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 (NO_x) were measured in heart homogenates. The p-AKT1/AKT1 ratio, p-glycogen-synthase kinase (GSK)3B/GSK3B ratio, 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 NO_x (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 (T_4) 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 bodyweight 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) T_4 (receiving $L-T_4$ (12 mg/l in drinking water; Ladenson *et al.* 1986)); and IV) T_4 +vitamin E (receiving T_4 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.

T_4 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 T_4 concentration was estimated by chemiluminescence using the Immunolite 2000 kit (Biomedical Technologies, Inc., Strougeron, 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 sulfonyl fluoride phenylmethylsulfonyl 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 (Llesuy *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 dinitrophenylhydrazine (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-amidinopropane) (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 (hydrosoluble vitamin E; Evelson *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 HRP. 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 (NO_x)

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 nitrite 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 nitrates 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 (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 2). 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 (NO_x)

Nitrates plus nitrites (NO_x) 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

	Control	Vitamin E	T ₄	T ₄ +vitamin E
Groups				
T ₄ (ng/ml)	3.8±0.1	3.9±0.2	24.5±0.8*‡	23.1±0.8*‡
Body weight (g)	232±25	211±15	201±18*	227±19
Heart weight (g)	0.70±0.10	0.65±0.05	0.97±0.10*	0.82±0.05†
Heart/body weight (mg/g)	3.0±0.02	3.1±0.02	4.9±0.05*	3.7±0.04†
Liver/body weight (mg/g)	33.0±0.4	34.0±0.5	50.0±0.8*	35.0±0.4†
LVSP (mmHg)	127±11	128±9.8	187±10*	149±27†,‡
LVEDP (mmHg)	4.5±0.7	5.0±0.8	10±1.3*	9.2±2.1‡

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

	Control	Vitamin E	T ₄	T ₄ + vitamin E
Groups				
Carbonyl groups (nmol/mg prot)	2.7 ± 0.1	1.9 ± 0.4*	4.1 ± 0.5*	2.9 ± 0.5 ^{†,‡}
GSH/GSSG (redox status)	15.0 ± 3.0	25.0 ± 5.0*	2.9 ± 0.5*	8.0 ± 2.0 ^{*,†,‡}
Vitamin C (μmol/mg prot)	14.0 ± 1.8	15.0 ± 2.1	9.2 ± 1.9*	12.6 ± 2.0 [†]
TRAP (U Trolox/mg prot)	30 ± 6	40 ± 12*	13 ± 4*	32 ± 5.0 ^{†,‡}

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 ($\pm dP/dt$) 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 peroxy 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 ($P < 0.05$).

Figure 2 Western blot analysis in cardiac homogenates using: (A) JUN and (B) FOS (one representative gel of five western blot experiments, showing two bands for each experimental group). Data as mean $\pm$ 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 H₂O₂, 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 H₂O₂. This primary production of superoxide radical and H₂O₂ 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 H₂O₂, to the establishment of oxidative stress (Adamo *et al.* 1989). We found that T₄ administration induced an elevation in myocardial H₂O₂ concentration. Vitamin E administration concomitant with T₄ attenuated the increase in H₂O₂, as these values did not differ from control.

Figure 3 Western blot analysis in cardiac homogenates using: (A) p-AKT1 (ser657) (p-AKT1 (ser657)/AKT1 ratio) and (B) p-GSK3B (ser9) (p-GSK3B (ser9)/GSK3B ratio) (one representative gel of five western blot experiments, showing two bands for each experimental group). Data as mean $\pm$ s.d. from ten animals in each group. *Significantly different from control ($P < 0.05$). #Significantly different from T₄ ($P < 0.05$).

An elevation in myocardial H_2O_2 by T_4 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 (NO_3^-) and nitrites (NO_2^-), which are chemical species derived from NO metabolism, were enhanced in the T_4 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 NO_x 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 H_2O_2 concentration is associated with elevated protein expression of transcription factors such as JUN and FOS in the T_4 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 H_2O_2 , 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 a 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 T_4 group, further corroborating previous data in literature (Kuzman *et al.* 2005). T_4 -induced oxidative stress could activate AKT1 phosphorylation especially through enhanced H_2O_2 concentration, leading to myocardial hypertrophy (Cai *et al.*

2003). This effect could be achieved directly by H_2O_2 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 T_4 . 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 H_2O_2 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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References

- Adamo AM, Llesuy SF, Pasquini JM & Boveris A 1989 Brain chemiluminescence and oxidative stress in hyperthyroid rats. *Biochemistry Journal* **263** 273–277.
- Akerboom T & Sies H 1981 Assay of glutathione disulfide and glutathione mixed disulfides in biological samples. *Methods in Enzymology* **77** 373–382.

