Nuclear α1-Adrenergic Receptors Signal Activated ERK Localization to Caveolae in Adult Cardiac Myocytes

Casey D. Wright, Quanhai Chen, Nichole L. Baye, Yuan Huang, Chastity L. Healy, Sivakanthan Kasinathan, Timothy D. O’Connell

Abstract—We previously identified an α1-AR-ERK (α1A-adrenergic receptor–extracellular signal-regulated kinase) survival signaling pathway in adult cardiac myocytes. Here, we investigated localization of α1-AR subtypes (α1A and α1B) and how their localization influences α1-AR signaling in cardiac myocytes. Using binding assays on myocyte subcellular fractions or a fluorescent α1-AR antagonist, we localized endogenous α1-ARs to the nucleus in wild-type adult cardiac myocytes. To clarify α1 subtype localization, we reconstituted α1 signaling in cultured α1A- and α1B-AR double knockout cardiac myocytes using α1-AR–green fluorescent protein (GFP) fusion proteins. Similar to endogenous α1-ARs and α1A- and α1B-GFP colocalized with LAP2 at the nuclear membrane. α1-AR nuclear localization was confirmed in vivo using α1-AR-GFP transgenic mice. The α1-signaling partners Gq and phospholipase Cβ1 also colocalized with α1-ARs only at the nuclear membrane. Furthermore, we observed rapid catecholamine uptake mediated by norepinephrine-uptake-2 and found that α1-mediated activation of ERK was not inhibited by a membrane impermeant α1-blocker, suggesting α1 signaling is initiated at the nucleus. Contrary to prior studies, we did not observe α1-AR localization to caveolae, but we found that α1-AR signaling initiated at the nucleus led to activated ERK localized to caveolae. In summary, our results show that nuclear α1-ARs transduce signals to caveolae at the plasma membrane in cardiac myocytes. (Circ Res. 2008;103:992-1000.)

Key Words: α1-adrenergic receptors ■ cardiac myocytes ■ ERK

Cardiovascular disease is the leading killer in the United States, accounting for 1.4 million deaths a year. Five million Americans experience heart failure, leading to 970,000 hospitalizations annually, a number that has tripled in the last 25 years.1 In heart failure, increased activation of the sympathetic nervous system is correlated with pathophysiological remodeling of the heart,2 which has led to the therapeutic use of β-adrenergic receptor (AR) antagonists in heart failure. However, the general consensus that inhibition of catecholamine activation of ARs is beneficial in heart failure is disputed by clinical trials with α1-AR antagonists in heart failure.5

All 3 α1-AR subtypes (A, B, and D) are expressed in the heart,6–9; however, cardiac myocytes only express the α1A and α1B.7 Using α1A- and α1B-AR double knockout mice (α1ABKO), we demonstrated previously that α1-ARs are required for postnatal hypertrophy and adaptation to pathological stress.9,10 In α1ABKO mice, we found that aortic constriction induced dilated cardiomyopathy that led to heart failure and death.9 More recently, we identified an α1A-AR-ERK (α1A-AR–extracellular signal-regulated kinase) survival signaling pathway in adult cardiac myocytes,11 and knockout of this α1A-ERK pathway could explain the maladaptive response to aortic constriction in α1ABKO mice. In summary, our data demonstrate that α1-ARs are protective in the heart, which agrees with clinical trials where α1-antagonists increased the incidence of heart failure.

Here, we examined the mechanisms of α1-AR survival signaling and expanded on our previous demonstration of α1-AR localization to the nucleus in adult cardiac myocytes.11 To circumvent the lack of α1-AR subtype–specific antibodies and ligands, we developed a reconstitution system by expressing α1-AR-GFP (α1-AR–green fluorescent protein) fusion proteins in cultured α1ABKO cardiac myocytes to examine α1-AR localization and survival signaling. With no endogenous α1-ARs, α1ABKO cardiac myocytes provide the ideal model for localization and signaling experiments. Earlier work in the mouse heart suggests that α1-ARs localize to the plasma membrane, possibly in caveolae and that

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α1-AR signaling can be modified by caveolin-3.12–15 These findings fit the classic “outside-in” model of G protein–coupled receptor (GPCR) localization and signaling because the immediate signaling partners (Gq and phospholipase [PL]C1) are also localized to caveolae.16,17 In contrast, our previous data using 1-AR-GFP fluorescent fusion proteins suggested a nuclear localization for 1-ARs in adult cardiac myocytes.11 Our identification of a GPCR (1-AR) localized to the nucleus is not without precedence. Recently, functional endothelin-A, endothelin-B and 1-ARs were identified on the nucleus in adult cardiac myocytes.18,19

