Increased Mature Interleukin-1β (IL-1β) Secretion from THP-1 Cells Induced by Nigericin Is a Result of Activation of p45 IL-1β-converting Enzyme Processing*

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Perregaux and Gabel (Perregaux, D., and Gabel, C. A. (1994) J. Biol. Chem. 269, 15195–15203) reported that potassium depletion of lipopolysaccharide-stimulated mouse macrophages induced by the potassium ionophore, nigericin, leads to the rapid release of mature interleukin-1β (IL-1β). We have now shown a similar phenomenon in lipopolysaccharide-stimulated human monocytic leukemia THP-1 cells. Rapid secretion of mature, 17-kDa IL-1β occurred, in the presence of nigericin (4–16 μM). No effects on the release of tumor necrosis factor-α, IL-6, or proIL-1β were seen. Addition of the irreversible interleukin-1β-converting enzyme (ICE) inhibitor, Z-Val-Ala-Asp-dichlorobenzoate, or a radicicol analog, inhibited nigericin-induced mature IL-1β release and activation of p45 ICE precursor. The radicicol analog itself did not inhibit ICE, but markedly, and very rapidly depleted intracellular levels of 31-kDa proIL-1β. By contrast, dexamethasone, cycloheximide, and the potassium ionophore, nigericin, rapidly depleted intracellular levels of 31-kDa proIL-1β. Upon addition of the K+/H+ ionophore, nigericin, rapid and complete processing of intracellular IL-1β occurred with the appearance of mature 17-kDa IL-1β in the medium. Similar effects were reported using human peripheral blood monocytes. Although in these studies marked leakage of the cytoplasmic enzyme, lactic acid dehydrogenase (LDH) occurred, suggesting substantial cell damage, it was argued that the effect of nigericin was not due simply to lysis, inasmuch as, unlike the effects of hypotonic shock, at no time were significant levels of proIL-1β detected in the culture medium. Furthermore, the nigericin-induced 17-kDa IL-1β was shown to have the expected N-terminal sequence. These results, together with studies by Waley et al. (5) showing that high extracellular concentrations of K+ or combinations of K+-channel blockers prevented the physiological release of IL-1β, suggest that a net reduction of intracellular K+ ion concentration is necessary for the processing of proIL-1β. Both Perregaux et al. (4) and Waley et al. (5) speculated that a reduction of K+ ion concentration might activate ICE or promote the processing of pro-ICE. Alternatively, it was suggested that nigericin-induced K+ depletion alters the cytoplasmic compartmentalization of ICE and IL-1β. So far, however, there has not been any direct evidence that nigericin-induced release of IL-1β is ICE-dependent.

In the present study, we show that nigericin evokes a massive and rapid release of 17-kDa IL-1β from prestimulated THP-1 cells under conditions where LDH leakage is absent. Under these conditions, the nigericin-induced secretion of IL-1β is almost completely blocked by the irreversible ICE inhibitor, 2-valyl-alanyl-3(S)-3-amino-4-oxo-5-(2,6-dichlorobenzoyloxopentanoic) acid (Z-VAD-DCB) (6), as well as a radical analog (7), demonstrating the ICE dependence of the process.

EXPERIMENTAL PROCEDURES

Compounds—Z-VAD-DCB was synthesized in our laboratories and radical analog A, C_{20}H_{24}O_8 (7S,12S,13S)-15E-4,12,13-trihydroxy-1,2-dime-thoxy-7-methyl-8,12,13,14-tetrahydro-7H-6-oxabenzocloctetradecene-5,11-dione), was isolated from the fungus strain F/87-2509.04. The chemical structures of both compounds are shown in Fig. 1.

Cytokine Production by THP-1 Cells and Biochemical Assays—Cells from the human monocytic leukemia cell line, THP-1, were grown in RPMI medium supplemented with 110 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 2 μg/ml NaHCO_3. Heat-treated fetal bovine serum (5%) was added before use. The cells were grown to a density of 5 × 10^6/ml and then stimulated with interferon-γ (100 units/ml). Three hours later, LPS (5 μg/ml) was added. This time point was designated time 0. Incubation continued for an additional 40 h. The media were then removed and clarified by centrifugation at 10 000 × g for 10 min. LDH measurements were performed immediately (8). Cytokine assays were performed using commercially available enzyme-linked immunosorbent assay kits (IL-1β, Cayman, Ann Arbor, MI; proIL-1β, Cistron, Biotechnology, Pine Brook, NJ; IL-6 and TNF-α, Innogenetics, Zwijndrecht, Belgium). DNA was assayed fluorometrically using the

