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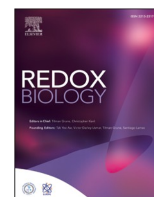

Distinct metabolic responses to heme in inflammatory human and mouse macrophages – Role of nitric oxide

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ABSTRACT

Activation of inflammation is tightly associated with metabolic reprogramming in macrophages. The iron-containing tetrapyrrole heme can induce pro-oxidant and pro-inflammatory effects in murine macrophages, but has been associated with polarization towards an anti-inflammatory phenotype in human macrophages. In the current study, we compared the regulatory responses to heme and the prototypical Toll-like receptor (TLR)4 ligand lipopolysaccharide (LPS) in human and mouse macrophages with a particular focus on alterations of cellular bioenergetics. In human macrophages, bulk RNA-sequencing analysis indicated that heme led to an anti-inflammatory transcriptional profile, whereas LPS induced a classical pro-inflammatory gene response. Co-stimulation of heme with LPS caused opposing regulatory patterns of inflammatory activation and cellular bioenergetics in human and mouse macrophages. Specifically, in LPS-stimulated murine, but not human macrophages, heme led to a marked suppression of oxidative phosphorylation and an up-regulation of glycolysis. The species-specific alterations in cellular bioenergetics and inflammatory responses to heme were critically dependent on the availability of nitric oxide (NO) that is generated in inflammatory mouse, but not human macrophages. Accordingly, studies with an inducible nitric oxide synthase (iNOS) inhibitor in mouse, and a pharmacological NO donor in human macrophages, reveal that NO is responsible for the opposing effects of heme in these cells. Taken together, the current findings indicate that NO is critical for the immunomodulatory role of heme in macrophages.

1. Introduction

Heme, an iron-containing tetrapyrrole, plays major biological roles in all aerobic organisms. It serves essential physiological functions as a prosthetic group of numerous hemoproteins for oxygen storage and transport (hemoglobin, myoglobin), electron transfer in the respiratory chain (cytochrome *c*) and in drug metabolism (cytochrome P450) [1–3]. Moreover, heme is a crucial signaling molecule and is involved in differentiation of tissue-specific iron-recycling macrophages [4,5]. In contrast, 'free', i.e. non-protein bound heme, can catalyze the Fenton reaction, an oxidation process activated by iron (II) in the presence of hydrogen peroxide that triggers pro-oxidant, pro-inflammatory and cytotoxic effects [6,7]. Clinically relevant disorders, in which free heme plays a critical pathophysiological role encompass hemolytic (e.g. sickle cell anemia, β-thalassemia or malaria [8–10]) and non-hemolytic conditions (e.g. ischemia-reperfusion injury, sepsis and atherosclerosis [3,11,12]).

The interaction between heme and macrophages is critical for the regulation of inflammatory responses [13]. Heme can mediate opposing pro- [13] or anti-inflammatory phenotypical alterations in macrophages

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[14–16] depending on the experimental setting used or the composition of the cellular microenvironment. Pro-inflammatory effects of heme in macrophages have been associated with activation of Toll-like receptor (TLR)4 signaling causing secretion of pro-inflammatory cytokines and activation of the inflammasome [17,18]. The mechanisms of heme-dependent TLR4 signaling have remained ill defined [16,19] and TLR2 has recently also been shown to mediate pro-inflammatory effects of heme [20]. Notably, heme can also cause anti-inflammatory effects in macrophages that may involve heme oxygenase-1 (HO-1)-dependent catabolism of heme. Enzymatic degradation of heme by HO-1 produces carbon monoxide (CO) and bilirubin [21], both of which exhibit potent anti-inflammatory and antioxidant properties [22,23]. Moreover, in human macrophages, heme can induce the so-called *Mhem* phenotype that is distinct from the prototypical M1/M2 polarization. This phenotype is characterized by increased expression of HO-1 and the anti-inflammatory cytokine interleukin (IL)-10 that has been associated with atheroprotective effects [12,24].

In macrophages, modulation of mitochondrial function and cellular bioenergetics strongly contributes to inflammatory activation. In particular, activated mouse macrophages undergo so-called metabolic reprogramming (also termed metabolic repurposing of mitochondria), in which they shift from ATP production via oxidative phosphorylation (OxPhos) to that via glycolysis upon LPS stimulation [25]. Notably, while a comparable bioenergetics switch from mitochondrial-dependent energy production to glucose utilization has recently been observed for heme-treated murine macrophages, others have reported that under similar conditions heme leads to a different metabolic rewiring by increasing glycolysis at the expense of impaired mitochondrial fatty acid β-oxidation [9,26]. Furthermore, it is not known whether heme induces metabolic reprogramming in human macrophages. This is important given that various conditions of hemolysis result in increased levels of free heme and human macrophages are less susceptible to mitochondrial dysfunction by lipopolysaccharide (LPS) compared to mouse cells [27]. Nitric oxide (NO) over-production has previously been shown to cause mitochondrial dysfunction in macrophages upon inflammatory activation [28,29]. Remarkably, NO appears to promote inflammation only in activated mouse, but not human macrophages because the latter do not produce NO in response to stimulation with LPS [30].

The major goal of the present study was to compare the effect of heme with that of the prototypical TLR4 ligand LPS on inflammatory activation and mitochondrial function in primary human monocyte-derived macrophages (hMDMs), and mouse bone marrow-derived macrophages (mBMDMs). It is demonstrated that the regulatory response to heme involving mitochondrial metabolism and inflammatory activation in LPS-activated hMDMs is different from that in mBMDMs. Finally, we show that the presence or absence of NO largely determines the pro-inflammatory effects of heme in mouse macrophages and its anti-inflammatory activity in human cells.

2. Results

2.1. Differential transcriptomics patterns in heme- and LPS-treated human macrophages

Heme has previously been demonstrated to activate TLR4, the receptor sensing the pro-inflammatory compound LPS [31]. To assess the comparative effects of heme and LPS on RNA expression, bulk transcriptomics was performed in hMDMs from four individual donors treated with either heme or LPS (Fig. S1 “Data processing”). Unbiased principal component analysis (PCA) revealed that heme and LPS caused differential transcriptional expression patterns in these cells (Fig. S2A). The distinct regulatory patterns based on hierarchical clustering are shown as heatmap (Fig. 1A). Unsurprisingly, genes up-regulated by LPS stimulation were predominantly associated with ‘cytokine-mediated signaling’ and ‘cellular response to cytokine stimuli’, as determined by gene ontology analysis (Fig. 1B–S2B). Furthermore, LPS induced the specific expression of pro-inflammatory genes such as IL-1β, IL-6 and cyclooxygenase-2 (COX-2), synonymous with prostaglandin-endoperoxide synthase-2 (PTGS2), in hMDMs (Fig. 1C). By contrast, exposure to heme did not alter expression of pro-inflammatory genes, but led to an up-regulation of the inducible heme-degrading enzyme HO-1 and various NF-E2-related factor-2 (Nrf2)-inducible antioxidant genes such as glutamate-cysteine ligase modifier subunit (GCLM) and NAD(P)H dehydrogenase quinone 1 (NQO1) (Fig. 1C and D) indicating that heme and LPS cause markedly different profiles of gene expression in hMDMs. Interestingly, we also observed differential effects of heme and LPS on glycolysis-related genes including Slc2a1, Pfkfb3, Hif1α and ACOD1 (Fig. 1C). Genes that were significantly up- or down-regulated by heme are shown with the highest adjusted p-value in heme-treated hMDMs relative to LPS (Fig. 1E). Collectively, these data indicate that heme does not activate classical TLR4-mediated cytokine signaling, but causes an increased expression of distinct anti-inflammatory genes in human macrophages.

