Heme modulates intestinal epithelial cell activation: involvement of NADPHox-derived ROS signaling

Pedro Barcellos-de-Souza, João Alfredo Moraes, Julio Cesar Madureira-de-Freitas-Junior, José Andrés Morgado-Díaz, Christina Barja-Fidalgo, and Maria Augusta Arruda

1Laboratório de Farmacologia Celular e Molecular, Departamento de Biologia Celular, Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil; 2Divisão de Biologia Celular, Instituto Nacional de Câncer, Rio de Janeiro Brazil; and 3Vice-Diretoria de Pesquisa, Ensino e Inovação, Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil

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Barcellos-De-Souza P, Moraes JA, de-Freitas-Junior JC, Morgado-Diaz JA, Barja-Fidalgo C, Arruda MA. Heme modulates intestinal epithelial cell activation: involvement of NADPHox-derived ROS signaling. Am J Physiol Cell Physiol 304: C170–C179, 2013. First published October 31, 2012; doi:10.1152/ajpcell.00078.2012.—In many gut chronic inflammatory conditions, intestinal epithelium (IE) is deprived of the protection of the mucus secreted by IE-specialized cells. In these events, bleeding and subsequent lysis of erythrocytes are common. This may lead to the release of high amounts of heme in the intestinal lumen, which interacts with IE. Previous works from our group have shown that heme itself is a proinflammatory molecule, activating a number of phlogistic signaling events in a nicotinamide adenine dinucleotide phosphate oxidase (NADPHox)-dependent manner. In this study, we aim to evaluate the effects of heme upon a well-established nontransformed small intestine epithelial cell lineage (IEC 6). Our results show that free heme evokes intracellular reactive oxygen species (ROS) production by IEC 6 cells, which is inhibited both by pharmacological inhibition with diphenyleneiodonium (10 μM), a NADPHox inhibitor, and small interfering RNA-mediated suppression of NOX1, a constitutive NADPHox isoform present in intestinal epithelial cells. Focal adhesion kinase phosphorylation and actin cytoskeleton polymerization are also induced by heme in a NADPHox-dependent manner. Heme increases monolayer permeability and redistributes key modulators of cell-cell adhesion as zona occludens-1 and E-cadherin proteins via NADPHox signaling. Heme promotes IEC 6 cell migration and proliferation, phenomena also regulated by NADPHox-derived ROS. Heme, in NADPHox-activating concentrations, is able to induce mRNA expression of IL-6, a cytokine implicated in inflammatory and tumorigenic responses. These data indicate a prominent role for heme-derived signaling in the pathophysiology of intestinal mucosa dysfunction and address an important role of NADPHox activity on the pathogenesis of intestinal inflammatory conditions.

heme; NADPH oxidase; intestinal epithelial cell; ROS signaling; inflammation

INTESTINAL EPITHELIUM (IE) works as an intrinsic barrier against microbial invaders. However, the role of IE in immunity is beyond physical intervention, since it is recognized that IE is able to distinguish potentially pathogenic microorganisms from endogenous bacterial flora, as well as coordinate the proper biological response against them (6, 13). Several IE cells features characterize them as active players in immune and inflammatory responses, and their regulatory role had been already identified, since these cells are able to express Toll-like receptors, major histocompatibility complex components, pro-inflammatory cytokines, and chemokines (27, 37).

In many pathological situations related to chronic inflammatory conditions, such as inflammatory bowel diseases, there is a decrease in mucus production by IE-specialized cells, leading to a lesser protection to the epithelial cells. Under these circumstances, notably in duodenal ulcers, blood vessels rupture is common, which allow the lysis of erythrocytes and consequent release of high amounts of free heme (ferriprotoporphyrin IX) to the extracellular milieu. This molecule thus interacts directly with IE (34, 38).

Our group has characterized free heme as a proinflammatory and antiapoptotic molecule exerting most of its effects via NADPHox complex activation, which is a specialized reactive oxygen species (ROS) generator (1, 2, 12). It has also been shown that heme is able to activate redox-sensitive pathways in vascular smooth muscle cells (15, 21), supporting the link between heme and NADPHox activation. The NADPHox family consists of seven members (NOX1–5 and DUOX1–2) that are distinctly expressed by different tissues. Although they share highly conserved structural patterns, each member of this family can be involved in distinct biological responses (4). The role of NADPHox-derived ROS signaling is well documented and is addressed to regulate precise signaling pathways, instead of generating extensive and nonspecific oxidative stress (28).

