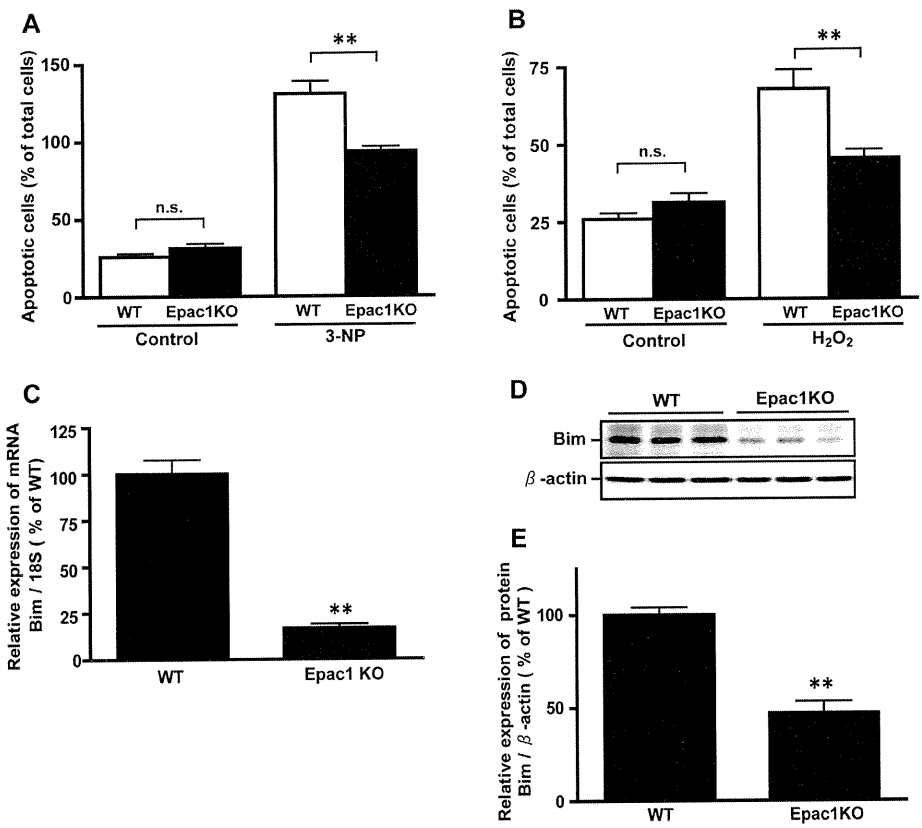

FIGURE 8. **The effect of 3-NP and hydroperoxide on apoptosis in cortical neurons from WT and Epac1 KO mice.** *A* and *B*, apoptosis was evaluated by means of TUNEL staining 48 h after the addition of the indicated reagents in cortical neurons from WT and Epac1 KO mice. The results are presented as percentages of the total cell number. *C*, the expression of endogenous Bim mRNA in cortical neurons from WT and Epac 1KO mice was quantified using real-time RT-PCR. The data are normalized to 18 S ribosomal RNA. $n = 6-8$; **, $p < 0.01$ *versus* WT. *D*, shown is a representative immunoblot of endogenous Bim protein expression in cortical neurons from WT and Epac1 KO mice. β-actin served as an internal control. *E*, the pixel intensity of the representative bands obtained in each experiment was calculated as described. $n = 4$; **, $p < 0.01$ *versus* WT mice. *n.s.*, not significant.

identified. In neuronal cells activation of cAMP/PKA signaling inhibited apoptosis induced by KCl in cerebella granule neurons (42) or by human immunodeficiency virus protein gp120 in the brain (43), promoting survival pathways in multiple neuronal cells (44, 45); these findings are in agreement with ours (supplemental Fig. 4). In cardiac myocytes, on the other hand, activation of cAMP signaling through such triggers as β-adrenergic receptor stimulation increased apoptosis (7, 8). In these studies the role of cAMP has been described primarily in terms of the activation of PKA.

Recently, several studies have suggested a contribution of either Epac alone or both Epac and PKA to apoptosis in restricted cell types including B-cell chronic lymphocytic leukemia (12), human leukocytes (11), immature B lymphoma cells (46), RINm5F β-cells (47), and H9c2 cells (48), showing that Epac and PKA play a protective role in apoptosis either alone and/or in concert in immune cells. However, the role of Epac in neuronal and myocardial apoptosis remains unknown despite the importance of cell death in tissues composed of post-mitotic cells. Our results show that stimulation and overexpression of Epac induces apoptosis in neurons but not in cardiac myocytes, implying that there are cell type-based differences in the effect of Epac activation on cell survival.

Epac-induced Apoptosis through Increased Bim Expression in Neuronal Cells—Our study demonstrated that Epac-induced apoptosis is mediated through the regulation of Bim, which acts on mitochondria as a pro-apoptotic factor, leading to disruption of the mitochondrial membrane potential. Bim binds to Bcl-2 and neutralizes its pro-survival function, resulting in apoptosis in multiple cell types (38, 49, 50). Bim is known to be expressed in neurons, hematopoietic cells, germ cells, lymphoid tissues, myeloid cells, and epithelial cells but not in cardiac myocytes, skeletal muscle, or neural-supporting cells, including glial, astrocytes, and oligodendrocytes (35). In agreement with these reports, our results show that Bim protein was highly expressed in primary culture of mouse cortical neurons but not in mouse cardiac myocytes. In cortical neurons we found that an Epac-selective cAMP analog increased Bim protein at the transcriptional level. When Bim was silenced, Epac-induced apoptosis was attenuated in neuronal cells. It should be noted that we were not able to exclude the possibility of off-target effects of the siRNAs because the rescue experiment that might exclude them is technically difficult. However, our results together with other data indicating that the suppression of the p38 MAPK pathway inhibits the elevation of Bim mRNA expression and Epac-induced apoptosis suggest that Epac-induced apoptosis is at least partly mediated by increased Bim expression. In fact, gene transfer of Bim to cardiac myocytes, which do not express Bim protein, induced apoptosis (supplemental Fig. 5). Taken together, the evidence strongly suggests that the expression of Bim is responsible for Epac-triggered apoptosis in neuronal cells, whereas Epac does not induce apoptosis in cardiac myocytes due to a lack of endogenous Bim expression. Further investigation is needed to identify the precise mechanism of Epac-induced transcriptional regulation of Bim in neuronal cells.

Changes in Epac Expression in Pathological/Physiological Conditions—Recent studies have indicated that the expression profile of Epac is altered during chronic degenerative inflammatory diseases. Epac1 mRNA, but not Epac2 mRNA, was increased in a mouse vascular injury model and was decreased in cardiac fibroblasts activated by transforming growth factor β (17, 23). Studies have reported the up-regulation of Epac1 mRNA and down-regulation of Epac2 mRNA in Alzheimer disease (13) and the up-regulation of Epac1 protein expression in inflamed rat neurons (14). These stud-

