Heme Induces Neutrophil Migration and Reactive Oxygen Species Generation through Signaling Pathways Characteristic of Chemotactic Receptors*

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Hemolysis or extensive cell damage can lead to high concentrations of free heme, causing oxidative stress and inflammation. Considering that heme induces neutrophil chemotaxis, we hypothesize that heme activates a G protein-coupled receptor. Here we show that similar to heme, several heme analogs were able to induce neutrophil migration in vitro and in vivo. Porphyrins, molecules lacking the vinyl groups in their rings, were not chemotactic for neutrophils and selectively inhibited heme-induced migration. Moreover, migration of neutrophils induced by heme was abolished by pretreatment with pertussis toxin, an inhibitor of Go inhibitory protein, and with inhibitors of phosphoinositide 3-kinase, phospholipase Cβ, mitogen-activated protein kinases, or Rho kinase. The induction of reactive oxygen species by heme was dependent of Go inhibitory protein and phosphoinositide 3-kinase and partially dependent of phospholipase Cβ, protein kinase C, mitogen-activated protein kinases, and Rho kinase. Together, our results indicate that heme activates neutrophils through signaling pathways that are characteristic of chemotactic molecules and suggest that mesoporphyrins might prove valuable in the treatment of the inflammatory consequences of hemorrhagic and hemolytic disorders.

The immune/inflammatory responses triggered by molecules of infectious agents or host origin are amplified by cytokines, lipid mediators, and reactive oxygen species (1). Diseases of increased intra- and extravascular hemolysis or extensive cell damage can lead to high levels of free heme (2–4). As the pro-thetic moiety of inactive apo-heme proteins, heme provides a wide range of biological functions determined in part by the polypeptide associated to it (5). Once released from heme proteins, free heme can amplify the cell damage and the inflammatory response (6). A hallmark of inflammation is the recruitment of leukocytes out of the vasculature to tissues. This process depends on the interplay of selected molecules on leukocytes and endothelial cells occurring in several stages, the so-called three steps paradigm of cell migration (7). Heme seems to affect this process in several ways: (a) inducing cell adhesion molecule expression on endothelial cells; (b) increasing vascular permeability; (c) enhancing chemokine expression and secretion; (d) inducing migration of leukocytes, especially neutrophils (6, 8). It has been assumed and widely accepted that the mechanism by which heme exerts its damaging effects relies on its amphipathic nature, allowing heme to intercalate the cell organelle membranes, and on its pro-oxidant effects (6, 9, 10). The induction of neutrophil migration by heme is dependent on protein kinase C activation (8), and heme is able to delay neutrophil apoptosis in vitro dependently of phosphoinositide 3-kinase (PI3K), extracellular-signaling kinase (ERK), and NF-κB pathways (11).

The ability of heme to induce neutrophil migration in vitro indicates that heme is a chemotactant. To date, the nature of neutrophil chemotactic molecules includes a variety of peptides (for example, formyl-Met-Leu-Phe, C3a and C5a, and CXC chemokines) and lipids (for example, PAF and LTB₄), which are, without exception, ligands of seven transmembrane receptors coupled to a Go inhibitory (Goi) protein sensitive to pertussis toxin (PTX) (12). Binding and activation of these receptors activate Goi protein, PI3K, and MAPKs, stimulating neutrophil migration (13, 14). Thus, we hypothesize that heme acts on neutrophil migration by activating a Goi-coupled receptor. In this study, we show the structural requirements by which porphyrins affect neutrophil migration in vitro and promote inflammation in vivo. Several heme analogs were able to induce neutrophil migration, whereas mesoporphyrins, molecules lacking the vinyl groups in their rings, were not chemotactic for neutrophils.

The abbreviations used are: PI3K, phosphoinositide 3-kinase; ERK, extracellular-signaling kinase; MAPK, mitogen-activated protein kinase; GPCR, G protein-coupled receptor; LTB₄, leukotriene B₄; LPS, lipopolysaccharide; PKC, protein kinase C; PAF, platelet-activating factor; PLCβ, phospholipase Cβ; PPIX, protoporphyrin IX; PTX, pertussis toxin; RhoK, Rho kinase; ROS, reactive oxygen species; TLR4, Toll-like receptor 4.
trophils and selectively inhibited the migration induced by heme. The neutrophil migration and ROS production induced by heme were inhibited by pertussis toxin, thus indicating the involvement of a Gαi protein. The use of selective inhibitors indicates that heme activates neutrophils through signaling pathways that are characteristic of chemoattractant molecules. Together, these results suggest that heme activates neutrophils through activation of a G protein-coupled receptor (GPCR).

