Early Atorvastatin Reduces Hemorrhage after Acute Cerebral Ischemia in Diabetic Rats

Hazem F. Elewa, Anna Kozak, Azza B. El-Remessy, Reginald F. Frye, Maribeth H. Johnson, Advive Ergul, and Susan C. Fagan

Program in Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Augusta, Georgia (H.F.E., A.K., A.B.E., A.E., S.C.F.); Charlie Norwood Veterans Administration Medical Center, Augusta, Georgia (H.F.E., A.K., A.B.E., A.E., S.C.F.); College of Pharmacy, University of Florida, Gainesville, Florida (R.F.F.); and Departments of Biostatistics (M.H.J.), Physiology (A.E.), and Neurology (S.C.F.), Medical College of Georgia, Augusta, Georgia

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ABSTRACT

Ischemic stroke is a leading cause of death in the United States, and diabetes mellitus is a major risk factor for stroke. Our previous work showed that type II diabetic rats [Goto-Kakizaki (GK)] have more bleeding after stroke than their normoglycemic controls (Wistar). Our aim was to evaluate the vascular protective properties of acute atorvastatin therapy after experimental ischemic stroke in diabetes and to explore the effect of stroke in GK rats compared with their normoglycemic controls. Fifty male Wistar and 40 GK rats (270–305 g) underwent 3 h of middle cerebral artery occlusion followed by reperfusion for 21 h. Animals received atorvastatin (5 mg/kg), atorvastatin (15 mg/kg), or vehicle, administered by oral gavage, one dose 5 min after reperfusion and a second dose after 12 h. At 24 h, functional outcome was measured, and brain

tissue was analyzed for infarct volume, hemoglobin content, and molecular biomarkers. Plasma was collected for analysis of atorvastatin concentrations. Atorvastatin-treated groups had significantly lower bleeding rates ($p = 0.0011$) and infarct volume ($p = 0.0007$) compared with controls. There was a significant reduction in hemoglobin content and infarct volume only in the higher dose group (15 mg/kg) ($p < 0.05$), and these benefits were more than 4 times greater in the diabetic animals. Atorvastatin (15 mg/kg) improved neurological outcome in both Wistar and GK rats ($p = 0.029$) at a peak concentration of 27 to 77 ng/ml and was associated with an increase in Akt phosphorylation ($p = 0.0007$). We concluded that atorvastatin is a vascular protective agent after experimental ischemic stroke, especially in diabetes.

Stroke is the third leading cause of death in the United States and affects approximately 750,000 individuals each year (Idris et al., 2006). Type II diabetes mellitus is a disease that affects more than 17 million Americans. In diabetic patients, the risk of stroke is increased by 2- to 4-fold (Folsom et al., 1999; Idris et al., 2006; Martini and Kent, 2007). Not only is there an increase in the risk of stroke recurrence in diabetics but also these patients have increased hospital and long-term stroke mortality and a worsening of overall stroke

outcomes (Folsom et al., 1999; Idris et al., 2006). One of the contributing factors to this dramatic prognosis is that hyperglycemia is associated with intracerebral hemorrhage in acute ischemic stroke patients treated with thrombolytic agents (Martini and Kent, 2007), and this has been confirmed in animal models (Kawai et al., 1997; Quast et al., 1997). In addition, many of the detrimental pathways involved in vascular damage associated with stroke are known to be up-regulated in the diabetic vasculature, including oxidative stress, endothelial dysfunction, and inflammation (Fagan et al., 2005; Martini and Kent, 2007). A vascular protective agent, administered after stroke onset, may improve the safety and efficacy of thrombolytic therapy in diabetic patients. We have identified statins as possible vascular protective agents in the acute stroke period (Fagan et al., 2004), and statins are routinely prescribed for lipid lowering in these patients. Statins also have other pleiotropic effects, including reduction of oxidative stress, improvement of the

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ABBREVIATIONS: GK, Goto-Kakizaki; MCAO, middle cerebral artery occlusion; MCA, middle cerebral artery; eNOS, endothelial nitric-oxide synthase; p, phosphorylated; BG, blood glucose; MAP, mean arterial blood pressure; ANOVA, analysis of variance; BP, blood pressure; ROS, reactive oxygen species.

