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Thyroxine pretreatment increases basal myocardial heat-shock protein 27 expression and accelerates translocation and phosphorylation of this protein upon ischaemia

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Abstract

Thyroxine pretreatment increases the tolerance of the heart to ischaemia, and heat-shock protein 27 (HSP27) is considered to play an important role in cardioprotection. The present study investigated whether long-term thyroxine administration can induce changes in the expression, translocation and phosphorylation of HSP27 at baseline and upon ischaemic stress. L-Thyroxine (T_4) was administered to Wistar rats (25 μg/100 g/day s.c.) for 2 weeks, while normal animals served as controls. Hearts from normal and thyroxine-treated rats were perfused in Langendorff mode and subjected to 10 or 20 min of zero-flow global ischaemia only or to 20 min of ischaemia followed by 45 min of reperfusion. Total and phospho-HSP27 expression were assessed at different times in the Triton-soluble (cytosol-membrane), S fraction, and the Triton-insoluble (cytoskeleton-nucleus) fraction, P fraction. Postischaemic recovery of left ventricular developed pressure at 45 min of reperfusion was expressed as % of the initial value. In hearts from thyroxine-treated animals, the levels of basal total HSP27 and phospho-HSP27 in the P fraction were significantly increased as compared to normal. In response to ischaemia, in hearts from thyroxine-treated rats, the levels of total HSP27 and phospho-HSP27 were found to be significantly increased in the P fraction at 10 and 20 min of ischaemia as compared to preischaemic values, whereas in normal hearts, the levels of total HSP27 and phospho-HSP27 were significantly increased at 20 min only. Postischaemic functional recovery was significantly greater in thyroxine-treated than in untreated hearts. In summary, long-term thyroxine pretreatment results in an increased basal expression and phosphorylation of HSP27 and in an earlier and sustained redistribution of HSP27 from the S to the P fraction in response to ischaemia. This effect might be of important therapeutic relevance.
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1. Introduction

Over the past years, tremendous progress has been made in understanding the pathophysiology of ischaemic heart disease and this has led to several therapeutic approaches to protect the myocardium from ischaemic injury. Important molecules that are found to play a crucial role in cardioprotection have been pharmacologically targeted with the hope of altering the response of the heart to ischaemia. Among these molecules, small heat-shock proteins have recently begun to receive attention as modulators of the response of the cell to ischaemic stress, mainly due to their effect on the stability of the cytoskeleton (Lavoie et al., 1993b; Huot et al., 1996; Guay et al., 1997; James et al., 2001). The cytoskeleton has been shown to play a crucial role in cell damage in response to stressful conditions (Steenbergen et al., 1987; Ganote and Armstrong, 1993). Disruption of this structure and disaggregation of actin filaments are immediate effects of a variety of stresses in eukaryotes, and stability of the cytoskeleton has been shown to increase cell survival after stress (Lavoie et al., 1993a; Huot et al., 1996). In fact, several studies have demonstrated that changes in small heat-shock proteins, and particularly in heat-shock protein 27 (HSP27), are associated with strengthening of the cyto-

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skeleton and increased tolerance of the cell to stress-induced injury (Lavoie et al., 1993a; Huot et al., 1996; Guay et al., 1997). Cells overexpressing HSP27 or other small heat-shock proteins are found to be resistant to ischaemic stress and other stresses (Landry et al., 1989; Lavoie et al., 1993a; Huot et al., 1996; Martin et al., 1997; Fortin et al., 2000; Ray et al., 2001). More importantly, established means of cardioprotection, such as ischaemic preconditioning, have been shown to involve alterations in the translocation and phosphorylation of small heat-shock proteins (Sakamoto et al., 2000; Eaton et al., 2001).

HSP27, like other heat-shock proteins, forms large multimeric complexes and phosphorylation, induced by stress, growth factors or pharmacological kinase activation, is the cue for these complexes to break down, a process crucial for translocation and protection (Lambert et al., 1999; Rogalla et al., 1999). Furthermore, the same function of HSP27 can be activated by growth factor stimulation and the stress response (Arrigo and Landry, 1994). The latter seems to be of importance since growth factors could potentially modulate the response of the cell to stress by regulating the function of cytoskeletal proteins such as HSP27. In fact, it is now realized that growth and the response to stress share common intracellular pathways, and this could be exploited therapeutically (Sugden and Clerk, 1998).

