HO-2 provides endogenous protection against oxidative stress and apoptosis caused by TNF-α in cerebral vascular endothelial cells

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APOPTOSIS, or programmed cell death, occurs by series of cellular events, including caspase cascade activation, chromosome condensation, DNA fragmentation, membrane blebbing, and cell detachment (14, 47). The pleiotropic cytokine tumor necrosis factor-α (TNF-α) plays a major role in inflammatory responses causing apoptosis by signaling through death receptors. The receptor of TNF-α (TNFR1) recruits cytoplasmic signaling molecules and forms the death-induced signaling complex, which activates serine proteases, known as caspasps, that execute the terminal phases of apoptosis (1, 8, 62). In the brain, TNF-α produced by astrocytes, microglia, and endothelial cells contribute to inflammation, sepsis, stroke, and seizures (3, 21, 23, 37, 42). Seizure-induced neuronal degeneration has been linked to TNFR1-mediated apoptosis (4, 57).

Endothelial cells from cerebral microvessels release vasoactive factors and messenger molecules that play key roles in the regulation of cerebral blood flow. Inflammation and oxidative stress following ischemia, seizures, and brain trauma can cause severe endothelial cell injury (6, 29, 31, 64). Endothelial cells are also involved in inflammatory and immune reactions by producing and responding to cytokines (19, 29, 31, 64, 68). Exposure of endothelial cells to TNF-α may result in endothelial dysfunction (1, 8, 14, 24, 62). However, very little is known about apoptotic effects of TNF-α in cerebral endothelial cells comprising the blood-brain barrier.

Generation of reactive oxygen species (ROS) plays a crucial role in endothelial death induced by TNF-α (12, 15, 52). Endogenous antioxidant stress defense mechanisms are essential for protection from TNF-α-induced apoptosis. Heme oxygenase (HO) metabolizes pro-oxidant heme to carbon monoxide (CO), a gaseous messenger molecule, and biliverdin/bilirubin, potent antioxidants (35, 36). Induction of the stress-responsive HO-1 protected vascular endothelial cells from TNF-α-mediated apoptosis (7, 8).

Cerebral vascular endothelial cells are characterized by high expression and activity of the constitutive HO-2 isoform (48). In cerebral vasculature, HO-1 is not expressed under baseline conditions in vivo and is not induced by epileptic seizures (49), hydrogen peroxide-induced oxidative stress or the excitatory neurotransmitter, glutamate, in vitro (50). The significance of constitutive HO-2 in oxidative stress-induced injury and vascular cell death is poorly understood. HO-2 plays an important role in the regulation of cerebral blood flow in newborn pigs and rats (9, 32, 39, 48). HO-2 activity is critical in preventing seizure-related cerebral vascular injury in vivo (9, 49). Evidence in vitro suggests that HO-2 also contributes to protection against oxidative stress. HO-2 gene deletion increased hemin-induced injury in astrocytes (11) and sensitized cerebral vascular endothelial cells to glutamate-induced apoptosis (50). However, HO-2 did not reduce oxidative stress-mediated damage caused by arachidonic acid, hydrogen peroxide, and peroxynitrite in cerebral endothelial cells (50). Moreover, in astrocytes, HO-2 deletion sensitized the cells to oxidative...
stress-related hemin injury (11). Therefore, it appears HO-2 provides a cell- and stimuli-specific cytoprotection, rather than general antioxidant protection.

The present study, conducted in primary cultures of CMVEC from newborn pigs and wild-type (WT) and HO-2 knockout (KO) mice, addresses the hypotheses that 1) TNF-α causes cerebral vascular endothelial death by apoptosis, 2) HO-2 provides endogenous protection against TNF-α-induced endothelial apoptosis, and 3) CO and bilirubin, the products of HO-2 pathway, protect cerebral vascular endothelium from oxidative stress and apoptosis caused by TNF-α.

METHODS

All protocols and procedures that involve piglets were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee, Memphis. Protocols involving CMVEC isolation from mice were approved by the institutional animal care and use committee at Thomas Jefferson University. All procedures were conducted aseptically.

HO-2 KO mice. The HO-2 KO mice used in this study were descendents of those produced by Poss et al. (50) by replacing a region of mouse HO-2 sequence that corresponds to rat HO-2 exons 4 and 5 with a neomycin resistance cassette containing a pgk promoter. The HO-2 KO mice were cross-bred with WT C57Bl/6 × 129/Sv mice (50). All mice were the product of heterozygous matings, so the HO2-KO and HO2-WT animals are of the same genetic background. The genotype was identified by PCR as previously (50). The mice were bred and then euthanized at 3–4 mo of age at Thomas Jefferson University for isolation of cerebral vascular endothelial cells.

