Enhanced Cardiac Function in Transgenic Mice Expressing a Ca\(^{2+}\)-Stimulated Adenylyl Cyclase

Larissa Lipskaia, Nicole Defer, Giovanni Esposito, Iman Hajar, Marie-Claude Garel, Howard A. Rockman, Jacques Hanoune

Abstract—The predominant functional adenylyl cyclases normally expressed in cardiac tissue and coupled to \(\beta\)-adrenergic receptors are inhibited by micromolar Ca\(^{2+}\) concentration. To modify the overall balance of activities, we have generated transgenic mice expressing the Ca\(^{2+}\)-stimulatable adenylyl cyclase type 8 (AC8) specifically in the heart. AC activity is increased by at least 7-fold in heart membranes from transgenic animals and is stimulated by Ca\(^{2+}\) in the same range of concentration that inhibits the endogenous activity. Moreover, the in vivo basal protein kinase A activity was augmented 4-fold. Overexpression of AC8 in the heart has no detrimental consequences on global cardiac function. Basal heart rate and contractile function, measured by noninvasive echocardiography, were unchanged. In contrast, on release of parasympathetic tone, the intrinsic contractility is heightened and unresponsive to further \(\beta\)-adrenergic receptor stimulation. AC8 transgenic mice thus represent an original model to investigate the relative influence of Ca\(^{2+}\) and cAMP on cardiac function within a phenotype of enhanced cardiac contractility and relaxation. (Circ Res. 2000;86:795-801.)

Key Words: adenylyl cyclase ■ transgenesis ■ cardiac function

In the heart, the force of contraction is dependent on the influx of Ca\(^{2+}\) ions through voltage-dependent channels.\(^1,2\) \(\beta\)-Adrenoceptor stimulation augments the amplitude of the L-type Ca\(^{2+}\) current and the force of contraction\(^3\) through binding to \(\beta\)-adrenergic receptors (\(\beta\)-ARs), stimulation of adenylyl cyclase (AC), and increase in the concentration of cAMP. To understand the physiological and pathological consequences of this cascade, murine models have been created with an enhanced efficacy of the \(\beta\)-AR–Gs-AC signaling pathway.\(^4-6\) Overexpression of the \(\beta\)-AR resulted in a maximal activation of the \(\beta\)-AR signaling pathway, even in the absence of the agonist.\(^7,9\) Gs\(\alpha\) overexpression resulted in cardiomyopathy and substantial cardiac histological abnormalities.\(^8\)

Another approach to enhance \(\beta\)-AR–Gs-AC signaling would be to bypass the potential deleterious consequences of the receptor or G protein and directly to increase the expression of the effector, AC. At least 9 isoforms of AC are known.\(^9\) There is a significant heterogeneity in the distribution and biochemical properties of the different isoforms, and each tissue or cell type possesses a unique combination of these isoforms. In the heart, the Ca\(^{2+}\)-inhibitable isoforms AC5 and AC6 are the most abundant.\(^10,11\) Elevation of Ca\(^{2+}\) concentration might inhibit cAMP synthesis and thereby provide a sensitive negative feedback.\(^12\) In contrast, AC1 and AC8, which are essentially expressed in the central nervous system, are activated by Ca\(^{2+}\) through the Ca\(^{2+}\)/calmodulin complex.\(^13-15\)

In this study, we describe transgenic mice overexpressing the Ca\(^{2+}\)/calmodulin-activatable isoform AC8 specifically targeted to cardiomyocytes. Surprisingly, we observed that AC8 overexpression has no effect on the viability of the animals but leads to a higher basal intrinsic contractility that is unresponsive to further \(\beta\)-AR stimulation.

Materials and Methods

Generation of Transgenic Mice

For the construction of transgenic mice, the murine \(\alpha\)-myosin heavy chain (MHC) promoter\(^16\) was ligated to the cDNA coding for human AC8.\(^18\) Mice were screened for the presence of the transgene by Southern blot performed on tail genomic DNA. Two founders were identified and propagated by crossbreeding with C57BL/6 wild-type mice. Number of transgene copies was determined by slot-blot analysis. The care and use of animals were in accordance with institutional guidelines.

