Verotoxin (Shiga Toxin) Sensitizes Renal Epithelial Cells to Increased Heme Toxicity: Possible Implications for the Hemolytic Uremic Syndrome

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*Escherichia coli*–derived verotoxins (VT; Shiga toxins) are causally related to the pathogenesis of enteropathic hemolytic uremic syndrome (HUS). Profound hemolysis is a defining feature of the disease, but it is not known whether the acute intravascular release of heme proteins contributes to HUS pathology. This study examined the biologic effects of heme and VT by means of tubular epithelial-derived ACHN cells. Hemin at concentrations ≥200 μM caused cell rounding, spike formation, and detachment that was morphologically distinct from verocytotoxicity. VT caused apoptosis at concentrations >100 pM, as demonstrated by nuclear segmentation and poly-(ADP-ribose) polymerase cleavage, whereas hemin-mediated injury of ACHN cells grown in serum-containing medium lacked attributes of programmed cell death. Pretreatment of ACHN monolayers with sublethal concentrations (1 to 10 pM) of VT for 12 to 18 h led to superadditive hemin-mediated cytotoxicity. This effect was not limited to ACHN cells, but was similarly noted in microvascular endothelial cells. Heme catabolism is regulated by (inducible) heme oxygenase-1 (HO-1). VT abrogated HO-1 expression in ACHN cells. Stimulation of cells for 6 h with CdCl2, which markedly increased HO-1 expression before the addition of VT, blunted subsequent heme injury. In conclusion, VT augments hemin-induced toxicity in renal tubular epithelial cells that can be reversed by prior induction of HO-1. It is proposed that VT subverts the physiologic defense against heme proteins by interfering with the regulated expression of HO-1 and that this mechanism contributes to the renal pathology in patients with *Escherichia coli*–associated HUS.
hemoglobin-haptoglobin complex is rapidly cleared by mononuclear phagocytes endowed with a high affinity CD163 scavenger receptor (18). Unbound hemoglobin is filtered in the renal glomerulus as αβ dimers, and reabsorbed and catabolized in proximal tubule cells. Spontaneous oxidation to met[Fe³⁺] hemoglobin destabilizes extrarerythrocytic hemoglobin, which results in the release of the heme moiety (19–21). Oxidized (ferric) protoporphyrin IX (hemin) is commonly isolated as a halide (hemin chloride) (18). It is a highly hydrophobic molecule capable of inducing adverse biologic effects. It intercalates with the membrane lipid bilayer and potentiates oxidant-mediated injury (20,22–24). The exact mechanism or mechanisms of heme toxicity and the predominant pathway of cell death remains to be defined.

To protect the integrity of the surrounding tissue, excess free heme is degraded, largely by two (microsomal) heme oxygenase (HO) isoforms, HO-1 (inducible), and HO-2 (constitutive). HO catalyzes the initial and rate-limiting step in heme catabolism, the oxidative cleavage of the protoporphyrin ring by three sequential monoxygenase reactions (25), thereby generating several biologically important products: ferrous iron, which stimulates the induction of ferritin, a high capacity intracellular iron store; biliverdin, the precursor of the (antioxidant) bilirubin; and the gas carbon monoxide (CO) (26). CO is increasingly recognized as an important messenger molecule with vasorelaxant, antithrombotic, antiinflammatory and antiapoptotic properties (24,26,27). Potent inducers of HO-1, both in vitro and in vivo, are heavy metals, oxidative stress, and its substrate, heme (28,29).

Acute kidney failure due to massive release of hemoglobin or myoglobin, as in blood transfusion accidents and rhabdomyolysis, demonstrates the potential of heme proteins to cause renal injury (29–31). We reasoned that because of the profound hemolysis observed in patients with HUS, the kidneys would be burdened with large amounts of hemoglobin and heme. This study tests the hypothesis that VT interferes with the regulated response to, and compromises the cellular response against, excess heme in renal tubular epithelial cells. We further examined if induction of the heme-degrading enzyme, HO-1 modifies the cell response to hemin or VT.

