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Inhibition of Interleukin-1β Production by SKF86002: Evidence of Two Sites of *In Vitro* Activity and of a Time and System Dependence

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SUMMARY

Cytokine-suppressing anti-inflammatory drugs (CSAIDs) are reported to inhibit production of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) by affecting a stress-induced kinase. To gain a better understanding of the selectivity and cellular dynamics of this type of inhibitor, we studied *in vitro* the prototype member of this class of agents, SKF86002. Lipopolysaccharide (LPS)-activated human monocytes treated with SKF86002 produced less proIL-1β but normal amounts of the noncytokine lysozyme. Two-dimensional gel analysis indicated that only eight polypeptides produced by monocytes were decreased by SKF86002. Inhibition of IL-1β production was achieved by affecting two separate steps in this cytokine's biogenesis. First, SKF86002 lowered proIL-1β synthesis. By pulse-chase analysis, this effect was localized to a posttranscriptional site of action; maximal inhibition was observed when SKF86002 was added at the time of cytokine translation. Exposure of monocytes to SKF86002 for >2 hr led to a loss of IL-1β inhibitory activity, suggesting that these cells adapted to this agent. Moreover, LPS-activated monocytes that were pretreated with granulocyte-macrophage colony-stimulating factor were less sensitive to the proIL-1β inhibitory effect of SKF86002, and production of proIL-1β by cytokine-stimulated human fibroblasts was impaired only modestly by the CSAID. A second effect of SKF86002 was to inhibit release of IL-1β into the medium in response to high concentrations of LPS; this effect is observed only with freshly isolated human monocytes as other IL-1β-producing cells do not release significant cytokine in response to LPS. The ability of SKF86002 to inhibit this posttranslational mechanism was mimicked by lysosomotrophic agents such as chloroquine, quinacrine, and methylamine. In contrast, chloroquine and quinacrine were not effective inhibitors of monocyte proIL-1β translation. Thus, SKF86002 inhibits IL-1β production by affecting at least two distinct steps in the biosynthesis of this cytokine. Manifestation of these two effects, however, is dependent on the length of time for which cells are exposed to this agent and the nature of the cytokine-producing cellular system.

IL-1 is an important proinflammatory cytokine produced by a variety of cells, including monocytes and macrophages (1). This cytokine is synthesized as two distinct species, IL-1α and IL-1β, both of which appear to bind to the same IL-1 receptors to promote their biological activities (2). These activities include up-regulation of adhesion molecule expression on endothelial cells (3), stimulation of matrix metalloproteinase expression (4) and cartilage degradation (5), and increased production of prostaglandins by target cells (6). Importantly, IL-1 is found in synovial effusions from rheumatoid arthritis patients (7), suggesting that this cytokine is produced at local sites of inflammation where it may serve an important role in the maintenance and progression of this disorder. Thus, agents that control IL-1 production represent attractive candidates for new therapies for the treatment of rheumatoid arthritis and other inflammatory disorders.

A new class of compound recently has emerged that inhibits cytokine production (8, 9). The prototype of these CSAIDs is SKF86002. *In vitro*, this agent has been shown to inhibit production of IL-1 and several additional cytokines, including TNF-α and IL-8 (8–10). Moreover, these cytokine-inhibiting activities have been observed *in vivo* (8, 11). The mechanism by which SKF86002 inhibits cytokine production is unclear. This agent is reported to inhibit arachidonic acid metabolism via both cyclooxygenase and 5-lipoxygenase pathways (12), but the cytokine inhibitory activity appears to be unrelated to these effects (8, 9). Inhibition of monocyte cytokine production is reported to occur in the absence of a change in message levels,

ABBREVIATIONS: CSAID, cytokine-suppressing anti-inflammatory drug; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; MAP, mitogen-activated protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

suggesting that SKF86002 alters a posttranscriptional step in cytokine biogenesis (13). Recently, a CSAID binding protein was isolated from THP-1 cells and identified as a stress-inducible protein kinase, and CSAIDs were shown to inhibit the stress-induced kinase activity *in vitro* (14). Thus, inhibition of a specific protein kinase may be involved in the mechanism by which CSAIDs suppress cytokine production.

To gain a better understanding of the mode of action of CSAIDs, we analyzed the ability of SKF86002 to inhibit IL-1β production by human monocytes and fibroblasts *in vitro*. Our results indicate that this agent acts at two separate steps in the biosynthetic pathway, corresponding to a translational and a posttranslational event. These two activities may occur via distinct mechanisms as chloroquine mimics one more effectively than the other. SKF86002, therefore, represents an important new tool with which to dissect and modulate cytokine biosynthetic pathways.

Materials and Methods

Monocyte isolation and induction of IL-1β production. Human monocytes were isolated from blood of normal volunteers by lymphocyte separation medium (Organon Teknika, Westchester, PA) centrifugation. Mononuclear cells from the gradient were collected and washed with RPMI 1640 medium containing 5% FBS, 25 mM HEPES, pH 7.2, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (maintenance medium). Then, 1×10^7 cells were seeded per well of six-well multidishes. After a 2-hr incubation, nonadherent cells were removed, and the adherent monocytes were washed twice with maintenance medium. Monocytes were incubated overnight in maintenance medium; these cells are referred to as aged monocytes and, where indicated, 1.5 ng/ml of human recombinant GM-CSF (R&D Systems, Minneapolis, MN) was added to the culture medium. The aged and GM-CSF-treated monocytes then were stimulated with 10 ng/ml LPS (*Escherichia coli* serotype O55:B5, Sigma Chemical Co., St. Louis, MO) for 2 hr, after which they were washed once with methionine-free RPMI, and 1 ml of this medium (containing 83 μCi/ml [^{35}S]methionine [Amersham], 1% dialyzed FBS, 25 mM HEPES, and 10 ng/ml LPS) was added, and the cells were labeled for 60 min in the absence or presence of 10 μM SKF86002. Where indicated, cultures were chased after labeling with 10 ng/ml LPS for 4 hr in the absence or presence of SKF86002. Chase media were collected and adjusted to 1% Triton X-100, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride. Cells were harvested by detergent extraction in 1 ml of a lysis buffer composed of 25 mM HEPES, pH 7, 150 mM NaCl, 1 mg/ml ovalbumin, 1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.1 mM phenylmethylsulfonyl fluoride. Both the cell extracts and media samples were clarified by ultracentrifugation (50,000 rpm in a Beckman TLA 100.3 rotor for 30 min).

Immunoprecipitation. Of each cell extract (or medium sample), 0.5 ml was incubated with 3 μl of anti-human IL-1β (Collaborative, Bedford, MA) or 5 μl of anti-human lysozyme (The Binding Site, San Diego, CA). To recover immune complexes, 0.25 ml of 10% suspension of protein A Sepharose in phosphate-buffered saline was added, and the solutions were mixed for 30 min at 4°. The beads were collected by centrifugation and washed five times with 1 ml of 10 mM Tris, pH 8, 10 mM EDTA, 1% Triton X-100, 0.4% deoxycholate, and 0.1% SDS and once with 50 mM Tris, pH 6.8. The final pellet was suspended in 0.1 ml of SDS disaggregation buffer and boiled for 3 min. Beads were removed by centrifugation, and the supernatants were recovered and analyzed with SDS-gel electrophoresis and autoradiography.

