Selective Neumicrovascular Endothelial Cell Death by 8-Iso-Prostaglandin F$_{2\alpha}$
Possible Role in Ischemic Brain Injury

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Background and Purpose—Free radical–induced peroxidation is an important factor in the genesis of hypoxic-ischemic encephalopathy, including that of the preterm infant. Isoprostanes are major peroxidation products. Since microvascular dysfunction seems to contribute to ischemic encephalopathies, we studied the cytotoxicity of 8-iso-prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) on cerebral microvascular cells.

Methods—Microvascular endothelial, astroglial, and smooth muscle cells from newborn brain were cultured. The cytotoxicity of 8-iso-PGF$_{2\alpha}$ on these cells was determined by MTT assays and lactate dehydrogenase (LDH) release, propidium iodide incorporation, and DNA fragmentation (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling [TUNEL]). In addition, effects of intraventricular injections of 8-iso-PGF$_{2\alpha}$ and possible involvement of thrombomodulin in 8-iso-PGF$_{2\alpha}$–induced cytotoxicity were determined.

Results—8-Iso-PGF$_{2\alpha}$–induced time- and concentration-dependent endothelial cell death (EC$_{50}$=0.1 nmol/L) but exerted little effect on smooth muscle and astroglial cells; endothelial cell death seemed mostly of oncotic nature (propidium iodide incorporation and LDH release). Cell death was associated with increased endothelial thromboxane A$_2$ (TXA$_2$) formation and was prevented by TXA$_2$ synthase inhibitors (CGS12970 and U63557A); TXA$_2$ mimetics U46619 and I-BOP also caused endothelial cell death. Intraventricular injection of 8-iso-PGF$_{2\alpha}$ induced periventricular damage, which was attenuated by CGS12970 pretreatment.

Conclusions—These data disclose a novel action of 8-iso-PGF$_{2\alpha}$ involving TXA$_2$ in oxidant stress–induced cerebral microvascular injury and brain damage. (Stroke. 2003;34:776-782.)

Key Words: apoptosis ■ brain damage ■ necrosis ■ oxidants ■ thromboxanes ■ white matter

Free radical–induced peroxidation plays a significant role in the pathogenesis of hypoxic-ischemic encephalopathy, including periventricular leukomalacia in premature infants.1,2 Ischemic encephalopathies and atherosclerotic lesions are associated with endothelial damage leading to cell death,3,4 which further promotes microcirculatory disturbances. Distinct cell types are variably affected by free radicals. Pericytes,5 smooth muscle cells,6 and perivascular astrocytes7 are relatively resistant to peroxidation-induced injury, whereas endothelial cells seem particularly susceptible.8 However, the exact mechanisms by which oxidant stress leads to endothelial cell death are likely complex and not well known.

Isoprostanes are a series of prostaglandin-like compounds generated by a free radical nonenzymatic process and are abundantly produced during oxidative stress. Isoprostanes are stable products of arachidonic acid peroxidation formed in greater abundance than the cyclooxygenase-derived prostaglandins.9,10 An abundantly produced isoprostane in vivo is 8-iso-prostaglandin F$_{2\alpha}$ (8-iso-PGF$_{2\alpha}$) (or 15-F$_{2\alpha}$–isoprostane).9 8-Iso-PGF$_{2\alpha}$ produces several effects, including vasoconstriction of different vascular beds,9,11–13 induction of immediate-early genes14 and increased vascular permeability15; it is noteworthy that these effects can also be produced by oxidative stress.16 In contrast to 15-series isoprostanes, 5-series isoprostanes do not seem bioactive.17 Whether 8-iso-PGF$_{2\alpha}$ has direct cytotoxic effects on cerebral microvascular endothelium has never been studied.

We recently reported that thromboxane A$_2$ (TXA$_2$) contributes to the vasoconstriction by 8-iso-PGF$_{2\alpha}$.12,13 TXA$_2$ has
also been suggested to elicit retinal microvascular endothelial cytotoxicity. In the present study we investigated the interactions of 8-iso-PGF2α and TXA2 on cerebral microvascular endothelial cell survival (in vitro) as well as their effects in brain injury (in vivo).

