Role of SREBP-1 in the Development of Parasympathetic Dysfunction in the Hearts of Type 1 Diabetic Akita Mice


Abstract—Diabetic autonomic neuropathy, a major complication of diabetes mellitus, is characterized in part by impaired cardiac parasympathetic responsiveness. Parasympathetic stimulation of the heart involves activation of an acetylcholine-gated K⁺ current, $I_{K_{Ac}}$, via a $(GIRK1)_{2}/(GIRK4)_{2}$ K⁺ channel. Sterol regulatory element binding protein (SREBP)-1 is a lipid-sensitive transcription factor. We describe a unique SREBP-1–dependent mechanism for insulin regulation of cardiac parasympathetic response in a mouse model for diabetic autonomic neuropathy. Compared to wild-type mice, Ins2Akita type 1 diabetic mice demonstrated a decrease in the negative chronotropic response to carbachol characterized by a 2.4-fold decrease in duration of bradycardia; a 52±8% decrease in atrial expression of GIRK1 ($P<0.01$) and a 31.3±2.1% decrease in SREBP-1 ($P<0.05$). Myocytes from atria of Akita mice exhibited a markedly decreased carbachol stimulation of $I_{K_{Ac}}$ with a peak value of $−181±31$ pA/pF compared to $451±62$ pA/pF ($P<0.01$) for cells from wild-type mice. Insulin treatment of Akita mice reversed the impairment in parasympathetic response and increased the expression of GIRK1, SREBP-1, and $I_{K_{Ac}}$ activity in atrial myocytes from these mice to levels in wild-type mice. Insulin treatment of cultured atrial myocytes stimulated GIRK1 expression 2.68±0.12-fold ($P<0.01$), whereas overexpression of dominant negative SREBP-1 reversed this insulin effect. Finally, adenoviral expression of SREBP-1 in Akita atrial myocytes reversed the impaired $I_{K_{Ac}}$ to levels in cells from wild-type. These results support a unique molecular mechanism for insulin regulation of GIRK1 expression and parasympathetic response via SREBP-1, which might play a role in the pathogenesis of diabetic autonomic neuropathy in response to insulin deficiency in the diabetic heart. (Circ Res. 2009;104:00-00.)

Key Words: diabetic autonomic neuropathy ■ SREBP ■ insulin deficiency ■ GIRK channel

Diabetes mellitus is associated with severe debilitating complications that include a diabetic autonomic neuropathy (DAN) characterized by impairment of vascular reflexes, occasional hypotension, and decreased sympathetic and parasympathetic responsiveness of the heart.1 Approximately 50% of patients with diabetes for 10 years or more demonstrate an impaired response of the heart to parasympathetic stimulation.2 The presence of DAN is a significant risk factor, as demonstrated by a 5-fold higher 5-year mortality compared with diabetics without DAN.3

Parasympathetic regulation of the heart has both a neuronal component involving vagal stimulation of parasympathetic ganglia in the atria and atroventricular node, followed by release of acetylcholine, and a molecular component involving an intrinsic cardiac signaling pathway that mediates the parasympathetic response to acetylcholine. The latter involves the binding of acetylcholine to M2 muscarinic receptors on the surface of cardiomyocytes and dissociation of the heterotrimeric G protein G$_{i2}$ into G$_{i2}$ and G$\beta\gamma$ subunits. G$\beta\gamma$ binds to and activates the G protein–coupled inward rectifying K⁺ channel (GIRK1)$_{2}$(GIRK4)$_{2}$, the channel that is responsible for $I_{K_{Ac}}$ (acetylcholine-gated K⁺ current), resulting in a decrease in the rate of diastolic depolarization and a decrease in heart rate (negative chronotropic effect).4,5 Changes in levels of expression of M2 and G$_{i2}$ in the heart have been shown to play a role in determining the magnitude of the parasympathetic response.6–8

Sterol regulatory element binding proteins (SREBPs) are a family of 3 transcription factors that regulate expression of genes involved in lipid homeostasis and glucose metabolism.
SREBPs are synthesized in the endoplasmic reticulum as 130-kDa precursor molecules that are transported to the Golgi, where they are processed via a 2-step sequential proteolytic cleavage to produce a 480-aa transcription factor that is transported to the nucleus.9,10 The expression, transport, and processing of these proteins are subject to feedback regulation by sterols. SREBP-1 levels have been shown to be regulated by insulin.10–12

