

Protein Kinase C- η and Phospholipase D2 Pathway Regulates Foam Cell Formation via Regulator of G Protein Signaling 2

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ABSTRACT

Regulator of G protein signaling 2 (RGS2) is a GTPase-activating protein for $G\alpha_q$, which is involved in regulating various vascular functions. To understand how RGS2 regulates foam cell formation, the present study identified signaling pathways controlled by lipopolysaccharide (LPS) and discovered new mechanisms whereby protein kinase C (PKC)- η and phospholipase D (PLD) 2 regulate RGS2 expression. The toll-like receptor (TLR) 4 agonist LPS caused foam cell formation of Raw264.7 macrophages and dramatically decreased RGS2 mRNA expression. RGS2 down-regulation by LPS was partially recovered by TLR4 small interfering RNA (siRNA). Peritoneal macrophages were separated from wild-type and TLR4 mutant mice, and treatment with LPS showed RGS2 expression decrease in wild-type macrophages but no change in TLR4 mu-

tant macrophages. RGS2 overexpression was suppressed, whereas RGS2 down-regulation accelerated foam cell formation by LPS. Treatment of PKC- η pseudosubstrate weakened foam cell formation and recovered RGS2 down-regulation by LPS. In addition, LPS or phorbol 12-myristate 13-acetate stimulated PLD activity, and the pretreatment of PLD inhibitor weakened foam cell formation and recovered RGS2 down-regulation. Inhibition of PLD2 expression by siRNA also weakened foam cell formation and partially recovered LPS-mediated RGS2 down-regulation. On the other hand, PLD2 overexpression intensified RGS2 down-regulation and foam cell formation by LPS. These results suggest that LPS causes foam cell formation by increasing PKC- η and PLD2 activity by down-regulating RGS2 expression via TLR4 dependently.

Introduction

Regulators of G-protein signaling (RGS) proteins are a large and diverse family initially identified as GTPase-activating proteins (GAPs) of heterotrimeric G-protein $G\alpha$ subunits. RGS proteins are important proteins regulating G-protein-coupled receptor-induced signaling by enhancing GTP hydrolysis, thereby terminating the G-protein activation (Kimple et al., 2009). Although RGS proteins were first known as negative regulators of G-protein, they are currently recognized as multifunctional proteins controlling G-protein signaling. More than 30 types of RGS and RGS-like proteins

have been identified and are divided into six distinct subfamilies according to their amino acid sequences (Manzur and Ganss, 2009). Among them, RGS2 has a preferred interaction with $G\alpha_{q/11}$ and is recognized as the most important regulator in cardiovascular pathophysiology. Many contractile responses are mediated via G_q in blood vessels, and RGS2 interrupts G_q -mediated vasoconstriction (Hendriks-Balk et al., 2009). Moreover, the vascular role of RGS2 is evidence that the increased RGS2 expression is related to hypotension and that decreased RGS2 expression is related to hypertension (Le and Coffman, 2003). A strong hypertensive phenotype has been observed in RGS2 gene-deficient mice as well (Heximer et al., 2003).

Reports of in vivo experiments show that RGS2 is selectively down-regulated in the early processes of cardiac hypertrophy. It has also been confirmed in neonatal rat cardiomyocytes that hypertrophy by endothelin-1 is increased when RGS2 expression is suppressed through small interfer-

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ABBREVIATIONS: RGS, regulator of G protein signaling; PKC, protein kinase C; PLD, phospholipase D; TLR, toll-like receptor; GAP, GTPase activating protein; PA, phosphatidic acid; DAG, diacylglycerol; LDL, low-density lipoprotein; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; PS, pseudosubstrate; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; BuOH, butanol; ABCA1, ATP-binding cassette transporter 1.

ing RNA (siRNA) methods. However, there are only indirect reports and no direct reports of RGS in atherosclerosis development. Peroxisome proliferator-activated receptor δ suppresses lesion formation induced by monocyte chemotactic protein-1 or chemokine (C-X-C motif) ligand 2, and such suppression suggests the functional possibilities of RGS in atherosclerosis through reports of up-regulation of RGS4 and RGS5 (Barish et al., 2008). The importance of RGS5 in vascular remodeling has been reviewed previously (Manzur and Ganss, 2009). It is noteworthy that one study reported that RGS5 expressions was dramatically down-regulated in the fibrous cap of primate atherosclerotic plaque-derived smooth muscle cells and human advance atherosclerotic plaques (Adams et al., 2006). However, there have not been any reports of RGS2 in atherosclerosis.

The participation of cell surface receptors by ligands induces hydrolysis of phospholipase-mediated cellular phospholipids, produces lipid-derived products, and essentially functions by using various signaling pathways among the cells. Among enzymes that hydrolyze phospholipids, phospholipase D (PLD) is known to be an essential second-messenger source, which contains phosphatidic acid (PA) (Lee et al., 2008a). PLD has two known isoforms, PLD1 and PLD2 (Donaldson, 2009). In mammalian cells, activation of phosphatidylcholine-specific PLD controls signal transduction pathways that regulate various physiological processes, including membrane trafficking, cytoskeleton rearrangement, phagocytosis, and mast cell degranulation (Kanaho et al., 2009). In addition, PLD is known to control cellular responses related to inflammation (Zhao and Natarajan, 2009). Some studies have suggested a role for PLD in tumor necrosis factor-mediated cellular cytotoxicity (Oprins et al., 2001), and other results established the key roles of PLD1 in tumor necrosis factor- α signaling (Sethu et al., 2008). However, there have been an inadequate number of studies concerning the potential function of PLD in RGS2 expression. Therefore, studies on how LPS-mediated PLD activity controls RGS2 expression and thereby controls foam cell formation are important.

Protein kinase C (PKC), a family of lipid-regulated serine/threonine kinases, is one of the important factors controlling PLD activation mediating cellular responses and cellular functions (Casabona, 1997). PKC has at least 10 different isoforms and is divided into three major groups according to its activation requirements and downstream targets. Classical isoforms (α , β I, β II, and γ) require diacylglycerol (DAG) and calcium for activation, whereas novel isoforms (δ , ϵ , η , and θ) only need DAG for activation. Atypical isoforms (ι and ζ) are PKCs that require neither. Among them, PKC- η is usually expressed in epithelial tissues and has been reported to have essential functions in the differentiation of this tissue (Kashiwagi et al., 2002). Functions of PKC- η related to blood vessels have also been reported (Redig et al., 2009). Nonsynonymous single nucleotide polymorphism of PKC- η is also related to cerebral infarction development. PKC- η is mostly reported to be expressed in vascular endothelial cells and foamy macrophages of human atherosclerotic lesions (Kubo et al., 2007). However, it is still unclear how PKC- η participates in atherosclerosis progression.

The present results demonstrate that PKC- η and PLD2 play a key role in signaling, and both are important for LPS-mediated RGS2 down-regulation. Furthermore, the

physiological importance of PKC- η and PLD2 in the LPS-mediated signaling pathways is emphasized by their role in mediating foam cell formation.

