

Cerebrovascular Dysfunction in Zucker Obese Rats Is Mediated by Oxidative Stress and Protein Kinase C

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Insulin resistance (IR) impairs vascular function in the peripheral and coronary circulations, but its effects on cerebral arteries are virtually unexplored. We examined the vascular responses of the basilar artery (BA) and its side branches through a cranial window in Zucker lean (ZL) and IR Zucker obese (ZO) rats. Nitric oxide (NO) and K⁺ channel-mediated dilator responses, elicited by acetylcholine, iloprost, cromakalim, and elevated [K⁺], were greatly diminished in the ZO rats compared with ZL rats. In contrast, sodium nitroprusside induced similar relaxations in the two experimental groups. Expressions of the K⁺ channel pore-forming subunits were not affected by IR, while endothelial NO synthase was up-regulated in the ZO arteries compared with ZL arteries. Protein kinase C (PKC) activity and production of superoxide anion were increased in the cerebral arteries of ZO rats, and pretreatment with superoxide dismutase restored all examined dilator responses. In contrast, application of PKC inhibitors improved only receptor-linked NO-mediated relaxation, but not K⁺ channel-dependent responses. Thus, IR induces in ZO rats cerebrovascular dysfunction, which is mediated by oxidative stress and partly by PKC activation. The revealed impairment of NO and K⁺ channel-dependent dilator responses may be responsible for the increased risk of cerebrovascular events and neurodegenerative disorders in IR. *Diabetes* 53:1352–1359, 2004

Obesity is a major and growing health problem in the world, and obese subjects are at high risk for developing insulin resistance (IR) and cardiovascular diseases (1–4). The adverse effects of IR on the peripheral and coronary circulations have been studied extensively, and vascular dysfunction emerges as a major factor in the development of hypertension and coronary artery disease (5–10). In sharp contrast, changes in regulatory mechanisms of the cerebral circulation are virtually unexplored, despite the fact that IR, as well as

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BA, basilar artery; BK_{Ca} channel, large conductance calcium-activated K⁺ channel; CSF, cerebrospinal fluid; eNOS, endothelial nitric oxide synthase; IR, insulin resistance; K_{ATP} channel, ATP-dependent K⁺ channel; K_{ir} channel, inward rectifier K⁺ channel; MCA, middle cerebral artery; PKC, protein kinase C; SNP, sodium nitroprusside; SOD, superoxide dismutase.

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type 2 diabetes, increases the prevalence of cerebrovascular events, and IR patients with stroke are subject to more severe progression, slower recovery, and higher mortality (11–14). In addition, obesity and IR in older people are risk factors for dementia, particularly Alzheimer's disease (15–17), which might be related to cerebrovascular dysfunction and chronic hypoperfusion of the brain (18,19).

In previous studies (20,21), we provided evidence that IR in a fructose-fed rat model led to impairment of dilator responses in isolated middle cerebral arteries (MCAs). However, the *in vitro* nature of these experiments prevented the analysis of the IR-induced mechanisms of cerebrovascular dysfunction. These pathological alterations possibly involve protein kinase C (PKC) activation and production of superoxide anion (O₂^{-•}), which are key factors in the development of endothelial dysfunction and reduced nitric oxide (NO)-mediated relaxation in the peripheral and coronary circulations (8,22–26). In addition, these same mechanisms might also impair K⁺ channel-mediated dilator responses in IR, since they were shown to inhibit K⁺ channel function in other vascular diseases (27–30). Responsiveness of the cerebral arteries can also be affected by pathological stimuli, which result in the downregulation of the endothelial NO synthase (eNOS), as reported in the peripheral circulation of diabetic animals (31), or decreased K⁺ channel density on the vascular smooth muscle cells as a result of reduced expression of the K⁺ channel pore-forming subunits.

Thus, in the present study, we evaluated dilator responses of the basilar artery (BA) and its side branches *in vivo*, with the use of a well-established genetic model for IR, the (*fa/fa*) Zucker obese (ZO) and its control counterpart, the Zucker lean (ZL), rats. We examined NO- and prostacyclin-mediated relaxations and dilation in response to the activation of the large conductance calcium-activated K⁺ (BK_{Ca}) channel, the ATP-dependent K⁺ (K_{ATP}) channel, and the inward rectifier K⁺ (K_{ir}) channel. We examined whether IR alters the expression of eNOS and the pore-forming subunits of the smooth muscle K⁺ channels and assessed PKC activity and the rate of O₂^{-•} production in the cerebral arteries of the ZL and ZO rats. Furthermore, we tested whether treatment with PKC inhibitors or superoxide dismutase (SOD) can restore the impaired cerebrovascular function in ZO rats.

RESEARCH DESIGN AND METHODS

The experimental protocol was approved by the Animal Care and Use Committee at Wake Forest University School of Medicine. Experiments were performed on male 12-week-old ZL (*n* = 49) and ZO (*n* = 51) rats (Harlan, Indianapolis, IN). Animals were fed standard rat diet and drank tap water *ad libitum*. Rats were anesthetized with pentobarbital sodium (70–80 mg/kg *i.p.*),

TABLE 1
Characterization of IR in the ZO rats

Group	Body weight (g)	Blood pressure (mmHg)	Glucose (mg/dl)	Insulin (ng/ml)	Total cholesterol (mg/dl)	Triglycerides (mg/dl)
ZL (<i>n</i> = 8)	304 ± 3	106 ± 1	107 ± 10	0.92 ± 0.14	51 ± 2	32 ± 4
ZO (<i>n</i> = 8)	444 ± 6*	106 ± 4	105 ± 9	11.71 ± 2.04*	79 ± 12*	310 ± 65*

