β3 Adrenergic Stimulation of the Cardiac Na⁺-K⁺ Pump by Reversal of an Inhibitory Oxidative Modification

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Background—Inhibition of L-type Ca²⁺ current contributes to negative inotropy of β3 adrenergic receptor (β3 AR) activation, but effects on other determinants of excitation-contraction coupling are not known. Of these, the Na⁺-K⁺ pump is of particular interest because of adverse effects attributed to high cardiac myocyte Na⁺ levels and upregulation of the β3 AR in heart failure.

Methods and Results—We voltage clamped rabbit ventricular myocytes and identified electrogenic Na⁺-K⁺ pump current (Iₚ) as the shift in holding current induced by ouabain. The synthetic β3 AR agonists BRL37344 and CL316,243 and the natural agonist norepinephrine increased Iₚ. Pump stimulation was insensitive to the β1/β2 AR antagonist nadolol and the protein kinase A inhibitor H-89 but sensitive to the β3 AR antagonist L-748,337. Blockade of nitric oxide synthase abolished pump stimulation and an increase in fluorescence of myocytes loaded with a nitric oxide–sensitive dye. Exposure of myocytes to β3 AR agonists decreased β1 Na⁺-K⁺ pump subunit glutathionylation, an oxidative modification that causes pump inhibition. The in vivo relevance of this was indicated by an increase in myocardial β1 pump subunit glutathionylation with elimination of β3 AR–mediated signaling in β3 AR−/− mice. The in vivo effect of BRL37344 on contractility of the nonfailing and failing heart in sheep was consistent with a beneficial effect of Na⁺-K⁺ pump stimulation in heart failure.

Conclusions—The β3 AR mediates decreased β1 subunit glutathionylation and Na⁺-K⁺ pump stimulation in the heart. Upregulation of the receptor in heart failure may be a beneficial mechanism that facilitates the export of excess Na⁺. (Circulation. 2010;122:2699-2708.)

Key Words: heart failure ■ molecular biology ■ myocardium ■ nitric oxide synthase ■ receptors, adrenergic

The β3 adrenergic receptor (β3 AR) differs from β1/β2 ARs in its molecular structure, genetic coding, and affinities for adrenergic ligands. Cellular responses to receptor activation also differ. In cardiac tissue, β3 AR activation decreases contractility, contrasting with the increase mediated by the β1/β2 AR. β3 AR agonists BRL37344 and CL316,243 and the natural agonist norepinephrine increased Iₚ. Pump stimulation was insensitive to the β1/β2 AR antagonist nadolol and the protein kinase A inhibitor H-89 but sensitive to the β3 AR antagonist L-748,337. Blockade of nitric oxide synthase abolished pump stimulation and an increase in fluorescence of myocytes loaded with a nitric oxide–sensitive dye. Exposure of myocytes to β3 AR agonists decreased β1 Na⁺-K⁺ pump subunit glutathionylation, an oxidative modification that causes pump inhibition. The in vivo relevance of this was indicated by an increase in myocardial β1 pump subunit glutathionylation with elimination of β3 AR–mediated signaling in β3 AR−/− mice. The in vivo effect of BRL37344 on contractility of the nonfailing and failing heart in sheep was consistent with a beneficial effect of Na⁺-K⁺ pump stimulation in heart failure.

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Cardiac β3 AR is upregulated in heart failure, and the negative inotropic effect it mediates in nonfailing hearts

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supports the widely held view that receptor upregulation is harmful with disease progression.\textsuperscript{2,6,11–14} It has been proposed that blockade of $\beta_1$ AR activation in heart failure might be beneficial.\textsuperscript{6,15–17} Because the Na\textsuperscript{+}–K\textsuperscript{+} pump effectively is the only export route for the excess intracellular Na\textsuperscript{+}, the therapeutic success of such an approach might be critically dependent on the effect of $\beta_1$ AR activation on pump activity. We examined whether $\beta_1$ AR activation regulates the Na\textsuperscript{+}–K\textsuperscript{+} pump in ventricular myocytes and alters glutathionylation of the $\beta_1$ subunit of the pump, an oxidative modification that is causally related to pump inhibition.\textsuperscript{18} We also examined $\beta_1$ AR pump subunit glutathionylation in the myocardium of $\beta_1$ AR–/– mice and the effect of a $\beta_1$ AR agonist on in vivo cardiac contractility in a large-animal model before and after induction of stable severe heart failure.

**Methods**

**Measurement of Na\textsuperscript{+}–K\textsuperscript{+} Pump Activity**

Electrogenic Na\textsuperscript{+}–K\textsuperscript{+} pump current ($I_p$) was measured in freshly isolated voltage-clamped rabbit ventricular myocytes. Solutions and voltage clamp protocol were designed to minimize nonpump membrane currents. Patch pipette solutions perfusing the intracellular compartment included 10 mmol/L Na\textsuperscript{+}, a concentration near physiological intracellular levels. While we established the whole-cell configuration, myocytes were superfused with solution that included Na\textsuperscript{+} and Ca\textsuperscript{2+}. Two to 3 minutes later, we switched to a superfusate that was nominally Ca\textsuperscript{2+} free and had 2 mmol/L BaCl\textsubscript{2} and 0.2 mmol/L CdCl\textsubscript{2} added. When indicated, the solution was Na\textsuperscript{+} free to eliminate transmembrane Na\textsuperscript{+} influx, which might cause an increase in the intracellular Na\textsuperscript{+} concentration and secondary pump stimulation. $I_p$ was identified at a holding potential of $-40$ mV as the difference between holding current before and after Na\textsuperscript{+}–K\textsuperscript{+} pump blockade with 100 $\mu$mol/L ouabain.

