Differential Vascular Responses of Aorta to Potassium Ion Channel Opener, Citrus Flavonoid Naringenin in Type 1 and Type 2 Diabetes Mellitus in Rats

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ABSTRACT

Background: Diabetes mellitus is associated with many cardiovascular dysfunction and impairment of potassium ion channel dysfunction which can be ameliorated by antioxidants.

Aim: We compared the vascular reactivity in aorta from streptozotocin-induced and Goto-Kakizaki (GK) diabetic rat.

Methodology: Diabetes mellitus (DM) was induced in Sprague Dawley rats by intraperitoneal injection of streptozotocin (STZ) at 65 mg/kg body weight. After four weeks of DM, vascular reactivity of the aortic rings from STZ-induced Sprague Dawley and age-matched GK and control rats to phenylephrine, acetylcholine, levocromakalim and naringenin were studied using standard organ bath procedure.

Results: The phenylephrine-induced contraction and sensitivity were significantly (P<0.05) increased in STZ-diabetic aortic rings when compared with GK rats and control. Relaxation responses to acetylcholine, levocromakalim and naringenin were significantly (P<0.05) attenuated in STZ-diabetic aorta when compared with GK rats. Maximal relaxation and sensitivity of aorta to acetylcholine, levocromakalim and naringenin were significantly (P<0.05) decreased in STZ-diabetic aorta when compared with GK-diabetic and control groups.

Conclusion: These results suggest that at early stage of DM, there is exaggerated vascular response to α-adrenergic receptor and attenuated responses to endothelial-dependent, KATP-potassium ion channel and naringenin in Type 1 and not Type 2 diabetes probably due to dysfunction of receptor mechanisms.

Keywords: acetylcholine, aorta, diabetes, endothelium, naringenin, potassium ion channel, vasoconstriction

1. INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder that represents a serious public health concern and is usually manifests as type 1 and type 2. Type 1 diabetes is characterized by defective insulin secretion in pancreatic beta-cells in response to glucose and by deficiencies in the action of insulin on its target tissues while type 2 is characterized with insulin resistance. Diabetes mellitus is associated with a wide range of circulatory manifestations such as alterations in endothelial function and cardiovascular disease (Hermans, 2007; Kar and Holt, 2008). Most of the complications in diabetes are due to increased

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hyperglycaemia and increased generation of oxygen-derived free radicals, which may lead to vascular
dysfunction (Singh et al., 2009; Kaneto et al., 2010).

Vascular dysfunction affect various membranes ion channels and evidence of vascular potassium ion
channel dysfunction due to oxidative stress in diabetes mellitus has been reported (Matsumoto et al.,
2004; Buboz et al., 2007). These include attenuation of ATP-dependent potassium ion channel (KATP) -
mediated vasorelaxation and increased vascular tone (Irat et al., 2006). Potassium ion channels
activation under normal condition produces hyperpolarization of the cell membrane, closure of voltage-
dependent calcium ion channels and vascular relaxation of smooth muscle cells (Nichols et al., 1991;
Faraci and Heistad, 1998). Potassium ion channel openers especially the KATP and BKCa-channel
openers have been useful in significantly improving diabetic vasculopathy(Matsumoto et al., 2004; Liu
and Gutterman, 2002).

In DM studies, a number of animal models have been used. STZ and alloxan are widely used to induce
Type 1 diabetes mellitus. The Goto-Kakizaki (GK) rats is a widely accepted, non-obese model of Type 2
DM that have elevated fasting blood glucose, impaired response to glucose, and increased
glycatedhaemoglobin levels at an early age (Goto et al., 1976; Yasuda et al., 2002). The GK rats are
model of hyperglycemia without the confounding effects of obesity or hypertension (Debin et al., 2009).