Materials and Methods

Animals
Two- to 3-week-old Sprague-Dawley rats (Charles River, St Constant, Quebec) and Yorkshire piglets (Fermes Ménard, L’Ange-Gardien, Quebec) were used according to a protocol of the Hôpital Sainte-Justine Animal Care Committee.

Brain Microvessel Preparation and Assessment of Viability
Brain microvessels from rats and piglets were isolated as previously reported. The isolated brain microvessels were seeded on glass coverslips and incubated overnight in Dulbecco’s minimum essential medium (DMEM). Microvessels were then treated with fresh DMEM in the absence or presence of 8-iso-PGF2α (100 nmol/L) and CGS12970 (1 μmol/L) for an additional 48 hours. Type of cell death (oncotic or apoptotic necrosis) was assessed by loading microvessels for 30 minutes with propidium iodide (PI) (1 μg/mL) and Hoechst 33342, as described above; the percentage of PI-positive PI/H9262 cytototoxicity. 18 In the present study we investigated the also led to 25% to 30% cellular PI incorporation (Figure 2B). Cytotoxicity by 8-iso-PGF2α was detected at 24 hours EC50 = 0.1 nmol/L; 8-iso-PGF2α was ineffective (Figure 1A). Smooth muscle cells (Figure 1C), astrocytes (Figure 1D), and human umbilical vein endothelial cells (93.2 ± 2.4% survival, 48 hours after addition of 8-iso-PGF2α) were not significantly affected by isoprostanes.

Measurement of Lactate Dehydrogenase and Thromboxane B2
Lactate dehydrogenase (LDH) activity was measured according to a described protocol. 18 Thromboxane B2 (TXB2, a stable TXA2 metabolite) in culture media of endothelial cells treated up to 48 hours with 8-iso-PGF2α (100 nmol/L) was measured by radioimmunoassay. 12,13

Brain Intraventricular Delivery and Histology
Rats (aged 21 days) were infused in the lateral ventricles with the use of Alzet micro-osmotic pumps (Alza). Rats were anesthetized with a mixture of ketamine and xylazine (90 and 2 mg/kg IP, respectively); osmotic pumps (infusion rate: 0.5 μL/h) were implanted subcutaneously in the nuchal region. Pumps were connected to a cannula for drug delivery in the lateral ventricle of the right hemisphere (stereotaxic coordinates: 1.5 mm para-midline and 1.5 mm posterior to bregma, depth of 4.5 mm). Pups were randomly selected to receive for 1 week either artificial cerebrospinal fluid (aCSF, vehicle), H2O2, 8-iso-PGF2α, urotensin II, U46619, CGS12970, or 8-iso-PGF2α plus CGS12970. On the basis of the rapid turnover of CSF (approximately every 1 hour), the estimated volume of CSF of 21-day-old rats (150 μL), and a drug infusion rate of 50 pmol/h, we predicted ventricular drug concentrations of 0.1 μmol/L.

At the end of the treatment period, rats were anesthetized with halothane (1.5%) and perfused-fixed through the left ventricle with saline followed by 4% formaldehyde. Brains were collected and fixed for an additional 2 hours in 4% formaldehyde followed by an overnight immersion in 20% sucrose in 0.1 mol/L phosphate buffer. Brain sections of 20 μm were cut with a cryostat (Microm International, HM500 O), mounted on Superfrost Plus slides, and stained with hematoxylin. Periventricular regions of interest were photographed (DMC Ie, Polaroid). Extent of brain injury was quantified by measuring the maximal cross-sectional length (serial sections) of the necrotic area.