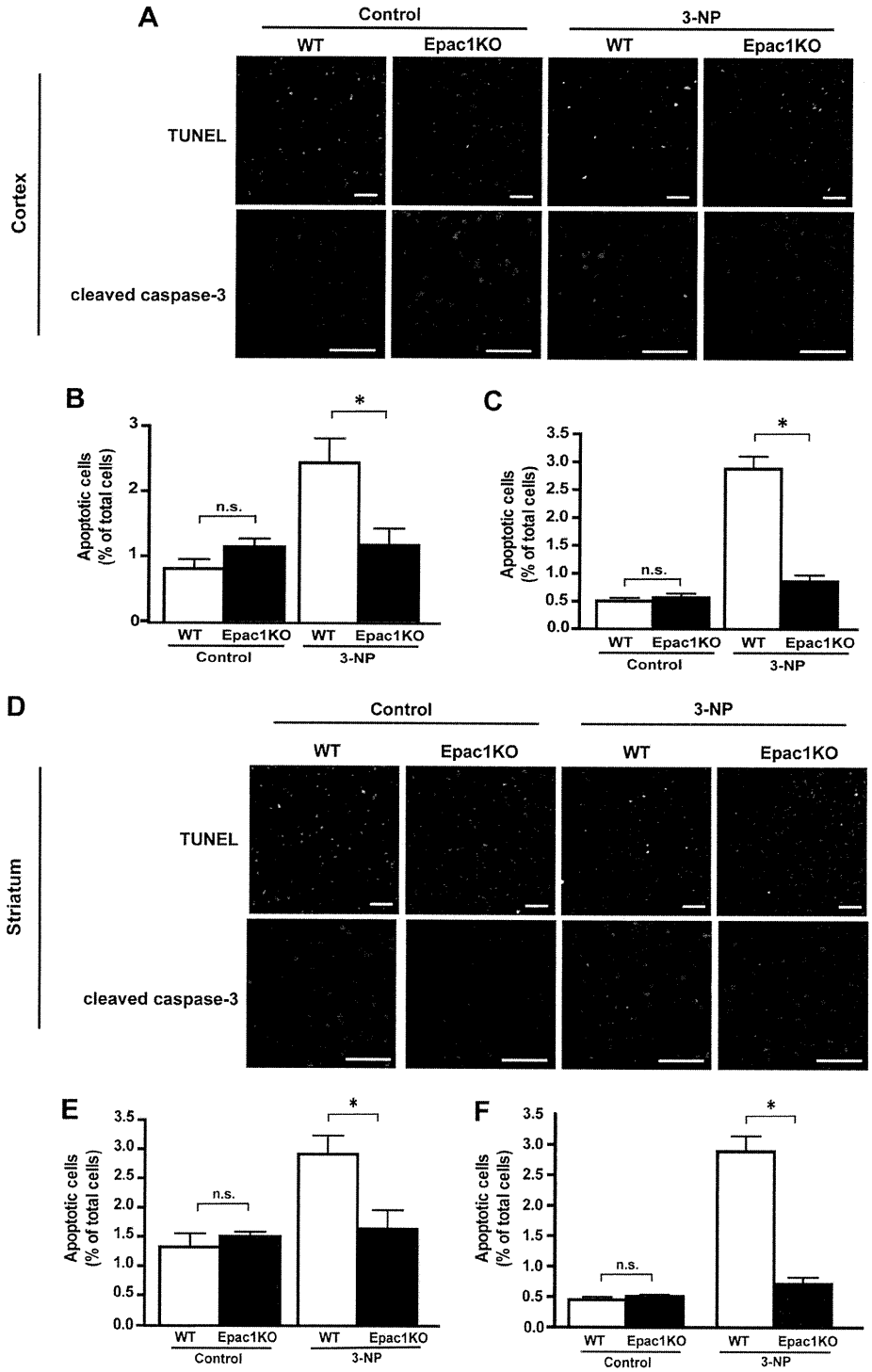

FIGURE 9. **Deletion of Epac1 suppressed 3-NP-induced brain cell apoptosis *in vivo*.** *A* and *D*, representative images of TUNEL staining and immunohistochemistry of cleaved caspase 3 of cortical and striatal sections from WT or Epac1 KO mice 24 h after injection of 3-NP. *Scale bar*, 100 μm. *B* and *E*, TUNEL-positive cells were quantified by counting nuclei in five randomly chosen fields. *C* and *F*, cleaved caspase 3-positive cells were quantified by counting nuclei in 5 randomly chosen fields. Deletion of Epac abrogated 3-NP-induced apoptosis in both the cortex and the striatum *in vivo*. The results are presented as percentages of the total cell number. $n = 5$ from 2 independent experiments. *, $p < 0.05$. *n.s.*, not significant.

ies indicate that the stoichiometry of Epac and especially that of Epac1 can be changed and selectively activated in disease conditions including neurodegenerative disorders.

Approximately half of all neurons in the nervous system undergo apoptosis during embryonic and early postnatal development (51), a period when Epac1 is highly expressed in the brain (15). Our results indicate that Epac1-induced neuronal apoptosis may be involved in the mechanisms underlying neuronal development. Nevertheless, Epac1 KO mice showed normal development up to at least 12 months of age, although no detailed assessment of their behavior, cognition, or learning memory has been made. Further studies using Epac2 KO mice and Epac1 and Epac2 double KO mice will need to be conducted given our observation that overexpression of Epac2 induced neuronal apoptosis *in vitro*.

The Effect of Epac1 Deletion on Apoptosis in Vivo—The mechanisms of neurological disorders such as Alzheimer disease, Huntington disease, and Parkinson disease are thought to stem from mitochondrial dysfunction (52). 3-NP, an irreversible inhibitor of the mitochondrial enzyme succinate dehydrogenase, is often administered systemically to treat these conditions and is considered to possess unique chemical and pharmacological traits that are accordingly considered in the generation of models of mitochondrial disorders and degenerative disorders (26, 39). The mechanisms of 3-NP toxicity are also thought to involve enhanced production of reactive oxygen species, including hydrogen peroxide, which can cause oxidative damage to DNA, lipids, and proteins (53). In the present study both 3-NP and hydrogen peroxide failed to induce apoptosis in cultured cortical neurons from Epac1 KO mice, and 3-NP-induced neuronal apoptosis was abolished in Epac1 KO mice *in vivo*. In contrast, there was no difference between Epac1 KO and WT mice in terms of 3-NP-induced apoptosis. Cardiac myocytes from Epac1 KO mice did not differ in their sensitivity to cAMP analogs (supplemental Figs. 6 and 7). Although this and our previous studies show that the inhibition of Epac1 protects 3-NP-mediated neuronal apoptosis *in vivo*

and *in vitro*, the relevance of Epac2 to this phenomenon needs to be examined in future studies. A recent study has demonstrated that Epac is involved in the secretion of an amyloid precursor protein, which has been known to induce apoptosis leading to Alzheimer disease (54). Together with our data, this indicates that selective inhibition of the Epac signal may prove useful as a therapeutic strategy in treating neurodegenerative diseases.

In conclusion, Epac induces neuronal apoptosis through increased Bim expression. Because disruption of Epac1 exerts a protective effect on neuronal apoptosis *in vivo*, inhibition of Epac may be a useful tactic in the treatment of neurodegenerative diseases.

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Review

Accessory proteins for heterotrimeric G-protein: Implication in the cardiovascular system

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Abstract

The G-protein signaling system plays an important role in controlling cellular responses to numerous hormones and neurotransmitters involved in homeostasis of the cardiovascular system. In addition to traditional determinants of G-protein signaling such as the G-protein-coupled receptor (GPCR), heterotrimeric G-proteins and effectors, accumulating data indicate the existence of entities that directly regulate the activation status of G-proteins independent of GPCR. To date, there have been a number of reports on accessory proteins that influence GDP dissociation, affect nucleotide exchange at the Gα subunit, alter subunit interactions within heterotrimeric Gαβγ independent of nucleotide exchange, or form complexes with Gα or Gβγ independent of the typical Gαβγ heterotrimer. Such proteins may provide an additional signal input to the G-protein signaling system in the absence of GPCR or may act as an alternative binding partner of G-protein subunits serving unknown roles of G-proteins in cells. Accumulating information suggests that accessory proteins for G-proteins are actually involved in the regulation of the signaling system to maintain homeostasis and the dynamic responses to physiological and pathological challenges. It is likely that alterations in signal processing may be achieved by the modulation of signal processing within the cell using accessory proteins for G-proteins. The loss of regulation of this system, leading to inappropriate activation or inactivation of G-protein signaling, is strongly implicated in various human diseases. In this review, we update current information and discuss different accessory proteins for heterotrimeric G-proteins in terms of their involvement in the regulation of the cardiovascular system. Such information may contribute to uncovering mechanisms underlying cardiovascular disease as well as the development of novel therapeutic approaches to human disease.

