Increased efferent cardiac sympathetic nerve activity and defective intrinsic heart rate regulation in type 2 diabetes

Running title: cardiac SNA and responsiveness in diabetes

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Word Count: 4236
Number of tables: 2
Number of figures: 7
Abstract

Elevated sympathetic nerve activity (SNA) coupled with dysregulated β-adrenoceptor (β-AR) signaling is postulated as a major driving force for cardiac dysfunction in type 2 diabetes, however cardiac SNA has never been assessed directly in diabetes. Our aim was to measure the sympathetic input to and the β-AR responsiveness of the type 2 diabetic heart. *In vivo* recording of SNA of the left efferent cardiac sympathetic branch of the stellate ganglion in Zucker Diabetic Fatty rats revealed an elevated resting cardiac SNA and doubled firing rate compared to non-diabetic rats. *Ex vivo*, in isolated denervated hearts, the intrinsic heart rate was markedly reduced. Contractile and relaxation responses to β-AR stimulation with dobutamine were compromised in externally paced diabetic hearts, but not in diabetic hearts allowed to regulate their own heart rate. Protein levels of left ventricular β1-AR and Gs were reduced, whereas left ventricular and right atrial β2-AR and Gi levels were increased. The elevated resting cardiac SNA in type 2 diabetes, combined with the reduced cardiac β-AR responsiveness, suggest that maintenance of normal cardiovascular function requires elevated cardiac sympathetic input to compensate for changes in the intrinsic properties of the diabetic heart.
Introduction

Diabetes mellitus is a strong independent risk factor for the development of cardiovascular complications and congestive heart failure (1-3). Cardiac autonomic dysfunction (CAD) is an undervalued, but significant, cause of morbidity and mortality in diabetes, most likely because CAD pathology is poorly understood (4). Among the characteristics of CAD, diabetic patients have an elevated resting heart rate (HR), but a paradoxically lower peak HR, which may result from impaired cardiac sympathetic innervation (4-6). Uptake of the sympathetic neurotransmitter noradrenaline (NA), using metaiodobenzylguanidine (MIBG), is reduced in type 2 diabetic vs. non-diabetic subjects screened for ischemic heart disease (7). Moreover, MIBG uptake is lower in type 2 diabetic patients with cardiac autonomic dysfunction than those without (7). These data suggest that diabetes progressively reduces sympathetic nervous input to the heart, limiting cardiac reserve.

An alternative explanation is that sympathetic nervous input is normal or elevated in diabetes, but the β-adrenoceptor (AR) responsiveness of the heart is reduced, a mechanism well described in heart failure (8; 9). Isolated heart preparations from type 1 diabetic rat models describe a reduced inotropic (contraction), lusitropic (relaxation) and chronotropic (rate) β-AR responsiveness (10-13), which is associated with downregulation of cardiac β<sub>1</sub>-ARs (11; 14-16). Increased cardiac NA content and spillover in type 1 diabetic rats also suggests elevated sympathetic nervous activity (SNA) in diabetes (17; 18). Moreover, diabetes in humans is associated with augmented activation of SNA in other organs, such as skeletal muscle (19; 20). However, central control of SNA to peripheral organs is differentially regulated (21-23). Importantly, obese humans with insulin-resistance show increased skeletal muscle SNA and renal NA spillover, but reduced cardiac NA spillover (24). Thus, while these studies suggest a key role for the sympathetic system in cardiac dysfunction in diabetes, direct measurements of the sympathetic nervous input to the diabetic heart have not yet been reported. Furthermore, it is unclear whether diabetic cardiac dysfunction results from changes in sympathetic nervous input to the heart (cardiac SNA), a reduction in β-AR responsiveness of the heart, or both. Identifying underlying processes of diabetic autonomic
dysfunction is important, as traditional treatment of patients with type 2 diabetes with beta-blockers is of benefit, however to a lesser extent than to non-diabetic patients (25); and it is even more critical for the development of potential new therapeutic targets that interrupt myocardial autonomic signaling, such as β-ARKct (26), β3-AR agonists (27), or renal denervation (28). This study uses direct in vivo recordings of efferent cardiac SNA and β-AR responsiveness of isolated denervated hearts from Zucker type 2 Diabetic Fatty (ZDF) rats to establish whether type 2 diabetes 1) increases cardiac SNA and 2) reduces cardiac β-AR responsiveness. Finally, we tested whether type 2 diabetes reduced the expression of β1- and β2-ARs and their downstream signaling G proteins.
Research Design and Methods

Animals

All experiments were approved and conducted in accordance with the guidelines of the Animal Ethics Committee of the University of Otago, New Zealand. Experiments were conducted on 20-week old male type 2 diabetes Zucker Diabetic Fatty (ZDF) (fa/fa, n = 26) rats and their non-diabetic littermates (+/+, n = 26).

