Propylthiouracil-induced hypothyroidism is associated with increased tolerance of the isolated rat heart to ischaemia-reperfusion

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Abstract

The present study investigated the response of the hypothyroid heart to ischaemia-reperfusion. Hypothyroidism was induced in Wistar rats by oral administration of propylthiouracil (0.05%) for 3 weeks (HYPO rats), while normal animals (NORM) served as controls. Isolated hearts from NORM and HYPO animals were perfused in Langendorff mode and subjected to zero-flow global ischaemia followed by reperfusion (I/R). Post-ischaemic recovery of left ventricular developed pressure was expressed as % of the initial value (LVDP%). Basal expression of protein kinase C ϵ (PKC ϵ) and PKC δ and phosphorylation of p46 and p54 c-jun NH₂-terminal kinases (JNKs) in response to I/R were assessed by Western blotting. LVDP% was found to be significantly

higher in HYPO hearts than in NORM. At baseline, PKC ϵ expression was 1.4-fold more in HYPO than in NORM hearts, $P < 0.05$, while PKC δ was not changed. Furthermore, basal phospho-p54 and -p46 JNK levels were 2.2- and 2.6-fold more in HYPO than in NORM hearts, $P < 0.05$. In response to I/R, in NORM hearts, phospho-p54 and -p46 JNK levels were 5.5- and 6.0-fold more as compared with the baseline values, $P < 0.05$, while they were not significantly altered in HYPO hearts. HYPO hearts seem to display a phenotype of cardioprotection against ischaemia-reperfusion and this is associated with basal PKC ϵ overexpression and attenuated JNK activation after I/R.

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Introduction

Hypothyroidism is a common clinical condition with various consequences on the cardiovascular system and has been associated with increased cardiovascular morbidity (Hak *et al.* 2000, Vanderpump *et al.* 2002). Furthermore, circulating thyroid hormone levels have been demonstrated to decline (3,5,3'-triiodothyronine (T₃) more and to a lesser extent L-thyroxine (T₄)) in various conditions such as acute myocardial infarction (Franklyn *et al.* 1984), congestive heart failure (Hamilton *et al.* 1998) or diabetes (Yue *et al.* 1998). Abnormal thyroid function also occurs after cardiac surgery requiring cardiopulmonary bypass (Bartkowski *et al.* 2002) or chronic administration of amiodarone (Klein & Ojamaa 2001).

It has been long realized that the heart is one of the most thyroid hormone-responsive tissues (Klein & Ojamaa 2001). In fact, thyroid hormone is shown to regulate the transcription of various myocyte-specific genes that encode important structural and regulatory proteins

including myosin heavy chain isoforms α and β , sarcoplasmic reticulum calcium activated ATPase (SR Ca²⁺-ATPase), phospholamban, the β -adrenergic receptor, adenyl cyclase isoforms and various membrane ion channels (Klein & Ojamaa 2001). Furthermore, recent research has revealed that thyroid hormone can interfere with the regulation of important intracellular signalling transduction pathways (Fryer *et al.* 1998, Pantos *et al.* 2001, 2002a, 2003a) that are thought to be involved in protection against ischaemia-reperfusion (I/R) (Speechly-Dick *et al.* 1994, Kawamura *et al.* 1998, Zhao *et al.* 1998, Pantos *et al.* 2000, 2001, Fryer *et al.* 2001). In fact, chronic administration of T₄ results in changes in cardioprotective molecules such as protein kinase C (PKC) and mitogen-activated protein kinases (Fryer *et al.* 1998, Pantos *et al.* 2001, 2002a, 2003a) and this was shown to be associated with increased post-ischaemic recovery of function (Buser *et al.* 1990, Pantos *et al.* 2002a, 2003a,c).

On the basis of this evidence, thyroid hormone seems to be an important regulator of cardiac performance as well as

of the response of the heart to ischaemic stresses and consequently one could anticipate that low thyroid hormone states might lead to impaired myocardial performance and increased susceptibility of the heart to ischaemia. This hypothesis, although of clinical relevance, has not been previously adequately explored. Therefore, the present study investigated the response of the isolated rat heart to I/R in an experimental model of propylthiouracil-induced hypothyroidism.

Materials and Methods

Animals

Forty-two Wistar male rats, 270–320 g were used for this study. The rats were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85–23, revised 1985). Anaesthesia was achieved with i.p. injection of ketamine hydrochloric acid (150 mg/kg).

