

# Tumor Necrosis Factor- $\alpha$ Induces Cyclooxygenase-2 Expression and Prostaglandin Release in Brain Microvessel Endothelial Cells

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## ABSTRACT

Primary cultured bovine brain microvessel endothelial cells (BBMECs), were used as an *in vitro* model of the blood-brain barrier to examine the involvement of eicosanoids in the permeability and cytoskeletal structural changes observed following exposure to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Compared with control monolayers, BBMECs exposed to TNF- $\alpha$  formed actin filament tangles and extracellular gaps with a resultant increase in permeability. Both the permeability and cytoskeletal changes observed with TNF- $\alpha$  were significantly reduced following pretreatment with NS-398 or indomethacin, inhibitors of cyclooxygenase (COX). Western blot analysis showed that TNF- $\alpha$  had no apparent effect on the expression of COX-1, but did induce the expression of COX-2 in the BBMECs. The in-

duction of COX-2 expression occurred within the same time frame (2–4 h following TNF- $\alpha$  exposure) as the permeability increases observed with the cytokine. Consistent with the increased expression of COX-2, BBMEC monolayers exposed to TNF- $\alpha$  had significantly greater secretion and release of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and prostaglandin F<sub>2 $\alpha$</sub>  (PGF<sub>2 $\alpha$</sub> ). Furthermore, BBMEC monolayers treated with PGE<sub>2</sub> or PGF<sub>2 $\alpha$</sub>  showed significant increases in permeability and cytoskeletal structural changes when compared with control monolayers. Together, these results suggest that the TNF- $\alpha$ -induced permeability and cytoskeletal structural effects are due, in part, to an induction of the COX-2 system and increased release of prostaglandins in the cerebral microvasculature.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a proinflammatory cytokine that is released during infection or tissue trauma. As a mediator of inflammatory responses, TNF- $\alpha$  has been shown to enhance microvascular permeability in the peripheral circulation (Brett et al., 1989; Beynon et al., 1993) and more recently in the cerebral microvasculature (Deli et al., 1995; de Vries et al., 1996; Mark and Miller, 1999). Several clinical reports indicate that the levels of TNF- $\alpha$  are elevated in the plasma and cerebrospinal fluid of patients suffering from inflammatory neurological conditions such as bacterial and viral meningitis, Alzheimer's disease, multiple sclerosis, acquired immune deficiency syndrome (AIDS)-related dementia, and Guillain-Barré syndrome (Grimaldi et al., 1991; Sharief and Hentges, 1991; Quagliariello and Scheld, 1992; Sharief et al., 1992, 1993). The resulting damage to the brain in these neurological diseases is associated with disruptions in the blood-brain barrier (BBB) that allow both an increased

number of immunoactive cells and macromolecules access to the brain extracellular fluid and the development of cerebral edema (Davson et al., 1993; Andjelkovic and Pachter, 1998).

Both *in vitro* and *in vivo* studies have shown that TNF- $\alpha$  elicits increased permeability in the brain microvessel endothelial cells that form the BBB (Kim et al., 1992; Wright and Merchant, 1992; Claudio et al., 1994; Deli et al., 1995; Abraham et al., 1996; de Vries et al., 1996; Mark and Miller, 1999). TNF- $\alpha$  has also been shown to stimulate cytoskeletal structural changes such as actin filament clumping and extracellular gap formation in the cerebral microvasculature (Claudio et al., 1994; Deli et al., 1995; Mark and Miller, 1999). These results suggest that the increased permeability in the brain microvessel endothelial cells is due to reorganization of the cytoskeleton, resulting in an enhanced paracellular diffusion of macromolecules across the BBB. However, the intracellular signaling pathway(s) connecting TNF- $\alpha$  to the cytoskeletal structural changes and permeability increases in the brain microvasculature are not well understood.

In the current study, the contributions of the prostaglandin

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**ABBREVIATIONS:** TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; AIDS, acquired immune deficiency syndrome; BBB, blood-brain barrier; PG, prostaglandin; COX, cyclooxygenase; BBMEC, bovine brain microvessel endothelial cell; BSA, bovine serum albumin; BCA, bicinchoninic acid; FDX, fluorescein-conjugated dextran; PBS, phosphate-buffered saline; DPBS, Dulbecco's phosphate-buffered saline; NF- $\kappa$ B, nuclear factor- $\kappa$ B; LPS, lipopolysaccharide.

(PG) signaling pathway to the TNF- $\alpha$ -induced changes in brain microvasculature were examined. The reasons for focusing on the PG pathway are 3-fold. First, TNF- $\alpha$  has been shown to cause the release of prostaglandins from various peripheral tissues (Vara et al., 1996; Fournier et al., 1997; Mollace et al., 1998). Second, there have been reports of an up-regulation in the inducible form of cyclooxygenase (COX)-2, the enzyme responsible for the production of PGs, following proinflammatory stimuli such as bacterial lipopolysaccharide and TNF- $\alpha$  (Gierse et al., 1995; Jobin et al., 1998; Quan et al., 1998; Chen et al., 1999). Finally, selected PGs, such as PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  produce increases in peripheral microvessel endothelial cell permeability (Gulati et al., 1983; Payne et al., 1994; Lozano et al., 1996) and have been linked to alterations in BBB integrity during inflammation in the central nervous system (Jaworowicz et al., 1998; Mollace et al., 1998).

In the current study, primary cultured bovine brain microvessel endothelial cells (BBMECs) were used as an *in vitro* model of the BBB to examine the effects of TNF- $\alpha$  stimulation on cyclooxygenase protein expression and the release of prostaglandins. The BBMEC model has previously been shown to exhibit the barrier properties and morphological characteristics (i.e., tight intercellular junctions, no fenestra, reduced pinocytotic vesicles, marker enzymes, transport systems, and functional membrane polarity) representative of the *in vivo* BBB (Audus and Borchardt, 1987; Miller et al., 1994; Stein et al., 1997). In addition, both increases in BBMEC monolayer permeability and altered cytoskeletal structure have been reported following exposure to TNF- $\alpha$  (Deli et al., 1995; Mark and Miller, 1999). In the present study, increases in the expression of COX-2 and the release of PG from TNF- $\alpha$  treated BBMEC monolayers were correlated with the permeability and cytoskeletal changes observed with the cytokine. Furthermore, inhibitors of COX significantly reduced the effects of TNF- $\alpha$  on permeability, PG release, and cytoskeletal structure in BBMEC monolayers. Together, the results of the present study indicate that the increased permeability and cytoskeletal structural changes observed in the BBMECs following exposure to TNF- $\alpha$  involves the induction of the COX-2 enzyme and the increased release of both PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub> .

