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Identification and Characterization of a Novel Class of Interleukin-1 Post-Translational Processing Inhibitors

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ABSTRACT

Lipopolysaccharide (LPS)-activated monocytes and macrophages produce large quantities of pro-interleukin (IL)-1β but externalize little mature cytokine. Efficient post-translational processing of the procytokine occurs in vitro when these cells encounter a secretion stimulus such as ATP, cytolytic T cells, or hypotonic stress. Each of these stimuli promotes rapid conversion of 31-kDa pro-IL-1β to its mature 17-kDa species and release of the 17-kDa cytokine. In this study, two novel pharmacological agents, CP-424,174 and CP-412,245, are identified as potent inhibitors of stimulus-coupled IL-1β post-translational processing. These agents, both diarylsulfonylureas, block formation of mature IL-1β without increasing the amount of procytokine that is released extracellularly, and they inhibit independently of the secretion stimulus used. Conditioned medium derived from LPS-activated/ATP-treated human monocytes maintained in the absence and presence of CP-424,174 contained comparable quantities of IL-6, tumor necrosis factor-α (TNFα), and IL-1RA, but 30-fold less IL-1β was generated in the test agent's presence. As a result of this decrease, monocyte conditioned medium prepared in the presence of CP-424,174 demonstrated a greatly diminished capacity to promote an IL-1-dependent response (induction of serum amyloid A synthesis by Hep3B cells). Oral administration of CP-424,174 to mice resulted in inhibition of IL-1 in the absence of an effect on IL-6 and TNFα. These novel agents, therefore, act as selective cytokine release inhibitors and define a new therapeutic approach for controlling IL-1 production in inflammatory diseases.

Interleukin (IL)-1 is a multipotential proinflammatory cytokine produced in abundance by activated monocytes and macrophages. Biological activity attributed to this cytokine is derived from two distinct polypeptides, IL-1α and IL-1β, that share <30% sequence identity (Dinarello, 1998). Despite their low sequence homology, both IL-1α and IL-1β bind to the same receptors on target cells and elicit comparable responses (Sims and Dower, 1994). Relative quantities of the two cytokine species produced in response to an activation stimulus are cell-dependent. For example, human monocytes produce large quantities of IL-1β in response to LPS challenge but little IL-1α (Demczuk et al., 1987). On the other hand, LPS-activated mouse peritoneal macrophages generate large quantities of both IL-1α and IL-1β (Perregaux and Gabel, 1998a). Molecular mechanisms that govern expression of the two cytokine species are not well understood.

Cells that produce IL-1 also closely regulate its post-translational processing. Both forms of IL-1 are synthesized as procytokines containing amino terminal propeptide extensions (Hazuda et al., 1991). In the case of pro-IL-1β, removal of the propeptide segment is necessary for biological activity; in contrast, pro-IL-1α is competent to bind to IL-1 receptors (Mosley et al., 1987; Hazuda et al., 1991). Proteolytic activation of pro-IL-1β is facilitated by caspase-1, the founding member of a family of cytoplasmically disposed cysteine proteases involved in apoptotic processes (Cerretti et al., 1992; Thornberry et al., 1992). Importantly, macrophages isolated from mice engineered to lack caspase-1 are impaired in the generation of mature, active IL-1β (Kuida et al., 1995; Li et al., 1995). Likewise, inhibitors of caspase-1 prevent proteolytic activation of pro-IL-1β, and these agents suppress inflammatory processes in vivo (Thornberry et al., 1992; Miller et al., 1995; Ku et al., 1996). Caspase-1 also is required for proteolytic activation of pro-IL-18 (Yong et al., 1997). Pro-IL-1α, on the other hand, can be cleaved to a 17-kDa mature species, but this processing proceeds independently of caspase-1 (Carruth et al., 1991).

Unlike most secreted cytokines, which are synthesized in the rough endoplasmic reticulum and processed in the Golgi apparatus during their transport to the cell surface, IL-1 (both α and β) appears to be synthesized on free polysomes within the cytosol. This atypical localization results from the absence of signal peptides on the precursor polypeptides that are required for entry into the endoplasmic reticulum

ABBREVIATIONS: IL, interleukin; LPS, lipopolysaccharide; CRID, cytokine release inhibitory drug; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethylsulfonyl fluoride; CTL, cytotoxic T lymphocyte; SFM, serum-free medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; LDH, lactate dehydrogenase; SAA, serum amyloid A.

(Rubartelli et al., 1990). Caspase-1 also is produced as an inactive precursor and appears to coexist with pro-IL-1 within the cytosolic compartment of LPS-activated monocytes (Ayala et al., 1994; Miossec et al., 1996). Mechanisms controlling caspase-1 activation and, in turn, proteolytic cleavage of pro-IL-1β remain to be delineated. Stimuli that initiate synthesis of pro-IL-1β are not necessarily sufficient to promote IL-1 post-translational processing. Thus, LPS promotes synthesis of large quantities of pro-IL-1β in both monocytes and macrophages, but few of the newly synthesized polypeptides are externalized as mature active molecules (Hogquist et al., 1991a; Chin and Kostura, 1993; Perregaux et al., 1994). Efficient post-translational processing requires that LPS-activated cells encounter a secondary stimulus such as ATP (Hogquist et al., 1991b; Perregaux and Gabel, 1994), nigericin (Perregaux and Gabel, 1994), cytolytic T cells (Hogquist et al., 1991b), or bacterial toxins (Walev et al., 1996). All of these secondary effectors promote major changes to the ionic composition of the IL-1-producing cell, and these changes appear necessary for cytokine post-translational processing (Perregaux and Gabel, 1994; Walev et al., 1995; Perregaux et al., 1996). Moreover, the aforementioned secretion stimuli promote cell death, suggesting that formation and release of active IL-1 represent terminal cellular processes (Hogquist et al., 1991b; Perregaux and Gabel, 1994). Ultimate sacrifice of the cytokine-producing cell may ensure that a mediator with the potency and scope of IL-1 is externalized only under situations of great stress to an organism.

In this study we identify a novel series of agents that inhibit stimulus-coupled IL-1 post-translational processing. These agents act independently of the type of stimulus used to activate monocytes and macrophages, and they arrest the cytokine-producing cell such that pro-IL-1β is neither cleaved to its mature species nor released extracellularly. As a result of this arrest, conditioned medium derived from LPS-activated human monocytes is greatly attenuated in terms of its ability to generate an IL-1-dependent signaling response after application to target cells. The effectiveness of these novel agents at suppressing IL-1 post-translational processing and a monocyte's signaling capacity demonstrate that control of IL-1 production in inflammatory disorders such as rheumatoid arthritis may be achieved with the use of cytokine release inhibitory drugs (CRIDs).

Materials and Methods

Human Monocyte Isolation. Blood collected from normal volunteers in the presence of heparin was fractionated using lymphocyte separation medium obtained from Organon Technica (Westchester, PA). The region of the resulting gradient containing banded mononuclear cells was harvested, diluted with 10 ml of maintenance medium (RPMI 1640, 5% FBS, 25 mM HEPES, pH 7.2, 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, 1×10^7 mononuclear cells were added to each well of six-well multidishes in a total volume of 2 ml of maintenance medium. Alternatively, in experiments where IL-1β production was measured by ELISA, 2×10^5 mononuclear cells were seeded into each well of 96-well plates in a total volume of 0.1 ml. Monocytes were allowed to adhere for 2 h, after which the supernatants were discarded and the attached cells were rinsed twice and then incubated in maintenance medium overnight at 37°C in a 5% CO_2 environment.

ATP-Induced IL-1β Post-Translational Processing. In the ELISA format, cultured monocytes in 96-well plates were activated with 10 ng/ml LPS (*Escherichia coli* serotype 055:B5; Sigma, St. Louis, MO). After a 2-h incubation, the activation medium was removed, the cells were rinsed twice with 0.1 ml of chase medium (RPMI 1640, 1% FBS, 20 mM HEPES, 5 mM $NaHCO_3$, pH 6.9), and then 0.1 ml of chase medium containing a test agent was added and the plate was incubated for 30 min; each test agent concentration was evaluated in triplicate wells. ATP was introduced (from a 100 mM stock solution, pH 7) to achieve a final concentration of 2 mM, and the plate was incubated at 37°C for an additional 3 h. Media were harvested and clarified by centrifugation, and their IL-1β content was determined by ELISA (R & D Systems; Minneapolis, MN).

In the metabolic format, cultured monocytes were incubated with 10 ng/ml LPS for 2 h and then labeled for 60 min in 1 ml of methionine-free RPMI 1640, containing 1% dialyzed FBS, 25 mM HEPES, pH 7.2, and 83 μCi/ml [^{35}S]methionine (Amersham Pharmacia Biotech, Arlington Heights, IL; 1000 Ci/mmol). The pulse medium subsequently was discarded, the radiolabeled cells were rinsed once with 2 ml of chase medium, and then 1 ml of chase medium, with or without a test agent, was added to each well. Where indicated, ATP was added (from a 100 mM stock solution, pH 7) to achieve a final concentration of 2 mM. Radiolabeled monocytes were treated with ATP at 37°C for various times after which the medium was recovered and clarified by centrifugation; the resulting supernatants were harvested and adjusted to 1% in Triton X-100, 0.1 mM PMSF, 1 mM iodoacetic acid, 1 μg/ml pepstatin, and 1 μg/ml leupeptin by addition of concentrated stock solutions of these reagents. Adherent monocytes 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 PMSF, 1 mM iodoacetic acid, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 mg/ml ovalbumin; 50 μl of this extraction buffer also was added to the pellets obtained after clarification of the media supernatants, and these samples were combined with their corresponding cell extracts. 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 by using a TLA 45 rotor (Beckman Instruments, Palo Alto, CA).