Several studies suggest that excessive levels of heme, both from hemolysis and from a diet rich in red meat, are able to irritate colon mucosa, causing inflammation and therefore deregulating normal proliferation/exfoliation rates; a scenario that increases colon cancer probabilities (22, 25, 34). Despite evidence of heme initiated-inflammation, the molecular mechanisms underlying this effect on IE had not been depicted.

This study aims to elucidate the putative role of NADPHox-derived ROS in intestinal epithelial cell activation by heme. We used a well-established nontransformed small intestine lineage, IEC 6, and assessed classical parameters of cell activation, such as intracellular redox status, focal adhesion kinase (FAK) activation, actin cytoskeleton polymerization, monolayer permeability, cell migration, proliferation, and interleukin 6 (IL-6) expression. To investigate NADPHox involvement in heme effect on IEC 6, both pharmacological intervention, using diphenyleneiodonium (DPI), a flavoprotein inhibitor extensively used as NADPHox negative modulator, and loss of function experiments, silencing the constitutive NADPHox isoform NOX1, present in IEC 6 cells, were performed.
MATERIAL AND METHODS

Reagents

DPI, Trolox, mitomycin c, BSA, PMSF, benzamidine, phorbole myristate acetate (PMA), DNase, RNase, and soybean trypsin inhibitor were purchased from Sigma-Aldrich (St. Louis, MO). DMEM was acquired from GIBCO-BRL (Carlsbad, CA). PVDF was obtained from Amersham (Piscataway, NJ). FAK (catalog no. 558) antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). E-cadherin antibody (cat. no. 610182) was from BD Biosciences (San Jose, CA). Zona occludens-1 (ZO-1) antibody (cat. no. 40–2300) was obtained from Zymed. Enhanced chemiluminescence system (ECL) was obtained from Pierce Biotechnology (Rockford, IL). Peroxidase-conjugated streptavidin was from (Caltag Laboratories, Burlingame, CA). AlexaFluor 555, CM-H2DCFDA, DAPI Pro Long gold antifade reagent with DAPI, rhodamine-conjugated phalloidin, phosphorylated FAK antibody (cat. no. 44–652G), and MitoSOX Red were acquired from Molecular Probes (Eugene, OR). Mito-TEMPO was purchase from Enzo Life Sciences (Farmingdale, NY).

Cell Culture

IEC 6 cells, a nontransformed rat small intestine duodenum cell lineage, were kindly gifted by Dr. Bruno Diaz (Instituto de Biofisica, Universidade Federal do Rio de Janeiro) and originally obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM medium containing 10% FBS, 50 U/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37°C in a 5% CO2 air atmosphere. The cells were dissociated with 0.1%/0.01% trypsin/EDTA and then seeded into new culture flasks between the 3th and 12th passages.

NOX1 Silencing

IEC 6 were transfected with either a nontargeting control small interfering (si)RNA (siRNA negative control no. 1; Ambion, Austin, TX), as a control for nonsequence-specific effects or a NOX1-specific siRNA sequence (Silencer Predesigned siRNA NOX1 ID no. 55585; Ambion). The NOX1 siRNA location is 725 and targets rat mRNA for NOX1 (NM_053683.1). Twenty micromoles of either the nontargeting or NOX1-specific duplex were diluted in 1 ml Opti-MEM I (Ambion) reduced serum medium and 1 ml of Lipofectamine 2000 (Ambion) diluted in Opti-MEM I was added. The mixture was gently mixed and allowed to incubate for 5 min at room temperature, and the final concentration of siRNA was 10 μM. IEC 6 were grown in six-well plate culture, and when they were at 70% of confluency, they were incubated with RNAi duplex-Lipofectamine 2000 complexes antibiotics-serum-free. Twenty-four hours after transfection, attenuation of NOX1 expression was verified by Western blotting of cell lysates probed with antibody against NOX1 (ab131088; Abcam, Cambridge, MA).

ROS Generation

Intracellular ROS generation assay. Aliquots of 2.5 × 10^4 IEC 6 cells (and IEC 6 silenced with siRNA scramble or siRNA NOX1 when mentioned) were seeded in a clear- bottom black 96-well plate overnight in DMEM medium containing 10% FBS. The cells were washed three times with PBS, and the medium was replaced by HBSS (0.137 M NaCl, 5.4 mM KCl, 0.25 mM NaH2PO4, 0.44 mM KH2PO4, 1.3 mM CaCl2, 1.0 mM MgSO4, and 4.2 mM NaHCO3, pH 7.2) medium with CM-H2DCFDA fluorescent probe (10 μM) for 30 min. After probe removal with PBS washes, HBSS medium was added and cells were pretreated or not with DPI (10 μM), Mito-TEMPO (5 mM), or Trolox (100 μM) for 15 min and incubated in the absence or presence with different concentrations of heme (0.03–100 μM) for 2 h. Plates were analyzed in Envision 2104 multilabel plate reader (PerkinElmer, Waltham, MA). Fluorescence was monitored for different times at excitation and emission wavelengths of 495 and 530 nm, respectively.

