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T3 and T4 decrease ROS levels and increase endothelial nitric oxide synthase expression in the myocardium of infarcted rats

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Abstract Myocardial infarction leads to a reduction in nitric oxide (NO) bioavailability and an increase in reactive oxygen species (ROS) levels. This scenario has been shown to be detrimental to the heart. Recent studies have shown that thyroid hormone (TH) administration presents positive effects after ischaemic injury. Based on this, the aim of this study was to evaluate the effect of TH on NO bioavailability as well as on endothelial nitric oxide synthase (eNOS) expression after myocardial infarction. Male Wistar rats were divided into three groups: Sham-operated (SHAM), infarcted (AMI) and infarcted + TH (AMIT). During 26 days, the AMIT group received T3 and T4 (2 and 8 μg/100 g/day, respectively) by gavage, while SHAM and AMI rats received saline. After this, the rats underwent echocardiographic analysis were sacrificed, and the left ventricle was collected for biochemical and molecular analysis. Statistical analysis: one-way ANOVA with Student–Newman–Keuls post test. AMI rats presented a 38 % increase in ROS levels. TH administration prevented these alterations in AMIT rats. The AMIT group presented an increase in eNOS expression, in NOS activity and in nitrite levels. TH administration also increased PGC-1α expression in the AMIT group. In conclusion, TH effects seem to involve a modulation of eNOS expression and an improvement in NO bioavailability in the infarcted heart.

Keywords Heart failure · Thyroid hormones · Oxidative stress · Nitric oxide

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Introduction

Myocardial infarction results in the loss of viable cardiomyocytes and the development of heart failure, which is a major cause of mortality and morbidity [1]. Much evidence suggests the involvement of reactive oxygen species (ROS) in the impairment of cardiac function found in the post-infarction period [2, 3]. High levels of ROS are correlated with dilatation of the cardiac chambers and with an increase in left ventricle end-diastolic pressure after ischaemic injury [4]. Two important antioxidant defences against ROS in the heart are the thioredoxin (TRX) and glutaredoxin (GRX) systems [5]. In the cells, TRX can act as an antioxidant enzyme, donating hydrogen to oxidized proteins. In this process, this enzyme becomes oxidized and in turn can be regenerated by TRX reductase [5]. In infarcted mice, the exogenous administration of TRX1 was shown to reduce cardiomyocyte apoptosis [6]. GRX, in turn, can catalyse the reduction of mixed disulphides, regenerating the activities of important cellular molecules [5, 7, 8].

Myocardial infarction has also been associated with a decrease in nitric oxide (NO) bioavailability, which can be evaluated by the balance between ROS and NO levels [9, 10]. In fact, high levels of ROS can cause an increase in the ROS/NO balance which seems to be detrimental to the heart [9]. In the cardiac tissue, NO is mainly produced by endothelial nitric oxide synthase (eNOS) which is an important enzyme in terms of cardioprotection [11]. One study showed that

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transplantation of adipose tissue-derived stem cells embedded with eNOS was capable of restoring NO bioavailability in the heart [12]. eNOS-derived NO can cause beneficial effects such as an increase in guanosine 3,5-monophosphate (cGMP)-dependent expression of peroxisome proliferator-activated receptor gamma (PPAR gamma) co-activator 1a (PGC-1α) [13]. This transcriptional factor is a strong promoter of mitochondrial biogenesis and is related with positive effects on cardiac cell metabolism [14].

Several studies have evaluated the use of thyroid hormones as a therapeutic tool for the treatment of complications of acute myocardial infarction [14–16]. In an experimental model of infarction, thyroid hormone administration was capable of promoting favourable ventricle remodelling and decreasing cardiac wall stress [15]. Recently, the effects of T3 and T4 after myocardial infarction were associated with an improvement in the redox status of the myocardium [17].

Despite the fact that the ROS/NO balance seems to be relevant for the infarcted heart [9–12], there are no studies evaluating the effects of T3 and T4 in this balance, as well as on eNOS expression, after myocardial infarction. Based on this, the aim of this study was to investigate the effects of thyroid hormones in the ROS/NO balance as well as on eNOS expression after myocardial infarction.

