Erythrophagocytosis induces heat shock protein synthesis by human monocytes–macrophages
(phagocytes/oxygen free radicals/respiratory burst/heme oxygenase/1,25-dihydroxyvitamin D3)

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ABSTRACT Exposure of cells to elevated temperatures and other environmental stresses results in the expression of specific genes encoding the so-called heat shock proteins (HSPs). Since exogenous H2O2 induces in human monocytes the synthesis of HSPs, and previous induction of HSPs protects these cells from oxidative injury, we investigated whether HSP synthesis was also induced during generation of reactive oxygen species by the phagocyte itself during phagocytosis. As a model system, we analyzed the effects of erythrophagocytosis on protein synthesis by the human premonocytic line U937, in which phagocytosis is induced during differentiation with 1,25-dihydroxyvitamin D3. Exposure to whole erythrocytes, but not to erythrocyte ghosts, induced in the phagocytic cells only the synthesis of the 70- and 83- to 90-kDa HSPs and a 32-kDa oxidation-related stress protein identical by partial peptide mapping to heme oxygenase. The radioprotective aminothiol N-(2-mercaptoethyl)-1,3-propanediamine (WR-1065), which can substitute for glutathione as hydrogen donor, prevented this induction. These results suggest that oxygen free radicals generated in the presence of hemoglobin-derived iron and consecutive glutathione depletion are involved in induction of stress protein synthesis during erythrophagocytosis. HSPs synthesized during phagocytosis may play a role in the phagocyte’s defense mechanisms and in protective immunity.

Exposure of prokaryotic and eukaryotic cells to elevated temperatures or other types of cellular injury, including oxidative injury, results in the selective and reversible expression of specific conserved genes encoding a small number of polypeptides called stress, or heat shock, proteins (HSPs) (1–3). We have investigated the relationships between oxidative injury and HSPs in human phagocytes and found that exogenous hydrogen peroxide (H2O2) induces in human monocytes the synthesis of HSPs (4), that preexposure to temperatures inducing the synthesis of HSPs partially protects monocytes from H2O2-induced cell death (5), and that in human neutrophils induction of a heat shock response by heat or cadmium is associated with an inhibition of superoxide production (6). These observations suggested potential implications of HSPs in the phagocytes’ biology (7). We therefore asked the question whether HSP synthesis was also induced in association with the generation of reactive oxygen species by the phagocyte itself during phagocytosis. The analysis of the stress response in phagocytes is of particular relevance since it has been shown that several immunodominant antigens of intracellular microorganisms such as mycobacteria or plasmodia are indeed, by sequence homology, HSPs, and the potential role of HSPs in protective immunity and/or immunopathology is an area of intense interest (8–11).

As a model system, we used the human premonocytic line U937 and phagocytosis of erythrocytes. In their undifferentiated state, U937 cells are nonphagocytic and are unable to generate superoxide (O2−) but acquire Fc receptors, phagocytosis, and a functional NADPH oxidase upon differentiation with the steroid hormone 1,25-dihydroxyvitamin D3 [1,25-(OH)2D3] (12, 13). Erythrophagocytosis probably allows in vivo clearance of senescent or parasitized erythrocytes (14, 15) and triggers in monocytes–macrophages the respiratory burst enzyme NADPH oxidase (16), thus leading to generation of O2− and other toxic oxygen derivatives. We found that during erythrophagocytosis, differentiated U937 cells and normal human monocytes and macrophages synthesized the 70-kDa and 83- to 90-kDa HSPs, as well as heme oxygenase. It has been shown before that human heme oxygenase is induced by hemin as well as by a variety of oxidative stresses unrelated to heme metabolism such as exposure to H2O2, cadmium, or ionizing radiation, but not by heat (17–19). In our experiments, induction of both HSPs and heme oxygenase was prevented when we used hemoglobin-free erythrocyte ghosts or the aminothiol N-(2-mercaptoethyl)-1,3-propanediamine (WR-1065), suggesting a role for hemoglobin-derived iron and oxygen free radicals generated during erythrophagocytosis in this induction.

