Protective Effect of Propylthiouracil Independent of Its Hypothyroid Effect on Atherogenesis in Cholesterol-Fed Rabbits

PTEN Induction and Inhibition of Vascular Smooth Muscle Cell Proliferation and Migration

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Background—Propylthiouracil (PTU) is used to treat hyperthyroid patients by its hypothyroid effect. PTU also is found to have potent antioxidant and immunosuppressive effects. These findings suggest that PTU may play a role in the prevention of atherosclerosis.

Methods and Results—This study evaluates the effect of PTU on atherosclerotic change in rabbits fed a high-cholesterol diet. The pronounced atherosclerotic lesions in the aortas of rabbits fed a 2% cholesterol diet for 12 weeks were significantly attenuated by the concurrent addition of 0.1% PTU to the drinking water. However, exogenous supplementation of thyroid hormone in hypothyroid PTU-treated rabbits did not abrogate the protective effect of PTU on atherogenesis. Immunohistochemical analysis showed that PTU administration apparently reduced the intimal smooth muscle cell/macrophage ratio in the atherosclerotic plaques of rabbits fed a 2% cholesterol diet. In vitro, the addition of PTU to the medium of cultured rat vascular smooth muscle cells led to a dose-dependent inhibition of cell proliferation and migration. Furthermore, this study confirmed that PTU dose-dependently increased expression of PTEN, a tumor suppressor gene known to be involved in the coordinate inhibition of VSMC proliferation and migration.

Conclusions—This study demonstrated that PTU inhibited the development of atherosclerosis through a thyroid-independent mechanism that may be explained, at least in part, by the ability of PTU to inhibit vascular smooth muscle cell proliferation and migration. Furthermore, PTEN induction, via disruption of the phosphatidylinositol 3–kinase-mediated pathway, plays a crucial role in mediating the inhibitory action on vascular smooth muscle cell proliferation and migration. (Circulation. 2004;110:1313-1319.)

Key Words: atherosclerosis ■ propylthiouracil ■ muscle, smooth, vascular ■ tumor suppressor proteins

Atherosclerosis involves a complex interaction between the cells of the arterial wall and various blood components, such as lipoproteins, platelets, and monocyte-derived macrophages. The pathogenesis of atherosclerosis is generally accepted as being a reaction to injury. In response to vascular injury, such as in the condition of hypercholesterolemia, vascular smooth muscle cells (VSMCs) can migrate and proliferate. These proliferating VSMCs deposit the extracellular matrix and accumulate lipid in the intimal layer. A high-fat diet has been reported to induce hypercholesterolemia, thus promoting atherogenesis in both humans and experimental animals.

Propylthiouracil (PTU) is used routinely in the treatment of hyperthyroidism. PTU exerts its hypothyroid effect by inhibiting iodide oxidation, moniodotyrosine iodination, and coupling steps in thyroxine production, as well as the peripheral conversion of thyroxine (T₄) to triiodothyronine (T₃). Beyond the hypothyroid effect, PTU has been demonstrated to reduce alcohol-induced hepatocyte damage and severe alcoholic liver disease. Although the mechanism by which PTU operates has yet to be elucidated, evidence exists that PTU may act as an antioxidant. Several antioxidants, such as probucol, butylated hydroxytoluene, and vitamin E, have been shown to prevent the progression of atherosclerosis. Therefore, we hypothesized that PTU may inhibit the development of atherosclerosis. Accordingly, investigating the effect of PTU on atherogenesis in the aortas of rabbits fed a high-cholesterol diet provided a very interesting means of testing this hypothesis. The present study especially focused on the interaction of hypercholesterolemia, PTU, and thyroid hormone. Specifically, this study sought to determine whether the atherogenic response of rabbits to a high-cholesterol diet could be modified by concurrent treatment with PTU and, if so, whether the effect was associated with the hypothyroid effect of PTU. The findings of this study confirmed that PTU, independent of its hypothyroid effect, protected against atherosclerosis.
Methods

