The role of redox signaling in cardiac hypertrophy induced by experimental hyperthyroidism

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Abstract

This study was conducted to test whether oxidative stress activates the intracellular protein kinase B (AKT1) signaling pathway, which culminates with cardiac hypertrophy in experimental hyperthyroidism. Male Wistar rats were divided into four groups: control, vitamin E, thyroxine (T4), and T4 + vitamin E. Hyperthyroidism was induced by T4 administration (12 mg/l in drinking water for 28 days). Vitamin E treatment was given during the same period via s.c. injections (20 mg/kg per day). Morphometric and hemodynamic parameters were evaluated at the end of the 4-week treatment period. Protein oxidation, redox state (reduced glutathione, GSH/glutathione disulfide, GSSG), vitamin C, total radical-trapping antioxidant potential (TRAP), hydrogen peroxide (H2O2), and nitric oxide metabolites (NOX) were measured in heart homogenates. The p-AKT1/AKT1, p-GSK3B/GSK3B, FOS, and JUN myocardial protein expression were also quantified by western blot after 4 weeks. Increases in biochemical parameters, such as protein oxidation (41%), H2O2 (62%), and NOX (218%), and increase in the left ventricular end-diastolic pressure were observed in the T4 group. T4 treatment also caused a decrease in GSH/GSSG ratio (83%), vitamin C (34%), and TRAP (55%). These alterations were attenuated by vitamin E administration to the hyperthyroid rats. Expression of p-AKT1/AKT1, p-GSK3B/GSK3B, FOS, and JUN were elevated in the T4 group (by 69, 37, 130, and 33% respectively), whereas vitamin E administration promoted a significant reduction in their expression. These results indicate that oxidative stress plays an important role in cardiac hypertrophy, and suggest redox activation of AKT1 and JUN/FOS signaling pathways with H2O2 acting as a possible intracellular mediator in this adaptive response to experimental hyperthyroidism.

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Introduction

Cardiac hypertrophy is an adaptive response to a variety of stimuli, including volume and pressure overload and neurohormonal activation (Donatelli et al. 2003, Hu et al. 2003). Although cardiomyocyte growth can be a beneficial adaptation, chronic exposure to such stimuli can lead to maladaptive hypertrophy resulting in dilated cardiomyopathy and heart failure (Li et al. 2007). In a previous study, we demonstrated a positive correlation between cardiac hypertrophy and oxidative stress in experimental hyperthyroidism, indicating that reactive oxygen species (ROS) may contribute to the progression to heart failure in this model (Araujo et al. 2006).

ROS are integral to the induction and maintenance of many signal transduction pathways involved in cell growth and differentiation (Valko et al. 2007). This signal transduction modality is triggered by numerous extracellular signaling factors such as hormones, growth factors, cytokines, and neurotransmitters (Thannickal et al. 2000). ROS not only modulate a variety of transcription factors (e.g., activator protein 1 (JUN)) but also act as second messengers in the coordination of important cellular functions such as proliferation and programmed cell death (Kaur et al. 2008). For example, IGF1 interacts with its receptor (IGF1R), which is abundantly expressed in myocardium, to stimulate proliferation of cardiomyocytes both in vivo and in vitro (Das et al. 2004). Our own group has also demonstrated that IGF1R expression can be modulated via alterations in the cellular redox state, controlling cardiomyocyte growth in experimental hyperthyroidism (Araujo et al. 2007). IGF may also activate the AKT1 (protein kinase B) signaling pathway, which has been implicated in the regulation of cardiac growth (Antos et al. 2002). The AKT1 family of serine/threonine protein kinases is stimulated classically by tyrosine kinase receptors, mediated by the action of phosphatidylinositol 3-kinase. AKT1 pathway induction has been implicated in cardiac mass increase, through the activation of FRAP1-dependent pro-growth pathways and suppression of...
GSK3B-dependent atrophy phenomenon (Antos et al. 2002). The increase in basal AKT1 expression also results in improvement in cardiac contractility and hypertrophy (Condorelli et al. 2002). Importantly, it was recently demonstrated that thyroxine (T4) activates the AKT1 signaling pathway in myocardium and that this process contributes to the cardiac hypertrophy associated with this model of hyperthyroidism (Kenessey & Ojamaa 2006). However, the mechanisms of AKT1 pathway activation in experimental hyperthyroidism remain unknown.