Hypotonic Stress-Induced IL-1β Post-Translational Processing. Human monocytes were isolated as described above and used on the day of their isolation. LPS stimulated/[^{35}S]methionine-labeled cells were maintained in an isotonic (132 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.6 mM KCl, 1.4 mM KH_2PO_4, 20 mM HEPES, pH 7.1, 5 mM glucose, and 1% FBS) or hypotonic (27 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.53 mM KCl, 0.29 mM KH_2PO_4, 20 mM HEPES, pH 7.1, 5 mM glucose, and 1% FBS) medium in the absence or presence of 2 μM CP-412,245. After the indicated incubation times at 37°C, media and cell-associated samples were harvested separately and processed as described above.

Preparation of Cytolytic T Lymphocytes (CTLs) and Coculture with [^{35}S]Methionine-Labeled Macrophages. Spleens from C57/Bl mice (Jackson Laboratories, Bar Harbor, MN) were suspended in RPMI 1640 medium and minced through a metal strainer. The resulting cell suspension was passed through a filter of sterile Nitex (110 μm; Tetko Inc., Briarcliff Manor, NY), after which cells were collected by centrifugation and resuspended in RPMI medium. Spleens from BALB/c mice (Jackson Laboratories; three spleens in 5 ml of RPMI) were minced through a metal strainer after dilution with 5 ml of 155 mM ammonium chloride, 1 mM EDTA, 10 mM potassium bicarbonate, pH 7.2. The resulting cell suspension was passed through a Nitex filter, cells were collected by centrifugation, and the cell pellet was suspended in 2 ml of RPMI 1640. This BALB/c spleen cell suspension was irradiated for 23 min at 80 kV and 5 mA. To elicit a mixed lymphocyte response, T-75 flasks were seeded with 2.5×10^6 C57/Bl spleen cells/ml and 0.5×10^6 irradiated BALB/c cells/ml; each T-75 flask received 50 ml total volume of RPMI 1640 medium containing 10% fetal bovine serum, 1% nonessential amino

acids, 1% penicillin/streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 0.01 mM β-mercaptoethanol. After 5 days at 37°C in a 5% CO_2 environment, cells were harvested by centrifugation and washed twice with RPMI 1640, 1% FBS, 25 mM HEPES, pH 7.3. Prior to their addition to macrophage target cells, an appropriate number of cells from the activated CTL preparation was collected by centrifugation and suspended in 0.5 ml of RPMI 1640 containing 25 mM HEPES, pH 7.3, 1 μg/ml LPS, 1% FBS, and test agent, where indicated.

Resident macrophages were isolated from BALB/c mice by peritoneal lavage and 1×10^6 cells were plated into each well of Natrix-coated six-well multidishes (Collaborative Research, Bedford, MA). After 2 h of adherence, the medium was removed and replaced with 2 ml of RPMI 1640 containing 5% FBS, and the cells were incubated overnight at 37°C. Fresh maintenance medium (2 ml) containing 1 μg/ml LPS was added to each well, the macrophages were stimulated for 75 min, and they then were pulse-labeled for 60 min in 1 ml of methionine-free α-minimal essential medium containing 1% dialyzed FBS, 25 mM HEPES, 5 mM $NaHCO_3$, pH 7.3, and 83 μCi/ml [^{35}S]methionine. Pulse media were removed and the cells were rinsed once with RPMI 1640, 25 mM HEPES, pH 7.3, 1 μg/ml LPS, and 1% FBS. At this point, 1 ml of the CTL cell suspension (with or without a test agent) was added to achieve a ratio of spleen cells to macrophages of 20:1. Cocultures were incubated at 37°C for 4 h after which cells and media were harvested separately.

Immunoprecipitation of IL-1β and Analysis of Radiolabeled Cytokine Product. IL-1β was immunoprecipitated from detergent extracts of cell and media samples by addition of 3 μl of a rabbit anti-human IL-1β serum (Collaborative Research) or 3 μl of goat anti-mouse IL-1β (Perregaux et al., 1998a). After a 2-h incubation at 4°C, 0.25 ml of a 10% suspension of Protein A- (human) or Protein G-Sepharose (mouse) was added and the resulting immune complexes were recovered by centrifugation. The bead-bound complexes were washed five times with 10 mM Tris, pH 8, 10 mM EDTA, 1% Triton X-100, 0.4% deoxycholate, 0.1% SDS and once with 50 mM Tris, pH 6.8. The final pellets were suspended in 0.1 ml of SDS disaggregation buffer and boiled for 3 min; beads were removed by centrifugation, and the disaggregated immunoprecipitate supernatants were stored at −20°C prior to analysis by SDS gel electrophoresis and autoradiography. Gels were soaked in Amplify (Amersham Pharmacia Biotech) prior to drying. Quantitation of the amount of radioactivity associated with the various species of IL-1β was determined with an Ambis Image Analysis System (San Diego, CA).

Isolation of Human Monocyte Conditioned Medium and Hepatocyte Bioassay. Mononuclear cells isolated from heparinized blood obtained from an individual normal volunteer were seeded into tissue culture dishes and maintained in macrophage serum-free medium (SFM; Invitrogen, Carlsbad, CA). After a 2-h incubation, nonadherent cells were removed, the attached monocytes were rinsed twice with SFM, and the cultures were incubated overnight in SFM containing 100 ng/ml recombinant human macrophage colony-stimulating factor (R & D Systems). This medium subsequently was discarded and replaced with RPMI 1640 medium containing 1% FBS, 25 mM HEPES, pH 6.9, and 10 ng/ml LPS, and the cultures were incubated for 3 h at 37°C. In some cases, CP-424,174 was added to the culture medium at the time of LPS addition, but in others the test agent was coadministered with the ATP secretion stimulus. CP-424,174 was introduced from a dimethyl sulfoxide stock solution to achieve the desired final concentration; in all cases, the final dimethyl sulfoxide vehicle concentration was 0.2% and the control cultures received vehicle alone. ATP (from a 100 mM stock solution previously adjusted to pH 7) was introduced to achieve a 2 mM final concentration, and the cultures were incubated for an additional 3 h. Conditioned media subsequently were harvested and clarified by centrifugation.

The Hep3B bioassay was performed as detailed previously (Laliberte et al., 1997). Briefly, Hep3B cells (2×10^5/well) were seeded into six-well cluster dishes and maintained overnight in RPMI 1640 medium containing 10% FBS. Maintenance media then were replaced with RPMI 1640 containing 10% human AB serum (Invitrogen), 1 μM dexamethasone, 0.1% Redu Serum (Upstate Biotechnology, Lake Placid, NY), 20 mM HEPES, pH 7, and, where indicated, 20% monocyte conditioned medium and/or effector cytokines. Recombinant human cytokines were used at the following concentrations: IL-6, 50 ng/ml (Collaborative Research); IL-1β, 10 ng/ml (Collaborative Research); and IL-1RA, 10 μg/ml (R & D Systems). Hepatoma cell cultures were stimulated for 20 h after which the activation media were removed and the cells were incubated in 1 ml of methionine-free RPMI 1640 medium containing 1% penicillin/streptomycin, 20 mM HEPES, pH 7.3, 1 μM dexamethasone, 0.1% Redu Serum, and 160 μCi of [^{35}S]methionine. After a 60-min incubation at 37°C, the pulse-medium was discarded, the cell monolayers were rinsed to remove free [^{35}S]methionine, and the cells were solubilized by detergent extraction. Cell extracts were clarified by centrifugation, and serum amyloid A was recovered from the resulting supernatants by immunoprecipitation; a sheep antiserum was obtained from Calbiochem (San Diego, CA). Resulting immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (15% polyacrylamide) and autoradiography.

In Vivo LPS/ATP Challenge Assay. Male Swiss-Webster mice, 6 to 10 weeks of age, were obtained from Taconic Farms (Germantown, NY). Mice were maintained for 1 week before use in a temperature-controlled room with a 12-h light/dark cycle and were allowed free access to standard laboratory chow and water. All procedures were approved by the Institutional Animal Care and Use Committee. Mice were injected with 1 μg of LPS (*E. coli* 055:B5; Sigma) in 0.5 ml of PBS. Sixty minutes later, the mice were dosed with test agent (as a 0.5% suspension in methyl cellulose) or vehicle by gavage, followed 60 min later by an i.p. injection of ATP (0.5 ml of a 30 mM solution in PBS neutralized to pH 7.3 with NaOH). After an additional 15-min incubation, the mice were euthanized by cervical dislocation, and the peritoneal cavity was lavaged with 3 ml of ice-cold PBS containing 10 U/ml heparin sodium salt (ICN Biochemicals, Cleveland, OH), 0.25 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM EDTA. Samples were maintained on ice prior to clarification by centrifugation; the resulting supernatants were harvested and stored at −20°C. ELISA kits for the measurement of murine cytokines were obtained from Genzyme (Cambridge, MA; IL-1β) and Endogen (Boston, MA; IL-1α, IL-6, and TNFα).

Other Procedures. Trichloroacetic acid (TCA) precipitation analysis was performed by spotting duplicate aliquots of media samples and cell extracts onto 1-cm glass fiber filter circles. One of these filters was air-dried to yield the total radioactivity. The duplicate filters were placed into individual wells of six-well cluster plates containing 2 ml of cold 5% TCA and incubated for 30 min at 4°C with gentle agitation. Solutions were removed by aspiration and the filters were washed twice with 2 ml of 5% TCA, twice with 95% ethanol, and once with absolute ethanol (all at 4°C, 30 min/wash). These filters then were air-dried, after which all filters were placed into 4 ml of scintillation fluid for radioactivity counting. The difference in the quantity of radioactivity recovered from the filters with and without TCA precipitation is attributed to TCA-soluble components. Aliquots of media samples and cell extracts were assessed for lactate dehydrogenase (LDH) content by using pyruvate as substrate and a colorometric pyruvate detection assay (Sigma).

