

Rifampin Inhibits Prostaglandin E₂ Production and Arachidonic Acid Release in Human Alveolar Epithelial Cells[∇]

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Rifampin, a potent antimicrobial agent, is a major drug in the treatment of tuberculosis. There is evidence that rifampin also serves as an immunomodulator. Based on findings that arachidonic acid and its metabolites are involved in the pathogenesis of *Mycobacterium tuberculosis* infections, we investigated whether rifampin affects prostaglandin E₂ (PGE₂) production in human alveolar epithelial cells stimulated with interleukin-1 β . Rifampin caused a dose-dependent inhibition of PGE₂ production. At doses of 100, 50, and 25 μ g/ml, it inhibited PGE₂ production by 75%, 59%, and 45%, respectively ($P < 0.001$). Regarding the mechanism involved, rifampin caused a time- and dose-dependent inhibition of arachidonic acid release from the alveolar cells. At doses of 100, 50, 25, and 10 μ g/ml, it significantly inhibited the release of arachidonic acid by 93%, 64%, 58%, and 35%, respectively ($P < 0.001$). Rifampin did not affect the phosphorylation of cytosolic phospholipase A₂ or the expression of cyclooxygenase-2. The inhibition of PGE₂, and presumably other arachidonic acid products, probably contributes to the efficacy of rifampin in the treatment of tuberculosis and may explain some of its adverse effects.

The eicosanoids, a group of lipid mediators such as prostaglandins, thromboxane, leukotrienes, and lipoxins, are involved in diverse processes of the immune and inflammatory responses and in many physiological functions (3, 13, 28). The first step in eicosanoid production in response to bacterial products or inflammatory cytokines is the release of arachidonic acid (AA) from the cell membrane by the family of phospholipases A₂ (PLA₂). AA is then further metabolized by three major biosynthetic pathways: the cyclooxygenases (COX), which produce prostaglandins and thromboxane; the 5-lipoxygenase, which produces leukotrienes; and the 15-lipoxygenase, which produces lipoxins. Two COX isoforms, the constitutive COX-1 and the inducible COX-2, convert AA to an unstable intermediate, prostaglandin H₂, which is further metabolized by a series of specific synthases to various prostaglandins (3).

Among the prostaglandins, prostaglandin E₂ (PGE₂) is best known as a major regulator of the immune response and plays a key role in inflammation and in chronic inflammatory diseases. In the immune system, PGE₂ is active in the regulation of T- and B-cell responses, activation of antigen-presenting cells, and modulation of cytokine production. In addition, it serves as a potent vasodilator and has been implicated in the induction of inflammatory symptoms, such as edema, pain, and fever. Although studies have shown that PGE₂ promotes inflammation, there is growing evidence that it also exerts strong immunosuppressive effects, including shifting of the T-helper (Th) cell response from Th1 to Th2 cytokine production and inhibition of leukocyte chemotaxis and phagocytosis (1, 10, 13, 22, 28).

Recent data from animal models have demonstrated that AA and its metabolites play a crucial role in the pathogenesis of *Mycobacterium tuberculosis* infection. Free AA apparently stimulates phagosome maturation in infected macrophages and promotes apoptosis and antimicrobial activity, whereas its products, PGE₂ and lipoxin 4, downregulate cell-mediated immunity, resulting in mycobacterial growth and disease progression (2, 5, 7). One study found that treatment of mice with COX inhibitor at the late stage of mycobacterial disease increased the expressions of the proinflammatory cytokines and inducible nitric oxide synthase (iNOS) and decreased the bacterial loads (18). In another study, mice deficient in 5-lipoxygenase showed increased interleukin-12 (IL-12), gamma interferon (IFN- γ), and iNOS expression and were more resistant to *M. tuberculosis* infection than wild-type mice (2).

Rifampin is a potent antimicrobial agent and a major drug in the treatment of tuberculosis. Its antibacterial activity is mediated by the inhibition of bacterial RNA polymerase. There is evidence that rifampin also modulates the host immune response, influencing such functions as lymphocyte migration, cytokine production, antigen presentation, and phagocytosis (9, 14, 17, 21, 31). Recently, our group has demonstrated that rifampin augments iNOS expression and nitric oxide (NO) production in alveolar epithelial cells stimulated by cytokines (30).

