Hypothyroidism and Oxidative Stress: Differential Effect on the Heart of Virgin and Pregnant Rats

Authors Y. C. Viglianco, M. J. Coria, L. B. Oliveros, M. S. Gimenez*

Affiliation Department of Biochemistry and Biological Sciences, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, IMIBIO-San Luis CONICET, Argentina

Key words
- thyroid hormone
- thyroid hormone receptor
- redox state
- myocardium

Abstract

The present study investigates the effects of hypothyroidism on both the redox state and the thyroid hormone receptors expression in the heart ventricle of virgin and pregnant rats.

Hypothyroid state was induced by 6-*n*-propyl-2-thiouracil in drinking water given to Wistar rats starting 8 days before mating until day 21 of pregnancy or for 30 days in virgin rats. Serum paraoxonase-1 (PON-1) activity, serum and heart nitrites, and thiobarbituric acid-reactive substances (TBARS) were analyzed. Heart protein oxidation, as carbonyls, and copper-zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GPx), and catalase (CAT) activities, were determined. In addition, heart expressions of NADPH oxidase (NOX-2), CAT, SOD, GPx, and thyroid receptors (TRα and TRβ) mRNA were assessed by RT-PCR. Inducible and endothelial Nitric Oxide Synthase (iNOS and eNOS) were determined by Western blot. Hypothyroidism in the heart of virgin rats decreased TRα and TRβ expressions, and induced oxidative stress, leading to a decrease of nitrites and an increase of carbonyls, NOX-2 mRNA, and GPx activity. A decreased PON-1 activity suggested low protection against oxidative stress in blood circulation. Pregnancy reduced TRα and TRβ mRNA expressions and induced oxidative stress by increasing nitrite and TBARS levels, SOD and CAT activities and NOX-2, eNOS and iNOS expressions, while hypothyroidism, emphasized the decreases of TRα mRNA levels and did not alter the redox state in the heart. TR expressions and redox balance of rat hearts depend on the physiological state. Pregnancy per se seems to protect the heart against oxidative stress induced by hypothyroidism.

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Correspondence
M. S. Gimenez
Department of Biochemistry and Biological Sciences
Chacabuco 917
National University of San Luis
5700
San Luis
Argentina
Tel.: +54/2652/446 909
Fax: +54/2652/431 301
marisofigime44@gmail.com

Introduction

Thyroid disorders are associated with profound alterations in the biochemical and physiological functioning of cardiac muscles [1]. The actions of the thyroid hormone triiodothyronine (T_3) are mediated by nuclear receptors [thyroid receptors (TRs)]. In the heart, 3 isoforms of TRs are expressed: α1, β1, and α2, where the latter form does not bind T_3. TRα and TRβ appear to play different roles in the physiology of the thyroid hormone (TH) action on the heart [2]. It is known that the cardiac phenotype is regulated by T_3 and predominantly mediated by TRα, where the lack of TRα cannot be compensated by TRβ in the heart [3]. In the presence of thyroid dysfunction, abnormal expressions of TR subtypes in the myocardium are closely associated with the pathogenesis of heart lesions [4].

Thyroid hormones are some of the most important factors involved in the regulation of basal metabolic state and in the oxidative metabolism; therefore, they play a crucial role in inducing generalized oxidative stress [5]. Hypermetabolic state in hyperthyroidism is associated with increases in free radical production and lipid peroxide levels [6]. Although it has been suggested that the hypometabolic state of hypothyroidism is associated with a decrease in oxidative stress, the data reported in the literature are controversial [7,8].

It is known that the generation of reactive oxygen species (ROS) triggers cell dysfunction, lipid peroxidation, and DNA mutagenesis, and can lead to irreversible cell damage or death. The NADPH oxidase (NOX) is a major source of $O_2^{\bullet -}$ in

*Member of CONICET (National Investigations Council of Science and Technology), Argentina.

vascular cells and myocytes [9]. Nitric oxide (NO) is another important reactive molecule controlling cardiovascular homeostasis. Enhanced superoxide production increases NO inactivation and leads to an accumulation of peroxynitrites and hydrogen peroxide [10]. It has been observed that hypothyroidism produces a heterogeneous tissue response in nitric oxide synthase (NOS) activity [11].

The first line of defence towards $O_2^{\bullet -}$ and H_2O_2 mediated injuries are antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) [12]. The antioxidant defence system is influenced by the thyroid hormone status. However, there are controversial results about the activity of antioxidant enzymes in the heart and hypothyroid state [13, 14].

