RESEARCH COMMUNICATION

Lipopolysaccharide-induced change of ADP-ribosylation of a cytosolic protein in bone-marrow-derived macrophages

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Treatment of bone-marrow-derived macrophages with nanogram quantities of bacterial lipopolysaccharide (LPS) or with the synthetic bacterial lipopeptide analogue *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl] (Pam₃)Cys-Ala-Gly results in a change of ADP-ribosylation of a cytosolic 33 kDa protein. The immunostimulant-induced change is both dose- and time-dependent. It is not observed in macrophages from an LPS-

unresponsive C3H/HeJ mouse strain upon treatment with LPS. Non-endotoxic LPS from *Rhodopseudomonas pallustris*, the inactive lipopeptide analogue Pam₃CysOH, and LPS in the presence of polymyxin B fail to induce the change of ADP-ribosylation of the protein. These observations indicate that reversible protein modification by ADP-ribosylation might play a role in macrophage activation.

INTRODUCTION

Lipopolysaccharide (LPS) and bacterial lipopeptides are potent macrophage activators [1,2]. In contrast with the biological effects initiated by the activators, little is known about the intracellular mechanisms by which they mediate these events. Covalent protein modification represents one mechanism underlying stimuli-induced alterations of intracellular functions. This mode of modification applies to phosphorylation–dephosphorylation which is a well-studied mechanism involved in signal transduction in macrophages [3,4]. Like phosphorylation, ADP-ribosylation constitutes another covalent modification by which cells regulate protein functions [5,6]. This post-translational protein modification is catalysed by enzymes that transfer ADP-ribose from NAD to proteins. It has been reported that this regulation affects processes in the plasma membrane, in ribosomes, mitochondria, the cell nucleus and in the cytoplasm [7]. Lately, the possible involvement of ADP-ribosylation reactions in stimuli-mediated signal transduction cascades has gained increasing interest. Brüne et al. [8] were able to demonstrate agonist-induced ADP-ribosylation of a specific cytosolic 42 kDa protein in platelets, and Halldórsson et al. [9] showed, by the use of inhibitors, that ADP-ribosylation is involved in histamine-induced endothelial production of prostacyclin. To study whether this mode of regulation applies to activation of macrophages, we measured the effect of LPS on the ADP-ribosylation of cytosolic proteins in bone-marrow-derived macrophages. We present evidence that stimulation with LPS leads to a change of the ADP-ribosylation of a cytosolic protein. This intracellular event might be related to functional activation of macrophages.

EXPERIMENTAL

Reagents

N-Palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl] (Pam₃)Cys-Ala-Gly and Pam₃CysOH, prepared by chemical synthesis [10,11], were kindly provided by G. Jung, Tübingen,

Germany. LPS from *Rhodopseudomonas pallustris* was generously donated by Dr. Weckesser, Freiburg, Germany; [³²P]NAD was purchased from Amersham Buchler Corp. (Braunschweig, Germany).

Bone-marrow-derived macrophage culture

Macrophages derived from bone marrow of BALB/c mice (aged 8–12 weeks) were obtained as described [12]. Briefly, cells (1 × 10⁵/ml) from the femur of BALB/c mice were suspended in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal-calf serum (FCS), 30% (v/v) L 929 conditioned medium, 5% (v/v) horse serum, 1 mM sodium pyruvate, 2 mM glutamine, 60 μM 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were cultured for 7 days in 87 cm² Teflon bags (Heraeus, Hanau, Germany) in 10% CO₂ at 37 °C before use.

Stimulation of macrophages and preparation of culture supernatant

Bone-marrow-derived macrophages taken from cultures on day 8 were washed twice in RPMI-1640 [Phenol Red-free; 5% (v/v) FCS]. The cells were suspended in 2 ml of RPMI-1640 at a density of 5 × 10⁶/ml in six-well culture plates (Falcon 3046, Becton-Dickinson, Heidelberg, Germany) and incubated at 37 °C in 5% CO₂ either in the presence or absence of various stimuli. After different incubation times, the supernatants were collected, centrifuged and analysed for NO₂[−] concentration.

