Increased platelet-activating factor-induced periventricular brain microvascular constriction associated with immaturity

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OXIDANT STRESS PLAYS a major role in the pathogenesis of various disorders, such as hypoxic-ischemic encephalopathies (38, 42), including periventricular leukomalacia in premature subjects (41, 42). Free radicals can alter brain hemodynamics by causing vasoconstriction (19, 37) and increasing thromboxane A2 (TXA2) formation (1, 26). Although TXA2 has been implicated in peroxidation-induced vasoconstriction (1, 23), the mechanisms of TXA2 production by brain vasculature during oxidant stresses are complex and not fully understood.

Oxidation leads to the activation of a number of pathways and the formation of various factors. An early and important event following peroxidation is the activation of phospholipase A2 (3, 7), which leads to the synthesis of agents with major vascular actions, including platelet-activating factor (PAF) (30, 35). PAF is a phospholipid with diverse biological functions mediated by a G protein-coupled receptor. The production and release of PAF in the brain have been reported under various pathological conditions, including oxidant stress-induced ischemic injury in newborn (4, 25). PAF is a modulator of vasomotor tone and induces pulmonary, coronary, and cerebral vasoconstriction (5, 10, 22). The mechanism of the vascular action of PAF is controversial, but many investigators have shown that some of its effects might be mediated through the formation of cyclooxygenase products of arachidonic acid metabolism in response to activation of PAF receptors (11, 22). However, the direct effects of PAF on brain intraparenchymal vasculature, implicated in the genesis of periventricular ischemic encephalopathies, remain unknown.

Because oxidant stress-induced encephalopathies are mostly localized to the periventricular region in immature subjects compared with older ones (42), we postulated that the constrictor effects of PAF on the microvessels of the periventricular brain region are more pronounced in immature than older subjects, and in this process we evaluated the effects of PAF on these microvessels as well as the role of TXA2 in these vascular responses.

MATERIALS AND METHODS

Tissue preparation. Animals were used according to a protocol of the Animal Care Committee of Hôpital Sainte-Justine along with the principles of the Guide for the Care

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and Use of Experimental Animals of the Canadian Council on Animal Care. Brains from fetal (78–90 days gestation (term 114 days)] and adult (6–8 mo old) pigs were obtained from an abattoir (St-Hélène, Quebec, Canada) immediately after exsanguination of sows transported in ice-cold buffer to the laboratory. Newborn pigs (1–3 days old) were acquired from Fermes Ménard (L’Ange-Gardien, Quebec, Canada). Animals were anesthetized with halothane (−2.5–5%) and killed with pentobarbital sodium (120 mg/kg). The brains were removed and placed immediately in ice-cold Krebs buffer (pH 7.4) of the following composition (in mM): 120 NaCl, 4.5 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 27 NaHCO₃, 1.0 KH₂PO₄, and 10 glucose; 1.5 U/ml heparin was added to the buffer. For biochemical measurements, tissues were frozen in liquid N₂ and stored at −80°C.

Vasomotor response of brain periventricular microvessels. Slices of brain (1-mm thick) exposing the periventricular brain region were prepared as previously described (12, 20) to study relatively undisturbed penetrating microvessels (30–50 μm) reported to contribute significantly to the control of cerebral vascular resistance (17). The brain slices were immersed securely in a 20-mL conical centrifuge tube containing Krebs buffer (pH 7.4) equilibrated with 95% O₂-5% CO₂ and maintained at 37°C. The preparations were washed two to three times with fresh buffer and allowed to equilibrate for 45 min before starting the experiment.

