Hemin induces active chloride secretion in Caco-2 cells

Aliye Uc, Russell F. Husted, Radhamma L. Giriyappa, Bradley E. Britigan, and John B. Stokes. Hemin induces active chloride secretion in Caco-2 cells. Am J Physiol Gastrointest Liver Physiol 289: G202–G208, 2005. First published March 31, 2005; doi:10.1152/ajpgi.00518.2004.—Enterocytes maintain fluid-electrolyte homeostasis by keeping a tight barrier and regulating ion channels. Carbon monoxide (CO), a product of heme degradation, modulates electrolyte transport in kidney and lung epithelium, but its role in regulating intestinal fluid-electrolyte homeostasis has not been studied. The major source of endogenous CO formation comes from the degradation of heme via heme oxygenase. We hypothesized that heme activates electrolyte transport in intestinal epithelial cells. Basolateral haimin treatment increased baseline Caco-2 cell short-circuit currents (Isc) twofold (control = 1.96 ± 0.14 μA/cm2 vs. hemin = 4.07 ± 0.16 μA/cm2, P < 0.01); apical hemin had no effect. Hemin-induced Isc was caused by Cl− secretion because it was inhibited in Cl−-free medium, with ouabain, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), or DIDS. Apical electrogenic Na+ channel inhibitor ben- zamid had no effect on hemin-induced Isc. Hemin did not alter the ability of Caco-2 cells to respond maximally to forskolin, but a soluble guanylate cyclase inhibitor, [1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) inhibited the effects of hemin. A CO-releasing molecule, tricarboxylichlororuthenium II, induced active Cl− secretion that was also inhibited with ODQ. We conclude that hemin induces active Cl− secretion in Caco-2 cells via a cGMP-dependent pathway. These effects are probably the consequence of CO formation. Heme and CO may be important regulators of intestinal fluid-electrolyte homeostasis.

heme oxygenase; electrolyte transport; carbon monoxide; tricarbo-
ylchlororuthenium II; cyclic guanosine 5’-monophosphate

Enterocytes are exposed to heme mainly from the apical (luminal) surface. Because the majority of dietary iron is not bioavailable, two-thirds of iron absorbed through intestines is in the heme form (7). After its absorption as an intact molecule, heme is degraded by the intestinal epithelial cell HO-1 to biliverdin, CO, and Fe2+ (38, 45); biliverdin is then converted to bilirubin by biliverdin reductase (6). It is not known if heme acquired by intestinal epithelial cells can influence fluid and electrolyte transport by inducing cGMP directly or via its metabolite CO.

Hemin induces guanylate cyclase activity and, therefore, cGMP formation in the intestinal mucosa, lung, glioma cells, and neuroblastoma cells (12, 48). Although the role of heme in intestinal ion transport has not been established, it is well recognized in other systems. In vascular smooth muscle cells, CO or hemin induce dilatation by increasing cGMP levels or by directly activating calcium-dependent K+ channels (49–51). Hemin stimulates calcium-dependent K+ channels in rat kidney thick ascending limb (27) and controls Na+ and fluid transport in the loop of Henle (52). In fetal lung cells, heme and CO induce epithelial cell Na+ transport, an important function for fluid absorption and postnatal pulmonary adaptation (39). The effects of heme on electrolyte transport do not always depend on the generation of CO. Heme can inhibit calcium-dependent Slo1 voltage-activated (BK) channels by directly associating with a heme binding amino acid sequence motif (44).

We hypothesized that heme, via its ability to induce intracellular cGMP, might activate ion transport in a human intestinal epithelia cell model Caco-2 cells (19, 20). Our study shows that hemin induces active Cl− secretion in Caco-2 cells via a cGMP-dependent mechanism. A CO-releasing molecule, tricarboxylichlororuthenium II (33), causes similar effects, suggesting that heme response is secondary to CO formation. We postulate that heme and CO are important regulators of intestinal fluid-electrolyte homeostasis.

