Beneficial effects of heme oxygenase-1 upregulation in the development of experimental inflammation induced by zymosan

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Abbreviations: AG = aminoguanidine, CO = carbon monoxide, FITC = fluorescein-isothiocyanate, Hem = heme, HO-1 = heme oxygenase-1, IL-1β = interleukin-1β, NOS-2 = nitric oxide synthase-2, LT = leukotriene, NO = nitric oxide, PG = prostaglandin, TNF-α = tumor necrosis factor-α, ZnPPIX = zinc protoporphyrin IX, Zymo = zymosan.

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ABSTRACT

Heme oxygenase-1 (HO-1) is part of the integrated response to oxidative stress. This enzyme may exert anti-inflammatory effects in some animal models, although the precise mechanisms are not fully understood. We have examined the role of HO-1 in the inflammatory response induced by zymosan in the mouse air pouch.

Zymosan administration induced HO-1 protein expression in leukocytes migrating to exudates, with maximal levels in the late phase of this response (24-48 h). This was accompanied by ferritin induction and bilirubin accumulation, indicating that this enzyme is active in our model. HO-1 expression by zymosan treatment was partly reduced by aminoguanidine, suggesting the participation of endogenous nitric oxide (NO) in the mechanisms leading to HO-1 synthesis in the zymosan-injected mouse air pouch. Upregulation of HO-1 by hemin administration resulted in inhibition of nitric oxide synthase-2 (NOS-2) activity, cellular infiltration into the air pouch exudate and plasmatic exudation. Leukotriene B4 (LTB4) levels in exudates were significantly decreased in the early phase of this response (4 h), whereas interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were inhibited at all time points. Inhibition of HO-1 activity by zinc protoporphyrin IX (ZnPPIX) prevented most of the effects caused by hemin administration. Our results indicate that HO-1 exerts anti-inflammatory effects on the response to zymosan in the mouse air pouch and support a role for this enzyme in the modulation of inflammatory processes.

Keywords: Heme oxygenase-1; zymosan; mouse air pouch; interleukin-1β, tumor necrosis factor-α; nitric oxide.
Heme oxygenase (HO) catalyzes heme degradation to iron, sequestered by ferritin, carbon monoxide (CO) and biliverdin, which is reduced to bilirubin by biliverdin reductase (Abraham et al., 1988). CO exhibits some properties similar to those of nitric oxide (NO), including generation of cGMP, regulation of vascular tone, neuronal signaling and modulation of apoptosis (Morita et al., 1995; Maines, 1997; Baranano and Snyder, 2001; Petrache et al., 2000), whereas biliverdin and bilirubin are antioxidant molecules. Three isozymes have been identified, the constitutive HO-2 and HO-3 (Maines et al., 1986; McCoubrey et al., 1997) and HO-1, which is induced by various stimuli such as heme, cytokines, mitogens, metals, reactive oxygen species, heat shock, UV radiations, hypoxia or hyperoxia (Maines et al., 1986; Otterbein and Choi, 2000).

The synthesis of HO-1 is elicited under a number of conditions where this enzyme may play a cytoprotective role. Moreover, HO-1 has been associated with the adaptive response to hypoxia (Yet et al., 1999) and also protection against ischemia reperfusion injury (Shimizu et al., 2000) and hyperoxia-induced lung injury (Otterbein et al., 1999).

HO-1 has shown beneficial effects in vascular diseases such as atherosclerosis by reducing vascular constriction and excessive cell proliferation (Duckers et al., 2001). Animal models also suggest that HO-1 can be protective in renovascular hypertension, cardiac hypertrophy (Wiesel et al., 2001) and endotoxic shock. The pathophysiological changes in this state involve the production of pro-inflammatory cytokines, which can be downregulated by CO administration. It is likely that CO mediates the protection afforded by HO-1 against lipopolysaccharide treatment by inhibiting the pro-inflammatory response of monocytes through the mitogen-activated protein kinase pathway (Otterbein et al., 2000).
Several lines of evidence indicate a link between HO-1 and inflammation (Willis et al., 2000; Otterbein et al., 2000), where tissues are exposed to stress factors with the potential to induce the synthesis of this protein. Thus, the presence of HO-1 may be part of an adaptive response against injury during inflammatory processes. Whereas the identification of mechanisms involved in HO-1 protection against cellular stress has been the subject of sustained research efforts, the role of this enzyme in inflammation is still to be fully understood.