Isolation of cerebral microvessels and cell cultures. Cerebral cortex was obtained from anesthetized newborn pigs and HO-2 KO or WT mice (3 to 4 mo old) (48). CMVEC were isolated from cerebral vessels (60–300 μm) by collagenase-dispase digestion, and separated by centrifugation in a Percoll gradient (48). CMVEC were plated on Matrigel-coated plates or glass coverslips, and cultured in DMEM by centrifugation in a Percoll gradient (48). CMVEC were plated on chromium mesoporphyrin (CrMP, 20 μM) and/or transferrin (20 μM), parthenolide (40–60 μM), superoxide dismutase (SOD) or its permeable analogue (PEG-SOD, 1,000 U), PEG-catalase (500 U), bilirubin (0.1–1 μM) or CORM-A1 (1–50 μM). All protoporphyrin solutions were freshly prepared by dissolving in Dulbecco’s phosphate-buffered saline (DPBS) containing 0.5% ethanolamine. Bilirubin was dissolved in distilled water with the addition of 1–2 drops of NaOH, and pH was adjusted to 7.4 with Tris-HCl. To stabilize bilirubin, a bilirubin/albumin mixture (molar ratio, 1:5) was prepared (18). A CO donor, sodium borocarbonate (CORM-A1), a boron-based carbonylating agent that slowly releases CO in neutral and acidified aqueous solutions (41), was diluted in DPBS immediately before the experiment.

Quantitative detection of DNA fragmentation by ELISA. DNA fragmentation was detected with an apoptosis detection kit (Roche Applied Science, Indianapolis, IN), which allows quantitative detection of cytoplasmic histone-associated-DNA fragments (mono- and oligonucleosomes). Quiescent CMVEC were treated with TNF-α (15 ng/ml) and/or CHX (10 μg/ml) for 3 h. The detached cells were collected for counting. The attached cells were washed with DPBS and lysed with the solution provided. Aliquots of the nuclei-free supernatant were placed in streptavidin-coated wells and incubated with anti-histone-biotin antibody and anti-DNA peroxidase-conjugated antibody for 2 h at room temperature. Immunobilized DNA fragments were visualized with 2,2’-azino-di(3-ethylbenzthiazolin-sulfonate), and the absorbance at 405 nm was detected (5).

Protein detection. CMVEC were lysed with the mammalian protein extraction reagent buffer (M-PER; Pierce, Rockford, IL). The protein contents were measured by the bicinchoninic acid method using a kit from Pierce, or the dot-blot method which allows determination of proteins dissolved in Laemmli sample buffer by their ability to quantitatively bind amidoblack 10B (25).

HO-1 overexpression in porcine CMVEC. Cobalt protoporphyrin (CoPP) is a potent and selective HO-1 inducer in vitro and in vivo (49). Quiescent CMVEC were treated with 20 μM of CoPP in serum-
depleted DMEM (0.1% FBS) for 3 h. After treatment, CoPP was removed from the media, and the incubation continued overnight in fresh DMEM with 0.1% FBS. Under these conditions, HO-1 expression was upregulated 10-fold.

**Western blot analysis.** CMVEC were solubilized in Laemmli sample buffer (100°C for 10 min). Proteins (20–50 μg/lane) were separated by 15% SDS-PAGE, transferred to PVDF membranes, and blocked with 5% BSA-0.1% Tween 20. Caspase-3 is expressed as an inactive 32-kDa precursor from which a variety of cleaved active subunits (11–19 kDa) are proteolytically generated during apoptosis. Caspase-3 activation was detected by formation of the 17- and 19-kDa proteolytic fragments using highly selective polyclonal antibodies (caspase-3-Asp175, Cell Signaling, Beverly, MA). To detect HO-1, membranes were probed with polyclonal antibody against human HO-1 (1:5,000) (SPA 895, Stressgen, Victoria, Canada), followed by peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO). To normalize caspase-3 and HO-1 expression to actin, the membranes were reprobed with monoclonal anti-actin (Roche Molecular Biochemicals, Indianapolis, IN) (1:10,000), followed by peroxidase-conjugated anti-mouse IgG (1:10,000). The immunocomplexes were visualized with the Western lightning chemiluminescence kit (Perkin Elmer Life Sciences, Boston, MA) and quantified by digital densitometry using NIH Image J 1.33 software.