Keywords: Heterotrimeric G-protein; Signal transduction; Activator of G-protein signaling (AGS); Regulator of G-protein signaling (RGS); Cardiovascular disease

Contents

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1. Introduction

Signal processing via heterotrimeric G-proteins is one of the most widely used systems for information transfer across the cell membrane. This system is essential for maintaining tissue homeostasis, but is also involved in the development of cardiac hypertrophy [1], remodeling of the heart [1], ischemic preconditioning [2,3], and angiogenesis [4]. In addition, many of the therapeutic interventions are used in the management of cardiovascular disease targeting G-protein signaling systems via cell surface G-protein-coupled receptors (GPCRs).

In this system, seven transmembrane domain GPCRs are activated by extracellular stimuli, such as hormones and neurotransmitters, inducing a conformational change in the Gα subunit, and then catalyzing GDP release from Gα (Fig. 1). Binding of GTP to Gα destabilizes Gαβγ complex, leading to a structural rearrangement of Gα-GTP, Gβγ and the receptor. This is followed by Gα-GTP disassociation from the receptor and Gβγ. Both subunits, Gα-GTP and Gβγ, stimulate distinct downstream effector molecules including adenylyl cyclases, phospholipases, ion channels, and protein kinases [5–7]. The activation of the signaling pathway is terminated when Gα hydrolyzes GTP to GDP by its intrinsic guanosine triphosphatase (GTPase) activity; it then reassociates with Gβγ, thus completing the cycle. Reassociation of Gβγ with Gα-GDP terminates interactions of effector molecules. Gβγ facilitates the coupling of Gα to GPCR and also acts as a guanine nucleotide inhibitor (GDI) for Gα-GDP, slowing spontaneous exchange of GDP for GTP (Table 1).

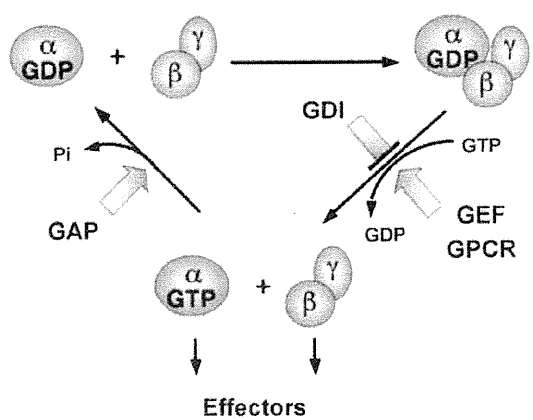

Fig. 1. Schematic diagram indicating G-protein activation/deactivation cycle and influences of Gα regulatory protein. GEF: guanine nucleotide exchange factor; GDI: guanine nucleotide dissociation inhibitor; GAP: GTPase-activating protein; GPCR: G-protein-coupled receptor.

Table 1
Abbreviations.

AGS, activator of G-protein signaling
ERK, extracellular signal-regulated kinase
GAP, GTPase-activating protein
GDI, guanine nucleotide dissociation inhibitor
GEF, guanine nucleotide exchange factor
GDP, guanosine-5′-diphosphate
GIRK, G-protein-activated inwardly rectifying potassium channel
GPCR, G-protein-coupled receptor
GPR, G-protein regulatory
GRK, G-protein-coupled receptor kinase
GTP, guanosine-5′-triphosphate
GTPγS, guanosine-5′-O-(3-thiotriphosphate)
HSP-90, heat shock protein 90
KATP, ATP-sensitive potassium
MAP, mitogen-activated protein
MEK, MAP/ERK kinase
NG-GPA, NG108-15 G-protein activator
NMDA, N-methyl-D-aspartate
Pcp2, Purkinje cell protein-2
PBP, phosphatidylethanolamine-binding protein
RACK1, receptor for activated C kinase 1
RGS, regulator of G-protein signaling
Ric, resistance to inhibitors of cholinesterase
RKIP, Raf-1 kinase inhibitor protein
siRNA, small interfering RNA
SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor

These events are dynamically regulated to optimize signal specificity, maximize signal efficiency and integrate diverse stimuli. As a consequence of this central role in signal integration, the subtle adaptations or maladaptations that occur in response to both physiological and pathophysiological challenges can play a key role in the manner in which the effector cell responds to such challenges. Regulations of the G-protein system include the segregation of specific signaling molecules in cell microdomains, receptor phosphorylation and internalization, and crosstalk between signaling pathways. In addition to these, a number of proteins that regulate the basal activation state of G-proteins independently of cell surface GPCR have been discovered. Such accessory proteins influence the activation or deactivation of the Gα subunit and alter subunit interactions within heterotrimeric Gαβγ independent of nucleotide exchange, or form complexes with

Gα or Gβγ independent of the typical Gαβγ heterotrimer. Accessory proteins for G-proteins may provide an additional signal input to the G-protein signaling system in the absence of GPCR or may act as an alternative binding partner of G-protein subunits serving unknown roles of G-proteins in cells. In previous work, we evolved the observation of such accessory proteins and tuning of the signaling system concerned based on cell-specific differences [8,9] and the subsequent biochemical characterization of such differences [9–12]. We later addressed the impact of accessory proteins in terms of system adaptation of the GPCR system [13] and identified novel accessory proteins for G-proteins induced in the myocardium in response to transient ischemia [14].

An adaptation of the signaling system is critical for maintaining homeostasis and the dynamic responses to physiological and pathological challenges. It is likely that subtle alterations in signal processing may be achieved by discrete modulation of signal processing within the cell using accessory proteins for G-proteins. Current information suggests that accessory proteins for G-proteins are actually involved in the regulation of the signaling system for maintaining homeostasis and the dynamic responses to physiological and pathological challenges. The loss of regulation of this system, leading to inappropriate activation or inactivation of G-protein signaling, is strongly implicated in various human diseases.

In this review, we update current information and discuss various accessory proteins for heterotrimeric G-proteins in terms of their involvement in the regulation of the cardiovascular system (Table 2). Such information may contribute to uncovering the mechanisms underlying cardiovascular disease as well as the development of a novel therapeutic approach to human disease.

2. Guanine nucleotide exchange factors (GEFs) for Gα subunit

The first class of accessory proteins facilitates guanine nucleotide exchange of Gα subunits such as activated G-protein-coupled receptors. Proteins have been identified as nonreceptor GEFs that increase GTPγS binding to the Gα subunit similar to activated GPCR, and include GAP43 [15,16], NG-GPA [8,9], β-APP [17], presenilin I [18], AGS1 [19–23], PBP/RKIP [24–28], and Ric-8 [29]. Among 16 of the Gα genes identified [30,31], most of them preferentially activate the $G\alpha_i$ family; however, Ric-8A can also activate $G\alpha_q$ in vitro [29]. Although the regulation of GEF and its physiological relevance are not completely understood at present, its potential association with cardiovascular disease has been reported in several proteins (Table 2).

2.1. Activator of G-protein signaling 1 (AGS1, DexRas1, and RASD1)

AGS1 was initially discovered as dexametasone-inducible, ras-related cDNA (DexRas) in mouse corticotroph cells [21]. AGS1 is a member of the Ras family of monomeric GTPases which is most closely related to Rhes (60% amino acid identity), a Ras-related protein with unique insert regions [32,33]. Purified AGS1 directly binds to $G\alpha_i$, and increases GTPγS binding to purified $G\alpha_{i1}$ and $G\alpha_{i2}$ in vitro [22].

In mammalian cells, transfected AGS1 activates the Erk1/2 pathway without receptor stimulation [22]. AGS1 also inhibits cAMP accumulation in response to forskolin or a constitutive active $G\alpha_s$ [34], which is also consistent with the regulation of $G\alpha_i$ by AGS1, indicating the function of AGS1 as a direct Gα activator. However, when transiently co-transfected with a $G\alpha_i$-coupled receptor, AGS1 blocks the downstream signaling of the agonist-stimulated receptor, as well as the receptor-mediated heterologous sensitization of adenylyl cyclase [35].

In human tissues, AGS1 is highly enriched in heart, brain, liver, skeletal muscle and bone marrow, with lower expression in pancreas, kidney, thymus, lung and placenta [36,37]. AGS1 expression is induced by various stimuli related to stress. Moreover, AGS1 expression is up-regulated in cultured cells following glucocorticoid treatment [21] and in whole animals following severe blood loss [38], suggesting that AGS1 plays a role in mediating the stress response. The disruption of AGS1 in mice suggests its importance in the signaling of the NMDA-mediated circadian cycle [39].

