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 (PKCε) and PKCδ 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, PKCε expression was 1·4-fold more in HYPO than in NORM hearts, P < 0·05, while PKCδ 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 PKCε overexpression and attenuated JNK activation after I/R.


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 T4-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, adenylyl 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 PKCe or PKCβ (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 Leaudro, CA, USA). For comparisons between groups, five samples from each group were loaded on the same gel. Optical densities of PKCe, PKCβ, 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,
hearts from NORM and HYPO animals were also subjected to 20 min of stabilization, 30 min of zero-flow global ischemia 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 ± 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 ischemia (Fig. 1; Table 2).

**Ischemic contracture profile**

Profiles of ischemic contracture are shown in Fig. 2. Within 20 min of ischemia, 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 ischemia, ischemic contracture reached a maximum at 25·3 ± 1·4 min in NORM hearts, while in HYPO hearts it did not reach a peak.

**PKCε and PKCβ protein expression at baseline**

PKCβ protein expression at baseline was not different between NORM and HYPO hearts, \( P > 0·05 \). However, PKCε protein expression at baseline was 1·4-fold more in HYPO-Base than in NORM-Base hearts, \( P < 0·05 \) (Fig. 3).

**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 ± S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>NORM</th>
<th>HYPO</th>
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<tbody>
<tr>
<td>BW1</td>
<td>305 ± 7·9</td>
<td>307 ± 2·6</td>
</tr>
<tr>
<td>BW2</td>
<td>338 ± 9·9</td>
<td>268 ± 9·1*</td>
</tr>
<tr>
<td>LVW</td>
<td>831 ± 27·7</td>
<td>675 ± 20·2*</td>
</tr>
<tr>
<td>LVW/BW</td>
<td>2·4 ± 0·05</td>
<td>2·5 ± 0·09</td>
</tr>
<tr>
<td>T3</td>
<td>0·87 ± 0·04</td>
<td>0·23 ± 0·05*</td>
</tr>
<tr>
<td>T4</td>
<td>52·50 ± 2·63</td>
<td>19·97 ± 0·38*</td>
</tr>
</tbody>
</table>

\(* P < 0·05 \) vs NORM.

**Table 2** Left ventricular developed pressure (LVDP, mmHg), \( + \text{dp/dt} \) (mmHg/s) and \( - \text{dp/dt} \) (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 ischemia. I/R = ischemia/reperfusion. The values are means ± S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>NORM-20I/R ((n=8))</th>
<th>HYPO-20I/R ((n=8))</th>
<th>NORM-30I/R ((n=8))</th>
<th>HYPO-30I/R ((n=8))</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVDP (baseline)</td>
<td>129·3 ± 4·5</td>
<td>103·8 ± 2·3*</td>
<td>133·9 ± 4·5</td>
<td>109·0 ± 3·6**</td>
</tr>
<tr>
<td>+ dp/dt (baseline)</td>
<td>5150 ± 280</td>
<td>3417 ± 135*</td>
<td>4476 ± 227</td>
<td>3200 ± 259**</td>
</tr>
<tr>
<td>− dp/dt (baseline)</td>
<td>2615 ± 112</td>
<td>1851 ± 47*</td>
<td>2461 ± 149</td>
<td>1731 ± 85**</td>
</tr>
<tr>
<td>LVDP at 45 min R</td>
<td>80·1 ± 6·5</td>
<td>96·3 ± 3·6*</td>
<td>156·3 ± 3·1</td>
<td>65·9 ± 10·8**</td>
</tr>
<tr>
<td>LVEDP at 45 min R</td>
<td>52·8 ± 5·6</td>
<td>12·3 ± 3·4*</td>
<td>105·8 ± 3·6</td>
<td>36·0 ± 7·7**</td>
</tr>
<tr>
<td>LVDP%</td>
<td>60·6 ± 5·1</td>
<td>93·1 ± 4·1*</td>
<td>115·2 ± 2·1</td>
<td>61·3 ± 11·0**</td>
</tr>
</tbody>
</table>

\(* P < 0·05 \) vs NORM-20I/R; \( * * P < 0·05 \) vs NORM-30I/R.
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 PKCδ 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 *T₄* and *T₃* 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 Ca²⁺-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 isom. 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

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**Figure 1** Post-ischaemic recovery of function, LVDP%, (upper panel) and left ventricular end-diastolic pressure at 45 min of reperfusion, LVEDP%, (bottom panel) in normal hearts (NORM) and hearts from hypothyroid rats (HYPO) subjected to 20 or 30 min of ischaemia. (Bar=S.E.M.)
cardioprotection has been demonstrated by various studies (Speechly-Dick et al. 1994, Kawamura et al. 1998). In fact, PKCε has been shown to be mainly involved in cardioprotective means such as ischaemic preconditioning (Fryer et al. 2002) while PKCδ has been implicated in pharmacological preconditioning (Fryer et al. 2001). Interestingly, chronic T4 administration is shown to upregulate PKCδ (Fryer et al. 1998, Pantos et al. 2002a) and induce pharmacological preconditioning (Pantos et al. 2002a), while cells overexpressing PKCδ (Zhao et al. 1998) or hearts from mice overexpressing PKCδ (Cross et al. 2002) are found to be less susceptible to ischaemia (Cross et al. 2002). In the present study, PKCε expression was found to be increased in HYPO hearts while PKCδ expression remained unchanged. On the basis of these data, it could be suggested that PKCε 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 PKCε as regards the response to ischaemia as well as ATP utilization during I/R; in a transgenic model overexpressing PKCε 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, PKCε 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 (Gabai et al. 1998). This has been clearly demonstrated in
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=SEM.)

Figure 3
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 evi-
dence support this notion. Recent studies demonstrate that
in established paradigms of cardioprotection such as ischae-
mic preconditioning and heat stress pretreatment, JNK
activation is also found to be attenuated during the
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
noted that carewiled administration at reperfusion is
shown to increase tolerance of the heart to ischaemia while
JNK activation is attenuated (Yue et al. 1998).

An 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 alter-
tations in thyroid hormone can induce adaptive changes
in the myocardium with important physiological conse-
quences as regards the cardioprotection. Different underly-
ing mechanisms seem to exist between the
hypothyroid- and hyperthyroid-induced cardioprotection
and this issue needs to be further investigated.

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

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