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Thyroxine Deiodination Associated With NADPH-Dependent Lipid Peroxidation in a Submicrosomal System (39044)

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The enzymatic system which is presumably responsible for the deiodination of thyroxine still remains unidentified, even though many workers have addressed themselves to this problem. Thyroxine is readily deiodinated by tissue preparations if appropriate additions are included, but unfortunately these systems have turned out to be non-enzymatic, as in the case of the Fe^{2+}-induced microsomal deiodination system (1–3). Poyer and McCay (4) have demonstrated that oxidative degradation of phospholipid in hepatic microsomes is effected by at least two different reactions, i.e. one that is NADPH-dependent (enzymatic) and another which is ascorbate-dependent (nonenzymatic). We have recently found that in the above two systems lipid peroxidation is correlated with thyroxine degradation (5).

The studies herein reported represent attempts to isolate microsomal components essential for NADPH-dependent lipid peroxidation during thyroxine degradation and to reproduce any such reactions in a simulated system.

Materials and Methods. ADP, NADPH and ^{131}I-labeled thyroxine (3′ and 5′) were obtained from Sigma, Oriental and Abbott Laboratories, respectively. The radioactive thyroxine was mixed with the corresponding nonradioactive compound to obtain a certain concentration. All other chemicals were of reagent grade. The ^{131}I-labeled thyroxine (3 and 5) (6), rat liver microsomes (7) and their deoxycholate–trypsin-treated microsomal particles (lipoprotein) (1) were prepared by established methods. As mentioned in a previous report from our laboratory (2), neither aminopyrine demethylation activity nor cytochrome P_{450} was found in the lipoprotein particles. Rat liver microsomes, suspended in 0.1 *M* potassium phosphate buffer (pH 7.5), were treated with trypsin at 2°C for 12 hours to obtain a trypsin-solubilized-extract (7) from which cytochrome P_{450} (8) and aminopyrine demethylation activity (9) could be excluded. NADPH-cytochrome *c* reductase and other components in trypsin-solubilized extract were separated by Sephadex G-100 gel filtration (7). The reductase was further purified by DEAE cellulose column chromatography (7). The enzyme at this stage was found to have a specific activity between 16 and 20, under the specified conditions (7).

Thyroxine deiodination activity and malondialdehyde formation were measured as previously described (1, 2). NADPH-cytochrome *c* reductase activity was defined by the method of Omura and Takesue (7). Protein was determined by the phenol–biuret method (10) using bovine serum albumin as the standard. NADPH content of the reaction mixture was determined from the optical density of its alcohol extract at 340 nm (11). The molecular weight of a factor was determined by gel filtration on a column (2.5 × 50 cm) of Sephadex G-25, calibrated according to the method of Andrews (12) with bacitracin, glucagon and cytochrome b_5. Paper chromatography and its radioautography of radioactive compounds were carried out as described previously (2).

Standard reaction mixture (a reconstructed system) consisted of 0.12 m*M* $Fe(NO_3)_3$, 2 m*M* ADP, 0.16 m*M* NADPH, Fraction D (see below), lipoprotein, cytochrome *c* reductase, 0.125 *M* Tris–HCl buffer (pH 6.8) and 2.5 μM L-thyroxine containing a trace amount of ^{131}I-labeled L-thyroxine (3′ and 5′) or none, in a final volume of 5 ml. Unless otherwise noted, incubation was carried out at 37° and the reaction was initiated by the addition of NADPH.

