Anti-inflammatory potential of ursolic acid in *Mycobacterium tuberculosis*-sensitized and Concanavalin A-stimulated cells

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Abstract. Ursolic acid (3-β-3-hydroxy-urs-12-ene-28-oic-acid; UA) is a triterpenoid carboxylic acid with various pharmaceutical properties. It is commonly found in apples, basil, berries, rosemary, peppermint, lavender, oregano, thyme, hawthorn and prunes. In the present study, the activities of UA against the *Mycobacterium tuberculosis* H37Rv-induced release of a panel of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 from RAW 264.7 murine macrophages, A549 alveolar epithelial cells and in concanavalin A (Con A)-stimulated rat splenocytes were investigated. In addition, the present study examined the ability of UA to reduce the expression levels of the inflammatory mediators, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in the stimulated cells. The reduction of nitric oxide (NO) release by UA was also examined in the stimulated cells. UA significantly inhibited the mRNA expression levels of *TNF-α*, *IL-1β* and *IL-6* in the stimulated cells. The expression levels of COX-2 and iNOS were also suppressed by UA, as was the release of NO at a significant level. The data indicated the potency of UA on different cell types, which may assist in the development of anti-inflammatory drugs. In the case of adjunct host-directed immune therapy for tuberculosis, UA may be used, in addition to established antibiotic therapies, to improve treatment efficacy and outcome due to their anti-inflammatory potential. Further detailed investigations are required to establish its use as an anti-inflammatory.

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Introduction

Inflammation is a complex biological response, acting as a self-defense mechanism against harmful environmental insults, which involves a network of cellular responses, cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), and humoral factors (1,2). However, their persistence may lead to chronic inflammation, which may be associated with several diseases, including cancer (1,3), neoplasia, inflammatory bowel disease, ulcerative colitis (4,5), arthritis (6), asthma and Alzheimer's disease (7). The detrimental effects of chronic inflammation can be controlled by conventional treatments, however, resistance to the drugs used and their side-effects necessitate the development of novel anti-inflammatory drugs (8). Previous studies have investigated the use of adjunct immunotherapies to improve treatment success for tuberculosis (TB). Among these, the immunosuppression-mediated reduction of TNF-α levels by current antibiotic therapies may eventually be effective in treating highly contagious TB (9).

In general, when *M. tuberculosis* enters the lung, it interacts with macrophages. Macrophages have been the most commonly examined cells in investigations of TB, although epithelial cells are being increasingly examined in TB, as they are essential in the immune response during pulmonary tuberculosis (10-13). Nitric oxide (NO) is an important mediator in cell signaling, neurotransmission and in the host defense mechanism (14,15). *M. tuberculosis* infection induces the activity of inducible nitric oxide synthase (iNOS) in A549 cells, which leads to the production of a significant level of NO (13).

A natural triterpenoid carboxylic acid, 3-β-3-hydroxy-urs -12-ene-28-oic-acid (uracil; UA), is present in a wide variety of foods (16). Its biochemical and pharmacological effects include anti-inflammatory, antioxidant, antiproliferative, anticancer, antimutagenic, antihypertensive, and antiviral properties (17,18). UA can also inhibit the immunoregulatory transcription factor, nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), in response to a wide variety of carcinogens and inflammatory agents (19). There is also evidence supporting the anti-inflammatory effects of UA (2).

In the present study, *M. tuberculosis*-induced RAW 264.7 mouse monocyte macrophages, A549 type II alveolar cells and

mitogen, concanavalin A-induced rat splenocytes were used to examine the effect of UA on immune regulation. The aim of the present study was to investigate the anti-inflammatory potential of UA, a candidate drug for controlling inflammation-associated diseases.

Materials and methods

Cell culture. RAW 264.7 mouse monocyte macrophages and A549 type II alveolar cells, purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified incubator with 5% CO_2 and sub-cultured every 2-3 days.

Reagents, treatment conditions and durations. The UA, N^G-monomethyl-L-arginine (L-NMMA), and concanavalin A (Con A) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Throughout the present study, the cells ($1x10^5$ cells/ml) were treated with 10 μg/ml UA and 5 mM L-NMMA for 6 h following *M. tuberculosis* infection, following which they were incubated for the specified durations. Immediately prior to infection or treatment, the medium were replaced with serum-free DMEM. In the case of the splenocytes, the cells ($1x10^6$ cells/ml) were co-treated with 5 μg/ml Con A and 10 μg/ml UA for the specified durations.

Animals and primary splenocyte collection. Female Wistar rats (190-220 g; 8-weeks old; n=8) were purchased from Nara Biotech, Ltd. (Pyeongtaek-si, Korea). The animals were housed in a solid-bottomed cage at 23±2˚C, maintained under a 12 h light-dark cycle and fed standard rodent chow (Purina Rodent Chow; Purina Co., Ltd., Seoul, Korea) and water *ad libitum*. The present study was performed according to the guidelines of the United States National Institutes of Health, and was approved by the ethics committee of Soonchunhyang University (Asan, Korea; SCH14-0031).

