CHAPTER VI
DISCUSSION

NO supersensitivity has been proposed as a mechanism of migraine pathogenesis. However, the mechanism of the vascular supersensitivity to NO was still unknown. In this study, a series of experiments was conducted to investigate the relationship between hyposerotonergic condition and NO supersensitivity. An animal model was developed to investigate the association between these two transmitters. The hyposerotonergic condition was developed by using PCPA, a tryptophanhydroxylase inhibitor, at the dose of 300 mg/kg, ip. The control animals received NSS, ip injection at the same volume as experimental rats. NTG was used as a NO donor since this drug can directly liberate NO without endothelial mechanism. Various doses of NTG were infused intravenously for the period of five minutes in order to select the appropriate dose in this study. The minimal doses that cause cerebral microvessels dilatation were 8 mg/kg BW and 10 mg/kg BW and were chosen for this experiment. The accessible results were divided into three main parts. The first part was the effect of NTG on vasomotor response. The second was the effect of NTG on endothelial ultrastructural changes. The last was the effect of NTG on the stimulation of neural pain pathway. The cerebrovascular response was studied using intravital pial microvascular monitoring. The endothelial ultrastructural changes were performed by transmission electron microscopic study and the neural activity in pain pathway was evaluated by Fos immunoreactivity.

The present study showed that infusion of NTG produced a transient decrease in systemic blood pressure. The blood pressure rapidly became

normal after stopping NTG infusion. This effect is agreed with the work of Knowles and Moncada (1994). They summarized that the systemic blood pressure of patients who received NTG was transiently decrease and return to normal value after 5-15 min after received NTG sublingually. When NTG is exposed to mammalian tissues, NO is released. The precise biochemical process responsible for this has not been defined. Several lines of evidence suggested that cellular thiols are involved in this process (Needleman et al., 1971; Ignarro et al., 1981). Whether or not a nitrosothiol is an intermediate in this process is not shown conclusively by these observations. Sulfhydryl groups may have other important roles such as serving as cofactor or donating reducing equivalents that lead to the formation of NO directly from NTG. The NO released is such a powerful vasodilator. Thus, infusion of NTG produced a drop in blood pressure. Since, NO has very short half-life (4-5 sec), this hypotension state is transient. Although infusion of NTG caused transient drop in systemic blood pressure, the difference in blood pressure was only 10 per cent which was unlikely to produce pathological changes. Other physiologic parameters including blood pH and arterial blood gas were kept stable throughout this study. Therefore, the observed changes in this study were likely to be resulted from direct effect of NO, not systemic effects.

The effects of NTG on vasomotor response

The effects of NTG on vasomotor response were studied over a period of 1 hour after NTG infusion. The vasomotor response of microvessels was divided into two main groups (diameter about 10-30 μm and 30-60 μm). After infusion of NTG 8 or 10 mg/kg BW, the significant pial arteriole dilatation was observed almost immediately after both

concentration of NTG infusion and reached its peak about 15-30 minutes. This pial arteriole dilatation persisted for at least 60 minutes. The pattern of vasomotor response of NTG with or without PCPA pretreatment are similar. The observation of prolonged effect of NO-donor on pial vessels demonstrated in this study is of interest. In physiologic condition, the vasodilating effect of NO is rather short since this molecule can be degraded very rapidlly after release. The prolonged vasodilatation resulted from other indirect mechanisms is an alternative explanation. Direct activation of perivascular sensory nerve fibers and/or initiation of perivascular neurogenic inflammation by NO may be other possibilities and also the direct cytotoxic effect of NO should be considered. NO is thought to be involved in the prolonged vasodilatation at several levels. In the vascular smooth muscle, its appeared to act as a cGMP stimulator and causes a potent smooth muscle relaxation. In the periphery, NO is thought to be a pain mediator. Wei and coworkers (1992) showed that local application of NTG to the pial surface induced CGRP release from perivascular nerve fibers. This implies that the prolonged dilatation in NTG-treated rats may be due to formation of another vasodilating substance indirectly induced by NO. In its central role, NO participates in the levels of sensitive trigeminal fibers and operates the release of SP and CGRP. SP stimulates the process of neurogenic inflammation and CGRP causes prolonged vasodilatation. Huang et al., (1993) demonstrated that the stimulation of trigeminovascular axons just 5 minutes caused vasodilatation and neuroginic inflammation that persists for hours. These effects are thought to be a consequence result from the release of CGRP and SP after trigeminal nerve stimulation.

Another mechanism that causes a prolonged vasodilatation in this study is cytotoxic effect of NO itself. This prolonged vasodilatation may

indicate an abnormal function of microvascular wall which was possibly injured by high doses of NO. Based on a recent *in vitro* study, stimulation of NOS has been observed during oxidative endothelial cell injury (Shimizu et al., 1998). Bouloumie (1997) suggested that the development of endothelial dysfunction was linked to an overproduction of NOS expression that might lead to the formation of endothelium derived radicals, peroxynitrite. Inhibition of NO generation can attenuate cerebrovascular endothelial cell injury after exposure to hemolysate (Kwan et al., 1997).

