Human Monocyte Interleukin-1β Posttranslational Processing

EVIDENCE OF A VOLUME-REGULATED RESPONSE*

David G. Perregaux, Ronald E. Laliberte, and Christopher A. Gabel‡

From the Department of Cancer, Immunology, and Infectious Diseases, Central Research, Pfizer Inc., Groton, Connecticut 06340

(Received for publication, June 28, 1996, and in revised form, August 29, 1996)

Interleukin (IL)-1β produced by monocytes and macrophages is not released via the normal secretory apparatus, and prior to its release, this cytokine must be proteolytically processed to generate a mature biologically active species. Biochemical mechanisms that regulate these posttranslational steps are not well understood. Lipopolysaccharide (LPS) is a poor activator of IL-1 posttranslational processing despite serving as a potent inducer of IL-1 synthesis. For example, freshly isolated human monocytes treated with LPS released <30% of their newly synthesized IL-1β as the mature 17-kDa cytokine species, and monocytes that were aged overnight in culture prior to LPS treatment released no 17-kDa cytokine. In contrast, addition of extracellular ATP promoted IL-1β posttranslational processing from both monocyte populations. Previous studies indicated that ATP, acting via surface P2Z-type receptors, promoted major intracellular ionic changes. To explore whether these ionic changes were required for cytokine posttranslational processing, LPS-stimulated human monocytes were maintained in ionically altered media. Hypotonic conditions promoted an efficient and selective release of mature 17-kDa IL-1β from LPS-activated monocytes in the absence of ATP. In contrast, hypertonic conditions blocked the ATP-induced posttranslational processing reactions. Both hypotonic stress- and ATP-induced processing were blocked when NaI was substituted for NaCl within the medium; substitution with NaSCN or NaNO3 also blocked the ATP response, but these salts were less inhibitory against the hypotonic stimulus. Sodium glucuronate substitution did not inhibit cytokine processing induced by either stimulus. Removal of divalent cations from the medium did not affect the ATP response, but pretreatment of monocytes with the phosphatase inhibitor okadaic acid dose-dependently suppressed ATP-induced IL-1β posttranslational processing. A volume-induced change to the intracellular ionic environment, therefore, may represent a key element of the mechanism by which IL-1β posttranslational processing is initiated. The strong dependence of this cytokine release mechanism on chloride anions suggests that selective anion transporters function as important components of this response.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 860-441-5483; Fax: 860-441-5719.

1 The abbreviations used are: IL-1β, interleukin 1β; ICE, interleukin 1 converting enzyme; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; RVD, regulated volume decrease; DIDS, 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid; FBS, fetal bovine serum; SITS, 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid.

© 1996 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.
mature IL-1β from LPS-stimulated human monocytes and mouse peritoneal macrophages was shown to be impaired by agents that disrupted anion transport processes, including DIDS, ethacrynic acid, and tenidap, a new anti-inflammatory/anti-arthritis agent (16). Changes to the intracellular ionic environment, therefore, appear to be required for activation of the posttranslational maturation of pro-IL-1β. In the present study we have continued to explore ionic conditions required for this unusual cytokine processing event. ATP and hypotonic stress are employed as alternative signals to activate human monocyte IL-1β posttranslational processing. Sensitivity of these cellular activation processes to changes of the ionic environment and to pharmacological effectors suggests that an anion transporter is a necessary component of the mechanism by which both hypotonic stress and ATP promote IL-1β posttranslational processing.

**EXPERIMENTAL PROCEDURES**

**Human Monocytes**—Mononuclear cells were isolated from blood (100 ml) drawn from normal volunteers as described previously (16). In an average experiment, 1 × 10⁷ mononuclear cells were added to each well of 6-well dishes in a total volume of 2 ml of RPMI 1640 containing 10% FBS, 25 mM Hepes, pH 7.2, and 1% penicillin/streptomycin (Maintenance Medium). Monocytes were allowed to adhere for 2 h, after which the supernatants were discarded and the attached cells were rinsed once with 2 ml of Maintenance Medium.

