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Ursolic Acid Activates Intracellular Killing Effect of Macrophages During *Mycobacterium tuberculosis* Infection

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Tuberculosis is one of the most threatening infectious diseases to public health all over the world, for which *Mycobacterium tuberculosis* (MTB) is the etiological agent of pathogenesis. Ursolic acid (UA) has immunomodulatory function and exhibits antimycobacterial activity. However, the intracellular killing effect of UA has yet to be elucidated. The aim of this study was to evaluate the intracellular killing effect of UA during mycobacterial infection. The intracellular killing activity of UA was evaluated in the macrophage cell line THP-1 by the MGIT 960 system as well as by CFU count. The production of reactive oxygen species (ROS) and the level of nitric oxide (NO) were measured using DCF-DA and Griess reagent, respectively. Phagocytosis was observed by a fluorescence-based staining method, and the colony forming units were enumerated on 7H11 agar medium following infection. In addition, MRP8 mRNA expression was measured by qRT-PCR. UA significantly decreased the number of intracellular *Mycobacterium* through generation of ROS and NO. In addition, it profoundly activated the phagocytosis process of THP-1 cells during MTB-infection. Furthermore, our data demonstrated that UA activated the phagocytosis process in human monocyte cells through MRP8 induction. These data suggest that UA firmly contributes to the intracellular killing effect of macrophages during mycobacterial infection.

Keywords: Mycobacteria, ursolic acid, macrophages, intracellular, phagocytosis

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is the second most common infectious disease after the incidence of HIV/AIDS infection [11]. The situation is getting worse day by day owing to the emergence of multidrug-resistant (MDR) TB strains [16], which cause disease that is essentially untreatable with the existing therapeutics agents. Such findings stress the urgency for developing new anti-TB agents that are effective against drug-resistant mycobacteria while being economical at the same time.

Treatment of tuberculosis can be challenging since the infection sometimes persists or reoccurs even after prolonged treatment with antimicrobial agents [16]. Several aspects of bacterial pathogenesis may be involved in the persistence of tuberculosis infections. An important feature reported by several authors is the ability of the bacteria to invade and survive inside cells. Virulent mycobacteria can avoid being killed by phagocytosis through different mechanisms [4].

Many drugs, including the commonly used antimycobacterial compounds such as rifampicin and isoniazid, exhibit poor intracellular activity against *Mycobacterium*. In addition, rises in the incidence of drug resistant *Mycobacterium* and the emergence of MDR strains [16] have further complicated the therapy used for these infections. Many previous studies have shown that the existing antimycobacterial drugs have either no or poor intracellular killing effects owing to the incapability of invading and accumulating in

macrophages. Thus, it is very necessary to find new candidate drugs that have immunomodulatory effects or intracellular killing effects. In this context, ursolic acid (UA) is considered as a potentially ideal drug since it combines a number of desirable properties, such as high bactericidal potency, immunomodulation properties [8, 20], *etc*.

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid), a natural pentacyclic triterpenoid, has been found in various plants, including apples, basil, cranberries, peppermint, rosemary, oregano, and prunes [2, 10]. UA has been reported to possess many biological activities, including antioxidant, anti-inflammatory, trypanocidal, antirheumatic, antiviral, antibacterial, and antitumoral properties [7, 8, 22]. One study previously reported UA to have antimycobacterial activity at concentrations as low as 25 μg/ml [8]. However, the intracellular killing effect of UA has not yet been evaluated. Using human macrophage THP-1 as an *in vitro* model may provide valuable information on the intracellular properties of UA. Thus, the objective of this study was to apply an *in vitro* method for testing the intracellular effects of UA against *Mycobacterium tuberculosis*. To the best of our knowledge, this study represents the first application of a combined *in vitro* approach to the study of the antimycobacterial properties governing the intracellular activity of UA.

Materials and Methods

Preparation of Antimicrobial Agents

Ursolic acid, rifampicin (RIF), and isoniazid (IN) were dissolved in dimethylsulfoxide (DMSO) to the final DMSO concentration of no more than 0.01%. The stock solutions were prepared in aseptic conditions and stored at −20°C.