Animals and Experimental Protocol
Sixty male New Zealand White rabbits (Laboratory Animal Center of National Taiwan University Hospital, Taipei, Taiwan) weighing 2.2 to 2.5 kg were randomly assigned to 1 of 6 diet groups: (1) control chow; (2) 0.1% PTU (Sigma) in the drinking water; (3) 0.1% PTU in the drinking water plus 15 μg of T3 (Sigma) per week intramuscularly; (4) 2% cholesterol diet (Purina Mills Inc); (5) 2% cholesterol diet with 0.1% PTU in the drinking water; and (6) 2% cholesterol diet with 0.1% PTU in the drinking water plus 15 μg of T3 per week intramuscularly. The T3 dose was designed to normalize thyroid hormone levels.

At the end of 12 weeks of study, the rabbits were euthanized, and the aortas and femoral arteries were removed, stained with Sudan III, or fixed with 4% paraformaldehyde and paraffin-embedded for hematoxylin-eosin staining or immunohistochemical analysis.

Biochemical Measurement
Thyroid hormone index (ie, T3, T4, and thyrotropin) was obtained with the use of the Automated Chemiluminescence System (Centaur, Bayer). Lipids (serum total cholesterol and lipoproteins [HDL, LDL, and VLDL cholesterol]) were measured enzymatically with a Hitachi Automatic Analyzer (model 7450).

Morphometric Measurement
The specimens were stained with hematoxylin-eosin. The intimal and medial areas were measured for each cross section, and the intima/media area ratio was determined with the use of IMAGE-PRO PLUS (Media Cybernetics Inc).

Immunohistochemistry
The arterial sections were incubated with mouse monoclonal antibodies against specific cell markers for VSMCs (α-actin; Dako Corp) or rabbit macrophages (RAM 11; Dako Corp). Subsequently, immunohistochemistry was performed with the Dako LSAB peroxidase kit and Dako liquid DAB plus substrate-chromogen system according to the procedures provided by the manufacturer.

Serum PTU Levels
Serum PTU levels were measured by high-performance liquid chromatography (HPLC) with mephenoxalone (Tung Yang Chemical Industries) as an internal standard. One hundred microliters of serum was placed into a tube containing 25 μL of mephenoxalone (1 mg/mL). The contents of each tube were extracted with 2 mL of methyl tert-butyl ether. Tube contents were mixed and centrifuged for 10 minutes at 3000 rpm at 25°C. The supernatant was transferred to a glass tube, dried under nitrogen gas, and reconstituted with 300 μL of mobile phase. HPLC was performed with a Hitachi HPLC system. An ODS column (Cosmosil SC18-MS, 4.6×250 mm, 5 μm; Nacalai Tesque) was used for the chromatographic separation, with a UV detector set at 275 nm. The mobile phase (acetonitrile/water/phosphoric acid, 40/80/0.5, vol/vol/vol) was degassed and filtered before use. The column flow rate was 1.0 mL/min, and the column temperature was maintained at 40°C.

Cell Culture
Rat VSMCs (passages 5 to 10) were prepared by enzymatic digestion of the thoracic aortic media from Sprague-Dawley rats and cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum as described.10

Cell Proliferation Assays
Cells were plated at a density of 10,000 cells/cm² in 24-well plates. After 24 hours, the cells were treated with PTU dissolved in 100% dimethyl sulfoxide at final concentrations of 0.05 to 5 μmol/L for 24 to 72 hours. Cells were then washed, followed by the addition of 1 mL DMEM containing 0.05 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma). After incubation at 37°C for 1 hour, MTT assay to determine the mitochondrial activity of VSMCs was performed as described.11 For the thymidine incorporation study, 90% of confluent cells were deprived of serum for 48 hours to make them quiescent. The cells were then fed with the fresh 10% serum and labeled with methyl-[3H]thymidine at 1 μCi/mL from 12 to 24 hours after serum stimulation, after which thymidine incorporation was measured by trichloroacetic acid (TCA) counting.12

Cytotoxicity and Apoptosis
To determine cytotoxicity, VSMCs were gently trypsinized, and their ability to exclude trypan blue was evaluated. To assess apoptosis, the morphology of propidium iodide–stained DNA in the cells was examined by fluorescent microscopy. Cells with condensed or fragmented chromatin were identified as apoptotic cells.

