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Original Contribution

THYROID STATUS MODULATES GLYCOXIDATIVE AND LIPOXIDATIVE MODIFICATION OF TISSUE PROTEINS

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Abstract—Steady state protein modification by carbonyl compounds is related to the rate of carbonyl adduct formation and the half-life of the protein. Thyroid hormones are physiologic modulators of both tissue oxidative stress and protein degradation. The levels of the glycation product N$^{\varepsilon}$-fructoselysine (FL) and those of the oxidation products, N$^{\varepsilon}$-(carboxymethyl)lysine (CML) and malondialdehyde-lysine (MDA-lys), identified by GC/MS in liver proteins, decreased significantly in hyperthyroid rats, as well as (less acutely) in hypothyroid animals. Immunoblotting of liver proteins for advanced glycation end-products (AGE) is in agreement with the results obtained by GC/MS. Cytosolic proteolytic activity against carboxymethylated foreign proteins measured in vitro was significantly increased in hypo- and hyperthyroidism. Oxidative damage to DNA, estimated as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8oxodG), did not show significant differences between groups. The results suggests that the steady state levels of these markers depend on the levels of thyroid hormones, presumably through their combined effects on the rates of protein degradation and oxidative stress, whereas DNA is more protected from oxidative damage.

Keywords—Carbonyl stress, N$^{\varepsilon}$-(carboxymethyl)lysine, N$^{\varepsilon}$-fructoselysine, Malondialdehyde-lysine, Proteolytic activity, 8-oxo-7,8-dihydro-2′-deoxyguanosine, Free radicals

INTRODUCTION

Carbonyl-amine reactions in vivo lead to nonenzymatic post-translational modification of proteins both by reducing sugars (glycation) and by lipid peroxidation-derived aldehydes. An increase in carbonyl stress is believed to be related to the development of diabetic complications and the aging process through the formation and accumulation of carbonyl-amine derived reaction products on extracellular matrix proteins [1,2]. Glucose, in its open chain, aldehyde form, reacts with epsilon-amino groups of lysine residues on proteins, yielding the Amadori compound N$^{\varepsilon}$-fructoselysine (FL) [3]. Subsequent oxidative reactions lead to the formation of glycoxidation products such as Nε-(carboxymethyl)lysine (CML) and pentosidine, among others [4-6]. Similarly, lipid peroxidation–derived aldehydes, like malondialdehyde (MDA) and 4-hydroxynonenal, formed from oxidized polyunsaturated fatty acids (PUFA), may react with proteins generating MDA-lysine and hydroxynonenal-lsysine adducts [7]. Further, CML is also formed from oxidized PUFA [8]. Regardless of its source, the steady state level of modification of a given protein is modulated by both the rate of carbonyl compound formation and the half-life of the protein [1,9].

To shed light on the role of these two factors in cellular protein damage, we sought a physiologic approach for influencing each of them. Thyroid hormones are physiologic modulators of both tissue oxidative stress and protein degradation. Thyroid hormones can affect oxidative stress through their effects on mitochondrial respiration [10], with hypothyroidism known to decrease and hyperthyroidism to increase respiratory activity [11,

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12]. Mitochondria are considered a major source of free radicals, such as superoxide, in healthy tissues [13,14] because they are responsible for more than 90% of cellular oxygen consumption, and these highly reactive oxidants can modify lipids, carbohydrates, proteins, and DNA.

Thyroid hormones also affect protein synthesis and degradation both in the whole body and in tissues such as skeletal muscle [15–19] and liver [15,20]. Thus, hyperthyroidism leads to a general loss of body mass caused by increased proteolysis, with protein synthesis remaining largely unaffected. Strikingly, the lack of thyroid hormones also leads to a relatively increased rate of protein breakdown, resulting in a general loss of protein, resulting from the hypothyroidism-induced decrease in protein synthesis.

The effects of changes in basal metabolic rate and protein degradation induced by thyroid status on the steady state levels of glycoxidation and lipoxidation products have not been explored. To evaluate how oxidative stress and/or protein degradation can modulate modification of protein by carbonyl-amine reactions, we have studied the effects in adult male rat livers of both increased and decreased T_3 levels on the concentration of FL, CML, and MDA-lysine protein adducts. Cytosolic proteolytic activity on carboxyxmethylated homogeneous and heterogeneous proteins has also been evaluated. Because the other two main cellular macromolecules, lipids and DNA, may also be targets of oxidative damage, the above data have been compared with fatty acid composition and the content of genomic 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in the rat liver.

MATERIALS AND METHODS

Materials

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. [$^2H_8$]lysine was purchased from MSD Isotopes (Rahway, NJ, USA). FL and [$^{13}C_6$]FL, CML and [$^2H_4$]CML, and MDA-lysine and [$^2H_8$]MDA-lysine, were prepared as previously described [7,21]. The rabbit antiserum used for immunoblotting was that described by Reddy et al [22]. The antiserum was raised against advanced glycation end-product (AGE) modified key hole limpet hemocyanin (KLH) incubated with glucose; CML is the primary epitope recognized by this antiserum.