Phagocytosis of zymosan, a yeast cell wall derivative, results in cellular activation and production of inflammatory mediators. We have previously shown that zymosan induces HO-1 in RAW 264.7 mouse macrophages and endogenous NO plays a positive modulatory role in HO-1 expression (Vicente et al., 2001). In vivo, zymosan administration induces HO-1 in leukocytes present in the mouse air pouch (Vicente et al., 2003), a structure resembling synovial tissue (Edwards et al., 1981). This is a useful model for assessing the contribution of the mediators involved in acute inflammation as well as the effects of anti-inflammatory agents (Posadas et al., 2000).

In this study, we have investigated the potential role of HO-1 in reducing inflammatory responses by studying the consequences of HO-1 upregulation or inhibition in the zymosan-injected mouse air pouch.
METHODS

Materials: [5,6,8,11,12,14,15(n)-3H] prostaglandin E₂ (PGE₂), [5,6,8,9,11,12,14,15(n)-3H] leukotriene B₄ (LTB₄) and L-[2,3,4,5-3H] arginine monohydrochloride were from Amersham Biosciences (Barcelona, Spain). The antibody against LTB₄ was kindly provided by Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). For tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) quantification we have used a DuoSet ELISA Development System from R&D System (Minneapolis, MN). Histamine was measured using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The rest of the reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Mouse air pouch: All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. The protocols were approved by the Institutional Animal Care and Use Committee. The air pouch was produced in female Swiss mice (25-30 g) as previously described (Edwards et al., 1981; Posadas et al., 2000). Six days after the initial air injection, 1ml of sterile saline or 1ml of 1% w/v zymosan in saline was injected into the air pouch. Hemin (50 nmol/pouch) and zinc protoporphyrin IX (ZnPPIX, 100 nmol/pouch) were administered 30 min before zymosan and then at 8 h intervals. At the indicated time points, animals were killed by cervical dislocation and the exudate in the pouch was collected. Leukocytes present in the exudates were measured using a Coulter counter. After centrifugation of exudates, the supernatants were used to assay bilirubin (Turcanu et al., 1998), LTB₄ and PGE₂ levels by radioimmunoassay (Moroney et al., 1988), nitrite by a fluorometric method (Misko et al., 1993), cytokine levels by time-resolved fluoroimmunoassay (Pennanen et al., 1995) and histamine by enzyme immunoassay. Cell pellets were used for Western blot analysis or nitric oxide synthase-2 (NOS-2) activity assay by the L-[2,3,4,5-3H]
arginine method, as reported (Posadas et al., 2000). Protein was quantified by the DC Bio-Rad protein reagent (Richmond, CA) using bovine serum albumin as standard. Separate sets of experiments were performed for the above assays, plasmatic exudation and zymosan phagocytosis.

**Western Blot Analysis:** Protein expression was studied in cell pellets obtained by centrifugation of air pouch exudates. Cells were lysed in 100 µl of buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl and 25 mM Tris, pH 7.4) and centrifuged at 4°C for 5 min at 10,000g. Equal amounts of protein (25 µg) were separated by 12.5 % SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Barcelona, Spain) for 90 min at 125 mA. Membranes were blocked in phosphate-buffered saline (0.02 M, pH 7.0)-Tween-20 (0.1 %) containing 3% non-fat dry milk. For HO-1 we used a polyclonal antibody (1:1000) (Alcaraz et al., 2000). For NOS-2, ferritin or β-actin expression, membranes were incubated with specific polyclonal antibodies (1:1000, Cayman Chemical, Ann Arbor, MI, Dako, Gloustrup, Denmark, or Sigma Chemical Co., St. Louis, MO). Membranes were then incubated with peroxidase-conjugated goat anti-rabbit IgG (1:20000, Dako, Gloustrup, Denmark). The immunoreactive bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Barcelona, Spain). Band intensity was quantified using computer-assisted densitometry.

**Determination of bilirubin:** Bilirubin was quantified spectrophotometrically according to Turcanu et al. (1998). Briefly, BaCl₂. 2H₂O (250 mg) was added to 0.5 ml of air pouch exudate. After vortexing, 0.75 ml of benzene was added, then it was vortexed again vigorously. Samples were then centrifuged at 13,000g for 30 min. The upper benzene layer was collected and the absorbance at 450 nm with reference wavelength at
600 nm was measured. The quantity of bilirubin produced was calculated using molecular extinction coefficient $\varepsilon_{450} = 27.3 \text{ mM}^{-1} \text{ cm}^{-1}$.

