane mimetic, U46619 (10^{-8} M). To check for a functional endothelium in control arteries, responses to ACh (10^{-6} M) were

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Abbreviations: ACh, acetylcholine; **PGI_2,** prostacyclin; **PGIS,** prostacyclin synthase; **PRAs,** resistance pulmonary arteries; **ROS,** reactive oxygen species; **TXA_2,** thromboxane; **TXB_2,** stable metabolite of thromboxane; **TXAS,** thromboxane synthase; **X+XO,** xanthine plus xanthine oxidase

evaluated. Responses to A23187 were used to check for a functional endothelium in hypoxic arteries as we previously found that hypoxic arteries constricted to ACh but dilated to the calcium ionophore, A23187 (23).

To evaluate responses to ROS, xanthine (X, 10^{-4} M) was added to the reservoir, after which changes in vessel diameter to cumulative doses of xanthine oxidase (XO, 0.0001–0.005 IU/mL) were measured. Changes in vessel diameter to cumulative doses of H_2O_2 (10^{-6}–10^{-3} M) were measured in other vessels. To evaluate the influence of prostanoid production on ROS responses, the cyclooxygenases inhibitor, indomethacin (10^{-5} M), was added to the reservoir 20 min before measuring changes in vessel diameter to H_2O_2 or X+XO. To evaluate the influence of thromboxane production, similar studies were performed after adding the thromboxane synthase inhibitor, dazoxiben (10^{-5} M).

For all of the above studies, vessel responses to the vehicle used for solubilization of the ROS, indomethacin, or dazoxiben were evaluated.

RIA of the stable metabolite of TXA_2, TXB_2, and enzyme immunoassay of the stable metabolite of PGI_2, 6-keto-$PGF1_\alpha$. These studies were performed to assess whether ROS stimulated production of either PGI_2 and/or TXA_2 differs between PRAs of hypoxic piglets and PRAs of comparable age control piglets. Dissected PRAs (≤300 μm diameter) were first incubated for 15 min at 37°C in HEPES buffer. The supernatant was discarded and replaced with new HEPES buffer containing vehicle, H_2O_2 (10^{-3} M), or xanthine (10^{-4} M) plus xanthine oxidase (0.005 IU/mL). The vessels were incubated for a second 15-min time period at 37°C. After the second incubation period, the supernatant was collected and stored at −20°C until the time of assay. The vessels were dried for at least 72 h. TXB_2 synthesis was measured by the method of Campbell and Ojeda (24). The antibody for TXB_2 was from Dr. Pfister's laboratory. Sensitivity of the assay is 1 pg/0.3 mL for TXB_2. 6-keto-$PGF1_\alpha$ synthesis was measured by enzyme immunoassay using kits (Cayman Chemical Co, Ann Arbor, MI). Sensitivity of the assay is 11 pg/mL. RIA determinations of TXB_2 and enzyme immunoassay determinations of 6-keto-$PGF1_\alpha$ were normalized to vessel dry weight.

Immunoblot analysis of PGI_2 synthase (PGIS) and TXA_2 synthase (TXAS). These studies were performed to determine whether differences in PGI_2 and TXA_2 production involve altered expression of their synthases, PGIS and TXAS, respectively. Pulmonary arteries (≤300 μm diameter) were dissected from lungs of control and hypoxic piglets, frozen in liquid nitrogen and stored at −80°C until use for immunoblot analysis.

We performed preliminary studies to determine the dynamic range of the immunoblot analysis. Accordingly, 10 μg protein for PGIS and 30 μg protein for TXAS were used to compare protein abundances between pulmonary artery homogenates from control and hypoxic piglets.

Frozen samples of small pulmonary arteries from both groups of piglets were used for immunoblot analysis of PGIS (antibody from Caymen Chemical Co.) and TXAS (antibody from Dr. Pfister's laboratory) using our previously published methods (9,22). The membranes were developed using enhanced chemiluminescence reagents and captured on X-Ray film. Similar procedures were followed to reprobe the membranes for β-actin (Sigma Chemical Co.-Aldrich, St. Louis, MO). The bands for each protein were quantified using densitometry.