**Immunostaining.** Control or treated cells were fixed with 3.7% paraformaldehyde (pH 7.4, 20 min), permeabilized with 0.1% Triton X-100 (20 min), and blocked with 5% BSA/PBS (1 h), at room temperature. The cells were incubated with monoclonal antibodies against NF-κB p65 (1:30) (BD Transduction, San Diego, CA), or β-catenin (1:20) (Cell Signaling) for 1 h at 37°C, followed by FITC-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h at 37°C. For F-actin staining, cells were incubated with rhodamine-phalloidin (1:100, Molecular Probes, Eugene, OR). Coverslips were mounted using antifade mounting medium (Vector Laboratories). Slides were viewed using an Image Deconvolution system consisting of Nikon Diaphot microscope with fluorescence filter coupled to MacQuadra 950 computer with a Mac processor 601 in conjunction with a cooled charge-coupled device camera (model 250 CH; Nikon). Images were deconvolved using IPLab Spectrum software for image collection. Digital processing of the images was done using Photoshop (Adobe Systems).

**Cytotoxicity assay.** Cytotoxicity was estimated by release of a cytosolic adenylate kinase (AK) activity into the medium (cytolysis) detected using the ToxiLight bioluminescent cytotoxicity kit (Cambrex, Rockland, ME).

**Detection of oxidant generation.** To assess intracellular ROS, we used two independent oxidant-sensitive probes, dihydrodihydrodiammine-123 (DHR123) and dihydroethidium (DHE) (50). DHR123, a cell-permeable nonfluorescent compound, is oxidized to fluorescent rhodamine 123 (Rh123) by a variety of ROS, especially H2O2 and peroxynitrite, and accumulates in mitochondria. DHE, a superoxide-sensitive probe, is oxidized by O2·− to ethidium and oxyethidium, intercalates with DNA in the nucleus and emits red fluorescence. To visualize ROS formation in live cells by fluorescence microscopy, CMVEC were preloaded with 50 μM DHR123 (20 min at 37°C) and incubated with 15 ng/ml TNF-α for an additional 20 min. Cells were rapidly rinsed 3 times with DPBS and observed under a Nikon Diaphot microscope with a fluorescence filter. For spectroscopic measurements of ROS generation, CMVEC were incubated with 15 ng/ml TNF-α (alone or with antioxidants as indicated) for 3 h. DHR123 (50 μM) or DHE (20 μM) were added to the incubation media for additional 20 min. To detect overall cytokine-induced oxidative stress, both attached and floating cells were collected by scraping and aspiration. Collected cells were diluted 10 times with ice-cold PBS and pelleted at 3,000 g for 10 min at 4°C. The process was repeated twice to remove free probes. The cell pellet was disrupted by sonication in 100 μl of ice-cold DPBS, and cell debris was pelleted. Supernatant was transferred to Falcon 96-well black microplates in duplicates, and fluorescence was measured with a Fusion αHT Universal Microplate Analyzer (Packard Instrumental) using excitation and emission wavelengths 470/525 nm for Rh123, or 490/605 nm for ethidium.
Statistical analysis. Data are presented as means ± SE of absolute values or percent of control. Data were analyzed by analysis of variance, followed by the Tukey-Kramer multiple-comparison test to isolate differences between groups. P < 0.05 was considered significant in all statistical tests.

Materials. Cell culture reagents were purchased from Life Technologies (Gaithersburg, MD), Hyclone (South Logan, UT) and Amersham Pharmacia Biotech (Piscataway, NJ). Human and rat TNF-α and Matrigel were from BD Biosciences (Bedford, MA). CORM A-1 was a generous gift from Tyco Healthcare/Mallincrodt (Petten, The Netherlands). SnPP and CoPP were purchased from Frontier Scientific (Logan, UT). DHR123 and DHE were from Molecular Probes. All other reagents were from Sigma.

RESULTS

Apoptotic effects of TNF-α in mouse CMVEC. Primary cultures of CMVEC isolated from H02-KO and H02-WT mice brains were established (50). Genotypes of H02-KO and WT mice were confirmed by PCR, immunoblotting, and immunostaining for HO-2 (50). HO-1 expression was not upregulated in HO-2 gene-deleted CMVEC (50). We compared the apoptotic effects of rat TNF-α and CHX, alone or in combination, in WT and H02-KO mouse CMVEC. Following overnight serum withdrawal, H02-KO CMVEC had increased apoptosis as indicated by DNA fragmentation (Fig. 1A), cell detachment (Fig. 1B), and caspase-3 activity (Fig. 1C). TNF-α (15 ng/ml) combined with CHX (10 μg/ml) increased apoptosis in mouse CMVEC, whereas TNF-α or CHX were without effects (Figs. 1, A–C). H02-KO CMVEC showed much greater sensitivity to TNF/CHX induced apoptosis evidenced by greater DNA fragmentation (Fig. 1A), cell detachment (Fig. 1B), and caspase-3 activity (Fig. 1C) compared with WT CMVEC. These data indicate that HO-2 gene deletion increases the sensitivity of CMVEC to TNF/CHX induced apoptosis.

