The protective effect of AT1-antibody against myocardial ischemia-reperfusion injury through inhibition of inflammation and apoptosis

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Abstract

Myocardial ischemia and reperfusion (I/R) injury is known to occur on restoration of coronary flow after a period of myocardial ischemia. Angiotensin II (Ang II) is the most important bioactive substance of the renin-angioensin system (RAS), and exerts its physiological actions through AT1 receptors by regulating vascular tension and blood flow, and promoting cell growth and proliferation. The aim of the present study is to investigate the effects and its possible underlying mechanisms of AT1-AB on myocardial ischemia/reperfusion (I/R) injury. To test this hypothesis, we randomly assigned four groups (six mice per group) for experiment. To produce myocardial injury, the left anterior coronary artery (LAD) was occluded for 30 min, followed by 120 min of reperfusion in anesthetized mice. In the mice heart subjected to I/R injury, pretreatment AT1-AB (1mcg/gm) decreased the pathological scores of myocardium, and significantly attenuated I/R-induced increases of myocardial TNFα, IL-1β, IL-6. Further, 1mcg/gm AT1-AB significantly attenuated cTn-I and reduced apoptosis index of cardiac muscle cell of mice subjected to I/R injury. Therefore, these results demonstrate that AT1-AB exhibits significant protective effect against myocardial I/R injury which is related to inhibition of the release of inflammatory cytokines and the apoptosis of cardiac muscle cell.

Keywords: AT1-AB; Ischemia/reperfusion; cTn-I; Pro-inflammatory cytokine; Apoptosis

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Introduction

Ischemic heart disease is the leading cause of death in the Western world and will become the leading cause of mortality of the whole world by 2020 [1] at present, the main treatment of acute myocardial infarction is the re-delivery of oxygen and metabolites back to the ischemic tissue known as recanalization therapy [2]. This strategic process is known as “reperfusion” and is a pre-requisite towards salvaging viable myocardium following ischemia. However, this therapeutic strategy is limited and cannot salvage all the viable myocardium, and this is because reperfusion itself can paradoxically lead to myocardial complications and irreversible cell “injuries” such as necrosis (unprogrammed cell death) and apoptosis (programmed cell death) [3]. Thus, reperfusion can cause further injury to the myocardium and act like a “double-edged sword” [4].

It is accepted that the production of reactive oxygen species (ROS) plays an important role in the development (I/R) injury in cardiac cell. (I/R) also have been found to induce myocytes necrosis and apoptosis that seem to be the prevalent modes of cell death during the ischemic period and the reperfusion [5]. Apoptosis is a form of programmed cell suicide which allows for the orderly removal of unwanted, improperly functioning, or injured cells, in balance with the production of new cells by mitosis [6]. Apoptosis is differentiated from necrosis, also referred to as passive cell death because necrosis is a non-energy dependent process. Necrosis is pathologic or accidental cell death that occurs as a result of insult from harmful events such as hypoxia, toxicity, or infection. With necrosis, cell death transpires marked by the presence of inflammation, resulting in damage to adjacent cells; however, in the normal process of apoptosis, cells are usually affected individually [7].

Apoptosis of cardiomyocytes may further be influenced by Ang II, which binds to the AT1 receptor [8]. Thereby, it enhances intracellular calcium and stimulates calcium dependent endogenic endonucleases, which cause DNA laddering, cell shrinking and formation of apoptotic bodies. Mechanical stretching of cardiomyocytes in vitro causes Ang II release, increased Bax expression, and apoptosis [8] and there is Cumulative evidence which suggests that Ang II is a major contributor to cardiomyocyte (CM) apoptosis and left ventricular (LV) dysfunction after acute reperfused MI. Importantly,
blockade of the Ang II type 1 receptor (AT\(_1\)R) limits CM apoptosis and LV dysfunction after acute reperfused MI [9]. Messadi-Laribi et al. (2007) [10] found that Cardiac AT1R gene expression increased after IR in our experimental conditions and their results were consistent with other observations of cardiac AT1R induction in IR [11, 12].

