The Small GTPases Regulate HMC05-Induced NQO-1 Expression with an Antioxidant Effect in Smooth Muscle Cells

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Recently, Banhabackchulchunmatang (HMC05) has been implicated as a preventive and/or therapeutic candidate for cardiovascular diseases due to its inhibition of atherosclerosis lesions and its reduction of neointima formation. Knowledge of the mechanism of HMC05 in smooth muscle cells (SMC) is limited. However, SMC may be a potential target for HMC05 therapy because they are supported by the HMC05-mediated preservation of medial smooth muscle cell layers in pathogenic progression. Therefore, in the present study, we hypothesized that the effect of HMC05 is associated with reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H):quinone oxidoreductase-1 (NQO-1) gene regulation, which precipitates an antioxidant effect in SMC. HMC05 significantly increased NQO-1 gene expression in a dose- and time-dependent manner. The reactive oxygen species-mediated toxicity that was generated by xanthine/xanthine oxidase was suppressed by HMC05. The knockdown of the NQO-1 gene abrogated the HMC05-mediated cytoprotection. Interestingly, pretreatment with a chemical inhibitor of geranylgeranyltransferase 1 or farnesyltransferase abolished the NQO-1 gene induction and cytoprotection by HMC05. The transfection of dominant negative RhoA or Ras suppressed HMC05-induced gene expression. Berberine and hesperidin, which are found in large quantities in HMC05, also induced NQO-1 gene expression. Taken together, this is the first study to demonstrate that HMC05 is efficacious in protection against oxidative stress through NQO-1 gene induction via the regulation of RhoA and/or Ras, and that berberine and hesperidin are major components of NQO-1 gene induction. This study provides mechanistic targets of HMC05 in reducing atherosclerotic lesions in atherosclerosis.

Key words HMC05; isoprenylation; smooth muscle cell; reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H):quinone oxidoreductase-1 (NQO-1)

HMC05 is a water extract of Banhabackchulchunmatang that has been used for headaches and hypertension in traditional herbal medicines.1) A pilot study on the reduction of blood pressure by HMC05 was performed in a randomized, single-blind cross over trial.2) Recently, HMC05 was demonstrated to lead to potent inhibition of atherosclerosis lesions in high-fat and high-cholesterol-fed apoE-knockout mice, which was partially attributed to anti-inflammatory effects.3) Neointimal formation was also reduced by HMC05 via the inhibition of PDGF-mediated cellular signaling in balloon-injured carotid arteries.3) Therefore, HMC05 is a candidate for anti-atherosclerosis agent that has also been used as an herbal medicine. HMC05 shows no toxicity in humans and is standardized based on berberine and hesperidin. Interestingly, the medial smooth muscle cell layer was preserved via the administration of HMC05 at both balloon injury sites and atherosclerotic lesions; this indicates that smooth muscle cells (SMC) may be a promising target of HMC05 for the prevention of cardiovascular diseases.

Reactive oxygen species (ROS) play a critical role in pathophysiology of vascular disease.4) ROS production in atherosclerotic lesions accelerates the migration of SMC from the media to the intima, which leads to the development of a fibrous thickened layer. SMC are therefore promising in targeting vascular redox signaling to prevent ROS. Reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H):quinone oxidoreductase-1 (NQO-1) (EC 1.6.99.2) is a homodimeric flavoprotein that catalyzes a two-electron reduction of electrophilic quinone substrates.5) Inducible NQO-1 expression plays a major role as a potential O$_2^·$ scavenger in SMC.6) Several phytochemicals inhibiting oxidative stress have a beneficial effect in atherosclerosis and restenosis via their direct antioxidant actions.7–9) Therefore, NQO-1 induction is likely to be of key importance in protecting against oxidative stress and suppressing atherosclerosis development.7)

Rho guanosine 5′-triphosphatases (GTPases) participate in cell adhesion, vesicle trafficking, cell cycle progression, gene expression and differentiation.10,11) Members of the Rho small GTPase and Rho kinase play a role in cardiovascular physiology.12,13) Geranylgeranylation, which is required for membrane targeting and the activity of the Rho protein, represses interleukin (IL)-1β-induced nitric oxide synthase 2 (NOS2) in SMC.14) Targeting geranylgeranyltransferase type I (GGTase-I) activating RhoA increased macrophage reverse cholesterol transport and reduced atherosclerosis development.15) Previously, we found that G3z mediated a positive signal for Nrf2 phosphorylation by tBH2 via Rho-protein kinase Cδ (PKCδ)-signaling, which led to target gene induction that included NQO-1.16)