Recent evidence point to a non-transcriptional action of thyroid hormone through membrane-initiated processes or the cytosolic thyroid hormone receptor (Kenessey & Ojamaa 2006). Tanaka et al. (2005) demonstrated that conformational changes in membrane proteins due to cellular oxidative stress may activate or inhibit the AKT1 pathway, inducing phosphorylation and activation of endothelial nitric oxide synthase (NOS3). Thus, the oxidation of the –SH group on protein cysteine residues can be very important in the modulation of signaling pathways, such as AKT1.

Thus, our objective was to demonstrate the role of oxidative stress in modulating the signaling pathways involved in cardiac hypertrophy in experimental hyperthyroidism.

**Materials and methods**

**Animals**

Forty male Wistar rats (200 ± 20 g) were obtained from the Central Animal House of the Universidade Federal do Rio Grande do Sul, Brazil. Animals were housed in plastic cages (five animals each) and received ad libitum water and pelleted food. They were maintained under standard laboratory conditions (controlled temperature of 21 °C, 12 h light:12 h darkness cycle). Animals were weighed weekly to follow body weight gain during the experimental protocol (28 days). They were divided into four groups (n = 10/group) as follows: I) control (receiving water ad libitum and s.c. injections of mineral oil); II) vitamin E (receiving s.c. injections of vitamin E (20 mg/kg per day, in mineral oil; Chitra & Mathur 2004)); III) T4 (receiving l-T4 (12 mg/l in drinking water; Ladenson et al. 1986)); and IV) T4 + vitamin E (receiving T4 and vitamin E, according to the conditions as previously described). All the animals were carefully monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and Brazilian College of Animal Experimentation.

**T4 concentration**

Blood samples were collected, through the right carotid artery cannulated with a PE 50 catheter, and immediately centrifuged at 1000 g for 10 min. Serum T4 concentration was estimated by chemiluminescence using the Immunolite 2000 kit (Biomedical Technologies, Inc., Strougerton, MA, USA) at Weinmann Clinical Analysis Laboratory.

**Hemodynamic and cardiac hypertrophy development measurements**

Cardiac hemodynamic parameters were assessed at the end of the fourth week of treatment. In brief, rats were anesthetized (ketamine 90 mg/kg; xylazine 10 mg/kg, i.p.) and the right carotid artery was cannulated with a PE 50 catheter connected to a strain gauge transducer (Narco Biosystem Pulse Transducer RP-155, Houston, TX, USA) linked to a pressure amplifier (HP 8805C, Hewlett Packard). Pressure readings were taken in a microcomputer equipped with an analog-to-digital conversion board (CODAS 1 kHz sampling frequency, Dataq Instruments, Inc., Akron, OH, USA). The catheter was advanced into the left ventricle (LV) to record the left ventricular systolic pressure (LVSP, mmHg) and the left ventricular end-diastolic pressure (LVEDP, mmHg).

Cardiac hypertrophy was evaluated by heart weight (mg) to body weight (g) ratio.

**Tissue preparation**

At the end of the 4-week treatment period, rats were decapitated and the hearts were rapidly excised, weighed, and homogenized (1:15% w/v KCl and phenyl methyl sulfonl fluoride phenylmethylsulfon fluoride 20 mmol/l) in Ultra-Turrax. The suspension was centrifuged at 1000 g for 10 min at 0–4 °C to remove cell debris (Ljesuy et al. 1985) and supernatants used for the assay of protein oxidation (carbonyl groups), total radical-trapping antioxidant potential (TRAP), and NO metabolites. At the time of killing, some cardiac tissue samples were also removed and frozen at −80 °C for the evaluation of glutathione content and protein expression.