endothelial function, increase in NO bioavailability, and inhibition of the inflammatory and cell death mediators (Liao, 2002; Endres, 2005; Liao and Laufs, 2005). Many studies have demonstrated that statins reduce stroke risk in both primary and secondary prevention trials (4S, 1994; WOSCOPS, 1998; Plehn et al., 1999; Sever et al., 2003; CARDS et al., 2004; Amarenco et al., 2006). Statins have also been shown to be neuroprotective when administered acutely after experimental stroke (Sironi et al., 2003; Kilic et al., 2005); yet, poststroke effects of statins in diabetes have not been evaluated. Recent data from our group demonstrated that type II diabetic rats [Goto-Kakizaki (GK)] are more susceptible to vascular damage after experimental cerebral ischemia than nondiabetic rats (Wistar) (Ergul et al., 2007) and may serve as a good model to test potential vascular protective agents after stroke in diabetes. In this study, we evaluated whether acute atorvastatin therapy is vascular protective in GK rats after experimental ischemic stroke. A set of normoglycemic rats (Wistar) was used to confirm previous reports showing postischemic neuroprotective effects of statins (Sironi et al., 2003; Kilic et al., 2005; Zhang et al., 2005). We also explored the effect of stroke in the diabetic GK rats compared with their normoglycemic controls.

Materials and Methods

The Institutional Animal Care and Use Committee of the Augusta VA Medical Center (August, GA) approved the protocol. Male Wistar rats ($n = 50$; Charles River Laboratories, Inc., Wilmington, MA) and GK rats ($n = 40$; Taconic Farms, Germantown, NY), within a narrow range of body weight (270–305 g), were used.

Experimental Cerebral Ischemia

Anesthesia was performed by using 2% isoflurane via inhalation. Cerebral ischemia was induced using the intraluminal suture middle cerebral artery occlusion (MCAO) model (Longa et al., 1989). Nineteen- to 21-mm 3-0 surgical nylon filament was introduced from the external carotid artery lumen into the internal carotid artery to block the origin of the right MCA. The animals were kept under anesthesia for only 15 min for the surgical procedure. Temperature was maintained at 37.0–38.0°C by using a controlled heating system. The suture was removed after 3 h of occlusion, and the animals were returned to their cages. At reperfusion, animals received atorvastatin (5 mg/kg) (Pfizer, Inc., New York, NY), atorvastatin (15 mg/kg), or methyl cellulose (0.5%) (Sigma Chemical Co., St. Louis, MO), administered twice daily by oral gavage, the first dose 5 min after reperfusion and the second dose after 12 h. Atorvastatin doses chosen were shown previously by others to be neuroprotective (Hayashi et al., 2004; Yrjänheikki et al., 2005).

Physiological Monitoring

Blood glucose level and body weight were measured in all animals before MCAO and before sacrifice. We also measured hemoglobin A1c at baseline by using the A1c+ kit (Metrika, Waltham, MA) in a group of 12 rats. In a fourth group ($n = 17$), cerebral perfusion was measured using a PeriScan PIM 3 system (Perimed AB, Stockholm, Sweden). A skin incision was performed, and the skull was exposed and cleaned. Whole brain scan was performed using the PIM 3 scanning camera to measure cerebral perfusion in both hemispheres (mainly the cortex) at baseline, after MCAO, and at reperfusion. The change in mean perfusion of the ischemic hemisphere is expressed as a percentage of baseline.

Assessment of Infarct Volume and Hemoglobin Content

At 24 h after the onset of MCAO, anesthesia was performed with ketamine (44 mg/kg) and xylazine (13 mg/kg) intramuscular (cocktail), animals were then perfused with saline, sacrificed, and their brains were removed. The brain tissue was sliced into seven 2-mm-thick slices in the coronal plane and stained with a 2% solution of 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co.) for 15 to 20 min. Images of the stained sections were taken. Using image analysis software (KS300; Carl Zeiss, Oberkochen, Germany), infarction zones were measured, and infarct volume was calculated. The ischemic and nonischemic hemispheres of the slices for the enzyme-linked immunosorbent assay were separated and processed, using the nonischemic side as a control. After homogenizing the slices in the core of the infarct and taking the supernatants, enzyme-linked immunosorbent assay was performed to measure the hemoglobin in the brain tissue (Hilali et al., 2004).

Neurological Assessment

Neurological function was measured before reperfusion and at 24 h (just before sacrifice) using the Bederson score (Bederson et al., 1986). Only animals with a score of 3 before reperfusion were included in all analyses. Before sacrifice, animals were tested again using the Bederson score. In a subset of controls and the high-dose atorvastatin group, spontaneous activity of the rats in their cages was recorded continuously using telemetry. Data were recorded every 10 min for 48 h before the stroke and until sacrifice at 24 h after the onset of stroke. Percentage of change in activity after reperfusion (after treatment) compared with baseline (before stroke) was calculated.

