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Original Article

Acute exposure of methylglyoxal leads to activation of K_{ATP} channels expressed in HEK293 cells

Yang YANG[§, *], Anuhya S KONDURU, Ningren CUI, Lei YU, Timothy C TROWER, Weiwei SHI, Yun SHI, Chun JIANG[*]

Department of Biology, Georgia State University, Atlanta, GA 30302–4010, USA

Aim: Highly reactive carbonyl methylglyoxal (MGO) is one of the metabolites excessively produced in diabetes. We have showed that prolonged exposure of vascular smooth muscle cells to MGO leads to instability of the mRNA encoding ATP-sensitive potassium (K_{ATP}) channel. In the present study we investigated the effects of MGO on the activity of K_{ATP} channels.
Methods: Kir6.1/SUR2B, Kir6.2/SUR2B or Kir6.2Δ36 (a truncated Kir6.2 isoform) alone was expressed in HEK293 cells. Whole-cell currents were recorded in the cells with an Axopatch 200B amplifier. Macroscopic currents and single-channel currents were recorded in giant inside-out patches and normal inside-out patches, respectively. Data were analyzed using Clampfit 9 software.
Results: The basal activity of Kir6.1/SUR2B channels was low. The specific K_{ATP} channel opener pinacidil (10 μmol/L) could fully activate Kir6.1/SUR2B channels, which was inhibited by the specific K_{ATP} channel blocker glibenclamide (10 μmol/L). MGO (0.1–10 mmol/L) dose-dependently activated Kir6.1/SUR2B channels with an EC_{50} of 1.7 mmol/L. The activation of Kir6.1/SUR2B channels by MGO was reversible upon washout, and could be inhibited completely by glibenclamide. Kir6.2Δ36 channels expressed in HEK293 cells could open automatically, and the channel activity was enhanced in the presence of MGO (3 mmol/L). Single channel recordings showed that MGO (3 mmol/L) markedly increased the open probability of Kir6.1/SUR2B channels, leaving the channel conductance unaltered.
Conclusion: Acute application of MGO activates K_{ATP} channels through direct, non-covalent and reversible interactions with the Kir6 subunits.

Keywords: methylglyoxal; reactive carbonyl species; ATP-sensitive potassium channel; Kir6.1; Kir6.2; Kir6.2Δ36; sulfonylurea receptor; diabetes; vascular dysfunction

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Introduction

Diabetes affects approximately 25 million people in the United States[1]. Hyperglycemia, a major hallmark of diabetes, serves as a trigger for the development and progression of many diabetes-associated complications on a systemic level. One of the key consequences of hyperglycemia is alteration of the metabolic pathways, leading to the production and accumulation of metabolic byproducts. Among these metabolites is the highly reactive carbonyl methylglyoxal (MGO), which also plays a key role in mediating oxidative stress and carbonyl stress[2]. MGO is produced mainly by the transformation of triosephosphate intermediates during glycolysis and is also produced in limited quantities via protein catabolism and fatty acid oxidation mechanisms[2]. Patients with type I diabetes have been reported to have a 5–6-fold increase in plasma MGO levels, whereas in type II patients, a 2–3-fold increase has been observed[3]. Excess MGO, when present as a result of either impaired carbonyl clearance mechanisms, increased availability of precursor molecules or both, is observed in many pathological conditions, including diabetes. Increased levels of MGO can lead to structural and functional changes in the cell by attacking molecules such as transporters, ion channels, transcription factors and signal transduction components at both the mRNA and protein levels[4–7].

Diabetic vascular complication is a severe event associated with many diabetic patients, and hyperglycemia is suggested to be a major contributor. Despite numerous studies hinting towards the importance of MGO in the regulation of the vascular functions in carbonyl stress[8, 9], the response pattern of key molecules in the vasculature to the presence of reactive carbonyl species is yet to be fully understood. The K_{ATP} chan-

§ Now in *Center for Neuroscience & Regeneration/Rehabilitation Research, Department of Neurology, Yale University School of Medicine & Veterans Affairs Connecticut Healthcare System, USA.*
* To whom correspondence should be addressed.
E-mail yang.yang.neurology@yale.edu (Yang YANG);
cjiang@gsu.edu (Chun JIANG)
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nel is a major regulator of vascular tone and a critical pharmacological target for treating diabetes[10–13]. This channel couples the cellular metabolism to membrane excitability[14]. Activation of the K_{ATP} channel produces hyperpolarization in smooth muscle cells, which lowers the activity of voltage-dependent calcium channels (VDCC), leading to vasorelaxation. *Kcnj8* (encodes Kir6.1, a vascular K_{ATP} channel pore forming subunit) null mice show vasospasm in coronary arteries and sudden death[15, 16]. In diabetic patients, the response of K_{ATP} channel to stimuli is impaired, resulting in defective vasodilation of the vascular rings[17, 18].