## Experimental Procedures

**Materials and Statistics.** Transwell polycarbonate membrane inserts, rat tail collagen (type I), minimal essential medium and Ham's F-12 medium were purchased from Fisher (St. Louis, MO). NS-398 was purchased from Research Biochemicals International (Natick, MA). Bovine fibronectin, bovine serum albumin (BSA), equine serum, fluorescein, and indomethacin were purchased from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Indianapolis, IN). BODIPY 581/591 phalloidin and fluorescein-labeled dextran (FDX-3000, 3000 mol. wt.) were purchased from Molecular Probes (Eugene, OR). All other nutrients, salts, and antibiotics used in the culture medium or assay buffers were of cell culture quality from Sigma.

Statistical analysis of the data was performed using single-factor analysis of variance with Newman-Keuls' multirange post hoc comparison of the means where appropriate. Statistical significance with a *p* value < 0.05 is indicated with an asterisk unless otherwise noted.

**Isolation and Culturing of BBMECs.** Primary BBMECs were collected from the gray matter of fresh bovine cerebral cortices using enzymatic digestion and centrifugal separation methods as previ-

ously described (Miller et al., 1992). The BBMECs were seeded (50,000 cells/cm<sup>2</sup>) on collagen-coated, fibronectin-treated, 75 cm<sup>2</sup> tissue culture flasks or 6-well Transwell polycarbonate membrane inserts (0.4- $\mu$ m pore/24-mm diameter). The culture media consisted of: 45% minimal essential medium, 45% Ham's F-12 nutrient mix, 10 mM HEPES, 13 mM sodium bicarbonate, 50  $\mu$ g/ml gentamicin, 10% equine serum, 2.5  $\mu$ g/ml amphotericin B, and 100  $\mu$ g/ml heparin. The BBMECs were cultured in a humidified 37°C incubator with 5% CO<sub>2</sub>, with media replacement occurring every other day until the monolayers reached confluency (approximately 11–14 days).

**Cytokine/Eicosanoid Preparation and Treatment of BBMECs.** Lyophilized recombinant human TNF- $\alpha$  ( $1.1 \times 10^5$  U/ $\mu$ g; R&D Systems, Minneapolis, MN) was reconstituted to 10  $\mu$ g/ml using 0.1% BSA in phosphate-buffered saline (PBS) and kept at -20°C until used. PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  (Sigma) were diluted to 1  $\mu$ g/ml using 0.1% BSA in PBS and kept at -20°C until used. The final concentrations of TNF- $\alpha$  (100 ng/ml), PGE<sub>2</sub>, and PGF<sub>2 $\alpha$</sub>  (10 ng/ml) were made by diluting the stock solution with culture medium containing 10% equine serum.

**Enzyme Immunoassay for Determination of Eicosanoids.** The effects of TNF- $\alpha$  on the release of eicosanoids was determined by measuring the amounts of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  secreted into the culture medium of BBMEC monolayers following exposure to TNF- $\alpha$ . Baseline levels of the eicosanoids were established by exposing BBMEC monolayers to low serum (5%) culture medium for 12 h. Baseline samples (time 0) were collected, after which the monolayers were treated with either low serum culture media alone or low serum culture media containing TNF- $\alpha$  (100 ng/ml). Samples (75  $\mu$ l) of the culture medium exposed to the BBMECs were collected at various time points (0.5–24 h). In separate studies, PGE<sub>2</sub> release was determined following TNF- $\alpha$  (100 ng/ml) exposure in the presence and absence of the COX inhibitor, indomethacin (10  $\mu$ M). For these studies, BBMEC monolayers were exposed to culture medium containing 10% equine serum for 24 h prior to treatment with cytokine or COX inhibitors. Samples were removed as previously described. The concentrations of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  were determined using enzyme immunoassay kits (Cayman Chemicals and Assay Designs; Ann Arbor, MI) and fell within the kits detection limits. Although the detection kits have cross-reactivity with their respective subtypes (e.g., PGF<sub>1 $\alpha$</sub>  is 20% cross-reactive with PGF<sub>2 $\alpha$</sub> ), there is no cross-reactivity with other eicosanoids (i.e., PGF is not cross-reactive with PGE). The concentration of eicosanoids was corrected for the amount of cellular protein that was determined using the BCA protein assay. The results are presented as the concentration of prostaglandin per microgram of protein.

**Effects of COX Inhibitors on BBMEC Permeability.** Confluent BBMEC monolayers were pretreated 1 h with culture medium alone or culture media containing indomethacin (nonselective cyclooxygenase inhibitor; 0.1–10  $\mu$ M), or NS-398 (COX-2 selective inhibitor; 0.01–10  $\mu$ M). Following the pretreatment period, TNF- $\alpha$  was added to the BBMEC monolayers to give a final concentration of 100 ng/ml of TNF- $\alpha$ , and the monolayers were then incubated at 37°C for an additional 6 h.

Following the 6-h incubation period, the BBMEC monolayers were inserted into side-by-side diffusion chambers to determine changes in permeability as previously described (Mark and Miller, 1999). Briefly, assay buffer was added to both receiver and donor compartments, and permeability across BBMEC monolayers was determined by adding fluorescein (1  $\mu$ M) or fluorescein-conjugated dextran (5  $\mu$ M FDX-3000; 3000 mol. wt.) to the donor compartment. Aliquots (3 ml) were removed from the receiver chamber at 0, 5, 15, 30, 60, and 120 min, and replaced with fresh assay buffer. Samples from the receiver compartment were measured spectrofluorometrically, and the permeability was expressed as the percent transfer of the fluorescent marker across the BBMEC monolayers. Changes in the BBMEC monolayer permeability following TNF- $\alpha$  treatment were compared with control monolayers receiving no cytokine and monolayers pretreated with the various COX inhibitors.

