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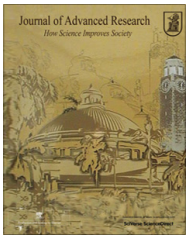

ORIGINAL ARTICLE

# The effect of streptozotocin-induced diabetes on the EDHF-type relaxation and cardiac function in rats

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**KEYWORDS**

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**Abstract** The endothelium-derived hyperpolarizing factor (EDHF) response is a critical for the functioning of small blood vessels. We investigated the effect of streptozotocin-induced diabetes on the EDHF response and its possible role in the regulation of cardiac function. The vasorelaxant response to ACh- or NS309- (direct opener endothelial small- ($SK_{Ca}$)- and intermediate-conductance ($IK_{Ca}$) calcium-activated potassium channels; main components of EDHF response) were measured in pressurized mesenteric arteries (diameter 300–350 μm). The response to 1 μM ACh was reduced in diabetes (84.8 ± 2.8% control vs 22.5 ± 5.8% diabetics; $n \geqslant 8$; $P < 0.001$). NS309 (1 μM) relaxations were also decreased in diabetic arteries (78.5 ± 8.7% control vs 32.1 ± 5.8% diabetics; $n \geqslant 5$; $P < 0.001$). $SK_{Ca}$ and $IK_{Ca}$-mediated EDHF relaxations in response ACh or NS309 were also significantly reduced by diabetes. Ruthenium red, RuR, a blocker of TRP channels, strongly depress the response to ACh and NS309 in control and diabetic arteries. RuR decreased $SK_{Ca}$ and $IK_{Ca}$-mediated EDHF vasodilatation in response to NS309 but not to ACh. An elevation in systolic blood pressure was observed in diabetic animals. ECG recording of control hearts showed shortening of PR interval. RuR reduced PR interval and R wave amplitude in diabetic hearts. In conclusion, the reduced EDHF-type relaxations in STZ-induced diabetes is due impairment of $K_{Ca}$ channels function. TRP channels possibly contribute to EDHF vasodilatation

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*via* direct opening of endothelial $K_{Ca}$. It is possible that EDHF and TRP channels contribute to the regulation of cardiac function and therefore can be considered as therapeutic targets to improve cardiovascular complications of diabetes.



## Introduction

Endothelial cells have an essential role in the control of tone of the underlying smooth muscle cells *via* the release of various vasodilators [1,2]. These include nitric oxide (NO), prostacyclin and the endothelium-derived hyperpolarizing factor (EDHF) [3]. Although the exact mechanism by which EDHF acts is controversial [4,5], it is well-established that endothelial small-conductance, ($SK_{Ca}$) and the intermediate-conductance, calcium-activated potassium channel ($IK_{Ca}$) are essential for the initiation of the EDHF pathway [5]. The activation of these channels requires an increase in the intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$ of endothelial cells [6]. The hyperpolarization-induced by the activation of endothelial $K_{Ca}$ channels increases the driving force for $Ca^{2+}$ influx *via* cation channels belonging to transient receptor potential ion channels (TRP channels) which sustain the $Ca^{2+}$ signal [7].

The contribution of EDHF to the relaxation of blood vessels depends on the size of the blood vessel being of major importance in small arteries [8].

Complications of diabetes (such as nephropathy and retinopathy) are due to dysfunction of small blood vessels [9]. Thus, the impairment of the EDHF responses could have an important impact on the microvasculature. Indeed, Wigg et al. [10] reported a selective impairment of the EDHF-mediated relaxation in the mesenteric artery whereas Shi et al. [11] reported an augmented contribution of EDHF and reduced contribution of NO to endothelium-dependent relaxations. Leo et al. [12] showed an impairment of both, NO and EDHF-dependent relaxation of rat mesenteric arteries. These studies showed a reduced responsiveness to the endothelium dependent vasodilator acetylcholine (ACh) which induces the activation of endothelial $K_{Ca}$ channels by a global increase in $[Ca^{2+}]_i$ [13,14].

NS309 is a selective opener of both the $SK_{Ca}$ and $IK_{Ca}$ channels acting by enhancing the sensitively of $K_{Ca}$ channels to intracellular $Ca^{2+}$ [15]. This compound hyperpolarizes smooth muscle cells of rat mesenteric arteries [16] and human endothelial cells [17]. Recently, it has been demonstrated that there is a reduction in EDHF-type relaxation upon ACh or NS309 stimulation of mesenteric small arteries from ZDF rat; an animal model of type II diabetes [18].

Changes in the heart rate are accompanied by alterations in both $[Ca^{2+}]_i$ and action potential duration (APD) [19]. The expression of different subtypes of $SK_{Ca}$ channels were demonstrated in rat, murine and human hearts [20–22]. It was hypothesized that based on the high calcium-sensitivity of these channels, they may be involved in the modification of APD of cardiac tissues particularly during cardiac repolarisation. Indeed, based on the observation that the inhibition of $SK_{Ca}$ channels lengthens the APD, it was suggested that these channels can represent an antiarrhythmic mechanism [21].

