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E. BRZEZIŃSKA-ŚLEBODZIŃSKA, B. PIETRAS

THE PROTECTIVE ROLE OF SOME ANTIOXIDANTS AND SCAVENGERS ON THE FREE RADICALS-INDUCED INHIBITION OF THE LIVER IODOTHYRONINE 5'-MONODEIODINASE ACTIVITY AND THIOLS CONTENT

Institute of Animal Reproduction and Food Research Polish Academy of Sciences, Department of Developmental and Experimental Endocrinology, Poznań, Poland

The influence of free radicals on iodothyronine 5'-monodeiodinase activity, the enzyme responsible for the deiodination of thyroxine to most active thyroid hormone 3,3',5-triiodothyronine (T_3), was examined in rabbit's liver. Incubation of the liver homogenate with the xanthine oxidase based free radical generating system (FRGS) caused a reduction in 5'-monodeiodinase activity to the 53.9% of initial value taken as 100%, and on increase (52.9% over the control value) in the level of lipid peroxidation by-product malondialdehyde. The inhibitory effect of FRGS on 5'-monodeiodinase activity was blocked by free radical scavengers: catalase (91.2%), thiourea (88.8%), superoxide dismutase (85%) and by some antioxidants; Trolox (the water soluble α-tocopherol analog, 81.4%) and glutathione (77.7%). These results suggest that oxygen radicals, hydrogen peroxide and hydroxyl radicals were involved in the inhibition of the 5'-monodeiodinase activity. The same scavengers significantly decreased the malondialdehyde formation. In the presence of the FRGS the amount of total SH groups (the cofactor of the deiodination reaction) was decreased in the liver homogenate to 51% of the initial value, and a positive relationship between the total SH groups levels and the 5'-monodeiodinase activity in the presence of free radical scavengers was observed. It suggests, that active oxygen radicals generated by FRGS may inactivate 5'-monodeiodinase, at least in part, by reduction of thiol cofactors.

Key words: *5'-monodeiodinase, triiodothyronine, free radicals, scavengers.*

INTRODUCTION

Toxic and highly reactive oxygen metabolites (free radicals, FR) generated during normal metabolism, when enter into uncontrolled reactions cause metabolic disorders and impair health. Free radicals may cause membranous lipid peroxidation (1), cellular enzyme inhibition (2, 3) and the nucleic acid damage (4, 5).

The work of Huang *et al.* (6) suggested that free radicals may influence 5'-monodeiodinase activity (5'-MD), the enzyme responsible for conversion of

prohormone thyroxine (T_4) to 3, 3'5-triiodothyronine (T_3), and indirect reduce serum T_3 level. The most active thyroid hormone — T_3 is involved in the modulation of protein, lipid and carbohydrate metabolism and play important role in every stage of mammalian growth and development. The reducing of extrathyroidal 5'-monodeiodination of T_4 to T_3 may change the thyroid hormone metabolism from activating (T_3) to inactivating (deiodination of T_4 in 5-position) pathway, so frequent observed in nonthyroidal illnesses (7).

In this study we partly reexamined the observations of Huang *et al.* (6) and by using some antioxidants and free radicals scavengers we have tried to answer the question what types of oxidative damage are involved in this process. As the most important mechanism of tissue damage by free radicals is lipid peroxidation, we have measured the by-product of lipid peroxidation — malondialdehyde (MDA) in the presence or absence of a xanthine oxidase based free radical-generating system (FRGS) and scavengers, and the influence of the FRGS on total SH groups content (the cofactor of deiodinating reaction).

Because the hepatic 5'-MD was found to be a selenoenzyme (8, 9) we also examined the effect of sodium selenite on the liver 5'-MD activity.

MATERIALS AND METHODS

Materials and reagents

Liver tissue was obtained from adult New Zealand rabbits and kept at −20°C until analysis.

Xanthine, xanthine oxidase, sodium ascorbate, ADP, ferric chloride ($FeCl_3$), catalase, superoxide dismutase (SOD), thiourea, tert — butanol, GSH, sodium selenite, dithiothreitol (DTT), butylated hydroxytoluene (BHT), 1,1,3,3,-tatraethoxypropane (MDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6-hydroxy-2,5,7,8-tetramethylchromam-2-carboxylic acid (Trolox) was obtained from Aldrich (Milwaukee, WI, USA), vitamin E (α-tocopherol acetate) from Merck (Darmstadt, Germany) and tri-chloroacetic acid (TBA) was purchased from Loba Feinchemie (Fischamend, Germany). All other chemicals were of analytical grade.

