Lipid peroxidation levels in rat cardiac muscle are affected by age and thyroid status

R Shinohara[1], T Mano[2], A Nagasaka[2], R Hayashi[2], K Uchimura[2], I Nakano[2], F Watanabe[2], T Tsugawa[2], M Makino[2], H Kakizawa[2], M Nagata[2], K Iwase[3], Y Ishizuki[4] and M Itoh[2]

[1]Department of Biochemistry, Fujita Health University School of Hygiene, Toyoake, Aichi 470–1192, Japan

[2]Department of Internal Medicine and Surgery, Fujita Health University School of Medicine, Toyoake, Aichi 470–1192, Japan

[3]Department of Internal Medicine and Surgery, Fujita Health University School of Hygiene and Medicine, Toyoake, Aichi 470–1192, Japan

[4]Ishizuki Thyroid Clinic, Naka-ku, Nagoya 460–0007, Japan

(Requests for offprints should be addressed to A Nagasaka, Department of Internal Medicine and Surgery, Fujita Health University School of Medicine, Toyoake, Aichi 470–1192, Japan)

Abstract

Free radicals, hydroxyperoxides and H_2O_2 are all known to damage cell components. This study was designed to compare the concentrations of hydroxyperoxide and free radical scavengers in the cardiac muscles of old rats in the hyper- or hypothyroid condition, to determine whether rates of peroxidation would differ with age, thyroid status, or both.

Rats were rendered hyper- or hypothyroid by administration of L-thyroxine or methimazole for 4 weeks. Among the old rats, the lipid peroxide (LPO) concentrations, measured as thiobarbituric acid (TBA) reactants, were significantly greater in the hyperthyroid than in the euthyroid state and the LPO concentrations measured as TBA+Fe^{3+} reactants, which may be precursors of LPO, were significantly greater in the hyperthyroid state, whereas in young rats, the LPO concentrations measured by TBA or TBA+Fe^{3+} methods did not differ significantly in the hyperthyroid state. In the euthyroid state, the concentration of LPO measured as TBA+Fe^{3+} reactants was significantly reduced with age. Xanthine oxidase (XOD) activity also was markedly increased with age, being more pronounced in the hyperthyroid than in the euthyroid state. The Mn and Cu/Zn superoxide dismutase activities were greater in the hyperthyroid than in the euthyroid state. Glutathione peroxidase activity decreased with age in the euthyroid and, particularly, in the hyperthyroid state. Catalase activity was not affected in the old rats. Concentrations of α-tocopherol in the old rats were high in the hyperthyroid state and low in the hypothyroid state, whereas the levels of β- and γ-tocopherols in these rats were unchanged in both conditions as compared with the euthyroid state findings.

Data suggest that the site of free radical generation differs in older rats, with additional shifts in the location of intracellular lipid peroxidation being noted during hyperthyroidism. Thus, as rats age, the reduction of the free radical scavenger system and the increase in LPO and XOD activities might induce myocardial dysfunction.

Journal of Endocrinology (2000) **164,** 97–102

Introduction

Lipid peroxidation *in vivo* is a fundamentally deteriorative reaction that is involved in aging processes (Barber & Bernheim 1967, Muscari *et al.* 1990, Yoshikawa *et al.* 1990) and the development of atherosclerosis (Hartoroft 1965, Perkins *et al.* 1965). Lipid peroxidation occurs in polyunsaturated lipids and involves the direct reaction of oxygen and lipid to form free radical intermediates and semistable peroxides. These free radicals, hydroxyperoxides and H_2O_2, which damage the cell components (Tappel 1973) are putatively produced by the xanthine/xanthine oxidase (XOD) reaction, the mitochondrial electron transport system, the peroxidation of arachidonic acids, and activated neutrophils.

