Glycogen Synthase Kinase-3β Inhibition Ameliorates Cardiac Parasympathetic Dysfunction in Type 1 Diabetic Akita Mice

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ABSTRACT

Decreased heart rate variability (HRV) is a major risk factor for sudden death and cardiovascular disease. We previously demonstrated that parasympathetic dysfunction in the heart of Akita type I diabetic mice was due to a decrease in the level of the Sterol Response Element Binding-Protein (SREBP-1). Here we demonstrate that hyperactivity of GSK3β in the atrium of the Akita mouse results in decreased SREBP-1, attenuation of parasympathetic modulation of heart rate, measured as a decrease in the high frequency (HF) fraction of HRV in the presence of propranolol, and a decrease in expression of the GIRK4 subunit of I_{K_Ach}, the ion channel which mediates the heart rate response to parasympathetic stimulation. Treatment of atrial myocytes with the GSK3β inhibitor Kenpaullone increased levels of SREBP-1 and expression of GIRK4 and I_{K_Ach}, while a dominant active-GSK3β mutant decreased SREBP-1 and GIRK4 expression. In Akita mice treated with GSK3β inhibitors Li⁺ and/or CHIR-99021, Li⁺ increased I_{K_Ach}, and both Li⁺ and CHIR-99021 partially reversed the decrease in HF fraction while increasing GIRK4 and SREBP-1 expression. These data support the conclusion that increased GSK3β activity in the type I diabetic heart plays a critical role in parasympathetic dysfunction via an effect on SREBP-1, supporting GSK3β as a new therapeutic target for Diabetic Autonomic Neuropathy.
Diabetic Autonomic Neuropathy (DAN) is a major complication of diabetes mellitus and has been associated with a marked increase in the incidence of sudden death in diabetics (1; 2). Risk factors for sudden death include clinical manifestations of parasympathetic dysfunction, such as a decreased high frequency (HF) component of heart rate variability (HRV) and increased dispersion of QT intervals (2-4). Fifty percent of patients with diabetes for 10 years or more have an impaired response of the heart to parasympathetic stimulation, characterized by a reduction in the HF component of HRV (5). Studies of type 1 diabetics who die suddenly in their sleep, “dead in bed syndrome”, suggested that HRV analysis of diabetic patients who lack clinical evidence of autonomic neuropathy often demonstrate decreased parasympathetic tone (6). Hence, decreased HRV is an important risk factor for arrhythmia and sudden death in diabetics.

Parasympathetic modulation of heart rate is mediated via acetylcholine binding to M2 muscarinic receptors released in response to vagal stimulation resulting in hyperpolarization of the myocyte membrane and prolonged diastolic depolarization via the activation of inward rectifying K+ channels (I\textsubscript{K\textsubscript{Ach}}) located primarily in the atria. I\textsubscript{K\textsubscript{Ach}} is a heterotetrameric G-Protein Coupled Inward Rectifying K+ Channel (GIRK) composed of (GIRK1)\textsubscript{2}/(GIRK4)\textsubscript{2} subunits, activated in response to the binding of the βγ-subunit of the heterotrimeric G-protein, G\textsubscript{i2}, which is released following the binding of acetylcholine to the M2 muscarinic receptor (7; 8). The GIRK4 subunit is essential for the formation of functional channels (9), and may regulate the expression of GIRK1 while protecting GIRK1 from proteolytic degradation. Thomas et al. demonstrated that treatment of chick embryonic atrial myocytes with muscarinic agonists decreased levels of GIRK1 and GIRK4 proteins and mRNAs (10). RFamide-related peptides have been shown to induce an outward current in Xenopus oocytes that was dependent on the expression of GIRK1 and GIRK4 and associated with pain in the rat (11). Most interestingly,
chronic atrial fibrillation in humans has been associated with the downregulation of GIRK4, I\textsubscript{K\textsubscript{Ach}} and decreased muscarinic receptor mediated shortening of the action potential duration (12). However, none of these studies directly addressed the mechanism of regulation of GIRK4 expression.

Sterol regulatory element binding proteins (SREBPs) are lipid sensitive transcription factors which regulate the expression of enzymes involved in cholesterol metabolism, fatty acid synthesis and glycolysis (13-15). We have demonstrated that SREBP-1 up-regulates both the expression of G\textsubscript{a12} and GIRK1 in atrial myocytes and the negative chronotropic response of the heart to the acetylcholine analogue carbamylcholine (16; 17).

The Akita type 1 diabetic mouse is characterized by a point mutation in the pro-insulin \textit{ins2} \textit{(Ins2\textsuperscript{Cys96Tyr})} gene which interferes with insulin processing resulting in the destruction of pancreatic β cells and the development of the diabetic phenotype (18) and secondary effects of diabetes (19). Recently, we have demonstrated that the hypoinsulinemia in the Akita mouse is associated with a decrease in the response of heart rate to carbamylcholine and decreased expression of SREBP-1 and GIRK1 (20), supporting the conclusion that insulin might regulate the parasympathetic response and I\textsubscript{K\textsubscript{Ach}} via the control of SREBP-1.

Glycogen synthase kinase (GSK) 3β is a serine/threonine kinase originally identified as an enzyme that phosphorylates and down regulates glycogen synthase (21). GSK3β is highly active in the basal state and inactivated by phosphorylation at the regulatory Ser9 residue in response to insulin stimulation of the IR (insulin receptor)/IRSs (insulin receptor substrate)/PI3 kinase (PI3K)/Akt cascade (22) (outlined in Fig. 1A). Insulin deficiency results in decreased phosphorylation and hyperactivity of GSK3β and has been implicated in diabetic nephropathy and/or retinopathy (23). Using inhibitors of specific steps in the insulin cascade as well as
inhibitors of GSK3β activity, the goals of this study are to determine whether increased GSK3β activity in the Akita mouse heart plays a unique role in regulating the autonomic response of the heart via the regulation of SREBP-1 (Fig. 1A) and hence might serve as a new therapeutic target for the treatment of DAN.

RESEARCH DESIGN AND METHODS

Materials and animals

Akita type 1 diabetic mice (C57BL/6-Ins2\textsuperscript{Akita/J}) were obtained from the Jackson laboratories. Ad-GFP-DN-SREBP-1 was a gift from Dr. Bruce Spiegelman, Dana Farber Cancer Institute and Harvard Medical School, Boston. Ad-GFP-βgal was a kind gift from Dr. Anthony Rosenzweig, Beth Israel Deaconess Hospital and Harvard Medical School, Boston. The dominant active (DA)-GSK3β (S9A)-expressing adenovirus was kindly provided by Dr. Thomas Force, Jefferson Heart Institute, Philadelphia, PA. The GIRK4 specific antibody for mouse was from Santa Cruz and a custom antibody generated by Chi Scientific (Maynard, MA). The peptide antigen was KKPRQRYMEKSGKC from the N-terminal region (34~47) of chick GIRK4 (NCBI accession #: AAB95313). SREBP-1 and β-actin antibody were from Santa Cruz. β-catenin, p-Akt (Ser473), Akt, p-GSK3β (Ser9) and GSK3β were from Cell Signaling Technology, Beverly, MA. The PI3K inhibitor LY294002 and the GSK3β inhibitor Kenpaullone were from Calbiochem. CHIR-99021 was a kind gift from the Broad Institute, Cambridge, MA. The heterozygous male diabetic Akita \textit{Ins2}\textsuperscript{Cys96Tyr} mice and littermate wild type mice were from The Jackson Laboratory, Bar Harbor, ME. To monitor the progression of disease, measurements of urine glucose, protein and ketones were made with Keto-Diastix Reagent Strips for Urinalysis (Bayer). Glucose was monitored using an Accu-Chek glucometer. Body weight and serum glucose levels are
summarized in Supplementary Table S1. Akita mice demonstrated both decreased body weight and marked hyperglycemia compared to WT. Insulin pellets releasing 0.1 units/implant/day, or placebo pellets (Linshin, Canada), were implanted subcutaneously. Serum glucose was monitored daily until stabilized, 10 days, and the number of pellets adjusted to maintain blood glucose levels at 100-150 mg/dL, as described previously (20). For Li\textsuperscript{+} experiments mice were fed a normal chow diet or a diet supplemented with 0.2% LiCl (Harlan Laboratories) for 7 days. Serum levels of Li\textsuperscript{+} on this diet have been shown to approximate those in patients on Li\textsuperscript{+} therapy (24). All vertebrate animal-related procedures described here were approved by the Tufts Medical Center Institutional Animal Care Committee.

