

Red Alert: Labile Heme is an Alarmin

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Abstract

Alarmins are a heterogeneous group of endogenous molecules that signal cellular damage when sensed extracellularly. Heme is an endogenous molecule that acts as a prosthetic group of hemoproteins, such as hemoglobin and myoglobin. When released from damaged red blood cells or muscle cells, oxidized hemoglobin and myoglobin release their prosthetic heme groups, respectively. This generates labile heme, which is sensed by pattern recognition receptors (PRR) expressed by innate immune cells and possibly regulatory T cells (T_{REG}). The ensuing adaptive response, which alerts for the occurrence of red blood cell or muscle cell damage, regulates the pathologic outcome of hemolysis or rhabdomyolysis, respectively. In conclusion, we propose that labile heme is an alarmin.

Introduction

This opinion article builds up on several assumptions. First, that all living organisms can sense events that alert for a possible disruption of homeostasis [1]. Second, that such sensors trigger adaptive responses that contribute to maintain or restore homeostasis [2,3]. Third, that host/microbe interactions often lead to disruption of homeostasis. Fourth, that sensing microorganisms alert for a possible disruption of homeostasis. Based on these general principles pattern recognition receptors (PRRs) were proposed to act as *bona fide* homeostatic sensors [1].

Engagement of PRR by pathogenic microorganisms is essential to elicit adaptive immune responses conferring resistance to infections [4,5]. In addition, PRR also play a pivotal role in maintaining steady state interactions with commensal microorganisms, as

illustrated originally for host/microbiota interactions [6]. Moreover, PRR can sense endogenous molecules released from damaged cells [7], presumably reporting on cellular damage and on possible disruption of homeostasis. The endogenous molecules recognized by PRR were originally designated as danger associated molecular patterns, based on the assumption that these are sensed when released from damaged cells [8,9]. The term alarmin is also used to refer to any endogenous molecule that signals cellular damage [10]. We shall argue herein that labile heme is a prototypical alarmin.

Labile Heme

Heme is an evolutionarily conserved molecular structure composed of a tetrapyrrole ring surrounding a single iron (Fe) atom (*Figure 1*). Heme acts essentially as a prosthetic group in a number of hemoproteins. These include hemoglobin (Hb) and myoglobin (Mb), which contain the largest pool of bioavailable heme in mammals [11]. When damaged, red blood cells and muscle cells release Hb and Mb, respectively. Extracellular Hb and Mb are readily oxidized, via a process catalyzed by the transition of iron-heme from ferrous (Fe^{++}) to ferric (Fe^{+++}) state. This gives rise to methemoglobin [12-14] and metmyoglobin [15], respectively, which can release their prosthetic heme groups and generate labile heme, that is, redox active heme that is loosely bound to proteins or molecules, other than hemoproteins.

Under homeostatic conditions, extracellular Hb and labile heme are scavenged in plasma by haptoglobin and hemopexin, respectively [16,17]. Whether extracellular Mb is also scavenged in plasma is, to the best of our knowledge, not established. The resulting haptoglobin/Hb and hemopexin/heme complexes are captured by monocyte/macrophages ($\text{M}\phi$) via the CD163 and CD91 receptors, respectively. This is associated with the induction of heme oxygenase-1 (HO-1) expression, a heme-catabolizing enzyme that generates carbon monoxide (CO), labile iron and biliverdin (*reviewed in* [11]). The end-products of heme catabolism can modulate $\text{M}\phi$ polarization, as demonstrated originally for CO [18]. Of note, when generated by $\text{M}\phi$ CO acts as a “microbial metabolic sensor” that fine-tunes $\text{M}\phi$ responses to live versus dead bacteria [19].

When the “scavenging capacity” of haptoglobin and hemopexin is exhausted, labile heme accumulates in plasma, where it becomes loosely bound to other plasma molecules that fail to restrain its redox activity. As such, labile heme becomes pro-

oxidant and presumably therefore pathogenic. This pathogenic effect is perhaps best illustrated in the context of hemolytic conditions such as sickle cell disease, caused by mutations in the β chain of Hb [20-22], malaria caused by *Plasmodium* infection [14,23-25] or severe sepsis caused by systemic infections [26].