A comprehensive concept of AGS1 function in the context of the whole signaling system is still being developed.

2.2. Growth-associated protein 43 (GAP43)

GAP43 enriched in the neuronal growth cone plays an important role during development and axonal regeneration. GAP43 increases the rate of GDP dissociation and GTPγS association to purified brain heterotrimer G-proteins and purified $G\alpha_o$ free of Gβγ [15,16]. In oocytes, GAP43 enhanced GPCR signaling in response to an agonist and was able to cause a transient current flow without receptor activation [40]. GAP43 is involved in the innervation of cardiomyocytes and left stellate ganglions [41]. The upregulation of GAP43 was reported in several experimental models, including myocardial infarction [41,42] and right ventricular hypertrophy [43].

2.3. Phosphatidylethanolamine-binding protein (PBP)/Raf kinase inhibitor protein (RKIP)

PBP or RKIP is a 23-kDa soluble protein that inhibits the activation of MEK [24,25] by RAF1 and also blocks GRK2 activity [26], suggesting a role in signal crosstalk in cells. PBP/RKIP facilitates binding of GTPγS to purified $G\alpha_{i1}$, and appears to enhance $G\alpha_{i/o}$-mediated signaling in vivo [28]. Lorenz et al. demonstrated that PKC activation following GPCR activation triggered RKIP dissociation from Raf-1 to associate with GRK2 [26]. Thus, the GPCR signal is enhanced both by removing an inhibitor from Raf-1 and by blocking receptor internalization via GRK2.

Table 2
Accessory proteins for heterotrimeric G-protein involved in the regulation of cardiovascular system.

Proteins	Function on G-protein	Association with cardiovascular system	References
Guanine nucleotide exchange factors (GEFs) for Gα subunit			
AGS1	GEF for $G\alpha_i$	Relative high level expression in human heart; up-regulated in response to biological stress	[19–23,35–39]
GAP-43	GEF for brain G-protein and $G\alpha_o$	Enriched in stellate ganglion; upregulation in myocardial infarction and right ventricular hypertrophy	[15,16,40–43]
PBP/RKIP	GEF for purified $G\alpha_{i1}$	Involved in β-adrenergic signaling and contractility of cardiomyocyte	[24–28]
Presenilin	GEF and GAP for $G\alpha_o$	Critical factor for cardiac development and familial DCM	[18,44–46]
Tubulin	Transactivate Gα subunit, interact with Gβγ subunit	Multiple roles including protein sorting; microtubule regulation	[47–52]
Guanine nucleotide dissociation inhibitors (GDIs) for Gα subunit			
AGS3	GDI for $G\alpha_{i/o}$, stabilize Gα-GDP complex	Short variant expressed in the heart; AGS3 null mice exhibited lower arterial pressure and an enhanced gain of the baroreceptor reflex	[13,32,33,55–56]
Caveolin	Inhibit GTPase activity and GTPγS binding to $G\alpha_o$ Interact with $G\alpha_{i2}$	Involved in various signaling system via its scaffolding function	[111–114]
GTPase-activating proteins (GAPs) for Gα subunit			
RGS2	GAP for $G\alpha_q \gg G\alpha_{i/o}$	Deletion of RGS2 caused strong hypertension in mice	[70,76–82]
RGS4	GAP for $G\alpha_{i/o}$, $G\alpha_q$	RGS4 overexpressing mice delayed hypertrophy in response to the pressure stress or $G\alpha_q$ pathway stimulation	[66,69,71–75]
Hax-1	Enhances $G\alpha_{13}$-mediated Rac activity	Inhibits apoptosis of adult cardiomyocyte through interaction with caspase-9	[115]
Accessory proteins interact with Gβγ subunits			
AGS8	Interact with Gβγ subunit	Identified in repetitive transient ischemia model of rat heart; increased expression in response to ischemia/hypoxia	[14,90]
Phosducin and phosducin-like protein	Interact with Gβγ subunit	Expression in the heart; overexpression of part of phosducin improved contractivity of failing heart	[102–110]
RACK1	Interact with Gβγ subunit	Suggested involvement in reduced contractility of senescent cardiomyocyte	[92–96]
Syntaxin1	Interact with Gβγ subunit	Modulate K^+ and Ca^{2+} channel activity; involvement in atrial natriuretic peptide release from cardiomyocyte	[97–101]
GRK2	Interact with Gβγ subunit	Regulation of cell surface GPCR	[116,117]
Others			
HSP-90	Alter $G\alpha_{12}$ signaling	Involved in multiple signaling cascades via chaperone function	[118]

A physiologic role for this mechanism was shown in cardiomyocytes in which the downregulation of RKIP restrains β-adrenergic signaling and contractile activity. Thus, the relative importance of these different actions of PBP/RKIP needs to be addressed.

2.4. Presenilin-1

Presenilin-1 is the gene responsible for the development of early-onset familial Alzheimer's disease and is involved in multiple cellular events [44]. The C-terminus of presenilin directly interacts with and enhances both GTPγS binding and GTP hydrolysis of $G\alpha_o$ [18]. Presenilin-1 is also expressed in the heart and is critical for cardiac development [45]. Li et al. reported familial DCM and heart failure associated with missense mutation of presenilin [46]. The role of the interaction of these proteins with G-proteins in the development of heart failure is not well understood at present.

2.5. Tubulin

Tubulin activates the Gα subunit by different mechanisms, and thus it increases GTP binding to Gα subunits by the transfer of GTP bound on tubulin to the GDP bound on

Gα [47–51]. Tubulin has also been reported to interact with GPCR and Gβγ [52]. From its functions, tubulin has been suggested to have multiple roles in trafficking, G-protein activation, and the control of microtubule dynamics [52]. Furthermore, tubulin and G-protein interaction may influence microtubule polymerization and alter cardiomyocyte architecture.

3. Guanine nucleotide dissociation inhibitors (GDIs) for Gα subunit

The second class of accessory protein is a group of guanine nucleotide dissociation inhibitors (GDIs). The majority of this subgroup of accessory proteins shares a common structural feature termed the G-protein regulatory (GPR) or GoLoco motif [32,33,53]. The GPR motif is a 20–25 amino acid cassette that serves as a docking site for $G\alpha_{i/o}$ and $G\alpha_t$. The interaction of GPR motifs with $G\alpha_{i/o}$ stabilizes the GDP-bound conformation of Gα and interferes with Gβγ for binding to $G\alpha_i$, which results in inhibition of Gα-mediated signaling and prolongation of Gβγ-mediated signaling. The GPR motif is found in AGS3 (GPSM1), LGN (GPSM2), AGS4 (GPSM3), Purkinje cell protein-2 (Pcp2)/L7, WAVE-1 and the GTPase-activating proteins RGS12, RGS14, RAP1GAPI (partial GPR motif), and Rap1GAPII [32,33,53]. The GPR proteins are also known as crucial players in asymmetric cell division during embryogenesis [54].

3.1. Activator of G-protein signaling 3 (AGS3)

AGS3 (GPSM1) is one of the proteins extensively studied in GDIs for Gα subunit. This protein was identified as a receptor-independent activator of G-protein signaling in a yeast-based functional screen of mammalian cDNAs. AGS3-LONG is a 650 amino acid protein containing 7 tetratricopeptide repeats as well as 4 GPR motifs, and is preferentially expressed in the brain. AGS3 has a short form enriched in the heart lacking the tetratricopeptide repeat motif domains and contains only three GPR motifs [55]. The impact of AGS3-SHORT in signal integration in cells was investigated in the sensitization of adenylyl cyclase. This sensitization is known as a transient enhancement of the activity of adenylyl cyclase following sustained stimulation of $G\alpha_i$-coupled receptors, and as an example of an adaptive response of cells [56,57]. The sensitization of $G\alpha_s$ regulation of adenylyl cyclase observed with sustained activation of $G\alpha_i$-coupled GPCR was blocked by the overexpression of heart-type AGS3 (AGS3-SHORT) in CHO cells [13]. This observation was associated with an increase in the stability of the $G\alpha_i$ subunit in the membrane. Interventions against AGS3 utilizing GPR-related components indicated that AGS3 also plays an important role in adaptive neuronal events associated with the drug-seeking behavior and locomotor sensitization following cocaine withdrawal [58,59]. These data suggested that AGS3 and GPR proteins influenced the adaptation process of the G-protein signaling system.

