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Selective Induction of the Glucose-Regulated Protein grp78 in Human Monocytes by Bacterial Extracts (OM-85): A Role for Calcium as Second Messenger

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Heat shock/stress proteins (HSP) act as molecular chaperones, protect cells from injury, and are involved in the immune response. We investigated the effects of the immunomodulating bacterial extracts OM-85 on the stress response in normal human peripheral blood monocytes. While OM-85 did not induce the classical HSP, we show here, using 2D gel electrophoresis combined with immunoblotting, the induction of the glucose regulated protein grp78 (the immunoglobulin heavy chain binding protein BiP) along with the described accumulation of pro-interleukin-1β. The increased Ca^{2+} mobilization observed with OM-85 is the likely second messenger for grp78 induction. Recent studies are in favor of a protective role of grp78 against cytokine-mediated cytotoxicity and apoptosis. We suggest that grp78 induction following exposure to OM-85 explains, at least in part, the immunodulatory and protective effects of the bacterial extracts.

Heat shock/stress proteins (HSP) are a set of universally induced proteins usually named and classified into families according to their apparent molecular weight and respective inducers (1). Besides the classical HSP, stress proteins include the glucose-regulated proteins (GRP), which are induced by alterations in calcium metabolism or glucose deprivation (2). Both HSP and GRP function as molecular chaperones and exert protective functions against inflammation, reactive oxygen species (ROS) and apoptosis (3, 4). Grp78/BiP specifically chaperones the heavy chains of immunoglobulins in the endoplasmic reticulum and thus contributes to the regulation of the synthesis and the secretion of antibodies (5). HSP/GRP might also contribute to antigen processing and to their association with the major histocompatibility class II molecules (6-8).

Bacterial extracts and in particular OM-85 have been used for the prevention of recurrent infections of the upper respiratory tract in both children and adults (9-11). The protective effects of OM-85 have been based on its capacity to modulate humoral and cellular immune defense mechanisms (12). We have proposed that the biological effects of bacterial extracts could be modulated by both their own stress proteins (13) or those induced in host cells (14).

Although exposure of human phagocytes to either bacteria or bacterial extracts is associated with a respiratory burst, i.e., the production of superoxide (O_{2-}) *via* NADPH oxidase activation, the stress response patterns induced in host cells are distinct (14-16). While OM-85 activates the production of O_{2-}, classical HSP are not induced in monocytes. Since the differential induction of HSP synthesis during phagocytosis appears to be connected to the nature of the second messengers generated (PKC activation, Ca^{2+} mobilization . . .)

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Abbreviations: HS: heat shock; HSP: HS proteins; GRP: glucose regulated proteins; PKC: protein kinase C; PMA: phorbol myristate acetate, ICE: IL-1 converting enzyme.

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rather than to the activation of NADPH oxidase and the resulting production of O2- (17), we also investigated the effects of OM-85 on Ca^{2+} mobilization, and, using an indirect approach, on PKC activation (18).

Here we report that OM-85 specifically induced the synthesis and overexpression of grp78, which paralleled the modifications in cytosolic Ca^{2+} induced by the bacterial extract. Grp78 might play a role in the immunomodulatory effects of OM-85.

MATERIAL AND METHODS

Cells and bacterial extracts. Monocytes were isolated from peripheral blood of healthy donors by gradient centrifugation and purified by adherence (14, 18). The cells were cultured in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 1% L-glutamine (Gibco) and 10% fetal calf serum (Gibco). Cells were incubated at 37°C in a humidified incubator (95% air and 5% CO_2). OM-85 (provided by OM Laboratories Geneva) was prepared from 8 different bacteria species as described (14).

Reagents and exposure to stress. In these experiments RPMI1640 was replaced by RPMI without methionine (Gibco). Monocytes were incubated for 4h at 37°C with 50ng/ml phorbol ester (PMA; Sigma) or 10μM A23187 (Sigma) or 1mg/ml OM-85. For HS, cells in 25 mM Hepes-buffered RPMI without methionine (Gibco) were incubated in a water-bath at 44°C for 20 min. After HS, cells were allowed to recover at 37°C for 3.5h. The effect of Staurosporine (SS, Calbiochem) used at 200nM was tested by preincubating the cells for 15 min with the inhibitor before stimulation with PMA, A23187, OM-85 or HS.

Protein synthesis. After HS and recovery or after stimulation with PMA, A23187 or OM-85, cells were labeled with 6 μCi/ml ^{35}S-methionine (specific activity >1000 Ci/mmol; Amersham Laboratories, Buckinghamshire, England) for 90 min, then washed and lyzed in SDS. Proteins from samples corresponding to equal cell numbers were resolved by SDS-PAGE as described (19) and revealed by autoradiography.