Two-step release assay for fresh human monocytes. Human mononuclear cells were isolated by lymphocyte separation medium centrifugation as described, and a total of 1.5×10^5 cells were seeded into each well of a 96-well plate. After 2 hr of adherence, the medium was replaced with 0.1 ml of a DMEM-based medium containing 5% FBS, 25 mM HEPES, pH 7.2, 100 units/ml penicillin, 100 μg/ml streptomycin, and 200 pg/ml LPS. After a 2.5-hr incubation, the medium was replaced with 0.1 ml of DMEM, 5% FBS, 25 mM HEPES, pH 7.2, 100 units/ml penicillin, and 100 μg/ml streptomycin (containing the indicated test agent). The monocytes were incubated for 20 min to allow equilibration of the test agent; then 100 ng/ml LPS was introduced, and the cultures were incubated at 37° for an additional 2 hr. Media were harvested and clarified by centrifugation, and aliquots were used for determination of released IL-1β; this determination was performed with an ELISA kit (R&D Systems). Chloroquine, quinacrine, 4-aminopyridine, lidocaine, methylamine, ephedrine, and benzylamine were obtained from Sigma; 4-aminoquinaldine was obtained from Pfaltz and Bauer (Flushing, NY). SKF86002 was synthesized following a protocol previously detailed (15); the isolated product possessed the reported melting point.

Quantification. Regions of the dried gel corresponding to immunoprecipitated IL-1β were excised and solubilized in 0.5 ml of 100 mM Tris, pH 8, and 20 mM $CaCl_2$ containing 5 mg/ml pronase. These pronase digests were incubated at 56° for at least 4 hr, after which they were cooled, and associated radioactivity was determined by liquid scintillation counting. Alternatively, radioactivity associated with immunoprecipitated IL-1β was determined directly with an Ambis image acquisition and analysis system.

Cell samples to be analyzed by two-dimensional gel electrophoresis were solubilized in 0.3% SDS, 200 mM dithiothreitol, 28 mM Tris·HCl, and 22 mM Tris base, after which the lysates were treated with DNAse I (1 mg/ml) and RNAse A (0.25 mg/ml) for 8 min at 4°; these two enzymes were obtained from Boehringer Mannheim (Indianapolis, IN). Polypeptides within these samples were then precipitated with acetone and resuspended in 0.06% SDS, 120 mM dithiothreitol, 5.6 mM Tris·HCl, 4.4 mM Tris base, 7.92 M urea, 3.2% NP-40, and 1.8% Ampholytes (pH 3–10; Millipore Corp., Bedford, MA) and boiled for 3 min. Trichloroacetic acid-precipitable counts were determined at this point, and an equal quantity of radioactivity was applied to individual isoelectric focusing gels; a 3–10 carrier Ampholyte system was used. The second dimension consisted of a 12.5% polyacrylamide slab gel. Gels were fixed and dried before image analysis (16).

LDH Assay. LDH activity within cell extracts was determined with a colorometric pyruvate detection assay (Sigma).

Results

SKF86002 is a selective inhibitor of monocyte protein production. To examine the selectivity of SKF86002 as an inhibitor, we labeled LPS-activated human monocytes with [^{35}S]methionine in the absence and presence of 10 μM SKF86002, and production of proIL-1β and lysozyme, a representative noncytokine product, was determined by immunoprecipitation. In the absence of the CSAID, monocytes produced large quantities of 31-kDa proIL-1β (Fig. 1); the immunoprecpitates also contained a smaller amount of a 28-kDa IL-1β species, which is believed to arise from proteolysis of the 31-kDa procytokine species (17). Likewise, the radiolabeled monocytes produced abundant quantities of 14-kDa lysozyme (Fig. 1). In the presence of SKF86002, a differential effect was observed in the production of these two protein products (Fig. 1). ProIL-1β production was reduced by 85%, but production of lysozyme was not inhibited; a slight stimulation in the recovery of [^{35}S]methionine-labeled lysozyme was consistently observed (Fig. 1). Inhibition of proIL-1β production by SKF86002 was dose dependent (Fig. 1); the estimated IC_{50} was 0.6 μM. Thus, SKF86002-treated

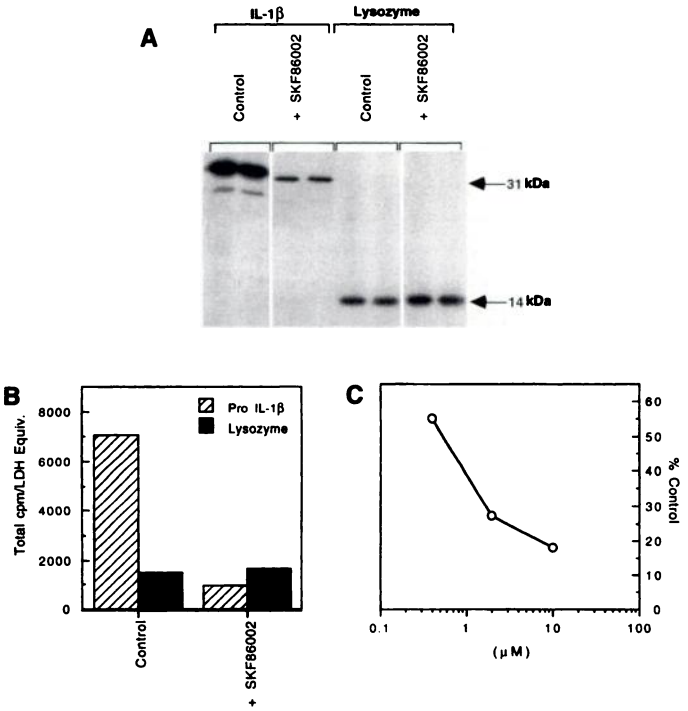

Fig. 1. SKF86002 inhibits proIL-1β but not lysozyme production by LPS-activated monocytes. Monocytes were activated with LPS for 2 hr and then labeled with [^{35}S]methionine for 60 min in the absence or presence of 10 μM SKF86002. ProIL-1β and lysozyme were recovered from detergent extracts of the radiolabeled cells by immunoprecipitation, and the resulting immunoprecipitates were analyzed with SDS-gel electrophoresis and autoradiography (A). Regions of the dried gel containing 31-kDa proIL-1β and 14-kDa lysozyme were excised, and the radioactivity was solubilized by pronase digestion; recovery of radioactivity (normalized to the quantity of cell-associated LDH activity) is indicated as a function of treatment (B). Each bar is the average of duplicate determinations. This experiment was repeated twice. In a separate experiment, production of [^{35}S]methionine-labeled proIL-1β was determined as a function of SKF86002 concentration (C). *Points*, averages of duplicate determinations.

monocytes produced less of the inflammatory cytokine IL-1β but the usual or greater amount of lysozyme.

