Disruption of Type 5 Adenylyl Cyclase Enhances Desensitization of Cyclic Adenosine Monophosphate Signal and Increases Akt Signal With Chronic Catecholamine Stress

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Background—Desensitization of the cyclic adenosine monophosphate signal protects cardiac myocytes against catecholamine stress, thus preventing the development of apoptosis. Molecular mechanisms of desensitization have been well studied at the level of adrenergic receptors but less so at the level of the effector enzyme, adenylyl cyclase (AC). Methods and Results—When the effects of long-term (1 to 2 weeks) isoproterenol infusion were compared between type 5 AC-null mice (AC5KO) and wild-type controls, we found that the subsequent responses of left ventricular ejection fraction to sudden intravenous isoproterenol challenge were reduced in AC5KO compared with wild-type mice (ie, physiological desensitization was more effective in AC5KO), consistent with enhanced downregulation of AC catalytic activity in AC5KO. One mechanism for the less effective desensitization in wild-type mice was paradoxical upregulation of type 5 AC protein expression. The number of apoptotic myocytes was similar at baseline but was significantly less in AC5KO after infusion. This was accompanied by a 4-fold greater increase in Bel-2 and a 3-fold greater increase in phospho-Akt in AC5KO. The latter is most likely mediated by increased membrane localization of phosphoinositide-dependent protein kinase 1, which is known to be inhibited by the cyclic adenosine monophosphate signal. Conclusions—The absence of type 5 AC results in more effective desensitization after long-term catecholamine stress and protects against the development of myocyte apoptosis and deterioration of cardiac function, potentially elucidating a novel approach to the therapy of heart failure. (Circulation. 2007;116:1776-1783.)

Key Words: catecholamines ■ heart failure ■ apoptosis ■ signal transduction

Clinical Perspective p 1783

AC is the target enzyme of β-AR stimulation and is composed of at least 9 isoforms,7 of which are expressed in the heart; the type 5 and type 6 isoforms are the major cardiac isoforms.3,4 We have previously demonstrated that the disruption of type 5 AC does not alter the expression of β-AR/Gsα/protein kinase A,4 but both significantly prevented the development of myocyte apoptosis and heart failure in response to pressure-overload stress5 and prolonged longevity.6 This beneficial effect, for which the exact molecular mechanisms remain unknown, does not appear to result from a simple loss of cardiac AC catalytic activity, because the...
Okumura et al  Catecholamine Stress and Type 5 Adenylyl Cyclase 1777

decrease in AC activity was modest, and the development of hypertrophy in type 5 AC-null mice (AC5KO) remained unchanged. These findings with type 5 AC are in contrast to those from other laboratories, because several studies demonstrated that increasing cardiac AC expression with type 6 AC, another major isoform, prevented the development of heart failure.\(^7\) It has thus prompted us to hypothesize that the disruption of type 5 AC may activate protective mechanisms that would lead to the preservation of cardiac myocyte viability in response to various stresses and that the role of type 5 AC is different from that of type 6 AC. The contribution of each AC isoform to the process of global desensitization, a major protective mechanism against catecholamine stress, may not be identical, as implicated by different contributions between 2 major \(\beta\)-AR subtypes in the heart (\(\beta_1\) versus \(\beta_2\)) to the development of desensitization, apoptosis, and heart failure.\(^{1,2}\)

The goal of the present investigation was to examine the role of type 5 AC in response to chronic catecholamine stress. First, we examined the development of physiological desensitization and myocardial apoptosis in AC5KO and wild-type (WT) littermates in response to long-term isoproterenol infusion. In addition, we studied changes in molecules involved in cardiac apoptotic signaling, eg, Akt (or protein kinase B)/GSK (glycogen synthase kinase),\(^8\) as well as changes in AC activity and its expression, in an isoform-specific manner. We demonstrated that the absence of type 5 AC was accompanied by enhanced Akt/GSK signaling, improved myocyte viability, and a greater degree of downregulation of AC signaling after long-term isoproterenol infusion.