The treatment with Trolox (100 μM) was considered as 100% of ROS production inhibition. The slope of the ROS-generated curves were calculated by determining the linear regression of each curve, using the equation: slope = ΔF/Δt.

Mitochondrial-superoxide (O2 *)-specific probe assay, IEC 6 cells (3 × 10^4/well) were placed on coverslips inside a 24-well plate overnight incubated with DMEM 10% FBS. After 24 h, cells were three times-PBS washed and incubated with DMEM 1% FBS. After pretreatment or not with Mito-TEMPO (5 mM), cells were treated with heme (10 and 30 μM) or FeCP (6 μM), an uncoupler of mitochondrial oxidative phosphorylation for 1 h. Medium was removed, and cells were incubated in HBSS with MitoSOX Red (5 μM) for 15 min. Cells were then washed with PBS three times, fixed, and counterstained with DAPI Pro Long. Cells were observed under epifluorescence microscope and magnified ×600. Mean fluorescence was calculated quantifying fluorescence intensity in four different fields.

Cell Extracts Preparation and Western Blot Analysis

For whole cell extracts, IEC 6 (2 × 10^6 cells) were resuspended in lysis buffer (50 mM HEPES pH 6.4, 1 mM MgCl2, 10 mM EDTA, 1% Triton X-100, 1 μg/ml DNase, and 0.5 μg/ml RNase) containing the following protease inhibitors: 1 mM PMSF, 1 mM benzamidine, 1 μM leupeptin, and 1 μM soybean trypsin inhibitor. Total protein content was determined by BCA method (30).

Lysates were denatured in sample buffer containing SDS and 2-mercaptoethanol and heated in boiling water for 5 min. Whole cell extracts (30 μg of protein) were submitted to 8% or 12% SDS-PAGE, and proteins were transferred to PVDF membranes. Rainbow molecular weight markers (Amersham Pharmacia Biotech) were run in parallel to estimate molecular weights. Membranes were blocked with Tween- PBS (0.1%) containing 2% BSA for 2 h and incubated for ~15 h with anti-FAK (1:1000) and anti-pFAK (1:1,000). After three washes (10 min each) at room temperature, PVDF membranes were incubated with anti-rabbit or anti-mouse IgG antibodies conjugated to biotin (1:10,000) for 1 h. After Tween-PBS washes, membranes were incubated with streptavidin conjugated to peroxidase (1:10,000). After Tween-PBS washes, immunoreactive proteins were visualized by ECL system. Protein bands were quantified by optical densitometry by Scion Image software (Scion, Frederick, MD).

Actin Cytoskeleton Polymerization

IEC 6 cells (3 × 10^5/ml) were seeded on coverslips inside a 24-well plate overnight in DMEM 10%. After 15 h, cells were washed three times with PBS and incubated in 1% FBS DMEM. Cells were pretreated or not with DPI (10 μM) for 15 min and then stimulated with heme (10 μM) for different time points (0–30 min). After each time point, cells were fixed and incubated with rhodamine-conjugated phalloidin overnight. Cells were washed with PBS and incubated for 5 min with DAPI (1:1000) at room temperature. Coverslips were mounted on a slide with the use of a 20 μM N-propylgalate and 80% glycerol solution in PBS before examination under microscope equipped for epifluorescence at ×1,000 magnification.

Transcellular Electric Resistance Assessment

IEC 6 cells (2 × 10^5) were seeded in a 0.4-μm Transwell insert and incubated in DMEM 10% FBS for 4 days to form a confluent monolayer. Cells were washed three times with PBS, incubated in DMEM 1% FBS, pretreated or not with DPI (10 μM), and stimulated with heme (0.1–30 μM) for 0–6 h. At determined time points, transepithelial electric resistance (TER) was assessed with Millicell-ERS (Millipore, Billerica, MA). All TER values were normalized for the area of the filter (0.6 cm²) and were obtained after background subtraction (i.e., filter and bath solution).
Immunocytochemistry

