Long-term thyroxine administration increases heat stress protein-70 mRNA expression and attenuates p38 MAP kinase activity in response to ischaemia

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Abstract

The present study was undertaken to investigate heat stress protein (HSP)-70 mRNA induction and p38 MAP kinase (MAPK) activity in response to ischaemic stress in the hyperthyroid rat heart.

L-Thyroxine (T₄) (25 µg/100 g body weight) was administered to Wistar rats for 2 days (THYRacute) or 14 days (THYR), while animals treated similarly with normal saline served as controls (NORMacute and NORM). In addition, abdominal aortic banding was performed in another group of rats to produce constriction-induced hypertrophy (HYP), while sham-operated (SOP) animals served as controls. Isolated rat hearts were perfused in a Langendorff mode. Hearts from NORMacute (n=6), THYRacute animals (n=8), NORM (n=6), THYR (n=6), SOP (n=5) and HYP (n=7) animals were subjected to 20 min of zero-flow global ischaemia followed by 45 min of reperfusion. HSP70 mRNA expression and phosphorylated p38 MAPK protein expression were detected in response to ischaemia and protein kinase C-ε (PKCε) protein expression was detected at baseline. Thyroid hormones were measured in plasma.

Long-term T₄ administration and aortic constriction resulted in the development of cardiac hypertrophy. Thyroid hormones were increased in both THYR and THYRacute as compared with normal groups (P<0.05). HSP70 mRNA induction was increased 2.3-fold in THYR as compared with NORM hearts (P<0.05), whereas there was not any difference between THYRacute and NORMacute hearts (P>0.05). Phosphorylated p38 MAPK protein expression was 2.2-fold more in NORM than in THYR hearts (P<0.05), but it was not different between NORMacute and THYRacute hearts (P>0.05). HSP70 mRNA induction was 1.8-fold greater in HYP than in SOP hearts (P<0.05), whereas phosphorylated p38 MAPK protein expression was similar between the two groups (P>0.05). PKCε protein expression at baseline was 1.7-fold more in NORM than in THYR hearts (P<0.05), and not different between NORMacute and THYRacute hearts (P>0.05) as well as HYP and SOP hearts (P>0.05).

This study shows that HSP70 mRNA expression is increased, whereas p38 MAPK activation is attenuated in response to ischaemia in long-term T₄-treated rat hearts as compared with normal and acute hyperthyroid hearts.


Introduction

Hyperthyroidism is associated with various effects on the myocardium that could potentially render the heart less tolerant to ischaemic stress. In hyperthyroidism, cardiac hypertrophy develops and alterations in cardiac energy metabolism occur (Klein 1980, Polikar et al. 1993). In fact, hearts with l-thyroxine (T₄)-induced cardiac hypertrophy has been found to have a shift towards isomyosin V1, the myosin with the fast ATPase activity and consequently decreased thermodynamic efficiency (Hoh & Egerton 1979). Furthermore, the levels of creatinine, phosphocreatine, ATP and glycogen are reduced in hyperthyroid hearts (Van der Vusse et al. 1998). However, hearts from T₄-treated animals are reported to tolerate ischaemia as well as normal hearts or even better, but the exact mechanism of this response is unclear (Buser et al. 1990, Pantos et al. 1999, 2000).

The adaptive response of the hyperthyroid heart to ischaemia and its molecular basis have not been adequately studied. It is likely that the occurring functional and metabolic changes in the hyperthyroid heart can induce changes in gene expression of various molecules that can confer cardioprotection against ischaemic insult. Heat
stress proteins (HSPs) and intracellular mitogen–activated protein kinases (MAPKs) have been found to play an essential role in the adaptive response of the normal heart to ischaemia (Gray et al. 1999, Bogoyevitch 2000). In fact, ischaemia can lead to the induction of HSPs and activation of the intracellular kinases as a cellular response to stress. HSP70, a member of the HSP family, has been associated with preservation of high energy phosphates, anti-apoptotic effects and increased tolerance to ischaemic stress (Gray et al. 1999). p38 MAPK, a member of the MAPK family, has been shown to be one of the signal transducers of apoptosis (Zechner et al. 1998, Yaoita et al. 2000). Furthermore, inhibition of p38 MAPK is associated with reduced apoptosis (Zechner et al. 1998, Ma et al. 1999) and increased post–ischaemic functional recovery (Ma et al. 1999). Similar studies on hyperthyroid hearts do not appear in the current literature. However, in those hearts, the study of the pattern of HSP70 mRNA expression and p38 MAPK activation in response to ischaemia could be of importance, since hyperthyroid hearts seem to have an increased tolerance to ischaemia through an undefined mechanism (Buser et al. 1990, Pantos et al. 1999, 2000).