**Imaging of Intracellular Nitric Oxide by Diaminofluorescein–2 Diacetate**

To detect nitric oxide production, we loaded myocytes with the nitric oxide (NO)–sensitive dye diaminofluorescein–2 diacetate (DAF-2DA). Myocytes representative of each experimental condition were selected randomly for measurement of fluorescent intensity with a laser-scanning confocal microscope. The average of each experiment was normalized against its control.

**Immunodetection of S-Glutathionylated Proteins and Protein Coimmunoprecipitation in Rabbit Ventricular Myocytes**

To detect S-glutathionylation of the $\beta_1$ Na\textsuperscript{+}–K\textsuperscript{+} pump subunit, aliquots of myocytes were loaded with biotinylated glutathione (GSH). After lysis, glutathionylated protein was precipitated with streptavidin–Sepharose beads,\textsuperscript{18} separated by gel electrophoresis, transferred to a membrane, and probed with antibodies to the $\beta_1$ subunit of the Na\textsuperscript{+}–K\textsuperscript{+} pump. Glutathionylation was also detected with an antibody against glutathionylated protein (anti-GSH antibody).\textsuperscript{18} Standard techniques were used for immunodetection of coimmunoprecipitation of $\alpha_1$ and $\beta_1$ pump subunits in rabbit myocytes and for detection of the $\beta_1$ AR in myocardium from mice and sheep.

**Heart Failure Model and In Vivo Studies of Cardiac Contractility**

Global damage to left ventricular (LV) function was induced by repeated selective coronary microembolization in sheep at 2-week intervals until ejection fraction remained stable at $<35\%$ over at least 4 weeks, as described previously.\textsuperscript{19} The effects of BRL37344 (BRL) on standard hemodynamic variables and LV end-systolic pressure-volume relationships (ESPVRs) of continuous LV pressure-volume loops were determined at baseline and after induction of heart failure. Because ESPVR is relatively load independent, it was chosen a priori as the primary index of cardiac contractility.

**Activation and Blockade of $\beta$ ARs and Downstream Pathways**

The $\beta_1$ AR was activated with the synthetic agonists BRL (1 to 100 nmol/L) and CL316,243 (CL; 10 to 1000 nmol/L) or the natural agonist norepinephrine (10 nmol/L). Nadolol (1000 nmol/L), L-748,337 (L-7; 1000 nmol/L), and prazosin (1000 nmol/L) were used to block $\beta_1$/$\beta_2$ ARs, $\beta_3$ AR, and $\alpha$ ARs. Protein kinase A (PKA) was inhibited by inclusion of H-89 (500 nmol/L) in patch pipette solutions perfusing the intracellular compartment of voltage-clamped myocytes or by exposing myocytes to the membrane permeable inhibitor KT5720 (200 nmol/L) in extracellular solutions. Forskolin (100 nmol/L) was used to activate adenyl cyclase, NO synthase (NOS) was inhibited by inclusion of N\textsuperscript{6}-nitro-L-arginine methyl ester (L-NAME) in pipette solutions (10 $\mu$mol/L) or extracellular solutions (100 to 1000 $\mu$mol/L). The heat shock protein antagonist radicicol (10 $\mu$mol/L) and 1H-(1,2,4)-oxadiazolo[4,3-$d$]quinoxalin-1-one (ODQ; 10 $\mu$mol/L) were included in pipettes to inhibit NOS activation or NO-activated “soluble” guanylyl cyclase.

**Statistical Analysis**

Results are expressed as mean±SE. Statistical comparisons were made with ANOVA after a D’Agostino-Pearson normality test had been used to examine whether the distribution of residuals deviated from normality. A posthoc Bonferroni test was used as appropriate. Relationships between dose of BRL and hemodynamic variables before and after induction of heart failure were compared with nonparametric Spearman correlation and Wilcoxon matched-pairs signed-ranks test. An expanded Methods section is available in the online-only Data Supplement.

**Results**

$\beta_1$ AR Agonists Stimulate the Na\textsuperscript{+}–K\textsuperscript{+} Pump Current

We exposed patch-clamped myocytes to a control superfusate or a superfusate containing 10 nmol/L BRL, switching to the BRL-containing superfusate after the whole-cell configuration had been established. Figure 1A shows the timing of changes in the composition of superfusates and holding currents of a control myocyte and a myocyte exposed to BRL. BRL stimulated the Na\textsuperscript{+}–K\textsuperscript{+} pump, as shown by the increase in the ouabain-induced shift in holding current, $I_p$ (Figure 1A and 1B). To rule out a BRL-induced transmembrane Na\textsuperscript{+} influx and Na\textsuperscript{+}–K\textsuperscript{+} pump stimulation secondary to an increase in the intracellular Na\textsuperscript{+} concentration, we also measured $I_p$ in Na\textsuperscript{+}–free superfusates. BRL increased $I_p$, indicating that pump stimulation was not due to enhanced Na\textsuperscript{+} influx (Figure 1C).