When freshly isolated monocytes were employed, the adherent cells immediately were exposed to 10 ng/ml LPS (Escherichia coli serotype 055:B5, Sigma) for 2 h. Alternatively, the adherent monocytes were incubated overnight at 37 °C in a 5% CO₂ environment in Maintenance Medium prior to LPS activation; where indicated, 5 ng/ml recombinant human granulocyte macrophage colony stimulating factor (R & D Systems, Minneapolis, MN) was added to the culture medium. In all cases LPS-stimulated cells were labeled for 60 min in 1 ml of Pulse Medium (methionine-free RPMI 1640, 1% dialyzed FBS, 25 mM Hepes, pH 7.2, 83 μCi/ml [35S]methionine (Amersham Corp., 1000 Ci/mmol). The Pulse Medium subsequently was discarded; the radiolabeled cells were washed once with 2 ml of Chase Medium (RPMI 1640, 1% FBS, 20 mM Hepes, 5 mM NaHCO₃, pH 6.9), and 1 ml of Chase Medium that contained various effector molecules was added to each well. Where indicated, ATP was added (from a 100 mM stock solution previously adjusted to pH 7 with NaOH) to a final concentration of 1–5 mM. Radiolabeled monocytes were chased at 37 °C for various times after which the cells recovered and clarified by centrifugation to remove cells that detached from the plate during the chase and cell debris; the resulting supernatants were harvested and adjusted to 1% in Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetate acid, 1 μg/ml pepstatin, and 1 μg/ml leupeptin by addition of concentrated stock solutions of these reagents. Cells were solubilized by addition of 1 ml of an extraction buffer composed of 25 mM Hepes, pH 7, 1% Triton X-100, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetate acid, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 mg/ml ovalbumin. After 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 Beckman tabletop ultracentrifuge using a TLA 40 rotor (Beckman Instruments, Palo Alto, CA).

**Immuno precipitation of IL-1β and Analysis of Radiolabeled Cytokine Product**—IL-1β was immunoprecipitated from detergent extracts of cell and media samples as detailed previously (16). Disaggregated immunoprecipitates were analyzed by SDS-gel electrophoresis and autoradiography (22); gels were soaked in Amplify (Amersham Corp.) prior to drying. Quantitation of the amount of radioactivity associated with the various species of IL-1β was determined with the use of an Ambis Image Analysis System (San Diego, CA).

**Lactate Dehydrogenase (LDH) Assays**—Aliquots of media samples and cell extracts were assayed for LDH using pyruvate as substrate and a colorimetric pyruvate detection assay (Sigma).

**Isotonic Media**—A modified Dulbecco's based formulation was employed to prepare isotonic medium (20 mM Hepes, pH 7.2, 137 mM NaCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 1.5 mM KH₂PO₄, 2.7 mM KCl, and 5 mM glucose). In some cases, NaCl was substituted with NaI, NaSCN, NaNO₃, or sodium gluturonate; all sodium salts were obtained from Sigma.

### RESULTS

**Monocyte Populations Differ in Their Release of IL-1β**

Freshly isolated human monocytes are heterogeneous with respect to their size and functional properties (23, 24). Moreover, when maintained in culture these cells may change their biochemical properties (25, 26), including their ability to respond to extracellular ATP (27–29). Based on this known heterogeneity, a comparison of the IL-1β posttranslational processing capacity of freshly isolated monocytes and monocytes that were aged overnight in culture was performed. Each population was stimulated for 2 h with LPS, labeled for 60 min with [35S]methionine, and chased in the absence or presence of ATP. In the absence of ATP, freshly isolated monocytes released proteolytically processed radiolabeled 17-kDa IL-1β (Table I); based on recovery of total radiolabeled IL-1β from these cultures, however, the 17-kDa species generally accounted for <30% of the newly synthesized cytokine. High concentrations of LPS are known to favor mature IL-1β release (15), but even at a concentration of 1 μg/ml only 26% of the total radiolabeled cytokine was recovered as the extracellular mature species (experiment 2; Table I). Aged monocytes, on the other hand, did not release significant mature IL-1β in the presence of LPS (Table I). In contrast, both monocyte populations responded to ATP and released proteolytically processed 17-kDa IL-1β; the nucleotide triphosphate, however, did not affect both populations equally. Thus, freshly isolated monocytes consistently released >90% of their IL-1β as the 17-kDa mature species, whereas 60% was radiolabeled cytokine was released as the mature species from aged monocytes (Table I).

In the presence of ATP an apparent over-recovery of radiolabeled IL-1β consistently was observed. For example, a total of 3970 counts were recovered as IL-1β from LPS-treated fresh monocytes in the absence of ATP (experiment 1; Table I). Following ATP treatment, however, total radioactivity recovered as immunoprecipitable IL-1β from an identical culture increased >4-fold (Table I). Since radiolabeled methionine was removed from the culture medium prior to the addition of ATP, new synthesis of IL-1β cannot account for this over-recovery.

### Table I

| Experiment | Monocyte population | Treatment | Total IL-1β/LDH | % of total as 17 kDa IL-1β |
|------------|---------------------|-----------|----------------|--------------------------|
| 1          | Fresh               | No ATP    | 3,970          | 15                       |
|            |                     | + 2 mM ATP| 17,700         | 92                       |
| 2          | Fresh               | No ATP    | 81,300         | 26                       |
|            |                     | + 5 mM ATP| 296,000        | 85                       |
| 3          | Aged                | No ATP    | 11,400         | 1                        |
|            |                     | + 2 mM ATP| 32,200         | 63                       |
| 4          | Aged                | No ATP    | 5,400          | 1                        |
|            |                     | + 2 mM ATP| 10,050         | 55                       |
Moreover, the antibody employed to capture radiolabeled IL-1β was not limiting. Treatment of monocyte extracts (derived from cells not exposed to ATP) with increasing polyclonal antibody did not yield additional radiolabeled IL-1β. Rather, it appeared that conditions which led to an increase in IL-1β posttranslational maturation also led to an increase in cytokine recovery. This over-recovery may result from a decrease in the intracellular turnover of newly synthesized cytokine and/or a change in IL-1β that allows recognition by the antibody. Similar differences were observed in the quantity of radiolabeled IL-1β recovered in the absence or presence of ATP from aged monocyte cultures (Table I). Although the cause of this over-recovery remains to be determined, the results indicate that ATP is a potent inducer of IL-1β release and maturation and that the extent of these posttranslational processing reactions varies between different monocyte populations. These differences suggest that the ATP response is not passive but, rather, requires active participation of the IL-1β producing cell.