Mycobacterium Culture

Avirulent *M. tuberculosis* strain H37Ra was purchased from the American Type Culture Collection and grown at 37°C and 120 rpm in Middlebrook 7H9 broth (Difco Laboratories, USA) supplemented with 10% oleic acid albumin dextrose catalase (OADC) (BD and Company, USA) and 0.5% glycerol (Samchun Chemicals, Korea). Middlebrook 7H10 was used for determination of the number of colony forming units (CFU).

Cell Culture

The THP-1 human monocytic cell line (ATCC) was used for the intracellular experiments. Cells were grown in RPMI 1640 medium supplemented with 10% decomplemented fetal bovine serum (Gibco BRL, Life Technologies, Scotland) at 37°C in an atmosphere of 5% CO_2. Differentiation of THP-1 monocytes was achieved by adding 100 nM of phorbol-12-myristate-13-acetate (PMA) for 48 h at 37°C in 5% CO_2.

Drug Cytotoxicity Assay

In order to check the effects of drugs, cell viability was assessed using the colorimetric-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. THP-1 cells were transformed into macrophages using 100 nm PMA. Cell viability was evaluated in a time- and dose-dependent fashion. The percentage of viable cells was determined prior to treatment and after 3, 6, 12, and 24 h of treatment with compounds by adding 20 μl of MTT solution. Those compound concentrations that did not affect cell viability by <90% (IC_{90}) after 24 h of incubation were considered non-toxic.

Cell Infection

Infection of THP-1 cells by *M. tuberculosis* and enumeration of viable intracellular bacteria were carried out as described previously [1]. In brief, bacterial cultures exhibiting logarithmic growth were collected by centrifugation at 14,000 rpm for 5 min. Cells were then resuspended in RPMI 1640 supplemented with 10% fresh human serum and incubated for 30 min at 37°C to allow opsonization. Infection of monocytes was carried out by replacing the culture medium with the bacterial suspension at a bacterium-to-monocyte ratio of 10:1. Bacteria were allowed to undergo phagocytosis for 3 h at 37°C and 5% CO_2. The macrophages were then washed three times with pre-warmed 1× phosphate-buffered saline (PBS) at 37°C to remove extracellular bacteria from the cells, and then 1 ml of amikacin (200 μg/ml) was added to each well followed by incubation under the same conditions as above for 1 h. After washing, different concentrations of drugs were added to the cells and incubation was carried out at 37°C for another 48 h. After incubation, 0.05% Triton X100 was added to the cells and they were spun down again, lysed, and resuspended in Middlebrook 7H9 medium before spreading dilutions on Middlebrook 7H10 agar medium for CFU determination and analysis using the MGIT system. Four dilutions of each homogenate were spread on duplicate plates containing BactoMiddlebrook 7H10 agar enriched with OADC, also from Difco. The incubation time was 28 days, and MGIT growth was observed for up to 42 days.

Phagocytosis Assay

Phagocytosis activity assays were performed on THP-1 cells using a fluorescent technique. First, 5×10^5 cells were seeded in 12-well plates and differentiated using PMA. After differentiation, the media were replaced with fresh medium containing Zymosan A Bioparticles at the ratio of 100 particles/cell. The control was left untreated with Zymosan A Bioparticles. The cells were then incubated for 1 h at 37°C to allow uptake of the particles. After incubation, phagocytosis was stopped by adding cold 1× PBS, after which the cells were washed four times with cold PBS. The cells were then fixed using 4% paraformaldehyde for 20 min and washed twice with PBS containing 2% FBS. Finally, the intensity of fluorescence was observed using an Axiovert-25 microscope (Carl Zeiss) with a filter range of Ex/Em: 490/525 nm. The results were proportional to fluorescence intensity.

