Hemolysis-induced lethality involves inflammasome activation by heme


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The increase of extracellular heme is a hallmark of hemolysis or extensive cell damage. Heme has prooxidant, cytotoxic, and inflammatory effects, playing a central role in the pathogenesis of malaria, sepsis, and sickle cell disease. However, the mechanisms by which heme is sensed by innate immune cells contributing to these diseases are not fully characterized. We found that heme, but not porphyrins without iron, activated LPS-primed macrophages promoting the processing of IL-1β dependent on nucleotide-binding domain and leucine rich repeat containing family, pyrin domain containing 3 (NLRP3). The activation of NLRP3 by heme required spleen tyrosine kinase, NADPH oxidase-2, mitochondrial reactive oxygen species, and K+ efflux, whereas it was independent of heme internalization, lysosomal damage, ATP release, the purinergic receptor P2X7, and cell death. Importantly, our results indicated the participation of macrophages, NLRP3 inflammasome components, and IL-1R in the lethality caused by sterile hemolysis. Thus, understanding the molecular pathways affected by heme in innate immune cells might prove useful to identify new therapeutic targets for diseases that have heme release.

Significance

Heme causes inflammation in sterile and infectious conditions, contributing to the pathogenesis of hemolytic diseases, subarachnoid hemorrhage, malaria, and sepsis, but the mechanisms by which heme operates in different conditions are not completely understood. Blocking the prooxidant effects of heme protects cells from death and prevents tissue damage and lethality in models of malaria and sepsis (12, 13, 15). Importantly, two recent studies demonstrated the pathogenic role of heme-induced TLR4 activation in a mouse model of sickle cell disease (29, 30). These results highlight the great potential of understanding the molecular mechanisms of heme-induced inflammation and cell death as a way to identify new therapeutic targets.
Hemolysis and heme synergize with microbial molecules for the induction of inflammatory cytokine production and inflammation in a mechanism dependent on ROS and Syk (24). Processing of pro–IL-1β is dependent on caspase-1 activity, requiring assembly of the inflammasome, a cytosolic multiprotein complex composed of a NOD-like receptor, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1 (31–33). The most extensively studied inflammasome is the nucleotide-binding domain and leucine rich repeat containing family, pyrin domain containing 3 (NLRP3). NLRP3 and pro–IL-1β expression are increased in innate immune cells primed with NF-κB inducers such as TLR agonists and TNF (34, 35). NLRP3 inflammasome is activated by several structurally nonrelated stimuli, such as endogenous and microbial molecules, pore-forming toxins, and particulate matter (34, 35). The activation of NLRP3 involves K+ efflux, increase of ROS and Syk phosphorylation. Importantly, critical roles of NLRP3 have been demonstrated in a vast number of diseases (34, 36). We hypothesize that heme causes the activation of the inflammasome and secretion of IL-1β. Here we found that heme triggered the processing and secretion of IL-1β dependently on NLRP3 inflammasome in vitro and in vivo. The activation of NLRP3 by heme was dependent on Syk, ROS, and K+ efflux, but independent of lysosomal leakage, ATP release, or cell death. Finally, our results indicated the critical role of macrophages, the NLRP3 inflammasome, and IL-1R to the lethality caused by sterile hemolysis.

Results

Heme Induces IL-1β Processing and Caspase-1 Activation. To examine the ability of heme to act as a second signal, triggering inflammasome activation, we pretreated bone-marrow–derived macrophages (BMMs) with LPS (signal 1) and stimulated with heme. Heme caused a dose-dependent secretion of IL-1β, including the processed form (Fig. 1A). In the absence of prestimulation with LPS, heme was unable to induce the secretion of IL-1β by BMMs or by thioglycollate-elicited peritoneal macrophages (Fig. S1 A and B). We have previously shown that the synergistic effect of heme on cytokine production triggered by microbial molecules only occurs in the presence of serum (24). Thus, we tested the requirement of serum on heme-induced IL-1β processing. Using BMMs, we observed that heme caused a robust processing of IL-1β and caspase-1 in the presence of serum, but only a modest processing in its absence, whereas ATP was highly effective in activating the inflammasome in both conditions (Fig. 1B). We measured lactate dehydrogenase (LDH) in the supernatants of macrophages stimulated with heme to quantify cell death. As expected, low amounts of LDH were present in the supernatants of BMMs stimulated with heme in the presence of serum, whereas high concentrations of LDH were observed in macrophages stimulated with heme without serum (Fig. 1C). These results indicate that macrophage death is not involved in inflammasome activation by heme. Interestingly, heme induced IL-1β processing by thioglycollate-elicited peritoneal macrophages both in the presence and absence of serum (Fig. S1B). Next, we analyzed the effect of heme on in vivo production of IL-1β. Injection of heme in the peritoneal cavity of mice also caused a significant increase in IL-1β (Fig. 1D). These results indicate that heme promotes the processing of caspase-1 and IL-1β in primed macrophages and the production of IL-1β in vivo.

