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Cerebrovascular responsiveness to N^G-nitro-L-arginine methyl ester in spontaneously diabetic rats

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1 There is evidence that endothelial dysfunction is associated with diabetes mellitus. The purpose of the present study was to assess local cerebral blood flow (LCBF) and cerebrovascular responsiveness to the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) in spontaneously diabetic insulin-dependent BioBred (BB) rats.

2 Diabetic rats, and non-diabetic controls, were treated with L-NAME (30 mg kg^{-1}, i.v.) or saline, 20 min prior to the measurement of LCBF by the fully quantitative [^{14}C]-iodoantipyrine autoradiographic technique.

3 There were no significant differences in physiological parameters (blood pH, PCO_2, and PO_2, rectal temperature, arterial blood pressure, or plasma glucose) between any of the groups of rats, and no difference in either the extent or the temporal characteristics of the hypertensive response to L-NAME between diabetic and non-diabetic rats.

4 In diabetic rats, a global reduction in basal LCBF was observed, although significant reductions (between −20 and −30%) were found in only 5 (mainly subcortical) out of the 13 brain regions measured. Following L-NAME injection, significant reductions in LCBF (between −20 and −40%) were found in the non-diabetic animals. In diabetic animals treated with L-NAME, a significant reduction in LCBF was measured only in the hypothalamus (−33%).

5 The cerebrovascular response to acute L-NAME is attenuated in spontaneously diabetic insulin-dependent BB rats. This would be consistent with the endothelial dysfunction in cerebral vessels, known to be associated with diabetes mellitus and it is possible that a loss of NO-induced dilator tone, amongst other factors, may underlie the observed reductions of basal LCBF in these animals.

Keywords: Cerebral blood flow; diabetes mellitus; L-NAME; nitric oxide; nitric oxide synthase inhibition; quantitative autoradiography

Introduction

Diabetes mellitus is a metabolic disorder associated with functional and structural abnormalities in a variety of organs and systems of the body. In the cardiovascular system, diabetes is associated with the development of hypertension, accelerated atherogenesis, thrombosis and ischaemia, which are associated with both macro- and microvascular changes (Colwell, 1991). These cardiovascular complications were thought to be largely independent of the degree of diabetic control (Kannel & McGee, 1979), suggesting that it was the disease process itself which was responsible and not exposure to hyperglycaemia or insulin treatment. However, more recent work (Reichard *et al.*, 1993) suggests a more complex causal relationship between the disease process and therapeutic intervention.

Although the cerebral circulation has been found to be subject to vascular pathology similar to that found in the periphery (Aronson, 1973; Grunnet, 1963), the effects upon cerebrovascular physiology were previously thought to be rather subtle, and often went unrecognised. More recently however, it has become apparent that neither the brain nor its vasculature are spared from the effects of diabetic pathology (Mooradian, 1988; McCall, 1992), and altered blood/brain transport, cerebral blood flow, and brain metabolism, as well as effects on neurones and glia, are all associated with the disease process. Pathophysiological effects of the disease upon the cerebral circulation are manifest in an impaired autoregulatory response to alterations in systemic blood pressure (Kastrup *et al.*, 1986), and altered CO_2 reactivity (Griffith *et al.*, 1987).

In the streptozotocin-induced animal model of diabetes there is clear evidence, from both *in vitro* and *in situ* (cranial window) studies, of impaired cerebrovascular responsiveness to a variety of vasoactive compounds including ADP (Mayhan, 1989), 5-hydroxytryptamine (Rosenblum & Levasseur, 1984; Mayhan, 1989), β-adrenoceptor agonists (Mayhan, 1994), and acetylcholine (Mayhan *et al.*, 1991). Studies performed *in vivo* showed a reduced effect of muscarinic agonists upon blood flow (Pelligrino *et al.*, 1992). Interestingly, however, the streptozotocin rat model does not appear to display the same reduced cerebrovascular CO_2 reactivity found in human subjects (Pelligrino & Albrecht, 1991). Further *in vivo* studies of peripheral vascular beds in streptozotocin-treated rats have revealed a complex endothelial dysfunction with the pressor response to L-NAME being attenuated (Kiff *et al.*, 1991a) whilst vasodilator responses to acetylcholine remained intact (Kiff *et al.*, 1991b).