Drugs. H_2O_2, xanthine, xanthine oxidase, and indomethacin were from Sigma Chemical Co.-Aldrich. Dazoxiben was a generous gift from Pfizer (Groton, CT). Concentrations for each drug listed in cannulated artery protocols and vessel incubations for TXB_2 and 6-keto-$PGF1_\alpha$ determinations are final concentrations in the vessel bath or incubation solutions.

Statistics. Data are presented as mean ± SEM. For cannulated artery studies, the % change from baseline diameter in response to each dose of H_2O_2 or X+XO was compared between control and hypoxic groups or between untreated and treated vessels using linear mixed effects models. Concentration of H_2O_2 and X+XO were included as unordered categorical variables. We included a random intercept in the regression model to account for taking repeated observations on the same vessel. All models were fit using the R statistics program. For the RIA and ELISA assay, a Wilcoxon signed-rank test was used to compare basal- and ROS-stimulated values for both groups. In addition, the % change between basal- and ROS-stimulated values of prostacyclin and thromboxane were calculated and compared using a Wilcoxon signed-rank test. For Western blot analysis, a Mann-Whitney *U* test was used to compare densitometry values for PGIS and TXAS between control and hypoxic piglets.

RESULTS

As shown in Figure 1, responses to X+XO differed between PRAs from hypoxic and comparable age normoxic piglets. Arteries from normoxic piglets had minimal responses to X+XO, whereas arteries from hypoxic piglets constricted

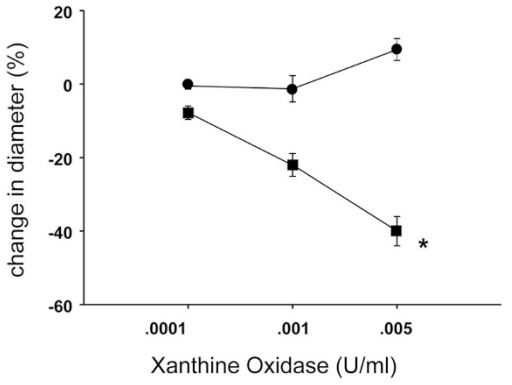

Figure 1. Responses to X+XO in PRA from piglets raised in normoxia (n = 26 arteries, ●) and hypoxia (n = 21 arteries, ■). All values are mean ± SEM *$p < 0.05$ by the linear mixed effects model.

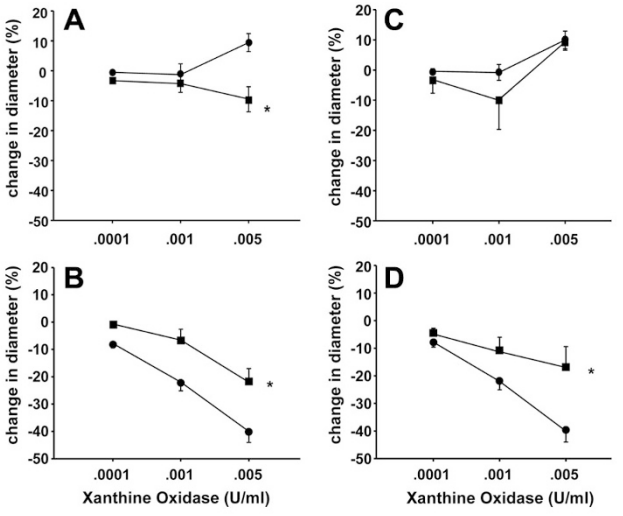

Figure 2. X+XO-induced responses in untreated and indomethacin-treated PRA from piglets raised in (*A*) normoxia (n = 26 untreated arteries, ●; n = 10 treated arteries, ■) and (*B*) hypoxia (n = 21 untreated arteries, ●; n = 8 treated arteries, ■), and X+XO-induced responses in untreated and dazoxiben-treated PRA from piglets raised in (*C*) normoxia (n = 26 untreated arteries, ●, solid line; n = 5 treated arteries, ■) and (*D*) hypoxia (n = 26 untreated arteries, ●; n = 6 treated arteries, ■). All values are mean ± SEM *$p < 0.05$ by the linear mixed effects model.

to X+XO. Both indomethacin treatment (Fig. 2*B*) and dazoxiben treatment (Fig. 2*D*) markedly reduced the degree of PRA constriction to X+XO in PRAs from hypoxic piglets. In normoxic piglets, treatment with indomethacin (Fig. 2*A*) or dazoxiben (Fig. 2*C*) had minimal to no impact on PRA responses to X+XO.

