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Propylthiouracil and thiamazole do not alter in vitro neutrophil oxidative burst

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Abstract

Propylthiouracil and thiamazole are thionamides used in the treatment of hyperthyroidism. In addition to reducing thyroid hormone synthesis, these drugs have other activities that improve the hypermetabolic state of the patients as well as adverse and toxic effects. The capacity of these 2 drugs to interfere with the production of reactive oxygen species of human neutrophils exposed in vitro to these drugs was evaluated. The production of reactive oxygen species was assessed by chemiluminescence assays and the cells were stimulated with zymosan particles opsonized with a pool of normal human serum. No alteration was found in the chemiluminescence response of treated human neutrophils when compared to controls.

The results show that these drugs, at the studied concentrations and with the experimental approach used, have no direct effect on the production of oxidative burst of neutrophils. We conclude that if these drugs have any action on the oxidative metabolism of neutrophils these might include some metabolization steps that do not take place in this in vitro model.

1. Introduction

Propylthiouracil (PTU) and thiamazole are members of a drug family called thionamides. These drugs are thyrostatic and are used in the treatment of hyperthyroidism to inhibit the iodine organification that is one of the main steps in the formation of thyroid hormones, T3 and T4. Propylthiouracil has an additional action that is the impairment of the T4 to T3 conversion in extrathyroid tissues.

Despite the widespread use and study of these drugs, there are many controversies about their complete mechanism of action and interference with blood cells activities. There are many suggestions about which effects these drugs may have apart from controlling T3 and T4 levels. Some authors have indicated an immunosuppression function involving the lymphocytes [1,2], monocytes [3], and complement system [4], whereas others do not agree with the immunosuppressive action of thionamide drugs [5]. In vivo and in vitro studies have suggested that they may act as free radical scavengers and this may be one of the mechanisms by which the drugs control some of the thyrotoxic symptoms of Graves' disease [6-9] and, once more, other studies suggested that there is no such antioxidant action [10]. Investigators have demonstrated the accumulation of PTU in the thyroid gland and in polymorphonuclear cells (PMN) [11-13]. Lim and Lindsay [14] showed that PTU accumulation is accompanied by an increase in H_2O_2 availability in phagocytizing PMN, suggesting that PTU accumulation is related to its oxidation products.

A number of adverse, many times life-threatening, effects are also ascribed to antithyroid drugs. Propylthiouracil can cause agranulocytosis in part due the reactive metabolites generated from oxidative neutrophil metabolism [15-19]. Hepatotoxicity [20,21] and glomerulonephritis [22,23], vasculitis [24-26], and pulmonary toxicity [27,28] are other adverse effects frequently addressed to these drugs.

In previous researches of our laboratory, we have observed enhanced oxidative burst of neutrophils from patients with Graves' disease [29,30] in chemiluminescence

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(CL) experiments. Cells CL production in response to phagocytosis of immunoglobulin G immune complexes, mediated by Fcγ and complement receptors, from the patients, were compared with those of cells from healthy donors. Most of the patients were taking PTU or thiamazole at the time of assessment (up to 600 mg/d of PTU and up to 120 mg/d of thiamazole). Because of the controversial and sometimes opposite results found in the literature, concerning the effects of these drugs on blood cells, depending on each experimental model and blood cells used, in this investigation we studied the effect of them on the oxidative burst of the in vitro treated peripheral blood human neutrophils using serum-opsonized zymosan as a cell stimulus. The main purpose of these tests was to establish if there is a direct action of thionamides on the cells that does not require metabolization or accumulation steps to produce some of the effects listed above. This approach was chosen because most of the studies dealt with cells cultured in presence of the drugs and we wanted find out if in these peripheral cells the brief contact with the drug could change their activity. This can help to address if the parental drugs contribute to the reduction or even enhancement of the oxidative stress of neutrophils from hyperthyroid human patients.

2. Materials and methods

2.1. Chemicals

Luminol and zymosan were purchased from Sigma Chemical Co (St Louis, Mo). Thiamazole and PTU were purchased from Eli Lilly do Brasil LTDA (São Paulo, Brazil) and Searle do Brasil LTDA (São Paulo, Brazil), respectively.

