ATP Treatment of Human Monocytes Promotes Caspase-1 Maturation and Externalization*

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Mechanisms that regulate conversion of prointerleukin-1β (pro-IL-1β) to its mature form by the cysteine protease caspase-1 are not well understood. In this study, we demonstrate that mature caspase-1 subunits are produced when human monocytes are treated with ATP and, like mature IL-1β, are released extracellularly. Characterization of the pharmacological sensitivity of this stimulus-coupled response revealed that some caspase-1 inhibitors allow pro-IL-1β secretion, whereas others do not. Two nonselective alkyating agents, N-ethylmaleimide and phenylarsine oxide, also blocked maturation and release of pro-IL-1β. Two inhibitors of anion transport, glyburide and ethacrynic acid, blocked maturation of both caspase-1 and pro-IL-1β and prevented release of the propolypeptides. Procaspase-3 was detected in monocyte extracts, but its proteolytic activation was not efficient in the presence of ATP. Maturation of procaspase-1 and release of the mature enzyme subunits therefore accompany stimulus-coupled human monocyte IL-1 post-translational processing. Agents that appear to selectively inhibit mature caspase-1 do not prevent ATP-treated cells from releasing their cytosolic components. On the other hand, anion transport inhibitors and alkyating agents arrest ATP-treated monocytes in a state where membrane latency is maintained. The data provided support the hypothesis that stimulus-coupled IL-1 post-translational processing involves a commitment to cell death.

Interleukin (IL)1-1 is an important inflammatory mediator produced in abundance by activated monocytes and macrophages (1). IL-1 biological activity is derived from two related but distinct polypeptides, IL-1α and IL-1β (1, 2). Human IL-1β is synthesized as a 31-kDa procytokine that is incompetent to bind to the type 1 IL-1 receptor (3). To gain activity, pro-IL-1β must be cleaved by caspase-1 (also known as interleukin-1-converting enzyme) to yield a 17-kDa carboxyl terminus-deleted polypeptide (4, 5). Human IL-1α also is produced as a 31-kDa procytokine, but, unlike pro-IL-1β, pro-IL-1α is fully competent to engage and to activate type 1 IL-1 receptors (3).

Although cleavage is not required, pro-IL-1α may be proteolytically processed by a calpain-like protease to generate a 17-kDa polypeptide that retains full activity (6); pro-IL-1α is not a substrate of caspase-1 (7).

Caspase-1 is the founding member of a family of intracellular cysteine proteases, many of which are involved in apoptotic processes (8–10). Overexpression and/or activation of caspase-1 is associated with a proapoptotic response in several cell systems (11, 12), and thymocytes isolated from mice engineered to lack caspase-1 are less sensitive to FAS antibody-induced apoptosis (13). In other cell systems, however, absence of caspase-1 does not prevent apoptosis (13–15). Caspase-1 itself is synthesized as a 45-kDa propolypeptide that must be activated via proteolytic cleavage to generate the 10- and 20-kDa subunits of the mature enzyme (4, 16, 17); a heterotetramer containing two of each of these subunits constitutes the active protease (18, 19). Procaspase-1 and pro-IL-1β appear to coexist within the cytoplasm of LPS-activated monocytes (20, 21); mechanisms regulating caspase-1 activation and the subsequent cleavage of the procytokine are not well understood. Evidence that caspase-1 is required for pro-IL-1β post-translational processing has been provided by studies showing that selective inhibitors of this protease block mature cytokine formation and that macrophages derived from caspase-1-deficient mice are impaired in the production of mature cytokine (4, 13, 22–24). The presence of mature caspase-1 subunits within monocytes and/or macrophages has been difficult to demonstrate, however.

IL-1β is released from monocytes and macrophages via an atypical secretory mechanism that does not involve the endoplasmic reticulum and Golgi complex (25). Release of IL-1β from cells stimulated to produce this cytokine generally is an inefficient process (26–28); the majority of newly synthesized cytokine molecules remain cell-associated and/or are degraded. To promote efficient proteolytic cleavage of pro-IL-1β and release of the 17-kDa mature polypeptide, the cytokine-producing cells must be treated with a secretion stimulus such as ATP, cytolytic T-cells, potassium-selective ionophores (e.g. nigericin), or bacterial toxins (24, 27, 29–35). In the presence of these secretion-promoting stimuli, LPS-activated monocytes and macrophages appear to die (27, 29, 34). However, not all agents that promote cell death activate IL-1 proteolytic maturation; therefore, the post-translational processing response is considered to be an active process.