Determination of nitrite

In the cell-culture supernatant, NO₂[−] was quantified by diazotization [13] and measurement of absorbance at 550 nm. The sensitivity of this method allowed the detection of ±0.1 μM NO₂[−]. This assay was performed in a sample volume of 0.26 ml on 96-well microtitre plates (Falcon 3072, Becton-Dickinson, Heidelberg, Germany) using a microplate reader (MR 600,

Abbreviations used: LPS, lipopolysaccharide; Pam₃, *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]; FCS, fetal-calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Dynatech, Alexandria, VA, U.S.A.). NO_2^- values were corrected for the background levels of medium determined in each case. Absence of interference by medium and buffer was ascertained using aq. standard NO_2^- solutions.

Preparation of cytosolic supernatant for [^{32}P]ADP-ribosylation

After bone-marrow-derived macrophages ($5 \times 10^6/\text{ml}$) had been stimulated as described above they were scraped off the plastic dishes with a 'rubber policeman' and washed twice in ice-cold RPMI-1640 (Gibco, Eggenstein, Germany). To disrupt the cells, the cell pellet was resuspended in ice-cold permeabilizing buffer, containing 10 mM Tris/HCl, pH 7.8, 1 mM EDTA, 4 mM MgCl_2 and 30 mM 2-mercaptoethanol, left on ice for 15 min and sonicated (3×30 s, 40 W, Branson Sonifier, Danbury, U.S.A.). To obtain the cytosolic supernatant, the homogenate was centrifuged for 1 h at 100000 g.

[^{32}P]ADP-ribosylation *in vitro*

To aliquots of cytosolic supernatant (about 30 μg) in a volume of 50 μl of permeabilizing buffer, 25 μl of an ADP-ribosylation reaction mixture was added. The mixture contained: 100 mM Tris/HCl, pH 7.8, 120 mM MgCl_2 , 1.4 μM [^{32}P]NAD (specific radioactivity 286 Ci/mmol, 30 $\mu\text{Ci}/\text{assay}$), 0.1 mM GTP, 0.1 mM ATP, 10 mM NaF, 0.01 % leupeptin, 0.54 mM NADP, 0.4 mM isobutylmethylxanthine, 0.1 % lubrol. The reaction mixtures were incubated for 30 min at 25 °C. The reactions were terminated by precipitating proteins with methanol according to Wessel and Flügge [14].

After centrifugation for 2 min at 10000 g the pellets were solubilized in SDS/PAGE sample buffer and boiled for 3 min. Proteins were separated by SDS/PAGE (10 % polyacrylamide gel) [15]. The gels were stained with Coomassie Blue (Serva Blue R, Serva Heidelberg, Germany), dried and autoradiography was performed using a DuPont Cronex film 4 (Dreieich, Germany).

RESULTS

In previous studies [16] we have shown that stimulating bone-marrow-derived macrophages for 24 h with LPS results in a slight decrease in overall ADP-ribosyltransferase activity. To determine whether LPS influences the ADP-ribosylation state of distinct proteins we incubated the macrophages with different concentrations of LPS and separated the cytosolic proteins by SDS/PAGE. As can be seen in Figure 1, there are a number of endogenously ADP-ribosylated cytosolic proteins in unstimulated macrophages (lane 1). Incubating the cells for 24 h in the presence of 0.1 $\mu\text{g}/\text{ml}$ LPS leads to the disappearance of ADP-ribosylation of a protein with an apparent molecular mass of 33 kDa. The labelling gradually declined in response to increasing LPS concentrations. At concentrations of as little as 1 ng/ml LPS the band was hardly visible. In the presence of polymyxin B LPS did not induce changes in ADP-ribosylation (Figure 2). Polymyxin B is an antibiotic which binds to the lipid A moiety of LPS and thus prevents its activity [17].

Not only LPS but also the synthetic bacterial lipopeptide Pam₃Cys-Ala-Gly, a potent macrophage activator [2], induced disappearance of the 33 kDa radioactive band (Figure 1). In contrast, the non-endotoxic LPS isolated from *Rhodopseudomonas pallustris* did not influence the ADP-ribosylation state of the protein (Figure 1). Neither did the inactive lipopeptide analogue Pam₃CysOH show any effect (Figure 3).