cell-associated, 31-kDa IL-1β. Upon addition of the K+/H+ ionophore, nigericin, rapid and complete processing of intracellular IL-1β occurred with the appearance of mature 17-kDa IL-1β in the medium. Similar effects were reported using human peripheral blood monocytes. Although in these studies marked leakage of the cytoplasmic enzyme, lactic acid dehydrogenase (LDH) occurred, suggesting substantial cell damage, it was argued that the effect of nigericin was not due simply to lysis, inasmuch as, unlike the effects of hypotonic shock, at no time were significant levels of proIL-1β detected in the culture medium. Furthermore, the nigericin-induced 17-kDa IL-1β was shown to have the expected N-terminal sequence. These results, together with studies by Waley et al. (5) showing that high extracellular concentrations of K+ or combinations of K+-channel blockers prevented the physiological release of IL-1β, suggest that a net reduction of intracellular K+ ion concentration is necessary for the processing of proIL-1β. Both Perregaux et al. (4) and Waley et al. (5) speculated that a reduction of K+ ion concentration might activate ICE or promote the processing of pro-ICE. Alternatively, it was suggested that nigericin-induced K+ depletion alters the cytoplasmic compartmentalization of ICE and IL-1β. So far, however, there has not been any direct evidence that nigericin-induced release of IL-1β is ICE-dependent.

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Interleukin-1β (IL-1β) is produced as an inactive 31-kDa precursor protein through the enzymatic cleavage of IL-1β-converting enzyme (ICE), which cleaves the IL-1β precursor between Asp-116 and Ala-117 (1). ICE itself is produced as a 45-kDa precursor, which has recently been shown to be converted autocatalytically to an active p10/p20 heterodimer (2). The physiological control of ICE processing, and hence IL-1β conversion and secretion, is still unknown. Studies by Perregaux et al. (3, 4) suggest that IL-1β processing is controlled by intracellular potassium concentration. Mouse peritoneal macrophages stimulated with LPS produce massive amounts of

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‡ The following abbreviations are used: IL, interleukin; ICE, interleukin-1β-converting enzyme; LDH, lactic acid dehydrogenase; Z-VAD-AMC, carbobenzoxy-Val-Ala-Asp-aminomethyl coumarin; Z-VAD-DCB, 2-valyl-alanyl-3(S)-3-amino-4-oxo-5-(2,6-dichlorobenzoyloxopentanoic) acid; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.
Processing. Radicicol analog A was added at 5 μM final concentration to nigericin, a consistent, rapid, concentration-dependent increase in IL-1 release of IL-1β following dialysis led to the induction of autocatalytic processing. Radicicol analog A was added at 5 μM final concentration to this last step. Cations, where mentioned, were present at the indicated concentrations in the refolding mixture, the dialysis buffer, as well as during the last incubation at room temperature. Western blot analysis was performed using anti-N-terminal p45 ICE or anti-p10 ICE subunit antibodies raised in our laboratories and shown to cross-react with p45 ICE. Detection was performed using an anti-rabbit IgG-POD (Sigma) with the chemiluminescence detection system of Boehringer Mannheim. Western blots were analyzed with a Molecular Dynamics Computing Densitometer 300A using Image Quant software.

**RESULTS**

Nigericin-induced IL-1β Secretion from THP-1 Cells—Preliminary experiments showed that when THP-1 cells, pre-stimulated with 5 μg/ml LPS for 39.5 h, were exposed for 30 min to nigericin, a consistent, rapid, concentration-dependent release of IL-1β into the medium occurred (Fig. 2A). This increase in total cumulative IL-1β in the medium varied by 2–5-fold in different experiments. Measurement of IL-1β levels at 30, 39.5, and again at 40 h in control cultures (no nigericin), showed that secreted IL-1β levels were at their peak and that IL-1β release over this time was negligible. Nigericin thus stimulated a massive and rapid release of IL-1β over and above the normal steady-state levels. When the ICE inhibitor Z-VAD-DCB was added to the cultures at 39 h (30 min before nigericin), it was found to substantially block the nigericin-induced IL-1β release (Fig. 2A and Table I). The effect of nigericin was not caused by cytotoxicity because, as shown in Fig. 2B, even at the highest concentration used (16 μM), there was no increase in LDH leakage over the 30 min period of exposure. Because longer exposure to nigericin eventually does lead to signs of cytotoxicity, the 30-min exposure was adhered to for all experiments. The specificity of the effect on IL-1β is further indicated in Fig. 2B, as TNF-α levels were unaltered by nigericin even at the highest concentration. Additional studies (results not shown) indicated that nigericin does also not affect the amount of IL-6 secreted by THP-1 cells.

