Tanshinone II A attenuates atherosclerotic calcification in rat model by inhibition of oxidative stress

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Abstract

Aim: We have previously proved that oxidized low-density lipoprotein (oxLDL), a proatherogenic lipoprotein, plays a pivotal role in the development of atherosclerotic calcification (AC). The present study was performed to investigate whether tanshinone II A (TS II A), an antioxidant which has been shown to inhibit in vitro oxidation of LDL, has the effects to inhibit AC in rat model and by which, if any, mechanisms.

Methods: Rat AC model was induced by excessive vitamin D2 (VD) and high cholesterol diet (HCD), which was proven to be successful histopathologically and biochemically.

Results: Administration of AC rats with TS II A (35, 70 mg/kg) dose-dependently attenuated the AC pathological changes, meanwhile reduced the vessel contents of lipid and calcium. However, TS II A had no effects on serum levels of lipids, calcium and 25-OH VD. Further studies revealed that TS II A decreased serum concentration of oxLDL, reduced the superoxide anion production and malondialdehyde (MDA) in vessel. In addition, TS II A increased vessel Cu/Zn SOD activity, upregulated vessel mRNA and protein expression of Cu/Zn SOD.

Conclusion: The results suggested that TS II A significantly attenuated the AC in rat model, which might be attributed to its inhibition of oxLDL production independent of the serum levels of lipids, calcium and 25-OH VD, and that increasing of Cu/Zn SOD activity as well as mRNA and protein expression by TS II A might protect LDL against oxidation induced by superoxide anion in vessel.

Keywords: Tanshinone II A; Atherosclerotic calcification; Oxidized low-density lipoprotein; Cu/Zn superoxide dismutase

1. Introduction

Vascular calcification is a common and clinically significant component of several human diseases, including atherosclerosis, aortic stenosis, and diabetes (Proudfoot et al., 2002) and is well known to be present in 80% of vascular lesions and more than 90% of patients with coronary artery disease (Bostrom, 2001). Vascular calcification is an important risk factor for cardiovascular events because it causes decreased aortic compliance and elastic recoil, which results in cardiac ischemia in severe cases due to impaired reverse aortic flow and coronary perfusion (Wallin et al., 2001). Vascular calcification has been believed as an end-stage process of “passive” mineral precipitation. However, there is a growing awareness that vascular calcification is a biological phenomenon similar with bone formation and osteoporosis. Those similarities are supported by accumulating evidences such as increased activity of alkaline phosphatase (ALP), a marker of osteogenic differentiation of osteoblastic cells, in the calcified vessels (Laroche and Delmotte, 2005). Hypercholesterolemia plays an important role in the initiation and progression of atherosclerosis and has positive correlation with cardiovascular disease, largely depending on the oxidation of LDL, the main cholesterol carrier in plasma (Hsu, 2003). LDL is oxidized underneath vessel intima mainly by reactive oxygen system (ROS) especially superoxide anion produced by vascular cells. Indeed, studies have shown that the degree of LDL oxidation is directly proportional to the rate of superoxide production (Fang et al., 1998). Several studies have shown that an increase in activity of Cu/Zn SOD, the specific scavenger of superoxide anion, is capable of preventing LDL oxidation in culture cells (Heinecke et al., 1986; Steinbrecher, 1988). Atherosclerotic calcification (AC) is seen as early as the second decade of life, just after the fatty streak stage, and
many studies have demonstrated a clear association between lipid accumulation and atherosclerotic calcification (Kramsch and Chan, 1978). Calcification co-localizes with cholesterol crystals in human atherosclerosis (Sarig et al., 1994) and is present in the intima of cholesterol-fed rabbits (Hsu et al., 2002) and ApoE knockout mice (Rattazzi et al., 2005). Microscopic calcification in aortic valves seems to form in the area in which lipoproteins have been retained (O'Brien et al., 1996). The early deposits of calcification occur within and around isolated vascular smooth muscle cells (VSMCs) from the lipid core. The studies in vitro indicated that oxidized low-density lipoprotein (oxLDL) is involved in the calcification of VSMCs by stimulating the osteoblastic differentiation of vascular cells (Parhami et al., 1997; Mody et al., 2001). We have previously demonstrated that hypercholesterolemia greatly accelerates the vascular calcification in rat model by increasing oxLDL production (Tang et al., 2006), suggesting that inhibition of oxLDL production by antioxidants might be beneficial to the patients with AC related diseases.

