Estrogen Decreases Infarct Size After Temporary Focal Ischemia in a Genetic Model of Type 1 Diabetes Mellitus

Thomas K. Toung, MD; Patricia D. Hurn, PhD; Richard J. Traystman, PhD; Frederick E. Sieber, MD

Background and Purpose—It is unclear how genetic type 1 diabetes mellitus (DM) influences infarct size when blood glucose is tightly controlled. The aim of this study was to determine the effect of genetic type 1 DM, as occurs in BB rats, on infarct size after transient unilateral middle cerebral artery occlusion (MCAO) in male and female rats. In addition, studies suggest that male type 1 DM rats have a higher incidence of end-organ complications than do females. A second aim of this study was to determine the effect of chronic 17β-estradiol (E2) administration on infarct size in male BB rats.

Methods—Diabetic male (MDiab, n=14) and female (FDiab, n=8) BB rats were studied and compared with background strain Wistar rats (MWist, n=16; FWist, n=14). Two additional male cohorts (MWist+E2, n=15; MDiab+E2, n=14) received subcutaneous 25 μg E2 implants 7 to 10 days before MCAO. Rats underwent 1 hour of MCAO followed by 22 hours of reperfusion. Physiological variables were controlled among groups, and the intraischemic laser Doppler flow signal was reduced similarly in all animals. Infarction volume was evaluated by 2,3,5-triphenyltetrazolium chloride staining and image analysis.

Results—Preischemic blood glucose was 94±5, 127±13, 90±15, 63±18, 122±8, and 81±14 mg/dL in MWist, FWist, MDiab, FDiab, MWist+E2, and MDiab+E2 rats, respectively (mean±SE). Intraischemic laser Doppler flow was reduced to 20% to 25% of baseline in all groups. Striatal infarct size (percentage of ipsilateral caudate putamen) was increased in male diabetic rats relative to nondiabetic MWist rats (41±3% versus 28±3%). Striatal injury was not increased in FDiab rats, and infarction volume was smaller than that in FWist rats (23±4% in FWist versus 13±3% in FDiab). Chronic estrogen treatment reduced cortical and striatal infarction in MDiab+E2 rats compared with untreated MDiab rats.

Conclusions—Type 1 DM is associated with increased infarct size after temporary MCAO, despite tight control of blood glucose. The deleterious effect of DM is evident only in male rats; female diabetic BB rats sustain small infarcts. Chronic E2 treatment reduced injury in the male BB rat, providing neuroprotection even in the presence of DM. These data suggest that genetic diabetes even with mild glucose elevation plays a role in determining neuropathology in experimental stroke. However, factors such as reproductive steroids also determine outcome in DM stroke. (Stroke. 2000;31:2701-2706.)

Key Words: brain ■ cerebral ischemia ■ complications ■ diabetes mellitus ■ estrogen ■ ischemia ■ middle cerebral artery ■ stroke ■ rats

It is unclear how genetic type 1 diabetes mellitus (DM) influences infarct size when blood glucose is tightly controlled. This issue is important because DM is an independent risk factor for stroke.1 In addition, DM is strongly related to early stroke progression and associated with poorer stroke outcome.2 Transient ischemia, whether global or focal, is associated with greater neurophysiological damage in DM hyperglycemic animals. In particular, the preischemic blood glucose is an important determinant of neurological outcome after temporary ischemia in DM.3 Insulin-based blood glucose management has been shown to decrease infarct size. However, it is still unresolved whether DM, independent of the blood glucose level, affects infarct size after temporary brain ischemia. The aim of the present study was to determine the effect of genetic type 1 DM, as occurs in male and female BB rats, on infarct size after transient middle cerebral artery occlusion (MCAO).

