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REVIEW

Apoptosis in Heart Failure

– The Role of the β-Adrenergic Receptor-Mediated Signaling Pathway and p53-Mediated Signaling Pathway in the Apoptosis of Cardiomyocytes –

Takayuki Fujita, MD; Yoshihiro Ishikawa, MD

The heart works as a driving force to deliver oxygen and nutrients to the whole body. Interrupting this function for only several minutes can cause critical and permanent damage to the human body. Thus, heart failure (HF) or attenuated cardiac function is an important factor that affects both patient's the quality of life and longevity. Numerous clinical and basic studies have been performed to clarify the complex pathophysiology of HF and to develop effective therapies. Modulating the β-adrenergic receptor-mediated signaling pathway has been one of the most crucial targets for HF therapy. Impressively, recent reports identified p53, a well-known tumor suppressor, as a major player in the development of HF. The present review highlights the apoptosis of cardiomyocytes, which is one of the important mechanisms that leads to HF and can be induced by both β-adrenergic signaling and p53. Consideration of the cross-talk among these major pathways will be important when developing effective and safe therapies for HF. (*Circ J* 2011; **75:** 1811 – 1818)

Key Words: Adrenergic signaling; Apoptosis; Heart failure; p53

Because the heart works as a driving force to deliver oxygen and nutrients to the whole body, cardiac function is a critical factor affecting quality of life and longevity. In addition, interrupting heart function for only a few minutes can cause critical and permanent damage to the human body. A report from the United States indicated that the lifetime risk of developing congestive heart failure (HF) is approximately 20%.[1] The most common cause of HF in Western countries is coronary artery disease (CAD). Although controlling the established risk factors for CAD has become more common in general healthcare and treating with several cardioprotective agents, including β blockers, RAS inhibitors, antiplatelet agents, and statins, improves the survival rate, the current prognosis for HF is still not acceptable. Therefore, further developments in HF treatment are one of the greatest issues for extending the healthy life of humans.

Numerous clinical and basic studies have been performed to clarify the complex pathophysiology of HF. These studies have identified several mechanisms that affect cardiac function, and some therapies were developed based on these results. Many years ago, the β-adrenergic receptor (β-AR)-mediated signaling pathway was identified as one of the most important pathways that regulates cardiac function. Modulating this pathway has been one of the most crucial targets for HF therapy.[2] On the other hand, recent reports identified p53, a well-known tumor suppressor, as a major player in the development of HF.[3–5] The present review focuses on these 2 pathways.

Both pathways can induce the apoptosis of cardiomyocytes. It is well known that cardiomyocytes undergo apoptosis in response to harmful stimuli, including ischemia,[6] reperfusion,[7] oxidative stress,[8] stretching,[9] rapid pacing,[10] etc. Although some signaling mechanisms for inducing apoptosis in cardiac myocytes may be specific, such as those with Bim induction by EPAC,[11] others may be shared among different cell types. Since a 1997 report showed that failing hearts were associated with an increased number of apoptotic cardiomyocytes, the importance of apoptosis in the development of HF has been extensively examined and established in many reports.[12] The cell death of terminally differentiated cells, which cannot proliferate, directly affects tissue function. When attempting to develop an effective antiapoptotic therapy for the heart, we reviewed reports that examined the importance of each pathway in cardiomyocyte apoptosis. Based on these findings, we speculate that there is cross-talk among these pathways. Therefore, it will be important to take that into consideration when developing more effective and safe therapies.

β-AR-Mediated Apoptosis of Cardiomyocytes

The positive inotropic effect of β-AR stimulation is one of the most effective measures for maintaining cardiac output during urgent care of HF. The β-AR stimulation induces protein kinase A (PKA) activation through G protein, adenylyl

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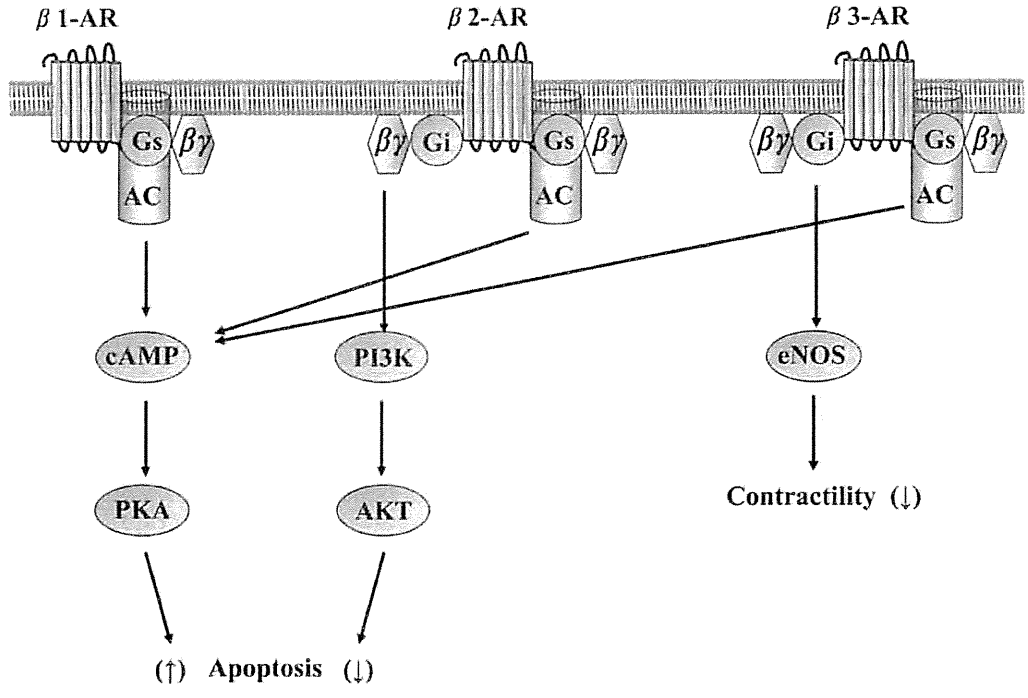

Figure 1. The β-adrenergic receptor (β-AR) receptor-mediated signaling pathway for apoptosis. Three β-AR subtypes (β1-AR, β2-AR, and β3-AR) are expressed in cardiomyocytes. Although all 3 subtypes are coupled to Gs, β2-AR and β3-AR are also linked to the Gi protein. β1-AR is thought to be mainly involved in the apoptosis of cardiomyocytes. β2-AR exerts antiapoptotic effects through Giβγ, PI3K, and AKT activation. β3-AR negatively modulates ventricular contractility through endothelial nitric oxide synthase (eNOS) activation.

cyclase (AC) and cyclic adenosine monophosphate (cAMP).[2] PKA-mediated phosphorylation of many calcium-handling molecules enhances ventricular wall motion.[13] However, long-term stimulation of these receptors can lead to the deterioration of cardiac function. In addition, the prognosis of HF patients improves with β-AR blocking therapy.[14] One of the mechanisms that contributes to this phenomenon is thought to be the induction of apoptosis upon β-AR stimulation.[15] Failing hearts have been shown to have desensitized β-adrenergic receptor signaling. This response may help maintain cardiac function.[16]

Three β-AR subtypes (β1-AR, β2-AR, and β3-AR) are expressed in cardiomyocytes (**Figure 1**). Norepinephrine or isoproterenol stimulates all β-AR subtypes and induces apoptosis in rat cardiomyocytes. However, not all subtypes of β-AR-mediated signaling induce cardiomyocyte apoptosis. It is thought that the β1-AR-mediated pathway mainly contributes to apoptosis.[17] Although all 3 subtypes are coupled to Gs, β2-AR and β3-AR are also linked to the Gi protein. The β2-AR exerts antiapoptotic effects through Giβγ, phosphatidyl inositol-3 kinase (PI3K), and AKT activation.[18] In a rat model of myocardial infarction, treating with β2-AR agonists for 2 weeks preserved cardiac contractility and reduced the number of apoptotic cardiomyocytes.[19] The β3-AR expression is upregulated in the failing heart.[20] It is reported that β3-AR negatively modulates ventricular contractility by activating endothelial nitric oxide synthase.[21] Although the role of β3-AR-mediated signaling in cardiomyocyte apoptosis is still unknown, it is possible that β3-AR exerts antiapoptotic effects through nitric oxide.[22]

Several mechanisms of β-AR stimulation-induced apoptosis have been reported.

Inducible cAMP Early Repressor (ICER)

ICERs are a group of proteins that are produced from the cAMP responsive element modulator (CREM) gene and known to induce apoptosis. PKA, which is activated by β-AR stimulation, is a key molecule that maintains ICER expression. PKA activates the cAMP-responsive element binding protein (CREB), which transactivates ICER. In addition, PKA stabilizes ICER by reducing ubiquitination.[23] Moreover, ICER attenuates phosphodiesterase (PDE) 3A transcription by interacting with the promoter region of the PDE3A gene. The downregulation of PDE3A results in elevated cAMP levels. Consequently, cAMP–PKA–ICER–PDE forms a positive feedback loop that maintains ICER expression.

ICER promotes apoptosis by downregulating Bcl-2, which is an antiapoptotic protein. Consistent with this function, isoproterenol-treated cardiomyocytes were shown to have induced ICER expression, enhanced apoptosis, and decreased Bcl-2 expression.[24] In addition, similar results were obtained in cardiomyocytes that overexpressed ICER.