Recently, Lanier's group carried out an initial characterization of AGS3 null mice [60]. In these mice, the full-length AGS3 was disrupted, but a short variant form of AGS3 was still expressed in the heart. Interesting alterations were observed in the regulation of blood pressure and metabolic functions in AGS3 null mice. Thus, the mean arterial pressure was significantly lower in AGS3 null mice, and its diurnal variations were reduced. Moreover, AGS3 null mice showed enhanced gain of the baroreceptor reflex. Altered vascular control was also observed in terms of the suppression of recovery of arterial pressure following administration of sodium nitroprusside. In the analysis of metabolic homeostasis, AGS3 null mice exhibited a lean phenotype, reduced fat mass and increased nocturnal energy expenditure. The first observation of a genetically modified animal of AGS3 suggests the potential of AGS3 and perhaps other GPR proteins as a therapeutic target in human disease.

4. GTPase-activating proteins (GAPs) for Gα subunit

The majority of proteins of this group share 120–130 amino acids of the regulator of G-protein signaling (RGS) homology domain, which mediates the GTPase-accelerating activity at Gα subunits [61,62]. At least 30 mammalian proteins are known to share an RGS or RGS-like domain [63,64]. Most of the RGS proteins are GAPs for $G\alpha_{i/o}$ and $G\alpha_{q/11}$ family members with some exceptions for GAPs for either $G\alpha_{12/13}$ or $G\alpha_s$ family members [64,65]. The change in the expression of RGS proteins in human heart failure has also been reported. Mittmann et al. indicated an increase in RGS4 mRNA but no change in RGS2 or RGS3 in the myocardium from terminally failing human hearts with dilated or ischemic cardiomyopathy [66], whereas Owen et al. found an apparent upregulation of RGS3 and RGS4 proteins and mRNA in end-stage failing hearts of humans [67]. Takeishi et al. also identified an apparent decrease of RGS2 protein in human failing heart following treatment with left ventricular assist device [68]. Although these results which might reflect the background of the patients were not consistent, alterations in RGS2, RGS3 and RGS4 expression suggested their potential to alter cardiovascular pathophysiology. Among the RGS protein families, the physiological roles of RGS2 and RGS4 in the cardiovascular system were extensively studied in mice deficient for RGS2 [69] or overexpressing RGS4 [70].

4.1. RGS4

RGS4 has a GAP activity for $G\alpha_{i/o}$ and $G\alpha_q$ and appears to negatively regulate signaling events mediated by Gα subunits. Its association with schizophrenia has also been reported in some populations [71–73]. On the other hand, the role of RGS4 in cardiovascular function was previously examined utilizing transgenic mice overexpressing RGS4

[70]. The overexpression of RGS4 did not affect basal cardiac function or chronotropic response to dobutamine, but significantly reduced the ability of the heart to adapt to an increase in cardiac afterload induced by transverse aortic constriction. In cardiomyocytes, the overexpression of RGS4 inhibited phenylephrine- and endotherin-1-induced hypertrophy or contraction [66,74]. The co-overexpression of RGS4 and $G\alpha_q$ in transgenic mice delayed the $G\alpha_q$-mediated onset of cardiac hypertrophy, which exhibits a phenotype similar to human cardiac hypertrophy [75]. The increase of RGS4 in a failing heart may decrease contractile response; however, this increase reduces the adverse effect of $G\alpha_q$ signaling. At present, the overall direction toward protection of the heart by RGS4 remains controversial.

4.2. RGS2

RGS2 has unique binding and some forms of selectivity for $G\alpha_{q/11}$ rather than for $G\alpha_i$ [76,77]. It appears to attenuate signaling events mediated by $G\alpha_{q/11}$-coupled receptor, including vasocontraction and cardiac hypertrophy [69,76,78]. It has also been shown that *rgs2*$^{+/-}$ and *rgs2*$^{-/-}$ mice exhibit a similar and strong hypertensive phenotype, suggesting that both copies of the gene are essential for normal cardiovascular function. Further analysis suggested that this phenotype may be attributed to enhanced and/or prolonged vascular contractile responses to GPCR stimulation [69,79].

The overexpression of RGS2 in cardiomyocytes eliminated any increase in cell size and genetic markers of hypertrophy in response to α_1-adrenergic stimulation [80,81]. A recent study found RGS2 to be selectively downregulated during the early onset of cardiac hypertrophy with enhanced $G\alpha_{q/11}$ signaling [82]. SiRNA-mediated RGS2 knockdown increased phenylephrine- and endothelin-1-induced PLCβ stimulation and exacerbated the hypertrophic effect [82]. Interestingly, no overt changes were observed in the cardiac function of *rgs2*$^{-/-}$ mice up to 6 months of age [69]. These lines of evidence implied a beneficial effect of RGS2 in protecting the heart, although more studies are needed to clarify the role of RGS2 in hypertrophy-associated GPCR signals.

5. Accessory proteins interact with subunits of Gβγ

There are 5 Gβ and 12 Gγ subunits reported in humans and mice, offering potential of a large diversity of combinations of Gβγ dimers [6,30]. $G\beta_{1-4}$ share 82–92% homology with each other and $G\beta_5$ has approximately 50% divergent homology from others [6,30]. In contrast to the Gβ subunit, Gγ shares only 15–30% sequence identity within subunits [6,30]. The $G\beta_5$ subunit is unique since it can form a unique complex with RGS7 apart from the gamma subunit. Other RGS proteins containing the Gγ-like (GGL) domain, namely, RGS6, RGS7, RGS9, and RGS11 are also predicted to be able to assemble with $G\beta_5$ [83–85]. Although $G\beta_5$ is preferentially expressed in neuronal systems, a relatively higher expression in the heart was observed in humans than in mice [86]. However, the functional and/or physiological roles of this interesting complex are still not clear.

Gβ subunit and Gγ subunit are tightly associated and usually act as a heterodimer. It was once considered that the Gβγ subunit inhibited the guanine nucleotide exchange of Gα and terminated the G-protein signal by sequestrating Gα subunits. However, it is now known that Gβγ regulates many signaling molecules, including G-protein-activated inwardly rectifying potassium channel (GIRK), Ca channels, $PLC\beta_2$ and β_3, PLCε, and GRK2 [6,7]. Interestingly, mutational and structural studies have indicated that effector proteins and the Gα subunit share an overlapping interface on the surface of Gβγ [7,87]. The Gβ subunit does not have a catalytic site, and does not change its conformation when it dissociates from the Gα subunit on activation of a heterotrimer. Thus, the activation of Gβγ-mediated signaling is initiated by protein–protein interaction of Gβγ without sharing the interface for signal transduction.

Number of Gβγ-interacting proteins continues to increase. Because Gβγ-interacting proteins share a common region of the surface, they would cover up and inhibit the Gβγ signal to other molecules. Thus, the binding of Gβγ-interacting proteins to this sharing site is principally expected to exclude interaction of other proteins, resulting in the shutdown of Gβγ signaling as Gα does. Recently, Bonacci et al. have developed small molecules targeting the sharing interface of Gβγ and have also identified a compound selectively altering the Gβγ signaling pathway in cells [14,88].

5.1. Activator of G-protein signaling 8 (AGS8)

AGS8 was isolated utilizing a yeast-based functional screen expressing human Gα in place of the yeast Gα from a cDNA library generated from a rat model of repetitive transient ischemia of the heart [14]. In this model, rat hearts were subjected to repetitive transient ischemia by the inflation of an implanted pneumatic snare around the proximal left anterior descending coronary artery (40 s occlusion, 20 min interval, 8 h/day) that induced coronary collateral growth in 2 weeks [89].