IEC 6 cells were plated (3 × 10^5 cells) on glass coverslips inside a 24-well plate and incubated for 4 days in DMEM containing 10% FBS to form a confluent monolayer. The cells were washed three times with PBS, and the medium was replaced by DMEM 1% FBS. Cells were pretreated or not with DPI (10 μM) for 15 min and incubated in the presence or absence of heme (10 μM) for 30 min. The monolayers were washed twice with PBS and fixed with 4% paraformaldehyde/4% sucrose in PBS for 20 min. Cells were permeabilized by Triton X-100, incubated for 30 min with 5% BSA in PBS, and incubated with polyclonal rabbit anti-ZO-1 (1:60) or polyclonal mouse anti-E-cadherin antibodies (1:300) for 2 h at 4°C. Subsequently, the cells were washed three times with PBS and incubated 1 h with biotin-conjugated anti-rabbit IgG or anti-mouse IgG (1:500), followed by three times wash and incubation with AlexaFluor 555-conjugated streptavidin (1:500) for 1 h at room temperature. Then, IEC 6 cells were washed three times with PBS and cells nuclei were counterstained with DAPI Pro Long at room temperature. Coverslips examination was performed under microscope equipped for epifluorescence and magnified X1,600.

Wound Healing Assay

IEC 6 cells were seeded (1 × 10^4/ml) in a six-well plate in DMEM 10% FBS for 3 h to allow cell adherence. Cells were incubated with serum-free DMEM medium overnight to synchronize cell cycle. Cells were then incubated with proliferation blocker mitomycin c (2 μg/ml) for 1.5 h. After medium removal, cells were incubated with DMEM 1% FBS and a scratch was made by a pipette tip in cell monolayers. After the treatment, cells were lysed with 0.2 N NaOH and the fluorescence and magnified X1,600.

Analysis of IL-6 mRNA Expression by RT-PCR

IEC 6 cells (2 × 10^6/well) were seeded in a six-well plate and incubated with DMEM 10% FBS overnight. Cells were washed three times with PBS and incubated in DMEM 1% FBS. Cells were stimulated with heme (0.1–30 μM) or TNF-α (5 μg/ml; positive control) for 4 h. Total cellular RNA was extracted with Trizol, chloroform was added, the aqueous phase was obtained, and the RNA was precipitated with isopropanol. The quantity of RNA was routinely tested by determining A260, whereas the RNA purity was assessed by A260/A280. Synthesis of full-length cDNA templates for RT-PCR was performed according to GoTaq polymerase kit (Promega) after RNA samples had been denatured at 95°C for 2 min. Semiquantitative PCR amplification of the resulting cDNA was performed using specific primers for rat IL-6 and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as housekeeping gene (Applied Biosystems). A master mix consisting of Taq DNA polymerase and deoxyribonucleotides was used in the appropriate buffer system. The sequence of the forward primer used for IL-6 amplification was 5’-CGCTAGCC-ACCTCCACAAGA-3’ and of the reverse primer was 5’-GTGAATG- TGTCCCTTAGCCAC-3’ resulting in a PCR product of 339 bp. IL-6 amplification was performed for 35 cycles with an annealing temperature of 56.5°C. The sequence of the forward primer for GAPDH amplification was 5’-TCAAAGGGAACCATCACCCTCT-3’ and of the reverse primer was 5’-ACGATACATCCAGCACCACG- CATCA-3’ resulting in a PCR product of 82 bp. GAPDH amplification was performed for 26 cycles with an annealing temperature of 50°C. Identical amounts of cDNA were used for IL-6 and the corresponding GAPDH amplifications. Controls using RNA samples without reverse transcription were used to demonstrate absence of contaminating DNA. PCR products were analyzed on GelRed-stained 1.5% agarose gels.

Statistical Analysis

Statistical significance was evaluated by ANOVA followed by Bonferroni comparison test by using Prism version 5.00 (GraphPad, La Jolla, CA).

RESULTS

Heme Impact on Intracellular ROS Generation

To assess heme-promoted ROS generation, we analyzed the fluorescence intensity of the intracellular oxidation-sensitive probe CM-H2DCFDA in IEC 6 cells incubated with heme in a concentration range from 0.03 to 100 μM over a 2-h period. Heme (3–100 μM) was able to increase an expressive probe oxidation, and concentrations ≥10 μM induced a robust ROS production (Fig. 1A). ROS generation was higher in early time points (5–35 min) as indicated through curve slope calculation (Table 1).

