Thromboxane inhibition reduces an early stage of chronic hypoxia-induced pulmonary hypertension in piglets

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Thromboxane inhibition reduces an early stage of chronic hypoxia-induced pulmonary hypertension in piglets. J Appl Physiol 99: 670–676, 2005. First published March 31, 2005; doi:10.1152/japplphysiol.01337.2004.—The pulmonary vasoconstrictor, thromboxane, may contribute to the development of pulmonary hypertension. Our objective was to determine whether a combined thromboxane synthase inhibitor-receptor antagonist, terbogrel, prevents pulmonary hypertension and the development of aberrant pulmonary arterial responses in newborn piglets exposed to 3 days of hypoxia. Piglets were maintained in room air (control) or 11% O2 (hypoxic) for 3 days. Some hypoxic piglets received terbogrel (10 mg/kg po bid). Pulmonary arterial pressure, pulmonary wedge pressure, and cardiac output were measured in anesthetized animals. A cannulated artery technique was used to measure responses to acetylcholine. Pulmonary vascular resistance for terbogrel-treated hypoxic piglets was almost one-half the value of untreated hypoxic piglets but remained greater than values for control piglets. Dilation to acetylcholine in preconstricted pulmonary arteries was greater for terbogrel-treated hypoxic than for untreated hypoxic piglets, but it was less for pulmonary arteries from both groups of hypoxic piglets than for control piglets. Terbogrel may ameliorate pulmonary artery dysfunction and attenuate the development of chronic hypoxia-induced pulmonary hypertension in newborns.

METHODS

Animals. A total of 14 hypoxic piglets and a total of 6 control piglets were studied. We have previously found no differences in pulmonary vascular resistance or pulmonary vascular responses between piglets raised in a room-air environment and piglets raised on a farm (17). Therefore, for this study, all of the control piglets were studied on the day of arrival from the farm at 6–7 days of age. For the hypoxic piglets, newborn pigs were placed in a hypoxic normobaric chamber on the evening of the day of arrival from the farm at 3 days of age. They were maintained in the hypoxic chamber for 3–3.5 days so that they were studied at age 6–7 days. Normobaric hypoxia was produced by delivering compressed air and N2 to an incubator (Therbecare). O2 content was regulated at 10–11% O2 (Po2 66–74 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. Some of the hypoxic piglets (n = 7) received terbogrel (10 mg/kg po bid). The first dose of terbogrel was given immediately before the piglet was placed into the hypoxic chamber.

The choice of terbogrel dosing was based on pharmokinetic studies in rats showing that 10 mg/kg po doses of terbogrel were rapidly and well (90%) absorbed with a systemic availability of ~30%, and terminal half-life in the range of 10 h (42).

Measurements in anesthetized animals. On the day of study, all piglets were weighed and anesthetized with ketamine (30 mg/kg im) and pentobarbital sodium (10 mg/kg/ iv). Additional intravenous pentobarbital sodium was given as needed via an ear vein to maintain anesthesia during placement of the catheters. First, the trachea of the piglet was cannulated so that the animal could be ventilated if necessary. Then, a catheter was placed into the aortic arch via the left femoral artery, and a catheter that served as an injection port was placed into the left ventricle via the left carotid artery. Cardiac output was measured at
end expiration as the mean of three injections of 3 ml of 0.9% saline (0°C). Next, another catheter was placed through the right external jugular vein into the pulmonary artery to monitor pulmonary arterial pressure. To obtain pulmonary wedge pressure, the pulmonary arterial catheter was advanced into a distal pulmonary vessel. In some animals, wedge pressure was unable to be obtained, and left ventricular end-diastolic pressure was then measured. We have previously found no difference between measurements of pulmonary wedge pressure and left ventricular end-diastolic pressure in anesthetized piglets (17). The zero reference for the vascular catheters was the midthorax. After blood gases were measured, all animals were given heparin (1,000 IU/kg iv) and additional anesthesia (3–5 mg/kg of pentobarbital sodium 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 for either cannulated artery studies or for incubation for TXA2 and prostacyclin (PGI2) determinations. 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.

Cannulated artery studies. Immediately before use, segments of 100- to 300-μm-diameter pulmonary arteries were dissected from a lung lobe. The system used to study cannulated arteries has been described in detail previously (20, 35). 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, tied in place, and filled with PSS. The distal end of the artery was then tied onto the distal cannula. 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 containing a gas mixture as the reservoir, and connected to the cannula with polyethylene tubing.