Structural Motifs Involved in Inflammasome Activation by Heme. To define the structural requirement of heme to activate the inflammasome, we used several heme analogs. Protoporphyrin IX (PPIX) is a direct precursor of heme that lacks the atom of iron. CoPPIX (cobalt) and SnPPIX (tin) are heme analogs with a metal substitution. Treatment of BMMs or thioglycollate-elicited peritoneal macrophages with different porphyrins demonstrated that heme, but not its analogs lacking the atom of iron, caused IL-1β secretion (Fig. 2A and Fig. S1B). Incubation of macrophages with heme in the presence of the iron chelator deferoxamine (DFO) abrogated heme-induced processing of IL-1β and caspase-1 and IL-1β secretion, although it had no effect on macrophages stimulated with ATP (Fig. 2 B and C). Treatment with DFO did not affect the amount of pro–IL-1β and pro-caspase-1, as observed in cell extracts. Incubation of DFO with heme inhibited inflammasome activation, although pretreatment with DFO, followed by media change before stimulation with heme, was ineffective in blocking IL-1β processing (Fig. 2D). Stimulation of LPS-primed macrophages with iron (Fe2+ or Fe3+) did not cause IL-1β maturation (Fig. 2E). Ferritin is an endogenous iron chelator that binds intracellular iron and protects cells from iron-induced oxidative damage (1, 37, 38). The stimulation of macrophages from ferritin heavy-chain-deficient (Fth−/−) mice with heme or ATP caused a similar processing of IL-1β compared with wild-type macrophages (Fig. 2F). The presence of excess iron even in the absence of ferritin was not capable of inducing IL-1β processing. The catabolism of heme by HO-1 causes the intracellular release of the atom of iron from the porphyrin ring (2). Thus, we tested the involvement of enzymatic release of intracellular iron from heme by HO-1 on inflammasome activation. Treatment of wild-type and Hmox1−/− macrophages with heme or ATP demonstrated that HO-1 is dispensable for inflammasome activation by these stimuli (Fig. 2G). Together, these results suggest that the iron
Heme Causes Activation of NLRP3 Inflammasome Dependent on K⁺ Efflux and ROS. Considering the essential role of NLRP3 inflammasome on macrophage activation by damage-associated signals, we tested the role of NLRP3, ASC, and caspase-1 on heme-induced IL-1β processing. BMMs deficient in Nlrp3, Asc, and Caspase-1 were unable to process and secrete IL-1β when stimulated with heme or ATP (Fig. 3A). The heme-induced IL-1β processing by macrophages in the presence of serum was independent of ATP release or the purinergic receptor P2X7, while ATP-induced inflammasome activation requires P2X7 receptor (39). This result was based on the following observations: using BMMs from P2X7-deficient mice heme-induced activation of IL-1β secretion was preserved, whereas the effect of ATP was abrogated (Fig. 3B), the use of oxidized-ATP (o-ATP), an antagonist of P2X7 receptor, and the treatment with apyrase, that degrades ATP, inhibited the effect of ATP but not of heme (Fig. 3C).

