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*J Immunol* 2004; 173:2023-2030; doi: 10.4049/jimmunol.173.3.2023

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Heme Inhibits Human Neutrophil Apoptosis: Involvement of Phosphoinositide 3-Kinase, MAPK, and NF-κB

Maria Augusta Arruda,* Adriano G. Rossi,† Marta S. de Freitas,* Christina Barja-Fidalgo,†* and Aurélio V. Graça-Souza†

High levels of free heme are found in pathological states of increased hemolysis, such as sickle cell disease, malaria, and ischemia reperfusion. The hemolytic events are often associated with an inflammatory response that usually turns into chronic inflammation. We recently reported that heme is a proinflammatory molecule, able to induce neutrophil migration, reactive oxygen species generation, and IL-8 expression. In this study, we show that heme (1–50 μM) delays human neutrophil spontaneous apoptosis in vitro. This effect requires heme oxygenase activity, and depends on reactive oxygen species production and on de novo protein synthesis. Inhibition of ERK and PI3K pathways abolished heme-protective effects upon human neutrophils, suggesting the involvement of the Ras/Raf/MAPK and PI3K pathway on this effect. Confirming the involvement of these pathways in the modulation of the antiapoptotic effect, heme induces Akt phosphorylation and ERK-2 nuclear translocation in neutrophils. Furthermore, inhibition of NF-κB translocation reversed heme antiapoptotic effect. NF-κB (p65 subunit) nuclear translocation and IκB degradation were also observed in heme-treated cells, indicating that free heme may regulate neutrophil life span modulating signaling pathways involved in cell survival. Our data suggest that free heme associated with hemolytic episodes might play an important role in the development of chronic inflammation by interfering with the longevity of neutrophils.


Severe hemolysis occurring during pathological states such as sickle cell disease, ischemia reperfusion, and malaria results in high levels of free heme (up to 20 μM). Under these conditions, the physiological mechanisms of removing free heme from the circulation, especially its binding to hemopexin, collapse, allowing nonspecific heme uptake and heme-catalyzed oxidation reactions (1, 2). We have recently reported that heme is a proinflammatory molecule able to induce neutrophil migration in vivo and in vitro (3). Interaction of free heme with human neutrophils leads to actin cytoskeleton reorganization and reactive oxygen species (ROS)2 generation through the induction of protein kinase C (PKC) activity, and also increases IL-8 expression (3). These findings attested to a prominent role for free heme in the development of inflammation associated with hemolytic diseases. In agreement, it has been shown that increased levels of heme in plasma are accompanied by a rise in cytokine and chemokine concentrations, as well as enhanced leukocyte function (4–8), events often associated with an inflammatory response that usually develops into chronic inflammation (9, 10).

Neutrophil apoptosis and subsequent clearance by phagocytes are critical to the resolution of acute inflammation (11, 12). These terminally differentiated cells constitute the first line of host defense against invading microorganism, being promptly recruited to inflamed loci in response to infection or tissue injury. Once activated, neutrophils are able to phagocytose, to release granular lytic enzymes and antimicrobial polypeptides into the phagolysosome, and to generate large amounts of ROS as well as reactive nitrogen species (13). Under normal conditions, neutrophils have a very short t1/2, being committed to programmed cell death (apoptosis). During this process, cell membrane integrity is maintained, avoiding the release of proinflammatory and potentially cytotoxic agents and the subsequent amplification of the inflammatory response. Apoptotic neutrophils also express surface markers that allow their recognition and nonphlogistic ingestion by professional phagocytes such as macrophages, or potential phagocytes such as fibroblasts and mesangial cells (14).

Culturing peripheral blood neutrophils in vitro reproducibly results in spontaneous apoptosis of >50% of the cells within 24 h. These apoptotic cells exhibit the classical features associated with this phenomenon, such as cytoplasmic condensation, phosphatidylserine exposure on the outer leaflet of the plasma membrane, and internucleosomal DNA cleavage, followed by chromatin condensation. Ultimately, it is the activation of caspases, a family of redox-sensitive cysteine proteases, that coordinates the structural dismantling of the cell (15).