ICER includes a DNA-binding domain for a cAMP-responsive element (CRE), but lacks the CREM transactivation domain. Therefore, ICER inhibits CRE-mediated transcription by CREM/CREB. Inhibiting CRE-mediated transactiva-

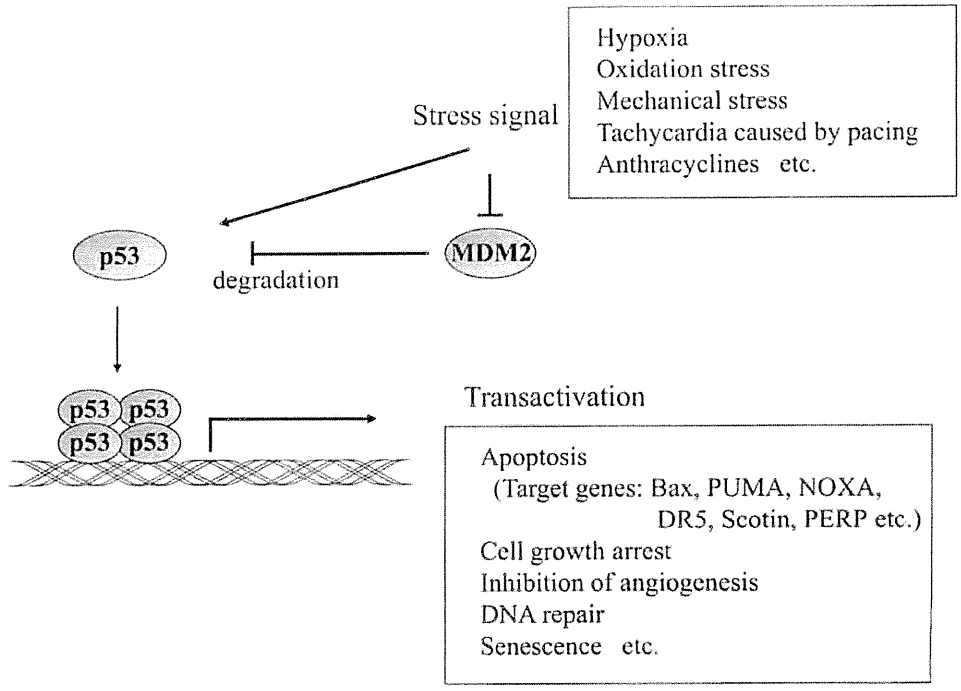

Figure 2. p53-mediated signaling pathway for apoptosis. Stresses that cause heart failure, including hypoxia, tachycardia caused by pacing, oxidative stress, mechanical stress, and anthracyclines, induce the accumulation of p53. The p53 expression level is regulated by MDM2 and MDM4, which promote the ubiquitination and degradation of p53. Accumulated p53 forms tetramers and activates the transcription of various molecules that induce apoptosis and cell growth arrest and inhibit angiogenesis, DNA repair, senescence, etc.

tion of antiapoptotic signaling is thought to be another mechanism of ICER-induced apoptosis.

Ca^{2+}/Calmodulin Kinase (CaMK), Calcineurin

β-AR stimulation increases intracellular Ca^{2+} through the L-type Ca^{2+} channel, which is essential for the proapoptotic effects of β-adrenergic stimuli. The elevated intracellular Ca^{2+} levels induce the activation of Ca^{2+}-dependent kinase, CaMK, and the phosphatase, calcineurin. Both of these proteins reportedly mediate β-adrenergic signaling-induced apoptosis. The increase in the intracellular Ca^{2+} concentration and CaMK activity is induced in a PKA-independent manner in cardiomyocytes.[25] However, the detailed mechanisms that lead to the proapoptotic effects of these proteins remain controversial. Calcineurin-independent induction of apoptosis was also observed in isoproterenol-treated cardiomyocytes.[26,27]

Exchange Protein Directly Activated by cAMP (EPAC)

cAMP, which can be induced by β-AR stimulation, activates EPAC independently of PKA.[28] EPAC, a guanine nucleotide exchange factor for the Ras-like GTPase, is involved in several cellular processes, including cell differentiation, cell proliferation, cell survival, etc. EPAC was shown to exert proapoptotic effects by inducing Bim in neuronal cells.[11] Bim directly binds to the antiapoptotic protein Bcl-2, thereby inhibiting its function.[29] However, EPAC may not play a central role in cardiomyocyte apoptosis. Overexpressing EPAC in cardiomyocytes does not induce significant apoptosis and 1 reason for this finding may be that the heart does not express Bim.

p53-Mediated Apoptosis of Cardiomyocytes

p53 is one of the most famous proteins and a major tumor suppressor, which is a group of proteins that have been well studied in cancer research.[30] Mutations in the p53 gene that attenuate p53 function have been found in 50% of human cancers.[31] This finding indicates the importance of p53 in preventing cancer. p53 mainly functions as a transcription factor and induces a variety of molecules that induce apoptosis (Bax, p53 upregulated modulator of apoptosis (PUMA), NOXA, Death receptor 5 (DR5), Scotin, p53 apoptosis effector related to PMP-22 (PERP) etc.), arrest cell growth, inhibit angiogenesis, function in DNA repair, regulate senescence, etc (Figure 2). Accumulating evidence has elucidated the importance of p53 in various cellular responses. p53 is recognized as a key molecule in the adaptation to a wide variety of harmful stimuli, including hypoxia, oxidative stress, infection, etc. In the cardiovascular system, p53 was recently shown to have a crucial function in the development of HF, arteriosclerosis, cell senescence, metabolism, etc.

We review those reports on the relationship between p53 and HF with particular emphasis on the apoptosis of cardiomyocytes.

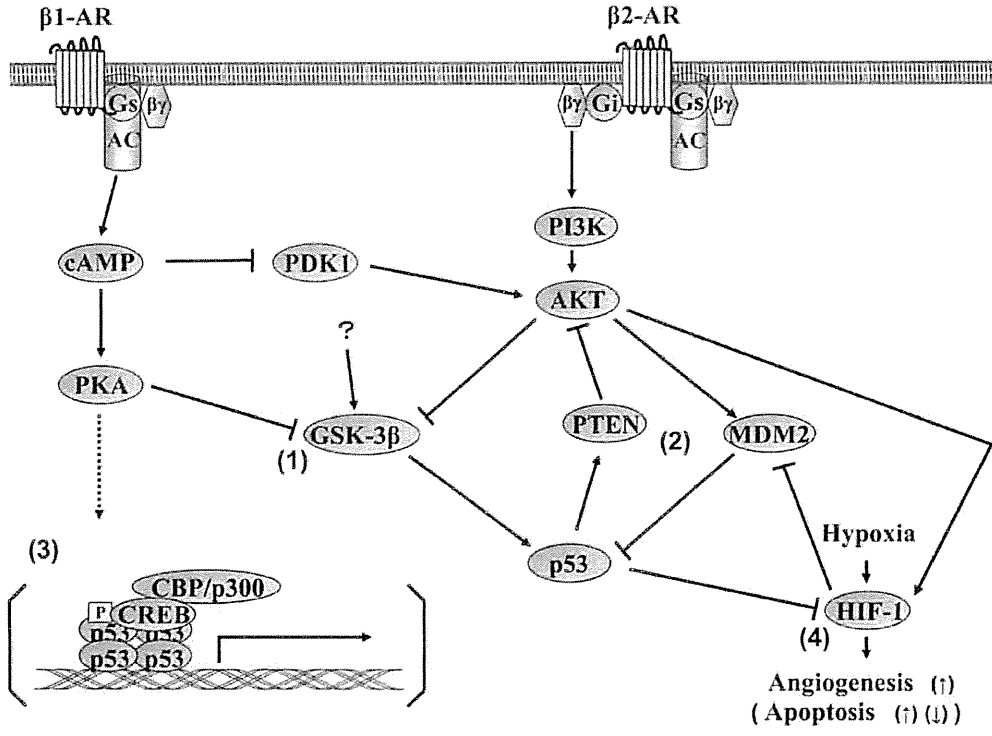

Figure 3. Cross-talk between the β-adrenergic receptor (β-AR)–mediated proapoptotic signaling pathway and p53-mediated signaling pathways. (1) Glycogen synthase kinase (GSK)-3β, (2) phosphatase and tensin homolog (PTEN)–AKT–MDM2–p53 loop, (3) cyclic adenosine monophosphate response element-binding protein (CBP)/p300–cyclic adenosine monophosphate-responsive element binding protein (CREB)–p53 complex, (4) hypoxia-inducible factor-1 (HIF-1).

p53 Expression During Stresses That Cause HF

A number of reports indicate that p53 expression is upregulated in the heart by the stresses that cause HF. Specifically, reports have shown that p53 is upregulated in the heart by ischemia,[32,33] oxidative stress,[34] mechanical stress,[35] and tachycardia caused by pacing.[36] Anthracyclines are anticancer agents that have been shown to cause cardiomyopathy, which leads to HF. Many reports demonstrated that treating with anthracyclines also induces p53 expression in cardiomyocytes.[37] In addition, involvement of telomere dysfunction induced p53 upregulation in the development of HF has been suggested.[38,39] Although not all reports support these findings,[40] accumulating evidence indicates that p53 plays an important role in stress-induced apoptosis in the heart.

Roles of p53 in the Development of HF

Several studies have been conducted to clarify the roles of p53 in the development of HF. Many studies indicate that suppressing the function of p53 induces preferable effects on cardiac function. The function of p53 was attenuated by knocking out p53[3] or PUMA,[41] which mediates the proapoptotic effects of p53, and overexpressing MDM2,[33] which induces the ubiquitination and downregulation of p53. An examination of these models showed that these direct or indirect changes in p53 function resulted in decreased cardiomyocyte apoptosis, reduced myocardial infarct size, or a better survival rate after myocardial infarction. In addition, p53-deficient mice have decreased susceptibility to anthracycline-induced myocardial apoptosis and HF.[42] On the other hand, knock-out mice for MDM4, which inhibits the accumulation of p53, develop cardiomyopathy.[43] In addition, overexpressing CHIP, which induces the degradation of p53, attenuated the accumulation of p53 and reduced cardiomyocyte apoptosis after myocardial infarction.[4] These findings indicate that p53 promotes the deterioration of cardiac function.

Recently, both apoptosis and inhibited angiogenesis were suggested to lead to the harmful effects of p53 on cardiac function. In a pressure overloaded mouse model, the cardiac condition transitions from an initial compensatory hypertrophy state to decompensatory HF several weeks after aortic banding. During this transition, p53 is upregulated, hypoxia-inducible factor-1 (HIF-1) expression is attenuated, and microvessels are reduced in the heart. HIF-1 is an established and major inducer of angiogenesis, and p53 was shown to play a pivotal role in downregulating HIF-1 expression during this transition.[5,44]

Potential Crosstalk Between the β-AR-Mediated Signaling Pathway and the p53-Mediated Signaling Pathway

Although there are only a few reports on the relationship between β-AR stimulation and the p53 expression level, p53 was shown to be upregulated in the presence of isoproterenol

in rat cultured cardiomyocytes.[45] In addition, p53 mRNA was also upregulated in cardiomyocytes that were isolated from a murine heart after long-term β-AR stimulation.[46]

On the other hand, p53 affects the expression level or activity of several molecules that can be involved in β-AR-mediated proapoptotic signal transduction, such as GSK-3β and HIF-1.

Accumulated findings obtained from studies of each pathway indicate that there are several possible cross-talk points between the β-AR- and p53-mediated signaling pathways during the induction of apoptosis (Figure 3).

