Prevention of Barrier Disruption by Heme Oxygenase-1 in Intestinal Bleeding Model

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In this study we investigated the effect of free heme, the local level of which was increased by bleeding, on the intestinal barrier function, using human epithelial colorectal adenocarcinoma cells (Caco-2). Our results show that the addition of hemin to the culture medium markedly disrupted the barrier function, which was significantly improved by glutamine supplementation. Although hemin treatment caused the increased expression of heme oxygenase (HO)-1, the inhibition of HO activity resulted in the aggravation of hemin-induced barrier dysfunction. Up-regulation of HO-1 by pretreatment with a low concentration of hemin almost completely prevented hemin-induced barrier dysfunction. Taken together, these observations indicate that an abnormally high level of intracellular free heme causes barrier dysfunction, probably through the modulation of proteins forming tight junctions.

Key words intestinal barrier function; heme oxygenase; free heme; glutamine

Lower gastrointestinal hemorrhage constitutes a very frequent clinical problem in the developed world.13 Massive gastrointestinal bleeding followed by hemolysis results in high levels of free heme, causing toxicity leading to tissue injury. It has been reported that the predominant component of hemoglobin that causes cell injury is heme.2 Heme is readily oxidized and subsequently dissociated from methemoglobin.3 During hemolytic events, a sudden local increase in heme has been reported to show a toxic effect leading to cell damage.4 Heme exerts a dual role. In small amounts, it acts by itself or in a functional group of heme proteins facilitating diverse and indispensable cellular functions, whereas in excessive amounts, free heme can cause severe cell or tissue damage.5–7 An excess of free heme may constitute a major threat because heme catalyzes the formation of reactive oxygen species (ROS), resulting in oxidative stress and, subsequently, cell injury.8 Although large amounts of heme act as pro-oxidative and pro-inflammatory modulators, other studies suggest that a low concentration of heme may be protective through the fast up-regulation of heme oxygenase (HO)-1.9 HO-1, which is the rate-limiting enzyme in heme catabolism, has been known to be induced by various oxidative stresses.10

One of the essential functions of the intestinal epithelium is to maintain a selective barrier that excludes potentially harmful agents. Breakdown of the barrier is implicated in bacterial translocation, leading to sepsis, and in the pathogenesis of acute illnesses such as multiple organ failure. Although it is well-known that colon diverticular disease is associated with massive bleeding, changes in the barrier function after intestinal hemorrhage have not been studied in detail. In this study, we closely investigated the relationships among free heme, the intestinal barrier function, and HO-1 induction, using the filter-grown human epithelial colorectal adenocarcinoma cell line (Caco-2), which has been extensively validated as a useful in vitro model system to study the intestinal epithelial barrier function.11 Previously, we reported the functional contribution of heat shock protein (Hsp)70 on intestinal epithelial cells, focusing on barrier dysfunction caused by ethanol treatment.12 Our results showed that induced Hsp70 significantly ameliorated the disruption of the barrier function caused by ethanol in Caco-2, and the Hsp70 induction was modulated by glutamine supplementation. The non-nutritive effects of glutamine have been reviewed.13 Evidence suggests that glutamine helps to maintain intestinal mucosal integrity, especially during stresses such as radiation therapy14 and experimental sepsis.15 In fact, we observed that glutamine pretreatment significantly ameliorated tissue injury in the lower intestine in rats with lipopolysaccharide (LPS)-induced sepsis via enhanced HO-1 induction.16

In this study, we investigated the physiological role of HO-1, which is also known as Hsp32, in the intestinal barrier function, intending to elucidate the disorder accompanying intestinal bleeding. We report here that hemin (ferriheme chloride) at more than 50 µM causes significant barrier disruption, which is prevented by the pre-induction of HO-1 with a low concentration of hemin.