Materials and methods

Ethical approval

The study and animal care procedures were approved by the Ethics Committee for animal research at this University (Universidade Federal do Rio Grande do Sul—UFRGS; process number 23262).

Animals

Male Wistar rats (354 ± 47 g) were obtained from the Central Animal House of the Universidade Federal do Rio Grande do Sul, Brazil. The animals were housed in plastic cages and received water and pelleted food ad libitum. They were maintained under standard laboratory conditions (controlled temperature of 21 °C, 12 h light/dark cycle) and divided into 3 groups (5–7 animals per group): (SHAM), infarcted (AMI) and infarcted plus thyroid hormone administration (AMIT).

Surgical procedure for myocardial infarction

Rats were anesthetized with ketamine (90 mg kg^{-1}, i.p.) and xylazine (20 mg kg^{-1}, i.p.), and myocardial infarction was produced by a method similar to one previously described [18]. Rats were submitted to a surgical ligature of the descending anterior branches of the left coronary artery, or to a sham-operation. The mortality of infarcted rats was approximately 40 %.

T3 and T4 administration

Rats were allowed to recover for 48 h (2 days) after the surgery. After this period, the AMIT group received T3 (2 μg/100 g/day) and T4 (8 μg/100 g/day), diluted in saline by gavage, while the SHAM and AMI groups received just saline. The period of hormonal administration was 26 days after the recovery period.

Measurement of T3 and T4 in plasma

Anesthetized rats were submitted to blood collection from the retro-orbital plexus 28 days after the surgery. The blood was centrifuged at 1000×*g* for 10 min. Quantitative plasma T3 and T4 measurements were performed by chemiluminescence using ROCHE cobas e-411 analyser kits at TECSA Veterinary Laboratory.

Morphometric analysis

Twenty-eight days after the surgery, anesthetized rats were killed by cervical dislocation. The heart was rapidly excised and the scar area was removed and discarded. The left ventricle (LV) was separated, weighed and immediately frozen in liquid nitrogen. This tissue was used for biochemical and Western blot analysis. LV hypertrophy was evaluated by the LV weight (in mg) to body weight (in g) ratio and the LV weight (in mg) to tibia length (in cm) ratio [19].

Echocardiographic analysis of infarction size

Rats were submitted to echocardiographic analysis 28 days after the surgery. Rats were anesthetized and placed in the left lateral decubitus position (45°) to obtain cardiac images. A Philips HD7 XE ultrasound system with a L2-13 MHz transducer was used. On each echocardiographic transverse plane, basal, middle and apical, the arch corresponding to the segments with infarction (I) and the total endocardial perimeter (EP) were measured at end-diastole. Infarction size (IS) was estimated as % IS = (I/EP) × 100 [20, 21]. LV systolic and diastolic diameters (cm) and systolic posterior wall thickness (cm) were measured using the M-Mode in three planes: basal, middle and apical. A final value for each animal was obtained by taking the average of all three planes [20, 21]. The wall tension index was evaluated as previously described [15, 16].

Tissue preparation

The LV was homogenized (1.15 % w/v KCl and phenyl methyl sulfonyl fluoride PMSF 20 mmol/L) in Ultra-Turrax. The suspension was centrifuged at $1000 \times g$ for 10 min at 4 °C to remove nuclei and cell debris, and the supernatants were used for biochemical measurements [22].

Determination of ROS levels

ROS generation was measured by 2′,7′-dichlorofluorescin diacetate (DCFH-DA) fluorescence emission (Sigma-Aldrich, USA). DCFH-DA is membrane permeable and is rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of intracellular ROS. The samples were excited at 488 nm; emission was collected with a 525-nm-long pass filter and expressed as nmols per milligram of protein [23].

GRX activity

GRX activity was assayed through the protocol established by Holmgren and Åslund [24]. The amount of GSSG produced was measured spectrophotometrically at 340 nm after the addition of glutathione reductase and NADPH. It was expressed as nmols per milligram of protein.