MATERIALS AND METHODS

Reagents, Cells, and Assay for Superoxide Production. Sheep erythrocytes (SRBCs) (BioMérieux, Charbonnier les Bains, France) were opsonized with a rabbit antiserum (20). Resealed ghosts were prepared as described (21) and used at the same concentration as SRBCs or opsonized SRBCs [SRBC(IgG)]. 1,25-(OH)2D3 was provided by U. Fisher (Hoffmann-La Roche). All other reagents were from Sigma.

Myeloplasma-free U937 cells were grown in RPMI 1640 medium (GIBCO) with 10% fetal calf serum/1% glutamine, with or without 1,25-(OH)2D3 (10 ng/ml) for 72 hr, then counted, centrifuged, and resuspended at 0.8 × 10⁶ cells per ml in RPMI 1640 medium without methionine and incubated with or without SRBCs for 3 hr at 37°C. Peripheral blood monocytes from normal volunteers were isolated by gradient centrifugation and purified by adherence (4), and human alveolar macrophages were obtained as described (22) from bronchoalveolar lavage of lungs excised during surgery for carcinoma, then isolated by adherence, cultured, and stimulated like U937 cells. Normal human foreskin fibroblasts were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Cocultures with SRBCs were done in tissue culture plates, and we used a high erythrocyte/

Abbreviations: HSP, heat shock protein; 1,25-(OH)2D3, 1,25-dihydroxyvitamin D3; SRBC, sheep erythrocyte; SRBC(IgG), opsonized SRBC.
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phagocyte ratio to maximize cell–cell contacts between the adherent phagocytes (or the fibroblasts) and the floating SRBCs. Exposure to heat was as described (4, 5). O2 production was assayed by the superoxide dismutase-inhibitable reduction of ferricytochrome c as described (13). Generation of H2O2 was measured according to Pick and Keisari (23). Percent phagocytic cells (cells containing one or more erythrocyte or erythrocyte ghost) were counted using a phase-contrast microscope (Olympus) and counting 200 cells (or 400 for low percentages of phagocytic indexes as in the case of nonopsonized SRBCs).

**Protein Analysis.** After exposure to SRBCs or heat, cells were labeled with 20 μCi of [35S]methionine (1 Ci = 37 GBq) (Amersham) for 90 min at 37°C, recovered, and washed twice with phosphate-buffered saline (GIBCO), and SRBCs were lysed with 155 mM NH4Cl 10 mM KHCO3. Aliquots were taken for microscopy analysis of phagocytosis and remaining cells were lysed in lysis buffer (24). Proteins from aliquots corresponding to equal cell numbers were resolved by NaDodSO4/PAGE in slab gels with 10% acrylamide (24). Two-dimensional gel electrophoresis was as described (25). Quantitative appraisal of labeled proteins was performed by scanning densitometry of autoradiographs (Genoscan TM laser densitometer, Genoﬁt, Geneva).

For Western blot analysis of the 70-kDa protein induced in 1,25-(OH)2D3-treated U937 cells treated by heat shock or by incubation with SRBC(IgG), cells were exposed to 45°C for 20 min and allowed to recover for 160 min at 37°C, or incubated 180 min with 3.2 × 106 SRBC(IgG) per ml, or maintained at 37°C (control) and labeled as described. Equal amounts of proteins were electrophoresed, transferred to nitrocellulose paper, and probed with a mouse monoclonal anti-human HSP70 antibody (kindly provided by W. Welch, Cold Spring Harbor Laboratory) (26). Bound HSP70 antibodies were revealed by using Auroprobe BPlsus Immunogold reagent and intense BL silver enhancement system (Janssen Pharmaceutica).