Animals and treatments

Twelve week-old male Wistar rats were obtained from Iffa-Creddo (Lyon, France). Throughout the experiment they were maintained at 23 ± 2°C, 12:12 h (light: dark) cycle, 50 ± 10% relative humidity, and were fed ad libitum. Rats were rendered hypothyroid by the administration of 0.05% 6-n-propyl-2-thiouracil in their drinking water for 4–5 weeks. Hyperthyroidism was induced by one daily intraperitoneal injection of 150 μg T_3/kg body mass in 250 μl 0.5 mM NaOH for 10 days. Paired litter-mate controls were injected with vehicle (0.5 mM NaOH). Animal body weight, oxygen consumption and rectal temperature were monitored through the experimental period. After treatment, animals were killed by decapitation and a blood sample was obtained. Livers and sera were stored at −80°C until further analysis. Blood glucose was determined using the GOD-PAP (Glucose oxidase) method (Boehringer, Germany). The thyroid status of all animals was confirmed by the measurement of serum T_3 and T_4 concentrations by radioimmunoassay (Commercial RIA kits, CIS Bio International, France) and by the estimation of the basal metabolic rate. The oxygen consumption of each animal was measured at rest in a 19-l closed circuit system respirometer at 22 ± 1°C just before the beginning and at the end of the treatments.

Measurement of FL, CML, and MDA-lys in tissue proteins

After thawing, liver samples were minced and washed in phosphate buffer to remove all traces of blood. The samples (~0.25 g) were homogenized in 2.5 ml 50 mM phosphate buffer pH 7.4 using a Polytron homogenizer (Brinkmann, Switzerland) for 15 s at a setting of 8, and then centrifuged at 750 × g for 10 min at 4°C. The supernatants were delipidated using 10 ml methanol/diethyl ether (1:10 v/v) and precipitated proteins were dried under a stream of nitrogen. MDA-lys and CML were measured in protein samples (1–2 mg) after overnight reduction with a final concentration of 500 mM $NaBH_4$ in 0.2M borate buffer, pH 9.2, containing 1 drop of hexanol as an antifoam reagent. Proteins were precipitated with 10% trichloroacetic acid and subsequent centrifugation. FL was measured on nonreduced protein samples. Isotopically labeled internal standards were added, and the samples were hydrolyzed at 110°C for 24 h in 6N HCl, then dried in vacuo. Residues were rehydrated in 1 ml of 1% trifluoroacetic acid and applied to a 1 ml C-18 solid extraction column (Supelco, Bellefonte, PA, USA), equilibrated with the same solvent. The first milliliter of the flow through, and two additional milliliters of 1% trifluoroacetic acid were all pooled and dried in vacuo. The N,O-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared as previously described [21]. Gas chromatography/mass spectrometry analyses were carried out on a Hewlett Packard (Palo Alto, CA, USA) model 6890 gas chromatograph equipped with a 30m HP-5MS capillary col-

umn coupled to a Hewlett Packard model 6890 mass selective detector. The injection port was maintained at 275°C; the temperature program was 2 min at 150°C, then 5°C/min to 225°C, then 25°C/min to 300°C, and finally held at 300°C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and nondeuterated standards. Analyses were carried out by selected ion monitoring gas chromatography/mass spectrometry. The ions used were lysine and [$^2H_8$]lysine, m/z 180 and 187, respectively; FL and [$^{13}C_6$]FL, m/z 110 and 116, respectively; MDA-lysine and [$^2H_8$]MDA-lys, m/z 474 and 482, respectively; and CML and [$^2H_4$]CML , m/z 392 and 396, respectively. The amount of products is expressed as the ratio mmol FL, or CML or MDA-lys / mol lysine.

Immunoblotting of N^{ε}(carboxymethylated)-proteins

Protein, 10 μg, was separated by SDS-polyacrylamide gel electrophoresis according to Laemmli [23], using 9% acrylamide as resolving gel. Proteins were transferred to PVDF membranes (Immobilon-P Millipore, Bedford, MA, USA) using a Semidry system (Semi-Phor, Hoefer Scientific Instruments, San Francisco, CA, USA). After transfer, the membrane was incubated with blocking buffer (0.2% I-Block, 0.1% Tween-20 in phosphate-buffered saline [PBS] pH 7.35) overnight, and then reacted for 1 h at room temperature with anti-AGE rabbit serum (1:2000 in blocking buffer). After washings, membranes were incubated for 1 h at room temperature with a goat antirabbit antibody conjugated with alkaline phosphatase (1:25,000) in blocking buffer (Tropix, Bedford, MA, USA), washed, and then reacted with the CSPD chemiluminescent substrate for alkaline phosphatase (Tropix, Bedford, MA, USA). Luminescence was detected using a Lumi-Imager (Boehringer, Germany).

