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Suppression of vascular cell adhesion molecule-1 expression by crocetin contributes to attenuation of atherosclerosis in hypercholesterolemic rabbits

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Abstract

To elucidate the molecular mechanism by which antioxidants alleviate atherosclerosis, we investigated the effect of crocetin, a naturally occurred carotinoid with potent antioxidant power, on vascular cell adhesion molecule-1 (VCAM-1) expression in atherosclerotic rabbits. Twenty-four male New Zealand White rabbits were allocated to three groups fed on standard diet (control group), high lipid diet (HLD group) or high lipid diet supplemented with crocetin (crocetin group), respectively. After 8 weeks of treatment, rabbits in HLD group developed severe hypercholesterolemia and atherosclerosis in aortas, together with a significantly up-regulated expression of both protein and mRNA for VCAM-1. In contrast, supplementation with crocetin resulted in markedly ameliorated atherosclerosis, coupled with a significantly decreased VCAM-1 expression, though plasma lipids level remained comparable to that of HLD group. Regression analysis revealed a positive correlation between VCAM-1 expression and the extent of atherosclerosis ($P < 0.01$). In addition, immunohistochemical analysis showed an increased activation of nuclear factor kappa B (NF-κB), a redox sensitive transcription factor essential for VCAM-1 expression, in aortas from rabbits fed on high lipid diet, which was evidently suppressed by crocetin supplementation. These findings suggest that the antiatherosclerotic effect of crocetin might be attributed, at least in part, to the suppressed expression of VCAM-1, which might result from reduced NF-κB activation. This study provides a further insight into the molecular mechanism by which antioxidants attenuate atherosclerosis and suggests a potential target for the treatment of atherosclerosis with antioxidants.

Keywords: Atherosclerosis; Cell adhesion molecule; Transcription factor; Reactive oxygen species; Antioxidants; Crocetin

1. Introduction

Atherosclerosis, a chronic inflammatory disease of arteries, is characterized by infiltration of leukocytes, deposition of lipids and thickening of vascular wall. Fatty streak, the earliest recognizable lesion of atherosclerosis, consists mainly of lipid-laden macrophages (foam cells) derived primarily from recruited monocytes [1]. The recruitment and subsequent transendothelial migration of circulating monocytes, an initial event in the pathogenesis of atherosclerosis, is mediated predominantly by cellular adhesion molecules [2]. Vascular cell adhesion molecule-1 (VCAM-1), a cytokine-inducible member of immunoglobulin gene superfamily, is implicated in atherogenesis by favoring firm adhesion of monocytes to vascular endothelium, which facilitates subsequent infiltration into arterial wall [3]. Several lines of evidence support the concept that VCAM-1 plays a pivotal role in the progression of atherosclerosis. Expression of VCAM-1 was up-regulated in arterial endothelial cells mainly at lesion-prone areas and atherosclerotic plaques in hyperlipidemic animals [4]. Time course studies demonstrated that expression of VCAM-1 precedes subendothelial accumulation of macrophages in hypercholesterolemic rabbits [5]. In humans, focal expression of VCAM-1 was detected mainly in atherosclerotic plaques [6]. Furthermore, circulating VCAM-1, which might derive from proteolytic cleavage and reflect the expression of membrane-bound VCAM-1, was elevated in patients with hyperlipoproteinemia [7] and

Abbreviations: HDL, high density lipoprotein; HLD, high lipid diet; ICAM-1, intercellular adhesion molecule-1; IκB, inhibitory kappa B; LDL, low density lipoprotein; NF-κB, nuclear factor kappa B; Ox-LDL, oxidized LDL; ROS, reactive oxygen species; TC, total cholesterol; VCAM-1, vascular cell adhesion molecule-1

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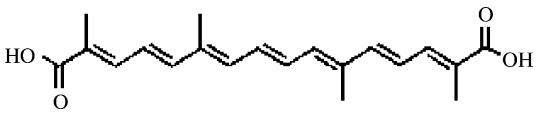

Fig. 1. Chemical structure of crocetin.

correlated significantly with carotid intima-media thickness and the extent of peripheral atherosclerosis [8,9]. On the contrary, genetic deficiency of VCAM-1 significantly diminishes adhesion of monocytes to arterial endoihelium and attenuates atherosclerosis in $LDLR^{-/-}$ and $Apoe^{-/-}$ mice [10,11]. Taken together, these observations indicated clearly the critical role of VCAM-1 in the initiation and progression of atherosclerosis.

