Research article

Thyroid hormone effect in human hepatocytes

Eliana A. Miler, María del Carmen Ríos de Molina, Gabriela Domínguez, Liliana N. Guerra

Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

We have already demonstrated that a combined treatment of methimazole and an antioxidant mixture improved the condition of hyperthyroid patients both biochemically and clinically. Elevated thyroid hormone levels might trigger signs and symptoms of hyperthyroidism through the increase of free radicals. To study the direct effect of thyroid hormone on cellular markers of oxidative stress, we carried out *in vitro* assays in which 0.1–20.0 nM T3 (6.5–1300.0 ng/dl) doses were added to culture media of the human hepatocyte cell line Hep G2 for 1–24 h. T3 increased malondialdehyde (MDA) and intracellular oxidized glutathione (GSSG) levels; SOD activity was also higher with hormone treatment, whereas catalase and glutathione peroxidase activities showed no variation at different T3 doses and during all experimental times. When ascorbic acid was added to the culture, the MDA level decreased and SOD activity was increased. With higher doses of T3 (*e.g.* 200 nM), cell death occurred (69% of apoptotic cells). The increase in SOD activity was not enough to overcome the effect of T3 since MDA and GSSG remained high during a 24-h experiment. We showed a beneficial effect of ascorbic acid when cells were exposed to a T3 dose of 20 nM, a higher level of hormone than that achieved in hyperthyroidism.

Keywords: thyroxine, human hepatocytes, oxidative stress

Introduction

The liver is an important target organ for the thyroid hormones. These hormones (T3 and T4) affect gene expression through a diverse range of cellular pathways and functions, including lipogenesis, cell proliferation and apoptosis. It has been estimated that about 8% of hepatic genes are regulated by the thyroid hormone (T3) *in vivo*.[1] Hepatic oxidative stress was observed in experimental animals made hyperthyroid by T3 administration.[2,3] Free radical-mediated oxidative stress has been related to the aetiopathogenesis of Graves' disease.[4–8] By the time effective treatment for hyperthyroidism starts, concurrent liver damage (as shown by fatty acid changes, hepatic necrosis and cirrhosis) is usually observed.[9] Oxidative stress results from an imbalance between reactive oxygen species (ROS) and antioxidant activity.[10,11] To prevent the accumulation of these toxic molecules and their deleterious effects, several enzymatic systems scavenge ROS (mainly catalase, superoxide dismutase and glutathione peroxidase). These enzymes are induced as part of a concerted response to protect cells against oxidative damage.[12] Cells also protect themselves with antioxidant systems involving a cascade of functional redox molecules such as vitamin C. Ascorbate is presumed to be an excellent reducing agent, since at high concentrations it is able to work as an antioxidant in free radical-mediated oxidation processes.[13]

In previous reports, we have shown that hyperthyroid patients had higher malondialdehyde (MDA) content and superoxide dismutase (SOD) activity compared to euthyroid controls.[8,14] We have reported

Correspondence to: Liliana N. Guerra, Departamento de Química Biológica Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires Ciudad Universitaria, Pabellón 2 (1428), Buenos Aires, Argentina, Fax: +54 11 4576 3342; E-mail: liliana@qb.fcen.uba.ar
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that a combined treatment with methimazole and an antioxidant mixture (including vitamin C and vitamin E) improved the clinical manifestations of hyperthyroidism.[8] Even though we studied the effect of thyroid hormone in the oxidative metabolism of patients, we could not demonstrate that the hormone exerts a direct effect on oxidative metabolism by modifying some oxidative stress parameters. Once it has been recognized that oxidative stress plays a significant role in the hyperthyroidism, a major question is to distinguish whether it is a causal factor and responsible for promoting tissue injury or a consequence of the primary disease.[15] In order to study the direct effect of thyroid hormone on some oxidative stress markers, we carried out assays using a cell line, Hep G2 (derived from human hepatocytes), treated with T3 as an *in vitro* model.

Materials and methods

Cell culture and drugs

Hep G2 cells were cultured in Modified Eagle Medium (MEM) supplemented with 10% fetal bovine serum. T3 (3,3′,5-triiodo-L-thyronine sodium salt) was purchased from Sigma (St Louis, MO, USA); it was dissolved in 1 N NaOH and then sterile medium was added to give a 100x stock solution (no change in the pH of the medium was observed).