To estimate the total number of monocyte proteins affected by SKF86002, LPS-activated human monocytes again were labeled with [^{35}S]methionine in the absence or presence of this agent, after which the total spectrum of radiolabeled products was analyzed by two-dimensional gel electrophoresis. [^{35}S]Methionine-labeled control monocytes yielded a complex two-dimensional map of radiolabeled polypeptides (Fig. 2); consistently, >500 polypeptide species were discernible. Polypeptide spots denoted 2 and 3 in the two-dimensional gel map were shown by immunoprecipitation to correspond to proIL-1β; it is not known whether this doublet corresponds to the 28- and 31-kDa species observed on the one-dimensional gel (Fig. 1). LPS-activated monocytes that were labeled with [^{35}S]methionine in the presence of SKF86002 yielded a two-dimensional map very similar to that observed for the control LPS-activated cells. Important differences, however, were noted between these maps. For example, the doublet corresponding to proIL-1β was significantly reduced in the presence of the CSAID (Fig. 2). In addition, production of six other polypeptides (denoted as spots 1 and 4–8) was consistently reduced in the presence of this agent (Fig. 2). Radioactivity associated with one polypeptide species (spot 9; 44.4-kDa) was increased slightly in the presence of SKF86002 (Fig. 2; Table 1). Changes in these affected species are summarized in Table 1. All other radiolabeled monocyte proteins appeared to be unaffected by the CSAID. Thus, SKF86002 was an effective inhibitor of proIL-1β and several other monocyte protein products, but this inhibition was selective as this agent did not alter production of most monocyte polypeptides.

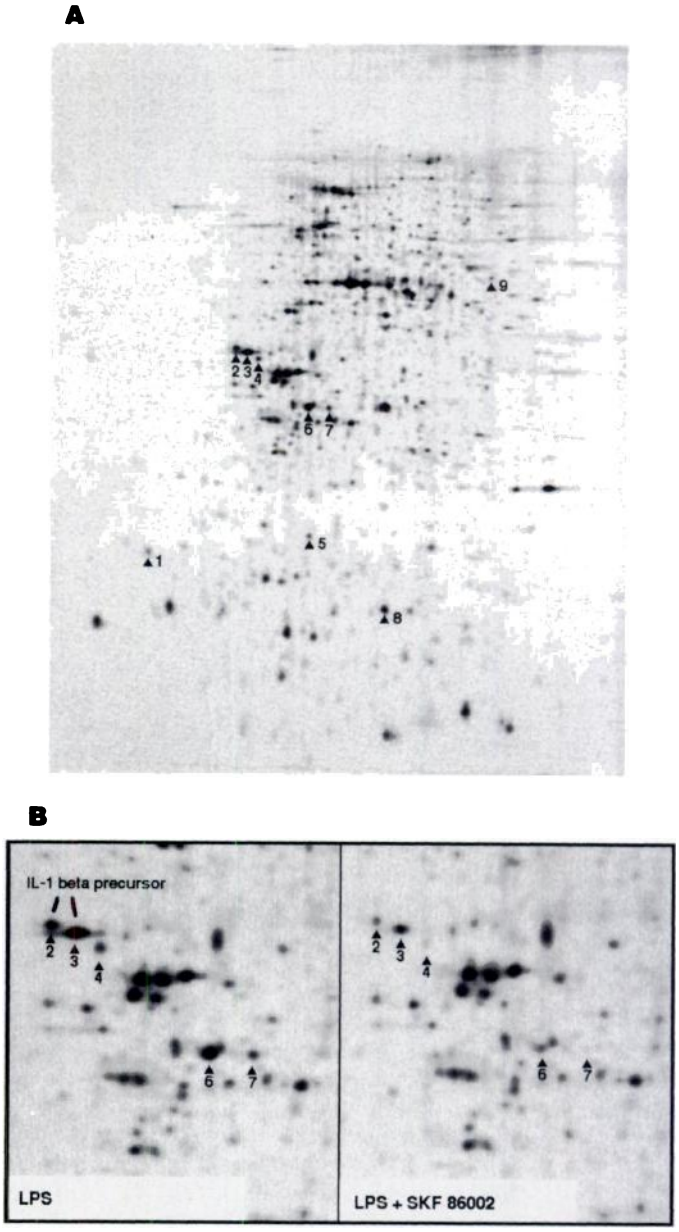

Fig. 2. Analysis of monocyte protein production by two-dimensional gel electrophoresis. Extracts of monocytes labeled with [^{35}S]methionine in the absence or presence of 10 μM SKF86002 were analyzed with two-dimensional gel electrophoresis. A, Radiolabeled polypeptide map produced by control LPS-activated monocytes. *Numbered arrowheads*, polypeptides affected by SKF86002. *B*, Magnified view of the region of the maps containing IL-1β for control (LPS) and treated (LPS+SKF86002) cells. Polypeptides denoted *2* and *3* were shown by immunoprecipitation to correspond to proIL-1β.

SKF86002 acts as a reversible translational inhibitor. To determine whether the cytokine inhibitory effect was reversible, LPS-activated monocytes were treated with

TABLE 1

Quantitative changes identified with two-dimensional gel electrophoresis

Spot	Apparent molecular mass	pI	Inhibition by SKF86002	
			Exp. 1	Exp. 2
	kDa		*% Control*	
1	20.6	<4.2	16	3
2	36	4.36	38	26
3	35.6	4.39	20	23
4	35	4.45	23	25
5	21.5	4.87	34	18
6	30.6	4.86	24	19
7	30.6	5.03	28	27
8	17.6	>5.2	16	16
9	44.4	>5.2	120	150

LPS-activated monocytes were incubated with [^{35}S]methionine for 60 min in the absence or presence of 10 μM SKF86002. After labeling, cells were harvested and analyzed with two-dimensional gel electrophoresis. Autoradiograms of dried two-dimensional gels were collected using 3–5-day exposures on BAS-III imaging plates, which then were scanned with a BAS-2000 Bio-Image analyzer (Fuji Medical Systems, Stamford, CT). Two-dimensional gel images were quantified and matched with the use of PDQUEST (PDI Inc., Huntington Station, NY). The change in the intensity of the indicated polypeptide spot is indicated relative to the nontreated control. pI = isoelectric point.

SKF86002 either before or at the time of the pulse labeling with [^{35}S]methionine. LPS promotes a rapid induction of IL-1β mRNA in cultured monocytes, with maximal levels being achieved 2–4 hr after stimulation (18). Once activated, these cells continue to express high steady state levels of IL-1β mRNA for >8 hr (18). When LPS-activated cells were treated with SKF86002 during the 60-min incubation with [^{35}S]methionine, the quantity of radiolabeled proIL-1β was reduced relative to cells labeled in the absence of the CSAID (Fig. 3). In contrast, when SKF86002 was present for the final 60 min of LPS activation but then removed during the 60-min labeling period, the quantity of [^{35}S]methionine-labeled proIL-1β recovered was 81% of that produced by the control cells (Fig. 3). Thus, SKF86002 acts as a reversible inhibitor.

