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A POSSIBLE MECHANISM FOR INCREASED CEREBROVASCULAR PERMEABILITY IN DIABETIC RATS: EFFECTS OF INSULIN AND 2-DEOXY-GLUCOSE

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Abstract—1. Cerebrovascular permeability estimated as Evans blue extravasation significantly increased during insulin-induced hypoglycemia in both non-diabetic and diabetic rat groups.

2. Cerebrovascular permeability also increased significantly during 2-deoxy-glucose-induced intracellular glycopenia in both groups.

3. When a blood glucose level was fixed, cerebrovascular permeability showed no more significant change despite the hyperinsulinemic state in both groups.

4. Results suggest that hypoglycemia and/or intracellular glycopenia seems to be the major factor for increases in cerebrovascular permeability.

INTRODUCTION

It has been well known that microvascular permeability in whole body increases in the diabetic state (Parving, 1976; Palmberg, 1977; Mogensen, 1983; Mogensen *et al.*, 1983). Similarly, it was reported that blood–brain barrier permeability increased in diabetic animals (Stauber *et al.*, 1981; Lorenzi *et al.*, 1986). Recently, we reported that cerebrovascular permeability significantly increased along with insulin-induced hypoglycemia in diabetic rats (Tanaka *et al.*, 1992). However, it has not been clarified whether this increase in cerebrovascular permeability during insulin-induced hypoglycemia was provoked by hypoglycemia itself or action of insulin other than hypoglycemia. The present study, therefore, was designed to investigate the possible mechanisms for the increase in cerebrovascular permeability during insulin-induced hypoglycemia in diabetic rats. For this purpose, in this study, 2-deoxy-glucose (2DG)-induced intracellular glycopenia and hyperinsulinemic glucose clamp were drawn up in diabetic and non-diabetic rats, since 2DG can produce the intracellular glycopenia without insulin administration (Brown, 1962; Sokoloff *et al.*, 1977) and the hyperinsulinemic glucose clamp does not induce hypoglycemia but can maintain a fixed level of blood glucose despite hyperinsulinemia (DeFronzo *et al.*, 1979; Kraegen *et al.*, 1983).

MATERIALS AND METHODS

Induction of experimental diabetes

Six-week-old male Wistar rats were injected intraperitoneally (i.p.) with 80 mg/kg streptozotocin (STZ: Sigma, St Louis, Mo.) dissolved in 0.01 mol/l citrate buffer (pH 4.5) for induction of experimental diabetes as a diabetic group ($n = 24$). Rats with a plasma glucose level more than 19.4 mmol/l were included in this diabetic group. The control group ($n = 24$) was injected with buffered vehicle. All animals were allowed free access to rat chow and water. Rats of the diabetic and control groups were used for experiments 12 weeks after STZ or vehicle injection.

Effects of insulin on cerebrovascular permeability

The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) after a 24 hr fast at 9:00 a.m. Blood samples were obtained from the femoral vein for determination of plasma glucose following glucose oxidase method and of glycated hemoglobin following affinity column method, respectively. Two hundred U/kg of rapid-acting insulin (Humalin R, Eli Lilly and Co., Indianapolis, Ind.) was administered intraperitoneally to diabetic rats ($n = 4$) and non-diabetic rats ($n = 4$). The tip of the tail was amputated and blood glucose was monitored using enzymatic test strip (Glucostix, Miles Laboratories, Elkhart, Ind.). Ninety minutes after achievement of hypoglycemia below 2.2 mmol/l, 0.1 g/kg of Evans blue (EB, Sigma, St Louis, Mo.) was injected intravenously. Thirty minutes after injection of EB, the chest was opened through a midsternal thoracotomy, and a catheter was inserted into the ascending aorta through the left ventricle to perfuse with 500 ml of saline at 100 cm H_2O pressure. Then the animal was decapitated and the whole brain was removed immediately.

Four rats each of the diabetic and control groups received the perfusion and removal of the brain without EB injection for determination of tissue blank.

The basal level of EB extravasation was measured using four rats each of the diabetic and control groups which were not administered with insulin.

The EB extravasation was measured following the method of Rössner and Tempel (1966). Briefly, the isolated brain was homogenized with 60% trichloroacetic acid at 3 ml/g wet tissue, and centrifuged at 6200 *g* for 20 min. The supernatant was diluted 4-fold with 100% ethanol, and colorimetry was made by means of a spectrophotometer (UVIDEC-40, Nihon Bunkou, Tokyo, Japan) at 620 nm of the absorption for EB. The remainder, which was obtained

by subtracting the absorption of tissue blank from the total EB absorption measured above, was applied to the straight line for calibration of standard EB doses. EB extravasation was thus defined and expressed in terms of μg/g wet tissue. The values of tissue blanks obtained from the control and diabetic groups were 1.31 ± 0.04 μg/g wet tissue ($n = 4$) and 1.48 ± 0.03 μg/g wet tissue ($n = 4$), respectively. There was no significant difference between these two values.