MATERIALS AND METHODS

Tissue culture. Caco-2 cells were kindly provided by Dr. Jeffrey Field’s core facility (University of Iowa, Iowa City, Iowa) and used between passages 30 and 55. Stock cultures were grown to confluency at 37°C in 10% CO2 using DMEM (Cellgro; Mediatech, Herndon, VA) containing 4.5 g/l glucose, 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 15 mM HEPES. Fresh medium was added every 2 days. Cells were released from stock plates by brief trypsin-EDTA treatment and plated at 30,000 cells/cm2 on Millicell PCF filters (Millipore, Billerica, MA). Development of a confluent monolayer was confirmed when transepithelial resistance was stable for two successive days (about 14–16 days after seeding).
HEMATE INDUCES CL\(^-\) SECRETION IN CACO-2 CELLS

Fig. 1. A: hemin induces short-circuit currents (I\(\text{sc}\)) in Caco-2 cells. Caco-2 cells were treated with hemin (100 \(\mu\)M) in the basolateral solution for 24 h and then placed in Ussing chambers. Hemin-treated cells had an increased I\(\text{sc}\) compared with control cells (*\(P < 0.01\), \(n = 7\)). B: basolateral, not apical hemin, induces I\(\text{sc}\) in Caco-2 cells. Caco-2 cells were treated with apical or basolateral hemin (100 \(\mu\)M) for 24 h. I\(\text{sc}\) was measured in Ussing chambers. Basolateral hemin increased I\(\text{sc}\); apical hemin had no effect (*\(P < 0.01\); \(n = 24\)).

Electrical measurements. Transmonolayer voltage, transepithelial resistance, and short-circuit current (I\(\text{sc}\)) were measured in Ussing chambers constructed to accommodate Millicell filters (Jim’s Instruments, Iowa City, IA) (26). Cells were bathed in a Krebs-Ringer bicarbonate solution consisting of (in mM) 115 NaCl, 25 NaHCO\(_3\), 5 KCl, 5 Na-HEPES, 5 H-HEPES, 1.5 CaCl\(_2\)-2H\(_2\)O, 1 MgCl\(_2\)-6H\(_2\)O, 1 Na\(_2\)HPO\(_4\), and 5 glucose. Cl\(^-\) and HCO\(_3\)\(^-\)-free Na\(^+\)-HEPES solution contained (in mM) 1.5 CaNO\(_3\), 4 H\(_2\)O, 5 K gluconate, 1 MgSO\(_4\)-7H\(_2\)O, 1 Na\(_2\)HPO\(_4\), 5 Na-HEPES, 5 H-HEPES, 140 Na isethionate, and 5 glucose. Krebs-Ringer bicarbonate solution was gassed with 5% CO\(_2\) at 37°C to maintain pH at 7.4. Cl\(^-\) and HCO\(_3\)\(^-\)-free Na\(^+\)-HEPES solution was gassed with air, instead of 5% CO\(_2\). A positive I\(\text{sc}\) value represents a flow of positive charge from the luminal (apical) to the basolateral solution (absorption) or a flow of a negative charge from the basolateral solution to the apical solution (secretion).

Chemicals. Hemin, forskolin, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), benzamil, DIDS, and tricarbonyldichlororuthenium II were obtained from Sigma Aldrich (St. Louis, MO). Hemin (3 mM; Sigma Aldrich) was prepared in 1 N NaOH and diluted to 0.5 mM in phenol red-free MEM with L-glutamine (GIBCO-Invitrogen, Carlsbad, CA) and 10% FCS. The pH was adjusted to 7.4 by slow addition of 12 N HCl. For convenience, we will use heme (Fe\(^{2+}\)) and hemin (Fe\(^{3+}\)) interchangeably.

Statistics. Within-subject analyses (i.e., comparing I\(\text{sc}\) at baseline and after intervention) were performed using paired-sample Student’s t-tests. Between-subject analyses (i.e., I\(\text{sc}\) between control and hemin groups) were performed using two-sample t-tests. Results are expressed as means ± SE. Statistical significance was defined as \(P < 0.05\) (two-tailed analysis).

RESULTS

Hemin induces I\(\text{sc}\) in Caco-2 cells. Because heme can induce guanylate cyclase activity (12, 48), leading to cGMP formation, we hypothesized that heme could activate electrolyte transport in an in vitro intestinal epithelial cell model, Caco-2 cells. To test this hypothesis, cells were treated with hemin (100 \(\mu\)M) or the same volume of its carrier from the basolateral side for 24 h and I\(\text{sc}\) were measured in Ussing chambers. Basolateral hemin exposure increased baseline I\(\text{sc}\) twofold (1.96 ± 0.14 vs. 4.07 ± 0.16 \(\mu\)A/cm\(^2\), \(n = 7\), \(P < 0.01\)) (Fig. 1A). Adding hemin to the apical or basolateral solution did not have any acute effect for the first hour (n = 3). Hemin-induced current was also not inhibited in dark.