**Protein oxidation assay**

Tissue samples were incubated with 2,4 dinitrophenyl-hydrazine (10 mol/l) in 2.5 mol/l HCl solution for 1 h at room temperature, in the darkness. About 20% TCA (w/v) was added to the samples, which were then left on ice for 10 min and centrifuged for 5 min at 1000 g to collect protein precipitates. The pellets were washed once with 10% TCA (w/v) and then thrice with ethanol:ethyl acetate (1:1) (v/v). The final precipitates were dissolved in 6 mol/l guanidine hydrochloride solution, left for 10 min at 37 °C, their absorbance values measured at 360 nm, and the results expressed in nmol/mg protein (Reznick & Packer 1994).
**Determination of oxidized and reduced glutathione concentration**

To determine oxidized and reduced glutathione concentration, tissue was deproteinized with 2 mol/l perchloric acid, centrifuged for 10 min at 1000 g, and the supernatant was neutralized with 2 mol/l potassium hydroxide. The reaction medium contained 100 mmol/l phosphate buffer (pH 7.2), 2 mmol/l nicotinamide dinucleotide phosphate acid, 0.2 U/ml glutathione reductase, and 70 μmol/l 5,5′ dithiobis (2-nitrobenzoic acid). To determine reduced glutathione, the supernatant was neutralized with 2 mol/l potassium hydroxide, to react with 70 μmol/l 5,5′ dithiobis (2-nitro benzoic acid), and the absorbance values measured at 420 nm (Akerboom & Sies 1981).

**Vitamin C determination**

To determine vitamin C concentration, samples were incubated for 30 min at 25 °C with tungstic acid reagent (suspension of sodium tungstate and sodium hydrogen phosphate anhydrous in deionized water, mixed with heating to dissolve and slowly added concentrated sulfuric acid. The solution was heated for 2 h with reflux condenser to not allow it to boil, and after this the solution was cooled down). This mixture was centrifuged for 15 min at 1000 g. Absorbance values of the supernatant and the vitamin C standard solution (diluted in 50 mmol/l oxalic acid solution) were measured at 700 nm (Kyaw 1978).

**TRAP**

TRAP, which indicates the total antioxidant capacity present in a homogenate, was measured by chemiluminescence using 2,2′-azo-bis(2-aminopropane) (ABAP, a source of alkyl peroxy free radicals) and luminol. A mixture consisting of 20 mmol/l ABAP, 40 μmol/l luminol, and 50 mmol/l phosphate buffer (pH = 7-4) was incubated to achieve a steady-state luminescence from the free radical-mediated luminol oxidation. A calibration curve was obtained by using different concentrations (between 0-2 and 1 μmol/l) of Trolox (hydrophilic vitamin E; Eyelson et al. 2001). Luminescence was measured in a liquid scintillation counter using the out-of-coincidence mode and the results were expressed in units of Trolox/mg protein.

**Determination of hydrogen peroxide**

Hydrogen peroxide was measured via its horseradish peroxidase (HRP)-mediated oxidation of phenol red, leading to the formation of a compound measurable at 610 nm. Slices of fresh tissue from ventricles were incubated for 30 min at 37 °C in phosphate buffer 10 mmol/l (NaCl 140 mmol/l and dextrose 5 mmol/l). The supernatants were transferred to tubes with 0-28 mmol/l phenol red and 8-5 U/ml HRPO. After 5-min incubation, 1 mol/l NaOH was added and the solution’s absorbance values measured at 610 nm. The results were expressed in nmoles H₂O₂/g tissue (Pick & Keisari 1980).

**Determination of NO metabolites (NOₓ)**

Nitrites (NO₂) 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 sulfanilamide (1%). Nitrates (NO₃) were determined as total nitrites (initial nitrite plus nitrate reduced from nitrate) after its reduction using nitrate reductase from Aspergillus species in the presence of DUOX1. A standard curve was established with a set of serial dilutions (10⁻⁸–10⁻³ mol/l) of sodium nitrite. Results were expressed as mmol/mg protein of nitrites plus nitrites (Granger et al. 1999).