Recording of cardiac SNA and β-AR responsiveness in vivo

Recordings of cardiac SNA, arterial blood pressure (ABP) and left ventricular cardiac function were performed in 12 non-diabetic and 12 type 2 diabetic ZDF rats in vivo, as previously described (29). In brief, animals were anesthetized with urethane (1.5 g/kg, intraperitoneal), intubated and ventilated (tidal volume ~ 3.5 ml; breathing rate ~80/min). A blood sample was taken to measure blood glucose and insulin. A left thoracotomy was performed between the first and second rib exposing the stellate ganglion. The cardiac sympathetic nerve was identified as a branch from the stellate ganglion and dissected free of surrounding connective tissue. After cutting the nerve, the proximal section was placed on a pair of platinum recording electrodes to measure nerve activity. Consequently, the nerve activity being measured was from the efferent sympathetic nerves. The recorded signal was filtered (low cut-off 0.1 kHz; high cut-off 1 kHz), amplified and subsequently passed through an amplitude discriminator to quantify nerve discharge frequency (impulse frequency). The raw signal was rectified and integrated (1-sec resetting interval) online, and the integrated nerve signal was displayed in real time. At the end of the experiments, a post-mortem nerve activity measure was performed for background subtraction of noise level, which was not different between groups.

Systemic ABP was measured through a femoral artery cannula, and HR was derived from the arterial systolic peaks. The right carotid artery was cannulated with a 1.5 F Millar pressure-volume (P-V) catheter (model SPR-869), which was then advanced into the left ventricle (LV) for the
continuous measurement of LV pressure (LVP) and LV volume (LVV), which combined provided LV end-diastolic pressure (LVEDP), LV end-systolic pressure (LVESP), LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV). From these measurements stroke volume (SV), cardiac output (CO), maximum rate of contraction (+dP/dt\textsubscript{max}) and relaxation (-dP/dt\textsubscript{max}) were derived. The Millar P-V catheter was calibrated using a sphygmomanometer (pressure), and volumetric cuvettes (volume) and hypertonic saline injections (for volumetric corrections), as previously described in detail (30).

Baseline cardiac SNA was recorded, and administering dobutamine (β\textsubscript{1}-agonist; 0.6 to 10 µg/kg/min) tested the in vivo cardiac response to β-AR stimulation.

**Immunohistochemistry**

After measuring the cardiac SNA, frozen sections of isolated left sympathetic cardiac nerve of 2 non-diabetic and 3 diabetic ZDF rats that had been fixed in 4% paraformaldehyde were cut at 12 µm, and processed as described previously (31). Sections were incubated with anti-neurofilament 160 (NF) polyclonal primary antibody (AB64300, Abcam, dilution 1:500 in Tris immunodiluent) for axon staining, or incubated with anti-tyrosine hydroxylase (TH) polyclonal primary antibody (AB152, Millipore, dilution 1:1000) for staining of sympathetic axons. Primary antibodies were detected using incubation with anti-species secondary antibody (AlexaFluor 488 conjugated Goat anti-rabbit IgG, Life technologies, dilution 1:500 in Tris immunodiluent). The total axon number (NF) and the number of sympathetic axons (TH) per nerve were determined for each of the nerves.

**Ex vivo cardiac β-AR responsiveness**

Intrinsic cardiac function and responsiveness to β-AR agonist stimulation were determined using Langendorff-perfused isolated hearts in 14 non-diabetic and 14 type 2 diabetic ZDF rats ex vivo as previously described (32). In brief, animals were anesthetized with pentobarbital (60 mg/kg), the
heart was excised, mounted on a Langendorff apparatus, and the aorta was retrograde perfused with Krebs-Henseleit buffer (KHB) at 37°C a constant pressure of 80 mmHg. The KHB contained the following concentrations in mM: 118.5 NaCl, 4.7 KCl, 1.2 MgSO₄·7H₂O, 1.2 KH₂PO₄·H₂O, 1.4 CaCl₂, 25.0 NaHCO₃ and 11.0 glucose and was continuously equilibrated with 95% O₂ and 5% CO₂ (pH 7.4). A custom-made balloon-tipped catheter connected to a hydrostatic pressure transducer was inserted into the LV to measure isovolumetric LV pressure. Two stimulating electrodes, one at the apex and the other on the right atrium, were used for pacing of the heart when required. The HR, LV developed pressure (Pdev), maximum rate of pressure development (+dP/dtmax), maximum rate of relaxation (-dP/dtmax) and time constant of relaxation (Tau) were calculated from the LV pressure trace.

Diabetic and non-diabetic rats were divided into two groups: unpaced and paced. Isolated hearts from the unpaced group were allowed to beat at their own intrinsic rate enabling simultaneous determination of both inotropic (contraction), lusitropic (relaxation) and chronotropic (rate) properties. The hearts from the paced (to exclude the influence of rate) group were paced at 5 Hz (~300 bpm, physiological resting HR) to exclude chronotropic properties. Hearts from both unpaced and paced groups were exposed to dobutamine (β₁-agonist; 1×10⁻⁹ - 1×10⁻⁶ M, 5 minutes/dose) to assess responsiveness to β-AR agonist stimulation. Maximal developed pressure (Pmax) was determined with a post-extra-systolic rest potentiation protocol (33).