TNF-α-induced NF-κB translocation in mouse CMVEC. Transcription factor NF-κB, a critical mediator of inflammatory apoptotic responses, resides in the cytoplasm of unstimulated cells. Upon initiation of apoptosis, NF-κB translocates to the nucleus (40, 52). In WT CMVEC, NF-κB is in the cytoplasm, whereas in H02-KO CMVEC nuclear localization is already detectable under basal condition (Fig. 2, A and D). In H02-KO CMVEC, nuclear translocation of NF-κB was observed after a 15-min treatment with TNF-α/CHX in 40 ± 10% cells (5 microscopic fields in 3 independent preparations), whereas in WT CMVEC, NF-κB remained in the cytoplasm (no cells in 5 microscopic fields in 3 independent preparations) (Fig. 2, B and E). After a 1-h treatment with TNF-α/CHX, complete cytosolic-nuclear translocation of NF-κB was observed in both WT and H02-KO cells (90 ± 5% and 95 ± 5% of the total cells, respectively, 5 microscopic fields in 3 independent preparations; Fig. 2, C and F). These data indicate the HO2 gene deletion accelerates the cytokine-induced NF-κB nuclear translocation, a key indicator of apoptosis.

TNF-α-induced cytotoxicity in H02-KO CMVEC. Cytolysis, a major indicator of cytotoxicity, was measured by release of cytosolic adenylate kinase (AK) into the media. In WT CMVEC, no cytotoxic effects were observed following a 3-h treatment with rat TNF-α (15 ng/ml) and/or CHX (10 μg/ml). In contrast, in H02-KO CMVEC, AK activity was increased to 230 ± 50% following a 3-h incubation with TNF-α/CHX (P < 0.05 compared with baseline values, N = 4 independent cultures). Neither TNF-α nor CHX alone had cytotoxic effects in H02-KO CMVEC.

TNF-α induces apoptosis in CMVEC from newborn piglets. We investigated whether TNF-α, alone or in combination with CHX, causes apoptosis in CMVEC from newborn pigs that constitutively express high HO-2 activity. In porcine CMVEC, human TNF-α alone (15 ng/ml, 3 h) caused significant DNA fragmentation and cell detachment (threefold above baseline; see Fig. 3, A and B). CHX (10 μg/ml) alone increased DNA fragmentation and cell detachment and greatly potentiated TNF-α-induced apoptosis. These data suggest that in contrast to mouse WT cells, TNF-α alone is a potent inducer of apoptosis in piglet CMVEC.

TNF-α does not upregulate HO-1 in mouse or piglet CMVEC. In piglet cerebral microvessels, HO-2 is highly expressed (Fig. 4A), while HO-1 is not detectable (49). In cultured mouse and
piglet CMVEC, baseline HO-1 protein level is detectable (Fig. 4, B and C). Prolonged (6–12 h) treatment with TNF-α or TNF-α/CHX did not upregulate HO-1 expression in HO2-KO (mouse) or HO2-WT (mouse and piglet) cells (Fig. 4, B and C).

Inhibition of HO-2 activity sensitizes piglet CMVEC to TNF-α-induced apoptosis. To investigate whether constitutive HO-2 plays a role in CMVEC survival, we used SnPP (20 μM) that effectively inhibits HO activity in CMVEC (33–35, 38). SnPP increased the basal level of apoptosis caused by serum deprivation as evidenced by DNA fragmentation (Fig. 5A) and caspase-3 activation (Fig. 5B). Furthermore, SnPP increased DNA fragmentation and caspase-3 activation caused by TNF-α alone or in combination with CHX (Fig. 5, A and B). In contrast, SnPP did not enhance apoptotic changes induced by CHX alone (Fig. 5, A and B).

