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Physiological role of ROCKs in the cardiovascular system

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Abstract

Rho-associated kinases (ROCKs), the immediate downstream targets of RhoA, are ubiquitously expressed serine-threonine protein kinases that are involved in diverse cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, and gene expression. Recent studies have shown that ROCKs may play a pivotal role in cardiovascular diseases such as vasospastic angina, ischemic stroke, and heart failure. Indeed, inhibition of ROCKs by statins or other selective inhibitors leads to the upregulation and activation of endothelial nitric oxide synthase (eNOS) and reduction of vascular inflammation and atherosclerosis. Thus inhibition of ROCKs may contribute to some of the cholesterol-independent beneficial effects of statin therapy. Currently, two ROCK isoforms have been identified, ROCK1 and ROCK2. Because ROCK inhibitors are nonselective with respect to ROCK1 and ROCK2 and also, in some cases, may be nonspecific with respect to other ROCK-related kinases such as myristolated alanine-rich C kinase substrate (MARCKS), protein kinase A, and protein kinase C, the precise role of ROCKs in cardiovascular disease remains unknown. However, with the recent development of ROCK1- and ROCK2-knockout mice, further dissection of ROCK signaling pathways is now possible. Herein we review what is known about the physiological role of ROCKs in the cardiovascular system and speculate about how inhibition of ROCKs could provide cardiovascular benefits.

Keywords

Rho GTPase; Rho-kinase; vascular endothelium; contraction; actin cytoskeleton; nitric oxide; statins

The small GTP-binding proteins belonging to the Ras, Rho, Rab, and Ran subfamilies act as molecular “on-off” switches that control multiple signaling pathways (24,36,116). Among them, the Rho GTPases are implicated in a variety of physiological functions associated with changes in the actin cytoskeleton, such as cell adhesion, motility, migration, and contraction (77,109). Downstream targets of Rho GTPases that are involved in actin cytoskeletal reorganization include citron kinase, p140mDia, protein kinase N (PKN), p21-activated protein kinase (PAK), rhophilin, and rhotekin. The Rho-associated kinases (ROCKs) were found to be one of the first downstream targets of RhoA (41,60,68). ROCKs are important regulators of cell growth, migration, and apoptosis via control of actin cytoskeletal assembly. They regulate cell contraction through serine-threonine phosphorylation of adducin, ezrin-radixin-moesin (ERM) proteins, LIM kinase, myosin light chain phosphatase (MLCP), and Na/H exchanger (NHE)1 (21,87). For example, ROCKs increase myosin light chain (MLC) phosphorylation through phosphorylating the myosin binding subunit (MBS) on MLCP and inhibiting the activity of MLCP, leading to vascular smooth muscle cell (VSMC) contraction (49,53,102). ROCKs can also phosphorylate and inhibit LIM kinase, which phosphorylates the

cofilin/actindepolymerizing factor complex involved in the depolymerization and severing of actin filaments (64). Thus the overall physiological effects of ROCKs are to enhance actin-myosin association through increasing MLC phosphorylation and preventing actin depolymerization.

In the mammalian system, ROCKs consist of two isoforms, ROCK1 and ROCK2. ROCK1, which is also known as ROK β and p160ROCK, is located on chromosome 18 and encodes a 1,354-amino acid protein (41,68). ROCK2, which is also known as ROK α and often referred to as Rho-kinase, is located on chromosome 12 and contains 1,388 amino acids (87). Y-27632 and fasudil are selective ROCK inhibitors that target their ATP-dependent kinase domains and therefore are equipotent in terms of inhibiting both ROCK1 and ROCK2. However, at higher concentrations, these ROCK inhibitors could also inhibit other kinases such as PKA and PKC (88). Thus current commercially available ROCK inhibitors are not entirely specific for ROCKs compared with other serine-threonine kinases in vivo and cannot distinguish between ROCK1 and ROCK2. Nevertheless, with Y-27632 and fasudil, some evidence is emerging regarding the physiological role of ROCKs, particularly in cardiovascular disease (38,94, 100).