Glycogen Synthase Kinase-3β (GSK-3β)

GSK-3β is a Ser/Thr protein kinase that phosphorylates and regulates many molecules that have a role in cell death, cell proliferation, cell growth, etc.[47] Several reports indicate that GSK-3β has proapoptotic effects in cardiomyocytes.[48] GSK-3β phosphorylates p53 and Bax, which facilitates proapoptotic signaling. In addition, 1 study reported that GSK-3β had a proapoptotic role in the isoproterenol-induced apoptosis of cultured adult rat cardiomyocytes.[49] These findings suggest that GSK-3β may have an important role in β-adrenergic signaling-induced p53 activation. On the other hand, GSK-3β can be inactivated via PKA- and AKT-mediated phosphorylation, which can be facilitated by the β-adrenergic signaling pathway.[50] Therefore, in contrast, β-AR stimulation can mediate antiapoptotic effects through GSK-3β. Although the mechanism by which β-AR stimulation induces apoptosis through the GSK-3β pathway is still unclear, studies suggest that a potent GSK-3β-activating pathway can overcome the effect on PKA- and AKT-mediated GSK-3β phosphorylation.

Phosphatase and Tensin Homolog (PTEN)–AKT–MDM2–p53 Loop

AKT (also known as protein kinase B) is involved in the development of hypertrophy, contractility, cell survival and inhibition of apoptosis in cardiomyocytes. The role of AKT in the heart was examined by developing a mouse model in which active AKT is specifically overexpressed in the heart. These mice had cardiac hypertrophy, increased contractility,[51] reduced infarct size and apoptosis after ischemia/reperfusion.[52] AKT is activated upon β-AR stimulation through PI3K and CaMK.[50] Therefore, β-AR stimuli-induced AKT activation may have a negative role in β-AR-induced apoptosis. Although β-AR signaling induces AKT activation through $G\beta\gamma$, at the same time $Gs\alpha$ that is also released from β-AR can inactivate AKT by inhibiting the membrane translocation of phosphoinositide-dependent protein kinase 1 (PDK1).[53] The expression levels of the molecules that are involved in these β-AR-induced pathways, including AC, are thought to be one of the deciding factors of the consequential effects on the role of these β-AR-induced pathways in AKT activity.[54]

AKT inhibits the accumulation of p53 by activating MDM2. When MDM2 is phosphorylated by AKT, MDM2 is translocated into the nucleus and promotes the degradation of p53. On the other hand, p53 inactivates AKT by transactivating PTEN. PTEN is a phosphatidylinositide phosphatase and a known antitumor molecule that inhibits AKT. PTEN overexpression causes apoptosis accompanied by AKT inactivation in cardiomyocytes.[55] Through this positive feedback loop (PTEN–AKT–MDM2–p53 loop),[56] β-AR-induced antiapoptotic signaling via AKT and p53-mediated proapoptotic signaling may eventually negatively affect each other. Regarding the β-AR-induced and p53-induced proapoptotic effects, p53 promotes β-AR-induced apoptosis, while β-AR signaling may inhibit p53-induced apoptosis through the signaling loop.

Calcineurin and Nuclear Factor of Activated T Cell (NFAT)

NFAT is a transcription factor that induces a number of molecules that cause apoptosis, cardiac hypertrophy, cell cycle control, etc. NFAT is activated by the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, which dephosphorylates NFAT, causing it to translocate from the cytoplasm to the nucleus. Carcinoma cells were shown to undergo p53-induced apoptosis through the calcineurin-dependent signaling pathway.[57] In addition, a previous report showed that both p53 and NFAT were involved in angiotensin II-induced apoptosis in vascular smooth muscle cells.[57,58] On the other hand, other reports have shown that calcineurin has a pivotal role in β-AR stimuli-induced apoptosis in cardiomyocytes.[26] Thus, calcineurin and NFAT may be involved in both the β-AR- and p53-mediated proapoptotic pathways.

Cyclic AMP Response Element-Binding Protein (CBP)/p300

CBP/p300 functions as a cofactor for several transcription factors, including p53, and facilitates their function. In addition, CBP/p300 was also shown to have histone acetyltransferase (HAT) activity. p53 is activated by CBP/p300 through acetylation.[59] When CBP/p300 activates p53, these 2 molecules form a tripartite complex with CREB. The formation of this complex is facilitated by the phosphorylation of CREB by PKA, CaMK, and protein kinase C.[60,61] PKA and CaMK are activated by β-AR signaling. Taken together, it can be speculated that there may be a situation in which β-adrenergic stimuli affect the p53 induced transactivation through the formation of the CBP/p300–CREB–p53 complex.

HIF-1

HIF-1 expression is induced by hypoxia and it predominantly functions as a transcription factor. HIF-1 transactivates a number of proteins that are involved in angiogenesis, cell proliferation, metabolism, cell survival, apoptosis, etc. HIF-1 can induce not only proapoptotic proteins such as BNIP3 and NIX, but also antiapoptotic proteins such as erythropoietin.[62] In addition, HIF-1 promotes the accumulation of p53 by directly interacting with MDM2.[63] Recent reports indicate that HIF-1 helps preserve cardiac function after hypoxic stress. HIF-1 overexpression attenuated cardiac damage after myocardial ischemia/reperfusion injury in cultured cardiomyocytes and a mouse model.[64,65] Although the enhancement of angiogenesis by HIF-1 is likely to be the important mechanism, several reports suggest that HIF-1 may modulate the apoptotic signal in cardiomyocytes.[66–69] Although the role of HIF-1 in the development of apoptosis is not well elucidated, HIF-1 is thought to be a potential factor in the apoptosis of cardiomyocytes.

HIF-1 expression is upregulated by the PI3K–AKT pathway,[70] which can be activated by $G\beta\gamma$ protein-mediated signaling. In addition, Forskolin, an AC activator that can be activated by Gs protein, also induces HIF-1 expression in cancer cells.[71] On the other hand, some reports indicate that p53 downregulates HIF-1 expression. In addition, this downregulation is inhibited by AKT activation,[72] which can be induced by β-AR stimulation. Taken together, β-AR signaling upregulates, while p53 downregulates HIF-1 expression. Therefore, HIF-1-mediated regulation of apoptosis may be a type of competitive cross-talk between the β-AR- and p53-mediated signaling pathways.

However, only a few reports have examined this pathway in cardiomyocytes, so further studies are required in order to determine the importance of this pathway in the development of HF.

Conclusions

It is crucial to maintain the number of cardiomyocytes in order to maintain the function of a failing heart. Although there have been several attempts to develop therapies that regenerate cardiomyocytes using stem cells or progenitor cells, currently there is not a clinically established method to increase in the number of cardiomyocytes.[73,74] Therefore, preventing cell death in the failing heart is still a promising approach to manage and prevent HF.

In this review, we focused on apoptosis as one mechanism of cell death in the failing heart. The β-AR- and p53-mediated signaling pathways are 2 major inducers of apoptosis. Many approaches, including gene therapy, have been developed to modulate the signaling of these pathways. Considering their effects on apoptosis, controlling these pathways could be a promising strategy to preserve cardiac function.

When attempting to establish HF therapies that modulate signal transduction, there are several important issues to be considered.

First, it is important to determine when and where signaling should be modulated. As the use of β-AR agonists or antagonists to treat HF depends on the disease state, the timing should be considered when modulating the p53 signaling pathways. p53 is a major tumor suppressor and may exacerbate HF by inducing apoptosis and inhibiting angiogenesis. However, p53 may also cause preferable effects on the heart. For example, p53 may inhibit the development of arteriosclerosis.[75] In addition, p53 may prevent the proliferation of vascular smooth muscle cells, which is pivotal in coronary restenosis after stent implantation.[76]

The β-antagonists have several side effects, including bronchial asthma, glucose intolerance, and Raynaud's phenomenon, because they affect tissues other than the heart. To avoid these effects, therapies that modulate specific subtypes of AC are near development.[77] In the same way, we should control p53 function in a tissue-specific manner.

Second, it is important to consider the possibility of cross-talk with other pathways that are involved in the development of HF. Modulating certain signaling pathways may affect others. Understanding the cross-talk among several important pathways would be useful in choosing the time and the method of therapeutic intervention to obtain the maximum effect. In this review, we noted 5 possible cross-talk points between the β-AR- and p53-mediated signaling pathways for apoptosis. Some of these are thought to facilitate another pathway. For example, β-adrenergic signaling may enhance the activity of p53 through several pathways. One of the mechanisms by which β blockers help preserve cardiac function may be by attenuating p53 function. On the other hand, potential points of competitive cross-talk have also been identified. For instance, p53 may downregulate HIF-1, while β-AR signaling may upregulate HIF-1. If there is a situation in which HIF-1 plays a significant role in regulating the apoptosis of cardiomyocytes, understanding these potentially interconnected pathways may lead to the development of more effective therapies that prevent apoptosis. Therefore, it will be important to examine the contribution of each pathway, as well as the cross-talk points under various conditions.

On the whole, to selectively inhibit the cAMP signaling pathway while preserving the PI3K–AKT pathway seems to be effective for inhibiting the apoptosis that is induced by these 2 pathways. This fact reminds us of the β1-selective β blockers. However, many pathways and molecules other than these 2 pathways are involved in the apoptosis of cardiomyocytes. Moreover, many mechanisms other than apoptosis are involved in the pathogenesis of HF. Cell death including necrosis, autophagy as well as Ca^{2+} handling, oxidative stress, metabolic state, etc have been identified as important factors that affect the development of HF. This may be one of the reasons why the advantages of β1-selective β blockers compared with nonselective β blockers for HF therapy seem not to be significant in clinical studies. In the COMET trial, the β1, β2, α1 blocker, carvedilol, extended the longevity of chronic HF patients better than the β1-selective blocker metoprolol.[78] Clarifying the relationships and roles of each signaling pathway in the various phases of HF development will lead to the development of more effective and sound treatments.

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Differential Regulation of Vascular Tone and Remodeling via Stimulation of Type 2 and Type 6 Adenylyl Cyclases in the Ductus Arteriosus

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Rationale: Prostaglandin (PG)E_2, which increases intracellular cAMP via activation of adenylyl cyclases (ACs), induces vasodilation and hyaluronan-mediated intimal thickening (IT) in the ductus arteriosus (DA) during late gestation. After birth, however, differential regulation of vasodilation and IT is preferable for treatment of patients with patent DA and DA-dependent congenital cardiac malformations.

Objective: Our objectives were to examine whether AC isoforms play differential roles in DA vasodilation and IT.

