Oxidative stress impairs IKCa- and SKCa-mediated vasodilatation in mesenteric arteries from diabetic rats

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Abstract: Objective To investigate the role of oxidative stress in impaired intermediate-conductance Ca²⁺-activated potassium channels (IKCa) and small-conductance Ca²⁺-activated potassium channels (SKCa) in mesenteric arteries of diabetic rats. Methods Rat diabetic model was induced by high fat and high glucose diet and streptozotocin (STZ) injection. Endothelial function of mesenteric arteries was assessed with use of wire myography. Expression levels of IKCa and SKCa in cultured human umbilical vein endothelial cells (HUVECs) treated with H2O2 and antioxidant alpha lipoic acid (ALA) were measured using Western blotting. Results IKCa and SKCa-mediated vasodilatation in response to acetylcholine was impaired in the third-order mesenteric arterioles of diabetic rats. In cultured HUVECs, H2O2 significantly decreased the protein expression of IKCa and SKCa. ALA alleviated the impairment of both vasodilatation function of the mesenteric arterioles and enhanced the expression of IKCa and SKCa challenged with H2O2 in cultured HUVECs. Conclusion Our data demonstrated for the first time that impaired IKCa- and SKCa-mediated vasodilatation in diabetes was induced, at least in part, by oxidative stress via down-regulation of IKCa and SKCa channels.

Key words: diabetes; Ca²⁺-activated potassium channels; oxidative stress; endothelial cells

INTRODUCTION

Diabetes mellitus is a serious and growing health problem. Vascular complications cause the majority of diabetes-related deaths. Oxidative stress plays a key mediatory role in the development of diabetic vascular diseases. The endothelium mediates the dilation of the arterioles by releasing an array of relaxing factors including nitric oxide (NO), prostacyclin (PGI2) and endothelium-derived hyperpolarizing factor (EDHF) from the endothelial cells. The contribution of EDHF to vasodilatation increases as the vessel size decreases, and EDHF activity plays a predominant role in the resistance vessels. Endothelial intermediate-conductance Ca²⁺-activated potassium channels (IKCa) and small-conductance Ca²⁺-activated potassium channels (SKCa) are pivotal in mediating the effects of EDHF in many small vascular beds. In diabetic rats, EDHF-mediated relaxation is impaired in the resistance arteries involving the down-regulation of IKCa and/or SKCa. Opening IKCa and SKCa channels restores attenuated EDHF-type relaxation in mesenteric small arteries in Zucker diabetic fatty (ZDF) rats. However, the mechanisms behind the impairment of IKCa- and SKCa-mediated relaxation in diabetes remain poorly understood.

Oxidative stress plays a key mediatory role in the development and progression of diabetes and its complications due to increased production of free radicals and impaired antioxidant defenses. It has been reported that oxidative stress may constitute the key and common event in the pathogenesis of secondary diabetic complications. But the role of oxidative stress in impaired IKCa- and SKCa-mediated relaxation in diabetic resistance arteries remains unclear.

In this study, using streptozotocin (STZ)-induced diabetic rats and cultured human umbilical vein endothelial cells (HUVECs), we found that oxidative stress was responsible for, at least in part, the impairment of IKCa- and SKCa-mediated relaxation in mesenteric arteries via down-regulation of IKCa and SKCa channels.

METHODS

Animals

The experimental protocols were approved by the Institutional Animal Care and Use Committee of Xi’an Jiaotong University and carried out in line with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA. Thirty-five male Sprague-Dawley rats (weighing 173±18 g) were housed at 22±2 °C with a humidity of 55±5%
and a 12/12 h light/dark cycle. Diabetes was induced by a single intraperitoneal injection of 30 mg/kg STZ after 8 weeks on a modified high fat and high glucose diet (10% grease, 20% sucrose, 1% bile salt, and 2.5% cholesterol) [3]. Blood glucose was measured using One Touch Sure Step Glucose Meter (LifeScan Inc., Milpitas, CA, USA) 1 week after STZ injection. Only the rats with a blood glucose of >11.1 mmol/L were included in diabetes mellitus (DM) group and alpha lipoic acid (ALA) group. The rats in DM and ALA groups were fed with high-fat and high-glucose diet for another 4 weeks after STZ injection with standard rat chow for two additional months. The rats in ALA group were treated with ALA (100 mg·kg·day⁻¹, intragastrically administered) after the elevation of blood glucose. Age-matched rats fed with standard chow and injected with the drug vehicle citric acid buffer served as the control.

