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Original Research

Anti-inflammatory effect of a curcumin-aspirin derivative on *Ureaplasma*-induced cytokine expressions in neonatal monocytes

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Abstract: One of the major contributors to death in infants is infant respiratory distress syndrome (IRDS). The transition from early acute inflammation to fibrotic hyperplasia is important in the development of IRDS. However, there are no available and effective remedies for suppression of the early inflammation. In this study, the effect of a curcumin-aspirin (C-A) derivative on the expressions of interleukin (IL)-10, IL-8, tumor necrosis factor (TNF)-α, IL-1β, toll-like receptor (TLR)4 and TLR2 was investigated in neonatal monocytes exposed to *Ureaplasma parvum.* The techniques used were qPCR and flow cytometry. The results revealed that C-A exerted anti-inflammatory effects on stimulated monocytes and significantly suppressed *Ureaplasma*-stimulated changes in IL-8, TNF-α and IL-1β levels. It did not produce any inflammation or apoptosis in the neonatal cells. Moreover, C-A enhanced IL-10 expression and suppressed the expressions of TLR2 and TLR4, indicating that curcumin-aspirin derivative might hold therapeutic potential for early IRDS.

Key words: Infant respiratory syndrome; *Ureaplasma parvum*; Neonatal monocytes; Anti-inflammation; Cytokines.

Introduction

Infant respiratory distress syndrome (IRDS) with progressive respiratory failure affects about 1 % of neonates, and is a major contributor to death in newborn infants (1). It is characterized by early acute inflammation and massive fibrosis in the late stage, as well as pulmonary edema, prominent leukocyte accumulation and excessive deposition of extracellular matrix. Preterm babies with this condition are more vulnerable due to the structural immaturity of their lungs (2). The primary cause of IRDS is developmental insufficiency which in most cases, is due to a genetic defect in with lung development. Despite great improvements made from studies on the pathophysiology of IRDS, its mortality rate remains significant at 35 – 40 % (3). In the transition phase of IRDS (from inflammation to early fibro-proliferation), only supportive treatments and protective lung ventilation have been shown to be clinically beneficial by reducing morbidity and mortality([4,5]). Therefore, it is necessary to develop treatments for suppressing fibrotic hyperplasia associated with inflammation in IRDS.

Aerobic, anaerobic and atypical bacteria such as *Ureaplasma* species (spp.) are potential pathogens with *ureaplasma,* being the predominant microorganisms in the respiratory tract of very immature infants who develop bronchopulmonary dysplasia (BPD) ([6,7]). Relevant epidemiological and experimental data have associated perinatal *Ureaplasma* infection with lung inflammation and adverse pulmonary outcome in this cohort ([8,9]). Among them, *Ureaplasma parvum* (*U. parvum*) which represents 48 – 86 % of clinical isolates, is considered the most common isolated species ([10-12]). Clinical studies have reported increased levels of TNF-α and IL-1β in the trachea of *Ureaplasma*-colonized premature infants ([13,14]). Enhanced lung fibrosis and higher levels of pro-inflammatory mediators have been documented in perinatal *Ureaplasma* infection (15). It has been suggested that the mobilization of neutrophils and monocytes to lung inflammation areas is carried out mainly by IL-8 ([16,17]). Interleukin (IL)-10 inhibits the expressions of mediators of inflammation in macrophages and monocytes, while simultaneously upregulating the expressions of factors associated with anti-inflammation ([18,19]). Toll-like receptors (TLRs) are involved in early sensing of pathogens and pathogen-induced signaling (20), with TLR2 and TLR4 providing inborn immunity against neonatal sepsis- and chorioamnionitis-associated pathogens ([21,22]). Moreover, TLR2 and TLR4 are involved in the etiology of neonatal inflammatory diseases.

