Oxytocin protects cardiomyocytes from apoptosis induced by ischemia–reperfusion in rat heart: Role of mitochondrial ATP-dependent potassium channel and permeability transition pore

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ABSTRACT

The current study examines the protective effect of oxytocin (OT) on cardiomyocyte apoptosis mediated by mitochondrial ATP-dependent potassium (mitoKATP) channel and permeability transition pore (mPTP) in the preconditioned myocardium of anesthetized rats. Eighty rats were equally divided into eight groups. The hearts of all animals except for the sham group were subjected to 25 min ischemia and 120 min reperfusion. Oxytocin, 5-hydroxydecanoate (5-HD), a specific inhibitor of the mitoKATP channel, and atracyloside (ATRC), an mPTP opener, were used prior to ischemia. Hemodynamic parameters were recorded throughout the experiment. Evaluations were made by infarct size, plasma lactate dehydrogenase level (LDH), transmission electron microscopy (TEM) and immunohistochemistry studies. OT prevented mean arterial pressure drop during early phase of ischemia and reperfusion. Treatment with OT before IR induction normalizes cardiomyocytes both in light microscopy and TEM observations. In addition, OT significantly reduced TUNEL- and increased Bcl-2-labeled positive cell number relative to IR (p < 0.05). However, 5HD or ATRC inhibited the protective effects of OT on cardiomyocytes damaged by IR (p < 0.05). Ultrastructural changes including extensive myofibril loss, sarcomelemal disruption and mitochondrial swelling due to amorphous dens bodies indicate necrosis induction in SHD and ATRC as well as in IR groups. Restoration of immunohistochemistry parameters and protection against IR-induced ultrastructural changes confirm OT cardioprotective effects via mitoKATP channel and mPTP modulation in apoptosis induced by ischemia–reperfusion.

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1. Introduction

Previous studies have shown OT cardioprotective effects against ischemia–reperfusion-induced injury in hearts of anesthetized rats [1,2]. However, the mechanism(s) underlying its infarct-sparing effect in cardiomyocytes remain controversial. Mitochondria are now known to play a crucial role in the development and progression of post ischemic myocardial damage through initiating different signaling cascades that ultimately bring about apoptosis and necrotic cell death in cardiomyocytes [5]. Mitochondrial permeability transition pore (mPTP) opening was first recognized as the underlying factor for heart damage which occurs during reperfusion after a period of ischemia [3]. A growing body of evidence indicates that oxygen-derived free radicals (ROS) production during ischemia and reperfusion triggers ischemic preconditioning (IPC) signaling pathway through mitochondrial ATP-dependent potassium (mitoKATP) channel and mPTP activation [7]. Upon elevated ROS, cross talk between mPTP and mitoKATP channel initiates apoptosis and necrosis in IR condition[7]. Though mPTP is generally linked to late apoptosis and necrosis, few studies were undertaken to investigate interrelationship between mitoKATP channel and mPTP in OT cardioprotective effects against progressive apoptosis during IR. Based on these observations, we hypothesized that ROS originating from mitochondria upon mitoKATP channel opening and mPTP closing may mediate attenuation of apoptosis and necrosis in OT cardioprotective effects. Therefore, the current...
study examines modulatory role of mitoKATP channels and mPTP in cardioprotective effects of OT against I/R induced cardiomyocyte apoptosis in anesthetized rats.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (300–350 g) were maintained in animal quarters under standardized conditions with a 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 performed according to the guidelines of the animal and human ethical committee of Tehran University of Medical Sciences.

2.2. Surgical preparation

The preparation used in the present study has previously been described [1]. Animals were anesthetized with sodium thiopental (60 mg/kg, IP) and maintained with supplementary doses (30 mg/kg, IP) as required. Body temperature was measured using a rectal thermometer and maintained at 37 °C. Rats were ventilated (Stroke volume 1.2 ml, 100 g−1, 60–70 min, PEEP 3–5 cm H2O) with an air/oxygen mixture using a tracheotomy tube and a rodent ventilator (Harvard Apparatus, model 683). A heparinized catheter (100 U/ml) was fixed into the right carotid artery for blood sampling and pressure monitoring. Lateral tail vein was also cannulated for drug administration. Standard limb lead-ll and arterial blood pressure were continuously monitored using a computerized Power Lab data acquisition system (AD Instruments, Australia).