Although HMC05 was proposed as a potential candidate for preventive cardiovascular diseases,1,3 the effects of HMC05 on the expression of antioxidant enzymes, scavenging of ROS in SMC and upstream signaling of the protective effect remain unclear. Therefore, in the present study, we determined whether NQO-1 gene induction is required for the antioxidant effect exerted by HMC05. Furthermore, we investigated the role of small GTPase in NQO-1 induction with an antioxidant effect of HMC05 in SMC.
MATERIALS AND METHODS

Reagents  Anti-NQO-1 antibody was obtained from Upstate (Scotland, U.K.). Berberine, hesperidin, xanthine, xanthine oxidase (XO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other reagents in the molecular studies were acquired from Sigma-Aldrich (St. Louis, MO, U.S.A.). All materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, CA, U.S.A.). NQO-1 small interfering RNA (siRNA) and non-targeting scrambled RNA (control siRNA) were obtained from Bioneer Co. (Daejoen, South Korea). Chemical inhibitors were purchased from Calbiochem (La Jolla, CA, U.S.A.).

HMC05 Preparation  HMC05 is mixture of herbal drug. The herbs had a moisture content of <10% by weight, and were dried. The composition of the mixture was as follows: Rhizoma of Pinellia ternate TÉN. ex BREITENB. (9 g), Rhizoma of Atractylodes japonica KOIZ. (12 g), Rhizoma of Gastrodia elata BLUME (6 g), Pericarpium of Citrus unshiu MARCOW (6 g), Poria cocos WOLF (9 g), Fructus of Crataegus pinnatifida BUNGÉ var. typica C.K. SCHNEIDER (9 g), Herba of Siegesbeckia pubescens (9 g). HMC05 extraction and quality control were carried out as described in the previous report.[5] The content of berberine and hesperidin in HMC05 was 1.93±0.01% and 1.02±0.11%, respectively.

Primary SMC Culture  All procedures were performed in accordance with the Korean Laws of Laboratory Animal Care (Health & Welfare Committee Registration No. 9025, revised 2010) and were based on the protocol approved by the Institutional Animal Care and Use Committee of Dongguk University (2011-03-004). Rat aortic SMC were isolated from 7-week-old male Sprague-Dawley rats (Orient Co., Seoul, Korea). The aortic strips were cut into small pieces and placed in six-well cell culture plates. These explants were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 50 units/mL penicillin and 50 µg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO2. The cells from passages 5 to 7 were used in the experiments. The purity of the SMC was assayed by α-smooth muscle actin staining and was determined to be >96% (Supplementary Fig. 1).
Reverse Transcription-Polymerase Chain Reaction Amplification (RT-PCR) Total RNA was isolated from cells with TRIzol (Invitrogen). RT-PCR was conducted as previously described. The specific primer set for NQO-1, glyceraldehyde-3-phosphate dehydrogenase and β-actin were designed to have a Tm of approximately 55°C and GC content of ca. 50%; BLAST searches were used to confirm the specificity of the selected nucleotide sequence. The primers used were as follows: NQO-1, 5′-ACC ACTCTACCTTTGCTC AA-3′ (forward) and 5′-CCT CTTTTT CCT ATC CTGGT-3′ (reverse); and β-actin, 5′-TCT ATG AGG GTT ACG CGC TT-3′ (forward) and 5′-TAA GTG CAC GCGA CCTTTCC-3′ (reverse). Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator. The densitometric intensities of each lane were integrated using Gelpro 3.1 software (Media Cybernetics Inc., Bethesda, MD, U.S.A.). The number of amplification cycles was empirically determined for each primer pair to identify the logarithmic phase which was confirmed by the real time PCR analyses.

Immunoblotting SDS-PAGE and immunoblot analyses were performed in lysates from cells according to previously published procedures. The resulting image was developed using the ECL® chemiluminescence detection kit (Amersham, U.K.). Equal loading of proteins was verified by β-actin immunoblotting. Changes in the protein levels were determined using a Gelpro analyzer (Media Cybernetics Inc., Bethesda, MD, U.S.A.).

