The role of nitric oxide, reactive oxygen species, and protein kinase C in oxytocin-induced cardioprotection in ischemic rat heart

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ABSTRACT

Ischemia–reperfusion injury is a common complication of heart disease that is the leading cause of death worldwide. Here, we plan to elucidate oxytocin cardioprotection effects against ischemia–reperfusion via nitric oxide (NO), reactive oxygen species (ROS), and protein kinase C (PKC) in anesthetized rat pre-conditioned myocardium. Forty-eight Sprague-Dawley rats were equally divided into eight groups. All animals were subjected to 25 min ischemia and 120 min reperfusion. Oxytocin (OT), L-NAME (LNA), a nitric oxide synthase inhibitor), chelerythrine (CHE, a PKC enzyme inhibitor), and N-acetylcysteine (NAC, a ROS scavenger) were used prior to ischemia. Results showed that mean arterial pressure significantly reduced during the first 10 min of ischemia and reperfusion in IR, LNA, CHE, and NAC groups (p < 0.05). OT prevented mean arterial pressure decline during early phase of ischemia and reperfusion. Cardioprotective effects of OT in infarct size, plasma levels of creatine kinase-MB and lactate dehydrogenase, severity and incidence of ventricular arrhythmias were abolished by OT, L-NAME, chelerythrine, and N-acetylcysteine (p < 0.05). The present study showed that OT pretreatment reduces myocardial infarct size and ventricular arrhythmias, and improves mean arterial pressure via NO production, PKC activation, and ROS balance. These findings provide new insight into therapeutic strategies for ischemic heart disease.

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1. Introduction

In spite of advances in management and care of heart diseases, ischemia–reperfusion (IR) injury continues to be a major cause of mortality and morbidity. There has been increased interest in the mechanisms involved in oxytocin-induced cardioprotection in recent years. It is not carefully understood how oxytocin (OT) protects the myocardium from ischemia–reperfusion (IR) injury, but the overall mechanism is likely to be multifactorial. In previous studies, we showed protective effects of OT on myocardial injury in ischemic reperfused heart of anesthetized rats, mediatory roles of mitochondrial ATP-dependent potassium (mitoKATP) channel [2] and the mitochondrial permeability transition pore (mPTP) [1]. We have also depicted that cardioprotective effects of ischemic preconditioning (IPC) can be induced by endogenous OT [3].

The potential intracardiac role of OT during ischemia–reperfusion is confirmed by its receptor expression in cardiomyocytes [1–3], and direct cardioprotection or stimulated protection via cellular mediators such as natriuretic peptides (NPs) [14] and nitric oxide (NO) [9,10]. Previous studies have indicated that NO mediates the activation of mitoKATP channels in cardiomyocytes [25,27]. Increased NO bioavailability may play an important role in OT-dependent cardioprotective mechanisms through the regulation of mitoKATP channels and NO producing enzymes [10]. In addition, we had previously showed the mediatory roles of mitoKATP channels and mPTP in protective effects of OT on ischemic reperfused heart [1,2]. Indeed, pretreatment with a mitoKATP channel opener appears to increase ROS production and PKC activity before ischemia and reduce IR injuries in adult isolated cardiomyocyte [19,24]. Moreover, the protection afforded by mitoKATP channel openers against the reperfusion-induced apoptosis or necrosis appears to necessitate stimulation of PKC activity by ROS [18] and NO-dependent pathways [15,25]. A further detailed understanding of the many signaling steps and the final cytoprotective mechanisms underlying oxytocin-induced preconditioning may lead to improvements in the management and care of ischemia–reperfusion injury. Therefore, in order to...
clarify the mechanisms leading to OT-mediated cardioprotection, we investigated the OT effects against ischemia–reperfusion via NO, PKC, and ROS modulation in preconditioned myocardium.

2. Materials and methods

2.1. Materials

Oxytocin, 2,3,5-triphenyltetrazolium chloride, Nω-nitro-l-arginine methyl ester (L-NAME), N-acetylcysteine, chelerythrine, and Evans blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Male Sprague-Dawley rats (300–350 g) were maintained in animal quarters under standardized conditions 12 h light/dark cycle, 20–22 °C ambient temperature, and 40–50% humidity with free access to rat chow and water. All experimental procedures were done according to the guidelines of animal and human ethical committee of Tehran University of Medical Sciences.

2.3. Surgical preparation

The preparation used in the present study was as previously described [2]. The animals were anesthetized with Sodium Thiopental (60 mg kg⁻¹, IP) and maintained with supplementary doses (~30 mg kg⁻¹, IP) if required. Body temperature was measured by rectal thermometer and maintained at 37 °C.