Primary splenocytes were collected from the rats following sacrifice by cervical dislocation, and aseptic collection of the spleen was performed. Each spleen was immersed in RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated FBS and a 1% (v/v) antibiotic/antimycotic cocktail, comprising 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were passed through a cell strainer (BD Biosciences, Durham, NC, USA) to form a single cell suspension. Erythrocytes were excluded from the resulting cell suspension using lysis buffer (Life Technologies, Seoul, Korea), containing 0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA (pH 7.3). The cells were washed once with phosphate-buffered saline (PBS) and cultured at a density of 10^6 cells/ml. Cell viability was determined according to the exclusion of Trypan blue (Sigma-Aldrich). The cells were maintained under standard culture conditions at 37˚C.

Mycobacterium infection/invasion. The *M. tuberculosis* H37Rv strain was purchased from ATCC and was grown in Middlebrook 7H11 agar (Becton Dickinson, Sparks, MD, USA) or Ogawa medium for ~3 weeks. Isolated colonies were inoculated in Middlebrook 7H9 broth (Becton Dickinson) in am incubator with agitation for 15 days. To avoid clumping, the bacterial suspension was vortexed vigorously with glass beads, and then passed through an 8-μm filter to form a single cell suspension. The suspension was allowed to stand for several minutes to settle and two-thirds of the clear upper portion of suspension was used for quantification at 600 nm using the UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan) adjusted with McFarland standards. A 0.5 McFarland standard was prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate with 9.95 ml of 1% sulfuric acid. Subsequently, 10 μl of the suspension was inoculated in Middlebrook 7H11 agar or Ogawa medium at 37˚C in an atmosphere of 5% CO_2 for 3-4 weeks to quantify the bacterial number in colony forming units (cfu). Following count determination, the bacterial suspension was aliquoted and stored at -76˚C as a single volume

For *in vitro* infection, the RAW 264.7 ($2x10^5$ cells) and A549 ($2x10^5$ cells) cells we re grown in six-well plates overnight and infected with *M. tuberculosis* H37Rv at a 1:10 ratio for 3 h in the aforementioned cell culture conditions. The cells were washed with warm PBS three to five times to remove extracellular bacteria. To confirm successful invasion of the bacteria, the final wash medium and cell extract (100 μl), following cell dissolution with 0.1% Triton X-100 (Sigma-Aldrich), were inoculated in Middlebrook 7H11 agar for colony counting, Middlebrook 7H9 broth for a resazurin assay (20) and in a Bactec MGIT system (Becton Dickinson) for the determination of time to detection (TTD) (21), as described previously (22). Prior to proceeding, it was confirmed that the bacteria present in the final wash medium were absent or few in number, compared with the high numbers of viable bacteria in the extracted cell suspension, which confirmed successful bacterial invasion.

Cell viability assay. The cell viability assay performed in the present study was based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) to formazan crystals by the mitochondrial dehydrogenase enzyme (23). In brief, the cells ($1x10^5$ cells/ml) were seeded overnight to achieve ~80% confluence and were treated with 10 μg/ml UA for 0, 6, 12, 24, 48 and 72 h. At the determined time, 20 μl of 5 mg/ml MTT reagent was added. Following 4 h incubation at 37˚C, the medium were aspirated, and 100 μl of dimethylsulfoxide (Samchun Pure Chemical Co., Ltd., Pyeongtaek, Korea) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a Victor™ X3 multilabel reader (Perkin Elmer, Inc., Waltham, MA, USA).

Splenocyte viability was determined using an MTS-based assay (24). In brief, the cells were treated for the indicated times periods, and the detection reagent was prepared using MTS and phenazine methosulfate (Sigma-Aldrich) at a ratio of 20:1, which was added at a 1:5 ratio of reagent mixture to cell culture. The absorbance was detected at 492 nm using the Victor™ X3 multilabel reader.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cells were infected

Table I. List of primers used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Mouse		
TNF-α	TGTCTCAGCCTCTTCTCATT	AGATGATCTGAGTGTGAGGG
IL-6	TTGCCTTCTTGGGACTGATG	CCACGATTTCCCAGAGAACA
IL-1β	GGGCTGCTTCCAAACCTTTG	TGATACTGCCTGCCTGAAGCTC
iNOS	CCCTTCCGAAGTTTCTGGCAGCAGC	GGCTGTCAGAGCCTCGTGGCTTTGG
COX-2	CCAGCACTTCACCCATCAGTT	ACCCAGGTCCTCGCTTATGA
L32	GCCAGGAGACGACAAAAAT	AATCCTCTTGCCCTGATCC
Human		
TNF-α	TCTTCTCGAACCCCGAGTGA	CCTCTGATGGCACCACCAG
iNOS	CCTCTGATGGCACCACCAG	ACCCTGCCAACGTGGAATTCACTCAG
COX-2	TTCAAATGAGATTGTGGGAAAATTGCT	AGATCATCTCTGCCTGAGTATCTT
GAPDH	TCCCATCACCATCTTCCA	CATCACGCCACAGTTTCC
Rat		
TNF-α	GACCCTCACACTCAGATCATCTTCT	TGCTACGACGTGGGCTACG
IL-6	CGAGCCCACCAGGAACGAAAGTC	CTGGCTGGAAGTCTCTTGCGGAG
IL-1β	CCCTGCAGCTGGAGAGTGTGG	TGTGCTCTGCTTGAGAGGTGCT
iNOS	GTGCTAATGCGGAAGGTCATG	GCTTCCGACTTTCCTGTCTCAGTA
COX-2	GTGTCCCTTTGCCTCTTTCAAT	GAGGCACTTGCGTTGATGGT
GAPDH	ATGATTCTACCCACGGCAAG	CTGGAAGATGGTGATGGGTT