**Prostaglandin Effects on Permeability.** To determine the effects of various prostaglandins on BBMEC permeability, confluent BBMEC monolayers were exposed to either culture medium alone, or culture media containing PGE<sub>2</sub> (10 ng/ml) or PGF<sub>2α</sub> (10 ng/ml). Following either a 60- or 90-min induction period, the monolayers were placed into side-by-side diffusion chambers, and permeability was measured using FDX-3000 as previously described.

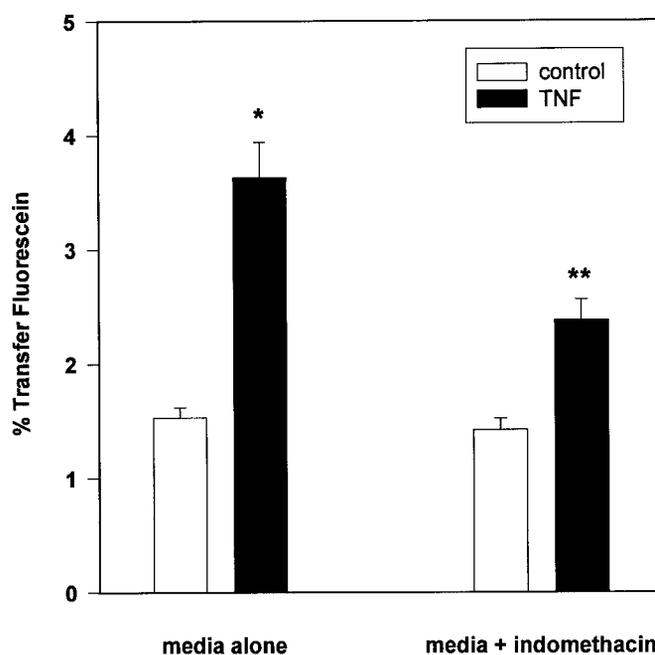
In addition, permeability studies were also performed with PGE<sub>2</sub> (10 ng/ml) in the presence of the COX inhibitor indomethacin (1 μM). For these studies, BBMEC monolayers were treated with either PGE<sub>2</sub> alone or in the presence of indomethacin for 90 min. Following the 90-min treatment, permeability studies were performed as described.

**F-Actin Staining of BBMEC Cytoskeleton.** Confluent BBMEC monolayers were treated with TNF-α (100 ng/ml; 6 h) either alone or in the presence of zileuton (5 μM) or indomethacin (1 μM). In the prostaglandin studies, the BBMEC monolayers were exposed to either PGE<sub>2</sub> or PGF<sub>2α</sub> (10 ng/ml; 60 and 90 min). Following treatment, the BBMEC monolayers were rinsed with phosphate-buffered saline solution (containing 1 mM CaCl<sub>2</sub>; DPBS), permeabilized, and fixed in 3.7% formaldehyde with 1% Triton X-100 at 25°C for 10 min. The monolayers were rinsed with 4°C DPBS and BODIPY 581/591 phalloidin stain was applied to the luminal side of each monolayer. The monolayers were incubated with the stain for 30 min at 25°C, after which they were rinsed with PBS. The polycarbonate inserts were removed and placed onto glass slides and mounted with 50% glycerol in DPBS and sealed with coverslips. Photographs were taken using a fluorescent microscope with a fluorescein isothiocyanate filter. Changes in F-actin staining following exposure to TNF-α or prostaglandins were compared with BBMEC monolayers that were exposed to culture medium alone.

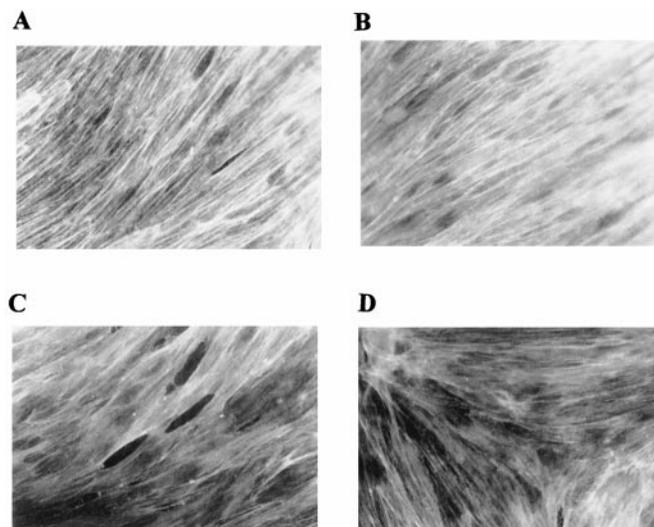
**Western Blot Analysis of COX-1 and COX-2 in BBMECs.** Confluent BBMEC monolayers were treated with TNF-α (100 ng/ml) or culture medium alone for 2, 4, or 6 h. The cells were solubilized with 1% SDS/1% protease inhibitor, and the protein was quantitated using the BCA method. COX-1 (10 ng; 70 kDa) and COX-2 (5 ng; 72 kDa) protein standards (Cayman Chemicals, Ann Arbor, MI) were used as positive controls. The samples (25–30 μg) of the BBMEC lysates were loaded onto a 7.5% polyacrylamide gel and electrophoresed at 45–65 V. The proteins were transferred from the polyacrylamide gel to a polyvinylidene fluoride membrane at 4°C for 1 h with 240 mA. Following the transfer, the membrane was blocked with Tween-20/1% BSA and then incubated overnight at 4°C with the respective ovine COX-1 or COX-2 primary antibody (1:1000 dilution). After incubating with the primary antibody, the membrane was washed a series of times with blocking buffer consisting of 1% BSA with Tween-20 (0.3–3%). The membrane was incubated with anti-mouse secondary antibody (1:1500 dilution) for 30 min at 4°C and then washed a final series of times with blocking buffer [1% BSA with Tween-20 (0–3%)]. The protein bands were developed using the enzyme chemiluminescence method (Amersham Pharmacia Biotech, Cleveland, OH).

