Epithelial cell polarity and hypoxia influence heme oxygenase-1 expression by heme in renal epithelial cells

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Basireddy, Mahesh, Jason T. Lindsay, Anupam Agarwal, and Daniel F. Balkovetz. Epithelial cell polarity and hypoxia influence heme oxygenase-1 expression by heme in renal epithelial cells. Am J Physiol Renal Physiol 291: F790–F795, 2006.—Induction of heme oxygenase-1 (HO-1) in renal tubules occurs as an adaptive and beneficial response in acute renal failure (ARF) following ischemia and nephrotoxins. Using an in vitro model of polarized Madin-Darby canine kidney (MDCK) epithelial cells, we examined apical and basolateral cell surface sensitivity to HO-1 induction by heme. Basolateral exposure to 5 μM hemin (heme chloride) resulted in higher HO-1 induction than did apical exposure. The peak induction of HO-1 by basolateral application of hemin occurred between 12 and 18 h of exposure and was dose dependent. Similar cell surface sensitivity to hemin-induced HO-1 expression was observed using a mouse cortical collecting duct cell line (94D cells). Hepatocyte growth factor (HGF) is known to decrease cell polarity of MDCK cells. Following pretreatment with HGF, apically applied hemin gave greater stimulation of HO-1 expression, whereas HGF alone did not induce HO-1. We also examined the effect of hypoxia on hemin-mediated HO-1 induction. MDCK cells were subjected to hypoxia (1% O2) for 24 h to simulate the effects of ischemic ARF. Under hypoxic conditions, both apical and basolateral surfaces of MDCK were more sensitive to HO-1 induction by hemin. Hypoxia alone did not induce HO-1 and appeared to potentiate both apical and basolateral sensitivity to hemin-mediated induction. These data demonstrate that the induction of HO-1 expression in polarized renal epithelia by heme is achieved primarily via basolateral exposure. However, under conditions of altered renal epithelial cell polarity and hypoxia, increased HO-1 induction occurs following apical exposure to heme.

Acute renal failure; kidney; hepatocyte growth factor

Heme oxygenase-1 (HO-1) is a 32-kDa microsomal enzyme that catalyzes the rate-limiting step in heme degradation, producing equimolar amounts of biliverdin, iron, and carbon monoxide (38). Two isoforms of HO have been identified: an inducible enzyme, HO-1, and a constitutive isoform, HO-2 (24). Several reviews have emphasized the importance of HO-1 as a potent cytoprotective enzyme as well as the biological effects of the HO-1 reaction product(s), which possess important antioxidant, anti-inflammatory, and antiapoptotic functions (9, 13, 14). HO-1 induction in the kidney provides an important adaptive and protective mechanism in response to acute renal injury secondary to ischemia-reperfusion, nephrotoxins (e.g., cisplatin), glomerulonephritis, renal transplant rejection, and rhabdomyolysis (18, 33). Therefore, the regulation of HO-1 in renal tubular epithelium is of critical importance.

HO-1 is dramatically induced after exposure to a wide variety of stimuli including heme (33). Heme serves as the prosthetic moiety for heme proteins such as hemoglobin, myoglobin, cytochromes, catalase, peroxidase, and respiratory burst oxidase (18, 29). Interestingly, heme exhibits a dual function by serving as a substrate for and inducer of HO-1 in vivo and in cultured cells (2, 3). In various models of renal disease, HO-1 expression is restricted to the renal epithelia lining the nephron (19, 26, 28). However, the relative cell surface (apical or basolateral) sensitivity of the renal tubular epithelium to heme-mediated induction of HO-1 is not understood.

The mammalian renal tubule is lined with a simple epithelium that is polarized with regard to cell surfaces and includes apical (facing the tubular lumen) and basolateral (facing the interstitium and vasa recta) surfaces. This renal epithelial cell polarity is important and necessary for renal physiology and host defense mechanisms.

Under normal, nonstressed conditions, levels of HO-1 in the renal tubule epithelium are low or undetectable (28). The epithelial cells lining the renal tubule are exposed to inducers of HO-1 such as heme from both the apical and basolateral surface during stressful conditions such as rhabdomyolysis. Because of water reabsorption, the concentration of heme becomes much higher in the renal tubule lumen especially in the distal nephron segments. Therefore, the apical surface would be exposed to relatively higher concentrations of heme. Because of these differences in concentrations of heme exposed to the apical and basolateral surface of the epithelium lining the renal tubule, we hypothesized that there would be differences in the sensitivity of HO-1 induction by heme on the apical and basolateral surfaces. To test this hypothesis, we have examined the role of epithelial cell polarity in the induction of HO-1 using an in vitro model of polarized renal epithelial cells. The data demonstrate that the apical surface of renal epithelial cells is relatively resistant to HO-1 induction compared with basolateral surface exposure. In addition, alteration of epithelial cell polarity with HGF or hypoxia increases apical sensitivity of HO-1 induction to heme.

