The Cathepsin B Inhibitor z-FA.fmk Inhibits Cytokine Production in Macrophages Stimulated by Lipopolysaccharide*

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Cathepsin B has previously been shown to proteolytically activate the proinflammatory caspase-11 in vitro. Here we show that cathepsin B is not involved in activation of caspase-11 induced by lipopolysaccharide (LPS) and subsequent maturation of interleukin (IL)-1β in macrophages. Nevertheless, we found that the cathepsin B inhibitor benzoylcarbonyl-Phe-Ala-fluoromethylketone (z-FA.fmk) prevents LPS-induced production of IL-1α, IL-1β, and tumor necrosis factor at the transcriptional level. The latter was not because of cathepsin B inhibition, but was mediated by inhibition of the transactivation potential of the nuclear factor κB (NF-κB). z-FA.fmk did not prevent LPS-induced activation of p38 mitogen-activated protein kinase, which was shown to be involved in NF-κB transactivation in response to LPS. These results suggest that the previously described therapeutic effect of z-FA.fmk in the treatment of rheumatoid arthritis might not only result from inhibition of cathepsin B but also implicate an important contribution from the inhibition of NF-κB-dependent gene expression.

Chronic inflammatory diseases are caused by prolonged production of several proinflammatory cytokines such as interleukin (IL)-1, IL-2, IL-6, IL-8, and tumor necrosis factor (TNF), as well as genes encoding nitric oxide synthase, cell adhesion molecules, and immunoreceptors (1). The expression of many of these genes is regulated by the nuclear factor κB (NF-κB). z-FA.fmk did not prevent LPS-induced activation of p38 mitogen-activated protein kinase, which was shown to be involved in NF-κB transactivation in response to LPS. These results suggest that the previously described therapeutic effect of z-FA.fmk in the treatment of rheumatoid arthritis might not only result from inhibition of cathepsin B but also implicate an important contribution from the inhibition of NF-κB-dependent gene expression.

In addition to transcriptional regulation, translational or post-translational regulation is also involved in the production of a number of inflammatory mediators. For example, the maturation and secretion of IL-1β is controlled by caspase-1 (2), which itself is activated by caspase-11 (3). The critical role of caspase-1 and caspase-11 in inflammation is well illustrated by the finding that caspase-1- or caspase-11-deficient mice are resistant to LPS-induced septic shock (3). Recently, we showed that caspase-11 could be activated by cathepsin B in vitro (4). Cathepsin B is implicated in a number of inflammatory diseases and pathological conditions, such as bronchitis, rheumatoid arthritis, acute pancreatitis, and cancer progression (5, 6). In the case of rheumatoid arthritis, it is well documented that cytokines induce the secretion of lysosomal proteases of the cathepsin family by the main cellular population of rheumatoid synovial membranes and fluids (7–9). Inhibitors of these proteases abrogate the articular degradation and maintenance of rheumatoid lesions and have already proven their therapeutic potential in diseases of excessive bone resorption, such as rheumatoid arthritis or osteoporosis (10). Benzoylcarbonyl-Phe-Ala-fluoromethylketone (z-FA.fmk) is a peptide inhibitor that irreversibly alkylates the active cysteine residue in cathepsin B, thereby inhibiting its proteolytic activity. In vivo, z-FA.fmk has been shown to inhibit the severity of inflammation, as well as the extent of cartilage and bone damage in adjuvant-induced arthritis (11). In the present study we demonstrate that z-FA.fmk is also a potent inhibitor of NF-κB-dependent gene expression in macrophages, which might contribute to its described anti-inflammatory activity.

MATERIALS AND METHODS

Cells and Reagents—The murine macrophage cell lines MΦ/4 (12) and pU5.18 (ATCC, Manassas, VA) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 1 mM sodium pyruvate. z-FA.fmk and SB203580 were obtained from Calbiochem-Novabiochem. Ef4 was purchased from Roche Molecular Biochemicals. LPS originated from Sigma, and anti-IL-1β antibody was from R & D Systems (Minneapolis, MN). Polyclonal anti-caspase-11 antibodies were raised in rabbits against purified murine caspase-11. Antibodies against p35, p65, and IκBα were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal phosphospecific and polyclonal p38 mitogen-activated protein (MAP) kinase antibodies were purchased from New England Biolabs (Beverly, MA).