In addition, Nio et al. (1995) [13] have also suggested that myocardial infarction in rats leads to increase in AT1aR gene transcription and protein expression and that therapy with AT1R, but not AT2R, antagonists was effective in blocking the increased expression of Ang II receptor subtypes after myocardial infarction. Furthermore, Baichun et al. (1998) [14] demonstrated that ischemia followed by reperfusion results in cardiac dysfunction in isolated perfused rat hearts, and the cardiac dysfunction is associated with at least a twofold increase in total Ang II receptor expression in the myocardium immediately following ischemia-reperfusion. Since AT2 receptor expression was unchanged, the marked increase in Ang II expression could be accounted for in its entirety by an increase in AT1 receptor expression [15].

**Method**

**Chemicals and instruments**

The materials used in this study are pure AT1-AB powder, ketamine (Hikma, Jordan), Xylazine (RompunTM, 2% vials, Bayer AG, Leverkusen, Germany), ethanol (Fluka, Switzerland) and normal saline (KSA). Mouse tumor necrosis factor-α (TNF-α), interleulin-6 (IL-6), interleukin-1beta (IL-1β) enzyme linked immunosorbent assay (ELISA) kits were purchased from Bioscience, Inc. USA. Mous cardiac troponin I ELISA kit was purchased from Life diagnostics Inc., USA. Mouse (Bcl-2) Elisa kit, mouse caspase 3 Elisa kit, was purchased from R&D Systems, Inc. USA. The instruments used in this study were High Intensity Ultrasonic Liquid Processor (Sonics & materials Inc., USA), Vascular Clamp (Biotechno, Germany) and ventilator (Harvard. USA).

**Animals**

Thirty adult males Swiss Albino mice weighing 28-33 g were purchased from Animal Resource Center, the National Center for Drug Control and Researches. The animals were apparently healthy and they were housed in the animal house of College of Medicine/University of Kufa in a temperature-controlled (24 ± 2°C) room with ambient humidity and alternating 12-h light/12-h dark cycles and were allowed free
access to water and standard chow diet until the start of experiments. The mice were left for two weeks without interference for acclimatization. They had no manifestation of any illness upon examination.

Design of the study

Animals were randomly divided into four groups (six mice/group) assigned as I, II, III, IV, Group I (sham): mice were subjected for all surgical procedure without ligation of left anterior descending (LAD) coronary artery. Group II (control): mice were subjected for entire surgical procedure with ligation of (LAD). Group III (control vehicle): Mice were pretreated with 1% D.W (vehicle for AT1-AB) then subjected to entire surgical procedure with ligation of (LAD). Group IV (AT1-AB treated group): Mice pretreated with AT 1-AB (1mcg/gm) of body weight at 30 min before LAD ligation via IV injection [15]. Then subjected to entire surgical procedure and ligation.

Surgical procedure and left anterior descending coronary artery (LAD) ligation

Mouse anesthetizes with 100mg/kg ketamine and 5mg/kg xylazine [16]. When the animals became unconscious (within 5-10 min), they were placed in supine position with their limbs fixed with stickers to ensure their immobilization during surgery and head extended with traction suture attached to the upper incisor teeth. Hair in the neck and chest regions was shaved and the skin was sterilized. All operative procedures were carried out in clean conditions. Longitudinal nick incision was made; trachea was reached by removing salivary glands via simultaneously pulling each part sideward with forceps. With the same maneuver, the paratracheal muscles on the midline fascia were split to expose the trachea in the larynx area with stay sutures applied to each side of split strap muscle. The trachea was intubated with a cannula sized either 22 G or 20 G according to the weight of animal with the small catheter reserved for the smaller animal. Mechanical ventilation was then achieved by connecting the endotracheal tube to scientific ventilator supplied with 100% oxygen at a respiratory rate of 50/min with a tidal volume of 20 mL/kg body weight [17].

Once steady breathing is established, animal's left limbs were fixed with right side limb, left thoracotomy was made between the 3rd and 4th rib and pericardiotomy was performed by using hemostats or round end scissors to open the space, without cutting the tissue so that the risk of bleeding can be reduced. A chest retractor was positioned within the fourth intercostal space in order to spread the ribs so that the left ventricle (LV) is exposed. Maximal care was taken not to damage the lung. A wet piece of small gauze, soaked with normal saline was inserted into the thorax to push back the lungs
away and expose the heart to have a clear view, the pericardium was removed using electronic microscope and LAD was easily detected. The LAD was transient ligated using a 6-0 prolene suture for a 30-minute ischemic period without exteriorization of the heart [18], the animal ventilator gradually was decreases. When spontaneous breathing was sufficient, decision was made for gentle and careful extubation after freeing the rat from tapes.