Curcumin (Cur) is a natural polyphenol which effect against various pro-inflammatory diseases has been established in multiple pathways such as NF-κB and STAT3 ([23,24]). Asha et.at reported that Cur played anti-inflammatory role via PPAR-γ activation([25]), and involved downregulation of MMP-9 in blood mononuclear cells([26]). Aspirin, a typical non-steroidal anti-inflammatory drug is important for mitigating inflammation and alleviating pain, that could inhibit the activity of the so-called prostaglandin synthetase enzyme system and suppress essential fatty acid metabolism ([27,28]). Therefore, the effect of a derivative of curcumin and aspirin (C-A, Figure 1) on *Ureaplasma*-induced cytokine expressions in human neonatal monocytes was investigated in the present study.

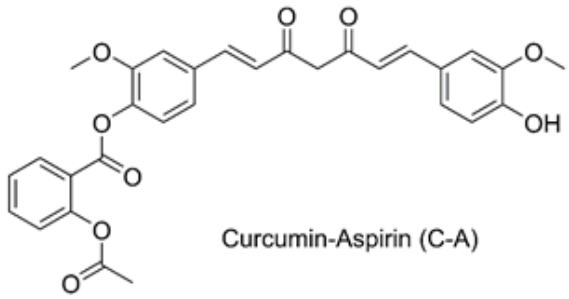

Figure 1. Structure of curcumin-aspirin.

Materials and Methods

Materials and reagents

The derivative of curcumin and aspirin was designed and kindly provided by Professor Rongdong Li of Hunan University of Chinese Medicine. A stock solution of the derivative was prepared in DMSO. The MTT used was bought from Sigma Chemical Co. (St. Louis, USA).

Ureaplasma parvum and culturing protocol

Ureaplasma parvum serovar 3 was provided by the American Tissue Culture Collection (ATCC). It was cultured in *Ureaplasma* Enrichment Broth® (Oxoid GmbH, Thermo Fisher Scientific, Wesel, Germany) at 37 °C until late log phase. Frozen stocks of 0.5 ml aliquots were prepared from mid-logarithmic-phase broth culture and kept at -80 °C until used for stimulation experiments. For each experiment, *U. parvum* was inoculated in PPLO-based medium containing 10 % horse serum (v/ v), 1 % urea (w/ v) and 0.0002 % phenol red (w/ v). The medium was diluted 1 in 10 at pH 6.5, and for inoculation of cell culture. Incubation was performed for 14 - 18 h until colour change to obtain titres of 10^8 - 10^9 CCU/ml. The CCU/ml was determined in duplicate by serial 10-fold dilutions in PPLO-based medium.

Enrichment of neonatal CD14$^+$ monocytes

Monocytes from cord blood mononuclear cells were donated by Professor Xiao Han, Nanjing Medical University. Parents of the preterm baby granted consent for the study prior to birth of the baby. The study received permission from the ethical committee of Nanjing Medical University, and was executed in strict compliance with the Helsinki Declaration. Venous blood samples were taken from the umbilical cord using *in utero* method, and were immediately used for isolation of mononuclear cells through the Ficoll-Paque procedure (25 min at 530 g) after diluting 1 in 3 with physiological saline. The enrichment of the neonatal CD14$^+$ monocytes was effected using magnetic-activated cell sorting (MACS) with CD14 MicroBeads® (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cytometric analysis was used purity assessment, and the results indicated purity in excess of > 90 %.

Stimulation assays on CD14$^+$ monocytes

The monocytes were cultured at 1 x 10^6 cells/ml for 2 h in 24-well plates in RPMI1640 medium to which 10 % FBS was added. Then, *Uroplasma parvum* suspensions were centrifuged and aliquots of viable organisms were added to the wells at a concentration of 10^8 -10^9 CCU/ml. The stimulation was done at 37 °C for 12 h in a 5 % CO_2 environment. Unstimulated cells served as negative control. To assess the levels of intracellular cytokines, Brefeldin A (10 µg/ml) was put in the incubation medium to enhance retention of cytokines. For mRNA analysis and accumulation of intracellular proteins by flow cytometry, detection rates had peaked at 12 h incubation. Cell viability was > 95 % after 12 h cell culture and *Uroplasma parvum* exposure.

qRT-PCR studies

NucleoSpin® RNA Kit (Macherey-Nagel) was used for extraction of total RNA from CD14$^+$ monocytes in line with the kit protocol after 12 h-treatment of C-A. The RNA extract was subjected to elution in zero-nuclease water (60 µl and preserved at -80 °C. Total RNA (0.11 - 0.52 µg) were subjected to reverse-transcription to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, CA). The first strand cDNAs were preserved at -80 °C. Using appropriate primers, the cDNAs were analyzed for mRNA expressions of the various TLRs and ILs, as well as TNF-α, using TNFiTaq™ Universal SYBR Green Supermix after diluting 10 times with zero-nuclease, deionized water. The relative expressions of the mRNAs were quantified using the $^{\Delta\Delta}$CT method.