Many caspase-1 inhibitors described in the literature are peptides whose sequences correspond to those found at the cleavage sites of natural polypeptide substrates (36). These pharmacological agents often contain reactive functional groups that facilitate covalent attachment to the catalytic cysteine residue (Cys285) of caspase-1. For example, the acid aldehyde moiety of the inhibitor acetyl-Tyr-Val-Ala-Asp aldehyde (YVAD-CHO) forms a reversible covalent adduct with the en-
zyme (4, 36). On the other hand, caspase inhibitors such as acetyl-Tyr-Val-Ala-Asp chloromethyl ketone (YVAD-CMK) and benzoyloxycarbonyl-Val-Ala-Asp acetyl氧化胆汁酸 (ZVAD-DCB) form stable adducts that inactivate caspase-1 irreversibly (36–38). Although these peptides are potent inhibitors of purified caspase-1, much higher concentrations are required to prevent maturation of pro-IL-1β by intact cells due, in part, to the necessity that these agents must penetrate the plasma membrane to access the intracellular protease. For example, the Kₐ of YVAD-CHO against caspase-1 is 0.76 μM, yet 5000-fold greater concentrations are required to inhibit IL-1β processing by human blood monocytes (4). Evidence demonstrating that the various caspase inhibitors react exclusively with caspase-1 at these higher concentrations is lacking. The inhibitor acetyl-Tyr-Val-Lys(biotin)-Asp (acyl氧化胆汁酸) methyl ketone displayed selectivity for mature caspase-1 subunits when assessed against an extract of THP-1 cells (38), but similar studies employing intact cells have not been reported.

In this study, ATP is employed as a stimulus to initiate IL-1β post-translational processing by LPS-activated human monocytes. This nucleotide triphosphate is shown to promote formation of mature caspase-1 subunits in addition to mature IL-1β and to elicit externalization of the mature products. In contrast, procaspase-3 is not efficiently processed by ATP-treated cells, suggesting that procaspase-1 and procaspase-3 require distinct signals for their maturation. The ATP-induced response is characterized with respect to its sensitivity to several distinct pharmacological agents. The results demonstrate that caspase-1 inhibitors do not act equivalently when evaluated in an intact cell system and provide insights into the novel cellular process employed by monocytes to generate and export the leaderless polypeptide IL-1β.

**Experimental Procedures**

**Human Monocyte Isolation**—Blood collected from normal volunteers in the presence of heparin was fractionated using lymphocyte separation medium obtained from ICN (Aurora, OH). The region of the resulting gradient containing banded mononuclear cells was harvested and diluted with 10 ml of maintenance medium (RPMI 1640 medium, 5% fetal bovine serum, 25 mM Heps, pH 7.2, and 1% penicillin/streptomycin), and cells were collected by centrifugation. The resulting cell pellet was suspended in 10 ml of maintenance medium, and a cell count was performed. In an average metabolic experiment, 1×10⁷ mononuclear cells were added to each well of 6-well multi-dishes in a total volume of 2 ml to achieve a final concentration of 2 mM, and the plate was incubated at 37 °C. In an average metabolic experiment, 1×10⁷ mononuclear cells were added to each well of 6-well multi-dishes in a total volume of 2 ml to achieve a final concentration of 2 mM, and the plate was incubated at 37 °C. In a 30-min incubation on ice, both the media and cell extracts were clarified by centrifugation at 45,000 rpm for 30 min in a tabletop ultracentrifuge using a TLA 45 rotor (Beckman Instruments).