The present study was designed to investigate the pattern of HSP70 mRNA expression and p38 MAPK activation in response to ischaemia in isolated hyperthyroid rat hearts. In addition, in order to define the role of cardiac hypertrophy per se in that particular response, the expression of HSP70 mRNA and the activation of p38 MAPK were also assessed in another model of cardiac hypertrophy, the aortic banding-induced cardiac hypertrophy (HYP).

Materials and Methods

Animals

Seventy-four Wistar male rats, 280–320 g body weight (BW), 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).

T₄ administration

Hyperthyroidism was induced in rats by T₄ administration. T₄ (Sigma Chemicals, St Louis MO, USA) was dissolved in 99% ethanol by adding a small volume (20 μl) of 25% NaOH and diluted 33-fold by adding 0.9% NaCl to obtain a stock solution of 1 mg/ml. Before each injection a fresh solution was made in 0.9% NaCl to a concentration of 50 μg T₄/ml. T₄ (25 μg/100 g BW) was administered s.c. once daily for 14 days. This treatment results in long-term moderate hyperthyroidism (Grofte et al. 1997). Normal rats were treated with s.c. injections of normal saline given once daily for 14 days (Pantos et al. 1999, 2001).

T₄ and normal saline were also administered once daily for 2 days and this treatment has been shown to result in acute hyperthyroidism (Grofte et al. 1997, Pantos et al. 2000).

Abdominal aortic stenosis

Abdominal aortic stenosis was induced using Weck haemoclips (Pilling Medical, Le Faget, France) placed above the renal arteries near the diaphragm using modified Weck Forcesps. The diameter of the clips was calibrated to produce cardiac hypertrophy with a reasonably low mortality (approximately 15%). A moderate hypertrophy was obtained after 4 weeks in most animals. Sham-operated (SOP) animals served as controls (Pantos et al. 1996).

Isolated heart preparation

A non-ejecting isolated rat heart preparation was perfused at a constant flow according to the Langendorff technique. 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. Since the balloon was not compressible, left ventricular contraction was isovolumic. As intraventricular volume was maintained at a constant value, diastolic fibre length, which represented preload, did not change. Thus, the left ventricular peak systolic pressure and the left ventricular developed pressure (LVDp), defined as the difference between left ventricular peak systolic pressure and LVDp, represented contractility indexes obtained under isometric conditions.

Rats were anaesthetized with ketamine hydrochloric acid, and heparin (1000 IU/kg) was given i.v. before thoracotomy. The hearts were rapidly excised, placed in ice-cold Krebs–Henseleit buffer (composition in mM: sodium chloride 118, potassium chloride 4.7, potassium phosphate monobasic 1.2, magnesium sulphate 1.2, calcium chloride 1.4, sodium bicarbonate 25 and glucose 11) and mounted on the aortic cannula of the Langendorff perfusion system. Perfusion with oxygenated (95% O₂/5% CO₂) Krebs–Henseleit buffer was established within 60 s after thoracotomy. The perfusion apparatus was heated to ensure a temperature of 37 °C throughout the experiment. Hearts were paced at 320 beats/min with a Harvard pacemaker (Edenbridge, Kent, UK). The pacemaker was turned off during the period of ischaemia. The water-filled balloon, connected to a pressure transducer and coupled to a Gould RS 3400 recorder (Valley View,
The purity of the isolated RNAs. The isolated RNAs had an OD of the preparation at 260 nm. The ratio of the RNA was estimated by measuring the optical density using the method described by Chomczynsky & Sacchi (1987). The concentration of Total RNA was extracted from myocardial ventricles. Isolation of RNA and Northern blot analysis