Because carbonyl stress has been suggested to play a critical role in the development of diabetes-associated vascular complications[19], we previously tested if MGO, a major reactive carbonyl species, regulated the K_{ATP} channel. Interestingly, we found that prolonged exposure of vascular smooth muscle cells to MGO leads to mRNA instability of the K_{ATP} channel, which likely contributes to the dysfunction of the vasculature[5]. However, it is not yet known if acute MGO exposure may also exert an effect on the K_{ATP} channel gating. Therefore, in this current study, we investigated the effect of acute exposure of MGO on the K_{ATP} channel activity. We found that acute MGO treatment causes the activation of the vascular K_{ATP} channel in a receptor-independent mechanism, mediated through non-covalent interactions with the pore-forming Kir subunit of the K_{ATP} channel.

Materials and methods

Chemicals and reagents

All reagents and chemicals used in this study were purchased from Sigma-Aldrich unless stated otherwise. Reagents were freshly made and prepared in high-concentration stocks in double-distilled water or dimethyl sulfoxide (DMSO). MGO was freshly made and used within 4 h of preparation. The final concentration of DMSO in the solutions used for experiments was less than 0.1%, which did not have any detectable effect on the channel activity.

Cell culture and heterologous expression of K_{ATP} channels

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM/F12 with 10% fetal bovine serum and penicillin/streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere.

K_{ATP} channels were expressed in HEK293 cells as previously described[20–22]. Rat Kir6.1 (GenBank No D42145), mouse Kir6.2 (GenBank No D50581) and SUR2B (GenBank No D86038, mRNA isoform NM_011511) coding sequences were cloned into pcDNA3.1 (a eukaryotic expression vector). HEK293 cells cultured in 35-mm petri dishes were transfected with 1 μg Kir6.1 (or Kir6.2) and 3 μg SUR2B using Lipofectamine^{2000} (Invitrogen Inc, Carlsbad, CA, USA). Kir6.2Δ36 (a truncated Kir6.2 isoform, 3 μg) was transfected into HEK293 cells without SUR2B. Green fluorescent protein (GFP) cDNA (0.4 μg, pEGFP-N2, Clontech, Palo Alto, CA, USA) was included in the cDNA transfection mixture to facilitate the identification of positively transfected cells. One day after transfection, cells were disassociated with 0.25% trypsin, split and transferred to cover slips. Electrophysiology experiments were performed on the cells grown on the cover slips for 2 continuous days.

Electrophysiology

Patch clamp experiments were performed at room temperature as described previously[23–25]. In brief, 1.2-mm borosilicate glass capillaries were fire-polished to make patch pipettes of 2–5 MΩ resistance. Whole-cell currents were recorded in voltage clamps with a holding potential of 0 mV that was stepped to -80 mV every 3 s. The bath solution contained (in mmol/L): KCl 10, potassium gluconate 135, EGTA 5, glucose 5, and HEPES 10 (pH=7.4). The pipette was filled with a solution containing (in mmol/L): KCl 10, potassium gluconate 133, EGTA 5, glucose 5, K_2ATP 1, NaADP 0.5, $MgCl_2$ 1, and HEPES 10 (pH=7.4). All solutions containing ATP and/or ADP were freshly made and used within 4 h to avoid nucleotide degradation. The recordings were obtained with an Axopatch 200B amplifier (Axon Instruments Inc, Foster City, CA, USA), low-pass filtered (2 kHz, Bessel 4-pole filter, -3 dB) and digitized (10 kHz, 16-bit resolution) with Clampex 9 (Axon Instruments Inc, USA). Macroscopic currents were recorded with giant inside-out patches and single-channel currents were recorded with normal inside-out patches with a constant single voltage of -80 or -60 mV. Symmetric high K^+ (145 mmol/L in total) was used in both bath and pipette solutions to make the reverse potential of K^+ close to 0 mV. K_2ATP 1 mmol/L and NaADP 0.5 mmol/L was also included in the bath solution for maintaining the channel activity. For inside-out patch experiments, a higher sampling rate (20 kHz) was used to digitize the recorded currents. The data were analyzed using Clampfit 9 software (Axon Instruments Inc, USA).

