Free Radical Biology & Medicine, Vol. 14, pp. 669–675, 1993
Printed in the USA.

0891-5849/93 $6.00 + .00

Brief Communication

ZYMOSAN-INDUCED LUMINOL-AMPLIFIED CHEMILUMINESCENCE OF WHOLE BLOOD PHAGOCYTES IN EXPERIMENTAL AND HUMAN HYPERTHYROIDISM

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(*Received* 14 *October* 1992; *Accepted* 28 *December* 1992)

Abstract—Luminol-amplified CL of whole blood phagocytes was studied in rats given 3 consecutive doses of 0.1 mg L-triiodothyronine T_3/kg or in hyperthyroid patients, after stimulation by zymosan. In both cases, CL was significantly increased, an effect which was produced independently of the opsonization of the zymosan particles and markedly inhibited by azide. The in vitro addition of T_3 or L-thyroxine (T_4) to whole blood phagocytes from normal rats did not modify the opsonized zymosan-dependent CL, when assayed at the concentrations found in euthyroid subjects or in hyperthyroid patients. Administration of propylthiouracil (400 mg/day for 2–3 months) to hyperthyroid patients reduced the CL response observed prior to treatment, to values comparable to those found in the euthyroid group. These data indicate that hyperthyroidism elicits an enhanced respiratory burst activity of whole blood phagocytes, probably related to adaptive changes induced by thyroid hormone on the mieloperoxidase-H_2O_2 system, rather than to direct actions of the hormone molecule or changes in the opsonic capacity of plasma.

Keywords—Chemiluminescence, Whole blood phagocytes, Respiratory burst activity, Hyperthyroidism, Free radicals

INTRODUCTION

Thyroid hormone administration to experimental animals has been found to induce an oxidative stress condition, possibly related to the calorigenic action exerted on several target tissues.[1–3] In the liver, this condition is characterized by an enhanced oxygen uptake and free radical activity,[1] together with a diminution of antioxidant mechanisms such as the activity of superoxide dismutase and catalase,[4] as well as the content of hepatic glutathione.[4,5] As a consequence of this prooxidant activity, lipid peroxidation indicators are increased in the liver of hyperthyroid animals,[1,4,6,7] in agreement with earlier observations on the dependency of the spontaneous cellular lipid peroxidative rate on the specific metabolic rate of the individuals.[8]

Hyperthyroidism in man is also associated with a prooxidant condition characterized by increments in circulating and urinary lipid peroxidative indexes, which is suppressed by PTU treatment.[9,10] Furthermore, erythrocytes from hyperthyroid patients were found to be more susceptible to *tert*-butyl hydroperoxide-induced oxidative stress in vitro, than those of euthyroid subjects, as they exhibited enhanced rates of oxygen uptake and light emission after the addition of the hydroperoxide.[9] In this respect, circulating phagocytes are known to increase their oxygen uptake upon stimulation, as a requirement for cellular production of reactive oxygen species and hypochlorous acid involved in microbicidal action.[11] Phagocyte microbicidal oxidative activity is also associated with the generation of electronically excited states due to the oxygenation of certain biological substrates, which decay to ground state by photon emission, thus resulting in a CL response.[12] Because human polymorphonuclear leukocytes are known to possess saturable nuclear binding sites for T_3 (ref. 13) and display a calorigenic response in thyrotoxic patients,[14] the pres-

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Table 1. Age, Hematologic Parameters and Serum Thyroid Hormone Levels in Euthyroid Subjects and Hyperthyroid Patients

Parameters	Euthyroid subjects (n = 15)	Hyperthyroid patients (n = 15)
Age (years)	30 ± 2	33 ± 2
Hemoglobin (g/dl)	14.3 ± 0.3	13.7 ± 0.3
Hematocrit (%)	43 ± 1	42 ± 1
Red blood cell count (cells/mm^3) × 10^{-6}	5.0 ± 0.1	4.8 ± 0.1
White blood cell count (cells/mm^3)	5973 ± 325	6273 ± 352
Phagocyte count[a] (cells/mm^3)	3873 ± 259	3720 ± 287
Serum T_3[b] (ng/dl)	143 ± 10	513 ± 38[d]
(pmol/phagocyte) × 10^6	0.24 ± 0.02	0.89 ± 0.08[d]
Serum T_4[c] (μg/dl)	8.6 ± 0.3	20.1 ± 0.9[d]
(pmol/phagocyte) × 10^6	12.7 ± 0.9	30.3 ± 2.2[d]

