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Overexpression of the Cardiac β_2-Adrenergic Receptor and Expression of a β-Adrenergic Receptor Kinase-1 (βARK1) Inhibitor Both Increase Myocardial Contractility but Have Differential Effects on Susceptibility to Ischemic Injury

Heather R. Cross, Charles Steenbergen, Robert J. Lefkowitz, Walter J. Koch, Elizabeth Murphy

Abstract—Cardiac β_2-adrenergic receptor (β_2AR) overexpression is a potential contractile therapy for heart failure. Cardiac contractility was elevated in mice overexpressing β_2ARs (TG4s) with no adverse effects under normal conditions. To assess the consequences of β_2AR overexpression during ischemia, perfused hearts from TG4 and wild-type mice were subjected to 20-minute ischemia and 40-minute reperfusion. During ischemia, ATP and pH fell lower in TG4 hearts than wild type. Ischemic injury was greater in TG4 hearts, as indicated by lower postischemic recoveries of contractile function, ATP, and phosphocreatine. Because β_2ARs, unlike β_1ARs, couple to G_i as well as G_s, we pretreated mice with the G_i inhibitor pertussis toxin (PTX). PTX treatment increased basal contractility in TG4 hearts and abolished the contractile resistance to isoproterenol. During ischemia, ATP fell lower in TG4+PTX than in TG4 hearts. Recoveries of contractile function and ATP were lower in TG4+PTX than in TG4 hearts. We also studied mice that overexpressed either βARK1 (TGβARK1) or a βARK1 inhibitor (TGβARKct). Recoveries of function, ATP, and phosphocreatine were higher in TGβARK1 hearts than in wild-type hearts. Despite basal contractility being elevated in TGβARKct hearts to the same level as that of TG4s, ischemic injury was not increased. In summary, β_2AR overexpression increased ischemic injury, whereas βARK1 overexpression was protective. Ischemic injury in the β_2AR overexpressors was exacerbated by PTX treatment, implying that it was G_s not G_i activity that enhanced injury. Unlike β_2AR overexpression, basal contractility was increased by βARK1 inhibitor expression without increasing ischemic injury, thus implicating a safer potential therapy for heart failure. **(*Circ Res*. 1999;85:1077-1084.)**

Key Words: adrenergic signaling ■ energetics ■ G proteins ■ ischemia ■ NMR spectroscopy

Cardiac β-adrenergic receptors (βARs) mediate the myocardial contractile response to the sympathetic transmitters epinephrine and norepinephrine. βARs are thought to be coupled primarily to the stimulatory guanine nucleotide binding protein G_s. Binding of agonists to βARs stimulates dissociation of the G_α subunit from the $G_{\beta\gamma}$ subunit (Figure 1). The stimulatory subunit $G_{s\alpha}$ binds and activates adenylate cyclase, causing production of cAMP and activation of protein kinase A (PKA). In the heart, PKA phosphorylates and activates L-type Ca^{2+} channels, the sarcoplasmic reticular Ca^{2+} ATPase inhibitor phospholamban, and the myofibrillar protein troponin I. The net result of these phosphorylations is an increase in cardiac contractility.[1] Desensitization and downregulation of βARs is mediated via phosphorylation of the activated receptors by kinases such as βAR kinase-1 (βARK1).[2] βARK1 is targeted to the βARs via its affinity for membrane-bound $G_{\beta\gamma}$ subunits.[3] $G_{\beta\gamma}$ binding is also necessary for βARK1 activation.[4] βARK1 has been shown to phosphorylate and uncouple other receptors such as angiotensin II in in vivo studies[5] and endothelin, M2 muscarinic cholinergic, α_{2A}-adrenergic, thrombin, dopamine, and δ- and κ-opioid receptors in in vitro studies.[6–8]

β_1 and β_2ARs exist in the myocardium, with β_1AR being the most abundant subtype.[9] β_1ARs are coupled solely to G_s and operate as described above. Recent findings have, however, revealed that β_2ARs are coupled to the inhibitory G protein G_i, in addition to G_s.[10] Although not shown directly for the β_2AR, on stimulation of other G_i-coupled receptors, $G_{i\alpha}$ dissociates from $G_{\beta\gamma}$, and $G_{i\alpha}$ then binds to adenylate cyclase and inhibits its activity, thereby opposing the action of $G_{s\alpha}$. Consistent with a similar mechanism for the β_2AR, $G_{i\alpha}$ activation appears to prevent many of the $G_{s\alpha}$-mediated downstream events of β-adrenergic signaling. β_2AR stimulation does not lead to PKA-mediated phosphorylation of phospholamban[11,12] or troponin I.[13] There is evidence in rat[11] and dog[12] that the increased cytosolic Ca^{2+} transients and increased contractility observed on β_2AR stimulation are dissociated from an increase in cAMP and instead are caused