Agents that promote neutrophil responsiveness, such as IL-8, GM-CSF, LPS, and leukotriene B4, also delay human neutrophil apoptosis (16–19). These stimuli promote neutrophil survival by modulating intracellular signaling pathways, including the MAPK, especially ERK and PI3K/Akt pathways (20, 21). Evidence has shown that activation of NF-κB pathway has a protective effect in several cell types, regulating the expression of antiapoptotic genes (22). In human neutrophils, NF-κB activation seems to regulate spontaneous apoptosis and the antiapoptotic effect of TNF-α, a

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0022-1767/04/$02.00

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†Departamento de Farmacologia, Instituto de Biologia, Universidade do Estado do Rio de Janeiro, and ‡Departamento de Bioquímica Médica, Instituto de Ciências Biológicas/Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; and §Centro de Inovação em Saúde, Universidade Federal do Rio de Janeiro, Av 28 de setembro 87-Vila Izabel, Rio de Janeiro, RJ, 20551-030 Brazil.

*Address correspondence and reprint requests to Dr. Christina Barja-Fidalgo, Departamento de Farmacologia, Instituto de Biologia, Universidade do Estado do Rio de Janeiro, Av 28 de setembro 87-Vila Izabel, Rio de Janeiro, RJ, 20551-030 Brazil. E-mail address: barja-fidalgo@uerj.br

1Abbreviations used in this paper: ROS, reactive oxygen species; BIM, bis-indoylmaleimide IV; CO, carbon monoxide; DPI, diphenyleneiodonium; HO, heme oxygenase; PDTC, pyrrolidine dithiocarbamate; PKC, protein kinase C; SnPPiX, tin protoporphyrin IX.
cytokine that exerts dual effects upon these cells. The modulation of all those pathways most likely regulates the balance between pro- and antiapoptotic proteins to influence neutrophil survival, especially the members of Bcl-2 family, which comprises both pro- and antiapoptotic members (23).

In the present study, we demonstrate that heme is able to prolong neutrophil life span by inhibiting apoptosis by a mechanism dependent on heme oxygenase (HO) activity and ROS generation. This effect depends on de novo protein synthesis and seems to be mediated by MAPK and PI3K/Akt pathways and involves NF-κB activation, indicating that heme may control neutrophil apoptosis through activation of these survival pathways. Our data support a role for heme as a proinflammatory mediator during hemolytic states, suggesting that this molecule is important in the development of chronic inflammation associated with hemolysis and hemoglobinemia.

Materials and Methods

Reagents

d Cycloheximide, diphenyleeniodionium (DPI), pyrrolidine dithiocarbamate (PDTC), and acapoycin (acetovanillone) were purchased from Sigma-Aldrich (St. Louis, MO). LYS49002, bis-indoylmaleimide IV (BIM), and PD98059 were from Calbiochem (San Diego, CA). Biliverdin and bilirubin were from Valeant Pharmaceuticals (Costa Mesa, CA). Tin protoporphyrin IX (SnPPIX) was from Porphyrin Products (Logan, UT). Human rF8– was a gift from F. Cunha (Faculdade de Medicina de Ribeirão Preto-Universidade de São Paulo, São Paulo, Brazil), and anti-IgG was donated by P. Bozza (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil).

Heme

Hemin (cell culture grade; Sigma-Aldrich) stock solutions were made in DMSO (culture grade; Sigma-Aldrich) and diluted in sterile PBS immediately before use. The final concentration of DMSO was kept lower than 0.01% for all assays.

Neutrophil isolation and culture

Neutrophils were isolated from EDTA (0.5%)-treated peripheral venous blood of healthy human volunteers by a method of dextran sedimentation and density gradient centrifugation, as previously described (24). Residual erythrocytes were removed by hypotonic lysis. Isolated neutrophils (5 × 10^6/ml) were incubated in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂ for 20 h, unless otherwise indicated. After all experimental conditions, >99% of cells were viable, as assessed by trypan blue dye exclusion.

Assessment of neutrophil apoptosis

Morphology. Cells were cytocentrifuged, stained with Diff-Quik, and counted under light microscopy (×1000) to determine the proportion of cells showing characteristic apoptotic morphology. At least 400 cells were counted per slide. The results were expressed as mean ± SD.

