Dominant Role for Calpain in Thromboxane-Induced Neurourothelial Endothelial Cytotoxicity

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ABSTRACT
Thromboxane A$_2$ (TXA$_2$) is an important lipid mediator generated during oxidative stress and implicated in ischemic neural injury. This autacoid was recently shown to partake in this injury process by directly inducing endothelial cytotoxicity. We explored the mechanisms for this TXA$_2$-evoked neural microvascular endothelial cell death. Stable TXA$_2$ mimetics 5-heptenoic acid, 7-[6-(3-hydroxy-1-octenyl)-2-oxabicyclo[2.2.1]hept-5-yl]-[1R-[1a,4a,5p,6a(1E,3S)]]-9,11-dedioxy-9α,11α-methanopyloxy (U46619) as well as [15-[1α,2α(3Z,3′E,3″S),4α]-7-[3-[3-hydroxy-4-(4-iodophenoxoy)]-1-butenyl]-7-oxabicyclo[2.1.1]-hept-2-yl]-5-heptenoic acid; I-BOP) induced a retinal microvascular degeneration in rat pups in vivo and in porcine retinal explants ex vivo and death of porcine brain endothelial cells (in culture). TXA$_2$ dependence of these effects was corroborated by antagonism using the selective TXA$_2$ receptor blocker (–)-6,8-difluoro-9-p-methylsulfonyl-benzyl-1,2,3,4-tetrahydrocarbazol-1-yl-acetic acid (L670596). In all cases, neurovascular endothelial cell death was prevented by pan-calpain and specific m-calpain inhibitors but not by caspase-3 or pan-caspase inhibitors. Correspondingly, TXA$_2$ (mimetics) augmented generation of known active m-calpain but not μ-calpain) form and increased the activity of m-calpain (cleavage of fluorogenic substrate N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; and of α-spectrin into specific fragments) but not of pan-caspase or specific caspase-3 (respectively, using sulforhodamine-Val-Arg-Asp-fluoromethyl ketone and detecting its active 17- and 12-kDa fragments). Interestingly, these effects were phospholipase C (PLC)-dependent [associated with increase in inositol triphosphate and inhibited by PLC blocker 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122)] and required calcium but were not associated with increased intracellular calcium. U46619-induced calpain activation resulted in translocation of Bax to the mitochondria, loss of polarization of the latter (using potentiometric probe 5,5′,6,6′-tetraethylbenzimidazolyl-carbocyanine iodide; JC-1) and in turn release of cytochrome c into the cytosol and depletion of cellular ATP; these effects were all blocked by calpain inhibitors. Overall, this work identifies (specifically) m-calpain as a dominant protease in TXA$_2$-induced neurovascular endothelial cell death.

Increasing evidence points to neural microvascular endothelial dysfunction and cytotoxicity in ischemic brain injury (Lee and Lo, 2003); an analogous profile is well described in ischemic cardiopathies. This microangiopathy is a major feature of diabetic complications, including retinopathy. Neurovascular endothelial cells seem particularly susceptible to the peroxidation involved in such types of injuries

ABBREVIATIONS: TXA$_2$, thromboxane A$_2$; TP, thromboxane receptor; Calp, calpain; U-46619, 5-heptenoic acid, 7-[6-(3-hydroxy-1-octenyl)-2-oxabicyclo[2.2.1]hept-5-yl]-[1R-[1α,4α,5β(3Z,6α(1E,3S)]]-9,11-dedioxy-9α,11α-methanopyloxy; I-BOP, [15-[1α,2α(3Z,3′E,3″S),4α]-7-[3-[3-hydroxy-4-(4-iodophenoxoy)]-1-butenyl]-7-oxabicyclo[2.1.1]-hept-2-yl]-5-heptenoic acid; L670596, (–)-6,8-difluoro-9-p-methylsulfonyl-benzyl-1,2,3,4-tetrahydrocarbazol-1-yl-acetic acid; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; JC-1, 5,5′,6,6′-tetraethylbenzimidazolyl-carbocyanine iodide; U73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; TRITC, tetramethylrhodamine B isothiocyanate; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; PI, propidium iodide; LDH, lactate dehydrogenase; SR, sulforhodamine; AMC, 7-amino-4-methylcoumarin; Ca$^{2+}$, intracellular calcium concentration; PKC, protein kinase C; Z, N-benzoyloxycarbonyl; Suc-LLVY-AMC, N-succinyl-Leu-Leu-Val-Tyr-7-AMC; DEVD, Asp-Glu-Val-Asp; fmk, fluoromethyl ketone; VAD, Val-Arg-Asp.
(Beauchamp et al., 2001; van Reyk et al., 2003). In the process of this oxidative stress, cytosolic phospholipase A₂ is activated and ultimately leads to the predominant generation of thromboxane (TXA₂) over that of other eicosanoids (Ogletree, 1987; Chemtob et al., 1995); TXA₂ in turn exerts cellular effects by interacting with its receptor, named TP.