Molecular Biomarkers

Lipid Peroxidation. Lipid peroxide concentration was determined by a method that measures the amount of thiobarbituric acid reactivity by the amount of malondialdehyde formed during acid hydrolysis of the lipid peroxide compound. Thirty microliters of sample was incubated with the reaction mixture containing 10 μ l of 8.1% SDS, 150 μ l of 20% acetic acid solution (buffered to pH 3.5), and 150 μ l of 0.8% thiobarbituric acid at 95°C for 1 h. After cooling, 1 ml of distilled water and 5 ml of a mixture of *n*-butanol and pyridine [15:1 (v/v)] were added, and the final mixture was shaken vigorously. After centrifugation at 1500g for 10 min, absorbance of the solvent layer was measured at 532 nm. Tetraethoxypropane was used to establish the standard curve, and lipid peroxide level is expressed in terms of micromoles per liter of malondialdehyde per milligram of protein.

Detection of Nitrotyrosine. Nitrotyrosine immunoreactivity was measured as an indicator of superoxide-dependent peroxynitrite formation by slot blot analysis. Brain homogenates (30 μ g) prepared for immunoblotting experiments were immobilized onto a nitrocellulose membrane using a slot blot microfiltration unit. After blocking with 5% nonfat milk, the membrane was incubated with an anti-nitrotyrosine antibody from Calbiochem (San Diego, CA) and visualized with the Super Signal kit (Pierce Chemical, Rockford, IL). The intensity of bands was analyzed by GelPro software (MediaCybernetics, Inc., Bethesda, MD).

Western Blot. Blots were performed for stroke and nonstroke sides of the brain homogenate. In brief, samples (50 μ g of protein of the brain homogenate) were separated on a 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked in 5% milk solution. Endothelial NO synthase (eNOS), phosphorylated eNOS (peNOS), serine/threonine protein kinase Akt (Akt), phosphorylated Akt (pAkt), and actin proteins were determined by probing the membranes with the corresponding primary (Cell Signaling Technology Inc., Danvers, MA) and secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Blood Pressure

In a subset of 24 rats (11 GK and 13 Wistar), telemetry transmitters (Data Sciences, Inc, St. Paul, MN) were implanted according to manufacturer's specifications under sodium pentobarbital anesthesia (65 mg/kg i.p.; Abbott Laboratories, Abbott Park, IL). A midline incision was used to expose the abdominal aorta that was briefly occluded to allow insertion of the transmitter catheter into the vessel. The catheter was secured in place with tissue glue. The transmitter body was sutured to the abdominal wall along the incision line as the incision was closed. The skin was closed using nonabsorbable suture (3-0). Rats were allowed to recover from surgery for 10 days and returned to individual housing for data acquisition before beginning the stroke protocol. The individual rat cages were placed on top of the telemetry receivers, and arterial pressure waveforms were continuously recorded throughout the study. Data were recorded every 10 min for 48 h before the stroke and until sacrifice at 24 h after the onset of stroke. Use of inhaled anesthetics during stroke surgery, with rapid recovery of consciousness, allowed frequent collection of arterial pressure data in awake animals close to the time of onset of ischemia.

Measurement of Plasma Atorvastatin Levels

The concentration of atorvastatin in plasma was determined by liquid chromatography tandem mass spectrometry as described previously (Jemal et al., 1999). The lower limit of quantification for atorvastatin was 0.2 ng/ml. The intra- and interday coefficients of variation were below 10% in the concentration range of 0.2 to 200 ng/ml for plasma samples. The atorvastatin maximal concentration (C_{max}) experiment was done on a subset of Wistar rats ($n = 3$), whereas trough levels were measured in both treated Wistar and GK rats.

Statistical Analysis

The distributions of all continuous variables were checked for normality before analysis, and a log transformation was found to be beneficial for infarct volume and excess Hgb. Baseline blood glucose (BG), weight, activity, and mean arterial blood pressure (MAP) were compared for strain and drug differences using a 2×2 ANOVA without interaction. Bederson scores were compared using a 2 strain (Wistar versus GK) $\times$ 2 drug (vehicle versus atorvastatin) ANOVA where the interaction between strain and drug was investigated. Log infarct volume and log excess Hgb values were analyzed using a 2 strain (Wistar versus GK) $\times$ 3 drug (vehicle versus 5 mg/kg atorvastatin versus 15 mg/kg atorvastatin) ANOVA where the interaction between strain and drug was investigated. A linear contrast was used to compare the two strains across the levels of drug, and Dunnett's test was used for comparisons of each atorvastatin dose with vehicle. All molecular biomarkers were analyzed using a 2 strain (Wistar versus GK) $\times$ 2 drug (vehicle versus atorvastatin 15 mg/kg) $\times$ 2 sides (stroke versus nonstroke) repeated measures ANOVA. Changes from baseline for BG, weight, activity, and MAP were analyzed using a 2 strain (Wistar versus GK) $\times$ 2 drug (vehicle versus atorvastatin) analysis of covariance where baseline values