## Results

**Effects of Eicosanoid Synthesis Inhibitors on TNF-α Permeability and Cell Morphology.** Confluent BBMEC monolayers exposed to TNF-α showed approximately a 2-fold increase in permeability, compared with control monolayers (Fig. 1). The increased permeability in the TNF-α treatment group was correlated with the development of large extracellular gaps in the BBMEC monolayers (Fig. 2). Indomethacin, a nonselective COX inhibitor, significantly decreased the effect of TNF-α on BBMEC monolayer permeability (Fig. 1). Furthermore, the indomethacin treatment also reduced the appearance of extracellular gaps in the BBMEC monolayers exposed to TNF-α (Fig. 2).



**Fig. 1.** Attenuation of TNF-α-induced permeability increases in BBMEC monolayers with cyclooxygenase inhibitor. The permeability of BBMEC monolayers to fluorescein was examined following 6-h exposure to TNF-α (100 ng/ml) under control conditions (culture medium alone) or following pretreatment with indomethacin (1 μM). The monolayers receiving no TNF are indicated by open bars, whereas those treated with TNF-α are indicated by closed bars. Values represent the mean ± S.E.M. of six BBMEC monolayers. \**p* < 0.05, compared with control; \*\**p* < 0.05, compared with TNF response in media alone.



**Fig. 2.** Actin filament staining of BBMEC monolayers using BODIPY-phalloidin-labeled antibodies. BBMEC monolayers were treated (6 h at 37°C) with culture media alone (A), culture media with indomethacin (B), culture media with TNF-α (C), or culture media containing TNF-α and indomethacin (D). BBMEC monolayers treated with TNF-α alone (C) showed clumping of actin filaments and the appearance of extracellular gaps (dark spots), compared with those treated with indomethacin (D). Photos were taken at a 40× magnification with a fluorescein isothiocyanate filter.

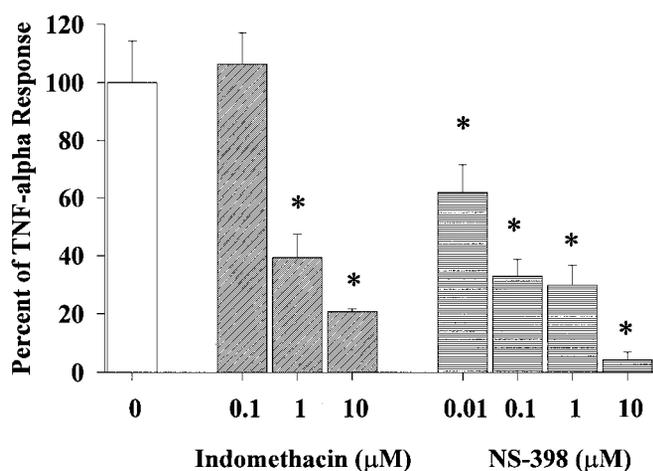
**Inhibition of TNF-α Effects on Permeability by Cyclooxygenase Inhibitors.** The permeability of BBMEC monolayers treated with TNF-α and either the nonselective COX inhibitor, indomethacin (0.1–100 μM), or the COX-2 selective inhibitor, NS-398 (0.01–10 μM), were compared

with monolayers receiving TNF- $\alpha$  alone. Both indomethacin and NS-398 inhibited the permeability increases produced in BBMEC monolayers by TNF- $\alpha$  in a concentration-dependent manner (Fig. 3).

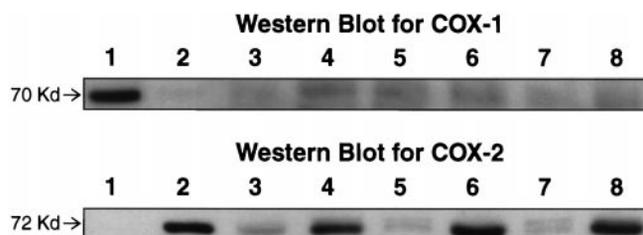
**TNF- $\alpha$  Induction of COX-2 Protein Expression in BBMECs.** Western blot analysis depicts the protein expression of the COX-1 and COX-2 enzymes in BBMEC monolayers treated with TNF- $\alpha$  (100 ng/ml), compared with control cells receiving culture medium alone. At all exposure times examined, BBMEC monolayers treated with TNF- $\alpha$  showed no significant difference in the expression of the COX-1 protein compared with the control monolayers not exposed to the cytokine (Fig. 4, top panel). In contrast, BBMEC monolayers treated with TNF- $\alpha$  for 2, 4, or 6 h showed a substantial increase in the expression of COX-2 protein when compared with control cells. The effects of TNF- $\alpha$  on COX-2 expression in BBMEC appears to increase as the induction time lengthens, with the 6 h TNF- $\alpha$  exposure showing the greatest intensity of COX-2 protein compared with the control (Fig. 4, bottom panel).

**Effects of TNF- $\alpha$  on Eicosanoid Production/Release.** Figures 5 and 6 show the time-release profiles for PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  in TNF- $\alpha$  treated BBMECs. The TNF- $\alpha$ -treated (100 ng/ml) BBMEC monolayers showed a significant increase of 2.5- to 3-fold, respectively, in the release of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  compared with control monolayers receiving only culture medium. The difference in the release of PGE<sub>2</sub> was statistically significant 4 h after stimulation by TNF- $\alpha$  (6.6 pg/ $\mu$ g protein versus 3.5 pg/ $\mu$ g protein, respectively), whereas the release of PGF<sub>2 $\alpha$</sub>  increased significantly within 2 h after TNF- $\alpha$  exposure (2.8 pg/ $\mu$ g protein versus 1.1 pg/ $\mu$ g protein, respectively). Furthermore, the TNF-induced release of PGE<sub>2</sub> from BBMEC monolayers was completely prevented by treatment with the COX inhibitor, indomethacin (Fig. 7).