Xanthine Oxidase Assay The SMC were cultured in 96-well plates and grown to 80–90% confluence. After serum starvation for 18 h, the cells were pretreated with HMC05 in the presence or absence of inhibitor for 1 h. The various concentrations of XO (10, 20, 30, 40 and 50 munit/mL) with 0.5 mM xanthine were added to each well at 1 h after HMC05 treatment, after which the cells were incubated for an additional 18 h. Next, MTT (5 mg/mL) was incubated for an additional 2 h. The cell culture medium was then aspirated and the cells were solubilized in 200 µL dimethyl sulfoxide. Finally, an absorbance of 550 nm was measured using an ELISA reader (Bio-Tek Instruments, Winooski, VT, U.S.A.).

Transfection of Plasmids and siRNA Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) was used to transfect the plasmids or siRNA into SMC, according to the manufacturer’s instructions. The SMC were transfected with empty vector (PCDNA) and dominant negative RhoA, RhoAN19 (DN-RhoA) or dominant negative Ras, RasN17 (DN-Ras) plasmid, and the inhibition of GTPase with exogenous plasmids was confirmed using immunoblotting and the activity kit. For the gene knockdown, the cells were transiently transfected with 10 nM of control or NQO-1 siRNA, and the suppression of gene expression was confirmed with the protein lysate samples.

Statistical Analysis One-way ANOVA procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman–Keuls test was used for comparisons of multiple group means. All statistical tests were two-sided.

RESULTS

Effects of HMC05 on the Expression of the NQO-1 Gene The inducible endogenous NQO-1 in the cardiovascular cells functions as a potential superoxide scavenger. Therefore, we investigated the effect of HMC05 on the expression of the NQO-1 gene in SMC (Fig. 1). In our study, there was no cytotoxicity with HMC05 at the dose we used (data not shown). The NQO-1 mRNA levels by HMC05 (0.01–1 mg/mL) at 6 h were significantly increased in a dose-dependent manner (1.3–2.8-fold) compared with the control (Fig. 1A, left). In the time course study, the NQO-1 mRNA was induced by HMC05 (1 mg/mL) with a maximal induction at 6 h (Fig. 1A, right). Next, to determine whether the changes in the levels of the NQO-1 mRNA by HMC05 were accompanied by changes in protein expression, the cells were treated with 0.01–1 mg/mL of HMC05 for the indicated times (6–24 h). The expression of the NQO-1 gene increased 2.5-fold from 0.01 mg/mL, initiated protein expression from 6 h and reached a 2.7-fold peak increase at 12 h (Fig. 1B). These results showed that HMC05 potently increased NQO-1 gene expression at an early time point with a saturated pattern at a dose of 0.1 mg/mL, which suggests that HMC05 may facilitate the scavenging of oxidative damage.

The Protective Effect of HMC05 on the Xanthine/XO-Induced Cytotoxicity and the Role of NQO-1 on Cytopro-
The gene induction of NQO-1 by HMC05 could protect the superoxide-mediated damage generated by xanthine/XO. To assess the cytoprotection of HMC05, cells were exposed to xanthine/XO pretreated with or without HMC05 for 1 h. We performed the MTT assays 18 h after the last treatment. As shown in Fig. 2A, the cells incubated with various concentrations of XO in the presence of 0.5 mM xanthine resulted in a significant decrease in cell viability. However, HMC05 potently suppressed xanthine/XO-induced cell death, indicating that HMC05 effectively scavenges the augmented superoxide radical, which then leads to cytotoxicity. Next, we performed NQO-1 gene knockdown with NQO-1-targeting siRNA to evaluate the role of NQO-1 in the protective effect of HMC05 against superoxide radical damage. The cell viability after treatment with xanthine and XO (30 m Unit/mL) showed 45% compared with the control level (Fig. 2B). NQO-1-specific siRNA transfected cells slightly enhanced the cell toxicity. HMC05 treatment resulted in 70% cell viability in xanthine/XO-mediated toxicity, whereas the treatment with HMC05 in the NQO-1-gene knockdown cells did not mitigate cell death; cell viability dropped to the same level as that in the presence of xanthine/XO. These results indicate that HMC05-mediated NQO-1 gene induction is critical for cytoprotection against oxidative stress.