Basolateral, not apical hemin induces I\(\text{sc}\). We have previously shown that heme-induced HO-1 expression shows polarity in Caco-2 cells (46). Cells exposed to hemin from the basolateral surface demonstrated a higher HO-1 induction than cells exposed to hemin from the apical surface (46). Moreover, hemin secretion is more rapid and active compared with its absorption (46). Therefore, we investigated whether hemin-induced I\(\text{sc}\) responses showed polarity between apical and basolateral surfaces of the cell. We found that the same concentration of apical hemin (100 \(\mu\)M) did not increase I\(\text{sc}\) in Caco-2 cells (Fig. 1B).

Hemin-induced I\(\text{sc}\) is Cl\(^-\) secretion. To determine the nature of the I\(\text{sc}\) induced by basolateral hemin exposure, Ussing chamber experiments were repeated in Cl\(^-\)- and HCO\(_3\)\(^-\)-free

Fig. 2. Hemin-induced I\(\text{sc}\) is Cl\(^-\)-dependent. Caco-2 cells were treated with basolateral hemin (100 \(\mu\)M) for 24 h, and I\(\text{sc}\) was measured in Ussing chambers with Krebs-Ringer bicarbonate or Cl\(^-\) and HCO\(_3\)\(^-\)-free Na\(^+\)-HEPES solution. I\(\text{sc}\) induced by hemin incubation was inhibited if Cl\(^-\) and HCO\(_3\)\(^-\) were not present. (*\(P < 0.01\) control vs. hemin; **\(P < 0.01\) Krebs-Ringer bicarbonate vs. Cl\(^-\) and HCO\(_3\)\(^-\)-free Na\(^+\)-HEPES solution, n = 4).
Na⁺-HEPES solution. Hemin-induced current was significantly inhibited if Cl⁻ and HCO₃⁻ were not present in Ussing chambers, suggesting that the $I_{sc}$ increase caused by hemin was due to active Cl⁻ secretion (Fig. 2).

We then examined the effect of ouabain on hemin-induced $I_{sc}$. Ouabain blocks Na⁺-K⁺-ATPase, a basolateral pump needed to maintain low intracellular Na⁺ levels (41). Ouabain produced a gradual inhibition of hemin-induced $I_{sc}$ (32.8 ± 0.55 min, n = 6) (Fig. 3). This inhibition indicates that the $I_{sc}$ was dependent on the Na⁺/K⁺ pump and does not represent an active transport process powered by a H⁺-ATPase. In addition, the gradual pattern of inhibition is typical of that seen by inhibitors of Cl⁻ secretion in which the entry of Cl⁻ is dependent on an intact Na⁺ gradient.

We tested the sensitivity of the $I_{sc}$ to an inhibitor of the cystic fibrosis transmembrane regulator (CFTR) NPPB (3). When applied from the apical surface (300 μM for 10 min), there was a modest effect in hemin-exposed monolayers (Table 1). There was also a modest inhibitory effect of 100 μM DIDS added to the apical solution (Table 1), a Cl⁻ transport inhibitor that usually does not affect CFTR (2). Bumetanide, an inhibitor of the Na⁺-K⁺-2Cl⁻ cotransporter (22) applied to the basolateral solution (100 μM) had minimal effect on $I_{sc}$, suggesting that the entry mechanism for Cl⁻ across the basolateral membrane is probably not entirely due to the activity of this cotransporter.

To determine whether Na⁺ absorption via the epithelial Na⁺ channel could account for any of the increase in $I_{sc}$, we added benzamil (32) (10 μM) to the apical solution. Whereas there was a small effect in the control condition, there was no effect in hemin-treated monolayers, indicating that Na⁺ absorption via electrogenic Na⁺ channel (ENaC) was not contributing to the hemin-stimulated $I_{sc}$. These results indicate that the hemin-stimulated $I_{sc}$ was largely the result of active Cl⁻ secretion and not Na⁺ absorption. The inhibitor profile is not completely typical of Cl⁻ secretion mediated by CFTR. There could be another kind of Cl⁻ ion channel on the apical membrane of these cells, or there could be a combination of CFTR and other Cl⁻ channels that mediate this Cl⁻ secretion.