Echocardiography

Echocardiography was performed in anesthetized mice (Avertin [tribromoethanol] 2.5%, 14 \(\mu\)L/g IP) using an ATL HDI 5000 (ATL Ultrasound, Bothell, Wash) echocardiograph as previously described.\(^17\) The following parameters were measured: left ventricular (LV) end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), posterior and septal wall thickness, heart rate, percentage of fractional shortening (\%FS) (calculated as [LVEDD–LVESD]/

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Hemodynamic Evaluation

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) and analyzed as previously described. Briefly, after endotracheal intubation, mice were connected to a rodent ventilator. After bilateral vagotomy, a 1.4F high-fidelity micromanometer catheter (Millar Instruments) was inserted into the right carotid artery and retrograde across the aortic valve into the LV. Hemodynamic measurements were recorded at baseline and 45 to 60 seconds after injection of incremental doses of isoproterenol (ISO). Doses of ISO were specifically chosen to maximize the contractile effect, the responses were averaged for each measurement.

Results

Figure 1. Genetic characterization of the transgenic mice. A, DNA construct used for generation of transgenic mice overexpressing human AC8 in the heart. B, Southern blot analysis of Hpal-digested genomic DNA from tail biopsies. Blots were hybridized with the 32P-labeled cDNA probe for AC8, washed (0.5× SSC, 0.1% SDS, at 65°C for 15 minutes), and exposed for 24 hours. Lanes 1 through 8 represent DNA from 13 littersmates after the crossing (AC8TM × C57BL/6). N indicates C57BL/6 control animal; P-AC8TM, transgenic parent (L7 founder); C, Cardiac-specific mRNA expression of the AC8 transgene. Shown are total RNAs (30 μg) from heart (H), kidney (K), brain (B), and skeletal muscle (M) of 1 AC8TM or of 1 control mouse. Autoradiograms were obtained after 2 hours (GAPDH) or 12 hours (AC8, AC5, and AC6) of exposure.

LVEDD×100), and mean velocity of circumferential fiber shortening (mean Vcfc).

RNA Preparations and Northern Blotting

Total RNA was extracted, and Northern blots were carried out as described. RNAs were hybridized with [32P]dCTP-labeled AC8, AC5, or AC6 cDNA probes. A rat GAPDH cDNA was used to control the equal RNA loading.

AC Assays

AC activity was measured as described on purified cardiac membranes. Hearts were homogenized in 10 volumes of ice-cold lysis buffer (in mmol/L, Tris-HCl [pH 7.6] 10, EDTA 0.1, DTT 0.5, and PMSF 0.5) and centrifuged at 500 g for 5 minutes. The supernatant was centrifuged at 15 000 g for 30 minutes and the pellet washed 3 times in the same buffer. For analysis of Ca2+ effect, the membranes were previously washed twice with 1 mmol/L EGTA. Brains were previously washed twice with 1 mmol/L EGTA. proteins (25 to 100 μg/lane) were separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with anti-calmodulin antibody as recommended by the manufacturer, and antigen was visualized using the enhanced chemiluminescence system from Amersham Pharmacia Biotech.

Statistical Analysis

All results are expressed as mean±SEM of at least 3 determinations. To examine the effect of ISO on changes in hemodynamic parameters between control and transgenic animals, a repeated-measures ANOVA was used. For echocardiographic data, a 1-factor ANOVA was used. Post hoc analysis with regard to differences in mean values between groups was conducted with a Scheffé test. P<0.05 was considered significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Generation and Genetic Characterization of the Transgenic Mice

The α-MHC-AC8-SV40 intron/pA transgene (Figure 1A) was microinjected into the pronuclei of fertilized mouse eggs. Two founders were obtained (L7 and L8). The transmission of the transgene was demonstrated by Southern blotting of the offspring (Figure 1B). Slot-blot analyses of genomic DNA from tail biopsies showed 7 to 8 copies of the transgene per genome (not shown). Both strains expressed AC8 mRNA and activity at a high level in the heart. For further studies, only the offspring of the L7 founder were used.