Previous studies indicated that SKF86002 did not alter cytokine mRNA levels, suggesting that a posttranscriptional step was the primary site of action (13). To determine whether the effect on human monocyte proIL-1β also was due to a posttranscriptional event, these cells were activated with LPS, labeled with [^{35}S]methionine, and chased for 4 hr; SKF86002 was added to these cultures at different times to determine when its maximum inhibitory effect was achieved. In the absence of a chase, monocytes treated simultaneously with LPS and 10 μM SKF86002 (for 2 hr) produced 61% of the radiolabeled proIL-1β generated by control non-SKF86002-treated monocytes during a 60-min pulse labeling (Table 2). When control [^{35}S]methionine-labeled monocytes subsequently were chased for 4 hr, the quantity of radiolabeled proIL-1β decreased (Table 2). This decline in intracellular cytokine was not accompanied by the appearance of extracellular IL-1β (data not shown), suggesting that the pulse-labeled cytokine was degraded intracellularly during the chase. When SKF86002 was added to monocyte cultures during the LPS exposure period and maintained throughout the entire pulse-chase protocol, recovery of proIL-1β again was reduced by 40% (Table 2). Therefore, the presence of SKF86002 during the chase did not increase its effectiveness as an inhibitor of proIL-1β production. Moreover, LPS-activated [^{35}S]methionine-labeled monocytes that were exposed

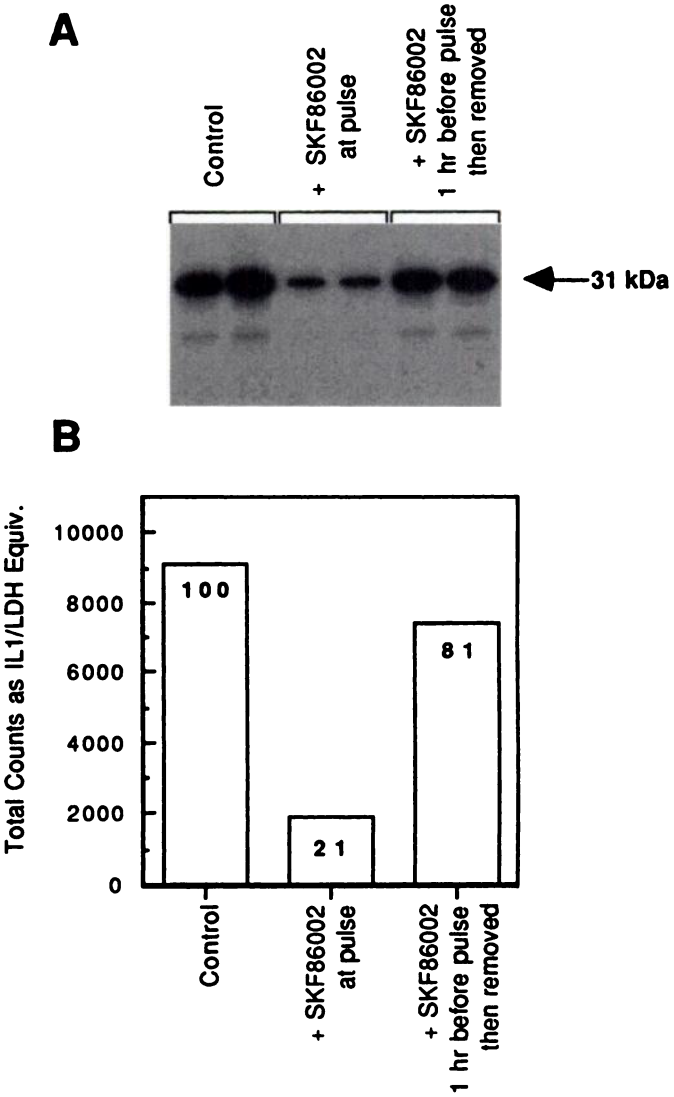

Fig. 3. SKF86002 is a reversible inhibitor of proIL-1β production. Monocytes were activated with LPS for 2 hr; where indicated, 10 μM SKF86002 was present during the final 60 min of this LPS-activation period. Activated cells then were labeled for 60 min with [^{35}S]methionine, after which they were harvested by detergent extraction, and proIL-1β was recovered by immunoprecipitation. A, Immunoprecipitates were analyzed by SDS-gel electrophoresis and autoradiography; B, radioactivity associated with the 31-kDa proIL-1β species was determined. *Bars*, averages of duplicate determinations; *numbers in bars*, percent values relative to the control. This experiment was performed once.

to the CSAID only during the 4-hr chase period yielded the same quantity of proIL-1β as recovered from control monocyte cultures (Table 2). Thus, SKF86002 did not affect the posttranslational intracellular degradation of proIL-1β, indicating that it did not lower intracellular cytokine levels by affecting a posttranslational process.

Surprisingly, monocyte cultures that were treated with SKF86002 after the 2-hr LPS activation produced less proIL-1β than did monocytes that were maintained in the presence of the CSAID throughout the entire activation period (Table 2). Although this was an unexpected result (see later), it suggested that SKF86002 was not acting as a transcriptional inhibitor. The known transcriptional inhibitor actinomycin, for example, totally inhibited proIL-1β production

TABLE 2
SKF86002 acts as a translational inhibitor

Condition	Time of SKF86002 exposure	Chase time	Total cpm/LDH equivalent	% Control
		hr		
Control	—	0	7276	100
+10 μM SKF86002	During LPS activation and pulse	0	4450	61
Control	—	4	2431	100
+10 μM SKF86002	During LPS activation, pulse, and chase	4	1475	61
+10 μM SKF86002	During pulse and chase	4	294	12
+10 μM SKF86002	During chase	4	2484	102

Human monocytes were activated for 2 hr with LPS and then labeled with [^{35}S]methionine for 1 hr and harvested immediately (0-hr chase) or chased for 4 hr before harvesting. SKF86002 was added to the cultures at the indicated times; once added, SKF86002 remained present throughout subsequent steps. Cells were harvested by detergent extraction, IL-1β was recovered by immunoprecipitation, and the immunoprecipitates were analyzed with SDS-gel electrophoresis and autoradiography. Regions of the dried gel corresponding to 31-kDa proIL-1β were excised, and the associated radioactivity was determined after pronase digestion. Each total cpm/unit of cell-associated LDH value is an average of duplicate determinations. This experiment was repeated three times.

when monocytes were incubated simultaneously with LPS and this agent (Fig. 4). Actinomycin is expected to prevent transcription of proIL-1β mRNA and, in turn, to inhibit [^{35}S]methionine-labeled protein production (19). In contrast, when monocytes were activated for 2 hr with LPS in the absence of actinomycin and then labeled for 60 min in the presence of this transcriptional inhibitor, the quantity of proIL-1β was reduced by only 60% (Fig. 4). Under these conditions, monocyte mRNA production was initiated in the absence of the transcriptional inhibitor, and protein subsequently was produced even though the addition of actinomycin to the pulse-labeling medium suppressed new transcriptional events. Importantly, when LPS-activated monocytes were pulse labeled in the presence of both actinomycin and SKF86002, the quantity of proIL-1β was reduced beyond the level recovered from cells treated with actinomycin only (Fig. 4). Because the concentration of actinomycin (5 μg/ml) used was sufficient to completely suppress proIL-1β transcription, the additional inhibition observed with SKF86002 indicates that this agent affects a posttranscriptional event in the production of proIL-1β. Together, these data provide evidence that SKF86002 affects proIL-1β production by altering a step involved in translation of this cytokine but does not affect transcriptional or posttranslational events.