Synthesis of CML-modified proteins

Bovine serum albumin (BSA) and hemolysate proteins were carboxymethylated as described previously [24]. Briefly, 10 mg BSA or hemolysate proteins were dissolved in 2 ml 0.3M sodium tetraborate pH 9.2 at 0°C and 1 mg $NaBH_3CN$ was added. Twelve μmoles glyoxylic acid were then added every 6 min under constant stirring. At the end of 90 min the proteins were dialyzed exhaustively against buffer. CML modification was assessed by both a change in electrophoretic mobility (increased net negative charge) and increased immunoreactivity to the anti-AGE antiserum, by immunoblotting.

Proteolytic activity assessment: labeling of proteins and proteolysis measurements

The degradation of fluorescein-isothiocyanate (FITC)-labeled foreign proteins by incubation with hepatic cytosol was evaluated. To obtain cytosolic fraction, liver samples (0.3 g) were washed and then homogenized with a Potter-Elvehjem device in 5 volumes of 5 mM n-[2-hydroxethyl]piperazine-N′-[2-ethanesulfonic acid] HEPES buffer (pH 7.35) containing 210 mM sucrose and 70 mM mannitol. The homogenate was centrifuged at 100,000 $\times$ *g* for 40 min, and the supernatant fraction was retained for study at −80°C until analyzed. To label proteins in order to measure their degradation rates, native and CML-rich proteins (7.5 mg) were dissolved in 1 ml 1M $NaHCO_3$ (pH 9) and 50 μl FITC (10 mg/ml) in DMSO. After 1 h at room temperature, unreacted FITC was quenched by adding 100 μl of a hydroxylamine solution (100 mg/ml, pH 9.8). FITC-labeled proteins were separated from low-molecular contaminants using PD-10 columns (Pharmacia, Uppsala, Sweden), previously equilibrated in PBS. After protein quantification by the Bradford method [25], labeled proteins were stored frozen at −80°C until analyzed. To quantify proteolysis, 100 μg of cytosolic protein and 3.75 μg of FITC-labeled native or CML-rich proteins were added to 125 μl of proteolysis buffer (50 mM Tris-HCl (pH 7.8), 20 mM KCl, 5 mM Mg acetate, and 0.5 mM dithiothreitol), total volume 200 μl, and the resulting mixture was incubated at 37°C. After 6 h proteolysis reached completion and samples were submitted to fluorescence analysis. Fluorescence (495 nm excitation/ 515 nm emission) from samples was measured in a Shimadzu RF-5000 spectrofluorimeter (Shimadzu Europe GmbH, Germany). Then, proteins were precipitated using the alkaline zinc-sulfate method [26] and fluorescence was again recorded. Proteolytic activity was estimated as the ratio fluorescence after protein precipitation/fluorescence before protein precipitation. All data are expressed relative to values obtained for euthyroid liver.

8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) assay

Liver samples were homogenized in 150 mM NaCl at a concentration of 500 mg of tissue/ml. After homogenization, 1.8 ml of buffer (150 mM NaCl, 10 mM Tris, 10 mM Na_2EDTA, pH 8.0) and 200 μl of 10% SDS were added to 200 μl of liver homogenate. Samples were rotated in an extraction bench for 15 min and then incubated at 37°C for 10 min. DNA extraction was performed as previously described [27]. Subsequently, the DNA was precipitated by addition of 5 ml of ice-cold 96% ethanol. The DNA was allowed to precipitate at −20°C overnight. After washing steps, the isolated DNA was digested to deoxynucleoside level by incubation with nuclease P1 and 1 U alkaline phosphatase. The

Table 1. Concentration of Serum Thyroid Hormones, Body Weight Gain, and Basal Metabolic Rate of Rats Differing in Thyroid Status

	Hypothyroid	Euthyroid	Hyperthyroid
Thyroid hormones (ng/ml)			
T_4	12.14 ± 0.43§	49.17 ± 2.50§	18.50 ± 1.90†
T_3	0.20 ± 0.01*	0.73 ± 0.05§	7.45 ± 0.20§
Body weight gain (g/week)	6.5 ± 1.30§	28.8 ± 2.10†	−5.6 ± 3.10§
Oxygen consumption (ml of O_2/h per g)			
Initial	1.35 ± 0.05	1.20 ± 0.12	1.28 ± 0.07
Final	0.98 ± 0.07*	1.18 ± 0.04†	1.48 ± 0.06§
Rectal temperature (°C)			
Initial	39.01 ± 0.1	39.07 ± 0.1	39.15 ± 0.1
Final	37.56 ± 0.2§	39.04 ± 0.1	39.42 ± 0.2§
Blood glucose (g/l)	1.29 ± 0.05	1.36 ± 0.04†	1.61 ± 0.04‡

Values are means ± S.E.M. from seven animals per group. Asterisks horizontally situated between two groups indicate statistically significant differences between these two groups. Asterisks situated at right of hyperthyroid group values indicate significant differences between hyperthyroid and hypothyroid groups.

