Impaired Response of Perforating Arteries to Hypercapnia in Chronic Hyperglycemia

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Diabetes mellitus increases the risk of cerebrovascular disease, the effects of hypercapnia on CBF (cerebral blood flow) and cerebrovascular reactivity during diabetes are still inconsistent. Here, we have established a new microangiographic technique using synchrotron radiation (SPring-8, Japan), which enabled us to visualize rat cerebral vessels with high spatial resolution in real time. 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. We found a significant vasodilatation of rat perforating arteries after hypercapnia with a maximum diameter of approximately 140% of baseline in normal Wistar rats. Chronic hyperglycemia impaired vasodilatation of perforating arteries in genetically diabetic GK rats. SNP (sodium nitroprusside) 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. 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.

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 (1). Hyperglycemia has been shown to increase neurologic deficits in models of hypoxic-ischemic-injury and it is possible that differences in the regulation of cerebral blood flow (CBF) could be responsible for this susceptibility (2).

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 (3-5), while in animal

experiments it has been found that CBF responses to hypercapnia of cortical arterioles are comparable to normal animals (6-10).

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 (11). 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. Therefore, the goal of the study presented here is 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 for animal experimentation at Kobe University Graduate School of Medicine. Six month-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 groups. The first group compromised control Wistar rats (n=13). The second group compromised genetically diabetic GK rats (CLEA, Tokyo, Japan) (n=10). 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 (SNP) (0.5 µg.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 (12, 13) (Fig. 1). 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 9.5×9.5 mm^2 and thus the pixel size was approximately 9.5 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 × 4 cm in the center of the platform for direct radiation of the head. After tracheotomy, each animal received pancuronium bromide (0.8 µmol/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
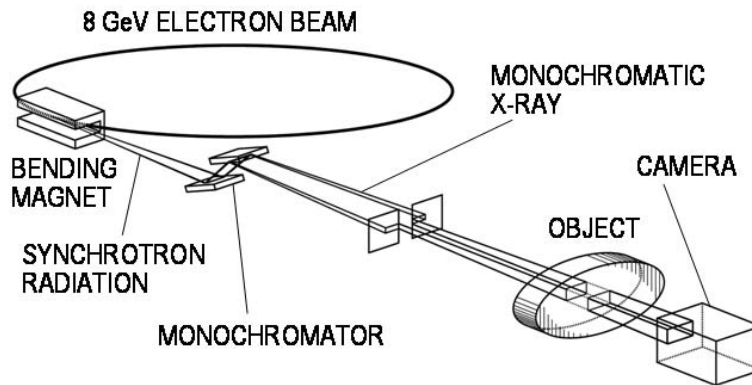
Figure 1 Illustration of the experimental arrangement at **BL20B2** beamline.

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 internal carotid artery (ICA), we chose a point at a distance of 665 μm from the posterior communicating artery (Pcom), for the medial cerebral artery (MCA), a point at a distance of 475 μm distant from the ICA bifurcation. Because perforating arteries have many anatomical variations in number and origin of the vessels (14), we selected the largest branches emerging from the MCA and determined a measuring point at 190 - 380 μm 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 (15).

Experimental protocol

The first angiogram was recorded to estimate the baseline diameter of the vessels. Hypercapnia was induced by inhalation of CO_2 at 12% mixed in air for 5min. The arterial blood gas 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 0.4 ml/min

Measurements of blood gases and glucose

Arterial blood gas tensions and pH were measured with an i-STAT G3 + Cartridge (Abbott Point-of-Care, East Windsor, NJ, USA) and blood glucose concentrations were measured by a Glutest-S analyzer (Shiga, Japan).

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 μm and 278±45.2 μm, in Wistar and GK rats, respectively. For MCA, 211.4 μm±9.9 and 190.0±8.2 μm in Wistar and GK rats, respectively. For perforating arteries, baseline diameters were 77.1±4.6 μm and 91.5±9.8 μm in Wistar and GK rats, respectively. Figure 2 represents the response of perforating arteries to hypercapnia in control rats at baseline (Fig. 2A) and after 5 min of CO_2 administration (Fig. 2B).
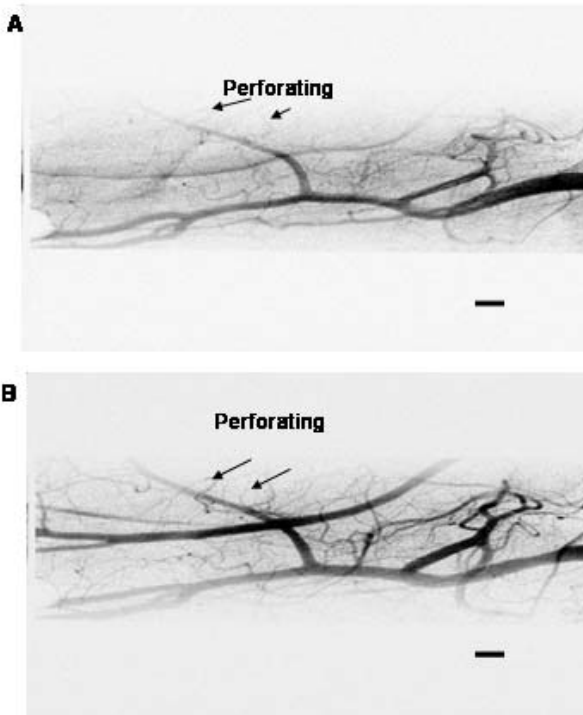
Table 1. Arterial Blood Values