Annexin V-binding assay

To measure phosphatidylserine exposure on apoptotic cell surface, a flow cytometric assay using annexin V binding (annexin V-FLUOS; Roche Molecular Biochemicals, Mannheim, Germany) was performed. A working solution of annexin V-FLUOS was made from stock annexin V-FLUOS (0.1 µg/ml) diluted 1/3000 in HBSS supplemented with 2.5 mM CaCl₂. Neutrophils (20 µl of 5 × 10⁹/ml) were added to 200 µl of a working solution of annexin V before being assessed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed on associated CellQuest (BD Biosciences) software. All experiments were performed at least three times.

DNA electrophoresis

DNA fragmentation was analyzed, as previously described (25). Briefly, neutrophils (5 × 10⁶ cells/ml) were taken after 20 h and lysed with 500 µl of lysing buffer (0.2% Triton X-100; 100 and 1 mM EDTA, pH 7.4). Cell lysates were then centrifuged at 13,000 × g, and the supernatants containing fragmented DNA were separated from the pellet. The supernatants obtained were treated with 50 µl of 5 M NaCl and 500 µl of isopropanol and left for 12 h at ~70°C. DNA pellets were washed with 70% ethanol, air dried, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). Fragmented DNA was separated on a 1% agarose gel electrophoresis containing 1 µg/ml ethidium bromide. The products of DNA fragmentation were visualized and documented under UV light.

Subcellular localization

Cells were cytocentrifuged and fixed with paraformaldehyde (4%), and then permeabilized with 0.5% Triton X-100 in PBS for 20 min. The slides were incubated with rabbit polyclonal anti-Bad Ab (Santa Cruz Biotechnology, Santa Cruz, CA; 1/200) at 4°C overnight, incubated at room temperature for 1 h with biotin-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology; 1/1000), and finally incubated with FITC-conjugated streptavidin for 1 h at room temperature. Slides were then mounted using N-propylglycol solution before examination under an Olympus BX51 microscope (Melville, NY) equipped for epifluorescence. Images were analyzed using Adobe Photoshop software (Adobe Systems, San Jose, CA).

Preparation of cell extracts

To obtain the whole cell extracts to analyze IKBα degradation, an indicator of NF-κB pathway activation, neutrophils (5 × 10⁶ cells/ml) 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, 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 (Sigma-Aldrich).

Preparation of nuclear extracts

For the analysis of NF-κB nuclear translocation, neutrophils (5 × 10⁶ cells/ml) were incubated with heme (3 µM) for 1 or 2 h at 37°C in a 5% CO₂ atmosphere. Nuclear extracts were obtained, as described earlier (26). Briefly, cells were lysed in ice-cold buffer A (10 mM HEPEs, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF), and after a 15-min incubation on ice, Nonidet P-40 was added to a final concentration of 0.5% (v/v). Nuclei were collected by centrifugation (1,810 × g; 5 min at 4°C). The nuclear pellet was suspended in ice-cold buffer C (0.5% (w/v) HEPEs, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml leupeptin, and 20% (v/v) glycerol) and incubated for 30 min. Nuclear proteins were collected in the supernatant after centrifugation (12,000 × g; 10 min at 4°C), and the immunoblotting for nuclear NF-κB and histone (H3) content was performed, as described below.

Immunoprecipitation

Neutrophils (5 × 10⁶ cells/ml) were incubated with heme (3 µM) for 5, 15, and 30 min at 37°C in a 5% CO₂ atmosphere in the presence of LYS200942 (3 µM) or BIM (10 mM). Cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1.5 mM EDTA, Triton X-100 (1%, v/v), glyceral (10%, v/v), aprotinin (10 µg/ml), leupeptin (10 µg/ml), pepstatin (2 µg/ml), and 1 mM PMSF. Lysates (2 µg of protein/µl) were incubated overnight at 4°C with polyclonal anti-Akt1 (1/200; Santa Cruz Biotechnology) Ab. After this time, protein A/G-agarose (20 µg protein/µl; Santa Cruz Biotechnology) was added, and samples were incubated at 4°C in a rotary shaker for 2 h. The content of total and phosphorylated Akt on serine residues was analyzed by Western blot, as described below.