The fourth rib was cut 3 mm below the left lateral sternal border. The pericardium was incised and a sling 6/0 silk Ethicon brand sewing stitch was placed around the left anterior descending (LAD) artery close to its origin 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 performing the intervention protocols. To induce transient myocardial ischemia, the 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 epicardial cyanosis and hyperemia, respectively.

2.3. Exclusion criteria

Data were excluded from the final analysis if arterial blood pressure was dropped less than 80 mmHg before prescriptions or if there was an inadequate blood O2 saturation level.

2.4. Experimental protocols

The hearts of all animals except for the sham group were subjected to 25 min ischemia and 120 min reperfusion (Fig. 1). Eighty rats were equally divided into eight groups: (I) sham group; rats underwent the same procedure as IR except that clamping was omitted, (II) IR group in which hearts subjected to 25 min of ischemia and 120 min of reperfusion, (III) OT group in which OT (0.03 μg/kg, IP) administered 25 min prior to ischemia, (IV) SHD + OT group in which SHD (10 mg/kg, iv), a specific mitoKATP channel inhibitor administered 10 min prior to OT administration, (V) SHD group in which SHD was used 35 min prior to ischemia, (VI) ATRC + OT group in which atracyloside (ATRC, an mPTP opener, 5 mg/kg, iv) was given 10 min prior to OT administration, (VII) ATRC group in which atracyloside was used 35 min prior to ischemia and

Fig. 1. Illustration of the experimental protocols. The hearts of all animals except for the sham group were subjected to 25 min ischemia and 120 min reperfusion. IR = ischemia-reperfusion; NS = normal saline; OT = oxytocin; ATRC = atracyloside and SHD = 5-hydroxydecanoic acid.

(VIII) ATRC + SHD + OT group in which atracyloside and SHD were used 10 min prior to OT administration.

2.5. Hemodynamic functions

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

2.6. Cardiac area at risk and infarct size determination

To identify area at risk (AAR), the coronary artery was reocluded. Evans blue dye (2 ml, 2%) was then injected through the lateral tail vein. The hearts were excised; atria and the roots of the great vessels were removed. Remaining tissues were frozen for 24 h and then 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 infarcted 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 ventricles and AAR, respectively. At the end of the experiment, small samples from the same location on the AAR were collected and fixed in 10% formaldehyde or 2.5% glutaraldehyde, and were processed for immunohistochemistry and transmission electron microscopy studies respectively.

2.7. Biochemical analysis

Blood samples were collected at the end of each experiment. Plasma samples were extracted and stored at −70 °C until they were assayed. Lactate dehydrogenase (LDH) isoenzyme level was analyzed using ELECSYS System (ELECSYS 2010, Roche, Germany) and commercial kit (Pars Azmoon, Iran).
Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>20 min baseline</th>
<th>Administer OT time</th>
<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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<tbody>
<tr>
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<td></td>
<td></td>
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<tr>
<td>SHAM</td>
<td>345 ± 12</td>
<td>343 ± 15</td>
<td></td>
<td>331 ± 14</td>
<td>325 ± 19</td>
<td>337 ± 19</td>
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<tr>
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<td>347 ± 11</td>
<td>344 ± 13</td>
<td></td>
<td>325 ± 21</td>
<td>330 ± 18</td>
<td>335 ± 15</td>
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<td>331 ± 14</td>
<td>325 ± 24</td>
<td>335 ± 25</td>
<td></td>
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<tr>
<td>5HD + OT</td>
<td>340 ± 11</td>
<td>347 ± 11</td>
<td>332 ± 16</td>
<td>327 ± 20</td>
<td>325 ± 17</td>
<td></td>
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<tr>
<td>SHD</td>
<td>343 ± 15</td>
<td>340 ± 13</td>
<td></td>
<td>335 ± 17</td>
<td>335 ± 18</td>
<td></td>
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<tr>
<td>ATRC + OT</td>
<td>347 ± 12</td>
<td>345 ± 12</td>
<td>335 ± 17</td>
<td>335 ± 18</td>
<td>335 ± 17</td>
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<tr>
<td>ATRC</td>
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<td>342 ± 12</td>
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<td>330 ± 22</td>
<td>321 ± 22</td>
</tr>
<tr>
<td>5HD + ATRC + OT</td>
<td>343 ± 10</td>
<td>335 ± 12</td>
<td>328 ± 16</td>
<td>322 ± 23</td>
<td>328 ± 22</td>
<td>325 ± 27</td>
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</table>

