Molecular Mechanisms Mediating Inhibition of Human Large Conductance Ca\(^{2+}\)-Activated K\(^+\) Channels by High Glucose

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Abstract—Diabetic vascular dysfunction is associated with an increase in reactive oxygen species (ROS). In this study, we hypothesized that hyperglycemia-induced ROS generation would impair the function of large conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels, which are major determinants in vasorelaxation. We found that when cultured in high glucose (HG) (22 mmol/L), HEK293 cells showed a reduction in expressed hSlo current densities, as well as slowed activation and deactivation kinetics. When human coronary smooth muscle cells were cultured in HG, similar findings were observed for the BK currents. HG enhanced superoxide dismutase and suppressed catalase (CAT) expression in HEK293 cells, leading to a significant increase in intracellular ROS. The effects of HG were mimicked by hydrogen peroxide (H\(_2\)O\(_2\)), and hSlo functions were restored by CAT gene transfer. Peroxynitrite inhibited hSlo current density but did not change channel kinetics. The hSloC911A mutant was insensitive to the effects of HG and H\(_2\)O\(_2\). Hence, imbalance of antioxidant enzymes plays a critical role in ROS generation in HG, impairing hSlo functions through H\(_2\)O\(_2\)-dependent oxidation at cysteine 911. This may represent an important fundamental mechanism that contributes to the impairment of vasodilation in diabetes. (Circ Res. 2006;99:607-616.)

Key Words: large conductance Ca\(^{2+}\)-activated K\(^+\) channels ■ hyperglycemia ■ catalase ■ reactive oxygen species ■ gene transfer

Diabetes is associated with a 2- to 4-fold increase in the risk of developing coronary artery disease and stroke, and there has been a 75% increase in mortality from cardiovascular diabetic complications over the last decade in the United States.\(^1,2\) Nearly 21 million Americans have diabetes, with another 41 million considered prediabetic. Multiple factors contribute to the development of diabetic vasculopathy.\(^1,2\) Endothelial dysfunction, reduced nitric oxide (NO) availability, and enhanced production of reactive oxygen species (ROS), such as superoxide anion (O\(_2^−\)) and hydrogen peroxide (H\(_2\)O\(_2\)), are thought to be major mechanisms that underlie the development of cardiovascular complications in diabetes.\(^1\) O\(_2^−\) is rapidly oxidized to H\(_2\)O\(_2\) by superoxide dismutase (SOD), and H\(_2\)O\(_2\) is further reduced to H\(_2\)O by catalase (CAT) and glutathione peroxidase (GPX).\(^3\) In addition, O\(_2^−\) reacts with NO extremely efficiently to form peroxynitrite (OONO\(^−\)). H\(_2\)O\(_2\) and OONO\(^−\) are highly reactive ROS. Abnormal ROS metabolism, leading to cellular oxidative stress, plays a central role in the progression of diabetic vascular dysfunction.\(^1,3\)

The large conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channel, abundantly expressed in vascular smooth muscle cells, plays a critical role in controlling vascular tone.\(^4\) The BK channel is composed of 4 pore-forming α subunits, each with 7 transmembrane domains (S0 to S6), including a highly conservative pore region between S5 and S6 and a voltage sensor located at S4.\(^5\) The cytoplasmic terminus has 4 hydrophobic segments (S7 to S10) that contain critical channel regulatory sites, including 2 regulators of K\(^+\) conductance (RCK1 and RCK2) domains and the Ca\(^{2+}\) binding motifs (Ca\(^{2+}\) bowl).\(^6,7\) Movement of the RCK domains affects Ca\(^{2+}\) binding affinity.\(^7\) There is evidence that BK channel–mediated vasodilation is impaired in hyperglycemia; however, the molecular mechanism has not been fully delineated.\(^8,9\) Hyperglycemia-induced ROS overproduction in diabetes is well documented.\(^10\) In vascular smooth muscle cells, a major source of ROS is NAD(P)H oxidase,\(^11\) which is present in HEK293 cells.\(^12\) OONO\(^−\) inhibits vascular BK channels in a dose-dependent manner, whereas O\(_2^−\) itself has no effect on BK channels.\(^13,14\) H\(_2\)O\(_2\) may either inhibit\(^15,16\) or activate BK channels through direct effects\(^17,18\) or through cGMP- and cAMP-dependent signaling pathways.\(^19,20\) Cysteine 911 of hSlo, which encodes the BK channel, is thought to be involved in channel regulation by redox and cysteine-modification reagents,\(^14\) although studies have produced conflicting results.\(^21\) Most of the previous studies used exogenously applied H\(_2\)O\(_2\) from 10 μmol/L to 167 mmol/L.\(^14,16,17\) Whether endogenously produced H\(_2\)O\(_2\) in hyperglycemia can
modulate BK channel function is unknown. In this study, we hypothesized that a high glucose (HG)-induced increase in ROS modulates hSlo channel function by oxidation of specific residues. We examined changes in the properties of hSlo channels, which encode human BK channel α subunits, in HG. The results may help elucidate the molecular mechanisms underlying diabetes-associated vascular dysfunction and the role of ROS in the regulation of BK current functions in blood vessels.

Materials and Methods

An expanded Materials and Methods section containing details for cell culture, transfection, vasoreactivity, Western blotting, and electrophysiology is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture and hSlo, CAT Transfection

HEK293 cells were cultured in DMEM containing normal glucose (NG) (5 mmol/L) or HG (22 mmol/L).22 hSlo cDNA and 2 recombinant adenoviruses, AdCAT carrying CAT and AdLacZ carrying β-galactosidase, were used for gene transfer.23

Animals and Vasoreactivity Measurements

Diabetes mellitus was produced in male Sprague–Dawley rats (~200 g. Harlan, Inc. Ind) by streptozotocin (60 mg/kg, IP). Control rats received vehicle injection. Blood glucose in excess of 300 mg/dL was considered diabetic. Animals were used for vasoreactivity experiments 4 weeks after development of diabetes. Handling and care of animals, as well as all animal procedures, were approved by the Institutional Animal Care and Use Committee, Mayo Foundation.

