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Increased resistance to hydrogen peroxide-induced cardiac contracture is associated with decreased myocardial oxidative stress in hypothyroid rats

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The purpose of this study was to determine whether decreased oxidative stress would increase the resistance to cardiac contracture induced by H_2O_2 in hypothyroid rats. Male Wistar rats were divided into two groups: control and hypothyroid. Hypothyroidism was induced via thyroidectomy. Four weeks post surgery, blood samples were collected to perform thyroid hormone assessments, and excised hearts were perfused at a constant flow with or without H_2O_2 (1 mmol/L), being divided into two sub-groups: control, hypothyroid, control + H_2O_2, hypothyroid + H_2O_2. Lipid peroxidation (LPO) was evaluated by chemiluminescence (CL) and thiobarbituric acid reactive substances (TBARS) methods, and protein oxidation by carbonyls assay in heart homogenates. Cardiac tissue was also screened for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities, and for total radical-trapping antioxidant potential (TRAP). Analyses of SOD and glutathione-*S*-transferase (GST) protein expression were also performed in heart homogenates. Hypothyroid hearts were found to be more resistant to H_2O_2-induced contracture (60% elevation in LVEDP) as compared to control. CL, TBARS, carbonyl, as well as SOD, CAT, GPx activities and TRAP levels were reduced (35, 30, 40, 30, 16, 25, and 33%, respectively) in the cardiac homogenates of the hypothyroid group as compared to controls. A decrease in SOD and GST protein levels by 20 and 16%, respectively, was also observed in the hypothyroid group. These results suggest that a hypometabolic state caused by thyroid hormone deficiency can lead to an improved response to H_2O_2 challenge and is associated with decreased oxidative myocardial damage. Copyright © 2009 John Wiley & Sons, Ltd.

KEY WORDS — thyroid hormones; protein expression; antioxidant enzymes; oxidative damage; heart function

INTRODUCTION

Thyroid hormones are some of the most important hormonal factors involved in the regulation of basal metabolic state and in oxidative metabolism.[1] Variations in thyroxine (T_4) and triiodothyronine (T_3) levels provide some of the main mechanisms for physiological modulation of the mitochondrial respiration process *in vivo*, as these variations can alter the function of various mitochondrial respiratory components.[2] An elevation in thyroid hormone levels leads to increased oxygen consumption and associated reactive oxygen species (ROS) generation and has been implicated in many pathological conditions.[3]

ROS have a high reactivity potential, making them toxic to biomembranes due to their ability to cause oxidative damage to cellular macromolecules such as proteins, lipids, and DNA.[4] Literature suggests that ROS may act as mediators in the pathogenesis of cardiac diseases associated with thyroid dysfunction, such as those seen with hyperthyroidism.[5,6]

By contrast, hypothyroidism is a disorder caused predominantly by the decreased thyroid hormone production that results from thyroid gland impairment. Less frequently, the disorder is caused by decreased thyroid-stimulating hormone (TSH) production.[7] Clinical manifestations such as fatigue, cold intolerance, decreased appetite, and bradycardia are generally the result of the hypometabolic state induced by hypothyroidism.[6] This depressed metabolic state is associated with decreased mitochondrial oxygen consumption and lesser ROS production. There are reports demonstrating that hypothyroidism induces increased myocardial glycogen content,[8] decreased ATP consumption, and increased beta-myosin high chain expression.[9] This energy conservation is thought to be cardioprotective, both under normal and pathophysiological conditions.

Indeed, several studies have shown that hypothyroid hearts are protected against ischemia-reperfusion, as they

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exhibit less ischemic contracture than euthyroid hearts.[10] Conversely, hydrogen peroxide (H_2O_2), an important ROS, has been implicated in the mechanism of ischemic cardiac contracture, leading to ventricular dysfunction.[11] Thus, H_2O_2 perfusion into the coronaries of isolated rat hearts has been proposed as a model to study cardiac contracture.[12] In this model, a dose dependent effect of H_2O_2 on changes in positive inotropic effect, cardiac arrhythmias, such as post-extrasystolic potentiation, and cardiac contracture was also observed.[13] The processes that seem to be involved in the cardiac contracture may be attributed to membrane lipid peroxidation (LPO), protein damage, reduction of the ATP level induced by H_2O_2 and LPO mediated by the release of cardioactive compounds such as prostaglandins and leukotrienes. These alterations of biomolecules may result in cytoplasmic calcium overload, inducing cardiac contracture.[14]