MAP

| Groups      |                 |                    |                            |                        |                          |                   |
|-------------|-----------------|--------------------|----------------------------|                        |                          |                   |
| SHAM        | 126 ± 10        | 124 ± 8            |                            | 111 ± 11               | 111 ± 11                 | 108 ± 10          |
| IR          | 124 ± 6         | 126 ± 8            |                            | 87 ± 12’               | 92 ± 11’                 |                   |
| OT          | 120 ± 4         | 122 ± 6            | 118 ± 9                    | 107 ± 13               | 105 ± 10                 | 111 ± 11          |
| SHD + OT    | 121 ± 8         | 124 ± 9            | 120 ± 8                    | 105 ± 12               | 104 ± 11                 | 107 ± 9           |
| SHD         | 116 ± 6         | 120 ± 5            |                            | 80 ± 6’                | 85 ± 11’                 | 98 ± 8            |
| ATRC + OT   | 118 ± 9         | 115 ± 11           | 114 ± 12                   | 100 ± 11               | 101 ± 13                 | 111 ± 13          |
| ATRC        | 123 ± 5         | 122 ± 13           |                            | 82 ± 10’               | 85 ± 10’                 | 99 ± 10           |
| 5HD + ATRC + OT | 120 ± 5     | 118 ± 13           | 114 ± 12                   | 102 ± 10               | 105 ± 10                 | 106 ± 12          |

Data are presented as mean ± SEM; N=10; MAP= mean arterial pressure (mmHg); HR=heart rate (beats/min); IR=ischemia–reperfusion; OT=oxytocin; 5HD=5-hydroxydecanoate; ATRC=atractyloside.

*p < 0.05 vs. baseline.

2.8. Immunohistochemistry

2.8.1. In situ nick end-labeling assay

In situ detection of cells with DNA strand breaks was performed in formalin-fixed, paraffin-embedded tissue sections by terminal deoxyxynucleotidyl transferase dUTP nick-end labeling (TUNEL) according to the manufacturer’s instructions (Roche, Germany). In each animal, 2–3 slides and in each slide, 5 microscopic fields were studied. Number of TUNEL-positive cells in each field were divided by apoptotic + non apoptotic cells and expressed in percentage value [4].

2.9. Bcl-2 detection

Immunohistochemistry was carried out on 5-μm tissue sections from formalin-fixed paraffin blocks using the avidin–biotin immunoperoxidase method. Sections were stained by monoclonal mouse antiRat/Rabbit Bcl-2 oncoprotein (DAKO Corporation, USA) using a K0673 LSAB2 detection system (DAKO Corporation, USA) according to the manufacturer’s instructions. In each animal, 2–3 slides and in each slide, 5 microscopic fields were studied. Number of Bcl2 positive cells in each field was divided by apoptotic + non apoptotic cells and expressed in percentage value [4].

2.10. Transmission electron microscopy

Hearts of experimental rats were fixed in 2.5% glutaraldehyde (Proscitech, Thuringowa, Australia) in PBS and processed for transmission electron microscopy (TEM). Samples were postfixed in 1% OsO4 (TAAB; Berkshire, UK) dehydrated through an ethanol series, equilibrated in propylene oxide, and embedded in araldite (Proscitech). Thin sections were stained with uranyl acetate and lead citrate. The specimens were observed with a LEO 906 (Zeiss, Germany) transmission electron microscope and images were recorded with a camera (Zeiss, Germany) for subsequent assessments.

2.11. Materials

Oxytocin, atractyloside, 5HD, 2,3,5-triphenyltetrazolium chloride and Evans blue were obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.12. Statistical analysis

Statistical analyses between groups were performed with one-way analysis of variance and the post hoc Tukey test. Hemodynamic data within and between groups were performed with two-way analysis of variance. Differences in infarct size, plasma LDH levels, apoptosis and Bcl-2 data were determined by one-way analysis of variance with SPSS 16.0 software. All data were expressed as mean ± SEM with p < 0.05 considered statistically significant.

3. Results

3.1. Hemodynamic data

Table 1 shows heart rate (HR) and mean arterial pressure (MAP) at the 20 min time point of baseline, OT administration, 15 min after OT administration, first 10 min time point of ischemia and reperfusion, and at the end of reperfusion. There were no significant differences between groups at baseline before treatment. MAP significantly reduced during first 10 min of ischemia and reperfusion in IR, 5HD and ATRC groups (p < 0.05). OT prevented MAP drop appeared during early phase of ischemia and reperfusion (Table 1). Hemodynamic parameters slightly but not significantly decreased in all groups compared with baseline at the end of reperfusion.

