Endothelin downregulates SERCA2 gene and protein expression in adult rat ventricular myocytes: regulation by pertussis toxin-sensitive G\textsubscript{i} protein and cAMP

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Hilal-Dandan R, He H, Martin JL, Brunton LL, Dillmann WH. Endothelin downregulates SERCA2 gene and protein expression in adult rat ventricular myocytes: regulation by pertussis toxin-sensitive G\textsubscript{i} protein and cAMP. Am J Physiol Heart Circ Physiol 296: H728–H734, 2009. First published January 16, 2009; doi:10.1152/ajpheart.00584.2008.—Downregulation of the sarcoplasmic reticulum calcium ATPase (SERCA2) is associated with diastolic dysfunction in the failing heart. Elevated plasma endothelin-1 (ET) levels are correlated with congestive heart failure suggesting that ET may play a pathophysiological role. We have investigated the ability of ET to regulate SERCA2 gene expression in isolated adult rat ventricular myocytes. We find that ET enhances net protein synthesis by ~40% but significantly downregulates SERCA2 mRNA expression, time dependently, by ~30–50%, and the expression of SERCA2 protein by ~50%. In myocytes, ET binds to ETA receptor that couples to G\textsubscript{q} and G\textsubscript{i} proteins. Inhibition of G\textsubscript{q}-PLC-induced phosphoinositide (PI) hydrolysis with U73122 (1 μM) or inhibition of G\textsubscript{i} protein with pertussis toxin (PTX) abolishes the ability of ET to downregulate SERCA2 mRNA gene expression. Further investigation suggests that ET coupling to PTX-sensitive G\textsubscript{i} with consequent lowering of cAMP is required for downregulation of SERCA2 mRNA levels. Increasing intracellular cAMP quantity using cAMP-specific PDE inhibitor Ro20-1724 or cAMP analog dibutyryl-cAMP reverses ET-induced downregulation of SERCA2 mRNA levels. The data indicate that, in adult myocytes, ET downregulates SERCA2 mRNA and protein levels, and the effect requires cross-talk between G\textsubscript{q} and PTX-sensitive G\textsubscript{i} pathways.

The contraction and relaxation cycle of cardiac muscle is tightly regulated by the release and re-uptake of Ca\textsuperscript{2+}. During excitation-contraction coupling, extracellular Ca\textsuperscript{2+} enters the myocyte through Ca\textsuperscript{2+} channels and induces Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) to enhance contraction; subsequent Ca\textsuperscript{2+} uptake via the SR Ca\textsuperscript{2+}-ATPase (SERCA2) reduces intracellular Ca\textsuperscript{2+} levels and facilitates muscle relaxation or diastole (4, 39). Thus cardiac muscle performance depends on the adequacy of SR and SERCA2 function.

Evidence in the literature suggests that alterations in SR function and expression levels of SERCA2 mRNA correlate with prolonged and incomplete diastole in the hypertrophied and failing heart muscle (1, 2, 5, 6, 30, 35, 36). Decreased expression of the mRNA encoding the SERCA2 gene is associated with slower removal of cytosolic Ca\textsuperscript{2+} (1, 2, 5, 6, 9, 30, 35, 36), whereas upregulation of SERCA2 mRNA expression, induced by thyroid hormone, is associated with an increase in the rate of tension development and enhancement in the rate of relaxation (9, 34). Furthermore, increasing the expression of SERCA2 gene in transgenic animals, or in myocytes through adenovirus expressing the SERCA2 gene, results in enhanced relaxation (8, 13, 15).

Mechanical, hormonal, and neuronal factors may contribute to decreases in SERCA2 gene expression and diastolic dysfunction in the hypertrophied and failing hearts (9). Endothelin-1 (ET) is one putative contributor to such dysfunction. ET is a potent vasoconstrictor and a growth peptide that has been linked to the pathophysiology of cardiac hypertrophy and failure (21, 25, 29, 38, 43). Clinically, the severity and prognosis of congestive heart failure correlates with elevated tissue and plasma immunoreactive levels of ET (25, 29). Antagonism of ET\textsubscript{A} receptor, the dominant receptor subtype in the heart, inhibits cardiac hypertrophy induced by aortic stenosis in rats, improves survival in rat models with chronic heart failure (by improving cardiac output and decreasing left ventricular hypertrophy), and has been clinically approved in the treatment of pulmonary hypertension (3, 21, 25, 29, 38, 43).