Total RNA was extracted from myocardial ventricles according to the procedure described by Chomczynsky & Sacchi (1987). The concentration of RNA was estimated by measuring the optical density (OD) of the preparation at 260 nm. The ratio of the isolated RNAs had ratios between 1:8 and 2:0. Twenty micrograms of RNA from each sample were subjected to 1% agarose-formaldehyde gel electrophoresis and then transferred to a nylon membrane (Amersham, Little Chalfont, Bucks, UK) by capillary blot. The membranes were baked at 80 °C for 2 h. Hybridization was carried out at 65 °C for 18 h using a human HSP70 cDNA probe labelled with fluorescein-11-dUTP and the random prime labelling module (Amersham). The membranes were washed using a solution of 1 × SSC–0.1% SDS at 65 °C for 15 min followed by a wash at 65 °C in 0.1% SSC–0.1% SDS for 15 min. The Gene Images CDP-Star detection module (Amersham) was used for detection. The autoradiograph was established by exposing the membrane to Hyperfilm–ECL (Amersham) at 250 mA and 4 °C. Blots were washed in TBST (3 × 5 min). Filters were then incubated with either either protein kinase-ε (PKCε) antibody (Transduction Laboratories, Lexington, KY, USA), dual phosphorylated p38 MAPK antibody or p38 MAPK antibody (New England Biolabs, Hitchin, Herts, UK) (dilution 1:1000) overnight at 4 °C. Blots were washed in TBST (3 × 5 min) and then exposed to horseradish peroxidase-conjugated second antibody at a dilution of 1:2000 for 1 h at room temperature. Filters were washed as above, incubated with ECL reagents and exposed to Hyperfilm paper (Amersham). Immunoblots and gels were quantified using the AlphaScan Imaging Densitometer (Alpha Innotech). Sample loadings were equalized relative to the OD of the actin band. ODs of PKCε, dual phosphorylated p38 and p38 MAPK immunoreactivity were expressed as a ratio of the actin OD to correct for slight variations in total protein loading.

Experimental protocol

Hearts from normal, acute and long-term T₄-treated animals, SOP and HYP animals that had not been subjected to any experimental procedure were used for the measurement of the PKCε levels at baseline. In order to detect the baseline HSP70 mRNA expression, hearts from all groups were perfused without being subjected to any ischaemic insult for a period equal to the duration of the experimental protocol.

Hearts from normal, hyperthyroid, SOP and HYP animals underwent an initial 30 min stabilization period. They were then subjected to 20 min of zero-flow global ischaemia followed by 45 min of reperfusion. Thus, there were the following experimental groups: hearts from normal animals with 2 day normal saline administration (NORM, n=6), hearts from hyperthyroid animals with 2 day T₄ administration (THYRacute, n=8), hearts from normal animals with 14 day normal saline administration (NORM, n=6), hearts from hyperthyroid animals with 14 day T₄ administration (THYR, n=6), hearts from SOP animals, (n=5), and hearts from HYP animals (n=7).

Measurement of thyroid hormones

Plasma T₄ and tri-iodothyronine (T₃) quantitative measurements were performed by using ¹²⁵I RIA kits.

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obtained from DiaSorin, Stillwater, MN, USA (CA 1535 M for T₄ and CA 1541 for T₃). T₄ and T₃ levels were expressed as pmol/ml plasma.

**Measurement of cardiac hypertrophy**

Cardiac hypertrophy was assessed by the measurement of left ventricular weight (LVW, in mg) and the ratio of LVW to BW (in g) (LVW/BW, in mg/g).

**Measurement of mechanical function**

Left ventricular systolic function was assessed by recording LVDP at the end of the stabilization period and at 45 min of reperfusion. Post-ischaemic function was assessed by the recovery of LVDP, which was expressed as per cent of the initial value (LVDP%). Diastolic function was assessed by monitoring isovolumic LVEDP as a measure of diastolic chamber distensibility. LVEDP was measured at 45 min of reperfusion (LVEDP45).