The cells were seeded and maintained for 24 h before different doses (0.1–200 nM) of thyroid hormone (T3) were added to the cultures; then, they were incubated for 1–24 h with the hormone. The level of T3 remained constant during the experiment. Control experiments with vehicle (sterile medium with 0.01 N NaOH) were carried out in parallel. Ascorbic acid (100 mg/l) was added to the culture as an antioxidant with the vehicle (control plates) or with the hormone (treatment plates); ascorbic acid remained for at least 24 h in the culture media (data not shown). Cell were detached from the culture dish with trypsin and then washed twice with 0.01 M phosphate-buffered saline at room temperature. For apoptosis assays, the cells were used immediately; for chemical determinations, the cells were lysed by two cycles of freezing and thawing. After a 5-min 10,000 *g* centrifugation, the supernatant was tested. In each experiment, the results shown are the averages of four different experiments (mean ± SD).

Chemical determinations

The malondialdehyde (MDA) concentration in cell extracts was determined by reaction with thiobarbituric acid and measuring absorbance at 535 nm, using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.[14] The results shown are the averages of four different experiments (mean ± SD).

Total glutathione (GSH plus GSSG) and glutathione disulfide (GSSG) concentrations were determined by a DTNB–GSSG reductase recycling procedure. GSH is oxidized by 5,5′-dithiobis-2-nitrobenzoic acid (DNTB) to give GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). GSSG is reduced to GSH by the action of the highly specific glutathione reductase and NADPH. The rate of TNB formation is followed at 405 nm and is proportional to the sum of GSH and GSSG present (total glutathione);[16] GSSG concentration was assessed with the same protocol with previously added 2-vinylpyridine.[16] The amount of reduced glutathione (GSH) in the same sample was obtained using a standard curve. The results shown are the averages of four different experiments (mean ± SD).

Protein was determined by the Bradford method using crystalline bovine serum albumin as standard.[17]

Enzyme activities were analyzed in cell extracts. Catalase activity was determined using hydrogen peroxide as substrate.[18] Superoxide dismutase (SOD) was determined using a RANDOX kit; the method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD was measured by the ability of cell extracts to inhibit this reaction.[19] Glutathione peroxidase was also determined using a RANDOX kit. The enzyme catalyses the oxidation of glutathione by cumene hydroperoxide; in the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH and decrease in absorbance at 340 nm which is measured.[20] In each activity determination, the results shown are the averages of four different experiments (mean ± SD).

Apoptosis assay

Evaluation of apoptosis was done by examining the differential uptake of two fluorescent DNA-binding dyes, Acridine Orange (AO) and ethidium bromide (EB). Acridine Orange (A 6014) and ethidium bromide (E 8751) were purchased from Sigma. We prepared a mixture of AO and EB (100 mg/ml each) in PBS. Cells were seeded for 24 h and then cultured for a further 24 h in the presence of: (i) vehicle; (ii) 20–400 nM T3; (iii) T3 and ascorbic acid (100 mg/l); or (iv) 50 μM H_2O_2 (apoptosis control). To an aliquot of 10^6 cells in 25 μl of PBD, 1 μl of AO and EB mixture was

added to cell suspension. AO is taken up by all cells and stains the nuclei bright green; EB is taken up only by cells that have lost membrane integrity and stains the nuclei bright orange. These dyes can be used to distinguish between apoptosis and necrosis, because apoptotic cells show condensed or fragmented bright green chromatin and necrotic cells have bright orange chromatin. Cells were assigned to the two categories described above by examination under a fluorescence microscope and counting of at least 200 cells per slide.[21]

Proliferation assay

Cells (10^6) were seeded on a plate and cultured in the presence of vehicle, 20 nM T3 or 200 nM T3. At the indicated times, cells were counted. The results shown are the averages of four different experiments (mean ± SD).

Statistical analysis

All results are expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance.[22]

Results

The normal human T3 level reported in the literature is 131.0 ± 5.4 ng/dl;[23] therefore, we decided to evaluate doses of T3 ranging from possible hypothyroidism (6.5 ng/dl = 0.1 nM) to possible hyperthyroidism (650 ng/dl = 10 nM). We also evaluated different doses of T3 which did not affect cell viability (1300 ng/dl, 2600 ng/dl and 6500 ng/dl; 20 nM, 40 nM and 100 nM, respectively) and a T3 dose of 200 nM which produced cellular toxicity (13,000 ng/dl = 200 nM); a 20-nM T3 dose is higher than the maximum value observed in human serum (maximal T3 serum level is 1000 ng/dl[23]). Control plates were performed by treating cells with vehicle.

