To the Editor: There is growing evidence that diabetes increases the risk of dementia for the elderly, and several studies have reported an association between diabetes and brain atrophy.[1-4] Moreover, hippocampal and amygdalar atrophy in diabetes have recently been shown to be associated with insulin resistance.[4] As the hippocampus is a key structure for memory formation, the degree of brain atrophy of the hippocampus may partially account for neuropsychological deficits in the memory of diabetic patients. Besides a weakened declarative memory, diabetes is often associated with impairment of cognitive speed and attention, which are frontal lobe-related brain functions.[5] However, it remains to be established whether morphometric changes occur in the frontal brain. This report concerns a preliminary study to investigate whether frontal brain atrophy (FBA) increases in elderly diabetics, and to explore the factors leading to the development of FBA.

The enrolment for this study comprised 67 patients with type 2 diabetes (aged 60-84) treated at Kobe University Hospital and 48 healthy individuals (aged 60-86) who underwent a medical examination at the Division of Health Consultation of the Minato Health Facilities, Kobe. Diabetes was diagnosed based on information from clinical charts regarding the medical history of diabetes, blood examination results, and the presence of diabetic complications. Patients suffering from alcohol abuse, hepatic diseases, dementia, and subjects with neurological deficits due to a previous stroke were excluded.[6] After an overnight fast, serum concentrations of blood glucose (FBG), HbA1c, total-cholesterol, triglyceride, and HDL-cholesterol were determined. All CT examinations were conducted with a third-generation scanner. FBA was identified with the aid of a planimeter applied to the CT section as described elsewhere.[7] Briefly, we manually outlined the frontal intracranial area (A) and pericerebral frontal area (B) after which FBA was calculated as a percentage expressed as (B)/(A). Statview ver. 5.0 was used for analysis of the data. Based on the hypotheses formulated in advance, 0.05 was selected as the level of significance.

Clinical features of control and diabetic subjects shown in Table 1 indicate that there were no differences in age, gender or serum levels of total-cholesterol,

triglyceride, and HDL-cholesterol. Serum concentrations of FBG and HbA1c, body mass index, and systolic/diastolic blood pressure, on the other hand, were significantly higher in diabetic patients. Five subjects had shown evidence of hypoglycemia during the preceding six months.[8] The FBA of diabetic patients was 16.8±0.5% and that of control subjects was 15.0±0.8%, for a significant difference after adjustment for age (ANCOVA: $p=0.018$). The association between clinical variables and FBA in diabetic subjects was tested by regression analysis, showing that FBA increased according to age (standardized $\beta= 0.21$, $p= 0.01$). After adjustment for age, men ($\beta= 0.22$, $p= 0.07$) and subjects with elevated diastolic blood pressure ($\beta= 0.37$, $p= 0.009$) were likely to have higher FBA, whereas HbA1c correlated negatively with FBA ($\beta= -0.26$, $p= 0.05$). The other indices of diabetes did not show any significant association with FBA. Multiple regression analysis, however, showed that age ($\beta= 0.46$, $p= 0.002$), diastolic blood pressure ($\beta= 0.38$, $p= 0.002$), and HbA1c ($\beta= -0.23$, $p= 0.04$) were significantly associated with FBA. Addition of other variables to the multiple regression analysis did not yield any significant correlation. Control subjects were subjected to a similar analysis, but except for age, no significant correlation was observed (data not shown).

Our study thus provides evidence that FBA increases in elderly diabetics and that age, diastolic blood pressure and lower HbA1c are independent risk factors. The finding of an association of higher blood pressure with brain atrophy agrees with previous results.[2] Our second finding that serum levels of HbA1c are negatively associated with FBA was unexpected. To the best of our knowledge, there have been no reports to date linking brain atrophy and HbA1c in elderly diabetics.[9] The elderly diabetics in our study had a longer history of diabetes with a higher prevalence of several diabetic vascular complications than did subjects of other studies.[1-2] It appears likely that strict blood glucose control combined with the limited cerebrovascular reserve capacity in elderly diabetics causes the disruption of cerebral glucose and energy metabolism homeostasis, resulting in subsequent neuronal degeneration. Because even a modest increase in the brain atrophy rate may lead to later cognitive impairment,[7,10] our observation suggests the importance of careful management of elderly diabetics considering brain atrophy. The

relationship between brain atrophy and HbA1c in elderly diabetics should be examined further in a prospective study.

