Original article

Effect of serotonin depletion on cortical spreading depression evoked cerebrovascular changes

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Background: The cortical spreading depression (CSD) is a phenomenon associated with several pathological conditions including migraine. It can induce alterations in both neural and vascular compartments. Serotonin (5-HT) depletion is known as a condition involved in migraine pathophysiology. The hyper-excitability of the cortical neurons to the CSD activation in the low 5-HT state has been previously reported. However, the cerebrovascular responses to CSD activation in this condition have never been studied yet.

Objectives: Determine the effect of 5-HT depletion on the cerebrovascular responses to CSD activation.

Methods: Wistar rats (weighing 250-300 grams) were divided into three groups: control, CSD, and low 5-HT with CSD group (five rats per group). To induce the low 5-HT state, the para-chlorophenylalanine was injected intraperitoneally into the rats three days before the experiment. CSD was induced by the application of solid KCl (3 mg) on the parietal cortex. NaCl instead of KCl was applied to the control group. Cerebral cortical blood flow was monitored using Laser Doppler flowmetry. The ultrastructure of cerebral microvessels was examined using electron microscopy to determine the cerebral microcirculatory responses to CSD.

Results: Depletion of serotonin induced a significant increase in the peak amplitude of CSD-evoked cerebral hyperaemia. This condition also enhanced the development of CSD-induced endothelial pinocytosis and microvillus formation in cerebrocortical microvessels.

Conclusion: 5-HT was an important neurotransmitter involved in the control of cerebrovascular responses to CSD activation. The hypersensitivity of the cerebrovascular responses observed in the 5-HT depleted state may explain the relationship between headache and 5-HT depletion.

Keywords: Cerebral blood flow, cortical spreading depression, endothelial cell, low serotonin, ultrastructural changes

The cortical spreading depression (CSD) is a depolarization wave moving across the surface of the cerebral cortex at the speed of 2-5mm/minute [1]. CSD is accompanied by series changes of the neurovascular responses. This includes the marked alteration of the ions homeostasis, the release of several neurotransmitters and the short-lasting increase in regional cerebral blood flow [2-5].

Many studies have indicated that CSD can occur following several pathological conditions such as head

trauma, stroke, exacerbates brain injuries and migraine. In migraine, several lines of evidence have indicated the tight association between CSD and the aura phase. This is the phenomenon happening prior to the headache phase in migraine with aura [6, 7]. Based on the CSD theory, CSD activation can induce the release of proinflammatory neuropeptides, which later provokes a neurogenic inflammation. This inflammation could activate the sensory C-fiber of the trigeminal system and finally cause pain [8]. Several neurotransmitters are involved in the pathway of this pain process. Among them, serotonin (5-HT) seems to be the important neurotransmitter involved in both mediating and modulating this process. The

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involvement of 5-HT in the migraine pathophysiology has been evident by the results of several studies that indicated the abnormality of the serotonin system in migraine patients. The decrease in the level of 5-HT in the brain, plasma, and platelets has been observed in these patients [9-11].

In the brain, 5-HT is a neurotransmitter involved in the control of both the neuronal function and the cerebral circulation. Therefore, the abnormality of neuronal and cerebrovascular responses could be expected in the condition with low 5-HT.

Previously, we have demonstrated that the low level of 5-HT could induce the hyper-excitability of the cortical neurons after CSD activation [12]. With the application of KCl (3 mg), the rats with low 5-HT could develop the CSD wave in the higher frequency than the rats of the control group. However, the effect of low 5-HT on the cerebrovascular responses to the CSD has never been studied yet. To clarify this point, we studied the effect of CSD on the alteration of cerebral cortical blood flow (CBF) and the ultrastructure of cerebral microvessels in the rats with and without 5-HT depletion. The CBF was measured using Laser Doppler flowmetry, while the ultrastructural changes of the cerebral endothelial cells were examined using electron microscopy.

Materials and methods

Study design

All the protocols in this experiment were approved by the Chulalongkorn University Animal Care and Use Committee. Male Wistar rats weighing 250-300 grams were divided into three groups (five animals each) as follows: control, CSD and low 5-HT with CSD. They were housed in a light/dark cycle with light on from 6.00 to 18.00. Food and water was provided ad libitum.

To induce the low 5-HT condition, the tryptophan hydroxylase inhibitor, para-chlorophenyl alanine (PCPA), at the dosage of 100 mg/kg of bodyweight, was intra-peritoneally injected into rats three days prior to the experiment. This PCPA treatment can deplete the 5-HT in the brain to < 20% of the normal amount [13]. In the control group, instead of PCPA, the physiological saline of the same volume was given to the rats three days prior to the experiment.