Results

Identification of Diarylsulfonylureas as Inhibitors of IL-1 Post-Translational Processing. Based on evidence obtained in previous studies indicating that ionic changes facilitate IL-1 post-translational processing (Perregaux and Gabel, 1994; Walev et al., 1995; Perregaux et al., 1996), a number of pharmacological agents known to block ion channel and/or ion transport activity were assessed as inhibitors

of human monocyte stimulus-coupled IL-1β post-translational processing. This assessment was performed in a two-step assay: blood-derived monocytes were activated with LPS to promote synthesis of pro-IL-1β after which they were treated with ATP to initiate cytokine post-translational processing in the absence or presence of a test agent. Concentrations of IL-1β within media recovered from these cultures then were determined by ELISA. Most agents tested were inactive but glyburide, a sulfonylurea-containing drug that is known to inhibit ATP-activated K^+ channels found in pancreatic β-cells (Ashcroft and Ashcroft, 1992), blocked IL-1β production in a dose-dependent manner (Fig. 1); the IC_{50} for this agent was determined to be 12 μM ($\pm$5 μM; n = 23). In contrast, the closely related sulfonylurea glipizide did not produce significant inhibition of ATP-induced IL-1β post-translational processing at concentrations $\leq$100 μM (Fig. 1). This selectivity led us to characterize a number of structurally related analogs in an attempt to find agents more potent than glyburide. A class of compounds designated as diarylsulfonylureas demonstrated improved activity in the cytokine production assay, and examples of two such compounds are shown in Fig. 2. These two agents, CP-424,174 and CP-412,245, blocked ATP-induced IL-1β post-translational processing with IC_{50} values of 0.21 μM ($\pm$0.06 μM; n = 20) and 0.26 μM ($\pm$0.05 μM; n = 4), respectively (Fig. 1). Not all diarylsulfonylureas were effective cytokine release inhibitors; the anticancer compound sulofenur (Talbot et al., 1993), for example, produced only modest inhibition when tested at concentrations $\leq$50 μM (Fig. 1).

An inhibitor of the stress-activated kinase p38α, SKF86002, is reported to block the low spontaneous release of IL-1β from human monocytes challenged with LPS only (Chin and Kostura, 1993). This agent did not affect ATP-induced IL-1β post-translational processing (data not shown), suggesting that diarylsulfonylureas disrupt a mechanism distinct from that affected by the stress kinase inhibitor.

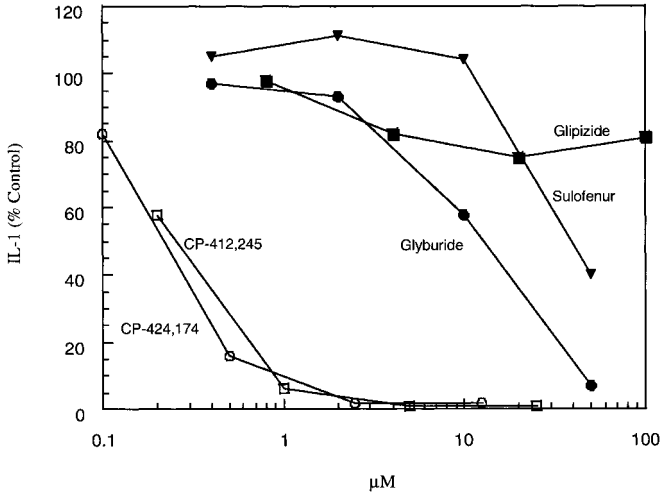

Fig. 1. Identification of inhibitors of stimulus-coupled IL-1β post-translational processing. LPS-activated human monocytes were incubated for 15 min with the indicated concentration of test agent after which 2 mM ATP was introduced into all culture media and cytokine processing was allowed to proceed for 3 h. At the end of this incubation, the quantity of IL-1β released into the medium was determined by ELISA. The amount of cytokine recovered, expressed as a percentage of that observed in the absence of test agent, is indicated as a function of test agent concentration. Each data point is an average of triplicate determinations within a single experiment.

Demonstration of IL-1β Post-Translational Processing Inhibition in a Metabolic Assay Format. To confirm CRID activity, LPS-activated/[^{35}S]methionine-labeled human monocytes were treated with ATP in the absence and presence of CP-424,174. IL-1β subsequently was recovered by immunoprecipitation from the media and cell-associated fractions, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography. ATP-treated cultures yielded large quantities of extracellular mature 17-kDa IL-1β and smaller quantities of 31-kDa pro-IL-1β (Fig. 3B). In contrast, cell-associated cytokine recovered from ATP-treated cells consisted almost exclusively of the procytokine (Fig. 3A). Addition of CP-424,174 to the medium led to loss of extracellular 17-kDa mature IL-1β (Fig. 3B). At 100 nM, little inhibition was observed, but 500 nM and 2.5 μM CP-424,174 inhibited production of the 17-kDa species nearly completely (Fig. 3C). Importantly, this decrease in the levels of extracellular 17-kDa cytokine was not compensated by an increase in the quantity of the 31-kDa species released (Fig. 3B), nor by an appearance of the 17-kDa species intracellularly (Fig. 3A). CP-424,174, therefore, inhibited both the post-translational proteolytic maturation of pro-IL-1β and the release of all cytokine species. ATP also promotes release of IL-1α from LPS-primed peritoneal macrophages (Perregaux and Gabel, 1998a), and externalization of this form of IL-1 was sensitive to inhibition by CP-424,174 (data not shown).

To ensure that the inhibition of cytokine post-translational processing was not the result of an irreversible toxic effect, cultures of LPS-activated/[^{35}S]methionine-labeled monocytes were treated for 15 min with 2.5 μM CP-424,174 after which the test agent was removed and the cells were treated with ATP to initiate cytokine post-translational processing. Monocytes pretreated with CP-424,174 yielded 68% as much 17-kDa IL-1β extracellularly as did cells that were not exposed to the test agent (Fig. 3, B and C). Addition of CP-424,174 to the monocyte cultures 15 min after initiation of the ATP response resulted in 60% inhibition of mature IL-1β production (Fig. 3, B and C).

Similar inhibitory effects were observed with CP-412,245. LPS-activated/[^{35}S]methionine-labeled monocytes exposed to ATP in the presence of this agent produced less 17-kDa mature IL-1β than did control ATP-treated cultures (Fig. 4A). At 250 nM, this agent produced 44% inhibition, and concentrations $\geq$1 μM inhibited mature cytokine formation $>$95% (Fig. 4B). The cytokine release inhibitory effect again was reversible, and monocytes that were pretreated with 4 μM CP-412,245 for 15 min and then exposed to ATP in its absence generated levels of 17-kDa IL-1β comparable with those produced by the control cells (Fig. 4A). Addition of CP-412,245 to the monocyte cultures 15 min after ATP resulted in 50% inhibition in mature cytokine production (Fig. 4, A and B).

Diarylsulfonylureas Prevent Murine Peritoneal Macrophage IL-1β Post-Translational Processing in Response to Allogeneic CTL Challenge. LPS-activated murine peritoneal macrophages release mature IL-1β when challenged with allogeneic CTLs in vitro (Hogquist et al., 1991b). To determine whether IL-1β post-translational processing induced by this type of cellular stimulus was CRID-

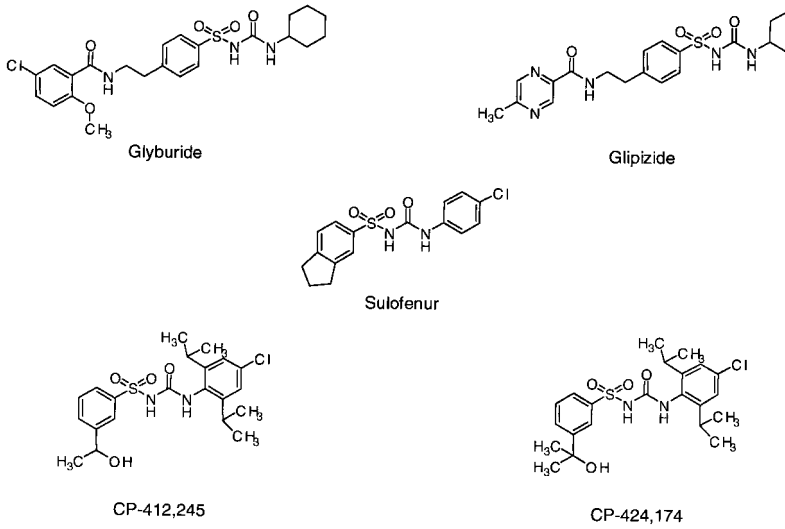

Fig. 2. Structures of pharmacological agents.

sensitive, LPS-activated/[^{35}S]methionine-labeled BALB/c peritoneal macrophages were cocultured with an effector CTL preparation (derived from C57 mice previously sensitized to irradiated BALB/c spleen cells in a mixed lymphocyte reaction) in the absence and presence of CP-424,174. In the absence of CTLs, radiolabeled macrophages released no cytokine extracellularly (Fig. 5). Addition of CTLs promoted formation and release of 17-kDa IL-1β (Fig. 5A); >80% of the radiolabeled IL-1β recovered after 3 h of coculture was recovered extracellularly as the 17-kDa species. When CP-424,174 was added to the cocultures, on the other hand, a concentration-dependent decrease in 17-kDa cytokine formation was observed (Fig. 5). CP-424,174 (1 μM) inhibited CTL-induced post-translational processing by 86%, with less inhibition observed at lower concentrations (Fig. 5B). CP-424,174-treated macrophages did not accumulate 17-kDa mature cytokine intracellularly (data not shown).