**ECG monitoring, heart rate and HRV analysis in conscious, unrestrained mice**

Anesthesia was induced with inhaled 1.5% isoflurane in oxygen. An ECG signal wireless radiofrequency transmitter was implanted in a subcutaneous pocket and electrodes sutured over the right pectoralis muscle and the lower left ribs in WT and Akita mice. The data were recorded at a sample rate of 5000 Hz with the use of a telemetry receiver and an analog-to-digital acquisition system (Data Sciences International). The ECG signal was analyzed using custom built software: Beat-to-beat heart rate data were computed, artifacts and non-sinus rhythms were removed after manual review. Composite heart rate plots and average heart rates and duration of bradycardia were computed as described previously (17). For HRV analysis, R-wave detection and beat annotation were both manually reviewed as above. All ectopic and post ectopic beats and artifacts were removed and replaced with intervals interpolated from adjacent normal beats, discarding segments where gaps accounted for over 15% of the recording segment. Frequency-domain analysis was performed after construction of an instantaneous RR-interval time series by
resampling at 10 Hz. The power spectra of detrended two-minute segments were computed for the frequency ranges of 0.5 to 1.5 Hz, designated as low-frequency power (LF), and 1.5 to 5 Hz, designated as high-frequency power (HF) as described previously (25; 26). HF fraction was computed as $\frac{HF}{(LF + HF)}$. The HF power has been shown to result predominantly from parasympathetic modulation of heart rate, while LF power has been shown to result from both sympathetic and parasympathetic modulation of heart rate (27). In order to minimize the effects of activity of the mice, we chose segments where heart rate and frequency domain parameters were relatively stationary, verified by employing Kalman-smoothing and wavelet-based visualization in addition to FFT-derived spectrograms and where noise due to mouse movement and the associated muscle activity and changes in entropy were minimal. We have developed a unique method for assessing parasympathetic modulation of heart rate in our mouse model, since we found the usual approach of administering atropine to be difficult in the presence of high baseline heart rates. In order to observe the parasympathetic influence on heart rate, we inhibited the sympathetic modulation of heart rate. Specifically, given that $\beta$-adrenergic receptor inhibition blocks the sympathetic component of HRV, leaving the parasympathetic component relatively unopposed, we injected mice with the $\beta$-adrenergic receptor blocker propranolol and computed the time course of the increase in HF fraction. Composite plots of HF fraction were computed from FFT power spectra over a three-minute sliding window of RR-interval data, repeated every 10 seconds and averaged to one HF fraction data point per minute per group (± SEM). Data outlined in Fig. 1B demonstrate that in response to propranolol, HF fraction increases with a time course similar to that for the decrease in LF power. For statistical comparisons between the groups, heart rate and frequency domain HRV parameters were computed for two-minute segments at the end of the baseline and propranolol phases. HF fraction increased from a mean
of 39.65±1.8% to 59.3±5.6% (n=13, \( P = 0.008 \), Fig. 1C), LF power decreased from 4.58±1.01 to 2.49±1.17 \( 10^{-6} \) ms\(^2\)/Hz, \( P = 0.074 \), while HF power was relatively unchanged 2.17±0.57 and 1.81±0.70 \( 10^{-6} \) ms\(^2\)/Hz, respectively, \( P = 0.431 \) (data not shown). These findings support the conclusion that the increase in HF fraction in response to propranolol is due primarily to a decrease in LF power. Absolute values for HF and LF for each study are given in Supplemental Table S3. Due to the large variability of the absolute values of HF and LF between individual mice and treatment groups, we used the normalized HF parameter computed as HF fraction = HF/(HF+LF) (27). Hence, the HF fraction measurements after administration of propranolol reflect the parasympathetic component of heart rate variability in the presence of sympathetic blockade. In these studies we compare differences in HF fraction after propranolol to assess parasympathetic dysfunction in a mouse model for type I diabetes.

**Cell culture, adult mouse atrial myocytes**

Atrial myocytes from chick embryos 14 days *in ovo* were prepared by a modification of the method of DeHaan (28) as described previously (29). Dissociated atrial myocytes from mouse atria were prepared by a retrograde Langendorff perfusion method as described (20) with some modifications. Cells were rod shaped with clearly defined striations.

**Adenoviral infection**

On the second culture day, cells had reached about 70% confluence and were infected with adenovirus at the indicated MOI. Following 2 days in culture, cells were harvested and whole cell extracts were used for western blot analysis, as outlined below.
Western blot analysis

To determine levels of gene expression in cultured atrial myocytes, cells were harvested and Western blot analysis carried out as described previously (17; 30). Western blot analysis of expression of proteins in atria of wild type and Akita diabetic mice was carried out on atrial homogenates. Protein concentration was determined by Bradford reagent (Bio-Rad). Each sample represents tissue from atria of a single mouse.

Cellular electrophysiology

Membrane currents were measured by the patch-clamp technique in whole-cell mode using an LM-EPC7 amplifier as described (20). In order to obtain and maintain good seal formation required for membrane current recording, we found it necessary to suppress contraction with high external K\(^+\) and 0 external Ca\(^{2+}\), which leads to persistent membrane depolarization and inactivation of voltage-activated Na\(^+\) channels. Both of these conditions have been shown to have no effect on I\(_{KAC}\) (17). Whole cell currents were elicited at room temperature in the presence and absence of 10 µM carbamylcholine introduced by focal perfusion over 10-15 seconds followed by washout. Currents returned to baseline within 10-15 seconds of washout. Currents were normalized to the cell capacitance determined via capacitance compensation and data presented as current density in pA/pF. Current-voltage (I-V) plots were constructed from a series of data points obtained from the carbamylcholine current responses at given voltages.

Echocardiography

Echocardiographic studies were performed as previously described (17). Briefly, a commercially available echocardiography system (Sonos 7500, Phillips Medical Systems) was utilized with a dynamically focused linear array transducer (15-6L Intraoperative Linear Array, Phillips Medical Systems) using a depth setting of 0.5–1.0 cm. Anesthesia was induced with inhaled 1.5%
isoflurane in oxygen and maintained with inhaled 1.0% isoflurane in oxygen. Animals were placed on a warming pad to maintain body temperature at 36.5 to 37.5° C.

Statistics

All values are expressed as mean ± SEM Statistical differences between mean values were calculated by independent or pairwise Student's t-test as appropriate. Normal distribution assumptions were verified using the Shapiro-Wilk test. A P value < 0.05 was considered significant.

RESULTS

Decreased heart rate variability in the type 1 diabetic Akita mouse

We previously reported that the negative chronotropic response to carbamylcholine in Akita mice was markedly blunted compared to wild type (WT) and that these effects were reversed by insulin treatment (20). In order to establish the clinical significance of these findings and their relevance as a cardiovascular risk factor, we determined whether the parasympathetic modulation of heart rate, was also decreased in the type 1 diabetic Akita mouse, compared to WT. Given that β-adrenergic receptor inhibition blocks the sympathetic component of HRV, leaving the parasympathetic component relatively unopposed, we compared the time course of the increase in HF fraction after the injection of the β-adrenergic receptor blocker propranolol in Akita and WT mice (described in Methods and Fig. 1B). The composite plot of HF fraction in WT mice increased continually over the time period studied, while HF fraction in the Akita mouse reached a plateau 10 minutes after propranolol injection (Fig. 2A). Fifteen minutes following propranolol injection, the 2-minute mean of HF fraction was significantly higher in WT mice, 70.9 ± 4.8%, compared to 48.6 ± 5.2% in Akita mice (n=10, P=0.005, Fig. 2B). For further validation of these
findings, we treated WT and Akita mice with atropine, since the increase in heart rate in response to muscarinic blockade by atropine also reflects the level of parasympathetic stimulation of the heart. Heart rate was measured over three minutes both before and after injection with atropine (0.5 mg/kg). Mean heart rate in WT mice increased by 185.1±25.9 beats per minute (bpm) and by 98.1±7.0 bpm in Akita mice (n=9, \( P=0.01 \), Fig. 2C), consistent with the increase in HF fraction after propranolol. Furthermore, in order to determine whether the observed differences in HF fraction between Akita and WT mice might be influenced by differences in sympathetic input to the heart, we compared the decrease in WT and Akita mice heart rate over 15 minutes following propranolol injection. Heart rate decreased by 65.8±22.5 bpm in WT mice and 79.1±22.6 bpm in Akita mice (n=10, \( P=0.628 \), Supplemental Table S4). Finally, baseline HF fraction measured prior to propranolol injection was not significantly different in the two groups; 34.5±2.3% vs. 37.2±2.4%, respectively, n=10, ns (data not shown).