Northern blot analysis revealed a high, cardiac-specific expression of the AC8 transgene in these animals (Figure 1C), which is consistent with the previously documented pattern of expression achieved with the murine α-MHC promoter. In contrast, no variation in the mRNA expression of the 2 major cardiac cyclases, AC5 and AC6, was detected.

Anatomical examination of 2-month-old animals showed no fibrosis or any obvious differences between hearts of AC8 transgenic mice (AC8TM; n=6) and control littermates (n=6) with respect to gross morphology or myocyte appearance (not shown). Body weight (control, 34±0.4 g; transgenic, 33±0.7 g), heart weight (control, 153.9±2.8 mg; transgenic, 156.9±7.8 mg), tibia length (control, 19±0.1; transgenic, 18.6±0.1 g), and LV wet weight (control, 113.4±2.16; transgenic, 117.8±6.6 g) were unchanged by transgene expression (control mice [CM], n=14; transgenic...
mice, n=12). No differences in behavior or exterior aspect were observed. Neonatal mortality was not different between transgenic and nontransgenic animals.

**AC Activity in Cardiac Membranes From AC8TM**

AC activity was assayed in cardiac membranes prepared from pools of 10 transgenic or 10 CM. Basal AC activity was increased at least 7-fold in AC8TM as compared with their littermates (156 ± 5.5 pmol cAMP/min mg⁻¹ proteins versus 21.0 ± 3.4 for control hearts [n=5; P<0.001]; Figures 2A and 2B). In the presence of NaF (10 mmol/L), AC activity was increased by 3-fold in AC8TM and by 8-fold in CM (470 ± 18.59 versus 178.43 ± 6.75 pmol cAMP/min mg⁻¹ proteins in cardiac membranes from AC8TM and CM, respectively).

To document the AC activity in cardiac membranes from AC8TM heart, the enzyme activity was assayed in the presence of increasing concentrations of Ca²⁺. As expected, micromolar concentrations of Ca²⁺ inhibited the FSK-stimulated AC activity in normal nontransgenic animals by ≈25% (Figure 2C). In membranes from AC8TM, forskolin (FSK)-stimulated AC activity was higher by 3- to 4-fold than in CM hearts; it was only slightly inhibited by Ca²⁺. Addition of calmodulin (1 μmol/L) had no effect on control membranes (Figure 2A) but evoked a 3-fold stimulation of AC activity in transgenic membranes (Figure 2B). This stimulation was completely abolished by the addition of the calmodulin inhibitor, W7 (100 μmol/L). Because the basal activity increased from 21.0 ± 3.4 to 156.26 ± 5.5 pmol cAMP/min mg⁻¹ proteins in membranes from control and transgenic hearts, respectively, and to 450.47 ± 39.0 pmol cAMP/min mg⁻¹ proteins in transgenic heart membranes under calmodulin stimulation, these results demonstrate that, in hearts from transgenic mice, AC8 represents the major part of the AC activity.

In mammalian cardiomyocytes, calmodulin plays an important role as a regulator of cell proliferation and function. Although its concentration decreases after birth in the heart, it remains high in the adult. We did not find any modification in the calmodulin expression in the heart of AC8TM as compared with their littermates (Figure 3).