Tissue preparation

The liver was homogenized in 4 vol (w/v) of ice — cold 0.2 Tris-HCl buffer containing 0.25 M sucrose and 5 mM EDTA (pH 9.0) for 5'-MD determination and 0.2 M Tris-HCl buffer containing 0.25 M sucrose (pH 7.4) for thiobarbituric acid reactive substances (TBARS) determination and centrifuged at 10 000 × g for 30 min. For total SH — groups assay, the liver was homogenized in 4 vol (w/v) of ice — cold 0.02 M EDTA and centrifuged at 10 000 × g for 30 min. The supernatant was used for incubation. Protein concentration in liver homogenate supernatant was estimated by the method of Lowry *et al.* (10) using bovine serum albumin as standard.

Incubation

The liver homogenate supernatant was preincubated for 30 min at 37°C without (control) or with a FRGS (1) comprised of xanthine (0.1 mM), xanthine oxidase (0.01 U/ml), $FeCl_3$ (0.1 mM), sodium ascorbate (2.2 mM) and ADP (0.8 mM). When the effect of FR scavengers was studied, the

homogenate was incubated for 30 min at 37°C with a scavenger, in the presence or absence of FRGS. The following FR scavenger and antioxidants were separately used: catalase (300 U/ml), SOD (600 U/ml), vitamin E (α-tocopherol acetate, 10 mg/ml), Trolox (5 μM), thiourea (0.15 M), tert — butanol (0.15 M), GSH (50 μM), sodium selenite (5 μM).

5'-Monodeiodinase determination

The 3,3',5'-triiodothyronine (reverse T_3, rT_3), the biologically less active product in T_4 metabolism, is widely used as a substrate for 5'-MD determination. The measurements of 5'-MD activity was based on release of $^{125}I^-$ from (^{125}I) rT_3 according to method of Leonard&Rosenberg (11) in modification of Jack *et al.* (12). The (^{125}I) rT_3 labelled in the outer ring only, specific activity 976—1260 μCi/μg, purchased from NEN (Du Pont — NEN, Belgium), was purified by Sephadex LH — 20 chromatography immediately before assay (13). The (^{125}I) rT_3 fraction was dried under nitrogen and dissolve in 0.1 M potassium phosphate buffer (pH 7.0). The assay mixture contained in total volume of 120 μl: 0.1 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, 10 mM DTT, liver homogenate supernatant and between 50 000—70 000 cpm (^{125}I) rT_3 mixed with unlabelled rT_3 to a final concentration of about 400 nmol/l. When the effect of FRGS and scavengers on the 5'-MD activity were studied, the homogenate (1.25 mg protein) was incubated at 37°C for 30 min with or without FRGS or scavengers, and after dilution by 0.1 M potassium phosphate buffer (pH 7.0), an aliquot of 5 μg protein was taken for 5'-MD assay. The assay mixture was incubated at 37°C for 2 min (blank value) and 12 min (sample). Reactions were terminated by addition of 0.5 ml of ice — cold horse serum followed by 0.5 ml of 10% (w/v) TCA. After centrifugation for 20 min at 2000 × g, radioactivity of free $^{125}I^-$ in 0.5 ml of supernatant was measured. The radioactivity of the blank tube was subtracted from that in the sample tube and the results expressed in pmol I^--liberated/mg protein × min.

Assay of TBARS

The amout of TBARS formed in the liver homogenate supernatant was determined by the method of Ledwożyw *et al.* (14). In short 0.25 ml of homogenate was mixed with 1.25 ml of 1.22 M TCA in 0.6 M HCl and allowed to stand for 15 min. To this mixture 0.75 ml of thiobarbituric acid soludion was added (obtained by dissolving 500 mg of thiobarbituric acid in 6 ml 1 M NaOH and then adding 69 ml H_2O) and thereafter heating for 30 min in a heating block. After cooling to room temperature 2 ml of n-butanol was added and the mixture was shaken vigorously for 3 min and centrifuged 20 min at 2000 × g. The organic layer was removed and its absorbance was measured at 532 nm. The results were expressed as nmoles of MDA/1 ml of homogenate using MDA as the standard.

Determination of total sulphydryl groups

The total sulphydryl groups were measured with Ellman's reagent according to the method of Sedlák (15).