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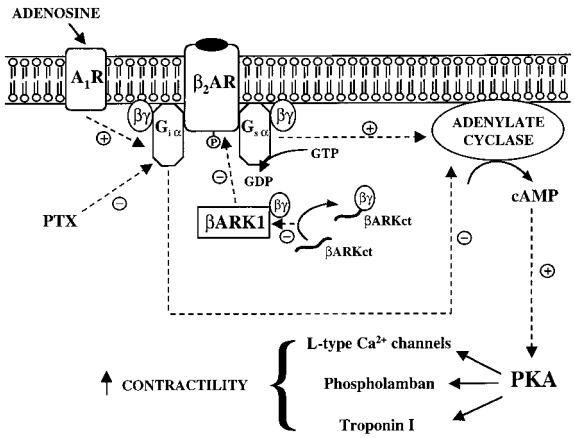

Figure 1. The β_2-adrenergic signaling cascade. Binding of an agonist to the β_2AR stimulates exchange of G_α-bound GDP for GTP; the G_α subunit then dissociates from the $G_{\beta\gamma}$ subunit and binds to adenylate cyclase. $G_{s\alpha}$ activates adenylate cyclase, causing production of cAMP and activation of PKA. PKA phosphorylates the L-type Ca^{2+} channel, phospholamban, and troponin I, leading to increased myocardial contractility. The β_2AR is also coupled to the inhibitory G protein, G_i. On receptor stimulation $G_{i\alpha}$ binds to adenylate cyclase and inhibits its activity, thereby opposing the action of $G_{s\alpha}$. $G_{i\alpha}$ activity can be inhibited by PTX, whereas adenosine can stimulate $G_{i\alpha}$ activity via activation of A_1 receptors. Desensitization and downregulation of the β_2AR is mediated via phosphorylation of the active receptor by βARK1. βARKct is a peptide that inhibits βARK1 activity by competitively binding $\beta\gamma$, $\beta\gamma$ being necessary for βARK1 activation.

by localized activation of the L-type Ca^{2+} channels. This implies that $G_{s\alpha}$ activation via β_2AR stimulation is only effective locally, at the sarcolemma, and any distant effects are attenuated, possibly by $G_{i\alpha}$-mediated inhibition of adenylate cyclase. This hypothesis was supported further by the observation that pertussis toxin (PTX), a G_i inhibitor, restores the ability of β_2AR stimulation to mediate phospholamban phosphorylation.[14]

The β-adrenergic signaling cascade is impaired in human congestive heart failure (CHF). β_1ARs are reduced by 50%, whereas βARK1 levels and activity are increased.[15] These changes contribute to the decreased basal and β-agonist–stimulated contractility observed in CHF patients.[9] β_2ARs, however, are not downregulated during CHF,[15] and in fact, β_2 sensitivity may be increased.[12] These observations led to the proposition that overexpression of β_2ARs in the myocardium could improve cardiac function and therefore be developed as a potential therapy for CHF.[16] To investigate this hypothesis, transgenic mice with a 200-fold overexpression of the β_2AR were created; these mice exhibited increased basal cardiac contractility without evidence of cardiac abnormalities or increased mortality.[16] Although this approach successfully increased cardiac function under normal physiological conditions, there may be consequences during pathological conditions such as ischemia.

By comparing the response to ischemia in isolated hearts from mice overexpressing the β_2AR with that of wild-type (WT) hearts, we aimed to determine the effect of β_2AR overexpression on ischemic injury. In addition, as the β_2AR activates both G_i and G_s, we aimed to distinguish between the effects of these 2 pathways by pretreating WT and β_2AR overexpressor mice with PTX to inhibit the G_i protein and studying the ischemic response. To assess further the role of β-adrenergic signaling in ischemic injury, we studied the ischemic response of mice overexpressing βARK1 and also of mice expressing a βARK1 inhibitor.[17] Finally, by monitoring basal contractility in mouse hearts from transgenic, WT, and PTX-treated animals, we hoped to determine the contribution of the various components of the β_2-adrenergic signaling cascade to the control of myocardial contractility.

Materials and Methods

Animals

Transgenic mice were developed by Milano et al[16] and Koch et al.[17] TG4 mice exhibit a 200-fold, cardiac-specific overexpression of the β_2AR.[16] TGβARK1 mice exhibit a 3-fold higher cardiac βARK1 activity than WT mice, and in mice expressing a βARK1 inhibitor peptide (TGβARKct mice), βARK1 activity was 50% lower than WT.[17]

Fifteen male adult heterozygous TG4 mice of body weight 28 ± 2 g; 7 heterozygous TGβARK1 mice of body weight 33 ± 1 g and 6 heterozygous TGβARKct mice of body weight 30 ± 1 g were used. Twenty-four WT mice of body weight 33 ± 2 g were used as controls. All animals were treated in accordance with NIH guidelines.

PTX Pretreatment

To inhibit the G_i protein, 8 WT and 6 TG4 mice were injected with 60 μg/kg PTX intraperitoneally, 24 hours before experimentation. To confirm inhibition of G_i, hearts were isolated, perfused, and stimulated with 10^{-8} mol/L isoproterenol before addition of 10^{-6} mol/L adenosine. As outlined in Figure 1, isoproterenol activates $G_{s\alpha}$ and subsequently contractility increases, whereas adenosine activates G_i and opposes the isoproterenol-induced stimulation of contractility.[18] In non-PTX–treated WT hearts, addition of 10^{-8} mol/L isoproterenol resulted in a 30% increase in contractility, and this increase was reversed fully by addition of 10^{-6} mol/L adenosine. In PTX-treated WT hearts, addition of 10^{-8} mol/L isoproterenol resulted in a 50% increase in contractility, and there was no decrease in contractility on subsequent addition of 10^{-6} mol/L adenosine, confirming the lack of G_i activity in PTX-treated hearts.