TRX reductase activity

TRX reductase activity was assayed by an in vitro reduction of dithionitrobenzoic acid (DTNB) to 50-thionitrobenzoic acid (TNB). The conversion of DTNB to TNB was measured spectrophotometrically at 412 nm. It was expressed as units per minute per milligram of protein [25].

Nitric oxide synthase (NOS) activity

NOS activity was assessed by measuring the NO-induced conversion of oxyhaemoglobin to methaemoglobin, as previously described [26]. The reaction medium was composed of mmol/L: $CaCl_2$ 1.8, KCl 2.7, $MgCl_2$ 0.23, NaCl 137, NaH_2PO_4 3.6, glucose 5.0, HEPES 10, pH 7.4, containing 2 µmol/L HbO_2 and 1 mmol/L L-arginine. It was expressed as nmols of NO per minute per milligram of protein.

Determination of nitrites

Nitrites (NO_2^-) were determined using the Griess reagent, in which a chromophore with a strong absorbance at 540 nm is formed by the reaction of nitrite with a mixture of naphthylethylenediamine (0.1 %) and sulphanilamide (1 %). The results were expressed as mmol/L per milligram of protein [27].

Determination of protein concentration

Protein was measured through the method of Lowry [28], using bovine serum albumin as standard.

Western blot analysis

Tissue homogenization, electrophoresis and protein transfer were performed as previously described [29]. Forty micrograms of protein were subjected to one-dimensional sodium docedyl sulphate–polyacrylamide gel electrophoresis in a discontinuous system using an 8–12 % (w/v) separating gel and stacking gel [29]. The immunodetection was processed in PVDF membranes using the following primary antibodies: GRX3 (37 kDa), TRX1 (12 kDa), eNOS (150 kDa) and PGC-1α (90 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA or Cell Signalling Technology, Beverly, MA). The bound primary antibodies were detected using anti-rabbit or anti-mouse horseradish peroxidase-conjugate secondary antibodies. The membranes were developed using chemiluminescence detection reagents and the autoradiographs generated were quantitatively measured with an image densitometer (Image-master VDS CI, Amersham Biosciences Europe, IT). The molecular weights of the bands were determined by reference to a standard molecular weight marker (RPN 800 rainbow full range Bio-Rad, CA, USA). The results were normalized by the Ponceau method [30].

Statistical analysis

Data were expressed as mean ± SEM. One-way ANOVA with Student-Newmann-Keuls post hoc test was used to compare multiple groups. Student's t test was used to compare the infarction size between the AMI and AMIT groups. The correlation between two variables was analysed by Pearson's correlation. Values of $P < 0.05$ were considered statistically significant.

Results

T3 and T4 levels in plasma

T3 and T4 levels are shown in Table 1. After 26 days of hormonal administration, T3 levels were significantly increased in the AMIT group when compared with AMI and SHAM. There was no difference in T3 levels between AMI and SHAM rats. The levels of T4 were not different among groups.

Morphometric and echocardiographic data

Body weight was no different between the groups. The LV weight was increased 21 and 44 % in AMI and AMIT rats, respectively, when compared with SHAM animals. AMIT rats presented the highest value of this parameter (Table 1). LV hypertrophy indicators (LV weight to body weight and LV weight to tibia length ratios) were increased in both infarcted groups when compared with SHAM. In AMIT rats, these parameters were augmented as compared to both the AMI and SHAM groups. LV diastolic and systolic diameters were also evaluated. These parameters were increased in the infarcted groups when compared with the SHAM group. There were no differences between AMI and AMIT rats in relation to these parameters. In terms of LV systolic posterior wall thickness, the AMI rats presented a decrease in these parameters when compared with SHAM rats. In the AMIT group, the hormonal treatment prevented this decrease. The wall tension index, which is indicative of cardiac wall stress, was also evaluated. This parameter was increased in the AMI rats when compared with the SHAM animals. T3 and T4 administration promoted a reduction of 15 % in this parameter in AMIT as compared to AMI rats. There was no significant difference in infarction size between the AMI and AMIT groups (Table 1).