EXPERIMENTAL PROCEDURES

Mice—C57BL/6 mice supplied by the breeding facilities of Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, Brazil) were housed in temperature-controlled rooms and given water and food ad libitum until use. Care and handling of the animals were in accordance with the National Institute of Health Guidelines.

Materials—Heme and analog molecules were purchased from Porphyrin Products (Logan, UT). Pertussis toxin, PD98059, SB203580, LY294002, U-73122, Y-27632, and bis-indoylmaleimide IV were from Calbiochem. LPS (O111:B4), thioglycollate, and calphostin C were from Sigma-Aldrich. LTB₄ and PAF were from Cayman Chemical (Ann Arbor, MI).

Heme and Analogs—For in vivo neutrophil migration experiments, heme and analog molecule stock solutions (3 mg/ml) were made in NaOH and diluted in endotoxin-free saline solution immediately before use. For in vitro neutrophil chemotaxis experiments, heme and analog molecule stock solutions (5 mM) were made in NaOH and diluted in RPMI 1640 medium immediately before use. All these procedures were performed in the dark to avoid generation of free radicals.

Human Neutrophil Isolation—Neutrophils were isolated from heparin-treated peripheral venous blood of healthy human volunteers using a gradient of Ficoll (Lymphoprep, Nycomed A/S). Erythrocytes were removed by hypotonic lysis, and neutrophils were resuspended in RPMI 1640 medium. Neutrophil purity and viability were always higher than 97 and 99%, respectively.

Neutrophil Chemotaxis—Chemotaxis was assayed in a 96-well chemotaxis microplate (ChemoTx System, NeuroProbe, Inc.) or in a Transwell system (Corning) using 5-μm polycarbonate membrane. Inducers of neutrophil chemotaxis were added to the bottom wells in RPMI 1640 medium in the presence of 1% fetal calf serum. Neutrophils suspended in RPMI 1640 medium (5 × 10⁴ cells/50 μl) were added to the top wells and incubated for 2 h at 37 °C under 5% CO₂ atmosphere. Following incubation, the migrated neutrophils were collected and counted on Neubauer chambers. Neutrophil migration toward RPMI 1640 medium alone (random chemotaxis) was used as negative control, and migration toward 1 nM leukotriene B₄ was used as positive control. To evaluate the involvement of inhibitory G protein, PI3K, MAPK (p38 and ERK), phospholipase Cβ (PLCβ), and Rho kinase (RhoK) on heme-induced neutrophil chemotaxis, the cells were pretreated with selective inhibitors at 37 °C under 5% CO₂ atmosphere. The trypan blue exclusion assay was used to evaluate the viability of cells treated with these inhibitors, and at the end of incubation, the cellular viability was always higher than 97%. The chemotactic indices were calculated as the ratio of the number of migrated cells to the total number of cells. Data are representative of two independent experiments, performed in triplicate for each sample and expressed as mean ± S.E. *p < 0.05 when compared with negative control.
Heme Activates Neutrophils throughout Gα, Protein and PI3K

FIGURE 2. Heme analogs are chemotactic for neutrophils in vivo. Mice were injected intraperitoneally with different doses of heme (3–300 μg/cavity) (A). After 4 h, animals were killed, and differential counts in the peritoneal fluid were determined. B, mice were injected with heme (30 μg/cavity). After 1.5, 4, 8, 12, 24, and 48 h, animals were killed, and cell counts in the peritoneal fluid were determined. Sal, saline. C, PPIX (3–300 μg/cavity) was injected intraperitoneally in mice. Mice received an injection of Zn-PPIX, Sn-PPIX, Mn-PPIX, and Co-PPIX (100 μg/cavity) (D) or mesoporphyrin (Meso) IX, iron mesoporphyrin IX, and palladium mesoporphyrin IX (100 μg/cavity) (E). The control group received endotoxin-free saline solution. Four hours later, differential counts in the peritoneal fluid were determined. Data are representative of two independent experiments (n = 5 for each group of treatment) and expressed as the mean ± S.E. of the number of cells in the peritoneal fluid. *, p < 0.05 when compared with saline-treated group.

migrated neutrophils in chemoattractant-containing wells divided by the number of neutrophils that migrated to RPMI 1640 medium alone.