**Hypotonic Stress Promotes Release of Mature IL-1β from LPS-activated Human Monocytes**

Based on previous observations suggesting that anion and potassium fluxes are necessary for the posttranslational maturation of IL-1β (14, 16, 20, 21), LPS-stimulated monocytes were subjected to hypotonic stress, a treatment known to promote KCl export in a wide variety of cells (30). Freshly isolated human monocytes were stimulated with LPS for 2 h, labeled with [35S]methionine for 60 min, and then chased for 3 h in media of differing ionic strength. The base medium in this experiment consisted of a standard Dulbecco’s formulation (Table II). Hypotonic media were prepared by varying the concentrations of NaCl, KCl, and K2HPO4; concentrations of other media components, including Ca2+, Mg2+, NaHCO3, glucose, and Hepes, were maintained at constant values (Table II). The chase medium varied in NaCl concentration from 132 to 27 mM.

In normal Dulbecco’s medium (Formulation A, Table II), LPS-stimulated cells released little mature IL-1β and the cell-associated cytokine persisted as the 17-kDa species (Fig. 1). Addition of 2 mM ATP to this medium, however, promoted complete release of the radiolabeled cytokine, and externalized IL-1β was processed efficiently to the 17-kDa species (Fig. 1). Importantly, as the concentration of NaCl within the chase medium decreased (in the absence of exogenous ATP), mature 17-kDa IL-1β increased extracellularly. Thus, in medium containing 55 mM NaCl (Formulation D) and 27 mM NaCl (Formulation E) mm NaCl, 85 and 97%, respectively, of the total radiolabeled IL-1β was recovered extracellularly as the 17-kDa species (Fig. 1).

To define the kinetics of this posttranslational processing, LPS-stimulated [35S]methionine-labeled monocytes were chased in hypotonic medium for various times. After a 10-min chase, only a small quantity of radiolabeled IL-1β was recovered in the medium, and the externalized polypeptides were not proteolytically processed (Fig. 2). Extracellular 17-kDa IL-1β, however, was readily detected after 20 min of hypotonic treatment, and the quantity of this species increased during longer chase times (Fig. 2). Importantly, beyond the 20 min time point extracellular IL-1 was recovered almost exclusively as the 17-kDa species (Fig. 2). In contrast, at all times intracellular cytokine was composed primarily of the 31-kDa species with only minor amounts of the 17-kDa mature form being present (data not shown). Externalization of the mature cytokine species, therefore, occurred more efficiently than release of the pro-IL-1β polypeptide species. Moreover, only 20% of the cytoplasmic constituent lactate dehydrogenase (LDH) was displaced to the medium after 3 h of hypotonic treatment (Fig. 2). Thus, hypotonic stress promoted formation of 17-kDa IL-1β and selective release of this species from LPS-activated human monocytes. Hypotonically stressed monocytes (in medium containing ≤55 mM NaCl) demonstrated the same ballooned appearance as previously observed after ATP or nigericin treatment (data not shown). Although viability was not assessed, cells demonstrating this dramatically altered appearance are assumed to be non-vital.

The sum of all radioactivity recovered as IL-1β (corrected for the 2-fold loss of [35S]-labeled methionine that accompany formation of the 17-kDa IL-1β species) again indicated that a 4-fold over-recovery occurred during the 3-h posttranslational maturation period (Fig. 2). Since quantities of radiolabeled polypeptides are expected to decrease during the chase as a result of protein turnover, this over-recovery (relative to the amount recovered from cells in the absence of a chase) cannot

---

**Table II**

| Component | A | B | C | D | E |
|-----------|---|---|---|---|---|
| NaCl      | 132| 110| 82 | 55 | 27 |
| KCl       | 2.6| 2.2| 1.6| 1.1| 0.54 |
| KH2PO4    | 1.4| 1.2| 0.9| 0.6| 0.3 |
| MgCl2     | 0.5| 0.5| 0.5| 0.5| 0.5 |
| CaCl2     | 0.9| 0.9| 0.9| 0.9| 0.9 |
| Hepes, pH 7.3 | 20 | 20 | 20 | 20 | 20 |
| NaHCO3, pH 7.3 | 5  | 5  | 5  | 5  | 5  |
| Glucose   | 5  | 5  | 5  | 5  | 5  |

**Hypotonic Stress Promotes Release of Mature IL-1β from LPS-activated Human Monocytes**

![Figure 1](image-url)
reflect differences in protein degradation. Rather, the marked increase in immunoprecipitable radioactivity suggests that pulse-labeled cytokine molecules initially exist within monocyte detergent extracts as forms that are inaccessible to and/or not recognized by the antibody and are converted during hypotonic stress to forms that are recognized immunologically.