To determine whether the ADP-ribosylation state of the

protein correlated with activation processes in macrophages, NO_2^- formation was measured as a parameter of macrophage activation. Macrophages have been shown to produce NO_2^- from L-arginine in response to immunostimulants [18,19]. Viability of the cells, as judged by Trypan Blue exclusion, was not affected by the immunostimulant. After 24 h of incubation about 70 % of the cells were viable in the presence or absence of stimulators. Table 1 shows that stimulating macrophages with Pam₃Cys-Ala-Gly (10 $\mu\text{g}/\text{ml}$) or LPS in the range of 1 ng/ml to 0.1 $\mu\text{g}/\text{ml}$ resulted in marked NO_2^- production. Hardly any NO_2^- formation was observed when the cells were incubated with LPS from *Rh. pallustris* or with Pam₃Cys-OH. The kinetics

Figure 1 Effect of LPS and Pam₃Cys-Ala-Gly on ADP-ribosylation of cytosolic proteins

Macrophages ($5 \times 10^6/\text{ml}$) were incubated in the presence or absence of different compounds. After 24 h cytosolic supernatants were prepared and incubated with [α - ^{32}P]NAD for 30 min. Proteins were separated on SDS/PAGE (10 % polyacrylamide) as described in the Experimental section. The autoradiograph is representative of four experiments.

Figure 2 Polymyxin B abolishes LPS-induced decline of ADP-ribosylation

Macrophages ($5 \times 10^6/\text{ml}$) were incubated for 24 h with 0.1 $\mu\text{g}/\text{ml}$ LPS in the presence of polymyxin B (5 $\mu\text{g}/\text{ml}$) and also without LPS. Cytosolic supernatants were prepared and ADP-ribosylation was carried out as described in the Experimental section.

Figure 3 Kinetics of LPS-induced decline of ADP-ribosylation of a 33 kDa protein and the effect of Pam₃CysOH on this 33 kDa protein

Macrophages (5×10^6 /ml) were treated for various times with LPS (0.1 µg/ml). Cytosolic supernatants were prepared and incubated with [α -³²P]NAD for 30 min. Proteins were resolved on 10% (w/v) polyacrylamide gels as described. Similar results were obtained in two separate experiments.

Table 1 Effect of LPS and lipopeptides on NO₂⁻ formation in bone-marrow-derived macrophages

Cells (5×10^6 /ml) were incubated for 24 h and the NO₂⁻ concentration was determined in the supernatant. Values are means $\pm$ S.D. ($n = 3$). Similar results were obtained in two further cultures.

Additions (µg/ml)	NO ₂ ⁻ formation (nmol/well)
None	6.2 $\pm$ 0.3
LPS from <i>Escherichia coli</i> (0.1)	116.6 $\pm$ 3.2
LPS from <i>E. coli</i> (0.01)	123.8 $\pm$ 1.3
LPS from <i>E. coli</i> (0.001)	58.5 $\pm$ 0.5
LPS from <i>E. coli</i> (0.0001)	9.8 $\pm$ 0.5
LPS from <i>Rh. pallustris</i> (50)	14.1 $\pm$ 1.1
Pam ₃ Cys-Ala-Gly (10)	100.1 $\pm$ 10.8
Pam ₃ CysOH (10)	14.5 $\pm$ 0.4

Figure 4 Effect of LPS on ADP-ribosylation of cytosolic proteins from C3H/HeJ mice

Macrophages (5×10^6 /ml) were incubated in the presence or absence of LPS (0.1 µg/ml) for 24 h. Cytosolic proteins were labelled as described in Figure 1. Identical results were obtained in a repeat of this experiment.

of the LPS-induced decline of ADP-ribosylation was examined in macrophages stimulated for 13, 16, 20 and 24 h. As shown in Figure 3, stimulation with LPS for 13 h did not induce the disappearance of the radioactive labelling of the 33 kDa protein. The ADP-ribosylation was still visible after 16 h, and by 20 h the labelled 33 kDa protein was no longer detectable.

We next tested the effect of LPS on ADP-ribosylation of cytosolic proteins from macrophages of the mouse strain C3H/HeJ, which is a functional LPS non-responder strain. As seen in Figure 4, in the C3H/HeJ strain the label did not disappear upon stimulation with LPS. Furthermore, LPS failed to induce NO₂⁻ formation in macrophages from C3H/HeJ mice. The concentrations were 1.01 ± 0.08 nmol of NO₂⁻/well per 24 h for unstimulated cells and 1.15 ± 0.02 for stimulated cells ($n = 3$).

