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Changes of heme oxygenase-carbon monoxide system in vascular calcification in rats

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Abstract

The aim of the present study was to investigate the change in heme oxygenase (HO)-carbon monoxide (CO)-cyclic guanosine monophosphate (cGMP) pathway in vascular calcification. Vascular calcification model was established in rats by using vitamin D_3 and nicotine. Vascular calcium content, alkaline phosphatase (ALP) activity, HO activity, HbCO formation and content of cGMP in vessels were measured. Immunochemistry (IH) for HO 1 expression and in situ hybridization (ISH) for HO 1 mRNA were observed. Compared to those of control rats, the aortic calcium content and vascular ALP activity in rats of the calcified group (VDN group) were obviously increased, but HO 1 activity, CO concentration and cGMP content in vessels of rats in VDN group were markedly decreased. Expressions of HO-1 protein and mRNA were significantly decreased compared to control rats. Vascular calcification might induce a down regulation in vascular HO-CO-cGMP pathway.

Keywords: Vascular calcification; Heme oxygenase; Carbon monoxide; Cyclic GMP

Introduction

Vascular calcification is often associated with the pathological process of atherosclerotic lesions, diabetes mellitus, vascular lesions of nephritic syndrome, vascular endothelial injury and aging. Although previously considered uncommon, it is now known to be present in 80% of significant lesions and in at least 90% of patients with coronary artery disease. Previously it was considered as a benign process, but it is now recognized as a major risk factor for cardiovascular events, and a major

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contributor to systolic hypertension, heart failure, plaque rupture and vascular stenosis [1]. Our previous research revealed that the diastolic function of calcified vessels were reduced, the stiffness was increased, and nitric oxide-nitric oxide synthase (NO-NOS) pathway changed [2]. Apart from endothelium-derived relaxing factor, there is nonendothelium-derived relaxing factor, carbon monoxide (CO), in vascular tissues. Similar to nitric oxide, CO was demonstrated to share many properties with NO, including relaxation of vessels, activation of guanylate cyclase, etc. [3]. Whether there are changes in HO-CO-cGMP pathway in the calcified vessels remains unclear. Hence, the purpose of the present study was to investigate if there was an alteration of HO-CO-cGMP pathway in calcified vessels.

Materials and Methods

Materials

All animal experiments in this study were performed with the approval of the Animal Care Committee of the First Hospital, Peking University. Male Wistar rats weighing from 200 to 220 g were provided by the Animal Department, Health Science Center of Peking University. Vitamin D_3, nicotine hydrogen tartrate, Tris, sucrose, phenylmethylsulfonyl fluoride (PMSF), EDTA, hemin, reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and alkaline phosphatase assay kit was obtained from Sigma Co; Radioimmunoassay kit for cGMP was purchased from NEN Life Science Products; polyclonal antibody against HO-1 was from StressGen; Other chemicals and reagents were of analytical grade.

Preparation of vascular calcification model in rats

Two-month-old male Wistar rats (n = 12) were randomly divided into the following groups (6 rats in each group): the control group and the calcified group (VDN group). The procedure of preparation for vascular calcification in rats was as previously described [4]. On day 1, the rats in VDN group received vitamin D_3 (300,000 IU/kg, i.m) and nicotine (25 mg/kg, p.o) at 9 a.m. on day 1. The nicotine administration was repeated at 6 p.m. on the same day. Control group followed a similar protocol except that animals received 0.15 M NaCl i.m and two gavages of distilled water (5 ml/Kg p.o) on day 1. Animals recovered for 6 weeks. During this period, food intake, water consumption and body weight were measured at 3-day intervals. Rats were housed under standard conditions (temperature 20 $\pm$ 1 °C, humidity 60 $\pm$ 10%, lights from 6 a.m. to 6 p.m.) and given standard rodent chow and water ad libitum.

After the above experiments, rats were deeply anesthetized with pentobarbital sodium and killed by an overdose of the anesthetics. The blood samples was pre-treated with EDTA-Na_2 (1 mg/ml), aprotinin (500 KIU) and heparin, and plasma was separated by centrifugation (1600 $\times$ g, 15 min). The heart and aorta were removed to cold (4 °C) PBS. Hearts and aorta were weighed, and were stored at $-$ 70 °C.