Thyroid hormone is one of the most important physiological regulators of the growth and development of all organs, including the heart. Owing to its regenerating effects, it has been recently used in nerve tissue repair and regeneration (Schenker et al., 2002). Interestingly, this effect was found to be mediated via regulation of cytoskeletal proteins (Schenker et al., 2002). This evidence could lead to the hypothesis that thyroid hormone can also be a regulator of small heat-shock proteins in the myocardium, with a subsequent beneficial effect on the response of the heart to ischaemia. Interestingly, thyroxine pretreatment has been recently found to result in increased tolerance of the isolated rat heart to sustained ischaemia (Pantos et al., 2000, 2001, 2002a,b, 2003a,b).

In the light of this evidence, the present study investigated whether long-term thyroxine pretreatment is associated with changes in the expression, translocation and phosphorylation of HSP27 under baseline conditions and in response to ischaemic stress. This issue has not been previously addressed and seems to be of clinical interest since thyroxine, besides being an inotrope, might prove to be a suitable agent for cardioprotection.

2. Materials and methods

2.1. Animals

Forty-six Wistar male rats, 280–330 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 intraperitoneal injection of ketamine hydrochloric acid (150 mg/kg).

2.2. Thyroxine administration

L-Thyroxine (T_4) (Sigma, St Louis MO, USA) was dissolved in 99% ethanol by adding a small volume (20 μl) of 25% NaOH and diluted 33 times by adding 0.9% NaCl to obtain a stock solution of 1 mg ml^{-1}. Before each injection, a fresh solution was made in 0.9% NaCl to a concentration of 50 μg T4 ml^{-1}. Thyroxine (25 μg 100 g^{-1} body weight) was administered subcutaneously once daily for 14 days. Normal rats were given subcutaneous injections of normal saline once daily for 14 days (Pantos et al., 2001, 2002a,b, 2003a,b).

2.3. 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 of 6 mm Hg in all groups and was held constant throughout the experiment. Since the balloon was not compressible, left ventricular contraction was isovolumic. As intraventricular volume was maintained at a constant value, diastolic fiber 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 left ventricular end-diastolic pressure, represented contractility indexes obtained under isometric conditions.

Rats were anaesthetized with ketamine hydrochloric acid, and heparin 1000 IU/kg was given intravenously before thoracotomy. The hearts were rapidly excised, placed in ice-cold Krebs-Henseleit buffer (composition in mmol/l: 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% O_2/5% CO_2) 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 per minute with a Harvard pacemaker. 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, was advanced into the left ventricle through an incision in the left atrium. Pressure signal was transferred to a personal computer using data analysis software (IOX, Emka Technologies) which allowed continuous monitoring and record-

ing of heart function. (Pantos et al., 2001, 2002a,b, 2003a,b,c).

2.4. Total protein preparation

Approximately 0.2 g of left ventricular tissue was homogenized in ice-cold buffer (homogenization buffer) containing 10 mM Tris–HCl pH = 7.5, 3 mM ethylenediaminetetraacetic acid, 1 mM phenylmethanesulphonyl fluoride, 30 μM leupeptin, 1 mM Na_3VO_4 and Triton X-100 0.1% with a Polytron homogenizer (Eaton et al., 2001). The resulting homogenate was centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant (Triton-soluble) corresponded to the cytosol-membrane fraction (fraction S). In order to purify the pellet, it was dissolved with homogenization buffer and centrifuged again (10,000 × *g* for 10 min, 4 °C). The supernatant was discarded. The pellet (Triton-insoluble) corresponded to the cytoskeleton-nucleus fraction (fraction P). Protein concentrations were determined by the bicinchoninic acid (BCA) method, using Bovine Serum Albumin as a standard (Walker, 1994).