* $p < .05$.
† $p < .01$.
‡ $p < .005$.
§ $p < .001$.

concentrations of 8-oxodG and deoxyguanosine (dG) were measured by high-performance liquid chromatography (HPLC) with on line electrochemical (ESA Coulochem II, Bedford, MA, USA) and ultraviolet (Biorad model 1806 UV, Madrid, Spain) detection, respectively, using a reverse-phase Beckman Ultrasphere ODS column (5 μm, 4.6 mm × 25 cm) (Beckman Instruments, Inc., Palo Alto, CA, USA), eluted with 3.3% acetonitrile in 50 mM phosphate buffer (pH 5.0) using a Waters 590 pump (Waters, Milford, MA, USA) at a flow rate of 1ml/min. Data are expressed as the ratio 8-oxodG/10^5dG.

Fatty acid analyses

Lipids were extracted from liver samples with chloroform:methanol (2:1 v/v) [28] in the presence of 0.01% butylated hydroxytoluene. The chloroform phase was separated and then evaporated under N_2. Lipids were transesterified by incubation in 2.5 ml of 5% methanolic HCl for 90 min at 75°C. The fatty acid methyl esters were extracted adding 2.5 ml of n-pentane and 1 ml of saturated NaCl. The n-pentane phase was separated, evaporated under N_2, and redissolved in 100 μl of carbon disulfide and 1 μl was used for GC/MS analysis. Separation was performed on an SP2330 capillary column (30 m × 0.25 mm × 0.20 μm) using a Hewlett Packard 5890 Series II gas chromatograph. A Hewlett Packard 5989A mass spectrometer was used as detector in the electron-impact mode. The injection port was maintained at 220°C, and the detector at 250°C; the temperature program was 2 min at 100°C, then 10°C/min to 200°C, then 5°C/min to 240°C, and finally held at 240°C for 10 min. Identification of methyl esters was made by comparison with authentic standards and on the basis of mass spectra.

Calculations and statistical methods

Average chain length (ACL) = [(Σmol%Total$_{14}$ × 14)+...+(Σmol%Total$_n$ × n)]/100 (n = carbon atom number); Double-bond index (DBI) = (Σmol% of unsaturated fatty acids × number of double bonds of each unsaturated fatty acid) indicates the total number of fatty acid double bonds; Saturated fatty acids (SFA) = Σmol% (14:0 + 16:0 + 18:0 + 22:0); Unsaturated fatty acids (UFA) = Σmol% (16:1 + 18:1 + 18:2 + 18:3 + 20:2 + 20:3 + 20:4 + 20:5 + 22:5 + 22:6); the peroxidizability Index (PI) = [(Σmol% Monoenoic × 0.025) + (Σmol% Dienoic × 1) + (Σmol% Trienoic × 2) + (Σmol% Tetraenoic × 4) + (Σmol% Pentaenoic × 6) + (Σmol% Hexaenoic × 8)] represents the sensitivity of fatty acids to oxidative damage [29].

All statistics were obtained using the SPSS software (SPSS, Chicago, IL, USA). Normality of variables was assessed using the Kolmogorov-Smirnoff test. Data were analyzed by One-Way Analysis of Variance (ANOVA). When necessary, after the ANOVA the Fisher's least significant difference (LSD) test was used to analyze significance between paired groups. The 0.05 level was selected as the point of minimal statistical significance for all comparisons.

RESULTS

Characterization of treated animals

As shown in Table 1, there are significant differences in thyroid hormone levels, body weight gain, basal metabolic rate, and rectal temperature among the experimental groups. The serum levels of thyroid hormones indi-

cate the effectiveness of the experimental treatments; the lowest T_3 serum concentrations were found in the hypothyroid group and the highest in the hyperthyroid animals. The significantly decreased T_4 levels in the hypothyroid compared with the euthyroid group are characteristic of animals treated with 6-n-propyl-2-thiouracil (an inhibitor of thyroid hormone synthesis). The decrease in serum T_4 in hyperthyroid rats is a result typically observed in T_3-treated animals because of hypothalamic-pituitary-thyroid axis negative feedback regulation. The differences in the thyroid status among groups at the end of treatment affected the metabolic state since the rate of growth, and as a consequence, the final body weight, were significantly lower in the hypo- and hyperthyroid group compared with the euthyroid group. Furthermore, the lowest final basal metabolic rate is found in the hypothyroid group and the highest in the hyperthyroid animals, following the progressive increase in serum T_3 status. Consistent with the decrease in basal metabolic rate, rectal temperature was significantly decreased in hypothyroid compared with eu- and hyperthyroid rats. Fasting blood glucose concentration was significantly increased in hyperthyroid group compared with the euthyroid group, while no significant changes were observed in the hypothyroid group.