Biochemical and Western blot data

AMI rats presented an increase of 38 % in ROS levels when compared with the SHAM group. Thyroid hormone administration prevented this increase in AMIT rats. In this group, the levels of ROS were no different from those in SHAM animals (Fig. 1). The levels of ROS were positively correlated with the wall tension index ($r = 0.51$, $P = 0.027$). T3 and T4 administration significantly increased eNOS expression and NOS activity in AMIT rats when compared with AMI and SHAM. There was no difference in these parameters between AMI and SHAM animals (Fig. 2a, b). The levels of nitrites, which are NO metabolites, were also increased in AMIT animals when compared with AMI and SHAM. There was no difference in these levels between the two non-treated groups (Fig. 2c). The levels of nitrites were positively correlated with NOS activity ($r = 0.55$, $P = 0.026$). AMI rats also presented an increase of 105 % in GRX activity when compared with SHAM. In AMIT rats, this enzyme activity was no different from in SHAM rats (Fig. 3a). GRX activity was positively correlated with the levels of ROS ($r = 0.50$, $P = 0.013$). GRX3 expression, however, was no different between groups (Fig. 3b). TRX reductase activity (Fig. 3c) and TRX1 expression (Fig. 3d) were also no different among groups. The protein expression of PGC-1α was increased in AMIT rats when compared with SHAM animals (Fig. 4). The expression of this transcription factor was positively correlated with NOS activity ($r = 0.67$, $P = 0.032$).

Discussion

The main finding of this study was to demonstrate that T3 and T4 administration decreased ROS levels and increased eNOS expression in the infarcted heart. This was

Table 1 Hormonal levels, morphometric and echocardiographic data of the experimental groups 28 days post-infarction

Parameters	SHAM	AMI	AMIT
Hormonal levels			
T3 (ng/dL)	98.8 ± 7.2	105.8 ± 8.0	218.9 ± 58.0*#
T4 (μg/dL)	5.1 ± 0.4	6.4 ± 0.3	6.5 ± 1.3
Morphometric data			
Body weight (g)	352 ± 32	345 ± 37	352 ± 56
LV weight (g)	0.70 ± 0.06	0.85 ± 0.11*	1.01 ± 0.68*#
LV/body weight (mg/g)	1.96 ± 0.021	2.41 ± 0.39*	3.19 ± 0.59*#
LV/tibia length (mg/cm)	22.5 ± 1.2	26.6 ± 2.9*	33.0 ± 3.9*#
Echocardiographic data			
LV diastolic diameter (cm)	0.68 ± 0.099	0.90 ± 0.048*	0.91 ± 0.069*
LV systolic diameter (cm)	0.35 ± 0.074	0.73 ± 0.046*	0.77 ± 0.07*
LV systolic posterior wall thickness (cm)	0.25 ± 0.008	0.21 ± 0.019*	0.23 ± 0.015
Wall tension index	2.12 ± 0.41	3.17 ± 0.32*	2.65 ± 0.57*#
Infarction size (%)	–	50.9 ± 14.3	54.8 ± 7.8

Values are expressed as mean ± SD from 5 to 7 animals per group. LV (left ventricle). One-way ANOVA (Student Newman–Keuls Method)

* Significantly different from SHAM group ($P < 0.05$); # significantly different from AMI group ($P < 0.05$)

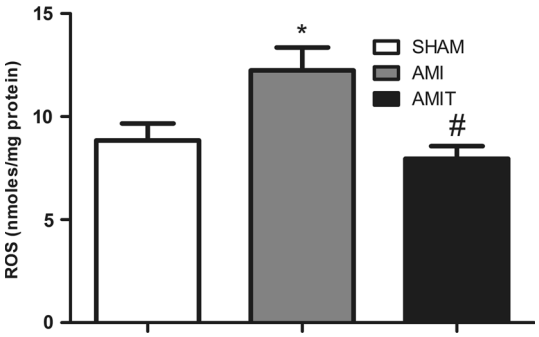

Fig. 1 Reactive oxygen species (ROS) levels in cardiac tissue. Values are expressed as mean ± SEM from 5 to 7 animals per group. One-way ANOVA (Student Newman–Keuls post hoc test). * Significantly different from SHAM group ($P < 0.05$); # significantly different from AMI group ($P < 0.05$)

associated with an increased PGC-1α expression in the cardiac tissue.

