Contribution of upregulated Hsps expression to the cardioprotection effect of oxytocin released in acute stress in ischemic reperfused hearts of the rat

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ABSTRACT

The heat shock proteins (HSPs) are expressed in normal cells but their expression is enhanced by a number of different stresses including heat and ischemia. The present study was aimed at determining the role of systemically released oxytocin in regulation of HSPs 70, 27 and 20 expression in stress-induced cardioprotection in isolated, perfused rat hearts. Rats were divided into four groups: IR (Ischemia/Reperfusion), St (stress); rats exposed to swim stress for 10 min, St+ATO; atosiban was used as an oxytocin receptor antagonist (1.5mg/kg,i.p.) prior to stress and ATO; atosiban was used prior to anesthesia. Hearts were isolated and subjected to 30 min regional ischemia and 60 min reperfusion (IR). Acute stress protocol consisted in swimming for 10 min. Ischemia-induced arrhythmias, Malondialdehyde in coronary effluent coronary flow, coronary flow and the expression of Hsp 70, 27 and 20 was measured in myocardium using real-time reverse transcriptase polymerase chain reaction (RT-PCR). The expression of Hsp 27 increased 4.5 folds by stress induction. Systemically administration of atosiban as an oxytocin antagonist prior to stress decreased Hsp27 mRNA levels. The malondialdehyde levels, which decreased in the St groups, increased by the administration of atosiban. These findings suggest that preconditioning effect of oxytocin at the periphery released in response to acute stress may be via Hsp27 over-expression as an early response.

Keywords: heart, heat shock proteins, ischemia, reperfusion, stress, oxytocin

INTRODUCTION

Cardiac preconditioning represents the most potent and steadily reproducible method of saving heart tissue from undergoing irreversible ischemic damage. Several recent clinical studies documented significant role of stress in evoking the severity of cardiovascular disease (1). As a neurohormone and as a neurotransmitter, oxytocin has also been involved in the stress response (2). On the other hand it is depicted preconditioning and post conditioning (3) effects of OT on the ischemic-reperfused heart injury. In rats, endogenous oxytocin plays an important role in cardiovascular responses to stress (4). Oxytocin is produced and released by the heart and acts on its cardiac receptors to decrease heart rate and force of contraction (5). It has been suggested that intrinsic OT system may play an important physiological role in regulation of vascular tone, as well as control of cardiac function (6). Our previous experiments indicated that activation of cardiac OT receptors by oxytocin released in response to stress may participate in cardioprotection induced by acute stress against myocardial ischemia reperfusion (IR) injury (7). In addition our previous study assessing the role of centrally released OT in stress provided the same result (8). The mechanism by which OT induces its cardioprotective effects is not yet known. So far the involvement of activation of mitochondrial ATP-dependent potassium channels (9), nitric oxide, reactive oxygen species, protein kinase C
(10), and protection against apoptosis (11) have been suggested. Recently we showed possible participation of Hsp27 over-expression as an early response (12).

Therefore, in view of the role of released oxytocin in stress-induced cardioprotection, we aimed to assess the expression and possible role of Hsp20, Hsp27 and Hsp70 in stress-induced cardioprotection.

MATERIALS AND METHODS

Animals
Male Wistar rats (200–250 g) were obtained from Tehran University of Medical Sciences and were housed in an air-conditioned colony room on a light/dark (12 h/12 h) cycle (lights on at 07:00 h) at 21–23°C with free access to food and water. The rats were housed individually in stainless steel cages. All experiments were conducted in accordance with the institutional guidelines of Tehran University of Medical Sciences (Tehran, Iran) and the national institutes of health guidelines for the care and use of laboratory animals.

Langendorff model of myocardial IR injury
The rats were anesthetized with sodium pentobarbital (60mg/kg, 15mg/0.5ml i.p.) and given heparinsodium (500 IU/0.5 ml i.p.). Hearts were rapidly excised and placed in ice-cold buffer, and mounted on a constant pressure (80mmHg) Langendorff perfusion apparatus.

Hearts were perfused retrogradely with modified Krebs–Henseleit bicarbonate buffer and ischemia was induced according to our previous article (7).

Coronary effluent was collected at the end of reperfusion to measure Malondialdehyde concentrations to determine the severity of oxidative damage. After completion of the reperfusion period, hearts were frozen and stored at -70°C until analysis.

Experimental protocol
All hearts were subjected to 30 min ischemia and 60 min reperfusion.