	WISTAR				GK			
	CO_2	O_2	pH	BS(mM)	CO_2	O_2	pH	BS(mM)
0 min.	30.9±4.1	107.1±5.6	7.5±0.1	6.5±0.5	33.3±2.7	101.8±6.5	7.4±0.1	15.2±1.4 *
5 min.	86.9±9.7	101.7±3.0	7.1±0.4	-	101.0±6.5	108.2±2.9	7.0±0.1	-
R15 min.	33.8±7.1	113.5±8.3	7.3±0.8	-	27.0±1.0	96±20.0	7.7±0.1	-

BS, blood sugar. 0 min, baseline. 5 min, after CO_2 administration. R15, room air.
Data are Mean±S.E. *P < 0.01 vs. control

Figure 2

A

B

Figure 2
Photographs of perforating arteries emerging from the MCA (arrows) at rest (A), and during hypercapnia (B). Diameters of perforating vessels in this example were 80.5 μm at rest and 116.15 μm during hypercapnia respectively. Scale bar=300 μm.

Table 2 shows the steady-state responses to hypercapnia of the diameters in ICA, MCA and perforating vessels of normal Wistar and GK rats. In ICA and MCA we found a significant increase of diameter after 5 min hypercapnia for Wistar rat (126% and 142% of baseline diameter, respectively), GK rat also tended to show vasodilatation but failed to be significant. The vessel diameter returned to baseline value after the CO_2 challenge (Table 2). Perforating vessels showed significant vasodilatation to hypercapnia in the control group (135%), but in the GK group, vasodilatation was observed at insignificant rate (112%). The vessel diameter returned to baseline value after the CO_2 challenge (Table 2).

Table 2. Effect of hypercapnia on cerebral blood vessels

	Wistar			GK		
	ICA	MCA	PERF	ICA	MCA	PERF
0 min.	100	100	100	100	100	100
5 min.	126±6.8*	142±11.0*	135±11.3*	117±18.3	114±7.7	112±17.3
R15 min.	99±8.3	100±11	106±9.5	82±3.5	101±14.5	98±8.0

Values are means ± SE in % of change of baseline diameter.
5 min., CO2 inhalation. R15, room air 15 mins. *P < 0.01 from 0 min

Nitric Oxide donor, SNP, caused a significant vasodilatation of perforating arteries of both, control and GK groups (126% and 120%, respectively) (Table 3). ICA and MCA arteries did not show any significant changes (Table 3).

Table 3. Effect of SNP on cerebral blood vessels

	Wistar			GK		
	ICA	MCA	PERF	ICA	MCA	PERF
0 min.	100	100	100	100	100	100
10 min.	101±2.3	103±2.7	126±3.9**	105±4.0	110±3.0	120±7.8+

Values are means ± SE in % change of baseline diameter
10 min, 10 min. infusion of SNP. **P < 0.001 from 0 min. +P < 0.01 from 0 min.

DISCUSSION

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

Previous experiments have reveled an intact CO_2 response of cerebral cortical arterioles in the diabetic dogs and STZ-induced diabetic rats (6, 8, 16). In contrast, human studies have (3-5) revealed that diabetic patients failed to respond normally to hypercapnia. Kadoi et al also suggested that the impaired response was related to severity of diabetes mellitus (5). 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 (17-19), 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 difference of animal models and/or the experimental technique such as anesthesia that could affect the vascular responses. Regional differences in the response of cerebral blood vessels have been specially noted in previous experiments (20).

Several mechanisms have been proposed for the hypercapnia induced cerebrovascular vasodilatation. Hypercapnia requires the development of extracellular acidosis (21-23). NO is a major mediator of endothelium-dependent relaxation in various vascular beds, and plays an essential role in regulation of the cerebral circulation. Iadecola et al. demonstrated that nitric oxide synthase (NOS) inhibitors attenuated the CBF response to hypercapnia that occurs only at $PaCO_2 < 100$ mmHg (24). You et al. suggested that the cerebral vasorelaxation elicited by CO_2 was not related with an increase in NOS activity (22). 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 it is partly responsible for the increase of cerebral blood flow during hypercapnia (23, 25, 26). 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. found that glibenclamide attenuated the dilatation of cerebral arterioles in response to a low concentration of acetylcholine and moderate hypercapnia (27).

In diabetes, functional impairment of NO and K_{ATP} channels-mediated vasodilatation have been suggested of pial arterioles and the basilar artery (28-29). 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 (19, 29-32). 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 (33, 34), and that the degree of vasodilatation varies in iliac and superior mesenteric arteries (35). 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 diabetes mellitus.

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.

ACKNOWLEDGEMENTS

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