Materials and Methods

Materials

Rabbit anti–heme oxygenase-1 specific for the N-terminal portion of human HO-1 was purchased from StressGen (Vancouver, BC, Canada); polyclonal anti-poly(ADP-ribose) polymerase (PARP) antibody were from Cell Signaling Technology (Beverly, MA); monoclonal anti–β-actin (N-terminus) antibody (mouse IgG1), goat anti-rabbit and rabbit anti-mouse horseradish peroxidase–conjugated antibodies were purchased from Sigma Chemicals (St. Louis, MO). Hemin (Fe[III] protoporphyrin IX chloride) was from Porphyrin Products (Logan, UT). Human recombinant TNF-α was from ICN Biomedicals (Irvine, CA). Cadmium chloride hemi(pentahydrate), cycloheximide, and DAPI (4',6-diamidino-2'-phenylindole dihydrochloride) and staurosporine (S 5921) were from Sigma. Crystalline protease- and Ig-free BSA was from Jackson Immuno Research Laboratories (West Grove, PA). All other chemical reagents were of analytical grade and were purchased from Fisher, unless indicated otherwise. Cell culture media and reagents were from Life Technologies/Life Technologies BRL (Grand Island, NY), unless indicated otherwise. FBS was from Cellgro Mediatech (Hendon, VA). Cell culture dishes were from TTP (The Technology Partnership, Royston, UK) and from Corning (Corning, NY). Highly purified verotoxins 1 and 2 were gifts from Dr. M. A. Karmali (Guelph, Ontario). Both toxins bind to the same globotriaosylceramide (Gb3) receptor and demonstrate similar biologic effects and cytotoxic activities by means of standardized Vero cell assays (6,12,13).

Cell Culture

Human renal carcinoma–derived tubular epithelial (ACHN) cells were obtained from the American Tissue Culture Collection (ATCC CRL-1611) and propagated in MEM with Earle salts and l-glutamine, supplemented with penicillin-streptomycin and 10% FBS. Cells were serially passaged every 5 to 7 d on 100-mm dishes with 0.05% trypsin-EDTA. To assess the effects of agents on ACHN cells, 100 mm dishes were seeded at a density of approximately 2 × 10⁵ cells and 96-well dishes at approximately 3 × 10⁴ cells/well and grown to confluence. Primary, pooled dermal human microvascular endothelial cells (HMVEC) were obtained at passage 3 from Clonetics/BioWhittacker (Walkersville, MD) and propagated (50 10⁵ cells per 35-mm dish) in endothelial growth medium containing a mixture of growth factors, hydrocortisone, 5% FBS, heparin, and gentamicin-ampthomycin (EGM2-MV, Clonetics). Cells were detached with 0.025% trypsin-EDTA and used through passages 5 to 7. ACHN (8,10,32) and HMVEC (11,12,33) have been previously shown to express Gb3 on their surface and to be sensitive to VT-induced cytotoxicity.

VT was added after dilution in tissue culture medium. Hemin was dissolved in 0.1 M NaOH to a concentration of 20 mM and further diluted in tissue culture medium under subdued light and immediately added to the cell culture. CdCl₂ was dissolved in H₂O to a concentration of 10 mM and further diluted in tissue culture medium. Because CdCl₂ caused ACHN cell toxicity at concentrations ≥100 μM, experiments were performed with 50 μM. Medium was replenished 6 h after the addition of CdCl₂ to further minimize its cytotoxic effect. All other agents remained in the dish throughout the experiment. Morphologic changes of (live) cell cultures were assessed with an inverted phase microscope.

Cytotoxicity Assays

Monolayer disruption was quantitated spectrophotometrically as described previously (12). Briefly, monolayers were fixed with 2% formalin in PBS and stained with 0.13% crystal violet, rinsed thoroughly, and dried. Cell-associated dye was eluted with 50% ethanol in water and monitored at 490 nm with an automated plate reader. The optical density directly correlates with the number of residual cells. The 50% cytotoxic dose (CD₅₀) was calculated as described previously (12). When appropriate, A₅₀₀ values were normalized to vehicle-treated controls.

Apoptosis Assays

Cell monolayers were grown in tissue culture dishes, treated with agents, and stained with DAPI (34). Briefly, nonadherent (floating) cells were collected and washed in ice-cold PBS at 200 °C and 4°C for 5 min, settled for 60 min on 12-mm glass cover slips previously coated with poly-L-lysine (1 mg/ml PBS) (Fisher) and fixed with
methanol for 1 min, followed by 0.1 μg/ml DAPI in methanol (2 min), rinsed sequentially in methanol and PBS, and mounted on glass slides with 2 μl Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Adherent (nondetached) cells were fixed and stained in situ. Cell preparations were viewed under incident ultraviolet (UV) light with a Zeiss Axiovert 10 microscope equipped with a filter for UV excitation (DAPI) and digital imaging fluorescence microscopy system (Zeiss Axioplan 2). Apoptotic cells were identified by segmented morphology of DAPI-stained cell nuclei. At least five different fields per slide were evaluated.

