Effect of Antithyroid Drugs on Hydroxyl Radical Formation and α-1-Proteinase Inhibitor Inactivation by Neutrophils: Therapeutic Implications

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ABSTRACT

The release of proteolytic enzymes and generation of strong oxidants such as the hydroxyl radical by activated neutrophils has been proposed to play an important role in mediating toxin-induced liver injury. The antithyroid drug propylthiouracil protects against liver injury induced by many hepatotoxic agents and markedly reduces mortality in patients with alcoholic liver disease. However, the mechanism(s) by which propylthiouracil protects against liver injury is not well understood. The present studies investigate the effect of antithyroid drugs on proteolytic enzyme activity and on hydroxyl radical generation from activated neutrophils. In the presence of hydrogen peroxide and chloride, neutrophil myeloperoxidase, an enzyme from the same gene superfamily as thyroid peroxidase, generates hypochlorous acid which inactivates α-1-proteinase inhibitor (A1PI) present in serum. This inactivation allows neutrophil-released proteolytic enzymes to attack cells. In the present study myeloperoxidase activity was inhibited fully at therapeutic concentrations by antithyroid drugs (propythiouracil and methimazole). Antithyroid drugs fully prevented hypochlorous acid formation, and prevented neutrophil-mediated inactivation of A1PI, with concomitant blockage of proteolytic activity. Conversely, generation of both superoxide and hydroxyl radicals by activated neutrophils was unaffected by propylthiouracil. The production of these oxygen radicals was fully inhibited by the NADPH oxidase inhibitor diphenylene iodonium chloride, however. These studies indicate that antithyroid drugs are unlikely to prevent cell injury by inhibiting hydroxyl radical generation or by scavenging hydroxyl radicals, but are likely to exert their hepatoprotective anti-inflammatory action by inhibiting neutrophil myeloperoxidase, an enzyme akin to thyroid peroxidase.

Propylthiouracil, a drug widely used in the treatment of hyperthyroidism, also protects against various forms of inflammatory liver injury in rats, including that induced by hypoxia-reoxygenation in ethanol-fed animals (Israel et al., 1975), acetaminophen (Raheja et al., 1982), carbon tetrachloride (Orrego et al., 1976), thioacetamide (Oren et al., 1986) and galactosamine (Cooper et al., 1984). In clinical studies PTU resulted in a 50% to 60% reduction in mortality rates from alcoholic liver disease (Orrego et al., 1987). However, subsequent use of PTU in the treatment of alcoholic liver disease has been limited mainly because of a lack of understanding regarding the mechanism of drug action.

A common finding in the aforementioned conditions is the presence of neutrophilic infiltrates in the liver. Reactive oxygen species and proteolytic enzymes are released by activated neutrophils during the respiratory burst, both of which have been proposed to play an important role in mediating the cell injury associated with inflammation (Smith, 1994). The formation of the highly toxic hydroxyl radical may play an important role in neutrophil-mediated tissue injury, as suggested by the observation that hydroxyl radical scavengers can protect target tissues from neutrophil-mediated damage in vitro and in vivo (Ward et al., 1983; Fox, 1984). NADPH oxidase (NADPH-FMN oxidoreductase, EC 1.6.99.1), an enzyme present in the neutrophil membrane, catalyzes the formation of superoxide which undergoes spontaneous and superoxide dismutase-catalyzed transformation to hydrogen peroxide, resulting in the formation of hydroxyl radical by the iron-catalyzed Haber-Weiss reaction. The rel-
evance of the latter reaction and a possible alternative mechanism for hydroxyl radical formation is considered under “Discussion.”

Hicks and co-workers (1992) showed that PTU has a powerful antioxidant action, scavenging hydroxyl radical and preventing liperoxidation, and proposed that PTU could protect against liver injury by such a mechanism.

An alternative mechanism is also conceivable. PTU and MMI, a related antithyroid drug, have been shown in vitro to inhibit myeloperoxidase (EC 1.11.1.7) (Taurog and Dorris, 1992), an enzyme present in neutrophil azurophilic granules that catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ions. Recent studies indicate that neutrophil myeloperoxidase is a member of the same gene superfamily as thyroid peroxidase (Kimura and Ikedo-Saito, 1988), which iodinates thyroglobulin, a precursor of thyroid hormones.

Hypochlorous acid oxidizes several biochemical targets (Arnhold et al., 1991), including the proteinase inhibitor a-1-antitrypsin (or A1PI). A1PI is generated by the liver and released into the plasma where it rapidly and irreversibly inactivates neutrophil-derived serine proteases such as elastase (Travis and Salvesen, 1983), which can otherwise degrade extracellular structural proteins and induce injury to host tissue (Gadek and Crystal, 1983; Wong and Travis, 1980).