Alveolar epithelial cells actively participate in the immune response in the lung by producing inflammatory mediators such as chemokines, cytokines, eicosanoids, and NO. It was reported that the pulmonary epithelial A549 cell line, which resembles alveolar epithelial cells, produces NO and IFN- γ upon infection with *M. tuberculosis*, suggesting that alveolar epithelial cells play an active role in innate immunity against mycobacteria (15, 19, 20).

In light of the increasing evidence of the involvement of lipid

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mediators, and particularly of PGE₂, in *M. tuberculosis* infection, we sought to determine whether rifampin affects PGE₂ production in A549 cells.

MATERIALS AND METHODS

Reagents. Cell culture medium and its supplements were obtained from Biological Industries (Beit HaEmek, Israel). Recombinant human IL-1 β was purchased from ProSpect-Tany TechnoGene Ltd. (Rehovot, Israel). Rifampin and A23187 were obtained from Sigma Chemical (St. Louis, MO). Stock solutions of rifampin (100 mg/ml) and A23187 (10 mM) were prepared in dimethyl sulfoxide and diluted at least 1:1,000 in culture media prior to use.

Cell culture. Human alveolar epithelial A549 cells were obtained from the American Type Culture Collection (ATCC) and maintained in F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), and nystatin (12.5 U/ml) (PSN) at 37°C in a humidified incubator with 5% CO₂. Cell exposure to IL-1 β and rifampin was conducted in serum- and PSN-free medium. Dimethyl sulfoxide, the vehicle of rifampin and A23187, at the highest concentration used (0.1%) had no effect on AA release (data not shown) or PGE₂ formation (16).

PGE₂ induction and determination. A549 cells were seeded in flat-bottomed 96-well microplates at a concentration of 1.5×10^5 cells/well and grown for 24 h. They were then incubated overnight in serum- and PSN-free medium. The cells were then exposed to IL-1 β (1 ng/ml) or IL-1 β with rifampin (10 to 100 μ g/ml) in fresh medium for 48 h. Supernatants were collected and centrifuged to remove cell debris and stored at -70°C. Each experiment was conducted in triplicate. The amount of PGE₂ was determined with an enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI), according to the manufacturer's instructions.

Measurement of cell viability. Cell viability was evaluated by the neutral red uptake viability assay (25). Briefly, after treatment, cells in 96-well microplates were incubated with neutral red dye at a final concentration of 0.05% in fresh medium for 1 h and washed with cold phosphate-buffered saline containing Ca²⁺ and Mg²⁺, followed by extraction of the neutral red with Sorenson's citrate buffer-ethanol mixture. Absorbance was read at 540 nm. Viability was assessed as the ratio of absorbance for treated cells to absorbance for untreated cells. No differences in viability among differently treated cells were found (data not shown).

Release of [³H]AA. Cells (2×10^6) were seeded in 3-cm plates and grown for 24 h. They were then labeled for 4 h with 0.5 μ Ci/ml of [5,6,8,9,11,12,14,15-³H]AA (7.36 TBq/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) in serum-free medium supplemented with L-glutamine. The cells were washed five times and incubated in serum-free medium with IL-1 β , IL-1 β with rifampin, or rifampin alone for the specified times; cells incubated with serum-free medium served as controls. The medium contained 1 mg/ml bovine serum albumin to absorb arachidonate metabolites. Unless otherwise specified, supernatants were collected after 4 h and centrifuged at $500 \times g$ for 5 min at 4°C, and 0.5 ml of supernatant was transferred to a scintillation vial containing 10 ml of Ultima Gold scintillation liquid (PerkinElmer, Boston, MA) and counted in a scintillation counter (Tri-Carb; Packard). In all cases, [³H]AA incorporation into cells was measured by harvesting cells in 0.5 ml NaOH (1 N) and a liquid scintillation counting. In each specimen, the release of [³H]AA was expressed as a percentage of the total radioactivity incorporated into cells (16). The inhibition of AA release was calculated according to the following formula: percent inhibition = $100 \times [(IL-1\beta - c'm) - (IL-1\beta - rif - c'rif)] / (IL-1\beta - c'm)$, where IL-1 β represents percent release of AA in cells treated with IL-1 β , IL-1 β -rif represents percent release in cells treated with IL-1 β together with rifampin, c'rif represents percent release in cells treated with rifampin alone, and c'm represents percent release in cells incubated in medium alone. The [³H]AA incorporation into the cells was estimated by subtracting the radioactivity in the supernatant at the end of the labeling time from the radioactivity added to the cells. The percentage of [³H]AA incorporation into the cells typically ranged from 70 to 80%.