- Antos CL, McKinsey TA, Frey N, Kutschke W, McAnaully J, Shelton JM, Richardson JA, Hill JA & Olson EN 2002 Activated glycogen synthase-3 beta suppresses cardiac hypertrophy *in vivo*. *PNAS* **99** 907–912.
- Araujo ASR, Ribeiro MFM, Enzweiler A, Schenkel P, Fernandes TRG, Partata WA, Irigoyen MC, Llesuy S & Belló-Klein A 2006 Myocardial antioxidant enzyme activities and concentration and glutathione metabolism in experimental hyperthyroidism. *Molecular and Cellular Endocrinology* **249** 133–139.
- Araujo ASR, Ribeiro MFM, Enzweiler A, Schenkel PC, Fernandes TRG, Partata WA, Irigoyen MC, Llesuy S & Belló-Klein A 2007 Oxidative stress activates insulin-like growth factor I receptor protein expression, mediating cardiac hypertrophy induced by thyroxine. *Molecular and Cellular Biochemistry* **303** 89–95.
- Cai H, Li Z, Davis ME, Kanner W, Harrison DG & Dudley SC 2003 Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. *Molecular Pharmacology* **63** 325–331.
- Chance B, Sies H & Boveris A 1979 Hydroperoxide metabolism in mammalian organs. *Physiological Reviews* **59** 527–625.
- Chitra KC & Mathur PP 2004 Vitamin E prevents nonylphenol-induced oxidative stress in testis of rats. *Indian Journal of Experimental Biology* **42** 220–223.
- Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, Russo MA, Gu Y, Dalton N, Chung C *et al.* 2002 Akt induces enhanced myocardial contractility and cell size *in vivo* in transgenic mice. *PNAS* **99** 12333–12338.
- Das DK, Maulik N & Engelman RM 2004 Redox regulation of angiotensin II signaling in the heart. *Journal of Cellular and Molecular Medicine* **8** 144–152.
- Donatelli M, Assennato P, Abbadi V, Bucalo ML, Compagno V, Lo Vecchio S, Messina L, Russo V, Schembri A, Torregrossa V *et al.* 2003 Cardiac changes in subclinical and overt hyperthyroid women: retrospective study. *International Journal of Cardiology* **90** 159–164.
- Evelson P, Travacio M, Repetto M, Escobar J, Llesuy S & Lissi EA 2001 Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. *Archives of Biochemistry and Biophysics* **388** 261–266.
- Granger DL, Anstey NM, Miller WC & Weinberg JB 1999 Measuring nitric oxide production in human clinical studies. *Methods in Enzymology* **301** 58–61.
- Hongisto V, Smeds N, Brecht S, Herdegen T, Courtney MJ & Coffey ET 2003 Lithium blocks the c-Jun stress response and protects neurons via its action on glycogen synthase kinase 3. *Molecular and Cellular Biology* **17** 6027–6036.
- Hu LW, Benvenuti LA, Liberti EA, Carneiro-Ramos MS & Barreto-Chaves ML 2003 Thyroxine-induced cardiac hypertrophy: influence of adrenergic nervous system versus renin–angiotensin system on myocyte remodeling. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **285** 1473–1480.
- Kaur P, Kaur G & Bansal MP 2008 Upregulation of AP1 by tertiary butyl hydroperoxide induced oxidative stress and subsequent effect on spermatogenesis in mice testis. *Molecular and Cellular Biochemistry* **308** 177–181.
- Kempna P, Reiter E, Arock M, Azzi A & Zingg JM 2004 Inhibition of HMC-1 mast cell proliferation by vitamin E: involvement of the protein kinase B pathway. *Journal of Biological Chemistry* **279** 50700–50709.
- Kenessey A & Ojamaa K 2006 Thyroid hormone stimulates protein synthesis in the cardiomyocyte by activating the Akt – mTOR and p70S6K pathways. *Journal of Biological Chemistry* **281** 20666–20672.
- Klein D, Kern RM & Sokol RZ 1995 A method for quantification and correction of proteins after transfer to immobilization membranes. *Biochemistry and Molecular Biology International* **36** 59–66.