Cytotoxic effects of TXA₂ have largely been attributed to its hemodynamic actions elicited through vasoconstriction and platelet aggregation (FitzGerald et al., 1987). But more recently, direct toxicity in response to activation of the TXA₂ receptor has been uncovered in thymocytes (Ushikubi et al., 1993), throphoblasts (Yusuf et al., 2001), renal tubule epithelial cells (Jariyawat et al., 1997), ventricular myocytes (Shizukuda and Buttrick, 2002) as well as in (rat, porcine, and human) neural microvascular endothelial cells (Lahaie et al., 1998; Beauchamp et al., 2001). In contrast, stimulation of receptors for related prostanoids prostaglandin E₂ and prostaglandin F₂₀ did not lead to toxicity. Of relevance, the major peroxidation products of the isoprostanes exert neurovascular endothelial cytotoxicity via TXA₂/TP pathway (Beauchamp et al., 2001; Brault et al., 2003). Furthermore, an important role for TXA₂ has been demonstrated in retinal vaso-obliteration association with ischemic retinopathies before platelet aggregation (which itself generates TXA₂) (Beauchamp et al., 2002). This TXA₂/TP-induced neurovascular endothelial cell death is delayed by 12 to 18 h, albeit it does not exhibit classic features of apoptosis such as chromatin condensation, terminal deoxynucleotidyl transferase dUTP nick-end labeling positivity, and frequently observed caspase dependence; accordingly, a role for other cysteine proteases may be inferred.

Caspases, calcium-dependent calpains, and cathepsins compose the three major groups of cysteine proteases. Conversely, ubiquitous calpain isoforms Calp I (μ-calpain) and Calp II (m-calpain) are abundantly expressed in the central nervous system (Ray et al., 2003), and calpain activity is increased in the process of cell death applied to neurodegeneration and ischemic central nervous system events (Saito et al., 1993; Majno and Joris, 1995). Interestingly, calpain activity has mostly been associated with necrosis, whereas caspase activity is largely associated with classic apoptosis (Wang, 2000). However, whether calpains participate in TXA₂-induced endothelial cytotoxicity and the mechanisms involved in this process have yet to be described.

Therefore, we investigated the mechanisms of TXA₂-induced neurovascular endothelial cell death, with particular emphasis on the role of calpains; for this purpose, established stable mimics of this prostanoid known to activate its TP receptor were used (U-46619 and at times I-BOP). Our findings reveal that TXA₂ (mimics) elicited a neuroretinal microvascular degeneration in vivo and in tissue explants (ex vivo) and in primary neural endothelial cells (cultures) via a mechanism dependent upon calpain activity and that degeneration was prevented specifically by μ-calpain inhibitor and mediated in turn by increased mitochondrial Bax/Bcl-2 ratio associated with loss of mitochondrial membrane polarity and consequent ATP depletion. In contrast, pan-caspase and more specifically caspase-3 activity and role were not involved. Thus, we hereby disclose an important mode of action of TXA₂ in eliciting neurovascular endothelial cytotoxicity.

**Materials and Methods**

**Chemicals and Materials.** L670596 was a gift of Merck Frosst (Pointe-Claire, QC, Canada). The following materials were purchased: ceramide, dimethyl sulfoxide, cycloheximide, staurosporine, Nonidet P-40, and 3,4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazo- lium bromide (MTT) (Sigma-Aldrich, St. Louis, MO); U-46619 and I-BOP (Cayman Chemical, Ann Arbor, MI); JC-1, valinomycin, Hoechst 33342, and propidium iodine (Invitrogen, Burlington, ON, Canada); calpain inhibitors IV and V, U73122 (Calbiochem, San Diego, CA); and Z-DEVD-fmk caspase-3 inhibitor (R&D Systems, Minneapolis, MN). Other materials were purchased from Fisher Scientific Co. (Montreal, QC, Canada).

TXA₂-elicited effects were tested using stable analogs of the prostanoids, namely, U-46619 and I-BOP, both of which are known to stimulate specifically the TP receptor (Beauchamp et al., 2001). TXA₂-elicited actions were further corroborated using the selective TXA₂ antagonist L670596 (Beauchamp et al., 2001).

**Animals.** Newborn Sprague-Dawley rats (Charles River Canada, Montreal, PQ, Canada) and 1- to 3-day-old Yorkshire piglets (Fermes Ménard, L’Ange-Gardien, QC, Canada) were used according to the protocol of the Hôpital Sainte-Justine Animal Care Committee and in accordance with regulations of the Canadian Council of Animal Care.

