The SUR2B subunit of rat vascular K_{ATP} channel is targeted by miR-9a-3p induced by prolonged exposure to methylglyoxal

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Li SS, Wu Y, Jin X, Jiang C. The SUR2B subunit of rat vascular K_{ATP} channel is targeted by miR-9a-3p induced by prolonged exposure to methylglyoxal. Am J Physiol Cell Physiol 308: C139–C145, 2015. First published October 29, 2014; doi:10.1152/ajpcell.00311.2014.—ATP-sensitive K^{+} (K_{ATP}) channels regulate plasma membrane excitability. The Kir6.1/SUR2B isoform of K_{ATP} channels is expressed in vascular smooth muscles and plays an important role in vascular tone regulation. This K_{ATP} channel is targeted by several reactive species. One of them is methylglyoxal (MGO), which is overly produced with persistent hyperglycemia and contributes to diabetic vascular complications. We have previously found that MGO causes posttranscriptional inhibition of K_{ATP} channel, aggravating vascular tone regulation. Here we show evidence for the underlying molecular mechanisms. We screened microRNA databases and found several candidates. Of them, miR-9a-3p, increased its expression level by ~240% when the cultured smooth muscle cell line was exposed to micromolar concentrations of MGO. Treatments with exogenous miR-9a-3p downregulated the SUR2B but not Kir6.1 mRNA. Antisense nucleotides of miR-9a-3p alleviated the effects of MGO. Quantitative PCR showed that the targeting sites of the miR-9a-3p were likely to be in the coding region of SUR2B. The effects of miR-9a-3p were mostly eliminated when the potential targeting site in SUR2B was site-specifically mutated. Our functional assays showed that K_{ATP} currents were impaired by miR-9a-3p induced with MGO treatment. These results suggest that MGO exposure raises the expression of miR-9a-3p, which subsequently downregulates the SUR2B mRNA, compromising K_{ATP} channel function in vascular smooth muscle.

MicroRNAs (miRs) are important regulators of gene expression especially in diseased conditions. In eukaryotes, miRs are encoded by nuclear DNA and transcribed as longer hairpin transcripts known as pre-miRs. After processed by the Drosha and Dicer enzymes, one strand of the hairpin duplex is loaded to an Argonaute family protein to form the core of miR-induced silencing complex that subsequently functions via base-pairing with complementary sequences of the target mRNA to regulate the mRNA life and its capability of translation. In the process, the binding of miRs to the target mRNA is important for their recognition. Most miR binding sites sufficient for the transcript silencing are located in the 3’-untranslated region (3’-UTR), while some are in the coding sequence (CDS). Our previous studies have suggested that MGO acts on the CDS of SUR2B mRNA, impairing their stability as well as K_{ATP} channel activity (44). Thus, it is possible that under diabetic conditions, the reactive carbonyl stress raises the expression of miRs that subsequently target the K_{ATP} channel, and impair the vascular tone regulation. To test this hypothesis, we performed the present studies.

MATERIALS AND METHODS

Reagents. Antibodies against Kir6.1 and GAPDH were purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal antibodies against SUR2B were purchased from Santa Cruz Biotechnology (Dallas, TX). All other chemicals and reagents were purchased from common commercial sources. Reagents were prepared in stocks with high-concentration in double-distilled water or DMSO. The final concentration of DMSO in experiments was <0.1% (vol/vol), which was tested to have no detectable effects.
Cell culture. HEK293 cells (CRL-1573; American Type Culture Collection, Manassas, VA) and rat VSM cells (A10 cell line, CRL-1476; American Type Culture Collection) were cultured in complete DMEM (10% FBS) in a 5% CO₂ atmosphere at 37°C. Two to four generations were used for experiments.

Cell transfection. After growth arrest, the cells were transfected using lipofectamine. Subsequently, the cells were switched to complete DMEM (10% FBS) and cultured in a 5% CO₂ atmosphere at 37°C for another 24–48 h.