Recent studies have suggested that hypochlorous acid generated by myeloperoxidase may contribute to the production of hydroxyl radical by a reaction that does not require the presence of a transition metal catalyst (1) (Candeias et al., 1993; Ramos et al., 1992). However, the relative contribution of this mechanism to the overall formation of hydroxyl radicals is not clear.

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\text{HOCI} + \text{O}_2^- \cdot \rightarrow \text{OH}^- + \text{O}_2 + \text{Cl}^- \quad (1)
\]

Because the generation of hypochlorous acid by myeloperoxidase may lead to either hydroxyl radical formation or to A1PI inactivation, an inhibitory effect of antithyroid drugs on myeloperoxidase might contribute a dual mechanism in the protection against tissue injury. The purpose of the present study was to determine the effect of therapeutically relevant concentrations of antithyroid drugs on the neutrophil-mediated inactivation of A1PI and on the formation of reactive oxygen species (superoxide, hypochlorous acid and hydroxyl radicals) in rat neutrophils. A second objective was to determine the relative contribution of myeloperoxidase to the generation of hydroxyl radicals by neutrophils. For this purpose we also have used chicken neutrophils, which are naturally devoid of myeloperoxidase (Rausch and Moore, 1975), to study the formation of reactive oxygen species.

**Methods**

**Reagents.** A1PI, ammonium chloride, catalase (38,000 U/mg protein, from bovine liver), DFO, EDTA, DTNB, DTPA, ferricytchrome c (from horse heart), heparin (176 U/mg protein, from porcine intestinal mucosa), MMI, PMA, porcine pancreatic elastase (52.8 U/mg protein), PTU, 4-POBN, sodium carbonate monohydrate (KHCO₃), SLAPN, superoxide dismutase (4000 U/mg protein, from bovine erythrocytes), tauroine and trypan blue (0.4%) were obtained from Sigma Chemical Co. (St. Louis, MO). d-PBS, HBSS without Ca²⁺, Mg²⁺ or phenol red and Tris HCl were purchased from Gibco (Grand Island, NY), dextran T-500 and Percoll, from Pharmacia (Piscataway, NJ), [1-13C]ethanol from Aldrich (St. Louis, MO), Chelex-100 chelating resin from Bio-Rad Laboratories (Richmond, CA), EDTA-coated Vacutainer tubes from Becton Dickinson (Franklin Lakes, NJ) and 100% ethanol from RDL Alcohols. Diphenylene iodonium chloride (Biomo Research Laboratories, Plymouth Meeting, PA) and PMA were prepared in dimethyl sulfoxide (Fisher, Fair Lawn, NJ) as 1 mg/ml stock solutions and stored at −20°C. HBSS was pretreated with Chelex-100 resin to remove residual iron contamination according to the method of Buettner (1988). Sterile stock solutions of Percoll were prepared weekly in 10× d-PBS, stored at 4°C and diluted to working concentrations with 1× d-PBS (pH 7.4). TNB was synthesized from DTNB according to the method of Riddles et al. (1983). DENH (0.5 M) was dissolved in 50 mM potassium phosphate buffer (pH 7.4) and titrated to pH 12 with 10 N NaOH. After 5 min, the pH was lowered to 7.4 with 10 N HCl, yielding a 1 mM solution of TNB.

**Hypochlorous acid formation.** The production of hypochlorous acid was determined from the formation of taurocholine and the subsequent oxidation of TNB to DTNB (Weiss et al., 1982).

**Discussion.**
Eppendorf tubes containing rat or chicken neutrophils (5 × 10^6 cells/ml), taurine (20 mM) and PMA (100 ng/ml) were incubated for 30 min at 37°C. The reaction was stopped by the addition of catalase (50 μg/ml), and the tubes were placed on ice for 10 min. Cells were removed by centrifugation at 14,000 × g at 4°C for 5 min. Supernatant (800 μl) was combined with 200 μl of TNB (0.2 mM final) and incubated in the dark at room temperature for 10 min. The concentration of DTNB in duplicate samples was determined spectrophotometrically from the loss of absorbance at 412 nm with an extinction coefficient of 13.6 mM^-1 cm^-1 (Ellman, 1959). Inhibitors were added as described above for O_2^- measurements.

