Glucose fluctuations increase the incidence of atrial fibrillation in diabetic rats

Shotaro Saito, Yasushi Teshima*, Akira Fukui, Hidekazu Kondo, Satoru Nishio, Mikiko Nakagawa, Tetsunori Saikawa, and Naohiko Takahashi

Aims
We investigated whether glucose fluctuations aggravate cardiac fibrosis and increase the occurrence of atrial fibrillation (AF) in rats with diabetes mellitus (DM).

Methods and results
Streptozotocin-induced diabetic rats were randomly divided into three groups: uncontrolled DM (U-STZ) group, controlled DM (C-STZ) group, and DM with glucose fluctuations (STZ-GF) group. Glucose fluctuations were induced by fasting for 24 h and additional regular insulin injections (0.5 IU/kg) administered three times per week for three consecutive weeks. C-STZ rats were administered long acting insulin (20 IU/kg) twice a day to control blood glucose levels. Cardiac fibrosis evaluated by Masson trichrome staining and the expressions of collagen type 1, collagen type 3, and α-smooth muscle actin were increased in U-STZ rats compared with C-STZ rats, which were more pronounced in STZ-GF rats. The inducibility of AF was significantly larger in U-STZ rats than C-STZ rats and was greatest in STZ-GF rats. To explore the mechanism of cardiac fibrosis, we investigated the levels of reactive oxygen species (ROS) and apoptosis. The expression of malondialdehyde, an indicator of ROS levels, was significantly upregulated in STZ-GF rats compared with U-STZ rats, along with increased thioredoxin-interacting protein (Txnip) expression in STZ-GF rats. Furthermore, caspase-3 expression and the number of TUNEL-positive cells were significantly increased in STZ-GF rats compared with U-STZ and C-STZ rats.

Conclusion
Glucose fluctuations increase the incidence of AF by promoting cardiac fibrosis. Increased ROS levels caused by upregulation of Txnip expression may be a mechanism whereby in glucose fluctuations induce fibrosis.

Keywords
Diabetes mellitus • Glucose fluctuation • Atrial fibrillation

1. Introduction
Diabetes mellitus (DM) is a major risk factor for cardiovascular diseases such as ischaemic heart disease, heart failure, and arrhythmias. For decades, risk of cardiovascular complications in DM has been believed to correlate with elevated glycated haemoglobin (HbA1c) and fasting glucose levels. However, several large-scale clinical trials have recently proposed adverse effects caused by intensive glycaemic control. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial revealed that intensive glycaemic control targeting a level of glycated haemoglobin \(< 6.0\%\) increased all-cause mortality more than standard therapy targeting 7.0–7.9\%. The Normoglycemia in Intensive Care Evaluation and Survival Using Glucose Algorithm Regulation (NICE-SUGAR) study also demonstrated that intensive glycaemic control caused high mortality in patients hospitalized in an intensive care unit. These results have raised concerns that hypoglycaemic episode could aggravate patient prognosis. Furthermore, a basic research showed that recurrent hypoglycaemic episodes increased mitochondrial free radical release and exacerbated cerebral ischaemic damage. In addition, intermittent high glucose exposure exacerbated oxidative stress and apoptosis in endothelial cells. On the basis of these observations, we hypothesized that glucose fluctuations may directly affect cardiomyocytes by increasing reactive oxygen species (ROS) levels and predispose to cardiac complications.

Atrial fibrillation (AF) is a common but critical arrhythmia because of the high risk of cerebral thrombosis associated with it. Hypertension, heart failure, hyperthyroidism, and DM are the known major risk factors for AF. Structural remodelling due to increased cardiac fibrosis by DM was associated with greater atrial arrhythmogenicity in rats. Increased fibrosis in myocardium also has been reported in human...
Because ROS facilitate apoptosis and cardiac fibrosis, glucose fluctuations may aggravate AF in accordance with ROS increase. In the present study, we investigated whether glucose fluctuations induced by repeated starvation increase the incidence of AF by facilitating atrial fibrosis more than persistent hyperglycaemia. We also explored the mechanisms involved in the observed effects.