Data are means ± SE. **P* < 0.01, ZO vs. ZL.

supplemented with 10–20 mg · kg⁻¹ · h⁻¹ i.v.) and were ventilated through the trachea with a mixture of room air and O₂. Depth of anesthesia was regularly monitored by applying pressure to a paw. If changes in heart rate or blood pressure were observed, additional pentobarbital was administered. A catheter was placed in a femoral artery to measure systemic arterial blood pressure and to obtain arterial blood samples, while a femoral vein was cannulated for infusion of supplemental anesthetics. Arterial blood gases during the experiments were pH = 7.43 ± 0.01, pCO₂ = 38 ± 1 mmHg, pO₂ = 104 ± 2 mmHg in the ZL rats and pH = 7.42 ± 0.01, pCO₂ = 38 ± 1 mmHg, pO₂ = 101 ± 3 mmHg in the ZO rats.

A ventral craniotomy was performed over the brain stem, as described previously (32), and the cranial window was superfused with artificial cerebrospinal fluid (CSF) at a rate of 3 ml/min. The CSF, which contained (in mmol/l) 2.95 KCl, 132 NaCl, 3.69 dextrose, 1.7 CaCl₂, 0.64 MgCl₂, and 23.2 NaHCO₃, was bubbled with 5% CO₂ in N₂ and maintained at 37–38°C. The gas tensions of the CSF sampled from the cranial window were pH = 7.38 ± 0.01, pCO₂ = 36 ± 1 mmHg, and pO₂ = 98 ± 3 mmHg in both the ZL and ZO groups. Changes in diameter of the BA and its side branches were observed at a sampling rate of 0.5 Hz with a microscope equipped with a charged coupling device (CCD) camera connected to a personal computer (PC) and analyzed using the Scion Image Software (Frederick, MD). Baseline diameters of the BAs and the side branches were similar in the ZL and ZO groups (269 ± 5 and 261 ± 5 μm and 99 ± 4 and 91 ± 4 μm, respectively). At the end of each study, animals were killed with 150–200 mg/kg pentobarbital sodium.

Measurement of vascular responses. Concentration-dependent dilator responses were evaluated in response to acetylcholine, iloprost, cromakalim, sodium nitroprusside (SNP) and to elevations of the extracellular K⁺ concentration. In some experiments the following inhibitors were used: N^ω-nitro-L-arginine methyl ester (L-NAME, 10 μmol/l) for the inhibition of NO synthesis and iberiotoxin (0.1 μmol/l), glibenclamide (10 μmol/l), and Ba²⁺ (100 μmol/l) to inhibit BK_{Ca}, K_{ATP}, and K_{ir} channel activation, respectively. SOD (150 units/ml) was used to scavenge O₂⁻, and Ro31-8220 (5 μmol/l) or chelerythrine (1 μmol/l) was used to inhibit PKC. All drugs were applied topically; treatment with inhibitors or SOD was started 30 min before the measurements and continued throughout the experiments.

Western immunoblotting. Protein samples of cerebral arteries (BAs and MCAs) from ZL and ZO rats were prepared as previously described (33). An equal amount of protein for each sample was separated by 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and blocked with 5% skimmed milk powder for eNOS, BK_{Ca}, Kir2.1, phospho-PKC (pan), and β-actin or with 3% BSA for Kir6.1 and 6.2 in Tris-buffered saline containing 0.1% Tween 20. Blots were incubated overnight at 4°C with one of the following antibodies: anti-eNOS (BD Transduction Laboratories, 1:2,500), anti-BK_{Ca} (BD Transduction Laboratories, 1:1,000), anti-Kir2.1 (Sigma, 1:500), anti-Kir6.1 and anti-Kir6.2 (Santa Cruz Biotechnology, 1:500), anti-phospho-PKC (pan) (Cell Signaling Tech, 1:1,200), and anti-β-actin (Sigma, 1:2,500). The bound antibodies were detected by chemiluminescence.

Detection of O₂⁻ production. At first, ~2-mm-long sections of the BAs from ZL and ZO rats were incubated at 37°C in PBS for 30 min and then for another 20 min in the presence of the O₂⁻-sensitive dye hydroethidine (5 μmol/l; Molecular Probes, Eugene, OR). Arteries were then rinsed in ice-cold PBS and transferred to the microscope stage in a chambered coverglass. For better visualization and to prevent movement, a coverslip was placed on top of the arteries. Fluorescent images were recorded by scanning the entire vascular wall from the adventitia to the endothelium in 2-μm-thick sections using a Zeiss LSM-510 laser scanning confocal system (Zeiss C-Apochromat 63×/NA 1.2 water-immersion objective, excitation λ = 488 nm, emission λ > 560 nm). In addition, O₂⁻ production was also measured with lucigenin-enhanced chemiluminescence assay. Cerebral arteries from ZL and ZO rats were dissected simultaneously and placed in a luminometer (BMG Fluostar Optima) in 37°C PBS. Scintillation counts were obtained for 20 min in the presence of lucigenin (5 μmol/l), and background-corrected values were normalized to protein content.

Characterization of IR in the ZO rats. In some experiments, systemic arterial blood pressure was measured, and blood samples were taken after 12 h of fasting from awake rats through an implanted femoral artery catheter.

Plasma insulin and glucose levels were measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Crystal Chem, Chicago, IL) and Trinder reagent (Sigma, St. Louis, MO), respectively. Triglyceride and total cholesterol were measured by an automatic analyzer (Technicon RA-1000).