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting

Samples were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by boiling for 5 min in Laemmli sample buffer containing 5% 2-mercaptoethanol. Aliquots (40 or 60 μg) were loaded onto 12% (w/v) acrylamide gels and subjected to SDS-PAGE in a Bio-Rad Mini Protean gel apparatus. For Western blotting, following SDS-PAGE, proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond ECL) at 80 mA and 4 °C, overnight using Towbin buffer. After transfer, the nitrocellulose membrane was blocked by incubation in 5% (w/v) non-fat dry milk dissolved in Tris-Buffered Saline-Tween (TBST) for 1.5 h for total HSP27 or for 1 h for phospho-HSP27 at room temperature. Filters were then incubated with specific antibodies against total HSP27 (Santa Cruz Biotechnologies, dilution 1:500) at room temperature for 1 h or with phosphorylated HSP27 antibody (New England Biolabs, dilution 1:1000), overnight at 4 °C. Blots were washed in TBST (4 × 5 min) and then exposed to horseradish peroxidase-conjugated second antibody at a dilution of 1:4000 for 1 h at room temperature. Filters were washed as above, incubated with Lumiglo reagents (New England Biolabs) and exposed to Hyperfilm paper (Amersham). Immunoblots and gels were quantified using an AlphaScan Imaging Densitometer (Alpha Innotech, USA), and optical densities for each sample were determined. For comparisons between groups, five samples from each group were loaded on the same gel. Phosphorylated and total HSP27 were traced from the same gel after stripping and reprobing. Actin was finally determined in each filter in order to normalize slight variations in protein loading.

2.6. Experimental protocols

(a) Hearts from normal, $n = 5$, and thyroxine-treated animals, $n = 5$, were subjected only to 20 min of stabilization. These experimental groups were designated as NORM-base and THYR-base, respectively.
(b) Hearts from normal, $n = 5$, and thyroxine-treated animals, $n = 5$, were subjected to 20 min of stabilization followed by 10 min of zero-flow global ischaemia. These experimental groups were designated as NORM-10I and THYR-10I, respectively.
(c) Hearts from normal, $n = 5$, and thyroxine-treated animals, $n = 5$, were subjected to 20 min of stabilization followed by 20 min of zero-flow global ischaemia. These experimental groups were designated as NORM-20I and THYR-20I, respectively.
(d) In order to define the functional response of the heart to ischaemia, hearts from normal, $n = 8$, and thyroxine-treated animals, $n = 8$, were subjected to 20 min of stabilization, 20 min of zero-flow global ischaemia and 45 min of reperfusion. These experimental groups were designated as NORM-I/R and THYR-I/R, respectively.

2.7. Measurement of thyroid hormones

Plasma T_4 and T_3 levels were measured by using ^{125}I radio immunofluorescence assay kits obtained from Dia-Sorin Stillwater, MN, USA (CA 1535 M for T_4 and CA 1541 for T_3). T_4 and T_3 levels are expressed as nmol/l of plasma.

2.8. Measurement of cardiac hypertrophy

Cardiac hypertrophy was quantified by measuring the left ventricular weight (LVW) in mg and the ratio of left ventricular weight (LVW) in mg to animal body weight (BW) in g; LVW/BW in mg/g.

2.9. 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. Postischaemic function was assessed by the recovery of LVDP, expressed as % of the initial value (LVDP%). Diastolic function was assessed by monitoring isovolumic left ventricular end-diastolic pressure (LVEDP) as a measure of diastolic chamber distensibility. Left ventricular end-diastolic pressure was measured at 45 min of reperfusion.

2.10. Statistics

Values are presented as means (S.E.M.). Unpaired *t*-test and Mann–Whitney test were used to evaluate differences between groups. A two-tailed test with a P value less than 0.05 was considered significant.

3. Results

3.1. Thyroid hormones

Thyroxine administration resulted in increased thyroid hormone levels in plasma. T_4 and T_3 concentrations were 45.8 (3.2) and 0.76 (0.08) nmol/l for normal animals, and 647.4 (128.8) and 5.92 (0.9) nmol/l for thyroxine-treated animals, $P < 0.05$.

3.2. Cardiac hypertrophy

Left ventricular weight (LVW) was increased in thyroxine-treated hearts as compared to normal hearts [832 (36.9) mg vs. 723 (26.7) mg $P < 0.05$]. The ratio of left ventricular weight to body weight (LVW/BW in mg/g) was 2.2 (0.06) for normal and 2.8 (0.1) for thyroxine-treated animals, $P < 0.05$.

3.3. Parameters of cardiac function

Postischaemic recovery of function was found to be increased in thyroxine-treated hearts than in normal hearts. In fact, LVDP% was 74.9 (4.4) for THYR-I/R and 55.7 (4.4) for NORM-I/R, $P < 0.05$, whereas LVEDP at 45 min of reperfusion was 39.9 (5.6) for THYR-I/R and 56.2 (3.9) for NORM-I/R, $P < 0.05$.