Reagents

1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34), 6,12,19,20,25,26-hexahydro-5,27:13,18:21, 24-Triethleno-11,7-metheno-7H-dibenzo[b,m] [1, 5, 12, 16] tetraazacyclotricosine-5,13-diiium(UCL-1684), ALA, indomethacin (INDO), N-nitro-l-arginine (L-NAME) and other chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-IKCa and anti-SKCa antibodies were from Alomone Labs Ltd. (Jerusalem, Israel). All drugs were dissolved in distilled water, with the exception of INDO, which was dissolved in 0.1 mol/L sodium carbonate, L-NAME, which was dissolved in 0.1 mol/L sodium bicarbonate, TRAM-34, UCL-1684 and ALA, which were dissolved in dimethyl sulfoxide (DMSO).

Isometric force measurements in mesenteric small arteries

After the rats were sacrificed, and the mesenteric arcade was isolated and immediately placed in ice-cold Krebs-Henseleit solution (KHS, containing, in mmol/L, NaCl 115, NaHCO₃ 25, KCl 4.6, NaH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 2.5, and glucose 10.0; pH 7.4) gassed with a mixture of 95% O₂ and 5% CO₂. The third-order branches of the superior mesenteric artery (internal diameter about 300 μm) were isolated with the fat and connective tissues removed, and cut into 2-mm-long rings. The arterial rings were threaded onto two stainless steel wires (40 μm in diameter) and mounted in 5 ml chambers of a multi-myograph system (model 610M, Danish Myo Technology, Aarhus, Denmark) containing KHS continuously aerated with 95% O₂ and 5% CO₂ at 37 °C for isometric force measurements. Tension signals were relayed to a PowerLab recording unit and saved to a Chart 7 for Windows software (ADInstruments Ltd, Aarhus, Denmark). After mounting, the arteries were allowed to equilibrate for 20 min before normalization. The passive tension-internal circumference was determined by stretching to 90% of Lₒₒ, which was defined as the circumference of the relaxed artery exposed to a transmural pressure of 100 mmHg [13]. The vessels of the rats in the control, DM and ALA groups were then allowed to equilibrate for at least 60 min with the bath solution changed every 15 min. After equilibration, the reactivity of the rings was examined thrice by administration of 60 mmol/L KCl (achieved by substitution of NaCl in KHS with an equimolar concentration of KCl). To assess the integrity of the endothelium, the mesenteric arteries were precontracted with phenylephrine (PE, 1 μmol/L) and a high dose of ACh (10 μmol/L) was used to relax the artery rings. ACh-induced relaxation was greater than 80% of the precontracted tone in all cases, indicating that the endothelium was functionally intact.

Myography protocol

After further washouts, the arteries were again precontracted with 1 μmol/L PE. The effect of the treatment on relaxant responses was determined by cumulative concentration-response curves to the endothelium dependent relaxant, ACh (0.1 nmol/L-10 μmol/L), and endothelium-independent relaxant, sodium nitroprusside (SNP, 0.01 nmol/L-10 μmol/L). In addition, the responses to ACh were examined after a 15-min incubation with 100 μmol/L L-NAME, a non-selective nitric oxide synthase (NOS) inhibitor, and with 10 μmol/L INDO, a blocker of cyclooxygenase (COX). The vessels were again washed and allowed to rest for 30 min in KHS before incubation with UCL-1684 (0.1 μmol/L), a selective blocker of SKCa, or with TRAM-34 (10 μmol/L), a selective blocker of IKCa, constantly in the presence of INDO and L-NAME, and the ACh concentration-response curve was repeated.

Cell culture and treatments

Human umbilical vein endothelial cell line (CRL-1730) was purchased from ATCC (Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12, Gibco) containing 10% fetal bovine serum (FBS, Invitrogen), penicillin (100 U/ml) and streptomycin (100 μg/ml) and incubated at 37 °C in a humidified atmosphere with 5% CO₂. The medium was changed twice weekly and the cells were subcultured when growing to 80% confluence.

Western blot analysis

HUVECs incubated with different stimuli were lysed with ice-cold modified RIPA buffer (60 mmol/L Tris-HCl, 0.25% SDS, 1 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 10 μg/ml aprotinin and leupeptin). The lysates were then centrifuged at 12 000 g for 15 min at 4 °C. After transferring the supernatant to a fresh ice-cold tube, the protein concentration was determined with BCA protein assay. Equal concentrations of proteins were mixed with SDS sample buffer and denatured at 100 °C for 10 min. The samples were separated on an SDS-10% polyacrylamide gel, and then transferred to a PVDF membrane at 300 mA for 2 h in a transfer buffer containing 20 mmol/L Tris, 150 mmol/L glycine, and 20% methanol. The membranes were blocked with 5% non-fat dried milk in TBST (0.1% Tween-20) for 1 h. After blocking, the blots were...
incubated with the primary antibodies for IKCa (1:200) or SKCa (1:200) at 4 °C overnight, and then incubated with HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. The bound antibodies were detected with an enhanced chemiluminescence detection system (ECL, Amersham, USA), and quantified by densitometry using a Chemi-Genius Bio Imaging System (Syngene, Cambridge, UK). After stripping, the membrane was reprobed with a loading control antibody (GAPDH) to normalize the amount of proteins. The ratio of band intensity to GAPDH was obtained to quantify the protein expression level.