2. Methods

All experimental procedures were performed in accordance with the guidelines of the Physiological Society of Oita University, Japan, for the care and use of laboratory animals, which follow the guidelines established by the U.S. National Institutes of Health.

2.1 Animal model

Eight-week-old male Sprague–Dawley rats received a single intravenous injection of streptozotocin (STZ; 60 mg/kg). One week after STZ injection, animals that showed glucose levels of $>$22.2 mmol/L were included in the study. STZ-induced DM rats were randomly divided into three groups: uncontrolled DM (U-STZ) group, controlled DM (C-STZ) group, and DM with glucose fluctuations (STZ-GF) group. C-STZ rats underwent subcutaneous injection of long acting insulin (insulin glargine; 20 IU/kg) twice a day to control blood glucose levels. Glucose level fluctuations were induced by 24 h of starvation, and if the blood glucose level was $<$5.5 mmol/L, regular insulin (insulin human; 0.5 IU/kg) was intraperitoneally injected to decrease the level to $\approx5.5$ mmol/L. Thereafter, rats were allowed free access to standard pelleted chow for 24 h to increase blood glucose levels. Rats were able to drink water freely even during the intervals without food. After 24 h of free access to food, rats were starved again to induce glucose fluctuations over a period of 3 weeks. At the age of 12 weeks, after 2 days of exposure to chow from last 24 h of starvation, hearts of the rats were rapidly excised under anaesthesia and immediately on a Langendorff perfusion apparatus for induction of AF ex vivo, or they were used for histological examination, western blotting, and immunohistochemical analyses. Rats were anaesthetized by an intraperitoneal injection of a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). The depth of anaesthesia was confirmed from a stable heart rate and a lack of flexor responses to a paw-pinch.

2.2 Haemodynamic parameters and echocardiography

Systolic blood pressure and heart rate were measured using the tail-cuff method. Transthoracic echocardiography (Hitachi Aloka Medical, Ltd, Tokyo, Japan) was then performed at the end of the sequential pattern of glucose fluctuations under anaesthesia by an intraperitoneal injection of a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) which was confirmed not to decrease the blood pressure. Measurements included left atrial dimension (LAD), left ventricular (LV) end-diastolic dimension, LV end-systolic dimension, LV fractional shortening (FS), LV ejection fraction (LVEF), and LV end-diastolic posterior wall thickness (LVPWth). We also measured peak early (E) and late (A) transmitral flow velocities. The deceleration time of the mitral E-wave (DcT) was measured from its peak to the time when the descent of the wave intercepted the baseline.

2.3 Laboratory data

Blood samples were collected from the inferior vena cava after echocardiography. Plasma glucose levels were measured by blood glucose-monitoring system (Terumo Co. Ltd, Tokyo, Japan). Plasma insulin levels were measured.

Figure 1 Blood glucose levels and body weight. (A) Body weight gain was significantly lower in U-STZ rats than in C-STZ rats, and this difference was more pronounced in STZ-GF rats. Body weights in STZ-GF rats fluctuated because of repetitive starvation. (B) Blood glucose levels in U-STZ rats were always $>$22.2 mmol/L. In contrast, glucose levels in C-STZ rats were maintained $\approx5.5$ mmol/L. After starvation and additional insulin injections, glucose levels were decreased $<$5.5 mmol/L in STZ-GF rats. Data are presented as mean ± SEM ($n=6$ per group).
using an insulin enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi Co. Ltd, Gunma, Japan) according to manufacturer’s protocol.

### 2.4 Histological studies

After excision, LA and LV were separately fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Masson trichrome staining was used to evaluate fibrosis. Micrographs were digitized using Photoshop 7.0 (Adobe systems Inc., San Jose, CA, USA), and areas of fibrosis were analysed using Scion image software (Scion Corp., Frederick, MD, USA). For each atrium and ventricle, three images with a magnification of ×400 were analysed and averaged (number of rats, six per group).

### 2.5 Quantitative RT–PCR analyses

Quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis was performed as described previously.14 Messenger RNA expression levels relative to actin were determined using comparative Ct method.