**Prostaglandin Effects on BBMEC Monolayer Permeability and Cell Morphology.** There was no difference in the permeability of BBMEC monolayers after a 60-min exposure to PGE<sub>2</sub> or PGF<sub>2 $\alpha$</sub>  when compared with control BBMEC



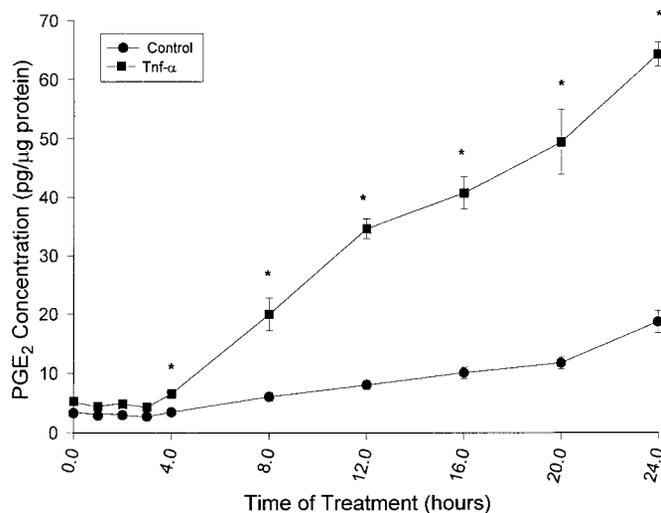
**Fig. 3.** Concentration-response of cyclooxygenase inhibitors on TNF- $\alpha$ -induced permeability. Confluent BBMEC monolayers were treated with TNF- $\alpha$  (100 ng/ml; open bar) and various concentrations of indomethacin (nonselective COX inhibitor) or NS-398 (selective COX-2 inhibitor). Permeability studies were performed with FDX-3000, and the results are reported as the percent of TNF- $\alpha$  permeability response in control monolayers receiving only TNF- $\alpha$ . Values represent the mean  $\pm$  S.E.M.;  $n = 3$ –6 monolayers per treatment group. \* $p < 0.05$ , compared with monolayers treated with TNF- $\alpha$  alone.



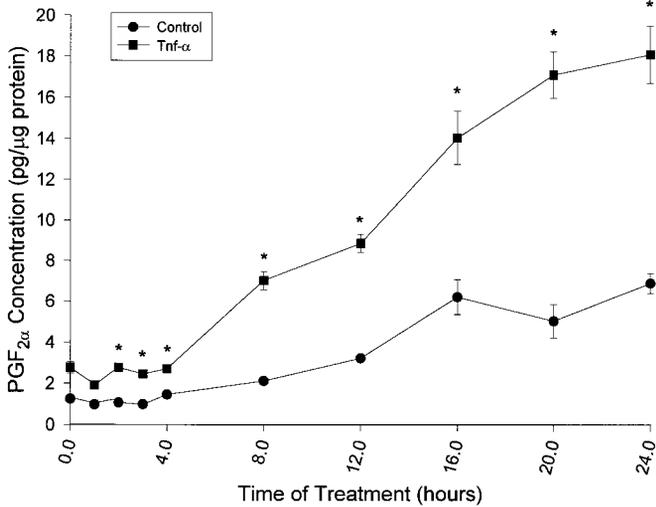
**Fig. 4.** Western blot analysis of BBMEC monolayers for COX-1 and COX-2 protein expression. Confluent BBMEC monolayers were treated with TNF- $\alpha$  (100 ng/ml) or culture medium alone for either 2, 4, or 6 h. The top panel demonstrates COX-1 protein expression, whereas the bottom panel demonstrates COX-2 protein expression using specific murine monoclonal antibodies for the respective proteins. The concentration of BBMEC lysate loaded in the top and bottom panels was 30 and 25  $\mu$ g, respectively. Concentration of the COX-1 and COX-2 standards used was 10 and 5 ng, respectively. 1, COX-1 standard; 2, COX-2 standard; 3, 2-h control BBMECs; 4, 2-h TNF- $\alpha$  BBMECs; 5, 4-h control BBMECs; 6, 4-h TNF- $\alpha$  BBMECs; 7, 6-h control BBMECs; 8, 6-h TNF- $\alpha$  BBMECs.

monolayers receiving culture medium alone. In contrast, after a 90-min exposure to 10 ng/ml of PGE<sub>2</sub> or PGF<sub>2 $\alpha$</sub> , there were significant increases in permeability (30% and 42%, respectively; Fig. 8) when compared with control monolayers. To further examine the permeability increases observed with the prostaglandins, BBMEC monolayers were exposed to PGE<sub>2</sub> for 90 min in the presence of the COX inhibitor, indomethacin. As can be seen in Fig. 9, PGE<sub>2</sub> treatment significantly enhanced BBMEC monolayer permeability. Furthermore, the permeability increases observed with PGE<sub>2</sub> were not inhibited by indomethacin. Indeed, permeability responses to the prostaglandin were significantly increased in the presence of indomethacin (Fig. 9). Treatment with indomethacin alone had no effect on BBMEC permeability (Fig. 9).

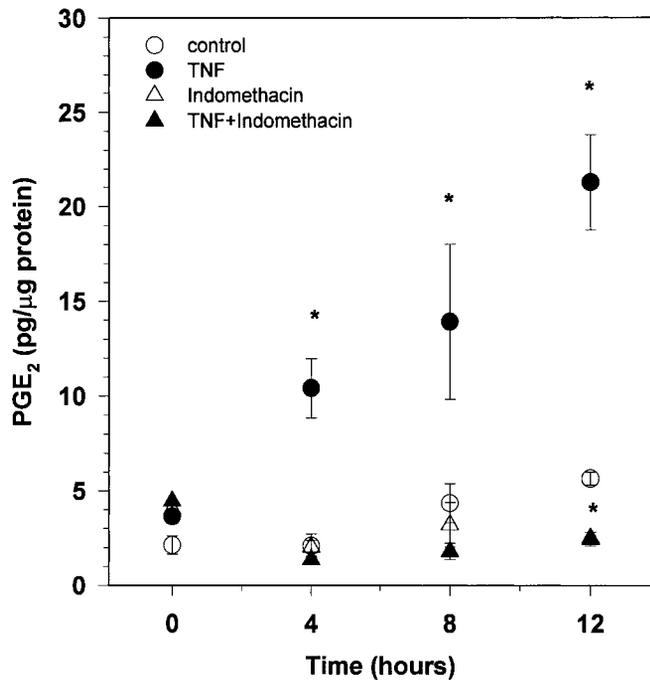
In addition to increasing permeability of the BBMEC monolayers, prostaglandin exposure was associated with changes in the cytoskeleton of the cells. Actin filament clumping was apparent in confluent BBMEC monolayers



