Vascular smooth muscle cell apoptosis is an early trigger for hypothyroid atherosclerosis

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Aims	Endothelial dysfunction is an initial and vascular smooth muscle cell (VSMC) apoptosis, a later step of atherosclerosis. Hypothyroidism accelerates atherosclerosis. However, the early events responsible for this pro-atherosclerotic effect are unclear.
Methods and results	Rats were resistant to induction of atherosclerosis by high cholesterol diet alone, but became susceptible in hypothyroid state achieved by administration of propylthiouracil (PTU) for 6 weeks. VSMC dysfunction and apoptosis were obvious within 1 week after PTU treatment, without signs of endothelial dysfunction. This early VSMC damage was caused by hypothyroidism but not the high cholesterol diet. In *ApoE* knockout mice, PTU-induced hypothyroidism triggered early VSMC apoptosis, increased oxidative stress, and accelerated atherosclerosis development. Thyroid hormone supplementation (T4, 10, or 50 μg/kg) prevented atherogenic phenotypes in hypothyroid rats and mice. In rats, thyroidectomy caused severe hypothyroidism 5 days after operation, which also led to rapid VSMC dysfunction and apoptosis. *In vitro* studies did not show a direct toxic effect of PTU on VSMCs. In contrast, thyroid hormone (T3, 0.75 μg/L plus T4, 50 nmol/L) exerted a direct protection against VSMC apoptosis, which was reduced by knockdown of TRα1, rather than TRβ1 and TRβ2 receptors. TRα1-mediated inhibition of apoptotic signalling of JNKs and caspase-3 contributed to the anti-apoptotic action of thyroid hormone.
Conclusion	These findings provide an *in vivo* example for VSMC apoptosis as an early trigger of hypothyroidism-associated atherosclerosis, and reveal activation of TRα1 receptors to prevent VSMC apoptosis as a therapeutic strategy in this disease.
Keywords	Atherosclerosis • Hypothyroidism • Vascular smooth muscle cell • Apoptosis • Thyroid hormone receptor • Metabolic cardiovascular disease

1. Introduction

Atherosclerosis is one of the leading causes of stroke, myocardial infarction, and cardiovascular death worldwide. High blood levels of cholesterol [especially low-density lipoprotein (LDL) cholesterol] are a principal risk factor, while endothelial dysfunction appears to be the first step or an early marker for atherosclerosis.[1−4] Hence, statin therapy to lower blood cholesterol and to improve endothelial function is successful in preventing and treating atherosclerosis.[5] However, even in patients treated with statins, a considerable residual cardiovascular risk remains.[3]

The lifetime risk of overt hypothyroidism is ∼5%, and this disease is usually preceded by subclinical hypothyroidism with a prevalence of 4−20%.[6−8] Clinical studies demonstrate the association of hypothyroidism with atherosclerosis.[6,9,10] Animal studies support hypothyroidism as an important risk factor for the disease. Indeed, when combined with a high-cholesterol diet, the suppression of thyroid function, using either thiourea hypothyroid drugs in dogs[11] and rats,[12] I[131] thyroid irradiation in dogs,[13] or surgical thyroidectomy in baboons,[14] accelerates the occurrence of experimental atherosclerosis. However, the mechanism underlying this acceleration of atherosclerosis by the hypothyroid state is still elusive.

If endothelial dysfunction plays a key role in the initiation of the atherosclerotic process,[1−4] apoptosis of vascular smooth muscle cells (VSMCs) contributes majorly to the late stage of the disease, especially

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as regards plaque instability.[15−17] Here, we report the surprising finding that VSMC dysfunction and apoptosis occur at a very early stage of hypothyroid atherosclerosis in the absence of demonstrable abnormal endothelial responses.

2. Methods

2.1 Animals, treatments, and thyroidectomy

Sprague−Dawley rats were purchased from Sino-British SIPPR/BK Lab Animal Ltd, Shanghai, China. ApoE knockout (ApoE$^{−/−}$) mice were purchased from Jackson Laboratories, Bar Harbor, Maine, USA. C57BL/6J mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Science, China. All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the ethical committee for animal experiments of the Second Military Medical University. All animals were anaesthetized with pentobarbital sodium (60 mg/kg, i.p.). Anaesthesia was monitored by pinching the toe.

Treatments started at the age of 8 weeks in male animals. Rats were fed a high-cholesterol high-fat (HC) plus propylthiouracil (PTU) diet (HC + PTU) modified from previous reports[12,18] for 3 days, or 1, 2, 4, 6, and 10 weeks. The HC + PTU diet contained 3% cholesterol, 0.5% sodium cholate, 3% sucrose, 6% fat from lard, and 0.2% PTU. In separate experiments, rats were fed an HC diet alone (16.5 g/day/rat) for 1 week, or treated with PTU alone (33 mg/day/rat, i.g.) for 3 days or 1 week. The amount of HC diet and the dose of PTU were calculated according to the HC + PTU diet consumption of the first week (average: 16.5 g/day/rat). Some PTU-treated rats were supplemented with T4 (50 μg/kg, s.c.)[19] every other day for 3 days or 1 week. Mice were fed an HC diet containing 0.15% cholesterol and 21% fat from lard[20] or treated with PTU (140 mg/kg/day, i.g.) for 6 weeks. In separate experiments, mice were treated with PTU (140 mg/kg/day, i.g.) and supplemented with T4 (10 or 50 μg/kg, every other day, s.c.) for 1 or 6 weeks. Vehicle controls were treated with 0.6% carboxymethylcellulose-natrium by oral gavage and received subcutaneous injection of saline solution. At the end of treatment, animals were fasted overnight and anaesthetized with pentobarbital sodium (60 mg/kg, i.p.). Blood was taken from the inferior vena cava to assay serum levels. Aortae and brachiocephalic arteries were collected for various examinations.

Thyroidectomy was performed in male, 8-week-old rats. Briefly, a 2 cm midline neck incision was made in the anterior laryngo-tracheal region under anaesthesia with pentobarbital sodium (60 mg/kg, i.p.) and aseptic conditions. The thyroid was identified and isolated in front of the thyroid cartilage and bilateral near-total thyroidectomy was performed. In sham operations, the thyroid was also identified by midline incision but not excised. Animals were fasted overnight and sacrificed under anaesthesia with pentobarbital sodium (60 mg/kg, i.p.) for different assays 5 days after surgery.

2.2 Atherosclerotic lesion analysis

In rats, whole aortae were removed and isolated as described.[21,22] The aorta was cut open longitudinally and stained with Oil Red O, a marker for the lipid plaque lesion.[23] Aortic plaque area was measured by computer-assisted image analysis system (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).[21] Aortic tissue sections were stained with Oil Red O plus haematoxylin.

In mice, tissue sections of thoracic aorta and brachiocephalic artery were stained with haematoxylin and eosin. In another set of experiments, the mouse heart was perfused using 10 mL of phosphate-buffered saline (PBS) and aortic arch was imaged. Then, the heart was perfused by 15 mL of 4% paraformaldehyde. The thoracic aorta was harvested and the adventitia was thoroughly cleaned under a dissecting microscope. The aorta was cut open longitudinally and stained with Oil Red O for analysis of aortic plaque area.

2.3 Aortic reactivity

Aortic rings (5 mm wide) with (E+) or without (E−) endothelium were prepared from the rat descending thoracic aortae.[22] Vascular reactivity was measured as isometric changes in tension using IT1−25 transducers and an IOX computerized system (EMKA Technologies, Paris, France). Contracting agents were added in cumulative concentrations. Some aortic rings were incubated with pharmacological inhibitors for 30 min before assessing vascular reactivity.[24] Acetylcholine-induced relaxations were measured in E+ aortic rings pre-contracted with phenylephrine or 5-hydroxytrptamine.

