Z Kardiol 93:109–115 (2004)
DOI 10.1007/s00392-004-0989-9

ORIGINAL PAPER

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Alteration of heme-oxygenase-carbon monoxide pathway in calcified rat vascular smooth muscle cells

Veränderungen des Heme-Oxigenase-Kohlenmonoxid-Wegs in kalzifizierten vaskulären Muskelzellen von Ratten

Zusammenfassung Das Ziel der vorliegenden Studie ist die Erforschung der Veränderungen von heme-oxygenase (HO)-carbon monoxide (CO)-cyclic guanosine monophosphate (cGMP) pathway in den vaskulären Muskelzellen. *Methoden* Die Kalzifikation der vaskulären Muskelzellen durch Inkubation der vaskulären Muskelzellen von Ratten mit β-Glycerophosphale. Der zelluläre Calcium-Gehalt, die ALP-Aktivitäten und die ^{45}Calcium-Deposition wurden gemessen. HO-Aktivitäten, die HbCO-Formation und der cGMP-Gehalt in den vaskulären Muskelzellen wurden bestimmt. Der Ausdruck der Immunocyto-Chemie für HO-1 wurde beobachtet. *Ergebnisse* Im Vergleich zu kontrollierten vaskulären Muskelzellen stiegen der zelluläre Calcium-Gehalt, die ALP-Aktivitäten und die ^{45}Ca-Deposition in den kalzifizierten vaskulären Muskelzellen deutlich an. Die Immunocyto-Chemie zeigt, dass der HO-1-Ausdruck in den kalzifizierten Zellen im Vergleich zu den nicht-kalzifizierten Zellen ist, und nicht im Gleichgewicht hielt. Interessanterweise ist jedoch das Positiv bei kalzifizierten Nodi stärker als das in vaskulären Muskelzellen. Im Vergleich mit vaskulären Muskelzellen nahm die Aktivität von HO-1 in kalzifizierten Zellen um 42,7% ab [$36{,}4 \pm 2{,}8$ pmol (mg Pr$\times$h)$^{-1}$ vs $63{,}5 \pm 5{,}3$ pmol (mg Pr$\times$h)$^{-1}$, $p < 0{,}01$]. Während sich die HbCO-Formation um 39,2% ($3{,}38 \pm 0{,}69$ µmol/mg Pr vs $5{,}56 \pm 0{,}48$ µmol/mg Pr, $p < 0{,}05$) verringerte. Der cGMP-Gehalt in kalzifizierten Zellen war um 78,1% ($4{,}3 \pm 0{,}51$ vs $19{,}6 \pm 1{,}2$ pmol/mg Pr, $p < 0{,}01$) niedriger als in nicht kalzifizierten Zellen. *Schlussfolgerung* Die Ergebnisse zeigen eine deutliche Veränderung von HO-CO-cAMP-pathway in den kalzifizierten vaskulären Zellen, was eine Unordnung der vaskulären Funktion erklären könnte.

Schlüsselwörter
Vaskuläre Kalzifikation –
Vaskuläre Muskelzellen –
Heme-oxygenase (HO) –
Carbon monoxide (CO) –
Cyclic guanosine monophosphate (cGMP)

Summary *Objective* The aim of the present study was to investigate the changes in heme-oxygenase (HO)-carbon monoxide (CO)-cyclic guanosine monophosphate (cGMP) pathway in clacified rat vascular smooth muscle cells (VSMCs). *Methods* Calcification of cultured rat VSMCs was induced by incubation of VSMCs with β-glycerophosphate. Cellular calcium content, ALP activities and ^{45}Ca uptake were measured. HO activity, HbCO formation and content of cGMP in VSMCs were determined. Immunocytochemistry for HO-1 expression was observed. *Results* In comparison of control VSMCs, the cellular calcium content, ALP activity and ^{45}Ca uptake in calcified VSMCs were obviously increased. Immunocytochemistry showed that HO-1 expression was weak and not well distributed in calcified cells as compared to non-calcified VSMCs, but interestingly, there