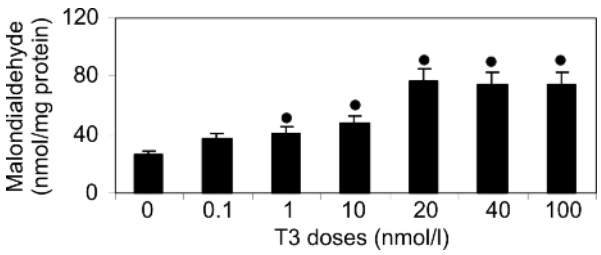

Figure 1 MDA content. Malondialdehyde is expressed as nmol MDA/mg protein. T3 doses included in the culture (24 h-treatment) are shown at the bottom. The results shown are the averages of four different experiments (mean ± SD). Experimental conditions were as described in Materials and methods. Dots indicate $P < 0.01$ versus control values (no T3)

We carried out experiments to determine T3 addition on some markers of oxidative stress (such as MDA and GSSG). T3 doses of 1–100 nM induced a significant increase in MDA levels (Fig. 1) with a maximum effect observed at 20 nM (26 ± 4 nmol MDA/mg protein [vehicle] versus 77 ± 6 nmol MDA/mg protein [20 nM T3]; $P < 0.01$). Doses of 20–100 nM T3 produced similar MDA levels. Since 20 nM T3 produced a maximum MDA level similar to higher doses of up to 100 nM, we determined glutathione levels at 20 nM T3. Reduced glutathione decreased with hormone treatment (336 ± 73 nmol

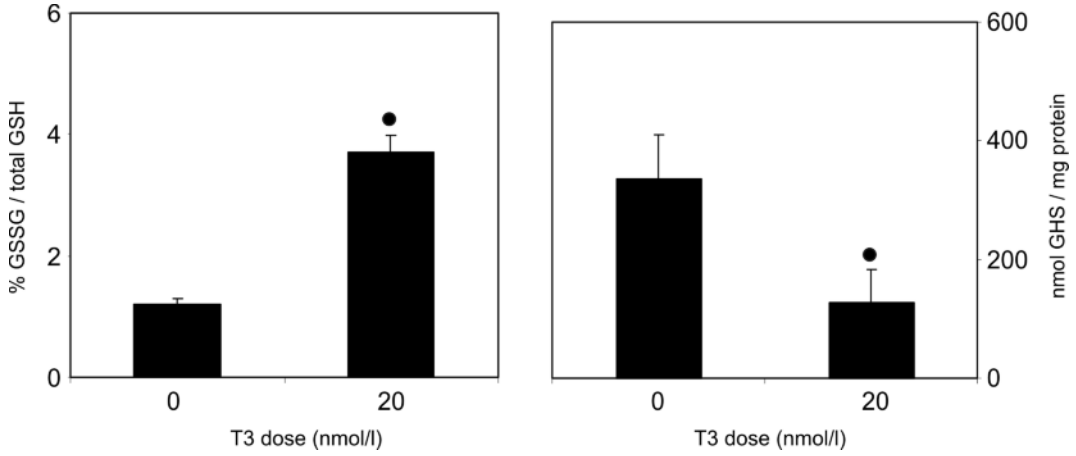

Figure 2 Glutathione levels. Reduced glutathione (GSH) is expressed as nmol/mg protein. Oxidized glutathione (GSSG) is expressed as percentage of total glutathione. A 20 nM T3 dose was included in the culture (24 h-treatment) and glutathione levels were determined. The results shown are the averages of four different experiments (mean ± SD). Experimental conditions were as described in Materials and methods. Dots indicate $P < 0.01$ versus control values (no T3)

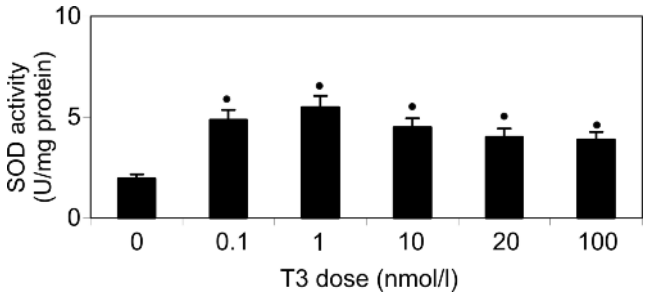

Figure 3 SOD activity. Superoxide dismutase activity is expressed as enzyme units/mg protein. T3 doses included in the culture (24 h-treatment) are shown at the bottom. The results shown are the averages of four different experiments (mean ± SD). Experimental conditions were as described in Materials and methods. Dots indicate $P < 0.01$ versus control values (no T3)