Western blot analysis

The total protein content in the cell extracts was determined by Bradford’s method (27). Cell lysates were denatured in sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 5% 2-ME, 10% glycerol, and 0.001% bromphenol blue) and heated in a boiling water bath for 3 min. Samples (30 µg of total protein) were resolved by 12% SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences). Rainbow markers (Amersham Biosciences, Upssala, Sweden) were run in parallel to estimate molecular weights. Membranes were blocked with Tween TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) containing 1% BSA and probed with polyclonal anti-Bcl-x, Santa Cruz Biotechnology (Santa Cruz; 1/500), polyclonal anti-Bad, Santa Cruz Biotechnology (1/500), monoclonal anti-phosphoserine (Sigma-Aldrich; 1/1000), polyclonal anti-Akt1 Ab (Santa Cruz Biotechnology; 1/1000), polyclonal anti-NF-κB Ab (Santa Cruz Biotechnology; 1/1000), polyclonal anti-iEκB Ab (Santa Cruz Biotechnology; 1/1000), or monoclonal anti-histone H3 Ab (Cell Signaling Technology, Beverly, MA; 1/1000). After extensive washing in Tween TBS, polyvinylidene difluoride sheets were incubated with biotin-conjugated anti-rabbit or anti-mice IgG (Santa Cruz Biotechnology, Amersham Biosciences, Uppsala, Sweden) and incubated with streptavidin for 1 h at room temperature. Slides were then mounted using N-propylglycol transparent solution before examination under an Olympus BX51 microscope (Melville, NY) equipped for epifluorescence. Images were analyzed using Adobe Photoshop software (Adobe Systems, San Jose, CA).
Biotechnology; 1:1000) Ab for 1 h and then incubated with HRP-conjugated streptavidin (CalTag Laboratories, Burlingame, CA; 1:1000). Immunoreactive proteins were visualized by 3,3′-diaminobenzidine (Sigma-Allenrich) staining. The bands were also quantified by densitometry using Scion Image Software (Scion, Frederick, MD).

Statistical analysis
Statistical significance was assessed by ANOVA, followed by Bonferroni’s t test, and p < 0.05 was taken as statistically significant.

Results
Heme delays human neutrophil apoptosis in vitro
The effect of heme in the modulation of spontaneous neutrophil apoptosis was simultaneously assessed by morphology (Fig. 1A), annexin V binding (Fig. 1B), and DNA electrophoresis (Fig. 1C). We have observed that incubation of human neutrophils with heme (3 μM) significantly delayed the apoptotic rate of these cells. Heme-mediated delay of apoptosis at 1–3 μM was similar to that observed with IL-8 (100 nM), a cytokine with a known ability to protect neutrophil from apoptosis. Heme was able to delay neutrophil apoptosis in vitro at all concentrations studied (1–50 μM; Fig. 2A), although its protective effects were less pronounced at the highest concentration used (50 μM). Because heme is able to evoke IL-8 synthesis in human neutrophils (3), we speculated whether the effect of free heme on neutrophil apoptosis would be mediated by an autocrine production of this antiapoptotic chemokine. Pretreatment of neutrophils with anti-IL-8 Ab did not inhibit the heme effect, indicating that heme inhibits human neutrophil apoptosis per se (Fig. 2B).

SnPPIX reverses heme effects on neutrophil apoptosis
We have previously observed that heme enhances HO-1 protein expression on neutrophils (A.V.G.-S., C. B.-F., and M. A. A., unpublished data). To investigate the involvement of HO in the inhibition induced by heme on neutrophil apoptosis, cells were co-incubated with SnPPIX (50 μM), a competitive inhibitor of this enzyme (Fig. 3). HO inhibition partially reversed heme effects on neutrophil apoptosis, suggesting that heme metabolites may play an important role on neutrophil survival under these conditions. However, neither biliverdin nor bilirubin (3 μM) protected human neutrophils from spontaneous apoptosis (51 and 53% of apoptotic cells, respectively), suggesting that other HO metabolites might be involved in heme effect.

Delaying of neutrophil apoptosis by heme requires ROS production
We have previously reported that heme evokes an oxidative burst in human neutrophils (3), which may lead to profound changes in the redox status of the cells. These alterations are known to modulate the activity of redox-sensitive proteins, including caspases.
formed in triplicate.

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nificantly inhibited the delay of apoptosis induced by heme (fig

/data shown are the results (mean ± SD) of three experiments, each performed in triplicate. * Indicates a significant difference (p < 0.001) between heme-treated and control neutrophils. #, Indicates that SnPPIX significantly inhibited the delay of apoptosis induced by heme (p < 0.05).

