

BRAP Activates Inflammatory Cascades and Increases the Risk for Carotid Atherosclerosis

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The *BRCA-1 associated protein* gene (*BRAP*) was recently identified as a susceptibility gene for myocardial infarction (MI). In the present study we aimed to decipher the association between the *BRAP* polymorphism and carotid atherosclerosis and the mechanism underlying its proatherogenic effect. A total of 1749 stroke/MI-free volunteers received carotid ultrasonic examinations for the measurement of intima-medial thickness (IMT) and plaque. The promoter polymorphism rs11066001 was selected because it affects the transcription of *BRAP*. We found that the GG genotype was associated with a 1.58-fold increased risk for having at least one plaque compared to carrying the A allele ($P = 0.021$). When subjects were divided by the cutoff value of IMT above the mean plus 1 standard deviation, there was an overrepresentation of the GG genotype in the subjects with thicker IMT ($P = 0.004$). The expression of *BRAP* increased significantly when human aortic smooth muscle cells (HASMCs) were treated with lipopolysaccharide (LPS). HASMCs were transfected with small interfering RNA against *BRAP* or scrambled sequences before treatment with LPS. Knockdown of *BRAP* led to attenuated HASMC proliferation and reduced secretion of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) in response to LPS. Downregulation of *BRAP* did not affect the protein levels of nuclear factor- κ B (NF- κ B), but prohibited its nuclear translocation. Coimmunoprecipitation experiments confirmed an interaction between *BRAP* and the two major components of the IKK signalosome, I κ B β and IKK β . Collectively, *BRAP* conferred a risk for carotid plaque and IMT. Inflammatory stimuli upregulated *BRAP* expression, and *BRAP* activated inflammatory cascades by regulating NF- κ B nuclear translocation.

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INTRODUCTION

Ultrasound measurements of intima-media thickness (IMT) and plaque at the carotid arteries are predictors of future cardiovascular risk (1,2). Each 1-standard deviation (SD) increment in IMT leads to a 1.26-fold increased risk for myocardial infarction (MI) (3), and the presence of plaque is associated with a 4.1-fold risk (4). Although both

phenotypes correlate well with pathologically and clinically defined atherosclerosis, they represent distinct traits with unique relationships to atherosclerosis (5). IMT is akin to a physical effect of adapting to aging and hypertensive stress, whereas plaque corresponds to a more pathogenic alteration in the vessel walls (6). These two phenotypes are used as independent surrogate markers

in genetic studies of cardiovascular diseases.

The *BRCA-1 associated protein* (*BRAP*) gene was recently found to be a susceptibility gene for MI (7), in which the polymorphism rs11066001 in this gene was significantly associated with MI risk in Japanese and Taiwanese populations (7). This finding was further replicated by an independent study in Japanese and Korean populations (8). In addition, rs11066001 was found to confer a risk for lower ankle-brachial index in the Taiwanese population (9). Because atherosclerosis is the common pathogenesis shared by MI and peripheral artery disease, these association studies high-

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lighted the effect of *BRAP* on the atherogenic process. *BRAP* was originally identified as a cytoplasmic protein that inhibited the nuclear translocation of the breast cancer suppressor protein BRCA-1 (10). It modulated the differentiation of monocytes through binding to the nuclear localization signal (NLS) of the cell-cycle inhibitor p21 (11). *BRAP* is also known to impede mitogenic signal propagation (IMP), the threshold modulator of the Raf–mitogen-activated protein kinase–extracellular signal regulated kinase (Raf-MEK-ERK) signal pathway (12). By repressing the Raf-MEK-ERK signal cascades, *BRAP* influences the secretion of interferon- γ in CD4 T cells (13). However, its role in the cardiovascular system remains to be explored.

Lines of evidence support the fact that atherosclerosis is a chronic inflammatory disease (14). Atherogenesis is initiated by accumulation of lipid components in the vessel walls. Recruitment of leukocytes and secretion of inflammatory cytokines subsequently open a vicious cycle of inflammatory response, leading to plaque formation and further thrombotic events (14). Many genes involved in the different stages of atherosclerosis are regulated by the transcription factor nuclear factor- κ B (NF- κ B) (15). For example, monocyte chemoattractant protein-1 (MCP-1), the crucial chemokine for monocyte recruitment, is activated by NF- κ B (16). Genes encoding vascular adhesion molecules, matrix metalloproteinases (MMPs), and interleukins are all downstream targets of NF- κ B. Genetic polymorphisms at the *MCP-1* (17), *MMP-3* (18,19), *MMP-9* (20) and *interleukin-6* (*IL-6*) (21) genes were found to be associated with carotid atherosclerosis. In our previous study (7), *BRAP* was found to be involved in the NF- κ B–dependent inflammatory pathway. Therefore, genetic variants in *BRAP* may also contribute to the disease susceptibility of carotid atherosclerosis.

The aims of the present study were to test for the influence of *BRAP* polymorphism on carotid atherosclerosis and to elucidate the mechanism underlying the

BRAP proatherogenic effect. We first tested the association between *BRAP* polymorphism and carotid atherosclerosis in human subjects. The single-nucleotide polymorphism (SNP) rs11066001 was selected because it affects the transcription of *BRAP* and has shown the strongest association with MI risks (7). In the second part of this study, we conducted *in vitro* experiments to clarify how *BRAP* influenced the activation of NF- κ B and promoted the atherosclerotic process.