TNF-α, tumor necrosis factor-α; IL, interleukin; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

and/or treated for the indicated time periods, following which the total RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA) at 260 nm. The assay used RNA at a 260:280 nm ratio of 1.8-2.0, indicating high purity. Subsequently, cDNA was prepared using 1,000 ng of the total RNA using Oligo dT_{15} Primer (Maxime™ RT PreMix kit; Intron Biotechnology, Inc., Seongnam, Korea) in a Veriti® 96-Well Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). Subsequently, qPCR was performed to amplify the cDNA using an iQ SYBR Green Supermix kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol, in a CFX96TM real-time PCR detection system (Bio-Rad Laboratories, Inc.). The temperature cycle followed for qPCR was as follows: 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec, 42˚C for 10 sec and 72˚C for 20 sec. A dissociation curve was acquired to ensure the specificity of the PCR product in every PCR assay. The assay results were normalized to the endogenous control gene, glyceraldehyde 3-phosphate dehydrogenase. The primers used were purchased from Bioneer Corporation (Seoul, Korea) and are listed in Table I, with the exception of human interleukin 1-β (*IL-1β*), and interleukin 6 (*IL-6*), which were also purchased from Bioneer Corporation (Seoul, Korea; cat. nos. N-1058 and N-1063, respectively). Relative quantification was obtained using the comparative threshold cycle (ΔΔCq) method. A CFX96™ real-time PCR detection system (Bio-Rad Laboratories, Inc.) with a default calculation system was used.

NO release assay. NO release was measured by detecting the concentration of nitrite produced, using the Griess reagent system (Promega Corporation, Madison, WI, USA). Briefly, nitrite standards were prepared, ranging between 100 and 1.56 μM (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 μM) by serial 2-fold dilutions. The cell-free supernatants were obtained by centrifugation at 2,000 x g for 1 min at room temperature. The nitrite standards and cell-free supernatants from the infected and/or treated samples (50 μl) were added to a 96-well tissue culture plate in triplicate. Subsequently, 50 μl sulphanilamide solution was added to each well, and incubated for 10 min at room temperature in the dark, followed by the addition of 50 μl N-1-napthylethylenediamine dihydrochloride. The absorbance was measured at 540 nm using the Victor™ X3 multilabel reader.

Statistical analysis. Data are presented as the mean ± standard deviation. At least three individual experiments were performed. Statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA), Differences between groups were analyzed using one-way analysis of variance, followed by the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of UA on cell viability. Prior to the experiments, it was necessary to evaluate the effect of UA on cell viability. Cell viability was determined using an MTT assay for the RAW

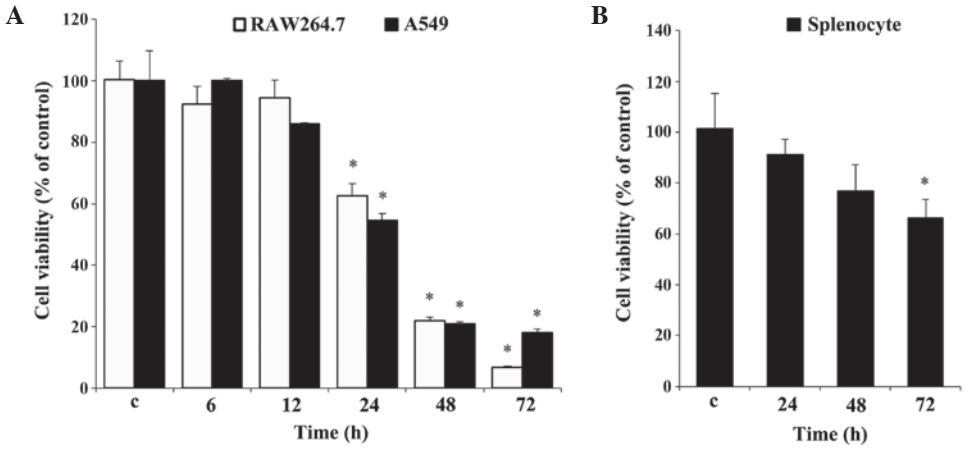

Figure 1. Effect of UA on cell viability using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (A) RAW 264.7 and A549 cells were treated with 10 μg/ml UA for 6-72 h and (B) rat splenocytes were treated with similar doses of UA for 24-72 h. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05, vs. control; UA, ursolic acid; c, control.

