

# Effect of a Chloride Channel Inhibitor, 5-Nitro-2-(3-Phenylpropylamino)-Benzoate, on Endothelin-1 Induced Vasoconstriction in Rabbit Basilar Artery

## Klor Kanalı İnhibitörü 5-Nitro-2-(3-Fenilpropilamino)-Benzoat'ın Tavşan Baziller Arterinde Endotelin-1 ile İndüklenen Vazokonstriksiyon Üzerine Etkileri

### ABSTRACT

**AIM:** Endothelin-1 (ET-1) has been implicated in the pathophysiology of cerebral vasospasm. Chloride (Cl<sup>-</sup>) channels exist in vascular smooth muscle and activation of these channels leads to depolarization and contraction. The aim of the present study was to test the effect of 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), a Cl<sup>-</sup> channel antagonist, on the ET-1-induced cerebral vasospasm in rabbit basilar artery and thus investigate the contribution of Cl<sup>-</sup> channels.

**MATERIAL and METHODS:** Thirty rabbits were divided into five groups and received intra-arterial injection of isotonic saline (Group I, n=6), ET-1 (group II, n=6), ET-1 plus NPPB (Group III, n=6), dimethylsulfate (DMSO4) (Group IV, n = 6) and NPPB (Group V, n=6). Pre and post injection basilar artery diameters were measured in each group and transmission electron microscopic investigations on basilar arteries were performed.

**RESULTS:** The mean pre-injection and post-injection vessel diameters were 0.8833mm and 0.7000mm in ET-1 group, 0.6833mm and 0.8500mm in ET-1 + NPPB group. NPPB administered prior to ET-1 injection, prevented the ET-1-induced vasoconstriction. Additionally, NPPB prevents the ET-1 induced changes in vessel wall and neurons in the brain stem.

**CONCLUSION:** The results of this study add further insights to our armamentarium against cerebral vasospasm.

**KEYWORDS:** 5-nitro-2-(3-phenylpropylamino)-benzoate, Cl channel, Endothelin-1, Cerebral vasospasm

### ÖZ

**AMAÇ:** Damar düz kasında Klor (Cl<sup>-</sup>) kanalları bulunmaktadır ve bu kanalların aktivasyonu depolarizasyon ve damar düz kasında kasılmaya neden olmaktadır. Bu çalışmanın amacı serebral vazospazmı Cl<sup>-</sup> kanallarının rolünü araştırmak ve bir Cl<sup>-</sup> kanal inhibitörü olan 5-nitro-2-(3-fenilpropilamino)-benzoat'ın (NPPB) ET-1 ile tetiklenen serebral vazospazmı etkinliğini değerlendirmektir.

**YÖNTEM ve GEREÇ:** Otuz adet tavşan beş gruba bölünmüş ve intraarteriyel izotonik salin (Grup I, n=6), ET-1 (Grup II, n=6), ET-1 ve NPPB (Grup III, n=6),

Fikret DOĞULU<sup>1</sup>  
Sureyya BARUN<sup>2</sup>  
Hakan EMMEZ<sup>3</sup>  
Cigdem ELMAS<sup>4</sup>  
Baran ONAL<sup>5</sup>  
Hayrunnisa BOLAY BELEN<sup>6</sup>  
Deniz ERDOĞAN<sup>7</sup>  
Erhan ILGIT<sup>8</sup>  
Yusuf SARIOĞLU<sup>9</sup>  
Gokhan KURT<sup>10</sup>  
Sevim ERCAN<sup>11</sup>  
M. Kemal BAYKANER<sup>12</sup>

1,3,10,12 Gazi University, Neurosurgery Department, Ankara, Turkey

2,9,11 Gazi University, Pharmacology Department, Ankara, Turkey

4,7 Gazi University, Medical Histology and Embryology Department, Ankara, Turkey

5,8 Gazi University, Radiology Department, Ankara, Turkey

6 Gazi University, Neurology Department, Ankara, Turkey

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Correspondence address:

Fikret DOĞULU

E-mail: alpob@yahoo.com

dimetilsulfat (DMSO4) (Grup IV, n = 6) and NPPB (Grup V, n=6) uygulanmıştır. Her grupta enjeksiyon öncesi ve sonrası baziler arter çapı ölçülmüş, baziler arter ve beyin sapında ışık ve elektron mikroskopuyla morfolojik değerlendirme yapılmıştır.