Chemicals. Acetylcholine, SNP, L-NAME, iberiotoxin, SOD, Ro31-8220, and lucigenin were purchased from Sigma. Cromakalim was obtained from Toocris (Ellisville, MO), chelerythrine from Calbiochem (San Diego, CA), and iloprost from Schering (Berlin, Germany). Drugs were dissolved in CSF, except for cromakalim and glibenclamide, which were dissolved in DMSO and CSF. The same concentration of DMSO alone had no effect on vessel diameter.

Statistical analysis. All data are expressed as means ± SE. The magnitudes of vascular responses were calculated as percentage of baseline diameter, which was measured for 1 min immediately before applying drugs. Effects of treatments and differences between ZL and ZO rats were evaluated using ANOVA, followed by Tukey's post hoc test. The criterion for significance was *P* < 0.05.

RESULTS

Characterization of IR in the ZO rats. At 12 weeks of age, ZO rats were significantly heavier than ZL rats. Mean arterial blood pressure and fasting glucose levels were similar in the ZL and ZO rats, while insulin was one magnitude higher in the ZO rats compared with ZL rats. Total cholesterol and triglyceride levels were also significantly elevated in the ZO group (Table 1).

Impaired dilator responses in the ZO rats. Acetylcholine-induced dose-dependent relaxations in the BAs were significantly reduced in the ZO rats compared with ZLs (Fig. 1A). These dilator responses were markedly inhibited by L-NAME in the ZL group (relaxation at 10⁻⁵ mol/l was 6 ± 2%, *n* = 6, *P* < 0.01 vs. untreated), while the already reduced responses in the ZO rats did not change noticeably (relaxation at 10⁻⁵ mol/l was 7 ± 1%, *n* = 6, NS). In contrast to acetylcholine, SNP-induced dilations were comparable in the BAs of ZL and ZO rats (at 10⁻⁸ mol/l: 14 ± 2 and 8 ± 2%; at 10⁻⁷ mol/l: 42 ± 3 and 33 ± 5%; at 10⁻⁶ mol/l: 52 ± 2 and 43 ± 9% for the ZL [*n* = 7] and ZO [*n* = 6] groups, respectively). Although these responses tended to be smaller in the ZO BAs, differences were not statistically significant.

Iloprost-induced dilation was reduced in the BAs of the ZO rats (Fig. 2A). Blockade of the BK_{Ca} channels with iberiotoxin inhibited these responses in the ZL BAs but was ineffective in the ZO arteries (relaxation at 10⁻⁶ mol/l was 9 ± 2% [*n* = 5, *P* < 0.01 vs. untreated] and 8 ± 3% [*n* = 5, NS] in the ZL and ZO rats, respectively). Similar to iloprost, relaxation to cromakalim was also reduced in the BAs of the ZO rats (Fig. 3A), while blockade of the K_{ATP} channels with glibenclamide inhibited dilator responses in both the ZL and ZO groups (relaxation at 10⁻⁶ mol/l was 6 ± 2 and 3 ± 2%, respectively [*n* = 5, *P* < 0.01 vs. untreated]). Increases in the extracellular K⁺ level induced dilation that was significantly diminished in the ZO rats compared with ZLs (Fig. 4A). These responses were attenuated by Ba²⁺. For example, relaxation to 15 mmol/l K⁺ was 19 ± 4% in the ZL (*n* = 5, *P* < 0.01 vs. untreated) and 8 ± 3% in the ZO (*n* = 5, *P* < 0.01 vs. untreated) rats.

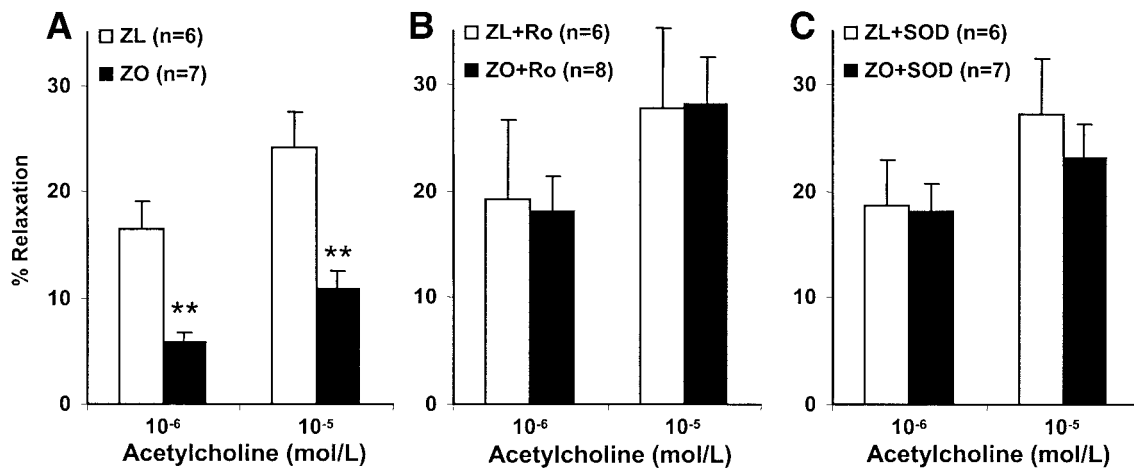


FIG. 1. Responses to acetylcholine in the BAs of naïve (A), Ro31-8220-treated (B), and SOD-treated (C) rats. Relaxation was impaired in the ZO rats compared with ZL rats; however PKC inhibition and elimination of superoxide restored responses to normal. ** $P < 0.01$ ZO vs. ZL.