Many studies have shown that supplementary estrogen administration is neuroprotective in stroke. Studies examining the epidemiology of DM have shown that male type 1 DM rats have a higher incidence of end-organ complications than do female rats.4 A second aim of the present study was...
Cerebral ischemia was accomplished by use of the intraluminal filament model (4-0 nylon monofilament suture) of proximal MCAO. The right common carotid artery was exposed through a lateral incision, separated from the vagus, and ligated. The external carotid artery was ligated, the occipital branch was cauterized, and the pterygopalatine artery was ligated. An occluding filament was advanced via the common carotid artery until the LDF signal displayed an abrupt and significant reduction, confirming ongoing ischemia; it was then secured in place. Ischemic LDF was determined over 5-minute periods throughout the 60-minute occlusion period, and then the suture was withdrawn with prompt restoration of blood flow. Each animal was recovered and supported with intravenous Ringer’s lactate solution and supplemental oxygen as needed. Insulin was given after surgery to maintain plasma glucose at the target level. After 22 hours of reperfusion, the animal was reanesthetized for harvesting of the brain. The tissue was sliced into seven 2-mm-thick coronal sections for 2,3,5-triphenyltetrazolium chloride staining and quantification via standard photography and digital planimetry (SigmaScan Pro, Jandel). The infarcted area was numerically integrated across each section and over the entire ipsilateral hemisphere. Infarct volume was measured separately in the cortex and caudate putamen and expressed as a volume percentage of the hemisphere. Infarct volume was measured separately in the cortex and caudate putamen and expressed as a volume percentage of the hemisphere.

### Physiological Data

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Values are mean±SE for baseline and at 60 minutes of MCAO. Arterial glucose was measured immediately before ischemia and on reperfusion. MAP indicates mean arterial blood pressure; PacO2, arterial carbon dioxide tension; PacO2, arterial oxygen tension; MWist, male nondiabetic Wistar rats; FWist, female nondiabetic Wistar rats; MDiab, male diabetic BB strain; FDiab, female diabetic BB strain; MWistE2, E2-treated male Wistar rats (25 μg implant); and MDiabe2, E2-treated male diabetic BB strain (25 μg implant).

### Materials and Methods

The present study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research. All protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University. All methods are as previously published.5,6

#### Diabetic Animal Model

Studies were conducted in type 1 DM male and female BB rats (Biotooling Laboratories, Ottawa, Ontario, Canada) and in age-matched background strain Wistar rats of both sexes. BB rats were obtained from the supplier after 5 weeks of DM and then were housed for 1 to 3 weeks while receiving insulin therapy with a goal of maintaining blood glucose <150 mg/dL. Additional groups of male BB and Wistar rats received subcutaneous implants of 17β-estradiol (E2, 25 μg) ~7 to 10 days before MCAO, as previously described.5,6 The 25-μg implant treatment was chosen on the basis of previous efficacy in reducing stroke injury after MCAO in male,5 female,7 and reproductively senescent female Wistar rats.8 One week of treatment with the implant yields a stable and physiological level of plasma E2 (ie, 10 to 20 pg/mL).5-8 Animals were housed with free access to water and food, and subcutaneous insulin was administered daily in response to blood glucose levels obtained from tail samples.

#### Reversible Unilateral MCAO

Male and female rats were anesthetized with 1% to 2% halothane delivered via face mask in oxygen-enriched air and instrumented with femoral artery catheters for physiological monitoring and blood gas measurement. Rectal and temporalis muscle temperatures were controlled at 37.5±0.5°C by use of heat lamps. Cortical perfusion was measured by laser Doppler flowmetry (LDF, Moor Instruments Ltd, model MBF3D) as previously described, with probe placement at 2 mm posterior and 6 mm lateral to the bregma. Unilateral focal cerebral ischemia was accomplished by use of the intraluminal filament model (4-0 nylon monofilament suture) of proximal MCAO. The right common carotid artery was exposed through a lateral incision, separated from the vagus, and ligated. The external carotid artery was ligated, the occipital branch was cauterized, and the pterygopalatine artery was ligated. An occluding filament was advanced via the common carotid artery until the LDF signal displayed an abrupt and significant reduction, confirming ongoing ischemia; it was then secured in place. Ischemic LDF was determined over 5-minute periods throughout the 60-minute occlusion period, and then the suture was withdrawn with prompt restoration of blood flow. Each animal was recovered and supported with intravenous Ringer’s lactate solution and supplemental oxygen as needed. Insulin was given after surgery to maintain plasma glucose at the target level. After 22 hours of reperfusion, the animal was reanesthetized for harvesting of the brain. The tissue was sliced into seven 2-mm-thick coronal sections for 2,3,5-triphenyltetrazolium chloride staining and quantification via standard photography and digital planimetry (SigmaScan Pro, Jandel). The infarcted area was numerically integrated across each section and over the entire ipsilateral hemisphere. Infarct volume was measured separately in the cortex and caudate putamen and expressed as a volume percentage of the ipsilateral structure.