[a] Polymorphonuclear leukocytes plus monocytes.
[b] Normal range: 65–185 ng T_3/dl [0.11–0.31 (pmol/phagocyte) × 10^6].
[c] Normal range: 4.5–11.5 μg T_4/dl [6.5–16.6 (pmol/phagocyte) × 10^6].
[d] $p < 0.05$, compared to values in the euthyroid group.

ent work was undertaken in order to evaluate the influence of hyperthyroidism on phagocyte respiratory burst metabolism. For this purpose, zymosan-induced luminol-amplified CL was measured as an index of respiratory burst activity in whole blood phagocytes, either in rats made hyperthyroid by treatment with T_3 or in hyperthyroid patients before and after PTU treatment.

MATERIALS AND METHODS

Animals

Female Sprague Dawley rats (Instituto de Salud Pública, Santiago, Chile) weighing 284 ± 8 g (n = 37) were fed ad libitum and received daily ip injections of either T_3 (0.1 mg Na-L-T_3/kg for 3 consecutive days) or equivalent volumes of T_3 diluent (0.1 N NaOH) (controls).[1] After treatment, serum T_3 levels measured by the Gamma Coat™ [^{125}I]T_3 Radioimmunoassay Kit (Baxter Healthcare Corp., Cambridge, MA) were significantly increased, expressed either as ng/dl (controls, 46 ± 4 [n = 9]; T_3-treated rats, 287 ± 35 [n = 9]; $p < 0.05$) or as (pmol/phagocyte) × 10^6 (controls, 0.092 ± 0.004 [n = 9]; T_3-treated rats, 0.582 ± 0.052 [n = 9]; $p < 0.05$). Phagocyte (polymorphonuclear leukocytes plus monocytes) count: (controls, 3738 ± 347 cells/mm^3 [n = 9]; T_3-treated rats, 3700 ± 431 [n = 9]; $p > 0.05$). In these conditions, the rectal temperature of hyperthyroid animals (measured with a thermocoupla Cole-Parmer model 8112-20, Cole-