Statistical analysis

Duncan's new multiple range test was applied. Difference between means considered significant when $P < 0.05$. All data are presented as means ± SEM.

RESULTS

FRGS caused a reduction in 5'-MD activity to 53.9±2.3% of the initial value (41.9±0.4 pmol I^-/mg protein/min; n = 63) of liver homogenate. The *Fig. 1* shows the effects of various free radicals scavengers and antioxidants on the FRGS-induced inhibition of hepatic 5'-MD. Most of the FR scavengers significantly diminished the FRGS-induced inhibition of the 5'-MD activity. For reducing the inhibitory effect of FR scavengers in the 5'-MD assay, a relatively large amount (1.25 mg) of tissue protein was preincubated with FRGS, in the presence or absence of FR scavengers, and a small fraction (5 μg protein) was taken for 5'-MD determination. When studied in this manner the FR scavengers alone did not inhibit 5'-MD activity. The FRGS-induced inhibition of 5'-MD activity was blocked nearly completely by catalase and thiourea (91.2±3.7% and 88.8±2.2% of initial value, respectively) and to a lesser extend by SOD (85.0±2.2%) and Trolox (81.4±2.8%). No effect of tert-butanol, the hydroxyl ion scavenger, was observed.

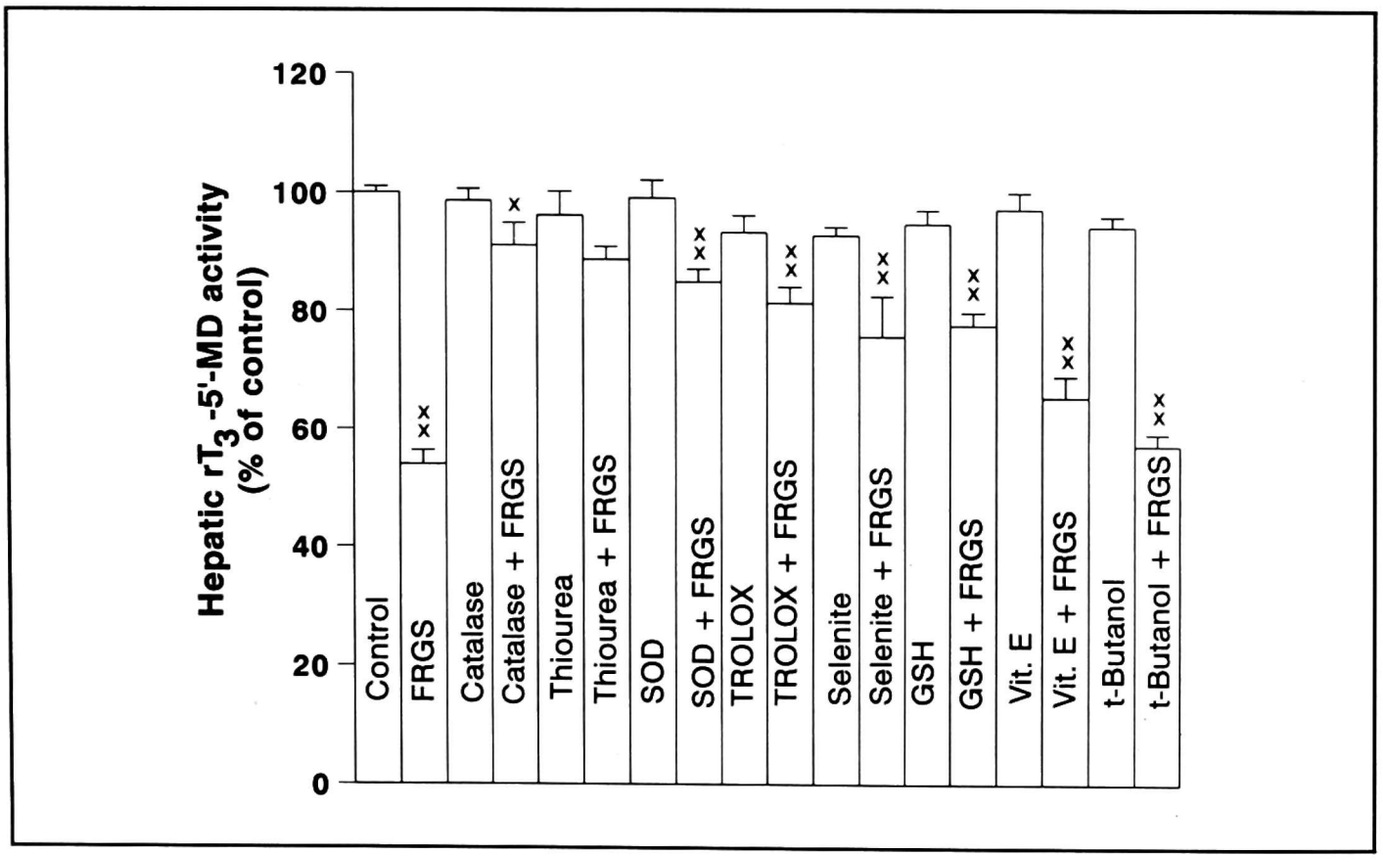

Fig. 1. Effects of free radical scavengers on FRGS — induced inhibition of hepatic 5'-MD. Control — the liver homogenate preincubated without FRGS. Means±SEM, n = 6—14 in each group. * $P < 0.05$, ** $P < 0.01$ vs control

