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Original Article

INTERACTION OF PROPYLTHIOURACIL WITH MODEL SYSTEMS GENERATING A SUPEROXIDE RADICAL

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Summary

Propylthiouracil is used against Grave's Disease and for developing animal models of hypothyroidism. Apart from depressed metabolism, free radical-induced tissue damage has been registered as an effect from this drug. Superoxide is essential for generation of free radicals in tissues. The mutual effects of Propylthiouracil and superoxide radical have not been well investigated.

The *in vitro* effects of Propylthiouracil on the free radicals in three model systems generating superoxide were measured using luminol-dependent chemiluminescence and spectrophotometry. The drug did not affect the formation of free radicals in the presence of potassium superoxide and in pyrogallol- oxygen solutions, while in the presence of the xanthine/xanthine oxidase system a distinct prooxidant effect was registered. The investigation of the system propylthiouracil/xanthine oxidase showed mild free radicals formation along with decreasing intensities of the drug's UV-specter.

Our *in vitro* investigation indicated that, along with the transformation of xanthine to uric acid over xanthine oxidase, some free radicals may be produced due to the interaction of propylthiouracil with the enzyme. It was proposed that this might contribute to the *in vivo* free radicals-induced tissue damage observed in the presence of propylthiouracil.

Keywords: propylthiouracil, xanthine oxidase, superoxide radical, free radicals formation

Introduction

Thyroid dysfunction has been related to increased formation of Reactive Oxygen Species (ROS) and tissues oxidative damage due to thyroid hormones imbalance [1, 2]. Despite the depressed metabolism due to hypothyroidism, increased ROS formation and oxidative stress (OS) were found both in hypothyroid humans [3, 4] and animals [2, 5]. Enhanced OS was found in tissues of Propylthiouracil (PTU)-induced animal models of hypothyroidism, too [2, 6, 7].

Superoxide radical, $O_2^{\cdot-}$, is involved in the formation of most of the ROS, which are responsible for the OS in tissues [8, 9]. The interaction with $O_2^{\cdot-}$ and the involvement of PTU in the OS via a superoxide pathway are not well investigated [10].

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In the present work, the interaction of superoxide with PTU was monitored in vitro, using three model systems that generate superoxide radical: potassium superoxide (KO_2), pyrogallol (PY), and xanthine/xanthine oxidase (X/XO). The effects were estimated using luminol-dependent chemiluminescence and UV-VIS spectroscopy.

Materials and Methods

Chemicals and solutions

All chemicals used were of finest grade (SIMA-ALDRICH). Deionized water and dehydrated dimethylsulfoxide (DMSO) were used as solvents. The 50 mM K, Na- phosphate buffer (PBS) of pH 7.45 and 50 mM TRISS-HCl of pH=8.2 were prepared and used as media in this investigation.

Standard aqueous solution of propylthiouracil of concentration 10^{-2}%wt was prepared, further diluted to 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3}%wt. The weight percentage was chosen because this type of concentration was used for the standard solutions when developing experimental hypothyroid animal models [2, 5]. Luminol was dissolved in a small amount of 10mM NaOH, diluted with PBS to a concentration of 1mM (pH being adjusted to a level of 7.4), and used as a chemiluminescent reagent. Solutions of KO_2 (1 mM in DMSO), xanthine (3 mM in H_2O), XO (100 UI/L, in PBS), MTT (3mg/ml in PBS), and pyrogallol (3mM, in TRIS-HCl with pH of 8.2) were prepared immediately prior use. All solutions were kept at room temperature. The pyrogallol solution was protected from light in a dark bottle in a light-free container.

Characteristic reactions for the free radicals formation in the presence of in vitro superoxide deriving model systems

The reactions to produce superoxide were as follows: decomposition of KO_2, pyrogallol autooxidation and transformation of xanthine into uric acid in the presence of xanthine oxidase.

The formation of free radicals was monitored using both luminol-dependent chemiluminescence (CL) and spectrophotometric measurements. The MTT-formazan (characteristic at 576 nm) formed by interactions of free radicals with MTT (Thiazolyl Blue Tetrazolium Bromide, SIGMA-ALDRICH) was measured using UV-VIS spectrophotometer. The formation of uric acid (UA) was estimated by monitoring the intensity of its characteristic band at 293 nm. The PY auto-oxidation was monitored by measuring the intensity of the 420 nm band, which is characteristic of the products of this reaction.