Medium Composition Affects the ATP and Hypotonic Stress Responses

To determine whether the ATP-activated IL-1β posttranslational processing mechanism also involved a volume-sensitive response, monocytes were treated with ATP in a hypertonic medium. Normal RPMI medium was made hypertonic by the addition of 0.2 M NaCl. LPS-activated [35S]methionine-labeled cells treated with ATP in this medium did not efficiently process IL-1β to the 17-kDa species nor did they release significant cytokine into their medium (Fig. 3). In contrast, cells maintained in normal RPMI demonstrated an efficient ATP response (Fig. 3). ATP-treated control cultures released 26% of their LDH into the medium, but monocytes treated with ATP in the hypertonic medium released only 15% of this cytoplasmic marker.

To explore the role of anions in the ATP-induced response, LPS-activated [35S]methionine-labeled monocytes were incubated in isotonic media that contained various sodium salts in place of NaCl; chaotropic anions (I⁻, NO₃⁻, and SCN⁻) and the membrane impermeant glucuronate anion were employed. In medium containing NaCl, monocytes demonstrated an efficient ATP response (Fig. 4); 17-kDa IL-1β was produced and externalized. In contrast, when monocytes were maintained in a NaI- or NaSCN-based medium, a marked decrease in IL-1β and release of 17-kDa IL-1β was observed (Fig. 4). In the presence of either of these salts, production of 17-kDa IL-1β was reduced by 97% relative to cells maintained in NaCl-containing medium. Likewise, cells maintained in a NaNO₃-based medium demonstrated an impaired response; 56% less 17-kDa IL-1β was produced by cells maintained in NaNO₃ relative to cells maintained in NaCl (Fig. 4). When the medium contained 137 mM sodium glucuronate, on the other hand, production of 17-kDa IL-1β was not impaired; rather, cells maintained in this medium consistently produced slightly greater amounts of the mature cytokine species (Fig. 4). The percentage of LDH released by ATP was 25, 10, 9, 16, and 31%, respectively, in media that contained 137 mM NaCl, NaI, NaSCN, NaNO₃, or sodium glucuronate. Thus, chaotropic an-
ions inhibited ATP-induced processing and release of IL-1β and the release of LDH.

To address the possibility that chaotropic anions impaired the ATP-induced cytokine response by blocking binding of ATP to its cell surface receptor, monocytes were treated with ATP at different times relative to the time of substitution of NaI for NaCl within the medium. When monocytes were placed in NaI either before or at the time of ATP addition, 17-kDa IL-1β production was blocked efficiently (data not shown). Likewise, when the medium substitution occurred within 15 min of ATP addition the cytokine response was inhibited by 87% (Fig. 5). When the NaI for NaCl substitution was delayed beyond 25 min of ATP addition, however, production of 17-kDa IL-1β was recovered efficiently from the NaI medium as from a NaCl medium; failure to recover 17-kDa IL-1β from the NaI medium, therefore, did not result from impaired immunoprecipitation.

Additional evidence that the anion effect on IL-1β processing was not due to inhibition of ATP binding to its receptor was obtained by asking if chaotropic anions altered cytokine processing promoted by hypotonic stress. LPS-activated [35S]methionine-labeled monocytes were treated with hypotonic medium that contained 55 mM concentrations of the individual sodium salts. As expected, cells incubated in hypotonic NaCl produced and externalized 17-kDa IL-1β (Fig. 6). In contrast, cells maintained in hypotonic NaI produced only 5% as much 17-kDa IL-1β (Fig. 6). Production of 17-kDa IL-1β also was reduced in the presence of hypotonic NaSCN, but the extent of inhibition (<30%) was not equivalent to that obtained in the NaI-based medium. Monocytes maintained in hypotonic NaNO$_3$ or sodium glucuronate produced quantities of 17-kDa IL-1β ≥ to that recovered from cells maintained in hypotonic NaCl (Fig. 6).

**Pharmacological Effectors**

Hypotonic stress promotes a regulated volume decrease (RVD) response in many cell types that is mediated by a net loss of KCl from the cytosol (30–32); the mechanism of KCl loss is cell type-dependent and may occur by the concerted action of separate K$^+$ and Cl$^-$ conducting channels or by an electroneutral KCl cotransporter (30–32). In an attempt to link IL-1β posttranslational processing events to a specific KCl release mechanism, known effectors of the RVD response were profiled in the human monocyte cytokine release assay.