Left coronary artery ligation promoted cardiac injury in the infarcted rats, leading to the loss of LV viable myocardium and the formation of a scar. The infarction size found in this work was not different between the two infarcted groups, indicating the reproducibility of the surgical method used in the present study [3, 4]. Despite the fact that infarction can induce a decrease in serum T3, a condition called 'low T3-syndrome' [31, 32], in the present study, the levels of thyroid hormones were no different between the AMI and SHAM groups. Other studies using a rat model of myocardial infarction also found no difference in the level of T3 after ischaemic injury [15, 16]. In AMI rats, morphometric measurements demonstrated an increased LV mass and left chamber hypertrophy. Similar results have also been described by other authors [3, 21]. Echocardiographic findings from the AMI group showed an increase in LV diastolic and systolic diameters and a decrease in systolic posterior wall thickness when compared with SHAM rats. These results indicate LV chamber dilatation and loss of cardiac wall thickness, which are both related with pathological cardiac remodelling [1, 4]. The alteration in these structural parameters of the heart promotes an increase in LV wall stress [33]. In fact, the wall tension index, which is indicative of cardiac wall stress, was higher in AMI rats when compared with SHAM animals. In AMIT rats, thyroid hormone administration was effective in promoting an increase in T3 levels. T4 levels, however, were unchanged in this group. The hormonal administration increased LV mass and promoted left chamber hypertrophy in AMIT rats. In this group, the LV hypertrophy indices were statistically higher than those presented by AMI. A possible explanation for these results is the fact that T3 and T4 administration prevented the loss of posterior wall thickness in AMIT rats. In this group, this parameter was no different from in SHAM animals. Therefore, this could be a possible explanation for the