264.7 and A549 cells (Fig. 1A), and using an MTS assay for the rat splenocytes (Fig. 1B). Measurements over time established that significant cytotoxicity was evident, beginning at 24 h, in the two cell lines. At 72 h, 10 μg/ml UA resulted in cytotoxicity towards the RAW 264.7 cells, which was almost 11% higher than that in the A549 cells (Fig. 1A). By contrast, the splenocytes showed less cytotoxicity under similar treatment conditions between 24 and 72 h, compared with the RAW 264.7 and A549 cells. No significant difference in splenocyte viability was found at any time point, with the exception of 72 h (Fig. 1B), at which the cell viability was decreased by almost 35%, which was marginal, compared with the other two cell types at 72 h (Fig. 1B).

UA suppresses infection- and Con A-induced cytokine expression. Cytokines are critical in the development and regulation of immune responses. Several phytochemicals are involved in the immunomodulatory activities of the immune system and, thus, are key in immunosuppression. The expression levels of genes encoding *TNF-α*, *IL-6* and *IL1-β* in different cells was evaluated following infection and/or treatment. The *M. tuberculosis*-infected RAW 264.7 cells showed a significant induction of the mRNA expression of *TNF-α* at 12 and 24 h (Fig. 2A). This induction was significantly reduced by treatment of the RAW 264.7 cells with UA. In the A549 cells, the induction of *TNF-α* by infection, and the reduction by UA were not significant (Fig. 2B). UA treatment alone reduced the mNRA levels of *TNF-α* in the RAW 264.7 cells at 12 h, however, the expression returned to a basal level at 24 h. In the A549 cells, UA did not induce significant changes at any time point (Fig. 2A and B). In the splenocytes, the Con A mitogen was used to investigate the immunoregulatory activity of UA. Con A increased the mRNA expression of *TNF-α* by ~2-fold at 12 h, which increased to 3-fold at 24 h. At both time points, UA significantly reduced the mRNA expression levels of*TNF-α*, compared with the control (Fig. 2C).

IL-6 acts as pro-inflammatory, as well as an anti-inflammatory, cytokine. In the RAW 264.7 cells, infection induced the mRNA expression of *IL-6* at 24 h, and was significantly reduced by UA treatment. Of note, the reduction was even lower than the basal level. Treatment with UA alone had no significant effect on the mRNA expression of *IL-6* (Fig. 3A). In the A549 cells, infection and/or treatment showed no significant change at 12 h, however, at 24 h the mRNA expression of *IL-6* increased almost 5-fold. UA significantly reduced this induced expression (Fig. 3B). No significant changes in the mRNA expression of *IL-6* mRNA were observed in the Con A and/or UA-treated splenocytes at 12 h. However, at 24 h, the expression of *IL-6* was significantly induced when treated with Con A, and UA successfully reduced this induced expression (Fig. 3C).

IL-1β is a pro-inflammatory cytokine, which was found to be significantly induced by *M. tuberculosis* H37Rv infection in RAW 264.7 cells (Fig. 4A) and A549 cells (Fig. 4B) at 12 and 24 h. Their induction was effectively reduced by UA treatment, however, in the RAW 264.7 cells, the reduction was below the basal level at 12 h, compared with the control (Fig. 4A and B). Compared with the RAW 264.7 and A549 cells, no significant changes were observed in the splenocytes treated with Con A and/or UA at 12 h. However, at 24 h, Con A induced the expression of *IL-1β*, whereas UA had no significant effect (Fig. 4C).

Infection- and Con A-induced expression of iNOS and COX-2 expression is regulated by UA. The *iNOS* and *COX-2* genes are usually induced by inflammation. To evaluate the effect of UA on their expression levels, the present study infected RAW 264.7 cells with *M. tuberculosis* H37Rv and/or treated the cells with UA. Infection induced the expression of the two genes significantly at 12 and 24 h. These levels of expression were significantly suppressed by UA at both time points. The gene expression of *iNOS* and *COX-2* were suppressed below the basal level at 24 h. No significant changes in the gene expression of *iNOS* or *COX-2* were observed in the cells treated with UA only (Fig. 5A and B).

