Upregulated Hsp27 Expression in the Cardioprotection Induced by Acute Stress and Oxytocin in Ischemic Reperfused Hearts of the Rat

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Abstract

In view of the cardioprotective effect of oxytocin (OT) released in response to stress, the aim of this study was to evaluate the role of heat shock proteins Hsps 70, 27 and 20 in stress-induced cardioprotection in isolated, perfused rat hearts. Rats were divided in two main groups: unstressed and stressed rats, and all of them were subjected to i.c.v. infusion of vehicle or drugs: unstressed rats [control: vehicle, OT (100 ng/5 µl), atosiban (ATO; 4.3 µg/5 µl) as OT antagonist, ATO+OT], and stressed rats [St: stress, OT+St, ATO+St]. After anesthesia, hearts were isolated and subjected to 30 min regional ischemia and 60 min subsequent reperfusion (IR). Acute stress protocol included swimming for 10 min before anesthesia. Malondialdehyde in coronary effluent was measured and the expression of Hsp 70, 27 and 20 was measured in myocardium using real-time reverse transcriptase polymerase chain reaction (RT-PCR). The malondialdehyde levels, which decreased in the St and OT groups, increased by the administration of atosiban as an OT antagonist. The expression of Hsp27 increased 4 to 5 folds by stress induction and i.c.v. infusion of OT. Central administration of atosiban prior to both stress and OT decreased Hsp27 mRNA levels. These findings suggest that endogenous OT may participate in stress-induced cardioprotection via Hsp27 over-expression as an early response.

Key Words: heart, heat shock proteins, ischemia, oxytocin, receptor, reperfusion, stress

Introduction

Oxytocin (OT) has been involved in stress response (20) and is well known for exerting effective physiological anti-stress effects (30). One of the important targets of OT is the cardiovascular system. Our previous experiments indicated that activation of cardiac OT receptors by OT released in response to stress may participate in cardioprotection induced by acute stress against myocardial ischemia reperfusion (IR) injury (28). There has been increased attention on the mechanisms involved in OT-induced cardioprotection in recent years. So far, the involvement of activation of mitochondrial ATP-dependent potassium channels (2), nitric oxide, reactive oxygen species, protein kinase C (14), and protection against apoptosis (1) have been suggested.

In this respect, heat shock proteins (Hsps) were identified as normally protective response to cellular stressors (36), and it is known that ischemia and reper-
fusion activate transcription of Hsp genes in the heart cells of various experimental animals (13). Moreover, it was also demonstrated that the levels of Hsps induced by mildly stressful procedures correlated with the level of protection, which was observed against the following stress (23, 32). These proteins are indeed induced by a wide range of stimuli which are potentially damaging to the cell. Hsps can also be induced by stimuli such as anoxia and/or ischemia and generators of free radicals such as hydrogen peroxide (34). There is now abundant experimental evidence that over-expression of individual Hsps, either in cultured cardiac cells in vitro or in the intact heart, have a protective effect (21). Hsp27 and αβ-crystallin are known to exist in detectable quantities in mammalian myocardium and are induced by oxidative stress, an important component of IR injury (7). Hence, a wide variety of stressful stimuli have been shown to increase Hsp70 synthesis in cardiac tissues (17, 24). It has been demonstrated that restraint results in marked induction of mRNA of both Hsp70 and Hsp27 in the vasculature of the rat (37). Moreover, it is now recognized that Hsp20 plays an important role in protecting against IR injury (15). Therefore, based on the role of released OT in stress-induced cardioprotection, we aimed to evaluate 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.

**Forced Swimming and Blood Sample Preparation**

For stress induction, the rats were forced to swim for 10 min in deep water at 19-20°C (19, 38). Blood samples for OT and corticosteroid analysis were taken from a tail cut after anesthesia in the control group, and 15 min after onset of the swim stress in the St group.

