Role of thromboxane in retinal microvascular degeneration in oxygen-induced retinopathy

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OIR is associated with vascular cell injury culminating in microvascular degeneration, which precedes an abnormal neovascularization (7, 8, 14, 42, 43, 54). This microvascular degeneration leads to ischemia (10), which is thought to contribute to the structural and functional changes observed in OIR (35, 55). Oxidant stress plays an important role in the retinal vasoobliteration of OIR (48, 52, 53). Endothelial cells seem particularly susceptible to peroxidation-induced injury (17, 34); pericytes, smooth muscle cells, and perivascular astrocytes are relatively resistant (9, 17, 25). The mediators of oxidant stress-induced cell death are complex and not fully known.

Thromboxane A2 (TxA2) is abundantly generated after an oxidant stress (15, 21, 24) and contributes to neurovascular injury, including injury to the retina (20, 30, 44); however, its specific role in retinal vasoobliteration in OIR has not been demonstrated. Many of the vascular actions of TxA2 have been attributed to vasoconstriction and platelet aggregation (21). However, endothelial cytotoxicity in OIR occurs before platelet aggregation (6, 14, 43). In addition, TxA2 generation and hemodynamic compromise after an oxidant stress to newborn retina are independent of platelet aggregation (15). Thus it is possible that TxA2 may also cause other effects on microvasculature, more specifically vascular endothelial cells.

It has recently been shown that TxA2 affects intercellular communication by modifying expression of intercellular adhesion molecules (31), as well as by affecting the distribution of gap junctions on endothelial cells (5). TxA2 can augment the effects of oxidant stress by increasing oxygen radical generation (40). Moreover, TxA2 has been found to cause death of immature murine thymocytes (65). However, direct evidence that TxA2 induces death of other primary cells, and especially in this context of neuroretinal microvascular...
endothelial cells, has never been reported. Corroboration of this inference may further our understanding of the pathogenesis of ROP. We, therefore, tested the hypothesis that TxA\(_2\) plays a role in the microvascular degeneration of OIR, to which direct cytotoxic actions of TxA\(_2\) on neuroretinal microvascular endothelial cells can contribute. Data support our hypothesis and disclose a previously undescribed function of TxA\(_2\).

**MATERIALS AND METHODS**

*Animals.* Newborn Sprague-Dawley rats (Charles River, St. Constant, Quebec) and 1- to 3-day-old Yorkshire piglets (Fermes Ménard, L'Ange-Gardien, Quebec) were used according to a protocol of the Hôpital Sainte-Justine Animal Care Committee.

*OIR.* OIR was induced as described by our laboratory (35) and others (48, 52, 54). Briefly, rats were placed in an 80 ± 5% oxygen environment from postnatal days 5–14; controls were maintained in room air (21% O\(_2\)). By 5 days of age, the retinal vasculature of rats reached ~30% of its distance to the periphery, which it attains by 14 days of age (28); this allows testing of the desired effect of hyperoxia on degeneration of existing vessels (7, 8). Pups were randomly selected to receive throughout the study period intraperitoneal injection of polyinosinic-polycytidylic acid (3, 16). Animals were killed on day 15, and retinal flat mounts were prepared for ADPase staining (35, 39). Retinas were photographed (MTI CCD-72, Dage, Michigan City, MI) and magnified on screen to ×100 to allow us to visualize the microvasculature more clearly. Vascular density was calculated for the full retinal surface by using the software program Image-Pro Plus 4.1 (Media Cybernetics, Silver Spring, MD). Vascular density in study groups was compared with that of untreated groups raised in 21% O\(_2\), and their values were set as 100%.

*Cell count on microvessels from the nervous system.* Neuroretinal microvessels from rats (10–14 days old) and piglets were isolated as previously reported (36). Isolated microvessels were dispersed in Hanks’ balanced salt solution media and filtered twice through 25-μm nylon mesh to obtain mostly capillaries. Filtrate predominantly contained endothelium, as these small microvessels were immunoreactive to factor VIII but not to smooth muscle-specific actin (36). Endothelial cells (stained with Hoechst 33342) were isolated as previously reported (36). Isolated microvessels were cultured (porcine) retinovascular endothelial cells 1 h before treatment with U-46619. Cell viability was estimated by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (38). At the end of the experiment, MTT (0.5 mg/ml in PBS, pH 7.2) was incubated with cells for 2.5 h at 37°C. The medium was then drained, the formazan product was solubilized with acidic (40 mM HCl) isopropanol, and optical density was measured at 600 nm. Characterization of the type of cell death (necrosis or apoptosis) was studied by using membrane-impermeable and -permeable DNA-binding dyes PI and Hoechst 33342, respectively (41, 45). Cells were loaded for 15 min at 37°C with PI and Hoechst 33342 (5 μg/ml) and visualized with an immersion objective (×400) placed directly onto the culture medium using red and ultraviolet filters. Images were acquired with a digital camera attached to a microscope (Axioskop, Zeiss, Germany). To enhance reproducibility, cells were only counted in microvessels containing >20 endothelial cells. The proportion of dying cells was estimated as the ratio of PI-positive cells relative to all cells (stained with Hoechst 33342).