Ischemia/Reperfusion Protocol

Hearts were isolated and perfused in the Langendorff mode as described previously.[19] All hearts were perfused for 30 minutes before being subjected to 20-minute no-flow ischemia and 40-minute reperfusion. Left ventricular developed pressure (LVDP), $\pm$dP/dt, and heart rate were monitored via a water-filled latex balloon inserted into the left ventricle. Recovery of contractile function was assessed by measurement of LVDP at the end of reperfusion and was expressed as a percentage of preischemic LVDP.

NMR Spectroscopy

Relative changes in concentrations of phosphorus metabolites were observed during the ischemia/reperfusion protocol by acquiring consecutive ^{31}P NMR spectra as described previously.[19]

The areas of the spectral peaks were expressed as a percentage of the peak areas of an initial, preischemic control spectrum from each heart. The ratios of the phosphocreatine (PCr)/ATP peaks in the preischemic control spectra were lower in the TG4 and TG4+PTX hearts, compared with the other groups. PCr levels decrease on β-adrenergic stimulation,[20] independent of the increased workload,[21] but ATP remains at normal levels. The lower PCr/ATP ratio therefore implies that preischemic PCr levels were lower in the 2 TG4 groups. PCr values were therefore normalized by expressing PCr peak areas as a percentage of the ATP peak area in the initial preischemic control spectrum. Intracellular pH was estimated from

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the chemical shift of the P_i peak relative to PCr using previously obtained titration curves.

Statistics

Results are expressed as mean±SEM. Significance ($P<0.05$) was determined by ANOVA followed by a Fisher post hoc test.

Results

Contractile Function

Myocardial functional parameters for the 6 groups of mice are shown in the Table.

Effects of Overexpression of the β_2AR

During the preischemic period, LVDP, +dP/dt, and −dP/dt were higher in the TG4 hearts, at 134 cm H_2O, 5.1 cm H_2O/ms, and −4.3 cm H_2O/ms, respectively, than in the WT hearts, at 113 cm H_2O, 4.2 cm H_2O/ms, and –3.4 cm H_2O/ms, respectively ($P<0.05$). Overexpression of the β_2AR, therefore, increased basal myocardial contractility.

Ischemic contracture began at 14 minutes and reached a maximum pressure at 19 minutes in the WT hearts. Contracture occurred earlier in TG4 hearts, beginning at 11 minutes and ending at 16 minutes ($P<0.01$).

By the end of the 40-minute reperfusion period, recovery of contractile function was significantly lower in the TG4 hearts, at 8% of initial LVDP, than in the WT hearts, at 31% of initial LVDP ($P<0.0001$). Overexpression of the β_2AR, therefore, resulted in increased ischemic injury.

Effects of Pretreatment With the Gi Inhibitor PTX

During the preischemic period, LVDP and −dP/dt were higher in the TG4+PTX hearts, at 157 cm H_2O and –5.6 cm H_2O/ms, respectively, than in the untreated TG4 hearts, at 134 cm H_2O and −4.3 cm H_2O/ms, respectively ($P<0.05$). There was no effect of PTX pretreatment on contractility in WT hearts. Pretreatment with PTX, therefore, had no effect on WT hearts, whereas, in the TG4 hearts, PTX pretreatment resulted in increased myocardial contractility. These results are consistent with the observation in guinea pig myocytes[22] that PTX treatment had no effect on contractility in unstimulated myocytes but increased myocyte sensitivity to isoproterenol stimulation.

Ischemic contracture began at 11 minutes and reached a maximum pressure at 16 minutes in the untreated TG4 hearts. Contracture occurred earlier in TG4+PTX hearts, beginning at 9 minutes and ending at 13 minutes ($P<0.05$). There was no effect of PTX pretreatment on the timing of contracture in WT hearts.

By the end of the 40-minute reperfusion period, recovery of contractile function was significantly lower in the TG4+PTX hearts, at 2% of initial LVDP, than in the untreated TG4 hearts, at 8% of initial LVDP ($P<0.05$). There was no effect of PTX pretreatment on postischemic recovery of contractile function in WT hearts. Therefore, pretreatment with PTX had no effect on WT hearts, whereas, in the TG4 hearts, PTX pretreatment resulted in increased ischemic injury. These results imply that G_i has no significant role in ischemic injury in unstimulated WT hearts, but given the findings in TG4 hearts, G_i may be expected to play a role in WT hearts if ischemia is preceded by β_2AR stimulation by, for example, release of epinephrine or norepinephrine or administration of β-agonists.