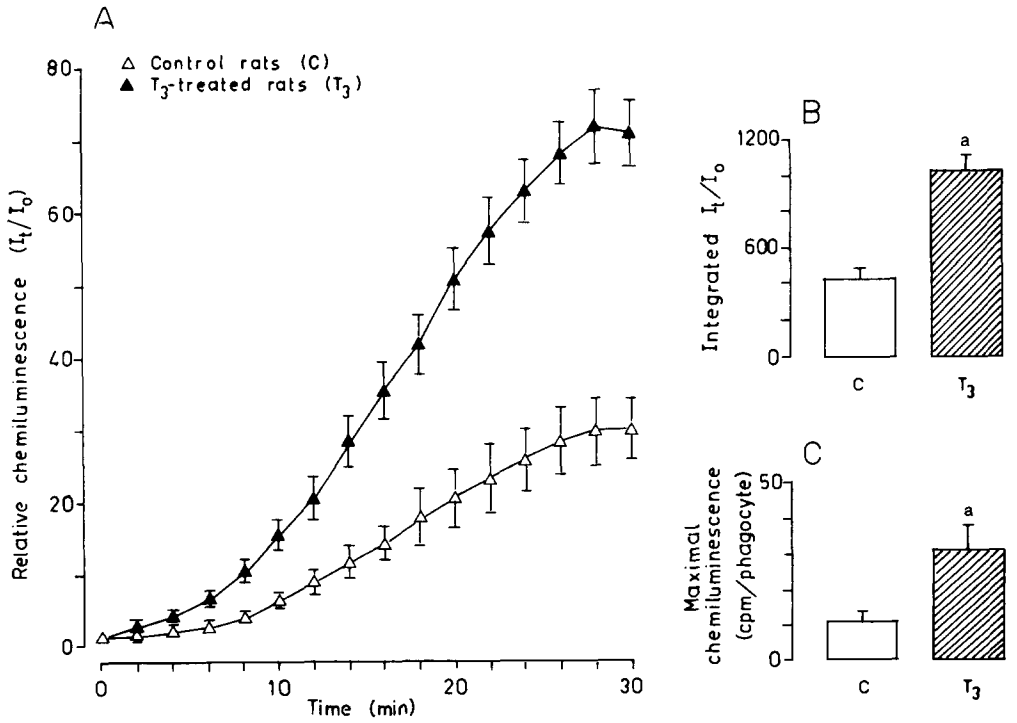

Fig. 1. Effect of the in vivo T_3 treatment (0.1 mg/kg for three consecutive days) on the opsonized zymosan-induced luminol-amplified light emission of whole blood phagocytes in the rat. Results are expressed either as relative chemiluminescence (I_t/I_0) (A), integrated I_t/I_0 (0–30 min) (B), or as maximal chemiluminescence (C). Values shown represent the means ± SE for four control rats and five T_3-treated animals. Significance of the effect of T_3 treatment compared to controls, $p < 0.05$.

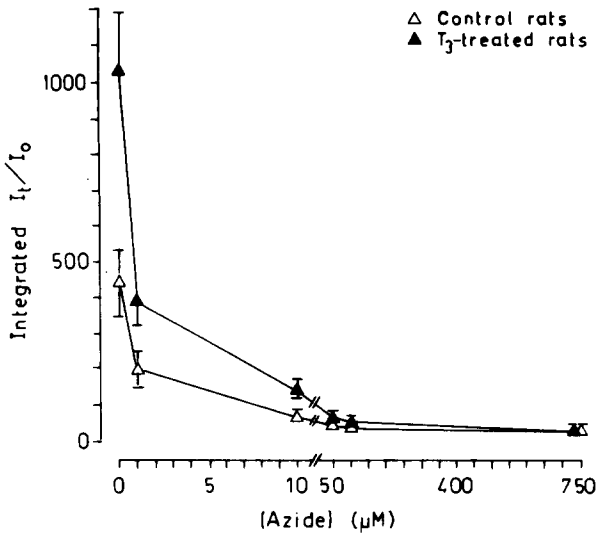

Fig. 2. Effect of the in vitro addition of azide in the 1–750 μM range on the opsonized zymosan-induced luminol-amplified integrated chemiluminescence (0–30 min) of whole blood phagocytes from control rats (n = 5) and T_3-treated animals (n = 4) (0.1 mg/kg for three consecutive days). Values shown correspond to means ± SE. Significance of the effect of azide at the different concentrations used compared to measurements in the absence of azide, $p < 0.05$.

Parmer Instrument Co., Chicago, IL) was significantly enhanced compared to controls (control rats, 36.7 ± 0.7°C [n = 9]; T_3-treated animals, 38.4 ± 0.1 [n = 9]; $p < 0.05$), suggesting a T_3-induced thermogenic state.

Patients

The study comprised 30 subjects (Table 1) referred to the Department of Endocrinology, San Juan de Dios Hospital (Santiago, Chile). Subjects with cardiac failure, thyroid storm or prestorm, immunologic alterations, hemolytic anemia, previous hepatic disease or positive HBsAg, drug hypersensitivity, alcohol or drug abuse history, cigarette smoking, or any other concomitant disease, were not included. A complete clinical history, a physical examination and laboratory tests, including hematologic parameters and serum T_3 and T_4 levels, were performed (Table 1). Serum T_3 and T_4 levels were determined with Radioimmunoassay Kits (Baxter Healthcare Corp., Cambridge, MA) in samples from each subject run in duplicate. Subjects were classified as hyperthyroid (n = 15; 12 women and 3 men) or euthyroid (n = 15; 11 women and 4 men) according to clinical criteria and their respective hormone levels in serum (Table 1). From the initial hyperthyroid group, a second evaluation was carried out in five patients after PTU treatment (400 mg/day for 2–3 months), antithyroid drug which was withdrawn at least 24 h before sampling. An informed consent was obtained from all subjects and the investigation was approved by the Committee of Ethics, Culture and History of the Faculty of Medicine, University of Chile.