*Cell cultures.* Because the U-46619-induced proportion of cell death of neuroretinal microvessels from rat and pig was similar (see Fig. 2), and because a large number of rat pup retinas would be required to isolate microvessels from their small eyes for endothelial cell culture, we cultured retinovascular endothelial, smooth muscle, and astroglial cells from piglets as described (29, 36). The retinal structures and the development of the pig retinal vasculature have characteristics similar to those of humans (11).

Microvessels were suspended in selective endothelial growth media (Cletonics). Confluent cells were trypsinized and subcultured. Cell viability was verified by trypsin blue exclusion and was >95%. Endothelial cells were identified by anatomic structure and positive reactivity to factor VIII and negative reactivity to smooth muscle-specific actin and glial fibrillary acidic protein antibodies (GFAP). A similar technique was used for smooth muscle cell culture. The latter were identified by their spindle-shaped appearance and positive reactivity to smooth muscle-specific actin and negative reactivity to factor VIII and GFAP antibodies.

Neuroretinal astroglial cells were also cultured (29, 36). Essentially, retinas were homogenized and filtered through 230- and 150-μm nylon mesh, and the filtrate was centrifuged at 1,000 g for 7 min, resuspended in DMEM with 10% fetal calf serum, and incubated for 30 min at 37°C. Macrophages were removed with a rotary shaker at 225 rpm for 3 h. Purity of astrocytes was assessed by immunoreactivity to GFAP (>95%).

To further investigate species independence and endothelial-type cytotoxicity to TxA\(_2\) mimetics, the effects of the latter were tested on endothelial cells from different tissues from humans. Specifically, effects of U-46619 were tested on human endothelial cells from adult brain (46), as well as on human endothelial cells from aorta, dermis, and umbilical vein.

*Cell viability assays.* Confluent cells (5–15 passages) were reseeded in DMEM (without fetal calf serum) for 24 h and then incubated for up to 48 h with stable specific TxA\(_2\) mimetics U-46619 and [15-[[1α,2α(Z,3β)](1E,3S*,4αS)]-7-[[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.1.1]-hept-2-yl]-5-heptenoic acid (1-BOP) or with the relevant peroxidation product S-iso-PO\(_2\)\(_8\)a, which elicits thromboxane production (29, 36). In some experiments, TxA\(_2\)-receptor antagonist L-670596 (0.1 μM) was selected as it provided maximum efficacy. For similar purposes, poly(ADP-
ribose)/polymerase inhibition using nicotinamide (12) (1–100 nM) was also tested. Cell viability was assessed by MTT assay.

Measurement of DNA fragmentation and lactate dehydrogenase. DNA fragmentation was determined by a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-based technique using a commercial kit (Apoptag). Endothelial cells were grown on coverslips and treated with U-46619 for different time periods. Cells were washed twice with cold PBS, fixed with 4% paraformaldehyde at room temperature for 10 min, washed twice in PBS, and postfixed in ethanol-acetic acid (2:1) for 5 min at −20°C. After they were washed, cells were incubated with terminal deoxynucleotidyl transferase and FITC-conjugated dUTP for 1 h at 37°C in a humid chamber. The reaction was stopped by incubation with stop wash buffer (from kit) for 10 min at room temperature. Nuclei were counterstained with PI, washed, and mounted with Immuno-mount. Cells were visualized under fluorescence microscope.

Lactate dehydrogenase (LDH) activity was measured as follows (2). Briefly, 800 µl of reaction medium (80 mM Tris-HCl, 200 mM NaCl, 0.2 mM NADH) were added to 200 µl of the culture medium in a spectrophotometer cuvette. The reaction was started by adding 1.5 mM pyruvate (final). LDH activity was measured by spectrophotometric intensity changes at 340 nm. The LDH content was determined by using the following equation: [optical density/ time (min)] × 9,682 = units LDH/liter (2), where A is change.