**Determination of G-protein and β-AR subtype protein expression**

Protein lysates from LV and right atrial (RA) tissue of in vivo and ex vivo hearts were separated on 12% SDS-polyacrylamide gels and transferred onto PVDF membranes for β₁-AR and nitrocellulose for β₂-AR, Gₛ and Gᵢ proteins, as described previously (34), which were subsequently probed with specific polyclonal antibodies against β₁-adrenoreceptor (rabbit, 1:500 dilution in 2% BSA; Novus Biologicals or 1:1000 dilution in 2% BSA; Genetex), β₂-adrenoreceptor (rabbit, 1:5000 dilution; Badrilla Ltd), VDAC1 (1:10,000 dilution in 2% milk, Novus Biologicals), Gₛ (rabbit, 1:1000 dilution, Novus Biologicals).
dilution; Abcam), G\textsubscript{i} (rabbit, 1:1000 dilution; Santa Cruz Biotechnologies) or GAPDH (rabbit, 1:25000 dilution; Badrilla Ltd). Proteins were visualized using an enhanced chemiluminescence detection system. The \(\beta_1\)- and \(\beta_2\)-AR protein expression was normalized to VDAC1 or GAPDH, and the \(G_\text{s}\) and \(G_\text{i}\) protein expression was normalized to GAPDH, to correct for protein loading.

Statistical analysis

Unpaired \(t\)-test was used to test for differences between groups for the \emph{in vivo} and \emph{ex vivo} variables and \(\beta_1\)- and \(\beta_2\)-AR protein expression levels. A non-parametric Mann-Whitney test was used for differences in nerve CSA and number of axons per nerve. Two-way ANOVA with repeated measures was used to test for \emph{in vivo} and \emph{ex vivo} \(\beta\)-AR responsiveness group comparisons, followed by a Bonferroni \emph{post hoc} analysis. \(P < 0.05\) was considered statistically significant. Results are presented as means ± SEM.
Results

ZDF(fa/fa) rats had higher non-fasting blood glucose, plasma insulin level and body mass compared to their littermates ZDF(+/+), confirming their type 2 diabetes and obese status (table 1). Type 2 diabetic ZDF rats showed no structural LV hypertrophy (similar to normalized heart mass), but they did show decreased LV filling (e.g. significant 16 ± 3% decrease in LVEDV; $p < 0.05$) and decreased SV (21 ± 4% decrease in SV; $p < 0.05$), suggesting diastolic dysfunction. There was no difference in in vivo resting HR, CO and MABP between type 2 diabetic and non-diabetic animals (table 1).

In vivo cardiac SNA

A representative direct recording of cardiac SNA in vivo in non-diabetic and type 2 diabetic ZDF rats is shown in Figure 1A. The direct cardiac SNA recordings revealed a 45% increase in resting cardiac SNA in the type 2 diabetic group, as shown from the averaged integrated cardiac SNA values (1.25 ± 0.17 and 1.87 ± 0.18 µV·s, non-diabetes vs. type 2 diabetes, respectively; $p < 0.05$, Figure 1B). Moreover, the mean nerve firing rate was twice as high in diabetic vs. non-diabetic rats (21.1 ± 4.1 and 45.0 ± 8.7 impulses/s, non-diabetes vs. type 2 diabetes, respectively; $p < 0.05$, Figure 1C). This elevated SNA, recorded in vivo from the left efferent cardiac sympathetic nerve, shows that the sympathetic input to the heart is increased in a type 2 diabetic rat model.

Cardiac sympathetic nerve

The cross sectional area of the left cardiac sympathetic nerve was not different between the non-diabetic and type 2 diabetic group (4399 ± 397 and 4268 ± 620 µm², non-diabetes (n=2) vs. type 2 diabetes (n=3), $p > 0.05$, Figure 2B). Moreover, the immunohistochemical NF and TH staining of the nerves revealed that neither the total number of axons (Figure 2C) nor the number of sympathetic axons (Figure 2D) per nerve were different between non-diabetic and diabetic group.
(1087 ± 196 and 989 ± 213 number of NF axons per nerve, and 984 ± 93 and 928 ± 181 number of TH axons per nerve; non-diabetes (n=2) vs. type 2 diabetes (n=3), p > 0.05).

In vivo hemodynamic responses to β-AR stimulation

Both the non-diabetic and type 2 diabetic ZDF animals responded to the β-AR agonist dobutamine with increases in HR, SV and CO (Figure 3A-C). No significant acceleration of the cardiac contractility (+dP/dt_{max}) or relaxation (-dP/dt_{max} and Tau) parameters were observed (Figure 3D-F), and the mean arterial blood pressure and total peripheral resistance (TPR) both decreased (Figure 3G-H) compared to saline injection. During incremental dobutamine infusion, the increase in stroke volume was attenuated in type 2 diabetic vs. non-diabetic rats (∆21.7 ± 3,3 vs. ∆6.7 ± 3,9 µl; at 10 µg/kg/min dobutamine; respectively; p < 0.05; figure 3B), indicating a reduced inotropic response to β-AR stimulation in type 2 diabetes in vivo. There were no differences in the response of any other hemodynamic variables between both groups.