were used as covariates before investigating the effects of strain, drug, and their interaction. For blood pressure (BP) data, the average of all measurements before MCAO was the baseline value. Values obtained during the 3 h of MCAO were averaged for the estimate of BP during stroke, the values for the period of the first 5 h after reperfusion were averaged for an estimate of the immediate effects of the drug, and all remaining values afterward were averaged for the delayed postreperfusion value. Means $\pm$ S.D. are presented for the various groups. Vertical error bars in the graphs represent S.E.M. SAS version 9.1.3 (SAS Institute, Cary, NC) was used for all analyses, and $p < 0.05$ was used to determine statistical significance.

Results

At stroke, the weights among groups were similar. However, before sacrifice, control Wistar rats lost more weight than the diabetic GK rats (-46.5 ± 12 versus -35.5 ± 7 g, respectively; $p = 0.002$), and change in weight was not affected by atorvastatin treatment. Baseline BG was significantly higher in the diabetic groups than in the normal rat groups (control and atorvastatin 15 mg/kg) (163.8 ± 56 and 149.5 ± 32 versus 107 ± 15 and 111.7 ± 22 mg/dl, respectively; $p < 0.0001$) (Table 1). Hemoglobin A1c, which is a parameter to monitor chronic glycemic control in people with diabetes, was significantly higher in GK rats ($n = 9$) than in Wistar rats ($n = 3$) (6.7 ± 0.34 versus $4.2 \pm 0.05\%$, respectively; $p = 0.0018$), indicating that the elevation in BG seen in the GK rats is due to chronic rather than acute hyperglycemia. As expected, BG decreased in all groups when measured again at sacrifice (24 h from stroke). After adjusting for baseline values, the decrease in serum glucose levels was found to be similar among the four different groups.

In a subset of untreated Wistar rats ($n = 8$) and GK rats ($n = 9$), there was a similar 30% decline in cerebral perfusion in the right hemisphere (compared with the contralateral hemisphere) 3 h after MCAO ($33.3 \pm 6.2\%$ in Wistar rats versus $33.6 \pm 9.9\%$ in GK rats) (Fig. 1). After reperfusion, blood perfusion in the ischemic hemisphere returned to the baseline level in both strains. Perfusion imaging was performed using PIM 3 Doppler scanner (Perimed AB).

Infarct Volume, Hemoglobin Content, and Behavior

As shown in Figs. 2 and 3, atorvastatin-treated groups had a significantly lowered bleeding rate ($p = 0.0011$) and infarct volume ($p = 0.0007$) compared with their controls. The degree of reduction in bleeding rate ($p = 0.0003$) and infarct volume ($p = 0.0002$) in both strains differed by the dose of atorvastatin given, indicating a positive dose-response effect. By comparing the two doses of atorvastatin to the control groups, there was a significant reduction in hemoglobin con-

TABLE 1

Weight, BG, and hemoglobin A1c levels among different treatment groups at baseline and at sacrifice
Wistar rats lost more weight than the diabetic GK rats ($p = 0.002$).

	W Control ($n = 7$)	W ATV ($n = 8$)	GK Control ($n = 7$)	GK ATV ($n = 5$)
Wt at stroke (S.D.)	290 (8.5) g 243.5 (14.3) g	286.1 (6.9) g 237.4 (11.1) g	286.3 (8) g 250.8 (9.1) g	289.2 (9.5) g 251.9 (14.8) g
BG at stroke (S.D.)	107 (14.7) mg/dl 101.2 (22) mg/dl	111.7 (22.3) mg/dl 109.5 (41.2) mg/dl	163.8 (56.1) [#] mg/dl 121.3 (38) mg/dl	149.5 (31.9) [#] mg/dl 130.4 (44.5) mg/dl
Hemoglobin A1c (S.D.)	W baseline ($n = 3$) 4.2 (0.05)	N.A. N.A.	GK baseline ($n = 9$) 6.7 (0.34) [*]	N.A. N.A.

ATV, atorvastatin, 15 mg/kg; C, control; N.A., not applicable; W, Wistar.