**Statistics**

Values are presented as means ± s.e.m. An unpaired *t*-test and a 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**

**Cardiac hypertrophy**

In acute hyperthyroidism, LVW was 887.8 ± 33.5 mg for NORMacute and 882.6 ± 33.1 mg for THYRacute hearts (*P*<0.05). LVW/BW was 2.5 ± 0.06 for NORMacute and 2.6 ± 0.10 for THYRacute hearts (*P*<0.05).

With long-term T₄ administration, LVW was increased in THYR as compared with normal hearts (1108 ± 46.8 vs 872.2 ± 23.3 mg, *P*<0.05). LVW/BW was 2.5 ± 0.10 for NORM and 3.1 ± 0.09 for THYR hearts (*P*<0.05).

LVW was 909.5 ± 25.5 mg for SOP and 988.2 ± 23.8 mg for HYP hearts (*P*<0.05). LVW/BW was 2.8 ± 0.06 for HYP and 2.5 ± 0.04 for SOP hearts (*P*<0.05).

**Thyroid hormones**

T₄ administration resulted in increased thyroid hormone levels in the blood. T₄ and T₃ were 47.1 ± 6.0 and 0.8 ± 0.1 pmol/ml for NORM, 1041.3 ± 69.8 and 6.0 ± 0.9 pmol/ml for THYRacute animals (P<0.05) and 723.2 ± 79.2 and 7.4 ± 0.9 pmol/ml for THYR animals (P<0.05).

**HSP70 mRNA expression at baseline and after ischaemia**

HSP70 mRNA expression was not detected at baseline in hearts from any of the experimental groups.

The induction of HSP70 mRNA by ischaemia was 2.3-fold greater in THYR as compared with NORM hearts (*P*<0.05), but there was no statistically significant difference between the THYR and NORM hearts (*P*>0.05), (Figs 1 and 2). The induction of HSP70 mRNA by ischaemia was 1.8-fold greater in HYP as compared with SOP hearts, (*P*<0.05) (Figs 1 and 2).

**Phosphorylated p38 MAPK protein expression in response to ischaemia**

Phosphorylated p38 MAPK protein expression was 2.2-fold more in NORM than in THYR hearts (*P*<0.05), but it was not different between NORMacute and

<table>
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<th>Group</th>
<th>n</th>
<th>LVDP</th>
<th>LVEDP45</th>
<th>LVDP%</th>
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<tr>
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<td>127.6 (2.3)*</td>
<td>53.3 (8.5)</td>
<td>54.9 (5.5)*</td>
</tr>
<tr>
<td>SOP</td>
<td>5</td>
<td>97.6 (3.4)</td>
<td>54.8 (6.6)</td>
<td>53.3 (5.8)</td>
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<tr>
<td>HYP</td>
<td>7</td>
<td>132.6 (5.6)**</td>
<td>60.6 (9.9)</td>
<td>43.4 (8.2)</td>
</tr>
</tbody>
</table>

*P<0.05 vs NORM; **P<0.05 vs SOP.

**Figure 1** Densitometric assessment of Northern blots showing the expression of HSP70 mRNA induced by ischaemia in hyperthyroid (THYRacute and THYR) and normal hearts (NORMacute and NORM), and hearts from SOP and HYP rats (columns are means of OD ratios ± s.e.m.). *P<0.05 vs NORM, **P<0.05 vs SOP.
THYR acute hearts, \((P>0.05)\) (Fig. 3). Phosphorylated p38 MAPK protein expression was not different between HYP and SOP hearts \((P>0.05)\) (Fig. 3).

**PKCε protein expression at baseline**

PKCε protein expression at baseline was 1.7-fold more in NORM than in THYR hearts \((P<0.05)\) and not different between NORMacute and THYRacute hearts \((P>0.05)\) (Fig. 4). In addition, PKCε protein expression at baseline was not different between SOP and HYP hearts \((P>0.05)\) (Fig. 4).

**Discussion**

In this study we investigated the expression of HSP70 mRNA and the activation of p38 MAPK in response to ischaemia in hyperthyroid rat hearts. These molecules are involved in the biochemical pathways that allow adaptation of the cell to various stresses such as ischaemia, hypoxia or heat.

