Atorvastatin Exerts Protective Effect on Cardiopulmonary Bypass Induced Renal Injury in Rats via PPAR-γ

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ABSTRACT

Background: To investigate the protective effect and possible mechanism of atorvastatin pretreatment on renal function after cardiopulmonary bypass (CPB) in rats.

Methods: Twenty-four adult male Sprague-Dawley (SD) rats randomly were divided into three groups: Sham operation group, CPB group, and administration group (N = 8 in each group). The caudal artery and right jugular vein were used to establish the CPB circuit for the CPB and administration groups. Drugs were administered by oral gavage one week before the operation. All rats were executed for succeeding experiments 72 h after the operation. Plasma levels of creatinine (Cre) and IL-8 at different time points and levels of TNF-α and MPO in renal tissue were detected by ELISA. Renal pathological changes were observed by HE staining. PPAR-γ expression was determined by immunohistochemistry and western blot.

Results: All rats survived the whole process without incident. Renal function of rats undergoing CPB was impaired to varying degrees based on the plasma Cre concentration, and atorvastatin pretreatment alleviated this effect. The concentrations of six inflammatory cytokines (IL-1β, IL-6, IL-8, IFN-γ, TNF-α, and MPO) were significantly elevated after CPB procedure, while atorvastatin pretreatment ameliorated the inflammatory condition caused by CPB. Further analysis showed that in both HK-2 cells and renal tissues, atorvastatin promoted the expression of PPAR-γ.

Conclusion: Atorvastatin pretreatment exerted protective effect on CPB-associated kidney injury and inflammation in rats. The activation of PPAR-γ may contribute to the protective effect of Atorvastatin.

INTRODUCTION

The concept of extracorporeal circulation dates back to about 50 years ago. Cardiopulmonary bypass (CPB) refers to the technique that maintains blood circulation and oxygenation of the body with a machine during surgery [Passaroni 2015]. CPB is usually applied in heart- or lung-related operations, when a surgeon must operate on still organs; the blood of a patient is diverted from the heart and lung to an extracorporeal oxygenation machine. The abrupt change of blood circuit in CPB frequently brings about significant changes in all organs of the body [Passaroni 2015]. These complications could arise immediately after surgery or later in the intensive care unit (ICU); acute kidney injury (AKI) is the most common and serious symptom [Hendrix 2019; Pickering 2015]. AKI affects approximately 5% of postoperative patients with CPB procedures, posing a high risk of prolonged hospitalization, rehospitalization, and postoperative morbidity and mortality [Issitt 2017]. Although the underlying mechanism is still under investigation, renal hypoperfusion, ischemia-reperfusion injury, and systemic inflammatory response could collectively contribute to the AKI after CPB surgery [Issitt 2017]. Besides, an increased uptake of lipid microemboli (LME) into renal vasculature partly accounts for CPB-associated AKI [Issitt 2017]. Strategies to ameliorate CPB-associated AKI have been extensively explored, such as nitric oxide administration [Hu 2019].

Statins function as a selective and competitive inhibitor of HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase and show powerful cholesterol-lowering abilities [Istvan 2001]. Statins mainly are prescribed for patients with hypercholesterolemia or as primary and secondary prevention of cardiovascular disease [Chou 2016]. Recent studies revealed a positive effect of statin prophylaxis on cardiac, renal, and pulmonary function in CPB [Chee 2017; Hua 2017; Kuhn 2013; Shao 2007]. For example, Chee et al. demonstrated that the use of high dose preoperative statins is an effective strategy to reduce cardiac and renal damage in cardiac surgery with CPB [Chee 2017]. The study by Hua et al. revealed that in patients who underwent cardiac surgery with CPB, perioperative Simvastatin administration lowers the levels of serum inflammatory cytokines and reduces the duration of assisted ventilation, which may be attributed to reduced myocardial injury through regulating myocardial autophagy [Hua 2017]. In a rat CPB model, Simvastatin improves the pulmonary function by suppressing inflammatory response [Shao 2007]. Although a
number of studies highlighted the anti-inflammatory function of statin as a key contributor of its protective effect on CBP-associated damages [Martinez-Comendador 2009; Ozguler 2015; Shao 2007], the detailed molecular mechanisms underlying the protective effect remain to be elucidated.