Vascular dysfunction is associated with both Type 1 and Type 2 diabetes. For instance, increased
contractile responses to adrenergic agonist and normal endothelium-dependent relaxation have been
reported in diabetic GK rats (Brondum et al., 2005) whereas attenuated acetylcholine (ACh) responses
have been shown in arteries of Zucker diabetic rats (Phillips et al., 2005). While some studies had
reported enhanced responsiveness of STZ- and GK-diabetic aorta to alpha adrenergic agonists (Orie et
al., 1993; Schulingkamp et al., 2005), other studies have shown decreased aortic responses in GK-
diabetic condition (Kobayashi et al., 2004).

The involvement of potassium ion channels in diabetic disease has been controversial probably due to
the stage of the disease and experimental models used for the study. Comparative study on the vascular
responses of aorta from Type 1 and 2 DM to adrenergic, nitric oxide and potassium ion dependent
channel activity in both types of diabetic models is lacking. The aim of this study was to compare the
adrenergic, endothelium-dependent and potassium ion channel responses of aorta from aged-matched
rat models of early Type 1 and Type 2 DM.

2. MATERIAL AND METHODS

2.1 Chemicals
Levcromakalim, phenylephrine, acetylcholine and (+/-)-naringenin were obtained from Sigma Chemical
Company (Poole, UK). Levcromakalim and (+/-)-naringenin were dissolved in dimethyl sulphoxide
(DMSO) while other drugs were dissolved in distilled water. All drugs were added directly into the organ
baths and the concentrations given are the final bath concentration in the bath solution. The solvents
used had no effect on tissue responses.

2.2 Experimental Animals
Male Sprague-Dawley, Wistar and GotoKakizaki (GK) rats (8-10 weeks old) weighing between 200-220 g
were obtained from the Biological Services Unit of the University College London for the study. The rats
were placed randomly into four groups namely STZ-control, STZ-induced diabetic, GK diabetic and Wistar
Control groups. All animals were allowed a standard laboratory diet and tap water ad libitum, and were
kept in room temperature controlled at 19-21°C. All studies were conducted in accordance with the Guide
for the Care and Use of Laboratory Animals of the University and conformed to the UK Animal Scientific
2.3 Induction of Diabetes Mellitus

DM was induced in Sprague Dawley rats by a single intraperitoneal injection of 65 mg/kg body weight streptozotocin (STZ) dissolved in citrate buffer (pH 4.5). Weight and age-matched Sprague Dawley control rats were injected with the citrate buffer vehicle alone. Body weight and basal blood glucose levels were measured just prior to STZ injection using animal balance and an automated glucose analyzer (glucometer Acucheck mini plus, Roche, Germany) respectively. After 48 h following STZ administration, blood samples were taken from the tail vein and hyperglycaemia was confirmed in animals by blood glucose above 10 mmol/L (Clark et al., 2004). The diabetic animals were experimented upon after four weeks of diabetes mellitus induction.

2.4 Artery Segment Preparation

After the rats were sacrificed by cervical dislocation, the aorta was rapidly removed and placed in cold (4ºC) physiological salt solution of the following composition (mmol/L): NaCl, 112; KCl 5; CaCl2 1.8; MgCl2 1, NaHCO3 25; KH2PO4 0.5; NaH2PO4 0.5; Glucose 10; pH 7.4. Each aorta was cleaned of connective tissues under the dissecting microscope and cut into segments (~3 mm long) and mounted in 20 ml organ baths at 37ºC containing physiological salt solution gassed with 95% O2 and 5% CO2. The aorta was connected to an isometric force transducer (Grass FT03), connected to a preamplifier Powerlab (AD Instruments Ltd, Australia) data acquisition unit and isometric contraction was recorded in a computer using AD Chart Software version 4.2.4 (AD Instruments Ltd). A passive tension of 1 g was applied to the tissue using a movable device. The rings were equilibrated for 90 min while being rinsed every 15 min. During the equilibration period, the rings were challenged with 1 µMol/L phenylephrine (PE) and the aorta was relaxed with 10 µMol/L acetylcholine to test the endothelial integrity.