Materials and Methods

Reagents. Cell culture reagents, including fetal bovine serum, were obtained from Invitrogen (Carlsbad, CA). RGS2 and PLD2 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). *Escherichia coli* LPS (0111:B4), phorbol 12-myristate 13-acetate (PMA), staurosporine, 1-butanol (BuOH), *t*-BuOH, low-density lipoprotein (LDL), and Oil-red O were from Sigma-Aldrich (St. Louis, MO). The reverse transcription-polymerase chain reaction (RT-PCR) kit was from Takara Bio (Shiga, Japan), siRNAs were from Invitrogen and angiotensin II was from Calbiochem (San Diego, CA). Pseudosubstrate (PS) peptide inhibitors were from Peptron (Seoul, South Korea). TLR4 wild-type mice (C3H/HeN) and TLR4 mutant mice (C3H/HeJ) were purchased from Central Lab Animal (Seoul, South Korea).

Cell Culture and Transfection. The Raw264.7 macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) at 37°C in 5% CO₂. Primary peritoneal macrophages were isolated from C3H/HeN or C3H/HeJ mice. For transfection, macrophages were plated in 35-mm diameter plates, grown overnight, and transfected using reagents (Amaya Biosystems, Gaithersburg, MD). Cells were nucleoporated according to the manufacturer's protocol and incubated for 24 h before LPS stimulation. The pcDNA3-RGS2, pcDNA3-PLD2, and pcDNA3 were transfected into cells using the FuGene HD transfection reagent (Roche Diagnostics, Basel, Switzerland).

Plasmid and siRNAs. The cDNA of mouse RGS2 was amplified with forward primer 5'-CGC AAG CTT ATA GAA TGC AAA GTG CCA TG-3' and reverse primer 5'-GGG GGA TCC TCC TGG TCT CAT GTA GCA TG-3'. The RGS2 PCR product and pcDNA3 (Invitrogen) were digested by HindIII and BamHI and then ligated with T4 ligase. The full-length PLD2 was also amplified by PCR. The cDNA encoding mouse PLD2 was amplified with the forward primer 5'-CCC GAA TTC ATG ACT GTA ACC CAG AAG AAC-3' and the reverse primer 5'-CCC CTC GAG CTA TGT CCA CAC TTC TAA AG-3'. The PCR product and pcDNA3 were digested by EcoRI and XhoI and ligated to pcDNA3 (Invitrogen). The constructs were confirmed by DNA sequencing. Stealth control and gene-specific siRNAs were designed by Block-IT Stealth RNA interference designer (Invitrogen) and by using the following gene-target sequences: TLR4, 5'-AAU GAU GGC ACC AUU GAA GCU GAG G-3'; RGS2, 5'-UGG AAG ACC CGU UUG AGC UAC UUC U-3'; and PLD2, 5'-AAG ACU UUG UGU CUC UGG AGG UCC C-3'. Macrophages were transfected with an siRNA at a final concentration of 100 to 200 pM using reagents (Amaya Biosystems). After transfection, the cells were treated with LPS to study the regulation of gene expression.

RT-PCR and Real-Time PCR. Total RNA was extracted from cells using TRIZOL reagent (Invitrogen). First-strand cDNA was synthesized by 1 μ g of total RNA, oligo(dT), and a reverse-transcriptase system (Promega, Madison, WI). The sequences of PCR primers used in the present study were as follows: RGS2, forward primer, 5'-ATG CAA AGT GCC ATG TTC CTG G-3'; reverse primer, 5'-TCA TGT AGC ATG GGG CTC CG-3', which is amplified as 636 base pairs; PLD2, forward primer, 5'-CTG GCC ATC TAT GAC CTT CAG C-3'; reverse primer, 5'-GCC ACC TCT TGG ACC ATC GAT A-3', which is amplified as 582 base pairs. Cycling conditions were 95°C for 5 min, followed by 26 to 33 cycles at 95°C for 1 min, 63°C for 1 min, and 72°C for 1 min. Real-time PCR was performed using a LightCycler 1.5 (Roche Diagnostics) with SYBR-Green I as the fluorescent dye according to the manufacturer's instructions. For semi-quantification, target genes were normalized versus β -actin.

Assay for Foam Cell Formation. For lipid uptake analysis, macrophages were cultured in six-well plates and then treated with LPS (100 ng/ml) and LDL (50 μ g/ml) for 24 h. Next, the cells were washed three times with phosphate-buffered saline, fixed with 10% formalin, and stained with Oil-red O. Intracellular lipid droplets were then detected by light microscopy using a DIAPHOT 300 light microscope (Nikon, Tokyo, Japan). Images were recorded with an AxioCam ICcl digital camera system (Carl Zeiss GmbH, Jena, Germany). Foam cells were observed under the light microscopy and counted by investigators, who were blinded to the genotypes of the stained cells. The foam cells were represented by the percentage of positive Oil-red O cells to total cells.

In Vitro PLD Assay. PLD activity was assayed by measuring the formation of phosphatidylbutanol, the product of PLD-mediated transphosphatidyl, in the presence of 1-butanol as described previously with a slight modification. Raw264.7 cells were labeled (3×10^6 cells/ml) with [3 H]myristic acid (5 μ Ci/ml; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) in the cell culture medium for 18 h. Unincorporated [3 H]myristic acid was removed by washing twice with DMEM. After the wash, the cells were incubated at 37°C for 15 min in DMEM containing 1-BuOH (0.5% final concentration). After LPS or PMA stimulation for the indicated times at 37°C, the cells were then lysed, and lipids were extracted with chloroform/methanol/1 M NaCl [1:1:1 (v/v/v)]. Phosphatidylbutanol was separated from total lipid with ethyl acetate/trimethylpentane/acetic acid (9:5:2) in a 60Å silica gel thin-layer chromatography plate.

Western Blot Analysis. Macrophages were cultured in wells of six-well plates and treated with LPS in the presence or absence of an inhibitor. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). Proteins were separated by 8% reducing SDS-polyacrylamide gel electrophoresis and immunoblotted onto nitrocellulose membranes in 20% methanol, 25 mM Tris, and 192 mM glycine. Membranes were then blocked with 5% nonfat dry milk and incubated overnight with primary antibody. The membranes were then washed, incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase, rewashed, and finally developed using an enhanced chemiluminescence system (GE Healthcare).

Statistical Analysis. Results are expressed as the mean \pm S.D. of at least three independent assays. Comparisons between the two groups were made by nonpaired two-tailed Student's *t* test using SPSS software version 12.0 (SPSS Inc., Chicago, IL). A *p* value < 0.05 was considered significant.