Endothelial, Smooth Muscle, and Astroglial Cell Cultures
To obtain sufficiently large quantities of endothelial, smooth muscle, and astroglial cells, these were cultured from pig brain as previously reported. 13 The isolated brain microvessels were seeded on glass coverslips and incubated overnight in Dulbecco’s minimum essential medium (DMEM). Microvessels were then treated with fresh DMEM in the absence or presence of 8-iso-PGF2α (100 nmol/L) and CGS12970 (1 μmol/L) for an additional 48 hours. Type of cell death (oncotic or apoptotic necrosis) was assessed by loading microvessels for 30 minutes with propidium iodide (PI) (1 μg/mL) and Hoechst 33342, as described above; the percentage of PI-positive cells (oncosis) relative to total cell number (Hoechst-positive cells).

Cell Viability Assays
MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were performed to assess cell viability as described. 18 Briefly, quiescent cells were treated with either 8-iso-PGF2α, 8-iso-prostaglandin E2 (PGE2), or TXA2 mimetics U46619 and 1-BOP ([1S-[1α, 2 α(Z)], 3β[1E, 3S*], 4 α]-7-[3-[3-hydroxy-4-(4-iodophenoxyl)-1-butenyl]-7-oxacyclo[2.1.1]-hept-2-yl]-5-heptenoic acid). Pretreatment (30 minutes) with the cyclooxygenase inhibitor ibuprofen (1 μmol/L), TXA2 synthase inhibitors U63557A (100 nmol/L) and CGS12970 (100 nmol/L), and effector caspase inhibitors Z-DEVD-FMK (50 μmol/L) was studied. The nature of cell death (apoptotic versus oncotic necrosis) was also assessed with PI and Hoechst 33342, as described above; the percentage of PI positivity was determined on 5 fields per well.

DNA Fragmentation Labeling
Labeling of fragmented DNA was performed by using a commercial kit based on terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) technique (Apoptag, Oncor), as previously described.

Chemicals and Materials
Cultures of astrocytes (Fisher Scientific). Antibodies to factor VIII and GFAP. Astrocytes were identified by their immunoreactivity to GFAP. In all cultures, purity was >95%. Subconfluent cultures of astrocytes (<5 passages) and of endothelial and smooth muscle cells (5 to 15 passages) were used. The effects of 8-iso-PGF2α were also studied on cultured human umbilical vein endothelial cells.

Statistical Analysis
Data were analyzed by 1-way ANOVA followed by post hoc Dunnett test for comparison among means. Values are presented as mean ± SEM. Statistical significance was set at P < 0.05.

Results
Effects of 8-IsopGF2α and 8-IsopPGF2α on Cell Viability
8-Isop-PGF2α caused a concentration-dependent (Figure 1A) and time-dependent (Figure 1B) death of cultured endothelial cells, as reflected by a decreased MTT reduction (Figure 1A and 1B). Cytotoxicity by 8-iso-PGF2α was detected at 24 hours (EC50 = 0.1 nmol/L); 8-iso-PGF2α was ineffective (Figure 1A). Smooth muscle cells (Figure 1C), astrocytes (Figure 1D), and human umbilical vein endothelial cells (93.2 ± 2.4% survival, 48 hours after addition of 8-iso-PGF2α) were not significantly affected by isoprostanes.

Nature of Cerebrovascular Endothelial Cell Death Induced by 8-Isop-PGF2α
Endothelial cell treatment with 8-iso-PGF2α for 24 to 48 hours also led to 25% to 30% cellular PI incorporation (Figure 2B).
and 2D), consistent with pathophysiologically significant cytotoxicity by other products of peroxidation. 4,20 8-Iso-PGF$_2\alpha$/H$_9251$ caused 8% TUNEL positivity (Figure 2B' and 2D) compared with approximately 30% with ceramide (Figure 2C). In accordance, 8-iso-PGF$_2\alpha$/H$_9251$ induced a time-dependent increase in LDH release in culture media (Figure 2E), indicative of cell membrane disruption and cytosolic protein leakage as seen during oncosis.

