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Neurotoxicity of advanced glycation endproducts during focal stroke and neuroprotective effects of aminoguanidine

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ABSTRACT **Cerebral infarction (stroke) is a potentially disastrous complication of diabetes mellitus, principally because the extent of cortical loss is greater in diabetic patients than in nondiabetic patients. The etiology of this enhanced neurotoxicity is poorly understood. We hypothesized that advanced glycation endproducts (AGEs), which have previously been implicated in the development of other diabetic complications, might contribute to neurotoxicity and brain damage during ischemic stroke. Using a rat model of focal cerebral ischemia, we show that systemically administered AGE-modified bovine serum albumin (AGE-BSA) significantly increased cerebral infarct size. The neurotoxic effects of AGE-BSA administration were dose- and time-related and associated with a paradoxical increase in cerebral blood flow. Aminoguanidine, an inhibitor of AGE cross-linking, attenuated infarct volume in AGE-treated animals. We conclude that AGEs may contribute to the increased severity of stroke associated with diabetes and other conditions characterized by AGE accumulation.**

Stroke damage caused by brain infarction is a devastating complication of diabetes mellitus, killing or permanently disabling more than 60% of diabetic patients who survive into their seventh decade (1–6). Diabetic patients are more likely to develop a stroke than nondiabetic patients, and moreover, strokes in diabetic patients are larger and more disabling than those in nondiabetic patients. Although a number of factors have been implicated in enhancing diabetic stroke-related neurotoxicity, a complete understanding of the biochemical basis for increased stroke size associated with diabetes remains elusive. Recent investigation into the pathogenesis of stroke indicates that a number of factors may directly influence the volume of brain infarction after occlusion of a cerebral artery (7–10). These studies suggest that such neurotoxic factors can transform ischemic but potentially viable brain tissue into an irretrievably infarcted lesion, resulting in larger strokes in terms of both parenchymal necrosis and corresponding functional impairment.

Advanced glycation endproducts (AGEs) have been implicated in the development of diabetic complications such as accelerated atherosclerosis, renal dysfunction, and neuropathy (11, 12). AGE modifications accumulate by nonenzymatic reactions as permanent adducts and cross-linking structures on long-lived body proteins (for instance, collagen) as a function of age and glucose concentration. AGE-modified proteins in tissues may subsequently undergo receptor-mediated or proteolytic cleavage into smaller, reactive AGE-peptides which are released into the circulation, where they may ultimately either reattach covalently to tissue proteins or be eliminated from the circulation by the kidneys (13, 14). Previous observations suggest that high levels of AGE-proteins and AGE-peptides may enhance tissue damage in part by binding to AGE-specific receptors present on macrophages, endothelial cells, and other cell types (15–19). AGE receptor-mediated responses mediate capillary leakage, cytokine production, enhanced procoagulant activity on the endothelial surface, and increased generation of reactive oxygen intermediates (11, 17–20). AGE-mediated tissue damage may also occur when circulating AGE-proteins or AGE-peptides react directly to covalently cross-link with basement membrane proteins in the subendothelial space (20–22).

In the present investigation we address the hypothesis that AGE-modified proteins or peptides are neurotoxic factors that amplify the volume of permanent damage and necrosis following focal cerebral ischemia. The results presented here show that administration of AGEs in clinically relevant amounts converted a typically small cerebral infarction following experimental occlusion of a middle cerebral artery (MCA) into a significantly larger stroke. The magnitude of AGE neurotoxicity depended on the dose of AGEs administered and the timing of AGE administration. Aminoguanidine, an inhibitor of AGE cross-linking, prevented this apparent neurotoxicity of exogenous AGEs. We conclude that the neurotoxicity-potentiating effects of AGEs may contribute importantly to the pathogenesis of diabetic stroke and that aminoguanidine may effectively inhibit AGE-mediated increases in infarct volume.