Proapoptotic role of NF-κB in TNF-α-induced apoptosis. The translocation of NF-κB from cytoplasmic to nuclear sites is the key event of apoptosis (40, 52). Human TNF-α (15 ng/ml, 3 h) and CHX (10 μg/ml, 3 h), alone or in combination, caused NF-κB nuclear translocation in piglet CMVEC (Fig. 6, A–D), consistent with apoptosis. To determine the role of NF-κB, we used TPCK, a serine/threonine protease inhibitor that selectively inhibits NF-κB signaling (65) and parthenolide, a sesquiterpene lactone, that inhibits NF-κB activation by directly interacting with p65 subunit of NF-κB and, to a minor degree, by inhibition of IκB-α degradation (22). TPCK (20–40 μM) and parthenolide (40–60 μM) blocked or greatly reduced DNA fragmentation response to TNF-α, CHX, and TNF/CHX (Fig. 7A). Both inhibitors dose-dependently inhibited caspase-3 activation: 1) in low concentrations, they prevented complete proteolysis of the zymogen to a 17-kDa fragment, resulting in accumulation of a 19-kDa band was accumulated; 2) in higher concentrations, both completely blocked caspase-3 activation evidenced by the absence of both the 17- and 19-kDa bands (Fig. 7B). These data indicate that NF-κB has pro-apoptotic role in TNF-α-evoked apoptosis signaling in CMVEC.

HO-1 overexpressing CMVEC are resistant to TNF-α-induced apoptosis. CoPP is a potent HO-1 inducer in CMVEC (49). We investigated whether HO-1 is protective against TNF-α-mediated cell death. HO-1 expression in piglet CMVEC was upregulated ~10-fold by a 3-h treatment with 20 μM CoPP, followed by overnight incubation in the starvation medium (Fig. 8B). HO-1 overexpressing CMVEC did not respond to TNF-α or TNF-α/CHX by apoptosis-related DNA fragmentation and caspase-3 activation (Fig. 8, A and B). Surprisingly, inhibition of HO-1 activity by SnPP did not restore apoptotic responses to TNF-α and/or CHX (Fig. 8, A and B).

Bilirubin and CO protect from TNF-α-induced apoptosis in piglet CMVEC. We investigated whether bilirubin or CO can protect piglet CMVEC from TNF-α-induced apoptosis. CMVEC were treated with TNF-α and/or CHX for 3 h in the absence or presence of bilirubin and CORM-A1, a spontaneous CO donor. Bilirubin (1 μM) inhibited DNA fragmentation (Fig. 9A) and caspase-3 activation (Fig. 9B) caused by TNF-α. Bilirubin was...
less effective in inhibiting TNF-α/CHX-induced apoptosis, although reduction of DNA fragmentation and caspase-3 activation were also observed (Fig. 9, A and B). CORM-A1 (50 μM) completely prevented DNA fragmentation and caspase-3 activation in response to TNF-α alone and greatly reduced apoptotic changes caused by a TNF-α/CHX treatment (Fig. 10, A and B). These data indicate that both bilirubin and CO are anti-apoptotic factors that attenuate TNF-α-induced endothelial death.

Effects of endogenous HO-2 inhibition and HO products on TNF-α-induced oxidative stress in piglet CMVEC. To determine whether HO-2 inhibition alters ROS formation, CMVEC were treated with HO inhibitors, SnPP or CrMP, for 3 h. ROS production was measured with two independent probes, DHR123 (total ROS formation) and DHE (a superoxide-sensitive probe). SnPP (20 μM) and CrMP (20 μM) greatly increased ROS formation. Rh123 fluorescence, 32 ± 3, 168 ± 12, and 234 ± 25 optical density units in control, SnPP-treated, and CrMP-treated cells, respectively (P < 0.05 compared with control values, n = 3 independent cultures in duplicates); ethidium fluorescence, 10 ± 2, 35 ± 6, and 31 ± 7 in control, SnPP-treated, and CrMP-treated cells, respectively (P < 0.05 compared with the control values, n = 3 independent cultures in duplicate). These data demonstrate antioxidant potential of endogenous HO-2 activity.

TNF-α (15 ng/ml, 3 h) greatly increased ROS formation detected by Rh123 and ethidium fluorescence microscopy (Fig. 11, A and B) and spectroscopy (Fig. 12, A and B). TNF-α also disrupted the junctional integrity of cerebral vascular endothelium as detected by β-catenin immunostaining. In control CMVEC, β-catenin, an integral component of cell-cell adherence junctions responsible for the maintenance of endothelial barrier functions, is localized to the CMVEC periphery (Fig. 11C). In TNF-α-treated cells, a notable disruption of the cell-cell contacts was observed as evidenced by punctate staining, membrane blebbing, and decreased appearance of β-catenin at the endothelial adherence junction areas (Figs. 11D).

