Beraprost sodium, a prostacyclin (PGI) analogue, ameliorates lipopolysaccharide-induced cellular injury in lung alveolar epithelial cells

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1. Introduction
Acute lung injury (ALI) is associated with significant morbidity and mortality in human patients (1). ALI is regarded as an increased inflammatory response that might cause morphological and functional damage to the respiratory membrane. Lung alveolar epithelial cells, which are characteristically the major cells with endothelial cells challenged by pathogenic bacteria (2,3), participate in the beginning and the development of ALI. Alveolar epithelial cells may also contribute to inflammatory events in ALI as they are an important source of cytokines (e.g., TNF-α, IL-1β, IL-6) under inflammatory conditions (4,5). Infection, and particularly gram-negative bacterial sepsis, is an important etiological factor for ALI (6), and endotoxin lipopolysaccharide (LPS) is an essential component of gram-negative bacterial cell walls (7). In the human lung, LPS results in the activation of a number of inflammatory pathways and therefore is often used as a valuable model to study potential processes associated with gram-negative sepsis (8,9). The present treatment for ALI comprises supportive and antiinflammatory treatments. Nevertheless, the consequences of these actions are not always adequate.

Cyclic adenosine monophosphate (cAMP) is an intracellular second messenger that mediates the effects of many hormones, β-adrenergic agonists, and prostaglandins (PGs) (8,10,11). Elevated cAMP concentrations in many cell types may suppress inflammation by inhibiting cytokine secretion and oxidative stress (12-14). Prostaglandin I₂ (PGI₂), a major product of arachidonic acid, causes a significant increase in the cAMP production of many cell types (13,15). PGI₂ analogues can attenuate inflammation via suppressing TNF-α, IL-1β, and IL-6 production in lungs, human monocytes, and endothelial cells (11,13,16,17). Beraprost sodium, a chemically stable PGI₂ analogue, has been shown to possess a similar pharmacological profile to PGI₂ (18). Beraprost mimics the biological properties of PGI₂ such as activation of adenylate cyclase and increasing intracellular cAMP concentrations, through activation of the PGI₂ receptor (19). Owing to its chemical characteristics, beraprost is
a more stable molecule with a longer life and a higher affinity to PGI₂ receptors than natural PGI₂ (20).

The A549 human alveolar epithelial cell line, which has many of the features of alveolar type II epithelial cells, is frequently used as a surrogate for human alveolar epithelium because of the limitations of primary cells. LPS is frequently used to initiate inflammation in A549 cells to simulate the pathogen-associated molecular pattern of ALI in vitro. The PGI₂ receptor is expressed in alveolar epithelial cells (21), but the effects of beraprost on inflammation and oxidative stress in these cells have not been documented. We applied LPS to stimulate A549 cells and investigated the effects of beraprost on the cell proliferation, expression of selected proinflammatory cytokines, lipid peroxidation, and antioxidative status.

2. Materials and methods

2.1. Cell culture

Adenocarcinomic human pulmonary alveolar basal epithelial cells (A549) were kindly provided by Dr J Mazella (CNRS, Valbonne, France). The cells were maintained in DMEM (low glucose, Sigma) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 100 IU/mL and 100 mg/mL, respectively, and incubated at 37 °C in a humidified atmosphere of 5% CO₂/95% air. Cells were digested with 0.25% trypsin and subcultured at 70% to 80% confluence.

2.2. Cell viability assay

The epithelial cells were cultured overnight at a density of 10 × 10⁴/well or 5 × 10⁴/well in a sterile 12-well or 24-well plate, respectively (Costar, Corning Inc., USA). The viability of the cells was analyzed based on the activity of mitochondrial dehydrogenase to reduce dimethylthiazol-diphenyltetrazolium bromide (MTT) (Fluka) to formazan. Briefly, A549 cells were seeded in 12-well plates and treated with DMSO or different concentrations of beraprost (1–100 µM) for 3–48 h (data not shown), and then 1 mg/mL MTT was added to each well with incubation for an additional 2 h in culture. The resulting formazan product was dissolved in acid-isopropanol, and the absorbance was measured at a wavelength of 570 nm.

2.3. Concentration and time response for LPS and beraprost

For the dose-response, cells were stimulated with 0.1–5 µg/mL of E. coli LPS serotype 0111:B4 (Sigma) for 24 h. In some experiments, the medium also contained beraprost (10 µM), in which case cells were pretreated for 30 min with this agent. This concentration of beraprost has been shown to be able to suppress LPS-induced cytokine expressions in vitro (11,16,22).