Sample preparation for 2D PAGE and western blot analysis. Cells were washed 3× with ice-cold NaCl (0.15 M) and resuspended in 200μl ISO buffer containing urea (8 mM), Chaps (4% w/v), Tris (40 mM) and DTE (10 mM). Cells were homogenized by short, low energy sonication on ice, and 10 μl were taken for liquid counting of ^{35}S. 2D PAGE was performed essentially as described by Bjellqvist (19). Prior to isoelectric focusing, protein samples in ISO buffer were centrifugated at 10.000 rpm for 30 min at room temperature. Equal amounts of of proteins (approximately 100 μg total protein) were loaded onto the gels. First-dimensional separation was done on sigmoidal pH3–10 gradient strips of 18 cm, rehydrated with urea (8M), CHAPS (2% w/v), DTE (10 mM) and a trace of bromophenol blue. The second dimension was run vertically on 16 cm 10% linear acrylamide/Bis gels according to Laemmli (20). After the second dimension, proteins were transfered to nitrocellulose membranes and probed with a mouse monoclonal antibody against human grp78 (SPA827, StressGen Biotechnologies, Victoria, Canada).

Superoxide measurement. Monocytes were washed three times with PBS and resuspended in a buffer containing 138 mM NaCl; 6 mM KCl; 1 mM MgSo4.7H2O; 1.1 mM CaCl2.2H2O; 0.2 mM EGTA; 5.5 mM glucose; 20 MM Hepes, pH 7.4. 106 cells were stimulated with 1.6×107 M PMA or 1mg/ml OM-85 for 30 min at 37°C and superoxide measured by the superoxide dismutase-inhibitable reduction of cytochrome c as previously described (21).

Measurement of cytosolic free calcium. Cells (8×10^6) were loaded with fura-2-AM at 37°C for 30 min. Prior to fluorescence measurement, cells were washed and resuspended at 8×10^6 cells/ml in a buffer containing 136 nM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2POO4, 5 MM NaHCO3, 20 mM Hepes, 5,5 mM glucose, 0.2 mM EDTA, 1.2 mM CaCl2; pH 7.4. Fluorescence was measured in a Perkin-Elmer spectrofluorimeter at an excitation wavelength. $[Ca^{2+}]_i$ was calculated using the following expression: $[Ca^{2+}]_i = K_d[(F - F_{min})/(F_{max} - F)]$. K_d was assumed to be 224 nM at 37°C (22).

RESULTS

OM-85 induces grp78 in human monocytes: Lack of inhibition of the stress response by SS. Cells were exposed to bacterial extracts (OM-85, 1 mg/ml, 4h) (figure 1, A and B). Using biometabolic labelling we showed that in contrast to HS (Figure 1A, lane 3), which induces classical HSP including hsp65, hsp70, hsp90 and hsp110, exposure of mϕ to OM-85 did not induce any of these HSP. However OM-85 favored the synthesis of a doublet at 75–78 kD (Figure 1A, lanes 6 and 7). This pattern was also observed with the calcium ionophore A23187 (Figure 1A, lanes 4 and 5) which is a well known selective inducer of grp78 (23, 24). In order to establish whether the 75–78 kD doublet induced by OM-85 was indeed the stress protein grp78, we used the combined technique of 2D-PAGE electrophoresis and Western blotting (figure 1B). We showed that grp78 was constitutively expressed in mϕ (figure 1B,1, circle)

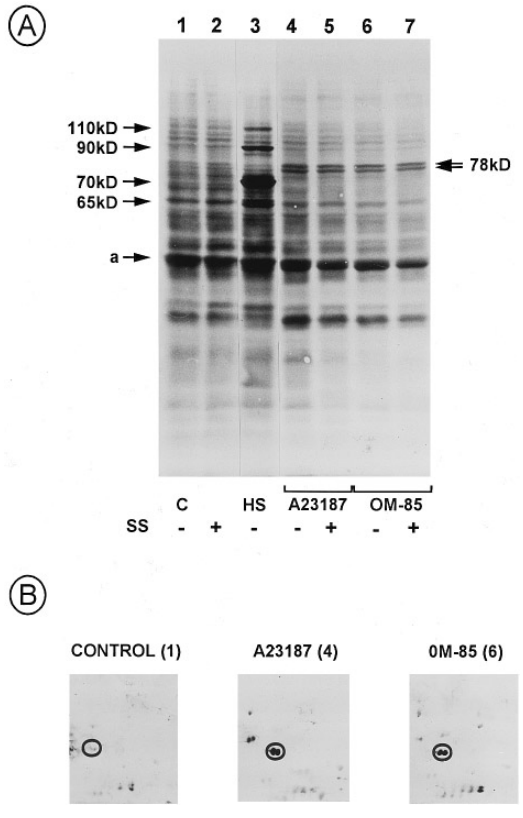


FIG. 1. Comparaison of HSP synthesis following exposure to HS, A23187 and OM-85. Human monocytes were incubated for 20 min at 44°C or for 4h with A23178 (7 μM) or OM-85 (1 mg/ml) in absence or in presence of SS (200 nM) before labelling with ^{35}S-methionine as described. Proteins were resolved by SDS-PAGE (10% polyacrylamide) and revealed by autoradiography. While HS induced classical HSP synthesis, A23187 and OM-85 induced the expression of a doublet at 75−78 kD, which was further confirmed to be grp78 by Western blot for grp78 on proteins separated by 2D-gel in human monocytes exposed to A23187(10μM) or to OM-85 (1 mg/ml) (B).Indeed, the 75−78 kD doublet induced by A23187 and OM-85 corresponded to the grp78.