HSP70 mRNA induction has been found to occur in normal hearts in response to ischaemia. Oxidant stress, intracellular ATP depletion and anaerobic metabolism by itself have been found to activate the heat shock transcription factor and increase the expression of HSP70 in response to ischaemic stress (Benjamin et al. 1992, Kukreja et al. 1994, Myrmel et al. 1994). Furthermore, this response can be modulated by various factors such as cardiac hypertrophy, ageing and neurohormonal alterations that accompany certain disease states (Nitta et al. 1994, Tajima et al. 1997). In fact, in an in vivo model of pressure overload cardiac hypertrophy, HSP70 mRNA expression in response to brief regional ischaemia was found to be attenuated in hypertrophied as compared with normal hearts, while a longer period of ischaemia abolished that response (Tajima et al. 1997). Furthermore, in ageing hearts, HSP70 mRNA induction by ischaemia was also shown to be attenuated in comparison with young hearts (Nitta et al. 1994).

In the present study, HSP70 mRNA induction in response to ischaemia was found to be altered in the presence of thyroid hormone excess. In fact, HSP70 mRNA induction was increased in hearts from THYR animals as compared with NORM hearts. Neurohormonal and other cellular changes that occur in hyperthyroidism can account for this response. Increased circulating thyroid hormone levels and/or the development of cardiac hypertrophy may play an essential role (Pantos et al. 1999, 2000). In order to address this issue, a model of acute hyperthyroidism was established and another model of cardiac hypertrophy was studied. In the model of acute hyperthyroidism, as it has been previously described, cardiac hypertrophy does not develop, but the circulating thyroid hormones are elevated to similar levels as in long-term administration of \(T_4\). Interestingly, in this experimental setting, we found that HSP70 mRNA induction by ischaemia was similar in THYRacute and NORMacute hearts. Hearts from HYP rats overexpressed HSP70 mRNA in response to ischaemia as compared with hearts from SOP rats. These data probably indicate that the development of cardiac hypertrophy rather than the increased levels of thyroid hormones might play an important role in the increased induction of HSP70 mRNA after sustained global ischaemia. However, the underlying mechanism of this response remains undefined. In cardiac hypertrophy, increased activation of various transcription factors has been shown to occur (Moalic et al. 1989). It is likely that such changes could alter the pattern of HSP70

![Figure 2](image_url)
mRNA induction by ischaemia in the hyperthyroid hearts.

The present study has also investigated the mode of p38 MAPK activation in the hyperthyroid heart subjected to ischaemic stress. p38 MAPK is one of the intracellular MAPKs that has been shown to be activated after ischaemia in normal hearts and the activation is maintained during the reperfusion period (Bogoyevitch et al. 1996, Yin et al. 1997). Both the duration and intensity of the stress seem to determine its regulation and the final cellular outcome (Mackay & Mochly-Rosen 1999, Bogoyevitch 2000). Transient activation of p38 MAPK seems not to have a deleterious effect on the cell, whereas sustained activation of p38 MAPK induces apoptosis (Mackay & Mochly-Rosen 1999). Activation of p38 MAPK has been found to promote cardiac myocyte death during extended periods of ischaemia (Mackay & Mochly-Rosen 1999), whereas inhibition of p38 MAPK can reduce apoptosis (Zechnet al. 1998, Ma et al. 1999) and delay ischaemic cell death (Mackay & Mochly-Rosen 1999, Barancik et al. 2000). In humans, it is also reported that the activity of p38 MAPK is increased in heart failure secondary to ischaemic heart disease (Cook et al. 1999). Furthermore, Nagarkatti & Shaaifi (1998) by using either SB203580, an inhibitor of p38 MAPK, or G0 6850, an inhibitor of PKC, during a lethal ischaemic insult, demonstrated increased cell survival as compared with cells subjected directly to the insult and suggested that this protective response involves both PKC and p38 MAPK.

In this study, we measured the levels of the dual phosphorylated p38 MAPK to assess the activity of p38 MAPK in response to ischaemia. It is known that p38 MAPK is activated by dual phosphorylation of threonine 180 and tyrosine 182 (Nagarkatti & Shaaifi 1998).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Phosphorylated p38 MAPK protein expression in hearts from THYRacute, THYR, NORMacute, NORM, SOP and HYP rats. Phosphorylated p38 MAPK was detected as a 40 kDa band. Ph-p38=P phosphorylated p38 MAPK, p38=total p38 MAPK (columns are means of OD ratios±S.E.M.). *P<0.05 vs NORM.}
\end{figure}
THYR hearts, dual phosphorylated p38 MAPK after ischaemia was less as compared with NORM hearts. Furthermore, in the experimental setting of the acute hyperthyroidism, this response was abolished. In fact, the levels of the phosphorylated p38 MAPK after ischaemia were similar in THYR acute and NORM acute hearts.