The Reversal of the Protective Effect of HMC05 through the Inhibition of Protein Isoprenylation Multiple kinases including phosphatidylinositol 3-kinases (PI3K), p38 mitogen-activated protein (MAP) kinase (p38 MAPK), Jun N-terminal kinase (JNK), and RhoA have been shown to play a role in oxidative injury is regulated by protein isoprenylation. The Role of RhoA and Ras on the HMC05-Mediated NQO-1 Induction Because the inhibition of GGT1 and FTase suppresses the prenylation of small GTPases such as Rho and Ras protein, respectively, we compared the changes in NQO-1 gene expression after transfecting cells with PCDNA or DN-RhoA or DN-Ras prior to the addition of HMC05. As shown in Fig. 4, we found that the inhibition of RhoA or Ras significantly reduced NQO-1 gene expression in response to HMC05. These results demonstrate that the

Fig. 3. The Upstream Signaling Pathways Involved in HMC05-Mediated NQO-1 Induction

(A) SMC were pretreated with MAPK inhibitors (30 µM PD98059: PD; 10 µM SB203580: SB and 3 µM JNK inhibitor II: JNKI), PI3K inhibitors (10 µM LY294001: LY and 1 µM wortmannin: Wort) and isoprenylation inhibitors (1 µM FTI-277; FTI and 1 µM GGTI-2133: GGTI) for 1 h following treatment with 1 mg/mL HMC05 for 12 h. NQO-1 expression was analyzed in the lysates via immunoblotting. The band intensities of NQO-1 were quantified and normalized to those of β-actin. Each value represents the mean ± S.E. from at least three separate experiments (*p < 0.05, significantly different from control; **p < 0.01, significantly different from HMC05).

(B) SMC were treated with HMC05 (1 mg/mL) after exposure to GGTI (1 µM) or FTI (1 µM) for 1 h. Xanthine and XO (30 m Unit/mL) were added 1 h after HMC05 treatment. Then, the MTT assay was conducted 18 h after the final treatment as described in Materials and Methods. The results were confirmed in repeated experiments. Each value represents the mean ± S.E. (**p < 0.01, significantly different from control; ***p < 0.001, significantly different from HMC05).

Fig. 4. RhoA and Ras-Mediated NQO-1 Induction by HMC05

SMC were transiently transfected with empty vector (PCDNA), DN-RhoA or DN-Ras and subsequently treated with HMC05 or vehicle for 6 h. The expression levels of mRNAs after HMC05 treatment were assessed by RT-PCR analyses. The equal loading of RNA in each lane was determined based on β-actin levels. Representative data are shown, and the graph indicates the relative densitometric intensities obtained from multiple analyses. Each value represents the mean ± S.E. (*p < 0.05, significantly different from control; **p < 0.01, significantly different from HMC05).
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tion in a balloon injury model. 1,3) A protective effect on the
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was increased 3-fold at 80 µM by 2.5 and 4-fold compared with the
control (Fig. 5). NQO-1 mRNA after hesperidin treatment
expression at 10 and 80 µM. These results demonstrate that
berberine and hesperidin are major components of HMC05
for the NQO-1 induction and that the potency of berberine is
higher than hesperidin for the gene induction.

DISCUSSION
Atherosclerosis is characterized by a thickening of the
arterial system. The progression of the disease is generally
believed to have both oxidative and inflammatory components
that ultimately lead to plaque formation in the arteries. Spe-
cifically, pro-inflammatory cytokines and growth factors by
foam cells increase ROS in the lesion with local inflammation
and accelerate the migration of SMC from the media to the in-
tima.21) Intimal SMC assist in forming foam cells and synthet-
size extracellular matrix protein that leads to the development
of a fibrous cap.22) Previously, HMC05 potently suppressed
the atherosclerotic lesion area and macrophage accumulation in
ApoE (+/−) mice fed with high-fat and high-cholesterol
diets and resulted in significant reduction in neointimal forma-
tion in a balloon injury model.1,3) A protective effect on the
SMC layer in the media of the aortic ring was found in the
HMC05-treated mice, implying that the target site of HMC05
may be in the SMC. ROS in SMC is one of key contributors
to the pathogenesis of atherosclerosis. NQO-1 limits the radi-
cal formation of electrophilic compounds through one-electron
reduction, thus generating ROS.23) Therefore, the induction of
NQO-1 can be a therapeutic target against oxidative injury-
mediated atherosclerosis development.19) We are the first to
demonstrate the up-regulation of the NQO-1 gene which was
selected in a DNA microarray analysis of HMC05 (data not shown). We found that the protection against xanthine/XO-
induced oxidative stress is attributed to HMC05-mediated
NQO-1 induction. HMC05 also significantly inhibited H2O2-
induced cell death (Supplementary Fig. 2). Other gene re-
 sponses to ROS stimuli were also increased by HMC05 (sub-
mitted). Detoxifying enzyme expressions including NQO-1
are known to be mediated through the antioxidant response
 element (ARE) in gene promoters; this was elucidated using
Nrf2 knockout mice.16) HMC05 also increased nuclear Nrf2
accumulation (data not shown). The HMC05-induced NQO-1
gene expression that blocks ROS production in SMC may be
in part attributed to the inhibition of the atherosclerotic lesion
area and the reduction of neointima formation.