Materials and Methods

Cell culture. MDCK strain II cells were used between passages 3 and 10. Cells were cultured as previously described (5, 6) in modified Eagle’s MEM containing Earl’s balanced salt solution and glutamine.
supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 
µg/ml streptomycin, and 0.25 µg/ml amphotericin B. The 94D mouse
cortical collecting duct cell line was developed as previously de-
cscribed (42). 94D cells were cultured in DMEM/F-12, 5% fetal calf 
serum, 1.3 µg/l sodium selenite, 1.3 µg/l 3,3′,5-triiodo-thyronine, 5 
mg/l insulin, 5 mg/l transferrin, 2.5 mM glutamine, 5 µM dexameth-
asone, 100 U/ml penicillin, and 100 µg/ml streptomycin at 5% CO2.
All renal cell lines were plated at confluency on Transwell filter units 
(Costar, Cambridge, MA) with a pore size of 0.4 µm. R. Schwall
generously provided recombinant human hepatocyte growth factor 
(HGF; Genentech, South San Francisco, CA). Because the c-met 
receptor is localized to the basolateral surface of MDCK cells (10), 
HGF was added to the basolateral compartment. Moreover, polarized 
MDCK cells do not respond to apically applied HGF (4). For hypoxia 
experiments, cells were placed in an incubator with 1% O2 and at 
37°C with 5% CO2. Hemin (heme chloride, Sigma) was dissolved in 
DMSO. A 1 mM stock solution of hemin in DMSO was diluted in the 
MDCK cell medium containing serum to give the desired concentra-
tion of hemin. Equal amounts of DMSO were added to controls. All 
results are representative of at least two to three independent exper-
iments.

Western blot analysis. Western blot analysis was performed as 
described previously with minor modifications (5). Cells were cul-
tured on 24-mm Transwell filters. Briefly, equal amounts of protein in 
MDCK cell lysates were run on appropriate (12%) acrylamide gels at 160 V for 45 min. Proteins were transferred to Immobilon P mem-
branes (Millipore, Bedford, MA). Membranes were blocked for 30 
min with PBS– (PBS without Ca2+ and Mg2+), 5% milk, and 0.1% 
Tween 20 (block solution). For HO-1 and actin protein analysis, 
membranes were probed with primary antibodies of interest (rabbit 
anti-HO-1, 1:5,000 from Stressgen, SPA-896; rabbit anti-α-actin, 
1:2,000 from Sigma; mouse anti-E-cadherin, 1:500 from Transduction 
Laboratories) for 45 min and then washed with PBS–, 0.1% Tween 20 
for 20 four times for 5 min each. Membranes were then probed with 
appropriate secondary horseradish peroxidase-labeled antibody (1: 
10,000 for both HO-1 and α-actin and 1:25,000 for E-cadherin) for 60 
min, washed with PBS–, 0.1% Tween 20 (4 × 5 min), and developed 
using the enhanced chemiluminescence kit (Amersham, Piscata-
way, NJ).

Immunofluorescent labeling of cells. MDCK strain II cells were 
grown on 6.5-mm-diameter Costar Transwell filters with 0.4-µm 
pores. Cells were washed with PBS+ (PBS containing Ca2+ and 
Mg2+) and then fixed with 4% paraformaldehyde for 20 min at 4°C. 
After filters were washed three times with PBS+, the cells were 
quenched with 75 mM NH4Cl and 20 mM glycine, pH 8.0, with KOH 
(quench solution) for 10 min at room temperature. Filters were 
then washed one time with PBS+ and permeabilized with PBS–, 0.7% fish 
skin gelatin, and 0.025% saponin (PFS) for 15 min at 37°C. Cells were 
exposed to primary antibody diluted in PFS [rabbit anti-HO-1 (1:500)] 
for 1 h at 37°C. Filters were then washed four times for 5 min each 
with PFS at room temperature and then labeled with the FITC-
conjugated secondary antibody diluted 1:100 in PFS for 1 h at 37°C.
Filters were rinsed four times for 5 min each with PFS, one time with 
PBS+, two times with PBS+ containing 0.1% Triton X-100, and one 
time with PBS–. Cells were postfixed in 4% PFA for 15 min at room 
temperature. Filters were cut from the support with a scalpel and 
mounted in Vectashield Mounting Medium (Burlingham, CA).
Immunofluorescent images were obtained with a laser-scanning confocal 
microscope (Leica LSCM, Heidelberg, Germany). The generated 
photomicrographs were captured and labeled using Adobe Photoshop.
Representative data are presented in RESULTS.