Methods and Results: AC2 and AC6 were more highly expressed in rat DA than in the aorta during the perinatal period. AC6-targeted siRNA counteracted PGE_1-induced hyaluronan production in rat DA smooth muscle cells. Overexpression of AC6 enhanced PGE_1-induced hyaluronan production and induced IT in DA explants. Furthermore, IT of the DA was less marked in mice lacking AC6 than in wild-type and AC5-deficient mice. Stimulation of AC2 attenuated AC6-induced hyaluronan production via inhibition of the p38 mitogen-activated protein kinase pathway and AC6-induced IT of the DA. An AC2/6 activator, 6-[*N*-(2-isothiocyanatoethyl) aminocarbonyl] forskolin (FD1), did not induce hyaluronan-mediated IT in DA explants, although an AC5/6 activator, 6-[3-(dimethylamino)propionyl]-14,15-dihydroforskolin (FD6) did. Moreover, FD1 induced longer vasodilation of the DA than did PGE_1 without significant adverse effects in vivo.

Conclusions: AC6 is responsible for hyaluronan-mediated IT of the DA and AC2 inhibited AC6-induced hyaluronan production. Stimulation of both AC2 and AC6 by FD1 induced longer vasodilation without hyaluronan-mediated IT in the DA in vivo. FD1 may be a novel alternative therapy to currently available PGE therapy for patients with DA-dependent congenital heart disease. (*Circ Res.* 2010;106:1882-1892.)

Key Words: patent ductus arteriosus ■ prostaglandins ■ smooth muscle ■ vasodilation ■ remodeling

Prostaglandin (PG)E_2 and PGE_1 play principal roles in maintaining the patency of the ductus arteriosus (DA) during gestation. PGE_1 is widely used to keep the DA open in patients with DA-dependent congenital heart diseases, because both PGE_1 and PGE_2 increase the intracellular concentration of cAMP, resulting in vasodilation in the DA.[1,2] On the other hand, we have demonstrated that PGE-EP4-cAMP signals during late gestation increased hyaluronan production in the DA and consequently induced intimal thickening (IT), which is critical for permanent closure of the DA after birth.[3] Therefore, the effects of $PGE_{1/2}$ on vasodilation and remodeling oppose each other in terms of regulation of the DA after birth. Differential regulation of vasodilation and IT in the DA would be preferable for patients who need PGE/anti-PGE therapy.

Because intracellular cAMP is synthesized by adenylyl cyclases (ACs), which are transmembrane enzymes activated by G protein-coupled receptors, including PGE receptors, ACs must play an important role in regulating vasodilation and remodeling in the DA. To date, nine different isoforms of membrane-bound forms of ACs (AC1 through AC9) have been identified in vertebrate tissues.[4] Most tissues express several AC isoforms, which exhibit remarkable diversities in their biochemical properties.[5,6] Because smooth muscle cells

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(SMCs) in the DA exert biological properties distinct from SMCs in other vessels such as the aorta, we hypothesized that such properties are attributable, at least in part, to the distinct roles of specific AC isoforms in the DA.

In addition to the role of $PGE_{1/2}$ in vasodilation, the PGE-AC-cAMP signal cascade has been shown to regulate vascular remodeling.[7,8] For example, cAMP markedly inhibits proliferation of SMCs[9] and reduces IT after arterial injury in vivo,[10] a process that shares many aspects with IT in the DA.[1,11] Interestingly, several studies have demonstrated that $PGE_{1/2}$ inhibits the proliferation of vascular SMCs,[7,8] whereas others have reported that PGE_2 stimulates the growth of vascular SMCs.[12,13] Such diversities in the effects of PGE signaling might be related to differential expression of AC isoforms among vascular tissues. It has been difficult, however, to evaluate the contribution of ACs to relevant phenomena in an AC isoform-dependent manner, because multiple isoforms of ACs are coexpressed. This is partially attributable to the lack of available AC isoform-selective pharmacological regulators. In previous studies, we synthesized more than 200 new derivatives of forskolin (a non–isoform-selective AC activator) and identified derivatives that are selective to specific AC isoforms.[6,14,15] Such AC isoform-selective activators enable us to explore the role of each AC isoform in vascular tone and remodeling especially in vivo. In the present study, using such AC isoform-specific activators, overexpression or selective silencing of AC isoforms and AC-isoform deficient-mice, we have investigated the role of AC isoforms and the availability of AC isoform-selective activators in regulating DA vascular tone and remodeling.

Non-standard Abbreviations and Acronyms

AC	adenylyl cyclase
Adv	adenovirus-mediated gene transfer
DA	ductus arteriosus
E	embryonic day
ERK	extracellular signal-related kinase
FD1	6-[*N*-(2-isothiocyanatoethyl) aminocarbonyl] forskolin
FD6	6-[3-(dimethylamino)propionyl]-14,15-dihydroforskolin
HAS2	hyaluronan synthase type 2
IT	intimal thickening
JNK	c-Jun N-terminal kinase
KO	knockout
MAPK	mitogen-activated protein kinase
PGE	prostaglandin E
PK	protein kinase
siRNA	small interfering RNA
SMC	smooth muscle cell

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Reagents

Forskolin derivatives: 6-[*N*-(2-isothiocyanatoethyl) aminocarbonyl] forskolin (FD1),[14,16] and 6-[3-(dimethylamino)propionyl]-14,15-dihydroforskolin (FD6)[14] were kindly provided by Nippon Kayaku Co, Ltd (Tokyo, Japan).

Animals and Tissues

All animals were cared for in compliance with the guiding principles of the American Physiological Society. The experiments were approved by the ethical committee of animal experiments at Yokohama City University School of Medicine. Wistar rat embryos were obtained from timed-pregnant mothers (Japan SLC Inc, Shizuoka, Japan). Pooled tissues of DA and aorta were obtained from rat embryos on embryonic day (E)19 (n>60) and E21 (n>60) and neonates on the day of birth (day0, n>60). Generation and phenotypes of AC5 knockout mice (AC5KO) and AC6 knockout mice (AC6KO) have been described previously.[17,18] All mice were littermates from heterozygote crosses.

Isolation and Culture of Rat Ductus Arteriosus Smooth Muscle Cells

Vascular SMCs were obtained from DA and aorta of Wistar rat embryos at E21 as previously described.[19]

Quantitative and Semiquantitative RT-PCR

Isolation of total RNA and generation of cDNA were performed and RT-PCR analysis was done as previously described.[19] The primers were designed based on the rat nucleotide sequences of AC isoforms. Each primer set was designed between multiple exons (Online Table I), and PCR products were confirmed by sequencing. The abundance of each gene was determined relative to the GAPDH transcript using TaqMan Rodent GAPDH control reagents kits (Applied Biosystems, Foster City, Calif).

Immunoblot Analysis

Proteins from whole cells were analyzed by immunoblotting as previously described.[19]

RNA Interference

Double-stranded small interfering (si)RNAs to the selected regions of AC2–7 and the negative siRNA used as a control were purchased from QIAGEN (Hilden, Germany) or Invitrogen (San Diego, Calif) (Online Table II). According to the instructions of the manufacturer, cells were transfected with siRNA (300 pmol), using Lipofectamin RNAiMAX (Invitrogen).

Adenovirus Construction

Full-length cDNA-encoding rat AC2 was cloned into the shuttle vector for construction of an adenoviral vector harboring AC2 through the use of an AdenoX adenovirus construction kit (Clontech, Tokyo, Japan). Adenovirus encoding murine AC6 driven by a cytomegalovirus promoter was generated by homologous recombination as previously described.[20] Adenovirus encoding MKK3 was kindly provided by Dr Yibin Wang (University of California, Los Angeles).[21]

cAMP Production by Radioimmunoassay

Rat ductus arteriosus smooth muscle cells (DASMCs) were serum-starved for 48 hour and assayed for cAMP production by RIA after incubation with drugs of interest (Online Data Supplement).

Quantitation of Hyaluronan

The amount of hyaluronan in the cell culture supernatant was measured by the latex agglutination method as previously described.[3]

Organ Culture

DA organ culture was performed as previously described.[3,22]

Measurement of Isometric Tension of the Vascular Rings of DA

Isometric tension of the vascular rings of DA was measured as previously described.[23]

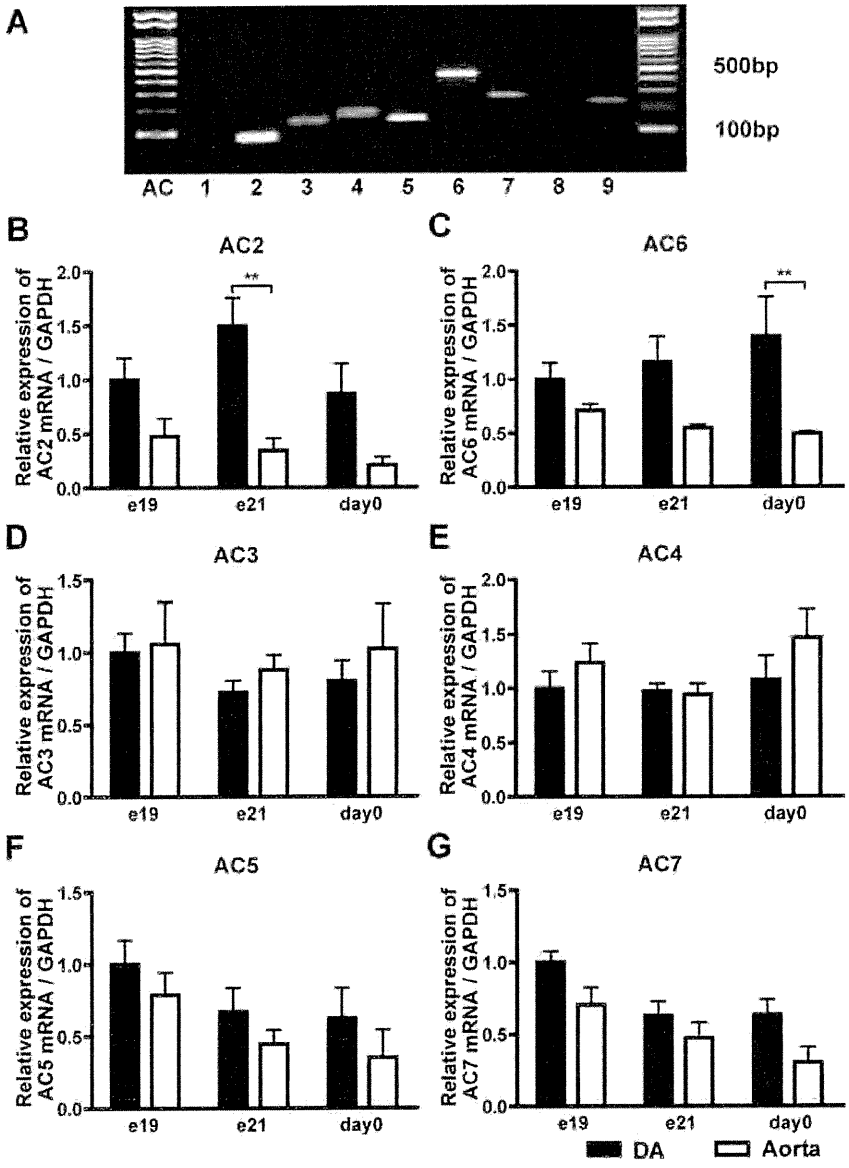

Figure 1. Multiple transcripts of AC isoforms in rat DA. A, mRNA expression of AC isoforms using semiquantitative RT-PCR in rat E21 DA. **B through G,** Quantitative RT-PCR analyses of AC2–7. $^{**}P<0.01$. Data are from 6 independent experiments.