### 2.6 Electrophysiological studies

Electrophysiological studies were carried out in isolated perfused hearts using a Langendorff apparatus as described previously.15,16 The interatrial conduction time (IACT) and the effective refractory period (ERP) of LA were measured. AF inducibility was tested using the S3 extrastimulus pacing method.

### 2.7 Western blot analysis

Western blot was performed as previously described.14 Antibodies for collagen type 1, collagen type 3, NADPH oxidase 4 (NOX4), gp91phox (NOX2), B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and Connexin 40 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for caspase-3, Connexin 43, and phosphorylated connexin 43 (Ser368) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies for α-smooth muscle actin (α-SMA) and GADPH were purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies for RAS-related C3 botulinus toxin substrate 1 (Rac1) and oxidized Ca2+/calmodulin-dependent protein kinase (CaMK II) (Met281/282) were purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies for malondialdehyde (MDA; Academy Bio-Medical, Houston, TX, USA), thioredoxin-interacting protein (Txnip; Medical & Biological Laboratories, Nagoya, Japan), phosphorylated CaMKII (Thr286) (Abcam, Cambridge, UK), and phosphorylated ryanodine receptor 2 (RyR2) (Ser2808) (Badrilla, Leeds, UK) were also used in the experiments.

### 2.8 Immunohistochemistry

Isolated LA were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections. For immunohistochemical staining of connexin 43 and connexin 40, sections were incubated overnight at 4°C with goat connexin 43 antibody (Cell Signaling Technology) and then incubated with biotin-conjugated rabbit anti-goat IgG (ABC reagent; Vector Laboratories). Samples were visualized with 4’,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei.

### 2.9 Assay for 8-hydroxy-2′-deoxyguanosine (8-OHdG), hydroxyproline, and advanced glycation end products

To evaluate the ROS levels in vivo, we also performed ELISA for 8-OHdG, an indicator of internal oxidative stress. DNA was extracted from the heart using a DNA Extractor TIS kit (Wako Pure Chemicals Industries, Osaka, Japan) and pre-treated with nuclelease. 8-OHdG assays were performed using 8-OHdG ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan) according to the manufacturer’s protocol. Cardiac collagen content was evaluated by quantification of hydroxyproline using a hydroxyproline assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer’s protocol. Levels of advanced glycation end products (AGEs) in LA were evaluated using OxiSelect™ Advanced Glycation End Product ELISA Kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer’s protocol.

### 2.10 Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining

Isolated LA were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5 μm sections, and stained. Apoptotic cells were detected with the in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay kit (TACS III TUNEL Apoptosis Detection Kit, Vivantis, Vietnam) according to manufacturer’s protocol.

#### Table 1 Physiological and echocardiographic findings

<table>
<thead>
<tr>
<th></th>
<th>U-STZ</th>
<th>C-STZ</th>
<th>STZ-GF</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>340.0 ± 21.9</td>
<td>388.2 ± 41.4*</td>
<td>283.3 ± 13.7**</td>
</tr>
<tr>
<td>HW (g)</td>
<td>1.12 ± 0.05</td>
<td>1.26 ± 0.04*</td>
<td>0.93 ± 0.05**</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.31 ± 0.09</td>
<td>3.24 ± 0.07</td>
<td>3.30 ± 0.07</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>131.0 ± 3.4</td>
<td>126.2 ± 11.1</td>
<td>125.0 ± 6.2</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>304.6</td>
<td>316.6</td>
<td>319.6 ± 16.3</td>
</tr>
<tr>
<td>LVPWth (mm)</td>
<td>1.40 ± 0.04</td>
<td>1.25 ± 0.04*</td>
<td>1.45 ± 0.05†</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>7.20 ± 0.18</td>
<td>7.48 ± 0.11</td>
<td>6.82 ± 0.24†</td>
</tr>
<tr>
<td>FS (%)</td>
<td>41.2 ± 1.4</td>
<td>44.8 ± 0.4*</td>
<td>41.0 ± 0.8†</td>
</tr>
<tr>
<td>LAD (mm)</td>
<td>3.42 ± 0.06</td>
<td>3.00 ± 0.15*</td>
<td>3.73 ± 0.11†</td>
</tr>
<tr>
<td>E (m/s)</td>
<td>0.94 ± 0.05</td>
<td>1.14 ± 0.05*</td>
<td>0.77 ± 0.05**</td>
</tr>
<tr>
<td>E/A</td>
<td>0.80 ± 0.05</td>
<td>0.99 ± 0.03*</td>
<td>0.89 ± 0.09</td>
</tr>
<tr>
<td>LAD (mm)</td>
<td>1.17 ± 0.03</td>
<td>1.15 ± 0.04</td>
<td>0.90 ± 0.08**</td>
</tr>
<tr>
<td>DcT (ms)</td>
<td>68.7 ± 5.8</td>
<td>60.2 ± 4.8</td>
<td>94.8 ± 11.1††</td>
</tr>
</tbody>
</table>