Received: 19 March 2003
Accepted: 24 June 2003

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was stronger staining in calcified nodules than in VSMCs. Compared with VSMCs, HO-1 activity in calcified cells decreased by 42.7% [36.4 ± 2.8 pmol (mg Pr×h)$^{-1}$ vs 63.5×5.3 pmol (mg Pr×h)$^{-1}$, $p < 0.01$], and HbCO formation decreased by 39.2% (3.38×0.69 μmol/mg Pr vs 5.56 ± 0.48 μmol/mg Pr, $p < 0.05$). The cGMP content in calcified VSMCs was 78.1% lower than that of non-calcified VSMCs (4.3 ± 0.51 vs 19.6 ± 1.2 pmol/mg, $p < 0.01$). *Conclusion* The results showed that HO-CO-cGMP pathway in calcified vascular cells obviously changed, which might contribute to disturbance of vascular function.

Key words
Vascular calcification – vascular smooth muscle cells – heme-oxygenase (HO) – carbon monoxide (CO) – cyclic guanosine monophosphate (cGMP)

Introduction

Calcification of vascular tissue is a common complication in aging, atherosclerosis, diabetes, renal failure, aortic stenosis, and prosthetic valve replacement. In arteries calcification is positively correlated with heart diseases, and increases risk of myocardial infarction, ischemic episodes in peripheral vascular disease, and dissection after angioplasty. In addition, the presence of calcification in arteries has recently been found to be a predictive marker for death and myocardial infarction in high-risk and asymptomatic patients. It is well known that vascular calcification induces severe consequences such as reduced diastolic function, potentiated vascular stiffness, thrombosis, and atherosclerotic plaque rupture, etc. (1). However, the mechanism responsible for calcified vascular dysfunction is unclear. Previously, it was widely accepted that the process of vascular calcification was a passive deposition of calcium in vascular extracellular matrix and cells. The view, however, has changed in recent years. The vascular calcification is considered as an active and regulative process similar to osteogenesis (2). During vascular calcification, the phenotype of vascular cells changed to an osteoblast-like phenotype, which could be a determinant in vascular damage (2). We supposed that phenotype-altered vascular cells not only had some properties of ostoblasts, but also changed their paracrine/autocrine functions, and that the paracrine/autocrine dysfunction of calcified vessels could play a crucial role in calcification-induced vascular damage (3). It is common knowledge that paracrine/autocrine factors secreted from vascular cells contribute to circulatory homeostasis, and mediate pathogenesis of cardiovascular diseases (4, 5). We previously observed that vascular nitric oxide (NO) generation was markedly decreased on the model of rat vascular calcification produced by administration of vitamin D_3 and nicotine (6). Apart from endothelium-derived relaxing factor, there is a nonendothelium-derived relaxing factor, endogenous carbon monoxide (CO), in vessels. Similar to NO, CO was demonstrated to share many properties with NO, including inhibition of platelet aggregation and of VSMCs proliferation, relaxation of vascular smooth muscle by activating soluble guanylate cyclase (sGC) and elevating intracellular levels of guanosine 3′,5′-cyclic monophosphate (cGMP), etc. (7). And CO has a much lower affinity for sGC than NO. Vascular CO is generated by heme metabolism through heme oxygenase (HO) catalysis. Decreased production or sensitivity to NO in atherosclerosis may be compensated for by an induction of HO-1, with bilirubin acting as a cellular antioxidant and CO as a vasodilator. Growing evidences demonstrated that vascular HO/CO pathway had a significant cytoprotective action against many cardiovascular diseases, such as atherosclerosis, hypertension, and vascular damage, etc. The present study was to observe if there was an alteration of the HO-CO-cGMP pathway in cultured calcified VSMCs, and to elucidate the mechanism of functional disturbance of calcified vessels.