Effects of Overexpression of βARK1 and the βARK1 Inhibitor

During the preischemic period, LVDP, +dP/dt and −dP/dt were lower in the TGβARK1 hearts, at 90 cm H_2O, 3.3 cm H_2O/ms, and –2.4 cm H_2O/ms, respectively, than the WT hearts, at 113 cm H_2O, 4.2 cm H_2O/ms, and –3.4 cm H_2O/ms, respectively ($P<0.05$). Heart rate was also lower in the TGβARK1 hearts, at 352 bpm, than in the WT hearts, at 400 bpm ($P<0.01$). Overexpression of βARK1, therefore, decreased basal myocardial contractility. LVDP and +dP/dt were significantly higher in the TGβARKct hearts than in the WT hearts, at 132 cm H_2O and 5.1 cm H_2O/ms, respectively ($P<0.05$).

By the end of the 40-minute reperfusion period, recovery of contractile function was significantly higher in the TGβARK1 hearts, at 48% of initial LVDP, than in the WT hearts, at 31% of initial LVDP ($P<0.01$). Recovery of function was similar in the TGβARKct hearts, at 27% of initial LVDP, as in WT. Therefore, overexpression of the βARK1 decreased ischemic injury, whereas expression of the βARK1 inhibitor had no significant effect on ischemic injury. It should be noted that use of shorter ischemic periods, at least in a working heart model, has been shown to give different results. Chen et al[23] demonstrated that after 6 minutes of ischemia, cardiac output in the TGβARK1 hearts was depressed during reperfusion relative to WT.

Myocardial Contractile Function During Ischemia and Reperfusion

	Preischemia				Ischemia (Contracture)			End Reperfusion (Functional Recovery)	
Group	Heart Rate, bpm	LVDP, cm H_2O	+dP/dt, cm H_2O/ms	−dP/dt, cm H_2O/ms	Start, min	End, min	Maximum, cm H_2O	(%, LVDP)	n
Wild type	400±8	113±3	4.2±0.1	−3.4±0.2	14±1	19±1	61±6	31±3	15
TG4	420±11	134±8*	5.1±0.4*	−4.3±0.4*	11±1*	16±1*	73±8	8±2*	8
Wild type, PTX treated	382±11	115±4	4.2±0.1	−3.2±0.2	14±1	19±1	59±6	30±3	6
TG4, PTX treated	447±7*	157±7*†	5.3±0.6*	−5.6±0.7*†	9±1*†	13±1*†	77±4	2±1*†	5
TGβARK1	352±23*	90±9*	3.3±0.3*	−2.4±0.2*	13±1	19±1	50±7	48±8*	7
TGβARKct	393±6	132±13*	5.1±0.4*	−3.8±0.3	14±1	18±1	60±7	27±6	5

Data are mean±SEM.

*Significantly different from wild type; †TG4, PTX-treated values significantly different from TG4 ($P<0.05$).

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Isoproterenol Stimulation of Contractile Function

In WT hearts there was an increase in contraction in response to isoproterenol with an EC_{50} of $\approx 10^{-6}$ mol/L. In TG4 hearts, however, there was no contractile response to isoproterenol doses in the range 10^{-8} to 10^{-4} mol/L, confirming original observations.[16] In PTX-treated TG4 hearts, there was a 20% increase in contractility in response to 10^{-8} mol/L isoproterenol. Therefore, the contractile resistance to isoproterenol observed in TG4 hearts was relieved by PTX pretreatment, supporting recent findings in myocytes.[24]

Phosphate Metabolite Levels and pH_i

Phosphate metabolite levels and pH_i were measured in all hearts to determine whether the manipulations of β_2AR signaling altered myocardial energetics or pH regulation.

Effects of Overexpression of the β_2AR

During the preischemic period, the PCr/ATP ratio was lower in the TG4 hearts, at 1.17±0.09, than in the WT hearts, at 1.51±0.08 ($P<0.01$), consistent with increased β-adrenergic signaling.

During ischemia, ATP levels fell lower in the TG4 hearts, reaching 16% of initial ATP, than in the WT hearts, in which ATP levels fell to 40% of initial ATP (Figure 2A; $P<0.0001$). During reperfusion, ATP levels increased in all hearts, however, ATP levels remained lower in the TG4 hearts, reaching 29% of initial ATP by the end of reperfusion, compared with 47% of initial ATP in the WT hearts ($P<0.01$).

PCr decreased rapidly in all hearts at the onset of ischemia (Figure 2B). At the end of ischemia, there was no significant difference in PCr levels between the TG4 and WT hearts. On reperfusion, PCr levels increased in all hearts. At the end of reperfusion, the PCr level was lower in the TG4 hearts, at 53% of initial ATP, than in WT hearts, at 89% of initial ATP ($P<0.01$).

Intracellular pH decreased during ischemia in all hearts (Figure 3). At the end of ischemia, pH was lower in the TG4 hearts, at pH 5.52, than in WT hearts, at pH 5.85 ($P<0.0001$). There were no significant differences in pH between the WT and TG4 hearts during reperfusion.

In summary, hearts from mice overexpressing the β_2AR, in addition to an increase in basal contractility, had a lower PCr/ATP ratio during basal perfusion than WT hearts. During ischemia, these hearts exhibited a greater loss of ATP and a lower pH_i than WT hearts. During reperfusion, recoveries of ATP and PCr were lower in β_2AR overexpressor hearts than in WT hearts, therefore correlating with the lower recovery of contractile function observed and indicating greater injury.