MATERIALS AND METHODS

Study Subjects

Stroke- and MI-free volunteers were enrolled from Kaohsiung Medical University Hospital (KMUH) from 2006 to 2009. The investigation conformed to the principles outlined in the Declaration of Helsinki. All study protocols and methods were approved by the local institutional review board of KMUH. Demographic data and histories of hypertension, diabetes mellitus, hypercholesterolemia and cigarette smoking were obtained from each subject. Body height and weight were measured for the calculation of body mass index (BMI). All subjects received carotid ultrasonic examinations for the measurement of carotid plaque and IMT. Among the 1749 subjects, 316 had only plaque data and 1433 had data on both phenotypes. Venous blood was collected for biochemical analyses and genomic DNA extraction. The SNP rs11066001 (270A>G at intron 3) was genotyped by using the TaqMan genotyping assay (Applied Biosystems, Foster City, CA, USA). Briefly, polymerase chain reaction (PCR) primers and two allele-specific probes were designed to detect the specific SNP target. The PCR reactions were performed in 96-well microplates with an ABI 7500 real-time PCR machine (Applied Biosystems). Allele discrimination was achieved by detection of fluorescence by use of ABI 7500 System SDS software version 1.2.3 (Applied Biosystems). The genotype calling rate was 96.1%.

IMT and Plaque Index

Ultrasonic examinations were assessed by using a Philips HD 11 ultrasonography system equipped with a 7.5–10-Hz transducer (Philips Medical Systems, Bothell, Washington, USA). An experienced technician who was blinded to the patients' clinical data performed all the ultrasonic measurements. Carotid plaque was defined as an area of focal protrusion into the lumen that was at least 50% greater than the surrounding wall thickness. The far walls of the carotid IMT were visualized bilaterally, and the IMT values were measured separately at the plaque-free area of the common carotid artery (CCA) 10–20 mm proximal to the tip of the flow divider, the carotid bifurcation (Bif) at the tip of the flow divider and extending 10 mm proximally, and the internal carotid artery (ICA) proximally 10 mm above the bulb. A computerized analyzing system (Philips Qlab quantification software) (22) automatically detected the echo interfaces. Manual corrections were performed when there was no automatic outlining of the lumen-intima or the media-adventia interfaces. All the ultrasonic data were interpreted by a single neurologist (HF Lin). The mean absolute difference and SD between two repeated measurements was 0.05 ± 0.04 mm.

Cell Culture and Small Interfering RNA Transfection

Primary HASMCs (Cascade Biologics, Portland, OR, USA) were grown in culture medium containing medium 231, smooth muscle cell growth supplement, fetal bovine serum (10%), amphotericin B (50 ng/mL) and gentamycin (50 μ g/mL) (all purchased from Cascade Biologics). The cells were incubated at 37°C in 95% air/5% CO₂ atmosphere. Passages 4 to 9 were used for experiments. After adherence, cells were starved in serum-free media for 24 h to induce quiescence. Cells were then incubated with lipopoly saccharide (LPS) (*Escherichia coli* O111:B4 1 μ g/mL; Sigma-Aldrich, St. Louis, MO, USA), oxidized LDL (ox-LDL 40 μ g/mL; Biomedical Technologies, Stoughton, MA, USA) or simvastatin (10 μ mol/L;

Sigma-Aldrich) for different periods of time as indicated.

Gene silencing was performed 24 h prior to the addition of LPS. Control small interfering RNA (siRNA) targeting scrambled sequences (12935-200), siRNA against *BRAP* (BRAP-HSS112138) and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Cells were transfected with 50 pmol control siRNA or siRNA against *BRAP* per 10^6 cells by using Lipofectamine 2000.

Measurements of HASMC Proliferation and Migration, and MCP-1/Interleukin-8 Concentration

To measure HASMC proliferation, microplates were incubated at 37°C for 24 h. After that, 0.5 mg/mL of dimethyl-thiazol-diphenyltetrazoliumbromide (Sigma-Aldrich) was added into each well and the cells were incubated for 2.5 h at 37°C. Spectrophotometric readings were done by use of an X340 spectrophotometer at 595 nm (BioTek Instruments, Winooski, VT, USA). Cell migration ability was evaluated by using a transwell assay (Millipore, Billerica, MA, USA). HASMCs were layered on the upper (inner) chamber of the 8- μ m-pore transwell filters, and culture medium with LPS 1 μ g/mL was added to the lower chamber. The filters were then stained with Crystal Blue, and the cells in the lower chamber were counted microscopically in a high-power field. The concentration of MCP-1 and interleukin-8 (IL-8) were measured by enzyme-linked immunosorbent assay (ELISA) (BD Biosciences Pharmingen, Franklin Lakes, NJ, USA). After transfection with control siRNA or siRNA against *BRAP*, HASMCs were treated with LPS 1 μ g/mL for different periods of time as indicated. The supernatants of conditioned medium were collected, and the concentrations of MCP-1 and IL-8 were quantified by ELISA kits according to the manufacturer's instructions.

Determination of *BRAP* and *NFKB1* Expression

Expression levels of *BRAP* and nuclear factor of *kappa* light polypeptide gene en-

hancer in B-cells 1 (NFKB1) in response to LPS stimulation were measured by quantitative reverse-transcription PCR (RT-PCR) and Western blot. Total RNA was extracted by using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The concentration and integrity of total RNA were determined by using a NanoPhotometerTM spectrophotometer (Implen GmbH, Munich, Germany). Reverse transcription was performed by using random hexamer primers and a Superscript II reverse transcriptase kit (Applied Biosystems). Quantitative RT-PCR was performed by using an ABI 7500 sequence detector with TaqMan gene expression assays (Applied Biosystems). Expression levels of the target genes were calculated by using the difference in the threshold cycle method with normalization to the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*.

