Atorvastatin reduces lipopolysaccharide-induced expression of C-reactive protein in human lung epithelial cells

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Abstract. Accumulating evidence suggests that statins possess anti-inflammatory properties and may decrease C-reactive protein (CRP) levels in plasma. However, no studies have as yet addressed whether or not statins regulate the expression of CRP in human lung epithelial cells (A549). In this study, we determined whether atorvastatin modulates the lipopolysaccharide (LPS)-induced expression of CRP in A549 cells. A549 cells were incubated in Dulbecco's modified Eagle's medium containing LPS in the absence or presence of various concentrations of atorvastatin. After incubation, the medium was collected and the amount of CRP was measured by an enzyme-linked immunosorbent assay. The cells were harvested and CRP messenger ribonucleic acid (mRNA) was analyzed by reverse transcription polymerase chain reaction. Incubation with LPS induced a significant time- and dose-dependent increase in CRP mRNA expression and CRP production in A549 cells, whereas atorvastatin significantly decreased LPS-induced CRP mRNA expression and CRP production in a dose-dependent manner. The present study revealed that A549 cells are capable of LPS-induced CRP expression, and that atorvastatin down-regulates the LPS-induced expression of CRP in cultured A549 cells. Our results suggest that statins ameliorate lung inflammation by regulating CRP production in human lung epithelial cells.

Introduction

C-reactive protein (CRP) is an acute inflammatory marker that is mainly produced by hepatocytes (1). In response to tissue damage or inflammation, serum levels of CRP may increase 1000-fold from baseline concentrations of less than 2 mg/l (2). Although the elevated serum level of CRP is predominantly produced by hepatocytes (1), a number of studies have shown the extrahepatic synthesis of CRP (3-6). Certain studies have noted that human lung epithelial cells express CRP (7-10). Additionally, our research team has observed that the CRP concentration in the sputum of patients with chronic obstructive pulmonary disease (COPD) is significantly higher than that in the plasma of these patients (11). This evidence suggests that lung epithelial cells are a source of locally produced CRP.

On the other hand, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (or statins) are extensively used in medical practice, and large clinical trials have demonstrated that this class of lipid-lowering drugs greatly reduces cardiovascular-related morbidity and mortality in patients with and without coronary disease (12-14). Previous *in vitro* and *in vivo* findings indicate that in addition to their established cholesterol-lowering properties, statins exert a number of cholesterol-independent pleiotropic effects such as anti-inflammatory properties by affecting endothelium function, leukocyte adherence, platelet aggregation and depressing production of inflammatory factors (15-17). Statins have been shown to ameliorate local acute inflammatory reaction and decrease plasma levels of inflammatory markers, such as CRP (18,19). Moreover, statins were previously shown to improve COPD survival (20,21). However, the exact mechanism has yet to be elucidated.

Epithelial and endothelial cell permeability are increased during lung inflammation. Consequently, proteins such as CRP, released from lung epithelial cells, have the potential to enter the circulatory system (22). Thus, inhibiting CRP production in these cells may reduce serum CRP levels. However, whether or not atorvastatin affects CRP expression in human lung epithelial cells has yet to be examined. Due to the significance of CRP in inflammatory diseases, we examined the effects of atorvastatin on lipopolysaccharide (LPS)-induced expression of CRP in cultured human lung epithelial cells. .