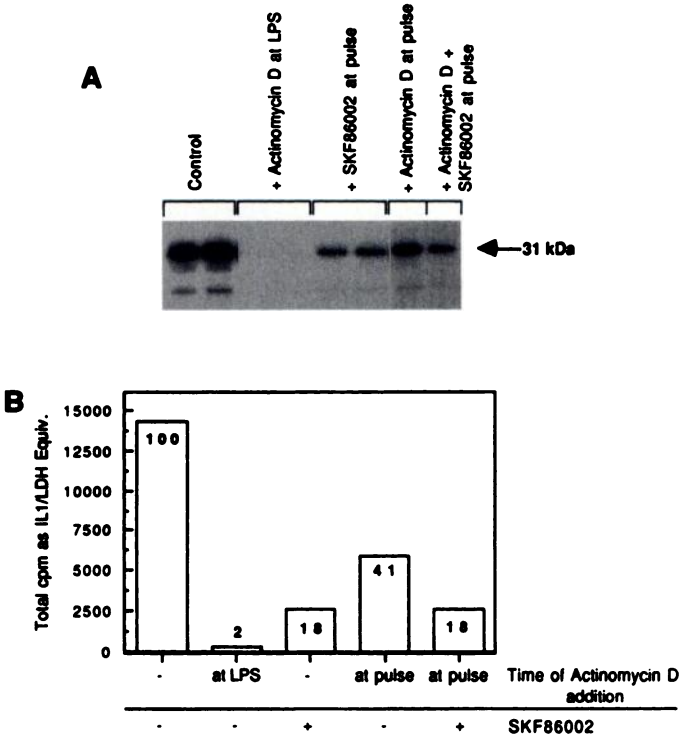

Fig. 4. SKF86002 inhibits proIL-1β production in the presence of actinomycin D. Monocytes were incubated with LPS for 2 hr in the absence or presence of actinomycin D, after which they were labeled for 60 min with [^{35}S]methionine in the absence or presence of actinomycin, SKF86002, or both agents simultaneously. ProIL-1β was immunoprecipitated from detergent extracts of the radiolabeled cells, and immunoprecipitates were analyzed with SDS-gel electrophoresis and autoradiography (A). Regions of the dried gel containing 31-kDa proIL-1β were excised, and the radioactivity was solubilized by pronase digestion and quantitated; total cpm recovered as proIL-1β normalized to LDH equivalents is indicated as a function of treatment (B), and numbers within the bars indicate the percent value relative to the control. This experiment was repeated three times.

Time and system dependence of SKF86002's inhibitory activity. As noted, monocytes exposed to SKF86002 during the entire 2-hr LPS activation and 1-hr pulse-labeling periods were less impaired in proIL-1β production than were cells that were exposed to this agent only during the 1-hr pulse-labeling period (Table 2). This suggested that length of exposure to SKF86002 was an important determinant in the ultimate cellular response. To explore this time dependence, human monocytes again were activated with LPS for 2 hr and labeled with [^{35}S]methionine for 60 min. In this experiment, however, different cultures were treated with 10 μM SKF86002 for 1, 2, 3, or 4 hr before the pulse labeling. When SKF86002 was added only at the time of the pulse, proIL-1β production was impaired by 89% relative to monocytes labeled in the absence of this agent (Fig. 5). Likewise, when monocytes were exposed to SKF86002 for 1 hr before the pulse (and maintained during the pulse), the quantity of proIL-1β was reduced by 88%. However, as the length of SKF86002 exposure time increased from 2 to 4 hr, a time-dependent decrease in the inhibitory effect was observed (Fig. 5). Thus, after 2 hr of preexposure to SKF86002, proIL-1β production was reduced by 54%, and after 4 hr of preexposure, only a 26% reduction in cytokine synthesis was observed (Fig. 5). It should be noted that the medium was changed at the time of the 1-hr pulse labeling and that fresh 10 μM SKF86002 was applied; therefore, loss of inhibitory

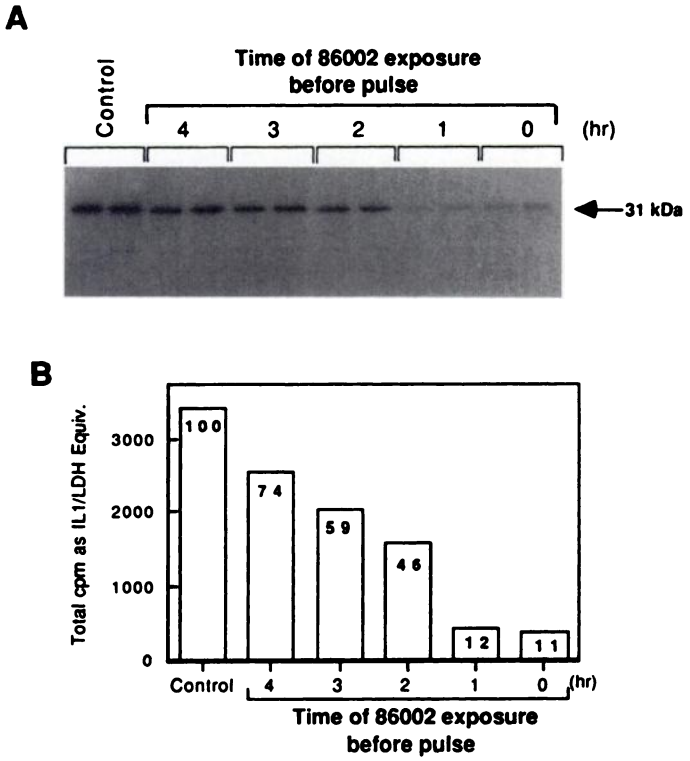

Fig. 5. Human monocytes adapt to the IL-1β inhibitory effect of SKF86002. Monocytes were incubated with 10 μM SKF86002 for the times indicated. In all cases, the cells were treated with LPS for 2 hr and labeled with [^{35}S]methionine for 60 min; fresh 10 μM SKF86002 was present within the pulse medium of all cultures except the control. After labeling, cells were harvested by detergent extraction, and IL-1β was recovered by immunoprecipitation. A, Immunoprecipitates were analyzed with SDS-gel electrophoresis and autoradiography; B, radioactivity associated with the 31-kDa proIL-1β species was determined after pronase solubilization. *Bars*, averages of duplicate determinations; *numbers in bars*, percent values relative to the control. This experiment was repeated twice.