n = 6 for each group. Data are presented as mean ± SEM.

#### Table 2 Serum parameters

<table>
<thead>
<tr>
<th></th>
<th>U-STZ</th>
<th>C-STZ</th>
<th>STZ-GF</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (mg/dL)</td>
<td>5.22 ± 0.10</td>
<td>5.12 ± 0.09</td>
<td>5.24 ± 0.10</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>22.9 ± 1.3</td>
<td>22.8 ± 1.9</td>
<td>28.6 ± 2.9</td>
</tr>
<tr>
<td>Cr (mg/dL)</td>
<td>0.21 ± 0.01</td>
<td>0.26 ± 0.04</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>210.3 ± 88.9</td>
<td>191.7 ± 37.1*</td>
<td>112.7 ± 19.1</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>87.3 ± 21.5</td>
<td>108.8 ± 4.8</td>
<td>65.7 ± 4.6†</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>20.6 ± 9.0</td>
<td>13.8 ± 1.1</td>
<td>82.1 ± 12</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.1 ± 0.2</td>
<td>5.9 ± 0.4*</td>
<td>7.0 ± 0.4†</td>
</tr>
<tr>
<td>Insulin (pg/mL)</td>
<td>54.7 ± 0.89</td>
<td>356.8 ± 3.06**</td>
<td>53.8 ± 0.06††</td>
</tr>
</tbody>
</table>

n = 6 for each group. Data are presented as mean ± SEM.

TP, total protein; BUN, blood urea nitrogen; Cr, creatinine; TG, triglycerides; TC, total cholesterol; LDL, low-density lipoprotein cholesterol; HbA1c, glycated haemoglobin.

*P < 0.05 vs. corresponding U-STZ.
**P < 0.01 vs. corresponding U-STZ.
†P < 0.05 vs. corresponding C-STZ.
‡P < 0.01 vs. corresponding C-STZ.
(TUNEL) method using an apoptosis kit (Medical Biological Lab, Nagoya, Japan), as described previously.17 Sections were treated with proteinase K and then treated with a mixture of terminal deoxynucleotidyl transferase (TdT), fluorescein isothiocyanate-dUTP, and TdT buffer II at 37°C for 1 h. After washing these slides with PBS, they were mounted with mounting medium using DAPI.

2.11 Exposure of cardiomyocytes to high- and low-glucose concentrations
Cardiac ventricular myocytes were prepared from 1- to 3-day-old Sprague–Dawley rats and cultured as described previously.18 The cardiomyocytes were randomly assigned to the following group: a group with normal (5.6 mmol/L) glucose concentration (NG), a group with high (25 mmol/L) and low (1.7 mmol/L) glucose concentrations (HL), or a group with normal and low glucose concentrations (NL).

2.12 Statistical analysis
Continuous data were assessed for normality using the Shapiro–Wilk test. Normally distributed data are expressed as mean ± SEM. Non-normally distributed variables are expressed as medians and interquartile ranges. Three-group comparisons were obtained with 1-way ANOVA followed by Bonferroni–Dunn test (for normally distributed data) or Kruskal–Wallis test with Dunn’s Multiple Comparison test (for non-normally distributed data). Repeated-measures analyses were performed with 2-way ANOVA and Bonferroni–Dunn tests. The incidence of AF or VF was compared among the three groups using Fisher’s exact test. Two-tailed P-values of <0.05 indicated statistical significance.