Sensing labile heme

The finding that when exposed *in vitro* to labile heme M ϕ secrete tumor necrosis factor (TNF) via a mechanism dependent on the expression of TLR4 and its adaptor signaling molecule MyD88, revealed that labile heme can be sensed by PRR [27]. More recently labile heme was shown to induce, again via a TLR4-dependent mechanism, the expression of molecules associated with endothelial cell activation, an effect that presumably contributes to the pathogenesis of sickle cell disease [20,22]. Labile heme acts irrespectively of the toll-Interleukin receptor (TIR) domain-containing adaptor-inducing interferon- β (IFN- β)(TRIF) and fails to induce the expression of co-stimulatory molecules that support dendritic cell immunogenicity. This suggests that in contrast to other alarmins, labile heme has little or no “adjuvant” activity.

Murine M ϕ also secrete interleukin 1 β (IL-1 β) when exposed *in vitro* to labile heme [28]. This occurs via a mechanism dependent on the expression of the NOD-like receptor 3 (NLRP3) inflammasome, a cytosolic complex that includes the adaptor protein apoptosis-associated speck-like protein (ASC) and caspase-1, which processes pro-IL-1 β into IL-1 β [28]. Activation of the NLRP3 inflammasome in response to heme occurs through a mechanism dependent on the phosphorylation of the spleen tyrosine kinase (Syk) as well as the production of reactive oxygen species (ROS) and cellular K⁺ efflux, while acting independently of lysosomal damage or cathepsin activity [28]. These findings add labile heme to a number of structurally unrelated endogenous molecules sensed by the NLRP3 inflammasome. The observation that mice in which the *Nlrp3*, *Asc*, *caspase 1/11* or *Il1r* alleles are deleted are more resilient to hemolysis [28], suggests that unfettered NLRP3 inflammasome activation and IL-1 β secretion in response to labile heme drives inflammation and tissue damage associated with hemolysis.

Labile heme is an Alarmin

Given the extraordinarily high intracellular heme content of red blood cells and muscle cells, as compared to any other cell type, sensing extracellular labile heme probably signals red blood cell and/or muscle cell damage, associated with hemolysis or rhabdomyolysis, respectively (*Figure 2*). In keeping with this notion, labile heme triggers cell autonomous and systemic adaptive responses that mitigate the pathogenic outcomes of hemolysis [25,29] and rhabdomyolysis [30]. This protective effect is mediated via a mechanism involving the expression of HO-1 [13,14]. A similar adaptive response, involving the expression of HO-1, contributes to the protective effect of sickle cell Hb against malaria [24,31]. Whether this involves heme sensing by PRR is likely, but remains to be established.

Alarmins can promote tissue damage repair, as illustrated in the context of muscle [32], intestinal [33] and lung [34] injury. The mechanisms via which this occurs are unclear but have been shown to involve the recruitment of tissue resident T_{REG} that can sense alarmins. Upon migration, tissue resident T_{REG} preserve the functional integrity of damaged tissues [32-34] via a mechanism involving the expression of molecules that act directly on parenchyma cells [34] and/or that polarize tissue-resident Mø towards tissue repair [32]. Whether labile heme, when released from damaged cells, acts in a similar manner is likely, but this has not been reported. In support of this notion, T_{REG} express TLR4 [35] and as such may sense labile heme. Moreover, induction of HO-1 in T_{REG} has been associated with enhanced T_{REG} function, which may contribute to tissue repair in a range of immune mediated inflammatory conditions where HO-1 is protective [13,36,37].

Labile heme as an amplifier of inflammation

Systemic infections are often associated with varying levels of hemolysis and in some cases with rhabdomyolysis as well. The extracellular Hb generated through hemolysis exerts anti-microbial effects, via the peroxidase activity of its prosthetic heme *b* groups, which uses hydrogen peroxide (H₂O₂) to oxidize molecules in microbes [38,39]. Moreover, binding of extracellular Hb to bacterial lipopolysaccharide (LPS) [40], alters the tertiary structure of Hb [39,41], enhancing its peroxidase and hence microbicidal activity [38,39]. Extracellular Hb enhances the pro-inflammatory activity of LPS [41] and when overtly oxidized can form large aggregates that act *per se* as a pro-inflammatory

agonist in endothelial cells [42]. This suggests that extracellular Hb acts as a soluble PRR and a cytotoxic molecule promoting resistance to systemic infections [40].

When released from oxidized Hb and probably from other hemoproteins as well, labile heme can exert cytotoxic effects on pathogens, as illustrated for bacteria [43] and protozoan parasites [44]. Labile heme also synergizes with LPS to induce cytokine production in Mø [45], suggesting that under suboptimal microbial sensing via PRR [45], labile heme provides an amplification system that boosts inflammation. Presumably this enhances resistance to infection, a feature shared with other alarmins.