Ex vivo animal and cardiac characteristics

The type 2 diabetic rats had elevated non-fasting blood glucose levels (Table 2), whereas their heart weight and LV V_{max} were not different, indicative of type 2 diabetes without cardiac structural remodeling. Interestingly, resting intrinsic HR was 32% lower in the unpaced isolated hearts of type 2 diabetic rats. Consistent with the in vivo data, the developed pressures in the LV (P_{dev}), the maximum rate of contraction (+dP/dt_{max}) and the maximum rate of relaxation (-dP/dt_{max}) were unchanged in type 2 diabetic animals, whereas late relaxation (Tau) was prolonged in the paced type 2 diabetic hearts. The maximal LV pressure (P_{max}) was not different between both groups. No differences in animal characteristics and resting cardiac function were observed between the unpaced and paced groups (Table 2).

Ex vivo cardiac responses to β-AR stimulation - paced vs. unpaced
A greater β-AR agonist concentration was required to achieve a target HR in unpaced type 2 diabetic vs. non-diabetic hearts (Figure 4A). In addition, diabetic hearts reached a lower peak HR than non-diabetic hearts at the highest dobutamine dose used. In contrast, LV pressure for a given β-AR agonist concentration was not different between groups (Figure 4C), suggesting altered chronotropic but preserved inotropic β-AR responsiveness.

When HR and LV pressures were normalized (as a percent of maximum), the relative chronotropic and inotropic β-AR responsiveness were not different between groups (Figure 4B and 4D). Moreover, the normal physiological lusitropic (relaxation) response to β-adrenergic stimulation was observed in both groups by an increased rate of maximum relaxation (-dP/dt_{max}, Figure 4E) and an accelerated late relaxation (Tau, Figure 4F).

When the dobutamine protocol was performed at a paced HR of 300 bpm (to exclude the influence of rate), type 2 diabetic hearts developed less LV pressure at higher concentrations of the β-AR agonist (Figure 5A), and the normalized LV pressure curve shifted to the right (Figure 5B), confirming reduced inotropic responsiveness to β-AR stimulation. When paced, the changes in maximum rate of relaxation (-dP/dt_{max}, Figure 5C) were greater during β-AR stimulation in the non-diabetic hearts compared to unpaced hearts, whereas the change in late relaxation (Tau) was absent in the diabetic heart (Figure 5D).

**β-AR subtype and G protein expression levels**

β_1-AR protein expression was 23% lower in the left ventricle (LV, Figure 6A+B) and 17% higher in the right atrium (RA, Figure 6E+F) of type 2 diabetic hearts compared to the non-diabetic hearts (LV: 1.41 ± 0.11 vs. 1.09 ± 0.07; RA: 0.88 ± 0.02 vs. 1.02 ± 0.04, A.U., non-diabetes vs. type 2 diabetes; *p* < 0.05 for both). β_2-AR protein expression level was 41% higher in the LV (Figure 6C+D) and 20% higher in the RA (Figure 6G+H) of the type 2 diabetic hearts compared to the non-diabetic hearts (LV: 1.26 ± 0.10 vs. 1.78 ± 0.19; RA: 0.82 ± 0.03 vs. 0.99 ± 0.2, A.U., non-diabetes vs. type 2 diabetes; *p* < 0.05 for both).
$G_s$ protein expression level was 28% lower in the left ventricle (LV, Figure 7A+B) and 39% lower in the right atrium (RA, Figure 7E+F) of type 2 diabetic hearts compared to the non-diabetic hearts (LV: 0.89 ± 0.04 vs. 0.64 ± 0.07; RA: 0.90 ± 0.07 vs. 0.54 ± 0.03, A.U., non-diabetes vs. type 2 diabetes; $p < 0.05$ for both). $G_i$ protein expression level was 30% higher in the LV (Figure 7C+D) and 27% higher in the RA (Figure 7G+H) of the type 2 diabetic hearts compared to the non-diabetic hearts (LV: 0.52 ± 0.03 vs. 0.68 ± 0.06; RA: 0.90 ± 0.04 vs. 1.14 ± 0.05, A.U., non-diabetes vs. type 2 diabetes; $p < 0.05$ for both).
Discussion

This study showed that resting and β-AR-stimulated hemodynamics of type 2 diabetic and non-diabetic rats were similar in vivo, despite the diabetic heart receiving a greater efferent SNA and having reduced β₁-AR expression. Ex vivo experiments (e.g., denervated hearts) revealed that the diabetic heart had a lower intrinsic HR and required a greater β-AR stimulation to achieve a given HR or contractile state (reduced β-AR responsiveness). These findings suggest that maintenance of normal cardiovascular hemodynamics requires elevated cardiac sympathetic input to compensate for changes in the intrinsic properties of the type 2 diabetic heart.