Statistical analysis

Data are presented as the mean±SEM. Differences in the mean values were evaluated with Student's *t*-test or ANOVA and were accepted to be statistically significant when $P<0.05$.

Results

Acute MGO treatment leads to activation of the Kir6.1/SUR2B isoform of the K_{ATP} channel

The Kir6.1/SUR2B isoform is the major isoform of the vascular K_{ATP} channel, although the presence of the Kir6.2 subunit has also been detected. To test if acute MGO treatment affected the activity of K_{ATP} channels, we expressed the Kir6.1/SUR2B channel in HEK293 cells and used whole cell voltage-clamp configuration to test the channel activity. Equal concentrations of K^+ (145 mmol/L) were applied to both sides of the patch membranes. Membrane potential was held at 0 mV, and a -80 mV command potential was given to the cells every 3 s. The activity of the Kir6.1/SUR2B channel was low at the basal level (Figure 1A); however, it could be fully activated by the K_{ATP} channel-specific opener Pinacidil (Pin, 10 μmol/L) and inhibited by the channel-specific blocker Glibenclamide (Glib, 10 μmol/L) (Figure 1A).

For quantitative analysis, the effect of MGO was normal-

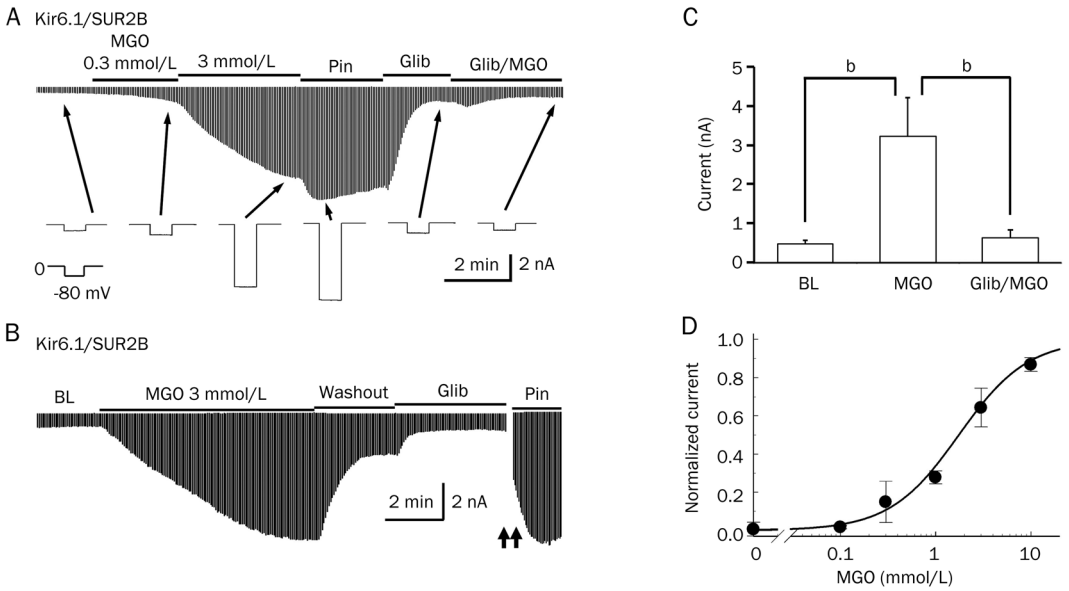

Figure 1. Acute MGO treatment led to activation of the Kir6.1/SUR2B channel. (A) The Kir6.1/SUR2B channel was expressed in HEK293 cells. Whole-cell currents were recorded from cells two days after transfection in a voltage-clamp configuration. MGO (0.3–3 mmol/L) treatment led to the activation of Kir6.1/SUR2B currents in a concentration-dependent manner. Pinacidil (Pin, 10 μmol/L), a K_{ATP} channel-specific opener, further opened the channel. The application of the K_{ATP} channel-specific inhibitor glibenclamide (Glib, 10 μmol/L) dramatically reduced the current. For quantitative analysis, the effect of MGO was normalized between the baseline current and the current activated by 10 μmol/L Pin. In the presence of Glib (10 μmol/L), the effect of the higher concentration of MGO (10 mmol/L) on channel activation was completely blocked. (B) The MGO-mediated channel activation was reversible. Following channel activation by MGO, washout with the bath solution returned the K_{ATP} channel currents to an almost baseline level. Glib caused a further reduction of the K_{ATP} channel currents. (C) Summary of the effect of MGO on the K_{ATP} channel currents in the presence and absence of Glib. $^{b}P<0.05$. (D) Dose-response relationship between the concentration of MGO and the normalized current. Data were described by the Hill-equation with an EC_{50} of 1.7 mmol/L.