The inflammatory response was induced in the splenocytes by treatment with the Con A mitogen. At 24 h, the expression of the *iNOS* gene was induced almost 5-fold, compared with the control, however, UA suppressed the expression by ~2-fold. No significant changes in the expression of *iNOS* were

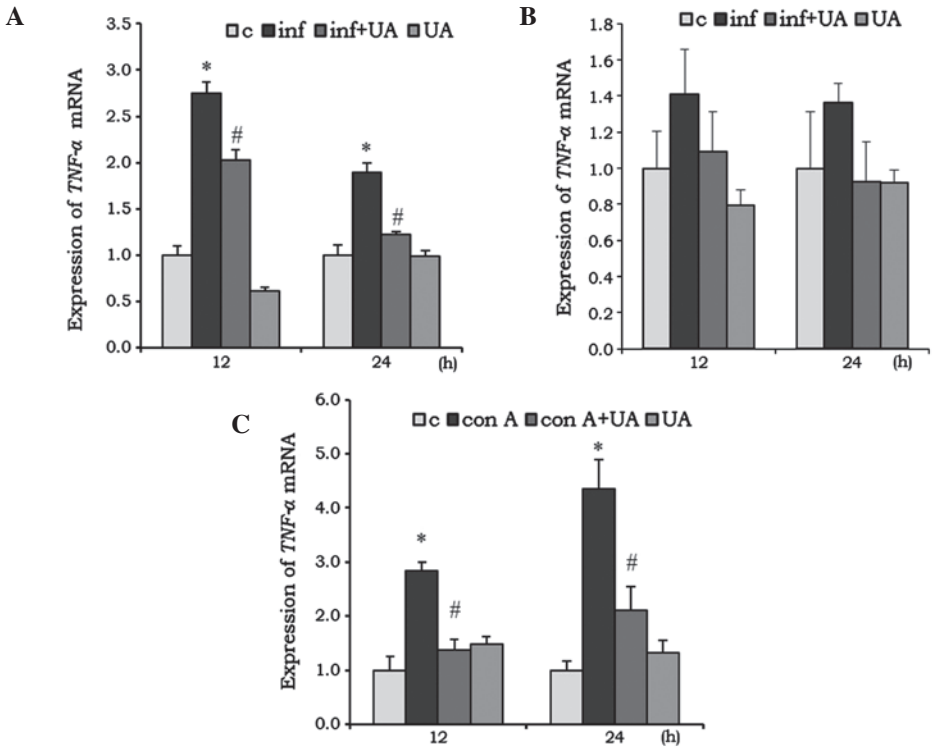

Figure 2. UA has an inhibitory effect on the expression of *TNF-α*. (A) RAW 264.7 cells and (B) A549 cells were infected with *M. tuberculosis* H37Rv (1:10) for 3 h and/or treated with 10 μg/ml UA for 6 h. (C) Rat splenocytes were co-treated with 5 μg/ml Con A and/or 10 μg/ml UA. Following 12 and 24 h treatment, the cells were harvested for total RNA collection, cDNA preparation and reverse transcription-quantitative polymerase chain reaction analysis. Data is presented as the mean ± standard deviation of three independent experiments. * P<0.05, vs. control; # P<0.05, inf+UA, vs. inf or Con A. TNF-α, tumor necrosis factor-α; c, control; inf, infection; UA, ursolic acid; Con A, concanavalin A.