Western blot analysis for cPLA₂ and COX-2. A549 cells were seeded in 3-cm dishes (2×10^6 /plate), grown for 24 h, and incubated in serum- and antibiotic-free medium for 24 h before exposure to IL-1 β and rifampin. At the indicated time, cells were washed twice with cold phosphate-buffered saline, lysed using a buffer (50 mM Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.1% bromophenol blue, 1.25% 2-mercaptoethanol), and denatured at 95°C. Equal amounts of protein (20 to 30 μ g) from total cell extracts estimated by bicinchoninic acid reagent (Pierce, Rockford, IL) were loaded onto 7.5% SDS-polyacrylamide gel electrophoresis gel for cytosolic PLA₂ (cPLA₂) and phosphorylated cPLA₂ and 10% SDS-polyacrylamide gel electrophoresis gel for COX-2 and transferred onto a polyvinylidene difluoride membrane (Amersham

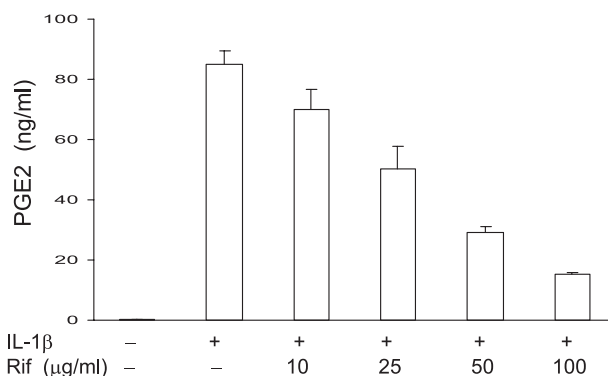


FIG. 1. Rifampin (Rif) inhibition of PGE₂ production in A549 cells. Cells were stimulated with IL-1 β (1 ng/ml) in the presence of rifampin for 48 h. Results are given as means \pm standard errors for one of three similar experiments performed in triplicate.

Biosciences, Piscataway, NJ). Nonspecific binding sites were blocked at room temperature with 5% milk in TBST (20 mM Tris [pH 7.8], 150 mM NaCl, 0.1% Tween 20). The membranes were then incubated overnight at 4°C with the following antibodies: mouse polyclonal anti-cPLA₂ diluted 1:1,000 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-cPLA₂ (Ser505) diluted 1:1,000 (Cell Signaling, Danvers, MA), rabbit anti-COX-2 diluted 1:1,000 (Santa Cruz), or goat anti-actin polyclonal diluted 1:200 (Santa Cruz). The membranes were then washed and incubated with a secondary antibody (Santa Cruz) as appropriate, namely, horseradish peroxidase-linked anti-rabbit (diluted 1:2,000), anti-mouse (diluted 1:5,000), or anti-goat immunoglobulin (diluted 1:10,000), at room temperature for 1 h and washed three times with TBST. Bound antibodies were detected by the enhanced-chemiluminescence method. Densitometry was performed using VersaDoc (Bio-Rad Laboratories, Inc., Hercules, CA).

Statistical analysis. Data were analyzed using BMDP software (BMDP statistical software [1993]; W. J. Dixon [chief editor], University of California Press, Los Angeles, CA). The results are presented as means \pm standard errors of the means. Statistical comparisons were performed using analysis of variance for multiple comparisons after verifying the normal distribution of the data tested. Since three or four comparisons were made, significance was a priori defined as *P* values of ≤ 0.01 .

RESULTS

Effect of rifampin on PGE₂ formation. The incubation of A549 cells with IL-1 β led to a time- and dose-dependent production of PGE₂. PGE₂ concentrations in the supernatants after 48 h of incubation with IL-1 β (1 ng/ml) ranged from 20 to 100 ng/ml (in four different experiments). The addition of rifampin to IL-1 β caused a marked concentration-dependent decrease in PGE₂ production (Fig. 1). Rifampin at 100, 50, and 25 μ g/ml reduced the levels of PGE₂ induced by IL-1 β by 75%, 59%, and 45%, respectively (*P* < 0.001 for IL-1 β versus IL-1 β with rifampin at 100, 50, and 25 μ g/ml; *n* = 9 for IL-1 β and IL-1 β with each dose of rifampin). Unstimulated A549 cells produced minor amounts of PGE₂ (30 to 70 pg/ml).