Previous studies (17-20) suggested a key role for increased sympathetic input to the diabetic heart in modulating cardiac dysfunction, however findings were all based on indirect measurements of cardiac SNA. This study, using direct recording of the left efferent cardiac sympathetic nerve, is the first to provide direct evidence that SNA is elevated in the diabetic heart. Ganguly and coworkers (17; 18) demonstrated a nearly two-fold increase in cardiac noradrenaline (NA) concentrations, enhanced catecholamine turnover, and increased initial rate of NA uptake after eight weeks of type 1 diabetes in rats. On the other hand, the reduced inotropic response of electrically stimulated left atria of type 1 diabetic rats has been related to an impairment of NA release from sympathetic nerve terminals (35). Cardiac NA spillover provides an estimation of cardiac SNA, however the relationship between actual SNA and the NA spillover is not linear and high rates of nerve discharge produce a plateau in the neurotransmitter release (36). In addition, only a variable fraction (estimated at 20%) of the NA released from the nerve terminals actually enters the plasma while the majority returned to the nerve varicosity via the NA transporter (37). Elevated ‘low frequency’ heart rate variability (HRV) is reported in diabetic patients and has been associated with increased cardiac sympathetic activity and cardiovascular morbidity and mortality (38). However, others have shown that atropine abolishes most of the low frequency HRV (39; 40), calling into question the association of HRV with cardiac SNA. Our data therefore are the first to directly
quantify the increase in sympathetic input to the type 2 diabetic heart, revealing a 45% increase in integrated efferent cardiac nerve activity and a doubling of its firing rate in diabetes. Chidsey and Braunwald (41) noted loss of cardiac sympathetic innervation in failing hearts, whereas Levin et al. (42) showed a marked lowering of cardiac NA levels in obese Zucker rats, both suggesting reduced cardiac sympathetic innervation. We were unable to demonstrate a difference in the nerve cross sectional area or in the number of NF-positive or TH positive axons within the left cardiac sympathetic nerve between non-diabetic and type 2 diabetic ZDF rats. This suggest that the increase in cardiac SNA in diabetes may not be due to atrophy or partial denervation, nor due to hypertrophy, of the cardiac nerve. Overall these data suggest that type 2 diabetes does not affect the numbers of sympathetic axons (structural input) within the cardiac sympathetic nerve, but increases its sympathetic nerve activity (throughput).

The increase in cardiac SNA may have been a consequence of cellular changes in the diabetic heart. The intrinsic HR was 32% lower in the isolated type 2 diabetic hearts at rest, whereas the chronotropic (rate), inotropic (contraction) and lusitropic (relaxation) responses to β-AR stimulation were the same as non-diabetic hearts. This lower intrinsic HR at rest is observed in many experimental rodent models of diabetes (12; 43; 44). Increased sympathetic input to the diabetic heart, which accelerates spontaneous depolarization and increases conduction velocity through the heart, could explain the observed changes in function and β-ARs and G protein expression in the diabetic heart, and why, under in vivo conditions, the HR was not different between non-diabetic and diabetic animals.

However, changes in the intrinsic HR are primarily determined by intracellular mechanisms within the sinoatrial node cells (vs. autonomic nervous system). Recently it was shown in rodents that the training-induced decrease in HR was not a consequence of changes in the activity of the autonomic function, but was caused by training-induced intrinsic electrophysiological changes within the sinoatrial node (45). Metabolic changes occurring during diabetes are also known to affect the modulation of the intrinsic heart rate, and therefore might provide an alternative
explanation for our finding that the intrinsic HR of the diabetic heart was reduced. For example, carnitine supplementation corrected the reduced free carnitine levels and normalized intrinsic HR in type 1 diabetic rats, suggesting that myocardial substrate availability plays an important role in HR regulation beyond autonomic tone (46).

When isolated hearts were paced to remove the confounding effects of rate, we found that the diabetic hearts had reduced inotropic and lusitropic responsiveness to β-AR stimulation. Interestingly, in the diabetic rats under in vivo conditions (no difference in HR at rest) and in their unpaced isolated hearts (HR reduced at rest), both the inotropic and lusitropic responses to β-AR stimulation were preserved. This suggests that changes in the intrinsic functions of the diabetic heart, by means of a reduction in absolute intrinsic HR, would ensure normal inotropic β-AR responsiveness (potentially by improving its lusitropic window). These findings therefore may indicate that metabolically altered chronotropic incompetence, rather than inotropic impediment is an important, and undervalued, feature of the diabetic heart (47).

The reduced inotropic and lusitropic responses to β-AR stimulation in the paced diabetic hearts have been observed in other experimental diabetes models (10-13; 15) and have been attributed to reductions in mRNA and protein levels of β₁-ARs (11; 14-16), the most predominant β-AR subtype in the healthy heart. β₁-AR stimulation increases cAMP via Gₛ coupled proteins, which augments contraction by increasing intracellular calcium flux during systole. Therefore, the reductions in β₁-AR (23%) and Gₛ (28%) in the diabetic LV’s are consistent with a blunted stimulatory response from the increased efferent SNA, and likely contributed to the observed changes in cardiac function of our diabetic hearts. Surprisingly, the β₁-AR expression in the RA was increased (17%) with an associated reduction of Gₛ expression (39%). This could indicate specific uncoupling of the β₁-AR form its downstream signaling proteins (48). Alternatively, β₂-ARs have a higher relative expression level in the sinoatrial node region (49) and, as a consequence of the co-localization of β₂-AR with the funny channels in the caveolae, have been shown to contribute more to chronotropic changes compared to β₁-ARs (50). We found that β₂-AR protein
expression was increased in the LV (40%) and in the RA (20%) of the diabetic hearts with concomitant increases of protein expression of $G_i$ (30% and 27% respectively), which to the best of our knowledge is the first report in a type 2 diabetic rodent model. It is unclear what the functional relevance of this shift from $\beta_1$-AR to $\beta_2$-AR in type 2 diabetes is, although similar relative results have been observed during heart failure (9). In the healthy heart, it is believed that $\beta_2$-ARs do not directly contribute to changes in contraction because their co-localization with $G_i$ protein-activated phosphodiesterases (PDE) inhibits the cAMP pathway and prevents the resultant increase in calcium fluxes. Interestingly, in heart failure redistribution of $\beta_2$-ARs changes this compartmentation of cAMP and might contribute to the failing myocardial phenotype (51). Moreover, recent evidence indicates that $\beta_2$-ARs modulate intracellular oxygen availability (52) and are linked to increases in metabolic kinases, such as AMP activated protein kinase (AMPK) (52; 53). AMPK is considered a critical sensor of cellular energy, activated by biguanide drugs (metformin and phenformin) and an attractive metabolic target for novel therapies in the treatment of type 2 diabetes (54). Therefore, the relation of $\beta_2$-ARs, their (un)coupling to $G_s$ and $G_i$ proteins, and their links to functional and metabolic challenges in type 2 diabetes, especially related to chronotropic modulation of cardiac function, warrants further research.