AGS8 (GeneBank accession # DQ256268) encodes an uncharacterized 1730 amino acid protein (KIAA1866, FNDC1 in humans) with 4 fibronectin-type III domains, 3 at the N-terminus and 1 C-terminal [14]. The amount of AGS8 mRNA was increased in the ischemic area of left ventricle by 3.5-fold compared with the non-ischemic area; however, this increase was not induced in cardiac hypertrophy or heart failure, suggesting a specific role of AGS8 in the signaling processes underlying ischemia [14]. Incidentally, hypoxic treatment of adult ventricular cardiomyocytes up-regulated AGS8 mRNA by 8-fold. This increase was not observed in cultures of aortic endothelial cells, aortic vascular smooth muscle cells or cardiac fibroblasts. Protein interaction assays with GST-AGS8 and purified G-protein subunits or brain

lysates indicated direct binding of AGS8 to Gβγ [14]. Interestingly, subsequent studies suggested that the interaction of AGS8 with Gβγ occurred in a manner that did not alter the regulation of the effector PLC-β_2 by Gβγ. The AGS8 binding site on the Gβγ subunit appears to reside at the common interface of Gβ overlapping with PLC-β_2 and the Gα subunit [7,90]. This observation suggested, in line with expectations, that the mechanism of Gβγ interaction to other molecules might be more complex. An alternative mode of Gβγ interaction and the formation of signaling components are postulated [7,90].

AGS8 serves as an ischemia-inducible binding partner for Gβγ in cardiomyocytes providing an unexpected mode of signal input to the G-protein system. Such mechanisms of signal regulation may provide intrinsic cues for the adaptation of the heart to ischemia.

5.2. Receptor for activated C kinase 1 (RACK1)

Gβ belongs to a large family of WD40 repeat proteins [91] and interacts with other WD40 proteins, such as the receptor for activated C kinase 1 (RACK1) [92]. RACK1 shares 57% amino acid similarity with $G\beta_1$ lacking N-terminal extension for binding the Gγ subunit, and usually does not interact with the Gα subunit [93].

RACK1 inhibited the Gβγ-mediated activation of phospholipase $C\beta_2$ and adenylyl cyclase, whereas it showed no obvious effect on Gβγ-mediated chemotaxis, MAP kinase activation or Gα-mediated signaling. The association of Gβγ with RACK1 induces the translocation of RACK1 from cytosol to the plasma membrane. This has been suggested as a mechanism for recruiting PKC and other RACK1-binding proteins to the plasma membrane [93,94]. PKC recruitment to G-protein-coupled receptors by Gβγ/RACK1 can contribute to the efficient modulation of signaling by PKC. Furthermore, the role of the translocation of RACK1 on α_1-adrenergic-mediated myocardial contraction was investigated in senescent rat hearts. It was suggested that the decreased level of RACKs in the senescent myocardium was involved in an impairment of the translocation of PKCα and PKCε following α_1-adrenergic receptor stimulation, resulting in a defective α1-adrenergic-receptor-mediated contraction in aged rat hearts [95]. The influence of RACK1 was also investigated in H9C2 stably expressing RACK1. RACK1 influenced cell size, cell survival under oxidative stress, adhesion, and migration of H9C2 cells [96].

5.3. Syntaxin 1A

Syntaxin 1A is a protein of the presynaptic vesicle release complex. Syntaxin 1A has been shown to directly interact with the Gβγ subunit and to modulate N-type calcium channels expressed in tsA-201 (HEK) cells [97,98]. In freshly isolated cardiomyocytes, inhibition of sytaxin 1A on the ATP-sensitive potassium channel (KATP channel, Kir6.2/SUR2A) was also demonstrated, which may serve as a "brake" to temper the fluctuation of low pH-induced KATP channel opening that could induce fatal re-entrant arrhythmias [99]. Syntaxin 1 may also play a role in the secretion of atrial natriuretic peptide in adult cardiac myocytes as in the soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARE) complex [100,101]. Although the roles of Gβγ in these functions are not fully identified, an interaction of syntaxin 1A with Gβγ has potentials to be involved.

5.4. Phosducin and phosducin-like protein

Phosducin is a 33-kDa cytosolic regulator of G-protein signaling, which was found in the retina and pineal gland as well as many other tissues including heart [102–104]. Phosducin-like protein, a protein sharing extensive amino acid sequence homology with phosducin, is also expressed in heart [105,106]. In the retina, phosducin interferes with the reassociation of $G\alpha_t$ and Gβγ following light-mediated G-protein activation. In an experimental system, phosducin and phosducin-like protein bound Gβγ [107–109] and effectively impeded Gβγ-mediated signaling. Although the overexpression of one part of phosducin improved the contractility of cardiomyocytes and a failing heart, its physiological relevance needs to be further determined [110].

6. Conclusions and perspective

A number of accessory proteins for heterotrimeric G-proteins have been identified in recent years. Each of them has unique selectivity for subunits and functions in the G-protein activation cycle or subunit association. Accumulating data indicates the involvement of these proteins in the regulation of the cardiovascular system. Also, the impact of several such proteins was compelling and has been characterized. However, the effects of many of these proteins on the cardiovascular system are described from their other functional properties rather than from their function on G-proteins.

The challenges facing this research field are to determine how these accessory proteins integrate into various signaling systems and to identify the role of the interaction of G-proteins to "other" functional properties of each accessory protein. Further precise information is expected to be elucidated on the basic mechanisms of accessory proteins on the activation of G-proteins as well as their role in G-protein-mediated signaling, especially in the face of physiological stress.

All these issues have important implications in relation to basic cell biology as well as to the understanding of the physiological response of the cardiovascular system to maintain homeostasis against stress. Further information will thus contribute to the development of novel therapeutics for human disease.

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Invited Review for the 2009 Hirosi Kuriyama Award

Regulation of vascular tone and remodeling of the ductus arteriosus

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Abstract

The ductus arteriosus (DA), a fetal arterial connection between the main pulmonary artery and the descending aorta, normally closes immediately after birth. The DA is a normal and essential fetal structure. However, it becomes abnormal if it remains patent after birth. Closure of the DA occurs in two phases: functional closure of the lumen within the first hours after birth by smooth muscle constriction, and anatomic occlusion of the lumen over the next several days due to extensive neointimal thickening in human DA. There are several events that promote the DA constriction immediately after birth: (a) an increase in arterial oxygen tension, (b) a dramatic decline in circulating prostaglandinE_2 (PGE_2), (c) a decrease in blood pressure within the DA lumen, and (d) a decrease in the number of PGE_2 receptors in the DA wall. Anatomical closure of the DA is associated with the formation of intimal thickening, which are characterized by (a) an area of subendothelial deposition of extracellular matrix, (b) the disassembly of the internal elastic lamina and loss of elastic fiber in the medial layer, and (c) migration into the subendothelial space of undifferentiated medial smooth muscle cells. In addition to the well-known vasodilatory role of PGE_2, our findings uncovered the role of PGE_2 in anatomical closure of the DA. Chronic PGE_2-EP4-cyclic AMP (cAMP)-protein kinase A (PKA) signaling during gestation induces vascular remodeling of the DA to promote hyaluronan-mediated intimal thickening and structural closure of the vascular lumen. A novel target of cAMP, Epac, has an acute promoting effect on smooth muscle cell migration without hyaluronan production and thus intimal thickening in the DA. Both EP4-cAMP downstream targets, Epac and PKA, regulate vascular remodeling in the DA.