Using our reconstitution system, we set out to clarify 1-AR localization and study the mechanisms behind 1-AR-ERK survival signaling in adult cardiac myocytes.10,11 In contrast, our previous data using 1-AR-GFP fluorescent fusion proteins suggested a nuclear localization for 1-ARs in adult cardiac myocytes.11 Our identification of a GPCR (1-AR) localized to the nucleus is not without precedence. Recently, functional endothelin-A, endothelin-B and β-ARs were identified on the nucleus in adult cardiac myocytes.18,19

Using our reconstitution system, we set out to clarify 1-AR localization and study the mechanisms behind 1-AR-ERK survival signaling in adult cardiac myocytes. In this report, we found that endogenous 1-ARs localized to the nucleus in wild-type (WT) adult mouse cardiac myocytes, which was confirmed in vivo using a cardiac-specific 1-AR transgenic mouse. Confocal microscopy and cellular fractionation demonstrated that the 1-AR subtypes, Gq, and PLCβ1 localized to the nuclear membrane in adult cardiac myocytes. Furthermore, we found no evidence that either 1-AR subtype localized to the plasma membrane or caveolae. Finally, activation of functional 1-ARs on the nucleus led to accumulation of activated (phosphorylated) ERK in caveolae at the plasma membrane. Our results present a provocative new model by demonstrating that 1-AR signaling initiated at the nucleus results in activated ERK localization in caveolae at the plasma membrane.

Materials and Methods

The procedures for generating adenoviral constructs,11 culture of adult mouse cardiac myocytes,10,11 localization of 1-ARs by confocal microscopy,11 isolation18,19 and enrichment20 of nuclei, and measurement of 1-AR expression have been described elsewhere. Methods describing the generation of 1-AR-GFP transgenic mice, catecholamine uptake assay, treatment of cardiac myocytes with CGP-12177A, and detection of the extraneuronal monoamine transporter (EMT/OCT3) are available in the expanded Materials and Methods section of the online data supplement at http://circres.ahajournals.org. The use of animals in this study conformed to the Public Health Service Guide for Care and Use of Laboratory Animals and was approved by The University of South Dakota Institutional Animal Care and Use Committee.

Results

Endogenous 1-Adrenergic Receptors Localize to the Nucleus in WT Adult Mouse Cardiac Myocytes

Here, we set out to determine 1-AR subtype localization in adult cardiac myocytes. To examine 1-AR subcellular distribution, we isolated membrane, cytosolic, and nuclear fractions from freshly isolated WT adult mouse cardiac myocytes and measured 1-AR binding with 3H-prazosin.
Membrane, cytosolic, and nuclear fractions were validated by Western blots for caveolin-3, GAPDH, and LAP2, respectively (Figure 1a). Binding assays with 3H-prazosin indicated that 80% of total α1-AR binding was detected in the nuclear fraction. Binding detected in the membrane fraction was likely a result of α1-ARs localized to endoplasmic reticulum, because SERCA2 was detected in the membrane fraction (Figure 1a). To verify this finding, we examined endogenous α1-AR localization in cultured WT adult cardiac myocytes by labeling α1-ARs with a fluorescent, subtype-nonselective α1-AR antagonist, BODIPY-prazosin, which overcomes the lack of subtype-specific α1-AR antibodies. BODIPY-prazosin labeling revealed that endogenous α1-ARs were concentrated around the nucleus but not on the plasma membrane in WT cardiac myocytes (Figure 1b). To verify that α1-ARs were expressed on the nucleus in cardiac myocytes, isolated nuclei from WT adult cardiac myocytes were also labeled with BODIPY-prazosin, and a strong signal was detected on the nuclear membrane (Figure 1b, insets). In summary, these data suggest that endogenous α1-ARs localize primarily to the nucleus in WT adult cardiac myocytes.

The α1A and α1B Subtypes Colocalize With the Nuclear Membrane Protein LAP2 in Adult Mouse Cardiac Myocytes

To test whether the α1A and α1B subtypes localize to the nucleus, we assessed colocalization of both α1 subtypes with the inner nuclear matrix protein LAP2. Again, to overcome the lack of subtype-specific antibodies, we expressed α1-fluorescent fusion proteins (α1-GFP) in cultured α1ABKO adult cardiac myocytes, which lack endogenous α1-ARs.11 In this reconstitution system, the α1-GFP fusion proteins are expressed at only 2.5- to 3-fold over endogenous α1-AR levels.11 Confocal microscopy revealed an intense GFP signal for both the α1A and α1B and, as expected, an intense fluorescent signal (red) for LAP2 on the nuclear membrane (Figure 2a). Using Imaris image analysis software, we demonstrated that both α1 subtypes colocalize with the nuclear matrix protein LAP2 (Figure 2a, yellow).