2.2. Opposing effects of heme on inflammation in human and mouse macrophages

To evaluate the effects of heme in inflammatory human macrophages, mRNA expression levels of TNF-α, IL-6 and COX-2 were determined in hMDMs treated with heme, LPS alone or the combination of heme + LPS. In contrast to LPS, heme alone did not appreciably affect the expression of TNF-α, IL-6 and COX-2, confirming our transcriptomics data in Fig. 1C. However, heme counter-acted the LPS-dependent induction of these pro-inflammatory genes in a concentration-dependent manner (Fig. 2A). In addition, heme led to an up-regulation of HO-1 expression (Fig. 2B), a decrease of the M1 cell surface marker CD64 and an increase of the M2 marker CD206 (Fig. 2C). Notably, the inhibitory effects of heme-on LPS-dependent cytokine up-regulation varied considerably in individual donors. Because regulation by heme is dependent on its interactions with serum proteins [32,33], we also examined the effects of increasing serum concentrations on responses to heme and LPS. Serum enhanced mRNA expression levels of TNF-α and IL-6 induced by LPS or heme + LPS, but decreased HO-1 in heme-treated hMDMs dose-dependently (Figs. S3A and S3B), indicating an association with lower HO-1 levels and increased inflammation. Serum proteins such as albumin are known to block the activity and uptake of heme [31,32]. Accordingly, our data indicate that the anti-inflammatory effects of heme are critically dependent on its interactions with heme-binding proteins. In contrast to these findings in human macrophages, previous reports showed that heme exerts opposing pro-inflammatory effects in mouse macrophages [17,31]. Therefore, we also determined the levels of TNF-α and IL-6 in response to heme, LPS or heme + LPS in mBMDMs. While heme alone had no effect in mBMDMs (Fig. 3A), we confirmed that it markedly enhanced the expression of pro-inflammatory genes and HO-1 in the presence of LPS (Fig. 3A and B). Collectively, the data demonstrate that heme has opposing effects in macrophages depending on the species considered: in LPS-activated hMDMs heme attenuates the pro-inflammatory response while exacerbating inflammation in mBMDMs.

2.3. Human and mouse macrophages exhibit distinct profiles of mitochondrial bioenergetics when treated with heme and LPS

Activation of macrophages is associated with alterations in mitochondrial metabolism [25,34]. Because human and mouse macrophages exhibit differential metabolic responses to LPS [27], the regulatory effects of heme and LPS on cellular bioenergetics were compared in hMDMs and mBMDMs. By means of extracellular flux analysis, using oxygen consumption rate (OCR) as a marker of mitochondrial function, a Mito Stress assay revealed no impairment of mitochondrial respiratory parameters in LPS- or heme-treated hMDMs (Fig. 4A). By contrast, in mBMDMs, LPS significantly reduced mitochondrial respiration while