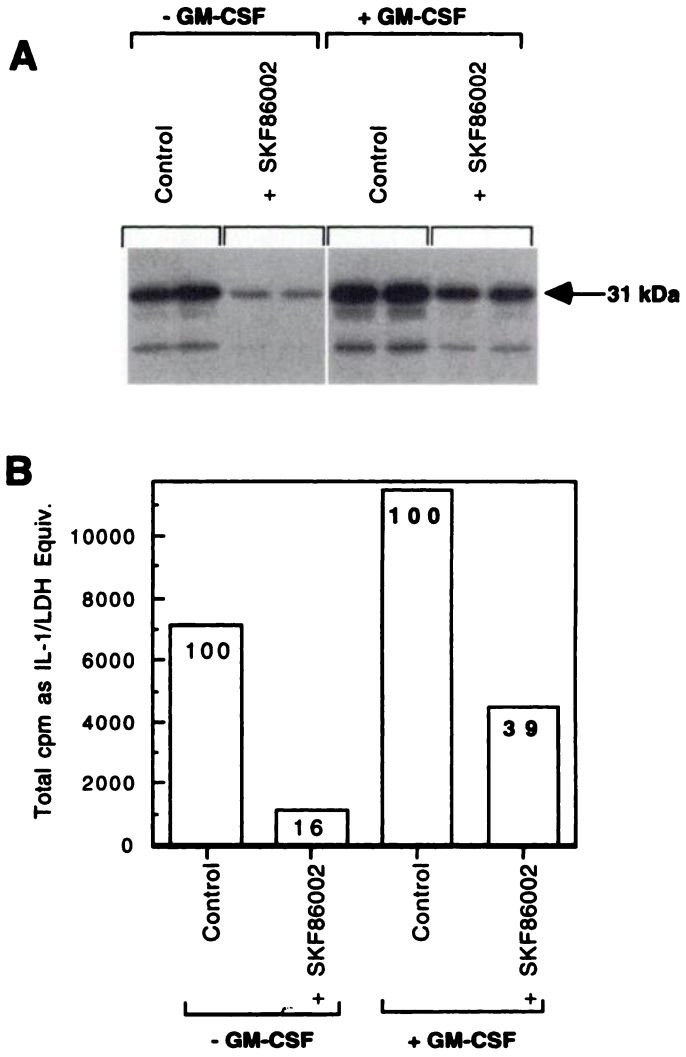

Fig. 6. GM-CSF-treated monocytes are less sensitive to SKF86002 inhibition. Monocytes were cultured for 2 hr in the absence (−) or presence (+) of 1.5 ng/ml human recombinant GM-CSF, after which they were activated with LPS (2 hr) and labeled with [^{35}S]methionine (60 min); the labeling was performed in the absence (*Control*) or presence of 10 μM SKF86002. Cells were harvested by detergent extraction, and IL-1β was recovered by immunoprecipitation. A, Immunoprecipitates were analyzed with SDS-gel electrophoresis and autoradiography; B, radioactivity associated with the 31-kDa proIL-1β species was determined after pronase solubilization. *Bars*, averages of duplicate determinations; *numbers in bars*, percent values relative to the control. This experiment was repeated twice.

activity cannot be attributed to depletion or breakdown of this agent. Rather, the time dependence suggests that monocytes "adapt" to the proIL-1β inhibitory effects of SKF86002.

The effectiveness of SKF86002 as an inhibitor of proIL-1β production also was diminished when monocytes were pretreated with GM-CSF. This cytokine is found at local sites of inflammation (20) and is known to alter monocyte properties (21, 22). For example, monocytes cultured *in vitro* in the presence of GM-CSF produce greater amounts of proIL-1β in response to LPS challenge than do cells cultured in its absence (22). Relative to monocytes cultured in the absence of GM-CSF, cultures treated with this cytokine yielded 1.6-fold more [^{35}S]methionine-labeled proIL-1β (Fig. 6). When LPS-activated monocytes that had not been treated with GM-CSF were labeled with [^{35}S]methionine in the presence of 10 μM SKF86002, an 84% reduction of proIL-1β production was observed relative to cells labeled in the absence of this agent (Fig. 6). On the other hand, when the GM-CSF-treated monocytes were labeled in the presence of the same concentration of SKF86002, the extent of inhibition was reduced to 61% (Fig. 6).

Finally, to determine whether SKF86002 inhibited proIL-1β production by a cell other than a monocyte, human fibroblasts were examined. These cells produce proIL-1β in response to stimulation with IL-1 and TNFα (23). After overnight culture with these two cytokines, human fibroblasts were labeled with [^{35}S]methionine for 60 min in the absence or presence of SKF86002. Nonstimulated fibroblasts produced no radiolabeled material that precipitated with anti-IL-1β serum (Fig. 7). The cytokine-stimulated fibroblasts, on the other hand, yielded an abundant quantity of the 31-kDa proIL-1β species (Fig. 7); no radiolabeled IL-1β was secreted into the medium (data not shown). Inclusion of SKF86002 in the medium during the 60-min pulse labeling decreased the quantity of [^{35}S]methionine-labeled proIL-1β produced, but the extent of inhibition was less than that observed with human monocytes (Fig. 7). Thus, 10 and 50 μM SKF86002 caused only a 23% and 34% reduction, respectively, in the production of proIL-1β (Fig. 7).

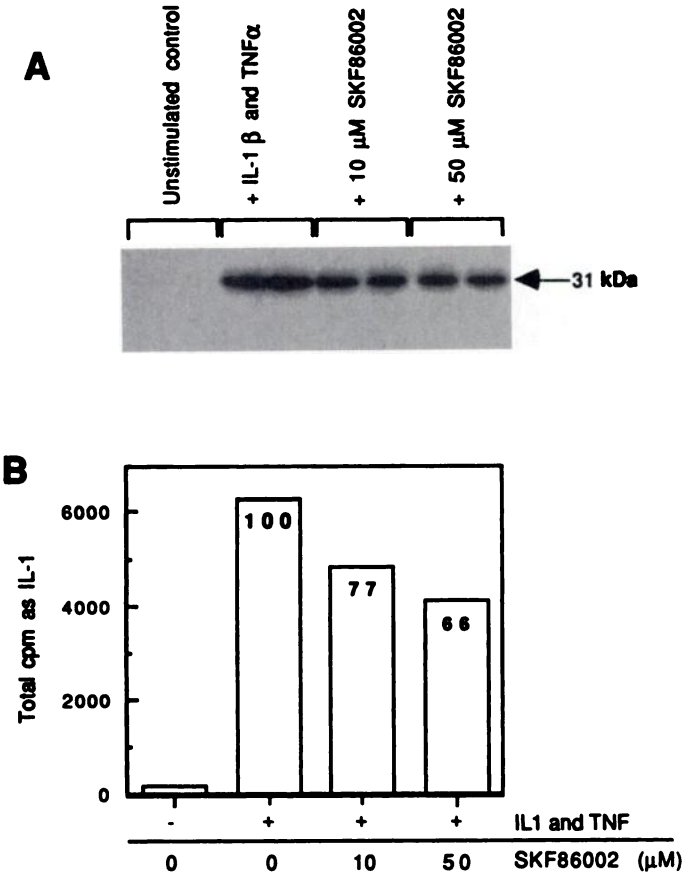

Fig. 7. SKF86002 is a weak inhibitor of human fibroblast proIL-1β production. Human fibroblasts (GM03652B) were cultured for 22 hr in α-minimal essential medium containing 10% FBS and, where indicated, 10 ng/ml human recombinant IL-1β and 20 ng/ml human recombinant TNF-α. These fibroblasts then were labeled with [^{35}S]methionine for 60 min in the absence or presence of the indicated concentration of SKF86002. Detergent extracts of the radiolabeled cells were prepared, and IL-1β was captured by immunoprecipitation. A, Immunoprecipitates were analyzed with SDS-gel electrophoresis and autoradiography; B, Regions of the dried gel containing the 31-kDa proIL-1β were excised, and the associated radioactivity (solubilized by pronase digestion) was determined. *Bars*, averages of duplicate determinations; *numbers in bars*, percent values relative to the control. This experiment was performed once.

