Phenylephrine Induces Early and Late Cardioprotection Through Mitochondrial Permeability Transition Pore in the Isolated Rat Heart

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Background. The aim of this study was to investigate the role of mitochondrial permeability transition pore (mPTP) in cardioprotection afforded by phenylephrine pretreatment in early and late phases.

Methods. Rat hearts were isolated and perfused with Krebs buffer in Langendorff preparation and subjected to 30 min regional ischemia followed by 60 min of reperfusion. Phenylephrine as a selective α1-adrenoceptor agonist and atractyloside as a specific opener of the mPTP were used. Seven groups (n = 6) of rats were randomly studied: (I) control: surgical procedure was performed with no ischemia/reperfusion, (II) ischemia/reperfusion: hearts underwent regional ischemia/reperfusion, (III) early phenylephrine: phenylephrine (50 μM) was perfused for 5 min prior to ischemia/reperfusion, (IV) late phenylephrine: rats were treated with phenylephrine (10 mg/kg, i.p) 24 h prior to ischemia/reperfusion, (V) early phenylephrine + atractyloside: hearts were perfused with phenylephrine as in group III and then atractyloside (20 mM) 5 min before reperfusion for 20 min, (VI) late phenylephrine + atractyloside: hearts were treated with phenylephrine as in group IV and then received atractyloside (20 mM), 5 min before reperfusion for 20 min, (VII) atractyloside-IR group: hearts were perfused with atractyloside (20 mM) 5 min before reperfusion for 20 min.

Results. Compared with ischemia/reperfusion group, perfusion of phenylephrine in early and late phases decreased myocardial infarct size (% of ischemia zone), reduced creatine kinase-MB (CK-MB) in the coronary effluent, and improved cardiac function. Administration of atractyloside abolished cardioprotective effects of phenylephrine in both early and late phases and returned infarct size, CK-MB and cardiac function to levels as seen in ischemia/reperfusion group.

Conclusion. These results suggest that administration of atractyloside as a specific opener of the mPTP abolishes phenylephrine-induced early and late cardioprotection in the isolated rat hearts.

Key Words: ischemia; reperfusion; preconditioning; alpha-1 adrenergic agonist; mPTP.

INTRODUCTION

Induction of brief periods of ischemia in the myocardium leads to an increased resistance to the injury due to a subsequent, more prolonged ischemic episode [1]. The cardioprotective effect of preconditioning occurs in two phases. The early phase lasts for 1 to 3 h while the late phase of protection occurs at least 12 h following the initial sublethal ischemic insult and has been shown to last up to 72 h [2]. Early studies have shown that stimulation of α1-adrenoceptors is one of the essential triggers of the early phase of ischemic preconditioning [3] and also exogenously activation of α1-adrenoceptor can exert both early [4–6] and late preconditioning [7, 8].

Experimental evidences suggest that mitochondrial permeability transition pore (mPTP) has an important role in mediating cell death following a prolonged myocardial ischemia/reperfusion [9–11]. The mPTP is a nonspecific pore of the inner mitochondrial membrane and three major components are seen in its structure: cyclophilin D, the adenine nucleotide translocase (ANT), and the voltage-dependent anion channel (VDAC) [12]. It has been shown that mPTP only opens in early reperfusion and remains closed during prolonged myocardial ischemia. Opening of
this pore initiates apoptosis as a result of efflux of proapoptotic factors such as cytochrome c due to increasing matrix volume and rupturing of the outer mitochondrial membrane [13]. In this manner, recent evidences have been indicated that inhibition of the mPTP by cyclosporine A induces a powerful cardioprotection against lethal ischemia/reperfusion injury [14, 15]. Also, it has been demonstrated that ischemic preconditioning inhibits mPTP opening in early [16] and late phases [17]. As well, participation of the mPTP inhibition was suggested in pharmacologic preconditioning afforded by diazoxide [13] and desflurane [18].

Therefore, in present study we investigated the possible role of the mPTP inhibition in early and late phases of phenylephrine-induced preconditioning in ischemia/reperfused rat heart.

MATERIALS AND METHODS

Preparation of Isolated Hearts

Male Wistar rats (200–250 g) were housed in an air-conditioned colony room on a light/dark cycle at 21–23 °C with free access to food and water. The animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and given heparin sodium (500 IU). Hearts were rapidly excised and placed in ice-cold buffer, and mounted on a constant pressure (80 mmHg) Langendorff-perfusion apparatus. 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.