Protein Extraction and Western Blot Analysis
Nonadherent cells were collected on ice. Adherent cells were detached by gentle scraping in ice-cold PBS, pooled with the nonadherent cells, pelleted, and lysed with modified Laemmli buffer in the presence of protease inhibitors (Protease Inhibitor Cocktail Set II; Calbiochem, San Diego CA). Cell lysates were cleared by centrifugation at 14,000 × g for 5 min at 4°C and stored at −70°C. Protein concentrations were measured with a modified Lowry (Bio-Rad detergent compatible) protein assay (Bio-Rad, Hercules, CA) with BSA as standard. Protein extracts were resolved by SDS-PAGE (12%) under reducing (0.02 M DTT) and denaturing conditions and transferred onto a polyvinylidifluoride membrane (Immun-Blot; Bio-Rad) by electroblotting. Membranes were blocked for 2 h in 50 mM Tris-buffered saline (TBS) containing 5% BSA and 0.1% Triton X-100 (TBS-T-BSA5%), followed by incubation with a polyclonal anti-human HO-1 antibody (1:2000 in TBS-BSA1%) for 1 h at room temperature, or anti-PARP antibody (1:8000 in TBS-BSA4%) overnight at 4°C. Antigen-antibody complexes were detected with horseradish peroxidase–conjugated secondary goat anti-rabbit antibody for 1 h at 25°C with luminol/peroxide-based chemiluminescence (SuperSignal West Pico; Pierce, Rockford IL) and brief exposure to autoradiography films. To ascertain equal loading, blots were stripped with 0.2 M glycine (pH 2.3) for 20 min at 25°C and reprobed with anti–β-actin 1:5000 in TBS-BSA1% in for 1 h. Alternatively, gels were stained with GelCode Blue (Pierce) after transfer.

Statistical Analyses
Data are expressed as mean ± SD. Experiments were performed at least three times, unless indicated otherwise. In experiments involving more than one potentially cytotoxic agent, a synergistic effect was assumed when the combined effect of the two agents was greater than the sum of both agents used alone. ANOVA was applied when comparisons involved multiple samples and treatments. Differences between means were determined by the two-sample t test. The level of significance was defined as P < 0.05.

Results
Hemin- and Verotoxin Cytotoxicity of Tubular Epithelial Cells
To determine the ability of heme proteins to cause renal tubular injury, we used human kidney-derived ACHN cells and Fe(III) protoporphyrin IX chloride (hemin) as a model system. In the first series of experiments, confluent cell monolayers were exposed to various hemin concentrations over 6 to 48 h. At concentrations > 100 μM, hemin treatment caused a distinct cytopathic effect, consisting of cell rounding and the appearance of spindle-form extensions or cell bridges not observed in vehicle-treated controls, and subsequent cell detachment (Figure 1A). Hemin-mediated changes in cell morphology and monolayer disruption were time and concentration dependent. At concentrations ≥200 μM, morphologic changes were observed as early as 6 h after the addition of hemin (Figure 1, A and B). In contrast, VT-induced cytotoxicity became apparent only over 24 to 72 h (Figure 1, A and C). Morphologic changes induced by VT1 and VT2 were identical and clearly distinguishable from changes induced by hemin (Figure 1A). CD50 was approximately 200 μM for hemin at 24 to 48 h (Figure 1B). The CD50 for VT1 and VT2 was approximately 0.1 nM and 0.5 nM, respectively, at 48 to 72 h (Figure 1C).