Materials and methods

Materials. A human lung epithelial cell line (A549) was purchased from American Type Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM), trypsin, fetal bovine serum (FBS) and LPS were purchased from

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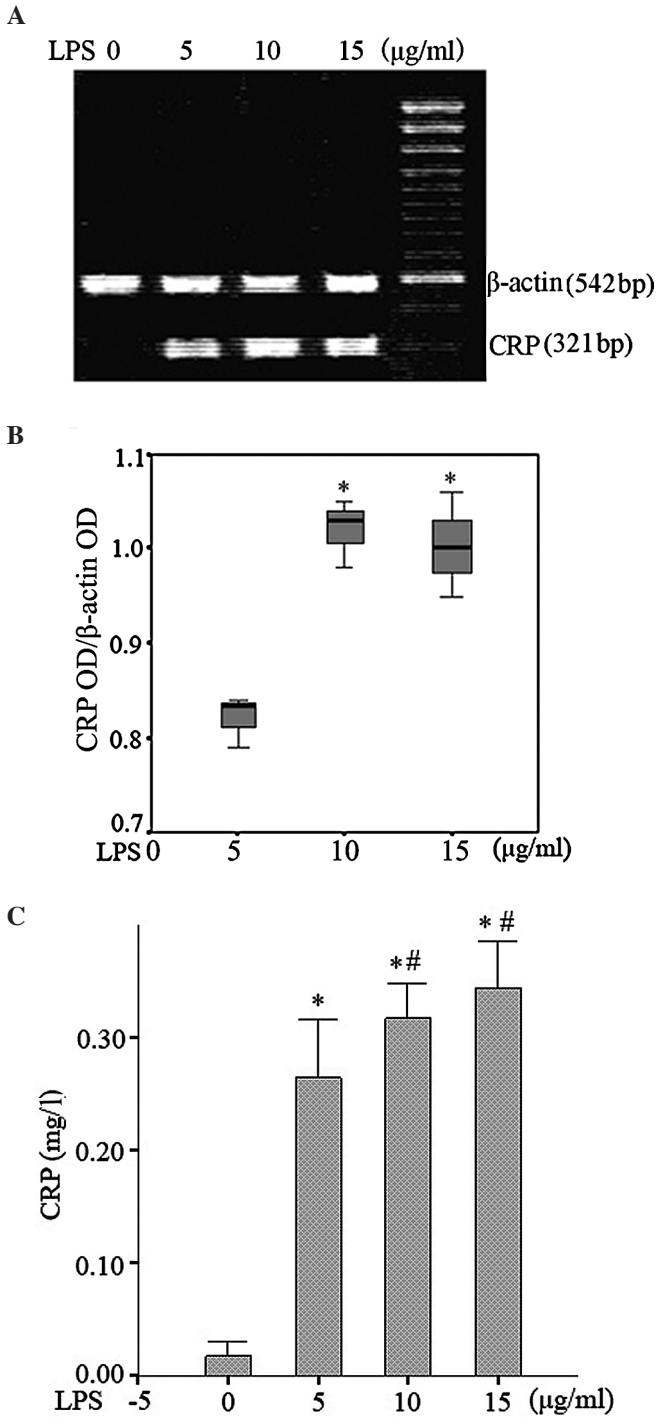

Figure 1. Dose-dependent effects of lipopolysaccharide (LPS) on C-reactive protein (CRP) mRNA expression and production. A549 cells were incubated with different concentrations of LPS for 24 h. (A) A representative gel of CRP mRNA expression determined by RT-PCR. (B) Relative density of gel (*p=0.01 vs. LPS 5 μg/ml). (C) CRP levels determined by ELISA at different LPS concentrations (*p<0.001 vs. non-LPS group; #p<0.05 vs. 5 μg/ml LPS group). OD, optical density.

Sigma-Aldrich (Sigma, St. Louis, MO, USA). TRIzol and electrophoresis reagents were purchased from Promega Co. (Promega, WI, USA). High sensitive CRP enzyme-linked immunosorbent assay (ELISA) kits were purchased from Daiichi Pure Chemicals Co., Ltd. (Daiichi Pure Chemicals, Japan). Atorvastatin was a gift from Beijing Honghui Medicine Co. (Honghui, China).