Effect of thyroid status on protein modification

The effect of thyroid status on protein modification by carbonyl compounds is shown in Figs. 1 and 2. Figure 1 (upper and middle panel) shows the effect of thyroid hormones on the levels of the glycation product FL and the oxidation product CML, respectively. FL and CML concentrations in the hypo- and hyperthyroid rats are significantly lower than those of euthyroid animals, although the magnitude of the hypothyroid-induced decrease was less severe than that induced by hyperthyroidism. Assuming that treatment of the animals was maintained long enough to achieve constant rates of protein synthesis and degradation, FL values could be used as a surrogate for protein turnover [9]. Thus, the CML/FL ratio can be considered as a marker of oxidative damage on tissue proteins, because the oxidation product CML may be derived from the reaction of lysine with products of glucose or fatty acid oxidation [6,8,30,31]. This ratio is significantly increased by hyper- but not by hypothyroidism (Fig. 1, lower panel), suggesting increased oxidative stress in hyperthyroidism. The effects of thyroid status on MDA-lys concentration is shown in Fig. 2 (upper panel). The data demonstrate that MDA-lys levels are significantly lower both in hyper- and hypothyroidism, being more pronounced in the latter than the former case. As for CML/FL, the MDA-lys/FL ratio was calculated to estimate protein turnover-corrected lipoxidative damage (Fig. 2, lower panel). This ratio is significantly lower in the hypothyroid and higher in the hyperthyroid rats compared with the euthyroid group, which is consistent with decreased and increased oxidative stress, respectively, in the two conditions.

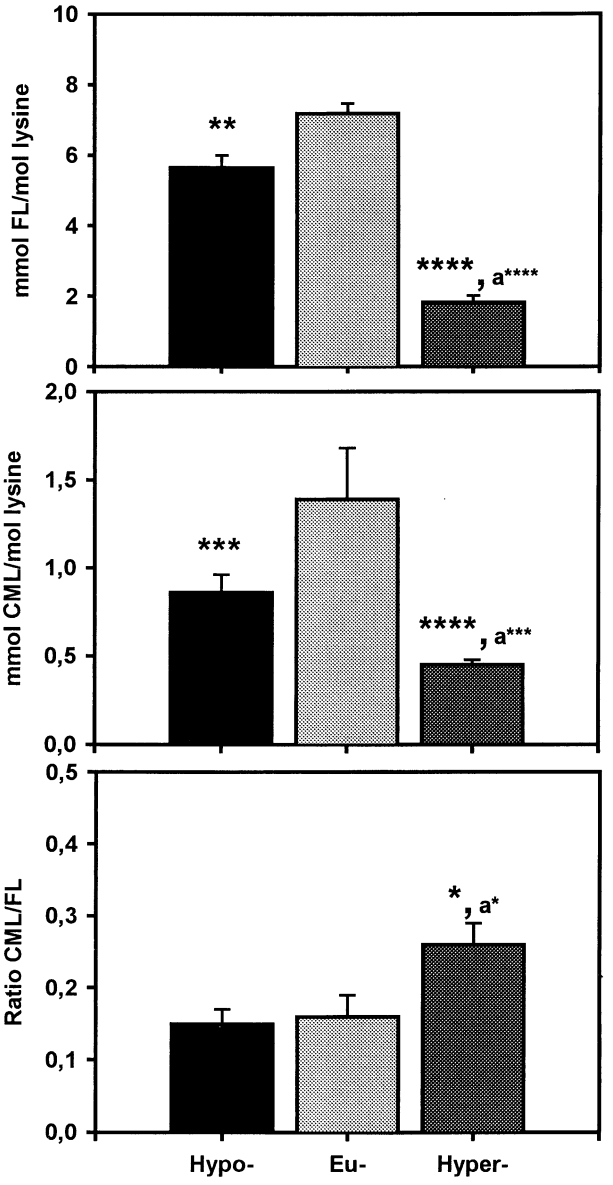

Fig. 1. Levels of N^{ϵ}-fructoselysine (mmol FL/mol lysine) (upper panel), N^{ϵ}-(carboxymethyl)lysine (mmol CML/mol lysine) (middle panel) and CML/FL ratio (lower panel) in liver proteins from rats differing in thyroid status. For each group, $n = 7$ animals. Values are expressed as mean ± S.E.M. Asterisks describe significant differences versus euthyroid group. Asterisks marked a*, a***, or a**** compare hyper- to hypothyroidism. *$p < .05$, **$p < .01$; ***$p < .005$, ****$p < .001$.

Detection of in vivo anti-AGE immunoreactive proteins

In preliminary assays, anti-AGE antiserum did not recognize native proteins, whereas CML-modified pro-