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors of ligand-activated transcription factors, which is composed of three isoforms, α, β/δ and γ [Laudet 1992]. PPARs can be activated by a number of endogenous factors (such as fatty acids and prostanoids) and exogenous ligands (such as plant extracts) [Kliewer 1997; O’Sullivan 2016; Wang 2014]. PPARγ exists in two isoforms (PPARγ1 and PPARγ2), which are expressed mainly in adipose tissue and intestines, liver and kidney [Vidal-Puig 1997; Grygiel-Gorniak 2014]. PPARγ is implicated in a number of biological functions, including the regulation of adipocyte differentiation, fatty acid storage, glucose metabolism, and the inhibition of inflammation [Kumari 2015]. It was reported that PPARγ exerts anti-inflammatory effects by suppressing inflammatory response genes [Ricote 1998; Welch 2003]. PPARγ activation shows powerful protection against ischemia reperfusion injury in multiple organs [Gui 2019; Zhuo 2019]. For example, the classic PPARγ agonist thiazolidinediones (TZDs) was reported to protect kidney from chronic kidney disease (CKD) by various mechanisms [Fogo 2011]. In this study, we studied the protective effect of atorvastatin on AKI in a rat CPB model and investigated whether PPARγ activation contributes to the protective effect of atorvastatin.

**MATERIALS AND METHODS**

**Cell culture and reagents:** HK-2 human proximal tubular epithelial cells were purchased from American Type Culture Collection (CRL-2190; ATCC, USA) and cultured in DF12 medium (DMEM/F12 1:1 mixture) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 1% penicillin/streptomycin (Life Technologies). Cells were grown at 37°C in the presence of 5% CO2. The medium was replaced every 48h. Cells were treated with Atorvastatin (Solarbio, Beijing, China) and GW9662 (Sigma, USA) at indicated concentrations.

**Animal experiment:** Twenty-four male Sprague-Dawley rats (6 month; 352.3±26.8g) were provided by the experimental animal center of Anhui Medical University. All animals were routinely fed and housed at a constant temperature (25±1°C) and constant humidity (55±5%), in a 12h light-dark cycle. After one week of acclimation, animals randomly were distributed into three groups: Sham operation group, cardiopulmonary bypass group (CPB group), and atorvastatin pretreatment group (administration group). One week prior to operation, the administration group was given atorvastatin (20mg/kg, Pfizer) daily by gavage.

Intraperitoneal anesthesia was performed with 3% Pento-barbital Sodium. Arterial blood samples from right femoral artery were collected for arterial blood gas analysis with an i-STAT® (Abbott point-of-care, Texas, USA) analyzer. A 16-G catheter was inserted into the right jugular vein as the venous drainage and advanced to the tail artery. The CPB circuit was maintained stably for 90min at a flow of 100ml/kg/min.

Approximately 0.5 mL venous blood samples were collected from the tails at three different time points: shortly before CPB (pre-CPB), after CPB (post-CPB), and 72h post-CPB. The blood samples were used for plasma Cre and IL-8 analysis. All experimental rats were executed 72h after CPB, and the bilateral renal tissue samples were surgically removed for further study.

**Enzyme linked immunosorbent assay (ELISA):** Plasma samples were prepared from 200μl of whole blood of all animals at each time point and then stored at -80°C until assayed. Plasma concentrations of Cre (Shanghai Tongwei, China) and inflammatory cytokines, including IL-1β, IL-6, IL-8, IFN-γ, tumor necrosis factor (TNF)-α (Wuhan Xinqidi Biological Technology Co.Ltd, China) were determined by commercial ELISA kits according to manufacturers’ instructions.

The levels of tumor necrosis factor (TNF)-α (Wuhan Xinqidi Biological Technology Co. Ltd, China) and myeloperoxidase (MPO) (Jianglai Biology, China) in renal tissue were determined by commercial ELISA kits. Tissue homogenates were prepared according to manufacturers’ instructions.