**Fig. 5.** Time-release profile for PGE<sub>2</sub> in BBMEC monolayers. Confluent BBMEC monolayers were treated with TNF- $\alpha$  (100 ng/ml) or culture medium alone. Samples of the supernatant were taken at the time points indicated (0–24 h) and analyzed for PGE<sub>2</sub>. Concentrations of PGE<sub>2</sub> were normalized to the amount of cellular protein in the monolayers. ●, control cells; ■, TNF- $\alpha$ -treated cells. Values represent the mean  $\pm$  S.E.M. of three monolayers per treatment group. \* $p < 0.05$ , compared with control values at the same time point.

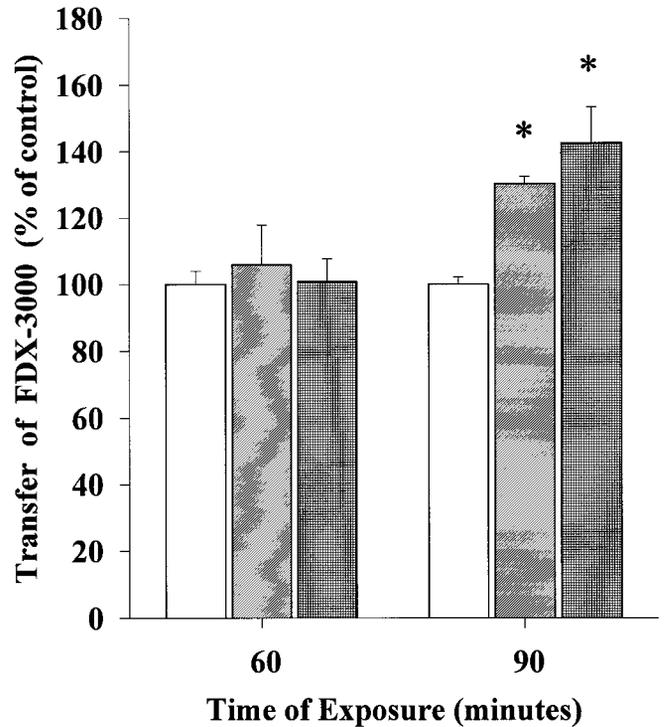


**Fig. 6.** Time-release profile for PGF<sub>2α</sub> in BBMEC monolayers. Confluent BBMEC monolayers were treated with TNF-α (100 ng/ml) or culture medium alone. Samples of the supernatant were taken at the time points indicated (0–24 h) and analyzed for PGF<sub>2α</sub>. Concentrations of PGF<sub>2α</sub> were normalized to the amount of cellular protein. ●, control cells; ■, TNF-α-treated cells. Values represent the mean ± S.E.M. of three monolayers per treatment group. \**p* < 0.05, compared with control values at the same time point.



**Fig. 7.** Inhibition of TNF-α-induced increases in PGE<sub>2</sub> release from BBMEC monolayers with indomethacin. Confluent BBMEC monolayers were treated with TNF-α (100 ng/ml) or culture medium alone in the presence and absence of indomethacin (10 μM). Samples of the supernatant were taken at the various time points and analyzed for PGE<sub>2</sub>. Concentrations of PGE<sub>2</sub> were normalized to the amount of cellular protein. Values represent the mean ± S.E.M. of three monolayers per treatment group. \**p* < 0.05, compared with control values at the same time point.

following 60 and 90 min of exposure to 10 ng/ml of PGE<sub>2</sub> or PGF<sub>2α</sub>, compared with control monolayers treated with culture medium alone (Fig. 10). Furthermore, extracellular gaps were apparent in the BBMEC monolayers following 90 min of exposure to PGE<sub>2</sub> (Fig. 10).

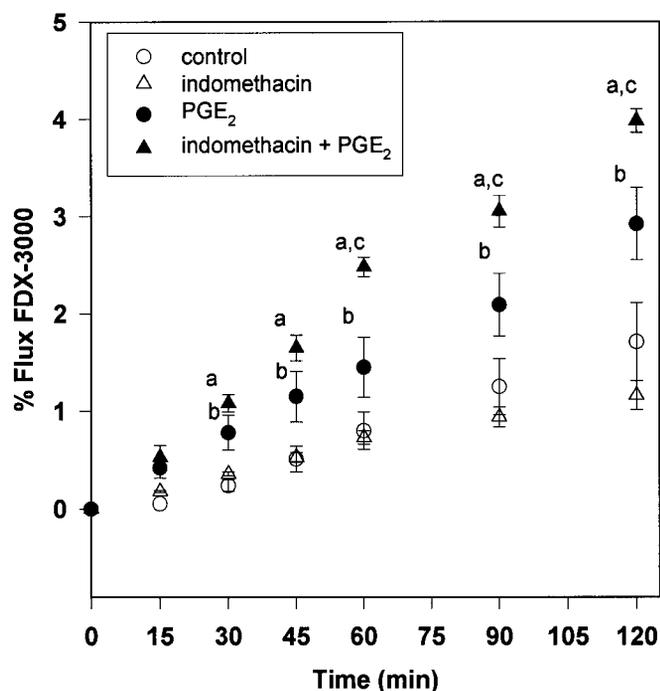


**Fig. 8.** Effects of prostaglandins on BBMEC monolayer permeability. Confluent BBMEC monolayers were treated with PGE<sub>2</sub> (10 ng/ml, diagonal bars), PGF<sub>2α</sub> (10 ng/ml, cross-hatched bars), or culture medium alone (open bars) for 60 or 90 min. Permeability was determined using FDX-3000, and data are presented as the percentage of FDX-3000 in the receiver compartment. Results are presented as the mean ± S.E.M. of 9–15 BBMEC monolayers. \**p* < 0.05, compared with culture medium alone.

