HMCO5, herbal extract, inhibits NF-κB expression in lipopolysaccharide treated macrophages and reduces atherosclerotic lesions in cholesterol fed mice

Ki Mo Kim a, Jin Yong Choi a, Si-Eun Yoo a, Mi Young Park a, Bok-Soo Lee a, Young Hye Ko b, Sang Hyun Sung c, Heung-Mook Shin d,∗∗, Jeong Euy Park a,∗

a Division of Cardiology, Samsung Medical Center & Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Seoul, South Korea
b Division of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea
c College of Pharmacy, Seoul National University, San 56-1, Slim-Dong, Gwanak-Gu, Seoul 151-742, South Korea
d Department of Physiology, Dongguk University College of Oriental Medicine, Sukjungdong, Kyongju, Kyongbuk 780-714, South Korea

Received 23 May 2007; received in revised form 25 July 2007; accepted 3 August 2007
Available online 19 August 2007

Abstract

HMCO5 is a herbal extract which comprises of eight different herbs. We studied whether this extract has anti-atherosclerotic effects. In lipopolysaccharide (LPS) stimulated RAW264.7 cells, HMCO5 inhibited NF-κB activation as well as iNOS promoter activity, inhibited the secretion of TNF-α and IL-1β, and directly inhibited the intracellular accumulation of reactive oxygen species. ApoE knock-out mice fed a high-fat high-cholesterol diet with HMCO5 for 10 weeks showed a significant reduction in atherosclerotic lesions. A notable finding was the preservation of the smooth muscle cell layer in the media of aorta in the HMCO5 co-treated mice. HMCO5 treated mice did not show significant decrease in serum level of cholesterol. These results suggest that HMCO5 has anti-atherosclerotic effects which in part may be attributable to the inhibition of production of NF-κB dependent pro-inflammatory cytokines.

© 2007 Published by Elsevier Ireland Ltd.

Keywords: HMCO5; Herb; Atherosclerosis; Inflammatory cytokines; NF-κB; Animal model

1. Introduction

Atherosclerosis is regarded as a chronic inflammatory disease of the vessel wall, characterized by the accumulation of lipid-laden macrophages and foam cells in the large arteries (Ross, 1993; Lusis, 2000). Macrophages and monocytes are the most important sources of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and NO through inducible nitric oxide synthase (iNOS) (Baueuerle and Henkel, 1994; Dinarello, 1996; Thurberg and Collins, 1998). NF-κB is a ubiquitous transcription factor that is involved in the regulation of the inflammatory response (Karin and Delhase, 2000). NF-κB exerts its activity by binding to gene promoters in the form of homo- or heterodimers consisting of NF-κB family proteins. Inactive NF-κB resides in the cytoplasm, where it is bound by its inhibitory subunit, IκB-α.

HMCO5 is a water extract of eight herbs, Pinelliae ternate Ten. Ex Breitenb., Atractylodes macrocephala Koidz., Gastrodia elata Blume, Citrus unshiu Marcow., Poria cocos Wolf, Crataegus pinnatifida Bunge var. typica C. K. Schneider, Siegesbeckia pubescens Makio., and Coptidis japonica Makino; it was standardized on the basis of berberine and hesperidine, the main constituent of C. japonica and C. unshiu. The safety of HMCO5 has been documented. HMCO5 is originated and modified from a popular traditional herbal medicine, Banhab-
ackchulchunmatang (BCT) (Shin and Morgan, 2003). Previous findings revealed that vasodilatory effect of BCT was associated with LC20 dephosphorylation via NO production as well as inhibition of PKCα activation (Shin and Morgan, 2003). In this study, HMCOS formula has also been developed to treat cardiovascular diseases on the basis of the known function of each herb, as described in the literature of traditional Chinese and Korean medicine (Hur, 1999). Pinellia ternate and Coptis japonica are known to have inhibitory effect on the smooth muscle cell proliferation and protective effects against peroxynitrite-induced oxidative damage (Ko et al., 2000; Xie et al., 2000; Yokozawa et al., 2005). Atractylodes macrocephala, Gastrodia elata and Citrus unshiu have been shown to improve the ability of learning and memory of rats as well as induce apoptosis in human colon cancer cells (Gao et al., 1995; Park et al., 2003; Kang et al., 2005). Poria cocos, Crataegus pinnatifida and Siegesbeckia pubescens have been shown to have preventive effects against atherosclerosis and have anti-inflammatory and anti-rheumatism effects (Xie et al., 2000; Hu et al., 2004; Lin and Lin, 2006). It also induces apoptosis in breast carcinoma cells (Jun et al., 2006). These findings indicate that each herbal extract plays a role in cell proliferation, apoptosis and atherosclerosis (Ko et al., 2000; Xie et al., 2000; Kang et al., 2005).