Bioinformatics prediction of miR targets. To identify the candidate miRs that potentially targeted rat Kir6.1 (Kcnj8), we used the miRWalk database (https://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html) that is supported by eight miRNA prediction programs on 3’-UTRs of all known genes of the human, mouse, and rat. A probability distribution of random matches of a subsequence (miR 5’-end sequence) in the given sequence was calculated by using Poisson distribution, where a low probability implies a significant hit (20, 35). The default P value (0.05), the mirSVR score ≤ 0 (33), plus at least six complementarity seeds were used for miR/Kcnj8 alignment. A number of miRs potentially target Kir6.1 3’-UTR. To limit the number we used the following criteria: 1) their high conservation across a wide range of mammals and 2) their potential involvement in diabetes in existing literature. On the basis of these criteria, three potential Kir6.1-targeting miRs were selected for further screening. Similarly, six potential miRs for SUR2B CDS targeting were predicted using the same database. The targeting site conservation was examined among rats, mice, and humans by comparing NCBI blastn alignment as the similar searches with human KCNJ8/ABCC9 showing no miR matches in the same 3’-UTR/coding region.

Synthesis of miR-9a-3p and anti-miR-9a-3p. Chemically synthesized and optimized double-strand nucleotides (m-9) were designed to mimic endogenous mature miR-9a-3p in VSM and scrambled RNAs (scmiR) was synthesized as negative control (Qiagen, Sample & Assay Technologies, Valencia, CA). Single-strand antisense oligonucleotides (anti-9) complementary to the mature miR-9a-3p were synthesized to specifically target and knock down endogenous miR-9a-3p in VSM (Sigma–Aldrich).

Construction of Kir6.1 and SUR2B mRNA expression vectors. The cDNAs encoding rat Kir6.1 mRNA (GenBank no. D42145.1) and mouse SUR2B mRNA isoform (GenBank no. D86038, mRNA isoform NM_011511) were cloned and inserted into pcDNA3.1 (a eukaryotic expression vector), respectively, as previously described (44), which were named with Kir and SUR, respectively.

Site-directed mutagenesis. The mutated pcDNA3.1 construct of the SUR2B (M-SUR) was obtained using the Stratagene QuikChange mutagenesis (New England BioLabs, Ipswich, MA) and used for real-time quantitative PCR (qPCR) and patch studies. The mutagenic oligonucleotide primer pair was designed according to the desired mutation in its seed match sequences. Briefly, the M-SUR was cloned with the primer pair (forward: CCCTAAATTACTTTTGGCCT- TATTTCCGTATG; reverse: CCAGTACAGGAATAACGCGAAAATGTAATTTAAGG), containing two site mutations in position 622 and 625 of SUR2B mRNA (Fig. 1B).

Heterologous expression of K\textsubscript{ATP} channel. K\textsubscript{ATP} channels were heterologously expressed in HEK293 cells as previously described (44). Briefly, the constructions of Kir6.1 and SUR2B mRNA expression vectors were cotransfected to the HEK293 cells (ratio of 1:3), and the pEGFP-N2 (Clontech, Palo Alto, CA) was transfected together to determine the positively transfected cells. One day after transfection, the cells were again transfected with synthesized m-9 or anti-9. After another day in culture, the cells were used for electrophysiological studies and luciferase analysis.

Patch-clamp studies. The single-cell voltage clamp was used to record whole cell K\textsubscript{ATP} currents in cells transfected with different agents. Patch-clamp protocols were performed as previously described (44). Briefly, the patch pipettes were made with 1.2 mm borosilicate glass capillaries (with resistance of 2–5 MΩ). Current records were filtered with low-pass (2 kHz, Bessel 4-pole filter, –3 dB), digitized (20 kHz, 16-bit resolution), and analyzed with Clampfit software (Axon Instruments, Union City, CA) The bath solution maintained (in mM) 10 KCl, 135 potassium gluconate, 5 EGTA, 5 glucose, and 10 HEPES (pH 7.4). The pipette solution contained (in mM) 133.0 K⁺ gluconate, 10.0 KCl, 5.0 EGTA, 5.0 glucose, 1 K₂ATP, 0.5 NaADP, and 10.0 HEPES (pH 7.4). The final Mg²⁺ concentration was adjusted to 1 mM. The membrane potential was held at 0 mV and stepped to −80 mV every 3–4 s.