2.2. Neutrophil isolation

Blood was drawn from healthy laboratory staff volunteers after a 12-hour fast. Blood samples were collected into sodium EDTA (EDTA-Na) tubes and PMN were isolated using gelatine gradient [31]. Briefly, the blood tubes were centrifuged at 755 × g to exclude plasma, the cells were suspended in a gelatine solution (2.5%, wt/vol) in 0.15 mol/L NaCl and incubated for 30 minutes at 37°C. The supernatant was collected and centrifuged at 270 × g for 10 minutes. The pellet was washed in 0.15 mol/L NaCl and the remaining erythrocytes were lysed with 0.83% NH_4Cl (wt/vol). The cells were washed again and suspended in Hanks balanced salt solution. The preparations usually consisted of about 98% neutrophils as seen in stained smears. Viability was always above 90% according to the trypan blue exclusion test.

2.3. Sera

Blood was drawn from healthy laboratory staff volunteers who had given verbal consent. Blood samples were collected into dry tubes and allowed to clot. The tubes were centrifuged and the sera aliquoted and frozen at −70°C until use.

2.4. Drug solutions

Thiamazole and PTU were dissolved in phosphate buffer solution. Thiamazole is highly soluble in water but PTU is

Table 1
Effect of different concentrations of PTU on cellular CL of human neutrophils

(A)

	PTU 0.387 mmol/L		PTU 0.78 mmol/L		PTU 2.6 mmol/L	
	Controls	Treated	Controls	Treated	Controls	Treated
	3714	2786	18440	16180	17440	20450
	14980	10910	831	1179	950	640
	6605	5964	3966	3070	4857	4529
	1130	1219	11870	21030	7645	10570
	24950	16060	4191	5609	3004	4229
Mean	10280	7388	7860	9414	6779	8084
SEM	4345	2728	3210	3890	2884	3479

(B)

	PTU 0.387 mmol/L		PTU 0.78 mmol/L		PTU 2.6 mmol/L	
	Controls	Treated	Controls	Treated	Controls	Treated
	847	915	836	1162	790	1710
	4848	4000	3460	6417	3628	4163
	607	608	2536	1642	1840	1688
	2628	2741	326	535	12240	13840
	18060	10250	12450	11830		
Mean	5398	3703	3922	4317	4625	5380
SEM	3255	1750	2206	2148	2605	2889

Control and treated cells (2 x 10^6) were stimulated with 1 mg opZy in 2 mL Hanks' solution, pH 7.2, 0.1% gelatine and 10^{-4} luminol at 37°C for 10 min. CL was calculated as AUC of the profile.
The control cells were incubated with 0.15 mmol/L NaCl. Sera used to opZy were a pool of NHS 1:2 in CFD/Mg^{+2}/gelatine (A) or in TEA/Mg^{+2}/gelatine (B). Each one of the data is individual values of the AUC from five independent experiments. Statistics: Paired t test.

not; thus, we used a warm bath to maximize the amount of soluble drug. The PTU solution was then filtered and the concentration determined by high-pressure liquid chromatography as described elsewhere [32].

2.5. Incubations of the cells and drugs

2.5.1. Thiamazole

Three concentrations of thiamazole were used and each drug concentration tested was incubated for 1 hour in a water bath at 37°C with the cells. The concentrations of the drug used were 0.175, 0.118, and 0.030 mmol/L. After the incubation period the PMN were isolated as described above.

2.5.2. Propylthiouracil

We studied 3 PTU concentrations and each one tested was incubated for 1 hour in a water bath at 37°C with anticoagulated whole blood. The concentrations of 0.387, 0.78, and 2.6 mmol/L were used. After the incubation period the PMN were isolated as described above.

For each of these treatments the controls were cells from the same individual as used in the tests that were submitted to the same incubation, dilution, and processing using NaCl 0.15 mol/L instead of the drug solution.