However, the biochemical and pharmacological effects of HMCOS extract in mouse models of atherosclerosis have not yet been demonstrated. We investigated the anti-inflammatory and anti-oxidant effects of HMCOS in RAW264.7 cells and its anti-atherogenic effects in apo E knockout mice.

2. Materials and methods

2.1. Animal model

All animal studies conformed to the Institutional Animal Care and Use Committee (IACUC) of Samsung Biomedical Research Institute (SBRI). apoE knock-out (−/−) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred at the Samsung Biomedical Research Institute under specific pathogen-free conditions. Eight-week-old apoE (−/−) mice (n = 17) were randomly divided into three groups including one reference (n = 5), high-fat-high-cholesterol (HFHC) diet only (n = 6), HFHC diet and HMCOS treated (200 mg/kg/day, n = 6) mice. The HFHC diet contained 1.25% cholesterol, 6% fat, 0.8% sodium chloride (CRF-1, Research Diets, Inc. New Brunswick, USA). To attenuate the atherogenic effects of the HFHC diet, the mice were fed with the HFHC diet for 1 week followed by 2 weeks of feeding normal chow. This schedule was repeated for 10 weeks. The indicated amount of HMCOS was dissolved in pure water and was administrated once a day through tube feeding. Reference mice were fed the normal chow.

In all experiments, body weights were monitored throughout the treatment period. After 10 weeks, the mice were sacrificed by CO2 inhalation. Total plasma cholesterol and triglyceride levels were measured using a 7020 Automatic Analyzer (Hitachi, Japan).

2.2. HMCOS preparation

All herbs used were purchased from a market that is specialized in herbs (Kyungdong herb market, Seoul Korea). All the herbs had been identified by Dr. Jong Hee Park, a professor of College of Pharmacy, Pusan National University. Voucher specimens have been deposited in Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

The herbs had a moisture content of <10% by weight, and were dried. The composition of the mixture was as follows: Pinelliae Rhizoma (9 g), Atractylodis Macrocephalae Rhizoma (12 g), Gastrodiae Rhizoma (6 g), Citri Pericarpium (6 g), Poria (9 g), Crataegi Fructus (9 g), Siegesbeckiae herba (9 g) and Coptidis Rhizoma (9 g). The herbs (69 g) were mixed, minced with a grinder (Rong Tong Iron Works, Taichung, Taiwan) and extracted with 500 ml of distilled water under reflux for 3 h by boiling the formula. The extract was filtered with 10 μm cartridge paper. The filtrate was concentrated to about 50 ml with a rotary evaporator at 50°C under vacuum and freeze-dried to dryness.

HMCOS was standardized on the basis of berberine and hesperidine, using HPLC. In brief, HPLC of HMCOS was performed using acetonitrile: 0.1% formic acid gradient over a period of 25 min: acetonitrile 0–10%, 0–10 min; 10–40%, 10–20 min; 40–90%, 20–25 min. These compounds were separated and quantified by Dionex Chromelon TM Chromatography Data System consisting of UVD 340U detector and Gemini C18 column (5 μm, 4.6 mm x 150 mm I.D.) by UV detection at 280 nm. HMCOS was found to be rich in berberine (2.14%) followed by hesperidine (1.18%). Major sources of berberine and hesperidine are Coptidis japonica Makino, which contains mainly isoquinoline alkaloids including berberine (3–7%) and Citrus unshiu Marcow, which contains mainly flavonoids including hesperidine (4%), respectively.