On the other hand, a paraoxonase 1 (PON-1) diminished activity has been associated to thyroid dysfunction and to atherosclerosis [15]. PON-1 is synthesized by the liver and circulates within high-density lipoprotein (HDL) particles, and protects low-density lipoproteins (LDL) against oxidative stress [16].

During the second and third trimesters of pregnancy, cardiac output increases to match placental blood flow resulting in a condition of sustained volume overload, where the heart responds by undergoing cardiac physiology hypertrophy [17]. Furthermore, the increase of the maternal cardiac metabolic rate and the heart workload results in a greater exposure to oxidative stress [18].

Considering that there is no experimental evidence of the influence of hypothyroidism on oxidative stress and thyroid hormone receptors in the heart of pregnant rats, and the controversial results regarding the effects of hypothyroidism on the heart of nonpregnant rats, the aim of the present study is to evaluate the redox state and the mRNA expression of thyroid hormone receptors in heart of virgin and pregnant rats, with 6-*n*-propyl-2-thiouracil (PTU)-induced hypothyroidism.

Materials and Methods

Animals

Virgin female Wistar rats, weighing 160–180 g at the onset of treatment, were used. They were bred in our animal facilities (National University of San Luis, San Luis, Argentina), and maintained in a 21–23 °C controlled environment with 12-h light/12-h dark cycles. Rats were given free access to food and water throughout the entire experiment. The experimental protocols were approved by the Committee for Care and Use of Laboratory Animals of the National University of San Luis.

The experiments were carried out in heart ventricles of virgin and pregnant hypothyroid rats. The hypothyroid state in virgin rats was induced by PTU (100 mg/l) in drinking water for 30 days. Subsequently, rats were sacrificed on diestrus of the estrous cycle, to avoid other hormonal influences. For the hypothyroidism study at the end of pregnancy, virgin rats were given PTU (100 mg/l) in the drinking water, 8 days before mating and the treatment continued until day 21 of pregnancy, at which point they were sacrificed. Female rats were mated with males on the evening of proestrus. The presence of sperm in the vaginal discharge was considered day 0 of pregnancy.

Rats were fasted overnight before sacrifice at 09:00 h by decapitation; then, the trunk blood samples were collected for serum separation. The heart was quickly excised, washed, and the ventricles were immediately placed in liquid nitrogen or used as fresh tissue as needed.

Thyroid hormones concentration

Serum thyroid hormone levels were determined by chemiluminescence assay. Free tetraiodothyronine (FT_4) and thyroid-stimulating hormone (TSH) were determined by using the commercial Bayer Health Care kit for the autoanalyzer ACS: 180 PLUS (Ciba Corning, Bayer Health Care, MA, USA). The intra-assay coefficient of variation was 1.54 % for the estimation of FT_4 at 1.42 ng/dl, and 3.01 % for TSH at 18.53 µIU/ml.

Thiobarbituric acid reactive substance (TBARS)

Lipid peroxidation in serum and heart homogenate were measured as malondialdehyde (MDA), which is the end product of lipid peroxidation and reacts with thiobarbituric acid (TBA), as TBARS, to produce a red coloured complex. For the determination, we used 1 ml of homogenate (fresh tissue in relation 1/10 (w/v) with PBS 30 mM, KCl 120 mM, pH 7.4), or 100 µl of serum, to which 1 ml of 20 % trichloroacetic acid (TCA, Sigma) was added, and incubated at ice bath for 30 min, to remove proteins. After centrifugation at 3 000 rpm for 10 min, 1 ml of supernatant was incubated with a 0.7 % TBA solution for 60 min at 100 °C to measure the TBARS content. The samples absorbance was measured spectrophotometrically at 535 nm [19]. An acid hydrolysis product of 1,1,3,3-tetramethoxypropane (TMP) was used as a standard. Protein concentration was determined by the Biuret reaction [20]. The TBARS levels in serum were expressed as nmol of MDA per ml serum, and in heart as nmol of TBARS per mg protein.

Measurement of PON-1/arylesterase activity

PON-1 activity toward phenyl acetate (arylesterase activity) was determined by measuring the initial rate of substrate hydrolysis in the assay mixture (0.7 ml) containing 2.8 mM of the substrate, 1 mM of $CaCl_2$ and 1 µl of serum in 20 mM Tris-HCl (pH 8). The absorbance was monitored for 2 min at 270 nm. A blank sample prepared as described above, but without serum, was included to correct for the spontaneous hydrolysis of phenyl acetate. Results were expressed as enzyme international units per ml of serum (1 unit corresponds to the amount of arylesterase required to hydrolyse 1 µmol phenyl acetate per min [21]).