The aim of the present study was, therefore, to investigate the effect of streptozotocin (STZ)-induced diabetes on the EDHF (and its main components $IK_{Ca}$ and $SK_{Ca}$)-mediated relaxation of mesenteric arteries using activators that work by two different mechanisms namely ACh (by causing a global increase in $[Ca^{2+}]_i$) and NS309 (acting by direct activation the $K_{Ca}$ channels). Both $K_{Ca}$ channels are activated by increase in $[Ca^{2+}]_i$ in order to initiate EDHF pathway. Therefore, we tested whether any change in NS309 or ACh-induced EDHF response is due to change in $Ca^{2+}$ influx mechanism especially TRP channels; one of the main pathways for $Ca^{2+}$ influx into the endothelial cells. The possible role of EDHF response in the regulation of cardiac function was also studied.

Our data suggest that the EDHF response is reduced in rats with (STZ)-induced diabetes. This is attributed to the impairment of direct opening of endothelial $K_{Ca}$ channels. TRP channels may be involved in the EDHF-mediated relaxations. EDHF response contributes to the regulation of the electrical conduction of normal hearts whereas the role of TRP channel is more prominent in diabetic hearts.

## Methodology

### *Animals*

Animal use in the present study was approved by The Animal Use Committee of Aleppo University and is in accordance with the institutional regulations. Male albino Wistar rats (220–300 g; $n = 25$) were maintained in the laboratory of the animal unit of Aleppo University under standard laboratory conditions, i.e. at 25 ± 2 °C with a 12-h dark-light cycle. They were fed with regular chow, and given free access to water. Diabetes was induced by a single intravenous injection of streptozotocin (STZ; 60 mg/kg of body weight, dissolved in citrate buffer, pH 4.5), into the tail vein. For controls, age-matched rats were injected with the same volume of citrate buffer only. All experiments were performed four weeks after the STZ injection; at that time the tail blood glucose level was above 350 mg/dl.

### *Preparation of mesenteric arteries*

Rats were decapitated. Small mesenteric arteries (second order branch; approximate diameter 300–350 μm) were rapidly removed and placed in ice-cold Krebs solution (composition in mM: NaCl 119, KCl 4.7, $CaCl_2$ 2.0, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.18, glucose 11) bubbled with 95% $O_2$ and 5% $CO_2$. The artery was carefully cleaned of fat and connective tissue, and cut into segments 1–2 mm in length, these were cannulated and mounted in the chamber of a pressure myograph (Model 111P; Danish Myo Technology, Aarhus, Denmark) containing 10 ml of oxygenated (95% $O_2$–5% $CO_2$) Krebs solution. The arteries were left for at least 30 min to adapt before application of drugs; the intraluminal pressure was held at 70 mm Hg and the temperature at 37 °C. The external diameter of the artery was recorded with CCD camera using MyoView software (Danish Myo Technology, Aarhus, Denmark). In order to study the EDHF-mediated response,

Krebs solution containing 300 μM *N*-nitro-L-arginine and 10 μM indomethacin (non-selective nitric oxide synthase and cyclooxygenase inhibitors, respectively) was used throughout the experiments. Arteries were pre-constricted with an approximate $EC_{50}$ concentration of phenylephrine (1 μM). $K_{Ca}$ and TRP channel inhibitors were applied intraluminally at least 20 min before the application of ACh or NS309.

*Body weight and biochemical measurements*

Body weights were determined before and after the induction of diabetes and at the day of the experiment. Glucose levels were measured in samples taken from blood *via* the tail vein using the glucose oxidase method (BioSed, Italy). Insulin levels were measured using Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem., Inc., IL, USA) with a microplate reader (Multiskan EX Microplate Photometer, Thermo Scientific, Schwerte, Germany).

*Tail-cuff blood pressure measurements*

Arterial blood pressure was measured non-invasively (Volume Pressure Recording; using a CODA 8-channel tail-cuff blood pressure system; Kent Scientific, Torrington, CT, USA). Blood pressure (BP; systolic, diastolic & mean) and heart rate (HR) measurements were performed after pre-warming the rats on a platform kept at 37 °C for 10 min. This allows to de-stressing the rat. However, the rat which exhibits any signs of disturbance after 10 min (such as moving the tail) was excluded from the study. The proximal occlusion cuff constricts the tail artery, while the distal cuff detects changes in tail artery volume when blood flow resumes as the occlusion cuff deflates. Measurements of average of three sessions (each consisting of 15 cycles) were used for statistical analysis.

*Electrocardiogram (ECG) recording*

Control and diabetic rats were anaesthetized with sodium pentobarbitone (50 mg $kg^{-1}$, i.p), were given heparin (250 IU i.v), and were killed by cervical dislocation. Their hearts were rapidly excised and placed immediately into an ice-cold perfusion buffer. These were cannulated through the aorta in a Langendorff system, perfused with oxygenated (95% $O_2$, 5% $CO_2$) Krebs-Henseleit solution (composition in mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, glucose 11.0, $NaHCO_3$ 25.0, pH 7.4) at 37 °C and allowed to stabilize for 30 min after being mounted. Initial perfusion pressure kept constant at 80 mmHg. The isolated hearts from control and diabetic rats were treated with: Krebs solution, Krebs solution plus 300 μM *N*-nitro-L-arginine and 10 μM indomethacin (in order to inhibit NO and prostaglandin synthesis) with and without 1 μM ruthenium red (a non-selective blocker of TRP channels; also known for its specific inhibition of TRPV channels in low micromolar concentrations) [23,24]. The ECG was recorded using an Animal BioAmp amplifier (Lab/8s, ADInstruments, Oxford, UK).

