Age-Related Changes in the Thyroid Hormone Effects on Malondialdehyde-Modified Proteins in the Rat Heart (44428)

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Abstract. **To determine the age-related changes in thyroid hormone (TH) effects on malondialdehyde (MDA)-modified proteins in cardiac tissue, rats at 4, 12, and 25 months of age were studied. Hyperthyroidism was induced with daily injection of L-triiodothyronine (15 μg/100 g) intraperitoneally for 10 days. Hypothyroidism was induced with 0.025% methimazole in the drinking water for 4 weeks. MDA proteins were measured with immunoblots using a specific anti-MDA antiserum. MDA was measured as thiobarbituric acid reactive substance.**

Hypothyroidism in 4-month-old rats was associated with significant reduction in MDA proteins compared to euthyroid rats (13.4 ± 5.9% vs. 99.8 ± 10.4% of controls $P < 0.001$). Hyperthyroidism did not result in a significant change of MDA proteins. In aged rats, neither hypothyroidism nor hyperthyroidism was associated with significant changes in cardiac MDA proteins. The changes in MDA proteins did not correlate with cardiac MDA concentrations. In young rats, the MDA concentrations (nmol/mg) were significantly reduced in hypothyroidism (2.71 ± 0.21) and were increased in hyperthyroidism (8.19 ± 0.78) compared to euthyroid values (5.06 ± 0.71) $P < 0.01$. In aged rats, cardiac MDA content was significantly increased during both hyperthyroidism and hypothyroidism. We conclude that alterations in MDA protein content is yet another potential biochemical effect of TH in cardiac tissue. This particular effect is significantly blunted with age. [P.S.E.B.M. 1999, Vol 222]

Thyroid disease is commonly associated with cardiovascular changes (1, 2). The biochemical effects of thyroid hormone (TH) on the heart are diverse and include modulation of β-adrenergic signal transduction (3–5), alterations in gene expression (6, 7), and enhancement of glucose utilization (8, 9), oxygen consumption (10, 11), along with increased lipid peroxidation (12–16). The latter is associated with increased formation of malondialdehyde MDA (17). This highly reactive aldehyde is known to modify various proteins (18–20), thereby altering their function, antigenicity, and possibly their turnover kinetics. Since aging is associated with changes in lipid peroxidation (21–23) and alterations in TH responsiveness (24), it was postulated that TH effects on MDA modification of proteins would be altered in aged rats. The purpose of this study was 1) to study the effect of TH on cardiac tissue MDA and MDA-modified proteins; and 2) to determine the age-related changes in TH effects on MDA-modified proteins in cardiac tissue.

Materials and Methods

Experimental Animals. Male Fischer 344 rats at 3, 12, and 24 months of age were obtained from the Harlan Laboratories (Indianapolis, IN). These animals had free access to standard laboratory rat chow (Teklad Laboratories, Madison, WI) and water.

All the animals were housed in individual cages. Body weight and food intake were measured every other day dur-

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ing induction of hyperthyroidism and hypothyroidism. Within 4 to 5 days of arrival at our animal facility, the food intake of all rats had stabilized, and the studies were initiated within 7 days.

Three different treatment groups were studied. One set of animals in each group was rendered hypothyroid with 0.025% methimazole (Sigma Chemicals, St. Louis, MO) in the drinking water for 4 weeks. The serum thyroxine level in all these rats on the day of sacrifice was less than 16.1 nmol/l (the lower limit of the assay used for these studies). To control for the potential antioxidant effects of methimazole, a group of thyroidectomized rats ($n = 10$) was also studied. These rats were obtained from Taconic (Germantown, NY). A subgroup of rats was rendered hyperthyroid with a daily dose of 15 μg T_3/100 g body weight intraperitoneally, for the last 10 days. Euthyroid untreated rats were observed as controls. To control for the stress effects of daily injections, a group of young rats was injected with vehicle daily for 10 days. The state of hypothyroidism and hyperthyroidism was further confirmed by measuring malic enzyme activity in the hepatic cytosol of these rats using the technique of Hsu and Lardy (25) as previously described from our laboratory (26). Ten rats in each experimental group were studied.

The animals were sacrificed by exsanguination through the abdominal aorta under pentobarbital anesthesia (45 mg/kg ip). The rat was perfused through the heart with 25–30 cc of phosphate-buffered saline (PBS).

Quantitation of MDA. The cardiac tissue was homogenized in 20 volumes of phosphate-buffered saline (PBS) at 4°C. The homogenates were centrifuged at 9000*g* for 10 min, and the supernatant (i.e., crude membrane and cytosolic elements) was used for the studies.