CP-424,174 Prevents Human Monocyte IL-1β Post-Translational Processing in Response to Hypotonic Stress. Hypotonic stress is an effective stimulus for promoting IL-1β post-translational processing from human monocytes (Walev et al., 1995; Perregaux et al., 1996), and CRIDs also impaired this type of stimulus-coupled processing. LPS-activated/[^{35}S]methionine-labeled monocytes subjected to hypotonic stress in the absence of CP-412,245 demonstrated a time-dependent release of mature 17-kDa IL-1β. After just 15 min of hypotonic exposure, 17-kDa IL-1β was detected extracellularly, and quantities of this species increased after 30 and 60 min of treatment. Extending the treatment time to 120 and 180 min produced little additional mature cytokine (Fig. 6). Based on recovery of total radiolabeled IL-1β from these cultures (sum of both intracellular and extracellular species and corrected for the 2-fold loss of [^{35}S]methionine as a result of caspase-1 cleavage), >80% was accounted for by the extracellular 17-kDa species after 60 min of treatment. Addition of CP-412,245 to the hypotonic medium effectively inhibited cytokine post-translational processing (Fig. 6A), and quantities of extracellular 17-kDa IL-1β recovered in the presence of this agent were minimal throughout the entire 3-h observation period (Fig. 6B). Additionally, CP-412,245-treated monocytes did not release pro-IL-1β (Fig. 6A). Hypotonic stress also promoted release of LDH; the time course for release of the cytoplasmic marker correlated with release of mature 17-kDa IL-1β (Fig. 6B). In the presence of CP-412,245, release of LDH was greatly attenuated (Fig. 6B).

To further characterize the hypotonic response, distribution of total [^{35}S]methionine-labeled radioactivity was examined. Cell-associated and media samples recovered after hypotonic stress were subjected to TCA precipitation; the quantity of both TCA-precipitable and TCA-soluble radiolabeled components was assessed (Fig. 7). As expected, prior to hypotonic stress (time 0) a large quantity of TCA-precipitable macromolecules was recovered from the cell-associated fraction and only a small quantity was recovered from the medium. In the absence of CP-412,245, levels of extracellular TCA-precipitable counts increased in a time-dependent manner as a result of hypotonic stress (Fig. 7B); a corresponding decline in the cell-associated species was observed (Fig. 7A). Monocytes maintained for 3 h in an isotonic medium released TCA-precipitable components (relative to the time 0 culture) but the quantities were reduced relative to those released by cultures subjected to hypotonic stress (Fig. 7B). TCA-soluble radiolabeled components also were released by hypotonically stressed monocytes in quantities greater than those released by cells maintained in isotonic medium (Fig. 7D). CP-412,245 prevented the hypotonic-induced release of TCA-precipitable components, and after 3 h of treatment levels of extracellular TCA-precipitable macromolecules generated in the presence of the test agent were comparable with those produced by monocytes maintained in isotonic medium (Fig. 7B). In contrast, CP-412,245 had a minimal effect on release of TCA-soluble radiolabeled components (Fig. 7D).

Exposure of LPS-activated monocytes to hypotonic medium caused many cells to demonstrate a large volume increase and cytoplasmic clearing (Fig. 8); similar changes to macrophage morphology have been observed after initiation of IL-1 processing with ATP or nigericin (Perregaux and Gabel, 1994). This change in morphology was time-dependent; few cells demonstrated the swollen state after 15 min of hypotonic treatment but many swollen cells were detected after 30 min (data not shown). The morphology change, therefore, coincided temporally with the appearance of mature IL-1β. After 180 min of hypotonic exposure many, but not all, monocytes possessed a swollen appearance (Fig. 8B). The morphology of monocytes maintained for 3 h in an isotonic medium, on the other hand, was similar to that dis-

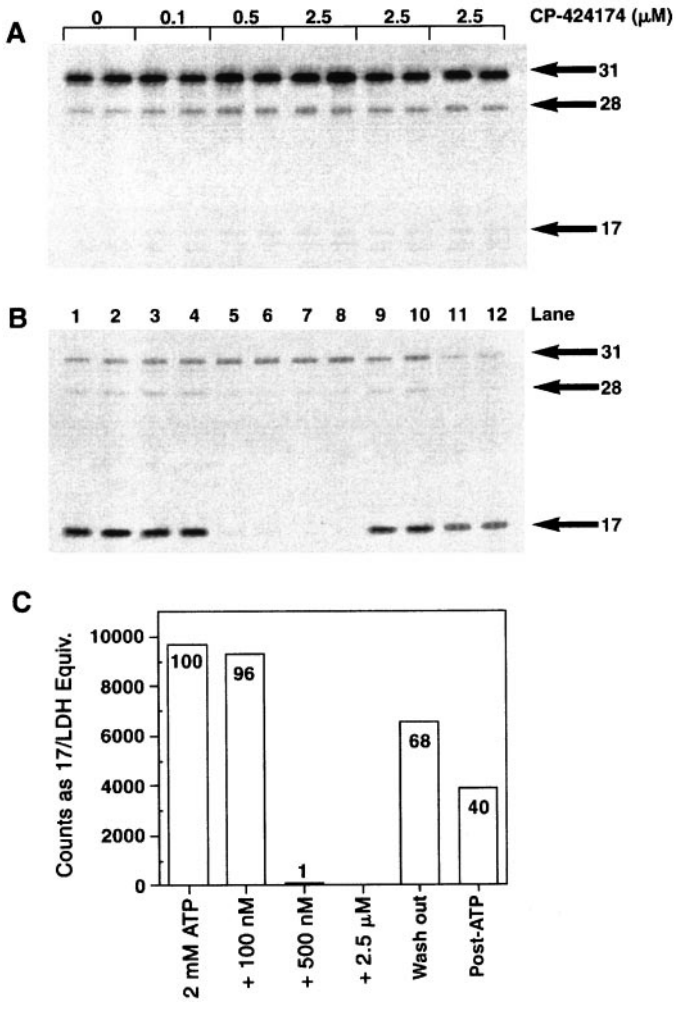

Fig. 3. CP-424,174 inhibits ATP-induced 17-kDa IL-1β production and cytokine externalization. LPS-activated/[^{35}S]methionine-labeled human monocytes were incubated with the indicated concentration of CP-424,174 for 15 min after which 2 mM ATP was introduced to the medium to promote cytokine post-translational processing. Where indicated, the preincubation medium (containing 2.5 μM CP-424,174) was replaced with test-agent-free medium prior to the introduction of ATP (washout), or cultures that had not been pretreated with test agent received 2.5 μM CP-424,174 15 min after ATP addition (post-ATP). Media and cells were separated after the ATP treatment and IL-1β was recovered from each by immunoprecipitation; the immunoprecipitates were analyzed by SDS-PAGE and autoradiograms for the cell-associated (A) and media (B) samples are shown. Arrows on the right indicate the migration positions of the 31-, 28-, and 17-kDa species of human IL-1β. The amount of radioactivity associated with the extracellular 17-kDa IL-1β species is indicated as a function of treatment (C); each column is the average of duplicate determinations normalized to total culture-associated LDH content. Numbers within the columns indicate the percentage of relative to the control (ATP only).

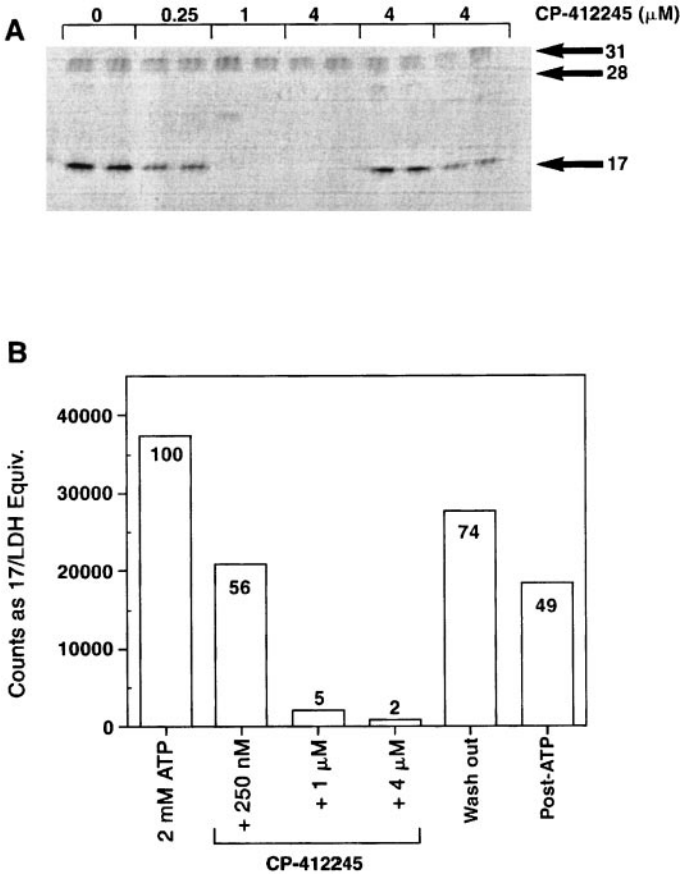

Fig. 4. CP-412,245 inhibits ATP-induced 17-kDa IL-1β production and cytokine externalization. LPS-activated/[^{35}S]methionine-labeled human monocytes were incubated with the indicated concentration of CP-412,245 for 15 min after which 2 mM ATP was introduced to the medium to promote cytokine post-translational processing. Where indicated, the preincubation medium (containing 4 μM CP-412,245) was replaced with test-agent-free medium prior to the introduction of ATP (washout), or cultures that had not been pretreated with test agent received 4 μM CP-424,174 15 min after ATP addition (post-ATP). Media and cells were separated after the ATP treatment and IL-1β was recovered from each by immunoprecipitation; the immunoprecipitates were analyzed by SDS-PAGE and the autoradiogram of the media samples is shown (A). Arrows on the right indicate the migration positions of the 31-, 28-, and 17-kDa species of IL-1β. The amount of radioactivity associated with the extracellular 17-kDa IL-1β species is indicated as a function of treatment (B); each column is the average of duplicate determinations normalized to the total culture-associated LDH content.

played by cells at the beginning of the treatment (Fig. 8, A and D). Cultures exposed to the hypotonic medium in the presence of 2 μM CP-412,245 contained few swollen cells but evidence of cytoplasmic clearing was apparent in some cells (Fig. 8C).

