Effect of Triiodothyronine on Reactive Oxygen Species Generation by Leukocytes, Indices of Oxidative Damage, and Antioxidant Reserve

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We have examined the effect of short-term triiodothyronine (T_3) administration on reactive oxygen species (ROS) generation by leukocytes in 9 euthyroid subjects. At a dose of 60 μg/d orally for 7 days, T_3 induced a significant increase in ROS generation by mononuclear cells (MNCs) from 183 ± 102 mV at baseline to 313 ± 111 mV on the seventh day ($P < .02$), and by polymorphonuclear leukocytes (PMNLs) from 195 ± 94 mV at baseline to 302 ± 104 mV on the seventh day ($P < .02$). There was also a significant increase in *meta*-tyrosine ($P < .001$) and *ortho*-tyrosine ($P < .001$), known indices of oxidative damage to proteins and amino acids. However, there was no increase in plasma thiobarbituric acid–reactive substances (TBARS), an index of oxidative damage to lipids, and in the level of carbonylated proteins, a less sensitive index to assess protein oxidation. There was no decrease in the level of antioxidants such as α-tocopherol, vitamin A, β-carotene, lycopene, and lutein/zeaxanthin. The stimulatory effect on ROS generation may reflect a generalized increase in metabolic activity or may be a specific effect on NADPH oxidase in leukocyte membranes. The absence of a significant change in TBARS, carbonylated proteins, α-tocopherol, vitamin A, β-carotene, lycopene, and lutein/zeaxanthin may reflect the short duration of the increased ROS load.

THYROID HORMONE increases the metabolic activity of almost all tissues of the body. Mitochondrial oxygen consumption has been shown to be increased in the hyperthyroid state,[1-3] suggesting that excessive amounts of reactive oxygen species (ROS) may be generated and H_2O_2 generation may be subsequently increased in the hyperthyroid state. In polymorphonuclear leukocytes (PMNLs) from both Graves' disease and toxic adenoma patients, a significantly increased mitochondrial oxygen consumption and a slightly increased superoxide anion generation were detected.[4]

An increase in Na-K adenosine triphosphatase (ATPase) has been suggested as one of the mechanisms by which thyroid hormone increases the metabolic rate. In rats, administration of triiodothyronine (T_3) stimulated Na-K ATPase activity in the liver and kidney, and this effect may account for the calorigenic effects of the thyroid hormone.[5] Studies have shown that thyroid hormone–induced oxidative stress stimulates cellular lipid peroxidation as determined by the biliary efflux of thiobarbituric acid–reactive substances (TBARS).[6] In this study, the rate of O_2 consumption by the liver increased 36%. The calorigenic effect of T_3 on rat liver tissue is accompanied by a stimulation of microsomal functions involving the univalent reduction of oxygen which might lead to a faster lipid peroxidative rate and cytochrome P-450 loss as secondary events of thyroid hormone action.[7] An increase of 20% to 23% in microsomal NADPH oxidase activity was observed under this experimental condition. We have previously demonstrated that short-term T_3 administration in humans increases leukocyte ^{86}Rb influx, similar to that found in hyperthyroidism.[8] The active transport of ^{86}Rb is mediated by the membrane enzyme Na-K ATPase, which is stimulated by T_3. NADPH oxidase is also a membrane enzyme; it is responsible for producing the superoxide ($O_2^{\cdot}$) radical that mediates bacterial killing following phagocytosis. It is possible that T_3 may stimulate $O_2^{\cdot}$ generation by NADPH oxidase.

It has been shown that exposure of human plasma to ROS significantly increases the level of *meta*-tyrosine and *ortho*-tyrosine,[9] which are biomarkers of oxidative stress and damage to amino acids and proteins. An increase in free radical generation can also lead to an increase in carbonylated proteins, although this is a less specific method to assess protein oxidation. There are various biological defense systems directed at free radicals: specific enzymes, eg, superoxide dismutase or glutathione peroxidase, and antioxidants, eg, the plasma proteins, uric acid, vitamin C, vitamin E, carotenoids, etc. These antioxidants may be depleted if subjected to an excessive oxidative load.