The BioBred (BB) rat strain provides a useful model for the study of insulin-dependent diabetes mellitus (IDDM). The involvement of genetic and immune aetiological factors in the pathogenesis of the disease, together with the dependence on exogenous insulin for prevention of ketoacidosis and the development of diabetic complications in a variety of organs (Marliss *et al.*, 1982), represent a condition more akin to the human disease process than that afforded by models of drug-induced diabetes (Eizirik *et al.*, 1994). Pathological changes in the retina, kidneys and peripheral nerves have been observed as early as 3 weeks following the onset of diabetes (Baird, 1989).

The purpose of this study was to measure the local cerebral blood flow (LCBF) in insulin-dependent diabetic BB rats to determine whether the decreases in CBF evident in human diabetes were paralleled in this animal model. Given the importance of NO in the regulation of normal cerebral blood

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flow (Faraci & Brian, 1994; Kimura *et al.*, 1994) and the fact that diabetes mellitus is associated with alterations of NO production and release in the extracerebral tissues (Bucala *et al.*, 1991; Corbett *et al.*, 1992; Cohen, 1993), diabetic rats were challenged with the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), to assess the involvement of the NO pathway in any perturbations in basal cerebrovascular control.

Methods

Animals

All rats were supplied from the British Diabetic Association BB (Edinburgh) U.K. Resource Unit. The BB/E colony consists of two lines created by selectively breeding for and against diabetes. In the high-incidence diabetes-prone (DP) main line, the incidence of IDDM is 50–60%, and the age at onset of diabetes is 96±18 days (mean±s.d.). In the diabetes-resistant (DR) subline, the incidence of diabetes is <1%.

All rats were maintained at 20°C on a 12 h light/dark cycle and fed rat and mouse Number 1 Expanded Feed (Special Diet Services, Witham, U.K.). Animals were weighed twice weekly from 40 days of age. Failure to gain weight, or loss of weight, was taken as an indication of the possible onset of diabetes, and such rats were tested for glycosuria. If glycosuria was detected, the blood glucose concentration was measured from a sample obtained by tail-tipping. A blood glucose concentration >18 mmol l^{-1} is invariably associated with ketonuria, weight loss and the requirement for daily injection of insulin to survive. These variables constituted our criteria for classifying an animal as having IDDM.

A total of 16 adult male BB rats (weight: 348–495 g) were used in this study from both the DP ($n=8$) and DR ($n=8$) sublines. At the time of the study all rats in the DP group were diabetic, and had been so for between 8 to 20 weeks. These animals had been treated since the onset of diabetes with a single daily subcutaneous injection of medium-acting insulin (2.4–4.0 iu) given at 09 h 00 min each day. Experiments were performed 4 h after the last insulin injection in the diabetic animals. Animals in the DR group were age-matched to those in DP group and served as controls for the effects of the disease processes.

Measurement of local cerebral blood flow

On the day of the experiment the animals were anaesthetized with halothane (1.5% in a gas mixture of 70% nitrous oxide and 30% oxygen) and prepared for the measurement of LCBF as described previously (Kelly *et al.*, 1994). Following surgery, general anaesthesia was withdrawn and 2 h allowed to elapse before any further experimental manipulation.

Equal numbers of non-diabetic and diabetic rats were injected (i.v.) with either L-NAME (30 mg kg^{-1}; $n=4$ from each group) or an equal volume of saline (1.0 ml; $n=4$ from each group) over 60 s via a femoral venous cannula. At this dose, L-NAME reduces LCBF significantly by 15 min post-injection and the effect is maintained stable for at least 3 h (Macrae *et al.*, 1993). The measurement of LCBF was started 20 min after the injection of L-NAME or saline by the fully quantitative [^{14}C]-iodoantipyrine autoradiographic technique. The protocols were in complete accordance with the methodology as originally published (Sakurada *et al.*, 1978) and as described previously from this laboratory (Kelly *et al.*, 1994). Autoradiographic images were analyzed by quantitative densitometry relative to ^{14}C-containing standards, and LCBF was calculated by the appropriate operational equation for the technique (Sakurada *et al.*, 1978). Areas of interest were chosen to represent brain areas in the vascular territories of the anterior, middle and posterior cerebral arteries. Arterial blood pressure and rectal temperature were monitored continuously in every animal throughout the experiments and heart rate was measured intermittently. Samples of arterial blood were withdrawn before and after L-NAME or saline injection, for the measurement of pH, P_{CO_2}, P_{O_2}, plasma glucose and haematocrit.