X+XO increased both 6-keto-$PGF1_\alpha$ and TXB_2 in PRA from normoxic and hypoxic piglets (Table 1). In PRA from normoxic piglets stimulated with X+XO, the relative increase in 6-keto-$PGF1_\alpha$ was greater than that of TXB_2 (Fig. 3*A*). This was not the case in PRA from hypoxic piglets (Fig. 3*B*). This differential impact of X+XO on PGI_2 and TXA_2 production in normoxic and hypoxic PRA could contribute to the altered vascular responses observed (Fig. 1).

H_2O_2 is known to be one of the ROS generated by X+XO (25,26). We therefore evaluated whether responses to H_2O_2

Table 1. *Basal- and X+XO-stimulated 6-keto-PGFI$_\alpha$ and TXB$_2$ production in PRAs for normoxic and hypoxic piglets*

	10-d normoxic (n = 14)	10-d hypoxic (n = 12)
6-keto-PGFI$_\alpha$ (pg/mg)		
Basal	38,774 ± 8,000	14,667 ± 1,116
X+XO	61,250* ± 1,115	25,810* ± 2,991
TXB$_2$ (pg/mg)		
Basal	13.7 ± 1.3	11.5 ± 2.4
X+XO	19.3* ± 2.4	21.2* ± 3.4

*Different from basal by Wilcoxon signed-rank test, $p < 0.05$.

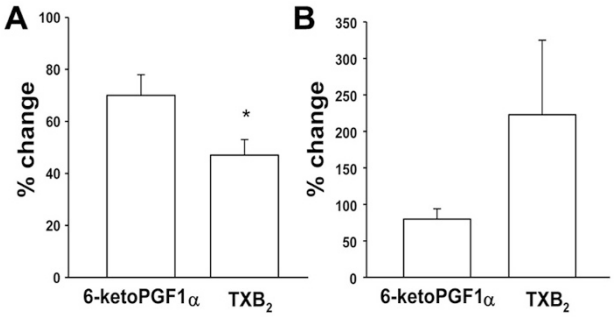

Figure 3. % change from basal values of 6-keto-PGF1$_\alpha$ and TXB$_2$ with X+XO stimulation in arteries from piglets raised in (*A*) normoxia ($n = 14$ arteries) and (*B*) hypoxia ($n = 12$ arteries). All values are mean ± SEM *$p <$ 0.05; Wilcoxon signed-rank test.

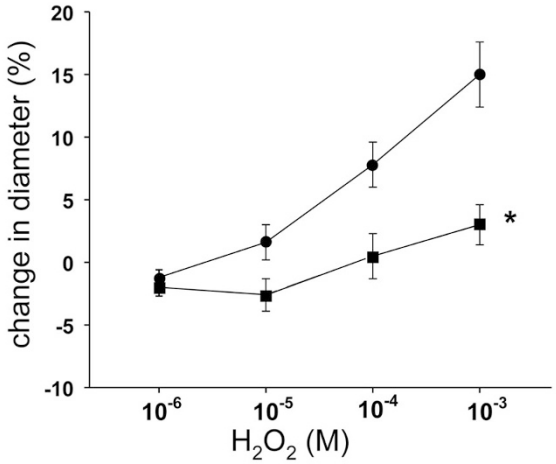

Figure 4. Responses to H_2O_2 in PRA from piglets raised in normoxia ($n = 31$ arteries, ●) and hypoxia ($n = 21$ arteries, ■). All values are mean ± SEM *$p < 0.05$ by the linear mixed effects model.

differed between PRAs from hypoxic and normoxic piglets. In response to all concentrations of H_2O_2, PRAs from both groups exhibited a brief, initial constriction (data not shown) with return to near baseline within 30 s. After return to near baseline, the higher concentrations of H_2O_2 elicited a dilator response in PRAs from both groups (Fig. 4). Dilation to H_2O_2 was greater in PRAs from normoxic than hypoxic piglets (Fig. 4). Neither indomethacin treatment (Fig. 5*A* and *B*) nor dazoxiben treatment (Fig. 5*C* and *D*) had a significant effect on responses to H_2O_2 in PRAs from normoxic or hypoxic piglets.