2.6. Zymosan opsonization

Zymosan was opsonized with a pool of NHS according to the method of Cheung et al [33]. To assess separately 2 of the complement activation pathways the sera were diluted in appropriate buffers and then used to opsonize zymosan. The dilution used for serum was 1:2 in complement fixation dilutor (CFD) with 0.1% (wt/vol) gelatine and 1:2 in triethylammonium (TEA)/ethyleneglycotetraacetic acid/Mg^{2+}/0.1% (wt/vol) gelatine solution.

2.7. Luminol-dependent CL assays

Neutrophils raise their oxidative metabolism during phagocytosis and a sensitive indicator of this is the light emission or CL generation [34]. Opsonized particles of zymosan were used as the stimulus to trigger the respiratory burst [33].

Neutrophils diluted at 2×10^6 cells/mL in Hanks balanced salt solution (pH 7.2) were mixed with an equal volume of opsonized zymosan (opZy) suspension at 1 mg/mL (final concentration of 0.5 mg/mL), in the presence of 10^{-4} mol/L of luminol in dimethyl sulfoxide. The reaction was monitored with a luminometer (Bio-Orbit 1250, Bio-Orbit, Finland) at 37°C for 10 minutes and recorded as millivolts.

The results are expressed as the integrated area under the curves (AUCs) of the CL profile (Tables 1 and 2). The CL profiles are presented in Fig. 1 as millivolts per minute.

2.8. Statistical analysis

Paired t tests were performed to analyze the difference between control and treated cells and between the various treatments. $P < .05$ was considered significant.

3. Results

3.1. Kinetics of CL production by human neutrophils treated with PTU or thiamazole

The kinetic profile of CL production from the experimental protocol using the in vitro treatment of neutrophils is presented in Fig. 1A and B as millivolts per minute for one of the tested individuals. Cell treatment with 118.25

Table 2
Effect of different concentrations of thiamazole on cellular CL of human neutrophils

(A)

	Thiamazole 0.03 mmol/L		Thiamazole 0.118 mmol/L		Thiamazole 0.175 mmol/L	
	Controls	Treated	Controls	Treated	Controls	Treated
	28110	19790	20100	19920	18330	22370
	15270	14520	10510	16840	9733	9471
	12570	15240	12290	10400	8555	6865
Mean	18665	16520	14300	15720	12210	12900
SEM	4794	1650	2945	2805	3081	4793

(B)

	Thiamazole 0.03 mmol/L		Thiamazole 0.118 mmol/L		Thiamazole 0.175 mmol/L	
	Controls	Treated	Controls	Treated	Controls	Treated
	16790	9139	10240	10180	13320	10790
	5280	5294	4896	7414	5465	4751
	5772	9859	4500	4447	3457	2648
Mean	9281	8097	6545	7347	7414	6063
SEM	3757	1417	1851	1655	3009	2440

Control and treated cells (2×10^6) were stimulated by 1 mg opZy in 2 mL Hanks' solution, pH 7.2, 0.1% gelatine and 10^{-4} luminol at 37°C for 10 min. CL was calculated as AUC of the profile.

The control cells were incubated with 0.15 mmol/L NaCl. Sera used to opsonize zymosan were a pool of NHS 1:2 in CFD/Mg^{+2}/gelatine (A) or in TEA/Mg^{+2}/gelatine (B). Each one of the data is individual values of the AUC from five independent experiments. Statistics: Paired t test.