**BULGULAR:** Grup I, grup IV ve grup V'de anlamlı anjiyografik ve morfolojik değişiklik gözlenmedi. ET-1 grubunda damar çapında anlamlı daralma ve morfolojik olarak belirgin patolojik değişiklikler saptandı. NPPB ve ET-1 grubunda ise ET-1 grubuna göre vazokonstriksiyonun daha az olduğu ve morfolojik olarak damar duvarı ve nöronlarda daha az etkilenme olduğu görüldü.

**SONUÇ:** Bu bulgular Cl kanallarının ET-1 ile tetiklenen vazospazmda rol oynadığını ve Cl kanal inhibitörlerinin serebral vazospazmı önlemede etkili olabileceğini düşündürmektedir.

**ANAHTAR SÖZCÜKLER:** Cl- kanal inhibitörleri, Serebral vazospazm, Endotelin-1

## INTRODUCTION

Endothelin-1 (ET-1), a peptide synthesized in endothelial cells, is one of the most potent vasoconstrictor agents that lead to profound and long-lasting effects in both arterial and venous smooth muscle (38). It has been implicated in the pathophysiology of cerebral vasospasm (39, 40). ET-1 is produced primarily by endothelium, but can also be produced by astrocytes and neurons. ET-1 is also involved in fibrosis, endothelial and smooth muscle proliferation, and inflammation (11). Following its release from endothelium, ET-1 produces contraction in vascular smooth muscle cells via activation of ET-1 receptor type A or type B (ETA and ETB) (32). Both receptor types increase intracellular concentrations of Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) through either mobilization of Ca<sup>2+</sup> from intracellular stores or stimulation of Ca<sup>2+</sup> influx through plasmalemma (2). Since ET-1 causes long-lasting vasoconstriction of large cerebral vessels both in-vivo and in-vitro, an experimental animal model has been established by using ET-1 to induce cerebral vasospasm (4, 38).

Chloride (Cl<sup>-</sup>) channels have been identified in several types of smooth muscle including vascular smooth muscle (25, 29, 37). Although their functional roles and expressions remain controversial, Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels are the most frequently studied Cl<sup>-</sup> channels. Many agonists, including norepinephrine (NE), methacholine, and histamine, also activate Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels (14). These agonists release Ca<sup>2+</sup> from the intracellular Ca<sup>2+</sup> store, which, in turn, activates the Ca<sup>2+</sup>-dependent Cl<sup>-</sup> current, thus depolarizing the cell membrane and ultimately resulting in contraction. Cl<sup>-</sup> efflux induces depolarization and contraction of smooth muscle cells (14, 25). Activation of Cl<sup>-</sup> channels leads to depolarization and activation of voltage-gated Ca<sup>2+</sup> channels (VGCC), resulting in Ca<sup>2+</sup> entry and

contraction (25). Chloride channels contribute to the regulation of intracellular pH and cell volume as well (9). Using isolated cerebral arteries, Nelson et al. (28) suggested that Cl<sup>-</sup> channels play a role in pressure-induced cerebral arterial myogenic tone and that the nonselective Cl<sup>-</sup> channel blockers IAA-94 and DIDS displayed dilated pressurized rat cerebral arteries. However, Nelson et al. (28) reported that the Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel blocker niflumic acid failed to show any inhibitory effect on the developed tone.

The aim of the present study was to test the effect of 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), a Cl<sup>-</sup> channel antagonist, on the ET-1-induced cerebral vasospasm in rabbit basilar artery to investigate the contribution of Cl<sup>-</sup> channels in cerebral vasospasm.