H_2O_2 increased 6-keto-PGF1$_\alpha$ in PRAs from normoxic piglets but had no significant effect on 6-keto-PGF1$_\alpha$ in PRAs

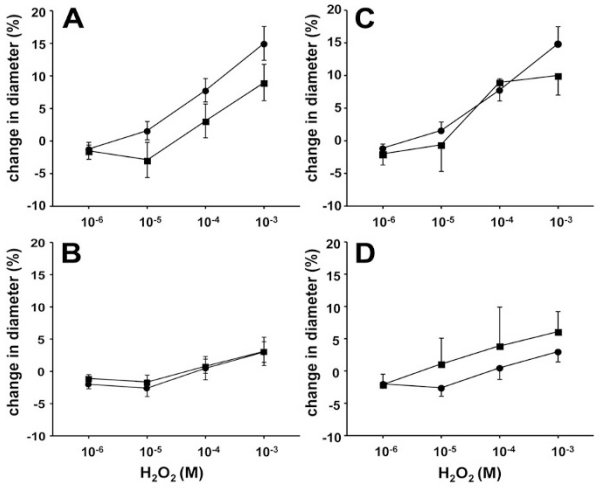

Figure 5. H_2O_2-induced responses in untreated and indomethacin-treated PRA from piglets raised in (*A*) normoxia ($n = 31$ untreated arteries, ●; $n = 18$ treated arteries, ■) and (*B*) hypoxia ($n = 21$ untreated arteries, ●; $n = 9$ treated arteries, ■) and H_2O_2-induced responses in untreated and dazoxiben-treated PRA from piglets raised in (*C*) normoxia ($n = 30$ untreated arteries, ●; $n = 6$ treated arteries, ■) and (*D*) hypoxia ($n = 21$ untreated arteries, ●; $n = 5$ treated arteries, ■). All values are mean ± SEM. No statistical differences between groups by the linear mixed effects model.

Table 2. *Basal- and H_2O_2-stimulated 6-keto-PGFI$_\alpha$ and TXB$_2$ production in PRAs for normoxic and hypoxic piglets*

	10-d normoxic (n = 14)	10-d hypoxic (n = 14)
6-keto-PGFI$_\alpha$ (pg/mg)		
Basal	15,135 ± 1,906	16,305 ± 2,514
H_2O_2 (10^{-3} M)	22,783* ± 1,916	15,762 ± 1,559
TXB$_2$ (pg/mg)		
Basal	15.9 ± 1.5	18.0 ± 2.0
H_2O_2 (10^{-3} M)	17.7 ± 1.1	26.4* ± 3.0

*Different from basal by Wilcoxon signed-rank test, $p < 0.05$.

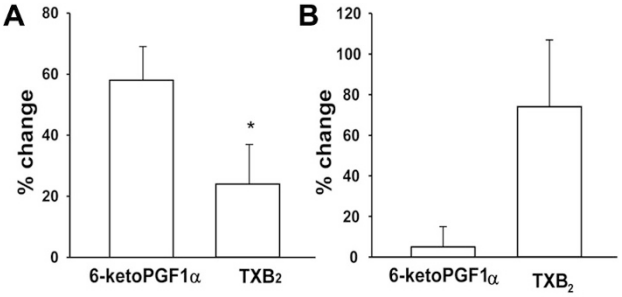

Figure 6. % change from basal values of 6-keto-PGF1$_\alpha$ and TXB$_2$ with H_2O_2 stimulation in arteries from piglets raised in (*A*) normoxia ($n = 14$ arteries) and (*B*) hypoxia ($n = 14$ arteries). All values are mean ± SEM *$p <$ 0.05; Wilcoxon signed-rank test.

from hypoxic piglets (Table 2). In contrast, H_2O_2 significantly increased TXB$_2$ from PRAs only in the hypoxic group (Table 2). Therefore, the relative magnitude of change in PGI$_2$ was greater than that of TXB$_2$ in normoxic PRA (Fig. 6*A*) but not in hypoxic PRA (Fig. 6*B*). The absence of an H_2O_2 effect on PGI$_2$ in hypoxic piglets (Table 2, Fig. 6*B*) might unmask a greater functional effect from the increase in TXA$_2$ (Fig. 6*B*)