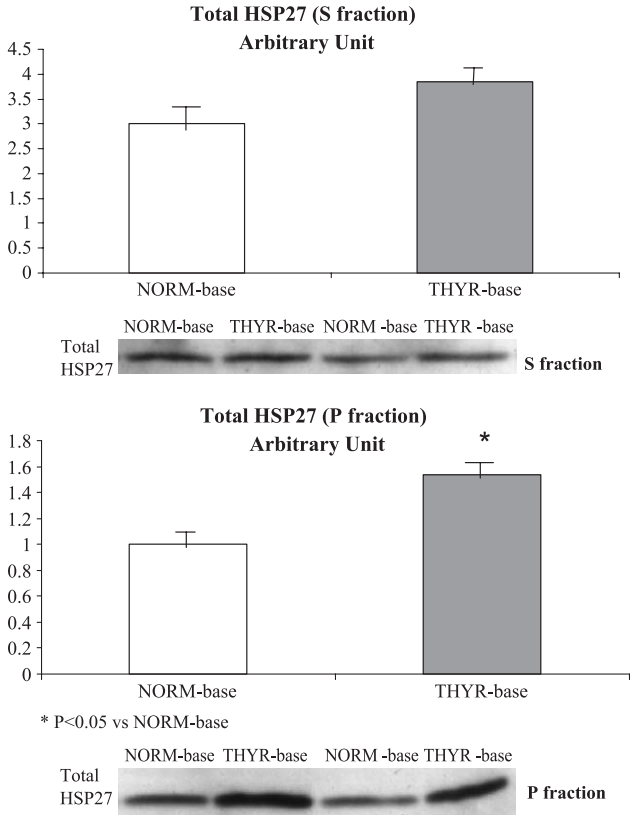

Fig. 1. Total Heat-shock Protein 27 expression at baseline in normal (NORM-base) and thyroxine-treated (THYR-base) hearts in the S and P fractions. (Columns are means of normalized optical densities, bar = S.E.M.).

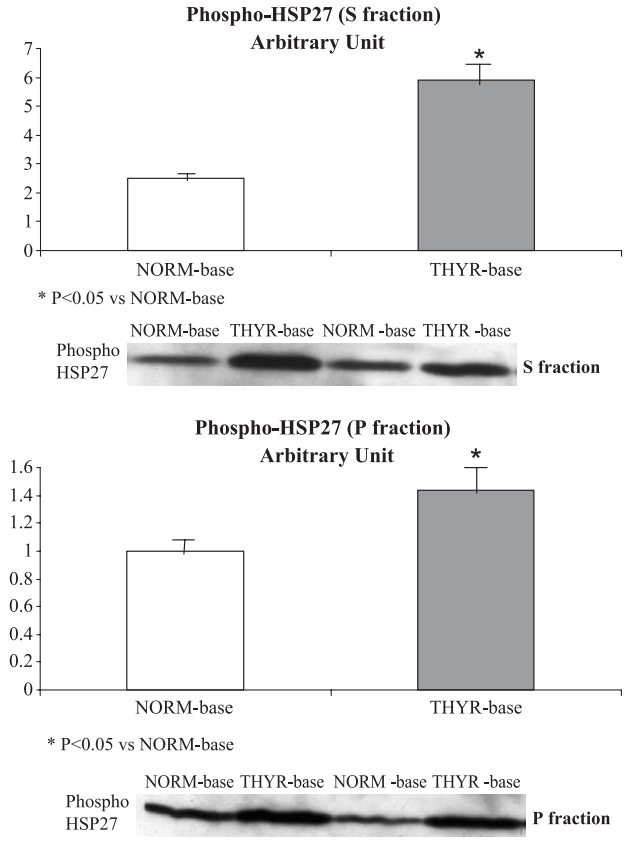

Fig. 2. Phosphorylated Heat-shock Protein 27 expression at baseline in normal (NORM-base) and thyroxine-treated (THYR-base) hearts in the S and P fractions. (Columns are means of normalized optical densities, bar = S.E.M.).

3.4. HSP27 changes at baseline in normal and thyroxine-treated hearts

In the S fraction, the levels of total HSP27 were not found to be different between NORM-base and THYR-base hearts, $P > 0.05$. In the P fraction though, the levels of total HSP27 were 1.5-fold higher in THYR-base than in NORM-base hearts, $P < 0.05$ (Fig. 1).