2.4. LPS stimulation of A549 cells

A549 cells were incubated in 12- or 24-well culture plates, and LPS (E. coli 0111:B4, Sigma) was added in the logarithmic phase. The final concentration of LPS was determined by MTT assays in which 0.1, 1.0, and 5.0 µg/mL were evaluated successively (Figure 1A). There were no significant differences in the rate of cell growth at 3 h after LPS treatment when all the concentrations were used (data not shown). However, 1 µg/mL LPS significantly induced cell proliferation rate (P < 0.05) at 24 h incubation. Therefore, 1 µg/mL was the concentration of LPS used in all subsequent experiments. Cells were collected from wells 24 h after adding LPS.

2.5. Determination of intracellular reduced glutathione (GSH) concentration

At the end of the incubations, cells were washed and lysed with ice-cold homogenization buffer (1% Triton X-100 (Merck), 50 mM HEPES (pH 7.2), 10 mM EDTA, 100 mM NaH₂PO₄·2H₂O) with a complete protease inhibitor mixture (aprotinin, phenylmethylsulfonyl fluoride, leupeptin, sodium fluoride) (Sigma, Germany). The detergent-insoluble proteins and tissue fragments were discarded by centrifugation at 12,000 × g for 10 min at 4 °C, and the cytosolic fraction was reserved for further analyses. The protein concentrations of the cell extracts were measured by the Bradford reagent using bovine serum albumin as a standard. GSH concentrations in cellular homogenates were determined according to the method developed by Sedlak and Lindsay (23). GSH is reacted with 5,5-dithiobis-2-nitrobenzoic acid, yielding the formation of a product that has a maximal absorbance at 410 nm. The data are given as µmol/mg protein.

2.6. Catalase activity analysis

Catalase activity was assessed using the method of Luck (24). Three milliliters of H₂O₂ phosphate buffer (12.5 mM H₂O₂ in 67.0 mM phosphate buffer (pH 7.0)) was pipetted directly into the cuvette. An appropriate amount of homogenate was added to the buffered solution. The contents were mixed and the decrease in absorbance was measured at 240 nm for 3 min. The activity was shown as k/mg protein in homogenates, where k is the degree constant of a first-order reaction.

2.7. Malondialdehyde analysis

Lipid peroxidation status was evaluated by measuring the concentration of malondialdehyde (MDA) in the cellular homogenates of the samples (25). The homogenate was precipitated with trichloroacetic acid and reacted with thiobarbituric acid in a boiling water bath for 30 min. Tubes were then immediately cooled in ice-cold water, and 4 mL of n-butanol was added. After centrifugation at 3000 rpm for 10 min, the absorbance intensity of the upper phase was measured by a spectrophotometer (Shimadzu UV-1208, Japan) at 535 nm. Values were compared with a series of standard solutions (1,1,3,3-tetramethoxypropane). Results were expressed as µmol per mg protein.
2.8. qRT-PCR analysis
Real-time PCR was implemented in a qPCR system (Stratagene Mx3005P, USA). Total RNA from A549 cells was extracted using TRIzol reagent (Sigma, USA). RNA was reverse-transcribed with a commercial kit (Fermentas, Lithuania). The primer sets obtained from Thermo Electron Corporation (Germany) were TNF-α (sense 5’-CAGAGGGAAGAGTTCCCCAG-3’, anti-sense 5’-CCTTGTGCTGTTAGGAGACG-3’) and IL-1β (sense 5’-GCAAGGGCCTTACGAGGCAGCGGC-3’, anti-sense 5’-GGTCATTCTCCTGGAAGGTCTGTGGGC-3’). The amount of RNA was normalized to β-actin amplification in a separate reaction (sense 5’-CATCTCGACAAGCTTCTCTGCTGGAAGGCTG-3’, anti-sense 5’-CTCGACATCTCTCTGTGGCCGC-3’). Quantifying the relative changes in mRNA expressions of target mRNA was considered using the 2ΔΔCt procedure, where ΔΔCt = (Ct gene studied − Ct β-actin)treated − (Ct gene studied  − Ct β-actin)control. The rise in fluorescent signal related to the exponential increase of PCR yields was detected by the laser detector.

2.9. Statistical analysis
One-way analysis of variance (ANOVA) and post hoc Duncan tests were carried out on the data to examine the differences among groups using SPSS for Windows v. 9.0. The results are presented as average ± standard error (SE). P < 0.05 was considered significant.

3. Results

3.1. Effect of beraprost sodium on cell growth
The effect of beraprost sodium on cell proliferation stimulated by LPS was investigated in A549 cells. The cells were incubated with various concentrations of LPS (0.1, 1, and 5 µg/mL) for 24-h periods (Figure 1A). LPS stimulated the proliferation of the cells in a dose-dependent manner, as measured by MTT assay. The highest increase in metabolic activity was induced by 1 µg/mL LPS stimulation. In response to 1 µg/mL LPS from E. coli 0111:B4, MTT activity was increased to 156% after 24 h of stimulation (P < 0.001) (Figure 1A). On the other hand, the cells pretreated with beraprost (10 µM) significantly inhibited (P < 0.001) cellular proliferation stimulated by LPS to the level of control (Figure 1B).