**Okadaic Acid Inhibits ATP-induced IL-1β Posttranslational Processing**—The protein phosphatase inhibitor okadaic acid impairs swelling activated KCl cotransport in rabbit red blood cells (33). To determine whether okadaic acid affected IL-1β posttranslational processing, LPS-activated [35S]methionine-labeled cells were preincubated with various concentrations of okadaic acid for 20 min and then treated with ATP. Okadaic acid impaired the ATP-induced release of IL-1β and the processing of IL-1β (Fig. 6).
Figure 6: Effect of chaotropic anions on the hypotonic stress response. LPS-stimulated [35S]methionine-labeled fresh monocytes were placed in an isotonic medium that contained 4.077 M of an individual sodium salt for 15 min, after which these media were replaced with hypotonic media containing only 55 mM of the same sodium salt. After a 1.5-h incubation, cells and media were separated, and IL-1β was recovered from the media samples by immunoprecipitation. An autoradiogram of the immunoprecipitates (A) and the absolute amount of radioactivity recovered as extracellular 17-kDa IL-1β (B) are indicated. Each condition was performed in duplicate, and the bars are the average of the two determinations; numbers within the bars indicate the percentage relative to the NaCl control.

Quinine Blocks Cytokine Posttranslational Processing—Quinine sensitivity is a criterion that often is employed to distinguish between the functioning of separate conductive channels or cotransporters in the pseudocleavage (34, 35). This agent is reported to block hypotonic stress-induced K+ channels, and quinine inhibition is reversed when gramicidin, a potassium conductive ionophore, is added to the system to provide an alternate K+ efflux mechanism (35). We previously reported that potassium selective ionophores such as nigericin and la-salocid can on their own promote maturation and release of IL-1β from monocytes and macrophages (20). Likewise, gramicidin promoted mature IL-1β release from LPS-activated human monocytes (Fig. 8). At a concentration of 2 μM, monocytes treated with this channel forming ionophore (36) efficiently externalized their newly synthesized IL-1β in the form of the 17-kDa species (Fig. 8). At a concentration of 1 mM, comparable with concentrations reported to inhibit RVD (34), quinine partially blocked both the gramicidin (39%) and ATP (58%) responses (Fig. 8). Quinine-treated monocytes did not release additional procytokine nor did they accumulate processed IL-1β intracellularly (data not shown). Quinine’s ability to impair both the ATP and gramicidin responses suggests that it is acting independently of a specific K+ channel. Monocytes activated by hypotonic stress also were treated with quinine; within the hypotonic medium, however, 1 mM quinine promoted release of LDH and 31-kDa pro-IL-1β suggesting that it was lytic under these conditions (data not shown).

Ca2+-free Media Do Not Affect the ATP Response—An RVD response mediated by separate conductive K+ and Cl− channels often is inhibited by removal of Ca2+ from the medium (34). LPS-stimulated [35S]methionine-labeled human monocytes treated with ATP in normal medium and in medium containing 10 mM EGTA yielded identical amounts of 17-kDa IL-1β (data not shown).

BuMethanide and Furosemide Do Not Impair the ATP Response—Agents that impair anion transport processes effectively suppress ATP-induced maturation and release of IL-1β; such agents include the stilbene derivatives DIDS and SITS and the anti-inflammatory agent tenidap (16). In addition, we noted previously that ethacrynic acid is a potent inhibitor of the ATP response of human monocytes; the IC50 against this response is 3 μM (16). Ethacrynic acid is used as a diuretic, and this effect, in part, is attributed to inhibition of kidney cotransporters (37). Two other diuretic agents, bumetanide and furosemide, are reported to be selective inhibitors of the Na+/K+/2Cl− cotransporter (38). At 100 μM, a concentration sufficient to impair Na+/K+/2Cl− cotransport (39, 40), neither of these agents inhibited ATP-induced cytokine posttranslational processing (Table III).

Discussion

Posttranslational maturation of IL-1β promoted by ATP is not observed with similar concentrations of AMP, UTP, or GTP, and ADP is less efficient than ATP (20). This selectivity suggests that ligation of specific purinoreceptors (P2) activates a signaling mechanism leading to the posttranslational processing of IL-1β (41, 42). Further evidence that the monocyte ATP response involves a signal transduction mechanism is indi-
IL-1β Posttranslational Processing

LPS-activated [35S]methionine-labeled aged human monocytes were pretreated for 15 min in RPMI, pH 6.9, medium in the absence or presence of bumetanide or furosemide and then treated for 2 h with 2 mM ATP. At the end of this treatment, cells and media were separated, and IL-1β was recovered by immunoprecipitation. The quantity of radioactivity associated with the extracellular 17-kDa IL-1β species is indicated.

| Experiment | Condition | 17 kDa IL-1β (counts) | Control |
|------------|-----------|-----------------------|---------|
| 1          | 2 mM ATP  | 7766                  | 100     |
|            | 2 mM ATP + 100 μM bumetanide | 6931       | 89      |
| 2          | 2 mM ATP  | 2496                  | 100     |
|            | 2 mM ATP + 100 μM furosemide  | 2472       | 99      |