Rapid Whole-Body Freezing Method

To study the in situ morphology and inner diameter of neonatal DA, a rapid whole-body freezing method was used as previously described.[24] The fetuses at E21 were delivered by cesarean section and intraperitoneally injected 1 hour after birth with PGE_1, FD1 or FD6 in 200 μL of saline. The minimal dose of FD1 (10.8 mg/kg of body weight) and FD6 (1.29 mg/kg of body weight) that caused maximal dilation in the DA were used.

Protein Kinase A Activity

Protein kinase (PK)A activity was measured using an assay kit (StressGen Biotechnologies, Ann Arbor, Mich) according to the instructions of the manufacturer, as described previously.[25]

Statistical Analysis

Data are shown as the means±SEM of independent experiments. Statistical analysis was performed between two groups by unpaired Student t test or between multi-groups by one-way ANOVA followed by Student–Newman–Keuls multiple comparison test. A value of $P<0.05$ was considered significant.

Results

Multiple Transcripts of AC Isoforms in Rat DA

First, we detected all isoforms except for AC1 and AC8 in rat E21 DA by semiquantitative analyses (Figure 1A). Next, quantitative RT-PCR analyses of AC2–7 showed that AC2, AC5, and AC6 were abundantly expressed in rat DA and that the expression levels of AC2 and AC6 were significantly higher in the DA than in the aorta during the perinatal period, whereas those of AC5 were comparable between the DA and the aorta. The expression of AC2 reached maximal level in E21 DA (Figure 1B), whereas that of AC6 was increased during development in rat DA (Figure 1C).

AC6 Is Responsible for Hyaluronan Production in DASMCs

We examined the contribution of AC2, AC5 and AC6 to PGE_1-induced cAMP production in DASMCs by using AC2-,

AC5-, and AC6-targeted siRNAs. The expression levels of ACs mRNAs using the siRNAs are shown in Online Figure I. Silencing of AC5 or AC6 dramatically decreased PGE_1-induced cAMP production and that of AC2 also decreased PGE_1-induced cAMP production by 58% (Figure 2A), indicating that AC2, AC5 and AC6 are major isoforms responsible for cAMP production by PGE_1 in DASMCs. We then examined the effect of ACs on hyaluronan production in DASMCs. AC6-targeted siRNA weakened PGE_1-induced hyaluronan production, whereas AC2-, and AC5-targeted siRNA did not (Figure 2B). Neither AC3-, AC4-, nor AC7-targeted siRNA weakened PGE_1-induced hyaluronan production (Online Figure II). Using adenovirus-mediated gene transfer of AC2 and AC6 (Adv.AC2 and Adv.AC6), efficacy of which is shown in Online Figure III, we found that the overexpression of AC6, but not of AC2, further enhanced PGE_1-induced hyaluronan production when compared with the overexpression of LacZ as a control (Figure 2C). Interestingly, co-overexpression of both AC2 and AC6 negated AC6-mediated enhancement of hyaluronan production.

AC6 Gene Transfer, but Not AC2, Promoted IT in Rat DA Explants

When AC6 was overexpressed in immature rat DA explants in which IT had not yet formed, prominent IT with strong hyaluronan deposition was observed in AC6-overexpressed DA explants, as compared to LacZ controls (Figure 3A, 3B, and 3D). The internal lumen of the DA treated with Adv.AC6 was almost completely closed (Figure 3C). However, overexpression of AC2 did not promote hyaluronan deposition and IT formation. Further, Adv.AC2 abrogated AC6 overexpression-induced hyaluronan production and IT ex vivo, which is consistent with the data in Figure 2C. Taken together, these results indicate that AC2 has an inhibitory effect on AC6-induced hyaluronan-mediated IT in DA explants.

AC6 Deficiency Decreased IT in Mouse DA

Although AC5 and AC6 share an extremely high amino acid homology, the above experiments suggested that AC6 is a major isoform for DA remodeling. We therefore examined whether AC6 indeed plays a major role in in vivo IT of the DA and found that genetic disruption of the AC6 isoform resulted in less IT during late gestation (E18.5) (Figure 4B, 4D, and 4F). It should be noted that DAs closed after birth in AC6KO mice (data not shown). The IT of AC5KO mice was normally developed at E18.5 (Figure 4A, 4C, and 4E), and the DA of AC5KO mice closed after birth (data not shown). These findings support the conclusion that AC6 plays a primary role in IT and, thus, the vascular remodeling in the mouse DA.

Effect of Isoform-Selective AC Activators on cAMP Accumulation in Rat DASMCs

Based on the findings of previous crystallographic studies and computer-assisted drug design, we identified forskolin derivatives (FD1 or FD6) that have enhanced selectivity for AC2 or AC5 in regulating tissue AC catalytic activity.[14]

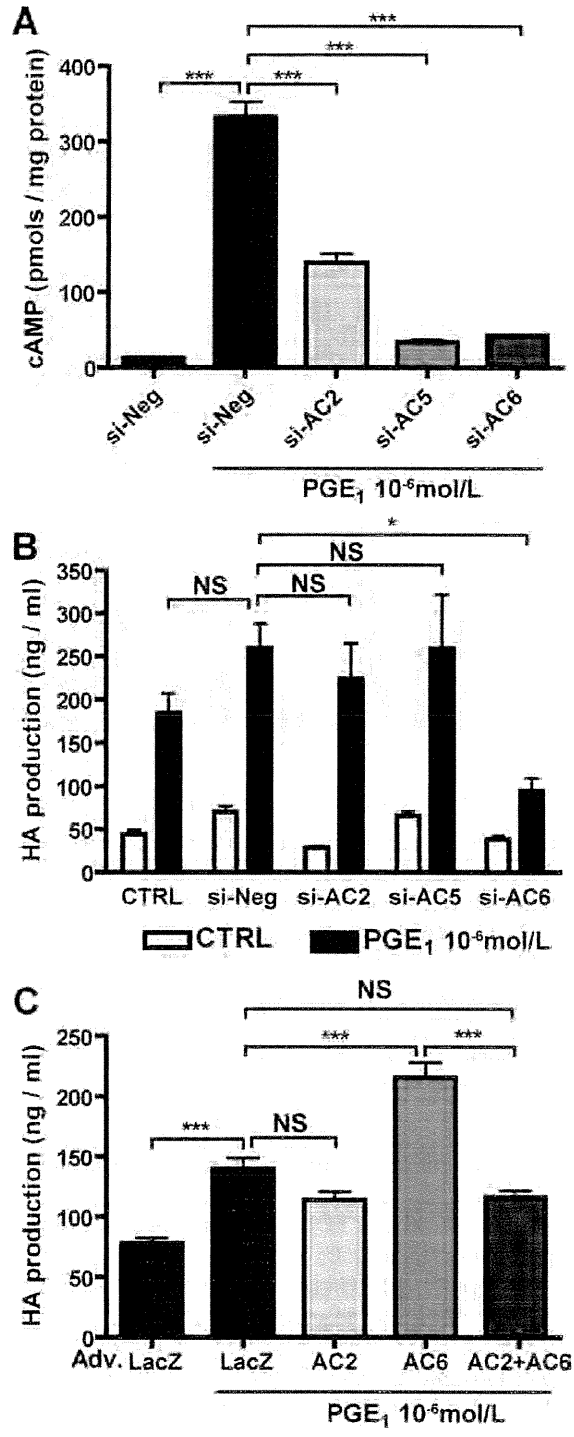

Figure 2. AC6 is responsible for hyaluronan production in DASMCs. A, PGE_1 induced cAMP accumulation in DASMCs in the cells treated with negative siRNA. AC2-, AC5-, and AC6-targeted siRNA decreased PGE_1-induced cAMP production (n=4). **B,** AC6-targeted siRNA attenuated PGE_1-induced hyaluronan (HA) production, whereas AC2- and AC5-targeted siRNA did not (n=7 to 11). **C,** Adv.AC6 enhanced PGE_1-induced hyaluronan production. Adv.AC2 abolished the Adv.AC6-induced enhancement of hyaluronan production (n=4). $^*P<0.05$ and $^{***}P<0.001$. NS indicates not significant.

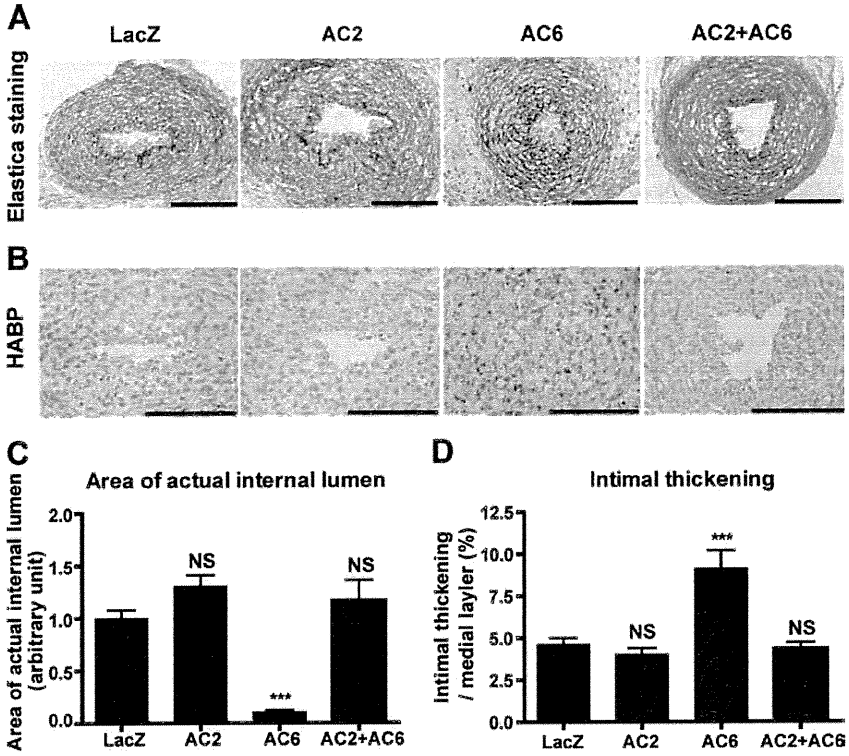

Figure 3. Adenovirus-mediated AC6 gene transfer promoted IT in rat DA explants. A, Elastica van Gieson staining for cultured DA explants overexpressed with Adv.LacZ, Adv.AC2, Adv.AC6, or Adv.AC2+Adv.AC6. **B,** Strong immunoreaction to hyaluronan in DA explants cultured with Adv.AC6. **Bars:** 100 μm. **C,** The area of the internal lumen of the DA treated with Adv.AC6 was significantly decreased (n=8 to 9). **D,** The ratio of IT to the thickness of the medial layer was increased in the DA treated with Adv.AC6, but not with Adv.AC2 (n=8 to 9). ***$P<0.001$. HABP indicates hyaluronan-binding protein.