2.4 Cell apoptosis

DNA laddering was determined according to the manufacturer's instructions [Genomic DNA Purification Kit, (Promega, Madison, WI, USA)]. TUNEL staining was performed according to the manufacturer's instructions [In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) or DeadEnd Fluorometric TUNEL Kit (Promega)].[22,25] TUNEL-positive cells were counted under fluorescence microscopy.

2.5 Western blotting

Western blotting was performed and visualized using Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA).[22,25] Rabbit anti-actin, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and TRα1 polyclonal antibodies and TRβ1/2 monoclonal antibody were purchased from Santa-Cruz (Santa Cruz, CA, USA). Rabbit anti-cleaved caspase-3, phospho-JNK (Thr183/Tyr185), and total JNK polyclonal antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Blots were incubated with specific primary antibodies and IRDye800CW-conjugated secondary antibody. All western blotting experiments were repeated at least three times.

2.6 Real-time PCR

Real-time PCR was performed using an ABI 7500 System (ABI, Foster City, CA, USA) and SYBR Premix Ex Taq Mixture (Takara, Otsu, Japan) as described previously.[25,26] Total RNA was extracted, using the standard Trizol RNA isolation protocol. The following primers were used: TRα1, forward 5′-ACC TGG ATG ATA CGG AAG TG-3′, reverse 5′-GTG TTT GCG GTG GTT GA-3′; TRβ1/2, forward 5′-ACC CAG ACA GCG AGA CTC TA-3′, reverse 5′-CAT CCA GGT TGA AAG ACG AC-3′. β-Actin was used as internal control, and the amount of RNA was calculated by the comparative threshold cycle method as recommended by the manufacturer.

2.7 RNA interference

Two methods for RNA interference (RNAi) were applied in the present study. For RNAi in primary VSMCs, a pDONR221-pAd/CMV/V5-DEST adenovirus system was used. Vectors pDONR221 and pAd/CMV/V5-DEST were purchased from Invitrogen (Carlsbad, CA, USA). The designed sequence targeting TRα1 (5′-GGGGACCACTTTGTACAAGAAAGCT GGGTC-3′) was synthesized and sub-cloned into adenovirus pDON-R221-EGFP plasmid. Then, the inserts in pDONR221 vector were transferred to pAd/CMV/V5-DEST vector via a recombination reaction using LR clonase II enzyme mix (Invitrogen). The plasmids were linearized with PacI and transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). The adenovirus was collected from the medium of HEK293 cells.

For RNAi in A10 VSMC cells, small interfering RNA (siRNA) was used. A10 cells were transfected with no-sense small interfering RNA (siRNA-NS, as control) or siRNAs pool (50 nM) targeting TRα1 or TRβ1/2 (Dharmacon, Lafayette, CO, USA). The sequences of the siRNA-TRα1 duplexes were as follows: 5′-GUGGGGAGAUGGCGGUUAA-3′, 5′-GGUUAUCA-CUACCGCUGUA-3′, 5′-GCUGCUGCGUCAUCGACAA-3′, 5′-UAACC UGGAUGAUACGGAA-3′; the sequences of the siRNA-TRβ1/2 duplexes were as follows: 5′-GGGACAAAGCCACGGGGUA-3′, 5′-AGAAAUCC AUUGGGCAUAA-3′, 5′-CUUUGUAACCAUUGCGUUU-3′, 5′-UGG UAUUCAUGAACGAGAA-3′. Transfections were performed in serum-free

medium for 5 h at 37°C using Lipofectamine 2000 reagent in Opti-MEM (Invitrogen). After 5 h of incubation, the medium was removed and replaced with DMEM plus 10% FBS. Cells were harvested for mRNA measurement 24 h and for protein measurement 64 h after transfection.

2.8 Cell culture study

Rats were anaesthetized with pentobarbital sodium (60 mg/kg, i.p.). VSMCs were isolated from the descending thoracic aortae and cultured in Dulbecco modified Eagle's medium with 10% foetal bovine serum.[22] Experiments were performed using cells between passages 3 and 8. Cells were grown to 60% confluence, and switched to serum-starved medium (0.2% foetal bovine serum). After incubation with PTU for 24 h, apoptosis was examined by TUNEL staining. Effects of T3 plus T4 (T3+T4) on H_2O_2-induced apoptosis were also studied; after 24-h incubation with T3 + T4, cells were co-incubated with H_2O_2 for an additional 12 h before TUNEL staining. These effects were further examined after knockdown of thyroid receptors. A10 VSMC cells were cultured in Dulbecco modified Eagle's medium with 10% foetal bovine serum. After 48 h of siRNAs transfection, the cells were switched to serum-starved medium supplemented with T3 + T4 for 16 h. Thereafter, cell apoptosis was induced by treating with H_2O_2 for an additional 2.5 h before TUNEL staining. Primary VSMCs were transfected with adeno-virus for 6 h. Three days later, the cells were switched to serum-starved medium supplemented with T3 + T4 for 16 h, and cell apoptosis was induced by treating with H_2O_2 for an additional 2.5 h before TUNEL staining.

2.9 Oxidative stress assays

Tissues were washed in ice-cold PBS and lysated in 20 mM HEPES buffer (pH 7.2), containing 1.5 mM EDTA, 50 mM sucrose, and 200 mM mannitol. The extracts were centrifuged at 3000 rpm for 5 min and the supernatant was collected. Total reactive oxygen species (ROS), malonaldehyde (MDA), and Mn-superoxide dismutase (MnSOD) activity were determined using

commercial kits (Biovision, Mountain View, CA, USA) according to the man-ufacturer's instruction.

2.10 Statistical analysis

Data are expressed as means ± SEM. Statistical significance was evaluated with unpaired Student's t-test or analysis of variance where appropriate, except for aortic reactivity, in which two-way analysis of variance for repeated measures was used to compare two curves. $P < 0.05$ was consid-ered to indicate statistically significant differences.

3. Results

3.1 The hypothyroid drug PTU promotes the development of atherosclerosis in rats

In line with previous studies,[12,27] HC diet alone did not induce aortic ath-erosclerotic lesion in rats even 10 weeks after treatment (data not shown), while gross atherosclerosis was produced by an HC + PTU diet. During the 10-week HC + PTU treatment, there was a progressive elevation in total and LDL cholesterol but a progressive reduction in thyroid hormone [triiodothyronine (T3) and thyroxine (T4)] in the serum (Figure 1A−D), with no significant changes in high-density lipopro-tein cholesterol, triglycerides, and glucose (data not shown). Aortic ath-erosclerosis lesions were visible at 6 and 10 weeks. Aortic plaques increased progressively (Figure 1E). Typical atherosclerotic lesions were observed under the microscope in the aortae examined with Oil Red O plus haematoxylin staining after 6 and 10 weeks of HC + PTU treatment (Figure 1F). These results confirm that the hypothyroid drug PTU renders the rat susceptible to atherosclerosis.

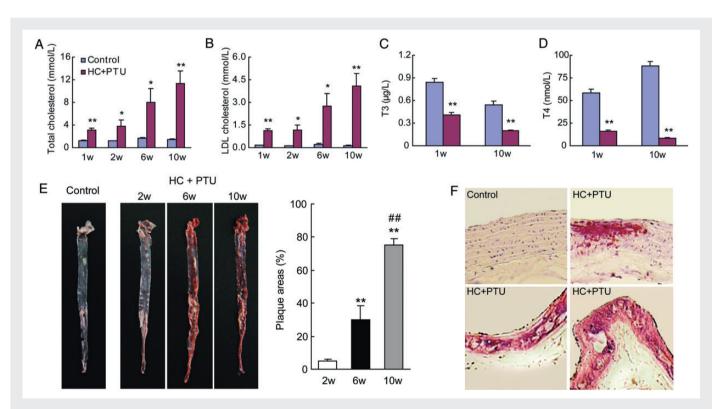**Figure 1** Serum characteristics and aortic lesions in HC + PTU-treated rats. (A−D) Serum levels of total and LDL cholesterol ($n = 5−10$) and T3 and T4 ($n = 10−15$). w, week(s). *$P < 0.05$, **$P < 0.01$ vs. control. (E) Aortic plaques indicated by Oil Red O staining. $n = 5−6$. **$P < 0.01$ vs. 2w; ##$P < 0.01$ vs. 6w. (F) Representative images of aortic sections stained with Oil Red O plus haematoxylin showing lipid accumulation and advanced atherosclerosis in rats treated with HC + PTU for 10 weeks.

3.2 Rapid onset of VSMC dysfunction and apoptosis in rats fed HC diet plus PTU

To know which vascular cell type is involved early in PTU-induced atherosclerosis, we characterized vascular function in aortae of rats fed HC + PTU diet. After 2, 6, and 10 weeks of HC + PTU treatment, contractions were significantly reduced both in aortic preparations with (E+) and without (E−) endothelium, in response to various contracting agents, including 5-hydroxytryptamine (*Figure 2A*), phenylephrine (*Figure 2B*), angiotensin II (*Figure 2C*), and KCl (*Figure 2D*), indicating that contractile function is remarkably impaired in a non-selective manner in VSMCs of HC + PTU-treated rats. Further experiments demonstrated that aortic contractions tended to be depressed as

early as after 3 days, and were decreased markedly after 1 week of HC + PTU treatment, indicating that the VSMC dysfunction occurs very early (*Figure 3A*).

Endothelial function has been determined by endothelium-dependent relaxation and/or endothelial anti-contractile effect. Unlike in normal controls, evaluating endothelial function by comparing acetylcholine-induced endothelium-dependent relaxations in E+ aortae of HC + PTU groups is rendered difficult because the pre-contractions developed in response to contracting agents are weak (*Figures 2* and *3A*). During these weak and variable pre-contractions, it was impossible to obtain full concentration response curves to acetylcholine. However, we tested, in such preparations with endothelium, the relaxations to the muscarinic agonist (10^{-5} M) during contractions
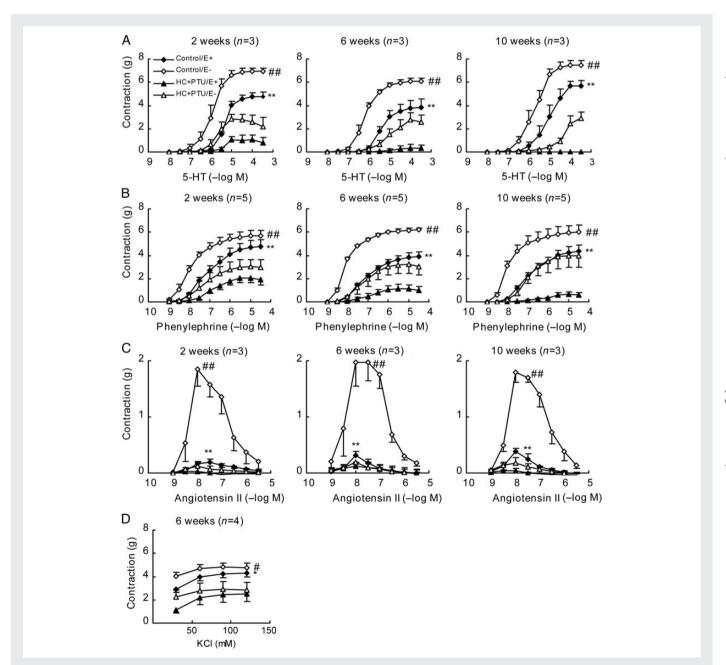

Figure 2 Impairment of VSMC contractile function in aortae of HC + PTU-treated rats in response to 5-hydroxytryptamine (5-HT, *A*), phenylephrine (*B*), angiotensin II (*C*), and KCl (*D*). Aortic rings with (E+) and without (E−) endothelium were prepared from rats fed an HC + PTU diet for 2, 6, and 10 weeks. *$P < 0.05$, **$P < 0.01$ Control/E+ vs. HC + PTU/E+; #$P < 0.05$, ##$P < 0.01$ Control/E− vs. HC + PTU/E−. $n = 3$–5.
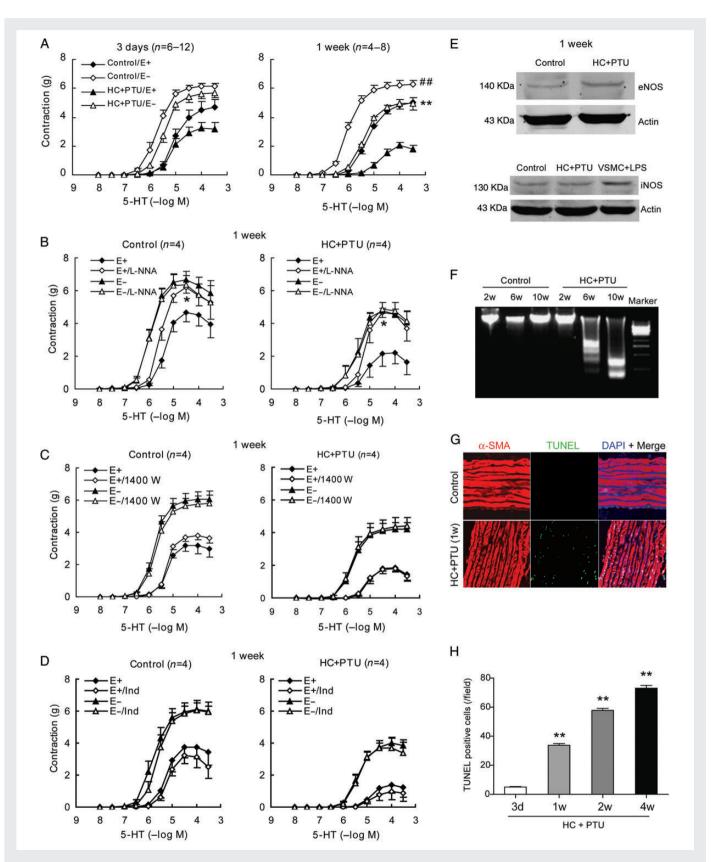
Figure 3 Rapid onset of VSMC dysfunction and apoptosis in aortae of HC + PTU-treated rats, without signs of endothelial dysfunction. (A) Aortic VSMC contraction was impaired within 1 week after HC + PTU treatment, while the endothelial anticontractile effect was preserved. **$P < 0.01$ Control/E+ vs. HC + PTU/E+; ##$P < 0.01$ Control/E− vs. HC + PTU/E−. $n = 6$–12 (3 days); $n = 4$–8 (1 week). (B–D) Endothelial anticontractile effects in both control and HC + PTU groups were blocked by L-NNA (300 μmol/L, B), but not by 1400 W (3 μmol/L, C) and indomethacin (10 μmol/L, D). *$P < 0.05$ E+/L-NNA vs. E+. $n = 4$. (E) Western blotting (repeated three times) showing an up-regulation of eNOS and no change of iNOS in aortae of HC + PTU-treated rats. LPS (10 μg/mL)-induced iNOS expression in cultured VSMCs as a positive control. (F) DNA fragmentation assay (repeated three times) in aortae. (G and H) Representative images and quantitative analysis of TUNEL assay in aortae. d, day(s). **$P < 0.01$ vs. 3d. $n = 8$–10. VSMCs were verified by α-smooth muscle actin (α-SMA) staining.

to either phenylephrine (10^{-6} or 3×10^{-5} M, after 2 weeks of treatment) or 5-hydroxytrptamine (3×10^{-4} M, after 3 days, 1 week, or 2 weeks of treatment); the response to the endothelium-dependent vasodilator was not reduced (data not shown). Furthermore, we determined the anticontractile effect of the presence of endothelial cells to evaluate endothelial function by comparing 5-hydroxytryptamine-induced contractions in E+ and E− aortae 1 week after treatment. The endothelial anticontractile effect (E+ vs. E−) was preserved in the HC + PTU group (Figure 3A). It was blocked by the non-selective nitric oxide synthase inhibitor L-NNA in both control and HC + PTU preparations (Figure 3B), but not by a selective inhibitor of iNOS 1400 W (Figure 3C) or by the cyclooxygenase inhibitor indomethacin (Figure 3D), indicating that NO produced by eNOS is responsible. Western blotting showed an up-regulation of eNOS protein and no change of iNOS protein in aortae from HC + PTU-treated rats (Figure 3E). Electron microscopy did not reveal endothelial damage in the treated rats (data not shown). These results indicate the absence of endothelial dysfunction at the early stage of HC + PTU treatment. They also exclude the possibility of up-regulation of iNOS as the common mechanism underlying the VSMC contractile dysfunction at the early stage of HC + PTU treatment.

We then examined the degree of aortic VSMC apoptosis. DNA laddering demonstrated that cell apoptosis was always present in aortae after 6 and 10 weeks of HC + PTU treatment (Figure 3F) and sometimes in aortae of rats treated for only 2 weeks (see Supplementary material online, Figure S1). Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) staining, a method more sensitive than DNA laddering,[28] detected apoptotic VSMCs in aortae as early as after 3 days of HC + PTU treatment, and it increased progressively within 2 weeks (Figure 3G and H), in parallel with the time course of VSMC contractile dysfunction (Figures 2A and 3A). These findings strongly suggest that triggered apoptosis of VSMCs is responsible for the VSMC dysfunction at the early stage of hypothyroidism-associated atherosclerosis.

3.3 Early VSMC dysfunction and apoptosis are caused by hypothyroidism rather than high-cholesterol diet

To find out what triggers VSMC dysfunction and apoptosis during HC + PTU treatment, rats were administered either HC or PTU alone. HC treatment for 1 week increased serum LDL cholesterol but had no effect on serum T3 and T4 (Figure 4A). Aortic contractions remained unchanged, and there was no apparent VSMC apoptosis in the aortae of HC-fed rats (Figure 4B and C).

PTU treatment for 1 week induced hypothyroidism (indicated by decreased serum T3 and T4 levels) and increased serum total and LDL cholesterol; these effects were prevented significantly by T4 supplementation (Figure 4A). The reduction in T4 occurred 3 days after PTU treatment, earlier than the changes of other serum parameters. The reductions in T3 and T4 following 1 week of PTU treatment (Figure 4A) were comparable with those observed after 1 week of combined HC + PTU treatment (Figure 1C and D). Aortic contractions tended to be depressed after 3 days, and were markedly reduced after 1 week of PTU treatment (Figure 4D). In aortae without endothelium (E−), the reductions in contractions caused by PTU alone (Figure 4D) were similar to those obtained with the combined treatment (Figure 3A), and were prevented by T4 (Figure 4D). VSMC apoptosis, detected after 3 days and aggravated after 1 week of PTU treatment

(Figure 4E and F), was prevented by T4 (Figure 4F, see Supplementary material online, Figure S2). Jun N-terminal kinases (JNKs), critical mediators in the apoptotic signalling pathway, and caspase-3, a major apoptotic executioner activated by cleavage, were activated in aortae from PTU-treated rats (Figure 4G, see Supplementary material online, Figure S2); these effects were prevented by T4 (Figure 4G, see Supplementary material online, Figure S2), further supporting the concept of hypothyroidism-induced apoptosis and the anti-apoptotic effect of thyroid hormone. It was also noted that T4 treatment reduced VSMC apoptosis partially and thus recovered VSMC contraction partially, which could be explained by still lower levels of T3 (Figure 4A), an active form of thyroid hormone, after T4 treatment.

3.4 Hypothyroidism induces early VSMC apoptosis and accelerates the development of atherosclerosis in *ApoE* knockout mice

HC diet is widely used to produce atherosclerotic lesion in $ApoE^{-/-}$ mice.[20,29] To test whether or not hypothyroidism can induce atherosclerosis in this mouse model, PTU was administered to 6- to 8-week-old $ApoE^{-/-}$ mice. The presence, or not, of atherosclerosis was examined in histological sections of thoracic aortae and brachiocephalic arteries. At the age of 14 weeks, no signs of atherosclerosis were found in $ApoE^{-/-}$ mice (Figure 5A−C), in accordance with previous findings reporting no evidence of endothelial damage or intimal hyperplasia in aortic sections from 4-month-old $ApoE^{-/-}$ mice.[30] Interestingly, PTU treatment alone induced atherosclerosis more efficiently than HC diet (Figure 5A−C).

Additional experiments were designed in $ApoE^{-/-}$ mice to examine VSMC apoptosis after 1 week and atherosclerotic lesions after 6 weeks of PTU treatment as well as the effects of T4 supplementation. Serum levels of thyroid hormone demonstrated a hypothyroid state in PTU-treated $ApoE^{-/-}$ mice, which was prevented by T4 supplementation (data not shown). Thoracic aortae displayed an up-regulation of eNOS and no alteration in iNOS 1 week after PTU treatment (see Supplementary material online, Figure S3). Aortic VSMC apoptosis was observed in $ApoE^{-/-}$ mice treated with PTU for 1 week (Figure 5D and E). This early VSMC apoptosis was significantly reduced by T4. Corresponding changes were observed in the active states of JNKs and caspase-3 in $ApoE^{-/-}$ aortae (Figure 5I and J). Judging from the levels of reactive oxygen species (ROS), MDA, MnSOD, and PTU also induced oxidative stress in $ApoE^{-/-}$ aortae, which was prevented by T4 (Figure 5F−H). Given that oxidative stress can induce cell apoptosis,[31] these results indicate that the effect of hypothyroidism on vascular oxidative stress may contribute to aortic VSMC apoptosis *in vivo*.

After 6 weeks of PTU treatment, $ApoE^{-/-}$ mice exhibited visible atherosclerotic lesions, mainly in the brachiocephalic artery and in the lesser curvature of the aortic arch (Figure 5K). T4 supplementation efficiently prevented the lesion formation. The PTU-induced atherosclerotic lesions and the preventive effect of T4 were further quantified by measurement of the aortic plaque area (Figure 5L). The preventive effects were comparable for the two doses of T4 (10 and 50 μg/kg) tested.

3.5 Deficiency of thyroid hormone triggers VSMC dysfunction and apoptosis

T4 supplementation prevented both the early changes (including T3 and T4 reduction, cholesterol elevation, vascular oxidative stress, VSMC apoptosis and dysfunction) and the later occurring atherosclerosis
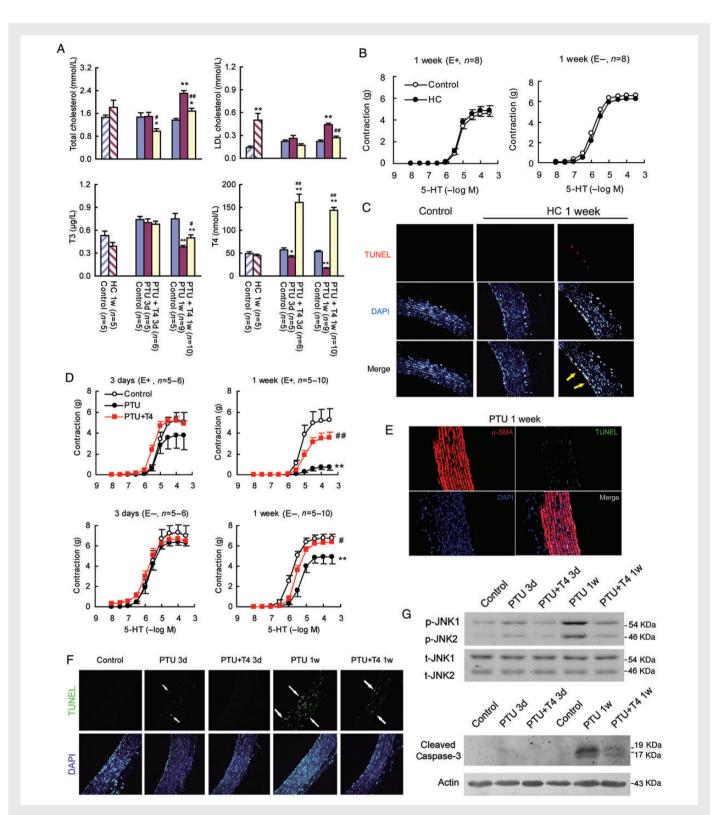
Figure 4 PTU-induced hypothyroidism triggers early VSMC dysfunction and apoptosis in rats, which is prevented by thyroid hormone T4. (*A*) Serum parameters in rats treated with HC or PTU alone. $n = 5{-}10$. (*B*) HC diet for 1 week had no effect on 5-hydroxytryptamine (5-HT)-induced aortic contractions in aortic rings both with (E+) and without (E−) endothelium. $n = 8$. (*C*) There was no aortic VSMC apoptosis in rats treated with HC diet for 1 week, although aortic endothelial apoptosis was seldom found. $n = 8$. (*D*) Impaired aortic VSMC contraction in PTU-treated rats. $n = 5{-}10$. (*E*) A large number of TUNEL-positive cells were observed in the media of aortae of rats treated with PTU (33 mg/day/rat, i.g.) for 1 week. DAPI staining indicates nuclei, α-SMA staining indicates VSMCs, and TUNEL staining indicates apoptosis. (*F* and *G*) TUNEL staining (*F*) and signalling assay (*G*) showing VSMC apoptosis in aortae of PTU-treated rats. See quantitative analysis in Supplementary material online, *Figure S2*. DAPI was used to stain the nuclei. Phosphorylation of JNKs and cleavage of caspase-3 indicated activation of apoptotic signalling. T4 prevented hypothyroidism (*A*), VSMC dysfunction (*D*), and apoptosis (*F* and *G*) in PTU-treated rats. $n = 5{-}10$ (*E*−*G*). Experiments were repeated at least three times (*E*−*G*). *$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. time-matched PTU-treated group.
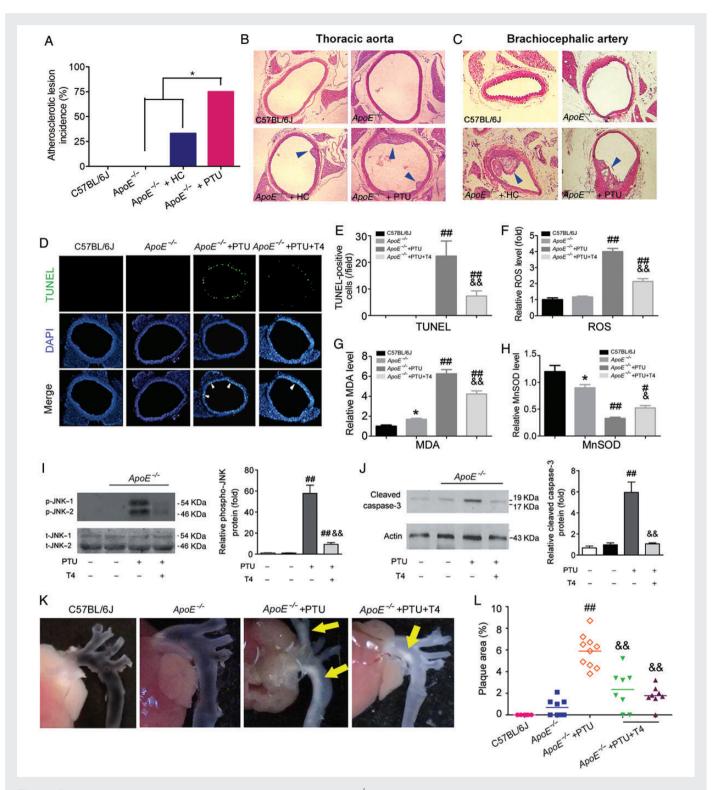
Figure 5 PTU-induced hypothyroidism promotes atherosclerosis in $ApoE^{-/-}$ mice, which is prevented by thyroid hormone T4. (*A*−*C*) Eight-week-old $ApoE^{-/-}$ mice were treated with HC or PTU for 6 weeks. Atherosclerosis was examined in arterial sections. *$P < 0.05$. $n = 6-8$. (*D* and *E*) $ApoE^{-/-}$ mice were treated with PTU or PTU + T4 for 1 week. T4 dose was 50 μg/kg. Aortic VSMC apoptosis was evaluated. $n = 6$. (*F*−*H*) Aortic oxidative stress including ROS, MDA, and MnSOD levels was determined. $n = 6$. (*I* and *J*) Aortic apoptotic signalling, including phosphorylated JNKs and cleaved caspase-3, was examined. $n = 6$. Experiments were repeated three times. (*K* and *L*) $ApoE^{-/-}$ mice were treated with PTU or PTU + T4 for 6 weeks. Arrows indicate atherosclerotic plaques in aortic arches (*K*). Plaque area was measured in thoracic aortae (*L*). T4 doses were 10 μg/kg (green) and 50 μg/kg (purple). $n = 8-10$. *$P < 0.05$ vs. C57BL/6J; #$P < 0.05$, ##$P < 0.01$ vs. $ApoE^{-/-}$; &$P < 0.05$, &&$P < 0.01$ vs. $ApoE^{-/-}$ + PTU.

produced by hypothyroidism *in vivo* (*Figures 4* and *5*). To exclude the direct toxic effect of PTU on VSMCs, we administered the drug to primary cultures of rat aortic VSMCs. PTU, at a concentration reached in rat serum, did not produce oxidative stress and cell apoptosis in cultured VSMCs (see Supplementary material online, *Figure S4*), suggesting that vascular changes induced *in vivo* by the compound are not due to its direct effect on the vascular wall. All the above data suggest that hypothyroidism plays a central role in mediating the pathological effects of PTU.

Further evidence was provided by performing thyroidectomy in rats. Thyroidectomy rapidly caused a hypothyroid state and led to cholesterol elevation after 5 days (*Figure 6A*). Like the aortae isolated from PTU-treated rats, those from the thyroidectomized rats exhibited impaired contractions in both preparations with (E+) and without (E−) endothelium (*Figure 6B*). In these aortae, VSMC apoptosis was detected by the TUNEL assay (*Figure 6C*).

In addition, T3 plus T4 (T3 + T4) had a direct effect against hydrogen peroxide-induced apoptosis in cultured VSMCs (*Figure 6D*), accompanied with corresponding changes in JNKs and caspase-3 (*Figure 6E*), similar to the *in vivo* anti-apoptotic effect of T4 in rats and mice (*Figures 4E−G* and *5D*, *E*, *I* and *J*). Here, two concentrations of combined T3 and T4 were used; one was the mean value of serum T3 (0.75 μg/L) and T4 (50 nmol/L) in normal control rats, termed 'normal T3 + T4', and

another was the mean value of serum T3 (0.38 μg/L) and T4 (17 nmol/L) in PTU-treated rats, termed 'reduced T3 + T4'. The analysis of the dose dependency of the effect of T3 + T4 indicated that their anti-apoptotic action was reduced in hypothyroidism compared with euthyroidism (*Figure 6D* and *E*). Therefore, in addition to a pro-apoptotic effect resulting from oxidative stress (*Figure 5F−H*), the reduced anti-apoptotic ability of the hypothyroid state *per se* (*Figure 6D* and *E*) may contribute to VSMC apoptosis.

3.6 Thyroid hormone protects VSMCs against apoptosis by activating TRα1 rather than TRβ receptors

Thyroid hormone-binding receptors include TRα1, TRβ1, and TRβ2.[32] TRα1 was detected in VSMCs, whereas TRβ1 and TRβ2 were almost absent (*Figure 7A*). We performed siRNA-mediated RNA interference (RNAi) in the A10 VSMC cell line. The anti-apoptotic effect of T3 + T4 was significantly inhibited by siRNA targeting TRα1, but not by siRNA targeting TRβ1 and TRβ2 (see Supplementary material online, *Figure S5*). To further corroborate the importance of TRα1 in mediating the anti-apoptotic effect of thyroid hormone, we used virus-mediated RNAi in primary cultures of rat aortic VSMCs. Control adenovirus (Ad-EGFP) and adenovirus targeting TRα1 (Ad-RNAi-TRα1)
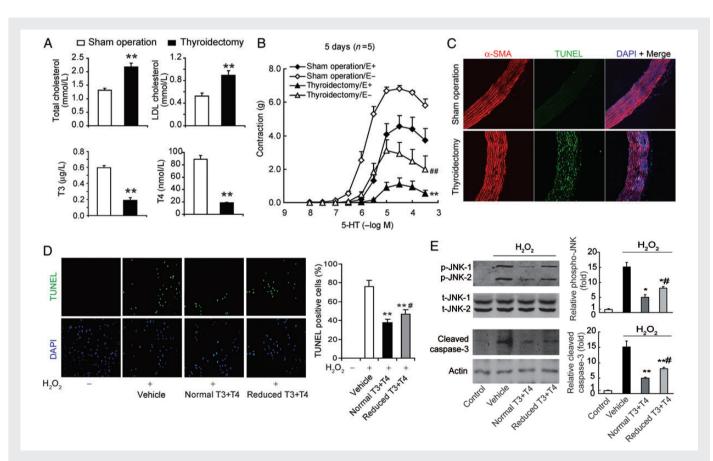

Figure 6 Deficiency of thyroid hormone triggers VSMC dysfunction and apoptosis. (A) Thyroidectomy increased serum cholesterol and induced hypothyroidism in rats 5 days after surgery. *P < 0.01 vs. Sham. *n* = 5. (B) Aortic VSMC contractile dysfunction after thyroidectomy. *P < 0.01 vs. Sham/E+; \#\#*P* < 0.01 vs. Sham/E−. *n* = 5. (C) Aortic VSMC apoptosis in thyroidectomized rats. α-SMA staining indicates VSMCs, DAPI staining indicates nuclei, and TUNEL staining indicates apoptosis. *n* = 5. (D and E) Thyroid hormone had an action against H$_2$O$_2$-induced apoptosis with corresponding changes of apoptotic signalling in primary culture of VSMCs. Normal T3 (0.75 μg/L) and T4 (50 nmol/L); reduced T3 (0.38 μg/L) and T4 (17 nmol/L). *n* = 6. Experiments were repeated three times. *P < 0.05, **P < 0.01 vs. vehicle; \#*P* < 0.05 vs. normal T3 + T4.
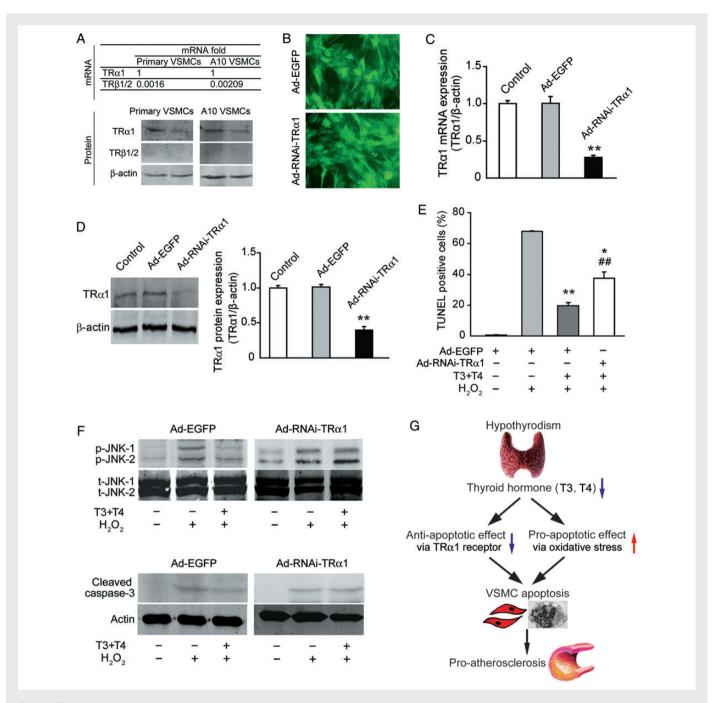
Figure 7 TRα1 receptors mediate the anti-apoptotic effect of thyroid hormone. (*A*) Expression of TRα1, TRβ1, and TRβ2 receptors in primary cultured VSMCs and A10 VSMC cell line. $n = 6$. Experiments were repeated three times. (*B*) Representative images for transfection of control adenovirus (Ad-EGFP) and adenovirus targeting TRα1 (Ad-RNAi-TRα1) in primary cultured VSMCs. (*C and D*) Efficiency of adenovirus-mediated knockdown of TRα1. **$P < 0.01$ vs. Ad-EGFP (control). (*E*) Knockdown of TRα1 blocked the anti-apoptotic effect of thyroid hormone (T3 + T4) in VSMCs. *$P < 0.05$, **$P < 0.01$ vs. H_2O_2, ##$P < 0.01$ Ad-RNAi-TRα1 vs. Ad-EGFP. (*F*) Influence of TRα1 knockdown on JNK and caspase-3 signalling in the presence and absence of thyroid hormone (T3 + T4) in VSMCs. See quantitative analysis in see Supplementary material online, *Figure S6*. $n = 6$ (*C–F*). Experiments were repeated three times (*C–F*). (*G*) Proposed mechanism for VSMC apoptosis as an early trigger in the pro-atherosclerotic effect of hypothyroidism.

transfected efficiently (*Figure 7B*). Ad-RNAi-TRα1 significantly reduced the mRNA (*Figure 7C*) and protein (*Figure 7D*) levels of TRα1, and markedly attenuated the anti-apoptotic effect of T3 + T4 in primary VSMCs (*Figure 7E*). Moreover, adenovirus targeting TRα1 blocked the inhibitory effects of T3 + T4 on the activation of JNKs and caspase-3 (*Figure 7F* and see Supplementary material online, *Figure S6*).

4. Discussion

We studied the early events in hypothyroidism-associated atherosclerosis in rodent models. The major findings included: (i) the hypothyroid drug PTU alone was able to induce atherosclerosis in $ApoE^{-/-}$ mice, in addition to the fact that it had a pro-atherosclerotic effect in rats fed HC

diet; (ii) hypothyroidism, whether induced pharmacologically or surgically, rapidly caused aortic VSMC dysfunction and apoptosis, while endothelial function was not impaired at the early stage; (iii) thyroid hormone protected VSMCs against apoptosis *in vivo* and *in vitro*, and postponed atherosclerosis in $ApoE^{-/-}$ mice. This anti-apoptotic effect of thyroid hormone was mediated by activation of TRα1 receptors and thereby inhibition of JNKs and caspase-3 signalling in VSMCs. Overall, our results provide evidence that VSMC apoptosis plays an early role in the development of atherosclerosis in the hypothyroid state.

Observational studies generally support the idea that hypothyroidism accelerates atherosclerosis.[6,9–14] A higher prevalence of coronary heart disease was found in patients with subclinical hypothyroidism (56% in patients with subclinical hypothyroidism vs. 16% in euthyroid persons).[33] Clinical studies also showed that people with subclinical hypothyroidism have a higher prevalence of cardiovascular mortality.[8,34] However, the mechanism linking hypothyroidism to atherosclerosis is unclear. Endothelial dysfunction, a vascular phenotype that is caused by many factors including hyperlipidaemia, has been widely recognized as the first step in atherosclerosis.[1–4] The endothelium is abnormal in the earliest stages of atherosclerosis, before plaque formation and certainly before clinical detection of the disease.[35,36] In the present study, studies in isolated aortae demonstrated that PTU alone or together with HC diet for 1 week severely impaired VSMC contractile function but had no effect on endothelial function. Further experiments using pharmacological inhibitors, molecular examinations, and electron microscopy did not show the presence of endothelial dysfunction. The major enzyme responsible for nitric oxide production in endothelial cells, eNOS, was even slightly up-regulated in aortae from rats or mice treated with PTU for 1 week, in line with a previous study reporting that PTU treatment in rats increased eNOS expression in the endothelial layer of the aorta.[19] Thus, it seems reasonable to conclude that hypothyroid drug PTU is not harmful for endothelial function at early stages. These results strongly indicate that short-term treatment with the hypothyroid drug PTU, at a dose which promotes atherosclerosis, impairs the functions of VSMCs but not that of endothelial cells.

The DNA ladder and TUNEL assays demonstrated that PTU induced aortic VSMC apoptosis within 3–7 days. These results raised the possibility that VSMC dysfunction and apoptosis play a critical role in hypothyroid atherosclerosis. Although the role of activated endothelium and inflammatory cell recruitment in the initiation and progression of early atherosclerosis is widely acknowledged, the role for VSMCs in the initiation of atherosclerosis has received less attention.[37] VSMCs are the major cellular component of the blood vessel wall where they normally exist in a contractile phenotype, but assume the differentiated state in response to atherogenic stimuli. Moreover, the extracellular matrix and cytokines produced by VSMCs affect the lipid content of the developing plaque.[37] Our data add a new perspective to the comprehension of the importance of VSMC dysfunction in the initiation and early progression of hypothyroid atherosclerosis.

PTU was the major tool used to induce hypothyroidism in the present study. Considering that PTU may have hypothyroid-independent effect on VSMCs,[38] we performed thyroidectomy to verify if the pro-apoptotic effect of hypothyroidism on VSMCs truly exists. Similar to PTU treatment, thyroidectomy increased serum lipid levels, reduced thyroid hormone levels, impaired VSMC contractile function, and induced VSMC apoptosis in rats. These results demonstrate that irrespective of its cause hypothyroidism results in VSMC dysfunction and apoptosis. Furthermore, thyroid hormone was protective in a dose-dependent manner against cell damage and apoptotic cascade activation induced by hydrogen peroxide. On the other hand, hypothyroidism also triggered oxidative stress in the aortic wall. Oxidative stress can induce cell apoptosis.[31] Therefore, the combination of the absence of anti-apoptotic effect (due to lack of thyroid hormone) and the presence of a pro-apoptotic effect (due to increased oxidative stress) leads to VSMC apoptosis, and ultimately promotes atherosclerosis (*Figure 7G*). This concept is further supported by the effects of thyroid hormone replacement therapy; T4 supplementation efficiently prevented the early VSMC apoptosis and the later occurring atherosclerosis in hypothyroid animals. In line with the present data obtained in a large conduit artery (aorta), T3 treatment in thyroidectomized rats causes phenotypic remodelling of VSMCs in coronary resistance arteries.[39] Due to obvious induction of aortic VSMC apoptosis in severe hypothyroidism, future work needs to pay attention on the development of aneurysmal dilation of such vessels. In addition to aortic VSMC apoptosis, hypothyroidism may cause apoptosis in other tissues, such as the brain,[40] liver,[41] and mammary tumour tissue.[42] One limitation for the present study was not to have obtained data on apoptosis in other tissues, especially cardiac and skeletal muscles.

Since thyroid hormone directly promotes cholesterol metabolism by the liver, hypothyroidism induces high blood levels of cholesterol.[32] In the present study in rodents, hypothyroidism, whether induced pharmacologically or surgically, led to increases in serum levels of total and LDL cholesterol (*Figures 4A* and *6A*), which improved upon thyroid hormone supplementation (*Figure 4A*). In humans, both primary and secondary hypothyroidism are commonly associated with an atherogenic lipid profile, which improves with thyroid hormone replacement.[43] In comparison with primary hypothyroidism, the lipid profile is more atherogenic in secondary hypothyroidism.[43]

Thyroid hormone binds to highly homologous receptors, TRα and TRβ, which are members of the nuclear hormone receptor family of ligand-gated transcription factors.[32] There are differences in expression pattern of TRα and TRβ. TRα is expressed abundantly in the brain and heart, whereas high level of TRβ is detected in the liver.[32] Additionally, activation of TRα boosts heart rate, whereas activation of TRβ lowers serum cholesterol levels.[32] Thus, selective activation of TRβ should have beneficial effects on serum cholesterol, and avoid deleterious effects on heart rate.[32] In the present study, TRα1, but not TRβ1 and TRβ2 receptors, were detected in VSMCs. Silencing of TRα1 blocked the anti-apoptotic effect of thyroid hormone, suggesting that activation of TRα receptors by the hormone may be beneficial. This direct protective effect of thyroid hormone on VSMCs may contribute to the prevention of hypothyroidism-induced atherosclerosis, in addition to its known beneficial action on liver cholesterol metabolism mediated by TRβ receptors.[32]

In summary, we used animal models to provide evidence that VSMC apoptosis plays an early role in the development of atherosclerosis in the hypothyroid state. We identified a previously unknown action of thyroid hormone against VSMC apoptosis that is mediated by TRα1 rather than TRβ receptor activation. Given that the burden of thyroid disease is considerable with an estimated 200 million of affected individuals worldwide, the present findings may help to understand better the cardiovascular outcome of thyroid disease and to improve the strategy to prevent metabolic cardiovascular complications.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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References

1. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med* 2011;**17**:1410–1422.

2. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation* 2004;**109**:III27–III32.

3. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature* 2011;**473**:317–325.

4. Vanhoutte PM. Endothelial dysfunction: the first step toward coronary arteriosclerosis. *Circ J* 2009;**73**:595–601.

5. Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J* 2012;**33**:829–837. 837a–837d.

6. Thvilum M, Brandt F, Brix TH, Hegedus L. A review of the evidence for and against increased mortality in hypothyroidism. *Nat Rev Endocrinol* 2012;**8**:417–424.

7. Cooper DS, Biondi B. Subclinical thyroid disease. *Lancet* 2012;**379**:1142–1154.

8. Tseng FY, Lin WY, Lin CC, Lee LT, Li TC, Sung PK, Huang KC. Subclinical hypothyroidism is associated with increased risk for all-cause and cardiovascular mortality in adults. *J Am Coll Cardiol* 2012;**60**:730–737.

9. Ichiki T. Thyroid hormone and atherosclerosis. *Vascul Pharmacol* 2010;**52**:151–156.

10. Squizzato A, Gerdes VE, Brandjes DP, Buller HR, Stam J. Thyroid diseases and cerebrovascular disease. *Stroke* 2005;**36**:2302–2310.

11. Steiner A, Kendall FE. Atherosclerosis and arteriosclerosis in dogs following ingestion of cholesterol and thiouracil. *Arch Pathol (Chic)* 1946;**42**:433–444.

12. Andrus SB, Fillios LC, Mann GV, Stare FJ. Experimental production of gross atherosclerosis in the rat. *J Exp Med* 1956;**104**:539–554.

13. Creech O Jr, Jordan GL Jr, De Bakey ME, Overton RC, Halpert B. The effect of chronic hypercholesterolemia on canine aortic transplants. *Surg Gynecol Obstet* 1955;**101**:607–614.

14. Gillman J, Gilbert C. Atherosis in the baboon (*Papio ursinus*); its pathogenesis and etiology. *Exp Med Surg* 1957;**15**:181–221.

15. Kavurma MM, Bhindi R, Lowe HC, Chesterman C, Khachigian LM. Vessel wall apoptosis and atherosclerotic plaque instability. *J Thromb Haemost* 2005;**3**:465–472.

16. Clarke MC, Figg N, Maguire JJ, Davenport AP, Goddard M, Littlewood TD, Bennett MR. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nat Med* 2006;**12**:1075–1080.

17. Gorenne I, Kumar S, Gray K, Figg N, Yu H, Mercer J, Bennett M. Vascular smooth muscle cell sirtuin 1 protects against DNA damage and inhibits atherosclerosis. *Circulation* 2013;**127**:386–396.

18. Go MF, Schonfeld G, Pfleger B, Cole TG, Sussman NL, Alpers DH. Regulation of intestinal and hepatic apoprotein synthesis after chronic fat and cholesterol feeding. *J Clin Invest* 1988;**81**:1615–1620.

19. Grieve DJ, Fletcher S, Pitsillides AA, Botham KM, Elliott J. Effects of oral propylthiouracil treatment on nitric oxide production in rat aorta. *Br J Pharmacol* 1999;**127**:1–8.

20. Johnson J, Carson K, Williams H, Karanam S, Newby A, Angelini G, George S, Jackson C. Plaque rupture after short periods of fat feeding in the apolipoprotein E-knockout mouse: model characterization and effects of pravastatin treatment. *Circulation* 2005;**111**:1422–1430.

21. Miao CY, Xie HH, Zhan LS, Su DF. Blood pressure variability is more important than blood pressure level in determination of end-organ damage in rats. *J Hypertens* 2006;**24**:1125–1135.

22. Wang P, Xu TY, Guan YF, Su DF, Fan GR, Miao CY. Perivascular adipose tissue-derived visfatin is a vascular smooth muscle cell growth factor: role of nicotinamide mononucleotide. *Cardiovasc Res* 2009;**81**:370–380.

23. McKinney B, Riley M. An orcein-oil red O stain for concomitant demonstration of elastic tissue and lipid. *Stain Technol* 1967;**42**:245–248.

24. Takaki A, Morikawa K, Tsutsui M, Murayama Y, Tekes E, Yamagishi H, Ohashi J, Yada T, Yanagihara N, Shimokawa H. Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. *J Exp Med* 2008;**205**:2053–2063.

25. Wang P, Xu TY, Guan YF, Tian WW, Viollet B, Rui YC, Zhai QW, Su DF, Miao CY. Nicotinamide phosphoribosyltransferase protects against ischemic stroke through SIRT1-dependent adenosine monophosphate-activated kinase pathway. *Ann Neurol* 2011;**69**:360–374.

26. Wang P, Zhang RY, Song J, Guan YF, Xu TY, Du H, Viollet B, Miao CY. Loss of AMP-activated protein kinase-alpha2 impairs the insulin-sensitizing effect of calorie restriction in skeletal muscle. *Diabetes* 2012;**61**:1051–1061.

27. Wojcicki J, Samochowiec L, Jaworska M, Hinek A. A search for a model of experimental atherosclerosis: comparative studies in rabbits, guinea pigs and rats. *Pol J Pharmacol Pharm* 1985;**37**:11–21.

28. Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, Brambilla C, Brambilla E. In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. *J Histochem Cytochem* 1996;**44**:959–968.

29. Bai B, Liang Y, Xu C, Lee MY, Xu A, Wu D, Vanhoutte PM, Wang Y. Cyclin-dependent kinase 5-mediated hyperphosphorylation of sirtuin-1 contributes to the development of endothelial senescence and atherosclerosis. *Circulation* 2012;**126**:729–740.

30. Wang YX, Halks-Miller M, Vergona R, Sullivan ME, Fitch R, Mallari C, Martin-McNulty B, da Cunha V, Freay A, Rubanyi GM, Kauser K. Increased aortic stiffness assessed by pulse wave velocity in apolipoprotein E-deficient mice. *Am J Physiol Heart Circ Physiol* 2000;**278**:H428–H434.

31. Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med* 2010;**48**:749–762.

32. Baxter JD, Webb P. Thyroid hormone mimetics: potential applications in atherosclerosis, obesity and type 2 diabetes. *Nat Rev Drug Discov* 2009;**8**:308–320.

33. Mya MM, Aronow WS. Subclinical hypothyroidism is associated with coronary artery disease in older persons. *J Gerontol A Biol Sci Med Sci* 2002;**57**:M658–M659.

34. Hak AE, Pols HA, Visser TJ, Drexhage HA, Hofman A, Witteman JC. Subclinical hypothyroidism is an independent risk factor for atherosclerosis and myocardial infarction in elderly women: the Rotterdam Study. *Ann Intern Med* 2000;**132**:270–278.

35. John S, Drobnik W, Lackner K, Schmieder RE. Soluble thrombomodulin and endothelial dysfunction in early atherosclerosis. *Lancet* 1999;**354**:1647.

36. Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK, Deanfield JE. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 1992;**340**:1111–1115.

37. Doran AC, Meller N, McNamara CA. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arterioscler Thromb Vasc Biol* 2008;**28**:812–819.

38. Chen WJ, Ho WJ, Chang GJ, Chen ST, Pang JH, Chou SH, Tsay PK, Kuo CT. Propylthiouracil, independent of its antithyroid effect, produces endothelium-dependent vasodilatation through induction of nitric oxide bioactivity. *Atherosclerosis* 2008;**196**:383–390.

39. Savinova OV, Liu Y, Aasen GA, Mao K, Weltman NY, Nedich BL, Liang Q, Gerdes AM. Thyroid hormone promotes remodeling of coronary resistance vessels. *PLoS One* 2011;**6**:e25054.

40. Cortes C, Eugenin E, Aliaga E, Carreno LJ, Bueno SM, Gonzalez PA, Gayol S, Naranjo D, Noches V, Marassi MP, Rosenthal D, Jadue C, Ibarra P, Keitel C, Wohllk N, Court F, Kalergis AM, Riedel CA. Hypothyroidism in the adult rat causes incremental changes in brain-derived neurotrophic factor, neuronal and astrocyte apoptosis, gliosis, and deterioration of postsynaptic density. *Thyroid* 2012;**22**:951–963.

41. Lopez-Fontal R, Zeini M, Traves PG, Gomez-Ferreria M, Aranda A, Saez GT, Cerda C, Martin-Sanz P, Hortelano S, Bosca L. Mice lacking thyroid hormone receptor Beta show enhanced apoptosis and delayed liver commitment for proliferation after partial hepatectomy. *PLoS One* 2010;**5**:e8710.

42. Lopez-Fontana CM, Sasso CV, Maselli ME, Santiano FE, Semino SN, Cuello Carrion FD, Jahn GA, Caron RW. Experimental hypothyroidism increases apoptosis in dimethylbenzanthracene-induced mammary tumors. *Oncol Rep* 2013;**30**:1651–1660.

43. O'Brien T, Dinneen SF, O'Brien PC, Palumbo PJ. Hyperlipidemia in patients with primary and secondary hypothyroidism. *Mayo Clin Proc* 1993;**68**:860–866.