For the measurement of IL-8 released by cells, HK2 cells were seeded at the density of 3 × 105/well in six-well plates. After 24-hour incubation, cells were treated with atorvastatin (10 μM) and GW9662 (5μM) for an additional 2h. Subsequently, the culture media were collected, and the cell debris was removed by centrifugation at 300g for 4 min. IL-8 concentration in 100 μl cell culture supernatants was determined by IL-8 ELISA kit (D8000C, R&D Systems).

**Western blot analysis:** Total proteins were extracted from the cultured HK-2 cells and tissues using RIPA lysis buffer containing protease inhibitor (Applygen, Beijing, China). SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed and proteins were transferred to PVDF membranes (Millipore, USA). After blocking with Tris-buffered saline containing 0.05% Tween-20 (TBST) containing 5% non-fat milk for 1h at room temperature, the membranes were incubated with anti-PPARγ (1:1000, Abcam, ab41928), IL-8 (1:500, Santa Cruz, sc-376750), and anti-β-actin (1:1000, Santa Cruz, sc-47778) antibodies at 4°C overnight, followed by incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 1h at 25°C. β-actin was used as an internal control. Proteins were detected with ECL chemiluminescence, and their intensity was analyzed with Image J software (NIH).

**Immunohistochemistry and H&E (hematoxylin and eosin) staining:** Immunohistochemical analysis was performed using standard protocols. Briefly, after deparaffinization and hydration, paraffin-embedded sections submersed in citrate unmasking solution were heated in a microwave for 15 min at a sub-boiling temperature (95-98°C). Afterward, the slides were cooled for 30 min, followed by incubation in 3% hydrogen peroxide (H2O2) for 10 min. Prior to antibody recognition, sections were blocked with 10% normal goat serum for 1h at room temperature. PPARγ antibody (1:500, Cell Signaling Technology, #24355) or normal rabbit IgG (Santa Cruz) was added to the sections overnight at 4°C. The
sections then were incubated with the corresponding secondary antibody (Solarbio, China). Sections were colored with DAB and counterstained with hematoxylin.

The pathomorphological changes of renal tissues were assessed by H&E staining. After deparaffinization and rehydration, cell nuclei were stained with hematoxylin solution for 5 min followed by differentiation in hydrochloric acid alcoholic (1% HCl in 70% ethanol) and then rinsed in distilled water. Then, the sections were stained with eosin solution for 3 min and followed by dehydration with graded alcohol and vitrification in xylene.

**Statistical analysis:** All experimental data were processed and analyzed using SPSS 21.0 (IBM, Armonk, NY, USA). Data were presented as mean ± SD. The statistical difference between two groups was compared using unpaired student’s t tests. Comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA) with Tukey’s post hoc test for pairwise comparison. Differences were considered statistically significant when \( P < 0.05 \).

**RESULTS**

**Assessment of CPB animal model:** All animals survived the whole course of experiment until being sacrificed. The results of blood gas analysis at different time points in each group are summarized in Table 1. (Table 1) These data all fell within an acceptable range, and no statistical difference was observed among the three groups \(( P > 0.05)\). In this rat model, CPB resulted in renal function impairment manifested as the elevation of plasma Cre level 72 h after operation. (Figure 1)

**Atorvastatin attenuates kidney injury by CPB:** As shown in the Elisa results from the plasma (Figure 1), Cre concentrations among all three groups did not immediately show significant changes before (Pre-CPB) or after CPB (Post-CPB). However, 72 hours after the CPB operation, plasma Cre concentrations in CPB and administration groups were significantly increased as compared with the Sham group (Figure 1), which implies the potential damage of CPB procedure to kidney function. However, the administration of Atorvastatin (administration group) attenuated the increase of Cre when compared to CPB group, suggesting a protective effect of atorvastatin on kidney function.

