Ursolic Acid Reduces *Mycobacterium tuberculosis*-Induced Nitric Oxide Release in Human Alveolar A549 cells

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Alveolar epithelial cells have been functionally implicated in *Mycobacterium tuberculosis* infection. This study investigated the role of ursolic acid (UA)—a triterpenoid carboxylic acid with potent antioxidant, anti-tumor, anti-inflammatory, and anti-tuberculosis properties in mycobacterial infection of alveolar epithelial A549 cells. We observed that *M. tuberculosis* successfully entered A549 cells. Cytotoxicity was mediated by nitric oxide (NO). A549 toxicity peaked along with NO generation 72 h after infection. The NO generated by mycobacterial infection in A549 cells was insufficient to kill mycobacteria, as made evident by the mycobacteria growth indicator tube time to detect (MGIT TTD) and viable cell count assays. Treatment of mycobacteria-infected cells with UA reduced the expression of inducible nitric oxide synthase, NO generation, and eventually improved cell viability. Moreover, UA was found to quench the translocation of the transcription factor, nuclear factor kappa B (NF-kB), from the cytosol to the nucleus in mycobacteria-infected cells. This study is the first to demonstrate the cytotoxic role of NO in the eradication of mycobacteria and the role of UA in reducing this cytotoxicity in A549 cells.

INTRODUCTION

*Mycobacterium tuberculosis* is the etiological agent of tuberculosis (TB). Inhalation of *M. tuberculosis* into the lungs leads to infection (Smith et al., 1966). The host response first involves cells of the innate immune system. Among these cells, macrophages and epithelial cells are vital in host defense. However, alveolar epithelial cells are also infected by mycobacteria (Bermudez and Young, 1994; Mapother and Sanger, 1984; Shepard, 1955). It is possible that both alveolar epithelial cells and mycobacteria play important roles in the quelling and establishment of TB infection, respectively.

Little is known about the role of alveolar epithelial cells in the pathogenesis of TB. One of the likely mediators of antimycobacterial activity is nitric oxide (NO), which is produced by oxidation of L-arginine by the enzyme nitric oxide synthase (NOS) (Stuer et al., 1989). In macrophages, NO, with other toxic superoxide radicals within acidic phagosomes, is vital in limiting mycobacteria (Chan et al., 1992). However, excessive generation of NO may lead to cytotoxic effects and DNA damage, which may lead to cell death through activation of p53 and poly (ADP-ribose) polymerase (Eizirik et al., 1996; Ignarro, 1998). The role of NO in alveolar epithelial cells during mycobacteria infection is not well elucidated.

Ursolic acid (UA; 3-beta-3-hydroxy-urs-12-ene-28-oic-acid) is a pentacyclic triterpenoid carboxylic acid with several biological and pharmacological effects, including anti-inflammatory, anti-oxidant, anti-proliferative, anti-cancer, anti-mutagenic, anti-atherosclerotic, anti-hypertensive, anti-leukemic, and antiviral activities in a number of experimental systems (Ikeda et al., 2008; Tsai and Yin, 2008). Additionally, recent studies demonstrated the anti-TB effects of UA through immunomodulation and activation of intracellular mycobactericidal activity (Jiménez-Arellanes et al., 2013; Podder et al., 2015). UA is found in a number of foods, including apples, basil, bilberries, cranberries, elder flower, peppermint, rosemary, lavender, oregano, thyme, hawthorn, and prunes (Liu, 1995). Natural compounds enriched in terpenoids have a strong potential to act as inhibitors of the activation of the immune-regulatory transcription factor, nuclear factor-kappa B (NF-kB) (De las Heras et al., 2003).

The present study involved alveolar epithelial A549 cells. This cell line has been used to study various intracellular pathogens such as *Mycoplasma pneumonia* and *Chlamydia pneumonia* (Yang et al., 2002; 2003). Infection of A549 cells by *M. tuberculosis* upregulates inducible nitric oxide synthase (iNOS) and increases NO levels (Roy et al., 2004). However, the excessive generation of NO can be cytotoxic for the neighboring cells (Eizirik et al., 1996; Ignarro, 2000), and administration of certain compounds may be required to improve cell viability. Since macrophages and epithelial cells in the lung may respond differently to mycobacterial infection and/or UA treatment, the present study explored the effect of UA on mycobacteria-infected lung epithelial cells. However, the role of infection
and/or UA treatment in the context of cell survival and NO generation in *M. tuberculosis*-infected epithelial cells was explored.