The emergence of ROCKs in cardiovascular disease coincides with the growing acceptance of the so-called “pleiotropic” or cholesterol-independent effects of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors or statins. In particular, accumulating evidence indicates that endothelial nitric oxide synthase (eNOS), which is an important mediator of endothelial function, is regulated by the RhoA/ROCK pathway (55,58,71,110,124). For example, inhibition of RhoA geranylgeranylation by statins decreases membrane GTP-bound active RhoA and subsequent ROCK activity, leading to the upregulation and activation of eNOS (58). Similarly, direct inhibition of the RhoA/ROCK pathway by ROCK inhibitors or dominant-negative mutant of RhoA has been shown to increase eNOS expression (88,124). Because eNOS is protective of the vasculature, these studies suggest that the RhoA/ROCK pathway may play a significant pathophysiological role in various aspects of cardiovascular disease. Indeed, ROCKs are activated in patients with cardiovascular disease or its associated risk factors, suggesting that ROCK inhibition may be a useful therapeutic target in the cardiovascular system (50,66,79,99).

REGULATION OF R_{HO}A

The Rho GTPases, Rho, Rac, and Cdc42, are involved in the regulation of vital cellular functions such as cytoskeleton organization, motility, proliferation, adhesion, apoptosis, and gene expression (10,76,77,109). In particular, RhoA regulates the assembly of actin stress fibers (85), whereas Rac and Cdc42 regulate actin polymerization and cellular protrusions (86). As with other Rho GTPases, RhoA functions as a molecular switch, cycling between an active GTP-bound form and an inactive GDP-bound form. The cycle between active and inactive forms is controlled by several regulatory proteins, including guanine dissociation inhibitors (GDIs), guanine nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPs) (24,73,95,116). Rho GEFs activate RhoA by catalyzing the exchange of GDP to GTP, whereas Rho GAPs inactivate RhoA by dephosphorylating GTP to GDP. The GDIs prevent the membrane translocation of RhoA from the cytosol. To date, numerous proteins have been identified as potential Rho GEFs and GAPs. In addition, numerous proteins function as downstream effectors of Rho GTPase, including ROCKs, suggesting the central importance of these proteins in cellular homeostasis (24).

ISOFORM AND STRUCTURE OF ROCKs

ROCK1 and ROCK2 are protein serine-threonine kinases that share 45–50% homology with other actin cytoskeletal kinases such as citron kinase, myotonic dystrophy kinase (DMPK),

and myotonic dystrophy-related cdc42-binding kinase (MRCK) (87). ROCKs consist of an amino-terminal kinase domain, followed by a mid-coiled-coil-forming lesion containing a Rho-binding domain (RBD), and a carboxyterminal cysteine-rich domain (CRD) located within the pleckstrin homology (PH) motif. ROCK1 and ROCK2 share overall 65% homology in amino acid sequence and 92% homology in their kinase domains. ROCK1 and ROCK2 are ubiquitously expressed in mouse tissues from early embryonic development to adulthood. ROCK1 mRNA is preferentially expressed in lung, liver, spleen, kidney, and testis, whereas ROCK2 mRNA is highly expressed in the heart and brain (22,75,121). In addition, immunolocalization and cell fractionation studies have shown that ROCK2 is distributed mainly in the cytoplasm (60,68). When activated by GTP-bound RhoA, ROCK2 translocates from cytoplasm to membranes. Indeed, a small amount of ROCK2 has been found in the membrane fraction, and some immunostaining is detectable at the cell periphery or membranes of growing cells (49). In contrast, little is known regarding the intracellular localization of ROCK1. However, a recent study suggests that ROCK1 may be colocalized to centrosomes (15).