Data (presented as mean±s.e.mean) were analyzed by Student's *t* test with Bonferroni correction applied to allow multiple pair-wise comparisons between appropriate groups. Acceptable levels of significance were set at $P<0.05$.

Results

Physiological variables

Prior to the injection of either L-NAME or saline there were no significant differences in blood gas tensions, pH, rectal temperature or mean arterial blood pressure (MABP) between non-diabetic and diabetic rats, although heart rate was significantly lower (−22%) in the diabetic group (Table 1). Abnormally high base excess in the diabetic animals confirmed the chronic metabolic disturbances of diabetes, but prior to any drug treatment there were no significant differences in either plasma glucose or body weight between untreated non-diabetic and insulin-treated diabetic rats. There was, however, considerable variation in plasma glucose in both non-diabetic and diabetic rats (coefficient of variation = 58 and 71% respectively). Whilst this possibly indicates a more variable glucose metabolism in the non-diabetic BB subline than would normally be expected, it must be stressed that plasma glucose in all animals was within the normal physiological range (Table 1). Although there was a trend towards increased haematocrit in diabetic rats, this was not significant.

Following the injection of L-NAME, MABP increased to a similar extent in both non-diabetic (+21%) and diabetic rats

Table 1 The effects of L-NAME upon physiological variables in non-diabetic and diabetic rats

	Non-diabetic		*Diabetic*	
	Pre L-NAME	*Post L-NAME*	*Pre L-NAME*	*Post L-NAME*
pH	7.38±0.02	7.38±0.02	7.40±0.01	7.39±0.01
P_{CO_2} (mmHg)	42.5±1.5	35.3±1.8	45.2±1.8	40.8±0.9
P_{CO_2} (mmHg)	89.6±2.7	100.1±1.9	85.9±0.9	92.1±4.4
Base excess	−0.13±0.7	−3.1±1	2.9±0.7	0.3±0.8
Haematocrit (%)	47.8±0.5	50.5±1.0	52.5±1.0	54.9±1.0
Plasma glucose (mmol l^{-1})	15±4.5	14±5	9.5±3.5	7.5±3.5
Heart rate (beats min^{-1})	405±15	293±8#	315±15*	255±15
MABP (mmHg)	117±3	142±2#	121±3	149±4#
Temperature (°C)	36.0±0.1	36.5±0.1	36.4±0.3	36.4±0.2

Data are presented as mean±s.e.mean (n = 4 in each group). There were no differences in the values obtained from the saline-treated animals (diabetic and non-diabetic) and those measured prior to injection of L-NAME. *Significant difference between diabetic and non-diabetic animals; #significant difference between pre- and post L-NAME.

(+23%), but the heart rate was reduced significantly only in the non-diabetic group (−28%) (Table 1). In diabetic rats, where heart rate was already significantly lower prior to treatment (−22% compared to non-diabetics), the effect of L-NAME (−19%) was not significant. As a result, heart rates were similar in the two L-NAME-treated groups (diabetic and non-diabetic) following L-NAME (see Table 1).

Local cerebral blood flow

In saline-treated, diabetic rats, mean LCBF was reduced in all 13 brain areas when compared to non-diabetic controls (Table 2). However, the extent of these reductions in LCBF were regionally heterogenous, ranging from −8% in parietal cortex (not significant) to −32% in piriform cortex ($P<0.05$). Using the conservative statistics required for multiple comparisons, the reductions were statistically significant in only five of the areas examined, and these were predominantly sub-cortical (Table 2). Taking each diabetic animal individually, there was no correlation between the extent of LCBF reduction and either duration of diabetes or plasma glucose status at the time of the experiment.