Tanshinone II A (TS II A) is a major component of Salvia miltiorrhiza Bunge known as Danshen which has long been used for prevention and treatment of cardiovascular diseases in China. Accumulating studies show that TS II A possesses many biological properties (Du et al., 2005; Kimm et al., 2004; Cao et al., 1996), largely depending on its anti-oxidative effects (Zhou et al., 1999; Niu et al., 2000). The present study was performed to investigate the effect of TS II A on AC in rat model induced by excessive vitamin D2 and high cholesterol diet. The results showed that TS II A effectively attenuated the rat AC and inhibition of oxLDL production might be one of the mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

Tanshinone II A (TS II A), extracted from Radix Salviae miltiorrhizae known as Danshen, was provided by professor Gu Lianquan (Institute of Pharmacy Synthesis, Sun Yat-sen University) and its purity is over 98% (assayed by HPLC). The structure of TS II A is shown in Fig. 1. Kits for the determination of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), calcium ions were from Beijing Zhongsheng Bioengineering Company (China). Kits for the detection of Cu/Zn superoxide dismutase (Cu/Zn SOD), malondialdehyde (MDA) and protein were produced by Nanjing Jiancheng Bioengineering Company (China). Detecting kits for serum oxLDL and 25-hydroxy vitamin D (25-OH VD) were the products of BBP Biomedicals, Inc. (USA) and IDS Inc. (UK) respectively. Dihydro-ethidium bromide (DHE), vitamin D3 (VD) and tetraethoxypropane were purchased from Sigma. dNTP, Moloney murine leukemia virus-transcriptase (MMLV), Taq DNA polymerase, RNAsin, oligo (dT)15 primer and oligonucleotides for Cu/Zn SOD and GAPDH were from Sangong Biotechnology (Shanghai, China). The sequences of oligonucleotide primers were as follows: Cu/Zn SOD-F: 5′-GGG TCT CCC GGG GAA GCA-3′, Cu/Zn SOD-R: 5′-ATT GGG CAA TCC CAA TCA CAC C-3′, GAPDH-F: 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′, GAPDH-R: 5′-TCT AGA CGG CAG GTC AGC ACC-3′. Other chemicals and reagents were of analytical grade.

2.2. Animal groups and rat model of atherosclerotic calcification (AC)

Male Sprague–Dawley rats (220±20 g) aged 6 weeks were purchased from the Experimental Animal Center, Sun Yat-sen University of China. The rats were housed under standard conditions (room temperature 20±1 °C, humidity 60±10%, lights from 6 a.m. to 6 p.m.) and given water freely. All experimental procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China (1998). Rats were randomly divided into control (Con) group, high cholesterol diet (HCD) group, HCD+TS II A (35) and HCD+TS II A (70) group, containing 8 animals in each group. HCD is composed of standard chow (94.3%), cholesterol (2%), lard (3%), cholic acid (0.5%), propylthiouracil (0.2%). The preparation for rat model of AC was as described by Kitagawa (Kitagawa et al., 1992) with some modifications. In short, except for the rats in the Con group, all rats were orally administered with 300,000 IU/kg/day vitamin D2 (VD) for 4 consecutive days followed by consuming HCD in HCD group and consuming HCD combined with orally administration of TS II A (35 mg/kg) in HCD+TS II A (35) group or TS II A (70 mg/kg) in HCD+TS II A (70) group for 12 weeks. The rats in the Con group consumed standard chow. All rats were weighed every 2 weeks. At the end of the 12th week, blood was collected from the abdominal aorta and serum separated was stored at −70 °C after the fasting rats were anesthetized with 30 mg/kg pentobarbital sodium. Thoracicabdominal aorta was isolated and put into ice-cold phosphate buffered saline (PBS). After removing the connective tissue carefully, it was stored at 70 °C. Aorta arch was removed and fixed in 10% formalin.