To confirm the confocal microscopy results, cellular fractions were isolated from cultured α1ABKO adult cardiac myocytes expressing the α1-GFP fusion proteins. Membrane, cytosolic, and nuclear fractions were validated by Western blots for caveolin-3, GAPDH, and LAP2, respectively (Figure 2b). As expected, both the α1A-GFP and α1B-GFP were detected predominantly in the isolated nuclear fraction (Figure 2b). In summary, the results obtained in our reconstitution system by confocal microscopy and subcellular fractionation demonstrated that the α1A and α1B subtypes were expressed in the nuclear membrane and confirmed the nuclear localization of endogenous α1-ARs in WT adult cardiac myocytes.

Prazosin Induces α1-Adrenergic Receptors to Move off the Nuclear Membrane in Adult Mouse Cardiac Myocytes

In cultured WT adult cardiac myocytes, BODIPY-prazosin labeling also identified a small population of nonnuclear α1-ARs (Figure 1b). We hypothesized that this was attributable to long-term incubation with BODIPY-prazosin, which induces β-arrestin–dependent receptor internalization (movement off the membrane).21 To test this, we expressed the α1-GFP fusion proteins in cultured α1ABKO cardiac myocytes and examined the α1-subtype localization throughout a 16-hour treatment with unlabeled prazosin. Indeed, prazosin induced translocation of both the α1A-GFP and α1B-GFP off the nucleus, whereas vehicle treatment had no effect (Figure 3). This effect was seen at 2 hours and was consistent throughout treatment for the α1A-GFP (Figure 3, time course). In summary, the nonnuclear α1-AR population observed with BODIPY-prazosin staining is likely attributable to α1-AR translocation off the nuclear membrane, possibly by receptor desensitization, rather than a subpopulation of nonnuclear α1-ARs.

α1-Adrenergic Receptors Localize to the Nucleus in Adult Mouse Cardiac Myocytes In Vivo

To determine whether the nuclear α1-AR localization we observed in cultured adult cardiac myocytes was similar in vivo, we generated cardiac-specific α1A-GFP transgenic mice and used an antibody against GFP to identify α1A-subtype localization in whole heart sections. Heart sections from the α1A-GFP transgenic mice stained for GFP, identified the α1A subtype at the nucleus (Figure 4), which parallels results from our culture model. Saturation binding analysis indicated that the α1A-GFP was overexpressed 7-fold in the heart (Figure 1a in the online data supplement). Functionally, α1A-GFP overexpression in
increased α1-mediated phosphorylation of ERK, as determined by Western blot (supplemental Figure Ib). However, we did not detect a hypercontractile phenotype, as previously reported in H9251A-Tg mice with 170-fold overexpression, nor did we detect a hypertrophic phenotype, as observed with overexpression of the α1B subtype (data not shown).12,22,23

The α1A and α1B Subtypes Colocalize With Gαq and PLCβ1 in the Nuclear Membrane in Adult Mouse Cardiac Myocytes

If functional α1-ARs are expressed on the nuclear membrane in adult cardiac myocytes, then the α1-signaling partners Gαq and PLCβ1 should colocalize to the nucleus as well. To test this, we used immunocytochemistry to determine whether Gαq and PLCβ1 were also expressed on the nuclear membrane. In cultured α1ABKO cardiac myocytes expressing α1A-GFP or α1B-GFP, we found that both Gαq and PLCβ1 localized to the nuclear membrane, as well as the plasma membrane (Figure 5a and 5b, respectively). However, image analysis revealed that both Gαq and PLCβ1 colocalized with the α1 subtypes only at the nuclear membrane.

To confirm the immunocytochemical analyses performed above, cellular fractionation and Western blot analyses were performed on freshly isolated WT adult mouse cardiac myocytes (Figure 5c). Western blots for caveolin-3, GAPDH, and LAP2 validated the purity of the membrane, cytosolic, and nuclear fractions (Figure 5c). Blotting the WT cell fractions for Gαq and PLCβ1 detected both signaling molecules in the nuclear fraction (Figure 5c). In summary, Gαq and PLCβ1 colocalized with both α1-AR subtypes only in the nuclear membrane (defined by LAP2 staining), which supports the hypothesis that α1-ARs could signal at the nucleus in adult cardiac myocytes.