Rats were divided into 4 groups: (1) IR (Ischemia/Reperfusion); hearts were subjected to 30 min ischemia and 60 min reperfusion, (2) St (stress); rats exposed to swim stress for 10 min before anesthesia, (3) St+ATO; Atosiban was used as an oxytocin receptor antagonist (1.5 mg/kg i.p.) 10 min prior to stress, (4) ATO; atosiban was used (1.5 mg/kg i.p.) 20 min prior to anesthesia. To investigate the effect of stress on IR-induced cardiac damage, we used the rats were forced to swim for 10 min in deep water at 19–20 °C (13, 14).

Blood samples for OT and corticosteroid analysis were taken from a tail cut after anesthesia in the IR group, and 15 min after onset of the swim stress in the St group.

RNA isolation and cDNA synthesis
Briefly, 90-100 mg cardiac ventricular tissues of the rats were cut into small pieces and homogenized by pressing through 20 gauge needles. Total RNA was extracted using Trirupere agent according to the manufacturer's instruction (Roche, Berlin, Germany) (15). Quantity and quality assessments were determined by NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop® Technologies, Technologies, Wilmington, DE, USA). Total RNA (1,000 ng) was subjected for cDNA synthesis using MMLV reverse transcriptase and random hexamers using a cDNA synthesis kit (TAKARA, Otsu, Shiga, Japan) according to the manufacturer's instruction (16).

Primer design and RT-PCR
The NCBI (National Center for Biotechnology Information) website was used to design specific primers for Hsp20, Hsp27, Hsp70 and β-actin, used as an internal control. The sequences of primers are listed in Table I. Specificity of the primers was evaluated using conventional RT-PCR (10).
Table I Sequences of primer used in RT-PCR.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP27 Forward</td>
<td>ACGAAGAAAGGCAGGATGAA</td>
<td>232</td>
</tr>
<tr>
<td>HSP27 Reverse</td>
<td>GCTCCAGACTGTTCCGACTC</td>
<td></td>
</tr>
<tr>
<td>HSP20 Forward</td>
<td>CATGGTCCACAAACCACCTT</td>
<td>252</td>
</tr>
<tr>
<td>HSP20 Reverse</td>
<td>GGTTGCCCAGTCGGAGATTAACG</td>
<td></td>
</tr>
<tr>
<td>B-Actin Forward</td>
<td>CACCCGCGAGTACAACCTTC</td>
<td>204</td>
</tr>
<tr>
<td>B-Actin Reverse</td>
<td>CACCCGCGAGTACAACCTTC</td>
<td></td>
</tr>
<tr>
<td>B-Actin Forward</td>
<td>CCCATACCCACCACATCACCC</td>
<td>250</td>
</tr>
</tbody>
</table>

The optimal annealing temperature for the primer was 60°C.

Real-time Quantitative polymerase chain reaction

Quantitative real-time PCR was performed using SYBR Green TAKARA in duplicate and the Rotor-GeneTM 6000 (Corbette Life ScienceTM, Germany) under the following conditions: 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and elongation at 72 °C for 20 s. Real-time PCR was carried out in a volume of 20 µl containing: 10 µl PCR Master Mix, 2 µl of cDNA which had been diluted 5 times to achieve 200 ng/µl, 1 µl mixed forward and reverse primers and 7 µl ddH₂O. To determine the maximum efficiency of each primer pair, serial dilution of mixed primers (mixed forward and reverse = 10pmol/µl) by 0.5, 1.5 and 2µl was performed and 0.5µl for each primer was considered as the best efficiency (mixed forward and reverse was 1µl). Finally, the expression level for hsp20, hsp27 and hsp70 of each sample was normalized by subtracting the cycle threshold (C<T) of housekeeping gene from the gene of interest to calculate the ∆C<T.

Malondialdehyde (MDA) analysis

The MDA level, used as a marker for assessment of oxidative stress, was calculated by a thiobarbitoric acid (TBA) method. In brief, 1.5 ml perfusate was added to 0.5 ml of a solution containing 30% trichloroacetic acid, 0.75% TBA and 0.5 N HCl, and then incubated in a water bath at 100 °C for 20 min. After cooling, the samples were centrifuged and lipid peroxidation was determined by spectrophotometerat 532 nm (17).