Several studies reported that multiple signaling pathways
including the MAPK cascade, PI3K and PKC are involved in
the regulation of Nrf2/ARE-dependent gene expression
including NQO-1.16,24) The activation of p38 MAPK has been
demonstrated to negatively regulate ARE-activity.25) Interest-
ingly, we observed that the induction of NQO-1 expression by
HMC05 was mediated by isoprenylation pathways without the
signals known to be upstream of NQO-1. Isoprenylations
have key roles in the membrane attachment and protein functional-
ity of the small GTPases.10,26) Conversely, inhibition of Rho
pathway has been believed to be a predominant mechanism
contributing to the beneficial effect on the cardiovascular
system, since statins inhibit the synthesis of farnesyl pyro-
phosphate and geranylgeranyl pyrophosphate, isoprenoids of
mevalonate via inhibition of 3-hydroxy-3-methylglutaryl co-
enzyme A reductase.27) However, statins rather induced GTP
loading of RhoA which imply that RhoA may be a potential
target for anti-atherosclerosis activity.28) In addition, modest
constitutively active RhoA seems to be beneficial to protect
against injuries (e.g. ischemia/reperfusion).29) We previously
reported that RhoA downstream of Got13 is important for
ARE-mediated detoxification enzyme induction.30) In the
present study, we found for the first time that the pathways
of GGtase 1 and Ftase are involved in the HMC05-induced
NQO-1 in SMC. Both RhoA and Ras, as effector molecules of
geranylgeranylation and farnesylation, respectively, appear to
play critical roles in NQO-1 induction by HMC05. Berberine
and hesperidin-mediated NQO-1 induction were also mediated
by small GTPase pathway (data not shown). A previous study
provided evidence that HMC05 inhibited NaF-induced GTP
RhoA activation in a muscle strip, which was not likely to
occur via PKC signaling, which is a major pathway for smooth
muscle function; this implies that other cells (not SMC) may
be responsible for that effect.30) Further studies on the up-
stream signaling of the RhoA by HMC05 are needed.

HMC05, an herbal mixture of Banhbachkachulchummatang, was
standardized on the basis of berberine and hesperidin at
1.93% and 1.02%, respectively. We found that both berberine
and hesperidin induced NQO-1 gene expression. It is likely
that berberine and hesperidin as major components of HMC05
cause NQO-1 gene induction with different efficacies. We
found the additive effect on the NQO-1 induction upon to
the low concentration of berberine+hesperidin, which imply

![Fig. 5. NQO-1 Gene Expression after Berberine or Hesperidin Treatment](Image)

The cells were pretreated with berberine or hesperidin at the indicated doses for 6 h. The NQO-1 mRNA levels in the SMC were determined via RT-PCR analyses. The levels of NQO-1 were normalized to β-actin. The relative changes in the NQO-1 were assessed from multiple analyses by scanning densitometry. Each value represents the mean±S.E. (n=3) as the fold change relative to control (*p<0.05, **p<0.01, significantly different from control).
that these components have the same action on the NQO-1 induction (Supplementary Fig. 3). Berberine has been reported to inhibit SMC proliferation and migration, which improve neointima formation after balloon injury.\(^3,32\) Berberine in high concentration showed cytotoxicity, whereas HMC05 shows good tolerability without adverse events in humans.\(^3,33\) The transient gastrointestinal adverse effect of berberine was reported.\(^33\) However, these side effects including diarrhea and constipation by berberine were disappeared and well tolerated at the dosage of 0.3 g three times a day which is lower than the content of berberine in 500 mg HMC05. HMC05 represents a promising new anti-atherosclerosis drug.

The present study is the first to demonstrate that HMC05 induces NQO-1 gene expression, which is conjunction with cytoprotection against ROS in SMC. This study also demonstrates that HMO05-mediated NQO-1 gene induction is attributed to small GTPase RhoA and Ras. Based on these results, the small GTPases in the SMC intimal layer becomes a new therapeutic target. HMC05 should be considered to be a potential anti-atherosclerotic agent.

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