Small G Proteins as Novel Therapeutic Targets in Cardiovascular Medicine

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Small G proteins are implicated in regulation of endothelial function, smooth muscle cell contraction, proliferation, and migration, as well as cardiomyocyte hypertrophy. Targeting small G proteins and their downstream signaling could constitute promising therapeutic approaches in cardiovascular disorders such as atherosclerosis, restenosis, hypertension, vasospasm, and cardiac hypertrophy.

In contrast to the heterotrimeric G proteins that are coupled to the seven-transmembrane-domain receptors, the small G proteins (also called small GTP-binding proteins) are monomeric proteins with a low molecular weight of 20–40 kDa and have intrinsic GTP-hydrolyzing activity. Therefore, they are also called GTPases (9). More than 100 small G proteins have been identified and comprise a superfamily. They are structurally divided into at least five subfamilies: Ras (Ras, Rap, Rad, Ral, Rln, and Rlt), Rh (Rho, Rac, Cdc42, and Rnd), and Rap (9). The small G proteins have a wide spectrum of functions, including regulation of gene expression, cell proliferation, cell migration, cytoskeletal rearrangement, intracellular vesicle trafficking and protein nucleocytoplasmic transportation.

General biochemical features of small G proteins

The small G proteins act as a molecular switch (9) and cycle between inactive GDP-bound and active GTP-bound forms (Fig. 1). The exchange of GDP from the inactive form for GTP is, on the one hand, stimulated by protein regulators, called guanine nucleotide exchange factors, upon stimulation by various agents or hormones (i.e., growth factors, cytokines, integrins, and G protein-coupled receptor ligands). On the other hand, the GDP/GTP exchange reaction is inhibited by another type of regulator, namely GDP-dissociation inhibitors, which prevents GDP dissociation from the GDP-bound form and keeps the small G protein inactive. Moreover, the small G proteins have slow intrinsic GTPase activity that can be accelerated by a family of GTPase-activating proteins, which returns the proteins to the GDP-bound state (Fig. 1).

Activation of small G proteins requires translocation from the cytoplasm to the cell membrane, where exchange of bound GDP for GTP occurs. Isoprenylation of small G proteins (defined as stable covalent modification of the proteins with two types of isoprenoids: either farnesyl or geranylgeranyl, catalyzed by protein farnesyl transferase or geranylgeranyl transferase, respectively) is the initial and necessary event for protein membrane targeting (Ref. 14; Fig. 1). Ras membrane translocation depends on farnesylation, whereas Rho membrane translocation depends on geranylgeranylation. Of particular clinical importance is the growing evidence showing that cholesterol-lowering drugs (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors or statins) block synthesis of isoprenoid intermediates, including geranylgeranyl pyrophosphate and farnesyl pyrophosphate, via inhibition of the l-mevalonic acid pathway. By preventing the synthesis of the isoprenoids, statins block small G protein function and exert many beneficial effects in prevention of cardiovascular diseases beyond their cholesterol-lowering properties (pleiotropic effects of statins; Ref. 14).

Multiple downstream effectors of small G proteins, some of them protein kinases, have been identified. They serve as important signal transduction molecules, transmitting extracellular signals into the cells and regulating multiple cellular functions (9). The Ras and Rh small G proteins are the most investigated molecules in the cardiovascular system. In this review, we will discuss the recent findings on the (physio)pathological role of small G proteins, focusing mainly on Ras and Rh and their effectors in cardiovascular disease. The perspectives of the small G proteins and the regulated signal transduction pathways as cardiovascular therapeutic targets are discussed.

Physiological role of small G proteins in the cardiovascular system

Endothelial cells. Under physiological conditions, endothelial cells are in the flat state, adhere tightly to each other, and serve as a dynamic barrier between the circulating blood and underlying vascular tissue. Moreover, endothelial cells play an important role in regulation of vascular contractility and integrity of vascular structure by releasing vasoactive substances, including relaxing factors and contracting factors (Fig. 2). One of the most important endothelium-derived relaxing factors is nitric oxide (NO), synthesized via endothelial NO synthase (eNOS) from l-arginine. NO is a potent vasodilator and platelet inhibitor. It also inhibits proliferation and migration of smooth muscle cells. On the other hand, endothelial cells also produce potent vasoconstrictors such as endothelin-1 (ET-1), which is produced from preproET-1 via sequential cleavage by a furin-like protease and ET-converting enzyme-1 (ECE-1; Fig. 2). Research in past years provided evidence for...
a negative regulatory effect of the small G protein RhoA and one of its downstream effectors, Rho kinase (ROCK), on eNOS gene expression by destabilizing eNOS mRNA and provided evidence for a positive regulatory effect on preproET-1 transcription (2, 6). Moreover, p42/44mapk, which lies downstream of the small G protein Ras and is activated by the Ras/Raf/MAPK kinase (MEK) cascade, upregulates ECE-1 gene expression in endothelial cells (2). Therefore, activation of Rho-ROCK and Ras-Raf-MEK-p42/44mapk pathways by cardiovascular risk factors, for example oxidized LDL and thrombin, causes imbalance of endothelial function favoring vascular occlusion. Endothelial cells and smooth muscle cells also express Rac1, a small G protein of the Rho subfamily and a component of NAD(P)H oxidase, the major source of reactive oxygen species (ROS) in the vascular tissue. Activation of Rac1 under pathological conditions produces ROS such as superoxide anion (O$_{2}^{-}$/G10$^{2-}$), which reacts with NO to generate a potent oxidant, peroxynitrate (OONO$^{-}$/G10$^{2-}$), and decreases bioavailability of NO, leading to endothelial dysfunction. Another important role of small G proteins, mainly RhoA and ROCK, in endothelial cells is regulation of endothelial barrier dysfunction by enhancing myosin light chain (MLC) phosphorylation, a mechanism similar to smooth muscle cell contraction (see below and Fig. 3).

