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Atorvastatin reduces lipopolysaccharide-induced expression of cyclooxygenase-2 in human pulmonary epithelial cells

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Abstract

Objective: To explore the effects of atorvastatin on expression of cyclooxygenase-2 (COX-2) in human pulmonary epithelial cells (A549).

Methods: A549 cells were incubated in DMEM medium containing lipopolysaccharide (LPS) in the presence or absence of atorvastatin. After incubation, the medium was collected and the amount of prostaglandin E₂ (PGE₂) was measured by enzyme-linked immunosorbent assay (ELISA). The cells were harvested, and COX-2 mRNA and protein were analyzed by RT-PCR and western-blot respectively.

Results: LPS increased the expression of COX-2 mRNA and production of PGE₂ in a dose- and time-dependent manner in A549. Induction of COX-2 mRNA and protein by LPS were inhibited by atorvastatin in a dose-dependent manner. Atorvastatin also significantly decreased LPS-induced production of PGE₂. There was a positive correlation between reduced of COX-2 mRNA and decreased of PGE₂ ($r = 0.947$, $P < 0.05$).

Conclusion: Atorvastatin down-regulates LPS-induced expression of the COX-2 and consequently inhibits production of PGE₂ in cultured A549 cells.

1. Introduction

Human pulmonary epithelial cell is one of major sources of productive inflammatory biomediators, such as prostaglandin E₂ (PGE₂), interleukin-6 (IL-6), in respiratory inflammatory diseases [1]. Cyclooxygenase-2 (COX-2) is an inducible enzyme that is expressed in response to inflammatory cytokines, and it is responsible for the syn-

thesis of pro-inflammatory PGs such as PGE₂. Increased expression of COX-2 and production of PGE₂ have been found in pulmonary inflammatory disorders [2].

Statins is a class of compounds that decreases cholesterol synthesis via inhibition of 3-hydroxy-3methylglutaryl coenzyme A (HMG-CoA) reductase. Recently, anti-

inflammatory effects of statins have been described [3]. For example, Atorvastatin reduces expression of the COX-2 in cultured vascular smooth muscle cells [4]. However, it is not clear whether Atorvastatin also affects COX-2 expression in human pulmonary epithelial cells. Because of importance of COX-2 in inflammatory respiratory diseases, we tested the effects of Atorvastatin on lipopolysaccharide (LPS)-induced expression of COX-2 in cultured human pulmonary epithelial cells.

2. Methods

2.1 Materials

Human pulmonary epithelial cell line (A549) was purchased from American Type Culture Collection (ATCC). Medium DMEM, trypsin, fetal bovine serum (FBS) and LPS were purchased from Sigma-Aldrich. ECL chemiluminescence reagents, COX-2 polyclonal antibody were purchased from Cayman Chemical Co. Anti-rabbit IgG, horseradish peroxidase linked whole antibody was obtained from Amersham LIFE SCIENCE. HECAMEG was from Vegatec (Villejuif, France). Trizol and electrophoresis reagents were from Promag Co. Atorvastatin was a gift from Beijing Honghui Medicine Co.

2.2 Cell culture

A549 cells were grown in DEME medium supplemented with 5% FBS, 100 u/ml penicillin, 100 u/ml streptomycin, and 50 µg/L amphotericin B. Cells were sub-cultured into six-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 other reagents. The cells were then incubated with various concentrations of LPS for 9 h, or 10 µg LPS for different times. For atorvastatin experiments, the cells were incubated in the serum-free medium containing 10 µg LPS in the presence or absence of different concentrations of atorvastatin for 9 h.

2.3 PGE₂ assay

After incubation, the medium was collected for measurement of PGE₂. PGE₂ was determined by enzyme-linked immunosorbent assays (ELISA, Shanghai Sun Biomedical Co. CV <10%).

2.4 RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR)

COX-2 mRNA was measured by RT-PCR as previously described [5]. Briefly, total RNA from different experimental conditions was obtained by Trizol method (Life technologies) 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 following primers were used for COX-2: forward: 5'-AAG CTG GGA AGC CTT CTC TA-3' and reverse: 5'-TTT CCA TCC TTG AAA AGG CGC-3',

which yielded products of 342 bp (50 sec at 55°C for annealing of the primers, 35 cycles), and CYCLOPHILIN A: forward: 5'-ATG GTC AAC CCC ACC GTG TTC TTC G-3' and reverse: 5'-CGT GTG AAG TCA CCA CCC TGA CAC A-3', which yielded products of 206 bp (50 sec at 55°C for annealing primers, 38 cycles). The DNA products from RT-PCR reactions were analyzed on a 4% polyacrylamide-urea gel in the same buffer. The polyacrylamide gels were dried and scanned using the ImageQuant densitometer (Gel Doc 2000, BioRad Co).