MDA in cardiac tissue was determined as thiobarbituric acid-reactive material (TBAR) as described previously (27). Each assay tube contained 5–10 mg protein equivalent of cardiac homogenates. MDA was measured in the supernatant after the tissue elements were precipitated with 5% trichloroacetic acid. This minimized the possible generation of MDA during the assay (28).

Preparation of MDA Conjugates. Briefly, MDA was released by acid hydrolysis of malondialdehyde bis (dimethylacetal) (Aldrich, Milwaukee, WI). This MDA solution was diluted with 0.01 *M* phosphate buffer, pH 6.4, and brought to pH 7.4 with 10 *N* NaOH to yield 200 m*M* MDA. Equal volumes of 10 mg/ml serum albumin (fraction V powder; Sigma, St. Louis, MO) in 0.01 *M* phosphate/0.01% EDTA (pH 7.4) and 200 m*M* MDA solutions were combined and incubated at 37°C for 72 hr as described previously (29). MDA-albumin conjugates were dialyzed against 0.01 *M* phosphate/0.01% EDTA buffer twice at 4°C for 48 hr. Protein concentrations were determined following dialysis using the method of Lowry *et al.* (30).

Production and Purification of Rabbit Anti-MDA Serum. The detailed description of the methods used in preparation of this antibody was published previously (29). Briefly, New Zealand white rabbits (6–8 weeks old, 2–3 kg) were immunized in four different subcutaneous sites with 1 mg of MDA-modified rabbit serum albumin (RSA) in complete Freund's adjuvant (Difco, Detroit, MI), and control animals received a comparable dose of RSA. Subsequently, rabbits were injected biweekly with the same dose of antigens in incomplete Freund's adjuvant (Difco). The specificity of anti-MDA-RSA serum has been published previously (29). The cross-reactivity with unmodified RSA, BSA, or RSA modified by glucose, acetaldehyde, formaldehyde, or glutaraldehyde was negligible (29). In addition, this antibody did not recognize free MDA.

Protein Electrophoresis and Immunoblotting. Tissue homogenates (50 μg protein) were resolved on 10% nonreducing sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to a nitrocellulose membrane by electroblotting as previously described (31). The membranes were probed with rabbit anti-MDA antisera (1:500) for 2 hr, or serum was preabsorbed with MDA-BSA using enhanced chemiluminescence (ECL) Western blotting technique (ECL Kit) as described in the manufacturer's protocol (Amersham Co., Arlington Heights, IL). The intensity of the total MDA-protein bands was measured with densitometry using a personal densitometer (Molecular Dynamics, Sunnyvale, CA). The results are presented as a percentage of specimen from a young control rat included in each gel as an internal control.

All results are expressed as mean ± SEM. The statistical analysis was carried out with Duncan's multiple range test. A $P < 0.05$ was considered the level of statistical significance.

Results

The mean (± SEM) body weight and heart weight of the rats studied are summarized in Table I. Some of these data have been included in a previously published manuscript (32). Hyperthyroidism in both young and aged rats was associated with significant body weight loss and significant cardiac hypertrophy. The body weight did not change significantly in methimazole-treated rats, and the heart weight was also not significantly altered (Table I). However, the body weight increased modestly in thyroidectomized rats from a baseline of 195.8 ± 2.1 g to 237.4 ± 6.5 g over 4 weeks of observation. This suggests that thyroidectomized rats were not as profoundly hypothyroid as the methimazole-treated group although the serum T_4 levels were reduced in both groups below 16.1 nmol/l.

The expected induction of hepatic malic enzyme (ME) in hyperthyroid young and aged rats is evident, and so is the expected downregulation in hypothyroid young rats. The age-related decline in basal and stimulated ME activity is also observed. These observations are consistent with previously published studies (26, 33). The reduction in hepatic cytosolic ME activity in thyroidectomized rats (12.8 ± 2.1 mU/mg) was not as great as in methimazole-treated rats (7.3 ± 1.2 mU/mg).