**Preparation of Isolated Hearts**

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

Hearts were perfused retrogradely with modified Krebs-Henseleit bicarbonate buffer and ischemia was induced according to our previous article (28). Coronary effluent was collected at the end of reperfusion to measure Malondialdehyde (MDA) 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 Design**

Rats were randomly divided into two main stressed and unstressed groups, and received i.c.v. infusion of vehicle or drugs before stress induction. Hearts were isolated and subjected to 30 min ischemia and 60 min reperfusion. Unstressed groups included: (1) control (n = 7), rats receiving vehicle; (2) OT (n = 7), for which OT (100 ng/5 µl/h, icv) was used; (3) ATO+OT (n = 5), atosiban (ATO) was administered (4.3 µg/5 µl/h, icv) prior to infusion of OT; (4) ATO (n = 6); rats receiving atosiban. Stressed groups, which were exposed to swim stress for 10 min before anesthesia, included: (1) St (stress) (n = 5), rats receiving vehicle 10 min prior to stress; (2) ATO+St (n = 5), rats receiving atosiban 10 min prior to stress; (3) OT+St (n = 6), infusion of OT was 10 min prior to stress.

**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 guage needles. Total RNA was extracted using Tripure reagent according to the manufacturer’s instruction (Roche, Berlin, Germany) (10). Quantity and quality assessments were determined by NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop® 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 (39).

**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 1. Specificity of the primers was evaluated using conventional RT-PCR (10).
Real-Time Quantitative Polymerase Chain Reaction

Quantitative real-time PCR was performed using SYBR GreenІ 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 pairs, serial dilution of mixed primers (mixed forward and reverse = 10 pmol/µl) by 0.5, 1, 1.5 and 2 µl was performed and 0.5 µl for each primer was considered as the best efficiency (mixed forward and reverse was 1 µl). 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 ΔC_T.

Malondialdehyde (MDA) Analysis

The MDA level, used as a marker for assessment of oxidative damage, was calculated by a tiobarbitoric 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 per-oxidation was determined by spectrophotometer at 532 nm (4).

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. Statistical significance was defined as P < 0.05.

Results

Measurement of MDA Levels in Coronary Effluent

Coronary effluent was collected at the end of reperfusion to measure Malondialdehyde (MDA) concentrations to determine the severity of oxidative damage.

The results showed significant interactions between treatment and stress on MDA (F (2,28) = 7.5, P < 0.001). All pairs of means were compared by Tukey’s post hoc test adjusting for multiple comparisons. The MDA levels showed a significantly decrease in the OT group, and following stress in St group compared to the control group (1.1 ± 0.3 and 1.1 ± 0.2 vs. 3 ± 0.1, both P < 0.05). Central administration of atosiban prior to stress resulted in 5-fold down-regulation of Hsp27 mRNA (0.18 ± 0.09, P < 0.01, Fig. 1) as compared with the stress group (as 1.0).

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).

Comparison to the control group, stressed hearts showed about 5-fold Hsp27 over-expression (1.0 vs. 5.6 ± 2.6, n = 5, P < 0.05, Fig. 2). I.c.v. infusion of atosiban prior to stress resulted in 5-fold down-regulation of Hsp27 mRNA (0.18 ± 0.09, P < 0.01, Fig. 2) as compared with the stress group (as 1.0). Central OT administration caused an increased ex-
pression of Hsp27 mRNA by about 4-fold (4.2 ± 4.18, P < 0.01, Fig. 2) in comparison to the control group, which was arbitrarily set as 1.0). Atosiban administration prior to OT led to a decrease in Hsp27 mRNA by about 19-fold (0.05 ± 0.17, P < 0.01, Fig. 2) compared to the OT group. Expression of Hsp27 was not different between unstressed and the atosiban group. In this study, we did not see any expression of Hsp70 or Hsp20 individually in cardiomyocytes between groups (Figs. 3 and 4).

**Discussion**

In this study, the protective effects of both stress and OT on MDA levels were shown. i.c.v. administration of atosiban as an antagonist of OT receptor abolished the mentioned effect of stress. Moreover, we observed that significantly increased Hsp27 expression by stress induction and central administration of OT. Infusion of atosiban prior to OT and stress showed a decreased level in Hsp27 mRNA. These findings suggest that centrally released OT in response to stress may participate in stress-induced cardioprotection via increasing Hsp27 expression, and that central exogenous OT via Hsp27 may have a cardioprotective effect.

Previous studies have showed that restraint results in marked induction of both Hsp70 and Hsp27 mRNA in the vasculature of the rat (22, 37), and that Hsp27 levels in most tissues increased significantly after the heart is stressed (18). It has also been confirmed that morphine withdrawal as a severe stressor is capable of inducing Hsp27 phosphorylation in the heart (3). In myocardial ischemia, the small Hsp27 protein was increased after 30-minute ischemia and subsequent reperfusion of the rat heart (11), which is in agreement with our results showing that swimming stress clearly increased Hsp27 mRNA levels in the cardiomyocytes. Many studies have shown that acute exercise protects the heart from IR injury (5, 8, 35). Therefore, the effect of acute exercise shown on our model should not be ignored.