Apparatus and data management

The luminal-dependent chemiluminescence was registered using a PC-connected LKB 1251 luminometer (Bioorbit, Turku, Finland) set at 310K, data being collected by Multiuse program, version 1.08 (Bioorbit, Turku, Finland). The Spectrophotometric measurements were collected using a software equipped apparatus Shimadzu version UV1601-1 (Japan), connected to a PC.

Microsoft Excel program was used for data processing, while statistical verification was done by INSTAT program package. Five parallel measurements were performed to calculate the average and standard deviation of each data point in the experiment. The effects of the PTU concentration on the formation of free radicals were presented by the scavenging indices: CL-SI for the luminol-dependent chemiluminescence as mentioned in [11, 12], and SPh-SI for the UV-VIS measurements, as mentioned in [13, 14]. The rate of MTT formazan formation was calculated using the VIS- characteristic adsorption band of the compound, with molar extinction coefficient of 13mM/cm [15].

Luminol-depending CL assays

The luminol-dependent CL in the presence of KO_2 and X/XO was measured as described elsewhere [11, 12]. Briefly, for the KO_2 model system, 1 ml of the sample contained 0.1mM luminol and the desirable concentration of the drug. In the control samples, the drug was omitted. CL was measured immediately after addition of 20µl KO_2, using the "flash assay" option of the data collection software. For the X/XO model system, 1 ml of the solution contained 0.1 mM luminol, 1mM xanthine and PTU of the desirable concentration (for the control measurement the drug was omitted). The CL was registered after adding 20µl of XO solution. The "standard luminescence assay" option of the data collection software was used for the measurements in the presence of this model system.

Each measurement was repeated five consecutive times.

Spectrophotometric measurements

The formation of free radicals in the presence of the desirable concentrations of PTU was monitored by measuring the amount of MTT-formazan formed by the interaction of MTT with the free radicals in the presence of the KO_2 and X/XO generated superoxide. Typically, one ml of the sample solution contained 20 µl KO_2, 50 µl MTT and the drug in the desired concentration, in PBS. For the control measurements, the drug was omitted. For the measurements in the presence of the X/XO system, 1 ml of the sample contained the drug in the desired concentration, 20 µl xanthine, 20 µl XO (100UI/ml), and 50 µl MTT if the MTT formazan was measured. For the control measurements the drug was omitted. The relative change of the intensity of the characteristic band of the MTT formazan at 576 nm was monitored for 10 minutes. The accumulation of UA for 10 minutes was estimated by measuring the intensity of its characteristic band at 293 nm. UV spectra of PTU in the presence of XO were recorded within 400 and 200 nm, against the XO specter. One ml of the sample contained the desired concentration of the drug (10^{-3} or 10^{-4} %wt) and 20 µl XO (100 UI/ml) in PBS. Spectra were recorded immediately and 10 minutes after introducing PTU to the solution.

Data management

For each experimental point, five parallel measurements were recorded and used to calculate the average value and standard deviation of the corresponding parameter. Data from the CL measurements were presented as CL scavenging indices (CL-SI), and those from the spectrophotometric measurements were presented as Spectrophotometric scavenging indices (SPh-SI). In both cases, the parameter observed in the presence of the drug was presented as a percentage of the same parameter in the absence of the drug. Only statistically significant relative differences were the subject of interpretations.

Results

The formation of MTT formazan in the model solutions of KO_2 and pyrogallol was not altered by propylthiouracil, while in the presence of the X/XO the formazan increased with increasing of the drug's concentration (Figure 1).