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Table 1. Clinical characteristics of control and diabetic subjects

	Control	Diabetes	P value
Number	48	67	
Age (years)	68.7±0.9	70.0±0.8	0.267*
Men (%)	23 (47.9)	24 (35.8)	0.193¶
BMI (kg/m^2)	20.6±0.4	26.2±0.4	0.001*
MMSE score	-	26.3±0.3	-
Duration of diabetes (years)	-	18.1±1.1	-
Fasting blood glucose (mg/dl)	94.9±0.9	164.8±9.6	0.015*
HbA1c (%)	5.2±0.1	8.1±0.2	<0.001*
Total-cholesterol (mg/dl)	198.4±4.6	214.7±5.0	0.292*
Triglyceride (mg/dl)	107.8±7.3	124.2±7.1	0.452*
HDL-cholesterol (mg/dl)	-	63.6±2.8	-
Insulin use (%)	-	38 (56.7)	-
Systolic blood pressure (mmHg)	110.3±1.9	135.1±2.6*	0.001*
Diastolic blood pressure (mmHg)	65.0±1.3	73.9±1.1*	0.009*
Diabetic retinopathy (%)	-	44 (65.7)	-
Symptomatic nephropathy (%)	-	37 (55.2)	-
Persistent proteinuria (%)	-	21 (31.3)	-
Coronary artery disease (%)	-	24 (35.8)	-

Data are given as means ± standard error or percentage.

*Mann-Whitney U test, ¶χ^2 test. BMI, body mass index; MMSE, mini-mental state test examination

Impaired response of perforating arteries to hypercapnia in chronic hyperglycemia

Ximena-Sayuri Oizumi [1], Taichi Akisaki [1], Yoshiyuki Kouta [1], Xiu Z. Song [1], Toshihiro Takata [1], Takeshi Kondoh [2], Keiji Umetani [3], Masatsugu Hirano [3], Katsuhito Yamasaki [3], Eiji Kohmura [2], Koichi Yokono [1] and Takashi Sakurai [1*]

[1] Department of Internal and Geriatric Medicine, Kobe University, Graduate School of Medicine, Kobe, Japan

[2] Department of Neurosurgery, Kobe University, Graduate School of Medicine, Kobe, Japan

[3] Japan Synchrotron Radiation Research Institute, Spring-8, Sayo-gun, Hyogo, Japan

*Corresponding author: Takashi Sakurai, M.D. Ph.D.,

Department of Internal and Geriatric Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

Phone: + 81-78-382-5901

Fax: + 81-78-382-5919

Introduction

Diabetes mellitus involves long-term vascular complications and hyperglycemia is recognized as the main cause in the pathogenesis of these diabetic vasculopathies. In brain, diabetes increases the risks of large and small cerebrovascular diseases and makes patients demonstrably more susceptible to cerebral ischemia (Karapanayiotides). Hyperglycemia has been shown to increase neurologic deficits in models of hypoxic-ischemic-injury and it is possible that differences in the regulation of CBF could be responsible for this susceptibility (LeBlanc 1993).

Hypercapnia is a potent dilator of cerebral blood vessels, but the effects of hypercapnia on CBF and cerebrovascular reactivity during diabetes are still inconsistent. In human studies, impaired vascular responses to hypercapnia have been reported (dandona et al 1978, dandona 1979, Griffith 1987, kadoi 2003), while in animal experiments, it has been found that CBF responses to hypercapnia of cortical arterioles are comparable to normal animals (kontos 1990, cenic 2000, sieber 1993, kawata 1998, rodriguez 1993).

Perforating arteries are terminal vessels directly emerging from the main cerebral arteries and particularly important because these arteries supply blood to brain structures such as basal ganglia, thalamus and hippocampus that are frequently impaired in diabetes (Groot 2000). Distinctive branching pattern and higher intraluminal pressure of perforating artery may suggest the differential regulation of vascular reactivity from that of the pial arterioles. However, to our knowledge, there is not any report about the vascular response of perforating arteries in vivo. In this study, we used a newly developed microangiographic technique and investigated the vascular response to hypercapnia of rat perforating arteries. There fore, the goal of the study presented here was to identify the effects of chronic hyperglycemia on hypercapnia-induced vascular responses (endothelium-dependent vasodilatation) and nitric oxide (NO) donor-induced vascular responses (endothelium-independent) of perforating arteries and of the deeply located large cerebral arteries.

Methods

Experimental design and animal preparation

All experimental procedures were performed following the guidelines set for animal experiments by the Kobe University Graduate School of Medicine. Six months-old Male Wistar-Kyoto and Goto-Kakizaki (GK) rats weighing 400 g -

450 g and 300 g - 350 g, respectively, were used. Animal rooms were controlled for temperature (23 ℃), humidity (55%) and light (12 h light-dark cycles).
In order to investigate the effects of hypercapnia on cerebral vascular reactivity, we divided the experimental animals into two. The first group compromised control Wistar rats (n=13). The second group compromised genetically diabetic GK rats (CLEA, Tokyo, Japan). For the hypercapnic challenge rats inhaled CO_2 at 12% mixed in air for 5 min. Then rats were allowed to a 15 min period of recovery under normal capnia. To investigate the endothelium-independent vascular vasodilatation we applied sodium nitroprusside (0.5 ug.kg -1.min-1, i.v.).

