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Alterations in connexin 43 during diabetic cardiomyopathy: competition of tyrosine nitration versus phosphorylation

Mandar S. JOSHI^{#1,2,5}, Michael J. MIHM^{#3}, Angela C. COOK³, Brandon L. SCHANBACHER^{4,5}, and John Anthony BAUER^{4,5}

¹ Baker IDI Heart and Diabetes Institute, 75 Commercial Road, Melbourne, Victoria 3150, Australia

² Department of Medicine, Central Clinical School, Monash University, Melbourne, Australia

³ The Ohio State University College of Pharmacy, 500 W 12th Ave, Columbus, OH 43210, USA

⁴ Centre for Perinatal Research, The Research Institute at Nationwide Children's Hospital, Columbus, OH 43205, USA

⁵ University of Kentucky College of Medicine, Department of Pediatrics, Lexington KY 40536, USA

These authors contributed equally to this work.

Abstract

Objective—Cardiac conduction abnormalities are observed early in the progression of Type I diabetes, but the mechanism(s) involved are undefined. Connexin 43, a critical component of ventricular gap junctions, depends on tyrosine phosphorylation status to modulate channel conductance - alterations in connexin 43 content, distributions, and/or phosphorylation status may be involved in cardiac rhythm disturbances. We tested the hypothesis that cardiac content/distribution of connexin 43 are altered in a rat model of Type I diabetic cardiomyopathy, investigating a mechanistic role for tyrosine.

Methods—We conducted electrocardiographic analyses during the progression of diabetic cardiomyopathy in rats dosed with streptozotocin (65mg/kg), at 3, 7, and 35 days post-induction of diabetes. Following functional analyses, we conducted immunohistochemical and immunoprecipitation studies to assess alterations in connexin 43.

Results—We observed significant evidence of ventricular conduction abnormalities (QRS complex, Q-T interval) as early as 7 days post-streptozotocin, persisting throughout the study. Connexin 43 levels were increased 7d post- streptozotocin and remained elevated throughout the study. Connexin 40 content was unchanged relative to controls throughout the study. Changes in Connexin 43 distribution were also observed; connexin 43 staining was dispersed from myocyte

Corresponding author: Prof. John Anthony Bauer Kentucky Children's Hospital/UK Healthcare University of Kentucky College of Medicine, Department of Pediatrics 800 Rose Street, MN 472 Lexington, KY 40536 Office: 859-218-2927 Fax: 859-257-3739 john.bauer@uky.edu.

Disclosures

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short axis junctions. Connexin 43 tyrosine phosphorylation declined during the progression of diabetes, with concurrent increases in tyrosine nitration.

Conclusions—These data suggest that alterations in connexin 43 content and distribution occur during experimental diabetes and likely contribute to alterations in cardiac function, and that oxidative modification of tyrosine-mediated signaling may play a mechanistic role.

Keywords

Diabetes; connexins; oxidative stress; cardiomyopathy; signal transduction

Introduction

Annually some 76,000 children aged less than 15 years develop type-1 diabetes (T1D) worldwide. Majority of the morbidity and mortality associated with this disease state is directly attributable to cardiovascular causes.¹⁻⁴ T1D is complicated by severe progressive cardiovascular diseases, with approximately 80% of deaths among diabetic patients due to coronary heart disease (CHD).^{3,5} A non-atherogenic cardiomyopathy in T1D has been recognized for over 20 years^{6,7} and this occurs in roughly 30% of all T1D patients. It presents as early diastolic and electrical abnormalities followed by later impairments in left ventricular ejection fraction,⁸ arrhythmias, and sudden cardiac death.^{6,9,10} The prevalence of a prolonged Q-T interval is increased in T1D, and this pro-arrhythmic condition is predictive of cardiovascular mortality in this patient population.¹¹⁻¹³ The mechanism(s) by which cardiac impulse conduction is altered in the diabetic heart remain poorly understood, the relation of these events relative to more traditional mechanisms of cardiac disease are not well defined, and therapies directed at controlling arrhythmias and sudden cardiac death in these patients are currently not optimized.