Effects of 2-deoxy-glucose (2DG) on cerebrovascular permeability

The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) after 24 hr fast at 9:00 a.m., and blood samples were obtained from the femoral vein for determination of plasma glucose and glycated hemoglobin following the methods described above. The 2DG-treated non-diabetic rats ($n = 8$) were injected intravenously with 0.8 mmol/kg of 2DG and the 2DG-treated diabetic rats ($n = 8$) were injected with 2.4 mmol/kg of 2DG. Threefold difference at the dose of 2DG was based on 3-fold difference seen in the values of plasma glucose and glycated hemoglobin of the non-diabetic and diabetic rats (Table 1). Four ($n = 4$) or 8 ($n = 4$) hr after 2DG injection, 0.1 g/kg of EB was administered intravenously. Thirty minutes after EB injection, the chest was opened and saline-perfusion and removal of the whole brain were achieved. Thereafter, in the same manner described above, the brain was homogenized and EB extravasation was defined by a spectrophotometry.

Effects of hyperinsulinemic glucose clamp on cerebrovascular permeability

The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) after 24 hr fast at 9:00 a.m. Blood samples for determination of plasma glucose and glycated hemoglobin were obtained from the femoral vein. Two hundred U/kg of rapid-acting insulin was administered intraperitoneally to the diabetic group ($n = 4$) and the control group ($n = 4$). A catheter was inserted into the left femoral vein, and 20% dextrose was infused using an infusion pump (TERUFUSION Syringe Pump model: STC-531, Tokyo, Japan) in order to maintain blood glucose at the level before insulin injection. Thus, the hyperinsulinemic glucose clamp was achieved. The tip of the tail was amputated and blood glucose was monitored every 10 min using the enzymatic test strip. Four hours after insulin injection, 0.1 g/kg of EB was administered intravenously. Thirty minutes after EB injection, the chest was opened, and then saline-perfusion and removal of the whole brain was followed in the same manner as described above. Thereafter, the brain was homogenized and EB extravasation was defined as mentioned above. Plasma insulin concentrations at the basal state and 4 hr after hyperinsulinemic glucose clamp were measured by the enzyme immunoassay (EIA) method using EIA kit "Immunoball IRI Neo" (Ono Pharmaceuticals, Tokyo, Japan).

Statistical analysis of the data

The unpaired Student's t-test was used for statistical comparison of the groups. A P value less than 0.05 was regarded as significant. The data were expressed as mean $\pm$ SEM.

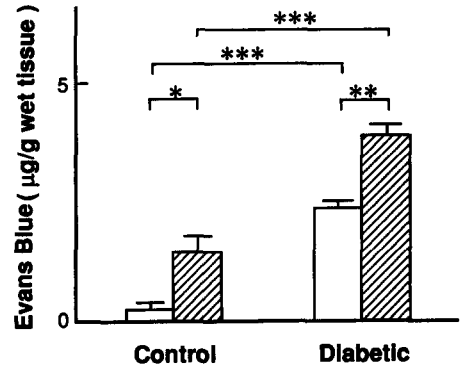

Fig. 1. Evans blue extravasation in the brain during insulin-induced hypoglycemia in non-diabetic (control) and diabetic rats. (□) Basal level ($n = 4$), (▨) during insulin-induced hypoglycemia ($n = 4$). Each column represents mean $\pm$ SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

RESULTS

General features of experimental rats

General features (values of body weight, wet brain weight, fasting plasma glucose and glycated hemoglobin) of non-diabetic (control, $n = 24$) and diabetic rats ($n = 24$) at the time of the experiment are summarized in Table 1. Body weight and wet brain weight in the diabetic group were significantly lower than those in the control group ($P < 0.001$), while fasting plasma glucose and glycated hemoglobin in the diabetic group were about threefold higher than those in the control group ($P < 0.001$).