Malondialdehyde (MDA), which is a by-product of lipid peroxidation, was measured in the liver homogenate without and in the presence of the FRGS and FR scavengers. As shown on the *Fig. 2.* the presence of FRGS increased lipid peroxidation followed by a rise in TBARS formation (expressed in nmoles

of MDA per ml of homogenate) by 152.9±2.0% ($P < 0.01$; $n = 17$) of control value (20.1±0.3 nmol MDA/ml of homogenate; $n = 16$). Some FR scavengers, such as thiourea and GSH, incubated with liver homogenate in the absence of FRGS influenced significantly ($P < 0.01$) the TBARS concentration. Thiourea diminished the TBARS level to 60.7±2.4% ($P < 0.01$; $n = 6$) and GSH to 80.6±3.0% ($P < 0.01$; $n = 9$) of control value. With exception of thiourea and GSH, the catalase, the most active scavenger, decreased TBARS production (in the presence of FRGS) to about the 107.3±7.2% of the control level, $P > 0.05$; $n = 6$), less active scavengers like SOD (118.3±8.52%, $P < 0.01$; $n = 6$), tert-butanol (119.7±2.7%, $P < 0.01$; $n = 6$), vitamin E (123.3±2.1%, $P < 0.01$; $n = 6$) and Trolox (128.7±10.0%, $P < 0.01$; $n = 6$) decreased TBARS production, but to the level still different from the control ($P < 0.01$). Sodium selenite restored the 5'-MD activity in the presence of FRGS to 75.7±65.9% of the initial value (*Fig. 1*), with no effect on the TBARS level (*Fig. 2*).