Protein oxidation assay

Heart homogenates (fresh tissue in a 1/10 (w/v) relation with buffer Hepes 50 mM, KCl 125 mM, pH 7.4) were incubated with 2,4-dinitrophenylhydrazine (10 mM) in 2 M HCl solution for 1 h at room temperature. About 20 % TCA (w/v) was added to the samples, and centrifuged to collect protein precipitates. The pellets were washed twice with ethanol/ethyl acetate (1:1) (v/v). The final precipitates were dissolved in 6 mol/l guanidine hydrochloride solution, and left for 15 min at 37 °C. Afterwards, their absorbance values were measured at 360 nm. Results were calculated using the extinction molar coefficient $E = 22\,000\ M^{-1}cm^{-1}$, and expressed as nmol of carbonyl groups/mg protein [22].

Nitrites

Nitrite levels were measured spectrophotometrically by the Griess method [23]. To determine the nitrites released from heart, the fresh ventricle slices (100 mg) were cut and incubated in 500 µl PBS buffer, pH 7.4, at 37 °C. After 2 h of incubation with stirring, the reaction was stopped by the addition of 50 µl of 5 %

Parameters	Control virgin	Hypothyroid virgin	Control pregnant	Hypothyroid pregnant
FT_4 (ng/dl)	2.85 ± 0.28	0.11 ± 0.01‡	1.54 ± 0.29§	<0.1 undetectable‡
TSH (μUI/ml)	1.14 ± 0.23	33.86 ± 5.10‡	0.68 ± 0.13	17.65 ± 1.24‡,§
Body weight (g)	203.7 ± 0.84	157.8 ± 4.10†	363.6 ± 5.04§	319 ± 18.46†,§
Heart weight (g)	0.76 ± 0.02	0.53 ± 0.02‡	0.86 ± 0.05¶	0.77 ± 0.05§
Heart/Body weight (mg/g)	0.38 ± 0.01	0.33 ± 0.01*	0.24 ± 0.03§	0.24 ± 0.03¶

Results are reported as mean ± SEM (n = 5)

FT_4: Free tetraiodothyronine; TSH: thyroid-stimulating hormone

‡p < 0.001, †p < 0.01 and *p < 0.05 vs. the respective control group, and §p < 0.001 and ¶p < 0.05 vs. the respective virgin group

Table 1 Assessment of the rats' hormonal state.

phosphoric acid. The supernatant (300 μl), which was diluted to 500 μl with distilled water, was used as a sample to measure nitrites. For serum nitrites measurement, 500 μl of serum were used as sample. The samples were immediately mixed with Griess reagent [sulfanilamide with *N*-(1-naphthyl)ethylenediamine HCl]. After 30 min of incubation at room temperature, absorbance was recorded at 540 nm, and μmoles of nitrite were determined using a standard curve. The results were expressed in μmol/mg protein of heart tissue or μmol/ml of serum.

Antioxidant enzyme activities

Homogenates were prepared with fresh heart ventricles in a 1/20 relation with 30 mM PBS buffer and 120 mM KCl, pH 7.4, containing 1X protease inhibitors; this was followed by centrifugation at 2 000 rpm for 15 min at 4 °C.

CAT activity was determined by measuring the decrease in absorption at 240 nm, in a reaction medium containing 50 mM phosphate buffer (pH 7.3) and 3 mM H_2O_2 [24]. The results were expressed as enzyme international units per mg protein (1 IU decomposes 1 μmol H_2O_2/min at pH 7 at 25 °C).

GPx activity was determined following NADPH oxidation at 340 nm in a reaction medium containing 0.2 mM GSH, 0.25 U/ml yeast glutathione reductase, 0.5 mM *tert*-butyl hydroperoxide (BHT), and 50 mM phosphate buffer (pH 7.2) [25]. Results were expressed as milli International Units/mg protein (1 IU oxidizes 1 μmol NADPH/min at pH 7.7 at 30 °C).

Copper-zinc superoxide dismutase (CuZnSOD) activity was determined on the basis of its inhibitory action on the rate of superoxide-dependent reduction of cytochrome c by xanthine-xanthine oxidase at 560 nm. The reaction medium contained 50 mM phosphate buffer (pH 7.8), 50 mM xanthine, 20 mM cytochrome c, and xanthine oxidase to detect 0.025 absorbance units/min [26]. One unit of SOD was defined as the amount of enzyme that inhibits cytochrome c reduction by 50 %.