EB extravasation during insulin-induced hypoglycemia

Values of EB extravasation during insulin-induced hypoglycemia in control and diabetic groups are presented in Fig. 1. The basal values of EB extravasation in control and diabetic groups were 0.24 ± 0.14 μg/g wet tissue ($n = 4$) and 2.36 ± 0.14 μg/g wet tissue ($n = 4$), respectively, showing that the diabetic group significantly increased cerebrovascular permeability at the basal state compared with the control group ($P < 0.001$). The values of EB extravasation in the insulin-hypoglycemic control group and the insulin-hypoglycemic diabetic group were 1.43 ± 0.28 μg/g wet tissue ($n = 4$) and 3.91 ± 0.20 μg/g wet tissue ($n = 4$), respectively. Thus, the values of EB extravasation during insulin-induced hypoglycemia in both control and diabetic groups significantly increased compared with each basal level ($P < 0.05$ and $P < 0.01$, respectively), and the value in the diabetic group was significantly higher than that in the control group ($P < 0.001$).

Table 1. General features of experimental rats

	Age (weeks)	Body weight (g)	Wet brain weight (g)	Fasting plasma glucose (mmol/l)	Glycated hemoglobin (%)
Control ($n = 24$)	18	363 ± 3	2.09 ± 0.01	6.0 ± 0.1	4.8 ± 0.1
Diabetic ($n = 24$)	18	$120 \pm 4^{***}$	$1.76 \pm 0.01^{***}$	$18.7 \pm 0.3^{***}$	$14.7 \pm 0.1^{***}$

Mean $\pm$ SEM. n = Number of rats. $^{***}P < 0.001$ vs control.

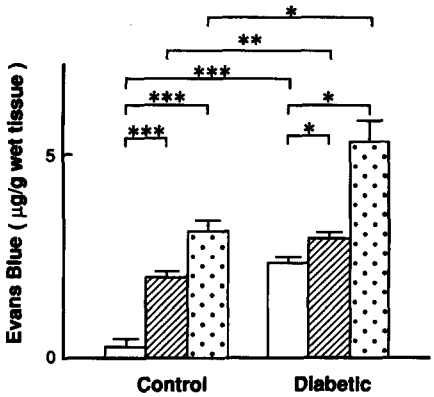

Fig. 2. Evans blue extravasation in the brain by 2-deoxy-glucose (2DG) injection in control and diabetic groups. (□) Basal level ($n = 4$), (■) 4 hr after 2DG injection ($n = 4$), (⊡) 8 hr after 2DG injection ($n = 4$). Each column represents mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

EB extravasation by 2DG injection

The values of EB extravasation at 4 hr after 2DG injection in the control and diabetic groups were $1.99 \pm 0.13\ \mu g/g$ wet tissue ($n = 4$) and $2.89 \pm 0.12\ \mu g/g$ wet tissue ($n = 4$), respectively (Fig. 2). These values of EB extravasation in the 2DG-treated control and diabetic groups were significantly higher than each basal level ($P < 0.001$ and $P < 0.05$, respectively), and the value in the 2DG-treated diabetic group was significantly higher than in the 2DG-treated control group ($P < 0.01$). Furthermore, the values of EB extravasation at 8 hr after 2DG injection in control and diabetic groups reached $3.13 \pm 0.27\ \mu g/g$ wet tissue ($n = 4$) and $5.34 \pm 0.46\ \mu g/g$ wet tissue ($n = 4$), respectively. Thus, the values of EB extravasation at 8 hr after 2DG injection in control and diabetic groups increased further significantly when compared with those at 4 hr after 2DG treatment ($P < 0.05$), and the value at 8 hr after 2DG injection in the diabetic group was significantly higher than that in the conrol group ($P < 0.05$).

Table 2. Plasma insulin levels in basal state and 4 hr after hyperinsulinemic glucose clamp

	Basal level (μU/ml)	After clamp (μU/ml)
Control ($n = 4$)	44.0 ± 1.6	1051.5 ± 11.3***
Diabetic ($n = 4$)	7.0 ± 0.9††	1100.3 ± 12.8***

Mean ± SEM. n = Number of rats.
††$P < 0.01$ vs control; ***$P < 0.001$ vs basal level.

EB extravasation in hyperinsulinemic glucose clamp

Plasma insulin concentrations in basal samples were $44.0 \pm 1.6\ \mu U/ml$ ($n = 4$) in the control group and $7.0 \pm 0.9\ \mu U/ml$ ($n = 4$) in the diabetic group, and there was a significant difference ($P < 0.01$) between these two groups. Plasma insulin levels at 4 hr after hyperinsulinemic glucose clamp reached $1051.5 \pm 11.3\ \mu U/ml$ ($n = 4$) in the control group and $1100.3 \pm 12.8\ \mu U/ml$ ($n = 4$) in the diabetic group. These values were significantly high compared with basal levels in each group ($P < 0.001$), but there was no significant difference in the values between the control and diabetic groups after hyperinsulinemic glucose clamp (Table 2).