We have previously used the circulating leukocytes, PMNLs, and mononuclear cells (MNCs) to investigate the effect of various disease states[10,11] and medications[12] on ROS generation. We now hypothesize that T_3 stimulates ROS generation by leukocytes and this increase in ROS generation may lead to higher levels of *meta*-tyrosine and *ortho*-tyrosine, suggesting increased oxidative stress to amino acids and proteins in thyrotoxicosis.

SUBJECTS AND METHODS

Experimental Subjects

Nine normal subjects (aged 34 ± 10 years, mean ± SD; 7 men and 2 women) were recruited from the staff of the Millard Fillmore Hospital. All subjects were euthyroid and within 10% of their ideal body weight. All subjects provided written informed consent as approved by the Millard Filmore Hospital Institutional Review Board, and confidentiality was maintained according to the Helsinki Declaration. Each subject received 60 μg T_3 (Cytomel, Jones Pharmaceuticals, St Louis, MO) orally every morning at 7 AM for 7 consecutive days. Blood samples were drawn just before the start of T_3 intake and 1 hour after the seventh T_3 dose. From the blood samples, total T_3, total thyroxine (T_4) and thyrotropin ([TSH] second generation) were determined by radioimmunoassay. ROS generation by PMNLS and MNCs, TBARS, carbonylated proteins, *ortho*-tyrosine, *meta*-tyrosine, and phenylalanine were also determined in all 9 subjects. Assays for α-tocopherol, lycopene, vitamin A, β-carotene, and lutein/zeaxanthin were performed in 8 subjects.

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Preparation of PMNLs and MNCs

Blood samples were collected with Na-EDTA as an anticoagulant. A 3.5-mL volume of the anticoagulated blood sample was carefully layered over 3.5 mL PMNL medium (Robbins Scientific, Sunnyvale, CA). The samples were centrifuged at 450 × *g* in a swing-out rotor for 30 minutes at 22°C. At the end of the centrifugation, 2 bands separated out at the top of the erythrocyte pellet. The top band consists of MNCs, while the bottom consists of PMNLs. The bands were harvested with a Pasteur pipette. The harvested cells were repeatedly washed with Hanks balanced salt solution (HBSS) and reconstituted to a concentration of 4×10^5 cells/mL in HBSS. This method yields greater than 95% pure PMNL and MNC suspensions.

Measurement of ROS

Five hundred microliters of PMNLs or MNCs (2×10^5 cells) were placed into a Chronolog Lumi-Aggregometer plastic flat-bottom cuvette to which a spin bar was added. Then, 15 μL 10-mmol/L luminol was added, followed by 1.0 μL 10-mmol/L *N*-formylmethionylleucinyl phenylalanine. Chemiluminescence was recorded for 15 minutes. (A protracted record after 15 minutes did not alter the relative amount of chemiluminescence produced by previous blood samples.) Our method, developed independently,[12] is similar to that published by Tosi and Hamedani.[13] In this assay system, the release of superoxide radical as measured by chemiluminescence has been shown to be linearly correlated with that measured by the ferricytochrome C method.[13] We further established that in our assay system, there is a dose-dependent inhibition of chemiluminescence by superoxide dismutase and catalase, as well as diphenylene iodonium ([DPI], data not shown), a specific inhibitor of NADPH oxidase, the enzyme responsible for the production of superoxide radicals. The specific inhibitory effect of DPI on NADPH oxidase has been established by Hancock and Jones.[14] Our assay system is exquisitely sensitive to DPI-induced inhibition at nanomolar concentrations.

TBARS Assay

TBARS levels were measured fluorometrically by the method described by Ohkawa et al.[15] Briefly, 100 μL plasma was precipitated with an equal volume of 8.1% sodium dodecyl sulfate. The reaction mixture was acidified with 0.75 mL 20% acetic acid (pH 3.5). Subsequently, 0.75 mL 0.53% thiobarbituric acid was added to the reaction mixture and allowed to form adducts with various oxidative products of lipid peroxidation at 95°C for 1 hour. After cooling, the samples were vortexed at 800 × *g* and the fluorescence was measured at an excitation wavelength of 535 nm and emission wavelength of 552 nm. 1,1,3,3-Tetramethoxypropane was used as the standard.