The Ins2Akita diabetic mouse is characterized by a point mutation in the proinsulin ins2 gene (Ins2Cys96Tyr), which interferes with the transport of ins1 and ins2, resulting in destruction of islet cells and development of the diabetic phenotype.13 Heterozygous males may survive untreated for up to a year, making them a good model for the study of secondary effects of diabetes.14

The autonomic dysfunction associated with diabetes mellitus has been attributed to effects of hyperglycemia on neuronal survival and neuronal function.15 Here, we show that the Akita type 1 diabetic mouse demonstrates a decreased response of the heart to parasympathetic stimulation characteristic of DAN associated with a decrease in expression of SREBP-1 and GIRK1 in the atrium. Our data support a new molecular mechanism for the impaired parasympathetic response in the diabetic heart and a unique relationship among insulin, lipid homeostasis, and the parasympathetic response, which might serve as a new therapeutic target for the treatment of DAN.

Materials and Methods
Detailed experimental protocols are described in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org.

Animals
The heterozygous male Akita Ins2Cys96Tyr mice and littermate wild-type (WT) mice were from The Jackson Laboratory. All vertebrate animal–related procedures described were approved by the Tufts Medical Center Institutional Animal Care Committee.

Cell Culture
Embryonic chick atrial myocytes were cultured as described.16 Atrial myocytes from mouse atria were prepared by a retrograde Langendorff perfusion method as described.17

ECG Monitoring in Conscious Mice
An implantable wireless radiofrequency transmitter was inserted, and the ECG signal was recorded with the use of a telemetry receiver and an analog-to-digital acquisition system (Data Sciences International).18

Western Blot Analysis
To determine GIRK1 and SREBP-1 levels, Western blot analysis was carried out as described previously.17 A GIRK1-specific antibody from Alomone Labs (Jerusalem, Israel) and SREBP-1 antibodies from Santa Cruz Biotechnology were used.

Cellular Electrophysiology
Membrane currents were measured by the patch-clamp technique in whole-cell mode using an LM-EPC7 amplifier as described.17

Echocardiography
Echocardiographic studies were performed as described previously.19

Results
Metabolic State and Left Ventricular Structure and Function in Akita Type 1 DM Mice
Blood glucose in Akita DM mice increased from 294±16 mg/dL (n=13) at 4 weeks of age, reaching a plateau varying from 406 to 732 mg/dL, with a mean of 585±18 mg/dL (n=33) at 4 months of age. In WT mice, glucose remained stable at 149±8 mg/dL (n=27) at all ages studied. Hemoglobin-A1c was 9.41±0.4 (n=9) in Akita mice compared with 4.37±0.09 (n=7) in WT. Although male Akita heterozygotes demonstrated a marked hyperglycemia, there was no significant difference in blood pH, serum electrolytes, or anion gap between Akita and WT mice 4 to 6 months of age (Online Table I). Echocardiographic analysis demonstrated no significant differences in left ventricular end diastolic dimension, left ventricular end systolic dimension, fractional shortening, ejection fraction, or resting heart rate in Akita DM mice compared to WT mice (Online Table II).
The Ins2Akita Mouse Demonstrates Parasympathetic Dysfunction

To determine whether type 1 diabetic Akita mice develop parasympathetic dysfunction, 10 conscious male WT mice 6 months of age with an average serum glucose level of 125±20 mg/dL and 10 littermate male Akita mice with average serum glucose 500 mg/dL were pretreated with propranolol to block β-adrenergic reflex responses to carbamylcholine. Propranolol blockade at this concentration has been shown to persist in mice for up to 2 hours. Animals were subsequently challenged with the nonhydrolyzable acetylcholine analog carbamylcholine, and heart rate was determined using implantable ECG transmitters. The effects of carbamylcholine on heart rate were significantly decreased in the Akita mouse. The duration of bradycardia was 5.2±0.51 minutes in the Akita mouse compared to 12.6±1.07 minutes in WT mice (P<0.001; Figure 1A and 1B). Following carbamylcholine treatment, heart rate recovered to 80% of pretreatment levels significantly faster in Akita mice, 7.4±1.03 minutes, compared to 13.6±1.96 minutes in WT mice (P<0.05; Figure 1A and 1D), and the slope of recovery of heart rate was much steeper 36.4±4.85 bpm in the Akita mouse compared to 23.5±2.73 bpm in the WT mouse (P<0.05). Finally, the absolute decrease in heart rate in response to carbamylcholine was 285.5±16.07 bpm in Akita mice, compared to 348.5±21.27 bpm in WT mice (P<0.05; Figure 1A and 1C). We also noted that compared to WT, there was a tendency to a decreased initial heart rate in the Akita mice, which might be attributable to a decreased intrinsic heart rate (heart rate in the absence of autonomic stimulation) in the diabetic heart.

