Effects of Ischemic Postconditioning on the Hemodynamic Parameters and Heart Nitric Oxide Levels of Hypothyroid Rats

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Abstract

Background: Ischemic postconditioning (IPost) is a method of protecting the heart against ischemia-reperfusion (IR) injury. However, the effectiveness of IPost in cases of ischemic heart disease accompanied by co-morbidities such as hypothyroidism remains unclear.

Objective: The aim of this study was to determine the effect of IPost on myocardial IR injury in hypothyroid male rats.

Methods: Propylthiouracil in drinking water (500 mg/L) was administered to male rats for 21 days to induce hypothyroidism. The hearts from control and hypothyroid rats were perfused in a Langendorff apparatus and exposed to 30 min of global ischemia, followed by 120 min of reperfusion. IPost was induced immediately following ischemia.

Results: Hypothyroidism and IPost significantly improved the left ventricular developed pressure (LVDP) and peak rates of positive and negative changes in left ventricular pressure (±dp/dt) during reperfusion in control rats (p < 0.05). However, IPost had no add-on effect on the recovery of LVDP and ±dp/dt in hypothyroid rats. Furthermore, hypothyroidism significantly decreased the basal NO metabolite (NOx) levels of the serum (72.5 ± 4.2 vs. 102.8 ± 3.7 µmol/L; p < 0.05) and heart (7.9 ± 1.6 vs. 18.8 ± 3.2 µmol/L; p < 0.05). Heart NOx concentration in the hypothyroid groups did not change after IR and IPost, whereas these were significantly (p < 0.05) higher and lower after IR and IPost, respectively, in the control groups.

Conclusions: Hypothyroidism protects the heart from IR injury, which may be due to a decrease in basal nitric oxide (NO) levels in the serum and heart and a decrease in NO after IR. IPost did not decrease the NO level and did not provide further cardioprotection in the hypothyroid group. (Arq Bras Cardiol. 2014; [online]. ahead print, pp. 0-0)

Keywords: Ischemic Postconditioning; Reperfusion; Hypothyroidism; Nitric Oxide; Rats.

Introduction

Acute myocardial infarction (AMI) is the principle cause of human mortality worldwide1,2, and its prevalence is increasing because of aging and co-morbid diseases such as obesity, diabetes, and thyroid disorders1-3. AMI is often induced by the partial occlusion of coronary arteries at the site of a ruptured atherosclerotic plaque1,4. Although reperfusion can rescue the ischemic myocardium from unavoidable death, it can also induce side effects, known as ischemia-reperfusion (IR) injuries4. The myocardial response to ischemia can be modulated by different interventions such as ischemic postconditioning (IPost)3,5. IPost is an effective mechanism of protecting the myocardium from IR injuries and is induced by cycles of brief IR periods that are immediately performed upon initiation of reperfusion after prolonged coronary artery occlusion1-4. Although IPost has various clinical applications, most investigations currently conducted on IPost involve healthy myocardium. Coronary artery disease frequently co-exists with other morbidities5; therefore, further research on the pathological condition before the use of the IPost in clinical conditions is necessary5.

Hypothyroidism is a major thyroid gland disease that also affects the heart and has been implicated in an increase in morbidity6. Low thyroid hormone levels could affect the response of the heart to IR injuries4. It is therefore essential to determine the clinical utility of cardioprotective interventions such as IPost in hypothyroid states to design future management strategies.

Nitric oxide (NO) is mainly synthesized by NO synthase enzymes in the heart and plays an important role in cardiac functions. Ischemia of the heart leads to an increase in NO production that might contribute to IR injury. However, no studies have examined the changes in NO content in the hearts of hypothyroid rats. Therefore, the aim of this study was to determine the response of the heart to IPost in a propylthiouracil-induced hypothyroidism rat model. In addition, changes in NO metabolites (NOx) following IR injury and IPost were also assessed.
Methods

Animals

Forty-eight 2-month-old male Wistar rats were obtained from the Laboratory Animal House of the Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences. We set a 2-sided $\alpha$ of 0.05 and a power of 90%, and the sample size of each group was calculated to be 8 using the formula:

$$n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2 \times (s_1^2 + s_2^2)}{(d^2)}$$