MATERIALS AND METHODS

Bovine serum albumin (BSA) (Fraction V, low endotoxin), glucose, and 2,3,5-triphenyl-2*H*-tetrazolium chloride were purchased from Sigma. Aminoguanidine was provided by Alteon (Ramsey, NJ). All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee as conforming with the recommendations of the National Institutes of Health. AGE-modified bovine serum albumin (AGE-BSA) was prepared by a modification of previously described methods (20–22); endotoxin content was measured by Limulus amoebocyte assay (E-toxate; Sigma) and found to be <0.2 ng/ml. AGE content in samples and in serum was assessed by ELISA (23, 24). The AGE-BSA (65 AGE units/ml) employed in these studies was prepared at a final concentration of 30 mg/ml in phosphate-buffered saline (PBS, pH 7.4).

Animal Model of Focal MCA Infarction. Focal cerebral ischemia was induced by a modification of previously described methods (25–28). Briefly, male Lewis rats (270–300 g) were anesthetized with ketamine (120 mg/kg; i.m.) and allowed to breathe spontaneously, and body temperature was maintained at 35.5–36.5°C. The ventral neck and the area between the right

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Abbreviations: AGE, advanced glycation endproducts; BSA, bovine serum albumin; MCA, middle cerebral artery; NO, nitric oxide; iNOS, inducible form of NO synthase.

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eye and ear were shaved, and the left common carotid artery exposed through a midline ventral cervical incision. The vagus nerve was preserved, and a loop of 4-0 silk placed around the artery for future manipulation. Next, the right common carotid artery was exposed and permanently occluded by double ligation with 4-0 silk.

A microsurgical craniotomy was performed via a 1-cm right scalp incision, and with the aid of the dissecting microscope, the right MCA was exposed through a 2-mm burr hole placed 2 mm rostral to the fusion of the zygoma with the temporal bone. Drilling, performed under a continuous drip of normal saline to avoid transmission of heat to the underlying cortex, continued until a thin shell of bone remained. This bone was carefully removed with a microhook and microforceps to avoid injury to the underlying structures. The right MCA was exposed by cutting the dura with a 30-gauge needle in a location approximately 1 mm from the rhinal fissure. The right MCA was elevated from the cortical surface by using a micromanipulator and a 20-μm tungsten wire hook, and an electrocautery tip was gently applied to the hook to sever the artery without underlying cortical injury. Within 5 min of severing the right MCA, the left common carotid artery was temporarily occluded for 30 min; the incisions were closed with a running vicryl suture, and the animals were returned to their cages for 24 h with unlimited access to food and water. After surgery, animals were somewhat clumsy but were able to walk, eat, and drink.

Measurement of Infarct Volume. Infarct volume was measured by using tetrazolium dye as previously described (25–28). Briefly, 24 h after the MCA was severed, animals were anesthetized and decapitated. Brains were quickly removed without perfusion and sectioned in the coronal plane at 1 mm intervals with a brain slicer. Slices were immersed and incubated in 2% (wt/vol) 2,3,5-triphenyl-2*H*-tetrazolium chloride/154 mM NaCl for 30 min at 37°C in the dark to stain for mitochondrial dehydrogenase activity. Infarct area in each slice was determined by planimetry, and infarct volume expressed as a percentage of the right hemisphere volume, calculated by the equation: (sum of infarct areas from all sections of a given animal) $\times$ (sum of total right hemisphere area from the same sections)$^{-1} \times 100$. Data were analyzed statistically by a factorial analysis of variance.