The formation of lipid peroxides is increased in the hyperthyroid state (Asayama *et al.* 1987). Lipofuscin granules, which may be the products of lipid peroxidation (Marzabadi *et al.* 1992), are seen in cardiac muscle in the hypothyroid state (Koobs *et al.* 1978, Nakano & Gotoh 1992). Lipid peroxidation may therefore influence the cardiac function of hyperthyroid and hypothyroid rats.

The free radical scavenger system consists of two famiies of enzymes, called the superoxide dismutases (SOD) (McCord *et al.* 1971). These enzymes catalyze the reaction $2H+2O_2 \rightarrow H_2O_2+O_2$ and help to minimize the cellular

0022–0795/00/0164–0097 *Printed in Great Britain*

Online version via http://www.endocrinology.org

injury mediated by various free radicals (Gregory & Fridovich 1973, Crapo & McCord 1976). The two main types of SOD are Mn-SOD and Cu/Zn-SOD. In this study, we investigated the changes in lipid peroxide (LPO) concentration along with changes in superoxide and LPO metabolism in cardiac muscles obtained from hyper- and hypothyroid rats (Mano *et al.* 1995*b*). In addition, we measured the tissue concentrations of Mn-SOD, Cu/Zn-SOD, XOD, glutathione peroxidase (GSH-PX) and catalase to determine whether they are modulated by lipid peroxidation.

Materials and Methods

Animals

Studies were conducted in two groups of male Wistar rats (Wistar-Imamichi Rat, obtained from Imamichi Institute for Animal Reproduction, Ibaraki, Japan), aged 8 weeks (young) and 1·5 years (old). They were fed a standard diet and distilled water throughout the experiment. The 30 old rats and 15 young rats were then treated as follows to induce hyper- or hypothyroidism. Subcutaneous injections of 200 μg L-thyroxine (L-T_4) were administered every other day for 30 days in six old rats and five young rats to induce hyperthyroidism, or 1 mg methimazole (MMI) was administered daily for 30 days in six old rats and five young rats to induce hypothyroidism. The other 12 old rats were divided into two groups: six old rats were treated with 200 μg L-T_4 injection every day for 15 days and then L-T_4-administration was withdrawn for 15 days (short-term L-T_4-treated), and the other six old rats were treated with 200 μg D-thyroxine (D-T_4) injection every other day for 30 days. The L-T_4 and D-T_4 were dissolved in 0·01N NaOH and diluted with saline; the MMI was dissolved in saline. Control animals received injections of an equivalent volume of saline. On the 30th day after each treatment, the basal heart rate was measured before the administration of ether anesthesia. Blood samples for the determination of plasma concentrations of triiodothyronine (T_3) and T_4 were obtained by cardiac puncture. The hearts were then removed, immediately frozen in liquid nitrogen, and stored at −80 °C until required for assay.

Samples of cardiac muscle

Rat cardiac muscles were homogenized at 4 °C in 50 mM Tris–HCl buffer, pH 7·5, to make a 10% (w/v) homogenate, using a polytron homogenizer for 5 min, followed by a sonic homogenizer for 3 min. The homogenates were then centrifuged at 1000 × ***g*** for 15 min at 4 °C. The resulting supernatants were collected and stored at 0 °C before use.

Measurement of LPO concentrations in rat cardiac muscle

Malondialdehyde (MDA), a secondary product of lipid peroxidation, was assayed by reacting the lipid peroxides with thiobarbituric acid (TBA) (Patton & Kurtz 1951). MDA was assayed by the method reported by Ohkawa *et al.* (1979), and the products obtained when 1 mM $FeCl_3$ was added before boiling were also measured.

Measurement of XOD activity in rat cardiac muscle

XOD was assayed as reported previously (Hashimoto 1974).