Carbonylated Protein Assay

Carbonyl levels were measured by the method described by Levine et al[16] with modifications. Briefly, 400 μL 10-mmol/L 2,4-dinitrophenylhydrazine (DNPH) in 2.5 mol/L HCl or 400 μL 2.5-mmol/L HCl was added to 100 μL serum and incubated for 1 hour. Samples were vortexed every 15 minutes, extracted with 500 μL 20% trichloroacetic acid (TCA), and then vortexed for 5 minutes at 14,000 × *g*. The precipitates were washed once with 1 mL 10% TCA followed by 3 washes with 1 mL ethanol:ethyl acetate (1:1) to remove the free DNPH and lipid contaminants. Precipitates from DNPH-treated samples were dissolved in 2 mL 6-mol/L guanidine hydrochloride solution at 37°C for 10 minutes. The carbonyl content was calculated from the peak absorbance (355 to 390 nm) using an absorption coefficient of 22,000 mol $L^{-1} \cdot cm^{-1}$ against pellets derived from the 2.5-mol/L HCl-treated samples which were also used to calculate the protein content against a bovine serum albumin standard dissolved in 6 mol/L guanidine hydrochloride and measured at 280 nm.

Ortho-Tyrosine, Meta-Tyrosine, and Phenylalanine Assay

Measurements of *ortho*-tyrosine, *meta*-tyrosine, and their precursor phenylalanine in serum were made using high-performance liquid chromatography (HPLC)-fluorometric detection as described by Ishimitsu et al[17] with modification. Briefly, venous blood was collected and centrifuged at 1,700 × *g* for 10 minutes to collect serum. A 0.5-mL serum sample was deproteinized by the addition of 125 μL 1.0-mol/L TCA. The mixture was centrifuged at 14,000 × *g* for 10 minutes at 4°C. The supernatant was collected and filtered through a 0.22-μm membrane filter (Millipore, Bedford, MA). The filtrate was lyophilized and then dissolved in 200 μL deionized water, and 100 μL of this solution was injected directly into a Shimadzu (Columbia, MD) HPLC system equipped with a 5-μL Supelco reversed-phase C18 column (4.6 mm ID × 25 cm; Supelco Bellefonte, PA) operated under isocratic conditions with an aqueous solution containing 250 mmol/L $KH_2PO_4 \cdot H_3PO_4$, pH 3.0, with 4% (vol/vol) methanol at a flow rate of 1 mL/min. The compounds were detected by a fluorometer (RF-10A; Shimadzu) with excitation at 275 nm and emission at 305 nm for *p*-, *m*-, and *o*-tyrosine and excitation at 258 nm and emission at 288 nm for phenylalanine. Analysis was performed in duplicate and the mean value is reported. Peaks were identified by coinjection with reference compounds (Sigma, St Louis, MO). The results for *ortho*-tyrosine and *meta*-tyrosine are expressed in absolute terms and as a ratio to their precursor phenylalanine.

Fat-Soluble Antioxidant Vitamins

Retinol, α-tocopherol, and carotenoid levels were measured in plasma simultaneously by HPLC on an LC-7A liquid chromatograph with a SPD-M6A photodiode array (Shimadzu Scientific Instruments).[1] HPLC-grade acetonitrile, methanol, dichloromethane, and triethylamine were purchased from J.T. Baker (Phillipsburg, NJ). Tocopherol acetate, α-tocopherol, lycopene, and β-carotene were purchased from Sigma. Vitamin A-alcohol (>95% *cis*-retinol) was purchased from Fluka Chemical (Ronkonkoma, NY). Lutein and zeaxanthin were purchased from Indofine Pure Biochemicals (Somerville, NJ).