*Drugs*

Acetylcholine (ACh; as chloride salt), indomethacin, $N^G$-nitro-L-arginine, NS309 (3-oxime-6,7-dichloro-1H-indole-2,3-dione) and ruthenium red were from Sigma–Aldrich, UK. Apamin was from Latoxan, USA, and 1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole (TRAM-34) from Enzo Life Sciences, UK.

*Data analysis*

All values are given as mean ± SEM. The number of animals is given by n. Data were analysed using analysis of variance (ANOVA) (GraphPad Prism software, version 4) followed by a Bonferroni's *post hoc*-test, where applicable. *P* values of less than 0.05 were considered to indicate statistically significant differences.

## Results

*Glucose level and body weight*

About threefold higher levels of glucose were measured in STZ-treated rats (diabetic rats) in comparison with untreated controls (Table 1). By contrast, insulin levels were significantly lower in diabetic rats. An approximately 40% reduction in body weight was observed in the latter (Table 1). Assessment of a potential cardiac hypertrophy induced by diabetes, as determined by the heart to body weight ratio, was negative (Table 1). The liver and lung to body weight ratios were not significantly changed.

*Blood pressure*

No significant changes were observed in diastolic and mean blood pressure (BP). In contrast, significant increases in heart rate (HR), systolic were observed (Table 2).

*Relaxations*

*Acetylcholine*

ACh induced a concentration-dependent relaxation of mesenteric arteries from control rats ($10^{-8}$–$10^{-5}$ M). ACh at 1 μM concentration (which produced submaximal relaxation of control arteries) was decreased by 75% in mesenteric arteries from STZ-diabetic rats (Fig. 1a, Table 3). ACh-induced relaxation mediated by $IK_{Ca}$ (in the presence of 100 nM apamin to block $SK_{Ca}$ channel activity) [25] was significantly reduced in diabetic arteries in comparison with controls (Fig. 1b, Table 3). Similarly, in the presence of 1 μM TRAM-34 (to block $IK_{Ca}$

**Table 1** Glucose and insulin levels, body weights, liver/body weight, lung/body weight and heart/body ratios, both in control and diabetic animals ($n = 25$). Data expressed as mean ± SEM.

| | Control | Diabetics |
|---|---|---|
| Glucose level (mg/dl) | 130.8 ± 11.2 | 433 ± 20.1* |
| Insulin level (μU/ml) | 19.2 ± 2.3 | 3.4 ± 0.3* |
| Body weight (g) | 259 ± 11 | 162 ± 6.1* |
| Liver/body weight ratio (g) | 38.4 ± 3.3 | 40.2 ± 2.4 |
| Lung/body weight ratio (g) | 5.0 ± 0.5 | 6.5 ± 0.4 |
| Heart/body weight ratio (g) | 3.5 ± 0.3 | 3.7 ± 0.5 |

* Significantly different from the control rats (Unpaired *t* test, $P < 0.001$).

**Table 2** Blood pressure parameters in control and diabetic rats ($n = 25$). Data expressed as mean ± SEM.

| | Control | Diabetics |
|---|---|---|
| HR (beats/min) | 340.8 ± 6.4 | 383.3 ± 10.0* |
| Diastolic pressure (mmHg) | 84.9 ± 2.5 | 92.4 ± 6.2 |
| Systolic pressure (mmHg) | 126.8 ± 2.8 | 148.0 ± 7.5* |
| Mean pressure (mmHg) | 96.2 ± 1.7 | 110.9 ± 8.5 |

* Significantly different from the control rats (unpaired $t$ test, $P < 0.05$).

channel activity and reveal $SK_{Ca}$-mediated responses) [26,27], the relaxation to ACh was reduced by 80% (Fig. 1c, Table 3). In the presence of TRAM-34 plus apamin, ACh-induced relaxation was completely abolished in diabetic but in control arteries (10.3 ± 7.1%; $n = 4$).

*Ns309*

NS309 induced a concentration-dependent relaxation of mesenteric arteries from control rats ($10^{-8}$–$10^{-5}$M). It was used at 1 μM concentration which produced submaximal relaxation of control arteries. This concentration is well below the $IC_{50}$ (10 μM) reported to inhibit voltage-dependent calcium channels in urinary bladder smooth muscle cells [28].

NS309-mediated relaxations were reduced by approximately 60% in arteries from diabetic rats (Fig. 2a, Table 3). In the presence of 100 nM apamin, the relaxation to 1 μM NS309 was decreased (Fig. 2b, Table 3). A reduction in the response to NS309 in the presence of 1 μM TRAM-34 was also observed in diabetic arteries in comparison with controls (Fig. 2c, Table 3).

We then tested whether the impairment of EDHF-type relaxation involves dysfunction of $Ca^{2+}$ influx mechanism mainly *via* TRP channels.