GSH/mg protein [vehicle] versus 127 ± 57 nmol GSH/mg protein [20 nM T3]; $P < 0.01$); however, the percentage of GSSG increased with the treatment (Fig. 2). Treatment with 200 nM T3 produced cell death; when GSH content was determined in remaining live cells, no significant difference compared to 20 nM treatment was observed (200 nM: 177 ± 79 nmol GSH/mg protein).

As a cell response to possible oxidative stress, we determined SOD, catalase and glutathione peroxidase activities. All the T3 doses assayed (0.1–100 nM) induced significant enhancement of SOD activity compared to control cells (1.97 ± 0.44 U/mg protein [vehicle] versus 4.03 ± 0.30 U/mg protein [20 nM T3]; $P < 0.01$; Fig. 3). We performed a time-course assay over 24 h for MDA and SOD (Fig. 4), using a T3 dose (20 nM) which induced maximum MDA levels after 24 h as previously shown. MDA increased 100% compared to control cells after 1 h; it continued to increase significantly for 3 h, and then reached an apparently stable level for 24 h. Superoxide dismutase activity in T3-treated cells reached a peak after 3 h (10.0 ± 6.0 U/mg protein [T3] versus 1.9 ± 0.4 U/mg protein [vehicle]; $P < 0.01$), and then decreased (10.0 ± 6.0 U/mg protein [3 h] versus 5.0 ± 0.3 U/mg protein [24 h]; $P < 0.01$). Addition of physiological dose of ascorbic acid to the culture (Fig. 4) resulted in a significant decrease in MDA after 3 h (96.6 ± 8.0 nmol MDA/mg protein [T3 alone] versus 65.8 ± 5.0 nmol MDA/mg protein [T3+ascorbic acid]; $P < 0.01$). When ascorbic acid was added for 24 h, SOD activity remained high, showing a similar value to 3 h of T3 treatment (9.0 ± 0.6 U/mg protein [T3+ascorbic acid]). Ascorbic acid did not affect MDA or enzyme activities in control plates significantly.

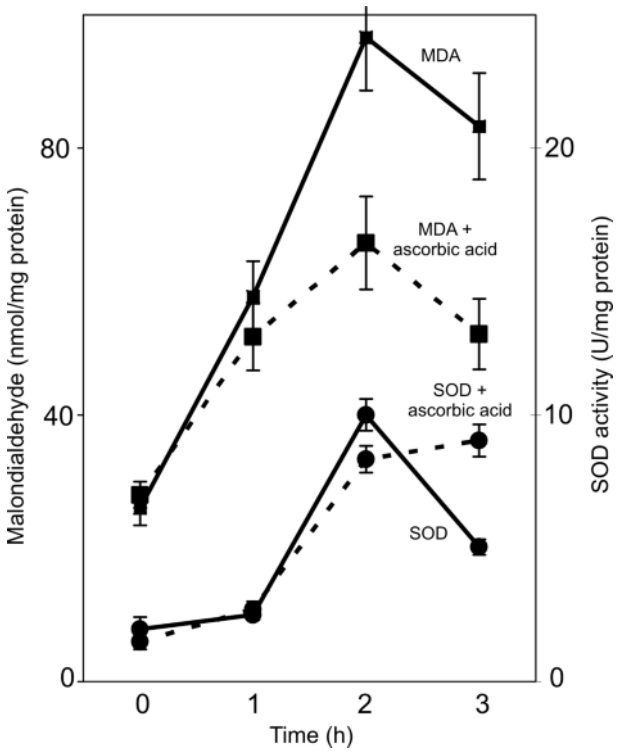

Figure 4 Time effect on SOD activity and MDA level. A dose of 20 nM T3 was added to the culture and SOD activity (circles, solid line) or MDA levels (squares, solid line) were determined. In addition, 20 nM T3 and 100 mg/l ascorbic acid (AA) were added to the culture and SOD activity (circles, dashed line) or MDA levels (squares, dashed line) were determined. Only three experimental times are shown (1 h, 3 h and 24 h). The results shown are the averages of four different experiments (mean ± SD). Experimental conditions were as described in Materials and methods

Glutathione peroxidase and catalase activities did not change significantly at any of the T3 doses tested (1–100 nM) compared to vehicle; Figure 5 shows the effect of a 20 nM T3 dose. A time course assay for these enzymes with 20 nM T3 demonstrated no change in their activities (data not shown). No effect of ascorbic acid on catalase or glutathione peroxidase activities was observed in either hormone-treated plates or control plates.