Finally, the mice was transferred into clean cage oxygenated with 100% oxygen and placed near a heating lamp. Cardiac reperfusion was allowed following 120 minutes of the LAD ligation [18]. Microsurgical scissors are used to cut the knot in the ligature. Proper ligation of the LAD was confirmed by observing blanching of myocardial tissue distal to the suture and dysfunction of the anterior wall as observed during the transient LAD ligation. Reperfusion was verified by the return of red color to the myocardial tissue and the demonstration of some recovery of anterior wall motion observed immediately following the transient LAD ligation.

**Samples collection**

At the end of reperfusion, blood was collected from the ventricles at the apical side. Hearts were cut from their main arteries (aorta and pulmonary artery), rinsed with normal saline to remove any blood, and stored in deep freeze (-20˚C) to (-80˚C).

The ventricles were cut from the atrio-ventricular junction and divided into two parts, lower (apical) and upper parts. The apical parts of the heart was further divided into two parts, one part used for apoptosis study while the other part was fixed in 10% formalin and processed by routine histological methods and embedded in paraffin blocks [19]. For subsequent histological examination, 5μm-thick horizontal sections were cut and stained with haematoxylin-eosin (H&E).

**Samples preparation**

*Preparation of Sample for TNF-α and IL-1β and IL-6 measurements*

The upper parts of the ventricles were washed with cold normal saline to remove any blood, stored in deep freeze (-20˚C), and then homogenized with high intensity liquid processor in 1:10 (w/v) phosphate buffered saline that contain 1% triton X-100 and protease inhibitor cocktail [20]. The homogenate was centrifuged with 2,500 g at 4˚C for 20 min. The supernatant was collected and used in TNF-α and IL-1β and IL-6 determination.
Preparation of sample for caspase 3 and Bcl-2 measurement

Rinse cardiac tissues two times with PBS, remove any remained PBS after the second rinse. Solubilize tissue in lysis buffer and allow samples to sit on ice for 15 minutes. Assay stored at ≤ -70°C. Before use, centrifuged samples at 2000 x g for 5 minutes and transfer the supernatant to a clean test tube. Sample protein concentration may be quantified using a total protein assay, dilution is made by IC diluent.

Preparation of sample for cTn-I measurement

At the end of reperfusion 0.5 ml blood was collected from the apex of the hearts. Blood samples were placed in a disodium EDTA (22 mg/ml) containing tube, mixed thoroughly and centrifuged at 3000 rpm for 15 min.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 for windows 7 (IBM, USA). Data were expressed as mean±SEM unless otherwise stated. One way Analysis of Variance (ANOVA) was used for multiple comparisons among all groups. Pearson correlation coefficient was used to assess the associations between two variables of study parameters. In all test; P<0.001 was considered statistically significant.

Results

Figure 1.

The levels of cardiac cytokines (TNF-α, IL-1β and IL-6) were found to be significantly elevated in the control group (II) and control vehicle (III) compared with the sham group (I). At the same time, cardiac cytokines were significantly decreased in AT1-AB treated group (IV) with respect to both control and control vehicle groups. *P<0.05 vs. sham group; **P<0.05 vs. Ctrl vehicle group.
Figure 2.

The level of plasma cTnI was significantly increased in control group (II) and control vehicle (III) compared to the sham group (I). On the other hand, cTn-I was significantly reduced in AT1-AB treated group (IV) with respect to both control (II) or control vehicle (III) groups. *P<0.05 vs. sham group, **P<0.05 vs. Ctrl vehicle group.

Figure 3.

The levels of cardiac pro apoptotic marker (caspase 3) were found to be significantly elevated in the control group (II) and control vehicle (III) compared with the sham group (I). While anti apoptotic (Bcl-2) were found to be significantly decrease in the control group (II) and control vehicle (III) compared with the sham group (I). At the same time, cardiac caspase 3 were significantly decreased in AT1-AB treated group (IV) with respect to both control and control vehicle groups. While cardiac Bcl-2 significantly elevated in AT1-AB treated group (IV) with respect to both control and control vehicle groups. *P<0.05 vs. sham group, **P<0.05 vs. Ctrl vehicle group.
Figure 4.
Component bar chart the relative frequency of different histopathology grading of abnormal heart changes among the four experimental groups. Score 0 (normal), no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), necrosis with the presence of contraction bands and neutrophil infiltrate; and score 4 (highly severe), widespread necrosis with the presence of contraction bands, neutrophil infiltrate, and hemorrhage.