Cell culture. A549 cells were grown in DMEM supplemented with 5% FBS, 100 μ/ml penicillin, 100 μ/ml streptomycin and 50 μg/l amphotericin B. Cultures were maintained at 37˚C in a humidified atmosphere of air and 5% CO_2 Cells were sub-cultured into 6-well plates and maintained until sub-confluence. The medium was then replaced by a serum-free culture medium for 24 h prior to the addition of LPS and/or atorvastatin. The cells were then incubated with various concentrations of LPS for 24 h, or 10 μg/ml LPS for various time periods. To investigate the effect of atorvastatin on the expression of CRP *in vitro*, the cells were incubated in the serum-free medium containing 10 μg/ml LPS in the absence (control group) or presence of an increased concentration of 10, 15 and 20 μmol/ml atorvastatin for 24 h.

RNA extraction and reverse transcriptase polymerase chain reaction. CRP messenger ribonucleic acid (mRNA) was measured by reverse transcriptase polymerase chain reaction (RT-PCR) as previously described (9). Briefly, total RNA from various experimental conditions was obtained using the TRIzol reagent method (Life Technologies, Carlsbad, CA, USA) and the concentration of RNA was determined by an absorbance at 260 nm. For RT-PCR, 100 ng of RNA from different experimental conditions was applied to the access RT-PCR System. The primers used for CRP were: forward: 5'-TTT TCT CGT ATG CCA CCA AG-3' and reverse: 5'-CTG GTG GGA GAC ATT GGA AA-3', which yielded products of 321 bp, and β-actin: forward: 5'-GTC ACC CAC ACT GTG CCC ATC-3' and reverse: 5'-ACA GAG TAC GCG CTC AGG AG-3', which yielded products of 542 bp. The PCR thermocycling conditions for the genes were: an initial denaturation for 4 min at 94˚C, 35 cycles with denaturation at 94˚C for 1 min, primer annealing at 55˚C for 1 min, primer extension at 72˚C for 1 min and 20 sec, and a final extension step at 72˚C for 10 min. Each PCR product (5 μl) was electrophoresed on 3-4 mm thick 1.5% agarose gels by electrophoresis and ethidium bromide stained bands were scanned by a Bio-Rad Gel Doc 2000 Imaging System (Bio-Rad Laboratories Ltd., UK). Optical density was analyzed using Quantity One 4.03 analysis software. The results for CRP mRNA levels were presented relative to the expression of β-actin.

CRP assay. After incubation, supernatants were collected to measure CRP. CRP was determined using ELISA kits as previously described (23). The high sensitivity human CRP ELISA kits are capable of detecting a minimal CRP concentration at 0.025 mg/l. The process was strictly operated according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using SPSS 10.0 Software. Data are presented as mean ± SD. The differences between the multiple treatment groups were analyzed by one-way ANOVA and the LSD test. For all tests, $p<0.05$ was considered to be statistically significant.

Results

Various concentrations of LPS induced up-regulation of CRP mRNA and CRP in A549 cells. The human lung epithelial cell line A549 was selected as a model to investigate whether