Effects of PTX Pretreatment

During the preischemic period, the PCr/ATP ratio was lower in the TG4+PTX hearts, at 1.11±0.07, than in the WT+PTX hearts, at 1.56±0.10 ($P<0.01$), again consistent with increased β-adrenergic signaling. There was no significant difference in PCr/ATP ratio between TG4 and TG4+PTX hearts.

During ischemia, ATP levels fell lower in the TG4+PTX hearts, reaching 6% of initial ATP, than in the TG4 hearts, in which ATP levels fell to 16% of initial ATP (Figure 2A; $P<0.05$). During reperfusion, ATP levels remained lower in the TG4+PTX hearts, reaching 18% of initial ATP, compared with 29% of initial ATP in the TG4 hearts ($P<0.05$). At no time during the protocol were there any differences in ATP levels between WT+PTX and WT hearts.

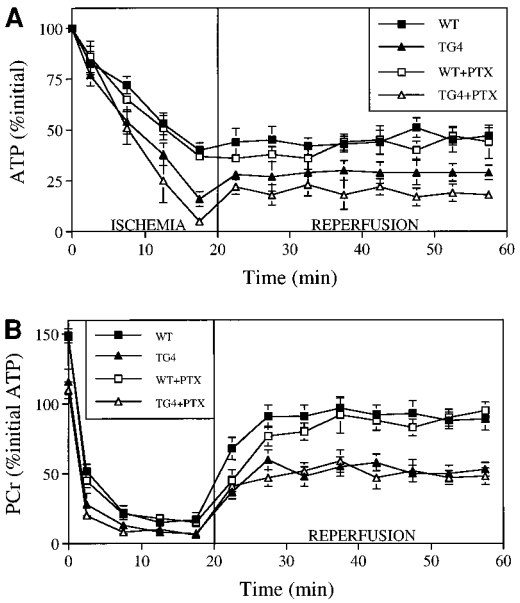

Figure 2. Myocardial intracellular levels of ATP (A) and PCr (B) during ischemia and reperfusion in WT, β_2AR overexpressor (TG4), PTX-treated WT (WT+PTX), and PTX-treated TG4 (TG4+PTX) mice. Points are mean±SEM. WT, n=15; TG4, n=8; WT+PTX, n=6; TG4+PTX, n=5.

PCr decreased rapidly in all hearts at the onset of ischemia and increased in all hearts during reperfusion (Figure 2B). At no time during the protocol were there any significant differences in PCr levels between TG4+PTX and TG4 hearts or between WT+PTX and WT hearts.

Intracellular pH decreased during ischemia in all hearts (Figure 3). Although pH was slightly lower at the end of ischemia in the TG4+PTX hearts, at pH 5.39, than in TG4 hearts, at pH 5.52, there were no significant differences in pH between any of the groups of hearts during the protocol.

To summarize, hearts from PTX-treated mice overexpressing the β_2AR exhibited a greater loss of ATP during ischemia than did untreated β_2AR overexpressor hearts. During reperfusion, recovery of ATP was also lower in PTX-treated β_2AR

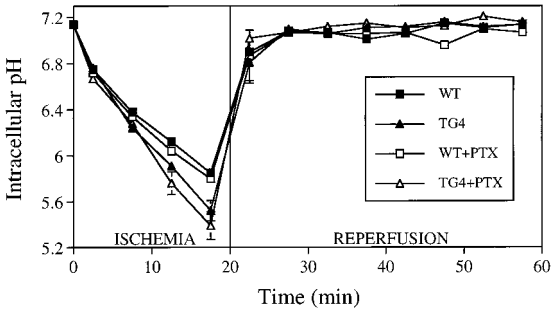

Figure 3. Myocardial pH_i during ischemia and reperfusion in WT, β_2AR overexpressor (TG4), PTX-treated WT (WT+PTX), and PTX-treated TG4 (TG4+PTX) mice. Points are mean±SEM. WT, n=15; TG4, n=8; WT+PTX, n=6; TG4+PTX, n=5.

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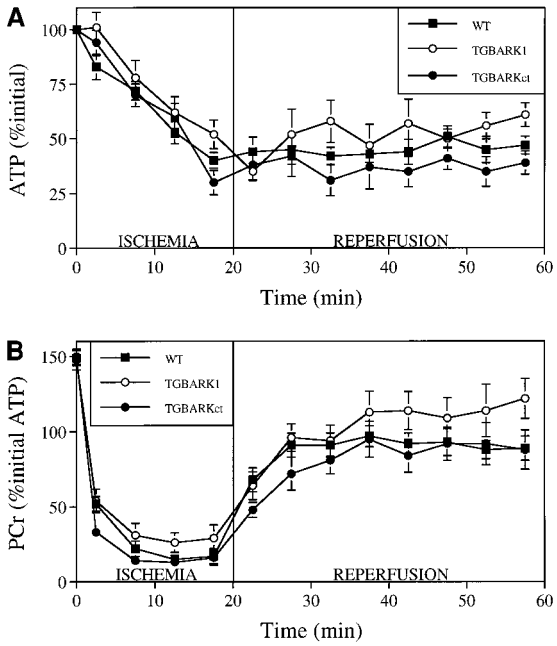

Figure 4. Myocardial intracellular levels of ATP (A) and PCr (B) during ischemia and reperfusion in WT, βARK1 overexpressor (TGβARK1), and βARK1 inhibitor (TGβARKct) mice. Points are mean±SEM. WT, n=15; TGβARK1, n=7; TGβARKct, n=5.

overexpressor hearts than in untreated β_2AR overexpressor hearts, therefore correlating with the lower recovery of contractile function observed in the PTX-treated hearts and indicating greater injury. There was no effect of PTX pretreatment, with respect to ischemic energetics or pH, on WT mice, consistent with the lack of functional effects of PTX pretreatment observed in these hearts.

