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β3-Adrenoceptor activation relieves oxidative inhibition of the cardiac Na+-K+ pump in hyperglycemia induced by insulin receptor blockade

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β3-Adrenoceptor activation relieves oxidative inhibition of the cardiac Na+-K+ pump in hyperglycemia induced by insulin receptor blockade. Am J Physiol Cell Physiol 309: C286–C295, 2015. First published June 10, 2015; doi:10.1152/ajpcell.00071.2015.—Dysregulated nitric oxide (NO)– and superoxide (O2•-)–dependent signaling contributes to the pathobiology of diabetes-induced cardiovascular complications. We examined if stimulation of β3-adrenergic receptors (β3-ARs), coupled to endothelial NO synthase (eNOS) activation, relieves oxidative inhibition of eNOS and the Na+-K+ pump induced by hyperglycemia. Hyperglycemia was established in male New Zealand White rabbits by infusion of the insulin receptor antagonist S961 for 7 days. Hyperglycemia increased tissue and blood indexes of oxidative stress. It induced glutathionylation of the Na+-K+ pump β1-subunit in cardiac myocytes, an oxidative modification causing pump inhibition, and reduced the electrogenic pump current in voltage-clamped myocytes. Hyperglycemia also increased glutathionylation of eNOS, which causes its uncoupling, and increased coimmunoprecipitation of cytosolic p47phox and membranous p22phox NADPH oxidase subunits, consistent with NADPH oxidase activation. Blocking translocation of p47phox to p22phox with the gp91ds-tat peptide in cardiac myocytes ex vivo abolished the hyperglycemia-induced increase in glutathionylation of the Na+-K+ pump β1-subunit and decrease in pump current. In vivo treatment with the β3-AR agonist CL316243 for 3 days eliminated the increase in indexes of oxidative stress, decreased coimmunoprecipitation of p22phox with p47phox, abolished the hyperglycemia-induced increase in glutathionylation of eNOS and the Na+-K+ pump β1-subunit, and abolished the decrease in pump current. CL316243 also increased coimmunoprecipitation of glutaredoxin-1 with the Na+-K+ pump β1-subunit, which may reflect facilitation of deglutathionylation. In vivo β3-AR activation relieves oxidative inhibition of key cardiac myocyte proteins in hyperglycemia and may be effective in targeting the deleterious cardiac effects of diabetes.

diabetes; hyperglycemia; β3-adrenergic receptors; Na+-K+ pump; endothelial nitric oxide synthase; glutathionylation

While a high prevalence of coronary artery disease predisposes patients with diabetes to ischemic cardiomyopathy, direct effects of diabetes on myocardial structure and function also contribute to an increased risk of heart failure (8, 37). Raised levels of reactive oxygen species (ROS), or “oxidative stress” due to increased activity of NADPH oxidase in cell membranes, contribute to the pathogenesis (28). The NADPH oxidase-derived ROS can quench nitric oxide (NO) and also uncouple endothelial NO synthase (eNOS), switching its function from NO to ROS generation (21). A “nitroso-redox imbalance” (51) then causes redox modifications of cell proteins and cell dysfunction.

The Na+-K+ pump is one of the important membrane proteins susceptible to redox modifications. We have shown that its activity is regulated by glutathionylation of Cys46 on the β1-subunit of the pump αβ-heterodimer (13, 17). Glutathionylation is a reversible oxidative modification whereby a disulfide bond is formed between glutathione (GSH) and protein cysteine residues. It has structural and functional effects on proteins reminiscent of phosphorylation and is involved in physiological and pathophysiological cell signaling (15). As reviewed elsewhere (29), oxidative signaling and down-stream glutathionylation and deglutathionylation of the Na+-K+ pump β1-subunit play important roles in receptor-coupled inhibition and stimulation of the Na+-K+ pump. Within this scheme, glutathionylation and the functional equivalent of pump inhibition are mediated by activation of NADPH oxidase, while receptor-coupled deglutathionylation and pump stimulation involve activation of NOS. This raises the possibility that NADPH oxidase activation in diabetes uncouples eNOS and disturbs the “nitroso-redox balance” (51) to the extent that it may cause the sarcosomal Na+-K+ pump inhibition that can occur with diabetes (24). Na+-K+ pump inhibition is expected to increase cytosolic Na+ levels, and such an increase has adverse effects in heart failure (1, 5, 35, 36). In support of such harmful effects, treatments that are beneficial in heart failure stimulate the Na+-K+ pump and, hence, Na+ export, while treatments that are harmful in clinical trials inhibit the pump, as we summarized previously (29).