Labile heme as a cytotoxic agonist

Labile heme is not only a cytotoxic to pathogens but also to host cells [44,46,47]. The very same structural features that render heme *b* a versatile redox active molecule when contained in the heme pockets of hemoproteins, drive its pathologic effects when released from those hemoproteins. The general principle being that the amphipathic nature of the tetrapyrrole ring of heme favors its interaction with nonpolar molecules such as phospholipids in cellular membranes. If not countered promptly, via heme cellular export [48] or catabolism [13,14], labile heme catalyzes lipid peroxidation, leading to programmed cell death [12-14,23,26,47]. Presumably this cytotoxic effect contributes critically to tissue damage associated with the pathogenesis of sickle cell disease [20-22], malaria [14,23-25] and severe sepsis [26].

Labile heme interacts synergistically with pro-inflammatory cytokines such as TNF [23] as well as other agonists such as Fas, ROS and reactive nitrogen species (RNS) [13,26] to induce programmed cell death in a variety of parenchyma cells [13,14,23,26,47,49,50]. Briefly, labile heme sensitizes parenchyma cells to undergo TNF-mediated programmed cell death via a mechanism that involves the iron contained in its tetrapyrrole ring, which reacts with hydrogen peroxide (H_2O_2) and catalyzes, via the Fenton chemistry, the production of hydroxyl radicals ($OH\cdot$) [13,51]. Sustained ROS production overrides the cytoprotective program triggered via activation of the nuclear factor kappa B (NF- κ B) family of transcription factors [52] and sustains the activation of the c-Jun N-terminal Kinase (JNK) leading to programmed cell death [49][53,54]. Presumably, this occurs via inhibition of redox-sensitive phosphatases that control JNK activation in response to TNF [55,56]. In keeping with this notion, antioxidants such as

the glutathione precursor N-acetyl cysteine suppress heme-driven JNK activation and the cytotoxic effect of labile heme and TNF [13,14,23,26,49,57]. Moreover, intracellular sequestration of labile iron by ferritin acts as an endogenous anti-oxidant that prevents sustained JNK activation and programmed cell death in response to heme and TNF [52]. This suggests that in parenchyma cells the pro-oxidant effect of labile heme is mediated, at least in part by iron, when released from the protoporphyrin ring of heme [13,23].

The effector mechanisms underlying the cytotoxic effect of labile heme in parenchyma are likely cell-type specific. Based on morphological and biochemical characteristics, including caspase-3 activation, membrane blebbing, nuclear shrinking/fragmentation, chromatin condensation and formation of apoptotic bodies, labile heme appears to sensitize hepatocytes to undergo apoptosis in response to TNF [23].

Mø also undergo programmed cell death when exposed to labile heme [58]. However, labile heme induces Mø to undergo necroptosis, rather than apoptosis. This occurs through a mechanism involving TLR4-driven NF- κ B activation, the production of TNF and TNF receptor 1 (TNFR1) signaling. Sustained ROS production drives JNK and receptor-interacting serine/threonine-protein kinase (RIPK) 1 and RIPK3 activation, leading to necroptosis [58]. The pro-oxidant effect of labile heme in Mø occurs independently of TLR4 [27] but involves the expression of NADPH oxidase-2 (NOX2)/gp91^{phox} [59-61] and the mitochondrial electron transport chain [28,62]. Whether ROS production also involves a receptor-mediated process that senses labile heme is not clear. A similar signal transduction pathway, leading to programmed cell death, applies to microglia [63] and to astrocytes [64].

The mechanism via which labile heme triggers programmed cell death in Mø contrasts to some extent with the one operating in parenchyma cells, which fail to produce TNF in response to labile heme. As such labile heme *per se* does not trigger programmed cell death in parenchyma cells. Heme becomes cytotoxic to parenchyma cells, only in the presence of exogenous TNF, ROS or RNS produced by bystander cells such as activated Mø.

Conclusion

Labile heme is a prototypical alarmin. In contrast to other alarmins however, labile heme is endowed with cytotoxicity, potentially amplifying the release of other alarmins from damaged cells. Presumably this explains the broad protective effects exerted by the adaptive response triggered in response to this alarmin, which converges at the level of heme catabolism by HO-1 and act in a protective manner against a variety of immune mediated inflammatory diseases.