To determine whether the increase in the AC activity observed in vitro in the heart membranes of transgenic mice corresponds to an increase in the AC activity in vivo, we measured the cAMP-dependent PKA activity on crude heart extracts from AC8TM and CM. In transgenic mice, PKA activity was found to be higher by 4-fold than that of control animals (2.14 ± 0.06 [n=3] versus 0.59 ± 0.04 pmol ATP/min μg⁻¹ protein [n=3]; P<0.0004), whereas the total PKA activity measured in the presence of an excess of cAMP was unchanged (transgenic, 7.52 ± 0.49 pmol ATP/min μg⁻¹ protein, n=3; control, 7.76 ± 0.43 pmol ATP/min μg⁻¹ protein, n=3; P=NS). This indicates that cAMP level in hearts of AC8TM was considerably increased as compared with CM, suggesting that AC8 was functionally active in vivo.

**Echocardiography and In Vivo Assessment of Cardiac Function in AC8-Overexpressing Mice**

To determine whether the marked overexpression of AC8 would affect the physiological phenotype, transthoracic echocardiography was performed. No differences in behavior or exterior aspect were observed. Neonatal mortality was not different between transgenic and nontransgenic animals.

**Figure 2. Effects of Ca²⁺ on basal and on FSK- and calmodulin-stimulated AC activities in cardiac membranes from AC8TM and CM.** Membranes (25 μg of proteins) prepared from hearts of CM (A) or transgenic AC8 littermates (B) were incubated with increasing amounts of Ca²⁺ in the presence (△ and ▲) or in the absence (□ and ■) of 1 μmol/L calmodulin. ● indicates W7 (100 μmol/L) added to the incubation medium. C, Effects of Ca²⁺ on FSK-stimulated AC activity. AC activity measured in the presence of 10 μmol/L FSK and 0.08 μmol/L free Ca²⁺ was taken as 100% (CM, 589.92 ± 11.99 pmol cAMP/min mg⁻¹ protein; AC8TM, 2054.74 ± 186.43 pmol cAMP/min mg⁻¹ protein).
Figure 3. Western blot analysis of calmodulin (CaM) expression. A, Expression in mouse brain and heart (50 μg protein/lane). Various concentrations of pure calmodulin were used as control as well as a positive control proposed by the manufacturer. B, Expression in mouse heart from 3 control and transgenic animals (50 μg/lane). C, Increasing amounts of cardiac extract from control and transgenic mouse hearts.

Cardiography was performed. Despite the increase in cardiac AC expression, basal heart rate and contractile functions were unchanged (Table); LV end-diastolic and end-systolic dimensions, heart rate, %FS, and mean Vcfc were similar between the 2 groups.

We further assessed the in vivo cardiac function by cardiac catheterization in intact anesthetized control and transgenic mice after bilateral vagotomy. The following parameters were measured: heart rate, %FS, and mean Vcfc were similar between the 2 groups.

Echocardiography Parameters in Control and AC8 Transgenic Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=10)</th>
<th>Transgenic (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>493±13</td>
<td>491±13</td>
</tr>
<tr>
<td>Velocity of circumferential fiber shortening, circ/s</td>
<td>7.76±0.54</td>
<td>8.05±0.34</td>
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<tr>
<td>Posterior wall thickness, mm</td>
<td>0.61±0.03</td>
<td>0.57±0.07</td>
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<tr>
<td>Septal wall thickness, mm</td>
<td>0.77±0.04</td>
<td>0.82±0.04</td>
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<tr>
<td>Left ventricular diastolic diameter, mm</td>
<td>4.00±0.09</td>
<td>3.88±0.09</td>
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<tr>
<td>Left ventricular systolic diameter, mm</td>
<td>2.50±0.09</td>
<td>2.37±0.10</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>37.4±1.94</td>
<td>39.2±1.42</td>
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Characterization of the AC Signaling in Transgenic Heart

