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Lipopolysaccharide-Induced Change of Phosphorylation of Two Cytosolic Proteins in Human Monocytes Is Prevented by Inhibitors of ADP-Ribosylation[1]

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Interaction of LPS with human monocytes causes altered phosphate labeling of cytosolic proteins of 36 kDa and 38 kDa (p36/38). This property, determined by in vitro studies, is shared by other monocyte activators. Phosphorylated p36/38 are distinct from p38, 42-kDa, and 44-kDa isoforms of mitogen-activated protein kinases expressed in monocytes. Occupation of LPS binding sites by a LPS antagonist, the synthetic tetraacylated bisphosphate precursor of *Escherichia coli* lipid A (also known as compound 406, lipid IV_a, or precursor Ia), prevents LPS-induced changes in the phosphate labeling of the two proteins. Abs against CD14 inhibit protein phosphorylation induced by low concentrations of LPS (10 ng/ml), whereas at high concentrations (1 μg/ml), the Abs fail to prevent phosphorylation. In addition to phosphorylation, ADP-ribosylation of proteins has been implicated in a number of biologic processes. Here we show that inhibitors of ADP-ribosylation, namely meta-iodobenzylguanidine and nicotinamide, inhibit LPS-initiated altered phosphorylation of p36/38. This loss of phosphate labeling of p36/38 is accompanied by an inhibition of TNF-α and IL-6 mRNA and protein production. The synthesis of IL-1 is not affected. This suggests that the inhibitors interfere with specific steps in IL-6 and TNF-α production, which are not required for IL-1 synthesis. Taken together, the data indicate that ADP-ribosylation may be involved in LPS-induced alteration of the phosphorylation state of two cytosolic proteins (p36/38) and that these proteins modulate cellular processes leading to TNF-α and IL-6 release. ***The Journal of Immunology,*** **1995, 155: 4899–4908.**

Interaction of LPS, a major outer membrane component of Gram-negative bacteria with cells of the monocytic lineage, induces multiple biologic responses including secretion of cytokines (1–3). This property is shared by a variety of other bacterial cell wall components such as bacterial lipopeptide (4), peptidoglycan (5, 6), muramyl dipeptide (7), and lipoteichoic acid (8). Little is known about the interactions of the activators with the plasma membrane and the subsequent molecular mechanisms by which the activators transfer their messages into the interior of the cell. A 55-kDa binding protein (CD14) for LPS has been identified on the cell surfaces of monocytes and macrophages (9, 10). This protein binds complexes consisting of LPS and serum-derived LPS binding protein (LBP)[3] (11). Engagement of CD14 by the LPS–LBP complex has been implicated in LPS-induced cellular responses (9). LPS was shown to rapidly increase tyrosine phosphorylation of several proteins in macrophages (12, 13), and this response was inhibited by Abs to CD14 (14, 15) which prevent binding of LPS–LBP complexes to CD14. Increased tyrosine phosphorylation appears to be a critical step in LPS-induced biologic responses (15, 16), indicating that this post-translational event constitutes an important mechanism involved in signal transduction. Similar to phosphorylation, ADP-ribosylation, i.e., post-translational modification of proteins by the enzymatic transfer of ADP-ribose from nicotinamide adenine dinucleotide, has been implicated in stimuli-induced cellular responses (17–19).

It is not known whether there is a link between phosphorylation and ADP-ribosylation events involved in signaling pathways. In view of the growing evidence that protein phosphorylation/dephosphorylation is a principal mechanism by which external signals can regulate intracellular events, we tested whether LPS induces altered phosphate labeling of cytosolic proteins in primary human monocytes and whether phosphorylation of proteins may be regulated by ADP-ribosylation. Here we show that stimulation with LPS leads to altered phosphorylation of two cytosolic proteins of 36 and 38 kDa, respectively, mediated by a pathway which at low but not at high LPS concentrations can be inhibited by the anti-CD14 Ab MEM-18. Phosphorylated p36/38 are distinct from the p38, 42-kDa, and 44-kDa isoforms of mitogen-activated protein kinases (MAPK) found in activated macrophages (13) and monocytes (20). In addition, we demonstrate that inhibitors of ADP-ribosyltransferases prevent phosphorylation of p36/38 as well as LPS-induced TNF-α and IL-6 production. These data suggest that ADP-ribosylation may play a role in LPS-induced altered phosphorylation of p36/38 and that phosphorylation of these two proteins may be linked to cytokine production.

Materials and Methods

Reagents

LPS of *Salmonella friedenau* (kindly provided by Dr. H. Brade, Borstel, Germany) was prepared by the phenol-water method, purified by repeated ultracentrifugation, and converted to sodium salt after electrodialysis as

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[3] Abbreviations used in this paper: LBP, LPS-binding protein; BCG, bacillus Calmette-Guérin; compound 406, a synthetic LPS antagonist; MAPK, mitogen-activated protein kinase; MIBG, meta-iodobenzylguanidine; Pam_3Cys-Ala-Gly, *N*-palmitoyl-*S*-(2,3-bis(palmitoyl)-(2*RS*)-propyl)-(*R*)-cysteinyl-alanyl-glycine; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcription PCR; T/PBS, Tris-buffered saline containing 0.05% Tween 20.

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described elsewhere (21). Synthetic tetraacylated bisphosphate precursor of *E. coli* lipid A (22; also known as compound 406, lipid IV_a, or precursor Ia) was purchased from ICN Medicals GmbH (Meckenheim, Germany). Synthetic bacterial lipopeptide *N*-palmitoyl-*S*-(2,3-bis(palmitoyl)-(2*RS*)-propyl)-(*R*)-cysteinyl-alanyl-glycine (Pam_3Cys-Ala-Gly) (kindly provided by Dr. G. Jung, Tübingen, Germany) was prepared by chemical synthesis and characterized by Drs. K.-H. Wiesmüller and G. Jung (23). Bacillus Calmette-Guérin (BCG; strain Connaught) was from Institut Mérieux GmbH (Leimen, Germany). [γ-^{32}P]ATP (sp. act. 5000 Ci/mmol) was from Amersham Buchler (Braunschweig, Germany). The anti-CD14 mAb MEM-18 (IgG1) was a kind gift of Dr. V. Horejsí (Prague, Czech Republic). The monoclonal anti-phosphotyrosine Ab (clone PT-66) was purchased from Sigma Chemical Co. (Munich, Germany). Anti-p38 Ab was kindly provided by Drs. R. J. Ulevitch and J. Han (Scripps Institute, La Jolla, CA). Anti-MAPK Ab (anti-rat MAPK R2 (*erk*-1-CT)) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Peroxidase-conjugated goat anti-mouse and anti-rabbit IgG and FITC-labeled goat anti-mouse IgG were from Dianova (Hamburg, Germany). The electrophoresis reagents were from Serva (Heidelberg, Germany). All other reagents were from Sigma unless otherwise stated.

