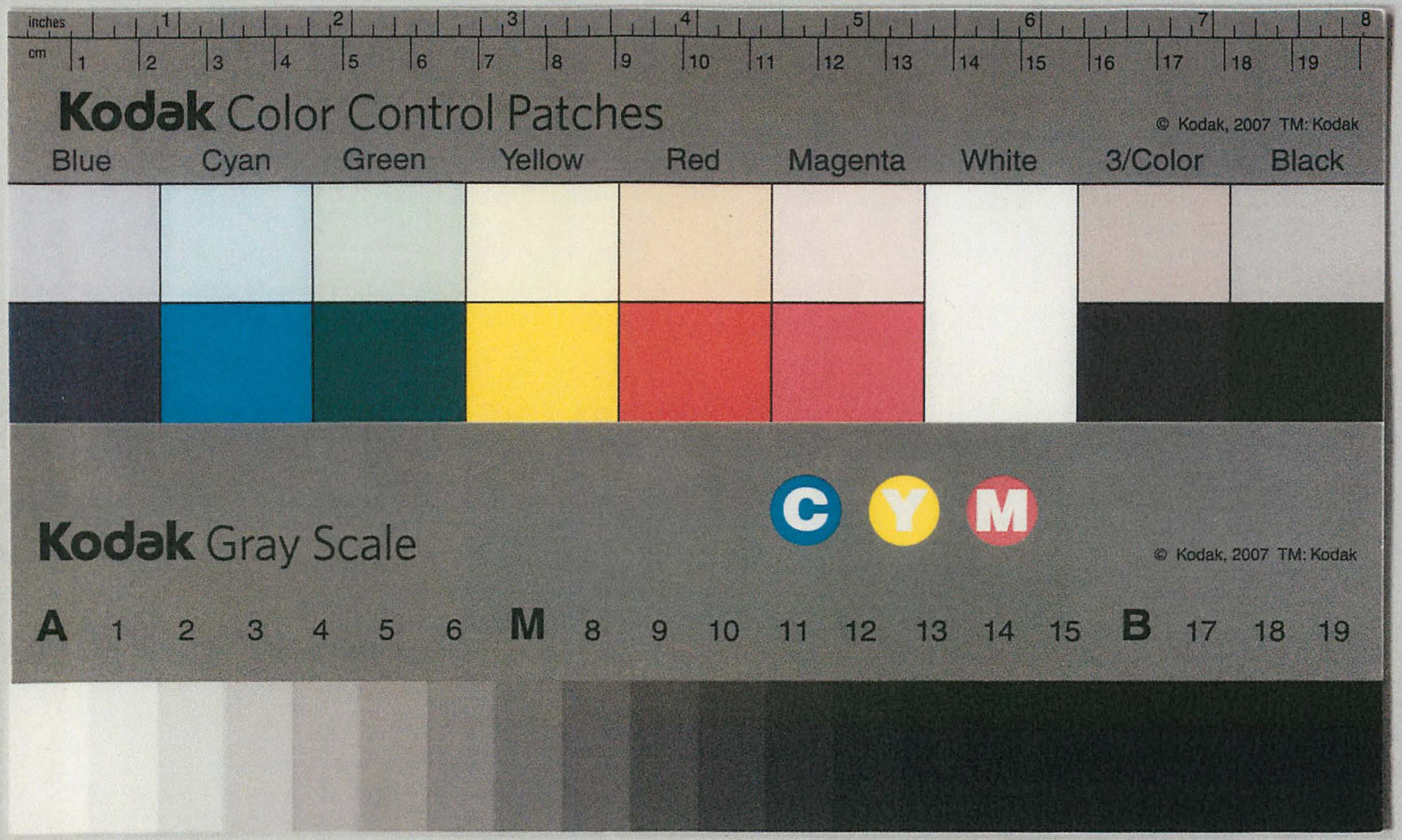
# Effects of Insulin-induced Hypoglycemia on Cerebrovascular Permeability in Diabetic Rats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# Effects of Insulin-induced Hypoglycemia on Cerebrovascular Permeability in Diabetic Rats

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Key words: cerebrovascular permeability, insulin-induced hypoglycemia, streptozotocin-diabetic rats, Evans blue, albumin tracer