Vitamins were extracted from 300 μL absolute ethanol containing 0.03% butylated hydroxytoluene and 50 μg/mL tocopherol acetate as internal standard. Samples were extracted twice with 3.0 mL hexane, and the upper phases were aspirated, pooled, and evaporated to dryness under nitrogen. The sample was dissolved in 300 μL mobile phase and injected onto a reversed-phase Supelcosil LC-18 column (150 mm × 4.6 mm; Supelco). Vitamins were eluted isocratically at a flow rate of 1.5 mL/min using a mobile phase of acetonitrile:methanol:dichloromethane (60:25:15 vol/vol/vol) which contained 0.1% triethylamine and 0.05 mol/L ammonium acetate. The eluant was monitored at 284, 292, 326, 450, and 478 nm, and each peak was quantified against a calibration curve of pure standard. The accuracy of this method was verified against National Institute of Standards and Technology (NIST, Gaithersburg, MD) SRM 968a fat-soluble vitamin control material and NIST round-robin proficiency testing. Results are expressed as micrograms per

Table 1. Thyroid Function Test Results for Nine Subjects at Baseline and Day 7: Serum T_3, TSH, and T_4, T_3 Resin Uptake, and Free T_4 Index (mean ± SD)

Parameter	Baseline	Day 7
Total T_3 (NR = 85-185 ng/dL)	118.33 ± 23.74	378.22 ± 161.82*
TSH (NR = 0.4-3.1 μIU/mL)	1.93 ± 0.67	0.16 ± 0.07*
Total T_4 (NR = 5.5-11.5 μg/dL)	8.71 ± 1.54	6.06 ± 0.59*
T_3 resin uptake (NR = 30%-40%)	34.63 ± 2.12	31.72 ± 3.28*
Free T_4 index (NR = 1.7-4.6)	3.00 ± 0.44	1.92 ± 0.28*

Abbreviation: NR, normal range.
*$P < .05$ *v* baseline.

Table 2. Results for Different Indices Measured at Baseline and Day 7 (mean ± SD)

Parameter	Baseline	Day 7
ROS-MNC (mV)	183 ± 102	313 ± 111*
ROS-PMNL (mV)	195 ± 94	302 ± 104*
Ortho-tyrosine (mmol/mol phenylalanine)	0.28 ± 0.03	0.34 ± 0.05*
Meta-tyrosine (mmol/mol phenylalanine)	0.28 ± 0.03	0.34 ± 0.04*
Ortho-tyrosine (absolute concentration in ng/mL)	3.36 ± 0.46	4.18 ± 0.74*
Meta-tyrosine (absolute concentration in ng/mL)	3.40 ± 0.47	4.28 ± 0.73*
Phenylalanine (μg/mL)	11.12 ± 0.59	11.31 ± 0.67
Carbonylated proteins (nmol/mg protein)	0.60 ± 0.10	0.72 ± 0.12
TBARS (μmol/L)	1.22 ± 0.21	1.15 ± 0.30
α-Tocopherol (μg/mL)	9.40 ± 3.05	8.16 ± 3.58
Lycopene (μg/mL)	0.39 ± 0.16	0.35 ± 0.11
Vitamin A (μg/mL)	0.41 ± 0.08	0.41 ± 0.12
β-Carotene (μg/mL)	0.16 ± 0.08	0.13 ± 0.06
Lutein/zeaxanthin (μg/mL)	0.15 ± 0.06	0.16 ± 0.06

*$P < .05$ *v* baseline.

milliliter. Lutein and zeaxanthin were quantified as a single peak and are reported as total lutein and zeaxanthin.

Statistical Analysis

A paired *T* test was used to compare the actual values at baseline versus day 7. The data on ROS generation were nonparametric, and the statistical comparisons were therefore made using the Mann-Whitney rank sum test. The software was Sigma Stat statistical software (Jandel, San Rafael, CA). For the graphs, Sigma Plot scientific graphing software was used (Jandel). All values are the mean ± SD.

RESULTS

T_3 administration to the subjects caused a decrease in TSH and total T_4 and an increase in total T_3 (Table 1). During the period of T_3 administration, 4 subjects had mild headache for 2 to 3 days, 1 subject complained of drowsiness, 1 had mild palpitation, and 1 had tachycardia, and the 9 subjects lost an average of 1.3 lbs. Despite these side effects, T_3 administration for 7 days was well tolerated.