Results. Presence of a factor for lipid peroxidation and thyroxine degradation in microsomes. Using the trypsin-solubilized extract obtained from hepatic microsomes,

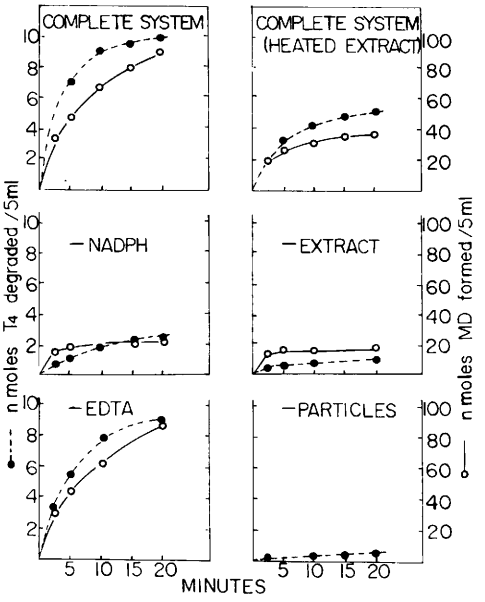


FIG. 1. Thyroxine (T_4) deiodination and malondialdehyde (MD) formation in a system composed of trypsin-solubilized extract and microsomal particles. The complete system consisted of 2 m*M* ADP, 0.12 *mM* $Fe(NO_3)_3$, 5 μM EDTA, 0.16 *mM* NADPH, 0.125 *M* Tris–HCl buffer (pH 6.8), 0.25 *M* NaCl, 0.2 mg of microsomal particles, trypsin-solubilized extract (containing 0.23 units of NADPH-cytochrome *c* reductase) in a final volume of 5 ml. Thyroxine was added to the above mixture at 2.5 μM, and omitted in the assay of malondialdehyde formation. Of these components, ADP and $Fe(NO_3)_3$ were dissolved in 0.25 *M* Tris–HCl buffer (pH 6.8), allowed to stand at room temperature over 2 hrs to ensure chelation, and added to the reaction mixture. The incubation was carried out at 37° for 20 min. Nonspecific malondialdehyde formation and nonspecific thyroxine degradation were determined without any components except for particles in 0.125 *M* Tris–HCl buffer (pH 6.8) and their values were subtracted from corresponding values measured in the experimental system.

the requirement of a factor in the NADPH-dependent peroxidation of microsomal lipid with thyroxine degradation was studied in systems in which the Fe^{3+}-ADP complex, NADPH and lipoprotein were present with or without EDTA (Fig. 1). In contrast to reported findings with purified cytochrome *c* reductase and liposomes (13), lipid peroxidation of lipoprotein by trypsin-solubilized extract (containing more than 90% of the microsomal cytochrome *c* reductase) occurred both with and without added EDTA. Heating the extract at 65° for 2 min depressed both the malondialdehyde formation and thyroxine degradation even in the presence of EDTA, indicating thereby that the reactions are heat instable.

When the extract was replaced with partially purified cytochrome *c* reductase (obtained by means of Sephadex G-100 column chromatography), both thyroxine degradation and lipid peroxidation in the NADPH-Fe^{3+}-ADP-lipoprotein system required EDTA for activity.

These findings suggest that trypsin-solubilized extracts contain a factor which performs a function (or functions) similar to that of EDTA.

Isolation and properties of a factor. A typical elution pattern of trypsin-solubilized extract on Sephadex G-100 is shown in Fig. 2. Four main components, NADPH-cytochrome *c* reductase, cytochrome b_5 and two other protein fractions were identified by enzymatic analysis, spectrophotometry and the phenol–biuret method. When cytochrome b_5, Fraction A or D was added individually to the system containing NADPH, Fe^{3+}-ADP complex, lipoprotein and purified cytochrome *c* reductase, only Fraction D was effective in promoting malondialdehyde formation. Exposure of Fraction D to 100° for 2 min did not alter its EDTA-like activity. Fraction D can be further purified by gel filtration on a column (2.5 x 50 cm) of Sephadex G-25 equilibrated with 10 m*M* phosphate buffer (pH 7.5), using the same solution as an elution buffer. At the end of the procedure, the specific activity (see below) increased two to three times over that of Fraction D. Elution of Fraction D with distilled water, instead of buffer, resulted in a greater loss of the activity. Attempts to purify the factor by Dowex 1 (acetate) column chromatography and electrofocusing method were unsuccessful. Analysis of HCl-digested factor by amino acid analyzer (Hitachi Model KLA-3B) and Integrator (JEOL model-DK) identified 17 amino acids among which lysine and glutamic acid were particularly abundant. The molecular weight of the factor calculated from the mole % of each amino acid in the HCl-digested fraction, assuming one mole of tyrosine in a