Von Kossa staining for calcification [5]

A 1-cm sample of the thoracic descending aorta, just below the aortic arch, was excised and immersed in 10% formol. Samples were dehydrated and embedded in paraffin. Six μm-thick sections were cut and stained with hematoxylin-eosin. The slides were deparaffinized and dehydrated before being immersed

in a lightprotected, silver nitrate solution (5 g in 100 ml distilled water) for 30 min. The slides were next immersed in D-19 Kodak developer for 15 min under exposure of a 100 W bulb, followed by immersion in a solution of 5% sodium thiosulfate in distilled water for 2 min. Finally, slides were counterstained with safranine (red staining).

Determination of vascular and myocardial calcium content and vascular alkaline phosphatase activity [6]

Myocardium (50 mg) of left ventricle and abdominal aorta (10 mg) were dissolved in HNO_3, then dried on oven and re-dissolved with a blank solution (27 nmol/L KCl, 27 μmol/L $LaCl_3$ in de-ionized water). The calcium content was measured with an atomic absorption spectrophotometer at 422.7 nm.

Homogenate of endothelium-denuded abdominal aorta (homogenized buffer: 20 mmol/L HEPES containing 0.2% NP-40, and 20mmol/Lol/L $MgCl_2$) was prepared by polytron. And after centrifugation 8000 × g for 10 min, protein content of the supernatant was determined by Bradford's method. Sample (200 μg protein/200 μl) was mixed with l ml reaction mixture (221 alkaline buffer: stock substrate solution 1:1). Stock substrate solution was prepared by dissolving the contents of a 100 mg capsule of Sigma 104 phosphatase substrate in 25 ml of ddH_2O. This mixture was then incubated for 30 min at 37 °C. The yellow color was indicative of alkaline phosphatase activity. The reaction was stopped by the addition of 20 μl of l mol/L NaOH, and absorbance was determined at 405 nm. Alkaline phosphatase activity was calculated using ρ-nitrophenol as a standard.

HO enzyme activity and HbCO formation

HO activity in vascular microsomes was measured by bilirubin generation [7]. Homogenates (1:5, wt/vol) of aorta were prepared in Tris-HCl buffer (50 mmol/L, pH 7.4, containing 250 mmol/L sucrose, 0.5 mmol/L EDTA). The microsomal fraction of aorta was obtained by centrifugation of the homogenates at 800 × *g* for 10 min and 13,500 × *g* for 20 min at 4 °C, followed by centrifugation of the 13500 × *g* supernatant of each tissue at 105,000 × *g* for 90 min at 4 °C; and then resuspension of the 105,000 × *g* pellet in 100 mmol/L phosphate buffer (pH 7.4, containing 0.1 mmol/L EDTA). 0.5mg of microsomal was added to a reaction mixture (0.4 ml) containing 0.6 mg of rat liver cytosol, 0.8 mmol/L NADPH, 1 mmol/L glucose-6-phosphate, and 0.2 unit glucose-6-phosphate dehydrogenase. Finally, 20 μl of 2.5 mmol/L hemin was added as the substrate. The mixtures were aerobically incubated for 10 min at 37 °C in the dark. The reaction was stopped by placement on ice, and the amount of bilirubin formed was measured with a double-beam spectrophotometer as ÄOD 464–530 nm [extinction coefficient, 40 $(nmol/L \cdot cm)^{-1}$ for bilirubin]. HO activity was expressed as pmol of bilirubin formed per mg protein in 60 min.

HbCO formation was measured by spectrophotometric method [8]. The principle of the method was to allow the CO in homogenates to interact with a solution of Hb and then to measure the HbCO formed by using the dithionite reduction method. One ml of the Hb solution was mixed with 0.5 ml of vascular homogenates or an equivalent amount of water, which was used as the blank to measure the endogenous CO present in the Hb solution. Each 0.1 ml of the sodium dithionite solution was added to both the test homogenates and water-containing blank solution, vortex-mix, and kept stand for 10 min. Read the absorbance of the test homogenates and water blank at 541 and 555 nm against a reference cuvette containing water and measure the ratio (R) of the 541 to 555 readings. Then the percent of HbCO was calculated from a standard curve derived by mixing different proportions of two Hb solutions containing 100% HbO_2 and 100% HbCO.

Determination of vascular cGMP content

Abdominal aorta was weighed, then homogenized by polytron for 4 times, 4 °C, 30 s each in an ice-cold buffer (0.2 mmol/L sodium acetate, pH 5.5). The homogenate was centrifuged at 2000 × g, 4 °C for 20 min. Supernatant was extracted with 4 volumes of sodium acetate buffer twice then extracted with ethanol thrice. Supernatant was then transferred to a fresh tube, evaporated at 60 °C in a vacuum oven until completely dried. The cGMP concentration was determined with commercially available RIA kit, presented as nmol/mg protein.