The present study also demonstrated an enhancing effect of PCPA on NTG-induced pial arteriolar response. Pretreatment with PCPA increased both magnitude and duration of NTG (8 mg/kg BW) – induced pial arteriole dilatation. Since PCPA is a strong 5-HT depletion agent, their findings imply that the cerebrovascular responses to NO can be facilitated in the hyposerotonergic condition. Base on initial molecular biological studies, the trigeminal ganglion and presynaptic nerve terminal may preferentially contain 5-HT_{1D} receptor subtype (Rebeck et al., 1994) whereas the blood vessels expressed 5-HT_{1B} receptor subtype (Hamel et al., 1993). The 5-HT_{1D} receptor is thought to be involved in modulating the activity of the trigeminovascular system by inhibiting the transmission of pain impulses into the brainstem from the trigeminal nerve to higher parts of the brain and inhibitory the release of agogenic peptide from perivascular nerve plexus. Since 5-HT_{1D} also expressed in presynaptic nerve terminals, 5-HT deficiency can reflected to ineffectiveness inhibitory response on the trigeminal nerve blocking neurogenic inflammation and pain transmission. This ineffectiveness can be related to the abnormallity of CGRP and SP release from nerve terminals after NO stimulation. CGRP and SP may then trigger the vasodilatation and

the sterile localized neurogenic inflammation. The smooth cells of the intracranial blood vessels expressed receptors of the 5-HT_{1B} subtype. Activation of 5-HT_{1B} receptors caused selective vasoconstriction of certain extra and/or intracranial blood vessels (Daholf and Hargreaves, 1998). Therefore, 5-HT deficiency trend to increase vessels caliber. From above reasons, we observed that the NTG-induced pial arteriole dilatation was more prominent in rats receiving PCPA-pretreatment. Unfortunately, we could not observed this effects in rats receiving higher dosage of NTG (10 mg/kg BW), since this dosage produced the maximal dilatation in both NTG-treated with and without PCPA pretreatment groups.

An enhanced microvascular response to NO in 5-HT depleted animals in this study imply the role of hyposerotonin in modulation of cranial vascular response to NO raises the possibility of hypothesis on a cause of NO supersensitivity in migraine pathogenesis. 5-HT has been implicated in the pathogenesis of migraine. Various abnormalities of serotonergic function during a migraine attack have been documented. For example, 5-HT level in plasma was increased during the migraine aura. At the onset of the headache, the 5-HT level in platelets fall rapidly and remain low during the attack (Sommerville, 1976). At the same time, increased amount of 5-HIAA, its metabolites, were detected in the urine during most attacks. A role for 5-HT is further supported by the finding that drug which cause the release of 5-HT from tissue stored, such as reserpine and fenfluramine can induce migraine attacks. Many lines of evidence raise the possibility of hyposerotonin as a crucial step in initiation of migraine attacks.

NO seems to be an important molecule in the development of migraine attacks since NTG causes a pulsating, dose-dependent headache

with several migraine-like characteristics in migraineurs. (Olesen et al., 1994). In addition, after administration of nitrate-containing compound, migraine patients developed typical migraine attacks, which was accompanied by a prolonged arterial vasodilatation compared to normal subjects (Ballantonio et al., 1997). Interestingly, in this experiment a prolonged NO-induced vasodilatation was also observed in animals receiving PCPA pretreatment. Involvement of NO in migraine is further confirmed by an observation that NTG infusion enhanced NO release during CSD, a physiologic phenomenon underlying migraine aura (Read et al., 1997). Besides the induction of dilatation in large intracranial arteries, NO may involve in development of vascular headache by initiating a perivascular neurogenic inflammation with liberation of vasoactive peptides. This proposed mechanism may explain the delayed NO-induced headache observed in migraine patients. Another possible mechanism involves the effect of NO in modulation of pain threshold. Thomsen et al., (1996) demonstrated that administration of NTG induced a decrease in both detection and tolerance thresholds in the temporal region as measured by pressure algometry.

The effects of NTG on endothelial ultrastructural changes

Morphological changes in the ultrastructure of endothelial cell observed in this study were characterized by increased microvilli, increased pinocytosis, mitochondrial swelling, doming of the endothelial surface and swelling of perivascular astrocytic footplate that result in partial separation of endothelial cells from the adjacent brain tissue. These ultrastructural changes may reflect some physiologic alterations of the cerebral microvessels after NO exposure.