These data suggest that the observed reduction in p38 MAPK activity in response to ischaemia in hearts with chronic T4 treatment is probably the result of the development of cardiac hypertrophy per se. However, as our data show, a similar response was not observed in hearts from HYP rats. Indeed, phosphorylated p38 MAPK levels in response to ischaemia were similar in hearts from HYP as compared with hearts from SOP animals. This observation probably indicates that the decreased p38 MAPK activation that occurs after ischaemia in long-term T4-treated hearts cannot be merely attributed to cardiac hypertrophy per se and other factors might also be involved. In fact, recent studies show that in hearts with different types of cardiac hypertrophy, various alterations exist in the intracellular PKC (Fryer et al. 1998, Jalili et al. 1999, Hamasaki et al. 2000), a molecule that has been previously demonstrated to be involved as well as p38 MAPK in the intracellular signalling transduction pathway that mediates the adaptive response of the cardiac cell to ischaemic stress (Nagarkatti & Shaafi 1998).

Interestingly, Fryer et al. (1998) found that the expression of the ε-isoform of PKC was decreased in hyperthyroid hearts and this modulation of the PKCε isoform was suggested to be a specific response to an activated renin-angiotensin system. Furthermore, Hamasaki et al.
In experimental models of T4- and aortic constriction-induced cardiac hypertrophy found that the protein expression of the PKCe was downregulated in the T4-induced cardiac hypertrophy, whereas PKCe protein expression was not decreased in hearts with aortic banding-induced cardiac hypertrophy. Similarly, in the present study, PKCe protein expression was found to be downregulated in the THYR as compared with NORM hearts, whereas in the THYR acute hearts it was similar to NORM acute hearts. Furthermore, PKCe protein expression was similar in hearts from HYP and SOP rats. This differential pattern of PKCe protein expression that is observed in hearts with T4- and aortic banding-induced hypertrophy seems to be identical to the pattern of activation of p38 MAPK that occurs in response to ischaemia. Thus, it could be suggested that in long-term T4-treated hearts the observed reduction of p38 MAPK activity might be the result of the decreased PKCe activation corresponding to the reduced PKCe protein expression. However, in the present study, we did not measure the activity of PKCe. Further studies are needed to elucidate the underlying mechanisms of the attenuated p38 MAPK activation induced by ischaemia in long-term T4-treated hearts.

It is apparent that the induction of HSP70 mRNA expression and the activation of p38 MAPK in response to ischaemic stress is altered after chronic T4 administration. However, the biological significance of this response is not known. In normal hearts, increased HSP expression has been found to confer cardioprotection against ischaemic insult (Marber et al. 1995, Suzuki et al. 1997). Furthermore, decreased activity of the p38 MAPK has been shown to reduce apoptosis and to protect the cell from lethal ischaemic stress (Zechner et al. 1998, Ma et al. 1999, Mackay & Moehly-Rosen 1999, Barancik et al. 2000). However, further work is needed to clarify the role of the HSPs and the p38 MAPK in the adaptive response of the hyperthyroid heart to ischaemia. Hyperthyroid hearts, as this study shows in accordance with previous reports (Buser et al. 1999, Pantos et al. 1999, 2000), have increased tolerance to ischaemia regardless of the development of cardiac hypertrophy and the decreased thermodynamic efficiency. Moreover, it has been recently reported that subclinical hypothyroidism is an independent risk factor for myocardial infarction in elderly women (Hak et al. 2000) and lower levels of T3 are associated with greater severity of acute myocardial infarctions (Kimura et al. 2000).

In conclusion, long-term T4 administration increases HSP70 mRNA induction and attenuates p38 MAPK activity in response to ischaemia. The biological significance of this adaptive response remains to be elucidated.

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