The cytosolic and nuclear fractions of HASMC protein were extracted separately by NE-PERTM nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA). Protein samples were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were incubated with autoantibodies against BRAP (1:1000; Abcam, Cambridge, UK), NF- κ B p65 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF- κ B p50 (1:1000; Santa Cruz Biotechnology), I κ B α (1:1000; Santa Cruz Biotechnology), phosphorylated I κ B α (1:1000; Invitrogen), I κ B β (1:1000; Abcam), phosphorylated I κ B β (1:1000; Cell Signaling Technology, Danvers, MA, USA) and β -actin (1:10000; Santa Cruz Biotechnology). The membrane was subsequently treated with goat anti-rabbit or goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen). The blots were visualized by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology) with an LAS-3000 imaging system (Fujifilm, Tokyo, Japan), and blot intensity was quantitatively measured by Gel-Pro analyzer 3.1 software.

Immunofluorescence Stain for NF- κ B Localization

After HASMCs were treated either with or without LPS 1 μ g/mL for 60 min, the cells were fixed with 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100 (GIBCO-BRL, Grand Island, NY, USA). Slides were washed with phosphate buffered saline (PBS) and incubated in blocking buffer (1% bovine serum albumin in PBS; GIBCO-BRL). The cells were then incubated with anti-NF- κ B p65 autoantibody (1:100; Santa Cruz Biotechnology) overnight at 4°C. Slides were later incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit autoantibody (1:1000; Invitrogen), and the nuclei were counterstained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI 1:100; Invitrogen). Fluorescence was detected with a Leica DMRE confocal microscope equipped with an argon laser source (Leica, Mannheim, Germany).

S-Tag Pull-Down Assay

An S-tagged BRAP expression plasmid was constructed by using a pTriEx-4 vector (Novagen, Darmstadt, Germany). Human embryonic kidney 293 (HEK293) cells (obtained from the Health Science Research Resources Bank, Osaka, Japan; JCRB9068) in 150-mm dishes were transiently transfected with BRAP-pTriEx-4 or pTriEx-4 vector. The cells were lysed and diluted 10-fold by using S-protein bind/wash buffer. The extracts were incubated with an S-protein agarose for 12–18 h at 4°C. The agarose was washed 3 \times in S-protein bind/wash buffer and one time in Tris/HCl buffer (10 mmol/L Tris, pH 8.0, with 150 mmol/L NaCl). Bound S-tagged proteins were eluted by glycine/HCl buffer (100 mmol/L glycine, 0.5 mol/L NaCl, pH 2.7) and neutralized by 1 mol/L Tris/HCl (pH 9.0). The protein complexes were concentrated and analyzed by SDS-PAGE and stained by using a silver stain MS kit (Wako Chemicals, Mountain View, CA, USA). The protein bands were analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI/TOF) mass spectrometry.

Table 1. Demographic data of the study participants (N = 1749).

Age, mean \pm SD, y	55.2 \pm 10.6 (19-87)
Sex, M	41.1%
Hypertension	34.4%
Diabetes	12.1%
Hypercholesterolemia	33.8%
Past and current smoker	18.6%
Total cholesterol, mean \pm SD, mg/dL	200.9 \pm 38.1
Triglyceride, mean \pm SD, mg/dL	122.3 \pm 81.4
HDL-cholesterol, mean \pm SD, mg/dL	56.5 \pm 15.2
Body mass index, mean \pm SD, Kg/m ²	24.5 \pm 3.5
CCA IMT value, mean \pm SD, mm	0.63 \pm 0.14
Bif IMT value, mean \pm SD, mm	0.66 \pm 0.13
ICA IMT value, mean \pm SD, mm	0.51 \pm 0.09
Presence/absence of plaque, n (%)	583 (33.3%)/1166 (66.7%)
<i>BRAP</i> SNP rs11066001	
AA	841 (48.1%)
AG	707 (40.4%)
GG	133 (7.6%)
Failed genotyping	68 (3.9%)

try at Shimadzu TechnoResearch, Kyoto, Japan.

Coimmunoprecipitation Experiments

We conducted coimmunoprecipitation (Co-IP) experiments to test the interactions between BRAP and three compo-

nents of the IKK-signalosome, which were (a) the inhibitor of kappa light polypeptide gene enhancer in B-cell, kinase beta (IKK β), (b) the nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha (NFKB1A, aka I κ B α), and (c) the nuclear factor of kappa

light polypeptide gene enhancer in B-cell inhibitor, beta (NFKB1B, aka I κ B β). Expression plasmids of Myc- or S-tagged BRAP or IKK β were transfected into COS7 cells (HSRRB; JCRB9127) by using Fugene6 (Roche, Mannheim, Germany). Immunoprecipitation was performed in lysis buffer, which contained 20 mmol/L Tris, pH 7.5, with 150 mmol/L NaCl, 0.4% Nonidet P-40, 5 μ g/mL of proteasome inhibitor MG-132 and a protease inhibitor tablet without ethylenediaminetetraacetic acid (Roche). Twenty-four h after transfection, cells were lysed and immunoprecipitation was done by using an anti-Myc tag (Santa Cruz Biotechnology) on S-protein agarose (Novagen). We visualized the immune complex by using HRP-conjugated S protein (Novagen) or anti-Myc antibody peroxidase conjugates (Santa Cruz Biotechnology).

COS7 cells were again transfected with expression plasmids of FLAG-tagged or S-tagged BRAP, NFKB1A or NFKB1B. Cells were lysed and immunoprecipitation was done using an anti-FLAG tag (Santa Cruz Biotechnology) on S-protein agarose. The immune complex was visu-

Table 2. The association between SNP rs11066001 in *BRAP* and carotid atherosclerosis.