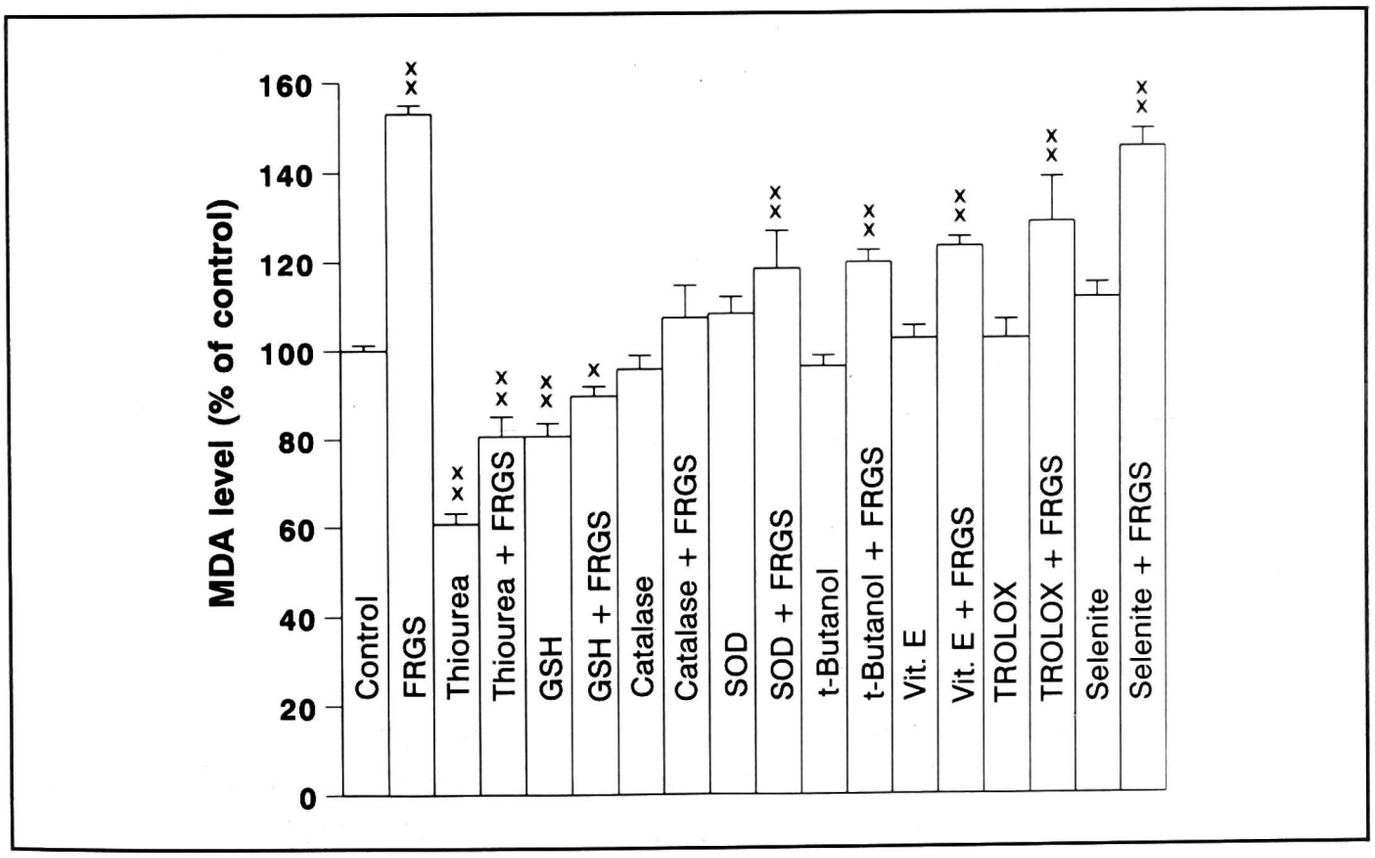

Fig. 2. Effects of free radical scavengers on FRGS-induced increase of lipid peroxidation by-product (MDA) measured by TBARS-test. Control — liver homogenate preincubated without FRGS. Mean±SEM, $n = 6$—18 in each group. * $P < 0.05$, ** $P < 0.01$ vs control.

In the presence of FRGS, the amount of total SH-group decreased in the liver homogenate to 55.0±1.3% ($P < 0.01$; $n = 11$) of control value (37.7±0.4 mg SH per 100 g of tissue preincubated without FRGS; $n = 17$). *Fig. 3* shows the effects of FR scavengers on FRGS-induced reduction of total SH-group content. The SH-groups were near completely protected by SOD (89.2±1.6%,

$P < 0.01$; $n = 6$) and by catalase ($83.0 \pm 1.6\%$; $P < 0.01$; $n = 10$). All scavengers used in the present study, diminished the FRGS-induced decrease of thiol content in the liver tissue.