Results

LPS Down-Regulates RGS2 mRNA Expression via TLR4 Signaling. We studied the effects of the LPS in converting Raw264.7 macrophages to foam cells. Confirmation of lipid droplets forming in foam cells by Oil-red O staining and the microscopy-based enumeration of Oil Red O-positive cells revealed a strong increase in foam cell formation by LPS (Fig. 1A). An mRNA microarray was used to identify the factors contributing to foam cell formation induced by LPS. In the mRNA microarray results, expression changes were evident in the RGS2 gene. Treatment with LPS was followed by the confirmation of the time-dependent diminution of RGS2 mRNA expression. However, there was no change in RGS4 mRNA expression (Fig. 1B). Down-regulation of RGS2 mRNA by LPS was also confirmed using real-time PCR (data not shown). Angiotensin II, which regulates hypertension, is also controlled by RGS2 (Hercule et al., 2007). Therefore, the expression changes of RGS2 mRNA by LPS and angiotensin II were compared. As expected, LPS decreased RGS2 expression dramatically, but angiotensin II had few effects (Fig.

1C). LPS also had no effect on RGS2 expression in smooth muscle cells (data not shown). These effects suggest that LPS and angiotensin II are involved in different mechanisms in the atherosclerosis process. The mechanism of LPS is known to be mediated through its receptor TLR4. To confirm this, cells in which the expression of TLR4 was abrogated by siRNA were compared with control siRNA cells. As expected, TLR4 mRNA expression decreased in the TLR4 siRNA-transfected cells. In these cells, LPS-induced RGS2 down-regula-

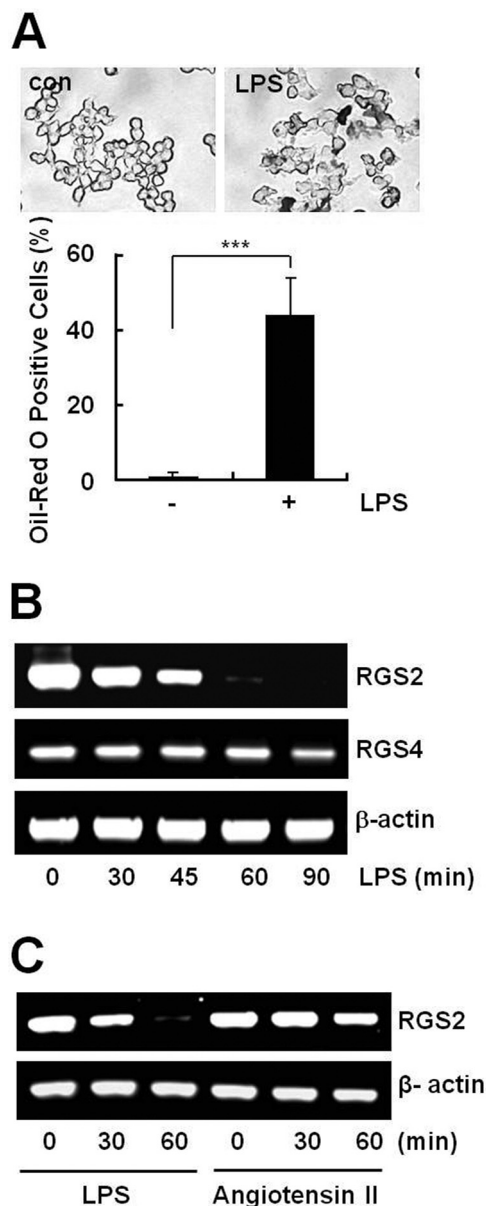


Fig. 1. LPS induces foam cell formation and RGS2 down-regulation in Raw264.7 cells. A, Raw264.7 cells were treated with LPS (100 ng/ml) containing LDL (50 μ g/ml) for 24 h. The cells were fixed with 10% formalin and stained with Oil-red O. The intracellular lipid droplets were then detected by light microscopy. Foam cells are expressed as the percentage of positive Oil-red O cells compared with total cells. Data represent mean \pm S.D., and experiments were repeated five times in duplicate. ***, *p* < 0.001 by two-tailed Student's *t* test. B, cells were stimulated with LPS for the indicated times. RGS2 and RGS4 mRNA expression was determined by RT-PCR and was normalized to β -actin. C, cells were treated with LPS (100 ng/ml) or angiotensin II (1 μ M) for the indicated times. RGS2 mRNA expression was determined by RT-PCR and was normalized to β -actin.

tion was comparatively less in TLR4 siRNA cells than in control siRNA cells (Fig. 2A). The roles of TLR4 were reconfirmed in C3H/HeN (TLR4 wild type) and C3H/HeJ (TLR4 mutant type) mouse-isolated peritoneal macrophages. Although LPS treatment diminished RGS2 expression in C3H/HeN macrophages, no changes in RGS2 expression were shown in C3H/HeJ macrophages (Fig. 2B). Furthermore, whereas LPS-induced foam cell formation increased in C3H/HeN, there were no effects in C3H/HeJ (Fig. 2C). These results provided evidence that LPS-mediated expression of RGS2 is regulated by TLR4.

RGS2 Overexpression Attenuates LPS-Induced Foam Cell Formation. An RGS2 overexpression system was used to confirm the direct relationship between foam cell formation and RGS2 down-regulation by LPS. Mouse RGS2 cDNA was cloned in the pcDNA3 vector and was then sequenced and transfected in Raw264.7 cells. The efficiency of RGS2 protein overexpression was checked by Western blot analysis (Fig. 3A). Foam cell formation by LPS was then analyzed using RGS2-overexpressing cells. In the vector-transfected cells, foam cell formation was increased approximately 25% by LPS, but RGS2-overexpressing cells strongly inhibited foam cell formation by LPS (Fig. 3B). RGS2 siRNA was used to confirm the effects of RGS2. RGS2 siRNA transfection successfully lowered RGS2 mRNA (Fig. 3C). LPS-induced foam cell formation shown in the control siRNA cells

was potentiated in RGS2 siRNA cells (Fig. 3D). These results suggest that RGS2 down-regulation is an essential step in LPS-induced foam cell formation.

PKC- η Is Important for LPS-Mediated RGS2 Down-Regulation and Foam Cell Formation. An experiment was performed to identify these mediators that are essential to the increase in foam cell formation after RGS2 down-regulation by LPS. With inhibitor screening, PKC mediation was identified. Staurosporine, a broad PKC inhibitor, not only recovered RGS2 down-regulation by LPS but also inhibited foam cell formation (Fig. 4A). Because there are various types of PKCs, experiments were performed to determine the isoforms that were most significant. Effects of several PKC isoforms were tested by using pseudosubstrate (PS) peptide inhibitors. PKC- α , PKC- β , PKC- ϵ , and PKC- θ inhibitors had no effect, whereas PKC- η inhibitor recovered RGS2 down-regulation by LPS (Fig. 4B); these effects were shown to be concentration-dependent (Fig. 4C) and attenuated foam cell formation (Fig. 4D). The RGS2 regulation by PKC was confirmed by treatment with PMA, a PKC activator. Treatment with PMA decreased RGS2 mRNA expression in a dose-dependent manner (Fig. 5A). However, PKC- η PS inhibitor also recovered RGS2 down-regulation with PMA (Fig. 5B) and attenuated foam cell formation (Fig. 5C). Taken together, these results suggest that PKC- η is an important mediator in RGS2 down-regulation and foam cell formation induced by LPS or with PMA.