^{*} $p = 0.0018$, hemoglobin A1c was significantly higher in GK rats.

[#] $p < 0.0001$, baseline BG was significantly higher in GK than Wistar rats.

Fig. 1. Change in cerebral perfusion 3 h after MCAO measured with PIM 3 scanner in Wistar rats (W) ($n = 8$) and GK rats ($n = 9$). Perfusion was more than 30% lower in the ischemic hemisphere compared with the contralateral hemisphere (averaged over the entire hemisphere) and was not different among strains.

Fig. 2. Effect of atorvastatin on the infarct volume in the ischemic hemisphere. Atorvastatin (15 mg/kg) significantly reduced infarct volume (cubic millimeters) in both Wistar and GK rats (third column, white and black) compared with their controls (first column, white and black) (*, $p < 0.05$). GK rats had significantly smaller infarct than Wistar rats (#, $p < 0.0001$). The degree of reduction in infarct volume was significantly related to dose in both strains ($p = 0.0002$). Vertical error bars indicate S.E.M. W, Wistar; C, control; ATV 5, atorvastatin 5 mg/kg; and ATV 15, atorvastatin 15 mg/kg.

tent and infarct volume only in the higher dose group (15 mg/kg) ($p < 0.05$). Infarct volume was reduced by approximately 10% with atorvastatin (5 mg/kg) and by more than 30% with atorvastatin (15 mg/kg) in the normal Wistar rats. Similarly, bleeding in the ischemic hemisphere was reduced by almost 40% with atorvastatin (5 mg/kg) and by 70% with the high-dose atorvastatin (15 mg/kg) in Wistar rats. In GK rats, infarct volume and hemoglobin content were reduced by approximately 15% with atorvastatin (5 mg/kg) and 80% with atorvastatin (15 mg/kg). Because neurovascular protection was achieved only with the higher dose of atorvastatin (15 mg/kg), all mechanistic studies included only this dose.

The neurovascular protection was associated with an improvement in the behavioral outcome in both diabetic and nondiabetic animals (Fig. 4A). Atorvastatin (15 mg/kg) significantly improved Bederson score in both Wistar and GK rats (2.8 ± 0.4 versus 2.5 ± 0.4 points in Wistar rats and 3.0 ± 0.1 versus 2.8 ± 0.3 points in GK rats ($p = 0.029$). Hemorrhage formation in diabetic rats tended to be higher than in normoglycemic rats, and they had significantly worse neurobehavioral outcome as shown by Bederson score ($p =$

Fig. 3. Effect of atorvastatin on the hemoglobin content in the ischemic hemisphere. Hemoglobin excess (micrograms of Hgb per gram of protein) decreased in atorvastatin (15 mg/kg) groups (third column, white and black) compared with their controls (first column, white and black) (*, $p < 0.05$). The degree of reduction in bleeding rate was significantly related to dose in both strains ($p = 0.0003$). Vertical error bars indicate S.E.M. W, Wistar; C, control; ATV 5, atorvastatin 5 mg/kg; and ATV 15, atorvastatin 15 mg/kg.

0.004); however, their infarct volume was significantly smaller than normal Wistar rats ($p < 0.0001$).

Using implanted transmitters, rats' spontaneous movement in the cages were recorded every 10 min throughout the experiment. By comparing rats' activity after treatments at reperfusion to their baseline activity levels before stroke, we found that there was a significant increase in the activity level after stroke in both Wistar and GK rats receiving vehicle compared with the atorvastatin-treated rats (70 ± 23 versus $-10 \pm 18\%$ change from baseline, respectively, in Wistar rats and 37 ± 32 versus $-6 \pm 14\%$ change from baseline, respectively, in GK rats; $p = 0.049$) (Fig. 4B). This is possibly due to an increase in the discomfort and loss of circadian rhythm in the control groups after stroke.

Molecular Biomarkers

Oxidative Stress Markers. To examine the differences between strains in oxidative stress levels and whether the beneficial effects of atorvastatin were associated with antioxidant effect, oxidative stress was assessed using a lipid peroxidation assay and nitrotyrosine slot blot. Both assays were done on both plasma and brain homogenate samples. Diabetes increased oxidative stress systemically as shown by plasma levels of lipid peroxides ($p < 0.0001$) (Fig. 5A) and nitrotyrosine ($p = 0.001$) (Fig. 5B) in control GK rats compared with Wistar rats. It is interesting that locally in the brain, diabetic animals had less oxidative stress than their normoglycemic controls as shown by brain lipid peroxides ($p = 0.001$) (Fig. 5C) and nitrotyrosine levels ($p = 0.036$) (Fig. 5D). Atorvastatin did not affect oxidative stress markers. As expected, stroke induced oxidative stress in both strains in the lipid peroxidation ($p = 0.021$) and in the nitrotyrosine formation ($p = 0.04$).