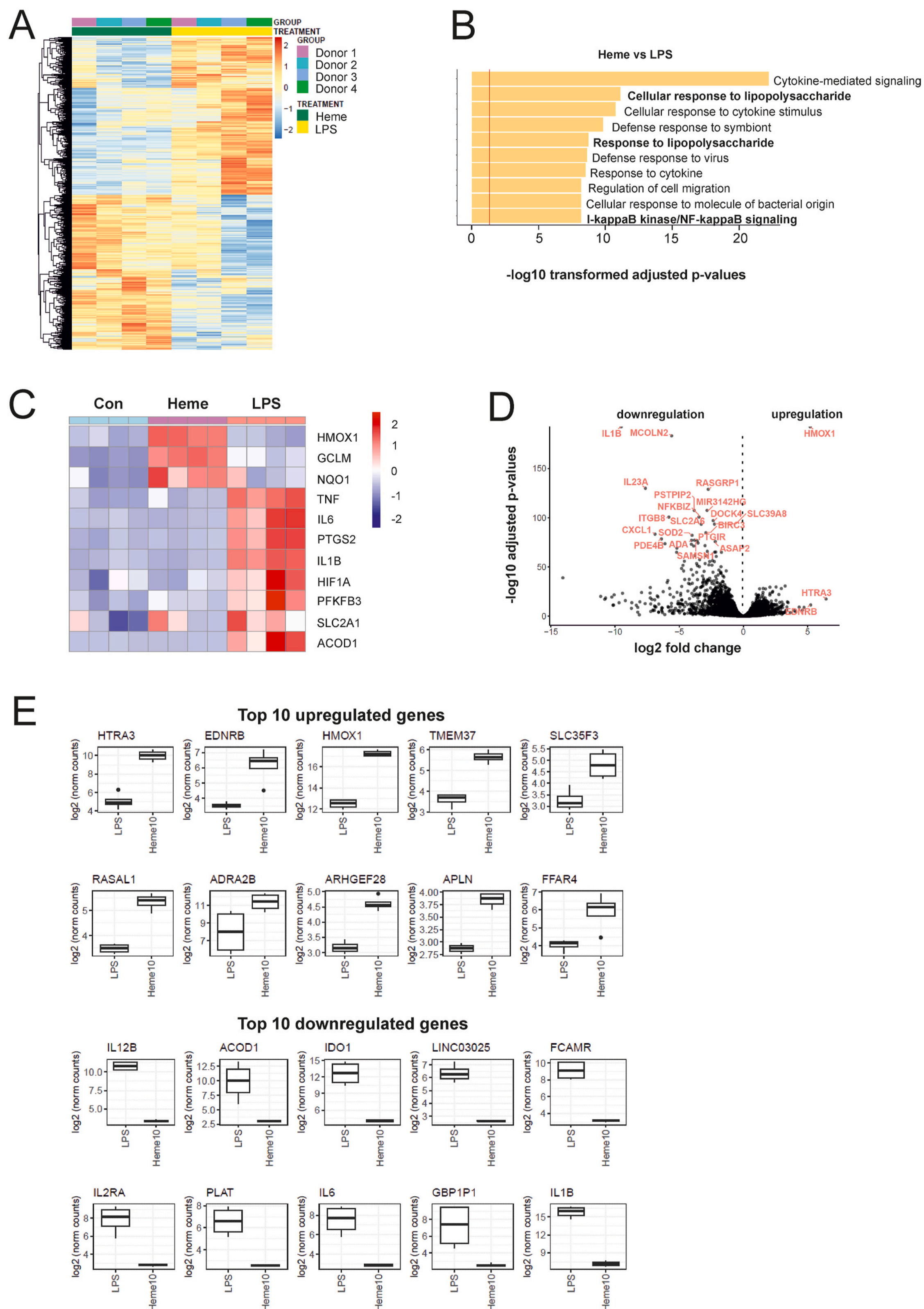

Fig. 1. Differential effect of heme and LPS on the gene expression profile of human macrophages. hMDMs were treated with heme (10 μM) and LPS (1 μg/ml) alone for 6 h and the bulk transcriptome was compared (n = 4). **(A)** Heatmap from differentially expressed genes (DEGs) in heme- vs LPS-treated cells. **(B)** Gene ontology cellular compartments (GOCC) analysis of differentially expressed genes in heme vs LPS. Gene Set Enrichment Analysis was performed using EnrichR. Significant GOCC database were defined as those having an adjusted p-value smaller than 0.05. **(C)** Heatmap representation of DEGs. The heatmap was scaled across genes and generated based on R package pheatmap v1.0.12. **(D)** Volcano plots for the DEGs of heme vs LPS. The x-axis represents the log2-fold change values, and the y-axis represents -log10 adjusted p-values. DESeq2 was used to calculate the normalized reads from raw counts. **(E)** Boxplots for the top 10 up-regulated and down-regulated DEGs of heme vs LPS. See also Figs. S1 and S2.

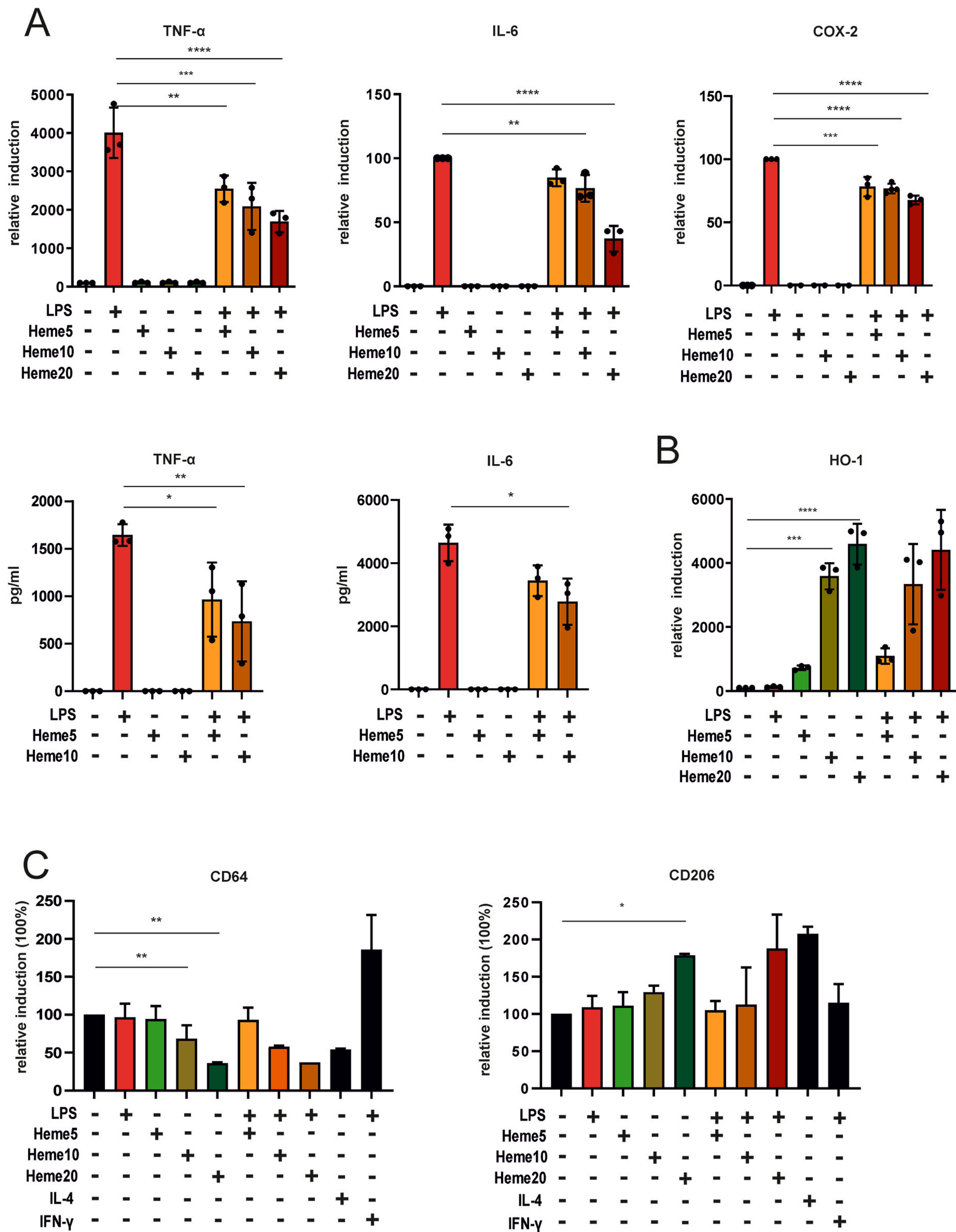

Fig. 2. Heme is anti-inflammatory in LPS-stimulated human macrophages. hMDMs were treated with various concentrations of heme and with LPS (1 μg/ml) alone or in combination for 16 h, as indicated. **(A)** Gene expression of TNF-α, IL-6, COX-2 were measured by real-time RT-PCR and cell culture supernatants were collected for ELISA to detect TNF-α and IL-6. **(B)** Gene expression of HO-1: values were normalized to the expression of HPRT and the respective ΔΔCT values are shown. **(C)** hMDMs were stimulated with IFN-γ (10 ng/ml), IL-4 (20 ng/ml), LPS or heme for 24 h. Expression of the M1 marker CD64 and the M2 marker CD206 were analyzed by flow cytometry and the fold induction of mean fluorescence intensity relative to unstimulated control macrophages is shown (n = 3). The values represent mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. See also Fig. S3.