The conduction of cardiac electrical impulses is mediated through intercellular gap junctions, which consist of specialized proteins that coordinate the mechanics of cardiac contractility. Connexins are essential protein components of cardiac gap junctions that assemble into hexameric hemichannels called connexons that conjoin between myocytes, creating low resistance channels for rapid ion transfer.^{14,15} Multiple isoforms of connexins are expressed differentially in a variety of tissues, providing selectivity regarding gap junction ion conductance.^{16,17} Connexin isoforms 43 (Cx43) and 40 (Cx40) appear to be the predominant isoforms expressed in adult cardiac tissue; as such, the relative content and/or distributions of Cx43 and Cx40 may have important implications on cardiac conduction.^{16,17} It has been demonstrated that Cx43 protein levels and distribution at the intercalated disk are altered in the diabetic heart, potentially contributing to cardiac dysfunction.^{13,18-20} Furthermore, since connexin turnover is remarkably fast relative to other cardiomyocyte proteins (half-life 1-2 hours), connexin distributions may change rapidly in response to cardiac injury.²¹ Gap junction resistance is further modulated by connexin phosphorylation status; connexins are phosphorylated at both serine and tyrosine sites that modulate channel conductivity.²² The content, distribution and phosphorylation status of cardiac connexin isoforms during the progression of diabetic cardiomyopathy, and their correlation to electrical conduction deficits, remain incompletely defined. Here we employed an experimental rat model of T1D to test the hypothesis that alterations in cardiac

connexin content and/or distribution may participate in electrical abnormalities associated with diabetic cardiomyopathy.

Several studies have demonstrated that oxidant related mechanisms participate in a wide array of diabetes-related complications. No previous studies have investigated such events as they relate to diabetes-related electrophysiological changes and/or connexin isoform status. For these reasons, we additionally investigated phosphorylation versus nitration status of connexin 43, testing the hypothesis that these may be competitive posttranslational modifications of this key protein in vivo.

Methods

Animals

All animal studies were performed in accordance with institutional guidelines.

Hyperglycemia was induced in Sprague-Dawley rats weighing 300-400g with a single dose of streptozotocin (STZ, 65 mg/kg i.p. prepared daily in citrate buffer pH 4.5) or vehicle control. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the NIH. Animals were studied longitudinally at 0, 3, 7, and 35 days post-STZ (n=6-10/time point). 6 age matched rats were treated with vehicle and studied at either 0 or 35 days, to evaluate control parameters. No statistical differences were observed between these two control groups (0 vs. 35 days) for any parameter evaluated, therefore these observations were pooled and served as control values; these control values are represented as time 0. Blood glucose was determined at each time point (0, 3, 7, 35 days) with a Glucometer Encore (Ames) clinical blood glucose monitor. Animals with blood glucose level <200mg/dl at day 3, were excluded from study. Following functional analyses, rats were sacrificed by Nembutal injection (100mg/kg), and hearts were rapidly excised, equatorially bisected just below the mitral valve, formalin-fixed, and processed for immunohistological analyses.

Electrocardiography

At 0, 3, 7, 35 days post-STZ, rats were studied non-invasively using an electrocardiographic data acquisition system (MP100, BIOPAC Systems, Inc.). Rats were anesthetized by light halothane inhalation (0.5-1% halothane) to maintain a stable plane of anesthesia while preserving physiologic heart rates. Animals were placed in the supine position on a heated gel pack during study. 3-lead recordings were collected with adhesive electrodes (BIOPAC Systems, Inc.) attached to the top of each paw. Electrocardiograms were collected digitally over a 180 second span at a sampling rate of 2000 Hz.

ECG signals were averaged over 150-200 beats in triplicate for each animal at each time point, using ACQKnowledge Software (BIOPAC Systems, Inc.). Mean P-wave duration, P-R interval, QRS complex duration, Q-T interval, and T-wave duration were determined. R-R interval was determined from the average of the entire 180 second acquisition, and was stable over the time in which waveforms were measured (p=NS). Rate-corrected QT interval was determined by Fridericia's method.

Connexin immunohistochemistry

Following fixation and paraffin embedding, cardiac tissues were prepared as 5µm cross-sections and mounted on slides for immunohistochemical analyses. Cardiac cross-sections were immunostained for connexin isoform content and distributions using specific antibodies directed against connexin isoforms 40 (1:100 dilution, Zymed, San Francisco, CA) and 43 (1:100 dilution, Zymed), as previously described.²³ Exposure of the tissue sections to 0.06% w/v diaminobenzidine followed by methyl green counterstaining provided visualization of immunoreactivity. Staining (isotypic) control tissues exposed for the same duration to non-immune rabbit IgG in place of primary antibody provided demonstration of antibody specificity.

Digital image analysis

Cross-sectional areas of each heart were visualized with an Olympus BX-40 microscope and captured using an Insight QE digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were then analyzed for extent of diaminobenzidine signal in each tissue using Image Pro Plus 4.0 (Silver Spring, MD), as previously described.²³ Extent of immunoreactivity was determined by measuring optical density of diaminobenzidine signal in each image. Integrated optical densities were determined for each image as a measure of staining intensity, and values were then averaged for each heart to provide a measure of connexin prevalence at each time point.

In parallel studies, we assessed the distributional changes in cardiac Cx43 in control and STZ-treated rats, over the same time course and in the same histological sections as described above. A digital imaging approach was developed to assess Cx43 staining intensities at the myocyte short axes versus the mid-myocyte region. In preliminary experiments, the mean distance from the short axis edge that completely encompassed Cx43 staining at the inter-myocyte junctions was determined for each control heart. The 95% confidence interval for this value was determined to be 12.5 µm from each short axis edge. This value was used to define the “short axis” staining for Cx43; all staining that was detected outside of this control confidence interval, but still contained within the myocyte, was defined as “mid-myocyte” staining for Cx43. Integrated optical densities of Cx43 staining were determined for each of these myocyte regions. Only longitudinally-oriented cardiac myocytes were assessed for Cx43 distributions by this digital imaging approach. Myocyte length, area and short axis areas analyzed were not different between treatment groups, and no age-dependant effects were observed. The longitudinal alignment of myocytes in cross-section was confirmed using a cell membrane stain, fluorescein wheat germ agglutinin (1:150 dilution in PBS) following diaminobenzidine application, and was visualized under a fluorescent microscope (Olympus BX60, FITC filter, Em 520nm).

Connexin immunoprecipitation and Western blotting

Cx43 was immunoprecipitated from LV homogenates, and then assessed for tyrosine phosphorylation and nitration status with western blotting methods. Following immunoprecipitation, Cx43 was isolated using SDS-PAGE, and probed for tyrosine phosphorylation (polyclonal anti-phospho-tyrosine, Upstate Biotechnology) and tyrosine nitration (polyclonal anti-nitro-tyrosine, Upstate Biotechnology), as we have previously

described²³. Visualization of Cx43 bands was achieved by enhanced chemiluminescence. Immunoblots for Cx43 phospho-tyrosine and nitro-tyrosine were digitally captured using a Epichemie3 Imaging System (UVP, Inc., Upland, CA). Protein band intensities were quantified by integrated optical density analysis, as previously described²³.

Statistical Analysis

All data are presented as mean $\pm$ SEM. Differences between treatment groups were assessed using One-way analyses of variance, with post hoc Dunnett's tests (comparisons against CTRL values) to evaluate significant comparisons. $p < 0.05$ described statistical significance.

Results

Rat model of hyperglycemia

Blood glucose concentrations were determined during STZ progression and are shown in Table 1. As expected, significant and rapid hyperglycemia was observed at 3 days post-STZ and persisted throughout the 35 day study. STZ-treated rats demonstrated significant cachexia during the progression of diabetes, as body weights were 30% lower than age matched controls at 35 days of diabetes. Despite this decrease in total body weight, heart weights remained unaffected, as no significant differences in cardiac wet weight were observed at any time point.

Cardiac electrophysiology

Control rats demonstrated a pronounced and detectable P-wave with each cardiac cycle, a narrow QRS complex, and a smooth S-T segment profile (Figure 1). By 7 days post-STZ, heart rate was significantly diminished, P-waves became poorly defined and/or absent in some animals and despite decreases in heart rate, T-wave duration was reduced. At 35 days post-STZ, heart rate was further decreased relative to age-matched control and P-waves were completely absent (indicative of atrial flutter/fibrillation). 35 day diabetic rats demonstrated a significantly widened QRS complex, and the S-T segment became depressed with a reversed curvature relative to control profiles.

As shown in Figure 2 a striking decrease in heart rate was detectable as early as 3 days, with a consistent increase in R-R interval observed throughout the entire study (Figure 2, upper left panel). Despite observable differences in P-wave amplitude and shape during the progression of diabetes, no significant changes in P-R interval (Figure 2, upper middle panel) or P-wave duration (Figure 2, upper right panel) were detected at 3 and 7 days post-STZ compared to control (P-wave at 35 days were undetectable in diabetic animals).

The QRS interval steadily increased during the progression of diabetes, and was statistically elevated compared to control at the 35 day time point (Figure 2, lower left panel, $p < 0.05$, 35 days post-STZ vs. age-matched control). Conversely, T-wave duration (Figure 2, lower middle panel) and corrected Q-T interval (Q-Tc, corrected for heart rate by Fridericia's method, Figure 2, lower right panel) demonstrated a bi-phasic response in diabetic animals, with an early decrease in Q-Tc interval, followed by a significant elongation of Q-Tc and T-wave interval at 35 days ($p < 0.05$ vs. age-matched control).

Cardiac immunohistochemistry

Shown in Figure 3 are representative photomicrographs from left ventricular cross-sections stained for Cx40 (Figure 3A, left panels) and Cx43 (Figure 3A, right panels). Connexin staining patterns were aligned with myocardial architecture at myocyte-myocyte junctions, with more prevalent staining observed for Cx43 compared to Cx40 (consistent with literature reports that Cx43 is the predominant isoform in adult ventricular tissue)^{16, 17}. Cx40 content was unchanged in diabetic hearts relative to control hearts, with no detectable alterations in Cx40 content or distribution at any time points studied (Figure 3B, left panel, $p = \text{NS}$). However, Cx43 levels exhibited a rapid increase as early as 3 days of diabetes, which were elevated 4-5 fold relative to control at 7 days, and remained elevated throughout the study (Figure 3B, right panel, $p < 0.05$ at 7, 35 days post-STZ).

Figure 4 illustrates representative alterations in Cx43 distribution in diabetic rats compared to controls. In control hearts, Cx43 staining was localized to the myocyte short axis regions, in discrete bands perpendicular to the myocyte long axis (Figure 4A). In contrast, Cx43 localization became highly disorganized in diabetic hearts; Cx43 staining on the myocyte short axis became wider and less linear and increased staining prevalence in the mid-myocyte regions was observed. Consistent with our whole heart histology (Figure 3), we observed significant increases in Cx43 prevalence in the short axis regions, at 7 and 35 days post-STZ (Figure 4B, right panel). Interestingly, we also saw striking increases (60-70 fold) in mid-myocyte staining for Cx43 (Figure 4B, middle panel; $p < 0.05$ at 7 and 35 days post-STZ). When expressed as a percentage of total Cx43 prevalence, mid-myocyte staining increased from approximately 3% of total staining in controls to over 20% by 3 days post-STZ (Figure 4B, left panel).

Connexin 43 immunoprecipitation

Representative immunoblots of Cx43 are shown in Figure 5A. Tyrosine phosphorylation of Cx43 decreased late in the progression of diabetes, with a statistically significant decrease observed at 35 days post-STZ (Figure 4B, closed circles). Conversely, tyrosine nitration of Cx43 showed time-dependent increases in STZ-treated rats, with significant increases at 7 and 35 days post-STZ (Figure 5B, open circles).

Discussion

A significant complication of diabetes is the development of cardiovascular disease and sudden cardiac death. Interestingly, a subset of diabetic patients develops a specific cardiomyopathy in the absence of clinically detectable atherosclerosis and/or coronary artery disease.^{6, 10-12, 24-26} In general, this unique form of cardiomyopathy presents with early reductions in diastolic performance and cardiac conduction abnormalities, followed by progressive impairment in systolic function, all developing in the absence of microvascular ischemia.^{11, 12, 24-27} Some investigators have hypothesized that this cardiomyopathy may also underlie more severe atherogenic cardiac disease in the broader diabetic population, but the cardiomyopathy is clinically undetectable under these circumstances.²⁷ The initiating events in this unique diabetic cardiomyopathy are unknown, and its participation in the development of more progressive cardiovascular disease states is undefined and thus far no

efforts have been made to specifically address this unique cardiac phenomenon therapeutically. We have previously demonstrated that the rat STZ model mimics this specific form of nonischemic cardiomyopathy, presenting with time dependent abnormalities in left ventricular contractility and relaxation.²⁸