The levels of phospho-HSP27 were found to be significantly increased in the thyroxine-treated hearts. In fact, in THYR-base hearts, the levels of phospho-HSP27 were 2.3-fold and 1.55-fold higher than in NORM-base hearts in the S and P fraction respectively, $P < 0.05$ (Fig. 2).

3.5. HSP27 changes in response to ischaemia in normal and thyroxine-treated hearts

In response to ischaemia, the amount of total HSP27 in the P fraction was increased in both groups but in a

different way. In normal hearts, at 10 min of ischaemia, the amount of HSP27 in the P fraction was slightly increased (1.25-fold more in NORM-10I vs. NORM-base $P>0.05$), whereas it was significantly increased at 20 min of ischaemia (1.9-fold higher in NORM-20I vs. NORM-base $P<0.05$). In thyroxine-treated hearts, the level of total HSP27 in the P fraction was significantly increased at 10 min of ischaemia (1.45-fold more in THYR-10I vs. THYR-base $P<0.05$) and no further increase was observed at 20 min of ischaemia (1.5-fold higher in THYR-20I vs. THYR-base $P<0.05$) (Fig. 3).

Increased phosphorylation of HSP27 was found to occur in the S fraction in normal hearts at 10 min of ischaemia; the levels of phospho-HSP27 were 1.4-fold higher in NORM-10I vs. NORM-base, $P<0.05$, returning back to baseline values at 20 min of ischaemia. In the S fraction of thyroxine-treated hearts, HSP27, already highly phosphorylated at baseline, was not further phosphorylated during ischaemia (Fig. 4).

In the P fraction, the levels of phospho-HSP27 were found to be significantly increased at 20 min of ischaemia in normal hearts (1.8-fold higher in NORM-20I vs. NORM-base, $P<0.05$) and at 10 min of ischaemia in thyroxine-treated hearts (1.75-fold higher in THYR-10I vs. THYR-base, $P<0.05$) without there being a further increase at 20 min of ischaemia (1.7-fold higher in THYR-20I vs. THYR-base, $P<0.05$) (Fig. 4).

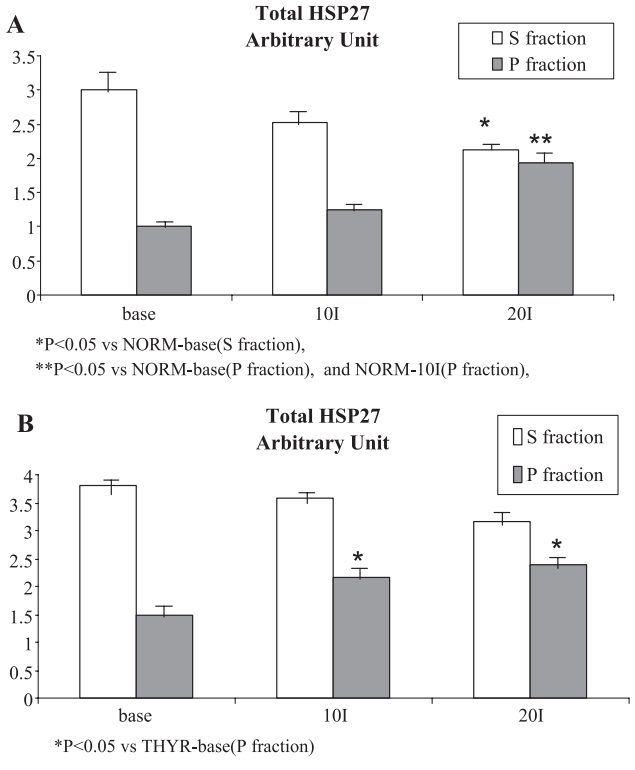

Fig. 3. Total Heat-shock Protein 27 expression at baseline (base) and after 10 min (10I) and 20 min of ischaemia (20I) in normal (A) and thyroxine-treated (B) hearts in the S and P fractions. HSP27 expression (S plus P fraction) was not significantly different between baseline and 10 min or 20 min of ischemia either in normal or in thyroxine-treated hearts. (Columns are means of normalized optical densities, bar = S.E.M.).

