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Long-term oral atazanavir attenuates myocardial infarction-induced cardiac fibrosis

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ABSTRACT

Atazanavir is an antiretroviral medication used to treat and prevent HIV/AIDS, but its effects on cardiac fibrosis are unknown. The aim of this study was to determine the effects of atazanavir on myocardial infarction (MI)-induced cardiac fibrosis in rats and used a TLR 9 antagonist, hydroxychloroquine (HCQ) to elucidate the potential mechanism *in vitro*. The results indicated that atazanavir significantly attenuated $CoCl_2$-induced neonatal rat cardiac fibroblast (rCFs) proliferation in a concentration-dependent manner. Treatment of rCFs with atazanavir 1–10 μM blocked $CoCl_2$-induced nuclear factor kappaB phosphorylation (p-NF-κB), p-IκBα and high-mobility group box 1 (HMGB1) expression. Treatment of rCFs with atazanavir 3 μM blocked HMGB1 downstream, p-NF-κB by blocking HMGB1 binds to toll-like receptor 9 (TLR 9). Intragastric administration of atazanavir sulfate 30 mg/k ameliorated changes in the left ventricular systolic pressure (LVSP), + dp/dtmax, and − dp/dtmax after 4 weeks. This was associated with attenuation of α-SMA, HMGB1, p-NF-κB, TLR 9, collagen I, collagen III expression and hydroxyproline (Hyp) content in ischemic myocardial tissue. Additionally, continuous intragastric administration of atazanavir for 28 days attenuated cardiac remodeling. These data suggested that the protective effect of atazanavir is likely due to blocking of myocardial inflammatory cascades through an HMGB1/TLR 9 signaling pathway.

1. Introduction

Myocardial infarction (MI) is the main pathogenic factor underlying heart failure (HF) (Samuel et al., 2008). In the post-MI phase, the heart undergoes extensive myocardial remodeling in response to the ischemic injury, leading to thickening or stiffening of regions of the heart, with progressive deterioration of cardiac function, which can progress to HF (Kirk and Cingolani, 2016). Cardiac fibrosis plays a major role in cardiac remodeling after MI and is a predisposing factor for HF. The pathological features of cardiac fibrosis include phenotypic changes in cardiac fibroblasts, excessive proliferation, and deposition of extracellular matrix (ECM) proteins such as collagen types I and III (Sutra et al., 2008).

Fibroblasts undergo dynamic phenotypic alterations and direct reparative response following MI, especially fibroblasts undergo myofibroblast transdifferentiation forming stress fibers and expressing contractile proteins (such as α-smooth muscle actin) in the infarct area at the proliferative phase of healing during hypoxia (Chen and Frangogiannis, 2013). In addition, previous studies have indicated that nuclear factor kappaB (NF-κB) plays a key role in inflammatory response during MI injury and subsequent cardiac hypertrophy and cardiac fibrosis. Suppression of NF-κB activation diminishes cardiac hypertrophy and cardiac fibrosis (Hamid et al., 2011; Zelarayan et al., 2009). High mobility group box-1 protein (HMGB1) is a key cytokine to play an extracellular role involving cellular activation and proinflammatory responses (Lotze and Tracey, 2005; Yang et al., 2005). HMGB1 binds to toll-like receptor 9 (TLR 9), interacts and preassociates with TLR9 to regulate inflammatory responses (Ivanov et al., 2007). Knockout TLR9 showed a reduction of fibrosis (Watanabe et al., 2007; Gäbele et al., 2008). HMGB1 plays a critical role to amplify fibrosis and involves the activation of NF-κB. Inhibiting HMGB1 blocks inflammatory response and may be a therapeutic target during cardiac fibrosis (Jiang et al., 2012; Su et al., 2014).

Atazanavir is an antiretroviral protease inhibitor class of drug that plays a key role in the treatment of HIV infection. In exploring novel pharmacological targets for cardiac fibrosis, we found that atazanavir attenuated hypoxia induced cardiac fibroblasts proliferation. This motivated us to experimentally examine the pharmacological activity of atazanavir in a cardiac fibrosis model. Therefore, this study utilized both *in vitro* and *in vivo* cardiac fibrosis models to characterize the role

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of atazanavir in signaling modulation that contributes potentially to the observed cardiac attenuation.

2. Materials and methods

2.1. Cell culture and expressional analysis

Rat cardiac fibroblasts (rCFs) from newborn (1- to 2-day-old) Sprague-Dawley rats were isolated according to previous method (Villarreal et al., 1993). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 100 kU/L penicillin and 100 mg/L streptomycin at 37 °C with 5% CO_2 in a humidified incubator. The cells were cultured to approximately 70% confluency and starved in serum-free DMEM overnight prior to the treatment. The cells were then treated with 3 μM atazanavir sulfate (purity > 99.0%; CAS No.: 229975-97-7; Hanxiang Biomedical Company, Shanghai, China) with or without cobalt chloride ($CoCl_2$; 100 μM) for 72 h and thereafter proteins were extracted.

2.2. rCFs proliferation assay and expressional assessment

To assess cellular proliferation, rCFs were maintained as described above. Cells were exposed to $CoCl_2$ at 100 μM to mimic hypoxia and treated with varying concentrations of atazanavir (0, 1, 3, 10 μM) with or without 3 μM hydroxychloroquine (HCQ), a TLR 9 antagonist, for 72 h. Cellular proliferation levels were determined via cell counting.