For partial peptide mapping of the 32-kDa protein induced in 1,25-(OH)2D3-treated U937 cells and monocytes–macrophages after incubation with SRBC(IgG), the 32-kDa protein was first localized on one-dimensional gel by autoradiography, then isolated by excision of gel slice, and treated, or not, with N-chlorosuccinimide as described (18). The resulting peptides were analyzed on 15% acrylamide gels.

**RESULTS**

**Exposure to SRBC(IgG) Induced Synthesis of HSPs in Phagocytic Cells Only.** After preincubation with 1,25-(OH)2D3, U937 cells became phagocytic, and 53% ± 12% (mean ± SD; n = 7) cells ingested one or more SRBC(IgG), whereas only 1.5 ± 0.5% ingested nonopsonized SRBCs. Fig. 1a shows that in 1,25-(OH)2D3-treated U937 cells, phagocytosis of SRBC(IgG) induced the synthesis of a 68- to 72-kDa and 32-kDa protein, together with an increase in an 83-kDa protein. Heat shock induces these cells the same 70- and 83-kDa proteins but not the 32-kDa protein (Fig. 1a; refs. 4 and 17). Identity of the 70-kDa protein induced by erythrophagocytosis to HSP70 was further established by Western blotting (Fig. 1b). The intensity of labeling of HSP70 increased with increasing erythrocyte/phagocyte ratio: by scanning densitometry, the band corresponding to HSP70 represented in 1,25-(OH)2D3-treated U937 cells 0% (Fig. 1a, lane 5), 2%, 8%, 19% (lane 7), and 23% (lane 6) of total protein synthesis. To establish that the effects of 1,25-(OH)2D3 on HSP synthesis during erythrophagocytosis were indeed related to the induction of phagocytosis and not to the previously described 1,25-(OH)2D3-mediated increase in HSP synthesis after exposure to heat (4), other normal human phagocytic and nonphagocytic cells were analyzed. Incubation with opsonized erythrocytes also induced synthesis of HSPs and p32 in peripheral blood monocytes (Fig. 1c) and alveolar macrophages (Fig. 1d) but not in nonphagocytic cells such as fibroblasts (not shown). Interestingly, in the more mature monocytes and alveolar macrophages, HSP synthesis was even more intense than in U937 cells (compare Fig. 1a and c), and, in these mature phagocytic cells, we also detected by metabolic labeling, the 60- to 65-kDa HSP homologous to the mycobacterial 65-kDa antigen to which

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**Fig. 1.** (a) Protein synthesis by U937 cells preincubated with (lanes 5–9) or without (lanes 1–4) 1,25-(OH)2D3 after incubation with different amounts of SRBC(IgG) (lanes 2–4 and 6–8) or after heat shock (45°C, 20 min) (lane 9). Cells were incubated with (lanes 2–4 and 6–8), or without (lanes 1, 5, and 9), 8 × 108 (lanes 2 and 6), 4 × 108 (lanes 3 and 7), or 1.6 × 108 SRBC(IgG) per ml (lanes 4 and 8) for 3 hr and then labeled and processed as described. Erythrophagocytosis increased the synthesis of the 83-kDa and induced the same 70-kDa proteins as heat shock, but although lane 9 was from an intentionally overexposed gel, the 32-kDa protein appeared to be induced during erythrophagocytosis only. (b) Western blot analysis of the 70-kDa protein induced in 1,25-(OH)2D3-treated U937 cells exposed to heat shock (lane 1) or incubated with SRBC(IgG) (lane 2). Lane 3, control. U937 cells treated with 1,25-(OH)2D3 (10 ng/ml, 72 hr) were exposed to 45°C for 20 min and allowed to recover for 160 min at 37°C (lane 4), incubated 180 min with 3.2 × 106 SRBC(IgG) per ml (lane 2), or maintained at 37°C (lane 3) and then labeled and processed as described. (c) Protein synthesis by peripheral blood monocytes after heat shock (lane 5), incubation with increasing amounts of SRBC(IgG) (from lane 4 to lane 2, as for U937 cells). Lane 1, control. (d) Protein synthesis by human alveolar macrophages after heat shock (lane 2), or incubation with SRBC(IgG) (lane 3). Lane 1, control.
Table 1. Effects of preincubation with 1,25-(OH)2D3 on O2- generation by U937 cells stimulated with SRBC(IgG) or opsonized zymosan