**Western Analysis**—Mononuclear cells (3×10⁶ cells) in 10 ml of maintenance medium containing 10 ng/ml LPS were added to 10-cm dishes. These cultures were incubated for 2.5 h at 37 °C, after which non-adherent cells were discarded, and the adherent monocytes were washed three times with serum-free maintenance medium containing 10 ng/ml LPS. A test agent (in a total volume of 6.65 ml of maintenance medium containing 10 ng/ml LPS and 0.2% MeSO) was introduced for 10 min; 5 mM ATP was then added (from a 100 mM stock solution), and the cultures were incubated for an additional 90 min. Media were harvested, supplemented with protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM iodoacetate), and clarified by centrifugation to remove cell debris. Adherent monocytes were solubilized by addition of 3 ml of 10% Triton, 0.1% SDS sample buffer was subsequently added to each sample, and the mixtures were boiled for 3 min. After centrifugation, the disagggregated protein samples were separated on 18% polyacrylamide gels (Novex, San Diego, CA). Proteins within the gels were transferred to nitrocellulose, and the blots were blocked by exposure to 5% nonfat milk in Tris-buffered saline containing 1% Tween (TBST). The blots were then incubated overnight at 4 °C with primary antibody in TBST containing 5% bovine serum albumin. After washing with TBST, these blots were incubated with appropriate conjugate (goat anti-rabbit, horseradish peroxidase (New England Biolabs Inc., Beverly, MA), or mouse anti-rabbit, ethacrynic acid was from Sigma. Rabbit anti-caspase-1 antibody and ELISA kits for measuring caspase-1 were obtained from Cistron Biotechnology, Inc. (Pine Brooks, NJ). Rabbit anti-caspase-3 antibody and A-431 nonstimulated cell samples (containing procaspase-3) were obtained from Upstate Biotechnol-
were treated with ATP in the presence of two inhibitors of ion transport, glyburide and ethacrynic acid (Fig. 1); these agents are known to block mature IL-1β production (39, 40). Estimated IC50 values for glyburide and ethacrynic acid were 11 and 2.3 μM, respectively. It should be noted that the ELISA employed for these studies shows a preference for mature IL-1β relative to pro-IL-1β, but both forms are detected.

The activity of the caspase antagonists was also evaluated in a metabolic assay format that allowed the fate of pro-IL-1β to be assessed. LPS-activated[^35S]methionine-labeled monocytes treated with ATP in the absence of an inhibitor released large quantities of 17-kDa mature IL-1β and smaller quantities of a 28-kDa species and of the 31-kDa precursor (Fig. 2A); the 28-kDa species represents an alternate caspase-1 cleavage product (7). Monocytes treated with ATP in the presence of YVAD-CHO generated less extracellular 17-kDa IL-1β (Fig. 2A). Reduction in extracellular 17-kDa IL-1β was greater when the test agent was present at 60 μM rather than at 6 μM, but both concentrations significantly reduced extracellular mature cytokine levels (Fig. 2A). Relative to the ATP-treated control cultures, quantities of extracellular 17-kDa IL-1β (determined by PhosphorImager analysis) were reduced by 66 and 87% at 6 and 60 μM YVAD-CHO, respectively. More important, the reduction in extracellular 17-kDa IL-1β caused by YVAD-CHO was accompanied by an increase in the quantity of extracellular pro-IL-1β (Fig. 2A); this observation is consistent with previous findings indicating that YVAD-CHO blocks maturation of pro-IL-1β, but does not inhibit cytokine release (4). After correcting for the loss of[^35S]methionine that occurs when pro-IL-1β is converted to its mature species (2), the quantity of radioactivity associated with all extracellular IL-1β species released from the YVAD-CHO-treated cultures represented 69 and 61% at 6 and 60 μM, respectively, of the quantity released by monocytes in the absence of the caspase inhibitor (Fig. 2B). YVAD-CMK similarly inhibited 17-kDa IL-1β formation in response to ATP stimulation, and the monocytes again compensated by releasing elevated quantities of pro-IL-1β (Fig. 2A); overall, quantities of radiolabeled IL-1β released from YVAD-CMK-treated cells accounted for >60% of the cytokine released from the control cultures (Fig. 2B). In contrast, ZVAD-DCB not only inhibited production of mature IL-1β, but also effectively blocked release of pro-IL-1β (Fig. 2A); in the presence of 6 and 60 μM concentrations of this test agent, <10% of the control level of radiolabeled cytokine was externalized (Fig. 2B). Recovery of total radiolabeled cytokine (sum of both intracellular and extracellular polypeptides and corrected for the loss of radioactivity incurred when the 31-kDa species was cleaved to its 17-kDa form) was comparable from all three cultures (data not shown), suggesting that ZVAD-DCB did not reduce overall cytokine content, but rather, inhibited release of pro-IL-1β.