2.3. Histological examinations

The cross-sectional areas for atherosclerotic lesions were quantified by evaluating the lesion size in the aortic sinus. Briefly, heart and aorta were perfused with phosphate-buffered saline (PBS) for 10 min, perfused with 4% paraformaldehyde for 5 min, and were then promptly removed. After fixation for 1 day in 10% buffered neutral formalin, hearts and aortas were embedded in OCT compound, and frozen at −70°C. All samples were sectioned using a cryostat at −20°C, and six consecutive 8 μm-thick sections were cut from the aorta where the valve cusp was visible. Plaques were stained with Oil red O and counterstained with hematoxylin. The lesion area (micrometer squared) of three sections was then quantified by computer-assisted morphometry (Image-Pro Plus, Silver Spring, MD), and the average lesion size was calculated in each animal.

2.4. Measurement of nitric oxide metabolites, cytokines, and PGE2

Nitrite, a stable oxidized product of NO, was measured in culture media using the Griess reagents. PGE2 concentrations
were determined by enzyme immunoassay (Amersham Pharmacia Biotech, Piscataway, NJ) and levels of TNF-α and IL-1β in the culture media were determined by ELISA (R&D Systems, Minneapolis, MN).

2.5. Western blot analysis

Cells were incubated with or without LPS in the presence or absence of HMC05. They were harvested, washed twice with ice-cold phosphate buffered saline (PBS). Cells were lysed with 1× Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M sodium dodecyl sulfate, 0.3 mM bromophenol blue) and boiled for 10 min. Protein content was measured with BCA protein assay reagent ( Pierce, Rockford, IL). The samples were diluted with 1× lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein (20 μg of protein) were separated on 8–12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in PBS containing 0.1% Tween 20 (PBST) for 2 h, and incubated with monoclonal mouse-iNOS or polyclonal COX-2 antibodies in PBS-T containing 1% non-fat milk for 2 h. After washing three times with PBS-T, the membranes were hybridized with horseradish peroxidase-conjugated secondary antibodies for 40 min. Following five washes with PBS-T, they were incubated with chemiluminescent solution for 2 min, and protein bands visualized on X-ray film.

2.6. RT-PCR

Total cellular RNA was extracted using the Trizol reagent (Gibco BRL Grand Island, NY, USA) according to the manufacturer’s instructions. For each RT-PCR, 1 μg of total RNA was used with Novegen One-step RT-PCR kit (EMD Biofacture’s instructions. For each RT-PCR, 1 μg of RNA was used with Novegen One-step RT-PCR kit (EMD Bioscience). The following sense and antisense primers were used with Novegen One-step RT-PCR kit (EMD Biofacture’s instructions. For each RT-PCR, 1 μg of RNA was used with Novegen One-step RT-PCR kit (EMD Bioscience). The following sense and antisense primers were used with Novegen One-step RT-PCR kit (EMD Bioscience). The following sense and antisense primers were used:

- iNOS primer, sense 5′-AGTTTGGGAGGTCTTTCGCCAGG-3′ and antisense 5′-GATTTCAAAGACCT-3′
- TNF-α primer, sense 5′-CTGAGACAG-3′ and antisense 5′-TCACAGAGCAAATGAC-TCC-3′
- IL-1β primer, sense 5′-ATGGGACACTTGTCAACAGC-3′ and antisense 5′-TTAGGAAGACCGGATT-3′
- β-actin primer, sense 5′-TCTTCTGTGTTCCGGTCCA-3′ and antisense 5′-GCTCCGAGTCCATCAC-3′

The reaction conditions were 40 cycles at reverse transcription at 60°C for 30 min, initial PCR activation at 94°C for 1 min, denaturation at 94°C for 1 min, annealing at 60°C for 90 s, and final extension at 60°C for 7 min. After amplification, the products were resolved by electrophoresis on 1% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

2.7. Electrophoretic mobility shift assay

Macrophages were treated with LPS in the presence of HMC05 extract for 2 h and nuclear extracts were prepared as described previously (Kim et al., 2003). A double stranded NF-κB-specific oligonucleotide (5′-AGTTTGGGAGGTCTTTCGCCAGG-3′) was labeled with [γ-32P] ATP by T4 polynucleotide kinase and purified on a G-50 Sephadex column. The nuclear extracts (10 μg of protein) were incubated with ~40,000 cpm (0.5 ng) of 32P-labeled oligonucleotide for 20 min at room temperature. Samples were separated on a 5% native polyacrylamide gel, were dried and subjected to autoradiography.