<table>
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<th>Stimulation</th>
<th>Preincubated without</th>
<th>Preincubated with</th>
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<tr>
<td>None</td>
<td>1.32 ± 1.50</td>
<td>0.40 ± 0.78</td>
</tr>
<tr>
<td>SRBC(IgG)</td>
<td>0.48 ± 0.54</td>
<td>14.52 ± 2.04</td>
</tr>
<tr>
<td>Opsonized zymosan</td>
<td>1.02 ± 0.36</td>
<td>22.4 ± 7.98</td>
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Mean ± SD of three experiments, in which each measure was done in duplicate. SRBC(IgG) were used at 8 × 10⁶ cells per ml, and opsonized zymosan was used at 3 mg/ml. Stimulation was for 60 min.

Specific T lymphocytes have been found in patients with autoimmune disease (reviewed in ref. 10).

Respective Roles of Phagocytosis, Hemoglobin, and Oxidative Burst in Stress Protein Induction. In 1,25-(OH)2D3-treated U937 cells, both phagocytosis of whole erythrocytes and generation of superoxide (O2-) were required for HSP synthesis. First, stimulation of 1,25-(OH)2D3-treated U937 cells with other phagocytic stimuli, either opsonized zymosan, which does trigger NADPH oxidase (Table 1), or latex beads, which do not induce O2- production, was not associated in these cells with detectable alterations in protein synthesis (Fig. 2, lane 3; data not shown for latex beads). Second, phagocytosis of opsonized erythrocytes depleted of hemoglobin (“resealed ghosts”; ref. 19) did not induce HSP synthesis (lane 5), although these opsonized erythrocyte ghosts did trigger respiratory burst in 1,25-(OH)2D3-treated U937 cells (Table 2) and were phagocytosed to a similar extent as whole erythrocytes. Indeed, there were for ghosts and SRBC(IgG), respectively, 45% and 56% phagocytic cells, with a mean of 1.7 ± 1.0 (range, 1–6; n = 200) opsonized erythrocyte ghosts and 2.3 ± 1.7 (range, 1–10; n = 200) SRBC (IgG) per phagocytic cell. The low grade phagocytosis achieved by 1,25-(OH)2D3-treated U937 cells with nonopsonized SRBCs was sufficient to result in induction of p32/heme oxygenase (Fig. 2, lane 2), but in this case HSP70 was barely detectable by one-dimensional gel electrophoresis.

Effects of Scavengers on Protein Synthesis. To assess the role of oxidants in the induction of stress proteins, we tested the effects of several scavengers on this induction during erythropagocytosis. Catalase did not inhibit induction of HSP70 or p32, whereas superoxide dismutase increased it (Fig. 3), suggesting that extracellular conversion of O2 to H2O2, which easily crosses cell membranes, probably increased oxidative damage. Indeed, addition of superoxide dismutase increased H2O2 production by 1,25-(OH)2D3-treated U937 cells exposed to (SRBC)IgG, which, in the absence of the enzyme, was 0.7 ± 0.1 nmol (mean ± SD; n = 3) and in its presence was 3.2 ± 0.3. Dimethyl sulfoxide (1%) was toxic to cells by itself and also increased HSP synthesis (not shown). The only compound tested that we found effective in preventing erythropagocytosis-induced synthesis of stress proteins was the free thiol WR-1065 (dephosphorylated from the radioprotective agent WR-2721) (27, 28). WR-1065 inhibited induction of HSP70 and p32 during erythropagocytosis (Fig. 3), but it had no effect on HSP synthesis after heat shock (not shown).