2.3. Vessel morphological observation

Aortic arch was fixed in 10% formalin followed by dehydration and embedding in paraffin. Six-micrometer thick sections were cut and some of the slides were stained with hematoxylin and eosin (H&E staining). Other slides were deparaffinized and dehydrated before being immersed in a light-
protected 5% AgNO₃ for 30 min and then immersed in a solution of 5% sodium thiosulfate for 2 min followed by counterstaining with eosin (von Kossa staining). The von Kossa staining was analyzed by Kontron IBAS 2.5 automatic image analyzing system (Germany) under the Zeiss Axiotron light microscopy (Germany) according to the double-blind experimental design. Ratio of the calcified area to the calcified and non-calcified area randomly selected was calculated. Photos were taken by JVC ky-F30B 3CCD (Japan).

2.4. Vessel levels of lipid and calcium

The accumulations of vascular lipids and calcium were measured using kit and atomic absorption spectrometry respectively. Briefly, thoracic aorta was freeze-dried to a constant weight using low temperature freeze drier (heto fd2.5 heto lab equipment, Japan). After weighted, the lipids were subsequently extracted at 50 °C for 20 min with chloroform–methanol (2:1) and the extracts were used for the determination of TC. The precipitation was dissolved in HNO₃ and then dried in an oven and re-dissolved with the blank solution (27 nmol/L KCl, 27 μmol/L LaCl₃ in de-ionized water). The calcium content was measured by the use of an atomic absorption spectrophotometer at 422.7 nm (Shimadzu, AA-670, Kyoto). Lipid and calcium content was expressed by mg/g dry tissue.

2.5. Serum levels of lipid files and calcium

Serum was diluted with 150 mmol/l NaCl, 1 mmol/l EDTA (pH 7.4), so that the OD measurement and lipid concentrations were brought into the normal range. Serum concentration of TC was assayed enzymatically by using commercial kits and serum HDL-C and LDL-C were determined by precipitation with phosphotungstic acid/magnesium chloride or with heparin/sodium citrate, respectively followed by assay same as TC. Serum calcium content was determined colorimetrically by the o-cresolphthalein complexone method.

2.6. Serum levels of oxLDL and 25-OH VD

Serum levels of oxLDL and 25-OH VD were assayed by competitive ELISA method following the manufacturer’s instruction as described previously (Tang et al., 2006).

2.7. Vessel levels of superoxide anion and autofluorescence of elastic lamellae in media (Szocs et al., 2002)

Frozen, enzymatically intact, 30-μm-thick sections of abdominal aorta were incubated with Dihydro-ethidium bromide (DHE, 10 μmol/L) in PBS for 30 min at 37 °C in a humidified chamber protected from light. DHE is oxidized on reaction with superoxide anion to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. For ethidium bromide detection, a 543-nm He–Ne laser (Olympus FV500 Laser Scanning Confocal Microscope) combined with a 560-nm long-pass filter was used. IPP (image pro plus) software was employed to analyze the images and to transform them into fluorescence intensity. For detecting the autofluorescence of the elastic lamellae in media, a 488-nm argon laser combined with a 500-to 550-nm band-pass filter was simultaneously used. Elastic lamellae were fluoresced green.

2.8. Vessel Cu/Zn SOD activity and MDA content

10% (w/v) homogenates of abdominal aortas (homogenized buffer: 20 mmol/L HEPES containing 0.2% NP-40 and 20 mmol/Lol/L MgCl₂) was prepared by the use of a Polytron followed by centrifugation at 8000 × g for10 min. The supernatant was used for determination of Cu/Zn SOD activity and MDA content. Cu/Zn SOD activity was determined using the hydroxylamine reduction assay of Oyanatui (Oyanatui, 1984). In this method, the reduction of hydroxylamine by peroxide anion was monitored at 550 nm utilizing the hypoxanthine/xanthine oxidase system as the source for superoxide anion. One unit of Cu/Zn SOD activity is defined as the amount of enzyme necessary to decrease the reduction of hydroxylamine by 50%. MDA content was measured as thiobarbituric acid reactive substances (TBARS) according to Yagi (Yagi, 1984). In short, the supernatant was added 10% (w/v) trichloroacetic acid (TCA) and 2-thiobarbituric acid (TBA) followed by an incubation at 95 °C for 1 h. After centrifugation, TBARS in the supernatant were determined at 532 nm. The concentrations of TBARS were calculated using tetraethoxypropane as a reference standard. The Cu/Zn SOD activity and MDA content were normalized to the protein content.

2.9. Immunohistochemical analysis of vessel Cu/Zn SOD and SMC α-actin expressions

Aorta sections treated as described in method above were used. The first antibodies were mouse anti-rat Cu/Zn SOD monoclonal antibody (Merck Company), rabbit anti-rat SMC α-actin polyclonal antibody (Cell Signaling) respectively. The secondary

Fig. 2. Body weights of rats. Rats were treated and body weight was monitored as described in Materials and methods. (mean±S.D., n=8. *P<0.05 compared with Con group; **P<0.01 compared with HCD group).
antibodies were sheep anti-mouse IgG-peroxidase in the case of Cu/Zn SOD and sheep anti-rabbit IgG-peroxidase in the cases of SMC α-actin (Santa Cruz Biotechnology). Binding was visualized using 3′,3′-diaminobenzadine (DAB) and 0.01% hydrogen peroxide as a chromogene. Sections were briefly air-dried and counterstained with hematoxylin and examined for positive staining (brown staining) by Zeiss Axiotron light microscopy (Germany) and analyzed by Kontron IBAS 2.5 automatic image analyzing system (Germany) according to double-blind experimental design. Photos were taken by JVC ky-F30B 3CCD (Japan).

2.10. Vessel mRNA expression of Cu/Zn SOD

Vessel mRNA expression of Cu/Zn SOD was assessed by RT-PCR. Total RNA of about 30 mg abdominal aorta was extracted following the standard techniques. Isolated total tissue RNA was then quantified by the use of an ultraviolet (UV)
spectrophotometer (DU-640, Beckman, USA). Reverse transcription to cDNA was accomplished by priming 2 μg of total RNA samples with MMLV and oligo (dT) 15 primer. The products were then used for the following PCR amplification: the PCR reaction mixture was in a 25-μL volume containing 2.5 mM dNTP 1 μL, 10-PCR buffer (20 mM MgCl₂, 500 mM KCl, 1.5 M Tris-HCl, pH 8.7), 2.5-μL cDNA, 200 nM of the appropriate rat Cu/Zn SOD-paired primers and 1.25 unit of Taq DNA polymerase. Amplification was performed in a Perkin Elmer Gene Amp PCR System 9600 and consisted of 33 cycles of denaturation at 95 °C, 30 s, annealing at 56 °C, 30 s and extension at 72 °C, 30 s (terminal extension at 72 °C, 7 min) after an initial denaturation step at 95 °C, 10 min. The amplified products were visualized in a 2% agarose gel by staining with

Fig. 5. Representative photos of autofluorescence in elastic lamellae determined using the laser confocal assay. Rats were treated and autofluorescence of elastic lamellae was detected as described in Materials and methods. Green color represents the elastic lamellae. Original magnification: ×200. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Representative photos of SMC α-actin expression (A) and the percentages of SMC α-actin positive (B) in aorta arch. Rats were treated and SMC α-actin expression in aorta arch was assayed as described in Materials and methods. I: intima; M: media. Original magnification: ×400. (mean±S.D., n=4. #P<0.01 compared with Con group; **P<0.01 compared with HCD group).
ethidium bromide under ultraviolet transillumination. Optical densities were analyzed using the Molecular Analyst computed program (Bio-Rad, Vilbert Lourmat, France). The final results are expressed as the ratio of Cu/Zn SOD PCR product to the GAPDH PCR product for each sample.