Conditioned Medium Derived from CP-424,174-Treated Monocytes Demonstrates Diminished Signaling Capacity. Conditioned medium harvested from human monocytes treated with LPS is a rich source of many cytokine products, including IL-1 (McNiff et al., 1995). To assess selectivity of the CRID effect, human monocytes were activated with LPS after which they were treated with ATP in the absence and presence of CP-424,174 and levels of three different cytokine products, IL-1β, IL-6, and the natural receptor antagonist of IL-1, IL-1RA (Arend et al., 1998), released to the medium were determined by ELISA. We demonstrated previously that the amount of IL-1β released into monocyte conditioned medium was greatly enhanced in the presence of ATP, whereas levels of IL-6 and IL-1RA were not significantly affected by the nucleotide triphosphate (Laliberte et al., 1997). Conditioned medium prepared in the absence and presence of CP-424,174 contained comparable quantities of IL-6 and IL-1RA (Table 1, experiment 1). IL-6 was present at 26 ng/ml in the absence of CP-424,174 and at 19 ng/ml in its presence; therefore, a 1.4-fold reduction occurred in the presence of the test agent. Likewise, IL-1RA levels were 128 and 137 ng/ml in the absence and presence of CP-424,174, respectively. In contrast, the quantity of IL-1β was reduced 30-fold

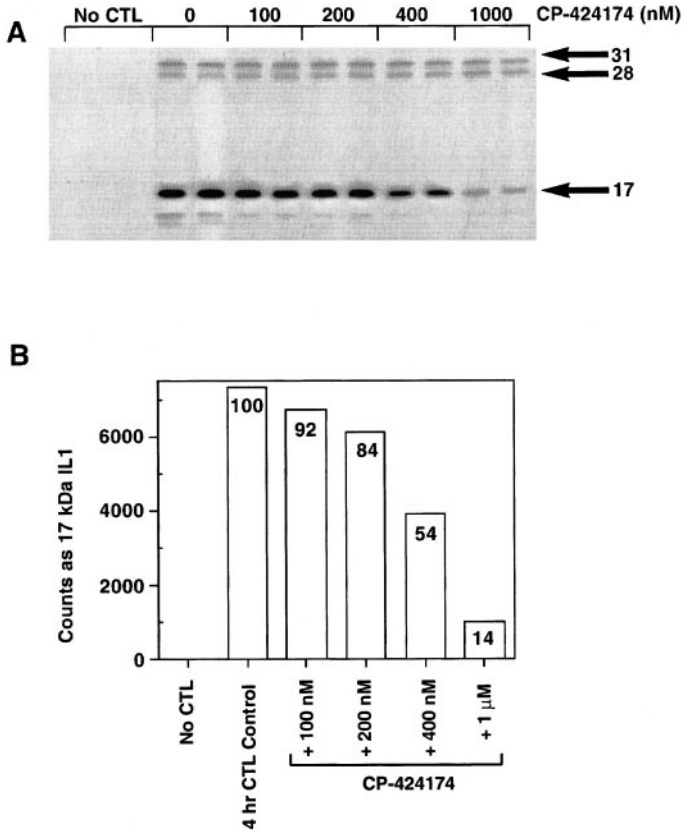

Fig. 5. CP-424,174 inhibits CTL-induced IL-1β post-translational processing from mouse peritoneal macrophages. LPS-activated/[^{35}S]methionine-labeled mouse peritoneal macrophages were cocultured in the absence (no CTL) or presence of allogeneic CTLs and the indicated concentration of CP-424,174. After 4 h of coculture, media were harvested and IL-1β was recovered by immunoprecipitation; these immunoprecipitates were analyzed by SDS-PAGE and an autoradiogram of the gel is indicated (A). Arrows denote the migration positions of 35-, 28-, and 17-kDa murine IL-1β species. The amount of radioactivity associated with the extracellular 17-kDa IL-1β species is indicated as a function of treatment (B); each column is the average of duplicate determinations.

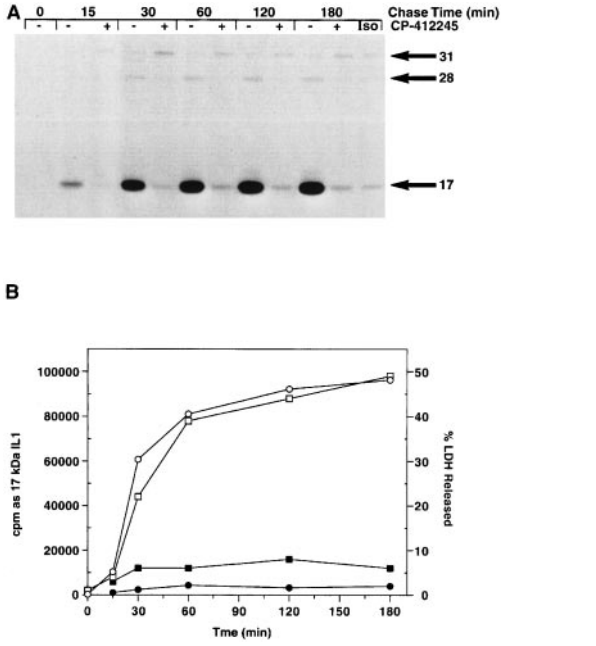

Fig. 6. Hypotonic stress-induced IL-1β post-translational processing is blocked by CP-412,245. Cultures of LPS-activated/[^{35}S]methionine-labeled human monocytes received either isotonic (lanes 1 and 12) or hypotonic medium (lanes 2–11) containing, where indicated, 2 μM CP-412,245. Individual cultures were harvested after the indicated times of hypotonic stress and IL-1β released into the medium was recovered by immunoprecipitation. The resulting immunoprecipitates were analyzed by SDS-PAGE and an autoradiogram is indicated (A). The amount of radioactivity associated with the extracellular 17-kDa species is indicated as a function of the time of hypotonic exposure (circles; B). Also shown is the percentage of total culture associated (sum of cell and medium) LDH released into the medium (squares). Solid symbols correspond to cultures incubated in the presence of test agent and open symbols represent those maintained in the absence of 2 μM CP-412,245.

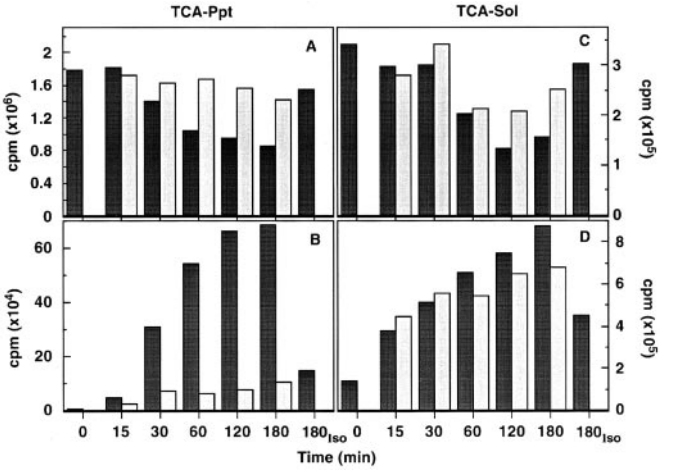

Fig. 7. Hypotonic stress promotes release of TCA-precipitable macromolecules via a CP-412,245-sensitive process. Samples of the detergent solubilized cell-associated (A and C) and media (B and D) fractions derived from the cultures described in Fig. 6 were subjected to TCA precipitation analysis. Quantities of TCA-precipitable (TCA-ppt; A and B) and TCA-soluble (TCA-sol; C and D) radioactivity are indicated as a function of the time of hypotonic stress; samples derived from cultures incubated for 180 min in an isotonic medium are indicated as 180_{ISO}. Solid columns correspond to cultures incubated in the absence of test agent, and open columns represent those maintained in the presence of 2 μM CP-412,245.

in the presence of CP-424,174 (Table 1, experiment 1). In a second experiment, CP-424,174 was introduced to the cultures simultaneously with LPS and maintained throughout the entire LPS and ATP treatment arms. Relative to control cultures, CP-424,174 caused a concentration-dependent inhibition of IL-1β production and achieved a maximal suppression of >99% (Table 1, experiment 2). In contrast, CP-424,174 produced only modest reductions in the secreted levels of IL-1RA and TNFα (Table 1, experiment 2); the latter cytokine is produced as a membrane-anchored precursor in the endoplasmic reticulum and is subsequently released by proteolysis to the medium. CP-424,174 reduced production of IL-6 by less than 2-fold (Table 1, experiment 2). Thus, CP-424,174 selectively impaired production of IL-1β by LPS-activated/ATP-treated monocytes.