The regulation of VCAM-1 expression is mediated, at least partly, by the redox-sensitive transcription factor nuclear factor-kappaB (NF-κB) [12], which is ubiquitously expressed and involved in the expression of several genes implicated in atherosclerosis. Previous studies demonstrated that reactive oxygen species (ROS) plays a key role in the signaling events leading to NF-κB activation [13], implying that antioxidants might effect as an inhibitor of NF-κB activation. Indeed, a large body of evidence indicated that antioxidants could suppress cytokine-induced NF-κB activation and VCAM-1 expression in cultured endothelial cells, resulting in reduced adhesion of monocytes to endothelial cells in vitro [14–16]. These findings suggest a potential target for antioxidants in the prevention of atherosclerosis. However, little evidence is available as to whether antioxidants could attenuate atherosclerosis by suppression of VCAM-1 expression in vivo, though antioxidants have been shown to alleviate atherosclerosis effectively in both animals and humans [17,18]. So, in this study, we investigated the effect of crocetin (Fig. 1, >98% HPLC), a carotenoid with potent antioxidant capacity [19], on VCAM-1 expression and NF-κB activation in atherosclerotic rabbits, with the hope to elucidate the molecular mechanism by which antioxidants attenuate atherosclerosis and to provide further evidence for using antioxidants in the prevention and therapy of atherosclerosis.

2. Materials and methods

2.1. Experimental protocol

Twenty-four male New Zealand White rabbits weighing 2.0–2.2 kg (Jinlin Rabbit Farm, Nanjing, China) were allocated randomly to the following groups with eight in each: control, high lipid diet (HLD) and crocetin groups. Except for the rabbits in the control group received a standard diet, those in the other two groups were fed a standard diet supplemented with 1.2% cholesterol (Xinbei Pharmaceutical Co., Nanjing, China) and 4% lard (high lipid diet) for 8 weeks. Crocetin (15 mg/kg body weight) was added to 10 g of high lipid diet and given to the animals in the morning to ensure that crocetin-containing diet was consumed completely. All the animals were provided with 120 g diet per day and kept under a circadian rhythm of 12 h light:12 h dark with free access to drinking water. Doses of crocetin were adjusted according to changes in body weight monitored weekly. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

2.2. Plasma lipids analysis

At the end of the experiment, fasting blood samples were collected from the marginal ear vein into EDTA-coated tubes followed by centrifugation at 1500 × *g*, 4 °C for 10 min. Plasma total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C) and high density lipoprotein-cholesterol (HDL-C) were determined enzymatically using commercially available kits (Nanjing Jiancheng Bioengineering Institute (NJBI), Nanjing, China). Oxidized LDL (Ox-LDL) was determined by sandwich ELISA as described previously [20].

2.3. Aortic cholesterol content

At the end of the experiment, rabbits were anaesthetized with pentobarbital sodium (50 mg/kg) and sacrificed by exsanguinations from abdominal aorta followed by collecting of the whole aortas. After removing of any adhering tissue, three segments from the thoracic aorta of each rabbit were weighted, pooled and homogenized in defined volume of distilled water. Lipids in homogenate were extracted two times by a mixture of chloroform and methanol (2:1, v/v) according to Koga et al. [21]. The combined lipid extract was evaporated to dryness under a stream of nitrogen and the residue was redissolved in isopropanol. Total cholesterol was determined enzymatically using commercial kits (Nanjing Jiancheng Bioengineering Institute).

2.4. Analysis of aortic atherosclerotic lesions

For each rabbit, three segments from different regions of aortic arch were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4) at room temperature overnight. The regions of the segments were standardized for all rabbits. Paraffin-embedded tissue sections (5 μm) were stained with hematoxylin and eosin (H&E). Samples were examined under microscope blindly by two experienced pathologists to evaluate the presence of fatty streak, atheroma and fibrous plaque. Lesions were evaluated semi-quantitatively on a four-point scale (1, absent; 2, mild; 3, moderate; 4, intense) for each damage according to

Ramirez-Tortosa et al. [22]. The extent of lesions in each rabbit was expressed as the average score of three separate segments, which denotes the mean of the three lesions.