Cell separation

PBMC from healthy volunteer donors were obtained by Ficoll-Isopaque (Pharmacia, Freiburg, Germany) density gradient centrifugation (24). After repeated washing in HBSS (Biochrom, Berlin, Germany), monocytes were isolated by counterflow elutriation using the JE-6B elutriator system (Beckman Instruments, Palo Alto, CA) as described previously (25). The cell preparations were >94% monocytes as determined by morphology and immunofluorescence staining with a mAb against CD14 (MEM-18).

Stimulation of the cells

Monocytes (4×10^6/ml) were suspended in RPMI 1640 supplemented with 10% (v/v) human serum and antibiotics in Falcon test tubes (Becton Dickinson, Heidelberg, Germany) and incubated with LPS, Pam_3Cys-Ala-Gly, or BCG at 37°C. In some experiments, monocytes were preincubated with compound 406 for 30 min, with the anti-CD14 mAb MEM-18 for 20 min, and with meta-iodobenzylguanidine (MIBG) or nicotinamide for 15 min.

Preparation of cytosol

After stimulation, cells were chilled on ice for 10 min and subsequently pelleted by centrifugation at $800 \times g$. Supernatants were harvested and analyzed for cytokine concentrations. The cells were resuspended in ice-cold buffer containing 10 mM Tris/HCl, pH 7.8, 1 mM EDTA, 4 mM $MgCl_2$, 30 mM 2-ME, and 1 mM vanadate. Subsequently, cells were sonicated (10 strokes, intensity 1.1, duty cycle 80%; Branson Sonifier 250, Danbury, CT) and cytosol was obtained by ultracentrifugation for 1 h at $100{,}000 \times g$ (Beckman Instruments). Protein concentration of the cytosol was determined by the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany).

In vitro phosphorylation of cytosolic proteins

To aliquots of the cytosolic supernatant (30 μg protein in a volume of 50 μl buffer) was added 25 μl of a phosphorylation reaction mixture containing 100 mM Tris/HCl, pH 7.8, 120 mM $MgCl_2$, 0.01% leupeptin, 0.1 mM PMSF, and [γ-^{32}P]ATP (5 μCi/aliquot). The reaction mixtures were incubated for 10 min at 37°C. The reactions were terminated by precipitating proteins with methanol according to Wessel and Flügge (26).

Electrophoresis

Pellets were dissolved in 85 μl Laemmli sample buffer (27), boiled for 5 min, and separated on 10 to 12% SDS-PAGE (SE-600; Hoefer Scientific Instruments, San Francisco, CA) at 70 mA for 4 to 5 h. The gels were stained with Coomassie blue (28), dried, and autoradiography was performed using a DuPont Cronex 4 film (DuPont, Bad Homburg, Germany) in a Kodak exposure cassette with Kodak X-OMAT intensifying screens (Eastman Kodak Co., New Haven, CT) at −70°C for 3 to 5 days.

Immunoblotting

Following electrophoresis, the gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 30 min and then the proteins were electrotransferred (Trans Blot; Bio-Rad Laboratories) to polyvinylidene difluoride (PVDF) membranes (Amersham Buchler) at 0.4 A for 14 h at 4°C. The membranes were blocked with PBS containing 5% dried milk for 3 h; then the membranes were washed six times for 10 min with PBS containing 0.05% Tween 20 (T/PBS) before overnight incubation with anti-phosphotyrosine (1:2000), anti-p38 (1:10,000), or anti-MAPK Ab (1:4000 dilution) in blocking buffer. On the next day, membranes were washed six times with T/PBS before incubation with goat anti-mouse (for anti-phosphotyrosine) or goat anti-rabbit IgG coupled with peroxidase (1:2000 dilution) for 2 h. Subsequently, the membranes were washed six times with T/PBS before visualizing the stained proteins on film by enhanced chemiluminescence (Amersham Buchler) according to the supplier's instructions. The part of the membrane labeled with anti-phosphotyrosine was subsequently washed six times with T/PBS, dried, and subjected to autoradiography as described above.

Reverse transcription-PCR of cytokine mRNA

Monocytes (1×10^6/ml) were incubated in RPMI 1640 supplemented with 10% (v/v) human serum and antibiotics in Falcon test tubes in the presence or in the absence of various compounds. After 2 h, cells were washed twice in PBS and the mRNA (2×10^5 cells/assay) was isolated using oligo(dt)-coated magnetic beads (Dynal, Hamburg, Germany) according to the manufacturer's instructions. For cDNA preparation, subsequent RT was performed as described (29). Genomic DNA was prepared according to standard protocols (30). The PCR was conducted in an automatic DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). To amplify cytokine-specific cDNA fragments, gene-specific, intron-spanning primers for IL-1β, IL-6, TNF-α, and β-actin were used, and the step cycle program was set as described (31). Electrophoresis of the PCR products was performed on 1.5% agarose gels (Life Technologies, Inc., Gaithersburg, MD) containing 1 μg/ml ethidium bromide and m.w. marker VI (pBR328 DNA × *Bgl*I + pBR328 DNA × *Hin*fI; Boehringer, Mannheim, Germany).

Flow cytometry analysis

The binding of MEM-18 was determined by flow cytometric analysis using Epics Profile II (Coulter Electronics, Krefeld, Germany). Monocytes were prepared under the same conditions used for the in vitro phosphorylation and incubated with 7.5 μg/ml MEM-18 for 20 min at 4°C. Subsequently, the cells were incubated in the absence or in the presence of 10 or 1000 ng/ml LPS for an additional 4 h at 37°C. After washing, bound MEM-18 was detected with FITC-labeled goat anti-mouse Abs. After further washing, the cells were fixed with 500 μl of azide-PBS containing 1.5% (v/v) paraformaldehyde and stored at 4°C.

For analysis, the number of positive monocytes and the mean fluorescence was determined. Unlabeled cells were used as control and adjusted to give a signal of approximately 2% positive monocytes. The data were analyzed with Epics Elite software.

Detection of cytokines in culture supernatants

Supernatants were collected for measurement of IL-1, IL-6, and TNF-α. To evaluate IL-1 activity, an IL-1-dependent human fibroblast proliferation assay was used (32). Determination of TNF-α activity was based on the cytopathic effect of TNF-α on L929 (murine fibrosarcoma) cells (33). For determination of IL-6 release, the IL-6-dependent proliferation of murine B9.9–3A4 hybridoma cells was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide staining of the cells (34).