α1-Adrenergic Receptors Do Not Localize to Caveolae in Adult Mouse Cardiac Myocytes

The colocalization of the α1-AR subtypes, Gαq, and PLCβ1 to the nuclear membrane suggests that α1-ARs signal from the nucleus not the plasma membrane. However, previous research suggested that α1-ARs localize to caveolae in rats17 and adult mice.12 Furthermore, others demonstrated that increasing caveolin-3 expression inhibits α1-ERK signaling in cardiac myocytes, whereas knocking out caveolin-3 leads to hyperactivation of ERK signaling.14,15 Here, we immunolabeled caveolin-3 to determine whether the α1-AR subtypes localized to caveolae. In cultured α1ABKO cardiac myocytes expressing the α1-GFP fusion proteins, caveolin-3 localized to the plasma membrane and t-tubules, whereas both α1-AR subtypes remained on the nucleus (Figure 6). The failure to observe caveolin-3 colocalization with either α1-AR subtype provides evidence against a direct interaction of α1-ARs with caveolin-3 in adult cardiac myocytes. These results also suggest that the modulation of α1-AR signaling by caveolin-3 likely occurs with signaling molecules downstream of the receptor itself.

Figure 3. Prazosin induces α1-ARs to move off the nuclear membrane in adult mouse cardiac myocytes. Cultured α1ABKO cardiac myocytes were infected as in Figure 2. After 24 hours, myocytes were treated with prazosin (50 nmol/L) or vehicle (control) for 16 hours and then fixed with paraformaldehyde, and confocal images were captured. Final magnification, ×600.

Figure 4. α1-ARs localize to the nucleus in adult mouse cardiac myocytes in vivo. Sagittal sections from hearts of α1A-GFP transgenic mice (α1A-GFP Tg) or WT mice were stained with anti-GFP antibody (Santa Cruz Biotechnology) to detect α1A-GFP and visualized using the Mouse-on-Mouse Kit with an FITC-conjugated secondary antibody (Vector Laboratories). Final magnification, ×600.
Catecholamine Uptake and Activation of Nuclear α1-Adrenergic Receptors in Adult Mouse Cardiac Myocytes

To activate nuclear α1-ARs, norepinephrine or other catecholamines must enter the cardiac myocyte, a process known as norepinephrine-uptake-2,24 which is facilitated by extraneuronal monoamine transporter (EMT/OCT3).25 To measure catecholamine uptake in WT cardiac myocytes, we used a fluorescent catecholamine analog that fluoresces only when transported inside a cell. We found that catecholamine uptake occurred almost immediately, peaked at 30 minutes and was antagonized by addition of norepinephrine 15 minutes before catecholamine uptake measurement, indicating specificity (Figure 7a).

To demonstrate the functionality of nuclear α1-ARs, we examined α1A-AR mediated activation of ERK in cultured WT adult cardiac myocytes. Specifically, we compared the ability of CGP-12177A, a membrane impermeable α1-antagonist26–29 and prazosin, which freely crosses the plasma membrane, to block phenylephrine (PE) (α1-agonist) induced phosphorylation of ERK in cultured WT adult cardiac myocytes. Prazosin blocked PE-mediated activation of ERK, whereas CGP-12177A did not (Figure 7b), indicating that α1-AR mediated activation of ERK requires intracellular agonist and receptor.

In cultured WT adult cardiac myocytes, we detected EMT/OCT3 on both the plasma and nuclear membranes by immunocytochemistry (Figure 7c). Furthermore, we demonstrated that inhibiting EMT/OCT3 with corticosterone, an EMT/OCT3 antagonist, prevented α1-mediated activation of ERK (Figure 7c). Because our results indicate that α1-ARs, Gq, and PLC1 localize to the nucleus, the results with CGP-12177A and corticosterone inhibition of EMT/OCT3 are best explained by α1 signaling at the nucleus.

Phosphorylated ERK Localizes to Caveolae at the Plasma Membrane in Adult Mouse Cardiac Myocytes

To investigate the mechanism of α1-mediated activation of ERK, we examined phosphorylated ERK localization following PE treatment. In cultured α1ABKO cardiac myocytes expressing α1A-GFP or α1B-GFP, PE led to phospho-ERK plasma membrane localization within 5 minutes, which persisted for up to 3 hours (Figure 8c). The localization of phospho-ERK to the plasma membrane was surprising, because the receptor and its signaling molecules were localized to the nuclear membrane. However, short-term PE treatment, which activated ERK at the plasma membrane, did not translocate α1-ARs from the nucleus, suggesting α1-AR signaling was initiated at the nucleus, with a postreceptor signal translocated to the plasma membrane.

