Lipopolysaccharide Activates Caspase-1 (Interleukin-1–Converting Enzyme) in Cultured Monocytic and Endothelial Cells

By Ralf R. Schumann, Claus Belka, Dirk Reuter, Norbert Lamping, Carsten J. Kirschning, J oerg R. Weber, and Dagmar Pfeil

Interleukin-1β (IL-1β) is a pleiotropic proinflammatory cytokine. Mechanisms leading to its secretion include not only release of newly synthesized protein, but also cleavage of a preformed immature precursor protein into an active secretory form by the intracellular protease caspase-1 (formerly termed IL-1–converting enzyme [ICE]). Caspase-1 belongs to a rapidly growing family of cysteine proteases with substrate specificity for aspartate involved in cellular apoptosis. We have used an assay determining the caspase-1 activity based on cleavage of a fluorogenic peptide substrate to elucidate its role in lipopolysaccharide (LPS)-induced secretion of IL-1β. We show that LPS induces moderate caspase-1 activity in the monocytic cell line THP-1, in freshly isolated peripheral blood monocytes, and in human umbilical vein endothelial cells (HUVECs) in a time- and dose-dependent fashion. Caspase-1 activation by LPS was associated with cleavage of the IL-1β precursor protein that was followed by release of the mature IL-1β protein in monocytic cells. In contrast, subsequent release of IL-1β by HUVECs was not significant. LPS-induced caspase-1 activation appeared not to result from modulation of caspase-1 transcript accumulation and inhibition of caspase-1 activity was accomplished by two specific inhibitors, YVAD-CHO and YVAD-CMK, capable of alleviating the release of mature IL-1β. Taken together, these results show that LPS moderately activates caspase-1 and that caspase-1 activation contributes to LPS induction of IL-1β secretion.

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Consist of an equimolar ratio of 10- and 20-kD proteins, termed p10 and p20. LPS stimulation of human monocytes or THP-1 cells fails to change the amount of p45 or its activity and does not induce appearance of detectable p20 caspase-1. cDNA cloning showed that both forms (p10 and p20) were found within the p45 precursor protein containing four cleavage sites, leading to formation of the protein subunits that are flanked by Asp-X bonds, so that it is conceivable to assume that the proenzyme is activated autocatalytically. Cleavage and activation of the native p45 caspase-1 precursor has been recently characterized by the use of specific inhibitors and antibodies recognizing various regions of caspase-1. Low-level expression and instability led to problems not only in isolation and purification of caspase-1, but also in estimating its activity in cells or cell lysates.

One of the strongest inducers of IL-1 secretion is LPS (endotoxin), a component of cell walls of Gram-negative bacteria. LPS, bound by the LPS binding protein (LBP), is transported to its cellular receptor, the CD14 molecule, where it induces its specific cellular responses. Responses of CD14- cells, eg, endothelial cells, to LPS stimulation require the presence of soluble CD14 (sCD14).

To examine LPS-induced caspase-1 activation in different cell types, we have used a caspase-1 bioassay employing a fluorogenic substrate that contains a specific peptide sequence of IL-1β, acyl-Tyr-Val-Ala-Asp-AMC. Cleavage activity of caspase-1 is shown by increased concentrations of the fluorogenic moiety of the substrate, aminomethylcoumarin (AMC), which can be visualized by fluorometry. Using this assay, caspase-1 activity can be precisely monitored. We show that LPS induces biologically active caspase-1 in monocytic and endothelial cells, which spurs the generation and release of mature IL-1β.