**Effects of IL-1 Inhibitors on Nigericin-induced IL-1β Secretion**—In a second series of experiments we compared the effects of Z-VAD-DCB, dexamethasone, cycloheximide and radicicol analog A, a compound previously demonstrated to reduce IL-1β production by causing mRNA instability (7, 10), on nigericin-induced IL-1β release. Tables I and II show that, whereas Z-VAD-DCB was able to inhibit nigericin-induced release of IL-1β, dexamethasone or cycloheximide were without effect. The radicicol analog also blocked the effects of nigericin. Intra-cellular levels of unprocessed 31-kDa IL-1β were measured in cell lysates. Table I shows that Z-VAD-DCB had no significant effect on intracellular levels of proIL-1β. By contrast, both the translational inhibitor, cycloheximide (Table II), and dexamethasone (Table I) caused a statistically significant decrease, in intracellular proIL-1β only in the nigericin-treated cells. A third pattern of inhibition was observed with radicicol analog A, which markedly inhibited the levels of proIL-1β in both

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**Fig. 1.** Chemical structures of Z-VAD-DCB and radicicol analog A.

**Fig. 2.** A, nigericin-induced IL-1β release from THP-1 cells. Cells were stimulated as described under "Experimental Procedures." Nigericin was added 39.5 h after LPS, and measurements were made 30 min later. The concentrations of IL-1β (normalized against cellular DNA) in the medium harvested from THP-1 cultures, 40 h after stimulation with LPS are shown. Filled squares, concentration-related increase in IL-1β release. The effect of nigericin was specific for IL-1β. Another inflammatory cytokine produced by THP-1 cells, TNF-α (filled circles, ICE inhibitor, Z-VAD-DCB (1 μM) added to the cells 30 min before nigericin (p values relate to differences between nigericin ± ICE inhibitor). The result is representative of a series of experiments in which the stimulation index ± 16 μM nigericin varied from 2 to 5. In all cases, the nigericin effect was completely blocked by 1 μM Z-VAD-DCB. Given are means ± S.E., n = 4 separate cultures. B, specificity of nigericin for IL-1β release. The effect of nigericin was specific for IL-1β. Another inflammatory cytokine produced by THP-1 cells, TNF-α (filled symbols, shapes as described above) is unaffected. LDH leakage (open symbols, shapes as described above) does not increase significantly above the levels seen in the control cultures without nigericin. Given are means ± S.E., n = 4 separate cultures.
Table I

| THP-1 cells were treated as described in Fig. 2. Measurements were done by ELISA. The effect of Z-VAD-DCB (1 μM), dexamethasone (Dex, 1 nM), and radicicol analog A (1 μM) given 30 min prior to nigericin (39.5 h after LPS addition) on IL-1β secretion and intracellular and extracellular proIL-1β accumulation. | Effect of inhibitors on IL-1β secretion and intracellular and extracellular proIL-1β accumulation |
|---|---|
| | Control | Z-VAD-DCB | Nigericin | Nigericin + Z-VAD-DCB | Nigericin + dexamethasone | Nigericin + radicicol analog A |
| **Secreted IL-1β** | 1.06 ± 0.06 | 1.69 ± 0.04 (NS) | 1.09 ± 0.04 (NS) | 2.15 ± 0.16*** | 1.33 ± 0.06 (NS) | 2.17 ± 0.06*** |
| **Intracellular proIL-1β** | 6.03 ± 0.16 | 5.27 ± 0.54** (NS) | 5.63 ± 0.11 (NS) | 4.85 ± 0.12** | 4.90 ± 0.12** | 3.48 ± 0.11*** |
| **Extracellular proIL-1β** | 0.03 ± 0.03 | 0.39 ± 0.02 (NS) | 0.96 ± 0.02 (NS) | 0.98 ± 0.05 (NS) | 0.38 ± 0.02 (NS) | 0.39 ± 0.02 (NS) |