Effect of rifampin on IL-1 β -stimulated AA release. To examine the mechanism of PGE₂ inhibition by rifampin, we initially investigated whether rifampin affects the first step in PGE₂ formation, the release of AA from cell membranes. The incubation of cells with IL-1 β alone induced a time- and dose-dependent release of AA into the medium, which was evident already 2 h after the addition of IL-1 β and increased gradually over 20 h. There was also a minimal AA release over time from control, untreated cells. The effect of rifampin on IL-1 β -induced AA release was measured at 4 h after stimulation, when

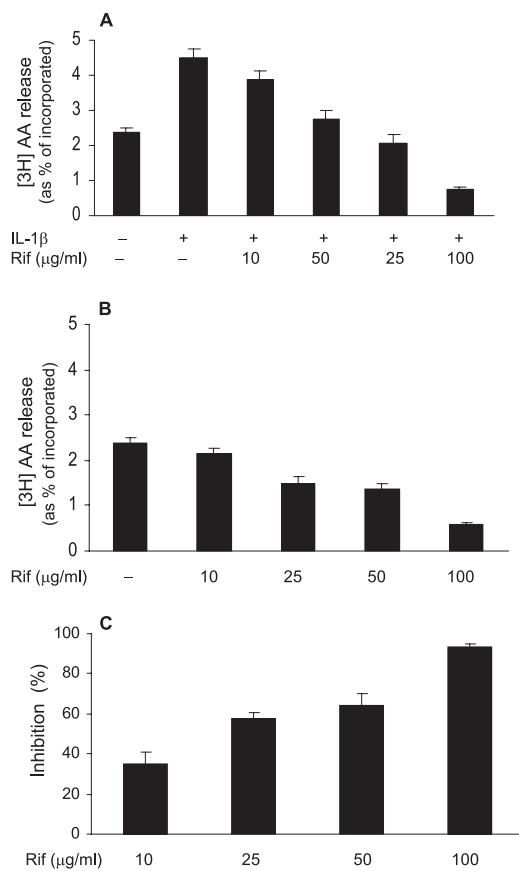


FIG. 2. Rifampin (Rif) inhibition of AA release from A549 cells. (A) Percent release of [³H]AA from cells incubated with IL-1β (1 ng/ml) alone or with different doses of rifampin. (B) Percent release of [³H]AA from cells incubated with medium alone or with different doses of rifampin. (C) Inhibition of IL-1β-stimulated AA release by rifampin. Amount of [³H]AA released into the medium 4 h after stimulation was determined and expressed as a percentage of the total radioactivity incorporated. Inhibition of [³H]AA by rifampin was calculated as described in Materials and Methods. Results are given as means ± standard errors. *P* was <0.001 for IL-1β versus IL-1β plus Rif at 100 (*n* = 5), 50 (*n* = 10), 25 (*n* = 8), and 10 (*n* = 6) μg/ml.

there was a significant increase in AA release from IL-1β-treated cells compared to that from control, unstimulated cells ($4.5\% \pm 0.2\%$ versus $2.4\% \pm 0.1\%$; *P* < 0.001). The incubation of A549 cells with IL-1β in the presence of rifampin caused a marked dose-dependent reduction in AA release (Fig. 2A). Rifampin also inhibited the spontaneous release of AA from the cells in a dose-dependent manner (Fig. 2B). This dose-dependent mechanism was taken into account when the inhibition by rifampin of IL-1β-induced release was calculated (see Materials and Methods). The inhibition of AA release by rifampin was significant at concentrations of 10 to 100 μg/ml (*P* < 0.001 for IL-1β versus IL-1β plus rifampin at 100, 50, 25, and 10 μg/ml) (Fig. 2C).