Data obtained from the side branches of the BA indicated a similar vascular impairment. Although the percent dilator responses were generally higher in these arteries compared with the BAs, relaxation to acetylcholine, iloprost, cromakalim, and K^+ were significantly reduced in the ZO rats (Table 2). Dilation to SNP, however, was similar in the ZL and ZO groups (at 10^{-8} mol/l: 14 ± 2 and $10 \pm 2\%$; at 10^{-7} mol/l: 57 ± 5 and $47 \pm 5\%$; at 10^{-6} mol/l: 81 ± 4 and $74 \pm 4\%$ for the ZL [$n = 8$] and ZO [$n = 6$] groups, respectively).

Expression of eNOS and the K^+ channel pore-forming subunits. Western blots with an eNOS-specific antibody revealed that despite the reduced NO-mediated responses, eNOS expression is increased in cerebral arteries from ZO rats compared with ZL rats (Fig. 5A). The densities of the eNOS immunoreactive bands normalized to β -actin were 1.7-fold higher in the ZO group ($n = 7$) compared with ZL ($n = 7$, $P < 0.05$). In contrast, expressions of the K^+ channel pore-forming subunits were similar in the cerebral arteries of ZL and ZO rats (Fig. 5B).

Role of PKC activation in the IR-induced vascular dysfunction. PKC activity in the cerebral arteries of the ZL and ZO rats was evaluated with Western blotting us-

ing an antibody, which detects PKC α , β_1 , β_2 , δ , ϵ , and η isoforms when phosphorylated at a COOH-terminal residue homologous to Ser660 of PKC β_2 . The autophosphorylation of this amino acid at the hydrophobic site indicates enzyme activation (34). We found, as shown on Fig. 5C, that densities of the phospho-PKC-specific immunoreactive bands normalized to β -actin were 1.6-fold higher in the ZO group ($n = 6$) compared with the ZL group ($n = 6$, $P < 0.05$). Furthermore, in the in vivo experiments, topical application of the PKC inhibitor Ro31-8220 restored dilator responses to acetylcholine in the ZO BAs (Fig. 1B). In contrast, the differences in responses to iloprost (Fig. 2B) and cromakalim (Fig. 3B) between the ZL and ZO rats were not abolished by this treatment. Although the K^+ -induced dilation was increased to some extent in the ZO rats, responses in the ZL rats were augmented even more in the presence of Ro31-8220. Thus, the differences between the two groups became even larger (Fig. 4B). Similar results were obtained with a structurally different PKC inhibitor, chelerythrine. Acetylcholine-induced responses were restored in the ZO rats (relaxation at 10^{-5} mol/l was $24 \pm 5\%$ in the ZL [$n = 6$] and $20 \pm 2\%$ in the ZO rats [$n = 6$]), while K^+ channel-mediated responses were

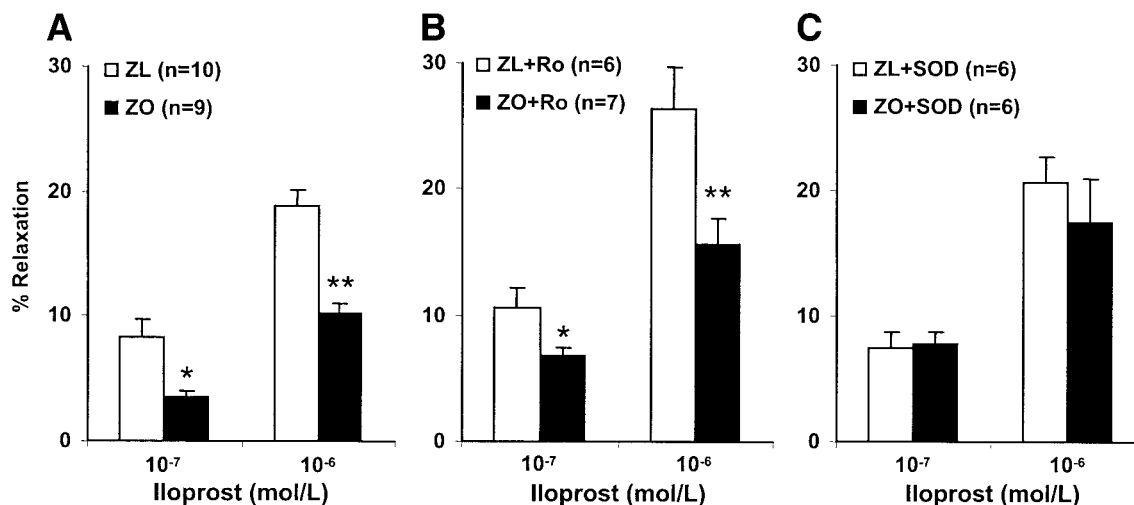


FIG. 2. Responses to iloprost in the BAs of naïve (A), Ro31-8220-treated (B), and SOD-treated (C) rats. Relaxation was impaired in the ZO rats compared with ZL rats. PKC inhibition did not reduce the difference between the two groups; however, SOD treatment restored relaxation to normal. * $P < 0.05$, ** $P < 0.01$ ZO vs. ZL.