**Role of Potassium Ions in Regulation of IL-1β**

Based on findings that 5-dimethyl amiloride was able to suppress IL-1β secretion from LPS-activated monocytes with an IC₅₀ = 3.5 μM, it was reported that extracellular Na⁺ and high intracellular pH was required for IL-1β secretion (12). It was possible, therefore, that the effects of nigericin were caused by...
a secondary compensatory influx of Na$^+$ ions. If this were indeed so, one would expect 5-dimethyl amiloride to reverse or neutralize the effects of nigericin. We thus tested IL-1β secretion from THP-1 cells in the presence of both nigericin and 5-dimethyl amiloride given at the same time. No reversal of the effects of nigericin by 5-dimethyl amiloride up to 30 μM was observed, suggesting that nigericin exerts its effects directly through K$^+$ efflux. It may well be that the efflux of K$^+$ is a stronger signal for ICE activation than extracellular Na$^+$ levels or changes in intracellular pH. It is also quite possible that THP-1 cells react differently to these changes, because 5-dimethyl amiloride, when given alone at 30 μM before LPS stimulation, decreased mature IL-1β secretion only slightly, which is in contrast to the strong inhibition reported on monocytes (12).

**DISCUSSION**

This study expands previous findings that nigericin is able to specifically induce the release of mature IL-1β from mononuclear cells to THP-1 cells. The process was extremely rapid, with 2–5 times as much IL-1β being released in 30 min as the cumulative release of IL-1β over the previous 39.5 h. Unlike the studies of Perregaux et al. (3, 4), there was no evidence of cytotoxicity over the time course used, as indicated by the lack

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**TABLE II**

|                | Control | Nigericin | CHX | Nigericin + CHX |
|----------------|---------|-----------|-----|----------------|
| Secreted IL-1β | 2.78 ± 0.16 | 5.68 ± 0.3*** | 2.81 ± 0.08 (NS) | 6.19 ± 0.24*** |
| Intracellular proIL-1β | 8.8 ± 0.36 | 7.31 ± 0.18*** | 8.18 ± 0.13 (NS) | 5.55 ± 0.27*** |
| Extracellular proIL-1β | 0.65 ± 0.03 | 1.79 ± 0.09** | 0.69 ± 0.01 (NS) | 2.03 ± 0.04*** |
of any increased LDH in the medium. On a molar basis, over 80% of the IL-1β released by LPS-stimulated cells is in the processed 17-kDa form. Under the influence of nigericin, the ratio of released 17-kDa to 31-kDa IL-1β in the medium remains the same, further substantiating the conclusion that the increase is not a result of cell lysis or simple leakage. Furthermore, the lack of effect on other cytokines such as TNF-α and IL-6 (latter not shown) demonstrates that the effects are specific.

When comparing intracellular levels of proIL-1β at 39 and 40 h after LPS stimulation no measurable increase could be observed (results not shown). Steady-state synthesis of proIL-1β at this time is apparently occurring at a very slow rate. Intracellular levels of proIL-1β were only affected by nigericin in the presence of inhibitors of de novo synthesis (dexamethasone and radicicol analog A, Table I; cycloheximide, Table II). The most likely explanation for a lack of an effect with nigericin alone is the presence of a homeostatic mechanism designed to maintain constant levels of intracellular proIL-1β. This would also explain why the presence of an ICE inhibitor does not increase intracellular proIL-1β levels. Because ICE inhibitors do not lead to a decrease in proIL-1β levels inside the cell, no signal is generated to induce an increase in the rate of proIL-1β synthesis.

The addition of an irreversible ICE inhibitor, Z-VAD-DCB, substantially blocks the nigericin-induced release of mature IL-1β, suggesting that nigericin is dependent upon mechanisms that operate during the physiological release of IL-1β. Analyses of the cellular levels of p45 ICE by Western blotting clearly show that nigericin induces the autocatalytic processing of p45 ICE (Fig. 3). The nigericin-induced processing of p45 ICE is prevented in the presence of the ICE inhibitor, which is consistent with the observations that this inhibitor prevents the autocatalysis of recombinant p45 ICE in a cell-free system (2). Furthermore, although Z-VAD-DCB also inhibits other caspases, ICE, with the exception of caspase 4 (which cleaves proIL-1β 250-fold less effectively), is the only caspase known to correctly cleave proIL-1β to its mature form (13). Also, unlike other caspases, again with the exception of caspase 4, no enzyme has so far been described to process p45 ICE other than ICE itself whereas in vitro ICE can also cleave pro-caspase 4. This, together with the inhibition of p45 ICE autoprocessing, but not p10/20 ICE activity by radicicol analog A (see below), further reduces the likelihood of a nigericin-induced activation of an enzymatic cascade upstream of ICE.