To determine whether phosphorylated ERK localized to caveolae at the plasma membrane, we colocalized phospho-ERK and caveolin-3 following α1-AR stimulation in cultured α1ABKO cardiac myocytes expressing the α1A-GFP (Figure 8d). Following PE treatment, phospho-ERK and caveolin-3 colocalized at the plasma membrane. To verify the caveolar...
localization of phospho-ERK, we disrupted the assembly of caveolae using filipin.31 Increasing concentrations of filipin reduced the colocalization of phospho-ERK and caveolin-3, suggesting an interaction between phospho-ERK and caveolin-3 (Figure 8d). A putative caveolin-binding domain is present in ERK and previous research has shown an interaction between ERK and caveolins,32–34 but we could not confirm a direct interaction between ERK and caveolin-3 by coimmunoprecipitation. However, our results demonstrating a loss of phospho-ERK and caveolin-3 colocalization following filipin treatment indicates that ERK likely localizes to caveolae, possibly via some intermediate binding partner. Furthermore, although α1-ARs do not localize to caveolae (Figure 6), α1-AR signaling could still be modified indirectly by caveolin-3 at the plasma membrane.14,15 In summary, our data suggest a novel model for α1-AR function in cardiac myocytes, where activation of nuclear α1-ARs leads to activated ERK localized to caveolae at the plasma membrane.

Discussion

Here, we examined the localization of both the α1A and α1B subtypes and the mechanisms regulating α1A-AR-ERK survival signaling in adult cardiac myocytes. Our data indicate that both the α1A and α1B subtypes localize to and signal at the nuclear membrane. Furthermore, our data suggest that α1-signal transduction from the nucleus to the plasma membrane results in activated ERK localization to caveolae in adult cardiac myocytes. The following lines of evidence support this novel paradigm for α1-AR signaling in cardiac myocytes.

Using binding assays on subcellular fractions and a fluorescent α1 antagonist, we localized endogenous α1-ARs to the nucleus in WT adult cardiac myocytes, which was confirmed using α1-GFP fusion proteins in cultured α1ABKO cardiac myocytes and in vivo with α1-AR-GFP transgenic mice (Figures 1 through 4). Furthermore, we colocalized the α1-signaling partners Goq and PLCβ1 with both α1-AR subtypes in the nuclear membrane (Figure 5), suggesting the possibility of α1 signaling at the nucleus. We failed to detect functional α1-ARs at the plasma membrane using confocal microscopy or functional studies examining α1-AR-mediated activation of ERK (Figures 6 and 7b). However, activation of nuclear α1-ARs led to accumulation of activated (phosphorylated) ERK at the plasma membrane in adult cardiac myocytes, whereas the receptor remained at the nucleus (Figure 8a). Finally, we demonstrated that phosphorylated ERK localizes to caveolae at the plasma membrane, which might provide a mechanism for caveolin-3 regulation of α1-AR signaling (Figure 8d).

Previously, others hypothesized that α1-ARs are expressed in caveolae at the plasma membrane in cardiac myocytes.16,17,35 These reports relied on isolation of caveolar membrane fractions, followed by immunobLOTS to detect α1-signaling partners and functional examination of inositol phosphate generation. However, some of these reports acknowledged the failure to identify α1-ARs in caveolae, possibly because of basic (pH 11) conditions used to isolate the caveolar membrane fractions.16,35 Alternatively, our data indicated that α1-ARs are expressed on the nuclear membrane in cardiac myocytes. The use of BODIPY-prazosin and α1-GFP fusion proteins in our reconstitution system avoided the technical challenges of isolating caveolar membrane fractions. Prior research also demonstrated that overexpression of caveolin-3 suppressed α1-AR signaling,15 and caveolin-3 knockout increased activation of ERK.14 In contrast to others,12,17 we demonstrated that these effects were probably not attributable to a direct interaction of α1-ARs and caveolin-3. However, our finding that activated ERK localized to caveolae following α1-AR stimulation provides a mechanism where caveolin-3 might regulate α1-AR signaling. Earlier studies in cardiac-specific α1A-AR transgenic mice suggested that α1-ARs are expressed on the plasma membrane in cardiac myocytes in vivo based on immunohistochemical detection of the α1A subtype in ventricular tissue sections.12 However, we observed α1A localization to the nucleus in our α1A-GFP Tg mouse. This discrepancy might
Nuclear localization of GPCRs in adult cardiac myocytes is not unique to α1-ARs. Functional endothelin-A and -B receptors and β-ARs, as well as their cognate signaling proteins, are also localized to the nuclear membrane of adult cardiac myocytes.18,19 Both of these reports directly demonstrated the functionality of their respective receptors on isolated nuclei, detecting a calcium transient in response to endothelin treatment18 and cAMP accumulation in response to cAMP agonist,36 we recently demonstrated that 3H-norepinephrine is taken up by neonatal rat cardiac myocytes and accumulates in the nucleus.36 We found that CGP-12177A, a membrane impermeable α1-AR antagonist, was unable to block PE-induced activation of ERK in cardiac myocytes, whereas prazosin did (Figure 7b). Finally, we also identified EMT/OCT3 on both the plasma and nuclear membranes and confirmed its role in catecholamine uptake in adult cardiac myocytes (Figure 7c).