2.8. iNOS promoter activity assay

A murine iNOS promoter-luciferase construct was transiently transfected by using the transfection reagent Lipofectamine 2000 (Invitrogen Life Technologies). After harvest cells were lysed in reporter lysis buffer (Promega, Madison, WI). Twenty microlitre of cell extract was mixed with 100 μl of the luciferase assay reagent and the emitted light intensity was measured using the luminometer AutoLumat LB953 (EG and G Berthold, Bad Wildbad, Germany). Fold induction was calculated as intensity value from each experimental group divided by value from control group after normalization of transfection efficiency by β-gal assay.

2.9. Measurement of intracellular ROS

Macrophages cultured in a 60 mm-dish at 4 × 10^5 cells/dish were treated with LPS (1 μg/ml) for 45 min. DCF-DA (5 μM) was added to the cultures and incubated for 20 min. The cells were harvested and washed three times with PBS. And intracellular ROS was quantified by flow cytometry using a FACScan (Becton-Dickinson, Mountain View, CA) with a 488 nm excitation beam.

2.10. Statistical methods

The data are expressed as mean ± S.D. Statistical significance was determined using the Student’s t-test, and a P value of less than 0.01 was considered significant.

3. Results

3.1. HMC05 inhibits the production of NO and PGE2 by suppressing iNOS and COX-2 expression

RAW264.7 cells were stimulated with LPS in the presence or absence of HMC05 for 16 h, and the levels of NO and PGE2 were measured in the culture medium by Griess reagents and ELISA kit, respectively. LPS-stimulated cells increased the accumulation of nitrite, a stable oxidized product of NO, and PGE2 in the culture medium while reference cells did not (Fig. 1A and C). These increases were significantly reduced in a dose dependent manner by co-treatment with HMC05. No cytotoxic effects of HMC05 were observed under the same experimental conditions as assessed by lactate dehydrogenase release (data not shown). We next examined the effects of HMC05 on the levels of iNOS and COX-2 proteins and mRNAs in LPS-stimulated cells. These experiments
Fig. 1. HMCO5 extract inhibits production of NO and PGE$_2$ as well as expression of iNOS and COX-2 in RAW264.7 cells (A and C, top). RAW264.7 cells were stimulated with LPS (1 $\mu$g/ml) in the presence or absence of different concentrations of HMCO5 extract. Nitrite and PGE$_2$ levels were measured by biochemical assay with culture supernatants after 16 h treatment. (A and C, bottom) Levels of iNOS and COX-2 were measured by Western blot with cells after 16 h treatment. (B and D) mRNA levels of iNOS and COX-2 were determined by RT-PCR from cells after 8 h treatment. 

showed that the LPS-mediated increases in the protein and mRNA levels of these enzymes were suppressed in a dose-dependent manner by HMCO5 treatment (Fig. 1B and D). These results indicate that HMCO5 inhibits NO and PGE$_2$ production by the suppression of iNOS and COX-2 expression at the transcriptional level in LPS-stimulated RAW264.7 cells.

3.2. HMCO5 suppresses production of TNF-α and IL-1β

Levels of TNF-α and IL-1β were measured in culture media by ELISA. Stimulation of RAW264.7 cells with LPS dramatically increased secreted TNF-α and IL-1β levels, and these increases were substantially inhibited by HMCO5 (Fig. 2A and C). TNF-α, and IL-1β are expressed in the inactive pro-form; they are then cleaved into the active forms by TNF-α converting enzyme and interleukin-1β-converting enzyme, and secreted (Thornberry et al., 1992; Black et al., 1997). Western blot analyses showed that the HMCO5 treatment reduced intracellular levels of the pro-forms (Fig. 2B and D), and also reduced levels of the mRNAs. Thus, HMCO5 inhibits expression of TNF-α and IL-1β, probably at the transcriptional level.