The source of ROS is of extreme importance to the delicate regulation of signaling pathways and to promote precise cellular responses (16, 26, 33). To investigate whether heme-induced ROS generation was from NADPHox or mitochondrial origin, we preincubated cells with DPI or Mito-TEMPO, a specific mitochondrial O2− scavenger before heme stimulation (Fig. 1B). We observed that most of ROS generated by heme at 10 μM (56%) at 35 min of incubation was from NADPHox origin whereas a small percent (5%) was from mitochondrial source. A distinct profile occurs in heme at 30 μM (56%) at 35 min of incubation which confirms a prominent NADPHox activation in this model.

To confirm higher mitochondrial-derived ROS production in heme at 10 μM, we assessed mitochondrial O2− generation using the fluorescent probe MitoSOX Red, a cell-permeable fluorogenic probe that is rapidly and selectively targeted to the mitochondria, where it is
readily oxidized by superoxide but not by other ROS- or reactive nitrogen species-generating systems. As observed in Fig. 1E, heme at 30 μM was able to induce mitochondrial ROS accumulation, which was even higher than the mitochondrial uncoupling agent FccP (6 μM). Moreover, mitochondrial ROS accumulation by heme at 30 μM was significantly inhibited by mito-TEMPO pretreatment (Fig. 1F).

**Heme Promotes FAK Phosphorylation Through NADPHox Activation**

FAK is a tyrosine kinase that is a key regulator of specialized structures called focal adhesions. Focal adhesions complexes localize to cell contacts and/or stabilize lamellipodia to the underlying matrix and have an important role in intestinal monolayer

Table 1. Slopes of heme-evoked ROS generation curves in IEC 6 cells

<table>
<thead>
<tr>
<th>Time interval, min</th>
<th>Heme, μM</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>30</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–35</td>
<td></td>
<td>0.6774 ± 0.201</td>
<td>1.148 ± 0.4290</td>
<td>4.292 ± 2.042</td>
<td>7.816 ± 2.565*</td>
<td>18.27 ± 1.6206*</td>
<td>25.39 ± 7.4951*</td>
<td>28.31 ± 11.523*</td>
</tr>
<tr>
<td>35–65</td>
<td></td>
<td>0.5662 ± 0.3124</td>
<td>1.052 ± 0.4125</td>
<td>3.958 ± 2.557</td>
<td>7.740 ± 3.902*</td>
<td>12.86 ± 2.829*</td>
<td>19.01 ± 8.623*</td>
<td>7.757 ± 11.57</td>
</tr>
<tr>
<td>65–95</td>
<td></td>
<td>0.5284 ± 0.4804</td>
<td>0.2734 ± 0.4220</td>
<td>1.476 ± 3.781</td>
<td>4.096 ± 6.437</td>
<td>7.333 ± 5.541</td>
<td>8.303 ± 12.67</td>
<td>5.377 ± 13.88</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3–7 independent experiments. IEC 6 cells (2.5 × 10⁵ well) were placed in a clear-bottom black 96-well plate and incubated with DMEM 10% FBS overnight. Cells were then incubated in HBSS with fluorescent probe CM-H₂DCFDA (10 nM) for 30 min. After probe removal, HBSS was added and cells were stimulated with heme (0.03 - 100 μM) and plate fluorescence was read at determined time intervals. Table shows the curve slope from each treatment during three time intervals: 5–35 min, 35–65 min, and 65–95 min. ROS, reactive oxygen species. *P < 0.05; †P < 0.01; ‡P < 0.001, significant difference between stimulated and control groups.
regulation, once it allows the promotion of tissue restitution by driving cell migration, and maintains tissue integrity by strengthening cell anchorage (32). Focal adhesions also integrate multiple intracellular and extracellular signals, coordinating distinct responses and functioning as a signaling nidus points. Therefore, FAK activation is related to several cellular responses as migration, proliferation, cell survival, and others (17).

To determine whether heme stimulation activates FAK, cells were treated with heme (10 μM) for different time points (0–120 min) and cell lysates were submitted to immunoblotting. Heme induced a transient but robust FAK phosphorylation peaking between 15 and 60 min. NADPHox activity was crucial for this event, since pretreatment with DPI reverted heme-induced FAK phosphorylation (Fig. 2A). NADPHox-derived ROS role in FAK activation was supported by the fact that only heme at 10 μM, a concentration that induces maximal NADPHox-derived ROS accumulation, was able to promote FAK phosphorylation (Fig. 2B).

