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

Benedek Erdös, MD; Steve A. Simandle, PhD; James A. Snipes, BS; Allison W. Miller, PharmD; David W. Busija, PhD

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 10−5 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 Kc 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;35:964-969.)

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 (Ks), and voltage-dependent (Kc) 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.

Received November 10, 2003; final revision received December 10, 2003; accepted December 15, 2003.

From the Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, NC (B.E., S.A.S., J.A.S., A.W.M., D.W.B.), and Institute of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary (B.E.).

Correspondence to Benedek Erdös, MD, Department of Physiology and Pharmacology, Wake Forest University Health Sciences, Medical Center Blvd, Winston-Salem, NC 27157-1083. E-mail berdos@wfubmc.edu

© 2004 American Heart Association, Inc.

Stroke is available at http://www.strokeaha.org DOI: 10.1161/01.STR.0000119753.05670.F1
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, KH2PO4 1.18, MgSO4 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. 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^{-8} to 10^{-6} mol/L), calcitonin gene-related peptide (CGRP; 10^{-10} to 3x10^{-8} mol/L), and pinacidil (10^{-6} to 10^{-5} mol/L). Furthermore, responses to increases in K+ concentration in the Krebs’ solution (4 to 12 mmol/L; osmolality 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 BKCa, KATP, K+, and Kir 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. 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 BKCa 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-BKCa (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.
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 KATP 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, Ba²⁺, 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. Ba²⁺ (100 μmol/L) completely inhibited the K⁺-induced responses in both groups. Furthermore, at normal K⁺ concentration, Ba²⁺ induced dose-dependent constrictions in both the control and IR arteries (Figure 3B). For example, constriction to 120 μmol/L Ba²⁺ 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 μmol/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 Kri6.2 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 IR MCAs.
IR MCAs (Figure 5B). For example, relaxation to 10^{-5} \text{ mol/L} \text{ pinacidil was 57} \pm 10\% \ (n=6) \text{ in control and 64} \pm 9\% \text{ in IR (n=6) arteries.}

**Discussion**

The major finding of this study is that IR induces a typespecific K^+ channel dysfunction in the MCAs of fructose-fed rats involving the BK_{Ca} and K_{ATP} channels, whereas the function of the K_{a} and K_{c} channels remains intact. The revealed alterations in BK_{Ca} and K_{ATP} channel–mediated relaxation are due to the oxidative stress elicited by IR and can be restored by scavenging ROS. IR does not influence the expression of the pore-forming subunits of the examined K^+ channels; thus, changes in channel density are unlikely to play a significant role in the reduced K^+ channel–mediated responses.

**Effect of IR on K^+ Channel–Mediated Relaxation**

BK_{Ca} channel dysfunction has been reported previously in mesenteric and coronary arteries of fructose-fed IR rats.^{21–23} We demonstrated in this study that dilator responses to iloprost, a stable prostacyclin analog that acts via opening of BK_{Ca} channels, are also reduced in the MCAs of IR rats compared with controls. This finding supports our earlier observations that the contribution of BK_{Ca} channels in the control of resting vascular tone is reduced and that endothelium-dependent relaxation mediated by a cyclooxygenase-derived substance such as prostacyclin and the activation of the BK_{Ca} channels is impaired in the MCAs of IR rats.^{9,10}

We have previously found that K_{ATP} channel function is altered by IR in the cerebral arteries.^{10} We extended these earlier studies by using the selective K_{ATP} opener pinacidil and CGRP, an endogenous substance released from perivascular nerves that dilates cerebral arteries via opening K_{ATP} channels.^{24} Similar to our previous results with cromakalim,^{10} pinacidil-induced dilator responses were reduced in the MCAs of IR rats compared with controls. However, relaxation to CGRP, which was also mediated by K_{ATP} channels in the control MCAs as indicated by the diminished responses in the presence of glibenclamide, was similar in the control and IR groups. This was a surprising finding, considering the previously revealed impairment of the K_{ATP} channels, but could be explained by a shift to an alternative vascular signaling mechanism in response to chronic attenuation of the K_{ATP} 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 K_{ATP} 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, Ba^{2+}, 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 K_{a} and K_{c} 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.^{25–28} Our data indicate that unlike the BK_{Ca} and K_{ATP} channels, K_{a} and K_{c} channel–mediated vascular responses are not affected by IR.

**Mechanisms of K^+ Channel Impairment in IR**

We considered 2 mechanisms to account for impaired BK_{Ca} and K_{ATP} channel function in IR. First, we examined whether IR alters the expression of and, as a result, the density of these K^+ 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 BK_{Ca} channels is altered in cerebral arteries in pathophysiological conditions.^{20} However, using immunoblot analysis, we found that levels of the pore-forming subunits of the BK_{Ca}, K_{ATP}, and
K⁺ channels are not detectably affected by IR. These findings are consistent with the results of a previous study²⁹ in which the expression of the BKCa 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 humans³⁻⁵ and animals¹¹,¹²,¹⁶ and because K⁺ channel function is inhibited by ROS in various pathophysiological conditions,¹⁷⁻¹⁹,³⁰ our second approach was to examine the contribution of ROS to BKCa and KATP channel dysfunction in IR. We found that coapplication of SOD and catalase restored normal dilator responses in the IR MCAs to iloprost, pinacidil, and cro- 

potassium 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 arteries²³ that revealed that agonist-induced activation of the BKCa 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 BKCa 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 BKCa and KATP channels is mediated by ROS. Why Kᵦ and Kᵥ channels, which have been previously reported to be impaired by ROS or by conditions consistent with ROS formation such as ischemia/reperfusion,¹⁹,³⁰,³¹ 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,¹¹,¹²,¹⁵ ROS produced in response to IR is also able to affect vascular smooth muscle function by inhibiting K⁺ channel–mediated hyperpolarization and relaxation.

In summary, we demonstrated the diverse effects of IR on vascular smooth muscle K⁺ channels. For example, Kᵦ and Kᵥ channels are largely unaffected by IR, and stimuli that activate these channels will exert normal dilator responses. On the other hand, BKCa and KATP 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 BKCa and KATP channel function may prevent appropriate compensatory vascular responses in pathophysiological conditions such as cerebrovascular occlusive disease, hemorrhagic stroke, or changes in blood pressure.

Acknowledgments

This research was supported by NIH grants HL-30260, HL-46558, HL-50587 (D.W.B.); HL-66074, HL-65380 (A.W.M.); AHA MidAtlantic Affiliate Grant 9951272U (D.W.B.), AHA Bugher Foundation Award 0270114N (D.W.B.), and the Hungarian OTKA (T 29169, T 90665, T 97334, T 37885) and ETT 218/2001. Dr Erdős was supported by a Hungarian National Eotvos scholarship.

References

20. Liu Y, Hudetz AG, Knaus HG, Rusch NJ. Increased expression of Ca²⁺-sensitive K⁺ channels in the cerebral microcirculation of genetically


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

Benedek Erdös, Steve A. Simandle, James A. Snipes, Allison W. Miller and David W. Busija

*Stroke*. 2004;35:964-969; originally published online February 19, 2004; doi: 10.1161/01.STR.0000119753.05670.F1

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/35/4/964

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/