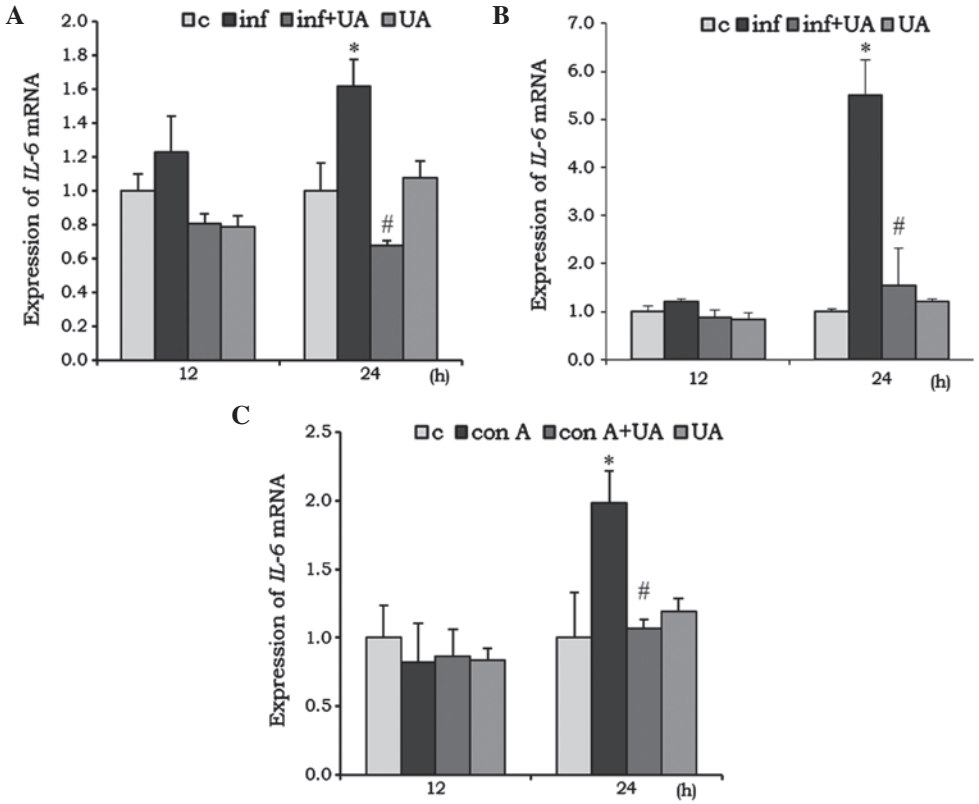

Figure 3. UA has an inhibitory effect on the expression of *IL-6* in RAW 264.7 cells, A549 cells and rat splenocytes. (A) RAW 264.7 cells and (B) A549 cells were infected with *M. tuberculosis* H37Rv for 3 h and/or treated with 10 μg/ml UA for 6 h. (C) Rat splenocytes were treated with 5 μg/ml Con A and/or 10 μg/ml UA. Following 12 and 24 h of incubation, the cells were harvested for total RNA collection, cDNA preparation and reverse transcription-quantitative polymerase chain reaction analysis of *IL-6* mRNA. Data is presented as the mean ± standard deviation of three independent experiment. *P<0.05, vs, control; #P<0.05, inf+UA, vs. inf or Con A. IL, interleukin; c, control; inf, infection; UA, ursolic acid; Con A, concanavalin A.

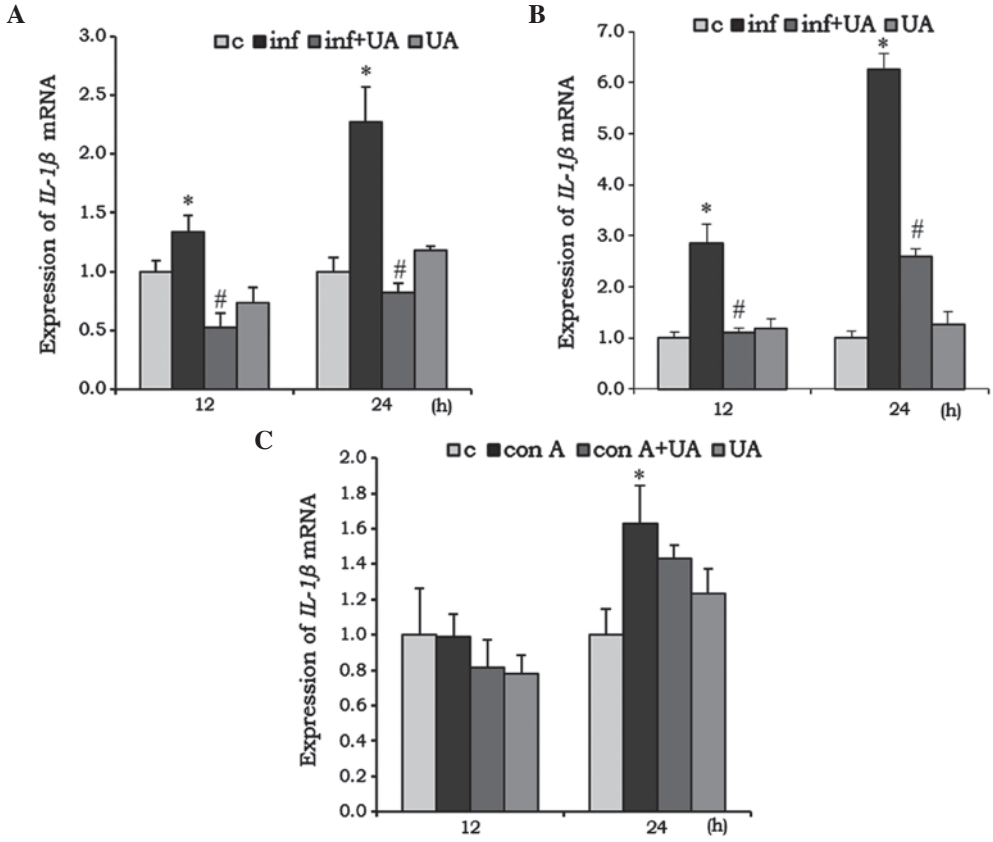

Figure 4. Effect of UA on the mRNA expression of *IL-1β*. (A) RAW 264.7 cells, (B) A549 cells were infected and/or treated with UA, and (C) rat splenocytes were infected and/or treated with Con A. Following indicated period of incubation, the cells were harvested for total RNA collection, cDNA preparation and reverse transcription-quantitative polymerase chain reaction analysis for *IL-1β*. Data is presented as the mean ± standard deviation of three independent experiments *P<0.05, vs. control; $^{\#}$P<0.05 vs. Inf or Con A. IL, interleukin; c, control; inf, infection; UA, ursolic acid; Con A, concanavalin A.

evident at 12 h in the samples treated with Con A and/or UA (Fig. 5C). However, at 12 and 24 h, the expression of *COX-2* was induced significantly by Con A treatment. UA treatment reduced this induced expression significantly at 24 h, but not at 12 h (Fig. 5D). Treatment of the splenocytes with UA alone did not significantly alter the gene expression levels of *iNOS* or *COX-2* from the basal level (Fig. 5C and D).

