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Gender-based Differences in Na^+–K^+ Adenosine Triphosphatase Activity Occur in the Microcirculation of the Diabetic Rat Brain

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SEVERAL mechanisms are thought to play a role in the development of diabetic microvascular disease. Among these mechanisms, protein kinase C (PKC) stimulation *via* increases in intracellular diacylglycerol (DAG) have been shown to play a role in development of diabetic microangiopathy.[1] In addition, it is thought that PKC stimulation also may lead indirectly to decreases in Na^+–K^+ adenosine triphosphatase (NKase) activity.[2] In animal models of diabetes mellitus (DM), changes in DAG-stimulated PKC activity have been shown in vascular beds of organs most susceptible to microangiopathy, *i.e.*, the retina and kidney. However, it is unknown whether these biochemical changes occur in the cerebrovasculature. The aim of this study was to determine whether increases in PKC activity and expression as well as decreases in NKase activity and expression occur in intracerebral microvessels of type 1 DM rats.

Studies examining the epidemiology of DM have shown that male type 1 DM rats have a higher incidence of end-organ complications than females.[3] A second aim of this study was to determine if gender-based increases in PKC activity and expression and decreases in NKase activity and expression occur in intracerebral microvessels of type 1 DM rats.

Materials and Methods

All procedures were approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions, Baltimore, Maryland.

Animal Model

Two groups of BB (biobreeding; Biomedical Research Models, Inc., Worcester, MA) rats were studied: DM prone (DPBB; n = 12) and DM resistant (DRBB; n = 12). There were six males and six females in each group. The BB rat is a genetic model of spontaneously developing autoimmune type 1 DM originally found in a closed colony of outbred Wistar rats.[4] From the animals originally identified as DM, two groups of animals were produced. The first was composed of the progeny of diabetic × diabetic matings from which the DPBB strain was developed. The second was composed of progeny selected to remain DM-free. In particular, the DRBB strain is derived from DP rats in the fifth generation of inbreeding.[4] Once the diabetic state is seen in the DPBB rats, insulin must be administered on a daily basis for continued survival. In the current study, insulin management throughout the DM period was based on AM whole blood glucose measurements, which were obtained *via* tail tipping to assist with insulin management (Yellow Springs Instrument Co., Yellow Springs, OH).

We selected the animals to be studied based on two criterion: (1) This study examined DM of 6–8 weeks duration. This length of DM was based on previous work in the BB rat documenting endothelial dysfunction by 8 weeks of diabetes.[5] (2) We wanted to match the weights of the DM and non-DM groups. This is because, in our laboratory, we have found that failure to match animal weight results in unacceptably large standard errors in infarction volume and in intraischemic laser Doppler flowmetry during focal brain ischemia protocols in rats.

Obtaining these endpoints produced a difference in age between the DRBB and DPBB groups. However, in terms of the main outcome variables of the study, PKC and NKase activity, it has been clearly shown in the Wistar rat strain that aging does not alter either PKC or NKase activity in the brain, neither on a regional nor on a global basis.[6,7]

Microvessel Preparation

Cerebral microvessel homogenates were prepared using the methods of Harder *et al.*[8] Briefly, rats were anesthetized with halothane, and the right common carotid artery was cannulated. The cerebral circulation was initially flushed with 20 ml cold HEPES-buffered physiologic salt solution, followed by 40 ml cold suspension of iron oxide particles (Aldrich, Milwaukee, WI) in HEPES-buffered physiologic salt solution at a concentration of

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10 mg/ml. The brain was removed quickly, placed on ice, and pressed through a 200-μm sieve (tissue grinder kit; Sigma, St. Louis, MO). The vascular tree and microvessel pieces laden with iron oxide were collected from the sieve surface, suspended in buffered saline, and separated using a side pull magnet (Biomag separator; Advanced Magnetics, Cambridge, MA). After isolation of the vascular tissue, the pellet was homogenized immediately in TRIS-EGTA buffer (10 mM TRIS HCL, 1 mM EGTA; pH 7.4) containing protease inhibitors (trypsin inhibitors [50 μg/ml], aprotinin [1 μg/ml], leupeptin [1 μg/ml], and pepstatin [1 μg/ml]). The side pull magnet was used to clear the homogenate of iron oxide particles. To obtain S2 and P2 fractions, whole homogenates were spun at 2,100 rpm for 10 min, after which the supernatant was centrifuged at 54,000 rpm for 20 min. After the high-speed spin, the supernatant was frozen as the S2 fraction. The pellet was resuspended and underwent a second high-speed spin, and the remaining pellet was resuspended and stored as the P2 fraction. Homogenates (whole, S2, and P2) were analyzed for protein content using the Lowry assay and stored in 10% glycerol at −70°C.