MATERIALS AND METHODS

Cell Culture and Treatments Caco-2 were kindly provided by Dr. Tooru Taniguchi, Nagoya City University Graduate School of Medical Sciences. Caco-2 were seeded onto 6-transwell plates with a pore size of 0.4 µm (BD Falcon®) at a density of 25000 cells/cm² and maintained at 37°C in a culture medium composed of Dulbecco’s modified Eagle’s medium (DMEM) containing 4 mM glutamine with 100 µM penicillin, 100 µg/mL streptomycin, and supplemented with heat-inactivated 10% fetal bovine serum. For the epithelial resistance and paracellular permeability studies, Caco-2 were seeded onto 24-transwell plates with a pore size of 0.4 µm (BD Falcon®) at a density of 150000 cells/cm² and were cultured as described above.16 Fifteen hours before the experiment, cells were rinsed with phosphate-buffered saline (PBS), and then cultured in medium containing 0 or 8 mM glutamine.

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according to the experimental protocol described in the figure legends. Heat shock loading was performed by exposing cells to 42°C for 1 h, followed by recovery at 37°C for 4 h. Heme arginate (HA), which is a water-soluble and stable reaction product of hemin and l-arginate, was used for hemin treatment at a final concentration of 20, 50, or 100 µM. Sn mesoporphyrin (SnMP), which is a specific inhibitor of HO, was kindly provided by Dr. George S. Drummond, The Rockefeller University. SnMP was dissolved in the vehicle used for hemin solution, and was used at concentrations of 0 to 50 µM.

Iron ascorbate was prepared as previously described. When filter-grown cells were treated with the chemicals described above, the culture medium was changed on the apical and/or basolateral side. Cell viability was assessed by counting cells attached to the filter at 24 h after treatment.

**Measurement of Transepithelial Electric Resistance (TEER)**

TEER of confluent monolayers was measured as an indicator of monolayer integrity, as described previously, using a Millicell-ERS ohm-meter (Millipore Corporation, Billerica, MA, U.S.A.). TEER was calculated as ohms per square centimeter by multiplying it by the surface area of the insert (0.3 cm²) and subtracting the internal resistance of the insert before calculations (25 Ω·cm²). The filter-grown Caco-2 monolayers had reached an epithelial resistance of 7–10 kΩ·cm² by 2 weeks after seeding.

**Unidirectional Flux of Inulin**

Transwells with the cell monolayers were incubated under different experimental conditions in the presence of fluorescein isothiocyanate (FITC)-inulin (0.1 mg/mL) (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.) in the basal well. Twenty-four hours after treatment, 10 µL each of apical and basal media were withdrawn, and fluorescence was measured using a fluorescence plate reader (PerkinElmer, Inc., 1420 Multilabel Counter). FITC-inulin flux into the apical well was calculated as the ratio to the fluorescence in the basal well.

**Western Blot Analysis**

Whole cell extracts were prepared from cultured cells in NP-40 lysis buffer (150 mM NaCl, 0.01 g/mL Nonidet P-40, 50 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL pepstatin, and 1 mM dithiothreitol) and subjected to Western blotting using a polyclonal antibody for heme-oxygenase-1 (Hsp32) (SPA-896; Stressgen), a mouse monoclonal IgG for Hsp70 (W27; Santa Cruz, CA, U.S.A.), and β-actin (AC-15; Sigma-Aldrich), which were obtained from commercial sources. Signals were visualized using ECL Western blotting detection reagents (General Electric, Fairfield, CT, U.S.A.). Chemiluminescence was detected using a LAS-4000 IR multi-color image reader (FUJIFILM).
Malondialdehyde (MDA) Formation Assay The extent of membrane peroxidation was estimated by measuring MDA formed in the culture medium with a spectrophotometric method.22)

RESULTS

Barrier Dysfunction Induced by Hemin Treatment We examined the effect of hemin loading on the barrier function using a filter-grown Caco-2 monolayer. Exposure to hemin at a concentration of 50 or 100 µM resulted in decreased TEER by Caco-2 (Fig. 1A). The concentration-dependent disruption of tight junction (TJ) by hemin was also confirmed by a progressive increase in the epithelial permeability of paracellular marker FITC-inulin flux from the basal to apical medium (Fig. 1B). The TEER changes were more sensitive to hemin-dependent TJ disruption, compared with FITC-inulin flux changes. On the basis of these observations, we measured TEER to assess the barrier disruption in the following experiments. Next, we examined whether hemin affected the barrier function from the apical or basal side. The results clearly showed that hemin exposure from the basal membrane disrupted the barrier function (Fig. 1C). Specific HO-1 induction was also observed when cells were treated with hemin from the basal side, not from the apical side (Fig. 1D). Hsp70 was not induced by hemin treatment, suggesting that HO-1 induction was not through a stress-inducible mechanism (Fig. 1D).