**Heme Modulation of Actin Cytoskeleton: Involvement of NADPHox-Derived ROS**

It is known that a rapid disassembly at the rear end and assembly of the focal adhesions at the leading edge of the cells allow the traction force required for cell migration. Previous works demonstrated that FAK activation promotes focal adhesion complexes arrangement, which mediates adhesion turnover, and subsequently cell migration (10). Thus we sought to investigate heme ability to induce actin polymerization by using rhodamine-conjugated phalloidin, which binds to filamentous actin (F-actin).

Heme promotes a redistribution of F-actin from the cell border to stress fibers formation across the cytoplasm (Fig. 3), what may contribute to weakening cell-cell contacts and enhancing migratory ability. Inhibition of NADPHox activity attenuates heme induced-stress fibers formation, suggesting a role for ROS signaling mediated by this enzyme.

**Role of NADPHox-Derived ROS on Heme-Induced Monolayer Permeability**

Adherent cell migration requires the undermining of the junctions that maintain cells together, which in turn increases the monolayer permeability. We measured TER of IEC 6 monolayers after incubation with heme (10 μM) and observed a transient decrease (5–30 min) in the resistance, which reflects an increase in permeability (Fig. 4A). Only heme at 10 μM was able to induce this event (Fig. 4B). The role of NADPHox-derived ROS in heme-promoted permeability was addressed by pretreatment of monolayers with DPI, which reverted heme effects (Fig. 4C).

To elucidate molecular aspects underlying this phenomenon, the distribution of the signal transduction regulator of tight junctions ZO-1 and the adherens junction scaffold protein E-cadherin, were assessed. Heme (10 μM) decreased cell border expression of these two proteins, an event dependent on NADPHox activity (Fig. 5, A and B).

**Heme Promotion of Wound Healing: Involvement of NADPHox-Derived ROS**

Since heme treatment was able to promote events that are crucial to migration, we investigated whether heme directly increased cell migration. For this we performed wound healing assay, which is a well-established method to study directional cell migration in vitro.

Heme (10 μM) incubation for 1 h significantly stimulated IEC 6 cell migration towards wounded area after 20 h (37% above control, Fig. 6A). Cell proliferation role in this event was excluded since cells were incubated with mytomicin c before heme stimulation. This phenomenon was dependent on NADPHox activity since DPI pretreatment abolished heme-induced cell migration. Silencing the NADPHox isoform NOX1 also inhibited this response (38%; Fig. 6B).

**Heme Effect on IEC 6 Cell Proliferation**

Although it was not observed a role for cell proliferation in wound healing assay, heme was able to promote IEC 6 cell proliferation. After 48 h of incubation, [H³]thymidine incorporation was increased in cells stimulated with heme (10 μM; Fig. 7A). Noteworthy, the heme 10-μM treatment was not toxic to IEC 6 cell; on the other hand, higher concentrations of heme (30 and 100 μM) affected cell viability, as assessed in trypan blue assay (data not shown). Heme-induced IEC 6 proliferation was an event dependent on NADPHox activity (Fig. 7B, A and B).
on NADPHox-derived ROS signaling once it was abrogated by pretreatment with DPI (Fig. 7B).

**Heme Induction of IL-6 mRNA Expression**

IEC 6 ability to secrete proinflammatory cytokines as IL-6 has already been described (19, 20). IL-6 is usually considered an inducer of inflammation acute phase proteins, and its expression increase has been related to pathophysiological disorders of the intestine (3, 31).

IEC 6 cells stimulated with heme (1–10 µM) for 4 h displayed increased IL-6 mRNA synthesis compared with control (Fig. 8). This result endorses heme role as a putative proinflammatory molecule, activating IL-6 de novo synthesis on intestinal epithelial cell.

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**Fig. 3.** Heme modulation of actin cytoskeleton-involvement of NADPHox-derived ROS. IEC 6 cells (3 × 10^5/ml) were pretreated or not with DPI (10 µM) for 15 min, and then stimulated with heme (10 µM) for different time points (0–30 min). After each time point, cells were fixed, permeabilized and incubated with rhodamine-conjugated phalloidin overnight. Then, cells were washed with PBS and analyzed under fluorescence microscope with ×1000 magnification. Data are representative of 3 independent experiments.