**Exudation assay:** Plasmatic exudation was assessed by measuring fluorescein-isothiocyanate (FITC)-labeled albumin in exudates. FITC-albumin (200 µl, 2 mg/ml in sterile saline) was injected iv after zymosan administration. At the indicated time points, animals were killed by cervical dislocation and the exudate in the pouch was collected. The level of FITC-albumin in the exudates was measured fluorometrically (Victor2, Wallac, Turku, Finland) and the results expressed as fluorescence units per µl of exudate.

**Flow Cytometry Analyses:** Leukocytes obtained by the centrifugation of air pouch exudates were washed twice in phosphate-buffered saline and stained with FITC-labeled specific antibodies for neutrophils (Ly-6G, eBioscence, San Diego, CA, 1:200) or macrophages (MOMA-2, Biospec, Oxford, England, 1:200), or incubated with the HO-1 antibody (Alcaraz et al., 2000) (1:50) followed by incubation with FITC-conjugated goat anti-rabbit IgG (1:200). We followed the process viewing the cells under a fluorescence microscope. Stained cells were analyzed by flow cytometry measuring the fluorescence emission at 530 nm (FL1) in a Coulter Epics XL-MCL cytometer (Miami, FL).

In another set of experiments, cells were stained with the Vybrant Apoptosis Assay Kit (Molecular Probes, Leiden, The Netherlands) for the differential analysis of apoptotic and necrotic cells, according to the manufacturer’s protocol. Stained cells were analyzed by measuring the fluorescence emission at 530 nm (FL1) and 575 nm (FL3).

Phagocytosis of zymosan particles was assessed by injecting FITC-zymosan into the air pouch and recovering exudates as above. The number of cells with phagocytosed
fluorescent particles was determined by measuring the fluorescence emission at 530 nm (FL1).

**Statistical analysis:** The results are presented as mean ± S.E.M. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's $t$-test for multiple comparisons.
RESULTS

HO-1 expression and activity induced by zymosan. Zymosan administration into the mouse air pouch induces an acute inflammatory response characterized by plasmatic exudation and migration of leukocytes to the cavity. In this paper we have shown that zymosan administration induces HO-1 protein expression in leukocytes migrating to air pouch exudates, reaching the highest levels 24-48 h after zymosan (Fig. 1A). Our results indicate that HO-1 is active in these conditions, resulting in ferritin induction (Fig. 1B) and bilirubin accumulation (Fig. 1C), which exhibited a time-dependent upward trend. In this experimental model, leukocytes present in the exudates are predominantly neutrophils. Flow cytometry analysis indicated that there were 81.4 ± 2.4 % neutrophils, 10.2 ± 2.1 % lymphocytes and 8.4 ± 1.8 % macrophages. Under these conditions, nearly all the cells (93.5 ± 4.0 %) were stained with anti- HO-1 antibody at 24 h.

Endogenous NO production. Zymosan increases NO production and nitrite accumulation, which peaks at 12 h (Posadas et al., 2000; Vicente et al., 2003). Maximal NO production would thus take place before maximal HO-1 protein expression (24-48 h). We examined whether the NO pathway could be responsible for HO-1 induction by zymosan in this response. We selected the drug and conditions that previously demonstrated the ability to effectively inhibit NO production in this experimental model (Posadas et al., 2000). Fig. 2 shows that aminoguanidine administration strongly inhibited NOS-2 activity in leukocytes present in exudates and NO production assessed by nitrite levels in exudates. As a result, HO-1 protein expression was partly decreased by this inhibitor, whereas NOS-2 expression was not affected.
In another set of experiments we administered hemin, a substrate analog widely used as an HO-1 inducer, to study the influence of HO-1 upregulation on NOS-2 activity. NOS-2 activity induced by zymosan in leukocytes present in the air pouch at 24 h was inhibited in animals treated with hemin (Table 1). At a later time (48 h), NOS-2 activity in the zymosan group was suppressed by hemin.