Effects of Overexpression of βARK1 and the βARK1 Inhibitor

During the preischemic period, PCr/ATP ratios were the same in the TGβARK1 hearts, at 1.51±0.09, as in WT hearts, at 1.51±0.08. Despite the increase in contractility and the presumed increase in β-adrenergic signaling in TGβARKct hearts, PCr/ATP ratios were the same as in WT hearts, at 1.49±0.07.

During ischemia, there were no significant differences in ATP levels between the groups of hearts (Figure 4A). Although ATP levels fluctuated in the TGβARK1 hearts during reperfusion, at the end of reperfusion ATP levels were higher in the TGβARK1 hearts, at 61% of initial ATP, compared with 47% of initial ATP in the WT hearts ($P<0.05$). ATP levels were the same in the TGβARKct as in WT hearts.

PCr decreased during ischemia and increased during reperfusion in all hearts (Figure 4B). At the end of reperfusion, PCr levels were higher in the TGβARK1 hearts, at 122% of initial ATP, than in the WT hearts, at 89% of initial ATP ($P<0.05$). There were no differences in PCr levels between TGβARKct and WT hearts.

Intracellular pH in the TGβARK1 and TGβARKct hearts is shown in Figure 5. At no time during the protocol were there any significant differences in pH between the WT and TGβARK1 or TGβARKct hearts.

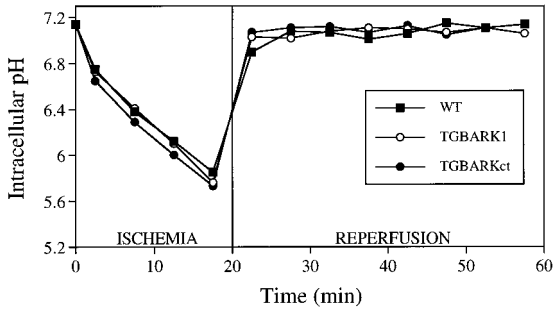

Figure 5. Myocardial pH_i during ischemia and reperfusion in WT, βARK1 overexpressor (TGβARK1), and βARK1 inhibitor (TGβARKct) mice. Points are mean±SEM. WT, n=15; TGβARK1, n=7; TGβARKct, n=5.

In summary, recoveries of energy metabolites during reperfusion were higher in βARK1 overexpressor hearts than in WT hearts, therefore correlating with the higher recovery of contractile function observed in these hearts and indicating less injury. Despite the increase in contractility and the presumed increase in β-adrenergic signaling observed in hearts overexpressing the βARK1 inhibitor, PCr/ATP ratios during basal perfusion were the same as in WT hearts, and there were no significant differences in energy metabolites or pH_i during ischemia and reperfusion when compared with WT hearts.

Discussion

Effects of β_2AR Signaling on Basal Contractility

In the present study, perfused hearts from β_2AR overexpressor mice exhibited increased peak contraction (LVDP), as well as increased rate of contraction (+dP/dt) and rate of relaxation (−dP/dt), compared with WT hearts. This is consistent with in vivo findings[16] and supports the hypothesis that overexpression of β_2ARs can improve cardiac function.

As discussed previously, β_2ARs can couple to both G_i and G_s. Treatment of β_2AR overexpressor hearts with PTX, a G_i inhibitor, will allow us to determine whether the coupling of the β_2AR to G_i modulates contractility in these hearts. PTX-treated β_2AR overexpressor hearts exhibited increased LVDP and ±dP/dt compared with untreated overexpressor hearts, implying that coupling of the β_2AR to G_i resulted in attenuation of contractility. This is consistent with the role of $G_{i\alpha}$ in inhibiting cAMP production by adenylate cyclase and therefore opposing the action of $G_{s\alpha}$. As described earlier, $G_{i\alpha}$ activation by the β_2AR prevents many of the $G_{s\alpha}$-mediated downstream events, which would otherwise contribute to increased myocardial contractility, such as phosphorylation of phospholamban and troponin I.[11] A $G_{i\alpha}$-mediated decrease in phosphorylation of phospholamban and troponin I is consistent with the observations of the present study. Phosphorylation of both of these proteins results in an increased rate of relaxation. In the PTX-treated β_2AR overexpressor hearts, the rate of relaxation (−dP/dt) was greatly increased compared with untreated hearts and, unusually, was greater than the rate of contraction. These observations confirm the functional role of dual G_s/G_i coupling of cardiac β_2ARs in a whole-heart model.