To determine whether the abnormality of HRV in the Akita mouse was due to hypoinsulinemia, we compared the time course of the effect of propranolol injection on HF fraction before and after implantation of slow-release insulin pellets. Following insulin treatment, HF fraction increased over time with a brief plateau 8 min after propranolol injection similar to that seen in Akita mice prior to insulin treatment. However, unlike untreated Akita mice, after reaching this plateau, HF fraction in insulin treated mice continued to increase over time (Fig. 2D). At 15 minutes following propranolol injection, the 2 minute average HF fraction was significantly higher; 75.2±4.6% post-insulin compared to 51.5±3.0% pre-insulin (\( P=0.004 \), n=8) (Fig. 2E). There was no significant difference in the decrease in mean heart rate in response to propranolol in Akita mice before and after insulin treatment consistent with the conclusion that insulin had no effect on the sympathetic response of the heart (see Supplemental Table S4). Baseline HF fraction was not significantly different before and after insulin treatment (38.6±4.0% vs.
42.0±3.4%, n=8, ns; data not shown). These data are consistent with the conclusion that parasympathetic modulation of heart rate in the Akita mouse was impaired secondary to hypoinsulinemia.

**Decreased HRV in Akita diabetic mice is associated with decreased expression of GIRK4 and decreased insulin signaling.**

Although GIRK4 expression in the atrium has been shown to be altered in the presence of AF (12), mechanisms of regulation of GIRK4 expression and their role in parasympathetic signaling have not been studied. To determine whether decreased expression of GIRK4 might be associated with the decreased HRV in the Akita mouse, we compared levels of GIRK4 expression in the atria of WT and Akita mice. Data in Fig. 3A demonstrate that GIRK4 expression in atria of Akita mice was decreased 0.54±0.07 fold compared to WT (n=14, P=0.00002). To determine if this effect were due to decreased insulin levels in the Akita mouse, Akita mice were treated for 10 days with either placebo or slow release insulin pellets. After 2 days, glucose levels in insulin treated mice reached those in WT mice (Supplemental Table S1). Data summarized in Fig. 3B demonstrate that GIRK4 expression in atria of placebo treated Akita mice was 0.46±0.07 fold lower than in WT mice (1.00±0.07 fold, n=5, P=0.0003). Insulin treatment resulted in an increase from 0.46±0.07 to 0.94±0.05 fold (n=6, P=0.0002). To identify downstream kinases in the insulin signaling pathway which might play a role in the regulation of GIRK4 expression and the associated decrease in HRV and heart rate response in Akita mice (see pathway outlined in Fig. 1A), the levels of p-Akt and p-GSK3β in atria of WT and placebo treated Akita mice were compared. p-Akt and p-GSK3β were decreased 0.62±0.06 (n=11, P=0.004) and 0.38±0.06 fold (n=8, P=0.0003), respectively, in the atria of Akita mice compared
to levels in atria of WT mice (1.0±0.11, n=8; Fig. 3C and D). Insulin treatment stimulated p-Akt and p-GSK3β levels from 0.62±0.06 to 1.95±0.38 fold (P=0.005) and from 0.38±0.06 to 0.68±0.08 fold (P=0.007), respectively.

**Insulin regulates the expression of GIRK4 via PI3K/Akt.**

Since it was not possible to obtain mouse atrial myocytes in sufficient quantities for western blot analysis, cultured embryonic chick atrial myocytes were used to determine the role of insulin signaling in the control of GIRK4 expression. Insulin treatment of embryonic atrial myocytes increased GIRK4 protein level 1.72±0.19 fold (n=12, P=0.003) compared to control in parallel with increased phosphorylation of both Akt and GSK3β (Fig. 4A and B) consistent with the effects of insulin treatment on GIRK4 in atria of Akita mice (Fig. 3). Treatment of atrial myocytes with the PI3K inhibitor LY294002 reversed insulin stimulation of GIRK4 expression and inhibited insulin stimulation of p-Akt and p-GSK3β (Fig. 4A and B) consistent with the conclusion that insulin regulation of the expression of GIRK4 is dependent on the activation of the PI3K/Akt/GSK3β pathway (Fig. 1A).

**Insulin regulation of GIRK4 in atrial myocytes is dependent on SREBP-1.**

We have previously demonstrated that parasympathetic dysfunction in the type 1 diabetic Akita mouse is associated with an insulin dependent decrease in levels of SREBP-1 in the atrium of the Akita mouse (20). To determine whether SREBP-1 played a role in insulin regulation of GIRK4 expression, we used cultured chick atrial myocytes. First we determined the role of PI3K on levels of SREBP-1 and the role of SREBP-1 in GIRK4 expression. Insulin treatment of chick atrial myocytes increased expression of the 60-kDa nuclear form of SREBP-1 (nSREBP-1)
1.65±0.11 fold (n=6, \( P=0.0003 \)), compared to control. The PI3K inhibitor LY294002 completely reversed this effect (Fig. 4A and B), consistent with the conclusion that insulin regulation of SREBP-1 is dependent on the activation of PI3K. In order to determine whether the PI3K dependent increase in nSREBP-1 in response to insulin might play a role in the insulin regulation of GIRK4 expression demonstrated in Fig. 3B and 4A and B, chick atrial myocytes were infected with an adenovirus expressing either GFP (Ad-GFP) or DN-SREBP-1 (Ad-GFP-DN-SREBP-1) followed by incubation for 16 hours with insulin. Fluorescence microscopy demonstrated that GFP was expressed in 90% of atrial myocytes infected with Ad-GFP (17; 20). Insulin treatment of cells infected with Ad-GFP increased GIRK4 expression 1.52±0.13 fold (n=8, \( P=0.006 \)) compared to control cells. Insulin treatment of cells infected with Ad-GFP-DN-SREBP-1 resulted in a decreased GIRK4 expression, 0.75±0.11 fold compared to cells treated with Ad-GFP (n=8, \( P=0.006 \)). (Fig. 4C and D). Taken together, these data support the conclusion that insulin stimulation of GIRK4 expression in atrial myocytes is dependent on SREBP-1 via the PI3K/Akt pathway.