Microangiography and image analysis

Microangiographic imaging of the rat brain was performed at the third generation synchrotron radiation facility SPring-8 in Hyogo, Japan. The experimental arrangement for X-ray imaging using monochromatic synchrotron radiation X-rays at the SPring-8 BL20B2 beamline has been fully described elsewhere (umetani 1, umetani2). In brief, we used monochromatic synchrotron radiation as an X-ray source, which was obtained from an 8 GeV electron storage ring (Beamline BL20B2, SPring-8, Hyogo, Japan). X-Ray was monochromatized at 17 keV energy using a silicon double-crystal monochromator. The camera head incorporates an X-ray direct-sensing pick-up tube (Saticon). Absorbed X-rays in the photoconductive layer of the tube are directly converted into electron-hole pairs, and signal charges are read out by electron beam scanning. The digital images were acquired as 1024×1024 pixels with 10-bit resolution after analog-to-digital conversion. The field of view was 9×9 mm^2 and thus the pixel size was approximately 9 μm.
Under anesthesia (pentobarbital sodium, 50 mg/kg i.p.), rats were placed in the supine position in a stereotaxic frame with a window of 3 cm x 4 cm in the center of the platform for direct radiation of the head. After tracheotomy, each animal received pancuronium bromide (0.8 μ mol/kg) and was mechanically ventilated with room air using a ventilator (SAR 830/P ventilator, California, U.S.A.) at a rate of 60-70 respirations/min. One femoral artery and one femoral vein were cannulated with PE-50 tubing (Natsume Manufacturing, Tokyo, Japan). The artery line was to measure systemic arterial blood pressure using a disposable pressure monitoring kit (Life Kit, Nihon Kohden, Tokyo, Japan) connected to a computer (Unique Acquisition, Unique Medical Company, Osaka, Japan). A femoral vein catheter was implanted for drug administration. Rectal

temperature was maintained at 36-37 ℃ with a heating pad. The right external carotid artery (ECA) was cannulated. The PE-50 tube inserted was connected to an automated injector (Auto Injector 120S, Nihon Kohden, Tokyo, Japan) that was programmed to reproducibly deliver 0.2 ml of nonionic contrast media (Iomeprol, Daiichi Pharmaceutical Company, Tokyo, Japan) in 0.4 second for each microangiographic imaging.

For consistent measurement of the vascular diameters of these arteries, we established an exact measuring point for each vessel. For the measurement of the ICA, we chose a point at a distance of 665 um from the posterior communicating artery (Pcom), for MCA, a point at a distance of 475 um distant from the ICA bifurcation. Because perforating arteries have many anatomical variations in number and origin of the vessels (rieke 1981), we selected the largest branches emerging from the MCA and determined a measuring point at 190 – 380 um distant from the MCA for each of the perforating arteries. Measurements of vessel diameters after repetitive angiography were made consistently at the same point. On the stored digital images, vessel diameters were measured semi automatically with a software (Image-Pro Plus ver.4.0, Media Cybernetics Inc., Silver Spring, MD, USA) combined with a program developed for this study (hirano).

Experimental protocol

The first angiogram was recorded to estimate the baseline diameter of the vessels. Hypercapnia was induced by inhalation of CO2 at 12% mixed in air for 5min. The arterial blood gases were analyzed, and the inhalation was returned to normal room air. An additional angiograph was made at 15 min under normocapnia and arterial blood gases were analyzed. On separate experiments, an infusion pump was connected to the vein catheter and SNP was injected continuously at a flow rate of.....

Measurements of blood gases, glucose osmolarity and insulin

Arterial blood gas tensions and pH were measured with an i-STAT G3 + Cartridge (Abbott Point-of-Care, East Windsor, NJ, USA).

Statistical analysis

Values are expressed as mean ± standard error. One way analysis of variance (ANOVA) was used for the comparison of more than two groups. Post-hoc

comparisons between mean values were made with Scheffe's test. P value <0.05 was accepted as statistically significant.