Quantitative Immunoblot Analysis

For Western blotting, whole, membrane-enriched (P2), and cytosolic-enriched (S2) homogenate fractions (10 μg each) were probed with antibodies to PKC βII (Gibco BRL, Rockville, MD) and NKase α_1 subunit (Upstate Biotechnology, Lake Placid, NY). Previous studies in rodents have shown that the principle isoform involved in diabetic vascular changes is PKC βII.[9] In addition, the NKase α_1 subunit has increased expression in the brain vasculature and has been shown to be effected by insulin management in DM.[10] Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system. Immunoblots for each animal were quantified densitometrically as previously described.[11] To control for differences in protein loading per lane, residual proteins in electroblotted gels were stained with Comassie blue, dried, and quantified densitometrically, and the optical densities were used as a correction factor.

PKC and NKase Enzyme Activity

To evaluate whether changes in PKC or NKase expression are associated with a gain or loss of function, biochemical assays of PKC and NKase were performed in whole homogenates. PKC determinations were done for each rat microvessel preparation in triplicate with a commercially available PKC enzyme assay system (Amersham, Pharmacia Brotech, Piscataway, NJ) that measures the transfer of ^{32}P from adenosine triphosphate (ATP) to a specific PKC substrate peptide in fresh frozen brain homogenates. NKase determinations were performed for each microvessel preparation (n = 24) using the method of Lin-Morales.[12] Linearity of PKC and NKase assays both were verified by using different amounts of protein in a constant reaction volume.

Table 1. Rat Demographics

		Diabetic (DPBB)	Nondiabetic (DRBB)
N	All	12	12
	Male	6	6
	Female	6	6
Weight (g)	All	307 ± 78	270 ± 74
	Male	379 ± 30	338 ± 23
	Female	236 ± 15*	202 ± 21
Days diabetic	All	54 ± 15	—
	Male	52 ± 6	—
	Female	56 ± 20	—
Age (days)	All	129 ± 12*	76 ± 9
	Male	131 ± 11*	78 ± 9
	Female	127 ± 14*	74 ± 9
Average AM glucose during days diabetic (mg/dl; whole blood)	All	304 ± 108	—
	Male	366 ± 144	—
	Female	263 ± 60	—
Glucose at sacrifice death (mg/dl; whole blood)	All	192 ± 129	116 ± 47
	Male	228 ± 155	100 ± 52
	Female	156 ± 96	132 ± 38
Insulin (U/day)	All	2.7 ± 0.4	—
	Male	3.1 ± 0.4	—
	Female	2.4 ± 0.2	—

Mean ± SD.

* $P < 0.05$ from respective nondiabetic group, Mann–Whitney U test.

Diacylglycerol

Diacylglycerol determinations were done for each microvessel preparation (n = 24; n = 12 each group) with a commercially available assay system (Amersham) that uses DAG kinase to measure the conversion of DAG to [^{32}P]-phosphatidic acid in the presence of [^{32}P]-γ-ATP in fresh frozen brain homogenates.

Statistics

Between-group comparisons were made using the Mann–Whitney test. To test relations between enzyme activity and blood glucose or DAG, linear regression using the Spearman coefficient was performed. A value of P less than 0.05 was considered significant.

Results

Table 1 summarizes the animal demographics for these experiments. Female DM rats weighed more than non-DM females. In addition, DM rats of both sexes were older than non-DM rats. In the DM animals, the glucose measurement at death was similar to the average AM glucose measurement obtained during the 6- to 8-week

Table 2. Biochemical Measurements of Microvessel Momogenates

		Diabetic (BBDP)	Nondiabetic (BBDR)
Na^+–K^+ ATPase activity (μmol Pi · mg^{-1} · h^{-1})	All	2.1 ± 0.9*	3.7 ± 1.4
	Male	2.0 ± 0.6*	4.2 ± 1.5
	Female	2.2 ± 1.2	3.2 ± 1.3
Protein kinase C activity (pmol Pi · μg^{-1} · min^{-1})	All	1.7 ± 0.5	1.3 ± 0.4
	Male	2.0 ± 0.2	1.5 ± 0.6
	Female	1.3 ± 0.4	1.2 ± 0.2
Diacylglycerol (nmol/mg protein)	All	9.1 ± 1.3	8.0 ± 2.9
	Male	8.2 ± 1.3	9.2 ± 3.1
	Female	10.0 ± 0.6*	6.8 ± 2.2
Protein kinase C βII isoform expression (optical density)	All	0.25 ± 0.09	0.23 ± 0.10
	Male	0.28 ± 0.11	0.22 ± 0.09
	Female	0.25 ± 0.07	0.24 ± 0.10
Na^+–K^+ ATPase α_1 subunit expression (optical density)	All	0.29 ± 0.04	0.30 ± 0.07
	Male	0.31 ± 0.03	0.30 ± 0.07
	Female	0.28 ± 0.03	0.31 ± 0.06

Mean ± SD.

* $P < 0.05$ from respective nondiabetic group, Mann–Whitney U test.

period of DM. This indicates that the blood glucose at death was similar to the chronic blood glucose.