Recently, ROCK1-deficient (ROCK1^{-/-}) and ROCK2-deficient (ROCK2^{-/-}) mice have been generated (98,111). Both mutant ROCK mice were generated by knocking in a *lacZ* reporter gene. In the ROCK2^{-/-} mouse, LacZ staining was observed in many locations throughout the embryo, including the dorsal root ganglions, heart, liver, and umbilical blood vessels. In the placenta, strong LacZ staining was also observed in the labyrinth layer. In the ROCK1^{-/-} mouse, LacZ staining was also detected throughout the embryo, including the aorta, dorsal root ganglia, heart, skin, and umbilical blood vessels. ROCK2^{-/-} embryos are embryonically lethal because of placental dysfunction and intrauterine growth retardation caused by thrombus formation in the labyrinth layer of the placenta. ROCK1^{-/-} mice, however, exhibit a completely different phenotype compared with ROCK2^{-/-} mice. They die postnatally because of an abnormality of filamentous actin accumulation that leads to impairment in umbilical ring closure. The ROCK1^{-/-} mice also exhibit eyes open at birth (EOB) due to disorganization of actin filaments in the epithelial cells of the eyelid. Interestingly, a substantial proportion of ROCK2^{-/-} mouse embryos exhibit EOB and omphalocele, indicating that both ROCK1 and ROCK2 may play important roles in mediating eye and umbilical ring closure. Thus further studies regarding the specific roles of ROCK1 and ROCK2 with haploinsufficient or conditional ROCK1- and ROCK2-knockout mice are needed. Recently, it was reported that haploinsufficient ROCK1-knockout mice are viable and healthy at birth and exhibit decreased cardiac fibrosis, but not hypertrophy, in response to angiotensin II infusion (90).

REGULATION OF ROCKs

The carboxy-terminal regions of ROCKs, which contain the PH domain and the RBD, serve as an autoregulatory inhibitor of the amino-terminal kinase domain (4). The interaction of GTP-bound RhoA to the RBD of ROCKs increases ROCK activity through derepression of the carboxy-terminal RBD-PH domains on the amino-terminal kinase domain, leading to an active “open” kinase conformation. This open conformation could also be caused by the binding of arachidonic acid to the PH domain (28) or by cleavage of the carboxy terminus by caspase-3 (18,96). Such a regulatory mechanism could also be found with DMPK and MRCK activation (4,13) and is consistent with studies showing that overexpression of various carboxy-terminal constructs of ROCKs or kinase-defective forms of full-length ROCKs function as dominant-negative ROCK mutants (3,41,59). Interestingly, ROCKs could also be activated independently of RhoA through amino-terminal transphosphorylation caused by protein oligomerization (13,113). Other small GTP-binding proteins such as Gem and Rad specifically regulate either ROCK1- or ROCK2-mediated cell rounding and neurite retraction (120). Although further studies are needed to uncover the precise mechanism, these results indicate that ROCK1 and ROCK2 may have different physiological roles in cellular function.

DOWNSTREAM TARGETS OF ROCKs

ROCKs phosphorylate various targets and mediate a broad range of cellular responses that involve the actin cytoskeleton in response to GTP-bound RhoA by activators of RhoA such as lysophosphatidic acid (LPA) or sphingosine-1 phosphate (S1P), which stimulate Rho GEFs. ROCKs control assembly of the actin cytoskeleton and cell contractility via phosphorylation of various downstream target proteins, including MBS on MLCP, MLC, ERM proteins, protein LIM kinase, and adducin (Fig. 1). The consensus amino acid sequences for phosphorylation are R/KXS/T or R/KXXS/T (R, arginine; K, lysine; X, any amino acid; S, serine; T, threonine) (46,106). Of interest, ROCKs can be autophosphorylated, suggesting that the function of ROCKs may be dependent in part on autoregulation (41,60). MBS on MLCP is an important downstream target protein of ROCKs. Phosphorylation of MBS on MLCP by ROCKs leads to the phosphorylation of MLC and subsequent contraction of VSMCs (103). MLCP holoenzyme is composed of three subunits: a catalytic subunit (PP1 δ), a MBS composed of a 58-kDa head and a 32-kDa tail region, and a small noncatalytic subunit, M21. ROCK2 phosphorylates MBS at Thr⁶⁹⁷, Ser⁸⁵⁴, and Thr⁸⁵⁵ (46). The functional significance of MBS phosphorylation at Ser⁸⁵⁴, however, is not known. Phosphorylation of Thr⁶⁹⁷ or Thr⁸⁵⁵ attenuates MLCP activity and, in some instances, the dissociation of MLCP from myosin (27,119). In addition, MLC is one of the major downstream target proteins of ROCKs. ROCK2 phosphorylates Ser¹⁹ of MLC, the same residue that is phosphorylated by MLC kinase. Thus ROCK2 may increase cellular contractility via dual effects on MLC kinase and MLCP. Indeed, ROCK2 can alter the sensitivity of VSMC contraction in response to changes in Ca²⁺ concentration (5). ROCKs also phosphorylate ERM proteins, which serve as cross-linkers between actin filaments and membrane proteins at the cell surface. ROCK-mediated phosphorylation of ERM proteins, namely, Thr⁵⁶⁷ of ezrin, Thr⁵⁶⁴ of radixin, and Thr⁵⁵⁸ of moesin, leads to the disruption of the head-to-tail association of ERM proteins and actin cytoskeletal reorganization (69). However, it is not known whether phosphorylation of MBS on MLCP or ERM proteins is specific to ROCK isoforms. Nevertheless, ROCK1 phosphorylates LIM kinase-1 at Thr⁵⁰⁸ and LIM kinase-2 at Thr⁵⁰⁵, which inhibits cofilin-mediated actin filament disassembly by phosphorylating cofilin (64,81,106). Adducin, which is a membrane skeletal protein that associates with and promotes the association of spectrin with F-actin, is also a downstream target of ROCK2 (32,48). Adducin is localized at cell-cell contact sites and is thought to participate in the assembly of the spectrin-actin network by capping the fast-growing ends of actin filaments and recruiting spectrin to the filament ends. The phosphorylation of α -adducin by ROCK2 enhances the binding activity of α -adducin to F-actin, thereby increasing the contractile response.