**Western blot analysis**

Tissue homogenization, electrophoresis, and protein transference were performed as described elsewhere (Laemmli 1970, Araujo et al. 2006). The membranes were processed for immunodetection using rabbit anti-total AKT1 polyclonal antibody, rabbit anti-phospho-AKT1 (ser657; 60 kDa), rabbit anti-total GSK3B polyclonal antibody, rabbit anti-phospho-GSK3B (ser9; 47 kDa), rabbit anti-JUN polyclonal antibody (39 kDa), and goat anti-FOS polyclonal antibody, rabbit anti-phospho-GSK3B (ser9; 47 kDa), rabbit anti-phospho-AKT1 (ser657; 60 kDa), rabbit anti-total GSK3B polyclonal antibody, goat anti-FOS polyclonal antibody, rabbit anti-total AKT1 polyclonal antibody, rabbit anti-phospho-AKT1 (ser657; 60 kDa), rabbit anti-total GSK3B polyclonal antibody, rabbit anti-phospho-GSK3B (ser9; 47 kDa), rabbit anti-JUN polyclonal antibody (39 kDa), and goat anti-FOS polyclonal antibody (62 kDa; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The bound primary antibodies were detected using rabbit anti-goat or goat anti-rabbit HRP-conjugate secondary antibodies and membranes were revealed for chemiluminescence. The autoradiographies generated were quantitatively analyzed for the protein levels with an image densitometer (Image master VDS CI, Amersham Biosciences Europe). The molecular weights of the bands were determined by reference to a standard molecular weight marker (RPN 800 rainbow full range Bio-Rad). The results from each membrane were normalized via the Ponceau red staining method (Klein et al. 1995).

**Determination of protein concentration**

Protein was measured by the method of Lowry et al. (1951), using BSA as the standard.
**Statistical analysis**

Data were expressed as mean ± s.d. To compare multiple groups, we used two-way ANOVA with Student–Newmann–Keuls *post hoc* test. The correlation between two variables was analyzed by Pearson’s correlation. Values of *P*<0·05 were considered statistically significant.

**Results**

**Cardiac remodeling in hyperthyroidism**

At the end of the 4-week post-treatment period, serum T4 levels were significantly increased by 512 and 500% in T4 and T4 + vitamin E groups respectively, as compared with their respective controls (Table 1). T4 group exhibited cardiac hypertrophy (60% higher than control) that was significantly reduced in T4 + vitamin E group (23% lower than T4; Table 1). Data from cardiac catheterization revealed that T4 induced an elevation in LVSP by 44% compared with control. Nevertheless, LVSP was significantly reduced (by 20%) in the T4 + vitamin E group, when compared with the T4 group (Table 1). LVEDP was increased by 128% in the T4 group, compared with the control and vitamin E groups, but no significant change was observed in relation to the T4 + vitamin E group (Table 1).

**Oxidative stress parameters**

T4 group exhibited increased (41%) myocardial oxidative damage to proteins, measured through carbonyl assay, as compared with control (*P*<0·05; Table 2). However, the T4 + vitamin E group showed reduced protein oxidation by 29%, relative to the T4 group. No difference was found between the control and T4 + vitamin E groups in terms of protein oxidation (*P*<0·05). The redox status (GSH/GSSG ratio) was significantly reduced (83%) in the T4 group as compared with control (*P*<0·05; Table 1). GSH/GSSG ratio was negatively correlated with cardiac hypertrophy redox status (*r* = −0·85; *P*<0·05). There was, however, a partial improvement of these values with vitamin E treatment, but GSH/GSSG values were still minor in T4 + vitamin E compared with control. Vitamin C was consumed in hyperthyroid (34%) rats in comparison with controls. When vitamin E was given to the hyperthyroid rats, vitamin C values returned to control levels (Table 2). TRAP was also reduced (by 55%) in the T4 group as compared with control, but these values were normalized with vitamin E treatment (*P*<0·05; Table 2). Hydrogen peroxide levels were increased (62%) in the T4 group as compared with control, and decreased (57%) in the T4 + vitamin E group as compared with T4 group (*P*<0·05). However, hydrogen peroxide levels in the T4 + vitamin E group were not significantly different as compared with the vitamin E group (Fig. 1A).

**NO metabolites (NOX)**

Nitrates plus nitrites (NOX) levels showed an elevation (218%) in the T4 group relative to control. Vitamin E administration to the T4 + vitamin E group reduced these values by 56% in comparison with the T4 group (*P*<0·05; Fig. 1B), but these values were still much higher (150%) than the vitamin E group.

**JUN and FOS protein expressions**

JUN and FOS myocardial protein concentrations in the T4 group were higher (33 and 130% respectively) than the control (Fig. 2A and B). In the T4 + vitamin E group, JUN and FOS protein expressions decreased by 25 and 43% respectively (Fig. 2A and B), as compared with the T4 group. However, there were no significant differences in JUN and FOS protein expressions, between the T4 + vitamin E and vitamin E groups (Fig. 2A and B). JUN and FOS protein levels were positively correlated with hypertrophy index values (*r* = 0·79, *P*<0·05 and *r* = 0·88, *P*<0·05 respectively).