2.11. Western blotting of vessel Cu/Zn SOD

Vessel protein expression of Cu/Zn SOD was determined by Western blots using β-actin as an internal standard. Proteins were extracted from frozen thoracic aorta with an ice-cold extraction buffer containing 120 mM NaCl, 25 mM KCl, 2 mM CaCl₂, 15 mM Tris–Cl pH 7.5, 0.5% Triton, 1 mM PMSF, 0.1 mM DTT, 10 μM leupeptin and 1 μM pepstatin. Protein concentrations in sample were determined by Bio-Rad protein assay using BSA as a standard. Supernatant protein (30 μg) was separated with 12% SDS-PAGE and then transferred to a nitrocellulose membrane (45 min, 100 V). membranes were then incubated for 2 h with primary antibody against Cu/Zn SOD (Merck Company) and β-actin (Santa Cruz Biotechnology) in blocking buffer. After six washes (6 × 5 min) in TBS/Tween under gentle agitation, blots were incubated for 45 min with horseradish peroxidase-labelled antibody (Santa Cruz Biotechnology). After further washes, blots were revealed by enhanced chemiluminescence detection reagents (Amersham). After immunoblotting, the film was scanned and densitometric analyses were performed using NIH Image software (ver. 1.62). Result was expressed as ratio between Cu/Zn SOD and β-actin.

2.12. Statistical analysis

Data are expressed as mean ± S.D. and were compared with analysis of Student’s t-test. A significant difference was considered as P < 0.05.

3. Results

3.1. Body weight

The body weights of the rats aged 6 weeks showed no differences among the four groups at the beginning of experiment (Fig. 2). The rats in the Con group grew gradually with body weight of about 380 ± 38 g at the end of 12 weeks. However, the
rats in HCD group experienced a slight reduction in body weight in the first 2 weeks due to VD toxicity-induced reduction of food intake, followed by a relatively fast growth in the next 2 weeks. And then their body weight kept stable because of poor appetite caused by HCD with 226±12 g at the end of the experiment. Nevertheless, the rats in HCD+TS II A groups showed a significant increase in body weight at the corresponding weeks compared with those in HCD group with 244±24 g in HCD+TS II A (35) group and 246±12 g in HCD+TS II A (70) group respectively at the end of experiment, suggesting a protection of TS II A against body weight losing caused by both VD and HCD (Fig. 2).

3.2. Histopathological observation by H&E staining

H&E staining of aorta arch (Fig. 3A) showed that no pathological changes were present in the rats in the Con group. While the rats fed with HCD developed severe atherosclerotic calcification characterized by thickened intima and by the co-localization of lipid and calcium deposition under the endothelium (blue color). Two doses of TS II A significantly decreased the thickness of intima, reduced aortic atherosclerotic calcification.

3.3. Vascular calcification observation by von Kossa staining

Von Kossa staining of aorta arch (Fig. 4A) showed that no calcium deposition in vessel was present in the rats in the Con group. While the rats fed with HCD developed severe atherosclerotic calcification (yellow color), locating in both intima and media. Two doses of TS II A significantly reduced atherosclerotic calcification. An analysis of the staining (Fig. 4B) demonstrated that TS II A (35) and TS II A (70) significantly reduced the vascular calcification by 40% and 57.5% respectively compared with HCD (P<0.01).

3.4. Autofluorescence of the elastic lamellae

The autofluorescence of elastic lamellae by laser confocal assay (Fig. 5) clearly exhibited that the elastic lamellae in normal vessel lined up in order. Rats fed with HCD showed severe disorders or fragmentations of elastic lamellae. TS II A (35) and TS II A (70) significantly improved these disorders and protected elastic fiber against disruptions.

3.5. Immunohistochemical analysis of SMC α-actin expression in aorta arch

SMC α-actin in vessel is responsible for the normal vasomotor and lost of SMC α-actin expression reflects the trans-differentiation of SMC to osteoblast-like calcifying vessel cells, resulting in vascular calcification and consequently the dysfunction of vasomotor. Immunohistochemical analysis of SMC α-actin expression (Fig. 6) showed a strong positive exiting in media in the Con group. However, the positive was almost lost especially in the proliferated or calcified SMC, with only 30% that of the Con group. TS II A (35) and TS II A (70) significantly increased the α-actin expression by 58% and 131% (P<0.05 or P<0.01).