3.2. Area at risk and infarct size measurement

AAR was not different between groups. Infarct size was significantly decreased in OT group compared to IR (p < 0.05) (Fig. 2). Administration of 5HD and ATRC in 5HD + OT and ATRC + OT groups increased infarct size to 44 ± 3.5% and 45 ± 3.7% respectively in comparison with OT group (p < 0.05). 5HD and atractyloside alone, and in combination had no significant effect on infarct size compared to IR group (Fig. 2).

3.3. Biochemical analysis

OT significantly decreased plasma LDH level at the end of reperfusion period in comparison with IR group (p < 0.05). 5HD and atractyloside administration in 5HD + OT and ATRC + OT groups significantly increased plasma LDH level compared to OT group.
(p < 0.05). 5HD and atracyloside alone, and in combination had no significant effect on LDH plasma level compared to IR group (Fig. 3).

3.4 Immunohistochemistry

The percentage of TUNEL-positive cardiomyocytes and Bcl-2 immunoreactive cells were higher and lower respectively in the IR than in the sham group (p < 0.05). Treatment with OT before ischemia–reperfusion induction caused a significant decrease in TUNEL-positive cell numbers compared with IR group but resulted in an increase in Bcl-2-labeled cells (p < 0.05) (Table 2). Treatment with 5HD, atracyloside alone and in combination significantly increased apoptotic cell number and decreased Bcl-2-labeled cells compared with OT group (p < 0.05) (Table 2).

3.5 Transmission electron microscopy

In sham group, cardiomyocytes had a large euchromatin nucleus with a smooth surface. The cytoplasm was full of microfilaments with regular repeated sarcomeres and striation. Mitochondria were abundant and contained numerous cristas (Figs. 4a and 5a). Ultrastructural features of cardiomyocytes in tissues taken from IR rats, showed less myofibril condensation. Most of nuclei had a marginal chromatin condensation without a moon-like shape. Nuclear deformities in the form of irregular peripheral infoldings were usually observed. The mitochondria in IR rats were condensed with inner wrinkled crista and heterogeneity both in size and shape. Intra-cytoplasmic vacuoles could also be seen in some cardiac cells (Figs. 4b and 5b).

OT administration before IR induction caused pretty normalization of the ultrastructure of the cardiomyocytes both in myofibrils and their striation, nuclei and mitochondrial morphology in comparison with IR group (Fig. 4c). Both in 5HD and atracyloside treated rats, cardiomyocytes intra-cytoplasmic myofilament shrinkage and nuclear deformity were noted. Striations disappeared and myofibrils were lysed in some cardiomyocytes. Prominent small amorphous electron dense bodies were observed in a majority of mitochondria. Partially or completely degenerated crista was observed in some cardiac cells. These features could be considered as signs of necrosis (Figs. 4d, e, f and 5c). In 5HD + OT, ATRC + OT and 5HD + ATRC + OT groups, the nucleous and myofibril morphology were not different from IR group. Most of mitochondria had degenerated crista or electron dense bodies (Figs. 4d, e, h and 5c–e).

4. Discussion

In this study, OT causes hemodynamic improvement during early phase of ischemia and reperfusion. The protective effects of OT on infarct size and LDH level were abolished by 5HD and atracyloside. OT significantly reduced TUNEL-positive-cell number though it had a reverse effect on Bcl-2 labeled positive cell number relative to the IR group. Strikingly, treatment with 5HD and atracyloside significantly increased apoptotic cell number in comparison with OT group. These findings strongly focus on apoptosis, mitoKATP channel and mPTP as requisites for the infarct-sparing effects of OT in IR injuries.

In the present study, OT reversed MAP drop observed during first 10 min of ischemia and reperfusion. Transient opening of mPTP and massive production of ROS associated with hemodynamic instability (stunning) happens before apoptosis and necrosis in IR [6]. Our previous unpublished data also showed ROS track in OT hemodynamic anti-stunning effect and this may indirectly be mediated through ROS and mPTP opening. In our previous study, we showed that OT myocardial effects were disregarded of hemodynamic changes and concluded that the observation would be a direct cytoprotection at the cellular level [1]. The present study also confirms this idea. Despite transit increase of MAP in 10 min first of ischemia and reperfusion, infarct size was increased in ATRC + OT.
and 5HD + OT groups. Therefore, OT cardioprotective effects do not likely related to hemodynamic alterations.