Key words: cyclic AMP, exchange protein activated by cyclic AMP (EPAC), protein kinase A, intimal thickening, the ductus arteriosus, vascular remodeling, hyaluronan

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Introduction

The ductus arteriosus (DA), a fetal arterial connection between the pulmonary artery and the descending aorta, is indispensable for fetal life. It shunts deoxygenated blood from the main pulmonary artery to the descending aorta. Over half of the blood flow in the descending aorta is diverted to the umbilico-placental circulation (Heymann and Rudolph, 1975), where gaseous exchange takes place. Although patency of the DA is required for fetal survival, the persistence of a patent DA after birth is a major cause of morbidity and mortality, especially in premature infants, leading to severe complications, including pulmonary hypertension, right ventricular dysfunction, postnatal infections and respiratory failure (Hermes-DeSantis and Clyman, 2006). The incidence of DA patency has been estimated to be one in 500 in term newborns and accounts for the majority of all cases of congenital heart diseases in preterm newborns (Mitchell *et al.*, 1971). In preterm babies with birth weights <1,500 g, the incidence of a patent DA exceeds 30% (Van Overmeire *et al.*, 2004). In addition, the presence of a patent DA is more serious in premature infants than in full-term infants since premature infants with a patent DA are more likely to develop problems such as intraventricular hemorrhage, necrotizing enterocolitis, bronchopulmonary dysplasia and congestive heart failure. Therefore, it is important to understand the precise mechanisms of regulation of the DA.

Closure of the human DA is believed to occur in two phases: (1) functional closure of the lumen within the first hours after birth by smooth muscle constriction, and (2) anatomic occlusion of the lumen over the next several days due to extensive neointimal thickening and loss of smooth muscle cells from the inner muscle media (Smith, 1998; Clyman, 2006; Yokoyama *et al.*, 2006b). Although its process is similar in mammalian DA, the time course of two phases is variable among species. There are several events that promote the DA constriction immediately after birth: (a) an increase in arterial oxygen tension, (b) a dramatic decline in circulating prostaglandinE_2 (PGE_2) because of metabolism in the now functioning lungs and elimination of the placental source, (c) a decrease in blood pressure within the DA lumen, and (d) a decrease in the number of PGE_2 receptors in the DA wall (Smith, 1998; Clyman, 2006).

The DA later undergoes permanent closure through structural remodeling and fibrosis. The resulting fibrous band with no lumen persists as the ligamentum arteriosum (Fay and Cooke, 1972). Anatomical closure of the DA is associated with the formation of intimal thickening, which are characterized by (a) an area of subendothelial deposition of extracellular matrix, (b) the disassembly of the internal elastic lamina and loss of elastic fiber in the medial layer, and (c) migration into the subendothelial space of undifferentiated medial smooth muscle cells (Smith, 1998). Some of these changes begin about halfway through gestation and some occur after functional closure of the DA in the neonate (Slomp *et al.*, 1997; Yokoyama *et al.*, 2006b). This cascade of events is thought to orchestrate the subsequent luminal DA reorganization, leading finally to complete obliteration of the DA. In this report, we review the current state of knowledge of the mechanisms of regulating vascular tone and remodeling of the DA.

1. Functional closure of the DA

1-1. Oxygen-induced contraction

During the fetal life, the DA is exposed to an oxygen tension that has been estimated as between 18 to 28 mmHg (Heymann and Rudolph, 1975). After birth, the DA is exposed to arterial blood and arterial oxygen tension rises rapidly after delivery. Rising oxygen tension significantly contracts the DA (Smith, 1998). With the exception of the pulmonary artery, most vascular smooth muscles relax in a low oxygen environment and contract in response to increasing oxygen tension. However, the response of the DA to oxygen is much greater in magnitude, although qualitatively similar to other vessels (Heymann and Rudolph, 1975; Smith and McGrath, 1988).

Several mechanisms have been proposed to explain the contractile effect of raising oxygen tension in the DA. The increase in oxygen tension inhibits ductal smooth muscle voltage-dependent potassium channels (Michelakis *et al.*, 2000; Reeve *et al.*, 2001), such as Kv1.5 and Kv2.1, which results in membrane depolarization, an influx of calcium and DA constriction (Nakanishi *et al.*, 1993; Leonhardt *et al.*, 2003). The inhibition of potassium channels is associated with production of diffusible redox mediator (H_2O_2) by a mitochondrial O_2-sensor, electron transport chain complexes I or III in the DA (Archer *et al.*, 2004). It has been reported that ATP-sensitive potassium channel was inhibited by the raising oxygen tension, resulting in membrane depolarization (Nakanishi *et al.*, 1993). In addition to involvement of potassium channels, recent study reported that depolarization-independent DA contraction is caused by release of calcium from the IP_3-sensitive store in the sarcoplasmic reticulum. Subsequent calcium entry through store-operated channels increases an influx of calcium and DA constriction (Hong *et al.*, 2006). Schematic illustration of the functional closure of the DA is shown in Figure 1.

Calcium entry trough L-type voltage-dependent calcium channels is involved in oxygen-induced DA contraction (Tristani-Firouzi *et al.*, 1996). Our previous study demonstrated that among L-type calcium channels, Cav1.2 was predominant isoform and expression level of Cav1.2 was higher in the rat DA than in the aorta (Yokoyama *et al.*, 2006a). Calcium influx through T-type voltage-dependent calcium channels, especially Cav3.1, also promoted oxygenation-induced DA constriction (Nakanishi *et al.*, 1993; Akaike *et al.*, 2009).

Coceani *et al.* reported that cytochrome P_{450} was the oxygen sensor and its activation promoted DA contraction through production of endothelin-1 (ET-1) from endothelium and smooth muscle of the DA (Coceani and Kelsey, 1991; Coceani *et al.*, 1992). They demonstrated stimulation of endothelin A receptor (ET_A) is associated with oxygen-induced DA contraction using mice with genetic disruption of ET_A (Coceani *et al.*, 1999). However, the DA was normally closed after birth in ET_A knockout mice. Consequently, ET-1- ET_A signaling plays a role in oxygen-induced contraction, but not in anatomical closure in the DA.

Vitamin A and/or retinoic acid signaling is a candidate for the activator of oxygen sensitivity, because the retinoic acid response element is strongly expressed in the mouse DA (Colbert *et al.*, 1996), and maternally administrated vitamin A accelerated development of the oxygen-sensing mechanism of the rat DA (Wu *et al.*, 2001). Our study demonstrated that

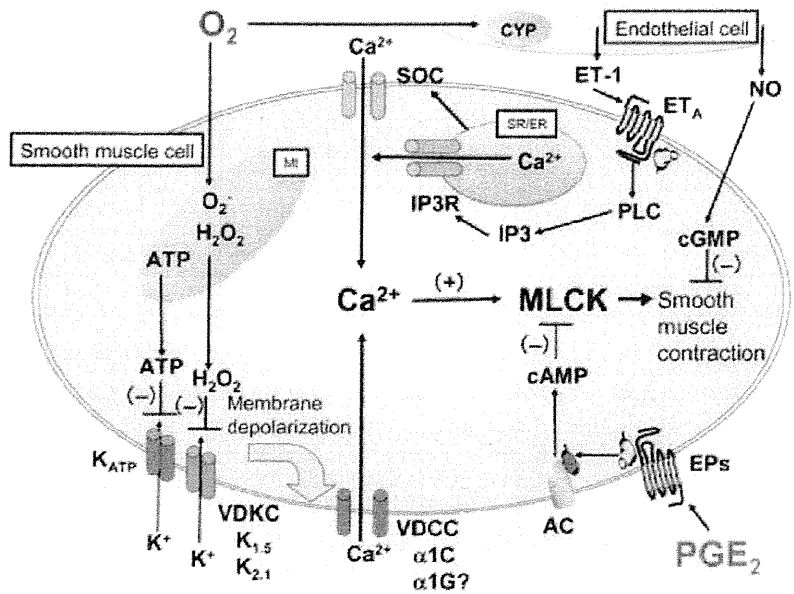

Fig. 1. A schematic model of functional closure of the DA. K^+: potassium ion, Ca^{2+}: calcium ion, O_2: oxygen, O_2^-: superoxide anion, H_2O_2: hydrogen peroxide, ATP: adenosine triphosphate, K_{ATP}: ATP-dependent potassium channel, VDCK: voltage-dependent potassium channel, VDCC: voltage-dependent calcium channel, SOC: store-operated calcium channel, Mt: mitochondria, SR/ER: sarco/endoplasmic reticulum, IP3: inositol triphosphate, IP3R: IP3 receptor, PLC: phopholipase C, MLCK: myosin light chain kinase, CYP: cytochrome P_{450}.

maternally administrated vitamin A increased the expression levels of Cav1.2 and Cav3.1 in the rat DA (Yokoyama *et al.*, 2006a).