2.5. Immunohistochemistry

After deparaffinized and hydrated gradually, sections of 4 μm thick were incubated with 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Then, the sections were rinsed and subjected to microwave for antigen retrieval. After preincubated with 20% normal goat serum for 30 min at room temperature, the sections were incubated with mouse monoclonal antibody against rabbit VCAM-1 (Santa Cruz Biotechnology, CA, USA) or NF-κB p65 subunit (Santa Cruz Biotechnology) at 4 °C overnight. The sections were then incubated with biotinylated secondary antibody (Zhongshan Golden Bridge Biotechnology Co., Beijing, China) for 30 min followed by incubation with peroxidase-conjugated streptavidin for 30 min at room temperature. The peroxidase was visualized by incubation with diaminobenzidine in dark for 10 min. The extent of VCAM-1 expression was evaluated quantitatively by two paihologists according to the five-grade evaluation of Koga et al. [21].

2.6. Analysis of VCAM-1 mRNA expression

Total RNA was extracted from thoracic aorta of each rabbit with Tripure isolation reagent (Roche Applied Science, IN, USA) according to the manufacturer's instruction. The quality of isolated RNA was examined by the ratio of A_{260}/A_{280} and gel electrophoresis. Reverse-transcription was performed at 37 °C for 60 min with M-MLV reverse transcriptase (Promega Corporation, WI, USA) using oligo (dT) (Promega Corporation) as primer in a 25-μl volume of reaction mixture containing 2 μg of total RNA. Two microliters of the single-stranded cDNA was used as templates for PCR with 1 U of Taq polymerase (TaKaRa Biomedicals, Shiga, Japan) in a 25 μl reaction system. Primers for VCAM-1 and GAPDH were designed on the basis of the mRNA sequence in GeneBank and synthesized by (Sagon Biotechnology, Shanghai, China). The primers for VCAM-1 were 5′-ACT TTC AGG TGG GGC AG-3′ and 5′-CAG CGG GCT GTC TAT CTG-3′ with a PCR product of 265 bp. The primers for GAPDH were 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ and 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ with a PCR product of 580 bp. The conditions of amplification was 94 °C, 30 s; 54 °C, 40 s; 72 °C, 40 s; 35 cycles for VCAM-1 and 94 °C, 30 s; 60 °C, 40 s; 72 °C, 50 s; 35 cycles for GAPDH, respectively. Intensity of VCAM-1 was standardized to that of GAPDH, which was used as an internal standard.

2.7. Statistical analysis

All data were presented as mean ± S.D. of eight animals. Significance of difference between data from two different groups was analysed by ANOVA. A value of $P < 0.05$ was considered statistically significant. All statistical analysis was performed using SPSS statistical software package (SPSS 10.0 for Windows).

3. Results

3.1. Plasma lipids

After 8 weeks of feeding on high lipid diet, rabbits developed severe hypercholesterolemia with TC, LDL-C and HDL-C all increased significantly compared to those in control group ($P < 0.01$). Ox-LDL, an index of lipids peroxidation, was also increased markedly in HLD group ($P < 0.01$). No significant differences were observed between croectin group and HLD group with respect to levels of TC, LDL-C and HDL-C. However, the level of Ox-LDL was notably decreased in crocetin-supplemented group compared to that in HLD group ($P < 0.01$) (Table 1).

3.2. Tissue cholesterol content

Fig. 2 shows the cholesterol content in thoracic aorta from rabbits in different groups. The accumulation of cholesterol in aortas was notably increased in HLD group, while supplementation with crocetin resulted in a significant decrease in cholesterol deposition in aortas (1.87 ± 0.37, 8.75 ± 2.16, 4.97 ± 1.53 mg/g tissue in control, HLD and crocetin groups, respectively).

3.3. Histological examination

As shown in Fig. 3, none of the three variables for atherosclerotic lesions were observed in sections from rabbits in control group. In HLD group, nearly all animals

Table 1
Plasma lipids profile in different groups (mean ± S.D., $n = 8$)

	TC (mmol/l)	LDL-C (mmol/l)	HDL-C (mmol/l)	Ox-LDL (μg/dl)
Control	1.25 ± 0.20	0.63 ± 0.12	0.35 ± 0.086	6.10 ± 1.04
HLD	17.36 ± 2.60*	14.67 ± 2.76*	0.85 ± 0.17*	16.58 ± 4.15*
Crocetin	15.98 ± 2.61	13.65 ± 2.54	0.72 ± 0.23	11.10 ± 2.05#

* $P < 0.01$ vs. control group.
# $P < 0.01$ vs. HLD group.