**Intravitreous Injections.** Retinovascular degeneration was studied in rat pups as reported previously (Beauchamp et al., 2001). Retinal explants (1-3-mm² fragments, placed on a Transwell membrane, (Whatman, Maidstone, UK), and left to float on the surface of DMEM (2% fetal bovine serum) culture medium at 37°C with 5% CO₂ in six-well plates (three explants per membrane). Retinal explants were treated with vehicle or U-46619 (1 μM) in absence or presence of TXA₂ receptor blocker L670596 (estimated final concentration 1 μM (30-μl ocular volume) as described previously; Lahaie et al., 1998; Beauchamp et al., 2001), pan-calpain inhibitor (Calp V; final concentration, 1 μM), or caspase-3 inhibitor Z-DEVD-fmk (final concentration, 50 μM). Some preparations were treated with the pan-caspase inhibitor V-Z-VAD-fmk (50 μM). Rats were euthanized on postnatal day 10, and retinas were isolated for endothelial cell staining with the TRITC-conjugated lectin Griffonia simplicifolia (Sigma-Aldrich). Retinas were visualized using a fluorescent Nikon Eclipse E800 microscope and photographed with a Nikon digital camera DMX1200. Vascular density was determined using a computer software (Image-Pro Plus 4.1; Media Cybernetics, Inc., Silver Spring, MD) as reported previously (Lahaie et al., 1998; Beauchamp et al., 2001).

**Retinal Explants.** To ascertain that the vasculotoxic effects of TXA₂ are hemodynamic-independent and can be reproduced in different species, the effects of the TXA₂ analog U-46619 were tested on retinal explants of 1- to 3-day-old pigs. Dissected retinas were cut into 5-mm² fragments, placed on a Transwell membrane, (Whatman, Maidstone, UK), and left to float on the surface of DMEM (2% fetal bovine serum) culture medium at 37°C with 5% CO₂ in six-well plates (three explants per membrane). Retinal explants were treated with vehicle or U-46619 (1 μM) in the absence or presence of L670596 (1 μM), m-calpain inhibitor Calp IV (Angliker et al., 1992) (k₂ = 28,900 M⁻¹ s⁻¹ (1 μM), pan-calpain inhibitor Calp V (Èszer et al., 1994) (1 μM), or Z-DEVD-fmk (50 μM). Some preparations were treated with the pan-caspase inhibitor V-Z-VAD-fmk (50 μM). μ-Calpain inhibitors are not yet available. After 3 days of incubation, the medium was removed, and the explants were fixed at room temperature with formalin and permeabilized with ice-cold 100% methanol. Tissues were washed three times with 1% Triton X-100 in PBS and stained with 1:100 TRITC-lectin G. simplicifolia (in 1% Triton X) overnight. Explants were flat mounted and visualized with a fluorescent microscope, and microvasculature was quantified as described above for the intravitreal injection experiments.

**Cells.** Neural microvessels (≤25 μm) were isolated from piglet brains as detailed previously (Lahaie et al., 1998; Beauchamp et al., 2001). Microvessels were suspended in selective endothelial growth media (Cambrex Bio Science Walkersville, Walkersville, MD), and endothelial cells were grown to confluence as reported.
previously (Lahaie et al., 1998; Beauchamp et al., 2001). Endothelial cells were identified morphologically and by their positive reactivity to FVIII and negative reactivity to smooth muscle-specific actin and glial fibrillary acidic protein. Only low passage (less than seven) cell cultures were used.

**Cell Viability Assay.** Cells at approximately 80% confluence were reseeded in DMEM (without fetal bovine serum) for 18 to 24 h. Cells were incubated for up to 24 to 36 h with stable TXA2 mimetics U-46619 and at times with I-BOP in the absence or presence of the selective TXA2 receptor blocker L670596 (1 μM), Calp IV (1 μM), Calp V (1 μM), or caspase-3 inhibitor Z-DEVD-fmk (50 μM). Cell viability was determined by mitochondrial-dependent reduction of MTT in cells containing approximately 60,000 cells as described in detail previously (Lahaie et al., 1998; Beauchamp et al., 2001). Reliability of assay was confirmed by cell counting. At the end of the experiment, MTT (0.5 mg/ml in PBS, pH 7.2) was incubated with cells for 2 h at 37°C. The medium was then drained, and the formazan product was solubilized with acidified (40 mM HCl) isopropanol. Optical density was measured at 600 nm. Cell death was further established using membrane-impermeable DNA-binding dye propidium iodide (PI) (McGahon et al., 1995) and released lactate dehydrogenase activity (LDH) (Lahaie et al., 1998; Beauchamp et al., 2001). LDH activity was measured spectrophotometrically at 340 nm as follows (Allain et al., 1973). In brief, 800 μl of reaction medium (80 mM Tris-HCl, 200 mM NaCl, and 0.2 mM NADH) was added to 200 μl of the culture medium in a spectrophotometer cuvette. The reaction was started by adding 1.5 mM pyruvate (final). LDH content was calculated as \[
\frac{\text{LDH content}}{\text{LDH/volume}} \times 9682 = \text{units of LDH/volume}.
\]