ized between the baseline current and the current activated by 10 μmol/L Pin as a percentage value. The Kir6.1/SUR2B channel was steadily activated in response to an increasing dosage of MGO. A 300 μmol/L MGO treatment increased the channel activity moderately (22.0%±10.6%, n=4) while a 3 mmol/L MGO treatment markedly increased the channel activity (57.4%±14.3%, n=4, Figure 1A). Interestingly, MGO-mediated channel activation was reversible: the application of the control bath solution caused a significant decrease (from 57.4%±14.3% to 29.3%±10.7%, $P<0.05$) in MGO induced channel activity (Figure 1B), thereby suggesting an involvement of non-covalent interactions between MGO and K_{ATP} channels. In the presence of Glib, an MGO concentration as high as 10 mmol/L was not able to induce an increase in the channel activity, suggesting that Glib and MGO target the same molecule (the K_{ATP} channel) (Figure 1C). The relationship between MGO dosage and K_{ATP} channel activities was described using the Hill equation with EC_{50} approximately 1.7 mmol/L (Figure 1D).

MGO activates the Kir6.1 and Kir6.2 subunit vascular K_{ATP} channel by targeting independently of the SUR subunit

To determine whether the effect of MGO on the K_{ATP} channel activity was isoform-specific, we additionally tested the effect of MGO on the Kir6.2/SUR2B isoform of the K_{ATP} channel, another isoform found in vascular smooth muscle cells. In whole cell patch configuration, we found that the application of MGO (3 mmol/L) caused an increase in the Kir6.2/SUR2B activity (65.4%±7.9%, n=5, Figure 2A). This response was similar to what we observed in the Kir6.1/SUR2B recording (57.4%±14.3%, n=4). These data thus indicate that both the Kir6.1- and Kir6.2-containing channels can be targeted by MGO.

Naturally occurring K_{ATP} channels need both Kir and SUR subunits to be functional. However, previous studies have suggested that the truncation of several dozen amino acids in the C-terminus of the Kir6.2 subunit (Kir6.2Δ36) results in detectable currents, thereby eliminating the need for the SUR subunit to be present for the K_{ATP} channel to be functional[26, 27]. This SUR-independent K_{ATP} channel functioning is isoform-specific, as the truncated Kir6.1 isoform cannot express by itself without the SUR subunit. To further investigate if the presence of Kir subunit alone was sufficient for MGO to exert its activating effect, we took advantage of the SUR-independent Kir6.2Δ36 channel. Because of the absence of the SUR subunit, the Kir6.2Δ36 channel was not sensitive to regulation by either Pin or Glib, but could still be blocked effectively by Ba^{++}. In this condition, we quantified the effect of MGO against the Ba^{++} mediated channel blocking and expressed the changes in currents as fold changes. In the