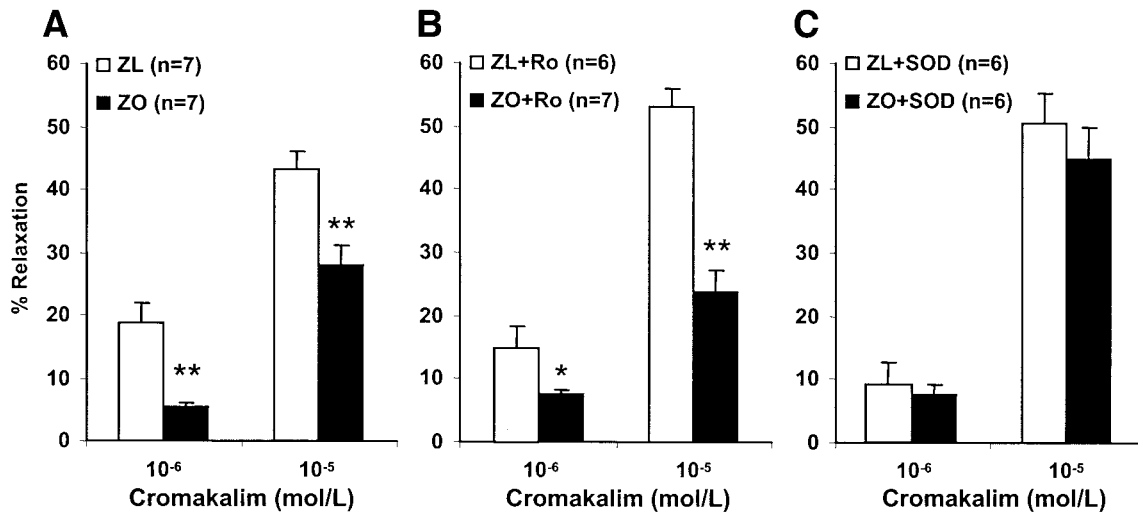


FIG. 3. Responses to cromakalim in the BAs of naïve (A), Ro31-8220-treated (B), and SOD-treated (C) rats. Relaxation was impaired in the ZO rats compared with ZL rats. PKC inhibition did not improve dilation in the ZO BAs; however, SOD treatment restored relaxation to normal. * $P < 0.05$, ** $P < 0.01$ ZO vs. ZL.

unaltered by this treatment. Relaxation to 10^{-6} mol/l iloprost was $20 \pm 4\%$ in the ZL ($n = 8$) and $13 \pm 1\%$ in the ZO rats ($n = 6$, $P < 0.05$). Relaxation to 10^{-5} mol/l cromakalim was $40 \pm 1\%$ in the ZL ($n = 6$) and $29 \pm 5\%$ in the ZO rats ($n = 6$, $P < 0.05$), while K^+ -induced dilation (at 15 mmol/l) was $48 \pm 3\%$ in the ZL ($n = 6$) and $34 \pm 6\%$ in the ZO rats ($n = 7$, $P < 0.05$). PKC inhibition either with Ro31-8220 or chelerythrine had a similar effect on the dilator responses of the side branches (Table 2).

Role of O_2^{-*} in the IR-induced vascular dysfunction. O_2^{-*} production was first assessed with the fluorescent dye hydroethidine and confocal microscopy. As shown on Fig. 6, fluorescent intensity in the ZO BAs compared with ZLs was markedly elevated in all layers of the vascular wall, approximately by $123 \pm 12\%$ in the endothelium, by $162 \pm 20\%$ in the media, and by $154 \pm 18\%$ in the adventitia ($n = 6$, $P < 0.01$). Increased O_2^{-*} production was indicated also by the lucigenin-enhanced chemiluminescence assays; scintillation counts were 144 ± 26 and 671 ± 43

$\text{min}^{-1} \cdot \text{mg protein}^{-1}$ in the cerebral artery preparations from ZL and ZO rats, respectively ($n = 6$, $P < 0.01$).

In addition, the SOD treatment greatly enhanced the reduced dilation in the ZO rats in response to acetylcholine (Fig. 1C), iloprost (Fig. 2C), and cromakalim (Fig. 3C). K^+ -induced responses were augmented in both the ZL and ZO BAs, but the increase was more significant in the ZO rats, so the differences diminished between the two groups (Fig. 4C). Data obtained from the side branches showed a similar improvement in the vascular function after SOD application (Table 2).

DISCUSSION

The major findings of this study are as follows: first, relaxations of the BA and its side branches are severely impaired in response to both endothelial NO-mediated and smooth muscle K^+ channel-dependent dilators in the ZO rats; in contrast, dilation to exogenous NO remained