**Nonselective Thiol Reagents Block ATP-induced IL-1β Post-translational Processing——LPS-activated[^35S]methionine-labeled monocytes were pretreated with the indicated test agent for 15 min, after which 2 mM ATP was introduced to initiate IL-1β post-translational processing.** After a 2.5-h incubation, media were harvested, IL-1β was recovered by immunoprecipitation, and these immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the dried gel is shown in A; the migration positions of the 31-kDa precursor and 17-kDa mature forms of IL-1β are indicated. Radioactivity associated with the individual 31- and 17-kDa species was determined by AMBIS image analysis. The total quantity of[^35S]-labeled IL-1β released by an individual culture was then determined by multiplying counts recovered as the 17-kDa species by 2 to correct for methionines lost as a result of caspase-1 proteolysis and summing this value with the number of counts recovered as the 31-kDa species. The amount of IL-1β released (expressed as a percentage of that released in the absence of an effector) is indicated in B; each bar is the average of duplicate determinations.

**FIG. 2. Demonstration that caspase-1 inhibitors block mature IL-1β formation.** LPS-activated[^35S]methionine-labeled monocytes were treated with the indicated test agent for 15 min, after which 2 mM ATP was introduced to initiate IL-1β post-translational processing. After a 2.5-h incubation, media were harvested, IL-1β was recovered by immunoprecipitation, and these immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the dried gel is shown in A; the migration positions of the 31-kDa precursor and 17-kDa mature forms of IL-1β are indicated. Radioactivity associated with the individual 31- and 17-kDa species was determined by AMBIS image analysis. The total quantity of[^35S]-labeled IL-1β released by an individual culture was then determined by multiplying counts recovered as the 17-kDa species by 2 to correct for methionines lost as a result of caspase-1 proteolysis and summing this value with the number of counts recovered as the 31-kDa species. The amount of IL-1β released (expressed as a percentage of that released in the absence of an effector) is indicated in B; each bar is the average of duplicate determinations.
which the medium was replaced with fresh medium containing 2 mM ATP; some cultures received medium devoid of ATP (−ATP). Following a 2.5-h incubation, cell and media fractions were harvested separately, and the cells were solubilized by detergent extraction. IL-1β was subsequently recovered by immunoprecipitation, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis; autoradiograms of the media (A) and cell-associated (B) samples are shown. The migration positions of the 31-kDa precursor and 17-kDa mature forms of IL-1β are indicated.

dehydrogenase activity were diminished, suggesting that the cells and/or the analyses were compromised. Externalization of pro-IL-1β required ATP, as NEM-treated cells incubated in the absence of the nucleotide triphosphate released minimal radiolabeled cytokine (Fig. 3A).

PAO pretreatment also potently blocked IL-1β post-translational processing in response to ATP activation. Relative to control ATP-treated cultures, monocytes exposed to the nucleotide triphosphate following treatment with 0.4 μM PAO yielded significantly less 17-kDa IL-1β, and this inhibition occurred without an increase in the amount of procytokine externalized (Fig. 4A). Higher PAO concentrations totally suppressed 17-kDa mature IL-1β production (Fig. 4A). A small increase was observed in the quantity of 31-kDa pro-IL-1β released from cultures treated with 1.2 or 3.6 μM PAO (Fig. 4A); at these concentrations, however, the increase in extracellular pro-IL-1β accounted for <10% of the 17-kDa mature IL-1β generated by cells in the absence of PAO treatment. Monocytes pretreated with 10.8 and 32.4 μM PAO, on the other hand, released no 17-kDa IL-1β, and at these concentrations, the quantities of 31-kDa pro-IL-1β released were comparable to those generated by control cultures in response to ATP treatment (Fig. 4A).

The ability of PAO to modify protein sulfhydryl groups is greatly enhanced when vicinal disulfide groups are involved (41, 42). The stable adduct formed between PAO and vicinal disulfides can be reversed by treatment with dimercaptopyrano1 (DMP) (41). To determine whether the PAO-induced block in IL-1β post-translational processing was DMP-reversible, LPS-activated/[35S]methionine-labeled monocytes were pretreated with PAO for 15 min, after which they were treated successively with DMP and ATP. Monocytes not treated with PAO or DMP released large quantities of 17-kDa IL-1β in response to ATP (Fig. 4B). Nucleotide triphosphate-treated cells also released polypeptides corresponding to pro-IL-1β and the 28-kDa cleavage product (Fig. 4B). The relative abundance of the pro-IL-1β species released in this experiment was greater than that observed in Fig. 4A. This difference reflects the efficiency at which monocytes isolated from individual donors proteolytically process their released cytokine product (43). When these monocytes were pretreated with PAO, 17-kDa IL-1β production was completely suppressed, with only a modest increase in the quantity of extracellular 31-kDa IL-1β. DMP treatment (10 or 100 μM) restored formation of 17-kDa IL-1β by PAO-arrested cells (Fig. 4B). Thus, PAO inhibition of monocyte IL-1β post-translational processing may involve an interaction with vicinal sulfhydryl groups.