Cytotoxicity assays. Cytotoxicity was determined by using the 
LIVE/DEAD viability/cytotoxicity assay (Molecular Probes, Eugene, OR) (41) and the clonogenic assay as previously described (11).

Measurement of transepithelial electrical resistance. For transep-
ithelial electrical resistance (TER) experiments, MDCK strain II cell 
monolayers were grown on 12-mm-diameter, 0.4-µm pore-size filters, 
and electrical resistance was measured with the EVOM electrical 
resistance system (World Precision Instruments, New Haven, CT). All 
TER experiments were performed at least three times. The TER 
results are expressed as the measured resistance (Ω) multiplied by the 
area of the filter (1 cm2).

RESULTS

Effect of apical or basolateral surface exposure to hemin on HO-1 induction. We exposed the apical, basolateral, or both surfaces of filter-grown, polarized MDCK II cells to 5 µM 
hemin for 16 h. Following hemin exposure, cell lysates were 
prepared and analyzed for HO-1 expression by Western blot 
analysis. Figure 1A demonstrates greater induction of HO-1 
protein expression following basolateral surface exposure to hemin. 
To a lesser degree, HO-1 protein induction was also 
observed following apical surface exposure to hemin. Quantita-
tion of HO-1 band densities following apical or basolateral 
exposure to hemin for 18 h was performed in six independent 
experiments. On average, basolateral exposure to hemin 
induced HO-1 expression 15-fold greater than that observed 
following apical exposure to hemin. Combined apical and 
basolateral exposure to hemin resulted in similar induction of 
HO-1 protein as observed in the cells exposed only to 
basolateral hemin. These results demonstrate that in a polarized 
renal epithelial cell line, the basolateral surface is more sensi-
tive than the apical surface to hemin-induced HO-1 protein expression. We also examined apical and basolateral hemin exposure on HO-1 protein expression in a mouse cortical collecting duct cell line (94D) (42). In polarized 94D cells grown on filters, basolateral surface hemin exposure also induced higher levels of HO-1 protein expression than did apical surface exposure (data not shown).

The time course of HO-1 induction by apical or basolateral surface exposure to hemin was examined (Fig. 1B). Again, basolateral exposure to hemin resulted in markedly higher levels of HO-1 protein induction than seen with apical exposure. Regardless of the cell surface exposed to hemin, induction of HO-1 protein was detected after 6 h of exposure, and the maximal stimulation of HO-1 protein was observed after exposure to hemin between 12 and 18 h and remained elevated for 24 h. This time course of HO-1 induction is similar to that observed in other model systems (32).

We next examined the hemin dose response of hemin-mediated HO-1 protein induction by either apical or basolateral exposure. Polarized MDCK II cells were apically or basolaterally exposed to increasing concentrations of hemin (0.005, 0.05, 0.5, or 5 μM) for 16 h. Prolonged incubation with >10 μM resulted in cell toxicity. The results in Fig. 1C confirm that the basolateral surface exposure to hemin gives greater induction of HO-1 protein expression than observed following apical surface exposure. However, at hemin concentrations below 5 μM, little to no HO-1 protein was observed in MDCK II cells regardless of the cell surface exposure. These data suggest that a threshold concentration of 5 μM is required to induce expression of HO-1 protein in MDCK II cells.