(a) The application of 1 μM ruthenium red (non-selective blocker of TRP channel blocker [29]) reduced the relaxant effect of ACh (Fig. 1a), suggesting an involvement of these channels in EDHF-mediated relaxation of mesenteric arteries. ACh-mediated relaxations in the presence of 100 nM apamin (Fig. 1b) or in the presence of 1 μM TRAM-34 (Fig. 1c) were not affected by ruthenium red.

In the presence of both TRAM-34 and apamin the remaining relaxant response to ACh (10.3 ± 7.1%; $n = 4$) was not affected by ruthenium red (9.1 ± 2.3%; $n = 4$).

Ruthenium red did not produce any effect when applied alone indicating the absence of non-specific effects on endothelial or smooth muscle cells at the concentration used.

The above results do not exclude the possibility that $Ca^{2+}$ influx through TRP channels is involved in the activation of $K_{Ca}$ channels and consequently the EDHF response. Therefore, a selective opener of $IK_{Ca}$ and $SK_{Ca}$ channels (NS309) was used [30].

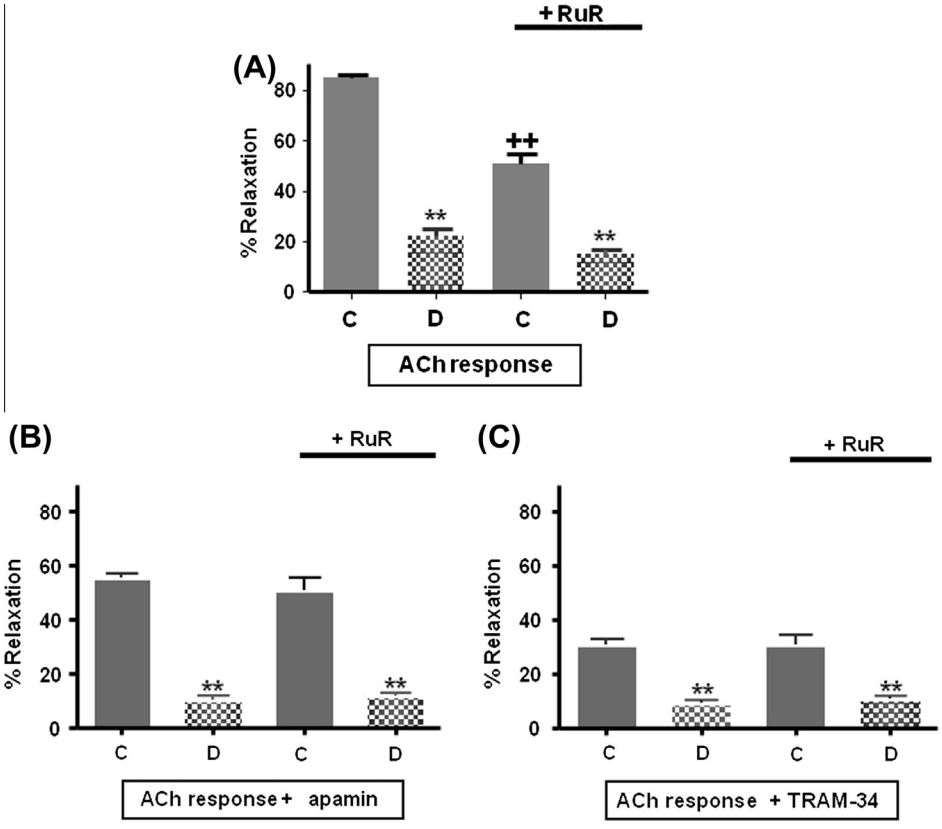


**Fig. 1** Changes (in%) of the EDHF-mediated relaxation of mesenteric arteries in control and diabetic rats in response to ACh. (A) ACh- (1 μM) induced relaxation of mesenteric arteries from control rats was significantly reduced in diabetics. The $IK_{Ca}$ response, in the presence of 100 nM apamin (B) and $SK_{Ca}$ response, in presence of 1 μM TRAM-34 (C), were also affected by diabetes. RuR (1 μM) produced a decrease in the response to ACh in arteries from both control and diabetic animals (A) but did not affect either $IK_{Ca}$ (B) or $SK_{Ca}$ (C) -mediated responses. Results shown are means ± s.e.mean ($n \geqslant 5$). One-way ANOVA; $^*P < 0.05$ was considered significant.

**Table 3** Changes (in%) of the EDHF- [induced by 1 μM ACh or 1 μM NS309, +1 μM TRAM-34, +100 nM apamin in the presence the (+) and in the absence (−) of 1 μM ruthenium red] mediated relaxations of mesenteric arteries from control and diabetic rats. Data expressed as mean ± SEM.