Expression and Phosphorylation of eNOS

To examine the strain differences and whether the beneficial effects of atorvastatin were associated with a change in eNOS expression or activity, eNOS and peNOS protein amounts were quantified by immunoblotting in both the ischemic and the contralateral hemispheres of the brains. There

Fig. 4. A, effect of atorvastatin 15 mg/kg on Bederson score in Wistar rats and GK rats subjected to MCAO. The animals were tested before reperfusion and treatment (white bars), and only animals with a score of 3 were further examined. Before sacrifice, Bederson score was significantly reduced with atorvastatin treatment in both strains (*, $p = 0.0291$). Bederson score was also significantly higher in GK rats compared with Wistar rats (#, $p = 0.004$). Vertical error bars indicate S.E.M. W, Wistar; C, control; and ATV 15, atorvastatin 15 mg/kg. B, effect of atorvastatin 15 mg/kg on activity level in Wistar and GK rats. Using implanted transmitters, rats' movement in the cages were recorded every 10 min throughout the experiment. There is a significant increase in the activity level in both Wistar and GK rats receiving methylcellulose compared with the atorvastatin-treated animals (*, $p = 0.049$). Vertical error bars indicate S.E.M.. W, Wistar; C, control; and ATV 15, atorvastatin 15 mg/kg.

were no differences between the diabetic and nondiabetic animals in eNOS expression or its phosphorylation. Stroke induced eNOS expression in both Wistar rats ($p = 0.002$) and GK rats ($p = 0.015$), but eNOS phosphorylation was less in the stroke side in Wistar rats ($p = 0.007$) and GK rats ($p = 0.011$). Atorvastatin did not affect eNOS expression, but there was a trend toward an increase in eNOS phosphorylation in the ischemic hemisphere in GK rats treated with atorvastatin compared with control (69 ± 37 versus 35 ± 23 ; $p = \text{N.S.}$) (Fig. 6, A and B).

Brain Akt Phosphorylation

To further explore the strain differences and the mechanisms related to the neurovascular protection with atorvastatin, expression of pAkt was quantified similar to eNOS and peNOS. There was no difference between diabetic and nondiabetic rats. Atorvastatin (15 mg/kg) significantly increased Akt phosphorylation in the ischemic hemisphere in comparison with controls for both strains ($p = 0.006$ for the interaction) (Fig. 6C).

Blood Pressure

To explore the effect of diabetes, stroke, atorvastatin treatment, and their interactions on blood pressure, we implanted the animals with BP transmitters, and MAP was recorded every 10 min on telemetry before stroke, during the onset of ischemia, at reperfusion and then during the following 21 h until sacrifice next day. Baseline blood pressure was elevated in diabetes as shown in Fig. 7 (MAP was 112 ± 7 mm Hg in GK rats versus 97 ± 5 mm Hg in Wistar rats; $p < 0.0001$). In addition, onset of stroke in diabetes caused significantly higher elevation in blood pressure than in normoglycemic rats (34 ± 5 mm Hg in GK rats versus 26 ± 6 mm Hg in Wistar rats; $p = 0.02$). It is interesting that atorvastatin (15 mg/kg) lowered blood pressure in the diabetic rats for the first five postreperfusion hours (141 ± 9 mm Hg in control GK rats versus 133 ± 9 mm Hg in treated GK rats), whereas there was no treatment effect on the MAP in the normoglycemic Wistar rats ($p = 0.023$ for the interaction).

Atorvastatin Concentrations after Oral Gavage

Administration of atorvastatin (15 mg/kg) in Wistar rats ($n = 3$) by oral gavage achieved an average (C_{max}) (range) of 53.9 (27–77) ng/ml after 30 min. We also checked the trough levels (before sacrifice) of atorvastatin (15 mg/kg) in Wistar rats ($n = 8$) and GK rats ($n = 5$), and they were 7.5 (1.4) and 5.5 (0.8) ng/ml, respectively. Trough levels of atorvastatin (5 mg/kg) in Wistar rats ($n = 4$) were proportionately lower at 2.5 (0.7) ng/ml.