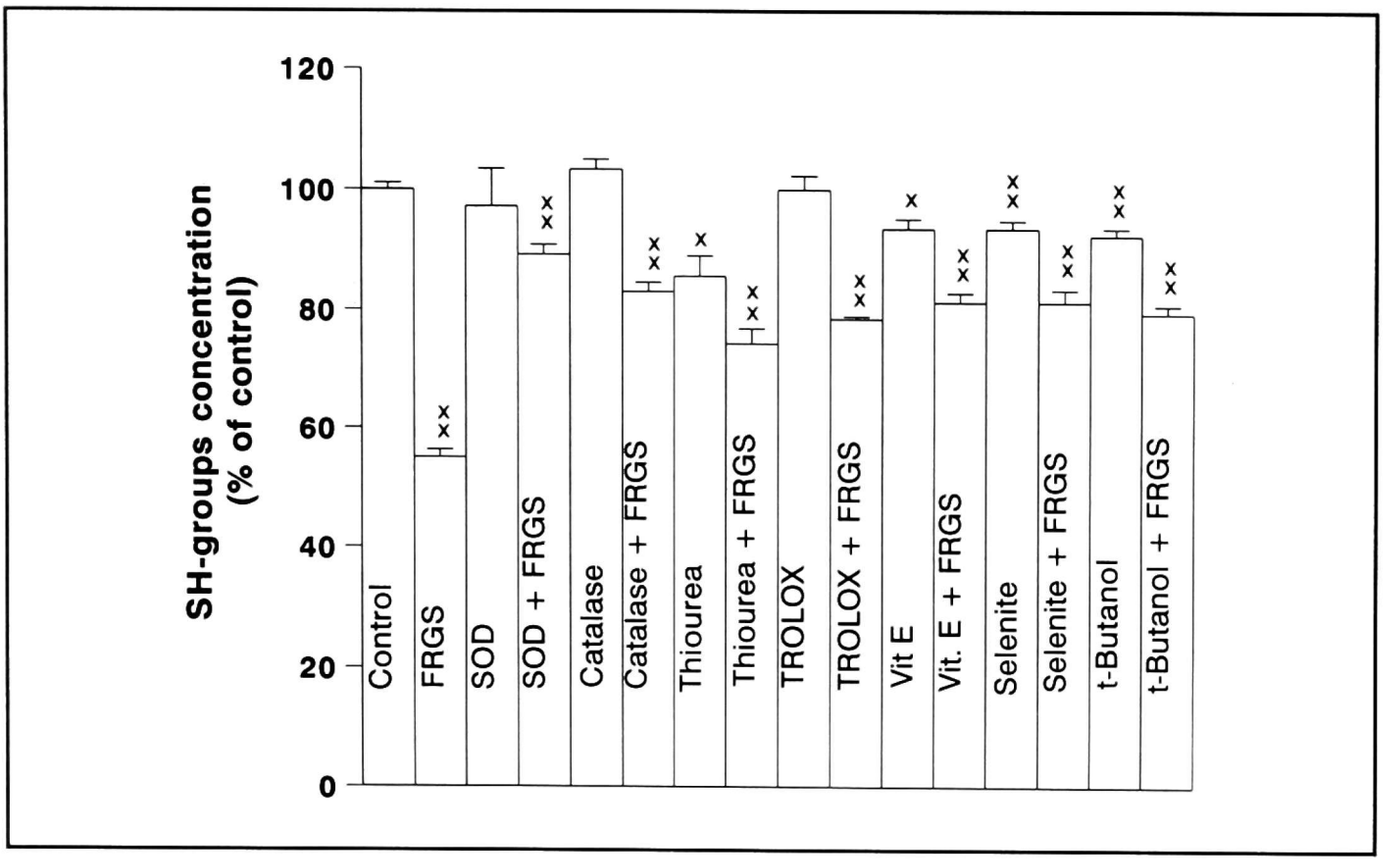

Fig. 3. Effects of free radicals scavengers on FRGS-induced reduction of total SH — group content in the liver homogenate. Control — liver homogenate preincubated without FRGS. Means $\pm$ SEM, $n = 6$—18 in each group. * $P < 0.05$, ** $P < 0.01$ vs control.

DISCUSSION

This study confirmed the previous observation of Huang *et al.* (6) that the generation of free radicals may cause a reduction of extrathyroidal 5'-monodeiodination of T_4 to T_3. The present data indicate that about 54% decrease in the 5'-MD activity, and 53% increase in the TBARS level in the presence of FRGS, suggest that FR influences the liver 5'-MD by inducing the lipid peroxidation processes. The increased lipid peroxidation in the liver microsomes might imply the peroxidative breakdown of endoplasmic reticulum membrane and the alterations in its enzymic, physical and structural changes (16). Our observation that catalase and SOD inhibited the effect of FRGS on 5'-MD by 85—90% indicates that hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$) may contribute to the reduced 5'-MD activity. As thiourea, the scavenger of the highly active hydroxyl radical, prevented of the 5'-MD inactivation by FRGS in about 90%, it indicates that hydroxyl radicals are involved in 5'-MD inhibition. On the other side it is difficult to explain why

tert-butanol, the well known $OH^{\cdot}$ scavenger, had no protective effect on 5'-MD inactivation. In studies on the effect of the free radical scavengers on the humic acid inhibition of the rat liver 5'-MD activity, Huang *et al.* (17) observed the 24% and 42% inhibition of the enzyme activity in the presence of 0.15 M tert-butanol and 0.15 M thiourea, respectively. We did not confirm this observation.