Measurement of Cathepsin B Activity—To determine cathepsin B activity in total cell extracts, cells were lysed in 10 mM Tris/HCl, pH 7, 1% Nonidet P-40, 200 mM NaCl, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Cleared cell extracts (25 μg of cellular protein) were incubated for 1 h at 30 °C in cell-free system buffer (10 mM Heps/NaOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH2PO4, 0.5 mM EGTA, 2 mM MgCl2, 5 mM pyruvate, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) with 50 μM of the fluorogenic cathepsin B substrate benzoylcarbonyl-Ala-Arg-Arg-afc; z-FA.fmk, benzoylcarbonyl-Phe-Ala-fmk.

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§ The abbreviations used are: IL, interleukin; TNF, tumor necrosis factor; afc, 7-amino-4-trifluoromethylcoumarin; EMSA, electrophoretic mobility shift assay; fmk, fluoromethylketone; LPS, lipopolysaccharide; MAP, mitogen-activated protein; NF-κB, nuclear factor κB; z-ARR.afc, benzoylcarbonyl-Ala-Arg-Arg-afc; z-FA.fmk, benzoylcarbonyl-Phe-Ala-fmk. This paper is available on line at http://www.jbc.org

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Fig. 1. Effect of z-FA.fmk and E64 on the production of IL-1β and the activation of caspase-11 in LPS-stimulated Mf4/4 cells. A, cathepsin B activity. Cells were treated for 48 h with 50 μM of the indicated inhibitors, and cathepsin B activity was measured in cell extracts (25 μg of cellular protein) with the fluorogenic substrate z-ARR.afc as described under Materials and Methods. Data are expressed as increase in fluorescence as a function of time (ΔF/min). B, IL-1β production in cell supernatant. Cells were incubated with 50 μM of the indicated inhibitors 1 h before stimulation with 1 μg/ml LPS for 48 h. MeSO₂, used as a solvent for the inhibitors, served as a negative control. Results are representative of three independent experiments (S.D. <10%). C, up-regulation of IL-1β (upper panel) and activation/up-regulation of caspase-11 (lower panel). Lysates were prepared from cells treated as in B. 100 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-IL-1β and anti-caspase-11. The faster migrating band detected with anti-caspase-11 corresponds to the p36 subunit generated by proteolytic removal of the C-terminal p10 subunit.

RESULTS

z-FA.fmk Inhibits IL-1β Production in LPS-stimulated Macrophages Independently of Cathepsin B—In line with our previous findings that cathepsin B can activate caspase-11 in vitro (4), we studied the effect of two cathepsin B inhibitors, viz. E64 and z-FA.fmk, on the LPS-induced activation of procaspase-11 and production of IL-1β by macrophages, which is known to require caspase-11 (3). Mf4/4 cells were preincubated for 1 h with 50 μM E64 or z-FA.fmk and subsequently stimulated with 1 μg/ml LPS for 48 h. As expected, pretreatment of Mf4/4 cells with z-FA.fmk or E64 completely inhibited cathepsin B activity over a period of 1 to 72 h as measured with the fluorogenic substrate z-ARR.afc (see Fig. 1A and data not shown). However, although the LPS-induced production of IL-1β could be completely inhibited by z-FA.fmk, E64 treatment had no effect at all. Similarly, pretreatment of the cells with the cathepsin B-specific inhibitor CA-074Me had no effect (data not shown). Identical results were obtained when IL-1β was measured in the supernatant in an IL-1-dependent CTLL proliferation assay (Fig. 1B) or in cell extracts by Western blotting with IL-1β-specific antibody (Fig. 1C, upper panel). The slightly lower IL-1β levels, detected with the bioassay in the supernatant of E64-treated cells, are because of a limited toxic effect of E64 on the CTLL cells used for the bioassay. In line with the above results, proteolytic activation of procaspase-11 with the generation of the p36 subunit still occurs in LPS-stimulated macrophages in the presence of z-FA.fmk or E64 (Fig. 1C, lower panel). Similar observations were made in pU5.18 macrophages. Measurement of a dose response in this cell line demonstrated that z-FA.fmk inhibits LPS-induced IL-1β production at a dose of 25–50 μM (Fig. 2), which is over 100 times higher than that required to inhibit cathepsin B (data not shown). In conclusion, these results exclude the involvement of cathepsin B in the LPS-induced signaling pathway leading to IL-1β production by macrophages, which is in contrast to the previously described effect of cathepsin B in vitro (4). Moreover, our observations also demonstrate that z-FA.fmk is able to inhibit LPS-induced IL-1 production by a novel mechanism.