Table 1. Effects of channel modulators on hemin-induced $I_{sc}$ in Caco-2 cells

<table>
<thead>
<tr>
<th>$I_{sc}$ (%)</th>
<th>Baseline</th>
<th>Benzamil</th>
<th>DIDS</th>
<th>Bumetanide</th>
<th>NPPB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±5.3</td>
<td>79.1±14.3*</td>
<td>92.1±10.5</td>
<td>82.3±8.6*</td>
<td>97.7±15.4</td>
</tr>
<tr>
<td>Hemin</td>
<td>194.4±8.8</td>
<td>194.8±29.8</td>
<td>154.5±25.3†</td>
<td>183.7+23.7†</td>
<td>161.5±12.4†</td>
</tr>
</tbody>
</table>

Values are mean percentages ± SE; n = 4. Caco-2 cells were treated with hemin 100 μM basolaterally for 24 h and $I_{sc}$ was measured in Ussing chambers. The responses to benzamil (10 μM apical), DIDS (100 μM apical), bumetanide (100 μM basolateral), and NPPB (300 μM apical) were measured. *P < 0.05 compared with control; †P < 0.01 compared with hemin.
Sodium can enter the enterocyte across the apical membrane via the amiloride-sensitive ENaC, the Na⁺-glucose cotransporter, a Na⁺-amino acid cotransporter and/or a neutral Na⁺/H⁺ exchanger (17). Sodium exits the cell across the basolateral membrane via ouabain-sensitive basolateral Na⁺-K⁺-ATPase, which maintains low intracellular Na⁺ levels (42). In our study, hemin-induced \( I_{sc} \) was not secondary to an increase in ENaC activity, since it was not inhibited by benzamil. A small amount of hemin-induced \( I_{sc} \), which persisted after the removal of Cl⁻ (Fig. 4) may be due to Na⁺-glucose cotransport or Na⁺-amino acid cotransport because these transport pathways are also electrogenic. However, the majority of the \( I_{sc} \) induced by hemin is Cl⁻ secretion.

It is not clear if hemin induces Cl⁻ secretion through CFTR activation. The hemin-induced current was minimally inhibited by NPPB, a generally effective but partial CFTR inhibitor (3). CFTR inhibitors may inhibit several kinds of Cl⁻ transporters and indirectly alter the activity of CFTR or the transepithelial secretion of Cl⁻ (41). The lack of specific inhibitors of specific Cl⁻ channels render the experiments directed at determining

**DISCUSSION**

In our study, heme increased \( I_{sc} \) in Caco-2 cells via a cGMP-dependent mechanism. A CO-releasing molecule, tricarbonyldichlororuthenium II (34) gave similar results, suggesting that heme effects were secondary to CO formation.

In our experimental model, hemin-induced \( I_{sc} \) was the result of an active Cl⁻ secretion; it was abolished if Cl⁻ was removed from the Ussing chambers and slowly inhibited with ouabain. In Cl⁻ secreting epithelial cells, basolateral membrane transporters accumulate Cl⁻ intracellularly by a variety of transport systems, most of which are dependent on Na⁺, such as Na⁺-K⁺-2Cl⁻ cotransporter (14, 16, 18). Transepithelial Cl⁻ secretion is also dependent on the activation of apical Cl⁻ channels, the most well characterized of which is CFTR (5). In our study, ouabain inhibited hemin-induced \( I_{sc} \) over time, suggesting that hemin-induced Cl⁻ secretion required a Na⁺ gradient maintained by basolateral Na⁺-K⁺-ATPase. The basolateral entry mechanism for hemin-induced Cl⁻ secretion was not via the Na⁺-K⁺-2Cl⁻ cotransporter, because it was not abolished with bumetanide.
Figure 7. A: CO-induced \( I_{sc} \) is Cl\(^{-}\)-dependent. Caco-2 cells mounted in Ussing chambers were treated with tricarbonyldichlororuthenium II (100 \( \mu \)M) apically in Krebs-Ringer bicarbonate or Cl\(^{-}\) and HCO\(_3\)^{-}-free Na\(^{+}\)-HEPES solution. CO-induced \( I_{sc} \) was inhibited if Cl\(^{-}\} and HCO\(_3\)^{-} were not present in Ussing chambers. (* \( P < 0.01 \) control vs. CO in Krebs-Ringer bicarbonate solution; \( **P < 0.01 \) CO in Krebs-Ringer bicarbonate vs. CO in Cl\(^{-}\) and HCO\(_3\)^{-}-free Na\(^{+}\)-HEPES solution; \( n = 14 \)). B: CO induces \( I_{sc} \) via cGMP. Confluent Caco-2 cells mounted in Ussing chambers were treated with tricarbonyldichlororuthenium II (100 \( \mu \)M) apically. CO-induced \( I_{sc} \) was inhibited by ODQ (25 \( \mu \)M) (* \( P < 0.01 \) control vs. CO; \( **P < 0.01 \) CO vs. CO + ODQ; \( n = 11 \)).