Real-time quantitative PCR. The qPCR analysis was performed with high-capacity cDNA Reverse Transcription Kit and Fast SYBR Green Master Mix (Applied Biosystems, Life Technologies, New York, NY) following the manufacturer’s instructions. Primers specific for Kir6.1, SUR2B, miRs, RNU6B, and GAPDH were synthesized from Sigma (Sigma). The qPCR was performed with a Fast Real-time PCR system (Applied Biosystems 7500) for 40 cycles. The fold increase relative to control samples was determined by the 2⁻ΔΔCT method. Expression levels of target mRNAs were determined using total RNA from A10 or HEK cells. GAPDH was used as an internal control for SUR2B mRNA expression. RNU6 was used for normalization of miR-9a-3p expression.

Western blot analysis. Proteins extracted from A10 and HEK cells were detected using a standard Western blot protocol. GAPDH was used as an internal control.

Statistical methods. Data are expressed as means ± SE. Comparisons of data were accomplished by one-way ANOVA followed by post hoc Dunnett’s test or Student’s t-test. The differences between means were considered significantly different when P ≤ 0.05.

RESULTS

Expression profiling of candidate miRNAs in reactive carbonyl stress. In diabetic patients, persistent hyperglycemia leads to overproduction of MGO to ~400 μM (23, 30). In our previous study, we found that exposure to 300 μM MGO...
causes disruption of vascular $K_{\text{ATP}}$ channels (44). Therefore, we used this concentration of MGO in the present study.

We first screened the potential miR candidates targeting mRNAs of rat Kir6.1 and SUR2B subunits using bioinformatics, as our previous experiments were mostly done in the rat A10 VSM line. On the basis of their mammalian conservation and potential involvement in diabetes in existing literature, nine diabetes-associated miRs were found. Among them, three miRs were potentially targeting Kir6.1, and six miRs were potentially targeting SUR2B. Subsequently, the expression profiles of the nine mature miRs were determined in A10 VSM line following MGO exposure. Our qPCR analysis showed that the MGO treatment resulted in a significant increase of miR-9a-3p level by 2.4–6 folds ($n = 3$ separated experiments with 3–6 samples each), while none of the other miRs increased their expression significantly (Fig. 2). We did not further study miRs that showed reductions in their expression as according to current literature they did not seem to have a direct effect on $K_{\text{ATP}}$ channel inhibition by MGO exposure. Because of this and because miR-9a-3p is highly conserved in mammals (Fig. 1A), all further studies were performed on miR-9a-3p.

Inhibition of $K_{\text{ATP}}$ channel expression by MGO and miR-9a-3p. To show whether miR-9a-3p affects $K_{\text{ATP}}$ channel expression, one chemically synthesized and optimized double-strand nucleotide (m-9) was designed with an identical sequence to the mature endogenous miR-9a-3p. Also synthesized was one single-strand nucleotide complementary to the mature miR-9a-3p (anti-9). We then transfected the A10 cells with these synthetic nucleotides and studied their effects on the expression of SUR2B subunit after MGO exposure.

Our qPCR analysis showed that the m-9 transfection caused suppression of the SUR2B mRNA expression (Fig. 3), which resembled the effect of MGO. Meanwhile, the effect of MGO was markedly diminished when the cells were transfected with anti-9 (Fig. 3). Consistent with qPCR results, Western blot analysis showed that m-9 inhibited SUR2B expression at the protein level, while anti-9 partially blocked the MGO effect (Fig. 4, A and B). To determine whether m-9 also acts on Kir6.1 in the same conditions, we tested the expression of Kir6.1 at protein level. Our data showed that m-9 had no effect on Kir6.1 protein expression (Fig. 4, C and D). These results suggest that miR-9a-3p regulates vascular $K_{\text{ATP}}$ channel expression, which appears necessary for MGO to produce its effect.

