Potassium Channel Dysfunction in Cerebral Arteries of Insulin-Resistant Rats Is Mediated by Reactive Oxygen Species

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Background and Purpose—Insulin resistance (IR) increases the risk of stroke in humans. One possible underlying factor is cerebrovascular dysfunction resulting from altered K+ channel function. Thus, the goal of this study was to examine K+ channel–mediated relaxation in IR cerebral arteries.

Methods—Experiments were performed on pressurized isolated middle cerebral arteries (MCAs) from fructose-fed IR and control rats.

Results—Dilator responses to iloprost, which are BK Ca channel mediated, were reduced in the IR compared with control arteries (19±2% versus 33±2% at 10−6 mol/L). Similarly, relaxation to the KATP opener pinacidil was diminished in the IR MCAs (17±2%) compared with controls (38±2% at 10−3 mol/L). IR also reduced the KATP channel–dependent component in calcitonin gene-related peptide–induced dilation; however, the magnitude of the relaxation remained unchanged in IR because of a nonspecified K+ channel–mediated compensatory mechanism. In contrast, Ks channel–mediated relaxation elicited by increases in extracellular [K+] (4 to 12 mmol/L) was similar in the control and IR arteries. Blockade of the Ks and Kr channels with Ba2+ and 4-aminopyridine, respectively, constricted the MCAs in both experimental groups with no significant difference. Pretreatment of arteries with superoxide dismutase (200 U/mL) plus catalase (150 U/mL) restored the dilatory responses to iloprost and pinacidil in the IR arteries. Immunoblots showed that the expressions of the pore-forming subunits of the examined K+ channels are not altered by IR.

Conclusions—IR induces a type-specific K+ channel dysfunction mediated by reactive oxygen species. The alteration of KATP and BKCa channel–dependent vascular responses may be responsible for the increased risk of cerebrovascular events in IR. (Stroke. 2004;35:●●●●●.)

Key Words: diabetes mellitus • insulin resistance • middle cerebral artery • potassium channels
• oxidative stress • rats

Insulin resistance (IR), a major and growing medical problem in the world, is associated with vascular dysfunction, arterial hypertension, and stroke.1–6 It is estimated that ≈24% of Americans have IR,7 and there is an escalating epidemic of IR in many developing countries, especially Asia and Africa.8 Despite considerable attention given to IR (>18,000 scientific papers and numerous newspaper articles), effects on the cerebral circulation are virtually unexplored. In previous studies, we provided evidence that IR in a fructose-fed rat model led to impairment of endothelium-dependent dilator responses in isolated middle cerebral arteries (MCAs)9 and that it can be related to the dysfunction of smooth muscle K+ channels.10 However, our evidence for impaired K+ channel function was indirect; we did not examine all relevant types of K+ channels; and the mechanisms of K+ channel dysfunction remained to be clarified.

The goal of this study was to directly evaluate K+ channel–mediated vascular responses in isolated MCAs from IR fructose-fed and control rats in response to agonists and inhibitors of the large-conductance Ca2+-activated (BKCa), ATP-dependent (KATP), inward rectifier (Kr), and voltage-dependent (Kv) potassium channels. Because IR-induced oxidative stress is thought to be a major factor leading to vascular dysfunction in the peripheral and coronary circulations11–16 and reactive oxygen species (ROS) were shown to inhibit K+ channel function in the cerebral vasculature,17–19 we also examined whether the altered K+ channel–mediated responses in IR can be restored by treatment of the arteries with ROS scavengers. Finally, we tested the hypothesis that IR alters the expression of and thus the density of the K+ channels in the cerebral arteries, leading to reduced total K+ efflux when widespread channel activation occurs.
Materials and Methods

Animal Preparation
Protocols were approved by the Animal Care and Use Committee at Wake Forest University Health Sciences. Male Sprague-Dawley rats were obtained at 6 weeks of age and randomized into control (n = 46) and IR (n = 48) groups. Animals in the IR group were fed a fructose-rich diet containing (as percentage of total calorie intake) 66% fructose, 22% casein, and 12% lard (Harlan Teklad). Control animals received standard rat chow that contained no fructose, 26% casein, and 14% lard. After 4 weeks, fasted rats were anesthetized with pentobarbital sodium (50 mg/kg IP) and anticoagulated with heparin sodium (500 U IP). After decapitation, the brain was immediately removed and placed in cold, oxygenated modified Krebs-Ringer bicarbonate solution containing (mmol/L) NaCl 119, KCl 4.7, NaHCO3 24, KH 2 PO 4 1.18, MgSO 4 1.17, EDTA 0.026, CaCl2 1.6, and glucose 5.5.