where $\mu_1, \mu_2, s_1^2$ and $s_2^2$ are the means and variances of the two groups, respectively, and $d^2 = (\mu_1 - \mu_2)^2$. Rats were housed in an animal room with a temperature of 22 ± 3°C and a relative humidity of 50 ± 6% and given free access to standard rat chow (Pars Co., Tehran) and tap water during the study. The animals were adapted to an inverse 12:12 h light/dark cycle. All experimental procedures employed, as well as rat care and handling, were in accordance with guidelines provided by the local ethics committee of the Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences. Hypothyroidism was induced by adding propylthiouracil (PTU) (500 mg/L) to the drinking water for 21 days. To determine the efficacy of the PTU treatment, changes in serum total T4, T3, and TSH levels and citrate synthase (CS) activity in the soleus muscle (Srere et al) were assessed in two study groups (control and hypothyroid). After the establishment of the hypothyroid model, animals in the control and hypothyroid groups were further divided into three subgroups [i.e., control, control-IR (C-IR), and control-IPost (C-IPost), and hypothyroid, hypothyroid-IR (H-IR), and hypothyroid-IPost (H-IPost)] for in vitro experiments. Serum total T4, total T3, and TSH levels were measured at end of the treatment period using enzyme-linked immunosorbent assay (ELISA) kits. Inter-assay coefficients of variation were 3.2% for total T4, total T3, and TSH levels.

Experimental protocol

All rats were anesthetized by intraperitoneal injection of ketamine/xylazine (50 mg/kg and 10 mg/kg, respectively). The hearts of the control and hypothyroid rats were immediately isolated and placed in an ice-cold perfusion buffer; after aorta cannulation using Krebs–Henseleit buffer (containing 118 mM NaCl, 25 mM NaHCO$_3$, 4.7 mM KCl, 1.2 mM MgCl$_2$, 2.5 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, and 11 mM glucose at a constant pressure (75 mmHg) and a pH level of 7.4), and the Krebs solution was oxygenated with 95% O$_2$ and 5% CO$_2$. The hearts were stabilized for 20 min to obtain the baseline data. In the C-IR and H-IR groups, after 20 min of stabilization, the hearts were subjected to 30 min of global ischemia, followed by 120 min of reperfusion. IPost was induced by 6 cycles of 10-s reperfusion-10-s ischemia immediately following the 30-min global ischemia; a latex balloon was inserted into the left ventricle to measure various hemodynamic parameters, including left ventricular developed pressure (LVDP) as an index of systolic function, the peak rates of positive changes in the left ventricular pressure (+dp/dt) as an index of contraction, negative changes in the left ventricular pressure (−dp/dt) as an index of relaxation, and left ventricular end diastolic pressure (LVEDP) as an index of contracture. Initially, the average LVEDP was adjusted to 5–10 mmHg in all hearts by filling the latex balloon with water; LVDP, LVEDP, and ±dp/dt were digitalized by a data acquisition system (PowerLab, AD Instruments, Australia). Post-ischemic hemodynamic parameters were assessed by the recovery of LVDP, LVEDP, and ±dp/dt and expressed in relation to their baseline values.

Measurement of NOx

Serum and heart NOx were measured using the Griess method. Briefly, after 2 h of reperfusion, myocardial samples from the left ventricle (LV) of the hearts were rinsed, homogenized in PBS (1:5, w/v), and centrifuged at 15,000 g for 20 min. The supernatant was deproteinized by adding zinc sulfate (15 mg/mL). The serum samples were also deproteinized by zinc sulfate (15 mg/mL) and centrifuged at 10,000 g for 10 min. A 100-μL aliquot of the supernatant or serum sample was transferred to a microplate well and 100 μL of vanadium (III) chloride (8 mg/mL) was added to each well to reduce nitrate to nitrite. Afterwards, 50 μL of sulfanilamide (2%) and 50 μL of N-(naphthyl) ethylenediamine (0.1%) were added to the samples and incubated for 30 min at 37°C; absorbance was read at a wavelength of 540 nm using an ELISA reader (BioTek, Powerwave XS2). NOx concentrations were determined from the linear standard curve established using 0–100 μM sodium nitrate. Serum and tissue NOx levels were expressed as μmol/L. Inter-assay coefficient of variation was 4.1%.

Statistical analysis

All values were expressed as the mean ± SEM. Statistical analysis was performed using the SPSS software (SPSS, Chicago, IL, USA; version 20); repeated measurement ANOVA was used to compare hemodynamic parameters at various time points. One-way ANOVA with Tukey’s post-hoc test was used to compare heart NOx levels among different groups. The student’s sample t-test was used to compare serum NOx levels, T3, T4, TSH, and CS activity between control and hypothyroid groups. Two-sided p-values < 0.05 were considered statistically significant.

Results

Serum thyroid hormone levels and soleus muscle CS activity significantly decreased, whereas serum TSH significantly increased in hypothyroid rats. In addition, weight changes were significantly lower in hypothyroid rats (Table 1).