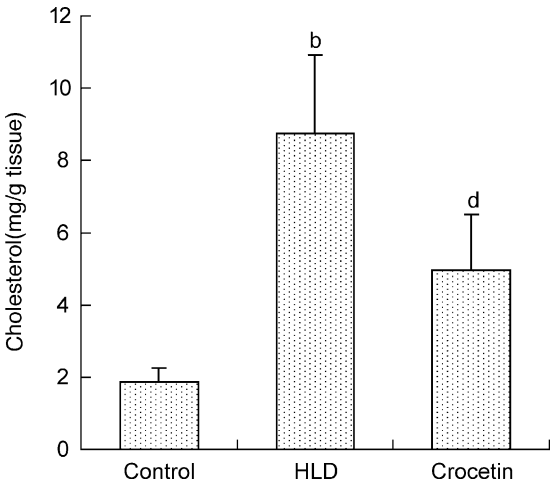

Fig. 2. Cholesterol content in thoracic aortas from rabbits fed on standard diet (control), high lipid diet (HLD) or high lipid diet supplemented with crocetin (crocetin) for 8 weeks. Thoracic aortas were homogenized in distilled water and total cholesterol was extracted with chloroform/methanol (2:1) for two times. The combined extract was evaporated and the residues were redissolved in isopropanol. Total cholesterol was determined enzymatically. Data were presented as mean ± S.D. of eight animals, (b) $P < 0.01$ vs. control group; (d) $P < 0.01$ vs. HLD group.

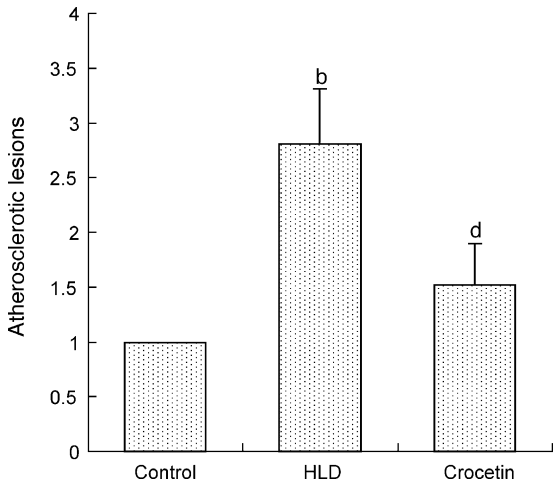

Fig. 4. Effect of crocetin on aortic atherosclerotic lesions. Three separate sections of aortic arch from each rabbit were stained with hematoxylin and eosin (H&E). Samples were evaluated for fatty streak, atheroma and fibrous plaque semi-quantitatively on a four-point scale (1, absent; 2, mild; 3, moderate; 4, intense). The score for each rabbit represented the average score of three sections denoting the mean of the three lesions. Data were presented as mean ± S.D. of eight animals, (b) $P < 0.01$ vs. control group; (d) $P < 0.01$ vs. HLD group.

developed fatty streaks in aortic arch, with the accumulation of lipids localized mainly in the areas subjacent to the endothelium. In two animals, lipids deposition formed a nearly complete ring, resulting in stenotic lumens. Moreover, deposition of lipids in the media was observed in two rabbits. By contrast, rabbits supplemented with crocetin showed much thinner layer of lipids deposition within intima and no complete lipid ring was found in this group. Atheroma, which consists of a large amount of foam cells, was present in six of the eight rabbits in HLD group, while