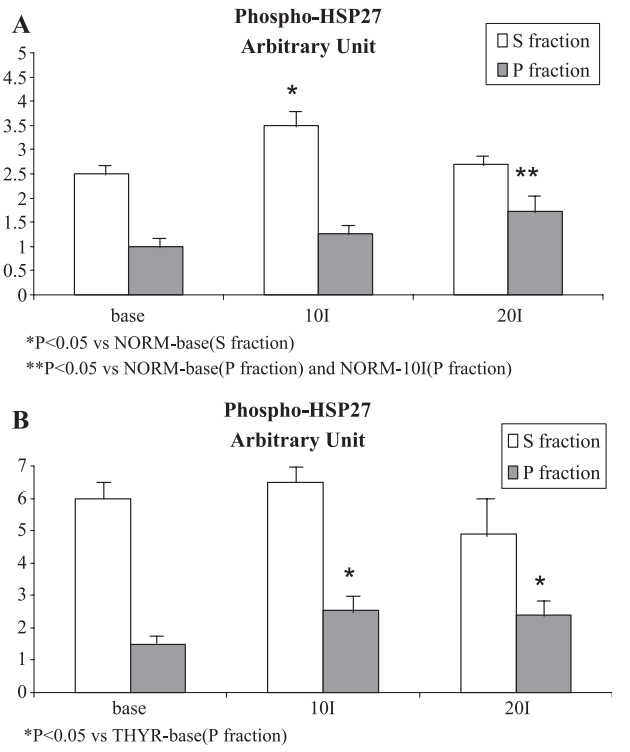

Fig. 4. Phosphorylated Heat-shock Protein 27 expression at baseline (base) and after 10 min (10I) and 20 min of ischaemia (20I) in normal (A) and thyroxine-treated (B) hearts in the S and P fractions. (Columns are means of normalized optical densities, bar = S.E.M.).

4. Discussion

The present study has shown that after long-term thyroxine administration various changes can occur in HSP27 content, phosphorylation and location in the rat heart under steady-state conditions and upon ischaemic stress. Furthermore, these changes were found to be associated with increased resistance of the heart to ischaemia.

Basal HSP27 expression was found to be increased in response to long-term thyroxine treatment, particularly in the insoluble fraction, indicating that thyroid hormones might be involved in the regulation of HSP27 expression in the unstressed rat myocardium. This finding seems to be consistent with recent data showing that the expression of heat-shock proteins in cardiac myocytes can be regulated by hormones (Knowlton and Sun, 2001). In fact, it has been demonstrated that the levels of heat-shock protein 72 in adult cardiac myocytes are raised after treatment with 17β-estradiol, progesterone or the glucocorticoid dexamethasone, whereas HSP27 expression is shown not to be altered. In fact, HSP27 expression was found to be decreased by increasing the dose of progesterone (Knowlton and Sun, 2001). HSP27, apart from being overexpressed, was also

found to be more phosphorylated in thyroxine-treated hearts, indicating that this protein was in a highly activated state. Several studies demonstrate that HSP27 activation is dependent on its phosphorylation and that phosphorylation modulates the function of this protein at the level of the cytoskeleton, by modifying its structure (Benndorf et al., 1994). In fact, HSP27 binds to actin filaments, thereby inhibiting actin polymerization, and this is found to be repressed by phosphorylation (Lavoie et al., 1993b, 1995; Huot et al., 1996).

The physiological significance of the regulation of HSP27 by thyroid hormone and the underlying mechanisms of this response remain largely unknown. However, in view of the available literature, small heat-shock proteins may regulate dynamic changes in the cytoskeleton. In fact, phosphorylation of HSP20 was recently shown to increase the myocyte shortening rate by increasing calcium uptake and more rapid lengthening (Pipkin et al., 2003). Furthermore, treatment with sodium nitroprusside was found to increase the phosphorylation of HSP20 (Pipkin et al., 2003). Based on this evidence, one could suggest that the increased phosphorylation of HSP27 in the thyroxine-treated heart might contribute to the increased contractility that occurs in these hearts. In support of this notion is the fact that contractility is depressed in hearts from hypothyroid rats (Ohga et al., 2002) and the phosphorylation of HSP27 is reduced (unpublished data). Furthermore, HSP27 seems to play a regulatory role in the growth or differentiation of certain tissues and is shown to have a homeostatic function at the actin cytoskeleton level, contributing to the stability of the cytoskeleton. This is thought to constitute an important arm of an adaptive response of the cell to growth or differentiation (Arrigo and Landry, 1994; Lavoie et al., 1993b). In fact, overexpression of wild-type HSP27 in serum-starved growth-arrested cells is shown to cause an enhanced accumulation of F-actin in response to growth factors (Lavoie et al., 1993b). This effect is thought to contribute to cytoskeleton stabilization during the growth process and at the same time seems to be of benefit to cells when they are exposed to stress (Gabai and Kabakov, 1993). On the basis of these data, it seems possible that the observed HSP27 overexpression in thyroxine-treated hearts might be part of an adaptive response of the heart to thyroxine-induced cardiac hypertrophy. This characteristic could be potentially beneficial for hearts under stressful conditions such as ischaemia. In fact, as shown in this and previous studies, thyroxine pretreatment can increase the tolerance of the heart to ischaemia (Pantos et al., 2000, 2001, 2002a,b, 2003a,b). This is consistent with the reports of other studies showing that cells from different tissues that overexpress HSP27 are more resistant to a variety of stresses (Geum et al., 2002; Wyttenbach et al., 2002; Coss et al., 2002), and that overexpression of HSP27 in adult cardiomyocytes increases survival after simulated ischaemia (Martin et al., 1997). Decreasing the high levels of endogenous HSP27 present in neonatal cardiomyocytes can render them more susceptible to damage caused by ischaemic stress (Martin et al., 1997).