Materials and Methods

Culture and cell treatment. Human umbilical vein endothelial cells (HUVECs) were obtained and characterized as described before. Cells were maintained and stimulated by LPS as indicated. Monocytes were obtained from healthy voluntary donors, separated by density gradient centrifugation, and washed with RPMI 1640 medium. Separated cells were resuspended in RPMI1640 containing 1% penicillin/streptomycin, 1% glutamine, 1% sodium pyruvate, and 10% human AB serum. Aliquots of 5 mL were incubated for 2 hours at 37°C in a humidified atmosphere of 5% CO2 in air. Nonadherent cells were discarded and adherent cells were washed with RPMI 1640 before stimulation with LPS (100 ng/mL) in RPMI. The human monocyte leukemia cell line THP-1 was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium containing 1% penicillin/streptomycin, 1% glutamine, 1% sodium pyruvate, and 10% fetal calf serum. To induce differentiation associated with increased surface expression of CD14, cells were exposed to 80 mmol/L DH-VD3 for 3 days at 37°C. Cells were stimulated with different concentrations of LPS using a medium containing 10% AB serum. Viability and function of cells were optimized by maintenance of cells in the log phase of growth and monitoring for greater than 95% viability as indicated by trypan blue exclusion.

Inhibition of enzyme activities. Caspase-1 activity was inhibited in vivo and in vitro by addition of the reversible inhibitor acyl-Tyr-Val-Ala-Asp-CH0 or the irreversible inhibitor acyl-Tyr-Val-Ala-Asp-chloromethylketone at concentrations of 0.2 to 20 µmol/L. The inhibitors were applied 2 hours before LPS stimulation (in vivo) and 5 minutes before caspase-1 substrate addition (in vitro), respectively. Viability of cells was monitored by trypan blue exclusion and calculated by the percentage of trypan blue-positive cells divided by protein content.

Lysis of cells. After washing with ice-cold phosphate-buffered saline (PBS) nonadherent cells were resuspended at 10⁷ cells/100 µL of ice-cold lysis buffer, and adherent cells were scraped off the plate into 100 µL lysis buffer/10 cm² culture plate surface. Lysis buffer consisted of 20 mmol/L Tris-acetate, pH 7.5, 1% Triton X-100, 0.1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaVO₃, 10 mmol/L Na-α-glycerophosphate, 50 mmol/L NaF, 5 mmol/L Na-pyrophosphate, 1 mmol/L benzamidine, 270 mmol/L sucrose, 1 mmol/L phenylmethylsulfonyl fluoride, and 20 mmol/L leupeptin. Lysates were placed on ice for 20 minutes, followed by centrifugation at 10,000g for 15 minutes at 4°C. The postmitochondrial supernatant fraction was removed, frozen in liquid nitrogen, and stored in aliquots at −80°C. Protein concentration of samples was determined by the Bradford method using bovine serum albumin (BSA) as standard.

Electrophoresis and immunoblotting. Protein samples of 50 µg were prepared for electrophoresis as described. Samples were separated on 12% or 15%, respectively, sodium dodecyl sulfate (SDS)-polyacrylamide gels using a buffer system as previously described. After electrophoresis, the gel was soaked in transfer buffer containing 48 mmol/L Tris, 39 mmol/L glycine, and 0.04% SDS for several minutes and the proteins were transferred to Hybond-C extra membranes by semidy blotting. Membranes were blocked overnight at 4°C with 5% BSA in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST). They were washed intensively according to the manufacturer’s instructions (Amersham, Braunschweig, Germany), incubated with polyclonal rabbit anti-human IL-1β antiserum, and incubated for 2 hours at room temperature. After washing, membranes were incubated with horseradish peroxidase-linked protein A (protein A-POD) in TBST (1:10,000) containing 1% BSA for 90 minutes at room temperature. After a final washing, blots were developed using the ECL system (Amersham) and immunoreactive proteins were visualized on film.

Quantification of mIL-1β levels by enzyme immunoassay (EIA) and immunoprecipitation of IL-1β. The TiterZyme IL-1β EIA kit (Biemann GmbH Diagnostica, Bad Nauheim, Germany) was used for quantitative determination of human IL-1β levels in cell culture supernatants. This assay was performed according to manufacturer’s guidelines. A total of 1.5 mL supernatant also was incubated with 15 µg of a polyclonal anti-IL-1β antibody (Genzyme, Cambridge, MA), followed by protein A/G plus agarose (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) addition and centrifugation according to the manufacturer’s instructions. The pellet was taken up in sample buffer and run on a 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by a IL-1β ECL Western blot as described above.

