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Group B Streptococcus Promotes Oxygen Radical-Dependent Thromboxane Accumulation in Young Piglets

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ABSTRACT. Both thromboxane A_2 and oxygen-derived free radicals appear to play central roles in group B streptococcus (GBS)-induced pulmonary hypertension in piglets. This study tested the hypothesis that GBS promotes oxygen radical-dependent thromboxane accumulation and pulmonary hypertension in infant piglets. Piglets 4–12 d old were anesthetized and prepared for assessment of pulmonary arterial pressure and arterial blood gases. In control animals, GBS (10^8 organisms/kg/min for 15 min) increased mean pulmonary artery pressure by 30 ± 1.5 torr and reduced arterial P_{O_2} by 100 ± 20 torr. Thromboxane A_2, radioimmunoassayed in venous blood as thromboxane B_2, increased by 2452 ± 800 pg/mL. A second group of piglets was treated with dimethylthiourea (DMTU: 750 mg/kg), a putative oxygen radical scavenger. In these animals, GBS increased pulmonary arterial pressure by only 7 ± 1 torr and reduced arterial P_{O_2} by a modest 10 ± 8 torr. Importantly, thromboxane B_2 content in venous blood failed to increase above control levels in DMTU-treated animals. The protective effects of DMTU in GBS-treated piglets could not be ascribed to inhibition of cyclooxygenase or thromboxane synthase because the oxygen radical scavenger failed to attenuate increases in pulmonary arterial pressure and venous thromboxane B_2 content or reductions in arterial P_{O_2} caused by i.v. infusions of arachidonic acid. DMTU also did not ameliorate pulmonary hypertension evoked by the thromboxane mimetic U44069, thereby suggesting that the scavenger did not act as an end-organ antagonist of thromboxane receptors. These observations suggest that GBS promotes accumulation of thromboxane A_2 and attendant pulmonary hypertension through an oxygen radical-dependent mechanism. (*Pediatr Res* 27: 349–352, 1990)

Abbreviations

Ppa, mean pulmonary arterial pressure
DMTU, dimethylthiourea
GBS, group B streptococci
cfu, colony-forming unit

In recent years the chemical mediators of GBS-induced pulmonary hypertension and arterial hypoxemia in infant piglets have come under increased scrutiny. Multiple lines of evidence point to products of arachidonic acid metabolism, both thromboxane A_2 and sulfidopeptide leukotrienes, as important participants in the adverse cardiopulmonary response (1–4). Studies in our laboratory indicate that DMTU, a scavenger of hydroxyl radical and, at higher doses, hypochlorous acid (5, 6), suppresses GBS-induced pulmonary hypertension and arterial hypoxemia in young piglets (7), thereby suggesting that toxic oxygen radicals also participate in this model of sepsis-related pulmonary dysfunction. These two lines of evidence raise the question as to whether generation of oxygen radicals and eicosanoids in the setting of GBS sepsis may be functionally interrelated. That oxygen radical free generation may lead to production of arachidonic acid metabolites is supported by previous reports showing that N-acetylcysteine, an oxygen radical scavenger that also directly influences neutrophil function, suppresses endotoxin-induced accumulation of thromboxane B_2 and prostaglandin $F_{2\alpha}$, as well as development of edematous lung injury in intact, adult sheep (8).

In view of the above considerations, our study tested the hypothesis that GBS promotes oxygen radical-dependent accumulation of thromboxane A_2 in infant piglets. We reasoned that if this hypothesis is valid, then a scavenger of oxygen radicals, specifically the hydroxyl radical scavenger noted above, DMTU, should suppress GBS-induced accumulation of thromboxane B_2 and prevent attendant increases in mean Ppa and arterial hypoxemia. Additional experiments were conducted to verify that DMTU does not act as a cyclooxygenase or thromboxane synthase inhibitor or as a thromboxane receptor antagonist in the pulmonary circulation.