Western blot analysis for iNOS and eNOS

Hearts were homogenized as described previously. Fifty μg of protein was mixed with 3 μl of sample buffer (250 mM Tris-HCl, 4 % SDS, 4 % β-mercaptoethanol, 0.002 % bromophenol blue and 40 % glycerol), boiled for 2–3 min and loaded into a 12 % SDS-PAGE gel. Protein molecular mass markers were always loaded on each gel. The separated proteins were transferred to PVDF membranes (Immobilon, Millipore USA) using BioRad blot system. After being blocked overnight with 5 % milk-TBS (20 mMTris, 0.9 % NaCl, pH 7.4) at 4 °C, membranes were incubated with a primary rabbit anti-iNOS and an anti-eNOS polyclonal antibody solution (Santa Cruz Biotechnology) for 1 h at room temperature. After washing 3 times with TTBS (0.05 % Tween 20, TBS 1X), membranes were incubated with a goat-anti-rabbit IgG secondary antibody and linked to peroxidase for 2 h at room temperature. Membranes were washed again and the color was developed using a Vectastain ABC-detection system (Vector Labs). Actin was determined as an internal control using an anti-actin antibody (Santa Cruz, Biotechnology). Band intensities were quantified using NIH ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/).

RNA isolation and semi-quantitative RT-PCR analysis

Total RNA was isolated from frozen heart ventricle by using TRIzol (Invitrogen) as indicated by the manufacturers. Three μg of total RNA were reverse-transcribed using random primer hexamers (Biodynamics, SRL) and M-MRV reverse transcriptase (Promega) at 37 °C for 1 h. PCR amplification was carried out using specific oligonucleotide primers (**Table 1S**). A cDNA aliquot (1/10 of the RT reaction product) was amplified with a PCR master mix using Taq DNA polymerase. The reaction samples were heated to 95 °C for 5 min (10 min for thyroid receptors), followed by 35 temperature cycles; each cycle consisted of: 94 °C for 60 s (95 °C for 15 s for thyroid receptors), 60 °C for 60 s, and 72 °C for 60 s. After 35 reaction cycles, the extension reaction (72 °C) was continued for another 5 min. Bands were resolved in a 2 % agarose gel containing GelRed (Genbiotech) to visualize the band. Bands intensities were quantified using NIH ImageJ software. Relative amounts of mRNA were expressed as the ratio of band intensity for the target genes relative to that for β-actin.

Statistical analyses

Statistical analyses were performed using two-way ANOVA followed by the Bonferroni post test. All results were expressed as the mean ± S.E.M. Differences between means were considered significant at $p < 0.05$ level.

Results

Hypothyroidism development

Administration of PTU in the drinking water to virgin and pregnant rats, produced a decrease in serum FT_4 and an increase in serum TSH, confirming the hypothyroid state (**Table 1**). Circulating FT_4 and TSH levels were always higher in virgin than in pregnant rats.

Total body weight was reduced in hypothyroid virgin and pregnant rats, in relation to their respective controls. Heart weight and heart/body weight ratio were lower in hypothyroid virgin rats, while they were not modified in pregnant rats, compared with their respective controls (**Table 1**).

Hypothyroid state on the expression of heart thyroid hormone receptors

Hypothyroid virgin rats exhibited a decrease of TRα and TRβ mRNA expressions, while hypothyroid pregnant rats showed a decrease of TRα mRNA expression in the heart, compared with their respective controls. However, the mRNA levels of TRα and

TRβ in the hearts of both, hypothyroid, and control rats were lower in pregnant than in virgin rats (**Fig. 1**).

Hypothyroid state on serum oxidative stress parameters

Serum levels of TBARS in both virgin and pregnant rats were not affected by hypothyroidism. The PON-1 activity decreased in hypothyroid virgin and pregnant rats, compared with their respective controls. The concentration of nitrites was significantly lower in hypothyroid pregnant rats, without change in virgin rats, compared with their respective controls. All these serum oxidative parameters were increased in pregnant rats, compared with virgin rats (**Table 2**).

Hypothyroid state on heart oxidative stress parameters

In the heart of virgin and pregnant hypothyroid rats, TBARS levels did not differ significantly from the control values (**Table 2**). Heart carbonyl groups content was increased in hypothyroid virgin rats, while it remained unchanged in hypothyroid pregnant rats, compared with their respective controls. Hypothyroidism decreased nitrite levels in the heart of both virgin and pregnant rats. The pregnancy produced an increase of TBARS and nitrite contents in the heart of hypothyroid and control rats (**Table 2**).

