Heme Induces Heme Oxygenase 1 via Nrf2
Role in the Homeostatic Macrophage Response to Intraplaque Hemorrhage

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Objective—Intraplaque hemorrhage (IPH) is an important progression event in advanced atherosclerosis, in large part because of the delivery of prooxidant hemoglobin in erythrocytes. We have previously defined a novel macrophage phenotype (hemorrhage-associated-mac) in human advanced plaques with IPH. These may be atheroprotective in view of raised heme oxygenase 1 (HO-1), CD163, and interleukin-10 expression and suppressed oxidative stress.

Methods and Results—We have used a combination of small interfering RNA and pharmacological reagents, protein analysis, and oxidative stress measurements to dissect the pathway leading to the development of this phenotype. We found that erythrocytes, hemoglobin, or purified heme similarly induced CD163 and suppressed human leukocyte antigen and reactive oxygen species. HO-1 was required for the development of each of these features. Challenge of macrophages with purified heme provoked nuclear translocation of Nrf2, and Nrf2 small interfering RNA resulted in significant inhibition of the ability of heme to induce HO-1 protein. Furthermore, tert-butyl-hydroquinone, which activates Nrf2, upregulated CD163, suppressed human leukocyte antigen, and induced interleukin-10, further supporting a role for Nrf2-mediated signaling. However, an inducible protein transactivator is also probably necessary, as heme-induced HO-1 mRNA expression was fully inhibited by the protein synthesis inhibitor cycloheximide.

Conclusion—Our experiments define an Nrf2-mediated pathway by which heme induces a homeostatic macrophage response following IPH. (Arterioscler Thromb Vasc Biol. 2011;31:2685-2691.)

Key Words: hemoglobin ■ macrophages ■ reactive oxygen species

Intraplaque hemorrhage is a key determinant of atherosclerosis progression and plaque destabilization. Intraplaque hemorrhage results in the delivery of both cholesterol-enriched membrane lipids and hemoglobin (Hb) to the plaque interior, an environment already challenged by accumulated cholesterol and oxysterols. As the iron in heme is an effective peroxidant catalyst via H2O2 coordination and Fenton chemistry, Hb deposition enhances the lipid peroxidation of cholesterol. This in turn can activate macrophage release of H2O2 and free radicals. Because intraplaque hemorrhage induces monocyte recruitment, how monocytes respond to Hb is critical in determining the outcome of hemorrhage.

Heme is catabolized by cytoprotective heme oxygenases into free ferric ions, biliverdin, and carbon monoxide (CO). Low levels of CO, like nitric oxide, engage protective cell signaling pathways such as soluble guanylate cyclase. Free ferric ions are normally quickly chelated by coinduced ferritin, where they are safely locked out of Fenton activity. Biliverdin is further converted by biliverdin reductase to bilirubin, an endogenous antioxidant. Heme oxygenase (HO) has 2 isoforms, with HO-1 being inducible by heme and many other agonists and HO-2 being constitutively expressed. The inducibility of HO-1 by heme provides a key negative feedback loop, protecting phagocytes from oxidative stress associated with free iron. Surprisingly, there is relatively little published information on how heme induces HO-1 compared with the large investment of effort in inflammatory or pharmacological stimuli.

We have recently identified a discrete phenotype of macrophages, designated hemorrhage-associated (HA)-mac, that are localized around hematoma in human plaques with fatal coronary thrombosis. Compared with more typical macrophage foam cells within the same plaque, these are characterized by suppressed oxidative stress, myeloperoxidase and activation marker human leukocyte antigen (HLA-DR), and increased HO-1 expression and interleukin-10. Culturing human blood-derived monocytes with Hb-haptoglobin (Hp) complexes (Hb:Hp) over 4 to 8 days reproduced these phenotypic features, which were prevented by inhibiting the antiinflammatory cytokine interleukin-10 (IL-10) or by inhibiting CD163 dependent endocytosis with cytochalasin-D or with CD163-small interfering RNA (siRNA).
The basic biochemistry of the transcription factor Nrf2 has been extensively reviewed. Nrf2 is activated by oxidative stress and electrophiles because of conversion of cystine residues to cysteine (ie, disulfide cross-linking) in an inhibitory partner Keap-1. Activated Nrf2 translocates to the nucleus and binds to antioxidant response elements. The electrophile tert-butylhydroquinone (t-BHQ) is extensively used as a selective Nrf2 activator.

CD163 is a macrophage scavenger receptor for Hb:Hp complexes, and it functions primarily as a means for their internalization. However, CD163 may also have signaling functions, as suggested by the effects of ligation by antibodies. If macrophage CD163 functions primarily as an endocytic rather than a signaling receptor, then other modes of entry of erythrocyte degradation products should induce HA-mac features. In this study, we demonstrated that this indeed occurs following exposure of monocytes to oxidatively damaged erythrocytes (OxRBCs) or free heme and that this involves a final common pathway mediated by activation of Nrf2.