Results

LPS changes the phosporylation state of a 36-kDa and a 38-kDa cytosolic protein

In previous studies it was shown that LPS rapidly induces protein phosphorylation in human macrophages (15). To investigate whether this post-translational protein modification also plays a role in the activation of human monocytes, we incubated freshly isolated monocytes with different concentrations of LPS and separated the cytosolic proteins by SDS-PAGE. As illustrated in Figure 1, incubating the cells for 4 h in the presence of as little as 0.1 ng LPS/ml was sufficient to increase phosphorylation of two cytosolic proteins with apparent molecular masses of 36 and 38 kDa. Maximal phosphorylation was achieved at doses of LPS between 10 and 1000 ng/ml. In the cytosol of unstimulated monocytes, weak phosphorylation of the 38-kDa protein was frequently observed, whereas in the minority of experiments phosphorylation of the 36-kDa protein was seen. As determined by Coomassie blue staining, we did not observe any differences between cytosolic proteins of stimulated and unstimulated monocytes (data not

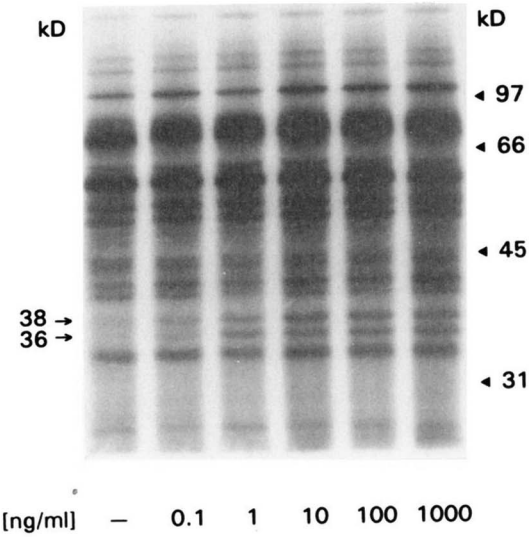

FIGURE 1. Dose dependency of LPS-induced altered phosphate labeling of p36/38. Monocytes (4×10^6/ml) were incubated in the presence or in the absence of different concentrations of LPS. After 4 h, cytosolic supernatants were prepared and incubated with [γ-^{32}P]ATP for 10 min. Proteins were separated on SDS-PAGE as described in *Materials and Methods*. The autoradiography is representative of three experiments.

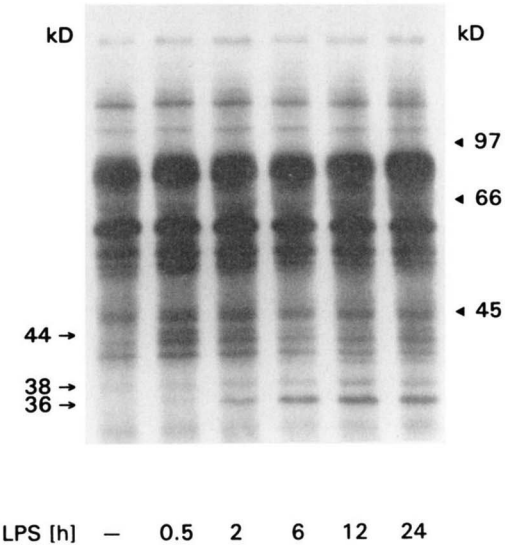

FIGURE 2. Time course of LPS-induced altered phosphate labeling of p36/38. Monocytes (4×10^6/ml) were treated for various times with LPS (10 ng/ml). Cytosolic supernatants were prepared and incubated with [γ-^{32}P]ATP for 10 min. Proteins were resolved on SDS-PAGE as described in *Materials and Methods*. Similar results were obtained in three separate experiments.

shown). The kinetics of the LPS-induced increase in altered phosphorylation of cytosolic proteins was examined in monocytes stimulated for 0.5, 2, 6, 12, and 24 h (Fig. 2). Increased phosphorylation was clearly visible 2 h after incubation, and after 24 h, the labeled proteins were still detectable. To test whether other extracellular ligands known to activate monocytes also would trigger this biochemical event, phosphorylation patterns of cytosolic proteins from monocytes incubated with the synthetic bacterial lipopeptide Pam_3Cys-Ala-Gly (4) or BCG (35) were studied. As can be seen in Figure 3, *lane 3*, stimulation with Pam_3Cys-Ala-Gly resulted in the appearance of phosphorylated p36/38. Similar results were obtained with BCG (6×10^5/ml; data not shown). Thus, altered phosphorylation of these two proteins seems to be a common event associated with monocyte activation.

Next, we conducted experiments to ascertain whether p36/38 are related to the p38, 42-kDa, and 44-kDa isoforms of MAPK and whether p36/38 are tyrosine phosphorylated in response to LPS. After in vitro phosphorylation, duplicate samples of cytosol from stimulated and unstimulated cells were resolved by SDS-PAGE and electroblotted to PVDF membrane. The membrane was reprobed by anti-phosphotyrosine and anti-MAPK Abs. Subsequently, the membrane was subjected to autoradiography. Bands detected on the Western blot corresponded to the p38 (Fig. 4), 42-kDa, and 44-kDa (data not shown) isoforms of MAPK. No anti-MAPK reactivity was noted in the region corresponding to p36/38. The p38 isoform of MAPK described by Han et al. has been cloned, and on the basis of the cDNA sequence, its molecular mass appears to be 41.5 kDa (36). LPS-induced tyrosine phosphorylation of a 41-kDa protein has also been shown in 70Z/3 cells transfected with CD14 (37). As can be seen in Figure 4, altered phosphorylation of the 38-kDa but not the 36-kDa protein may be partly a result of tyrosine phosphorylation. Judged by their mobility on SDS-PAGE and by their lack of reaction with the anti-MAPK Abs, p36/38 appear to be distinct from p38, 42-kDa, and 44-kDa MAPK.

LPS-induced change of phosporylation state of p36/38 can be inhibited by a synthetic LPS-antagonist and by CD14 Abs

The LPS-induced altered phosphate labeling of p36/38 could be completely inhibited by compound 406, a synthetic LPS antagonist (Fig. 3, *lane 5*). Compound 406 has been shown to block LPS-induced production of IL-6, IL-1, and TNF-α in a competitive manner (38–40). Compound 406 had no effect on the phosphorylation pattern of the cytosolic proteins by itself (Fig. 3, *lane 4*). Although compound 406 blocked LPS-induced altered phosphorylation, it did not prevent induction of this response mediated by Pam_3Cys-Ala-Gly (Fig. 3, *lane 6*) and BCG (data not shown). When determining the production of IL-1, IL-6, and TNF-α after incubating monocytes with LPS, Pam_3Cys-Ala-Gly, or BCG, only LPS-induced cytokine production was prevented by compound 406 (Fig. 5). These data lend support to the proposed mechanism of compound 406 by which it competes with LPS for the same binding sites but lacks the capacity to induce signaling (40–42). Furthermore, the data imply the existence of binding sites specific for LPS.

