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The Effect of Global Brain Ischemia in Normal and Diabetic Animals

The Influence of Calcium Channel Blockers

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Diabetes with hypertension is characterized by increased cerebrovascular pathology and poorer outcomes following stroke. In this study we evaluated the effect of global brain ischemia on brain metabolic parameters in normal and diabetic rats treated with a dihydropyridine calcium antagonist, felodipine. Normal and diabetic rats were treated daily with felodipine (5 mg/kg) or saline. After 4 wk global ischemia was produced by occluding the carotid arteries for 1 h. In other groups the occlusion was removed and the animals were allowed to reperfuse for an additional 2 h. Following 1 h global ischemia, with or without reperfusion, the animals and controls were killed by decapitation. Cerebral water, lactate, ATP, and glutamate were measured. Global ischemia with or without reperfusion increased cerebral water and lactate, but decreased ATP. Treatment with felodipine decreased lactate, but increased water content. Ischemia in diabetics with or without reperfusion decreased water and lactate. Treated diabetics had higher ATP levels after reperfusion. Glutamate levels were increased in diabetics and were further increased by treatment. We conclude that the enhanced CNS damage following cerebral ischemia in diabetes is not correlated with ATP or lactate levels and may be mediated in part by increased glutamate. Calcium channel antagonist may augment this process.

Key Words: Calcium channel blocker; cerebral ischemia; glutamate; lactate; diabetes.

Introduction

Epidemiological studies demonstrate that diabetes mellitus carries up to a sixfold increased risk of thrombotic stroke and causes 7% of deaths due to stroke *(1–3)*. Increased risk of cerebrovascular incidence in diabetic individuals persists even when it is corrected for other concomitant risk factors, i.e., hypertension *(4,5)*. Morbidity is worse, mortality is higher, cerebral edema more likely, and final neurological status is more severely impaired in hyperglycemic patients *(3,6,7)*. The cause for this specific grave outcome of stroke in diabetes needs further clarification. Ischemia is known to induce cellular acidosis and increase nerve cell dysfunction *(8,9)*; one characteristic consequence of these events is intracellular calcium overload in the nervous tissue *(10,11)*. Cellular calcium overload represents an important biochemical event during ischemia, hypoglycemia and hypoxia *(12)*. Consequently, pharmacological intervention that will ameliorate these changes in cellular calcium metabolism is expected also to decrease the cerebral ischemic damage. Diabetes can be characterized as a condition in which cell calcium homeostasis is impaired *(13)*. Studies by us and others in experimental diabetic models and in diabetic patients demonstrate that abnormal intracellular calcium metabolism is a common defect in insulinopenic and noninsulinopenic diabetes *(14,15)*. Abnormal intracellular calcium regulation in diabetic animals has also been described in cardiac muscle, aorta, skeletal muscle, kidney, liver, erythrocytes, lens, and osteoblasts *(14–17)*. Altered intracellular calcium metabolism has also been observed in diabetic patients and occult increases in plasma calcium levels have been described *(18)*. Thus, defective cell handling of Ca^{2+} in diabetes has been detected in all tissues tested so far suggesting that abnormalities in intracellular calcium metabolism constitutes a fundamental disorder in the diabetic state. Therefore, ischemia-induced changes in intracellular calcium load in the nervous tissue would likely be even more severe in diabetes. Consequently, it would be expected that calcium channel antagonists will be particularly effective in reducing ischemia-induced nervous tissue damage in diabetes.

In line with this assumption are findings by us and by others that calcium channel antagonists decrease the incidence of calcium-dependent alterations in diabetes *(16,19)*. In the present study we evaluated the effect of global ischemia on metabolic indexes of brain activity. Additionally, we sought to determine the influence of diabetes and/or an dihydropyridine calcium antagonist on these responses.

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Table 1
Brain Water in Normal and Diabetic Rats with and without Treatment with Felodipine Following Global Ischemia or Ischemia with Reperfusion[a]

	Basal	Ischemia	Ischemia + reperfusion
Normal	76 ± 3% (9)	86 ± 3%* (7)	83 ± 4% (4)
Normal treated	84 ± 4%$^{+}$ (5)	88 ± 3% (5)	86 ± 4% (5)
Diabetic	74 ± 2% (8)	75 ± 2%$^{+}$ (7)	83 ± 4% (9)
Diabetic treated	70 ± 2%$^{\#\$}$ (5)	74 ± 5%$^{+}$ (5)	83 ± 4%* (5)

[a]Data are expressed as % brain wt ± SE.
*$p < 0.05$ vs basal.
$^{+}p < 0.05$ vs normal.
$^{\#}p < 0.05$ vs diabetic.
$^{\$}p < 0.05$ vs normal treated.