Results

Table 1 shows the effects of CO_2 inhalation on the average pH, PaO_2, $PaCO_2$ of arterial blood gas from control and GK rats. We determined the initial diameter of each vessel before induction of hypercapnia, there was no statistical difference between the two groups. Baseline diameter for ICA was 232±17.8 um and 278±45.2 um, for Wistar and GK, respectively. For MCA, 211.4 um± 9.9 and 190.0±8.2 um for Wistar and GK, respectively. For perforating baseline diameters were 77.1±4.6 um and 91.5±9.8 um for Wistar and GK, respectively. Figure 1 shows the steady-state responses to hypercapnia of the diameters in ICA, MCA and perforating vessels (Figs. 1A, B and C, respectively) of normal Wistar and GK rats. In ICA we found a significant increase of diameter after 5 min hypercapnia for Wistar rat (126%), GK rat also showed vasodilatation but this failed to be significant (117%). The vessel diameter returned to baseline value after the CO_2 challenge (Fig. 1A). MCA showed a significant increase in diameter in control (142%), GK rat showed 114% dilatation. The vessel diameter returned to baseline value after the CO_2 challenge (Fig 1B). Perforating vessels showed significant vasodilation to hypercapnia in the control group (135%), in the GK group vasodilation was present (112%), but failed to be significant. The vessel diameter returned to baseline value after the CO_2 challenge (Fig 1C).

Nitric Oxide donor, sodium nitroprusside, caused a significant vasodilatation of perforating arteries of both, control and Gk groups (126%-120%, respectively) (Fig 2). ICA and MCA arteries did not show any significant change (Fig 2).

Discussion

This study is the first to directly investigate the effects of chronic hyperglycemia on the response of perforating arteries to hypercapnia and NO donor. We found a significant vasodilatation of rat perforating arteries after hypercapnia with a maximum diameter of approximately 140% of baseline in normal Wistar rats. In contrast, chronic hyperglycemia impaired vasodilatation of perforating arteries in genetically diabetic GK rats. Second, SNP caused a similar vasodilatation of perforating vessels in normal and chronic hyperglycemia, indicating that

endothelium-dependent vasodilatation of perforating arteries may be specifically impaired in chronic hyperglycemia.

Previous experiments have found an intact CO_2 response of cerebral cortical arterioles in the diabetic dogs and STZ-induced diabetic rats (sieber 1993, simpson 1990, wang 1994). In contrast, human studies have (dandona 1978, Griffith 1987, kadoi 2003) observed that diabetic patients failed to respond normally to hypercapnia. Kadoi et al 2003 also suggested that the impaired response was related to severity of diabetes mellitus. The interpretation of clinical studies is complicated by the association of diabetes with microangiopathy and large vessel diseases. Therefore, we investigated the morphological changes in cerebral arteries in brains from 6-month and 12-month-old GK rats prior to this angiographic experiment. It has been reported that diabetes produces thickening of the arterial wall, perivascular and interstitial fibrosis, microaneurysms, arteriolar hyalinosis, and atheromatosis (Muruganandan, velasquez, yu), which could account for the pathogenesis of diabetic cerebrovascular disorders. In 6-month-old GK rats, however, cerebral arteries, including the MCA and perforating vessels did not show such microscopic alterations, nor could we find any significant microscopic changes in the of 12-month-old GK rats (data not shown). Thus, we think that the impaired reactivity to hypercapnia of deeply located vessels including perforating arteries could be due to hyperglycemia, rather than diabetic microangiopathy in GK brains.

In another set of experiments, we have analyzed the effects of hyperglycemia on the blood pressure induced vascular dilatation (autoregulation) of perforating arteries. We have found that autoregulatory responses were reversibly impaired in GK rats (data not shown), which supported the notion that perforating arteries of GK rats had functional loss of endothelium-dependent vasodilatation, rather than structural deficits of cerebral blood vessels. The discrepancy between previous findings and our findings may be related to the differences in experimental methods, including anesthesia, the diabetic animal model used, the severity of hyperglycemia, and the regional differences in CBF regulation. Regional differences in the response of cerebral blood vessels to vasoactive agonists have been specially noted in previous experiments (2, 16 from autoregulation paper).

Several mechanisms have been proposed for the hypercapnia induced cerebrovascular vasodilatation. Hypercapnia requires the development of