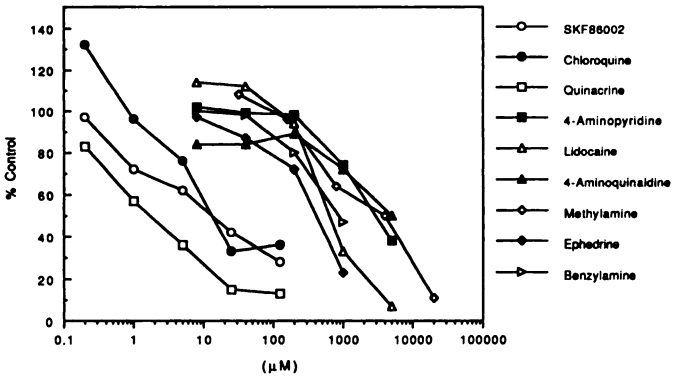

Fig. 8. SKF86002 and lysosomotrophic agents prevent LPS-induced release of IL-1β from fresh human monocytes. Monocytes were activated with a low dose (200 pg/ml) of LPS and then treated with a high dose (100 ng/ml) of LPS in the presence of the indicated test agent to promote IL-1 release (24). After the 2-step reaction, media supernatants were harvested and assayed for their content of IL-1β by ELISA. The quantity (expressed as a percent of the LPS-treated controls) is indicated as a function of test agent concentration. Each data point is the mean of triplicate determinations. SKF86002 was tested in this assay on two separate occasions and yielded IC_{50} values of 4 and 14 μM.

SKF86002 inhibits LPS-induced release of mature IL-1β from human monocytes by a different mechanism. When treated with levels of LPS in excess of 100 ng/ml, human monocytes release mature IL-1β into their medium (24); this release, however, is inefficient (25) and does not occur when monocytes are cultured overnight before LPS exposure (26). SKF86002 recently was reported to block this LPS-induced release mechanism; inhibition was demonstrated by activating monocytes with a low dose of LPS and subsequently stimulating release of IL-1 with a higher LPS concentration in the presence and absence of SKF86002 (24). With a similar two-step release assay, SKF86002 blocked LPS-induced release of immunoreactive IL-1β (Fig. 8); the IC_{50} for this activity under our culture conditions was approximately 10 μM. We observed, however, that the potency of SKF86002 as an inhibitor of this response was dependent on the medium in which the monocytes were maintained (data not shown); this medium dependence may account for the higher IC_{50} value observed in the present study compared with that previously reported (24). Structural features of SKF86002 suggest a potential to act as a lypophilic weak base (8); this bicyclic imidazole contains two weakly basic amines. Based on this, other weak base-like compounds were profiled in the LPS-induced release assay (Fig. 8). Although the potency of the different compounds varied, every weak base tested suppressed release of IL-1β promoted by high LPS concentrations. The most potent compounds tested were quinacrine and chloroquine, which possessed IC_{50} values of 2 and 12 μM, respectively. On the other hand, concentrations in excess of 1 mM of methylamine and 4-aminopyridine were required to suppress this response (Fig. 8).

Mature IL-1β release from monocytes and macrophages also can be achieved by treating these cells with ATP (26, 27). This release mechanism is inhibited by agents that block anion transport (26). Neither SKF86002 nor chloroquine, however, inhibited the ATP-induced release of mature IL-1β from LPS-activated human monocytes (data not shown)

In contrast to chloroquine's ability to mimic SKF86002's activity as an inhibitor of the LPS-induced posttranslational release of IL-1 from fresh monocytes, this agent was not an effective inhibitor of proIL-1 translation. In three separate experiments, the amount of proIL-1β recovered from aged LPS-activated monocytes treated with 50 μM chloroquine during a 60-min pulse labeling was reduced by no more than 36% relative to non-chloroquine-treated monocytes (Table 3). Increasing the chloroquine concentration to 100 μM did not lead to a significant increase in its inhibitory activity (Table 3). Likewise, 10 μM quinacrine lowered proIL-1β production by only 33%; 50 μM quinacrine significantly lowered proIL-1β production, but at this concentration total cell-associated radioactivity also was reduced, suggesting that the higher concentration impaired cell viability (Table 3). In contrast, LPS-activated monocytes labeled in the presence of 10 μM SKF86002 consistently produced 86–90% less proIL-1β than nontreated control cells (Table 3).

TABLE 3

Chloroquine and quinacrine are poor inhibitors of proIL-1β synthesis

Exp.	Condition	Total cell associated	ProIL-1β	
		cpm/LDH equivalent (×10⁶)	*Counts/LDH equivalent*	*% Control*
1	Control	0.73	5377	100
	+50 μM Chloroquine	0.62	5018	93
2	Control	5.47	2992	100
	+10 μM SKF86002	4.73	308	10
	+50 μM Chloroquine	5.39	1914	64
3	Control	2.46	5864	100
	+10 μM SKF86002	2.24	842	14
	+50 μM Chloroquine	2.35	4436	76
	+100 μM Chloroquine	2.06	3734	64
	+10 μM Quinacrine	2.30	3933	67
	+50 μM Quinacrine	0.70	933	16

Human monocytes were activated for 2 hr with LPS and then labeled for 60 min with [^{35}S]methionine in the absence or presence of indicated effector. The cells were washed twice with phosphate-buffered saline and harvested by detergent extraction. IL-1β was recovered by immunoprecipitation, and the immunoprecipitates were analyzed by SDS-gel electrophoresis and autoradiography. Radioactivity associated with proIL-1β (sum of 31- and 28-kDa species) was determined by pronase digestion (expt. 1) or by scanning the dried gel with a gas-phase radioactivity detector (expts. 2 and 3). Total cell-associated radioactivity recovered in the detergent extracts also was determined. Each value is an average of duplicate determinations.

Discussion

SKF86002 is the prototype member of a class of bicyclic imidazoles that act as cytokine inhibitors and possess anti-inflammatory activities (8). These agents do not inhibit phosphodiesterases, suggesting that their CSAID activity is not dependent on changes in cAMP levels (8). Likewise, their cytokine inhibitory activity occurs in the absence of major changes in mRNA levels of the affected cytokines (13), suggesting that they are not transcriptional inhibitors. Rather, it has been proposed that these agents inhibit cytokine translation via inhibition of a stress-activated protein kinase (14). LPS stimulation is known to induce activation of a stress-induced MAP-like kinase (28), but the exact role that this kinase serves in the signal transduction cascade remains unclear.

In the present study, SKF86002 acted as a very selective inhibitor of human monocyte protein production. In the presence of SKF86002, LPS-activated cells were impaired in their synthesis of proIL-1β but not in their production of lysozyme. Moreover, by two-dimensional gel analysis, only a relatively small number of monocyte protein products (approximately eight) were affected by the CSAID. At present, the identity of the affected polypeptides observed on the two-dimensional gel map (other than IL-1β) is unknown. Therefore, SKF86002 did not universally suppress all protein synthetic activity.