3. Results
3.1 Blood glucose fluctuations and physiological characteristics in STZ-induced DM rats
Blood glucose levels and body weight changes of STZ-induced DM rats during the experimental period are shown in Figure 1. Body weight gain was significantly less in U-STZ group than in C-STZ group, and this difference was more marked between STZ-GF and C-STZ groups. Body weights in STZ-GF group fluctuated because of repetitive starvation. Blood glucose levels in U-STZ rats were always >22.2 mmol/L. In contrast, glucose levels in C-STZ rats were maintained around 5.5 mmol/L.

Figure 2 Levels of ROS. (A and B) MDA expression and levels of 8-OHdG were significantly larger in U-STZ rats than in C-STZ rats, and this difference was more pronounced in STZ-GF rats. Data are presented as mean ± SEM (n = 6 or 7 per group). *P < 0.05 vs. U-STZ, **P < 0.01 vs. U-STZ, †P < 0.05 vs. C-STZ, and ‡P < 0.01 vs. C-STZ. (C) Quantitative results obtained by confocal microscopy in cultured neonatal cardiomyocytes stained with CM-H2DCFDA. Levels of ROS were significantly higher in HL than in HG and NL. Data are presented as mean ± SEM (n = 6 per group). CM-H2DCFDA; 5-(and-6)-chloromethyl-1-2,7 ′-dichlorodihydrofluorescein diacetate, acetyl ester. *P < 0.05 vs. NG and †P < 0.01 vs. HL.
by periodic insulin injection. After starvation and additional insulin injections, glucose levels were decreased to 5.5 mmol/L in STZ-GF rats.

After 3 weeks of glucose fluctuations, heart weights were significantly lower in U-STZ rats than in C-STZ rats, and the differences were more pronounced between STZ-GF and C-STZ rats. However, the heart-to-body weight ratio did not differ among the three groups on Day 22. In addition, systolic blood pressure values and heart rates did not significantly differ among the three groups (Table 1). In blood chemistry data, plasma insulin levels were extremely low in all three groups. HbA1c levels were higher in U-STZ and STZ-GF than in C-STZ rats. However, there was no significant difference in HbA1c levels between U-STZ and STZ-GF rats (Table 2).

### 3.2 Oxidative stress

To investigate ROS levels in an animal model, we assayed MDA expression and 8-OHdG levels in rat heart. MDA expression and levels of 8-OHdG were significantly higher in U-STZ rats than in C-STZ rats, and the differences were more marked between STZ-GF and C-STZ rats. However, the heart-to-body weight ratio did not differ among the three groups on Day 22. In addition, systolic blood pressure values and heart rates did not significantly differ among the three groups (Table 1). In blood chemistry data, plasma insulin levels were extremely low in all three groups. HbA1c levels were higher in U-STZ and STZ-GF than in C-STZ rats. However, there was no significant difference in HbA1c levels between U-STZ and STZ-GF rats (Table 2).

#### Figure 3

Mechanisms of ROS increase. (A) Txnip expression was upregulated in U-STZ compared with C-STZ rats, with highest expression seen in STZ-GF rats. (B) Levels of AGEs in LA were significantly higher in U-STZ rats than in C-STZ rats, which were more increased in STZ-GF rats. (C) Protein expressions of NOX2, Rac1, and NOX4 were significantly upregulated in STZ-GF rats compared with C-STZ rats. Data are presented as mean ± SEM (n = 6 or 7 per group). *P < 0.05 vs. U-STZ, **P < 0.01 vs. U-STZ, ***P < 0.05 vs. C-STZ, and †P < 0.01 vs. C-STZ.

### 3.3 Apoptosis

Loss of functional cells through apoptosis is a critical step in LA structural remodelling. Next we investigated whether glucose fluctuations could increase apoptosis in LA. The number of TUNEL-positive cardiomyocytes was significantly increased in U-STZ rats compared with C-STZ rats, which were greater in STZ-GF rats (Figure 4A). Caspase-3 and Bax protein expression was significantly increased and Bcl-2 expression was decreased in U-STZ rats compared with C-STZ rats, and these changes were more pronounced in STZ-GF rats (Figure 4B and C).