Microvilli of the endothelial cells have been termed in different reports as "*endothelial microvilli*", "*endothelial projections*" and "*hair-like projections*". Functions of the endothelial microvilli are unknown, although they are assumed to relate to the augmented cellular activity. An increase in number of microvilli causing surface area expansion, may imply an increase in transendothelial transport. These microvilli may contain pinocytic vesicles. Therefore the augmented vesicular transport of the endothelial cell must be accelerated by the increased numbers of microvilli. The presence of profused microvilli on the endothelial surface was also reported by Hazama et al., (1978) in spontaneously hypertensive rats. Still and Dennison (1974) also showed the presence of microvilli in the arterial endothelium of the hypertensive rat. Lossinski et al., (1995) reported an increased endothelial microvilli in the osmotically impaired blood brain barrier.

An increase in microvillous formation which expands the luminal surface area may imply increased cellular activity regarding uptake and transcellular transportation. The hypothesis of NO-induced derangement of the blood brain barrier can be supported by the observation of a co-existing increase in the density of cytoplasmic pinocytic vesicles. Unlike endothelial cell in other organs, brain endothelial cells possess only few pinocytic vesicles during the resting state. An increasing number of these vesicles reflects an enhanced cellular transport activity. Such increase may lead to increased permeability of cerebral microvessel and in the extreme state may cause impairment of blood brain barrier. An increase in cerebral microvessel pinocytosis was previously observed in other conditions, including concussive brain injury and hypertensive cerebrovascular diseases (Wei et al., 1980; Hazama et al., 1978). Fukui et al., (1995) also reported that microvessels of rat heart induced by

endotoxin administration had increased numbers of pinocytic vesicles. In both conditions, increased pinocytosis was related to an increase in cerebral vascular permeability.

In this study, a greater number of pinocytic vesicles was observed in cerebral arterioles of the NTG-group as compared to control. Interestingly, increased pinocytic vesicles coincided with increased numbers of microvilli. It is also possible that the vessel from NTG-treated rats tend to increase in permeability of endothelial cells. In addition, the capillary endothelial cells alteration frequently shows signs of much pinocytic activity.

Morphological abnormalities of mitochondria obtained from NTG-treated rats were observed in this study. In comparison with the controls, a greater diameter of mitochondria was demonstrated in NTG-treated group. This alteration may result from the injury processes of the endothelial cells in response to toxic oxidized metabolites derived from NTG. An overproduction of NO that liberated from NTG might lead to the formation of endothelium-derived radicals, peroxynitrite. These free radicals augment Ca^{2+} influx and inhibit Ca^{2+} pump, thereby influencing membrane conductance to ions including Ca^{2+}, mitochondrial respiration. As a consequence, massive uptake of calcium by the mitochondria occurs. The uptake of calcium by mitochondria of injured cells is paralleled to progressive mitochondria swelling (Kristian and Siesjo, 1998).

Another abnormality possibly found in the endothelium of cerebral microvessels was a formation of endothelial membrane bleb (dome-like). In endothelial cell obtained from NTG-treated rats, dome-like protrusions into lumen were observed frequently. Such membrane bleb varied considerably in size and shape. Most of them were circular, balloon-like.

The presence of perivascular astrocytic footplate swelling demonstrated in this study may indicate the compensatory response of astrocytes to an excessive increase in blood-brain barrier permeability. Such increase may result in the leakage of intravascular substances into interstitial tissue and cause partial separation of the cerebral microvessels from the adjacent brain tissue. However, this study could not demonstrate any leakage of macromolecule from pial vessels in fluorescein study.

Although exposure to NO donor can result in various morphological changes in endothelial cells as reported in this study, it is unlikely that such features are specific to NO-induced cellular injuries. Ballooning of endothelial mitochondria can be present in several conditions, e.g. ethanol poisoning, etc (Romansky and Stamenov 1995; Hirakawa et al., 1994). Focal swelling of endothelial cell has been previously reported in the dural vessels after trigeminal stimulation (Dimitriadou et al., 1992). Therefore these ultrastructural deformities are likely to be non-specific changes of endothelial cells in response to various forms of intense stimuli.

The mechanisms by which NO can induce endothelial cell injury are not fully understood. NO can interact with oxygen molecules and results in peroxynitrite as well as other tissue damaging radicals (Beckman et al., 1990). These radicals will lead to endothelial cell injury via the process of lipid peroxidation as well as peroxidation of sulfhydryls (Radi et al., 1991a and 1991b). However, recent studies showed the interaction between NO and reactive oxygen intermediates in peroxide-mediated endothelial toxicity to be complex. Administration of NO donor can attenuate neutrophil-endothelial cell adhesion as well as neutrophil-mediated endothelial cell killing (Kausalya and Nath, 1998). Peroxidant-