**Smooth muscle function.**

**Smooth muscle contractility.** The major regulatory mechanism of smooth muscle contraction is the phosphorylation state of the 20-kDa MLC, which is controlled by a balanced activity of two enzymes, namely the Ca$^{2+}$-calmodulin-dependent MLC kinase (MLCK) and the Ca$^{2+}$-independent MLC phosphatase (MLCP) (Fig. 3). On stimulation by vasoconstrictor hormones, intracellular Ca$^{2+}$ concentration increases and activation of MLCP occurs. Activated MLCK phosphorylates MLC and produces smooth muscle contraction. In addition, MLCP will be phosphorylated, resulting in inactivation of the enzyme (which cannot dephosphorylate the MLC), leading to hyperphosphorylation of MLC, allowing an increase in sensitivity of the smooth muscle to a constant Ca$^{2+}$ concentration (12). Smooth muscle MLCP is composed of three subunits: the catalytic subunit of type 1 protein phosphatase, the regulatory subunit myosin phosphatase target protein-1 (MYPT-1), and a small noncatalytic subunit M20 of unknown function. MYPT-1 contains various phosphorylation sites, and phosphorylation of MYPT-1 leads to inactivation of MLCP and therefore to enhanced smooth muscle sensitivity to Ca$^{2+}$. The role of small G proteins in smooth muscle cell contraction is best investigated with Rho. Studies demonstrate that RhoA plays an important role in increased Ca$^{2+}$ sensitivity of smooth muscle cells via activation of ROCK, which directly phosphorylates MYPT-1 (12). Moreover, a new endogenous myosin phosphatase-associated kinase has been characterized in rabbit bladder smooth muscle cells. This enzyme, similar to ROCK, enhances Ca$^{2+}$ sensitivity by directly phosphorylating MYPT-1, and it is therefore referred to as MYPT-1 kinase. It is interesting to investigate whether MYPT-1 kinase plays a role in vascular constriction in the cardiovascular system.

In addition to Rho/ROCK, PKC also enhances smooth muscle sensitivity to Ca$^{2+}$ involving an inhibitor protein for MLCP called CPI-17, that is only expressed in smooth muscle. Phosphorylation of CPI-17 at Thr$^{38}$ by PKC converts this protein to a potent inhibitor of MLCP (12). The CPI-17-MLCP signaling pathway may be as significant as the Rho-ROCK-MLCP pathway in G protein-coupled agonist-induced Ca$^{2+}$ sensitization of smooth muscle contraction. It should be mentioned that CPI-17 can also be phosphorylated at Thr$^{38}$ by ROCK or protein kinase N (PKN), another RhoA downstream effector. Phosphorylation of CPI-17 by PKN dramatically increases...
The Ras small G protein is well known to promote cell proliferation and migration at least in part via the Rho-ROCK pathway. One of the mechanisms of Rho-induced cell proliferation and migration may be through reduction of expression of the cyclin-dependent kinase inhibitors such as p21Cip1 or p27Kip1, most likely via phosphoinositol 3-kinase (PI3K), as demonstrated recently in rat aortic smooth muscle cells (11). Indeed, direct inhibition of Rho by Clostridium botulinum C3 transferase, which ADP-ribosylates and inactivates Rho, or by a dominant-negative Rho mutant increases p27Kip1 and inhibits retinoblastoma protein hyperphosphorylation and smooth muscle cell proliferation in response to growth stimuli. In contrast to this observation and the results from rat smooth muscle cells, inhibition of Rho by statins did not prevent PDGF-induced p27Kip1 down-regulation in human smooth muscle cells (17). This may be due to the species difference, and it would be interesting to investigate the exact mechanisms by which Rho regulates smooth muscle cell cycle progression in humans.