Bilirubin (1 μM) and CORM-A1 (1–50 μM) inhibited Rh123 and ethidium fluorescence responses to TNF-α (Fig. 12, A and B). SOD and its permeable derivative PEG-SOD (1,000 U/ml) blocked TNF-α-induced Rh123 and ethidium fluorescence signals (Fig. 12, A and B). The cell-permeable derivative of catalase, 500 U/ml PEG-catalase, also reduced Rh123 fluorescence (Fig. 12A). Overall, these data indicate that endogenous HO-2 metabolites exert antioxidant effects in serum-starved and TNF-α-treated CMVEC.

DISCUSSION

Our major novel findings in mouse HO-2 gene-deleted and in HO-2-expressing mouse and newborn pig cerebral vascular endothelial cells are as follows: 1) the pro-inflammatory cytokine, TNF-α, alone (in piglet cells) or in combination with the protein synthesis inhibitor CHX (in piglet and mouse cells), causes oxidative stress-mediated apoptosis detected by caspase-3 activation, NF-κB cytoplasmic-nuclear translocation, cell-cell contact disruption, DNA fragmentation and cell detachment, 2) HO2 gene deletion and inhibition of HO-2 activity greatly potentiate TNF-α and/or TNF-α/CHX-induced apoptosis, 3) products of endogenous HO-2 activity, CO and...
Fig. 7. NF-κB inhibitors block TNF-α-induced apoptosis in CMVEC from newborn piglets. Quiescent CMVEC were treated for 3 h with human TNF-α (15 ng/ml), CHX (10 μg/ml), or TNF/CHX in the absence or presence of NF-κB inhibitors N-tosyl-L-phenylalanine (TPCK; 20 and 40 μM) or parthenolide (40 and 60 μM). A: DNA fragmentation. B: caspase-3 activation (representative blots). Data represent an average of 3 independent experiments. Values are means ± SE. *P < 0.05 compared with control values.

Fig. 8. Apoptotic effects of TNF-α in HO-1-overexpressing piglet CMVEC. To overexpress HO-1, CMVEC were treated with cobalt protoporphyrin (CoPP; 20 μM). CMVEC (control or CoPP pretreated) were incubated with human TNF-α (15 ng/ml) and/or CHX (10 μg/ml) for 3 h in the absence or presence of 1 μM bilirubin/5 μM albumin. A: DNA fragmentation. B: representative blots of 17-kDa caspase-3 active fragment and actin. Data represent average of 3 independent experiments. Values are means ± SE. *P < 0.05 compared with the control values. †P < 0.05 compared with the corresponding values in the absence of bilirubin.

Fig. 9. Bilirubin protects newborn pig CMVEC from TNF-α-induced apoptosis. Confluent CMVEC from newborn pigs were incubated with human TNF-α (15 ng/ml) or TNF/CHX (10 μg/ml) for 3 h in the absence or presence of 1 μM bilirubin/5 μM albumin. A: DNA fragmentation. B: representative blots of 17-kDa caspase-3 active fragment and actin. Data represent average of 3 independent experiments. Values are means ± SE. *P < 0.05 compared with the control values.
TNF-α-INDUCED ENDOTHELIAL APOPTOSIS

Fig. 10. CORM-A1 (CO-releasing agent) protects CMVEC from newborn piglets from TNF-α-induced apoptosis. Confluent CMVEC were incubated with human TNF-α (15 ng/ml) or TNF/CHX (10 μg/ml) for 3 h in the absence or presence of 50 μM CORM-A1. A: DNA fragmentation. B: representative blots of 17-kDa caspase-3 active fragment and actin. Data represent average of 3 independent experiments. Values are means ± SE. *P < 0.05 compared with the control values. †P < 0.05 compared with the corresponding values in the absence of CORM-A1.

Fig. 11. Immunofluorescence detection of reactive oxygen species (ROS) (A, B) and β-catenin (C, D) in newborn pig. Live CMVEC preloaded with dihydroorhodamine 123 (DHR123) (50 μM) were incubated with either Krebs buffer (control, A) or human TNF-α (15 ng/ml, B) for 20 min, and Rh123 fluorescence was detected by microscopy. For β-catenin immunostaining, confluent quiescent CMVEC were untreated (C) or treated with human TNF-α (15 ng/ml) for 3 h (D). Cells were immunostained for β-catenin and visualized with FITC-conjugated anti-mouse IgG. Scale bar, 20 μm.
Control and treated cells were loaded with 50 (1,000 U/ml), 13 h in the absence or presence of PEG-catalase (500 U/ml), SOD or PEG-SOD. Data represent average of 3 independent experiments. Values are means of cerebral vascular endothelium to TNF-