**Abstract**

We studied the effect of insulin-induced hypoglycemia in both normal and streptozotocin-diabetic rats with two different durations of diabetes : 3 months and 6 months. The study was carried out in order to investigate : (a) whether diabetes alters cerebrovascular permeability, (b) whether the alteration of cerebrovascular permeability is changed by insulin-induced hypoglycemia, and (c) the relationship between the duration of the diabetic state and blood-brain barrier permeability. Evans blue fluorescence was used as an albumin tracer. In the basal condition, diabetic rats showed a significant increase in cerebrovascular permeability compared with normal rats. Insulin-induced hypoglycemia significantly enhanced cerebrovascular permeability in both normal rats and diabetic rats. The 6-month diabetic rats demonstrated a significant increase in cerebrovascular permeability compared with the 3-month diabetic rats.

In summary, insulin-induced hypoglycemia was thus demonstrated to be associated with a significant increase of cerebrovascular permeability in diabetic rats in relation to the duration of their diabetic state. Although the clinical significance of the findings still remains unknown, the results suggest the possibility that a similar phenomenon might also occur in the brains of long-term diabetic patients with severe and prolonged hypoglycemia.

## Introduction

Since the use of intensive insulin therapy was first introduced, insulin-induced hypoglycemia has constituted one of the major problems in the treatment of diabetes[1]. Severe hypoglycemia induced by the administration of insulin can lead to cerebral damage, particularly to cortical neurons, both in man and in experimental animals[2,3]. The mechanism of this phenomenon in normal rats and mice is partially explained by the increased permeability of the blood-brain barrier (BBB) after the insult of insulin-induced hypoglycemia[4,5]. On the other hand, an increased vascular permeability is usually observed even in the early stage of diabetic nephropathy[6], and it is also known that

an increased vascular permeability has a close relationship to diabetic microvascular angiopathy[7]. However, there are still few reports available concerning the permeability of the BBB in the basal or insulin-induced hypoglycemic diabetic state. In the present study we used Evans blue (EB) fluorescence, which is known as an albumin tracer[8], for quantification of protein leakage induced by insulin-induced hypoglycemia in the brains of streptozotocin (STZ)-diabetic rats with different durations of the diabetic state.

## Materials and Methods

*I Experimental animals*

Six-week-old male Wistar rats were injected intraperitoneally with 80㎎/㎏ STZ (Sigma, St. Louis, MO, U.S.A.) dissolved in 0.01mol/l citrate buffer (pH4.5) for use as the diabetic group (n=24). The presence of diabetes was determined by body weight loss, positive glycosuria, positive ketonuria, and hyperglycemia. The control group (n=24) was injected with the equivalent volume of citrate buffer. Following the injection, all the animals were allowed food and water ad libitum. In the first experiment (STUDY 1), the diabetic and control groups were evaluated after 3 months, and in the second experiment (STUDY 2), the two groups were evaluated after 6 months.

*II Induction of vascular protein leakage*

The animals were anesthetized with an intraperitoneal injection of 50㎎/㎏ pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL, U. S. A.) after a 24 hour fast. A 200U/㎏ dose of regular insulin (Humalin R U-100, Eli Lilly and Co., Indianapolis, IN, U.S.A.) was administered intraperitoneally to hypoglycemic groups for preparing the hypoglycemic diabetic group (n=4) and the hypoglycemic control group (n=4). For monitoring the blood glucose, the tip of the tail was amputated and blood was drawn onto an enzymatic test strip (Glucostix, Miles Laboratories, Elkhart, IN, U.S.A.) until blood glucose decreased to below 2.2mmol/l. Blood was simultaneously drawn into a heparinized microhematocrit capillary tube for glucose oxdase method in order to check the accuracy of the result obtained by the test strip. Ninety minutes after hypoglycemia was recognized, a blood sample was obtained from the femoral vein for determining glycated hemoglobin (affinity column method), and then 0.1㎖/㎏ EB (Sigma, St. Louis, MO, U. S. A.) as an 8% solution in saline was injected via the same route. The non-hypoglycemic groups received an equivalent amount of saline intraperitoneally, and the EB was then injected to create the non-hypoglycemic diabetic group (n=4) and the non-hypoglycemic control group (n=4).

*III Removal of the brain*

Thirty minutes after EB had been administered, for removal of the intravascularly localized dye, the chest was opened and the animal was perfused with saline through the left ventricle at 100㎝ $H_2O$ pressure until colorless perfusion fluid was obtained from the right atrium, the brain was then removed. For an accurate determination of EB extravasation, the brain tissue blank was measured using 4 brains each from the diabetic and control group after the same perfusion and removal of the brain without administering EB.