z-FA.fmk Inhibits the LPS-induced Production of IL-1β and Other Cytokines at the Transcriptional Level—It has previously been demonstrated that the LPS-induced expression of several...
cytokines can be regulated at the transcriptional, translational, and post-translational levels (16). In the case of IL-1β, we demonstrated by Western blotting that z-FA.fmk inhibits synthesis of the inactive IL-1β precursor (Fig. 1C). To investigate whether z-FA.fmk inhibits IL-1β synthesis at the transcriptional level, mRNA was isolated from Mf4/4 cells that were stimulated with LPS for 3 h in the absence or presence of 50 μM z-FA.fmk and analyzed for the expression of several cytokine mRNAs in a RiboQuant assay. This revealed that z-FA.fmk inhibits the LPS-induced synthesis of IL-1β at the mRNA level (Fig. 3). Also, the expression of IL-1α and TNF was strongly inhibited by z-FA.fmk, whereas IL-6 synthesis, as well as transcription of the housekeeping genes L32 and glyceraldehyde-3-phosphate dehydrogenase, were not affected (Fig. 3).

**z-FA.fmk Inhibits NF-κB-mediated Gene Expression in LPS-stimulated Macrophages**—It has previously been demonstrated that the transcription of the TNF and IL-1β genes is, at least partly, dependent on the activation of the transcription factor NF-κB (17). To investigate whether z-FA.fmk inhibits LPS-induced IL-1 and TNF production by preventing LPS-induced NF-κB activation, we analyzed its effect on NF-κB-dependent luciferase reporter gene expression in Mf4/4 cells that were transiently transfected with p(κB)3LUC and stimulated with LPS. Pretreatment of Mf4/4 cells with 50 μM z-FA.fmk for 1 h significantly inhibited LPS-induced luciferase reporter gene expression (Fig. 4). As a control, expression of a noninducible cytomegalovirus-regulated luciferase reporter was not blocked by z-FA.fmk (data not shown). These results suggest that inhibition of NF-κB activity by z-FA.fmk contributes to the inhibitory effect of z-FA.fmk on the expression of IL-1β and several other cytokines in LPS-stimulated macrophages.

We subsequently analyzed the effect of z-FA.fmk on LPS-induced NF-κB activation by measuring the amount of nuclear NF-κB in EMSA. Mf4/4 cells stimulated with LPS translocate NF-κB into the nucleus, resulting in a defined band in EMSA (Fig. 4B, lane 2). Pretreatment of Mf4/4 cells with z-FA.fmk reduced the nuclear levels of NF-κB by 50% compared to the untreated control (Fig. 4B, lane 3). This is consistent with the EMSA data showing that z-FA.fmk reduces NF-κB activation by 50% compared to the untreated control. Pretreatment with z-FA.fmk for 1 h protected against NF-κB nuclear translocation after LPS stimulation (Fig. 4C). Pretreatment with z-FA.fmk reduced the nuclear levels of NF-κB by 50% compared to the untreated control (Fig. 4C). Pretreatment with z-FA.fmk reduced the nuclear levels of NF-κB by 50% compared to the untreated control (Fig. 4C). Pretreatment with z-FA.fmk reduced the nuclear levels of NF-κB by 50% compared to the untreated control (Fig. 4C). Pretreatment with z-FA.fmk reduced the nuclear levels of NF-κB by 50% compared to the untreated control (Fig. 4C). Pretreatment with z-FA.fmk reduced the nuclear levels of NF-κB by 50% compared to the untreated control (Fig. 4C).