One candidate involved in LPS binding on monocytes is the membrane protein CD14. At concentrations higher than 10 ng/ml, LPS may activate certain receptors directly without help from LBP or CD14 (42, 43). To test whether LPS-induced altered phosphorylation of p36/38 is mediated by CD14, monocytes were incubated with 10 and 1000 ng/ml LPS in the absence and in the presence of anti-CD14 Abs (7.5 μg/ml). After stimulation, phosphorylation patterns of cytosolic proteins were analyzed. As illustrated in Figure 6, LPS at concentrations of 10 and 1000 ng/ml induced altered phosphorylation of the 36- and 38-kDa proteins. Induction of the phosphorylation response achieved by doses of LPS of 10 ng/ml was completely abolished by anti-CD14 Abs, whereas the Abs had no effect on cells stimulated with 1 μg/ml LPS. Measuring cytokine production in the same preparations revealed that complete inhibition by the Ab was achieved at low concentrations, and partial inhibition at high concentrations of LPS (Fig. 7). To exclude

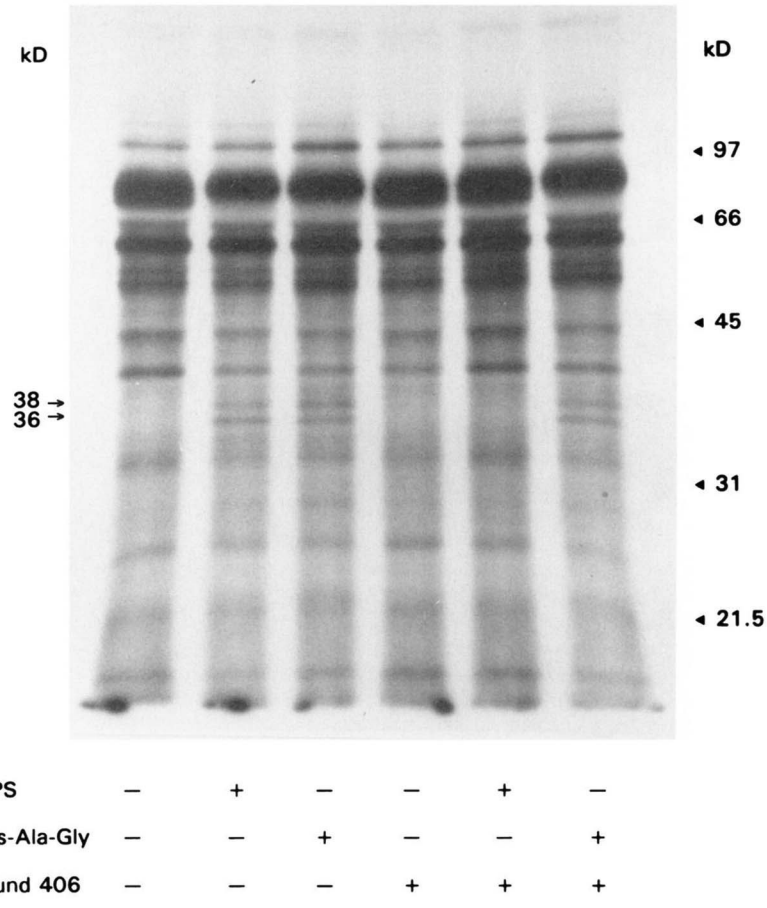

FIGURE 3. Compound 406 inhibits LPS- but not lipopeptide-induced altered phosphate labeling of p36/38. Monocytes (4×10^6/ml) were incubated for 30 min with either medium or compound 406 (10 μg/ml). The cells were then stimulated with either LPS (10 ng/ml) or Pam_3Cys-Ala-Gly (10 μg/ml). After 4 h, cytosolic supernatants were prepared and incubated with [γ-^{32}P]ATP for 10 min. Proteins were separated on SDS-PAGE as described in *Materials and Methods*. The autoradiography is representative of two experiments.

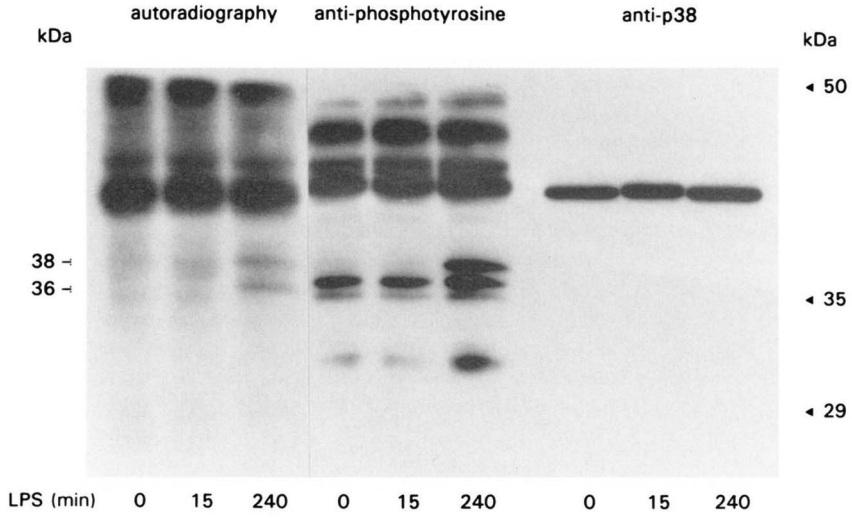

FIGURE 4. Autoradiography, anti-phosphotyrosine, and anti-p38 immunoblot of cytosolic proteins. Monocytes (4×10^6/ml) were incubated in the presence or in the absence of LPS (10 ng/ml). After 15 and 240 min, cytosolic supernatants were prepared. Aliquots were incubated for 10 min in the presence of [γ-^{32}P]ATP (*lanes 4–6*). Proteins (*lanes 4–9*) were resolved on SDS-PAGE, electroblotted to PVDF membrane and analyzed by anti-phosphotyrosine (*lanes 4–6*) or anti-p38 Ab (*lanes 7–9*). Subsequently, *lanes 4–6* were subjected to autoradiography (results shown in *lanes 1–3*).

the possibility that excess LPS (1 μg/ml) displaced anti-CD14 Abs from their binding sites, we conducted flow cytometry studies measuring the binding of anti-CD14 Abs in the absence or in the presence of LPS. As shown in Figure 6*B*, excess LPS does not displace binding of anti-CD14 Abs. Furthermore, when increasing the MEM-18 concentration (7.5 to 100 μg/ml), the partial inhibition of cytokine release and phosphorylation could not be overcome by any concentration tested (data not shown). Because MEM-18 and LPS bind to the same epitope on CD14 (44), one cannot exclude the possibility that LPS-induced altered phosphorylation of p36/38, as well as cytokine production, may be mediated by other than CD14-dependent mechanisms. Previous data by Lynn et al. (45) suggest that CD14 is not absolutely necessary for activation of mononuclear phagocytes by LPS.