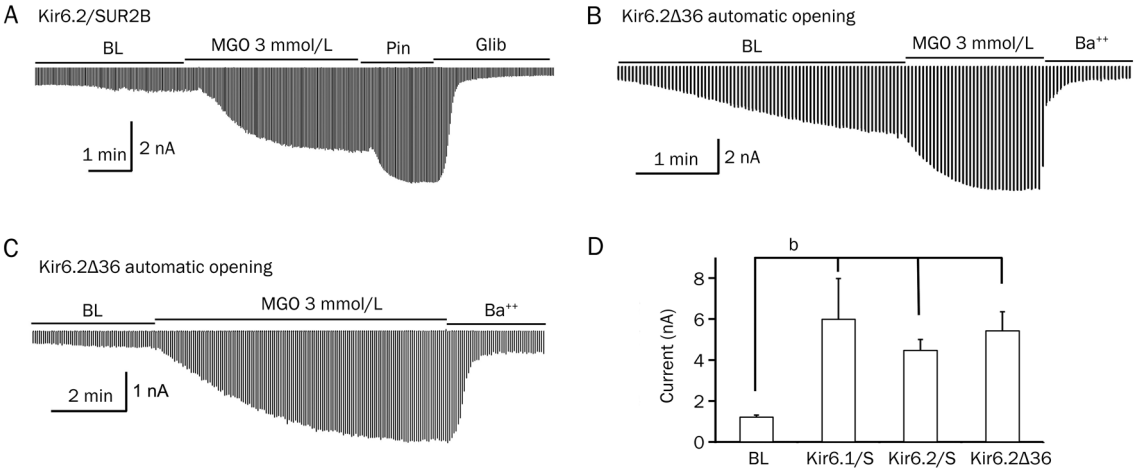

Figure 2. MGO-mediated activation was dependent on the Kir but not the SUR subunit of the K_{ATP} channel. (A) MGO (3 mmol/L) activated the Kir6.2/SUR2B isoform of the vascular K_{ATP} channel to a similar extent as seen in the Kir6.1 isoform. (B) A truncated form of the Kir6.2 subunit (Kir6.2Δ36) channel, capable of expressing itself without the need for the SUR subunit, opened automatically, and the channel activity was further enhanced in the presence of MGO. (C) In the presence of 1.0 mmol/L ATP and 0.5 mmol/L ADP, the basal currents of the Kir6.2Δ36 channel remained small and the application of MGO (3 mmol/L) augmented the channel activity significantly. (D) Summary of the effect of MGO on the Kir6.1/SUR2B (Kir6.1/S), Kir6.2/SUR2B (Kir6.2/S) and Kir6.2Δ36 channels (note that unlike the Kir6.2 isoform, the truncated Kir6.1 channel was not able to express by itself); $^bP<0.05$.

absence of ATP and ADP in the pipette solution, the Kir6.2Δ36 channels readily opened and the application of MGO led to a 3.87±1.81-fold increase in the channel currents (n=4, P<0.05, Figure 2B, 2D). In the presence of 1.0 mmol/L ATP and 0.5 mmol/L ADP, the basal currents of the Kir6.2Δ36 channel remained small and the application of MGO (3 mmol/L) was able to augment the channel activity by 3.35±0.6-fold (n=5, P<0.05, Figure 2C, 2D). Additionally, in both of the above experimental conditions, Ba^{++} treatment almost completely blocked the MGO-induced channel activity (Figure 2B, 2C). These data thus suggest that MGO is targeting the Kir rather than the SUR subunit.

Biophysical properties of MGO-mediated K_{ATP} channel activation

MGO-mediated activation of the K_{ATP} channel might be due to the direct modulation of the channel protein or involve the activation of other signaling pathways that regulate the K_{ATP} channel activity. To investigate these two possibilities, we studied the channel activity in giant inside-out patch configurations, in which the cytosolic components of the cell were excluded. In this condition, a 3 mmol/L MGO treatment led to a significant increase in the channel activity (52.7%±12.1%, n=6, P<0.05, Figure 3A, 3B), which was comparable to the results found in our whole-cell patch study.

Moreover, we performed single-channel patch experiments to study the effect of MGO treatment on channel open probability (P_O) and channel conductance. In the single channel study, a few channel openings were observed and the P_O had a basal level average of 0.183±0.069 (n=4, Figure 3C). With a 3 mmol/L MGO treatment, the P_O of the Kir6.1/SUR2B channel increased to 0.444±0.084 (n=7, Figure 3D). Pin treatment further augmented the channel P_O to 0.675±0.159 (n=6). In addition, we also tested the channel conductance using a ramp protocol with voltage ranging from -100 mV to 100 mV. Three active channels were observed with both MGO and Pin treatments (Figure 3E, 3F). The straight lines in Figure 3E and 3F represent a slope conductance of 36 pS for all three channels, indicating that the channel conductance remains unchanged with MGO treatment (Figure 3E, 3F). Taken together, these data indicate that the modulation of vascular K_{ATP} channels by MGO is mediated via an increase in the P_O of the channel without changing the channel conductance or recruitment of other cellular signaling machinery.