Phenotype	Genotype			Statistics (recessive model)
	AA	AG	GG	
Carotid plaque				Logistic regression ^a
Absence	563 (50.6%)	472 (42.4%)	77 (6.9%)	Reference
Presence	278 (48.9%)	235 (41.3%)	56 (9.8%)	OR = 1.58 (1.08–2.34); P = 0.021
Carotid IMT				Multivariate regression ^a
Continuous IMT data	AA (n = 700)	AG (n = 571)	GG (n = 100)	
CCA, mean \pm SD, mm	0.62 \pm 0.14	0.63 \pm 0.14	0.65 \pm 0.16	P = 0.089
Bif, mean \pm SD, mm	0.65 \pm 0.13	0.66 \pm 0.13	0.66 \pm 0.13	P = 0.732
ICA, mean \pm SD, mm	0.51 \pm 0.10	0.51 \pm 0.09	0.51 \pm 0.10	P = 0.834
Dichotomized IMT, \geq mean + 1 SD versus < mean + 1 SD				Logistic regression ^a
CCA	46.0% versus 52.0%	43.1% versus 41.4%	10.9% versus 6.6%	OR = 2.25 (1.30–3.89), P = 0.004
Bif	49.0% versus 51.5%	43.7% versus 41.2%	7.3% versus 7.3%	OR = 1.12 (0.61–2.03), P = 0.720
ICA	54.5% versus 50.5%	39.6% versus 42.0%	5.9% versus 7.5%	OR = 0.77 (0.40–1.50); P = 0.445

^aP value obtained from multivariate regression analysis with adjustment for age, sex, hypertension, diabetes, hyperlipidemia, BMI and smoking. Only covariates with P < 0.05 were kept in the regression models.

alized by using HRP-conjugated S protein or anti-FLAG antibody peroxide conjugates (Santa Cruz Biotechnology).

Statistical Analysis

Genotype distributions were tested for Hardy-Weinberg equilibrium (HWE) by using the goodness-of-fit test. Logistic regression and χ^2 squared tests were used to compare the genotype distributions between subjects with at least one plaque and those with no plaques. We analyzed both the continuous IMT data as well as the dichotomized IMT data. For the continuous IMT data, ANOVA and Student *t* test were used to compare the mean IMT values across different genotypes. For the dichotomized IMT data, subjects with IMT values above the mean plus 1 SD were defined as high-risk individuals, and the rest of study subjects were defined as reference individuals. Logistic regression with adjustment for other cardiovascular risk factors (diabetes, hypertension, hypercholesterolemia, BMI and smoking) were used to estimate the odds ratio (OR) and 95% confidence interval (CI) for the risk genotype. Only covariates with a *P* value <0.05 were kept in the regression model. Because we tested the *BRAP* effect on two phenotypes (that is, IMT and plaque), the Bonferroni-corrected *P* value was adopted for multiple testing correction. All statistical analyses were performed with SPSS statistical software (version 13.0).

For the cellular experiments, variables were presented as mean \pm SD. Student *t* test was used to compare the variables between the treatment and control groups. All experiments were performed at least 3 \times with technical duplicates in each sample.