Migration Assay
VSMC migration was assayed with the use of Transwell filters (6.5-mm diameter, 8-μm pore size, polycarbonate membranes) (Corning). The basal chamber of each Transwell contained 500 μL of high-serum medium in the presence or absence of PTU (0.05 to 5 mmol/L). The apical chamber of each Transwell contained VSMCs (1×10⁵) in 200 μL of nonserum medium. After a 5-hour incubation, cells that migrated from the apical to the basal chamber surface were stained with 5% Liu’s stain and were counted.

Western Blot Analysis
Samples of 30 μg protein per lane were subjected to 12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated for 2 hours with monoclonal anti-phospho-PTEN, anti-Akt, anti–phospho-Akt (Ser473) (Cell Signaling), or anti-tubulin antibodies (NeoMarkers). The membrane was then incubated in PBS containing goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma) for 1 hour. Membranes were washed, and positive signals were developed with the use of Dako liquid DAB plus substrate-chromogen system.

Statistical Analysis
Data are expressed as mean and SD. We used 1-way ANOVA with the Tukey multiple comparison test to compare continuous data among groups. All probability values presented are 2 sided, and the significant level was set at 0.05.

Results
The Table compares the values of the 57 rabbits in 6 groups, each given a different diet/drug combination. Three animals did not complete the study. Two animals in the cholesterol diet group died because of cerebral vascular attack, and 1 in the cholesterol/PTU/T3 group died because of poor intake with unknown cause. At the end of the PTU treatment, the hypothyroid PTU-treated rabbits displayed no reduction in body weight, despite a decrease in water and chow intake. Hypothyroidism was confirmed by elevated thyrotropin levels. The PTU and cholesterol/PTU groups each exhibited a significant elevation of serum thyrotropin (7.2±0.9 and 6.8±0.8 μIU/mL, respectively) compared with the control, PTU/T3, cholesterol, and cholesterol/PTU/T3 groups (0.30±0.05, 0.32±0.04, 0.35±0.02, and 0.29±0.05 μIU/mL, respectively; P<0.01) during euthanasia. As expected, serum T3 and T4 decreased to unmeasurable levels after 4 weeks of treatment with PTU. This hypothyroid effect could be reversed by a concomitant supplementation with T3 (Figure 1).

Plasma lipid levels, especially serum cholesterol levels, rose rapidly after high-cholesterol feeding. However, there was no significant difference in cholesterol and lipoprotein levels among the 3 groups (groups 4 to 6) fed a high-cholesterol diet. However, compared with the control group, rabbits that consumed PTU only (group 2) showed a slight increase in serum cholesterol, LDL, and VLDL levels.
Effect of PTU on Atherosclerosis

Figure 2A shows representative examples of fatty-streak distribution in the aortas of cholesterol-fed and cholesterol-fed/PTU-treated rabbits. The presence of 2% cholesterol in the diet resulted in the formation of grossly visible, yellow, atheromatous lesions, predominantly in the aortic arch and thoracic aorta. The extent of sudanophilic areas was markedly less in cholesterol-fed/PTU-treated rabbits. In aortas, the extent of atherosclerosis in terms of intima/media area ratio was decreased significantly in the ascending aorta, aortic arch, and femoral arteries (Figure 2B). There was an inverse relationship between levels of serum PTU and intima/media area ratio in 12 cholesterol-fed/PTU-treated rabbits (P<0.01, r=-0.606). Furthermore, rabbits treated with PTU only (groups 2 and 3) had no visible atherosclerotic lesion in their aortas and arteries (data not shown). The absolute area of the media did not differ significantly among the 6 groups.