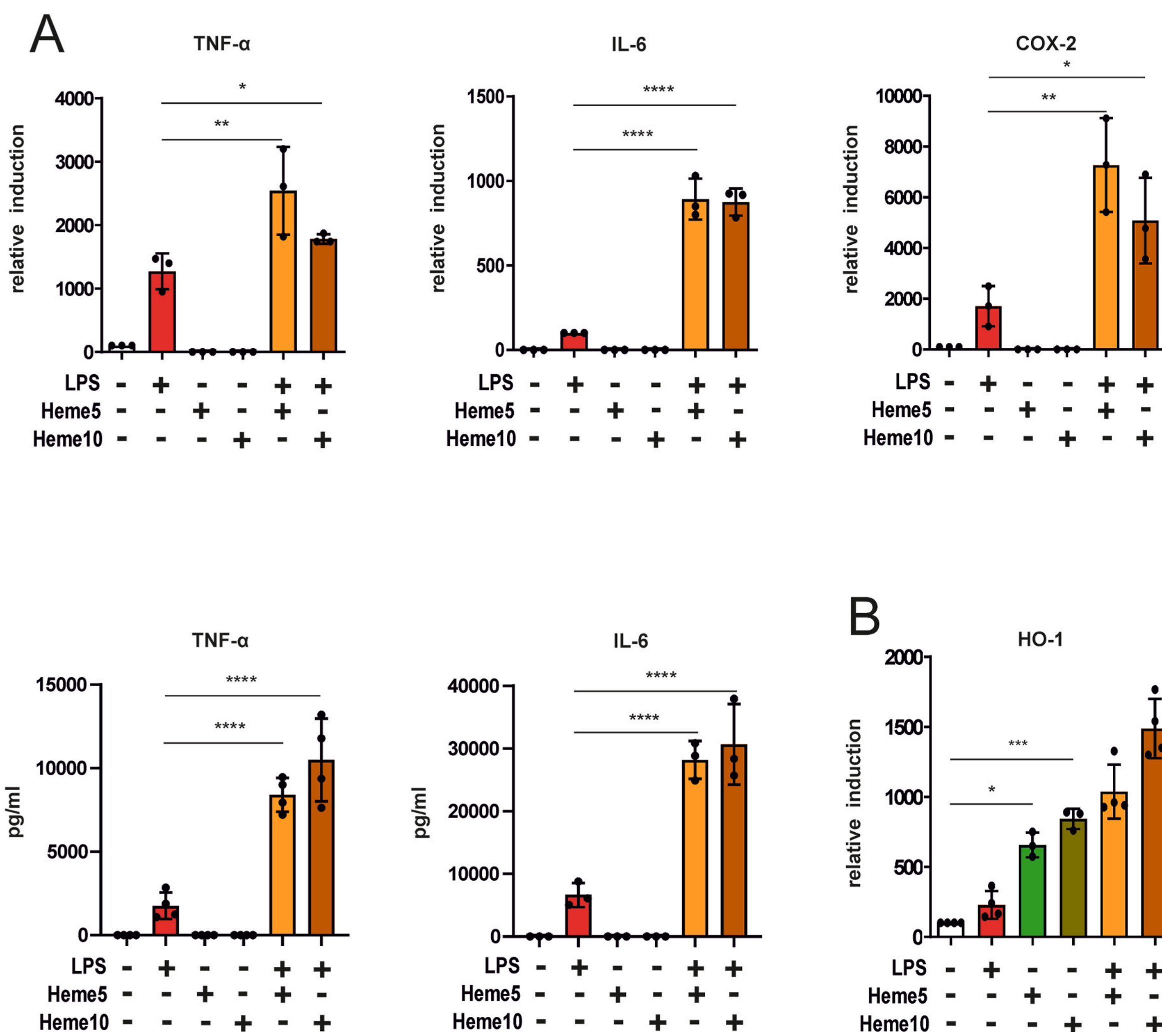

Fig. 3. Heme is pro-inflammatory in LPS stimulated mouse macrophages. mBMDMs were treated with heme (5 or 10 μM) and LPS (1 μg/ml) for 16 h. **(A)** Gene expression of TNF-α, IL-6, COX-2 were measured by real-time RT-PCR and culture supernatants were collected for ELISA to detect TNF-α and IL-6. **(B)** Gene expression of HO-1: values were normalized to the expression of HPRT and the respective ΔΔCT values are shown. The values represent mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

heme had no effect (Fig. 4B). Remarkably, exposure of hMDMs to heme significantly reduced basal glycolysis, as indicated by a decreased extracellular acidification rate (ECAR) (Fig. 4C). Consistent with this observation, heme-treated hMDMs exhibited lower levels of glycolysis-related genes (Fig. 1C). In contrast, heme stimulation in mBMDMs led to a minor increase in basal glycolysis that was markedly lower compared to the effect by LPS (Fig. 4C). These bioenergetic alterations were associated with changes in intracellular ATP levels, which were increased by heme in human, but not mouse macrophages (Fig. 4D). Taken together, these findings indicate that heme alone does not differentially affect OxPhos in human and mouse macrophages.

We also examined the influence of heme on LPS-activated macrophages. In hMDMs, we found that simultaneous treatment with heme + LPS did not affect the mitochondrial respiratory parameters and glycolysis (Fig. 5A and B). By contrast, in mBMDMs heme + LPS caused mitochondrial dysfunction and increased glycolysis (Fig. 5C and D). These findings suggest that heme causes species-specific regulatory differences in bioenergetics of LPS-activated human and mouse macrophages.

2.4. Availability of NO is critical for the pro-inflammatory effects of heme in LPS-activated mouse macrophages

A key difference between LPS-activated human and murine macrophages is the increased expression of inducible nitric oxide synthase (iNOS) and consequent NO production that only occurs in mouse cells [30,35,36]. Accordingly, a number of metabolic changes in mouse macrophages, including impairment of mitochondrial respiration and mitochondrial function due to inflammatory activation, have been attributed to NO [37,38]. Hence, we examined whether pharmacological inhibition of iNOS enzyme activity by treatment with 1400W may affect the secretion of pro-inflammatory cytokines elicited by heme + LPS in mBMDMs (Fig. 6A). As expected, exposure to 1400W decreased nitrite to levels measured in control cells (Fig. 6B). Importantly, 1400W also decreased the mRNA expression and secretion of TNF-α and IL-6 in mBMDMs stimulated with LPS and heme + LPS (Fig. 6C and D). To further prove that NO plays a role in enhancing the pro-inflammatory response human macrophages were pre-treated with the NO donor DETA-NONOate (DETA) to compensate for their lack of iNOS before the inflammatory challenge. As shown in Fig. 6E, the presence of NO exacerbated LPS-induced TNF-α, IL-6 and COX-2 expression. More interestingly, under these conditions heme lost its anti-inflammatory effect in human cells and acquired a pro-inflammatory nature. Indeed,