Cerebral microvessels were visualized and recorded using a video camera (model CCD72, MTI) mounted on a dissecting microscope (model M-400, Nikon), as reported previously (12, 20). Vascular diameter was measured using a digital image analyzer (Sigma Scan software, Jandel Scientific, Corte Madera, CA) and repeated three times with a variability of <1%. Vascular diameter was recorded before and after topical application of increasing concentrations of test agents (C-PAF, thromboxane mimetic U-46619, and PGF₂α) in the presence and absence of a 20-min pretreatment with the following agents at known effective concentrations (1, 15, 20): TXA₂ synthase inhibitor CGS-12970 (1 μM); TXA₂ receptor antagonist L-670596 (0.1 μM); PAF receptor antagonist THG-315 (1 μM); non-voltage-dependent Ca²⁺ entry and receptor-mediated Ca²⁺ channel blocker SK&F-96365 (29) (20 μM); L-type voltage-gated Ca²⁺ channel blocker nifedipine (5 μM); and N-type voltage-gated Ca²⁺ channel blocker ω-conotoxin (36) (10 μM). Focus was placed on receptor-operated as well as N- and L-types voltage-gated Ca²⁺ channels since endothelial cells are not excitable and are essentially devoid of voltage-gated Ca²⁺ channels (18), whereas smooth muscle cells and astrocytes contain voltage-gated Ca²⁺ channels, mostly L and N type (14, 34).

Removal of the endothelium. The endothelium of newborn pig microvessels was chemically removed by intracarotid perfusion with 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulphonate (CHAPS; 5 mg/l for 2 min) (32). After denudation, the vasomotor response of brain periventricular microvessels to C-PAF was studied. The removal of the endothelium was considered successful since the vasodilatory response to substance P (1 μM) (12) was absent while tissues responded normally to endothelium-independent stimulants U-46619 (0.2 μM) and sodium nitroprusside (1 μM).

Preparation of brain microvessel membrane. Microvessels from fetal, newborn, and adult brain were prepared as previously described (20, 24). Briefly, periventricular brain region was homogenized, preserving microvascular structure by homogenizing tissues in 5 mM Tris·HCl buffer (pH 7.4) containing 1.1 mM acetylsalicylic acid, 0.5 mM EGTA, 1 mM benazamide, 0.1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml soybean trypsin inhibitor with three up-and-down strokes with a tissue grinder (Wheaton, Teflon type). The homogenate was filtered through a nylon mesh filter (70 μm) and rinsed with the buffer above. Microvessels were collected from the nylon mesh, resuspended in the buffer above, homogenized with a hand pestle, centrifuged at 1,000 g for 15 min, and refiltered as above. The purified microvessels were collected, recentrifuged at 100,000 g for 45 min, and the pellet was stored at −80°C until used. The morphology and purity of microvessels were confirmed by light microscopy and a 15-fold higher level of γ-glutamyl transpeptidase activity when compared with brain parenchyma, as described previously (24). The calibers of microvessels used ranged from 20 to 60 μm in diameter.

Microvascular endothelial cell culture. Microvessels were suspended in selective endothelial or smooth muscle growth media (Clonetics). Confluent individual endothelial cells were trypsinized, centrifuged, resuspended in culture flasks, and subcultured; cell viability was verified by trypan blue exclusion and was >90%. Endothelial cells were identified by their cobblestone morphology at confluence, positive reactivity to factor VIII antibody, and negative reactivity to smooth muscle-specific actin and glial fibrillary acidic protein (GFAP) antibodies (Dako, Carpinteria, CA). Confluent cultures of endothelial cells from passages 5-15 were used for experiments.

[³H]PAF binding assay. Membranes were suspended in assay buffer, and proteins were measured by the dye-binding method using BSA as the standard. Saturation binding experiments were performed by incubating 200 μg of brain microvessel membrane proteins for 30 min at 37°C with increasing concentrations of [³H]PAF in the presence or absence of 25 μM unlabeled PAF; specific binding reached equilibrium within 10 to 15 min and remained stable for at least 30 min, as we previously reported (24). Reactions were terminated by the addition of 2.5 ml ice-cold 5 mM Tris·HCl buffer (pH 7.4). The incubates were rapidly filtered through Whatman GF/C glass filter disks and washed three times with 2.5 ml of the same buffer. The radioactive on the filter disks was counted with a beta counter (Beckman LS6000IC). Receptor densities (maximal binding; B_max) and dissociation constants (K_D) were determined from the saturation isotherms (24) using a computer program (Prism, GraphPad).