All supplementary materials are available online at www.molmed.org

RESULTS

BRAP Genetic Polymorphism and Carotid Atherosclerosis

The demographic features of the study participants are shown in Table 1. The

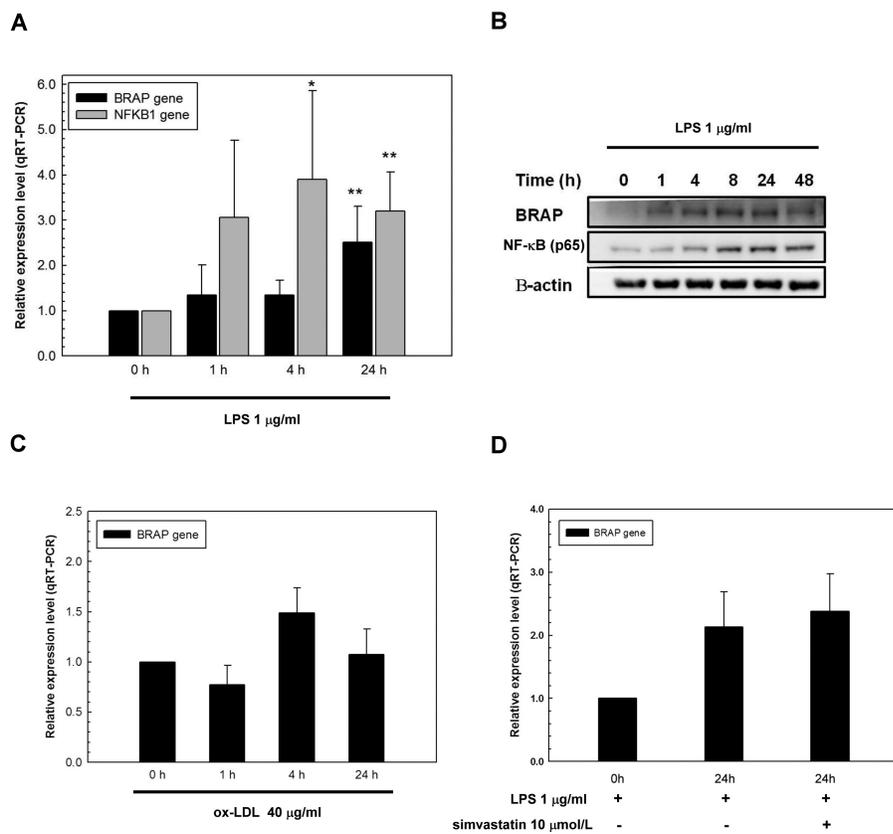


Figure 1. LPS upregulated the expression of *BRAP* and *NFKB1*. HASMCs treated with 1 μ g/mL LPS led to a significant increase of *BRAP* and *NFKB1* expression detected by (A) qRT-PCR and (B) Western blot. (C) There was no change in *BRAP* mRNA levels in cells treated with 40 μ g/mL ox-LDL. (D) Pretreatment of the cells with 10 μ mol/L simvastatin did not reverse the LPS-induced *BRAP* upregulation in HASMCs. Each experiment was repeated three times and each sample was studied in duplicate. Data were analyzed by pairwise comparison between the cells treated with LPS for different periods of time and the cells at time zero (Student *t* test, **P* < 0.05, ***P* < 0.01). Error bars: SD.

genotype distribution of rs11066001 was in HWE. We used two intermediate phenotypes (that is, carotid IMT and plaque) to assess the *BRAP* effect on atherosclerosis. When subjects with at least one plaque were compared with those without any plaque, the frequency of minor homozygote GG was overrepresented in the former (9.8% versus 6.9%, Table 2). Compared with the subjects with the AG or AA genotype, those carrying the GG genotype had a 1.58-fold greater risk for having at least one plaque (nominal *P* = 0.021; Bonferroni-corrected *P* = 0.042).

Continuous IMT data indicated that subjects carrying the GG genotype had a thicker IMT at CCA than those with the

AA or AG genotype (0.65 ± 0.16 mm versus 0.63 ± 0.14 mm respectively, Table 2). However, the association was not statistically significant (nominal *P* = 0.089). For the dichotomized IMT data, subjects with the GG genotype had an OR of 2.25 (nominal *P* value = 0.004, Bonferroni-corrected *P* value = 0.008) for a thicker CCA IMT in comparison to the A allele carriers. The average IMT values at Bif or ICA were not significantly different among individuals with AA, AG or GG genotype.

We further tested the associations between rs11066001 and other cardiovascular risk factors (that is, sex, hypertension, diabetes, hypercholesterolemia, smoking

status and BMI). The genotype distribution did not show any significant difference between subjects with or without any of the risk factors (nominal $P = 0.21$ – 0.86 , Supplementary Table 1).

LPS Induced BRAP Upregulation and HASMC Proatherogenic Changes

We conducted a series of *in vitro* experiments to elucidate the BRAP mechanism underlying atherosclerosis. LPS stimulation significantly increased the mRNA levels and protein amount of BRAP in HASMCs (Figures 1A, B), whereas the ox-LDL treatment did not cause any BRAP expression changes (Figure 1C). Augmented mRNA and protein levels of *NFKB1* were observed along with the increase of BRAP expression (Figures 1A, B). Treating HASMCs with 10 $\mu\text{mol/L}$ simvastatin prior to the LPS stimulation did not reverse the upregulation of BRAP expression (Figure 1D).

LPS enhanced the proliferation and migration of HASMCs (Supplementary Figures 1A, B). After the cells were treated with LPS, there was a time-dependent increment in cell proliferation and migration. The concentrations of MCP-1 and IL-8 in the culture medium also increased gradually after HASMCs were exposed to LPS (Supplementary Figures 1C, D).

Knockdown of BRAP Attenuated the Proatherogenic Effect

We then knocked down BRAP by siRNA to clarify the relationship between BRAP expression and the proatherogenic phenotypes observed in HASMCs. BRAP siRNA significantly suppressed BRAP mRNA levels and protein amounts (Figures 2A, B). When BRAP was downregulated, the secretion of inflammatory cytokines (MCP-1 and IL-8) was reduced in response to LPS stimulation (Figures 2C, D). In addition, LPS-induced HASMC proliferation was decreased in the presence of BRAP siRNA compared with transfection with control siRNA (Supplementary Figure 2A). The migration ability of cells transfected with BRAP siRNA