Inhibitors of ADP-ribosylation inhibit LPS-induced altered phosphate labeling of p36/38 and cytokine production

In addition to phosphorylation, ADP-ribosylation constitutes an important mechanism in the post-translational modification of cellular proteins (46, 47). Similar to phosphorylation, ADP-ribosylation seems to be involved in signal transduction mechanisms (17–19). We were interested to see whether there is a cross-reaction between these two biochemical events. Therefore we pretreated monocytes with inhibitors of ADP-ribosylation, namely MIBG

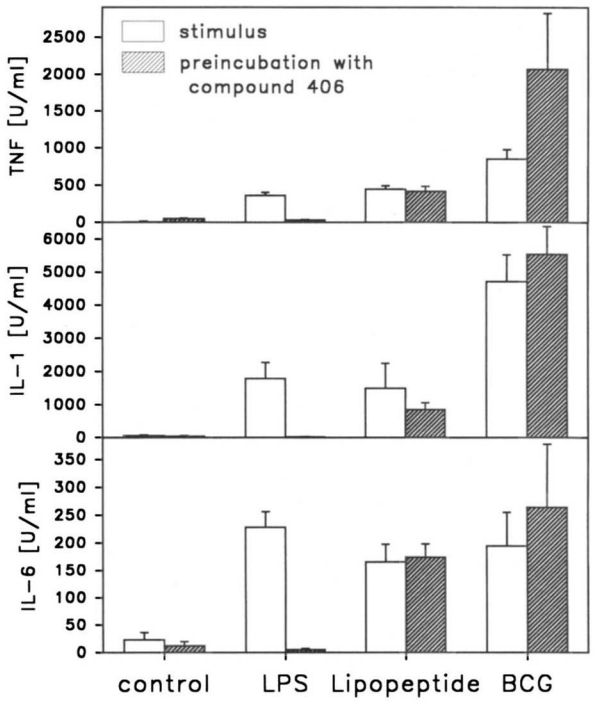

FIGURE 5. Compound 406 inhibits LPS- but not Pam_3Cys-Ala-Gly- or BCG-induced cytokine release. Monoytes (4×10^6/ml) were incubated in the presence or in the absence of LPS (10 ng/ml), Pam_3Cys-Ala-Gly (10 μg/ml), or BCG (6×10^5/ml) for 4 h. Compound 406 (10 μg/ml) was added 30 min before the addition of the substances. Supernatants were harvested and analyzed for cytokine content as described in *Materials and Methods*. Values are means ± SD of one out of two experiments.

and nicotinamide, for 15 min and then exposed the cells to LPS. After 4 h of incubation, cytokine production was measured and the phosphorylation pattern of cytosolic proteins was analyzed. Metaiodobenzylguanidine (MIBG) is a functional analogue of noradrenalin, derived from the neuron blocking agents guanethidine and bretyllium. It inhibits mono(ADP-ribosyl)transferase and has no effect on poly(ADP-ribose)polymerase activity (48). Nicotinamide inhibits both enzymes. Whereas low doses (μM) of nicotinamide are sufficient to inhibit the poly(ADP-ribose)polymerase, higher concentrations (mM) are needed to block the mono(ADP-ribosyl)transferase (49). The effects of MIBG on the LPS-induced altered phosphorylation of cytosolic proteins of monocytes are shown in Figure 8. Increasing concentrations of MIBG lead to a diminished phosphorylation of p36/38. At 0.5 mM, phosphorylation of the 38-kDa protein was hardly visible and phosphorylation of the 36-kDa protein was no longer detectable. To block phosphorylation of p36 and to diminish phosphorylation of p38 by pretreatment of the cells with nicotinamide, concentrations of 25 mM were needed (Fig. 9). Whereas in the presence of 25 and 50 mM nicotinamide complete disappearance of phosphorylated p36 was observed, phosphorylation of p38 never completely vanished. As assessed by trypan blue staining, neither 1 mM MIBG nor 50 mM nicotinamide affected the viability of the cells (data not shown). When added to the phosphorylation assay directly, a slight overall decrease in the phosphorylation activity at concentrations of 1 mM MIBG and 50 mM nicotinamide was observed (data not shown). Because MIBG and nicotinamide when given to intact cells at the same concentrations only caused disappearance of the phosphate labeling of distinct proteins, it seems likely that the inhibitors do not affect protein phosphorylation in

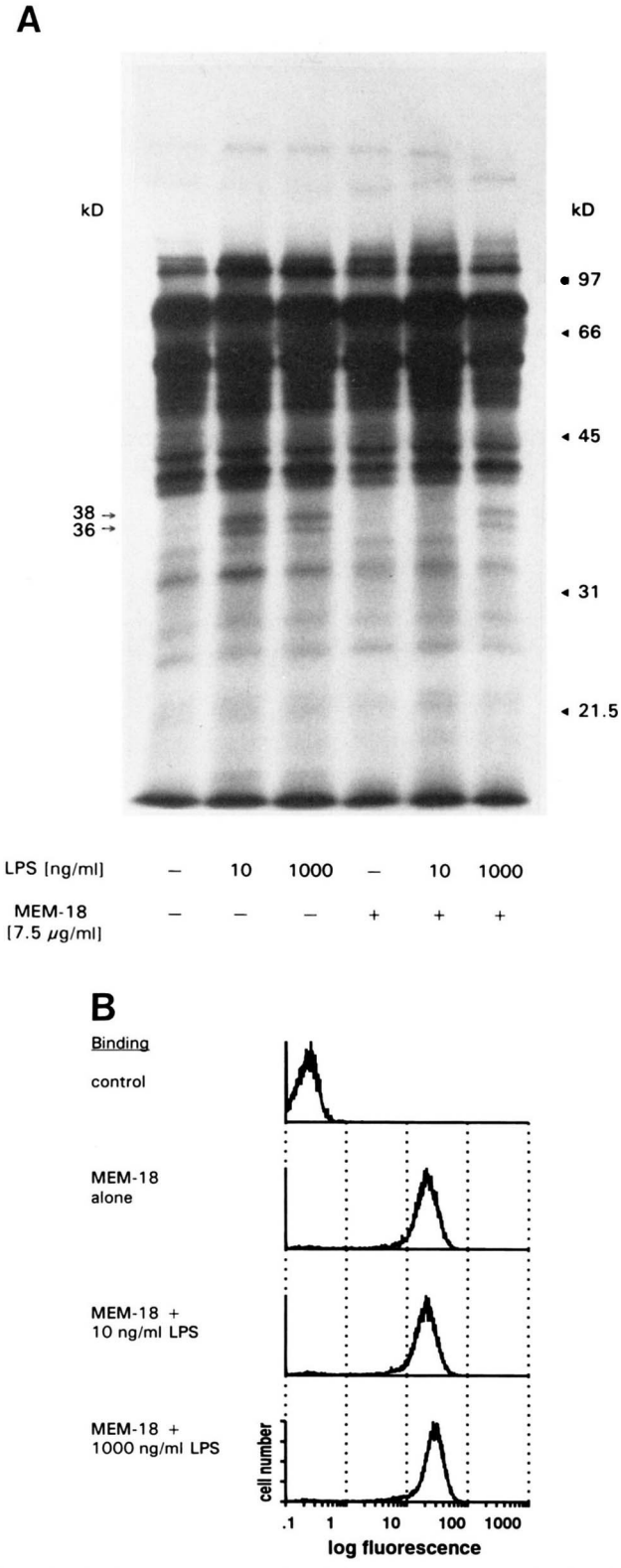

FIGURE 6. Effect of low and high LPS concentrations on phosphorylation of p36/38 and on binding of MEM-18 after preincubation with MEM-18. Monocytes (4×10^6/ml) were incubated for 20 min with either medium or MEM-18 (7.5 μg/ml). The cells were then stimulated with LPS at low (10 ng/ml) or high (1 μg/ml) concentrations for 4 h. *A*, Cytosolic supernatants were prepared and incubated with [γ-^{32}P]ATP for 10 min. Proteins were separated on SDS-PAGE as described in *Materials and Methods*. The autoradiography is representative of two experiments. *B*, Binding of MEM-18 was detected with FITC-conjugated goat anti-mouse Abs and analyzed by flow cytometry. The results are expressed as the log fluorescence of the mean fluorescence intensity.