After 90 min equilibration period, PE (10^-9-10^-5Mol/L) was added cumulatively to the bath until a maximal response was achieved. A plateau response was allowed to develop before the addition of the next dose of the same agonist. For relaxation studies, aortic rings from both the diabetic and control rats were precontracted with 1 µMol/L PE. When the PE contraction had stabilized, relaxation responses were elicited in a cumulative manner using one of the following: acetylcholine (10^-9-10^-5Mol/L), KATP channel opener, levromakalim (10^-9-10^-5Mol/L) or a flavonoid, (+/-)-naringenin (10^-8-10^-4Mol/L). A subsequent concentration was added to the organ bath after the previous concentration had reached its steady state. Each aortic ring was used once for each drug protocol.

2.5 Statistics

All results are reported as mean ± SEM and n represents the number of animals per group. Concentration-response curves from rat aortae were computer fitted to a sigmoidal curve using nonlinear regression (Prism software version 5.0, GraphPad Software, San Diego, CA, USA) to calculate the sensitivity of each agonist. Statistical analysis of the data was performed by one way analysis of variance (ANOVA) followed by Bonferroni’s test or Student’s unpaired t-tests as applicable. Relaxation responses were given as a percentage of the contraction induced by PE. The sensitivity of the drugs was expressed in terms of their pD2, which is defined as the negative logarithm of the EC50 or IC50 for the agonist used. In all comparisons, P<0.05 was considered significant.

3. RESULTS

3.1 Body Weight and Blood Glucose Level

As indicated in Table 1, blood glucose levels were significantly (P<0.01) elevated in STZ-induced diabetic rats (32.9±0.57 mmol/L) when compared with controls (6.9 ±0.38). The blood glucose level in the GK rats (12.9 ±1.6 mmol/L) was significantly (P<0.01) higher than the Wistar control rats(7.4 ± 0.51 mmol/L). However, the blood glucose level in STZ-diabetic rats were significantly (P<0.01) higher than the level in GK diabetic rats. In contrast, body weight was comparable between the age- matched control and GK rats whereas it was significantly (P<0.05) reduced in STZ-diabetic group compared with control.
Table 1. Body weight and blood glucose levels in age-matched control and diabetic rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>STZ-Control</th>
<th>STZ-induced DM</th>
<th>Wistar Control</th>
<th>GK diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>430 ± 26.2</td>
<td>286 ±19.6**</td>
<td>344± 5</td>
<td>350 ±10.3</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>6.9 ± 0.38</td>
<td>32.9 ±0.57**†</td>
<td>7.4 ± 0.51</td>
<td>12.9 ±1.6‡§</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SE; ** = P = .01 significant differences vs. STZ-control rats; † = P = .01 significant differences vs. GK rat; ‡ = P = .01 GK diabetes vs. STZ-control rats, § = P = .01 vs. Wistar control group; n=6 in each group.

3.2 Contraction with Phenylephrine

Exposure of aortic strips to phenylephrine led to a concentration-dependent rise in tension in all experimental groups. Aortas from the STZ diabetic rats showed significant (P=.05) increases in maximum contractile forces compared with those of age-matched control and GK rats (Fig. 1). There was a notable shift of the phenylephrine concentration-response curve of STZ-diabetic to the left of control. In contrast, aortas from GK diabetic rats showed a significant (P<.05) decrease in maximum contraction induced by phenylephrine and displayed no greater sensitivity to phenylephrine than the Wistar control rats (Table 2).