Material 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 SD rats weighing from 150 to 180 g were provided by the Animal Department, Health Science Center of Peking University. β-Glycerophosphate, collagenase (type I), FBS, cane sugar, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), hemin, reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, anti-a-smooth muscle actin antibody (1A4), and alkaline phosphatase assay kit were obtained from Sigma Co. The $^{45}CaCl_2$ and cGMP assay kit were produced by Amersham. Polyclonal anti-HO-1 antibody (SPA 895) was purchased from StreessGen Co. Other chemicals and reagents were of analytical grade.

Cell culture

VSMCs were obtained by an explant method originally described by Campbell JH and Campbell GR (8). Briefly, medial tissue was separated from segments of rat aorta. Small pieces of tissue were placed in a 10-cm culture dish and cultured in DMEM media containing 20% FBS and antibiotic supplements. Cells migrated from the explants were collected and maintained in DMEM containing 10 mmol/L sodium pyruvate supplemented with 15% FBS (culture medium). VSMCs of the fifth to the eighth passages were used.

Cell calcification in vitro

After confluence, the cells were inculated on 12 well-plates (1×10^4 cells/ml) in DMEM containing 10 mmol/L sodium pyruvate supplemented with 15% FBS in the absence (culture medium) or in the presence of 10 mmol/L β-glycerophosphate (calcifying medium). The medium was replaced with fresh medium every 3 days (9).

Cell counting and cell viability assay

Cells were dispersed with 0.5 mg/ml collagenase in DMEM for 45 ~ 60 min at 37 °C, then 0.1% trypsin in 2 mmol/L EDTA in BPS was used as to ensure that the cells were dispersed. The cells were counted by hemocytometry. Cellular viability was assayed by Trypan blue dye-exclusive test, and was presented as %.

Von Kossa staining for calcification

Cells were fixed in 0.1% glutaraldehyde in PBS for 15 minutes at room temperature, then washed for twice with ddH_2O and incubated with 5% silver nitrate for 30 minutes at room temperature in the dark. Silver nitrate was removed and cells were rinsed twice with ddH_2O. Next, cells were air dried and exposed to sunlight until color development was complete. Cells were rinsed with ddH_2O and prepared for the light microcope.

Assay of cellular calcium content

After incubation, the medium was removed and the cell layer was washed for five times with cold PBS. Scraped cells were collected, and dissolved in HNO_3 and diluted with a blank solution (27 nmol/L KCl, 27 μmol/L $LaCl_3$); the calcium content was measured on an atomic absorption spectrophotometer at 422.7 nm (10).

Measurement of alkaline phosphatase (ALP) activity

Assay of cell-associated ALP activity was performed with ALP assay kit from Sigma. The cells were lysed, then 1 ml of reaction mixture was added, and incubated for 30 min at 37 °C. ALP activity was determined at 405 nm. It was calculated using ρ-nitrophenol as a standard, according to the kit's instructions (Sigma). One unit of ALP activity was defined as the activity producing 1 nmol ρ-nitrophenol for 30 min (10).

Calcification (^{45}Ca accumulation) assay

Twenty-four hours before the end of treatment, 37 kBq/ml $^{45}CaCl_2$ was added into cultured cells of different groups. At the end of the incubation, the medium was removed and the cell layer was washed for five times with cold PBS, scraped into borosilicate tubes containing 0.5 ml perchloric acid, and spun vigorously. Then, 0.5 ml H_2O_2 was added and the suspensions were incubated for 60 min at 80 °C. The mixture was then dissolved in 1.0 ml of ethylene glycol monoethyl ether and spun vigorously, and radioactivity was measured by β-scintillation counting (10).