induced endothelial cell injury can be significantly ameliorated by administration of S-nitroso-N-acetylpenicillamine (SNAP), a NO donor (Degnim et al., 1998). These observations reflect the inverse relationship between NO and reactive oxygen intermediates and implying a cytoprotective effect of NO rather than cytotoxic effect. On the other hand, Hidaka et al (1997) demonstrated that high levels of NO released by activated monocytes contribute to endothelial cell injury. It has been known that reactive oxygen species participate in regulation or execution of apoptotic process (Kroemer et al., 1995). These radicals may play roles both in the induction and in the effector stages. Similar to other classes of free radicals, recent *in vitro* study demonstrated that administration of excessive dose of SNAP can produce apoptosis of endothelial cell. This process is probably via cGMP-independent mechanism (Shen et al., 1997). However, structural changes typical for endothelial cell apoptosis such as chromatin clumping, decreased pinocytic vesicles, etc. were not evident in this study.

This experiment demonstrated that the NO-induced pathological changes were more pronounced in animals receiving PCPA pretreatment. This observation indicated that endothelial responses to NO are more severe in the hyposerotonergic state, and thus provided a further evidence in support of the role of 5-HT depletion on facilitation of NO response. Endothelial cell activation (increase in vesicle number and vacuole formation) and neurogenic plasma extravasation have been previously observed in microvessels of the dura mater following electrical trigeminal ganglion stimulation, an animal model for migraine headache (Dimitriadou et al., 1992). Whether or not blood-brain barrier is normal during the attack of migraine is still a question.

The effects of NO on c-fos expression in neural pain pathway

The present study demonstrated that administration of NTG 10 mg/kg BW causes an increase in the number of Fos positive cells in some area of the brainstem, TNC, NTS, LRN and IO of experimental rats with and without PCPA-pretreatment. An increase in Fos immunoreactivity reflext an increase in number of activated neuron in these area. The expression of Fos immunoreactivity is maximum 2 hours after NTG infusion. Billitt et al., (1990) also found that the expression of Fos positive cells is maximum 2 hours after trigeminal ganglion stimulation. Fos expression has been used to trace nociceptive pathways in CNS (Hunt et al., 1987). Stimulation of the trigeminal ganglion electrically or chemically results in increased Fos immunoreactivity in TNC, which is correlated with the stimulus intensity (Shepheard et al., 1995). The number of Fos positive cells in TNC is reduced if C-fibers are destroyed by neonatal capsaicin treatment or by trigeminal nerve transection prior to afferent stimulation (Nozaki et al., 1992). In TNC, the number of Fos positive cells was most prominent in the ventrolateral segment of TNC corresponding with inputs from the ophthalmic division (Shigenaga et al., 1986). This pattern is in agreement with the view that C-fibers are involved. Vasodilatation induced by NTG infusion may stimulate dural and blood vessel nociceptors or may directly stimulate the primary afferent fibers of trigeminal nerve, resulting in subsequent c-*fos* expression in the brainstem nuclei.

Apart from TNC, the systemic administration of NTG also induced Fos expression in NTS, LRN and IO. We have observed the numerous Fos expression in the NTS, which plays a pivotal role in the response to cardiovascular changes, like the transiently decrease in blood pressure caused by NTG administration. The sustained Fos expression in the LRN

seem to represent a complex reflex response to the vasodilatation induced in cerebral vessels, as it is well known that some of the activated areas are involved in the regulation of cranial vessels. Moreover, many lines of evidence demonstrated that trigeminal brainstem nuclear complex (TBNC) has also been shown to project to the IO and NTS (Figure 2.2). This pathway may be important in the coordination of somatic and visceral reflexes. Numerous investigators have demonstrated that the neurons in the TBNC project to the adjacent recticular formation and this connection provides the processing of nociceptive information. In addition, the recticular formation has its own projection to the thalamus (Sessle, 1986).

Beside in the brainstem, Fos positive neurons were also in the epithalamus (habenular) and the hypothalamus (paraventricular and supraoptic) of NTG-treated rats with and without PCPA-pretreatment. Hypothalamic neurons partly function as thermoreceptors, osmoreceptors or chemoreceptors and therefore may be activated by variety of stimuli irrelevant to the actual arteriolar vasomotor responses, endothelial ultrastructural changes and the stimulation of pain pathway in normal rats. (Strassman et al., 1994) The habenular nucleus receives afferents from the periventricular neurons of hypothalamus, periaqueductal gray matter, reticular formation and interpeduncular nucleus of the midbraine. It links the hypothalamus and limbic control mechanisms with the rostral part of the brainstem.

We can not demonstrate any difference of the number of Fos positive cells in the TNC, NTS, LRN and IO in the brainstem, as well as epithalamus and hypothalamus of NTG (10 mg/kg BW)-treated with and without PCPA-pretreatment rats. This may due to the higher dosage of NTG in this experiment. For further study, the lower dose than this experiment should be selected.