(28). In Fig. 4, we show that the incubation of cells with DPI and apocynin, two different NADPH oxidase inhibitors, reversed the inhibition of apoptosis in neutrophils treated with heme. These results suggest that alterations in the redox potential of these cells, mediated by NADPH oxidase activity, might be critical for the heme-induced antiapoptotic effect.

Heme-induced delay of neutrophil apoptosis requires protein synthesis

The balance between levels of pro- and antiapoptotic proteins has a pivotal role in the modulation of apoptosis. To determine the requirement of de novo protein synthesis on the heme-induced effect on neutrophil apoptosis, cells were pretreated with cyclo-

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–heme (1 × 10^7/ml) or in the presence of DMSO (23). Fig. 6 shows that heme induced the degradation of Bad, a proapoptotic Bcl-2 member, which reached lower levels 30 min after heme treatment. In contrast, heme induced the synthesis of the antiapoptotic protein Bcl-xL (Fig. 6B), reinforcing the regulation of prosurvival signaling by heme.

Heme delays neutrophil apoptosis via PI3K- and ERK-dependent pathways

Because the Ras/Raf/MAPK and PI3K pathways have been reported to be actively involved in regulating the antiapoptotic effect of other proinflammatory agents, we investigated the participation of these signaling pathways in heme-mediated delay of neutrophil apoptosis in vitro. Pretreatment of cells with the PI3K inhibitor LY294002 (3 μM) or the MEK1/2 inhibitor PD98059 (10 μM) totally reversed the delay of apoptosis promoted by heme (3 μM) and IL-8 (100 nM; Fig. 7), suggesting a pivotal role of these signaling pathways in the modulation of heme effect on neutrophils.

Heme activates ERK-2 nuclear translocation

The phosphorylation of ERK-1/2 regulatory sites can drive its translocation to the nucleus, where ERK exerts part of its biological activity. Confirming the involvement of the ERK pathway in

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of three experiments, each performed in triplicate. #, Indicates that DPI and apocynin significantly inhibited the delay of apoptosis induced by heme (p < 0.05).

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/heme (1–10 μM; Fig. 5). As we have previously observed that apocynin is cytotoxic at later time points (e.g., 20 h) (data not shown), we evaluated apoptosis after 5-h incubation in which there is no detectable cytotoxicity. Cycloheximide completely abolished the survival effect induced by heme on human neutrophil apoptosis, suggesting that the effects of heme rely on newly synthesized antiapoptotic proteins.

Heme induces Bad degradation and Bcl-xL expression on human neutrophils

The balance between the expression of anti- and proapoptotic proteins of Bcl-2 family has been shown to be a prominent feature on the control of apoptosis. As mature neutrophils exhibit a very short life span, the expression of proapoptotic Bcl-2 members is constitutively high, whereas antiapoptotic members’ levels are very low or not detectable (23). Fig. 6A shows that heme induced the degradation of Bad, a proapoptotic Bcl-2 member, which reached lower levels 30 min after heme treatment. In contrast, heme induced the synthesis of the antiapoptotic protein Bcl-xL (Fig. 6B), reinforcing the regulation of prosurvival signaling by heme.

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heme antiapoptotic effects, Fig. 8 shows that heme (3 μM), as well as IL-8 (100 nM), promoted ERK-2 translocation to the nucleus in human neutrophils, after 1 h of incubation, as detected by immunofluorescence microscopic analysis.

**Heme induces Akt activation**

Akt phosphorylation on serine residues is a key event in PI3K/Akt signaling cascade. Once activated, Akt is able to promote cell survival phosphorylating proapoptotic Bcl-2 family members, especially Bad, inducing their degradation by the proteasome (20). Fig. 9 shows that heme (3 μM) induced Akt phosphorylation on serine residues. This effect was highly significant after 5 min of incubation, peaking after 15 min and decreasing thereafter. The time course of Akt phosphorylation seems to be modulated hierarchically by PI3K and PKC. Although LY294002 (3 μM; □) inhibited only the early effect observed at 5 min after incubation with heme, BIM, a PKC inhibitor (10 nM; ◊), exclusively inhibited heme-induced Akt phosphorylation on later time points (15–30 min). These results strongly suggest that heme triggers Akt signaling in human neutrophils in a PI3K-dependent manner, but requires PKC activity to sustain this effect.