Arrhythmia and sudden cardiac death are prevalent and serious cardiac complications of diabetes that are difficult to control therapeutically in diabetic (and non-diabetic) patients.^{1, 2, 6, 7, 9-12} Many of the currently employed pharmacological approaches for arrhythmia control have limited efficacies, life-threatening toxicities, and can influence glycemic control. Connexins are integral protein components of intercellular gap junctions, and are involved in the dynamic regulation of channel conductance (Figure 6).^{14, 15} Connexin isoform 43 (Cx43) is the predominant isoform in the cardiac left ventricle, expressed throughout the myocardium, while connexin isoform 40 (Cx40) is expressed selectively in the myocytes that are localized in the conduction system, i.e., the His bundle, the bundle branches, and the Purkinje fibers.^{16, 17, 29} These connexin isoforms provide the electrical connections that are essential for the coordinated excitation of the myocardial syncytium, and are aligned predominantly along the myocyte short axes, providing directed pathways for impulse conduction.^{14, 15} Therefore, both the content and the intracellular distribution of connexin isoforms are likely to be important for normal cardiac excitation and contraction. Very few studies describe the role for connexin content and distribution of cardiac connexin isoforms in diabetic cardiomyopathy. Here we established the time course of ECG changes in the rat STZ model of Type I diabetic cardiomyopathy, and tested the hypothesis that alterations in connexin isoform content and distribution may play a functional role in these changes.

ECG waveforms were acquired under light inhalation anesthesia, maintaining physiological heart rates to provide for more reliable and physiologically relevant cardiac performance assessments.^{28, 30} Diabetes (35 day post-STZ) was associated with the absence of normal P-wave morphology, consistent with atrial flutter or fibrillation. Widening of the QRS complex was also observed, which is often associated with impaired AV conduction or heart block. Interestingly, we observed a bi-phasic time-course of Q-T interval alterations, predominantly mediated by a shortening (7 days), followed by significant elongation (at 35 days) of the T-wave duration. This early Q-T interval response in rats is not currently described in the literature, and may represent a compensatory effort of the heart to maintain normal rhythm in the face of failing conduction. Q-Tc prolongation can predispose the heart to arrhythmia (torsades de pointes), and has been described as an independent risk factor for mortality in Type I diabetic patients.^{11, 12} These observations are highly consistent with observations in diabetic patients, as diabetes is an independent risk factor for both atrial and ventricular arrhythmias, and suggest that the STZ-treated rat is appropriate for the mechanistic study of diabetic cardiac conduction pathways.

Our immunohistochemistry data provide the first evidence that cardiac connexins are altered during diabetic cardiomyopathy, and suggest that these alterations may mediate some of the electrophysiological abnormalities associated with this condition. These alterations developed in the absence of significant cardiac structural remodeling, as this rat model does not develop overt ventricular hypertrophy or increased fibrotic deposition in our hands.²⁸

How these alterations relate to the bi-phasic changes in ECG performance is likely complex, we postulate that concurrent changes in Cx43 content, cellular distribution, and phosphorylation status all contribute to this time dependent phenomenon.

Since these studies were performed using *in situ* methods, we had the opportunity to assess both the content and distribution of Cx43 isoforms throughout the myocardium. In addition to the changes in cardiac Cx43 content, we also observed evidence of altered Cx43 distributions, as Cx43 staining appeared to migrate from its strict alignment with individual myocyte short axis connections to the mid-myocyte regions. We developed an imaging approach to assess these distributional changes quantitatively, using Cx43 staining in control rats to define the short axes distributions expected in normal healthy myocardium, and establishing 95% confidence intervals for control Cx43 distributions in myocytes that exhibited longitudinal alignment in our cross-sections. We defined Cx43 intramyocyte staining that fell outside this interval as “mid-myocyte” staining. Cx43 which has been distributed to the mid-myocyte regions is unlikely to mediate normal cardiac conduction, since this staining was virtually absent in control myocytes, and since these proteins would not provide myocyte-to-myocyte connections through the intercalated disk regions of the myocyte short axes. Using this approach, we found that in control hearts, only 2-3% of total Cx43 staining was found in the mid-myocyte region (as defined by our preliminary studies); however, by 7 days post-STZ, over 20% of Cx43 was localized to the mid-myocyte region. This corresponded to a >4000% increase in mid-myocyte Cx43 content (as determined by integrated optical density analysis) by 35 days post-induction of diabetes. Few papers have attempted to quantify intracellular connexin distributions in cardiac sections;^{18, 31} therefore altered myocyte connexin distribution may be a general and important characteristic in various settings of cardiac arrhythmia.