3.2. Beraprost sodium protects alveolar epithelial cells against LPS-induced oxidative stress
In A549 cells, we measured the lipid peroxidation levels (MDA) as a marker of oxidative stress (Table). The levels of this marker were compared between the groups that underwent 24 h of beraprost sodium treatment for LPS exposure and the control group (nonexposed group). A significantly increased MDA level was observed in the LPS-exposed group (P < 0.001) in comparison to the control group, suggesting LPS-induced oxidative damage. However, beraprost significantly (P < 0.001) reduced lipid peroxidation induced by LPS.

Reduced glutathione (GSH), a major nonprotein thiol involved in many aspects of cellular metabolism and regulation, plays an important role in cellular antioxidant defense systems by scavenging free radicals and other reactive oxygen species (ROS), removing hydrogen and lipid peroxides, and precluding oxidation of biomolecules. LPS-induced oxidative stress caused significant depletion of GSH levels in the cells (P < 0.001) (Table). However, beraprost-treated cells demonstrated higher GSH levels than the untreated control cells (P < 0.05). Similar to this result, beraprost elevated LPS-decreased GSH concentration to the control level (P < 0.001).

We measured the antioxidant catalase enzyme activity that may reflect intracellular defenses. Catalase is a crucial enzyme in cellular antioxidant defense systems, as it has critical roles in the elimination of H₂O₂ in living organisms. Catalase converts H₂O₂ to water and molecular oxygen.
Inadequate elimination of ROS results in oxidative stress that may cause severe metabolic malfunctions and can damage biological macromolecules. In the present study, LPS significantly (P < 0.001) reduced catalase activity in the cells (Table). Similar to the protection of the cells from lipid peroxidation, beraprost elevated LPS-inhibited catalase activity (P < 0.001).

3.3. Effect of beraprost sodium on proinflammatory cytokine production by A549 cells stimulated with LPS

A549 cells in culture are known to express genes of proinflammatory cytokines such as TNF-α and IL-1β (26), which trigger inflammatory responses. To determine the effect of beraprost on LPS-induced proinflammatory cytokine gene expressions, qRT-PCR was conducted in A549 cells at 24 h. We found that LPS (1 µg/mL) increased production of TNF-α and IL-1β expressions by 8- and 2.5-fold, respectively (P < 0.001). In contrast, 10 µM beraprost pretreatment 30 min before LPS significantly reversed these cytokine mRNA expressions (Figures 2A and 2B) (P < 0.001).

4. Discussion

We used the A549 cell line as representative alveolar epithelial cells that have been implicated in the pathogenesis of sepsis-induced ALI (27,28). Epithelial cells produce various immune effectors such as cytokines and antimicrobial peptides in response to inflammatory stimuli (28). Although most PGs have proinflammatory actions, PG1 (11) and PGE, (29) are reported as having antiinflammatory effects that are both potent and context-dependent. PGI2 analogues can regulate cytokine expression of T cells, monocytes, and macrophages via IP receptor (11). PGI2 analogues play a role in airway remodeling induced by repeated allergen challenge in mice (30). Though the PGI1 receptor is expressed in alveolar epithelial cells (21), the role of PGI1 analogues against endotoxin-induced inflammation and oxidative events remains to be revealed in these cells.

Table. Effects of beraprost sodium (BPS) on oxidant-antioxidant parameters of LPS-induced human pulmonary alveolar epithelial cells (A549).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>BPS</th>
<th>+ BPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA levels (µmol/mg protein)</td>
<td>0.08 ± 0.003</td>
<td>0.29 ± 0.007*</td>
<td>0.07 ± 0.003</td>
<td>0.14 ± 0.004^*</td>
</tr>
<tr>
<td>GSH levels (µmol/mg protein)</td>
<td>1.27 ± 0.02</td>
<td>0.64 ± 0.01*</td>
<td>1.65 ± 0.03*</td>
<td>1.30 ± 0.02*</td>
</tr>
<tr>
<td>Catalase activity (k/mg protein)</td>
<td>0.47 ± 0.09</td>
<td>0.15 ± 0.03*</td>
<td>1.44 ± 0.05##</td>
<td>0.62 ± 0.05¥</td>
</tr>
</tbody>
</table>

Malondialdehyde (MDA) and reduced glutathione (GSH) levels and catalase activity were measured in the cellular homogenates in the absence or presence of 1 µg/mL LPS and/or 10 µM beraprost sodium for 24 h. *: P < 0.001 vs. control, #: P < 0.05 vs. control, *#: P < 0.001 vs. control, ¥: P < 0.001 vs. LPS. Values are expressed as the means ± SE from at least 3 different experiments.