Chemiluminescence

Blood samples from experimental animals (cardiac puncture) and human subjects were obtained with heparinized plastic syringes and were processed immediately. Plasma, obtained by centrifugation of blood aliquots at 2500 × g for 10 min at 4°C, was used to opsonize zymosan (OZ), as described by Allen et al.[15] Non-opsonized zymosan (NOZ) was prepared by the same procedure,[15] except that plasma was replaced by an equivalent volume of CVB pH 7.25, containing 130 mM NaCl, 4.3 mM KCl, 5 mM sodium 5,5-diethylbarbiturate, 0.5 mM $MgCl_2$, 0.45 mM $CaCl_2$, 1 mg/ml albumin and 5.6 mM D-glucose, previously sterilized by filtration.[15] Both OZ and NOZ were resuspended in CVB to give a final concentration of 2.5 mg/ml.

Visible CL was measured in a Beckman LS-6000TA liquid scintillation spectrometer, using single photon monitoring (Beckman Instruments Corp., Fullerton, CA), at 23–24°C. Measurements were carried out in a reaction medium (final volume of 2.0 ml) containing CVB, 0.05 ml of 0.8 mM luminol and 0.01 ml of whole blood. The reaction was started by the addition of 0.05 ml of 2.5 mg/ml OZ or NOZ (time zero), and light emission was recorded at 2 min intervals until peak values were observed. Backgrounds consisting of CVB alone or in the presence of either luminol, whole blood, zymosan, or luminol plus zymosan did not emit light, except for that containing luminol plus whole blood, which was subtracted from the values obtained in the complete reaction medium. Zymosan-induced luminol-amplified light emission from whole blood phagocytes was expressed either as relative CL (light emission at any given time t (I_t) over that at time zero (I_0)), integrated I_t/I_0 (area under the time curves of I_t/I_0 between 0–30 min (in animal studies using OZ), 0–50 min (in human studies using OZ) and 0–70 min (in human studies using NOZ), or maximal CL in cpm/phagocyte.

Studies on the influence of sodium azide (1–750 μM) on the OZ-induced luminol-amplified CL of whole blood phagocytes were carried out in samples from control rats and T_3 treated animals. The influence of the in vitro addition of T_3 and T_4 on whole blood phagocyte CL was assessed in a separate group of control rats. For this purpose, experiments were carried out in the complete reaction medium de-