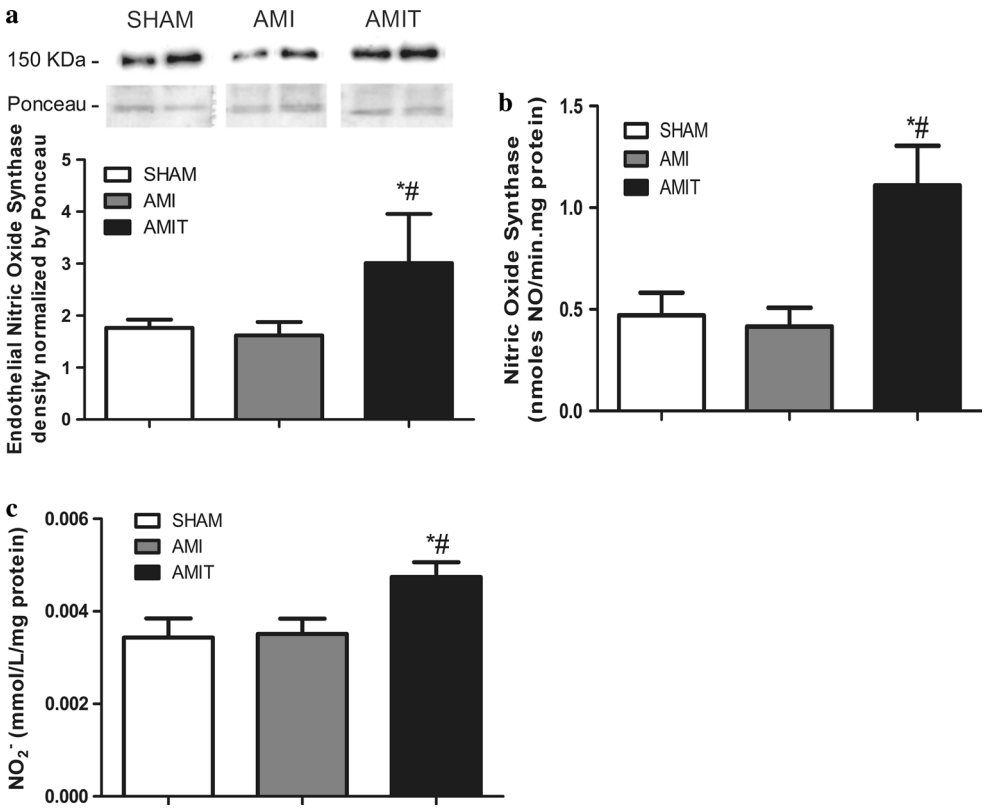

Fig. 2 Endothelial nitric oxide synthase protein expression (**a**), nitric oxide synthase activity (**b**) and nitrite (NO_2^-) levels (**c**) in cardiac tissue. The representative Western blot lanes were derived from the same blot. Values are expressed as mean ± SEM from 5 to 7 animals per group. One-way ANOVA (Student Newman–Keuls post hoc test). * Significantly different from SHAM group ($P < 0.05$); # significantly different from AMI group ($P < 0.05$)

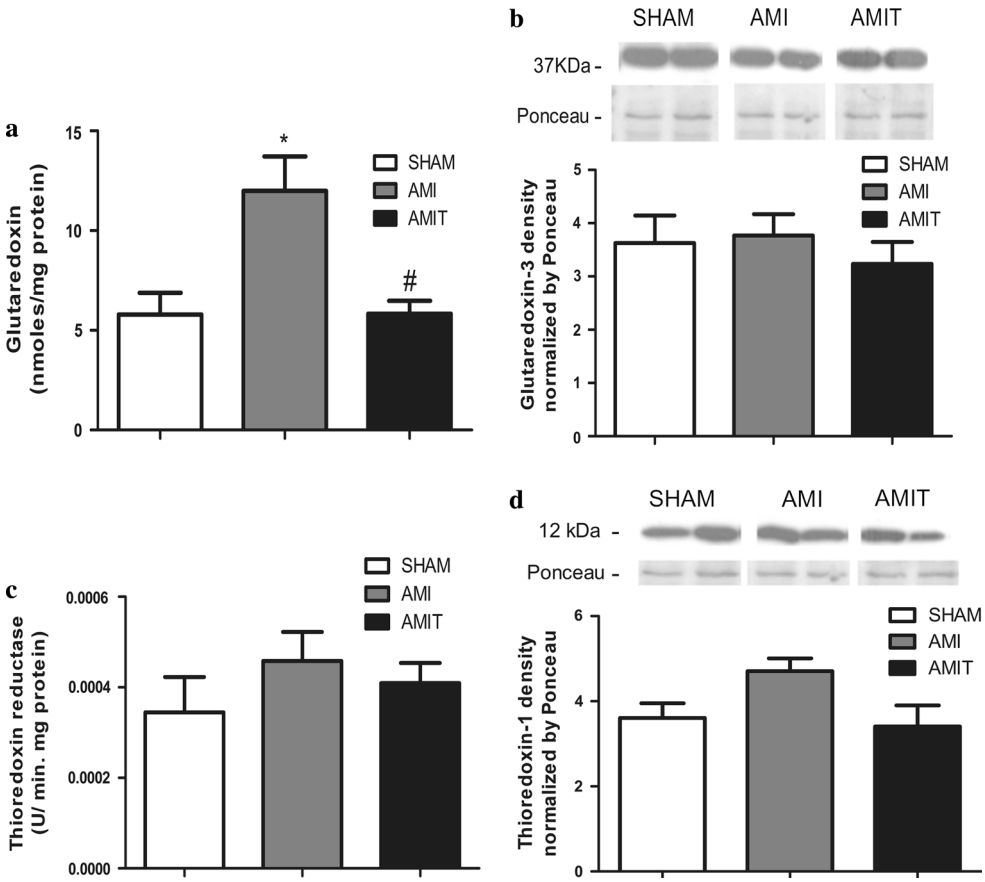

Fig. 3 Glutaredoxin activity (**a**), glutaredoxin-3 protein expression (**b**), thioredoxin reductase activity (**c**) and thioredoxin-1 protein expression (**d**) in cardiac tissue. The representative Western blot lanes were derived from the same blot. Values are expressed as mean ± SEM from 5 to 7 animals per group. One-way ANOVA (Student Newman–Keuls post hoc test). * Significantly different from SHAM group ($P < 0.05$); # significantly different from AMI group ($P < 0.05$)

