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Research paper

# Involvement of inducible nitric oxide synthase in the loss of cardioprotection by ischemic postconditioning in hypothyroid rats

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ABSTRACT

Cardioprotection by ischemic postconditioning (IPost) is negated in hypothyroidism; the underlying mechanisms however are unknown. This study aimed at determining whether changes in Bax, Bcl-2, eNOS, and iNOS gene expressions are involved in the negating effects of IPost against ischemia–reperfusion (IR) injury in hypothyroidism. The hearts from control and hypothyroid rats were perfused in Langendorff apparatus and exposed to 30 min ischemia, followed by 120 min reperfusion and IPost. In a subgroup of hypothyroid rats, ischemia duration was extended to 40 min. Hemodynamic parameters, infarct size, and gene expressions were measured. Compared to controls, hypothyroid rats with 30 min ischemia had higher recovery of post-ischemic LVDP and ±dp/dt, confirmed by decreased CK and LDH levels (187 ± 16 vs. 485 ± 41 and 191 ± 9 vs. 702 ± 48 U/L, respectively; $p < 0.05$), decreased infarct size (6.7 ± 1.1 vs. 46.1 ± 1.7%; $p < 0.05$), and a reduced DNA laddering pattern. Recovery of post-ischemic LVDP and ±dp/dt decreased and infarct size increased following extension of ischemia period in hypothyroid rats. IPost increased eNOS and Bcl-2 expression by 3.2-fold and 3.7-fold and decreased Bax and iNOS expression by 79% and 38%, respectively; it also reduced IR-induced DNA laddering pattern in controls, whereas no change was observed in hypothyroid rats, regardless of the ischemia period. In conclusion, hearts from hypothyroid rats were resistant to IR injury, partly due to the lower expression of iNOS and subsequent reduction in apoptosis after IR. In hypothyroid rats, IPost was not associated with further reduction in iNOS expression and failed to provide additional cardioprotection against ischemia.



## 1. Introduction

Ischemic heart disease is one of main causes of death worldwide (Go et al., 2013). Although reperfusion therapy can effectively rescue ischemic heart patients from expected death, it can however induce further injury, known as ischemia–reperfusion (IR) injury (Yin et al., 2012; Badalzadeh et al., 2014). Ischemic postconditioning (IPost) includes short episodes of IR that are applied before start of reperfusion and is a cardioprotective strategy against IR injury in normal conditions (Zhao et al., 2003). IPost reduces myocardial infarct size and preserves left ventricular systolic and diastolic functions partly due to increased endothelial nitric oxidase (eNOS) and decreased inducible NOS (iNOS) expressions, subsequently inhibiting myocardial apoptosis, as indicated by the decrease in the Bax-to-Bcl-2 ratio (Wang et al., 2006; Hausenloy, 2009; Inamura et al., 2010; Fan et al., 2011; He et al., 2011; Wei et al., 2014).

Experimental studies indicate that the cardioprotection provided by IPost is lost in pathologic states, including diabetes and metabolic syndrome (Badalzadeh et al., 2012; Wu et al., 2015b), findings supported by the results of a recent meta-analysis indicating that IPost following percutaneous coronary intervention is not associated with any reductions in infarct size or in clinical outcomes among patients with ST-elevation myocardial infarction (Khalili et al., 2014). It has been proposed that pathologic states associated with myocardial infarction can attenuate the protective effect of IPost, although the underlying mechanisms have not yet been elucidated (Ferdinandy et al., 2014).

We previously reported that IPost failed to provide cardioprotection against IR injury in both hypothyroid (Jeddi et al., 2015) and hyperthyroid (Zaman et al., 2014) rats; the underlying mechanism in hyperthyroid rats is, at least in part, due to the inability to prevent NO overproduction by iNOS (Zaman et al., 2015). The mechanisms underlying inability of IPost to provide cardioprotection against IR in hypothyroid rats have not been clearly elucidated; the aim of this study is

*Abbreviations:* IR, ischemia–reperfusion; IPost, ischemic postconditioning; LVDP, left ventricular developed pressure; +dP/dt, peak rates of positive; -dp/dt, negative changes in left ventricular pressure; LVEDP, left ventricular end diastolic pressure; CK, creatine kinase; LDH, lactate dehydrogenase; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase.

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therefore to determine whether changes in the expression of Bax, Bcl-2, eNOS, and iNOS genes are involved in negating cardioprotective effects of IPost against IR injury in hypothyroid rats.

## 2. Materials and methods

### 2.1. Animals

In this study, 56 male Wistar rats (2 months old, weight 200–250 g), obtained from the laboratory animal house of the Research Institute for Endocrine Sciences (RIES), were housed in an animal room with a temperature of 22 ± 2 °C, relative humidity 50 ± 6%, and given free access to standard rat chow (Pars Co., Tehran) and tap water during the study. The rats were adapted to an inverse 12:12 h light/dark cycle. This study was performed in accordance with guidelines for the care and use of laboratory animals verified by the RIES ethics committee. All experimental procedures employed, as well as rat care and handling, were in accordance with guidelines provided by the local ethics committee of RIES, Shahid Beheshti University of Medical Sciences.

### 2.2. Induction of hypothyroidism

Hypothyroidism was induced by administration of propylthiouracil (500 mg/L) in drinking water for 21 days. At the end of the treatment phase, serum total $T_4$ (thyroxine) and $T_3$ (triiodothyronine) were measured using commercial Elisa kits (Diaplus Inc., USA), and thyroid-stimulating hormone (TSH) was measured using commercial Elisa kits (Demeditec, Germany) for determining the efficiency of treatment with propylthiouracil.