The induction of HO-1 may reflect the severity of cell injury. To assess whether the lesser induction of HO-1 with apical exposure, compared with basolateral exposure, was due to less cell injury that occurred with apical exposure, we examined the effect of cell surface exposure to hemin on cell viability using the LIVE/DEAD viability/cytotoxicity assay (Molecular Probes) and the clonogenic assay. At the concentrations used (5 μM), we did not detect an effect of hemin exposure on cell viability. In both assays, polarized MDCK strain II cells were exposed to vehicle or 5 μM hemin on the apical, the basolateral, or both surfaces for 16 h. With the LIVE/DEAD assay, the mean percentage of dead cells in the monolayers ± SD (n = 5) was 0.195 ± 0.18, 0.15 ± 0.07, 0.28 ± 0.08, and 0.22 ± 0.10% for vehicle, apical, basolateral, and apical + basolateral hemin exposure, respectively. The percentages of dead cells in the all of the hemin exposed monolayers were not statistically different from the vehicle-exposed cell monolayers (P > 0.05). In the clonogenic assay, cell monolayers were exposed to vehicle or hemin on the apical, basolateral, or apical + basolateral surface for 16 h and trypsinized into a single-cell suspension. Cells were counted using a hemocytometer, equal numbers of cells were plated, and cell colonies were counted 5 days thereafter. The number of colonies ± SD (n = 3) were 174.5 ± 19.7, 193.1 ± 12.1, 197.1 ± 13.0, and 187.7 ± 25.0 for vehicle, apical, basolateral, and apical + basolateral hemin exposure, respectively, and were not statistically different from the vehicle-exposed cells (P > 0.05).

Effect of HGF pretreatment on HO-1 induction by apical or basolateral exposure to hemin. Treatment of polarized MDCK II cells with HGF reduces epithelial cell polarity (5, 6, 8, 40, 41). Using this model system, we have demonstrated the importance of cell polarity in renal epithelial cell resistance to bacterial pathogens (41), apical surface susceptibility to adenoviral based gene delivery vectors (8), and production of antimicrobial factors (40). In addition, other investigators have provided evidence that HGF, by itself, is also an inducer of HO-1 in HepG2 cells (36). Therefore, we examined the effects of HGF treatment of MDCK II cells (100 ng/ml for 24 and 48 h) on cell surface sensitivity of hemin induction of HO-1 as well as on HO-1 induction. Figure 2A shows that HGF treatment alone for periods extending to 48 h did not induce detectable levels of HO-1 as determined by Western blotting. However, HGF-treated MDCK II cells expressed higher levels of HO-1 following apical exposure to hemin by Western blotting (Fig. 2A) and confocal microscopy (Fig. 2B), which showed a perinuclear, endoplasmic reticulum staining pattern for HO-1 following induction. These data provide evidence that altering cell polarity with HGF increases the sensitivity of the apical (or luminal) surface to HO-1 induction by hemin.

We have previously shown that HGF alters MDCK II cell polarity and that removal of HGF restores epithelial cell polarity (8). We examined whether removal of HGF from MDCK cells previously treated with HGF for 48 h restores the relatively low induction of HO-1 by apical exposure to hemin. These results are shown in Fig. 2C. Following a 72-h washout period in medium not containing HGF, the MDCK cells express lower levels of HO-1 with apical exposure to hemin compared with MDCK cells treated with HGF for 48 h. These data correlate with our previously published morphological data showing that MDCK cell polarity is restored following removal of HGF stimulation (8).

Effect of hypoxia on HO-1 induction by apical and basolateral exposure to hemin. Hypoxia is a potent inducer of HO-1 in rat, bovine, mouse, and monkey cells but is a repressor in human cells (21, 22, 33). We examined the effects of hypoxia on HO-1 induction in general as well as cell surface sensitivity of induction by hemin in MDCK II cells. The results are shown in Fig. 3 and demonstrate that hypoxia leads to greater stimulation of HO-1 protein expression in MDCK II cells following either apical or basolateral stimulation with hemin. Hypoxia alone did not stimulate HO-1 protein expression in MDCK II cells. We next examined the dose response of hemin-mediated HO-1 induction under normoxic and hypoxic conditions. The apical or basolateral surface of polarized MDCK II cells was exposed to increasing concentrations of hemin for 24 h under normoxic and hypoxic conditions. Western blot analysis demonstrated that under hypoxic conditions, HO-1 induction was observed following exposure to hemin at lower concentrations on both the apical and basolateral surface of the renal epithelial cells (Fig. 4). These data provide evidence that hypoxia increases both the apical and basolateral cell surface sensitivity to hemin-mediated HO-1 induction and that hypoxia alone does not stimulate HO-1 induction in MDCK II cells.