Exposure of myocytes to 1 or 10 nmol/L BRL increased $I_p$, but stimulation was lost at a concentration of 100 nmol/L (Figure 2A). Because BRL has imperfect selectivity for the $\beta_1$ AR, we blocked $\beta_1$/$\beta_2$ ARs with nadolol. This restored the BRL-induced pump stimulation that was observed at the lower concentrations (Figure 2A), indicating first that $\beta_1$/$\beta_2$ ARs do not mediate pump stimulation and second that nonspecific activation of $\beta_1$ AR by BRL leads to the loss of pump stimulation at 100 nmol/L of BRL. In an independent set of experiments, we included H-89 in pipette solutions and nadolol in the superfusate. The concentration of H-89 we used abolishes the effect of forskolin-induced PKA activation.
to induce a decrease in $I_p$\textsuperscript{20} The combined blockade of $\beta_1/\beta_2$ ARs and the downstream pathway coupled to them had no effect on the BRL-induced increase in $I_p$ (Figure 2A). The increase also persisted in the presence of the $\alpha$ AR antagonist prazosin (Figure 2A).

Although BRL is regarded as the classic agonist for the rodent $\beta_3$ AR, CL is more specific albeit less potent.\textsuperscript{21} CL increased $I_p$, and in contrast to the effect of BRL, there was no reduction in Na$^+$-K$^+$ pump stimulation with an increase in CL concentration (Figure 2B). L-7, an antagonist for the $\beta_3$ AR,\textsuperscript{2,22} abolished the CL-induced increase in $I_p$ (Figure 2B). The $\beta_3$ AR has a high affinity for the naturally occurring catecholamine norepinephrine and is sometimes referred to as the “noradrenergic” receptor.\textsuperscript{3} Norepinephrine increased $I_p$, an effect that was insensitive to nadolol and H-89 (Figure 2C).

**Figure 1.** Effect of the $\beta_3$ adrenergic receptor agonist BRL on Na$^+$-K$^+$ pump current. A, Protocol for measurement of $I_p$ and examples of membrane currents. Stable currents before and after exposure of patch-clamped myocytes to ouabain were sampled with an electronic cursor to identify $I_p$. The superfusate contained Na$^+$ throughout the experiments illustrated and 10 nmol/L BRL as indicated. $C_m$ indicates membrane capacitance (pF). B, Effect of BRL on $I_p$ measured in Na$^+$- containing superfusate. C, $I_p$ in Na$^+$-free superfusates to eliminate Na$^+$ influx and hence secondary pump stimulation. Numbers of myocytes studied are indicated in parentheses within columns. *Statistical significance relative to control.

**Figure 2.** Inhibition of $\beta_1/\beta_2$ AR-mediated signaling. A, An increase in $I_p$ at 1 or 10 nmol/L BRL was not observed at 100 nmol/L. Data for 10 nmol/L BRL are also shown in Figure 1B. Pump stimulation occurred at 100 nmol/L BRL when myocytes were exposed to the $\beta_1/\beta_2$ AR antagonist nadolol with or without the protein kinase A inhibitor H-89 in patch pipette solutions. Prazosin had no effect on BRL-induced pump stimulation. B, Effect of the $\beta_3$ AR agonist CL and antagonist L-7. C, Effect of norepinephrine (NE). *Statistical significance relative to control experiments.
NO Dependence of Na\(^+\)-K\(^+\) Pump Stimulation

Because the \(\beta_1\) AR is coupled to activation of NOS,\(^{23}\) we examined NO-sensitive fluorescence of DAF-2DA–loaded myocytes. Exposure to BRL for 10 minutes augmented fluorescence (Figure 3A). The augmentation was blocked by preincubation with L-NAME, consistent with coupling of the \(\beta_1\) AR and NOS in rabbit cardiac myocytes. In functional Na\(^+\)-K\(^+\) pump studies on voltage-clamped myocytes, we inhibited NOS or blocked its activation by including L-NAME or radicicol in patch pipette solutions. Both compounds abolished the BRL-induced increase in \(I_p\) (Figure 3B). We included ODQ in pipette solutions to inhibit NO-activated guanylyl cyclase. ODQ abolished the norepinephrine-induced increase in \(I_p\) (Figure 3C).

\(\beta_3\) AR Agonists Reduce Glutathionylation of the \(\beta_1\) Subunit of the Na\(^+\)-K\(^+\) Pump

Oxidant cellular signaling can inhibit the Na\(^+\)-K\(^+\) pump via reversible glutathionylation of its \(\beta_1\) subunit.\(^{18}\) Because there is glutathionylation at baseline, we examined whether the converse applies, that \(\beta_1\) AR agonist-induced pump stimulation is associated with a decrease in glutathionylation. BRL and CL decreased \(\beta_1\) subunit glutathionylation, as detected with the biotin-GSH technique (Figure 4A) and with the independent GSH antibody technique (Figure 4B). The \(\alpha/\beta\) subunit interaction is critical for Na\(^+\)-K\(^+\) pump function,\(^{24}\) and glutathionylation is associated with a decrease in coimmunoprecipitation of the subunits.\(^{18}\) The decrease in glutathionylation induced by \(\beta_1\) AR agonists was associated with the converse, ie, an increase in subunit coimmunoprecipitation (Figure 4C).