Assessment of ventricular arrhythmias

Ischemia-induced ventricular arrhythmias were determined in accordance with the Lambeth Conventions (18). In this regard, three forms of ventricular arrhythmias were analyzed as below: ventricular ectopic beat (VEB), was identified as premature QRS complex, ventricular tachycardia (VT) was defined as four or more seriate VEBs, ventricular fibrillation (VF), was characterized as undetectable QRS complex. Multipar forms of VEBs such as bigeminy and salvos (couplet and triplet) were counted at separate episodes. The incidence, time of occurrence and duration of arrhythmias were used to identify arrhythmias severity according to the following scoring system (19); 0: 0–49 VEBs, 1: 50–499 VEBs, 2: >499 VEBs and/or 1 episode of spontaneously reverting VT or VF, 3: >1 episode of VT or VF or both with a total duration <60 s, 4: VT or VF or both 60–120 s total duration, 5: VT or VF or both >120 s duration, 6: fatal VF starting at >15 min after occlusion, 7: fatal VF starting between 4 and 14 min 59 s, 8: fatal VF starting between 1 and 3min 59 s, 9: fatal VF starting <1 min after occlusion.

Statistical analysis

Differences in MDA levels were evaluated by two-way ANOVA (stress and treatment as factors). When significant interaction was found, Tukey’s post hoc test was used for comparison between pair groups. Relative expression data were analyzed by LinReg 11 to determine the individual amplification efficiency and cycle threshold for each reaction tube, and REST 2009 to calculate expression levels by analyzing output of data obtained from LinReg 11. Arrhythmia scores were analyzed with Kruskal–Wallis test followed by post-hoc test Mann-Whitney, and the incidences of VT or VF were compared by Fisher exact test. All data were expressed as mean±SEM. Statistical significance was defined as P < 0.05.

RESULTS

Measurement of MDA levels in coronary effluent

The MDA levels showed a decrease following stress compared to IR group (0.57±0.23 vs 3.05± 0.82 nmol/ml, P<0.01) and peripheral administration of atosiban prior to stress in ATO+St group significantly increased MDA level compared to St group (3.57±0.83 vs 0.57±0.23, P<0.01, Fig. 1).
Myocardial expression of Hsp mRNAs
The expression of Hsp 70, 27 and 20 was measured in myocardium using real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Compared to the IR group, stressed hearts showed about 4.5-fold Hsp27 over-expression (1.0 vs. 4.5±2.6, \(P<0.05\), Fig. 2.). Peripherally infusion of atosiban prior to stress resulted in about 5-fold and 16 down-regulation of Hsp27 and Hsp20 mRNA respectively (0.21±0.15, \(P<0.05\) and 0.06±0.03, \(P<0.001\), Fig. 2.) as compared with the stress
Expression of Hsp27 was not different between IR and the atosiban group. In this study, we did not see any expression of Hsp70 or Hsp20 individually in cardiomyocytes between groups (Figs. 3 & 4).

**Ventricular arrhythmias during ischemia**

Severity of arrhythmias

The stress used in this study had no effect on ischemia-induced ventricular arrhythmias severity (2.08±0.3 vs. 2±0.42, p<0.05). But administration of atosiban prior to stress in ATO+St group intensified severity of arrhythmia compared to St group (3.87±0.3 vs. 2.08±0.3, p<0.01, Fig.5).

Incidence of VT and VF

In IR group incidences of VT and VF occurred in 66.6 and 22.2% of hearts respectively. Administration of stress prior to ischemia significantly decreased incidences of VF with respect to IR group (p<0.05). Peripheral administration of atosiban prior to stress in ATO+St group significantly increased VT and VF incidences compared to St group (both p<0.01). Incidences of VT and VT did not change by administration of atosiban compared to IR (Fig.6).
DISCUSSION

We observed that stress significantly decreased MDA levels and incidence of VF in the isolated rat heart subjected to an ischemia-reperfusion sequence. Systemic administration of atosiban (as an antagonist of OT receptor) eliminated the mentioned effect of stress. Furthermore, we demonstrate that significantly increased Hsp27 expression by stress induction. Infusion of atosiban prior to stress showed a decreased level in Hsp27 mRNA. As we reported in previous study that the plasma levels of oxytocin was significantly increased by our model of stress (7), these findings suggest that systemically released OT in response to stress may participate in stress-induced cardioprotection via increasing Hsp27 expression.

It has become evident that acute exercise protects the heart from IR injury (20-22). On the other hand previous studies have showed that restraint results in marked induction of both Hsp70 and Hsp27 mRNA in the vasculature of the rat (23, 24), and that Hsp27 levels in most tissues increased significantly after the heart is stressed (25). It has also been confirmed that morphine withdrawal as a severe stressor is capable of inducing Hsp27 phosphorylation in the heart (26). In myocardial ischemia, the small Hsp27 protein was increased after 30-minute ischemia and subsequent reperfusion of the rat heart (27), which is in agreement with our results showing that swimming stress clearly increased Hsp27 mRNA levels in the cardiomyocytes. Therefore, the effect of swim stress as an acute exercise shown on our model should not be ignored.