Although the homology of the kinase domain between ROCK1 and ROCK2 is 92%, ROCK1 and ROCK2 may serve different functions and may have different downstream target proteins. Most of the downstream targets of ROCKs are cellular proteins associated with the regulation of the actin cytoskeleton. The difficulty with studying the roles of ROCKs is due to the lack of specific inhibitors that can distinguish not only the roles of ROCK isoforms but also the roles of ROCKs from other serine-threonine kinases, such as PKA, PKC, and citron kinase. Therefore, a genetic approach using conditional or haploinsufficient ROCK1- and ROCK2-knockout mice offers the best opportunity to dissect the functions of ROCK1 and ROCK2.

FUNCTIONS OF ROCKs

ROCKs are important regulators of cellular apoptosis, growth, metabolism, and migration via control of the actin cytoskeletal assembly and cell contraction (87). ROCK1 and ROCK2 are differentially expressed and regulated in specific tissues. For instance, only ROCK1 is cleaved by caspase-3 during apoptosis (18,96). On the other hand, smooth muscle-specific basic calponin is phosphorylated only by ROCK2 (44). Furthermore, ROCK1 is highly expressed

ubiquitously, whereas ROCK2 is preferentially expressed in cardiac and brain tissues (22,75, 121). With the deletion of ROCK1 and ROCK2 alleles in mice, distinguishing the physiological roles of ROCK isoforms is now feasible.

Stimulation of tyrosine kinase and G protein-coupled receptors recruits and activates Rho GEFs, leading to activation of RhoA, the direct upstream activator of ROCKs (37,52). ROCKs are pivotal downstream effectors of RhoA in regulating the actin cytoskeleton. Inhibitors of ROCKs such as Y-27632 and fasudil, or overexpression of dominant-negative mutants of ROCK, cause loss of actin stress fibers and focal adhesion complexes (41,114). This is predominantly due to the phosphorylation and inhibition of MLCP by ROCKs, which increase MLC phosphorylation and cellular contraction by facilitating interaction of myosin with F-actin. Thus ROCKs regulate cell polarity and migration through cellular contractions, protrusions, and focal adhesions. By affecting tight and adherens junctions through actin cytoskeletal contractions, ROCKs can also regulate macrophage phagocytic activity and endothelial cell permeability (122,123).