The intimal plaques in the cholesterol-fed rabbits contained large numbers of VSMCs and macrophage-derived foam cells. The intimal lesions of the cholesterol-fed rabbits that received 0.1% PTU in their drinking water had fewer VSMCs than those of the rabbits that received cholesterol alone. Overall, PTU could reduce the VSMC/macrophage ratio in the atherosclerotic lesions induced by a high-cholesterol diet (Figure 3).

Effects of PTU on Proliferation

PTU significantly inhibited VSMC proliferation in a concentration-dependent manner (Figure 4). PTU decreased the mitochondrial activity of VSMCs to 99%, 86%, 75%, and 37% of that in the control in MTT assay and inhibited thymidine incorporation into the VSMCs to 99%, 87%, 69%, and 41% of that in the control at final concentrations of 0.05, 0.5, 2.5, and 5 mmol/L, respectively (IC50 at 4.2 mmol/L).

As shown in Figure 4, the inhibitory effects on VSMC proliferation after 24 hours of PTU treatment were observed at much higher concentrations than the physiological concentrations of PTU in clinical use (1 to 10 μg/mL). Therefore, it was necessary to determine the long-term effects of PTU on the proliferation of VSMCs grown in physiological concentrations. Time-course experiments showed that the antiproliferative effect of PTU was indeed observed when VSMCs were exposed to the physiological concentration of PTU (0.05 mmol/L to 8.5 μg/mL) for ≥2 days (data not shown).

Examination of Cytotoxicity and Apoptosis

PTU had no cytotoxic effect on VSMCs, as assessed by the trypan blue exclusion test. After treatment for 24 hours, the number of cells that excluded trypan blue was 94.4±0.4% compared with 94.2±0.6% under control conditions (n=5; data not shown). By examining the propidium iodide–stained DNA with a fluorescent microscope, PTU even at the highest concentration (5 mmol/L) did not cause any pyknosis or karyorrhexis (typical changes of apoptosis) in VSMCs compared with the control VSMCs (data not shown).

Effects of PTU on Migration

In a Transwell assay, PTU showed a concentration-dependent inhibitory effect on serum-induced VSMC migration (Figure
5). PTU significantly decreased migratory activity to 80%, 71%, 57%, and 39% of the control at final concentrations of 0.05, 0.5, 2.5, and 5 mmol/L, respectively (IC$_{50}$ at 3.4 mmol/L).

**Effects of PTU on PTEN Expression**

The lipid products of phosphatidylinositol 3 (PI3)–kinase, a lipid kinase that phosphorylates phosphatidylinositol, are potent regulators mediating signal transduction in cell proliferation and migration.$^{13}$ PTEN, which functions as a phosphatidylinositol 3'-phosphatase for hydrolyzing the lipid products of PI3-kinase, is therefore thought to play a role in these cellular processes.$^{14,15}$ Overexpression of PTEN, which disrupts the PI3-kinase–mediated signaling through intermediates such as Akt, p70$^S6K$, and FAK, results in suppression of VSMC proliferation and migration.$^{16}$ To elucidate the mech-

![Figure 2](image_url)

Figure 2. A, Representative illustration of the sudanophilia distribution in the aortas of 3 cholesterol-fed rabbits assigned randomly to groups receiving either cholesterol (CHO) alone, CHO/PTU, or CHO/PTU/T3. B, Quantitative analysis of dietary hypercholesterolemia-induced atherosclerotic lesions determined by measuring intima/media area ratios of the aortic lesions found in 3 treatment groups. Data are expressed separately as ascending aorta (AsAo), aortic arch (AoArch), and femoral arteries (Femoral). *†$P<0.01$, significant differences among corresponding groups by Tukey multiple-comparison test.

![Figure 3](image_url)

Figure 3. Localization of VSMCs (arrowhead) in atheromas of the cholesterol-fed (CHO) (A) and cholesterol-fed/PTU-treated (CHO+PTU) (B) groups (magnification of objective lens $\times 100$). Rabbits in the CHO/PTU group had significantly fewer $\alpha$-smooth muscle actin–positive cells than those in the CHO alone group. In addition, decreased number of VSMCs resulted in decreased VSMC/macrophage ratio.