Involvement of TXA$_2$ in 8-iso-PGF$_2\alpha$/H$_9251$–Induced Endothelial Cell Death

Treatment of endothelial cells with 8-iso-PGF$_2\alpha$/H$_9251$ for 6 hours produced a 20-fold increase in TXB$_2$ levels (Figure 3A), as reported in neural tissue, 12,13,18 which gradually decreased to near control values by 48 hours (Figure 3A). Ibuprofen, U63557A, and CGS12970 decreased TXB2 levels of 8-iso-PGF$_2\alpha$/H$_9251$–treated preparations to 120 pg/10$^6$ cells (data not shown). These treatments abolished cytotoxicity of 8-iso-PGF$_2\alpha$/H$_9251$ (Figure 3B), while the TXA$_2$ mimetics U46619 and I-BOP caused endothelial cell death (Figure 3C). Consistent with the nature of cell death evoked by 8-iso-PGF$_2\alpha$/H$_9251$, caspase inhibitor Z-DEVD-FMK (50 μmol/L) diminished negligibly 8-iso-PGF$_2\alpha$/H$_9251$- and U46619-induced cell death, whereas, as expected, it prevented ceramide-elicited cell death 21 but not that caused by H$_2$O$_2$ (0.5 mmol/L). 22

Effects of 8-IsopGF$_2\alpha$/H$_9251$ on Isolated Cerebral Microvessels

8-iso-PGF$_2\alpha$/H$_9251$ caused a comparable increase in the proportion of rat and pig cerebral microvascular cells incorporating PI, which was markedly attenuated by CGS12970 (Figure 4).

In Vivo Effects of 8-IsopGF$_2\alpha$/H$_9251$ on Periventricular Brain Tissue

Because endothelial cell death is associated with compromised regional circulation,3,4 we tested the in vivo effects of 8-iso-PGF$_2\alpha$/H$_9251$ on periventricular brain tissue. Control (aCSF-treated) rats exhibited normal periventricular brain histology; the site of cannula implantation is clearly distinguishable (Figure 5A, indicated by asterisk). H$_2$O$_2$ (Figure 5B and 5G) caused extensive necrosis of the periventricular area near the infusion site, resulting in a cystlike formation of the pericorpus callosum area. Similarly, 8-iso-PGF$_2\alpha$/H$_9251$ and U46619, but not 8-iso-PGE$_2$ (data not shown), also led to brain necrosis (Figure 5C, 5E, 5G). Cotreatment with CGS12970 prevented effects of 8-iso-PGF$_2\alpha$/H$_9251$ (Figure 5D and 5G), as observed on endothelial cells (Figure 3B) and isolated microvessels (Figure 4A and 4B); CGS12970 alone did not...
affect brain histology (data not shown). A vasoconstrictor equipotent to U46619, namely, urotensin II, did not alter brain histology (Figure 5F).

**Discussion**

In this study we report that 8-iso-PGF$_2\alpha$, a major and stable product of lipid peroxidation, was potent in causing cell death of cultured brain microvascular endothelial cells as well as of ex vivo preparation of microvessels but not of human umbilical vein endothelium, smooth muscle, or astroglia; another related isoprostane, 8-iso-PGE$_2$, did not trigger endothelial cell death. 8-iso-PGF$_2\alpha$-induced cytotoxicity was TXA$_2$ dependent and mostly of oncotic nature. Associated with these effects, intraventricular injection of 8-iso-PGF$_2\alpha$ caused cystlike formations in the periventricular area.