**Table 1** Hormone concentration, morphometric, and hemodynamic parameters after 4-week treatment with thyroxine and/or vitamin E

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Vitamin E</th>
<th>T4</th>
<th>T4 + vitamin E</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 (ng/ml)</td>
<td>3·8 ± 0·1</td>
<td>3·9 ± 0·2</td>
<td>24·5 ± 0·8‡</td>
<td>23·1 ± 0·8‡</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>232 ± 25</td>
<td>211 ± 15</td>
<td>201 ± 18</td>
<td>227 ± 19</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0·70 ± 0·10</td>
<td>0·65 ± 0·05</td>
<td>0·97 ± 0·10*</td>
<td>0·82 ± 0·05†</td>
</tr>
<tr>
<td>Heart/body weight (mg/g)</td>
<td>3·0 ± 0·02</td>
<td>3·1 ± 0·02</td>
<td>4·9 ± 0·05*</td>
<td>3·7 ± 0·04†</td>
</tr>
<tr>
<td>Liver/body weight (mg/g)</td>
<td>33·0 ± 0·4</td>
<td>34·0 ± 0·5</td>
<td>50·0 ± 0·8*</td>
<td>35·0 ± 0·4†</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>127 ± 11</td>
<td>128 ± 9·8</td>
<td>187 ± 10*</td>
<td>149 ± 27‡</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>4·5 ± 0·7</td>
<td>5·0 ± 0·8</td>
<td>10 ± 1·3*</td>
<td>9·2 ± 2·1‡</td>
</tr>
</tbody>
</table>

LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure. Values are expressed as mean ± s.d. of ten animals per group. *Significantly different from control (*P*<0·05). †Significantly different from T4 (*P*<0·05). ‡Significantly different from vitamin E (*P*<0·05).
Table 2 Myocardial markers of oxidative damage to proteins (carbonyl groups), redox status (GSH/GSSG), and total radical-trapping antioxidant potential (TRAP) capacity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Vitamin E</th>
<th>T4</th>
<th>T4 + vitamin E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonyl groups (nmol/mg prot)</td>
<td>2.7 ± 0.1</td>
<td>1.9 ± 0.4*</td>
<td>4.1 ± 0.5*</td>
<td>2.9 ± 0.5†‡</td>
</tr>
<tr>
<td>GSH/GSSG (redox status)</td>
<td>15.0 ± 3.0</td>
<td>25.0 ± 5.0*</td>
<td>2.9 ± 0.5*</td>
<td>8.0 ± 2.0*†‡</td>
</tr>
<tr>
<td>Vitamin C (μmol/mg prot)</td>
<td>14.0 ± 1.8</td>
<td>15.0 ± 2.1</td>
<td>9.2 ± 1.9*</td>
<td>12.6 ± 2.0‡</td>
</tr>
<tr>
<td>TRAP (U Trolox/mg prot)</td>
<td>30 ± 6</td>
<td>40 ± 12*</td>
<td>13 ± 4*</td>
<td>32 ± 5.0†‡</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.d. of ten animals per group. *Significantly different from control (P<0.05). †Significantly different from T4 (P<0.05). ‡Significantly different from vitamin E (P<0.05).

p-AKT1/AKT1 and p-GSK3B/GSK3B protein expressions

L-T4 treatment resulted in an elevated p-AKT1/AKT1 protein levels (69%) as compared with control (P<0.05; Fig. 3A). In the T4 + vitamin E group, p-AKT1/AKT1 protein expression was reduced by 23% in comparison with T4 (Fig. 3A). Phosphorylated protein levels (p-AKT1) were positively correlated with hypertrophy index values (r=0.86, P<0.05). The T4 group also featured a 37% increase in p-GSK3B/GSK3B protein expressions (Fig. 3B) as compared with control (P<0.05). The hyperthyroid group treated with vitamin E showed a significant decrease (30%) in p-GSK3B/GSK3B levels as compared with the T4 group (P<0.05). These values were not significantly different between the T4 + vitamin E and vitamin E groups.