Experimental hypothyroidism

Hypothyroidism was induced in rats by administration of 6-*n*-propyl-2-thiouracil in drinking water to a final concentration of 0.05% for 3 weeks (Cernohorsky *et al.* 1998, Shenoy *et al.* 2001). These animals were designated as HYPO. Untreated rats were used as controls and were designated as NORM.

Isolated heart preparation

A non-ejecting isolated rat heart preparation was perfused at constant coronary flow according to the Langendorff technique, as previously described (Pantos *et al.* 2000, 2002b, 2003b). In this model, coronary flow per gram of cardiac tissue was similar in all the experimental groups. Rats were anaesthetized with i.p. injection of ketamine hydrochloric acid and heparin (1000 IU/kg body weight) was given i.v. before thoracotomy. The hearts were perfused with oxygenated (95% O₂/5% CO₂) Krebs–Henseleit buffer at a constant temperature of 37 °C and were paced at 320 bpm with a Harvard pacemaker. The pacemaker was turned off during the period of ischaemia. An intraventricular balloon allowed measurement of contractility under isovolumic conditions. Left ventricular balloon volume was adjusted to produce an average initial left ventricular end-diastolic pressure (LVEDP) of 6 mmHg in all groups and was held constant thereafter throughout the experiment. Pressure signal was transferred to a personal computer using data analysis software (IOX; Emka Technologies, Paris, France). Cardiac function was assessed by left ventricular peak systolic pressure and the left ventricular developed pressure (LVDP), defined as the

difference between left ventricular peak systolic pressure and LVEDP. LVDP and its positive and negative first derivative (+dp/dt, –dp/dt) were measured at the end of the stabilization and reperfusion period respectively. Post-ischaemic cardiac function was assessed by the recovery of LVDP which was expressed as % of the initial value (LVDP%) and by LVEDP at 45 min of reperfusion. Ischaemic contracture was assessed by measurement of the observed increase in left ventricular pressure at various time points during ischaemia.

Total protein preparation

Isolation of total protein content and Western blotting have been performed as previously described (Pantos *et al.* 2001, 2002a, 2003a). Approximately 0.2 g frozen tissue was homogenized in ice-cold Tris–sucrose buffer (0.35 M sucrose, 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride) with a Polytron homogenizer and the resulting homogenate was centrifuged at 15 000 *g* for 20 min at 4 °C. The supernatant, representing the total cell extract, was used for immunoblotting. Protein concentrations were determined by the bicinchoninic acid method using BSA (Walker 1994).

SDS-PAGE and immunoblotting

After boiling for 5 min in Laemmli sample buffer, protein aliquots (40 µg) were loaded onto 10% (w/v) acrylamide gels and subjected to SDS-PAGE. After Western blotting, filters were probed with specific antibodies against either PKCε or PKCδ (Transduction Laboratories, Lexington, KY, USA, dilution 1:1000), or total c-jun NH₂-terminal kinases (JNKs) or dual phospho-JNKs (New England Biolabs, Hitchin, Herts, UK, dilution 1:1000), or actin (Sigma, 1:1000) overnight at 4 °C and immunoreactivity was detected by enhanced chemiluminescence. Immunoblots were quantified using the AlphaScan Imaging Densitometer (Alpha Innotech Corporation, San Leandro, CA, USA). For comparisons between groups, five samples from each group were loaded on the same gel. Optical densities of PKCε, PKCδ, dual phospho-JNKs and total JNK immunoreactivity were expressed as a ratio of the actin optical density to correct for slight variations in total protein loading.

Experimental protocol

Hearts from NORM and HYPO rats were subjected only to 20 min of stabilization, NORM-Base, *n*=5 and HYPO-Base, *n*=5.

Hearts from NORM and HYPO rats were subjected to 20 min of stabilization, 20 min of zero-flow global ischaemia and 45 min of reperfusion, NORM-20I/R, *n*=8, and HYPO-20I/R, *n*=8. Since ischaemic contracture did not reach a plateau within 20 min of ischaemia,

Table 1 Initial body weight (BW1 in g), body weight after 3 weeks of treatment (BW2 in g), left ventricular weight (LVW in mg), the ratio of left ventricular weight to body weight (LVW/BW in mg/g), T₃ and T₄ levels in plasma (nmol/l) for NORM and HYPO rats. The values are means $\pm$ S.E.M.