**GSK3β regulates the level of SREBP-1 and the expression of GIRK4.**

Data presented in Fig. 3C and D demonstrated that compared with WT, pGSK3β was markedly decreased in the atrium of Akita mice and that this effect was reversed by insulin. Hence GSK3β activity is markedly increased in atria of Akita mice. Furthermore, insulin stimulation of GIRK4 expression and nSREBP-1 levels in chick atrial myocytes was associated with a marked increase in pGSK3β (Fig. 4A and B) which was inhibited by LY294002. Given that insulin inactivates GSK3β via a PI3K/Akt dependent pathway, we used chick atrial myocytes to determine the effect of inhibition of GSK3β on insulin regulation of SREBP-1 levels and GIRK4 expression.
Cells were treated with increasing doses of Kenpaullone, a competitive GSK3β inhibitor which binds to the ATP binding site of GSK3β (31). Incubation with Kenpaullone for 24 hours increased the nuclear form of SREBP-1 and the expression of GIRK4 in a dose dependent manner (Fig. 5A and B) at concentrations of Kenpaullone which are selective for GSK3β inhibition (31). Compared to control, Kenpaullone increased GIRK4 1.69±0.18 fold (n=5, P=0.018) at 2 µM and 3.09±0.35 fold (n=7, P=0.011) at 5 µM while nSREBP-1 increased 3.21±0.58 fold (n=6, P=0.033) at 5 µM Kenpaullone. Furthermore, Kenpaullone increased levels of β-catenin in a dose dependent manner consistent with its inhibitory effect on GSK3β activity (Fig. 5A). Given that Kenpaullone inhibits both GSK3α and GSK3β, we determined the effect of adenoviral expression of an HA-tagged dominant active (DA) GSK3β (S9A) mutant on GIRK4 and SREBP-1 levels in atrial myocytes (32). Western blot analysis demonstrated that in chick atrial myocytes infected with HA-DA-GSK3β, GSK3β migrated as a doublet consistent with expression of the more slowly migrating HA-tagged form. Overexpression of DA-GSK3β decreased the levels of nSREBP-1, GIRK4, and β-catenin expression in a dose-dependent manner (Fig. 5C, D). These results suggest that GSK3β is a negative regulator for SREBP-1 and GIRK4 and are consistent with the hypothesis that increased GSK3β activity due to decreased insulin levels might result in decreased SREBP-1 dependent GIRK4 expression.

**Overexpression of a constitutively activated Akt (myristoylated Akt) in atrial myocytes mimics the effect of insulin on GSK3β, nSREBP-1 and GIRK4.**

To further delineate the role of the PI3K/Akt/GSK3β pathway in insulin regulation of SREBP-1 dependent expression of GIRK4, atrial myocytes were infected with an adenovirus expressing the activated myristoylated form of Akt (Myr-Akt) or an adenoviral control vector expressing...
GFP. Expression of the Myr-Akt resulted in an increase in the phosphorylation of GSK3β (Fig. 5E), a 1.71±0.18 fold (n=10, P=0.003) increase in nSREBP-1 and 3.88±0.55 fold (n=6, P=0.003) increase in GIRK4 expression compared to cells infected with an adenoviral vector expressing GFP alone (Fig. 5E and F).

Inhibition of GSK3β increases $I_{K_{ACh}}$ in HL-1 cells.

In order to determine the physiological relevance of GSK3β regulation of GIRK4 expression in atrial myocytes (Fig. 5), we determined the effect of the inhibition of GSK3β activity on the response of $I_{K_{ACh}}$ to parasympathetic stimulation in HL-1 cells, an immortalized mouse atrial myocyte line. HL-1 cells have been shown to demonstrate contractile, morphologic, biochemical and electrophysiological properties characteristic of atrial myocytes (33). To establish the presence of the insulin signaling pathway in these cells, they were treated with insulin and the phosphorylation of downstream kinases determined. Insulin treatment as well as expression of Myr-Akt resulted in increases in p-Akt and p-GSK3β, nSREBP-1 and the expression of GIRK4 (Supplementary Fig. S1a and b). Finally, treatment of HL-1 cells with Kenpaullone increased the levels of both nSREBP-1 and GIRK4 (Supplementary Fig. S1c) consistent with the conclusion that HL-1 cells were an appropriate model for the study of insulin regulation of GIRK4 expression via an Akt/GSK3β dependent pathway.

To determine whether GSK3β played a role in the regulation of $I_{K_{ACh}}$, we compared membrane currents from HL-1 cells cultured either with vehicle or Kenpaullone (Supplementary Fig. S2). The current-voltage (I-V) relationships demonstrated inward rectification with a reversal potential at -28 mV consistent with a $K^+$-dependent current at 50 mM extracellular $K^+$. The peak inward current increased from -22.4±3.2 pA/pF in cells cultured in vehicle to -53.2±5.8
pA/pF (n=15; P<0.001) in cells treated with Kenpaullone with no change in reversal potential (Supplementary Fig. S2b and c).

**Lithium treatment reverses the parasympathetic dysfunction in the Akita mouse.**

These data suggested that insulin regulates GIRK4 expression via an Akt/GSK3β dependent pathway and that GSK3β regulates $I_{K_{Ach}}$ in HL-1 cells. To determine whether the decrease in HF fraction in the Akita mouse described in Fig. 2 and the impaired heart rate response of the Akita mouse to carbamylcholine described previously (20) might be associated with increased GSK3β activity in the diabetic heart, male Akita mice 4 months of age were fed a diet containing Li$^+$, a GSK3β inhibitor, for 7 days.

The heart rate response to the acetylcholine analogue carbamylcholine was compared in mice prior to and 7 days after the initiation of Li$^+$ treatment. Mice were treated first with propranolol to block the reflex response followed by carbamylcholine (Fig. 6A). There was no significant effect of Li$^+$ on the response of heart rate to propranolol. However, the duration of bradycardia following carbamylcholine administration plotted here as heart rate plateau increased from 5.9±0.6 minutes pre-Li$^+$ to 9.5±1.5 minutes post-Li$^+$ (n=11, P<0.05, Fig. 6A and B, left panel). Furthermore, the absolute decrease in heart rate in response to carbamylcholine was increased from 244±20 bpm pre-Li$^+$ to 304±14 bpm (n=11, P<0.05) following Li$^+$ treatment (Fig. 6A and B, right panel). To determine the effect of Li$^+$ treatment on HF fraction, we compared the time course of the increase in HF fraction in response to propranolol injection in Akita mice before and after Li$^+$ treatment. Composite plots of HF fraction demonstrated that prior to Li$^+$ treatment HF fraction reached a plateau 8 minutes after propranolol injection. Following Li$^+$ treatment, HF fraction also reached a relative plateau, but continued to increase.
during a second phase (Fig. 6C). Fifteen minutes following propranolol injection, the 2-minute mean of HF fraction increased from 50.6±4.4% pre-Li+ to 73.5±3.2% (n=10, P < 0.001) post-Li+ (Fig. 6D). There was no significant difference in the decrease in mean heart rate in response to propranolol in Akita mice before and after Li+ treatment consistent with the conclusion that Li+ had no effect on the sympathetic response of the heart (see Supplemental Table S4).

Echocardiographic analysis demonstrated that Li+ had no effect on left ventricular end diastolic dimension, left ventricular end systolic dimension, fractional shortening, ejection fraction, or resting heart rate (see Supplemental Table S2). Hence, changes in autonomic response in Li+-treated mice were not associated with changes in ventricular function.

**Li+ treatment increases I_{K_Ach} in atrial myocytes from Akita mice in parallel with increased nSREBP-1 levels and GIRK4 expression in the atrium.**

In order to determine whether Li+ might increase parasympathetic responsiveness in Akita mice via an effect on I_{K_Ach}, we measured I_{K_Ach} in atrial myocytes from untreated Akita and Li+-treated Akita mice. Carbamylcholine-stimulated peak inward current increased from -188.7±15.4 pA/pF (n=12) in atrial myocytes from untreated Akita mice to -370±39.6 pA/pF (n=12, P=0.006) in atrial myocytes from Li+-treated Akita mice; Fig. 7A, B and C). We had previously demonstrated a peak inward current of -451±62 pA/pF in WT mice (20). Hence Li+ treatment partially reversed the abnormality in I_{K_Ach} in Akita mice. We further compared the levels of nSREBP-1 and GIRK4 in extracts of atria from WT, untreated and Li+-treated Akita mice. Expression of nSREBP-1 and GIRK4 were 0.48±0.14 (n=6, P=0.014) and 0.48±0.09 (n=5, P=0.003) fold, respectively, in Akita compared to WT atria (Fig. 7D and E) consistent with our prior observations (20) and data summarized in Fig. 3. However, Li+ treatment increased expression of GIRK4 from 0.48±0.14 to
1.17±0.10 fold (n=4, \( P=0.007 \)) and nSREBP1 expression from 0.48±0.09 to 1.75±0.44 (n=5, \( P=0.007 \)) fold, respectively (Fig. 7D and E). Thus Li\(^+\)-treatment reversed the effects of insulin deficiency on both \( I_{KAch} \) and gene expression.