Determination of Reactive Oxygen Species (ROS) Production

Intracellular ROS was determined using a fluorescence-based dye, 2′,7′-dichlorofluoresecin diacetate (DCFH-DA). In short, monocyte THP-1 cells (2×10^5) were seeded in a 24-well tissue culture plate. After conversion of the monocytes to macrophages, the cells were treated with RPMI 1640 medium containing 10 μM DCFH-DA for 30 min. The cells were then washed twice with 1× PBS to remove extra DCFH-DA. Next, the cells were treated with different concentrations of UA, 2 μg/ml RIF, and 2 μg/ml IN, and then incubated for an additional 1 h. The drug-containing media were then removed and the cells were rinsed with 1× PBS before measurement of the fluorescence intensity of DCF using an Axiovert-25 microscope with a filter range of Ex/Em: 490/525 nm. At least five regions in each culture plate were observed to determine the fluorescence intensity.

Determination of Nitric Oxide (NO) Production

NO production was determined using the Griess reagent. Briefly, THP-1 cells were plated in 24-well plates and cells were differentiated using PMA. After successful differentiation, THP-1 cells were incubated with different concentrations of UA (2.5–10 μg/ml), 2 μg/ml of RIF, and 2 μg/ml of IN for 24 h. The supernatants were collected, and 100 μl of each supernatant was added to triplicate wells in a 96-well plate. Then, 20 μl/well of the Griess reagent (6 mg/ml) was added to the samples and incubated for 30 min at room temperature. The reaction products were estimated colorimetrically at 550 nm.

Real-Time PCR Analysis of MRP8 Expression in Human THP-1cells

Total mRNA was isolated from macrophage THP-1 cells using the RNeasy Mini Kit as per the manufacturer's protocol (Qiagen USA), and and cDNA was synthesized using 1 μg of RNA and the Maxime RT PreMix kit (Intron Biotechnology, Seoul, Korea). Quantitative real-time (qRT)-PCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) with iQ SYBR Green Supermix (Bio-Rad, USA). The MRP8 primer had been used to check the expression label of the *MRP8* gene. The forward primer had the sequence 5′-ACCTGAAGAAATTGCTAGAGACCGAGTG-3′ and the reverse primer was 5′-CCACGCCCATCTTTATCACCA GAATGAG-3′.

Statistical Analysis

Each experiment was repeated at least three times, with negligible differences in the results of individual experiments. Statistical significance of the results from different experiments was evaluated using the paired Student's t-test. Data in graphs are presented as the mean ± SD Means were considered to be significantly different if $*p < 0.05$.

Results

Macrophage Viability

The viability of the macrophage THP-1 cultures with or without various drug treatments did not differ significantly. At 24 h, the mean viability was almost 100% in macrophages treated with 2.5–10 μg/ml UA (Fig. 1A). However, cytotoxicity was found at higher concentrations of UA. Cells were also examined morphologically after treatment with 2.5–10 μg/ml UA, and no morphological changes were observed by phage contrast microscopic analysis (data not shown). To check for cytoprotection activity of UA, the cells were pretreated with 1–10 μg/ml UA prior to MTB infection. The data showed that UA-pretreated cells were protected from cell death due to treatment with *Mycobacterium* (Fig. 1B).