Measurement of Regional Cerebral Blood Flow. Cerebral blood flow was measured by using microspheres (29, 30). Briefly, rats were anesthetized with ketamine, and the femoral artery was cannulated with PE-50 polyethylene tubing (Clay Adams). The right MCA was exposed and severed as outlined above. Thirty minutes after injection (i.v.) of BSA or AGE-BSA, ^{141}Ce-labeled microspheres (15 μm) were administered into the left ventricle of the heart as a suspension [300,000–400,000 microspheres per ml in 10% (wt/vol) dextran; PBS; 0.3 ml per injection] via a 23-gauge needle connected to PE-50 polyethylene tubing. The microspheres were infused over 20 sec, and simultaneously, a reference arterial blood sample was withdrawn from the femoral artery catheter at a rate of 420 μl/min for 1 min. The anesthetized rats were then decapitated, the brains were removed, and the hemispheres were separated. A region of each hemisphere from 3 to 7 mm caudal to the frontal pole was excised, weighed, and the radioactivity was measured in a γ-counter. Cerebral blood flow was calculated from the equation: cerebral blood flow = [(reference blood withdrawal rate in ml/min) (cpm in the excised region)] [(excised region wt) (cpm in reference blood)]$^{-1}$.

RESULTS

Serum AGE Clearance Following Administration of Exogenous AGE-BSA. Circulating AGE levels are elevated in diabetic patients (13, 14, 23), but the influence of circulating AGEs on the pathogenesis of cerebral infarction is unknown. As an initial step in establishing an animal model where the neurotoxic effects of elevated, circulating AGE levels could be directly assessed, we first examined the serum clearance of AGEs in rats given exogenous AGE-BSA. Lewis rats received AGE-BSA (235 mg/kg) as a bolus injection via a surgically implanted carotid artery catheter, and total serum AGE levels were determined by ELISA. Peak AGE levels (120 $\pm$ 11 units/ml; mean $\pm$ SE; $n = 4$ animals per group) were detected 10 min after administration. Within 120 min, circulating AGE levels had fallen to baseline (20 $\pm$ 3 units/ml). The half-life of AGE in the serum was estimated to be 60 min.

Neurotoxic Effects of AGEs During Focal Cerebral Ischemia. Animal models of MCA occlusion have been widely used for the study of stroke, because they closely mimic the effects of focal stroke in humans (25, 31). In the present model, the MCA was microsurgically interrupted just distal to the lenticulostriate vessels, thereby limiting the zone of infarction to the cortex (Fig. 1). Systemically administered AGEs were markedly neurotoxic, as evidenced by significantly larger infarct volumes in AGE-treated animals (3.2% $\pm$ 1.1% in the presence of AGE-BSA) as compared to BSA-treated controls (0.6% $\pm$ 0.2% in the presence of BSA; $P < 0.05$). The neurotoxic effects of AGEs in this model were readily apparent as increased areas of infarction present on comparable stereotactic brain sections (Fig. 1). Moreover, AGE administration caused the zone of infarction to extend over a larger number of stereotactic brain sections (Fig. 1).