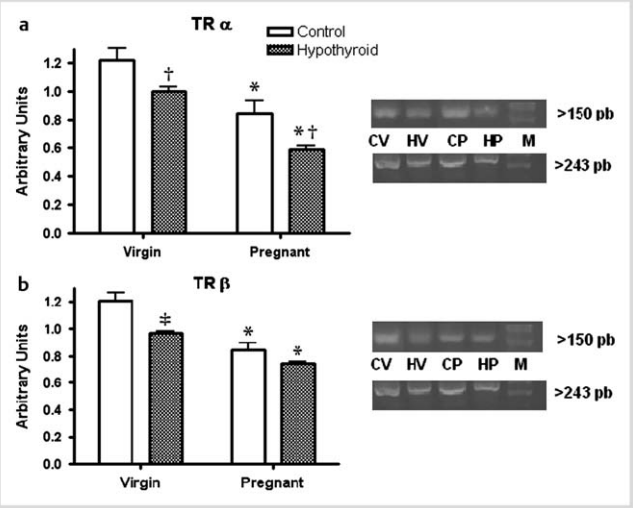

Fig. 1 Thyroid receptors expression in the heart of hypothyroid rats. Representative RT-PCR analysis for **a** TRα (150 pb) and **b** TRβ (150 pb). Transcript levels were measured by RT-PCR and normalized against β-actin mRNA levels (243 pb). M: molecular weight marker. Values are expressed as mean ± SEM (n = 5). *p < 0.01 vs. the respective virgin group, and †p < 0.05 and ‡p < 0.01 vs. the respective control group.

Hypothyroid state on heart eNOS and iNOS protein expression

Western blot analysis showed that hypothyroidism did not modify iNOS and eNOS protein expressions in heart of virgin rats, while it showed a decrease of iNOS, without change of eNOS, in heart of pregnant rats, compared with their respective controls. Pregnancy produced an increase of iNOS and eNOS protein expressions in hypothyroid and control rats (**Fig. 2**).

Hypothyroid state on heart antioxidant enzymes activities

The activities of both SOD and CAT, were not affected by the hypothyroid state in virgin and pregnant rats, but they were increased in pregnant rats compared with virgins. The GPx activity was increased in heart of hypothyroid virgin rats, while it was unchanged in pregnant rats, compared with their respective controls (**Fig. 3**).

Hypothyroid state on heart antioxidant enzymes mRNA levels

To evaluate the effects of the thyroid hormone on mRNA abundance of oxidant and antioxidant enzymes, the mRNA expression of NOX, SOD, CAT, and GPx were measured using semiquantitative RT-PCR of total RNA, prepared from heart of virgin and pregnant rats. Heart NOX-2 mRNA expression was increased, while the antioxidant enzymes SOD, CAT and GPx mRNA expressions were not influenced by thyroid status in virgin rats, compared with control. The mRNA levels of NOX-2, SOD, CAT, and GPx did not show any significant change in heart of hypothyroid pregnant rats, compared with their control. Finally, NOX-2, SOD, and CAT mRNA were higher in pregnant than in virgin rats (**Fig. 4**).

Discussion

This study describes, for the first time, the effects of hypothyroidism on the redox balance and thyroid receptors expression in heart of pregnant rats. Normal pregnancy is characterized by significant changes in the cardiovascular system [17]. Pregnant rats showed a higher PON-1 serum activity, associated to an increase of HDLc levels (**Table 2S**), compared with virgin rats.

Table 2 Hypothyroidism on serum and heart oxidative stress parameters.

Parameters	Control virgin	Hypothyroid virgin	Control pregnant	Hypothyroid pregnant
Serum				
TBARS (nmol/ml)	5.72 ± 1.73	3.25 ± 0.61	21.73 ± 0.91§	18.13 ± 2.10§
PON-1 (U/ml)	726.1 ± 39.09	355.5 ± 37.25‡	1 265 ± 142.41§	767.1 ± 75.10‡,§
Nitrites (μmol/ml)	13.60 ± 4.28	13.41 ± 4.95	106.51 ± 16.70§	37.15 ± 6.12‡,§
Heart				
TBARS (nmol/mg protein)	0.18 ± 0.01	0.18 ± 0.01	0.31 ± 0.01§	0.34 ± 0.02§
Carbonyl G. (nmol/mg protein)	6.48 ± 0.95	10.41 ± 1.20*	7.93 ± 0.43	5.83 ± 0.39¶
Nitrites (μmol/mg protein)	1.14 ± 0.13	0.57 ± 0.02*	2.22 ± 0.09§	1.49 ± 0.16§,*