3.6. Vessel levels of cholesterol and calcium

Vascular total cholesterol (TC) and calcium content represent the formation of foam cell and degree of atherosclerotic calcification. TC content of thoracic aorta in HCD group (10.98±1.52 mg/g dry tissue) was significantly higher than those in the Con group (6.8±0.69 mg/g dry tissue) (P<0.01). Both TS II A (35) and TS II A (70) significantly reduced TC content by 26.4% and 32.4% respectively compared with HCD (P<0.01) (Fig. 7A). The calcium content of thoracic aorta in HCD group increased about 10 times that of the...
Con group (12.47±2.36 mg/g dry tissue in HCD group vs 1.3±0.25 mg/g dry tissue in the Con group). TS II A (35) and TS II A (70) reduced calcium content in aorta by 42% and 60.3% respectively compared with HCD treatment (P<0.01) (Fig. 7B).

3.7. Serum levels of lipids, calcium and 25-OH VD

Serum levels of TC, LDL-C, calcium and 25-OH VD in HCD group increased while HDL-C decreased compared with those in the Con group (P<0.01). No significant differences in those biochemical parameters were detected between TS II A groups and HCD group (P>0.05), suggesting that the complementation of TS II A to rat did not affect those parameters (Table 1.).

3.8. Serum contents of oxLDL

Because oxLDL is mainly formed by oxidized modification of LDL under the vessel endothelium and releases to blood, the serum oxLDL concentration indirectly represents its production in vessel. The results showed that serum level of oxLDL increased by 105.9% in HCD group compared with that in the Con group, suggesting an increased production of oxLDL under vessel endothelia. Both TS II A (35) and TS II A (70) treatments decreased oxLDL content by 33.6% and 42.7% respectively compared with HCD treatments (P<0.01) (Fig. 8).
3.9. Vessel superoxide anion production, Cu/Zn SOD activity and MDA content

Superoxide anion produced by various vessel cells is the main source to oxidize LDL into oxLDL, and Cu/Zn SOD is the specific scavenger of superoxide anion. Vessel MDA partly reflects the vascular oxidation state. The present results indicated that the vessel production of superoxide anion and MDA content significantly increased while Cu/Zn SOD activity decreased in HCD group compared with those in the Con group (P<0.01), suggesting an imbalance between vessel oxidation and anti-oxidation states, which was prone to LDL oxidation. Both TS II A (35) and TS II A (70) treatments remarkably increased vessel Cu/Zn SOD activity and reduced the MDA content, leading to a decreasing of superoxide anion (P<0.05 or P<0.01) (Fig. 9, Table 2).

3.10. Immunohistochemical analysis of vessel Cu/Zn SOD

The results of immunohistochemical study showed that vessel expressions of Cu/Zn SOD in HCD group were significantly downregulated by 53% compared with that in the Con group. However, those downregulations were significantly inhibited by 52% and 136% by TS II A (35) and TS II A (70) respectively (P<0.05 or P<0.01) (Fig. 11).

3.12. Western blotting of vessel Cu/Zn SOD

The results of western blotting showed that vessel protein expressions of Cu/Zn SOD in HCD group were significantly downregulated 55% compared with that in the Con group. However, those downregulations were significantly inhibited by 45% and 82% by TS II A (35) and TS II A (70) respectively (P<0.05 or P<0.01). The results were well consistent with those of vessel Cu/Zn SOD activity and mRNA expression (Fig. 12).