extracellular acidosis (kontos 1977, You 1994, Tian 1995). NO is a major mediator of endothelium-dependent relaxation in various vascular beds, and plays an essential role in regulation of the cerebral circulation. Ladecola et al (1994), demonstrated that nitric oxide synthase (NOS) inhibitors attenuated the CBF response to hypercapnia that occurs only at $PaCO_2$ < 100 mmHg. You et al. suggested that the cerebral vasorelaxation elicited by CO_2 was not related with an increase in NOS activity. This might indicate that the cerebral vasodilatation elicited by hypercapnia has NO-dependent and NO-independent components. It is likely that NO plays a role in the response to hypercapnic acidosis and this partly responsible for the increase of cerebral blood flow during hypercapnia (Tian 1995, Iadecola 1992, wang 1992). There is enough evidence of the existence of ATP-sensitive potassium channels (K_{ATP}) in cerebral blood vessels, and therefore their implication in the vasodilatation of cerebral arteries to hypercapnia has also been investigated. Faraci et al (1994) found that glibenclamide attenuated the dilatation of cerebral arterioles in response to a low concentration of acetylcholine and moderate hypercapnia. In diabetes, functional impairment of NO and K_{ATP} channels-mediated vasodilatation have been suggested of pial arterioles and the basilar artery (mayhan 1993, matsumoto 2004). Diabetes is associated with an increased generation of oxygen-derived free radicals in vascular tissues, and reactive oxygen species could influence the structure and activity of K_{ATP} channels (faraci 1998, matsumoto 2004, yu 2002, Niedowicz 2004, erdos 2005). Continuous production of reactive oxygen species produces an impaired vascular response of perforating arteries during chronic hyperglycemia.
There is considerable controversy regarding the effects of NO donors on vascular reactivity. It has been reported that application of intracarotid SNP fails to augment CBF (Young 2002, Joshi 2002), and that the degree of vasodilatation varies in iliac and superior mesenteric arteries (Martines-Nieves 1998). The discrepancy between experiments seems to be inconsistent across animal species and vascular bed examined. However, we could find that application of SNP increased the vascular diameter similarly in control and diabetic rats, suggesting that impaired vasodilatation of perforating arteries to hypercapnia is due to the deficit in NO production/release in vascular endothelium in part during chronic hyperglycemia.
In summary, we could demonstrate the in vivo evidences for the first time that responses of rat perforating arteries to hypercapnia are specifically disrupted

during chronic hyperglycemia. Possible impairment of endothelium-dependent vasodilatation in perforating vessels during chronic hyperglycemia may cause decreased vascular reserve capacity of perforating artery, resulting in the increased ischemic insults and cerebrovascular diseases in diabetes. Further studies are needed to know the cellular mechanism of hyperglycemic impacts on cerebrovascular reactivity.

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Title: Neuroprotective Effect of D-Fructose-1,6-Bisphosphate Against β-Amyloid Induced Neurotoxicity in Rat Hippocampal Organotypic Slice Culture: Involvement of PLC and MEK/ERK Signaling Pathways.

Authors: XiuZhen Song [a], Bin Wu [b], Toshihiro Takata [a], XiaoNan Wang [a], XimenaSayuri Oizumi [a], Taichi Akisaki [a], Koichi Yokono [a] and Takashi Sakurai [a] *,

a :Department of Internal and Geriatric Medicine,
Kobe University Graduate School of Medicine,
7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
b : Open Research Center of Lifestyle-Related diseases
Mukogawa-Women's University
6-46 Ikebiraki-chome, Nishinomiya 663-8558,Japan
* Corresponding author: Takashi Sakurai
E-mail: sakurai@med.kobe-u.ac.jp
Tel: +81-78-382-5901
Fax: +81-78-382-5919

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1. Introduction

Alzheimer's disease (AD) is a progressive senile dementia characterized by deposition of amyloid β-peptide (Aβ) in the form of senile plaques and the microtubule associated protein tau as paired helical filaments. Aβ is a 4 kDa peptide of 39-42 residues which has multi neurotoxic effects leading to the dysfunction and death of neurons(Yanagisawa,2000). Both in vitro and in vivo studies have confirmed the crucial role of Aβ in the development of AD. Progressive neuronal loss in AD is considered to be a consequence of the neurotoxic properties of Aβ (Iwata et al.,2005). From this point of view, preventing the Aβ-induced neurotoxicity is of great important for the development of potent therapeutic strategies.

D-fructose-1,6-bisphosphate (FBP), an endogenous intermediate of glycolytic pathway, can protect organ system from lethal injury accompanying ischemia or shock (Markov et al.,1983). There is some other evidence that FBP attenuates brain damage induced by hypoxia-ischemia (Izumi et al.,2003), insulin induced hypoglycemia (Fairas et al., 1986) and cardiogenic shock (Zhang et al.,1988). FBP is also reported provide protection of neurons against simulated ischemia in hippocampal slices (Liniger et al., 2001). The mechanisms by which FBP protects the brain neurons are not well understood. Possible mechanisms of protection include anaerobic metabolism of FBP to yield ATP (Gobbel et al.,1994) or it's ability to reduce ATP loss (Gregory et al, 1990), calcium chelation (Hassinen et al.,1991) and modulation of second messenger system.

Up to date, whether the neurotoxicity of Aβ in hippocampus could be antagonized by FBP is not well documented. We conducted this study to examine if FBP has the neuroprotective effect against Aβ-induced toxicity using organic hippocampal slices. Furthermore, to determine whether FBP serves as an alternative energy sources to preserve neuronal survival, we examined the effects of exogenous FBP on ATP levels in organic hippocampal slices during Aβ neurotoxication. Recently many studies also indicate that FBP exerts its neuroprotective effect by modulating intracellular signaling pathways. A few intracellular signaling pathways, such as PLC and MEK/ERK (Fahlman et al., 2002) are reported to be associated with the neuroprotective effects of FBP, and thus we investigated here if these pathways involved in the effects of FBP on the Aβ-induced neurotoxicity.