In summary, we found that the α1A-ERK signaling in cardiac myocytes are different from the α1B-ERK signaling in cardiac myocytes. α1A-ERK signaling in cardiac myocytes is transduced from the nucleus to the plasma membrane whereas α1B-ERK signaling is not. This novel model does not fit the classic outside-in GPCR signaling model or the “caveola signaling hypothesis” as described previously.33 However, some details of this new model remain to be determined. First, the mechanism regulating α1 signaling is unknown but could involve protein kinase C, which is activated by α1-ARs, translocates to caveolae when activated,37 and could lead to ERK phosphorylation. Second, potential downstream targets of α1-ERK signaling in cardiac myocytes are unidentified. Previous work in neonatal cardiac myocytes suggested that targets could include the survival/transcription factor GATA4 and the Bcl-2 family member Bad.38,39 However, we recently demonstrated that α1 signaling does not activate GATA4 and that GATA4 was not required for α1-mediated survival signaling in adult cardiac myocytes.40

In summary, we found that the α1A and α1B subtypes localized to and activated signaling at the nuclear membrane

Figure 8. Phosphorylated ERK localizes to caveolae at the plasma membrane in adult mouse cardiac myocytes. a, Cultured α1ABKO cardiac myocytes were infected as in Figure 2. After 40 hours, myocytes were treated with 20 μmol/L PE for 15 minutes. Myocytes were fixed and stained with antibodies against P-ERK and caveolin-3, and images were captured by confocal microscopy and pseudocolored using Imaris software (GFP, pseudocolored magenta; P-ERK, green). Final magnification, ×600.
in adult cardiac myocytes. Activation of these nuclear α1-ARs led to accumulation of activated ERK at the plasma membrane, where caveolin-3 might regulate downstream α1-AR signaling. These results present a provocative new model for α1-AR signaling, where nuclear α1-ARs signal to the plasma membrane. This unique α1-AR-ERK survival-signaling pathway challenges the classic model of α1-AR localization and signaling in adult cardiac myocytes.

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Disclosures

None.

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SUPPLEMENTAL METHODS

Generation of adenoviral constructs

To generate α1A-GFP and α1B-GFP fusion proteins, cDNAs for the human α1A-AR (NM000680) and α1B-AR (NM000679) were amplified by polymerase chain reaction with primers designed to remove the stop codon and insert Bgl II and Mlu I restriction sites 5’ and 3’, respectively. The amplified α1A and α1B products were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and then subcloned into the Bgl II–Mlu I restriction sites in the multicloning site of the humanized pGFP²-N3 vector (BioSignal Packard, Montreal, Quebec, Canada), with GFP at the C-terminus.¹⁻⁴

To generate adenoviruses expressing the α1A-GFP and α1B-GFP fusion proteins under control of the cytomegalovirus promoter, the α1A-AR-GFP² and α1B-AR-GFP² were amplified by polymerase chain reaction with primers designed to insert Pme I and Xba I restriction sites at the 5’ and 3’ ends, respectively. The amplified α1A-GFP and α1B-GFP products were cloned into the pCR2.1-TOPO vector (Invitrogen) and then subcloned into the Pme I–Xba I restriction sites in the Ad5CMV K-NpA vector (ViraQuest, North Liberty, IA) under control of the cytomegalovirus promoter. The Ad5 plasmids with α1-AR inserts were then recombined with an adenoviral cell line. Clones positive for recombination were transfected into HEK293 cells. Viral products evident 7 to 10 days after transfection were amplified, purified through 2 rounds of CsCl gradients, and dialyzed against a 3% sucrose/phosphate buffer solution. Viral titer was determined by observing plaque formation in agarose overlay assays.⁵

Culture of adult mouse cardiac myocytes.