Consistent with the functional studies shown in Figures 2B and 3B, the decrease in \(\beta_1\) Na\(^+\)-K\(^+\) pump subunit glutathionylation induced by CL was blocked by preincubation of myocytes with L-NAME (Figure 5A) or the \(\beta_1\) AR antagonist L-7 (Figure 5B). We used the membrane-permeable compound KT5720 to examine the effect of PKA inhibition on the CL-induced decrease in \(\beta_1\) subunit glutathionylation. Inhibition of a forskolin-induced increase in glutathionylation\(^{26}\) was used as a positive control. The PKA inhibitor had no effect on the CL-induced decrease in \(\beta_1\) Na\(^+\)-K\(^+\) pump subunit glutathionylation (Figure 5C), consistent with the functional results shown in Figure 2A.

Mice Lacking the \(\beta_3\) AR Have Increased Glutathionylation of the \(\beta_1\) Na\(^+\)-K\(^+\) Pump Subunit

\(\beta_3\) AR \(^{-/-}\) mice have an exacerbated phenotype of pathological cardiac remodeling after transaortic constriction that is mediated by an increase in oxidative stress.\(^{25}\) We therefore examined whether \(\beta_3\) AR signaling affects downstream glutathionylation of the \(\beta_1\) Na\(^+\)-K\(^+\) pump subunit in vivo. The \(\beta_3\) AR was detected in the myocardium from wild-type mice but not \(\beta_3\) AR \(^{-/-}\) mice (Figure 6A). Consistent with the decrease in glutathionylation of the \(\beta_1\) Na\(^+\)-K\(^+\) pump subunit in isolated rabbit myocytes induced by \(\beta_3\) AR agonists in vitro (Figure 4A and 4B), there was an increase in \(\beta_1\) subunit glutathionylation induced by \(\beta_3\) AR agonists in mice but not in \(\beta_3\) AR \(^{-/-}\) mice (Figure 6B).
glutathionylation in the myocardium of mice lacking the β3 AR (Figure 6B). As previously shown for rabbit myocytes,18 glutathionylated β1 subunit was not detected in the immuno- 
 blot when myocardial lysate had been preincubated with 1 mmol/L dithiothreitol (data not shown), supportive of a 
mixed disulfide bond between the β1 subunit and glutathione.

Effects of BRL on Contractility of the Normal and 
Failing Heart
We examined the effect of BRL on cardiac contractility in 
sheep before and after induction of severe, stable heart failure 
as defined previously.19 Expression of the β3 AR in sheep 
heart was ascertained by immunoblotting. Myocardium from 
wild-type and β3 AR−/− mouse as positive and negative 
controls (Figure 7A). After determining standard hemody- 
namic variables and LV ESPVR in sheep at a drug-free 
baseline, we gave BRL intravenously in incremental doses 
ranging from 0.05 to 2.5 μg/kg. Each dose was given over a 
period of 8 minutes, and hemodynamic parameters were 
determined after an additional 5 minutes. When the measure- 
ments were completed, we gave a larger dose of BRL, 
repeating the timing of the protocol. Hemodynamic param- 
eters are summarized in Table I in the online-only Data 
Supplement. Variability of the reference level for pressure 
measurements relative to the tip of the LV Millar catheter 
contributes to the coefficient of variation of pressures, and 
each measurement at the different dose levels in failing and 
nonfailing hearts was normalized to the corresponding drug-
free baseline value. The normalized parameter at each BRL 
dose in a nonfailing heart was matched with its corresponding value at the same dose 
after induction of heart failure for each of 4 sheep. There was 
a positive correlation between the rate of LV systolic pressure
increase (dP/dtmax) for the failing and nonfailing heart with increasing BRL dose (Spearman correlation coefficient, 0.72; \(P<0.05\)). There was no correlation with dose for peak systolic pressure, stroke work, LV diastolic pressure decay (dP/dtmin), LV end-diastolic pressure, or cardiac output. A paired replicate rank test indicated that peak systolic pressure and dP/dtmin were significantly higher with BRL administration for the failing than the nonfailing heart, whereas LV end-diastolic pressure was significantly lower. There was no difference between the effects of BRL on stroke work and cardiac outputs in failing and nonfailing heart.

Figure 7 shows examples of LV pressure-volume loops in nonfailing (B) and failing (C) heart. BRL induced a decrease in ESPVR in the nonfailing but an increase in the failing heart. Each ESPVR measured was normalized to the value at the drug-free baseline. There was a negative relationship between the dose of BRL and ESPVR in the nonfailing heart, consistent with a negative inotropic effect of the drug (\(P<0.05\)). However, there was a significant shift of the relationship between the dose of BRL and ESPVR in a positive direction after induction of heart failure (paired replicate rank test, \(P<0.05\)). The Spearman correlation coefficient between values ESPVR for failing and nonfailing hearts at different doses of BRL was \(-0.2\) (Figure 7C).