In our study, heme induced active Cl\(^{-}\} secretion in Caco-2 cells via a cGMP-dependent pathway. This was probably not a direct effect of heme, because short heme treatments did not have an effect on \( I_{sc} \). We therefore hypothesized that CO, a heme degradation product known to induce cGMP (49–51), was responsible for heme-induced Cl\(^{-}\} secretion. A CO-releasing molecule, tricarbonyldichlororuthenium II (33) induced active Cl\(^{-}\} secretion via a cGMP-dependent pathway, supporting our hypothesis. The effect of CO on intestinal electrolyte transport is not surprising because CO is known to modulate ion channels in cardiovascular, pulmonary, and renal systems (27, 39, 49–52). Enterocytes acquire and degrade heme to extract its iron; CO, biliverdin, and bilirubin are formed during that process (37). Our findings suggest that heme acquired by intestinal epithelial cells can influence fluid and electrolyte transport by inducing cGMP, possibly via its metabolite CO. We have not yet investigated the effects of HO-1 inhibition and other heme degradation products (e.g., bilirubin and biliverdin) on electrolyte transport. The effect of hemin on electrolyte transport was observed only if cells were treated with hemin from the basolateral surface. We have previously shown (46) that cells exposed to hemin from the basolateral side demonstrated a higher HO-1 induction than cells exposed to hemin from the apical surface. It is possible that basolateral heme, by inducing higher levels of HO-1, increases CO concentrations inside the cell, thereby causing an increased Cl\(^{-}\} secretion. It seems most likely that heme enhances Cl\(^{-}\} secretion via its metabolic by-products rather than causing a direct effect. Although heme has been shown to bind and activate a K\(^{+}\} channel (44), such an effect on Cl\(^{-}\} secretion seems unlikely in our system. We have not observed Cl\(^{-}\} secretion with apical heme exposure, probably because heme does not induce high levels of HO-1 after apical treatment (46). Moreover, heme effect on Cl\(^{-}\} secretion is not an immediate event; it requires more than 1 h to develop. The blockade of the heme-induced Cl\(^{-}\} secretion by cGMP antagonists is also consistent with a metabolic effect of heme. Thus while CO is the most likely candidate, we cannot eliminate a possible role for bilirubin or biliverdin. In this regard, we note that jaundiced infants develop secretory diarrhea while they undergo phototherapy (9); the cause of this phototherapy-induced diarrhea is known, but a photooxidation product of bilirubin may be playing a role. In our experimental model, hemin-induced current was not inhibited in total darkness, making a photooxidation product of heme degradation unlikely to play a role here.

It must be noted that the effect of long-term heme treatment was relatively modest compared with the CO donor. This difference could be caused by relatively lower concentrations of CO after 24-h hemin treatment, because cells were not replenished with hemin during the Ussing chamber experiments. Therefore, there was relatively less heme as a substrate to produce CO, and the current was not as high as it was following incubation with the CO donor.

If heme and its degradation products are involved in maintaining fluid and electrolyte transport by the intestine, they may have a role in the treatment of certain disorders. A recent report showed that curcumin corrected the rectal potential difference defect of mice with \( \Delta F508 \) cystic fibrosis, a common genetic disorder characterized by abnormal Cl\(^{-}\} secretion (11). The authors provided evidence that curcumin permitted the defective \( \Delta F508 \) CFTR protein to reach the apical membrane by disrupting the degradation of the misfolded protein from the endoplasmic reticulum control system. Interestingly, curcumin is also an inducer of HO-1 (24, 34). It is not known whether curcumin, by inducing HO-1 and possibly CO, played a role in correcting the secretory functions of the epithelial cells in this model. The role of curcumin in correcting the defects in cystic fibrosis is not clear because the findings of Egan et al. (11) could not be reproduced by other researchers (10, 28, 42). Nevertheless, curcumin seems to increase CFTR channel activity in patch clamp studies if the channel is phosphorylated and ATP is present (4).

Another interesting model is rapid intestinal transit and diarrhea observed during gastrointestinal hemorrhage. It is not known if large amounts of blood in the intestinal lumen cause...
rapid transit by scavenging NO and CO and therefore stimulating intestinal contractions (53) or by inducing electrolyte secretion directly via heme and/or its degradation products. It seems possible that induction of HO-1 and the subsequent enhancement of heme metabolism may play a role in this clinical setting.

In summary, heme induces active Cl\textsuperscript{−} secretion in Caco-2 cells via a cGMP-dependent mechanism. Heme-induced effect is probably secondary to CO formation. Heme degradation, a natural function of enterocytes to extract iron from heme may also influence fluid and electrolyte transport.

**GRANTS**

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