Determination of cytokine protein expressions using flow cytometry

Using appropriate antibodies, the protein expressions of TLR2, TLR4, TNF-α, IL-1β, IL-8 and IL-10 in the CD14$^+$ monocytes were assayed after 24 h-treatment of C-A through intracellular cytokine staining incorporating methanol permeabilization, using eFluor® 780, APC-H7 ([29]). Flow cytometric analysis was carried out with FACSCanto™ II (BD Biosciences), and the marker for positive cells was established using fluorescence minus one (FMO).

Statistical analysis

Results are presented as mean ± standard deviation (SD). Statistical analyses were carried out using non-parametric Kruskal-Wallis test and Dunn's multiple comparison post hoc-test. All analyses were done with GraphPad Prism6 (San Diego, CA). Values of $p \leq 0.05$ were taken as evidence of statistical significance.

Results

Influence of U. parvum stimulation and C-A exposure on viability of neonatal CD14$^+$ monocytes

At first, the effects of *U. parvum* and C-A exposure on viability of neonatal CD14$^+$ monocytes was assessed by flow cytometry. Exposure to 50 µg/ml C-A or *U. parvum* stimulation had no effect on viability (Figure 2).

Effect of C-A on U. parvum-induced changes in TNF-α, IL-1β and IL-8 in neonatal monocytes

The expressions of TNF-α, IL-1β and IL-8 mRNA in native or *U. parvum*-stimulated neonatal monocytes was determined using RT-PCR. At 12 h, the expressions of TNF-α, IL-1β and IL-8 mRNA were very low in native neonatal monocytes, without treatment and after administration of C-A (Figures 3A-3C). Therefore, C-A

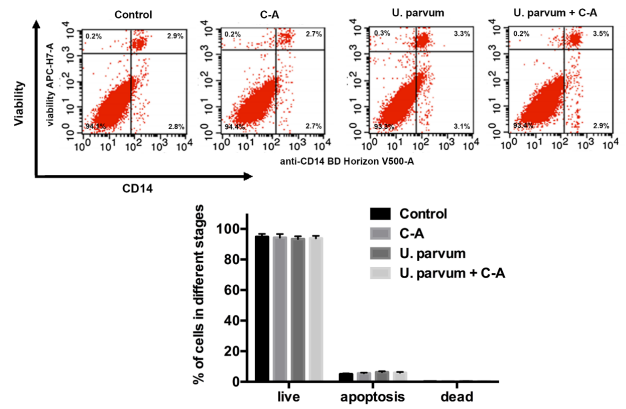

Figure 2. Flow cytometric analysis of apoptosis in the various groups: Control, C-A treatment, *U. parvum* stimulation, and simultaneous addition *U. parvum* and C-A.