**Effect of hemin administration on leukocyte functions and plasmatic exudation.**

We then investigated whether HO-1 activity can modulate various parameters of the inflammatory response to zymosan in the mouse air pouch. In our experimental conditions, hemin augmented HO-1 expression and activity, as indicated by Western blot analysis of HO-1 and ferritin (Fig. 3A), and bilirubin accumulation (Fig. 3B). We first determined the consequences of HO-1 upregulation on the number of infiltrating leukocytes, plasmatic exudation and phagocytosis of zymosan. As shown in Fig. 4A, cellular infiltration into the air pouch exudate was reduced by hemin at 12, 24 and 48 h. Although reduced apoptosis may play a role in the accumulation of leukocytes at late time points, we found no evidence of differences in the number of cells showing apoptosis or necrosis after treatment with either zymosan or zymosan + hemin in cells present in air pouch exudates at all time points (data not shown).

Hemin was also able to reduce exudation at 6 and 24 h, as indicated by the decreased levels of FITC-albumin in the exudates (Fig. 4B). In our experimental conditions histamine was not detected in zymosan-injected air pouch exudates at time points from 4 h to 48 h (the detection limit of the enzyme immunoassay was 0.5 nM). On the other hand, the phagocytosis assay performed with FITC-zymosan did not reveal any difference in the number of cells showing phagocytosed particles between the zymosan-injected group and the zymosan + hemin-injected group (44.8 ± 2.6 % and 44.5 ± 1.6 %, respectively, at 24 h).
Effect of hemin administration on eicosanoids and cytokine production. The levels of eicosanoids LTB₄ and PGE₂ and cytokines IL-1β and TNF-α were measured at different times throughout this inflammatory process. LTB₄ was present in the first phase of the response (4 h) and decreased in the presence of hemin (Fig. 5A), while at 12 h, LTB₄ levels were undetectable. In contrast, PGE₂ levels did not change significantly (Fig. 5B). Interestingly, IL-1β (Fig. 6A) and TNF-α (Fig. 6B) decreased at all time points except at 48 h, when the inflammatory response is too low to observe any difference in cytokine levels.

Effect of HO-1 activity inhibition. In order to investigate whether the reduction of inflammatory parameters by hemin was related to the increase in HO-1 expression and activity that occurs after its administration, we assessed the effects of HO-1 inhibition by ZnPPIX in animals treated with zymosan + hemin. For this purpose, we selected a time point (24 h) when HO-1 expression and activity were high and accompanied by significant levels of inflammatory mediators. As shown in Fig. 7, bilirubin present in exudates was reduced in animals treated with ZnPPIX, indicating the inhibition of HO-1 activity. In the presence of ZnPPIX there was an increase in cell migration into the air pouch and also in IL-1β and TNF-α levels, in comparison with the group treated with zymosan + hemin. However, at this time point the effect of hemin on plasmatic exudation was lower than at earlier times (6 h) and ZnPPIX did not modify this effect significantly. Since PGE₂ production was not affected by hemin, the inhibition of HO-1 activity did not change the levels of this eicosanoid.
DISCUSSION

Our results describe that HO-1 is induced in migrating leukocytes during the late phase of the in vivo response to zymosan in the mouse air pouch, where maximal HO-1 expression and activity coincide with the decline in inflammation. We have also shown that endogenous NO production can partly mediate HO-1 expression in this model of inflammation, which is in line with our previous study in the macrophage cell line RAW 264.7 (Vicente et al., 2001). In turn, HO-1 activity could reduce NO production by different mechanisms, such as a decrease in heme availability, release of iron (Weiss et al., 1994) and interaction of CO with the heme group of NOS-2 (Fukuto and Chaudhuri, 1995), leading to inhibition of NOS-2 expression or activity. Our results suggest that HO-1 induction results in the inhibition of NOS-2 enzyme activity. This could be the main mechanism of regulation of NOS-2 by HO-1 in the zymosan-injected mouse air pouch, since we have previously reported that hemin administration does not affect NOS-2 protein expression in this model (Vicente et al., 2003). HO-1 could therefore be part of a general mechanism of defense against NO cytotoxicity, as suggested in studies using other animal models (Datta et al., 2002; Wei et al., 2003).

Since histamine regulation can be part of the anti-inflammatory mechanisms of HO-1 (Di Bello et al., 1998; Takamiya et al., 2002) and NO has been proposed as a secondary mediator in histamine-induced allergic responses (Meijer et al., 1996), we have examined the possible participation of histamine in the inflammatory response to zymosan. Nevertheless, we have not detected histamine levels in exudates from zymosan-injected air pouches and thus our results do not support a role for this mediator in our inflammatory model.