UA suppresses NO release in RAW 264.7 and A549 cells. NO is a signaling molecule, which is important in modulating the release of various inflammatory mediators from a wide range of cells (25). Infection of RAW 264.7 (Fig. 6A) and A549 (Fig. 6B) cells with *M. tuberculosis* H37Rv produced a significant release of NO into the culture medium at 24 and 48 h, respectively. The alveolar epithelial A549 cells did not appear to release NO prior to 48 h. In addition, treatment with UA or the NO synthase inhibitor significantly reduced the release of NO in the two cell types at these time points. Of note, UA and the NO synthase inhibitor also induced NO release in the two cell type, although without statistical significance (Fig. 6A and B).

Discussion

Inflammation is a healing process in the body, however, when it is prolonged, various diseases can result (26). Immunomodulatory therapy of TB reduces excessive inflammation and restricts pathology. Pro- and anti-inflammatory drugs may have significant potential in tailoring TB treatment, when administered with routine anti-TB drugs (27-29). In the present study, *M. tuberculosis* H37Rv was selected to infect mouse monocyte macrophages, RAW 264.7 and A549 type II alveolar cells. In the majority of previous studies, alveolar macrophages were considered for TB-associated studies. Alveolar epithelial cells are now also considered to be involved in pathogenesis and in the innate immune response (30,31). Natural compounds are a valuable source for a wide array of prospective biomedical uses, due to their limited side effects. UA shows anti-inflammatory potential in RAW 264.7 cells by attenuating the expression of *iNOS* and *COX-2* (32,33). The anti-inflammatory potential of UA has also been reported in activated T cells, B cells and macrophages (34). The mechanism underlying these effects has been attributed to the inhibition of mitogen-induced phosphorylation of extracellular signal-regulated kinase and c-Jun, N-terminal kinase, and the suppression of immunoregulatory transcription factors, NF-κB and nuclear factor of activated T cells and activator protein-1. Notably, UA mitigates lipopolysaccharide-induced expression of TNF-α, IL-1β and IL-6 in splenic adherent macrophages (34). In the present study, UA was investigated for its potential anti-inflammatory activity in differentially stimulated cells. A comparative investigation was performed to determine the gene expression levels of the *TNF-α*, *IL-1β* and *IL-6* cytokines, the *iNOS* and *COX-2*

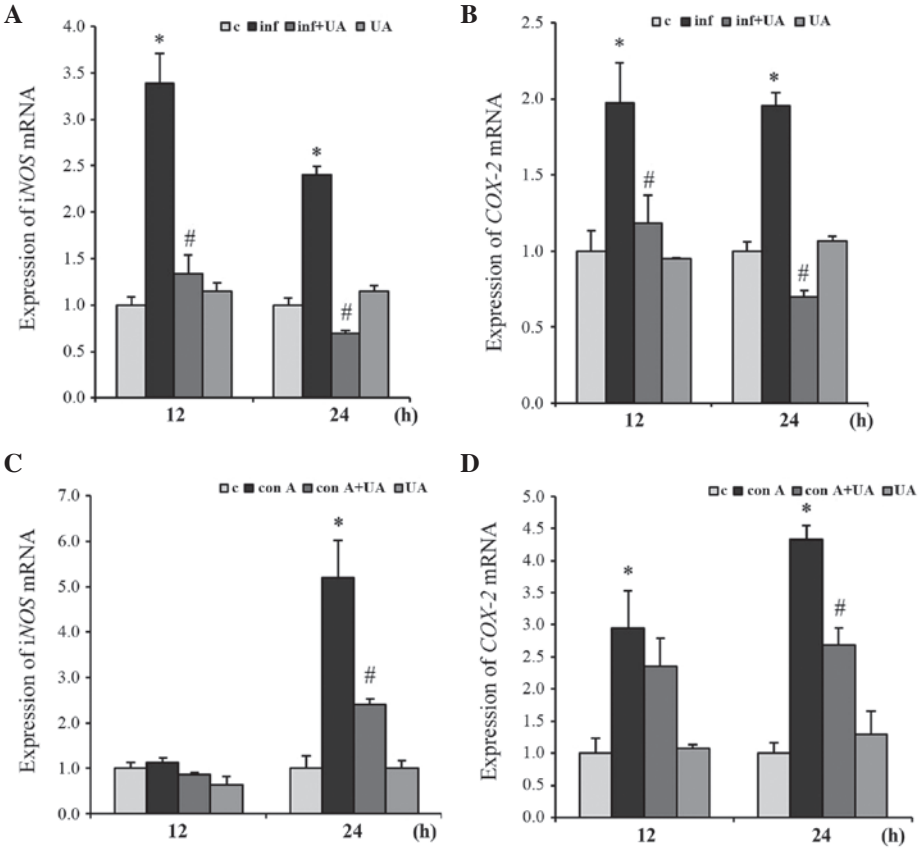

Figure 5. UA suppresses the expression of the *iNOS* and *COX-2* inflammatory mediators. (A and B) RAW 264.7 cells were infected and/or treated with UA. (C and D) rat splenocytes were treated with con A and/or UA. Following incubation, the cells were harvested, total RNA was collected, cDNA was prepared and reverse transcription-quantitative polymerase chain reaction analysis was performed for *iNOS* and *COX-2*. Data is presented as the mean ± standard deviation of three independent experiments *P<0.05, vs. control; $^{\#}$P<0.05, vs. inf or Con A. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; c, control; inf, infection; UA, ursolic acid; Con A, concanavalin A.