The rats were ventilated through a tracheotomy tube with air-and-oxygen mixture by a rodent ventilator (model 683, Harvard Apparatus) (stroke volume approximately 1.2 ml 100 g⁻¹, 60–70 stroke min⁻¹). A positive end-expiratory pressure was applied to prevent alveolar atelectasis (3–5 cm H2O).

Heparinized catheter (100 U/ml) was fixed into the right carotid artery for blood sampling and pressure monitoring. The lateral tail vein was cannulated to inject Evans blue dye and other drugs. Electrocardiogram standard limb lead-II and arterial blood pressure were continuously monitored using a computerized data acquisition system (Power Lab data acquisition system, four channels, AD Instruments).

The fourth rib was cut 3 mm below left lateral sternal border. The pericardium was incised and a sling (6-0 silk Ethicon) was placed around the left anterior descending (LAD) artery close to its origin right below the left atrial appendage. Both ends of the ligature were passed through a small plastic tube. Heart rate and blood pressure were allowed to stabilize for 20 min before the intervention protocols. To induce transient myocardial ischemia, LAD was occluded by applying tension to the plastic tube-silk string. Reperfusion was then initiated by releasing the ligature and removing the tension. Regional ischemia and reperfusion were confirmed by myocardial cyanosis and hyperemia, respectively.

2.4. Exclusion criteria

Data were excluded from the final analysis if arterial blood pressure was less than 80 mmHg before drug administration or when the O2 saturation dropped below 90% in the presence of O2 administration.

2.5. Experimental protocols

Forty-eight rats were divided into eight groups (n = 6): (i) IR; hearts were subjected to 25 min ischemia and 120 min reperfusion, (ii) OT; oxytocin was administered (0.03 µg/kg, IP) 25 min prior to ischemia, (iii) LNA + OT; L-NAME (LNA) was used as a nitric oxide synthase inhibitor (2 mg kg⁻¹, IP) 10 min prior to OT administration, (iv) LNA; L-NAME was used 35 min prior to ischemia, (v) CHE + OT; chelerythrine (CHE) was used as a PKC enzyme inhibitor (5 mg kg⁻¹, IP) 10 min prior to OT administration, (vi) CHE; chelerythrine was used 35 min prior to ischemia, (vii) NAC + OT; N-acetylcysteine (NAC) was used as a ROS scavenger (200 mg kg⁻¹, IP) 10 min prior to OT administration, and (viii) NAC; N-acetylcysteine was used 35 min prior to ischemia.

2.6. Hemodynamic functions

Hemodynamic parameters such as mean arterial pressure (MAP) and heart rate (HR) were continuously monitored and recorded by Power Lab data acquisition system.

2.7. Cardiac area at risk and infarct size determination

To identify area at risk (AAR), the coronary artery was reoccluded. Evans blue dye (2 ml 2%) was then injected through lateral tail vein. The heart was excised; atria and the roots of the great vessels were removed. Remaining tissues were frozen for 24 h. Then they were cut into 2 mm slices. All pieces were incubated in 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC, 0.1 M phosphate buffer, pH 7.4, Sigma Chemical Co., St. Louis, MO, USA) at 37 °C for 15 min to visualize the infarct area. Then they were fixed for 2 days in 10% formalin to enhance the contrast. Non ischemic area, area at risk and infarcted area were colored blue, brick red, and pale respectively. Sections were scanned to determine normal area, AAR, and infarct size (IS) by calculating pixels occupying each area using Adobe Photoshop software (Adobe Systems Seattle, WA). Total AAR and IS are expressed as the percentage of total ventricle and AAR, respectively.

2.8. Assessment of ventricular arrhythmias

Ischemia-induced ventricular arrhythmias were counted during the occlusion period and determined in accordance with the Lambeth Conventions [30]. Ventricular ectopic beats (VEBs) were diagnosed as a distinctive and identifiable premature QRS complexes. Ventricular tachycardia (VT) was defined as a run of four or more consecutive VEBs. Ventricular fibrillation (VF) was defined as unidentifiable and low voltage QRS complexes. Other multipart forms of VEBs, such as bigeminy and salvos (two or three consecutive VEBs), were not separately determined and were included as single VEB. VF lasting for more than 5 min was measured as irreversible [2]. The incidence, time of occurrence, and duration of arrhythmias were used to identify arrhythmias severity according to the following scoring system [23]: 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 3 min 59 s, 9: fatal VF starting <1 min after occlusion.

2.9. Biochemical analysis

Blood samples were collected at the end of each experiment. Plasma samples were extracted and stored at ~70 °C till final assay. Lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) isoenzyme level were analyzed using ELECSYS System (ELECSYS 2010, Roche, Germany) and commercial kit (Pars Azmoon, Iran).
2.10. Statistical analysis

Statistical analysis of hemodynamic data within and between groups was performed with two-way analysis of variance. Differences in infarct size, LDH and CK-MB plasma levels were determined by one-way analysis of variance. Groups were individually compared using Dunnet’s test. ARRhythmia scores were analyzed with Kruskal–Wallis test, 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.