**Caspase and Calpain Activity.** Pan-caspase reactivity was determined using the fluorogenic substrate SR-VAD-fmk (BIOMOL Research Laboratories, Plymouth Meeting, PA) which fluoresces (red) upon binding to the active enzyme (Grabarek et al., 2002). Essentially, microvascular endothelial cells were seeded on cover slips and treated as described above; staurosporine (1 μM) was used as positive control activator of caspase. At the end of drug exposure period, SR-VAD-fmk was added to the cell media for 1 h at 37°C. Cells were counterstained with Hoechst dye 33342. Cells were photographed under fluorescent microscopy and quantified with the Image-Pro software described above. In addition, caspase-3 activity was determined by membrane-impermeable DNA-binding dye propidium iodide (PI) (McGahon et al., 1995) and released lactate dehydrogenase activity (LDH) (Lahaie et al., 1998; Beauchamp et al., 2001). LDH activity was measured spectrophotometrically at 340 nm as follows (Allain et al., 1973). In brief, 800 μl of reaction medium (80 mM Tris-HCl, 200 mM NaCl, and 0.2 mM NADH) was added to 200 μl of the culture medium in a spectrophotometer cuvette. The reaction was started by adding 1.5 mM pyruvate (final). LDH content was calculated as \[
\frac{\text{LDH content}}{\text{LDH/volume}} \times 9682 = \text{units of LDH/volume}.
\]

**Inositol Phosphate Measurements.** Endothelial cells grown in 12-well plates were labeled with 1 to 2 μCi/ml [3H]myo-inositol (17 Ci/mmol; GE Healthcare, Little Chalfont, Buckinghamshire, UK) overnight. The cells were preincubated in DMEM containing 10 mM LiCl with or without the phospholipase C (PLC) inhibitor U73122 (0.1 μM) or L670596 (1 μM) and treated with U-46619 (0.1 μM) for 30 min at 37°C. The reaction was terminated by addition of 0.5 volume of NaOH (100 mM), followed by acidification with 2 mM formic acid. Total inositol phosphates were separated by using Dowex AG1X8 (formate form) and 1.2 M ammonium formate in 0.1 N formic acid as the eluant. Radioactivity of phosphoinositides was determined in liquid scintillation cocktail.

**Intracellular Ca2+ Measurements.** Intracellular Ca2+ concentration ([Ca2+]i) was measured using the fluorescent Ca2+ indicator fura 2-acetoxymethyl ester as described previously. Cells were reseeded in Hanks’ balanced salt solution with Ca2+ (2.5 mM) and 1% fetal bovine serum, and they were then stimulated with U-46619 (0.1 μM), EGTA (5 mM), or bradykinin (1 μM). [Ca2+]i was determined in 2 ml of fura 2-acetoxymethyl ester-loaded cell suspension and measured using a spectrophotofluorometer (model LS 50; PerkinElmer Life and Analytical Sciences, Beaconsfield, UK); excitation wavelengths were 340 and 380 nm, and emission was at 510 nm. Calibration of the fluorescent signal was determined on 2 ml of cell suspension by sequential addition of 0.2% Triton X-100 to obtain the maximal fluorescence ratio (Rmax) and of 5 mM EGTA to obtain the minimal fluorescence ratio (Rmin). Autofluorescence was determined by measuring fluorescence from nonloaded cells and subtracting it from the fluorescence produced by fura 2-loaded cells to calculate the fluorescence ratio R corresponding to the values produced at

![Fig. 1. TXA2-mediated retinal microvascular vasodilation. Rat pups on postnatal day 7 were injected intravitreally (1 μl) with vehicle or U-46619 in the absence or presence of TXA2 receptor antagonist L670596 (estimated final concentration 1 μM (30-μl ocular volume) as described previously; Lahaie et al., 1998; Beauchamp et al., 2001, 2002), pan-caspase inhibitor Calp V (final concentration, 1 μM), or Z-DEVD-fmk caspase-3 inhibitor (final concentration, 50 μM). Retinas were removed on postnatal day 10, and endothelium was stained with TRITC-conjugated lectin G. simplicifolia to determine vessel density. A, representative retinal flat mounts depicting vascular network. A, compiled vessel density of retinas treated as described above. Values are mean ± S.E.M. of three to four experiments. *, P < 0.05 compared with all other values without asterisks.](image-url)
340 and 380 nm. The $[Ca^{2+}]_i$ was calculated from the equation 
$$[Ca^{2+}]_i = K_d \cdot \frac{(R - R_{\text{min}})(R_{\text{max}} - R)}{S_{\text{f2}}/S_{\text{b2}}},$$ 
where $K_d$ (224 nM) is the effective dissociation constant of the fura 2-Ca$^{2+}$ complex, and $S_{\text{f2}}/S_{\text{b2}}$ is the ratio of fluorescence intensity at 380-nm wavelength in the presence of EGTA to that in the presence of Triton X-100 (Gryniewicz et al., 1985).

**Western Blot.** Microvascular endothelial cells were treated with U-46619 in the absence or presence of inhibitors as described for cell viability assays. Twenty micrograms of protein from the lysate, mitochondrial, or cytosol soluble fractions was loaded on SDS-polyacrylamide gel electrophoresis (8 or 12%) and probed with Bax, Belo, cytochrome c (BD Biosciences PharMingen, San Diego, CA), non-erythroid α-spectrin, active caspase-3 (Chemicon International, Temecula, CA), latent and active m-calpain (Chemicon International), and β-actin (Abcam, Cambridge, UK).