- Kuzman AJ, Vogelsang KA, Thomas AT & Martin Gerdes A 2005 L-Thyroxine activates Akt signaling in the heart. *Journal of Molecular and Cellular Cardiology* **39** 251–258.
- Kyaw A 1978 A simple colorimetric method for ascorbic acid determination in blood plasma. *Clinica Chimica Acta* **86** 153.
- Ladenson PW, Kieffer JD, Farwell AP & Ridgway EC 1986 Modulation of myocardial L-triiodothyronine receptors in normal, hypothyroid and hyperthyroid rats. *Metabolism* **35** 5–12.
- Laemmli V 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.
- Li HL, Huang Y, Zhang CN, Liu G, Wei YS, Wang AB, Liu YQ, Hui RT, Wei C, Williams GM *et al.* 2006 Epigallocatechin-3 gallate inhibits cardiac hypertrophy through blocking reactive oxidative species-dependent and -independent signal pathways. *Free Radical Biology and Medicine* **40** 1756–1775.
- Li HJ, Yin H, Yao YY, Shen B, Bader M, Chao L & Chao J 2007 Tissue kallikrein protects against pressure overload-induced cardiac hypertrophy through kinin B2 receptor and glycogen synthase kinase-3 β activation. *Cardiovascular Research* **73** 130–142.
- Llesuy SF, Milei J, Molina H, Boveris A & Milei S 1985 Comparison of lipid peroxidation and myocardial damage induced by adriamycin and 4'-epidriamycin in mice. *Tumori* **71** 241–249.
- Lowry OH, Rosebrough AL, Farr AL & Randall R 1951 Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193** 265–275.
- Navarro F, Navas P, Burgess JR, Bello RI, De Cabo R, Arroyo A & Villalba JM 1998 Vitamin E and selenium deficiency induces expression of the ubiquinone-dependent antioxidant system at the plasma membrane. *FASEB Journal* **12** 1665–1673.
- Pick E & Keisari Y 1980 A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *Journal of Immunological Methods* **38** 161–170.
- Reznick AZ & Packer L 1994 Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology* **233** 357–363.
- Rodríguez-Gómez I, Wangenstein R, Moreno JM, Chamorro V, Osuna A & Vargas F 2005 Effects of chronic inhibition of inducible nitric oxide synthase in hyperthyroid rats. *American Journal of Physiology. Endocrinology and Metabolism* **288** E1252–E1257.
- Saraiva RM & Hare JM 2006 Nitric oxide signaling in the cardiovascular system: implications for heart failure. *Current Opinion in Cardiology* **21** 221–228.
- Soriani M, Pietraforte D & Minetti M 1994 Antioxidant potential of anaerobic human plasma: role of serum albumin and thiols as scavengers of carbon radicals. *Archives of Biochemistry and Biophysics* **312** 180–188.
- Tanaka T, Nakamura H, Yodoi J & Bloom ET 2005 Redox regulation of the signaling pathways leading to eNOS phosphorylation. *Free Radical Biology and Medicine* **38** 1231–1242.
- Thannickal VJ, Day RM, Klinz SJ, Bastien MC, Larios JM & Fanburg BL 2000 Ras-dependent and -independent regulation of reactive oxygen species by mitogenic growth factors and TGF- β 1. *FASEB Journal* **14** 1741–1748.
- Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M & Telser J 2007 Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cellular Biology* **39** 44–84.
- Venditti P & Di Meo S 2006 Thyroid hormones-induced oxidative stress. *Cellular and Molecular Life Sciences* **63** 414–434.
- Venditti P, Bari A, Di Stefano L & Di Meo S 2007 Vitamin E attenuates cold-induced rat liver oxidative damage reducing H₂O₂ mitochondrial release. *International Journal of Biochemistry and Cellular Biology* **39** 1731–1742.

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