MATERIALS AND METHODS

Animal model. Twenty-five infant piglets unselected as to gender and ranging in age and weight from 5–14 d and 2–4.5 kg, respectively, were used in these studies. Animals were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneally) and placed on a heat-exchanging pad to maintain body temperature at 38 ±1°C. Catheters were introduced into the femoral artery and vein for measurement of mean systemic arterial pressure and administration of pharmacologic agents, respectively. Mean Ppa was measured from a specially formed polyethylene catheter inserted into the right external jugular vein and advanced into the pulmonary artery. Mean systemic arterial pressure and Ppa were monitored using Statham pressure transducers (Gould, Inc., Oxnard, CA) zeroed at midthoracic level and a Grass polygraph (Quincy, MA). Arterial blood gases were determined in 1.0-mL samples taken from the femoral arterial catheter using an Instrument Laboratories blood gas analyzer (Lexington, MA).

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After catheter introduction, a tracheostomy was performed and the animals were connected to a Harvard small animal ventilator (Harvard Apparatus Co., Inc., South Natick, MA). The animals were then paralyzed with pancuronium bromide (0.75 mg/kg i.v. followed by supplemental doses of 0.4 mg/kg as needed) and ventilated at 45 breaths/min with a mixture of 30% O_2 in N_2 using a tidal volume of 7 mL/kg. At the outset of a 20-min acclimitization period, ventilator settings were adjusted to attain baseline arterial P_{O_2}, P_{CO_2}, and pH of approximately 100 torr, 40 torr, and 7.4, respectively, and were not changed during the subsequent experimental protocols.

Bacterial preparation. Cultures of GBS (*Streptococcus agalacticae*, serotype II, American Type Culture Collection no. 13813, Difco Laboratories, Detroit, MI) were inoculated in brain-heart infusion broth containing 7% heat-inactivated FCS and grown to log phase. The medium was centrifuged at 2000 × *g* for 15 min and the pellet washed and resuspended in Hanks' balanced salt solution. Concentration of bacteria in the slurry was determined by quantitative culture and by relating OD to cfu. GBS was administered to the piglets as a 15-min i.v. infusion of 10^8 cfu/kg/min. Inoculums were routinely tested for purity.

Thromboxane B_2 RIA. The concentration of thromboxane A_2 in blood was estimated by RIA of its stable degradation product, thromboxane B_2. Blood samples (1.0 mL) were collected into tubes containing 2 mg EDTA and 1 μM indomethacin (Sigma Chemical Co., St. Louis, MO) and stored on ice. Subsequently, they were centrifuged and the plasma frozen at −70°C until extraction and RIA by previously-described methods (9). All assays were performed in duplicate.

Vasoactive agents. In addition to GBS, two other stimuli were used to elevate pulmonary arterial pressure in infant piglets. Arachidonic acid (Nu Cek Prep, Eliasan, MN) was delivered as an i.v. infusion of 5 mg over a 2-min period. Changes in Ppa and arterial P_{O_2} were monitored during the infusion and for 20 min thereafter. Some animals received i.v. injections of 1 mg/kg indomethacin before administration of arachidonic acid to confirm the role of cyclooxygenase in hemodynamic and blood gas changes. The stable thromboxane mimetic U44069 (10), obtained from the Upjohn Co. (Kalamazoo, MI), was administered as i.v. infusions of 100 and 300 ng/kg/min. DMTU (Aldrich Chemicals, Milwaukee, WI) was dissolved in saline and administered intravenously at a dose of 750 mg/kg over a 20-min period, as employed in previous studies (7).

Statistical analysis. Data are presented as the mean ± SEM. Time and experimental group-dependent differences between mean values were assessed using a two-way analysis of variance combined with Neumann-Kuels test when appropriate. $p \leq 0.05$ were considered to denote statistical significance.

RESULTS

Changes in Ppa and P_{O_2} evoked by GBS in control piglets and in piglets treated with DMTU are shown in Figure 1. In these studies, infusion of DMTU or its vehicle was initiated 10 min prior to infusion of GBS and continued for 10 min into the 15-min infusion of GBS. As expected, GBS promoted significant increases in Ppa that persisted for at least 5 min after termination of the bacterial infusion. Similarly, arterial P_{O_2} dropped substantially after GBS infusion. Baseline Ppa did not differ from controls in piglets receiving DMTU, but baseline arterial P_{O_2} was, for unexplained reasons, slightly lower in the treated animals. Consistent with our previous observations (7), DMTU significantly inhibited both the increase in Ppa and the decline in arterial P_{O_2} normally caused by GBS infusion.