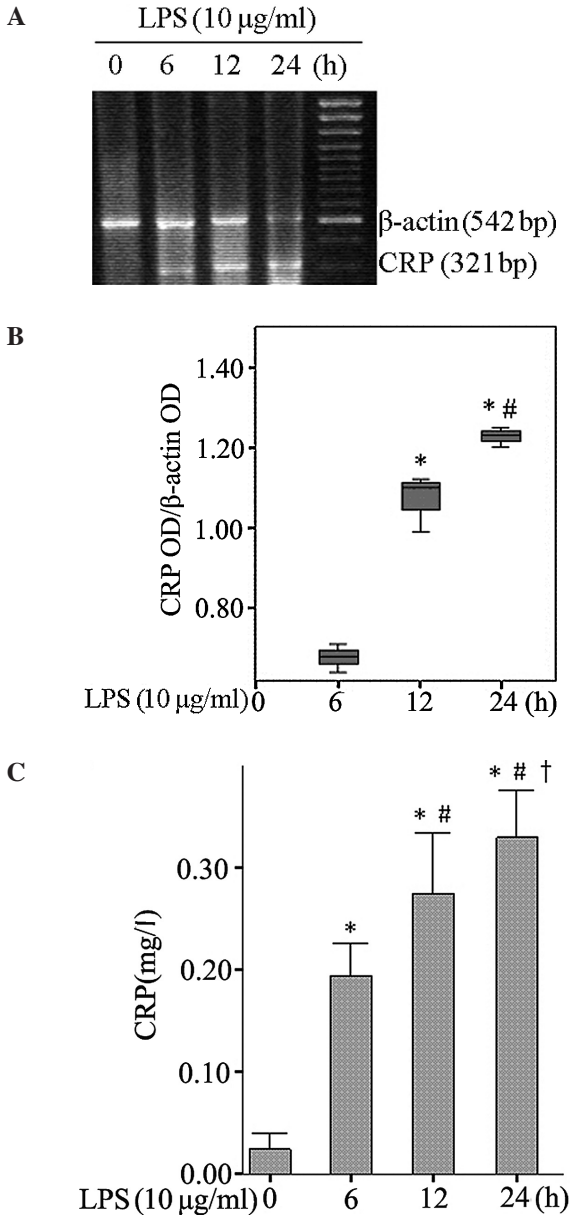

Figure 2. Time-dependent effects of lipopolysaccharide (LPS) on C-reactive protein (CRP) mRNA expression and CRP production. A549 cells were incubated with LPS (10 μg/ml) for various time periods. (A) A representative gel of CRP mRNA expression determined by RT-PCR. (B) Relative density of gel (*p=0.00 vs. 6 h group; $^{\#}$p=0.007 vs. 12 h group). (C) CRP levels determined by ELISA at different time points (*p=0.00 vs. control group; $^{\#}$p=0.00 vs. 6 h group; †p<0.05 vs. 12 h group). The data were representative of four independent experiments. OD, optical density.