Determination of heme oxygenase activity

The method used for the measurement of HO activity was according to the protocol published by Morita T. et al. (11). Briefly, cells were lysed and centrifuged, and microsomal fractions were resuspended in 100 mmol/L of potassium phosphate buffer (pH 7.4) containing 2 mmol/L $MgCl_2$. The supernatant (400 μL) was added into an NADPH-generating system containing 0.8 mmol/L NADPH, 2 mmol/L glucose-6-phosphate, 0.2 U glucose-6-phosphate-1-dehydrogenase, and 2 mg protein of rat liver cytosol. Finally, 20 μl 2.5 mmol/L hemin was added as the substrate. The reaction was conducted for 1 h at 37 °C in the dark and terminated by placement on ice, and the amount of bilirubin formed was measured with a double-beam spectrophotometer as ΔOD 464 and 530 nm (extinction coefficient, 40 mmol L^{-1} cm^{-1} for bilirubin). HO activity was expressed as pmol of bilirubin formed per mg of cell protein per hour.

Measurement of carboxyhemoglobin (HbCO) levels in conditioned medium

The relative amount of CO released into the medium was measured by adding Hb (50 mmol/L) for the last hour of incubation, and HbCO was measured spectrophotometrically.

Radioimmunoassay of cGMP

Cellular cGMP was extracted by rapid aspiration of medium, washes with ice-cold phosphate-buffered saline, and the addition of ice-cold ethanol to 65% (vol/vol). The cells were harvested and centrifuged and 2000×g for 5 min at 4 °C. Supernatants were transferred to fresh tubes, evaporated at 60 °C in a vacuum oven until completely dry, and kept at –80 °C until assay. The cGMP concentration of cell extracts was determined by using commercially available RIA kits. The unit of cGMP was pmol/mg Pr.

Histochemical analysis

HO-1 immunoreactivity was detected in VSMCs using polyclonal anti-HO-1 antibody (1:2000 dilution). Briefly, VSMCs were cultured on 10-well heavy Teflon-coated microscope glass slides for 24 h and fixed with cold methanol. After eliminating endogenous peroxidase activity by 0.3% hydrogen peroxide for 5 min, cells were preincubated with normal goat serum and then tested by the ABC method. The brown granules in VSMCs under the microscope were defined as the positive signals. For negative controls, the first antibodies were omitted or the cells were treated with the immunoglobulin fraction of nonimmune goat serum as a substitute for the primary antibody.

Statistical analysis

The values of various parameters were averaged from four independent experiments with duplication. Results of some experiments were normalized to total protein determined by Bradford's method. The data were expressed as means ± SD. Comparisons across two groups were made using Student's test. $P < 0.05$ was considered statistically significant.

Results

Cellular morphology

During spontaneous differentiation, calcified VSMCs displayed distinct morphological transitions. At 5–7 days after plating, cells aggregated into ridge-like structures closely resembling embryonic condensations. From 9–11 days after plating, these condensations formed multicellular nodules, which became increasingly darkened after 14 days in culture due to mineralization, identified by strongly positive von Kossa straining (Fig. 1).

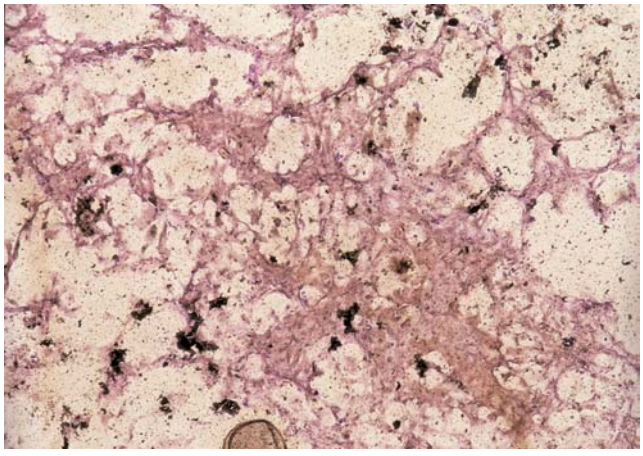


Fig. 1 von Kossa's straining of calcified 14 day old cells

Expression of osteoblastic differentiation and mineralization markers

Compared with control VSMCs, calcium content increased by 9.5 fold ($p < 0.01$), ALP activity increased by 2.9 fold ($p < 0.01$), and ^{45}Ca uptake increased by 11.0 fold ($p < 0.01$) in calcified VSMCs (Table 1).