Methods

**Mice**

Generation of AC5KO mice was described previously.\(^4\) Long-term infusion of isoproterenol (Sigma, St. Louis, Mo) was performed for 7 to 14 days at a dose of 30 to 60 mg·kg\(^{-1}\)·d\(^{-1}\) with a miniosmotic pump (ALZET model 2001, DURECT Corp, Cupertino, Calif).\(^9\) Control mice received vehicle rather than isoproterenol in pumps. Pumps were removed 24 hours before biochemical and physiological studies. All experiments were performed in 4- to 5-month-old male homozygous AC5KO and WT littermates. In addition, the effects of long-term isoproterenol on myocyte apoptosis were also examined in type 5 AC transgenic mice (AC5TG). In the hearts of these mice, forskolin-stimulated AC activity was increased 8-fold. This study was approved by the Animal Care and Use Committee at New Jersey Medical School.

**Physiological Studies**

Mice were anesthetized with 2.5% tribromoethanol (0.010 to 0.015 mL per gram of body weight) injected intraperitoneally, and echocardiography was performed with ultrasonography (ACUSON Sequoia C256, Siemens Medical Solutions, Malvern, Pa).\(^4\)

**AC Assay**

Hearts were dissected from the mice, and membrane preparations were made as described previously.\(^10\) This crude membrane preparation was used in the AC assay. AC activity was measured by a modification of the method of Salomon et al.\(^11\)

**\(\beta\)-AR Binding Assay**

\(\beta\)-AR density was measured by antagonist binding with \(^{125}\)I-cyanopindolol.\(^{12-14}\) Selective \(\beta_1\)-AR binding was determined by the method of Bristow et al.\(^12\)

**Evaluation of Apoptosis**

DNA fragmentation was detected in situ by terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining.\(^5,15\)

**Western Blotting**

Western blotting was conducted with commercially available antibodies, except for type 5 and type 6 AC. Western blotting for type 5 and 6 AC was performed as previously described.\(^3\) These antibodies can discriminate the 2 isoforms, and the molecular weight of the type 5 AC band is greater than that of the type 6 AC band, as expected from the nucleotide sequences of each complementary DNA we have isolated.\(^16,17\) Expression of these AC proteins was quantified by densitometry.

**Statistical Analysis**

All data are reported as mean ± SEM. Comparisons between AC5KO and WT values were done with appropriate contrasts in 2-way ANOVA models with interaction. Bonferroni corrections were used to adjust the overall error rate when multiple comparisons were performed. A repeated-measures ANOVA was performed to compare the 2 curves for left ventricular ejection fraction (LVEF) in response to sudden isoproterenol challenges in AC5KO and WT. In addition, comparisons for data in cytosol versus membrane fractions between WT and AC5KO were done with multivariate ANOVA (MANOVA), because measurements on the variables of interest were made on the same samples.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Enhanced Desensitization of Cardiac Function in AC5KO**

The effects of long-term isoproterenol on cardiac function (LVEF) were examined by echocardiography (Figure 1). Physiological desensitization was determined by short-term administration of isoproterenol (0 to 0.40 µg·kg\(^{-1}\)·min\(^{-1}\) IV for 5 minutes) 1 day after long-term isoproterenol infusion was completed. Basal LVEF before long-term isoproterenol was similar between WT (72 ± 1.7%) and AC5KO (71 ± 1.6%) mice, as reported previously.\(^4\) After long-term isoproterenol, however, the deterioration of basal LVEF was greater in WT mice (56 ± 1.7%) than in AC5KO mice (65 ± 1.6%; \(P < 0.05, n = 8\) to 10; Figure 1A). These differences could be due in part to alterations in hemodynamics (eg, heart rate). However, heart rates were similar in WT and AC5KO mice at baseline after long-term isoproterenol (345 ± 10 versus 371 ± 14 bpm). LVEF responses to short-term isoproterenol were reduced in AC5KO compared with WT mice (\(P < 0.01;\)) Figure 1B); the effects of isoproterenol appeared saturated in AC5KO mice even at a low dose of isoproterenol (0.13 µg·kg\(^{-1}\)·min\(^{-1}\) for 5 minutes), and at a high dose of isoproterenol (0.40 µg·kg\(^{-1}\)·min\(^{-1}\) for 5 minutes), AC5KO mice did not experience a further increase in LVEF. In contrast, isoproterenol increased LVEF dose dependently in WT mice. These results demonstrate more effective desensitization in AC5KO.