The nature of cell death induced by 8-iso-PGF$_2\alpha$, seems to be primarily oncotic rather than apoptotic necrosis (Figure 2). This inference is supported by the data that 8-iso-PGF$_2\alpha$ caused <8% of cell DNA fragmentation, a time-dependent increase in PI incorporation and LDH release, indicative of membrane disruption (Figure 2), and its effects, as well as those of its mediator TXA$_2$, were unaltered by caspase inhibitor (Figure 3D). However, a process of cell death intermediate between apoptosis and necrosis cannot be excluded in view of the relatively long lag time between 8-iso-PGF$_2\alpha$ exposure and detection of cell death (Figure 1).
A major finding of this study is the important role of TXA₂ in 8-iso-PGF₂α-induced cerebral microvascular endothelial cell death (Figures 3 and 4) and brain white matter damage (Figure 5). We have previously shown that 8-iso-PGF₂α increases TXA₂ production in neurovascular tissue by stimulating via distinct channels calcium entry in endothelium and astroglia. TXA₂ in turn stimulates TXA₂ receptors on periventricular microvessel smooth muscle to evoke constriction. The contribution of TXA₂ in ischemic lesions has been attributed largely to its ability to cause vasoconstriction and platelet aggregation. Our findings do not support a primary role for vasoconstriction in 8-iso-PGF₂α-induced brain damage since the equivalently effective vasoconstrictor 8-iso-PGE₂ (data not shown) and the potent vasoconstrictor urotensin II did not affect brain histology even after 7-day treatment (Figure 5F). On the other hand, an effect of TXA₂ in retinovascular endothelial cell death has recently been reported. Interestingly, stimulation of calcium entry in cells by TXA₂ and 8-iso-PGF₂α is associated with both oncotic and apoptotic cell death. Calcium can activate specific phospholipases and proteases, disrupt mitochondrial permeability transition pore, resulting in arrest of ATP production.
and trigger the generation of reactive oxygen species; the latter in turn can further augment intracellular calcium and sustain this self-destructive cycle as triggered by TXA2 and 8-iso-PGF2α. The vulnerability of endothelial cells compared with other vascular and perivascular cells to the toxic actions of 8-iso-PGF2α is consistent with that observed with oxidants. This increased susceptibility of endothelial cells to 8-iso-PGF2α cannot be explained simply by a limited ability of the other vascular and perivascular cells to produce TXA2 since the latter cells generate this eicosanoid. Similarly, diminished TXA2 receptor density cannot explain lack of cytotoxicity to astroglial and smooth muscle cells because the latter abundantly express TXA2 receptors. However, TXA2 may activate cell-specific mechanisms resulting in different effects on distinct cell types. For instance, in smooth muscle cells TXA2 causes proliferation, as observed with 8-iso-PGF2α and oxidants.

8-iso-PGF2α-induced cystlike formations of the periventricular white matter (Figure 5C). Because these lesions seemed circumscribed near the site of injection and since the most abundant cells in brain, namely, astrocytes, were unaffected, a circulatory compromise secondary to microvascular degeneration is a likely significant contributory mechanism. Such a circulatory compromise secondary to microvascular degeneration: (1) oxidant stress, which greatly increases 8-iso-PGF2α generation in neural tissue, is implicated in hypoxic-ischemic brain injury such as periventricular leukomalacia; (2) 8-iso-PGF2α-induced cerebral vasoconstriction is greater in fetus and newborn than in juvenile; (3) rat pups injected into the vitreous with 8-iso-PGF2α exhibit retinal microvascular degeneration; and (4) intraventricular administration of 8-iso-PGF2α in normal young rats induces cystlike formations in periventricular white matter (Figure 5C). Despite the potentially greater vulnerability of the younger subject to peroxidation, cytotoxic effects of 8-iso-PGF2α may also be observed in mature vasculature. Indeed, the animals tested were not newborns (aged 21 days), endothelial cells in culture cannot be given a postconceptional age, and, interestingly, the cytotoxic effects of a major 8-iso-PGF2α mediator, TXA2, could be reproduced in cultured endothelial cells from adult human cerebral microvasculature.

In conclusion, our findings identified a new pathological effect of 8-iso-PGF2α in mediating cerebrocerebrovascular endothelial cell death through a TXA2-dependent process. This study provides additional insights into the mechanisms of oxidant stress–induced brain injury by involving the actions of 8-iso-PGF2α not only through its vasomotor effects but also through microvascular degeneration. The present observations may have important implications for other vascular disorders associated with peroxidation and TXA2 generation such as ischemic brain damage, atherosclerosis, and diabetes.

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