ROCKs could regulate other cellular functions independent of their effects on the actin cytoskeleton. For example, ROCKs inhibit insulin signaling via direct phosphorylation of insulin receptor substrate-1 (IRS-1). Phosphorylation of IRS-1 uncouples the insulin receptor from phosphatidylinositol 3-kinase (PI3K) and potentially Glut4 activation (26). ROCKs could also regulate cell size by enhancing IGF-induced cAMP response element binding protein (CREB) phosphorylation (104), which may be the underlying mechanism by which ROCK inhibitors reduce cardiac hypertrophy (80,83). However, a recent study in haploinsufficient ROCK1^{+/-} mice indicates that ROCK1 is required for the development of cardiac fibrosis, not hypertrophy (90). Interestingly, ROCKs may be involved in tissue differentiation from adipocytes to myocyte. In p190-B Rho GAP-deficient mice in which the RhoA/ROCK pathway is chronically activated, there is a defect in adipogenesis, with a predilection toward myogenesis (104,105). Treatment of p190-B Rho GAP-deficient mice with Y-27632 restores normal adipogenesis (105), suggesting that ROCKs are involved in the regulation of the myocyte differentiation.

REGULATION OF eNOS BY ROCKs

The vascular endothelium secretes vasoactive substances such as NO, prostacyclin, endothelium-derived hyperpolarizing factor, and endothelins (63,84,117). In particular, endothelium-derived NO plays an important role in the regulation of vascular tone, inhibition of platelet aggregation, suppression of smooth muscle cell proliferation, and prevention of leukocyte recruitment to the vessel wall (115,118). Increased bioavailability of NO is, in part, dependent on increased expression and activity of eNOS as well as on decreased inactivation of NO by reactive oxygen species (ROS), specifically the superoxide anion (Fig. 2). Although various conditions and factors such as laminar shear stress (19), oxygen tension (62), and TGF- β 1 (93) can regulate eNOS expression at the transcriptional level, eNOS expression can be also regulated at the posttranscriptional level. For example, chronic hypoxia (55), TNF- α (125), thrombin (25), oxidized LDL (61), and cellular proliferation (6,31) are known to decrease eNOS mRNA stability. Chronic hypoxia and cellular proliferation are known to activate RhoA and ROCKs (110). In contrast, statins, which have been shown to increase eNOS mRNA stability, inhibit RhoA geranylgeranylation (58) and ROCK activity (110). Thus RhoA/ROCK inversely regulates eNOS expression through alteration in eNOS mRNA stability.

A recent study has shown that 3'-untranslated region complexes (3'-UTR) of eNOS mRNA, which consist of a UC-rich region and a AU-rich region, regulate the stability of eNOS mRNA by multiple cytosolic proteins binding to the 3'-UTR of eNOS mRNA (42). For example, cytosolic proteins, which bind to the eNOS mRNA 3'-UTR after TNF- α stimulation, lead to

the reduction of eNOS mRNA half-life in human endothelial cells (2,125). Because the RhoA/ROCK pathway and the actin cytoskeleton also regulate eNOS mRNA half-life (88,110), it is likely that cytosolic proteins, which bind to the 3'-UTR eNOS mRNA, are actin cytoskeleton-associated proteins.

The RhoA/ROCK pathway may also be important in regulating eNOS activity. The activity of eNOS is dependent on intracellular Ca^{2+} concentration (11). There is increasing evidence that eNOS activity is also partly dependent on eNOS-interacting proteins and posttranslational modification of eNOS (11,20). For example, eNOS activity could be regulated in part through association with caveolin-1 (35), porin (107), dynamin (12), G protein-coupled receptors (43), heat shock protein 90 (91), and various protein kinases (14,23,30,34). In addition, the regulation of eNOS activity can occur via eNOS phosphorylation. The phosphorylation of Ser¹¹⁷⁷ of eNOS leads to the rapid activation of eNOS by fluid shear stress, insulin, estrogen, bradykinin, and VEGF (9,23,30,47,54). Interestingly, inhibition of RhoA or ROCKs leads to the rapid activation of PI3K/Akt and phosphorylation of eNOS (71,124), suggesting the potential role of ROCKs in regulating eNOS activation in addition to eNOS expression. Although the phosphorylation of Ser¹¹⁷⁷ leads to increased eNOS activity, the phosphorylation of Thr⁴⁹⁵ is associated with a decrease in eNOS activity (30). In addition, although other phosphorylation sites have been identified on eNOS proteins, such as Ser¹¹⁴, Ser⁶³³, and other tyrosine residues, the precise functional roles of these phosphorylation sites remain to be determined (29,108).