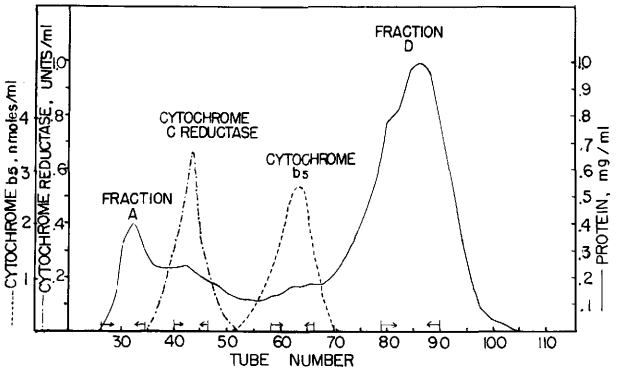


FIG. 2. Typical elution pattern (gel filtration) of a trypsin-solubilized extract. The trypsin-solubilized extract (110 mg protein/20 ml) was applied to a Sephadex G-100 column (2.5 × 65 cm) and eluted with 10 *mM* phosphate buffer (pH 7.5). Four ml fractions were collected. The arrows identify fractions which were pooled.

single peptide molecule, gave values of 9,380 to 10,225 which correspond to four times the molecular weight (2800) determined by Sephadex G-25 gel filtration. This finding and data obtained by electrofocusing and Dowex 1 (acetate) column chromatography of the factor suggest that the factor (Fraction D) is a mixture of peptides rather than a single peptide.

Reduction of Fe^{3+} and consumption of NADPH. Rates of Fe^{3+} reduction and NADPH consumption by NADPH-cytochrome *c* reductase system (see below) were measured by color development produced by Fe^{2+}-*o*-phenanthroline chelation (14) and the optical density at 340 nm (11), respectively. The addition of Fraction D to the system inhibited the apparent reduction of Fe^{3+}, whereas the addition of bovine albumin (0.5–1.5 mg/3 ml) instead of Fraction D had no effect. However, NADPH consumption was greatly enhanced by the addition of Fraction D. Such discrepancy may be attributable to the competition between the factor and *o*-phenanthroline for chelating Fe^{2+}, identical to that manifested by EDTA (14). For convenience, one unit of the activity can be defined as the amount of the factor which produces 50% inhibition of initial rate of Fe^{3+} reduction in the NADPH-cytochrome *c* reductase system (3 ml) in which 0.25 unit of cytochrome *c* reductase/ml, 0.1 m*M* Fe^{3+}, 1.67 m*M* ADP, 1.2 m*M* *o*-phenanthroline, 0.125 *M* Tris–HCl buffer (pH 6.8), 0.16 m*M* NADPH and Fraction D, are present; specific activity is defined as units/mg protein/3 ml of incubation system.

Thyroxine degradation and malondialdehyde formation in the reconstructed system. Thyroxine degradation and malondialdehyde formation in the reconstructed system (consisting of Fraction D, lipoprotein, Fe^{3+}, ADP, NADPH and NADPH-cytochrome *c* reductase) were studied as a function of time (Fig. 3). No detectable initial phase for rapid formation of malondialdehyde (initial rate) was observed in the presence of thyroxine at the concentration tested. On the other hand, malondialdehyde accumulation after 1 min-incubation increased linearly up to 12 min-incubation (slope), both in the presence and absence of thyroxine, but the slope decreased with increasing amounts of thyroxine. The inhibition of lipid peroxidation, calculated from the slope, reached its maximum (80%) when the concentration of the hormone was 10 μM (Fig. 3, inset), whereas (80%) inhibition of the initial rate of lipid peroxidation was achieved by 2.5 μM thyroxine. No inhibition of cytochrome *c* reductase activity in the assay system (7) was observed in the presence of thyroxine at a concentration of 10 μM. These results suggest that thyroxine inhibits both the initial and subsequent reaction for lipid peroxidation.