Results are reported as mean ± SEM (n = 5)

Lipid peroxidation was measured as TBARS (thiobarbituric acid reactive substances). Serum PON-1 (Paraoxonase-1) activity, was measured using phenyl acetate as substrate. Nitrites were measured using the Griess reagent. Heart protein oxidation was determined by carbonyl-groups

‡p < 0.001 and * p < 0.05 vs. the respective control group, and §p < 0.001 and ¶p < 0.05 vs. the respective virgin group

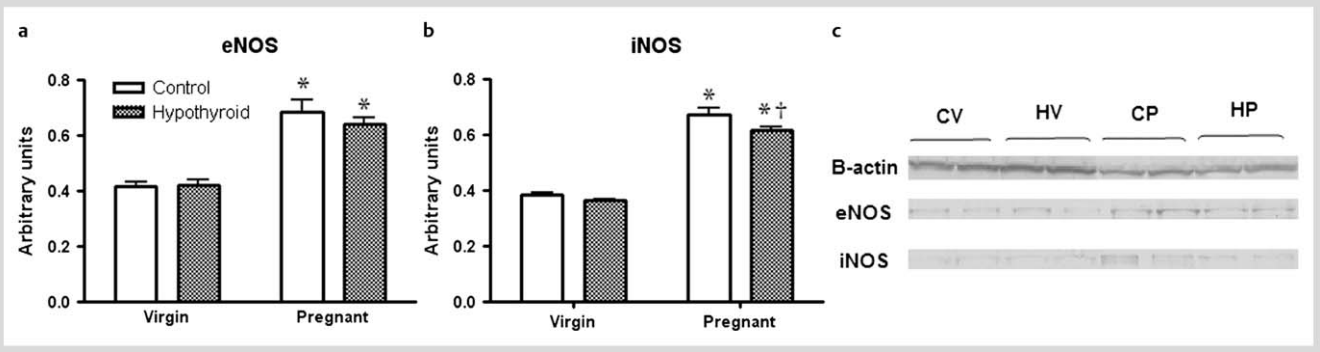

Fig. 2 Western blot analysis in cardiac homogenates using **a** eNOS, endothelial nitric oxide synthase antibody, and **b** iNOS, inducible nitric oxide synthase antibody. Representative inmunoblot shows eNOS (140 kDa), iNOS (130 kDa), and β-actin (43 kDa) **c**. Protein levels in relation to β-actin are expressed as arbitrary units. Results are reported as mean ± SEM (n = 4). *p < 0.001 vs. the respective virgin group, and †p < 0.05 vs. the respective control group.

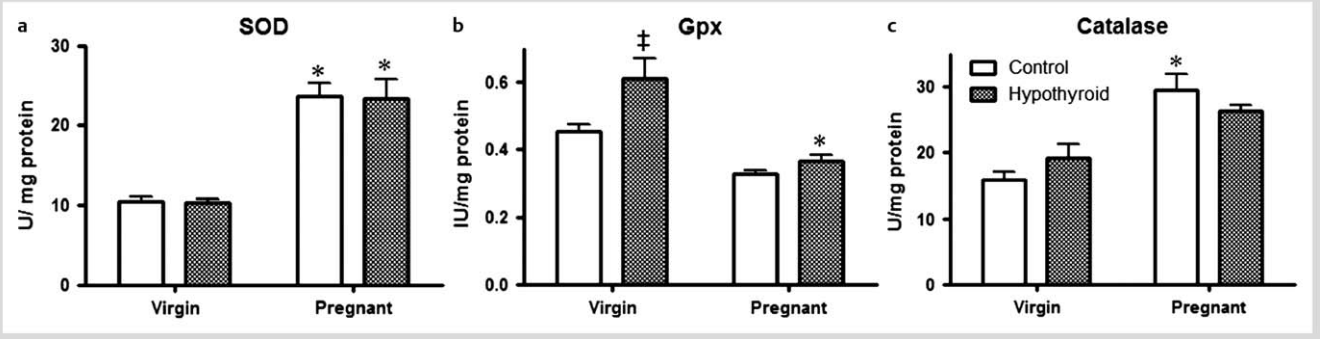

Fig. 3 Enzyme activities in the rat heart of **a** SOD, superoxide dismutase; **b** CAT, catalase; and **c** GPx, glutathione peroxidase. Results are reported as mean ± SEM (n = 5). ‡p < 0.05 vs. the respective control group, and *p < 0.001 vs. the respective virgin group.