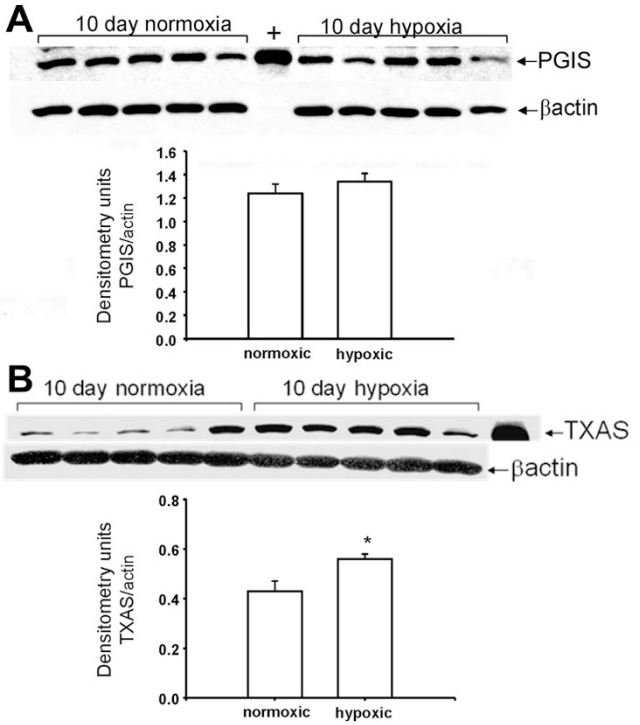

Figure 7. Immunoblot results and corresponding densitometry for (*A*) PGIS and (*B*) TXAS in PRA homogenates from piglets raised in normoxia (control, $n = 5$) and hypoxia ($n = 5$). All values are means ± SEM $*p < 0.05$, Mann-Whitney *U* test.

and thereby contribute to the difference in responses to H_2O_2 noted between normoxic and hypoxic piglets (Fig. 4).

As shown in Figure 7, immunoblot analyses demonstrate that TXAS expression was increased (Fig. 7*B*) while PGIS expression was unchanged (Fig. 7*A*) in PRA homogenates from piglets exposed to 10 d of hypoxia.

DISCUSSION

One of the major findings in this study is that responses to ROS differ between PRAs from normoxic, normotensive piglets and piglets with chronic hypoxia-induced pulmonary hypertension. We also provide evidence that the prostanoid constrictor, thromboxane, is involved in the disparate functional responses to ROS found between normoxic and hypoxic vessels. These data provide an important, new mechanistic link between ROS and the prostanoid pathway in mediating aberrant pulmonary vascular responses in piglets with chronic hypoxia-induced pulmonary hypertension.

The ability of ROS to modify vascular tone has been a topic of great interest for the past several decades (10,27–39). Yet, the effect of ROS on vascular tone from animals with either systemic (40–44) or pulmonary hypertension has not been well studied (10). Others have found that responses to ROS differ between normotensive and hypertensive beds of the systemic circulation (40–45). Ours is the first report of a differential impact of ROS on pulmonary vascular responses in normoxic, normotensive animals and those with chronic hypoxia-induced pulmonary hypertension.

Like us, other investigators have been interested in evaluating a role for prostanoids in mediating vascular responses to ROS (30–32,37,39–50). Supportive of this possibility, prostanoid inhibitors have been shown to alter responses to ROS in both the systemic (31,32,39–49) and pulmonary circulations (30,37,50). Our findings with the cyclooxygenases inhibitor, indomethacin, clearly implicate a role for prostanoids in mediating PRA responses to X+XO in the neonate, particularly in the hypertensive pulmonary circulation (Fig. 2). Moreover, findings with the thromboxane synthase inhibitor, dazoxiben, point to a contribution from thromboxane in mediating the constrictor response to X+XO in hypoxic arteries (Fig. 2). However, the failure of indomethacin and dazoxiben treatments to completely abolish constriction to X+XO in hypoxic vessels implicates additional constrictor(s). Furthermore, our data suggest that pulmonary arterial responses to H_2O_2 largely depend on other vasoactive metabolites as neither indomethacin nor dazoxiben significantly altered vascular responses. Other investigators have also provided evidence that nonprostanoid metabolites are involved with H_2O_2 responses in the pulmonary circulation (28,51). These vasoactive metabolites could include other arachidonic acid metabolites, including those of nonenzymatic, *i.e.* isoprostanes (52), and enzymatic, *i.e.* leukotriene (28), pathways.