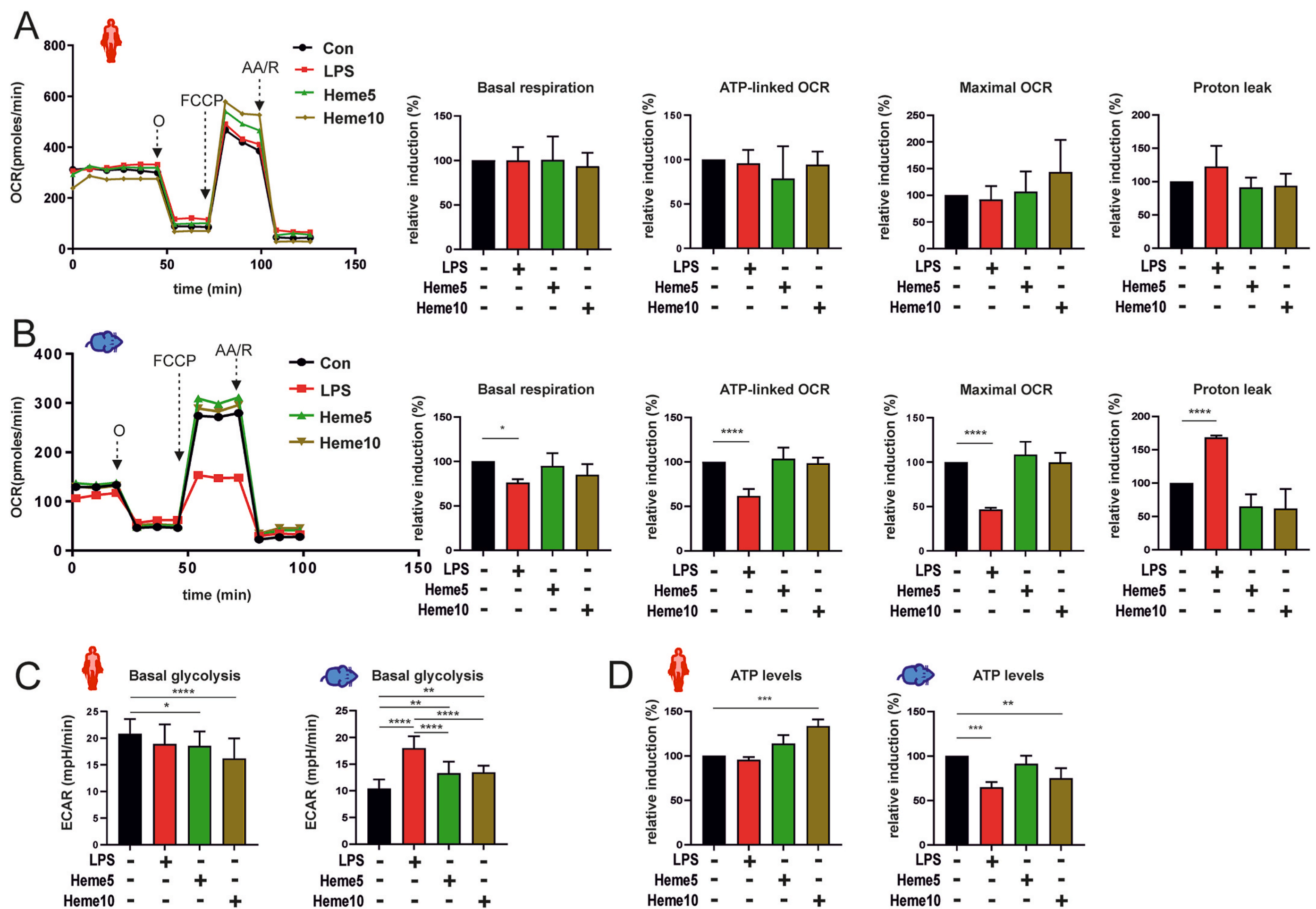

Fig. 4. Heme differentially affects bioenergetics in human and mouse macrophages. (A–B) hMDMs and mBMDMs were treated with heme (5 or 10 μM) or LPS (1 μg/ml) alone for 16 h and subjected to a Mito Stress test consisting of the sequential addition of oligomycin (1 mg/ml), FCCP (0.7 μM), and rotenone (1 μM) plus antimycin A (1 μM). The Mito Stress profile and the values of the calculated respiratory parameters are shown as mean ± SEM of three independent experiments. **(C)** Extracellular acidification rate (ECAR), an index of glycolysis, is shown. **(D)** ATP levels were assessed in hMDMs and mBMDMs by an ATP assay 16 h after heme or LPS treatment. Statistical analysis was performed using one-way ANOVA with Tukey's test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Con, control; FCCP, carbonyl cyanide *p*-trifluoro methoxyphenylhydrazone.

combined treatment with DETA + heme + LPS led to an enhanced expression of inflammatory markers in LPS-activated hMDMs (Fig. 6E). These data clearly indicate that the absence of NO production in activated human macrophages precludes the manifestation of the pro-inflammatory activity of heme upon LPS challenge. To address whether mitochondrial dysfunction is associated with the pro-inflammatory effects of heme in LPS-activated macrophages, hMDMs were pre-treated with the OxPhos inhibitor oligomycin. We observed that oligomycin reversed the anti-inflammatory effects induced by heme in hMDMs, enhancing the LPS-mediated expression of TNF-α and IL-6 (Fig. S4). Thus, when mitochondrial function is preserved in LPS-treated hMDMs, heme mediates anti-inflammatory activities. In contrast, if mitochondrial function is compromised, heme also becomes pro-inflammatory in human cells. Collectively, these data indicate that NO plays a crucial role in mediating the pro-inflammatory effects of heme in activated macrophages.

3. Discussion

The major findings of the current study are that 1) heme possesses a pro-inflammatory nature in LPS-activated murine macrophages; 2) heme exerts anti-inflammatory effects in LPS-activated human macrophages that are independent of TLR4 signaling; 3) availability of NO is critical for determining the immunomodulatory effects of heme.

3.1. *Anti-inflammatory effects of heme in human macrophages*

Heme is a damage-associated molecular pattern (DAMP) that has been proposed to activate TLR4 signaling [6,31,39]. The current findings in primary human macrophages clearly show that only a portion of the heme-mediated effects occur via TLR4 signaling, demonstrating that the regulatory effects of heme are distinct from those elicited by the prototypical TLR4 activator LPS [19]. Specifically, RNA sequencing data from hMDMs indicated that heme did not affect the classical MyD88/NF-κB pathway that is normally engaged by LPS [40], but rather the Kelch-like ECH-associated protein 1 (Keap1)/Nrf2 cascade [41]. These findings are consistent with previous reports showing that heme counteracts the pro-inflammatory activation of hMDMs [12,16] via the Nrf2 pathway [16,42]. Nrf2 may mediate the anti-inflammatory effects of heme via HO-1-dependent and/or -independent mechanisms [16]. For example, Nrf2 inhibits LPS-dependent up-regulation of IL-6 and IL-1β in macrophages via a transcriptional mechanism that involves the proximal promoter region of these pro-inflammatory cytokine genes [43]. Independently, BACH1, a heme-regulated nuclear repressor, has previously been demonstrated to affect mitochondrial function and inflammatory responses in macrophages [44]. Therefore, BACH1 likely plays a critical role for the heme-dependent immunomodulation of these cells.