Ventricular cardiac myocytes from adult male mice were cultured as previously described.⁵⁻⁷ Briefly, hearts were removed, cannulated and perfused with collagenase type II (Worthington Biochemical Corp., Lakewood, NJ) to dissociate ventricular myocytes. Myocytes were plated at a density of 50 rod-shaped myocytes per square millimeter on laminin-coated culture dishes or coverslips. Myocytes were cultured in MEM with Hank’s Balanced Salt Solution, 1 mg/ml bovine serum albumin, 10 mM 2,3-butanedione monoxime, and 100 U/ml penicillin in a 2% CO₂ incubator at 37°C. Following myocyte plating for 1 hr, the culture medium was changed and adenovirus was added.
Isolation of intact nuclei from adult mouse cardiac myocytes.

To isolate nuclei, we used procedures previously described to isolate\textsuperscript{13,14} and enrich\textsuperscript{15} nuclei with some modifications. Briefly, cultured α1ABKO myocytes expressing α1-fluorescent fusion proteins or WT myocytes were resuspended in hypotonic lysis buffer (10 mM K-HEPES (pH 7.9), 1.5 mM MgCl\textsubscript{2}, 10 mM KCl, 1 mM DTT, 0.2 mM Na\textsubscript{3}VO\textsubscript{4}, 1% Protease Inhibitor Cocktail (Sigma P-8340)), incubated for 15 minutes on ice and homogenized using a dounce homogenizer. Whole cell lysates were centrifuged at 60xg for 5 minutes to collect unbroken cells. The supernatant was collected and the dounce homogenization and centrifugation was repeated on the pellet. The second supernatant was combined with the original supernatant and the pellet was sonicated (Sonic Dismembrator, 3 pulses for 5 sec, setting 2) in hypotonic lysis buffer and recombined with the dounce supernatants. The total homogenate was centrifuged at 2,000xg to separate crude nuclei and unbroken cells (pellet) from cell membrane and cytosolic proteins (supernatant). The supernatant of the total homogenate was centrifuged at 100,000xg for 90 minutes to separate cell membrane fraction (pelleted in tube) from soluble cytosolic proteins (supernatant). Following ultracentrifugation, the cell membrane pellet was resuspended in storage buffer (20 mM Na-HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl\textsubscript{2}, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT, 25% Glycerol, 1% Protease Inhibitor Cocktail). The nuclei and cell pellet from the total homogenate was resuspended in 500 µl of hypotonic lysis buffer, combined with 5.5 ml of 2.4 M sucrose and layered on top of 6 ml of a 2.4 M sucrose cushion and centrifuged at 100,000xg for 90 minutes to purify nuclei. The pelleted nuclei were resuspended in storage buffer and protein content determined for all fractions. The purity of the resulting protein fractions, cell membrane, cytosolic, and nuclear, was verified by Western blots using caveolin-3 (Santa Cruz; membrane marker), GAPDH (Research Diagnostics Inc, Concord, MA; cytosolic marker), and LAP2 (Cell Signaling; nuclear marker). All cell fractions were immunoblotted for Gαq, PLCβ1 (Santa Cruz), and GFP (marking α1A- or α1B-GFP; Santa Cruz).

Measurement of α1-AR expression.

α1-AR levels and binding affinity in cultured WT and α1ABKO myocytes expressing α1-ARs were measured by saturation binding with $^{3}$H-prazosin (0.04-1.2 nM), where 10 mM phentolamine (RBI, Natick, MA) defined non-specific binding. After 40 hr in culture, myocytes were counted, harvested with a cell scraper and distributed at 20,000 myocytes per tube for
binding assays (6 concentrations of prazosin in duplicate, with both specific and non-specific curves, 480,000 total myocytes per analysis). Equilibrium binding reactions were incubated at 30°C for 1 hr. Bound ligand was separated from free ligand by filtration, and filters were counted (5 min) to determine bound ligand. Total receptor number (Bmax) and binding affinity (KD) were calculated by nonlinear regression using Graph Pad PRISM 4.0 (Graph Pad, San Diego, CA).^8

Localization of α1-ARs in adult mouse cardiac myocytes by confocal microscopy

WT or α1ABKO myocytes were cultured on laminin coated glass coverslips. α1ABKO myocytes were infected with adenovirus expressing α1-GFPs. For uninfected WT myocytes, 50 nM BODIPY prazosin (Molecular Probes, Eugene, OR) was added to the culture after 24 hours. After an additional 16 hours, myocytes were fixed with 4% paraformaldehyde and mounted on slides with fluoromount G (Electron Microscopy Sciences, Hatfield, Pa). For immunocytochemistry, myocytes were blocked for 1 hr before addition of primary antibody directed against LAP2 (BD Biosciences, San Jose, CA), Gαq (Santa Cruz Biotechnology, Santa Cruz, CA), PLCβ1 (Santa Cruz), phosphorylated ERK (Cell Signaling Technology, Beverly, MA), or caveolin-3 (Santa Cruz). Samples were incubated with conjugated secondary antibodies (Alexa Fluor 405 or 594 anti-goat, Texas red anti-mouse, Texas red anti-rabbit; Invitrogen, Carlsbad, CA) for 1 hr before mounting with Fluoromount G. Fluorescent images were captured by confocal microscopy using Fluoview software (Olympus BX50 confocal microscope; Olympus America Inc., Melville, NY). Images were processed for publication using Imaris software (Bitplane Scientific Solutions, St. Paul, MN).