In keeping with previous observations, L-NAME treatment produced reductions in LCBF throughout the brain in non-diabetic animals (Table 2). Only in parietal (−10%) and cingulate areas of cortex (−19%) and in nucleus accumbens (−21%) did the effects of L-NAME fail to reach statistical significance. Elsewhere, significant ($P<0.05$) reductions in LCBF were measured, ranging from −21% in the molecular layer of the hippocampus to −44% in piriform cortex (Table 2). In contrast, L-NAME treatment had no significant effect upon LCBF in diabetic rats, when compared to the appropriate saline-treated (diabetic) group. The one exception to this was the hypothalamus, where a significant (−33%) decrease in LCBF was observed (Table 2). In contrast to the significant differences in flow between the saline-treated non-diabetic and diabetic groups, there were no significant differences in LCBF between the groups treated with L-NAME (Table 2).

The cerebrovascular response to L-NAME will be influenced not only by the direct inhibition of NOS in the blood vessels of the brain, but also indirectly via autoregulatory responses to peripheral hypertension. Following L-NAME

Table 2 Local cerebral blood flow in diabetic and non-diabetic animals, treated with saline or L-NAME

	Saline		*L-NAME*	
	Non-diabetic	*Diabetic*	*Non-diabetic*	*Diabetic*
Neocortex				
Parietal	146±5	134±12	131±7	115±6
Cingulate	148±9	127±16	120±8	113±13
Occipital	133±2	99±11	83±9#	84±13
Piriform	111±6	76±6*	62±6#	66±6
Corpus callosum	39±2	32±1	27±1#	25+4
Basal ganglia				
Striatum	125±5	87±3*	93±2#	84±9
Globus pallidus	73±1	54±3*	50±2#	47±5
Accumbens	124±10	107±6	98±4	89±10
Thalamus				
Hypothalamus	95±3	80±6	59±5#	54±5#
Lateral geniculate	142±7	109±6*	84±2#	86±10
Hippocampus				
CA 2,3	94±5	74±7	60±3#	66±9
Molecular layer	90±2	68±8	70±11#	61±6
Dentate gyrus	92±4	72±4*	59±2#	67±10

Data are presented as mean local cerebral blood flow (ml $100g^{-1}\,min^{-1}$)±s.e.mean (n = 4 in each group). *Significant difference between diabetic and non-diabetic animals; #significant difference between saline and L-NAME-treated animals.

Table 3 Cerebrovascular resistance in diabetic and non-diabetic animals, treated with saline or L-NAME

	Non-diabetic			*Diabetic*		
	Saline	*L-NAME*	*% change*	*Saline*	*L-NAME*	*% change*
Neocortex						
Parietal	0.76±0.05	1.10±0.08#	45	0.84±0.08	1.32±0.10#	57
Cingulate	0.76±0.07	1.20±1.10#	58	0.90±0.12	1.37±0.16	52
Occipital	0.82±0.04	1.79±0.23#	118	1.15±0.15	1.93±0.37	67
Piriform	1.00±0.10	2.35±0.27#	135	1.46±0.15	2.33±0.30	60
Corpus callosum	2.86±0.13	5.34±0.26#	87	3.40±0.17	6.28±0.93	85
Basal ganglia						
Striatum	0.88±0.05	1.53±0.05#	74	1.26±0.07*	1.84±0.22	46
Globus pallidus	1.51±0.07	2.84±0.12#	88	2.03±0.12*	3.33±0.30#	64
Accumbens	0.91±0.10	1.47±0.07#	62	1.03±0.06	1.76±0.24	71
Thalamus						
Hypothalamus	1.16±0.07	2.49±0.24#	115	1.39±0.13	2.85±0.37#	105
Lateral geniculate	0.77±0.04	1.70±0.05#	121	1.05±0.05*	1.83±0.25	74
Hippocampus						
CA 2,3	1.16±0.01	2.40±0.08#	107	1.52±0.15	2.42±0.39	59
Molecular layer	1.22±0.05	2.37±0.24#	94	1.68±0.23	2.36±0.52	40
Dentate gyrus	1.20±0.05	2.43±0.10#	103	1.52±0.10*	2.43±0.40	60

Cerebrovascular resistances were calculated by dividing mean arterial blood pressure (mmHg) by LCBF values (ml $100g^{-1}\,min^{-1}$) for each individual animal and are presented as mean±s.e.mean (n = 4 in each group). *Significant difference between diabetic and non-diabetic animals; #significant difference between saline and L-NAME-treated animals.

treatment, calculated mean vascular resistance values were increased in all brain regions in both non-diabetic and diabetic rats (Table 3). In non-diabetic rats, these increases in resistance paralleled a decrease in LCBF in the majority of brain regions (Table 2), but in diabetic rats, whilst there was only moderate change in LCBF (with the exception of the hypothalamus), ranging between −3% and −21% (Table 2), cerebrovascular resistance increased by between 40 and 74% (Table 3). This increased resistance, with no change in flow, is likely to be the result of autoregulatory cerebrovascular constriction in response to peripheral hypertension.