**AT1-AB reduced myocardial inflammatory response**

The levels of myocardial cytokines (TNF-α, IL-1β and IL-6) were found to be significantly elevated ($P<0.001$) in control group (II) and control vehicle (III) compared with sham group (I). At the same time, cardiac cytokines were significantly decreased ($P<0.001$) in AT1-AB treated group (IV) with respect to both control and control vehicle groups, (Figure 1).

**Effect of AT1-AB on the myocardial injury**

The level of plasma cTn-I was also significantly increased ($P<0.001$) in control group (II) and control vehicle (III) compared to the sham group (I). On the other hand, cTn-I was significantly reduced ($P<0.001$) in AT1-AB
treated group (IV) with respect to both control (II) or control vehicle (III) groups, (Figure 2).

**Role of AT1-AB on cardiac Caspase 3, Bcl-2 after myocardial injury**

Exposure of myocytes to ischemia/reperfusion injury through LAD ligation significantly increased the pro apoptotic marker (caspase-3) levels ($P<0.001$) in the control group (II) compared to the sham group. Pretreatment with AT1-AB (group IV) significantly reduced ($P<0.001$) caspase-3 levels when compared to the control group (I) and control vehicle group (II), (Figure 5). while the level of cardiac Bcl-2 was significantly decrease ($P<0.001$) in control group (II) and control vehicle (III) compared to the sham group (I). On the other hand, Bcl-2 was significantly increase ($P<0.001$) in AT1-AB treated group (IV) with respect to both control (II) or control vehicle (III) groups, Figure (6).

**Figure 5.**

A representative photomicrograph of a section of the heart tissue section stained with Haematoxylin and Eosin (X 40). A, the sham group shows normal architecture (score 0); no interstitial edema, no diffuse myocardial cell swelling and necrosis, no neutrophils infiltration, no hemorrhage, no capillary compression and no evidence of apoptosis. B, cardiac section for the control group showed hemorrhage, necrosis and neutrophil infiltration. C, cardiac section for the control group showed contraction band and Hemorrhage D, cardiac section for the control vehicle group showed interstitial edema, hemorrhage and PMN infiltration. E, cardiac section of moderate injury showed hemorrhage and PMN infiltration. F, cardiac section after treatment with AT1-AB show almost normal cardiac structure.
Discussion

The major findings of the present study are as follows. Firstly, that the inflammatory cytokine (TNF-α, IL-1β, IL-6) and apoptosis play important role in the pathology of myocardial I/R. Secondly, AT1-AB pretreatment played a protective role against myocardial I/R injury, the protective effects of AT1-AB during myocardial I/R injury were correlated with the attenuation of inflammation and apoptosis. Thirdly, AT1-AB ameliorate myocardial I/R injury as evidenced by reduce the release of cardiac specific enzyme troponin I and Myocardial damage. A full spectrum of important molecules involved in the cellular response to stress are induced by the AT1 receptor these include the proinflammatory mediators Interleukin-1 beta (IL-1β).

Tumor Necrosis Factor-alpha (TNF-α), Interleukin-6 (IL-6) [21]. Nio et al (1995) suggested that myocardial infarction in rats leads to increase in AT1aR gene transcription and protein expression and that therapy with AT1R, but not AT2R, antagonists was effective in blocking the increased expression of Ang II receptor subtypes after myocardial infarction [22]. Harada et al (1998) have reported amelioration of reperfusion arrhythmias in AT1aR knockout mice [23]. To the best of our knowledge, there is no data available about effect of AT1-AB on caspase-3 and Bcl2 in regional ischemia reperfusion injury by ligation of LAD coronary artery. To the best of our knowledge, there is no data available about effect of AT1-AB on cTn-I in regional ischemia reperfusion injury by ligation of LAD coronary artery. The total severity scores mean of this group showed that 16.7% of the group had no damage, 66.7% had mild cardiac injury and 16.7% had moderate cardiac injury. No other studies are yet available to compare our results with.

Competing interests

The authors declare that there is no conflict of interest.

Author Contributions

All authors wrote, read and approved the final manuscript.
References