An alternative explanation for the z-FA.fmk-induced mobility shift could be that z-FA.fmk inhibits the potential proteolytic processing of one of the components of the NF-κB complex during nuclear extract preparation, even in the presence of general protease inhibitors such as Pefabloc, aprotinin, and leupeptin. To check this possibility we analyzed nuclear extracts of LPS-treated Mf4/4 cells for the presence of intact p50 and p65 by immunoblotting. As expected, LPS induced nuclear translocation of p65 and p50, even in the presence of z-FA.fmk. However, in the absence of z-FA.fmk, p65 was cleaved to products of 31 and 33 kDa (Fig. 5A). Cleavage products of p50 could not be detected (Fig. 5B), although p50 levels were slightly increased in LPS-stimulated cells pretreated with z-FA.fmk. In contrast, p65 cleavage was not detected when cells were directly lysed in Laemmli buffer (Fig. 5C). Analysis of the same extract revealed that 1xR-luciferase reporter gene expression was not affected by z-FA.fmk and z-FA.fmk pretreatment.

In conclusion, these results further demonstrate that inhibition of NF-κB-mediated gene expression by z-FA.fmk is not because of inhibition of the nuclear translocation of NF-κB in response to LPS. Moreover, the specific degradation of p65 in nuclear extracts of LPS-treated cells and its inhibition by z-FA.fmk pretreatment demonstrates that the slower migrating NF-κB complex, visible in EMSA of LPS-treated cells pretreated with z-FA.fmk (Fig. 4B), results from the z-FA.fmk-mediated inhibition of p65 proteolysis during nuclear extraction. These data also demonstrate the risk of misinterpretation of mobility shifts liable to occur in EMSA.

**z-FA.fmk Has No Effect on the LPS-induced Activation of p38 MAP Kinase**—We and others previously demonstrated that p38 MAP kinase plays an essential role in the transactivation of NF-κB in response to TNF, whereas it is not involved in the pathway leading to nuclear translocation of NF-κB (18, 19). Because z-FA.fmk also seems to interfere specifically with the transactivation of NF-κB in response to LPS, we analyzed whether p38 MAP kinase is also involved in NF-κB-dependent gene expression in response to LPS and whether z-FA.fmk affects the activation of p38 MAP kinase. Pretreatment of LPS-stimulated Mf4/4 cells with the p38 MAP kinase inhibitor SB203580 showed that p38 MAP kinase was also involved in the enhanced transcriptional activity of NF-κB in response to LPS (Fig. 6A). However, z-FA.fmk did not affect the LPS-induced phosphorylation of p38 MAP kinase in Mf4/4 cells, excluding the fact that z-FA.fmk prevents NF-κB transactivation by inhibiting the LPS-induced activation of p38 MAP kinase.