The role of ET in regulating SERCA2 mRNA and protein levels has not been examined in adult cardiac myocytes. In this study, we investigate the effects of ET on the fully differentiated adult myocytes where hypertrophy would more likely represent a pathological condition rather than a pattern of growth (as opposed to effects on neonatal cardiac myocytes). The study shows that ET induces downregulation of SERCA2 mRNA and protein abundance levels in cardiac myocytes mediated through G\textsubscript{q} and G\textsubscript{i} signaling. These findings suggest that, by downregulating SERCA2 expression levels, ET may contribute to reduced cardiac contractility associated with cardiac hypertrophy and failure.

MATERIALS AND METHODS

Animal protocols were approved by the Animal Subject Committee of the University of California San Diego, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Isolation and culture of adult ventricular myocytes. Ventricular myocytes from 250- to 300-g male Sprague-Dawley rats were prepared by collagenase dissociation method (17). The preparation provided ~10 × 10\textsuperscript{6} myocytes/heart with 70–80% viability, as assessed by rod-shaped morphology and exclusion of trypan blue.

Isolated adult ventricular myocytes were cultured at 37°C on plates coated with 2 μg/cm\textsuperscript{2} of laminin (Sigma, St. Louis, MO) at a density of 2 × 10\textsuperscript{4} cells/cm\textsuperscript{2} in serum-free Medium 199 (Earls’ Salts; Gibco, Grand Island, NY) supplemented with 30 mM tau-
raine, 2 mM creatine, 2 mM carnitine, 1% BSA, and 100 U/ml of penicillin and streptomycin.

Measurement of protein synthesis. [3H]phenylalanine incorporation was used to measure the rate of protein synthesis in myocytes. Phenylalanine is not readily metabolized in myocytes and equilibrates readily across plasma membrane (12). Freshly isolated myocytes were suspended at a density of 10^5 cells/aliquot in Minimum Essential Medium containing 50 μM Ca^{2+}, treated with ET (10 nM) or diluent for 10 min before adding 10 μCi/ml L-[2,3,4,5,6] [3H]phenylalanine (Amersham Life Sciences, Arlington Heights, IL). Incubations were stopped after 1 h by adding ice cold 10% trichloroacetic acid (TCA) for 30 min. Cell precipitates were washed three times with ice-cold 10% TCA. Proteins were solubilized with 0.4 N NaOH and quantified by liquid scintillation spectrometry. Total protein was determined by the method of Bradford (7). Signals ranged from 7,000 to 12,000 counts·min⁻¹·aliquot⁻¹·10^6 cells, depending on the drug treatment.

Northern analysis of specific mRNAs. For Northern blot analysis, cultured myocytes were treated with ET (10 nM) or control vehicle in the presence or absence of specified inhibitors for 24–48 h. The cells were then lysed, and total RNA was extracted using TRIZOL RNA isolation reagent (Life Technologies, Grand Island, NY). RNA (5 μg/sample) was loaded and electrophoresed on 1% agarose/2.2 M formaldehyde gels. RNA was visualized by ethidium bromide staining. RNA gels were transferred onto Hybred-N nylon membranes and hybridized with specific cDNA probes. Hybridizations were carried out overnight at 42°C. Membranes were exposed to Kodak XAR5 film (Eastman Kodak, Rochester, NY) at −70°C. Northern blots were quantified using an AMBIS radioisotopescanner. Probing with Gαi2 cDNA was used for normalizing data for RNA loading.