**MATERIALS AND METHODS**

**Alveolar epithelial cells, chemicals, and treatment conditions**

Type II human alveolar A549 cells were purchased from American Type Culture Collection (USA). Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) antibiotic/antimycotic cocktail (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Invitrogen, USA) in a humidified atmosphere of 5% CO₂ at 37°C. Cells were seeded (5 × 10⁵ cells/ml) in six-well tissue culture plates overnight until they reached a confluency of 75-85%. Immediately before infection or treatment, cells were replaced with serum-free DMEM media. UA and the competitive nitric oxide synthase inhibitor, N⁵-monomethyl-L-arginine (L-NMMA), were obtained from Sigma-Aldrich (USA). Throughout the study, cells were treated with 10 μg/ml UA and 5 mM L-NMMA for 6 h following establishment of *M. tuberculosis* infection and then incubated for the desired times.

**Mycobacteria**

*M. tuberculosis* H37Rv was purchased from ATCC and cultured in Middlebrook 7H11 agar for 22 days. Isolated colonies were inoculated in Middlebrook 7H9 broth in a shaking incubator for 15 days. Because of the tendency of mycobacteria to form clumps, the suspension was vortexed vigorously and passed through an 8-μm filter to form a single-cell suspension. The suspension was allowed to stand for 3 min, the upper two-thirds was removed, and the cell concentration was measured at 600 nm using McFarland standards. In addition, 10 μl bacterial suspension was inoculated in Middlebrook 7H11 agar to establish the exact number of bacteria in the inoculum. The measured bacterial suspension was dispensed in aliquots and kept as a single lot at -20°C. Infection of A549 cells was carried out using this lot at an infecting ratio of cells to bacteria of 1:10.

**Infection of A549 cells by *M. tuberculosis* H37Rv and resazurin assay**

A549 cells (1 × 10⁵ cells/ml) were grown in a six-well tissue culture plate overnight. The culture medium was removed and replenished by warm DMEM without FBS. Cells were infected with *M. tuberculosis* H37Rv (multiplicity of infection 10) for the desired time at 37°C in a 5% CO₂ atmosphere. Following incubation, cells were washed three times with warm phosphate-buffered saline (PBS) to remove extracellular bacteria. The final extracellular wash medium was collected and 100 μl was inoculated in 4.9 ml of Middlebrook 7H9 broth followed by growth in a shaking incubator for 15 days. The intracellular bacteria were released by incubation of cells with 1 ml 0.1% Triton X-100 in sterile water for 10 min. Samples were harvested and vigorously vortexed. One hundred microliters was inoculated in 4.9 ml of Middlebrook 7H9 broth and incubated for 15 days as before described. Following incubation, a resazurin assay was performed. In brief, 200 μl of culture grown in 7H9 medium was added to a 96-well tissue culture plate with unincubated medium as the negative control for mycobacterial growth. Then, 30 μl of 0.02% resazurin was added and incubated for 24 h. The representative picture was taken, and fluorescence intensity was measured in a Victor™ X3 Multilabel reader (Perkin Elmer, USA) at excitation and emission wavelengths of 530 and 590 nm, respectively. Both the final wash medium and suspension following cell lysis were also plated on 7H11 agar to confirm the absence of extracellular mycobacteria (data not shown).

**Cell viability assay**

The cell viability assay is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystal by mitochondrial dehydrogenase enzyme. A549 cells were grown in a 96-well tissue culture plate overnight to a confluency of about 80%. The cells were infected and/or treated as required. Following incubation, 20 μl of 5 mg/ml MTT were added to 200 μl of cell suspension and incubated at 37°C for 4 h. The medium was aspirated and the purple formazan crystal was dissolved by adding 100 μl of dimethyl sulfoxide. After 30 min of incubation at 37°C in the dark, the absorbance was measured at 570 nm using a Victor™ X3 Multilabel reader (Perkin Elmer, USA).