Hearts were perfused retrogradely with modified Krebs-Henseleit bicarbonate buffer containing (in mmol/L): NaHCO3 25; KCl 4.7; NaCl 118.5; MgSO4 1.2; KH2PO4 1.2; glucose 11; CaCl2 2.5 gassed with 95% O2/5% CO2 (pH 7.35–7.45 at 37 °C). A latex, fluid-filled, isovolumic balloon was introduced into the left ventricle through the left atrial appendage and inflated to give a preload of 8 to 10 mmHg and connected to a pressure transducer (Harvard, March-Hugsteten, Germany).

A surgical needle was passed under the origin of the left anterior descending coronary artery, and the ends of the suture were passed through a pipette tip to form a snare. Regional ischemia was induced by tightening the snare and reperfusion was performed by releasing the ends of the suture. The perfusion apparatus was water-jacketed to maintain a constant perfusion temperature of 37 °C. Heart rate (HR), left ventricular developed pressure and coronary flow were monitored with a homemade program (Ossilograph Monitor, Biomed, Tehran, Iran). Left ventricular function was assessed by left ventricular developed pressure, rate pressure product – heart rate × left ventricular developed pressure and coronary flow. In addition, coronary effluent was collected for CK-MB (Creatine Kinase-MB) measurement at regular intervals.

Experimental Protocol

After heart isolation and prior to baseline period, all hearts were perfused and allowed to stabilize for 30 min in which heart rate and left ventricular developed pressure (difference between left ventricular systolic and diastolic pressure) were maintained at the same level for three continuous measurement periods timed 5 min apart. As seen in Fig. 1, all animals were randomly divided into experimental groups as follow: (I) Control group: hearts were perfused with no regional ischemia/reperfusion; (II) ischemic/reperfusion group: hearts underwent 30 min of regional ischemia followed by 60 min reperfusion; (III) early phenylephrine group: hearts were perfused for 5 min with phenylephrine (50 μM) 15 min prior to regional ischemia; (IV) late phenylephrine group, rats were treated with phenylephrine (10mg/kg, i.p) 24 h prior to regional ischemia; (V) early phenylephrine+attractyloside group: hearts were perfused with phenylephrine as in group III and then attractyloside (as a specific mPTP opener, 20 mM) 5 min before reperfusion for 20 min; (VI) late phenylephrine+attractyloside group: hearts were treated with phenylephrine as in group IV and then received attractyloside, 5 min before reperfusion for 20 min; (VII) attractyloside-IR group: hearts were perfused with attractyloside (20 mM) 5 min before reperfusion for 20 min. Administrations of drugs in early phase were performed via the second arm of perfusate cannula which was connected to the main perfusion cannula and the experimental conditions were constant throughout the experiment.

Infarct Size Measurement

After completion of the reperfusion period, the left coronary artery was reoccluded, and Evans blue dye was infused via the aorta to differentiate the ischemic zone from the nonischemic zone. Hearts were frozen for overnight and then sliced into 2-mm transverse sections from apex to base. Slices were then incubated with 1% triphenyl tetrazolium chloride (TTC in 0.1 M phosphate buffer, pH 7.4) for a period of 20 min at 37 °C. TTC reacts with viable tissue, producing a red formazan derivative, which is distinct from the white necrotic tissue once fixed in 10% formalin for 24 h. The areas of the left ventricle and infarcted tissues were measured by way of a planimetry from the scanned hearts by using Photoshop program (Ver. 7.0, Adobe System, San Jose, CA, USA). Volumes were obtained by multiplying the area by the thickness of the slices. Area at risk was expressed as a percentage of left ventricular volume for each heart. The infarct size was determined by using computer-aided planimetry and expressed as a percentage of Area at risk.

Measurement of Creatine Kinase-MB (CK-MB)

The level of CK-MB was calculated in coronary effluent samples at 5 and 60 min of reperfusion with a specific CK-MB Kit (Pars Azmoon, Tehran, Iran), using an autoanalyzer (Roche Hitachi Modular DP Systems; Mannheim, Germany).

Chemicals

Phenylephrine, attractyloside, and triphenyltetrazolium chloride were obtained from Sigma-Aldrich (Deisenhofen, Germany) and general laboratory chemicals were acquired from Merck (Darmstadt, Germany). Stock solutions of phenylephrine and attractyloside were dissolved in distilled water and added to the Krebs-Henseleit bicarbonate (KHB) buffer and equilibrated with O2(95%)–CO2 (5%) (pH 7.4 at 37 °C).

Statistical Analyses

Data are expressed as means ± SEM. Statistical comparison of means between groups was made by one-way ANOVA and a subsequent Tukey test. Significant differences were determined as P < 0.05.

RESULTS

Hemodynamic Function

Table 1 illustrates the hemodynamic parameters (heart rate (HR), left ventricular developed pressure
(LVDP)] and coronary flow (CF) in experimental groups in different periods. Since heart rate and left ventricular developed pressure may recover to different degrees, rate pressure product (RPP) was calculated via multiplying heart rate by left ventricular developed pressure and presented as reliable left ventricular function parameter for the isolated heart.