$I_{K_{ACh}}$ Is Decreased in Dissociated Atrial Myocytes From Akita DM Mice

To determine whether the decreased response of heart rate to carbamylcholine in the Akita mouse was associated with a decrease in $I_{K_{ACh}}$, we compared membrane currents in atrial myocytes from Akita and WT mice. Rod-shaped cells from atria of Akita mice 4 months of age remained viable for up to 48 hours in culture medium, demonstrating clearly defined striations and spontaneous contractions with stable resting membrane potentials. The current–voltage (I-V) relationships derived from membrane currents in these cells demonstrated that myocytes from atria of adult Akita mice exhibited a markedly decreased carbamylcholine stimulation of $I_{K_{ACh}}$ with a peak value of $-181±31$ pA/pF (n=5) compared to $-451±62$ pA/pF (n=5, P<0.01) for cells from WT mice (Figure 2A and 2B).

Atria of Akita DM Mice Demonstrate Decreased Expression of GIRK1

To determine whether the decrease in parasympathetic responsiveness in the Akita mouse heart and the decrease in $I_{K_{ACh}}$ in atrial myocytes from these mice were caused by a decrease in expression of molecular components of the parasympathetic response pathway, the level of expression of GIRK1 was compared in extracts from atria of WT and Akita mice. Western blot analysis demonstrated 2 bands that had been previously shown to correspond to the glycosylated (64-kDa) and unglycosylated (52-kDa) GIRK1 bands determined by autoradiographs from 6 WT and 6 Akita mice normalized to the expression of Gβ. **P<0.01.
Insulin Treatment Reverses the Parasympathetic Dysfunction in the Akita Mouse

To determine whether the decreased response of the Akita mouse heart to parasympathetic stimulation might be an insulin-dependent reversible process, we measured the effect of insulin treatment on parasympathetic responsiveness and $I_{K_{ACh}}$ and GIRK1 expression in Akita mice. Implantation of insulin pellets in Akita mice decreased serum glucose from 14 to 118 mg/dL (n=7), with a 3- to 4-day time course. Duration of bradycardia was increased by 7.4 ± 2.67 minutes (n=7, $P<0.05$) in insulin-treated mice, more than 2-fold higher than pretreatment levels (Figure 3A and 3B). The recovery time of heart rate to 80% of resting levels following carbamylcholine injection increased from a pretreatment level of 7.4 ± 2.84 minutes to 16.8 ± 2.59 minutes in insulin-treated mice (n=7, $P<0.05$; Figure 3A and 3D). Finally, the absolute decrease in heart rate in response to carbamylcholine was 114.71 ± 32.51 bpm greater in insulin-treated mice compared to untreated Akita mice (n=7, $P<0.05$; Figure 3A and 3C). Thus, insulin treatment significantly reversed the parasympathetic dysfunction in the Akita mouse.

These findings suggested that insulin might increase parasympathetic responsiveness in Akita mice via an effect on $I_{K_{ACh}}$. To test this hypothesis, $I_{K_{ACh}}$ was determined in atrial myocytes from placebo and insulin-treated Akita mice. Carbamylcholine-stimulated peak inward current was $-209.1 ± 22.2$ pA/pF (n=8) in atrial myocytes from placebo-treated mice and $-520.2 ± 40.1$ pA/pF (n=9, $P<0.01$; Figure 4A and 4B) in atrial myocytes from insulin-treated mice, which was not significantly different from that seen in WT mice (see Figure 2B). Given the decreased level of expression of GIRK1 in the atria of Akita mice compared with WT, one explanation for the increase in $I_{K_{ACh}}$ in response to insulin might be an increase in the expression of GIRK1. Analysis of extracts of atria from placebo and insulin-treated Akita mice demonstrated that insulin treatment increased GIRK1 expression 2.74 ± 0.11-fold (n=4, $P<0.01$) compared to placebo (Figure 4C and 4D).