The β1-subunit in a proportion of Na+-K+ pumps in cardiac myocytes isolated from normal rabbits is glutathionylated at baseline, and we previously showed that in vitro exposure of the myocytes to β3-adrenergic receptor (β3-AR) agonists decreases glutathionylation and increases electrogenic Na+-K+ pump current (Ip) measured in voltage-clamped myocytes. These effects are NOS-dependent (9). In the present study we examine if in vivo treatment of rabbits with the β3-AR agonist CL316243 (CL) can reverse hyperglycemia-induced pump inhibition through NO- and ROS-dependent signaling. We previously studied the effect of alloxan-induced type 1 diabetes on the Na+-K+ pump in cardiac myocytes (24). However,
nonspecific intrinsic oxidant effects of alloxan (39) are undesirable when NO- and ROS-dependent signaling is examined. In this study we induced hyperglycemia in rabbits by infusion of S961.

S961 and the closely related S661 are synthetic peptides that competitively displace insulin from purified human, rat, and pig insulin receptors with affinities similar to the affinity of insulin itself and with a selectivity for the insulin receptor vs. the insulin-like growth factor 1 receptor that is higher than the selectivity for insulin. In a cellular assay, S961 blocks insulin action with an IC_{50} of 1.3 nM, and in vivo intravenous bolus administration of S661 to rats transiently raises blood glucose nearly fivefold. The increase is reduced or eliminated in a dose-dependent manner by administration of insulin, consistent with competition for binding to the insulin receptor (41). We modified the protocol that induces transient hyperglycemia with bolus administration (41) to a protocol that induces sustained hyperglycemia by administration of S961 via osmotic mini-pumps.¹

**METHODS**

Animals, tissues, and cells. Pharmacology and physiological effects on the heart mediated by the β_3-ARs are highly species-dependent, and since rabbits provide a relevant model for the human receptor (3), we used male New Zealand White rabbits (2.2–2.6 kg body wt). S961 (a gift from Novo Nordisk, Denmark), with the peptide sequence GSLDESFYDWERQLGSSGGLSLEEEWAGIQCEVWGRGCPNS and a disulfide bridge connecting with the peptide sequence GSLDESFYDWERQLGSSGGLSLEEEWAGIQCEVWGRGCPNS and a disulfide bridge connecting the two cysteine residues (41), was infused for 7 days at a rate of 12 g·kg⁻¹·h⁻¹ via osmotic mini-pumps (Alzet, Palo Alto, CA) implanted subcutaneously under general anesthesia. The selective β_3-AR agonist CL (Sigma-Aldrich, St. Louis, MO) was infused via mini-pumps at a rate of 40 μg·kg⁻¹·h⁻¹ with bolus administration (41) to a protocol that induces selective β_3-ARs are highly species-dependent, and since rabbits provide a relevant model for the human receptor (3), we used male New Zealand White rabbits (2.2–2.6 kg body wt). S961 (a gift from Novo Nordisk, Denmark), with the peptide sequence GSLDESFYDWERQLGSSGGLSLEEEWAGIQCEVWGRGCPNS and a disulfide bridge connecting the two cysteine residues (41), was infused for 7 days at a rate of 12 g·kg⁻¹·h⁻¹ via osmotic mini-pumps (Alzet, Palo Alto, CA) implanted subcutaneously under general anesthesia. The selective β_3-AR agonist CL (Sigma-Aldrich, St. Louis, MO) was infused via mini-pumps at a rate of 40 μg·kg⁻¹·h⁻¹ with bolus administration (41) to a protocol that induces selective β_3-AR agonist CL (Sigma-Aldrich, St. Louis, MO) was infused via mini-pumps at a rate of 40 μg·kg⁻¹·h⁻¹ with bolus administration (41) to a protocol that induces sustained hyperglycemia by administration of S961 via osmotic mini-pumps.¹

