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Heme oxygenase-1 induction and regulation in unstimulated mouse peritoneal macrophages

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Abstract

Heme oxygenase-1 (HO-1) is a stress protein induced by a variety of stimuli in inflammatory cells. This study was set up to investigate the induction of this protein in unstimulated macrophages. Resident mouse peritoneal macrophages purified by adhesion and cultured in basal conditions strongly induced HO-1 in a time-dependent manner, with a peak at 20 hr. At the same time, low levels of nitrite accumulated in the culture medium and expression of nitric oxide synthase-2 (NOS-2) and NOS-3 protein was detected. Inhibition of NO production and/or NOS expression by incubating macrophages with different drugs inhibiting NOS activity or modulating the redox state of the cell, such as *N*-acetylcysteine (NAC) resulted in inhibition of HO-1 expression, suggesting that NO is an endogenous mediator of this stress response. In conclusion, mouse peritoneal macrophages cultured in basal conditions develop an adaptive response with up-regulation of HO-1 as a very sensitive marker of oxidative stress.

Keywords: Heme oxygenase-1; Macrophage; Nitric oxide; Nitric oxide synthase; *N*-acetylcysteine

1. Introduction

HO-1 is a stress responsive protein induced by a variety of stimuli in many cell types. In macrophages and other inflammatory cells, HO-1 is induced by inflammatory mediators such as cytokines, radicals, or hypoxia and it can be regarded as a protective mechanism against injury, due to the antioxidant and antiinflammatory properties of products formed by HO-1 activity, e.g. bilirubin and carbon monoxide [1]. NO, a crucial component of innate immunity, is synthesized by three NOS isozymes. The constitutive enzymes NOS-1 and -3 generate small amounts of NO over short periods of time, whereas the calcium-independent NOS-2 produces sustained NO production following the activation of inflammatory cells [2]. This leads to NO overproduction and tissue injury which have been implicated in a number of diseases such as endotoxic shock, arthritis, or asthma [3–5]. Although the involvement of NOS-2 in macrophage responses is well established, little is known of the role that constitutive isozymes may play in these cells in either basal or activated states. It is interesting to note that NOS-3 has been detected in naive unstimulated rat alveolar macrophages where it may play a role in the regulation of cellular metabolism [6].

NO affects the activation of inflammatory cells in several ways. Recently, we have shown that HO-1 is induced by exogenous NO in the RAW 264.7 mouse macrophage-like cell line [7], whereas endogenous NO could be a mediator of HO-1 induction in the inflammatory response to zymosan [8]. In the present work, we have investigated whether HO-1 is induced in primary cells in culture. We have used resident mouse peritoneal macrophages purified by adhesion and cultured in standard conditions. We have also determined the participation of NO and cellular redox status in HO-1 induction.

2. Materials and methods

2.1. Cell culture

Cells were harvested from female Swiss mice (25–30 g) by peritoneal lavage. Cells from seven animals were pooled and resuspended at 3×10^6 cells/mL in Dulbecco's

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Abbreviations: BSO, buthionine sulfoximine; HO-1, heme oxygenase-1; L-NAME, L-N^G-nitroarginine methyl ester; NAC, *N*-acetylcysteine; NO, nitric oxide; NOS, nitric oxide synthase; PI, propidium iodide.