We observed modifications in cell viability during our experiments; therefore, we decided to perform an apoptosis assay. T3 concentrations between 0.1–100 nM did not induce cell death. Figure 6 shows three T3 dose effects: 200 nM T3 produced 69% apoptosis after 24 h of treatment. Ascorbic acid (100 mg/l) partially reversed this, with increases in apoptotic cell numbers at higher doses of T3 (*e.g.* 400 nM) as shown in Figure 6A. A proliferation assay showed a higher hormone

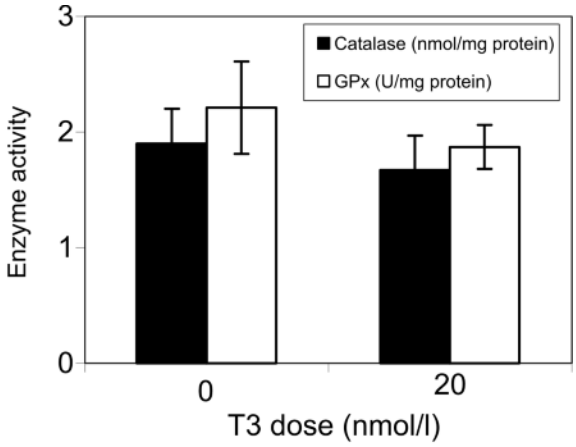

Figure 5 **Catalase and glutathione peroxidase activities. Catalase activity is expressed as nmol enzyme/mg protein. Glutathione peroxidase is expressed as unit/mg protein. A 20 nM T3 dose was included in the culture (24 h-treatment) and enzyme activities were determined. The results shown are the averages of four different experiments (mean ± SD). Experimental conditions were as described in Materials and methods. No significant differences between control (T3 dose, 0 nmol/l) and a 20 nM T3 dose (24 h treatment) were observed**

effect after 48 h compared to control cells; nevertheless, cell numbers remained almost constant between 24 h and 48 h of hormone treatment at both indicated doses (Fig. 6B). Figure 6C shows typical bubbles and condensed chromatin on the cells treated with 200 nM T3; it is also possible to see some necrotic cells (orange color). At 20 nM, very few cells presented bubbles or fragmented chromatin, similar to control cells (vehicle).

Discussion

Although the effect of thyroid hormones (T3 and T4) on oxidative metabolism in Graves' disease has been demonstrated,[4,5,14,24,25] how the thyroid modifies basal metabolism in the cell remains obscure. Reactive oxygen species (ROS) are generated under physiological conditions during normal metabolic events; to prevent this increase in ROS and possible deleterious effects, several enzymatic systems detoxify ROS. Our group demonstrated that lipoperoxidation and SOD activity are increased in hyperthyroid patients;[8,14] these results are in agreement with Videla