On the seventh day, there was significant increase in ROS generation by MNCs ($P < .02$) and PMNLs ($P < .02$). The mean ROS generation by MNCs was 183 ± 102 mV at baseline and 313 ± 111 mV on the seventh day, an increase of 71% (Table 2 and Fig 1). The mean ROS generation by PMNLs was 195 ± 94 mV at baseline and 302 ± 104 mV on the seventh day, an increase of 55% (Table 2 and Fig 2). ROS generation by both PMNLs and MNCs decreased in 1 subject.

Ortho-tyrosine at baseline was 0.28 ± 0.03 mmol/mol phenylalanine, which significantly increased to 0.34 ± 0.05 mmol/mol phenylalanine on the seventh day ($P < .001$; Table 2 and Fig 3). *Meta*-tyrosine at baseline was 0.28 ± 0.03 mmol/mol phenylalanine, which increased to 0.34 ± 0.04 mmol/mol phenylalanine on the seventh day ($P < .001$; Table 2 and Fig 4). The absolute concentration of *ortho*-tyrosine and *meta*-tyrosine

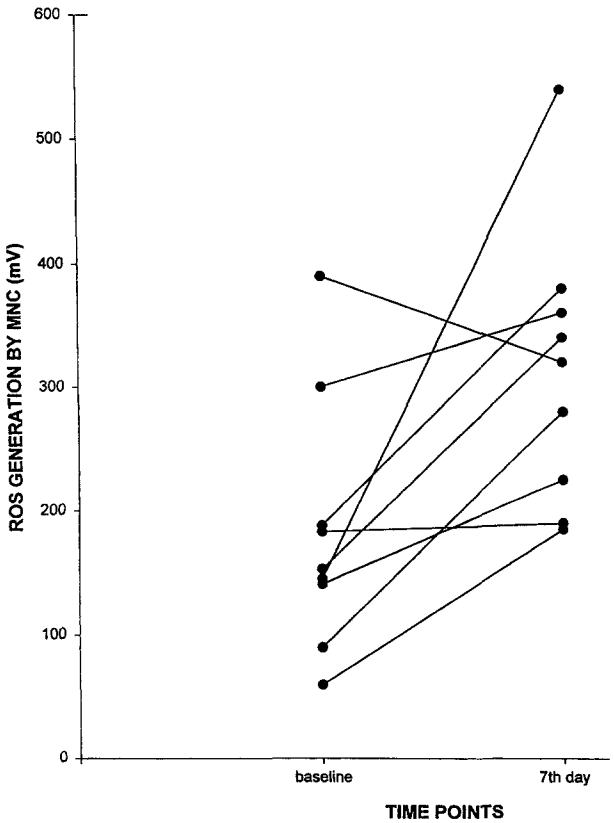

Fig 1. ROS generation by MNCs at baseline and day 7. Change from baseline was significant on day 7 ($P < .02$).