To document whether cardiac myocytes overexpressing AC8 were responsive to β-adrenergic stimulation in vitro, AC assays were performed in the presence of ISO and in the presence or absence of calmodulin (Figure 5). In control heart, β-agonist stimulation increased AC activity by 2-fold (Figure 5A). Addition of calmodulin (1 μmol/L) had no effect on the ISO-stimulated AC activity. In membranes from AC8TM, the AC activity was only poorly stimulated by 10 mmol/L ISO, from 219.6±2.99 to 262.25±5.94 pmol cAMP/min mg⁻¹ protein in the absence of calmodulin, and increased from 600.0±11.35 to 685.0±14.58 pmol cAMP/min mg⁻¹ protein in the presence of 1 μmol/L calmodulin (Figure 5B). The apparent affinity toward ISO was not different in control and transgenic mice. Thus, ISO stimulation does not appear to affect AC8 activity directly, and it is very likely that in AC8TM, the ISO-stimulated AC activity essentially corresponds to the effect of ISO on the endogenous isoforms. Furthermore, radioligand binding assays indicated a similar β-AR number in control (57.95±3.10 fmol 125I-CYP bound/mg protein) and transgenic (59.39±1.96 fmol 125I-CYP bound/mg protein) animals, with no difference in the apparent affinity.

The GTPγS dose-response curve is shown in Figure 5C. The activity assayed on membranes from CM increased from 13.75±3.25 pmol cAMP/min mg⁻¹ protein in the absence of GTPγS to 126.67±3.48 in the presence of 10 μmol/L GTPγS. Addition of calmodulin does not affect this activity. In cardiac membranes from transgenic animals, the activity increased from 150.33±4.63 (GTPγS=0) to 320.67±41.09 (GTPγS=10 μmol/L) pmol cAMP/min mg⁻¹ protein in the absence of calmodulin and from 313.0±7.21 (GTPγS=0) to 641.0±38.8 (GTPγS=10 μmol/L) pmol cAMP/min mg⁻¹ protein in the presence of 1 μmol/L calmodulin. Thus, the increased AC activity in transgenic mice did not affect either the number of β-ARs or the GTPγS responsiveness of AC activity.

Figure 6 shows the effect of increasing Ca²⁺ concentration on ISO-stimulated AC activity. As expected, Ca²⁺ inhibited the ISO-stimulated endogenous activity by ∼30% in heart membranes from CM and to a lesser extent in heart membranes from AC8TM. Whereas calmodulin had no effect on inhibition of ISO-stimulated AC activity in CM, in the AC8TM, Ca²⁺ and calmodulin increased the ISO-stimulated activity from 265.0±5.34 to 463.0±32.25 pmol cAMP/min mg⁻¹ protein.

Discussion

In the heart, the inotropic effect of β-adrenergic agonists is mediated by the stimulation of AC activity and the subsequent phosphorylation of specific proteins by cAMP-dependent protein kinase. The prevalence of AC5 and AC6 in the cardiomyocytes hints at a crucial role for the susceptibility of these ACs to Ca²⁺ inhibition in the regulation of cardiac function. It has been proposed that elevated [Ca²⁺]i could inhibit cAMP synthesis by AC5 and AC6 and thereby provide sensitive negative feedback.12,30–32 We have developed an in vivo model of transgenic mice overexpressing the Ca²⁺-stimulatable isoform AC8 specifically in cardiomyocytes. Because of the presence of calmodulin in the heart,28,29 the activity of this isoform should be activated
by Ca\(^{2+}\) when the activity of the endogenous isoforms, AC5 and AC6, are inhibited.

Two transgenic lines have been obtained, both expressing AC8 at high levels in cardiomyocytes. For both, AC activity was increased \(\approx 7\)-fold and was strongly activated by Ca\(^{2+}/\)calmodulin, with AC8 representing at least 80% of the total activity in the cardiomyocyte membranes. ISO does not stimulate directly AC8 activity in heart membranes from transgenic mice. The inability of AC8 to respond to ISO by increased cAMP accumulation has already been described in AC8-transfected HEK293 cells.\(^{14}\) Furthermore, Fagan et al\(^{33}\) demonstrated that HEK 293 cells possess the capability to localize transfected AC appropriately, suggesting that the targeting information is encoded within the protein sequence. In this context, AC8 would appear to function as a "pure Ca\(^{2+}\)" detector.\(^{34,35}\) Baker et al\(^{36}\) have demonstrated that the Gs-coupled receptor, 5-HT7 receptor, stimulates AC8 activity in vivo, by increasing [Ca\(^{2+}\)]\(_i\) concentration. We cannot exclude a similar mode of action for the \(\beta\)-AR to account for some effect on AC8 activity in vivo.