Our previously studies showed that activation of cardiac OT receptors by stress induced oxytocin released may participate in cardioprotection and inhibition of myocardial IR injury (7). Hence we suggested that centrally release of endogenous oxytocin in response to stress could also play a role in induction a preconditioning effect in ischemic-reperfused rat heart via brain receptors (8). In our studies, we observed that stress significantly, increased plasma oxytocin and rate pressure product (RPP), decreased infarct size, CK and LDH in the isolated rat heart subjected to an ischemia-reperfusion sequence and stress-induced myocardial tolerance to ischemia was abolished by administration of atosiban prior to stress exposure. In the present study, we showed that upregulated Hsp27 expression of the myocardium by stress could be blocked by systemic administration of atosiban as an oxytocin receptor antagonist. These observations imply that oxytocin probably has a direct cardioprotective effects and that the Hsp27 upregulation may be transmitted in part by the actions of systemic stress released oxytocin in the heart. Earlier studies have shown that Hsps, specifically Hsp70 and small Hsps such as Hsp27, are able to protect the heart against injuries associated with IR (28).

The mechanisms responsible for the cardioprotective action of oxytocin are not understood. Involvements of activation of mitochondrial ATP-dependent potassium channels (9), nitric oxide, reactive oxygen species, protein kinase C (10, 29), protection against apoptosis (11) and intracellular signaling (30) have been suggested. Since our previous results had shown that stress significantly reduced infarct size (7), it may be concluded that stress-induced
systemic endogenous OT may protect the heart by induction of Hsp27 expression. Additional knockdown and overexpression experiments on Hsp27 are needed to demonstrate this prediction, which was a limitation of our study.

It has become evident that heat shock proteins are involved in the mechanism of preconditioning (PC), and it has been reported that PC may increase gene expression by increasing transcription factors (31). Increased levels of Hsp27 are showed to participate in cardioprotection by maintaining the integrity of microtubules and actin cytoskeleton, and can protect endothelium from ischemia (32). Moreover Hsp27 can act as an endogenous cytoprotective stress response protein, evoking cardioprotection to ischemic injury via its role as a molecular chaperone (33). Several protective functions have been attributed to Hsps, which include repairing ion channels, restoring redox balance, interacting with nitric oxide-induced protection, preventing proinflammatory cytokines, and inhibiting apoptosis pathway activation (34). Hsp27 is reported to play a role as a downstream effector of p38 MAPK during an ischemic or β-adrenergic preconditioning protocol (35) and since oxytocin treatment under physiological conditions in vivo leads to activation of p38-MAPK (30), it may be suggested that oxytocin participates in the induction of Hsp27 expression. In this study, we did not find any increase in Hsp70 or Hsp20 individually in cardiomyocytes which was probably due to the different durations and stress patterns used. Presently we also showed that stress via systemic actions reduced the level of MDA, an oxidative stress marker, in coronary effluent. Dusunceli et. al. reported that OT treatment may inhibit IR-induced increases in MDA levels (36). Since systemic administration of atosiban prior to stress in this experiment abolished the reported effect of stress, it seems that stress induced OT release also plays a role in the preservation of antioxidant capacity against oxidative stress reduces cardiac ischemia/reperfusion injuries. In the present study, our model of stress could not cause a significant attenuation in severity of arrhythmias whoever diluted incidence of VF, showing that the changes in MDA levels don’t correlate with the antiarrhythmic effects of stress. In this context some studies showed that preconditioning reduces infarct size but accelerates time to ventricular fibrillation in ischemic heart (37-39). Peripherally blockade of oxytocin receptor significantly eludes the effects of stress on ventricular arrhythmias. This observation implies beneficial effects of peripheral oxytocin released on arrhythmia in stress condition. In this line some studies showed that adaptation to short term non-damaging stress effect largely limits or prevents cardiac arrhythmias in acute ischemia and reperfusion (40). A very large number of studies demonstrated that a mild stress resulted in protection against exposure to a subsequent more severe stress (41, 42) representing cardiac preconditioning. The present study showed that administration of stress prior to ischemia-reperfusion may have preconditioning effect.

CONCLUSION

These findings suggest that systemically released OT in response to stress may participate in stress-induced cardioprotection via increasing of Hsp27 expression in myocardiocytes and reduction of lipid peroxidation.

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Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES


