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Osteopontin gene expression in the aorta and the heart of propylthiouracil-induced hypothyroid mice

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Summary

It is known that there is abnormal osteopontin (OPN) expression at the sites of atherosclerotic lesions. In the Apolipoprotein E gene knockout (ApoE-KO) mouse, a model of the atherosclerotic process, altered cholesterol metabolism with associated increase in OPN expression is evident at 12–22 weeks in the aorta and at 22 weeks in the heart. In this study, we analyzed another animal model of hypothyroid mice created by ingestion of propylthiouracil (PTU). After 2 weeks of PTU ingestion, the animals had significant decreases in thyroid hormones (T3 and T4) and immediate increases in blood lipids/cholesterol. Hypothyroid mice showed 1.3-, 1.5-, 2-fold increases in blood levels of total cholesterol, triglycerides, and low density lipoprotein-cholesterol respectively. Semi-quantitative RT-PCR analysis showed that hypothyroid mice had 1.4- to 2-fold increases of OPN mRNA expression in the aorta and 1.5-fold increases in the heart. Hypothyroid animals treated with T3 (5 μg/day for 6 days) or statin (0.2 mg/30 g for 2 weeks) reduce blood lipids and aortic OPN mRNA expression. Data obtained with ELISA analyses showed 1.5- and 1.7-fold increases in OPN protein in the aorta (10 weeks) and the heart (22 weeks), respectively. This increase is close to the mRNA expression in both tissues of hypothyroid mice. In addition, western blots showed several variants of OPN protein expressed in the aorta and the heart. The decrease in the 70 kDa OPN is accompanied by an increase in 45 kDa OPN in the aorta of hypothyroid mice. In contrast, only 45 kDa OPN is found in the heart of control and hypothyroid mice. These data indicate that the increase of OPN mRNA and protein expression occurs in cardiovascular tissues of hypothyroid mice.

Introduction

Osteopontin (OPN) is a secreted phosphoprotein, originally isolated from mineralized rat bone matrix [1–3]. OPN was also found in epithelial lining cells [4], secretions of body fluids [5], kidneys [6], uterus and placenta [7]. OPN contains an integrin-binding arginine–glycine–aspartic acid (RGD) sequence and additional sequences for cell adhesion. By binding to a variety of receptors (e.g. multiple forms of integrin receptors and CD44), OPN could mediate both physiological and pathological process such as bio-minerialization, inflammation, leukocyte recruitment, and cell survival [8].

OPN has been implicated in a number of diseases including atherosclerosis [9], coronary restenosis [10], aortic valve calcification [11], myocardial infarction [12], and heart failure [13]. Most

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of these cardiovascular diseases are clearly linked with adverse lipid and lipoprotein levels [14]. Studies with transgenic animal models of Apolipoprotein E-deficient (ApoE -/-) and OPN null-out (OPN -/-) mice have suggested that OPN may cause atherosclerosis as a result of altered cholesterol metabolism [15–17]. However it is not known if high blood cholesterol levels cause elevation of OPN expression in affected tissues during the development of cardiovascular disease.

Clinical studies have shown an inverse correlation between serum levels in cholesterol and thyroid hormone [18]. Studies with hypothyroidism also indicate that decreases in thyroid hormone can lead to a reduction in receptor-mediated low-density lipoprotein (LDL) metabolism and increases in the blood levels of highly atherogenic low-density lipoprotein-cholesterol (LDL-C) [19–21]. Recent evidence indicates that hypothyroidism may result in accelerated atherosclerosis and coronary artery disease, via increases in circulating levels of LDL-C particles, induction of diastolic hypertension, altered coagulability, and direct effects on increased total peripheral vascular resistance [22, 23]. Molecular mapping studies have found that the promoter of the LDL receptor gene contains a thyroid hormone responsive element, indicating that triiodothyroxine (T3) could modulate gene expression of the LDL receptor [24]. Apparently, hypothyroidism could offset the circulating levels of lipids and that may further promote the development of cardiovascular diseases.

In this study, we created the animal model of hypothyroid mice induced by an anti-thyroid drug, propylthiouracil (PTU), to determine if the disturbance by thyroid secretions would affect OPN expression in cardiovascular tissues. After 2 weeks of PTU ingestion, the animals had significant decreases in thyroid hormones (T3 and T4) and moderate increases in total cholesterol, LDL-C, and triglyceride levels. All hypothyroid animals had decreased heart rate but no significant change in blood pressure. Semi-quantitative RT-PCR, ELISA analyses, and western blotting were used to further determine the expression of OPN mRNA and protein in the aorta and the heart of PTU-induced hypothyroid and control mice. The data reported here indicate that the hypothyroid mice had increased OPN expression in the aorta and the heart. Supplementing with T3 (5 μg/day for 6 days) or statin (0.2 mg/30 g for 2 weeks) causes a reduction of blood lipids/cholesterol and a decrease in aortic expression of OPN mRNA. Thus, hypothyroidism could alter lipid metabolism and OPN expression in vasculature, and that may accelerate the development of cardiovascular diseases.

Materials and methods

Experimental animals

Male ICR mice (body weight: 25–30 g; 4-week-old) were raised in the usual laboratory conditions recommended by the Animal Care and Use Committee of National Chung-Hsing University (Taichung, Taiwan). The experimental animals (at 5-week-old) had a regular diet supplemented with 0.15% 6-propyl-2-thiouracil (PTU) in drinking water for 2, 4, 6, 8, 10, and 22 weeks before the experiment. Female ApoE knockout mice (ApoE-KO) and C57BL/6 mice were raised in the animal center of National Cheng-Kung University Medical College following the institutional guidelines. Six-weeks-old ApoE-KO mice were fed atherogenic diet containing 0.15% cholesterol to accelerate atherosclerosis formation (Test Diet, USA).

In vivo measurements of heart rates and blood pressure

Mice (body weight: 25–40 g) were anesthetized with urethane (1.2 g/kg, by intra-peritoneal injection). Under a dissecting microscope, a polyethylene 10 catheter connected to a liquid pressure transducer (Ohmeda P23XL) was surgically inserted into the right carotid artery. Blood pressure was continuously recorded on a physiological recorder (Gould TA 240) for 30 min. Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) was calculated from the chart record. The pulse pressure (PP) was calculated as the difference between SBP and DBP, and the mean arterial pressure (MAP) was calculated as the sum of DBP and 1/3 PP.

Measurements of thyroid hormones (T3 and T4) and lipids (total cholesterol, triglyceride, HDL-C, and LDL-C) in serum

Total serum triiodothyroxine (T3) (ng/dl) and thyroxine (T4) (μg/dl) levels were measured by

using a two-step chemo-luminescent micro-particle immunoassay method, according to the manufacturer's instructions. Cholesterol and triglyceride reagent kits (Olympus Diagnostica GmbH, Ireland) were used to examine an enzymatic color test for the quantitative determination of total cholesterol and triglyceride in serum. The reagent kits of Cholestest N HDL and Cholestest LDL (Daiichi Pure Chemicals, Japan) were used for measuring HDL-C and LDL-C in serum, respectively.

RNA extraction

Cardiac tissue or aorta (50–100 mg) was homogenized with 1 ml TRI Reagent to extract total RNA (Molecular Research Center, Cincinnati, USA). The quality of isolated RNA was examined by ethidium bromide staining of RNA separated in an agarose gel (2 kb 18S rRNA, and 5 kb 28S rRNA). The concentration of RNA was determined spectrophotometrically on the basis of an extinction coefficient (1 μg/ml) of 0.024.

Semi-quantitative reverse transcription polymerization chain reaction (semi-quantitative RT-PCR)

One μg of total RNA in a final volume of 25 μl buffer solutions was used to synthesize the first-strand cDNA with oligo-$(dT)_{18}$ primers (0.25 μg) and MMLV-RTase (12.5 U) at 37 °C for 60 min. For further PCR amplification, an aliquot (1/10) of the RT product was adjusted to contain 0.1 μg of each primer and additional buffer was added for a total volume of 25 μl. PCR was performed for 30 cycles (94 °C, 1 min; 54 °C, 1 min; 72 °C, 1 min). Based on the reported gene sequences of mouse OPN (NCBI accession number: NM 009263) and β-actin (NCBI accession number: NM 007393), the primers specific for OPN (Forward: 5′-ATGATGATGACGATGGAGAC-3′; Reverse: 5′-TGTCCTTGTGGCTGTGAAAC3-3′) and β-actin (Forward: 5′-ACACCCGCCACCAGTTCGCC-3′; Reverse: 5′-CGTACATGGCTGGGGTGTTG-3′) were synthesized. β-actin is a universal transcript and was used as an internal control of RT-PCR.

Enzyme-linked immunosorbent assay (ELISA)

Homogenized tissues were analyzed for the total protein concentration by using a micro-plate assay with the bicinchoninic acid reagent (Pierce, Rockford, IL). The protein levels of OPN expression in tissues were determined by the enzyme-linked immunosorbent assay (ELISA). Additions of 50 μl samples of porcine OPN recombinant proteins were captured on the ELISA micro-plate (Microlon 200, Greiner, Germany) at 37 °C overnight, followed by addition of 100 μl methanol for 10 min. After removal of methanol, the plates were blocked out non-specific binding with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) for 10 min, and washed four times with 400 μl 0.05% Tween-20 in PBS (PBST). A primary antibody, goat anti-mouse OPN polyclonal antibody (1:1000, Santa Cruz), was added to react with antigen containing samples on the plates at 37 °C for 90 min. After four washes with PBST, a secondary antibody, donkey anti-goat IgG conjugated to alkaline phosphatase (AP) (1:5000, Santa Cruz), was added for a further 90 min of incubation. The plates were washed again thoroughly with PBS, and 100 μl of substrate solution containing 0.1% *p*-nitrophenyl phosphate (ImmunoPure PNPP, Pierce) was added for development at 37 °C for 30 min. Optical density at 405 nm was read with a Micro-plate Reader (Anthos 2000, Australia).

Western blot

The tissues were homogenized with the denaturing solution (8 M urea, 2 M thiourea, 50 mM Tris–OH, pH 6.8, 75 mM dithiothreitol, 3% SDS), and the homogenate was centrifuged at $13{,}200 \times g$ (4 °C) for 15 min. The supernatant was diluted twice with sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), and incubated in a water bath (100 °C) for 10 min. The samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 150 V for 2 h, and electro-transferred to polyvinylidene difluoride membranes (PVDF) (Millipore) at constant current (210 mA) for 3 h. The blots were probed with primary antibodies recognizing mouse against OPN (1:1000) (goat anti-mouse OPN polyclonal antibody) (Santa Cruz) and washed with PBST three times for 10 min, followed by the reaction with AP-conjugated second antibodies (1:5000) (rabbit anti-goat IgG-AP) (Biorad) at 4 °C for 2 h. The colorimetric detection was visualized by BCIP-NBT substrate

reaction. Band intensities were photographed by a Kodak digital science EDAS 290 camera and analyzed by a computer-program with Kodak digital science 1 D image analysis software.

Statistics

Quantitative values are presented as the mean ± SEM. Statistical comparisons were made using a non-paired Student's *t*-test, with a p value < 0.05 regarded as significant.

Results

PTU-induced hypothyroid mice

In this study, hypothyroid mice were generated by inhibiting the hormone-producing functions of the thyroid gland with additions of an anti-thyroid drug, 6-propyl-2-thiouracil (PTU), to the drinking water. After 2 weeks or longer, PTU-treated mice showed 58.6% and 35.7% decreases in the circulating levels of thyroxine (T4) and triiodothyronine (T3) respectively (Table 1).

After 2 weeks or longer of PTU treatment all mice have increases in the blood levels of lipids (triglyceride: 1.5-fold; total cholesterol: 1.3-fold) and changes of lipoprotein-cholesterol profiles (HDL-C and LDL-C) in serum, as shown in Table 2. There were 2-fold increases in LDL-C levels but no significant changes in HDL-C concentrations. However, the ratio of HDL-C to total cholesterol was significantly decreased (~20%) in hypothyroid mice. The significant increase in LDL-C in hypothyroid mice could be attributed to the decreased number of LDL receptor in liver cells as reported in many other studies [19–26]. It is of note that there is species-difference of lipoprotein-cholesterol profile in human and mice. As shown here, the blood lipoprotein-cholesterol profile in mice contains a greater HDL-C/total cholesterol (~60%) and a lower LDL-C/total cholesterol (~4%), indicating that the major carrier of cholesterol is HDL instead of LDL.

Table 1. Body weight and serum concentrations of thyroxine (T4) and triiodothyronine (T3) of control and PTU-treated mice at 2, 4, 6, 8, 10, and 22 weeks.

	Body weight (g)		T4 (μg/dl)		T3 (ng/dl)	
	Control	PTU	Control	PTU	Control	PTU
2 weeks	37.0 ± 0.7	34.8 ± 0.7*	3.75 ± 0.34	1.48 ± 0.08*	55.73 ± 2.25	39.77 ± 2.94*
4 weeks	37.7 ± 1.1	35.1 ± 0.9*	3.91 ± 0.12	1.45 ± 0.19*	63.57 ± 2.19	37.60 ± 3.15*
6 weeks	38.9 ± 1.5	36.8 ± 1.3	3.36 ± 0.30	1.17 ± 0.07*	55.27 ± 3.15	35.48 ± 1.91*
8 weeks	39.3 ± 1.6	40.5 ± 1.8	2.82 ± 0.17	1.38 ± 0.13*	58.32 ± 4.45	38.39 ± 3.76*
10 weeks	38.8 ± 1.6	39.8 ± 1.1	3.20 ± 0.16	1.35 ± 0.14*	64.03 ± 2.01	38.70 ± 4.91*
22 weeks	37.7 ± 1.2	43.0 ± 0.7*	2.57 ± 0.25	1.27 ± 0.16*	55.22 ± 6.32	31.23 ± 3.79*

Values are mean ± SEM ($n = 5$). p values were calculated with the Student's *t*-test, with * indicating a significant difference between control and hypothyroid mice.

Table 2. Serum concentrations of high density lipoprotein-cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglyceride in control and hypothyroid mice.

	HDL-C (mg/ml)		LDL-C (mg/ml)		TC (mg/ml)		Triglyceride (mg/ml)	
	Control	PTU	Control	PTU	Control	PTU	Control	PTU
2 weeks	61.7 ± 2.3	62.3 ± 3.0	4.7 ± 0.3	7.3 ± 0.3*	106.0 ± 4.0	124.3 ± 6.2	78.3 ± 11.7	128.0 ± 28.1
4 weeks	62.7 ± 2.7	67.1 ± 2.7	4.3 ± 0.3	8.3 ± 0.3*	107.7 ± 4.8	132.0 ± 4.7*	98.5 ± 20.5	182.0 ± 45.8
6 weeks	60.7 ± 3.3	63.0 ± 2.7	6.0 ± 0.6	10.0 ± 0.6*	105.7 ± 5.4	130.0 ± 5.3*	118.3 ± 11.4	151.7 ± 8.2
8 weeks	56.0 ± 2.3	60.1 ± 2.9	5.7 ± 0.3	10.3 ± 0.3*	99.3 ± 4.1	128.3 ± 2.7*	97.5 ± 5.1	184.8 ± 31.4*
10 weeks	64.3 ± 2.7	65.7 ± 3.8	4.7 ± 0.3	9.5 ± 0.6*	103.3 ± 4.3	127.3 ± 7.7*	103.8 ± 10.0	150.4 ± 16.5*
22 weeks	66.0 ± 4.1	67.7 ± 2.7	6.9 ± 0.7	14.0 ± 0.6*	107.0 ± 6.6	132.0 ± 5.1*	92.8 ± 6.2	153.2 ± 10.3*

Values are mean ± SEM ($n = 5$), with * indicating a significant difference between control and hypothyroid mice.

Table 3. Heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) in control and PTU-treated mice at times indicated.

	HR (Beats/min)		SBP (mm Hg)		DBP (mm Hg)		MAP (mm Hg)	
	Control	PTU	Control	PTU	Control	PTU	Control	PTU
2 weeks	528 ± 21	412 ± 18*	64.0 ± 1.5	56.3 ± 3.0*	48.3 ± 1.9	41.0 ± 2.1*	54.7 ± 1.7	45.3 ± 2.5*
4 weeks	508 ± 23	413 ± 32*	62.0 ± 2.1	55.3 ± 1.8*	49.0 ± 1.7	40.7 ± 1.5*	53.0 ± 1.9	44.7 ± 1.6*
8 weeks	568 ± 10	508 ± 15*	74.3 ± 2.3	68.3 ± 2.0	61.0 ± 1.5	64.0 ± 3.7	64.5 ± 2.1	59.9 ± 2.1
22 weeks	591 ± 25	530 ± 20*	72.7 ± 5.3	64.8 ± 4.8	61.0 ± 8.4	59.3 ± 5.4	62.8 ± 6.4	60.4 ± 4.9

Values are mean ± SEM ($n = 5$), with * indicating a significant difference.

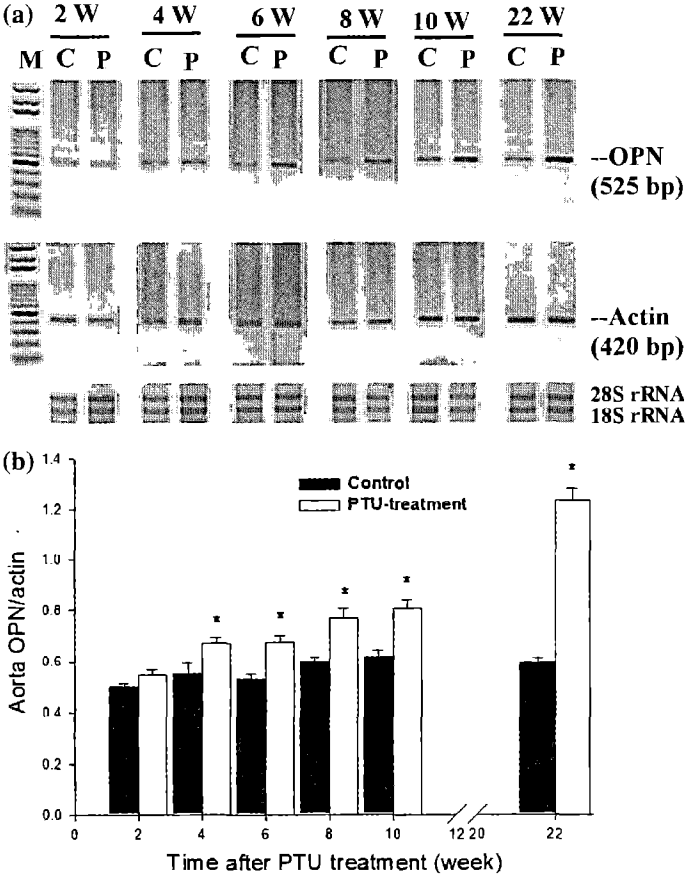

Figure 1. (a) Agarose gels illustrating the time course of changes in OPN mRNA in the aorta of control (C) and hypothyroid (P) mice. (b) Histogram illustrating the expression of OPN mRNA relative to the expression of β-actin mRNA in the aorta of control and hypothyroid mice. Values are expressed as mean ± SEM ($n = 5$) with * indicating a significant difference between control and experimental animals.

Hypothyroidism has several cardiovascular effects including decreased cardiac output and contractility, decreased heart rate, and increased systemic vascular resistance [27]. Hypothyroid mice had a decrease in heart rate but no significant change in blood pressure (Table 3).

Semi-quantitative RT-PCR analysis of OPN mRNA expression in the aorta and the heart

One major goal of this study was to determine if the hypothyroid associated changes in blood lipids would alter OPN expression in cardiac and vas-

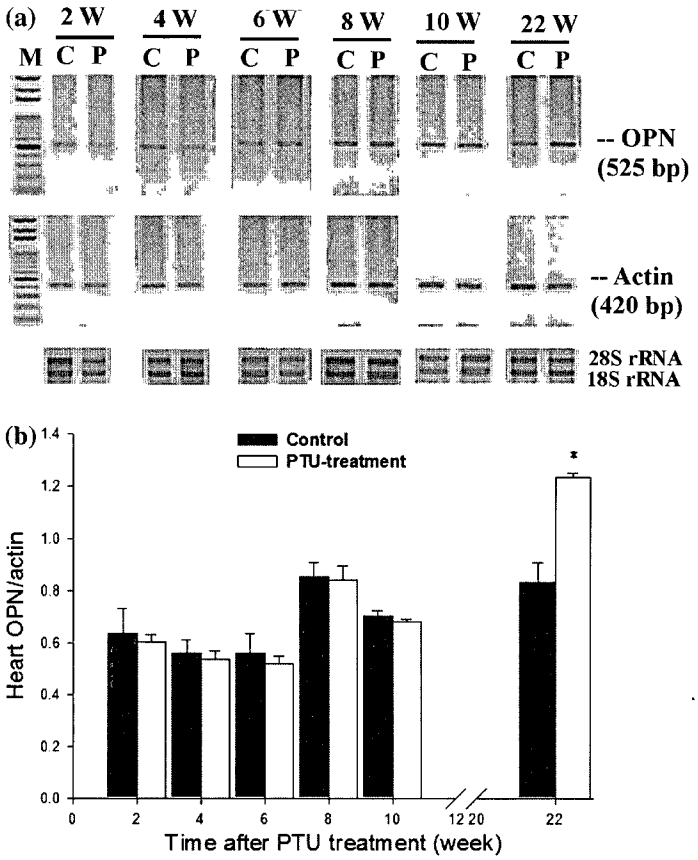

Figure 2. (a) Agarose gels illustrating the expression of OPN mRNA in the heart of control (C) and hypothyroid (P) mice. (b) Histogram illustrating the expression of OPN mRNA relative to β-actin mRNA in the heart. Experimental details as in Figure 2.

Table 4. Effects of T3 and statin on serum concentrations of HDL-C, LDL-C, TC, and TG, and the OPN mRNA expression in the aorta of hypothyroid mice. Hypothyroid mice after PTU ingestion for 8 weeks were abdominally injected with T3 (5 μg/day) for 6 days in a row, or fed with statin (0.2 mg/30 g body weight) for the two consecutive weeks. Values are mean ± SEM ($n=5$), with * indicating a significant difference between PTU- and control or PTU-mice with T3 or statin treatments.

	HDL-C (mg/ml)	LDL-C (mg/ml)	TC (mg/ml)	Triglyceride (mg/ml)	OPN mRNA/β-actin mRNA (aorta)
Control	57.25 ± 2.32	4.75 ± 0.25*	92.75 ± 2.29*	124.75 ± 9.05*	0.17 ± 0.03*
PTU	58.33 ± 2.03	8.60 ± 1.47	114.40 ± 2.54	184.00 ± 19.98	0.85 ± 0.03
PTU + T3	28.00 ± 1.77*	3.70 ± 0.21*	50.28 ± 2.90*	102.13 ± 8.15*	0.38 ± 0.03*
PTU + statin	57.33 ± 3.23	8.00 ± 0.37	94.00 ± 2.22*	123.00 ± 6.86*	0.59 ± 0.03*

cular tissues. The effect of hypothyroidism on OPN mRNA expression in the aorta is shown in Figure 1. The PCR product of OPN cDNA (~525 bp) was significantly increased after 4 weeks or longer of PTU ingestion, as compared with that in controls. As an internal standard, the β-actin mRNA was the same in both experimental and control mice. After short (4–10 weeks) and long (22 weeks) periods of PTU ingestion the aortic OPN mRNA/β-actin mRNA ratio was increased, relative to controls, 1.4- and 2.2-fold, respectively. In contrast, the same ratio in the heart was increased 1.5-fold only after 22 weeks of PTU ingestion (Figure 2).

Table 4 shows that both T3 and statin treatments reduce blood lipids and OPN mRNA expressions in hypothyroid animals. Hypothyroid mice supplemented with T3 (5 μg/day for 6 days)