Effects of Glutamine on the Hemin-Induced Barrier Dysfunction Next, we examined the TEER changes after hemin treatment, in the presence or absence of glutamine. Basal level of TEER was not affected by glutamine supplementation (data not shown). Treatment with 50 µM HA caused significant disruption of the barrier in Caco-2 in glutamine-free medium, while it brought about only a slight decrease in TEER until after 24 h of hemin treatment in glutamine-supplemented medium (Fig. 2A). The cell viability under hemin treatment was not affected by glutamine supplementation (Fig. 2B), suggesting that the barrier dysfunction caused by hemin in glutamine-free medium was not caused by the cytotoxic effect of hemin. Ferrous iron, one of the heme degradation products, also caused barrier dysfunction in Caco-2 at the concentration of 1 mM (Fig. 2C). In contrast to hemin-induced disruption of the barrier, glutamine supplementation did not improve TEER at all when the cells were treated with ferrous iron. Since MDA formation levels were well correlated with barrier dysfunction (Fig. 2D), iron-induced barrier dysfunction

Fig. 2. Effect of Glutamine on Hemin- or Ferrous Iron-Induced Barrier Dysfunction

(A) Filter-grown Caco-2 monolayers were treated with 50 µM HA in the absence (Gln(−)) or presence of 8 mM glutamine (Gln(+)). TEER was measured at 6, 9, and 24 h after HA treatment. Data represent the mean ± S.E. from four independent experiments (n=4). (B) In the same set of experiments as (A), cells attached to the filter were harvested at 24 h after 100 µM HA treatment and counted to assess cell attachment. Data represent the mean ± S.E. from four independent experiments (n=4). (C) Filter-grown Caco-2 monolayers were treated with 1 mM ferrous iron in the absence or presence of 8 mM glutamine. TEER was measured at 6, 9, and 24 h after ferrous iron treatment. Data represent the mean ± S.E. from four independent experiments (n=4). (D) In the same set of experiments as (C), culture medium was collected and MDA formation was determined as described in Materials and Methods. Data represent the mean ± S.E. from four independent experiments (n=4).
was thought to be brought about by membrane peroxidation, unlike hemin-induced phenomena.

**Aggravation of Hemin-Induced Barrier Dysfunction by HO Inhibitor** Since hemin treatment caused significant barrier dysfunction, accompanied by HO-1 induction (Fig. 1), it was of interest to confirm whether hemin or its degradation products catalyzed by HO-1 were responsible for barrier disruption. To examine the contribution of HO activity to hemin-induced barrier dysfunction, cells were treated with 50 μM HA and/or 5 μM SnMP in the absence or presence of 8 mM glutamine. TEER was measured at 24 h after HA treatment. Data represent the mean ± S.E. from four independent experiments (n=4).

**Up-Regulation of HO-1 Prior to Hemin Treatment Prevents Hemin-Induced Barrier Dysfunction** Because HO-1 degrades free heme in cells, we expected that HO-1 induction prior to hemin treatment might protect the barrier function. To investigate this, we pretreated the cells with a low concentration of hemin, which significantly induced HO-1 but did not cause barrier dysfunction (Figs. 4A, B, lane 3). Analysis of TEER showed that pretreatment with 20 μM HA markedly prevented hemin-induced injury of the barrier, irrespective of glutamine supplementation (Figs. 4A, B, lane 4). On the other hand, heat shock loading prior to hemin treatment resulted in significant induction of Hsp70, but only partial protection against hemin-induced barrier dysfunction (Figs. 4A, B, lane 6). Western blot analysis revealed decreased occludin levels in hemin-treated cells, which were well correlated with decreased TEER (Figs. 4A, B, lane 6). HSP70 expression did not change under these conditions. Removal of glutamine from the medium further aggravated hemin-induced barrier dysfunction (Fig. 3C).
DISCUSSION