Isolation and Cannulation of the Arteries
These methods have been described previously in detail.9,10 Briefly, a section of the MCA was transferred to a vessel chamber and mounted between 2 glass micropipettes. Oxygenated (20% O2/5% CO2/75% N2; 37°C) Krebs’ solution was continuously circulated through the vessel bath. The lumen of the artery was filled with Krebs’ solution; 1 micropipette was clamped off, and the other was connected to an elevated reservoir to maintain a constant intraluminal pressure of 80 mm Hg. Drugs were added abuminally into the bath solution. Only 1 experiment was performed per artery.

Measurement of Vascular Responses
Concentration-response experiments were performed with the following drugs: iloprost (10^{-6} to 10^{-5} mol/L), calcitonin gene-related peptide (CGRP; 10^{-10} to 3×10^{-8} mol/L), and pinacidil (10^{-8} to 10^{-6} mol/L). Furthermore, responses to increases in K+ concentration in the Krebs’ solution (4 to 12 mmol/L; osmolarity was adjusted with NaCl) were examined. Roles of the different K+ channels were evaluated with the nonselective K+ channel inhibitor tetraethyl ammonium chloride (TEA; 2.5 mmol/L) and with these selective inhibitors of the BK Ca channel, Kir6.1, Kir6.2, and K+ channels: iberiotoxin (0.1 μmol/L), glibenclamide (10 μmol/L), 4-aminopyridine (4-AP; 0.1 to 1 mmol/L), and Ba2+ (20 to 120 μmol/L), respectively. To scavenge ROS, arteries were treated with the combination of superoxide dismutase (SOD; 200 U/mL) and catalase (150 U/mL).

Chemicals
TEA, iberiotoxin, glibenclamide, pinacidil, and 4-AP were purchased from Sigma. CGRP was obtained from American Peptide Inc. Iloprost was purchased from Schering AG. Drugs were dissolved in Krebs’ solution except for pinacidil and glibenclamide, which were dissolved in dimethyl sulfoxide and Krebs’ solution. The same concentration of dimethyl sulfoxide alone had no effect on vessel diameter.

Evaluation of K+ Channel Expression by Immunoblotting
Protein samples of MCAs from control and IR rats were prepared as previously described.20 An equal amount of protein for each sample was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and blocked with 5% skimmed milk powder (for BK Ca and Kir2.1) or 3% bovine serum albumin (for Kir6.1 and 6.2) in Tris-buffered saline containing 0.1% Tween 20. Blots were incubated overnight at 4°C with 1 of the following antibodies: anti-BK Ca (BD Transduction Laboratories, 1:1000 dilution), anti-Kir2.1 (Sigma, 1:500 dilution), anti-Kir6.1, or anti-Kir6.2 (Santa Cruz Biotechnology Inc, 1:500 dilution). The bound antibodies were detected by chemiluminescence.

Results
The maximal intraluminal diameter of the MCAs did not differ between the 2 groups (230±2 μm for control [n = 83] versus 226±1 μm for IR [n = 78]). The spontaneously developed tone was also similar; the MCAs constricted to 69±1% and 71±1% of the initial diameter in the control and IR groups, respectively.