4. Discussion

Vascular calcification is a common feature of atherosclerotic lesions. In the past, it was considered a passive, degenerative process of aging and was presumed unregulated, inevitable and untreatable. In recent years, vascular calcification has been regarded as an actively regulated, cell-mediated process and shares several features with skeletal bone formation at the cellular and molecular levels. Evidences for this hypothesis include the presence of osteoblast-like calcifying vascular cells in the artery wall that undergo osteoblastic differentiation and calcification in vitro (Demer and Tintut, 2003). Pathologic examinations reveal the co-localization of vascular calcification with atherosclerotic lesion in general, which is termed as atherosclerotic calcification (AC). The close link between atherosclerotic lesion and vascular calcification suggests that atherogenic lipids may regulate vascular cell differentiation and mineralization (Parhami et al., 2002). Nevertheless, the underlying mechanism remains unclear. Hypercholesterolemia is a major risk factor for atherosclerosis, which largely depends on the production of oxLDL derived from oxidized modification of LDL, the main carrier of cholesterol. A large body of evidences showed that oxLDL is involved in the very early yet critical steps of atherogenesis such as endothelial injury, expression of adhesion molecules, and leukocyte recruitment and retention as well as foam cell and thrombus formation (Meisinger et al., 2005; Steinberg and Lewis, 1997; Berliner et al., 1995). The studies in vitro demonstrated that oxLDL could promote vascular calcification (Parhami et al., 1997). Our previous in vivo study (Tang et al., 2006) revealed that oxLDL plays a bridge role between hypercholesterolemia and vascular calcification in rat, suggesting a potential action for those agents such as antioxidants to prevent or attenuate the formation of AC.

In the present study, we have observed the effects of tanshinone II A (TS II A), an ingredient of Salvia miltiorrhiza Bunge known as danshen which is widely used for prevention and/or treatment of cardiovascular diseases in China, on the AC in rats induced by orally administrating excessive vitamin D2 for 4 days followed by feeding high cholesterol diet (HCD) for 12 weeks. The results showed that TS II A significantly attenuated the AC pathological changes in rats. Pathologically, TS II A alleviated the AC by simultaneously reducing lipids and calcium deposition underneath the intima, by improving disorders or ruptures of elastic lamellae shown as H&E staining, von Kossa staining and...
autofluorescence assay, and by upregulating vessel smooth muscle cells (SMC) α-actin expression shown as immunohistochemical analysis, which downregulation meant the transdifferentiation of SMCs to osteoblast-like calcifying vascular cells (Bobryshev, 2005). Biochemically, TS II A simultaneously reduced the vessel cholesterol and calcium contents. Those results suggested that improvement of AC by TS II A might be closely related to the reduction of lipids deposition in intima.

Accumulating studies (Hsu et al., 2002; Rattazzi et al., 2005) showed that rabbit fed with high cholesterol diet or ApoE knock out (ApoE KO) mice develops atherosclerosis lesion accompanied by vascular calcification, suggesting that hypercholesterolemia is an important risk factor for the formation of AC. Thus, we firstly observed the effect of TS II A on the serum cholesterol level including TC, LDL-C and HDL-C in AC rats. The results showed that TS II A had no influences on the serum cholesterol level, suggesting that other actions rather than cholesterol lowering effect of TS II A were attributed to its inhibition of AC.

Administration of excessive VD to rats could enhance intestinal calcium absorption and bone resorption, leading to hypercalcemia (Garabedian et al., 1974; Krohn et al., 2003). The present study showed that the calcium deposition in aorta was accompanied by a rise of serum calcium level in HCD treated rats, suggesting likely a causal relationship between hypercalcemia and aortic calcification. However, TS II A dose-dependently decreased the calcium content in vessel without affecting the serum calcium levels, suggesting that serum calcium level could not explain the mechanism that TS II A reduced the calcium deposition and that other factors might be responsible for those phenomena. This was consistent with the report by Hass et al. (Hass et al., 1960) which showed that there was no persistent rise in serum calcium levels in VD treated rats although the rats developed arterial calcification in a time dependent manner. R. porta et al. (Porta et al., 1994) found in the rat model treated with VD that calcium antagonist nifedipine rather than defibrotide could reduce serum calcium, however, both remarkably prevented the aorta calcinosis. In addition, age-dependent aortic calcification in humans is not related to the changes in blood calcium levels. The present study and other reports suggest a poor correlation between serum and tissue calcium concentration and other factors should be responsible for vessel calcification. Serum 25-OH VD, the active form of VD when orally given, is a major regulator of intestinal calcium absorption. The results in the present study showed that TS II A also had no effect on serum 25-OH VD levels, suggesting that the action of TS II A on AC might be independent of circulating levels of 25-OH VD.

