Rifampin Augments Cytokine-Induced Nitric Oxide Production in Human Alveolar Epithelial Cells

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Rifampin increased nitric oxide production and inducible nitric oxide synthase expression in alveolar cells stimulated with cytokines. Nitric oxide concentrations after induction with cytokines, cytokines with 10 μg/ml rifampin, and cytokines with 50 μg/ml rifampin were 3.2, 4.5, and 8.8 μM, respectively (P < 0.02 versus cytokines alone). This indicates that rifampin modulates the immune response.

In recent years, it has become apparent that antibiotics, in addition to having antibacterial activity, modulate the production of cytokines and inflammatory mediators (13) and may therefore have a significant influence on innate immunity and inflammation.

Nitric oxide (NO) is one of the main mediators of the host defense against infectious diseases. It is produced by inducible NO synthase (iNOS) at the site of infection in response to defense against infectious diseases. It is produced by inducible inflammation.

Recent data from animal models and human studies show that NO takes part in the immune defense against Mycobacterium tuberculosis. In mice deficient in iNOS (iNOS−/−) or mice treated with iNOS inhibitors, infection with M. tuberculosis was associated with significantly higher rates of bacterial dissemination and mortality (4, 8). There is also evidence that in humans, NO is synthesized by macrophage and pulmonary alveolar epithelial cells infected with M. tuberculosis (3, 14) and that the NO produced is bactericidal against M. tuberculosis (10).

Rifampin is recognized as one of the most effective drugs in the treatment of mycobacterial infections. Several authors reported that rifampin also exerts immunosuppressive effects and can modulate cytokine induction (2, 12). Studies of mouse macrophages showed that rifampin either had no effect on NO production (9) or inhibited NO, TNF-α, and IL-10 production (5). Little is known about the effect of rifampin on NO production in human cells.

In view of the important role of NO in controlling tuberculosis, we investigated whether rifampin influences the release of NO in human alveolar epithelial cells stimulated with IL-1β, IFN-γ, and TNF-α.

For determination of NO production, human alveolar epithelial A549 cells (maintained in F-12 medium) were seeded in flat-bottomed microwell plates at a concentration of 1.5 × 104 cells/well and grown for 24 h. They were then incubated in serum-free medium for 24 h before stimulation. The cells were exposed to a mixture of IL-1β, IFN-γ, and TNF-α (100 ng/ml each; ProSpect-Tany TechnoGene Ltd., Rehovot, Israel), alone or together with rifampin (10 to 100 μg/ml; Sigma Chemical). Each experiment was conducted in triplicate.

Incubation of A549 cells with IL-1β, IFN-γ, and TNF-α led to time-dependent release of NO. When A549 cells were stimulated with cytokines in the presence of rifampin, there was a marked concentration-dependent augmentation of NO production (Fig. 1). The NO concentrations in cell supernatant after 48 h incubation with cytokines alone were 3.2 ± 0.27 μM. After the addition of rifampin, values rose to 10.1 ± 1.38 μM with 100 μg/ml rifampin (P = 0.04), 8.8 ± 0.74 μM with 50 μg/ml rifampin (P = 0.004), and 4.5 ± 0.43 μM with 10 μg/ml rifampin (P = 0.03) (12 to 14 cell cultures) (by analysis of variance for repeated measures). Unstimulated A549 cells or cells treated with rifampin alone did not produce detectable amounts of NO (Fig. 1). The nitrite (NO2−) concentrations were measured as an indicator of NO production by the spectrophotometry method based on the Griess reaction (16). Because the red color of rifampin interferes with the color of the Griess reaction mixture, controls of rifampin in medium, at the relevant concentrations, were included in each assay. Optical density values of rifampin alone were subtracted from the optical density values of the cell supernatants.

To evaluate whether the increase in NO concentration in the rifampin-treated cells was due to the increase in iNOS, we examined the expression of iNOS protein in total cell extracts 20 h after exposure and compared the findings to β-actin expression. Equal amounts of protein (50 to 70 μg), from total cell extracts estimated by BCA reagent (Pierce, Rockford, IL), were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and examined by Western blot analysis. Incubation with a specific anti-NOS2 rabbit polyclonal antibody (diluted 1:1,200) and an anti-actin goat polyclonal antibody (diluted 1:200) was performed in parallel for 20 h at 4°C. The blots were then incubated with a secondary antibody, horseradish peroxidase-linked anti-rabbit (diluted 1:2,500) or anti-goat (diluted 1:10,000) immunoglobulin, for 1 h at room temperature. The blots were detected by the enhanced chemiluminescence method. Densitometry was performed using VersaDoc (Bio-Rad Laboratories, Inc.). Antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).
As shown in Fig. 2, the coinoculation with rifampin significantly increased the expression of iNOS protein in a concentration-dependent manner. Rifampin alone did not induce iNOS expression (data not shown).

There is growing evidence of the crucial role of NO in the host defense against M. tuberculosis. Interestingly, drugs that cure M. tuberculosis infection appear to be more successful in immunocompetent patients than in immunosuppressed patients (11, 14), suggesting that they might exert their effect also via activation of the immune system. The results of our study indicate that the elevation of NO production is probably one of the mechanisms by which rifampin activates the immune host response.

To the best of our knowledge, this is the first report showing that rifampin enhances the production of NO. Rifampin is usually described as an immunosuppressive drug, based on in vitro studies, although clinical observations did not support this notion (2, 11). Pahlevan et al. (12) showed that, in vitro, in the human Jurkat T-cell line, rifampin analogues, including rifampin, inhibited TNF-α- and phorbol myristate acetate-induced activation of the transcription nuclear factor κB, which is involved in immunostimulation, thereby explaining the mechanism of its immunosuppressive effect. However, they report that rifamides were unable to block lipopolysaccharide-induced TNF production from human peripheral blood monocytes. Other studies showed that rifampin had no effect on NO but inhibited TNF-α production and suppressed phagocytosis in mouse macrophages (9) or that rifampin inhibited NO, TNF-α, and IL-1β in murine macrophages infected with M. tuberculosis (5). This is in agreement with our observation that rifampin inhibited NO release in murine macrophages stimulated with lipopolysaccharide (unpublished data).

The discrepancy between the results is not surprising, since there is a distinct difference between the production of NO in mice and in humans and the mechanisms regulating NO production are cell and species specific (6).

The increase in NO levels was significant at rifampin concentrations of 10 to 100 μg/ml. These are pharmacologically relevant concentrations, since the peak concentration of rifampin in the serum, following a 600-mg dose, is 10 μg/ml, but in other tissues and fluids, rifampin may reach higher levels (12, 15).

The considerable increase in NO release induced by rifampin in cytokine-stimulated cells may have clinical implications. In addition to its multiple activities in the immune system, which include modulation of cytokine production, NO mediates major functions, such as smooth muscle relaxation and neurotransmission. High levels of NO can be deleterious to the host; thus, it is possible that some of the many adverse effects of rifampin, such as gastrointestinal discomfort, hypersensitivity reactions (eosinophilia), and influenza-like syndromes (arthralgias and myalgias) (15), are consequences of the increase in NO production. Further investigation of the action of rifampin on other human cells may help to elucidate its benefits and disadvantages in other infections.

**REFERENCES**