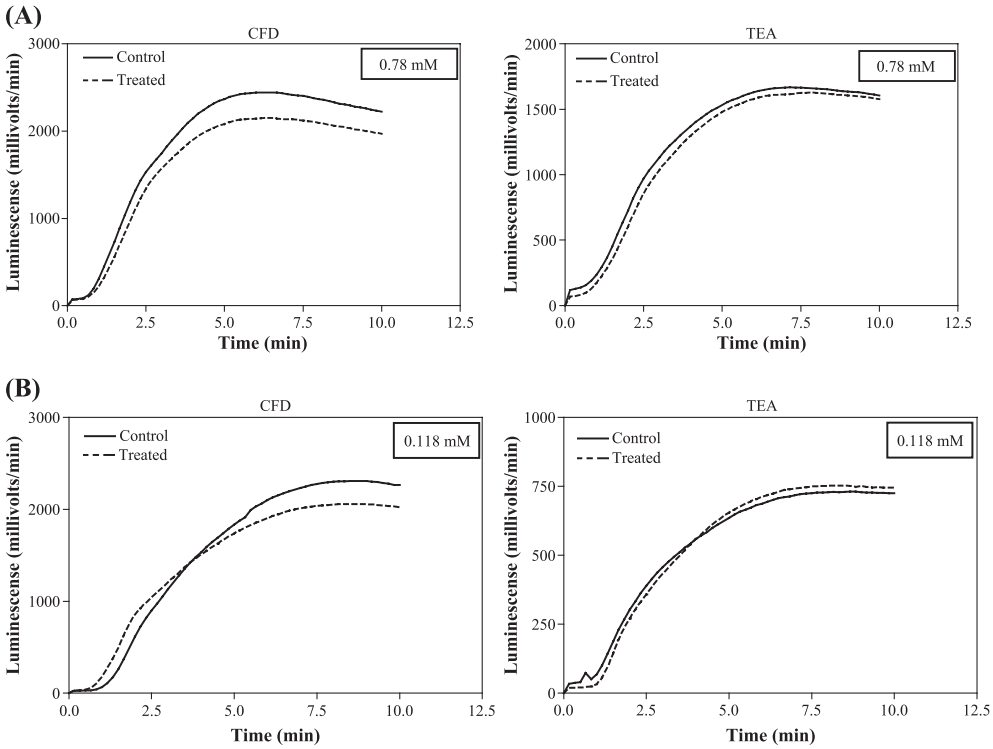

Fig. 1. Representation of the kinetic profiles of CL production by human neutrophils stimulated with opZy with a pool of NHS 1:2 in CFD/Mg^{2+}/gelatine or in TEA/Mg^{2+}/gelatine. The cells (4.5 mL of whole blood) were treated in vitro with PTU (A) or thiamazole (B) and isolated after incubation time. The profiles shown were obtained from isolated treated or control neutrophils (2×10^6) stimulated with 1 mg opZy in 2 mL of Hanks balanced salt solution (pH 7.2), 0.1% gelatine, and 10^{-4} mol/L luminol. Data are presented as millivolts per minute and the concentrations of the drugs used in the exemplified profiles are in the figure.

μmol/L thiamazole is shown in A and treatment with 0.78 mmol/L PTU is shown in B. This figure presents a comparison of CL profiles between treated and control neutrophils and the results indicate the same capacity of stimulus binding to the cells to induce nicotinamide adenine dinucleotide phosphate (reduced form)-oxidase activity.

3.2. Effect of PTU on neutrophil CL

The neutrophil capacity of generating the oxidative burst was assessed after 1 hour of cell incubation with PTU at the 3 concentrations tested. The cells were stimulated with opZy with a pool of NHS diluted 1:2 in CFD or TEA. Chemiluminescence values were calculated as AUC of the CL profiles over a period of 10 minutes. The AUC values for the treated and control cells stimulated with opZy with a pool of NHS are presented in Table 1A and B.

No differences in respiratory burst generation were found even when the highest concentration of PTU was used. The data show that the in vitro treatment of cells with PTU had no significant effect on the oxidative burst of human neutrophils during phagocytosis of complement-opZy particles.

3.3. Effect of thiamazole on neutrophil CL

The capacity of neutrophils to generate the oxidative burst was also assessed after 1-hour incubation of the cells with thiamazole at the 3 concentrations tested. The cells were stimulated with opZy with a pool of NHS diluted 1:2 in CFD or TEA.

The AUC of the CL profiles over a period of 10 minutes for the test and controls cells stimulated with opZy with a pool of NHS are presented in Table 2A and B.

Differences in respiratory burst generation at any of the thiamazole concentrations studied in vitro compared to controls were not found. The in vitro treatments of cells with thiamazole also did not produce a significant effect on the oxidative burst of human neutrophils during phagocytosis of complement-opZy particles.