#### Statistical Analysis

All values are reported as mean±SE; all physiological variables, infarct volumes, and residual LDF were analyzed by 1-way ANOVA and post hoc Newman-Keuls test. The relationship between preischemic glucose and infarction was analyzed by Pearson correlation. Statistical significance was confirmed at value of P≤0.05.

#### Results

Six groups were studied: nondiabetic male (n=16, mean body weight 310±15 g) and female (n=14, 262±13 g) Wistar rats, diabetic male (n=14, mean body weight 355±7 g) and female (n=8, mean body weight 299±5 g) BB rats, and E2-treated male Wistar (n=15, mean body weight 341±10 g) and BB (n=14, mean body weight 373±7 g) rats. Physiological variables were controlled during the peri-ischemic period, as shown in the Table. Preischemic glucose was higher in diabetic males than in females; otherwise, there

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To determine whether chronic preischemic estrogen treatment reduced infarction size in male type 1 DM rats treated with transient MCAO.
were no baseline differences among treatment groups. There was no difference in intraischemic reduction of the LDF signal among groups (Figure 1).

Figure 2 displays infarction volumes for the 2 brain regions damaged by 1 hour of MCAO, the cortex and caudate putamen, as a percentage of the respective ipsilateral structure. Cortical injury was small in all treatment groups, particularly in diabetic females, in which no injury was present, and in female nondiabetic Wistar rats (Figure 2). In the striatum, injury volumes were larger, and differences linked to diabetic strain and to sex were apparent. Diabetic males sustained larger striatal infarction compared with their male Wistar counterparts (Figure 2). Diabetes-linked exacerbation of stroke injury was sex dependent in that female diabetic rats did not demonstrate increased striatal injury. Furthermore, striatal infarction was surprisingly smaller in diabetic BB than in Wistar females.

Figure 3 shows the effect of estrogen treatment in male diabetic and Wistar rats compared with their untreated male counterparts in the same strain. When untreated and E2-treated Wistar males were compared, E2 treatment did not alter the relatively small damage sustained after 60 minutes of MCAO in the cortex or caudate putamen. However, when untreated and E2-treated male diabetic BB rats were compared, E2 clearly produced a robust protection in both the cortex and striatum.

Figure 4 plots individual animal values for preischemic plasma glucose concentration and striatal infarction within all experimental groups. There was no strong correlation between these variables ($r=0.191$), in part because hyperglycemia was limited by the study design. Therefore, only limited ranges of glucose were present (<200 mg/dL), and most animals fell in the 75- to 150-mg/dL range. The correlation was not improved by comparison by sex or estrogen treatment. Although many females or estrogen-treated males had normal or low preischemic glucose levels, others sustained high glucose levels but small infarctions.

**Discussion**

The present study shows that type 1 DM is associated with increased infarct size after temporary MCAO, despite tight control of blood glucose. The deleterious effect of DM is evident only in males, whereas female diabetic BB rats sustain small infarcts. Chronic E2 treatment reduced injury in the male BB rat, providing neuroprotection even in the presence of DM. These data suggest that genetic DM even with mild glucose elevation plays a role in determining neuropathology in experimental stroke. However, factors such as reproductive steroids also determine outcome in DM stroke.
Transient ischemia, whether global or focal, is associated with greater neuropathologic damage in DM hyperglycemic animals. During temporary focal ischemia, DM hyperglycemia increases infarct size. Similarly, with transient global ischemia, the level of DM hyperglycemia also affects the neurological outcome. In the rat forebrain ischemia model, preischemic treatment of DM hyperglycemia with insulin leads to a neurological outcome and histopathology similar to that in non-DM normoglycemic rats. However, untreated hyperglycemia leads to a dismal neurological and histopathologic outcome. These results agree with studies using magnetic resonance spectroscopy, which suggest that the most hyperglycemic DM animals are at highest risk for poor neurological outcome. Similarly, in dogs, the preischemic blood glucose level is an important determinant of morbidity and neuropathologic damage after global brain ischemia in insulin-dependent animals. It is unclear whether the above-mentioned results represent a neuroprotective effect of insulin or a neurotoxic effect of hyperglycemia. However, they emphasize the potential importance of insulin-based blood glucose control in determining neurological outcome after transient brain ischemic insults in DM. In the present study, intensive insulin-based blood glucose management was undertaken 1 to 3 weeks before MCAO to eliminate hyperglycemic effects. We targeted blood glucose at <200 mg/dL immediately before the onset of MCAO, and preischemic glucose values were similar in the Wistar and diabetic male groups. Despite this insulin-intensive management of blood glucose, ischemic injury was greater in type 1 DM males.