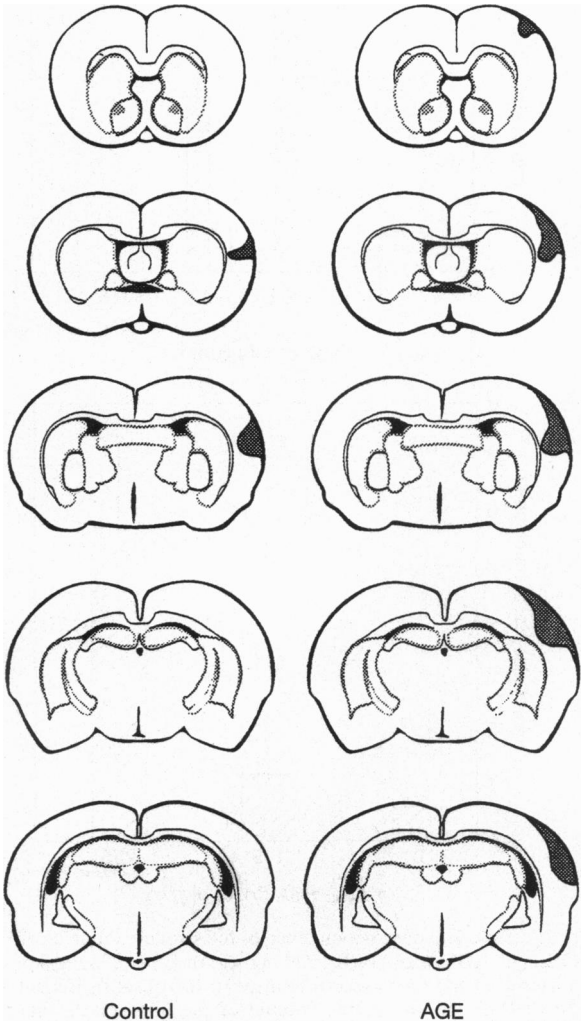

FIG. 1. Pattern of cerebral infarction observed at five stereotactic levels. Note that the infarcted region (stippled area) on each section is limited to the cortex. Shown are representative animals that received either BSA (Control) or AGE-BSA (AGE) 30 min prior to the onset of ischemia.

We performed experiments to bracket the time course of the neurotoxic effects of AGE-BSA in this model. Administration of AGE-BSA 2 h before cutting the artery caused a significant increase in infarct volume (5.4% ± 2.0%; $P < 0.05$ versus controls) (Fig. 2*A*). Stroke volume was also significantly increased by administering AGE-BSA 30 min prior to cutting the artery. The stroke-enhancing effects of AGEs were not observed, however, when the AGE-BSA was administered either 24 h prior to cutting the artery or 30 min after the artery was cut (Fig. 2*A*). These results suggest that the neurotoxicity of a single dose of AGEs, systemically administered in conjunction with focal cerebral ischemia, is a temporally restricted event, occurring when circulating AGE levels are increased immediately prior to the interruption of cerebral blood flow.

Separate experiments were performed to evaluate the relationship of AGE dose to the volume of resultant cerebral infarction. As a control for both total injectate volume and total quantity of albumin delivered, all animals received equal amounts of total albumin (235 mg/kg). Two hours before the MCA was microsurgically divided, control animals received BSA only, but experimental animals received AGE-BSA mixed with BSA. As shown in Fig. 2*B*, the neurotoxic effects of AGE-BSA were dose-related, with larger doses of AGE-BSA causing larger cerebral infarctions. Considered together, these data indicate that exposure to elevated serum AGE levels just prior to cessation of cerebral blood flow results in larger strokes, the size of which is ultimately dependent upon the dose of AGEs administered and the timing of AGE administration.