Immunohistochemical staining [9]

Samples of the thoracic aorta were excised and immediately fixed with 4% paraformaldehyde in 0.1 M phosphate-bufferd saline (PBS; pH7.4). Vessels were fixed for ≥ 24 h and processed for paraffin embedding. Sections (6 μm) were cut and mounted on chrome alum-coated slides. Sections were deparaffinized in toluene, rehydrated in a graded series of ethanol, and washed in 0.01 M PBS. Endogenous peroxidase activity was quenched by incubating sections in 0.3% (vol/vol) H_2O_2 in methanol for 30 min at room temperature. An indirect immunohistochemical method was used to detect HO-1 by using a commercially available avidin-biotin peroxidase(ABC-HRP) immunostaining kit. Rabbit polyclonal antibody against rat HO-1 was applied for 1 h at room temperature and then overnight (4 °C) at a dilution of 1:1000 in a humidified chamber before staining. Immunoreactive sites were visualized by incubating sections with 0.05% (wt/vol) diaminobenzidine (DAB), 0.003% (vol/vol) H_2O_2 in 50 mmol/L Tris-HCl (pH 7.6) for 5 min. Sections were then counterstained with hematoxylin, dehydrated in ethanol, mounted with permount, coverslipped, and examined under a light microscope. The presence of HO-1 were indicated by the development of a brown color within the cytoplasm. Leica Q550IW system (Germany) were used to analyze the mean optical density (OD) and the area of HO-1 staining of the tissues.

In Situ Hybridization of HO-1

An in situ hybridization method was used to detect HO-1 mRNA by using a commercially available HO-1 mRNA ISH detection kit. Dig-labled probe to HO-1 (oligo probe) was obtained from Boster Biological Technology Co, Ltd. The sequence of HO-1 is

(1) 5′—AGAAT GCTGA GTTCA TGAGG AACTT TCAGA—3′
(2) 5′—GCTGC TGGTG GCCCA CGCCT ACACC CGCTA—3′
(3) 5′—TTCCT GCTCA ACATC CAGCT CTTTG AGGAG—3′

Samples of the thoracic aorta were excised and immediately fixed with 4% paraformaldehyde in 0.1 M PBS (pH7.4). Aortic fragments were fixed for ≥24 h and processed for paraffin embedding. Sections (6 μm) were cut and mounted on chrome alum-coated slides. Sections were deparaffinized in toluene, rehydrated in a graded series of ethanol, and washed in 0.1 M PBS. Complex digestive juice was added, and digested 20 min at 37 °C. Slides were prehybridized for 1 hour at 37 °C. Then hybridization was carried out overnight at 4 °C. After hybridization, the slides were washed with 2 × SSC, for four times, each for 5 min. The positive expression was visualized by incubating sections

with DAB for 5 min. Sections were then counterstained with hematoxylin, dehydrated in ethanol, mounted with permount, coverslipped, and examined under a light microscope. The presence of HO-1 was indicated by the development of a brown color within the cytoplasm. Leica Q550IW system (Germany) were used to analyze the mean optical density (OD) and the area of HO-1 staining of the tissues.

Statistical analysis

The results were expressed as means ± SD. Results of some experiments were normalized to total protein determined by Bradford's method. The statistical analysis of the data were performed using Student's t test. A p value of less than 0.05 was considered statistically significant.

Results

Deposition of Vascular Calcification

There were no calcified deposits in control aorta but apparent calcified deposits among the elastin fibers in the medial layer in calcified aorta. (Fig. 1).

Vascular and Myocardial Calcium Contents and Vascular Alkaline Phosphatase Activity

Compared to control group, vascular and myocardial calcium contents increased by 11.1 fold ($p < 0.01$) and 3.3 fold ($p < 0.01$), respectively, and vascular ALP activity increased by 14.2 fold ($p < 0.01$) in rats of VDN group (Table 1).