*IV Extraction and quantification of Evans blue*

After the wet weight was measured, each

brain was dissected into coronal sections, and then the EB staining of each section was graded as follows : GRADE 0, no staining ; GRADE 1+, just noticeable staining ; GRADE 2+, moderate blue staining ; GRADE 3+, dark blue staining[9]. The EB extraction was done to quantify the EB extravasation[10]. The brain was homogenized in 60% trichloroacetic acid (3mℓ/g tissue), and centrifuged at 6200g for 20 minutes. The supernatant was diluted fourfold with 100% ethanol. A colorimetric measurement was performed in a spectrophotometer (UVIDEC-40, Nihon Bunkou, Tokyo, Japan) at the absorption maximum for EB (620nm). Calculation was based on external standards in the solvent. That which remained in a subtraction of the tissue blank was defined as EB extravasation. The EB concentration in the terminal perfusion fluid was similarly determined.

*V Statistical analysis*

All the results were expressed by EB μg/g wet tissue in the form of means ± SEM. Statistical analysis was assessed by the unpaired Student's t-test. P value of less than 5% was defined as a significant difference.

## Results

*I General features of experimental animals*

The data are summarized in Table 1. In STUDY 1, the values of body weight were 349±4g and 127±8g, the level of plasma glucose and glycated hemoglobin were 5.6±0.1 mmol/l and 18.6± 0.6mmol/l, 4.3±0.1% and 14.2± 0.3% for the control and diabetic groups, respectively. There were significant differences between the values of body weight, plasma glucose, and glycated hemoglobin in the diabetic group compared with the control group in STUDY 1 ($p<0.01$).

Table 1 Characteristics of the experimental rats

| | Age (weeks) | Diabetes duration (weeks) | Weight (g) | Fasting plasma glucose (mmol/ℓ) | Glycated hemoglobin (%) |
|---|---|---|---|---|---|
| STUDY 1 CONTROL (n=12) | 18 | 0 | 349±4 | 5.6±0.1 | 4.3±0.1 |
| DIABETIC (n=12) | 18 | 12 | 127±8[a] | 18.6±0.6[a] | 14.2±0.3[a] |
| STUDY 2 CONTROL (n=12) | 30 | 0 | 407±5[a] | 5.4±0.1 | 4.5±0.1 |
| DIABETIC (n=12) | 30 | 24 | 181±12[bc] | 18.4±0.5[b] | 14.9±0.2[bd] |

[a] $p<0.01$ vs CONTROL in STUDY 1, [b] $p<0.01$ vs CONTROL in STUDY 2
[c] $p<0.01$ vs DIABETIC in STUDY 1, [d] $p<0.05$ vs DIABETIC in STUDY 1

In STUDY 2, for the control and diabetic groups respectively, the values of body weight were 407±5g and 181±12g, the levels of plasma glucose were 5.4±0.1mmol/l and 18.4±0.5mmol/l, and glycated hemoglobin levels were 4.5±0.1% and 14.9±0.2%. There were significant differences between the values of body weight, plasma glucose, and glycated hemoglobin in the diabetic group compared with the control group in STUDY 2 ($p<0.01$). There were also significant differences between the values of body weight ($p<0.01$) and glycated hemoglobin ($p<0.05$) in the 6-month diabetic group compared with the 3-month diabetic group.

*II Accuracy in the quantification of Evans blue*

Absorbance analysis of EB standards in 60% trichloroacetic acid-100% ethanol (1 : 3, volume/volume) revealed a linear relationship between absorbance and concentration at 0.1-5.0 μg/mℓ. Recovery of the EB that had been exogenously added in the amounts of 2μg, 4μg, 6μg, 8μg, 10μg to whole brains was 2.14μg (107%), 4.01μg (100%), 5.85μg (98%), 8.13μg (102%), 9.50μg (95%), respectively (Table 2). No absorbance

quenching due to tissue constituents occurred. The EB concentration in the terminal perfusion fluid was never detectable.