As reported by others (1–3), GBS infusion was associated with profound increases in blood thromboxane B_2 content (Fig. 2), which were apparent both during and 5 min after termination of the GBS infusion. In contrast, although baseline thromboxane B_2 did not differ between controls and piglets receiving DMTU, GBS failed to increase levels of the prostanoid in treated animals.

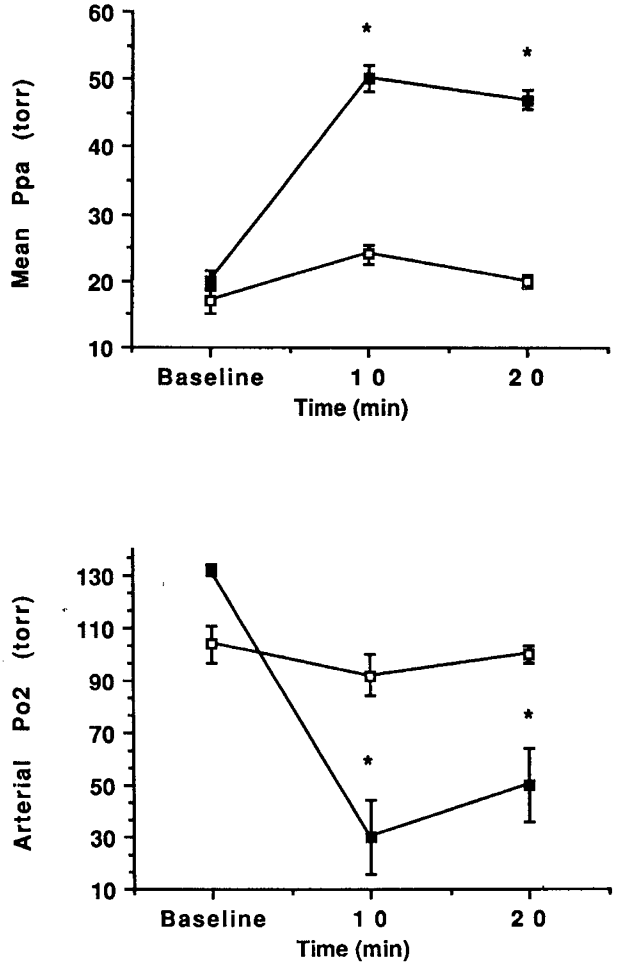

Fig. 1. Changes in mean Ppa (*top panel*) and arterial P_{O_2} (*bottom panel*) in infant piglets infused with GBS (10^8 cfu/kg/min for 15 min) in the absence (■) and presence (□) of dimethylthiourea (750 mg/kg). n = 6 for each experimental group. *Significantly ($p < 0.05$) reduced in comparison to piglets not receiving DMTU.

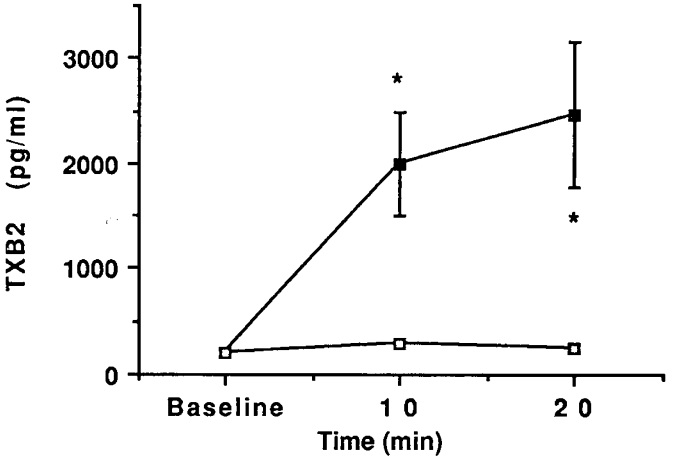

Fig. 2. Changes in blood thromboxane B_2 levels in infant piglets infused with GBS (10^8 cfu/kg/min for 15 min) in the absence (■) and presence (□) of DMTU (750 mg/kg). n = 6 for each experimental group. *Significantly ($p < 0.05$) reduced in comparison to piglets not receiving DMTU.