**Caspase-1 Is Activated and Released from ATP-treated Human Monocytes**—LPS-activated human monocytes treated with ATP release lactate dehydrogenase in addition to mature IL-1β (39); release of lactate dehydrogenase is assumed to reflect loss of plasma membrane latency. To determine whether caspase-1 also is externalized, the medium conditioned by LPS-activated monocytes in the absence and presence of ATP was...
assessed for caspase-1 antigen by ELISA. As shown in Fig. 5A, ATP promoted a 16-fold increase in the level of extracellular caspase-1 antigen. The employed ELISA kit detects both mature and procaspase-1 subunits, so the nature of the secreted enzyme could not be ascertained from this analysis. To make this assessment, samples of the media and cell extracts were subjected to Western analysis. The medium harvested from LPS-activated monocytes maintained in the absence of ATP contained few immunopositive proteins, but the corresponding cell lysate contained a major immunoreactive polypeptide in the region expected for procaspase-1 (Fig. 5B). After 2.5 h of treatment, media were harvested, cells were solubilized by detergent extraction, and samples of each (20% of the medium and 5% of the cell-associated sample) were subjected to Western analysis for caspase-1. Representative blots are indicated in B. The migration positions of mature and procaspase-1 are indicated by arrows; each blot also contained recombinant mature caspase-1 (standard (STD)).

Several other inhibitors of IL-1β post-translational processing were analyzed for their effect on ATP-induced caspase-1 activation. Monocytes treated with ATP in the absence of a test agent released large quantities of ELISA-positive caspase-1 antigen (Fig. 6A) and of the 20-kDa mature caspase-1 subunit (Fig. 6C). These cells also released large quantities of IL-1β (Fig. 6B). In contrast, LPS-activated monocytes maintained in the absence of ATP released no significant quantities of caspase antigen, mature caspase-1 subunits, or IL-1β (Fig. 6). Monocytes treated with ATP in the presence of ZVAD-DCB released less ELISA-positive caspase-1 antigen (Fig. 6A) and less of the 20-kDa mature caspase-1 subunit (Fig. 6C) than did cells treated with the nucleotide triphosphate in the absence of this test agent. Likewise, the ZVAD-DCB-treated cells released reduced quantities of IL-1β (Fig. 6B). In contrast, ATP-treated monocytes maintained in the presence of YVAD-CHO released quantities of the caspase-1 antigen and of the 20-kDa caspase-1 subunit that were comparable to those produced by the control ATP-treated cultures (Fig. 6A, C).}

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**Fig. 5.** ATP promotes externalization of caspase-1. A, LPS-activated monocytes were incubated for 2.5 h in the absence (−) or presence (+) of 5 mM ATP. Media were subsequently harvested and analyzed for caspase-1 content by ELISA. The amount of caspase-1 antigen is indicated in A as a function of treatment; each bar is an average of duplicate values. B, LPS-activated monocytes were incubated in medium containing no effector (−ATP), 5 mM ATP (+ATP), or 5 mM ATP and 10 μM ethacrynic acid (+ATP/EA). After 2.5 h of treatment, media were harvested, cells were solubilized by detergent extraction, and samples of each (20% of the medium and 5% of the cell-associated sample) were subjected to Western analysis for caspase-1. Representative blots are indicated in B. The migration positions of mature and procaspase-1 are indicated by arrows; each blot also contained recombinant mature caspase-1 (standard (STD)).