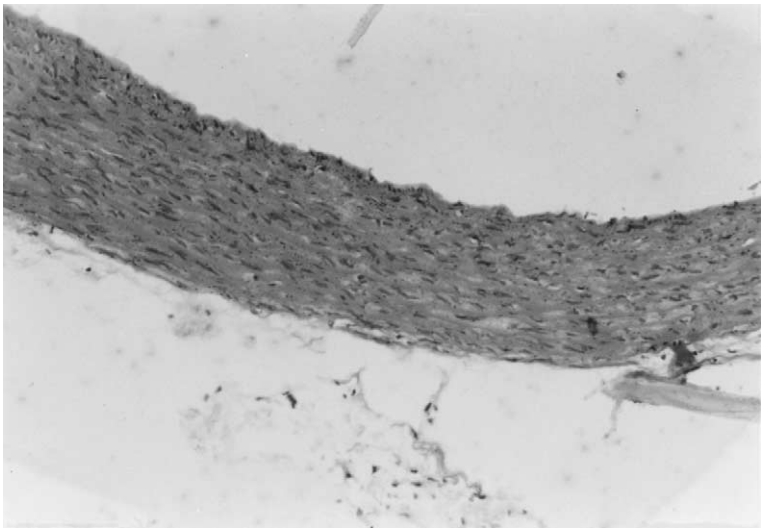

Fig. 1. von Kossa's staining. calcified aorta × 400. There are apparent black speckles among the elastin fibers in the medial layer.

Table 1
Vascular and myocardial calcium content and vascular alkaline phosphatase (ALP) activity in rats. (n = 6, $\bar{x} \pm s$)

	Vasc Ca (μmol/g dw)	Myo Ca (μmol/g dw)	Vasc ALP (U/mg pr)
Control	11.2 ± 0.86	2.26 ± 0.15	42.0 ± 3.9
VDN	124.0 ± 5.42*	7.43 ± 0.53*	596.0 ± 21.1*

Vasc: vascular; Myo: myocardial.
*$p < 0.01$, VDN vs. control.

HO-1 Activity, HbCO Formation and cGMP Content in Vessels

In rats of VDN group, vascular HO-1 activity and HbCO formation were 2.83 ± 0.97 nmol $(mg \cdot h)^{-1}$ and 113.64 ± 3.43 μmol/mg Pr, respectively, and in rats of control group they were 7.14 ± 1.16 nmol

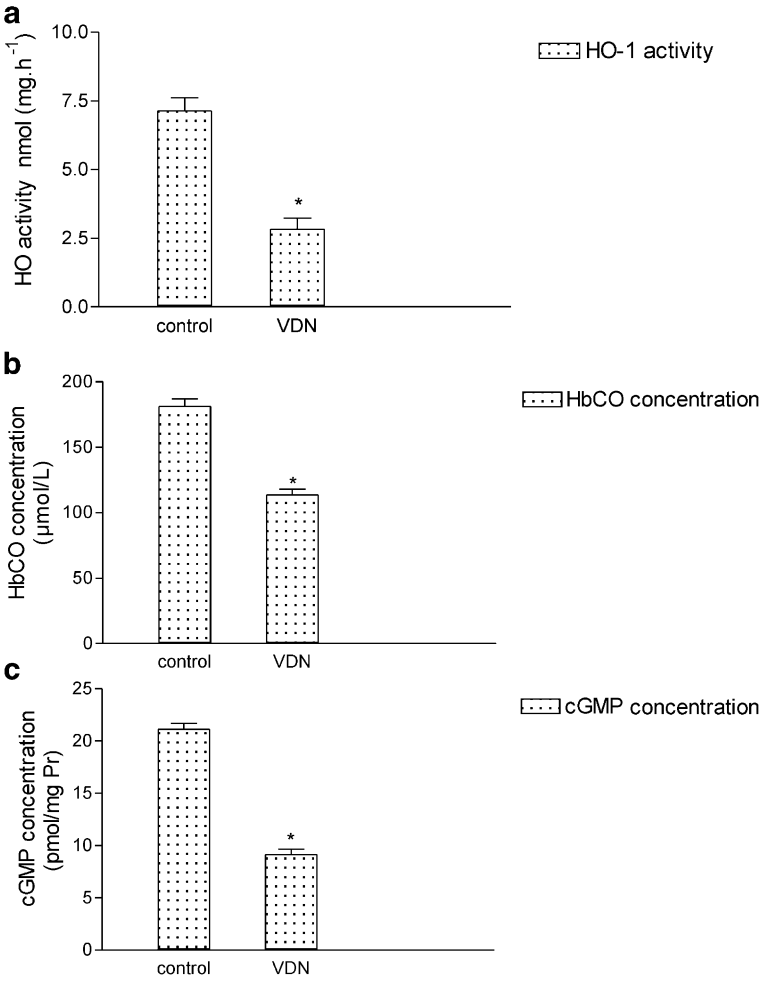

Fig. 2. Changes of HO activity [nmol $(mg \cdot h)^{-1}$] and HbCO formation (μmol/mg Pr) and cGMP content (pmol/mg Pr) from aorta in rats. (n = 6, $\bar{x} \pm s$). *:$p < 0.01$, VDN vs. control.