Thromboxane and 8-iso-PGF2α assays. TXB2 (stable TxA2 metabolite) in retinas of animals exposed to hyperoxia was extracted by using octadecylsilyl silica columns and was measured by radioimmunoassay (15, 29, 36). Cross-reactivity of the antibody for other prostanoids is <2%, and interassay variability was <5%. Similar measurements were made on culture media of cells.

Isoprostanes were extracted from tissues as for TXB2 (15, 29, 36), reflecting the free active unesterified isoprostane (36), and was measured by immunoassay technique with a commercial kit, as described by the manufacturer (Cayman Chemical, Ann Arbor, MI) and previously utilized (36). The cross-reactivity of the antibody with TXB2, PGF2α, and 8-iso-PGF2α is ≤0.1%; intra- and interassay variability was ≤5%.

Chromosomes and materials. CGS-22652 and CGS-12970 were gifts from Ciba-Geigy (Summit, NJ), and L-670596 from Merck Frosste (Pointe-Claire, Quebec). Brain endothelial cells were generously provided by the National Research Council of Canada (Ottawa, Ontario). The following materials were purchased: human aortic, dural, and umbilical vein endothelial cells (Clonetics); ceramide, DMSO, nicotinamide, MTT, and PI (Sigma Chemical, St. Louis, MO); U-46619, I-BOP, 16,16-dimethyl-PGE2, and 8-iso-PGF2α (Cayman Chemical); Hoechst 33342 (Polysciences, Warrington, PA); Z-DEVD-FMK (R&D Systems, Minneapolis, MN); TXB2 radioimmunoassay kit (Amersham); antibodies to factor VIII, smooth muscle-specific actin, and GFAP (Dako, Capoitera, CA); FITC-conjugated goat anti-rabbit antibody (Jackson Immunoresearch Laboratories, West Grove, PA); Apoptag direct fluorescein kit (Intergen, Gaithersburg, MD); and other materials were purchased from Fisher (Montreal, Quebec).

Statistical analysis. Data were analyzed by one- or two-way ANOVA factoring for treatment and concentration or time, followed by the Tukey-Kramer method for comparison among means. Statistical significance was set at P < 0.05. Values are presented as means ± SE.

RESULTS

Role of TxA2 in hyperoxia-induced retinal microvascular degeneration. Control [vehicle (DMSO)-treated] animals exhibited a fully vascularized retina at 15 days of age as expected (6, 8, 14, 28). Exposure of rat pups to 80% O2 caused a marked increase in retinal TXB2 levels compared with levels observed in animals exposed to 21% O2 (Fig. 1A). The hyperoxia induced the expected retinal vasoobliteration (Fig. 1, B and C) (6–8, 54). TxA2 synthase inhibitor CGS-12970 decreased TXB2 levels and significantly attenuated the 80% O2-induced decrease in retinal vessel density. This vasoobliteration was similarly diminished by TxA2 receptor antagonist CGS-22652. CGS-12970 and CGS-22652 did not affect retinal vessel density of control rats maintained at 21% O2.

Effect of U-46619 on isolated retinal microvessels. The effect of the stable TxA2 mimic U-46619 on microvascular cell death was tested directly on retinal microvessels (<25 µm) containing primarily endothelial cells (factor VIII positive and smooth muscle actin negative). U-46619 increased PI incorporation in rat retinal microvasculature, which is indicative of cell death (Fig. 2A), such that the proportion of PI-positive cells relative to all cells (which stain to Hoechst 33342) was significantly augmented by U-46619. To ascertain these observations, this effect was also tested in the pig, which, although debated, has been used to produce an OIR (60, 62). U-46619 also caused increased PI incorporation in newborn pig retinal microvessels (Fig. 2B).