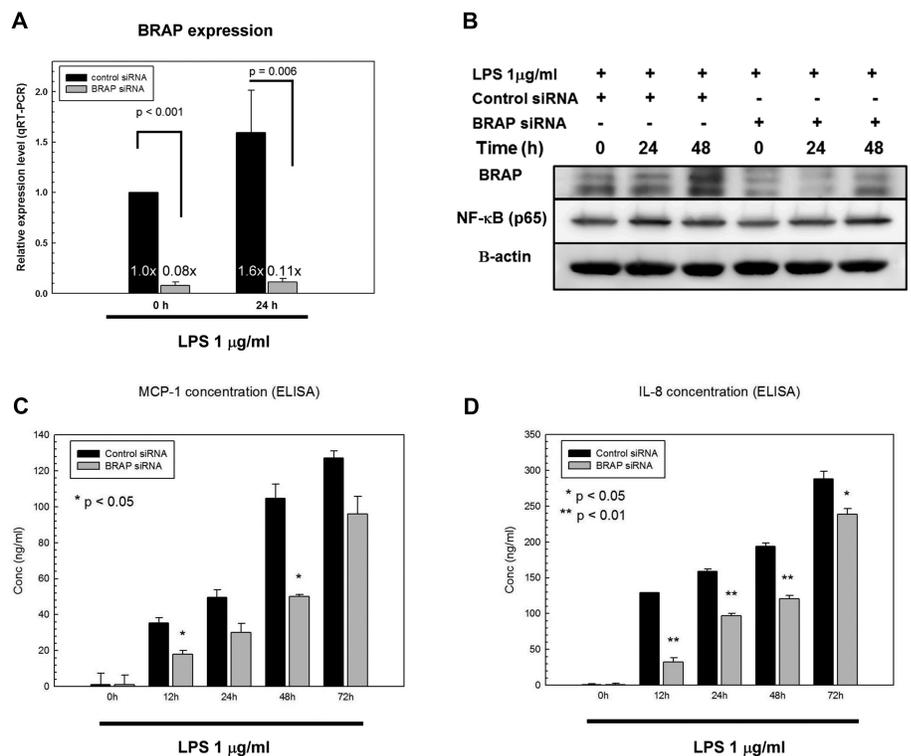


Figure 2. Knockdown of BRAP attenuated the LPS atherogenic effect. HASMCs were transfected with either control siRNA or BRAP siRNA 24 h prior to LPS stimulation. (A) The mRNA levels and (B) protein amounts of BRAP were reduced in cells with BRAP knockdown. The secretion of (C) MCP-1 and (D) IL-8 of HASMCs significantly declined in cells with BRAP knockdown. Each experiment was repeated three times and each sample was studied in duplicate. Data were analyzed by pairwise comparison between cells treated with LPS for different periods of time and cells at time zero (Student *t* test, * $P < 0.05$, ** $P < 0.01$). Error bars: SD.

was inferior to that of the control groups, but there was no significant difference (Supplementary Figure 2B).

BRAP Influenced the Nuclear Translocation of NF- κ B

We hypothesized that the proatherogenic effect of BRAP might be attributable to its influence on NF- κ B. Knocking down BRAP did not influence *NFKB1* mRNA levels (Figure 3A). However, the nuclear translocation of NF- κ B p65 and p50 decreased markedly in cells transfected with BRAP siRNA (Figure 3B). When the HASMCs were treated with LPS, the nuclear/cytoplasm ratio of NF- κ B protein increased gradually in a time-dependent manner (Figure 3C, Supplementary Table 2). On the contrary, there was no change in the nu-

clear/cytoplasm ratio of NF- κ B protein in HASMCs with BRAP knockdown prior to LPS stimulation. Similar findings were also demonstrated by immunofluorescence stains (Supplementary Figure 3).

BRAP Interacted with IKBKB and NFKBIB, but Not NFKBIA

During identification of proteins that interact with BRAP by using an S-tag pull-down assay and MALDI/TOF mass analyses, we found IKBKB (aka IKK β) as a possible binding partner of BRAP protein. To examine the interaction between BRAP protein and IKBKB, we constructed plasmids expressing Myc-tagged or S-tagged BRAP or IKBKB in COS7 cells. The Co-IP experiments confirmed their interaction in protein blot

analysis (Figure 4A). We further transfected COS7 cells with plasmids containing FLAG-tagged or S-tagged BRAP, NFKBIA (aka I κ B α) or NFKBIB (aka I κ B β). The protein blot analysis showed that BRAP interacted with NFKBIB, but not with NFKBIA (Figures 4B, C).

BRAP Affected NFKBIB Degradation, but Not NFKBIA

We then used Western blot to evaluate the influence of *BRAP* silencing on I κ B degradation. For HASMCs transfected with control siRNA, the protein amount of I κ B α and I κ B β decreased gradually after the cells were treated with LPS (Figure 3D, Supplementary Table 3). The LPS stimulation increased the phosphorylation of I κ B α and I κ B β in a time-dependent manner, which was parallel to the degradation of I κ B α and I κ B β (Figure 3D). When the HASMCs were transfected with *BRAP* siRNA, there was no significant change in the total amount of I κ B β or in the concentrations of phosphorylated I κ B β . This result suggested that *BRAP* silencing would attenuate the phosphorylation and degradation of I κ B β in response to the LPS stimulation. On the other hand, *BRAP* silencing did not affect the LPS-induced degradation of I κ B α (Figure 3D, Supplementary Table 3). There was a time-dependent decrement in the total I κ B α amount in response to the LPS treatment if the HASMCs were transfected with *BRAP* siRNA or with control siRNA. These findings were coherent with the results from Co-IP experiments, in which BRAP protein interacted with I κ B β , but not with I κ B α .

DISCUSSION

Carotid plaque and IMT are surrogate markers of cardiovascular diseases. The major pathways that are implicated in atherosclerosis include abnormal lipid metabolism, endothelial dysfunction, thrombosis/platelet aggregation and arterial inflammation (23). The results of the present study demonstrated that *BRAP*'s effect on cardiovascular diseases is primarily mediated by its proinflam-