We previously showed that activation of cardiac OT receptors by OT released in response to stress may participate in cardioprotection and inhibition of myocardial IR injury (28). Infusion of exogenous OT and centrally release of endogenous OT in response to stress could also play a role in induction a preconditioning effect in ischemic-reperfused rat heart via brain receptors (29). In both studies, we observed that stress and exogenous OT significantly decreased infarct size, CK and LDH and increased rate pressure product (RPP) 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 and central infusion of OT could be blocked by atosiban.
OT receptors in the heart; second, since OT has a biphasic dose-dependent effect against I/R injury as we have previously reported (16), combination of exogenous OT and endogenous OT release in response to stress could have provided a higher dose which showed less activity.

Our study also showed that both stress and OT via central actions reduced the level of MDA, an oxidative stress marker, in coronary effluent in comparison with their control groups. It has been reported that OT treatment may abolish IR-induced increases in MDA levels (12). In agreement with these studies, since atosiban prior to stress abolished the stated effect of stress, it seems that stress induced OT release via the maintenance of antioxidant capacity against oxidative stress reduces cardiac ischemia/reperfusion injuries. Atosiban prior to OT did not significantly increase MDA levels in coronary effluent despite reduced Hsp27 levels. However, MDA levels may be increased in the ventricle or plasma. These findings suggest that central OT release in response to stress may participate in stress-induced cardioprotection via increasing of Hsp27 expression in myocardocytes and reduction of lipid peroxidation.

**Acknowledgments**

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**References**


8. Brown, D.A., Lynch, J.M., Armstrong, C.J., Caruso, N.M. and as an OT receptor antagonist. According to our previous study, central infusion of exogenous OT had no effects on OT plasma concentration (29). Since central administration of atosiban that could not cross the blood-brain barrier (27) eliminated the direct stimulatory influence of OT on brain receptors, these observations imply that the Hsp27 upregulation may be transmitted in part by the actions of OT in the brain, although OT probably also has a direct cardioprotective effects.

Numerous studies have shown that Hsps, specifically Hsp70 and small Hsps such as Hsp27, are capable of protection against irreversible injuries associated with I/R (6). As our previous results had shown that both stress and OT significantly reduced infarct size (28), it may be concluded that stress-induced endogenous OT may protect the heart by induction of Hsp27 expression. Additional knock-down and overexpression experiments on Hsp27 are needed to demonstrate this prediction, which was a limitation of our study. The mechanisms responsible for the cardioprotective action of OT are not understood. Involvements of activation of mitochondrial ATP-dependent potassium channels (2), nitric oxide, reactive oxygen species, protein kinase C (14, 25), protection against apoptosis (1) and intracellular signaling (31) have been suggested.

Heat shock proteins are possibly involved in the mechanism of preconditioning (PC), and it has been suggested that PC may increase gene expression by influencing transcription factors (9). Elevated levels of Hsp27 are reported to participate in cardioprotection by maintaining the integrity of microtubules and actin cytoskeleton, and can protect endothelium from ischemia (21). Hsp27 can also act as an endogenous cytoprotective stress response protein, eliciting cardioprotection to ischemic injury via its role as a molecular chaperone (33). In this setting, different protective functions have been attributed to Hsps, which include repairing ion channels, restoring redox balance, interacting with nitric oxide-induced protection, inhibiting proinflammatory cytokines, and preventing apoptosis pathway activation (13). Hsp27 is confirmed to play a role as a downstream effector of p38 MAPK during an ischemic or β-adrenergic preconditioning protocol (26). Since OT treatment under physiological conditions in vivo leads to activation of p38-MAPK (31), it may be suggested that OT participates in the induction of Hsp27 expression. In this study, we were unable to demonstrate any increase in Hsp70 or Hsp20 individually in cardiomyocytes which was probably due to the different durations and stress patterns used.

Administration of OT prior to stress did not increase the protection of stress. There are two possible explanations for these findings; first, swimming could have stimulated the release of OT in multiple ways and the release could be sufficient enough to saturate