Effects of thromboxane mimetics on cell viability. Effects of TxA2 (mimetics) were tested directly on retinovascular endothelial cells from the piglet because a large number of rat pup retinas would otherwise be needed. U-46619 and I-BOP caused time- (Fig. 3, A and B) and concentration-dependent (Fig. 3C) death of retinovascular endothelial cells in culture (as reflected by a decrease in MTT). EC50 for U-46619 and I-BOP was ~50 and 5 nM, respectively (48 h). Selective TxA2 receptor antagonist L-670596 completely prevented U-46619-induced endothelial cell death (Fig. 3D), substantiating the selectivity of TxA2 mimetic actions. Other major prostanoids with vasoconstrictor properties comparable to TxA2, namely 16,16-dimethyl PGE2 (stable analogs of PGE2) and fenprostalene (stable analogs of PGF2α), did not affect endothelial cell viability (Fig. 3D).

In all experiments presented above, media contained U-46619 or I-BOP throughout the study period. To determine whether a shorter duration of exposure to TxA2 mimetics could induce cell death, the media were changed after different exposure times with a new one excluding U-46619; control cells were subjected to similar media changes. Cell death was quantified (by MTT assay) 48 h after the addition of U-46619 or the vehicle. Exposure to U-46619 (0.5 µM) for 4 h was sufficient to trigger cell death of the same magnitude as was observed after a longer duration of exposure (Fig. 3E).
In contrast to effects observed on retinovascular endothelial cells, U-46619 elicited negligible cell death of retinovascular smooth muscle cells and astrocytes (Fig. 3F).

Effect of endogenously generated thromboxane on cell death. OIR is associated with an oxidant stress (48, 52) and hence is expected to stimulate formation of the major peroxidation product 8-iso-PGF$_{2\alpha}$ (36, 57), which can generate thromboxane in the retina (36). Indeed, 8-iso-PGF$_{2\alpha}$ levels in the rat retina 1 day after exposure to hyperoxia were $396 \pm 21$ pg/mg protein and were significantly ($P < 0.01$) higher than in the control retina ($88 \pm 12$ pg/mg protein). We examined whether 8-iso-PGF$_{2\alpha}$ could induce a TxA$_2$-dependent endothelial cell death.

Fig. 1. A: effects of thromboxane A$_2$ (TxA$_2$) synthase inhibitor CGS-12970 and TxA$_2$-receptor antagonist CGS-22652 on hyperoxia-induced vasoobliteration in rats. Rats were exposed from postnatal days 5–14 to air or 80% O$_2$ (as described in MATERIALS AND METHODS). TxB$_2$, thromboxane B$_2$. B: pups were randomly assigned throughout study period to intraperitoneal injections of 50 μl of vehicle DMSO, selective TxA$_2$ synthase inhibitor CGS-12970 (10 mg·kg$^{-1}$·day$^{-1}$), or TxA$_2$-receptor antagonist CGS-22652 (10 mg·kg$^{-1}$·day$^{-1}$). Rats were killed on day 15, and retinal flat mounts were stained with ADPase. Vascular density was calculated for the total retinal surface, and results are presented as percent difference from untreated animals raised in air. Values in histogram are means ± SE of 4–5 retinas for A and 5–16 retinas for B. *P < 0.01 compared with all other values. C: original and negative photomicrographs presented are magnified to clearly display the microvasculature. Scale bar is 500 μm.

Fig. 2. Cytotoxic effects of U-46619 on freshly isolated rat pup (A) and newborn pig (B) neuroretinal microvessels. Microvessels (≤25 μm) containing primarily endothelium (factor VIII positive and smooth muscle actin negative) from 10- to 14-day-old rats and 1- to 3-day-old piglets were incubated for 24 h with U-46619 and then stained with Hoechst 33342 (identifies all cells) and propidium iodide (PI) as described in MATERIALS AND METHODS. Staining was visualized with an immersion objective placed directly onto the culture medium using red and ultraviolet filters. Note significant incorporation of PI in microvessel cells treated with U-46619, indicating cellular membrane disruption. Scale bars represent 20 μm. Values in histograms are means ± SE of proportion of PI-positive cells (relative to all cells stained with Hoechst 33342) in 3 experiments performed in quadruplicate. *P < 0.05 compared with saline-treated (control) microvessels (by ANOVA and comparison among means).
TxA₂-INDUCED ENDOTHELIAL CELL DEATH

Fig. 3. Time (A and B), concentration (C), and prostaglandin dependence and cell specificity of effects of TxA₂ mimetics U-46619 (A and C) and I-BOP (B and C) on cell viability. Cultured porcine neuroretinal microvascular endothelial cells were treated with U-46619 or I-BOP, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed at 48 h, unless otherwise indicated (in A). Cell viability in untreated cells was ≥95%.