Targeting at the CDS of SUR2B. Our bioinformatics analysis showed that a 9 seed-nucleotide region in the position 661–685 of rat SUR2B mRNA matches the miR-9a-3p, which also can be recognized in mouse (608–632) and human (2720–2744) SUR2B mRNAs (Fig. 5A). Several constructs were made to demonstrate whether such a potential binding site interacts directly with miR-9a-3p. The mouse SUR2B mRNA was cloned into the pcDNA3.1 vector (SUR). Site-directed mutagenesis of two nucleotides at positions 622 and 625 without changing the amino acids (M-SUR) was also carried out using the same vector (Fig. 1C). We then cotransfected the HEK293 cells together with the M-SUR and m-9. The cotransfection of HEK293 cells with the SUR alone or scmiR was used as negative control. As shown in Fig. 5B, the cotransfection of HEK293 cells with the SUR and m-9 resulted in downregulation of SUR mRNA expression compared with controls, which is consistent with our findings in the A10 cells. This effect of m-9 was abrogated when the miR-9a-3p binding site was mutated (Fig. 5B). Therefore, these results suggest that miR-9a-3p is likely to target the SUR2B CDS, and the position 608–632 seems to be an important target site.
Inhibition of functional $K_{\text{ATP}}$ currents by miR-9a-3p.

To prove that exogenous m-9 and anti-9 had a functional impact on $K_{\text{ATP}}$ channels, we studied its effects on heterologously expressed Kir6.1/SUR2B channels in HEK293 cells, in which $K_{\text{ATP}}$ currents were relatively large and sufficient for a long-term analysis. Equal high concentrations of K/H11001 (145 mM) were applied to both sides of the membranes. The membrane potential was held at 0 mV and stepped to $-80$ mV every 3 s in voltage clamp. Pinacidil (Pin), a specific $K_{\text{ATP}}$ opener, and glibenclamide (Glib), a $K_{\text{ATP}}$ inhibitor, were used to set a window of $K_{\text{ATP}}$ channel activity. At the basal level, $K_{\text{ATP}}$ channel activity was low. Exposure to 10 $\mu$M Pin strongly activated $K_{\text{ATP}}$ currents, which were subsequently suppressed by 10 $\mu$M Glib (Fig. 6A).

Consistent with our previous study (44), a marked inhibition in $K_{\text{ATP}}$ currents occurred after cells were treated with MGO for 12 h (Fig. 6B). In cells transfected with anti-9, the MGO effect was drastically attenuated (Fig. 6, C and D). A significant inhibition in $K_{\text{ATP}}$ currents was found after the cells were cotransfected with m-9 (Fig. 7B), to the degree similar to the effect of MGO on $K_{\text{ATP}}$ currents (Fig. 6B). $K_{\text{ATP}}$ current inhibition was reversed partially when the binding sites for miR-9a-3p were mutated in SUR2B (M-SUR) (Fig. 7, C and D). In contrast, scmiR had no effect on $K_{\text{ATP}}$ channel activity (data not shown).

**DISCUSSION**

This is the first demonstration of regulation of $K_{\text{ATP}}$ channel by miRs. The miR-9a-3p is upregulated in MGO-induced carbonyl stress. By targeting the CDS of SUR2B, the miR-9a-3p inhibits $K_{\text{ATP}}$ channel expression, leading to a reduction in $K_{\text{ATP}}$ channel activity.

In diabetic conditions, persistent hyperglycemia leads to overproduction of a variety of RCS including the highly reactive MGO, contributing to the development of diabetic complications. Acting on proteins, lipids, or nucleotides, excessive MGO can cause carbonyl stress and cell damage. Our previous studies have shown that MGO acts on $K_{\text{ATP}}$ channels in VSM cells, causing instability of Kir6.1 and SUR2B mRNAs, in which the 3'-UTR of Kir6.1 and the CDS of...
SUR2B are likely to be targeted (44). As a result, a loss of functional KATP channels occurs followed by dysregulation of vascular tone (44).

Emerging evidence suggests that miRs contribute to the pathogenesis of diabetes and diabetic complications (5, 15, 31, 43). Since each miR has its potential targeting genes, information of miR involvement in diabetes is helpful for identification of the targeted molecules as well. The expression of these miRs may be upregulated by several pathological conditions in diabetes including the carbonyl stress. Therefore, it is reasonable to believe that the instability of Kir6.1 and SUR2B mRNAs shown in our previous studies is attributable to certain miRs.