**NO release assay**

The concentration of nitrite produced as a means to measure NO was measured using Griess reagent system (Promega, USA). In brief, supernatants of infected and/or treated cells for specified time points were collected and centrifuged at 400 × g for 8 min to remove cells. In the meantime, nitrate standards were prepared by diluting 100 μM nitrate solution up to 1.56 μM by two-fold dilution. Cell-free 50 μl supernatants and nitrate standards were added in 96-well tissue culture plates in triplicate. Subsequently, 50 μM sulfanilamide solution was added to each well and incubated at room temperature for 5-10 min followed by addition of 50 μM N-1-naphthyletherylendiamine dihydrochloride. After 5-10 min, the absorbance was measured at 540 nm in the aforementioned Multilabel reader.

**MGIT 960 system and viability counts**

A549 cells were seeded, infected with *M. tuberculosis* H37Rv for 2 h, and incubated for 0-72 h. At each time point, cells were disrupted by 0.1% Triton X-100 and harvested. Two hundred microliters of cell lysate was inoculated in a BACTEC MGIT 960 tube (Becton Dickinson, USA) following supplementation with 800 μl growth supplement (Becton Dickinson). BACTEC MGIT 960 tubes were incubated at 37°C in the BACTEC MGIT 960 instrument, in which they were automatically monitored each hour for fluorescence development for 42 days or until a positive signal developed by time to detection (TTD). The same inoculum was used to inoculate Middlebrook 7H11 agar, which was incubated at 37°C for 3 weeks. These cultures were inspected weekly or until mycobacterial colonies were seen. The colonies were counted. Colony forming units (CFUs) were calculated taking the dilutions in consideration.

**Cytosolic and nuclear protein extraction**

Cytosolic and nuclear proteins were extracted according to the company protocol (BioVision, USA). Briefly, cells were harvested by centrifugation after the desired infection and/or treatment for desired time points. Cytosol extraction buffer (NEB), repeatedly vortexed, and kept on ice for 10 min. Finally, it was centrifuged at 16,000 × g for 10 min, and the supernatant containing the nuclear extract was collected.
Both the cytosolic and nuclear extracts were stored at -80°C until use.

**Western blot**

Cells were harvested following the desired infection and/or treatment, and proteins were collected by using RIPA lysis buffer containing protease inhibitor cocktail (Santa Cruz Biotechnology, USA). The protein concentration was quantified using a BCA protein assay kit (Pierce, USA). Proteins were separated using a 4-20% sodium dodecyl sulfate polyacrylamide electrophoresis gradient gel (Mini-PROTEAN® TGX® Precast Gel; Bio-Rad, USA) at 100 V for 1.30 h. The separated proteins were transferred to a Trans-Blot SD Semi-Dry Cell polyvinylidene fluoride membrane (Bio-Rad) at 15 V for 1 h. The membranes were blocked by incubation with 5% dried skim milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibody against iNOS, nuclear factor-kappa B (NF-κB), P65, Lamin B, α-tubulin (all from Santa Cruz Biotechnology), or β-actin (Abcam, USA). A second incubation was carried out with horseradish peroxidase-conjugated secondary anti-rabbit IgG and antimouse IgG (Santa Cruz Biotechnology) for 1.5 h at room temperature. The bound antibodies were visualized using enhanced chemiluminescence western blotting detection reagents (Bio-Rad) and images were acquired using a ChemiDoc™ XRS+ System with Image Lab™ software (Bio-Rad).

**gRT-PCR**

Cells were harvested following infection and/or treatment. Total mRNA was collected, quantified, and checked for purity, cDNA was prepared, and qRT-PCR was performed as described previously (Kim et al., 2013). The primers used for human tumor necrosis factor-α (TNF-α) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5'-TCTTTCGAAACCCCGAG-TGA-3', 5'-CTGTGATGGCCACACCAC-3' and 5'-TCCCAT-CACACTTCTCA-3', 5'-CATCAACGCCACATTTCC-3', respectively. The primer for human interleukin-6 (IL-6) was purchased from Bioneer, catalog no. N-1063 (Korea). The assay results were normalized to the endogenous control gene GAPDH.

**Statistical analysis**

At least three individual experiments were conducted. Differences between groups were analyzed using one-way analysis of variance followed by the Student’s t test, with a p-value < 0.05 considered as statistically significant. Data are expressed as the mean ± standard deviation (SD).