The chain-breaking antioxidants as vitamin E and GSH protected 5'-MD against the FR damage less activity. The better results were obtained in the presence of Trolox, the water-soluble vitamin E analog. Our earlier results showed that Trolox, reacting with peroxyl radicals (18), depressed the iron-ascorbate-dependent peroxidation of lipids in semen plasma (19).

Almost all the antioxidants tested in the present study inhibited the FRGS-induced lipid peroxidation in the liver homogenate as shown by TBARS determinations. Cytotoxic aldehydes (e. g. MDA in this paper), was used as an indicator of free radicals induced lipid peroxidation (20), provide the basis for the thiobarbituric acid test for measuring lipid peroxidation end product in body fluids. It cannot be excluded that a marked increase in TBARS level, after preincubation of liver homogenate with FRGS, and the parallel decrease in 5'-MD activity, may be caused by an unknown process (apart from lipid peroxidation) directly influencing the 5'-MD. As it was shown in a pilot experiment, no effect of MDA (the product of lipid peroxidation), added to the incubation mixture, on the liver 5'-MD activity was observed.

It cannot be excluded that FR may ifluence 5'-MD not only by inducing lipid peroxidation but also by oxidative damage to protein component of the enzyme (21) or by reduction of thiol cofactors necessary for the enzymatic iodothyronines deiodination. There is a strong relationship between the sulfhydryl status of cells, mostly the GSSG to GSH ratio, and the 5'-MD activity (22). We found the positive relationship between total SH group levels and 5'-MD activity in the liver homogenate preincubated with FRGS in the presence of FR scavengers ($r = 0.69$; $P < 0.05$; $n = 9$). It is possible that active oxygen radicals, generated by FRGS, inactivate 5'-MD at least in part by oxidazing the SH group to disulfide form which may be further converted to sulfenic, sulfinic of sulfonic acids.

The role of Zn in the antioxidant defense system in tissues is well known. The first line of free radical defense system (enzymatic) is CuZnSOD, responsible for the superoxide anion radical neutralization; the second is the protection of sulfhydryl groups against oxidation (23). In fact, the inhibitory effect of FRGS on 5'-MD activity and on SH group contents was diminished in 85.0 ± 2.2 and $80.2 \pm 1.6\%$ respectively, by SOD. This confims the eassential role of SH groups content in 5'-MD activity and suggests that the free radicals influence the enzyme activity (in part of course) by decreasing the thiols content.

Sodium selenite restored the 5'-MD activity in 75%. As it was established, the 5'-MD is a selenoprotein with a single selenium molecule as selenocysteine at its active site (9). Until recently, the role of selenium in metabolism has been concentrated on the function of glutathione peroxidases (GSH-px) and cell antioxidant systems involved in the prevention of free radical mediated damage in cells (24). The discovery of the role for selenium as an essential component of 5'-MD (8, 9) has revealed the importance of this element in thyroid hormone metabolism (25), suggesting that selenium deficiency can decrease T_4 to T_3 convertion in tissues by inhibiting the 5'-MD activity. Behne *et al.* (26) proposed protein-specific hierarchy whereby proteins other than GSH-px have priority for selenium over GSH-px and found that under conditions of selenium depletion, 5'-MD is preferentially supplied with selenium compared to the cellular GSH-px. The cellular form of GSH-px might even represent an intracellular storage form of selenium under some conditions (27).