As previously mentioned, there is strong evidence in the literature that thyroid hormones modulate oxidative stress in cardiac tissue.[15] However, there is a paucity of information regarding the reduction of rigor contracture in hypothyroidism associated with a specific oxidative stress profile, including the expression of antioxidant enzymes. Thus, the purpose of this study was to evaluate not only the protective effect of hypothyroidism against myocardial contracture induced by H_2O_2, but also the role of oxidative stress in this process, *vis-a-vis* the myocardial concentration and activity of antioxidant enzymes, non-enzymatic antioxidants and oxidative damage in hypothyroid rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (250 ± 50 g) were obtained from the Central Animal House of the Universidade Federal do Rio Grande do Sul, Brazil. Animals were housed in plastic cages (four animals in each) and received water and pelleted food *ad libitum*. The animals were maintained under standard laboratory conditions (controlled temperature of 21°C, 12 h light/dark cycle). Animals were weighed weekly to follow body weight gain during the course of the experimental protocol (28 days). All animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and Brazilian College of Animal Experimentation.

Experimental groups and protocol

Animals were divided in to two groups; *control* ($n = 6$/each), where rats were subjected to the simulation of thyroidectomy, and *hypothyroid* ($n = 6$/each), where rats were thyroidectomized. For heart perfusion evaluation, each group was additionally divided in two sub-group, that is, with or without H_2O_2 using a Langendorff system. Four groups ($n = 6$/each) were used: control, hypothyroid, control + H_2O_2, and hypothyroid + H_2O_2.[12]

Thyroid hormones concentration

At 4 weeks post-surgery, blood samples were collected from the retroorbital venous plexi and immediately centrifuged at $1000 \times g$ for 10 min. Serum thyroid hormone concentration was estimated via chemiluminescence (CL) using the Immunolite 2000 kit (Biomedical Technologies, Inc., Strougerton, MA, USA) at Weinmann Clinical Analysis Laboratory.

Isolated working heart using a Langendorff system

At the end of the experimental protocol, rats were sacrificed by cervical dislocation and hearts were excised and perfused using a Langendorff apparatus. The aorta was retroperfused with a Krebs–Henseleit solution as described elsewhere.[11] The solution was maintained at 31°C, gassed with 95% O_2 and 5% CO_2, and used for perfusion at a constant flow (10 mL/min). A water filled latex balloon connected to a pressure transducer (Isotec P23 XL – Hugo Sachs Elektroniks, March, Freiburg, Germany) was placed inside the left ventricle. Signals were processed using an analog-to-digital conversion system and analyzed with the Isoheart software (Hugo Sachs Elektroniks, Germany). A stabilization period of 10 min was required to reach steady-state cardiac function. After stabilization, the balloon volume was adjusted in the left ventricle to generate a diastolic pressure of 10 mmHg. Hearts were then perfused with a H_2O_2 solution (1 mmol/L) for 20 min.[13] Heat rate (beats per minute), left ventricle end diastolic pressure – LVEDP (mmHg), onset of rigor contracture (minutes), and peak LVEDP (mmHg) were also monitored. Cardiac contracture was considered to have occurred when the relaxation phase (LVEDP) was significantly increased, and was determined by measuring the baseline elevation of myograms. The peak of LVEDP was used as a parameter of the severity of the contracture.

Tissue preparation

For the oxidative stress evaluation, hearts were rapidly excised, weighed, and homogenized (1.15% w/v KCl and phenyl methyl sulfonyl fluoride PMSF 20 mmol/L) in Ultra-Turrax. The resulting suspension was centrifuged at $1000 \times g$ for 10 min at 0–4°C to remove the nuclei and cell debris[16] and the supernatants were used for oxidative damage and enzymatic activity assays. At the time of the sacrifice, cardiac tissue samples were also removed and frozen at −80°C for protein expression evaluation.