Data presented previously suggested that potassium and anion fluxes were necessary components of the mechanism by which ATP promoted IL-1β posttranslational processing (14, 16, 20, 21). A remarkable feature of the release process induced by ATP is the dramatic morphological change that accompanies cytokine externalization. In the continuous presence of this nucleotide triphosphate LPS-stimulated monocytes and macrophages swell extensively (14, 16, 20). Mammalian cells possess a remarkable ability to regulate their cytoplasmic volume (30, 31), and this regulation is easily visualized when cells are placed within an osmotically altered media. For example, Ehrlich ascites tumor cells initially swell after suspension in a hypotonic medium due to an influx of water. This swelling soon subsides, however, and within minutes a cell’s volume normalizes despite its persistence within the hypotonic medium (30). This RVD response often occurs as a result of activation of K⁺ and Cl⁻ channels or K⁺/Cl⁻ cotransporters within the plasma membrane. These ion transporters facilitate net loss of KCl followed by the passive movement of H₂O and a near restoration of the cell’s original volume (30, 31).

LPS-stimulated monocytes subjected to hypotonic stress responded by activating both the proteolytic maturation of pro-IL-1β and the release of the mature 17-kDa cytokine. This activation was not instantaneous, and monocytes suspended in 55 mM NaCl-containing buffer required >10 min of treatment to initiate the processing reactions. This time dependence is consistent with the notion that the hypotonic conditions triggered changes in the intracellular ionic environment which, in turn, activated the posttranslational processing reactions. Importantly, the employed hypotonic conditions did not simply lyse the monocytes. When mouse peritoneal macrophages are suspended in an extreme hypotonic medium, for example, they lyse and release their content of both LDH and IL-1β, the released cytokine, however, is not processed to the mature 17-kDa species (14). Lysis, therefore, is not sufficient to promote correct posttranslational processing. In contrast, after 30 min of moderate hypotonic exposure, virtually all of the recovered IL-1β released by human monocytes was processed to the 17-kDa mature species. Cell-associated cytokine, on the other hand, remained predominantly as higher molecular mass species. Moreover, <10% of the total LDH associated with the

![Figure 8. Gramicidin promotes IL-1β posttranslational processing. LPS-stimulated [35S]methionine-labeled aged monocytes (previously cultured in the presence of 5 ng/ml granulocyte macrophage colony-stimulating factor) were placed in RPMI medium and treated for 3 h with either 2 mM ATP or 2 μM gramicidin in the presence or absence of 1 mM quinine; cultures treated with quinine were pretreated with this agent for 15 min prior to the addition of the release stimulus. Media IL-1β immunoprecipitates were analyzed by SDS-gel electrophoresis and autoradiography (A); the region of the autoradiogram that contained 31- and 17-kDa IL-1β is shown. The amount of radioactivity associated with extracellular 17-kDa IL-1β is indicated in B; each bar is the average of the duplicate determinations. Numbers within the bars indicate the percentage relative to the ATP or gramicidin-treated controls.](image)
monocyte cultures was recovered in the medium after 30 min of hypotonic treatment. This selective externalization of the 17-kDa cytokine species, therefore, suggests that the majority of the monocytes possessed intact membranes during the initial period of hypotonic treatment. With prolonged treatment times, however, the amount of extracellular LDH increased indicating that membrane integrity was compromised. The ability of hypotonic stress to promote IL-1β posttranslational processing also has been reported by an independent group (21).

As with ATP-treated cells, monocytes subjected to hypotonic stress developed a swollen appearance that correlated temporally with the release of mature IL-1β. Therefore, if hypotonic stress activated an RVD-like response in monocytes, this response apparently failed as the volume did not normalize. Swelling induced by ATP treatment of human monocytes was suppressed when these cells were maintained in the presence of a hypertonic medium; likewise, the hypertonic medium blocked ATP-induced posttranslational processing of IL-1β. Volume-activated changes, therefore, appear to be important elements of both the ATP- and hypotonic-induced cytokine responses.

Substitution of chaotropic anions for Cl⁻ ions in the medium also impairs the ATP and hypotonic responses. Iodide anions, for example, completely suppressed both responses. The thio-cyanate anion was an effective inhibitor of the ATP response but was less effective against the hypotonic stress response, and nitrate anions partially suppressed the ATP response without affecting the hypotonic response. In contrast, gluconate anions did not inhibit the ATP or hypotonic stress-induced posttranslational processing of IL-1β. The employed chaotropic anions are, to varying degrees, permeant to biological membranes, and they are expected to enter the cytosol (54, 55); the gluconate anion, on the other hand, is membrane-impermeant. Cotransporters possess a very high selectivity for Cl⁻ ions, and chaotropic anions often inhibit cotransport function (54–56). Likewise, some Cl⁻ channels are inhibited by I⁻ anions (57) although others are less discriminating and will facilitate transport of divergent anions such as I⁻ and SCN⁻ (30, 58). Based on the remarkable inhibition observed in the presence of the chaotropic anions, therefore, function of a selective chloride transporter is suggested. If ATP and hypotonic stress were initiating IL-1β posttranslational processing simply by opening non-selective pores in the membrane, then one would not expect to observe this strong anion dependence. The degree of inhibition obtained in the presence of chaotropic anions is likely to reflect their ability to penetrate the monocyte membrane and access the anion transporter under the different experimental situations.