Homology of p32 to Heme Oxygenase. In human cells, p32, for which Keyse and Tyrrell have recently established iden-

![Fig. 2. Protein synthesis by 1,25-(OH)2D3-treated U937 cells after incubation with nonopsonized SRBCs (lane 2), SRBC(IgG) (lane 6), resealed ghosts of nonopsonized SRBCs (lane 4), resealed ghosts of SRBC(IgG) (lane 5) (all used at 3.2 × 10⁶ cells per ml), or with opsonized zymosan (lane 3). Lane 1, control. On corresponding panels, phagocytosis by 1,25-(OH)2D3-treated U937 cells of nonopsonized SRBC (2) and SRBC(IgG) (6), ghosts of nonopsonized SRBC (4) and ghosts from SRBC(IgG) (arrow in 3). For cytology, aliquots of cells were taken after erythrocyte lysis, cytocentrifuged, and colored with May–Grünwald–Giemsa stain. Photomicrographs were taken with a Zeiss Axiophot microscope. (×750.)](image-url)
Table 2. 1,25-(OH)2D3-treated U937 cells generate O2- in response to opsonized erythrocyte ghosts

<table>
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<tr>
<th></th>
<th>Nonopsonized</th>
<th>Opsonized</th>
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<tbody>
<tr>
<td></td>
<td>8 × 10^6 cells</td>
<td>3.2 × 10^6 cells</td>
</tr>
<tr>
<td>SRBCs</td>
<td>2.12 ± 0.73</td>
<td>ND</td>
</tr>
<tr>
<td>Ghosts</td>
<td>0.54 ± 0.2</td>
<td>9.79 ± 1.71</td>
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Mean ± SD (n = 3). Stimulation was as described for 60 min. After stimulation with ghosts, cells were centrifuged at 10,000 rpm for 5 min. ND, not done.

tity to heme oxygenase (19), is induced—besides by hemin—selectively by oxidative injury (e.g., H2O2, UV radiation, heavy metals) and not by heat shock (17–19, 29), as was the case in our experiments (Fig. 1 a, c, and d). Cadmium also induces stress protein (HSP70, -83, and p32) synthesis in human neutrophils (6) and in both control and 1,25-(OH)2D3-treated U937 cells and monocytes (not shown). To establish the homology of the 32-kDa protein induced during erythrophagocytosis to heme oxygenase, we used chemical peptide mapping as described in ref. 18, and we obtained, after cleavage of tryptophanyl peptide bonds, similar resulting peptides (Fig. 4; ref. 16).

Furthermore, two-dimensional gel electrophoresis revealed multiple pl values for p32 in both 1,25-(OH)2D3-treated U937 cells and human monocytes (Fig. 4 b and c). These pl values were similar to those found in human polymorphonuclear leukocytes exposed to cadmium (ref. 6; unpublished data).

**DISCUSSION**

In this report, we established that synthesis of HSPs and heme oxygenase is induced during phagocytosis of erythrocytes, a physiological function of human monocytes—macrophages associated with activation of the respiratory burst. Although it has been known for a long time that erythrophagocytosis induces heme oxygenase activity in rodent and porcine macrophages (30, 31), synthesis of the classical HSPs had not been reported before during phagocytosis. Our observations constitute evidence for the induction of HSPs during a normal human cellular function distinct from development—i.e., phagocytosis.