Administration of hemin to the zymosan-injected group increased HO-1 expression and activity that were associated with bilirubin production and anti-inflammatory
effects. Some of these effects appeared at 4 h, which coincides with the presence of bilirubin, an index of HO-1 activity. Our results indicate that the reduction in the number of cells present in exudates after hemin administration was not the consequence of increased apoptosis. Therefore, hemin inhibited leukocyte migration, a crucial step in the response to zymosan, and this inhibition was accompanied by reduced plasmatic exudation. Administration of zymosan resulted in the early synthesis and release of LTB₄, a strong chemotactic agent able to increase vascular permeability and edema. The reduction of this eicosanoid after hemin administration could participate in the inhibition of cell migration and plasmatic exudation. In contrast, our results suggest that modification of phagocytic activity is not involved in the anti-inflammatory mechanisms of HO-1. It is known that HO-1 induction in microvascular endothelial cells confers resistance to oxidative stress and inhibits upregulation of adhesion molecules, resulting in decreased leukocyte recruitment (Hayashi et al., 1999). In this context, the control of inflammatory responses by HO-1 could be related to inhibitory effects on P- and E-selectin expression in vascular endothelial cells mediated by the antioxidant molecules biliverdin or bilirubin (Vachharajani et al., 2000). We have also demonstrated the induction of ferritin in this experimental model. This protein chelates intracellular free iron, preventing its participation in the generation of reactive oxygen species, which can be an important mechanism affording cytoprotection from oxidative injury (Balla et al., 1992).

High levels of cytokines are present in the initial phase of the response to zymosan (4 h) before the expression of NOS-2 and HO-1, suggesting a possible role for cytokines in the modulation of both enzymes in this experimental model. Interestingly, cytokine levels were reduced by hemin treatment, which cannot be explained by an inhibitory effect on cell migration since it can be observed at 4 h, when cell migration is not yet
affected. Inhibitory effects on TNF-\(\alpha\) and IL-1\(\beta\) production may be due to CO generated by HO-1, as reported in mice treated with lipopolysaccharide (Otterbein et al., 2000). This inhibitory effect has been observed at all time points and is of relevance since both pro-inflammatory cytokines mediate important processes, including endothelial adhesiveness, leukocyte recruitment and cell activation (Collins et al., 1995). Surprisingly, we have not observed any inhibition of PGE\(_2\) production, likely due to a direct effect of hemin in the air pouch as co-factor of cyclooxygenase, which would counteract the influence of HO-1 activity on this enzyme. In line with this observation, we have previously shown that induction of HO-1 by hemin results in a reduction of cyclooxygenase-2 expression in mouse macrophages (Vicente et al., 2003), and other authors have demonstrated that HO-1 plays a negative regulatory role on this enzyme in endothelial cells (Haider et al., 2002).

In this study, HO-1 activity contributed to the effects of hemin administration because inhibition of enzyme activity by ZnPPIX prevented hemin-induced reduction of most inflammatory parameters. Nevertheless, this HO-1 inhibitor did not counteract the effect of hemin on plasma exudation at 24 h, which could be due to the fact that plasmatic exudation is more relevant at early times in this experimental model, making it difficult to detect differences between treatments at 24 h.

While the involvement of HO-1 in mediating protective effects has focused primarily on cardiovascular diseases (Yet et al., 1999; Melo et al., 2002; Mayer et al., 2003), it is becoming apparent that this enzyme plays a broader role in the modulation of inflammatory processes. Our data support the view that HO-1 exerts anti-inflammatory effects \textit{in vivo} since HO-1 induction resulted in decreased cell migration, exudation and production of inflammatory mediators. These effects may be dependent on metabolites derived from HO-1 activity and likely contribute to the resolution of inflammation.
REFERENCES


*Prostaglandins* **52**:431-446.


FOOTNOTES

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FIGURE LEGENDS

Figure 1. Time course of HO-1 and ferritin protein expression and bilirubin levels in the zymosan-injected mouse air pouch. At different time points after zymosan administration, air pouch exudates were collected to measure protein expression in leukocytes and bilirubin levels in supernatants. A) HO-1 expression. B) Ferritin expression. C) Bilirubin levels. In A) and B) Protein band intensity is represented as arbitrary units and the mean ± S.E.M of three similar experiments was calculated. In C), the data show the mean ± S.E.M of 12-18 animals. In animals receiving saline, we did not detect any protein expression or bilirubin.