Discussion

The main objective of this study was to demonstrate the participation of oxidative stress in modulating the intracellular signaling pathway involved in the elaboration of cardiac hypertrophy in experimental hyperthyroidism. We focused on the survival AKT1 pathway, proposing hydrogen peroxide as a signaling candidate, since it was previously demonstrated that ROS may lead to AKT1 phosphorylation (Cai et al. 2003).

Initially, we confirmed our model of chronic hyperthyroidism by means of hormone, morphometric, and hemodynamic measurements (Table 1). Here, we demonstrate increased LVEDP accompanied by liver congestion in the hyperthyroid group. In our previous study, LVEDP was found to be increased significantly only in the last week of T4 treatment, accompanied by lung and liver congestions (Araujo et al. 2006). We also demonstrated an amelioration in ventricular performance (±dP/dt) promoted by vitamin E, as well as a reduction in heart to body weight ratio and organ congestions, suggesting that the rats were in a compensatory stage of heart failure (Araujo et al. 2007).

Given that our data indicate a protective effect of vitamin E in preventing cardiac hypertrophy, this suggests that oxidative stress is involved in the remodeling process in hyperthyroidism-induced cardiac hypertrophy. Vitamin E, a lipophilic and chain-breaking antioxidant, incorporates into the lipid bilayer, where it can interfere with the formation of lipid peroxides and carbonyl groups (through its action as an alkyl, alcoxyl, and peroxyl radicals scavenger) to attenuate lipid peroxidation and protein oxidation respectively. Previously, we have demonstrated a reduction in myocardial lipid peroxidation (LPO) when vitamin E was administrated to hyperthyroid rats (Araujo et al. 2007) and, in the present study, we show a reduction in protein oxidation products (Table 2). In this regard, vitamin E by virtue of its free radical scavenging action would reduce the production of carbon-centered radicals capable of reacting directly with biomolecules, such as –SH groups of proteins (Soriani et al. 1994) resulting in reducing the consumption and exhaustion of GSH thereby improving the GSH/GSSG ratio (Table 2). In fact, Venditti et al. (2007) have also...

Figure 1 (A) Hydrogen peroxide (mmol/g tissue), and (B) nitrates plus nitrites (mmol/mg prot) in cardiac tissue. Data as mean ± s.d. from ten animals in each group. *Significantly different from control (P<0.05). #Significantly different from T4 (P<0.05).
demonstrated a better preservation of GSH content in liver homogenates when vitamin E was given to hyperthyroid rats. Although this effect is probably a reflection of an indirect action of vitamin E by reducing cellular oxidation products, a direct effect of GSH on vitamin E recycling (from the chromanoxyl radical of tocopherol) has also been documented (Venditti et al. 2007). Endogenous storage of antioxidants such as vitamin E decreases gradually as a result of reacting with free radicals. When vitamin E is given exogenously, intracellular antioxidants may be preserved. GSH, the most abundant intracellular antioxidant, is preserved because the need of vitamin E recycling is diminished. However, the most efficient mechanism to recycle vitamin E is through vitamin C. As shown in Table 2, a significant reduction of vitamin C levels in the myocardium of hyperthyroid rats was probably a result of its utilization in the replenishment of vitamin E, which is very demanding in this oxidative stress situation. Although vitamin E is a liposoluble antioxidant (not measured by TRAP), it preserves the hydrosoluble antioxidants, such as vitamin C and GSH, evaluated globally by TRAP (Table 2).

In order to assess the role of ROS as possible mediators in hypertrophic response to hyperthyroidism, we measured the steady-state concentration of H$_2$O$_2$, the most stable ROS, in cardiac tissue. An important fraction of oxygen uptake of mammalian cells proceeds through univalent and bivalent reduction of oxygen, yielding superoxide radical and H$_2$O$_2$. This primary production of superoxide radical and H$_2$O$_2$ in the mitochondrial membranes is the most important physiological source of these oxygen species in eukaryotic cells that are relatively devoid of the microsomal electron transport chain (Chance et al. 1979). Brain homogenates from hyperthyroid animals show increased oxygen consumption, which could lead to an increased steady-state level of oxygen intermediates, such as superoxide radical and H$_2$O$_2$, to the establishment of oxidative stress (Adamo et al. 1989). We found that T$_4$ administration induced an elevation in myocardial H$_2$O$_2$ concentration. Vitamin E administration concomitant with T$_4$ attenuated the increase in H$_2$O$_2$, as these values did not differ from control.
An elevation in myocardial H2O2 by T4 has also been previously described (Venditti & Di Meo 2006). Vitamin E may act to reduce this ROS formation, especially by neutralizing the superoxide radical (Navarro et al. 1998, Venditti et al. 2007).