Methods
A full description of the Methods is found in the supplemental materials, available online at http://atvb.ahajournals.org. These are previously described, with modification where indicated. Blood was collected from normal volunteers, and monocytes were aseptically purified and cultured as previously described. RNA was spin-column purified and analyzed by quantitative reverse transcription–polymerase chain reaction using standard molecular biology methods. Western blotting was by standard methods using a precast gel system (Novex, Invitrogen), commercial antibodies, and chemiluminescence.

Results
We tested whether CD163-independent entry of Hb would drive undifferentiated monocytes to the HA-mac phenotype. Oxidatively damaged erythrocytes (OxRBCs) contain Hb, are pathophysiologically relevant, and are known to be phagocytosed via macrophage scavenger receptor A (macrophage scavenger receptor class A, CD204) rather than by CD163. On the other hand, heme does not require a receptor to enter cells, as it is lipid soluble and freely membrane permeant. As shown in Figure 1, culturing monocytes for 6 days with OxRBCs or with purified heme increased surface CD163 and decreased surface HLA-DR in the same manner as Hb:Hp complexes. This result is therefore consistent with a common mechanism of monocyte response to Hb that is not dependent on CD163.

Because macrophage oxidants play an important role in driving atherosclerosis, we developed an assay using aminophenyl fluorescein, which has been previously validated to detect hypochlorite, hydroxyl free radical, and peroxynitrite (collectively termed highly reactive oxygen species [hROS]). As part of basic assay characterization, we found that oxidized low-density lipoprotein (OxLDL) evoked macrophage hROS (Supplemental Figure I) but other agonists (tumor necrosis factor, lipopolysaccharide, palmitoyl-oxo-valeryl-phosphatidylcholine, interferon-γ) had minimal effect under the assay conditions used (not shown). OxLDL-evoked hROS were at least partly dependent on myeloperoxidase, evidenced by suppression by the selective myeloperoxidase inhibitor 4-amino-benzoic acid hydrazide (Supplemental Figure IA) and were maximal with 30 μM of polystyrene microbeads, which are phago-
cytosable but otherwise inert, to these cultures at a ratio of 1 bead/cell also evoked hROS (Supplemental Figure IIG), indicating that phagocytosis itself may lead to hROS generation. Together, this set of experiments validated OxLDL as a plaque-relevant phagocytic stimulus for a macrophage oxidative burst and generation of pathogenic reactive species (hROS). This was then used as a standard assay for macrophage oxidative stress.

When human monocyte-derived macrophages were incubated with OxRBCs, heme, or Hb:Hp for 2 days and then challenged with OxLDL, OxRBCs and heme suppressed hROS to an even greater extent than Hb:Hp complexes (ANOVA, P<0.05, Figure 1D). Importantly, this effect required macrophages, because heme and hydrogen peroxide were strongly synergistic for aminophenyl fluorescein fluorescence in a cell-free system (Figure 1E). This is consistent with the known Fenton chemistry of heme in the presence of hydrogen peroxide and provides a positive control for the assay.

Because the effect of Hb:Hp, OxRBCs, and heme appeared similar, we next asked whether erythrocytes and Hb:Hp complexes liberated heme once within the macrophages. As porphyrins have characteristic visible-light absorption profiles, we first used visible spectrophotometry (Supplemental Figure II). Reference spectra for Hb and heme from 400 to 800 nm were consistent with those previously published. Hb had characteristic Soret peaks, whereas heme had a broader absorption profile, with a shoulder from 400 to 450 nm (Supplemental Figure II A and II B). We found that the absorption profile of Hb:Hp complexes became like that of heme after coincubation with macrophages (Supplemental Figure II C). We then carried out the same procedure for OxRBCs and again found a change in the overall spectrum following incubation with macrophages, in a pattern consistent with release of heme (Supplemental Figure II D). Measurement of free heme using a commercially available chemical assay broadly corroborated the spectroscopy data, with a maximum heme release from Hb:Hp complexes at 3 days and generation of relatively large concentrations of intracellular heme in response to OxRBC treatment (Supplemental Figure II E and II F).

As heme is potentially prooxidant, we questioned whether there the heme-induced antioxidant state is preceded by oxidative stress (Supplemental Figure III). Indeed, treatment with purified heme (10 μmol/L) increased generation of hROS at 1 hour, and this returned to baseline at 4 hours and reversed to an antioxidant effect by 48 hours. However, oxidative stress alone is unlikely to explain the development of the HA-mac phenotype, as 2 other oxidative stress stimulants, H2O2 (100 nmol/L-10 μmol/L) and the purified synthetic oxylipid palmitoyl-oxo-valeryl-phosphatidylcholine, failed to lead to late suppression of hROS generation or HLA-DR expression or to an increase in CD163 (Supplemental Figure IV).