![Figure 4](image_url)

Figure 4. Effects of PTU on cell proliferation in VSMCs. Mitochondrial activity and thymidine incorporation of VSMCs were determined by MTT assay and TCA counting, respectively, as described in Methods. Each value (mean±SD [n=6]) is expressed as a percentage of the mitochondrial activity or thymidine incorporation of control cells maintained in medium containing 0.1% dimethyl sulfoxide (DMSO) as vehicle. *†‡$P<0.01$, significant differences among groups by Tukey multiple-comparison test.
anism responsible for the inhibition of VSMC proliferation and migration by PTU, the effects of PTU on PTEN gene expression were assessed by Western blot analysis at the protein level. As shown in Figure 6, a low level of PTEN could be detected in serum-treated VSMCs, whereas the PTEN level increased markedly in the serum-deprived condition. The induction of PTEN gene expression was dependent on PTU concentration; increased PTEN expression was initially detectable at 0.5 mmol/L (EC50 at 6.9 mmol/L). Furthermore, PTU had no effect on PTEN phosphorylation. As expected, PTU blocked serum-induced Akt phosphorylation (IC50 at 5.6 mmol/L). These findings indicate that PTU inhibits VSMC proliferation and migration via induction of PTEN and disruption of the PI3-kinase pathway.

Discussion

This study provided the first demonstration of the effect of an antithyroid drug on the attenuation of atherosclerosis in cholesterol-fed rabbits. The findings of this study conflict with those obtained with the use of animal models of hypothyroidism produced by either thyroidectomy or exposure to antithyroid chemicals, including thiourea or methylthiouracil. In cholesterol-fed animals, coexisting hypothyroidism induced by thyroidectomy appears to accelerate atherosclerosis, whereas administration of thyroid hormone reduces atherosclerosis. Thiourea and methylthiouracil are potential antithyroid metabolites formed in animals. The response of the aorta to the 2% cholesterol diet was unaffected or even aggravated by concurrent treatment with thiourea18 or methylthiouracil.19 Notably, a parallel study also found that methimazole, another antithyroid drug used in clinical practice, had no protective effect on atherosclerosis in cholesterol-fed rabbits compared with rabbits treated with PTU (data not shown). Thiourea, methylthiouracil, methimazole, and PTU belong to a class of structures known as thionamides. Thionamides are characterized by a thio carbamamide backbone in the chemical structure. Despite having the same action in decreasing thyroid activity, these compounds have a different effect on atherosclerosis in cholesterol-fed animals. Therefore, we suggest that the antiatherosclerotic effect of PTU operates through a structure other than the thio carbamamide backbone, which has been thought to be responsible for the hypothyroid effect of PTU. Furthermore, the in vivo data presented here demonstrate that the antiatherosclerotic effect of PTU does not involve a thyroid-mediated mechanism. This conclusion is drawn on the basis of 2 findings. First, PTU has a different effect from other experimental models of hypothyroidism on atherogenesis in cholesterol-fed animals. Second, the antiatherosclerotic effect of PTU could not be reversed by thyroid hormone supplementation.

The experimental protocol in this study followed the designs published previously by using exogenous administration of T3 to restore the euthyroid status in hypothyroid PTU-treated rats. However, whether maintaining the circulating T3 levels to a normal range may convert intracellular hypothyroid status to the euthyroid status should be further addressed. This inference had been clarified in our previous study, at least in an in vitro condition.

Hypothyroid patients generally have hypercholesterolemia. In the present study, PTU, possibly through its effect on thyroid hormone, had a mild hypercholesterolemic effect in rabbits treated with PTU only. However, serum lipid concentrations were unaffected by PTU in cholesterol-fed rabbits. The antiatherosclerotic effects of PTU found in this study suggest that the inhibition of atherosclerosis is not related to changes in lipid subclass concentration because these variables were unaffected by the PTU treatment.