However, the ability of cAMP production via AC6 of FD1 and FD6 has not been demonstrated. FD1 enhanced LacZ control-induced cAMP accumulation in DASMCs infected with Adv.AC2 or Adv.AC6 (Figure 5A). FD6 enhanced cAMP accumulation in DASMCs with Adv.AC6, but not with Adv.AC2. These data suggest that FD1 stimulates both AC2 and AC6 and that FD6 stimulates AC5 and AC6. We confirmed that FD1 (AC2/6 stimulator) and FD6 (AC5/6 stimulator) increased cAMP accumulation in DASMCs in a dose-dependent manner (Figure 5B).

The Effects of Isoform-Selective AC Activators on DASMC Hyaluronan Production

We then found that FD6 significantly increased hyaluronan production (Figure 5C) and transcripts of hyaluronan synthase type 2 (HAS2) in DASMCs at 10^{-5} mol/L (Figure 5D). In contrast, FD1, in doses up to $10^{-5.5}$ mol/L, did not increase hyaluronan production or HAS2 transcripts up. It should be noted that production of cAMP by FD1 at a concentration of $10^{-5.5}$ mol/L was equivalent to that by FD6 at 10^{-5} mol/L (Figure 5B and 5C) and that FD1 significantly decreased DASMC viability at a concentration higher than 10^{-5} mol/L. Silencing of AC6, but not of AC5, abolished FD6-induced hyaluronan production (Figure 5E), indicating that AC6 is responsible for FD6-induced hyaluronan production. Furthermore, to examine whether the effect of FD6 on hyaluronan production is specific to DASMCs, we found that FD6 did not induce hyaluronan production in SMCs from the rat aorta (Figure 5F), because expression of AC6 mRNA in aortic SMCs was approximately 60% lower than in DASMCs. However, when AC6 was overexpressed in the aortic SMCs, hyaluronan production was significantly increased by 1.4±0.1-fold (n=6) in the presence of FD6 (10^{-5} mol/L), suggesting that this data can provide insight into a more general vascular remodeling by AC6.

Involvement of MKK3-p38 MAPK in AC6-Induced Hyaluronan Production

To examine the mechanism by which AC2 inhibits AC6-induced hyaluronan production, we focused on several signal pathways such as p38 mitogen-activated protein kinase (MAPK). We found that FD6 increased phosphorylation of p38 protein in DASMCs, whereas FD1 and N6-Benzoyladenosine-cAMP (Bnz-cAMP), a PKA selective cAMP analog, did not (Figure 6A and 6B). FD6-induced phosphorylation of p38 and MKK3/6 was negated in DASMCs treated with AC6-targeted siRNA (Figure 6C). FD1 increased phosphorylation of p38 and MKK3/6 when AC2 expression was downregulated by AC2-targeted siRNA (Figure 6C). FD6-induced hyaluronan production was attenuated by SB203580, a p38 inhibitor, or H89, a PKA inhibitor. Combined treatment of SB203580 and H89 further inhibited hyaluronan production (Figure 6D). In contrast, SB203580 did not affect PKA-induced hyaluronan production (Figure 6E). These data suggest that p38 MAPK and PKA independently regulate hyaluronan production. Furthermore, overexpression of MKK3, the efficacy of which is demonstrated by Adv.MKK3 (Figure 6F), enhanced FD6-induced hyaluronan production in DASMCs (Figure 6G). Extracellular signal-related kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK) were not phosphorylated by FD6 (data not shown). Phospholipase C, PKC, IP3 receptor, PI3-kinase, and Epac signaling were not

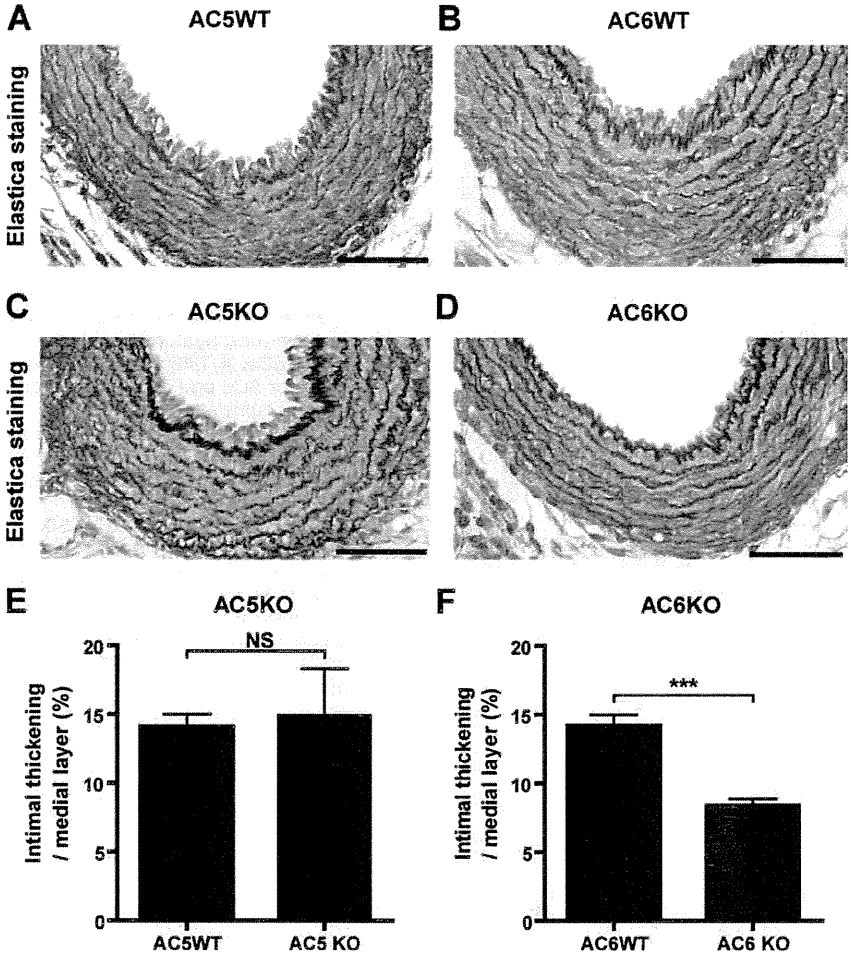

Figure 4. Impaired IT in the mouse DA attributable to AC6, but not AC5, deficiency. A, C, and E, DAs from AC5KO mice at E18.5 were stained with elastica van Gieson stain. Both AC5KO and wild-type (WT) mice showed IT in the DA (n=4 to 5). **B, D, and F,** DAs from AC6KO mice at E18.5 had less IT compared to wild-type mice (n=8). **Bars:** 50 μm. ***$P<0.001$.

involved in AC6-induced hyaluronan production, as shown using specific agonists or inhibitors for each pathway (Online Figure IV). These data indicate that stimulation of AC6 promotes hyaluronan production via both p38 and PKA pathways and that AC2 inhibits the AC6-activated MKK3-p38 pathway.

The Effects of Isoform-Selective AC Activators on IT Ex Vivo

We then examined the effects on FD1 and FD6 on IT using DA explants. Consistent with other data (Figures 5 and 6), 48 hour incubation with FD6 significantly induced IT, increased hyaluronan production, and narrowed the internal lumen in DA explants (Figure 7). It should be noted that FD1 and FD6, similarly to forskolin and PGE_1, inhibited proliferation of DASMCs. Overexpression of AC2 or AC6 also inhibited DNA synthesis in DASMCs (Online Figure V), indicating that FD6 does not directly promote IT by proliferation of DASMCs.

The Effects of Isoform-Selective AC Activators on Vasodilation

Because $PGE_{1/2}$ strongly dilates the DA via activation of ACs, AC activators should be potent vasodilators for the DA as well. We found that FD1 and FD6 similarly attenuated indomethacin-induced contraction in DA explants (Figure 8A). We then examined the vasodilatory effect of FD1 and FD6 in vivo using a whole-body freezing method. Here, the DA closed completely 1 hour after birth (Figure 8B) when PGE_1, FD1, or FD6 were administrated by intraperitoneal injection. Consistent with previous data,[24] PGE_1 caused maximal dilatation 30 minutes after injection and then the DA completely closed within 2 hour (Figure 8C and 8D). FD1 induced maximal dilatation of the DA up to 4 hour and then the DA gradually contracted, suggesting that FD1 has a longer vasodilatory effect on the DA than dose PGE_1. Although FD6 also dilated the DA 30 minutes after injection, all neonates died approximately 4 hours after injection because of suppression of respiration, which is the same side effect caused by PGE_1 and was not caused by FD1. We also found that FD1 and FD6 did not affect the diameter of the ascending aorta, whereas FD6, but not FD1, significantly dilated the main pulmonary artery up to 4 hour after administration (Online Figure VI, A through C). Using cultured DASMCs, AC6-targeted siRNA attenuated forskolin-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP), whereas AC2-targeted siRNA had no effect, suggesting that AC6 is primarily involved in DA vasodilation (Online Figure VI, D).