Table I. The Mean Body Weight, Heart Weight, and Hepatic Malic Enzyme Activity of Rats at the Day of the Experiment

	Basal body weight (g)	Body weight on day of death (g)	Heart weight (mg)	Malic enzyme (mU/mg)
4-month-old				
Hyperthyroid (n = 10)	279.3 ± 5.9	237.6 ± 3.6*†	912.2 ± 42.3†	79.9 ± 4.8†
Euthyroid (n = 10)	289.3 ± 4.9	304.6 ± 5.2	636.5 ± 16.6	17.2 ± 1.6
Hypothyroid (n = 10)	295.6 ± 4.7	278.1 ± 5.3†	633.4 ± 42.8	7.3 ± 1.2†
25-month-old				
Hyperthyroid (n = 10)	451.1 ± 5.3	387.4 ± 5.3*†	1617.4 ± 123.8†	26.7 ± 2.4†
Euthyroid (n = 10)	451.1 ± 6.8	449.0 ± 6.3	1206.1 ± 116.3	3.1 ± 0.3
Hypothyroid (n = 10)	438.8 ± 4.6	393.6 ± 5.8*†	1116.3 ± 141.6	2.1 ± 0.4

Note. Hyperthyroid rats received 15 μg/100 g of body weight of L-T3 daily intraperitoneally for 10 days. Hypothyroid rats were maintained on methimazole 0.025% in drinking water for 4 weeks. (n = 10 in each group). The body weight data were reported previously (32).
Mean (± SEM) body weight (g) and heart weight (mg).
* $P < 0.05$ compared to baseline weight.
† $P < 0.05$ compared to euthyroid state within the same age group.

The age-related changes at basal euthyroid conditions were studied. The MDA content of cardiac tissue (nmol/mg protein) of 25-month-old rats (3.86 ± 0.69) was not significantly different from that of 12-month-old (4.03 ± 0.81) or 4-month-old rats (5.06 ± 0.71). The MDA-protein content in euthyroid 25-month-old rats (20.4 ± 1.7 arbitrary units) and of 12-month-old (18.8 ± 1.4) was significantly higher than that in 4-month-old rats (14.3 ± 0.87) $P < 0.05$.

In 4-month-old rats, hyperthyroidism was associated with a significant increase in cardiac MDA concentration compared to controls (8.19 ± 0.78 vs. 5.06 ± 0.71 nmol/mg/protein), $P < 0.01$, whereas methimazole-induced hypothyroidism was associated with a significant reduction in MDA content of the heart (2.71 ± 0.21 nmol/mg) $P < 0.01$ (Fig. 1A). The cardiac tissue MDA content of thyroidectomized rats was also reduced (3.42 ± 0.18 nmol/mg) $P < 0.05$. However, in 25-month-old rats, cardiac MDA content of hyperthyroid rats (8.20 ± 0.79 nmol/mg) and methimazole-treated hypothyroid rats (11.05 ± 0.97 nmol/mg) was significantly higher compared to age-matched euthyroid controls (3.86 ± 0.69 nmol/mg) $P < 0.01$ (Fig. 1A).

The malic enzyme activity and cardiac MDA content of vehicle-injected euthyroid rats were not different from untreated control rats (data not shown).

A representative immunoblot of cardiac proteins using an anti-MDA-protein antiserum is shown in Figure 2. The multiple bands representing various proteins modified by MDA are evident. The specificity of these bands was demonstrated with lack of any signal when an antiserum preadsorbed with MDA-BSA was used. The significant decrease in MDA proteins in methimazole-treated hypothyroid state is evident (Fig. 2A). The shift in electrophoretic profile of