Our study further showed that the increased basal expression and phosphorylation of HSP27 in thyroxine-treated hearts was followed by acceleration of the translocation and phosphorylation of this protein upon subsequent ischaemia. In fact, within 20 min of zero-flow global ischaemia, in both groups, the amount of soluble HSP27 was shown to be progressively decreased, whereas HSP27 in the insoluble fraction was increased as compared to preischaemic values. The pattern of HSP27 redistribution was not found to be similar in the two groups: at 10 min of ischaemia, the amount of total and phospho-HSP27 in the insoluble fraction was significantly increased as compared to preischaemic values only in thyroxine-treated hearts and not in normal hearts. In normal hearts, a significant increase in the amount of total and phospho-HSP27 was found to occur in the insoluble fraction only at the end of the 20 min of ischaemia. The physiological consequences of this response are not clear. However, recent studies show that small heat-shock proteins are translocated in response to ischaemic stress, and this seems to be a part of an adaptive protective mechanism guarding against stress (Barbato et al., 1996; Golenhofen et al., 1999; Sakamoto et al., 2000; Eaton et al., 2001). In fact, enhanced translocation and phosphorylation of small heat-shock proteins upon ischaemia has been linked to ischaemic preconditioning induced cardioprotection. The translocation and phosphorylation of αB crystallin was found to be accelerated during subsequent zero-flow global ischaemia following brief episodes of ischaemia and reperfusion (ischaemic preconditioning) (Eaton et al., 2001). Interestingly, as shown in this study, the pattern of translocation and phosphorylation of HSP27 upon ischaemia in thyroxine-treated hearts closely resembles that of ischaemic preconditioning. Since the functional role of the translocation and phosphorylation of small heat-shock proteins is to induce myocardial protection or adaptation to ischaemia (Sussman, 2002), the fact that these events are brought forward and enhanced in both preconditioning and thyroxine pretreatment might explain partly the protection conferred by these two interventions. It is also of note that in other tissues, for instance, organotypic hippocampal cultures, HSP27 was also shown to be modulated by preconditioning (Valentim et al., 2003).

It appears from this study as well as from the previous studies that thyroid hormone can potentially modify the response of the heart to ischaemia, due to its effects on important intracellular signalling transduction pathways that are involved in cardioprotection (Pantos et al., 2001, 2002a, 2003a). This prompts further studies to identify whether this response is mediated by certain type(s) of thyroid nuclear receptors, in the hope of developing thyroid analogues that could potentially be suitable pharmacological agents for the treatment of ischaemic heart disease.

4.1. Study limitations

In the present study, the pattern of activation of HSP27 was not studied during reperfusion. Whether reperfusion modifies HSP27 activation and whether this corresponds to a physiological effect is not known. To our knowledge, this has not been previously addressed with regard to cardioprotective interventions. It is conceivable that further investigations are needed to address this issue.

In summary, long-term thyroxine pretreatment increases the tolerance of the heart to ischaemia. An increased basal expression and phosphorylation of HSP27 after thyroxine pretreatment can occur, followed by an accelerated translocation and phosphorylation of this protein in response to prolonged ischaemic stress. This might be of important therapeutic significance since small heat-shock proteins are now thought of as potential drug targets.

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