PLD2 Is Involved in PKC- η -Mediated RGS2 Down-Regulation. The next experiment was designed to identify the contribution of PLD by studying the downstream molecule of PKC- η . First, the changes in PLD activity by LPS were uncertain in Raw264.7 cells. LPS treatment increased the production of phosphatidylbutanol, a product of PLD activity, which was concentration-dependent (Fig. 6A). Next,

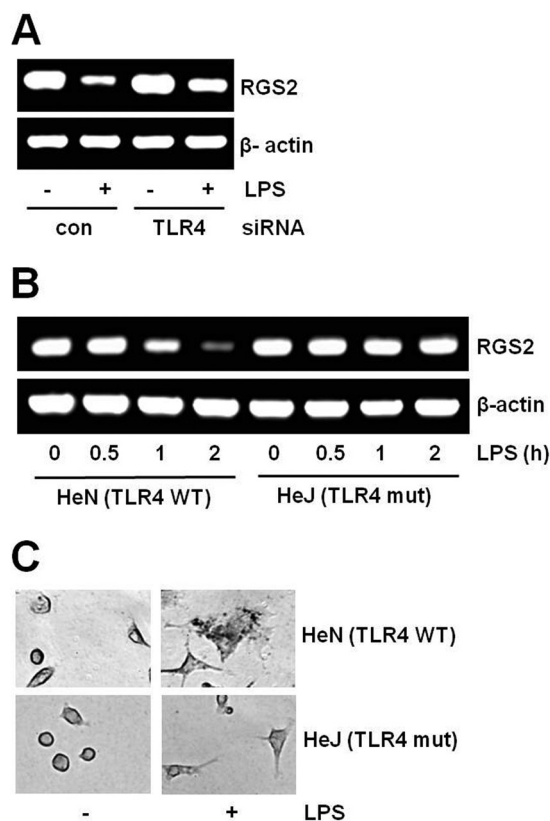


Fig. 2. TLR4 siRNA or mutation recovers LPS-induced RGS2 down-regulation and foam cell formation. A, Raw264.7 cells were transfected with either control siRNA or TLR4 siRNA for 24 h. The cells were stimulated with vehicle or LPS for 45 min. RGS2 mRNA expression was determined by RT-PCR and was normalized to β -actin. B and C, peritoneal macrophages were isolated from TLR4 wild-type or mutant mice and incubated with vehicle or LPS. RGS2 mRNA and foam cell formation was determined by RT-PCR (B) or Oil-red O staining (C), respectively.

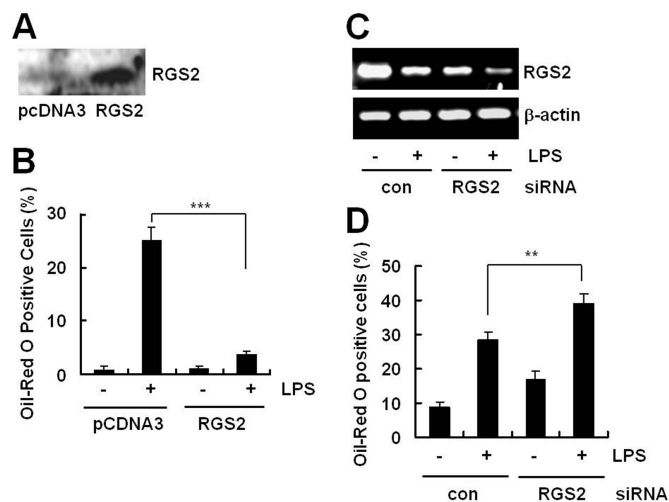


Fig. 3. RGS2 overexpression inhibits and RGS2 down-regulation potentiates LPS-induced foam cell formation. A and B, Raw264.7 cells were transfected with a pcDNA3 vector expressing mouse RGS2 or vector alone. A, the cell lysates were analyzed for RGS2 by Western blotting. B, gene-transfected cells were stimulated with or without LPS for 24 h, and foam cell formation was determined by Oil-red O staining. C and D, Raw264.7 cells were transfected with either control siRNA or RGS2 siRNA for 24 h. C, RGS2 mRNA was determined by RT-PCR. D, siRNA-transfected cells were stimulated with or without LPS. Foam cell formation was determined by Oil-red O staining. Data represent mean \pm S.D., and experiments were repeated four or five times in duplicate. **, $p < 0.01$, or ***, $p < 0.001$ by two-tailed Student's t test.

a PLD inhibitor was used to determine whether the increase of PLD activity by LPS had an effect on RGS2 expression. Pretreatment with 1-BuOH, a PLD inhibitor, reversed the decrease in RGS2 expression by LPS. However, pretreatment with the control (*t*-BuOH), a control of 1-BuOH, showed no effect by LPS (Fig. 6B). The effects of 1-BuOH and *t*-BuOH on foam cell formation were assessed. Although 1-BuOH suppressed LPS-induced foam cell formation, *t*-BuOH had a comparatively reduced effect (Fig. 6C). The effects of PLD inhibitor showed similar results in both Raw264.7 cells and primary macrophages (data not shown). A third experiment assessed whether the increase in PLD activity by PMA affected RGS2 expression. PMA also increased PLD activity (Fig. 6D), whereas 1-BuOH reversed the decrease in RGS2 expression by PMA (Fig. 6E). These results provided support for the suggestion that PLD activity, increased by LPS or PMA, diminishes RGS2 expression and induces foam cell formation. Between two known PLD types, we tested the contribution of PLD2. The PLD2 siRNA method was used to confirm the function of PLD2. PLD2 siRNA transfection successfully decreased PLD2 mRNA and protein expression (Fig. 7A). RGS2 expression and foam cell formation by LPS was compared in control siRNA cells and PLD2 siRNA cells. LPS-induced RGS2 down-regulation shown in the control siRNA cells was partially recovered in PLD2 siRNA cells (Fig. 7B). These results suggest that PLD2 participates in the RGS2 down-regulation by LPS and thereby controls foam cell formation (Fig. 7C). To reconfirm the importance of PLD2, the PLD2 overexpression system was used. The mouse PLD2 gene was cloned into the pcDNA3 vector and transfected into Raw264.7 cells. The efficiency of PLD2 protein overexpression was determined by Western blot analysis (Fig. 8A). LPS-induced RGS2 expression and foam cell formation was compared in control vector-transfected cells and in PLD2-overexpressing cells. RT-PCR consistently demonstrated that RGS2 down-regulation by LPS was stronger in PLD2-

overexpressing cells than in vector-transfected cells (Fig. 8B). These results were also shown through real-time PCR (data not shown). Foam cells were also more strongly produced in PLD2-overexpressing cells (Fig. 8C). Therefore, we suggest that PLD2 is an essential factor in LPS-induced RGS2 down-regulation and foam cell formation.