Table 2. Parameters of the concentration-dependent responses to various drugs in the thoracic aorta of age-matched control and diabetic rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>STZ-Control</th>
<th>STZ-DM</th>
<th>Wistar Control</th>
<th>GK diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylephrine –logEC(_{50})</td>
<td>7.39±0.10</td>
<td>7.42±0.13</td>
<td>7.43± 0.11</td>
<td>7.41 ±0.13</td>
</tr>
<tr>
<td>Phenylephrine Max (g)</td>
<td>1.42±0.21</td>
<td>2.03 ±0.07 **†</td>
<td>2.07± 0.12</td>
<td>1.47±0.14‡</td>
</tr>
<tr>
<td>Acetylcholine –log IC(_{50})</td>
<td>7.73±0.11</td>
<td>7.31±0.07*</td>
<td>7.41 ±0.07</td>
<td>7.69 ± 0.13‡</td>
</tr>
<tr>
<td>Acetylcholine –Max (%)</td>
<td>95.14±2.9</td>
<td>90.04±2.01*†</td>
<td>92.67 ± 3.2</td>
<td>92.30 ± 2.22</td>
</tr>
<tr>
<td>Levcromakalim –log IC(_{50})</td>
<td>7.41±0.12</td>
<td>7.16±0.09†</td>
<td>7.39 ± 0.08</td>
<td>7.53±0.11‡</td>
</tr>
<tr>
<td>Levcromakalim Max (%)</td>
<td>97.63±1.15</td>
<td>92.45±1.27*†</td>
<td>93.17 ± 2.5</td>
<td>97.6 ± 0.88‡</td>
</tr>
<tr>
<td>Naringenin –log IC(_{50})</td>
<td>4.6±0.50</td>
<td>3.7±0.37†</td>
<td>3.39 ± 0.05</td>
<td>4.93±0.35‡</td>
</tr>
<tr>
<td>Naringenin Max (%)</td>
<td>86.49±3.0</td>
<td>77.77±2.24**†</td>
<td>87.17 ± 3.8</td>
<td>90.98 ±2.23</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SE; * = P < 0.05 significant differences vs. STZ-control rats; † = P < 0.05 significant differences vs. GK rat; ‡ = P <0.05 vs. Wistar control; –LogEC\(_{50}\) or –LogIC\(_{50}\) = negative logarithm of the molar concentration of each drug causing 50% of the maximal contraction or relaxation; max =maximal contraction or relaxation to each drug (percentage).
Fig. 1. Concentration-response curves for phenylephrine-induced vasoconstriction of aortic strips obtained from age-matched controls and streptozotocin-induced (A) and GK diabetic (B) rats. Each data point represents the mean ± SEM of 6 experiments; * = P < 0.05 STZ-diabetic vs. STZ-control and GK-diabetic vs. Wistar Control (WC) group, n = 6.

3.3 Relaxation to acetylcholine

The cumulative ACh concentration-response curves on PE-pre-constricted aortic rings in both diabetic and control aortas are presented in Fig 2. DM significantly reduced the sensitivity to ACh in STZ rat aorta compared with control (Fig 2A). However in GK rat aorta, the sensitivity, but not maximum relaxation was significantly (P<0.05) increased compared with Wistar control (Fig 2B). The sensitivity to ACh was significantly (P<0.05) decreased in STZ-diabetic aorta when compared with GK diabetic rats.
Fig. 2. Concentration-response curves for acetylcholine-induced vasorelaxation of aortic strips obtained from age-matched streptozotocin-induced diabetes (A) and GK diabetic (B) rats.

Each data point represents the mean ± SEM of 6 experiments; *=P< 0.05 STZ-diabetic vs. STZ-control and GK-diabetic vs. Wistar control (WC) group, n = 6.

3.4 Relation to Levcromakalim

The cumulative dose-response curve to the ATP-dependent potassium ion channel opener, levcromakalim in aorta shows that the aortic rings from all experimental groups relaxed to levcromakalim. The relaxation response of aorta from STZ-diabetic rat was significantly (P<0.05) reduced with a shift of the dose-response curve to the right of STZ-control (Fig. 3A). DM significantly (P<0.05) caused a reduction in maximum relaxation in STZ model when compared with STZ-control (Table 2). In contrast, the sensitivity (-logIC50), GK-diabetic, 7.53 ± 0.11 vs. Wistar control, 7.39± 0.08, n = 6, P > 0.05)

and maximum relaxation (GK-diabetic, 97.63 ± 0.88% vs. Wistar control, 93.17 ± 2.25%, n = 6, P > 0.05)
to levcromakalim were not affected (Fig. 3B). However, the sensitivity and maximum relaxation was significantly (P<0.05) greater in GK diabetic model when compared with STZ diabetic model.

Fig. 3. Concentration-relaxation response curves for levcromakalim of aortic strips obtained from age-matched controls and streptozotocin-induced (A) and GK (B) rats.