**Downregulation of AC Activity in AC5KO**

We previously demonstrated that total AC activities were decreased by \(\approx 30\%\) in AC5KO mice relative to WT mice, which indicates that type 5 AC is responsible for \(\approx 30\%\) of total AC activity in the mouse heart.\(^4\) We examined forskolin-
Long-Term Isoproterenol
Expression of Type 5 AC Increased but That of Type 6 AC Decreased After
Heart Apoptosis Was Reduced in AC5KO Mice

We also examined the development of myocyte apoptosis after long-term isoproterenol, which has been shown to be augmented. Before long-term isoproterenol, no difference existed in the number of TUNEL-positive cells between the WT and AC5KO mice (0.05±0.02% versus 0.04±0.01%), which suggests that the lack of type 5 AC did not alter the viability of cardiac myocytes at baseline. Long-term isoproterenol increased the number of TUNEL-positive cells in WT by 4.5-fold (0.21±0.04%; Figure 4A). However, the increase in apoptosis was significantly smaller in AC5KO mice (0.11±0.01%) after long-term isoproterenol than in WT mice (0.21±0.04%; Figure 4A), which suggests that the lack of type 5 AC significantly reduced the development of apoptosis (~53%) induced by long-term isoproterenol and preserved cardiac function compared with that in WT mice after isoproterenol. Conversely, myocyte apoptosis was increased 3-fold (0.69±0.33%) in ACSTG hearts compared with WT hearts (0.21±0.04%) after long-term isoproterenol.

Expression of Bcl-2 Was Enhanced in AC5KO Hearts After Long-Term Isoproterenol

To determine the cellular mechanisms for the differences in developing apoptosis between WT and AC5KO mice, we examined Bcl-2, an inhibitor of apoptosis (Figure 4B). Bcl-2 expression was barely detectable in hearts from the vehicle group in both WT and AC5KO mice (data not shown), which suggests that the lack of type 5 AC did not alter Bcl-2 expression at baseline, as has been reported previously. Bcl-2 protein expression was increased after long-term isoproterenol in both WT and AC5KO, but the magnitude of this

stimulated (100 μmol/L each) AC activity, which represents the total amount of AC activity with minimal involvement of the receptor and G proteins (Figure 2). Forskolin-stimulated AC activity decreased, but only by 11%. However, it decreased by 30% in AC5KO mice (P<0.01), demonstrating more effective desensitization at the AC level in AC5KO, which is consistent with the physiological studies (Figure 1).

In contrast, total β-AR density, measured with [125I]cyanopindolol, was reduced with long-term isoproterenol similarly in AC5KO (17.4±1.8 to 10.3±1.9 fmol/mg; n=4) and WT (18.5±1.2 to 11.1±2.7 fmol/mg; n=4) mice. β2-AR density also decreased similarly in AC5KO (10.8±1.9 to 5.6±1.1 fmol/mg; n=4) and WT (12.6±1.1 to 5.4±1.8 fmol/mg; n=4) mice.

Protein Expression of Type 5 AC Increased but That of Type 6 AC Decreased After Long-Term Isoproterenol

To examine the mechanisms for the enhanced AC downregulation in AC5KO mice, we examined protein expression of the 2 major AC isoforms (types 5 and 6 AC) in the heart after long-term isoproterenol (Figure 3). Other AC isoforms in the heart were not reliably detected with available antibodies. Expression of type 6 AC decreased after long-term isoproterenol in WT, whereas type 5 AC unexpectedly increased after long-term isoproterenol (Figure 3B). In AC5KO mice, the decrease in type 6 AC was modest and not significant.