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An interesting observation in the β_2AR overexpressor hearts was that, unlike WT, there was no myocardial contractile response to isoproterenol. Originally this was attributed to the β_2AR overexpressor hearts being already maximally stimulated.[16] However, we found that treatment of the β_2AR overexpressors with PTX restored the isoproterenol sensitivity of contraction. Thus, the level of G_i activation in the β_2AR overexpressor hearts on stimulation by isoproterenol appears to be sufficient to prevent any increase in contraction, supporting recent findings in myocytes.[24] G_i activity therefore attenuates contractility in the β_2AR overexpressor hearts under stimulated, as well as basal, conditions.

Hearts with a 3-fold overexpression of βARK1 exhibited decreased LVDP, ±dP/dt and heart rate compared with WT hearts. This confirms that high levels of βARK1 can indeed contribute to decreased contractility, as predicted in CHF. As βARK1 phosphorylates and desensitizes βARs, the observation of lower contractility in βARK1 overexpressor hearts also indicates that β_1 and/or β_2ARs may be activated, presumably by endogenous catecholamines, and contribute to basal contractility in the perfused heart.

We also measured contractility in hearts overexpressing a βARK1 inhibitor. The βARK1 inhibitor is a peptide (βARKct) corresponding to the $G_{\beta\gamma}$ binding region of βARK1. As $G_{\beta\gamma}$ binding is required for activation of βARK1,[4] expression of the βARKct results in competition for $G_{\beta\gamma}$, and βARK1 activation is attenuated.[25] Consequently, $G_{\beta\gamma}$-stimulated βARK1 activity in extracts from hearts expressing the βARKct were 50% lower than in WT hearts.[17] We found that expression of this βARK1 inhibitor peptide increased LVDP and +dP/dt compared with WT hearts, consistent with findings in vivo.[17] This implies that, even at normal levels, βARK1 may modulate contractility in the perfused heart. These findings also implicate expression of a βARK1 inhibitor as an alternate gene therapy approach for increasing contractility in heart failure patients.

Effects of β_2AR Signaling on Ischemic Injury

To determine the consequences of β_2AR overexpression with respect to ischemic injury, we compared the response to ischemia in hearts from mice overexpressing the β_2AR with that of WT hearts. The postischemic recoveries of both contractile function and the energy metabolites, ATP and PCr, were less in the β_2AR overexpressor hearts, indicating greater injury. During ischemia, ATP levels fell lower in the β_2AR overexpressor hearts than in WT hearts, reflecting greater ischemic energy utilization. The faster rate of ATP utilization in the β_2AR overexpressor hearts is also consistent with the earlier onset of contracture observed. As H^+ are produced by ATP degradation, the greater ATP utilization in the β_2AR overexpressors may contribute to the lower pH also observed in these hearts. Low ischemic ATP levels and pH often correlate with increased ischemic injury. ATP is required to maintain function of proteins such as the Na^+/K^+-ATPase, activity of which is necessary to prevent ischemic Na^+ overload and consequently, via Na^+/Ca^{2+} exchange, Ca^{2+} overload and injury.[19,26–28] Low pH activates the Na^+/H^+ exchanger, which also contributes to Na^+ overload and injury.[29,30] Although the low ischemic ATP and pH may be sufficient to cause the increased injury observed in the β_2AR overexpressor hearts, increased Ca^{2+} influx through L-type Ca^{2+}channels, which are activated by β_2AR signaling, may also contribute. In summary, β_2AR overexpression resulted in greater ischemic energy utilization and increased ischemic injury. These results suggest that a negative consequence of overexpressing β_2ARs in CHF patients may be an increase in susceptibility to ischemic injury. There is some indication, however, that lower levels of β_2AR expression may prove to be less deleterious.[31]

As the β_2AR is coupled to both G_i and G_s, we determined whether the increased ischemic injury observed in the β_2AR overexpressor hearts was mediated through activation of G_i or G_s. WT and β_2AR overexpressor mice were pretreated with PTX to inhibit the G_i protein, and the response to myocardial ischemia was then studied. The postischemic recoveries of both contractile function and ATP were less in the PTX-treated β_2AR overexpressor hearts compared with untreated hearts, indicating greater injury. During ischemia, ATP levels fell lower and the onset of contracture was earlier in the PTX-treated hearts, reflecting greater ischemic energy utilization. Therefore, PTX treatment increased ischemic energy utilization and exacerbated injury in the β_2AR overexpressor hearts. These results indicate that it is the activation of G_s by β_2ARs that leads to greater energy demand and injury and also suggests that G_i activation attenuates the increased energy demand and is protective. The suggestion of a protective effect of G_i activity with respect to ischemic injury is consistent with findings that pretreatment of hearts with adenosine, a G_i activator, results in greater recovery from ischemia.[32]

To further assess the role of βAR signaling in ischemic injury, we studied the ischemic response of mice overexpressing βARK1 and also of mice expressing a βARK1 inhibitor.[17] We found that hearts with a 3-fold overexpression of βARK1 exhibited higher postischemic recoveries of contractile function and energy metabolites, indicating less injury. The observation of increased ischemic injury in the β_2AR overexpressor hearts indicated that increased β_2AR signaling can be detrimental. As βARK1 desensitizes βARs, the observation of less injury in βARK1 overexpressor hearts also indicates that even normal levels of β_1 and/or β_2AR signaling can enhance ischemic injury. Schomig et al[33] demonstrated extracellular accumulation of endogenous myocardial catecholamines after 10-minute ischemia, even in the isolated perfused heart. Our findings indicate that this ischemic catecholamine release may activate βAR signaling and exacerbate injury.