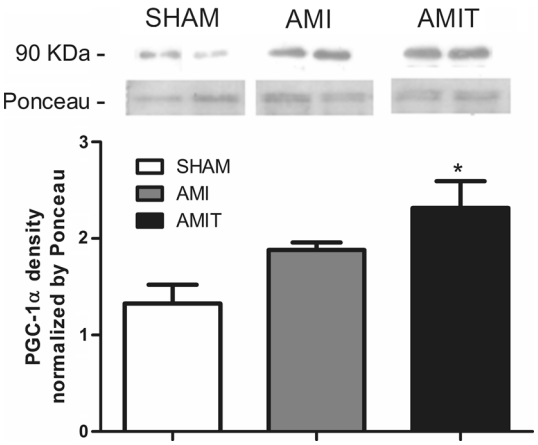

Fig. 4 PGC-1α protein expression in cardiac tissue. The representative Western blot lanes were derived from the same blot. Values are expressed as mean ± SEM from 5 to 7 animals per group. One-way ANOVA (Student Newman–Keuls post hoc test). * Significantly different from SHAM group ($P < 0.05$); # significantly different from AMI group ($P < 0.05$)

higher LV mass found in the infarcted treated rats when compared with AMI animals. This result was similar to what has been demonstrated in previous studies [15, 16]. LV systolic and diastolic diameters were increased in AMIT rats when compared with SHAM and these parameters were no different from those in AMI rats. However, thyroid hormone administration promoted an improvement in the wall tension index in AMIT rats when compared with AMI. Since thyroid hormones prevented the loss of cardiac wall, it is possible that this response could be compensating for LV dilatation, attenuating cardiac wall stress in AMIT animals. This positive effect of hormonal administration has also been previously described [15, 16].

In the present study, myocardial infarction promoted an increase in ROS levels in the AMI group. In heart cells, mitochondrial electron-transport chain is a major source of ROS [2, 34]. After myocardial infarction, the degradation of the adenine nucleotide cellular pool leaves the mitochondrial electron carriers in a reduced state, leading to an increase in electron leakage from the respiratory chain, which in turn reacts with the residual oxygen trapped in the mitochondria to produce ROS [34]. Previous studies have shown that ROS production is increased after myocardial infarction [2, 3]. As a response to ROS levels, the enzymatic activity of GRX was increased in the AMI group. This protein is a key player in the defence against oxidative stress in mammalian cells [5]. The GRX system can catalyse both the formation and reduction of mixed

disulphides between protein thiols and GSH, and its activity can be induced by oxidative stress [35]. However, in the present study, the expression of isoform GRX3 was no different between groups. In the AMIT group, ROS levels were significantly decreased when compared with AMI and were no different from those in SHAM animals. The activity of GRX was also decreased in AMIT rats when compared with AMI and was no different in SHAM animals. This result probably reflects an adaptation of the antioxidant system to the low levels of ROS [34]. In fact, the levels of ROS were positively correlated with GRX activity. The TRX system, however, was changed neither by myocardial infarction nor by thyroid hormone administration. In the present study, TRX reductase activity and TRX1 expression were no different among groups. In view of this, the decrease in ROS levels caused by T3 and T4 administration does not seem to involve a modulation of the TRX or GRX systems. In previous work from our group, it was demonstrated that T3 and T4 administration was capable of decreasing xanthine oxidase expression [17], which is an important source of ROS. This mechanism could be a possible explanation for the lower levels of ROS found in AMIT rats. Besides that, LV wall stress is also related to ROS production in the heart [36]. A study performed in patients with coronary heart disease showed that cardiac wall stress represents a primary determinant of myocardial oxygen consumption [36], which is related to ROS production. So, it is possible that the decrease in the wall tension index in AMIT rats could also be related to the low levels of ROS in this group. In fact, in the present study, the levels of ROS were positively correlated with the wall tension index.