Removal of extracellular Ca²⁺ by addition of the calcium chelator EGTA did not impair the ATP response; entry of extracellular Ca²⁺, therefore, is not necessary, and this divalent cation independence suggests that a Ca²⁺-activated K⁺ channel is not operative in the cytokine response. These data, however, do not eliminate the possibility that release of Ca²⁺ from intracellular stores is sufficient for channel activation.

Quinidine inhibited ATP-induced posttranslational processing of IL-1β, but this inhibitory effect also was observed when gramicidin served as the stimulus. Since gramicidin is expected to facilitate K⁺ loss independently of a K⁺ channel, quinidine's inhibitory effect does not appear to result from inhibition of a specific K⁺ channel; high concentrations of quinidine required to block cytokine posttranslational processing probably affected multiple cellular components. Finally, anion transport inhibitors such as DIDS, 5-nitro-2-(3-phenylpropylamino)benzoic acid, and ethacrynic acid suppress ATP-induced IL-1β posttranslational processing (16); these agents are not selective, and they are reported to impair cotransporters as well as anion channels (59, 60). Two specific blockers of the Na⁺/K⁺2Cl⁻ cotransporter, bumetanide and furosemide, did not inhibit the ATP response at concentrations that were sufficient to inhibit cotransport functions.

Taken together, these data suggest a role for a specific anion transporter in the IL-1β response, but the nature of this transporter (channel versus cotransporter) remains to be determined. A number of different Cl⁻ channels have been cloned (57), but the monocyte repertoire of such channels is unknown; these cells are reported to possess a SITS-inhibitable chloride channel (45). A K⁺/Cl⁻ cotransporter was cloned recently, but it is not known whether monocytes express this protein (61); moreover, this cloned cotransporter was inhibited by bumetanide suggesting that it is not involved in the bumetanide-insensitive monocyte cytokine response. The apparent diversity in the nature of agents that promote posttranslational maturation of IL-1β may be explained based on a shared ability of these agents to activate chloride and potassium transport. Ionophores such as gramicidin and nigericin are expected to depolarize monocytes. Likewise, ATP stimulation of murine macrophages causes depolarization as a result of the opening of a large non-selective pore in the membrane, and hypotonic stress also may elicit depolarization (34, 47). Thus, membrane depolarization may serve as the common trigger that activates cytokine posttranslational processing. The exact nature of the ionic events that underlie subsequent steps culminating in cytokine posttranslational processing remains unclear, but lowering intracellular concentrations of KCl as a result of activation of KCl efflux and/or an increase in cell volume may facilitate ICE activation and conversion of pro-IL-1β to its mature counterpart. Catalytic activity of ICE is inhibited by ionic conditions in excess of 50 mm (62), and within LPS-activated cells this enzyme is inactive (63). After initiation of IL-1β posttranslational processing with nigericin, however, active ICE is observed (64). In addition, ionic signals may regulate IL-1 export (65).