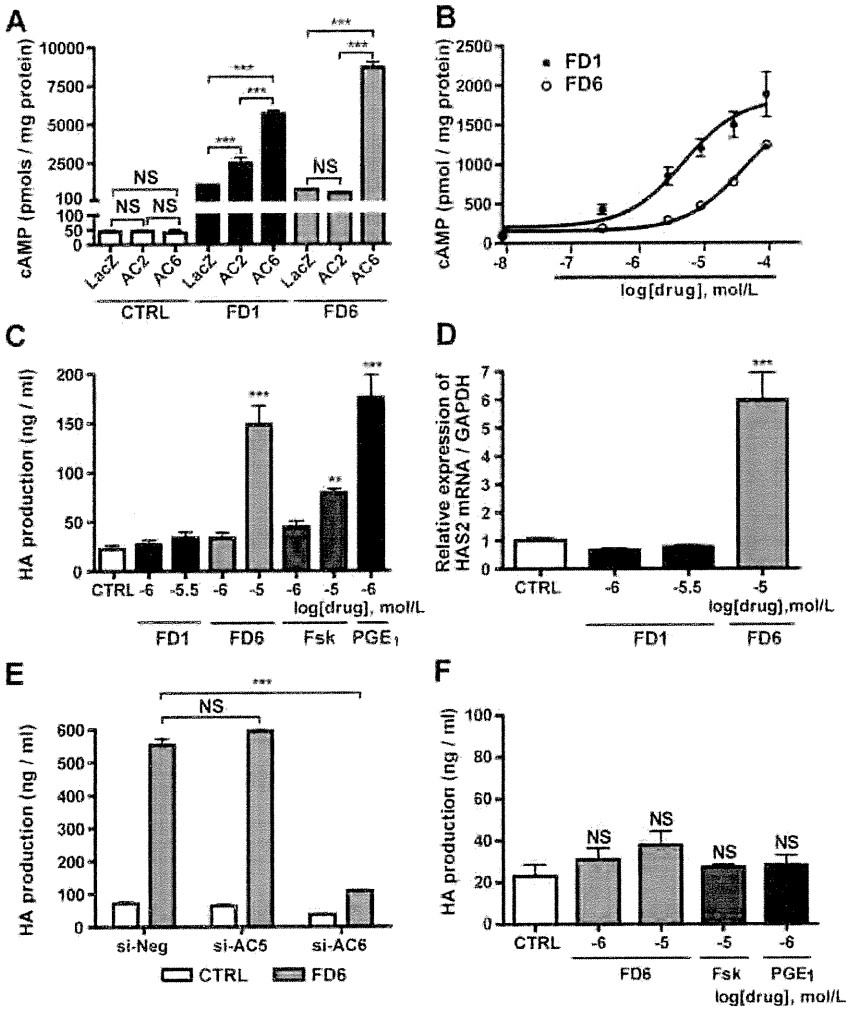

Figure 5. The effects of FD1 and FD6 on cAMP and hyaluronan production in DASMCs. A, Effect of overexpression of AC2 or AC6 on FD1- or FD6-induced cAMP accumulation (n=6). **B,** FD1 and FD6 increased cAMP accumulation in DASMCs in a dose-dependent manner (n=4). **C,** FD6, but not FD1, increased hyaluronan production (n=8 to 11). **D,** FD6 significantly increased HAS2 mRNA (n=6). **E,** AC6-targeted siRNA negated FD6-induced hyaluronan production (n=4). **F,** FD6, PGE_1, and forskolin did not induce hyaluronan production in aortic SMCs (n=6). $^{**}P<0.01$ and $^{***}P<0.001$ vs control (CTRL). Fsk indicates forskolin.

Taken together, these results reveal that AC6 play a primary role in hyaluronan-mediated vascular remodeling in the DA through activation of the PKA and MKK3-p38 MAPK pathways and that AC2 has an inhibitory effect on AC6-mediated hyaluronan production and IT.

Discussion

The Effect of AC2, AC5, and AC6 on Vascular Remodeling

The present study demonstrated for the first time that AC plays a significant role in vascular remodeling in the DA. Intimal thickening during development is a critical form of vascular remodeling for postnatal closure of the DA.[3,26] We found that hyaluronan induced by PGE-EP4-cAMP signaling is a prominent constituent of the extracellular matrix of IT in the DA.[3] It seems a worthwhile endeavor to investigate mechanisms leading to an increase in hyaluronan, because insights into its regulatory mechanisms and signaling pathways might eventually lead to ways of controlling hyaluronan-mediated IT in the DA. However, the isoform-selective role of AC in DA vascular remodeling has not previously been reported. Importantly, we found that AC6 is responsible for hyaluronan-mediated IT in the DA via activation of the MKK3-p38 and PKA pathways and that AC2 has an inhibitory effect on AC6-mediated hyaluronan production and DA remodeling. We also demonstrated that simultaneous stimulation of AC2 and AC6 by FD1 produces a longer vasodilatory effect than does PGE_1 without inducing hyaluronan-mediated IT.

It is important to identify whether the source of hyaluronan is from SMCs or endothelial cells of the DA. Using cell sorting analysis, we found that the expression levels of HAS1, HAS2 and AC6 mRNAs in CD31-positive/CD45-negative endothelial cells from E21 rat DA were significantly lower than those in CD31-negative/CD45-negative SMCs from E21 rat DA (Online Figure VII). Therefore, we believe that DASMCs are a major source of hyaluronan production.

DA IT in AC6-Deficient Mice

Our results also showed that the DA of AC6KO mice had less fully formed IT during late gestation. These results support the belief that AC6 plays a role in EP4-mediated hyaluronan synthesis. Nevertheless, the DA of AC6KO mice eventually closed after birth, similarly to wild-type mice, whereas the DA remained open in EP4KO mice. These results suggest

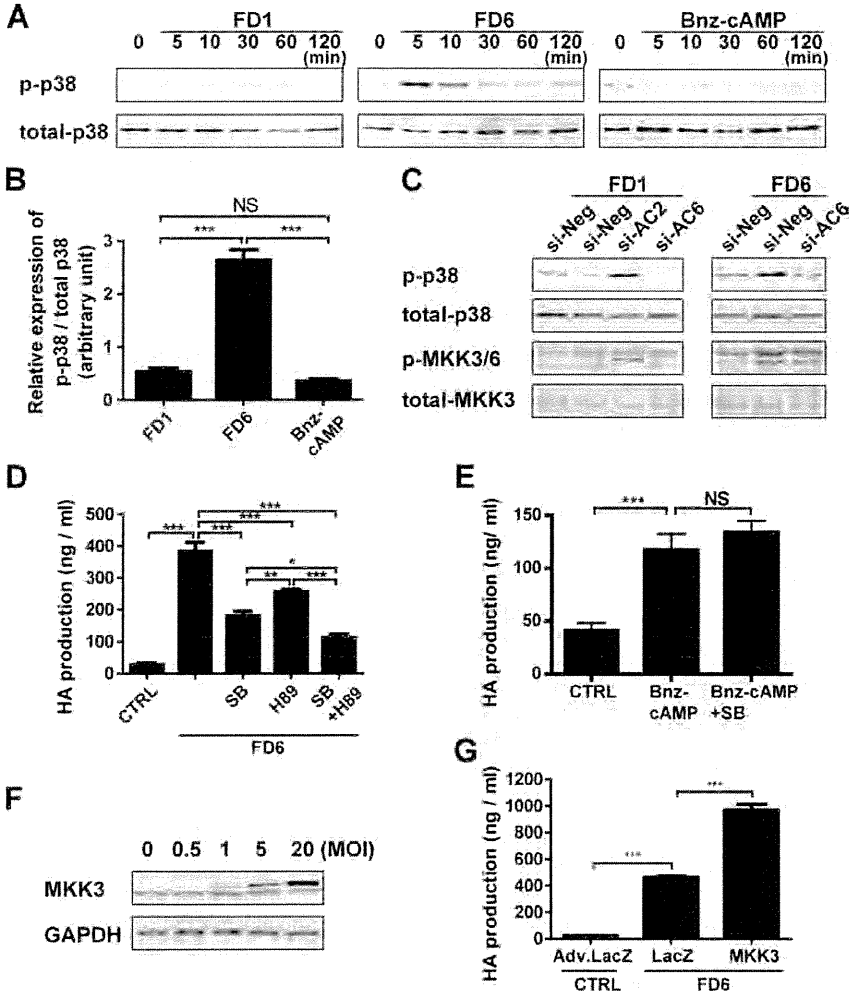

Figure 6. The signaling pathway of AC6-induced hyaluronan production in DASMC. A, Phosphorylation of p38 protein (p-p38) by FD1 ($10^{-5.5}$ mol/L), FD6 (10^{-5} mol/L), or Bnz-cAMP (10^{-5} mol/L) (n=4). **B,** Quantification of the ratio of p-p38 to total p38 after 5 minutes stimulation by FD1, FD6 or BnzcAMP. (n=4). **C,** Phosphorylation of p38 and MKK3/6 induced by 5 minutes treatment of FD1 ($10^{-5.5}$ mol/L) or FD6 (10^{-5} mol/L) in DASMCs treated with si-negative, si-AC2, or si-AC6 RNA (n=4). **D,** FD6-induced hyaluronan production was attenuated by SB203580 (SB) (2×10^5 mol/L) and H89 (10^6 mol/L) (n=6). **E,** SB203580 (2×10^5 mol/L) did not affect Bnz-cAMP-induced hyaluronan production (n=6). **F,** MKK3 protein expression by Adv.MKK3. MOI indicates multiplicity of infection. **G,** Adv.MKK3 enhanced FD6-induced hyaluronan production (n=6). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

that other AC isoforms might compensate for deficiency of AC6. Alternatively, other EP4 signal pathways in addition to the AC-cAMP pathway may be involved in the patency of EP4KO mice. Further study is required to determine how multiple signaling pathways contribute to yield IT in the DA. We assume that the closure of the DA in AC6KO mice may be delayed after birth because of insufficient IT, which will also be addressed in our future studies.

Interaction of AC2 and AC6 Signaling in Hyaluronan-Mediated IT

AC isoforms have specific patterns of regulation by G proteins, protein kinases and calcium/calmodulin. For example, cAMP production by AC2 is stimulated by several signals including G_s-α and $\beta\gamma$-subunits, and by PKC. In contrast, cAMP production by AC6 is only stimulated by G_s-α and inhibited by G_i-α, PKA, and low concentrations of Ca^{2+}.[4,27] Moreover, different AC isoforms have different effects on cAMP-mediated responses independent of their rate of synthesis of cAMP.[28] To the best of our knowledge, this is the first study to show how the effect of an AC isoform counteracts the effect of another isoform independent of cAMP production. We demonstrated that overexpression of AC2 by itself has little effect on hyaluronan production and appears to have an inhibitory effect on AC6-induced hyaluronan and IT. These data are consistent with the other experiments in which activation of both AC2 and AC6 by FD1 did not induce hyaluronan and IT in DASMCs and DA explants. The response of FD6 to hyaluronan production was much greater than that of FD1, even though FD6 produced less cAMP than FD1 at the same concentrations, suggesting that this process is not simply dependent on the amount of intracellular cAMP.