Despite the high AC and PKA activities, the basal cardiac function of the transgene-positive animals, as measured by echocardiography, was not affected. In contrast, when the phenotype was evaluated by invasive hemodynamics, LV dP/dt\(_{\text{max}}\) (an index of contractility) was increased and found to be unresponsive to further \(\beta\)-AR stimulation. Our physiological data demonstrate that overexpression of AC8 does not have deleterious consequences on global cardiac function, because chamber size and fractional shortening are normal. Furthermore, heart rate is not affected as long as the autonomic nervous system is intact. However, release of parasympathetic tone shows that the intrinsic contractility is heightened, in part related to the higher heart rate, with loss of normal \(\beta\)-AR function as shown by the lack of responsiveness to catecholamine stimulation. Because echocardiography is most sensitive for the determination of chamber dimension and not contractile function, it is not surprising that echo parameters of %FS and Vcfc are the same. For instance, overexpression of the \(\beta\)-AR results in a marked increase in dP/dt max\(^4\) but has no effect on %FS or Vcfc.\(^{37}\) Importantly, under certain conditions, these mice lose normal regulation of \(\beta\)-AR coupling. Whether this will have an impact in the conscious animal will require further study.

Cardiac overexpression of the \(\beta\)-AR, Gs, or \(\beta\)-AR kinase inhibitor only slightly increases the basal AC activity and the \(\beta\)-AR signaling.\(^{4–6,38}\) On the other hand, unlike our AC8 mice, AC6 overexpression in mice resulted in a strong amplification of the \(\beta\)-AR signaling,\(^{39}\) as evidenced by cAMP accumulation in isolated cardiomyocytes and physiological assessment of cardiac function, although the echocardiographic parameters were unchanged. The overall published literature points to a very complex relationship

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**Figure 4.** In vivo assessment of LV contractile function in response to \(\beta\)-agonist stimulation. Parameters measured were LV end-systolic and end-diastolic pressure, LV dP/dt\(_{\text{max}}\) and LV dP/dt\(_{\text{min}}\), and heart rate. Four measured parameters are shown at baseline and after progressive doses of ISO in wild-type mice (○, \(n=14\)) and AC8TM (●, \(n=12\)). A, Heart rate. B, LV systolic pressure. C, LV dP/dt\(_{\text{max}}\). D, LV dP/dt\(_{\text{min}}\). Data were analyzed with a repeated-measures ANOVA. \(*p<0.0001, †p<0.05\) vs AC8TM.
Figure 5. Effect of ISO or GTPγS on basal or Ca2+/calmodulin-stimulated AC activities. AC activity was measured in the presence of indicated concentrations of ISO and 10 μmol/L GTP or in the presence of GTPγS. Effect of ISO and GTPγS on Ca2+/calmodulin-stimulated AC activity was assayed in the presence of GTP (10 μmol/L), calmodulin (1 μmol/L), and 1.7 μmol/L free Ca2+. M, Control (basal); □, control (1 μmol/L calmodulin); ■, AC8TM (basal); ▲, AC8TM (1 μmol/L calmodulin).

Figure 6. Effect of Ca2+ and calmodulin on ISO-stimulated AC activities. AC activity was measured in the presence of ISO (5 μmol/L), GTP (10 μmol/L), and indicated concentrations of free Ca2+, with or without addition of calmodulin (1 μmol/L). M, Control (basal); □, control (1 μmol/L calmodulin); ■, AC8TM (basal); ▲, AC8TM (1 μmol/L calmodulin).
between β₁- and β₂-ARs, Ca²⁺, and cardiac contraction, which could explain the differences observed in the various models of transgenic animals. At present, the relationship between Ca²⁺, cAMP, and the various parameters of cardiac function still needs to be clarified. From this point of view, AC8TM represent an original model in which the AC activity is stimulatable by Ca²⁺. These mice can be used to investigate in more detail the relative influence of Ca²⁺ and cAMP on cardiac function within a phenotype of enhanced contractility and relaxation.