Western blot analysis. Myocytes were treated with ET (10 nM) or a control vehicle for 72 h. Treatments were terminated by rapid washing of the cells with cold phosphate buffered saline (PBS) and addition of SDS lysis buffer. The samples were electrophoresed on 8% polyacrylamide gels; the gels were transferred to nitrocellulose (PBI) membranes and immobilized by baking at 80°C in a vacuum oven. cDNAs for SERCA2 and glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) were labeled by random priming with [γ-32P]-dCTP. Hybridizations were carried out overnight at 42°C. Membranes were exposed to Kodak XAR5 film (Eastman Kodak, Rochester, NY) at −70°C. Northern blots were quantified using an AMBIS radioisotopescanner. Probing with GAPDH cDNA was used for normalizing data for protein loading. Chemiluminescence was used for detection (ECL, Amersham Life Sciences).

Construction of transgenic encoding adenovirus vectors. The constitutively active GTPase-deficient mutant Gαi2 2Q205L (mGi) was generously provided by Dr. Gary Johnson (University on North Carolina). The cDNA for rat Gαi2 2Q205L (1.7 Kb) was cloned into the shuttle vector pCMVPLPA, which contains a CMV promoter, a polylinker, SV40 polyadenylation sequences, and flanking adenoviral sequences from which E1A and E1B genes have been deleted. The resulting plasmids were co-transfected (calcium phosphate precipitation) into 293 cells (human embryonic kidney transformed with E1A/E1B) along with plasmid JM17, which contains the entire human adenovirus 5 genome with an additional 4 Kb of pBR322 sequence that makes the construct too large to be packaged into adenovirus capsid. Homologous rescue recombination results in adenoviral vectors encoding the Gαi2 transgene but without E12A/E1B sequences (and thus non-replicative in untransformed mammalian cells) (13,15). Recombinant adenovirus containing the correct Gαi2 2Q205L insert was confirmed by Southern blot analysis. Recombinant adenovirus was propagated in 293 cells. Lysates were harvested, purified over cesium chloride gradient, de-salted, and titered by optical density measurements at 260 nm. The optimal dose for infecting myocytes in cultures was 100 viral particles per cell to avoid cytotoxic effects. A time span of 24–48 h was adequate for transgene expression as determined in myocytes infected with recombinant adenovirus containing the lac Z gene. The recombinant adenovirus construct containing the shuttle vector PACCVMPLPA with the Gαi2 backbone control (−) was used as an empty vector control.

Analysis of data. Statistical comparisons were performed with the program InStat from GraphPad Software (San Diego, CA). Data are expressed as means ± SE, as noted in legends to individual figures.

RESULTS

ET enhances net protein synthesis. ET induces hypertrophic or growth responses in neonatal cardiac myocytes marked by increase in cell size, organization of the actin filaments, and enhanced protein synthesis (18, 37). In adult rat ventricular myocytes, the effect of ET on cell size and organization is less prominent, so we employed [3H]phenylalanine incorporation as a measure of increased protein synthesis and thus of a hypertrophic response. One hour of stimulation with ET induced a significant increase (43 ± 12%) in [3H]phenylalanine incorporation compared with control cells (Fig. 1), indicating that ET activates measurable hypertrophic response in these myocytes.

ET downregulates SERCA2 gene and protein levels via ETa receptor. Treatment of myocytes with ET (10 nM for 24 h) significantly reduced the levels of SERCA2 mRNA by 33 ± 8.7% (Fig. 2, A and B). The ability of ET to downregulate SERCA2 mRNA levels was time dependent: SERCA2 levels were further reduced by 54 ± 5.7% after 48 h of incubation with ET (Fig. 2C).

In previous studies, we have shown that BQ-123, a specific ETa receptor antagonist, competes with ET binding on adult myocytes with an IC_{50} of ~9 nM and demonstrated that BQ-123 (10 μM) abolished the ability of ET (10 nM) to induce phosphoinositide (PI) hydrolysis and to lower isoproterenol-stimulated cAMP formation (17). In the present study, BQ-123 (10 μM), also inhibited the ability of ET to downregulate SERCA2 mRNA levels (Fig. 3), suggesting that the effect of ET is mediated through the ETa receptor.