Measurement of SOD activity in rat cardiac muscle

Nitrite, which is a product of the oxidation of hydroxylammonium chloride by SOD, was measured under the following conditions. The incubation mixture (0·8 ml) contained 0·5 ml distilled water, 0·1 ml boric acid buffer (0·156 mM), 0·1 ml xanthine (2 mM) and 0·1 ml NH_2OH (10 mM). The reaction was started by adding 20 U/ml XOD (Boehringer Mannheim, Mannheim, Germany) to the mixture, with heating in a water bath at 37 °C for 30 min. The nitrite concentration was determined as follows. After the addition to the reaction mixture of 2·0 ml 3·6 mM *N*-1-naphthyl-*N*′-diethylethylenediamine oxalate, 38 μM sulfanilic acid and 2·0 ml 100% acetic acid solutions, distilled water was added to obtain a final volume of 400 ml. After an incubation at 37 °C for 45 min, the absorbance of the reaction mixture was measured at 550 nm by spectrophotometer (Weisiger & Fridovich 1973, Elstner & Heupel 1976). To discriminate Cu, Zn-SOD and Mn-SOD, we used 1 mM cyanide ion, which inactivates Cu, Zn-SOD. SOD activities were proportional as a function of the enzyme volume.

Measurement of catalase and GSH-PX activities in rat cardiac muscle

Catalase and GSH-PX activities were assayed by a method reported previously (Hochstein & Utley 1968, Aebi 1984).

Measurement of cholesterol, triglyceride, co-enzyme Q, and tochopherol concentrations in rat cardiac muscle

Assays for cholesterol and triglyceride were performed as reported previously (Fletcher 1968, Zlatkis & Zak 1969). Concentration of co-enzyme Q and of tocopherols were determined as reported previously (McCord *et al.* 1971).

Measurement of serum concentrations of T_3 and T_4

T_3 and T_4 concentrations in the sera of the hyper-, hypo- and euthyroid rats were measured with an

Table 1 Thyroid status of the rats studied. Values are expressed as mean ± S.E.M.

	T_4 (μg/l)	T_3 (μg/l)	BW (g)	HR (beats/min)
Group (n=6 each group)				
Rats aged 1·5 years				
Saline-treated (euthyroid)	37·2 ± 1·5	0·71 ± 0·29	421 ± 10	264 ± 7
L-T_4-treated (hyperthyroid)	152·0 ± 8·4*	4·46 ± 2·27*	380 ± 11*	342 ± 14*
MMI-treated (hypothyroid)	26·2 ± 3·0*	0·45 ± 0·04*	435 ± 6	235 ± 18
Short-term L-T_4-treated	39·5 ± 2·4	0·63 ± 0·03	399 ± 8	274 ± 19
D-T_4-treated	37·4 ± 2·0	0·66 ± 0·04	417 ± 9	251 ± 11
Rats aged 8 weeks				
Saline-treated (euthyroid)	40·2 ± 2·3	0·65 ± 0·08	348 ± 9	272 ± 20
L-T_4-treated (hyperthyroid)	182·1 ± 15·1*	3·42 ± 0·40*	310 ± 6*	340 ± 15*
MMI-treated (hypothyroid)	28·0 ± 4·9*	0·48 ± 0·19	339 ± 17	240 ± 17

BW, body weight; HR, heart rate. *P<0·05 compared with saline-treated rats.

immunochemiluminescence kit according to the manufacturer's instructions (Amersham Co. Ltd, Amersham, Bucks, UK).

Protein assays

Protein concentration in cardiac muscle was determined with a Bio-Rad protein assay kit, with bovine serum albumin used as a standard (Bradford 1976).

Statistical analyses

Data are expressed as mean ± S.E.M Differences between data sets were evaluated by analysis of variance (ANOVA) *post hoc* test or Welch's test.

The intra-assay and interassay coefficients of variation for these assays were within 7·5%. A level of P<0·05 was accepted as statistically significant.

Results

The thyroid status of the rats was confirmed by measuring serum concentrations of T_4 and T_3. The concentrations of these hormones were significantly increased in the hyperthyroid rats and low in the hypothyroid rats, compared with the concentrations in the euthyroid rats. In the young hypothyroid rats, the serum concentrations of T_3 were also low, but not to a statistically significant extent (Table 1).