Wynn has reported a 73% inhibition of initial rate of lecithin peroxidation induced with Fe^{2+} alone at 1×10^{-7} *M* thyroxine (15). The relatively high concentrations of thyroxine required to inhibit lipid peroxida-

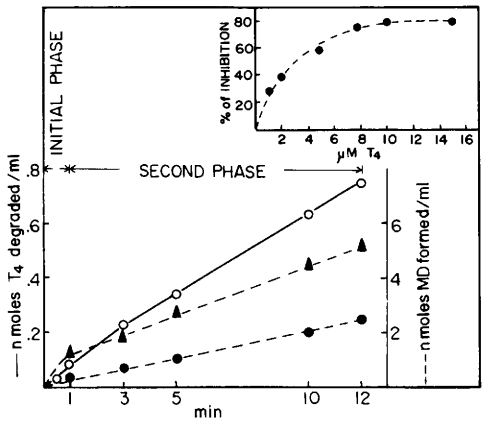


FIG. 3. Time course studied of thyroxine degradation and malondialdehyde formation in the reconstructed system, with note of inhibition of malondialdehyde accumulation with thyroxine.

The reaction mixture consisted of 0.65 mg of microsomal particles, 0.2 units of purified NADPH-cytochrome *c* reductase, 1.2 mg of Fraction D (specific activity, 2.5) and 2.5 μM thyroxine (or none) in a final volume of 5 ml. The incubation media was prepared as described in the legend for Fig. 1. Nonenzymatic production of malondialdehyde and nonenzymatic thyroxine degradation were measured without NADPH and NADPH-cytochrome *c* reductase and the values were subtracted from their corresponding values measured in the experimental system. Enzymatic deiodination was usually linear during 3 min of incubation. Inhibition (inset) was expressed as the depression of slope for malondialdehyde accumulation (calculated by dividing the amount of malondialdehyde formed between 1 min and 3 min by the time interval) in the presence of thyroxine. (○——○); Thyroxine deiodination, (●-----●); Malondialdehyde formation in the system with thyroxine, (▲------▲); Malondialdehyde formation in the system without thyroxine.

tion in our system may be attributable to the fact that our system contains microsomal protein which conjugates thyroxine to a large extent (1), thereby suppressing the lipid-thyroxine interaction essential for thyroxine degradation and for protection of lipid peroxidation.

With the reconstructed system containing 2.4 units of Fraction D and 0.3 unit of NADPH-cytochrome *c* reductase (but Fe^{3+} in various concentrations), both intial rates of thyroxine degradation and of malondialdehyde formation (in the absence of thyroxine) were found to be partially dependent on added Fe^{3+}. Both activities reached their maximum at 180 μM Fe^{3+} and then decreased at concentrations above 240 μM Fe^{3+}, whereas there was little if any activities when iron was not added.

The main radioactive products from ^{131}I-labeled thyroxine (3′ and 5′) and from ^{131}I-labeled thyroxine (3 and 5) in the reconstructed system were found to be inorganic iodide and diiodotyrosine, respectively, i.e. identical to those observed in the Fe^{2+} induced microsomal lipoprotein system (2).