There are limited reports of ROS-stimulated prostanoid production in the pulmonary (37,48,53) and systemic circulations (42,43,45,46,49). It has been reported that H_2O_2-stimulated TXB_2 production is increased in mesenteric arteries from rats with systemic hypertension (42,45). All other reports to date have been limited to measurements of ROS-stimulated prostanoid production in the circulatory bed from either normotensive (37,46,48,49,53) or hypertensive (43) animals but not from both. To our knowledge, we are the first to show that the effect of ROS on prostanoid production differs between the normotensive and hypertensive pulmonary vascular bed.

Our finding that hypoxia increases TXAS expression provides a mechanism for the differential effects of ROS on prostanoid production in pulmonary vessels from piglets exposed to normoxia *versus* hypoxia for 10 d. ROS stimulate the release of arachidonic acid that is metabolized to PGH_2 by cyclooxygenases. PGH_2 is subsequently metabolized to a number of prostanoids, including PGI_2 and TXA_2, in accordance with the presence and abundance of their synthases. In the case of 10 d exposure to hypoxia, the increase in TXAS expression would lead to preferential metabolism of PGH_2 to TXA_2. Thus, the net effect of hypoxia on expression of PGIS and TXAS would result in a change in ROS-stimulated production of these prostanoids favoring the constrictor, TXA_2.

Other mechanisms by which ROS mediate vascular tone could also contribute to the different functional effects of ROS on hypoxic and normoxic vessels. For example, it has been shown that the same concentration of H_2O_2 can act as a relaxing factor or as a vasoconstrictor depending on whether hyperpolarization is compromised or not (34,35). We previously found that PRA smooth muscle cells from hypoxic piglets are depolarized (54). Therefore, hypoxia-related differences in the resting membrane potential of smooth muscle

cells could contribute to the differential responses to ROS observed in this study. H_2O_2 has also been found to directly relax smooth muscle by hyperpolarization through K_{Ca} channel activation (49). Hence, differences between K_{Ca} channel expression between normoxic and hypoxic PRAs could also contribute to differences in responses to H_2O_2. This possibility remains to be explored.

There are limitations of our study. One is that our findings were generated with exogenous ROS. Precise levels of endogenous ROS in normoxic and hypoxic vessels are not known. Indeed, there continues to be great controversy as to whether and which ROS levels are altered by hypoxia (34,55). In addition, we are not certain which of the ROS generated by X+XO, which include superoxide, H_2O_2, and hydroxyl radicals, underlie the different responses to X+XO in PRAs from normoxic and hypoxic piglets. Nonetheless, we have previously provided evidence suggesting that endogenous ROS mediate vasodilation in the PRAs from normoxic piglets, whereas endogenous ROS seem to mediate vasoconstriction in PRAs from hypoxic piglets (8,9). Findings in this study add to these previous findings and provide further support to the notion that ROS elicit aberrant responses in hypoxic vessels.

In conclusion, our study demonstrates differential functional effects of ROS on the normoxic and hypoxic pulmonary circulation. Although the mechanisms mediating ROS responses differ with the specific ROS studied, we provide evidence of a role for thromboxane in the altered vascular responses to ROS generated by X+XO in PRA from hypoxic piglets. These findings may have potential therapeutic implications in the NICU. Although precise ROS levels are not known, local concentrations of ROS are certain to be increased in the presence of activated inflammatory cells, such as neutrophils, reaching millimolar concentrations (4,15,56,57). Thus, our findings may be particularly applicable to situations of increased inflammatory stress where the aberrant pulmonary vascular response to ROS which are mediated, at least in part, by prostanoid production, could help explain the poor ability of infants with chronic pulmonary hypertension to tolerate acute episodes of infection. Taken together, our findings continue to support the pursuit of both prostanoid and ROS signaling as important therapeutic targets for the treatment of chronic hypoxia-induced pulmonary hypertension in newborns.

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