**Effect of the GSK3 inhibitor CHIR-99021 on autonomic dysfunction and GIRK4 expression.**

Although Li\(^+\) has been shown to inhibit GSK3\(\beta\) function, it has also been shown to inhibit IMPase (34), inositol monophosphatase and structurally related phosphomonoesterases as well as \( \beta \)-arrestin-2–Akt complex formation (34). Furthermore, Li\(^+\) has been shown to compete for Na\(^+\) and affect both membrane potential and Na\(^+\) currents (35) and has been shown to effect ventricular repolarization (36; 37). For these reasons we studied the effect of a specific GSK3\(\beta\) inhibitor, CHIR-99021, an aminopyrimidine which competitively inhibits GSK3 activity by competition with binding of ATP to the ATP binding site (37). The time course of increase in HF fraction in response to the injection of propranolol was computed before and after a 14 day treatment with 50mg/kg CHIR-99021 given IP daily, Fig. 8A. At 15 minutes following propranolol injection, the 2 minute average HF fraction increased from 46.8±2.9\% prior to CHIR-99021 treatment compared to 67.8±5.1\% following CHIR-99021 treatment (n=6, \( P=0.034 \), Fig. 8B). There was no significant difference in the decrease in mean heart rate in response to propranolol in Akita mice before and after CHIR-99021 treatment consistent with the conclusion that CHIR-99021 had no effect on the sympathetic response of the heart (see Supplemental Table S4). Baseline HF fraction was not significantly different before and after CHIR-99021 treatment (data not shown). Western blot analysis of atrial homogenates demonstrated that GIRK4 expression was decreased in atria of placebo treated Akita mice to 0.28±0.06 fold (n=6,
\( P=0.00002 \) of levels in WT mice, while CHIR-99021 treatment increased GIRK4 levels from 0.28±0.06 fold to 1.08±0.14 fold (n=5) of those in WT mice which was significantly higher than placebo (\( P=0.0003 \)). Finally, compared to WT, SREBP-1 in placebo treated Akitas was decreased to 0.53±0.07 (n=6, \( P=0.006 \)), while treatment of Akitas with CHIR-99021 increased SREBP-1 from 0.53±0.07 to 1.17±0.11 fold (n=5, \( P=0.0008 \)) (Fig. 8C and D). These data strongly support the role of hyperactivity of GSK3β in autonomic dysfunction in the type I diabetic Akita mouse.

**DISCUSSION**

Cardiac parasympathetic dysfunction has been described in a number of animal models for diabetes. Baroreceptor mediated bradycardia has been shown to be impaired in diabetic rabbits (38). HRV has been shown to be decreased in streptozotocin treated rats (39) in association with degeneration of autonomic neurons (40) and decreased autonomic responsiveness of the heart. Thus studies have implicated both neuronal dysfunction and/or abnormalities of the response of the heart to parasympathetic signaling in the pathogenesis of parasympathetic dysfunction (41). We have previously presented data demonstrating that parasympathetic dysfunction in the type I diabetic heart is at least in part due to decreased expression of proteins in atrial myocytes which mediate the response of the heart to parasympathetic stimulation (20). Here we demonstrate a molecular mechanism for a novel relationship between the insulin signaling pathway and the regulation of the response of the heart to parasympathetic stimulation in the atrial myocardium. These data demonstrate that insulin regulates the expression of the GIRK4 subunit of \( I_{K\text{Ach}} \), which is responsible for the hyperpolarization and resulting negative chronotropic response of the heart to parasympathetic stimulation, via an SREBP-1 dependent mechanism. Although
studies of the function of GSK3β are limited by the lack of inhibitors that differentiate between GSK3α and GSK3β, a combination of experiments using the GSK3β inhibitor Kenpaullone, which increases both SREBP-1 and GIRK4 and the overexpression of a DA-GSK3β, which interferes with the expression of SREBP-1 and GIRK4, strongly supports the conclusion that GSK3β plays a role in the regulation of their expression. Taken together with the finding that decreased insulin levels in the Akita mouse result in a marked decrease in p-GSK3β and a resulting hyperactivity of GSK3β, these data support the conclusion that the decreased expression of SREBP-1 and GIRK4 in the Akita mouse is due at least in part to increased GSK3β activity. The physiologic relevance of GSK3β regulation of SREBP-1 and GIRK4 is supported by the finding that Kenpaullone treatment of HL-1 cells not only increases the levels of SREBP-1 and GIRK4, but also increases the activity of $I_{K_ATP}$. Furthermore, the decrease in HF fraction and the attenuated negative chronotropic response to parasympathetic stimulation in the Akita type I diabetic mouse and the decrease in $I_{K_ATP}$ in atrial myocytes from the Akita mouse were in part reversed by treatment of mice with Li⁺, an inhibitor of GSK3β in parallel with an increase in levels of atrial nSREBP-1 and GIRK4. Although the interpretation of the Li⁺ data is complicated by effects on membrane potential and Na⁺ currents (34-36), the role of GSK3β in the regulation of HRV and expression of genes involved in the parasympathetic response of the heart were corroborated by studies in Akita mice treated with CHIR-99021, a specific GSK3β inhibitor that also increased HF fraction and the expression of nSREBP-1 and GIRK4.

The finding that neither Li⁺ nor CHIR-99021 had an effect on glucose levels taken together with our prior observation that increased SREBP-1 levels in insulin treated Akita mice was not mimicked by phloridizin treatment which normalizes glucose levels by inhibiting glucose reuptake in the kidney, supported the conclusion that the decreased expression of genes
involved in parasympathetic signaling reported here is dependent on hypoinsulinemia and not on hyperglycemia (20). These data strongly support the conclusion that parasympathetic dysfunction in the Akita mouse heart may be due at least in part to hyperactivity of GSK3β in response to decreased insulin levels, resulting in decreased nSREBP-1 dependent GIRK4 expression, attenuation of $I_{K,\text{Ach}}$ and a decrease in HF fraction (Fig. 8E).

Modulation of heart rate response to parasympathetic simulation involves not only $I_{K,\text{Ach}}$, but also the hyperpolarization dependent $I_{\text{(f)}}$ current as well as L-Type Ca$^{2+}$ currents. It has been suggested that L-Type Ca$^{2+}$ channel activity might be decreased in the Akita mouse heart (42). Such a decrease in L-type Ca$^{2+}$ currents might affect the balance between the response of the heart rate to sympathetic and parasympathetic stimulation. However, unpublished data from our laboratory suggests that resting L-type Ca$^{2+}$ currents are relatively unchanged in ventricular myocytes from Akita mice compared to WT. Although no data have appeared regarding $I_{\text{(f)/HCN}}$ pacemaker currents in the Akita mouse heart, such a decrease in a hyperpolarization dependent pacemaker current might also result in a decreased heart rate response to sympathetic stimulation. However, decreases in either $I_{\text{(f)}}$ and/or L-Type Ca$^{2+}$ currents might decrease the response of the heart rate to sympathetic stimulation, thus attenuating the effect of parasympathetic dysfunction observed in these studies.

The impaired response of the heart to autonomic stimulation in diabetics has been attributed to the development of neuronal dysfunction. These conclusions are based on the observations that autonomic dysfunction in animal and cell culture models of diabetics have demonstrated an increase in apoptosis in superior cervical ganglia, dorsal root ganglia and Schwann cells from streptozotocin treated rats. In vitro studies demonstrated increased caspase activity and an increase in reactive oxygen species under conditions of hyperglycemia which
were reversed by insulin-like growth factor-1 (43; 44). More recently, Yang et al have suggested that Akita mice might demonstrate abnormalities of autonomic innervation (45). Although direct measurements of vagal nerve function in the Akita mouse are beyond the scope of the studies reported here, our data do demonstrate a direct effect of insulin on the regulation of the expression of genes involved in the response of the heart to parasympathetic stimulation. Hence, although the decrease in HF fraction might be due in part to neuronal dysfunction, our data support the conclusion that the end organ might also demonstrate an impaired response to vagal stimulation, since the increase in HF fraction in Li+ treated Akita mice was associated with increased expression of GIRK4 in the Akita atrium and an increase in I_{K_ACh} in atrial myocytes from the Akita mouse. The finding that patients with type I diabetics develop autonomic dysfunction in the presence of insulin replacement therapy, might reflect the presence of irreversible neuropathy. Hence in our mouse model, early initiation of insulin treatment might attenuate the development of irreversible changes in innervation.