**Heme induces a redox-sensitive NF-κB activation**

It is well established that NF-κB modulates prosurvival signaling pathways, inhibiting apoptosis of several cell types (29–31). NF-κB activation requires IκB phosphorylation and degradation in the cytoplasm and subsequent translocation of NF-κB to the nucleus. This process can be regulated by redox-sensitive mechanisms. In Fig. 10A, we show that PDTC (100 nM), an antioxidant able to inhibit NF-κB activation, abrogated the delay of neutrophil apoptosis induced by heme, suggesting that NF-κB activation modulates heme-mediated cell survival.

To confirm that NF-κB activation occurs in neutrophils stimulated with heme, we evaluated the degradation of cytoplasmic IκB and the translocation of NF-κB p65 subunit to the nucleus in these

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**FIGURE 6.** Heme induces Bad degradation and Bcl-xL synthesis. Neutrophils (5 × 10^6/ml) were cultured in the absence or the presence of heme (3 μM). At the indicated time points, Bad (A) or Bcl-xL (B) protein expression was assessed by Western blot analysis, as described in Materials and Methods. Quantification of band OD is expressed in arbitrary units.

**FIGURE 7.** Inhibition of PI3K and ERK pathway abrogates the anti-apoptotic effect of heme on neutrophils. Neutrophils (5 × 10^6/ml) were cultured with heme (3 μM) or IL-8 (100 nM), in the absence (■) or in the presence of LY294002 (3 μM; □) or PD98059 (10 μM; □). After 20 h, cells were cytocentrifuged, and the number of apoptotic cells was determined microscopically. Data shown are the results (mean ± SD) of three experiments, each performed in triplicate. *, Indicates a significant difference (p < 0.01) between heme- or IL-8-treated and control neutrophils. #, Indicates that PD98059 and LY294002 significantly inhibited the delay of apoptosis induced by IL-8 and heme (p < 0.05). The results are expressed as mean ± SD.

**FIGURE 8.** Heme induces ERK nuclear translocation. Neutrophils (5 × 10^6/ml) were incubated for 1 h with medium alone (control; A), IL-8 (100 nM; B), or heme (3 μM; C). Cytocentrifuge preparations were fixed, and subcellular localization of endogenous ERK-2 was detected by staining with anti-ERK-2 Ab. Quantification of nuclear fluorescence intensity (arrowheads) is shown in D. Experiments were performed three times with similar results.
cells. After 1 h incubation with 3 μM heme, the levels of IκB significantly decreased in neutrophils treated with heme (Fig. 10B). This is followed by a significant increase of the NF-κB p65 subunit in nuclear extracts of neutrophils, which was more prominent after 2 h incubation with heme and comparable to that evoked by LPS (1 μg/ml; Fig. 10C). The translocation of NF-κB evoked by heme and LPS was inhibited when cells were treated with the NADPH oxidase inhibitor DPI, providing an additional evidence for the role of ROS on NF-κB activation.

Discussion

Chronic inflammation, an important feature in hemolytic diseases, involves an intense neutrophil activation accompanied by increased survival rate of these cells (12), events that may lead to an undesirable persistence of inflammation. In this study, we show, for the first time, that free heme increases neutrophil survival, providing evidence that this effect may contribute to the inflammatory process often associated with hemolytic episodes.

It was previously reported that erythrocytes are able to inhibit apoptosis of human neutrophils even when these cells are physically separated (32), suggesting the existence of a diffusible molecule able to retard neutrophil apoptosis. Our findings strongly suggest that the ability of erythrocytes to prevent neutrophil apoptosis may be directly linked to an increase in free heme concentrations released by lysed RBC.