3.3. HMCO5 suppresses NF-κB activation and nuclear translocation of NF-κB

NF-κB is an important transcription factor for the stimulation of various inflammatory genes including iNOS, COX-2, TNF-α and IL-1β in macrophages treated with LPS (Rhodus et al., 2005). We tested whether HMCO5 regulates NF-κB activation in LPS-stimulated RAW264.7 cells. As shown in Fig. 3A, we found that the increase in LPS-induced NF-κB-DNA binding was markedly inhibited by HMCO5 treatment. We further examined whether HMCO5 suppresses iNOS promoter activity through the inhibition of NF-κB activation (Fig. 3B). LPS treatment of RAW264.7 cells resulted in a three-fold increase in iNOS promoter activity that was suppressed by the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) as well as by HMCO5.

To determine whether the inhibitory effect of HMCO5 on LPS-induced NF-κB activation was due to inhibition of IκB-α phosphorylation and NF-κB translocation, cytosolic and nuclear IκB-α p65 subunit levels were measured following treatment with LPS in the presence or absence of HMCO5. While LPS treatment increased nuclear p65, HMCO5 co-treatment inhibited this translocation (Fig. 3C). We also found that the IκB-α phosphorylation in response to LPS was suppressed by HMCO5 (Fig. 3D).

3.4. HMCO5 and NADPH oxidase inhibitor suppress LPS-induced increase in ROS level

Macrophages stimulated with LPS generate ROS via the activation of membrane bound NADPH oxidase (Bokoch and Knaus, 2003), and ROS plays an important role in NF-κB activation (Flohe et al., 1997). We examined whether HMCO5 inhibits ROS generation and NO production in RAW264.7 cells stimu-
lated with LPS. Treatment with HMCO5 as well as the NADPH oxidase inhibitor diphenylene iodonium (DPI) significantly reduced the intracellular levels of ROS in RAW264.7 cells stimulated with LPS (Fig. 4A). Moreover, treatment with HMCO5, DPI, and PDTC suppressed the LPS-induced increase in NO production and iNOS expression compared with the reference cells (Fig. 4B). This result suggests that the antioxidant activity of HMCO5 contributes to the suppression of NF-κB-dependent iNOS expression.

3.5. Anti-atherogenic effect of HMCO5 on apoE (−/−) mice

We investigated whether HMCO5 affects atherogenesis in apoE (−/−) mice by quantifying the areas of lesions formed in the aortic sinuses of mice treated with a high fat and high cholesterol (HFHC) diet and co-treated with the HMCO5. Fig. 5A and C show the longitudinal aorta exhibiting positive oil red-O stained lesions. Also, sections of the aortic root from apoE (−/−) mice in the normal diet (ND), HFHC diet, and HFHC diet co-treated HMCO5 groups are shown. The mice fed HFHC for 10 weeks developed much more atherosclerotic lesions in the aorta compared with the mice fed normal chow. The mice fed HFHC and co-treated with HMCO5 showed significantly less atherosclerotic lesions compared with mice fed HFHC only. Co-treatment with HMCO5 reduced the atherosclerotic lesion area to 52.2% of that of the HFHC-fed mice in the aortic sinuses (Fig. 5D). Plasma levels of total cholesterol showed no significant differences between the HMCO5 co-treated and HFHC groups (Fig. 5B). Mean plasma cholesterol levels were 453 ± 48 mg/dl in the reference group, 723 ± 194 mg/dl in the HFHC with distilled water treated group, 783 ± 229 mg/dl in the HFHC with HMCO5 treated group. Mean plasma triglyceride levels were 71 ± 7 mg/dl in the reference group, 58 ± 8 mg/dl in the HFHC with distilled water treated group, and 70 ± 5 mg/dl in the HFHC with HMCO5 treated group. The body weights of each group were not statistically different, nor were any notable histological lesions found in other parenchymal organs of each group.

3.6. Effect of HMCO5 on macrophage accumulation

The aortic arches, from HMCO5 treated mice, showed decreased recruitment of macrophages into the arterial wall. MOMA-2 positive areas in the mice co-treated with HMCO5 (n = 6) was significantly reduced to 10.2 ± 3.4% of the total cross-sectional area of aortic sinuses compared with 38.2 ± 4.5% in the HFHC only treated group (p<0.01) (Fig. 6A, B).