**Atorvastatin treatment alleviated inflammatory reaction caused by CPB:** To explore whether inflammatory responses were involved in CPB-associated AKI, we monitored plasma concentrations of inflammatory cytokines, including IL-1β, IL-6, IL-8, IFN-γ, and TNF-α. As revealed in Figure 2, all of these cytokines displayed significant increase 72 hours post-CPB as compared with the Sham group. The pretreatment of Atorvastatin (administration group) significantly impaired the elevation of these cytokines 72 hours post-CPB when compared with the CPB group. (Figure 2) Together, these data indicate that Atorvastatin pretreatment could alleviate the post-CPB inflammation.

To further validate the anti-inflammatory effect of Atorvastatin pretreatment in kidneys, we further remeasured the level

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**Table 1. Physiological data over the study period of each group**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Pre-catheterization</th>
<th>Post-catheterization</th>
<th>Pre-CPB</th>
<th>Post-CPB</th>
<th>Pre-CPB</th>
<th>Post-CPB</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH</td>
<td>8</td>
<td>7.30±0.03</td>
<td>7.42±0.04</td>
<td>7.29±0.05</td>
<td>7.38±0.04</td>
<td>7.50±0.02</td>
<td>7.49±0.01</td>
</tr>
<tr>
<td>pO2 (mmHg)</td>
<td>8</td>
<td>150±12.5</td>
<td>162±10.3</td>
<td>158±23.0</td>
<td>102±13.0</td>
<td>203±11.9</td>
<td>159±15.2</td>
</tr>
<tr>
<td>pCO2 (kPa)</td>
<td>8</td>
<td>39.82±6.02</td>
<td>38.94±5.03</td>
<td>40.17±4.81</td>
<td>42.15±5.04</td>
<td>39.88±3.94</td>
<td>38.77±5.76</td>
</tr>
<tr>
<td>SpO2 (%)</td>
<td>8</td>
<td>96.0±1.21</td>
<td>99.85±0.11</td>
<td>96.05±1.75</td>
<td>99.89±0.11</td>
<td>96.56±0.18</td>
<td>98.13±0.12</td>
</tr>
<tr>
<td>BE</td>
<td>8</td>
<td>-1.52±1.8</td>
<td>1.53±0.42</td>
<td>-2.54±0.28</td>
<td>-2.07±1.34</td>
<td>-1.96±1.58</td>
<td>1.98±1.58</td>
</tr>
<tr>
<td>Hb(g/L)</td>
<td>8</td>
<td>123.03±11.12</td>
<td>89.50±0.38</td>
<td>128.45±10.3</td>
<td>89.56±12.03</td>
<td>125.47±14.30</td>
<td>91.25±10.75</td>
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<tr>
<td>Hct (%)</td>
<td>8</td>
<td>35.45±4.15</td>
<td>24.01±4.13</td>
<td>35.48±4.85</td>
<td>27.01±4.89</td>
<td>34.46±2.01</td>
<td>25.13±3.20</td>
</tr>
<tr>
<td>K⁺ (mmol/L)</td>
<td>8</td>
<td>4.38±0.45</td>
<td>4.10±0.15</td>
<td>5.01±0.33</td>
<td>4.69±0.81</td>
<td>4.10±0.89</td>
<td>5.01±0.12</td>
</tr>
<tr>
<td>Ca²⁺ (mmol/L)</td>
<td>8</td>
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<td>1.00±0.04</td>
<td>1.02±0.15</td>
<td>0.98±0.19</td>
<td>1.00±0.11</td>
<td>1.02±0.12</td>
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</table>
of TNF-α and MPO in renal tissue after the rats were sacrificed. When compared with the Sham group, TNF-α and MPO concentrations in the CPB and administration groups were significantly elevated. Atorvastatin pretreatment in the administration group significantly impaired the elevation of TNF-α and MPO. (Figure 3) These data strongly support a protective role of atorvastatin in renal function by inhibiting inflammation.