Limitations

Surgery and anesthetic agents modulate the SNS and therefore caution is needed when interpreting data from anesthetized animals during surgery. Whilst direct assessment of cardiac SNA in conscious sheep is possible (55), no ovine diabetic model exists. Unfortunately in rodents due to the limited accessibility of the cardiac nerves, telemetric recordings are not feasible. We used urethane because of its minimal effects on autonomic, cardiovascular and respiratory systems (56).

SNA measurements in other cardiac diseases, such as myocardial infarction (29; 57) or more severe heart failure (58; 59), have found larger changes in cardiac SNA. Nevertheless, our relative
small, but significant, change in cardiac SNA in type 2 diabetes, if maintained over long periods during the progression of diabetes, may still have very detrimental effects on cardiac function.

Acute central administration of leptin activates the SNS (60), increases lumbar and renal SNS activity (61) and increases arterial blood pressure (62), all consistent with the increased cardiac sympathetic activity. However, this suggests that a leptin-deficient animal would, if anything, have reduced cardiac sympathetic activity. Therefore, we believe that the development of type 2 diabetes independently explains the changes in cardiac SNA and resultant changes in myocardial β-adrenergic responsiveness in our study.

In conclusion, this is the first study to demonstrate, with direct measurement of the efferent cardiac sympathetic nerves, elevated resting cardiac SNA in type 2 diabetes in vivo. In contrast, the intrinsic properties of the diabetic heart showed lower HR and rate-independent contractility. In this context, the elevated cardiac SNA may have been a compensatory response to a diabetes–induced lower intrinsic HR rate; ensuring normal hemodynamics in vivo. The proportion of β₁/β₂-AR and associated Gₛ and Gᵢ proteins in the left ventricle was reduced by diabetes, consistent with other models of high cardiac SNA, such as congestive heart failure. Thereby, in our type 2 diabetic animal model without any other cardiovascular disease, we demonstrated that sympathetic hyperactivity and a defective intrinsic HR regulation are associated with cardiac dysfunction and β-AR dysregulation in type 2 diabetes.
Author’s contribution


Acknowledgements

R.R.L. 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.

Part of this study has been published as abstract: HPA Thaung et al. New Zealand Medical Journal, 125 (1367), 2012; and HPA Thaung et al. Proceedings of the Australian Physiological Society, 43-193P, 2012.

Funding

The work was supported by grants from HealthCare Otago Charitable Trust, the New Zealand National Heart Foundation Grant No1491 - NH Taylor Charitable Trust, and by the Department of Physiology from the University of Otago.

Duality of Interest

None
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Figure legends

**Figure 1** - Direct *in vivo* recordings of cardiac SNA: increased sympathetic input to the heart in type 2 diabetic ZDF rats. A. Representative LabChart recordings showing ABP, raw efferent cardiac SNA and the derived integrated cardiac SNA in non-diabetic and type 2 diabetic ZDF rats. B. Increased integrated resting cardiac SNA, and C. a trend towards an increased nerve firing rate in type 2 diabetic ZDF rats. Abbreviations: SNA, sympathetic nerve activity; ABP, arterial blood pressure. Unpaired t-test, *p* < 0.05, significantly different from non-diabetic rats, n = 11 per group. Data are presented as mean ± SEM.

**Figure 2** - A. Example of left cardiac sympathetic nerves immunohistochemical stained for NF and TH from non-diabetic (control) and type 2 diabetic ZDF rats; scale bar is 50µm. B. The CSA of the cardiac sympathetic nerves, C. the total number of axons per nerve (stained with NF), and D. the total number of sympathetic axons per nerve (stained with TH) were not different between non-diabetic and type 2 diabetic ZDF rats. Abbreviations: CSA, cross sectional area; NF, neurofilament; TH, tyrosine hydroxylase. Non-diabetes (n=2) vs. type 2 diabetes (n=3), *p* > 0.05, non-parametric Mann-Whitney test. Data are presented as mean ± SEM.