3.7. Effects of HMCO5 on smooth muscle layer of aorta

A notable finding was the preservation of the smooth muscle cell layer in the media of mice co-treated with HMCO5 (Fig. 6C).
Fig. 3. HMCO5 extract suppresses NF-κB activation, iNOS promoter activity, IκB-α phosphorylation and nuclear translocation of NF-κB. RAW264.7 cells were incubated with LPS in the presence or absence of HMCO5 extract for 2 h and cytosolic (CE) and nuclear extracts (NE) were prepared. (A) RAW264.7 cells were treated with LPS in the presence or absence of HMCO5 extract for 2 h. Nuclear NF-κB activity was analyzed by EMSA in the presence or absence of excess cold probe. (B) RAW264.7 cells were transiently transfected by a lipofectamine method with iNOS promoter. Cells were treated with LPS in the presence or absence of HMCO5 extract for 12 h and luciferase activity in cell extract was measured with a luminometer. Data shown are mean ± S.D. (n = 3). (C) Nuclear extract samples of 20 μg protein were separated on SDS-PAGE and the NF-κB p65 subunit, and equal amounts of nuclear protein were visualized by Western blot analysis. (D) Whole cell lysates from RAW264.7 cells treated with LPS in the presence or absence HMCO5 extract for 1 h were separated on SDS-PAGE and IκB-α phosphorylation was visualized by Western blot analysis. PDTC, pyrrolidine dithiocarbamate, NF-κB inhibitor.

Fig. 4. Effect of HMCO5 extract on ROS generation, and iNOS expression. (A) RAW264.7 cells were treated with LPS in the presence or absence of HMCO5 extract for 45 min, incubated with 5 μM of DCF-DA for an additional 20 min, and intracellular levels of ROS analyzed by fluorescence activated cell sorter (FACS). (B) The nitrite levels of culture media were measured by Griess reagents and iNOS level was determined by Western blot analysis as described in Fig. 1. PDTC, pyrrolidine dithiocarbamate, NF-κB inhibitor; DPI, diphenylene iodonium, NADPH oxidase inhibitor.
Fig. 5. Effects of HMCO5 extract on atherosclerotic lesions in apoE (−/−) mice fed a high fat high cholesterol (HFHC) for 10 weeks. (A and C) Representative en face Oil red-O stained aortas from apoE (−/−) mice fed an HFHC chow from 6 week of age and cotreated daily with (200 mg/kg) HMCO5 extract for 10 weeks. (a) Control group fed a normal diet (original magnification 40×). (b) HFHC fed diet (original magnification 40×). (c) HMCO5 extract cotreated with HFHC groups. (B) Total plasma cholesterol levels in apoE (−/−) mice treated with normal diet, HFHC, and HFHC and HMCO5 extract cotreated group. (D) Oil red-O stained aortic sinus lesion areas were quantitated by computer assisted morphometry. All result are shown as mean ± S.D. (*) Indicates P < 0.01 compared with the HFHC group.

Fig. 6. Effects of HMCO5 extract on macrophages accumulation. (A) Upper panel (a, b, c) shows cross-sections which are stained with rat monoclonal antibody to mouse macrophages (MOMA-2) in the aortic sinus of apoE (−/−) mice fed a normal diet (a) high-fat high cholesterol (HFHC) diet (b) and HFHC and HMCO5 extract (c). (B) Quantification of the areas stained positively for MOMA-2 antibody. Three sections from each animal were stained to determine the mean, which were expressed as a percentage of the total lesion area. All results are shown as mean ± S.D. (*) Indicates P < 0.01 compared with the HFHC group. (C) Lower panel (a, b, c) shows the same cross-sections as upper panel which are stained with smooth muscle α-actin specific antibody.
4. Discussion

HMCO5 is a water extract of a herbal mixture. In RAW264.7 cells, HMCO5 showed suppression of NF-κB activation. HMCO5 also inhibited the expression of iNOS, COX-2, TNF-α, and IL-1β, as well as production of NO and PGE2 in LPS stimulated RAW264.7 cells. HMCO5 inhibited LPS-induced IkB-α phosphorylation and nuclear translocation of cytosolic NF-κB p65. These results may account for its anti-inflammatory action by suppressing NF-κB activation. Expression of inflammatory cytokines is regulated by NF-κB activation (Barnes and Karin, 1997). Thus, modulation of NF-κB activation has become a potential therapeutic target for chronic inflammation.