$(mg \cdot h)^{-1}$ and 181.26 ± 3.74 μmol/mg Pr, respectively, 60.4% ($p < 0.01$) and 37.3% ($p < 0.01$) lower than those in rats of control group, respectively. The aortic cGMP contents in rats of VDN group and control group were 9.13 ± 1.29 pmol/mg Pr and 21.13 ± 1.4 pmol/mg Pr, respectively. It was 56.8% lower in VDN group than that of control rats ($p < 0.01$). There was no significant difference in myocardial cGMP content between VDN group and control group ($p > 0.05$) (Fig. 2).

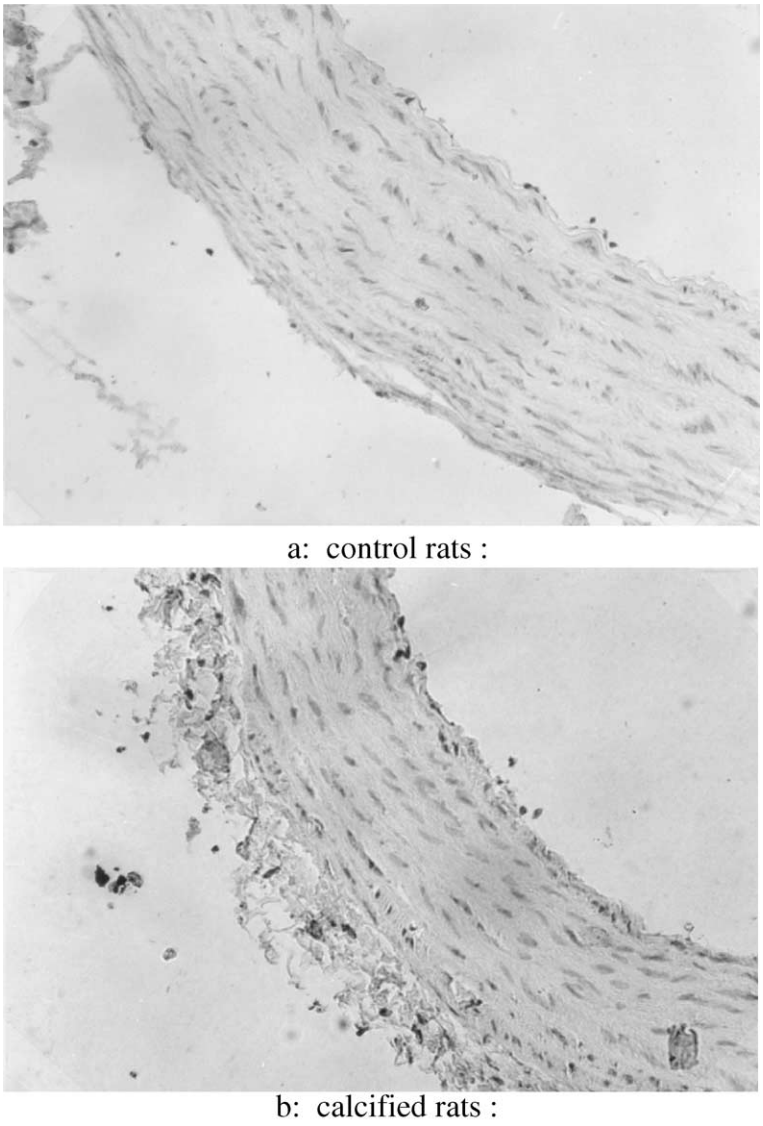

Fig. 3. Immunocytochemical localization of HO-1 expression in rat aorta. Aorta from control rats or calcified rats were stained using a rabbit anti-HO-1 antibody. The brown color represents positive staining for HO-1. Magnification for all photomicrographs is 200 ×.

HO-1 Immunohistochemistry and in situ Hybridization of Calcified Vessels

HO protein was identically distributed in the endothelium, medial and adventitial layers in the control vessels by using the HO-1 antibody (Fig. 3a). However, in calcified vessels, there was positive staining of HO-1 in the endothelium and adventitial layers, and only trace HO-1 immunoreactivity in the medial layer (Fig. 3b). The mean OD was 0.33 ± 0.01 in control group vs. 0.21 ± 0.02 in VDN group ($p <$