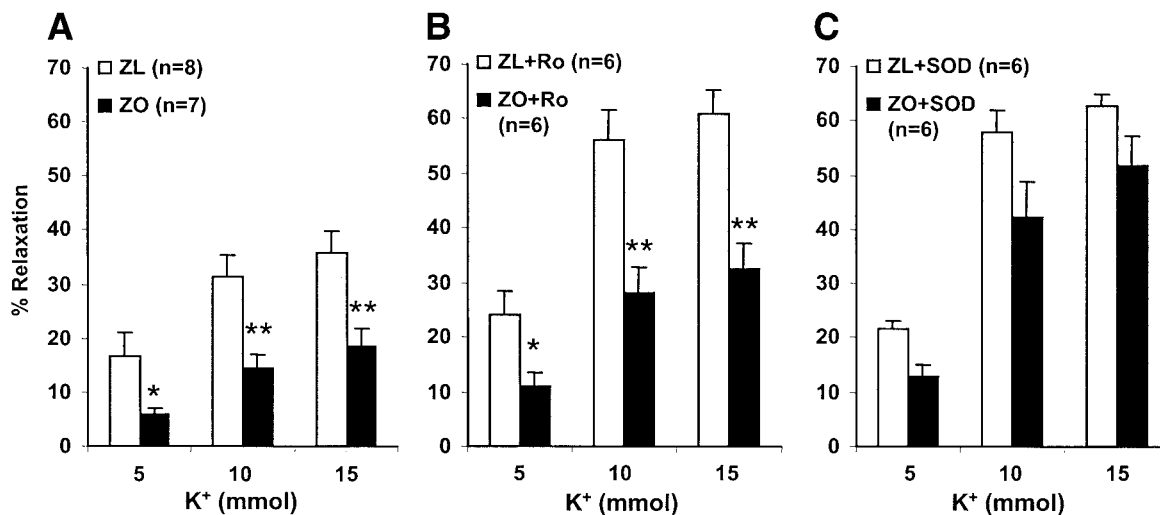


FIG. 4. Responses to increases in the extracellular K^+ concentration in the BAs of naïve (A), Ro31-8220-treated (B), and SOD-treated (C) rats. Relaxation was impaired in the ZO rats compared with ZL rats. Ro31-8220 augmented the dilator responses in the ZO BAs to some extent; however, this effect was more pronounced in the ZL arteries. Thus, differences between the two groups became even more significant. In contrast, relaxations of both ZL and ZO BAs were enhanced in the presence of SOD in a way that the differences diminished between the ZL and ZO groups. * $P < 0.05$, ** $P < 0.01$ ZO vs. ZL.

TABLE 2
Percent relaxation of the BA side branches

	Native		Ro31-8220		Chelerythrine		SOD	
	ZL	ZO	ZL	ZO	ZL	ZO	ZL	ZO
Acetylcholine (10^{-5} mol/l)	39 ± 5 (n = 10)	9 ± 2* (n = 7)	33 ± 4 (n = 8)	26 ± 3 (n = 7)	35 ± 6 (n = 9)	24 ± 2 (n = 9)	32 ± 5 (n = 7)	26 ± 4 (n = 9)
Iloprost (10^{-6} mol/l)	37 ± 4 (n = 13)	21 ± 3† (n = 9)	32 ± 3 (n = 6)	22 ± 2† (n = 6)	33 ± 4 (n = 10)	19 ± 4† (n = 6)	33 ± 3 (n = 8)	31 ± 6 (n = 9)
Cromakalim (10^{-5} mol/l)	72 ± 6 (n = 11)	31 ± 7* (n = 6)	58 ± 12 (n = 6)	25 ± 4† (n = 7)	50 ± 4 (n = 10)	27 ± 6† (n = 6)	74 ± 7 (n = 8)	63 ± 5 (n = 8)
K ⁺ (15 mmol/l)	74 ± 2 (n = 9)	42 ± 4* (n = 7)	78 ± 2 (n = 6)	44 ± 4* (n = 6)	67 ± 4 (n = 10)	43 ± 7† (n = 9)	77 ± 3 (n = 8)	60 ± 6 (n = 9)

Data are means ± SE. *P < 0.01, ZO vs. ZL; †P < 0.05, ZO vs. ZL.

substantially intact. Second, eNOS is upregulated in the cerebral arteries of ZO rats compared with ZL rats in spite of the reduced NO-dependent responsiveness, while expression of the K⁺ channel pore-forming subunits are similar in the ZL and ZO groups. Third, PKC activity and O₂^{-*} production are increased in the cerebral arteries of the ZO rats, and inhibition of PKC restores dilator responses to acetylcholine but not to K⁺ channel-dependent dilators. Topical treatment with SOD, however, restores both endothelium-dependent and K⁺ channel-mediated dilations.

NO-mediated relaxation in IR. NO-mediated dilation has been studied previously in various circulatory beds of IR and type 2 diabetic animals and humans, and while the majority of these studies have reported reduced NO-mediated responsiveness (3,8,22–24,35,36), others have found that this regulatory mechanism remained intact in IR (37,38). Our previous in vitro observations on isolated MCAs have suggested that NO-mediated dilation remained unchanged in fructose-fed IR rats (20). However, experiments from the present study showed that in the ZO rats, endothelial NO-mediated relaxation to acetylcholine was severely impaired compared with ZL rats. This discrepancy between our in vitro and in vivo results indicates that severity of IR (ZO rats are in a more progressed state of IR compared with fructose-fed rats) or factors that are present in vivo, but not in a controlled environment of an in vitro experimental setup (e.g., fluctuating serum levels of insulin, glucose, and lipids), may have a significant effect on NO-mediated vascular responses. In contrast to acetylcholine, SNP elicited comparable relaxations in the ZL and ZO rats, indicating that responsiveness of the vascular smooth muscle cells to NO is not affected significantly. Thus, diminished NO-dependent responses are presumably due to decreased production or increased inactivation of NO.

One possible mechanism for the reduced NO biosynthesis would be a decreased expression of eNOS. However, we found that despite the diminished relaxation to acetylcholine, paradoxically, eNOS is upregulated in the cerebral arteries. Such an increase in eNOS expression indicates the presence of a compensatory mechanism, which would attempt to maintain normal NO level by upregulating eNOS and which is probably induced by oxidative stress (39) or PKC activation (40). Furthermore, increased expression of eNOS can also be the result of the marked hyperinsulinemia observed in the ZO rats. Insulin induces eNOS expression in endothelial cells (41), and if hyperinsulinemia overcomes the reduced sensitivity to insulin in the ZO rats, it might induce the upregulation of eNOS (42). Similar compensatory eNOS upregulation has also been described in other models of vascular dysfunction, but in accordance with our findings, it usually represents only a futile compensatory mechanism that goes along with reduced synthesis of NO and increased production of O₂^{-*} due to the uncoupling of the eNOS enzyme in response to PKC activation, oxidative stress, or decreased availability of the tetra-hydro-biopterin cofactor of the enzyme (24,26,31). This alteration in eNOS function, combined with the observed upregulation of the enzyme, may lead to a significant increase in O₂^{-*} release from the endothelium of cerebral arteries, leading to further deterioration of