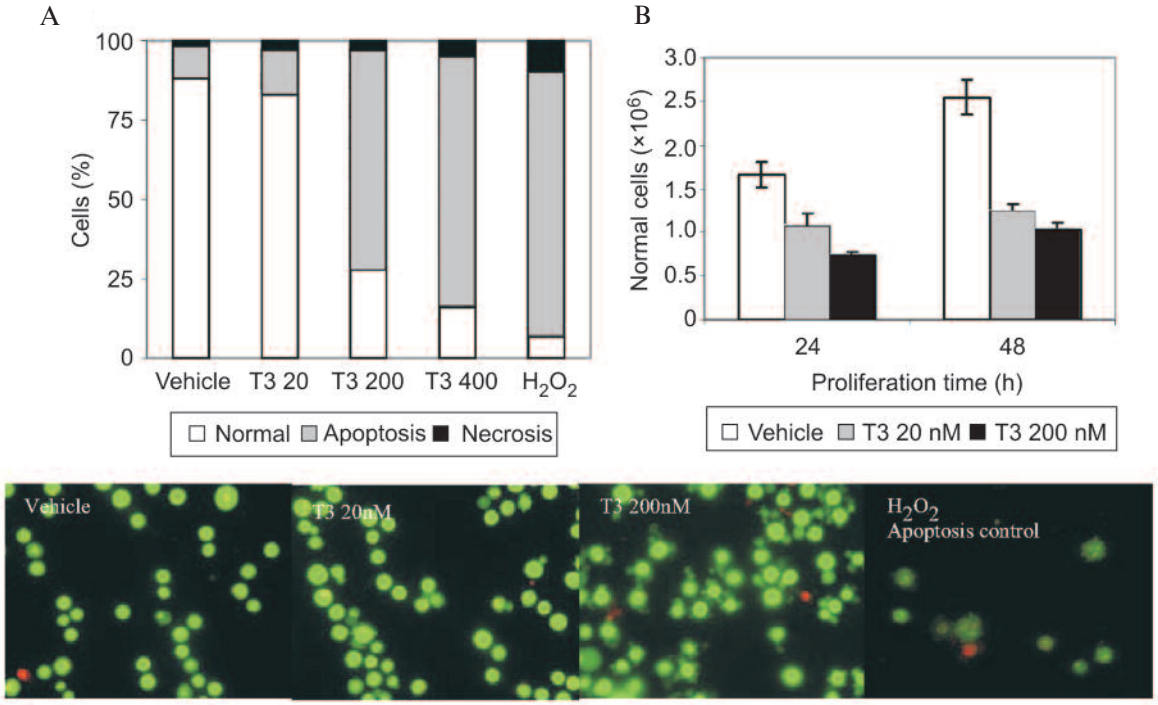

Figure 6 **(A) Apoptosis assay. The cells were cultured for 24 h in the presence of: (i) vehicle (PBS); (ii) 20 nM T3; (iii) 200 nM T3; (iv) 400 nM T3; (v) or 50 μM H_2O_2 (apoptosis control). Cells were stained by AO/EB fluorescent DNA binding after the treatment. Three categories of cell viability distribution distinguished by AO/EB staining after 24 h treatment are shown – normal, apoptotic and necrotic. The results shown are the averages of four different experiments. Experimental conditions were as described in Materials and methods. (B) Proliferation assay. Cells were cultured in the presence of: (i) vehicle (PBS); (ii) 20 nM T3; or (iii) 200 nM T3. The results shown are the averages of four different experiments (mean ± SD). Experimental conditions were as described in Materials and methods. (C) Representative views of AO/EB staining. The technique shows normal cells (bright green chromatin), apoptotic cells (bright condensed or fragmented chromatin and bubbles around the cells) and necrotic cells (orange chromatin)**

et al.[26] who showed increases in lipid and protein oxidation after thyroid hormone treatment but contradict their findings of reduced hepatic superoxide dismutase activity following hormone treatment. Gomes *et al*.[9] also showed progressive diminution of liver SOD activity after daily doses of T3 for 1–3 days. With the aim of clarifying the T3 effect on some oxidative stress markers, we used an *in vitro* model. Due to the importance of thyroid hormone in liver function, we tested the effect of different T3 doses on a human liver cell line (Hep G2). Liver is an organ central to both hormone and lipid homeostasis[27] and Hep G2 cells are a typical hepatocyte model.[28]

In agreement with previous reports which described thyroid hormones as physiological modulators of tissue oxidative stress,[24,29] we observed high MDA and GSSG levels following T3 treatment which could be a consequence of oxidative status induced by the hormone. Even though the T3 dose at which MDA levels reached a maximum was 20 nM (1300 ng/dl), MDA began to increase in treated cells at 1 nM T3 (65 ng/dl). The SOD activity increase at 0.1 nM T3 could be an effective response to hormone exposure, since the MDA level is similar to that observed in control cells at this hormone dose. However, SOD activity is not sufficient to prevent oxidative damage produced by this higher dose of hormone treatment and the MDA content significantly increased at 1 nM T3. No significant difference in SOD activity was observed with the T3 doses tested. As protein oxidation is increased by T3,[26] enzyme activity may decrease because of a direct oxidative effect of ROS on proteins. Our results suggest a direct effect of T3 on oxidative stress parameters, as MDA levels increased after 1 h of treatment and SOD activity was raised relatively early after hormone treatment (3 h). SOD activity showed a temporal response, since a progressive decreased activity occurs at later incubation periods (3–24 h). Protection could involve superoxide dismutase, since the treatment did not affect glutathione peroxidase and catalase activities; the early and temporary increases in SOD activity, observed here, may be part of mechanism of liver preconditioning by T3.[30] Nevertheless, this antioxidant system is not able to produce a significant decrease in MDA levels after 24 h. It is not known if all target genes are regulated directly by T3, since other intracellular factors might be rapidly induced to regulate some of the target enzymes we have studied.