REFERENCES

1. Kellogg EW III, Fridovich I. Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. *J Biol Chem* 1975; 250: 8812—8817.
2. Hexum TD, Fried R. Effects of superoxide radicals on transport (Na+K) adenosine triphosphatase and protection by superoxide dismutase. *Neurochem Res* 1979; 4: 73—78.
3. Chan PH, Yurko M, Fishman RA. Phospholipid degradation and cellular edema induced by free radicals in brain cortical slices. *J Neurochem* 1982; 38: 525—530.
4. Pasquali-Ronchetti I, Bini A, Batti B *et al.* Ultrastructural and biochemical changes induced by progressive lipid peroxidation on isolated microsomes in rat liver endoplasmic reticulum. *Lab Invest* 1980; 42: 457—468.
5. Summerfield FW, Tappel AL. Determination of malonaldehyde-DNA crosslinks by fluorescence and incorporation of tritium. *Biochem Biophys Res Commun* 1981; 111: 77—82.
6. Huang T-S, Boado RJ, Chopra IJ, Solomon DH, Chua Teco GN. The effect of free radicals on hepatic 5'-monodeiodination of thyroxine and 3,3',5'-triiodothyronine. *Endocrinology* 1987; 121: 498—503.
7. Chopra IJ, Huang TS, Beredo A *et al.* Evidence for an inhibitor of extrathyroidal conversion of thyroxine to 3,5,3'-triiodothyronine in sera of patients with nonthyroidal illnesses. *J Clin Endocrinol Metab* 1985; 60: 666—672.
8. Behne D, Kyriakopoulos A, Meinhold H, Kohrle J. Identification of type I iodothyronine 5'-deiodinase as a selenoenzyme. *Bioch Biophys Res Commun* 1990; 173: 1143—1149.
9. Berry M, Banu L, Larsen P. Type I iodothyronine deiodinase is a selenocysteine-containing enzyme. *Nature* 1991; 349: 438—440.
10. Hartree EF. Determination of protein: A midification of the Lowry method that gives a linear photometric response. *Anal Bioch* 1972; 48: 422—427.
11. Leonard JL, Rosenberg IN. Iodothyronine deiodinase from rat kidney: substrate specificity and the 5'-deiodination of reverse triiodothyronine. *Endocrinology* 1980; 107: 1376—1383.
12. Jack LJW, Kahl S, Germain DLSt, Capuco AV. Tissue distribution and regulation of 5'-deiodinase processes in lactating rats. *J Endocrinol* 1994; 142: 205—215.
13. Nakamura Y, Chopra IJ, Solomon DH. Preparation of high-specific-activity radioactive iodothyronine and their analogues. *J Nuclear Med* 1977; 18: 1112—1115.

14. Ledwożyw A, Michalak J, Stępień A, Kądziołka A. The relationship between plasma triglycerides, cholesterol, total lipids and lipid peroxidation products during human atherosclerosis. *Clin Chim Acta* 1986; 155: 175—184.
15. Sedlák J. Determination and content of sulfhydryl groups in the thyroid and adrenals of rats. *Endocrinol Exp* 1970; 4: 3—11.
16. Slater TF. Free radical mechanisms in tissue injury. *Biochem J* 1984; 222: 1—15.
17. Huang T-S, Lu FJ, Chopra IJ. Inhibition of hepatic thyroxine 5'-monodeiodinase by humic acids. *Environ Toxicol Chem* 1993; 12: 1267—1271.
18. Glazer AN. Phycoerythrin fluorescence — based assay for reactive oxygen species. In *Methods in Enzymology*, L Parker, AN Glazer (eds) Academic Press, San Diego, CA 1990, V. 186, pp. 161—168.
19. Brzezińska-Ślebodzińska E, Ślebodziński AB, Pietras B, Wieczorek G. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. *Biol Trace El Res* 1995; 47: 69—74.
20. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: An overview. In *Methods in Enzymology*, L Parker, AN Glazer (eds) Academic Press, San Diego, CA 1990, V. 186, pp. 1—20.
21. Freeman BA, Crapo JD. Biology of disease: free radicals and tissue injury. *Lab Invest* 1982; 47: 412—426.
22. Sato K, Mimura H, Wakai K *et al.* Modulating effect of glutathione disulfide on thyroxine-5'-deiodination by rat hepatocytes in primary culture: effect of glucose. *Endocrinology* 1983; 113: 878—886.
23. Bray TM. Bettger WJ. The physiological role of zinc as an antioxidant. *Free Radical Biol Med* 1990; 8: 281—291.
24. Zachara BA. Mammalian selenoproteins. *J. Trace Elem Electrolytes Health Dis* 1992; 6: 137—151.
25. Arthur JR. Selenium deficiency, thyroid hormone metabolism and thyroid hormone deiodinases. *Am J Clin Nutr* 1993; 57: 236S-239S.
26. Behne D, Hilmert H, Scheid S, Gessner H, Elger W. Evidence for specific selenium target tissues and new biologically important selenoproteins. *Biochim Biophys Acta* 1988; 966: 12—21.
27. Evenson JK, Thompson KH. Weiss SL, Sunde RA. Effect of selenium status on 75Se-metabolism and incorporation into selenoproteins. *Selenium in Biology and Medicine.* Abstract Book: No 64 1992.

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Author's address: E. Brzezińska-Ślebodzińska, Institute of Animal Reproduction and Food Research Polish Academy of Sciences, Department of Developmental and Experimental Endocrinology, 250 Grunwaldzka Str., 60-166 Poznań, Poland.