**Figure 3** - Preserved hemodynamic responses to β-AR stimulation *in vivo* in type 2 diabetic ZDF rats. In both the non-diabetic (closed circles) and type 2 diabetic (open circles) ZDF rats incrementing doses of β-AR agonist (dobutamine) resulted in an increase in A. heart rate (HR), B. stroke volume (SV) and C. cardiac output (CO) compared to saline injection. No change in the cardiac contractile parameter D. speed of contraction (+dP/dt\textsubscript{max}), or the relaxation parameters E. speed of relaxation (-dP/dt\textsubscript{max}) and F. time constant of relaxation (Tau) were observed compared to saline injection, whereas G. the mean arterial blood pressure (MABP) and H. the calculated total peripheral resistance (TPR) both decreased with β-AR agonist stimulation compared to saline
injection. The type 2 diabetic rats only had a lower SV compared to the non-diabetic animals. Two-way ANOVA repeated measures; * significant vs. saline (baseline), # significant vs. non-diabetes, both p < 0.05; n = 7 per group; data are mean ± SEM.

**Figure 4** - Altered chronotropic but preserved inotropic and lusitropic β-AR responsiveness in unpaced isolated type 2 diabetic ZDF hearts. A. Unpaced isolated type 2 diabetic hearts (open circles) required a higher β-AR agonist (dobutamine) concentration to achieve a given absolute heart rate (HR) compared to non-diabetic hearts (closed circles), B. however after normalization the HR response was not different between groups. C. Developed LV pressure (LVP_{dev}) increased with β-AR agonist stimulation in both groups, but showed no difference between non-diabetic and type 2 diabetic hearts, D. even after normalization. E. Both non-diabetic and type 2 diabetic hearts showed increased speed of early relaxation (-dF/dt_{max}) and F. a faster late relaxation (Tau) due to β-adrenergic stimulation. Thus, altered chronotropic but preserved inotropic and lusitropic β-AR responsiveness in unpaced isolated hearts of 20-week old male type 2 diabetic ZDF rats. Two-way ANOVA repeated measures; * p < 0.05 vs. baseline, # p <0.05 vs. non-diabetes; n = 7 per group; for normalization data were fitted with a four-parameter sigmoidal dose-response curve with variable slope; data are mean ± SEM.

**Figure 5** - Altered inotropic and lusitropic β-AR responsiveness in paced isolated type 2 diabetic ZDF hearts. A. In isolated non-diabetic (closed circles) and type 2 diabetic (open circles) hearts paced at fixed heart rate of 300 bpm, developed LV pressure (LVP_{dev}) increased with β-AR agonist stimulation, but less in the type 2 diabetic group; B. consequently the normalized dose-response curve shifted to the left in the type 2 diabetic hearts, indicative of reduced inotropic β-AR responsiveness. C. β-AR agonist stimulation increased the speed of early relaxation (-dF/dt_{max}) and D. accelerated late relaxation (Tau) in the non-diabetic hearts. In the type 2 diabetic hearts, the change in early relaxation was impaired and the change in late relaxation was absent. Two-way
ANOVA repeated measures; * p < 0.05 vs. baseline, # p < 0.05 vs. non-diabetes; n = 7 per group; for normalization data were fitted with a four-parameter sigmoidal dose-response curve with variable slope; data are mean ± SEM.

**Figure 6** - Left ventricular (LV) and right atrial (RA) β₁-and β₂-adrenoceptor (AR) protein expression levels in type 2 diabetic ZDF rats. Panels A, C, E and G show representative images of β₁- and β₂-AR Western blots in LV and RA, respectively. Panels B, D, F and H show quantitative analysis of the protein expression (mean ± SEM; 3 replicates). Shown are reduced β₁-AR protein expression levels in LV (A+B) and increased β₁-AR levels in RA (E+F) in type 2 diabetic group relative to non-diabetic group, normalized to VDAC or GAPDH protein expression levels; Unpaired *t*-test, * p <0.05 vs. non-diabetes; LV: non-diabetes n = 17, type 2 diabetes n = 20; RA: non-diabetes n = 7, type 2 diabetes n = 6 (mean ± SEM; 3 replicates). The β₂-AR protein expression levels were elevated in LV (C+D) and in RA (G+H) in type 2 diabetic group relative to non-diabetic group, normalized to VDAC or GAPDH protein expression levels; Unpaired *t*-test, * p <0.05 vs. non-diabetes; LV: non-diabetes n = 17, type 2 diabetes n = 17; RA: non-diabetes n = 7, type 2 diabetes n = 6 (mean ± SEM; 3 replicates).