**Discussion**

In vitro exposure of rabbit ventricular myocytes to \(\beta_1\) AR agonists induced a decrease in glutathionylation of the \(\beta_1\) subunit of the Na\(^{+}\)-K\(^{-}\) pump that was functionally important, as indicated by an increase in Na\(^{+}\)-K\(^{-}\) pump current. The in vivo relevance of this finding was supported by an increase in glutathionylation of the pump subunit in the myocardium from \(\beta_1\) AR\(^{-/-}\) mice. These findings are likely to be important for our understanding of \(\beta_1\) AR upregulation in heart failure.

There is a complicated species dependence of the structure, ligand affinity, and expression of the \(\beta_1\) AR.7,22 We used rabbit ventricular myocytes that express receptors with a high degree of functional similarity to the human receptor.7 It is unlikely that Na\(^{+}\)-K\(^{-}\) pump stimulation was due to nonspecific activation of \(\beta_1/\beta_2\) ARs because PKA, expected to be activated downstream from these receptors, mediates an increase in glutathionylation of the \(\beta_1\) subunit in the myocardium from \(\beta_1\) AR\(^{-/-}\) mice. These findings are likely to be important for our understanding of \(\beta_1\) AR upregulation in heart failure.
It may provide an alternative explanation for the absence of an effect of isoproterenol on Na\textsuperscript{+}-K\textsuperscript{+} pump activity in PLM\textsuperscript{−/−} myocytes. Additional factors contribute to controversies around the effects of adrenergic signaling on the cardiac myocyte Na\textsuperscript{+}-K\textsuperscript{+} pump, as discussed previously.\textsuperscript{20}

Glutathionylation is emerging as an important mechanism for the regulation of protein function.\textsuperscript{28} Oxidative stimuli, including activation of adenyl cyclase that is coupled to the \( \beta_{1}/\beta_{2} \) ARs\textsuperscript{20} or exposure to angiotensin II,\textsuperscript{18,29} can induce glutathionylation of the \( \beta_{1} \) Na\textsuperscript{+}-K\textsuperscript{+} pump subunit and cause pump inhibition in myocytes. Oxidation-induced glutathionylation of the \( \beta_{1} \) subunit expressed in Xenopus oocytes and pump inhibition is abolished by mutation of a specific cysteine, indicating a causal relationship between glutathionylation and inhibition.\textsuperscript{18} The glutathionylation is associated with a decrease in coimmunoprecipitation of the \( \alpha_{1} \) and \( \beta_{1} \) subunits,\textsuperscript{18} the interaction of which is critical for overall Na\textsuperscript{+}-K\textsuperscript{+} pump activity.\textsuperscript{24} Conversely, in the present study, deglutathionylation was associated with an increase in \( \alpha_{1}/\beta_{1} \) subunit coimmunoprecipitation and pump stimulation. These results support a central role for the glutathionylation state of the \( \beta_{1} \) subunit in determining Na\textsuperscript{+}-K\textsuperscript{+} pump activity. To the best of our knowledge, the \( \beta_{1} \) AR–mediated effect we report here is the first example of receptor-coupled reversal of an oxidative modification of a key cellular protein causing a change in function.

Deglutathionylation is catalyzed by glutaredoxin 1 (GRx1) with exclusive selectivity for the protein-glutathione “mixed” disulfide.\textsuperscript{30} Coimmunoprecipitation of GRx1 with the \( \beta_{1} \) Na\textsuperscript{+}-K\textsuperscript{+} pump subunit and functional studies implicate GRx1 at the molecular level,\textsuperscript{14} but at the cellular level, regulation of deglutathionylation is poorly understood.\textsuperscript{28} Signaling mediated by NO-activated guanylyl cyclase is implicated here, but to the best of our knowledge, no posttranslational modification is reported to activate GRx1. In contrast, as its name implies, PLM can be phosphorylated in response to cellular signaling. Phosphorylation of serines 63 and 68 in the cytoplasmic terminal is firmly established, as reviewed previously.\textsuperscript{31} Recently, phosphorylation of an amino acid in position 69 has also been reported. The amino acid can be a serine or threonine, depending on the species. Some details of the mechanism remain uncertain, but it has been established that selective phosphorylation of serine 69 is induced by cyclic guanosine monophosphate–dependent signaling.\textsuperscript{32} PLM-dependent deglutathionylation of the \( \beta_{1} \) Na\textsuperscript{+}-K\textsuperscript{+} pump subunit (S. Biebert, PhD, C. C. Liu, PhD, G. A. Figtree, MB, BS, DPhil., A. Garcia, BSc, E. J. Hamilton, BSc, F. M. Marassi, PhD, K. J. Sweadner, PhD, F. Cornelius, PhD, K. Geering, PhD and H. H. Rasmussen, MD, DMSc, unpublished data, 2010) may somehow be facilitated by phosphorylation of serine 69, perhaps by a promotion of access of GRx1 to the \( \beta_{1} \) pump subunit. Unfortunately, our current understanding of the 3-dimensional crystal structure of the complex of \( \alpha \) and \( \beta \) subunits with FXYD\textsuperscript{24} does not indicate how this might occur. The cytoplasmic terminal of FXYD proteins where phosphorylation sites reside remains to be resolved, and although the location of the reactive cysteine in the \( \beta_{1} \) subunit is unlikely to be accessible to GRx1 in the E\textsubscript{2} conformational state that was used to determine the
3-dimensional structure of the α/β/FXYD complex, its location in the E1 conformation remains unknown. Large movements of the subunits relative to each other are known to occur during the E2→E1 transition in the pump cycle.