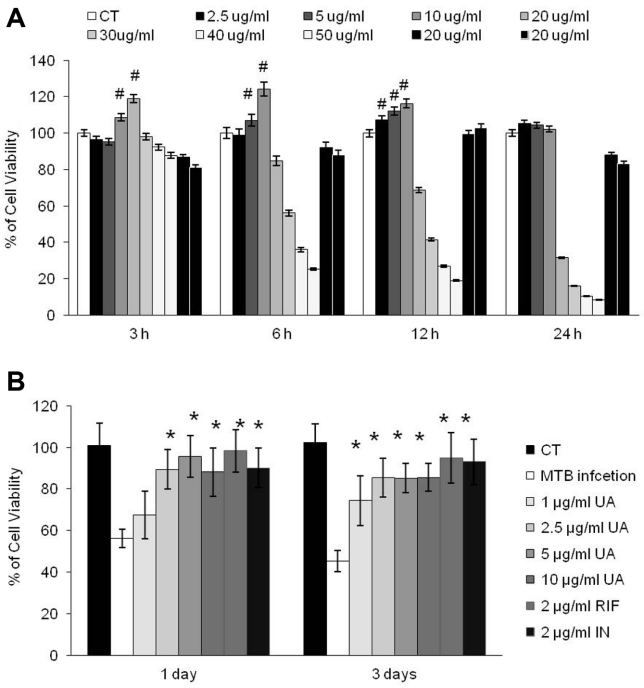

Fig. 1. Effect of ursolic acid (UA) on viability of THP-1 cells in *M. tuberculosis* (MTB) infection conditions.
(**A**) Cells were treated with 2.5, 5, 10, 20, 30, 40, and 50 μg/ml of UA for different time periods and (**B**) with various concentrations of UA during *Mycobacterium* infection to detect cell viability *via* the MTT assay. The results were expressed as means ± SD of three different experiments. $*P < 0.05$, compared with the infection group. $^{\#}P < 0.05$, compared with the control group.

Intracellular Killing of Mycobacteria in UA-Treated Macrophages

Having demonstrated the bactericidal activity of UA, we next sought to assess whether UA can activate the phagocytosis activity of macrophages. Using the Zymosen A-based phagocytosis assay, our data showed that THP-1 cells treated with UA internalized Zymosen A particles in a

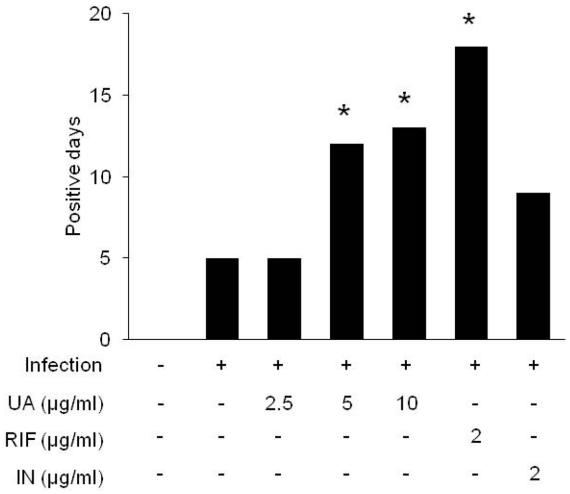

Fig. 2. Ursolic acid (UA) shows intracellual killing effects during *M. tuberculosis* infection.

Cells were pretreated with 2.5, 5, and 10 μg/ml of UA for 24 h before *Mycobacterium* infection and cell lysate was collected and inoculated into MGIT tubes.

Table 1. Colony forming units (CFU) were quantified on 7H11 agar medium.

Sample	CFU/ml ($\times 10^3$)
Medium only	Nil
No infection	Nil
Infection	18
Infection + 10 μg/ml UA	6
Infection + 2 μg/ml RIF	4

Cells were subjected to different concentrations of drug prior to infection. Afterwards, cell lysate was collected and plated on 7H11 agar media for CFU assessment.

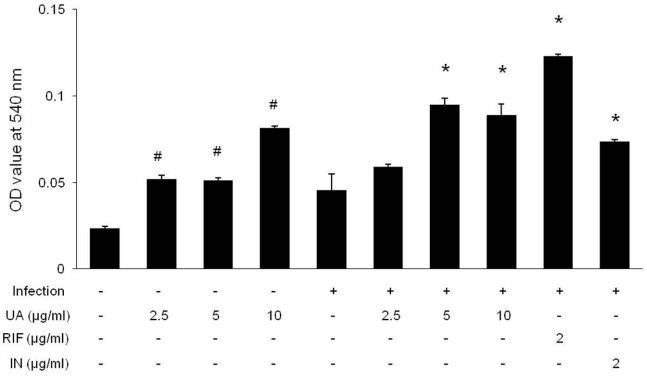

Fig. 4. Ursolic acid (UA) induces nitric oxide in human macrophages.