Fraga *et al*.[31] demonstrated that antioxidant mixture consumption is associated with effective antioxidant action in healthy humans. The addition of a physiological dose of ascorbic acid to the culture resulted in a significant decrease in MDA levels after 3 h. A possible free radical scavenger effect caused by ascorbic acid and, consequently, a decrease in lipid peroxidation could occur. Since ascorbic acid alone did not affect MDA content in vehicle-treated cells (data not shown), MDA levels could be regulated by the endogenous cellular antioxidant system and by ascorbic acid;[32] this may support previous results[14] regarding the benefit of antioxidants in the treatment of hyperthyroidism. Since SOD activity decreased after 24 h in T3-treated cells, but remained almost at the same level in the presence of ascorbic acid, a possible oxidative effect on the enzyme leading to proteolysis in T3-treated cells could be speculated; a possible pro-oxidant effect could cause the decrease in SOD activity and ascorbic acid could overcome the hormonal effect. Furthermore, the increase in the level of SOD activity by supplementing ascorbic acid to the hormone treatment may be considered as a protection for SOD levels which was consumed by T3 rather than triggering oxidative stress.

An increasing number of biological investigations have focused on the biological impact of apoptosis induced by oxidative stress.[33] In this *in vitro* system, we observed that hormone treatment did not stimulate proliferation, as control cell numbers almost doubled after 24 h but 20 nM T3 (1300 ng/dl) treated cells did not proliferate during the same experimental time. In addition, treatment with 200 nM T3 (13,000 ng/dl) showed a decrease in cell number during the experiment. Therefore, we decided to evaluate a possible hormone effect on apoptosis. We have shown that apoptosis could be a consequence of high-dose T3 treatment (200 nM); lower doses produced important lipoperoxidation and high GSSG content but also stimulated SOD activity. Therefore, no significant difference between control plates and 20 nM T3 plates was observed for apoptosis test. We did not show an increase in molecules that could modify redox homeostasis in the cell; nevertheless, we considered that pro-oxidant status could not be controlled by cellular antioxidant system and cell death occurred at 200 nM T3 after 24 h of treatment. T3 hormone is known as a hepatocyte mitogen. *In vivo* hormone administration triggers liver CDK-2 expression and cellular proliferation through a cascade associated with JNK phosphorylation and AP-1 activation.[34] A recent study has demonstrated that T3 also has anticarcinogenic effects.[35] Moreover, other authors have reported changes in genes involved in apoptosis and cell cycle progression, 6 h after T3 administration.[1] Since enhanced lymphocyte apoptosis has been observed after *in vitro* treatment with thyroid hormone,[36] *in vitro* and *in vivo* systems may be dissimilar in T3 treatment responses.

Conclusions

This study has addressed the benefit of antioxidants in hyperthyroidism by avoiding hormonal deleterious effects; ascorbic acid was effective in decreasing the number of apoptotic cells and diminishing lipoperoxidation levels, as well as maintaining SOD activity.

Acknowledgements

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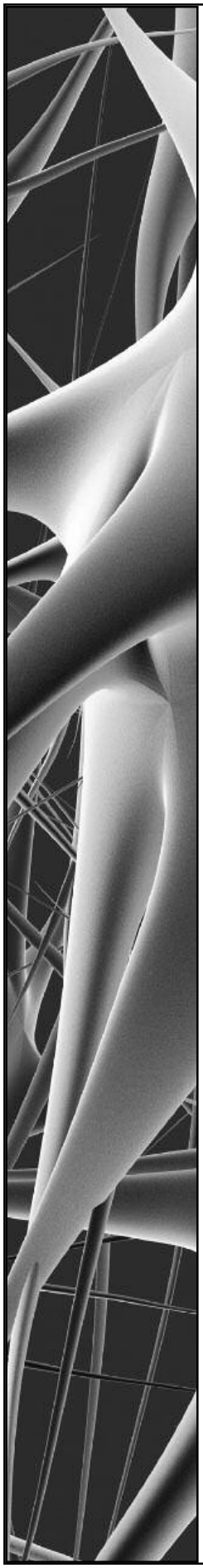

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