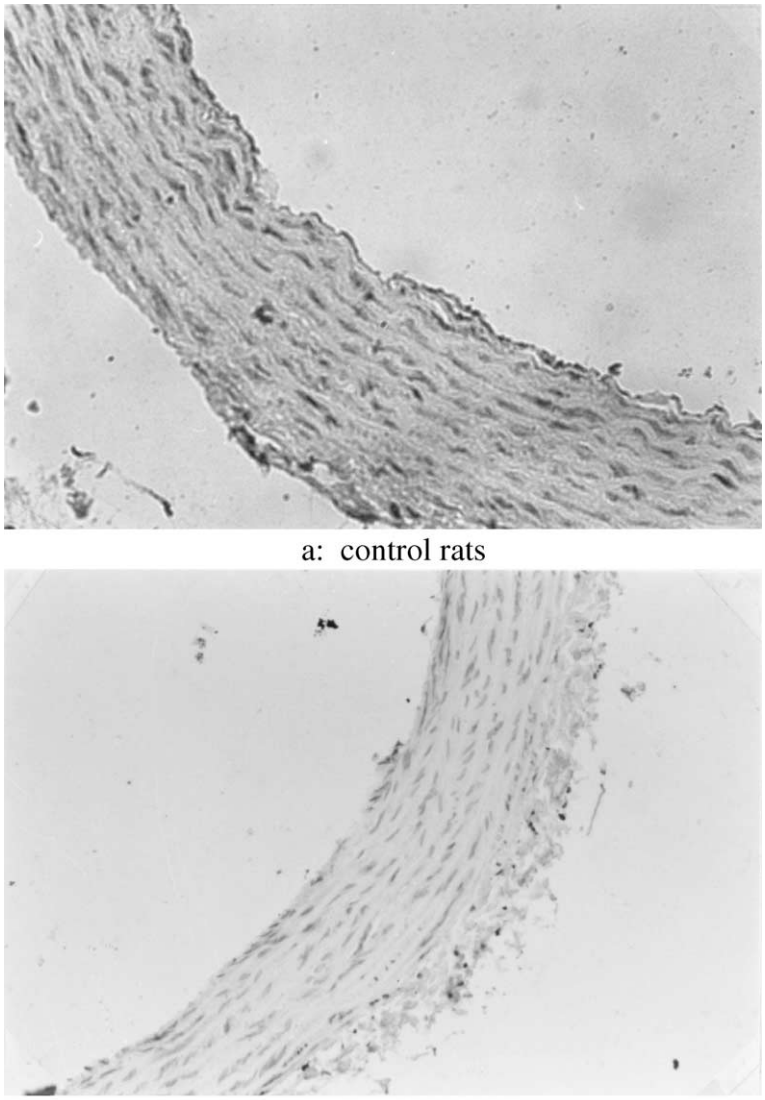

Fig. 4. mRNA localization of HO-1 expression in rat aorta. Aorta from control rats or calcified rats were hybrid using a Dig-labeled probe to HO-1. The brown color represents positive staining for HO-1 mRNA. Magnification for all photomicrographs is 200 ×.

0.05) and the area of HO-1 staining was 157.75 ± 8.89 in control group vs. 156.14 ± 6.61 in VDN group ($p > 0.05$). HO-1 mRNA expression was present in the endothelium, medial and adventitial layers in the control vessels (Fig. 4a). In the calcified vessels, there was apparent staining of HO-1 in the adventitial and medial layers, and only trace mRNA expression in the endothelium (Fig. 4b). The mean OD was 0.35 ± 0.02 in control group *vs* 0.13 ± 0.01 in VDN group ($p < 0.01$), and the area of HO-1 staining was 385.25 ± 5.78 in control group *vs* 367.94 ± 8.62 in VDN group ($p > 0.05$).

Discussion

Previously vascular calcification was considered to be a passive process of calcium deposition. However, at present it is increasingly recognized as an active, regulated process similar to bone formation and osteoporosis [10,11]. Arterial calcium deposition could induce arterial rigidification and decrease arterial distensibility, which correlates not only with calcium accumulation in the extracellular matrix, but also with the turbulence of paracrine/autocrine in vascular cells. The levels of endothelins (ET-1), angiotensin II (Ang II), and platelet-derived growth factor-β (PDGF-β) in calcified vessels were obviously increased, but the contents of C-type natriuretic peptide (CNP) [12], parathyroid hormone (PTH), parathyroid hormone-related peptide (PTHrP) and adrenomedullin (ADM) [13] in calcified vessels were markedly reduced.