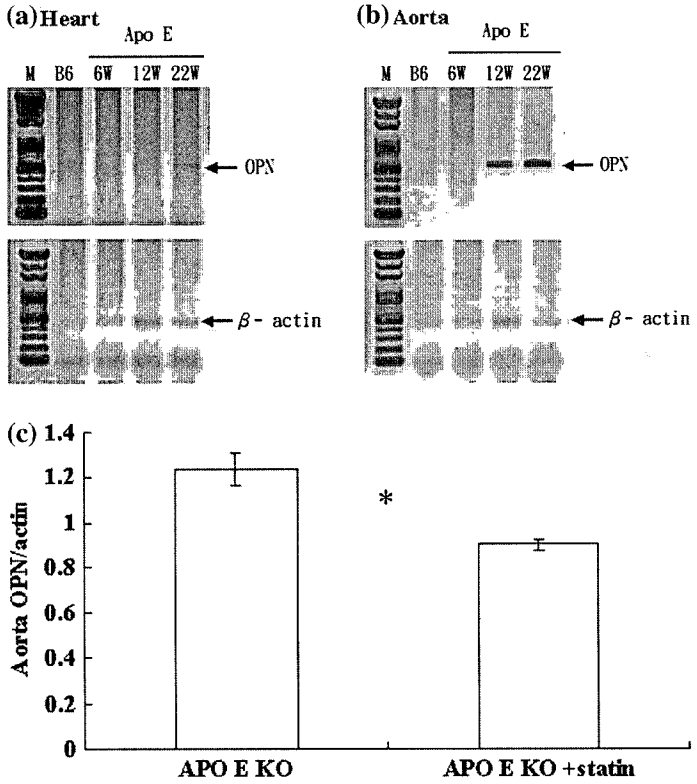

Figure 3. Agarose gels illustrating the OPN mRNA expression in the heart (a) and the aorta (b) of C57 BL6 control (B6) and Apo E gene knockout (Apo E) mice. (c) Statin effect on reduction of OPN mRNA expression in the aorta of Apo E knockout mice (Apo E KO). Apo E knockout mice (16-week-old) were fed with statin (0.2 mg/30 g) for the two consecutive weeks. Each group contains three animals. Values are mean ± SEM, with * indicating a significant difference.

have decreases in the blood levels of lipids (triglyceride: 44.5%; total cholesterol: 55.7%) and changes of lipoprotein-cholesterol profiles (HDL-C: 52.4%; LDL-C: 57.0%) in serum, as compared to PTU-treated mice. In parallel, these changes in blood lipids are associated with a 55.3% of reduction in OPN mRNA expression in the aorta of hypothyroid mice. In contrast, hypothyroid mice with statin treatment cause decreases in blood lipids (triglyceride: 33.2%; total cholesterol: 17.8%) but no significant effect on the contents of HDL-C and LDL-C. The ratio of HDL-C to total cholesterol was increased by 10% (statin + PTU: 61% vs. PTU: 51%), while no changes in the ratio of LDL-C to total cholesterol (statin + PTU: 8.5% vs. PTU: 7.5%). With statin treatment, hypothyroid mice have 30.6% reduction of OPN mRNA expression in the aorta.

In addition, with another animal model of defective cholesterol metabolism, the Apolipoprotein E-deficient mouse, we have observed 2.0-fold (1.98 ± 0.08, $n=4$) and 3.0-fold (2.95 ± 0.31, $n=4$) increases in OPN mRNA expression in the aorta after 12 and 22 weeks, respectively, and a 1.5-fold (1.52 ± 0.01, $n=4$) increase in the heart after 22 weeks (Figure 3a and b). Treatment with statin for 2 weeks diminished the OPN mRNA expression in the aorta of ApoE-KO mice (~26.6%) (Figure 3c). Apparently, altered lipid metabolism may contribute to enhanced OPN mRNA expression in both the heart and vascular tissues.

Enzyme-linked immunosorbent assay of OPN protein expression in the aorta and the heart

To determine if the OPN protein levels increased along with the OPN mRNA levels we used ELISA to quantitate, in μg/mg of tissue, the OPN content in the aorta and heart. As shown in Figure 4a, there was a greater OPN content in the aorta of hypothyroid mice than that of controls (PTU: 1.07 ± 0.08 μg OPN/mg aorta; Control: 0.71 ± 0.05 μg OPN/mg aorta). Figure 5b shows the comparison of the OPN content in the heart of

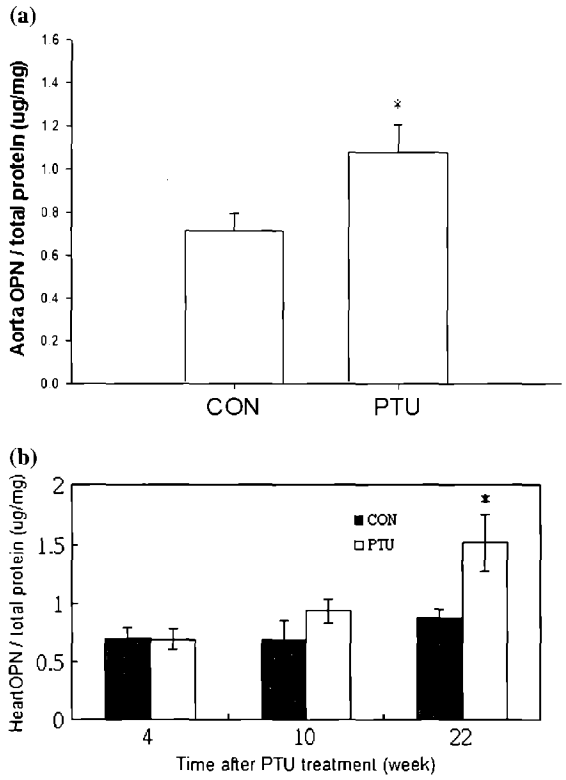

Figure 4. ELISA analysis of OPN protein content, relative to total protein, in the aorta (a) and heart (b) of control and hypothyroid mice. The aorta was analyzed after 10 weeks of PTU ingestion while the heart was analyzed after 4, 10, and 22 weeks. Values are mean ± SEM ($n = 5$), with * indicating a significant difference between control and experimental animals.