Fig. 12. Effects of TNF-α on ROS production. Confluent quiescent CMVEC from newborn pigs were untreated or treated with human TNF-α (15 ng/ml) for 3 h in the absence or presence of PEG-catalase (500 U/ml), SOD or PEG-SOD (1,000 U/ml), 1 µM bilirubin/5 µM albumin (BR) or CORM-A1 (1–50 µM). Control and treated cells were loaded with 50 µM DHR123 (A) or 20 µM dihydroethidium (DHE) (B). Fluorescence of the total cell lysates was measured in fusion oHT microplate analyzer and expressed in optical density units. Data represent average of 3 independent experiments. Values are means ± SE. *P < 0.05 compared with the control value.

found species- and/or age-specific differences in the responses of cerebral vascular endothelium to TNF-α. In adult mouse CMVEC, TNF-α initiated apoptosis only when the cells were sensitized by cycloheximide, a protein synthesis inhibitor. In newborn pig CMVEC, TNF-α alone was sufficient to induce apoptosis, although cycloheximide also greatly potentiated cell death. We therefore conclude that inhibition of de novo protein synthesis is required for the induction of cell death in mouse and, to a lesser extent, in piglet CMVEC.

We provide evidence that in cerebral vascular endothelium, apoptotic responses to TNF-α are mediated by oxidative stress. Oxidative stress is the consequence of an imbalance between the formation of ROS and the antioxidant capacity of the cell (43). ROS include superoxide anion radical, hydrogen peroxide, peroxynitrite, and hydroxyl radical that readily react with various biological molecules including membrane lipids, cytoskeletal proteins, and DNA. TNF-α rapidly and steadily increased ROS formation in CMVEC as detected using DHR123 (overall ROS production, preferentially hydrogen peroxide and peroxynitrite) and a superoxide-selective probe, DHE. SOD and its permeable analogue, PEG-SOD, as well as PEG-catalase blocked both the ROS and apoptosis responses to TNF-α, indicating that superoxide and hydrogen peroxide are among the leading factors in the cytokine-induced endothelial damage.

HO-1 gene belongs to a group of antioxidant stress response elements, which can be induced by a variety of oxidative stress factors and is important in cell survival against oxidative stress (7, 13, 44, 45, 66). HO-1 is not expressed in cerebral vessels under physiological conditions, whereas in cultured mouse and pig CMVEC, basal HO-1 protein level is detectable due to the presence of growth-stimulating factors in the culture media. In contrast to other cell types, in cerebral vascular endothelium in vitro HO-1 is induced only by severe oxidative stress induced by peroxynitrite, but not hydrogen peroxide or excitotoxic glutamate (50). Our current study in pig and mouse CMVEC showed that HO-1 is not induced during oxidative stress conditions caused by apoptotic concentrations of TNF-α alone or even combined with CHX. CoPP is the most potent and selective HO-1 inducer in cerebral vascular endothelium in vivo and in vitro (49). Antia apoptotic effects of HO-1 are clearly indicated by complete resistance of HO-1 overexpressing CMVEC to TNF-α-induced apoptosis. Surprisingly, we found that in HO-1-overexpressing CMVEC, the HO inhibitor, SnPP, did not reverse apoptosis protection to the cytokine. In contrast, SnPP greatly potentiated apoptotic responses in control CMVEC. It is possible that HO-1 and HO-2 isoforms have distinct sensitivity and/or selectivity to pharmacological inhibitors. Still it is also conceivable that HO-1 has anti-apoptotic endothelial protective functions that are not related to heme metabolizing activity. Indeed, in cerebral vascular endothelium HO-1 is localized to the nuclear envelope and the nucleus (47, 50), indicating possible nuclear functions of the isoform. However, because HO-1 is not inducible by TNF-α, HO-1 cannot be considered a physiologically significant anti-apoptotic endogenous factor that may protect from cerebrovascular endothelial damage induced by TNF-α-mediated inflammation.

The cerebral microcirculation is characterized by a high expression and activity of constitutive HO-2 (35, 38). HO-2-produced CO is a paracrine and autocrine relaxing factor in the cerebral circulation that contributes to vasodilator responses to glutamate, hypoxia, and seizures in newborn pigs (9, 33–35, 49) and adult rats (39). Recently, we demonstrated that inhibition of HO-2 activity or HO-2 gene deletion exaggerated oxidative stress and apoptosis induced by glutamate in cerebral vascular endothelium (50). Our present data in newborn pigs and WT and HO-2 gene-deleted mice show that pharmacological inhibition or complete elimination of HO-2 activity causes excessive ROS formation during basal conditions and in response to TNF-α stimulation, indicating that HO-2 is an antioxidant and antiapoptotic factor in cerebral circulation.