A decrease in intracellular Na\(^{+}\) levels with Na\(^{+}\)-K\(^{+}\) pump stimulation enhances Ca\(^{2+}\) export via Na\(^{+}\)-Ca\(^{2+}\) exchange and decreases cytosolic Ca\(^{2+}\) available for uptake into the sarcoplasmic reticulum. This should reduce the amplitude of cytosolic Ca\(^{2+}\) transients and hence contractility, as shown in rabbit cardiac myocytes exposed to β\(_3\) AR agonists in concentrations similar to those we used. However, this cannot necessarily be attributed solely to Na\(^{+}\)-K\(^{+}\) pump stimulation because Ca\(^{2+}\) channel inhibition is expected to have a similar effect. Heart disease introduces additional confounders with changes in the relative abundance of β ARs and uncertainty about interaction with the effect that other “neurohormones” have on the Na\(^{+}\)-K\(^{+}\) pump, Na\(^{+}\) influx, and intracellular Na\(^{+}\). Our in vivo studies on failing and nonfailing sheep heart indicated a differential effect of BRL consistent with Na\(^{+}\)-K\(^{+}\) pump stimulation and a decrease in intracellular Na\(^{+}\) that might have a negative inotropic effect in the normal heart but not in the failing heart. Any upregulation of the β\(_3\) AR with heart failure did not accentuate a negative inotropic effect of receptor activation; there was no adverse acute effect on hemodynamic variables of BRL in our large-animal model of severe heart failure.

The view that upregulation of the β\(_3\) AR in severe heart failure is maladaptive was well reasoned on the basis...
of available evidence but should now be reconsidered. Accentuation of the phenotype that results from transaortic constriction in β3 AR−/− mice suggests that upregulation of the β3 AR can be a useful compensatory mechanism. The increase in oxidative stress implicated in the phenotype is in good agreement with the increase in glutathionylation of the β3 Na+/K+ pump subunit in the myocardium of β3 AR−/− mice we found and with the decrease in its glutathionylation induced by β3 AR activation in isolated rabbit cardiac myocytes. The causal relationship between β3 subunit glutathionylation and pump inhibition suggests that adverse effects of myocardial oxidative stress and raised myocyte Na+ levels may be closely interrelated. Activation of the β3 AR may reverse these abnormalities.

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Disclosures

Drs Bundgaard and Rasmussen are named inventors on a patent application submitted by Royal North Shore Hospital and University of Sydney for the use of β3 AR agonists in heart failure. The other authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

The β3 adrenergic receptor differs from the β1 and β2 types in its structure, ligand affinities, and coupling to intracellular signaling pathways. Its expression in the myocardium is upregulated in heart failure, and because it mediates a negative inotropic effect in the normal heart, this upregulation has been thought to be maladaptive. This has led to the suggestion that β3 adrenergic receptor antagonists might be useful. The β receptor antagonists of proven efficacy target β1 and β2 receptors, and we have reported that the downstream cAMP-dependent pathways coupled to these receptors induce an oxidative modification on a subunit of the membrane Na+/K+ pump that inhibits pump activity. Because raised intracellular Na+ levels and increased myocardial oxidative stress contribute adversely to the phenotype of contractile abnormalities and cardiac arrhythmias in heart failure, the “β-blockers” may limit the oxidative Na+/K+ pump inhibition and hence reduce myocyte Na+ overload. Here, we show that selective β3 adrenergic receptor agonists reverse the oxidative molecular modification that inhibits the cardiac Na+/K+ pump and hence stimulate pump activity. Activation of upregulated β3 adrenergic receptors with the increase in catecholamine levels typically seen in heart failure may be a useful compensatory mechanism, consistent with a recent report of β3 adrenergic receptor knockout mice suffering an accentuated cardiac phenotype in an aortic constriction model. It may also explain the adverse outcome in a previous large human heart failure trial when raised catecholamine levels were specifically targeted in heart failure with the use of a centrally acting sympatholytic drug.
β3 Adrenergic Stimulation of the Cardiac Na⁺-K⁺ Pump by Reversal of an Inhibitory Oxidative Modification

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals and cells. Single ventricular myocytes were isolated from 64 male White New Zealand rabbits as described previously \(^1\). To reduce effects of between-rabbit variability we used myocytes from each rabbit for several different types of experiments and/or experimental conditions within the same type of experiment. This allowed the use of at least 3 different rabbits for each set of conditions. Four, ~15 week old \(\beta_3 \text{AR}^{-/-}\) male mice and 4 age-matched FVB background wild-type controls were used for measurement of \(\beta_1\) subunit glutathionylation in the myocardium. They were killed by cervical dislocation. The heart was dissected out and frozen immediately. Invasive hemodynamic studies were performed on 4 sheep before and after induction of heart failure. Procedures and anesthesia are described in a separate section below. The institutional review committee for animal research had approved experimental protocols.