Insulin Regulates the Expression of GIRK1 in Cultured Chick Atrial Myocytes via an SREBP-1–Dependent Mechanism

Although culture of adult mouse atrial myocytes yielded sufficient cells for measurements of $I_{K_{ACh}}$, the number of cells was not sufficient for Western blot analysis to determine the role of insulin in the regulation of either GIRK1 or SREBP-1 expression. Furthermore, antibodies have not proven effective for immunohistochemical analysis of either SREBP-1 and GIRK1 expression in mouse atrial tissues. Embryonic chick atrial myocytes constitute an excellent model for the study of the role of SREBP-1 in the regulation of $I_{K_{ACh}}$.17,22 Hence, embryonic chick atrial myocytes were incubated with either insulin or vehicle, and the effect on GIRK1 expression was determined. Insulin increased GIRK1 expression 2.68 ± 0.12-fold compared to control (n=4,
SREBP-1 Levels Are Decreased in the Atrium of Akita Mice

The finding that SREBP-1 was regulated by insulin in cultured chick atrial myocytes suggested that insulin deficiency in Akita mice might result in a decrease in SREBP-1 levels in the atrium. Levels of the 60-kDa form of SREBP-1 in atria of Akita mice were decreased by 31.3±2.1% (n=3, P<0.01). This effect was specific, as demonstrated by the finding that the expression of Goα was unaffected by insulin (Figure 5A and 5B).

We had previously demonstrated that GIRK1 expression was regulated by insulin in rat and adipocytes. Although insulin had been shown to increase SREBP-1 expression in liver and adipocytes, the effect of insulin on SREBP-1 expression in the heart had not been determined. Insulin treatment of cultured chick atrial myocytes resulted in a small increase in levels of the 130-kDa precursor form of SREBP-1 and a 1.9±0.2-fold increase (n=3, P<0.05) in the 60-kDa nuclear form of SREBP-1 (Figure 5C and 5D). To determine whether this increase in SREBP-1 might play a role in insulin regulation of GIRK1 expression, cultured atrial myocytes were infected with an adenoviral vector expressing GFP plus DN-SREBP-1 or GFP plus βgal at the time of plating (multiplicity of infection of 20). On the third culture day, cells were incubated with or without insulin for 16 hours and harvested, and expression of GIRK1 was determined by Western blot analysis. E, Mean intensity of the precursor (130-kDa) and processed (60-kDa) SREBP-1, determined by densitometric scanning of 3 independent experiments similar to that in A (*P<0.05). The relative intensity of GIRK1 normalized to Gβ in the absence of insulin was taken as 1. C, SREBP-1 levels in chick atrial myocytes cultured with and without insulin, determined by Western blot analysis. D, Mean intensity of the precursor (130-kDa) and processed (60-kDa) SREBP-1, determined by densitometric scanning of 3 independent experiments similar to that in C (*P<0.05). E, Effect of DN-SREBP-1 on GIRK1 expression in atrial myocytes. Embryonic chick atrial myocytes were infected with an adenoviral vector expressing GFP plus DN-SREBP-1 or GFP plus βgal at the time of plating (multiplicity of infection of 20). On the third culture day, cells were incubated with or without insulin for 16 hours and harvested, and expression of GIRK1 was determined by Western blot analysis. F, Densitometric scanning of 3 experiments, similar to that in E. Data are normalized to the expression of Gβ. *P<0.05.

Expression of SREBP-1 in Atrial Myocytes From Akita Mice Rescues the Impaired I_{K_Ach} Response to Carbamylcholine

To determine whether the decreased levels of SREBP-1 in atria of Akita mice might play a role in the impaired response of the Akita mouse to parasympathetic stimulation, the effect of adenoviral expression of SREBP-1 in atrial myocytes from Akita mice on I_{K_Ach} was determined. Florescence microscopy revealed that 90% of cells infected with adenovirus expressing either GFP plus βgal or GFP plus SREBP-1 were GFP-positive (Figure 7, inset). Expression of GFP with βgal had no effect on peak inward currents in I-V curves derived from measurements of I_{K_Ach} (compare DM cells in Figure 2A and 2B and control cells in Figure 7A and 7B). However, infection of atrial myocytes from Akita mice with adenovirus...
expressing GFP plus SREBP-1 increased $I_{K_{Ach}}$ from $-186.2 \pm 17 (n=6)$ to $-380.6 \pm 32.9 (n=7, P<0.01)$, which was not significantly different from $I_{K_{Ach}}$ in atrial myocytes from WT mice (Figure 2B). These findings supported the conclusion that SREBP-1 regulates the response of the heart to parasympathetic stimulation and that decreased SREBP-1 in the diabetic heart results in parasympathetic dysfunction.