Previous studies used chemical or surgically induced DM. These animal models may not accurately reflect type 1 DM, which entails complex genetic and immunologic interactions.

In the present study, a genetic rodent model of type 1 DM was used, simulating some complexity of human DM. There are several reasons to suspect that DM, even with intensive insulin-based blood glucose management, would be associated with worsened neurological outcome after stroke. In particular, DM alters cerebral blood flow and reactivity. These effects have been observed in larger vessels and in the microvasculature. Autopsy studies suggest that diabetics are particularly prone to cerebral small-artery disease, lacunar infarction, and large-artery atherosclerotic occlusive disease. DM-induced vascular abnormalities may have relevance to the present results with the present model of MCAO, which would simulate this latter pathology. Although the reduction of LDF during vascular occlusion was similar among all groups in the study (~20% to 25% of baseline signal), absolute intraischemic cerebral blood flow was not measured. If type 1 DM altered baseline cerebral blood flow, then differences in intraischemic blood flow cannot be excluded and may have influenced tissue outcome. Last, in our 1-hour MCAO model, the effect of DM was most striking in the striatum, which contains the core of the ischemic lesion, rather than the cortex. A moderate intensity of ischemic duration was chosen to best ensure survivorship within the insulin-dependent diabetic cohort. Consequently, cortical injury levels were small in all treatment groups within the study and may have been somewhat insensitive to the effects of genetic strain or treatment. Alternatively, genetic diabetes may recruit potentially salvageable tissue into the core of the lesion by compromising collateral circulation and vasodilatory capacity during MCAO.

The present data strongly suggest that sex and estrogen availability have large influences on the outcome from stroke in the diabetic strain. Although DM exacerbates stroke damage in the male, the female is spared. Our laboratory and others have demonstrated that female animals sustain reduced tissue damage from experimental stroke; loss of endogenous reproductive steroids in females results in ischemic damage, which becomes indistinguishable from that of age-matched males. Furthermore, exogenous estrogen treatment has been widely shown to be neuroprotective in ischemic injury both in vivo and in vitro (for review, see Reference 18). The anti-ischemic mechanism(s) of the steroid is under heavy investigation; however, both vascular and non-vascular sites of action have been demonstrated. Cellular mechanisms are likely multifactorial, including preservation of vascular tone and residual cerebral blood flow during cerebral ischemia, induction of protective gene products, antioxidant activity, modification of inflammatory processes, and reduction of glutamate excitotoxicity. The present data further these observations by demonstrating that genetically diabetic female rodents are also protected from ischemic damage relative to the male of the same strain and that estrogen treatment strongly protects the male diabetic brain during MCAO. Estrogen implants reduced infarction volume in the diabetic brain to levels not different from those observed in the nondiabetic brain. These findings emphasize that outcome from DM stroke, like non-DM stroke, is sex dependent. Furthermore, the deleterious effect of DM in the

Figure 4. Lack of relationship between plasma glucose sampled immediately before to occlusion and ipsilateral (ipsi) striatal infarction volume. Data are compiled from individual animals within all treatment groups. Sex or estrogen treatment was not associated with selective reduction of plasma glucose and decreased infarction. Rat groups are as follows: male Wistar with and without E2 treatment (MWist and MWistE2, respectively), n = 16 and 15, respectively; male diabetic BB strain with or without E2 treatment (MDiab and MDiabE2, respectively), n = 14 and 14, respectively; female Wistar (FWist), n = 14; and female diabetic BB strain (FDiab), n = 8.
male brain confronted with an ischemic stress is responsive to a neuroprotectant, such as estrogen.