The body weights and heart rates were determined in each age group during the experiment. The weight of the whole heart was also measured at the time of decapitation. The body weight of the hyperthyroid rats was significantly less than that of either the hypo- or euthyroid rats (P<0·05). The short-term L-T_4-treated or D-T_4-treated rats did not show any changes in these hormones, body weight and heart rate (Table 1). Body weight did not differ significantly between the hypothyroid and euthyroid rats, regardless of age. Organ weights were not significantly affected by thyroid status, except for the heart, which was significantly heavier in the rats treated with L-thyroxine than in the saline-treated (euthyroid) young rats (data not shown).

In both age groups, the heart rate of the hyperthyroid animals significantly exceeded that of the euthyroid animals. Conversely, the heart rate of the hypothyroid animals did not differ significantly from that of the euthyroid animals in either age group.

Under the experimental conditions, the serum concentrations of thyroid hormone did not affect the protein : wet weight ratio of organ tissue (data not shown).

LPO concentrations

The concentrations of LPO, estimated as TBA reactants, were greater in the old hyperthyroid rats than in all the other groups. However, the concentrations of LPO, estimated as Fe-added TBA (TBA+Fe^{3+}) reactants, were increased in the old T_4-treated rats compared with those in the age-matched saline-treated rats. These concentrations were not changed in the short-term L-T_4-treated or D-T_4-treated rats. They were lower in the saline-treated and MMI-treated aged rats compared with the correspondingly treated young rats (Table 2).

XOD concentrations

The concentration of XOD was low, but detectable, in rat cardiac muscle. The concentration in the old hyperthyroid rats exceeded that in the age-matched euthyroid rats, and the concentrations in the short-term L-T_4-treated or D-T_4-treated rats were not different from that of the saline-treated rats. The concentration of XOD in the old rats significantly exceeded that in the young rats across all conditions of thyroid activity (Table 3).

Table 2 LPO concentrations in cardiac muscle from old (1·5 years) and young (8 weeks) rats. Values are mean ± S.E.M. of six animals studied

	Lipid peroxide (nmol/mg protein)			
	TBA		TBA ± Fe^{3+}	
	Old	Young	Old	Young
Group				
Saline-treated	0·623 ± 0·03	0·88 ± 0·15	7·03 ± 1·00†	12·45 ± 1·42
L-T_4-treated	0·857 ± 0·08*	1·19 ± 0·26	12·23 ± 0·90*	11·29 ± 0·35
MMI-treated	0·626 ± 0·07	0·87 ± 0·11	8·41 ± 0·22†	12·18 ± 1·65
Short-term L-T_4-treated	0·77 ± 0·09		8·20 ± 1·08	
D-T_4-treated	0·63 ± 0·08		8·45 ± 0·76	

TBA, measured as thiobarbituric acid reactants; TBA+Fe^{3+}, measured as thiobarbituric acid reactants when the reaction mixture contains Fe^{3+}.
P<0·05 compared with *saline-treated rats or †8-week-old rats.

SOD concentrations

The total SOD concentrations were not changed in the old rats, but were significantly increased after L-T_4 treatment in the young hyperthyroid rats. The Mn-SOD concentrations were significantly increased only in the old hyperthyroid rats relative to the remaining groups. The Cu/Zn-SOD levels were not changed in the old or the young rats. None of these concentrations was affected by the short-term L-T_4 treatment or D-T_4 treatment (Table 4).

GSH-PX and catalase concentrations

GSH-PX concentration showed a reduction with age, with a significant difference in values between the old euthyroid and hyperthyroid rats.

GSH-PX and catalase concentrations were not different between the saline-treated rats and the short-term L-T_4-treated or D-T_4-treated rats (Table 5).