PAF receptor antagonists (aporidine B 2 [stable TXA 2 metabolite] was assessed on homogenates from fetal, newborn, and adult pig brains stimulated for 15 min with C-PAF (1 μM); PAF-stimulated microvascular endothelial cell cultures were treated with selective endothelial or smooth muscle growth media (Clonetics). Confluent individual endothelial cells were trypsinized, centrifuged, resuspended in culture flasks, and subcultured; cell viability was verified by trypan blue exclusion and was >90%. Endothelial cells were identified by their cobblestone morphology at confluence, positive reactivity to factor VIII antibody, and negative reactivity to smooth muscle-specific actin and glial fibrillary acidic protein (GFAP) antibodies (Dako, Carpinteria, CA). Confluent cultures of endothelial cells from passages 5-15 were used for experiments.

TXA₂ synthase assay. Effects of C-PAF on thromboxane formation were studied in fetus, newborn, and adult pig brain slices stimulated (15 min) with C-PAF at 0.1, 1, and 10 μM; the reaction was terminated with liquid N₂. Thromboxane B₂ (stable TXA₂ metabolite) was assessed on homogenized tissue by radioimmunossay as previously described (1, 20). TXB₂ concentration was also measured in newborn pig brain slices stimulated for 15 min with C-PAF (1 μM) in the presence of CGS-12970 (1 μM), SK&F-96365 (20 μM), ω-conotoxin (10 μM), or nifedipine (5 μM).

Immunoblotting of thromboxane synthase. TXA₂ synthase immunoreactivity on brain was determined as we previously described for other membrane-bound enzymes (33).
Briefly, homogenized tissues from the periventricular regions of all age groups studied were preabsorbed with 50 ml of immunoprecipitin for 30 min and then centrifuged at 12,000 g for 10 min to remove the immunoprecipitin. The supernatant was incubated with porcine TXA2 synthase-specific polyclonal antibodies (Cayman Chemicals) for 1.5 h, and immune complexes were collected by incubation with 50 ml immunoprecipitin for 30 min, followed by centrifugation. Immune precipitates were denatured in SDS buffer, centrifuged at 12,000 g for 15 min to remove the immunoprecipitin, and the samples were loaded on SDS-polyacrylamide gels. The proteins were electrophoretically transferred to nitrocellulose membranes and incubated with TXA2 synthase-specific polyclonal antibodies (Cayman Chemicals) for 1.5 h, and immune complexes were collected by incubation with 50 ml immunoprecipitin for 30 min, followed by centrifugation. Immune precipitates were denatured in SDS buffer, centrifuged at 12,000 g for 15 min to remove the immunoprecipitin, and the samples were loaded on SDS-polyacrylamide gels. The proteins were electrophoretically transferred to nitrocellulose membranes and incubated with TXA2 synthase-specific antibodies. After being washed, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody followed by several washes. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham) as recommended by the supplier and analyzed by densitometry.

**Chemicals.** L-670596 and CGS-12970 were generous gifts from Merck-Frosst (Pointe-Claire, PQ, Canada) and Ciba-Geigy (Summit, NJ), respectively. THG-315 was a gift from Geigy (Summit, NJ), respectively. THG-315 was a gift from Geigy (Summit, NJ).

**Statistics.** All results are expressed as means ± SE. Results were analyzed using Student’s t-test and two-way ANOVA factoring for concentrations and age or treatments. Post-ANOVA comparisons among means were performed using the Tukey-Kramer method. P values < 0.05 were considered to be significant.

### RESULTS

**Effects of PAF on brain periventricular microvessels.** C-PAF caused concentration-dependent constriction of periventricular microvessels from fetal and newborn pigs, whereas vasoconstriction of adult pig microvessels was negligible (Fig. 1A). Emax values for fetus, newborn, and adult were 29.27 ± 2.6, 22.14 ± 3.2, and 2.3 ± 0.8%. The EC50 values of C-PAF on fetal and newborn pig microvessels were comparable: 25.96 ± 0.9 and 19.95 ± 1.4 nM. In contrast, PGF2α was more