and that its expression was increased after OM-85 or A23187 treatment (figure 1B,4 and 6) (the numbers refer to the the corresponding lanes in figure 1A). The position of grp78 was determined by a previously described 2D-map of mϕ and by western-blot using specific antibodies against grp78 which also confirmed the induction of grp78 by A23187 and OM-85 (figure 2B). Then, to determine whether the OM-85-induced 75−78kD doublet could be mediated by PKC as previously shown for PMA-induced HSP (17), we tested the effects of SS on stress proetin synthesis. This PKC inhibitor SS, which inhibits the PMA-induced HSP expression (17), did not prevent the induction of the 75−78 kD doublet induced by A23187 or OM-85.

Second messengers possibly involved in grp78 induction by OM-85: Superoxide production and cytosolic free calcium. As an approach to further understand the nature of the messenger involved in grp78 induction, we first compared the O_{2-} production by mϕ exposed to PMA and OM-85, and analyzed the effect of SS on O_{2-} production (figure 3). OM-85 stimulated the respiratory burst enzyme NADPH oxidase in mϕ, but in contrast to PMA, there was no significant inhibition of this effect by SS, further confirming that the signal cascade activated by OM-85 does not involve PKC.

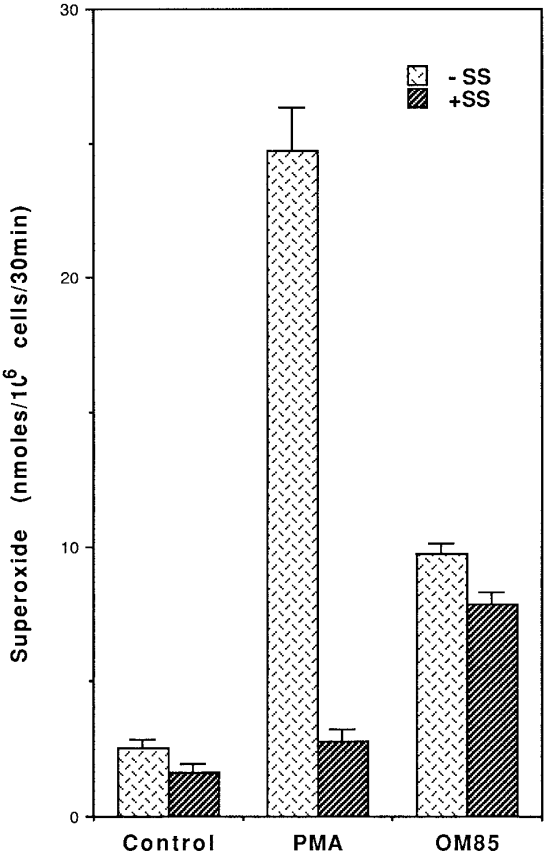


FIG. 2. OM-85 mediated superoxide production does not involve PKC. Monocytes were preincubated 15 min with SS (200nM) and then stimulated for 30 min at 37°C with either 1.6×10^{-7} PMA or 1 mg/ml OM-85. The results represent means+/−SEM from 6 different experiments in which each sample was performed in triplicates. ONly PMA-stimulated superoxide production was significatively inhibited by SS.

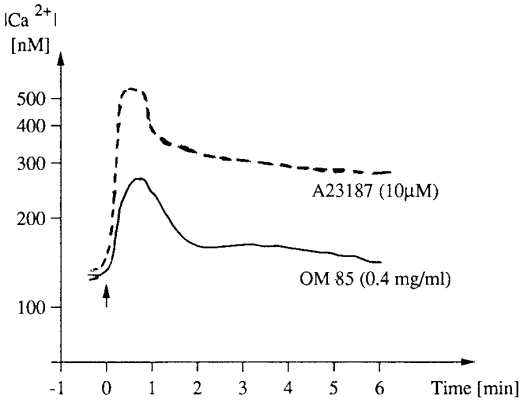


FIG. 3. OM-85 induces a rapid rise in cytosolic free calcium in human monocytes. Monocytes were loaded with fura-2 AM as described. The rise in $[Ca^{2+}]_i$ after exposure to A23187 or OM-85 was measured on 8×10^6 cells. Both stimuli, A23187 and OM-85 induced in increase in intracellular Ca^{2+}.