**RESULTS**

*M. tuberculosis* infection and cell viability assay of UA in A549 cells

To detect successful infection of *M. tuberculosis* inside cells, cell monolayers were infected with *M. tuberculosis* H37Rv for 2, 3, and 4 h and then washed three times. Supernatants were collected. Cells were lysed using Triton-X 100 and inoculated in 7H9 media for 15 days. A resazurin assay was performed with un-inoculated medium as a control. The supernatants and un-inoculated medium were free of bacteria and remained blue, but the extracts obtained after infected A549 cells disruption were pink (Fig. 1A), indicating that bacteria successfully entered into the cells following infection. Fluorescence intensity data are presented in Fig. 1A. The effect of UA on A549 cells viability was assessed by MTT analysis (Fig. 1B). Both concentration-dependent (2.5-80 µg/ml), and time-dependent (6-24 h) effects of UA were observed. UA at a concentration of 10 µg/ml did not show any cytotoxic effect at 6 h; these conditions were used in the remaining experiments.

**Effect of UA on NO release and cell viability in infected A549 cells**

NO production was induced in A549 cells upon exposure to *M. tuberculosis* H37Rv (Fig. 2A). Induction was not evident earlier than 48 h (data not shown) and reached a significant level at 72 h. The induction of NO levels is the indicator of active participation of the alveolar epithelial cells in the innate immune response during *M. tuberculosis* infection. L-NMMA was used to confirm the effect of NO generation during infection. L-NMMA treatment (5 mM) partially reversed *M. tuberculosis*-induced NO release in A549 cells. As the kinetics showed significant induction of NO production at 48 and 72 h, we chose those time...
Ursolic Acid Reduces Mycobacteria-Induced NO Release
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The innate immune system is the first line of defense against pathogens before the adaptive immune system takes over. Once inhaled, mycobacteria enter the lung and infect macrophages (Ellner, 1997; Fenton and Vermeulen, 1996; Fulton et al., 1998; Rich et al., 1997). Thus far, studies have mainly focused on the pathogenesis of mycobacteria in alveolar macrophages. However, it is likely that mycobacteria also invade alveolar epithelial cells during TB infection. Mycobacteria can successfully invade and replicate within type II alveolar epithelial cells (Bermudez and Goodman, 1996; Garcia-Perez et al., 2003). Therefore, epithelial cells are not innocent bystanders; rather, they have significant roles in innate immunity and inflammatory responses (Gribar et al., 2008). Our data demonstrate that mycobacteria successfully infect and replicate in alveolar epithelial A549 cells. Along with anti-carcinogenic, anti-inflammatory, antioxidant, and pro-apoptotic properties, UA also has anti-TB potential (Jiménez-Arellanes et al., 2013; Podder et al., 2015). Our goal was to detect the role of UA in mycobacteria-infected alveolar epithelial A549 cells in the context of NO generation and cell viability. NO plays an important role in inflammation, where it is produced by iNOS, which is responsive to interferon-gamma (IFN-γ). IFN-γ stimulation of A549 cells increases NO production (Guzik et al., 2003; Xie and Nathan, 1994). Therefore, NO that is produced due to mycobacterial infection in A549 cells may be attributed to the de novo synthesis of IFN-γ in these cells. Nonetheless, susceptibility to NO differs markedly from cell to cell. Increased NO-mediated cell death may be related to DNA or mitochondrial damage (Murphy, 1999). Presently, increased production of NO was critical for cell viability and mycobacterial activity. With increasing NO concentrations, cytotoxicity also increased with time, even when NO-mediated cell death was reduced at 72 h in M. tuberculosis H37Rv-infected A549 cells (Fig. 4A). UA treatment significantly decreased NF-kB accumulation in the nuclei of infected cells (Fig. 4B). The densitometry analyses are presented beneath the respective Western blot data.

DISCUSSION

Up-regulation of iNOS was observed in M. tuberculosis H37Rv-infected A549 cells by Western blot (Fig. 3A). The induction started at 24 h and continued until 72 h. UA (10 μg/ml) successfully suppressed the upregulation of iNOS at 72 h (Fig. 3B). Densitometry analyses of each western blot data are shown in Fig. 3A and 3B. Expression of TNF-α and IL-6 was induced following infection of the cells with M. tuberculosis H37Rv, which was significantly suppressed by UA treatment at the indicated time points, as determined by qRT-PCR (Figs. 3C and 3D).