**A1PI activity.** The elastase inhibitory activity of A1PI was measured as described previously (Travis and Johnson, 1982). Reaction mixtures contained neutrophils (1 × 10^6 cells), A1PI (138 μg/ml), PMA (100 ng/ml) and HBSS (pH 7.4) in a total volume of 250 μl. Additional samples were prepared as described above, with the addition of PTU (100 μM), DPI (10 μM) or desferrioxamine (100 μM). Samples were incubated for 30 min at 37°C, placed on ice for 3 min and centrifuged at 14,000 × g for 5 min. Porcine pancreatic elastase (1.5 U/ml) was combined with 100 μl of the cell-free supernatant and 0.2 M Tris buffer (pH 8.0) in a total volume of 500 μl. After a 10-min incubation at room temperature, samples were heated and the increase in absorbance at 410 nm was measured spectrophotometrically in duplicate samples during a 3-min interval. The rate of formation of β-nitroanilide was calculated with an extinction coefficient of 8.8 mM^-1 cm^-1 (Bieth et al., 1974). Results were expressed as a percentage in comparison with samples incubated with A1PI in the absence of neutrophils and inhibitors. In these samples elastase activity was inhibited by 50% to 75%. Inhibitors had no effect on the activity of A1PI in the absence of activated neutrophils and did not interfere directly with the assay of elastase activity.

**ESR/spin trapping of α-hydroxyethyl radicals.** The ability of stimulated neutrophils to form hydroxyl radicals was measured by spin trapping of the α-hydroxyethyl radical with 4-POBN and ESR detection of the stable 4-POBN/α-hydroxyethyl spin-trap adduct (Ramos et al., 1992). Rat or chicken neutrophils (2 × 10^7 cells/ml) were combined with 4-POBN (20 mM), ethanol (170 mM), DTPA (100 μM), PMA (100 ng/ml) and HBSS (pH 7.4) in a total volume of 300 μl. Inhibition studies were carried out with the addition of PTU (100 μM), DPI (10 μM) or desferrioxamine (100 μM). Samples were vortexed, immediately transferred to a quartz ESR flat cell cuvette and placed in the cavity of a Bruker Instruments ER200D/ESP 3220 spectrometer system. Spectra were recorded for 15 min at 37.0 ± 0.5°C with instrument settings as follows: microwave frequency, 9.63 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; sweep width, 65 G; sweep time, 84 sec; number of scans, 10; time constant, 52 nsec.

**Cytotoxicity.** Neutrophil viability in the presence of test compounds was assessed by trypan blue exclusion. Rat neutrophils (5 × 10^6 cells/ml) were suspended in HBSS with PMA (100 ng/ml), PTU (100 μM), DPI (10 μM) or desferrioxamine (100 μM) in a total volume of 500 μl and incubated for 30 min at 37°C. An aliquot of the mixture was combined with 1.0% trypan blue in 0.9% saline, and the percentage of trypan blue-negative neutrophils was determined with a hemocytometer. The viability of rat neutrophils incubated with these agents did not differ significantly from that of control neutrophils. All viabilities exceeded 90%.

**Statistics.** Results are expressed as the mean ± standard error for a minimum of three experiments. Differences between groups were determined by analysis of variance followed by Neuman-Keuls post hoc tests. Results were considered significant at P < .05.

**Results**

Hypochlorous acid was generated by rat neutrophils (1 × 10^6 cells/ml) activated with PMA (100 ng/ml) at the rate of 16.1 ± 2.7 nmol/10^6 cells/30 min, but was not formed by nonactivated neutrophils. PTU and MMI inhibited the synthesis of hypochlorous acid by rat neutrophils (fig. 1) with a K_i of 6 μM for PTU and 20 μM for MMI. The NADPH oxidase inhibitor DPI also inhibited hypochlorous acid formation with a K_i of 0.20 μM.

Rat neutrophils stimulated with PMA generated superoxide at a rate of 23.9 ± 2.2 nmol/10^6 cells/30 min. In the absence of PMA the synthesis of superoxide was negligible (<5%) (not shown). The antithyroid agents PTU and MMI (up to 100 μM studied) did not have an effect on superoxide synthesis (fig. 2). The NADPH oxidase inhibitor DPI (10 μM) completely inhibited the synthesis of superoxide (apparent K_i = 0.20 μM).

Figure 3 shows the inhibitory effect of activated rat neutrophils on the activity of A1PI in the elastase assay. PMA-activated neutrophils reduced the inhibitory potency of A1PI to 35% of control values, thus markedly increasing elastase activity. PTU (100 μM) inhibited the inactivation of A1PI exerted by stimulated neutrophils by about 90%, which was not significantly different from A1PI activity in the absence of activated neutrophils. The NADPH oxidase inhibitor DPI (10 μM) blocked neutrophil-mediated A1PI inactivation by approximately 60%, whereas the iron chelator desferrioxamine (100 μM) had no effect on the neutrophil-induced inactivation of A1PI.