The resistance of connexin channels is modulated in part by connexin protein phosphorylation status at both serine and tyrosine sites.²² Tyrosine phosphorylation of Cx43 causes decreased channel conductance under most circumstances. Recent studies have shown that tyrosine signaling can be highly sensitive to oxidative environments, particularly via the formation of reactive nitrogen species (RNS) that can selectively interact with protein-bound tyrosine residues.³² Reactive nitrogen species are a family of biologically relevant oxidants derived from the interaction of nitrogen based intermediates (e.g. nitric oxide) with reactive oxygen species (superoxide anion, hydroxyl radical, hydrogen peroxide).³³ RNS can have profound cellular effects and toxicities due to the distinct reactivities of RNS relative to their reactive oxygen precursors.³⁴ These reactivities include the avid capacity to cause nitration of tyrosine residues, resulting in the stable formation of 3-nitrotyrosine residues (3NT).³⁵ Protein-3NT formation has been demonstrated to be a potent structural and functional post-translational protein modification, and has been observed in a wide array of acute and chronic cardiovascular disease states, including diabetes.^{23, 36-38} RNS formation and attendant protein nitration have been shown to modify the function of multiple proteins that depend on tyrosine phosphorylation for their activity. Following immunoprecipitation from rat left ventricles, we sequentially probed for tyrosine phosphorylation versus tyrosine nitration signal in Cx43. We found that while the tyrosine phosphorylation of Cx43 progressively decreased during the progression of diabetes,

tyrosine nitration significantly increased over this same time course. Given the high rate of connexin protein turnover in the cardiac myocyte, these data suggest that diabetes is associated with a chronic elevation in cardiac RNS formation. Since multiple connexin proteins with multiple tyrosine phosphorylation sites are present in each gap junction, it is difficult to determine whether the directional changes in phospho-tyrosine and nitro-tyrosine occur due to direct competition for tyrosine sites. Controversy exists in the literature as to whether tyrosine nitration directly blocks or can actually increase (primarily via interactions with phosphatases) other tyrosine phosphorylation pathways³⁹ and these interactions are likely to be highly context-dependent and specific to the proteins studied. Further investigations defining the exact tyrosine residues involved in these interactions will provide important insights into the mechanisms involved, and are ongoing in our laboratory. Interestingly, we observed that the time course of Cx43 tyrosine nitration strongly paralleled increases in content and mid-myocyte prevalence, suggesting that these phenomena may be linked. Cardiac RNS formation and attendant post-translational protein modifications thus may have important effects on connexin protein processing, trafficking and functionality in this setting, and may represent an addressable site for modulating impulse conduction in the heart.

In summary, this study describes first-time evidence that significant alterations in cardiac Cx43 content, distribution and tyrosine phosphorylation status occurred in an experimental model of Type I diabetic cardiomyopathy. The interactions of these changes with the cardiac conduction deficits that occur during diabetes are likely to be complex and multifactorial, but may be mediated in part by increased cardiac RNS formation that is associated with the highly pro-oxidative environment of diabetes. Our observations additionally support the potential for specific protein nitration events (e.g. to key proteins involved in myocyte performance) as contributors to cardiovascular pathogenesis. Given the lack of safe and effective pharmacological approaches for arrhythmia and rhythm control that are specific to diabetic patients, further studies defining the contribution of these alterations to conduction deficits in this setting, and the capacity of RNS to modulate Cx43 protein trafficking and function, are warranted.

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Significant findings of the study:

- Evidence of left ventricular conduction abnormalities during diabetes progression.
- Increased levels of connexin 43 but impaired trafficking away from short axis.
- Reduced connexin phosphorylation with concurrent increase in nitration.

What this study adds:

- This study demonstrates that abnormalities in cardiac conduction occur early during diabetes progression.
- Functional deficits are associated with altered connexin 43 content and distribution.
- Evidence for role of connexin 43 nitration in cardiac conduction abnormalities.

Figure 1. Representative signal-averaged ECGs from control and diabetic rats

Three lead ECGs were collected in anesthetized rats at 3, 7, and 35 days following treatment with STZ or vehicle control. Waveforms were signal averaged over 150-200 beats. *Panel A* Representative signal averaged waveform, illustrating the start and endpoints for each ECG parameter collected. *Panel B* Representative signal-averaged waveforms from control rats and diabetic rats at 7 and 35 days post-STZ showing altered P-wave morphology, abnormal S-T segment shape and QRS broadening at advanced stages of diabetic cardiomyopathy.