No differences were obtained between the experimental groups for RPP at baseline period. RPP (as a percentage of an individual baseline) reduced during regional ischemia period, and recovered partially throughout reperfusion period.

Compared with ischemia/reperfusion group, pretreatment with phenylephrine in both early and late phases increased the recovery of rate pressure product (70.9% ± 1.7% and 68.2% ± 1% versus 47% ± 1.5% in IR group, P < 0.001) at the end of reperfusion period. Administration of atracyloside significantly abolished the recovery effect of phenylephrine on RPP in early and late phases (45.2% ± 1.7% and 44.5% ± 1.2% respectively, P < 0.001) and returned this parameter as seen in ischemia/reperfusion group (Fig. 2). Perfusion of atracyloside alone had no effect on hemodynamic parameters.

Infarct Size and Area at Risk

Figure 3 illustrates the ratio of area at risk to total left ventricular areas and ratio of infarct size to area at risk. There were no significant differences in the ratio of area at risk to total left ventricular areas between the hearts in all experimental groups. The ratio of infarct size to area at risk decreased considerably from 37.8% ± 0.7% in ischemia/reperfusion group to 8.8% ± 0.3% and 8.85% ± 0.3% in phenylephrine early preconditioning and phenylephrine late preconditioning groups respectively. Phenylephrine-induced infarct limitation was inhibited by administration of atracyloside in early and late phases, where the ratio of infarct size to area at risk was increased to 32.8% ± 1.5% and 33.1% ± 1.4%, respectively. Perfusion of atracyloside alone had no effect on infarct size.

CK-MB Levels

CK-MB levels (IU/L) in coronary artery effluent were markedly declined by phenylephrine-induced preconditioning in early and late phases compared with ischemia/reperfusion group at 5 min (30.3 ± 15.8, 38.2 ± 3...
**TABLE 1**

<table>
<thead>
<tr>
<th>Groups</th>
<th>HR (b.p.min)</th>
<th>LVDP (mmHg)</th>
<th>CF (ml/min)</th>
<th>LVDP % CF (ml/min)</th>
<th>HR (b.p.min)</th>
<th>LVDP % CF (ml/min)</th>
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</thead>
<tbody>
<tr>
<td>Control (Con)</td>
<td>283 ± 38</td>
<td>80 ± 30/8</td>
<td>90 ± 0.42</td>
<td>0.042 ± 0.77</td>
<td>298 ± 34</td>
<td>82 ± 33</td>
</tr>
<tr>
<td>Ischemia/reperfusion (IR)</td>
<td>283 ± 44</td>
<td>82 ± 30/8</td>
<td>90 ± 0.42</td>
<td>0.042 ± 0.77</td>
<td>263 ± 31</td>
<td>80 ± 30</td>
</tr>
<tr>
<td>Early phenylephrine (early PE)</td>
<td>298 ± 34</td>
<td>82 ± 30/8</td>
<td>90 ± 0.42</td>
<td>0.042 ± 0.77</td>
<td>263 ± 31</td>
<td>80 ± 30</td>
</tr>
<tr>
<td>Late phenylephrine (late PE)</td>
<td>298 ± 34</td>
<td>82 ± 30/8</td>
<td>90 ± 0.42</td>
<td>0.042 ± 0.77</td>
<td>263 ± 31</td>
<td>80 ± 30</td>
</tr>
<tr>
<td>Early phenylephrine+ atractyloside (early PE+Atr)</td>
<td>298 ± 34</td>
<td>82 ± 30/8</td>
<td>90 ± 0.42</td>
<td>0.042 ± 0.77</td>
<td>263 ± 31</td>
<td>80 ± 30</td>
</tr>
<tr>
<td>Late phenylephrine+ atractyloside (late PE+Atr)</td>
<td>298 ± 34</td>
<td>82 ± 30/8</td>
<td>90 ± 0.42</td>
<td>0.042 ± 0.77</td>
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<td>80 ± 30</td>
</tr>
<tr>
<td>Atr (Atr)+</td>
<td>298 ± 34</td>
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<td>80 ± 30</td>
</tr>
</tbody>
</table>

- **HR (b.p.min)**: beats per minute
- **LVDP (mmHg)**: left ventricular developed pressure
- **CF (ml/min)**: coronary flow
- **LVDP % CF (ml/min)**: percentage of LVDP

**FIG. 2.** Recovery of RPP (rate pressure product, basal value %) in control (Con), ischemia/reperfusion (IR), early phenylephrine (early PE), late phenylephrine (late PE), early phenylephrine+ atractyloside (early PE+Atr), late phenylephrine+ atractyloside (late PE+Atr) groups. Data are presented as mean ± SEM.