The next important question is the mechanism how AC2 and AC6 differentially regulate vascular remodeling of the DA. Our results demonstrated that AC6 induced hyaluronan production via the MKK3-p38 MAPK and PKA pathways and that AC2 inhibited the MKK3-p38 pathway, resulting in inhibition of AC6-induced hyaluronan production. PKC, phospholipase C, PI3-kinase, Epac, and other MAPK pathways including ERK and JNK were not involved in AC6-induced hyaluronan production. In addition, we also found that AC2 is primarily localized in the caveolae fraction, whereas AC5/6 localized in the caveolae and the noncaveolae fractions (Online Figure VIII). This differential localization may change the effect of AC2 and AC6 on the downstream signal pathways. Identification of the upstream target linking AC6 and the MKK3-p38 pathways will be addressed in future studies.

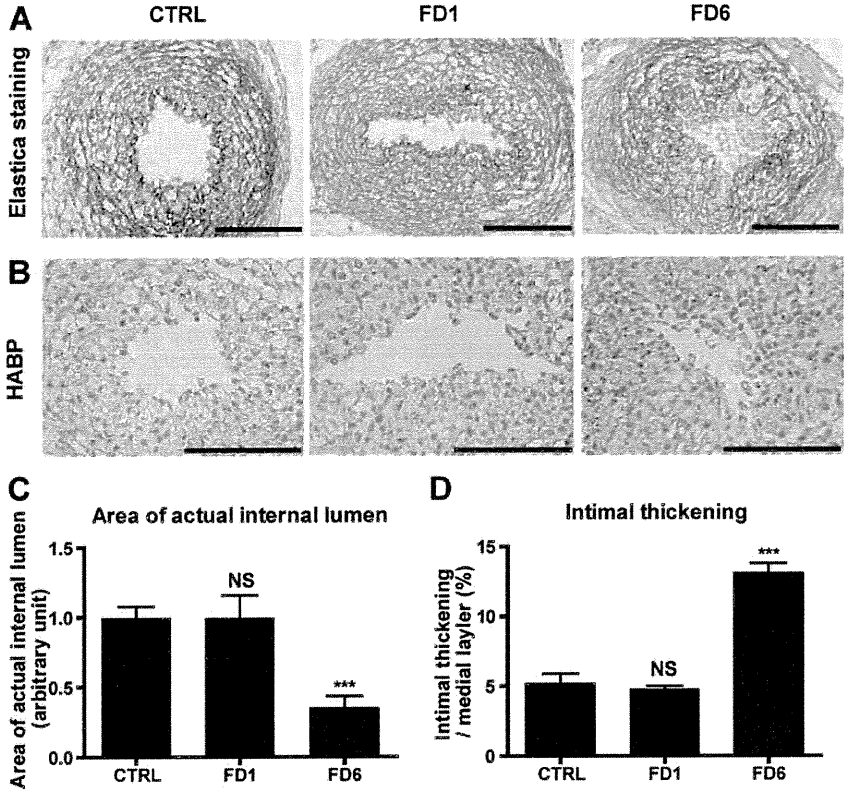

Figure 7. IT in rat DA explants is promoted by FD6. A, Elastica van Gieson staining for DA explants treated with FD1 or FD6. **B,** Strong immunoreaction to hyaluronan in DA explants cultured with FD6. **C,** The area of the internal lumen of the DA treated with FD6 was significantly decreased (n=6 to 7). **D,** The increased IT in the DA treated with FD6 (n=6 to 7). ***$P<0.001$ vs control (CTRL). **Bars:** 100 μm. HABP indicates hyaluronan-binding protein.

Clinical Implications of Using AC Isoform-Selective Modulations

The manipulation of the contractile state of the DA is important for patients with patent DA and complicated congenital heart diseases. All currently available pharmacological therapies rely on synthetic PGE_1 to dilate the DA and prostaglandin H synthase inhibitors to close it. Because these therapies basically change the plasma and/or local concentra-

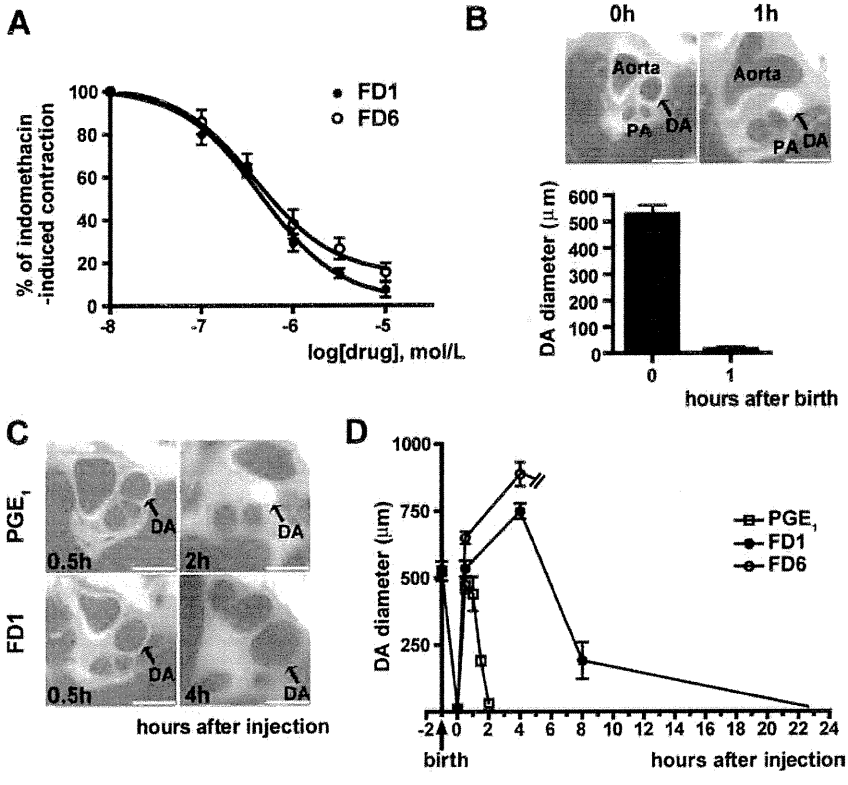

Figure 8. The effects of FD1 and FD6 on vasodilation. A, FD1 and FD6 similarly attenuated indomethacin-induced contraction in DA explants in a dose-dependent manner (n=4 to 10). **B,** The whole-body freezing method revealed that the rat DA opened widely after birth and closed 1 hour after birth **(arrow)** (n=5). **C,** Representative images of rat DAs treated with PGE_1 and FD1 using the whole-body freezing method. **D,** The vasodilating effects of PGE_1, FD1, or FD6 were compared (n=5 to 7). FD1 had a longer action of duration than did PGE_1. All rats injected with FD6 died 4 hours after injection because of apnea. **Bars:** 1 mm. PA indicates pulmonary artery.

tion of PGE, they broadly affect the contractile state and the cellular responses in other types of smooth muscle and tissues, resulting in severe adverse effects on systemic organs. Moreover, PGE_1 has a short duration of action and induces hyaluronan-mediated IT in the DA. In cases of DA-dependent congenital heart diseases, opening of the DA without induction of IT is of particular necessity until the hemodynamics can be improved through surgery. Although differential regulation of vasodilation and IT is preferable for treatment of patients with DA-dependent congenital cardiac malformations, such a treatment is not currently available. Therefore, selective manipulation should be a desirable direction for novel therapeutic strategies. In the present study, we demonstrated that AC isoform-selective activators differentially regulated vascular tone and remodeling in the DA. Our data imply that AC2/6-selective manipulation could be a novel means of achieving DA vasodilation with only minimal effects on the pulmonary arteries and aorta. Moreover, the AC2/6 activator, FD1 has longer pharmacological effects than does PGE_1. Recent studies from other authors have indicated that specific agonists/antagonists for EP4 specifically regulate ductal prostaglandin signals,[29] which could potentially yield a DA-selective vasodilator or vasoconstrictor. However, it should be noted that the EP4 receptor underwent short-term agonist-induced desensitization,[30] which is a common biological phenomenon involving reduction of responsiveness despite continuous agonist induction. Direct activation of AC may overcome the disadvantages of agonist-induced desensitization and FD1 may be beneficial for patients with DA-dependent congenital heart diseases.

In conclusion, we have shown that AC2 and AC6 exert distinct regulation of vascular tone and play an important role in DA remodeling. AC isoform-selective pharmacotherapy using FD1 may serve as a novel therapeutic strategy for patients with DA-dependent congenital heart diseases and as an alternative to currently available PGE therapy.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Prostaglandin (PG)E-adenylyl cyclase (AC)-cAMP signaling opens the ductus arteriosus (DA) by vasodilation and closes it by hyaluronan-mediated intimal thickening.
- Differential regulation of vasodilation and remodeling of the DA is required for patients with DA-dependent congenital heart diseases after birth.

What New Information Does This Article Contribute?

- AC type 6 (AC6) is involved in vasodilation and hyaluronan-mediated intimal thickening.
- AC type 2 (AC2) inhibits AC6-induced intimal thickening.
- Stimulation of both AC2 and AC6 by the new forskolin derivative FD1 induced long-lasting vasodilation without intimal thickening in the DA.

PGE plays 2 opposing roles in the DA: it induces opening of the DA by vasodilation, and closure by hyaluronan-mediated intimal thickening. Dilation of the DA, but not intimal thickening, is necessary in patients with DA-dependent congenital heart diseases after birth. However, the currently available PGE therapy is not able to differentially regulate vasodilation and intimal thickening in the DA. Our results suggest that AC6 plays a primary role in hyaluronan-mediated vascular remodeling and vasodilation in the DA and that AC2 has an inhibitory effect on AC6-mediated vascular remodeling. We found that stimulation of both AC2 and AC6 by the forskolin derivative FD1 induced long-lasting vasodilation without intimal thickening in the DA. For the first time, we demonstrated that AC2 and AC6 exert distinct regulation of vascular tone and remodeling. In particular, our identification of the interaction of two signaling pathways of AC isoforms in vascular remodeling is novel. AC isoform-selective pharmacotherapy using FD1 may yield a new therapeutic strategy for patients with DA-dependent congenital heart diseases who require DA opening, but not DA closure, through hyaluronan-mediated neointimal thickening. This may become an alternative to the currently available PGE therapy.