**Mitochondrial and Cytosol Fraction Isolation.** Cell fractionation was performed as described in detail previously (Gobeil et al., 2003). Stimulated cells were rinsed with PBS and concentrated by centrifugation (500 g). Cells were suspended in lysis buffer, pH 7.4 (10 mM Tris-HCl, 10 mM NaCl, 3 MgCl$_2$, and 30 mM sucrose) and homogenized with a glass homogenizer (300 strokes). Lysate was centrifuged at 700 g for 10 min at 4°C to remove nuclei and debris, and the supernatant was recentrifuged at 10,000 g for 15 min. The mitochondrial-containing pellet was resuspended in phosphate buffer and used for corresponding experiments (see below), whereas the supernatant was centrifuged again at 120,000 g for 1 h to obtain the S100 cytosolic fraction.

**Mitochondrial Membrane Potential.** Mitochondrial membrane depolarization was determined using the potentiometric probe JC-1 (Smiley et al., 1991). JC-1 selectively enters the polarized mitochondria and is driven by the mitochondrial membrane electrochemical gradient. In polarized membranes, it forms red fluorescent aggregates and when depolarized, JC-1 stays dispersed as a monomer and fluoresces in green. Microvascular endothelial cells were split in six-well plates and treated as described above for viability assays. After the 24-h incubation period, cells were trypsinized, centrifuged, and resuspended in 500 µl of PBS. JC-1 (1 mM) was added for 15 min at 37°C; valinomycin (100 nM) was used as a positive control of mitochondrial depolarization. Monomers and aggregates of JC-1 were detected in the FL1 and FL2 channels, respectively, with a FACScalibur (BD Biosciences, San Jose, CA). For microscopic visualization, cells were photographed as described above.

**ATP Assay.** ATP content was determined using a commercial kit (Calbiochem). Microvascular endothelial cells were split in 12-wells plates, starved, and treated. Cells were suspended in boiling 100 mM Tris-HCl, pH 7.75, 4 mM EDTA buffer for 10 min to inactivate released ATPases. The suspension was centrifuged and cooled. Fifty milliliters of cell suspension was mixed with 50 ml of HEPES buffer and 25 ml luciferin-luciferase solution. ATP was determined by measuring the light generated, using the following formula described in the manufacturer's manual:
$$\text{D-luciferin} + \text{ATP-Mg}^{2+} + \text{O}_2 \rightarrow \text{oxyluciferin} + \text{AMP + CO}_2 + \text{Ppi-Mg}^{2+} + \text{light}.$$
Statistical Analysis. Data were analyzed by one- or two-way analysis of variance factoring for treatment and/or time or concentration. Comparison among means was analyzed by Tukey-Kramer method. Statistical significance was set at \( P < 0.05 \). Values are presented as mean ± S.E.M.

Results

TXA<sub>2</sub>-Induced Neural Microvascular Degeneration: Role of Calpain. U-46619 injected intravitreally into rat pups induced a significant decrease in retinal vessel density (Fig. 1). These effects were prevented by the selective TXA<sub>2</sub> receptor blocker L670596 and by the pan-calcium inhibitor Calp V but not by the caspase-3 inhibitor Z-DEVD-fmk; the pan-caspase inhibitor Z-VAD-fmk was also inefficient. To ascertain that the observed effects of TXA<sub>2</sub> were not simply due to hemodynamic changes (TXA<sub>2</sub>-induced vasoconstriction and ensuing ischemia) and to show that they can be reproduced in higher species, experiments were conducted on porcine retinal explants. U-46619 caused a marked loss (degeneration) of microvasculature (compared with vehicle-treated controls) (Fig. 2) equivalent to that detected in vivo (Fig. 1); I-BOP induced similar effects (data not shown). Retinal microvascular density was relatively preserved by cotreatment with L670596, pan-calcium inhibitor Calp V, and m-calcium inhibitor Calp IV (Fig. 2) (μ-calcium inhibitors are not yet available).

Cytotoxic Effects of TXA<sub>2</sub> and Role of Calpain Specifically Apply to the Neural Microvascular Endothelium. To ascertain that the cytotoxic effects of TXA<sub>2</sub> and thus far presumed (pharmacological) role of calpain specifically apply to the neural microvascular endothelium, the latter was studied on primary neurovascular endothelial cell cultures. TXA<sub>2</sub> analogs U-46619 as well as I-BOP induced a concentration- and time-dependent decrease in cell viability, determined by MTT assay and LDH release (Fig. 3, A and B) (and confirmed by direct cell counting); accordingly, cellular PI incorporation was increased by U-46619 (Fig. 3D). This U-46619-triggered cytotoxicity was largely prevented by the selective TXA<sub>2</sub> receptor blocker L670596, Calp IV, and Calp V as well as by the PLC and PKC inhibitors U73122 and calphostin-C, respectively (Fig. 3, B–D), whereas Z-DEVD-fmk (or Z-VAD-fmk; data not shown) was ineffective, consistent with in vivo and ex vivo observations (Figs. 1 and 2).