2.5 Western Blot analysis

After incubation, A549 cells were washed twice in phosphate-buffered saline, lysed in 200 µl lysis buffer (20 mM Tris/HCL, pH 7.5, 20 mM HECAMEG, 1 mM benzamidine). Protein content was determined by a microbicinchoninic acid assay (Pierce) with bovine serum albumin as standard. Western blot analysis was performed as described previously [6]. Briefly, the protein was separated by electrophoresis on a 10% polyacrylamide gel at 180 V for 45 min. After transfer to nitrocellulose, the membrane was blocked, incubated with a specific rabbit polyclonal antibody against COX-2 (1:1000). The blots were then incubated with a horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:5000). Antibody labeling was detected by enhanced chemiluminescence. The films were scanned using an Arcus II Agfa scanner, and densitometric analysis was performed using Sigma Gel software.

2.6 Statistical analysis

Statistical analysis was performed with SPSS analysis (SPSS10.0 Software). PGE₂ and RT-PCR data are presented as mean ± S.D., and the differences between the multiple treatment groups were analyzed by the one-way ANOVA, LSD test. Data were correlated by nonparametric Spearman's rank method. Probability values of 0.05 or less were considered to be statistically significant.

3. Results

3.1 Dose- and time-dependent effects of LPS on COX-2 mRNA expression and PGE₂ production

To determine the concentration dependent effect of LPS, A549 cells were incubated with various concentrations of LPS for 9 h. RT-PCR analysis indicated that LPS increased the expression of COX-2 mRNA in a concentration-dependent manner (Figure 1A, top). Concentrations as low as 5 µg/ml LPS were effective in inducing expression of the COX-2 mRNA. To determine the time-dependent effect, A549 cells were incubated with LPS (10 µg/ml) for different times. The expression of COX-2 mRNA was increased by LPS as early as 6 h, the earliest time point tested. It reached a maximum induction by 9 h of incubation, and remained stable for at least 12 h, the longest time tested (Figure 1B, middle). LPS also increased the