Given the role of insulin in the inhibition of GSK3β activity and the recent findings suggesting that GSK3β inhibitors might mimic the effects of insulin on glycogen synthase activity and gluconeogenesis in the liver (22), a role for hyperactivity of GSK3β in the pathogenesis of insulin resistance and the secondary effects of diabetes has been suggested. Thus GSK3β has been implicated in the phosphorylation and proteosomal degradation of the Insulin Receptor Substrate 1 (IRS1) in response to hyperglycemia and hence might play a role in the development of insulin insensitivity (46). Recently, inhibition of GSK3β has been implicated in the replication and survival of pancreatic β-cells (47). Furthermore, hyperactive GSK3β has been implicated in the development of diabetic nephropathy and/or retinopathy (23). Our data are consistent with such a role of GSK3β in DAN. The relationship between GSK3β activity and the
level of SREBP-1 in the hypoinsulinemic Akita mouse is consistent with recent findings demonstrating that GSK3β plays a role in regulating the phosphorylation, ubiquitination and subsequent proteasomal degradation of SREBP-1 (48).

The analysis of heart rate and HRV has been complicated by the variation of activity levels in conscious mice. In order to minimize the effects of activity of the mice on HRV, we chose segments of the record for HRV analysis in which heart rate and frequency domain parameters were relatively stationary and where noise due to muscle activity and changes in entropy were minimal. We further developed a unique approach for the study of parasympathetic modulation of heart rate by computing HF fraction after the inhibition of the sympathetic contribution to the power spectrum in response to propranolol injection. Using this approach, differences in HF fraction after sympathetic blockade in WT and Akita mice would be due primarily to differences in the parasympathetic contribution to HF power. However, differential effects of propranolol between groups on sympathetic-vagal interactions at the neuro-effector junction in the SA node, might complicate the interpretation of HRV analysis.

Abnormalities of heart rate response to parasympathetic stimulation and decreased HRV have both been associated with cardiovascular disease. Specifically, parasympathetic stimulation of the heart has been shown to play a protective role in the development of arrhythmias and sudden death (49). Furthermore, the decreased HRV associated with DAN has been shown to be a significant risk factor for sudden death and cardiovascular disease (6; 50-53). Taken together these measurements of parasympathetic function are a critical predictor of clinical risk for sudden death and heart disease in the diabetic population. The finding that inhibition of GSK3β in part reverses the abnormality of HRV in the Akita mouse offers important new insight into the
pathogenesis of DAN and the treatment and prevention of sudden death in the diabetic population.

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Y.Z. designed and performed most of the experiments and quantified the results in association with J.B.G., C.M.W. and H.J.P. C.M.W. carried out the statistical analyses and produced the figures. C.M.W. also developed the concepts and design of the heart rate and HRV experiments, wrote the software and performed the analysis of heart rate and HRV data. C.M.W., J.B.G. and Y.Z. wrote the manuscript. K.P. and C.M.W. carried out western blot analyses. K.P. also generated the chick atrial myocyte cultures and carried out the studies in HL-1 cells. C.D. carried out measurements of $I_{K_{ACh}}$. B.W. generated the atrial myocyte cultures, J.K. provided the Myr-Akt construct and was involved in the evaluation of signaling pathways, M.A. implanted EKG transmitters and assisted with measurements of heart rate. W.C. provided the HL-1 cells and advice as to maintaining them in culture. R.B. carried out the echocardiographic measurements and calculated left ventricular parameters.

J.B.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

FIG. 1. **A**: Schematic representation of the insulin signaling cascade. Insulin binding to the insulin receptor results in phosphorylation of Insulin receptor substrate (IRS1/2) which in turn activates PI3kinase, which converts Akt to the activated phosphorylated form. pAkt phosphorylates and inactivates GSK3β whose role in regulation of SREBP-1 and GIRK4 is suggested. LY294002 is a PI3K inhibitor; Kenpaullone, Li+ and CHIR-99021 are GSK3β inhibitors. **B**: Time course of changes in HF fraction and the LF power in response to propranolol. EKG was monitored in 4 month old WT male mice and recorded continuously for 5 minutes prior to injection of 1mg/Kg propranolol IP and continued for 15 minutes. Composite plots of HF fraction and LF power were computed as described in Methods. **C**: Quantitation of HF fraction at baseline and 15 min after propranolol. Results are the mean ± SEM. **P < 0.01.**

FIG. 2. Comparison of HF fraction of HRV in WT, Akita and Insulin treated Akita mice, as well as heart rate change after atropine injection in WT and Akita mice. EKGs were monitored in 4 month old male mice prior to the injection of 1 mg/Kg propranolol IP and continued for 15 minutes as described in Methods. **A**: Comparison of group wise averaged (± SEM) composite plots of HF fraction prior to and over the duration of the propranolol phase in WT and Akita (DM) mice. **B**: Quantitation of HF fraction at 15 min after propranolol injection. **C**: Comparison of the increase in heart rate, averaged over 3 minutes before and 3 minutes after the injection of atropine in Akita and WT mice. **D**: Comparison of composite plots of the response of Akita mice to propranolol prior to (pre-Insulin) and 10 days after insulin treatment (post-Insulin). Each mouse served as its own control. **E**: Quantitation of HF fraction at 15 min after propranolol
injection pre- and post-insulin treatment. Results are reported as mean ±SEM, statistical comparisons were done by Student’s $t$ test throughout the figure. * $P < 0.05$, ** $P < 0.01$.

**FIG. 3.** GIRK4 expression and insulin signaling are decreased in atria of Akita diabetic mice. Western blot analysis of GIRK4 expression in atria of *A*: WT and Akita (DM) mice and *B*: WT mice and placebo and insulin treated Akita mice: Bar graphs represent densitometry analysis of western blots normalized to β-actin. *C*: Western blot analysis of p-Akt, Akt, p-GSK3β and GSK3β in atria of age-matched WT, placebo and insulin treated Akita mice. *D*: Bar graphs of levels of p-Akt and p-GSK3β in placebo (DM+Placebo) and insulin treated Akita mice normalized to the expression of the β subunit of Gα12. **$P<0.01$, *** $P<0.001$.

**FIG. 4.** Insulin stimulation of GIRK4 expression is dependent on PI3K and SREBP-1. Embryonic chick atrial myocytes were incubated for 16 hours with either vehicle or 100 nM insulin with or without 10 µM LY294002. *A*: Effect of LY294002 on expression of GIRK4, p-Akt, p-GSK3β, and nSREBP-1 as determined by Western blot analysis. *B*: Densitometric analysis of GIRK4 and nSREBP-1 expression from experiments similar to those in panel A normalized to β-actin. *C*: Effect of infection of embryonic chick atrial myocytes with Adenovirus expressing either GFP or GFP-DN-SREBP-1 on insulin stimulated GIRK4 expression. *D*: Densitometric analysis of GIRK4 expression in cells infected with Ad-GFP and the effect of insulin in cells infected with Ad-GFP and with Ad-GFP-DN-SREBP-1. Data are normalized to β-actin. **$P<0.01$, *** $P<0.001$, brackets indicate groups compared in statistical tests.
FIG. 5. GSK3β regulation of nSREBP-1 and GIRK4 levels in chick atrial myocytes. 

**A:** Western blots demonstrating dose dependence of Kenpaullone inhibition of GSK3β on GIRK4 and nSREBP-1 levels. 

**B:** Densitometric analysis of western blots similar to those in **A**, normalized to β-actin. 

**C:** Western blot analysis of levels of GIRK4 and nSREBP-1 protein in atrial myocytes infected with increasing MOI of an adenovirus expressing an HA tagged dominant active GSK3β. 