**Discussion**

The parasympathetic response of the heart involves both a neuronal component carrying input from the central nervous system via the vagus nerve and an intrinsic molecular cardiac response pathway. In the present study, carbamylcholine was injected into the Akita DM mouse to bypass vagal stimulation and directly activate the intrinsic parasympathetic signaling pathway in the heart. Thus, the finding of an impaired parasympathetic response to carbamylcholine in the type 1 diabetic Akita mouse supports the conclusion that the parasympathetic dysfunction in the diabetic heart may involve not only a neuropathy but also an abnormality of the intrinsic downstream parasympathetic signaling pathway in the heart. This pathway involves acetylcholine binding to the $M_3$ muscarinic receptor, dissociation of the heterotrimeric G protein $G_{i2}$ into $G_{i2}$ and $G_{i3}$ subunits, $G_{i3}$ activation of (GIRK1)$_2$/GIRK4$_2$, and increased $I_{K_{Ach}}$, resulting in hyperpolarization of the myocyte membrane and a decrease in heart rate. Changes in the level of expression of $G_{i2}$ have been shown to modulate the parasympathetic response in the heart and in atrial myocytes during embryonic development of the chick heart. Viral expression of $G_{i2}$ in the mouse atrioventricular node was shown to increase parasympathetic response. The finding that insulin treatment reversed parasympathetic dysfunction and stimulated GIRK1 expression in the atrium of the Akita mouse, whereas increasing $I_{K_{Ach}}$ in atrial myocytes from these mice supported a role of decreased GIRK1 expression in the parasympathetic dysfunction in the diabetic heart. Studies comparing GIRK4 expression suggested a similar decrease in GIRK4 expression in atria of Akita mice compared with WT (data not shown). Thus, data presented in the present study are the first to suggest that regulation of GIRK expression might constitute a molecular

**Figure 6.** Insulin regulates SREBP-1 levels in Akita mice. A, Expression of SREBP-1 in nuclear extracts from atria of age-matched male WT and Akita mice, determined by Western blot analysis. B, Densitometric analysis of the autoradiograph in A normalized to the expression of $G_{i3}$ (*$P<0.05$). C, Effect of insulin treatment on SREBP-1 in atria of Akita mice. Atria of age-matched male Akita mice treated for 1 week with slow-release insulin pellets or placebo pellets were homogenized as in A, and the level of SREBP-1 was determined by Western blot analysis. D, Mean intensity of SREBP-1, determined as in B from 4 placebo- and 4 insulin-treated Akita mice (*$P<0.05$). E, Effect of phloridzin treatment of Akita mice on levels of SREBP-1. Age-matched male Akita mice were treated with phloridzin or vehicle and SREBP-1 and determined as in C, F, Mean intensity of SREBP-1, determined as in B from 7 phloridzin-treated and 6 vehicle-treated Akita mice.

**Figure 7.** Adenoviral expression of SREBP-1 restores the impaired $I_{K_{Ach}}$ in atrial myocytes from Akita mice to levels similar to those in cells from WT mice. A, I-V plots of $I_{K_{Ach}}$ from dissociated atrial myocytes from Akita mice infected with Ad-GFP-Gal (multiplicity of infection of 20). Forty-eight hours following infection, $I_{K_{Ach}}$ was measured in $G_{i3}$-expressing and non-$G_{i3}$-expressing cells, as described in Figure 2A. B, Mean of peak inward currents from 7 Ad-GFP-$G_{i3}$-infected and 7 Ad-GFP- SREBP-1c-infected cells from 4 separate Akita mice. **$P<0.01$. Inset, Fluorescence in a typical atrial myocyte infected with an adenovirus expressing GFP (90% of cells).
mechanism in the pathogenesis of the parasympathetic dysfunction in the diabetic heart.

Although heart rate is under the control of the sinoatrial node in which pacemaker currents (I_p) and Ca currents, as well as I_{K_Ach}, contribute to the negative chronotropic response, in this study, we measured I_{K_Ach} in atrial myocytes because I_{K_Ach} is a critical mediator of parasympathetic signaling.26 The use of atrial myocytes for measurement of I_{K_Ach} is supported by the findings of Lomax et al, who demonstrated that I_{K_Ach} has very similar electrophysiological properties in both sinoatrial node and atrium and that the major difference was the distribution of I_{K_Ach} in a gradient across the superventricular structures of the mouse atrium with the highest density in the sinoatrial node.27