HO-1 activity, HbCO formation and cGMP content in cells

To observe an alteration of HO-CO-cGMP system in calcified VSMCs, HO-1 activity, HbCO formation and cGMP content were measured. Compared with VSMCs, HO-1 activity in calcified cells decreased by 42.7% [36.4 ± 2.8 pmol (mg Pr×h)$^{-1}$ vs 63.5 ± 5.3 pmol (mg Pr×h)$^{-1}$, $p < 0.01$], and HbCO formation decreased by 39.2% (3.38 ± 0.69 μmol/mg Pr vs 5.56 ± 0.48 μmol/mg Pr, $p < 0.05$). The cGMP content in calcified VSMCs was 78.1% lower than that of non-calcified VSMCs (4.3 ± 0.51 vs 19.6 ± 1.2 pmol/mg Pr, $p < 0.01$) (Fig. 2).

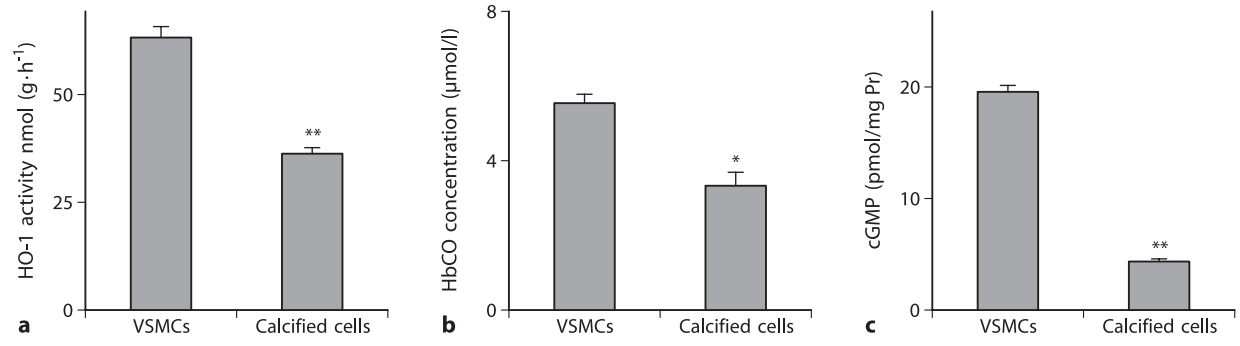
HO-1 immunocytohistochemistry

Expression of HO-1 was shown mainly in intracellular cytoplasm (Fig. 3). Immunocytohistochemistry showed that HO-1 expression was not well dis-

Table 1 Calcium content and ALP activity and ^{45}Ca uptake in cultured cells

	Ca content (nmol/mg Pr)	ALP activity (U/mg Pr)	^{45}Ca uptake (10^3 cpm/mg×Pr)
Control VSMCs	12.6 ± 0.73	2.13 ± 0.12	48.0 ± 3.9
Calcified VSMCs	132 ± 2.42 [1]	8.29 ± 0.53 [1]	573.0 ± 21.1 [1]

[1]: $p < 0.01$, data shown were representative of four separate experiments



* p < 0.05, ** p < 0.01, calcified cells vs VSMCs; the results represent 4 separate experiments with identical results

Fig. 2 Changes of HO activity [nmol (mg×h)$^{-1}$] (**a**) HbCO formation (µmol/mg Pr; **b**) and cGMP content (pmol/mg Pr; **c**) in cells). *: p < 0.05, **: p < 0.01, Calcified cells vs VSMCs; the results represent 4 separate experiments with identical results