Oxidative damage measurements

LPO was measured by the *tert*-butyl hydroperoxide-initiated CL assay, as previously described by Gonzalez Flecha *et al.*[17] The assay was carried out with an LKB Rack Beta Liquid Scintillation Spectrometer 1215 (LKB Producter AB, Bromma, Sweden) in the out-of-coincidence mode at room temperature. Heart supernatant fractions were diluted in

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140 mmol/L KCl and 20 mmol/L phosphate buffer, pH 7.4, which was added to the samples placed in scintillation vials. At this moment, 3 mmol/L of *tert*-butylhydroperoxide was added, and CL was determined by the maximal emission levels. The results were reported as counts per second (cps)/mg of protein. LPO was also estimated by the TBARS assay. Results were expressed as micromoles per milligram of protein (μmol/mg protein).[18] Protein oxidation was performed by carbonyl assay and the results expressed as nmol/mg protein.[19]

Determination of antioxidant defenses

Antioxidant defenses were evaluated by measuring enzyme activity. Superoxide dismutase (SOD) activity, expressed as units per milligram of protein, was determined based on the inhibition of the superoxide radical reaction with pyrogallol.[20] Catalase (CAT) activity was determined by following the decrease in H_2O_2 absorbance at 240 nm and was expressed as picomoles of H_2O_2 reduced per minute per milligram of protein.[21] Glutathione peroxidase (GPx) catalyze the reduction of H_2O_2 using reduced glutathione. Its activity was measured by following NADPH oxidation at 340 nm and was expressed as nanomoles of peroxide/hydroperoxide reduced per minute per milligram of protein.[22] Total radical-trapping antioxidant potential (TRAP), which indicates total antioxidant capacity present in homogenates, was also screened. TRAP was measured via CL using 2,2′-azo-bis(2-amidinopropane) (ABAP) a source of alkyl peroxyl free radicals) and luminol. Luminescence was measured in a liquid scintillation counter in the out-of-coincidence mode and results were expressed in units of Trolox/mg protein.[23]

Western blot analysis

Tissue sample preparation, electrophoresis, and protein transference were performed as described previously.[5,24] The membranes were processed for immunodetection using sheep anti-Cu/Zn SOD polyclonal antibody and rabbit anti-GST polyclonal antibody as primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The bound primary antibodies were detected using rabbit anti-sheep or goat anti-rabbit horseradish peroxidase-conjugate secondary antibodies and membranes were revealed via CL. The autoradiographs generated were quantitatively analyzed with an image densitometer (Imagemaster VDS CI, Amersham Biosciences Europe, IT). The molecular weights of the bands were determined by to a molecular weight marker standard (RPN 800 rainbow full range Bio-Rad, CA, USA). The results from each membrane were normalized through Ponceau red method.[25]

Determination of protein concentration

Protein concentration was measured by the Lowry method,[26] using bovine serum albumin as a standard.

Statistical analysis

Data were expressed as mean ± SD and compared using the unpaired Student's *t*-test for oxidative stress data. Cardiac contracture data were compared by one-way ANOVA followed by the Student–Newmann–Keuls test. Values of $p < 0.05$ were considered significant.

RESULTS

Hypothyroidism development

T_4 and T_3 levels were significantly decreased (96 and 90%, respectively) in the hypothyroid group. To assess cardiac muscle mass, animals were examined for heart and body weight. The heart to body weight ratio was lower (17%) in hypothyroid rats than in control ones ($p < 0.05$). No difference was found in total protein concentration between groups (Table 1).

Cardiac function data

It was found that the hypothyroid group, which was perfused solely with Krebs–Henseleit, showed cardiac relaxation characterized by a reduction in LVEDP (40%) (Figure 1A). As expected, H_2O_2 induced an elevation in the LVEDP by 98% in control + H_2O_2 after 20 min, compared to control. However, H_2O_2-induced LVEDP elevation was only 60% in the hypothyroid + H_2O_2 group as compared to its control (control + H_2O_2) (Figure 1B). Peak of LVEDP during H_2O_2-induced contracture in the hypothyroid group was almost half that of the control group (Figure 1B). Heart rate was significantly lower in the hypothyroid group as compared to control during 20 min of Krebs–Henseleit perfusion. When these groups have been perfused with H_2O_2, heart rate was significantly lower in all groups from the tenth minute of perfusion (Figure 2A). Time to onset of contracture was markedly longer in the hypothyroid group than in control (Figure 2B).