On the day of the experiment, rats were anesthetized with pentobarbital (50 mg/kg IP). The heart was rapidly removed from the thorax. Small coronary arteries were isolated from control and diabetic rats and vasoreactivity was measured using videomicroscopy.8

Electrophysiology

hSlo currents were recorded in HEK293 cells 72 hours after hSlo transfection using standard patch clamp techniques at room temperature (23°C).24,25

Intracellular ROS Measurement

Products of intracellular H₂O₂ and O₂⁻ were detected as previously described.26 Briefly, attached HEK293 cells were exposed to ROS resulting from hypoxyhemoglobin production. Cells were treated with the fluorescent reagent, 5-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCF-DA) for 30 minutes at 37°C in the dark, washed twice with PBS, and incubated in NG or HG medium for another 30 minutes. Cells were trypsinized (0.1%) and washed with PBS. After centrifugation (1200 rpm for 10 minutes), the cell pellet was suspended in 200 μL of PBS. Formation of ROS was detected by signals from the fluorescent reaction products of CM-DCF and ethidium using flow cytometry (Becton Dickinson FACScan System and CellQuest Software).

Western Blot Analysis

Protein expressions of manganese SOD (MnSOD), copper zinc SOD (CuZnSOD), CAT, and GPX-1 in HEK293 cells cultured with NG and HG were determined using Western blot.27

Chemicals

OONO⁻ was purchased from Upstate Cell Signaling and Solutions (Lake Placid, NY). All other chemicals were obtained from Sigma-Aldrich (St Louis, Mo).

Statistical Analysis

Data are presented as mean±SEM. Student t test was used to compare data between 2 groups. Paired t test was used to compare data before and after treatment. One-way ANOVA, followed by Tukey test analysis was used to compare data from multiple groups using SigmaStat software (Jandel, San Rafael, Calif). Statistical significance was defined as P<0.05.

Results

Inhibition of hSlo Currents by HG

Acute exposure of hSlo expressed in HEK293 cells to medium containing NG or HG had no effects on current density or kinetics. However, when hSlo was expressed in HEK293 cells that had been exposed to HG for 1 week, reduction in current density and slowing of channel activation were observed. These changes became stable in cells after 2 weeks culture with HG. Figure 1A shows representative whole-cell currents of hSlo 72 hours after transfection in HEK293 cells that had been cultured in NG or HG for 2 weeks. HG significantly reduced current amplitudes (Figure 1A) and current/voltage (I-V) curves showed that HG reduced the maximal current density of hSlo by 47.1%, with 671.6±105.1 pA/pF (n=10) in NG and 355.6±102.3 pA/pF in HG (+200 mV; n=10; P=0.045) (Figure 1B). Normalized conductance/voltage (g-V) curves of hSlo in NG and HG were fitted using a Boltzmann equation (Figure 1C). The voltage at half-maximal activation (V½) of hSlo activation was not different between NG and HG (113.5±10.8 mV in NG versus 97.5±9.2 mV in HG; n=10 for both; P=NS), and neither was the slope factor k (34.8±1.6 mV/e-fold in NG versus 31.4±2.3 mV/e-fold in HG; n=10 for both; P=NS). Single-channel recordings showed that cells in HG reduced hSlo open probability (Po) by 60%, prolonging channel closed time constants (τc) by 100% without changing channel open time constants (τo) (Figure 1D; Table I in the online data supplement). HG slowed the time course of channel activation and increased the activation time constants (τo) compared with those in NG (detailed analysis is presented in supplemental Table II).

HG also reduced hSlo tail current amplitudes (Figure 2A) and slowed the time course of channel deactivation. The activation time constant (τa) and deactivation time constant (τd) was fitted from the hSlo currents in NG and HG (detailed analysis of the channel deactivation kinetics is presented in Table II and Figure III of the online data supplement). The relationships between time constants (τ) and voltages (τ-V curve) were fitted using the voltage-dependent relaxation equation,28 as shown in Figure 2B. Increases in τa and τd by HG resulted in shifting the τ-V curve upward. The voltage-dependent free energy (ΔG) required for transition from channel closed state (C) to open state (O) was calculated by curve fitting using rate theory analysis29 to be 2.6 kJ/mol in NG and 4.9 kJ/mol in HG, an increase of 1.9-fold in HG, and ΔG from O to C was increased by 1.3-fold in HG (4.5 kJ/mol in NG and 5.8 kJ/mol in HG), suggesting that channel activation and deactivation transitions in HG are less favorable thermodynamically compared with those in NG. Similar results were obtained from the BK channels of human coronary artery smooth muscle cells cultured with HG (see the online data supplement). In addition, vasoreactivity studies showed that epoxyeicosatrienoic acid (EET)-induced vasodilation through BK channel activation was impaired in...
coronary arteries of streptozotocin-induced diabetic rats (Figure 2C). In controls, BK channel activation accounted for 39.3% of the 11,12-EET–mediated (1 μmol/L) vasodilation (63.8 ± 4.2% at baseline versus 24.5 ± 2.1% with 100 nmol/L iberiotoxin [IBTX]; n = 5). In diabetic vessels, BK channels contributed only 9.5% of the EET-mediated vasodilation, with 31.9 ± 2.1% dilation at baseline (n = 5; P < 0.01 versus control) and 22.4 ± 2.0% with IBTX (n = 5; P = NS versus baseline). These results suggest that BK channel dysfunction contributes to the impairment of vasodilation in diabetes (Figure 2C).