Recently, we reported the functional contribution of Hsp70 on intestinal epithelial cells, focusing on barrier dysfunction caused by ethanol treatment. Our results showed that induced Hsp70 significantly ameliorated disruption of the barrier function of Caco-2. Of particular interest is the fact that glutamine serves as an enhancer of Hsp70 induction through HSF1 activation, resulting in the progressive recovery from barrier dysfunction caused by ethanol. In this study, we investigated the physiological role of HO-1, which is also known as Hsp32, in the intestinal barrier function, focusing on the disorder accompanying intestinal bleeding. Although it was unclear whether or not free heme interacted with intestinal epithelial cells, our results clearly showed for the first time that exposure to heme from the basal membrane significantly disrupted the barrier function accompanying HO-1 induction (Fig. 1). Because heme was transported more effectively from the basal than apical membrane in Caco-2, our results indicate that the increase in intracellular free heme or its degradation products might affect TJ permeability, regardless of cell detachment (Figs. 1, 2). Although iron released from heme is known to be responsible for oxidative damage through the ferryl oxidation status, the addition of SnMP, a specific competitive inhibitor of HO, resulted in the aggravation of barrier dysfunction (Figs. 3A, C). HO activity was inhibited by SnMP, failing to degrade the intracellular free heme, which induced HO-1 protein expression (Fig. 3B). Furthermore, the induction of HO-1 and in some cases the subsequent increase in ferritin has been suggested to prevent cell and tissue injury in other diseases mediated by hemoglobin and oxidative stress. Taken together, these results suggested that increased concentration of intracellular free heme, not iron, was the major cause of barrier dysfunction.

It has been reported that TJ protein expression and cellular localization in Caco-2 monolayers rely on glutamine, which has been shown to maintain TEER and reduce permeability in intestinal cell culture monolayers. In this study, we also demonstrated the beneficial effect of glutamine on hemin-induced barrier dysfunction in Caco-2, but not on iron-induced injury (Fig. 2). Since hemin-induced injury did not accompany Hsp70 induction (Figs. 1, 2), unlike ethanol-induced injury, an unknown mechanism may exist to protect the barrier function involving glutamine.

It was reported that a low concentration of heme exhibits anti-inflammatory and cytoprotective actions via up-regulating HO-1 and stimulating the formation of HO-1 end-products, such as CO and bilirubin, whereas a large amount of heme is deleterious to tissue due to its pro-oxidative and pro-inflammatory functions. In the present study, we succeeded in showing that an increase in HO-1 induced by a low concentration of heme completely prevented barrier dysfunction caused by high-concentration heme treatment (Fig. 4). Western blot analysis of occludin, which was known as a typical TJ protein, showed interesting correlation with the barrier function. In detail, in the most injured barrier caused by heme under glutamine-free condition (Fig. 4A, lane 2, Gln(−)), the expression of occludin was significantly decreased (Fig. 4B, lane 2, Gln(−)), while pre-HO-1 induction resulted in maintaining not only barrier function (Fig. 4A, lane 3, Gln(−)) but also occludin levels (Fig. 4B, lane 3, Gln(−)). Since occludin expression was not well correlated with barrier function under glutamine-supplemented condition, there might be other molecules participating in injuring barrier by free heme. These results indicate that HO-1 induction prior to toxic heme exposure is effective to protect against barrier disruption, partially through occludin maintenance. Although the mechanism by which heme disrupts the intestinal barrier function is unclear, the present findings provide direct evidence for the importance of the prompt induction of HO-1 in the protection of TJ permeability against intestinal bleeding.

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Conflict of Interest The authors declare no conflict of interest.

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