Figure 1. Left ventricular function. Echocardiographic measurements of left ventricular function were performed in WT and AC5KO mice after long-term isoproterenol (ISO) infusion. A, LVEF was not different between WT (72±1.7%) and AC5KO (71±1.6%) mice with vehicle. After long-term isoproterenol infusion, LVEF was decreased in both WT and AC5KO mice, but the magnitude of the decrease was greater in WT (56±1.7%) than in AC5KO (63±1.6%) mice. B, Absolute changes in LVEF in response to sudden isoproterenol challenge were less in AC5KO than in WT mice after long-term isoproterenol. **P<0.01; *P<0.05; NS, not significant. n=8 to 10.

Figure 2. AC activity. Steady-state AC activity was determined as maximal cAMP production in WT and AC5KO mice after long-term isoproterenol (ISO) or vehicle. Stimulation was performed at the level of the AC catalytic unit (forskolin; 100 μmol/L). Forskolin-stimulated AC activity was decreased significantly in both WT and AC5KO mice, with the decrease greater in AC5KO mice. *P<0.05; **P<0.01, n=4 to 5.
This is reminiscent of the activation of Bcl-2 in response to long-term isoproterenol, which suggests that the lack of type 5 AC potentiated the increase in PDK1 in the membrane. We examined changes in phosphoinositide-dependent protein kinase 1 (PDK1), an upstream molecule of Akt signaling, which phosphorylates and thus activates Akt after long-term isoproterenol. Akt Signaling Was Increased in AC5KO Hearts

Figure 4. Comparison of TUNEL staining and Bcl-2 expression after long-term isoproterenol (ISO) between WT and AC5KO. A, TUNEL-positive myocytes in left ventricular myocardium were counted in WT and AC5KO hearts and are expressed as percent of total myocytes. The number of TUNEL-positive myocytes was significantly smaller in AC5KO than in WT hearts after long-term isoproterenol. **P<0.01; n=7 to 10 for each. B, Expression of Bcl-2 was compared between WT and AC5KO. Protein expression of Bcl-2 was determined by Western blot analysis, which showed a greater expression in AC5KO than in WT. The Bcl-2 expression level in WT mice after long-term isoproterenol was taken as 100%. **P<0.01, n=6 for each. Representative immunoblots are shown.

We then examined changes in Akt signaling, which is involved in apoptotic pathways in the heart. Long-term isoproterenol increased the activation of Akt, as demonstrated by an increased amount of phospho-Akt (Ser-473) in both WT and AC5KO, presumably through the release of Gsα from β-AR on activation with isoproterenol. We initially thought that the degree of Akt activation was similar between AC5KO and WT hearts because the amount of released Gsα was similar as well; however, the magnitude of Akt activation was much greater in AC5KO (Figure 5A and 5D). Phosphorylation of GSK3α/β (Ser-21/Ser-9), a target molecule of Akt-mediated phosphorylation, was increased after long-term isoproterenol in both WT and AC5KO (Figure 5B and 5D). GSK3α/β phosphorylation was greater in AC5KO mice before long-term isoproterenol, which implies that molecules other than Akt are also involved. We also examined changes in forkhead transcription factor (FKHR), another target of Akt signaling, which interacts with the promoter region of various regulators of apoptosis, such as Fas (or CD95/Apo-1) ligand. We found that FKHR was phosphorylated (Ser-256) and thus activated after long-term isoproterenol and that the degree of activation was significantly greater in AC5KO mice than in WT mice (Figure 5C and 5D). Thus, Akt and its downstream target molecules were all activated to a greater degree in AC5KO than in WT mice after long-term isoproterenol.

We next examined changes in phosphoinositide-dependent protein kinase 1 (PDK1), an upstream molecule of Akt signaling, which phosphorylates and thus activates Akt after its translocation to the membrane via Gβγ/Pi3K (phosphatidylinositol 3-kinase) activation. We examined the amount of PDK1 in the membrane versus cytosol after long-term isoproterenol. In vehicle, the amount of PDK1 in the membrane was similar between AC5KO and WT, whereas that in cytosol was greater in AC5KO mice (Figure 6A). After long-term isoproterenol, the amount of membrane PDK1 was significantly greater in AC5KO than in WT mice, and the amount of cytosol PDK1 was greater in WT, which suggests a greater magnitude of PDK1 membrane translocation in AC5KO mice (Figure 6B). These findings suggest that an increase in PDK1 in the membrane contributes, at least in part, to the enhanced expression of Bcl-2.
part, to enhanced Akt signaling in AC5KO. Because cAMP signaling is known to inhibit the membrane translocation of PDK1, it is possible to speculate that this inhibition by cAMP was smaller in AC5KO because of the lack of type 5 AC and/or that type 5 AC is a major regulator of PDK1 translocation.