**D:** Densitometric analysis of western blots similar to those in panel **C**. 

**E:** Effect of adenoviral expression of myristoylated Akt (Myr-Akt) on the phosphorylation of GSK3β and levels of nSREBP-1 and GIRK4 proteins. Chick atrial myocytes were infected with Ad-Myr-Akt or Ad-GFP at an MOI of 50 pfu/cell for 3 hours, followed by incubation for 48 hours in fresh medium. Cells were harvested and levels of nSREBP-1, GIRK4 and pGSK3β were determined. 

**F:** Densitometric analysis of western blots similar to those in **E**. Data are normalized to β-actin, *P<0.05, **P<0.01.

FIG. 6. Li⁺ treatment of the Akita mouse increases both the negative chronotropic response of the heart to the parasympathetic receptor agonist carbamylcholine and the HF fraction of HRV. 

**A:** Negative chronotropic response of male Akita mice 4 months of age to carbamylcholine before and after a 7 day treatment with Li⁺. Mice were pretreated with propranolol, 1 mg/Kg IP, to block the β-adrenergic reflex response to carbamylcholine, followed 20 minutes later by 0.2 mg/kg carbamylcholine, IP. Heart rate was recorded as described in Methods. Data are the composite mean heart rates obtained from moving average beat data of 11 Akita mice before Li⁺ (pre-Li⁺) and 7 days following Li⁺ (post-Li⁺). 

**B:** **Left panel:** Duration of bradycardia following carbamylcholine injection defined as the elapsed time from the carbamylcholine induced bradycardia until the initiation of recovery given here as the heart rate plateau. 

**Right panel:**
Magnitude of the negative chronotropic response to carbamylcholine. The difference between baseline heart rate immediately prior to carbamylcholine injection and the lowest heart rate following carbamylcholine injection were used to compute the heart rate response. 

C: Composite plots of the averaged (± SEM) time course of the increase in HF fraction following injection of propranolol in mice pre-Li+ and post-Li+. D: Comparison of the magnitude of HF fraction pre-Li+ and post-Li+ computed 15 min after propranolol injection. Note: for these experiments each mouse served as its own control. *P<0.05, **P<0.01

FIG. 7. Li+ treatment increases I\text{K\textsubscript{Ach}} in atrial myocytes and increases levels of nSREBP-1 and GIRK4 expression in the Akita atrium. I\text{K\textsubscript{Ach}} was determined as described in Methods, and I-V plots were constructed. A: I-V relationship of the carbamylcholine-induced whole-cell currents elicited from a 1-second voltage ramp with a continuously changing voltage from +50 to -110 mV (1); Current from a typical atrial myocyte with and without 20 µmol/L carbamylcholine (2); Current generated by subtracting the trace obtained prior to and after the addition of carbamylcholine (3). B: I-V plots constructed from a series of data points as in A3. Data are the mean ± SEM of 12 recordings each from cells from 4 untreated Akita mice and 4 Li+-treated Akita mice. C: Quantitation of peak inward currents from A, **P=0.006 compared to control. D: Levels of nSREBP-1 and GIRK4 in atria from WT, (DM) and Li+-treated Akita mice (DM+Li) determined by Western blot analysis of atrial extracts of 5 mice in each group. E: Densitometric analysis of nSREBP-1 and GIRK4 from D: Data were normalized to the expression of β-actin. *P<0.05, **P<0.01
FIG. 8 The GSK3β inhibitor CHIR-99021 increases HF fraction and levels of expression of nSREBP-1 and GIRK4 in Akita mice. A: Composite plots of the averaged (± SEM) time course of the increase in HF fraction following injection of propranolol in mice pre- and post- treatment with CHIR-99021. B: Comparison of the magnitude of HF fraction pre-CHIR-99021 and post-CHIR-99021 computed 15 min after propranolol injection. CHIR-99021 had no effect on baseline HF fraction, data not shown. Note: for these experiments each mouse served as its own control. C: Levels of nSREBP-1 and GIRK4 in atria from WT, placebo (DM+Placebo) and CHIR-99021 -treated Akita mice (DM+CHIR-99021) determined by Western blot analysis. D: Densitometric analysis of nSREBP-1 and GIRK4 from C. Data were normalized to the expression of β-actin. E: Schematic representation of the proposed effect of hypoinsulinemia on GSK3β and the development of parasympathetic dysfunction. *P<0.05, **P<0.01, ***P<0.001.
Figure 1

A

\[ \text{Insulin/IRS1} \rightarrow \text{PI3K} \rightarrow \text{p-Akt} \rightarrow \text{p-GSK3\beta} \rightarrow \text{nSREBP1} \rightarrow \text{GRK1/4} \]

LY294002

Rapamycin, Li+, Chex 00021

B

![Graph showing time (minutes) vs. LF and HF fraction with Propranolol treatment](image)

C

![Bar graph comparing Baseline to Propranolol](image)

219x201mm (300 x 300 DPI)
Figure 4

A

|        | LY294002 | Insulin | n-Akt | p-GSK3β | GIRK4 | β-actin | nSREBP-1 | β-actin |
|--------|----------|---------|-------|---------|-------|---------|----------|---------|
|        | -        | -       | -     | -       | -     | -       | -        | -       |

B

|        | Control | Insulin | Insulin+LY |
|--------|---------|---------|------------|
| GIRK4  | ![Graph](image1) |
| SREBP1 | ![Graph](image2) |

C

| Ad-virus | GFP | GFP | DN-SREBP-1 |
|----------|-----|-----|------------|
| Insulin  | -   | +   | +          |

D

|        | Control | Insulin | Insulin+DN SREBP |
|--------|---------|---------|------------------|
| GIRK4  | ![Graph](image3) |

182x176mm (300 x 300 DPI)
Figure 5

A

Kenpaullone - 2 μM 5 μM

nSREBP-1

GIRK4

β-catenin

β-actin

B

Relative expression

GIRK vs SREBP1

Control 2μM Kenpaullone 5μM Kenpaullone

C

DA-GSK3β (MOI)

nSREBP-1

GIRK4

β-catenin

GSK3β

HA tag

β-actin

D

Relative expression

GIRK vs SREBP1

Control MOI 25 MOI 50 MOI 100

E

Ad-virus

nSREBP-1

GIRK4

p-GSK3β

GSK3β

Akt

β-actin

F

Relative expression

GIRK4 vs SREBP1

Control DA-Myr-Akt

241x305mm (300 x 300 DPI)
Figure 6

A

Propranolol

Carbachol

Heart rate (bpm)

0 20 40 60 80

Time (minutes)

red - pre-Li⁺
blue - post-Li⁺

B

HR Plateau (min)

2 4 10 12

pre-Li⁺ post-Li⁺

HR Response (bpm)

0 50 100 150 200 250 300 350

pre-Li⁺ post-Li⁺

C

Propranolol

Time (minutes)

0 5 10 15 20

HF fraction (%)

red - pre-Li⁺
blue - post-Li⁺

D

HF fraction (%)

0 20 40 60 80 100

pre-Li⁺ post-Li⁺

***
Figure 7

A

1

2

3

- Carbachol
- Carbachol
+ Carbachol
+ Carbachol

B

\[ \text{I (pA/pF)} \]

\[ \text{V (mV)} \]

\[ \text{DM} \]

\[ \text{DM+Li}^+ \]

C

\[ \text{DM} \]

\[ \text{DM+Li}^+ \]

D

\[ \text{WT} \]

\[ \text{DM} \]

\[ \text{DM+Li}^+ \]

nSREBP-1

GIRK4

β-actin

E

\[ \text{Relative expression} \]

\[ \text{GIRK4} \]

\[ \text{SREBP1} \]

\[ \text{DWT} \]

\[ \text{DM} \]

\[ \text{DM+Lithium} \]
Figure 8

A

Propranolol

HF fraction (%)

Time (minutes)

0 5 10 15 20

preCHIR postCHIR

B

HF fraction (%)

preCHIR postCHIR

C

WT DM+Placebo DM+CHIR-99021

nSREBP-1 GIRK4 β-actin

D

Relative expression

GIRK4 SREBP1

*** ***

E

Niasia Mellitus
Insulin Deficiency

p-Akt ↓

p-GSK3β ↓

GSK3β activity ↑

nSREBP-1 ↓

GIRK1, GIRK4 ↓

I_{KAD} ↓

HRV, Chronotropic Response ↓

Cardiac Diabetic Autonomic Dysfunction
**Supplementary Data**

**Culture of HL-1 cells.**
The mouse atrial cardiomyocyte cell line, HL-1 cells (1) were grown as monolayers in Claycomb Media (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum, 0.1 mmol/L norepinephrine, 2 mmol/L L-glutamine, and 100 U/mL penicillin/streptomycin. All coverslips, dishes and flasks were precoated with 0.00125% fibronectin in 0.02% gelatin (BD Bioscience). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

**Supplementary Figure S1.** Regulation of the insulin signaling pathway and expression of nSREBP-1 and GIRK4 in the HL-1 mouse atrial myocyte cell line.