Statistical analysis

The results are presented as Mean±SE. The animal characteristics and Western blotting results were compared using one-way analysis of variance (ANOVA) with Bonferroni’s post test. The concentration-response curves for isolated rat mesenteric arteries were computer fitted to a sigmoidal curve using nonlinear regression to calculate the sensitivity of each agonist (pEC50). The maximum relaxation (Rmax) to ACh or SNP was measured as a percentage of precontraction to PE. Group pEC50 and Rmax values were compared via one-way ANOVA showing decrements in both IKCa and SKCa-mediated relaxation, especially the latter. SKCa-mediated maximum relaxation in the arteries of DM rats was reduced by 57.06% (compared with that of the control rats), while IKCa-mediated maximum relaxation was reduced by 34.53%. The sensitivity to ACh in the arteries of DM rats incubated with UCL-1684 or TRAM-34 further reduced the maximum relaxation by 29.75% or 64.09% in the arteries of the control rats, indicating that SKCa contributed more than IKCa to EDHF-mediated relaxation in normal small arteries. Compared with the arteries of the control rats, the arteries of DM rats showed decrements in both IKCa- and SKCa-mediated relaxation, especially the latter. SKCa-mediated maximum relaxation in the arteries of DM rats was reduced by 57.06% (compared with that of the control rats), while IKCa-mediated maximum relaxation was reduced by 34.53%. The sensitivity to ACh in the arteries of DM rats incubated with UCL-1684 or TRAM-34 also decreased (Fig.1C, 1D and Tab.2, P<0.05 or P<0.01). ALA treatment partly reversed the decrease of IKCa- and SKCa-mediated relaxation (Fig.1C, 1D and Tab.2, P<0.01). The untreated control arteries maintained an endothelium-dependent ACh relaxation throughout the experiment.

Role of oxidative stress in down-regulation of IKCa and SKCa channels

To investigate whether the suppression of IKCa- and SKCa-mediated relaxation in diabetes was related to a decreased expression of the channels by oxidative stress, HUVECs were cultured in the medium without (control) or with 40 μmol/L H2O2 for 24 h to determine the changes in the expression levels of KCa2.3 and KCa3.1 channels by Western blotting. Incubation with 40 μmol/L H2O2 significantly decreased the expression of SKCa by 59.82% and IKCa by 55.37% (Fig.2A and B, P<0.01). Pretreatment with 50 μmol/L ALA obviously reversed the down-regulation of IKCa and SKCa channels induced by H2O2 (Fig.2A and B, P<0.01), indicating that the oxidation products down-regulated the expression of IKCa and SKCa channels.

DISCUSSION

EDHF can lead to endothelial-dependent vasodilation via NO- and prostaglandin-independent
mechanisms, particularly in resistance arteries where the contribution of NO appears to be less important than in conduit vessels, and where EDHF appears to play a major role in regulating tissue blood flow. In the present study, we found that blocking NOS and COX with L-NAME and INDO significantly reduced ACh-induced EDHF-mediated vasodilation in the third-order mesenteric arteries of high fat and glucose-fed and STZ-treated rats. This finding is consistent with the results of other studies. Decreased EDHF-mediated responses with no or minor alterations in NO-dependent responses have been reported in resistance arteries of STZ-treated diabetic rats and other animal models of diabetes. But there are also evidence that supports a role for unchanged or augmented EDHF activity in maintaining endothelial dependent relaxation in diabetes when endothelial production of NO and prostaglandins are compromised, particularly during the early stages of disease progression. While the differences in the diabetes models and in the duration or severity of diabetes may contribute to such discrepancies, the differences in the vascular bed, size of the vessels and conditions of the studies seem to be a much more important source of the disparity.

The nature of EDHF and EDHF-signaling pathways is not fully understood yet. However, endothelial hyperpolarization mediated by KCa has been suggested to play a critical role in initializing EDHF-dilator responses in resistance-sized arteries of many species, including humans. Findings from IKCa- and/or SKCa-deficient mice indicate that endothelial IKc and SKc are indeed fundamental components of EDHF-signaling pathway in vivo. Our data showed that incubation with the selective blocker of IKc and SKc, TRAM-34 and UCL-1684, respectively, significantly reduced ACh-induced NO- and prostaglandin-independent relaxation in the third-order mesenteric arteries of rats, which further confirmed the contribution of endothelial Kc to EDHF-type relaxation. There are reports indicating that impaired EDHF-mediated relaxations in small mesenteric arteries in animal models of diabetes involve endothelial Kc channels.