To investigate the involvement of the NF-kB pathway, cytosolic and nuclear extracts were collected after A549 cells were infected and/or treated with UA. Western blots showed that the nuclear NF-kB levels started to increase at 48 h, which was very significantly evident at 72 h. Conversely, the cytosolic levels were reduced at 72 h in M. tuberculosis H37Rv-infected A549 cells (Fig. 4A). UA treatment significantly decreased NF-κB accumulation in the nuclei of infected cells (Fig. 4B). The densitometry analyses are presented beneath the respective Western blot data.

To correlate NO generation with intracellular mycobactericidal activity, we investigated the involvement of the NF-κB pathway, cytosolic and nuclear extracts were collected after A549 cells were infected and/or treated with UA. Western blots showed that the nuclear NF-kB levels started to increase at 48 h, which was very significantly evident at 72 h. Conversely, the cytosolic levels were reduced at 72 h in M. tuberculosis H37Rv-infected A549 cells (Fig. 4A). UA treatment significantly decreased NF-κB accumulation in the nuclei of infected cells (Fig. 4B). The densitometry analyses are presented beneath the respective Western blot data.
ern blot analysis was performed to detect the iNOS expression. A549 cells were infected with M. tuberculosis H37Rv for 2 h, washed three times and incubated for 0-72 h. Western blot data. A549 cells were infected with M. tuberculosis H37Rv-infected and/or 10 μg/ml UA-treated A549 cells after 72 h by Western blot (B). β-actin was the endogenous control. The densitometry analyses are presented beneath the respective Western blot data. A549 cells were infected with M. tuberculosis H37Rv and/or treated with 10 μg/ml UA for 48 and 72 h. Cells were harvested, the total mRNA was collected, cDNA was prepared, and qRT-PCR was performed to detect TNF-α and IL-6 mRNA expression. The data are presented as mean ± S.D. of three independent experiments. * denotes significant differences relative to control (p < 0.05) and # denotes significant difference between ‘inf’ and ‘inf + UA’ (p < 0.05). C, inf, and UA stand for control (normal A549 cells), infection, and ursolic acid, respectively.

though the level of NO was insufficient to kill intracellular mycobacteria. A previous report provided a similar interpretation, where the increased NO generated due to mycobacterial infection was insufficient to kill intracellular mycobacteria in A549 cells (Roy et al., 2004). Hence, surrounding inflammatory cytokines from monocytes/macrophages might be required to generate the increased amount of NO to kill intracellular mycobacteria. This NO level would be highly cytotoxic to normal cells. Another potential reason for A549 epithelial cell death is virulent M. tuberculosis H37Rv infection resulting in apoptosis and necrosis (Danelishvili et al., 2003).

The anti-carcinogenic, anti-inflammatory, and pro-apoptotic activities of UA are due to its ability to inhibit the immunoregulatory transcription factor, NF-kB, in response to a variety of carcinogens and inflammatory agents (Shishodia et al., 2003). After taking into account that UA is a triterpenoid carboxylic acid, it is not surprising that this compound possesses potent anti-inflammatory and cytotoxic activities and is a potent inhibitor of NF-kB activation (De las Heras et al., 2003; Yang et al., 2002).

2003). UA partially inhibited the activation of NF-kB and its downstream pro-inflammatory cytokines, TNF-α and IL-6, in mycobacteria-infected alveolar epithelial cells. The observations indicate the anti-inflammatory potential of UA and support the previous description of the activation of NF-kB 48 h after M. tuberculosis H37Rv infection in human THP-1 cells (Dhiman et al., 2007).

Our results strongly suggest that alveolar epithelial cells act as the first line of defense against mycobacteria by inducing NO generation. The levels of NO generated directly following infection of A549 cells with mycobacteria are insufficient to kill intracellular mycobacteria. However the bacterial count increased with time. Interestingly, the increased production of NO due to mycobacterial infection showed an increasing cytotoxicity in A549 cells. This cytotoxic effect was partially reversed by treating cells with UA following mycobacterial infection, which is attributed to the reduction of NO production in infected A549 cells. The induced activation of the immunoregulatory transcription factor, NF-kB following infection was also significantly quenched by UA. Taken together, our data reveal the critical role of NO in mycobacterial infection and the protective role of UA in mycobacteria-infected A549 cells.

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