Fig. 1. Effects of S961 on blood glucose and plasma insulin levels. **A**: blood glucose after subcutaneous injection of S961 at 15 μg·kg⁻¹·h⁻¹ (●), 30 μg·kg⁻¹·h⁻¹ (▲), or 75 μg·kg⁻¹·h⁻¹ (▲); n = 2 for each dose. **B**: blood glucose in a rabbit during subcutaneous infusion of S961 at 12 μg·kg⁻¹·h⁻¹ via osmotic mini-pump. **C**: blood glucose during 7 days of infusion of S961 at 12 μg·kg⁻¹·h⁻¹ via osmotic mini-pump. **D**: blood glucose levels in 7 control rabbits and 8 rabbits infused with S961 at 12 μg·kg⁻¹·h⁻¹ for 7 days in 5 rabbits. **E**: plasma insulin levels before and after infusion of S961 at 12 μg·kg⁻¹·h⁻¹ for 7 days in 5 rabbits. *P < 0.05.

¹ This article is the topic of an Editorial Focus by Stephen M. Black (7a).
NA+-K+ pump β₁-subunit (Upstate Biotechnology) or an anti-eNOS antibody (Sigma-Aldrich) in Western blots. Glutathionylation of the Na+-K+ pump β₁-subunit was also detected by the biotinylated GSH ethyl ester technique in cardiac myocytes. The glutathionylated subfraction in lysate from myocytes that had been loaded with biotinylated GSH (500 μM, 1 h at room temperature) was precipitated with streptavidin-Sepharose beads (GE Healthcare Biosciences), as described elsewhere (13). Coimmunoprecipitation protocols were used to examine the association of proteins using antibodies against the α₁-subunit (Upstate Biotechnology), p47phox and p22phox subunits of NADPH oxidase, and glutaredoxin-1 (Grx1; Santa Cruz Biotechnology).

Measurement of lipid peroxidation in plasma. Plasma lipid peroxidation levels were analyzed by the thiobarbituric acid-reactive substances (TBARS) assay, which reacts with malondialdehyde (MDA), using the OxiSelect TBARS assay kit (Cell Biolabs). Briefly, fresh plasma was mixed with butylated hydroxytoluene to arrest oxidation, mixed with sodium dodecyl sulfate lysis solution, and then incubated with thiobarbituric acid at 95°C for 1 h before centrifugation. The TBARS-MDA adduct was measured by fluorometry in the supernantant by absorption at 530 nm. Lipid peroxidation level was determined from a standard curve.

High-performance liquid chromatography analysis of dihydroethidium oxidation products. High-performance liquid chromatography was used to separate the O₂⁻−-dependent 2-hydroxyethidium (2-OH-E+⁰) product from the nonspecific product ethidium (E⁺) following dihydroethidium oxidation (50) in rabbit aorta. Rabbit aorta was incubated with dihydroethidium (50 μM, 30 min at 37°C) in Krebs buffer containing diethylenetriaminepentaacetic acid (100 μM) in darkness. Aortic segments were lysed in methanol, and after protein precipitation by addition of HClO₄ (200 mM) in methanol at 4°C and centrifugation, the oxidation products in the supernatant (50 μl) were separated by high-performance liquid chromatography (Shimadzu). Products were quantified by fluorescence and electrochemical oxidation, and their mean value was normalized to protein concentration.