To determine whether decreased levels of SERCA2 mRNA gene were followed by downregulation in the corresponding SERCA2 protein, we measured SERCA2 protein levels by Western blot analysis after 72 h of treatment with ET. As indicated in Fig. 4, A and B, ET induced a significant decrease...
ET downregulates SERCA2 expression in adult myocytes

Fig. 2. ET downregulates SERCA2 mRNA levels. A: adult myocytes were cultured overnight in the presence of ET (10 nM) or diluent. mRNA was collected and analyzed for expression of SERCA2 and GAPDH by Northern analysis, as described in MATERIALS AND METHODS. ET treatment significantly downregulates SERCA2 mRNA in adult myocytes. Probing with cDNA for GAPDH indicates equal loading. B: summary of analysis of Northern blots. ET treatment significantly lowers SERCA2 mRNA levels by 33 ± 8.7% in adult myocytes (n = 5; *P < 0.05). C: effect of ET on SERCA2 mRNA levels is time dependent. ET lowers SERCA2 mRNA levels by 54 ± 5.7% (n = 3; *P < 0.05) after 48 h of treatment.

Fig. 3. Effect of ET on SERCA2 mRNA levels is mediated through ETA receptor. Adult myocytes were cultured overnight in the presence of ET (10 nM) or diluent. BQ-123 was added 15 min before addition of ET. Myocytes were lysed, and mRNA was collected and analyzed for expression of SERCA2 and GAPDH by Northern analysis, as described in MATERIALS AND METHODS. The ability of ET treatment to downregulate SERCA2 mRNA levels is antagonized with BQ-123. Probing with cDNA for GAPDH indicates equal loading. This is a representative example of three replicate experiments.

Effect of ET on SERCA2 mRNA levels is mediated by multiple G proteins; reduced cAMP is required. The ability of ET to downregulate SERCA2 mRNA levels in the adult ventricular myocytes appears to be mediated through coupling to both Gq and PTX-sensitive G1 proteins (Fig. 5, A and B). Either addition of U73122 (1 μM), which blocks Gq-phospholipase C activation of PI hydrolysis (Fig. 5C), or pretreatment with pertussis toxin (PTX; 0.1 μg) to block G1 activation and the inhibitory effect of ET on cAMP accumulation (19) prevented the capability of ET to downregulate SERCA2 levels.

We have previously shown that ET couples to PTX-sensitive G1 pathway in myocytes and inhibits the activity of adenyl cyclase with consequent lowering of cAMP formation (17, 19). Since ET-induced downregulation of SERCA2 mRNA levels was reversed with PTX, we wanted to determine whether lowering of cAMP is an essential signal. This was accomplished by increasing intracellular cAMP levels using Ro20-1724 (100 μM), a cAMP-specific phosphodiesterase inhibitor (17, 19), or by introducing cAMP into myocytes using the cAMP analog dibutryl-cAMP (db-cAMP; 100 μM) (Fig. 6). Both treatments reversed the ET effect (Fig. 6), suggesting that lowering of intracellular cAMP levels is an important signal, mediated through ET coupling to PTX-sensitive G1 protein, to allow downregulation of SERCA2 expression levels in myocytes.

Expression of constitutively active Ga12 lowers SERCA2 mRNA and protein levels. We have previously reported that expression of constitutively active form of Ga12 (mGa12), packaged into adenoviral vectors, in myocytes can induce hypertrophy (16). When mGa12 was expressed in cultured adult myocytes, the levels of both SERCA2 mRNA (Fig. 7A) and protein (Fig. 7B) were downregulated, suggesting that activation of Ga12 pathway is involved in regulating SERCA2 abundance.

DISCUSSION

This study demonstrates that ET induces a rapid hypertrophic response in myocytes, as marked by significant increase [3H]phenylalanine uptake and incorporation into protein (within 1 h of stimulation). Prolonged incubation with ET (24–72 h) leads to significant downregulation in SERCA2 mRNA and protein levels. This effect of ET on SERCA2

decrease SERCA2 protein levels were relatively specific for SERCA2 protein, since ET increased protein synthesis (Fig. 1).