To demonstrate that the observed reduction in IL-1β was biologically significant, the signaling capacity of the monocyte conditioned media prepared in the absence and presence of CP-42,4174 was compared. For this purpose, a bioassay was used; Hep3B cells produce the acute phase protein SAA in response to stimulation by monocyte conditioned medium (McNiff et al., 1995), and both IL-1 and IL-6 are required for the activation process (Ganapathi et al., 1991). Hep3B cells were incubated overnight in the presence of various effector cocktails, after which media were removed and the cells were pulse-labeled with [^{35}S]methionine; SAA subsequently was

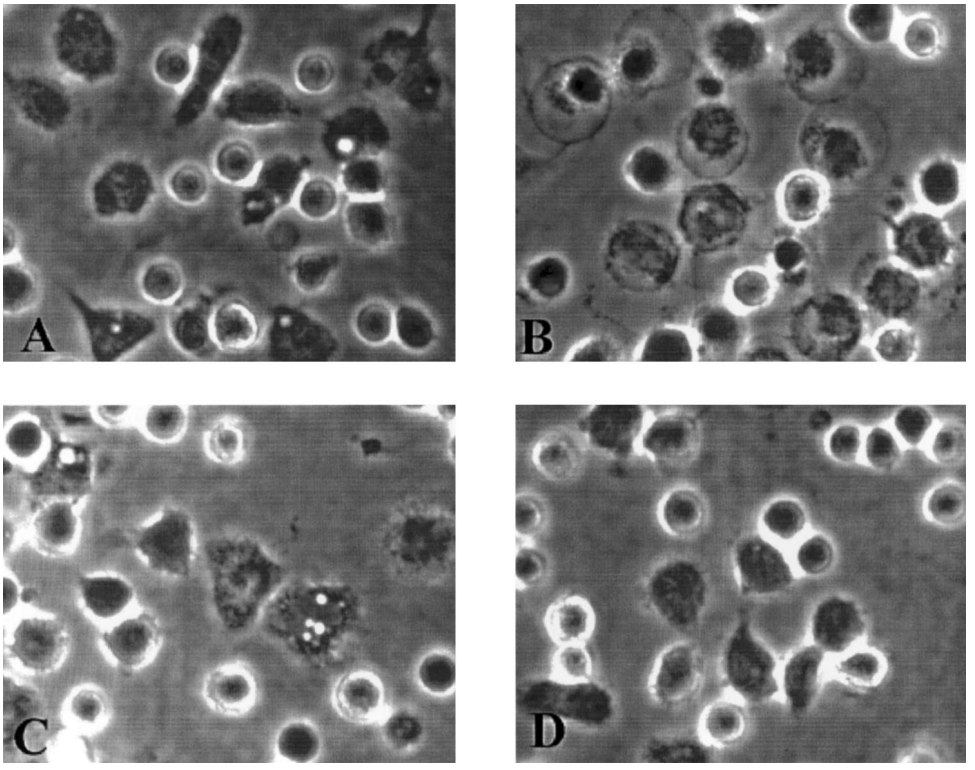

Fig. 8. CP-412,245 blocks hypotonic stress-induced morphology changes. LPS-activated monocytes were maintained in a hypotonic (B and C) or an isotonic (A and D) medium for 0 (A) and 180 min (B–D), in the absence (A, B, and D) and presence of 2 μM CP-412,245 (C). At the end of the treatment, cells were photographed by phase microscopy with a 63× objective.

TABLE 1

Cytokine content of monocyte conditioned medium

Media from LPS/ATP-treated human monocytes prepared in the absence (−) or presence (+) of the indicated concentration of CP-424,174 were harvested and samples of each were assessed for cytokine content by using specific ELISAs for IL-6, IL-1RA, TNFα, and IL-1β. Concentrations were estimated based on comparison of the assay response generated with conditioned media samples to the response observed with recombinant cytokine standards provided in each ELISA kit. In experiment 1, CP-424,174 was added to the culture just prior to addition of ATP, whereas in experiment 2 the test agent was added simultaneously with LPS and maintained throughout the entire LPS and ATP treatments. In experiment 1, mononuclear cells isolated from 200 ml of blood were seeded into two 15-cm dishes, and 25 ml of SFM were added per culture. In experiment 2, mononuclear cells from 70 ml of blood were seeded into four 6-cm dishes, and 3 ml of SFM were used per culture.

Experiment	Culture	Concentration			
		IL-6	IL-1RA	TNFα	IL-1β
		ng/ml			
1	−CP-424,174	26	128	N.D.	31
	+1 μM CP-424,174	19	137	N.D.	0.97
2	−CP-424,174	1.9	5.0	4.1	1.4
	+0.2 μM CP-424,174	1.4	4.0	3.6	0.39
	+1 μM CP-424,174	1.1	3.6	3.5	0.006
	+5 μM CP-424,174	1.0	4.0	3.6	0.012

N.D., not determined.

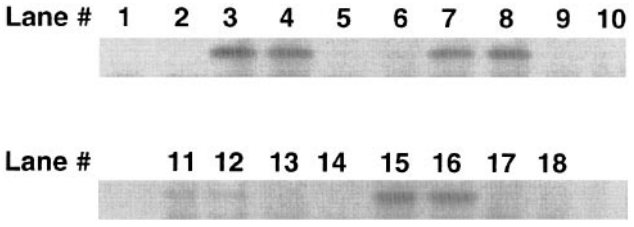

Fig. 9. Conditioned medium derived from LPS/ATP-activated human monocytes in the presence of CP-424,174 is a poor inducer of Hep3B cell SAA synthesis. Hep3B cells were incubated in the presence of recombinant cytokines and/or conditioned medium harvested from LPS-activated/ATP-treated human monocytes prepared in the absence or presence of 1 μM CP-424,174 (generated in experiment 1, Table 1). After overnight culture, growth media were removed and the hepatoma cells were pulse-labeled with [^{35}S]methionine for 60 min. The radiolabeled cells subsequently were solubilized by detergent extraction, SAA was recovered by immunoprecipitation, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography; the region of the autoradiogram corresponding to the migration position of a 12-kDa acute phase SAA standard is shown. Lanes correspond to Hep3B cultures incubated with: no effector (lanes 1 and 2), rIL-1/rIL-6 (lanes 3 and 4), rIL-1/rIL-6/rIL-1RA (lanes 5 and 6), 20% monocyte conditioned medium prepared in the absence of CP-424,174 (lanes 7 and 8), 20% monocyte conditioned medium prepared in the absence of CP-424,174 + 10 μg/ml rIL-1RA (lanes 9 and 10), 20% monocyte conditioned medium prepared in the presence of 1 μM CP-424,174 (lanes 11 and 12), 20% monocyte conditioned medium prepared in the presence of 1 μM CP-424,174 + 10 μg/ml rIL-1RA (lanes 13 and 14), 20% monocyte conditioned medium prepared in the absence of CP-424,174 but supplemented with 0.2 μM the diarylsulfonylurea and 0.4 mM ATP (lanes 15 and 16), rIL-6 only (lanes 17 and 18).

recovered from detergent lysates of the cells by immunoprecipitation. Hep3B cells incubated in the absence of any effector generated no detectable radiolabeled SAA product (Fig. 9, lanes 1 and 2). In contrast, cells cultured in the presence of both IL-1 and IL-6 yielded a radiolabeled 12-kDa polypeptide that comigrated with an SAA standard (Fig. 9, lanes 3 and 4). Hep3B cells cultured with IL-6 alone (Fig. 9, lanes 17 and 18) or a combination of recombinant IL-1, IL-6, and IL-1RA (Fig. 9, lanes 5 and 6) did not produce significant quantities of the 12-kDa polypeptide, confirming the importance of IL-1 in the activation process. Hep3B cells cultured in the presence of 20% monocyte conditioned medium produced in the absence of CP-424,174 generated a strong SAA signal (Fig. 9, lanes 7 and 8). Addition of recombinant IL-1RA to this conditioned medium led to loss of the radiolabeled SAA product (Fig. 9, lanes 9 and 10), confirming that IL-1 within the conditioned medium is a major driver of SAA synthesis. Hepatocytes cultured with 20% conditioned medium derived from monocytes incubated in the presence of CP-424,174, on the other hand, produced only minimal quantities of radiolabeled SAA (Fig. 9, lanes 11 and 12). The small amount of SAA produced was reduced by addition of recombinant IL-1RA (Fig. 9, lanes

13 and 14). To rule out the possibility that ATP and/or CP-424,174 carried into the bioassay with monocyte conditioned medium affected SAA production, hepatoma cells were cultured in the presence of 20% monocyte conditioned medium (originally collected in the absence of CP-424,174) supplemented with 0.4 mM ATP and 0.2 μM CP-424,174. After overnight incubation, these hepatocytes produced quantities of radiolabeled SAA comparable with those generated by cultures maintained in the absence of the supplemented effectors (Fig. 9, lanes 15 and 16). Therefore, CP-424,174 does not directly affect SAA synthesis by Hep3B cells but, rather, lowers acute phase protein production via its effect on monocyte IL-1 production.

Oral Administration of CP-424,174 to Mice Selectively Blocks IL-1 Production. LPS priming of peritoneal cells in vivo generates small quantities of extracellular IL-1, and these quantities are greatly enhanced when ATP is injected into the peritoneal cavity (Griffiths et al., 1995). This system was used to determine whether CP-424,174 could inhibit IL-1 production in vivo. After oral administration, CP-424,174 inhibited ATP-induced IL-1α and IL-1β release from LPS-primed peritoneal cells (Fig. 10A); the ED_{50} for inhibition of both cytokines was similar (15 $\pm$ 3 mg/kg for IL-1β and 14 $\pm$ 3 mg/kg for IL-1α). Importantly, as observed in vitro, inhibition of IL-1 production in vivo is a selective process. Mice treated with CP-424,174 produced levels of IL-6 and TNFα that were comparable with those generated by vehicle-treated animals (Fig. 10B). CP-424,174, therefore, is orally bioavailable in mice and capable of achieving levels at peripheral locations sufficient for the selective inhibition of stimulus-coupled IL-1 production.