A role of lipid metabolism in the regulation of the parasympathetic response of the heart is supported by prior studies in which growth of atrial myocytes in lipoprotein-depleted serum (LPDS) resulted in an increased response to muscarinic stimulation in parallel with an increase in the expression of mRNAs coding for M_3, G_{q2} and GIRK1.6,22,28 It has subsequently been demonstrated that growth of atrial myocytes in LPDS results in an increase in levels of SREBP-1 and that SREBP-1 regulates the expression of G_{q2} in atrial myocytes in response to LPDS.16 Furthermore, both the negative chronotropic response to parasympathetic stimulation and GIRK1 expression were decreased in atria of an SREBP-1 knockout mouse.17 Taken together with the findings reported here that atria of type 1 diabetic Akita mice demonstrate decreased levels of SREBP-1 and GIRK1 and that insulin treatment increased the levels of SREBP-1 and GIRK1 in Akita mice, these data further support the conclusion that insulin might regulate GIRK1 expression via an effect on SREBP-1. The finding that insulin stimulated the expression of GIRK1 in cultured atrial myocytes and that overexpression of a DN-SREBP-1 inhibited insulin-stimulated GIRK1 expression in these cells also supported the conclusion that insulin regulation of GIRK1 expression was dependent on SREBP-1.

Decreased I_{K_Ach} in atrial myocytes from Akita mice would account for the decreased negative chronotropic response of these mice to carbachol. Furthermore, insulin treatment of Akita mice restored the heart rate response to carbachol in the intact mouse and increased I_{K_Ach} in atrial myocytes from these mice to levels similar to those in atrial myocytes from WT mice. Finally, the finding that viral expression of SREBP-1 in atrial myocytes from Akita mice mimicked the effect of insulin and restored I_{K_Ach} to levels similar to those in cells from WT mice was consistent with a role of SREBP-1 in insulin regulation of the parasympathetic response. Hence, these data suggest a mechanism for parasympathetic dysfunction in type 1 diabetes in which decreased insulin levels result in decreased SREBP-1, which in turn results in decreased expression of GIRK1, attenuation of I_{K_Ach}, and parasympathetic dysfunction. The finding that glucose lowering by chloridizin23 had no effect on SREBP-1 levels and that streptozotocin-treated mice, which demonstrate a less marked hyperglycemia compared with Akita mice, supports the conclusion that the decreased SREBP-1, the associated decrease in GIRK1 expression, and parasympathetic dysfunction in the type 1 diabetic Akita mouse are attributable to hypoinsulinemia and not to hyperglycemia.

Insulin regulation of parasympathetic signaling in the heart via SREBP-1 constitutes a unique role for both insulin and SREBP-1 in a pathway not directly related to lipid, glucose, or fatty acid metabolism.

The finding that the decreased negative chronotropic response of the heart in the Akita mouse is attributable, in part, not only to a decrease in the magnitude of the initial carbachol response but also to a decrease in the duration of bradycardia and an increase in recovery of the heart rate from bradycardia suggested that the impaired parasympathetic response might also involve differences in desensitization of the diabetic heart to carbachol compared to WT. Although this study demonstrates an impaired negative chronotropic response to parasympathetic stimulation, data suggest that the basal heart rate in the Akita mouse is lower relative to WT. One explanation for this finding is that intrinsic heart rate, heart rate in the absence of autonomic stimulation, is decreased in the diabetic heart. Measurement of heart rate in the presence of propranolol and atropine demonstrated a trend to lower intrinsic heart rates in the Akita mice (data not shown). The mechanism for the lower intrinsic heart rate in the Akita mouse remains to be further studied.

These studies suggest that the cardiac autonomic dysfunction in diabetes, previously considered to be secondary to a “neuropathy,” might require a redefinition to include a unique intracardiac molecular abnormality resulting in parasympathetic dysfunction. It is interesting to speculate that parasympathetic dysfunction in the diabetic heart might result in an autonomic imbalance that might predispose the heart to the development of ventricular arrhythmias and sudden death. Hence, the regulation of the intrinsic cardiac parasympathetic signaling pathway by insulin and SREBP-1 might offer new therapeutic targets for the treatment and prevention of autonomic dysfunction and sudden death in the diabetic population.

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Disclosures
None.