**Fig. 4.** Role of NADPHox-derived ROS on heme-induced monolayer permeability. A: IEC 6 cells (2 × 10^4/well) were placed in a 0.4-µm Transwell insert and incubated in DMEM 10% FBS for 4 days to form a monolayer. Then, cells were incubated in DMEM 1% FBS and stimulated with heme at 10 µM (dashed lines) or not (control, solid lines) for 6 h or heme (B: 0.1–30 µM) for 30 min. Data shown are the results (means ± SE) of 3–5 independent experiment. *P < 0.05; **P < 0.01, significant difference between heme-treated and control groups. C: under the same experimental conditions, cells were pretreated or not with DPI (10 µM) and cultured in the absence or in the presence of heme (10 µM) for 30 min. Data shown are the results (means ± SE) of 3 independent experiments. *P < 0.05, significant difference between control and heme-stimulated groups. **P < 0.05, DPI significantly reverted heme effect.
DISCUSSION

Intestinal epithelium is constituted by a cell monolayer that represents a highly selective barrier between the hostile luminal gut environment and underlying lamina propria immune cells. As conditions involving mucosal inflammation are important causes of morbidity and mortality, intense research efforts to comprehend the mechanisms that lead to their development are of paramount importance (37).

During inflammatory onset and progression, intestinal epithelial cells become more susceptible to external insults, once the protective mucous-bicarbonate-phospholipids barrier is disturbed (11, 29). In these situations, erythrocytes are often released from ruptured blood vessels and destructed in the hostile gut environment, allowing hemoglobin to escape. Although hemoglobin has high affinity to serum proteins like haptoglobin, the amount of cell-free hemoglobin can exceed these scavenging mechanisms binding capacities throughout extensive hemolitics episodes, which results in methemalbumin generation and free heme accumulation (23).

Heme molecule is the prosthetic group of hemoproteins and is important for the execution of various biological processes. Nevertheless, free heme released from hemoproteins is a known prooxidant and causes oxidative stress and inflammation per se (2, 35).

In this study, we proposed to assess free heme ability to regulate classical cell features related to inflammatory situations. As observed in other cell types (2, 15, 36), heme induced intracellular ROS generation in IEC 6 cells. Heme (≥10 µM) promoted a strong ROS production that was higher in the first 35 min of incubation; however, the source of ROS generation was distinct between the tested concentrations. In a concentration easily found at hemorrhagic sites (10 µM), heme primarily activated NADPHox to generate ROS. Interestingly, NOX1 silencing lead to a significant inhibition of ROS, suggesting that this NADPHox isoform, which is constitutively expressed in intestinal epithelial cells, plays a role in the redox-sensitive effects of heme. However, higher concentrations of heme (30 and 100 µM) incited the production of mitochondria-derived ROS. As heme at 30 and 100 µM is detrimental to cell viability over 24 h (data not shown), it is possible that these mitochondrial ROS are a result of an imbalance in mitochondrial electrons transport chain as a result of mitochondrial collapse, leading to cell death. However, we cannot rule out the involvement of other ROS-generating systems.

FAK is a scaffold protein integrating the extracellular and the intracellular environments and signaling for a number of events such as migration, proliferation, and cell survival among others (7). Heme-incited NADPHox-derived ROS promotes FAK phosphorylation in a transient manner. In line with FAK activation, heme also induced actin cytoskeleton polymerization, which was susceptible to NADPHox modulation.

Evidence supports a role for ROS modulation on FAK activation and focal adhesion complex organization, as it was described that NADPHox subunits colocalize and physically interact with focal adhesions complex (33). Moreover, Chiarugi et al. (8) showed that ROS generation evoked by integrin binding...
leads to oxidative inactivation of a phosphatase that downregulates FAK, providing this kinase activation and allowing cell spreading events. These interactions with signaling platforms propose localized and specific effects promoted by NADPHox, which are critical to a finely regulation of signaling pathways.

We also observed a transient increase in monolayer permeability that was accompanied by loss of ZO-1 and E-cadherin on the border of the cells, indicating monolayer disruption, what may facilitate cell migration. These events strongly relied on NADPHox-dependent signaling.

Fig. 6. Heme promotion of wound healing: role for NADPHox-derived ROS.

A: IEC 6 cells (1 × 10^5/ml) were placed in a 6-well plate and incubated with serum-free DMEM medium overnight. Cells were incubated with mitomycin c (2 µg/ml) for 1.5 h. After medium removal, cells were incubated with DMEM 1% FBS, and a scratch was made by a pipette tip in cell monolayer. Following pretreated or not with DPI (10 µM) for 15 min before heme stimulation (0.03–100 µM) for 48 h. In the last 24 h, [H3]timidine (1 µCi) was added to the wells. After the treatment, cells were lysed and the obtained supernatants were adsorbed in solid filters. Next, H3-incorporation was counted in a liquid scintillation spectrometer. Representative data are shown (n = 3). *P < 0.01; ***P < 0.001, significant difference from the control group.