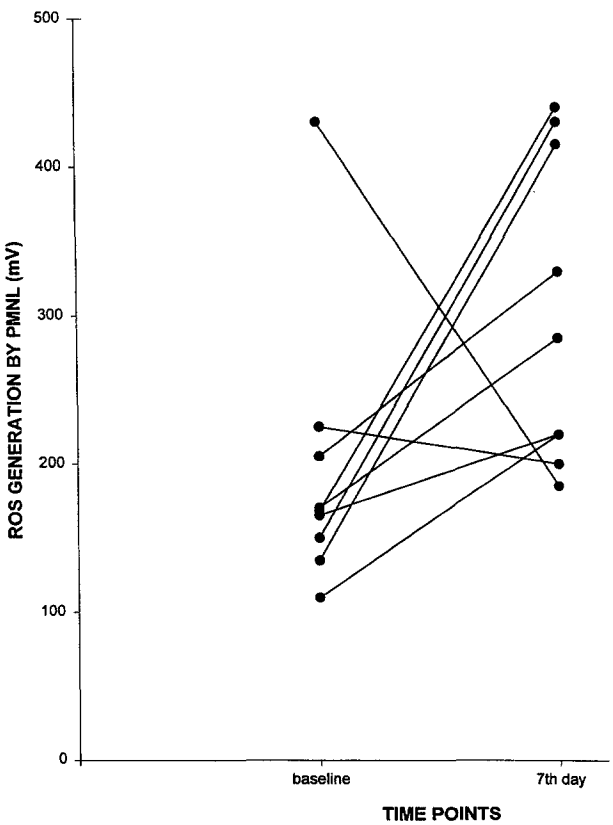

Fig 2. ROS generation by PMNLs at baseline and day 7. Change from baseline was significant on day 7 ($P < .02$).

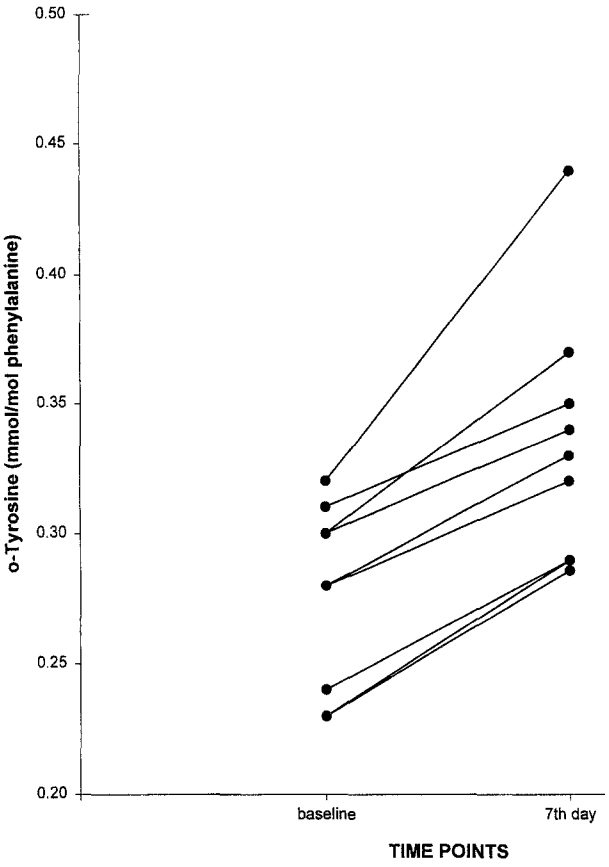

Fig 3. *Ortho*-tyrosine levels (mmol/mol phenylalanine) at baseline and day 7. Change from baseline was significant on day 7 ($P < .001$).

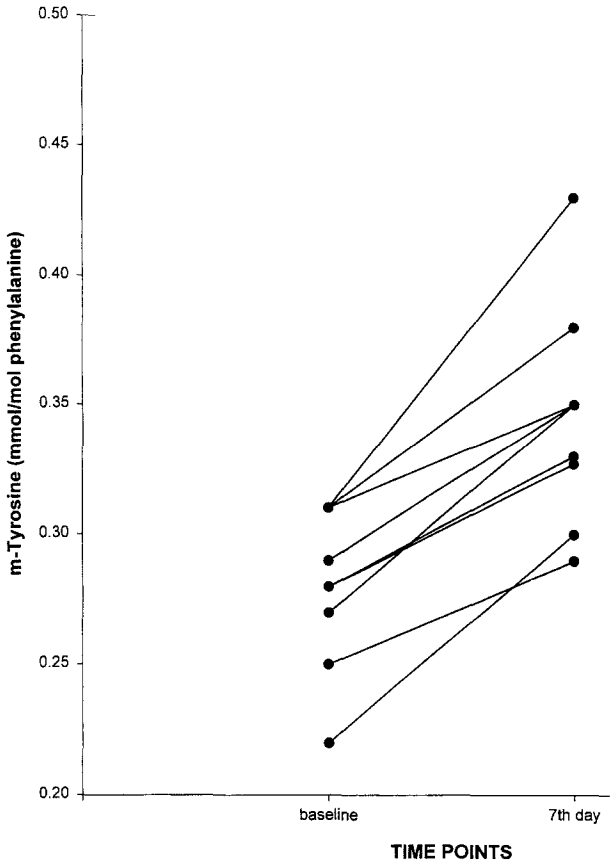

Fig 4. *Meta*-tyrosine levels (mmol/mol phenylalanine) at baseline and day 7. Change from baseline was significant on day 7 ($P < .001$).