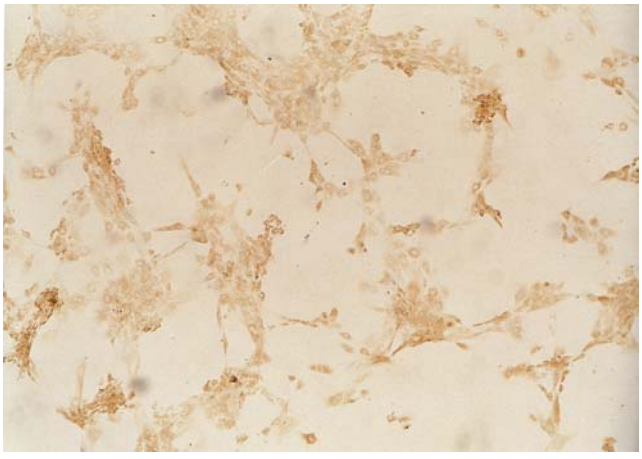


Fig. 3 a Immunocytochemical localization of HO-1 expression in normal cells. The brown color represented positive staining for HO-1. Magnification for all photomicrographs was 200×

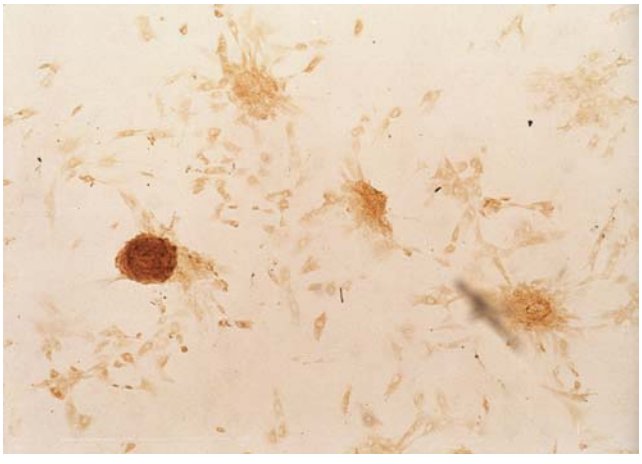


Fig. 3 b Immunocytochemical localization of HO-1 expression in calcified cells. The brown color represented positive staining for HO-1. Magnification for all photomicrographs was 200×

tributed in calcified VSMCs. In general, HO-1 labelling was weaker than that of VSMCs, but interestingly, there eas stronger staining in calcified nodules than VSMCs. The grey level of HO-1 expression was 0.39 ± 0.01 in VSMCs vs 0.18 ± 0.01 in calcified VSMCs.

Discussion

Vascular calcification is often associated with the pathological process of atherosclerotic lesions, diabetes mellitus, vascular lesions of nephritic syndrome, vascular endothelial injury and aging. It is considered as a common pathological property of cardiovascular diseases, which presents in 80% of significant lesions and in at least 90% of patients with coronay artery disease. Previously vascular calcification was thought as a benign process, but it is now recognized as a major risk factor for cardiovascular events, and a major contributor to systolic hypertension, heart failure, plaque rupture and vascular stenosis (1). In addition, it was considered as an active, regulated process similar to bone formation and osteoporosis. Calcium deposition in arteries could induce vascular rigidification and decrease its distensibility, which correlates not only with calcium accumulation in the extracellular matrix, but also with the turbulence of paracrine/autocrine in vascular cells. The vascular cells, including VSMCs, pericyte, macrophage, transformed into osteoblast-like phenotype (2). It is well known that in calcified vascular cells ALP activity increased; matrix vesicles and the nucleation sites for formation of hydroxyapatite were detectable; and bone morphogenetic proteins (BMPs) including BMP-2 and bone matrix proteins such as osteopontin, osteonectin, and osteocalcin overexpressed. However, the function of vascular cells with osteoblast phenotype, especially its paracrine/autocrine remains unclear.

HO/CO pathway plays an important role in regulating cardiovascular homeostasis (12). During 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. Bilirubin and biliverdin have protective effects against cardiovascular diseases through scavenging free radicals. 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 has not been found in cardiovascular system. Endogenous CO is a gaseous signal molecule that shares some of the properties of NO, such as relaxing vessels, binding to the heme moiety of cytosolic guanylyl cyclase to produce cGMP, etc.