HG Produced Antioxidant Enzyme Imbalance and Increased Intracellular ROS Production in HEK293 Cells

Western blot analysis showed that CAT expression was reduced by 44% in HG cells (0.37 ± 0.04 versus 0.66 ± 0.07 in NG cells; n = 3 for both; P = 0.023), whereas CuZnSOD expression increased by 36% (0.83 ± 0.06 versus 0.61 ± 0.05 in NG cells; n = 3 for both; P = 0.048) (Figure 3A and 3B). The level of GPX-1 expression in HEK293 cells was very low. There was no significant difference in MnSOD and GPX-1 expression between NG and HG cells. This altered antioxidant enzyme profile in HG cells could result in reduced ability to scavenge H$_2$O$_2$ sufficiently, favoring the accumulation of H$_2$O$_2$.

Production of total peroxides was measured in HEK293 cells after 2 weeks in NG and HG, using the fluorescent ROS indicator CM-H$_2$DCF-DA (Figure 3C). Cells cultured in NG (NG cells) showed fluorescence signals of 66.9 ± 9.1 arbitrary units (n = 4) in NG medium and 124.6 ± 21.3 arbitrary units (n = 4; P = 0.02) in HG medium, suggesting HG could instantaneously increase intracellular ROS production in NG cells. However, the fluorescence signals from cells cultured in HG (HG cells) were 153.0 ± 17.3 arbitrary units when measured in NG medium (n = 4; P = 0.002 versus HG cells in NG medium), comparable to that of NG cells measured in HG medium (n = 4; P = NS). The fluorescence signals in HG cells further increased to 219.6 ± 43.8 arbitrary units when placed in HG medium (n = 4; P = 0.048 versus NG cells with HG medium). Thus, intracellular ROS increased...
3.3-fold in HG cells in HG medium, compared with NG cells in NG medium. These results suggest that intracellular ROS was persistently elevated in HG cells and the ability to scavenge intracellular ROS generated was impaired. Group data are summarized in Figure 3D.

Adenoviral Expression of CAT Reversed the Effects of HG on hSlo
To determine whether the effects of HG on hSlo could be alleviated by increase in CAT activities, we overexpressed CAT by adenoviral gene transfer into HG cells before transfection of hSlo. Figure 4A shows raw tracings of hSlo 72 hours after transfection in HG cells infected with adenoviral-CAT (AdCAT). There was a 4-fold increase in CAT expression in AdCAT-infected HG cells compared with HG cells infected with control adenovirus (AdLacZ) (Figure 4B). Interestingly, hSlo current density in HG cells was enhanced by AdCAT infection (890.7 ± 125.5 pA/pF, +200 mV [n=6] versus 355.6 ± 102.3 pA/pF in HG control [n=10]; P=0.006), attaining levels similar to that in NG cells (dashed line) (n=6; P=NS) (Figure 4C). Enhanced CAT expression had no effect on the g-V curve (Figure 4D). V1/2 and k were 93.0 ± 8.6 mV and 36.7 ± 1.8 mV/e-fold (n=6; P=NS versus HG control for both), respectively, in HG cells infected with AdCAT, showing no difference from those in HG control. However, CAT overexpression reduced $\tau_\alpha$ and $\tau_0$ in parallel (Figure 4E) to levels comparable to those in NG. Hence, enhanced CAT activity restored hSlo current density and kinetics to that of NG cells, indicating that reduced CAT activity is the major culprit in hSlo inhibition by HG.

Figure 2. Effects of HG on hSlo deactivation. A, Whole-cell hSlo tail currents were recorded from cells 72 hours after transfection. HG inhibited hSlo tail current amplitude and slowed the time course of tail current deactivation. B, $\tau$-V curves for cells in NG (○) and HG (●) (n=10 for both). *P<0.05. C, Vasodilation of small coronary arteries in control and type 1 diabetic rats by 11,12-EETs. 11,12-EET (1 μmol/L) induced vasodilation of coronary arteries from control and diabetic rats with and without pretreatment with IBTX (100 nmol/L). Contribution of BK channels to EET-mediated vasodilation is shown in the inset.

Exogenous $\text{H}_2\text{O}_2$ Mimicked the Effects of HG on hSlo Kinetics
NAD(P)H oxidase is present in HEK293 cells and intracellular $\text{H}_2\text{O}_2$ in HEK293 cells is under physiological conditions. Because the ROS florescence signal increased 3.3-fold in HG, the average cellular concentration of ROS could be raised to the 10$^{-4}$ mol/L range in HG cells. Figure 5A shows that H$_2$O$_2$ dose-dependently inhibited the inside-out macroscopic currents of hSlo with an IC$_{50}$ of 4 mmol/L. The H$_2$O$_2$ effect was reversed by 2 mmol/L dithiothreitol (DTT). Hence, in subsequent experiments, we applied 4 mmol/L $\text{H}_2\text{O}_2$ to determine the effects of $\text{H}_2\text{O}_2$ on hSlo kinetics. Similar to HG, $\text{H}_2\text{O}_2$ reduced hSlo current density and increased $\tau_\alpha$ and $\tau_0$ in a voltage-dependent manner (Figure 5B). I-V curves normalized to baseline showed that $\text{H}_2\text{O}_2$ reduced the maximal current density by 44.7 ± 8.1% (+200 mV; n=3; P=0.01 versus baseline) (Figure 5C). $V_{1/2}$ was unaltered by $\text{H}_2\text{O}_2$ (131.0 ± 7.0 mV at baseline versus 134.6 ± 6.3 mV with $\text{H}_2\text{O}_2$; n=3; P=NS.
versus baseline), as was \( k \) (17.7 ± 2.6 mV/e-fold at baseline versus 19.3 ± 2.3 mV/e-fold with \( H_2O_2 \); \( n = 3; \ P = N/S \)) (Figure 5D). Thus, the effects of \( H_2O_2 \) on hSlo channel kinetics were similar to those of HG. The \( \Delta G \) for channel activation was calculated to be 2.0 kJ/mol at baseline and 4.4 kJ/mol with \( H_2O_2 \), whereas the \( \Delta G \) for channel deactivation was 4.8 kJ/mol at baseline and 5.5 kJ/mol with \( H_2O_2 \). These values are similar to those obtained in HG. The \( \gamma-V \) curve and kinetics parameters in response to \( H_2O_2 \) are shown in Figure V, E, in the online data supplement.