Oxidative modification hypothesis of atherosclerosis emphasizes the pivotal role of oxidized LDL (oxLDL) in initiation and progression of atherosclerosis. Several lines of evidence indicate that enhanced serum levels of oxLDL are strongly predictive for coronary heart disease (Anselmi et al., 2006; Dominguez-Rodriguez et al., 2005; Meisinger et al., 2005). The studies in vivo and in vitro indicated that modified forms of LDL including oxLDL are involved in calcification of vessel or VSMCs by stimulating the osteoblastic differentiation of vascular cells. Proudfoot et al. (Proudfoot et al., 2002) found that treatment of cultured VSMCs with acetylated low-density lipoprotein (acLDL) increased nodule calcification by 3-fold whereas lipoprotein-deficient serum significantly inhibited nodule calcification. Those results are further supported by other studies in which oxLDL and isoprostanes stimulated alkaline phosphatase (ALP) activity, which is known to be a key regulator/initiator of matrix vesicle calcification via generation of local phosphate ions, in calcifying vascular cells (Pathami et al., 1997; Mody et al., 2001). Study from our laboratory also demonstrated that oxLDL accelerated the vascular calcification in AC rat model. Those investigations suggest a casual role of oxLDL in the vascular calcification. Because oxLDL is mainly formed by oxidized modification of LDL, the main carrier of cholesterol, under the vessel intima and releases to blood, serum oxLDL concentration indirectly represents its production in vessel. Therefore, we secondly measured the serum levels of oxLDL in order to further investigate the anti-AC mechanism of TS II A. The present study showed that TS II A lowered serum content of oxLDL in a dose dependent manner, implying that the production of oxLDL in vessel was significantly inhibited. Those results might, at least partly, explain the mechanism by which TS II A exerted its anti-AC action.

LDL is oxidized underneath vessel intima mainly by reactive oxygen system (ROS), especially the superoxide anion, produced by vascular cells. Niu et al. (Niu et al., 2000). L reported that TS II A inhibits LDL oxidation in vitro, which appears to be related to its peroxyl radical scavenging and LDL biding activity. To further investigate the mechanisms by which TS II A inhibited the oxLDL production under intima in vivo, we finally detected vessel levels of superoxide anion, MDA content and activity, mRNA and protein expressions of Cu/SOD, the specific scavenger of superoxide anion. Present results showed that HCD treatment decreased vessel activity, mRNA and protein expression of Cu/Zn SOD while increased the vessel production of superoxide anion and MDA, which was in favor of LDL oxidation in vessel. TS II A increased Cu/Zn SOD activity as well as mRNA and protein expression while reduced superoxide anion production and MDA content. Those results might partly explain the inhibiting action of TS II A on oxLDL production. Indeed, many studies have shown that the degree of LDL oxidation is directly proportional to the rate of superoxide production (Fang et al., 1998). Several studies have shown that an increase in Cu/Zn SOD activity is capable of preventing LDL oxidation in culture cells. Heinecke et al. (Heinecke et al., 1986) have reported that addition of Cu/Zn SOD to the cultured medium inhibits the LDL oxidation induced by human and monkey smooth muscle cells (SMCs). Steinbrecher (Steinbrecher, 1988) reported that Cu/Zn SOD attenuates the LDL oxidation in rabbit endothelial cells and cultured SMCs.

In summary, the present study has demonstrated that TS II A significantly attenuated AC formation in a rat model both pathologically and biochemically, which was related to the inhibition of oxLDL production under intima. This study also suggested that the increasing of Cu/Zn SOD activity as well as mRNA and protein expressions by TS II A might be responsible for the action to inhibit the oxLDL production. These observations should offer further insight into the anti-AC mechanisms.
of antioxidants and provide a potential target for antioxidants in the prevention and treatment of AC.

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