Bilirubin, a product of HO/biliverdin reductase reactions, was long considered a toxic metabolite of heme catabolism responsible for the clinical manifestation of jaundice, particularly in neonates. Recently, the potent biological properties of bilirubin as a major physiological antioxidant have been recognized (61). Bilirubin is an effective scavenger of superoxide and peroxyl radicals (38, 61). Antioxidant properties of bilirubin have been also suggested based on the ability of biliverdin/bilirubin to undergo the redox cycling (2, 18, 36, 38, 61). We confirm the antioxidant potency of bilirubin in our experimen-
tal system by measuring ROS formation in TNF-α-treated cells. Increasing evidence suggests that bilirubin acts as a powerful chain-breaking antioxidant that may exert cytoprotective effects (17, 18, 61). In nervous tissue, bilirubin serves as an important cytoprotective factor (2, 17, 18). Our previous (50) and present data demonstrate that bilirubin is an extremely efficient antiapoptotic factor that protects cerebrovascular endothelium from oxidative stress-related apoptosis induced by glutamate and TNF-α. Therefore, bilirubin can provide physiological cytoprotection against oxidant-mediated cell and tissue injury.

While accumulating evidence indicates that CO may also act as anti-apoptotic cytoprotective factor (28, 35, 50, 67), the possibility of antioxidant properties of CO needs to be addressed. Recently, we demonstrated that CO is a potent antioxidant and antiapoptotic factor that reduces glutamate-induced apoptosis in cerebral vascular endothelium (50). Our present data provide direct evidence that CO administered in the form of a CO-releasing molecule, CORM-A1, blunts severe oxidative stress caused by TNF-α by reducing hydrogen peroxide and superoxide anion formation. Astoundingly, CORM-A1 was even more effective than SOD, catalase, or bilirubin, in preventing oxidative stress-related apoptosis in response to TNF-α in cerebral vascular endothelium. On the basis of CO chemistry, it is unlikely that CO acts as a ROS scavenger. However, due to its heme-binding capacity, CO may inhibit oxidant-producing enzymes, including heme-containing mitochondrial cytochromes and the electron transport chain components, thus decreasing ROS formation. Recent papers present experimental evidence that CO and CO-releasing molecules do inhibit mitochondrial function and ROS generation (16, 30, 54, 55, 58, 59). Anti-oxidant properties of CO may also be due to interaction with the heme proteins of the NF-κB signaling pathway (7, 8).

Nuclear transcription factor NF-κB plays a key role in controlling apoptosis (26). The cellular consequences of the NF-κB pathway, that include translocation to the nuclear sites upon activation, are complex and may produce both pro- and anti-apoptotic effects in a stimuli-dependent manner (20, 26, 40, 60). Cytoplasmic-nuclear NF-κB translocation was observed in CMVEC in response to TNF-α, CHX, or TNF-α/CHX. HO-2 KO CMVEC showed accelerated NF-κB translocation consistent with exaggerated apoptotic responses, including DNA fragmentation. NF-κB signaling inhibitors, TPCK and parthenolide, blocked DNA fragmentation and caspase-3 activation initiated by TNF-α, CHX, or TNF-α/CHX. These findings suggest that NF-κB plays a proapoptotic role in TNF-α- and TNF-α/CHX-induced apoptosis in cerebral vascular endothelium.

We present evidence that TNF-α may cause disruption of the blood-brain barrier. β-Catenin, a component of adherence junctions, is responsible for the maintenance of endothelial barrier functions. Functional disassembly and modifications of the cadherin/catenin complex may lead to increased paracellular permeability of the endothelial barrier. β-Catenin integrity is important for the survival from TNF-α-induced apoptosis (46, 56). TNF-α-mediated apoptosis in CMVEC was accompanied by loss of cell-cell contacts and disruption of β-catenin at the cell periphery. These results suggest that progression of apoptosis in cerebral vascular endothelium is concomitant with the loss of integrity of adherence junctions resulting in cell detachment from the substratum.

Overall, the pleiotropic cytokine TNF-α causes oxidative stress-mediated apoptosis in adult mouse and newborn piglet cerebral vascular endothelial cells. CO and bilirubin, products of heme degradation by the HO pathway, are potent antioxidants and antiapoptotic factors against endothelial injury caused by TNF-α. HO-1 is not induced in cerebral vascular endothelium by TNF-α, and, therefore, is not physiologically relevant to endogenous protection against the cytokine-induced cerebrovascular injury. On the other hand, the products of HO-2 activity appear to be essential components in endogenous defense against TNF-α-induced apoptosis in the cerebral vascular endothelium.

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