**DISCUSSION**

Cathepsin B is a cysteine protease implicated in a number of inflammatory diseases and pathological conditions, such as bronchitis, rheumatoid arthritis, acute pancreatitis, and cancer progression (5, 6). The main function of cathepsin B is the degradation of proteins that have entered the lysosomal system from outside the cell (via endocytosis or phagocytosis) or from other compartments within the cell (autophagy). In addition,
activation of a number of proteins such as procollagenase, proplasminogen activator, and prorenin has been described (20–22). Recently, we demonstrated that purified cathepsin B is also able to proteolytically activate the proinflammatory caspase-11 in vitro (4). The latter results prompted us to investigate the role of cathepsin B in the activation of caspase-11 and the subsequent production of IL-1 in LPS-stimulated macrophages. For these experiments we made use of z-FA.fmk and E64, which are frequently used inhibitors of cathepsin B (23, 24). However, none of these inhibitors was able to prevent the LPS-induced processing of caspase-11 in macrophages. To our surprise, z-FA.fmk completely inhibited the LPS-induced production of IL-1β. Also, IL-1α and TNF synthesis was abolished by pretreatment with z-FA.fmk, whereas IL-6 and caspase-11 production was not affected. Because E64 and CA-074Me did not modulate the LPS-induced production of these cytokines, we conclude that this effect of z-FA.fmk is independent of cathepsin B. Moreover, we were unable to detect an effect of LPS on cathepsin B activity in the cells studied (data not shown). z-FA.fmk was found to interfere with the production of IL-1 and TNF by preventing the transcription activation potential of NF-κB. It is still unclear why the expression of IL-6 is only minimally affected by z-FA.fmk, because IL-6 expression has previously been shown to be NF-κB-dependent (25). This suggests the involvement of gene-specific regulatory mechanisms of NF-κB-dependent gene expression, as previously demonstrated in the case of TNF (26). This also fits with our observation that z-FA.fmk does not prevent the IκB kinase.
pathway leading to degradation of the NF-κB inhibitor IκB and subsequent nuclear translocation of NF-κB. In contrast, z-FA.fmk seems to specifically interfere with a signaling pathway or molecule that leads to the transactivation of NF-κB and that might indeed be gene promoter-specific. The p38 MAP kinase pathway has previously been shown to fulfill such an NF-κB transactivation function in response to TNF-α (18). Here we could demonstrate a similar role for p38 MAP kinase in activating NF-κB-dependent gene expression in response to LPS. However, z-FA.fmk did not interfere with the LPS-induced activation of p38 MAP kinase. Although we cannot exclude an effect of z-FA.fmk on the activation of downstream substrates of p38 MAP kinase, the above observations make it rather unlikely that z-FA.fmk modulates NF-κB-dependent gene expression by interfering with the p38 MAP kinase pathway. The fluoromethyl ketone group of z-FA.fmk is known to be very reactive with activated nucleophiles (27), and the specificity of drugs like z-FA.fmk is only controlled by the structure of the peptidyl portion of the reagent. Therefore, side reactions with other proteins susceptible to alkylation are likely to occur. In this case, we have previously shown that fluoromethyl ketone peptide inhibitors of caspases can nonspecifically inhibit the activity of several cathepsins (28). Recently, it has been reported that inhibition of NF-κB-dependent gene expression by several reagents, including helenalin and arsenite, is because of the irreversible alkylation of a cysteine residue in the p65 NF-κB subunit or the IκB kinase α and β subunits, respectively (29–31). In all cases, this resulted in decreased DNA binding of NF-κB. As z-FA.fmk did not affect the nuclear translocation and DNA binding of NF-κB, it is unlikely that z-FA.fmk acts in a similar way. Moreover, other fmk-based peptide inhibitors, such as benzoylxy carbonyl-Asp(OMe)-Glu (OMe)-Val-Val-Asp(OMe)-fmk and benzoylxy carbonyl-Val-Ala-Asp(OMe)-fmk, did not have an effect on NF-κB-dependent gene expression in response to LPS (data not shown).

Our observations illustrate that results obtained with the so-called specific cathepsin B inhibitor z-FA.fmk should be interpreted with care and verified by other inhibitors before any conclusion on the role of cathepsin B can be drawn. For example, cathepsin B has been implicated in rheumatoid arthritis, but z-FA.fmk has been used successfully to treat that disease (11). However, the role of NF-κB and IL-1 in rheumatoid arthritis is also well documented (32). Therefore, it is very likely that the therapeutic effect of z-FA.fmk in this disease is at least partially mediated by its inhibitory effect on NF-κB-dependent gene expression. These results, as well as the fact that z-FA.fmk can be used in vivo without severe side effects (11, 33–35), suggest z-FA.fmk to be a promising new tool for the treatment of a number of other inflammatory diseases.

Our studies also revealed that the band pattern obtained in EMSA for NF-κB is inhibited by several reagents, including helenalin and arsenite, is because of the irreversible alkylation of a cysteine residue in the p65 NF-κB subunit or the IκB kinase α and β subunits, respectively (29–31). In all cases, this resulted in decreased DNA binding of NF-κB. As z-FA.fmk did not affect the nuclear translocation and DNA binding of NF-κB, it is unlikely that z-FA.fmk acts in a similar way. Moreover, other fmk-based peptide inhibitors, such as benzoylxy carbonyl-Asp(OMe)-Glu (OMe)-Val-Val-Asp(OMe)-fmk and benzoylxy carbonyl-Val-Ala-Asp(OMe)-fmk, did not have an effect on NF-κB-dependent gene expression in response to LPS (data not shown).

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