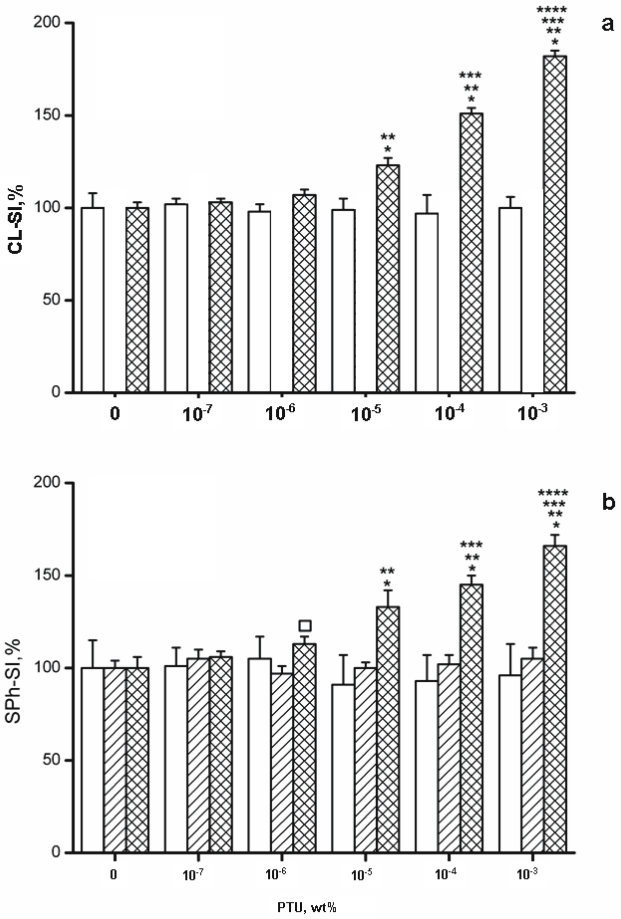

Figure 1. Effect of the concentration of propylthiouracil on the CL-SI (a) and SPh-SI (b) in the presence of superoxide derived from the KO_2 (▭), Pyrogallol (▤) and X/XO (▤) model systems. Statistically significant differences:
* - different than the control (0 %wt PTU), - different from 10^{-7} %wt PTU,
** - different from 10^{-6} %wt PTU,
*** - different from 10^{-5} %wt PTU,
**** - different from 10^{-4} %wt PTU

The effect of PTU on the formation of MTT formazan in the X/XO solution became statistically significant at concentrations of the drug higher than 10^{-6} %wt. In aqueous and PBS solutions containing both xanthine and propylthiouracil no MTT formazan was formed.

In order to better understand the increased MTT formazan formation in the solution containing PTU, xanthine and xanthine oxidase, the formations of MTT formazan and uric acid were estimated at different PTU concentrations (Figure 2).

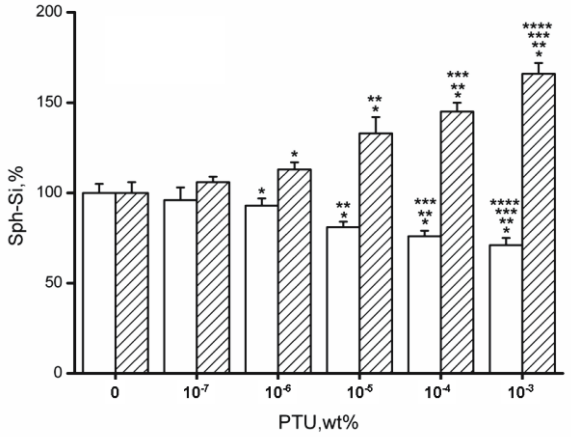

Figure 2. Effect of the PTU concentration on the spectrophotometric scavenging indices of MTT formazan (▤) and uric acid (▭), in the presence of superoxide radical generated by the X/XO model system; statistically significant differences:
* - different than the control (0 %wt PTU),
** - different from 10^{-6} %wt PTU,
*** - different from 10^{-5} %wt PTU,
**** -different from 10^{-4} %wt PTU

The SPh-SI of the uric acid decreased, while the SPh-SI of MTT formazan increased with increasing of the concentration of PTU. This indicated a possible involvement of the XO in a transformation of PTU resulting in free radicals.

To check the possibility for having interactions between propylthiouracil and xanthine oxidase, the transformation of MTT to MTT formazan was investigated in a system, containing the drug and the enzyme, in the absence of xanthine. The rates of formation of MTT formazan were calculated in pmoles/min. UV spectra of the propylthiouracil (at concentrations of 10^{-3} and 10^{-4}%wt) were recorded against the xanthine oxidase spectrum, in the reaction medium, immediately after adding the drug to the xanthine oxidase solution, and 10 minutes later. The results are presented in Figure 3a. These results were compared with the rates of formation of MTT formazan in the presence of various concentrations of PTU at fixed amounts of xanthine and XO (Figure 3b).