Different models have been proposed to explain the activation of NLRP3: K⁺ efflux, generation of ROS, mitochondrial dysfunction, and lysosomal damage with cathepsin release (34, 35). Thus, we tested the role of these pathways on heme-induced inflammasome activation in the presence of serum. High concentration of K⁺ in the culture medium blocks the K⁺ efflux and is able to abrogate inflammasome assembly and caspase-1 processing induced by all known NLRP3 activators (40, 41). Similarly, processing of IL-1β triggered by heme was blocked by high concentrations of K⁺ (Fig. 3D and E). Treatment of LPS-primed macrophages with N-acetyl cysteine, a precursor of the antioxidant glutathione, inhibited heme-induced secretion of IL-1β (Fig. 3F). As previously shown, antioxidants also inhibited ATP-induced IL-1β secretion (42, 43). Treatment with bafilomycin, an inhibitor of the vacuolar type H⁺-ATPase that prevents the phagosomes maturation and fusion to lysosome, had no effect on heme or ATP-induced inflammasome activation (Fig. 3G). As expected, bafilomycin blocked the inflammasome activation by silica crystals (44). Similar results were obtained with the cathepsin inhibitor CA-074, able to block IL-1β secretion triggered by silica but not by heme or ATP (Fig. S2B). Particular matter has been shown to activate the NLRP3 inflammasome in a mechanism that requires internalization and is inhibited by drugs that cause cytoskeleton disturbance (43, 44). Because hemozoin is a crystal formed by heme able to activate the NLRP3 inflammasome (45–47), we tested the necessity of endocytosis for heme-induced inflammasome activation. Treatment with cytochalasin D, which blocks actin polymerization, abolished silica-induced inflammasome activation but had no
Heme activates the NLRP3 inflammasome through the canonical pathway. (A) WT, Nr3p−/−, Asc−/−, and Caspase−1−/− BMMs primed with LPS were stimulated with heme or ATP to analyze IL-1β maturation in cellular supernatants by Western blot. (B) WT and P2X7−/− BMMs primed with LPS were stimulated with heme or ATP to analyze IL-1β secretion in cellular supernatants by ELISA. (C) BMMs primed with LPS were incubated with apyrase (10 units/ml) or o-ATP (100 μM) for 30 min before stimulation with heme or ATP to analyze IL-1β secretion in cellular supernatants by ELISA. (D) WT and gp91phox−/− BMMs primed with LPS were stimulated with various concentrations of KCl for 15 min before stimulation with heme or ATP to analyze IL-1β maturation in cellular supernatants by ELISA. (E and F) BMMs primed with LPS were incubated with 10 mM of N-acetyl cysteine (NAC) for 30 min before stimulation with heme or ATP to analyze IL-1β secretion in cellular supernatants by ELISA. Data are from one representative of two independent experiments (B, C, and E) and data represent mean ± SE of four (F) independent experiments. *P < 0.05.

Mitochondrial ROS and NOX2 Are Involved in Heme-Induced Inflammasome Activation. Heme is a potent inducer of ROS production, activating different sources of ROS generation in macrophages (19, 24). Recent studies indicate an essential role of mitochondrial ROS (mtROS) on NLRP3 activation induced by several stimuli (48, 49). We observed that heme caused the generation of mtROS by macrophages using a probe for mitochondrial superoxide, MitoSOX (Fig. 4A). The selectivity of this response was confirmed using MitoTEMPO, a specific scavenger of mitochondrial superoxide (Fig. 4A). Treatment of LPS-stimulated macrophages with MitoTEMPO caused the abrogation of heme- and ATP-induced IL-1β and caspase-1 processing (Fig. 4B). The NADPH oxidase inhibitor apocynin blocked heme-induced IL-1β processing/secretion but was ineffective on ATP-induced inflammasome activation (Fig. 4C). Part of the controversy related to the role of ROS on NLRP3 activation is due to results obtained with nonselective drugs. Thus, we tested the role of NADPH oxidase 2 (NOX2) using macrophages deficient in the membrane subunit gp91phox (gp91phox−/−). Heme-induced IL-1β and caspase-1 processing was abrogated in gp91phox−/− macrophages (Fig. 4E and F). As previously demonstrated, ATP-induced secretion of IL-1β was not affected by gp91phox deficiency (44). These results demonstrated a selective role of NOX2 on heme-induced NLRP3 activation, an effect that is not observed with ATP and that occurred despite similar amounts of pro-IL-1β and procaspase-1. To evaluate whether NOX2 affects heme-induced mtROS generation, we stimulated wild-type and gp91phox−/− macrophages with heme and analyzed mtROS. Heme caused mtROS generation in wild-type but not in gp91phox−/− macrophages (Fig. 4G). The role of NOX2 on mtROS was selective to heme because LPS + ATP caused a similar mtROS induction on wild type and gp91phox−/− (Fig. S3A). Treatment of macrophages with high concentrations of K⁺ had no effect on heme-induced mtROS generation (Fig. S3B).