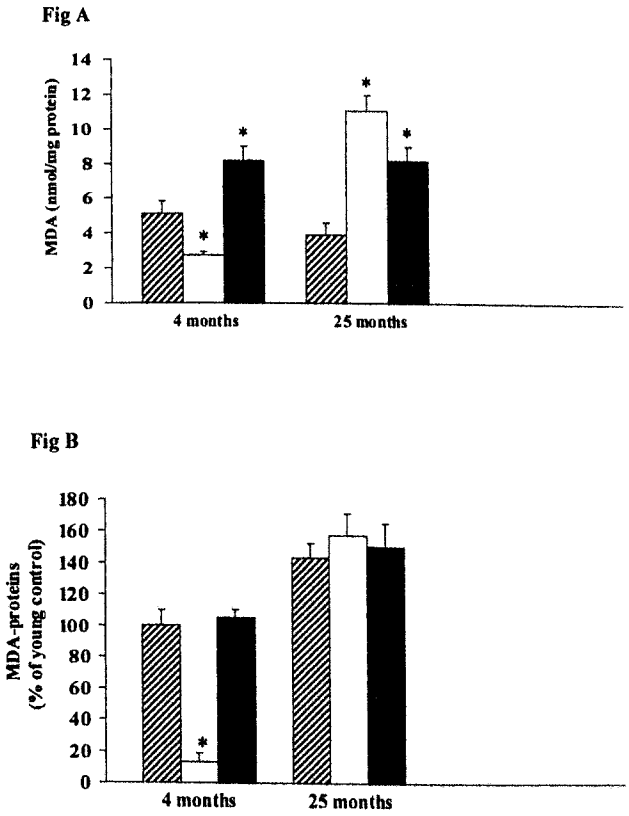

Figure 1. (A) The mean (± SEM) of malondialdehyde (MDA) in the heart of 4- and 25-month-old rats at various thyroidal states (n = 10 in each group). *$P < 0.01$ compared to euthyroid values. (B) The mean ± SEM of the quantitative estimates of MDA-protein content of cardiac tissue from 4-month-old and 25-month-old rats at various thyroidal states (n = 10 in each group). *$P < 0.01$ compared to euthyroid values. ▨ Euthyroid, □ Methimazole-treated hypothyroid, ■ Hyperthyroid.

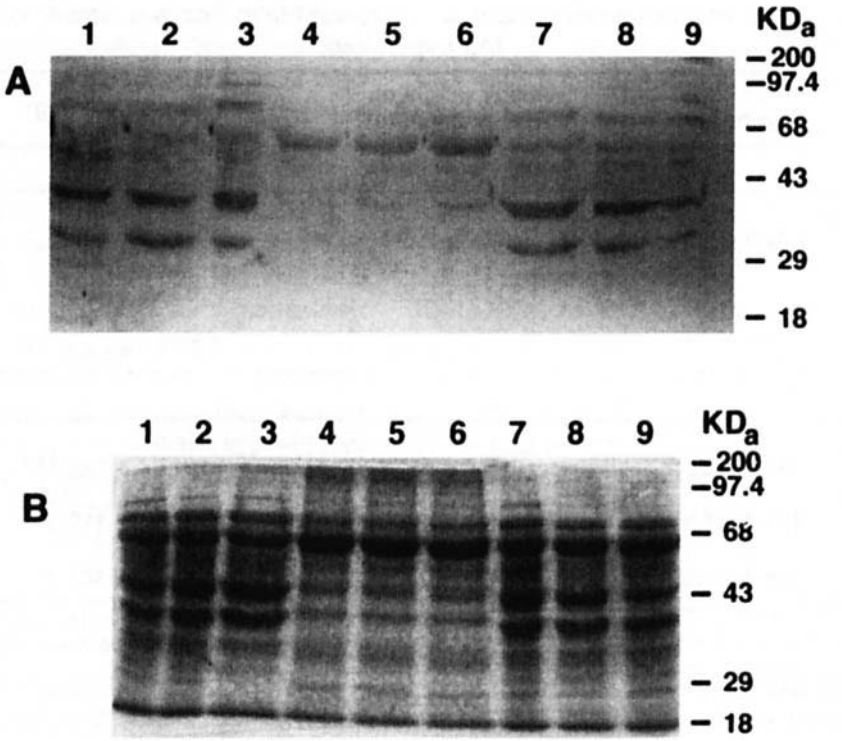

Figure 2. (A) A representative immunoblot of cardiac proteins (50 μg) from 4-month-old rats subjected to 10% SDS-PAGE and probed with anti-MDA antiserum as described in the text. Cardiac proteins from euthyroid (Lanes 1–3), methimazole-treated hypothyroid (Lanes 4–6), and hyperthyroid rats (Lanes 7–9) are shown. The reduction in MDA proteins in the heart of methimazole-treated hypothyroid rats is apparent. (B) The electrophoretic profile of cardiac proteins from euthyroid (Lanes 1–3), hypothyroid (Lanes 4–6), and hyperthyroid (Lanes 7–9) rats. The 10% SDS-PAGE stained with Coomassie blue.

cardiac proteins in profoundly hypothyroid rats is also apparent (Fig. 2B). However, there was no change in MDA-protein profile or intensity in cardiac tissue of thyroidectomized rats (data not shown).

There were no differences in the profile of MDA-protein bands of euthyroid 25-month-old rats compared to euthyroid 4-month-old rats. Similar results have been observed previously (23).

The quantitative estimates of MDA proteins from all the gels are summarized in Figure 1B. The MDA protein of methimazole-treated hypothyroid 4-month-old rats (13.4 ± 5.9% of controls) was significantly reduced compared to euthyroid rats (99.8 ± 10.4%) or hyperthyroid rats (104.6 ± 9.8%) $P < 0.01$. In 25-month-old rats neither hyperthyroidism (157.4 ± 14.4% of controls) nor hypothyroidism (150.5 ± 14.5% of controls) was associated with significant changes compared to euthyroid aged rats (143.1 ± 8.7% of controls). There was no correlation between the tissue concentration of MDA and tissue concentration of MDA proteins.