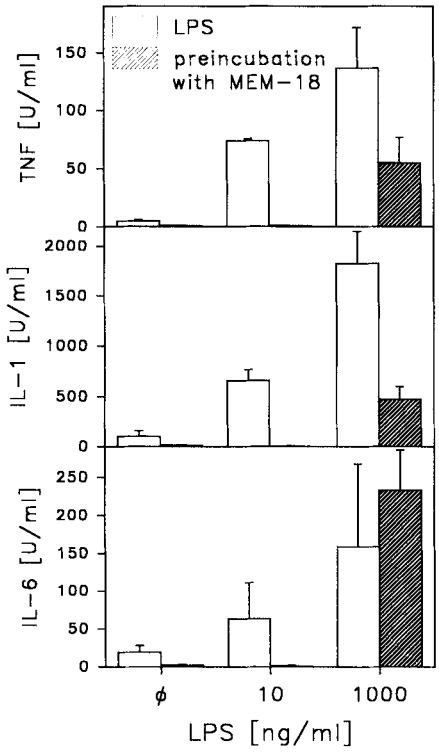

FIGURE 7. Effect of αCD14 mAb MEM-18 on LPS-induced cytokine release. Monocytes (4×10^6/ml) were incubated in the presence or in the absence of LPS (10 or 1000 ng/ml) for 4 h. The αCD14-mAb MEM-18 (7.5 μg/ml) was added 20 min before the addition of LPS. Supernatants were harvested and analyzed for cytokine content as described in *Materials and Methods*. Values are means ± SD of one out of two experiments.

general. The MIBG- and nicotinamide-induced disappearance of the phosphate labeling of p36/38 was accompanied by an inhibition of LPS-induced TNF-α and IL-6 production (Fig. 10). Treatment with 25 mM nicotinamide or 0.5 mM MIBG abolished TNF-α and IL-6 release completely. In contrast to TNF-α and IL-6 production, IL-1 production was not suppressed at any concentrations of nicotinamide or MIBG tested. High concentrations of nicotinamide (50 mM) and MIBG (0.5 mM) even increased IL-1 release. A comparison of the concentrations of MIBG and nicotinamide needed to block TNF-α and IL-6 release with those needed to prevent the phosphate labeling of p36/38 revealed that disappearance of the phosphate labeling was always accompanied by complete inhibition of the TNF-α and IL-6 release.

Inhibition of IL-6 and TNF-α mRNA expression by MIBG and nicotinamide

The next experiments were conducted to examine whether inhibitors of ADP-ribosylation would affect LPS-induced mRNA expression of cytokines. Monocytes were stimulated with LPS in the presence or in the absence of MIBG and nicotinamide. Two hours after incubation, mRNA expression of IL-1β, IL-6, and TNF-α was determined by RT-PCR. As shown in Figure 11, LPS induced the mRNA expression of all three cytokines (*lane 10*). At concentrations of 0.5 mM MIBG, the mRNA expression of TNF-α and IL-6 (*lane 4*) was strongly diminished, and at 1 mM it was no longer detectable (*lane 5*). The upper cDNA fragment obtained by TNF-α-specific RT-PCR could be shown to be from genomic DNA (*lane D*). Fifty millimolar nicotinamide suppressed mRNA expression of IL-6 and markedly reduced TNF-α mRNA expression (*lane 9*). These data show that inhibitors of ADP-ribosylation prevent LPS-induced mRNA expression of the cytokines IL-6 and TNF-α. However, they have no effect on mRNA expression of IL-1.

Discussion

Our data document that LPS initiates changes of the phosphorylation state of a 36- and a 38-kDa cytosolic protein in human monocytes. There are a number of proteins that undergo tyrosine phosphorylation in response to LPS (12, 13, 37, 50). Some of these proteins have been identified as the 42-kDa and 44-kDa isoforms of MAPK (13, 20, 51, 52). MAPKs are a family of enzymes that participate in the signal transduction pathways activated by a variety of extracellular ligands (53–55). Recently, another member of the MAPK family, p38, has been shown to be a target of LPS action (14, 50). Similar to its counterpart in yeast, the product of the *Saccharomyces cerevisae HOG1* gene, p38 is also phosphorylated in response to osmotic stress (50), the activation of p38 being mediated by the dual phosphorylation motif Thr-Gly-Tyr.

The onset of phosphorylation of MAPK (p38/42/44) is rather rapid (13, 14). Maximal phosphorylation occurred at 15 min and tyrosine-phosphorylated p38 was still detectable 120 to 180 min after stimulation (14). In contrast to phosphorylation of MAPK, altered phosphorylation of p36/38 does not occur until 30 to 60 min of incubation, and the peak of phosphorylation is observed between 2 and 6 h. This difference in kinetics, along with Western blot experiments that show that Abs of p38, 42-kDa, and 44-kDa MAPK isoforms do not react with p36/38, indicate that p36/38 are distinct from MAP kinases and that alteration of the phosphate labeling of p36/38 occurs downstream of the activation of the Ras/raf/MAP kinase cascade. Moreover, immunoblotting with anti-phosphotyrosine Abs revealed that LPS does not induce tyrosine phosphorylation of the 36-kDa protein.

The changes in the phosphorylation state of p36/38 as measured under in vitro conditions could represent an enhanced phosphorylation mediated by a protein kinase or a suppression of a specific phosphatase(s). Alternatively, with respect to p36, it is possible that LPS elicits augmented dephosphorylation of this protein in intact monocytes so that it serves as substrate in vitro. If this holds true, LPS could have an enhancing effect on phosphatase activity.