Acknowledgments

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References

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ENHANCED CARDIAC FUNCTION IN
TRANSGENIC MICE EXPRESSING A CALCIUM-
STIMULATED ADENYLYL CYCLASE

by
Larissa Lipskaia*, Nicole Defer*, Giovanni Esposito**, Iman Hajjar**, Marie-Claude Garel**, Howard A. Rockman** and Jacques Hanoune*

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METHODS (For Online Supplementary informations)

Generation and Identification of Transgenic Mice. The use of animals was in accordance with institutional guidelines. Transgenic mice were created using the \textit{\textalpha-myosin heavy chain} (\textit{\textalpha-MHC}) promoter to direct cardiac specific overexpression of AC8. A blunt-KpnI 5.5 kb \textit{\textalpha-MHC} promoter \textsuperscript{1} cassette was ligated into pGEM-9Zf(-) plasmid, previously modified to contain the 698 bp \textit{SV-40 intron/poly A} sequence. A 3.9 kb KpnI-blunt fragment corresponding to the entire coding region for human AC8 \textsuperscript{2} was inserted in the appropriated site downstream of the promoter. The resultant recombinant plasmid, pGEM- \textit{MHC-AC8-SV40} was digested with NotI and Nsil to generate a linear DNA fragment, consisting of the \textit{\textalpha-MHC} promoter, the coding sequence for human AC8, and the \textit{SV-40 intron/polyA} signal. This
fragment was purified by electrophoresis and microinjected into the pronuclei of fertilized eggs from superovulated (C57BL/6 X DBA) F1 female mice. The eggs were then reimplanted into pseudogestant mice. Transgenic founders were screened for the presence of human AC8 DNA by Southern blotting analysis performed on genomic DNA extracted from tail biopsies, digested with HpaI enzyme, and using as probe a 477 bp fragment specific for AC8. Filters were washed at decreasing concentrations of SSC with the final wash in 0.5 X SSC / 0.1 % SDS at 65°C, and exposed to XAR film for 24 hr. Number of transgene copies was determined by slot-blot analysis, and quantified by Instant Imager. The AC8TM line was propagated by mating with C57BL/6 mice.

**Transthoracic echocardiography.** Echocardiography was performed in anesthetized mice (Avertin 2.5%, 14 μl/g intraperitoneally) using an ATL HDI 5000 echocardiograph (ATL, Bothell, WA) as previously described. The following parameters were measured: Left ventricular end diastolic dimension (LVDD); Left ventricular end systolic dimension (LVSD); Posterior and Septal wall thickness (PW); Heart rate (HR);%Fractional shortening (%FS) calculated as (LVEDD-LVESD)/LVEDD×100; Mean velocity of circumferential fiber shortening (meanVcfc).