Experiments were designed to further characterize the cytotoxic effect induced by hemin and VT in ACHN cells. Cell monolayers were treated with 100 or 200 μM hemin for up to 30 h, or with 0.1 to 5 nM VT2 for 48 h, respectively. Replicate monolayers were treated with TNF-α (10 ng/ml) and cycloheximide (10 μg/ml) for control purposes. Cells were stained with DAPI and monitored by microscopy for the presence of morphologic changes via phase contrast and incident UV light. Nonadherent (floating) cells and adherent cells were evaluated separately. Apoptosis, defined by nuclear segmentation and visualized by DAPI staining, was evident in approximately 30% (range, 10% to 50%) of the detached cells from VT1 and VT2-treated dishes (Figure 2A). Of the adherent cells <1% were apoptotic. In contrast, apoptotic cells were consistently absent among vehicle- and hemin-treated monolayers, both in preparations from detached and residual (adherent) cells (Figure 2A). In another experiment, ACHN cell monolayers were treated with vehicle, hemin (100 to 200 μM; 24 h), or VT2 (1 nM; 72 h) and monitored for PARP cleavage by Western blot test. As shown in Figure 2B, the large 89 kD fragment of the intact 116 kD PARP protein was noted in the extracts from VT-treated cells, whereas PARP cleavage was consistently lacking in hemin- and vehicle-treated cells.

Combined Effects of VT and Hemin on ACHN Cells
VT is central to the development of human HUS and VT-producing *Escherichia coli*-induced systemic disease in experimental animals (35,36). We therefore investigated the combined effect of VT and hemin on tubular epithelial cells and asked whether VT alters the susceptibility of these cells to hemin toxicity. ACHN cell monolayers were treated for various intervals with VT before hemin was added. In a typical experiment, depicted in Figure 3, pretreatment of cells with VT 18 h before the addition of hemin caused increased monolayer disruption and cell loss, whereas treatment with hemin or VT alone had only minor effects on monolayer integrity. Comparable effects were observed when replicate monolayers were pretreated with cycloheximide instead of VT (Figure 3A). In a similar experiment, cells were grown in 96-well plates and residual monolayers stained with crystal violet for quantitative spectrophotometry. Results, depicted in Figure 3B, showed that preincubation of cell monolayers with VT increased the cytopathic effect of hemin. Further analysis of the A490 values revealed that sequentially added VT and hemin exerted, at least in part, supradditive or synergistic cytotoxic effects (Figure 3B).

In separate experiments, endothelial cell monolayers
(HMVEC) were exposed to VT and/or hemin and monitored by time-lapse video microscopy. Sequential treatment of HMVEC with VT2 and hemin at concentrations that failed to induce microscopically discernible cytotoxicity when added alone resulted in increased cell injury (Figure 3C).

**HO-1 Induction Modulates Hemin and VT Toxicity**

HO-1 induction is critical for the effective degradation of excess heme. We therefore tested to see whether the stimulation of ACHN cells with an inducer of HO-1, such as CdCl₂, will enhance HO-1 expression and enzymatic activity and protect these cells from hemin-mediated toxicity. CdCl₂ increased HO-1 expression in a time and concentration-dependent manner up to about 50 μM. HO-1 expression diminished with greater CdCl₂ concentrations, possibly as a result of increasing toxicity of the compound (Figure 4A). As shown in Figure 4B, CdCl₂-induced HO-1 protein was evident after 6 h of incubation. HO-1 levels peaked after 9 h, were sustained for at least 12 h more, and decreased to baseline >36 h after stimulation (Figure 5, lanes 5 to 7).

Because VT is known to block nascent peptide synthesis (6), we determined whether VT modifies expression levels HO-1 protein. When cell monolayers were pretreated with VT2 for 9 h and subsequently stimulated with CdCl₂, HO-1 expression was blocked (Figure 5, lanes 10 and 11). VT also diminished the induction of HO-1 expression by hemin in a time- and concentration-dependent manner (Figure 5, lanes 1 to 4). Inhibition of hemin-induced HO-1 expression was complete when VT pretreatment was extended to 18 h (results not shown). In contrast, when cadmium was used to stimulate HO-1 expression, elevated HO-1 protein levels were maintained for at least 9 h after the addition of VT (Figure 5, lanes 8 and 9).

We then asked whether induction of HO-1 expression by CdCl₂ protects cells from the combined cytotoxicity of VT and hemin. On the basis of the HO-1 induction experiments described above (Figure 4 and 5), monolayers were incubated with CdCl₂ for 6 h, followed by VT2 and hemin. As shown in Figure 6A, CdCl₂ treatment resulted in blunting of hemin-induced cytotoxicity. CdCl₂ failed to prevent the cytotoxic effect of VT alone (results not shown). However, the prior induction of HO-1 reversed the combined cytotoxic effect of hemin and VT in ACHN cells. Results from a typical experiments that used various VT2 (10 to 1000 pM) concentrations...
and hemin over 6 and 48 h are depicted in Figure 6, B and C. Taken together, results indicate that the induction of HO-1 protects ACHN cells from augmented hemin toxicity in the presence of VT.