To induce the CSD, 3 mg of solid potassium chloride was placed on the cerebrocortical surface of the rat to elicit CSD. Solid sodium chloride at the same weight was applied on the cortex of the control animals. The CBF was continuously monitored for

60 minutes using Laser Doppler flowmetry. After completion of the CBF recording, all rats were sacrificed using an excessive dose of pentobarbital. The brains were immediately removed. The ultrastructure of the cerebral microvessels was examined using electron microscopy.

Surgical operation and induction of CSD

The rats were anaesthetized with pentobarbital sodium (50 mg/kg body weight, intraperitoneally) and were mechanically ventilated by a positive pressure ventilator (Rodent ventilator model 683, Harvard Apparatus, South Natick, USA) via the tracheostomy opening. The blood pressure was monitored continuously with an intra-arterial pressure transducer (Nihon model TP-300T, Nihon Khoden, Tokyo, Japan). It was placed in a femoral artery and recorded on a polygraph (Nihon RM 6000, Nihon Khoden, Tokyo, Japan). Arterial blood was collected periodically for the determination of pH, PaO₂ and PaCO₂ by a pH/blood gas analyser (238 pH/blood gas analyzer, Ciba Corning Diagnostics, Essex, UK). The blood gas and pH were controlled in the physiologic range throughout the experiment.

After tracheostomy and cannulation, the rat was placed on a surgical frame with the head fixed to the head holder. Craniotomy (2 mm in diameter) was performed in the parietal bone at 7 mm posterior and 1 mm lateral to the bregma. The dura was opened to expose the cortical surface. Three milligram of solid KCl was placed directly on the surface of the parietal cortex. Sodium chloride was given to the rats of the control group.

CBF monitoring

To measure the cortical blood flow, an anterior craniotomy (7 mm in diameter) was performed in the frontal bone at 1 mm anterior and 1 mm lateral to the bregma. A glassless window was placed over the craniotomy opening. The cortical surface was prevented from drying and hypothermia by superfusion with artificial cerebrospinal fluid (NaCl 118 mM, KCl 4 mM, Na₂HPO₄·H₂O 1 mM, NaHCO₃ 25 mM, CaCl₂·2H₂O 1.5 mM, MgSO₄·7H₂O 1.2 mM, dextrose 5 mM in distilled water, pH 7.4, 37°C). The fiber optic needle probe of the Laser Doppler flowmeter (ALF 21, Advance, Tokyo, Japan) was placed perpendicularly with a distance of 2 mm above the cortical surface. The wavelength of the laser beam was 780 nm. The data were recorded and the

amplitude of each hyperemic peak was calculated as a percent change from the baseline value.

Ultrastructure examination

Portions of the frontal cortex were removed and cut into multiple cubes (1x1 mm). All specimens were immediately immersed in 2.5% glutaraldehyde for four hours and post-fixed with 1% osmium tetroxide. The tissues were dehydrated through graded series of ethanol. They were passed through two changes of propylene oxide, before being embedded in plastic media (Epon 812; Electron Microscopy Sciences, Ft. Washington, USA). After polymerization, semi-thin and ultrathin sections were cut using ultramicrotome with a glass knife. The semi-thin sections (0.5 μm thick) were stained with toluidine blue in order to select suitable sections for electron microscopy. The ultrathin sections (70-90 nm thick) were stained with uranyl acetate and lead citrate and were examined under the transmission electron microscope (JEM 1210; JEOL, Tokyo, Japan). The measured variables included the number of endothelial pinocytic vesicles and the number of microvilli.

Ten capillaries (diameter: 8-10 μm) and five small arterioles (diameter: 15-20 μm) per sample were selected. The number of microvilli was counted and reported as an average number of microvilli per vessel. To evaluate the alteration in pinocytic vesicle formation, two electron micrographs that covered the area of endothelial cells were taken from every capillary at the direct magnification of 20,000 times while four electron micrographs were taken at the same magnification from every selected arteriole.

A 200x200 nm square grid was fixed to the electron micrograph. Then, the number of endothelial pinocytic vesicles was counted and reported as an average number of pinocytic vesicles per μm^2 .

Statistical analysis

All data were expressed as mean $\pm$ standard deviation (SD), and were analyzed for possible statistical significance using ANOVA for repeated measurements with the post hoc Tukey test. The result of CBF was presented in percent change from the baseline value. The average of the peak amplitude of the hyperemic cycle was compared for possible statistically significant difference between groups using the Kruskal-Wallis method. Probability values of less than 0.05 were considered statistically

significant.