### 3.4 Fibrosis in LA

Figure 5A shows representative images and the quantitative ratio of the area of fibrosis to the area of the reference tissue obtained by Masson trichrome staining of LA. LA fibrosis was increased in U-STZ rats compared with in C-STZ rats, which was more pronounced in STZ-GF rats. Next, we examined the expression of molecules related to profibrotic and proinflammatory signaling. Expressions of collagen 1, collagen 3, α-SMA, TNF-α, and TGF-β1 were significantly upregulated in LA of U-STZ rats than in C-STZ rats, with even more pronounced upregulation in STZ-GF rats (Figure 5B, C, and Supplementary material online, Figure S1).

### 3.5 Fibrosis and diastolic dysfunction in LV

Figure 6A shows representative images and quantitative results of Masson trichrome staining of LV. Similar to the results of LA histological assay, LV fibrosis was increased in U-STZ compared with in C-STZ rats, which was more pronounced in STZ-GF rats. Figure 5B and C demonstrate more
abundant collagen expression in the hearts of STZ-GF than those in the hearts of U-STZ or C-STZ rats. Echocardiographic analysis revealed that LVPWth was increased and LVEF was decreased in the U-STZ and STZ-GF groups compared with the C-STZ group (Table 1). Diastolic function, as evaluated by DcT, amplitude of E waves, and the E/A ratio, was impaired in U-STZ; this impairment was more pronounced in STZ-GF rats (Table 1). Similarly, LAD was larger in U-STZ rats than in C-STZ rats, with even greater enlargement seen in STZ-GF rats (Table 1).

### 3.6 Electrophysiological studies and AF induction

ERP of LA and IACT were measured in each group for basic cycle lengths (BCL) of 200, 150, 120, and 90 ms. IACT was significantly prolonged at all BCL tested in U-STZ rats than in C-STZ rats; this prolongation was even greater in STZ-GF rats (Figure 7A). There were no significant differences in ERP of LA among each group (Figure 7B). Figure 7C shows representative ECG recordings of AF triggered by S3 extrastimulus pacing, which was observed in a STZ-GF rat. These were characterized by a rapid and chaotic atrial rhythm and an irregular ventricular response. The incidence of AF induced by double extrastimuli under Langendorff perfusion was significantly increased in U-STZ rats compared with C-STZ rats, and the increase was more pronounced in STZ-GF rats (Figure 7C).

### 4. Discussion

ROS are generated as natural byproducts of oxygen metabolism, and moderate levels of ROS function as intracellular signalling molecules. However, high levels of ROS are detrimental to cardiomyocytes. There is accumulating evidence that ROS production and oxidative stress are increased in the hearts of diabetes mellitus. Oxidative stress induces various cardiovascular complications such as cardiomyopathy, cardiac hypertrophy, endothelial dysfunction, and cardiac fibrosis. The levels of 8-OHdG and MDA expression, indicators of ROS levels, were significantly higher in STZ-GF rats than in U-STZ and C-STZ rats in the present study. These results were consistent with previous reports showing that intermittent exposure to high glucose increased ROS levels in endothelial cells and induced apoptosis. A clinical study also revealed that glucose fluctuations increase oxidative stress in patients with type 2 diabetes. Although the sequential pattern of glucose fluctuations in the present study, hyperglycaemia to hypoglycaemia, may differ from those in previous studies of intermittent hyperglycaemia, both patterns of glucose fluctuations increased oxidative stress. Taken together, extent rather than the pattern of fluctuations may be more critical to ROS increase. The results of in vitro experiments using cultured cardiomyocytes also support this idea. ROS levels in cardiomyocytes alternately exposed to normal and low glucose concentrations were significantly less than those in cardiomyocytes alternately exposed to high and low glucose concentrations (Figure 2C). These results indicate that fluctuations of glucose concentrations per se rather than repetitive hypoglycaemia have deleterious effects on cardiomyocytes.