| | Ruthenium red | Controls (*n*) | Diabetics (*n*) |
|---|---|---|---|
| *ACh* | | | |
| ACh | − | 84.8 ± 2.8% (8) | 22.5 ± 5.8%* (10) |
| +TRAM-34 | − | 55.5 ± 3.7% (5) | 10.8 ± 3.5%* (6) |
| +apamin | − | 31.1 ± 3.3% (5) | 9.5 ± 2.5%* (6) |
| ACh | + | 50.6 ± 8.7%§ (4) | 15.7 ± 3.3% (5) |
| +TRAM-34 | + | 50.7 ± 11.3% (4) | 12.5 ± 2.3% (5) |
| +apamin | + | 30.0 ± 9.0% (5) | 10.6 ± 2.7% (6) |
| *NS309* | | | |
| NS309 | − | 78.5 ± 8.7%* (5) | 32.1 ± 5.8%* (6) |
| +TRAM-34 | − | 27.0 ± 4.4%* (5) | 14.8 ± 2.7%* (6) |
| +apamin | − | 52.6 ± 8.9%* (4) | 25.4 ± 5.7%* (5) |
| NS309 | + | 39.9 ± 9.5%§ (5) | 19.8 ± 3.6%§ (5) |
| +TRAM-34 | + | 13.9 ± 4.0%§ (5) | 8.2 ± 0.7%§ (5) |
| +apamin | + | 16.6 ± 1.0%§ (5) | 8.9 ± 0.3%§ (5) |

* Significantly different from the ACh or NS309 response in the mesenteric arteries from control rats (Bonferroni's test, $P < 0.001$).
§ Significantly different from the ACh or NS309 response in the mesenteric arteries from control and diabetic rats after treatment with ruthenium red (Bonferroni's test, $P < 0.05$).

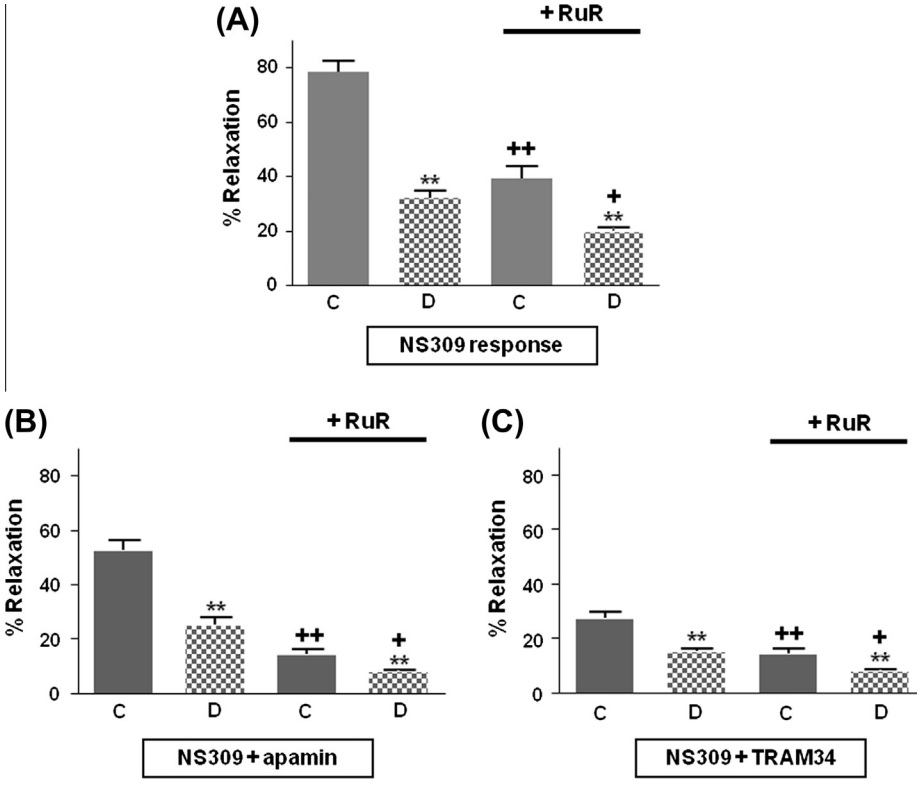


**Fig. 2** Changes (in%) of NS309-induced responses of mesenteric arteries from control and diabetic rats in response to NS309. (A) NS309 (1 μM)-induced relaxations were reduced in diabetics arteries. The $IK_{Ca}$ response, in the presence of 100 nM apamin (B) and the $SK_{Ca}$ response, in presence of 1 μM TRAM-34 (C) were also affected by diabetes. Relaxations of arteries mediated by NS309, opening of $IK_{Ca}$ (B) or $SK_{Ca}$ (C) were markedly reduced by RuR. Results shown are means ± s.e.mean ($n \geqslant 5$). One-way ANOVA; $^*P < 0.05$ was considered significant.

(a) Ruthenium red application resulted in a significant decrease in relaxation to NS309 (Fig. 2a). The application of ruthenium red reduced the relaxant response of 1uM NS309 in the presence of 100 nM apamin (Fig. 2b). A similar reduction in relaxation to NS309 was also detected in the presence of 1 μM TRAM-34 (Fig. 2c).

*Electrocardiograms parameters of isolated hearts*

There was no significant alteration in P duration, QT interval and QRS interval between hearts from control and diabetic rats (Table 4). The application of NO and COX inhibitors (to reveal EDHF pathway) before and after treatment with 1 μM ruthenium red was not accompanied by a change in the above ECG parameters (Table 4). Similarly, ST and T wave amplitude was not significantly changed between groups studied. However, a significant decrease in PR interval was obtained in hearts isolated from control rats after the infusion of NO and COX inhibitors. In addition, following the application of ruthenium red a significant decrease in PR interval and R amplitude was observed in diabetic hearts in comparison with diabetic hearts that were infused with NO and COX inhibitors (Table 4).