Not surprisingly, 1 μM dexamethasone (a concentration shown to give >80% inhibition of IL-1β secretion if added before LPS stimulation) and cycloheximide do not prevent nigericin-induced IL-1β processing indicating that nigericin-induced secretion of IL-1β comes from a pre-existing pool of proIL-1β.

Radicicol analog A had a profound effect on IL-1β levels. A 1-h exposure led to a dramatic reduction in intracellular proIL-1β. Previous studies (10) have shown that radicicol analog A induces the rapid degradation of cytokine mRNAs (including IL-1β), which have in common the AUUUA instability motif in the 3′-untranslated region. In contrast to dexamethasone and cycloheximide, the effect of radicicol analog A on released proIL-1β/ICE from nigericin-treated cells therefore reflects a drastically reduced pool of proIL-1β in the cells that is available for processing (Tables I and II). Because over the time period measured in our experiments there is no detectable increase in IL-1β secretion, or proIL-1β leakage in control cells, it is not surprising that no effect is seen with radicicol analog alone on the secretion of mature IL-1β or on extracellular levels of proIL-1β. The effect of radicicol analog A on the nigericin-induced release of mature IL-1β is twofold. First, as mentioned...
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before, radicicol analog A induces rapid degradation of IL-1β mRNA and possibly also inhibits transcription and translation. Thus, in the presence of radicicol analog A, synthesis of proIL-1β is decreased. Because the rate of proIL-1β production is decreased and the rate of proIL-1β consumption either by export of processed IL-1β or intracellular degradation of proIL-1β, is unaffected by radicicol analog A, intracellular proIL-1β levels are expected to decrease whereas the levels of mature IL-1β secreted remain at the control levels because radicicol analog A does not inhibit pre-existing mature ICE. Therefore, also with the addition of nigericin, extracellular proIL-1β levels do not increase. Second, because radicicol analog A also blocks p45 ICE processing (Fig. 4) but does not inhibit ICE activity (results not shown), the increase in IL-1β secretion in the presence of nigericin is a result of the presence of more active ICE, a result of increased p45 ICE processing triggered by the lowering of K⁺ levels by nigericin. Radicicol analog A blocking p45 ICE processing inhibits the effects of nigericin, whereas cycloheximide, which also inhibits protein synthesis but does not inhibit ICE activity or ICE processing (results not shown), does not influence the effects of nigericin.

So far, however, there are no clues as to how a reduction in K⁺ ion concentration may induce p45 ICE processing. Autoprocessing of p45 ICE, in vitro is not sensitive to K⁺ alone (Table III). Ionic strength-dependent inhibition of ICE activity in vitro was also seen (results not shown). Mature ICE was sensitive to salt concentrations equivalent to the intracellular concentration of potassium. Such concentrations caused approximately 50% inhibition of ICE activity using the synthetic substrate Z-VAD-AMC. However, this effect was not ion-specific, so it is unlikely that this explains the specific effects of reducing K⁺ ion concentration in whole cells. We have also eliminated the possibility that nigericin has a direct effect on ICE activity in a cell-free system (results not shown). Although it remains unclear how nigericin induces ICE processing, this effect does not appear to require metabolically active cells because nigericin-induced proIL-1β processing continued in azide-treated cells (results not shown).

Whether K⁺ ion flux plays a role in the physiological processing of ICE and IL-1β secretion in response to pro-inflammatory stimuli, is not clear. Walev et al. (5) showed that a variety of manipulations that resulted in reduced intracellular levels of K⁺ could trigger IL-1β processing. Furthermore, high extracellular concentrations of K⁺ could reverse these effects. Combinations of tetraethylammonium and 4-aminopyridine (potassium channel blockers) could also inhibit the physiological release of LPS-induced IL-1β. Because no single channel blocker was effective, even at high concentration, this suggests that multiple channels are involved and therefore potassium channel blockers are unlikely to be leads in the search for cytokine release inhibitors.

Taken together, our results show that nigericin-induced K⁺ efflux induces rapid p45 ICE processing leading to an increase in active ICE, which in turn results in a higher secretion of mature IL-1β. Because nigericin treatment specifically leads to mature IL-1β release, a better understanding of the mechanism by which K⁺ ions control p45 ICE activation and proIL-1β processing might lead to the identification of new anti-inflammatory drug targets.

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