3. Results

3.1. Hemodynamic data

Table 1 shows heart rate and mean arterial pressure during 20 min baseline, immediately before OT administration, 15 min post OT administration, the first 10 min of ischemia and reperfusion, and at the end of reperfusion. No significant differences were seen between groups at baseline. Mean arterial pressure significantly reduced during the first 10 min of ischemia and reperfusion in IR, L-NAME, CHE, and NAC groups (p < 0.05). Oxytocin administration prevented mean arterial pressure decline during early phase of ischemia and reperfusion (Table 1). Hemodynamic parameters slightly but not significantly decreased in all groups in comparison with baseline at the end of reperfusion.

3.2. Area at risk and infarct size measurement

AAR was not different among groups. Infarct size was significantly decreased in IR group compared to IR (p < 0.05). Administration of L-NAME, chelerythrine, and N-acetylcysteine in LNA + OT, CHE + OT, and NAC + OT groups increased infarct size to 44 ± 4, 41 ± 1.9%, and 42 ± 2.5% respectively in comparison with OT group (p < 0.05). L-NAME, N-acetylcysteine, and chelerythrine alone had no significant effect on infarct size compared to IR group (Fig. 1).

3.3. Ventricular arrhythmias during ischemia

3.3.1. Severity of arrhythmias

OT use prior to ischemia significantly declined ventricular arrhythmias severity compared to IR group (p < 0.05).

Administration of L-NAME, chelerythrine, and N-acetylcysteine in LNA + OT, CHE + OT, and NAC + OT groups was intensified severity of arrhythmias compared to OT group (3.4 ± 0.8, 3.7 ± 0.6 and 3.5 ± 0.6 vs. 0.9 ± 0.2 respectively, p < 0.05). L-NAME, N-acetylcysteine, and chelerythrine alone had no significant effect on arrhythmias severity compared to IR group (Fig. 2).

3.3.2. Incidences of VT and VF

In IR group, all animals were experienced VT, while VF occurred in 67% of cases. OT use attenuated VT incidence to 22% and abolished VF incidence completely compared to IR group (p < 0.05). In comparison with OT group, administration of L-NAME, chelerythrine, and N-acetylcysteine restored the incidence of VT to 100%, 100%, and 100%, and VF to 57%, 57%, and 67% in LNA + OT, CHE + OT, and NAC + OT groups respectively (p < 0.05). L-NAME, N-acetylcysteine, and chelerythrine alone did not significantly change incidences of VT and VF in comparison with IR group (Fig. 3).

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>20 min baseline</th>
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<th>15 min after administer OT</th>
<th>First 10 min ischemia</th>
<th>First 10 min reperfusion</th>
<th>End of reperfusion</th>
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Data are presented as mean ± SEM; Number of six animals in each group; MAP, mean arterial pressure (mmHg); HR, heart rate (beats/min); IR, ischemia–reperfusion; OT, oxytocin; LNA, L-NAME; CHE, chelerythrine; NAC, N-acetylcysteine.

*p < 0.05 vs. baseline.
Fig. 2. Distribution of the arrhythmia score during 25 min ischemia in IR, OT, LNA + OT, LNA, CHE + OT, CHE, NAC + OT, and NAC groups. Data are presented as mean ± SEM. *p < 0.05 vs. IR group. #p < 0.05 vs. OT group. OT, oxytocin; LNA, L-NAME; CHE, chelerythrine; NAC, N-acetylcysteine.

Fig. 3. The incidence of ventricular fibrillation (VF) and ventricular tachycardia (VT) during 25 min ischemia in IR, OT, LNA + OT, LNA, CHE + OT, CHE, NAC + OT, and NAC groups. Data are presented as mean ± SEM. *p < 0.05 vs. IR group. #p < 0.05 vs. OT group. OT, oxytocin; LNA, L-NAME; CHE, chelerythrine; NAC, N-acetylcysteine.

3.4. Biochemical analysis

Oxytocin significantly decreased plasma LDH and CK-MB levels at the end of reperfusion period compared with IR group (p < 0.05). L-NAME, N-acetylcysteine, and chelerythrine administration in LNA + OT, NAC + OT, and CHE + OT groups significantly increased LDH and CK-MB plasma level compared to OT group (p < 0.05). L-NAME, N-acetylcysteine, and chelerythrine alone had no significant effect on LDH and CK-MB plasma level compared to IR group (Figs. 4 and 5).

4. Discussion

The results showed that the protective effects of OT on infarct size, CK-MB, and LDH plasma level, severity and incidence of ventricular arrhythmia were abolished by L-NAME, a nitric oxide synthase inhibitor, chelerythrine, a PKC inhibitor, and N-acetylcysteine, a ROS scavenger. These findings suggest that activation of NO, ROS, and PKC is a requisite for the infarct-sparing effects of OT in IR injuries.