Additional experiments were conducted to determine if DMTU suppressed changes in Ppa and blood thromboxane B_2 content evoked by arachidonic acid. We reasoned that if the protective actions of DMTU noted above were related to direct

impairment of thromboxane synthesis, either by inhibition of cyclooxygenase or thomboxane synthase, then DMTU should prevent hemodynamic changes and thromboxane B_2 accumulation promoted by arachidonic acid, the precursor to thromboxane synthesis. As shown in Figure 3, infusion of arachidonic acid into control piglets increased Ppa and decreased arterial Po_2, with the decline in Po_2 occurring slightly after the increase in Ppa. Although the elevated Ppa reverted to baseline values within 5 min, the arterial hypoxia was present when Ppa was normalized and persisted for at least 10 min thereafter. Pretreatment with indomethacin (1 mg/kg, i.v.) was, for unexplained reasons, associated with a slight reduction in baseline Ppa. As expected, the cyclooxygenase inhibitor abolished arachidonic acid-induced increases in Ppa and decreases in arterial Po_2, thereby confirming the ability of cyclooxygenase blockade to protect against the actions of arachidonic acid infusion. DMTU treatment, initiated 15 min before arachidonic acid, failed to influence either baseline Ppa or arterial Po_2. The scavenger also failed to moderate the increase in Ppa and decrease in arterial Po_2 caused by arachidonic acid infusion. Data presented in Figure 4 indicate that increases in Ppa and decreases in arterial Po_2 evoked by arachidonic acid were associated with an increased blood content of thromboxane B_2. Indomethacin failed to influence baseline contents of the prostanoid but prevented the increase normally evoked by arachidonic acid infusion. Neither baseline thromboxane B_2 levels nor the increase associated with arachidonic acid infusion were suppressed by DMTU treatment.

An additional mechanism by which DMTU could protect against GBS-induced pulmonary hypertension is by antagonism of thromboxane A_2 receptor sites. To test this hypothesis, the ability of DMTU to inhibit increases in Ppa evoked by the thromboxane mimetic, U44069, were examined. We found that 6-min infusions of 100 and 300 ng/kg/min U44069 caused dose-related increases in Ppa (20 ± 8 and 29 ± 10 torr, respectively), but that DMTU did not alter either baseline Ppa or the increases normally evoked by U44069 (data not shown).