	Group	
	NORM	HYPO
BW1	305 $\pm$ 7.9	307 $\pm$ 2.6
BW2	338 $\pm$ 9.9	268 $\pm$ 9.1*
LVW	831 $\pm$ 27.7	675 $\pm$ 20.2*
LVW/BW2	2.4 $\pm$ 0.05	2.5 $\pm$ 0.09
T ₃	0.87 $\pm$ 0.04	0.23 $\pm$ 0.05*
T ₄	52.50 $\pm$ 2.63	19.97 $\pm$ 0.38*

* $P < 0.05$ vs NORM.

hearts from NORM and HYPO animals were also subjected to 20 min of stabilization, 30 min of zero-flow global ischaemia and 45 min of reperfusion, NORM-30I/R, $n=8$, and HYPO-30I/R, $n=8$.

Measurement of thyroid hormones

Plasma T₄ and T₃ quantitative measurements were performed by using ¹²⁵I RIA kits obtained from DiaSorin, Stillwater, MN, USA (CA 1535 M for T₄ and CA 1541 for T₃). T₄ and T₃ levels were expressed as nmol/l of plasma.

Statistics

Values are presented as means $\pm$ S.E.M. The unpaired *t*-test and Mann-Whitney test were used for differences between groups. A two-tailed test with a *P* value less than 0.05 was considered significant.

Results

Thyroid hormones and alterations in animal body weight and heart weight

Propylthiouracil administration resulted in a significant decrease of thyroid hormone levels in plasma (Table 1). Animal body weight and left ventricular weight were significantly decreased in HYPO compared with NORM rats (Table 1).

Basal and post-ischaemic cardiac function

Basal cardiac contractility was found to be significantly reduced in HYPO as compared with NORM rats (Table 2). Post-ischaemic recovery of function was found to be significantly improved in hearts from HYPO animals as compared with NORM hearts after either 20 or 30 min of ischaemia (Fig. 1; Table 2).

Ischaemic contracture profile

Profiles of ischaemic contracture are shown in Fig. 2. Within 20 min of ischaemia, neither NORM nor HYPO hearts reached a plateau, although HYPO hearts displayed a significant attenuation of the rise of diastolic pressure. Within 30 min of ischaemia, ischaemic contracture reached a maximum at 25.3 ± 1.4 min in NORM hearts, while in HYPO hearts it did not reach a peak.

PKC ϵ and PKC δ protein expression at baseline

PKC δ protein expression at baseline was not different between NORM and HYPO hearts, $P > 0.05$. However, PKC ϵ protein expression at baseline was 1.4-fold more in HYPO-Base than in NORM-Base hearts, $P < 0.05$ (Fig. 3).

Table 2 Left ventricular developed pressure (LVDP, mmHg), +dp/dt (mmHg/s) and - dp/dt (mmHg/s) at the end of the stabilization period for NORM and HYPO hearts as well as LVDP%, LVDP and left ventricular end-diastolic pressure (LVEDP, mmHg) at 45 min of reperfusion (R) for NORM and HYPO hearts subjected to 20 or 30 min of ischaemia. I/R=ischaemia/reperfusion. The values are means $\pm$ S.E.M.

	Group			
	NORM-20I/R ($n=8$)	HYPO-20I/R ($n=8$)	NORM-30I/R ($n=8$)	HYPO-30I/R ($n=8$)
LVDP (baseline)	129.3 $\pm$ 4.5	103.8 $\pm$ 2.3*	133.9 $\pm$ 4.5	109.0 $\pm$ 3.6**
+dp/dt (baseline)	5150 $\pm$ 280	3417 $\pm$ 135*	4476 $\pm$ 227	3200 $\pm$ 259**
- dp/dt (baseline)	2615 $\pm$ 112	1851 $\pm$ 47*	2461 $\pm$ 149	1731 $\pm$ 85**
LVDP at 45 min R	80.1 $\pm$ 6.5	96.3 $\pm$ 3.6*	15.6 $\pm$ 3.1	65.9 $\pm$ 10.8**
LVEDP at 45 min R	52.8 $\pm$ 5.6	12.3 $\pm$ 3.4*	105.8 $\pm$ 3.6	36.0 $\pm$ 7.7**
LVDP%	60.6 $\pm$ 5.1	93.1 $\pm$ 4.1*	11.5 $\pm$ 2.1	61.3 $\pm$ 11.0**

* $P < 0.05$ vs NORM-20I/R; ** $P < 0.05$ vs NORM-30I/R.

Figure 1 Post-ischaemic recovery of function, LVDP%, (upper panel) and left ventricular end-diastolic pressure at 45 min of reperfusion, LVEDP45, (bottom panel) in normal hearts (NORM) and hearts from hypothyroid rats (HYPO) subjected to 20 or 30 min of ischaemia. (Bar = S.E.M.)