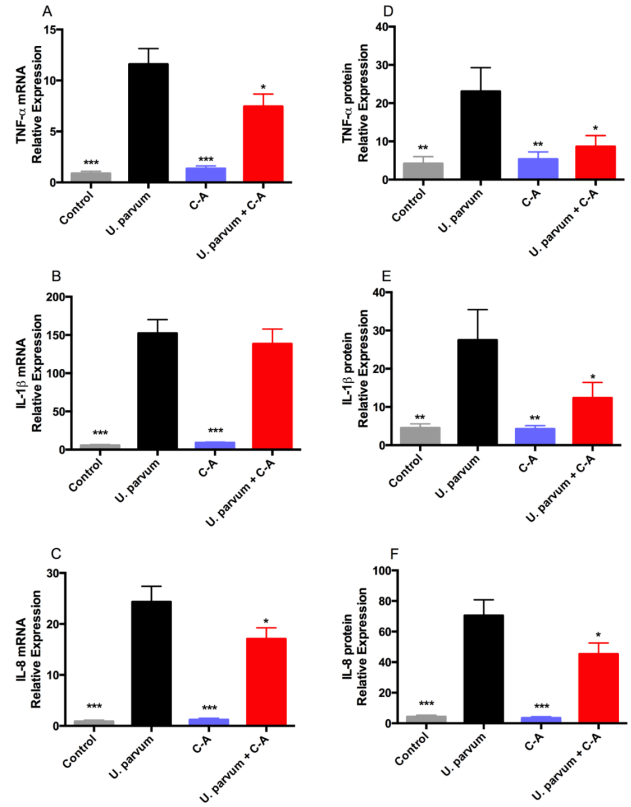

Figure 3. Effect of CHF5633 on TNF-α, IL-8 and IL-1β expressions in neonatal monocytes stimulated with *U. parvum* (n = 5). Each row shows results of mRNA (A, B, C) and protein expressions (D, E, F). Data are expressed as mean ±SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, relative to *U. parvum*-stimulated controls.

did not produce inflammation in unstimulated neonatal monocytes. However, incubation of neonatal CD14$^+$ monocytes for 12 h with *U. parvum* led to significantly increased expressions of TNF-α, IL-1β and IL-8 mRNA, when compared with unstimulated controls (Figures 3A-3C). Flow cytometric analysis also showed marked upregulation of intracellular TNF-α, IL-1β and IL-8 protein levels in *U. parvum*-stimulated CD14$^+$ monocytes. The C-A treatment caused significant reductions in the *U. parvum*-induced increases in TNF-α and IL-8 mRNA, but did not affect IL-1β mRNA expression. The *U. parvum*-induced upregulation of protein expressions of TNF-α, IL-1β and IL-8 were significantly reduced by C-A administration. Simultaneous exposure to C-A and *U. parvum* significantly reduced *U. parvum*-induced upregulations of TNF-α and IL-8 mRNA, and intracellular protein levels of TNF-α, IL-1β and IL-8 in neonatal monocytes (Figures 3D-3F).

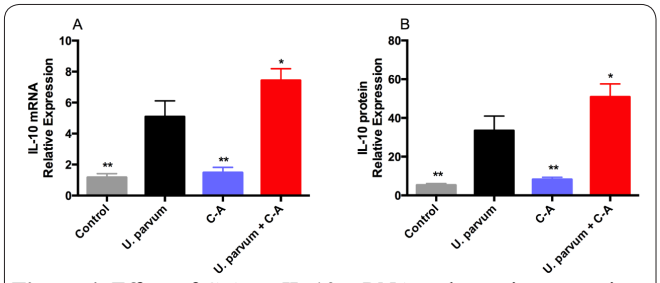

Figure 4. Effect of C-A on IL-10 mRNA and protein expressions in neonatal monocytes stimulated with *U. parvum* (n = 5). Results are indicated as mean ± SD. * $p < 0.05$, ** $p < 0.01$, relative to *U. parvum*-stimulated control.

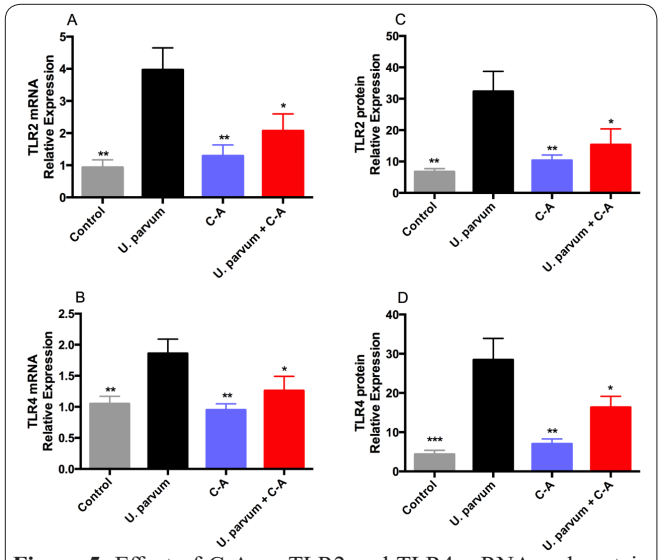

Figure 5. Effect of C-A on TLR2 and TLR4 mRNA and protein expressions in neonatal monocytes stimulated with *U. parvum* (n = 5). Results are presented as mean ± SD (* $p < 0.05$, ** $p < 0.01$, relative to *U. parvum*-stimulated control.