Since methimazole was used to cause hypothyroidism in these rats, the potential effect of methimazole on MDA modification of proteins was tested *in vitro.* Bovine serum albumin (BSA 10 mg/ml) was incubated with 0.1 *M* MDA for 24 hr at 37°C in the presence or absence of 5 m*M* methimazole. Methimazole did not reduce the MDA binding to BSA as measured with immunoblotting.

Discussion

The MDA content of young euthyroid rat heart was similar to that reported previously by Ohkawa *et al.* (34). There was a modest trend of an age-related decrease in cardiac MDA content, although the changes did not achieve statistical significance. Hyperthyroidism was associated with a significant increase in cardiac MDA content in both young and aged rats. Hypothyroidism in young rats was associated with reduced MDA content in the heart. Unexpectedly, hypothyroidism in aged rats was associated with increased cardiac MDA content. In a previously published study, hypothyroidism did not cause a significant change in cardiac MDA content whereas hyperthyroidism was associated with a significant increase in MDA content (15). The discrepancy may be related to the differences in the age of animals studied. In the present study, young postpubertal and aged rats were used whereas Asayma *et al.* (15) used mostly prepubertal animals. TH responsiveness of tissues is markedly altered during pubertal development and much slowly thereafter during senescence (24).

The cause of our observed increase of cardiac MDA in aged hypothyroid rats is not known. It is possible that in this age group, hypothyroid-related changes in fatty acid composition of cells may provide sufficient substrate for lipid peroxidation thereby increasing MDA production despite the expected decrease in oxygen consumption during hypo-

thyroidism. However, this explanation remains highly speculative at this time.

It is noteworthy that there was no clear correlation between cardiac MDA content and the concentration of MDA proteins. A similar dissociation between MDA and MDA-protein concentrations was reported earlier in diabetic rats where MDA proteins in tissues were reduced despite increased MDA production (35). These observations underscore the fact that the availability of MDA is only one determinant of MDA-protein accumulation. Other factors, notably protein turnover kinetics, are important determinants of MDA protein content of tissues. Alternatively, it is possible that these discrepancies between MDA and MDA-protein measurements could be related to the lack of specificity in the TBA method used for measuring MDA (36). These methodologic uncertainties may explain the lack of a strong correlation between MDA and MDA-protein content, but should not alter the age-related differences observed.

The decrease in MDA and MDA proteins in hypothyroid young rats is consistent with the reduced oxygen consumption and decreased generation of free radicals in the heart of these rats (10, 37). The lack of a significant increase in MDA proteins in hyperthyroid rats could not be explained. It is possible that hyperthyroidism for longer periods may have resulted in the expected increase in MDA proteins. Another potential explanation could be related to alterations in MDA or MDA-protein degradation. It is not known whether there is an age-related change in the effects of TH on MDA or MDA-protein degradation.

Although methimazole did not alter MDA modification of proteins *in vitro,* it is possible that methimazole alters MDA generation through its antioxidant properties (39, 40). However, it is not clear why such an effect would be evident only in young rats and only in cardiac tissue. In a previous study, methimazole did not alter MDA content of plasma or cerebrum (32). However, the lack of changes in MDA-protein content of cardiac tissue of thyroidectomized rats suggests that methimazole may indeed have interfered with MDA modification of cardiac proteins *in vivo* possibly through free radical scavenging effects (39, 40). Alternatively, thyroidectomy in these rats may have not achieved the same degree of hypothyroidism as achieved with methimazole treatment. This latter speculation is supported by the modest reduction in hepatic malic enzyme activity of thyroidectomized rats compared to methimazole-treated rats.

The electrophoretic profile of cardiac proteins, when stained with Coomassie blue, was altered in hypothyroidism (Fig. 2B). In general, the lower molecular weight protein bands were reduced whereas protein bands over 60 KDa molecular weight were increased. These changes may be related partly to alterations in MDA modifications of proteins that often result in either degradation of certain proteins and often aggregation with apparent changes in molecular size (38). However, the magnitude of such changes in other tissues is not always sufficient to result in discernible changes in Coomassie blue stained gels (32).

The biological implications of MDA-protein modification are multiple. These modifications may change the biological functions of the proteins, alter their turnover kinetics, change antigenicity, and initiate an autoimmune response with generation of anti MDA-protein antibodies (20). The present study clearly shows that altered thyroid function can be associated with changes in MDA proteins. It is not known whether similar changes occur in humans during clinical thyroid disease. Such changes may interfere with cardiac contractility, alter cardiac electrophysiology, and contribute to the cardiomyopathy associated with thyroid disease.

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