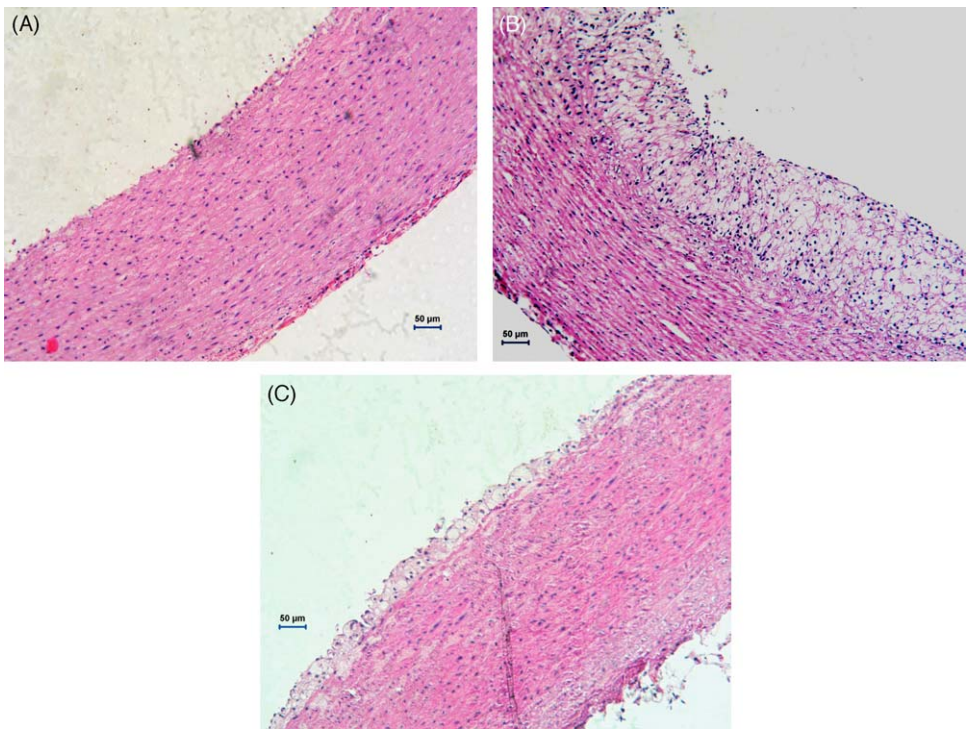

Fig. 3. Representative photomicrograph of aortic arch from rabbits in different groups. Rabbits were fed on standard diet (control group), high lipid diet (HLD group) or high lipid diet supplemented with crocetin (crocetin group) for 8 weeks. No atherosclerotic lesion was observed in control group (A). Rabbits in HLD group developed severe atherosclerotic lesions (B), which was significantly attenuated by crocetin supplementation (C) (H&E ×100). The bars represent 50 μm.

only three of the eight animals in crocetin-treated group developed evidently smaller atheroma. Two rabbit in HLD group developed fibrous plaque, whereas this lesion was absent in crocetin-supplemented rabbits. Semi-quantitative analysis indicated that rabbits in HLD group developed severe atherosclerotic lesions ($P < 0.01$), which was significantly attenuated by supplementation with crocetin ($P < 0.01$) (Fig. 4).

3.4. Immunohistochemical examination

As shown in Fig. 5, little VCAM-1 was detected in aortic sections from control group (A). Eight weeks of high lipid diet induced a significant increase of VCAM-1 expression on endothelial cells (B), especially at lesion areas. Three rabbits from HLD group exhibited nearly complete stained rings. In contrast, only irregular patches of staining for VCAM-1 were observed in sections from crocetin-supplemented animals (C), and two rabbits had merely spotty staining for VCAM-1 on endothelia. Semi-quantitative analysis indicated that the increased VCAM-1 expression induced by high lipid diet was markedly suppressed by crocetin-supplementation ($P < 0.01$) (Fig. 6).

Fig. 5 also showed the representative results of examination of NF-κB activation. Little activated NF-κB was detected in sections from control group (D), while in HLD group, a significantly increased activation of NF-κB was observed (E). Supplementation with crocetin resulted in a significant decrease in NF-κB activation (F). The activated NF-κB was localized mainly in endothelial cells and