A putative mechanism by which the organism can control the deleterious effect of large amounts of heme is through the HO activity. HO are ubiquitous enzymes able to catalyze the initial and rate-limiting step in the oxidative degradation of heme to bilirubin, producing equimolar amounts of biliverdin, free iron (Fe), and carbon monoxide (CO) (33). The expression of the inducible HO isoform (HO-1) is positively modulated by several inflammatory mediators and oxidative stress and also by heme itself. HO-1 has also been highlighted as one of the major inducible enzymes during the inflammatory response, especially during the resolution phase of inflammation (34). Its expression is associated with the inhibition of apoptosis of several cell types such as fibroblasts, endothelial cells, pancreatic β cells, and hepatocytes (35), and could be related to the action of one or more catalytic by-products generated by HO-1 (36). Recent reports have shown that the induction of HO-1 activity inhibited TNF-α-induced apoptosis in endothelial cell, via CO generation (37), whereas E-selectin and VCAM expression promoted by this stimulus is negatively modulated by HO-1 activity via bilirubin and Fe (38). In agreement

FIGURE 9. Heme induces Akt phosphorylation via PI3K and PKC. Neutrophils (5 × 10⁶/ml) were incubated with heme (3 μM) for the indicated time points in the absence or in the presence of LY294002 (3 μM; □) or BIM (10 nM; □). Cells were then harvested, cell extracts were immunoprecipitated with anti-Akt, and Western blots were performed for phosphoserine detection. Quantification of band OD is expressed in arbitrary units.

FIGURE 10. Heme-mediated delay of apoptosis requires NF-κB activation. A, Neutrophils (5 × 10⁶/ml) were incubated with heme (3 μM) in the absence (□) or in the presence (■) of PDTC (100 nM). After 20 h, cells were cytocentrifuged, and the number of apoptotic cells was determined microscopically. Data shown are the results (mean ± SD) of three experiments, each performed in triplicate. *, Indicates a significant difference (p < 0.01) between heme-treated and control neutrophils. #, Indicates that PDTC significantly inhibited the delay of apoptosis induced by heme (p < 0.05). B, Western blot analysis of whole cell extracts prepared from non-stimulated or heme-stimulated (3 μM) neutrophils for the indicated time points. C, Western blot analysis of nuclear extracts prepared from neutrophils stimulated or not with heme (3 μM) or LPS (1 μg/ml) for 1 or 2 h in the absence (■) or the presence of DPI (□). Quantification of band OD is expressed in arbitrary units.
with these findings, we showed that HO activity is, at least in part, required for heme effects upon human neutrophils because the delay of neutrophil apoptosis was partially reversed by a competitive inhibitor of this enzyme. However, neither biliverdin nor its metabolite, bilirubin, was able to influence on neutrophil life span, suggesting that probably CO and/or Fe might be the effector molecules generated by HO-1. The role of heme and HO-1 in modulating neutrophil spontaneous apoptosis points to a new and intriguing role of this system during the inflammatory response.

We have shown that heme is also able to trigger oxidative burst in neutrophils (3). This effect appears to be closely related to the antiapoptotic effect of heme because the inhibition of neutrophil apoptosis is reversed by DPI and apocynin, two NADPH oxidase inhibitors, providing evidence that alterations in redox potential mediate heme effects on human neutrophils. Data concerning the relationship between ROS generation, and the subsequent alterations in the redox potential, and the apoptotic process of different cells have been conflicting. Several reports have shown that antioxidants could both elicit or delay apoptosis (39, 40). As neutrophils can change their redox potential through their primary function of killing invading microorganisms, ROS generation as well as the presence of intracellular antioxidant molecules may interfere with the regulation of apoptosis in these cells. Although ROS and glutathione were shown to block caspase activity, both can inhibit, as well as trigger, apoptosis in human neutrophils, depending on the experimental conditions (41, 42). These data indicate that the regulation of neutrophil survival most likely involves a delicate balance in the redox status of the cell rather than the prevalence of intracellular oxidants or antioxidants. This may probably explain the reduced inhibition of neutrophil apoptosis when the cells were incubated with a higher concentration of heme (50 μM; Fig. 2), which was able to generate higher superoxide anion production (3).

Evidence has shown that the antiapoptotic effect of some agents require de novo protein synthesis, although it was recently reported that cAMP inhibits neutrophil apoptosis via a novel, reversible, and transcriptionally independent mechanism (43). Heme inhibition of neutrophil apoptosis was blocked by cycloheximide, suggesting that newly synthesized antiapoptotic proteins are likely to be involved in this effect. Neutrophils constitutively express proapoptotic proteins, including Bax, Bid, Bak, and Bad, while the expression of antiapoptotic Bcl members (Bcl-xL, A1, and Mcl-1) is very low or undetectable in resting cells (23). These antiapoptotic proteins are highly and transiently expressed when neutrophils are exposed to survival factors, such as IL-8 and GM-CSF, which act through the activation of MAPK, PI3K, NF-κB, and other distinct signaling pathways (20, 21, 23). We have shown that exposition of neutrophils to heme induced the degradation of Bad reducing after to 30 min to very low levels. In contrast, heme treatment increased the levels of Bcl-xL, corroborating to heme antiapoptotic effects. In parallel, we have also shown that the inhibition of ERK and PI3K pathways, which have been directly correlated to the expression of those proteins, successfully reversed heme-induced delay of neutrophil apoptosis. Involvement of ERK activation in the heme-mediated antiapoptotic effects was supported by the observation that heme induces ERK-2 nuclear translocation, a prominent feature of ERK activation.