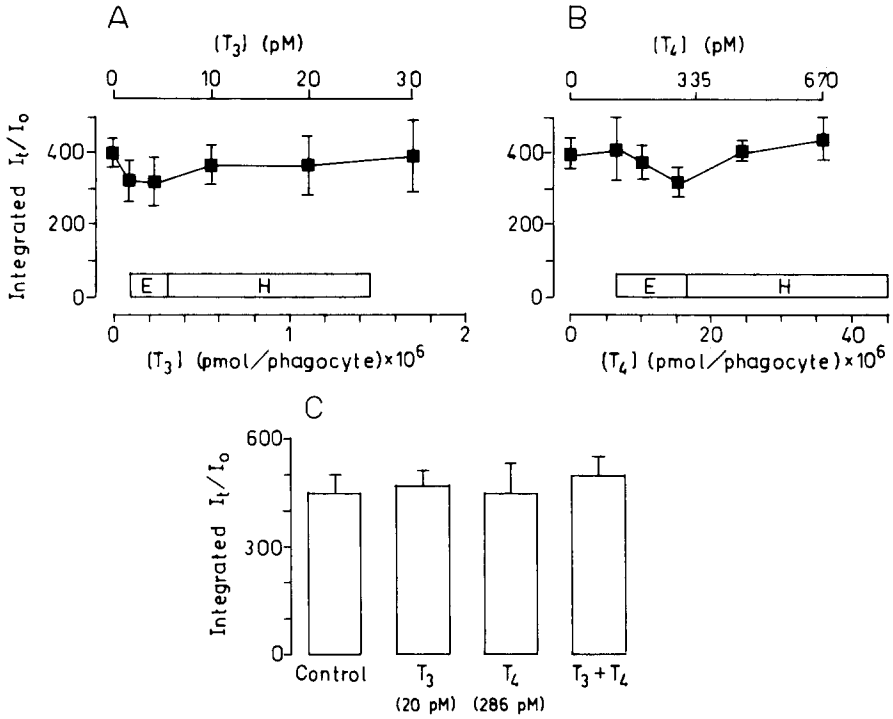

Fig. 3. Effect of the in vitro addition of T_3 (A), T_4 (B), and T_3 plus T_4 (C) on the opsonized zymosan-induced luminol-amplified chemiluminescence (0–30 min) of whole blood phagocytes from normal rats. Thyroid hormones were added to the reaction medium at the picomolar concentrations shown, to give values in (pmol/phagocyte) × 10^6 in the ranges calculated for euthyroid subjects (E) and hyperthyroid patients (H), as described in Materials and Methods. Values shown correspond to means ± SE for 4–14 animals per each experimental condition.

scribed above, in the absence and presence of picomolar concentrations of T_3, T_4 or T_3 plus T_4, to give values in the range of those observed in euthyroid subjects (0.11–0.31 (pmol T_3/phagocyte) × 10^6 or 6.5–16.6 (pmol T_4/phagocyte) × 10^6) and in hyperthyroid patients (0.31–1.45 (pmol T_3/phagocyte) × 10^6 or 16.6–45.0 (pmol T_4/phagocyte) × 10^6), calculated according to the respective hormones levels in serum, the phagocyte count, and assuming a hematocrit of 42% (from Table 1).

All reagents used were obtained from Sigma Chemical Co. (St. Louis, MO). Values shown correspond to the means ± SE for the indicated number of separate observations. The significance of the differences between mean values was determined by one-way analysis of variance, using the random model.

RESULTS AND DISCUSSION

The administration of 0.1 mg T_3/kg to fed rats for 3 consecutive days elicited an enhancement in their metabolic rate, as evidenced by the significant elevation in the rectal temperature of the animals.[1,5] In these conditions, OZ-induced luminol-amplified CL of whole blood phagocytes from hyperthyroid rats was significantly increased compared to control animals, expressed either as relative CL (Fig. 1A), integrated light emission (Fig. 1B) or maximal CL (Fig. 1C). Phagocyte CL stimulated by OZ in control animals and that induced by T_3 treatment in vivo, were found to be drastically diminished (91% and 94%, respectively) by azide, a potent mieloperoxidase inhibitor[16] which does not impair cellular production of superoxide radicals or H_2O_2,[17] with a median inhibitory concentration of 0.6 μM (Fig. 2). Contrarily to the effect of the in vivo T_3 treatment (Figs. 1 and 2), the addition of T_3, T_4, or T_3 plus T_4, in the concentration ranges observed in euthyroid subjects and hyperthyroid patients (Table 1), did not produce significant changes in the OZ-induced CL of whole blood phagocytes from control rats (Fig. 3). Thyroid hormones are presumably carried into phagocytes through binding sites on the plasma membrane or bound to the OZ particles, to be subsequently degraded.[18] These findings indicate that hyperthyroidism in the rat leads to an enhancement in the respiratory burst activity of circulating phagocytes as evaluated by the CL response. The effect does not seem to be elicited by a direct action of the hormones, which exhibit phenolic estructures suitable for free radical interactions, in agreement with the lack of prooxidant or antioxidant behaviour previously reported when assayed in biolog-