Heme-induced ROS is largely dependent on Syk activation (24). Thus, we analyzed the involvement of Syk on heme-induced inflammasome activation. Treatment with piceatannol, an inhibitor of Syk, abrogated the processing of IL-1β induced by heme but did not affect ATP-induced inflammasome activation (Fig. 4H). Treatment with piceatannol was effective in blocking the generation of mtROS by macrophages stimulated with heme (Fig. 4I). The inhibitory effect of DFO on heme-induced inflammasome activation prompted us to characterize the mechanism. The simultaneous incubation of macrophages with DFO and heme caused the inhibition of heme-induced Syk phosphorylation and mtROS generation (Fig. S3 C and D). Together these results indicate that DFO blocked the early events of macrophage activation by heme and that heme caused mtROS-dependent NLRP3 inflammasome activation, an event that required Syk.
Inflammasome Components Are Essential to Heme-Induced Inflammation and Lethality Caused by Hemolysis. The in vitro activation of the inflammasome by heme suggested a possible participation of this pathway causing inflammation on hemolytic conditions. In fact, instillation of heme in the peritoneal cavity of wild-type mice caused the recruitment of neutrophils, which was significantly reduced in \( \text{Asc}^{-/-} \) mice (Fig. 5A). Next, we analyzed if lyzed red blood cells also cause neutrophil recruitment dependent on inflammasome. The injection of lyzed erythrocytes in the peritoneal cavity promoted neutrophil recruitment in wild-type mice, an effect significantly reduced in \( \text{Asc}^{-/-} \) mice (Fig. 5A). Several studies documented that during the hemolytic process or rhabdomyolysis, the high amounts of hemoglobin, myoglobin, and subsequently free heme promote inflammation and tissue damage (3–5, 37). We used Phz to characterize the involvement of macrophages and inflammasome components on the pathogenesis of hemolytic diseases. The challenge with a high dose of Phz caused hemolysis in mice evident by the dark brown coloration of urine as early as 4 h after challenge. This dose of Phz caused 70–100% lethality within 9 d (Fig. 5B–E). Because macrophages are considered an important target of the inflammatory effects of heme and the main source of IL-1β production upon inflammasome activation, we initially analyzed the participation of macrophages in the pathogenesis of hemolysis. We used clodronate liposomes to deplete macrophages and after 2 d of treatment we challenged mice with Phz. Mice depleted of macrophages were significantly more resistant to lethality induced by hemolysis compared with mice that received control liposomes (Fig. 5B). The role of inflammasome components was also characterized in this model. The lack of \( \text{Nlrp3}, \text{Asc}, \) or \( \text{Caspase-1} \) strikingly prevented hemolysis-induced lethality (Fig. 5C). These results indicate an essential role of the NLRP3 inflammasome on heme-induced inflammation and to the lethal effects of hemolysis. To define the importance of IL-1 in the lethality caused by hemolysis, we compared the effect of Phz in wild-type and \( \text{Il1r}^{-/-} \) mice. Our results demonstrated that IL-1R receptor was essential to the hemolysis-induced lethality (Fig. 5D). These results suggest that heme promotes inflammasome activation and IL-1β secretion by macrophages that participate in the inflammatory response, tissue damage, and lethality induced by hemolysis. In the presence of serum, inflammasome activation was independent of cell death, ATP, and functional P2X7, whereas in the absence of serum, it correlated with cell death. Thus, we tested the role of P2X7 on hemolysis pathogenesis. The challenge of wild-type and