## Discussion

In mesenteric arteries from control rats, ACh produced a relaxation which was largely due to the opening of $SK_{Ca}$ channels. In control arteries, the relaxant response to ACh required the opening of both $IK_{Ca}$ and $SK_{Ca}$ channels as evident by significant reduction in the relaxation induced by ACh in the presence of both TRAM-34 plus apamin. The remaining ACh response could be to due to the involvement of additional pathways (independent of endothelial hyperpolarization). An example is the release of epoxyeicosatrienoic acids (EETs) acting on the potassium channels located on the smooth muscle cells [5]. In contrast, in diabetic arteries the ACh-induced relaxation appeared to be due to EDHF, since ACh failed to produce any response in the presence of TRAM-34 and apamin. This indicates that the contribution of EDHF relaxation of small arteries is becoming more important in pathological conditions. These results are in agreement with recent findings by Leo et al. [12] who showed that endothelium-dependent relaxation was abolished in diabetic arteries, but only slightly attenuated in normal arteries.

The induction of diabetes impaired the EDHF pathway in response to ACh. In the presence of either TRAM-34 or apamin, $SK_{Ca}$ or $IK_{Ca}$- mediated relaxations in response to ACh were also compromised in mesenteric arteries from diabetic rats. Our results are in agreement with previous findings [10,12]. It is possible that the impairment of the EDHF response and $K_{Ca}$-mediated relaxation of mesenteric arteries contribute to the development of elevated systolic blood pressure observed in this study, since these arteries are considered to play an important role in regulating blood pressure [30].

Previous studies [10,12] showed that the most commonly observed effect in the resistance arteries is a reduced responsiveness to the endothelium dependent vasodilator ACh [10] which induces the activation of endothelial $K_{Ca}$ channels by a global increase in $[Ca^{2+}]_i$ [13,14]. This led us test the following hypothesis:

> Is the impairment of $SK_{Ca}$ or $IK_{Ca}$- mediated relaxation in response to ACh (and consequently the EDHF response) is due to impairment of global increase in $[Ca^{2+}]_i$ which will in turn affect the activity of $K_{Ca}$ channels or is it due to the compromised function of the $K_{Ca}$ channels per se?

In order to address this possibility, NS309 (a direct opener of $K_{Ca}$ channels) was used. Results showed that NS309 induced a relaxation of mesenteric arteries from control rats. The response to NS309 was largely attenuated in diabetic rats. In addition, $SK_{Ca}$ or $IK_{Ca}$- mediated relaxation in response to NS309 was also reduced in diabetics. This indicates that impairment of the EDHF response is due to dysfunction of $SK_{Ca}$ or $IK_{Ca}$ channels.

It was also observed that NS309, which acts by increasing the channel sensitivity for $Ca^{2+}$, appears to produce its relaxant response largely *via* $IK_{Ca}$ channels. This is in agreement with study by Strobaek et al. [15] and consistent with results of a study in rat mesenteric arteries and human umbilical vein endothelial cells in which $IK_{Ca}$ channels play the prominent role with respect to the response to NS309 [27].

> Is the impairment of the EDHF relaxation in response to ACh or NS309 is related to a change in the $Ca^{2+}$ influx mechanism required for the activation of $K_{Ca}$ channels?

In order to test this possibility, we examined the EDHF-mediated relaxation of mesenteric arteries from control and diabetic rats in the presence of ruthenium red which is a non-selective blocker of TRP channels. These are considered one of the main pathways for $Ca^{2+}$ entry into the endothelial cells.

Results showed that ACh- and NS309 produced relaxations were inhibited by ruthenium red. This suggests that TRP channels are involved in the EDHF-mediated relaxation in response to ACh, or *via* NS309. Ruthenium red also reduced EDHF-mediated relaxation induced by ACh and NS309 in mesenteric arteries from diabetic rats, which suggests that TRP-mediated dilatations of those arteries are also impaired.

**Table 4** Summary of ECG parameters of the hearts obtained from control and diabetic rats without and following treatment with: NO and cyclooxygenase inhibitors (EDHF) and NO and cyclooxygenase inhibitors + 1 μM ruthenium red [EDHF + RuR] ($n = 5$). Data expressed as mean ± SEM.