Exogenous OONO\(^-\) Inhibited hSlo Current Density but Not Channel Kinetics

Because OONO\(^-\) can also be overproduced in HG, we examined the effects of OONO\(^-\) on hSlo. We found that OONO\(^-\) (0.1 mmol/L) inhibited hSlo current density by as much as 4 mmol/L \( H_2O_2 \) but did not alter channel activation or deactivation kinetics, and the effects of OONO\(^-\) were completely reversed by 2 mmol/L DTT (Figure 6A). Normalized I-V curves in the presence and absence of 0.1 mmol/L OONO\(^-\) are shown in Figure 6B. OONO\(^-\) inhibited the maximal hSlo activity by 31.4 ± 16.7\% (\( n = 4; \ P = 0.01 \)), comparable to the effects of 4 mmol/L \( H_2O_2 \) and HG (\( n = 4; \ P = N/S \)). Neither channel \( V_{1/2} \) (162.8 ± 11.8 mV at baseline and 170.5 ± 13.1 mV with OONO\(^-\); \( n = 4; \ P = N/S \)) nor \( k \) (21.5 ± 1.7 mV/e-fold at baseline and 22.0 ± 1.4 mV/e-fold with OONO\(^-\); \( n = 4; \ P = N/S \)) was altered by OONO\(^-\). Unlike HG and \( H_2O_2 \), OONO\(^-\) had no effect on \( \tau_0 \) and \( \tau_e \) (Figure 6C). A comparison between the effects of \( H_2O_2 \) and OONO\(^-\) on hSlo channel kinetics is given in Figure VI, D, and Table II in the online data supplement.

**hSloC911A Mutant Was Insensitive to \( H_2O_2 \) and HG**

Because the effects of HG on hSlo were mainly mediated by \( H_2O_2 \) in HEK293 cells, we determined the role of cysteine 911 in HG- and \( H_2O_2 \)-mediated hSlo modulations. hSlo with a single substitution at cysteine 911 by alanine (hSloC911A) was expressed in HEK293 cells. The time course of the effects of 4 mmol/L \( H_2O_2 \) and 0.1 mmol/L OONO\(^-\) on hSloC911A inside-out macroscopic currents is illustrated in Figure 7A (top and bottom, respectively). Relative to hSlo wild type (wt), hSloC911A was insensitive to \( H_2O_2 \) inhibition, although \( H_2O_2 \) produced a very small but still significant inhibition in current density and the effect was reversed by 2 mmol/L DTT. However, the effects of OONO\(^-\) on...
hSloC911A were unchanged from wt, suggesting that C911 is the molecular site for H$_2$O$_2$ modulation, but not for OONO$^-$. Moreover, H$_2$O$_2$ failed to slow hSloC911A activation or deactivation (Figure 7B and 7D) and only produced a maximal current inhibition of 14.3±2.4% (+200 mV; n=4; P=0.048) (Figure 7C), compared with 44.7±8.1% in wt (n=4; P=0.011). H$_2$O$_2$ did not alter the channel $V_{1/2}$ (130.3±16.1 mV at baseline and 137.3±27.4 mV with H$_2$O$_2$; n=4; P=NS) or $k$ (17.7±2.6 mV/e-fold at baseline and 19.7±2.3 mV/e-fold with H$_2$O$_2$; n=4; P=NS). H$_2$O$_2$ did not

**Figure 4.** Effects of HG on hSlo kinetics after AdCAT infection. A, Raw current tracings of hSlo were recorded from cells in HG after AdCAT infection. AdCAT infection restored hSlo current density and channel kinetics in HG. B, Western blots showing that HG cells had a 4-fold increase in CAT expression 72 hours after AdCAT infection compared with those infected with AdLacZ. C, hSlo I-V curves in HG cells after AdCAT (●) and AdLacZ infection (○) (n=6). Dashed line represents the I-V curve of NG. *P<0.05 vs AdLacZ. D, Normalized g-V curves in cells infected with AdCAT (●) and AdLacZ (○) (n=6). E, τ-V curves from cells infected with AdCAT (●) and AdLacZ (○). Overexpression of AdCAT accelerated and normalized hSlo activation and deactivation kinetics in HG.

hSloC911A were unchanged from wt, suggesting that C911 is the molecular site for H$_2$O$_2$ modulation, but not for OONO$^-$. Moreover, H$_2$O$_2$ failed to slow hSloC911A activation or deactivation (Figure 7B and 7D) and only produced a maximal current inhibition of 14.3±2.4% (+200 mV; n=4; P=0.048) (Figure 7C), compared with 44.7±8.1% in wt (n=4; P=0.011). H$_2$O$_2$ did not alter the channel $V_{1/2}$ (130.3±16.1 mV at baseline and 137.3±27.4 mV with H$_2$O$_2$; n=4; P=NS) or $k$ (17.7±2.6 mV/e-fold at baseline and 19.7±2.3 mV/e-fold with H$_2$O$_2$; n=4; P=NS). H$_2$O$_2$ did not