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Note: While preparing this manuscript another manuscript putting forward similar notions was published elsewhere: "*Heme as a danger molecule in pathogen recognition*" By Barbara Wegiel, Carl J. Hauser, Leo E. Otterbein, *Free Radical Biology and Medicine*. Volume 89, Received 13 July 2015, Accepted 8 August 2015, Available online 9 October 2015"

BOX1: Heme as a TLR4 agonist:

When assigning an endogenous molecule as a putative TLR4-ligand one should consider the possibility that this molecule may be contaminated by LPS (i.e. endotoxin), a promiscuous TLR4-ligand present in many commercially available reagents. In the case of labile heme this possibility was excluded by a series of independent observations; i) LPS, e.g. lipid A, antagonists fail to inhibit the pro-inflammatory activity of labile heme and antibodies that block the binding of LPS to TLR4 and/or to MD-2 fail to inhibit the pro-inflammatory activity of labile heme [27]; ii) Protoporphyrin IX, a tetrapyrrole ring identical to heme but lacking the iron molecule, acts as competitive inhibitor of labile heme while not interfering with the activity of LPS [27], iii) Serum blocks the pro-inflammatory activity of labile-heme while supporting that of LPS [27]; iv) The pro-inflammatory activity of labile heme acts irrespectively of the Toll-Interleukin receptor (TIR) domain-containing adaptor-inducing interferon- β (IFN β) (TRIF) or MD-2 [27,58]. This argues that labile heme is an endogenous TLR4 ligand that binds to TLR4 in a distinct manner from LPS [65].

Figure legends

Figure 1: Specific alterations in the vinyl and methyl groups of the tetrapyrrole ring of heme define heme *a* ($C_{49}H_{56}O_6N_4Fe$), *b* ($C_{34}H_{32}O_4N_4Fe$) and *c* ($C_{34}H_{36}O_4N_4S_2Fe$). Heme *a* associates, via hydrogen bonds formed by the formyl and hydroxyfarnesylethyl groups of the tetrapyrrole ring, with arginines and serines in hemoproteins such as cytochrome c oxidase. Heme *c* is less abundant and binds covalently, through the tetrapyrrole vinyl groups, to cysteines in hemoproteins such as cytochrome c. Heme *b*, the most abundant form of heme, associates via *van der Waals* contacts and salt-bridges in the propionate group of the tetrapyrrole ring, to hydrophobic amino acids in hemoproteins, such as hemoglobin (Hb) and myoglobin (Mb).

Figure 2: Labile heme acts as a *bona fide* inflammatory agonist, inducing the activation and migration of polymorphonuclear (PMN) cells [59,60,66] as well as the activation of M ϕ [59,61,62,67-69] and endothelial cells [20]. In some cases this is mediated via a mechanism involving heme sensing by PRR [20,27,28] as well as G coupled proteins

(GCP) in PMN cells [59]. This argues that extracellular labile heme acts as an alarmin, that is, an endogenous molecule that when sensed in the extracellular space signals cellular damage [10].

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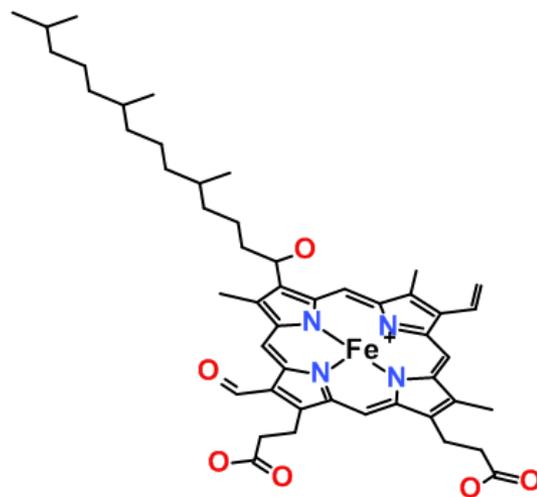
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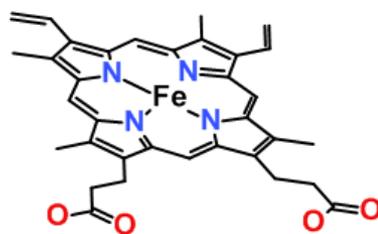
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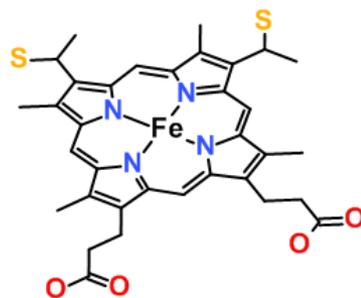
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Heme a



Heme b



Heme c