We next tested whether phagocytosed Hb:Hp complexes or erythrocytes traffic into lysosomes. Using live-cell confocal microscopy, Alexa 488–labeled Hb:Hp complexes were seen to internalize and traffic to lysosomes, as indicated by colocalization with the lysosomal marker LysoTracker Red. The images were taken with a ×63 objective after 20 minutes. Figure 2A shows green-labeled Hb and red labeled lysosomes superimposed as yellow, indicating that Hb colocalizes with lysosomes as far as can be determined by optical microscopy. We then extended this analysis to calcein-labeled OxRBCs. A low-magnification image taken seconds after addition showed labeled erythrocytes surrounding the macrophages (Figure 2B). In contrast, images taken after 20 minutes showed OxRBCs (green-labeled) in lysosomes (red-labeled), with yellow colored areas in the overlay indicating colocalization (Figure 2C). In contrast, noninternalized erythrocytes were entirely green at this point, indicating that they were devoid of lysosomal coating (Figure 2D).

To ask whether generation of HA-mac features requires lysosomal function, we tested the effect of chloroquine. In unstimulated macrophages, chloroquine suppressed HLA-DR with minimal effect on surface CD163 in a concentration-dependent manner from 100 nmol/L to 10 μmol/L (Figure 3A). The lowest effective concentration was chosen for subsequent experiments (1 μmol/L). In contrast, chloroquine (1 μmol/L) instead increased HLA-DR in macrophages cultured in the presence of OxRBCs (Figure 3B) or with Hb:Hp (Figure 3C). In parallel, chloroquine (1 μmol/L) inhibited the induction of surface CD163 with OxRBCs or

Figure 2. Lysosomal colocalization of ingested erythrocytes or hemoglobin (Hb):haptoglobin (Hp) complexes. A, Hb was labeled with Alexa 488 (green), conjugated with Hb (1:1 molar ratio), and added to macrophages labeled with the lysosomal marker LysoTracker Red. The images were taken with a ×63 objective after 20 minutes. B to D, Autologous erythrocytes were oxidatively damaged (1 mmol/L H2O2 for 20 minutes), labeled with calcein (green) and added to LysoTracker Red–labeled macrophages. Images were acquired after 1 minute (B), 20 minutes (C), and 22 minutes (D) with a ×20 objective (B) or a ×63 objective (C and D). Red indicates lysosomes of varying sizes, some below optical resolution; larger lysosomes are pointed to by arrowheads (Ly); e, some of the calcein-labeled erythrocytes; Mϕ, macrophage. Colocalization of green and red is indicated by yellow (open arrows indicate representative areas).
Hb:Hp complexes (Figure 3B and 3C). As a control, chloroquine (1 μmol/L) had minimal effect on either CD163 or on HLA-DR in heme-treated macrophages (Figure 3D). With the specificity provisos of a pharmacological inhibitor, this therefore suggests that inhibiting lysosomes prevents the CD163hi HLA-DRlo phenotype on challenge with complex hemorrhage products that are a source of heme but does not suppress equivalent responses to heme itself.

An important antiinflammatory and antioxidant gene induced by heme is HO-1.7 As shown in Supplemental Figure V, macrophages differentiated with Hb:Hp complexes had increased HO-1 as assessed by fluorescence image analysis (Supplemental Figure V). This increased HO-1 was in part colocalized with CD68 (macrolsin), a specific marker of macrophage lysosomes (Supplemental Figure V). HO-1 was not identified in unstimulated cells.

We then determined the contribution of HO-1 to the antioxidant profile of heme-stimulated cells, using OxLDL or serum deprivation (serum-free medium17,18) as stimuli for oxidative stress. Heme suppressed macrophage hROS (Figure 4A), and this protection was strongly reversed in the presence of the specific HO-1 inhibitor 10 μmol/L zinc protoporphyrin19,20 (Figure 4B). Zinc protoporphyrin also reduced macrophage survival in culture over 6 days, whereas both heme and the specific HO-1 activator cobalt protoporphyrin19,20 had the opposite effect, each in a concentration-dependent manner over 100 nmol/L to 10 μmol/L. D and E, human leukocyte antigen (HLA-DR) and CD163 expression in cells differentiated over 6 days with heme (10 μmol/L) and varying concentrations of ZnPP (D) or CoPP (10 μmol/L) (E). Flow cytometry histograms for intracellular interleukin-10 (IL-10) on monocytes incubated with CoPP (10 μmol/L) over 2 days. Values are mean±SE, with 5 subjects. P<0.05, ANOVA with the Bonferroni correction where appropriate. Iso indicates staining with isotype-matched control antibody.

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Lysosomes are required for macrophage response to erythrocytes and hemoglobin (Hb):haptoglobin (Hp) complexes. Freshly isolated monocytes were cultured with chloroquine (10 μmol/L), a lysosomal inhibitor, either alone (A) or in combination with oxidatively damaged erythrocytes (OxRBCs) (10:1 erythrocytes:macrophages) (B), Hb:Hp complexes (100 nmol/L) (C), or purified heme (10 μmol/L) (D) for 6 days. Expression of CD163 and human leukocyte antigen (HLA-DR) was then assessed by flow cytometry. Data are representative of 4 donors. Iso indicates staining with isotype-matched control antibody.