K+ Channel–Mediated Vascular Responses in IR
Iloprost induced concentration-dependent relaxation in both control and IR MCAs; however, responses were significantly reduced in the IR group (Figure 1). Maximum relaxation to iloprost (10^{-6} mol/L) was 33±2% in control MCAs (n = 7) while only 19±2% in the IR arteries (n = 6, P < 0.01). Application of the nonselective TEA or the BK Ca-selective inhibitor iberiotoxin inhibited these responses in both control and IR arteries (Figure 1). Glibenclamide had no effect on the iloprost-induced dilation in either of the 2 experimental groups; maximum relaxations were 32±3% and 20±3% in the control (n = 6) and IR (n = 6) arteries, respectively.
Pinacidil induced dose-dependent relaxations in the MCAs (Figure 2A); however, maximum dilation in the IR arteries (17±2%, n=7) was significantly reduced compared with controls (38±2%, n=6, \(P<0.01\)). Glibenclamide completely inhibited these relaxations in both experimental groups. CGRP-induced dilator responses were comparable between the control and IR arteries (Figure 2B). Maximum relaxations in response to CGRP (10\(^{-8}\) mol/L) were 33±2% in the control (n=9) and 27±2% in IR (n=9) MCAs. Glibenclamide significantly diminished the CGRP-induced dilation in the control arteries, but the blockade of the K\(_{ATP}\) channels had no effect in the IR MCAs (relaxation at 10\(^{-8}\) mol/L, 12±2% in control [n=8] versus 27±3% in IR [n=6] arteries). Other K\(^+\) channel inhibitors (ie, TEA, Ba\(^{2+}\), 4-AP, ouabain) had no effect on the CGRP-induced responses in either the control or IR groups (data not shown); however, an increase in extracellular K\(^+\) level to 50 mmol/L completely inhibited the vascular responses to CGRP in both control and IR arteries (Figure 2B).

Elevations of the K\(^+\) concentration in the Krebs’ solution caused relaxation in the control and IR MCAs with similar dose-response relationships (Figure 3A). For example, the relaxation at 8 mmol/L K\(^+\) concentration was 42±7% in the control (n=10) and 45±9% in the IR (n=8) MCAs. Ba\(^{2+}\) (100 \(\mu\)mol/L) completely inhibited the K\(^+\)-induced responses in both groups. Furthermore, at normal K\(^+\) concentration, Ba\(^{2+}\) induced dose-dependent constrictions in both the control and IR arteries (Figure 3B). For example, constriction to 120 \(\mu\)mol/L Ba\(^{2+}\) was 15±1% in control (n=7) and 20±2% in IR (n=6) MCAs.

4-AP increased the spontaneously developed tone similarly in the control and IR arteries. Constriction to 100, 400, 700, and 1000 \(\mu\)mol/L 4-AP was 3±1% and 4±1%, 7±1% and 6±1%, 9±1% and 9±1%, and 12±1% and 13±2% in the control (n=6) and IR (n=6) MCAs, respectively.

**Mechanisms of K\(^+\) Channel Impairment in IR**

Western immunoblots with antibodies directed against the BKCa, Kir2.1, and Kir6.1 to Kir6.2 subunits revealed that IR had no detectable effect on the expression of these proteins (Figure 4). Pretreatment of the arteries with a combination of SOD and catalase restored the impaired vascular responses to iloprost in the IR MCAs (Figure 5A). For example, relaxation to 10\(^{-6}\) mol/L iloprost was 40±12% (n=6) in control and 46±6% in IR (n=6) arteries. After the same treatment, pinacidil-induced relaxation increased in both the control and IR groups, but this increase was more significant in the IR arteries, resulting in comparable responses in the control and
expression of the pore-forming subunits of the examined K⁺ channel can be restored by scavenging ROS. IR does not influence the relaxation are due to the oxidative stress elicited by IR and the presence of glibenclamide, was similar in the control and IR groups. This was a surprising finding, considering the previously revealed impairment of the KATP channels, but could be explained by a shift to an alternative vascular signaling mechanism in response to chronic attenuation of the KATP channels by IR. This hypothesis is supported by the finding that in contrast to control arteries, glibenclamide treatment had no effect on the CGRP-induced dilation in the IR MCAs. These results could not be attributed to a decreased effectiveness of glibenclamide on KATP channels of the IR arteries because the same treatment completely abolished the pinacidil-induced dilation even in the IR MCAs. On the other hand, a high concentration of K⁺, which sets the equilibrium potential to K⁺ equal to the membrane potential, completely abolished dilator responses to CGRP in both groups, suggesting that the relaxation was mediated by K⁺ channel activation and subsequent hyperpolarization. Yet, none of the other K⁺ channel inhibitors (ie, TEA, Ba²⁺, 4-AP, ouabain) was able to alter CGRP-induced relaxation in the IR MCAs. Thus, although the exact nature of the dilator mechanism mediating responses to CGRP in IR MCAs is not known at this time, these findings indicate a role of a compensating signaling pathway, which involves the opening of a presently undefined K⁺ channel and mediates responses to endogenously present substances such as CGRP but not to selective openers and inhibitors of well-defined K⁺ channels.