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*Fig. 1. Vasoconstrictor response to C-platelet-activating factor (C-PAF), PGF2α, and U-46619 on brain periventricular microvessels of fetal, neonatal, and adult pigs. Constriction is the percent reduction in vascular diameter from basal values that were 38.4, 3.8, and 35.1 mg, respectively, for fetus, newborn, and adult. Effects of agents were studied in situ on brain slices as described in MATERIALS AND METHODS. Data are means ± SE of 5–6 separate experiments. *p < 0.01 compared with fetal and newborn pigs; †p < 0.05 compared with fetal and adult pigs (2-way ANOVA and comparison among means test).*
effective on adult than fetal and newborn pig microvessels ($P < 0.05$) (Fig. 1B), and U-46619 was equivalently effective on tissues of all three age groups. Because PAF has been shown to increase the production of vasodilator prostaglandins and nitric oxide (30) in several tissues, we tested whether there were ontogenic changes in relaxant response to C-PAF; C-PAF did not elicit vasorelaxation, whereas substance P (1 μM) relaxed brain microvessels of all ages (data not shown).

Specific $[^{3}H]$PAF binding on brain microvessels and inositol phosphate production. To explain the difference in the response of fetal, newborn, and adult brain microvessels to C-PAF, we compared PAF receptor density and the production of the second messenger inositol phosphate in fetal, newborn, and adult tissues. Maximum specific binding of $[^{3}H]$PAF to brain microvessel membranes was greater in fetus ($B_{max}$ 551.5 ± 36.2 fmol/mg protein) than in newborn ($B_{max}$ 423.1 ± 60.6 fmol/mg protein), which was three times greater than in adult ($B_{max}$ 180.0 ± 37.3 fmol/mg protein) (Fig. 2A). Dissociation constants ($K_{d}$, nM) were comparable: 28.61 ± 5.2, 27.27 ± 4.8, and 18.11 ± 4.1, respectively, in fetal, newborn, and adult tissues. PAF-induced inositol phosphate production exhibited an age-dependent profile that was greater in fetal than in newborn and minimally present in adult tissues (Fig. 2B).

$TXA_2$-mediated vasoconstriction to PAF. The vasoconstrictor effects of PAF on the microvessels from fetal and newborn pigs were almost fully inhibited by $TXA_2$ synthase inhibitor CGS-12970 and $TXA_2$ receptor antagonist L-670596 (Fig. 3, A and B). The role of $TXA_2$ in PAF-induced constriction was not studied in adults given the negligible vasoconstriction in this age group. In addition, PAF-induced constriction was virtually abolished by endothelial denudation (Fig. 3C) as demonstrated in newborn tissues. Moreover, TXB$_2$ levels increased dose dependently after stimulation of the fetal and newborn periventricular tissue with PAF (Fig. 4A). In contrast, TXB$_2$ levels were only mildly increased by PAF stimulation in adult tissue (Fig. 4A). A similar developmental pattern of immunoreactive $TXA_2$ synthase expression was observed (Fig. 4, B and C). Hence, developmental differences in PAF-induced constriction appear to depend on ontogenic differences in $TXA_2$ formation, which seems to be generated largely by the endothelium.