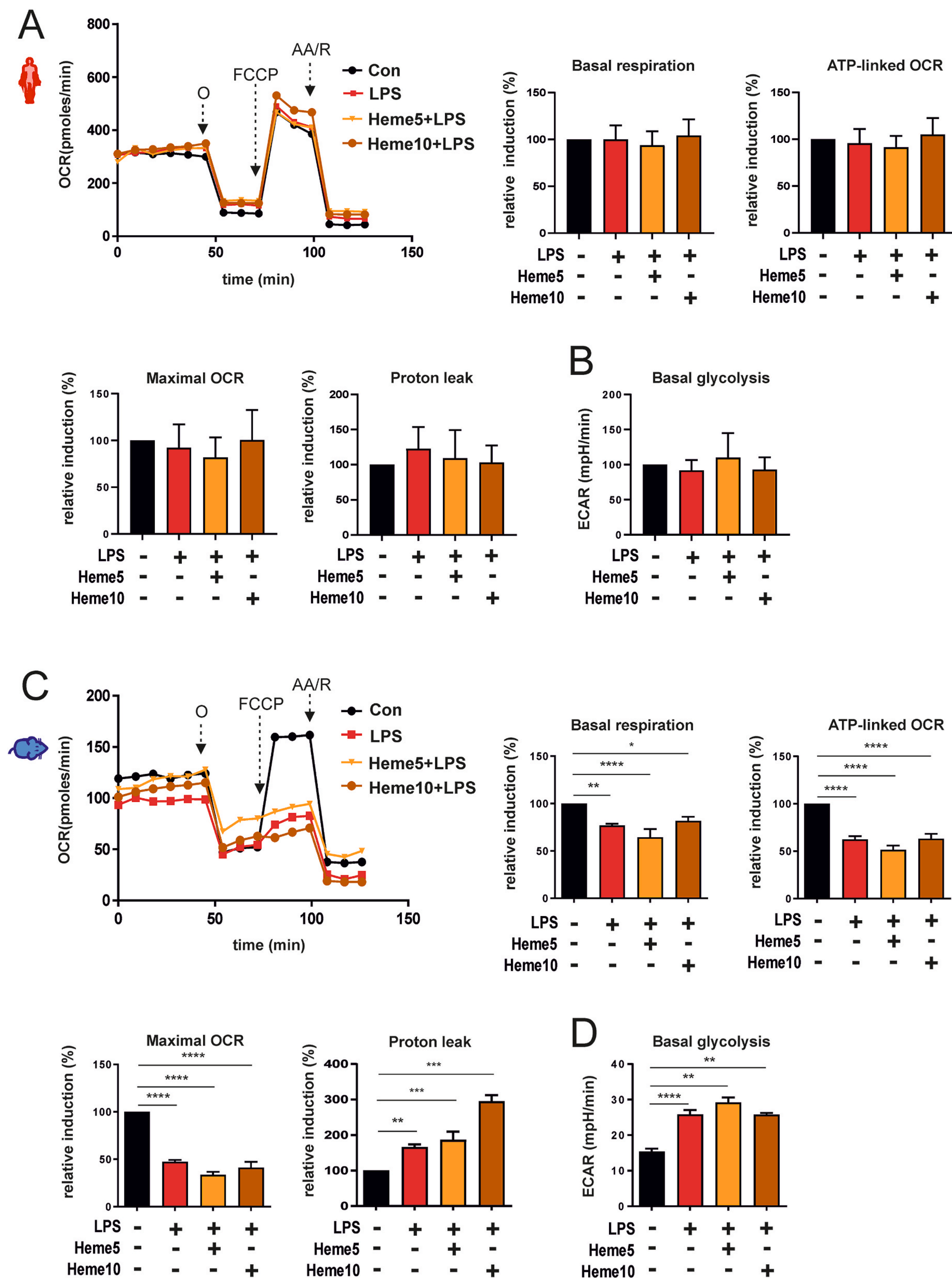

Fig. 5. Heme differentially affects mitochondrial bioenergetics in LPS-activated human and mouse macrophages. hMDMs and mBMDMs were treated with heme (5 or 10 μM) or LPS (1 μg/ml) alone for 16 h and subjected to a Mito Stress test after the sequential addition of oligomycin (1 mg/ml), FCCP (0.7 μM), and rotenone (1 μM) plus antimycin A (1 μM). **(A)** Mito-stress profile in hMDMs and respiratory parameters. **(B)** Extracellular acidification rate (ECAR), an index of glycolysis, is shown. **(C)** The Mito-Stress profile in mBMDMs and the values of the calculated respiratory parameters are shown as mean ± SEM of three independent experiments. **(D)** ECAR: statistical analysis was performed using one-way ANOVA with Tukey's test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Con, control; FCCP, carbonyl cyanide *p*-trifluoro methoxyphenylhydrazone. See also Fig. S4.