**Morphological evidence for the protective action of atorvastatin:** Next, we performed the hematoxylin/eosin (HE) staining of the microstructure of the rat kidney. Figure 4A represented a typical microscopic structure of kidney with clear glomerular structure, intact proximal convoluted tubules (PCT), and no expansion and hyperemia in capillary. (Figure 4) While after CPB, glomeruli atrophy was observed, and the structure of PCT was blurring with degeneration and desquamation of some epithelial cells (Figure 4B). In the

![Graph showing the difference in TNF-α and MPO concentration in renal tissues after rat sacrifice.](image)

**Figure 3.** Difference in TNF-α and MPO concentration in renal tissues after rat sacrifice. Statistics: One-Way ANOVA, **P < 0.001, *P < 0.01, *P < 0.05.**

![Graphs showing ELISA measurement of inflammatory cytokines (IL-1β, IL-6, IL-8, IFN-γ, and TNF-α) in plasma among three groups at different time points.](images)

**Figure 2.** ELISA measurement of inflammatory cytokines (IL-1β, IL-6, IL-8, IFN-γ, and TNF-α) in plasma among three groups at different time points. Statistics: One-Way ANOVA, **P < 0.001, *P < 0.01, *P < 0.05.**
Figure 4. The microscopic structure of kidney by H&E staining, containing tubules, interstitium and glomerulus. (A) Sham Group, (B) CPB group, (C) Administration group

Figure 5. The expression of PPAR-γ protein by western blot. (A) Atorvastatin treatment increased PPAR-γ level in HK-2 cells in a dose-dependent manner. (B) CPB slightly increased the level of PPAR-γ, and atorvastatin pretreatment largely promoted the level of PPAR-γ in renal tissue.

Figure 6. The expression of PPAR-γ protein in different renal tissues by IHC staining (×400). (A) Sham group, (B) CPB group, (C) Administration group

Figure 7. (A) ELISA measurement of IL-8 secreted from HK-2 cells in the presence of Atorvastatin (Ator, 10μM) and GW9662 5μM for 2h. (B) The protein level of PPAR-γ was determined by western blot in the presence of Atorvastatin (Ator, 10 μM) and GW9662 5 μM in HK-2 cells.
administration group, however, the glomeruli were uniform and normal in size, although the epithelial cells of the PCT showed slight degeneration (Figure 4C). In summary, an overall improvement was observed in the kidney microstructure of the atorvastatin administration group.

PPAR-γ was responsible for this protective action of atorvastatin: PPAR-γ has long been reported to protect against ischemia-reperfusion injury and possess strong anti-inflammatory abilities [Gui 2019; Zhuo 2019]. To investigate whether PPAR-γ is involved in inflammation inhibition by atorvastatin, we treated HK-2 cells with different concentrations of atorvastatin (0, 10, 20, 40 and 80μM, respectively) for 2h, and performed western blot to detect PPAR-γ. The results showed that the amount of PPAR-γ protein increased in an atorvastatin dose-dependent manner (Figure 5A). (Figure 5) The expression of PPAR-γ protein in renal tissue also was detected by western blot (Figure 5B). CPB alone slightly promoted the expression of PPAR-γ, while atorvastatin pretreatment remarkably increased the amount of PPAR-γ. Furthermore, IHC staining revealed a more ubiquitous and denser distribution of PPAR-γ in administration group as compared with the Sham and CPB groups. (Figure 6)

Next, we sought to examine whether PPAR-γ activity contributes to the anti-inflammatory effect of atorvastatin. We treated HK-2 cells with atorvastatin (10 μM) in the presence or absence of a canonical PPAR-γ inhibitor GW9662 (5 μM). As shown in Figure 7, atorvastatin treatment significantly inhibited the secretion of IL-8, which was abrogated by PPAR-γ inhibitor GW9662, although the protein level of PPAR-γ was not affected by GW9662. (Figure 7) All these data above indicate that atorvastatin induced anti-inflammation depends on the increased activity of PPAR-γ.

**DISCUSSION**

Cardiopulmonary bypass (CPB) has significantly assisted the performance of many otherwise infeasible surgical plans. However, a number of complications have ensued after the surgery with CPB, such as arrhythmias, respiratory failure, renal failure, and neurological injury [Passaroni 2015]. CPB per se is an independent risk factor for acute kidney injury (AKI) [Sethi 2011]. The present study investigated whether and how atorvastatin, a widely-used cholesterol-reducing medication, might protect against CPB-associated renal injury.