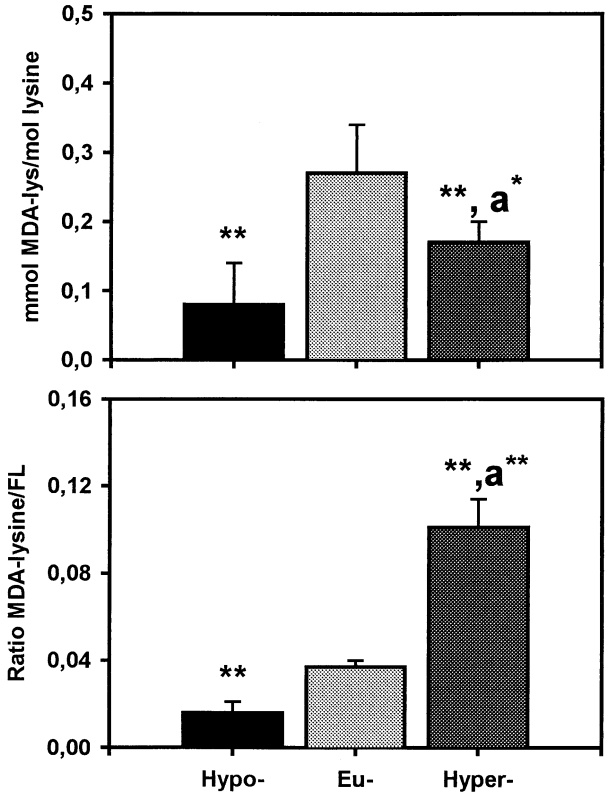

Fig. 2. Levels of MDA-lysine (mmol MDA-lys/mol lysine) (upper panel), and MDA-lys/FL (lower panel) ratios in liver proteins from rats differing in thyroid status. For each group, $n = 7$ animals. Values are expressed as mean ± S.E.M. Asterisks describe significant differences versus euthyroid group. Asterisks marked a* or a** compare hyper- to hypothyroidism. $*p < .05$, $**p < .01$.

teins were strongly stained (data not shown). Preincubation of anti-AGE antiserum with CML-modified proteins suppressed immunostaining (data not shown). Thus, carboxymethylated proteins were visualized by immunostaining on SDS-PAGE separated liver homogenate proteins (Fig. 3). Three prominent protein bands were detected with molecular weights of about 77.3, 36.7 and 33.4 kD, respectively. The overall immunostaining patterns differed between groups in the same manner that CML levels differed when measured by GC/MS; i.e., the signal for both hypo- and hyperthyroid groups is less marked than that of the euthyroid group, indicating a decreased steady state level of protein modification.

Proteolytic activity on carboxymethylated proteins

Oxidative stress seems to increase the intracellular proteolysis mediated by an ATP-independent proteasome complex pathway by conferring increased proteolytic susceptibility to oxidatively modified proteins [32]. MDA-modified proteins also exhibit accelerated rate of

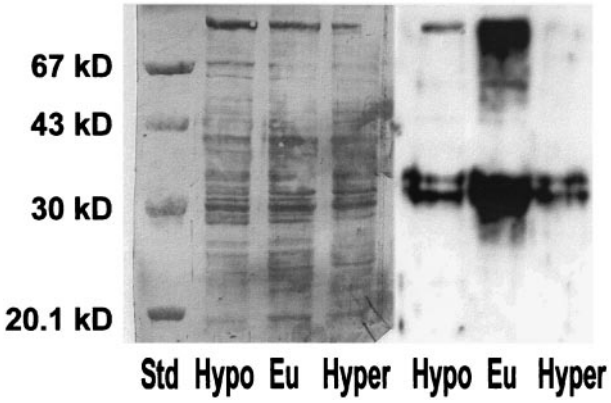

Fig. 3. Carboxymethylated modified proteins from rats differing in thyroid status. SDS-PAGE analyses of extracts from liver of rats with hypo-, eu- and hyperthyroidism. (Left) Coomassie Blue protein stain; (right) anti-AGE immunoblotting. Molecular weight markers are shown on the left of protein stain.

proteolysis [33]. Because the cellular pathways for degradation of glyco- and lipoxidated proteins have not been dissected, it has been assumed that similarly to oxidatively modified proteins, the proteasome activity may be relevant. Thus, the degradation of native and carboxymethylated foreign proteins (BSA and hemolysate protein) added to liver cytosol was studied, to evaluate whether the decreased CML/mol lysine values may be derived, even partially, from changes of the cytosol proteolytic activities. In agreement with the quantification of CML, hypo- and hyperthyroidism induced a significantly increased proteolytic activity for carboxymethylated proteins compared with the euthyroid group (Fig. 4). No differences were observed for native proteins (Fig. 4).

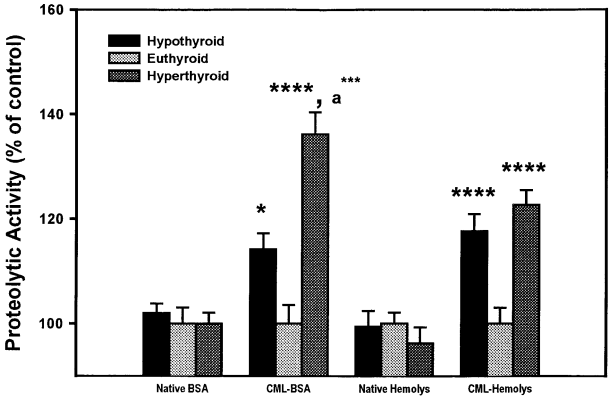

Fig. 4. Proteolytic activity of liver cytosol on native and carboxymethylated BSA and hemolysate proteins from rats differing in thyroid status. Values reported are mean ± S.E.M. for $n = 7$ animals. Asterisks describe significant differences versus euthyroid group. Asterisk marked a*** compares hyper- to hypothyroidism. $*p < .05$, $***p < .005$, $****p < .001$.