* Each data point represents the mean ± SEM of 6 experiments; *= P< 0.05 STZ-diabetic vs. STZ-control, n = 6.

3.5 Relaxation to (+/-)-naringenin

The relaxation induced by the flavonoid, (+/-)-naringenin was significantly reduced in the aorta from STZ-diabetic rat when compared with age-matched control (Fig 4A). The maximal relaxation of aortic rings to (+/-)-naringenin was also significantly (P<0.05) reduced in STZ-diabetic group when compared with STZ-control groups. In contrast, relaxation of aortic rings from GK rats to (+/-)-naringenin was significantly enhanced with a significant (P<0.05) increase in sensitivity compared with Wistar control and STZ-diabetic groups. However, the maximum relaxation or aortic rings in GK was not significantly different from Wistar control (Fig. 4B).

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Each data point represents the mean ± SEM of 6 experiments; *=P< 0.05 STZ-diabetic vs. STZ-control and GK-diabetic vs. Wistar control (WC) group, n = 6.
Fig. 4. Concentration-relaxation curves for naringenin of aortic strips obtained from age-matched controls and streptozotocin-induced (A) and GK diabetic (B) rats.

Each data point represents the mean ± SEM of 6 experiments; *=P< 0.05, STZ-diabetic vs. STZ-control and GK vs. Wistar control (WC) group respectively, n = 6.

4. DISCUSSION

The main findings in the present study, which compared vascular reactivity of aortas from GK (Type 2 diabetes) rats with those from age-matched streptozotocin (Type 1 diabetes) control rats, is that phenylephrine-induced contraction is increased and endothelium-dependent relaxation is attenuated in STZ-induced DM whereas it is preserved in GK diabetic rat. Similarly, the KATP channel function and naringenin relaxation are enhanced in GK model while it is attenuated in STZ model. These results suggest that in the early stage of diabetes both endothelium-dependent relaxation and potassium ion channel-mediated relaxation are preserved in Type 2 diabetic model whereas the vasoreactivity is altered in Type 1 diabetes.

The result of decreased phenylephrine-induced contraction in Type 2 diabetes is in agreement with a previous studies conducted on the aorta and mesenteric artery of streptozotocin-induced, Zucker and GK rats that showed a decrease in contractile responses to alpha adrenergic agonist in early diabetic condition (Kobayashi et al., 2004). Various mechanisms for the decreased phenylephrine response include receptor desensitization or signalling pathways, alteration in calcium sensitivity/handling mechanisms and inhibition of contractile effects of alpha-adrenergic agonists in vascular smooth muscle by NO (Vanhoutte and Miller, 1989, Kobayashi et al., 2004). Therefore, the reduced contractility to phenylephrine in the GK aorta may be due to exaggerated NO-dependent relaxation that inhibits the contractile mechanism of the alph-adrenoceptor in the endothelium of the GK rats.
Although a previous study had reported that down-regulation of the alpha-1 adrenoceptor is not likely to mediate the enhanced contractile responses in arteries from STZ diabetes (Weber and Macleod, 1997), an increased alpha receptors density have been reported in STZ-diabetic rats (Schulingkamp et al., 2005). Another study reported that there is a reduced adrenergic-induced contractile response seen at an early stage in the GK diabetic aorta due to NO-dependent relaxation mediated via an increased expression of the alpha 2D-adrenoceptor in the endothelium (Kobayashi et al., 2004). Taken together, the observed differences in contractile responses to phenylephrine found between the STZ-diabetic and GK diabetic models could probably be due to differences in the adrenergic receptor expressions and coupling mechanisms that mediate contractile responsiveness elicited by alpha-1 adrenoceptor stimulation.