To examine changes in expression, the cells were seeded into 6-well flat bottom plates and maintained as described above, with one well per plate maintained as an untreated control. Cells were treated with 3 μM atazanavir sulfate with or without $CoCl_2$ (100 μM) for 72 h and thereafter the supernatants were collected and the proteins were extracted. Collagen I and collagen III were examined by ELISA kits. The expression levels of TLR 9, HMGB1, p-NF-κB, p-IκBα and total NF-κB were examined by Western blot and normalized and displayed as described above. To investigate the possible mechanism of reduction in rCF proliferation, cells were treated with 3 μM atazanavir sulfate with or without 3 μM HCQ for 72 h, TLR 9 and expression levels of HMGB1 and p-NF-κB were examined using Western blot as described above.

2.3. Ethics approval and consent to participate

All animal experimental procedures in this study were performed in accordance with the Institutional Animal Care and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Maryland, USA). The protocol was approved by the Committee on the Ethics of Animal Experiments of Binzhou Medical University (Permit Number: SCXK 20140013).

2.4. Induction of myocardial infarction (MI) model and experimental assessment

An MI model were induced according to a previously procedure (Jiang et al., 2010). Briefly, Rats were anesthetized with ketamine 100 mg/kg (i.m.) and xylazine 10 mg/kg (i.m.) and ventilated with room air using a rodent respirator. The chest was opened by middle thoracotomy and the left coronary artery was ligated at 2–3 mm from its origin between the left atrium and pulmonary artery conus using a 6-0 prolene suture. A successful operation was confirmed by the occurrence of ST-segment elevation in an electrocardiogram. This operation was performed by an experimenter who was blinded to the group assignments of the animals to avoid subjective bias of the experimenter on the outcome. The sham-operated group underwent thoracotomy and cardiac exposure without coronary ligation. Thirty rats were divided into three groups including (I) non-MI rats; (II) MI rats received saline alone; (III) MI rats received intragastric administration of atazanavir sulfate (30 mg/kg) plus ritonavir (10 mg/kg). Atazanavir is a low oral bioavailability compound and, clinically, is generally coadministrated with Ritonavir, which boosts the oral bioavailability of atazanavir by inhibiting cytochrome P450 (CYP) 3A4, and P-glycoprotein via the same metabolic pathway (Le Tiec et al., 2005, 021567s026lbl). The rats were administered daily via intragastric administration of corresponding drug for continuous 28 days after MI 24 h. Treatment was orally administered on a daily basis for atazanavir-treated animals, while animals in the vehicle-treated and sham groups were given an equal volume of saline. At day 29, determine hemodynamics and analyze histopathological change.

2.5. Determination of hemodynamics

Twenty eight days after MI, the rats were anesthetized with sodium pentobarbital (40 mg/kg) through intraperitoneal injection and a Millar vessel was inserted into the left ventricular cavity via the right common carotid artery. The pressure was transduced and amplified by a pressure transducer. Left ventricular systolic pressure (LVSP) and $\pm$ dp/dt_{max} were recorded and programmed by using a biotic signal collection and processing system (BIOPIC, American).

2.6. Histological examination

After fixation, three cross-sections through the ventricles were obtained and embedded. Paraffin sections were stained with Masson's trichrome and aniline blue for determination of collagen volume fraction, stained with Masson's trichrome for measurement of infarct size, the infarct size was expressed as previously described (Fishbein et al., 1978). The sections of HE staining measured myocyte size. For the measurement of cardiomyocyte cross-sectional area and diameter in the noninfarcted LV, a total of 30 myocytes sectioned transversely for area and longitudinally for diameter at the level of the nucleus were randomly chosen from each section at ×400 magnifications, and traced. To measure collagen volume fraction, 16 fields in the border and remote myocardium of the noninfarcted LV walls per section were scanned at a magnification of ×200. The interstitial collagen volume fraction was measured while omitting fibrosis of the perivascular, epi-, and endocardial areas from the study. The collagen volume fraction was obtained by calculating the mean ratio of connective tissue to the total tissue area of all the measurements of the section. The collagen-positive areas from all sections were determined by a single investigator who was unaware of the experimental groups.

2.7. Measurement of hydroxyproline (Hyp)

Frozen heart tissue samples were washed with saline and hydrolyzed with 6 mol/L hydrochloric acid at 100 °C for 5 h. The Hyp content was determined by adding p-dimethylaminobenzaldehyde and was quantified on a spectrophotometer at 560 nm and expressed as milligrams per gram of the wet heart tissue.

2.8. Western blots analysis of myocardial tissue

The heart samples (area at risk) were suspended in a buffer that contained 10 mM Tris (pH7.5),1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% Triton X-100 and lysed by homogenization. Nuclei were recovered by microcentrifugation at 6288 *g* for 5 min. The supernatant was collected and stored at − 80 °C for Western blot analysis. Nuclear proteins were extracted at 4 °C by gently resuspending the nuclei pellet in buffer that contained 20 mM Tris (pH7.5), 20% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, and 0.1% Triton X-100, followed by 1 h incubation with occasional vortexing at 4 °C. After microcentrifugation at 18,894 *g* for 15 min, the supernatant was collected. Protein concentrations of the extracts were measured by BCA assay. Equal amounts of cell protein (50 μg) were separated by SDS-PAGE and analyzed by western blot using specific antibodies to collagen I, collagen III, TLR 9, p-NF-κB,

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