We also demonstrated that Txnip expression increased in STZ-GF rats. Txnip is a ubiquitously expressed protein that binds and inhibits thioredoxin, thereby inducing oxidative stress and apoptosis. Txnip expression is strongly induced by hyperglycaemia, and the
overexpression of Txnip induces apoptosis in cardiomyocytes. In the present study, both oxidative stress and apoptotic cardiomyocytes were significantly increased along with the increase in Txnip expression in STZ-GF rats. Furthermore, a previous report showed that Txnip knockdown decreased procollagen type I expression and scar formation in LV. Hence, upregulation of Txnip expression may be a mechanism whereby cardiac fibrosis is induced in response to glucose fluctuations.

NADPH oxidase (NOX) is a major source of ROS production and the heart expresses primarily NOX2 and NOX4. Activation of NOX2 requires combination with p47phox, p67phox, p40phox, and Rac1. In the present study, the expression of Rac1, and NOX4 was significantly upregulated in STZ-GF rats compared with U-STZ and C-STZ rats, indicating a possible contribution of NOX to increased ROS levels induced by glucose fluctuations. Furthermore, the activation of NOX increases mitochondrial ROS production and vice versa, creating a vicious feed-forward cycle that further augments ROS production and oxidative stress.

ROS facilitate apoptosis and inflammation, leading to cardiac fibrosis. ROS stimulate TNF-α expression, a promoter of apoptosis, and TNF-α in turn stimulates ROS formation. In the present study, we observed that atrial TNF-α expression was significantly upregulated in STZ-GF rats, in accordance with an increase in apoptosis. ROS are also essential to TGF-β-mediated profibrotic signalling, and we showed that atrial TGF-β1 expression was upregulated in STZ-GF rats. The link between ROS and cytokines may comprise a key axis in the progress of apoptosis and atrial fibrosis.

Increased atrial fibrosis and atrial dilatation are central features of atrial structural remodelling in AF. Fibroblasts proliferation and differentiation into myofibroblasts play a critical role in the progression of fibrosis, and involve altered expression of several ion channel proteins. Delayed cardiac conduction by increased deposition of collagen may promote reentry and increase AF susceptibility. Moreover, increased cardiomyocyte–myofibroblast interaction through paracrine factors such as angiotensin II and TGF-β1 may also facilitate heterogeneous conduction.

In the present study, STZ-GF rats showed prolonged IACT. On the other hand, no significant difference was observed in ERP of LA. Prolongation of action potential duration (APD) in atrium and ventricle has been observed in diabetic animal models. Meanwhile, in other studies, there were no significant changes in ERP in DM compared with control, and oxidative stress could be related to ERP shortening. Taken together, potential alterations in ERP might be masked by offsetting effects of oxidative stress induced by DM in the present results. Conduction abnormalities are also exacerbated by impaired cell-to-cell coupling via gap junctions. AF is reportedly associated with the decrease in connexin 43 and/or
Figure 6  Fibrosis and diastolic dysfunction in LV. (A) Representative images and quantitative results of Masson trichrome staining of LV. LV fibrosis was increased in U-STZ rats compared with C-STZ rats, and the difference was more pronounced between STZ-GF and C-STZ rats. (B) Quantification of hydroxyproline in LV as a marker of collagen content. (C) The expression of COL1 and COL3 was significantly upregulated in LV of U-STZ rats than C-STZ rats, and was highest in STZ-GF rats. Data are presented as mean ± SEM (n = 6 or 7 per group). COL1, collagen type 1; COL3, collagen type 3. Scale bar = 500 μm.

*P < 0.05 vs. U-STZ, **P < 0.01 vs. U-STZ, †P < 0.05 vs. C-STZ, and ‡P < 0.01 vs. C-STZ.