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protein levels appears to be specific: the protein expression levels of phospholamban (a protein that regulates SERCA2 pump) and \(\alpha\)-actin (as a measure of contractile proteins) do not significantly change in response to ET.

ET downregulates SERCA2 mRNA levels by binding to the ETA receptor since the effect of ET is eliminated in the presence of the specific ETA receptor antagonist BQ-123. This is in agreement with our previous findings that ET binds and mediates its effects in myocytes through a single population of ETA receptors (17, 18). The involvement of the ETA receptor in mediating myocyte hypertrophy is supported by experimental and clinical studies showing that antagonism of the ETA rather than the ETB receptor is effective in reducing left ventricular hypertrophy and in improving cardiac function (21, 25).

Studies on hormonal regulation of SERCA2 mRNA levels have mostly been executed using neonatal cardiac myocytes. Cytokines as well as \(\alpha\)-adrenergic receptor stimulation and ET have been reported to diminish SERCA2 mRNA expression in neonatal cardiac myocytes (26, 32, 41, 42, 44). Additionally, angiotensin II (AngII) reportedly downregulates SERCA2 mRNA levels in neonatal myocytes, but in the same study AngII was reported to have no effect on SERCA2 mRNA.
expression levels in the adult myocytes (22), suggesting distinct and disparate responses between neonatal and adult ventricular myocytes. It is important to note that neonatal myocytes are not fully differentiated, and SERCA2 mRNA abundance has been reported to change with development (11). In contrast to neonatal myocytes, adult rat myocytes are terminally differentiated, and ET-induced downregulation of SERCA2 mRNA and protein levels more likely reflects a pathophysiologic consequence than a pattern of growth and development.

The hypertrophic effects of ET on myocytes are mediated through coupling to the ET\(_A\) receptor and activation of both \(G_\text{q}\) and the PTX-sensitive \(G_\text{i}\) pathways (18). Our data indicate that inhibition of ET-coupled \(G_\text{q}\)-PLC pathway with U73122 abolishes ET-induced PI hydrolysis and prevents ET-induced downregulation of SERCA2 mRNA levels. The hypertrophic effects of ET mediated through \(G_\text{q}\) activation and stimulation of PLC-PI hydrolysis/PKC and MAPK have been well established (37). We have previously reported that, in adult myocytes, ET activates \(G_\text{q}\)-phospholipase C pathways to induce PI hydrolysis and activation of PKC\(\delta\) and \(\epsilon\) and the MAPK cascade (19, 33). Several studies indicate that \(G_\text{q}\)-coupled signaling, phorbol ester stimulation, specific activation of PKC\(\delta\) and \(\epsilon\) isoforms, and activation of Raf/MEK/MAPK pathways (14, 16, 20, 31, 44) can downregulate SERCA2 mRNA gene expression. Thus our data support a role for ET-coupled \(G_\text{q}\)-PLC pathway in mediating downregulation of SERCA2 mRNA gene expression levels in myocytes.

We have previously shown that PTX-sensitive \(G_\text{i}\) protein can mediate hypertrophic effects in myocytes (16, 18). The hypertrophic effects of ET mediated through coupling to PTX-sensitive \(G_\text{i}\) are not as clearly understood as those mediated via coupling to \(G_\text{q}\) (37). Our data indicate that inhibition of \(G_\text{i}\) with PTX attenuates the ability of ET to downregulate SERCA2 mRNA levels. Activation of the PTX-sensitive \(G_\text{i}\) protein inhibits adenylyl cyclase with consequent lowering of cAMP formation. Cardiac hypertrophy and failure are frequently marked by elevated \(G_\text{i}\) protein as well as elevated plasma ET levels (10). Several studies have shown that upregulation of \(G_\text{i}\) protein in the hypertrophied and failing hearts is associated with lower cAMP levels and a blunted \(\beta\)-adrenergic response (10, 23). We wanted to determine whether increased activated \(G_\text{i}\) expression in cardiac myocytes would affect SERCA2 abundance. To investigate the role of \(G_\text{i}\) activation on SERCA2 expression, we induced the expression of constitutively active \(G_{\text{i}2}\) (mGi) in myocytes through adenovirus vectors. The expression of mGi gene in myocytes caused significant downregulation in SERCA2 mRNA and protein levels. Our findings support recent studies by McKloskey et al., which showed that transgenic mice with increased expression in \(G_\text{i}\) signaling exhibit impaired calcium handling and a significant decrease in SERCA2 levels (23). Thus increased expression of \(G_\text{i}\) protein in the hypertrophied and failing hearts, in conjunction with elevated ET levels, may constitute one of the mechanisms that regulate SERCA2 levels.