Interestingly, use of shorter ischemic periods, at least in a working heart model, has been shown to give different results. Chen et al[23] demonstrated that after 6 minutes of ischemia, cardiac output in the TGβARK1 hearts was depressed during reperfusion relative to WT. If an increase in extracellular catecholamines is an important factor in myocardial ischemic injury, as proposed above, then the 6-minute ischemic duration used in the Chen et al[23] study would not be sufficient for catecholamine accumulation, which may explain why overexpression of βARK1 was not protective in their study but does appear to be protective after 20-minute

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ischemia. Regardless of the mechanism, a short period of ischemia has limited clinical relevance; therefore, we believe the protective effect observed in our study, with clinically relevant longer durations of ischemia, is the more important effect. Other factors that may contribute to the differing findings of our study and that of Chen et al,[23] in addition to the different ischemia protocols, are the different model systems and different methods of function measurement used.

An intriguing observation was that, despite contractility being elevated to the same extent as in β_2AR overexpressor hearts, hearts expressing the βARK1 inhibitor did not exhibit increased ATP depletion during ischemia, and ischemic injury was not increased, as assessed by recovery of postischemic contractile function and energy metabolites. Even during basal perfusion, unlike the β_2AR overexpressor hearts, PCr/ATP ratios were not decreased relative to WT. Therefore it appears that expression of the βARK1 inhibitor results in increased contractility without depletion of energy metabolites during basal perfusion or increased energy utilization during ischemia. The reason for this difference between βARK1 inhibitor and β_2AR overexpressor hearts is unclear. As the βARK1 inhibitor relieves βARK1-mediated desensitization of the β_2AR, one may expect βARK1 inhibitor and β_2AR overexpressor hearts to have similar responses to ischemia as well as similar alterations in contractility. As βARK1 desensitizes receptors other than the βARs,[5–8] the increased contractility observed in the βARK1 inhibitor hearts may be due to increased activity of a receptor that does not concomitantly increase energy utilization. Interestingly, agonist activation of the endothelin-B receptor, a βARK1 substrate, increases contractility without decreasing the PCr/ATP ratio.[34] Furthermore, β-adrenergic stimulation has been shown to increase ATP utilization via the actomyosin ATPase, an event not observed on stimulation with endothelin.[35] Alternatively, the difference may be related to the ability of the βARK1 inhibitor (βARKct) to also inhibit $G_{\beta\gamma}$-mediated effects.[36] Interestingly, differences between the effects of β_2AR overexpression and βARK1 inhibitor expression have also been observed in an in vivo model. When β_2AR overexpressor mice were crossed with a mouse model of cardiomyopathy and failure, deterioration of myocardial function and mortality were increased.[37] However, when βARK1 inhibitor mice were crossed with the same model, contractility was increased and myocardial degeneration and failure were prevented. Regardless of the mechanism, our observation that basal contractility can be increased by expression of the βARK1 inhibitor without a concomitant increase in ischemic injury suggests a novel and safe therapeutic strategy for heart failure.

In summary, by comparing basal contractility in mice overexpressing the β_2AR to that of WT mice, we confirmed that overexpression of the cardiac β_2AR increases contractility. Pretreatment of β_2AR overexpressors with the G_i inhibitor, PTX, increased contractility and abolished the contractile resistance to isoproterenol, indicating that coupling of the β_2AR to G_i attenuates contractility in the β_2AR overexpressor hearts under both basal and stimulated conditions. These observations confirm the functional role of dual G_s/G_i coupling of cardiac β_2ARs in a whole-heart model. We also demonstrated that overexpression of βARK1 resulted in decreased contractility and expression of an inhibitor of βARK1 increased contractility, suggesting that, at normal levels or when overexpressed, βARK1 modulates contractility in the perfused heart. These findings also implicate expression of the βARK1 inhibitor as an alternate gene therapy approach for increasing contractility in heart failure patients.

By comparing the ischemic response of hearts overexpressing the β_2AR with that of WT hearts, we also demonstrated that β_2AR overexpression resulted in greater ischemic energy utilization and increased ischemic injury. These results suggest that a negative consequence of overexpressing β_2ARs in CHF patients, at least at high levels, may be an increase in susceptibility to ischemic injury. The increased energy utilization and injury observed in the β_2AR overexpressors was exacerbated by PTX treatment, which indicates that it is the activation of G_s by β_2ARs that leads to injury and also suggests that G_i activation is partially protective. βARK1 activity was also shown to be protective, as βARK1 overexpression decreased ischemic injury. A major finding of this study, however, was that, despite increasing basal contractility, expression of the βARK1 inhibitor had no effect on ischemic injury. This finding implicates expression of the βARK1 inhibitor as a novel and safe potential therapy for heart failure.

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