Discussion

This is, to our knowledge, the first study of local cerebral blood flow in sponanteously diabetic insulin-dependent BB rats. The global tendency towards reduced cerebral blood flow which we have observed in these animals parallels to some extent that found originally in human diabetic patients (Kety *et al.*, 1948), but with the greater spatial resolution afforded by the use of quantitative autoradiography in our studies, we have been able to identify a degree of regional heterogeneity in the effects of diabetes upon cerebral blood flow. Whether this apparent differential susceptibility to the disease processes in different parts of the cerebrovascular bed reflects regional variations in vascular pathology, remains to be determined. Regional differences in LCBF have also been described recently in human diabetics when compared to healthy control subjects (Grill *et al.*, 1990; Macleod *et al.*, 1994). In these human studies a relative sparing of flow in fronto-parietal cortex and large decreases in the caudate nucleus show remarkable similarities to the results described here. However, even the most sophisticated imaging techniques currently availabe in man do not have the spatial resolution of animal brain autoradiography, nor can the experimental conditions be as rigorously controlled. It may, therefore, be impossible to find exact parallels between the effects of diabetes upon LCBF described in this study and those in diabetic humans.

A number of studies have examined cerebral blood flow in untreated streptozotocin-induced diabetic rats, with varying results (Duckrow *et al.*, 1987; Harik & LaManna, 1988; Jakobsen *et al.*, 1990; Pelligrino & Albrecht, 1991). Although in general terms reductions in LCBF were found when streptozotocin diabetic rats were compared to controls, the results were often too variable to reach statistical significance, and no clear consensus emerges on the susceptibility of particular regions of the brain to the condition. Interestingly however, if the rats were treated with insulin to normalize glycaemia at the time of the measurement, any differences in LCBF between diabetics and controls were eliminated (Pelligrino & Albrecht, 1991). A similar effect has also been described in peripheral nerve blood flow (Kihara & Low, 1995). In contrast, in the present study of spontaneously diabetic, insulin-dependent rats, significant decreases in LCBF were evident despite the fact that there was no difference in plasma glucose levels between diabetic and non-diabetic animals at the time of the study. This is not to say, however, that the BB rats have not experienced periods of hyperglycaemia. The measurement of LCBF in the diabetic animals was conducted around 4 h after the injection (s.c.) of medium-acting insulin, and the evidence from the physiological data suggests that the hormone was acting to normalize plasma glucose. Over a longer time scale, plasma glucose concentrations in BB/E rats are quite unstable and fluctuate in the course of any 24 h cycle between 3 and 22 mmol l^{-1}. In a parallel study using groups of rats from the same colony, we have found (unpublished observations) that glycosylated haemoglobin (HbA_1) values were elevated ($7.46 \pm 0.87\%$) compared to non-diabetic controls ($3.13 \pm 0.24\%$). There is no doubt, therefore, that our diabetic animals are hyperglycaemic for a large part of the time, although maximum plasma glucose levels are unlikely to reach those found in streptozotocin-treated rats.