Statistical analysis. Values are means ± SE. Student’s t-test was used for comparison between two groups. For multiple comparisons, one-way analysis of variance was used, with Tukey’s post hoc analysis for multiple comparisons. P < 0.05 was considered statistically significant.

RESULTS

Effect of S961 on blood glucose, insulin levels, and oxidative stress. We first examined if transient hyperglycemia induced by a bolus injection of S661 in rats (41) can be reproduced by a bolus injection of S961 in rabbits. We administered S961 subcutaneously at 15, 150, or 300 μg/kg to a rabbit. The two larger doses induced transient hyperglycemia (Fig. 1A). We next examined the effect of continuous subcutaneous infusion of S961. The time course of blood glucose levels in a rabbit infused with S961 at 12 μg·kg⁻¹·h⁻¹ is shown in Fig. 1B. Additional higher and lower rates of infusion were used, and blood glucose levels were sampled daily over the following 7 days. Mean levels sampled in one rabbit for each rate of infusion increased with an increase in the rate of infusion (Fig. 1C).

On the basis of results shown in Fig. 1C, we selected an infusion rate of 12 μg·kg⁻¹·h⁻¹ for the study. The mean blood glucose levels in a number of rabbits treated for 7 days with this rate of infusion compared with levels in controls are shown in Fig. 1D. The S961-induced increase in blood glucose levels was accompanied by an increase in plasma insulin levels (Fig. 1E), consistent with a counterregulatory response to the competitive insulin receptor blockade in vitro studies (41). There were no effects of S961-induced hyperglycemia on serum Na⁺, K⁺, cholesterol, or triglycerides or on indexes of renal function (data not shown). The level of 2-OH-E+ in aorta (Fig. 2A) and the level of MDA in plasma (Fig. 2B) as indexes of oxidative stress were increased with S961-induced hyperglycemia relative to control.

Na⁺-K⁺ pump current and glutathionylation of the Na⁺-K⁺ pump β₁-subunit. Figure 3A illustrates the experimental protocol used to measure Iₚ in myocytes. Traces of currents in myocytes from a control rabbit and a rabbit treated with CL are shown. Iₚ for each myocyte was identified as the mean value of holding currents sampled with an electronic cursor before and after exposure to ouabain. Sampling of currents and criteria for their stability are described elsewhere (46). Iₚ was reduced in myocytes from S961-treated rabbits relative to Iₚ of myocytes from control rabbits. In vivo treatment with CL for 3 days reversed Na⁺-K⁺ pump inhibition (Fig. 3B). CL had no effect on plasma glucose, insulin levels, or heart rate, but it reduced mean arterial pressure in control and S961-treated rabbits (18) and reduced levels of MDA (Fig. 2B).

In a subset of experiments, we omitted L-arginine from patch-pipette solutions used to voltage-clamp myocytes. L-Arginine deficiency uncouples NOS (52), and omission of L-arginine from pipette solutions reduces Iₚ relative to Iₚ in L-arginine-containing solutions (48). There was no significant differ-
Rabbits in each group is indicated within columns. Shaded columns indicate an increase in eNOS in the myocardium from the baseline. The signal for glutathionylation of eNOS was consistent with an increase in glutathionylation of eNOS that was abolished by CL treatment. To independently support a role of oxidative stress (10), and we examined if infusion of S961 is associated with glutathionylation of eNOS. S961 increased glutathionylation of the subunit, as detected by two independent techniques. Treatment with CL reversed the increase by 10.22 ± 0.33.