Nitric acid production in THP-1 cells treated with different concentrations of UA for 24 h. Rifampicin (RIF) and isonazid (IN) were used as positive controls. Experiments were performed in triplicate. The results were expressed as means ± SD of three different experiments. $^*P < 0.05$, compared with the infection group. $^{\#}p < 0.05$, compared with the control group.

dose-dependent manner. This activity indicates that UA activates the phagocytosis process (Fig. 3). Herein, 2.5 to 10 μg/ml doses of UA were chosen owing to the lack of toxicity on THP-1 cells. THP-1 cells were treated with UA to check for intracellular killing of the *Mycobacterium*. As we hypothesized that UA may stimulate the intracellular activity of macrophages, THP-1 cells were pretreated with UA and the intracellular killing effect was observed through the MGIT system and CFU count. After 3 h of infection, the intracellular bacterial burden was found to be significantly less in pretreated cells compared with untreated cells. In the MGIT system, positive growth for the 10 μg/ml UA treatment was observed at 18 days, while positive growth without drug treatment was observed at 5 days (Fig. 2). In addition, the number of CFUs was profoundly reduced by UA on the 7H11 agar plate (Table 1). These results indicated that UA increased phagocytosis activity and killed intracellular *Mycobacterium*.

UA Induces ROS and NO in Macrophages

Mycobacterium tuberculosis can stay inside macrophages for longer periods of time. However, THP-1 cells can kill

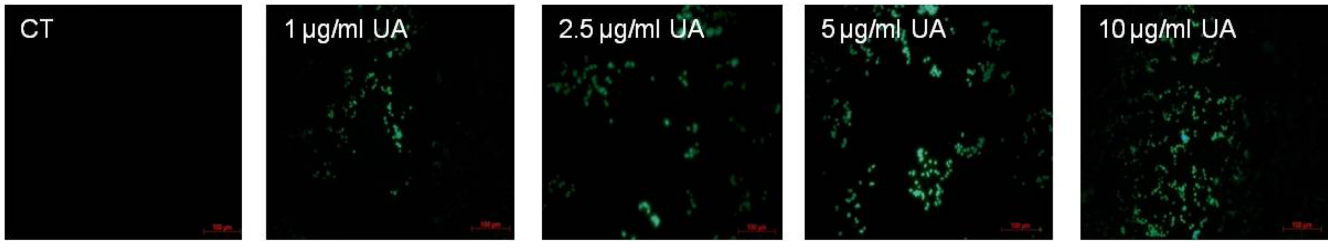

Fig. 3. Ursolic acid (UA) increases phagocytosis activity of THP-1 cells.

Cells were pretreated with 2.5, 5, and 10 μg/ml of UA for 24 h before Zymozen A treatment, and images were taken using fluorescence microscopy. Experiments were performed in triplicate.