**Discussion**

Acute intravascular hemolysis is a defining feature of hemolytic uremic syndromes. The etiology of the hemolytic...
Figure 3. Combined effects of verotoxin and hemin. (A) ACHN cell monolayers were treated with vehicle or VT1 10 pM for 18 h, followed by the addition of 100 or 200 μM hemin (or vehicle) for another 12 h. Additional dishes were treated with cycloheximide (CHX) 10 μg/ml for 30 min instead of VT. Monolayers were photographed in situ with an inverted Zeiss microscope with a ×10 objective. (B) ACHN monolayers were treated with diluent or VT1 (1 or 10 pM) for 18 h in 96-well dishes, followed by vehicle or hemin (100 or 200 μM) for another 6 or 48 h. Residual cells were quantitated by crystal violet staining. Shown are means and SD of triplicate determinations. * P < 0.05; ** P < 0.01; *** P < 0.005. Results represent at least three similar experiments. (C) Human microvascular endothelial cell (HMVEC) monolayers were incubated with 0.1 pM VT2 (top), or sequentially with VT2 and hemin (50 μM) after 18 h (bottom). Hemin failed to induce microscopically discernible monolayer changes at the used concentration when added alone (data not shown). Images were acquired via time-lapse video microscopy. The number of hours elapsed from the addition of VT and of hemin is indicated. For comparison, the cytotoxic effect of a 100-fold higher concentration of VT2 on HMVEC is shown on the right.
process is unclear, and its contribution to the pathogenesis of HUS is not known. Here we show that hemoglobin-derived hemin imparts enhanced toxicity toward human renal epithelial (and endothelial) cells in the presence of verotoxin, the principal causative agent of the classic, enteropathic HUS.

Release of hemoglobin during hemolysis can exceed the rate of haptoglobin production in the liver resulting in rising plasma concentrations of free (unbound) hemoglobin (38). Free hemoglobin is in part filtered in the glomerulus and reabsorbed by proximal renal tubule cells, where it accumulates in the apical regions to be degraded (18,39). Extracellular hemin is released upon oxidation of (ferrous) hemoglobin to (ferric) methemoglobin (19,21,40). Similar to haptoglobin, the plasma glycoprotein hemopexin, which binds heme with high affinity, becomes saturated and depleted during profound hemolysis (38) or (therapeutic) infusion of hemin (41). Heme-mediated toxicity has been coupled to oxidative and nonoxidative mechanisms. It causes peroxidation of various cellular components, including membrane lipids (40); activates neutrophils and induces monocyte chemoattractant protein-1 (24,42); and potentiates cell killing by polymorphonuclear leukocytes and other sources of reactive oxygen intermediates (43).

Induction of the microsomal heme oxygenase system, e.g., by heavy metals, oxidative stress, hemoglobin, and heme (26,37), is an effective and widely conserved mechanism to limit heme protein-mediated tissue injury (18). Recent cDNA microarray studies in human microvascular endothelial cells revealed that HO-1 overexpression is associated with a decrease in mRNA levels for antiproliferative and proapoptotic genes (44). The physiologic and clinical importance of HO-1 has become strikingly evident in the case of a child with hereditary HO-1 deficiency (45) and in HO-1 knockout mice (46). A prominent feature of HO-1 deficiency is chronic oxidative stress and—at least in the reported child—chronic intravascular hemolytic anemia with profound schistocytosis (45,46). This experiment of nature also suggests that inducible HO-1 is necessary to effectively remove heme (45). Although constitutively expressed HO-2 appears to play a protective role in lung tissue and neurons, it is not sufficient to metabolize excess heme (45,47,48).