*P < 0.05 compared with values at 12 h (A and B) and other concentrations of U-46619 at corresponding time as well as to 10⁻¹¹ M TxA₂ mimetics (C) (1- or 2-way ANOVA factoring for time and concentrations). D: effects of (1 μM) contractile prostanoids fenprostalene and 16,16-dimethyl-PGE₂ and of U-46619 in presence of selective TxA₂-receptor blocker L-670596 (0.1 μM) (22). *P < 0.01 compared with control (vehicle-treated) value. E: effects of variable exposure time to U-46619 (0.5 μM) on cell viability. Cell death was assayed at 48 h; cell death was quantified (by MTT assay) 48 h after addition of U-46619 or vehicle to media containing the cells. *P < 0.01 compared with control (vehicle-treated) value. F: effects of U-46619 (1 μM) on retinal smooth muscle and astroglial cells. +P < 0.01 compared with control values. Values are means ± SE of 3–6 (A, B, C, F), 3–4 (D), or 4–7 (E) experiments, each performed in quadruplicate and expressed as percentage of control.

Fig. 4. Effects of 8-iso-PGF₂α on thromboxane generation (A) and TxA₂-dependent death of retinal microvessel endothelial cells (B). Cultured endothelial cells were exposed to 8-iso-PGF₂α (100 nM) for 48 h. TxB₂ levels in media were measured at indicated times, and cell death was determined by MTT assay at 48 h in the absence and presence of TxA₂ synthase inhibitor CGS-12970 (1 μM). *P < 0.01 compared with other values at corresponding times or concentrations and to initial values (2-way ANOVA factoring for time or concentration and treatment).
(Fig. 3) was consistent with a corresponding increase in PI incorporation (Fig. 5, b and d). The number of cells with intact membranes and fragmented or condensed chromatin (Fig. 5a) as well as TUNEL-positive cells (Fig. 5c) (indicative of apoptosis) was ≤8%, even after 48-h exposure to U-46619 (Fig. 5d). In accordance with these observations, the caspase inhibitor Z-DEVD-FMK (50 μM) only slightly reduced U-46619-triggered cell death, whereas, as anticipated, cell death induced by ceramide (27) was totally prevented by Z-DEVD-FMK and that by H2O2 (0.5 mM) was unaffected (23) (Fig. 5e). Similarly, inhibition with nicotinamide (12) (1–100 nM) of the poly(ADP-ribose)polymerase also involved in apoptosis did not prevent U-46619-induced cell death.

![Fig. 5. Effects of U-46619 on chromatin condensation PI incorporation, DNA fragmentation, and lactate dehydrogenase (LDH) release by porcine cerebrovascular endothelial cells, and contribution of caspase in U-46619-induced cell death.](image)

- **a**: Chromatin staining of cells with membrane-permeable Hoechst 33342 (identifies cells) was done 48 h after exposure to U-46619 (0.5 μM). Solid arrow indicates few cells containing condensed chromatin (intense fluorescence).
- **b**: Chromatin staining of the same cells as in a using membrane-impermeable PI, which reflects membrane disruption, which is indicative of necrotic cell death. Open arrow points to a cell without chromatin condensation in a and the same cell permeable to PI in b. Other PI-positive cells are not indicated in a to avoid overcrowding.
- **c**: In situ staining of 3’OH DNA fragments (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining) of endothelial cells exposed for 24 h to U-46619 (0.5 μM). Note rare positively (green) stained nuclei. Scale bar represents 10 μm.
- **d**: Time course of PI incorporation (●) and TUNEL-positive cells (○) exposed to U-46619 (0.5 μM). Values are means ± SE of percentage of cells viewed in 5 fields/coverslip on 3 separate preparations, each performed in triplicate. *P < 0.01 compared with basal values (1-way ANOVA); †P < 0.01 compared with TUNEL-positive cells.
- **e**: Effects of effector caspase inhibitor Z-D(OMe)-E(OMe)-V-D(OMe)-FMK (Z-DEVD-FMK) (50 μM) on cell death induced by U-46619 (0.5 μM), ceramide (8 μM), or H2O2 (0.5 mM). Cells were preincubated (hatched bars) or not (open bars) with Z-DEVD-FMK, and viability was determined by MTT assay. Values are means ± SE of percentage of controls of 3 separate experiments, each performed in triplicate. *P < 0.01 compared with basal values (1-way ANOVA); †P < 0.05 compared with other treatments.
- **f**: LDH activity in the endothelial cell media at different time periods after addition of U-46619 (0.5 μM). Values are means ± SE. *P < 0.05 compared with other values (1-way ANOVA).
cell death (n = 4). In contrast, LDH release in response to U-46619 (indicative of necrosis) increased in a time-dependent manner (Fig. 5f).