TXA<sub>2</sub>-Mediated Changes in Caspase and Calpain Activities. Effects of TXA<sub>2</sub> (mimetic) on caspase and calpain activities were specifically studied. U-46619 analogs U-46619 and I-BOP on porcine neurovascular endothelial cell viability determined by MTT assay (see Materials and Methods). B, LDH activity in endothelial cell media at given times after treatment with U-46619 (0.1 μM) with or without L670596 (1 μM). C, cell viability determined by MTT assay after 24-h treatment with vehicle or U-46619 (0.1 μM) with or without (1 μM) L670596, Calp IV, Calp V, calphostin-C, U73122, or Z-DEVD-fmk (50 μM). D, PI (red) incorporation after 24-h treatment of cells with indicated treatments at concentrations described in C. Cells were counterstained with Hoescht 33342 (blue); inhibitors alone did not affect PI incorporation. Representative photomicrographs are shown on the left, and quantification in histogram format is presented on the right. Values are mean ± S.E.M. of three to six experiments. * \( P < 0.05 \) compared with other values without asterisks.

Fig. 3. A, time and concentration dependence of effects of TXA<sub>2</sub> analogs U-46619 and I-BOP on porcine neurovascular endothelial cell viability determined by MTT assay (see Materials and Methods). B, LDH activity in endothelial cell media at given times after treatment with U-46619 (0.1 μM) with or without L670596 (1 μM). C, cell viability determined by MTT assay after 24-h treatment with vehicle or U-46619 (0.1 μM) with or without (1 μM) L670596, Calp IV, Calp V, calphostin-C, U73122, or Z-DEVD-fmk (50 μM). D, PI (red) incorporation after 24-h treatment of cells with indicated treatments at concentrations described in C. Cells were counterstained with Hoescht 33342 (blue); inhibitors alone did not affect PI incorporation. Representative photomicrographs are shown on the left, and quantification in histogram format is presented on the right. Values are mean ± S.E.M. of three to six experiments. * \( P < 0.05 \) compared with other values without asterisks.
failed to activate caspase-3 in contrast to staurosporine as determined by increased immunoreactivity of the 17- and 12-kDa fragments (Fig. 4C). In addition, pan-caspase activity determined using the enzyme-binding fluorescent substrate SR-VAD-fmk did not reveal any activity upon treatment [24 h as well as 12 h (data not shown)] with U-46619 (Fig. 4, A and B); the positive control staurosporine (Gao et al., 2000) readily evoked caspase activation, which was inhibited by Z-DEVD-fmk.

In contrast, U-46619 evoked a significant increase in the formation of the 58-kDa m-calpain active fragment within 8 h (Fig. 5A) (Weber et al., 2004; Park and Ferreira, 2005), whereas μ-calpain was unaltered. Generation of m-calpain active fragment was inhibited by treatment with PKC and PLC inhibitors calphostin-C and U73122, respectively; correspondingly, U-46619 evoked an increase in inositol phosphate generation, which was blocked by U73122 (Fig. 5B). This increase in calpain activity was further confirmed by induced hydrolysis of cytoskeletal α-spectrin, which generates a Calp IV-sensitive 145- to 150-kDa-specific fragment of spectrin detectable by Western blot (Fig. 5E).

Calpain activity is calcium-dependent (Sato and Kawashima, 2001). Interestingly, however, U-46619 does not elicit net calcium transients in neurovascular endothelial cells (Lahaie et al., 1998) as presently corroborated (Fig. 5F). In contrast, calpain activation can be PKC-dependent (Fig. 5, A and D) as long as cellular calcium is maintained. Indeed, depletion of calcium stores with thapsigargin and concurrent extracellular calcium chelation (with EGTA) precluded U-46619-induced calpain activation (Fig. 5D).

**TXA2-Induced Mitochondrial Dysfunction.** Cytotoxicity secondary to calpain activation is reported to occur in a number of instances via sequential activation of Bax, leading to mitochondrial dysfunction (Chen et al., 2002; Liu et al., 2004). Therefore, we determined the immunoreactivity of the procell death protein Bax relative to that of the anticell death protein Bcl-2 in cytosol and mitochondria of endothelial cells treated with the TXA2 mimetic U-46619. Bax expression in mitochondria increased, whereas that in cytosol decreased over time after stimulation with U-46619, consistent with a translocation of Bax to the mitochondria (Fig. 6A). This led to a corresponding rise in Bax/Bcl-2 ratio in the mitochondria, which was virtually abrogated by L670596, Calp IV, and Calp V (Fig. 6A). Therefore, U-46619 caused (at 18 and 24 h) mitochondrial membrane depolarization [appearance of green and loss of red fluorescence (colors are superimposed in Fig. 6B)], associated with release of cytochrome c in the cytosol (Fig. 6C) and ATP depletion (Fig. 6D). U-46619-induced changes in mitochondrial function were markedly attenuated by the mitochondrial permeability transition pore blocker bongkrekic acid (Halestrap and Brennerb, 2003) as well as by L670596, Calp IV, and Calp V (Fig. 6B). Valinomycin served as a positive control.