Assay of Reactive Oxygen Species Generation—Human neutrophils (10⁶ cells/microtub) were pretreated with selective inhibitors of Gα, protein, PI3K, PLCβ, RhoK, and MAPKs. After that, cells were stimulated with 30 μM heme for 1 h at 37 °C under 5% CO₂ atmosphere and incubated with 2 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) in Hanks’ balanced saline solution for 30 min at 37 °C under 5% CO₂. ROS production was analyzed by flow cytometry, using FACSCalibur (BD Biosciences).

Acute Peritonitis—Acute peritonitis was induced by an intraperitoneal injection of heme (3–300 μg/cavity), protoporphyrin IX (PPIX) (3–300 μg/cavity), Zn-, Sn-, Mn-, Co-PPIX, mesoporphyrin IX, iron and palladium mesoporphyrin. X (100 μg/cavity) in a volume of 200 μl. The control group received an intraperitoneal injection of endotoxin-free saline solution in a same volume. After 4 h of injection, the animals were killed under ether anesthesia, and their peritoneal cavities were rinsed with 3 ml of cold phosphate-buffered saline. Total leukocytes in the peritoneal fluid were determined on Neubauer chambers after dilution in Turk solution. Differential counting of leukocytes was carried out on Diff-Quik (Baxter Travenol Laboratories)-stained slices.

Statistical Analysis—Data are presented as mean ± S.E. Results were analyzed using a statistical software package (GraphPad Prism 4). Statistical differences among the experimental groups were evaluated by analysis of variance with Newman-Keuls correction or with the Student’s t test. The level of significance was set at p < 0.05.

RESULTS

Heme Analogs Are Chemotactic for Neutrophils in Vitro—We took advantage of the existence of several heme analogs to determine the structural determinants on heme required to neutrophil migration. Initially, we characterized the dose-response effect of heme on human neutrophil migration in vitro. A typical bell-shape curve is observed, with the dose of 3 μM heme consistently inducing the best response (Fig. 1A). The lack of the atom of iron did not affect the ability of PPIX to cause a concentration-dependent chemotactic effect on neutrophils, similar to the observed effect of heme (Fig. 1B). This result indicates that the porphyrin ring is the essential part of the heme molecule involved in the activation of neutrophils. On the other hand, mesoporphyrin IX was unable to induce the migration of neutrophils in vitro (Fig. 1C). Mesoporphyrin IX has a similar structure of PPIX but lacks the two vinyl groups, which are substituted by two ethyl groups. These results indicate that the induction of neutrophil migration by heme and its analogs requires the presence of the two vinyl groups but not the atom of iron.

Heme Analogs Are Chemotactic for Neutrophils in Vivo—Intraperitoneal injection of heme induced in a dose-dependent fashion the recruitment of neutrophils 4 h after injection (Fig. 2A). The effect of heme (30 μg/cavity) in vivo was selective to neutrophils, and no increase of mononuclear cells recruitment
was observed from 90 min to 48 h (Fig. 2B). Similar to heme, PPIX caused a dose-dependent recruitment of neutrophils to the peritoneal cavity (Fig. 2C). Furthermore, analogs of heme with a metal substitution were still capable of recruiting neutrophils (Fig. 2D). Again, similar to the in vitro chemotaxis assay, mesoporphyrins were not able to induce neutrophil migration in vivo (Fig. 2E).

**Mesoporphyrin IX Inhibits Heme-induced Neutrophil Migration in Vitro**—On the basis of the result showing that mesoporphyrins were not able to induce neutrophil migration in vitro, we investigated whether the mesoporphyrin IX could act as an antagonist of heme. When heme was added together with increasing concentrations of mesoporphyrin IX, an almost complete inhibition of the heme-induced neutrophil migration was observed (Fig. 3A), suggesting that mesoporphyrins might act as antagonists of heme. In addition, when PAF, a neutrophil chemoattractant molecule, was added with mesoporphyrin IX, no inhibition was observed (Fig. 3B).