**Fig. 6.** Comparison of pharmacological sensitivity of ATP-induced IL-1 and caspase-1 processing. LPS-activated monocytes were incubated for 90 min in the absence (−) or presence (+) of ATP and the indicated effectors. After this treatment, media were harvested and assessed for caspase-1 content by ELISA (A), IL-1β content by ELISA (B), and the 20-kDa caspase-1 subunit by Western analysis (C). Effector concentrations were as follows: YVAD-CHO, 20 μM; ZVAD-DCB, 20 μM; glyburide, 50 μM; and ethacrynic acid, 20 μM.
Acid yielded reduced quantities of caspase-3 antigen, 20-kDa mature caspase-3 subunits, and IL-1β (Fig. 6). Caspase-3 Is Inefficiently Activated by ATP Treatment—Pro-apoptotic stimuli often lead to activation of procaspase-3, after which the mature protease functions in the execution phase of the death response (8, 44, 45). Agents that promote stimulus-coupled IL-1β post-translational processing such as ATP and nigericin have been reported to initiate changes in the target cell population that are characteristic of an apoptotic death response (29, 46, 47).

To assess whether LPS/ATP-treated monocytes generate mature caspase-3, cell-associated and medium fractions were subjected to Western analysis using an anti-caspase-3 antiserum. In the absence of ATP, the major immunodetectable protein in the monocyte extracts comigrated with a 32-kDa procaspase-3 standard (Fig. 7); no immunopositive species corresponding to the recombinant 17-kDa mature caspase-3 subunit was detected. The medium recovered from these cells contained small quantities of antigenic polypeptides that comigrated with procaspase-3 (Fig. 7). Following ATP treatment, quantities of extracellular caspase-3 polypeptides increased; the major extracellular species displayed an apparent mass of 32 kDa, but several smaller species were also detected (Fig. 7). These smaller species did not comigrate with the recombinant 17-kDa mature caspase-3 subunit (Fig. 7). In the presence of ethacrynic acid, fewer caspase-3 polypeptides were released to the medium (Fig. 7). Cells treated with YVAD-CHO, on the other hand, continued to release quantities of caspase-3 antigenic polypeptides comparable to those released from control ATP-treated cultures (Fig. 7).

**DISCUSSION**

The involvement of caspase-1 in the post-translational processing of pro-IL-1β is accepted based on evidence derived from several independent experimental systems. For example, production of 17-kDa IL-1β by heat-killed Staphylococcus aureus-treated human blood monocytes is blocked by the caspase-1 inhibitor YVAD-CHO, and ZVAD-DCB impairs mature cytokine production by LPS-activated murine peritoneal macrophages (4, 23). Likewise, peritoneal macrophages isolated from caspase-1-deficient mice generate reduced levels of 17-kDa mature IL-1β compared with their wild-type counterparts (3, 22). Despite its recognized involvement in the maturation pathway of IL-1β, few studies have detected active caspase-1 within monocytes and/or macrophages. Nigericin treatment of murine peritoneal macrophages was reported to increase caspase-1 activity (23), and THP-1 cells treated with this ionophore were reported to possess less procaspase-1 than non-ionophore-stimulated cells (24); the presence of mature caspase-1 subunits was not, however, demonstrated in either cell system. On the other hand, procaspase-1 is readily detected in THP-1 monocyte cells both by immunocytochemistry and by Western analysis (17, 20); the proenzyme appears to be distributed uniformly within the cytoplasm (20). Human monocytes have been reported to possess mature caspase-1 at their surface as part of an IL-1 transport apparatus; however, the low level of the mature enzyme precluded its detection by Western analysis (48). The difficulty in demonstrating mature caspase-1 subunits is surprising based on many reports noting that the related enzyme caspase-3 is readily detected as its mature form following induction of apoptosis (45, 49). Factors and/or mechanisms regulating procaspase-1 activation are not well understood. The proenzyme demonstrates autocatalysis in vitro (18, 50), and this process appears to be dependent on oligomerization of the proenzyme subunits (51). Whether procaspase-1 activation within intact cells occurs autocatalytically or requires participation of an unidentified protease remains to be established. Recently, ATP was reported to initiate maturation of caspase-1 in N13 murine microglial cells. This maturation was blocked by lactacystin, suggesting that the proteasome may participate in the activation mechanism (52). New findings presented in this study extend our understanding of ATP-induced IL-1β post-translational processing by demonstrating that 1) the caspase-1 inhibitor ZVAD-DCB, in contrast to YVAD-CHO and YVAD-CMK, inhibits not only formation of human mature IL-1β, but also release of the procytokine species; 2) human monocytes produce and externalize 20-kDa mature caspase-1 subunits; and 3) procaspase-3 is not efficiently activated when monocytes are stimulated to process IL-1β.