2 Materials and methods

The experiments were conducted according to the guideline of animal experimentation at the Kobe University School of Medicine and conform to relevant National Institution of Health guidelines.

2.1 Preparation of organotypic hippocampal slices

Hippocampal slices were made from the septal half of the hippocampus using a standard method (Sakaguchi et al., 1994). Briefly, 9-11 days Wistar rats (Hartley, SLC, Japan) were anesthetized with 98% Diethyl Ether and decapitated. The hippocampi were rapidly dissected at 4-6℃ and cut into 450μm slices using a Mcillwain Tissue Chopper (Mickle Laboratory Engineering Co.Ltd, UK). Slices were then transferred

onto 30-μm diameter-pored membrane （Millicell-CM, Millipore, Bedford, MA, USA）, and put into a six-well microplate (Costar Corning Inc, NY,USA) with 1ml slice culture media per well. The culture media were contain 50% Eagles minimal essential medium (MEM) (Gibco,CA,USA), 25% Hanks' Balanced Salt Solution (HBSS) (Gibco,CA,USA), 25% heat inactivated horse serum (Gibco,CA,USA) containing 1% penicillin/streptomycin. Slices were kept in culture for 14 days before study and the six-well micropaltes were stored at 37℃ in a 95% humidified atmosphere with 5% CO_2 incubator (Sanyo, Tokyo, Japan) untill use.

2.2 Treatment of hippocampal slices

Slices in six-well micropaltes at day 14 were washed, and the basic medium was replaced with various agents for the treatment. The basic medium contained 90mM NaCl, 4mM KCl, 0.1mM $MgCl_2$, 0.1mM KH_2PO_4 , 0.5 mM $MgSO_4$, 0.1 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 14 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 10 mM glucose, about 2 mM essential and non-essential amino acids, 0.02 mM vitamins. To establish the Aβ-induced neurotoxicity, slices were treated with three kinds of Aβ pepetides ($A\beta_{25-35}$, $A\beta_{1-40}$, and $A\beta_{1-42}$) in various concentrations. $A\beta_{25-35}$, $A\beta_{1-40}$, and $A\beta_{1-42}$ (Peptide Institute Inc. Japan, Osaka) were dissolved in sterilized distill water. To assure full contact between Aβ and the culture, treatment media was applied from underneath the insert onto the culture by pipetting at first 4h. Various concentrations of FBP (Sigma, St.Louis, MO,USA) were added to the culture with or without $A\beta_{25-35}$ for determining the FBP's effect against the Aβ induced neurotoxicity. To determine whether a signaling pathway is involved in the neruoprotective effect of FBP, a few signaling pathway-specific inhibitors were used, including a phospholipase-C (PLC) inhibitor, U0126 (Wako, Osaka, Japan), a mitogen activated extracellular signal protein kinase (MEK1/2) inhibitor, U-73122 (Wako, Osaka, Japan), an extracellular signal activated protein kinase (ERK) inhibitor, PD98059 (Wako, Osaka, Japan), and a protein kinaseC (PKC) inhibitor, chelerythrine (Calbiochem Merck,Tokyo, Japan). Each pathway-specific inhibitors (10 μM) was added into the slice culture with or without various concentrations of FBP and $A\beta_{25-35}$.

2.3 Assessment of cell death in hippocampal slices

Propidium iodide (PI) method was applied for the assessment of neuron death in hippocampal slices at 24h, 48h, and 72h after each treatment in the CA1 region of the hippocampus. To label the nuclei of dead neurons, 4.6μg /ml PI (Sigma, Louis.St, Mo,USA) was added to the wells of the culture microplates for 15 min. After 15 min, digital imagines of PI fluorescence were obtained with an inverted fluorescence microscope equipped with a digital camera (Olympus IX70, Tokyo, Japan). After the final image, all the neurons were killed by adding 10 μM N-Methyl-D-Aspartic Acid （NMDA） and the final PI fluorescence intensity was adjusted equivalent to 100% cell death. The mean intensity (green values) of the PI fluorescence were measured using an image program MacScope (Ver 2.6.1, Mitani Inc, Osaka, Japan).

2.4 Measurement of ATP levels.

Hippocampal slices were dissected under a microscope at 48h after each treatment. Four slices were immediately homogenized in 0.5 N perchloric acid with 1 mM thylenediaminetetra acetic acid and centrifuged for 15min at 2000rpm. The

supernatant was neutralized with 2M $KHCO_3$, recentrifuged and stored at -30℃ until assay of ATP. ATP was quantitated enzymatically and fluorometrically by measuring the production of nicotinamide adenine dinucleotide phosphate hydride (Sakurai et al., 2002). Protein content of the slices was determined by the method of Lowry and Passonneau (Okada et al.,1974).