Table 2
Brain Lactate Content in Normal and Diabetic Rats with and without Treatment with Felodipine Following Global Ischemia or Ischemia with Reperfusion[a]

	Basal	Ischemia	Ischemia + reperfusion
Normal	0.26 ± 0.09 (10)	0.92 ± 0.28*$^{+}$ (10)	0.38 ± 0.13 (7)
Normal treated	0.10 ± 0.02 (9)	0.25 ± 0.14^{+} (8)	0.28 ± 0.09 (8)
Diabetic	0.08 ± 0.04 (9)	0.59 ± 0.23*$^{+}$ (11)	0.23 ± 0.15* (10)
Diabetic treated	0.18 ± 0.03 (9)	0.25 ± 0.05$^{\#}$ (9)	0.20 ± 0.06 (10)

[a]Data expressed as mg/g brain ± SE.
*$p < 0.05$ vs basal.
$^{+}p < 0.05$ vs normal.
$^{\#}p < 0.05$ vs diabetic.

Results

The blood glucose in the different animal models demonstrate that both diabetic and diabetic treated animals had significantly higher glucoses than normals (normal 117 ± 4; normal treated 121 ± 8; diabetic 881 ± 16*; diabetic treated 621 ± 20*; *$p < 0.01$ vs. normal). The animals were killed by high-energy focused irradiation, and we evaluated cerebral water content, ATP concentration, lactate concentration, and glutamate concentration in normal and diabetic rats with or without treatment with a calcium channel blocker felodipine. The determinations were made following 1 h global ischemia or 1 h global ischemia following 2 h reperfusion.

The cerebral water content was increased from 76% to 86% following 1 h global ischemia and also following 2 h reperfusion (76% to 83%) (Table 1). Felodipine treatment in normal rats was associated with an increased cerebral water content under basal conditions; however, the water content was not increased following 1 h global ischemia or following the 2 h reperfusion period (Table 1). It can also be noted that diabetes was associated with a decreased cerebral water content following 1 h global ischemia compared to normals. Felodipine treatment in the ischemic reperfused diabetic animals had increased water content when compared to basal levels.

Global ischemia and ischemia plus reperfusion were both associated with an increase in lactate levels in normal rats (Table 2). The lactate levels were lower in felodipine-treated rats under basal, ischemic, and ischemic plus reperfusion conditions when compared to normals. Diabetes was also associated with an increase in tissue lactate levels following global ischemia and after reperfusion. However, these levels were lower than those found in normals. Treatment with felodipine did not significantly alter lactate levels in diabetic animals.

Global ischemia and ischemia following reperfusion resulted in a significant decrease in ATP levels in normal rats. In animals that were diabetic, ischemia was associated with a decrease in ATP and reached significant levels following reperfusion (Table 3). Felodipine-treatment in normal animals did not affect the ischemia-induced decrease in ATP levels. Diabetes animals had cerebral ATP contents that were comparable to those of normal rats. Felodipine-treated diabetic reperfused animals had lower ATP levels (Table 3) compared to basal levels.

Glutamate levels were not altered by global ischemia or ischemia with reperfusion in normal animals. Felodipine treatment also did not result in any changes in glutamate levels in normal animals. Diabetic animals tended to have higher glutamate levels and felodipine treatment increased it to a greater extent especially under basal and reperfused conditions (Table 4).