control and hypothyroid mice at 4, 10, and 22 weeks. Comparing control and experimental animals, there was no significant increase in OPN content at 4 and 10 weeks (4 week: Control, 0.67 ± 0.04 μg OPN/mg heart; PTU, 0.67 ± 0.04 μg OPN/mg heart; 10 week: Control, 0.70 ± 0.1 μg OPN/mg heart; PTU, 0.90 ± 0.06 μg OPN/mg heart). However, after 22 weeks there was a significant increase (1.57-fold) in cardiac OPN content (Control: 0.89 ± 0.04 μg OPN/mg heart; PTU: 1.42 ± 0.12 μg OPN/mg heart). Thus, both mRNA and protein levels are increased in aorta and heart tissues of hypothyroid mice.

Western blotting analysis of the pattern of the OPN expression in the aorta and the heart

Due to extensive post-translational modifications, including phosphorylation, glycosylation, and proteinase cleavage, OPN (264–301 amino acids) has several molecular variants expressed in different tissues, ranging from 25 to 75 kDa [28]. It is believed that the extensive modification of OPN is important to its function [29, 30]. As shown in Figure 5, a wide array of OPN proteins representing the native 70 kDa form, 45- and 25-kDa cleavage fragments, and 140 kDa dimers were detected by Western blot analysis of the aorta and the heart of control and PTU-treated mice. The 45 kDa cleavage fragment was increased in the aorta of the hypothyroid mice, relative to controls, whereas in the heart it was increased in both experimental and control animals. The ratio of the 70 kDa native OPN to the 45 kDa cleavage fragment was dramatically reduced in the aorta of the hypothyroid animals (0.75 ± 0.2), as well as in the heart of both control (0.27 ± 0.05) and hypothyroid animals (0.3 ± 0.007), as compared with that of control aorta (Control: 5.93 ± 1) (Figure 5b). The decreased 70 kD native OPN and increased 45 kD cleavage fragment suggests that there may be an increased protease activity (e.g. thrombin) in the aorta of hypothyroid mice. The data reported in this study indicate that hypothyroid animals with moderate increases in blood lipids have an enhanced OPN gene expression in both cardiac and vascular tissues.

Discussion

The hypothyroid mice analyzed in this study displayed significant increases in blood levels of total cholesterol (1.3-fold), triglycerides (1.5-fold), and low density lipoprotein cholesterol (LDL-C) (2-fold), but no change in high density lipoprotein cholesterol (HDL-C). Supplement of T3 (5 μg/day for 6 days) cut back the blood levels of total cholesterol (0.54-fold), triglycerides (0.82-fold), HDL-C (0.49-fold), and LDL-C (0.78-fold). The hypothyroid associated increases in blood lipids/cholesterol were not as severe as those seen in other models of hypercholesterolemia or hyperlipidemia, such as the Apolipoprotein E-deficient mouse [31, 32] and the cholesterol-fed rabbit [33]. However, even with moderate increases in blood lipids there was enhanced OPN expression in both the aorta (Figure 1) and the heart (Figure 2). Treatments with T3 or a cholesterol inhibitor (statin) have reduced the aortic OPN mRNA