Basal hemodynamic parameters were significantly lower in the hypothyroid group than in the controls (Table 2). When ischemia was induced by pausing coronary perfusion, the LVDP, ±dp/dt, and heart rate rapidly decreased and ceased in the isolated hearts.
The hearts from the H-IR group showed significant recovery in post-ischemic LVDP and ± dp/dt after 30 min of ischemia and 120 min of reperfusion compared to those from the C-IR group. IPost significantly improved the LVDP and ± dp/dt during reperfusion in the C-IPost group. In contrast, IPost failed to increase recovery of LVDP and ± dp/dt in the H-IPost group (Figure 1). During the 30-min ischemia, the hypothyroid group displayed a significant decrease in LVEDP, compared to the controls. IPost significantly prevented the reperfusion-induced increase in LVEDP in the C-IPost group (Figure 2).

Serum and heart NOx levels were significantly lower in the hypothyroid group, compared to the control. IR and IPost had no effect on heart NOx levels in the hypothyroid group, whereas in the control group, IR induced a marked increase in heart NOx levels and IPost significantly decreased the IR-induced increase in heart NOx levels (Figure 3).

### Discussion

Our findings indicate that hypothyroidism decreases injuries induced by IR in the rat heart, which may be due to the reduction in NO. IPost provides protection against IR injury in control rats, whereas no add-on effect was observed in hypothyroid rats.

The decrease in weight change and CS activity in the soleus muscle and the decrease in the level of circulating thyroid hormone with elevated TSH levels all indicate that hypothyroidism has been successfully induced.

Baseline LVDP, LVDP, and ± dp/dt were lower in the hypothyroid groups, compared to the controls, and these results were similar to those of previous studies. Several changes, including upregulation of phospholamban and V3 isomyosin and downregulation of SR Ca\(^{2+}\)-ATPase and ryanodine receptor occur in the heart of hypothyroid rats; therefore, cardiac dysfunction is a frequent consequence of hypothyroidism. In response to IR, the hypothyroid group showed an increased recovery of LVDP, LVDP and ± dp/dt, which indicates that hypothyroidism can protect the heart from IR injury. These findings are supported by previous studies.

The protective mechanisms against IR in the hearts of the hypothyroid group have not been entirely elucidated and might be due to changes in metabolism. Previous studies have shown that in hypothyroid rats, ATP, oxygen, and glycogen levels slowly decrease during ischemia and are higher at the start of reperfusion. On the other hand, the hearts of hyperthyroid rats, despite having a high metabolism, were protected from IR injury; therefore, tolerance of the hypothyroid group to IR injury cannot be solely explained by the low metabolism during ischemia.

Different molecular pathways, including total c-jun NH\(_2\) terminal kinases, mitogen-activated protein kinases, and NO play an essential role in the response of the heart to IR injury. Our results showed that the baseline serum and heart levels of NOx were lower in the hypothyroid groups; NOx levels increased after IR in control group, whereas no change was observed in hypothyroid group. A decrease in heart NOx levels in lethal

### Table 1 – Characteristics of control and hypothyroid rats

|                     | Controls (n = 8) | Hypothyroid rats (n = 8) |
|---------------------|-----------------|-------------------------|
| Weight change (g)   | 20.3 ± 3.2      | 8.1 ± 3.5*              |
| T3 (nmol/L)         | 0.76 ± 0.06     | 0.20 ± 0.04*            |
| T4 (nmol/L)         | 49.43 ± 2.34    | 17.65 ± 3.42*           |
| TSH (ng/mL)         | 6.8 ± 0.6       | 29.62 ± 3.7             |
| Citrate synthase activity (µmol/mL/min) | 1.2 ± 0.3 | 0.45 ± 0.01* |

Data are expressed as mean ± SEM.; * p < 0.05.

### Table 2 – Baseline cardiac function

|                     | Controls (n = 8) | Hypothyroid rats (n = 8) |
|---------------------|-----------------|-------------------------|
| LVEDP (mmHg)        | 8.5 ± 2.2       | 8.8 ± 2.8               |
| LVDP (mmHg)         | 96.8 ± 7.6      | 74.0 ± 6.3              |
| +dp/dt (mmHg/s)     | 3135 ± 211      | 2352 ± 434*             |
| –dp/dt (mmHg/s)     | 2215 ± 185      | 1684 ± 124*             |
| Heart rate (pulse/min) | 283.3 ± 10.4 | 170.2 ± 11.3* |