Table 2 shows the biochemical measurements from the homogenates. NKase activity was decreased in the DM group, and the depressed activity was restricted to vessels obtained from males. However, PKC activity did not change with DM. Although DAG levels were increased in DM females, no correlation was found between DAG levels and PKC activity. PKC activity in DM correlated positively with blood glucose concentration at the time of death in all animals (Spearman coefficient $r = 0.709$; $P = 0.0118$). No correlation was evident between blood glucose at death and NKase activity. DM was not associated with changes in protein expression of either PKC βII or NKase α_1 subunit in the P2 fraction (table 2).

Discussion

These data suggest that, in type 1 DM, there is a gender-based predisposition toward decreased NKase activity in cerebral arterioles. This hyperglycemia-induced biochemical change in arteriolar vessels is present only in males. However, the functional vascular significance of altered enzymatic activity in the unstressed circulation of diabetic males remains unclear.

Patients with diabetes are at least 2 times more likely to have a stroke than nondiabetic patients. In addition, a higher prevalence of stroke is found in the patients with undiagnosed diabetes and glucose intolerance.[13,14] Cerebrovascular disease in the diabetic population follows the same pattern as in the nondiabetic population; however, diabetics are more likely to have increased morbidity and mortality after stroke.[13,14] Poorer stroke outcome with diabetes may be related to rheologic abnormalities, cerebral hemodynamic and vascular derangements, hyperglycemia, or other related risk factors.[13,14] The current study sought to determine whether similar biochemical changes in PKC and NKase, which occur in the microvasculature of the kidney and retina in DM, are present in the brain microvasculature.[5,6,9] The data indicate there is a gender-based predisposition toward depressed NKase activity in brain microvessels.

Inappropriate activation of protein kinase C has been implicated in the pathophysiology of many diseases, including DM. The chronic hyperglycemia of DM is thought to activate PKC *via* increased DAG levels. Among the various PKC isoforms, the βII and δ isoforms seem to be activated preferentially in the vasculatures of diabetic animals.[2] Experiments seeking to determine the expression of DAG-sensitive PKC isoforms in normal and diabetic rat glomerular cells have shown that the expression, compartmentalization, and activity of the α, βII, δ and ϵ isoforms are modulated independently by high glucose and insulin.[15] Changes in PKC βII have been shown clearly to occur in the retina of BB rats. However, no changes have been documented in the DM brain.[16,17] Our study sought to determine changes in the brain microvasculature as opposed to the whole brain. The current data indicate that the DM-induced changes in PKC found in organ systems susceptible to DM pathology do not occur in the DM brain. The close correlation between blood glucose at death and PKC activity indicates that increases in PKC activity with DM are influenced by the level of hyperglycemia at death. However, changes in DAG were independent of alterations in PKC activity, indicating that an alternative modulating mechanism must be at work.

Decreased NKase activity has been reported in both humans and animal models of DM. For example, erythrocyte NKase activity is decreased in type I diabetic patients.[18] In the streptozotocin DM rat, NKase activity is reduced in nerves, accompanied by a substantial reduction in α_1 isoform expression.[10] In this same diabetic model, brain NKase activity also decreases in a regionally specific manner.[19] Further, cerebral microvessel NKase activity is unaltered in streptozotocin DM,[7] in contrast to our current findings in the genetically diabetes-prone rat. Previous studies with the diabetic BB rat also reported an irreversible impairment of NKase function as early as 15 days after disease onset, despite insulin-based control of blood glucose at 6.7 mM.[20]

Our data extend these observations in that the impaired NKase activity observed in target organs for type

1 DM also is present in the cerebral microvasculature. This biochemical abnormality appears to be restricted to changes in enzymatic activity, rather than in protein expression (*i.e.*, unchanged NKase α_1 as quantified by Western blot analysis), and does not correlate with blood glucose. Further, depression of NKase activity is averted by some mechanism in female diabetic vessels. Whether this protection is linked to female reproductive steroids remains to be shown; however, recent findings indicate that female gender or estrogen administration reduces damage in experimental cerebrovascular occlusion.[21] Interactions between estrogen and DM could involve the steroid's well-known antioxidant properties.[22] For example, in nonvascular cell types, such as neurons, estrogen directly decreases oxidative impairment of membrane NKase activity.[23] Additional benefits conferred by steroid administration, which could have an impact on diabetic microvessels, include enhancement of cerebral perfusion under conditions of vascular stress,[24] protection of vascular endothelial integrity,[24] and enhancement of blood–brain barrier transport function.[25] Further studies are needed to determine whether exogenous estrogen administration restores NKase function in brain microvessels in diabetic males.

In conclusion, type 1 DM results in the biochemical alteration of NKase activity in cerebral microvessels, and the effect is gender specific. Whether this change in enzymatic activity is simply a disease marker or part of a vascular signaling derangement remains unclear.

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