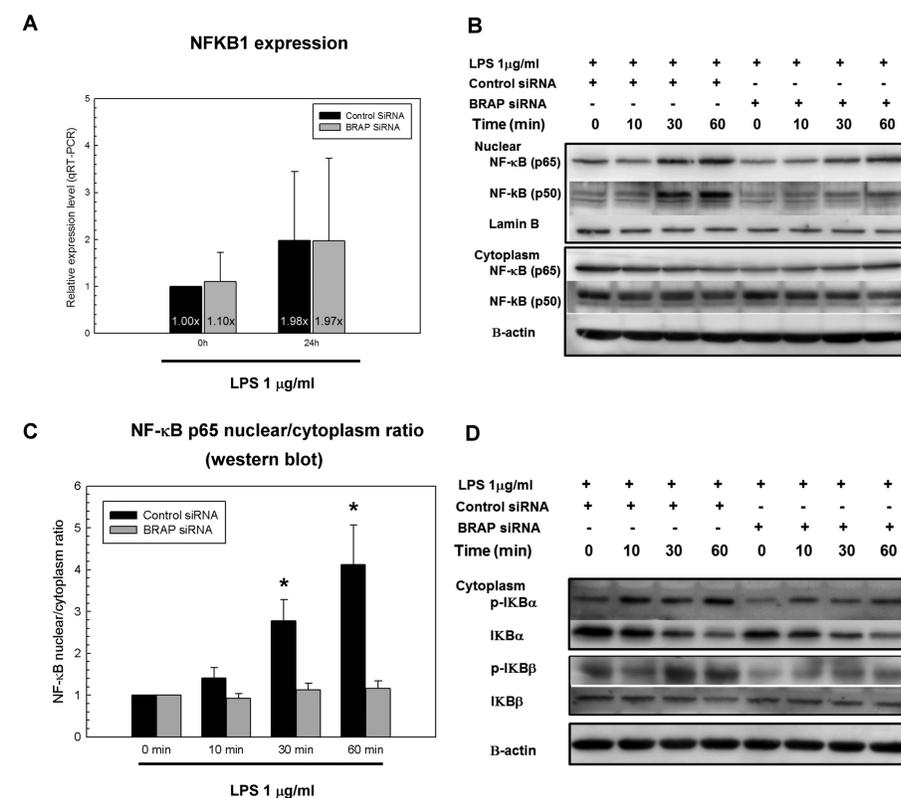


Figure 3. Knockdown of *BRAP* prohibited NF- κ B nuclear translocation and attenuated I κ B β degradation. (A) Knockdown of *BRAP* did not change the mRNA levels of *NFKB1* ($n = 3$). (B) Western blot showed that NF- κ B p50 and p65 increased gradually in the nucleus when the HASMCs were treated with LPS. Cells with *BRAP* silencing prohibited the nuclear translocation of NF- κ B p50 and p65. (C) There was a significant increment in the nuclear/cytoplasm ratio of NF- κ B p65 in the wide-type cells, but no change in the cells with *BRAP* knockdown. Data were analyzed by pairwise comparison between cells treated with LPS for different periods of time and cells at time zero (Student t test, $*P < 0.05$). (D) For the cells transfected with control siRNA, LPS induced the degradation and phosphorylation of I κ B α and I κ B β . *BRAP* silencing prohibited the degradation of I κ B β but did not influence the degradation of I κ B α . Each experiment was repeated at least three times. Error bars: SD.

matory activity. First, *BRAP* expression was induced by LPS but not ox-LDL. Second, knockdown of *BRAP* hampered LPS-induced cell proliferation and MCP-1/IL-8 secretion. In addition, reduced expression levels of *BRAP* inhibited NF- κ B activation by reducing the nuclear translocation of NF- κ B. We also demonstrated that BRAP interacted with two major components of the IKK-signalosome, NFKBIB and I κ BK β . The NFKBIB (aka I κ B β) sequesters the NF- κ B complex in the cytoplasm, and I κ BK β (aka IKK β) allows NF- κ B to translocate into nucleus by initiating the degrada-

tion of I κ B (24,25). Western blot analysis further confirmed that *BRAP* silencing could influence the degradation of I κ B β . Taken together, LPS-induced *BRAP* up-regulation activated the IKK signalosome, enhanced NF- κ B nuclear translocation and consequently increased the expression of inflammatory cytokines.

The present study is the first to demonstrate that *BRAP* is upregulated by an inflammatory stimulant, leading to the secretion of inflammatory cytokines. We are also the first to demonstrate that *BRAP* may influence NF- κ B nuclear translocation, a prerequisite of NF- κ B ac-

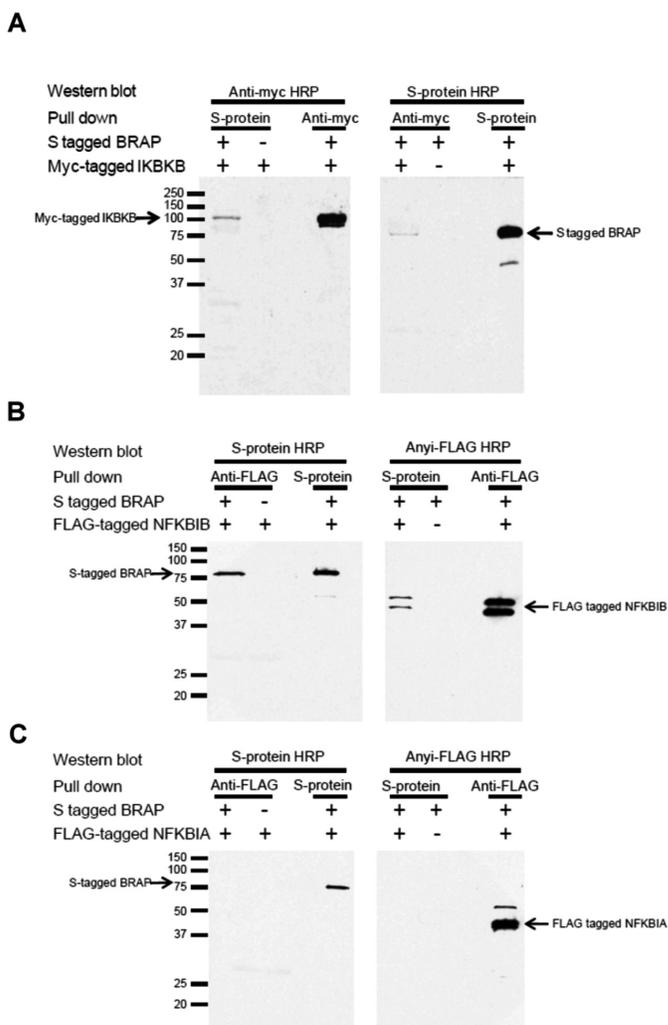


Figure 4. BRAP interacted with IKK β and NFKBIB, but not NFKBIA. Co-IP demonstrated interactions between (A) BRAP and IKK β (aka IKK β) and between (B) BRAP and NFKBIB (I κ B β). (C) BRAP did not bind to NFKBIA (I κ B α) directly.