Myocardial oxidative damage

There was a 35% decrease in LPO as indicated by CL in the cardiac tissue of the hypothyroid group as compared to the

Table 1. Thyroxine levels, morphometric, and hemodynamic parameters 4 weeks after surgery

Parameters	Control	Hypothyroid
T_4 (ng/mL)	3.00 ± 0.10	0.10 ± 0.02*
T_3 (ng/mL)	0.67 ± 0.07	0.06 ± 0.009*
Body weight gain (g/week)	16.7 ± 4.1	14.7 ± 1.2
Final body weight (g)	260 ± 25	255 ± 28
Heart weight (g)	0.78 ± 0.10	0.50 ± 0.10*
Heart/body weight (mg/g)	3.00 ± 0.02	2.50 ± 0.05*
Protein concentration in heart homogenate (mg/mL)	9.15 ± 0.90	8.89 ± 1.45

Values are expressed as mean ± SD of six animals per group.
*Significantly different from control ($p < 0.05$).
Student's *t*-test.

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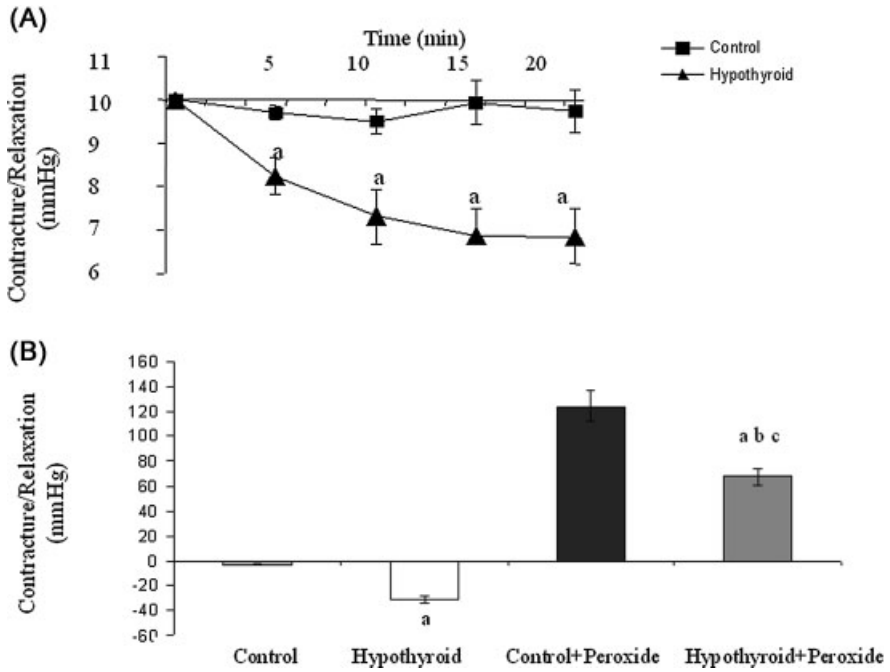

Figure 1. (A) Time course profile of cardiac contracture and relaxation (in %) from isolated working heart using a Langendorff apparatus and perfusion with hydrogen peroxide (H_2O_2) solution (1 mmol/L) for 5, 10, 15, and 20 min in control and hypothyroid rats. (B) Cardiac contracture and relaxation data (in %) of isolated working heart perfused with H_2O_2 solution (1 mmol/L) for 20 min: control, hypothyroid; control + peroxide, and hypothyroid + peroxide groups. Data as mean ± SD from six animals in each group. [a]Significantly different from the control ($p < 0.05$), [b]significantly different from the control + peroxide ($p < 0.05$), and [c]significantly different from the hypothyroid ($p < 0.05$). One-way ANOVA followed by the Student–Newmann–Keuls test

control ($p < 0.05$). TBARS levels were also reduced (30%) in hypothyroids as compared to controls ($p < 0.05$) (Table 2). Oxidative damage to proteins, assessed by the carbonyl assay, was diminished by 40% in the hypothyroid rats compared to controls (Table 2).