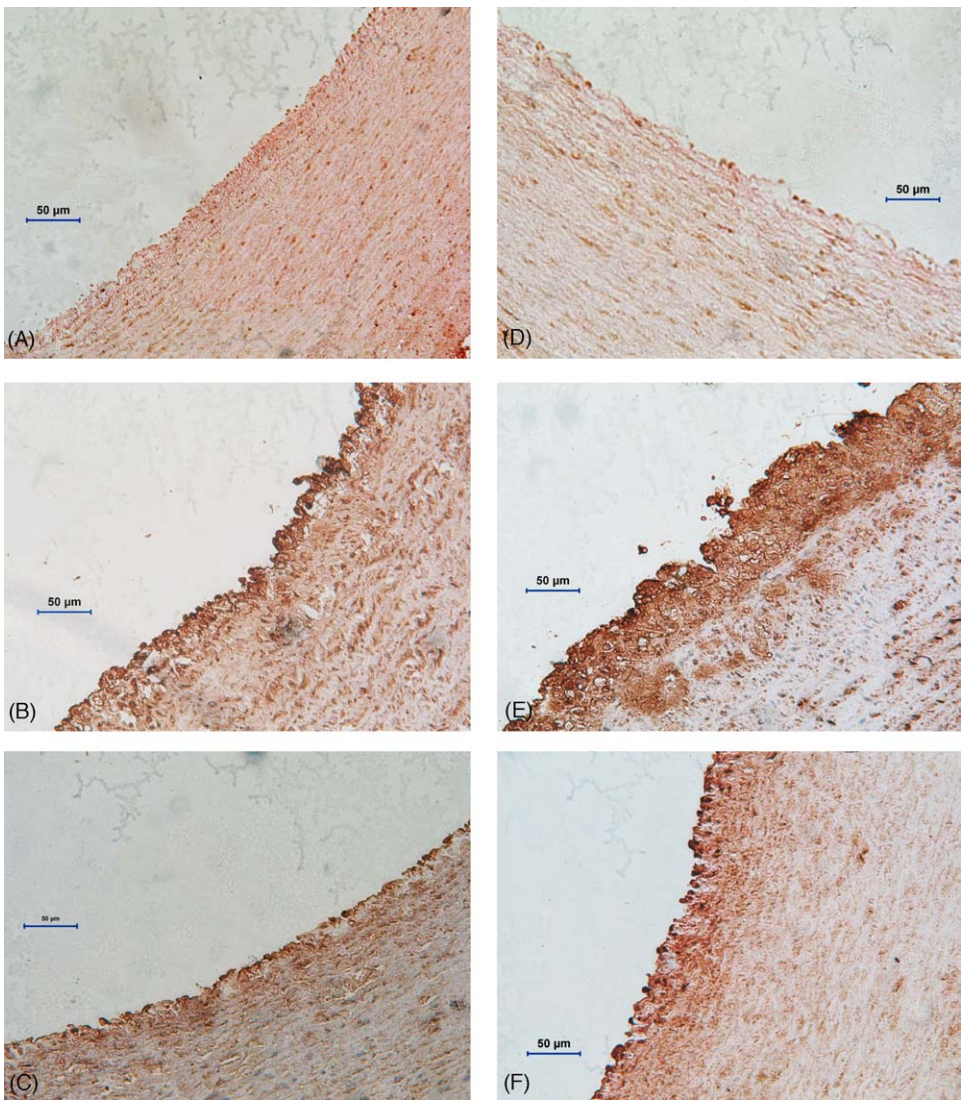

Fig. 5. Representative photomicrograph of immunohistochemical examination for VCAM-1 expression and NF-κB activation in aortas of rabbits fed on standard diet (A and D), high lipid diet (B and E) or high lipid diet supplemented with crocetin (C and F) for 8 weeks. Little VCAM-1 (A) or activated NF-κB (D) was detected in control group. Eight weeks of high lipid diet induced significant increase of VCAM-1 expression (B) and NF-κB activation (E) in aortas, especially at lesion areas. Supplementation with crocetin resulted in significant suppression of VCAM-1 expression (C) and NF-κB (F) activation (×200). The bars represent 50 μm.

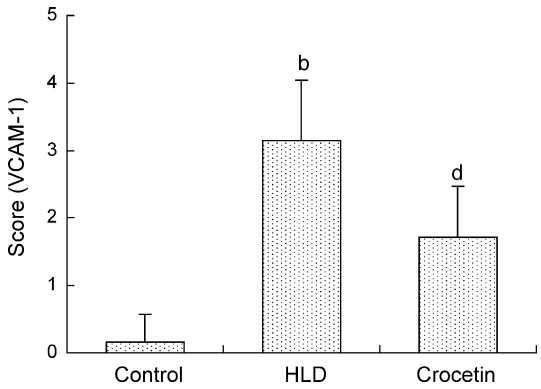

Fig. 6. Effect of crocetin on VCAM-1 expression on aortic endothelial cells. Rabbits were fed on standard diet (control), high lipid diet (HLD) or high lipid diet supplemented with crocetin (crocetin) for 8 weeks. Sections of rabbit aortic arch were subjected to immunohistochemical examination for VCAM-1 and evaluated by two pathologists on a five-grade evaluation. Data were presented as mean ± S.D. of eight animals, (b) $P < 0.01$ vs. control group; (d) $P < 0.01$ vs. HLD group.

macrophages close to atherosclerotic lesions. In some areas, activated NF-κB was also observed in smooth muscle cells in the media.