Figure 7  Electrophysiological studies and AF induction. (A and B) Interatrial conduction time (IACT) and effective refractory period (ERP) of LA. IACT in U-STZ rats was significantly prolonged compared with that in C-STZ rats at all basic cycle lengths tested; IACT was further prolonged in STZ-GF rats. There were no significant differences in ERP of LA among the three groups. (C) Representative ECG recordings of AF triggered by S3 extrastimulus pacing. The incidence of AF was significantly higher in U-STZ rats compared with in C-STZ, and was highest in STZ-GF rats. Data are presented as mean ± SEM (n = 18 per group). *P < 0.05 vs. U-STZ, **P < 0.01 vs. U-STZ, †P < 0.05 vs. C-STZ, and ‡P < 0.01 vs. C-STZ.
connexin 43 proteins, the principal atrial gap junction proteins, and/or the abnormal distribution of them. However, there were no significant changes in the expression and lateralization of them among each group in the present study (Supplementary material online, Figure S2). Ca\(^{2+}\)-handling abnormalities also contribute to AF-associated arrhythmogenesis by promoting focal ectopic activity and/or reentry. In the present study, oxidation of CaMKII, phosphorylation of CaMKII, and phosphorylation of RyR2 were not significantly different among each group (Figure 8). Na\(^{+}\)-current abnormalities may also affect the Ca\(^{2+}\)-handling abnormalities.

AGEs are considered to play a crucial role in the pathogenesis of cardiovascular disease. In the present study, AGEs levels in LA were significantly increased in STZ-GF rats more than U-STZ and C-STZ rats (Figure 3B). These results provide a possible mechanism that glucose fluctuations promote cardiac fibrosis via AGEs formation indicating cumulative toxicity.

LV dysfunction, which raises LA pressure, is another risk factor for AF. In the present study, we showed that profibrotic signalling was upregulated not only in LA but also in LV of STZ-GF rats. LV diastolic function was impaired and the area of LV fibrosis was significantly larger in STZ-GF rats. LV diastolic dysfunction increases the risk of AF by the following mechanisms: (i) increased atrial afterload; (ii) increased atrial stretch; and (iii) increased atrial wall stress as a result of dilatation.

Since we did not subject the animals to atrioventricular nodal ablation, the slight but significant decrease in LV diastolic function observed in the present study may contribute to the increased incidence of AF. There were no significant differences in the inducibility of VF among each group probably because of the difference of sensitivity to arrhythmia induction between atrium and ventricle (data not shown).

Patients with DM sometimes emerge heart failure without coronary artery disease and/or hypertension, termed ‘diabetic cardiomyopathy.’ Progression of LV fibrosis was proposed as one of the mechanisms of LV dysfunction in DM. Persistent hyperglycaemia augments oxidative stress and subsequently induces cardiac fibrosis, leading to diastolic and systolic dysfunction in LV. In the present study, we demonstrated that glucose fluctuations exacerbated not only LA fibrosis but also LV fibrosis and resulted in diastolic dysfunction in LV. These results indicate the possible aggravation of diabetic cardiomyopathy by glucose fluctuations, which may be another risk factor predisposing to AF.

Recent progress of blood glucose monitoring device enabled us to measure the continuous blood glucose levels for several days. These data revealed that blood glucose levels in diabetic patients changed more dynamically than expected. In particular, a study has shown that patients who are administered insulin injections and/or oral hypoglycaemic agents sometimes exhibit hypoglycaemia with or without subjective symptoms. These patients are exposed to hypoglycaemia-induced glucose fluctuations. The sequential changes in blood glucose levels used in the present study may be unusual in patients with DM; however, we accentuated the changes in blood glucose levels to clearly delineate the effects of hypoglycaemia-induced glucose fluctuations on cardiomyocytes.

In conclusion, our results demonstrate that glucose fluctuations increase the incidence of AF by promoting cardiac fibrosis. Increased ROS levels caused by upregulation of Txnip and NADPH oxidase expression may be a mechanism whereby glucose fluctuations induce fibrosis. The present results robustly support the idea that diabetic patients with frequent hypoglycaemic episodes may be at higher risk for AF than with persistent hyperglycaemia.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

**Conflict of interest:** none declared.

**Funding**

This study was supported by Grants-in-Aid for scientific Research (C) 22590209 (to Y.T.) and 23592672 (to N.T.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The project was also supported by Research Fund at the Discretion of the President, Oita University (to Y.T.).

**References**