Treatment of LPS-activated human monocytes with ATP in the presence of YVAD-CHO or YVAD-CMK prevented proteolytic maturation of pro-IL-1β, and as a consequence, pro-IL-1β was released extracellularly. Procytokine released in the presence of these agents represented >60% of the quantity of mature cytokine released in their absence; blockade of pro-IL-1β proteolytic maturation therefore minimally affected its release. In contrast, LPS-activated monocytes treated with ATP in the presence of ZVAD-DCB produced no mature cytokine and released minimal quantities of the procytokine. This agent was shown previously to block 17-kDa IL-1β production by nigericin-stimulated murine macrophages; in this system, the inhibitor-treated cells continued to release some pro-IL-1β, but absolute quantities were not reported (23). Release of pro-IL-1β from human monocytes may therefore be more sensitive to the inhibitory effects of ZVAD-DCB than is release of the procytokine from mouse macrophages. Since YVAD-CHO and YVAD-CMK did not block release of pro-IL-1β, inhibition of cytokine export observed in the presence of ZVAD-DCB likely is due to its interaction with cellular polypeptides distinct from caspase-1. In this regard, the nonselective thiol reagents NEM and PAO both blocked maturation of pro-IL-1β and release of the procytokine. The effect of PAO was reversed by dimercaptopropanol, suggesting that proteins containing vicinal sulfhydryl groups are involved in the cellular response. ZVAD-DCB and the nonselective thiol reagents may therefore modify thiol-dependent cellular proteins in addition to caspase-1 and, as a result, impair both proteolytic maturation and externalization of pro-IL-1β. Why should ZVAD-DCB be different from the other caspase-1 inhibitors? Both ZVAD-DCB and YVAD-CMK are expected to behave as irreversible inhibitors, yet only ZVAD-DCB impaired pro-IL-1β secretion; thus, irreversibility is not the explanation. The inherent reactivity of the acyloxydichlorobenzoate group and/or the addition of the benzyllox-
carbonyl group to the peptidic core may cause ZVAD-DCB to be less selective toward caspase-1. ZVAD-DCB, NEM, and PAO may impair other caspase family members and/or other thiol-dependent enzymes whose activity is required for membrane disruption. Additional work will be needed to clarify the molecular basis for the difference in inhibitory profiles.

Interestingly, ATP-treated monocytes maintained in the presence of concentrations of YVAD-CHO that were sufficient to block proteolytic maturation of pro-IL-1β still externalized processed 20-kDa caspase-1 subunits. Failure to block procaspase-1 conversion may indicate that inhibitor concentrations inside the cell were not sufficient to impair autocatalysis; in vitro studies have shown that 45-kDa procaspase-1 is labeled by a covalent caspase-1 inhibitor, but at concentrations >500-fold above those required to label the mature enzyme subunits (50). Alternatively, procaspase-1 may be activated by a YVAD-CHO-insensitive protease (52).

ATP appears to promote IL-1β post-translational processing by activation of a novel ligand-gated ion channel known as the P2X7 receptor (33, 39, 54). Ligation of this receptor opens a nonselective ion channel, which, after several minutes of activation, demonstrates pore-like properties and allows passage of molecules <900 Da (55). Continuous ligation of the P2X7 receptor ultimately results in cell death (56). In normal tissue culture media, millimolar concentrations of ATP are required to activate the P2X7 receptor; ATP4– is the active ligand, and divalent cations within media lower the concentration of the tetravalent species. Is it likely that the P2X7 receptor is involved in IL-1 processing in vivo? At sites of inflammation, ATP may be released from dying cells, leading to high local concentrations sufficient for receptor ligation. In addition, platelet granules contain high concentrations of ATP, and their discharge in the close proximity of a monocyte/macrophage, possibly in a microenvironment deficient in divalent cations, could promote P2X7 receptor activation. Alternatively, other ligands may exist for the P2X7 receptor that have not yet been identified; benzoylbenzoyl-ATP, for example, is known to be a more effective agonist than is ATP itself (55). We suspect that the P2X7 receptor represents one triggering mechanism by which monocytes and macrophages activate and release IL-1 and that other stimuli also operate in vivo, including cytolytic T-cells (29) and bacterial toxins (30–32).