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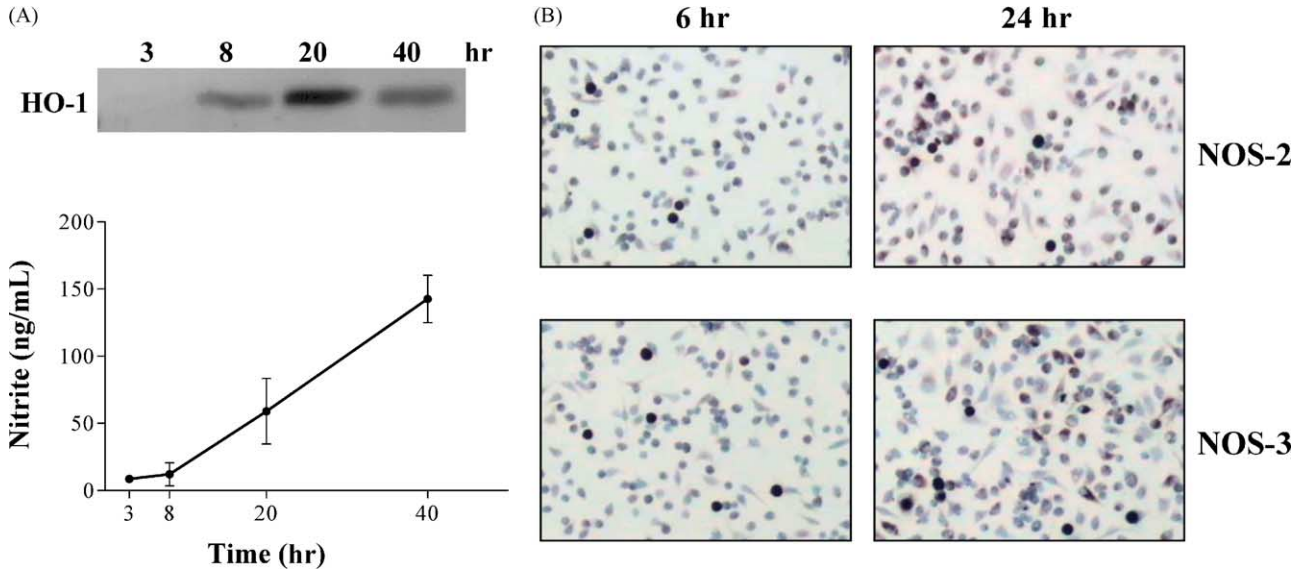

Fig. 1. (A) Time-dependent HO-1 expression and nitrite accumulation. Peritoneal macrophages were incubated in DMEM for 3, 8, 20, or 40 hr. HO-1 protein expression was determined by Western blot analysis. Results show a representative experiment of three. Nitrite levels were measured in culture supernatants. Data are the mean ± SEM of three experiments. (B) Immunodetection of NOS isoforms. NOS-2 and -3 were detected using specific antibodies in peritoneal macrophages cultured for 6 or 24 hr. Results show a representative experiment of three. The photomicrographs were obtained as video images (magnification, 200×).

modified Eagle's medium (DMEM, Life Technologies Inc.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were seeded in 24-well plates at 1.5×10^6 cells/well. After incubation at 37° for 2 hr, adherent cells were used to perform the following experiments. After washing, new medium was added and incubations proceeded at 37° for different times. Nitrite levels were determined in culture supernatants by a fluorometric method [9] and cells were used for Western blot.

2.2. Western blot analysis

Macrophages were lysed in buffer (1% Triton X-100, 1% deoxicholic acid, 20 mM NaCl, and 25 mM Tris, pH 7.5) and centrifuged at 4° for 10 min at 10,000 *g*. The protein content was determined by the DC Bio-Rad protein reagent. Samples were separated in 12.5% sodium dodecyl sulphate-poliacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech Europe GmbH). After blocking, they were incubated with a specific polyclonal antibody against HO-1 (1/1000) [10] and blots were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG (1/20,000, Dako). The immunoreactive bands were visualized by an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech Europe GmbH). The possibility of contamination of reagents with endotoxin was excluded after performing the Limulus assay (Sigma Chemical Co.).

2.3. Immunocytochemistry

Cells (6×10^6/well in 6-well plates) were fixed with Bouin's solution (Sigma Chemical Co.) and incubated with a polyclonal antibody (1/100) against HO-1 [10], followed by incubation with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) or with antibodies against NOS-2 (Cayman Chemical) or NOS-3 (Stressgen) followed by peroxidase-conjugated