## MATERIALS and METHODS

Thirty Albino rabbits of both sexes weighting 2.7-3.6 kg were divided into five groups. Protocols were performed in conformity with the guidelines of National Institutes of Health guidelines for animal care. All the animals were anesthetized by 30mg/kg sodium pentobarbital (iv) and ventilated using a respirator. A 5 French high flow multipurpose catheter was placed in the vertebral artery through the femoral artery in each animal. A control angiogram of the basilar artery was performed in each group by bolus injection of 10ml Omnipaque (300mg/ml, Nycomed AS, Oslo) with a power injector set at a rate of 6ml/sec, using an Advantx AFM-DX Hiline digital imaging system (General Electric, Milwaukee, Wisconsin, USA) under standard conditions. Throughout the investigation, a 16cm image intensifier with a focal spot size of 0.6mm on a 1024x1024 matrix and a image acquisition rate of 2 frames/sec was used. Fifteen minutes after the control angiogram, 0.6ml of isotonic saline in Group I, 0.6 ml of 10<sup>-9</sup> M ET-1 (Sigma, Germany) in Group II were slowly injected into the

vertebral arteries of the animals. Thirty minutes after the injection, control angiograms were performed to evaluate the effects of ET-1 and isotonic saline. In the ET-1 plus NPPB (1A/1540793, Tocris, USA) treated group (Group III), 0.1 ml 20mM NPPB was slowly injected after the control angiogram. 0.6ml, 10-9M ET-1 was slowly injected ten minutes after the NPPB administration. The last angiograms were performed thirty minutes after the injection of ET-1 to evaluate the effects of ET-1 in NPPB treated rabbits. In Group IV, the effect of dimethylsulfate (DMSO4) was tested by a control angiogram thirty minutes after the injection of 0.1ml DMSO4 since NPPB was dissolved in DMSO4. In Group IV, the effect of NPPB in the normal basilar artery was tested by a control angiogram 30 minutes after the injection of 0.1ml 20mM NPPB (Figure 1). On the subtraction images, the diameter of the basilar artery in pre and post injection angiograms was measured in each group and compared by quantitative analysis software (4). All animals were sacrificed immediately after the repeat angiography by exsanguination. Basilar arteries were rapidly removed and processed by methods described previously for ultrastructural investigation (5,8). The prefixative was 2% glutaraldehyde in 0.1 M Sorensen's phosphate (ph:7.4). The basilar arteries and brain stems were freshly dissected and immersed in the prefixative for 1 hour at 18°C. After a brief period of

washing in phosphate buffer, they were post fixated for one hour in 1% osmium tetroxide in phosphate buffer. They were then briefly rinsed in phosphate buffer and dehydrated with graded concentrations of ethanol. Specimens were stained en bloc using a saturated solution of uranyl acetate in 80% ethanol before completion of dehydration and embedding in Araldite. 1.0 μm thick sections were stained with toluidine-blue for light microscopy. Thin (silver-gray) sections were stained with uranyl acetate and lead citrate and examined with the electron microscope (EM 900 Carl Zeiss). The diameter of the basilar artery on the pre and post injection angiograms was measured in each of the two treatment groups. Basilar artery diameter values before and after treatments in each group were expressed as the mean ± standard error of the mean, and 95% confidence interval. Statistical comparison between the calibers of the basilar artery between the pre and post injection angiograms was calculated with Wilcoxon signed ranks test.

**RESULTS**

*Angiographic Findings*

The angiographic vessel caliber of the basilar artery in the ET-1 group reduced significantly ( $p < 0.05$ , 0.020) (Figure 2). In ET-1 + NPPB group, there was significant increase in the diameter of the vessel ( $p < 0.05$ , 0.023).