SKF86002's inhibition of proIL-1β production corresponded to a translational site of action. This agent did not alter the turnover of newly synthesized proIL-1β, indicating that it did not lower intracellular levels of proIL-1β by a posttranslational mechanism. Moreover, SKF86002 did not act as a transcriptional inhibitor. In contrast to actinomycin, which showed maximum inhibition when added to monocytes at the time of LPS activation, SKF86002 was most effective when added at the time of the biosynthetic labeling, consistent with a site of action directed toward a translational event. SKF86002 inhibition was observed even in the presence of maximally effective concentrations of actinomycin, further indicating a site of action for SKF86002 independent of transcription. Thus, in aged human monocytes, SKF86002's inhibition of proIL-1β production appears to result from impairment of this cytokine's translation. Because other monocyte proteins (e.g., lysozyme) were unaffected, this translational effect must be specific for proIL-1β and the other affected polypeptides. It has been suggested that this selectivity may involve the 3′ end of mRNAs that encode for cytokines such as proIL-1β and TNFα (14); these messages share a repeated AUUUA motif within their 3′ untranslated regions that affects stability and translatability (29).

The timing of SKF86002 sensitivity is interesting with respect to the proposal that CSAIDs act via inhibition of a stress-induced protein kinase. LPS application to CD14-positive cells is known to result in a rapid (within 15 min) activation of a MAP-like kinase (28). Once activated, many kinases phosphorylate their substrates, causing amplification and initiation of downstream signaling events, and, with time, the upstream signaling kinases may be inactivated. For example, LPS stimulation of mouse peritoneal macrophages leads to a rapid increase in tyrosine phosphorylation of several polypeptides and MAP kinase activation (30, 31); maximum activities are observed within 20–30 min of LPS addition, after which the levels of both tyrosine phosphorylation and MAP kinase decline so that by 60 min of LPS exposure MAP kinase activity is restored to a prestimulatory level (31). SKF86002 was maximally effective when added 2 hr after monocytes were activated with LPS, indicating that this agent did not have to be present during the initial activation stages to be effective. Moreover, when SKF86002 was added during LPS activation but subsequently removed during the radiolabeling phase, little inhibition of proIL-1β production was observed. Therefore, if an LPS-inducible kinase is the CSAID target, it appears that this enzyme does not act solely as an initiator of the LPS response as its activity is required for prolonged periods after stimulation. IL-1 recently was shown to activate a β-casein kinase that remained active for several hours after stimulation (32), signifying that long-lived kinases may be part of a stress-induced cascade.

The effectiveness of SKF86002 as an inhibitor of proIL-1β translation was dependent on the length of time for which monocytes were exposed to this agent. Treatment times of >2 hr led to loss of inhibitory activity. This response was not due to degradation or depletion of SKF86002, indicating that the decline in its effectiveness must result from a change in the sensitivity of the monocyte. The mechanism for this apparent adaptation response is unknown. Monocytes pretreated with GM-CSF also were more resistant to the inhibitory effects of SKF86002. This decreased sensitivity may result from a GM-CSF-induced increase in the number of CSAID-binding targets. Assuming that binding and inhibition occur to a rate-limiting enzyme in the biosynthetic pathway, then the same concentration of SKF86002 may provide less inhibition to GM-CSF-stimulated versus nonstimulated cells due to the enhanced number of binding sites. Alternatively, GM-CSF may alter the monocytes so that they become resistant to the inhibitory effects of the CSAID. Likewise, fibroblasts stimulated with a combination of IL-1β and TNF-α to produce proIL-1β demonstrated a reduced sensitivity to SKF86002

relative to that observed with LPS-activated monocytes. A combination of TNF-α and IL-1β was used to stimulate fibroblasts because these cells do not respond to LPS (23). Insensitivity of fibroblast IL-1β production to SKF86002 may indicate that these cells are resistant to the CSAID and/or that cytokine-induced IL-1β synthesis involves an activation mechanism distinct from that used by LPS. Importantly, IL-1 recently was shown to induce activation of a stress-induced kinase cascade (33, 34); assuming that the IL-1- and LPS-induced cascades involve the same stress-induced kinase, it is not clear why fibroblasts should be less sensitive to the inhibitory effect of SKF86002.

SKF86002 also interfered with the posttranslational release of IL-1β from monocytes that were treated with high concentrations of LPS. This inhibition can only be observed with freshly isolated human monocytes; neither aged human monocytes, murine peritoneal macrophages, nor human alveolar macrophages release IL-1β in response to LPS (26, 27, 35, 36). The extent of IL-1β release from LPS-treated fresh monocytes is variable and correlates with the release of the cytoplasmic enzyme marker LDH (25). SKF86002 inhibited LPS-induced release of immunoreactive IL-1β, confirming a previous observation (24). Moreover, a number of weak bases also inhibited the LPS-induced release mechanism. Compounds that demonstrated this activity are known to affect lysosomes and other intracellular compartments possessing low intravesicular pH values (37). This activity is believed to arise from partitioning of the nonprotonated lipophilic base into these organelles, followed by its protonation and subsequent entrapment; as a result, intravesicular pH rises and the organelles may swell (37). The concentration range in which each individual weak base demonstrated its activity with respect to inhibition of IL-1β release correlated well with its potency as a lysosomotrophic agent. For example, concentrations of 10–30 μM chloroquine, 0.5–1 mM ephedrine, and 1 to 10 mM methylamine are required to promote lysosomal vacuolization within mouse peritoneal macrophages (37). Likewise, these three agents inhibited IL-1β release with IC_{50} values of 12μM, 400μM, and 4 mM, respectively. The structure of SKF86002 possesses features that are similar to elements of the lysosomotrophic agents. Application of 10 μM SKF86002 to murine J774 cells did not result in an immediate change in lysosomal pH, but after longer incubations cytoplasmic vacuolization was observed (data not shown). Therefore, inhibition of LPS-induced IL-1β release from fresh monocytes appears to result from a weak base-like activity; it is not known whether this inhibition is due to a change in lysosomal and/or cytoplasmic pH or a resulting change in ionic homeostasis.

Importantly, the weak base chloroquine did not inhibit monocyte proIL-1β translation as effectively as did SKF86002. At concentrations greater than those required to mimic SKF86002's effect on IL-1β release, chloroquine inhibited proIL-1β synthesis by only 36%. Chloroquine and hydroxychloroquine are reported to inhibit IL-1 and TNF production (38, 39), and these agents are used as treatments for rheumatoid arthritis (40); their therapeutic mechanism of action remains unclear. The small inhibition of proIL-1β production observed *in vitro* in the presence of chloroquine and quinacrine may, under a different set of experimental conditions, result in a greater effect; these agents were tested only under conditions that resulted in maximal inhibition by SKF86002. It appears, therefore, that inhibition of proIL-1β translation and inhibition of LPS-induced cytokine release require different features of the CSAID molecule as chloroquine effectively mimicked only the latter of these two activities. In an intact cell, the overall effectiveness of SKF86002 is likely to depend on both of these activities and to be affected by the type of cell, its state of differentiation, and the length of time during which the cell is exposed to this CSAID.

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