Antioxidant defenses

SOD, CAT, and GPx activities were lower (30, 16, and 25%, respectively) in the hypothyroid group than in the control ($p < 0.05$) (Table 3). Thyroidectomy resulted in a 33% reduction in TRAP levels in cardiac tissue ($p < 0.05$) compared to the control (Table 3). Protein levels of antioxidant enzymes were analyzed by the Western blot technique. There was also a 20 and 16% decrease in Cu/Zn SOD and GST myocardial protein levels, respectively, in the hypothyroid rats as compared to the control group (Figure 3A and B) ($p < 0.05$).

DISCUSSION

In this study, we tested if hypothyroid hearts would be more resistant to cardiac contracture induced by a high dose of H_2O_2. In many acute myocardial events, there is a variable level of different ROS production, which participate in the myocardial damage. Therefore, perfusing isolated heart with H_2O_2, a very stable ROS, has been used a model of cardiac contracture. The initial finding of this study was that hypothyroid hearts can withstand H_2O_2-induced cardiac contracture better than control hearts. Secondly, we explored a possible mechanism of this cardioprotection and found a reduction in myocardial oxidation products in the hypothyroid rats.

This hypothyroidism model was induced by thyroidectomy due to few adverse effects. Propilthiouracil (PTU) is a drug alternative for the induction of hypothyroidism; however, its sulfhydril moiety composition can act as an antioxidant which may interfere with oxidative stress measurements.[27] PTU has been shown to act as a highly efficient scavenger of hydroxyl radicals, a potent inhibitor of LPO, and an inhibitor of H_2O_2 production in neutrophyls.[28] Therefore, the use of PTU as a model of hypothyroidism is not suitable when oxidative stress measurements are performed.

Evidence from the literature demonstrated that hypothyroid-induced metabolic depression is associated with a decrease in ROS production and an ameliorated oxidative stress profile.[29] Vargas *et al.*[6] also demonstrated that hypothyroidism is often associated with many cardiovascular changes, such as a decrease in cardiac output, heart rate, and pulse pressure. In the present study, the hypothyroid state was characterized by a profound decrease in thyroid hormone levels, cardiac mass reduction (the hypothyroid group demonstrated a 43% reduction in cardiac mass compared to the control), and bradycardia. This decrease in muscle mass could be due to decreased cardiac

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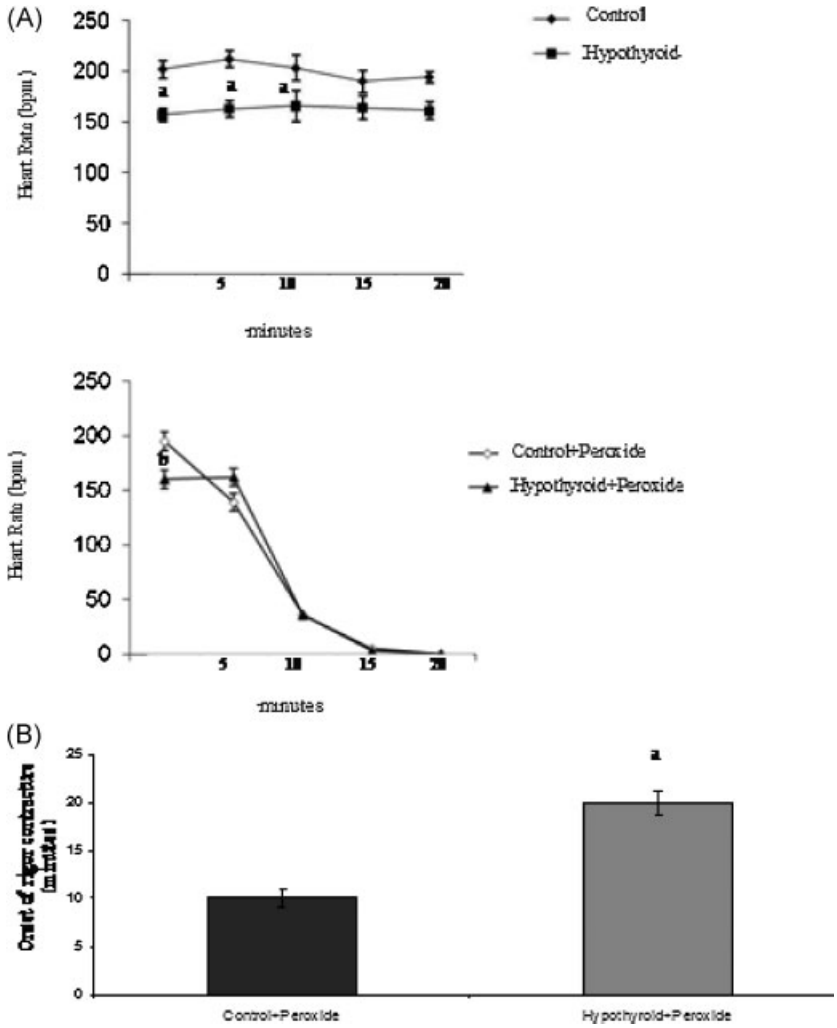