Discussion

In this study, we found that acute MGO exposure led to the activation of K_{ATP} channels through direct targeting of the Kir6.x subunits. Furthermore, we found that this effect was independent of the SUR subunit and that MGO did not cause covalent modifications to the K_{ATP} channel. Single channel analysis revealed that MGO treatment augmented the K_{ATP} channel open probability without altering the channel conductance. In addition, MGO-mediated activation of the K_{ATP} channel did not require signal transduction components including receptors, secondary messenger molecules or cytosolic effectors.

In several instances, the concentration of MGO has been determined to be in the sub-millimolar range: in cultured mammalian cells, an approximate 300 μmol/L MGO concentration was detected[28] while an approximate 400 μmol/L MGO concentration was reported in diabetic patients with poorly controlled hyperglycemia[29, 30]. In other studies, the concentration of MGO was reported to be much lower[31–33]. It is possible that the reported concentrations of MGO represent the average concentration on a systemic level instead of the local concentration at a specific site. The levels of MGO in

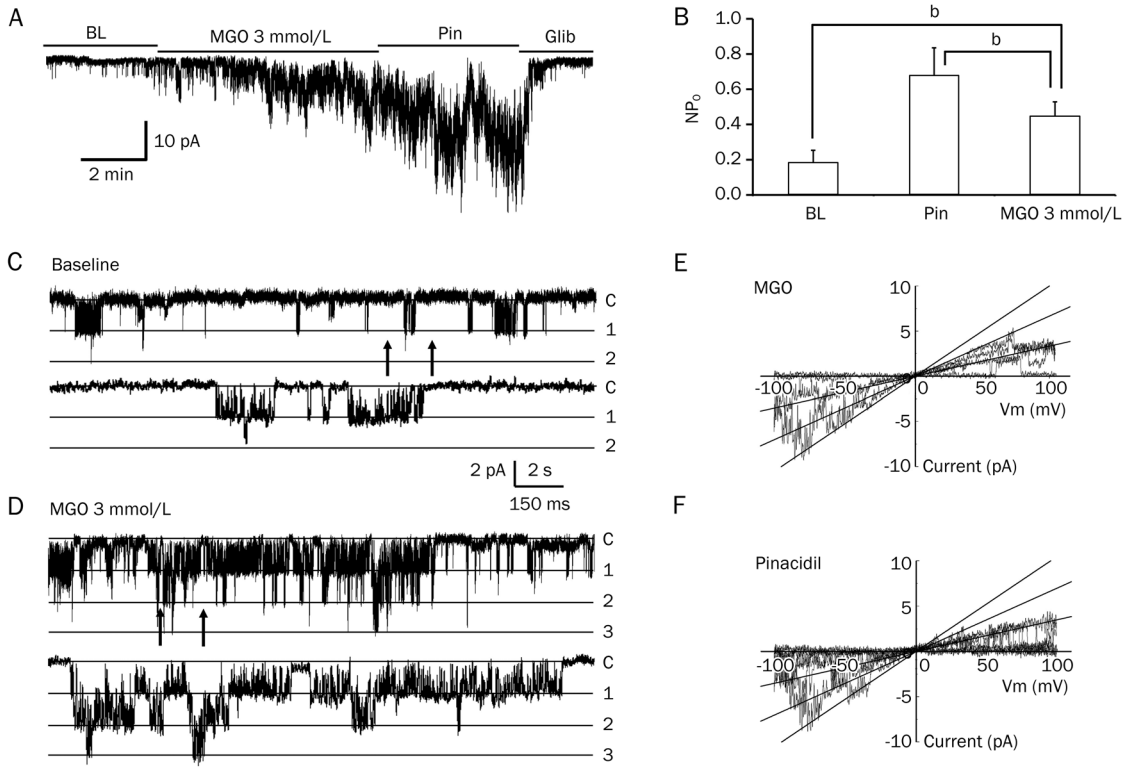

Figure 3. MGO affected the single-channel properties of the K_{ATP} channel. (A) In a giant inside-out patch configuration, 3 mmol/L MGO activated the Kir6.1/SUR2B currents in a manner similar to that seen in the whole cell configuration. This current could be further activated and inhibited by Pin and Glib, respectively. (B) Summary of the MGO treatment in the inside-out patch study. $^{b}P<0.05$. (C) Single-channel level currents were recorded in inside-out patches with a holding potential of -80 mV. The lower trace was an expansion from the upper trace between the arrows. The presence of two active channels was evident at the baseline level. The NP_0 averaged 0.183 (n=4) in the baseline. (D) Treatment of the inside-out patch with 3 mmol/L MGO augmented the single-channel activity, as a result of the appearance of one more active channel. The NP_0 averaged 0.444 with MGO treatment. (E, F) Single-channel conductance was measured with a ramp protocol with voltage ranging from -100 mV to 100 mV. Three active channels were observed with both MGO alone (E) and Pin alone (F) treatments. The straight lines represent a slope conductance of 36 pS for all three channels.