Results

Effect of 5-HT depletion on CSD-evoked changes in CBF

The induction of CSD by application of KCl caused repeated cycles of cerebral hyperemia while the application of NaCl had no effect on the CBF. The average duration of these cycles was 4.0 ± 0.5 minutes. The amplitude of each peak was calculated as a percent change from the baseline flow. The median number of the hyperemic cycles within one hour was 11 peaks (ranging from 10 to 13 peaks). Compared with the control group, the average amplitude of CSD-induced hyperemic waves in the low 5-HT group were higher, which was clearly observable starting from the first peak. In the low 5-HT group the average percent change from the baseline of CBF after CSD activation was $298\pm50\%$, which was significantly higher than those obtained from the group of normal 5-HT rats ($140\pm70\%$). The latency between two subsequent peaks was not different (**Fig. 1** and **Table 1**).

Effect of 5-HT depletion on the CSD-evoked changes in the ultrastructure of the cerebral microvessels

The ultrastructural examination in both capillary and small arteriole revealed that CSD induced the ultrastructural changes of cerebral endothelial cells compared with the rats with NaCl application. The changes were characterized by an increased number of pinocytic vesicles and the formation of endothelial microvilli. An increase in pinocytic vesicles was observed both in luminal and abluminal surfaces. Furthermore, the ultrastructural changes were enhanced in the rats with 5-HT depletion. In capillaries, the average number of pinocytic vesicles was significantly increased from 27 ± 10 vesicles per μm^2 in the CSD group to 45 ± 12 vesicles per μm^2 in the 5-HT depleted rats with CSD activation. The average number of microvilli was increased from 1.4 ± 1.3 microvilli/vessel in the CSD group to 3.7 ± 1.3 microvilli/vessel in the low 5-HT with CSD group. The study in arteriole had revealed a similar result as observed in capillary. The average number of pinocytic vesicles and microvilli obtained from the arteriole in 5-HT depleted rats were significantly higher than those measured in the CSD group (**Fig. 2, 3**, and **Table 2**).

Fig. 1 The recording example of CBF changes after the application of 3 mg NaCl in the control group (A), the application of 3 mg KCl in CSD group (B) and the application of 3 mg KCl in low 5-HT group.

Table 1. The effect of 5-HT depletion on CSD-evoked hyperaemic changes of the CBF. The data were expressed as mean $\pm$ S.D.

Group	Average of % change from baseline
Control	0
CSD	140 $\pm$ 70
Low 5-HT with CSD	298 $\pm$ 50 *

*p<0.01 compared with CSD group.

Besides the alteration in the endothelial cell, the swelling of the perivascular astrocytic footplates was frequently demonstrated around the capillary obtained from the low 5-HT with the CSD group (**Fig. 2**). This abnormality was not observed in the control and CSD group.

Discussion

The present results demonstrate that the cerebrovascular responses to the CSD activation increase in the rats with low 5-HT compared with the control group. In the control group, the induction of CSD can provoke the cyclical changes of CBF and the alteration of the ultrastructure of cerebral endothelial cells. However, in low 5-HT rats, those responses are increased. An increase in the CSD

induced cerebral hyperemia as well as an increase in the number of the pinocytic vesicles and microvilli are observed in the serotonin-depleted rats. These findings indicate the important role of 5-HT in the control of the cerebrovascular responses to the CSD activation.

It has been known that the CSD can induce activation in both the neural and vascular compartment [4]. This results in the alteration of the cortical neuron activities and cerebrovascular responses. In this study, the ultrastructural changes of cerebral endothelial cells and the cyclical hyperemic changes of the CBF were observed after CSD activation. Several studies have confirmed the cerebral hyperemia after the CSD activation, but the mechanism underlying this phenomenon and the function of this hyper-perfusion is still unclear. Since the repeated CSD does not induce

Fig. 2 Electronmicrographs showing the capillaries obtained from the control (A), CSD (B) and low 5-HT with CSD (C, D) groups. The microvilli and pinocytotic vesicle are hardly observed in the endothelial cell in the control group (A). Few microvilli, M, and pinocytotic vesicles, P, are observed in the capillary obtained from the CSD group (B). In the low HT group, the induction of CSD induces an increase in the pinocytotic vesicle and microvillus formation in the capillary endothelial cell. In this group, the swelling of astrocytic foot plate (a) is clearly demonstrated as well (C, D). Bar = 1000 nm.

the damage to the neural tissue under the physiological condition, the protection of neural tissue from injury by increasing the CBF is one hypothesis that could explain the function of CSD in healthy brain [14]. However, the induction of CSD in a physiological impaired condition can result in the damage of neural tissue [15].

In this study, the activation by CSD in the low 5-HT group could induce higher cerebrovascular responses than those observed from the normal 5-HT group. The CSD induced changes of CBF and ultrastructure of cerebral endothelial cells were significantly increased in this group.