We are unaware of previous studies examining Kᵣ and Kᵦ channel–mediated responses in IR arteries despite the fact that these ion channels play an important role in the regulation of vascular function both in the cerebral circulation and in other vascular beds. Our data indicate that unlike the BKCa and KATP channels, Kᵣ and Kᵦ channel–mediated vascular responses are not affected by IR.

Mechanisms of K⁺ Channel Impairment in IR

We considered 2 mechanisms to account for impaired BKCa and KATP channel function in IR. First, we examined whether IR alters the expression of and, as a result, the density of these ion channels in the cerebral arteries, leading to reduced total K⁺ efflux when widespread channel activation occurs. For example, a previous study had shown that expression of BKCa channels is altered in cerebral arteries in pathophysiological conditions. However, using immunoblot analysis, we found that levels of the pore-forming subunits of the BKCa, KATP, and
K<sub>c</sub> channels are not detectably affected by IR. These findings are consistent with the results of a previous study<sup>29</sup> in which the expression of the BK<sub>Ca</sub> subunit in mesenteric arteries was found to be unaffected by IR.

Because increased production of ROS has been reported in various circulatory beds of IR or diabetic humans<sup>13</sup>–15 and animals<sup>11,12,16</sup> and because K<sup>+</sup> channel function is inhibited by ROS in various pathophysiological conditions,<sup>17</sup>–19,30 our second approach was to examine the contribution of ROS to BK<sub>Ca</sub> and K<sub>ATP</sub> channel dysfunction in IR. We found that appplanation of SOD and catalase restored normal dilator responses in the IR MCAs to iloprost, pinacidil, and cro-makalim (not shown). Thus, it seems that continuous production of ROS, probably superoxide anion and/or hydrogen peroxide, exerts a constant inhibitory effect on BK<sub>Ca</sub> and K<sub>ATP</sub> channels in the cerebral arteries of IR rats. The finding that an acute treatment with ROS scavengers is able to restore these dilator responses also suggests that the ROS-induced inhibition is reversible, at least at this stage of IR. This view is supported by patch-clamp studies performed in myocytes from IR mesenteric arteries<sup>23</sup> that revealed that agonist-induced activation of the BK<sub>Ca</sub> channel was normal when the membrane patch was separated from the cell in an inside-out configuration. In the same study, when function of the BK<sub>Ca</sub> channel was examined in a cell-attached configuration, activation of the channel was impaired, implying an inhibitory effect of endogenous cytosolic substances. From our present findings, we suggest that this inhibition of the BK<sub>Ca</sub> and K<sub>ATP</sub> channels is mediated by ROS. Why K<sub>c</sub> and K<sub>k</sub> channels, which have been previously reported to be impaired by ROS or by conditions consistent with ROS formation such as ischemia/reperfusion,<sup>19,30–32</sup> are not similarly affected by IR is unclear but may involve different amounts, production sites, or types of ROS. To the best of our knowledge, our results are the first to show that in addition to the previously described ROS-induced endothelial dysfunction,<sup>11,12,15</sup> ROS produced in response to IR is also able to affect vascular smooth muscle function by inhibiting K<sup>+</sup> channel-mediated hyperpolarization and relaxation.

In summary, we demonstrated the diverse effects of IR on vascular smooth muscle K<sup>+</sup> channels. For example, K<sub>c</sub> and K<sub>k</sub> channels are largely unaffected by IR, and stimuli that activate these channels will exert normal dilator responses. On the other hand, BK<sub>Ca</sub> and K<sub>ATP</sub> channel functions are severely impaired in IR, and dilator responses of the cerebral arteries mediated by these ion channels are reduced. Furthermore, in the case of CGRP, IR may lead to a change in cellular signaling pathways mediating arterial dilation. The implication of these findings is that IR, even in the absence of diabetes, can compromise normal vasodilator function of the cerebral arteries. Thus, the cerebral resistance vessels will not be able to respond normally to a variety of endogenous metabolic stimuli, which could lead to a mismatch between blood flow and metabolic rate. Additionally, reduced BK<sub>Ca</sub> and K<sub>ATP</sub> channel function may prevent appropriate compensatory vascular responses in pathophysiological conditions such as cerebrovascular occlusive disease, hemorrhagic stroke, or changes in blood pressure.

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References

20. Liu Y, Hudetz AG, Knaus HG, Rusch NJ. Increased expression of Ca<sup>2+</sup>-sensitive K<sup>+</sup> channels in the cerebral microcirculation of genetically


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