The Ras small G protein is well known to promote cell proliferation at least in part via activation of the Raf-MEK-ERK cascade. Ras has also been shown to have a synergistic effect with Rho to stimulate smooth muscle cell growth. In hamster embryo fibroblasts, Ras activates RhoA and ERKs; the former downregulates p27Kip1 via cyclin E/Cdk2 and the latter stimulates cyclin D expression (7). This result may explain the synergistic effect between Ras and Rho to stimulate cell growth. Whether this is also true for vascular smooth muscle cells remains to be investigated.

Small G proteins as therapeutic targets for cardiovascular diseases

Atherosclerosis. Manifested atherosclerosis is associated with a decreased bioavailability of NO, either due to enhanced NO breakdown by increased ROS or decreased eNOS gene expression. Various atherogenic factors such as oxygenated LDL, IL-1β, TNF-α, and thrombin stimulate Rho or ROCK, leading to eNOS downregulation, increased endothelial barrier permeability, and enhanced vasoconstriction and smooth muscle cell proliferation or migration. They also stimulate the Ras-Raf-MEK-ERK pathway, which may synergize with Rho to promote smooth muscle cell proliferation and/or migration. The major evidence for the therapeutic potential of inhibiting small G proteins arises from experimental and clinical studies with statins that protect against atherosclerosis beyond cholesterol lowering (14). Experimental evidence suggests that statins upregulate eNOS gene expression and inhibit smooth muscle cell proliferation/migration via inhibition of the Rho-ROCK pathway. Inhibition of ROCK by statins may also contribute to the statins’ beneficial effects. A very recent study confirmed the potential role of Ras in pathogenesis of atherosclerotic plaques in apolipoprotein E knockout mice (4). Moreover, statins also reduce ROS generation, possibly through inhibition of membrane translocation of Rac1 (14), which is required for the assembly and activation of the superoxide-forming NAD(P)H oxidase. Interestingly, inhibition of ROCK either by specific inhibitors or by dominant-negative ROCK mutant gene transfer not only prevents vasospasm but also induces regression of coronary atherosclerosis in a pig model treated with IL-1β (10). Whether this approach also works with other atherosclerotic models of humans needs further investigation.

Restenosis. Restenosis after balloon angioplasty or stenting still remains a serious limitation of successful therapy for patients with atherosclerosis. Smooth muscle cell proliferation and/or migration from the media into the intima in response to local injury play an essential role in the process. A number of experimental studies demonstrate that targeting Ras either pharmacologically or genetically prevents neointimal formation in rat carotid artery or pig coronary artery after angioplasty in vivo, when the drugs or negative Ras mutants are delivered locally at the site of vascular injury (16). Recent research has been interested in the potential role of ROCK in restenosis, as demonstrated in pig coronary artery and rat carotid artery, in which inhibition of ROCK by the specific inhibitor Y-27632 or overexpression of dominant-negative ROCK mutant suppresses neointimal formation. These results from animal models may provide a novel therapeutic target for treatment of restenosis in humans.
**Hypertension.** Hypertension is a cardiovascular disorder characterized by increased peripheral vascular resistance and/or vascular structural remodeling. Decreased endothelial NO bioavailability due to excessive ROS production is considered to play an important role in increased vascular tone in hypertension. More recently, rapidly growing evidence from hypertensive animal models suggests that Rho and its downstream effector ROCK are essential for enhanced smooth muscle contractility in hypertension. A greater RhoA expression and an enhanced RhoA activity have been observed in aortas of hypertensive rats, such as N\(^\text{N}\)-nitro-L-arginine methyl ester-induced hypertension or genetic spontaneously hypertensive rats (SHR). Interestingly, the enhanced RhoA expression and activity was already observed in young SHR rats before the onset of hypertension (11). These results suggest that both genetic factors and blood pressure can upregulate RhoA expression. Moreover, Y-27632, the specific ROCK inhibitor, markedly decreases blood pressure in various hypertensive but not normotensive animals (15). Similarly, the ROCK inhibitor induces a more pronounced increase in forearm blood flow in hypertensive than normotensive subjects, implicating an increased Rho/ROCK activity in hypertension. The enhanced Rho/ROCK activity may also contribute to the vascular remodeling in hypertension due to enhanced smooth muscle cell proliferation and/or migration, since RhoA expression and activity is correlated with reduced cell cycle inhibitor p27Kip1 expression and enhanced DNA synthesis in SHR rat smooth muscle cells (11). It is interesting to note that patients treated with statins that inhibit RhoA have a lower incidence of high blood pressure, probably due to an enhanced eNOS gene expression, or a decreased angiotensin II AT\(_1\) receptor expression on smooth muscle cells or inhibition of ROS production via blockade of Rac1. Moreover, treatment of animals with farnesyl transferase inhibitors, which inhibit Ras GTPase activity, have also been shown to attenuate angiotensin II-induced hypertension and ameliorate pathological vascular structural changes in rats, indicating the potential role of Ras in pathogenesis of hypertensive vascular lesions.