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, 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 (Hitachi VCC-151) and television monitor. Vessel diameters were measured with a videoscaler (FORA IV).

Each artery was allowed to equilibrate for 40–60 min to establish basal tone. The arteries were equilibrated at transmural pressures similar to those in vivo (17): 15 cmH2O for control arteries and 25 basal tone. The arteries were equilibrated at transmural pressures measured with a videoscaler (FORA IV) and television monitor. Vessel diameters were verified by assessing dilatory responses to the calcium ionophore, A-23187 (20). The arteries were then washed with fresh PSS and allowed to return to their precontracted diameter, i.e., allowed to reestablish basal tone.

In one series of studies, the diameter of arteries from control, untreated hypoxic, and terbogrel-treated hypoxic piglets were continuously monitored while cumulative doses of ACh (10−8 to 10−5 M) were added to the reservoir at 10-min intervals. In another series of studies, the influence of elevated tone on ACh responses in arteries from each group of piglets was assessed. Tests for viability and a functional endothelium were done as described above, and then endothelin was added in increasing doses until the arterial diameter had decreased by 40–50%. The dose of endothelin needed to accomplish this tended to be greater (3–5 × 10−9 M endothelin) for both groups of hypoxic piglets than for the control piglets (1 × 10−9 M endothelin). After equilibration at the elevated tone, the dose-response curves to ACh were performed. After each study, vessel viability was retested using U-46619.

TXA2 and PGI2 determinations. Pulmonary arteries (20- to 600-μm diameter) were dissected from control, untreated hypoxic, and terbogrel-treated hypoxic lungs, and then they were 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. After incubation of the vessels in HEPES for 15 min, the media were collected and stored at −20°C until the time of assay for specific metabolites. The vessels were weighed and then dried to constant weight at 60°C for at least 72 h. Synthesis of the stable metabolites of TXA2 and PGI2, TXB2 and 6-keto-prostaglandin F1α, (6-keto-PGF1α), respectively, were measured by enzyme immunoassay following standard methods and using kits from Cayman laboratories. Enzyme immunoassay determinations of TXB2 and 6-keto-PGF1α were normalized to vessel dry weight.

Materials. Concentrations for each drug listed in the preceding paragraphs were expressed as final molar concentrations in the vessel bath. ACh was obtained from Sigma Chemical and was solubilized in saline. A-23187 was from Biomol and was solubilized in DMSO. Endothelin, from Calbiochem, and U-46619, from Cayman Chemical, were solubilized in ethanol.

Statistics. Data are presented as means ± SE. One-way ANOVA with post hoc multiple comparison test was used to compare measurements between control, untreated hypoxic and terbogrel-treated hypoxic piglets. P < 0.05 was considered significant.

RESULTS

The measured values of blood pH, Po2, and Pco2 obtained during hemodynamic measurements in anesthetized piglets breathing room air were similar between all groups of piglets (Table 1). Measurements of pulmonary arterial pressure, pulmonary wedge pressure, cardiac output, and aortic pressure in the anesthetized piglets are shown in Table 2. Pulmonary arterial pressure and pulmonary wedge pressure were greater in both untreated and terbogrel-treated groups of chronic hypoxic piglets than in control piglets. Cardiac output did not differ between untreated and terbogrel-treated groups of chronic hypoxic piglets and was less in untreated chronic hypoxic piglets than in control piglets. Most importantly, Fig. 1 illustrates that the calculated value of pulmonary vascular resistance [(pulmonary arterial pressure − pulmonary wedge pressure)/cardiac output] in terbogrel-treated chronic hypoxic piglets was intermediate in value between that in untreated chronic hypoxic piglets and control piglets.

After equilibration at baseline tone, the mean diameter of all vessels used in the cannulated artery studies was 230 ± 6 μm for arteries from control piglets, 250 ± 6 μm for arteries from untreated hypoxic arteries, and 260 ± 6 μm for arteries from terbogrel-treated hypoxic piglets. None of the vehicles significantly changed arterial diameter in the concentrations used to solubilize any of the agents.