Although PON-1 is a HDL associated enzyme with the ability to hydrolyze oxidized lipids in lipoproteins [16], an altered antioxidant protection in the circulating lipids of pregnant rats was observed. Here, pregnancy caused an increase of the serum TBARS, in agreement with previous studies [27] and an increase of TBARS in the heart. The altered redox environment in serum and heart was also supported by the higher serum and heart nitrite levels observed in pregnant rats, compared with virgin rats. This was associated to the increase in heart eNOS and iNOS protein expression, suggesting a high production of endogenous NO biosynthesis. Nitric oxide (NO) is a free-radical released in oxidative stress [12]. Therefore, the higher NOX-2 mRNA expression observed in the heart of pregnant rats, might increase the $O_2^{\bullet-}$ production, leading to peroxynitrite formation.

Moreover, the increase of the mRNA expression and activity of SOD and CAT, antioxidant enzymes that function together in a way linked to the formation and dissociation of H_2O_2, suggested an activation of the antioxidant response in the heart of pregnant rats. To the best of our knowledge there is no background information concerning any changes in oxidative state of the rat heart that may be related to the oestrous cycle, but there is some information regarding other tissues, such as rat liver [28] and mouse ovary [29]. It is known that estradiol is an antioxidant hormone [30] and that its circulating levels change during rat pregnancy. In addition, oxidative stress damage has been observed in cardiomyocytes and vascular smooth muscle cells in the aorta of the GH-transgenic rat [31]; and a variation of the growth hormone circulating levels has been observed during pregnancy [32]. Therefore, it is conceivable that cardiac redox state is under hormonal control.

It is known that TRs play an important role in mediating the cardiac physiological actions of T_3 [2]. We observed that pregnancy decreased the mRNA levels of both TRα and TRβ in the heart of euthyroid rats, while hypothyroidism induced an even bigger decrease of the heart TRα mRNA expression. In addition, hypothyroidism modified the TRs expression in the hearts of virgin rats by decreasing both TRα and TRβ mRNA levels. Therefore, it is reasonable to associate the TR expressions in the heart with the T4 circulating levels. In this sense, during hyperthyroidism, the upregulation of TRα1 mRNA expression in the rat heart has been related to the high level of thyroid hormones [4]. Thus, the low T_4 levels observed in pregnancy, and also in the hypothyroid state of pregnant and virgin rats, could be associated, at least in part, with the decreased TRα mRNA expression in the heart. Abnormal expression of TR subtypes in the myocardial and coronary arteries are closely associated with the pathogenesis of heart failure [33, 34]. Comparison of cardiac phenotype between TRα and TRβKO mice, clearly indicates that TRα exerts a predominant effect of cardiac electrophysiological phenomena, such as impulse generation and mechanical functions [4].

Thyroid hormones are important factors involved in the regulation of oxidative metabolism. We did not observe lipoperoxidation in serum of hypothyroid virgin rats, since TBARS concentration was not modified compared to control. This can possibly be related to the low levels of serum triglycerides [35] and heart triglycerides (**Fig. 1S**). However, parameters of oxidative stress, other than TBARS, were modified in the serum and heart of these animals.

A decrease of the serum PON-1 activity in hypothyroid virgin rats was observed, as it has been found in serum of hypothyroid