Similar to LPS, other monocyte and macrophage activators such as Pam_3Cys-Ala-Gly and BCG initiate the change of the phosphorylation state of p36/38, indicating that modification of these two proteins is a general intracellular event in a process of monocyte activation by a variety of extracellular stimuli. In the presence of LPS antagonist compound 406, only LPS-, but not Pam_3Cys-Ala-Gly- or BCG-induced alteration of phosphorylation of p36/38 was abolished. Furthermore, inhibition of the cytokine production by compound 406 was restricted to the action of LPS. Previously it has been shown that compound 406 inhibits binding of LPS on human monocytes and that it lacks the ability to induce cytokine production (39, 40). To ensure that compound 406, which behaves in a competititve manner, was present in sufficient amounts to inhibit the Pam_3Cys-Ala-Gly- and BCG-induced response, the concentrations of Pam_3Cys-Ala-Gly and BCG were reduced stepwise down to 100 ng/ml and 6×10^4/ml, respectively—the lowest concentrations still inducing cytokine release. At all concentrations, tested against a constant concentration of compound 406 (10 μg/ml), no inhibitory effect of compound 406 was observed (data not shown). However, when regarding the stoichiometry of agonist–antagonist interactions, one should keep in mind that lipids such as compound 406, LPS, and lipopeptides are amphiphiles in aqueous solution and that they form supramolecular structures

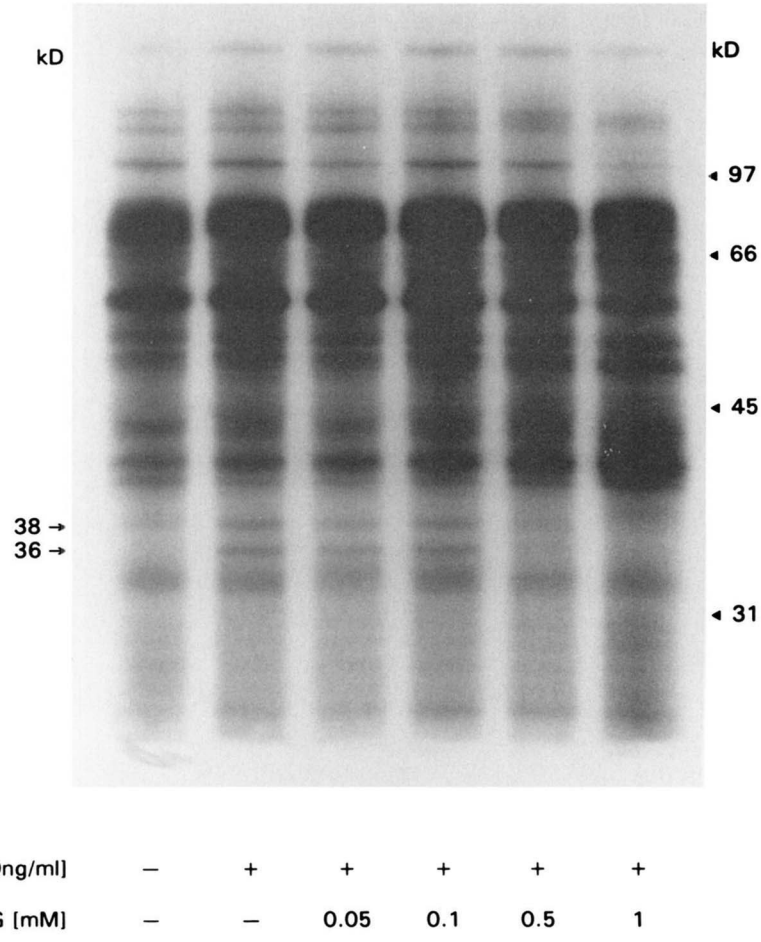

FIGURE 8. Inhibition of LPS-induced altered phosphate labeling of p36/38 by MIBG. Monocytes (4×10^6/ml) were incubated in the presence or in the absence of various concentrations of MIBG for 15 min. The cells were then stimulated with LPS (10 ng/ml). After 4 h, cytosolic supernatants were prepared and incubated with [γ-^{32}P]ATP for 10 min. Proteins were separated on SDS-PAGE as described in *Materials and Methods*. The autoradiography is representative of three experiments.

(56). It is necessary to compare the physical states of these structures in aqueous solution rather than compare the m.w.s. However, because the physical states of these structures are unknown, comparison of the bioactivities on a molar basis is not possible. The results mentioned previously and the present data suggest that LPS and compound 406 share a common ''receptor'' or a portion thereof, that the ''receptor'' is not used by the two other stimuli tested, and that, in addition to binding to the ''plasma membrane,'' structural information is needed for a compound to induce signal transduction resulting in cytokine production. To date, the only well-characterized receptor for LPS is CD14 (9). To address the role of CD14 in LPS-induced events, we measured protein phosphorylation and cytokine production in the cytosol of cells that were stimulated with 10 ng/ml and 1 μg/ml LPS in the presence of anti-CD14 Abs. We found that anti-CD14 (MEM-18) Abs completely inhibited the altered phosphorylation of p36/38 and cytokine production induced by 10 ng/ml LPS; partial inhibition was found after incubation with 1 μg/ml LPS. Because MEM-18 binding was not displaced by high LPS concentrations, the possibility cannot be excluded that mechanisms other than CD14-dependent mechanisms exist by which LPS can trigger the cells.

Although it has been shown that the region between amino acids 57 and 64 of CD14 is essential for proper binding of LPS (57) and that this region is also required for binding of MEM-18 (44), there is the possibility that other functional LPS binding sites exist in addition to amino acids 58–65 that are not recognized by MEM-18. Unless LPS exerts its effects in the presence of Abs against such putative binding sites, and the Abs cannot be displaced by LPS, it is too early to distinguish between a CD14-dependent and

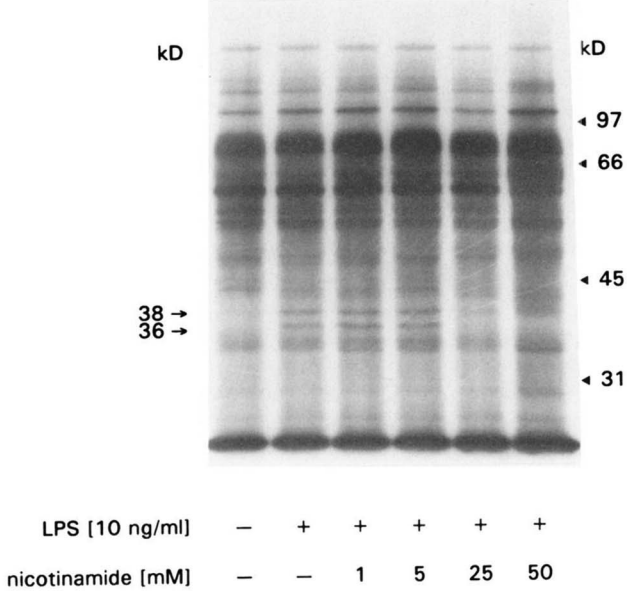

FIGURE 9. Inhibition of LPS-induced altered phosphate labeling of p36/38 by nicotinamide. Monocytes (4×10^6/ml) were incubated in the presence or in the absence of various concentrations of nicotinamide for 15 min. The cells were then stimulated with LPS (10 ng/ml). After 4 h, cytosolic supernatants were prepared and incubated with [γ-^{32}P]ATP for 10 min. Proteins were separated on SDS-PAGE as described in *Materials and Methods*. The autoradiography is representative of three experiments.