Phosphorylation of p54 and p46 JNKs after I/R

The levels of phospho-p54 and -p46 JNKs were found to be 2.2- and 2.6-fold more in HYPO-Base than in NORM-Base hearts respectively, $P < 0.05$. After I/R, the levels of phospho-p54 and -p46 JNKs were increased 5.5- and 6.0-fold respectively in NORM-20I/R as compared with NORM-Base hearts, $P < 0.05$. On the contrary, there was not a significant increase in the levels of the phospho-JNKs in HYPO-20I/R as compared with HYPO-Base. The levels of phospho-p54 and -p46 JNKs were 1.8- and 2.2-fold less in HYPO-20I/R hearts as compared with NORM-20I/R hearts respectively, $P < 0.05$ (Fig. 4).

Discussion

Recent research has pointed out the important role of thyroid hormone in the response of the cardiac cell to ischaemic stress. In fact, excess of thyroid hormone can result in increased tolerance of the heart against I/R (Buser *et al.* 1990, Walker *et al.* 1995, Liu *et al.* 1998, Pantos *et al.* 2000) and PKC δ and p38 mitogen-activated protein kinase are suggested to be important elements of this response (Pantos *et al.* 2001, 2002a,b). The present study has explored the possibility that decreased thyroid

hormone levels could potentially have detrimental effects on the tolerance of the heart to ischaemia.

An experimental model of hypothyroidism was induced by administration of propylthiouracil for a period of 3 weeks. This treatment resulted in short-term hypothyroidism with significant but not marked decrease of T_4 and T_3 levels in plasma. Animal body weight and heart weight were found to be reduced in HYPO rats whereas baseline myocardial functional parameters were impaired in HYPO hearts as compared with NORM. These findings are consistent with previous reports (Cernohorsky *et al.* 1998, Shenoy *et al.* 2001, Ohga *et al.* 2002). In fact, cardiac dysfunction is a common finding in the hypothyroidism and this has been attributed to various changes that occur in the myocardium (Ohga *et al.* 2002). Such changes include increased expression of V3 isomyosin, reduced expression of SR Ca^{2+} -ATPase and ryanodine receptor and enhanced expression of phospholamban (Arai *et al.* 1991, Kiss *et al.* 1994, Ohga *et al.* 2002).

In response to I/R, HYPO hearts displayed an increased post-ischaemic recovery of function as compared with NORM while ischaemic contracture occurred later in those hearts. Several studies have concluded similar results. Abe *et al.* (1992), using an isolated working heart model, demonstrated increased recovery of the pressure-rate product in HYPO hearts as compared with NORM. Furthermore, Eynan *et al.* (2002) showed an improved post-ischaemic recovery of function and delayed ischaemic contracture in isolated HYPO rat hearts subjected to zero-flow global ischaemia. Along the same line, Zhang *et al.* (2002) have recently demonstrated that hypothyroidism can be protective against I/R arrhythmias.

The mechanisms that underlie hypothyroidism-induced cardioprotection are not fully understood and changes in metabolism or energy utilization have been suggested to be implicated in this effect. In fact, it is thought that HYPO hearts have a higher efficiency and consume less oxygen in doing mechanical work due to the predominance of V3 myosin isoform. As a consequence, in HYPO hearts, ATP levels are found to decline more slowly during ischaemia and are higher at reperfusion (Abe *et al.* 1992). Furthermore, other studies show that pre-ischaemic myocardial glycogen levels are higher in those hearts whereas glycolysis during ischaemia is slowed (Eynan *et al.* 2002). However, it has been recently reported that hearts displaying opposite metabolic characteristics such as hyperthyroid hearts are also found to be more tolerant to ischaemia (Buser *et al.* 1990, Van der Vusse *et al.* 1998, Pantos *et al.* 2000, 2001, 2002a,b) indicating that the increased resistance of the HYPO heart to ischaemia cannot be merely explained on the basis of the metabolic changes that are observed in those hearts.