4. Discussion

One of the most important functions of neutrophils is the host defense against bacterial infection, a role based on functions such as phagocytosis, degranulation of proteases, and generation of reactive oxygen species (ROS). Phagocytosis can be triggered by ligands that bind to receptors for the Fc region of immunoglobulin G (FcγRII and FcγRIII) and to receptors for complement fragment C3b and its breakdown product iC3b (CR1 and CR3). These complement and Fc receptors have a synergistic action during the phagocytic response of human neutrophils [35,36]. There are phagocytic stimuli that bind to CR1 and FcR, activating

the oxidative burst concomitantly with the phagocytosis of the particles to which they are attached [37]. The mechanism of activation of the oxidative burst can be different for each stimulus. Neutrophils are important in host defense but they are also effectors of tissue damage [38]. The local phagocytosis promotes release of inflammatory mediators, enzymes, and ROS that damage tissue.

Hyperthyroidism determines a net enhancement in the oxidant capacity of PMN [29]. The oxidative stress induced by thyrotoxicosis can aggravate free radical–mediated tissue injury [39-42]. Experimental studies have reported changes in the protective system against ROS mainly in muscle, liver, and kidney [43,44].

Propylthiouracil and thiamazole are used in hyperthyroidism, primarily to decrease the levels of the thyroid hormones, but different drugs of the thionamide class have been reported to ameliorate the oxidant status in hyperthyroid conditions [3,7,9]. Once a reduction of the oxidative stress is achieved, an improvement in susceptibility to free radical damage is expected. In this investigation we studied the effect of these drugs in vitro treatment on the oxidative burst of neutrophils to assess the oxidative burst mediated by complement Fcγ receptors of neutrophils from healthy human donors. This approach can contribute to attribute the high CL responses found in those patients to the thyrotoxicosis state and rule out an action of these drugs in this system.

The measurement of luminol-dependent CL reflects a global cell process depending on the rate of production of ROS and myeloperoxidase activity. In this study we treated cells with thiamazole or PTU in vitro and assessed the capacity of neutrophils to produce luminol-dependent CL when stimulated with opZy. The drug concentrations used were calculated as an estimative of the doses taken by patients, so the thiamazole concentrations were to be correspondent to 90, 60, and 15 mg of the drug. The concentrations added to 4.5 mL of blood were found using a relation with the population median volume of 4500 mL of blood and considering the whole amount of the drug present in the blood stream. The same was done to the PTU concentrations and the doses to be compared were 300, 600, and 2000 mg. Actually, the highest concentrations for both drugs are not used in clinical trials but we wanted a overdose too see if we found any response. The total CL produced did not differ between control and treated cells. The shape of the CL profiles of treated and untreated cells with both drugs studied was very similar. The time to reach the peak of CL (t_{max}) and the time to reach the maximum value of CL (CL_{max}) were not the same for the treated and untreated cells, but were not significantly different. The t_{max} and CL_{max} reflect the rate and efficiency of binding of the stimulus (opZy) to the cells. In the present study, with the in vitro experimental design described, we did not find a reduction or enhancement of the oxidative burst of neutrophils treated with them. Because ROS can be generated via different molecular mechanisms, the observed differences reported in the literature in response to the drugs may be dependent on the mechanism of ROS generation. Differences in drug concentration used, in vivo and vitro experimental conditions, species variation and sensitivity among cell types, routes of administration, procedures, and methods must be also considered.

In conclusion, the present results show that these drugs at the studied concentrations and with the experimental approach used have no effect on the oxidative burst mediated by Fcγ and complement receptors during the neutrophil phagocytosis of complement-opIC when these cells are exposed to the drug in vitro.

Our results contrast with some previous reports, possibly because of differences in the drug concentrations used, in vivo and in vitro experimental conditions, species variation, and sensitivity among cell types. Several studies have shown the antioxidant activity of thionamides, but mainly on erythrocytes and studies focusing on CL production by human neutrophils were not done.

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