α1A-AR-GFP Transgenic Mice

α1A-AR-GFP transgenic mice (C57BL/6) were generated using the α-myosin heavy chain promoter to drive cardiac myocyte-specific expression of our α1A-AR-GFP construct (University of Connecticut Transgenic Core). To localize α1A-AR-GFP expression, hearts from WT and transgenic mice were arrested in diastole with 50 mM KCl, perfusion fixed with 4% paraformaldehyde, embedded in paraffin and sectioned. Sagittal sections (5-7 µm) were cleared of paraffin, rehydrated, permeabilized with 0.2% Triton X100, and processed for antibody staining with the Mouse-on-Mouse Immunodetection Kit (Vector Labs Inc., Burlingame, CA) using primary antibody to GFP (Santa Cruz Biotechnology, Santa Cruz, CA).
Measurement of Catecholamine Uptake in Isolated Adult Mouse Cardiac Myocytes.

To measure catecholamine uptake, we used the Neurotransmitter Uptake Assay (Molecular Devices, Sunnyvale, CA). Freshly isolated WT adult mouse cardiac myocytes were counted and 50,000 myocytes were used per assay condition (each condition was measured in triplicate). Myocytes suspended in perfusion buffer were dispersed in a 24-well clear bottom plate in a volume of 500 µl. For the inhibition of catecholamine uptake 50 nM or 10 µM norepinephrine was added to the appropriate wells 15 min prior to addition of the catecholamine uptake dye. Following the inhibition period, 500 µl of catecholamine uptake dye was added and measurements of fluorescence determined on a Fusion Microplate Analyzer (Perkin Elmer, Shelton, CT). Each well was measured in six locations for 0.25 sec with a 30 to 70 percent ratio of excitation to emission time.

Treatment of cultured cells with α1-AR antagonist.

WT adult mouse cardiac myocytes were cultured for 40 hr, treated for 15 min with 20-200 µM CGP-12177A, a membrane impermeable α1-AR antagonist,9-12 or 2 µM prazosin, which freely crosses the membrane, and subsequently treated for 15 min with 20 µM phenylephrine. Treatment was terminated by addition of Laemmli buffer and cells were collected by scraping for Western blot analysis of total ERK (T-ERK, Cell Signaling Technology) and phosphorylated ERK (P-ERK, Cell Signaling Technology).

Analysis of extraneuronal monoamine transporter (EMT/OCT3) expression and function.

WT adult mouse cardiac myocytes were cultured in either 35 mm dishes or on laminin coated coverslips. After 40 hrs, cardiac myocytes were harvested for Western blot analysis of EMT/OCT3 expression or fixed with 4% paraformaldehyde and stained with primary antibody to EMT/OCT3 (Santa Cruz) and an Alexa Fluor-594-conjugated secondary antibody (Invitrogen). EMT/OCT3 function was measured by using corticosterone, a specific EMT/OCT3 antagonist, to block α1-mediated activation of ERK. Cultured WT adult mouse cardiac myocytes were treated for 15 with 1 µM corticosterone and subsequently treated for 15 min with 20 µM phenylephrine. Phospho- and total ERK levels were measured by Western blot.
(a) α1-AR levels in WT and α1A-Tg cardiac myocytes were determined by saturation binding with $^3$H-prazosin (0.4-1.2 nM), where 10 mM phentolamine (RBI, Natick, MA) defined non-specific binding. (b) Cultured cardiac myocytes from WT and α1A-GFP Tg hearts were treated with 20 μM PE for 15 min and phospho- and total-ERK levels were determined by Western blot. Densitometric analysis compares the ratio phospho- to total-ERK (n=2).
REFERENCES for Supplemental Methods


Supplemental Figure 1. Overexpression and Hyperactivation of $\alpha$1A-GFP in Transgenic Mice

a. 

![Graph showing the effect of Prazosin on the ratio of P-ERK to T-ERK in WT and $\alpha$1A-Tg mice.](image)

b. 

![Western blots showing P-ERK and T-ERK levels in WT and $\alpha$1A-Tg mice with and without 20 $\mu$M PE](image)