**Figure 5.** Exogenous H$_2$O$_2$ reproduced the effects of HG on hSlo kinetics. A, Time course of inside-out macroscopic currents of hSlo, recorded at +160 mV with a holding potential of −60 mV, showing the effects of various concentrations of H$_2$O$_2$ and 2 mmol/L DTT applied to the cytoplasmic surface of hSlo. IC$_{50}$ of H$_2$O$_2$ on hSlo currents was ~4 mmol/L. B, Application of 4 mmol/L H$_2$O$_2$ suppressed hSlo current density and slowed its activation and deactivation kinetics. C, hSlo I-V curves at baseline (○) and after exposure to H$_2$O$_2$ (●) (n=6). Results were normalized to maximum current amplitude at baseline. D, τ-V curves composed of $\tau_a$ (~140 mV to +100 mV) and $\tau_d$ (+100 mV to +210 mV) before (○) and after (●) exposure to H$_2$O$_2$. H$_2$O$_2$ shifted the τ-V curves upwards, similar to the effects of HG. *P<0.05 vs baseline.
change the $\Delta G$ of hSloC911A activation (2.8 kJ/mol at baseline and 3.0 kJ/mol with $H_2O_2$) or deactivation (4.5 kJ/mol at baseline and 4.8 kJ/mol with $H_2O_2$). There was no difference between the current densities, $V_{1/2}$, and channel kinetics of hSloC911A in cells in NG and HG, which were similar to wt recordings in NG (Figure 8). Hence, the C911A mutation prevented downregulation of channel activities in HG (926.0 ± 193.2 pA/pF, $+200$ mV [n=6] versus 355.6 ± 102.3 pA/pF in wt [n=10]; $P=0.008$) and maintained normal $\tau$-$V$ curves. The $\Delta G$ for hSloC911A activation and deactivation in HG was 2.9 kJ/mol and 4.9 kJ/mol, respectively, similar to those of wt channel in NG. Our results indicate that $H_2O_2$ is the major ROS produced by HEK293 cells in HG and C911 is the major molecular site of redox regulation in hSlo by HG.

Discussion
In this study, we have made several important findings. First, HG reduced hSlo current density and altered channel kinetics. Second, HG enhanced SOD expression but suppressed CAT expression, resulting in a 3.3-fold increase in ROS generation in HEK293 cells. Third, $H_2O_2$ but not $OONO^-$ mimicked the effects of HG on hSlo channel kinetics. Fourth, hSloC911A was insensitive to HG or $H_2O_2$ modulation, suggesting that C911 in hSlo is the major molecular target of redox regulation by HG. We believe this is the first report identifying the molecular mechanism associated with impaired BK channel function in hyperglycemia.

Vascular $K^+$ channels are effectors of endothelium-derived relaxation factors (EDRF) and endothelium-derived hyperpolarizing factors (EDHF) and are critical determinants of vascular tone. Impaired BK channel–mediated vasodilation is known to be associated with vascular dysfunction in diabetic animals. However, the mechanisms through which vascular BK channels are modulated by hyperglycemia have not been delineated. In high fructose diet–induced, insulin-resistant rats, BK channel density in mesenteric smooth
muscle cells was reduced, but the channel Ca^{2+} sensitivity and the voltage sensitivity were unchanged.32 In early-stage type 2 diabetic rats, impaired coronary arterial smooth muscle BK channel activation by arachidonic acid was attributable to a decrease in prostacyclin bioavailability.8 In this study, we demonstrated that enhanced H_2O_2 formation in HEK293 cells cultured with HG directly inhibited channel activity through redox modulation at C911 on the BK channel α subunit. HG not only directly enhanced NAD(P)H oxidase, but also interrupted the mitochondria electron transport chain at complex III, resulting in accumulation of O_2^-•, which in turn formed H_2O_2 and OONO^-•.10 However, hSlo activity was not inhibited by acute exposure to HG but by prolonged culture in HG that reached stable effects after 14 days. This time frame was required for HG to produce the imbalance in SOD and CAT activities, resulting in the accumulation of H_2O_2. This was supported by direct measurements of intracellular ROS, showing a 3.3-fold increase in HEK293 cells after 14 days of culture in HG. We believe that the major ROS product in our system was H_2O_2. First, SOD expression was increased by 36%, whereas CAT expression was decreased by 44% in HG cells, hence resulting in net H_2O_2 accumulation. Second, gene transfer of CAT into HG cells abolished the HG effects on hSlo current density and channel kinetics. Third, the application of H_2O_2, but not OONO^-•, mimicked the effects of HG, suggesting that OONO^-• was not the key ROS participant in modulating hSlo properties in HEK293 cells. Whereas BK channel kinetics were not affected by OONO^-•, we found that 0.1 mmol/L OONO^-• produced as much hSlo current density inhibition as 4 mmol/L H_2O_2. Because OONO^-• is highly reactive and generated by both endothelial and vascular smooth muscle cells, we believe that in diabetic vessels, BK channels are also modulated by OONO^-•. Why relatively high concentrations of exogenous H_2O_2 are required to reproduce the HG results is not immediately fully understood. We believe that H_2O_2 acts directly on hSlo and not on membrane lipids because a single C911A mutation is sufficient to eliminate the HG and H_2O_2 effects.