Another important signaling molecule investigated was NO, which plays an essential role in cardiac and vascular functions (Saraiva & Hare 2006). Myocardial levels of nitrates (NO3⁻) and nitrites (NO2⁻), which are chemical species derived from NO metabolism, were enhanced in the T4 group. These values were reduced by vitamin E treatment, but were still higher than control. Oxidative stress could activate AKT1 pathway, inducing phosphorylation and activation of endothelial NOS3 (Tanaka et al. 2005). Thus, increased oxidative stress would explain the increased levels of nitrates and nitrites in hyperthyroid rats by means of AKT1 activation. Furthermore, AKT1 activation by increased NO levels has previously been reported (Cai et al. 2003). However, since even reducing oxidative stress, nitrite/nitrate levels persisted much higher than control; this indicates that other NO production pathways must be involved. In this regard, it has recently been demonstrated that thyroid hormones would activate the inducible NOS2 directly, enhancing additionally NOX levels (Rodríguez-Gómez et al. 2005). As superoxide radicals may be enhanced in hyperthyroid rats, peroxynitrite (ONOO⁻) could also be produced, and may act to trigger the hypertrophic response (Kuzman et al. 2005).

We have also demonstrated that increased H2O2 concentration is associated with elevated protein expression of transcription factors such as JUN and FOS in the T4 group. JUN and FOS proteins are the main components of homodimers and/or heterodimers of the transcription factor JUN, which translocates to the nucleus to stimulate the transcription of its target genes. Most cellular JUN is formed by JUN and FOS heterodimers, and this complex is believed to be the most stable and active form. JUN activity is induced in response to certain metals in the presence of H2O2, as well as by several cytokines and other physical and chemical stimuli (Valko et al. 2007). Recent data also suggest that low GSH concentration may activate JUN, which is transcriptional complex important to gene expression induction in cardiac hypertrophy (Kaur et al. 2008). In addition, a decreased JUN expression prevents the development of cardiac hypertrophy through a ROS-dependent pathway (Li et al. 2006). Our findings corroborate these data, since a positive correlation was seen between FOS/JUN and cardiac hypertrophy.

Our results also demonstrated increased AKT1 phosphorylation in the T4 group, further corroborating previous data in literature (Kuzman et al. 2005). T4-induced oxidative stress could activate AKT1 phosphorylation especially through enhanced H2O2 concentration, leading to myocardial hypertrophy (Cai et al. 2003). This effect could be achieved directly by H2O2 by altering protein conformation, and promoting higher susceptibility to phosphorylation, or secondarily in promoting redox status imbalance (GSH/GSSG ratio). Moreover, AKT1 activation leads to GSK3B phosphorylation, inducing its inhibition and reducing the signaling to apoptosis. GSK3B is a proapoptotic protein which utilizes many mechanisms to reduce cellular growth. One of these mechanisms acts through the inhibition of JUN activation by phosphorylation of JUN inhibitory sites (Hongisto et al. 2003). We observed that vitamin E attenuates AKT1 phosphorylation, promoting GSK3B uninhibition and decreased JUN activation, culminating in a reduced myocardial hypertrophy. These effects reinforce the role of the redox signaling in cardiac hypertrophy induced by T4. However, a direct molecular action of vitamin E in GSK3B and AKT1 phosphorylation cannot be ruled out, as there is previous evidence in the literature for its existence (Kempna et al. 2004).

In summary, our data strongly suggest H2O2 as a possible mediator of AKT1 pathway activation, providing evidence of an important role for oxidative stress in this intracellular signaling pathway in experimental hyperthyroidism. Vitamin E could be a prospective therapeutic strategy serving to reduce oxidative stress in the context of cardiac hypertrophy and the progression to heart failure in hyperthyroid patients.

Declaration 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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