Table 1. Data for control, chronic hypoxic, and terbogrel-treated chronic hypoxic piglets

<table>
<thead>
<tr>
<th>Weight, g</th>
<th>pH</th>
<th>Po2, Torr</th>
<th>Pco2, Torr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>2.250±5</td>
<td>7.41±0.01</td>
<td>86±2</td>
</tr>
<tr>
<td>Chronic hypoxic (n = 7)</td>
<td>2.167±121</td>
<td>7.41±0.02</td>
<td>95±7</td>
</tr>
<tr>
<td>Terbogrel-treated chronic hypoxia (n = 7)</td>
<td>2.208±140</td>
<td>7.37±0.02</td>
<td>89±3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals.
For control arteries studied at basal tone \((n = 8 \text{ arteries from 5 piglets})\), vessel diameter increased to all but the highest dose of ACh (Fig. 2A). For both groups of chronic hypoxic arteries studied at basal tone, the diameter decreased to all doses of ACh, and the magnitude of decrease was less for terbogrel-treated \(\left(n = 14 \text{ arteries from 7 piglets}\right)\) than for untreated \(\left(n = 7 \text{ arteries from 5 piglets}\right)\) arteries at the two lowest doses of ACh (Fig. 2A). When tone was elevated with endothelin, arteries from all groups of piglets dilated to all doses of ACh (Fig. 2B), although the dilation to the highest dose of ACh was less for both untreated and terbogrel-treated groups of chronic hypoxic arteries than for control arteries \(\left(n = 6 \text{ arteries from 6 piglets}\right)\). Notably, dilation to the highest dose of ACh was greater for arteries from terbogrel-treated \(\left(n = 7 \text{ arteries from 7 piglets}\right)\) chronic hypoxic piglets than for arteries from untreated \(\left(n = 7 \text{ arteries from 7 piglets}\right)\) chronic hypoxic piglets (Fig. 2B).

Enzyme immunoassay determinations of the media from incubated arteries showed that production of 6-keto-PGF\(_{1\alpha}\), the stable metabolite of PGI\(_2\), was less for both untreated \(\left(n = 12 \text{ samples from 6 piglets}\right)\) and terbogrel-treated \(\left(n = 12 \text{ samples from 4 piglets}\right)\) groups of chronic hypoxic piglets than for control piglets \(\left(n = 12 \text{ samples from 6 piglets}\right)\) (Fig. 3A). By comparison, production of TxB\(_2\), the stable metabolite of TxA\(_2\), was greater for untreated chronic hypoxic piglets \(\left(n = 12 \text{ samples from 6 piglets}\right)\) than for either terbogrel-treated chronic hypoxic piglets \(\left(n = 12 \text{ samples from 4 piglets}\right)\) or for control piglets \(\left(n = 12 \text{ samples from 6 piglets}\right)\) (Fig. 3B).

**DISCUSSION**

The findings of diminished dilation to ACh and elevated pulmonary vascular resistance in newborn piglets exposed to 3 days hypoxia are consistent with our previous findings (17, 20). The major new findings in this study are that treatment with a combined Tx synthase inhibitor-receptor antagonist, terbogrel, given immediately before and throughout 3 days of exposure to...
chronic hypoxic, due to in utero ligation of the ductus arteriosus than in lung homogenates of fetal lambs with pulmonary hypertension. Values are means ± SE. *Different from control.

A: values for the stable metabolite of prostacyclin [6-keto-prostaglandin F, (PGF,)] for incubated arteries from control, untreated chronic hypoxic, and terbogrel-treated chronic hypoxic piglets. Values are means ± SE. *Different from control, P < 0.05 (1-way ANOVA with post hoc multiple comparison test). B: values for the stable metabolite of thromboxane A, [thromboxane B, (TXB,)] for incubated arteries from control, untreated chronic hypoxic, and terbogrel-treated chronic hypoxic piglets. Values are means ± SE. *Different from control; †different from terbogrel-treated chronic hypoxic, P < 0.05 (1-way ANOVA with post hoc multiple comparison test).