Discussion

Accumulation of foam cells containing cholesterol in arterial intima is a key event in the early stages of atherosclerosis (Paulson et al., 2010). Exposure to TLR agonists such as LPS or CpG oligodeoxynucleotide converts macrophages into foam cells (Kalayoglu and Byrne, 1998). There are various types of essential factors that contribute to foam cell formation. Among them, scavenger receptors that uptake modified lipoproteins are known to be critical (Badimón et al., 2009). TLRs directly cause inflammatory responses and induce the expression of specific scavenger receptors, such as lectin-like oxidized low density lipoprotein receptor 1 (Lee et al., 2008b). Although it is clear that PKC-PLD signaling participates in mechanisms that increase atherosclerosis, few research stud-

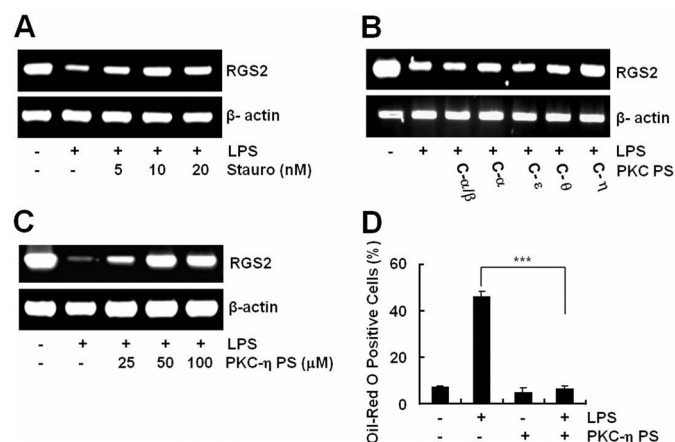


Fig. 4. PKC- η is involved in the LPS-induced RGS2 down-regulation and foam cell formation. A, Raw264.7 cells were pretreated with staurosporine and then stimulated with LPS for 45 min. RGS2 mRNA expression was determined by RT-PCR and was normalized to β -actin. B, cells were pretreated with various PKC PS inhibitors and stimulated with LPS for 45 min. RGS2 mRNA expression was determined by RT-PCR. C, before LPS stimulation for 45 min, cells were treated with the indicated doses of PKC- η PS inhibitor. RGS2 mRNA expression was determined by RT-PCR. D, cells were treated with LPS in the presence or absence of PKC- η PS for 24 h. Foam cell formation was determined by Oil-red O staining. Data represent mean \pm S.D. and experiments were repeated five times in duplicate. ***, $p < 0.001$ by two-tailed Student's t test.

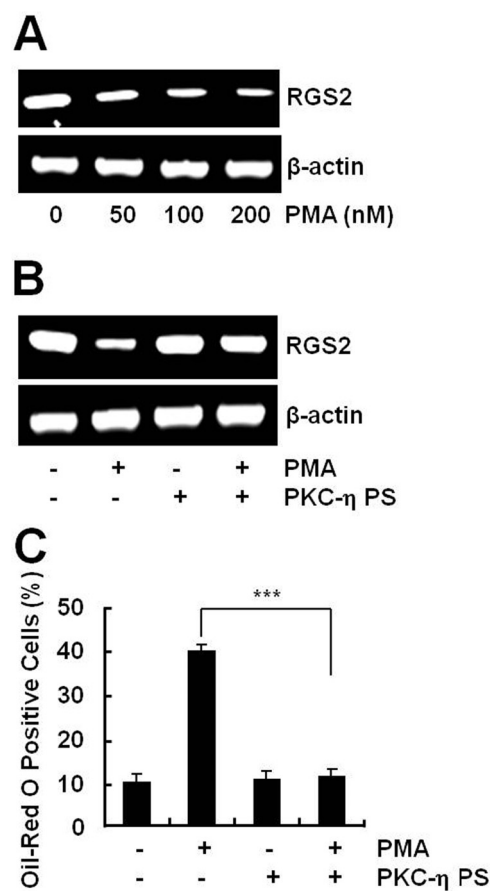


Fig. 5. PKC- η also mediates PMA-induced RGS2 down-regulation and foam cell formation. A, Raw264.7 cells were treated with the indicated doses of PMA for 45 min. RGS2 mRNA expression was determined by RT-PCR and was normalized to β -actin. B and C, cells were pretreated with PKC- η PS inhibitor and stimulated with PMA. RGS2 mRNA and foam cell formation were determined by RT-PCR (B) or Oil-red O staining (C), respectively. Data represent mean \pm S.D., and experiments were repeated five times in duplicate. ***, $p < 0.001$ by two-tailed Student's t test.

ies have addressed the function of RGS2 and the PKC-PLD pathway in the development of atherosclerosis.

The present results demonstrate that the PKC- η -PLD2 signaling pathway decreases LPS-induced RGS2 expression, thereby causing foam cell formation of macrophages. Multiple evidences support this conclusion. First, LPS decreased RGS2 expression and induced foam cell formation, and the overexpression of RGS2 suppressed LPS-induced foam cell formation. Second, it has been demonstrated using TLR4 siRNA and TLR4 mutant mice that the regulations of foam cell formation and RGS2 expression by LPS is mediated by TLR4. Third, the use of a PKC- η -specific inhibitor recovered RGS2 down-regulation and suppressed macrophage foam cell formation. Fourth, whereas PLD2 siRNA partially recovered LPS-induced RGS2 down-regulation and foam cell formation, PLD2 overexpression intensified those LPS responses.

It has been speculated that RGS signaling, which controls GAP activity, functions essentially in vascular pathologies such as hypertension, atherosclerosis, or angiogenesis (Riddle et al., 2005). RGS proteins were first known as negative regulators of G-protein signaling, but currently it is understood that these are multifunctional proteins, which control

G-protein signaling. So far, more than 30 types of RGS proteins have been found in mammals (Manzur and Ganss, 2009). Among them, recent studies have researched the in vivo cardiovascular function of RGS proteins (Cho et al., 2008). The most frequent research targets were RGS2 and RGS4, but recently RGS5 was implicated in vascular remodeling and is known to regulate blood pressure (Gu et al., 2009). RGS2-deficient mice are shown to be hypertensive and have prolonged vasoconstriction (Heximer et al., 2003). In preliminary human studies, elevation of RGS2 expression is related to hypotension, and the decrease in expression is related to hypertension (Hahntow et al., 2009). Although RGS2 has essential functions in hypertension, its functions in the atherosclerosis process have not been clarified. At present, the TLR4 agonist LPS rapidly decreased RGS2 mRNA expression and induced foam cell formation, but overexpression of RGS2 suppressed the production of foam cell formation.