The importance of sex or reproductive steroids in type 1 DM end-organ complications has not been extensively studied in human or animal models. Although premenopausal nondiabetic women have lower stroke rates than age-matched males (for review, see Reference 18), this relationship has not been specifically studied in diabetic women. Diabetes is a known risk factor for stroke in both men and women, but most epidemiological studies have not reported sex-specific relative risks. Whether estrogen treatment alters stroke outcome in human DM is also unclear; however, data from the Framingham study show that stroke damage is greatly increased in postmenopausal diabetic versus nondiabetic women.28 Several factors in the present rodent experiments must be considered relative to the neuroprotection of estrogen. First, because reischemic plasma glucose levels were, on average, lower in DM females than in males, we cannot exclude this factor as a source of the protection enjoyed by the female. Reischemic hyperglycemia is known to increase brain damage by fueling lactic acidosis and depressing intraischemic tissue pH,12,13,29 potentially enhancing oxygen radical–mediated injury mechanisms.29,30 However, glucose levels were relatively controlled in all the BB rats for 1 to 3 weeks before MCAO. The level of hyperglycemia present in any of the animals was mild and consequently not well correlated with increased infarction for males or females. Second, the observation that striatal damage in diabetic females was less severe than in nondiabetic females could suggest an overlap of tissue mechanisms by which estrogen protects brain and DM increases stroke damage. This is the first report of sex differences in DM stroke or of the efficacy of estrogen in reducing injury in the DM male, so further studies of DM stroke. Vascular and parenchymal mechanisms by which estrogen decreases infarct size in DM are needed to determine cellular mechanisms of interaction. Potential interactions between estrogen and DM could involve the ability of the steroid to enhance cerebral perfusion under conditions of vascular stress,5,19,20 protect vascular endothelial integrity,19 enhance glucose transport and increase blood-brain barrier expression of the glucose transporter GLUT1.15,16,18 and decrease oxidative impairment of membrane Na+,K+–ATPase activity.34 Last, estrogen treatment did not decrease infarction volume in nondiabetic male rats, contrary to previous reports of significant efficacy in the male brain.6,34 The lack of neuroprotection is likely related to the small degree of injury produced in nondiabetic animals after a single hour of MCAO. When the duration of MCAO is extended and larger tissue volumes are compromised, chronic and acute estrogen treatment ameliorates foci injury in the nondiabetic brain.6,34

In conclusion, genetic type 1 DM negatively influences infarct size when hyperglycemia is tightly controlled. This effect is limited to the male and can be altered by estrogen availability, suggesting that these factors be considered in further studies of DM stroke. Vascular and parenchymal mechanisms by which DM enhances stroke damage require further investigation.

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References
Diabetes mellitus increases the risk for stroke. The goal of this study was to examine effects of genetic, type 1 diabetes on infarct size in a rat model of focal cerebral ischemia in which relatively good control of plasma glucose levels were achieved by using insulin. In addition, the study examined the influence of chronic treatment with 17β-estradiol on infarct size during diabetes.

The results suggest that type 1 diabetes is associated with increased infarct size, even with relatively good control of plasma glucose levels. This increase in brain injury in response to ischemia was seen in males and could be reduced by 17β-estradiol. Thus, these findings support the concept that estrogen is neuroprotective. In contrast to the results in males, females rats with diabetes did not have increased infarct size following ischemia.

At the present time, it is unclear what mechanism(s) produce gender-specific increases in brain injury following ischemia during diabetes. What are some possibilities? There are several levels at which one might hypothesize that diabetes could exacerbate brain injury following ischemia. These include diabetes-induced vascular dysfunction and reductions in blood flow, increases in production of reactive oxygen species (or reductions in effectiveness of antioxidant mechanisms), or reprogramming of gene expression such that subsequent injury in response to ischemia is enhanced.

Although it is fairly well established that estrogen has neuroprotective effects in cerebral ischemia, the present work provides new evidence that this protective effect extends to the setting of diabetes. The mechanisms that account for neuroprotection during diabetes also remain to be defined.

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