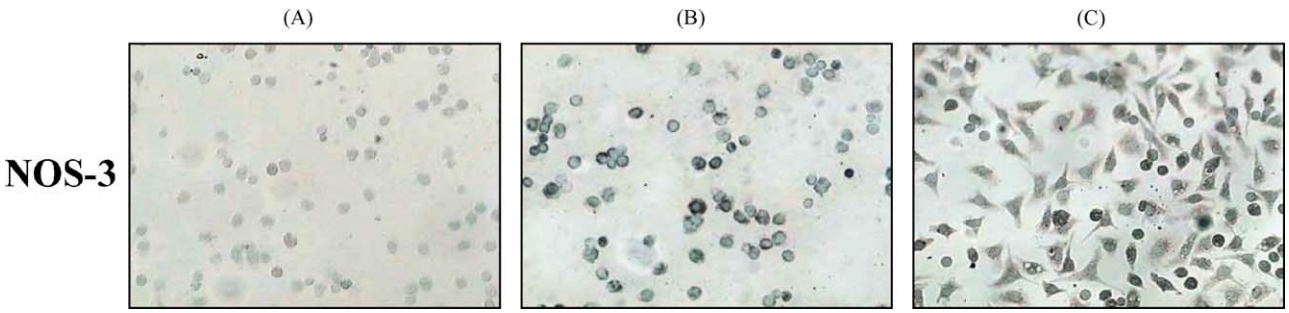

Fig. 2. Immunodetection of NOS-3 in macrophages. (A and B) cells were immunostained immediately after collection from peritoneum. (A) Negative control, cells not treated with the specific NOS-3 antibody. (C) Cells immunostained with specific NOS-3 antibody after the 2 hr adhesion time.

goat anti-rabbit IgG (1/1000, Dako). Cell viability was assessed by PI staining.

3. Results and discussion

We observed by Western blotting that HO-1 protein expression was strongly induced during incubation of resident peritoneal macrophages in a time-dependent manner, with a maximum at 20 hr (Fig. 1A). This was accompanied by accumulation of nitrite in culture supernatants. Our results by Western blot analysis indicated that NOS-2 was induced weakly in these cells (data not shown). However, to determine the isozymes involved in NO production in these conditions and to increase the sensitivity of analysis, we used immunocytochemistry. NOS-2 was immunodetected at 6 hr (4% of cells), while at 24 hr an increase in the number of cells expressing this enzyme was observed (12%, Fig. 1B). Interestingly, macrophages were immunostained when we used a specific antibody against NOS-3