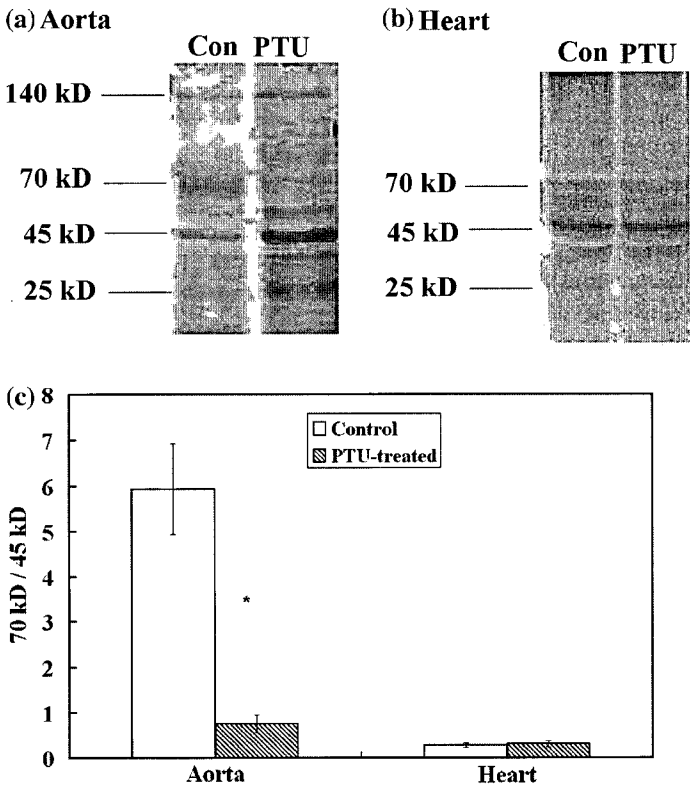

Figure 5. Western blot analysis of OPN variants (70 and 45 kDa) in the aorta (a) and heart (b), and histogram showing the 70 kDa/45 kDa ratios in the two tissues. The aorta and heart were analyzed after 10 and 22 weeks, respectively. Values are mean ± SEM ($n = 5$), with * indicating a significant difference.

expression by 55.3% and 30.5%, respectively (Table 4). In the ApoE-KO mouse, there are more marked increases in OPN mRNA expression (2.0- to 3.0-fold, Figure 3) in the aorta with similar increase (1.5-fold) in the heart. Supplement with statin caused a 26.6% reduction of OPN mRNA expression in the aorta of ApoE-KO mice. As a comparison, total cholesterol was increased 5-fold and triglycerides 1.7-fold in ApoE-KO mice [32]. In cholesterol-fed rabbits OPN expression was increased in Kupfer cells [34]. In addition, cholesterol feeding in the rabbit increased OPN expression in the aortic valve, an effect which was suppressed by administration of atorvastatin [33]. These results indicate that elevated levels of lipids and/or cholesterol could promote OPN expression in both cardiac and aorta tissues.

Many studies have suggested that the interplay between cholesterol and OPN may be involved in the development of cardiovascular diseases [15–17, 35–37]. However, the mechanism by which the OPN gene expression is up-regulated by the increase in circulating levels of lipids and/or cholesterol is not currently understood. Using low density-lipoprotein receptor (LDLR -/-) gene knockout mice, Towler et al. [38] have shown that high fat diets induce vascular mineralization in association with the up-regulation of OPN gene expression in the aorta, and a syndrome resembling type II diabetes. Studies also have shown that high glucose levels could activate OPN gene expression in diabetic human and rat vascular walls [39, 40]. In cultured rat aortic smooth muscle cell high glucose levels enhance OPN expression through protein kinase C (PKC) and hexosamine dependent pathways [41].

Recent studies indicate that Rho/Rho kinase pathway functions downstream of PKC and the hexosamine pathways and upstream of extracellular signal-regulated kinase (ERK) in activating glucose-dependent OPN expression [39]. Analysis of mouse OPN promoter activity in cultured aortic smooth muscle cells has shown that transcription factors acting on the regulatory element contain-

ing CCTCATGAC at −80 to −72 base pair upstream of the OPN gene are involved in the glucose-dependent regulation of arterial vascular smooth muscle cell OPN expression in diabetes [42]. Future studies will be needed to determine if the same signaling pathway in the cell is shared by the metabolism of glucose and lipids during the development of cardiovascular diseases.

Although hypothyroidism may not cause a severe increase in circulating levels of lipids (as shown in this study), evidence has been presented that hypothyroidism may promote the development of cardiovascular disease via a reduction in receptor-mediated LDL metabolism, and increases in blood levels of LDL-C [19–21]. In addition, clinical evidence also indicates that LDL is more susceptible to oxidation in patients with hypothyroidism [25, 26]. All these atherogenic stimuli may initiate the signal transduction pathway that causes elevation of OPN expression in a variety of cell types including endothelial cells [43], T-lymphocytes [44], macrophages [45], vascular smooth muscle cells [9], and cardiac myocytes [46]. In this context, oxidative stress has been shown to play key roles in regulating gene expression during atherosclerosis development and progression [47]. Whether OPN expression is modulated by oxidative stress warrants further investigation.

Finally, on the Western blot analysis we found a decrease in 70 kD native protein accompanied by an increase in 25 and 45 kD fragments in the aorta of hypothyroid mice (Figure 4a). It has been shown that upon treatment with serine proteases such as thrombin, the 70 kD protein is cleaved to 25 and 45 kD fragments [48, 49]. OPN is also a substrate for cleavage by matrix metalloproteinase such as MMP-3 (stromelysin-1) and MMP-7 (matrilysin) [29]. All these cleavage fragments would enhance integrin receptor-mediated cell adhesion and migration [49].

In addition, OPN can easily form a *dimmer* (~140 kD) in the aorta of hypothyroid mice (Figure 4a). The polymerized form of OPN is cross-linked by tissue transglutaminase, which is hypothesized to participate in cell adhesion, matrix assembly, and calcification [50]. Thus, changes in the patterns of OPN protein expression in aorta might suggest that alterations in vascular functions occur in hypothyroid mice. In contrast, both control and hypothyroid mice have the major 45-kD OPN present in heart tissues, although enhancement of OPN mRNA and protein was found in the heart of PTU-treated animals. In summary, hypothyroid associated changes in circulating lipids might affect the OPN gene expression in both heart and aorta.

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