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**Fig. 4.** Heme activates the inflammasome through ROS generation induced by mitochondria and NOX2. (A and B) BMMs were incubated with 500 μM of MitoTEMPO (MT) for 1 h before stimulation with heme or ATP. (A) BMMs were stimulated with heme for 1 h and then loaded with MitoSOX to analyze mtROS by flow cytometry. (B) BMMs incubated or not with MitoTEMPO were stimulated with heme or ATP to analyze IL-1β and caspase-1 maturation in cellular supernatants by Western blot. (C and D) BMMs primed with LPS were incubated with 100 μM of apocynin (Apo) for 1 h before stimulation with heme or ATP to analyze IL-1β secretion and maturation in cellular supernatants by ELISA and Western blot. (E and F) WT and \( \text{gp91phox}^{-/-} \) BMMs primed with LPS were stimulated with heme or ATP to analyze IL-1β secretion by ELISA, and IL-1β and caspase-1 maturation by Western blot. A line was included to identify that the Western blot was cut. (G) WT and \( \text{gp91phox}^{-/-} \) BMMs were stimulated with heme for 1 h to analyze mitochondrial ROS by flow cytometry by loading the cells with MitoSOX. (H) BMMs primed with LPS were incubated with 10 μM of piceatannol (PIC) for 1 h before stimulation with heme or ATP to analyze IL-1β maturation in cellular supernatants by Western blot. (I) BMMs were incubated with 10 μM of PIC for 1 h before stimulation with heme for 1 h to analyze mitochondrial ROS generation by flow cytometry. Data represent mean ± SE of two (C) or five (E) independent experiments. * \( P < 0.05. \)
In this study, we show that heme is an inducer of IL-1β secretion, Syk phosphorylation, mtROS generation, and NLRP3 activation in the presence of serum requires macrophages. The molecular mechanism by which heme promotes the NLRP3 activation in the presence of serum requires Syk phosphorylation, mtROS generation, and K+ efflux, although it is independent of lysosomal damage, cathepsin activity, heme internalization, heme catabolism by HO-1, cell death, release of ATP, or a functional P2X7 receptor (Fig. 6). In vivo, hemoxylation causes inflammasome activation and IL-1β secretion, both of which participate in the inflammatory response and contribute to the lethality. Together, these data confirm that the coordinated inflammatory response in sterile and infectious conditions (50). Thus, we treated macrophages with antioxidants after heme-induced inflammasome activation only when incubated together with heme in the culture media. (i) Iron was involved in the activation of NLRP3 induced by heme. The following observations suggested that the coordinated iron of the protoporphyrin ring, not free iron, was involved in this response: (i) Heme, but not CoPPIX, SnPPIX, or PPIX, promoted the secretion of mature IL-1β. (ii) Stimulation of LPS-treated macrophages with free iron (Fe2+ or Fe3+) did not cause secretion of mature IL-1β. (iii) The iron chelator DFO inhibited heme-induced inflammasome activation only when incubated together with heme in the culture media. (iv) The lack of HO-1, which cleaves the porphyrin ring releasing the iron inside the cells, or the lack of ferritin H, which stores labile iron, did not affect the heme-induced inflammasome activation. However, we cannot formally exclude the possibility that iron released from heme, through oxidative assault inside macrophages, is involved in Syk phosphorylation, mtROS generation, and inflammasome activation.

Discussion

In this study, we show that heme is an inducer of IL-1β processing through the activation of the NLRP3 inflammasome in macrophages. The molecular mechanism by which heme promotes the NLRP3 activation in the presence of serum requires Syk phosphorylation, ROS, and K+ efflux, although it is independent of lysosomal damage, cathepsin activity, heme internalization, heme catabolism by HO-1, cell death, release of ATP, or a functional P2X7 receptor (Fig. 6). In vivo, hemoxylation causes inflammasome activation and IL-1β secretion, both of which participate in the inflammatory response and contribute to the lethality. Together, these data confirm and extend the notion that heme is sensed by innate immune receptors affecting the inflammatory response in sterile and infectious conditions (50). The importance of ROS in the activation of NLRP3 inflammasome is a subject of debate. It was suggested that inhibitors of ROS block the priming of macrophages, impairing the pro–IL-1β and NLRP3 expression instead of affecting the NLRP3 activation (51). Thus, we treated macrophages with antioxidants after 4 h of stimulation with LPS to avoid any effect on signal 1, and saw that this treatment did not affect the amount of pro–IL-1β produced. Several reports indicate the participation of mitochondrial dysfunction on NLRP3 activation by stimuli, including ATP, pore forming toxins, and particulate matter (48, 49, 52, 53). We observed that macrophages stimulated with heme had increased mtROS, and the selective blockage with MitoTEMPO abrogated mtROS and inflammasome activation by heme. ROS generated by NOX was initially considered important to NLRP3 activation especially by crystals and other particles (43). However, monocyes from patients with chronic granulomatous disease (CGD) due to a genetic deficiency of p22phox, a component required for the function of NOX1 (CGD) due to a genetic deficiency of p22phox, a component required for the function of NOX1−4, produced equivalent amounts of IL-1β, compared with control subjects, when stimulated with uric acid or silica (54). Similar results were obtained with cells from patients with CGD carrying mutations in the CYBB (the NOX2 gene) or with macrophages from gp91phox−/− (44, 54). A recent study, however, demonstrated the requirement of NOX2 for activation of NLRP3 by heme. The mechanism involves the regulation of mtROS, which indicates that NOX2 is upstream of mtROS generation. In fact, a recent study demonstrated that angiotensin...
II induces superoxide production by the mitochondria dependent on NOX2 (56).