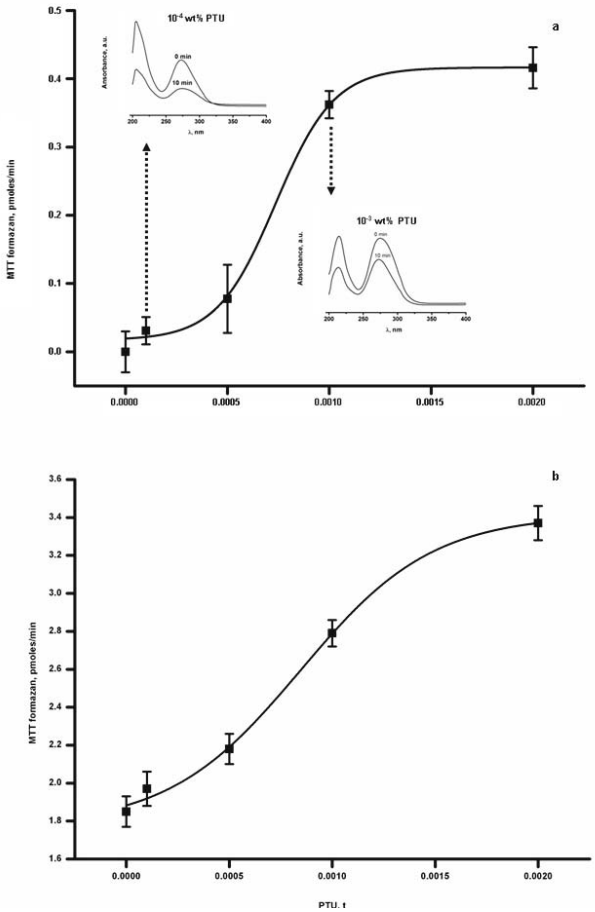

Figure 3. Effect of PTU concentration (% wt) on the rate of MTT formazan formation in the presence of XO (a) and X/XO (b)

In the presence of propylthiouracil and xanthine oxidase, small amount of MTT-formazan was formed, accompanied with relatively decreased intensities of the UV-spectra of the drug (Figure 3a). The maximum rate of MTT formazan formation of (0.4160.01) pmoles/min was reached at PTU concentrations above 0.001% wt (Figure 3a), with half maximal effective concentration EC_{50} of $7.24*10^{-4}$%wt. The rate of formation of MTT formazan in the X/XO solution alone was (1.850.01) pmoles/min (Figure 3b). The increase of the PTU concentration from 0 to 0.002%wt resulted in a relative increase of this rate to 3.370.02 pmoles/min, with EC_{50} of $8.57*10^{-4}$%wt. The increase of the PTU concentration from 0 to 0.002%wt in the X/XO model system resulted in a 3.65 times higher rate of MTT formazan formation, as compared to the corresponding rate in the absence of xanthine.

Discussion

At concentrations below 200μM (corresponding to 0.003%) propylthiouracil did not directly interact with superoxide derived from the X/XO system [11]. In our investigations, the maximal concentration of the drug was 0.002%.

The data presented in Figure 1 suggested no direct interaction of PTU molecule with the O_2^- derived from the KO_2 and pyrogallol solutions. In the presence of X/XO derived superoxide, the free radicals formation increased with increasing of the concentration of PTU up to 0.002% (Figure 1). Considering the observations of [11], the latter might be a result of interactions of propylthiouracil either with xanthine, or with xanthine oxidase, or both. The UV spectra of solutions containing xanthine and PTU revealed no interactions between the two molecules resulting in free radicals formation. In the system (PTU+X)/XO, the increase of the drug's concentration resulted in a decrease of the UA formation and an increase of the formation of MTT formazan (Figure 2). The lack of free radicals in solutions containing drug and xanthine, along with data in Figures 2 and 3, suggested that some PTU might react with the xanthine oxidase producing free radicals.

Our investigation suggested that at *in vitro* conditions propylthiouracil might act as a competitive inhibitor of the xanthine transformation to uric acid over xanthine oxidase. In the presence of xanthine oxidase some of the PTU molecules transform into free radicals over the xanthine oxidase.

Conclusions

Propylthiouracil at concentrations below 0.002 %wt did not interact directly with superoxide radical derived from model systems, containing potassium superoxide and pyrogallol.

Along with the transformation of xanthine to uric acid over xanthine oxidase, some free radicals may be produced due to an interaction of propylthiouracil with the enzyme. It was proposed that this might contribute to the *in vivo* free radicals-induced tissue damage observed in the presence of propylthiouracil.

Acknowledgement

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