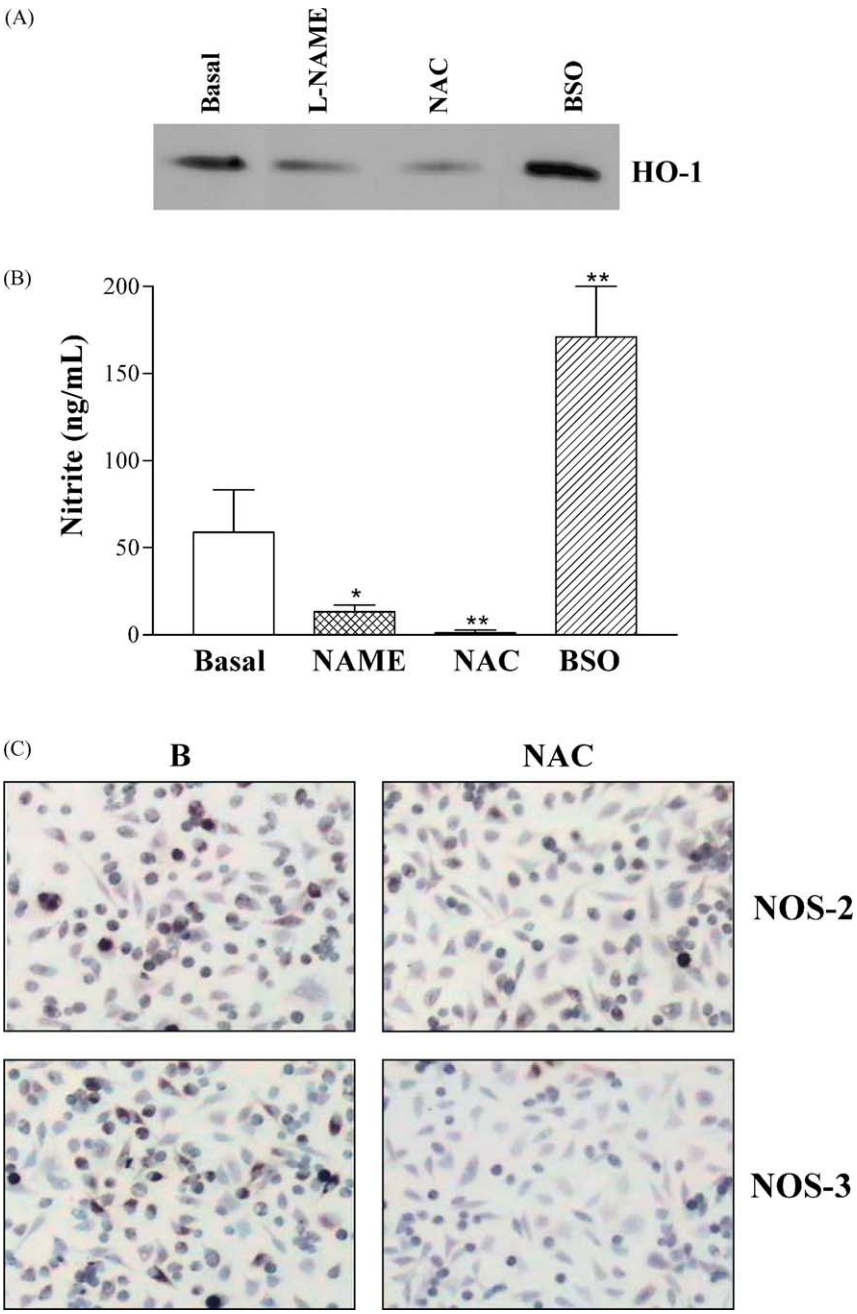

Fig. 3. Effect of L-NAME, NAC, and BSO on protein expression and nitrite levels. Cells were incubated with L-NAME (1 mM), NAC (10 mM), or BSO (50 μM) for 20 hr. (A) HO-1 expression was determined by Western blot analysis. (B) Nitrite levels (mean ± SEM of three experiments) were measured in culture supernatants after 20-hr incubation. $^{*}P < 0.05$; $^{**}P < 0.01$, Dunnett's *t*-test. (C) NOS-2 and -3 were immunodetected using specific antibodies in peritoneal macrophages cultured for 20 hr. Results show a representative experiment of three. The photomicrographs were obtained as video images (magnification, 200×).

(3 and 10% at 6 and 24 hr, respectively). Controls were performed to assess the presence of the constitutive isozyme NOS-3 in cells before seeding. As shown in Fig. 2, NOS-3 was detected in macrophages immediately after collection from peritoneum.

To determine if HO-1 expression was dependent on basal NO production, cells were incubated in the presence of L-NAME for 20 hr. As shown in Fig. 3A and B, this treatment inhibited nitrite levels and HO-1 expression. We also studied the possible contribution of changes in the redox state of these cells. Incubation of peritoneal macrophages with the antioxidant and glutathione precursor, NAC for 20 hr abolished nitrite production and HO-1 expression (Fig. 3A and B). In contrast, glutathione depletion by BSO treatment of cells, resulted in increased nitrite levels and HO-1 expression, suggesting an inverse relationship between glutathione levels and HO-1 induction. Incubation of cells with the permeable superoxide scavenger Tiron, did not exert any effect on these parameters (data not shown).

As observed in Fig. 3C, the reduction caused by NAC in nitrite levels could be due to inhibition of NOS expression since NAC treatment reduced the number of cells immunostained for NOS-2 (from 12 to 6%) and NOS-3 (from 10 to 3%). In this respect, the inhibition of NOS-2 induction by NAC has been reported in rat peritoneal macrophages stimulated by endotoxin or cytokines [11].

The potential relationship between HO-1 and cellular viability was studied by immunofluorescence. Fig. 4 shows that HO-1 expression increases with time in parallel with cellular death assessed by PI staining. At the time of maximal HO-1 expression (20 hr), NAC treatment resulted in strong inhibition in the number of cells positive for HO-1 and increased cell viability.