the body over a period of time could vary depending on the availability of the precursors and the efficiency of the carbonyl clearance system. During carbonyl stress, MGO levels in the body might follow a sudden burst pattern followed by a recession in the MGO spikes due to the carbonyl detoxification system "catching up" with the extent of carbonyl stress present. In our experimental condition, we found that the EC_{50} of MGO on the Kir6.x subunit activation appears to be 1.7 mmol/L. Our findings could thus be interpreted as the responses to an experimental condition that may represent the initial response of the vasculature to a rapid increase in the levels of MGO in a localized environment, as seen in pathological conditions.

Oxidative stress, characterized by the presence of excess reactive oxygen species (ROS) as a result of overproduction of ROS and impairment of cellular anti-oxidant machinery, has been long thought to be a major contributing factor for diabetes-associated vascular complications[34]. Several previous studies have suggested that important vascular tone regulators, including the vascular K_{ATP} channel, are targeted by ROS[35–37]. In agreement, we found that ROS modulates the vascular K_{ATP} channel by the covalent post-translational modification mechanism, *S*-glutathionylation[21, 38]. In addition to oxidative stress, carbonyl stress has recently been proposed to be an underappreciated stress that may play a more dominant role in the progression of diabetes-associated vascular complications via the action of reactive carbonyl species (RCS). Among a variety of RCS, MGO is highly reactive. Excess MGO readily reacts with nucleophilic groups on proteins and nucleic acids, leading to cellular dysfunction and propagation of carbonyl stress[39, 40]. Indeed, in our recent study, we found that a prolonged MGO treatment led to disruption of the K_{ATP} channel activity via mRNA instability, which was likely a contributing factor for the impairment of arterial function under carbonyl stress[5]. Interestingly, in the current study, we found that acute MGO treatment can activate the K_{ATP} channel. This differential response of the K_{ATP} channel to MGO treatment is dependent on the exposure time and works through different mechanisms of action[5, 21].

MGO is well known to interact with positively charged lysine residues of proteins to form advanced glycation adducts[41]. This type of modulation involves covalent interactions, which are not likely to be broken by a washout alone.

Interestingly, in our present study, we found that MGO-mediated K_{ATP} channel activation could be rapidly reversed with washout, suggesting an activation mechanism not involving the formation of a covalent bond. We suspect that MGO can interact with the residues near the gating area, affecting the channel gating directly. It is also possible that MGO can target the phospholipid bilayer of the membrane associated with the K_{ATP} channel, affecting the channel gating. Future studies addressing these questions will provide valuable information regarding carbonyl stress-mediated K_{ATP} channel activation.

It has been shown that MGO contributes to the development of vascular complications of diabetes[42], mainly through the formation of advanced glycation end products (AGEs) that interact with their cellular receptor, the receptor for advanced glycation end products (RAGEs)[43]. Interestingly, in this current study, this "classical" AGE/RAGE interaction does not appear to play a role in the MGO-mediated K_{ATP} channel activation, as our single channel preparation, which lacks all the signaling components, still yields similar results as our whole cell patch experiments.

In summary, our study demonstrated that acute MGO treatment led to reversible K_{ATP} channel activation in a signaling cascade-independent manner via interacting with the Kir6.x subunits, augmenting the channel-open probability. This finding may help us to better understand the response pattern of the vasculature to RCS and provide information necessary to design effective pharmacological treatments against carbonyl stress-mediated vascular dysfunction.

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Author contribution

Yang YANG and Chun JIANG designed the experiments. Yang YANG, Anuhya S KONDURU, Ningren CUI, Lei YU, Timothy C TROWER, Yun SHI, and Weiwei SHI performed the experiments; Yang YANG, Anuhya S KONDURU, and Chun JIANG analyzed the data; Yang YANG, Anuhya S KONDURU, and Chun JIANG wrote the manuscript.

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