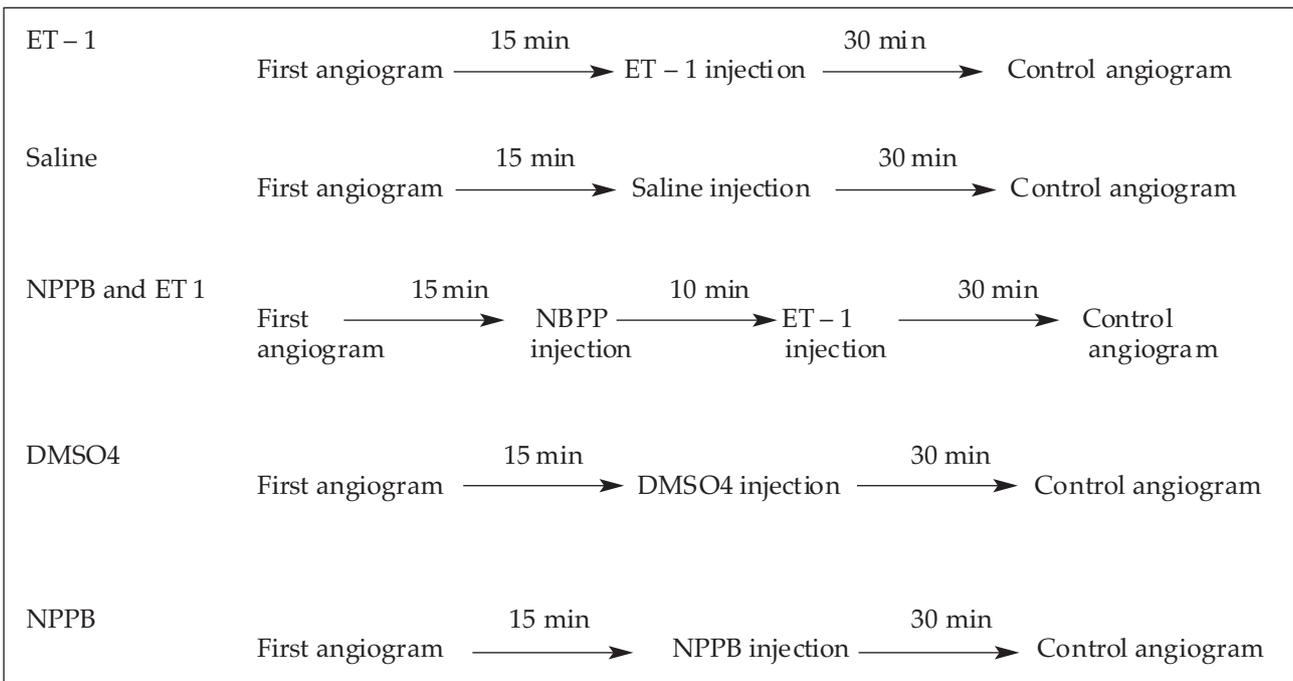
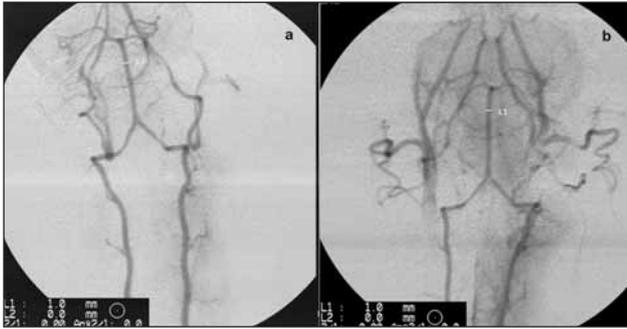


Figure 1: Timetable showing the injections and angiograms.



**Figure 2:** Measurements of basilar artery before (A) and after (B) the consecutive intra-arterial injection of NPPB + ET1. There is no reduction in the diameter of the vessel.

There was no statistically significant difference in the diameter of the vessels in NPPB, saline and DMSO4 groups. The mean values, standard deviation and median values of the diameter of the vessels are shown in (Table I).

**Morphological Findings**

Light and transmission electron microscopic evaluation of basilar arteries showed prominent vasoconstriction in blood vessels in ET-1 applied group (Group II). The lumen was narrowed and the vascular wall thickness was increased with the effect endothelin (Figure 3A,B). Lamina elastica interna (LEI) was electron lucent, damaged and deeply convoluted due to ET-1 induced vasospasm (Figure 3A,B). Because of the blood vessel construction, endothelial cells were established in close proximity to each other and seemed to have lost their squamous appearance and plumped to the lumen (Figure 3B). They had both large and small vacuoles luxuriantly and had convoluted nuclei (Figure 3B). Smooth muscle cells

that were located between and under the LEI infoldings had structural degeneration in certain places (Figure 3B). Intercellular spaces between the smooth muscle cells were broadened (Figure 3B).

In the NPPB plus ET-1 combined group (Group III), thickening of the vessel walls and narrowing of the lumen were not determined (Figure 3C). Convolution and density of LEI were normal (Figure 3C,D). Healthy structures of endothelial cells seemed to be protected. Cytoplasmic vacuoles were determined to be within normal limits (Figure 3D). Smooth muscle cells and collagens under endothelial cells were established to be normal (Figure 3D).