Discussion

Data from both animal and human studies suggest that hyperglycemia during acute ischemic stroke is associated with an increase in the brain injury due at least in part to an increase in blood-brain barrier permeability, edema, and hemorrhage formation. This finally leads to worsening in functional outcomes and an increase in mortality (Bruno et al., 2004). We have adapted our stroke model to a model of type II diabetes mellitus (GK). We found that GK rats are more vulnerable to reperfusion injury and hemorrhage formation. This is due to the pathological remodeling characterizing this model, which induces blood-brain barrier breakdown leading to an increase in hemorrhage and worsening of the functional outcomes after stroke (Ergul et al., 2007). In GK rats, laser Doppler revealed a drop in cerebral perfusion after MCAO similar to that in Wistar rats. This experiment indicates that the small infarct size seen in the latter group is not due to inability to occlude the MCA. Bederson test showed that diabetic rats were more impaired compared with Wistar rats regardless of the treatment, which may be due to the increased incidence of bleeding. Increased hemorrhage formation in GK rats confirms that they are a suitable model

Fig. 5. Effect of strain and treatment on oxidative stress markers in both plasma (A and B) and brain (C and D). In plasma, both lipid peroxidation (A) and nitrotyrosine slot blot (B) showed significant elevation in the diabetic animals. In the brain, there was less oxidative stress in diabetic rats as shown in the lipid peroxidation assay (C). There were no differences between treated and control animals in plasma or the brain. W, Wistar; C, control; ATV 15, atorvastatin 15 mg/kg; N, contralateral hemisphere; and S, ipsilateral hemisphere.

to test vascular protective agents. As expected, diabetic rats had more oxidative stress than their normoglycemic controls systemically. However, in the brain, oxidative stress markers were lower in GK rats. These results also raise the question of whether the infarct, bleeding, and oxidative stress pattern observed in GK rats is due to a compensatory response in this mild chronic diabetes model. As reviewed recently, our knowledge on the effect of diabetes of experimental ischemic stroke is limited to acute hyperglycemia or severe short-term diabetes models, both of which showed increased infarct size and worse functional outcomes (Ergul et al., 2009). In future studies, we plan to study the effects of the duration and degree of hyperglycemia on the neurovascular and functional outcomes after stroke in healthy compared with diabetic animals.

The most important finding in our study is that, in both strains, atorvastatin (15 mg/kg) was not only neuroprotective but also vascular protective and improved neurological function. This result differs from that of the Stroke Prevention by Aggressive Reduction in Cholesterol Levels trial (Amarenco

et al., 2006) in which atorvastatin was associated with an increase in hemorrhagic stroke, despite a robust reduction in overall stroke risk. This may be due to the difference between the acute versus the chronic effects of atorvastatin. In this project, we also found that the atorvastatin peak plasma concentration achieved after administering 15 mg/kg by oral route was similar to that reported after a dose of 80 mg/day atorvastatin is given to humans (Lins et al., 2003). This gives our results clinical relevance for future translational studies, especially that 80 mg/day atorvastatin was the dose shown to reduce stroke recurrence in the Stroke Prevention by Aggressive Reduction in Cholesterol Levels trial (Amarenco et al., 2006). The failure of the lower dose atorvastatin to achieve statistically significant protection in our study points to the importance of achieving high concentrations acutely in this population.

Another novel finding is that the vascular protection with atorvastatin (15 mg/kg) in the diabetic rats and their normoglycemic controls was associated with increased levels of phosphorylated Akt kinase in the brain homogenate, an ef-

Fig. 6. Effect of atorvastatin and stroke on eNOS, peNOS, and pAkt levels in the brain. A, quantitative data of percentage relative density showing increased eNOS expression in the ischemic hemisphere in Wistar rats (*, $p = 0.002$) and GK rats (#, $p = 0.015$). B, eNOS phosphorylation was less in the stroke side in Wistar rats (*, $p = 0.007$) and GK rats (#, $p = 0.011$). C, quantitative data of percentage of relative density showing increased activation of pAkt in atorvastatin-treated groups compared with their controls (*, $p = 0.0007$). Vertical error bars indicate S.E.M. W, Wistar; C, control; ATV 15, atorvastatin 15 mg/kg; N, contralateral hemisphere; and S, ipsilateral hemisphere.