Measurement of Na\(^{+}\)-K\(^{+}\) pump activity. We used the whole-cell patch clamp technique to measure \(I_p\) in freshly isolated ventricular myocytes (used on the day of isolation only). Solutions and voltage clamp protocol were designed to minimize non-pump membrane currents. Wide-tipped patch pipettes (4-5\(\mu\)m) were filled with solutions containing (in mmol/L): N-2-hydroxyethyl piperazine-N’-2-ethene-sulphonic acid (HEPES) 5; MgATP 2; ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA) 5; potassium glutamate 70, sodium glutamate 10 and tetramethylammonium chloride and (TMA-Cl) 80. The solutions were titrated to a pH of 7.20 at 35 °C with KOH. Patch pipettes filled with these solutions had resistances of 0.8-1.1 M\(\Omega\). The series resistance after formation of the whole-cell configuration had to be \(\leq 2.8\) M\(\Omega\)\(^3\) for an experiment to be defined as valid.
While we established the whole-cell configuration, myocytes were superfused with solution containing (in mmol/L): NaCl 140; KCl 5.6; CaCl$_2$ 2.16; MgCl$_2$ 1; glucose 10; NaH$_2$PO$_4$ 0.44; HEPES 10. It was titrated to a pH of 7.40 at 35 °C with NaOH. Two to three minutes after establishing the whole cell configuration we switched to a superfusate that was nominally Ca$^{2+}$-free and had 2 mmol/L BaCl$_2$ and 0.2 mmol/L CdCl$_2$ added. When indicated, Na$^+$ containing compounds were replaced with N-Methyl-D-glucamine (NMG.Cl) to eliminate any possible transmembrane Na$^+$ influx that might cause an increase in the intracellular Na$^+$ concentration and secondary pump stimulation.

$I_p$ was identified at a holding potential of -40 mV as the difference between holding current before and after Na$^+$–K$^+$ pump blockade with 100 µmol/L ouabain. Na$^+$-K$^+$ pump inhibition is expected to be saturated when rabbit ventricular myocytes are exposed to ouabain at this concentration. Since Na$^+$-K$^+$ pump currents are small, reliable measurement of $I_p$ depends on stability of membrane currents before and after myocytes are exposed to ouabain. A stable current plateau was identified when no drift could be identified on the digital display of the voltage clamp amplifier for at least 50 s, and the plateaus used to identify $I_p$ were defined by the means of current samples acquired with an electronic cursor at ~5 s intervals, as described. $I_p$ was normalized for membrane capacitance and hence cell size. The effect of ouabain is not reversible within the time frame stable holding currents can be reliably measured. Separate myocytes were therefore used for measurements of $I_p$ for each set of experimental conditions. Recordings were obtained using the continuous single-electrode mode of Axoclamp 2A or 2B amplifiers supported by AxoTape and pCLAMP software (Axon Instruments, Foster City, CA, USA).
**Imaging of intracellular nitric oxide by DAF-2DA**

To detect nitric oxide production we loaded myocytes with the nitric oxide (NO)-sensitive dye DAF-2DA \(^6,7\). Some myocytes were pre-incubated with the nitric oxide synthase inhibitor L-NAME (1 mmol/L) for 15 minutes prior to loading with DAF-2DA (1 µmol/L) for 30 minutes in the dark at 37 °C. They were then exposed to solution containing the β\(_3\) AR agonist BRL (10 nmol/L) or control solutions for 10 minutes prior to fixation in 2% paraformaldehyde on ice for 4 minutes. The myocytes were washed, mounted on poly-L-lysine coated glass slides in **Vectashield**\(^8\) and examined under a laser-scanning confocal microscope (Nikon C1). The excitation wavelength was 488 nm and the emission wavelength 530 nm. The fluorescence images were obtained using constant settings of scanning speed, pinhole diameter and voltage gain. Myocytes representative of each experimental condition were selected randomly for measurement of fluorescent intensity (Photoshop, Adobe). Only myocytes with clear striations and rod-like shape were included in the analysis. The average of each experiment was normalized against its control.

**Immunodetection of S-glutathionylated proteins and protein co-immunoprecipitation in rabbit ventricular myocytes**

To detect S-glutathionylation of specific pump-related proteins, isolated myocytes were loaded with biotinylated GSH (500 µmol/L; 1 hour) \(^8\). Biotinylated GSH ester was made by mixing 25 mmol/L sulfo-NHS-biotin with 25 mmol/L GSH ethyl ester in 50 mmol/L NaHCO\(_3\) at pH 8.5 for 2 h followed by the addition of 125 mmol/L NH\(_4\)HCO\(_3\) at pH 8.5 for 1 h. After incubation in biotinylated GSH ester, cells were washed 3 times with cold phosphate buffer and lysed in buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mmol/L NaCl, 50 µmol/L
diethylenetriaminepentaacetic acid, 2 mmol/L phenylmethylsulfonyl fluoride) containing 10 mmol/L N-ethylmaleimide to block further thiol reactions. The biotin-tag was used to precipitate S-glutathionylated proteins using methods we described previously \(^8\). Equal amount of protein from each experimental condition (approximately 0.5 mg) was mixed with streptavidin-Sepharose beads for 1 h. The beads were washed five times with lysis buffer with 0.1% SDS, and the final precipitate was incubated for 15 min with 40 µl of elution buffer (lysis buffer + 20 mmol/L DTT) to release S-glutathionylated proteins. After adding Laemmlı buffer, GSS-protein pulled down by this technique was separated by gel electrophoresis, transferred to a membrane and probed with antibodies to Na\(^+\)-K\(^+\) pump \(\alpha_1\) and \(\beta_1\) subunits.