2.5 Statistical analysis

Date was expressed as mean ± standard error of the mean (s.e.m) from three independent experiments. Statistical significance was established by ANOVA followed by post-hoc test using SPSS (Ver 12.0, SPSS. Inc., Chicago, USA) software. $P<0.05$ was considered to be statistically significant.

3. Results

3.1 Neurotoxicity of Aβ

Three different kinds of Aβ fragments, $A\beta_{25-35}$, $A\beta_{1-40}$, and $A\beta_{1-42}$, were applied to establish the neurotoxicity of Aβ. Cell death was evaluated at 48h after various concentrations of three Aβ fragments administration. $A\beta_{1-40}$, and $A\beta_{1-42}$ caused up to 40%-70% cell death at concentrations ranging from 0.5 μM-50 μM. $A\beta_{25-35}$ (50 μM) induced similar toxicity comparable to $A\beta_{1-40}$, and $A\beta_{1-42}$ at 25 μM (data not shown). Since $A\beta_{25-35}$ and full length $A\beta_{1-42}$ cause neuron death by similar mechanisms (Mattson, 1997), $A\beta_{25-35}$ 50 μM was used in all subsequent experiments.

3.2 Neuroprotective effect of FBP in hippocampal slices

Various concentration of FBP (0 mM, 1.7 mM, 3.5 mM, 7 mM, 10 mM) were added to the media. Compared with control group (FBP 0 mM), the addition of FBP significantly reduced the cell death in hippocampal slices at 24h, 48h and 72h after treatment (shown in Fig. 1). Interestingly, this neuroprotective effect of FBP was not in a dose-dependent manner. Compared with other FBP concentration groups, FBP 3.5 mM has better neuroprotective effect than those of other FBP groups (FBP 3.5 mM group *vs* other FBP concentration groups, all the $P<0.01$).

3.3 Neuroprotective effect of FBP against Aβ induced neurotoxicity in hippocampal slices.

As shown in Fig.2, treatment with FBP significantly decreased Aβ induced cell death in hippocampal slices at 24h, 48h and 72h (All the FBP concentration groups compare with control group, $P<0.01$). Similarly, this neuroprotective effect of FBP against Aβ was not in a dose-dependent manner. FBP 3.5 mM group has better neuroprotective effect than that of other FBP concentration groups (FBP 3.5mM+Aβ group *vs* other FBP concentration groups, all the $P<0.01$)

3.4 Neuroprotective effect of FBP against Aβ induced neurotoxicity was attenuated by PLC, MEK or ERK inhibitors

Some other studies suggested that the neuroprotection action of FBP against hypoxia was dependent on PLC, MEK/ERK pathways, and this was also found to be the case with the hippocampal slices when exposure to Aβ induced neurotoxicity. Protective effect of FBP 3.5 mM against Aβ induced neurotoxicity in hippocampal slices was abolished by PLC inhibitor, U73122, MEK inhibitor, U0126, and ERK inhibitor, PD98059 at 24h, 48h and 72h. However, administration of chelerythrine, a protein kinase C inhibitor, did not modulate the neuroprotection of FBP against Aβ

induced neurotoxicity in hippocampal slices (Fig. 3).

3.5 Effects of FBP on the ATP levels of hippocampal slices in the presence or absence of Aβ.

To test the hypothesis whether the neuroprotective action of FBP against Aβ induced neurotoxicity was due to its role as an alternative energy source, we examined the effect of various concentrations of FBP on the ATP levels in hippocampal slices in the presence or absence of Aβ. Compared with control group (FBP 0mM), FBP groups (concentration raging from 1.7 mM to 10 mM) had significant elevated ATP levels in hippocampal slices at 24h and 48h in the absence of Aβ (all the $P<0.001$) . The ATP levels were not significantly different among these FBP groups (Fig.4). With the presence of Aβ, the results were similar with those without Aβ, and the ATP levels were preserved at each concentration of FBP (compare with FBP 0 mM+Aβ group, all the $P<0.001$) . However, the difference of the ATP levels among these various FBP concentration groups did not reach to significance (Fig. 5).

3.6 Effects of PLC, MEK, ERK or PKC inhibitors on the ATP levels in hippocampal slices in the presence of FBP and Aβ.

To investigate whether energy metabolism is involved in the neuroprotective action of FBP against Aβ toxicity through specific signaling pathways, ATP levels were examined when co-treated with specific inhibitors. Compared with control group (FBP 3.5 mM+ Aβ), the addition of PLC inhibitor, MEK inhibitor, ERK inhibitor or PKC inhibitor did not cause significant difference in the ATP levels in hippocampal slices at 24h and 48h (Fig.6).