Figure 2. (A) Heart rate (in bpm) of isolated working heart perfused with H_2O_2 solution (1 mmol/L) for 20 min: control, hypothyroid; control + peroxide, and hypothyroid + peroxide groups. (B) Time to onset of rigor contracture (in minutes) in control + peroxide and hypothyroid + peroxide group. Data as mean ± SD from six animals in each group. [a]Significantly different from the control ($p < 0.05$), [b]significantly different from the control + peroxide ($p < 0.05$). One-way ANOVA followed by the Student–Newmann–Keuls test

muscle contractile protein expression.[30] Kuzman *et al.*[31] demonstrated that increased thyroid hormone levels provided intracellular signaling pathway activation for hypertrophy, indicating an important role for these hormones in cardiomyocyte growth.[31] In the present study, when the perfusion of the isolated heart in basal condition was performed, we observed that hypothyroid hearts exhibited cardiac relaxation. This effect may be due to thyroid hormone regulation of calcium handling proteins.[14] When control hearts were perfused with H_2O_2, an irreversible contracture known as ''stone heart'' appeared.

Table 2. Myocardial markers of oxidative damage to membrane lipids (CL and TBA-RS) and proteins (carbonyl groups) 4 weeks after surgery

Parameters	Control	Hypothyroid
LPO by CL (cps/mg protein 10^3)	170 ± 23	$118 \pm 21^*$
TBA-RS (μmol/mg protein)	2.2 ± 0.1	$1.3 \pm 0.2^*$
Carbonyl groups (nmol/mg protein)	3.5 ± 0.1	$2.1 \pm 0.3^*$

Values are expressed as mean ± SD of six animals per group.
*Significantly different from control ($p < 0.05$).
Student's *t*-test.

Table 3. Antioxidants: CAT, SOD, GPx, and TRAP in cardiac homogenates 4 weeks after treatment

Antioxidants	Control	Hypothyroid
SOD (U/mg protein)	6.4 ± 0.8	$4.5 \pm 0.7^*$
CAT (pmol/mg protein)	63.5 ± 6.0	$53.5 \pm 6.4^*$
GPx (nmol/min/mg protein)	46.6 ± 7.8	$34.8 \pm 5.0^*$
TRAP (U of Trolox/mg protein)	62 ± 10	$36 \pm 13^*$

Values are expressed as mean ± SD of six animals per group.
*Significantly different from control ($p < 0.05$).
Student's *t*-test.

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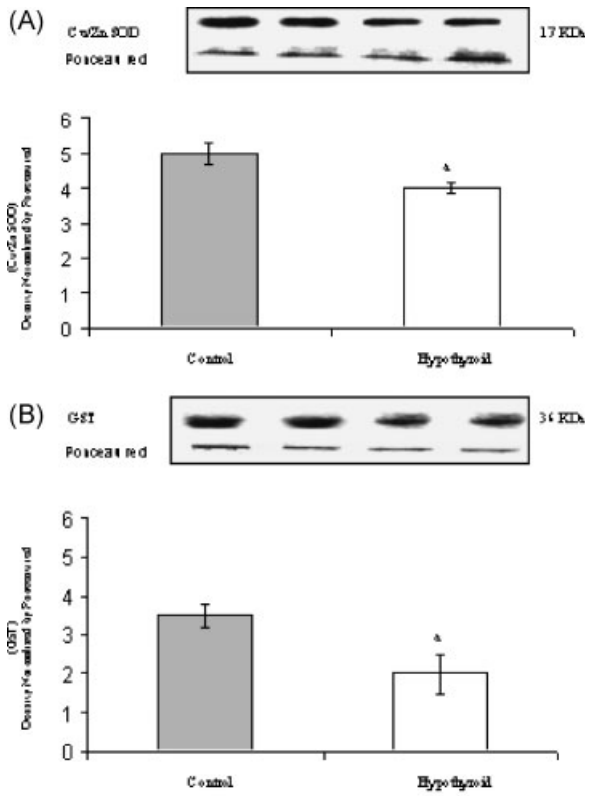