**Discussion**

Effects of chronic sympathetic stimulation, as occurs in the pathogenesis of heart failure, are ameliorated by desensitization mechanisms, which have been elucidated primarily at the level of β-AR and less at the level of AC. The present investigation demonstrated a novel role for type 5 AC, a major cardiac isoform, which appears to impair desensitization in response to catecholamine stress, resulting in enhanced apoptosis. In support of this concept, the physiological studies demonstrated more effective desensitization in AC5KO than WT mice in response to chronic catecholamine stress, as reflected by reduced LVEF responses to sudden isoproterenol challenges. The more effective desensitization, confirmed by measurement of AC activity, most likely was responsible for the reduced myocyte apoptosis, measured by TUNEL staining, in AC5KO mice. These results are not likely related to altered desensitization of the β-AR, which decreased similarly in AC5KO and WT mice with long-term isoproterenol.

We would have predicted that type 5 AC would be downregulated with chronic catecholamine stress in WT, as was type 6 AC; however, this did not occur, and type 5 AC was actually increased in WT mice. Because type 5 AC possesses a much higher enzyme catalytic activity than type 6 AC, the upregulation of type 5 AC in WT mice would overwhelm the downregulation of type 6 AC and potentially other AC isoforms in the total AC catalytic activity of WT mice. Accordingly, the upregulation of type 5 AC may account for a major difference between WT and AC5KO mice in their ability to desensitize. Type 5 AC may act to maintain cardiac responses to catecholamine stimulation at the cost of myocyte viability in advanced heart failure, and therefore, the inhibition of this enzyme isoform may be beneficial in preserving myocyte viability and thus function in heart failure.

We found that Bcl-2 was upregulated 4-fold more in AC5KO than in WT mice and that phospho-Akt also was increased 3-fold more in AC5KO mice after chronic catecholamine stress, which suggests that changes in these molecules...
account for the protection against apoptosis in AC5KO. Interestingly, this protective mechanism against apoptosis was upregulated during chronic catecholamine stress with an increase in apoptosis. It is possible that this Bcl-2 upregulation occurred as a compensatory mechanism in response to the apoptosis and that the increase in apoptosis may actually have been greater if this increase in Bcl-2 had not occurred. It has been reported that a cAMP-dependent mechanism also appears to enhance degradation of Bcl-2 through phosphorylation.26 Thus, the stability of Bcl-2 is enhanced by the reduced protein kinase A–mediated phosphorylation of Bcl-2 in AC5KO, and this mechanism might play an important role in protecting hearts from chronic catecholamine stress, because it is now evident that Bcl-2 itself plays a central role in regulating apoptosis in the cardiovascular system.27 The present data also show that the expression of PDK1, an upstream regulator of Akt located in the cardiac plasma membrane, was greater in AC5KO than in WT mice after isoproterenol. It is known that the cAMP-dependent signaling pathway inhibits Akt activity by blocking the membrane translocation of PDK1 and that it thus inhibits the coupling between PDK1 and Akt, as demonstrated in many cell types.23 It was thought, therefore, that a potential mechanism to induce greater activation of the Akt signal was the limited cAMP production in response to isoproterenol in AC5KO and WT mice, whereas it was significantly elevated after long-term isoproterenol in AC5KO compared with WT. The amount of expression in WT with vehicle was taken as 100% in each determination. *P<0.05, **P<0.01; n=4 to 5. Representative immunoblots are shown.