The rationale for the use of terbogrel to prevent pulmonary arterial dysfunctions is based on evidence provided by our laboratory (19, 20) and other investigators (2, 3, 15, 27). TXA, production has been found to be elevated in adults with pulmonary hypertension due to a variety of causes (15), in children with congenital heart disease associated with pulmonary vascular disease (3), in infants with congenital diaphragmatic hernia and pulmonary hypertension (21), and in infants with persistent pulmonary hypertension of the newborn (27). In regard to evidence from newborn animal models of pulmonary hypertension, a greater TXA, content was measured in lung homogenates of fetal lambs with pulmonary hypertension due to in utero ligation of the ductus arteriosus than in lung homogenates of age-matched controls (2). Our laboratory recently found that resistance-level pulmonary arteries of piglets with pulmonary hypertension resulting from 3 days exposure to chronic hypoxia produced greater amounts of TXA, than did resistance-level pulmonary arteries of comparable-age control piglets (19). We also provided evidence that TXA, is at least partly responsible for the abnormal pulmonary vascular responses to ACh that develop when newborn piglets are exposed to 3 days chronic hypoxia (20).

A number of other studies have also implicated the involvement of a cyclooxygenase (COX)-dependent contracting factor, such as TXA, in abnormal vascular responses. Many studies have evaluated the contribution from COX-dependent contracting factors to vascular dysfunction in systemic hypertension. For example, it has been shown that vasoconstrictor COX products are released by and contribute to impaired vascular responses in response to stimulation with ACh in cerebral arteries of spontaneously hypertensive rats (37), in aortas of rabbits exposed to high glucose (46), in the aorta of spontaneously hypertensive rats (25), and in renal arteries from cholesterol-fed rats (7). Relevant to the pulmonary circulation, rings of intrapulmonary arteries from adult rabbits (11), and 700- to 1,600-μm-diameter, conduit-level, pulmonary arteries from adult rats with pulmonary hypertension after 10 days of chronic hypoxia (36), also exhibit constrictor responses to ACh or its stable analog, methacholine, that is mediated, at least in part, by COX-dependent constrictors.

On the basis of the potential role of the COX-TXA, signaling pathway, a number of studies have evaluated the effect of TXA, synthase inhibitors on vascular dysfunction and development of systemic hypertension. In hyperinsulinemic spontaneously hypertensive rats (49), in rats chronically infused with insulin (33), and in fructose-fed rats (24), the development of hypertension was inhibited by treatment with a TXA, synthase inhibitor. Vascular function was improved by administration of a TXA, synthase inhibitor into coronary arteries of adult patients with angina (45) and by treatment of cholesterol-fed rabbits with the COX inhibitor, aspirin (43). By comparison to the large number of studies investigating the systemic circulation, there is a paucity of studies that have evaluated manipulation of the COX-TXA, signaling pathway and its influence on pulmonary vascular dysfunction and the development of pulmonary hypertension. Use of a competitive TXA, receptor antagonist inhibited the development of pulmonary hypertension in newborn rats exposed to hyperoxia (31). Treatment with the COX inhibitor, aspirin, reduced the severity of pulmonary hypertension in chronically hypoxic adult rats (34). In this latter study, because COX inhibition diminishes production of both COX-dependent dilators as well as constrictors, it is perhaps not surprising that aspirin only partially inhibited pulmonary hypertension (34).

It merits comment that altered production of COX-dependent dilators, including PGI, may be involved in the pathogenesis of pulmonary hypertension. PGI, production has been shown to be elevated in adult rats with chronic hypoxia-induced pulmonary hypertension (9, 41). The elevation in PGI, may serve in a compensatory role to counteract pulmonary hypertension. However, in many cases of pulmonary hypertension (15, 40, 48), including some animal models of pulmonary hypertension (6, 19), the evidence suggests that PGI, production is reduced. In fact, in some
studies, reductions in COX-dependent dilators, such as PGI2, are found in association with elevated production of COX-dependent constrictors (15, 19). In particular, we previously found that concomitant with increased TXA2 production, resistance-level pulmonary arterial PGI2 production was decreased in piglets exposed to 3 days of chronic hypoxia (19). Thus it seems that a shift in the balance of COX metabolites toward production of constrictors is involved in the pathogenesis of at least some forms of pulmonary hypertension (15), including the early stage of chronic hypoxia-induced pulmonary hypertension in newborn piglets (19).

Our finding that PG12 production remained reduced is one possible reason why pulmonary hypertension was ameliorated, not completely prevented, in newborn piglets treated with terbogrel throughout exposure to chronic hypoxia. Theoretically, when TXA2 synthase is inhibited, upstream metabolites might be diverted toward other arachidonic acid pathways, including PGI2 synthase, leading to an elevation in synthesis of other products, such as PG12, concurrent with diminished TXA2 production (42). Although not supported by our findings, results of other studies confirm this possibility. Adults with primary pulmonary hypertension treated with terbogrel showed slight increases in PG12 production (14, 23).