Effects of thromboxane mimic on endothelial cells from different human tissues. To further ascertain U-46619-induced neurovascular endothelial cytotoxicity, effects of this Tx2 mimetic were tested on human endothelial cells from (adult) brain (46) and other tissues. U-46619 caused death of endothelial cells from brain but not from aorta, dermis, or umbilical vein (Fig. 6). Hence data suggest that neurovascular endothelial cells are particularly susceptible to Tx2-elicited toxicity.

DISCUSSION

The mechanisms underlying the microvascular degeneration observed in the OIR, a model of ischemic retinopathy (10, 54) comparable to ROP (6–8, 42), are mostly unknown. The present study was conducted to determine whether Tx2 played a role in retinal microvascular obliteration in OIR and, if so, to determine whether Tx2 could contribute to microvascular degeneration by inducing endothelial cytotoxicity. Our data support this inference and provide evidence for a novel function for Tx2 as an inducer of retinovascular endothelial cell death.

The cytotoxic effect of Tx2 (Figs. 2 and 3) is relatively cell type selective because it was observed in neuroretinovascular endothelial cells but not in smooth muscle and astroglial cells. Also, Tx2 (mimetics) did not affect endothelial cells from nonneuronal tissues (Fig. 6). Inhibitors of endogenous Tx2 generation prevented cell death (Figs. 1 and 4); hence it may be inferred that Tx2 also contributes to the retinal microvascular degeneration observed in vivo. This cytotoxic action of Tx2 is unlikely to be caused entirely by its vasoconstrictor effects, because other vasoconstrictor prostanoids (PGE2 and PGF2α, analogs) did not cause cell death (Fig. 3D). Similarly, it is improbable that it is simply due to platelet aggregation (6–8, 14, 15, 42).

Cultures of endothelial cells were performed on retinal vessels of newborn pigs because of the small number of microvessels available in rat pup retinas. However, the newborn pig is born with a fully vascularized retina (11), in contrast to the rat (28) and premature infant (6). On the other hand, retinal structures and vasculature and their development in the pig have characteristics similar to those of the human (11). Despite differences between rat and pig retinas, Tx2-induced cell death was similar in the microvessels of rat and pig (Fig. 2). This implies a species-independent cytotoxic effect of Tx2 on neuroretinal vascular endothelial cells (Figs. 2 and 6).

An important role for Tx2 in retinal vasooobliteration of OIR is suggested by the data showing the prevention of the loss of ADPase staining in endothelial cells (39) by Tx2 synthase and receptor blockers CGS-12970 and CGS-22652 (Fig. 1). Although ADPase can also be found in pericytes and smooth muscle cells (39), because the Tx2 mimetics U-46619 and I-BOP induced death of endothelial but not smooth muscle cells (Fig. 3), the loss of vascular staining in OIR is more likely consistent with the requirement of endothelium for pericyte survival (19). One can argue that, whereas Tx2 production is stimulated by oxidant stress (15, 21, 24), because the latter is known to contribute to the retinal vasooobliteration in OIR (48, 52), the protective effects of Tx2 synthase and receptor blockers CGS-12970 and CGS-22652 on vasooobliteration may be explained by postulated antioxidant properties of these agents. However, these types of compounds do not block the oxidant stress-induced increase in peroxidation products (15). Furthermore, it should be noted that results obtained with CGS-12970 and CGS-22652 contrast with those reported with the cyclooxygenase inhibitor indomethacin, which was associated with greater vasooobliteration in OIR (47). This apparent discrepancy can be due to the lack of specificity of indomethacin, which compromises circulation (50), and/or by inhibition of the formation of the cytoprotective prostaglandins such as PGI2 and PGE2 (56, 66). All in all, based on evidence previously reported (47, 50, 56, 66) and especially presented in this study (Figs. 1–4), the prostanoid Tx2 seems to mediate the effects of oxidant stress (15, 29, 36) by contributing significantly to the microvascular degeneration in OIR and possibly ROP.