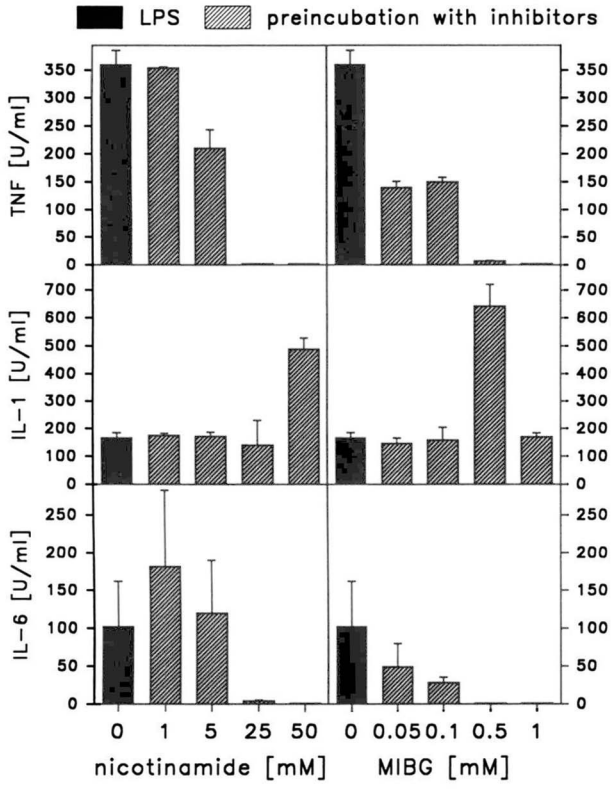

FIGURE 10. Effect of nicotinamide and MIBG on LPS-induced cytokine release. Monoytes (4×10^6/ml) were incubated in the absence or in the presence of different concentrations of nicotinamide or MIBG 15 min before the addition of LPS (10 ng/ml) for 4 h. Supernatants were harvested and analyzed for cytokine content as described in *Materials and Methods*. Values are means ± SD of one of four experiments.

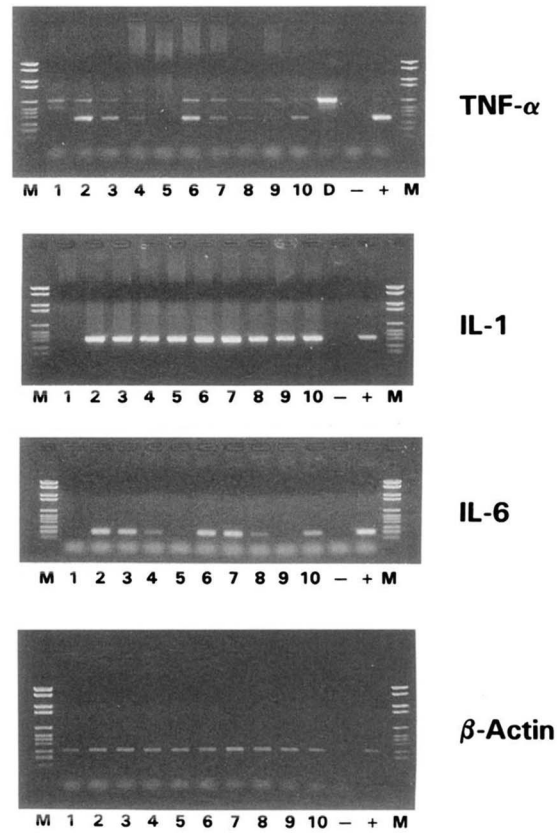

FIGURE 11. LPS-induced expression of TNF-α and IL-6 mRNA is inhibited by high concentrations of nicotinamide and MIBG. Monocytes (1×10^6/ml) were incubated for 15 min with either medium or various concentrations of nicotinamide or MIBG. The cells were then stimulated with LPS (10 ng/ml). After 2 h, mRNA was isolated. After RT, PCR was performed with specific primers for TNF-α, IL-6, IL-1, and β-actin. Data show one representative experiment out of three. Unstimulated monocytes (*lane 1*), LPS-stimulated monocytes (*lane 10*), LPS-stimulated monocytes in the presence of 0.05, 0.1, 0.5, and 1 mM MIBG (*lanes 2–5*), LPS-stimulated monocytes in the presence of 1, 5, 25, and 50 mM nicotinamide (*lanes 6–9*). Controls: no cDNA (*lane* —), defined cDNA of the concerned protein (*lane* +), DNA preparation for TNF-α (*lane D*). Marker: DNA m.w. marker VI (*lane M*).

-independent pathway. Furthermore, the absence of biophysical information regarding the precise interactions between CD14 and LPS, and between the CD14–LPS complex and downstream effector molecules, precludes conclusive interpretations about possible LPS-mediated mechanisms without the help of CD14.

LPS-induced altered phosphorylation of p36/38 can be prevented by nicotinamide and MIBG, inhibitors of ADP-ribosylation (48, 49). Whereas MIBG is supposed to be selective for mono(ADP-ribosyl)transferases (48), nicotinamide acts on both mono(ADP-ribosyl)transferase and poly(ADP-ribose)synthetase (58). The high doses of nicotinamide needed to inhibit p36/38 phosphorylation suggest involvement of a mono(ADP-ribosyl)transferase. Inhibition of phosphorylation of p36/38 by these two substances might imply that protein modification by ADP-ribosylation alters the rate of phosphorylation. Thus, mono(ADP-ribosyl)transferases may modify p36/38 directly or they may act on proteins involved in phosphorylation of the two proteins. A decrease in phosphorylation by ADP-ribosylation has been reported for histone H1 (59) and for phosphorylase kinase (60). Concomitant with the loss of phosphate labeling of p36/38, an inhibition of TNF-α and IL-6 but not IL-1 production at the mRNA and protein level could be observed. Disappearance of the phosphate labeling of p36/38 is always accompanied by a blockade of TNF-α and IL-6 production, suggesting that the two proteins may be involved in the production of these two cytokines. These results suggest that LPS stimulates the production of TNF-α and IL-6 through a pathway different from IL-1. Different biochemical pathways for LPS-induced TNF-α and nitric oxide production in mouse peritoneal macrophages has been suggested by Zhang and Morrison (61), who report that the signal transduction for both pathways is regulated by a pertussis toxin-sensitive factor. Nicotinamide and MIBG have been shown to suppress a series of cellular functions and biosynthetic processes (19, 47, 62). It is not clear whether they block these reactions by virtue of their ability to inhibit endogenous ADP-ribosylation or through putative nonspecific effects. However, the specificity of these inhibitors on protein phosphorylation, limited to the disappearance of labeling of p36/38, argues against the possibility that general unspecific phenomena are responsible for the phosphorylation processes described here.

Our results suggest that ADP-ribosylation may be involved in LPS-triggered events leading to altered phosphorylation of p36/38. Inhibition of phosphorylation is accompanied by inhibition of TNF-α and IL-6 production, indicating that the phosphorylation state of p36/38 may be part of a signal transduction pathway that mediates monocyte responses initiated by a variety of monocyte activators.

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