- *(P < 0.001)*, significant difference with Con group.
- *(P < 0.001)*, significant difference with IR group.
- *(P < 0.001)*, significant difference with PE group.

versus 135 ± 15, respectively and 60 min (26.4 ± 14.5, 28.5 ± 3.3 versus 73 ± 14, respectively) of reperfusion. Addition of atractyloside restored the levels of CK-MB as seen in ischemia/reperfusion group in early and late phenylephrine-induced preconditioning either at 5 min (89 ± 12 and 100 ± 11) or 60 min (72 ± 6 and 75 ± 18) of reperfusion (Fig. 4). CK-MB level was not changed in atractyloside-IR group.

**DISCUSSION**

The present study indicates that administration of phenylephrine (as an α1-adrenoceptor agonist) exerts early and late cardioprotection against ischemia/reperfusion injuries by recovery of postischemic developed pressure, reduction in infarct size and CK-MB release from coronary effluent in isolated rat heart. Opening

**FIG. 3.** The ratio of area at risk to total left ventricular areas (AAR/LV%) and ratio of infarct size to area at risk (IS/AAR) in Control (Con), ischemia/reperfusion (IR), early phenylephrine (early PE), late phenylephrine (late PE), early phenylephrine+ atractyloside (early PE+Atr), late phenylephrine+ atractyloside (late PE+Atr) groups. Data are presented as mean ± SEM.

- *(P < 0.001)*, significant difference with Con group.
- *(P < 0.001)*, significant difference with IR group.
- *(P < 0.001)*, significant difference with PE group.
the mPTP by using a specific mPTP opener (attractyloside) eliminated this cardioprotective effect. Therefore, for the first time, this result suggests the involvement of mPTP in the mechanism of phenylephrine-induced early and late preconditioning.

It has been demonstrated that the mPTP, which remains closed during prolonged myocardial ischemia, opens in the early minutes of reperfusion [16]. Increasing evidence suggests that interventions that reduce lethal reperfusion injury are associated with a decrease in mPTP opening and, in this way, it has been shown that direct pharmacologic inhibition of the mPTP opening by cyclosporine A (as a mPTP inhibitor) inhibits cardioprotection obtained by phenylephrine in a specific mPTP opener.

It has been suggested that preconditioning inhibits mPTP opening through reduction in mitochondrial Ca\(^{2+}\) overload and activation of protein kinase C (PKC) and mitogen activated protein kinase (MAPK) [9]. On the other hand, it has been reported that attenuation of mitochondrial Ca\(^{2+}\) overload possibly underlies the basis of the cardioprotection due to \(\alpha_1\)-adrenoceptor activation [20], and also phenylephrine-induced preconditioning is mediated by PKC activation [21].

In an early study, we concluded the contribution of mitochondrial ATP-sensitive potassium channel (mK\(_{\text{ATP}}\)) to the protection of phenylephrine-induced early and late preconditioning in isolated rat heart [22]. An important relation has been proposed between opening of mK\(_{\text{ATP}}\) channels and mPTP inhibition [19]. It has been reported that diazoxide (as a selective mK\(_{\text{ATP}}\) opener) induces preconditioning via inhibition of mPTP [13]. In this way, it is guessed that opening of mK\(_{\text{ATP}}\) could lead to mPTP inhibition via reducing mitochondrial Ca\(^{2+}\) load, enhancing mitochondrial energy production, or releasing of ROS [13]. Therefore, in our study, it seems that stimulation of \(\alpha_1\)-adrenoceptor by phenylephrine could lead to the mPTP inhibition via opening of mK\(_{\text{ATP}}\).

As well, it has been demonstrated that there is a close relationship between activity of Bcl-2 in the outer mitochondrial membrane and mPTP inhibition during late ischemic preconditioning [17]. Bcl-2 exerts its cardioprotection via inhibition of mitochondrial cytochrome c release into the cytosol [23] and prevention of the mPTP opening [24]. On the other hand, stimulation of \(\alpha_1\)-adrenoceptor inhibits apoptosis by increasing the levels of the Bcl-2 as an antiapoptotic protein [6]. So, it is thought that inhibition of the mPTP due to stimulation of \(\alpha_1\)-adrenoceptor is mediated by facilitating the release of antiapoptotic proteins, including Bcl-2 and activation of mK\(_{\text{ATP}}\) channel.

In conclusion, this study is the first to show that phenylephrine can induce early and late preconditioning via inhibition of the mPTP opening in the isolated rat heart.

REFERENCES