**Effect of the β3-AR on glutathionylation of eNOS**. Glutathionylation mediates uncoupling of eNOS under conditions of oxidative stress (10), and we examined if infusion of S961 is associated with glutathionylation of eNOS. S961 induced an increase in glutathionylation of eNOS that was abolished by treatment with CL (Fig. 4A). To independently support a role for β3-AR-dependent signaling in glutathionylation of eNOS, we examined glutathionylation of eNOS in the myocardium of mice lacking β3-ARs (β3-AR⁻/⁻). The abundance of eNOS in β3-AR⁻/⁻ mice and their wild-type littermates was similar, but the signal for glutathionylation of eNOS was consistent with an increase in eNOS in the myocardium from the β3-AR⁻/⁻ mice relative to their wild-type littermates (Fig. 4B). Glutathionylation of the myocardial Na⁺-K⁺ pump β1-subunit also is increased in β3-AR⁻/⁻ mice (9), consistent with an effect of glutathionylation-dependent eNOS uncoupling on the Na⁺-K⁺ pump.

**NADPH oxidase and effects of S961 and β3-AR activation with CL**. In view of the role NADPH oxidase can have in uncoupling of eNOS (21), we examined effects of blocking NADPH oxidase on Iₚ and glutathionylation of the Na⁺-K⁺ pump β1-subunit in myocytes isolated from control and S961-treated rabbits. In the functional studies, we blocked translocation of the cytosolic p47phox subunit to the membranous p22phox subunit necessary for NADPH oxidase activity by incubating myocytes with the gp91ds-tat peptide (5 μM) for 1 h at 37°C, and we included the peptide in patch-pipette solutions at the same concentration when we subsequently measured Iₚ in voltage-clamp experiments. The peptide induced a significant increase in Iₚ of myocytes from S961-treated rabbits (Fig. 5A). Exposure to the gp91ds-tat peptide had no effect on glutathionylation of the Na⁺-K⁺ pump β1-subunit in myocytes from the control rabbits but decreased glutathionylation in myocytes from S961-treated rabbits (Fig. 5B), corresponding to the increase in Iₚ (Fig. 5A).

Since results in Fig. 5 suggest that increased constitutive NADPH oxidase activity contributed to the hyperglycemia-
induced Na\textsuperscript{+}-K\textsuperscript{+} pump inhibition, we examined coimmunoprecipitation of the p47\textsuperscript{phox} subunit with the membranous p22\textsuperscript{phox} subunit and, as an index of p47\textsuperscript{phox} subunit translocation to the membrane, coimmunoprecipitation of the p47\textsuperscript{phox} subunit with the Na\textsuperscript{+}-K\textsuperscript{+} pump \(\alpha_1\)-subunit. We determined coimmunoprecipitation in lysate of myocytes from control and S961-treated rabbits. Hyperglycemia had no effect on expression of p22\textsuperscript{phox} and p47\textsuperscript{phox} NADPH oxidase subunits or the Na\textsuperscript{+}-K\textsuperscript{+} pump \(\alpha_1\)-subunit (Fig. 6). However, it increased coimmunoprecipitation of p47\textsuperscript{phox} with p22\textsuperscript{phox} and p47\textsuperscript{phox} with the Na\textsuperscript{+}-K\textsuperscript{+} pump \(\alpha_1\)-subunit. In vivo treatment with CL reversed these changes (Fig. 6).

Effect of \(\beta_3\)-AR activation on coimmunoprecipitation of Grx1 with the Na\textsuperscript{+}-K\textsuperscript{+} pump \(\beta_1\)-subunit. Grx1 mediates deglutathionylation of proteins, and it coimmunoprecipitates with the Na\textsuperscript{+}-K\textsuperscript{+} pump \(\beta_1\)-subunit in cardiac myocytes (7). Addition of recombinant Grx1 to patch-pipette solutions prevents an oxidation-induced decrease in \(I_p\) in voltage-clamped myocytes (13). We examined coimmunoprecipitation of Grx1 with the Na\textsuperscript{+}-K\textsuperscript{+} pump \(\beta_1\)-subunit in lysate of myocytes from control and S961-treated rabbits. S961 induced a decrease in coimmunoprecipitation of Grx1 with the Na\textsuperscript{+}-K\textsuperscript{+} pump \(\beta_1\)-subunit that was reversed by treatment with CL. Treatment of control rabbits with CL also increased coimmunoprecipitation of Grx1 with the Na\textsuperscript{+}-K\textsuperscript{+} pump \(\beta_1\)-subunit (Fig. 7).