We first established a rat model of CPB in which the caudal artery and right jugular vein were used to establish the CPB circuit. This was an effective method under our experiment condition. We initially planned two time points for the circulation duration (60 and 90 min). The incidence of renal injury as determined by plasma Cre at 60 min was lower than that of 90 min. Also, it was reported that longer CPB duration is associated with a higher risk of CPB associated AKI [Kumar 2012]. We therefore used a circulation duration of 90 min in our CPB experiment. Seventy-two hours after the CPB, renal injury induced by CPB was observed in the CPB and administration groups as manifested by the increased levels of plasma Cre concentration. Atorvastatin pretreatment one week prior to CPB significantly reduced the plasma Cre level. Therefore, atorvastatin pretreatment was a protective strategy in CPB-associated renal injury.

It is widely accepted that most postoperative complications are related to the systemic inflammatory response syndrome (SIRS) [Goldstein 2019]. Inflammatory cytokines like members of the tumor necrosis factor super family, interleukin family and interferon family, are well-established markers of inflammation and increased significantly after CPB [Goldstein 2019]. Among those, TNF-α, IL-6 and IL-8, were the most widely used indicators of inflammatory responses [Qin 2015]. Preoperative therapy with statin was reported to repress post-CPB inflammation to a certain degree [Morgan 2009]. In our study, plasma IL-1β, IL-6, IL-8, IFN-γ, and TNF-α levels were monitored to evaluate inflammatory responses. Seventy-two hours after CPB, these inflammatory cytokines were significantly increased, implying a systematic inflammation activation. Pretreatment with atorvastatin impaired the elevation of these cytokines in rats of the administration group. Similarly, TNF-α in renal tissue showed a blunted elevation in the administration group.

Surgical procedures and equipment used in CPB bring about CPB-specific inflammatory responses [Al-Fares 2019]. For example, hemodilution employed in CPB could lead to increased neutrophil activation and SIRS [Gourlay 2003]. Myeloperoxidase (MPO) is most abundantly expressed in neutrophil granules and serves as a marker of neutrophil activation [Odobasic 2016]. Our study also found that the expression of MPO in renal tissue also was significantly increased by CPB, which was abrogated by atorvastatin pretreatment. These data indicate the protective effect of atorvastatin on CPB-associated renal injury.

To elucidate the underlying mechanism, we proceeded with the analysis of expression level of PPAR-γ. PPARs are a class of nuclear transcription factors comprising α, β/δ and γ [Laudet 1992]. The biological activities of PPARs can be activated by a number of ligands, including both natural and synthetic substances [Grygiel-Gorniak 2014]. It was reported that quite a number of novel PPARs agonists exerts protective effect in ischemia-reperfusion injury of multiple organs [Chen 2017; Elshazly 2019; Li 2019]. PPAR-γ activation also induces anti-inflammatory effect through various pathways, such as the inhibition of NF-κB dependent inflammation [Ricote 1998; Scirpo 2015; Welch 2003]. We treated HK-2 cells with atorvastatin concentration gradient for 2h. The time and concentration were selected according to a previous study [Liu 2017]. The level of PPAR-γ protein increased with the increasing concentration of atorvastatin. Consistently, the amount of PPAR-γ protein in renal tissue also was significantly increased in the atorvastatin administered group. Of note, CPB group without atorvastatin pretreatment also showed a slight elevation of PPAR-γ, which we believe is an innate response to CPB. The employment of GW9662, a potent PPAR-γ antagonist, established the involvement of PPAR-γ activity in the anti-inflammatory effect of atorvastatin. PPAR-γ can be activated by both ligand and phosphorylation [Diradourian 2005]. Future experiments are required to investigate whether phosphorylation-dependent activation is involved in the activity of atorvastatin.
Atorvastatin pretreatment exerts a protective effect against CPB-induced renal injury in the animal model. This protective effect is likely mediated by the upregulation of PPAR-γ and the impaired inflammatory responses.

REFERENCES


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