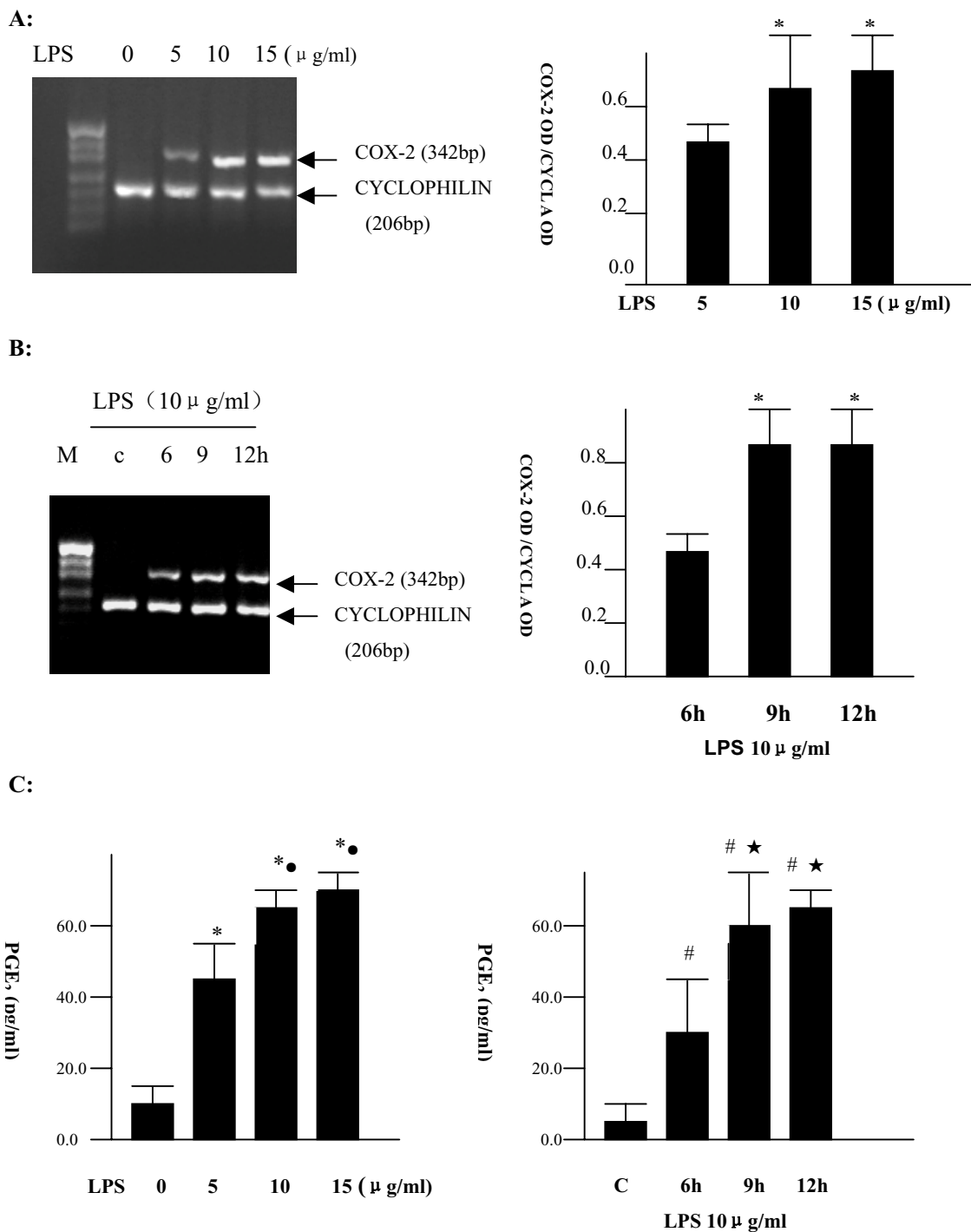


Figure 1

A: Dose-dependent effect of LPS on COX-2 mRNA expression. A549 cells were incubated with various concentrations of LPS for 9 h (top, * $P < 0.05$ vs LPS 5 μ g/ml). **B:** Time-dependent effect of LPS on COX-2 mRNA expression. A549 cells were incubated with LPS (10 μ g/ml) for various times (middle, * $P < 0.05$ vs 6 hrs group); **C:** Time- and dose-dependent effects of LPS on PGE₂ production (bottom, * $P < 0.05$ vs non-LPS group; * $P < 0.05$ vs 5 μ g/ml LPS group; # $P < 0.01$ vs non-LPS group; * $P < 0.05$ vs 6 h group). These data were representative of three separate experiments.

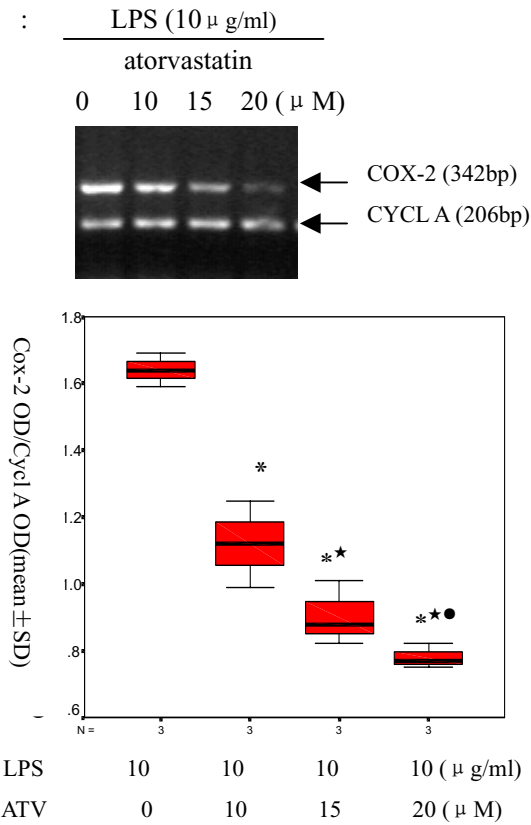


Figure 2
Effect of atorvastatin on LPS-induced expression of COX-2 mRNA. A549 cells were treated with various concentrations of atorvastatin in the presence of LPS (10 µg/ml) for 9 h. After incubation, total RNA were extracted and assayed by RT-PCR. A representative gel. (top) and relative density of gel. (bottom) are shown. * P < 0.05 vs non- atorvastatin. * P < 0.05 10 µM vs atorvastatin group; * P < 0.05 vs 15 µM atorvastatin group.

production of PGE₂, a major cyclooxygenase product in A549 cells, in a time- and dose-dependent manner (Figure 1C, **bottom**). LPS (5 µg/ml) caused a 3-fold increase in amount of PGE₂, and increased PGE₂ was also observed as early as 6 h of incubation.