DISCUSSION

Animal models of hyperglycemia are usually associated with obesity and elevated triglyceride levels (26). Such elevated levels can influence lipid peroxides detected by the TBARS assay, and it is important that infusion of S961 induced stable hyperglycemia in this study but did not increase serum triglyceride levels relative to levels in control rabbits (18). Although the prevalence of hypertriglyceridemia is increased in type 2 diabetes (40), the pattern of hyperglycemia and normal triglyceride levels induced by S961 is similar to that in type 2 diabetes, because in most patients with type 2 diabetes, triglyceride levels are not elevated. A strong correlation between increased TBARS levels and markers of insulin resistance and plasma glucose in humans (45) suggests that the S961 model of hyperglycemia reproduces the oxidative stress that is a central feature in the pathophysiology of human diabetes. The \(\beta_3\)-AR agonist CL reversed the increase in TBARS levels, but not the hyperglycemia.

Infusion of S961 induced a decrease in \(I_p\) of myocytes studied ex vivo and a corresponding increase in Na\textsuperscript{+}-K\textsuperscript{+} pump \(\beta_1\)-subunit glutathionylation. In vivo treatment with CL decreased Na\textsuperscript{+}-K\textsuperscript{+} pump \(\beta_1\)-subunit glutathionylation and increased \(I_p\) of myocytes isolated from control and S961-treated rabbits. This indicates that the effects of CL on glutathionylation and \(I_p\) in the complex in vivo milieu, even in a disease model, are similar to previously reported effects of exposure of myocytes from normal rabbits to \(\beta_3\)-AR agonists in vitro (9). The effects of in vitro exposure to CL are mediated by a NOS-dependent pathway (9).

While it is firmly established that \(\beta_3\)-AR signaling in the heart is mediated by NOS, the specific isof orm involved has varied between experimental models, as reviewed elsewhere (31). In this study, infusion of S961 was associated with an increase in glutathionylation of eNOS, which is expected to uncouple its function from synthesis of NO to synthesis of \(O_2^-\) (10). This is consistent with the increase in vascular eNOS glutathionylation with streptozocin-induced hyperglycemia in rats reported previously (42). Our finding in the present study of no effect of S961 when \(I_p\) was measured ex vivo using \(L\)-arginine-free patch-pipette solutions provides functional support for the role of NOS uncoupling in effects of S961-induced hyperglycemia on the Na\textsuperscript{+}-K\textsuperscript{+} pump, because with use of the wide-tipped patch pipettes needed for effective perfusion of the intracellular compartment and accurate measurements of \(I_p\) (19), relatively low-molecular-weight intracellular substances, such as \(L\)-arginine, are expected to be markedly depleted. This facilitates uncoupling of NOS and, as observed experimentally, is expected to abolish the difference in \(I_p\) between myocytes from control and diabetic rabbits that is due to uncoupling of NOS developed in vivo. The parallel effect of treatment with CL to reverse the S961-induced increase in eNOS glutathionylation and the decrease in \(I_p\) sug-
gests that glutathionylation of the eNOS isoform and Na⁺/H⁺ pump inhibition are interrelated.