In the present study, we found that calcified VSMCs had a higher calcium content, ALP activity and ^{45}Ca uptake than non-calcified VSMCs, which was in accordance with the results of previous research (9). To observe an alteration of HO-CO-cGMP system in calcified VSMCs, HO-1 activity, HbCO formation and cGMP content were measured. Compared to VSMCs, HO-1 expression was not well distributed in calcified VSMCs. In general, HO-1 labeling was weaker than that of VSMCs, but interestingly, there was stronger staining in calcified nodules. HO-1 activity in calcified cells decreased by 42.7% [36.4 ± 2.8 pmol (mg Pr$\times$h)$^{-1}$ vs 63.5 ± 5.3 pmol (mg Pr$\times$h)$^{-1}$, $p < 0.01$], and HbCO formation decreased by 39.2% (3.38 ± 0.69 μmol/mg Pr vs 5.56 ± 0.48 μmol/mg Pr, $p < 0.05$). The cGMP content in calcified VSMCs was 78.1% lower than that of non-calcified VSMCs (4.3 ± 0.51 pmol/mg Pr vs 19.6 ± 1.2 pmol/mg Pr, $p < 0.01$). The above results indicated that there was a significant change in HO-CO-cGMP pathway in calcified VSMCs.

The mechanism of lowered function of HO/CO pathway in calcified VSMCs is not clear yet. The nucleotide sequence of HO-1 gene appears to be organized in four introns and five exons. Many promoter elements present in 5′ regulatory region of HO-1 gene that bind to their respective inducers and cause transcription of the gene. The production of endothelium-derived vasoactive agents, such as endothelin-1(ET-1), platelet-derived growth factor-β (PDGF-β) and angiotensin II (Ang II), etc., can bind with specific DNA sequences within a few hundred base pairs upstream of the transcribed region to inhibit the expression of HO-1. ET-1 increased in calcified vessels (13). As we know, the concerted activity of heme oxygenase and NADPH-cytochrome P-450 reductase was required for the expression of heme oxygenase activity and heme degradation (14). Therefore, a reduced activity of heme oxygenase and/or NADPH-cytochrome P-450 reductase enzyme proteins would result in a decreased heme oxygenase rate. The inhibition of heme oxygenase activity by calcium overload could be caused mostly by the inhibition of the reductase.

Lowered function of HO/CO pathway in calcified VSMCs could have important influence on pathogenesis of cardiovascular disease related to vascular calcification. Brune B et al. (15) found that CO increased the cGMP levels in platelets, caused inhibition of the metabolism of arachidonate, and ultimately inhibited platelet aggregation. Morita T et al. (11) reported that hypoxia significantly increased the transcriptional rate of the gene encoding HO-1, resulting in correspondingly increased mRNA levels and HO-1 enzymatic activity. The activated HO-CO pathway served to increase cGMP levels by activating sGC, but NO was not responsible for the activation of sGC in this setting. They also found that VSMCs-derived CO inhibited ET-1, VEGF and PDGF-β generation under hypoxic conditions. Previous studies showed that lipid peroxidation was a crucial cause of vascular calcification and atherosclerosis (16). Biliverdin (17, 18), the metabolite of protoheme, could inhibit sGC activity in a dose-dependent fashion, and remove an excess of oxygen-derived free radidicals in vivo. The reduced biliverdin in calcified vessels could induce aggregation of oxidative products and therefore deteriorate calcification. CO has the function to relax vessels, inhibit VSMCs proliferation and calcium overload. Hence, a reduction of CO in calcified vessels could contribute to vascular stiffness and proliferation lesions, and accelerate the vascular calcification in a positive feedback manner. This hypothesis of paracrine/autocrine-dysfunction in calcified vascular vessels should be further studied. If this is true, we can raise the possibility that intervening in the heme-HO-CO-cGMP pathway or exogenously applying substrate of CO as a new strategy of prevention and therapy for atherosclerosis and hypertension may be a promising new research direction.

Acknowledgment This work was supported by a research grant from the National Major Basic Research Program (G2000056905).

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