fect that was shown by others to be involved in the acute neuro- and cardioprotection of statins (Wolfrum et al., 2004; Zhang et al., 2007; Prinz et al., 2008). The phosphatidylinositol-3 kinase/Akt signaling pathway is known to be very important in regulating cell growth, proliferation, and survival. The activation of the phosphatidylinositol-3 kinase/Akt pathway reduces thrombogenicity, vascular permeability, inflammation, and apoptosis and thereby preserves vascular function (Cantley, 2002). Statins induce Akt translocation to the plasma membrane of endothelial cells. Upon phosphorylation and subsequent activation of Akt at serine 473, it induces phosphorylation of Bad (member of the proapoptotic BCL2 protein family), forkhead transcription factor (AFX), caspase-9, eNOS, and inhibitor of nuclear factor- κ B (Datta et

al., 1997). Although others have shown the importance of eNOS in statin neuroprotection (Endres, 2005), our study did not show any treatment effect on eNOS expression or its activation. We think that these differences are due to the prophylactic versus the acute effects of statins in stroke, the route of administration and the dosage administered. In parallel to our results, a previous study showed that atorvastatin in combination with tissue-type plasminogen activator reduced the disruption of vascular integrity in the brain after embolic stroke in rats without affecting eNOS levels, and blocking NOS activity did not inhibit the beneficial effects of the combination treatment on stroke (Zhang et al., 2005). In addition, low doses of intravenous rosuvastatin after stroke provided neuroprotection and increased pAkt without alter-

Fig. 7. MAP (mm Hg) after acute stroke in the Wistar rats (triangles) and GK rats (rectangles). MAP was recorded every 10 min (telemetry) before stroke (baseline between 6:00 AM and 9:00 AM), during the onset of ischemia (at 10:00 AM; left arrow), reperfusion (at 1:00 PM; right arrow), and then during the following 21 h until sacrifice the next day. At reperfusion, the animals were treated with methylcellulose (gray) or with atorvastatin (15 mg/kg; black); another dose was given after 12 h. The black horizontal bar indicates nighttime in the light/dark cycles. Values shown are 1-h averages $\pm$ S.E.M. #, $p = 0.012$; *, $p = 0.023$.

ing eNOS expression or its phosphorylation (Prinz et al., 2008). However, in our study, it is also possible that the eNOS phosphorylation was transient, with an early peak after atorvastatin administration followed by a decline to baseline level, a pattern that would not be detected using Western blot on samples collected at 21 h after reperfusion and drug administration.

Last, our study showed that these diabetic rats had slightly elevated blood pressure at baseline compared with their normoglycemic controls. This is in contrast to our previous work, done in older GK animals, in which there was no difference in blood pressure (Harris et al., 2008). It is likely that the endothelial response to the hyperglycemia changes over time, and we are investigating this. The animals were also more sensitive to ischemia, leading to a higher increase in their MAP than normal rats during MCA occlusion. These results are probably mediated by the endothelial dysfunction and the altered nature of the diabetic vasculature in GK rats (Harris et al., 2005; Elgebaly et al., 2008). It is interesting that atorvastatin (15 mg/kg) significantly reduced MAP after reperfusion for 5 h, an effect that was only seen in GK rats. It is possible that this mild blood pressure lowering contributed to the robust protection seen in the diabetic animals in this study and may have improved their cerebral blood flow. A recent large, parallel-design randomized clinical trial showed that various statins lower BP

and that this reduction may contribute to the cardiovascular protection of statins (Golomb et al., 2008). Although BP lowering is not clinically accepted as a tactic of neurovascular protection, we as well as others have already shown that various pharmacologic agents that lower BP are vascular protective after ischemic stroke (Hosomi et al., 2005; Fagan et al., 2006; Elewa et al., 2007).

An interesting finding of our study is that we did not detect any effect of atorvastatin treatment on oxidative stress, which may have been due to the following reasons. First, statins are known to reduce oxidative stress through their inhibitory effect on the Rac, which inhibits NADPH oxidase and hence decreases ROS production (Endres, 2005). However, there are many other sources of oxidative stress and ROS in the brain after ischemia/reperfusion injury that are not inhibited by statins such as cyclooxygenase, myeloperoxidase, and the mitochondrial electron transport chain (Lo et al., 2003). In addition, ROS production seems to occur very early after ischemia, which makes it a very difficult target to reach with the delayed oral statin treatment (given at 3 h after the onset of stroke).

In conclusion, this is the first report of the robust vascular protective effect of acute, high-dose atorvastatin in diabetic and normoglycemic rats. Atorvastatin is a novel vascular protective agent, already safely administered to many acute

ischemic stroke patients, and it may be especially helpful when given at reperfusion in high-risk diabetic patients.

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Address correspondence to: Dr. Susan C. Fagan, College of Pharmacy, HM-1200 Medical College of Georgia, University of Georgia, 1120 15th St., Augusta, GA 30912-2450. E-mail: sfagan@mail.mcg.edu