In previous studies, we showed the role of mitoKATP channels and mPTP in infarct-sparing effects of OT [1,2]. The role of mitoKATP channels activation in cardioprotective signaling mechanisms has been supported by the findings that PKC and NO-mediated mechanisms facilitate the opening of mitoKATP channels [17,25,26]. In the present study, pretreatment with L-NAME abrogated cardioprotective effects of OT in anesthetized rat; indicating NO may involve in OT cardioprotective effects. Furthermore, OT is known to stimulate NO release during IR [12,22]. Three recent reports suggest that the protective effects of OT pretreatment during IR injury may be caused by NO release [10,12,29]. Consistent with these, our results confirm mediatory role of NO in OT-induced cardioprotection.

On the other hand, a board of experimental evidence suggests that the opening of mitoKATP channel generates ROS, and that these radicals represent a part of the cascade leading to cardioprotection [11,16,20,21]. Here, we revealed that N-acetylcysteine, a ROS scavenger and chelerythrine, a PKC inhibitor eliminated the protective effects of OT in rat heart. Chen et al. [7] showed that N-acetylcysteine blocks the effects of IPC and other cardioprotection agents. In addition, PKC is known to be activated by ROS [4] hence; it may work downstream to ROS and to close mPTP as end effectors in cardioprotection phenomena. Furthermore, cardioprotective effects of PKC have been shown to be omit by mitoKATP.
channel inhibitors [13] and mPFP openers [8]. Consistent with these, our results confirm mediatory role of ROS and PKC in OT-induced cardioprotection.

In the present study, OT administration before ischemia led to suppression of life-threatening arrhythmias. In previous study, we reported that 5-HD, a specific mitoKATP channel blocker, prevented OT benefits effects on life-threatening arrhythmias. Das and Sarkar [10] showed the activation of mitoKATP due to OT in prevention of ventricular arrhythmia during reperfusion. The authors, however, did not report arrhythmia score during ischemic phase preceding reperfusion. In this study, a new manifestation of OT anti-arrhythmic effects has demonstrated during ischemic phase.

Previous studies proved different mechanisms of arrhythmogenicity in ischemia and reperfusion phases [5]. In fact, occurrence of arrhythmia in ischemic phase is dependent to release of endogenous triggers factors originated from biochemical mediatory compounds such as NO and ROS [5]. Furthermore, pharmacological agents such as diazoxide [28] and pravastatin [6] would prevent arrhythmia in ischemic phase by a mechanism mediated through ROS and PKC pathways [5]. Consistent with these, in the present study, OT administration induced similar anti-arrhythmic profile as IPC and pharmacologic preconditioning during ischemia. In addition, pretreatment with L-NNAME, N-acetylcyesteine, and chelerythrine abrogate beneficial anti-arrhythmic effects of OT which brings more focus on critical role of NO and ROS in arrhythmogenic effects of OT. On the other hand, ROS and infarct size are two fundamental determinants of arrhythmogenicity during reperfusion phase [5]. In the present study, we did not measure superoxide and ROS levels in cardiomyocytes, but in preliminary experiment, we showed OT-induced decrease in plasma malondialdehyde activity (2.1 ± 0.3 vs. 3.7 ± 0.4 nM/ml). In addition, hemodynamic instability shown as MAP drop in the first phase of reperfusion is the sign of ROS release from mitochondria. Here, decreasing infarct size as well as compensation of hypotension during first 10 min of reperfusion possibly indicates ROS suppression during reperfusion phase by OT, and indirectly emphasizes to anti-arrhythmic role of OT in reperfusion phase. Therefore, a decrease in ROS release may be partly involved in OT inhibitory effect on life-threatening arrhythmias.

In summary, we may infer that OT as a triggering agent can initiate mitochondrial signaling cascade including NO, ROS, and PKC. Nevertheless, without analyzing the influences of OT in the presence of several inhibitors or direct kinase activity assay, we cannot determine whether the interactions among multiple mediators are in parallel or in sequence. Future studies are required to provide sufficient evidence related to downstream signaling pathways of OT and their possible links.

5. Conclusion

Our results indicate that pharmacological preconditioning of rat heart by pretreatment with L-NNAME, a non-specific NOS inhibitor, chelerythrine, a PKC inhibitor and N-acetylcysteine, a ROS scavenger abolished the beneficial effects of oxytocin in ischemia–reperfusion heart model. This may suggest that the benefits were achieved via NO release, PKC activation, and ROS balance. These observations have considerable relevance for future therapeutics approaches for oxytocin in myocardial ischemic injuries.

Conflict of interest
The author(s) declare(s) that they have no conflict of interest to disclose.

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