![Fig. 4. Caspase activity in porcine neurovascular endothelial cells treated with U-46619. A, representative micrographs of pan-caspase activity determined using the fluorogenic substrate SR-VAD-fmk; cells were counterstained with Hoescht 33342. Cells were treated with U-46619 (0.1 μM) for 12 h (data not shown) or 24 h, or with staurosporine (1 μM) for 1 h without or with caspase-3 inhibitor Z-DEVD-fmk (50 μM). Green fluorescence was not detected after treatment with U-46619 but was with staurosporine (positive control), which was prevented by Z-DEVD-fmk. B, histogramic representation of pan-caspase activity determined using SR-VAD-fmk. Values are mean ± S.E.M. of three experiments. * P < 0.01 compared with other values without asterisks. C, activated caspase-3 fragments determined by Western blot (representative of three experiments). U-46619 (0.1 μM) did not increase immunoreactivity to the caspase-3 fragments; the positive control staurosporine (1 μM; 1 h) induced an increase in caspase-3 fragment immunoreactivity. Values are mean ± S.E.M. of three experiments. *, P < 0.05 compared with other values without asterisks.](https://jpet.aspetjournals.org/doi/10.1124/jpet.106.105105)
Discussion

TXA₂ is an important lipid autacoid that participates in oxidative stress-induced neuroretinal injury (Lahaie et al., 1998; Beauchamp et al., 2001; Brault et al., 2003). This property of TXA₂ has recently been emphasized in relation to ischemic complications associated with cyclooxygenase-2 inhibitors. In addition to well known hemodynamic impairment induced by TXA₂ (FitzGerald et al., 1987), direct cytotoxic effects upon activation of its receptor have now been described, and these seem to contribute to neurovascular degeneration (Beauchamp et al., 2001; Brault et al., 2003). However, the mechanisms for TXA₂-induced neurovascular endothelial cytotoxicity are not known. We therefore conducted the present study to explore such mechanisms. Our findings reveal that TXA₂, mimetics and activators of the TP receptor) elicits a neurovascular endothelial injury in vivo, ex vivo, and directly on primary (cultured) cells via a mechanism dependent upon calpain, but not caspase activity, prevented by selective TP blocker and specifically by m-calpain inhibitor, and mediated in turn by an apparent translocation of Bax to the mitochondria associated with loss of mitochondrial membrane polarity and consequent cellular ATP depletion. These observations disclose a previously undescribed mechanism in neurovascular endothelial cytotoxicity elicited by the major autacoid TXA₂ and mediated mostly by m-calpain.

A dominant feature in this study is the role of calpain without that of caspase in TXA₂-induced neurovascular endothelial cell death. The TXA₂ mimetic U-46619 elicited early m-calpain activation (within 6 h) detected by distinct techniques (Fig. 5, A and C–E), whereas pan-caspase and caspase-3 activation were not detected at early and later
times (6 and 24 h) (Fig. 4, A–C). More importantly, calpain and specifically m-calpain inhibitors, but not caspase-3 or pan-caspase inhibitors (Beauchamp et al., 2001), prevented (to a similar extent) microvascular degeneration in vivo and ex vivo as well as endothelial cell death in vitro (Figs. 1–3). Findings point to a major role for m-calpain in TXA2-evoked neural microvascular endothelial cell death. Cell death often but not exclusively involves an interaction between calpains and caspases (Neumar et al., 2003; Rami, 2003). For example, the degradation of the endogenous calpain inhibitor calpastatin by caspase could potentiate the combined cytotoxic effects of calpains and caspases (Wang et al., 1998), but this explanation is unlikely because calpastatin immunoreactivity was unaltered in the first 24-h response to U-46619 (data not shown). Another potential mechanism of interaction between these protease systems could be through the release of cytochrome c in the cytosol following calpain-induced disruption of mitochondrial integrity, which in turn would predictably sequentially activate caspases-9 and -3 (Vindis et al., 2004). However, neither activation nor a role for caspases in U-46619-induced neurovascular endothelial cell death could be detected (Figs. 1, 2, 3C, and 4). Perhaps the explanation