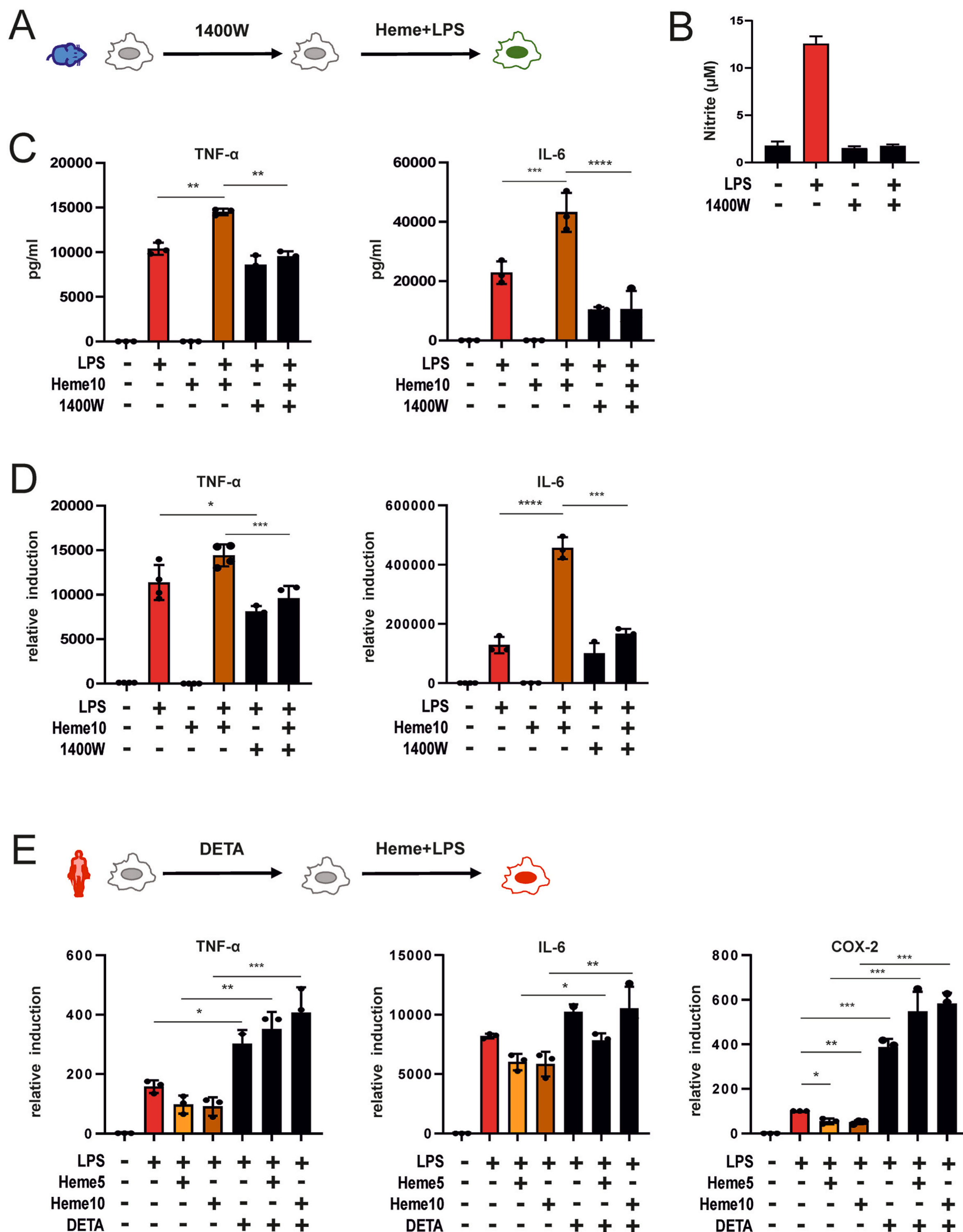

Fig. 6. Inhibition of NO activity reduces pro-inflammatory cytokine production in mouse macrophages. (A) mBMDMs were pre-treated with 1400W for 30 min followed by exposure to heme (10 μM) and LPS (1 μg/ml) alone or in combination for 16 h **(B)** Nitrite production was measured by the Griess assay. **(C)** Culture supernatants were collected for ELISA to detect TNF-α and IL-6. **(D)** Gene expression of TNF-α and IL-6 was measured by real-time RT-PCR. The values were normalized to the expression of HPRT and the respective ΔΔCT values are shown. **(E)** hMDMs were pre-treated with DETA (1 mM) for 1 h followed by stimulation with heme (10 μM) and LPS (1 μg/ml) for 16 h. Gene expression of TNF-α, IL-6 and COX-2 were measured by real-time RT-PCR. The values were normalized to the expression of HPRT and the respective ΔΔCT values are shown. The values are shown as mean of ±SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's test or unpaired *t*-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.2. NO availability is critical for the distinct regulatory effects of heme in inflammatory human and mouse macrophages

Our findings indicate that species-specific differences of iNOS gene expression [35,36] are critical for the opposing immunomodulatory effects of heme in human and mouse macrophages. The pro-inflammatory effects of heme are directly dependent on the availability of NO, which has previously been shown to cause mitochondrial dysfunction in mouse macrophages [28,29]. Accordingly, inhibition of iNOS activity decreased heme-induced TNF-α and IL-6 secretion in our model of LPS-activated mouse macrophages. In line with our current data, a previous study demonstrated that the NO donor DETA inhibits mitochondrial function in LPS + IFN-γ-activated human macrophages and enhances the secretion of pro-inflammatory cytokines [29]. This effect is possibly due to abrogation of mitochondrial respiration by NO. NO is known to affect OxPhos [28] through inhibition of the mitochondrial complexes I and IV [28,45]. Crucially, NO has also been shown to regulate enzymes of the tricarboxylic acid cycle and their metabolites, all of which are linked with a switch into a pro-inflammatory phenotype [38,46]. Of note, different nitrate concentrations in the cell culture media for human (RPMI 1640: nitrate 0.69 mM) and mouse macrophages (DMEM: nitrate 0.247 μM) do not appear to have an effect on the cellular availability of NO. In particular, higher nitrate levels in RPMI 1640 media do not affect the inflammatory response in human macrophages without addition of the NO donor DETA. Moreover, our current findings reveal that blocking of mitochondrial respiration in human cells by treatment with the OxPhos inhibitor oligomycin reversed the anti-inflammatory effects of heme. The effect of oligomycin in human cells is similar to the NO-dependent inhibition of respiration in mouse macrophages that contributes to an enhanced pro-inflammatory phenotype, as indicated by an increased TNFα and IL-6 production. From these data, we conclude that inhibition of mitochondrial respiration is responsible for the pro-inflammatory effects of heme in LPS-activated macrophages irrespective of the species.

Notably, the species-specific effects of heme on cellular bioenergetics in inflammatory human and mouse macrophages seem to agree with our previous report showing that LPS causes distinct cellular bioenergetics profiles in human and mouse macrophages [27]. The observation that mBMDMs respond to heme with a minor increase in glycolysis without affecting OxPhos is similar to findings reported by Pfefferle and colleagues [16], but in conflict to those of another study [26]. This could be due to variations in experimental conditions such as concentrations of heme and/or the presence of serum. Because heme alone did not affect mitochondrial respiration in our experimental setting, the dysfunction caused by combined treatment with heme + LPS appears to be primarily mediated by NO in mouse macrophages. The presence or absence of NO production is not the only difference between LPS-activated human and mouse macrophages. For example, the basal mitochondrial membrane potential is markedly higher in hMDMs compared to mBMDMs [27]. Thus, it might be worthwhile to investigate whether and how mitochondrial membrane potential regulates NO-dependent metabolic adaptation and inflammatory polarization in macrophages.

3.3. Heme and its interactions with NO

NO mediates LPS-dependent induction of the heme-degrading enzyme HO-1 in macrophages [47]. In the endothelium, NO enhances cellular uptake of heme resulting in increased HO-1 upregulation [48]. These findings could explain how an interaction of heme with NO may modulate inflammatory responses. Stuehr and colleagues have previously demonstrated that NO influences heme insertion into target apo-proteins and how this process may ultimately affect downstream events [49,50]. In particular, NO has been suggested to be a regulator of heme distribution within the cell and to be important for modulating various biological functions of hemoproteins [51]. Given the role of redox signaling in the regulation of mitochondrial function, it is conceivable that heme-NO complexes may enhance superoxide production [52]. Furthermore, in the presence of NO and superoxide (observed during inflammation), heme is known to facilitate the production of the damaging species peroxynitrite [53]. How the effects of heme and NO may differ in various cell types and/or species remains to be investigated in more detail.

3.4. Translation into the clinic

Beneficial immunomodulatory effects of heme have been demonstrated in various preclinical animal models. For example, heme treatment exerted salutary anti-inflammatory effects in acute lung injury, diabetes-induced nephropathy and colitis [15,54–56]. More recently, albumin-bound heme and liposomal encapsulated heme have been shown to induce an anti-inflammatory phenotype in macrophages, suggesting that the pharmacological actions of heme may be critically dependent on the formulation of this compound [16,57]. It is also noteworthy that administration of heme arginate, a pharmacological compound consisting of heme and arginine, has been approved for the treatment of acute intermittent porphyria in the clinic [58,59]. In addition, heme arginate has also been successfully applied in a clinical phase IIB trial as an inducer of HO-1 in renal transplantation [60].