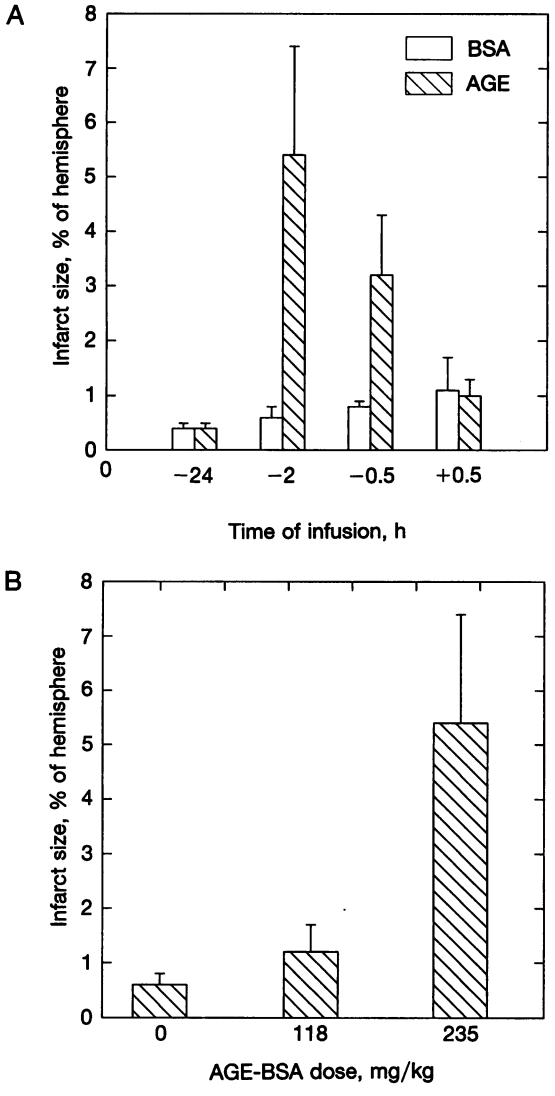

FIG. 2. Time and dose dependence of the stroke-enhancing effects of AGEs. (*A*) AGE-BSA (AGE; 235 mg/kg) or BSA (235 mg/kg) was administered at the times shown relative to the onset of ischemia at time = 0. Data shown are the volume of the hemisphere that was infarcted expressed as a percentage of the entire hemispheric volume. (Mean ± SE; $n = 6–8$ animals per group.) (*B*) Two hours before the onset of ischemia, AGE-BSA was administered in the doses shown. Data shown are the volume of the hemisphere that was infarcted expressed as a percentage of the entire hemispheric volume. (Mean ± SE; $n = 6–8$ animals per group.)

Effects of AGE Administration on Physiological Parameters. A number of physiological parameters are known to correlate with an increased volume of cerebral infarction in this model of focal stroke. For instance, a fall in blood pressure predictably reduces cerebral perfusion in the collateral vessels supplying the zone of ischemia and thereby increases infarct volume (7, 9). Other parameters which increase stroke size in this model are: bradycardia, hyperthermia, hyperglycemia, hypoxia, acidosis, and hypercarbia (7, 9). The effect of AGEs on each of these parameters was measured in a parallel group of animals at baseline (before the administration of BSA or AGE-BSA), when the MCA was cut ($t = 0$), and at hourly intervals thereafter for 2 h. In agreement with previous experience with this model, animals in both control and AGE groups developed a transient decrease in blood pressure and heart rate immediately after the MCA was occluded; these temporary effects normalized within 1 h. No statistically significant differences were observed between animals treated with AGE-BSA and those treated with BSA alone at any time point ($P > 0.05$; data not shown).

Effects of AGEs on Regional Cerebral Blood Flow. Blood flow to the ischemic cerebral cortex is derived from collateral blood vessels that are patent after blood flow through the occluded cerebral artery has ceased. Neuronal survival in the ischemic region is critically dependent upon the rate of regional cerebral blood flow from these collateral vessels, and any reduction of regional blood flow results in larger, more damaging infarctions (32, 33). Cerebral blood flow is regulated in part by nitric oxide (NO), a potent vasodilator that is produced in the blood vessel wall, and NO is implicated in the mediation of increased regional blood flow (34, 35). We have shown previously that chronic, repeated administration of AGE-BSA chemically inactivates the vasodilatory activity of NO in the systemic regulation of blood pressure, probably by local quenching (20, 36). On the basis of these observations, we considered it plausible in the present study that AGEs might diminish cerebral blood flow by inactivating NO in collateral cerebral vessels.

We performed experiments to measure the effects of AGEs on regional cerebral blood flow in this model. As shown in Table 1, microsurgical interruption of the MCA in BSA-treated controls reduced cerebral blood flow to a level that was 73% of the intact contralateral hemisphere. Surprisingly, rather than the predicted decrease, we found that the administration of AGEs actually increased absolute cerebral blood flow in both the normal and infarcted hemispheres. Administration of AGE-BSA did not change the proportional reduc-

Table 1. Effect of AGEs on regional cerebral blood flow

Treatment group ($n = 4$)	Left hemisphere (intact)	Right hemisphere (infarcted)	Ratio of infarct to intact, %
BSA	66 ± 5*	48 ± 3*	73
AGE-BSA	111 ± 6	86 ± 5	77
AGE-BSA + aminoguanidine	91 ± 11	63 ± 6	69

All data are expressed in ml/min per 100 g of tissue (mean ± SE). Cerebral blood flow was determined as described in *Materials and Methods*.