A potent inhibition of AA release by rifampin was evident already 2 h after stimulation, and it gradually increased with time (Fig. 3). Rifampin (50 μg/ml) inhibited AA release at 2, 6, and 19 h by 53%, 79%, and 95%, respectively (*P* < 0.001 for IL-1β with rifampin at 2 versus 19 h). The inhibition of the basal release of AA by rifampin was milder and slightly de-

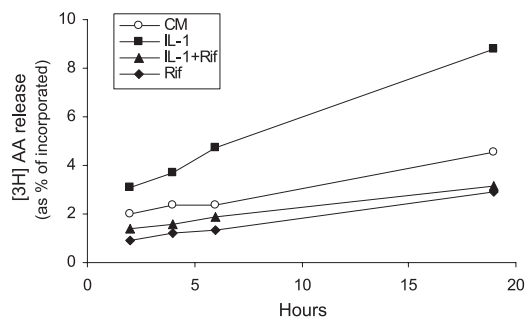


FIG. 3. Time course of [³H]AA release from A549 cells stimulated with IL-1β (1 ng/ml) in the presence or absence of rifampin (Rif; 50 μg/ml). Amounts of [³H]AA released into the medium at various times by cells incubated with medium alone (CM) or with the addition of IL-1β, IL-1β with rifampin, or rifampin alone were determined and expressed as percentages of the total radioactivity incorporated. Results are given as means for two independent experiments (*n* = 5 in each group).

creased with time, from 47% at 2 h to 34% at 19 h (*P* = 0.008 for rifampin alone at 2 versus 19 h).

Effect of IL-1β and rifampin on cPLA₂ expression and phosphorylation. cPLA₂ is the main enzyme that cleaves AA from phospholipids for eicosanoid generation (6), and it reportedly regulated AA release in A549 cells in response to IL-1β (15). The expression of cPLA₂ and its activity are regulated at the transcriptional level and by two posttranslational steps: elevation of intracellular calcium and phosphorylation. To investigate whether rifampin inhibits AA release through a decrease in cPLA₂ protein expression or phosphorylation, Western blot analysis was performed. As shown in Fig. 4, incubation with IL-1β did not increase total cPLA₂ levels up to 4 h, when a significant IL-1β-induced AA release was measured. An increase in IL-1β-induced cPLA₂ phosphorylation was observed at 5 min, with a maximal effect at 15 min, which declined at 30 min (Fig. 4). There was no further increase in the amount of phosphorylated cPLA₂ in IL-1β-treated cells up to 4 h after stimulation (data not shown). The addition of rifampin had no effect on IL-1β-induced cPLA₂ phosphorylation.

Effect of rifampin on Ca ionophore-stimulated AA release. It has been previously reported that an increase in calcium levels is sufficient to induce AA release in A549 cells (26). To examine the mechanism of rifampin inhibition of AA release, we investigated whether rifampin also inhibits the release of AA stimulated by Ca²⁺ ionophore A23187. Incubation of cells with A23187 caused an increase in AA release already at 45 min, earlier than in response to IL-1β: $0.92\% \pm 0.1\%$ in untreated cells, $2.59\% \pm 0.2\%$ with A23187, and $1.29\% \pm 0.1\%$ with IL-1β (*P* < 0.001 and *P* = 0.5 for untreated cells versus A23187 and IL-1β, respectively). The addition of rifampin to A23187 did not significantly inhibit AA release at 45 min, 2 h, or 4 h after stimulation. The decreases in AA release at 2 h and 4 h in the presence of rifampin (50 μg/ml) measured $5.5\% \pm 4.1\%$ (*n* = 6 for each group in two separate experiments) and $8.3\% \pm 4.7\%$ (*n* = 9 for each group in three separate experiments), respectively.

Effect of rifampin on IL-1β-induced expression of COX-2. We examined whether rifampin also affects the next steps that regulate PGE₂ formation, namely, the expression of COX-2 en-