Involvement of Ca$^{2+}$ on PAF-induced $TXA_2$ formation and vasoconstriction. Because removal of the endothelium completely abolished the $TXA_2$-dependent action of C-PAF, endothelial cells must contribute to the $TXA_2$ formation evoked by C-PAF (Fig. 3C). Because enzyme-catalyzed prostanoid formation is Ca$^{2+}$ dependent via phospholipase A$_2$, we attempted to identify the type of Ca$^{2+}$ channel involved in PAF-induced $TXA_2$ generation and vasoconstriction. PAF-induced increase in TXB$_2$ formation in periventricular tissue of newborn pigs was markedly inhibited by CGS-12970, putative receptor-operated Ca$^{2+}$ channel blocker SK&F-96365 (29), and by the Ca$^{2+}$ chelator EGTA, but not by selective N-type voltage-gated Ca$^{2+}$ channel blocker ω-conotoxin MVIIA (36) or L-type voltage-gated Ca$^{2+}$ channel blocker nifedipine (Fig. 5A); similar inhibition of PAF-induced increase in TXB$_2$ formation was observed in the fetus when we tested SK&F-96365 in contrast to ω-conotoxin MVIIA (not shown). Accordingly, vasoconstriction to C-PAF was also nearly abolished by SK&F-96365 but was unaffected by ω-conotoxin MVIIA in young animals (Fig. 5C). Vasoconstriction to C-PAF and $TXA_2$ mimic U-46619 was inhibited by nifedipine (Fig. 5C). The effects of C-PAF on Ca$^{2+}$ transients corroborated the data on TXB$_2$ formation. C-PAF induced an increase in Ca$^{2+}$ signals in endothelial cells, which was significantly reduced by SK&F-96365 and EGTA, but not by nifedipine or ω-conotoxin (Fig. 5B). In contrast, C-PAF did not affect Ca$^{2+}$ transients in smooth muscle cells. On the other hand, $TXA_2$ mimetic U-46619 (1 μM) induced Ca$^{2+}$ transients in smooth muscle cells, which were inhibited by nifedipine (5 μM), but not by SK&F-96365 (20 μM) (data not shown).
Fig. 3. A and B: contribution of thromboxane A2 (TXA2) in PAF-induced periventricular microvascular constriction of newborn and fetus; adults were virtually unresponsive to C-PAF. Tissues were pretreated 20 min with saline (control), thromboxane synthase inhibitor CGS-12970 (1 μM), thromboxane receptor blocker THG-315 (1 μM). Experimental preparations are similar to those for Fig 1. Data are means ± SE of 5–6 separate experiments. *P < 0.01 compared with saline-treated preparations (2-way ANOVA). C: deendothelialization of brain vasculature was performed by intracarotid perfusion with 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulphonate (5 mg/l for 2 min; see MATERIALS AND METHODS). Data are means ± SE of 5–6 separate experiments. *P < 0.01 compared with values for control (2-way ANOVA).

DISCUSSION

PAF is an important phospholipid with diverse physiological and pathological roles in vivo, including effects on circulation (5, 22, 30). Due to its potent actions on blood vessels, PAF might be a putative mediator in ischemic brain injury (25, 26). Little is known about the effects of PAF on brain intraparenchymal microvessels during development. The present study reveals that PAF causes greater constriction of fetus and newborn periventricular microvessels compared with those of adult animals as a result of a higher density of PAF receptor and greater thromboxane generation, principally of endothelial origin, via activation of receptor-operated Ca2+ channels.

PAF exerts its effects by interacting with a specific PAF receptor (30). Depending on the species and tissues, PAF causes constriction or dilation according to different coupling mechanisms (5, 22, 26). For instance, PAF is a potent constrictor of cerebral arterioles in newborn pigs (5) and produces vasodilatation in mesenteric circulation in dogs (30). In some tissues (e.g., coronary and cerebral vessels), vasoconstriction evoked by PAF is partly mediated by TXA2 (22, 26). In the present study, effects of PAF on periventricular microvasculature are also mediated via TXA2 (26). PAF stimulates calcium-dependent thromboxane production (Fig. 5, A and B); 2) perivascular vasoconstriction to PAF is thromboxane dependent (Fig. 3, A and B); 3) PAF-induced Ca2+ signals, thromboxane formation, and vasoconstriction are abrogated by non-voltage-gated calcium channel blocker SK&F-96365, consistent with absence of voltage-gated calcium channels in endothelium (40); 4) removal of endothelium abolishes effects of PAF as seen with TXA2 synthase and receptor blockers (Fig. 3A); and 5) PAF-induced Ca2+ transients and thromboxane formation were not inhibited by selective N-type voltage-gated Ca2+ channel blocker ω-conotoxin MVIIA (36) or L-type voltage-gated Ca2+ channel blocker nifedipine (Fig. 5, A and B), suggesting that perivascular astrocytes that contain N- and L-type voltage-gated Ca2+ channels (34, 43) are not contributors to the TXA2 formation (20). Accordingly, the efficacy of nifedipine in PAF-evoked vasoconstriction, but not thromboxane formation, concurs with the action of PAF-generated thromboxane on L-type voltage-gated Ca2+ channels in smooth muscle (21). Taken together, these data suggest that PAF increases the influx of calcium through receptor-operated channels in endothelial cells, and this, in turn, enhances the formation of thromboxane.