## Discussion

The current study examined the expression of COX and the release profiles of prostaglandins in BBMEC monolayers to determine the involvement of this particular pathway in the permeability and cytoskeletal effects observed following exposure to TNF-α. The rationale for selecting this particular pathway is 3-fold. First, the COX system has both a constitutively expressed isoform, COX-1, and an inducible isoform, COX-2, whose expression can be increased in response to a number of inflammatory stimuli (Gierse et al., 1995; Quan et al., 1998). Second, the prostaglandins that are formed via the COX system have been shown to increase permeability and alter the cytoskeletal structure of cells (Gulati et al., 1983; Payne et al., 1994; Lozano et al., 1996). Finally, there is evidence that indicates an increase in the release of prostaglandins from various cells following exposure to TNF-α (Chen et al., 1995; Vara et al., 1996; Fournier et al., 1997; Mollace et al., 1998).

The present study shows that TNF-α exposure produced dramatic changes in the expression of COX-2 in BBMEC monolayers. The expression of COX-1, the constitutive isoform, was not affected by TNF-α at any of the time points examined in this study. These results are supported by previous studies where Jobin et al. (1998) demonstrated TNF-α-induced changes in COX-2, but not COX-1 expression in intestinal epithelial cells. The current results are also consistent with the recent studies of Tsao et al. (1999), demonstrating the brain inflammation caused by either the administration of *Escherichia coli* or TNF-α was associated with



**Fig. 9.** Effects of indomethacin on PGE<sub>2</sub> induced increases in BBMEC monolayer permeability. Confluent BBMEC monolayers were treated for 90 min with PGE<sub>2</sub> (10 ng/ml) either alone, or in the presence of indomethacin (10  $\mu$ M). Following pretreatment, changes in the BBMEC monolayer permeability were examined using FDX-3000. Values represent the mean  $\pm$  S.E.M. of three monolayers. (a)  $p < 0.05$ , compared with control monolayers; (b)  $p < 0.05$ , compared with indomethacin-treated monolayers; and (c)  $p < 0.05$ , compared with PGE<sub>2</sub>-treated monolayers.

induction of COX-2 in the brain arterioles and infiltrating neutrophils.

The cellular mechanism for the increased expression of COX-2 in BBMEC monolayers following TNF- $\alpha$  treatment remains unclear. A likely signal transduction pathway for TNF involves the dephosphorylation of the inhibitor nuclear factor- $\kappa$ B (NF- $\kappa$ B) to form NF- $\kappa$ B. In support of this, are studies in intestinal epithelial cells indicating COX-2 induction by TNF- $\alpha$  occurs through activation of NF- $\kappa$ B (Jobin et al., 1998). Alternatively, other downstream elements could also be involved. Recent studies by Chen et al. (1999) demonstrated that TNF- $\alpha$ -induced increases in COX-2 expression in vascular smooth muscle cells occurred independent of NF- $\kappa$ B. Studies are currently ongoing to determine the role of NF- $\kappa$ B in the effects of TNF observed in the present study.

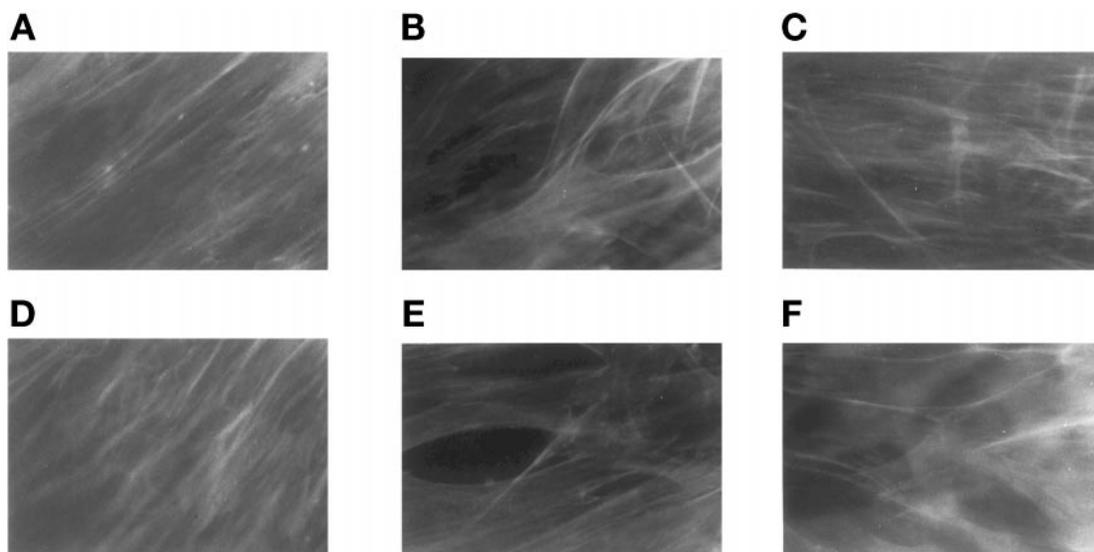
The time period at which the induction of COX-2 was observed in BBMEC monolayers correlates well with the 2- to 6-h lag time required for the appearance of permeability changes in microvessel endothelial cells following exposure to TNF- $\alpha$  (Brett et al., 1989; Abraham et al., 1996; Mark and Miller, 1999). This further supports that the induction of COX-2 is involved in the permeability and cytoskeletal alterations observed with TNF- $\alpha$  in BBMEC monolayers. A similar increase in COX-2 expression has been reported in brain microvessel endothelial cells following peripheral lipopolysaccharide (LPS) administration in rats (Quan et al., 1998). It is also interesting to note that the induction time required to induce COX-2 following LPS administration was comparable with the 2- to 6-h period for the time-induced effects observed in the present study with TNF- $\alpha$ . Since LPS causes the release of several proinflammatory mediators, including

TNF- $\alpha$ , increases in COX-2 observed following the LPS challenge may be attributable to TNF- $\alpha$ -induced effects in the brain endothelial cells.