The mechanism underlying BK channel regulation by H_2O_2 is complex. A putative EDHF, H_2O_2 has been shown to activate vascular BK channels directly or through second messenger signaling pathways, leading to vasodilation.19,20 However, activation of BK channels by H_2O_2 is observed only when applied extracellularly, whereas intracellularly applied
H$_2$O$_2$ inhibits BK channels. Hence, the paracrine and autocrine functions of H$_2$O$_2$ on BK channels have divergent outcomes. Contrary to its physiological signaling role, prolonged excessive production of H$_2$O$_2$ is detrimental to the maintenance of cellular homeostasis, resulting in the development of diseases such as diabetes and arteriosclerosis. Cysteine modification of hSlo is involved in channel regulation by H$_2$O$_2$. However, the molecular identity of the cysteine residue in hSlo that is the functional target of redox modulation has been controversial. Tang et al reported that C911 near the Ca$^{2+}/$H$^{+}$bowl in the RCK2 domain is the major target of cysteine-modification reagents such as MTSEA and of cytoplasmic application of H$_2$O$_2$. Oxidation of C911 also reduces channel Ca$^{2+}$ sensitivity as a result of an increase in the free energy for Ca$^{2+}$ binding, which was only observed in the presence of high free Ca$^{2+}$ ($>$1 $\mu$mol/L). Zhang and Horrigan showed that C430 in the RCK1 domain but not C911 was the functional residue modified by oxidation. The crystal structure of the RCK domains on the Ca$^{2+}$/K$^{+}$ channel, MthK, shows that RCK1 is coupled to the channel pore through the M2 transmembrane domain, which corresponds to S6 in the BK channel. Thus, oxidation of C430 may impair RCK1 movement and reduce channel Ca$^{2+}$ sensitivity. Both studies presented strong evidence that a single mutation virtually eliminates the effects of the cysteine-modification reagents on hSlo channel Ca$^{2+}$ sensitivity and voltage sensitivity. H$_2$O$_2$ only efficiently oxidizes cysteine residues that are deprotonated, whereas most cysteine residues in proteins have a pKa of 8.5, so they would not be targeted by H$_2$O$_2$ unless they are located in the vicinity of highly positively charged residues. The Ca$^{2+}$ bowl contains a high density of aspartic acids surrounded by positively charged amino acids. This critical location may explain why C911 is the prime target for H$_2$O$_2$ modulation. Our results show that HG and H$_2$O$_2$ inhibit hSlo mainly through C911 oxidation, because the C911A mutation abrogated the effects of HG and eliminated 80% of the H$_2$O$_2$ effects. The effects of cysteine-modifying reagents such as MTSET, MTSES, and NEM on hSlo channel kinetics can shift the steady-state I-V curves either leftward or rightward, depending on whether the channel activation or deactivation gate is modified by these reagents. Slowing channel activation can shift the steady-state I-V or g-V curves leftward, whereas slowing channel deactivation favors channels to stay in open state and shifts the curves rightward. We found that HG and H$_2$O$_2$ do not shift the I-V curve, perhaps because of a balanced slowing of channel activation and deactivation by HG and H$_2$O$_2$. Another explanation is that hSlo currents were recorded with 200 $\text{nmol/L}$ intracellular free Ca$^{2+}$, which may be insufficient to change the free energy of Ca$^{2+}$ binding.

To summarize, we have provided compelling evidence that HG inhibits BK channel function through H$_2$O$_2$ oxidation of C911. These results indicate that diabetes alters antioxidant enzyme profiles, enhances ROS generation and compromises BK channel function. Gene transfer of CAT ameliorates these functional impairments and has therapeutic potential in the treatment of diabetic vascular dysfunction.
Acknowledgments

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Disclosures

None.

References

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Molecular Mechanisms Mediating Inhibition of Human Large Conductance $\text{Ca}^{2+}$-Activated $\text{K}^+$ Channels by High Glucose

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METHODS

**hSlo and CAT cDNA transfection in HEK 293 cells**

hSlo wt and C911A mutant cDNA in pTracerCMV-2 were transfected into HEK293 cells using a FuGENE 6 transfection kit as previously described \(^1\). The iberiotoxin (IBTX, 100 nmol/L)-sensitive component (BK currents) accounts for at least 90% of total K\(^+\) currents in HEK293 cells 72 h after transfection (Figure 1 supplement). AdLacZ (2\(\times\)10\(^{10}\) pfu/ml) containing the histochemical marker β-galactosidase, and AdCAT (9\(\times\)10\(^{10}\) pfu/ml) carrying the catalase gene were transfected into HEK293 cells with a multiplicity of infection (MOI) of 50 for 4 h before hSlo cDNA was added to the culture medium \(^2\).

**Vasoreactivity Measurements**

Hearts were rapidly excised and placed in ice-cold Krebs solution that contained (in mmol/L): NaCl 118.3, KCl 4.7, CaCl\(_2\) 2.5, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25, and dextrose 11.1. The secondary and tertiary branches (50-200 \(\mu\)m in intraluminal diameter) of the right and left coronary arteries from the epicardial surface, as well as branches of the septal coronary arteries were carefully dissected and isolated free of surrounding myocardium and connective tissue under a dissecting microscope (Olympus SZ4045 Stereo Microscope, Olympus America Inc., Melville, NY). Isolated small coronary arteries (1-2 mm in length) were transferred to a custom-made vessel chamber filled with Krebs solution. The arteries were mounted and secured between two borosilicate glass micropipettes (30 \(\mu\)m diameter tips) with 10-O ophthalmic suture. The lumen of the vessel was filled with Krebs solution through the micropipettes and
maintained at a constant pressure (no flow) of 60 mmHg. The vessel chamber was transferred to an inverted light microscope stage (Olympus CK40) coupled to a video measurement system (VIA-100, Boeckeler Instruments, Inc., AZ) equipped with a video camera, monitor and calibrated video calipers for visualization and recording of the intraluminal diameter as described previously. Vessels were equilibrated for at least 30 min in oxygenated (20% O₂, 5% CO₂, balanced with N₂, 37°C) Krebs solution, which was continuously circulated through the vessel bath. Vessels were unacceptable for experiments if they demonstrated leaks, failed to produce >30% constriction to 60 mmol/L KCl or to graded doses of endothelin-1 (ET-1), or failed to produce 80% dilation with nitroprusside (100 µmol/L).

All compounds were added abluminally. During a 30 min equilibration period, some vessels were pretreated with 100 nmol/L of iberiotoxin (IBTX). Vessels were then constricted to 30-60% of baseline diameter with endothelin-1 and the effect of 1 µM 11,12-epoxyeicosatrienoic acid (11,12-EET) on vessel diameter was measured.