Fig. 6. TXA2-mediated mitochondrial dysfunction of porcine neurovascular endothelial cells. A, U-46619 (0.1 μM)-induced translocation of Bax to the mitochondria. Drug concentrations were as described in Fig. 3. Values are mean ± S.E.M. of three to four experiments. B, mitochondrial depolarization evoked by U-46619 (0.1 μM) in the presence or absence of L670596 (1 μM), Calp V (1 μM), or the adenine nucleotide translocator of the mitochondrial permeability transition pore bongkrekic acid (50 μM). Mitochondrial membrane depolarization was determined using the potentiometric probe JC-1 (see Materials and Methods). Some preparations were treated with valinomycin (100 nM), a known inducer of mitochondrial depolarization. Photomicrographs are merged images (orange-yellow) of polarized (red) and depolarized (green) mitochondria. Representative micrographs are shown on the left, and compiled data in histogram format are presented on the right; inhibitors alone did not affect mitochondrial depolarization. Values are mean ± S.E.M. of three experiments. *, P < 0.05 compared with other values without asterisks. C, effects of U-46619 (0.1 μM) on cytochrome c (Cyt C) release in the cytosol. The 15-kDa cytochrome c was detected in the cytosol by immunoblotting (representative of three experiments); β-actin (bottom band) was used as control. Cells were treated as indicated; concentration of inhibitors is presented in B. Note increase in cytosolic cytochrome c upon treatment with U-46619 alone and prevented by inhibitors. D, ATP cell content as a function of time after treatment with U-46619 (0.1 μM) with or without L670596 (1 μM) or Calp V (1 μM). Values are mean ± S.E.M. of three experiments. *, P < 0.05 compared with all other corresponding values.
lies in the inactivation of caspases-9 and -3 by calpains, despite the release of cytochrome c, as reported for certain types of cell death (Chua et al., 2000) consistent with present observations (Fig. 6C).

Cell death induced by calpains is for the most part believed to occur by causing mitochondrial dysfunction and ensuing ATP depletion (Liu et al., 2004) by modifying the expression of pro- and anticytotoxic small proteins such as Bax and Bcl-2 (Gao et al., 2000) on mitochondrial membrane (Cory et al., 2003) following kinase activation (Nomura et al., 2003; Tsuruta et al., 2004) or proteolysis (Chen et al., 2001); calpain-dependent cell death can also take place through the direct presence of calpain (-like) activity at the mitochondria, which impairs the permeability transition pore function (Gores et al., 1998). Although we cannot rule out the latter possibility, our findings support a TXA2-induced increase in Bax/Bcl-2 ratio at the mitochondria and associated loss of mitochondrial membrane polarity and cellular ATP depletion, which are prevented by calpain inhibitors (Fig. 5, A, B, and D).

Mechanisms for TXA2-induced activation of calpain seem complex and somewhat unexpected. Other than in humans, in other species TXA2 acts on a single receptor homologous to the human TPα receptor (Kinsella, 2001). TPα can couple to Gs, Gq/11, or G12/13 (Walsh et al., 2000) but apparently not to Gi; correspondingly, pertussis toxin did not effect calpain activation. Stimulation of Gs would lead to an increase in cAMP, but the latter is primarily involved in cell survival, including of the central nervous system (Cui and So, 2004), and thus exerts effects opposite to those we observed (Figs. 1–3). G12/13 effects are mostly mediated by Rho GTPases (Kurose, 2003), which are downstream of calpains (Sato and Kawashima, 2001). Conversely, our findings support coupling of TP to Gq/11, which leads to activation of phospholipase C to generate inositol triphosphate (Fig. 5B). But surprisingly, although calpains are well known to be activated by a rise in intracellular Ca2+ (Sato and Kawashima, 2001), U-46619 (and I-BOP) do not elicit Ca2+ transients in neurovascular endothelial cells (Lahaie et al., 1998; Fig. 5F). However, as long as intracellular Ca2+ concentrations are not depleted, other mechanisms partake in calpain activation (Fig. 5, A, C, and D–F) such as phosphorylation (Sato and Kawashima, 2001). Of relevance, diacylglycerol generated concurrently with inositol triphosphate during phospholipase C catalysis activates protein kinase C (Exton, 1993), which in turn can phosphorylate and activate calpain. Indeed, both the inhibitors of phospholipase C and PKC, U73122 and calphostin-C, respectively, prevented U-46619-induced activation of m-calpain (active fragment and activity) (Fig. 5, A and D) and ensuing endothelial cell death (Fig. 3C).

Endothelial cytotoxicity and ensuing impaired angiogenesis in response to TXA2 has been reported in a number of studies (Beauchamp et al., 2001; Ashton et al., 2003; Brault et al., 2003; Ashton and Ware, 2004). However, endothelial cells of various origins respond differently; for example, dermal and aortic endothelial cells are not susceptible to TXA2 (Beauchamp et al., 2001, 2002; Brault et al., 2003), whereas on cornea U-46619 induces angiogenesis in the presence (but not in the absence) of fibroblast growth factor (Daniel et al., 1999). Dissimilar actions of TXA2 on different endothelial cells probably reflect the heterogeneity of endothelium as the same receptor couples to different signaling partners in different cells (Gudermann et al., 1996); notably, for example, one would expect distinct phenotypes and corresponding functions in glomerular and brain endothelium.

In summary, we have identified a major mechanism in neurovascular endothelial cell death in response to TXA2, specifically (and principally) m-calpain. In this process, we have also uncovered a previously unreported role for calpain in endothelial cell death secondary to this important mediator of oxidant stress, namely, TXA2 (Beauchamp et al., 2001, 2002; Brault et al., 2003). Because preservation of microvascular especially in the salvageable ischemic penumbra is important, our findings provide an additional explanation for the efficacy of calpain inhibitors in ischemic encephalopathies and retinopathies (Tamada et al., 2002, Rami, 2003).

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References


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