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Heme oxygenase 1 (HO-1) is required for the macrophage response to heme. A, Monocytes were cultured in medium alone (open bars) or in the presence of heme (10 μmol/L) for 2 days. Then, 1 μmol/L aminophenyl fluorescein was added with oxidized low-density lipoprotein (OxLDL) (30 μg · mL⁻¹), or medium was removed, and cells were washed twice in serum-free medium (SFM) and then incubated in SFM with 1 μmol/L aminophenyl fluorescein. B, Monocytes were cultured and challenged as in A, but in the presence or absence of zinc protoporphyrin (ZnPP) (10 μmol/L). C, Normalized cell number of monocytes cultured over 6 days in the presence of heme, ZnPP, or cobalt protoporphyrin (CoPP) (each over a concentration-effect curve 100 nmol/L to 10 μmol/L). D and E, human leukocyte antigen (HLA-DR) and CD163 expression in cells differentiated over 6 days with heme (10 μmol/L) and varying concentrations of ZnPP (D) or CoPP (10 μmol/L) (E). Flow cytometry histograms for intracellular interleukin-10 (IL-10) on monocytes incubated with CoPP (10 μmol/L) over 2 days. Values are mean±SE, with 5 subjects. P<0.05, ANOVA with the Bonferroni correction where appropriate. Iso indicates staining with isotype-matched control antibody.
induces HO-1 and showed that this was specifically ablated by HO-1 siRNA (Figure 5B). Relative to control transfection, HO-1 siRNA reversed the antioxidant effect of Hb:Hp complexes on macrophage hROS triggered by OxLDL (Figure 5C). Similarly, heme-induced suppression of hROS was also reversed to a strongly prooxidant effect with HO-1 siRNA (Figure 5D). Furthermore, HO-1 siRNA reversed the suppression of HLA-DR and inhibited the induction of CD163 on day 6 macrophages by heme (Figure 5E and 5F). Finally, HO-1 siRNA inhibited heme-induced IL-10 secretion (Figure 5G). Thus, HO-1 induction is necessary for CD163 induction, HLA-DR suppression, and suppression of hROS induced by erythrocytes, Hb, and free heme.

We next assessed changes in levels of HO-1 mRNA in response to heme and found that these were near maximally elevated by 10 μmol/L heme (Supplemental Figure VI). In a time-course analysis, we found that there was minimal induction at 1 hour but near-maximal expression by 4 hours (Supplemental Figure VI). We therefore chose 10 μmol/L and 4 hours as standard conditions to test the role of Nrf2 in induction of HO-1. Purified heme (Figure 6A) or Hb:Hp (not shown) evoked clear-cut nuclear translocation of Nrf2 to the nucleus when added to monocytes. Moreover, Nrf2 siRNA, which suppressed Nrf2 protein by more than 89%, decreased the ability of heme to induce HO-1 protein by 50% (Figure 6B). Further support for a role of Nrf2 signaling came from the use of the known Nrf2 activator t-BHQ,21 which suppressed heme-induced hROS in a concentration-dependent manner, maximal by 10 μmol/L (Fig-
ure 6C). Similarly, 50 μmol/L t-BHQ evoked IL-10 secretion (control 71.8±21.8; t-BHQ 477±40 pg · mL⁻¹) and induced a subpopulation with upregulated CD163 and reciprocally suppressed HLA-DR (Figure 6D).

As established above, oxidant stress alone does not induce the HA-mac phenotype, suggesting that Nrf2 activation is not sufficient. Consistent with this, we found that heme induction of HO-1 mRNA was completely suppressed in monocytes from 12 separate donors by the protein synthesis inhibitor cycloheximide (100 μmol/L), without loss of cell viability (Supplemental Figure VI). As Nrf2-mediated transcriptional activation does not require de novo protein synthesis, these observations therefore indicate that the induction of HO-1 mRNA in monocytes by heme also requires synthesis of an induced intermediary protein transactivator.

**Discussion**

The data presented in this article provide fresh insights into the origins of the HA-mac phenotype in hemorrhaged plaques by showing that the phenotype is driven by internalization of Hb:Hp or erythrocytes complexes followed by the release of heme. We have established for the first time that heme induces HO-1 expression, in part via Nrf2. HO-1 is then required for the increase in CD163, suppression of HLA-DR and suppression of oxidative stress. We therefore propose that a heme→Nrf2→HO-1 pathway is part of a final common pathway for HA-mac differentiation. We have demonstrated that Hb:Hp complexes and oxidized erythrocytes are internalized by macrophages, sent to lysosomes, and then liberate heme. This induces HO-1, which is then itself trafficked to lysosomes. Using HO-1 inhibitors and inducers, we have found that the induced HO-1 is necessary for hemorrhage products to evoke the features of HA-mac. Taking these data together, we suggest a unifying schema whereby hemorrhage-related degradation products enter macrophages (Supplemental Figure VII). Heme is lipophilic and freely passes cell membranes. On the other hand, Hb:Hp complexes undergo receptor-mediated endocytosis via CD163, and OxRBCs are internalized via CD204/macrophage scavenger receptor class A.11,12 As far as can be determined with state-of-the-art reagents and microscopy, internalized products are passed to lysosomes, where the globin scaffold is proteolysed, liberating heme. This framework goes against the concept that CD163 ligation by Hb:Hp complexes initiates a classical protein phosphorylation signaling cascade that directly causes the HA-mac transcriptional response.