Table 2 Recovery of exogenously added Evans blue after incubation with whole brain for 24h at 36℃

| EVANS BLUE | |
|---|---|
| Added ($\mu$g) | Recovered ($\mu$g/whole brain) [a] |
| 2.00 | 2.14 |
| 4.00 | 4.01 |
| 6.00 | 5.85 |
| 8.00 | 8.13 |
| 10.00 | 9.50 |

[a] Mean values of 4 experiments

*III Analysis of STUDY 1 results*

The values of EB extravasation are graphically depicted in Fig.1 and summarized in Table 3. The tissue blanks obtained from the control and diabetic group were 1.02±0.18 $\mu$g/g tissue, and 1.27±0.18 $\mu$g/g tissue, respectively, which is not a significant difference. In the non-hypoglycemic and hypoglycemic control groups, the values obtained by colorimetric measurement were 1.04±0.03 $\mu$g/g tissue, and 1.91±0.06 $\mu$g/g tissue, respectively. In the non-hypoglycemic and hypoglycemic diabetic groups, the values were 3.45± 0.06 $\mu$g/g tissue, and 5.21±0.43 $\mu$g/g tissue, respectively. Hardly any EB extravasation, which is the remainder after a subtraction of the tissue blank, was obtained in the non-hypoglycemic control group. Although EB extravasation in the hypoglycemic control group was significantly increased ($p<0.01$), it was not recognizable in the gross inspection. In both the non-hypoglycemic and hypoglycemic diabetic groups, the EB extravasation was significantly increased ($p<0.01$) compared with the non-hypoglycemic and hypoglycemic control groups. EB extravasation in the hypoglycemic diabetic group was significantly increased ($p<0.01$) compared with the non-hypoglycemic diabetic group ; moreover, it was recognizable in the gross inspection.

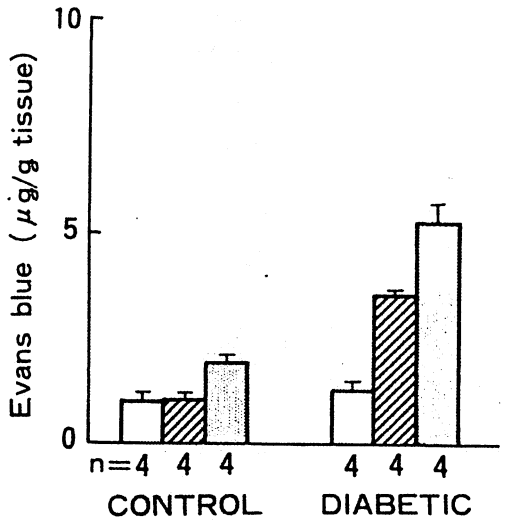


Fig.1 Evans blue extravasation in 3-month diabetic rats. Blank column, tissue blank; striped column, without hypoglycemia; stippled column, with hypoglycemia, n as indicated.

Table 3 Evans blue extravasation in 3-month diabetic rats

| | Hypoglycemia(−) | | Hypoglycemia(+) | |
|---|---|---|---|---|
| | GRADE | EVANS BLUE ($\mu$g/g tissue) | GRADE | EVANS BLUE ($\mu$g/g tissue) |
| CONTROL (n=4) | 0 | 0.02±0.03 | 0 | 0.89±0.06[b] |
| DIABETIC (n=4) | 0 | 2.18±0.06[a] | 1+ | 3.94±0.43[ab] |

GRADE : 0, no staining : 1+, just noticeable staining : 2+ moderate blue staining 3+, dark blue staining macroscopically

[a] $p<0.01$ vs CONTROL. [b] $p<0.01$ vs Hypoglycemia(−)

*IV Analysis of STUDY 2 results*

The values of EB extravasation are graphically depicted in Fig.2 and summarized in Table 4. The tissue blank obtained from the control group and the diabetic

groups were 1.08±0.07 μg/g tissue, and 1.14±0.09 μg/g tissue, respectively. There was no significant difference between the tissue blanks. In the non-hypoglycemic and hypoglycemic control groups, the values obtained by colorimetric measurement were 1.60±0.24 μg/g tissue, and 4.87±1.06 μg/g tissue, respectively. In the non-hypoglycemic and hypoglycemic diabetic groups, the values were 4.43±0.94 μg/g tissue, and 13.64±2.91 μg/g tissue, respectively. Hardly any EB extravasation was obtained in the non-hypoglycemic control group, but in the hypoglycemic control group it was significantly increased ($p<0.05$). In both the non-hypoglycemic and hypoglycemic diabetic groups, EB extravasation was significantly increased ($p<0.05$) compared with the non-hypoglycemic and hypoglycemic control groups. EB extravasation in the hypoglycemic diabetic group was significantly increased ($p<0.05$) compared with the non-hypoglycemic diabetic group. The macroscopic grading of EB extravasation was well correlated with the results of absorbance analysis.