Measurement of caspase-1 activity. The assay measuring caspase-1 activity in cell lysates is based on published protocols. Test volumes of 100 µL consisted of 100 µg protein in a final buffer of 50 mmol/L Tris-acetate, pH 7.4, containing 1 mmol/L dithiothreitol, 0.5 mmol/L EDTA, and 20% glycerol. Ten microliters of a stock solution of the acetylated and AMC-conjugated peptide substrate, acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (1 µg substrate/µL dimethyl sulfoxide), was added to the test tubes. The final concentration was 150 µmol/L. Ten minutes after incubation at 37°C, the reaction was terminated by quick-freezing in liquid nitrogen. Triplicates of samples and the substrate control containing no protein were thawed immediately before recording fluorescence intensity. The reaction product AMC was detected at λex = 350 nm and λem = 435 nm. The absorbance was correlated to caspase-1 concentration by using a standard curve of AMC. Specific activity was read as units per milligram of protein. One unit was defined as an activity that releases 1 pmol AMC per minute.

Statistical analysis of the results. Results shown are representative of at least three independent experiments. Statistical analysis was performed at a level of probability of .01 < P < .05 (caspase-1...
rehem and acyl-Tyr-Val-Ala-Asp-Chloromethylketone (YVAD-CHO), and an irreversible one, YVAD-CMK. To exclude
whether LPS effects on the caspase-1 activation are specific, LPS-induced caspase-1 activity is concentration- and
time-dependent and is associated with intracellular accumulation and release of mature IL-1β. Induction of caspase-1 activity
in DH-VD3–pretreated THP-1 cells was time-dependent and dependent on the concentrations of LPS used. As shown in Fig
1, the addition of LPS to THP-1 cell cultures increases caspase-1 activity, with 100 ng LPS/mL being the optimum
stimulatory concentration. Further increase of the LPS concentration was not associated with better inducibility of caspase-1.
Time- and dose-dependency of caspase-1 induction also correlated with levels of IL-1β detectable in supernatants of THP-1
cells as assessed by enzyme-linked immunosorbent assay (ELISA; Fig 1B). To examine whether LPS-induced activation of
caspase-1 was also associated with increases of intracellular levels of mature IL-1β, Western blot analysis was performed
using an anti–IL-1β antibody and cell lysates of stimulated and nonstimulated THP-1 cells. As shown in Fig 2, the addition of
LPS led to an increase of both cleavage products of caspase-1, the 18-kD mature IL-1β protein, and the 28 kD fragment. An
increase of the 31/33-kD precursor protein was also observed, indicating that LPS was inducing both synthesis of the IL-1β
precursor protein and activation of caspase-1, leading to enhanced cleavage of the precursor.

LPS induces moderate caspase-1 activity in freshly isolated peripheral blood monocytes and HUVECs, correlating with
release of mature IL-1β in monocytes only. To assess whether LPS induces caspase-1 activity also in freshly isolated monocytes
or in LPS-responsive, yet CD14− cells such as endothelial cells, experiments were performed with freshly isolated peripheral
blood-derived human monocytes and HUVECs. The results, which are shown in Fig 3, indicate that, in monocytes, an
approximately twofold, significant increase of caspase-1 activity can be achieved by stimulating cells with LPS and that this
increase is paralleled by a twofold enhanced IL-1β secretion (Fig 3A). In HUVECs, constitutive levels of IL-1β release could not be significantly enhanced by LPS. However, caspase-1 activity could be moderately increased by addition of LPS, which also was statistically significant (Fig 3B). To determine
whether mature IL-1β or proIL-1β was released into the supernatant of monocytes and HUVECs upon stimulation by
LPS, we performed an immunoprecipitation experiment, followed by Western blotting. In both cell types, no precursor
IL-1β molecule, which would migrate at 31 kD, could be detected (Fig 4). However, mature IL-1β at 18 kD gave rise to a
signal, and the previous result of an inducibility in monocytes but not in HUVECs was confirmed in this assay.