In the present study there appeared to be an attenuation of the effects of L-NAME upon LCBF in diabetic rats. It is tempting to speculate that the decreases in LCBF apparent in saline-treated diabetics may be the result of reduced dilatator influence of endogenous NO in determining basal cerebral blood flow, possibly mediated via disinhibition of endothelin release (Gardiner *et al.*, 1995; Kelly *et al.*, 1995; Richard *et al.*, 1995). There is certainly evidence from the peripheral circulation that endothelial NO systems are disrupted in diabetes (Cohen, 1993; Poston & Taylor, 1995), although there is clear evidence of variation in the defect between different vascular beds (Kiff *et al.*, 1991a). In the cerebral circulation the data are equally complex. Indirect evidence for reduced NO activity comes from studies of streptozotocin-induced diabetic rats where a significant, but regionally variable, impairment of endothelium-dependent vascular relaxation was observed following injection of a muscarinic receptor agonist. More direct evidence of an impairment of cerebrovascular NO systems in this diabetic model are however lacking, in that the effects of L-NAME upon cerebral blood flow (Pelligrino *et al.*, 1992) and pial vessel diameter (Mayhan *et al.*, 1991) were reported to be similar in both non-diabetic and diabetic rats. Whilst we similarly found no difference in LCBF values between diabetic and non-diabetic rats following L-NAME, with the exception of the hypothalamus, this did not represent a significant decrease in flow from saline-treated diabetic rats in which blood flow was already depressed. This we interpret as an attenuation of the cerebrovascular response to L-NAME.

It is interesting that whilst we found reduced basal LCBF and an attenuated cerebrovascular response to L-NAME in our diabetic rats, the saline-treated diabetic animals were not hypertensive and L-NAME-treated diabetics displayed the normal blood pressure response, i.e. hypertension. This might suggest that the disease process is more pronounced in the cerebral circulation than it is in other vascular beds. However, the aortae of diabetic BB rats do develop morphological defects in endothelial cells and abnormal endothelium-dependent responses to acetylcholine (Meraji *et al.*, 1987). Moreover, the hypertensive response to chronic L-NAME administration is attenuated in diabetic BB/E rats (Lindsay *et al.*, 1995). Thus it is possible that the mechanisms of NO dysfunction associated with diabetes develop differentially in different vascular beds.

Although there is evidence that the effectiveness of endogenous NO in influencing basal vascular tone may be altered by diabetes (Bucala & Cerami, 1992; Wascher *et al.*, 1994), it is not clear whether this represents a change in synthesis and release, or in activity. There is evidence for reduced levels of L-arginine in the plasma of diabetic rats (Mans *et al.*, 1987) which might reduce NO synthesis, although there is also *in vitro* evidence that hyperglycaemia may actually increase NO production (Wascher *et al.*, 1994). However, *in vivo*, elevated intracellular glucose is converted to sorbitol via the polyol pathway in the endothelium with resultant depletion of cellular NADPH and reduced NOS activity (Cohen, 1993). The same pathway increases the formation of free radicals that inactivate endogenous NO (Cameron & Cotter, 1995). Finally, increased formation of subendothelial advanced glycosylation end products by elevated glucose may quench and inactivate NO (Bucala *et al.*, 1991; Cohen, 1993).

Although hyperglycaemia is believed to be an important factor contributing to vascular dysfunction associated with diabetes, and would certainly account for dysfunction in endothelial NO systems (Cohen, 1993; Poston & Taylor, 1995), other mechanisms may also be involved. Rheological problems such as an increase in plasma viscosity (Barnes *et al.*, 1977) and increased adhesion of platelets to endothelial cells (MacMillan *et al.*, 1978; Wautier *et al.*, 1981) may contribute to cerebrovascular dysfunction in diabetes, and some aspects of diabetic vascular pathology, notably arteriosclerosis (Grunnet, 1963), may be related to the hypertension often associated with diabetes. Hypertension develops only at a later stage in BB rats and is not therefore an issue in these studies, but in a parallel study using groups of rats from the same colony, we have

indeed found increased blood viscosity in BB diabetic animals. However not only is the significance of blood viscosity in determining LCBF contested (Brown & Marshall, 1985; Waschke *et al.*, 1994), it is also unlikely that increased viscosity can explain the heterogeneity in the reduction of blood flow which we observed.

There is increasing evidence that diabetes adversely affects the outcome in experimental models of cerebral ischaemia (Nedergaard & Diemer, 1987; Sutherland *et al.*, 1992) and in human occlusive stroke (Jørgensen *et al.*, 1994). Although the crucial role of elevated plasma glucose in situations of cerebral ischaemia cannot be over-emphasized (Smith *et al.*, 1986), evidence from the present study that there may also be an already perturbed basal blood flow and reduced endothelial NO activity, could represent an important additional factor contributing to the morbidity of stroke in diabetic patients.

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