The role of Syk in activation of caspase-1 and secretion of IL-1β and IL-18 by macrophages was demonstrated in studies using activators of NLRP3 and AIM2 (45, 57, 58). The phosphorylation of Syk in response to Candida albicans participates in signal 1 and signal 2 required for inflammasome activation, and this role of Syk was attributed to its effect on CARD9 and ROS generation (57). Syk has been shown to interact with ASC present in the NLRP3 inflammasome (45). Nigericin-induced NLRP3 activation in macrophages, but not in dendritic cells, also requires Syk (58). This effect is related to the phosphorylation of ASC with subsequent formation of ASC specks, but is unrelated to CARD9 or mtROS generation. In the case of heme, we previously observed the pivotal role of Syk phosphorylation in the production of ROS by macrophages (24). Here, we found that Syk was essential to heme-induced mtROS and IL-1β processing, whereas activation of NLRP3 by ATP was not affected by Syk inhibition. Syk is activated by receptors and adaptor proteins that contain immunoreceptor tyrosine-based activation motifs (ITAMs), affecting several signaling pathways (59). The nature of a putative receptor involved in recognition of heme and triggering of Syk phosphorylation is unknown. Interestingly, it has been shown that molecules that intercalate the cell membrane interacting with lipid rafts, such as monosodium urate crystals, can activate Syk in a mechanism independent of specific receptors (60). DFO abrogated heme-induced Syk phosphorylation and mtROS generation, events that are upstream of NLRP3 activation by heme. These results suggest that the inhibitory effect of DFO might be related to its ability to physically interact with heme (61, 62), interfering with the association of heme with a receptor or with the cell membrane, but not related to its free iron chelating property. Future studies will define the molecular mechanism by which heme causes phosphorylation of Syk and if heme-induced Syk activation participates exclusively in ROS generation or also in ASC phosphorylation.

A recent study has described heme as an activator of NLRP3 (63), showing, however, important distinctions from our present work. Some of the discrepancies between the two studies are difficult to reconcile. We followed the protocols used in their study, including the use of thioglycollate-elicited peritoneal macrophages, but we were unable to observe processing or secretion of IL-1β by PPIX with or without pre-stimulation with LPS. We also did not confirm heme-induced inflammasome activation in the absence of signal 1. We observed that BMMs or thioglycollate-elicited peritoneal macrophages primed with LPS and treated with heme secreted mature IL-1β in the presence of serum, in which condition heme induced negligible amounts of TNF and caused few cell deaths in the 4 h of stimulation. Moreover, heme-induced inflammasome activation in the presence of serum was independent of macrophage death, ATP release, and functional P2X7 receptor. In the absence of serum, thioglycollate-elicited peritoneal macrophages stimulated with heme processed IL-1β and this involved required P2X receptor (63). We observed that in the absence of serum, inflammasome activation correlated with macrophage cell death determined by LDH release. It is well established that upon necrosis, macrophages release ATP that in turn activates the NLRP3 inflammasome (53, 64). Thus, heme-induced inflammasome activation has different mechanisms in the presence or absence of serum.

Injection of heme or lyzed red blood cells into the peritoneal cavity caused neutrophil recruitment in a mechanism dependent on inflammasome components. In a model of unilateral ureteral obstruction, heme and the active forms of caspase-1 and IL-1β are increased (63). It has been demonstrated that inflammation, renal dysfunction, and death triggered by transient renal artery occlusion, a model of tubular necrosis induced by ischemia–reperfusion, was dependent on NLRP3 (53), and intracerebral hemorrhage also activates the NLRP3 inflammasome in a mechanism dependent on mtROS (65). We observed that the pathogenesis of hemolysis was dependent on inflammasome components, demonstrated by the high resistance of mice lacking Nlrp3, Asc, or Caspase-1. In this model, the activation of the NLRP3 and lethality was likely unrelated to ATP, because wild-type and P2X7−/− mice displayed similar susceptibility. The increased resistance of P2X7−/− mice suggested that upon hemolysis, heme activates the inflammasome on macrophages, promoting the secretion of processed IL-1β that participates in inflammation and tissue damage. These results add a component of the innate immune system to the recently demonstrated essential participation of heme-induced TLR4 activation in sickle cell disease (29, 30). Moreover, inflammasome activation is likely important in malaria pathogenesis (45, 66). Thus, understanding the molecular signaling pathways affected by heme might prove useful to the identification of new options for treating pathological conditions that have increased extracellular heme.