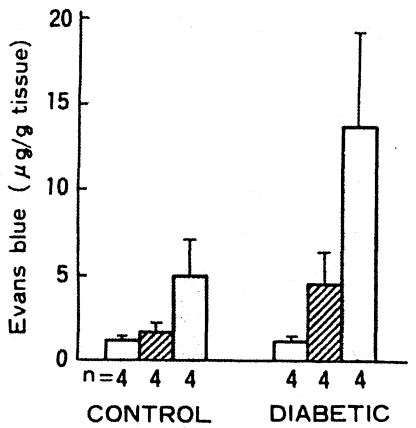


Fig. 2 Evans blue extravasation in 6-month diabetic rats. Blank column, tissue blank; striped column, without hypoglycemia; stippled column, with hypoglycemia, n as indicated.

Table 4 Evans blue extravasation in 6-month diabetic rats

| | Hypoglycemia(−) | | Hypoglycemia(+) | |
|---|---|---|---|---|
| | GRADE | EVANS BLUE (μg/g tissue) | GRADE | EVANS BLUE (μg/g tissue) |
| CONTROL (n=4) | 0 | 0.52±0.24 | 1+ | 3.79±1.06[b] |
| DIABETIC (n=4) | 1+ | 3.29±0.94[a] | 2+ | 12.50±2.91[ab] |

GRADE : 0 . no staining : 1+ . just noticeable staining : 2+ . moderate blue staining : 3+ . dark blue staining macroscopically

[a] $p<0.05$ vs CONTROL. [b] $p<0.05$ vs Hypoglycemia(−)

## V *Comparison of STUDY 1 and STUDY 2*

The values of EB extravasation are graphically depicted in Fig. 3. There were no significant differences between the values of the tissue blank in STUDY 1 and STUDY 2. EB extravasation in the non-hypoglycemic 6-month diabetic group increased in comparison with the non-hypoglycemic 3-month diabetic

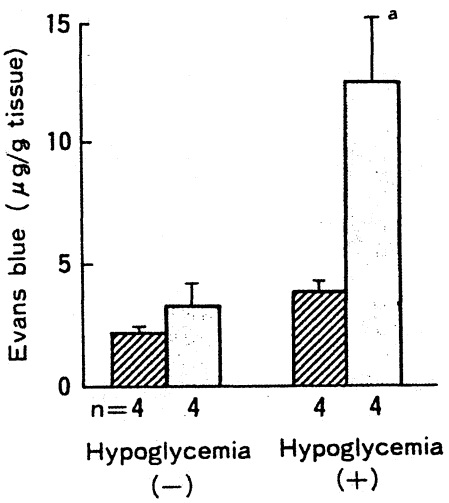


Fig. 3 Comparison of Evans blue extravasation between 3-month diabetic rats and 6-month diabetic rats. Striped column, 3-month diabetic rats; stippled column, 6-month diabetic rats, n as indicated. a $p<0.05$ vs 3-month diabetic rats.

group, but the difference was not statistically significant. An insignificant increase was also noted between the non-hypoglycemic control groups of the two studies. The increment of EB extravasation was significantly different, not only between the hypoglycemic diabetic groups ($p<0.05$), but between the hypoglycemic control groups as well ($p<0.05$).

## Discussion

It is known that insulin-induced hypoglycemia involves the increment of the permeability of BBB in normal rats and mice[4,5]. On the other hand, even though it is known that systemic vascular permeability in the diabetic state increases[6,7], there are still few reports concerning the permeability of the BBB in the basal or insulin-induced hypoglycemic diabetic state. In this study we were able to clarify that cerebrovascular permeability in diabetes was increased in relation to the duration of the diabetic state, and moreover, that it was intensified by insulin-induced hypoglycemia.

In severe and prolonged hypoglycemia, a moderate rise in blood pressure, seizures and coma occur[11]. Experimentally induced seizures per se have been observed to increase cerebrovascular permeability[12,13], and it has also been suggested that the increase in blood pressure associated with seizures might be responsible for this increased permeability[14]. In this experiment, therefore, we avoided these changes by the use of anesthesia with pentobarbital.