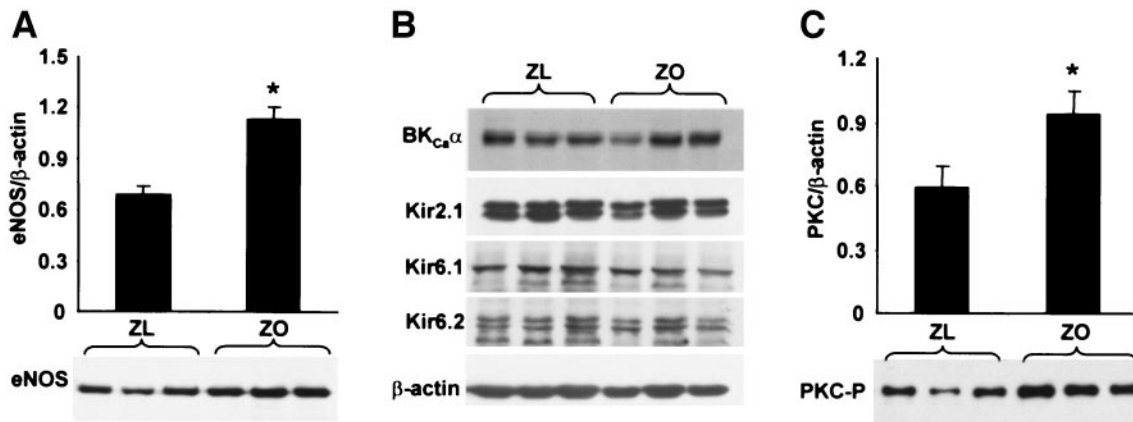


FIG. 5. Immunoblot analysis of eNOS expression in isolated cerebral arteries revealed that eNOS is upregulated in the ZO rats (A). Western blots using antibodies directed against the pore-forming subunits of the examined K^+ channels ($BK_{Ca}\alpha$ for the BK_{Ca} channel, Kir2.1 for the K_{ir} channel, and Kir6.1 and Kir6.2 subunits for the K_{ATP} channel) revealed that IR did not influence the expression of these proteins (B), while immunoblots with a phospho-PKC-specific antibody demonstrated that PKC activation is increased in the ZO rats compared with ZL rats (C). Each lane was loaded with an equal amount of protein, and densities of the immunoreactive bands were normalized to β -actin. * $P < 0.05$ ZO vs. ZL.

vascular function by, for example, scavenging NO and forming peroxynitrate. However, in addition to eNOS, excess O_2^{-*} can be produced by other enzymes like NAD(P)H-oxidase, which can be induced by PKC activation (25,27). Since increased PKC activation and O_2^{-*} production play such a multifaceted role in the regulation of NO signaling, we examined the contribution of these mechanisms to the revealed impairment of cerebrovascular responsiveness. Our findings confirmed that both PKC activation and O_2^{-*} production are indeed elevated in the BAs of ZO rats, and showed that acute, topical treatment with PKC inhibitors or SOD restored NO-mediated relaxation to acetylcholine.

K^+ channel-mediated relaxations in IR. Activation of K^+ channels on the vascular smooth muscle cells, with concomitant hyperpolarization and relaxation, is an important regulatory pathway in the cerebral circulation. BK_{Ca} and K_{ATP} channels are key targets of various vasoactive substances released either from the endothelium or from perivascular nerve endings, while K_{ir} channels are activated by increases in the extracellular K^+ concentra-

tion and involved in coupling cerebral blood flow to neuronal metabolism (43).

Previous *in vitro* studies have indicated that IR has an adverse effect on the function of the BK_{Ca} and K_{ATP} channels in the mesenteric and coronary arteries (38,44,45), and we obtained similar results in isolated MCAs of IR rats (21). Thus, in the present study, we examined dilator responses elicited by the activation of the BK_{Ca} , K_{ATP} , and K_{ir} channels.

We tested the BK_{Ca} channel-mediated responses with iloprost and found that relaxation to this prostacyclin analog is significantly diminished in the ZO rats compared with ZL rats. Since iberiotoxin inhibits these dilations in ZL rats, but has no effect in the ZO rats, these results indicate that the BK_{Ca} channel-mediated relaxation in response to iloprost is completely lost in IR and support our previous findings (21) that endothelium-dependent, cyclooxygenase- and prostacyclin-mediated dilation is impaired because of the dysfunction of this K^+ channel subtype. Relaxations to activation of the K_{ATP} channel by cromakalim and K_{ir} channels by increases in extracellular