Blocking translocation of the cytosolic p47phox subunit to the membranous p22phox subunit with the gp91ds-tat peptide reversed the decrease in \( I_p \) and the increase in glutathionylation of the Na⁺/H⁺ pump \( \beta_1 \)-subunit in myocytes isolated from rabbits treated with S961. However, the peptide had no significant effect on \( I_p \) or \( \beta_1 \)-subunit glutathionylation in myocytes from control rabbits. The lack of significant effect of gp91ds-tat on \( I_p \) in control myocytes, although shown here in only a small number of myocytes, is consistent with the same result in a previous report (47). S961 also increased coimmunoprecipitation of p47phox and p22phox NADPH oxidase subunits and the Na⁺/K⁺ pump α1-subunit in total myocyte lysates. α-Tubulin was used as loading control. Top right: p47phox, p22phox and the Na⁺/K⁺ pump α1-subunit immunoblot of p47phox immunoprecipitate from myocyte protein. Bottom: mean densitometry for coimmunoprecipitation (Co-IP) experiments from 5 rabbits in each group. *\( P < 0.05 \).
tation of the p47<sub>phox</sub> and p22<sub>phox</sub> subunits in myocyte lysate. These results strongly implicate an increased activity of NADPH oxidase in the effects of S961-induced hyperglycemia and are consistent with the increased myocardial oxidative stress shown to occur in streptozotocin-induced diabetes (42). NADPH oxidase is also a major source of diabetes-induced oxidative stress in vascular tissue in experimental animal models (20), as well as in humans (22), suggesting that effects of hyperglycemia on the Na<sup>+</sup>-K<sup>+</sup> pump reported here for the heart may be similar for blood vessels.

NADPH oxidase-derived ROS can uncouple eNOS, and, conversely, activation of NO production can downregulate the NOX2 isoform of NADPH oxidase (28). Cross talk between NOS and NADPH oxidase activity may contribute to the hyperglycemia-induced oxidative Na<sup>+</sup>-K<sup>+</sup> pump inhibition in the present study. Reversal of the uncoupling is heat shock protein 90 (Hsp90)-dependent (23, 43), and we previously showed that receptor-coupled, NOS-dependent stimulation of I<sub>p</sub> in vitro in cardiac myocytes, including stimulation mediated by the β<sub>3</sub>-AR, is blocked by the Hsp90 inhibitor radicicol, implicating Hsp90 in the mechanism for stimulation (9, 49). Recoupling of eNOS function by in vivo treatment with CL in this study would have reduced the role of uncoupled eNOS as a source of ROS, but it may also have inhibited NADPH oxidase-dependent ROS synthesis, because we have found that activation of the canonical NO-dependent signaling pathway

### Fig. 7. In vivo β<sub>3</sub>-AR activation and association of Grx1 with the Na<sup>+</sup>-K<sup>+</sup> pump in hyperglycemia. Top left: immunoblots of the Na<sup>+</sup>-K<sup>+</sup> pump β<sub>1</sub>-subunit and glutaredoxin-1 (Grx1) in total myocyte lysates. α-Tubulin was used as loading control. Top right: Na<sup>+</sup>-K<sup>+</sup> pump β<sub>1</sub>-subunit and Grx1 immunoblot of Na<sup>+</sup>-K<sup>+</sup> pump β<sub>1</sub>-subunit immunoprecipitate from myocyte protein. Bottom: densitometry for coimmunoprecipitation experiments from 5 rabbits in each group. *P < 0.05.

### Fig. 8. Proposed effects of S961-induced hyperglycemia and β<sub>3</sub>-AR stimulation on redox regulation of the Na<sup>+</sup>-K<sup>+</sup> pump. A: hyperglycemia induces oxidative stress by activating NADPH oxidase. This in turn causes glutathionylation and uncoupling of eNOS, and the uncoupled eNOS generates O<sub>2</sub><sup>-</sup>, rather than nitric oxide (NO). Elevated O<sub>2</sub><sup>-</sup> promotes glutathionylation of the Na<sup>+</sup>-K<sup>+</sup> pump β<sub>1</sub>-subunit and pump inhibition. B: activation of the β<sub>3</sub>-AR recouples eNOS, likely by promoting its association with heat shock protein 90 (Hsp90), and NO synthesized by eNOS suppresses hyperglycemia-induced NADPH oxidase activation via the canonical soluble guanylyl cyclase (sGC)-dependent pathway in steps that include dephosphorylation of the p47<sub>phox</sub> subunit and its dissociation from the membranous subunit (dashed line). These changes reduce the forward reaction rate for β<sub>1</sub>-GSS formation and restore Na<sup>+</sup>-K<sup>+</sup> pump function.
with the “soluble” guanylyl cyclase stimulator 3-(5’-hydroxymethyl-2’-furyl)-1-benzylindazole (widely known as YC-1) reverses NADPH oxidase-derived oxidative stress and oxidative pump inhibition in cardiac myocytes (11). Figure 8 provides a schematic summary of the effects of S961-induced hyperglycemia and CL on redox pathways and the Na\(^{+}\)-K\(^{+}\) pump in cardiac myocytes.