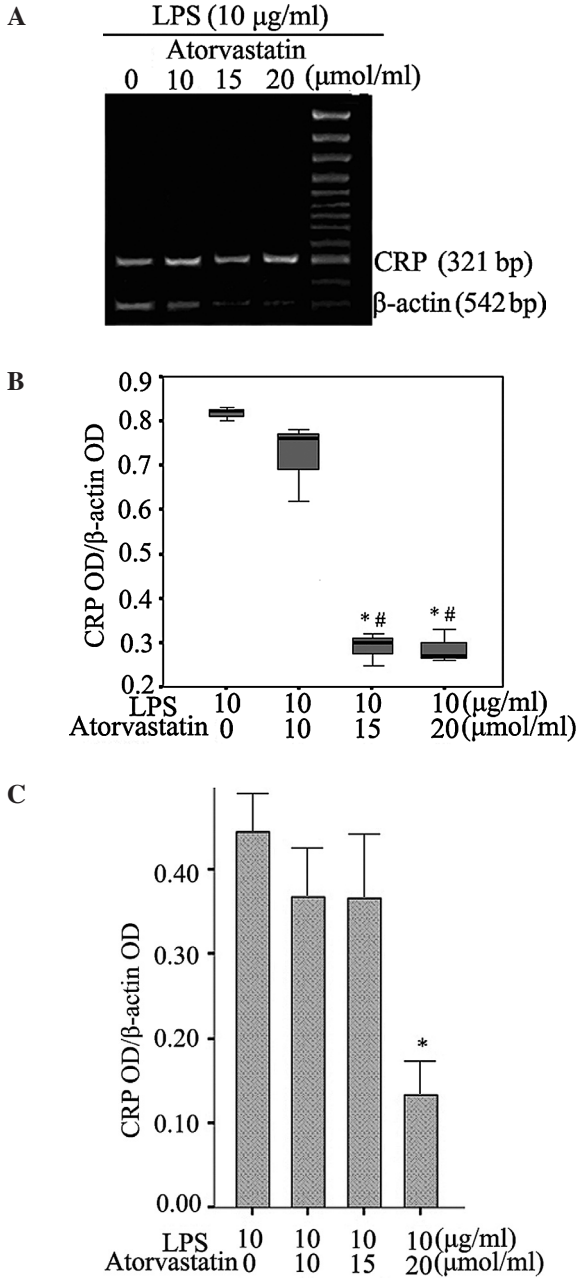

Figure 3. Atorvastatin reduces lipopolysaccharide (LPS)-induced C-reactive protein (CRP) mRNA expression and CRP production. A549 cells were treated with various concentrations of atorvastatin in the presence of LPS (10 μg/ml) for 24 h. (A) A representative gel of CRP mRNA expression determined by RT-PCP. (B) Relative density of gel (*p=0.00 vs. non-atorvastatin; $^{\#}$p<0.05 vs. 10 μmol/ml atorvastatin group). (C) CRP levels determined by ELISA in the presence of LPS (10 μg/ml) with various concentrations of atorvastatin ($^{\#}$p<0.05 vs. other groups). The data were representative of four independent experiments. OD, optical density.

LPS induces CRP production by human lung epithelial cells. A549 cells were incubated with various concentrations of LPS for 24 h. RT-PCR analysis indicated that CRP mRNA expression in the presence of LPS was significantly higher than that in the absence of LPS in a dose-dependent manner (p<0.05) (Fig. 1A). No CRP mRNA expression was noted in the absence of LPS. A concentration of 5 μg/ml LPS was effective in the induction of CRP mRNA expression. LPS (10 and 15 μg/ml) also increased CRP mRNA expression, but no significant difference was found between the two concentrations (p>0.05).

Consistent with CRP mRNA expression, ELISA analysis revealed that CRP levels in the presence of LPS were significantly higher than those in the absence of LPS in a dose-dependent manner (p<0.05) (Fig. 1B).

Time-dependent effects of LPS on CRP mRNA expression and CRP production. To determine the time-dependent effect of LPS, A549 cells were incubated with LPS (10 μg/ml) for various time periods. CRP mRNA levels were increased in the presence of LPS at 10 μg/ml in a time-dependent manner (p<0.05) (Fig. 2A). No CRP mRNA expression was found in the absence of LPS. The expression

of CRP mRNA was increased by LPS as early as 6 h, the earliest time point tested.

Similarly, CRP levels were increased in the presence of LPS at 10 μg/ml in a time-dependent manner ($p<0.05$) (Fig. 2B), significantly higher than those in the absence of LPS.

Atorvastatin decreases LPS-induced CRP mRNA levels and CRP production in A549 cells. To determine whether atorvastatin affects LPS-induced CRP mRNA expression and CRP production, A549 cells were incubated with LPS in the presence or absence of various concentrations of atorvastatin for 24 h. The expression of CRP mRNA induced by LPS was down-regulated by atorvastatin ($p<0.05$) (Fig. 3A). Consistent with this observation, LPS-induced CRP production was also inhibited by atorvastatin ($p<0.05$) (Fig. 3B). Atorvastatin inhibited LPS-induced CRP mRNA expression and CRP production in a dose-dependent manner.

Discussion

In the present study, we report for the first time that A549 cells are capable of LPS-induced CRP expression and that atorvastatin significantly decreased LPS-induced CRP expression in A549 cells. These results suggest that atorvastatin has direct anti-inflammatory effects on A549 cells.