tivation. We found that BRAP directly binds to I κ B β and IKK β , both of which are the major components of the IKK-signalosome. IKK β , the major subunit of I κ B kinase, phosphorylates I κ B in response to inflammatory cytokines, LPS and tumor necrosis factor- α (26). IKK β -dependent phosphorylation subsequently triggers polyubiquitination and degradation of I κ B. It exposes the NF- κ B NLS masked by I κ B and initiates the shuttle of NF- κ B into the nucleus (27). The transcription factor NF- κ B controls the activation of various atherogenic genes like *vascular cellular adhesion molecule-1* (*VCAM-1*), *E-selectin* and

MMPs (24). By regulating the nuclear translocation of NF- κ B, BRAP might influence other proatherogenic genes in addition to *MCP-1* and *IL-8* (16,28).

Notably, we found that BRAP bound to I κ B β but not I κ B α . It has been found that I κ B β is degraded only when cells are stimulated by LPS and interleukin-1 (IL-1), whereas I κ B α is degraded by all of the known inducers of NF- κ B (24). I κ B α replenishes quickly after NF- κ B activation, resulting in a rapid but transient effect on cells (29). I κ B β , on the other hand, is not resynthesized immediately and causes a slow but long-term activation of NF- κ B (29). Previous studies also

revealed that unphosphorylated I κ B β may compete with I κ B α and lead to persistent activation of NF- κ B (25,30). The present findings indicate that BRAP's effect on NF- κ B translocation might be mediated through influencing the IKK signalosome. However, we do not know whether BRAP was involved in the phosphorylation, ubiquitination or proteolysis of I κ B β . In our data, BRAP knockdown did not change the NF- κ B nuclear/cytoplasm ratio when the cells were not treated with LPS. Accordingly, BRAP by itself probably does not initiate the NF- κ B translocation. BRAP protein may be a chaperone of the IKK signalosome and modulates the activation of NF- κ B signal cascades. Further studies are warranted to clarify these possible functions of BRAP.

In the present study, the minor allele of rs11066001 in BRAP was associated with a thicker CCA IMT, as well as the presence of plaque. Our results are consistent with the detrimental effect of this minor allele observed in patients with MI (7). Because the minor allele G has greater transcription efficacy than the common allele A (7), individuals with the GG genotype may have higher levels of BRAP protein and thus manifest more severe atherogenic phenotypes. Statins, the widely used antiatherosclerotic drugs with antiinflammatory effects, did not reverse BRAP overexpression in response to LPS stimulation. A recent study showed that statins inhibited LPS-induced NF- κ B activation via inhibiting the Rho/Rho kinase signal pathway (31). Because NF- κ B activation could be regulated by various signal pathways, individuals of the GG genotype might be exposed to a higher risk of cardiovascular events despite receiving statin therapy.

We observed significant associations between SNP rs110660001, plaque and dichotomized IMT data. Although both IMT and plaque are predictors of cardiovascular risks, the two phenotypes measure different aspects of atherogenesis (5). IMT mainly indicates hypertensive hypertrophy of the vessel walls (6), whereas plaque reflects a later stage of

atherosclerosis in which lipid infiltration, inflammation, matrix overproduction, endothelium dysfunction and smooth muscle cell proliferation take place (32). In cellular experiments, knockdown of *BRAP* significantly reduced the secretion of inflammatory cytokines and had a modest effect on HASMC proliferation and a minor effect on the HASMC migration. Because we used the cutoff value of IMT above the mean plus 1 SD in analyzing IMT data, our results indicate that the effect of *BRAP* effect may be more evident when the atherosclerosis is advanced.

There were several limitations to the present study. We acknowledge that a significant threshold of nominal $P < 0.05$ may have led to false-positive results when several phenotypes were tested concurrently. We presented both the nominal P values and the Bonferroni-corrected P values. The significance threshold represented a trade-off between avoidance of false-positive associations while taking into account that a set of related phenotypes were tested in the present study. The validation of *BRAP*'s effect in HASMC further strengthened the association observed in human subjects. We analyzed both continuous and dichotomized IMT data because a certain threshold may be needed to detect the atherogenic effect (2). We selected the mean plus 1 SD as the cutoff level to dichotomize thick and thin IMT to be comparable to that observed in a previous study, which used the per-SD difference in IMT values to estimate the atherosclerotic risks (3,33). We used overexpression of the tagged protein rather than the endogenous *BRAP*/*NF- κ B* proteins in the Co-IP experiments. We acknowledge that overexpression might change protein cellular localization and thus the results might not truly reflect the protein-protein interaction *in vivo*. Further studies are warranted to validate our findings.

In summary, the results of the present study demonstrate that an SNP in *BRAP* confers a risk for carotid atherosclerosis. Through binding to the IKK signaling, *BRAP* enhances *NF- κ B* nuclear

translocation, initiates the transcription of downstream inflammatory cytokines and provokes the atherosclerotic process. Our results indicate that *BRAP* has an effect on cardiovascular disease. Further studies are warranted to identify treatment strategies for individuals at risk.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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