Effect of C-A on anti-inflammatory IL-10 induced by U. parvum in neonatal monocytes

The expression of IL-10 mRNA following *U. parvum* treatment alone was significantly increased at 12 h, when compared with control (Figure 4A), but was 25 % higher in *U. parvum*-stimulated neonatal monocytes exposed to C-A for 12 h (Figure 4A). A similar trend was also found in flow cytometric assays for intracellular IL-10 protein expression: IL-10 protein expression in CD14$^+$ monocytes was significantly increased by *U. parvum* alone, but simultaneous exposure to C-A and *U. parvum* for 12 h induced significantly higher expression of IL-10 protein than *U. parvum* (Figure 4B).

Effect of C-A on U. parvum-induced expressions of TLR2 and TLR4 mRNA in neonatal monocytes

As can be seen in Figure 5, on stimulation with *U. parvum*, there were significant upregulations in the expressions of TLR2 and TLR4 mRNAs in the monocytes, but their expressions were unaffected by C-A administration alone compared with control group. Exposure of *U. parvum*-stimulated neonatal monocytes to 50 µg/ml C-A significantly decreased TLR2 and TLR4 mRNA expressions (Figure 5A-B). Identical trends were seen in the protein expressions of TLR2 and TLR4, using flow cytometry analysis (Figure 5C-D).

Discussion

Studies have shown that cytokine responses are very robust in alveolar macrophages and monocytes ([30]). A monocyte model was employed in this investigation due to the fact that broncho-alveolar lavage results in very low levels of alveolar macrophages. We investigated potential effects of C-A on *Ureaplasma*-induced monocyte cytokine responses, the latter having been linked to serious pulmonary inflammation in premature babies. *Ureaplasma* upregulated the expressions of mRNAs and intracellular proteins of the pro-inflammatory factors TNF-α, IL-1β and IL-8 in neonatal monocytes. However, a similar effect was not seen in unstimulated, native monocytes.

Exposure to C-A down-regulated the mRNA and protein levels of TNF-α and IL-8, and also downregulated IL-1β protein synthesis, but did not affect IL-1β mRNA expression. The C-A exposure also led to significant enhancement of IL-10 mRNA and protein in neonatal monocytes stimulated with *U. parvum* for 12 h.

The presence of some inconsistencies in the results may be attributed to fast modifications and folding of some inflammation mRNA. Studies have reported slow translation of IL-10 transcripts. This might serve to maintain homeostasis in view of the fact that excessive production of IL-10 protein might enhance cellular inflammation. The present study appears to be the first investigation that reports C-A-induced significant upregulation of TLR2 and TLR4 expressions in neonatal monocytes. These results are important because the toll-like receptors sensitize monocytes to the presence of microbial pathogens.

The findings from this study confirm that C-A does not exert inflammatory and apoptotic influences on neonatal monocytes even in pre-existing *Ureaplasma* infection. In fact, C-A exerted anti-inflammatory features in stimulated monocytes and significantly suppressed *Ureaplasma*-mediated cytokine expressions. In addition, C-A promoted expression of IL-10, and suppressed TLR2 and TLR4 expressions, indicating stimulus-independent anti-inflammatory features of C-A. Further studies will be devoted to addressing the specific mechanisms involved in these cytokine responses. The anti-inflammatory characteristics of the curcumin-aspirin derivative might hold therapeutic potential for IRDS.

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Interest conflict
There is no conflict of interest to be declared by the author.

Author's contribution
We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors, all authors read and approved the manuscript for publication.
Jiang Yan conceived and designed the study, Jiang Yan, Li Heqin, Yi Xiangping, Ye Hui collected and analysed the data, Jiang Yan wrote the manuscript.

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