All cerebral vessels are surrounded by neurons and the astrocytic footplate. With this anatomical arrangement, both neurons and astrocytes play an important role in the adjustment of the CBF to local

metabolic activity [16-18]. In this study, besides the increase of pinocytotic vesicles and microvillus formation, the swelling of the astrocytic footplate around the cerebral vessels was clearly demonstrated in the low 5-HT group. These abnormalities were not detected in rats with a normal level of 5-HT. The increase in the number of pinocytotic vesicles and microvillus formation have been demonstrated in several conditions of an impaired blood brain barrier (BBB) such as in the concussive and traumatic brain injury, and hypertensive encephalopathy [19-22]. The swelling of the astrocytic footplate is known as another characteristic indicating the abnormality of the BBB. This abnormality was reported in parallel with the impairment of the BBB and the BBB breakdown [19, 23]. For these reasons, the higher degree of the ultrastructural alteration of the cerebral endothelial cells

Fig. 3 Electron micrographs showing the ultrastructure of an arteriolar endothelial cell obtained from the control group (A) which demonstrates a few number of pinocytotic vesicle and microvilli. In the CSD group, the induction of CSD can induce an increase in the pinocytotic vesicles, P, and microvilli, M, in the endothelial cell. These CSD-evoked ultrastructural changes are facilitated in the condition with low 5-HT (C). Bar = 400 nm.

Table 2. The effect of low 5-HT on CSD-evoked ultrastructural changes of the cerebral endothelial cells

Variables	Control group	CSD group	Low 5-HT with CSD group
Capillaries			
Pinocytotic vesicles (<i>number per μm^2</i>)	16 ± 7	27 ± 10	$45 \pm 12^{* \#}$
Microvilli (<i>number per vessel</i>)	1 ± 0.7	1.4 ± 1.3	$3.7 \pm 1.3^{* \#}$
Arterioles			
Pinocytotic vesicles (<i>number per μm^2</i>)	18 ± 7	$34 \pm 10^*$	$49 \pm 11^{* \#}$
Microvilli (<i>number per vessel</i>)	8 ± 4	$15 \pm 5^*$	$26.5 \pm 5^{* \#}$

^{*}p < 0.05 compared with the control group. [#]p < 0.05 compared with the CSD group.

and the swelling of astrocytic footplate observed in low 5-HT rats may imply that the activation by CSD in the 5-HT depleted state can induce higher cerebrovascular responses than in the normal 5-HT condition. If the activation is persistent, the cerebral endothelial cells in low 5-HT condition will damage quicker than those in the condition with normal 5-HT.

According to the current knowledge on the regulation of CBF, there are a number of neurotransmitters and substances involved in the hyperemic changes during CSD. Among them, nitric oxide (NO) is one of the important neurotransmitters involved in both vascular and neuronal alteration during CSD [24]. NO itself has a vasodilatation effect via the cGMP dependent mechanism. After release, NO can directly react with superoxide (O_2^-) resulting in the formation of the peroxynitrite ($ONOO^-$) which is the strong reactive oxygen free radical. $ONOO^-$ has been reported to be the crucial molecule in the alteration of the blood flow as well as the integrity of BBB in several pathological conditions [25]. The role of NO in the control of the integrity of BBB has been confirmed by the number of findings that suggest close relation between the increase in the synthesis/release of NO and an increase in the permeability of cerebral circulation in response to a variety of mediators [26-28].

Interestingly, there are several lines of evidence demonstrating the abnormality of NO production in the low 5-HT state. Ramos et al. revealed that the depletion of serotonin by the PCPA injection could induce an immediate increase in the nNOS activity in several parts of the brain (striatum, hippocampus, and parietal cortex) [29]. This hypothesis has been confirmed by the study of the postnatal development of the nitrergic system after depletion of 5-HT. The results showed that after depletion of serotonin by the para-chloroamphetamine treatment, the nNOS immunoreactivity in striatum, frontal cortex, and hippocampus was higher than those observed in the control rats. The results also demonstrated that the more serotonin was depleted, the higher the expression of NO marker was. This suggested the close relation between serotonin and the NO system [30]. Altogether, it can be suggested that the low 5-HT state can induce a larger increase in the NO production in the brain than in the control group. Furthermore, the increase of the level of the NO released after CSD activation can be at least one explanation for the increase in the CBF and the

ultrastructure of cerebral endothelial cells observed in the low 5-HT group.

In conclusion, 5-HT is the neurotransmitter involved in the control of cerebrovascular responses to CSD activation. The depletion of 5-HT can induce an increase in the CBF hyperperfusion as well as the damage of the cerebral endothelial cells from CSD activation. The increase in the production of NO might explain these abnormalities of the cerebrovascular responses observed in this study. However, since the hyper-excitability of the cortical neurons have been demonstrated in the 5-HT depleted state, the increase of several vasoactive neurotransmitters released after CSD activation cannot be excluded for these cerebrovascular responses in 5-HT depleted condition.

Abbreviations

CBF: cortical blood flow,
CSD: cortical spreading depression,
5-HT: serotonin,
PCPA: para-chlorophenylalanine,
NO: nitric oxide.

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