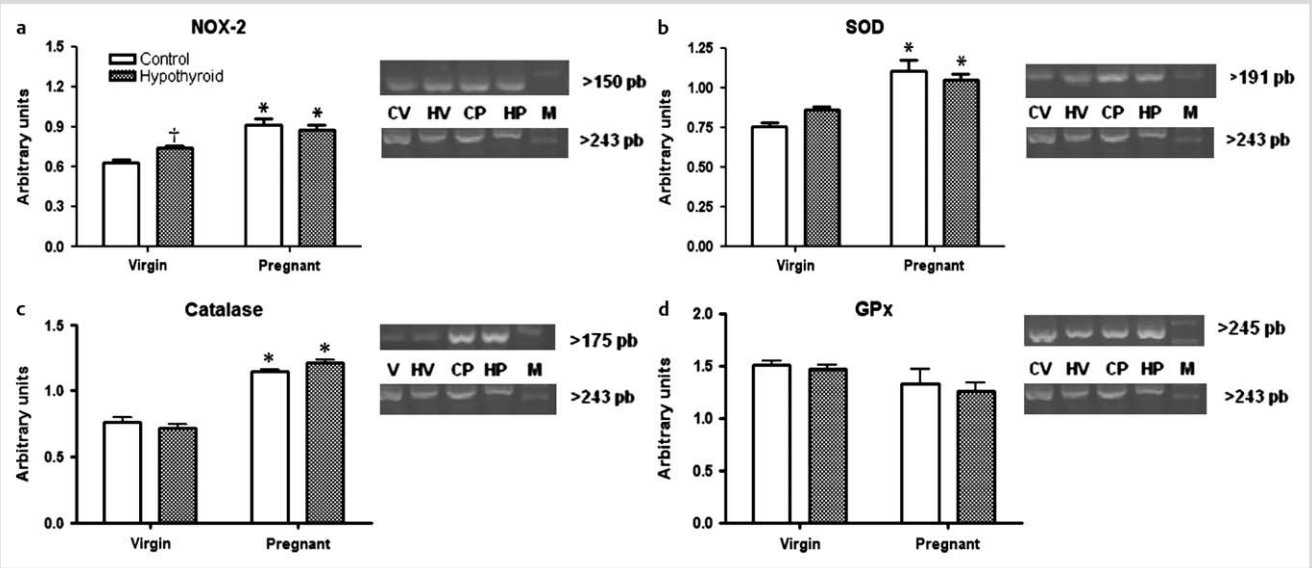

Fig. 4 Effect of hypothyroidism on the mRNA expression of the enzymes involved in the redox state of the heart. Representative RT-PCR analysis for **a** NOX-2, NADPH oxidase-2 (150 pb); **b** SOD, superoxide dismutase (191 pb); **c** CAT Catalase (175 pb); and **d** GPx, glutathione peroxidase (245 pb). Transcript levels were measured by RT-PCR and normalized against β-actin mRNA levels (243 pb). M: molecular weight marker. Values are expressed as mean ± SEM (n = 5). *p < 0.01 vs. the respective virgin group, and †p < 0.05 vs. the respective control group.

male rats [15]. An imbalance of the redox state in heart of hypothyroid virgin rats was suggested by the high mRNA expression of NOX-2, as well as the increase of carbonyls content that indicates myocardial protein oxidation. The superoxide anion could not be properly eliminated, since mRNA expression and activity of SOD and CAT, were not modified in the heart of virgin rats, as well as the protein (**Fig. 2S**) and mRNA levels of GPx. Only an increase in the GPx activity was observed in the heart of hypothyroid virgin rats, suggesting an enzyme postraductional modification. A high GPx activity has been recently reported in the liver of hypothyroid rats [36].

Hypothyroidism in pregnant rats caused a decrease of the serum PON-1 activity, as it was observed in hypothyroid virgin rats, and a marked reduction in serum and heart nitrites, as well as in the heart iNOS protein. The alteration of nitrites/iNOS could be explained, at least in part, by the low circulating estradiol levels that have been reported in hypothyroid rats, on days 20 and 21 of pregnancy, compared with euthyroid pregnant rats [32]. It is known that estradiol stimulates NOS activity and NO production in the heart [37]. Inhibition of serum NOS has been associated to pathological cardiovascular processes in pregnant rats [38]. Despite these changes, the TBARS content in serum and heart, the activity and mRNA expression of SOD, CAT, and GPx, and the levels of NOX-2 mRNA, were not affected in the heart of hypothyroid pregnant rats, indicating that pregnancy may counter the effects of hypothyroidism in the heart. The lack of change in the induction of heart SOD expression by the hypothyroid state, might be associated to the decrease of TRα in the heart of hypothyroid virgin and pregnant rats. It has been shown that unliganded TR activates the SOD-1 promoter, while T_3 reverses this effect [39].

In conclusion, our results demonstrate that hypothyroidism alters TR expressions and redox state of rat heart depending on the physiological state. While the hypothyroid condition decreases TRα and TRβ expressions and induces oxidative stress in the heart of virgin rats, it decreases TRα mRNA levels and does not alter the redox balance in the heart of pregnant rats. Pregnancy per se seems to protect the heart against oxidative stress induced by hypothyroidism.

Understanding the complex function of thyroid hormones and their receptors on redox balance in the heart could provide potential benefits in the prevention of cardiovascular injury induced by hypothyroidism.

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Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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