Furthermore, pre-incubation with HMCO5 repressed the transcription from the NF-κB-dependent promoter stimulated by LPS in RAW264.7 cells in a dose-dependent manner. This provides evidence for a mechanism by which HMCO5 treatment reduced the expression of TNF-α, COX-2, nitric oxide (NO) in our in vitro experiments. The data from nuclear extract of RAW264.7 cells showed that HMCO5 suppressed LPS-induced activation and subsequent DNA binding of NF-κB in a dose-dependent manner. Activation of NF-κB requires phosphorylation, dissociation and proteolytic degradation of IkBα before nuclear translocation of NF-κB, and then activation of gene transcription may occur (Ghosh and Karin, 2002). Therefore, we propose that HMCO5 extract inhibits IkBα phosphorylation, which prevents the phosphorylation and subsequent degradation of IkBα-α, and thereby blocks NF-κB activation, which in turn leads to decreased transcription of inflammatory cytokines.

HMCO5 also demonstrated a strong antioxidant action and inhibited intracellular ROS accumulation. Several studies have demonstrated that some antioxidants, N-acetyl cysteine, α-lipoate and α-tocopherol inhibit NF-κB activation (Schreck et al., 1991; Islam et al., 1998), and may be considered as potential anti-atherogenic agents.

In our experiment, supplementation of the HFHC diet with HMCO5 for 10 weeks in apoE (−/−), mice, resulted in significant decrease in atherosclerotic lesions (Figs. 5 and 6). As expected, lipid accumulation in aorta (Fig. 5) and macrophage accumulation (Fig. 6A and B) were significantly reduced by HMCO5 co-treatment. However, plasma cholesterol level did not show significant changes with HMCO5 co-treatment.

This can be partly attributed to the inhibition of NF-κB-dependent induction of inflammatory mediators, such as TNF-α, IL-1β, COX-2, and nitric oxide (NO) that have been implicated in the pathogenesis of atherosclerosis (Buttery et al., 1996; Ross, 1999). NO is considered as an important modulator of various vascular functions; such as vasorelaxation, smooth muscle cell growth, and inflammatory responses (Napoli et al., 2006). Impaired NO production or reduction of endothelial nitric oxide synthase (eNOS) bioactivity in endothelial cells together with increased generation of reactive oxygen species can cause endothelial damage and subsequent decreased sensitivity to its vasodilator function of smooth muscle cell. In fact, we further tested the possibility of HMCO5’s effect on phosphorylation of eNOS of endothelial cells. HMCO5 did not affect bFGF-induced phosphorylation of eNOS in HUVEC cells (data not shown). However, we could not rule out the possibility that HMCO5 may affect other functions of eNOS such as NO production or enzymatic activity. Meanwhile, HMCO5 clearly inhibited LPS-induced NO production by inhibiting iNOS expression in macrophages. Since overproduction of NO by iNOS in infiltrated macrophages also causes severe damage directly to the surrounding cells in arterial wall including apoptosis (Napoli and Ignarro, 2001; Braam and Verhaar, 2007), we think that the protective effect of HMCO5 on atherosclerosis is attributed to regulating macrophage function in the lesion.

Of particular interest was the finding of preservation of the smooth muscle cell layer in the media of the aortic wall in the HMCO5 co-treated mice. HMCO5 may affect smooth muscle cell survival directly by preventing their apoptosis or necrosis in HMCO5 co-treated mice. In the mice, which were not co-treated with HMCO5, the smooth muscle cell layer was observed to be significantly reduced in areas where atherosclerotic plaques were aggressively formed. Currently, the characterization of the active compounds of HMCO5 that may mediate the anti-inflammatory and anti-atherogenic activity of HMCO5 are under investigation.

5. Conclusion

HMCO5, herbal extract, showed anti-inflammatory effects on mouse monocyte cells line, and anti-atherosclerotic effects on apo E (−/−) mice.

Acknowledgements

This study was supported by grants of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (B050042AM081505N100020B) and The Korean Society of Hypertension.

References