Discussion

A limited number of potential therapeutic approaches have been proposed to regulate IL-1 activity and all have limitations. For example, recombinant IL-1RA, either as the protein or via its transgenic expression, has demonstrated anti-inflammatory activity in both animal models and human clinical trials (Arend et al., 1998; Bresnihan et al., 1998; Bendele et al., 1999). The relatively short in vivo half-life of the recombinant protein, however, necessitates that large quantities of the recombinant polypeptide be generated and/or used. Likewise, caspase-1 inhibitors have demonstrated efficacy in animal models of inflammatory diseases (Miller et al., 1995; Ku et al., 1996), and mice engineered to lack this enzyme display reduced inflammatory processes (Kuida et al., 1995; Li et al., 1995). Caspase-1 inhibitors, however, are not expected to directly affect production of IL-1α, and pro-IL-1β released in their presence may be processed by extracellular proteases to an active cytokine species (Hazuda et al., 1990). Peptide-based antagonists of IL-1 receptors also have been generated, and these can inhibit IL-1 signaling processes (Akeson et al., 1996). The peptidic nature of these antagonists carries metabolic liabilities that limit their in vivo utility. Finally, a number of nonselective inhibitors of anion transport (e.g., tenidap, ethacrynic acid, meclofenamic acid) have been shown to inhibit stimulus-coupled IL-1 production in vitro (Laliberte et al., 1994). Multiple other activities associated with the aforementioned anion transport inhibitors and their low potency, however, make them poor candidates as CRIDs. Nonetheless, activity of these latter agents is consistent with previous observations demonstrating that stimulus-coupled IL-1 processing requires changes to the ionic status of the cell, and agents that promote IL-1 post-translational processing share an ability to alter ionic homeostasis. For example, ATP ligation of the $P2X_7$ receptor leads to membrane depolarization and efflux of intracellular K^+ (Sung et al., 1985). Increasing extracellular K^+ inhibits ATP-induced IL-1β processing by human monocytes presumably by preventing cytoplasmic depletion of this cation (Perregaux and Gabel, 1994; Walev et al., 1995). In addition, extracellular Na^+ is required for post-translational processing of IL-1β (Perregaux and Gabel, 1998b), and an important role for anions in the cellular process is suggested by the finding that replacement of extracellular Cl^- with chaotropic anions (e.g., iodide or nitrate) blocks stimulus-coupled processing (Perregaux et al., 1996).

The ionic dependence of the stimulus-coupled response prompted us to screen a panel of known ion transport inhibitors for their ability to alter IL-1 production. Although most inhibitors evaluated were without effect, the sulfonylurea glyburide blocked IL-1β post-translational processing in a dose-dependent manner. This effect is considered selective because similar concentrations of the structurally related drug glipizide did not inhibit cytokine production. Concen-

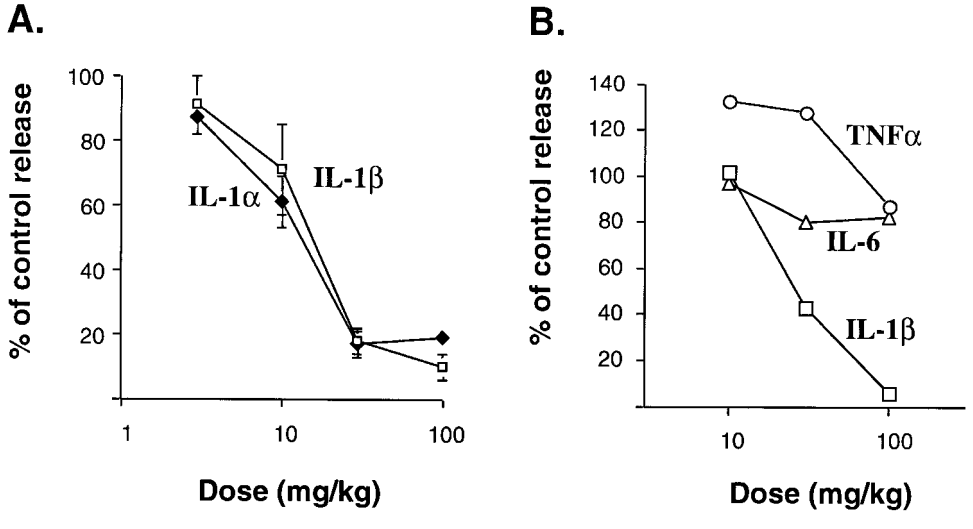

Fig. 10. Oral administration of CP-424,174 to mice results in selective inhibition of IL-1 production. LPS was injected intraperitoneally to promote cytokine synthesis. An hour later, animals received CP-424,174 or vehicle followed 60 min later by an injection of ATP. Animals were euthanized 15 min later, and cytokines in peritoneal lavage fluids were measured by ELISA. A, levels of IL-1α and IL-1β were measured and are indicated as a function of the dose of CP-424,174. The values (expressed as a percentage of cytokine measured in animals treated with vehicle only) are means of three separate experiments, and standard errors are indicated. B, levels of IL-1β, TNFα, and IL-6 were measured and are indicated (expressed as a percentage of cytokine levels measured in animals treated vehicle only) as a function of the dose of CP-424,174. Values are means of six animals in each dose group.

trations of glyburide required to inhibit the monocyte response are in great excess of those required to bind to the high-affinity sulfonylurea receptor associated with the pancreatic β-cell ATP-gated K^+ channel (Ashcroft and Ashcroft, 1992). This difference in potency, combined with the discordance between the shared ability of both glyburide and glipizide to bind to the β-cell sulfonylurea receptor but only glyburide blocks the monocyte response, indicates that glyburide's inhibitory effect is not due to inhibition of an ATP-gated K^+ channel. Glyburide also is known to inhibit several types of anion transporters, including CFTR at concentrations >1 μM (Sheppard and Welsh, 1992). Luciani et al. (1997) independently discovered that glyburide blocked IL-1 post-translational processing, and suggested that this effect was dependent on inhibition of a P-glycoprotein type of transporter, ABC-1 (Luciani et al., 1997). The actual molecular target of glyburide within monocytes remains to be established.

A search for structurally related compounds that were more potent than glyburide led to the discovery of diarylsulfonylureas CP-424,174 and CP-412,245. These agents act as reversible, selective inhibitors of stimulus-coupled post-translational processing; they do not inhibit production of cytokines such as IL-6, TNFα, and IL-1RA whose export is not dependent on a secretory stimulus. Cytokine products such as these rely on the constitutive secretory pathway involving the endoplasmic reticulum and Golgi apparatus and they do not accumulate within the cell. Importantly, CRIDs block IL-1 post-translational processing initiated by ATP, cytolytic T cells, and hypotonic stress, indicating that they interfere with a step common to all initiators of the cytokine post-translational response. Diarylsulfonylurea-arrested cells were impaired in several aspects of the IL-1 post-translational response; these agents block formation and release of mature 17-kDa IL-1β, and this blockade occurs without a compensatory increase in the quantity of pro-IL-1β recovered extracellularly, as is observed with some caspase-1 inhibitors (Thornberry et al., 1992). Lack of 17-kDa IL-1β formation suggests that caspase-1 is not activated in the presence of diarylsulfonylureas; recombinant caspase-1 activity, however, is not affected by these agents when tested in a cell-free assay (data not shown). Diarylsulfonylureas block IL-1β post-translational processing even when added 15 min after ATP; previous studies demonstrated that chloride removal from the medium during this same time period blocked the cellular response (Perregaux et al., 1996). This correspondence raises the possibility that CRIDs alter cytokine processing by affecting a chloride-dependent step, but their precise mode of action remains to be established.

The diarylsulfonylureas also block release of LDH and TCA-precipitable macromolecules from stimulus-activated monocytes. Release of these cellular components is probably the result of damage to the plasma membrane driven, in part, by an extensive increase in cell volume (Perregaux et al., 1996). This volume change appears to be an important component of the stimulus-coupled cytokine response; for example, monocytes treated with ATP in a hypertonic medium did not release mature IL-1β and they did not demonstrate the marked increase in cell volume (Perregaux et al., 1996). Remarkably, CP-412,245 blocks the cell volume increase as evidenced by its ability to reduce the number of swollen cells after hypotonic stress. Therefore, CRIDs appear to arrest monocytes/macrophages at an early step in the activation process that preserves cell membrane integrity. The ultimate fate of pro-IL-1β that is retained within the arrested cells and the long-term viability of the cells themselves remain to be determined.

Is an agent that inhibits IL-1 post-translational processing likely to suppress IL-1-dependent signaling processes? We demonstrated previously that the complex mixture of cytokines generated by LPS-activated human monocytes is more effective as an inducer of the acute phase protein SAA when prepared in the presence of ATP (Laliberte et al., 1997). Inclusion of the nucleotide triphosphate caused a large shift in the ratio of IL-1β to IL-1RA, resulting in an enhanced IL-1 signaling capacity as measured by the ability of the conditioned medium to promote hepatoma cell SAA synthesis. In contrast, in the absence of ATP the balance of IL-1 to IL-1RA within the conditioned medium favored the antagonist and little SAA synthesis was induced. A large excess of IL-1RA over IL-1 is required to impair the cytokine's signaling activity (Arend et al., 1998), and treatments that shift the agonist concentration in the presence of a fixed amount of antagonist are expected to alter signaling capacity. Indeed, conditioned medium derived from LPS-activated/ATP-treated monocytes maintained in the presence of CP-424,174 demonstrated a markedly reduced ability to promote SAA synthesis by hepatoma cells relative to medium prepared in the absence of this agent. The reduction in signaling capacity was not accompanied by a decrease in the quantities of IL-6 and/or IL-1RA, but was associated with a large decrease in IL-1β levels. Therefore, by reducing the ratio of IL-1β to IL-1RA, CP-424,174 suppressed the signaling capacity of monocyte conditioned medium with respect to an IL-1-dependent process. Stimulus-coupled IL-1 post-translational processing also has been demonstrated in vivo; LPS activated murine peritoneal macrophages generate minimal quantities of extracellular IL-1β in the absence of a secretion stimulus, and the subsequent injection of ATP evokes large quantities of mature cytokine (Griffiths et al., 1995). A stimulus-coupled response mechanism thus appears to be required for efficient production of extracellular IL-1 in vivo as well as in vitro. CP-424,174 impaired stimulus-coupled IL-1α and IL-1β production after oral administration to mice without affecting production of the LPS-inducible cytokines IL-6 and TNFα. Therefore, CRID-like agents are expected to dampen IL-1-dependent signaling events and to provide therapeutic utility to patients suffering from diseases such as rheumatoid arthritis where IL-1 is thought to serve as a major mediator of the inflammatory process.