Time-courses of changes in blood glucose during hyperinsulinemic glucose clamp in control and diabetic groups are illustrated in Fig. 3, showing confirmation of glucose clamp despite hyperinsulinemia. The values of EB extravasation after hyperinsulinemic glucose clamp in the control and diabetic groups were $0.64 \pm 0.14\ \mu g/g$ wet tissue ($n = 4$) and $2.95 \pm 0.34\ \mu g/g$ wet tissue ($n = 4$), respectively, as shown in Fig. 4. These values showed no more significant increase compared with each basal level.

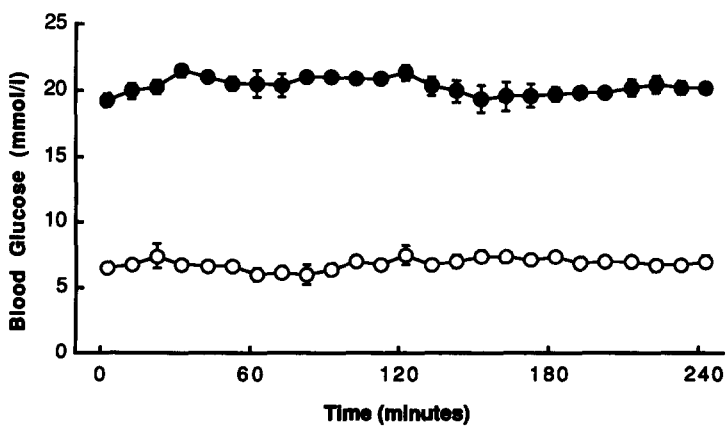

Fig. 3. Time-courses of changes in blood glucose levels during hyperinsulinemic glucose clamp. (○) Control group ($n = 4$), ● diabetic group ($n = 4$). Each point indicates mean ± SEM, and small SEM is included within circles.

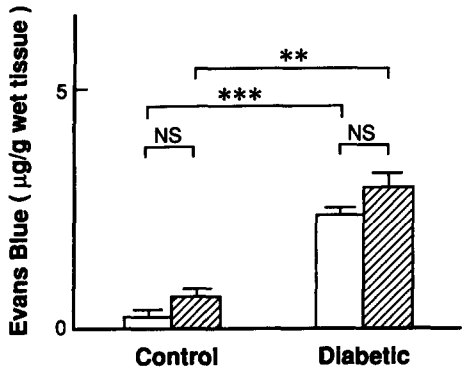

Fig. 4. Evans blue extravasation in the brain after hyperinsulinemic glucose clamp in control and diabetic rats. (□) Basal level ($n = 4$). (■) 4 hr after hyperinsulinemic glucose clamp ($n = 4$). Each column represents mean ± SEM. NS; not significant, $^{**}P < 0.01$, $^{***}P < 0.001$.

DISCUSSION

In the present study, under non-hypoglycemic basal conditions, cerebrovascular permeability significantly increased in diabetic rats compared with non-diabetic rats. Although the exact mechanisms for the increased cerebrovascular permeability in diabetes mellitus were not explained by the present results alone, the present findings are in accordance with previous reports which have suggested a possible participation of increased vascular permeability to plasma proteins in diabetes mellitus (Stauber *et al.*, 1981; Lorenzi *et al.*, 1986; Tanaka *et al.*, 1992), and at the same time they prove the appropriateness of the present experimental procedure. During insulin-induced hypoglycemia, cerebrovascular permeability further and significantly increased in both control and diabetic groups, although the degree of its increase was more significant in the diabetic group than in the control group. These results are similar with our previous report (Tanaka *et al.*, 1992).

2-Deoxy-glucose used in the present study is an analogue of glucose which is transported from blood to brain tissue by the same carrier that transports glucose into the cell, and is phosphorylated to 2-deoxyglucose-6-phosphate which is not further metabolized. Thus, 2-deoxyglucose-6-phosphate trapped in the tissue competitively inhibits glucose metabolism and induces intracellular glycopenia. As a result, it has been proposed that 2DG leads to inhibition of glycolysis and has a potency of producing intracellular glycopenia without reducing plasma glucose (Brown, 1962; Sokoloff *et al.*, 1977). In the present study, we utilized this property of 2DG and examined cerebrovascular permeability after 2DG injection. It was observed that EB extravasation after 2DG injection significantly increased more than basal levels in both control and diabetic groups. This observation suggests that 2DG-induced increase in cerebrovascular permeability would be resulted from intracellular glycopenia.