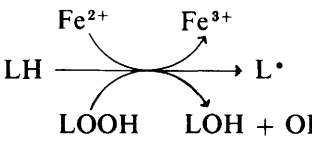
Discussion. The NADPH oxidase which appears to be involved in both microsomal lipid peroxidation and drug metabolism is NADPH-cytochrome *c* reductase (16, 17). A purified preparation of rat liver microsomal NADPH-cytochrome *c* reductase has been shown to catalyze the NADPH-dependent peroxidation of isolated microsomal lipid (13) or microsomal lipoprotein (14) in the presence of the Fe^{3+}-ADP complex and a critical concentration of EDTA. Even though iron is normally present in tissue in both the free and protein bound form, most of this metal is in trivalent form (18). The latter could be reduced to Fe^{2+} either by an enzymatic reaction (NADPH-NADPH-cytochrome *c* reductase system) or by a nonenzymatic process (reduction with ascorbate) as in the microsomal lipid peroxidation associated with thyroxine degradation. The role of the factor is probably that of a chelating agent for Fe^{3+}, as is the case with EDTA (14), which elevates the redox potential of Fe^{3+} and of its iron complex and thereby facilitates the transfer of one electron from NADPH to the trivalent iron. Even though precise formulation of the mechanism of iron-catalyzed oxidation is not yet possible, it is likely that Fe^{2+} or an Fe^{2+}-peptide complex plays an important role in the initial breakdown of the minimum concentration of lipid hydroperoxide present in the phospholipids of lipoprotein to form the alkoxy radicals essential for the chain reaction.

Even though the factor released by trypsin treatment from microsomes may have a possible chelating effect, the peptides in Fraction D could be an artifact of the true factor present in fresh microsomes or even some part of the natural factor segmented

after proteolysis. Therefore, it is possible that structural alteration of the microsomal electron transfer system produced by trypsinization may increase the requirement of Fe^{3+} concentration in this reconstructed system beyond that required by the microsomal system (4).

Hrycay and O'Brien (19, 20) have demonstrated that the NADPH-NADPH cytochrome *c* reductase–cytochrome P_{450} system (well known drug hydroxylation system) also catalyzes the cleavage of various organic hydroperoxides including linoleic acid hydroperoxide and emphasized that cytochrome P_{450} is an important microsomal site for the production of free radicals (possible initiator for lipid peroxidation) from endogenous lipid peroxides. However, this may not well be pertinent to our studies because cytochrome P_{450} is absent from our system.

From the experimental results presented here, it is difficult to explain in detail the relationship between lipid peroxidation and thyroxine degradation. The combination of electron (or hydrogen) donating ability of thyroxine and possible formation of lipid peroxy radicals in the process of lipid peroxidation (21) make it possible to propose the following scheme:



initiation

$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$
$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$
$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet}$

propagation

$2\,LO^{\bullet} + T_4 \rightarrow 2\,LOH + 2\,I^- + DIT + etc$
$2\,LOO^{\bullet} + T_4 \rightarrow 2\,LOOH + 2\,I^- + DIT + etc$

T_4 degradation

$LOO^{\bullet} + LOO^{\bullet} \rightarrow LOH + {}^1O_2 + aldehyde$ ..... termination (22)

where LH, $L^{\bullet}$, $LO^{\bullet}$, $LOO^{\bullet}$, LOOH, LOH and $^1O_2$ are lipid, lipid radical, lipid alkoxy radical, lipid peroxy radical, lipid hydroperoxide, alcohol and singlet oxygen, respectively. Thus thyroxine (T_4) could react with lipid peroxy radicals and/or alkoxy radical, thereby suppressing the initiation and propagative process of lipid peroxidation, identical with that manifested by antioxidants (23), while the hormone is oxidized by these radicals with formation of inorganic iodide (I^-), diiodotyrosine (DIT) and other compounds. A possible alternative means of thyroxine degradation is provided by excited oxygen ($^1O_2$) generated in lipid peroxidation (22), but the sensitivity of thyroxine for $^1O_2$ may be much more lower than that of the olefins (well known $^1O_2$ trappers (24)) in phospholipid; the interaction of $^1O_2$ and thyroxine in the present system will be of less significance.

Summary. A lipoprotein present in trypsin-treated microsomes can be oxidized with formation of malondialdehyde in a system which contains NADPH, ferric ion-ADP complex, NADPH-cytochrome *c* reductase and a factor. This factor, a mixture of peptides, can be isolated from hepatic microsomes by trypsin digestion and successive gel filtration through Sephadex G-100 and G-25 columns.

Lipid peroxidation in this system catalyzes the deiodination of thyroxine, as does NADPH-dependent lipid peroxidation in fresh hepatic microsomes. Thyroxine inhibits lipid peroxidation as it is deiodinated in this system.

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