4. Discussion

We have shown that exogenous FBP reduced Aβ induced cell toxicity in rat hippocampal slices. This neuroprotective effect of FBP might be the result of additional supply of ATP in the hippocampal slices. However, the neuroprotective action of FBP against Aβ induced neurotoxicity was not in a dose-dependent manner, and the ability of FBP to produce or preserve ATP was also not in a dose-dependent manner. The results therefore suggested that protective action of FBP against Aβ induced neurotoxicity in hippocampal slices was due to, at least in part, other than its role as an alternative energy substrate to yield additional ATP. Furthermore, co-treatment of specific signaling pathways inhibitors with FBP and Aβ redueced the cell viability without alternating of ATP levels, suggesting that protective action of FBP against Aβ induced neurotoxicity was not only due to the alternative energy source, but also a modulator for neuroprotective signaling pathways.

FBP has been shown to attenuate tissue damage resulted from myocardial or kidney ischemia (Fairas et al.,1986; Didlake et al.,1989). Many studies demonstrated that FBP has neuroprotective effect in central nervous system against hypoxia/ischemia (Sola et al.,1996; Takata et al.,1997). Here we showed that FBP attenuated the neuron death induced by Aβ in hippocampal slices in a non dose-dependent manner, and also the ability of FBP to preserve the ATP levels appeared not related to its concentrations. Because of its role as an intermediate products in glycolysis, it has been widely assumed that the protective effects of exogenous FBP are resulted from

its serving as an additional substrate for glycolysis (Hardin and Roberts ,1994). But our findings didn't seem to consist with this hypothesis since the increasing of FBP could not lead to the ATP elevation in a dose-dependent manner in hippocampal slices during Aβ exposure. To our knowledge, there are no specific transporters for FBP in the central nervous system. FBP is a highly negative charged molecular which is not easy to across the hippocampal cellular membranes. Considering the fact that FBP was not taken up by red blood cell (Rigobello et al., 1982) or myocardial tissue (Galzigna et al.,1989), it was hypothesized the FBP would first have to undergo hydrolysis to fructose in order to be utilized. However some other experiments have demonstrated that, unlike FBP, addition of fructose or fructose-6-phosphate did not have neuroprotective effects (Gregory et al., 1989). It seems that relatively small amounts of exogenous FBP could be metabolized by hippocampal slices.

While it seems only small amount of FBP could be uptaken by hippocampal culture cells, and they are probably insufficient to explain its role as an energy substrate to maintain ATP level in hippocampal slice cultures. However, we could not exclude the possibility that this level of FBP might be sufficient to regulate energy metabolism and to modulate intracellular second messenger system. Some investigators illustrated that exogenous FBP has biphasic effects on the neuronal cellular metabolism. On one hand, FBP promotes glucose metabolism in astrocytes via pentose phosphate pathway (PPP; Kelleher et al.,1995). PPP is quite active in the CNS (Zubairu et al.,1983; Larrabee 1980) and stimulation of PPP may lead to the increasing production of NADPH, synthesis of fatty acids, triglycerides, and phospholipids, then reduces oxygen radical injury of neural cell by regulating glutamine peroxidase (Lazzarino et al.,1987; Tavazzi et al.,1990). On the other hand, exogenous FBP may reduce the uptake of glucose from extracellular environment (Kelleher et al.,1995), and, moreover, it could inhibit the activity of phosphorfructokinase (PFK)-the key enzyme of glycolysis, therefore reduce the production of lactate and the activity of TCA cycle (Kelleher et al.,1995). Our findings that neither higher nor lower levels of FBP cause better neuroprotective effects could be partially explained by this dual effects of FBP on the metabolism of neural cells.

Besides its role for serving as a metabolism regulator in the neruoprotective effects of FBP, the possible involvement of FBP in several intracellular signaling pathways should be taken into account. Recent studies have shown that neuroprotective qualities of FBP on hypoxia/ischemia- induced toxicity in hippocampal slices are dependent on PLC (Donohoe et al.,2001) and MEK/ERK pathways (Fahlman et al.,2002). In consistent with these studies, our observations also imply that PLC and ERK/MEK pathways are involved in the neuroprotective effects of FBP against Aβ induced neurotoxicity in hippocampal slices. Co-administration of PLC or ERK/MEK pathway inhibitors attenuate the neuroprotective action of FBP, but without affecting the ATP levels in hippocampal slices. Intracellular signaling pathways play crucial roles in regulating the cellular response and survival following insults by neurotoxins such as Aβ. Since little of exogenous FBP could enter neurons to serve as a signal, it was hypothesized that FBP might initiate its neuroprotective signaling at or near the cell surface. On the cell surface, FBP stimulate lipolysis