It is now realized that intracellular molecules such as PKC and/or mitogen-activated protein kinases could play an important role in the adaptive response of the heart to ischaemia. The role of PKC and its isotypes in

Figure 2 Ischaemic contracture profiles of normal hearts (NORM) and hearts from hypothyroid rats (HYPO) subjected to 20 min (upper panel) or 30 min (bottom panel) of ischaemia. (Bar=S.E.M.)

cardioprotection has been demonstrated by various studies (Speechly-Dick *et al.* 1994, Kawamura *et al.* 1998). In fact, PKC ϵ has been shown to be mainly involved in cardioprotective means such as ischaemic preconditioning (Fryer *et al.* 2002) while PKC δ has been implicated in pharmacological preconditioning (Fryer *et al.* 2001). Interestingly, chronic T₄ administration is shown to upregulate PKC δ (Fryer *et al.* 1998, Pantos *et al.* 2002a) and induce pharmacological preconditioning (Pantos *et al.* 2002a), while cells overexpressing PKC δ (Zhao *et al.* 1998) or hearts from mice overexpressing PKC ϵ are found to be less susceptible to ischaemia (Cross *et al.* 2002). In the present study, PKC ϵ expression was found to be increased in HYPO hearts while PKC δ expression remained unchanged. On the basis of these data, it could be suggested that PKC ϵ overexpression is likely to be linked to the increased resistance of those hearts to ischaemia. In support of this notion is the fact that HYPO hearts closely resemble hearts from mice overexpressing PKC ϵ as regards

the response to ischaemia as well as ATP utilization during I/R; in a transgenic model overexpressing PKC ϵ in the myocardium, ATP levels were found to decline more slowly during ischaemia and to be higher at reperfusion while post-ischaemic recovery was significantly improved in those hearts (Cross *et al.* 2002). Furthermore, PKC ϵ overexpression is also shown to occur in hearts from diabetic rats that are found to be tolerant to ischaemia and abnormal thyroid function frequently coexist (Liu *et al.* 1999).

Recent research has emphasized the important role of JNK-dependent pathways in determining the response of the cell against various stresses (Bogoyevitch *et al.* 1996). JNKs are found to be activated in stressful conditions and this has been associated with cell death (Chen *et al.* 1996) while inhibition of JNK activation is shown to prevent cell injury induced by a variety of stresses, including heat shock, ethanol, UV irradiation, oxidative stress and other (Gabal *et al.* 1998). This has been clearly demonstrated in

Figure 3 Densitometric assessment of PKCδ (upper panels) and PKCε (bottom panels) expression in normal hearts (NORM, n=5) and hearts from hypothyroid rats (HYPO, n=5). (Columns are means of optical ratios, bar=S.E.M.)

Figure 4 (Upper panels) Densitometric assessment of phosphorylated JNKs in normal hearts (NORM) and hearts from hypothyroid rats (HYPO) at baseline (Base, $n=5$ for each group) and after 20 min of ischaemia and reperfusion (20I/R, $n=5$ for each group). (Columns are means of optical ratios, bar=s.e.m.) * $P<0.05$ vs NORM-Base. (Lower panel) Western blots showing phosphorylated and total JNKs in NORM and HYPO hearts at baseline (Base, $n=5$ for each group) and after 20 min of ischaemia and reperfusion (20I/R, $n=5$ for each group).

cell-based models by interruption of the JNK signalling pathway, either immediately upstream of JNK by expression of dominant negative mutants of the JNK activator SEK1 (Verheij *et al.* 1996), or immediately downstream of JNK, by an expression of a dominant negative mutant of

JNK substrate, c-jun (Gabai *et al.* 1997). In the present study, JNKs were found to be significantly activated in NORM hearts in response to the I/R sequence. In fact, the levels of phospho-p46 and -p54 JNK after I/R were found to be 6- and 5-fold more than the baseline values.

On the contrary, in HYPO hearts, the levels of phospho-p46 and -p54 JNK were not increased after I/R. On the basis of these data, it seems likely that inhibition of JNK activation during I/R might be an important element of HYPO-induced cardioprotection. Several lines of evidence support this notion. Recent studies demonstrate that in established paradigms of cardioprotection such as ischaemic preconditioning and heat stress pretreatment, JNK activation is also found to be attenuated during the subsequent I/R (Sato *et al.* 2000, Pantos *et al.* 2003b,c). The fact that both hypothyroidism and preconditioning reduce the JNK activation in response to I/R might indicate that JNK is an essential component of the protection conferred by these two interventions. It is also of note that carvedilol administration at reperfusion is shown to increase tolerance of the heart to ischaemia while JNK activation is attenuated (Yue *et al.* 1998).

It appears from this study and from previous studies that thyroid hormone can play an important role in the response of the heart to ischaemia. Long-standing alterations in thyroid hormone can induce adaptive changes in the myocardium with important physiological consequences as regards the cardioprotection. Different underlying mechanisms seem to exist between the hypothyroid- and hyperthyroid-induced cardioprotection and this issue needs to be further investigated.

In conclusion, propylthiouracil-induced hypothyroidism increases post-ischaemic recovery of function and this was associated with basal PKC ϵ overexpression and attenuated JNK activation in response to I/R.

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