**Electrophysiology**

hSlo transfected cells were identified by GFP expression under a UV microscope (Olympus, IX70). Whole-cell and inside-out single channel currents were recorded with an integrating amplifier (Axon patch 200B, Axon Instruments) and filtered with an 8-pole low pass Bessel filter (902 LPF, Frequency Devices Inc.) with a bandwidth (-3 dB) of 2 kHz and sampling rate of 50 kHz (12-bit resolution). Pipette resistance when filled with the pipette solution was 0.5~1 MΩ for whole-
cell and macroscopic current recordings, and 8~10 MΩ for single channel recordings. The pipette solution for whole-cell recordings contained (in mmol/L): KCl 140, MgCl₂ 0.5, Na₂ATP 5.0, Na₂GTP 0.5, HEPES 1.0, EGTA 1.0, CaCl₂ 0.465 (200 nmol/L free Ca²⁺), pH 7.35. The pipette solution for inside-out macroscopic current recordings contained (in mmol/L): NaCl 145, KCl 5.6, MgCl₂ 1.0, CaCl₂ 0.5, HEPES 10, pH 7.4. The bath solution for whole-cell recordings contained (in mmol/L): NaCl 145, KCl 5.6, MgCl₂ 1.0, CaCl₂ 0.5, HEPES 10, glucose 5.0 or 22, pH 7.4. The bath solution for inside-out macroscopic current recordings contained (in mmol/L): KCl 140, MgCl₂ 0.5, HEPES 1.0, EGTA 1.0, CaCl₂ 0.465, pH 7.35. The pipette solution for single channel recording contained (in mmol/L) KCl 140, CaCl₂ 1.0, HEPES 10, MgCl₂ 0.5, pH 7.4. The bath solution for single channel recording contained (in mmol/L): KCl 140, HEPES 10, MgCl₂ 1.0, EGTA 1.0, CaCl₂ 0.685 (free Ca²⁺~0.5 μM), pH 7.35.

To determine channel activation, hSlo currents were elicited from a holding potential of -60 mV with 50 ms duration testing pulses from -40 mV to +210 mV in 10 mV increments. To determine channel deactivation, hSlo tail currents were recorded using a protocol with a holding potential of -60 mV and a conditioning pulse of 50 ms at +160 mV, followed by testing potentials from -140 mV to +90 mV in 10 mV increments.

The conductance-voltage relationship (g-V curve) and the current-voltage relationship (I-V curves) of hSlo channels were characterized by the Boltzmann equation:

$$\frac{g}{g_{\text{max}}} = \frac{g}{1 + \exp \left( \frac{V_{1/2} - V}{k} \right)}$$
where $g_{\text{max}}$ represents the maximal $K^+$ conductance ($g$), $V_{1/2}$ is the voltage at which $g$ reaches half of $g_{\text{max}}$, $V_m$ is the membrane potential, and $k$ is the slope factor associated with the Boltzmann distribution.

Channel activation ($\tau_A$) and deactivation ($\tau_D$) time constants were fitted using a single exponential function. According to a two-state (closed state $\leftrightarrow$ open state) model, the time constant and voltage relationship ($\tau$-$V$ curve) was composed of $\tau_D$ ($-140$ mV to $+60$ mV) and $\tau_A$ ($+70$ mV to $+210$ mV) and was fitted using the voltage-dependent relaxation equation:

$$\tau(V) = \frac{1}{\alpha_0 \exp(V z_\alpha F / RT) + \beta_0 \exp(-V z_\beta F / RT)}$$

where $\alpha_0$ represents the forward rate constant from closed state to open state, and $\beta_0$ represents the backward rate constant from open state to closed state at 0 mV. $z_\alpha$ and $z_\beta$ represent the forward and the backward charge movements. $V$ is the membrane potential, $F$ is the Faraday constant, $R$ is the universal gas constant, and $T$ is the absolute temperature.

The free energy ($\Delta G$) required to overcome the energy barrier during transitions between closed-state and open-state was calculated using rate theory analysis:

$$\frac{1}{\tau} = k_B T / h \exp(-\Delta G \pm z \delta V / 2RT)$$

where $k_B$ is the Boltzmann constant, $T$ is the absolute temperature, $h$ is the Planck constant, $z$ is the charge movement, and $\delta$ is the position of the energy barrier with a single binding site in membrane, assuming the peak is halfway at an electrical distance ($\delta/2$) from the beginning. $V$ is the membrane potential, $F$ is the Faraday constant, and $R$ is the universal gas constant.
Single channel kinetics were analyzed using TAC software (Bruxton, Inc.) The components of channel open times ($\tau_o$) and closed time constants ($\tau_c$) were fitted with the sums of exponential probability density functions using the maximum likelihood with simple optimization. Any additional exponential component was added only when the probability exceeded 0.95$^7$.

**RESULTS**

**Effects of high glucose in native BK channel activities**

We examined the effects of HG on native IBTX-sensitive BK currents of human coronary artery smooth muscle cells, as shown in Figure 2 supplement. Similar to expressed hSlo channels, HG reduced BK current densities from $63.3\pm 7.6$ pA/pF (+200 mV, n=3) in NG to $38.1\pm 5.2$ pA/pF (+200 mV, n=4, p=0.036), and slowed the channel activation and deactivation time courses.

**Effects of high glucose on hSlo channel activation kinetics**

HG not only reduced hSlo current density but also slowed the time course of channel activation and deactivation (tail currents) compared to NG (Figure 3A supplement). The activation time constant ($\tau_A$) was increased from 0.72 ms in NG to 1.87 ms in HG at +200 mV, and from 1.41 ms in NG to 2.27 ms in HG at +100 mV. There was no difference between NG and HG in $\tau_A$ at voltages below +50 mV. The reciprocal of $\tau_A$ ($1/\tau_A$) as a function of voltage in NG and HG (n=10) is shown in Figure 3B supplement.