| | Controls | Diabetics | EDHF-C | EDHF-D | EDHF-C + RuR | EDHF-D + RuR |
|---|---|---|---|---|---|---|
| *P* duration (ms) | 15.3 ± 2.8 | 19.10 ± 1.4 | 16.8 ± 0.001 | 20.4 ± 0.004 | 20.4 ± 0.6 | 8.82 ± 0.002 |
| QT interval (ms) | 56.4 ± 3.8 | 54.4 ± 4.6 | 62.4 ± 5.6 | 59.1 ± 8.6 | 62.4 ± 5.5 | 51.2 ± 12.4 |
| PR interval (ms) | 44.1 ± 4.2* | 30.7 ± 4.7 | 14.6 ± 3.9* | 32.8 ± 0.008* | 14.6 ± 3.9 | 16.1 ± 2.9* |
| QRS interval (ms) | 24.7 ± 1.9 | 23.8 ± 3.1 | 29.4 ± 5.9 | 25.1 ± 2.7 | 29.4 ± 5.9 | 32.2 ± 10.1 |
| ST amplitude (mV) | 0.18 ± 0.01 | 0.12 ± 0.01 | 0.39 ± 0.09 | 0.14 ± 0.07 | 0.13 ± 0.02 | 0.08 ± 0.03 |
| *T* amplitude | 0.35 ± 0.16 | 0.28 ± 0.02 | 0.21 ± 0.06 | 0.16 ± 0.02 | 0.22 ± 0.01 | 0.05 ± 0.001 |
| *R* amplitude | 1.56 ± 0.55 | 0.78 ± 0.12 | 1.03 ± 0.23 | 0.62 ± 0.12* | 0.42 ± 0.12 | 0.018 ± 0.006* |

* Significantly different from the corresponding control ($P < 0.05$).

Ruthenium red did not affect $IK_{Ca}$ or $SK_{Ca}$ induced relaxations of mesenteric arteries caused by ACh whereas it markedly reduced those produced by NS309. This strongly indicates that above) and indicates that impairment of the EDHF response is due to compromised opening of $K_{Ca}$ channels. It is also possible that the activation of TRP channels leads to dilatation of arteries *via* a mechanism which involves direct opening of endothelial $IK_{Ca}$ and $SK_{Ca}$ channels, most likely associated with a near-membrane rather than a global increase in $[Ca^{2+}]_i$ [14,16,31]; see Fig. 3. Thus, it is possible that these channels may provide and maintain some level of $[Ca^{2+}]_i$ that is necessary for the activation of endothelial $IK_{Ca}$ and $SK_{Ca}$ by NS309 (see Fig. 3). This direct interaction between $K_{Ca}$ and TRP channels and the resulting adequate levels of $[Ca^{2+}]_i$ are possibly impaired in diabetes.

This interpretation is in agreement with the results of Earley et al. [32] in rat cerebral arteries. They showed that $Ca^{2+}$ influx *via* TRPA1 (which co-localizes with $K_{Ca}3.1$) produces a vasodilatation by a mechanism involving the opening of endothelial cell $IK_{Ca}$ and $SK_{Ca}$ channels [32]. Another channel of the TRP family, TRPV4, produces EDHF-mediated vasodilatation in small-sized Arteria gracilis vessels, in which EDHF plays a significant role [24].

It is unlikely that TRP channels-mediated relaxation of mesenteric arteries from control rats is due the opening of $K^+$ channels that are located on vascular smooth muscle cells since ruthenium red did not change the relaxant response to ACh following the blockade of endothelial $K_{Ca}$ channels.

Is there any effect of STZ-induced diabetes on the function of rat hearts?

The most known metabolic disturbance associated with STZ-induced diabetes is hypothyroidism [33–35]. This was linked to cardiovascular disturbances [34], whereas other studies showed no effect of hypothyroidism on diabetes-induced cardiac dysfunction [36,37]. However, in a study by Ramanadham et al. [38], cardiac dysfunction was observed in diabetic rats in the absence of hypothyroidism.

Zhang et al. [39] reported that, in contrary to previous studies, streptozotocin-induced diabetes protected the ex vivo heart against ischemia–reperfusion induced arrhythmias. They observed signs of clinical hypothyroidism (including decreased heart rate, prolonged QT interval and decreased rectal temperature) in STZ-diabetic rats. These signs were absent from our study. Moreover, there was no change in ECG parameters in hearts isolated from STZ-diabetic rats in comparison with those obtained from control rats which exclude the possibility that STZ is associated with metabolic disturbances that affect cardiac function.

Our data showed a significant weight loss in STZ-diabetic rats despite the fact that these animals exhibited normal food intake and grooming. Weight loss also does not seem to affect cardiac function as evident by comparable ECG parameters (such as P duration and PR interval) between control and STZ-rats. The weight loss in STZ-diabetic rats observed in our study is in agreement with previous findings by Wang et al. [40] in which STZ-induced weight loss was attributed to reduction in adipose tissue mass and gene expression of proteins that play important role in the regulation of adipocytes and adipose tissue function including leptin and adiponectin receptors.

Is there any role of the EDHF in the regulation of cardiac function in control and diabetic hearts?