As an additional parameter to evaluate the involvement of PI3K on heme antiapoptotic effect, we analyzed the phosphorylation of Akt on serine residues. Akt is the main downstream target of PI3K, which induce Akt translocation to the plasma membrane and subsequent phosphorylation by the PI3K-dependent kinase. Heme-induced Akt phosphorylation was dependent on PI3K and PKC activation. However, while PI3K activation is an essential and early event for Akt phosphorylation induced by heme, PKC appears to be involved in the late and sustained activation of Akt, which is probably associated with the modulation of antiapoptotic effect. A great deal of effort has been directed toward defining which PKC isoforms are involved in the regulation of the apoptotic process. It is now believed that classical (α, β1, β2, and γ) and atypical (ζ, μ, and λ) isoforms are associated with cell survival, whereas novel PKC isoenzymes (δ, ε, η, and θ) are considered proapoptotic (44, 45). Although the precise participation of different PKC member on heme antiapoptotic effect requires further investigation, our data suggest a prominent role for PI3K/Akt pathway on heme antiapoptotic effect, which is probably up-regulated by PKC.

Many inflammatory mediators regulate gene expression in target cells by influencing the activity of transcription factors, especially NF-κB, to evoke their proinflammatory response (46). NF-κB comprises a family of transcription factors that act to regulate genes involved in a variety of events such as inflammatory and immune responses, apoptosis, proliferation, and differentiation (47–50). NF-κB regulation seems to be highly cell specific and redox sensitive (51–53). Moreover, Asehnoune et al. (54) have recently shown that the antioxidant N-acetylcysteine and α-tocopherol prevented LPS-induced nuclear translocation of NF-κB on early events in TLR4 signaling. We have observed that heme activates NF-κB in polymorphonuclear cells, inducing its translocation to the nucleus. The inhibition of the heme-induced antiapoptotic effect by PDTC suggests that heme induces redox-sensitive NF-κB activation. The evidence that the inhibitor DPI impairs both heme- and LPS-induced NF-κB nuclear translocation points to a prominent role of ROS generated by NADPH oxidase on this phenomenon. Furthermore, this is the first report showing the importance of NADPH oxidase-derived ROS in NF-κB activation on human neutrophils. The involvement of NF-κB on heme cytoprotective effects reinforces the close relationship between activation status and enhanced granulocyte life span already observed for other antiapoptotic stimuli (52).

Taken together, our data strongly suggest that the protective effect of heme on human neutrophil apoptosis is modulated by PI3K and MAPK pathways and involves NF-κB activation in an NADPH oxidase-dependent manner. The activation of those pathways might lead to the transcription of HO-1 (55), whose activity could modulate the heme-induced antiapoptotic effect, among other antiapoptotic proteins, as previously discussed.

In summary, we are presenting novel data showing that free heme is able to delay human neutrophil apoptosis in vitro at concentrations found during hemolytic events in vivo. This effect is dependent on HO activity and ROS generation, requires de novo protein synthesis, and is modulated through PI3K, ERK, and NF-κB pathways. Furthermore, the data suggest that heme may contribute to the development of chronic inflammation associated with hemolytic episodes by delaying apoptosis and promoting functional longevity of neutrophils. The understanding of this process can lead to the establishment of new strategies to ameliorate tissue damage associated with severe hemolysis.

Acknowledgments

We thank Dr. I. M. Fierro for the critical reading of this manuscript. We also thank Dr. F. Q. Cunha for supplying human rIL-8, and Dr. P. Bozza for anti-IL-8 mAb, as well as Carla Gregório and Pedro Barcellos de Souza for excellent technical assistance.

References

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