Specific caspase-1 inhibitors block LPS-induced activation of caspase-1 and IL-1β release in THP-1 cells. To determine
whether LPS effects on the caspase-1 activation are specific, two peptide inhibitors were instrumental, a reversible inhibitor,
YVAD-CMK, and an irreversible one, YVAD-CHO. To exclude
cytotoxic effects, cell viability was assessed by trypan blue exclusion and no cytotoxicity of both inhibitors could be observed (Table 1). YV AD-CHO and YV AD-CMK were added in vitro to lysates of LPS-induced and control cells, respectively, and caspase-1 activity was assessed (Fig 5). Caspase-1 activity was found to be decreased in lysates of LPS-induced and control cells by both inhibitors in a concentration-dependent fashion. At concentrations of 0.2 µmol/L, LPS-induced increase in caspase-1 activity was blocked significantly by both inhibitors (.01 < P < .02 for YV AD-CHO and .01 < P < .05 for YV AD-CMK, respectively). This condition reflects a molar ratio of 1:750 (inhibitor to substrate). Additionally, caspase-1 inhibitors were added to the culture medium of cells, and caspase-1 activity as well as IL-1β release induced by LPS was measured. Figure 6 shows that treatment of THP-1 cells with caspase-1 inhibitors in vivo alleviates both LPS-induced caspase-1 activity and IL-1β release significantly (P < .01). To exclude that caspase-1 inhibitors affected IL-1β precursor synthesis, Western blot analyses were performed in the absence or presence of YV AD-CMK. Figure 7 shows that the addition of the caspase-1 inhibitor failed to significantly diminish levels of the IL-1β precursor protein. On the contrary, addition of YV AD-CMK led to a slight increase in constitutive levels of intracellular precursor protein. In the presence of LPS, YV AD-CMK slightly reduced IL-1 precursor levels. As controls, the transcriptional inhibitor of protein synthesis actinomycin D and the translation inhibitor cycloheximide strongly suppressed synthesis of the IL-1β precursor protein. In summary, it was shown that LPS-induced caspase-1 activity can be blocked specifically by the inhibitors YV AD-CHO and YV AD-CMK.
pretreated THP-1 cells. DH-VD3–pretreated THP-1 cells obtained experiments irreversible caspase-1 inhibitor YVAD-CHO. (B) Results adding YVAD-AMC. (A) Results obtained with the irreversible caspase-1 inhibitor YVAD-CHO before stimulation as described in the Materials and Methods. The inhibitors were added in vitro to the lysates 5 minutes before measurement of caspase-1 before addition YVAD-AMC. (A) Results obtained with the reversible caspase-1 inhibitor YVAD-CHO. (B) Results obtained with the irreversible caspase-1 inhibitor YVAD-CMK. Shown are mean values of three independent experiments ± SD. Differences in (A) were significant with .01 < P < .02 and in (B) with .01 < P < .05, respectively. (□) Without LPS; (■) with LPS.

Table 1. Caspase-1 Inhibitors Do Not Influence Cell Viability at Concentrations Used

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Inhibitor</th>
<th>Concentration (µg/mL)</th>
<th>Toxicity Quotient*</th>
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<tr>
<td></td>
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<tr>
<td>Endothelial cells</td>
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<tr>
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*Trypan blue OD595nm divided by protein content.

DISCUSSION

Induction of cytokine production by LPS has attracted much attention in the past because of the profound stimulatory effects that LPS exerts on cells of the monocyte/macrophage and the endothelial cell lineages. Although this stimulatory effect may have protective effects in the physiologic host response to inflammatory challenges, if unbalanced, it may contribute to acute and chronic pathologic states of the organism. For instance, a dramatic induction of IL-1β occurs during inflammation and sepsis and is caused by LPS. The regulation of this process has been studied by several groups, including our own.18,25,41,42 Secretion of IL-1β by monocytes is tightly regulated, and the amount of IL-1β secreted by these cells is much higher than that produced by any other cell type in the body.29,41 Processing of the precursor of IL-1β to the fully active and secretory cytokine is regulated by the specific cystein protease caspase-1. However, up to now, regulation of caspase-1 activity has not been studied in greater detail because of technical difficulties in measuring caspase-1 activity.18,25,41,42