### 2.3. Groups of the study

Control rats were randomly divided into 3 subgroups ($n = 7$ each): control (C), control-IR (C-IR), and control-IPost (C-IPost); hypothyroid rats were also divided into 3 subgroups ($n = 7$ each): hypothyroid (H), hypothyroid-IR (H-IR), and hypothyroid- IPost (H-IPost). Since hearts from hypothyroid rats were protected against IR injury, which could mask the cardioprotective effects of IPost, we also added two subgroups of hypothyroid rats in which ischemia duration (30 min in other groups) was extended to 40 min [hypothyroid-IR-40 min (H-IR40) and hypothyroid-IR-40 min-IPost (H-IPost40)].

### 2.4. Surgical preparation

All rats were anesthetized with an intraperitoneal injection of ketamine and xylazine (50 mg/kg and 10 mg/kg). The hearts of control and hypothyroid rats were rapidly excised and immersed in an ice-cold perfusion buffer; aortas were then cannulated, and the hearts were fixed on a constant-pressure mode of the Langendorff perfusion apparatus and perfused through the aorta with a Krebs–Henseleit solution (pH 7.4) containing (mmol/L): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $KH_2PO_4$ 1.2, and glucose 11; perfusion pressure of solution was adjusted constantly at 75 mmHg and Krebs solution was gassed with a mixture of 95% $O_2$ and 5% $CO_2$ at 37 °C.

### 2.5. Measurement of hemodynamic parameters

Isolated hearts were subjected to 20 min of stabilization to obtain baseline values; subsequently, in the C-IR and H-IR groups, hearts were exposed to a 30 min of global ischemia and 120 min of reperfusion. In the IPost groups (C-IPost, H-IPost, and H-IPost40), hearts were subjected to IPost before the start of reperfusion. IPost was induced by 6 cycles of 10-s reperfusion–10-s ischemia immediately following the 30 and 40 min global ischemia. A latex balloon was inserted to the left ventricle to allow measurement of hemodynamic parameters, including left ventricular developed pressure (LVDP), the peak rates of positive (+dP/dt) and negative (−dp/dt) changes in left ventricular pressure, and the left ventricular end diastolic pressure (LVEDP). Initially, average LVEDP was adjusted at 5–10 mmHg in all hearts by filling water in the latex balloon; hemodynamic parameters (LVEDP, LVDP, and ±dp/dt) were digitalized by a data acquisition system (Power Lab, AD instrument, Australia). Post-ischemic hemodynamic parameters were assessed by the recovery of LVEDP, LVDP, and ±dp/dt.

### 2.6. Measurement of creatine kinase (CK) and lactate dehydrogenase (LDH) activities

Samples of coronary flow were collected within 5–10 min after start of reperfusion to measure the myocardial enzyme leakage (i.e., CK-MB and LDH); CK-MB and LDH levels in the coronary flow were determined by the spectrophotometric method using CK-MB and LDH kits (Pars Azmoon, Iran), and the results are expressed as U/L (Amani et al., 2013).

### 2.7. Measurement of infarct size

At the end of the reperfusion period, infarct sizes were determined as described previously (Ghanbari et al., 2015; Zaman et al., 2015). In brief, the frozen heart samples were cut into thin slices and were incubated for 10 min in 1% of 2, 3, 5-triphenyltetrazolium chloride in phosphate buffer solution (20 mM, pH 7.4) at 37 °C. The slices were immersed in formalin (10%) for 24 h to identify viable myocardium discriminable from unstained necrotic tissue. The sections were then photographed using a digital camera (Samsung, Japan, version DV101). Infarct sizes were measured by Photoshop CS6 software (version 13) and have been expressed as percentage of the total area.

### 2.8. Measurement of nitrate + nitrite (NOx) content

Heart NOx were measured by the Griess method. In brief, samples from the left ventricle of hearts were rinsed, homogenized in PBS (1:5, *w/v*), and centrifuged for 20 min at 15,000*g*. Samples were deproteinized by adding 15 mg/ml zinc sulfate, 100 μL of the supernatant was transferred to a microplate well, and 100 μL vanadium (III) chloride (8 mg/ml) was added to each well to reduce nitrate to nitrite; 50 μL sulfanilamide (2%) and 50 μL N-1-(naphthyl)ethylenediamine (0.1%) were then added and samples were incubated for 30 min at 37 °C; absorbance was read at 540 nm using the ELISA reader (BioTek, Powerwave XS2). NOx concentrations were determined from the linear standard curve established by 0–100 μM sodium nitrate. Tissue NOx levels were expressed as μmol/L.

### 2.9. DNA fragmentation analysis

Frozen left ventricle tissue samples (20–30 mg each) were homogenated in 600 μL of lysis buffer, including the following: Tris–HCl (10 mmol/L), NaCl (150 mmol/L), EDTA (10 mmol/L), and 1% SDS, pH 8.0. The homogenates were then treated with 100 μg/mL proteinase K (Sigma-Aldrich) for 3–4 h at 56 °C. After incubation, the homogenates were precipitated and centrifuged at 16,000*g* for 5 min. The supernatant was removed, and the DNA was extracted with the phenol and chloroform method. After centrifugation at 16,000*g* for 10 min, supernatants containing DNA were precipitated with isopropanol; after re-centrifugation at 16,000*g* for 10 min, the DNA pellets were rinsed with 75% ethanol and again centrifuged at 12,000*g* for 5 min. The DNA pellet was re-suspended in Tris–EDTA buffer containing Tris–HCl 10 mmol/L, EDTA 1 mmol/L, and pH 8.0; 10 μg of the extracted DNA was loaded on 1.5% agarose gel containing Gel Red, and DNA electrophoresis was performed at 80 V for 1 to 2 h. The DNA ladders, as indicator of heart tissue apoptosis, were visualized under UV light and then photographed from gels (Dong et al., 2003).