**Mesoporphyrin IX Inhibits the Neutrophil Recruitment Induced by Heme**—Hemorrhagic episodes are associated with an inflammatory process, characterized by a prominent leukocyte infiltration, which can cause tissue injury (15–17). Blood or heme injection caused neutrophil accumulation into the peritoneal cavity of mice, and pretreatment with mesoporphyrin IX abolished this recruitment (Fig. 4A). Treatment with mesoporphyrin IX did not interfere with neutrophil migration induced by LPS or thioglycollate (Fig. 4B), thus suggesting that the inhibitory effect of mesoporphyrin IX is selective to heme.

**The Effect of Heme on Neutrophil Migration Is Dependent on Signaling Pathways Characteristic of Chemoattractants**—We used PTX to define the involvement of 

**DISCUSSION**

Several studies indicated the existence of cell surface proteins in mammalian cells able to bind heme, and recent publications identified specific cell surface proteins that directly interact with heme (20–24). Heme binds to Slo1 channels and inhibits transmembrane K⁺ currents by decreasing the frequency of channel opening (23). This effect occurred when heme was applied at the intracellular side of the cell membrane but was ineffective when applied to the channels from the extracellular side. Similarly, the cell surface receptor for feline leukemia virus is an exporter of intracellular heme, affecting erythropoiesis (22), whereas a heme importer has been recently identified in intestine epithelial cells (24).
Heme Activates Neutrophils throughout Gαi Protein and PI3K

We also demonstrated that heme induces the secretion of tumor necrosis factor-α on macrophages and dendritic cells dependently ofTLR4, CD14, and myeloid differentiation protein-88 (MYD88) (25). These studies clearly demonstrate that heme requires defined membrane receptors to affect selected cell functions.

The agonistic and antagonistic effects of heme and its analogs, together with the inhibitory effect of PTX, support the concept of a physical interaction of the porphyrin ring with a GPCR. PPIX, which lacks a metal atom, as well as porphyrins with metal substitutions, were all able to induce neutrophil migration. Previous studies demonstrated that PPIX and other metal porphyrins are unable to affect the K+ current (23) or the secretion of tumor necrosis factor-α by macrophages (25). In these situations, the iron is likely to be involved in the interaction of heme with the Slo1 channel and with TLR4. These results suggest that a putative GPCR involved in porphyrin recognition by neutrophils would be more promiscuous in its activating properties by these molecules when compared with the ion channel or TLR4.

Mesoporphyrin IX had a dose-dependent antagonistic effect on heme-induced neutrophil migration in vitro, suggesting that this analog competes by the same binding site of heme. This effect of mesoporphyrin IX seems to be selective to heme since no inhibition of neutrophil migration was observed when PAF was used as neutrophil chemotactant. Furthermore, mesoporphyrin IX inhibited the migration of neutrophils to the peritoneal cavity induced by blood or heme but not the recruitment induced by LPS or thioglycollate, again supporting the contention that mesoporphyrin IX is a selective antagonist of heme. These results also suggest that migration of neutrophils induced by hemorrhagic episodes might be dependent on heme activating a GPCR. The vinyl groups are known to be important to the association of heme with heme proteins, including cytochrome c (26). It has been suggested that the vinyl groups of heme form thioether bonds with cysteine residues of cytochrome c. Moreover, the vinyl groups are highly hydrophobic, thus facilitating the interaction of the porphyrin ring with selected protein regions or with cell membranes. Future studies are required to define the molecular mechanism involved in mesoporphyrin IX inhibitory effects on heme-induced neutrophil activation.

It has been previously shown that neutrophil migration induced by heme requires PKC activity (8). We now extended this observation by showing that besides the requirement of Gαi, heme also activates PI3K, PLCβ, RhoK, ERK, and p38 to induce the migration of neutrophils. These pathways were analyzed because they are known to be downstream of seven transmembrane receptors coupled to Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein (19). In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemotactants such as formyl-Met-Leu-Phe, LTB4, C5a, and interleukin-8 activate Gαi protein. In fact, various neutrophil chemo-
Heme Activates Neutrophils throughout Gαi Protein and PI3K

scenario: heme activates neutrophil Gαi protein, inducing migration and ROS production, which in turn activates adjacent cells through PKC, thus amplifying the inflammatory response. It will be important to further define and discriminate the signaling events involved in the effects of heme on neutrophil physiology.