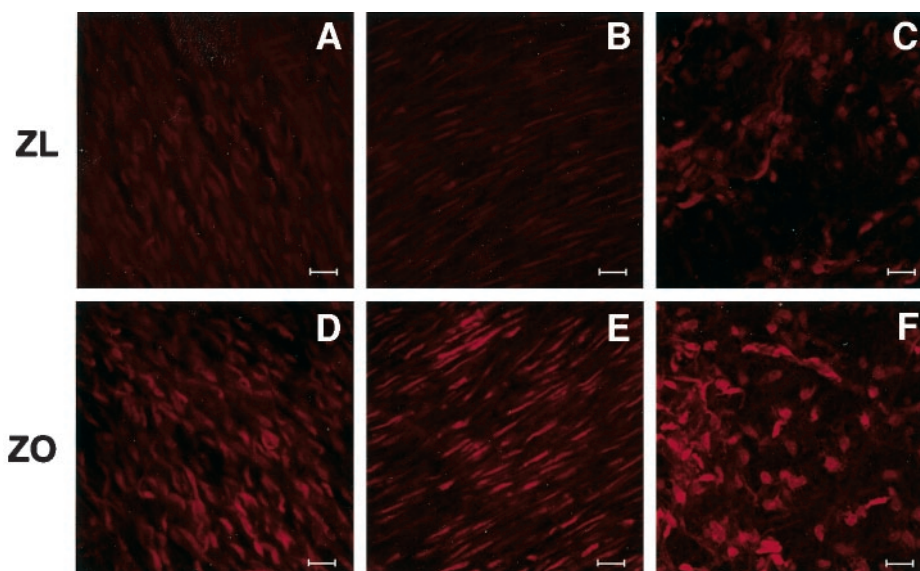


FIG. 6. Confocal microscopy images of BAs from ZL (upper row) and ZO rats (bottom row) after a 20-min incubation with the superoxide-sensitive dye hydroethidine. Fluorescent intensity was markedly increased in all layers of the ZO BAs, indicating O_2^{-*} overproduction in the endothelium (A), media (B), and adventitia (C) of the ZO arteries compared with ZLs. Bars in the right bottom corners of the images represent 20 μ m.

K^+ were also compromised in the ZO rats compared with ZL rats; however, the function of these channels is not completely lost in the ZO rats, as indicated by the finding that glibenclamide and Ba^{2+} inhibited the cromakalim- and K^+ -induced responses even in the ZO rats.

We considered three mechanisms to account for impaired K^+ 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. Using immunoblot analysis we found that levels of the $BK_{Ca}\alpha$, Kir6.1-6.2, and Kir2.1 proteins (the pore-forming subunits of the BK_{Ca} , K_{ATP} , and K_{ir} -channels, respectively) are not detectably affected by IR. These findings are consistent with the results of a previous study (46) in which the expression of the $BK_{Ca}\alpha$ subunit in mesenteric arteries was found to be unaffected by IR, although the same channel subunit has been found to be upregulated in other vascular disease states (33).

Next, we examined whether increased PKC activation is involved in the IR-induced K^+ channel dysfunction. We expected the involvement of PKC, because PKC activation has been shown to alter K^+ channel-mediated vascular responses in the cerebral arteries either by phosphorylation of certain regulatory sites of the channels or by inducing O_2^{-*} production (27,47–49). However, with topical application of PKC inhibitors, we were unable to abolish the differences in K^+ channel-mediated relaxation between the ZL and ZO rats. Although iloprost- and K^+ -induced dilations were augmented somewhat in the ZO rats by this treatment, similar or even more significant increases were seen in the ZL group. Thus, it seems that while PKC may indeed be involved in the regulation of these ion channels, increased PKC activity is not the major cause of reduced responsiveness in the IR arteries. One explanation for these findings might be that inhibition of PKC alone is not sufficient to restore vascular function, because the dominant inhibitory effect of O_2^{-*} prevents any improvement elicited by this treatment, and O_2^{-*} production in the smooth muscle cells is not mediated by PKC. Furthermore, Ro31-8220 and chelerythrine are not isoform-specific PKC inhibitors. Therefore, the failure of these drugs to improve K^+ channel-mediated relaxation might be due to opposing effects of the different PKC isoforms.

Since K^+ channel function has been shown to be inhibited by O_2^{-*} in various pathological conditions (29,30,50) and our experiments revealed that O_2^{-*} production is increased not only in the endothelium, but even in the smooth muscle cells, our third approach was to examine the contribution of O_2^{-*} to K^+ channel dysfunction in IR. We found that acute, topical treatment with SOD restored normal dilator responses to iloprost, cromakalim, and K^+ in the ZO rats, which indicates that production of O_2^{-*} exerts a constant inhibitory effect on BK_{Ca} , K_{ATP} , and K_{ir} channels in the cerebral arteries of ZO rats, and that the O_2^{-*} -induced inhibition is reversible, at least at this stage of IR. This view is supported by patch-clamp studies, which were performed in myocytes from IR mesenteric arteries (45), and revealed that agonist-induced activation of the BK_{Ca} channel was normal when the membrane patch was separated from the cell in an inside-out config-

uration but was impaired when examined in a cell-attached configuration, indicating the presence of endogenous inhibitory substances. Based on our present findings, we suggest that this intracellular inhibitor substance is O_2^{-*} and that it inhibits not only the BK_{Ca} but the K_{ATP} and K_{ir} channels as well.

Summary. We have shown for the first time in the in vivo cerebral circulation that IR causes widespread reductions of dilator responses in arteries of different sizes and that the underlying mechanisms of impairment involve cell-type selective actions of O_2^{-*} and PKC. Endothelial NO-dependent dilation to acetylcholine is severely reduced in IR despite augmented eNOS levels and could be restored by PKC inhibitors or SOD. Relaxation to smooth muscle K^+ channel activators is also markedly decreased in IR and could be restored by SOD but not by PKC inhibitors. In addition, we have shown that expressions of the pore-forming K^+ channel subunits are unchanged in IR, while O_2^{-*} production and PKC activity are increased in cerebral arteries from ZO rats.

The implication of these findings is that IR, even in the absence of diabetes, is able to compromise 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. The resulting chronic hypoperfusion of the brain might contribute to the development of general dementia and Alzheimer's disease in particular. Additionally, the reduced dilator ability of the cerebral arteries may increase neurological consequences and mortality due to occlusive events and hemorrhagic strokes by preventing appropriate compensatory responses in collateral arteries.

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