Hemin induces active chloride secretion in Caco-2 cells

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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 heme 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 heme 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 benzamil 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, tricarbonyldichlororuthenium 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; tricarbonyldichlororuthenium II; cyclic guanosine 5′-monophosphate

ENTEROCYES MAINTAIN fluid-electrolyte homeostasis by keeping a tight intestinal barrier and regulating ion channels (8, 36). Microbial agents, toxins, drugs, or inflammation can disrupt this intestinal barrier and induce secondary messengers, such as cAMP and cGMP (15, 23, 36). These cyclic nucleotides induce epithelial chloride (Cl−) secretion and play a major role in the intestinal fluid-electrolyte homeostasis (21, 25, 30, 43).

A messenger molecule, carbon monoxide (CO), increases intracellular cGMP levels and participates in vascular and gastrointestinal smooth muscle relaxation (13, 47, 50). It is not known if CO induces intestinal electrolyte secretion by stimulating intracellular cGMP production.

Endogenous CO is formed predominantly through the cleavage of heme via heme oxygenase (HO) (29, 37). To date, two major forms, HO-1, HO-2, which catalyze this reaction, have been identified (1, 31). HO-1 protein is inducible by numerous stimuli including heavy metals, heme, cytokines, hypoxia, or heat shock. HO-2 is a constitutively synthesized enzyme (37).

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+. 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 heme 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, tricarbonyldichlororuthenium 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).

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Heme induces I_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 heme (100 μM) or the same volume of its carrier from the basolateral side for 24 h and I_sc was measured in Ussing chambers. Basolateral heme exposure increased baseline I_sc twofold (1.96 ± 0.14 vs. 4.07 ± 0.16 μA/cm², n = 7, P < 0.01) (Fig. 1A). Adding heme 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 heme induces I_sc. We have previously shown that heme-induced HO-1 expression shows polarity in Caco-2 cells (46). Cells exposed to heme from the basolateral surface demonstrate a higher HO-1 induction than cells exposed to heme from the apical surface (46). Moreover, heme secretion is more rapid and active compared with its absorption (46). Therefore, we investigated whether heme-induced I_sc responses showed polarity between apical and basolateral surfaces of the cell. We found that the same concentration of apical heme (100 μM) did not increase I_sc in Caco-2 cells (Fig. 1B).

Hemin-induced I_sc is Cl⁻ secretion. To determine the nature of the I_sc induced by basolateral heme exposure, Ussing chamber experiments were repeated in Cl⁻- and HCO₃⁻-free solution. Basolateral hemin increased I_sc; apical hemin had no effect (*P < 0.01; n = 24).

Electrical measurements. Transemolayer voltage, transepithelial resistance, and short-circuit current (I_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 NaHCO₃, 5 KCl, 5 Na-HEPES, 5 H-HEPES, 1.5 CaCl₂·2H₂O, 1 MgCl₂·6H₂O, 1 Na₂HPO₄, and 5 glucose. Cl⁻- and HCO₃⁻-free Na⁺-HEPES solution contained (in mM) 1.5 Ca(NO₃)₂·4 H₂O, 5 K gluconate, 1 MgSO₄·7H₂O, 1 Na₂HPO₄, 5 Na-HEPES, 5 H-HEPES, 140 Na isethionate, and 5 glucose. Krebs-Ringer bicarbonate solution was gassed with 5% CO₂ at 37°C to maintain pH at 7.4. Cl⁻- and HCO₃⁻-free Na⁺-HEPES solution was gassed with air, instead of 5% CO₂. A positive I_sc value represents a flow of positive charge from the luminal (apical) to the basolateral solution (absorption) or a flow of 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 tricarboxyldichlororuthenium 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 (Fe²⁺) and hemin (Fe³⁺) interchangeably.

Results

Statistics. Within-subject analyses (i.e., comparing I_sc at baseline and after intervention) were performed using paired-sample Student’s t-tests. Between-subject analyses (i.e., I_sc between control and heme 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).
The entry mechanism for Cl⁻ secretion in Caco-2 cells was dependent on extracellular Na⁺ concentrations. Hemin-induced current was significantly inhibited if Cl⁻ was 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 benzamid 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 mV, 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 benzamid (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 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.

Hemin does not increase cAMP-related responses in Caco-2 cells. Cells treated with hemin responded to forskolin (apical and basolateral 10 μM) (19), an activator of adenylate cyclase known to increase intracellular cAMP levels (Fig. 4). The increase in forskolin-activated I_{sc} was not different between controls and hemin-treated cells (ΔI_{sc} 11 ± 2.3 μA/cm² for control cells, n = 10, P = not significant), suggesting that hemin did not alter the maximum capacity of Caco-2 cells to secrete cAMP.

Hemin-induced I_{sc} is inhibited with ODQ. We then investigated whether hemin-induced I_{sc} was secondary to cGMP formation. Caco-2 cells were treated with hemin 100 μM for 24 h, and the response to [1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), a soluble guanylate cyclase inhibitor, was examined in Ussing chambers. ODQ (25 μM) inhibited hemin-induced I_{sc}, but had no effect on control cells (Fig. 5), suggesting that hemin-induced I_{sc} was cGMP-dependent.

**CO increases Caco-2 cell I_{sc}**. We then postulated that CO, a degradation product of heme, played a major role in hemin-mediated increases of I_{sc} in Caco-2 cells treated with hemin 100 μM basolaterally for 24 h and I_{sc} was measured in Ussing chambers. The responses to benzamid (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.

**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>Benzamid</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.
Hemoglobin 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 benzamid. 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 CO effects were secondary to CO formation.

In our experimental model, hemin-induced \(I_{sc}\) was partially inhibited 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.

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that process (37). Our findings suggest that heme acquired by extract its iron; CO, biliverdin, and bilirubin are formed during (27, 39, 49–52). Enterocytes acquire and degrade heme to ion channels in cardiovascular, pulmonary, and renal systems transport is not surprising because CO is known to modulate our hypothesis. The effect of CO on intestinal electrolyte chambers. (*P < 0.01 control vs. CO; **P < 0.01 CO in Krebs-Ringer bicarbonate vs. CO in Cl−- and HCO3−-free Na+−HEPES solution; n = 14). B: CO induces Isc via cGMP. Confluent Caco-2 cells were treated with tricarbonyldichlororuthenium II (100 μM) apically. CO-induced Isc was inhibited by ODQ (25 μM) (*P < 0.01 control vs. CO; **P < 0.01 CO vs. CO + ODQ; n = 11).

the involvement of these channels in the heme-induced Cl− secretion difficult to interpret.

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 Isc. 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 heme 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 heme from the basolateral side demonstrated a higher HO-1 induction than cells exposed to heme 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 biliverdin or bilirubin. In this regard, we note that jaundiced infants develop secretory diarrhea while they undergo phototherapy (9); the cause of this phototherapy-induced diarrhea is unknown, but a photooxidation product of bilirubin may be playing a role. In our experimental model, heme-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 hemin 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 ΔF508 cystic fibrosis, a common genetic disorder characterized by abnormal Cl− secretion (11). The authors provided evidence that curcumin permitted the defective ΔF508 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⁻ 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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REFERENCES