Tab.2 Maximal ACh relaxation and pEC50 values of endothelium-intact mesenteric arteries in control, DM and ALA groups (Mean±SE, n=6)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DM</th>
<th>ALA</th>
<th>Control</th>
<th>DM</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEC50</td>
<td>7.79±0.22</td>
<td>7.23±0.42*</td>
<td>7.40±0.19</td>
<td>92.74±2.59</td>
<td>54.67±4.04**</td>
<td>79.42±4.18**</td>
</tr>
<tr>
<td>+L-NAME+INDO</td>
<td>7.72±0.25</td>
<td>7.07±0.30</td>
<td>7.15±0.41</td>
<td>63.54±2.74</td>
<td>26.72±2.66**</td>
<td>55.47±3.93**</td>
</tr>
<tr>
<td>+L-NAME+INDO+UCL-1684</td>
<td>7.80±0.42</td>
<td>6.94±0.33**</td>
<td>7.62±0.20**</td>
<td>22.82±2.53</td>
<td>14.94±1.44**</td>
<td>20.30±0.61**</td>
</tr>
<tr>
<td>+L-NAME+INDO+TRAM-34</td>
<td>8.07±0.28</td>
<td>7.02±0.50**</td>
<td>7.11±0.38</td>
<td>44.64±3.30</td>
<td>19.17±1.78**</td>
<td>39.95±3.40**</td>
</tr>
<tr>
<td>SNP</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>99.05±1.03</td>
<td>98.23±2.40</td>
<td>98.44±1.35</td>
</tr>
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Values were analyzed by one-way ANOVA with Tukey's post test. *P<0.05, **P<0.01 vs control; **P<0.01 vs DM.
In the present study, we used STZ-treated rats fed with high fat and glucose diet for 3 months as the animal model of diabetes. This diabetic model is characterized by elevated fasting blood glucose, serum cholesterol, glycosylated hemoglobin, blood serum insulin, and mean artery pressure as well as reduced body weight as previously described\textsuperscript{33}, resembling the state of patients with type 2 diabetes. Our data showed that this diabetic model exhibited significant decrements in both IK\textsubscript{Ca} and SK\textsubscript{Ca} channels. These results indicate that oxidative stress is involved in the damage of IK\textsubscript{Ca} and SK\textsubscript{Ca} channels in diabetic ZDF rats\textsuperscript{36}. Our data showed that H\textsubscript{2}O\textsubscript{2} reduced the expression of IK\textsubscript{Ca}, which was reversed by ALA. But how ROS altered the expression of IK\textsubscript{Ca} and SK\textsubscript{Ca} needs further investigation.

In conclusion, the present study provides novel experimental evidence that oxidative stress is responsible for, at least in part, impaired IK\textsubscript{Ca} and SK\textsubscript{Ca}-mediated relaxation in the mesenteric arteries of diabetic rats via down-regulation of IK\textsubscript{Ca} and SK\textsubscript{Ca} channels. These results may have clinical implications for the treatment of endothelial dysfunction in diabetic patients.

Acknowledgments

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REFERENCES

[5] Burnham MP, Johnson IT, Weston AH. Impaired small conductance Ca\textsuperscript{2+} -activated K\textsuperscript{+} channels with40 HUVECs treated without (control) or with40 μmol/L H\textsubscript{2}O\textsubscript{2} or with40 μmol/L H\textsubscript{2}O\textsubscript{2} + 50 μmol/L ALA for 24 h (n=5, one-way ANOVA with Bonferroni’s post test). \textsuperscript{**}P<0.01 vs control; \textsuperscript{###}P<0.01 vs H\textsubscript{2}O\textsubscript{2}.

ROS have been proposed to be regulators of K\textsuperscript{+} channel function in various tissues\textsuperscript{30-32}. For the vascular cells, inconsistent (i.e., inhibitory versus stimulatory) effects of ROS have been reported on the function of K\textsuperscript{+} channels and, in particular, on the function of endothelial K\textsubscript{Ca}\textsuperscript{35-36}. Brakemeier et al\textsuperscript{35} found that intracellular as well as extracellular challenge of endothelial BK\textsubscript{Ca} with H\textsubscript{2}O\textsubscript{2} and ROS resulted in a dose-dependent and irreversible channel inactivation. Such an inhibition of K\textsubscript{Ca} single-channel function by H\textsubscript{2}O\textsubscript{2} has also been reported for IK\textsubscript{Ca} in bovine aortic endothelial cells\textsuperscript{36}. Our data showed that H\textsubscript{2}O\textsubscript{2} reduced the expression of IK\textsubscript{Ca} and SK\textsubscript{Ca}, which was reversed by ALA. But how ROS altered the expression of IK\textsubscript{Ca} and SK\textsubscript{Ca} needs further investigation.