Table 2. Fatty acid composition (mol %) and general indexes related to membrane fatty acid composition of rat liver lipids

	Hypothyroid	Euthyroid	Hyperthyroid
14:0	0.73 ± 0.08	0.80 ± 0.04§	0.45 ± 0.01*
16:0	21.17 ± 0.69*	23.41 ± 0.63‡	20.61 ± 0.27
16:1n-7	1.71 ± 0.13§	3.21 ± 0.15‡	1.94 ± 0.20
18:0	20.59 ± 0.31*	19.09 ± 0.48§	23.55 ± 0.41§
18:1n-9	9.88 ± 0.37§	13.60 ± 0.31	15.28 ± 0.80§
18:2n-6	18.58 ± 0.51*	16.83 ± 0.44‡	14.08 ± 0.44§
18:3n-3	0.24 ± 0.02	0.21 ± 0.00§	0.13 ± 0.00‡
20:2n-6	0.63 ± 0.08‡	0.19 ± 0.00	0.20 ± 0.03‡
20:3n-6	0.48 ± 0.07	0.57 ± 0.07	0.50 ± 0.03
20:4n-6	18.27 ± 0.48	17.72 ± 0.44*	19.47 ± 0.58
20:5n-3	0.48 ± 0.04†	0.27 ± 0.03*	0.17 ± 0.00§
22:0	1.51 ± 0.33*	0.52 ± 0.08	0.33 ± 0.03†
22:5n-3	0.60 ± 0.06†	0.35 ± 0.02§	0.11 ± 0.00§
22:6n-3	5.05 ± 0.14§	3.14 ± 0.12	3.11 ± 0.31§
ACL	18.19 ± 0.02	17.97 ± 0.02	18.08 ± 0.02
SFA	44.02 ± 0.71	43.84 ± 0.43	44.95 ± 0.39
UFA	55.97 ± 0.71	56.15 ± 0.43	55.04 ± 0.39
PI	141.09 ± 2.88§	118.94 ± 2.05	120.54 ± 3.60‡
DBI	161.14 ± 2.76‡	146.24 ± 1.72	145.76 ± 2.88‡

Values are means ± S.E.M. from seven animals per group. For general indexes related to fatty acid composition see Materials & Methods.

* $p < .05$.
† $p < .01$.
‡ $p < .005$.
§ $p < .001$.

Fatty acid composition and peroxidizability index

The fatty acid composition of total liver lipids from rats differing in thyroid status is shown in Table 2. The peroxidizability index and the double-bond index (Table 2) were higher in the hypothyroid than in the eu- and hyperthyroid groups; i.e., the total number of liver fatty acid double bonds is significantly increased in hypothyroidism. This is mainly due to an increase in the proportions of various polyunsaturated fatty acids of the n-3 and n-6 series, such as 22:6n-3 and 18:2n-6, and to a decrease in monounsaturated n-7 and n-9 fatty acids, in agreement with previous reports [34]. Although some differences in fatty acid composition were present between the euthyroid and the hyperthyroid groups, they did not lead to changes in the general types of fatty acids or in their double-bond index and peroxidizability index. Thus, even though the potential substrate for MDA generation is higher in the hypothyroid state, MDA-lys levels are lower, suggesting protein degradation is the major determinant of the steady state level of protein modification.

Oxidative damage to genomic DNA

The hepatic steady state concentration of 8-oxodG in the genomic DNA was measured. The data (0.99 ± 0.12; 0.87 ± 0.17 and 0.80 ± 0.13 8-oxodG/10^5dG for hypo-, eu- and hyperthyroid groups, respectively; $p > .05$) suggest that large variations in thyroid status do not change the extent of oxidative damage to nuclear DNA.

DISCUSSION

The effectiveness of the experimental approach was confirmed by the plasma levels of thyroid hormones and the basal metabolic rate (oxygen consumption). The markedly lower rate of body growth of both hypo- and hyperthyroid animals in relation to euthyroid controls is consistent with the observation that these treatments alter the balance of protein turnover by increasing the ratio of protein degradation/protein synthesis [15-20]. This modulation of protein turnover is also reflected in the markers of protein carbonyl damage.

Interestingly, tissue FL and CML concentrations reported here are as high as those in long-lived extracellular proteins. FL concentration in skin collagen is about 4 mmol/mol lysine [35]. Because the formation of FL results from the nonenzymatic covalent attachment of glucose to the free amino groups of proteins [3], and the liver protein values are higher than those of human collagen, the results suggest that intracellular sugars such as glucose-phosphates may be more active in glycation reactions. Glucose levels, usually considered as the major determinant for the FL concentration, do not explain the differences observed in the present work, as glycemia pattern, influenced by the hepatic gluconeogenesis, is not reflected by the FL concentrations (particularly when considering values of the hyperthyroid group). Thus, the differences in FL values among experimental groups may be explained by a decrease in the half-life of liver proteins, mainly because the ratio protein degradation/protein synthesis is increased both by hyperthyroidism and hypothyroidism [15–20], as emphasized in the present study by the lack of body weight gain in these groups.