Taken together, these results indicate that the change in ADP-ribosylation of this protein is not an early event associated with rapid signal transduction but rather a late event possibly participating in the LPS-induced process of macrophage activation.

DISCUSSION

The present results show that LPS initiates the decline of ADP-ribosylation of a 33 kDa cytosolic protein in bone-marrow-derived macrophages. The effect is shared by another potent macrophage activator, the synthetic lipopeptide Pam₃Cys-Ala-Gly, indicating that it may represent a general mechanism underlying macrophage activation.

The decline may be due to activation or induction of an enzyme that hydrolyses protein-bound ADP-ribose [20], to suppressed activity of an enzyme catalysing the ADP-ribosylation of the 33 kDa protein, or to down-regulation of the 33 kDa substrate protein in response to LPS. Alternatively it is possible that LPS elicits augmented ADP-ribosylation of this protein in intact cells so that it will not serve as a substrate *in vitro*. This is not uncommon for potential kinase substrates and if it holds true for ADP-ribosyltransferase protein substrates, LPS could have an enhancing effect on ADP-ribosylation. Judged by the Coomassie Blue staining (Figure 5), we did not observe any differences between cytosolic proteins of stimulated and unstimulated macrophages, indicating that LPS did not induce disappearance

Figure 5 Comparison between protein staining and autoradiography of cytosolic proteins from bone-marrow-derived macrophages

Macrophages (5×10^6 /ml) were incubated for 24 h in the presence or absence of 0.1 µg/ml LPS. Cytosolic supernatants were prepared and ADP-ribosylation was carried out as described in the Experimental section. Protein staining and autoradiography of the same gel is shown. Similar results were observed in four other experiments.

of the 33 kDa substrate protein. Recently, it has been shown that sodium nitroprusside, a NO-generating compound, stimulates auto-ADP-ribosylation of a 37 kDa cytosolic protein, which has been identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [21,22]. NO induces the transfer of ADP-ribose from NAD⁺ to a cysteine residue of GAPDH, thereby inhibiting enzyme activity [23]. As macrophages produce large amounts of NO upon immunostimulation it is conceivable that increased endogenous ADP-ribosylation of GAPDH might also occur in macrophages. However, under the experimental conditions used in this study, changes in the ADP-ribosylation state of a 37 kDa protein were not observed.

The nature of the protein whose ADP-ribosylation declines in response to LPS is not known at present. The decline of ADP-ribosylation was correlated with the increase in NO₂⁻ formation. No effect on either ADP-ribosylation or NO₂⁻ formation was observed when the inactive lipopeptide Pam₃CysOH [24] or a non-endotoxic LPS from *Rh. pallustris* were used, indicating the specificity of the corresponding active compound. Furthermore, in C3H/HeJ-derived macrophages a 33 kDa protein was also found to be labelled. The C3H/HeJ strain of mice is well known to be unresponsive to LPS [25]. This unresponsiveness was attributed to a mutation of a single gene locus on chromosome 4 [26]. Neither a loss of the labelling of this 33 kDa protein nor an increase in NO₂⁻ formation was seen in these macrophages in response to LPS. In chicken polymorphonuclear leucocytes, which correspond to human neutrophils [27], a 33 kDa protein has been identified as an endogenous target protein for an arginine-specific ADP-ribosyltransferase [28]. The authors suggest that the protein is the product of the *mim-1* gene (*myb*-induced myeloid protein-1) [29], which was found to be a cellular gene activated by the nuclear oncogene product, v-Myb [30].

In summary, the present data, which are based on ADP-ribosylation studies performed extracellularly with cytosolic proteins, show that activation of macrophages is accompanied by changes in ADP-ribosylation. We have identified a 33 kDa protein which is ADP-ribosylated and which loses the label in response to LPS or Pam₃Cys-Ala-Gly. It would be interesting to know whether the change in ADP-ribosylation constituting a late intracellular event occurring in immunostimulant-induced macrophage activation also occurs in the intact cells. Characterizing the 33 kDa protein should provide us with more information concerning the role of ADP-ribosylation in stimuli-mediated intracellular events.

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