**Effects of high glucose on hSlo channel deactivation kinetics**

The hSlo current deactivation time constant ($\tau_D$) was fitted from the hSlo tail currents in NG and HG. HG reduced hSlo tail current amplitudes (Figure 2A in
text) and slowed the time course of channel deactivation (Figure 4A supplement). 

\( \tau_D \) increased from 0.5 ms in NG to 2.54 ms in HG at +90 mV, and from 0.48 ms to 1.54 ms at +40 mV; there was no difference between NG and HG at voltages below 0 mV. The finding that \( \Delta G \) is increased for channel transitions from open state to closed state and from closed state to open state indicated that hSlo has to overcome higher energy barriers in HG both for channel activation and deactivation (Figure 4B supplement).
Figure 1 (supplement): hSlo channel expression in HEK293 cells

Continuous recordings of whole-cell K⁺ currents elicited at +100 mV from a holding potential of -60 mV (left panel) show the response to 100 nmol/L IBTX, a specific BK channel blocker. The time course of the IBTX effects is displayed in the right panel. The IBTX-sensitive component (BK currents) accounts for at least 90% of total K⁺ currents in HEK293 cells 72 h after transfection.
Figure 2 (supplement): Effects of high glucose on native BK channels of cultured human coronary artery smooth muscle cells

A: Representative IBTX-sensitive BK currents recorded from human coronary artery smooth muscle cells cultured with NG and HG for 2 weeks. B: Normalized BK currents elicited at +200 mV in NG and HG. C: I-V curves showing voltage-dependent inhibition of BK channel density by HG. * p<0.05 vs. NG.
Figure 3 (supplement): A: Normalized hSlo current activation recorded at +50 mV, +100 mV and +200 mV in NG (solid lines) and HG (dashed lines). HG slowed the time course of channel activation at voltages greater than +50 mV. B: $1/\tau_A$ of NG (○) and HG (●) plotted as a function of voltage. The solid lines represent the best fit according to the rate theory model with a single binding site. * p<0.05 vs. NG.
Figure 4 (supplement): A: Normalized hSlo tail currents at –10 mV, +40 mV and +90 mV in NG (solid lines) and in HG (dashed lines). HG slowed the time course of channel deactivation in a voltage-dependent manner. B: Plots of $1/\tau_D$ as a function of voltage in NG (○) and HG (●) cells. The solid lines represent the best fit according to rate theory analysis. * p<0.05 vs. NG.
Table 1 (supplement)  Effects of high glucose on the open time and closed time constants of hSlo wt single channel kinetics

<table>
<thead>
<tr>
<th></th>
<th>Open Times Constants $\tau_O$ (ms)</th>
<th>Closed Time Constant $\tau_C$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau_{O1}$ ($A_{O1}$)</td>
<td>$\tau_{O2}$ ($A_{O2}$)</td>
</tr>
<tr>
<td>NG</td>
<td>2095 (0.16)</td>
<td>0.66 (0.64)</td>
</tr>
<tr>
<td>HG</td>
<td>2142 (0.24)</td>
<td>0.66 (0.61)</td>
</tr>
</tbody>
</table>

$\tau_{O1}$, $\tau_{O2}$ and $\tau_{O3}$ represent the slow, intermediate and fast components of channel open time constants and $A_{O1}$, $A_{O2}$ and $A_{O3}$ represent the corresponding weights respectively. Similarly, $\tau_{C1}$, $\tau_{C2}$, $\tau_{C3}$ and $\tau_{C4}$ represent the very slow, slow, intermediate and fast components of channel closed time constants. $A_{C1}$, $A_{C2}$, $A_{C3}$ and $A_{C4}$ are the relative weights. Here, $A_{O1}+A_{O2}+A_{O3}=1$ and $A_{C1}+A_{C2}+A_{C3}+A_{C4} =1$. Compared to NG, culture with HG prolonged each components of $\tau_C$ without changing the $\tau_O$ components.
Table 2 (supplement)  Activation and deactivation kinetics of hSlo wt and C911A mutant channels in response to high glucose, H$_2$O$_2$, OONO$^-$, and high glucose with CAT over-expression

<table>
<thead>
<tr>
<th></th>
<th>NG</th>
<th>HG</th>
<th>CAT+HG</th>
<th>C911A+HG</th>
<th>Baseline</th>
<th>H$_2$O$_2$</th>
<th>OONO$^-$</th>
<th>C911A+H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_0$ (s$^{-1}$)</td>
<td>235.5</td>
<td>187.5</td>
<td>298.7</td>
<td>275.6</td>
<td>109.8</td>
<td>88.5</td>
<td>113.7</td>
<td>164.2</td>
</tr>
<tr>
<td>$\beta_0$ (s$^{-1}$)</td>
<td>800.6</td>
<td>758.7</td>
<td>926.8</td>
<td>1024</td>
<td>981.5</td>
<td>686.8</td>
<td>875.4</td>
<td>883.7</td>
</tr>
<tr>
<td>$z_\alpha$ (e)</td>
<td>0.65</td>
<td>0.52</td>
<td>0.71</td>
<td>0.59</td>
<td>0.49</td>
<td>0.42</td>
<td>0.48</td>
<td>0.46</td>
</tr>
<tr>
<td>$z_\beta$ (e)</td>
<td>0.43</td>
<td>0.59</td>
<td>0.38</td>
<td>0.47</td>
<td>0.53</td>
<td>0.58</td>
<td>0.50</td>
<td>0.54</td>
</tr>
</tbody>
</table>

$\alpha_0$ represents the forward rate constant from closed to open state, $\beta_0$ represents the backward rate constant from open to closed state in a 2 state model, $z_\alpha$ represents the charge movement from closed state to open state, and $z_\beta$ represents the charge movement from open state to closed state.
REFERENCES


7. Lu T, Katakam PV, VanRollins M, Weintraub NL, Spector AA, Lee HC. Dihydroxyeicosatrienoic acids are potent activators of Ca(2+)-activated