Some investigators have reported that intravenous administration of 2DG increased insulin release in mice and dogs (Karlsson *et al.*, 1987; Guo *et al.*, 1988). According to their reports, however, the value of increased insulin did not correspond to hyperinsulinemia but was within a physiological range. Therefore, the influence of endogenous insulin that secreted in response to 2DG would be disregarded. On the other hand, Rapoport *et al.* (1972, 1980) have reported that blood–brain barrier permeability was increased by injection of hyperosmolar agents such as urea, sodium chloride and ethanol. However, since the osmolarity of 2DG administered in the present study was significantly lower than the calculated threshold osmolarity of hyperosmolar agents which have been reported to increase blood–brain barrier permeability, it is unlikely that cerebrovascular permeability increased by 2DG injection may be induced by its osmolarity.

The present results show that EB extravasation after hyperinsulinemic glucose clamp did not change significantly from basal levels in both control and diabetic groups. Some investigators have proven the existence of insulin receptors on blood–brain barrier (van-Houten and Posner, 1979; Pardridge *et al.*, 1985) and Frank *et al.* (1986) have suspected that the insulin receptor involves in a part of transendothelial transport system in brain. On the other hand, there are many reports that insulin has no influence on all the cerebral functions including glucose transport and metabolism (Betz *et al.*, 1979; Lund-Anderson, 1979; Goodner *et al.*, 1980; Hom *et al.*, 1984; Shapiro *et al.*, 1990). If insulin itself acts on glucose transport and metabolism in the brain, however, it's action seems not so significant, since the present results of hyperinsulinemic glucose clamp proved that the increment of cerebrovascular permeability after insulin injection would be introduced by hypoglycemia rather than hyperinsulinemia.

Generally, it has been reportedly known that the glucose utilization rate in whole brain reduces by about 20–30% during hypoglycemia (Eisenberg and Seltzer, 1962; Suda *et al.*, 1990), that the hexose distribution volume in the brain increases 2–3 times during hypoglycemia (Shapiro *et al.*, 1990), and that transport of glucose from blood to the brain increases during hypoglycemia (Lund-Anderson, 1979). These phenomena might be concrete features of self-protecting mechanisms of brain during hypoglycemia. On the other hand, the increase in EB extravasation caused by hypoglycemia and/or intracellular glycopenia may reflect a progress of dysfunction in the endothelial transport system of brain, since it is

considered that EB extravasation means increased permeability to protein that does not appear in the intact state. Therefore, it is speculated that prolonged hypoglycemia and/or intracellular glycopenia induces some functional disorders in the endothelium, resulting in increased EB extravasation.

In conclusion, it was observed in the present study that cerebrovascular permeability increased during insulin-induced hypoglycemia in both non-diabetic and diabetic rat groups, and the permeability in the diabetic group increased more significantly than that in the control group. Cerebrovascular permeability also increased during 2DG-induced intracellular glycopenia in both groups, and the increase was more significant in the diabetic group. On the other hand, when blood glucose was maintained at a fixed level, cerebrovascular permeability showed no more significant change despite hyperinsulinemic conditions in both groups (hyperinsulinemic glucose clamp). Therefore, hypoglycemia and/or intracellular glycopenia seems to be the major factor for increases in cerebrovascular permeability. Further extended studies are necessary to clarify the precise mechanisms for the increased permeability by hypoglycemia and/or intracellular glycopenia.

SUMMARY

In our previous study, increased cerebrovascular permeability was observed during insulin-induced hypoglycemia in the diabetic state. However, it has not been clarified whether the increase in cerebrovascular permeability during insulin-induced hypoglycemia was provoked by hypoglycemia itself or other action of insulin than hypoglycemia. To investigate the possible mechanisms for the increase in cerebrovascular permeability during insulin-induced hypoglycemia, cerebrovascular permeability during 2-deoxy-glucose (2DG)-induced intracellular glycopenia and after hyperinsulinemic glucose clamp was elucidated by measurement of extravasation of Evans blue (EB) dye as an albumin tracer.

Increased EB extravasation in the brain during insulin-induced hypoglycemia was confirmed in both non-diabetic and diabetic rats, and the degree of EB extravasation was more significant in the diabetic group than that in the control group. Cerebral EB extravasation also increased during 2DG-induced intracellular glycopenia in both groups, and the increase was more significant in the diabetic group. On the other hand, when blood glucose was maintained at a fixed level, the EB extravasation showed no more significant change despite hyperinsulinemic state in both groups (hyperinsulinemic glucose clamp).

These results suggest that hypoglycemia and/or intracellular glycopenia seems to be the major factor for increases in cerebrovascular permeability.

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