Tx2 exerts known functions that may contribute to vasooobliteration, notably platelet aggregation and vasoconstriction (21). Platelet aggregation is involved in other forms of ischemic neuropathies (51). However, studies of oxidant stress on impaired ocular hemodynamics (15) and many others on OIR reveal an early endothelial cytotoxicity that is independent of platelet aggregation (6–8, 14, 42), although this can be detected later (42). The degree of retinal vasoconstriction evoked by Tx2 is unlikely to result in vasooobliteration (1), as supported by the effects of other important retinal vasoconstrictors such as (analogs of) PGF2α, which are equally released under oxidant stresses (15) but did not cause cell death (Fig. 3D). Hence, effects of Tx2 other than platelet aggregation and vasoconstric-
tion probably also contribute to the retinal vasobilitation associated with OIR.

Indeed, a major finding of this study is the direct and relatively selective thromboxane-induced cytotoxicity to retinovascular endothelial cells (Fig. 3); this effect was observed by using different TxA2 mimetics and prevented by a selective TxA2-receptor antagonist (Fig. 3, A–D). Although oxidant stress can stimulate TxA2 generation in neural tissues over long durations (Figs. 1 and 4; Ref. 61), data suggest a triggering action of TxA2 in inducing retinovascular endothelial cell death; exposure of cells for only 4 h to U-46619 was sufficient to induce cell death detected 48 h later (Fig. 3E). The increased vulnerability of retinovascular endothelial cells to TxA2 cannot simply be explained by a limited expression of TxA2 receptors in astrocytes and certainly not in smooth muscle cells (26, 32, 33). Similarly, the effect of TxA2 on retinovascular endothelial cells also seems to distinguish itself from that on other endothelial cells (Fig. 6). Consistent with our observations, it has been reported that TxA2 causes migration of renal microvascular endothelial cells (18) but not of human umbilical vein endothelial cells (5), which are also known to contain TxA2 receptors (32). This probably reflects the heterogeneity of endothelium (63), such as, for instance, the distinct properties of glomerular and brain endothelium. Hence, differences in cellular phenotypes contribute to cell-specific TxA2-induced effects. Altogether, our data strongly suggest that TxA2 is cytotoxic to neuroretinal microvascular endothelium, which might contribute to the retinal vasobilitation of OIR.

Retinovascular endothelial cell death induced by TxA2 (using the mimetic U-46619) does not seem to be due primarily to apoptosis. Nuclear condensation and DNA fragmentation were observed in ≤8% of cells (Fig. 5, a, c, and d). Inhibition of major effector caspases only slightly reduced TxA2 mimetic-induced cell death (Fig. 5e); a similar inefficacy of the poly(ADP-ribose) polymerase inhibitor nicotinamide (12) was observed. On the other hand, U-46619 caused a time-dependent increase in PI incorporation and LDH release, indicative of membrane disruption and suggestive of necrosis (Fig. 5, b, d, and f). Nonetheless, because of the relatively long lag time (24 h) between TxA2 mimetic treatment and detection of cell death (Fig. 3, A and B), one cannot totally exclude a form of cell death intermediate between apoptosis and necrosis as proposed for other cells and termed “necrapoptosis” (37, 59).

The mechanisms for TxA2-induced cytotoxicity of neuroretinal vascular endothelial cells are not clear. A possibly important mechanism may involve cellular mobilization and incorporation of calcium by TxA2 (4). There is strong evidence that an increase in intracellular calcium can induce both necrotic and apoptotic cell death processes (49, 64). Changes in cellular calcium can activate specific phospholipases and proteases, disrupt mitochondrial permeability transition pores, which results in arrest in ATP production, and stimulate the generation of reactive oxygen species (49, 64), which can in turn sustain a self-destructive cycle (13).

Interestingly, we found that treatment of retinovascular endothelial cells with U-46619 caused a four-fold increase in hydroperoxides, consistent with other reports (40), and, more importantly, the induced cell death was prevented by the antioxidant U-74389G (data not shown). Studies on mechanisms of TxA2-induced neuroretinovascular endothelial cell death are presently under investigation.

In conclusion, this study unveils for the first time an important role for a specific factor in the retinal microvascular degeneration of OIR, namely TxA2. Also, TxA2 may contribute in this process through a previously undescribed function, specifically by directly inducing retinovascular endothelial cell death. We speculate that TxA2-induced microvascular endothelial degeneration could contribute to the pathogenesis of ROP and other ischemic retinopathies and perhaps encephalopathies (21, 24, 30, 44, 58).

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