REFERENCES
1. Dinarello, C. A. (1991) Blood 77, 1627–1652
2. Dinarello, C. A., Cannon, J. G., Wolf, S. M., Bernheim, H. A., Beutler, B., Cerami, A., Figari, I. S., Palladino, M. A., and O'Connor, J. V. (1986) J. Exp. Med. 163, 1433–1450
3. Bevilacqua, M. P., Stengelin, S., Ginbrone, M. A. Jr., and Seed, B. (1989) Science 243, 1160–1164
4. Dayer, J. M., de Rochemontex, B., Burras, B., Denczuk, S., and Dinarello, C. A. (1986) J. Clin. Invest. 77, 645–648
5. Stephenson, M. L., Goldring, M. B., Birkhead, J. R., Drane, S. M., Rahmsdorf, J. J., and Angel, P. (1987) Biochem. Biophys. Res. Commun. 144, 538–540
6. Dodge, G. R., and Poole, R. (1989) J. Clin. Invest. 83, 647–661
7. Burchett, S. K., Weaver, W. M., Westall, J. A., Larsen, A., Kronheim, S., and Wilson, C. B. (1988) J. Immunol. 140, 3473–3481
8. Wevers, M. D., and Herryk, D. J. (1989) J. Immunol. 143, 1635–1641
9. Schindler, R., Geffland, J. A., and Dinarello, C. A. (1990) Blood 76, 1631–1638
10. Arend, W. P., Gordon, D. F., Wood, W. M., Janson, R. W., Joslin, F. G., and Janeel, S. (1989) J. Immunol. 143, 118–126
11. Herryk, D. J., Allen, J. N., Marsh, C. B., and Wevers, M. D. (1992) J. Immunol. 149, 3052–3058
12. Landis, R. C., Friedman, M. L., Fisher, R. I., and Ellis, T. M. (1991) J. Immunol. 146, 128–135
13. Hogquist, K. A., Nett, M. A., Unanue, E. R., and Chaplin, D. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8485–8489
14. Perreagaua, D., Barberia, J., Lanzetti, A. J., Geoghegan, K. F., Carty, T. J., and Gabel, C. A. (1992) J. Immunol. 149, 1294–1303
15. Chin, J., and Kostura, M. J. (1993) J. Immunol. 147, 1433–1450
16. Lilliberte, R., Perreagaua, D., Svensson, L., Paoles, C. J., and Gabel, C. A. (1984) J. Immunol. 133, 2169–2175
17. Hogquist, K. A., Unanue, E. R., and Chaplin, D. D. (1991) J. Immunol. 147, 2181–2186
18. Young, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993) Cell 75, 641–652
19. Miura, M., Zhu, H., Rotello, R., Hartwig, E. A., and Yuan, J. (1993) Cell 75, 653–660
20. Perreagaua, D., and Gabel, C. A. (1994) J. Biol. Chem. 269, 15195–15203
21. Valew, I., Reske, K., Palmer, M., Valeva, A., and Bhakdi, S. (1995) EMBO J. 14, 1607–1614
22. Laemmli, U. K. (1970) Nature 227, 680–685
23. Lewis, C. E., McCarthy, S. P., Lorenzen, J., and McGee, O. D. (1989) *Immunology* 69, 402–408
24. Wang, S.-Y., Mak, K. L., Chen, L. Y., Chou, M. P., and Ho, C. K. (1992) *Immunology* 77, 298–303
25. Blanchard, D. K., and Djeu, J. Y. (1991) *J. Leukocyte Biol.* 50, 28–34
26. Tsai, V., Firestein, G. S., Arend, W., and Zvaifler, N. J. (1992) *Cell. Immunol.* 144, 203–216
27. Hickman, S. E., Khoury, J. E., Greenberg, S., Schieren, I., and Silverstein, S. C. (1994) *Blood* 84, 2452–2456
28. Blanchard, D. K., McMillen, S., and Djeu, J. Y. (1991) *J. Immunol.* 147, 2579–2585
29. Falzoni, S., Munerati, M., Ferrari, D., Spisani, S., Moretti, S., and Di Virgilio, F. (1995) *J. Clin. Invest.* 95, 1207–1216
30. Hoffmann, E. K., and Simonsen, L. O. (1989) *Physiol. Rev.* 69, 315–382
31. Grinstein, S., Rothstein, B., Sarkadi, B., and Gelfand, E. W. (1984) *Am. J. Physiol.* 246, C204–C215
32. Lauf, P. K., Bauer, J., Adragna, N. C., Fujise, H., Zade-Oppen, A. M. M., Ryu, K. H., and Delpire, E. (1992) *Am. J. Physiol.* 263, C917–C932
33. Jennings, M. L., and Schulz, R. K. (1991) *J. Gen. Physiol.* 97, 799–818
34. Sarkadi, B., and Parker, J. C. (1991) *Biochim. Biophys. Acta* 1071, 407–427
35. Gallin, E. K., Mason, T. M., and Moran, A. (1994) *J. Cell Physiol.* 159, 573–581
36. Pressman, B. C. (1976) *Annu. Rev. Biochem.* 45, 501–530
37. Palfrey, H. C., and Leung, S. (1993) *Am. J. Physiol.* 264, C1270–C1277
38. Steinberg, T. H., Newman, A., Wasserman, J. A., and Silverstein, S. C. (1987) *J. Biol. Chem.* 262, 17713–17722
39. Dubyak, G. R., and El-Moatassim, C. (1993) *J. Physiol. (Lond.)* 465, 386–390
40. El-Moatassim, C., Dornand, J., and Mani, J.-C. (1992) *Biochim. Biophys. Acta* 1134, 31–45
41. McMillen, S., and Djeu, J. Y. (1991) *J. Membr. Biol.* 126, 27–37
42. Ayala, J. M., Yamin, T.-T., Egger, L. A., Chin, J., Kostura, M. J., and Miller, D. K. (1994) *J. Immunol.* 153, 2592–2599
43. Miller, B. E., Krasney, P. A., Gauvin, D. M., Holbrook, K. B., Koonz, D. J., Abruzzese, R. V., Miller, R. E., Pagani, K. A., Dolle, R. E., Ator, M. A., and Gilman, S. C. (1995) *J. Immunol.* 154, 1331–1338
44. Siders, W. M., Kimovitz, J. C., and Mizel, S. B. (1993) *J. Biol. Chem.* 268, 22150–22154