CML values in rat liver proteins are as high as those reported in human extracellular-matrix proteins. We found CML values in the range of 0.5–1.4 mmol/mol lysine, depending on thyroid status, still well within the values for human skin collagen, at ages 18 and 85 years, which contain approximately 0.35 and 1.2 mmol CML/mol lysine, respectively [4,35]. These high levels may be attributable to the origin of CML from free radical reactions, suggesting higher oxidative stress in cells in comparison to extracellular matrix. CML may be produced from glyoxal formed on auto-oxidation of glucose, other nonglucose carbohydrates, or even PUFA [8,31,36], or by oxidative cleavage of Amadori adducts on proteins [6,30]. Thus, at a first glance, the decreased steady state CML levels might be viewed as an indication of decreased oxidative stress in hyperthyroidism. However,

the intracellular proteolytic activity on carboxymethylated BSA and hemolysate proteins was clearly increased in hyperthyroidism. Thus, a putative increase in CML formation (oxidative stress) would be masked by a stronger increase in CML–protein breakdown, finally leading to a decrease in steady state CML levels in hyperthyroid animals, similarly to FL. FL is representative of glycation, and CML of oxidation, so that CML/FL ratio may be indicative of overall oxidative damage. Thus, the increase in CML/FL ratio in hyper- but not in hypothyroidism is in agreement with previous studies [37,38], confirming the occurrence of an increased oxidative stress in hyperthyroidism in liver tissue.

The decrease in FL and CML levels in hypothyroid animals, without changes in the CML/FL ratio, suggests unchanged oxidative stress. In this condition, proteolysis is the likely major determinant in the steady state level of carbonyl damage, because proteolytic activity on carboxymethylated proteins is moderately, but significantly, increased in hypothyroidism. Nevertheless, contributions of the decreased body temperature and possible changes in concentrations of intracellular precursors may not be excluded.

The differences in CML levels induced by thyroid status was further confirmed using an anti-AGE based immunoblotting analysis. CML is the major epitope recognized by this antiserum [22]. Strikingly, only three bands were strongly reactive with the antiserum, suggesting a specificity for protein modification. Studies are in progress to identify these proteins.

Aldehydes generated during PUFA peroxidation vary in chain length, and range from 9-carbon compounds, such as 4-hydroxynonenal, to small 2- and 3-carbon reactive aldehydes, such as MDA, acrolein, and glyoxal. All of these carbonyl compounds may react with primary amino groups on proteins [39]. In this work, we have evaluated the MDA-lys adducts occurring on intracellular proteins. Fatty acid composition, oxidative stress, and protein degradation, are all factors that may be modulated by thyroid hormones, and thus ultimately affect MDA-lys levels in tissue proteins. Similar to results obtained for CML, the increase in protein degradation seems the most relevant variable in determining the decrease in MDA-lys levels in hyperthyroid animals, because the double-bond index remained unchanged but oxidative stress (CML/FL ratio) was increased in T_3-treated rats.

For hypothyroidism, despite a higher double-bond index (that is, a higher potential substrate for lipid peroxidation), lower MDA-lys values indicate a decrease in protein modification by MDA, possibly due in part to a lower free radical attack to cellular macromolecules. Consistent with this suggestion, a decrease in in vivo free radical generation in hypothyroid animals has been recently demonstrated [37,38]. Moreover, a moderate increase in protein degradation may also contribute to the decreased MDA-lysine levels, because MDA-modified proteins are subject to an accelerated rate of intracellular proteolysis [33].

Even though hypo- and hyperthyroidism alter oxidative stress in liver lipids and proteins, oxidative damage to DNA (8-oxodG) was not changed, in spite of the strong variation in circulating thyroid hormone levels and in total oxygen consumption among the three experimental groups. Similarly, it has been found that basal concentrations of liver 8-oxodG and urinary MDA-deoxyguanosine are not sensitive to some forms of externally imposed increased oxidative stress [40], or deficiency or supplementation with antioxidant vitamins in animals or humans under nonstressful conditions [41,42]. In contrast, liver protein oxidation and liver or urine lipid peroxidation products are sensitive to these manipulations [40,43]. Some authors suggested [40,41] that the reason for this may be that DNA is better protected than most cellular proteins and lipids against free radicals because these are mainly generated outside the nuclear compartment. Another possible explanation for the 8-oxodG results is that the repair of oxidative lesions to DNA is much faster than that of damage to other kinds of macromolecules [44], 8-oxodG being repaired in a few hours by a specific 8-oxoguanine DNA glycosylase/lyase [45,46]. The high rate of repair of DNA damaged by oxidative stress [47], which can also depend on thyroid hormone levels, may have contributed to the lack of changes in 8-oxodG among experimental groups.

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ABBREVIATIONS

AGE—Advanced glycation end-products
CML—N^{ε}(carboxymethyl)lysine
GC/MS—gas chromatography/mass spectrometry
FITC—fluorescein-isothiocyanate
FL—N^{ε}-fructoselysine
MDA-lys—malondialdehyde-lysine
8-oxodG—8-oxo-7,8-dihydro-2′-deoxyguanosine
T_4—thyroxine
T_3—triiodothyronine