**Hemodynamic Evaluation in Intact Anesthetized Mice.** Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) and analyzed as previously described. Briefly, after endotracheal intubation, mice were connected to a rodent ventilator. Following bilateral vagotomy, a 1.4 Fr high fidelity micromanometer catheter (Millar Instruments) was inserted into the right carotid artery and retrograde across the aortic valve into the LV. Hemodynamic measurements were recorded at baseline and 45-60 seconds after injection of incremental doses of isoproterenol (ISO). Doses of ISO were specifically chosen to maximize the contractile response but limit the increase in heart rate. Ten sequential beats were averaged for each measurement.
**Adenylyl Cyclase Assays.** AC activity was measured as described on purified cardiac membranes. Hearts were homogenized in 10 volumes of ice-cold lysis buffer [10 mmol/L Tris-HCl (pH 7.6), 0.1 mmol/L EDTA, 0.5 mmol/L DTT and 0.5 mmol/L PMSF (Phenyl methyl sulfonyl fluoride)] and centrifuged at 500 x g for 5 min at 4°C. The supernatant was centrifuged at 15000 x g for 30 min and the pellet washed three times in the same buffer. The standard AC assay contains 70 mmol/L Tris-HCl (pH 7.6), 5 mmol/L MgCl2, 1 mmol/L ATP (disodium salt), 1 x 10^6 cpm/assay [α-^32P] ATP (tetra (triethylammonium) salt) (Amersham), 1 mmol/L cAMP containing [8-^3H] cAMP (10 - 15 x 10^3 cpm/assay), 5 mmol/L phosphocreatine (disodium salt), 60 units/ml creatine phosphokinase and 20-40 μg of membrane protein in a final volume of 60 μl. The reactions were conducted for 10 min at 35°C and terminated by addition of 200 μl of 0.5 mol/L HCl, and neutralized by 200 μl of 10 mmol/L imidazole (pH 7.6). Cyclic AMP formed was separated by alumina column and corrected for recovery of added [^3H] cAMP. For analysis of calcium effect on AC activity the membranes were washed twice with 1 mmol/L EGTA. ISO-stimulated activities were measured in the presence of 10 μmol/L GTP. Data are presented as mean activities ± S.E.M. of five determinations. Free concentrations of Ca^{2+} were calculated as described by Cooper.

**cAMP-Dependent Protein Kinase Assay.** PKA activity was measured on crude myocardial extracts prepared as follows : hearts were rapidly excised, washed in 0.9% NaCl and homogenized in 5 volumes of ice-cold extraction buffer containing 25 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 1 mmol/L DTT, 1μmol/L leupeptin, 1μmol/L aprotinin, 0.5 mmol/L PMSF. The homogenate was centrifuged (14000 x g for 5 min at 4° C), and the supernatant was immediatly assaied for PKA activity using Signa TECTm cAMP-Dependent Protein Kinase (PKA) Assay System (Promega), according to the instructions of the manufacturer. Protein concentration was determined by the Bradford method. Assays were
performed in triplicate with or without exogenous cAMP (5 µmol/L). Addition of the PKA peptide inhibitor completely abolished the enzyme activity.

**Beta-AR Binding.** β-AR were estimated by saturation binding of $^{125}$I-iodocyanopindolol ($^{125}$I-CYP) as described. Reactions were performed in 250 µl of incubation buffer (75 mmol/L Tris-HCl, pH 7.4; 12.5 mmol/L MgCl$_2$; 2mmol/L EDTA; 0.1 mmol/L ascorbic acid) containing 25 µg of membrane proteins and 5-400 pmol/L of $^{125}$I-CYP alone or in the presence of 3 µmol/L of (-)-propranolol. All assays were performed in triplicate.

**Western Blotting.** Crude cardiac homogenates were prepared by homogenization of whole hearts in lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 µg EGTA; 1 mM PMSF; 1 µg/ml aprotinin, leupeptin, pepstatin; 1mM Na$_3$VO$_4$; 1 mM NaF), as described (Upstate-Biotechnology). Proteins (25-100 µg/lane) were separated on 12% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was incubated with anti-calcmodulin antibody as recommended by the manufacturer, and antigen visualized using the ECL system from Amersham (Amersham Pharmacia Biotech).

**Statistical Analysis.** All results are expressed as mean value ± SEM of at least three determinations. To examine the effect of ISO on changes in hemodynamic parameters between wild-type controls and transgenic animals, a repeated measures analysis of variance (ANOVA) was used. For echocardiographic data, a one factor ANOVA was used. Post hoc analysis with regard to differences in mean values between groups was conducted with a Scheffé test. $p < 0.05$ was considered significant.
REFERENCES