Discussion

We have observed that global ischemia is associated with an increase in brain water content in normal animals. This observation is in agreement with previous observations demonstrating enhanced water content following ischemia *(20–24)*. We also demonstrated that treatment with calcium channel antagonist increase basal water content but prevented the enhancement of water accumulation in response to ischemia *(22)*. Diabetes is associated with a lower brain water content but the water content was significantly increased following reperfusion. The above observations are also consistent with previous observations that demonstrated that chronic

Table 3
Brain ATP Content in Normal and Diabetic Rats with and without Treatment with Felodipine Following Global Ischemia or Ischemia with Reperfusion[a]

	Basal	Ischemia	Ischemia + reperfusion
Normal	1.89 ± 0.10 (10)	1.28 ± 0.16* (14)	1.17 ± 0.17* (9)
Normal treated	1.87 ± 0.11 (9)	1.22 ± 0.13* (11)	1.13 ± 0.18* (9)
Diabetic	1.78 ± 0.09 (11)	1.50 ± 0.17 (11)	1.45 ± 0.13* (14)
Diabetic treated	1.86 ± 0.08 (8)	1.70 ± 0.12*+ (8)	1.50 ± 0.13* (9)

[a]Data expressed as μmol/g brain ± SE.
*$p < 0.05$ vs basal.
+$p < 0.05$ vs normal treated.

Table 4
Brain Glutamate in Normal and Diabetic Rats with and without Treatment with Felodipine Following Global Ischemia or Ischemia with Reperfusion[a]

	Basal	Ischemia	Ischemia + Reperfusion
Normal	1.17 ± 0.06 (9)	1.16 ± 0.07 (8)	1.16 ± 0.09 (15)
Normal treated	1.14 ± 0.09 (6)	1.16 ± 0.05 (5)	1.23 ± 0.07 (5)
Diabetic	1.32 ± 0.05 (10)	1.34 ± 0.06+$ (13)	1.16 ± 0.11 (10)
Diabetic treated	1.85 ± 0.23+#$ (6)	1.33 ± 0.05+$ (9)	1.44 ± 0.04#+$ (9)

[a]Data expressed as mg/g brain wet weight ± SE.
*$p < 0.05$ vs basal.
+$p < 0.05$ vs normal.
#$p < 0.05$ vs diabetic.
$$p < 0.05$ vs normal treated.

hyperglycemia was associated with a decrease in brain water content and cell death in response to global ischemia *(7,8, 25)*. But this was not observed in all studies *(8)*.

The CNS lactate levels were increased following global brain ischemia in normal and diabetic animals and is consistent with the effects of decreased tissue perfusion *(26, 27)*. Treatment with calcium channel antagonist reduced the accumulation of lactate following ischemia in both normal and diabetic animals. This may be the result of blood vessel dilation and enhanced cerebral flow in these animals following felodipine treatment *(28)*. Although we did not monitor blood pressure, this has been suggested as a mechanism. As expected, reperfusion decreased the lactate levels in normal and diabetic animals. This further supports the concept of flow regulated lactate accumulation. The accumulation of lactate is associated with decreased pH, which could be a source of CNS injury *(29)*. Therefore, the felodipine treatment that prevented lactate accumulation could provide a protective effect against ischemia induced cerebral acidosis.

The diabetic animals revealed lower lactate levels at basal and at the ischemic conditions, although these levels did not reach significance. The explanation for that observation is not clear.

In this regard, the ATP content of the brain was evaluated as an index of the energy status of the brain. We observed that, as expected, it was significantly decreased following ischemia and did partially recover on reperfusion of 2 h, suggesting that full recovery might take longer. Diabetes was actually associated with higher levels of ATP following ischemia with or without reperfusion. The treatment with calcium channel antagonist did not affect the recovery of ATP levels in both the control or in the diabetic animals *(30)*. The decreased ATP during ischemia is consistent with previous observations that demonstrated decreased energy molecules following ischemia *(31)*.

In this study glutamate determination was used as an additional index of ischemic damage. The increased glutamate can be correlated to a glutamate-induced neurotoxicity *(32,33)*. The glutamate levels were not significantly changed in normals or in diabetic animals following the induction of the global ischemia, and calcium channel antagonist did not alter the glutamate levels in normals. To the contrary, both basal and ischemia and reperfused levels of glutamate were higher in the diabetic animals, and the calcium antagonist increased the glutamate levels further, significantly. These observations suggest that the enhanced glutamate released in diabetic animals may lead to enhanced neurotoxicity, and calcium channel blockers aggravate this phenomenon *(34)*. Glutamate, in addition to being a major neurotransmitter, has been demonstrated to exert potent neurotoxic effects during ischemia. Extracellular glutamate overload acts to enhance neuronal dysfunction *(32,35)*. The mechanism by which Ca^{2+} channel blocker may enhance glutamate accumulation remains unclear and deserves further investigation. However, these might be associated with defects in either calcium-mediated enhanced release or decreased reuptake mechanisms *(36,37)*.