3.5. Expression of VCAM-1 mRNA

As shown in Fig. 7, aortas from control group showed little expression of VCAM-1 mRNA. A significantly up-regulated expression of VCAM-1 mRNA was observed in aortas from HLD group, while supplementation with crocetin led to evident down-regulation of VCAM-1 mRNA expression. These findings were consistent with examinations for VCAM-1 protein expression and NF-κB activation.

4. Discussion

Hypercholesterolemia, an established risk factor for atherosclerosis, has been shown to increase production of reactive oxygen species (ROS) by both vascular endothelial cells and smooth muscle cells [23], which could in turn initiate several processes involved in atherogenesis, including endothelial dysfunction and LDL oxidation. Activated endothelial cells express a number of adhesion molecules, which facilitate the recruitment and transendothelial migration of circulating monocytes. Transformation of recruited monocytes into macrophages and subsequent uptake of Ox-LDL within intima results in foam cell formation and lipids deposition in arterial wall. Consistently, the present study demonstrated that rabbits fed on atherogenic diet developed serious hypercholesterolemia and atherosclerotic lesions in aorta, characterized by fatty streaks, atheromas and fibrous plaques. Cholesterol accumulation in arteries, an important feature of atherosclerosis, was also increased significantly in atherogenic diet-fed rabbits. As expected, supplementation with crocetin significantly ameliorated atherosclerosis and reduced cholesterol deposition in thoracic aorta. Inconsistent with previous report [24], however, no significant difference was observed between crocetin-supplemented group and HLD group regarding plasma lipids level. These findings suggested that the reduced atherosclerosis could not be attributed to the hypolipidemic effect of crocetin. This discrepancy could be explained by the fact that the dose of crocetin used in this study was much smaller than in the previous one (15 mg/kg versus 50 mg/kg and 100 mg/kg body weight). Consistent with a resent report [25], crocetin supplementation resulted in a significant increase in the resistance of LDL to in vitro oxidation (data not shown) and decreased plasma level of Ox-LDL, implying that crocetin could exert an antioxidant effect in vivo. Based on these results, it is reasonable to deduce that the alleviated atherosclerosis could be ascribed, at least in part, to the antioxidant capacity of crocetin.

To further explore the antiatherosclerotic mechanism of antioxidants, we observed the effect of crocetin on VCAM-1 expression in aortas. As an initial event in the pathogen-

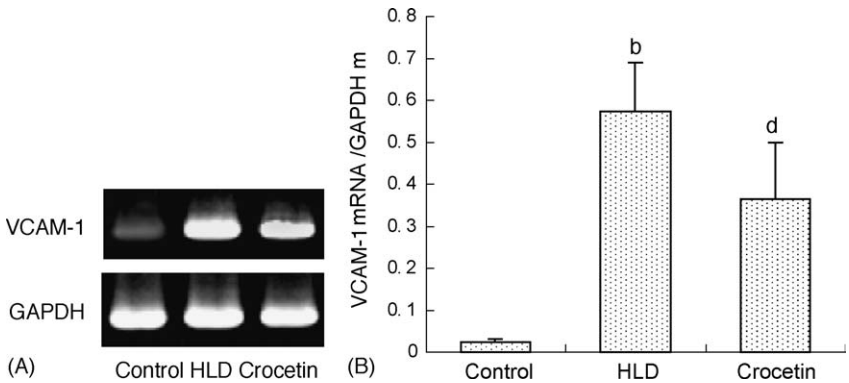

Fig. 7. Effect of crocetin on VCAM-1 mRNA expression in rabbit aorta. Rabbits were fed on standard diet, HLD or HLD supplemented with crocetin for 8 weeks. Total RNA was extracted from thoracic aorta of each rabbit and then subjected to reverse transcription. PCR was performed in a 25 μl reaction system using specific primers for VCAM-1 and GAPDH. (A) Representative result of agarose gel electrophoresis of PCR products. (B) Optical density analysis shown as ratio of VCAM-1 mRNA/GAPDH mRNA. Data represented mean ± S.D. of eight animals, (b) $P < 0.01$ vs. control group; (d) $P < 0.01$ vs. HLD group.