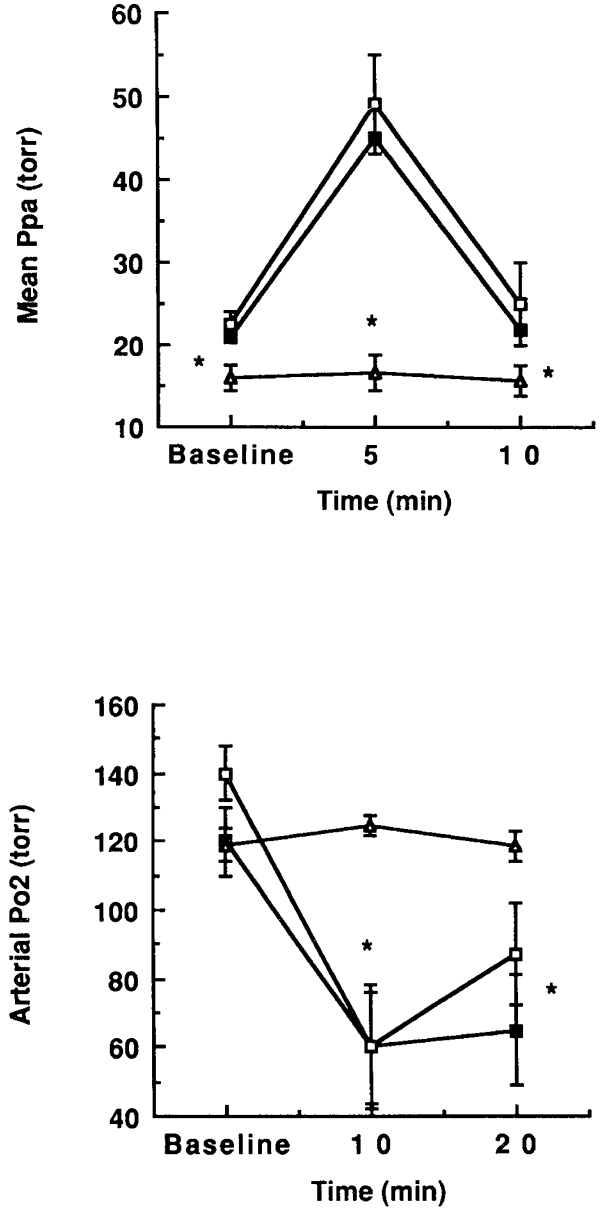

Fig. 3. Changes in mean Ppa (*top panel*) and arterial Po_2 (*bottom panel*) in infant piglets infused with arachidonic acid (5 mg over 2 min) in the absence (■) and presence of indomethacin (△: 1 mg/kg) or dimethylthiourea (□: 750 mg/kg). n = 3–5 for each experimental group. *Significantly inhibited relative to GBS alone or GBS plus DMTU. There were no differences between piglets receiving GBS and piglets receiving GBS plus DMTU.

DISCUSSION

Previous studies have provided compelling support for involvement of thromboxane in acute phase of infantile sepsis-related pulmonary hypertension (1–3). Blood thromboxane B_2 levels correlate with the severity of the GBS-induced pulmonary hypertensive response; thromboxane synthesis inhibitors prevent or reverse GBS-induced pulmonary hypertension; and gram-positive organisms other than GBS also increase thromboxane levels and promote pulmonary hypertension. In addition to this eicosanoid, studies from our laboratory have suggested that oxygen radicals also may participate in GBS-induced pulmonary hypertension (7). Against this background, the present experiments sought to determine if elaboration of thromboxane in the setting of GBS sepsis was dependent on free radical accumulation.

An important observation of our study is that DMTU attenuates GBS-induced increases in Ppa and arterial hypoxemia, as we have previously reported (7), and also prevents accumulation of thromboxane B_2 in systemic blood. Although this finding suggests that accumulation of thromboxane might be free-radical dependent, we also considered the possibilities that DMTU could be acting as an inhibitor of cyclooxygenase and/or thromboxane synthase or that the scavenger could antagonize thromboxane at its end-organ receptors. To examine the first option, we infused the precursor to thromboxane, arachidonic acid, and determined if DMTU influenced the ensuing pulmonary response and accumulation of thromboxane B_2. This appeared to be a reasonable strategy based on earlier reports by Hyman *et al.* (11) indicating that rapid i.v. bolus infusions of arachidonic acid into intact cats

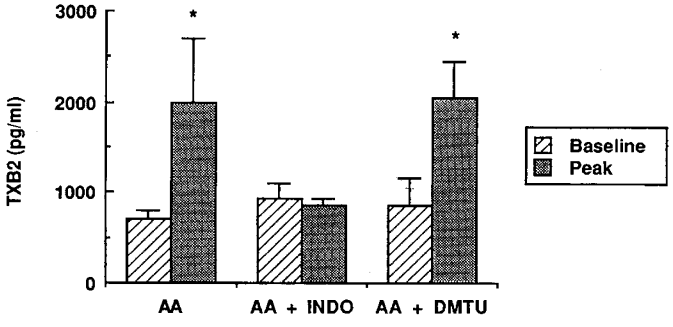

Fig. 4. Changes in blood thromboxane B_2 levels in infant piglets before and after infusion of arachidonic acid (5 mg over 2 min) in the absence and presence of indomethacin (*INDO:* 1 mg/kg) or DMTU (750 mg/kg). n = 3–5 for each experimental group. There were no significant differences between control piglets and piglets receiving DMTU either in the baseline state or after infusion of arachidonic acid.