Total cholesterol, triglyceride, co-enzyme Q, and tocopherol concentrations

No differences in the concentrations of total cholesterol, triglyceride, or co-enzyme Q were obtained between any of the rat groups (data not shown).

Table 3 XOD activity in cardiac muscle from old (1·5 years) and young (8 weeks) rats. Values are mean ± S.E.M. of six animals studied

	Activity (mIU/mg protein)	
	Old	Young
Group		
Saline-treated	0·17 ± 0·02†	0·11 ± 0·06
L-T_4-treated	0·22 ± 0·04*†	0·12 ± 0·06
MMI-treated	0·19 ± 0·02†	0·09 ± 0·03
Short-term L-T_4-treated	0·27 ± 0·10	
D-T_4-treated	0·20 ± 0·09	

P<0·05 compared with *saline-treated rats or †8-week-old rats.

The concentration of α-tocopherol was significantly increased in the old hyperthyroid rats, and was significantly decreased in the old hypothyroid rats, compared with the values obtained in euthyroid rats of that age (Table 6).

Discussion

Among the hyperthyroid animals, in the old rats Mn-SOD concentration was increased relative to either euthyroid or hypothyroid conditions, whereas in the young rats, total SOD concentrations were increased. These results corroborate the findings of Asayama *et al.* (1987), who demonstrated that Mn-SOD concentrations increased 4 weeks after T_4 administration.

The formation of superoxide activated by thyroid hormone may occur mainly in the mitochondrial respiratory system. However, the xanthine/XOD reactions, the arachidonic acid cascade, or both, may be involved in this process. An immunocytochemical study showed that Mn-SOD was present in mitochondria, and that Cu/Zn-SOD was localized in the cytoplasm (Slot *et al.* 1985). Thus, in hyperthyroidism, Mn-SOD may be induced to protect the mitochondria from oxidative injury by superoxide.

Among the old rats analyzed in our present study, the concentrations of XOD were increased in each thyroid state, with the hyperthyroid animals having the largest increase compared with that in the young rats. The old rats also exhibited lower concentrations of LPO than the young rats, but these were increased in the hyperthyroid state. The generation of LPO in the old rats may occur mainly via XOD activation, particularly in the activated mitochondrial respiration observed during the hyperthyroid state.

The LPO concentration measured by TBA was increased only in the old hyperthyroid rats. The LPO concentrations measured as TBA+Fe^{3+} may be the precursors of the concentrations measured as TBA reactants

Table 4 SOD acitivity in cardiac muscle from old (1·5 years) and young (8 weeks) rats, expressed as μg/mg protein, using purified bovine erythrocyte SOD (Sigma Chemical Co., St Louis, MO, USA) as the standard enzyme protein. Values are mean ± S.E.M. of six animals studied

	Total SOD (μg/mg protein)		**Mn-SOD** (μg/mg protein)		**Cu,Zn-SOD** (μg/mg protein)	
	Old	Young	Old	Young	Old	Young
Group						
Saline-treated	2·03 ± 0·01	1·86 ± 0·67	0·63 ± 0·05	0·64 ± 0·15	1·70 ± 0·18	1·31 ± 0·66
L-T_4-treated	2·10 ± 0·20	3·35 ± 0·69*	0·98 ± 0·13*	0·83 ± 0·31	1·84 ± 0·27	2·03 ± 0·58
MMI-treated	2·22 ± 0·16	2·53 ± 0·46	0·60 ± 0·07	0·75 ± 0·24	1·84 ± 0·18	1·78 ± 0·35
Short-term L-T_4-treated	2·27 ± 0·25		0·68 ± 0·07		1·86 ± 0·19	
D-T_4-treated	2·42 ± 0·22		0·65 ± 0·09		1·51 ± 0·26	

*P<0·05 compared with saline-treated rats.