*$P < 0.05$ versus AGE-BSA.

tion of cerebral blood flow between the intact and the infarcted hemisphere (Table 1). Since blood pressure was not altered, these results suggest that AGE-BSA administration mediated vasodilation of cerebral resistance vessels with a resultant increase in cerebral blood flow.

Neurotoxic Effects of AGEs Are Inhibited by Aminoguanidine. Because aminoguanidine inhibits the accumulation of exogenously administered AGEs in the subendothelium and attenuates the pathological effects of AGEs in tissues (20–22), we performed an experiment to assess whether aminoguanidine would also attenuate the neurotoxic effects of exogenously elevated serum AGEs in the present focal stroke model. Aminoguanidine treatment in conjunction with exogenous AGEs reduced total cerebral infarction volume by 78% compared to AGE-treated controls that did not receive aminoguanidine (Fig. 3). Aminoguanidine did not significantly reduce the AGE-mediated increases of regional cerebral blood flow observed in either the infarcted hemisphere or the intact hemisphere (Table 1).

DISCUSSION

Within minutes after cessation of local cerebral blood flow, a region of densely ischemic brain tissue dies. This infarcted core is surrounded, however, by a zone of ischemic but potentially viable tissue termed the "ischemic penumbra," which receives suboptimal perfusion via collateral blood vessels (7, 9). The volume of the penumbra that ultimately becomes infarcted after an acute arterial occlusion is determined by a variety of factors that mediate neurotoxicity within this zone during the hours following interrupted blood flow. The nature of these neurotoxic factors (including glutamate, superoxide radicals, and NO) is only partially understood (7, 9, 10, 37). Some of these factors are intrinsic to the locus of ischemia, and others are delivered to the penumbra via the circulation. The present results now indicate that AGE-proteins, which are intrinsic to normal tissues and serum and significantly increased as a consequence of senescence and diabetes (13, 14, 23, 24, 38), are neurotoxic in the ischemic penumbra.

The molecular basis of AGE-mediated neurotoxicity is unknown, but one possible mechanism is through AGE-mediated induction of secondary signaling pathways. It has previously been shown that cellular recognition and uptake of AGE-modified molecules induce the expression of mRNA for tumor necrosis factor α (TNF-α), interleukins 1 and 6, and other growth factors and the production of reactive oxygen intermediates (16–18, 39). These cytokines and reactive oxygen intermediates have been implicated in increasing tissue injury during ischemia (40–45). The AGE-mediated induction of these mediators is down-regulated within hours, which may account for a window of maximal neurotoxicity within hours after exposure to AGEs. Although serum TNF-α levels were not elevated in the present study (data not shown), it is plausible that the neurotoxicity of AGEs is mediated through TNF-α or another secondary signal produced locally in tissues (46). This proposed mechanism is also consistent with our findings that AGEs administered 24 h before interruption of cerebral blood flow do not enhance stroke volume, presumably because the short-lived, AGE-induced signals are already down-regulated before the tissue is made ischemic. When administered 30 min after the artery has already been occluded, the AGE-proteins have only restricted vascular access to the ischemic region and may not have triggered the production of sufficient neurotoxic secondary signals necessary to significantly affect infarct size.