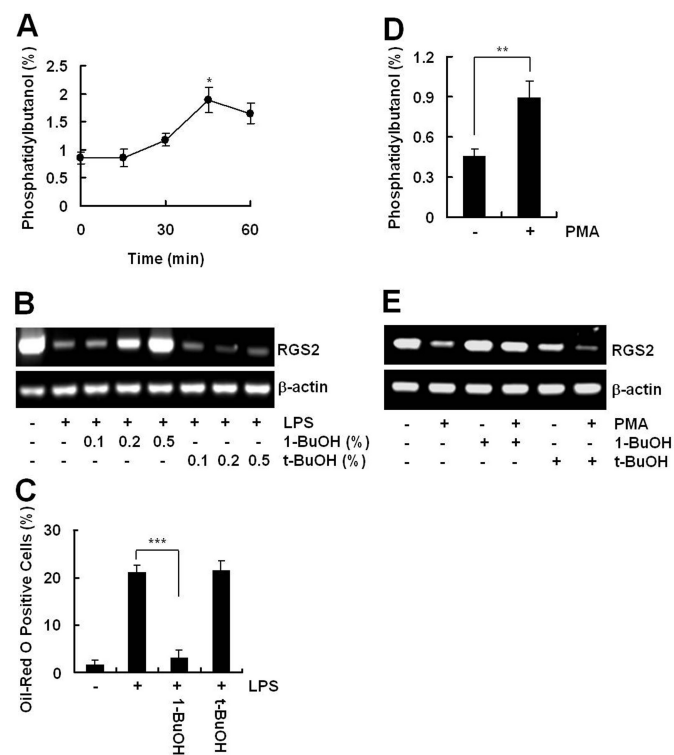


Fig. 6. PLD plays a role in the LPS-induced RGS2 down-regulation and foam cell formation. A, Raw264.7 cells were labeled with [3 H]myristic acid (5 μ Ci/ml) overnight and stimulated with the different times of LPS. The accumulated phosphatidylbutanol was extracted by thin-layer chromatography, and the radioactivity was counted. B, cells were pretreated with the indicated doses of 1-BuOH or *t*-BuOH and stimulated with LPS for 45 min. RGS2 mRNA expression was determined by RT-PCR. C, cells were pretreated with 1-BuOH or *t*-BuOH and stimulated with LPS for 24 h. Foam cell formation was determined by Oil-red O staining. Data represent mean \pm S.D., and experiments were repeated five times in duplicate. D, Raw264.7 cells were labeled with [3 H]myristic acid overnight and stimulated with PMA (100 nM). The accumulated phosphatidylbutanol was extracted by thin-layer chromatography, and the radioactivity was counted. E, cells were pretreated with the 1-BuOH or *t*-BuOH and stimulated with PMA. RGS2 mRNA expression was determined by RT-PCR. *, $p < 0.05$, **, $p < 0.01$, or ***, $p < 0.001$ by two-tailed Student's *t* test.

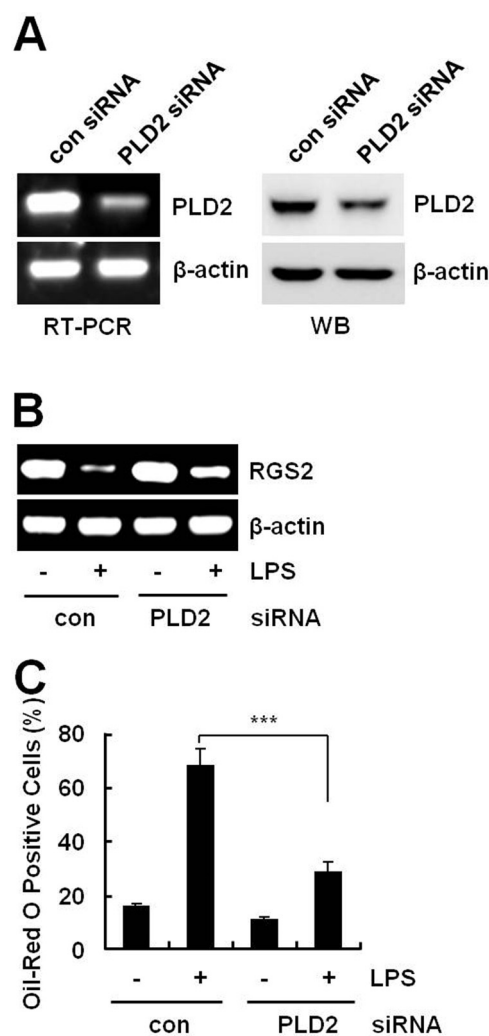


Fig. 7. PLD2 siRNA suppresses LPS-induced RGS2 down-regulation and foam cell formation. Raw264.7 cells were transfected with either control siRNA or PLD2 siRNA for 24 h. A, PLD2 mRNA and protein expression were determined by RT-PCR or Western blot, respectively. B and C, siRNA-transfected cells were stimulated with or without LPS. RGS2 mRNA and foam cell formation were determined by RT-PCR (B) or Oil-red O staining (C), respectively. Data represent mean \pm S.D., and experiments were repeated five times in duplicate. ***, $p < 0.001$ by two-tailed Student's *t* test.

RGS2 down-regulation by LPS has been reported previously (Riekenberg et al., 2009). From these results, it is appropriate to suggest that RGS2 may regulate atherosclerosis. RGS2 down-regulation by LPS showed cell specificity. Although LPS-induced RGS2 expression was decreased in macrophages, there was no effect on vascular smooth muscle cells (data not shown). In addition, RGS2 regulation by LPS differed from the results of angiotensin II. In previous reports, angiotensin II was reported to stimulate RGS2 expression (Hercule et al., 2007). The present results did not support this earlier observation. Although RGS2 down-regulation by angiotensin II was weak, it showed a time-dependent decrease. Under the same conditions, when the effects of angiotensin II and LPS were compared, angiotensin II was much weaker than that of LPS in RGS2 down-regulation. These results suggest that the mechanisms of LPS and angiotensin II are different in the processes of atherosclerosis. It is believed that LPS decreases GAP activity by decreasing RGS2 expression and foam cell formation, eventually contributing to atherosclerosis development.