An important observation in this study is the greater constriction evoked by PAF in the fetus compared with the newborn, which is markedly larger than that in adult pigs (Fig. 1A); results in the adult pig are in agreement with the unresponsiveness of mature rats (13). These findings are of interest because the constrictor responses to a number of agents, such as adrenergic agonists, serotonin, angiotensin, and prostaglandin F2α (Fig. 1B), are often reduced on the blood vessels of younger subjects compared with those of adult animals (2, 9, 39). Specific binding of [3H]PAF to brain microvessel membranes revealed a greater density of PAF receptor in younger than adult animals (Fig. 2A); mechanisms for this developmental change...
remain to be clarified. However, simply a lower density of PAF receptor in adults could not per se explain the lack of vasoconstriction to PAF (Fig. 1A). On the other hand, the virtual absence of PAF-induced thromboxane formation due to diminished thromboxane synthase protein expression and associated inositol phosphate production in mature animals is consistent with the vasomotor response to PAF (Figs. 2 and 4), whereas effects of thromboxane seem preserved throughout development (Fig. 1C), as previously reported (20).

Hence, developmental changes in PAF-induced brain microvascular constriction seem partly dependent on ontogenic differences in PAF receptor density, but maybe mostly due to the greater thromboxane synthase expression observed in younger subjects (Fig. 4B). The reason for increased expression of TXA2 synthase in the periventricular brain region of immature subjects is not clear. However, its role in the migration of FIG. 5.

A: effects of C-PAF on TXB2 production by periventricular tissues from newborn pig brain. Tissues were pretreated 20 min with saline or one of the following: CGS-12970 (10^{-6} M), SK&F-96365 (20 \mu M), EGTA (100 \mu M), \omega-conotoxin (10 \mu M), and nifedipine (5 \mu M). Data are means \pm SE of 5–6 separate experiments. *P < 0.01 compared with corresponding values for newborn and fetus; †P < 0.05 compared with corresponding values for fetus and adult; ‡P < 0.05 compared with C-PAF at 0.1 \mu M (2-way ANOVA and comparison among means test). B: representative immunoblot of 3 experiments. C: compiled densitometry of the immunoblots relative to that of the fetus set at 100%. †P < 0.05 compared with corresponding values for fetus and adult.

B: intracellular peak calcium transients [Ca^{2+}i] in newborn pig brain endothelial cells in response to C-PAF (0.1 and 1 \mu M) using fura 2-AM (see MATERIALS AND METHODS). Cells were pretreated 20 min with SK&F-96365 (20 \mu M), EGTA (100 \mu M), \omega-conotoxin (10 \mu M), and nifedipine (5 \mu M). Values are means \pm SE of 4–5 separate experiments. *P < 0.01 compared with basal value; †P < 0.01 compared with other values other than basal. C: vasoconstrictor response of periventricular microvessels of newborn pigs to C-PAF and U-46619 in the presence of saline, SK&F-96365 (20 \mu M), \omega-conotoxin (10 \mu M), or nifedipine (5 \mu M); effects of agents were studied in situ on brain slices as described in MATERIALS AND METHODS. Data are means \pm SE of 5–6 separate experiments. *P < 0.01 compared with C-PAF + saline as well as with C-PAF + \omega-conotoxin (2-way ANOVA and comparison among means tests).
of astrocytes from the germinal matrix in the periventricular region to others in the developing brain has been proposed (27). We speculate that the markedly greater PAF-induced brain microvascular constriction in the younger subjects may contribute to the hemodynamic compromise and periventricular brain injury observed in premature neonates exposed to oxidant stress. PAF antagonists, thromboxane synthase inhibitor, and/or receptor blockers may attenuate the deleterious effects of oxidant stress (4, 25, 28).

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