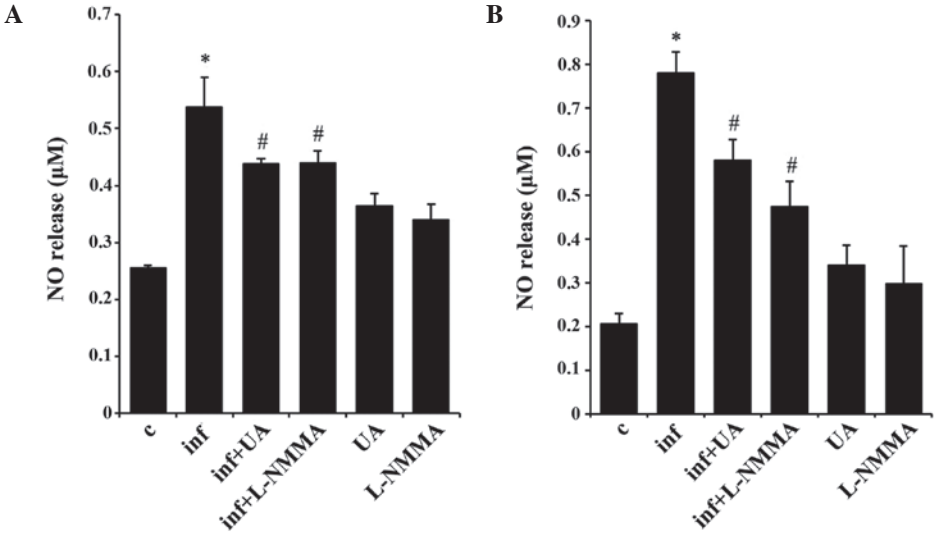

Figure 6. UA inhibits NO release. *M. tuberculosis* H37Rv infected (A) RAW 264.7 cells and (B) A549 (B) cells were treated with UA or L-NMMA for 6 h, or left untreated, and incubated for 24 and 48 h, respectively. Data is presented as the mean ± standard deviation of three independent experiments. *P<0.05, vs. control; $^{\#}$P<0.05, vs. inf. NO, nitric oxide; L-NMMA, N^{G}-monomethyl-L-arginine; c, control; inf, infection; UA, ursolic acid; Con A, concanavalin A.

inflammatory mediators, and the important signal transducer, NO. As a model, *M. tuberculosis* H37Rv was used to sensitize RAW 264.7 cells and A549 cells, and Con A-stimulated rat splenocytes.

The present study focused on the expression of *COX-2* and *iNOS*, as they are usually induced during inflammatory conditions, and are reduced by the effect of flavonoids (35,36). Abnormal upregulation of COX-2 and/or iNOS may be

associated with certain types of cancer or inflammatory disorders (37,38). In the present study, infection by *M. tuberculosis* H37Rv or treatment with Con A significantly increased the mRNA levels of *COX-2* and/or *iNOS*, which were downregulated by UA. NO, the product of iNOS, is crucial during inflammation. In addition to downregulating the expression levels of *COX-2* and *iNOS*, UA decreased the release of NO, which was induced following mycobacterial infection, in the cell culture medium. The infected A549 cells exhibited delayed NO release, compared with the RAW 264.7 cells. It has been reported that mycobacterial antigenic components produce significantly higher levels of NO at 48 h in A549 cells (39).

To detect anti-inflammatory activity, the present study examined the ability of UA to reduce the production of *TNF-α* in the mycobacteria-infected RAW 267.4 and A549 cells, and Con A-stimulated rat splenocytes. *TNF-α* is pivotal in inflammation, and its effect on the reduction of *IL-1β* and *IL-6* were examined in similar treatment conditions. All three cytokines were upregulated when stimulated by *M. tuberculosis* H37Rv in the RAW 267.4 cells, A549 cells and Con A-stimulated rat splenocytes. The three cytokines were induced by different stimuli in different cells, however, UA significantly reduced their expression levels at the transcriptional level in the present study. Upon stimulation, the expression levels of various cytokines, inflammatory mediators, including *iNOS* and *COX-2*, and NO release were induced at varying magnitudes and at different times.

UA induced marked inhibitory effects on the expression levels of cytokines and immunomodulatory mediators, and on NO release. These findings, and the results of previous reports suggest that UA has significant potential in mitigating the induced inflammatory response. Further detailed investigations of UA are required, to determine its potential as a functional remedy to control inflammation in various diseases.

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