Aortas from GK diabetic rats exhibited an enhanced endothelium-dependent relaxation to ACh in comparison with the age-matched STZ-diabetic rats suggesting that endothelium-dependent relaxation is preserved in early stage of Type 2 DM. The preserved ACh-mediated relaxation of aortic rings from the GK rats that we reported in this study confirms previous studies of this model of diabetes (Brondum et al., 2005). In contrast to preserved endothelial function in Type 2 DM, attenuated relaxation response was observed in STZ-diabetic aorta which is in agreement with previous studies (Orie et al., 1993; Majithiya and Balaraman, 2006). Endothelium-mediated response is via the nitric oxide pathway which is altered by endothelial dysfunction (Natali et al., 2005). In STZ-induced DM, the basal levels of NO production and expression of eNOS are reduced in diabetic arteries suggesting eNOS uncoupling (Leo et al., 2011) while there is enhanced NO production via overexpression of eNOS in GK diabetes (Zhong et al., 2012). It is therefore possible that the nitric oxide pathway is preserved through enhanced expression of eNOS in the early stage in Type 2 diabetes therefore contributing to the enhanced endothelium-dependent relaxation to ACh.

Vascular potassium ion channels play a major role in the modulation of vascular tone and local blood flow (Orie et al., 2009). Studies have shown that in Type 1 DM there is a diminished response to KATP channel openers causing vasoconstriction (Zimmermann et al., 1997) while KATP-sensitive K+ channel have been reported to be attenuated in Type 2 diabetes (Miura et al., 2003; Erdos et al., 2004). The mechanisms that underlie potassium ion channel dysfunction in diabetic vessels are dependent on the animal model, vessel bed, and stage of diabetes (Lu et al., 2005). Therefore, KATP and BKCa channels may provide a compensatory mechanism for preserving the dilator responses attributed to the dysfunction of the aorta in early stage in this model of Type 2 diabetes. However, other regulatory mechanisms may contribute to the observed preservation of potassium ion channel in Type 2 DM such as G-protein coupled receptor-evoked release of NO which is influenced by endothelial calcium-activated potassium ion channels and an influx of calcium ion (Misurski and Gopalakrishnan, 2002; Simonsen et al., 2009). Thus, the KATP channels may contribute to vasodilator mechanisms in the early stage of Type 2 DM disease where the level of reactive oxygen species is at a minimal level.

Naringenin, a naturally occurring flavanone present in citrus fruits (Cavia-Saiz et al., 2010) has been reported to induce concentration-dependent relaxation in aortic tissue from normal rats (Saponara et al., 2006). In this study, naringenin improved vasorelaxation in GK rats though aortic reactivity did not improve in STZ DM group. This result is different from a recent study that naringenin could prevent the functional changes in vascular reactivity in STZ diabetic rats through nitric oxide pathway (Fallahi et al., 2012). The difference could be probably due to experimental design.

The low level of hyperglycaemia in the GK group when compared with the STZ-diabetic rats reflects the low level of oxidative stress and may contribute to the differences in vascular activity in the two models of diabetes mellitus. The differences in responses between type 1 and type 2 DM may not be attributed to hyperglycaemia alone. It was observed in this study that the maximum contraction to PE and ACh in the STZ-DM was similar to Wistar control despite a great difference in their blood glucose levels. A recent study has reported that the lean GK had marked hyperglycemia with well-preserved endothelium function at early and later stages of DM suggesting that hyperglycemia may not cause vessel dysfunction but may be a preconditioning stimulus, inducing endothelial nitric oxide synthase and haemoxigenase that can exert both vasodilation as well as cardiovascular protection at early or late stage of type 2 diabetes (Zhong et al., 2012).
5. CONCLUSION

The present results suggest that phenylephrine-induced contraction, endothelium-dependent relaxation, KATP and naringenin-induced vasorelaxation are preserved in the early stages of Type 2 diabetes whereas there is exaggerated contractile response and a relaxant dysfunction involving the endothelium, KATP in Type 1 diabetes mellitus. The difference in vasoreactivity is probably due to differential receptor mechanisms that mediate these responses.

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COMPETING INTERESTS

Authors do not report any conflicts of interest

AUTHORS’ CONTRIBUTIONS

DUO performed the experiment, NNO designed the study and managed the analyses of the study, CRN wrote the second draft, LHC and EEO reviewed the manuscript. All authors read and approved the final manuscript.

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