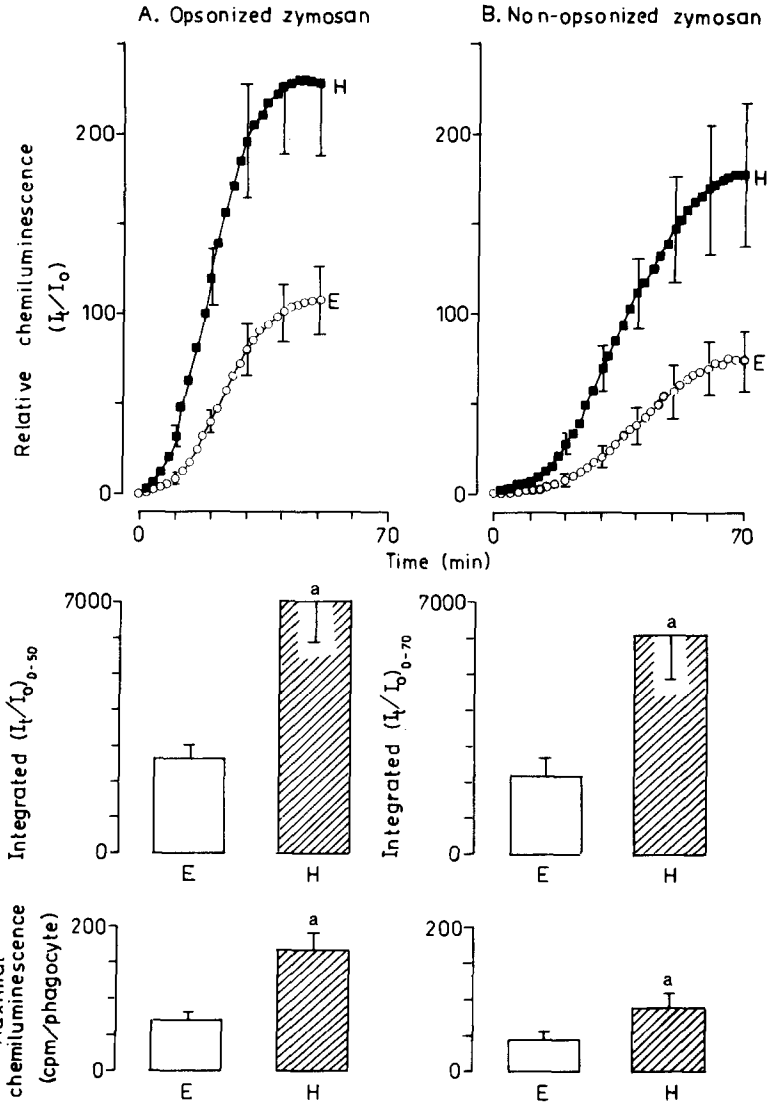

Fig. 4. Influence of hyperthyroidism on the opsonized (A) and non-opsonized (B) zymosan-induced luminol-amplified chemiluminescence from whole blood phagocytes in man. Results are expressed either as relative chemiluminescence (I_t/I_0), integrated I_t/I_0 (0–50 min in A; 0–70 min in B), or as maximal chemiluminescence. Values shown represent means ± SE for 15 euthyroid subjects (E) and 15 hyperthyroid patients (H). Significance of the effect of hyperthyroidism compared to the euthyroid group, $p < 0.05$.

ical systems at nanomolar concentrations.[19] It is suggested that T_3-induced respiratory burst activity in phagocytes could represent an adaptive response mediated by its interaction with nuclear binding sites,[13] with the mieloperoxidase-H_2O_2 system playing an important role in the observed increment of luminol-dependent CL.

In agreement with the studies in experimental animals, a significant increase in the respiratory burst activity of whole blood phagocytes was also found in hyperthyroid patients compared to that in euthyroid subjects (Fig. 4), in conditions of comparable values of hematologic parameters including total white blood cell and phagocyte counts (Table 1). This was evidenced by increments in the relative, integrated and maximal luminol-dependent CL, in response to either OZ (Fig. 4A) or NOZ (Fig. 4B), an effect which was diminished by 98% by 750 μM azide (OZ-induced integrated CL: euthyroid subjects (n = 15), 2620 ± 375 and 70 ± 24 in the absence and presence of azide, respectively; $p < 0.05$. Hyperthyroid patients (n = 15), 6966 ± 1095 and 85 ± 16 in the absence and presence of azide, respectively; $p < 0.05$. Phagocyte CL induced by OZ was slightly higher than that elicited by NOZ, and maximal values were achieved at shorter times after stimulation (Fig. 4). This could be due to the fact that zymosan stimulates phagocytes by interacting with different recognition mechanisms