It has been shown that LPS enhances both the rate of transcription of the IL-1β gene and accumulation of IL-1β transcripts. However, enhanced gene expression alone cannot account for the rapid and strong induction of IL-1β release by LPS seen in several cell types. We show here that activation of caspase-1 is another mechanism whereby LPS can induce IL-1β release. According to our results, LPS acts most likely on both levels: transcriptional induction of the IL-1β gene, followed by an intracellular increase of the 30-kD IL-1β precursor, which is paralleled by a moderate activation of caspase-1, leading to cleavage and release of the mature IL-1β protein. Activation of caspase-1 results from a catalytic event, deliberating an inactive form of caspase-1 into an active one. Reverse transcriptase-polymerase chain reaction experiments performed by us suggest that a transcriptional induction of the caspase-1 gene does not play a role during LPS-induced caspase-1 activation (data not shown). Others have recently shown that Langerhans cells and epidermal-derived dendritic cell lines constitutively display high levels of caspase-1 mRNA that are only moderately enhanced by LPS. Our data are also consistent with studies showing that LPS-induced and noninduced THP-1 cells contain comparable levels of caspase-1 protein, indicating that LPS does not effect levels of caspase-1.25

It has been suggested that caspase-1 is largely associated with the cell membrane and enhances directly the export of mature IL-1β after cleavage of the precursor protein.18 This hypothesis is consistent with our observation that mL-1β is found only in small quantities inside the cell, indicating that processing and secretion are temporarily linked. Our data also show that the kinetics of caspase-1 activation and secretion of IL-1β in monocytes, but not in endothelial cells, are similar, further suggesting that the state of caspase-1 activation in myeloid cells directly relates to secretion of IL-1β. In HUVECs, caspase-1 is also moderately induced; however, this induction does not lead to significant IL-1β release. Moreover, inhibition of caspase-1, which may have potential clinical ramifications as a tool during IL-1β-mediated processes, was shown by us to inhibit IL-1β release. Inhibition by two specific compounds, YVAD-CHO and YVAD-CMK, was almost as effective as the potent but toxic inhibitor of protein synthesis cycloheximide (data not shown). It was recently shown that caspase-1 (ICE) knock-out mice exhibit a striking resistance towards experimental induction of septic shock.43 This finding is supported by recent results showing that caspase-1 not only leads to cleavage and release of IL-1β but also of interferon-γ-inducing factor (IGIF; IL-18).20 Thus, specific caspase-1 inhibitors that act selectively and are
less toxic may be valuable tools to suppress caspase-1 activity and subsequent release of a number of cytokines and thus may be useful in interfering with the onset and course of sepsis and septic shock. However, in our experimental approach, we were able to inhibit only approximately 50% of LPS-induced caspase-1 activity, and it has to be shown in animal experiments whether this inhibition is sufficient to block the LPS effects in vivo. Experiments addressing this question are currently underway in our laboratory.

Activation of caspase-1–like proteases has also been linked to the induction of programmed cell death (apoptosis). During this process, activation of caspases ultimately leads to the cleavage of the poly-(ADP-ribose) polymerase (PARP) and thus to inhibition of DNA repair. Recent findings indicate that FAS-mediated apoptosis is mediated by other caspases, such as CPP32 or FLICE/Mach and not by caspase-1. On the other hand, it was recently found that apoptosis induced by infection, ie, human immunodeficiency virus infection of cells is strictly caspase-1–mediated. Several investigators furthermore showed that LPS can induce apoptosis in different cell types. Whether LPS-induced activation of caspase-1 mediates programmed cell death of monocytes/endothelial cells needs further analysis. Nonetheless, there were no signs of apoptosis induction in the cells observed over the observation period reported here. Completely elucidating the cellular events induced by LPS will aid in understanding inflammatory processes and may potentially lead to novel therapeutic strategies in sepsis and shock.

ACKNOWLEDGMENT

The authors thank Nicole Siegemund and Ina Krukenberg for their excellent technical assistance.

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