Nrf2 is a cap’n’collar transcription factor activated by oxidation of cysteines on its inhibitory partner, Keap.22 Thus, Nrf2 is activated by cellular oxidative stress or electrophiles, and its target genes include many that decrease oxidative stress (eg, superoxide dismutase, HO-1) or induce phase II metabolism (which catalobises exogenous electrophiles, eg, xenobiotics).22 Given this background, Nrf2 was a priori a strong candidate to mediate adaptive macrophage responses to heme,23 and this was supported both by experimental activation with t-BHQ and by gene silencing with siRNA. We cannot find a previous mechanistic dissection of the role of Nrf2 in HO-1 induction by heme, the physiological substrate for this enzyme.

Interestingly, Kadl et al have recently published a landmark article on Nrf2 and HO-1 in mouse macrophages.24 They stimulated mouse macrophages with the synthetic lipid 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine, which they had purchased and oxidized in-house by passive exposure to atmospheric oxygen. Oxidized 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine and palmitoyl-oxo-valeryl-phosphatidylcholine are defined oxylipids related to phosphatidylcholine; they are found in minimally modified low-density lipoprotein and have been extensively characterized as being bioactive.15 Oxidized 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine evoked a macrophage transcriptome pattern, designated by the authors as Mox, that included induction of HO-1, along with sulfiredoxin and thioredoxin genes. This did not occur in Nrf2-null mice, indicating that Nrf2 was required for HO-1 induction in this model.24 Nrf2-mediated induction of HO-1 via the antioxidant response element has been previously described in response to a wide variety of stimuli, including toxic electrophiles, dietary (vegetable) indirect antioxidants, and cigarette smoke.23,25–32 Although the Mox transcriptome analysis set the oxidized 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine–responding gene set apart from M1 and M2 subsets in the mouse, it remains to be seen whether this represents a genuinely committed stable lineage or a plastic state. Notwithstanding, our own transcriptome analysis of HA-Mac clearly distinguishes this putative atheroprotective phenotype from Mox, as well as M1 and M2 cells (J.J. Boyle et al, unpublished data, 2011).

A strength of our study has been the use of freshly isolated cells. Transcriptome analyses of plaque macrophages and pooled cell line microarray data indicate that gene expression in freshly isolated blood-derived monocytes is relevant to plaques.33 Moreover, a metaanalysis of microarray data giving a global map of human gene expression has revealed enormous differences between transformed cell lines and primary tissues.34 Thus, although human blood-derived monocytes are difficult to transfect, they are likely to be be far more informative about human pathophysiology. However, the use of fresh human cells has limitations, not least the difficulty of obtaining a complete knockdown using siRNA. We corroborated data with siRNA and extensively characterized pharmacological inhibitors, establishing concentration-dependent action with effects at appropriate concentrations.16,19–21 We cannot be sure to what degree the relatively modest reduction in HO-1 seen in cells treated with Nrf2 siRNA was due to gene silencing being only partial (89%) or to what degree it reflects the status of Nrf2 as just one of the transcription factors necessary for eliciting HA-mac features. The latter seems likely, as an experiment with cycloheximide showed that induction of HO-1 mRNA requires protein synthesis as an intermediary step. The identification of transactivators that operate in this system and that may deviate the stress response toward the HA-mac rather than Mox phenotype is the subject of a separate study (J.J. Boyle et al, unpublished data).
In conclusion, uncommitted monocyte-derived macrophages respond to intraplaque hemorrhage by internalizing Hb:Hp complexes, oxidized red blood cells, or both into lysosomes, resulting in the intracellular release of free heme. This in turn induces HO-1, in part via Nrf2, which leads to a phenotype similar to the one we have previously identified in the vicinity of hemorrhage in human plaques.

Sources of Funding
We thank the British Heart Foundation (Gerry Turner Intermediate Fellowship FS07/010 to J.J.B. and Professorial Core Support to D.O.H.) and the Imperial College Biomedical Research Centre for financial support.

Disclosures
None.

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Arterioscler Thromb Vasc Biol. 2011;31:2685-2691; originally published online August 25, 2011;
doi: 10.1161/ATVBAHA.111.225813

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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**Supplement Material**

**Methods**

**Cell culture and reagents**

Blood (45ml) was collected aseptically from normal human volunteers, with Ethical approval and informed consent. Monocytes were purified as described\(^1\), and were cultured at \(10^5\) cells per well in 24-well plates in Iscove’s modified Eagle’s medium supplemented with 10% autologous serum for the times specified.