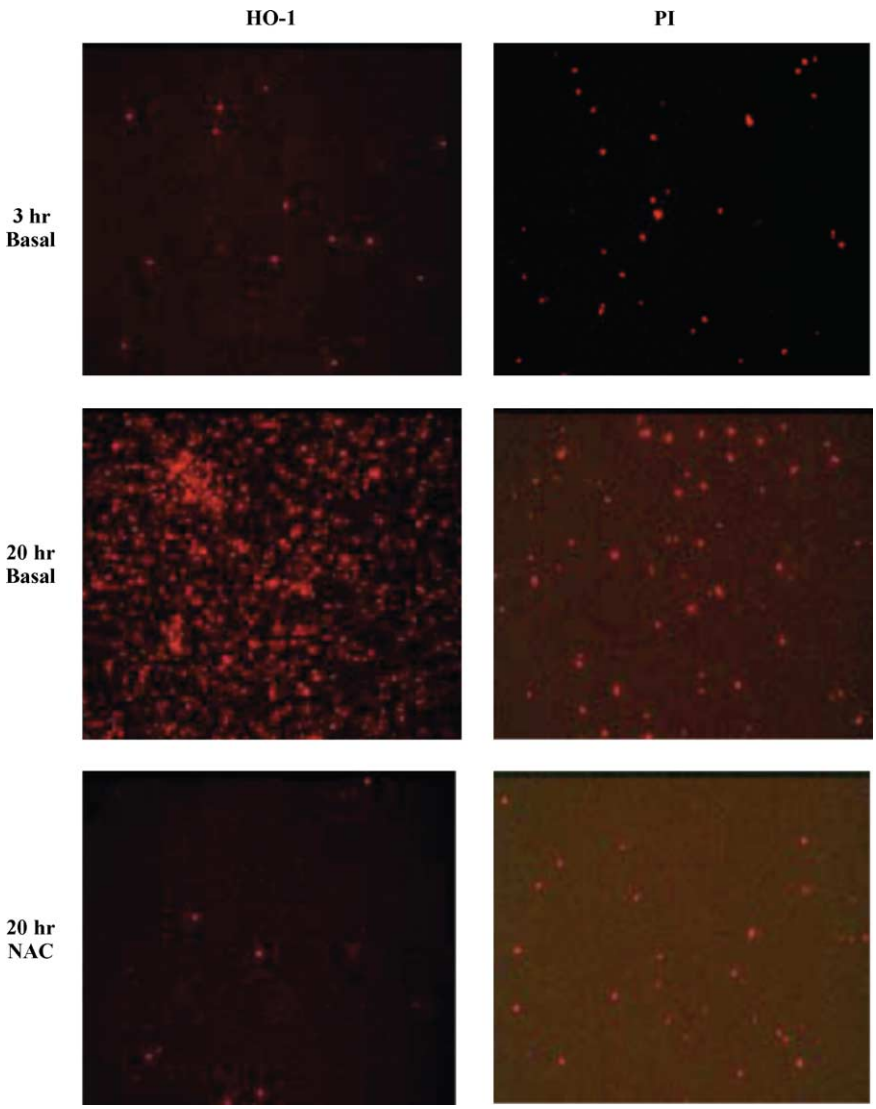

Fig. 4. Effect of NAC on HO-1 expression and cellular viability. Peritoneal macrophages were incubated in the absence or presence of NAC (10 mM) for 20 hr. HO-1 expression was determined by immunofluorescence detection. Dead cells were stained with PI and examined microscopically by epifluorescence. Results show a representative experiment of three. The photomicrographs were obtained as video images (magnification, 200×). The percentages of cells stained with PI were 3 (basal 3 hr), 6 (basal 20 hr), and 3 (NAC 20 hr).

The two major redox buffering systems in murine macrophages are the superoxide dismutase–catalase system and glutathione [12]. Our results do not support a role for superoxide in HO-1 induction although the participation of other reactive oxygen species cannot be excluded. During macrophage culture in basal conditions, oxidative stress and the consequent decrease in glutathione may occur. These conditions can activate mechanisms to counteract the deleterious effects of reactive oxygen species that can lead to the lose of cellular viability. We have shown that murine peritoneal macrophages cultured in basal conditions develop an adaptive response with up-regulation of NOS and HO-1. Our results suggest that HO-1 induction is a very sensitive marker of cellular stress and would be the result of endogenous NO production, likely due to the activity of constitutive and inducible NOS. This HO-1 induction does not take place in the mouse macrophage RAW 264.7 in the same culture conditions [8], which would underscore the differences between primary cells and immortalized cell lines. Since HO-1 is induced to a high extent and this enzyme activity results in the formation of antioxidant and antiinflammatory molecules, the response we describe here may have implications when interpreting findings from experimental studies on cellular metabolism or inflammatory mechanisms.

Acknowledgments

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