PKC is an important factor that can increase the chance of developing atherosclerosis (Harja et al., 2009). PMA, a PKC activator, can trigger the transformation of monocytes to macrophages (Salonen et al., 2006). PKC- β and - δ isoforms mediate cholesterol accumulation in PMA-activated macrophages (Ma et al., 2006). The importance of PKC- β in foam cell formation has been proven (Osto et al., 2008). The inhibition of PKC- β 1 presently decreased the expression of scavenger receptor A and suppressed foam cell formation. There are at least 11 different types of PKC isoforms. Therefore, the possibility of other types of isoforms controlling RGS2 expression and foam cell formation cannot be excluded. In addition, the most dominantly expressed PKC isoform in Raw264.7 cells is reported to be PKC- η (Pham et al., 2003). In our research on the effects of various PKC PS inhibitors on RGS2 expression, the contribution of PKC- η was mediated. Among the tested isoforms, PKC- η PS efficiently recovered the decrease in RGS2 expression by LPS and strongly inhibited foam cell formation as well. These results suggest the important relationship between PKC- η and RGS2 in atherosclerosis. Previous studies reported that PKC- η is essential in the differentiation of epithelial tissues (Kashiwagi et al., 2002) and regulates ADP-induced thromboxane generation in platelets (Bynagari et al., 2009), but the function of PKC- η in atherosclerosis development is not clear. However, the importance of PKC- η in cardiovascular pathology has been suggested previously (Kubo et al., 2007). These authors suggested the possibility of PKC- η function in atherosclerosis by showing that PKC- η overexpression in human monocytes caused nitric oxide production and inducible nitric-oxide synthase induction after exposure to endotoxin (Pham et al., 2003). PKC- η PS inhibited foam cell formation and RGS2 down-regulation by PMA, used as a PKC activator. With the treatment of LPS or PMA, the similar effects from PKC- η PS suggest that the function of PKC- η in RGS2 regulation and foam cell formation is a general phenomenon.

Known functions of PLD are varied and include involvement in signaling pathways, membrane fusion, vesicular trafficking, and cytoskeleton dynamics (Kanaho et al., 2009). Treatment with LPS increased PLD activity of macrophages. PLD2 inhibition by siRNA reversed the decrease of RGS2 expression and the increase of foam cell formation by LPS. On the other hand, an increase in the PLD2 level by overexpression intensified LPS-induced RGS2 down-regulation and worsened foam cell formation. These observations suggest that PLD2 activity by LPS weakens RGS2 expression and induces foam cell formation. There are no direct reports describing the relationship between PLD and atherosclerosis development, but some reports have supported the possibility of a PLD role. ATP-binding cassette transporter 1 (ABCA1) transports cellular cholesterol and phospholipids to high-density lipoprotein-apolipoproteins. The factors modulating ABCA1 activity have a profound impact on cholesterol transport and atherosclerosis. Previous reports showed that PLD2 activity, stimulated by unsaturated fatty acids, destabilize this transport by the phosphorylation of ABCA1 (Wang and Oram, 2005). Subsequent studies by the same author revealed ABCA1 destabilization mediated by PKC- η and suggested a model that mediates the PKC- η -PLD2 signaling pathway in the regulation of ABCA1 (Wang and Oram, 2007).

PLD2 is usually constitutively active and is modestly activated by ADP-ribosylation factor and PKC (Koch et al.,

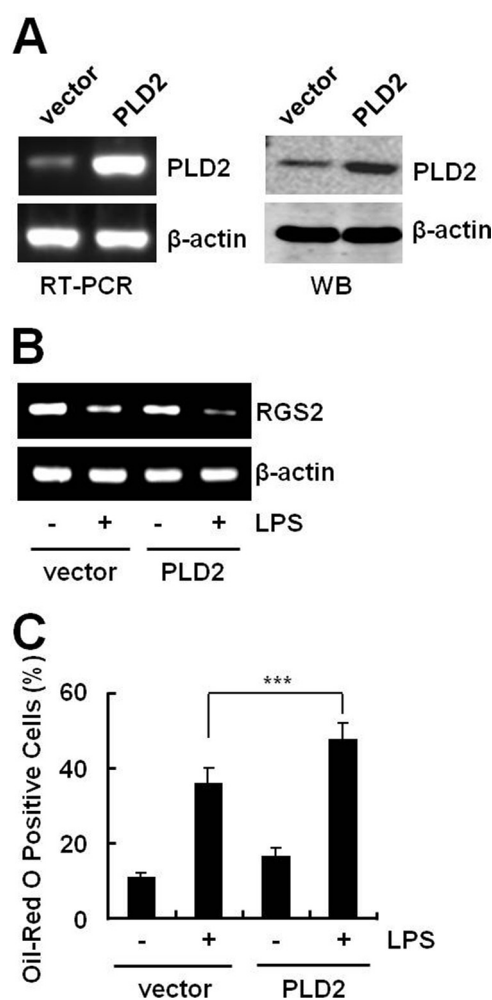


Fig. 8. PLD2 overexpression potentiates LPS-induced RGS2 down-regulation and foam cell formation. Raw264.7 cells were transfected with a pcDNA3 vector-expressing mouse PLD2 or vector alone. A, PLD2 mRNA and protein expression were determined by RT-PCR or Western blot, respectively. B and C, gene-transfected cells were stimulated with or without LPS. RGS2 mRNA and foam cell formation were determined by RT-PCR (B) or Oil-Red O staining (C), respectively. Data represent mean \pm S.D., and experiments were repeated five times in duplicate. ***, $p < 0.001$ by two-tailed Student's *t* test.

2003). How PLD2 activation affects RGS2 expression and foam cell formation has not yet been proven. We hypothesized the roles of second messengers PA and DAG, produced by PLD, and tested these molecules. Treatment with 1,2-dioctanoyl-*sn*-glycerol, a DAG analog, or dioleoyl PA had no effect on RGS2 expression and foam cell formation (data not shown). Further study of PLD2 products controlling LPS-mediated RGS2 down-regulation and foam cell formation is necessary.