The generation of hydroxyl radicals by neutrophils was determined from the reaction of hydroxyl radical with ethanol. Stimulation of rat neutrophils by PMA in the presence of ethanol (170 mM), the chelator DTPA (100 μM) and the spin-trap 4-POBN resulted in a 6-line ESR spectra (fig. 4), with hyperfine splitting constants characteristic of the α-hydroxyethyl adduct of 4-POBN (a_H = 15.6 G; a_A = 2.6 G) (Ramos et al., 1992). Neither PTU (100 μM) nor the iron-chelating agent desferrioxamine (100 μM) affected the synthesis of hydroxyl radicals. In contrast, the NADPH-oxidase inhibitor DPI (10 μM) fully inhibited the hydroxyl radical-generated ESR signal. To confirm the identity of the 4-POBN spin-trap adduct, [1,2-^3^C]ethanol was substituted for [^1^H]ethanol in the incubations. The resulting 12-line...
spectra confirmed that the trapped species in fact was derived from ethanol.

Myeloperoxidase-deficient chicken neutrophils stimulated with PMA (100 ng/ml) generated superoxide at a rate of 30.0 ± 6.3 nmol/30 min/10^6 cells, but did not generate hypochlorous acid (0.8 nmol/30 min/10^6 cells). The generation of hydroxyl radicals by chicken neutrophils (fig. 5) was of the same order of magnitude as that observed with rat neutrophils. PTU (100 μM) did not affect the generation of hydroxyl radicals but DPI (10 μM) fully inhibited the hydroxethyl radical-generated ESR signal, which indicates that in these cells, as in rat neutrophils, hydroxyl radical formation depends completely on NADPH oxidase activity but occurs independently of myeloperoxidase.

Discussion

This study offers several insights into the possible mechanism by which antithyroid drugs can protect against toxin-induced cell injury. In the present investigation, antithyroid drugs showed a concentration-dependent inhibition of myeloperoxidase-dependent hypochlorous acid formation in stimulated rat neutrophils. Hypochlorous acid formation was inhibited by PTU at concentrations (K_i < 56 μM) well below those used in the therapy of alcoholic liver disease (peak concentration of 43 μM after a 300-mg oral dose of PTU) (Orrego et al., 1987; Long et al., 1983).

PTU has been reported to inhibit the catalytic activities of both myeloperoxidase (Taurog and Dorris, 1992) and thyroid peroxidase (EC 1.11.1.7) (Taurog and Dorris, 1989). The latter enzyme plays an important role in the synthesis of thyroid hormones (DeGroot and Niepomniszcze, 1977) and displays considerable amino acid homology with myeloperoxidase (Kimura and Ikeda-Saito, 1988). In early studies we postulated that drug-induced hypothyroidism was
the main cause for the protection of alcohol-induced liver injury (Israel et al., 1975). However, subsequent studies showed that in some forms of toxin-induced liver injury, such as that produced by galactosamine and aceterminophen, PTU exerts a protective effect independently of circulating thyroid hormone levels (Raheja et al., 1982; Cooper et al., 1984). These observations are in line with an inhibition by antithyroid drugs of myeloperoxidase rather than thyroid peroxidase contributing to the hepatoprotective effect.

Inhibition of myeloperoxidase-dependent hypochlorous acid formation may be an important mechanism of hepatoprotection because it can promote tissue injury by 1) inactivating A1PI or 2) generating hydroxyl radicals. A reduction in A1PI activity, resulting from either its inactivation or deficiency, has been implicated in several disease states such as emphysema, cystic fibrosis and rheumatoid joint disease in which oxidized A1PI and/or elevated levels of free elastase are detectable in tissue fluids (Gadek and Crystal, 1983; Wong and Travis, 1980). In the present study PTU afforded nearly complete protection of A1PI against neutrophil-mediated damage, an effect which may account for the therapeutic effect of PTU in the treatment of alcoholic liver disease and other forms of toxin-induced liver injury. The possible involvement of neutrophil-mediated inactivation of A1PI as a mechanism in alcoholic liver injury has not yet been investigated extensively, but is suggested by the enhanced susceptibility to spontaneous and alcohol-induced liver disease in individuals with genetic A1PI deficiency. The incidence of the heterozygous A1PI variant in patients with alcoholic liver disease is reported to be 250% higher than in the healthy population, whereas homozygotes for the mutant A1PI allele frequently develop spontaneous liver fibrosis and cirrhosis (Pott et al., 1983). Acetaldehyde, generated during ethanol metabolism, also can inactivate A1PI and may help promote neutrophil-mediated proteolytic injury of the liver (Brecher et al., 1994).