Our study also indicates that the ability of ET to lower cAMP, through PTX-sensitive \(G_\text{i}\), plays an important role in mediating the hypertrophic effects of ET and the downregulation of SERCA2 mRNA levels. Increasing intracellular cAMP levels with cAMP-PDE inhibitor (Ro20-1724) or the cAMP analog db-cAMP inhibits ET-induced \(^{3}\text{H}\)phenylalanine incorporation [measuring fold change from control (mean \(\pm\) SE; \(n = 3\)): control = 1; +ET= 1.43 \(\pm\) 0.12; \(*P < 0.05; \)**

![Fig. 6. ET coupling to G and lowering of cAMP is required for SERCA2 downregulation. Myocytes were pretreated with PTX (0.1 \(\mu\)g/ml) or incubated in the presence of cAMP-specific phosphodiesterase inhibitor Ro20-1724 (100 \(\mu\)M) or cAMP analog db-cAMP (100 \(\mu\)M), and then ET was added as described in MATERIALS AND METHODS. These treatments reverse the ability of ET to lower SERCA2 mRNA levels. Data are representative of three replicate experiments.](http://ajpheart.physiology.org/)

![Fig. 7. Expression of constitutively activated Gi2 (mGi) downregulates SERCA2 mRNA and protein levels. A: Northern blot analysis of myocytes infected with adenovirus vector expressing mGi or control (−) empty adenovirus vector. Data indicate that, as mGi mRNA expression levels are elevated, the SERCA2 mRNA levels are downregulated. Data are representative of three replicate experiments. B: Western blot analysis of myocytes infected with adenovirus vector expressing mGi or control (−) empty adenovirus vector. mGi expression induces downregulation of SERCA2 protein levels. Data are representative of three replicate experiments.](http://ajpheart.physiology.org/)
ET DOWNREGULATES SERCA2 EXPRESSION IN ADULT MYOCYTES

Ro20-1724 + ET = 0.93 ± 0.13, and db-cAMP + ET = 1.15 ± 0.07; \( P > 0.05 \). Elevation of cellular cAMP, with the above treatments, also reverses the ability of ET to lower SERCA2 mRNA levels (Fig. 5, A and B).

Other work suggests plausible mechanisms by which cellular cAMP might regulate SERCA2 expression. Elevation of intracellular cAMP levels activates cAMP response element (CRE), which induces transcriptional regulations (27). SERCA2 gene has a CRE component, and transfection of constitutively activated CRE into myocytes has been shown to stimulate SERCA2 gene transcription (24). This is supported by studies from Muller et al. (28) reporting that transgenic animals lacking CRE-modulator (CREM) gene have selective downregulation of β-AR and SERCA2 levels, whereas transgenic mice expressing the CREM gene exhibit increased contractility and upregulation of SERCA2 levels. In rat PC13 cells, both cAMP and thyroid hormone have been reported to up-regulate SERCA2 levels (40). Our data are in line with the above findings, suggesting that the ability of ET to lower intracellular cAMP levels is a necessary requirement to down-regulate SERCA2 levels presumably by decreasing the activation of CRE of the SERCA2 gene. Future studies are required to further delineate this pathway.

In conclusion, our data indicate that ET must activate both Gq and Gi-coupled pathways to downregulate SERCA2 levels (inhibition of either Gq or Gi reverses the effects), suggesting that cross-talk between Gq and Gi signaling is necessary to mediate the effects of ET on SERCA2 levels.

GRANTS
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