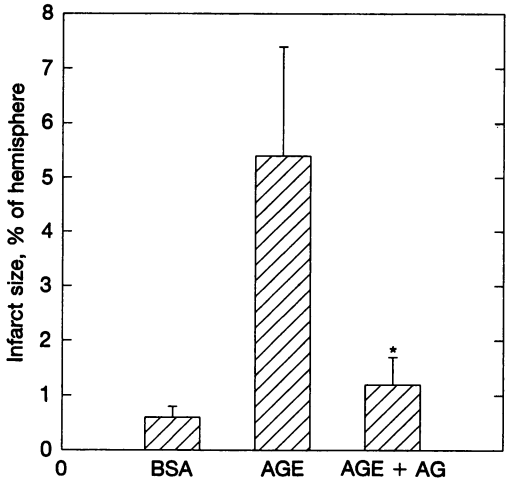

FIG. 3. Aminoguanidine attenuates the enhanced stroke volume associated with exogenous AGE administration. Two hours before the onset of ischemia, animals received either BSA (235 mg/kg), AGE-BSA (235 mg/kg) or AGE-BSA (235 mg/kg) and aminoguanidine (AG; 450 mg/kg). To control for the total volume injected, all solutions were given in a final volume of 3.2 ml/kg. Data shown are the volume of the hemisphere that was infarcted expressed as a percentage of the entire hemispheric volume. (Mean ± SE; n = 6–8 animals per group.) * ($P < 0.05$ vs. AGE).

Previous studies addressing the pathogenesis of increased stroke size in diabetic patients have implicated a number of factors, including increased procoagulant activities, micro- and macroangiopathy, hyperlipidemia, decreased red blood cell deformability, impairment of cerebrovascular autoregulation, and hyperglycemia (47–51). We now conclude that AGEs may also participate in the development of increased cerebral infarct damage in diabetic patients. AGE levels are known to be increased in serum proteins and low density lipoproteins of diabetic patients with or without renal dysfunction (13, 14, 24, 38). From the present data, we can now predict that the onset of cerebral ischemia in a patient with elevated serum AGE levels would be associated with the development of a larger cerebral infarction than would otherwise occur. It will be interesting to determine next whether serum AGE-protein or AGE-apolipoprotein B/low density lipoprotein levels can be used clinically to identify a subpopulation of diabetic patients at increased risk for catastrophic stroke.

Aminoguanidine confers protection against the neurotoxic effects of AGEs in focal cerebral ischemia. Although the molecular mechanism of neuroprotection cannot be precisely determined in this study, it is plausible that by reducing cross-linking between AGE-protein and basement membrane proteins, aminoguanidine reduced the production of secondary neurotoxic signals. Another possible neuroprotective mechanism of aminoguanidine is inhibition of the inducible form of NO synthase (iNOS), since NO has been implicated in mediating neuronal cell death during cerebral infarction (10, 44, 52–57). Recent investigations of the role of NO in focal stroke, however, indicate that the expression of iNOS does not begin to increase until more than 24 h after the onset of cerebral ischemia (58). Aminoguanidine inhibition of iNOS conferred late neuroprotection in this model (58). Because we observed an acute neuroprotective effect of aminoguanidine administered during the early periods of ischemia (and well before the reported period for increases in iNOS), it is unlikely that the protective effects of aminoguanidine in the present study were due to inhibiting iNOS. Further evidence suggesting that the effects of aminoguanidine were independent of NOS inhibition is obtained from our observations that aminoguanidine did not prevent the AGE-mediated increases in regional cerebral blood flow, a vascular response that is largely dependent upon NO.

Another potential mechanism for the neuroprotective effects of aminoguanidine is through its activity in abolishing the toxicity of polyamine metabolism by inhibiting the formation

of toxic aldehydes (59). Because the oxidation of polyamines is enhanced in cerebral ischemia (7, 9, 60–62) and the production of these toxic metabolites may further enhance neuronal killing, it is plausible that the neuroprotective effects of aminoguanidine are the result of inhibition of polyamine metabolism in focal cerebral ischemia. This potential mechanism of aminoguanidine-mediated neuroprotection may be effective even in the absence of exogenously added or abnormally elevated AGE levels. AGE-modified proteins and peptides are detectable in the circulation of normal (nondiabetic, nonsenescent) animals, including humans, and we have recently found that aminoguanidine attenuates focal cerebral infarction in normal animals during more prolonged ischemia (63).

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