Our results, however, do not prove the requirement of a GPCR for the observed heme effects on neutrophils. Because of its amphipathic nature, heme could also intercalate the lipid membrane activating the Gαi protein directly inside the cell. In fact, G proteins and ion channels, among other membrane proteins, are mechanosensitive to specific amphipathic molecules (29, 30). In addition, it has recently been shown that human neuroglobin, a heme protein, is able to bind directly to the α subunit of the Gαi protein in vitro (31). We are currently investigating the requirement and identity of a putative GPCR for the effect of heme on neutrophil chemotaxis and activation.

During hemolysis, free vascular hemoglobin became associated with its scavenger haptoglobin, which in turn binds to CD163 on macrophages, promoting the endocytosis of the complex (32). However, hemoglobin can be rapidly converted into methemoglobin, which liberates heme. The pro-oxidant and pro-inflammatory nature of heme requires an efficient system of transport and degradation to avoid cellular damage. Several species produce heme binding plasma proteins, such as hemopexin or albumin, which bind and remove the intravascular free heme (33). However, when large amounts of heme accumulate, the scavengers get overwhelmed or are unable to reach them, as in the case of extravascular deposition of heme (2–4). In the present study, we used concentrations of heme, in vitro and in vivo, compatible with concentrations observed during hemorrhagic and hemolytic episodes, indicating that the mechanism of neutrophil recruitment and activation we are describing might be clinically relevant. Interestingly, a recent study indicates that heme is critically involved in the pathogenesis of cerebral malaria affecting the blood-brain barrier integrity and causing neuro-inflammation (34). Considering the role of neutrophils in the pathogenesis of cerebral malaria, we speculate that the mechanism by which heme potentiates neuro-inflammation might involve activation of Gαi protein in these leukocytes. The molecular mechanisms of heme-induced inflammation during cerebral malaria require further characterization.

In conclusion, our results suggest that heme released in situations of hemorrhage or hemolysis acts as a chemoattractant, which can stimulate neutrophil migration and ROS generation through Gαi protein and selected downstream signaling pathways, thus amplifying the inflammatory response. The characterization of mesoporphyrins as selective antagonists of heme will allow defining the role of endogenous heme on inflammatory conditions triggered by intra- and extravascular hemolysis or extensive cell damage as in sickle cell anemia, trauma, malaria, hemorrhagic fevers, and leptospirosis. Furthermore, mesoporphyrins might prove valuable in the treatment of the inflammatory consequences of hemorrhagic and hemolytic disorders.

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REFERENCES

1. Nathan, C. (2002) Nature 420, 846–852
2. Muller-Eberhard, U., Javid, J., Liem, H. H., Hanstein, A., and Hanna, M. (1968) Blood 32, 811–815
3. Jacob, H. S. (1994) J. Lab. Clin. Med. 123, 808–816
4. Jeney, V., Balla, J., Yachie, A., Varga, Z., Vercellotti, G. M., Eaton, J. W., and Balla, G. (2002) Blood 100, 879–887
5. Dawson, J. H. (1988) Science 240, 433–439
6. Wagener, F. A., Volk, H. D., Willis, D., Abraham, N. G., Soares, M. P., Adema, G. J., and Figdor, C. G. (2003) Pharmacol. Rev. 55, 551–571
7. Springer, T. A. (1994) Cell 76, 301–314
8. Graça-Souza, A. V., Arruda, M. A., Freitas, M., Barja-Fidalgo, C., and Oliveira, P. (2002) Blood 99, 4160–4165
9. Balla, J., Jacob, H. S., Balla, G., Nath, K., Eaton, J. W., and Vercellotti, G. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9285–9289
10. Balla, J., Balla, G., Jeney, V., Kakuk, G., Jacob, H., and Vercellotti, G. (2000) Blood 95, 3442–3450
11. Arruda, M. A., Rossi, A. G., Freitas, M. S., Barja-Fidalgo, C., and Graça-Souza, A. V. (2004) J. Immunol. 173, 2023–2030
Heme Activates Neutrophils throughout Goi Protein and PI3K