TER is a surrogate marker for tight junction (TJ) function and paracellular permeability. We observed a 17% decrease in TER of MDCK II cells after 24 h of hypoxia (P < 0.01; data not shown). Therefore, hypoxia may increase the apical surface sensitivity to hemin-mediated HO-1 induction by inhibition of TJ and allowing paracellular movement of apically applied hemin to the basolateral surface (see Discussion).
sensitivity of MDCK II cells to HO-1 induction by hemin, we examined the effects of apical, basolateral, and or both surface stimulation to cadmium chloride (another known inducer of HO-1). Figure 5 shows that cadmium exposure does result in the induction of HO-1. Both apical or basolateral surface exposure alone to cadmium resulted in similar induction of HO-1. However, exposure of both the apical and basolateral surface to cadmium resulted in greater HO-1 induction than seen with cadmium exposure to either surface alone. These data show that cell polarity does not influence cadmium-mediated HO-1 induction in MDCK II cells and that the differential cell surface sensitivity to hemin-mediated HO-1 induction is relatively specific.

Fig. 2. Hepatocyte growth factor (HGF) treatment increases apical sensitivity of MDCK II cells to hemin induction of HO-1. A: MDCK II cells were treated with HGF (100 ng/ml) for 24 and 48 h and then exposed to hemin (5 μM) on the apical (A), basolateral (B), or both (A+B) surfaces for 16 h (C, control: vehicle). Cell lysates were prepared in RIPA buffer and probed for HO-1 and actin (protein loading control) expression. B: control (no HGF) and HGF-treated (100 ng/ml) MDCK II cells were apically or basolaterally exposed to hemin (5 μM) for 16 h and HO-1 expression was visualized by laser-based confocal microscopy. Bars = 20 μm. C: HGF washout for 72 h restores relative apical resistance to HO-1 induction by hemin. MDCK II cells were treated with HGF (100 ng/ml) for 48 h and then exposed to hemin (5 μM) on the apical (A), basolateral (B), or both (A+B) surfaces for 16 h (C, control: vehicle). Another set of 48-h HGF-pretreated MDCK II cell monolayers was rinsed in fresh medium and cultured in fresh medium without HGF for 72 h. After the 72-h HGF washout, the cells were then exposed to hemin (5 μM) on the apical (A), basolateral (B), or both (A+B) surfaces for 16 h (C, control: vehicle). Cell lysates were prepared in RIPA buffer and probed for HO-1 and actin (protein loading control) expression.

Fig. 3. Hypoxia increases HO-1 expression following apical (A), basolateral (B), or both (A+B) surface exposure to hemin. Polarized MDCK II cells were simultaneously exposed to hemin (5 μM) for 24 h on specific cell surfaces under normoxic and hypoxic (1% O2) conditions. Cell lysates were prepared in RIPA buffer and probed for HO-1 and actin (protein loading control) expression. C, control: vehicle.

Fig. 4. Dose-response of MDCK II cells to HO-1 induction by hemin [apical (A), basolateral (B), or both (A+B) surface exposure] under normoxic and hypoxic conditions. The above surfaces of polarized MDCK II cells were exposed to increasing concentrations of hemin for 24 h under normoxic and hypoxic conditions. Cell lysates were prepared in RIPA buffer and probed for HO-1 and actin (protein loading control) expression. To allow for direct comparison of expression of HO-1 and actin, blots were exposed to film for identical times. C, control: vehicle.
The finding that under hypoxic conditions both the apical and basolateral surface of renal epithelial cells are sensitized to hemin-mediated HO-1 induction may be accounted for by different mechanisms. First, because hypoxia inhibits TJ function as demonstrated by the TJ surrogate marker TER, apically applied hemin may increase accessibility to the hemin-sensitive basolateral surface and greater HO-1 induction. However, the increased basolateral sensitivity to hemin-induced HO-1 by hypoxia cannot be accounted for by alterations in TJ permeability (if this were the case, basolaterally applied hemin would be diluted by leaking into the apical compartment and resulting in less HO-1 induction). Another possibility is the induction of HIF-1 by hypoxia and augmentation of HO-1 expression following basolateral exposure to hemin. Induction of HIF-1 has been shown to also upregulate HO-1 expression in other cell types but not in all human cells (31, 36). Consistent with our cell culture data, it has been recently shown that ischemia-reperfusion of the rat kidney increases the sensitivity to HO-1 induction by hemin (12). Because HO-1 is a protective enzyme during ischemic renal injury (33), the augmentation of luminal hemin-mediated HO-1 induction by alterations in polarity and hypoxia are likely to be very relevant in the pathogenesis and repair during acute renal injury following ischemia-reperfusion and rhabdomyolysis.

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