CRP is generally thought to be produced only by hepatocytes (1) induced by interleukin-6 (IL-6). However, mounting evidence has revealed that other cells such as vascular smooth muscle cells (3), renal epithelial cells (4), adipocytes (5) and neuronal cells (6) also secrete CRP. More specifically, Gould and Weiser (7) reported that the human respiratory tract was able to produce CRP. Ramage *et al* reported that CRP was produced by A549 cells in response to cytokines, carbon particles and environmental air pollution particles (8,9). Consistent with these results, our study revealed that LPS induced CRP secretion in A549 cells. In addition, we previously reported that A549 cells were capable of producing inflammatory biomediators, such as cyclooxygenase-2, IL-6 and prostaglandin E2 in response to LPS (24). Results of this study provided further support for the hypothesis that A549 cells were able to produce CRP and other inflammatory factors in response to inflammatory cytokines. Based on the pathway that hepatocytes synthesize and secrete CRP induced by IL-6 (25), we postulate that IL-6 is involved in the LPS-induced CRP production of A549 cells, and that it may have a positive effect on CRP production in this process. In addition, the transcription factors STATs and NF-κB are involved in CRP expression (26). Thus, we hypothesize that cytokines IL-6 are released rapidly by A549 cells induced by LPS, and that those factors then act on the cells to up-regulate the production of CRP by affecting the expression of the transcription factors STATs and NF-κB. Further studies are required in order to clarify the definite signaling mechanisms for the induction of CRP.

In addition to their established cholesterol-lowering properties, statins exert anti-inflammatory actions, including reducing serum CRP levels (18,19). Previously, Arnaud *et al* reported that atorvastain decreased CRP production in human hepatocytes (25). In the present study, we report for the first time that atorvastatin is capable of reducing LPS-induced CRP production in A549 cells. These results provide new evidence of the direct anti-inflammatory effects of statins. The results also suggest that statins directly inhibit CRP expression in lung epithelial cells and hepatocytes that are capable of expressing CRP, thereby reducing serum CRP levels.

Numerous large observational studies have revealed a reduced incidence of pneumonia and improved pneumonia outcomes in patients receiving statins (27-30). These effects may be associated with reduced plasma CRP levels (27). More recently, results of a study showed that simvastatin not only reduced plasma CRP levels, but also decreased bronchoalveolar lavage fluid CRP levels of LPS-induced pulmonary inflammation in humans (31). This study confirmed that statins direct anti-inflammatory effects on lung epithelial cells. Thus, the results of this study are consistent with those of our study.

Inflammation in the lung itself as well as systemic inflammation have been shown to be involved in COPD (32). More specifically, mounting evidence suggests that serum CRP is a significant predictor of COPD outcome (33-37) and lung inflammation in COPD (9,10). Previous studies indicated that statins ameliorated COPD survival (20,21). Lee *et al* reported that pravastatin-treated COPD patients with a greater percentage decrease in CRP had a significant improvement in exercise time compared with those without CRP decrease (38). Based on these findings, we consider that statins are likely to improve COPD survival by reducing serum CRP levels. Our study as well as those of other authors revealed that human lung epithelial cells produced CRP (7-9). Epithelial and endothelial cell permeability may be increased during lung inflammation, allowing the movement of proteins across the lung-blood barrier (39). Therefore, CRP secreted by human lung epithelial cells may enter the systemic circulation and increase serum CRP levels in the presence or absence of a hepatic response. Thus, inhibiting CRP production in these cells may reduce serum CRP levels. We postulate that statins ameliorate COPD survival, partly through decreasing CRP expression in human lung epithelial cells.

The reasons that statins inhibited LPS-induced CRP expression in A549 cells are unclear. However, one specific mechanism may be involved. STAT3 is the transcription factor of CRP (40). Statins may inhibit STAT3 phosphorylation, thus inhibiting CRP gene expression (25). Further investigations are required to elucidate the mechanism of statin-inhibited CRP expression in A549 cells.

Although further studies are required, the present data reveal that A549 cells express CRP induced by LPS and that atorvastatin may reduce LPS-induced CRP expression in these cells. Our results indicate that statins may ameliorate the outcomes of COPD and pneumonia by regulating CRP production in human lung epithelial cells. These results may provide a further explanation of the beneficial effects of statins on lung inflammation in clinical trials.

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