References

Akeson AL, Woods CW, Hsieh LC, Bohnke RA, Ackermann BL, Chan KY, Robinson JL, Yanofsky SD, Jacobs JW, Barrett RW, et al. (1996) AF12198, a novel low molecular weight antagonist, selectively binds the human type I interleukin (IL)-1 receptor and blocks in vivo responses to IL-1. *J Biol Chem* **271:**30517–30523.

Arend WP, Malyak M, Guthridge CJ, and Gabay C (1998) Interleukin-1 receptor antagonist: role in biology. *Annu Rev Immunol* **16:**27–55.

Ashcroft SJH and Ashcroft FM (1992) The sulfonylurea receptor. *Biochim Biophys Acta* **1175:**45–59.

Ayala JM, Yamin T-T, Egger LA, Chin J, Kostura MJ, and Miller DK (1994) IL-1β-converting enzyme is present in monocytic cells as an inactive 45 kDa precursor. *J Immunol* **153:**2592–2599.

Bendele A, McAbee T, Sennello G, Frazier J, Chlipala E, and McCabe D (1999) Efficacy of sustained blood levels of interleukin-1 receptor antagonist in animal models of arthritis. *Arthritis Rheum* **42:**498–506.

Bresnihan B, Alvaro-Gracia JM, Cobby M, Doherty M, Domljan Z, Emery P, Nuki G, Pavelka K, Rau R, Bozman B, et al. (1998) Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis Rheum* **41:**2196–2204.

Carruth LM, Demczuk S, and Mizel SB (1991) Involvement of a calpain-like protease in the processing of murine interleukin 1α precursor. *J Biol Chem* **266:**12162–12167.

Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, Van Ness K, Greenstreet TA, March CJ, Kronheim SR, Druck T, Cannizzaro LA, et al. (1992) Molecular cloning of the interleukin-1β converting enzyme. *Science (Wash DC)* **256:**97–100.

Chin J and Kostura MJ (1993) Dissociation of IL-1β synthesis and secretion in human blood monocytes stimulated with bacterial cell wall products. *J Immunol* **151:**5574–5585.

Demczuk S, Baumberger C, Mach B, and Dayer JM (1987) Expression of human IL-1 alpha and beta messenger RNAs and IL-1 activity in human peripheral blood mononuclear cells. *J Mol Cell Immunol* **3:**255–265.

Dinarello CA (1998) Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. *Int Rev Immunol* **16:**457–499.

Ganapathi MK, Rzewnicki D, Samols D, Jiang SL, and Kushner I (1991) Effect of combinations of cytokines and hormones on synthesis of serum amyloid A and C-reactive protein in Hep3B cells. *J Immunol* **147:**1261–1265.

Griffiths RJ, Stam EJ, Downs J, and Otterness I (1995) ATP induces the release of IL-1 from LPS primed cells in vivo. *J Immunol* **154:**2821–2828.

Hazuda DJ, Strickler J, Kueppers F, Simon PL, and Young PR (1990) Processing of precursor interleukin 1β and inflammatory disease. *J Biol Chem* **265:**6318–6322.

Hazuda DJ, Strickler J, Simon P, and Young PR (1991) Structure-function mapping of interleukin 1 precursors. *J Biol Chem* **266:**7081–7086.

Hogquist KA, Unanue ER, and Chaplin DD (1991a) Release of IL-1 from mononuclear phagocytes. *J Immunol* **147:**2181–2186.

Hogquist KA, Nett MA, Unanue ER, and Chaplin DD (1991b) Interleukin 1 is processed and released during apoptosis. *Proc Natl Acad Sci USA* **88:**8485–8489.

Ku G, Faust T, Lauffer LL, Livingston DJ, and Harding MW (1996) Interleukin-1β converting enzyme inhibition blocks progression of type II collagen-induced arthritis in mice. *Cytokine* **8:**377–386.

Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS-S, and Flavell RA (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1β converting enzyme. *Science (Wash DC)* **267:**2000–2002.

Laliberte R, Perregaux D, Svensson L, Pazoles CJ, and Gabel CA (1994) Tenidap modulates cytoplasmic pH and inhibits anion transport in vitro. *J Immunol* **153:**2168–2179.

Laliberte RE, Perregaux DG, McNiff P, and Gabel CA (1997) Human monocyte ATP-induced IL-1β posttranslational processing is a dynamic process dependent on in vitro growth conditions. *J Leukoc Biol* **62:**227–239.

Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, McDowell J, Paskind M, Rodman L, Salfeld J, et al. (1995) Mice deficient in IL-1β-converting enzyme are defective in production of mature IL-1β and resistant to endotoxic shock. *Cell* **80:**401–411.

Luciani M-F, Hamon Y, Becq F, Rubartelli A, and Chimini G (1997) Interleukin-1β secretion is impaired by inhibitors of the ATP binding cassette transporter, ABC1. *Blood* **90:**2911–2915.

McNiff PA, Stewart C, Sullivan J, Showell HJ, and Gabel CA (1995) Synovial fluid from rheumatoid arthritis patients contains sufficient levels of IL-1β and IL-6 to promote production of serum amyloid A by Hep3B cells. *Cytokine* **7:**209–219.

Miller BE, Krasney PA, Gauvin DM, Holbrook KB, Koonz DJ, Abruzzese RV, Miller RE, Pagani KA, Dolle RE, Ator MA, et al. (1995) Inhibition of mature IL-1β production in murine macrophages and a murine model of inflammation by WIN 67694, an inhibitor of IL-1β converting enzyme. *J Immunol* **154:**1331–1338.

Miossec C, Decoen M-C, Durand L, Fassy F, and Hercend AD (1996) Use of monoclonal antibodies to study interleukin-1β-converting enzyme expression: only precursor forms are detected in interleukin-1β-secreting cells. *Eur J Immunol* **26:**1032–1042.

Mosley B, Urdal DL, Prickett KS, Larsen A, Cosman D, Conlon PJ, Gillis S, and Dower SK (1987) The interleukin-1 receptor binds human interleukin-1α precursor but not the interleukin-1β precursor. *J Biol Chem* **262:**2941–2944.

Perregaux D and Gabel CA (1994) Interleukin-1β maturation and release in response to ATP and nigericin. *J Biol Chem* **269:**15195–15203.

Perregaux DG and Gabel CA (1998a) Post-translational processing of murine IL-1: evidence that ATP-induced release of IL-1α and IL-1β occurs via a similar mechanism. *J Immunol* **160:**2469–2477.

Perregaux DG and Gabel CA (1998b) Human monocyte stimulus-coupled IL-1β posttranslational processing: modulation via monovalent cations. *Am J Physiol* **275:**C1538–C1547.

Perregaux DG, Laliberte RE, and Gabel CA (1996) Human monocyte interleukin-1β posttranslational processing. Evidence of a volume regulated response. *J Biol Chem* **271:**29830–29838.

Rubartelli A, Cozzolino F, Talio M, and Sitia R (1990) A novel secretory pathway for interleukin-1β, a protein lacking a signal sequence. *EMBO (Eur Mol Biol Organ) J* **9:**1503–1510.

Sheppard DN and Welsh MJ (1992) Effect of ATP-sensitive potassium channel regulators on cystic fibrosis transmembrane conductance regulator chloride currents. *J Gen Physiol* **100:**573–591.

Sims JE and Dower SK (1994) Interleukin-1 receptors. *Eur Cytokine Netw* **5:**539–546.

Sung S-S, Young JD-E, Origlio AM, Heiple JM, Kaback HR, and Silverstein SC (1985) Extracellular ATP perturbs transmembrane ion fluxes, elevates cytosolic [Ca^{2+}], and inhibits phagocytosis in mouse macrophages. *J Biol Chem* **260:**13442–13449.

Talbot DC, Smith IE, Nicolson MC, Powles TJ, Button D, and Walling J (1993) Phase II trial of the novel sulphonylurea sulofenur in advanced breast cancer. *Cancer Chemother Pharmacol* **31:**419–422.

Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, et al. (1992) A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. *Nature (Lond)* **356:**768–774.

Walev I, Reske K, Palmer M, Valeva A, and Bhakdi S (1995) Potassium-inhibited processing of IL-1β in human monocytes. *EMBO (Eur Mol Biol Organ) J* **14:**1607–1614.

Walev I, Weller U, Strauch S, Foster T, and Bhakdi S (1996) Selective killing of human monocytes and cytokine release provoked by sphingomyelinase (beta-toxin) of *Staphylococcus aureus*. *Infect Immun* **64:**2974–2979.

Yong G, Kuida K, Tsutsui H, Ku G, Hsiao K, Fleming MA, Hayashi N, Higashino K, Okamura H, Nakanishi K, et al. (1997) Activation of interferon-γ inducing factor mediated by interleukin-1β converting enzyme. *Science (Wash DC)* **275:**206–209.

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