Figure 2. Proinflammatory cytokine expression of A549 epithelial cells in the presence of lipopolysaccharide (LPS) and/or beraprost sodium (BPS, +BPS). A549 cells were cultured in standard medium without LPS (control) and in the presence of 1 µg/mL LPS from E. coli 0111:B4 and 10 µM BPS. Relative gene expression of proinflammatory cytokines IL-1β and TNF-α in A549 cells in standard medium (control) and in standard medium in the presence of LPS and BPS. The total mRNA was isolated from the cells after 24 h, and real-time qRT-PCR was performed using specific primers. Gene expression was calibrated to β-actin as a housekeeping gene (n = 3). *: P < 0.001 vs. control, **: P < 0.001 vs. LPS.
Our findings suggest that LPS from *E. coli* 0111:B4 can promote A549 cell proliferation and induce oxidative stress and inflammation, which is consistent with previous studies (6,31–33). Our data suggest that endotoxin is an applicable and powerful promoter of tumor cell proliferation. The escalation in metabolic activity was previously noted after 24 h of LPS treatment and A549 cells are well known to correspond to cellular proliferation (32). The obtained results indicate that beraprost treatment significantly reverse LPS-induced cell proliferation. Though the intracellular cAMP level was not measured in this study, it has already been shown that cAMP concentration is induced by PGI₂ analogues, including beraprost, as well as different cAMP-elevating agents (18,34–36). These agents have antiproliferative and antioxidant effects, as demonstrated by in vivo and in vitro studies. cAMP activates protein kinase A to initiate multiple phosphorylation reactions and thus regulates various functions within cells. Beraprost is able to elevate intracellular cAMP concentration and promotes peroxisome proliferator-activated receptor δ (PPARδ) activity (37,38). PPARδ modulates target gene expressions in response to ligand activation after heterodimerization with the receptor and the binding to peroxisome proliferator-responsive elements of target genes (38). Therefore, PPARδ-mediated modulation of gene transcription by PGI₂ may form the basis for its novel role as a regulator of gene expression. Thus, our data suggest that beraprost could be beneficial to control cell proliferation during endotoxin exposure in lungs.

Recent studies have reported that LPS enables the production of ROS, leading to lipid peroxidation in lungs and resulting in lung damage (39–41). Therefore we measured the antioxidant GSH levels and catalase enzyme activities, as well as lipid peroxidation levels that may reflect intracellular defenses. GSH, a major nonprotein thiol involved in many aspects of cellular metabolism and regulation, has an important role in the cellular antioxidant protection system by scavenging free radicals and preventing oxidation of biomolecules (40). LPS-induced oxidative stress can lead to significant depletion of GSH level in the cells (42). Our study indicated that exposing A549 cells to LPS depletes GSH levels and catalase activity, and results in lipid peroxidation. Pretreating the cells with 10 µM beraprost for 30 min before LPS exposure resulted in a significant decrease in lipid peroxidation and elevated GSH levels and catalase activity. This finding corroborates the ideas of Brown et al. and Mishima et al. (12,19), who suggested that beraprost or cAMP-elevating agents protect renal tubular cells against cisplatin-induced oxidative injury by obliterating ROS and the subsequent inhibition of TNF-α synthesis through a blockade of p38 MAPK activation. Based on the present study and previous data, it can be suggested that supporting the antioxidant system with beraprost sodium helps to ameliorate LPS-caused oxidative stress.

LPS-induced ALI is characterized by alveolar epithelial permeability, inflammatory mediator release, and a neutrophilic inflammatory response (13,40). TNF-α and IL-1β are suggested as important early mediators of ALI under pathophysiological conditions (43). In relation with its antiinflammatory activity, beraprost has been demonstrated to inhibit TNF-α and IL-1β release by downregulating NF-kB or p38 MAPK activation signaling via intracellular cAMP elevation (11,13,17,19,44,45). In this experiment, we found that pretreatment with beraprost (10 µM) effectively decreased the concentrations of TNF-α and IL-1β compared with the LPS group. These results indicated that the protective effect of beraprost on LPS-induced ALI could also be attributed to the inhibition of proinflammatory cytokine release.

Beraprost sodium is an orally active prostacyclin analogue used clinically for the treatment of peripheral arterial disease and pulmonary arterial hypertension. However, its inhibitory effect on the formation of inflammation and oxidative stress has not been revealed in pulmonary alveolar epithelial cells. The results obtained from this study show that beraprost sodium could provide a pivotal molecular basis for the design of new therapeutic strategies to cure endotoxin-induced lung injury, although further comprehensive studies are still required.

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**References**