3.5. Conclusion

In summary, the current study demonstrates that heme has opposing pro- and anti-inflammatory effects in mouse and human macrophages depending on the availability of NO and/or mitochondrial function. Notably, findings from pro-inflammatory activated mouse macrophages treated with heme cannot be necessarily translated to human cells. A better understanding of these complex regulatory events may lead to novel therapeutic approaches in the treatment of various inflammatory and hemolytic diseases with high levels of circulating heme.

4. Materials and methods

4.1. Reagents

Recombinant cytokine macrophage colony-stimulating factor (M-CSF) was purchased from PeproTech Inc., USA. Penicillin-streptomycin was purchased from C.C.Pro GmbH (Oberdorla, Germany). Lipopolysaccharide serotype 0111:B4 was from Invivogen (San Diego, CA, USA). Accutase was obtained from Capricorn Scientific (Ebsdorfergrund, Germany). 1400W was purchased from Cayman solutions (Michigan, USA). DETA was from Fisher Scientific (Schwerte, Germany) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless indicated otherwise.

4.2. Mice

Wild type C57BL/6J mice were used at 6–14 weeks of age. Mice were kept on a standard laboratory diet and were housed in cages under standardized environmental conditions (12 h light/dark cycle, 23 ± 1 °C and 55 ± 1 % relative humidity). All animal experiments were approved by the Committee for Animal Welfare.

4.3. Cell cultures

Human peripheral mononuclear blood cells were isolated from healthy donors by density centrifugation using lymphosep (c.c. pro GmbH, Thuringia, Germany). Adherent monocytes were then cultured and differentiated in RPMI 1640 medium (nitrate concentration: 0.69 mM) containing 5 % AB-serum, 100 U/ml penicillin, and 10 mg/ml streptomycin and 25 ng/ml recombinant human macrophage-colony stimulating factor (M-CSF). Mouse bone marrow cells were flushed from the tibia and femur of male C57BL6/J mice and cells were cultured

for 7 days in DMEM high glucose media (nitrate concentration: 0.247 μM) containing 10 % fetal bovine serum (FBS) (Merck), 100 U/ml penicillin, 100 mg/ml streptomycin and 25 ng/ml recombinant mouse M-CSF. On day 7, cells were plated in a 12-well plate at 3 x 10^5 cells per well. All treatments were performed in 1 % serum containing 12.5 ng/ml M-CSF and LPS was used at a concentration of 1 μg/ml.

4.4. Analysis of mRNA expression

Total RNA isolation was performed using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was carried out using high Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Primers for quantification of mRNA levels of HO-1, COX-2, IL-6, TNF-α and hypoxanthine phosphoribosyltransferase (HPRT) were from Applied Biosystems. Amplification was performed with TaqMan Gene Expression Master Mix on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Thermal cycling was performed at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. GAPDH was used as a control for normalization of cDNA values. The ΔΔCT method was used to semi-quantify mRNA levels.

4.5. RNA sequencing

RNA isolation was performed with an RNeasy Mini Kit according to the manufacturer's recommendations. RNA concentration was measured with Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) and the quality of total RNA was assessed by 1 % agarose gels or by utilizing Agilent 2100 bioanalyzer and Agilent RNA 6000 Nano Kit (Agilent Technologies, Boeblingen, Germany). TruSeq Stranded mRNA Kit (Illumina) was used for library preparation based on the recommendations from the manufacturer. The sequencing was performed on a NextSeq 500 sequencer using75 base read lengths in paired-end mode.

4.6. Bioinformatics analysis of transcriptomic data

High-quality reads were aligned against Hg38 human genome using STAR v2.4.0.1 [61] (See Data Processing in supplemental section). The quality control was performed using RseQC v2.6.4 [62]. The uniquely mapped reads were counted by HT-seq [63] v0.11.1 using Hg38 RefSeq as the reference genome to quantify the gene/isoform levels in raw counts. Normalization and differential expression analysis were performed on the raw counts using the R package DESeq2 v1.32.0.33 [64]. Low-expressed genes with the sum of normalized expression levels lower than ten across all samples of interest were removed. The multiple testing corrections were processed with the default Benjamin-Hochberg correction [65] method in DESeq2. The differentially expressed genes (DEGs) were defined as those with an adjusted p-value smaller than 0.05. A gene set enrichment analysis was applied to the DEGs using the R package Enrichr v3.0.34 [66]. Significant gene ontology cellular compartments (GOCC) terms were similarly defined as those with an adjusted p-value smaller than 0.05. The normalized gene expression levels and DEG results were visualized using R and the related packages, including ggplot2 v3.3.5 [67], pheatmap v1.0.12.3 [68], and ggrepel v0.9.1 [69].

4.7. Seahorse analysis

OCR and ECAR were measured using a Seahorse XF24 extracellular flux analyzer (Agilent, USA). Mito stress assay was performed as previously described [27]. Basal respiration was calculated by subtracting oxygen consumption rate (OCR) values after rotenone/antimycin addition from basal OCR values. Maximal OCR was calculated by subtracting OCR values after FCCP addition from OCR values after rotenone/antimycin addition. Basal glycolysis was measured before the addition of mitochondrial inhibitors.

4.8. Flow cytometry

Flow cytometry analysis was performed on a FACS Canto II flow cytometer and analyzed with FACS Diva Software (BD Biosciences, San Jose, CA, USA).

4.9. Other biochemical assays

Measurements of nitrite (Promega, Madison, Wisconsin, USA), ATP (Cayman Chemical, MI, USA) and cytokines (Biolegend, San Diego, CA, USA), were performed using the respective assay kits according to the manufacturers' instruction.

4.10. Statistical analysis

All statistical data analysis was performed using one-way ANOVA with Tukey's test or unpaired *t*-test as indicated in the figure legends using GraphPad Prism Version 8 (GraphPad Prism Software Inc.)

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CRediT authorship contribution statement

Pooja Pradhan: Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Vijith Vijayan:** Methodology, Investigation, Formal analysis, Conceptualization. **Bin Liu:** Validation, Methodology, Formal analysis, Data curation. **Beatriz Martinez-Delgado:** Supervision, Methodology, Investigation, Data curation. **Nerea Matamala:** Investigation, Formal analysis. **Christoph Nikolin:** Methodology, Investigation. **Robert Greite:** Resources, Investigation. **David S. DeLuca:** Resources, Investigation. **Sabina Janciauskiene:** Writing – review & editing, Methodology, Investigation. **Roberto Motterlini:** Writing – original draft, Validation, Resources, Methodology, Investigation. **Roberta Foresti:** Writing – original draft, Supervision, Methodology, Conceptualization. **Stephan Immenschuh:** Writing – original draft, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The accession number of the human bulk transcriptomics data is GEO:GSE255234 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE255234). All other data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2024.103191.

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