**Figure 7** - Left ventricular (LV) and right atrial (RA) Gₛ and Gᵢ protein expression levels in type 2 diabetic ZDF rats. Panels A, C, E and G show representative images of a Gₛ and Gᵢ Western blots in LV and RA, respectively. Panels B, D, F and H show quantitative analysis of the protein expression. Shown are reduced Gₛ protein expression levels in LV (A+B) and RA (E+F) in type 2 diabetic relative to non-diabetic group, normalized to GAPDH protein expression levels. Gᵢ protein expression levels are elevated in LV (C+D) and RA (G+H) in type 2 diabetic relative to non-diabetic group, normalized to GAPDH protein expression levels. Unpaired *t*-test, * p <0.05 vs. non-diabetes; non-diabetes n = 7, type 2 diabetes n = 6 (mean ± SEM; 3 replicates, except LV Gₛ duplicates).
Table 1 - Animal characteristics and *in vivo* hemodynamics in 20-week old male non-diabetic (+/+)
and type 2 diabetic (fa/fa) ZDF rats.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetes ZDF(+/+) n=12</th>
<th>Type 2 diabetes ZDF(fa/fa) n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>338 ± 6</td>
<td>412 ± 16**</td>
</tr>
<tr>
<td>Blood glucose level (mM)</td>
<td>8.0 ± 1.2</td>
<td>23.8 ± 2.9***</td>
</tr>
<tr>
<td>Plasma insulin level (ng/mL)</td>
<td>3.3 ± 0.5</td>
<td>20.5 ± 4.5**</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.83 ± 0.14</td>
<td>1.75 ± 0.05</td>
</tr>
<tr>
<td>Heart weight: tibia length ratio</td>
<td>0.064 ± 0.003</td>
<td>0.070 ± 0.003</td>
</tr>
<tr>
<td>Tibia length (mm)</td>
<td>27.2 ± 1.3</td>
<td>26.0 ± 0.6</td>
</tr>
<tr>
<td>EF (%)</td>
<td>63 ± 2</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>SV (µL)</td>
<td>109 ± 7</td>
<td>84 ± 6*</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>379 ± 10</td>
<td>378 ± 8</td>
</tr>
<tr>
<td>CO (mL/min)</td>
<td>41.0 ± 1.8</td>
<td>35.6 ± 4.2</td>
</tr>
<tr>
<td>LVEDV (µL)</td>
<td>121 ± 7</td>
<td>101 ± 4*</td>
</tr>
<tr>
<td>LVESV (µL)</td>
<td>48 ± 4</td>
<td>44 ± 4</td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>136 ± 6</td>
<td>130 ± 6</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>16 ± 4</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>LV +dP/dt max (mmHg/s)</td>
<td>9812 ± 549</td>
<td>11228 ± 1009</td>
</tr>
<tr>
<td>LV -dP/dt max (mmHg/s)</td>
<td>-5513 ± 217</td>
<td>-5263 ± 357</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>12.3 ± 1.2</td>
<td>13.1 ± 2.1</td>
</tr>
<tr>
<td>Mean ABP (mmHg)</td>
<td>80 ± 8</td>
<td>85 ± 7</td>
</tr>
</tbody>
</table>

Abbreviations: EF, ejection fraction; SV, stroke volume; HR, heart rate; CO, cardiac output; bpm, beats per minute; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-
systolic volume; LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic pressure; +dP/dt\text{max}, maximum rate of contraction; -dP/dt\text{max}, maximum rate of relaxation; Tau, time constant of relaxation; ABP, arterial blood pressure. Unpaired t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, significantly different from non-diabetes. Data are presented as mean ± SEM.
Table 2 - Animal characteristics and ex vivo cardiac function in unpaced and paced isolated hearts of 20-week old male non-diabetic and diabetic ZDF rats.

<table>
<thead>
<tr>
<th></th>
<th>Unpaced</th>
<th>Paced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-diabetes</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td></td>
<td>ZDF(+/-) n=7</td>
<td>ZDF(fa/fa) n=7</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>357 ± 7</td>
<td>401 ± 13*</td>
</tr>
<tr>
<td>Blood glucose level (mM)</td>
<td>10.5 ± 0.5</td>
<td>25.8 ± 2.7**</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.33 ± 0.14</td>
<td>1.5 ± 0.02</td>
</tr>
<tr>
<td>LV V_max (µL)</td>
<td>343 ± 16</td>
<td>357 ± 30</td>
</tr>
<tr>
<td>LV P_dev (mmHg)</td>
<td>142 ± 6</td>
<td>135 ± 9</td>
</tr>
<tr>
<td>+dP/dt_max (mmHg/s)</td>
<td>3577 ± 194</td>
<td>4099 ± 177</td>
</tr>
<tr>
<td>-dP/dt_max (mmHg/s)</td>
<td>-2353 ± 127</td>
<td>-2230 ± 106</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>74 ± 12</td>
<td>73 ± 18</td>
</tr>
<tr>
<td>P_max (mmHg)</td>
<td>338 ± 16</td>
<td>330 ± 23</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>245 ± 10</td>
<td>165 ± 9**</td>
</tr>
</tbody>
</table>

Abbreviations: LV, left ventricle; V_max, maximum volume; P_dev, developed pressure; +dP/dt_max, maximum rate of contraction; -dP/dt_max, maximum rate of relaxation; Tau, time constant of relaxation; P_max, maximal developed pressure; HR, heart rate; bpm, beats per minute; Unpaired t-test, * p < 0.05, ** p < 0.01, significantly different from non-diabetes. Data are presented as mean ± SEM.
A

Non-diabetes

Type 2 diabetes

ABP (mmHg)

Raw cSNA (µV)

Integrated cSNA (µV s)

Time (minutes)

1.37 1.38 1.39 1.40 1.41 1.42

1.47 1.48 1.49 1.50 1.51 1.52

B

Integrated cardiac SNA

C

Nerve firing rate

Integrated cSNA (µV s)

Non-diabetes  Type 2 diabetes

Impulses/s (Hz)

Non-diabetes  Type 2 diabetes

*