at baseline was 3.36 ± 0.46 ng/mL and 3.40 ± 0.47 ng/mL, which significantly increased on the seventh day to 4.18 ± 0.74 ng/mL ($P < .001$) and 4.28 ± 0.73 ng/mL ($P < .001$), respectively (Table 2). There were no significant changes noted in the concentration of phenylalanine, TBARS, carbonylated proteins, α-tocopherol, vitamin A, lycopene, β-carotene, and lutein/zeaxanthin (Table 2).

DISCUSSION

Our data demonstrate clearly that T_3 administration for 7 days at a dose of 60 μg/d orally, induces an increase in ROS generation by MNCs. This effect was highly predictable in each of the subjects except 1, with a mean increment of 71% on the seventh day. There was also an increase in the ROS generation by PMNLs, with a mean increment of 55%. To our knowledge, this is the first report on the effect of T_3 on ROS generation by PMNLs and MNCs in humans in vivo. This short-term T_3 administration also led to a significant increase in *meta*-tyrosine and *ortho*-tyrosine levels. *Meta*-tyrosine and *ortho*-tyrosine are formed by the ROS attack on phenylalanine and thus reflect ROS-induced damage of phenylalanine. Carbonylated protein is a relatively insensitive index of oxidative damage to proteins, and it did not increase significantly during this short period of T_3 administration. While we observed evidence of increased ROS generation, we were unable to demonstrate an increase in lipid peroxidation as reflected by TBARS. α-Tocopherol, vitamin A, β-carotene, lycopene, and lutein/zeaxanthin, the antioxidants measured in our study, did not show any change. It is possible that a period of 7 days is too short to induce significant changes in these indices. However, there was a trend for a depletion of α-tocopherol after 7 days ($P = .057$).

Thyroid hormone treatment of human T lymphocytes in vitro has induced a reduction of mitochondrial transmembrane potential and production of ROS.[18] In previous studies of Graves' disease patients, there were significantly reduced levels of superoxide dismutase and plasma thiol and elevated levels of lysate thiol.[19] Xanthine oxidase and glutathione peroxidase were also significantly higher in thyroid specimens of Graves' disease patients versus normal thyroid tissue.[20] Serum levels of vitamin A were significantly decreased in both hyperthyroidism and hypothyroidism, but vitamin A levels almost never decrease to subnormal values in hyperthyroidism.[21]

Based on polarographic measurement, the respiratory rate is higher in hepatocytes of hyperthyroid rats versus euthyroid animals.[22] In experimental hyperthyroidism in rats, vitamin E supplementation reduced the burden of oxidative stress.[23] These findings provide evidence for both an enhanced lipid peroxidation in experimental hyperthyroidism in rats and the protective role of vitamin E in the prevention of lipid peroxidation. Vitamin E–supplemented euthyroid rats also had decreased T_3

values, which suggests that vitamin E supplementation in the euthyroid state decreases either T_3 synthesis or T_4-T_3 conversion.[24] In hyperthyroid cats, the plasma α-tocopherol level was at least 30% lower than normal while the retinol concentration was 30% higher than normal.[25]

In conclusion, T_3 administration for 7 days in euthyroid subjects induced a predictable increase in ROS generation by MNCs and PMNLs. This increase in ROS generation was associated with increased levels of *meta*-tyrosine and *ortho*-tyrosine, suggesting an increase in oxidative damage to proteins and amino acids in the thyrotoxic state. The increase in ROS generation may reflect a generalized increase in metabolic activity or may be a specific effect on NADPH oxidase in leukocyte membranes.

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