The present investigation demonstrated opposite roles for type 5 AC relative to those for type 6 AC28 or type 8 AC.29 It is known that the intracoronary gene transfer of type 6 AC increases cardiac function and attenuates the deleterious effects of left ventricular remodeling in congestive heart failure.7 More recently, it has been shown that cardiac-specific overexpression of type 6 AC increased survival after myocardial infarction.30 Type 8 AC overexpression did not result in depressed cardiac function, despite a 7-fold increase in basal AC activity and a 4-fold increase in protein kinase A activity in the heart.29 In contrast, we have demonstrated that disruption of type 5 AC (AC5KO) plays a protective role in the development of heart failure in response to pressure overload6 and to chronic catecholamine stress (present study),

Figure 6. PDK1 expression in cytosolic and membrane fractions. The amounts of PDK1 in membrane (Mem) and cytosolic (Cyto) fractions were compared between vehicle (A) and isoproterenol (ISO; B). Note that membrane PDK1 expression was similar between AC5KO and WT in vehicle, whereas it was significantly elevated after long-term isoproterenol in AC5KO compared with WT. The amount of expression in WT with vehicle was taken as 100% in each determination. *P<0.05, **P<0.01; n=4 to 5. Representative immunoblots are shown.
which implies that type 5 AC is regulated differently from other AC subtypes. In further support of this concept, we found that not only did the frequency of myocyte apoptosis decrease in AC5KO with long-term isoproterenol, but the opposite occurred (ie, it was actually enhanced in AC5TG with long-term isoproterenol).

The results of the present study may have important implications for understanding the pathogenesis of heart failure. Sympathetic activation is a major compensatory mechanism in response to decreased left ventricular function of heart failure but is deleterious chronically because it leads to cell death and thus accelerates the progression of heart failure. Conversely, β-AR desensitization mechanisms in heart failure and β-AR blockade therapy are clearly effective in heart failure patients. The present data demonstrate that the disruption of type 5 AC plays a protective role in response to chronic β-AR stimulation and to chronic pressure overload, as shown previously, by attenuating the decline in cardiac function and protecting against the increased apoptosis. These findings, taken together, make the type 5 AC molecule potentially important to study for future β-AR blockade therapy, in which suppression of the activity of type 5 AC may be advantageous in the treatment of heart failure.

Acknowledgments

We thank Dr J. Tomlinson and Dr C.J. Homay for providing the type 5 and type 6 antibodies.

Sources of Funding

This study was supported by National Institutes of Health grants AG014121, HL033107, HL059139, HL069752, AG021337, AG028854, and HL069020 (Drs S. Vatner and D. Vatner); GM067773 (Dr Ishikawa); the Japanese Ministry of Education, Culture, Sports, Science (Drs Okumura and Ishikawa), and Technology; the Japan Space Forum (Dr Ishikawa); the Kitasen Kagaku Research Foundation (Dr Ishikawa); Fukuda Foundation for Medical Technology (Dr Okumura); Takeda Science Foundation (Dr Okumura); grant for research on autonomic nervous system and hyperthyroidism from Kimura Memorial Foundation/Pfizer Pharmaceuticals (Dr Okumura); grant for 2006 to 2007 strategic research project (No. K19027) of Yokohama City University, Japan (Dr Okumura); and Takeda Science Foundation (Dr Okumura).

Disclosures

None.

References

β-Adrenergic receptor (β-AR) blockers inhibit cardiac sympathetic activity via a reduction in cardiac adenylyl cyclase (AC) activity and have been shown to be effective in reducing the risk of death and hospitalization in patients with chronic heart failure. However, the American College of Cardiology/American Heart Association “2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult” recommends that while treating heart failure patients with β-AR blockers, physicians should monitor them closely, because the negative inotropic effects of β-AR blockers may initially exacerbate heart failure. β-AR blockers also can aggravate bronchospasm, because β-ARs are also expressed in the lungs. The present study indicates that the absence of type 5 AC, which is a major AC isoform in the heart, protects the heart in response to chronic sympathetic stress. This protection results in more effective desensitization, preservation of function, and less cell death, which suggests that inhibition of type 5 AC may be a better strategy than the current β-AR blockade therapy, because basal cardiac function is preserved. Accordingly, the next goal is to develop a specific type 5 AC inhibitor that can be used in the treatment of patients with heart failure.
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Circulation. 2007;116:1776-1783; originally published online September 24, 2007; doi: 10.1161/CIRCULATIONAHA.107.698662
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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