promoted thromboxane-dependent pulmonary vasoconstriction. In addition, we confirmed the involvement of cyclooxygenase by demonstrating that arachidonic acid-induced changes in both Ppa and arterial P_{O_2} were abolished by indomethacin. In the context of our study, we found that DMTU did not alter either the arachidonic acid-induced increase in blood thromboxane B_2 or the attendant increase in Ppa and decreases in P_{O_2}. To determine if DMTU acted as an end organ antagonist of thromboxane receptors, we used the thromboxane mimetic, U44069, a stable epoxymethano-analogue of PGH_2 believed to interact with the thromboxane receptor to promote contraction of isolated smooth muscle preparations (11). Similar to results of experiments with arachidonic acid as the provocative agent, DMTU also failed to attenuate increases in Ppa promoted by U44069. Collectively, these findings demonstrate that the protective effects of DMTU in GBS-induced pulmonary hypertension cannot be ascribed to inhibition of enzymes involved with thromboxane synthesis or to blockade of thromboxane receptors on target smooth muscle cells. Keeping in mind the limited information regarding the selectivity of DMTU as a free radical scavenger, results of our study suggest that induction of thromboxane synthesis by GBS is oxygen radical-dependent. Furthermore, inasmuch as DMTU is reasonably specific for hydroxyl radical (5, 6), as opposed to its precursors, superoxide anion and hydrogen peroxide, our observations point to hydroxyl radical as an immediate cause of thromboxane synthesis in the setting of GBS sepsis.

Considerable evidence suggests that oxygen-derived free radicals can promote arachidonic acid metabolism. Introduction of chemical oxygen radical-generating systems (*e.g.* xanthine plus xanthine oxidase or glucose plus glucose oxidase) to medium perfusing isolated lung preparations results in the appearance of arachidonic acid metabolites, including thromboxane, in lung perfusion medium (12, 13). Blockade of arachidonic acid metabolism in these systems attenuates increases in lung water and pulmonary vascular resistance, thus indicating that the actions of oxygen radicals were mediated in part by arachidonic acid metabolites. The mechanism by which oxygen radicals promote arachidonic acid metabolism has yet to be defined, but it has been known for some time that free radicals can attack membrane lipids and release arachidonic acid (14) or induce cyclooxygenase (15), and thereby promote synthesis of eicosanoids. Based on these considerations, we suggest that the protective effects of DMTU in piglets infected with GBS is related to inhibition of an early step in arachidonic acid metabolism, probably arachidonic acid release, which occurs secondarily to free radical accumulation. Additional studies, possibly using cell culture systems, will be necessary to test this hypothesis.

Generation of toxic oxygen radicals appears to be a characteristic response to sepsis or endotoxemia (16). An interesting question to emerge relates to the functional importance of free radical generation in the setting of GBS sepsis. Traditional pathogenic concepts suggest that free radical generation by pulmonary intravascular cells may be an important component of host-defense against bacteria. In support of this contention, we have found that GBS is avidly sequestered and killed in the lungs of infant piglets (17). Electron microscopy indicated that the organisms were localized exclusively within pulmonary intravascular macrophages, though other populations of phagocytes also may participate in the response to GBS. As with other cells of the reticuloendothelial system, pulmonary intravascular macrophages exhibit profound phagocytic activity (18) and are capable of generating a spectrum of arachidonic acid metabolites (19). We have reported that DMTU attenuates killing of GBS in the lungs of infant piglets as well as abolishing the adverse hemodynamic complications (20). Based on these considerations, we propose that activation of an oxygen radical-dependent host microbicidal mechanism plays a key role early in the response to GBS by initiating generation of thromboxane A_2. Many elements of this hypothesis remain untested, but it may provide a useful framework for future studies.

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