**Vasospasm.** Vasospasm plays an important role in ischemic stroke and stable coronary angina. Although imbalanced endothelium-dependent responses have been suggested to play an important role in enhanced vascular smooth muscle contraction, recent data imply that activation of ROCK in smooth muscle cells may play a more prominent role in the sustained constriction and vasospasm observed in various cardiovascular diseases via the mechanisms described above (Ref. 8; Fig. 3). Therefore, inhibition of the Rho-ROCK pathway may provide a promising approach to prevent vasospasm in various cardiovascular diseases.

**Myocardial hypertrophy.** Cardiac hypertrophy is an initial pathophysiological adaptive response to mechanical overload such as increase in blood pressure and hormonal stimuli (i.e., ET-1, angiotensin II, or catecholamines). The role of small G proteins in cardiac myocyte hypertrophy has attracted much attention in recent years. Experiments with cultured neonatal rat cardiomyocytes imply that Ras, Rho, and Rac1 are all involved in cellular hypertrophy (1). The downstream effectors of Ras, RhoA, or Rac1, which mediate myocyte hypertrophy, have not been completely elucidated. The Raf-MEK-ERK pathway, PI3K, and Ral-GDP dissociation stimulator are implicated in the effects of Ras, whereas ROCK and JNK may participate in the effects mediated by RhoA or Rac1. In addition, a cross-talk between the small G proteins may also exist, and they may cooperate with each other to promote myocyte hypertrophy. Inhibition of Ras, Rho, or Rac1 either by specific inhibitors or by statins has been shown to be effective in prevention of rat cardiac myocyte hypertrophy in response to angiotensin II in vitro or in vivo. These experimental results suggest that targeting small G proteins could constitute an interesting, novel pharmacological approach to the treatment of cardiac hypertrophy.

**Conclusions**

Studies from laboratories and clinical research both provide growing evidence that small G proteins and their downstream effectors are involved in the pathogenesis of various cardiovascular diseases (Fig. 4). Elucidation of functions of small G proteins and their downstream effectors in the cardiovascular system has resulted in the development of specific inhibitors such as ROCK inhibitors and isoprenylation inhibitors. Some of these drugs have already been widely studied for applications in oncology, but recently they have also been tested in various animal models of cardiovascular disease. They may also be used for treatment of some cardiovascular disorders such as in-stent restenosis, if a local delivery of the drugs to the vasculature (to achieve a relatively high local concentration of the drugs and avoid systemic side effects) is desirable. More
over, therapeutics that target multiple small G proteins may exhibit more powerful and efficient effects. However, adverse effects must be taken into account.

At this time, no solid data on adverse effects of such compounds are available except for statins. Although statins are well tolerated by most people, the voluntary withdrawal of the Bayer compound cerivastatin (Baycol) from the market on August 8, 2001 raised concerns about the safety of this class of drug. The best-documented adverse effect of statins is toxic myopathy (i.e., myalgia, myositis, and rhabdomyolysis) occurring rarely, in <0.1% of patients receiving statins (3). Severe myositis is rare, and fatal rhabdomyolysis occurs at a rate of <1 death/10^6 prescriptions that is increased with high dose of cerivastatin or in combination with gemfibrozil (13). There is no clear consensus of opinion on the mechanisms of statin-associated myopathy. Inhibition of synthesis of endogenous ubiquinone (coenzyme Q10) from the cholesterol synthetic pathway in the muscle by statins has been considered to be important in statin-associated myopathy (5). Ubiquinone serves as an essential electron carrier in the mitochondrial respiratory chain, in which it is essential for oxidative phosphorylation with concomitant production of ATP. Ubiquinone deficiency in the muscle would disturb cellular respiration and cause toxic myopathy. Other adverse effects such as polyneuropathy, gastrointestinal disturbances, and hepatic damage have been occasionally reported but have not been found in the large, blinded, randomized, controlled trials.

As for any therapeutic approaches, adverse effects of targeting small G proteins in the cardiovascular system will always be an important issue, since small G proteins are critically involved in a wide spectrum of cellular functions. Due to the fact that 1) a large number of small G proteins (>100) are identified and their functions are largely unknown, 2) more and more downstream effectors are being discovered, and 3) cross-talk between different G proteins or with other signal transduction mechanisms exists, exploration of the pathophysiological role of small G proteins, their effectors in the cardiovascular system, and their therapeutic potential will remain an active area in cardiovascular biology.

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