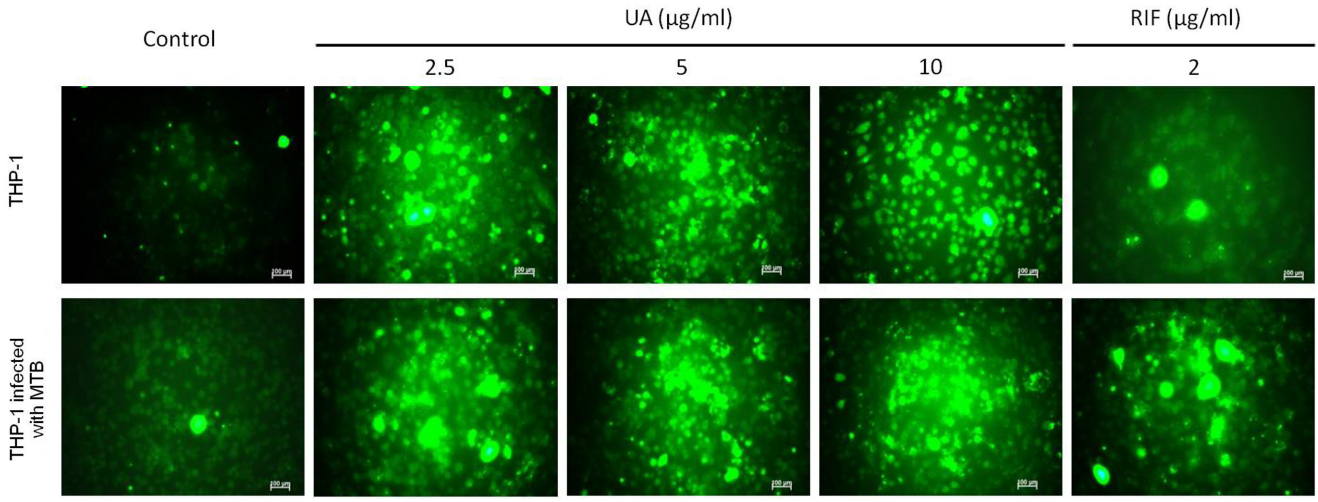

Fig. 5. Ursolic acid (UA) activates ROS in human macrophages.
ROS production was measured by DCFH-DA staining using fluorescence microscopy. The level of ROS generation is proportional to the intensity of fluorescence. Experiments were performed in triplicate. The fluorescence images were taken by observing five different regions from three individual experiments.

the *Mycobacterium* by producing intracellular ROS and NO. Cells treated with UA were observed to produce significantly higher levels of intracellular ROS and NO compared with control cells (Figs. 4 and 5). In Fig. 4, UA induced NO in a dose-dependent manner, and the same pattern was followed during the conditions of *Mycobacterium* infection. Rifampicin and isoniazid were used as controls, although NO was activated by RIF but not by IN. This finding demonstrated that UA triggers intracellular NO inside of cells, like RIF. In addition, intracellular ROS was also generated by UA treatment in both infected and uninfected conditions. ROS was generated in the same pattern as NO generation, as shown in Fig. 5. These results direct us to the conclusion that the intracellular killing effect of UA may be achieved by the generation of ROS and NO.

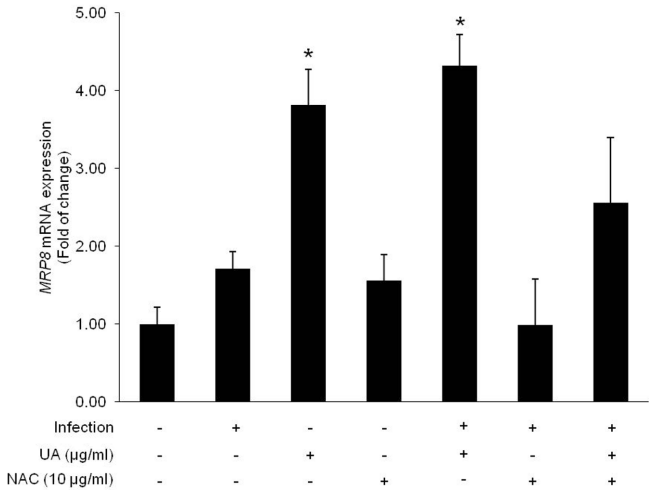

Fig. 6. MRP8 expression in THP1 cells after *M. tuberculosis* infection in presence of ursolic acid (UA) and *N*-acetylcysteine NAC.
THP1 cells were infected with *Mycobacterium* for 24 h in the presence and absence of UA; prior to infection, cells were also treated with NAC. mRNA expression levels of MRP8 were measured by real-time PCR. Data are shown as the mean ± SD of at least three independent experiments. Asterisks denote statistically significant difference compared with untreated control cells (*$p < 0.05$).

UA Facilitate *MRP8* Gene Expression in THP-1 Cells

To examine whether MRP8 and MRP14 play a role in the killing of *Mycobacterium*, the expressions of *MRP8* and *MRP14* were checked after *Mycobacterium* infection in the presence and of absence UA and *N*-acetylcysteine (NAC), which act as a ROS and NO inhibitor. Our result showed that UA significantly induced *MRP8* in THP-1 cells, but not in presence of NAC (Fig. 6). Treatment with antioxidant NAC, however, dramatically decreased the *MRP8* expression in *Mycobacterium*-infected cells. The results indicated that intracellular ROS and NO generation are involved in *MRP8* activation.