On the other hand, modifications of intracellular calcium have been described as a signalr leading to grp78 expression. In order to unravel the mechanism of selective induction of grp78 induction by OM-85, we thus investigated whether OM-85 induced a rise in $[Ca^{2+}]_i$ in mϕ. Fura-2 AM-loaded monocytes were stimulated by OM-85 (4 mg/ml) or A23187 (10 μM), and cytosolic Ca^{2+} was measured. As shown in figure 3, both stimuli induced a rise in $[Ca^{2+}]_i$ during the first minute of exposure of mϕ to OM-85 or A23187.

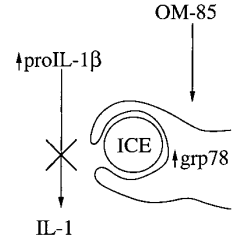
DISCUSSION

Although the bacterial extract OM-85 activates NADPH oxidase, thus leading to the generation of extracellular O_{2-}, this response is insufficient to induce the classical HSP (14). In this study we established that, in contrast, incubation of human monocytes with OM-85 induced in these cells the selective synthesis and overexpression of grp78. In order to provide a mechanistic base for the differences in HSP expression according to the type of receptor-mediated signal provided by the various inducers of NADPH oxidase, we compared the effect of OM-85 to those of A23187 and PMA on protein synthesis, superoxide production and cytosolic free Ca^{2+}.

Bacteria and bacterial extracts interact at the cell membrane with a number of receptors which are able to activate, in turn, NADPH oxidase, such as receptors for FMLP, C3 or IgG. According to the nature of ligand-receptor interactions, distinct second messengers are produced (inositol phosphate metabolism, diacylglycerol, PKC activation, arachidonic acid metabolism and Ca^{2+} mobilization). Both PMA and OM-85 were able to activate NADPH oxidase, but O_{2-} production is in itself insufficient to induce HSP synthesis, while other ROS are able to do so in human monocytes (17).

While PMA-induced HSP synthesis was suppressed by SS (17), this inhibitor had no effect on OM-85-induced overexpression of grp78 and only the PMA-mediated activation of NADPH oxidase was prevented by the PKC inhibitor, SS. Thus the effects of OM-85 are likely linked to other receptor-mediated events such as Ca^{2+} mobilization. Agents stimulating Ca^{2+} mobilization such as the calcium ionophore A23187 are classical inducers of grp78 (24). Using A23187 as a control, we found that OM-85 mimicked the effect of A23187 on both grp78 induction and the rise in intracellular Ca^{2+}. These observations are in good agreement with the involvement of Ca^{2+}, rather than PKC, as a second messenger for grp78 induction. They also confirm our hypothesis that during phagocytosis, the type of stress response observed depends upon the nature of the second messenger activated. We recently provided evidence for the involvement

TABLE I
Agents inducing grp78 also induce an increased expression of proIL-1β

Inducer	grp78	proIL-1β	Proposed mechanism for the observed association between grp78 and proIL-1β
A23187[1]	+	+	
OM-85[2]	+	+	
Cristals[3]	+	+	
Cathepsine G[4]	+	+	
Bacterial phagocytosis[5]	–	–	
Erythrophagocytosis[6]	–	–	
PMA[7]	–	–	
H_2O_2[8]	–	–	

1 and 2: ref. 14 and this paper; 3: Pizurki and Polla, in preparation; 4: Pinot and Polla, unpublished; 5: ref. 28; 6: ref. 29; 7: ref. 17; 8: ref. 17 and 30. For details, see text.

of phospholipase A2 and arachidonic acid metabolism in the OM-85 induced Ca^{2+} mobilization (Walti et al., submitted; see also ref 25).

In parallel to the induction of grp78, OM-85 also induces the accumulation of the intracellular precursor for interleukine-1β (14). Recent studies are in favor of a protective role of grp78 against cytokine-mediated apoptosis, in particular when mediated by IL-1 and TNFα. Tumor cells overexpressing grp78 become resistant to cytotoxic T cells and TNFα-mediated lysis (4). As the increased expression of grp78 parallels the increased expression of intracellular pro-interleukin-1β, we propose that grp78, induced by OM-85-mediated alterations in intracellular Ca^{2+}, binds, within the endoplasmic reticulum, to the IL-1 converting enzyme (ICE) and thus prevents the cleavage of proIL-1β into IL-1. Interestingly, all cellular stresses we found to induce grp78, also led to an increase in proIL-1β (Table I). Chaperoning and inhibition of ICE activity by grp78 (see insert in Table I) might explain the anti-apoptotic activity of grp78 (26, 27), and confer to OM-85, as an inducer of grp78, anti-apoptotic potential.

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