Because we limited our measurements to PG12 and TXA2 production, we cannot rule out the possibility that due in part to shunting within the metabolic pathway, production of other arachidonic acid dependent-constrictors was increased. These include both COX and non-COX products, such as lipoxygenases (50), cytochrome P-450 products (22, 51), and isoprostanes (32). In addition, constrictors other than metabolites of arachidonic acid pathways might be produced and explain why terbogrel failed to completely inhibit pulmonary hypertension. For example, there is evidence that reactive oxygen species (5, 12, 29, 47), such as superoxide (10, 30, 44, 47), are involved in aberrant vascular responses in both systemic and pulmonary hypertension. Endothelin might also contribute to the pathogenesis of pulmonary hypertension (16, 26).

Another possible reason why treatment with terbogrel only partly prevented pulmonary hypertension could be that the dose used was not high enough. Our choice of dose was based on pharmokinetic studies in rats (42). That our dose yielded adequate tissue levels to inhibit TXA2 synthesis is supported by our finding that resistance-level pulmonary arterial production of the stable metabolite of TXA2 was reduced below levels measured in arteries from untreated hypoxic piglets. However, it remains possible that production of metabolites that activate the prostaglandin H2 (PGH2)-TXA2 receptor was increased and that despite its receptor-antagonizing effects (42), the dose of terbogrel was not sufficient to completely block receptor activation.

Relevant to the above issues, our laboratory previously found that PGH2-TXA2 receptor antagonists abolished ACh-induced constriction in resistance-level arteries of piglets exposed to 3 days hypoxia (20). Hence, our findings in this study that some degree of constriction was elicited by ACh in the arteries from terbogrel-treated hypoxic piglets could indeed be interpreted to indicate that the PGH2/TXA2 receptors were not sufficiently blocked. Future studies evaluating dose responses to Tx receptor agonists and antagonists could help ascertain both optimal doses for and mechanisms underlying the ameliorative effect of terbogrel-treatment.

It should be considered that the mechanism by which terbogrel treatment ameliorated pulmonary hypertension may not be limited to a direct impact on the pulmonary vascular bed from blocking PGH2/TXA2 receptors and diminishing production of COX-dependent constrictors. Although not statistically different, cardiac output tended to be greater in terbogrel-treated hypoxic piglets than in untreated hypoxic piglets. Hence, it must be considered that terbogrel has hemodynamic influences that contribute to its beneficial effects.

It should also be considered that in addition to preventing the development of pulmonary hypertension, treatment with agents such as terbogrel, which inhibit TXA2, might inhibit progression once pulmonary hypertension is established. For example, in one study, 3-mo treatment with a TXA2 synthase inhibitor reduced pulmonary vascular resistance in patients with primary pulmonary hypertension (40). Terbogrel was recently tested for efficacy in adult patients with primary pulmonary hypertension (14, 23). Unfortunately, because of an unforeseen complication of leg pain, which confounded the primary end point of walking distance, the study was discontinued.

Limitations of methodology must always be considered. For example, in our study different doses of endothelin were required to elevate tone to the same degree in arteries from hypoxic and control piglets. Differential effects from endothelin on signal transduction pathways might contribute to the difference in dilator responses to ACh between control and hypoxic piglets. Notably, because doses needed to elevate tone were similar for both terbogrel-treated and untreated groups of hypoxic piglets, confounding influences due to endothelin are not likely to explain the difference in ACh responses between them.

To summarize, our findings show that treatment with a combined TXA2 synthase inhibitor-receptor antagonist partially inhibited the development of both aberrant pulmonary vascular responses and elevations in pulmonary vascular resistance in piglets exposed to 3 days of chronic hypoxia. These findings provide information important to devising other potentially more effective treatment strategies to inhibit the onset and progression of chronic hypoxia-induced pulmonary hypertension in newborns. For instance, combined use of a dilator, such as PGI2, along with a TXA2 synthase inhibitor-receptor antagonist might be more effective than either alone. In addition, it merits future consideration that targeting the TXA2 pathway might be useful in acutely lowering pulmonary vascular resistance in infants with established pulmonary hypertension, particularly those infants suffering from conditions associated with chronic hypoxia.

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