Following addition of ATP to LPS-activated monocytes, 17-kDa IL-1β is observed intracellularly, but the mature cytokine does not accumulate at this location and is externalized (57). Because of this transient behavior, detection of intracellular mature IL-1 is difficult and requires use of metabolically labeled cells and a pulse-chase analysis (34, 57). Similarly, 20-kDa mature caspase-1 subunits were detected in the medium of ATP-treated monocytes by Western blotting, but these subunits were not seen within detergent extracts of the ATP-treated cells. We assume that protease activation occurred intracellularly, followed by release of the mature subunits; the low abundance of the processed subunits and/or failure to look at earlier times post-ATP addition may account for our inability to detect the cell-associated mature species. Caspase-1 that remained cell-associated after ATP treatment consisted of the 45-kDa propolypeptide. This behavior parallels what is seen with IL-1β; cytokine that remains cell-associated following ATP treatment is composed of the 31-kDa prospecies (34, 57). This type of all-or-none behavior suggests that some monocytes respond to ATP and activate their entire complement of caspase-1 and, in turn, efficiently convert their pro-IL-1β to the 17-kDa species. However, the ionic imbalance generated by the opening of the P2X7 receptor channel causes responding cells to die and to release cytoplasmic constituents, including lactate dehydrogenase, procaspase-3, and mature caspase-1. In contrast, a subset of cells within the monocyte population does not respond to ATP (or responds nonproductively); these cells do not proteolytically cleave procaspase-1 and pro-IL-1β, and they do not release their cytoplasmic constituents. Heterogeneity within monocyte populations has been reported previously (58), and the ATP responsiveness of cultured human monocytes is known to be dynamic and subject to regulation by cytokines and serum-derived factors (43).

Several of the aforementioned stimuli that promote IL-1 post-translational processing have been reported to initiate both apoptotic and necrotic death responses. For example, mouse peritoneal macrophages treated with ATP demonstrate DNA laddering, a characteristic feature of many apoptotic processes (29, 48). Likewise, nigericin has been reported to promote apoptotic changes in THP-1 cells (46), and cytolytic T-cell killing of target cells is associated with apoptotic changes (59). On the other hand, nigericin has also been reported to promote necrosis of THP-1 cells (60). Procaspase-3 is activated by a wide variety of proapoptotic stimuli, and its activity is often required to complete the cell death pathway (8, 44, 45). Interestingly, monocytes possessed procaspase-3, but this protease was not activated to its 17-kDa mature form in response to ATP. Several slightly larger (~20 kDa) processed caspase-3 polypeptides were detected, but the proform represented the most abundant secreted product; processed caspase-3 subunits of multiple sizes have been reported (61). Stimuli that promote activation of procaspase-1 and efficient IL-1β post-translational processing are thus not necessarily sufficient to promote activation of procaspase-3. The ATP response may therefore not represent prototypical apoptosis. Likewise, stimuli that promote apoptosis do not necessarily lead to activation of procaspase-1 (45, 62). In contrast to our results with human monocytes, ATP treatment of murine N13 microglial cells has been reported to promote caspase-3 activation (52); different cell types may respond differently to the ATP stimulus.

Stimulus-coupled IL-1 post-translational processing is also blocked by agents that affect anion transport processes such as ethacrynic acid and glyburide (39, 40). These agents presumably disrupt cytokine processing by altering the sequence and/or magnitude of ionic changes that occur when monocytes and/or macrophages are activated by a secretion stimulus. Consistent with this hypothesis, stimulus-coupled IL-1 post-translational processing is blocked when extracellular chloride ions are replaced with chaotropic anions or when cells are maintained in a sodium-free medium (35, 63). Moreover, this unusual cellular response requires a change in cell volume (35), and both ATP- and nigericin-treated macrophages ultimately display morphological features characteristic of an osmotically driven oncosis type of death response (27, 34, 64). Glyburide and ethacrynic acid not only blocked proteolytic conversion of pro-IL-1β, but also blocked release of pro-IL-1β, activation of caspase-1, and release of procaspases to the medium. These agents therefore yield a pattern of suppression similar to that of ZVAD-DCB. Although ethacrynic acid can potentially alkylate protein sulfhydryl groups as a result of an α,β-unsaturated ketone constituent (53), glyburide does not possess chemical reactivity with protein sulfhydryl groups, and its effect cannot be attributed to covalent protein modification. Inhibitors of anion transport may therefore block ionic changes that are necessary for activated monocytes/macrophages to commit to the stimulus-coupled release pathway and consequently suppress caspase-1 activation, pro-IL-1β processing, and externalization of cytoplasmic constituents.