All reagents were from Sigma-Aldrich (Poole, UK) except where otherwise stated, and were of the highest grade. Cycloheximide was supplied predissolved in Tissue-Culture grade vials and added to cultures at 100µM final concentration on the basis of previous data indicating lack of cell death over 24h at this concentration. Protoporphyrin IX (PPIX) and hemin (ferric PPIX) were from Sigma-Aldrich and were tested by ourselves as endotoxin-free (Limulus kit, Cambrex). Cobalt PPIX and Zinc PPIX were from Frontier Scientific (Carnforth, Lancashire, UK). Porphyrins were dissolved in hybridoma grade DMSO at 25mM stock, and diluted in IMDM to 100mM, before addition to cultures at the indicated concentrations (normally 10µM, unless otherwise stated). Vehicle controls were equivalent dilutions of DMSO of equivalent grade. Haemoglobin A\(_0\) ferrous-stabilised (Sigma-Aldrich) and was tested limulus-negative by ourselves as before (Limulus kit, Cambrex) \(^1\).

Haptoglobin (Hp) was purified human Hp 1-1 (Sigma-Aldrich) reconstituted in 1mL tissue culture water and endotoxin-tested negative. Hb:Hp complexes were prepared as before by 1:1 molar mixing of Hb and Hp for 20mins prior to addition to cultures at 100nM \(^1\). Oxidised erythrocytes were prepared by a minor modification of published methods. Autologous erythrocyte pellets were a byproduct of monocyte purification, diluted 1:5 in PBS, 1mM (final) \(\text{H}_2\text{O}_2\) added from 1.0M stock (Sigma Aldrich) for 120min at 37°C, then red cells
washed three times in PBS by resuspension and centrifugation, then added to monocytes at the indicated ratios. Visual inspection confirmed that there was no significant contamination with neutrophils.

**Macrophage oxidative stress**

This was essentially as previously described. Macrophages were adherence-purified and cultured in 96 well plates in 10% AHS-IMDM the presence or absence of additional reagents: heme (Sigma-Aldrich); HbHp (Sigma-Aldrich); POVPC (Cayman Chemicals); H$_2$O$_2$ (Sigma-Aldrich) for the concentrations and times indicated. To measure oxidative stress, we used aminophenylfluorescein (APF, Molecular Probes, Invitrogen, Paisley, UK). This is highly characterised as an electrophilic adduction-specific reagent that acts as validated selective reporter for highly reactive oxygen species (hypochlorite, hydroxyl free radical, peroxynitrite). In its activation, electrophilic species adduct to a terminal amino, which rearranges causing scission to liberate free fluorescein. Medium was exchanged for a PBS containing 5μM APF and then 30μg.mL$^{-1}$ OCl-LDL added. The OCl-LDL itself was also prepared essentially as previously described. Human low density lipoproteins (LDL, Calbiochem) were purchased, diluted to 1mg.mL$^{-1}$ and treated with 1mM NaOCl (Sigma-Aldrich) for 24h. Controls indicated that this had no direct effect on APF although APF has an assay sensitivity on purified NaOCl of approximately 1-10μM. This indicated that there was no detectable free NaOCl remaining, indicating the NaOCl was completely reacted with the LDL. LDL oxidation was confirmed as before using fluorescence and electrophoretic mobility shift (not shown) and OxLDL particle size was measured by confocal microscopy and flow cytometry (below). At the indicated time points, typically 24h, APF conversion to fluorescein is measured by the fluorescein channel (Ex490 ± 10nm; Em 530 ± 10nm) on a 96-well plate reader (Biotek Synergy HT, Potton, UK) using automated sensitivity adjustment and empty wells as low instrument reference, and APF without cells as blank signal.
**Flow cytometry**

This was by minor modification of published methods. Cells were resuspended in 10% AHS IMDM, and stained with CD163-FITC (Bachem) and HLA-DR-PE (Sigma-Aldrich), 1:50 each for 20mins, washed once in PBS and then analysed on a Coulter EPICS-IV or a Coulter CyAN (High Wycombe, UK). Data was analysed postacquisitionally with WinMDI as before.

**RNA analysis**

Total RNA was extracted from cells using Qiagen RLT buffer and Qiagen RNAEasy Mini columns following manufacturer’s instructions; reverse transcribed using Invitrogen Superscript-II, Invitrogen oligo-dT primers and Promega dNTPs following manufacturer’s instructions. Realtime quantitative PCR was with a 96-well Biorad iCycler, Eurogentec Mesa x2 mastermix (SybrGreen, Taq, buffer) and primers at 100fmol/µL. Expression was measured using the maximum curvature and ΔΔCt method normalised to hprt.

**Western blotting**

Macrophages were lysed in 200µL western lysis buffer, after which lysates were heated for 5 mins at 80°C, Total proteins were measured using BCA (Pierce kit) and concentrations adjusted to enable equal loading. Lysates were added to sample buffer and run in precast gradient gels wet blotted (Novex system, Invitrogen) to PVDF (Immobilon-P, Millipore). Membranes were blocked in 4% milk proteins 30mins 0.1% Tween-20 PBS and then immunostained 16 hours at 4°C with primary antibody. Primary antibodies were as follows: HO-1, mouse monoclonal 1:2,000 OSA-110, Stressgen; Nrf2, rabbit polyclonal 1:2,000 Ab63252, Abcam; beta-actin 1:10,000 Ab8226. Antibodies were detected with horseradish peroxidase conjugated anti-rabbit (Dako 1:2000) or anti-mouse (Dako 1:2000) and visualised
with ECL-Plus (GE Healthcare) and Hyperfilm (GE Healthcare), processed on a Kodak X-O-Mat automated developer unit.