**Methods**

**Reagents and Materials.** Heme, PPIX, SnPPIX, and CoPPIX were purchased from Frontier Scientific. They were dissolved in NaOH (0.1 M) diluted in RPMI, and filtered just before stock solutions of porphyrins were prepared in the dark to avoid free radical generation. LPS 0111:B4 from *Escherichia coli* was obtained from InvivoGen. Apocynin, N-acetyl cysteine, deferoxamine, piceatannol, ATP, FeSO4, FeCl3, KCl, oxidized ATP, apyrase, cytochalasin D, anti-IL-1β, and Phez were obtained from Sigma-Aldrich. Clodronate liposomes and control liposomes were obtained from www.ClodronateLiposomes.com.
Anti-caspase-1 was obtained from Genentech. Protease inhibitors and luminol were obtained from Santa Cruz Biotechnology. X-ray films were from Kodak. ELISA for IL-1β detection was obtained from Peprotech. MitoSOX was obtained from Molecular Probes. MitoTEMPO CA-074, and baflomycin were obtained from Enzo Life Sciences. RPMI medium 1640 and penicillin–streptomycin were obtained from LGC Biotechnology. FCS was from Invitrogen, and nitrodiama was obtained from Roche.

Mice. C57BL/6 WT mice were obtained from the animal facility at the Universidade Federal do Rio de Janeiro (Rio de Janeiro, Brazil). P2X7−/− mice were obtained from the animal facility of the Instituto de Biofísica, Universidade Federal do Rio de Janeiro. Nlrp3−/−. Asc−/−, Caspase-1−/−, and Il1r−/− were obtained from the animal facility at the Universidade de São Paulo (Ribeirão Preto, SP, Brazil). gp91phox−/− mice were obtained from the animal facility at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil). Tbias and femurs of WT, Hmox1−/−, and Fth−/− mice were obtained from the animal facility at the Instituto Galvão de Ciência (Oeiras, Portugal). All experiments followed guidelines of the institutional ethical committee and underwent approval [approval ID: CEU/CCSUFRJ/IMP011 (Comissão de Ética no Uso de Animais do Centro de Ciências da Saúde da Universidade Federal do Rio de Janeiro, RJ, Brazil)].

Macrophages. BMMs were prepared using tibia and femur from 6- to 12-wk-old mice. Bone marrow was obtained using flushing bones with cold sterile RPMI. The differentiation medium was RPMI supplemented with 20% (vol/vol) heat-inactivated FCS and 30% (vol/vol) L929 cell supernatant. Initially, 5 × 105 bone marrow cells were suspended in 10 mL of differentiation medium, then seeded in 100-mm Petri dishes (BD Biosciences) at 37 °C in humidified 5% CO2. After 3 d, 10 mL of differentiation medium was added. Finally, after more days, cells were washed with cold RPMI and suspended and seeded at the required density for all experiments. Before any experiment, cells rested for at least 12 h. Peritoneal exudate cells were harvested by lavage from C57BL/6 mice (4–6 wk) 4 d after i.p. injection of 2 mL of 3% (wt/vol) thio-lemma in cold PBS solution in a same volume. After 5 h of injection, the animals were killed, and their peritoneal cavities were rinsed with 3 mL of cold PBS. Total leukocytes in the peritoneal fluid were determined on Neubauer chambers after dilution in Turk solution. Differential counting of leukocytes was carried out on DiffQuik (Baxter Travenol Laboratories)-stained slides. Also, the peritoneal fluid was centrifuged and the amount of IL-1β from the supernatant was quantified by ELISA.

IL-1β ELISA. Experiments to analyze IL-1β secretion were performed with 2 × 106 cells and the cellular supernatants were collected for ELISA analysis (Peprotech). All of the measurements were performed according to the manufacturer’s protocol.

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