Figure 2. Cardiac electrophysiological parameters

Average data from control and diabetic animals at 0, 3, 7, and 35 days post-STZ. At 35 days of diabetes, P-waves were undetectable in many of the waveforms studied, and these intervals were not calculated. QTc represents Q-T interval corrected for heart rate by Fridericia's method [$Q-Tc = Q-T/(R-R \text{ interval})^{1/3}$]. In control animals, no age-dependent effects (0 vs. 35 days) were observed in any parameter studied. ○, control ; ●, diabetic. *, $p < 0.05$ vs. pooled control (0 and 35 days).

Figure 3. Selective alterations in cardiac connexin isoform content during experimental diabetes Connexin isoform content for Cx40 and Cx43 was determined by immunohistochemistry, in left ventricular cross-sections from control and diabetic animals. *Panel A* Representative photomicrographs of connexin immunohistochemistry for Cx40 (800x magnification, left panels) and Cx43 (400x magnification, right panels), brown staining indicates connexin immunoprevalence. *Panel B* Average integrated optical densities for Cx40 (left panel) and Cx43 (right panel) by digital image analysis. In control animals, no age-dependent effects (0 vs. 35 days) were observed in any parameter studied, these values were pooled and represented as Time 0. Cx43 content was significantly increased by 7 days of diabetes, and remained elevated throughout the study, while no changes in Cx40 were observed at any time point studied. *, $p < 0.05$ vs. pooled control (plotted as time 0).

Figure 4. Cardiac connexin 43 distributional changes during experimental diabetes
Cx43 distribution was studied from histological cross-sections in myocytes that demonstrated longitudinal alignment. Control tissues were used to define normal ranges for short axis Cx43 staining; mid-myocyte regions describe staining that fell out of the 95% confidence interval for short axis regions in control hearts (see Methods). *Panel A* Representative photomicrographs of single cardiac myocytes obtained from histological cross-sections. Control myocytes exhibit Cx43 staining that is strictly confined to myocyte short axes; diabetic hearts show significant increases in mid-myocyte staining, away from short axis regions. *Panel B* Digital image analysis for Cx43 staining in short axis versus midmyocyte regions. Short axis staining was increased 4-5 fold by 35 days diabetes (consistent with whole heart histology, Figure 4). Increases in mid-myocyte staining was much more dramatic, with 40-50 fold increases in Cx43 integrated optical densities observed at 7 and 35 days post-STZ. The average percentage of total Cx43 staining that exhibited mid-myocyte localization (mid-myocyte IOD/total myocyte IOD) for each time point is shown in the lower panel. *, $p < 0.05$ vs. pooled control (plotted as time 0).

Figure 5. Cardiac connexin 43 tyrosine status was significantly altered in diabetic hearts
Cx43 was immunoprecipitated from cardiac left ventricular homogenates, then probed for tyrosine phosphorylation and nitration. *Panel A* Representative western blots from control and diabetic hearts at 3, 7, and 35 days post-STZ. *Panel B* Digital image analysis for band intensities, expressed as a percent of control staining. IOD, integrated optical density (band intensity x band area). *, $p < 0.05$ vs. control (plotted as time 0).

Figure 6. Connexins are gap junction proteins that facilitate impulse conduction in the heart Connexins are critical components of cardiac gap junctions. These channels consist of multiple isoforms; Cx43 and Cx40 predominate in cardiac tissue. Cardiac conduction is modulated by channel composition & distribution; connexin size, number, and spatial distribution determine ion flow and thus the conduction in the heart.

Table 1
GLYCEMIC CONTROL PARAMETERS IN CONTROL & STZ-TREATED RATS

	Vehicle Control		Streptozotocin-treated		
	Day 0	Day 35	Day 3	Day 7	Day 35
Blood Glucose (mg/dL)	106.6±4.5	90.0±2.8	425.7±26.7 [*] p<0.001	491.3±39.5 [*] p<0.001	535.2±37.6 [*] p<0.001
Body weight (grams)	374.4±7.0	432.5±10.6 [*]	351.3±8.3	344.2±9.2	315.8±17.4

^{*} - when compared to Day 0 vehicle control