Diabetes has consistently been associated with an increased incidence of stroke, and the resulting outcome tends to be more severe *(1,2)*. Hypertension is also associated with an increase in stroke and is a factor in decreased neurological outcome *(6,38)*. In the above studies, diabetes or the hyperglycemia of diabetes was not associated with increased water, lactate, or decreased ATP, and is not consistent with the poorer outcome associated with CNS ischemia. The hyper-

glycemia associated with diabetes, the increased blood pressure, and the altered tissue perfusion can increase tissue calcium levels, or the production of metabolites and free radicals *(10,13,39)*.

In other studies from our laboratory we have proposed that alterations in intracellular and extracellular calcium may pose a significant impact on the influence of diabetes on many systems *(16,40)*. We have demonstrated that calcium channel antagonist can slow or decrease some of the calcium-dependent changes associated with diabetes. However, in the above study we focused on the possible interaction of a calcium channel antagonist in diabetic and normal animals. In these animals cerebral lactate response to global ischemia was reduced suggesting a beneficial effect, but glutamate accumulation was enhanced suggesting a poorer outcome.

These studies demonstrate that there are multiple factors that may contribute to diabetes-induced decreased outcome following cerebral ischemia, especially the decreased ATP or increased glutamate. The hyperglycemia alone does not appear to significantly decrease ATP or increase lactate. However, treatment with calcium channel antagonist, although it tends to minimize lactate accumulation, could be a major factor in neurotoxicity by exacerbating glutamate accumulation.

Materials and Methods

Male Wistar rats, weighing 200–250 g, were divided into two groups. Diabetes was induced in one group by the infusion of streptozotocin (55 mg/kg diluted in citrate buffer saline, pH 4.5) via the tail vein. Diabetes was confirmed by fasting blood glucose in excess of 200 mg/dL. The second group received vehicle. Both non-diabetic and diabetic groups were subdivided and one-half were treated with felodipine (a dihydropyridine calcium channel antagonist) 5.0 mg/kg/d or saline given intraperitoneally for 4 wk.

Blood samples were collected on a weekly basis and before each study to evaluate the glycemic status. Plasma glucose was determined by a glucose analyzer (YSI, Yellow Springs, OH).

Global Ischemia and Reperfusion

On the day of the study the rats were anesthetized with sodium pentobarbital and placed on a heating pad throughout the subsequent surgical procedure. The animals were subjected to one of three procedures: (a) Global ischemia: global ischemia was induced by bilateral common carotid artery occlusion for 1.0 h with bulldog clips or ligated with surgical threads in the neck. (b) Global ischemia with reperfusion: in these animals following the 1 h occlusion, the clamps were removed and reperfusion (recirculation) was maintained for 2 h. (c) The third group were sham-operated and maintained for the appropriate time for controls.

Measurement

Cerebral ATP, Lactate, and Glutamate

Controls (sham-operated) 1 h global ischemia or 1 h global ischemia with 2 h reperfused animals were subjected to high-energy focused microwave irradiation *(19)* (5.0 W for 2.0–3.0 s) to prevent further metabolism, and then decapitated. One-half of the cerebrum was homogenized in 4 mL ice-cold 6% perchloric acid, and the homogenate centrifuged at 18,000*g*, and 0°C for 15 min. The supernatant was neutralized with 2 *M* K_2CO_3, and a sample used to determine tissue lactate and glutamate with a biochemistry analyzer (YSI 2700 select from YSI Incorporated, Yellow Springs OH). ATP concentration was determined by enzymatic assay (ATP Kit, Sigma Corp., St. Louis, MO). In brief, the extract was mixed with 12% TCA, centrifuged at 4000*g* for 10 min, and then add 1.0% PGA buffer I NADH vial and read vs water at 340 nm for initial unit then add 40 µL GAPD/PGK repeat reading to determine final units for calculation.

Cerebral Water

The weight (wet weight, WW) of the other half brain, the cerebral hemisphere, was determined after microwave irradiation. The cerebral hemispheres were then dried in an oven heated at 90°C for 24 h and the weight was again determined to obtain the dry weight (D). The cerebral water content was calculated as follows: cerebral water content (%) = $[(WW - D)/D] \times 100$.

Statistical analysis was performed on all data using a two-way ANOVA, followed by Fishers' pairwise comparison test.

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