The results of the present study indicate not only a mechanism by which PTU may protect the liver from toxin-induced injury, but also the possibility that PTU may be effective in treating a variety of other diseases characterized by neutrophil-mediated tissue injury. The recent demonstration (Elias et al., 1994) that psoriasis, an autoimmune condition characterized by neutrophil accumulation, also can be treated effectively with PTU supports this conclusion.

Because myeloperoxidase and hypochlorous acid previously have been shown to contribute to the formation of hydroxyl radicals by neutrophils (Ramos et al., 1992; Candiaseas et al., 1993), it was hypothesized that antithyroid drugs would inhibit hydroxyl radical formation and that this may be a potential mechanism of hepatoprotection. However, the data presented here showed that hydroxyl radical generation, measured by ESR as the hydroxyethyl radical, was not altered by PTU at concentrations sufficient to fully inhibit the myeloperoxidase-dependent production of hypochlorous acid. In addition, the lack of effect of PTU on hydroxethyl radical formation tends to rule out the possibility that PTU acts as a hydroxyl radical scavenger in vivo, as has been suggested on the basis of prior studies with cell-free systems (Hicks et al., 1992). These observations prompted additional studies to delineate the enzymatic mechanisms of hydroxyl radical production in neutrophils.

Is there a role for myeloperoxidase and hypochlorous acid in hydroxyl radical generation?

The lack of involvement of myeloperoxidase and hypochlorous acid on the formation of hydroxyl radicals was suggested by the inability of PTU to inhibit hydroxyl radical generation. This was confirmed further by the finding that myeloperoxidase-null chicken neutrophils, which did not generate hypochlorous acid, produced hydroxyl radical at rates that were similar to those observed in myeloperoxidase-rich rat neutrophils. In contrast, the production of superoxide anion was essential to generate hydroxyl radicals, as seen by the complete abolition of hydroxynyl radical production in the presence of the NADPH oxidase inhibitor DPI in both chicken and rat neutrophils. To minimize the contribution of hydroxyl radical formation by the iron-dependent Haber-Weiss reaction, the incubation buffers in the present study were treated with Chelex resin to remove free iron and contained the iron chelator DTPA. Hydroxyl radical formation was evident despite these procedures and was not affected by substitution of DTPA with the more potent iron chelator desferoxamine. Prior studies suggest that in neutrophils hydroxyl radical production by the Haber-Weiss reaction, which requires free intracellular iron as a catalyst, occurs only when exogenous ferrous iron (Fe^{2+}) is added to the neutrophil incubation medium (Ramos et al., 1992). In our experience, addition of ferrous iron to a physiological buffer containing ethanol-generated hydroxynyl radicals per se even in the absence of neutrophils (unpublished data). This observation confirms studies by Reinke et al. (1994) which show that autooxidation of Fe^{2+} generates species that react with ethanol to produce hydroxynyl radicals, without the involvement of a hydroxyl radical intermediate. The ability of neutrophils to endogenously generate hydroxyl radical via the Haber-Weiss reaction also has been questioned on the basis that the availability of free iron in the neutrophil may be limited by the iron-binding protein lactoferrin (Gutteridge et al., 1981). However, because the actual concentration of free iron in neutrophils is unknown and the ability of extracellular iron chelators to alter intracellular free iron substantially is not clear, hydroxyl radical synthesis in neutrophils by the Haber-Weiss reaction cannot be ruled out.

An alternative explanation for the ability of rat neutrophils to generate hydroxyl radical via superoxide in the absence of exogenous iron, is that the hydroxyl radical is not derived from the Haber-Weiss reaction, but rather from the decomposition of peroxynitrite. Peroxynitrite is formed by the reaction of superoxide and nitric oxide radicals and decomposes to produce hydroxyl radicals by a mechanism that is iron-independent (Beckman et al., 1990). A variety of cell types including neutrophils, which simultaneously generate nitric oxide and superoxide (Rodenas et al., 1995), in addition might produce hydroxyl radicals from the decomposition of peroxynitrite. Future studies should determine the relative role of this mechanism.

In summary, the data obtained in the present studies indicate that inhibition of neutrophil-mediated hypochlorous acid formation and A1PI inactivation, rather than decreased hydroxyl radical formation or a hydroxyl radical-scavenging effect, are the likely mechanisms by which antithyroid drugs protect against neutrophil-mediated tissue injury in a variety of pathological conditions.
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