DeJong[15] and other investigators[16-18] suggested the existence of diabetic encephalopathy as a diabetic microangiopathy. By electron microscopy Luse et al.[19] observed a thickening and reduplication of basement membranes together with the degeneration of neuronal bodies in the brains of diabetic Chinese hamsters.

The significant increase of EB extravasation in non-hypoglycemic diabetic rats observed in our study might suggest the possibility of increased vascular permeability due to diabetic microangiopathy, in addition to the possibility of effects due to the hyperglycemia itself[20].

Insulin-induced hypoglycemia led to a significant increase of EB extravasation in both normal and diabetic rats. The fact that the increment of cerebrovascular permeability in our experiment was less than that of previous reports[4,5] might possibly be explained as the effect of the anesthesia[21]. Some investigators have reported that chronic hyperglycemia represses the glucose transfer from blood to brain[22,23]. It may be possible, therefore, to speculate that in our experiment, a more severe hypoglycemia was induced in the brain of diabetic rats than in the controls. Various insults which increase the demand for energy in the brain activate the endothelial vesicular transport with a subsequent increase of cerebrovascular permeability[24]. Accordingly, this might explain why a significant increase of cerebrovascular permeability occurred in diabetic rats with insulin-induced hypoglycemia.

Westergaard[25] and Winkelmüller[26] have reported that insulin administration increases the concentration of tryptophan in the brain with a subsequent rise in the brain levels of serotonin which can in turn increase the BBB permeability. In addition, other investigators have reported that insulin binds to brain blood vessels with specificity[27] and activates the vesicular transport pathway of endothelial cells[28]. These reports suggest not only the role of hypoglycemia itself, but also the role of hyperinsulinemia in vascular permeability

in insulin-induced hypoglycemia. The significant increase of cerebrovascular permeability in hypoglycemic-diabetic rats observed in our study, however, might suggest that hypoglycemia, rather than hyperinsulinemia, has the major role.

One of the counter-regulatory hormones, epinephrine, can produce arteriolar dilatation and venoconstriction by sympathomimetic actions[29]. Accordingly, epinephrine might be responsible for the enhancement of EB extravasation in the hypoglycemic groups, even though its effect on cerebrovascular permeability has not been precisely understood.

A significant increase of EB extravasation in relation to age was observed in hypoglycemic control groups in our experiment. The association of capillary permeability with aging in healthy males has also been reported[30]. However, some report the association only in diabetic patients and not in controls[31]. The increase of vascular permeability in relation to the duration of the diabetic state in hypoglycemic diabetic groups has been presumed to arise from both diabetic microangiopathy and aging. Bent-Hansen et al.[32] showed the relationship between an increment of vascular permeability and progression of microangiopathy, a finding which supports the results we obtained of a more enhanced vascular permeability in diabetic groups.

The permeability of BBB is presumed to be affected by an active, energy-requiring process through enhanced pinocytosis within endothelial cells or by a passive leakage of protein tracers through vessel walls[24]. It might be possible, therefore, to hypothesize that a hypoglycemia-induced energy shortage in the cerebral cortex, which demands a relatively greater energy source, activates vesicular transport in endothelial cells. This is consistent with the results obtained of a significantly increased vascular permeability in diabetic rats with hypoglycemia. Sekimoto et al.[33] demonstrated the damage of the ultrastructure in cerebral endothelial cells from insulin-induced hypoglycemia. They observed a swelling of the cells and a dissolution of the marginal folds accompanied by the appearance of craters between the cells, which suggest the destruction of Na-K countertransport due to an energy shortage in the endothelial cells[4]. Therefore, destruction of the endothelial cells, which are relatively resistant to energy shortage[34], might possibly occur with subsequent bleeding in the parenchyma of the brain in prolonged hypoglycemia[5,33].

In conclusion, insulin-induced hypoglycemia was thus associated with a significant increase of cerebrovascular permeability in diabetic rats in relation to the duration of the diabetic state. Diabetic microangiopathy in the brain vasculature and a more severe energy shortage in the neuronal cells than in the controls were presumed to be involved as the mechanisms of this phenomenon. These results suggest the possibility that a similar phenomenon might also occur in the brains of long-term diabetic human patients with severe and prolonged hypoglycemia, although the clinical significance of these findings still remains unknown.

Portions of this work were presented at the 26th annual meeting of the European Association for the Study of Diabetes, Copenhagen, Denmark, September 1990.

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