CO is an important vasodilating agent [14]. HO is the enzyme that generates CO and biliverdin in heme metabolism. HO catalyzes the rate-limiting step in the oxidative degradation of heme to biliverdin, releasing equamolar amounts of CO and iron. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. Released iron from HO catalysis is sequestered into ferritin. Three isoforms of HO exist and are products of distinct genes. HO-1 isoform is inducible and ubiquitously distributed in mammalian tissues. In addition to heme, its major substrate, HO-1 synthesis is upregulated by a variety of non heme inducers, including heavy metals, cytokines, hormones, endotoxin, and heat shock. HO-2 (a non-inducible isozyme) is a constitutively expressed isoform predominantly found in the central nervous system, and is not inducible. HO-3, however, has not been found in cardiovascular system. CO is a gaseous molecule that shares some of the properties of NO, such as relaxing vessels, inhibiting proliferation of vascular smooth muscle cells, and binding to the heme moiety of cytosolic guanylyl cyclase to produce cGMP, etc.

In the present study, rats with vascular calcification had a higher vascular and myocardial calcium content and vascular ALP activity than controls, which was in accordance with the results of previous research [4]. There was a significant change in vascular HO-CO-cGMP pathway in rats of VDN group. Compared to control group, HO-1 activity decreased by 60.4%, CO content (HbCO formation) decreased by 37.3%, and the content of cGMP decreased by 56.8% in calcified vessels. The results of HO-1 immunohistochemistry showed that HO-1 immunoreactivity was localized in the endothelium, medial and adventitial layers in the control vessels. In the calcified vessels, there was positive staining of HO-1 in the endothelium and adventitial layers, and only trace HO-1 immunoreactivity in the medial layer. The results of HO-1 in situ hybridization showed that HO-1 mRNA was localized in the endothelium, medial and adventitial layers in the control vessels. In the calcified vessels, there was apparent staining of HO-1 mRNA in the adventitial and medial layers, and only trace HO-1 mRNA expression in the endothelium. The above results suggested that there was an obvious damage of HO-CO-cGMP pathway in calcified vessels.

The mechanism responsible for the damage in heme-HO-CO-cGMP pathway in the calcified vessel is not clear. The nucleotide sequence of HO-1 gene appears to be organized in four introns and five exons. Many promoter elements may be present in HO-1 gene that bind to their respective inducers and cause transcription of the gene. HO-1 gene also contains a heatshock element at the 5′-flanking region. A variety of factors could activate or inhibit the element to influence the preexisting HO-1 mRNA pool, and rapidly increase or decrease the expression of HO-1. Bacterial toxins; heme compound (e.g., hematin, hemoglobin); metal ions (e.g., aluminum, tin, lead, gold); hormones (e.g., epinephrine, insulin, dibutyryl AMP, glucagon) and stress, fever, starvation etc, have been identified by various investigators to induce an increase in translatable mRNA for HO-1. But angiotensin II (Ang II) may induce an decrease in translatable mRNA for HO-1. And it was demonstrated that Ang II decreased HO-1 mRNA levels, HO-1 protein expression and HO-1 activity in a calcium-dependent manner in vascular smooth muscle cells [15]. It is well known that vascular Ang II content is seriously increased in atherosclerosis, hypertension, and vascular calcificaton, etc. Hence, it might be suggested that the increased Ang II in calcified vessels could suppress the expression of HO-1 gene and protein. Previous research showed that the concerted activity of heme oxygenase and NADPH-cytochrome P-450 reductase was required for the expression of HO-1 activity and heme degradation [16]. What agents could inhibit HO-1 activity was not very clear. It has been identified that only certain non-physiological metalloporphyrins, such as Sn-, Zn-, Cr- and Mn-protoporphyrins and cadmium have such a function. In addition, intracellular calcium overload could effectively inhibit HO-1 activity. However, it needs further study to clarity whether intracellular calcium accumulation in verified vessels and/or production of endogenous inhibitors cause the inhibition of vascular HO-1 activity.

HO-1 is a kind of heat shock protein (HSP32). Recent studies indicated that vascular HO-CO-cGMP pathway might play a significantly protective role in the cardiovascular system. For instance, Christon et al. [17] demonstrated that HO-1 played an important role in the prevention of hypoxia-induced pulmonary hypertension and importantly inhibited the structural remodeling of the pulmonary vessels. Also, Tulis et al. [18] found that adenovirus-mediated HO-1 gene delivery inhibited balloon angioplasty-induced vascular neointima formation. On the other hand, CO has the function to relax vessels. Brune et al. [19] found that CO could inhibit platelet aggregation and the production of endothelium-derived vasoactive agents, such as endothelin-1 and platelet-derived growth factor-β. Furthermore, biliverdin [20], a metabolite of protoheme and a powerful antioxidant, could remove the supernumerary oxygen-derived free radicals in vivo, attenuate the vascular calcification. The above facts suggested that vascular HO-CO-cGMP pathway played an important role in the regulation of cardiovascular system.