References


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METHODS

Materials

Ad-GFP-DN-SREBP-1 and Ad-GFP-SREBP-1c (ADD-1) were gifts 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 heterozygous male Akita Ins2Cys96Tyr mice and the littermate wild type mice were from the Jackson Laboratories. The heterozygous Akita Ins2Cys96Tyr mice were selected by restriction digestion analysis of a PCR product of the Ins2 gene using genomic DNA of Akita mice as described.1 To monitor the progression of disease, measurements of urine glucose, protein and ketones were made with Keto-Diastix Reagent Strips for Urinalysis (Bayer). Serum glucose was determined by the hexokinase method using a Hitachi 747; hemoglobin-A1c by HPLC; blood gases and electrolytes measured in the clinical lab. Osmolarity was measured by freezing point depression (Idexx Laboratories, Grafton, MA). Phloridzin (0.4 g/kg, subcutaneously 3 times a day) was from Sigma-Aldrich. All vertebrate animal-related procedures described here were approved by the Institutional Animal Care Committee at Tufts Medical Center.

Cell culture

Embryonic chick atrial myocytes were cultured as described.2 For electrophysiological studies dissociated atrial myocytes from mouse atria were prepared by a retrograde Langendorf perfusion method as described.3

ECG monitoring in conscious mice
An implantable wireless radiofrequency transmitter was inserted and the ECG signal was recorded with the use of a telemetry receiver and an analog to digital acquisition system (Data Sciences International) as described. In one set of experiments, age-matched male WT and Akita mice (DM), 4-6 months old were studied, and in a second set of experiments, age-matched male Akita mice before and 1 week after implantation of slow-release insulin pellets (LinShin, Inc, Canada, 2U of insulin/day) were studied. Treatment regimens for propranolol and carbamylcholine are outlined in the text. The recording was analyzed using custom built software: Beat-to-beat heart rate data were obtained from the ECG signal by R-wave peak detection. Artifacts and non-sinus rhythms were detected and removed after manual review. Heart rate measurements for statistical analysis were obtained from moving average beat data. Baseline heart rate immediately prior to carbamylcholine injection and the lowest heart rate following carbamylcholine injection were used to compute the heart rate response and the 80% recovery point. The elapsed time from the carbamylcholine induced bradycardia until the initiation of recovery was defined as the plateau time of bradycardia. The recovery phase was characterized by the time elapsed until achieving 80% recovery of the baseline heart rate.

**Western blot analysis**

To determine GIRK1 and SREBP-1 levels, Western blot analysis has been done as described previously. A GIRK1 specific antibody from Alomone Labs (Jerusalem, Israel) and SREBP-1 antibodies from Santa Cruz were used. A rabbit polyclonal Gαs and a rabbit polyclonal Gβ antibodies were from Santa Cruz.

**Cellular Electrophysiology**

Membrane currents were measured by the patch-clamp technique in whole-cell mode using an LM-EPC7 amplifier as described. Briefly, immediately prior to seal formation, the
extracellular solution was replaced with a high K⁺ solution without Ca^{2+} to suppress contraction: 100 mM NaCl, 50 mM KCl, 1 mM MgCl₂, 5 mM HEPES, 5.5 mM D-glucose, adjusted to pH 7.4. Patch electrodes were pulled from 1.5 mm diameter Fischer brand glass capillaries and demonstrated a 2 MΩ resistance when filled with pipette solution: 140 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, 5 mM Mg₂ATP, 0.1 mM GTP, and pH adjusted to 7.2 with KOH. In order to obtain and maintain good seal formation required for membrane current recording, we found it necessary to suppress contractions with high external K⁺, which leads to persistent membrane depolarization and inactivation of voltage-activated Na⁺ channels, and 0 external Ca²⁺. Both of these conditions have been shown to have no effect I_{KAC}.⁵ It was necessary to suppress contraction, because of difficulty in maintaining seals on spontaneously beating cells. Data were acquired by an Axon Instrument Digitizer (Digidata 1322B) with pClamp 9 software. Whole cell currents were elicited at room temperature in the presence and absence of 20 μM carbamylcholine introduced by focal perfusion over 10-15 seconds followed by washout as described.³,⁶ Currents returned to baseline within 10-15 seconds of washout. In order to correct for differences in cell size 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.⁷ 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 2.5%
isoflurane in oxygen and maintained with inhaled 2.0% isoflurane in oxygen. Animals were placed on a warming pad to maintain body temperature at 36.5 to 37.5°C. Two-dimensional images and M-mode tracings (sweep speed 50–100 mm/s) were then 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)*100/ LVEDV; LVEDV (LV end diastolic volume) = (7 * LVEDd3)/(2.4 + LVEDd); LVESV (LV end systolic volume) = (7 * LVESd3)/(2.4 + LVESd); LVEDd, LV end diastolic diameter; LVESd, LV end systolic diameter.