12. Rot, A., and von Andrian, U. (2004) *Annu. Rev. Immunol.* 22, 891–928
13. Heit, B., Tavener, S., Raharjo, E., and Kubes, P. (2002) *J. Cell Biol.* 159, 91–102
14. Coxon, P. Y., Rane, M. J., Uriarte, S., Powell, D. W., Singh, S., Butt, W., Chen, Q., and McLeish, K. R. (2003) *Cell. Signal.* 15, 993–1001
15. Davenport, R. D., Strieter, R. M., Standiford, T. J., and Kunkel, S. L. (1990) *Blood* 76, 2439–2442
16. Davenport, R. D., and Kunkel, S. L. (1994) *Transfus. Med. Rev.* 8, 157–168
17. Haeger, M., Unander, B., Andersson, A., Tarkowski, A., Arnestad, J. P., and Bengtsson, A. (1996) *Acta Obstet. Gynecol. Scand.* 75, 695–701
18. Nishida, M., Maruyama, Y., Tanaka, R., Kontani, K., Nagao, T., and Kurose, H. (2000) *Nature* 408, 492–495
19. Neves, S. R., Ram, P. T., and Iyengar, R. (2002) *Science* 296, 1636–1639
20. Galbraith, R. A. (1990) *J. Hepatol.* 10, 305–310
21. Worthington, M. T., Cohn, S. M., Miller, S. K., Luo, R. Q., and Berg, C. L. (2001) *Am. J. Physiol.* 280, G1172–G1177
22. Quigley, J. G., Yang, Z., Worthington, M. T., Phillips, J. D., Sabo, K. M., Sabath, D. E., Berg, C. L., Sassa, S., Wood, B. L., and Akhowitz, J. L. (2004) *Cell* 118, 757–766
23. Tang, X., Xu, R., Reynolds, M., Garcia, M., Heinemann, S., and Hoshi, T. (2003) *Nature* 425, 531–535
24. Shayeghi, M., Latunde-Dada, G. O., Oakhill, J. S., Laftah, A. H., Takeuchi, K., Halliday, N., Khan, Y., Warley, A., McCann, F. E., Hider, R. C., Frazer, D. M., Anderson, G. J., Vulpe, C. D., Simpson, R. J., and McKie, A. T. (2005) *Cell* 122, 789–801
25. Figueiredo, R. T., Fernandez, P. L., Moura-Sa, D. S., Dutra, F. F., Alves, L. S., Oliveira, M. F., Oliveira, P. L., Graça-Souza, A. V., and Bozza, M. T. (2007) *J. Biol. Chem.* 282, 20221–20229
26. Daltrop, O., Allen, I., Willis, A., and Ferguson, S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 7872–7876
27. Kass, G. E. N., Duddy, S. K., and Orrenius, S. (1989) *Biochem. J.* 260, 499–507
28. Gopalakrishna, R., and Anderson, W. B. (1991) *Arch. Biochem. Biophys.* 285, 382–387
29. Gudi, S. R., Lee, A. A., Clark, C. B., and Frangos, J. A. (1998) *Am. J. Physiol.* 274, C1424–C1428
30. Suchyna, T. M., Besch, S. R., and Sachs, F. (2004) *Phys. Biol.* 1, 1–18
31. Wakasugi, K., Nakano, T., and Morishima, I. (2003) *J. Biol. Chem.* 278, 36505–36512
32. Kristiansen, M., Graversen, J. H., Jacobsen, C., Sonne, O., Hoffman, H. J., Law, S. K., and Moestrup, S. K. (2001) *Nature* 409, 198–201
33. Muller-Eberhard, U., and Fraig, M. (1993) *Am. J. Hematol.* 42, 59–62
34. Pamplona, A., Ferreira, A., Balla, J., Jeney, V., Balla, G., Epiphanio, S., Chora, A., Rodrigues, C. D., Gregoire, I. P., Cunha-Rodrigues, M., Portugal, S., Soares, M. P., and Mota, M. M. (2007) *Nat. Med.* 13, 703–710