ROCKs IN CARDIOVASCULAR DISEASE

Selective ROCK inhibitors such as fasudil have been shown to prevent cerebral vasospasm after subarachnoid hemorrhage. Furthermore, another ROCK inhibitor, Y-27632, has been shown to inhibit the development of atherosclerosis and arterial remodeling after vascular injury (65,94). We recently showed (89) that ROCK activity is involved in the expression of plasminogen activator inhibitor-1 (PAI-1) mediated by hyperglycemia, indicating that the RhoA/ROCK pathway may function as a key regulator of cardiovascular injury in patients with diabetes mellitus. Thus there is increasing evidence that inhibition of the RhoA/ROCK pathway may be beneficial in cardiovascular diseases and could be a potential therapeutic target (Fig. 1). Specifically, the RhoA/ROCK pathway has been shown to be involved in angiogenesis (40,51), atherosclerosis (65), cerebral and coronary vasospasm (45,92), cerebral ischemia (112), erectile dysfunction (8,17), glomerulosclerosis (78), hypertension (114), myocardial hypertrophy (38), myocardial ischemia-reperfusion injury (7,124), neointima formation (70, 94), pulmonary hypertension (1), and vascular remodeling (72). In many of these studies, the underlying protective mechanism of ROCK inhibition was mediated by the upregulation of eNOS. However, in other studies, the protective effects of ROCK inhibition were mediated by the acute activation of eNOS via the PI3K/Akt pathway (124). Moreover, ROCKs may also play a role in Alzheimer disease (74), bronchial asthma (16), cancers (101), demyelinating diseases (74), glaucoma (39), and osteoporosis (82). However, it remains to be determined how ROCKs are involved in these disease states.

CLINICAL IMPLICATIONS OF ROCKs

Inhibition of ROCKs by fasudil leads to beneficial effects in patients with systemic hypertension (66), pulmonary hypertension (33), vasospastic angina (67), stable effort angina (99), stroke (97), and chronic heart failure (50). Indeed, perhaps many of the so-called pleiotropic effects of statins are mediated by ROCK inhibition (55–58,110,124). Thus assessing ROCK activity in patients undergoing statin therapy may provide greater insights into the mechanism underlying statin nonresponders in statin clinical trials. Furthermore, the development of ROCK inhibitors could be useful therapeutically in patients without elevated

cholesterol levels or in cardiovascular conditions such as ischemic stroke and heart failure in which elevated serum cholesterol level is not a risk factor.

In conclusion, there is growing evidence that the RhoA/ROCK pathway plays an important pathophysiological role in cardiovascular diseases and that inhibition of ROCKs by ROCK inhibitors or statins may be beneficial. To date, a great number of cellular and physiological functions are mediated by ROCKs, and ROCK activity is often elevated in disorders of the cardiovascular system. Thus inhibition of ROCKs may be a potential therapeutic target in reducing cardiovascular disease. However, a greater understanding of the physiological role of each ROCK isoform in the cardiovascular system and the development of isoform-specific inhibitors are needed to resolve the specificity and safety of ROCK inhibitors.

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Fig. 1.

Role of Rho-associated kinases (ROCKs) in cardiovascular disease. Activated ROCKs, mediated through GTP-binding active RhoA, phosphorylate various downstream targets of ROCKs. Most of these processes lead to cardiovascular injury, including hypertension, pulmonary hypertension, erectile dysfunction, atherosclerosis, vasospasm, cerebral ischemia, neointima formation, myocardial hypertrophy, ischemia-reperfusion injury, and so forth. MLC, myosin light chain; MLCP, MLC phosphatase; ERM, ezrin-radixin-moesin proteins.

Fig. 2.

Regulation of endothelial nitric oxide synthase (eNOS) by ROCKs. Statins decrease the synthesis of isoprenoids, resulting in the indirect inhibition of the RhoA/ROCK pathway. The indirect inhibition of the RhoA/ROCK pathway by statins as well as the direct inhibition of ROCKs by ROCK inhibitors leads to increased eNOS expression and eNOS activity by stabilization of eNOS mRNA or phosphorylation of eNOS, respectively. Angiotensin II; ET-1, endothelin 1; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HSP, heat shock protein; ox-LDL, oxidized LDL; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; PP, pyrophosphate; GG, isoprenoid geranylgeranyl.