**Blood gases**

For blood gases mice were anesthetized with 2.5% isoflurane in oxygen, intubated and body temperature maintained using a rectal probe and a feedback heating pad. The heart was exposed via a midline sternotomy and blood obtained via a percutaneous intraluminal left ventricular stick using a 25 gauge needle and a 1cc heparinized syringe.

**Statistical analysis**

All values are expressed as mean ±SEM. Statistical differences between mean values were calculated by ANOVA, followed by Bonferroni's test for unpaired comparisons where appropriate. For comparison of WT and DM mice, Student's t-test was applied. The effect of insulin treatment on DM mice was assessed using a paired t-test. A 2-tailed p value ≤0.05 was considered significant.
REFERENCES


### Online Table I. Blood parameters of WT and Akita mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Akita DM</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose</td>
<td>585 ± 18</td>
<td>149 ± 8</td>
</tr>
<tr>
<td>Hemoglobin A1c</td>
<td>9.41 ± 0.4</td>
<td>4.37 ± 0.09</td>
</tr>
<tr>
<td>pH</td>
<td>7.35 ± 0.03</td>
<td>7.37 ± 0.03</td>
</tr>
<tr>
<td>pCO₂</td>
<td>38.93 ± 3.7</td>
<td>35.98 ± 2.5</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>19.45 ± 0.7</td>
<td>19.95 ± 1.0</td>
</tr>
<tr>
<td>Na⁺</td>
<td>144 ± 1.5</td>
<td>144 ± 1.8</td>
</tr>
<tr>
<td>K⁺</td>
<td>4.1 ± 0.09</td>
<td>4.4 ± 0.16</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>111 ± 1.1</td>
<td>111 ± 0.7</td>
</tr>
<tr>
<td>Anion Gap</td>
<td>17.5 ± 1.5</td>
<td>16.3 ± 1.5</td>
</tr>
</tbody>
</table>

1. Blood glucose (mg/dL), 33 Akita DM and 27 WT (P < 0.001); 2. Hemoglobin A1c (%), 9 Akita DM and 7 WT (P < 0.001); 3. Blood gases (mm Hg) and electrolytes (mmol/L), ns.; 4. Anion gap (mmol/L) = ([Na⁺] + [K⁺]) - ([Cl⁻] + [HCO₃⁻]), ns. Both WT and Akita mice did demonstrate a mild metabolic acidosis, which was probably secondary to the effects of general anesthesia.
### Online Table II. Echocardiographic analysis of LV structure and function of WT and Akita mice

<table>
<thead>
<tr>
<th></th>
<th>Akita DM</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDD</td>
<td>2.78±0.08</td>
<td>2.92±0.04</td>
</tr>
<tr>
<td>ESD</td>
<td>1.46±0.07</td>
<td>1.52±0.06</td>
</tr>
<tr>
<td>Post wall</td>
<td>1.18±0.04</td>
<td>1.21±0.03</td>
</tr>
<tr>
<td>Ant wall</td>
<td>1.17±0.03</td>
<td>1.13±0.03</td>
</tr>
<tr>
<td>HR</td>
<td>400±10</td>
<td>414±6</td>
</tr>
<tr>
<td>FS</td>
<td>47.3±2.36</td>
<td>48.0±1.45</td>
</tr>
<tr>
<td>EF</td>
<td>79.8±2.24</td>
<td>80.6±1.47</td>
</tr>
</tbody>
</table>

Akita DM (n=9) and WT mice (n=10). 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 (%). No statistically significant difference was found in these parameters.
**Online Figure I. Streptozotocin treatment decreases SREBP-1 levels in the heart.**

Twelve to sixteen weeks old male C57BL/6 mice were treated intraperitoneally (i.p.) with a single dose of streptozotocin (STZ) in citrate buffer (pH 4.5), 180 mg/kg body weight, as described previously. Control mice were injected with an equal volume of citrate buffer. A subset of STZ treated mice received insulin (Regular Iletin II porcine insulin, Eli Lilly). Blood glucose in control mice, STZ treated mice and STZ+insulin treated mice were 130±4 mg/dL (n=12), 457±12 mg/dL (n=10), 140±14 mg/dL (n=11), respectively. Expression of SREBP-1 from atria of control, STZ and STZ+insulin treated mice were determined by Western blot analysis as described in Methods. These data are typical of 3 similar experiments.
Online Figure I

Control  STZ  STZ + Insulin

SREBP-1

β-actin