1-2. Rapid withdrawal of the vasodilator effect of PGE_2

PGE_2 is produced in the placenta (Smith, 1998) and the DA (Clyman *et al.*, 1978; Coceani *et al.*, 1978) and contributes to the DA patency in utero. Stimulation of PGE_2 receptors activates adenylyl cyclases (Bouayad *et al.*, 2001). The increased intracellular concentrations of cyclic AMP (cAMP) inhibit myosin light chain kinase, resulting in the DA relaxation (Smith, 1998). The dilator effect of PGE_2 on the rabbit DA was mediated by the PGE_2 receptor, EP4 (Smith and McGrath, 1994). After birth, the high fetal circulating concentrations of PGE_2 dramatically decline because the placenta is removed and the lung promotes catabolism of PGE_2 (Smith, 1998). Further, the expressions of PGE_2 receptors were decreased in the DA wall (Smith, 1998; Clyman, 2006).

Both isoforms of the enzyme responsible for synthesizing PGE_2, cyclooxygenase (COX)-1 and COX-2, are expressed in the fetal DA (Takahashi *et al.*, 2000). Since COX-2 expression in the fetal DA significantly increased with advancing gestational age (Trivedi *et al.*, 2006), COX-2 inhibitor-induced DA contraction is weaker in preterm rats on the 19th day of gestation than in near-term on the 21st day (Toyoshima *et al.*, 2006). A COX inhibitor is widely used for the patent DA, however, this may not be a better therapy for premature infants with patent DA.

1-3. Other factors mediating contraction of the DA

It has been reported that nitric oxide (NO) plays a role in vasodilation of the DA. NO is synthesized by endothelial nitric oxide synthase (eNOS) in the luminal endothelium and the vasavasorum endothelium and induces relaxation of the DA through cyclic GMP (cGMP) signaling (Clyman, 2006). The relative importance of the cAMP and cGMP has been studied. Adenylyl cyclase stimulator, forskolin completely reversed the combined contractile effects of elevated oxygen tension, norepinephrin and COX inhibitor, whereas inhibition of cGMP signaling by sodium nitroprusside caused 4% of the effect of forskolin (Smith and McGrath, 1993). This implies that cAMP signaling is more important than cGMP signaling in near-term DA. On the other hand, in premature DA, the combined use of an NO synthase-inhibitor and COX inhibitor produces a much greater degree of the DA contraction than COX inhibitor alone (Seidner *et al.*, 2001).

2. Anatomical closure of the DA

2-1. Histological change during perinatal period

After birth, there is extensive remodeling of the DA wall, which leads to permanent closure of the DA. Intimal thickening, a characteristic developmental remodeling process in the DA, is required for postnatal DA closure (Rabinovitch, 1996; Mason *et al.*, 1999; Yokoyama *et al.*, 2006b). Intimal thickening starts with lifting of the endothelial cells (Gittenberger-de Groot *et al.*, 1985) and accumulations of hyaluronan in the subendothelial region, creating a space that is suitable for migration of smooth muscle cells through the fragmented elastic lamina into the subentothelial region (De Reeder *et al.*, 1988). Figure 2 shows histological change of the rat DA. Intimal thickening is developed in mature rat DA on the 21st day of gestation, while it is lacked in immature DA on the19th day of gestation. Since intimal thickening is poorly developed in human patent DA patients and animal models of patent DA (Gittenberger-de Groot *et al.*, 1980; Gittenberger-de Groot *et al.*, 1985; Tada *et al.*, 1985), this process plays an important role in permanent closure of the DA after birth.

2-2. Molecular mechanisms of regulating intimal thickening

PGE_2 plays a primary role in maintaining the patency of DA, however, previous studies have demonstrated that genetic disruption of the PGE receptor EP4 paradoxically results in fatal patent DA in mice (Nguyen *et al.*, 1997; Segi *et al.*, 1998). In addition, double mutant mice in which COX-1 and COX-2 are disrupted also exhibit patent DA (Loftin *et al.*, 2001). We found that intimal thickening was completely absent in the DA from EP4-disrupted neonatal mice (Yokoyama *et al.*, 2006b). Moreover, a marked reduction in hyaluronan production was found in EP4-disrupted DA, whereas a thick layer of hyaluronan deposit was present in wile-type DA. PGE_2-EP4-cAMP-protein kinase A (PKA) signaling up-regulates hyaluronan synthase type 2 mRNA, which increases hyaluronan production in the DA. Accumulation of hyaluronan then promotes smooth muscle cell migration into the subendothelial layer to form intimal thickening (Yokoyama *et al.*, 2006b). Signals through PGE_2-EP4 have two essential roles in DA development, namely, vascular dilation and intimal thickening.

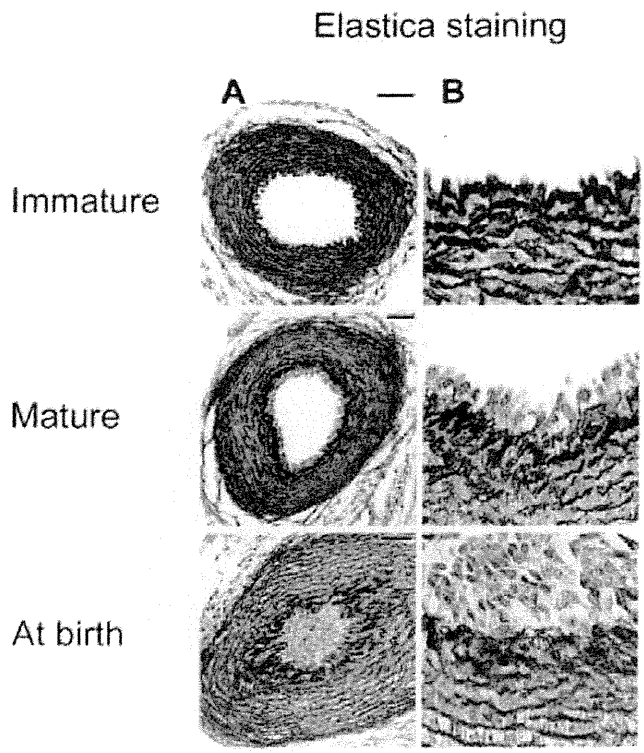

Fig. 2. Developmental changes in intimal thickening in rat DA. (A) Elastica van Gieson staining shows that intimal thickening was poor on the 19th day of gestation (immature), whereas it became apparent on the 21st day of gestation (mature) and 4 hours after birth (at birth). (B) DA intimal thickening are also shown at higher magnification.

A new target of cAMP, *i.e.*, an exchange protein activated by cAMP, has recently been discovered; it is called Epac (de Rooij *et al.*, 1998). Epac has been known to exhibit a distinct cAMP signaling pathway that is independent of PKA (Bos, 2003). Our previous study demonstrated that Epac, which is up-regulated during the perinatal period, had an acute promoting effect on smooth muscle cell migration without hyaluronan production and thus intimal thickening in the DA (Yokoyama *et al.*, 2008). Therefore, both EP4-cAMP downstream targets, Epac and PKA, induced intimal thickening in the DA (Fig. 3).

T-type voltage-dependent calcium channels, especially Cav3.1, promoted oxygenation-induced DA constriction (Akaike *et al.*, 2009). Our study revealed that Cav3.1 was significantly up-regulated in oxygenated rat DA tissue and in the region of intimal thickening of DA and that Cav1.3 promoted smooth muscle cell migration. These results indicate that Cav1.3 promotes oxygenation-induced DA closure through smooth muscle cell migration and vasoconstriction in rats (Akaike *et al.*, 2009). We also found that a novel spliced variant of the alpha1C-subunit was highly expressed in the neointima cushion of the DA (Yokoyama *et al.*, 2006a), although a role of the novel isoform is needed to be studied.