B: IEC 6 cells transfected or not with siRNA were treated with mitomycin c and after the scratching the cells were incubated or not with heme at 10 µM for 1 h. Procedures were followed as mentioned above. Images were captured at the beginning and after 20 h of incubation and clear areas were measured and compared. Data shown are the results (means ± SE) of 3 independent experiments (A). *P < 0.05 and **P < 0.01, significant difference between control and stimulated groups. #P < 0.05, DPI pretreatment significantly reverted heme effects of 4 independent fields (B). **P < 0.01 and ****P < 0.001, difference from nontransfected control. #P < 0.05, siRNA transfection reverted significantly heme effects.

Fig. 7. Heme effect on IEC 6 proliferation. After adherence in a 96-well plate (10^3/well) and cell cycle synchronization, IEC 6 cells (A and B) were incubated in DMEM 1% FBS. Cells were pretreated or not with DPI (10 µM) for 15 min before heme stimulation (0.03–100 µM) for 48 h. In the last 24 h, [H3]timidine (1 µCi) was added to the wells. After the treatment, cells were lysed and the obtained supernatants were adsorbed in solid filters. Next, H3-incorporation was counted in a liquid scintillation spectrometer. Representative data are shown (n = 3). **P < 0.01; ***P < 0.001, significant difference from the control group. #P < 0.05, DPI treatment significantly reverted heme effects.

Fig. 8. Heme induction of IL-6 mRNA expression. Adhered IEC 6 cells (1 × 10^5/well) were incubated in DMEM 1% FBS and stimulated with heme (0.1–30 µM) or TNF-α (5 µg/ml; for positive IL-6 expression) for 4 h. Total cellular RNA was harvested and IL-6 mRNA was semiquantitatively assessed by RT-PCR. GAPDH mRNA expression was used as internal control. PCR products were analyzed on Gel Red-stained agarose gels.
To investigate cell migration, we use the wound healing assay, which mimics epithelial cell restitution in vitro, a phenomenon where wounds or breaks in the epithelial lining are repaired by migration/proliferation of the surrounding epithelial cells. Cell migration towards wounded area after 20 h was enhanced when cells were incubated during the first hour with heme, the period when ROS generation induced by heme was maximal compared with control. Cell proliferation does not take an important role in this phenomenon once mitomycin pretreatment did not interfere with heme effects. Negative modulation of NADPHox activity suppressed heme-stimulated cell migration, proposing a role for NADPHox-derived ROS in activating cellular mobility apparatus. Silencing of NOX1 partially inhibited this response (Fig. 6B), suggesting that this isof orm is likely to be involved in heme-mediated migration, but it does not exclude the role of other NADPHox isoforms in heme chemokinetic effect.

Heme-induced cell proliferation was another biological response that was shown to be dependent on NADPHox-derived ROS signaling. Although our data do not present robust evidences that link heme activation of FAK and heme-induced cell proliferation, a recent work elegantly described FAK as a crucial regulator of proliferation and restitution in epithelial injury conditions (24), highlighting the role of FAK on IE homeostasis.

The onset of inflammation is orchestrated by several cytokines, which can act in an auto- or paracrine manner. It is depicted that cytokines like IL-6 are elevated in mucosal biopsies of inflammatory bowel disease patients (3, 31) and that IL-6 is critically involved in inflammation-associated tumorigenesis, once it drives proliferation of enterocytes and tumor initiating cells, in addition to protecting intestinal epithelial cell from apoptosis (5, 14). Heme expressively enhanced IL-6 mRNA expression in IEC 6 cells, which was maximal at 10 μM.

The enhanced mobility traits, cell proliferation, and IL-6 expression on IEC 6 cells tempt us to speculate that heme may be promoting cellular transformation. Notably, heme induced E-cadherin downregulation and this protein loss was depicted as a crucial promoter of epithelial-to-mesenchymal transition. Epithelial-to-mesenchymal transition is a cell transformation process that nowadays is considered a hallmark in aggravating chronic inflammatory disorders, preceding fibrosis. It is also described as an initiator of neoplasia (9).

In summary, our results show that heme is a potent activator of NADPHox on intestinal epithelial cells. NADPHox-dependent ROS generation evoked by heme regulates processes closely related to IE activation, such as cell migration, proliferation, and cytokine (IL-6) synthesis. These features are in agreement with the inflammatory scenario associated to hemolytic conditions of the intestine. The study of cellular aspects involved in the establishment of chronic inflammatory situations related to intestinal hemorrhage and the signaling pathways underlying them is important to comprehend and design better therapies to diseases affecting the intestinal mucosa.

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REFERENCES


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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