Separate experiments were performed using an antibody against glutathionylated protein (anti-GSH antibody) \(^8\). Myocytes were treated with ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 1% Triton X-100, 2 mM EDTA, and protease inhibitors (Complete EGTA-free, Roche Diagnostics). After 5 min at 4°C, the lysate was clarified by centrifugation at 16,000 g for 20 min. The supernatant (0.25–1 mg protein) was pre-cleared and incubated with the appropriate antibody and then with protein A/G plus agarose beads. The proteins bound to the collected beads were subjected to SDS-PAGE and probed with antibodies. This protocol was also used to detect co-immunoprecipitation of \(\alpha_1\) and \(\beta_1\) pump subunits \(^8\). Automatic exposure time was used to ensure densities of individual bands were within the linear range for seconds. In all cases this was in seconds to minutes range.

**Heart failure model and in vivo studies of cardiac contractility**

All procedures on sheep were performed under anesthesia, which was induced by thiopentone (15–20 mg/kg) and maintained with 1.5–2% isoflurane in 40% oxygen using a respirator (model
Expired CO₂ was monitored with a POET II monitor (Criticare Systems; Milwaukee, WI) and maintained at 30–35 mmHg.

We examined the effect of severe chronic ischemic heart failure on the hemodynamic effects of BRL in sheep using continuous LV pressure-volume loops obtained by conductance catheter measurement after brief inferior vena caval occlusion using a 23 ml Fogarty catheter. This procedure enables a rapid, purely mechanical, reduction in preload, which prevents reflex mechanisms and is easily reversed by deflation of the balloon, as previously described ⁹. Global damage to LV function was induced by repeated selective coronary microembolization at two week intervals until ejection fraction had remained stable at <35% over at least 4 weeks as determined by echocardiography. We used the slope of the ESPVR which determines end-systolic elastance as a sensitive and relatively load-independent descriptor of LV systolic function ¹⁰, ¹¹ that reflects intrinsic changes in response to inotropic agents (positive or negative) ¹². The construction of pressure-volume relations from continuous recording of these parameters allows derivation of indices of intrinsic systolic function which are better measures than frequently used alternatives. Details of heart failure induction and instrumentation for measurement of pressure-volume relationships have been described previously ¹¹. High cost of the model and significant morbidity and attrition of animals ¹¹ restricted the studies we could perform.

**Chemicals and Reagents**

TMA.Cl and NMG.Cl were purum grade and were obtained from Fluke Chemicals (Switzerland). All other chemicals used in Tyrodes solutions were analytical grade and were obtained from BDH (Australia). Ouabain, BRL 37344, CL 316243, norepinephrine, nadolol,
prazocin, forskolin, KT5720, L-NAME and DAF-2DA were obtained from Sigma Chemical Co., (St Louis, MO), L-748,337 from Tocris Bioscience, Ellisville, MO, USA, Vectashield® from Vector Laboratories Incorporated (Burlingame, Ca), H-89 and 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) from Calbiochem, La Jolla, CA and Sulfo-NHS-biotin from Pierce Protein Research Products. Protease inhibitors were obtained from Roche Diagnostics (Australia). Antibodies to the Na^+-K^+ pump’s α1 and β1 subunits were from BD Bioscience (North Ryde, NSW, Australia), anti-GSH antibody from Virogen (Watertown, MA, USA) Antibodies for β ARs were from Santa Cruz Biotechnology (Santa Cruz, CA, USA): β1 AR (V-19): sc-568; β2 AR (M-20): sc-570 and β3 AR (M-20): sc-1473.
Supplemental Figures

A. IB: 2nd anti-rabbit

B. IB: GAPDH

Supplemental Figure 1A. Secondary antibody control experiments for Western blot studies of expression of β₁,3 ARs presented in Figure 6A. Myocardial lysate from rat, β₃ AR⁻/⁻ and WT mice (n=4) was obtained and 15 µg of protein loaded in each lane under reducing conditions. After transfer, the membrane was incubated to the appropriate control secondary antibody as shown. B. GAPDH immunoblots demonstrate uniform sample loading. Selected data from the appropriate molecular weight is shown in Figure 6A (lower panel).
Supplemental Figure 2A. Secondary antibody control experiments for Western blot studies of expression of β3 AR in sheep myocardium presented in Figure 7A. Myocardium from rat, wild-type (WT) mice and β3 AR−/− mice was used as additional controls. B. GAPDH immunoblots for the same experiments. Selected data from the appropriate molecular weight is shown in Figure 7A (lower panel).
<table>
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<tr>
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<td><strong>HR</strong></td>
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<td>96 ± 6</td>
<td>94 ± 8</td>
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<td>95.8 ± 7.1</td>
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<td>-1365 ± 99</td>
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<td><strong>CO</strong></td>
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**Normalized**

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<td>1.36 ± 0.20</td>
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HR indicates heart rate (bpm); LVEDP, left ventricular end diastolic pressure (mmHg); PSP, peak systolic pressure (mmHg); SW, stroke work (mmHg.ml/100g.beat); LV dP/dt_{max} and LV dP/dt_{min}, first derivatives of LV pressure rise and decay (mmHg/sec); CO, Cardiac output (L/min). Data are expressed as mean±SEM.

* P<0.05 versus non-failing subjects.
References