In the present study, the infarct-sparing effect of OT was abrogated by a selective mitoKATP channel blocker and mPTP opener, suggesting that the cardioprotective effect of OT is caused by mitoKATP channel activation and mPTP blockade. Previous studies established the role of mitoKATP channel and mPTP as end-effectors of mitochondrial dysfunction preceded necrosis and apoptosis during IR injury [11,13,15]. MitoKATP channels are activated by a decreased ATP/ADP ratio in hypoxic/ischemic conditions. Its activation may shorten the action potential duration, reduce cellular calcium overload and preserve myocardial viability in ischemic situations [14,16]. Mitochondrial matrix volume may also increase while membrane potential remains nearly stable. This would be favorable for ATP generation due to the reduction in driving force and speculated to preserve mitochondrial function during IR [9]. In our study, it has not been determined the link between mitoKATP channel and mPTP in the infarct-sparing effect of OT. A growing body of evidence indicates that produced ROS during IR triggers IPC signaling pathway through mitoKATP channel and mPTP [7]. ROS has a dual effect on IPC in myocardial salvage. Massive mitochondrial ROS induces apoptosis and necrosis in cardiomyocytes, whereas its moderate release leads to mPTP opening and mediates IPC like effects [7]. Generation of ROS by mitochondria during reperfusion period facilitates mPTP opening due to apoptotic cell death and necrosis [17]. Our previous study showed that N-acetylcysteine, a ROS scavenger eliminated the infarct-sparing effect of OT in rat heart. This suggests that cardioprotective signaling pathway of OT, at least in part, may be mediated through ROS. Consistent with our hypothesis, transient opening of mPTP evokes an imbalance between close and open state which in turn has a critical role in cardiomyocyte survival or death [6,8]. Taken together, our results suggest that mitoKATP channel opening and mPTP closing by ROS may contribute to myocardial salvage by OT.

We also showed that IR induction is associated with cardiomyocyte apoptosis according to TEM and immunohistochemistry observations. The proto-oncogene Bcl-2 and other bcl-2 family members play key roles in cell death regulation and appear to govern the decision to die at multiple levels [10]. In our study, administration of OT significantly reduced cardiomyocyte apoptosis and increased Bcl-2 positive cells in comparison with IR. We identified signs of apoptosis and necrosis in the 5HD + OT and ATRC + OT groups. Therefore, in this ischemia–reperfusion model, cell death occurs via both apoptosis and necrosis. Apoptosis and necrosis share common mediators and pathways and result in final stages of cell death [12]. Signs such as mitochondrial amorphous

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**Fig. 4.** Transmission electron microscopic photomicrographs from rat cardiac cells. (a) Sham group, (b) IR group, cardiac cell nucleus with condensed chromatin and irregular folding of the nuclear surface (arrows), cell shrinkage, (7000×); (c) OT group; OT causing pretty normalization of the organization of myofibrils and mitochondria (7000×); (d) 5HD + OT group, shows a cardiomyocyte nuclei with its surface destination, note to lysis of myofibrils, and presence of two polymorphic nuclear cells in the endomysium as a sign of inflammation (3000×); (e) ATRC + OT group, indicating degeneration of cristae inside the mitochondria and reduced distance between Z lines (12,000×); (f) ATRC group, note to disappearing of cristae inside of some mitochondria (7000×); (g) 5HD group, note to swollen mitochondria with amorphous dense bodies (black arrows), blue arrows show intercalated disks (3000×); (h) ATRC + 5HD + OT group, note to less condensation of myofibrils and heterogeneity in size and shape of mitochondria (3000×). Nucleus (N), sarcoplasmic reticulum (SR), mitochondria (M). Z disks (Z), cardiomyocytes (Cm), and endomysium (E), polymorphic nuclear (PMN), IR = ischemia–reperfusion; OT = oxytocin; 5HD = 5-hydroxydecanoate and ATRC = atractyloside. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
density, crystal breakage and sarcolemmal disruption are considered to be irreversible signs of necrosis. Indeed, our observations indicate that the signs in most of the cardiac cells in 5HD + OT and ATRC + OT groups are irreversible. These findings strongly indicate mitoKATP channel and mPTP involvement in OT anti-apoptosis effects.

5. Conclusion

The significant restoration of altered immunohistochemistry parameters and prevention of IR-induced rise in ultrastructural changes confirm that oxytocin cardioprotective effects provide through mitoKATP channel and mPTP modulation in apoptosis induced by ischemia–reperfusion.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

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