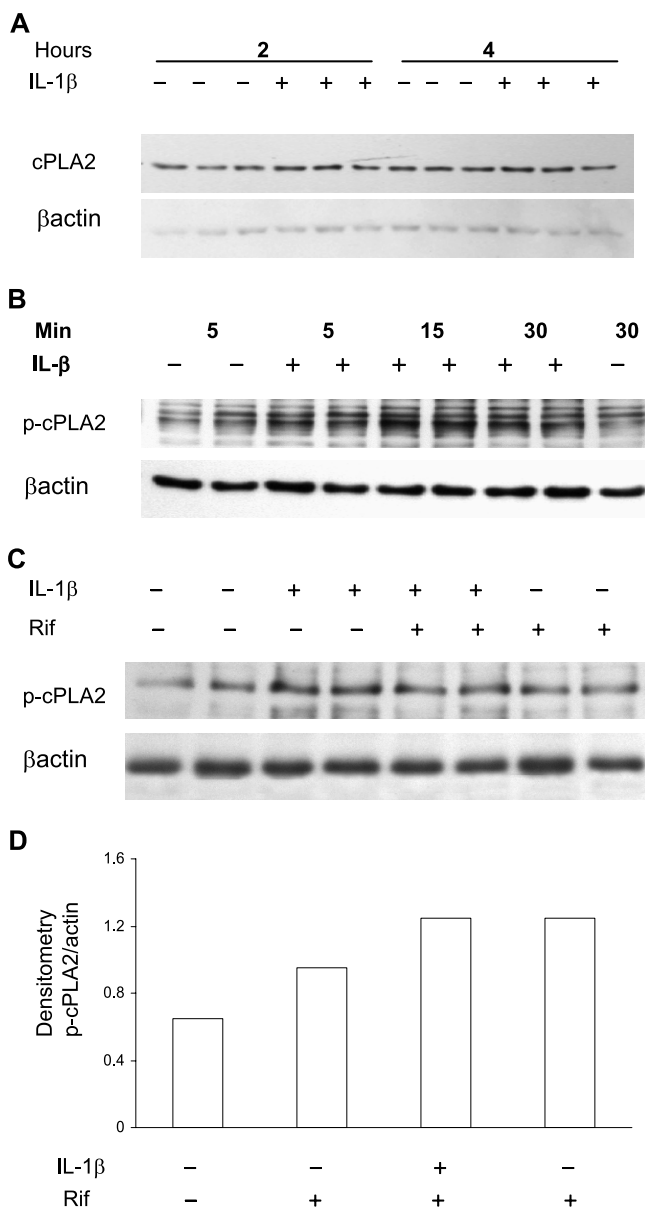


FIG. 4. Western blot analysis of effects of IL-1 β and rifampin (Rif) on cPLA₂ expression and phosphorylation in A549 cells. (A) Total cPLA₂ expression in cells treated with IL-1 β (10 ng/ml) for 2 and 4 h. (B) Phosphorylated cPLA₂ (p-cPLA₂) in cells treated with IL-1 β for different times. (C) Phosphorylated cPLA₂ in cells treated with IL-1 β or IL-1 β with rifampin (50 μ g/ml) for 15 min, compared to β -actin. (D) Densitometric analysis of the blot presented in panel C. Similar results were obtained in at least three separate experiments.

zyme, induced by IL-1 β in A549 cells. As shown in Fig. 5, a very low expression level of COX-2 was detected in control, untreated cells, which increased strongly in response to the addition of IL-1 β . The incubation of cells with IL-1 β together with rifampin did not significantly change the expression of COX-2.

DISCUSSION

The present study demonstrates that rifampin, one of the most effective drugs in treatment of tuberculosis, strongly in-

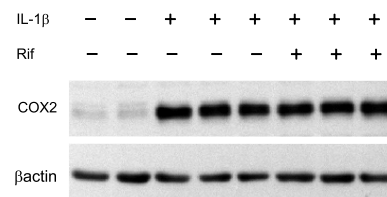


FIG. 5. Western blot analysis of the effects of IL-1 β and rifampin (Rif) on COX-2 expression in A549 cells. COX-2 expression at 24 h after stimulation with IL-1 β (1 ng/ml) or IL-1 β with rifampin (50 μ g/ml) is compared to β -actin expression. Similar results were obtained in at least three separate experiments.

hibits IL-1 β -induced AA release and PGE₂ production in human alveolar A549 cells.

AA and its metabolites, particularly PGE₂, are important mediators in the host response against mycobacterial infection. Although AA apparently promotes mycobacterial elimination, its products through the COX and lipoxygenase pathways, PGE₂ and lipoxin, suppress the immune response to mycobacteria (2, 5, 7). PGE₂ at high concentrations is a potent inhibitor of the Th-1 cytokine response and suppresses the production of tumor necrosis factor alpha (TNF- α), IFN- γ , and NO, which are critical for macrophage activation and mycobacterial killing (5, 18). Studies with animal models of tuberculosis and with human macrophages infected with *Mycobacterium* spp. demonstrated that PGE₂ inhibited the production of proinflammatory cytokines, such as TNF- α , IL-1 β , and NO; increased the levels of the anti-inflammatory cytokine IL-10; and suppressed macrophage and T-lymphocyte effector functions against mycobacteria (5, 24, 29). Recently, using a mouse model of pulmonary tuberculosis, Rangel Moreno et al. (18) showed that high concentrations of PGE₂ are present at the late stage of the disease and are involved in the downregulation of cell-mediated immunity, thereby contributing to disease progression. The inhibition of PGE₂ by a COX inhibitor at the late stage of the disease increased iNOS, TNF- α , and IFN- γ expression, with a significant decrement in bacillary load. By contrast, a low concentration of PGE₂ at the early stage of the disease increased iNOS expression and mycobacterial killing. Thus, rifampin inhibition of PGE₂ production most probably contributes to the immune defense against mycobacteria.