In the present study, COX inhibitors were able to attenuate both the permeability effects and the cytoskeletal rearrangement observed in the BBMEC following exposure to TNF- $\alpha$ . This is a critical finding, because an increased expression of COX-2 by itself does not prove definitively the importance of the eicosanoid pathway in the alterations in permeability and cytoskeletal structure produced by TNF- $\alpha$ . Previous studies reported that the nonselective COX inhibitor, indomethacin, could inhibit the effects of TNF- $\alpha$  on brain microvessel endothelial transendothelial electrical resistance, an indirect measure of cell permeability (Vries et al., 1996). The current studies extend those findings in the BBMEC by demonstrating that the permeability and cytoskeletal structure changes appear to be linked to TNF- $\alpha$  through a COX-2-dependent pathway. In addition, the attenuation of TNF- $\alpha$ -induced effects with the selective COX-2 inhibitor, NS-398 (Futaki et al., 1994; Gierse et al., 1995; Riendeau et al., 1997), provides further support for the role of COX-2 in the BBMEC response to TNF- $\alpha$ .

In conjunction with the increased expression of COX-2, there was a significant increase in the release of two selected prostaglandins, PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub> , from BBMEC monolayers exposed to TNF- $\alpha$ . The focus on these two particular prostaglandins is based on previous studies by Moore et al. (1988), indicating that PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  are the primary prostaglandins produced by the cerebral microvasculature. In the present study, statistically significant increases in PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> were noted at approximately 2 and 4 h, respectively, and continued to increase throughout the time period examined. The time course associated with increased release in prostaglandins is similar to both the time period for the induction of COX-2 by TNF- $\alpha$  in the present study, and the appearance of the permeability and cytoskeletal changes demonstrated previously in BBMEC monolayers (Mark and Miller, 1999). Furthermore, the enhanced release of prostaglandins from the BBMEC monolayers following TNF exposure was completely abolished by indomethacin treatment. These results, together with the finding that PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  cause permeability increases in the BBMEC monolayers, at concentrations within the range observed following TNF exposure (i.e., 10 ng/ml), provide compelling support for the role of COX-2 and prostaglandins in the TNF- $\alpha$ -induced response in BBMEC monolayers.

Although products of the COX pathway have effects on vascular permeability, the changes observed appear to be dependent on the particular prostaglandin or thromboxane used and the particular vascular bed examined (Taylor et al., 1987). For example, previous studies have demonstrated that PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  can produce alterations in cytoskeletal structure and increased permeability in pulmonary endothelial cells (Payne et al., 1994). In contrast, studies by Ma and Pedram (1996) demonstrated treatment of aortic endothelial cells with a PGE<sub>2</sub> analog caused a decrease in paracellular permeability, presumably through increases in cAMP. The increased permeability observed with PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  in the present study is the first report of the effects of these particular prostaglandins on brain microvessel endothelial cell permeability. Interestingly, the permeability increases produced by PGE<sub>2</sub> were increased in the presence of indomethacin.



**Fig. 10.** Actin filament staining of BBMEC monolayers treated with medium alone (A and D), PGE<sub>2</sub> (10 ng/ml; B and E), or PGF<sub>2α</sub> (10 ng/ml; C and F) for 60 (A, B, and C) and 90 min (D, E, and F). Control monolayers receiving culture medium alone show a diffuse distribution of the actin filaments, whereas the PGE<sub>2</sub>- and PGF<sub>2α</sub>-treated monolayers show clumping of the actin filaments and the appearance of extracellular gaps (dark spots). BODIPY-phalloidin was used to stain actin filaments. Photos were taken using a 100× oil immersion objective.

Since indomethacin itself did not affect BBMEC monolayer permeability, it is unlikely that the responses to PGE<sub>2</sub> observed in the presence of the COX inhibitor reflect a decreased basal level of endogenous prostaglandins. It is more likely that the enhanced permeability response to PGE<sub>2</sub> following indomethacin treatment is due to inhibition of the production of endogenous eicosanoids in response to the initial effects of PGE<sub>2</sub> that may have opposing effects on permeability. The observation that indomethacin does not decrease PGE<sub>2</sub> responses in BBMEC monolayers indicates that the permeability effects of the prostaglandins are mediated through direct interactions with the endothelial cells.

The present study suggests that TNF- $\alpha$  induced increases in brain endothelial cell permeability occur, at least in part, through the release of prostaglandins and their subsequent effects on the endothelial cells. Although significant increases in permeability were observed with PGE<sub>2</sub> and PGF<sub>2α</sub>, the magnitude of the response was substantially less than the 2-fold increase in BBMEC monolayer permeability observed following TNF- $\alpha$  treatment. Therefore, it may be likely that TNF- $\alpha$ 's activation of the COX-2 enzyme system results in the enhanced release of other prostaglandins and thromboxanes in addition to those currently studied. The combined effects of these eicosanoids may be required to produce the magnitude of permeability and cytoskeletal structural changes observed with TNF- $\alpha$  in BBMEC monolayers. Alternatively, other signaling pathways, in addition to the release of eicosanoids, may be contributing to the responses observed with TNF. The involvement of other signaling pathways and potential cross-talk with the arachidonic acid metabolite pathway are currently being evaluated.

The present study provides compelling evidence implicating COX-2 and prostaglandins in the TNF- $\alpha$ -induced permeability effects observed in brain microvessel endothelial cells. The usefulness of the current *in vitro* model for studying the BBB function and permeability at the cellular level (Miller et al., 1992) suggests COX-2 induction may be involved with the increased BBB permeability observed following TNF exposure. Given the observations that the levels of TNF- $\alpha$  are

higher under conditions such as stroke, multiple sclerosis, meningitis, Alzheimer's, and AIDS-related dementia, COX-2 inhibitors may be of therapeutic benefit in controlling the observed breakdown of the BBB. Although this study has presented significant evidence to support TNF- $\alpha$ -induced prostaglandin involvement in BBB permeability, further investigation is necessary to understand how TNF- $\alpha$ , COX-2, prostaglandins, and possibly other intracellular mediators are connected to cytoskeletal restructuring and increased permeability in the brain microvasculature.

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