Migration of VSMCs from the media to the intima, and their subsequent proliferation, is an important mechanism in atherogenesis. To determine whether PTU has a direct effect on atherogenesis, this study used cultured VSMCs to study the effect of PTU on the proliferation and migration of VSMCs.
these cells. The present results demonstrated that PTU has an inhibitory effect on VSMC proliferation and migration. These findings suggest that PTU selectively inhibited atherogenesis, in part through inhibiting VSMC proliferation and migration. The results obtained from the present in vitro model system demonstrating the inhibitory effects of PTU on VSMC proliferation and migration were consistent with the in vivo findings indicating a decrease in intimal VSMC/macrophage ratio.

The mechanism by which PTU exerts its inhibitory effects on VSMC proliferation and migration has been elucidated in this study. The tumor suppressor gene PTEN, which functions as a phosphatidylinositol 3′-phosphatase to hydrolyze the lipid products of PI3-kinase, has recently been found to play a role in VSMC proliferation and migration.14–16 Overexpression of PTEN, which disrupts the PI3-kinase-mediated signaling pathway but not the ERK pathway, inhibits growth factor-induced proliferation, migration, and survival of VSMCs.16 The results presented here showed that PTU enhanced PTEN expression in VSMCs. Because of the inverse relation between PTEN expression and the effects on the proliferation and migration, this study suggests that PTEN induction in VSMCs is associated with the effect of PTU on inhibiting VSMC proliferation and migration. Although PTEN was found to be involved in the formation of apoptosis, this study did not observe any evidence of apoptosis in VSMCs treated with PTU. A limitation of this study is that the mechanism of this disparity has not been clarified. One possible explanation is that apoptosis may be observed only in the condition of abnormally high levels of PTEN but not in a physiological condition. (The level of PTEN expression in PTU-treated VSMCs was below the basal expression level in the serum-deprived condition.)

PTU also possesses other actions that may contribute to its pronounced antiatherosclerotic effect. Nitric oxide, a potent vasodilator, inhibits leukocyte-endothelium adhesion and the proliferation of VSMCs, which may have a beneficial effect in preventing atherosclerosis.22 PTU was found to enhance the production of nitric oxide in rat aorta, although this effect was attributed to its hypothyroid activity.23 Oxidation of LDL is a key step in the development of atherosclerosis.2 The effect of PTU on lipid peroxidation has been investigated previously, PTU reduced the extent of lipid peroxidation in both in vivo24,25 and in vitro6 studies with this agent. Moreover, PTU, as do other thionamides, has an immunosuppressive action that is beneficial for the treatment of Graves’ disease.26,27 As is known, inflammatory processes may play a key role in the development of atherosclerotic lesions.2 However, the contribution of such antioxidant and immunosuppressive effects to the observed reduction in atherosclerotic progression during PTU treatment remains to be determined.

Serum concentrations of PTU achieved by administration via drinking water in this study were within the range reported in human subjects, given the standard dose of 300 to 600 mg/d. The physiological concentration of PTU in blood after normal intake of a therapeutic dose may be 1 to 10 μg/mL.4 This study demonstrated the inhibitory effect of PTU on VSMC proliferation and migration at physiological concentrations. It can therefore be assumed that PTU in the therapeutic range may have an inhibitory effect on VSMC growth and migration in vivo.

In conclusion, this study using cholesterol-fed rabbits demonstrated PTU to have a potent antiatherosclerotic effect, which clearly was independent of its hypothyroid effect. VSMC proliferation and migration play an important role in the pathogenesis of atherosclerotic lesions. The observed results showed that PTU inhibited VSMC proliferation and migration. PTU may be of potential benefit for preventing atherosclerotic changes in patients with hypercholesterolemia if its structure is modified to preserve the antiatherosclerotic effect while minimizing its hypothyroid effect. Furthermore, VSMC proliferation and migration are also thought to be the main causes of restenosis after angioplasty. Because PTU has adverse side effects affecting thyroid function during systemic application, local drug delivery via drug-eluting stents coated with PTU may be a promising approach for preventing restenosis.

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