3.2 Effect of atorvastatin on LPS-induced expression of COX-2 mRNA and protein

To determine whether atorvastatin affect the LPS-induced expression of COX-2, the cells were incubated with various concentrations of atorvastatin for 9 h in the presence of 10 µg/ml LPS. RT-PCR analysis indicated that LPS-

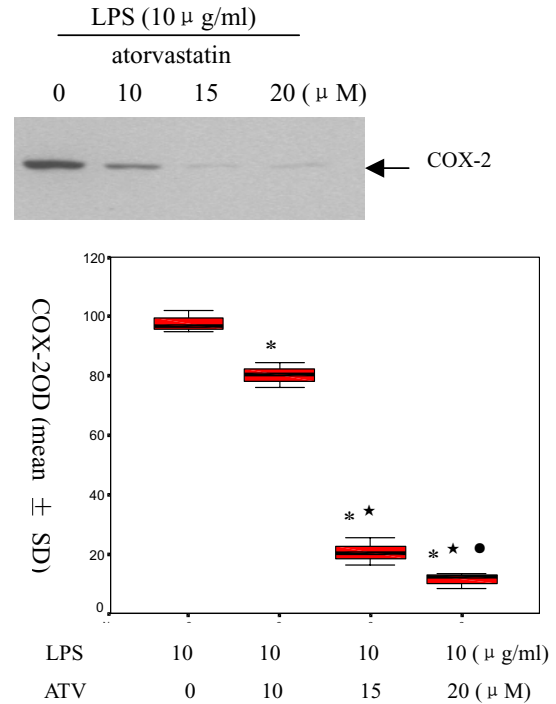


Figure 3
Effect of atorvastatin on LPS-induced expression of COX-2 protein. A549 cells were treated as described in Figure 2. After incubation, the cells were harvested, and sonicated. COX-2 protein in the cell lysates was detected by Western-blot using a specific antibody against COX-2. A representative western-blot gel. (top) and the density of COX-2 band (bottom) are shown. * P = 0.001 vs non atorvastatin; * P < 0.05 vs 10 µM atorvastatin; * P < 0.05 vs 15 µM atorvastatin group.

induced expression of the COX-2 mRNA was decreased significantly by atorvastatin (Figure 2). Consistent with this observation, LPS-induced expression of the COX-2 protein was also inhibited by atorvastatin (Figure 3). Atorvastatin inhibited LPS-induced expression of COX-2 mRNA and protein in a dose-dependent manner.

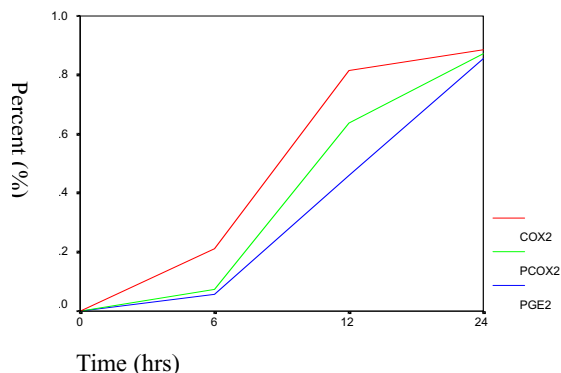
3.3 Effect of atorvastatin on LPS-induced PGE₂ production

Because atorvastatin decreased the expression of COX-2 mRNA and protein, we determined whether it also blocks PGE₂ production. A549 cells were incubated with various concentrations of atorvastatin in the presence of 10 µg/ml LPS for 9 hrs. After incubation, the medium was collected, and the amount of PGE₂ in the medium was detected by

Table 1: The effects of atorvastatin on amount of PGE₂ production

Atorvastatin PGE ₂ (pg/ml)	LPS (10 µg/ml)			
	0	10 µM	15 µM	20 µM
	58.3 ± 2.8	46.1 ± 3.0*	31.2 ± 0.7*	31.7 ± 3.6*

* P < 0.05 vs non-atorvastatin.