In U937 cells differentiated with 1,25-(OH)2D3, both phagocytosis of whole erythrocytes and O2- production were required for this induction, and we propose that after erythrophagocytosis, generation of hydroxyl radical (OH) in the presence of iron released from oxidized hemoglobin (32) by the metal-catalyzed Haber–Weiss reaction (O2- + H2O2 $\rightarrow$ OH' + O2 + OH-) (33) may participate in the induction of stress proteins. The extremely reactive OH' can induce DNA damage as well as protein degradation, both of which may signal for induction of HSP synthesis (34, 35). Although specific inducers such as heat or hemin regulate expression of HSPs or heme oxygenase separately (and even in the species in which heme oxygenase is induced by heat) (17, 36, 37), both were coordinately up-regulated during erythrophagocytosis, suggesting that oxidative injury is the likely inducer in our system. This hypothesis is further supported by the failure of human neutrophils to synthesize stress proteins during erythrophagocytosis, although these cells do respond to heat or cadmium: indeed, neutrophils' lactoferrin is responsible for the limitation of iron-catalyzed OH' formation, whereas monocytes lack lactoferrin (refs. 6 and 38; unpublished data). Moreover, the induction of both HSPs and heme oxygenase was prevented by the free thiol WR-1065, an agent that protects U937 cells from H2O2-induced calcium intrusion and cell death (B.S.P., Y. Donati, M. Kondo, Y. Tochon-Danguy, and J. P. Bonjour, unpublished data; ref. 5). Because WR-1065 can act in thiol disulfide exchange reactions and substitute for glutathione as hydrogen donor (28), the protective effects of WR-1065 suggest a role for glutathione depletion in the induction of stress proteins during erythrophagocytosis. Indeed, one common property attributed to the various inducers of p32/heme oxygenase is reactivity toward sulphydryl groups and depletion in cellular glutathione (18, 19, 29).

We are further investigating the effects of phagocytosis with or without respiratory burst on monocyte–macrophages' protein synthesis, using either monocytes from patients with chronic granulomatous disease or 17-hydroxy-wortmannin, a powerful inhibitor of the respiratory burst.

**Fig. 3.** Effects of scavengers on stress protein synthesis. 1,25-(OH)2D3-treated cells were incubated with nonopsonized SRBC (lane 2), or SRBC (IgG) (lanes 3–6) after preincubation, for 1 hr before adding SRBCs, with 5 mM WR-1065 (kindly provided by J. P. Bonjour, Geneva) (lane 4), superoxide dismutase (30 μg/ml) (lane 5), or catalase (4000 units/ml) (lane 6). Lane 1, control.

**Fig. 4.** (a) Partial peptide mapping of the 32-kDa protein induced in 1,25-(OH)2D3-treated U937 cells after incubation with SRBC (IgG). The 32-kDa protein induced by incubation with SRBC (IgG) was purified as described and then treated (lane 2) or not (lane 1) with N-chlorosuccinimide (15). (b) and (c) Two-dimensional gel electrophoresis of p32 in human monocytes after phagocytosis of SRBC (IgG) (c). (b) Control.
associated with phagocytosis (39). Preliminary results (S. Kantengwa and B.S.P., unpublished data) suggest that the state of maturation of the monocyte as well as the nature of the phagocytic stimulus are determining the phagocyte’s response.

Since we have previously shown that synthesis of HSPs protects U937 cells from H$_2$O$_2$-induced cell death (5), and because heme oxygenase has anti-oxidant potential (19, 36), we propose that induction of stress protein synthesis during erythropagocytosis represents a physiological mechanism for phagocyte autoprotection. Whether phagocytosis of microorganisms themselves constitutively expressing HSPs (such as plasmidia or mycobacteria; refs. 8–10) also induces host HSP synthesis, and the respective roles of pathogen and host HSPs in virulence, oxidative tissue injury, and immunopathology, remain to be determined. Synthesis of the 65-kDa HSP by mature monocytes–macrophages during phagocytosis and respiratory burst activation may be of particular relevance to autoimmune diseases (10, 11). Finally, since we have shown that induction of a heat shock response in phagocytes is associated with inhibition of NADPH oxidase activity (6), our observation could explain the decreased respiratory burst described in clinical situations associated with erythropagocytosis (40).

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