Discussion

The objective of the present study was to measure the ability of UA to activate the intracellular killing of

Mycobacterium in infected human macrophages. In order to improve tuberculosis treatment, the intracellular killing of *Mycobacterium* was measured in UA-pretreated macrophages. The intracellular antimicrobial activity of UA against *Mycobacterium tuberculosis* was measured using the THP-1 monocytic cell line, since it has been a reliable model to test intracellular infection with *M. tuberculosis*.

The cell viability study showed that up to 10 μg/ml of UA had no toxic effects on human macrophages. The cell viability was found to gradually decrease with continuing increase in the concentrations. The drug showed antimycobacterial activity against strain H37Ra, which complies with the results of another study [15]. To the best of our knowledge, no previous study has evaluated the intracellular killing effect of UA against *M. tuberculosis* infecting human macrophages. The cultured THP-1 macrophages have previously been shown to be a good model in terms of receptor expression, bacterial uptake, survival, and replication of *M. tuberculosis* [23]. These data indicate that THP-1 is one of the best models in terms of relevance for *in vivo* studies.

It is supposed that *Mycobacterium* resides in the compartment of macrophages because it is an intracellular pathogen [8, 19]. This makes it imperative to activate the macrophages or deliver therapeutic molecules to the target sites that would kill the intracellular *Mycobacterium*. In addition, macrophages may eradicate *Mycobacterium* by induction of ROS/RNS generation and pro-inflammatory cytokine production [3, 5, 14]. We observed herein that UA contributes to the killing of intracellular bacteria in macrophages by inducing the generation of ROS and NO in the macrophages. A previous study showed UA to cause ROS generation in U87MG cells [17]. Herein, increased ROS and NO production in macrophages was observed after UA treatment.

MRP8 belongs to the calcium-binding S100 protein family and usually functions as a heterodimeric protein complex to execute cytokine-like activities. MRP8 is strongly expressed in the myeloid cell lineage, such as neutrophils, monocytes, and macrophages, but is weakly expressed in endothelial and epithelium cells [6, 19]. Studies have demonstrated that MRP8 was dramatically up-regulated in THP-1 cells after mycobacteria challenge; the findings indicated that MRP8 has involvement in the host immune response to *Mycobacterium* infection [21]. Another study has showed that MRP8 executes antimicrobial activities against various extracelluar and intracellular organisms. For example, MRP8 directly inhibits the growth of *C. albicans*, through blocking glucose incorporation and interfering in mycelia growth [12]. Our data showed that UA (20 μg/ml) up-regulates *MRP8* expression in mycobacterial infection and exploits intracellular killing. In contrast, the same expression was not observed when cells were pretreated with NAC (a ROS and NO inhibitor). These findings are consistent with a previous study showing that MRP8 accelerates intracellular killing through ROS generation. One plausible explanation of this event could be that those intracellular ROS levels may attain intracellular killing effect through autophagy. For example, a previous study presented that MRP8 increased the expression level of Light chain 3 (*LC3*), an important autophagy gene, and participated in execution of autophagy [21]. Overall, it could be assumed that the killing effect of UA may be due to the generation of ROS and NO. This result complies with another study, which showed that intercellular killing events occurred with increased levels of ROS and NO [13]. Another possible method could be through the induction of several cytokines and chemokines by UA, which activate the cells to result in an increase in the killing efficiency of the macrophages [18].

It is noteworthy that there have been no reports measuring the intracellular killing effect of UA in human macrophages to date. Thus, to the best of our knowledge, this study constitutes the first that focused on evaluating the intracellular killing activity in THP-1 cells exposed to UA. At high concentrations of UA, higher cytotoxicity to human macrophages was observed, whereas treatment with UA at lower concentrations killed the intracellular mycobacterium without exhibiting any toxic effects on the *Macrophages*. However, other possible mechanisms still need further investigation. The intracellular killing effects could be achieved from to the increased ROS and NO production. Thus, UA can be regarded as a potential candidate for the treatment of TB with minimum side effects on human macrophages.

Acknowledgments

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