Both endothelial $K_{Ca}$ channels have high sensitivity to $[Ca^{2+}]_i$. which leads to the expectation that the opening of these channels (and consequently the generation of EDHF) may affect $Ca^{2+}$ handling, cardiac repolarisation and hence the electrical conduction of the cardiac myocytes [41]. In the presence of NO and COX inhibitors (in order to reveal

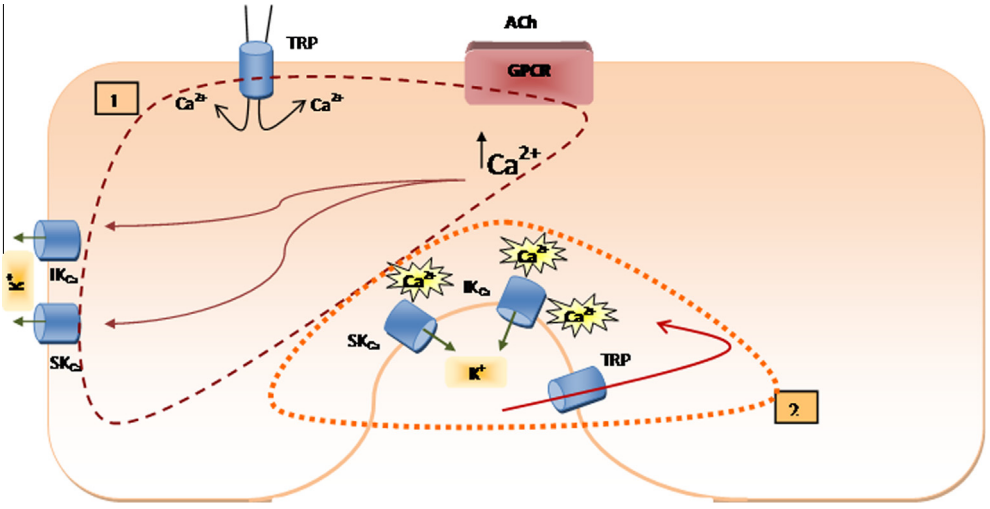


**Fig. 3** Summary of suggested pathways for the activation of $K_{Ca}$ channels and generation of EDHF. ACh leads to a global increase in $[Ca^{2+}]_i$ as a consequence of $Ca^{2+}$ release from inositol trisphosphate ($IP_3$) sensitive $Ca^{2+}$ stores within endothelial cells. The rise in $[Ca^{2+}]_i$ triggers the activation of $IK_{Ca}$ and $SK_{Ca}$ and consequently the generation of EDHF response. Depletion of intracellular stores triggers $Ca^{2+}$ entry *via* TRP channels, which consequently participates in the global increase of $[Ca^{2+}]_i$.(1). $K_{Ca}$ are possibly localized in close vicinity to TRP channels. The opening of TRP channels maintains some level of localized increase in $[Ca^{2+}]_i$, which is essential for $K_{Ca}$ channels to be activated by NS309 (2).

the EDHF pathway), the P wave duration (which represents the wave of depolarization of the atria that is created by sino-atrial nodal action potentials) [42] remained unchanged which indicates that EDHF does not contribute to the propagation of the action potential through the atria.

After atrial activation the action potentials reach the atrioventricular node and His bundle and the time during which they are activated corresponds the PR interval on the ECG [42]. In our study, the observed decrease in PR interval in hearts isolated from control rats, indicates that under normal conditions EDHF may contribute to the regulation of atrioventricular conduction.

NO and COX inhibitors had no significant effect on the duration of QRS complex (which represents the propagation of the action potential through the ventricles) [42]. The QT interval (representing the time for ventricular depolarization and repolarisation to occur) [42] was also not changed after the application of NO and COX inhibitors which suggest that EDHF does no contribute to the rate of propagation of excitation in the perfused rat heart. Since $Ca^{2+}$ influx is essential for the activation of $K_{Ca}$ channels and the EDHF response, we tested the effect of TRP channels inhibition (in the presence of both NO and COX inhibitors plus RuR) on the function of hearts from control and diabetic rats.

The inhibition of TRP channels seem to shorten the PR interval and decrease the R wave amplitude in diabetic hearts. These changes indicate that TRP channels may have a role in the regulation of the electrical conduction through the AV node as well as intraventricular conduction particularly in diabetes. Indeed, a recent study on the developing chick heart showed that TRPC channels have a role in the regulation ventricular activity and their inhibition leads to ventricular arrhythmias [43]. However, other ECG parameters were comparable between control and diabetic hearts in the presence and absence of NO and COX inhibitors and ruthenium red. Future studies are needed to elucidate the type of $K_{Ca}$ and/or TRP channels involved in there regulation of cardiac electrical activity in isolated myocytes.

There are other important questions that remain to be answered including which TRP channel/s are involved in the EDHF response and whether the function and/or protein expression of these channels are also affected by diabetes.

## Conclusion

The results of the present study demonstrate: (1) the impairment of EDHF-mediated relaxation of rat mesenteric arteries with streptozotocin-induced diabetes; (2) diabetes affects the direct opening of both $IK_{Ca}$ and $SK_{Ca}$ channels; (3) a possible involvement of TRP channels in the EDHF-mediated relaxation of rat mesenteric arteries; (4) TRP-induced relaxations are likely mediated *via* the opening of endothelial $K_{Ca}$ channels and are affected by diabetes (5) the EDHF response is possibly involved in the regulation of the electrical conduction between the atria and the ventricles in hearts from control rats. In contrast, TRP channels are more important in diabetic state and may be play a role in the regulation of ventricular activity of the heart.

Based on the findings of the resent study, it is possible that $K_{Ca}$-mediated EDHF response and TRP channels could provide potential therapeutic targets for the treatment of cardiovascular complications associated with diabetes.

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