(Wills 1964, Demopulos 1973). The LPO concentration estimated as TBA+Fe^{3+} reactants in the old hyperthyroid rats exceeded that in the old euthyroid rats, suggesting that the production of LPO in the cardiac muscle of the older hyperthyroid rats exceeded its turnover. In the old hypo- and euthyroid rats, the concentration of LPO measured as TBA+Fe^{3+} reactants was decreased, suggesting that the formation and turnover of LPO were both diminished. This may lead to the production of lipofuscin granules, which are found only in the myocardium of hypothyroid animals.

Salminen *et al.* (1988) reported that the unstimulated peroxidation rate decreases with age. Lemeshko *et al.* (1981) showed that an age-related decrease in the rate of lipid peroxidation in the rat liver was due to a decrease in enzymatic lipid peroxidation in the microsomes, and to an increase in the concentrations of NADPH-dependent antioxidants in the cytoplasm of liver cells. Barrett & Horton (1975) observed an increase in total peroxidation capacity in muscle mitochondria and microsomes between the age of 1 and 8 months. Therefore, the difference in the LPO concentrations observed between the old and young rats in our study may be due to the site of LPO generation. Combining these LPO data with our GSH-PX findings, it appears that lipid peroxidation was affected by aging, as shown in our previous study of the aged rat brain (Mano

Table 5 GSH-PX and catalase activity in cardiac muscle from old (1·5 years) and young (8 weeks) rats. Values are mean ± S.E.M. of six animals studied

	GSH-PX (mIU/mg protein)		**Catalase** (IU/mg protein)	
	Old	Young	Old	Young
Group				
Saline-treated	169 ± 11†	237 ± 15	17·68 ± 2·49	15·71 ± 2·33
L-T_4-treated	87 ± 27*†	207 ± 18	18·25 ± 1·84	18·25 ± 1·97
MMI-treated	172 ± 20†	256 ± 19	18·45 ± 2·04	14·22 ± 2·96
Short-term L-T_4-treated	179 ± 16		18·32 ± 2·51	
D-T_4-treated	168 ± 23		17·15 ± 2·22	

P<0·05 compared with *saline-treated rats or †8-week-old rats.

Table 6 Tocopherol (Toc.) concentrations in cardiac muscle from old rats (data in parentheses are those of young (8 week-old) rats. Values are mean ± S.E.M. of six animals studied

	Tocopherols (μg/l homogenate)		
	α-Toc.	β-Toc.	γ-Toc.
Group			
Saline-treated	140·3 ± 3·7 (130·0 ± 12·9)	7·01 ± 0·54 (5·57 ± 0·39)	5·96 ± 0·82 (8·62 ± 1·86)
L-T_4-treated	174·2 ± 10·4*	5·54 ± 0·54	5·37 ± 0·61
MMI-treated	130·2 ± 2·4*	7·58 ± 0·80	6·72 ± 1·12
Short-term L-T_4-treated	148·4 ± 9·2	7·34 ± 0·78	6·32 ± 0·93
D-T_4-treated	151·3 ± 8·9	6·98 ± 0·92	6·45 ± 1·01

*P<0·05 compared with saline-treated rats.

et al. 1995*a*). Such alterations in lipid peroxidation may cause cardiac dysfunction in an older hyperthyroid animal.

Though there were no differences in total cholesterol, triglyceride, or co-enzyme Q concentrations, the concentrations of α-tocopherol were slightly increased in the old hyperthyroid and hypothyroid rats, which suggests that this substance may act as a free radical scavenger.

These events in rats in an L-T_4-induced hyperthyroid state were reversed by withdrawing the short-term administered L-T_4. The D-T_4 treatment did not affect these indicators. These data imply that these changes may be specific for L-T_4-induced hyperthyroidism, but not for D-T_4 treatment, which does not induce the hyperthyroid state.

Acknowledgements

The authors thank Ms N Takekawa for her secretarial assistance.

This work was supported by a grant from the Ishizuki Thyroid Science Foundation, Nagoya, Japan.

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Received 18 May 1999
Accepted 31 August 1999