(a) Effect of insulin on phosphorylation of Akt and GSK3β, and the levels of nSREBP-1 and GIRK4. HL-1 cells were incubated for 16 hours with 100 nM insulin, harvested and levels of pAkt, GSK3β and GIRK4 determined by western blot analysis.

(b) Effect of adenoviral expression of myristoylated Akt on phosphorylation of GSK3β and levels of GIRK4 and nSREBP-1. HL-1 cells were infected with an adenovirus expressing a myristoylated Akt or an adenoviral control vector at an MOI of 100. Cells were incubated for 72 hours, harvested and levels of pAkt, GSK3β and GIRK4 determined as described.

(c) Effect of Kenpaullone on the levels of nSREBP-1 and GIRK4 in HL-1 cells. HL-1 cells were treated for 16 hours with 5 µM Kenpaullone, harvested and levels of GIRK4 and nSREBP-1 determined as described. Data are representative of results from 4 independent cultures.
Supplementary Figure S2. Inhibition of GSK3β by Kenpaullone increases $I_{K_{ach}}$ in HL-1 cells.
(a) I-V relationship of the carbamylcholine-induced whole-cell currents elicited from a 1-second voltage ramp with a continuously changing voltage from +50 to -110 mV [1]. Current in a typical atrial myocyte with and without 10 µmol/L carbamylcholine [2]. Current generated by subtracting the trace obtained prior to and after the addition of carbamylcholine [3].
(b) I-V plots constructed from a series of data points in (a)[3]. Data are the means ± SEM of 15 determinations.
(c) Mean of peak inward current from vehicle and Kenpaullone treated cells (± SEM, n=15), **$P < 0.001$. 

![Diagram](image-url)
Supplementary Table S1. Body weight and blood glucose of WT and Akita mice

|                                | WT (n=10) | Akita (n=8) | Akita+Insulin (n=8) | Akita+Li⁺ (n=8) | Akita+CHIR99021 (n=5) |
|--------------------------------|-----------|-------------|---------------------|----------------|----------------------|
| Blood glucose (mg/dL)          | 163 ± 6*  | 534 ± 16    | 150 ± 22*           | 511 ± 50       | 538 ± 28             |
| Body weight (g)                | 28.5 ± 1.7* | 22.7 ± 1.6  | 23.8 ± 1.4          | 24.0 ± 1.3     | 20.6 ± 1.6           |

*P<0.05 vs. Akita

Supplementary Table S2. Echocardiographic analysis of LV structure and function of Akita diabetic mice treated with Li⁺.

Two-dimensional images and M-mode tracings (sweep speed 50–100 mm/s) were recorded from the short axis view at the papillary muscle level. Using M-mode tracings, LV end-diastolic diameter (EDD) and end-systolic diameter (ESD) were measured to the nearest 0.1 mm, averaging three cardiac cycles. Fractional shortening (FS) was calculated using the standard equation: FS (%) = (EDD – ESD)/EDD X 100. Ejection fraction (EF) was calculated using the standard equation: EF (%) = (LVEDV - LVESV) X 100/ LVEDV; LVEDV (LV end diastolic volume) = (7 X LVEDd3)/(2.4 + LVEDd); LVESV (LV end systolic volume) = (7 X LVESd3)/(2.4 + LVESd); LVEDd, LV end diastolic diameter; LVESd, LV end systolic diameter. (N= 13); EDD, end-diastolic dimension (mm); ESD, end-systolic dimension (mm); Post wall, posterior wall thickness (mm); Ant wall, anterior wall thickness (mm); HR, heart rate (beats/min); FS, fractional shortening (%); EF, ejection fraction (%).

|                                | Pretreatment | Li⁺-treated | P value |
|--------------------------------|--------------|-------------|---------|
| EDD (mm)                       | 2.95±0.11    | 3.23±0.14   | 0.126   |
| ESD (mm)                       | 1.56±0.07    | 1.61±0.10   | 0.689   |
| Post wall (mm)                 | 1.20±0.04    | 1.19±0.06   | 0.818   |
| Ant wall (mm)                  | 1.09±0.04    | 1.05±0.08   | 0.576   |
| HR (beats/min)                 | 413±9        | 439±21      | 0.217   |
| FS (%)                         | 46.9±1.6     | 50.4±1.2    | 0.147   |
| EF (%)                         | 79.4±1.5     | 82.6±1.2    | 0.161   |
SUPPLEMENTARY DATA

**Supplementary Table S3.** LF and HF parameters (10⁻⁶ ms²/Hz) of WT and Akita mice before (baseline) and after propranolol for each of the reported studies. WT and Akita data correspond to Fig. 1C and Fig. 2B respectively. Values for treated Akita mice correspond to post-treatment data shown in Figures 2D, 6D and 8B respectively.

|               | WT (n=13) | Akita (n=10) | Akita+Insulin (n=8) | Akita+Li⁺ (n=10) | Akita+CHIR99021 (n=6) |
|---------------|-----------|--------------|---------------------|-------------------|-----------------------|
| **Baseline**  |           |              |                     |                   |                       |
| LF            | 4.58 ± 1.01 | 8.24 ± 5.01  | 8.37 ± 3.19         | 11.37 ± 4.74      | 19.78 ± 5.65          |
| HF            | 2.17 ± 0.57 | 3.39 ± 1.56  | 6.42 ± 2.46         | 7.94 ± 3.21       | 13.24 ± 4.44          |
| **Propranolol** |          |             |                     |                   |                       |
| LF            | 2.49 ± 1.17 | 3.48 ± 1.43  | 2.11 ± 0.98         | 6.45 ± 1.94       | 6.55 ± 1.52           |
| HF            | 1.81 ± 0.70 | 2.29 ± 0.87  | 9.44 ± 4.88         | 23.40 ± 9.27      | 15.22 ± 3.74          |

**Supplementary Table S4.** Decrease in heart rate (beats per minute, bpm) in response to propranolol in WT and Akita mice and for each of the reported studies before and after treatment. WT and Akita data correspond to Figure 2B. Values for treated Akita mice correspond to pre and post-treatment data shown in Figures 2E, 6D and 8B respectively. P-values are for comparisons between WT and Akita, as well as Akita mice pre- and post-treatment for each study.

|               | WT (n=10) | Akita (n=10) | Akita+Insulin (n=8) | Akita+Li⁺ (n=10) | Akita+CHIR99021 (n=6) |
|---------------|-----------|--------------|---------------------|-------------------|-----------------------|
| **Pre**       | 65.8 ± 22.5 | 79.1 ± 22.6  | 87.2 ± 20.3         | 74.5 ± 21.4       | 50.0 ± 36.0           |
| **Post**      | 82.8 ± 21.4 | 101.3 ± 20.0 | 153.0 ± 31.4        |                   |                       |
| **P-value**   | 0.682     | 0.902        | 0.454               | 0.147             |                       |

**References**
1. Claycomb WC, Lanson NA, Jr., Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ, Jr.: HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci U S A 1998;95:2979-2984