Figure 3. Western blot analysis in cardiac homogenates using Cu/Zn SOD antibody (A) and GST antibody (B). Data as mean ± SD from six animals in each group (one representative gel of five Western blot experiments, showing two bands for each experimental group). [a]Significantly different from the control ($p < 0.05$). One-way ANOVA followed by the Student–Newmann–Keuls test

H_2O_2 may not only reduce the level of ATP necessary for the functioning of the ionic pumps, but also inhibit glycolysis in order to reduce the ATP stores necessary to maintain the ionic gradients across the cell membrane.[8,9] The resulting energy loss and selective membrane changes contribute to intracellular calcium overload that impairs muscle relaxation. Alternatively, the hypothyroid group, when perfused with H_2O_2, showed minor cardiac contracture compared to that of the control. This could be due to the involvement of sarcoplasmic reticulum Ca^{2+} ATPase gene expression regulation by thyroid hormone.[32] H_2O_2 has also been shown to react with reduced ferrous ions, resulting in hydroxyl radical production via the Fenton reaction.[12] This radical can then initiate LPO and change membrane permeability, leading to the formation of hydrophilic ''pores.''[4] However, hypothyroidism has been implicated in elevated mitochondrial resistance to the opening of membrane permeability transition pores, blocking non-selective calcium overload that would promote cellular damage.[33] These results suggest that hypothyroidism may offer a protective effect to the myocardium, as already demonstrated by others.[34] Several mechanisms for this cardioprotection have been proposed, and in this study it has been shown that the oxidative stress profile changes due to hypothyroidism may play a role in this process.

Our results shown that LPO was reduced in cardiac tissue, as measured by both TBARS and CL assessments in the hypothyroid group. These changes are also associated with a lower protein oxidation in cardiac tissue. Other investigators have demonstrated a similar decrease in LPO in other tissues of thyroidectomized rats,[28] which may reflect the animal's hypometabolic state. A reduced ROS production *in vivo* was also observed in hypothyroidism.[29] According to Lopez-Torres *et al*.,[35] myocardial H_2O_2 production was diminished in the mitochondria of hypothyroid rats. Reduced oxidative damage products were also described in hypothyroidism[28] and TRAP levels were decreased in the hypothyroid group in this study. A decrease in TRAP, as measured by chemiluminescent methods, has also been reported previously in the myocardium of hypothyroid rats.[36] Literature also suggests that there can be an important reduction of co-enzyme Q9,[36] a non-enzymatic antioxidant, as well as in vitamins A, D, B_2, C, and GSH in hypothyroid rats.[37,38] In this study, there was a reduction not only in non-enzymatic antioxidant potential, but also in enzymatic antioxidant activity and protein expression. Das and Chainy[36] also demonstrated decreased CAT activity in hepatic and neural tissues of hypothyroid rats, corroborating what this study found in the heart. A reduction in the activity and protein expression of Cu/Zn SOD was also found. This could reflect diminished substrate availability (superoxide anion radical) in hypothyroidism. Additionally, GST protein expression was also depressed indicative of a less oxidative cellular environment.[39] This feature could be an adaptive response to the hypometabolic state caused by thyroid hormone withdrawal.[40]

In summary, thyroid hormone reduction, promoted by thyroidectomy, produced a cardiac mass loss which was accompanied by decreased oxidative damage to the myocardium and increased resistance to H_2O_2-induced contracture. This may be resultant of lower oxidative metabolism which would lead to less ROS production and, therefore, to preserve the membrane permeability characteristics, avoiding calcium overload. The functional relevance of this study is that, besides of the hypothyroidism state, a cardioprotection was seen even with an administration of a supraphysiological dose of H_2O_2.

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