With bioinformatics prediction, nine diabetes-associated miRs were selected for the profiling in carbonyl stress. With the information, we treated the A10 VSM cell line with 300 μM MGO, a concentration that is seen in serum of diabetic patients and also found effective for KATP disruption (44). With the MGO treatment, we have found that miR-9a-3p is upregulated, while none of the other eight miRs show a significant increase in their expression levels. miR-9a-3p is highly conserved in mammals and has potential targeting sites in the human SUR2B gene as well. Under diabetic conditions, miR-9 is suggested as being involved in insulin secretion by targeting Sirt1 in pancreatic β-islets (34). However, no previous study has reported that miR-9a-3p is regulated by RCS under diabetic conditions. Therefore, our current study provides the first evidence for the upregulation of miR-9a-3p in reactive carbonyl stress.

In A10 cells, we overexpressed m-9 with/without MGO treatment and found inverse correlations between the exoge-

![Fig. 6. Effects of MGO and miR-9a-3p on functional ATP-sensitive K⁺ (KATP) currents. One day after Kir6.1/SUR2B channels were expressed in HEK293 cells, the cells transfected with anti-9 were treated with 300 μM MGO and cultured for 12–24 h. Cells transfected with scmiR were used as negative control. A: KATP currents were recorded using symmetric K⁺ concentrations of internal and bath solutions. Inward K⁺ currents were elicited with voltage commands from 0 to −80 mV every 3 s. KATP currents were strongly activated by 10 μM pinacidil (Pin) and were inhibited by 10 μM glibenclamide (Glib). BL, baseline. B: MGO treatment significantly suppressed KATP current in HEK cells. C: MGO-induced reduction of KATP currents was reversed in anti-9-transfected cells. D: current density is represented in the bar graph. **P < 0.01 (n = 8–10 cells).](http://ajpcell.physiology.org/)

![Fig. 7. Inhibition of KATP currents by miR-9a-3p. Experiments were done as described in Fig. 6. Cells transfected with scmiR were used as negative control. A: KATP currents were recorded from the negative control (Ctl). B: Pin-induced currents became much smaller in m-9-transfected cells. C: m-9-induced current inhibition was abrogated when their targeting sites in SUR2B subunit were mutated. D: effect of miR-9a-3p on current density is represented in the bar graph. **P < 0.01 (n = 8–10 cells).](http://ajpcell.physiology.org/)
nous miR-9a-3p and SUR2B mRNA levels. Consistently, antagonizing the endogenous miR-9a-3p with anti-9 reversed the MGO-induced reduction of SUR2B expression. In HEK cells, our qPCR and Western analysis further proved that the CDS of SUR2B was directly targeted by miR-9a-3p. These results thus indicate that miR-9a-3p is involved in MGO-induced disruption of vascular K\textsubscript{ATP} channels by targeting at the CDS of SUR2B.

The SUR2B targeting by miR-9a-3p should have an impact on K\textsubscript{ATP} channel activity. Our data indeed show that miR-9a-3p inhibits functional K\textsubscript{ATP} currents to the degree similar to MGO treatment. The effects of MGO on K\textsubscript{ATP} channel can partially be reversed when the endogenous miR-9a-3p was knocked down with anti-9, further supporting that miR-9a-3p mediates the modification of K\textsubscript{ATP} channels in carbonyl stress. Our site-directed mutagenesis studies confirm that miR-9a-3p targets SUR2B mRNA at the CDS region.

In our previous studies, we have found that MGO causes instability of both Kir6.1 and SUR2B mRNAs (17). Although miR-9a-3p is likely to target SUR2B, the Kir6.1-targeting miR(s) remains to be demonstrated. Since none of the three conserved miRs that we have studied seems to be the player, and since miR-9a-3p does not affect Kir6.1 expression either, we speculate that the Kir6.1 may be targeted by miRs that are either nonconserved or missing in the database. Alternatively, the Kir6.1 inhibition by MGO may not be mediated by miRs at all.

Mouse models of loss-of-function of SUR2 genes have shown the critical role of the vascular K\textsubscript{ATP} channel in the coronary circulation. K\textsubscript{ATP} channel dysfunction in mice leads to coronary vasospasm and sudden death (9, 27). Notably, in SUR2\textsuperscript{\textminus/\textminus} mice, loss of hyperpolarizing K\textsubscript{ATP} current causes abnormally elevated [Ca\textsuperscript{2+}], leading to a reduction in coronary artery vasospasm (9). Therefore, vascular K\textsubscript{ATP} channel targeting by miR-9a-3p appears consistent with the adverse effects of MGO on vasculatures.