References

- Adams LD, Geary RL, Li J, Rossini A, and Schwartz SM (2006) Expression profiling identifies smooth muscle cell diversity within human intima and plaque fibrous cap: loss of RGS5 distinguishes the cap. *Arterioscler Thromb Vasc Biol* **26**:319–325.
- Badimón L, Vilahur G, and Padró T (2009) Lipoproteins, platelets and atherothrombosis. *Rev Esp Cardiol* **62**:1161–1178.
- Barish GD, Atkins AR, Downes M, Olson P, Chong LW, Nelson M, Zou Y, Hwang H, Kang H, Curtiss L, et al. (2008) PPARdelta regulates multiple proinflammatory pathways to suppress atherosclerosis. *Proc Natl Acad Sci USA* **105**:4271–4276.
- Bynagari YS, Nagy B Jr, Tuluc F, Bhavaraju K, Kim S, Vijayan KV, and Kunapuli SP (2009) Mechanism of activation and functional role of protein kinase Ceta in human platelets. *J Biol Chem* **284**:13413–13421.
- Casabona G (1997) Intracellular signal modulation: a pivotal role for protein kinase C. *Prog Neuropsychopharmacol Biol Psychiatry* **21**:407–425.
- Cho H, Park C, Hwang IY, Han SB, Schimel D, Despres D, and Kehrl JH (2008) Rgs5 targeting leads to chronic low blood pressure and a lean body habitus. *Mol Cell Biol* **28**:2590–2597.
- Donaldson JG (2009) Phospholipase D in endocytosis and endosomal recycling pathways. *Biochim Biophys Acta* **1791**:845–849.
- Gu S, Cifelli C, Wang S, and Heximer SP (2009) RGS proteins: identifying new GAPs in the understanding of blood pressure regulation and cardiovascular function. *Clin Sci* **116**:391–399.
- Hahntow IN, Mairuhu G, van Valkengoed IG, Baas F, Alewijnse AE, Koopmans RP, and Michel MC (2009) Are RGS2 gene polymorphisms associated with high blood pressure in an ethnicity- and gender-specific manner? *Am J Hypertens* **22**:80–86.
- Harja E, Chang JS, Lu Y, Leitges M, Zou YS, Schmidt AM, and Yan SF (2009) Mice deficient in PKC beta and apolipoprotein E display decreased atherosclerosis. *FASEB J* **23**:1081–1091.
- Hendriks-Balk MC, Tjon-Atsoi M, Hajji N, Alewijnse AE, and Peters SL (2009) LPS differentially affects vasoconstrictor responses: a potential role for RGS16? *J Physiol Biochem* **65**:71–83.
- Hercule HC, Tank J, Plehm R, Wellner M, da Costa Goncalves AC, Gollasch M, Diedrich A, Jordan J, Luft FC, and Gross V (2007) Regulator of G protein signaling 2 ameliorates angiotensin II-induced hypertension in mice. *Exp Physiol* **92**:1014–1022.
- Heximer SP, Knutsen RH, Sun X, Kaltenbronn KM, Rhee MH, Peng N, Oliveiras-Santos A, Penninger JM, Muslin AJ, Steinberg TH, et al. (2003) Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice. *J Clin Invest* **111**:445–452.
- Kalayoglu MV and Byrne GI (1998) A *Chlamydia pneumoniae* component that induces macrophage foam cell formation is chlamydial lipopolysaccharide. *Infect Immun* **66**:5067–5072.
- Kanaho Y, Funakoshi Y, and Hasegawa H (2009) Phospholipase D signalling and its involvement in neurite outgrowth. *Biochim Biophys Acta* **1791**:898–904.
- Kashiwagi M, Ohba M, Chida K, and Kuroki T (2002) Protein kinase C eta (PKC eta): its involvement in keratinocyte differentiation. *J Biochem* **132**:853–857.
- Kimple AJ, Soundararajan M, Hutsell SQ, Roos AK, Urban DJ, Setola V, Temple BR, Roth BL, Knapp S, Willard FS, et al. (2009) Structural determinants of G-protein alpha subunit selectivity by regulator of G-protein signaling 2 (RGS2). *J Biol Chem* **284**:19402–19411.
- Koch T, Brandenburg LO, Schulz S, Liang Y, Klein J, and Holtt V (2003) ADP-ribosylation factor-dependent phospholipase D2 activation is required for agonist-induced mu-opioid receptor endocytosis. *J Biol Chem* **278**:9979–9985.
- Kubo M, Hata J, Ninomiya T, Matsuda K, Yonemoto K, Nakano T, Matsushita T, Yamazaki K, Ohnishi Y, Saito S, et al. (2007) A nonsynonymous SNP in PRKCH (protein kinase C eta) increases the risk of cerebral infarction. *Nat Genet* **39**:212–217.
- Le TH and Coffman TM (2003) RGS2: a “turn-off” in hypertension. *J Clin Invest* **111**:441–443.
- Lee HY, Yea K, Kim J, Lee BD, Chae YC, Kim HS, Lee DW, Kim SH, Cho JH, Jin CJ, et al. (2008a) Epidermal growth factor increases insulin secretion and lowers blood glucose in diabetic mice. *J Cell Mol Med* **12**:1593–1604.
- Lee JG, Lim EJ, Park DW, Lee SH, Kim JR, and Baek SH (2008b) A combination of Lox-1 and Nox1 regulates TLR9-mediated foam cell formation. *Cell Signal* **20**:2266–2275.
- Ma HT, Lin WW, Zhao B, Wu WT, Huang W, Li Y, Jones NL, and Kruth HS (2006) Protein kinase C beta and delta isoenzymes mediate cholesterol accumulation in PMA-activated macrophages. *Biochem Biophys Res Commun* **349**:214–220.
- Manzur M and Ganss R (2009) Regulator of G protein signaling 5: a new player in vascular remodeling. *Trends Cardiovasc Med* **19**:26–30.
- Oprins JC, van der Burg C, Meijer HP, Munnik T, and Groot JA (2001) PLD pathway involved in carbachol-induced Cl^- secretion: possible role of TNF-alpha. *Am J Physiol Cell Physiol* **280**:C789–C795.
- Osto E, Kouroedov A, Mocharla P, Akhmedov A, Besler C, Rohrer L, von Eckardstein A, Iliceto S, Volpe M, Lüscher TF, et al. (2008) Inhibition of protein kinase Cbeta prevents foam cell formation by reducing scavenger receptor A expression in human macrophages. *Circulation* **118**:2174–2182.
- Paulson KE, Zhu SN, Chen M, Nurmohamed S, Jongstra-Bilen J, and Cybulsky MI (2010) Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis. *Circ Res* **106**:383–390.
- Pham TN, Brown BL, Dobson PR, and Richardson VJ (2003) Protein kinase C-eta (PKC-eta) is required for the development of inducible nitric oxide synthase (iNOS) positive phenotype in human monocytic cells. *Nitric Oxide* **9**:123–134.
- Redig AJ, Sassano A, Majchrzak-Kita B, Katsoulidis E, Liu H, Altman JK, Fish EN, Wickrema A, and Platanius LC (2009) Activation of protein kinase C(eta) by type I interferons. *J Biol Chem* **284**:10301–10314.
- Riddle EL, Schwartzman RA, Bond M, and Insel PA (2005) Multi-tasking RGS proteins in the heart: the next therapeutic target? *Circ Res* **96**:401–411.
- Riekenberg S, Farhat K, Debarry J, Heine H, Jung G, Wiesmüller KH, and Ulmer AJ (2009) Regulators of G-protein signalling are modulated by bacterial lipopeptides and lipopolysaccharide. *FEBS J* **276**:649–659.
- Salonen T, Sareila O, Jalonon U, Kankaanranta H, Tuominen R, and Moilanen E (2006) Inhibition of classical PKC isoenzymes downregulates STAT1 activation and iNOS expression in LPS-treated murine J774 macrophages. *Br J Pharmacol* **147**:790–799.
- Sethu S, Mendez-Corao G, and Melendez AJ (2008) Phospholipase D1 plays a key role in TNF-alpha signaling. *J Immunol* **180**:6027–6034.
- Wang Y and Oram JF (2005) Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a phospholipase D2 pathway. *J Biol Chem* **280**:35896–35903.
- Wang Y and Oram JF (2007) Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a protein kinase C delta pathway. *J Lipid Res* **48**:1062–1068.
- Zhao Y and Natarajan V (2009) Lysophosphatidic acid signaling in airway epithelium: role in airway inflammation and remodeling. *Cell Signal* **21**:367–377.

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