Data are expressed as the mean ± SEM. LVEDP: Left ventricular end diastolic pressure; LVDP: Left ventricular developed pressure; and the peak rates of positive and negative changes in left ventricular pressure (±dp/dt); *p < 0.05.
Figure 1 – Recovery of cardiac function after IR injury: A. Left ventricular developed pressure (LVDP); B. Peak rates of positive changes in left ventricular pressure (+dp/dt); C. Peak rates of negative changes in left ventricular pressure (−dp/dt); D. Heart rate; Control-IR, C-IR; Control-IPost, C-IPost; Hypothyroid-IR, H-IR; Hypothyroid-IPost, H-IPost; values are expressed as the mean ± SEM; (n = 8 rats); *p < 0.05 as compared to the C-IR group.
hypothyroid rats has been previously reported\(^\text{17}\), suggesting that low levels of NOx during the experimental period might be a significant element of hypothyroid-induced cardioprotection.

The function of NO in myocardial IR injury has not been clearly demonstrated and is a very complex issue. Some studies have described the protective role of NO, whereas others have reported a detrimental role\(^\text{18-20}\). Recent experimental studies have indicated that the NO level of heart tissues is within a low range at baseline and increases during ischemia because it triggers the enzymatic (through NO synthase 3) and non-enzymatic (tissue acidosis) production of NO. Although a small increase in NO content may be cardioprotective, a large increase appears to be detrimental\(^\text{18,21-23}\); the detrimental effect of NO in the heart in response to IR is mediated by peroxynitrite\(^\text{18}\). At high levels, NO reacts with superoxide and produces peroxynitrite, which is a highly toxic agent that could induce apoptosis in heart cells. Thus, it could be hypothesized that hypothyroidism protects the heart from ischemia by decreasing NO production and subsequently reducing the levels of nitro-oxidative stress\(^\text{18,20,24-28}\).

In the present study, IPost protected the heart against IR injury in the C-IPost group through the recovery of LVEDP, LVDP and ± dp/dt, whereas no significant effects on the hearts in the H-IPost group were observed, indicating that IPost might have lost its efficacy in a hypothyroid group. Our findings were similar to the results of previous studies on diabetic rats, which show that preconditioning and IPost lose their protective effects against IR injuries in the hearts of diabetic rats\(^\text{4,29}\).

The mechanism behind the effect of IPost in the unhealthy myocardium has not been established. Several studies have indicated that IPost and preconditioning protect the hearts of healthy rats from IR injury by decreasing its NO content during ischemia\(^\text{18,24,30,31}\). Similarly, in the present study, the application of six cycles of IPost significantly decreased the heart NOx levels after a 30-min ischemia in the C-Ipost group. However, this reduction was not observed in the H-IPost group, and the application of Ipost did not result in an additional decrease in the NOx level in the hearts of hypothyroid group. These results demonstrate the inefficiency of IPost in providing additional protective effects against IR injury in the hypothyroid group. Both IPost and hypothyroidism reduced the levels of NOx in response to IR and protected the heart from IR injury, indicating that NOx is a critical component of the protection response.

With regard to the limitations of this study, we did not measure NO synthase activity, which might play a role in the cardioprotective effects of IPost on hypothyroid rats\(^\text{16,32}\). In addition, our results were limited to male rats, whereas hypothyroidism is more prevalent among females\(^\text{33}\).
Conclusion

Hypothyroidism increased the recovery of LVEDP, LVDP, and ± dp/dt following IR in the rat heart, which might be due to a decrease in the basal levels of NOx and NOx after the IR period. Although IPost imparted protective effects, these were not related to further decreases in NOx levels. The abolished protective effect of IPost in the hypothyroid group could be attributable to the impairment of the NO pathway.

Author contributions

Conception and design of the research: Jeddi S, Ghasemi A; Acquisition of data: Jeddi S, Zaman J; Analysis and interpretation of the data: Jeddi S; Statistical analysis and Writing of the manuscript: Jeddi S, Zaman J, Ghasemi A; Obtaining financing: Ghasemi A; Critical revision of the manuscript for intellectual content: Ghasemi A.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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Figure 3 – Change in NOx levels in the control and hypothyroidism groups in the heart (above) and serum (below). Control-IR, C-IR; Control-IPost, C-IPost; Hypothyroid-IR, H-IR; Hypothyroid-IPost, H-IPost; values are expressed as the mean ± SEM; *(n = 8 rats); *p < 0.05 as compared to the control group. #p < 0.05 as compared to the C-IR group.

Heart NOx (µmol/L)

Control C-IR C-Ipost Hypothyroid H-IR H-Ipost

Serum NOx (µmol/L)

Control Hypothyroid
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