Recent studies indicate that the activity of the vascular isoform of K\textsubscript{ATP} channels is necessary for the systemic responses in diabetes. The increased metabolic rate requires a corresponding elevation in cardiac output to meet the metabolic demands. Therefore, K\textsubscript{ATP}-mediated vasodilation may act as compensatory role for several vital organs perfusion. Our studies indicate that miR-9a-3p modulates the K\textsubscript{ATP} channel, which compromises the compensatory vasodilation in several vital organs and leads to inadequate perfusion, contributing to tissue hypoxia and cell injury. Therefore, our studies on miR-mediated K\textsubscript{ATP} channel regulation in diabetes may help in the understanding of diabetic organ dysfunction.

A number of diabetes-associated miRs have been identified. Some miRs are involved in tissue dysfunction, including retina, kidney, peripheral nerves, heart, and the vasculature. For instance, some miRs modulate the renin-angiotensin-aldosterone system, like miR-181a, miR-663, miR-155, miR-29b, miR-129-3p, and miR-132, and oxidative stress like miR-377, miR-23a/b, miR-27a, miR-24, miR-335, miR-205, and miR-210 in diabetic nephropathy (19). Some miRs have been proved to be involved in vascular endothelial damage like miR-195, miR-503, and miR-146a in diabetic retinopathy (6, 8, 29), and some miRs like miR-34b, miR-34c, miR-199b, miR-210, miR-650, and miR-223 are shown to be dysregulated in diabetic ischemic heart failure patients (18). Therefore, our present study illustrating the role of MGO-induced miR-9a-3p in VSM cells helps in the understanding of the molecular mechanisms of vasculature dysfunction in diabetes.

At present, the mechanisms regulating miR expression and activity are still not fully understood. There is no information on the up- and downregulation of miRs by MGO in VSM cells either. Since the basic miR biogenesis pathways involve miR transcription, Drosha and Dicer processing, RNA editing, RNA modification, Argonaute loading, and RNA decay, MGO as a reactive carbonyl may act on one or some of these molecules affecting miR expression. Accumulating evidence suggests that aberrant DNA methylation of tumor suppressor genes occurs commonly in cancer, and events for miR hypermethylation/hypomethylation are found to play a role in human metastasis (24, 25). Indeed, MGO-mediated DNA demethylation has been found to alter the cellular redox balance in human cataract formation (32). Similar mechanisms may work in the VSM cells in reactive carbonyl stress. Thus, our findings in the present study may stimulate further studies of the regulation of miRs by RCS as well as the resulting vascular complications in diabetes.

Other unidentified targets may exist as miR-9a-3p sequence has multiple partial matches with Abcc9, Kir6.1, and other mRNAs. Indeed, miR-9a-3p seed match regions are found at three places in rat Abcc9 (675-AGCTTTAT-682, 5404-GCTTTAT-5398, and 5978-GCTTTAT-5972). Thus, we cannot rule out the possibility that other sites may also be targeted by miR-9a-3p in rat SUR2B and perhaps Kir6.1 as well.

In conclusion, the present study provides the first evidence for regulation of K\textsubscript{ATP} channels by miRs. Our results indicate that miR-9a-3p plays an important role in the regulation of vascular K\textsubscript{ATP} channels in reactive carbonyl stress, acting on the CDS of the SUR2B and inhibiting K\textsubscript{ATP} channel activity. Therefore, the stabilization of miR-9a-3p levels may be a novel strategy for clinical treatment of diabetic vascular complications.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
SS.L., Y.W., X.J., and C.J. conception and design of research; SS.L., Y.W., and X.J. performed experiments; SS.L., Y.W., X.J., and C.J. analyzed data; SS.L., Y.W., and X.J. interpreted results of experiments; SS.L., Y.W., and X.J. prepared figures; SS.L., Y.W., X.J., and C.J. drafted manuscript; SS.L., Y.W., X.J., and C.J. edited and revised manuscript; C.J. approved final version of manuscript.

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