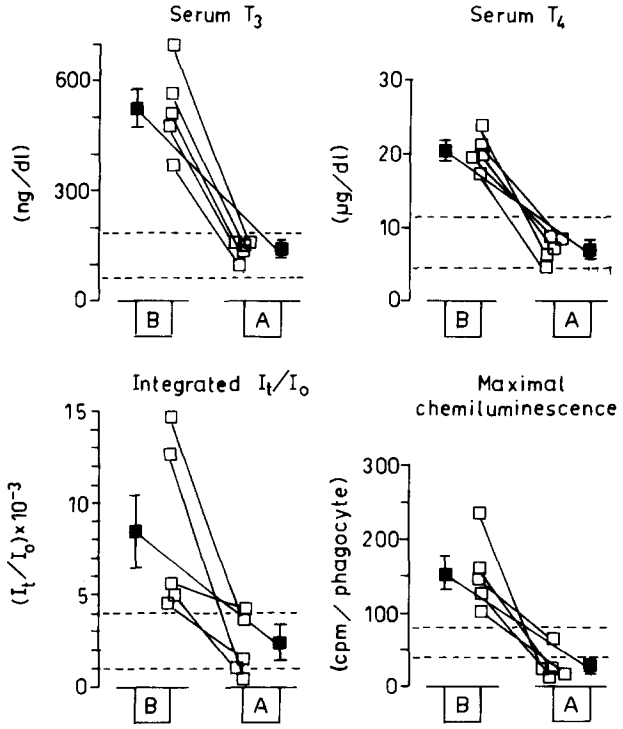

Fig. 5. Serum T_3 and T_4 levels, opsonized zymosan-induced luminol-amplified integrated chemiluminescence (0–50 min) and maximal light emission of whole blood phagocytes from 5 hyperthyroid patients before (B) and after (A) PTU treatment (400 mg/day for 2–3 months). Individual (□) and mean ± SE (■) values are shown, with values between broken lines representing means ± SE for euthyroid subjects (from Table 1 and Fig. 4). All values in untreated hyperthyroid patients were significantly different from those either in hyperthyroid patients after PTU treatment or in euthyroid subjects ($p < 0.05$), while values in hyperthyroid patients treated with PTU and euthyroid subjects were comparable ($p > 0.05$).

present on the cell surface, being recognized by iC3b and Fc receptors when opsonized[20,21] or by those interacting with its polysaccharide glucan and mannan components when unopsonized.[22] Thus, the increased respiratory burst activity of whole blood phagocytes from hyperthyroid patients does not seem to be related to differences in the opsonic capacity of plasma, but rather to changes in the intracellular mechanisms involved in the generation of reactive species and/or in antioxidant protection. This view is supported by the studies in hyperthyroid patients after treatment with PTU, which abolished the enhancement in phagocyte CL observed prior to treatment (Fig. 5), probably related to the attainment of the euthyroid state evidenced by the reduction in serum T_3 and T_4 levels to values within normal ranges (Fig. 5).

In conclusion, data obtained in experimental animals and man indicate that hyperthyroidism induces an enhanced respiratory burst activity of phagocytes in whole blood. The effect seems to be related to adaptive changes induced by thyroid hormone on the mieloperoxidase-H_2O_2 system of the phagocyte, rather than direct actions of the hormone molecule or changes in the opsonic capacity of plasma. This enhanced prooxidative activity may be of importance in determining toxic effects in cells such as erythrocytes, leukocytes, platelets, or endothelial cells,[20,23] which constitute immediate targets for the deleterious actions of the reactive species that could be released from activated phagocytes at higher rates. The intracellular mechanisms underlying the increased prooxidative capacity of phagocytes induced by thyroid hormone are currently under study in our laboratory.

Acknowledgements — The present work was supported by grants from DTI, Universidad de Chile (B-3241) and FONDECYT (1109/91). The authors thank E. Moncada, A. Kriz, C. Almeyda, and M. Suárez for expert technical assistance.

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ABBREVIATIONS

T_3—L-triiodothyronine
T_4—L-thyroxine
PTU—propylthiouracil
CL—chemiluminescence
CVB—complete veronal buffer
OZ—opsonized zymosan
NOZ—non-opsonized zymosan
ip—intraperitoneal