Figure 2. Effect of aminoguanidine on NOS-2 activity, nitrite levels, and HO-1 and NOS-2 protein expression in the response to zymosan at 24 h. Exudates from air pouches treated with either zymosan (Zymo) or zymosan+aminoguanidine (Zymo+AG) were collected to determine NOS-2 activity and protein expression in leukocytes, and nitrite levels in supernatants. AG (200 mg/kg, ip) was administered at 8 h and 20 h after zymosan. A) NOS-2 activity. B) Nitrite levels. The data show the mean ± S.E.M of 12-18 animals. **p<0.01. C) HO-1 and NOS-2 expression. Results are representative of three similar experiments.

Figure 3. Effect of hemin on HO-1 and ferritin protein expression and bilirubin levels. Hemin (Hem) was administered as indicated in Methods. At different time points, air pouch exudates were collected. A) Protein expression in leukocytes and B) Bilirubin levels in supernatants. The data show the mean ± S.E.M of 10-12 animals. ** p<0.01.

Figure 4. Effect of hemin on cellular infiltration and plasmatic exudation. A) Number of infiltrating leukocytes. Cells present in exudates were measured using a
Coulter counter. In another set of experiments, FITC-albumin was injected iv as indicated in Methods. B) Plasmatic exudation. FITC-albumin concentration was quantified in supernatants after centrifugation of exudates. The data show the mean ± S.E.M of 12-18 animals. * $p<0.05$, ** $p<0.01$.

Figure 5. Effect of hemin on LTB$_4$ and PGE$_2$ levels in exudates. A) LTB$_4$ levels. B) PGE$_2$ levels. The data show the mean ± S.E.M of 12-18 animals. ** $p<0.01$. In groups treated with either saline or hemin, LTB$_4$ and PGE$_2$ were not detected.

Figure 6. Effect of hemin on cytokine levels in exudates. A) IL-1$\beta$ levels. B) TNF-\(\alpha\) levels. The data show the mean ± S.E.M of 12-18 animals. * $p<0.05$, ** $p<0.01$. In groups treated with either saline or hemin, TNF-\(\alpha\) was not detected.

Figure 7. Effect of the HO-1 inhibitor ZnPPIX. Drugs were administered as indicated in Methods. At 24 h, air pouch exudates were collected to measure the number of leukocytes. After centrifugation, bilirubin, FITC-albumin, PGE$_2$, IL-1$\beta$ and TNF-\(\alpha\) levels were determined in supernatants. The data show the mean ± S.E.M of 12 animals. ** $p<0.01$ versus Zymo, #$p<0.01$ versus Zymo+Hem.
TABLE 1

Effect of hemin on NOS-2 activity

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<th>NOS-2 activity (pmol L-citrulline/mg protein/60 min)</th>
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<tr>
<td></td>
<td>24 h</td>
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<tr>
<td>Saline</td>
<td>56.0 ± 6.0</td>
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<tr>
<td>Hemin</td>
<td>108.5 ± 28.0</td>
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<tr>
<td>Zymosan</td>
<td>435.5 ± 63.0</td>
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<tr>
<td>Zymosan+Hemin</td>
<td>295.5 ± 40.5*</td>
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Results are the mean ± S.E.M. of 6 animals. *p<0.05, **p<0.01 compared with the zymosan group. NOS-2 activity was determined in cytosolic fractions from leukocytes present in pouch exudates.
Figure 1
Figure 2

A) NOS-2 activity (pmol L-citrulline/mg/60 min)

- Zymo
- Zymo+AG

B) Nitrite (ng/ml)

- Zymo
- Zymo+AG

C) HO-1

NOS-2

- Zymo
- Zymo+AG
**Figure 3**

A) Western blot analysis showing the expression of HO-1, Ferritin, and β-actin in Zymo and Zymo+Hem groups at different time points (4, 12, 24, 48 h).

B) Graph showing the concentration of Bilirubin (pmol/ml) over time (4, 12, 24, 48 h) for Zymo and Zymo+Hem groups. The data points are marked with asterisks to indicate significance. ** indicates p < 0.01.
Figure 4
**Figure 5**

A) 

B)
Figure 6
Figure 7