The results of the present study indicated that there were damages in vascular HO-CO-cGMP pathway in calcified vessels. Taking into consideration that vascular calcification is often associated with atherosclerosis, aged vessels, and hypertension in middle-aged and aged patients, which may raise the possibility that intervening heme-HO-CO-cGMP system and applying exogenous substrate of CO as a new strategy of prevention and therapy for atherosclerosis and hypertension may be a promising new research direction in this important area.

Acknowledgements

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References

[1] Blumenthal HT, Lansing AI, Wheeler PA. Calcification of the media of the human aorta and its relationship to intimal atherosclerosis, aging, and disease. Am J Pathol 1994;20:665–87.

[2] Li X, Yan N, Chen JS, Tang CS. Effects of overload calcium induced by vitamin D_3 on vascular L-arginine-nitric oxide pathway. Chinese Journal of Pathophysiology 1999;15:143–6.

[3] Siow RCM, Sato H, Mann GE. Heme oxygenase-carbon monoxide signalling pathway in atherosclerosis: anti-atherogenic actions of bilirubin and carbon monoxide? Cardiovascular Research 1999;41:385–94.

[4] Niederhoffer N, Bobryshev YV, Lartaud II, Giummol LP, Arkinson J. Aortic calcification produced by vitamin D_3 plus nicotine. J Vasc Res 1997;34:386–98.

[5] Balica M, Bostrom K, Shin V, Tillisch K, Demer LL. Calcifying subpopulation of bovine aortic smooth muscle cells is responsive to 17 beta-estradiol. Circulation 1997;95:1954–60.

[6] Hsu HHT, Tawfik O, Sun F. Effects of lectins on calcification by vesicles isolated from aortas of cholesterol-fed rabbits. Biochemica et Biophysica Acta 2000;1464:262–72.

[7] Morita T, Perrella MA, Lee ME, et al. Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. Proc Natl Acad Sci USA 1995;92:1475–9.

[8] Morita T, Kourembanas S. Endothelial cell expression of vasoconstrictors and growth factors is regulated by smooth muscle cell-derived carbon monoxide. J Clin Invest 1995;96:2676–82.

[9] Marks GS, Mclaughlin BE, Vreman HJ, Stevenson DK, Nakatsu K, Brien JF, Pang SC. Heme oxygenase activity and immunohistochemical localization in bovine pulmonary artery and vein. J Cardiovascular Pharmacology 1997;30:1–6.

[10] Shioi A, Mori K, Jono S, Wakikawa T, Hiura Y, Koyama H, Okuno Y, Nishizawa Y, Morii H. Mechanism of atherosclerotic calcification. Z Kardiol 2000;89(Suppl 2):75–9.

[11] Parhami F, Tintut Y, Patel JK, Mody N, Hemmol LA, Demer LL. Regulation of vascular calcification in atherosclerosis. Z Kardiol 2001;90(Suppl 3):27–30.

[12] Tang C, et al. The effect of C-Type natriuretic peptide(CNP), parathyroid hormone (PTH) and parathyroid hormone-related peptide(PTHrP) on the calcification of vascular smooth muscle cells (to be published).

[13] Zhang BH, Tang CS, Du JB. Effects of adrenomedullin on vascular calcification in rats. Z Kardiol 2002 [accepted].

[14] Maines MD. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997; 37:517–54.

[15] Ishizaka N, Griendling KK. Heme oxygenase-1 is regulated by angiotensin II in rat vascular smooth muscle cells. Hypertension 1997;29:790–5.

[16] Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J 1988;2:2557–68.

[17] Christion H, Morita T, Hsieh CM, Koike H, Arkonac B, Prrrella M, Kourembanas S. Prevention of hypoxic-induced pulmonary hypertension by enhancement of endogenous heme oxygenase-1. Circ Res 2001;86:1224–9.

[18] Tulis DA, Durante W, Liu XM, Evans AJ, Peyton KJ, Schafer AI. Adenovirus-mediated heme oxygenase-1 gene delivery inhibits injury-induced vascular neointima formation. Circulaion 2001;104:2710–5.

[19] Brune B, Ullrich V. Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. Mol Pharmacol 1987;32:497–504.

[20] Madhavan M, Wattigney WA, Srinivasan SR, Berenson GS. Serum bilirubin distribution and its relation to cardiovascular risk in children and young adults. Atherosclerosis 1997;131:107–13.