**Figure 4**

Time-dependent effects of atorvastatin on LPS-induced expression of COX-2 mRNA (red line), COX-2 protein (green line) and PGE₂ production (blue line). The A549 cells were incubated with 10 µM atorvastatin in the presence of LPS (10 µg/ml) for different times. After incubation, COX-2 mRNA and protein were analyzed by RT-PCR and western-blot respectively, and the amount of PGE₂ in the medium was determined by ELISA. Percent inhibitions by atorvastatin are shown.

ELISA. As shown in Table 1, atorvastatin decreased LPS-induced PGE₂ production in a dose-dependent manner.

3.4 Correlations

According to Spearman's non-parametric rank correlation method, data analysis revealed that atorvastatin-mediated reduction of LPS-induced expression of COX-2 is correlated with a decrease in PGE₂ production ($r = 0.947$, $P < 0.05$).

3.5 Time-dependent effects of atorvastatin on LPS-induced COX-2 expression and PGE₂ production

We further investigated the time-dependent effect of atorvastatin on expression of COX-2 and PGE₂ production. A549 cells were incubated with 10 µM atorvastatin in the presence of 10 µg/ml LPS for various times. Atorvastatin decreased the expression of COX-2 mRNA, protein, and PGE₂ in a time-dependent manner (Figure 4). The result

showed the similar time-dependent patterns in atorvastatin-mediated reduction of COX-2 mRNA, protein and PGE₂, further suggesting a relationship between COX-2 expression and PGE₂ production.

4. Discussion

Inflammatory cytokines as well as prostaglandins (PGs) play important roles in inflammatory process of respiratory system [7]. PGs are synthesized from arachidonic acid by a reaction catalyzed by cyclooxygenase. Two isoforms of this enzyme have been identified [8]. COX-1 is expressed constitutively in almost all tissues [9], and COX-2 is an inducible enzyme that is expressed in response to inflammatory cytokines [10]. Increased expression of COX-2 has been reported in human pulmonary epithelial cells under experimental inflammatory conditions [11]. In the present study, we also found that LPS induces expression of COX-2 mRNA and PGE₂ formation in a dose- and time-dependent manner in A549 cells. These results suggested that expression of the COX-2 could be induced in A549 cells. Because the COX-2 is responsible for the synthesis of pro-inflammatory PGs such as PGE₂ [10,11], an increased expression of COX-2 might play an important role in respiratory inflammatory processes.

HMG-CoA reductase inhibitors, which decrease the synthesis of cholesterol, have been shown to decrease the incidence of acute coronary events [12]. Recent studies suggest that the beneficial effects of statins on clinical events may be not related to its' effect on cholesterol synthesis. Statins affect endothelial cells, smooth muscle cells, monocyte- macrophage, vasomotor function, inflammatory responses, and plaque stability [13,14]. Anti-inflammatory action of statins might be related to the reduction of the production of pro-inflammatory cytokines. Statins inhibit the Ang II-induced secretion of interleukin-6 (IL-6) in cultured human vascular smooth muscle cells, and decrease production of IL-6, interleukin-1β in human umbilical vein endothelial cells [15]. Atorvastatin also down-regulates expression of COX-2 mRNA both *in vivo* and *in vitro* [4]. In this study, we found that atorvastatin significantly reduced LPS-induced expression of COX-2 mRNA in cultured A549 cells. Atorvastatin also significantly reduced LPS-induced PGE₂

production. The correlation analysis indicated that there is a positive correlation between reduced expression of COX-2 and decreased PGE₂. Furthermore, the patterns showing effects of atorvastatin on LPS-induced expression of COX-2 mRNA, protein and PGE₂ production in different times were similar. These suggest that decreased production of PGE₂ by atorvastatin is caused by down-regulation of COX-2 expression. In contrast to our observations, other study [16] showed that mevastatin and lovastatin increase expression of COX-2 and subsequent prostacyclin formation in human aortic smooth muscle cells. It appears that the effects of statins on expression of COX-2 might depend on the cell types or different statins used.

In conclusion, atorvastatin down-regulates LPS-induced expression of COX-2 and production of PGE₂ in cultured A549 cells. These results suggest that HMG-CoA reductase inhibitors might have beneficial effects against respiratory inflammation.

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