In the heart, NO levels seem to be relevant for regulating cardiomyocyte function, tissue vascularization and perfusion [9, 37]. Under physiological conditions, eNOS is the major source of NO in cardiac tissue. However, after heart failure, the bioavailability of eNOS-derived NO is severely reduced [38, 39]. In the present study, eNOS expression, NOS activity and nitrite levels were no different between the AMI and SHAM groups. However, since AMI rats presented high levels of ROS, this could cause a change in the ROS/NO balance in favour of ROS, resulting in decreased NO bioavailability in this group. In fact, it was previously suggested that the balance between ROS and NO is a more relevant parameter to establish NO bioavailability than its individual concentration itself [37]. In AMIT rats, however, T3 and T4 administration promoted an increase in eNOS expression, NOS activity and nitrite levels. In this group, thyroid hormone administration could be directly stimulating eNOS expression and its activity in the heart. This effect could lead to an increase in NO production, as was previously described [37]. In fact, NOS activity was positively correlated with nitrite levels, which are NO metabolites. Corroborating this, Spooner et al. showed that the thyroid hormone analogue 3,5 diiodothyropropionic acid (DITPA) was capable of increasing eNOS expression in the aortic tissue of infarcted rats, as well as increasing NO-dependent vasorelaxation [40]. Therefore, the decrease in ROS levels and increase in NO production indicate an improvement in NO bioavailability in the AMIT rats. Besides that, previous studies have shown that NO can act as an antioxidant molecule by regulating the pro-oxidant enzyme xanthine oxidase [41]. In view of this, an increase in NO bioavailability could be related to the reduction in ROS levels in AMIT rats. Therefore, the increase in eNOS expression and in NO bioavailability seems to represent an important part of T3 and T4 effects in the infarcted heart.

Moreover, thyroid hormone administration increased PGC-1α expression in AMIT rats. Similar results were also described in infarcted rats treated with T3 alone [14]. PGC-1α is a transcription factor involved mainly with the mitochondrial biogenesis process and cellular metabolism [42]. An increase in PGC-1α levels seems to be positive, since this factor's expression was recently related to a decrease in cardiac cell apoptosis [43]. In another study, the absence of this factor was related to pulmonary congestion in a model of heart failure in mice [44]. In addition, previous studies also showed that PGC-1α is directly involved with the regulation of myocardial mitochondrial antioxidant systems [43, 44]. Therefore, the increase in expression of this transcriptional factor could be another mechanism related to the normalization of ROS levels and, therefore, with the improvement in NO bioavailability in the AMIT rats. PGC-1α expression was shown to be regulated by eNOS-derived NO [13]. Therefore, in AMIT rats, it is possible that thyroid hormones could be stimulating PGC-1α expression mainly through an increase in NO levels. In fact, in the present study, NOS activity was positively correlated with PGC-1α expression. Therefore, the increase in PGC-1α expression could be a NO-dependent effect of T3 and T4 in the infarcted heart.

A limitation of the present study was the lack of a housekeeper protein expression to normalize Western blot analysis. Since several loading proteins (tubulin, GAPDH) are influenced by thyroid hormones administration, or by myocardial infarction [45, 46], Ponceau method was used for Western blot normalization.

Taken together, our data showed that T3 and T4 administration promoted a reduction in the levels of ROS, an increase in eNOS expression and an improvement in NO bioavailability after myocardial infarction. These effects seem to be beneficial for the heart, since they resulted in PGC-1α up-regulation [13]. These results may complement other studies that evaluate underlying mechanisms of T3 and T4 administration after myocardial infarction.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest. The authors declare that the experimental procedures were performed taking into consideration the welfare of animals and were approved by the Ethics Committee for animal research at Universidade Federal do Rio Grande do Sul.

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