Rifampin inhibition of AA release may also affect other molecular pathways relevant to antimycobacterial host defense. Most recently, lipoxin A4, a product of AA through the lipoxygenase pathway, has been implicated in host control of mycobacterial infection. Bafica et al. (2) reported that mice deficient in 5-lipoxygenase showed increases in IL-12, IFN- γ , and iNOS mRNA expression and significantly decreased bacterial burdens compared to wild-type mice. This phenomenon was reverted by the administration of the lipoxin A4 analog. The inhibition of AA release by rifampin most probably decreases the production of lipoxin 4 as well, in addition to presumably other products of the lipoxygenase pathway, which together with the inhibition of PGE₂ production enhance the immune response against mycobacterial infection. These may further contribute to the efficacy of rifampin in tuberculosis.

The inhibition of PGE₂ formation by rifampin may be beneficial in other infections as well. Aronoff et al. (1) demon-

strated that PGE₂ suppressed phagocytosis of *Klebsiella pneumoniae* and *Escherichia coli* by rat alveolar macrophages, suggesting that PGE₂ inhibition could be useful in the treatment of pneumonia. In another study, PGE₂ promoted the growth of *Trypanosoma cruzi* in infected macrophages, and COX inhibitors (aspirin, indomethacin) almost completely abolished parasitemia in infected mice (8). These authors proposed that COX inhibitors may serve as an additional pharmacological intervention in *Trypanosoma* infections. Our findings may imply that rifampin might also be useful in increasing the host responses against these infections.

The inhibition of AA release by rifampin may have many immunological and physiological consequences, given that AA by itself is an important signaling molecule and a precursor of many lipid mediators, including the endocannabinoids (3). It is recognized that rifampin is also immunosuppressive and exerts steroidlike activities, especially in the brain, and there is even a debate on whether it activates the glucocorticoid receptor (4, 11, 12, 27). In fact, the inhibitory effect of rifampin on AA and PGE₂ release in the present study closely resembled steroid activity. It is possible, therefore, that some of the adverse effects of rifampin, such as its psychotropic influence, stem from its ability to profoundly suppress the production of AA, PGE₂, and presumably other eicosanoids.

The inhibition of PGE₂ production is a result of the profound reduction in AA release caused by rifampin. However, the mechanism by which rifampin inhibits AA release is unclear. Rifampin did not reduce the IL-1 β -induced expression of COX-2, which indicates that it did not affect the IL-1 β receptor but rather interfered with signal transduction. cPLA₂ activity is regulated by two posttranslational steps: elevation of intracellular Ca, which allows enzyme translocation from the cytosol to the nuclear envelope and endoplasmic reticulum, and phosphorylation (6). Our study showed that rifampin had no effect on cPLA₂ phosphorylation. On the other hand, it also did not inhibit AA release by ionophore-stimulated-Ca influx from the outside environment. This may imply that rifampin does not directly inhibit cPLA₂ activity but rather may interfere with its activation, possibly through the inhibition of Ca mobilization from intracellular sources. Indeed, others have reported that rifampin may interfere with the entrance of calcium, magnesium, and zinc into the cells (9).

The inhibition of AA release and PGE₂ formation was achieved with pharmacologically relevant concentrations of rifampin, since its peak concentration in the serum following the usual 600-mg dose is 10 μ g/ml, whereas in other tissue and fluids, rifampin may reach higher levels (17, 23).

In conclusion, our findings indicate that in addition to its antibacterial activity, rifampin is a strong immunomodulatory agent. Its inhibitory effect on PGE₂ production, together with its ability to increase NO production, as demonstrated in our previous study (30), most likely contributes to the control of *M. tuberculosis* as well as of other infections and may explain some of the adverse effects of the drug.

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