Our results demonstrate enhanced renal tubule cell injury in the presence of VT. This effect was mirrored by cycloheximide, an antibiotic with peptide synthesis inhibiting activity (Figure 3A). Moreover, VT also led to exacerbation of hemin-induced injury in microvascular endothelial cells (Figure 3C). Although VT caused apoptosis of ACHN cells, in agreement with an earlier report (10), the mechanism of heme-induced cytotoxicity is not well understood. Cell death due to oxidative injury as well as oxidative stress–independent hemin toxicity has been described (21). Hemin produced peculiar morphologic changes in ACHN cells consisting of cell rounding and

Figure 4. Induction of heme oxygenase-1 (HO-1) by cadmium chloride (CdCl₂) in ACHN cells. Confluent monolayers were treated (A) for 6 h with the indicated CdCl₂ concentrations, or (B) with 50 μM CdCl₂ for the indicated time intervals. Total cellular extracts were resolved by SDS-PAGE, blotted, and developed with a polyclonal antibody to HO-1. Gels were stained after transfer to assess relative protein loading as described in Materials and Methods (A). Alternatively, blots were stripped and reprobed with a monoclonal antibody to β-actin as loading control (B).

Figure 5. Verotoxin (VT) abrogates induction of HO-1 protein expression in ACHN cells. Monolayers were sequentially treated with vehicle (−CdCl₂ (50 μM), VT2 (10 or 100 pM), or hemin (200 μM) for the indicated time intervals. Time 0 h corresponds to the addition of VT2. Total cellular protein was extracted at time X. Blots were developed with a polyclonal antibody to HO-1, stripped, and reprobed with a monoclonal antibody to β-actin as loading control.
the appearance of elongated cell processes, but failed to induce nuclear segmentation or PARP cleavage. We inferred that hemin causes nonapoptotic, likely necrotic death in ACHN cells in our model. Interestingly, after completion of our experiments, Gonzalez-Michaca et al. (48) reported that hemin induced apoptosis in immortalized rat proximal renal tubular cells under serum-deprived, but not serum-replete, culture conditions. Inhibiting HO-1 activity by zinc protoporphyrin further enhanced the proapoptotic effect of hemin in serum-deprived cultures (48). These results are of great interest in the context of the proposed hypothesis and warrant further scrutiny in an in vivo model of verotoxemia.

Although the HO system has been increasingly recognized as part of an adaptive cell response, specifically to oxidative stress, overexpression of HO-1 can also impart adverse effects because of increased release of redox-active iron (23). Observations in patients and animal models of hemolytic anemia and sickle cell disease (31,38) suggested a priori that HUS causes substrate-induced upregulation of HO-1 in response to excess hemoglobin. We found that VT repressed HO-1 expression in vitro in renal tubular epithelial (ACHN) (Figure 5) by translational inhibition. If VT represses HO-1 induction in vivo, it is expected that the lack of HO-1 protein induction in the presence of hemoglobinuria results in increased renal parenchymal toxicity (29,30). Nath et al. reported that infusion of hemoglobin caused renal failure and death within 1 to 2 wk in HO-1 knockout mice, but not in heterozygote littermates (28). A day after hemoglobin administration, HO-1-deficient mice maintained normal renal function and histologic appearance when challenged with the same dose of hemoglobin (28).
We hypothesize that VT blocks HO-1 expression in patients with *Escherichia coli*–induced HUS and that lack of an adequate HO-1 response would increase tissue sensitivity to heme protein toxicity. Our observation that treatment of ACHN cells with CdCl₂, a potent inducer of HO-1, affords cell protection when added before treatment with VT and hemin, further adds to the plausibility of our hypothesis. We posit that VT causes a state of vulnerability to heme protein-mediated injury when it is needed most, i.e., during the acute, hemolytic stage of the HUS. On the basis of the growing understanding of the physiology of the HO system (49), we further hypothesize that the repression of HO-1 leads to diminished local production of the gaseous signaling molecule carbon monoxide with its emergent vasorelaxant, anti-thrombotic, antiapoptotic, and anti-inflammatory activities (24,27,46,49,50).

In this scenario, excess heme/globin (and heme) release is expected to cause or worsen vasoconstriction, platelet aggregation, and acute renal failure (Figure 7). This model, if confirmed, may help understanding the central role of the kidney in enteropathic (*Escherichia coli*–induced) HUS. Furthermore, lack of adequate HO-1 expression may contribute to the intravascular hemolytic process (45).

In conclusion, our results led us to propose the hypothesis that hemeoglobin and hemoglobin-derived heme may play a previously unrecognized role in classical HUS. It is possible that VT-mediated repression of vital cell defense systems, particularly HO-1, contributes to the renal pathology of HUS.

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**References**


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**Figure 7.** Schematic diagram of the proposed pathophysiological model of the effects of heme and verotoxin in *Escherichia coli*–associated hemolytic uremic syndrome.