esis of atherosclerosis, expression of adhesion molecules, especially VCAM-1 [10,11], plays a central role in the recruitment of circulating monocytes into intima. A large number of studies indicated that up-regulated VCAM-1 expression was associated with an increased accumulation of foam cells and lipids within arterial wall [7,26]. In line with these reports, this study demonstrated that, after 8 weeks of feeding on atherogenic diet, expression of both protein and mRNA for VCAM-1 was markedly up-regulated in aortic endothelium, especially at areas adjacent to atherosclerotic lesions, whereas supplementation with crocetin resulted in a significant decrease in VCAM-1 expression. Regression analysis demonstrated that VCAM-1 expression correlated positively with the extent of atherosclerosis ($P < 0.01$) and cholesterol deposition in arterial wall ($P < 0.01$), implying that suppression of VCAM-1 expression might be one of the mechanisms by which crocetin inhibits atherosclerosis. These results differed from a recent study demonstrating that Vitamin E suppressed intercellular adhesion molecule-1 (ICAM-1), but not VCAM-1 expression in aortas of hypercholesterolemic rabbits [21]. ICAM-1, another member of immunoglobulin gene superfalimy relating closely to VCAM-1 in both structure and function, is also implicated in the recruitment of circulating leukocytes to intima. So, in this study, we also observed the effect of crocetin on ICAM-1 expression on aortic endothelium. The results showed that aortas of normal rabbits expressed a low level of ICAM-1, which was greatly upregulated in rabbits fed on high lipid diet. Supplementation with crocetin resulted in a significant reduction of ICAM-1 expression on aortic endothelium (data not shown). Since previous studies have demonstrated that it is VCAM-1 but not ICAM-1 that plays a crucial role in the initiation of atherosclerosis [11], whether suppression of ICAM-1 by crocetin plays a role in the alleviation of atherosclerosis calls for further study. Taken together, these findings indicated that suppression of VCAM-1 expression by antioxidants contributes to attenuation of atherosclerosis in hyperlipidemic rabbits.

Since expression of VCAM-1 is regulated at a transcriptional level by NF-κB [27], the suppressed expression of VCAM-1 prompted us to examine the influence of crocetin on NF-κB activation. As a post-transcriptionally regulated transcription factor, in quiescent cells, NF-κB is retained in cytoplasm in an inactivated form associated with its inhibitor, inhibitory KB (IκB), which masks the nuclear localization sequence of NF-κB. In response to diverse stimuli, including ROS, IκB is phosphorylated by IκB kinase (IKK) and detached from NF-κB, allowing NF-κB to translocate to nucleus, where it binds to target genes and promotes transcription [27]. So NF-κB cannot be monitored directly by detecting the expression of new mRNA or protein [28]. In this study, using an monoclonal antibody that recognizes specifically the IκB binding region on the p65 subunit, we detected activated NF-κB in aortas of atherogenic diet-fed rabbits, especially in the areas of atherosclerotic lesions, whereas little activated NF-κB was observed in control group. This finding was consistent with previous reports that activated NF-κB exists in atherosclerotic lesions of humans and hypercholesterolemic animals but is absent or nearly so in vessels devoid of atherosclerosis [29,30], implying that NF-κB activation might be involved in atherogenesis. Supplementation with crocetin resulted in a significant reduction of NF-κB activation, which was in accordance with VCAM-1 expression, suggesting that the suppressed expression of VCAM-1 might result from the reduced activation of NF-κB. These findings were supported by previous reports that diverse, while chemically distinct antioxidants, as well as overexpression of antioxidant enzymes, could attenuate atherosclerosis through inhibition of NF-κB activation [31,32].

In conclusion, the present study demonstrated that suppression of VCAM-1 expression, which probably resulted from reduced activation of NF-κB, contributes to alleviation of atherosclerosis in hyperlipidemic rabbits. This might be one of the molecular mechanisms by which antioxidants ameliorate atherosclerosis. These observations should offer further insight into the antiatherosclerotic mechanisms of antioxidants and provide a potential target for antioxidants in the prevention and treatment of atherosclerosis.

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