**Transfection**

Monocytes were adherence-purified at $10^5$ cells per well and rested overnight. Polyethyleneimine liposomes (Interferin, PolyPlus) were added as 1µl for each well to be transfected to 1.5ml eppendorfs, and then mixed with 100pmol in 1µL RNAase free water (Santa Cruz) for each well to be transfected, vortexed gently and briefly spun; then complexes left to form to 10mins before adding to the monocytes in medium (10% AHS IMDM). To show uptake, fluoresceinated siRNA oligos were used (Control 1 –FITC, Santa Cruz). HO-1 siRNA was with Dharmacon human HO-1 sequence 1. Cells were incubated with oligos for 18h before challenge with heme.

**Confocal microscopy**

For culture and immunostaining, PBMCs were purified and monocytes adherence-separated on the glass of 8-well chamber slides (Labtek-II, Nunc, VWR) at approximately 500 cells / mm$^2$ (matching the 24-well plates) and rested overnight in standard medium (10% AHS IMDM). For live cell imaging, monocytes were cultured the same way, but instead in 8-well chambered coverglasses (also, Nunc, VWR). Images were acquired on a Zeiss LSM510Meta. For the live cell images, a CO$_2$-chamber / temperature chamber unit was deployed. For immunofluorescence, cells were washed twice briefly in PBS, fixed in acetone 4°C 5min, air-dried, blocked in 10% normal goat serum (Dako), incubated overnight in primary antibody at 4°C in a humidified chamber (Nrf2, 1:100, source above; HO-1, sc1796 1:100 Santa Cruz; ); washed three times in PBS, incubated for 2h at room temperature in 1:100 goat polyclonal secondary labelled with Alexa 488 or Alexa 568 as indicated (Molecular Probes, Invitrogen);
washed three times in PBS, counterstained in 1:1000 TOPRO-3 in PBS (Molecular Probes, Invitrogen) and mounted in 80% PBS/glycerol.

Live cell imaging was with LysoTracker Red (Molecular Probes, Invitrogen) in line with manufacturer’s instructions, 1:1000 20mins. Erythrocytes were labelled by incubation in Calcein-AM 1:1000 20mins in PBS (Molecular Probes, Invitrogen) and then added to the live cell cultures. Hemoglobin was labelled with Alexa488 using a hydroxysuccinimide-based Alexa488 covalent labelling kit (Molecular Probes) and then added to haptoglobin 1-1 (Sigma-Aldrich) at equimolar concentration for 20mins Labelled complexes were added to the cells. Phagocytosis was visualised in imaged in real time at 37ºC 5% CO₂ with confocal acquisition and visual inspection.

**Heme assay**

A chemical assay kit for free heme (BioVision hemin assay kit, Cambridge Biosciences, Cambridge, UK) was purchased and used according to manufacturer’s instructions. Cultures of macrophages in 24-well plates (10⁵ cpw) were incubated with 100:1 erythrocytes:macrophages or 100nM HbHp complexes for the times indicated, washed twice in PBS and then hypotonically lysed in 100μL ultrapure water. Of this lysate, 1μL was added to 100μL reaction mix with standards as recommended by manufacturer.

**Supplemental Figures - Online**

**Supplemental Figure I:** In (A) the y-axis is hROS determined by fluorescence with 1μM APF (Ex.488nm Em535nm); x-axis is time (in seconds) since addition of 30μg.mL⁻¹ OxLDL. Treatments as indicated. 4-ABH = 4-aminobenzoic acid hydrazide. Representative of n=5 donors. Mean ± SE. (B) concentration-effect curve for OxLDL; x-axis, OxLDL added to cultures (final concentration), y-axis, hROS (APF-measured fluorescence); (C) maximum
hROS response occurs 2 days after macrophage isolation and culture, x-axis days of macrophage culture after which APF and OxLDL were added and fluorescence measured; (D) Using forward scatter (FSC) as an indicator of cells size, OxLDL had a range of particle sizes up to 10µm. The arrow shows 10µm beads superimposed; (E) shows confocal microscopy of Nile-Red stained OxLDL particles in the region of 1-10µm, scalebar as indicated, Ex.546, Em; (F) Induction of macrophage hROS is blocked by cytochalasin-D: y-axis, hROS measured as before; x-axis, cells were incubated with APF and OxLDL in the presence of absence of cytochalasin-D (1µM) as indicated; (G) phagocytosable microbeads also elicit hROS: y-axis, elicited hROS as before; x-axis, cells incubated with APF treated with the presence (beads) or absence (control) of phagocytosable 10µm polystyrene microbead;