Administration of S961 was associated with a decrease in coimmunoprecipitation of Grx1 with the Na\(^{+}\)-K\(^{+}\) pump \(\beta_1\)-subunit, while treatment with CL increased coimmunoprecipitation in myocyte lysate with or without prior administration of S961. Activation of the cytosolic Grx1 is poorly understood but is believed to involve its translocation to target proteins (16). The level of coimmunoprecipitation of Grx1 with the Na\(^{+}\)-K\(^{+}\) pump \(\beta_1\)-subunit in the different treatment groups was in the opposite direction of effects of the treatments on glutathionylation of the Na\(^{+}\)-K\(^{+}\) pump \(\beta_1\)-subunit, indicating that abundance of target disulfide bonds is an unlikely primary determinant of translocation of Grx1. Grx1 itself has highly reactive cysteine residues at its active site and is inhibited under conditions of oxidative stress (16). In vitro studies have suggested that the major role of Grx1 is to reverse effects of oxidative stress after the stress has subsided, rather than limit effects during the oxidative stress (34). The coimmunoprecipitation patterns in this study are consistent with, but do not prove, that a similar scheme operates in vivo.

While it had been a widely held early view that the \(\beta_3\)-AR mediates harmful effects in heart failure, indirect evidence from human clinical data suggests that receptor activation might actually be useful, as reviewed elsewhere (38). Such indirect evidence includes the observation that use of thiazolidinediones (TZDs) to treat type 2 diabetes increases the risk of developing heart failure (27, 44) and that TZDs in low, clinically relevant concentrations rapidly downregulate the \(\beta_3\)-AR in various cell types maintained in culture (4). A similar downregulation in vivo would reduce \(\beta_3\)-AR-mediated signaling activated by endogenous catecholamines and perhaps contribute to TZD-induced cardiac dysfunction. This, in combination with the effect of CL to reverse effects of S961-induced hyperglycemia on glutathionylation-induced Na\(^{+}\)-K\(^{+}\) pump inhibition in the present study, suggests that \(\beta_3\)-AR agonists might be useful in prevention and treatment of heart failure in diabetes.

Recent experimental evidence supports the efficacy of \(\beta_3\)-AR agonists in heart failure. After transverse aortic constriction, mice that genetically lack the receptor have improved survival compared with wild-type mice (30). Stress, exacerbation of pathological remodeling, and an enhanced NOS uncoupling, increased myocardial oxidative stress, exacerbation of pathological remodeling, and an enhanced NOS uncoupling, increased myocardial oxidative stress, exacerbation of pathological remodeling, and an enhanced NOS uncoupling, increased myocardial oxidative stress, exacerbation of pathological remodeling, and an enhanced NOS uncoupling.

Overexpression of the \(\beta_3\)-AR in heart failure (32) may be a useful compensatory mechanism, as suggested by the recent studies on heart failure in animal models. Overexpression of the receptor, as indicated by studies on the diabetic rat heart (2), might have a similar protective role, and we suggest that early-phase human studies examining the effect of selective \(\beta_3\)-AR agonists on cardiac dysfunction in diabetes might be justified by the common and serious long-term complication of overt heart failure. A clinically effective selective \(\beta_3\)-AR agonist, mirabegron, is in use for the treatment of overactive bladder syndrome, and other compounds are being developed.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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