Supplemental Figure II: Heme is released by Hb:Hp complexes and RBCs in macrophages The figure shows UV-Vis spectrophotometry and a chemical assay for free heme, Hb:Hp or OxRBCs in macrophages. Since heme is coloured and its absorption depends on whether it is free or associated with globin, spectrophotometry is as sensitive and specific as more costly analyses. (A) Spectra for purified cell-free solutions of Hb:Hp complexes and OxRBCs as indicated; y-axis, absorbance, x-axis, wavelength. The spectra for OxRBCs and control RBCs were identical (not shown); (B) Spectra for purified cell-free solutions of Hb:Hp complexes and purified heme as indicated: y-axis, absorbance; x-axis, wavelength. This is displayed below (A) to allow easy matching of wavelengths; (C) Spectra for macrophages loaded with 10µg.mL⁻¹ Hb:Hp complexes done serially over approximately 1 week as indicated. Note the loss of the Hb peak and the emergence of short-wavelength absorption consistent with heme. Data are representative of n=5 donors. (D) Spectra for cultures of 1:1 OxRBCs : macrophages measured serially over approximately 1 week as indicated. Note the loss of the Hb peak and the emergence of short-wavelength absorption
consistent with heme. Data are representative of n=5 donors; (E,F) y-axes shows free heme measured in lysates of macrophages (free heme assay kit, Biovision); x-axes shows time after addition of either 10μg.mL⁻¹ Hb;Hp complexes (E) or 100:1 OxRBCs:macrophages (F). Data are mean ± SE, n=5 donors.

**Supplemental Figure III** Time course of oxidative stress in macrophages after heme stimulation. Heme (10μM) was added to macrophages and oxidative stress was measured (y-axis) by adding 1μM APF and 30μg.mL⁻¹ OxLDL at the times indicated (x-axis). hROS are increased at 1 h but are suppressed at 48 h, * p<0.05, ANOVA.

**Supplemental Figure IV** Heme suppresses macrophage oxidative stress induces CD163 and suppresses HLA-DR, but oxylipid POVPC and H₂O₂ do not (A) shows macrophage oxidative stress measured by adding OxLDL / APF as before (y-axis) 48h after addition of H₂O₂ 10μM or POVPC 50μg.mL⁻¹. (B-E) show flow cytometric histograms of macrophages cultured in H₂O₂ (B,D) or POVPC (C,E) for 4 days at concentrations indicated, and then stained for CD163 (B,C) or HLA-DR (D,E) as indicated. Iso = staining with isotype-matched control antibody.

**Supplemental Figure V:** Induced macrophage HO-1 protein colocalises to lysosomes
Monocytes were stimulated with Hb:Hp complexes (100nM) for 6 days, fixed in acetone and double immunofluorescence stained for CD68/FITC (green) and HO-1/Alexa568 (red) as indicated. (A) Quantification of HO-1 by image intensity analysis. Data are mean ± SE, from five donors.* p<0.05, Student’s t-test; (B) Image of a macrophage, with yellow cytoplasmic
structures indicative of colocalisation of HO-1 and CD68; (C) High power of box in (B) showing close-up of a single lysosome (this is at the limit of optical resolution). The white arrow shows colocalisation (yellow). (D) Image of unstimulated cells, also with anti-HO-1 immunostaining (red); there is an absence of red (HO-1); CD68-green, nuclear dye = blue.

**Supplemental Figure VI - Online Induction of HO-1 mRNA requires protein synthesis**

HO-1 mRNA was measured in freshly isolated monocytes by RT-qPCR and normalised against HPRT. (A) Concentration-effect curve of heme on HO-1 mRNA at 4 hours following challenge; (B) time-course of HO-1 mRNA following stimulation with heme (10μM); (C) effect of cycloheximide (100μg/ml) on induction of HO-1 mRNA at 4 hours after heme (10μM) stimulation. *p<0.05, Student’ t-test, from twelve donors. A concentration-effect curve for cycloheximide was carried and this concentration of cycloheximide did not affect macrophage viability (not shown).

**Supplemental Figure VII - Schema of mechanism of HA-mac**

Heme enters cells directly by free diffusion, Hb:Hp enters via CD163 and OxRBCs enter via CD204 (MSR1). Complex heme sources are trafficked to lysosomes, where they release heme. Heme, irrespective of source, stimulates HO-1 in early macrophages via Nrf-2 and an unknown transactivator. HO-1 then contributes to the transcriptional response leading to the HA-mac phenotype. Line and arrow symbols = gene activation. Green lines = activates.

Reference List

Supplemental Figure I
Supplemental Figure II

A. Graph showing absorbance of Whole RBCs and Purified HbHp complexes.

B. Graph comparing absorbance of 1μM HbHp (8 heme moieties) and 8μM Free Heme.

C. Graph showing absorbance of T0 - HbHp complexes and various time points.

D. Graph showing absorbance of T0 - OxRBCs and various time points.

E. Bar chart showing free heme (fmol/10^6 cells) at different time points since HbHp 100nM.

F. Bar chart showing free heme (fmol/10^6 cells) at different time points since RBCs (100:1).
Supplemental Figure IV
Supplemental Figure V

A

Control

Hb:Hp

HC-1 level per cell (AFU)

B

CD68

HO-1

Nuc

D

CD68

HO-1

Supplemental Figure V
Supplemental Figure VI
Supplemental Figure VII