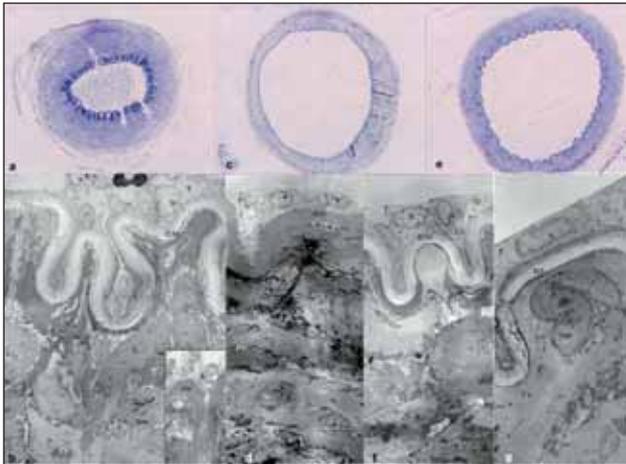
There were no degenerative findings in the group given solely NPPB (Group V) (Figure 3E,F). The diameters of both the wall and the lumen, and the degree of LEI convolution were within normal limits (Figure 3E). LEI were less electron lucent than the endothelin applied ones (Figure 3F). Endothelial cells had normal ultrastructural appearances (Figure 3F). Smooth muscle cells, collagen distribution and structure were also determined as normal (Figure 3F). Control group of basilar arteries had normal ultrastructural appearance (Figure 3G).

Light and transmission electron microscopic evaluation of the brain stem and vessels showed prominent vasoconstriction in ET-1 applied group (Group II) (Figure 4A,B). Vasoconstriction and perivascular edema were observed in thick sections of the brainstem in this group (Figure 4A). Same group’s electron microscopic figures represent evident edema and vasoconstriction in blood vessel wall (Figure 4B). Small and big vacuolar accumulation and mitochondrial crystalalysis in neuron cytoplasm were interpreted as prominent degenerative findings (Figure 4B). In addition, separation and leakage in myelin sheets of axons were observed (Figure 4B).

Thick sections of the NPPB and ET-1 applied group (Group III) represented normal structure of neurons, blood vessels and myelin structure in the brainstem like in NPPB group (Group V) and no edema in blood vessel wall (Figure 4C.) No degenerative findings or structural variations were seen in brain stem as well. Blood vessel wall and neurons were observed normal in electron microscopy (Figure 4D). Edema was not observed in blood vessel walls (Figure 4E). Neurons and blood vessel wall seemed to be normal in brainstem. Widening myelin lamellae in same places was evaluated as minimal degenerative finding (Figure 4F).

**Table I:** Table showing mean values, standart deviation and the median values of the groups.

		ET-1	ET-1 + NPPB	Saline	DMSO4	NPPB
Mean	pre	,8833	,6833	,8667	,8833	,8667
	post	,7000	,8500	,8667	,9000	,9167
N	pre	6	6	6	6	6
	post	6	6	6	6	6
Standart Deviation	pre	,07528	,07528	,08165	,09832	,05164
	post	,06325	,08367	,10328	,14142	,07528
Median	pre	,9000	,7000	,8500	,8500	,9000
	post	,7000	,8000	,9000	,9000	,9000

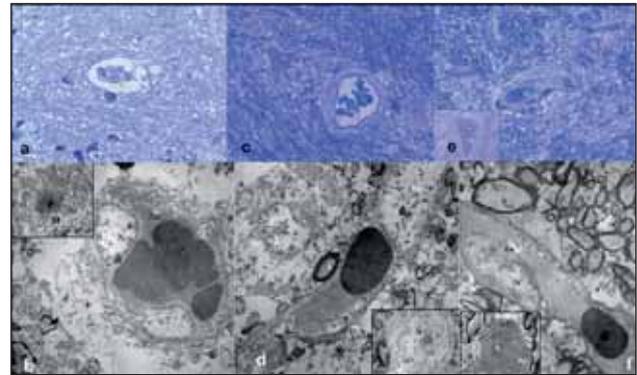


**Figure 3:** Comparative findings on light and electron micrographs of cross sectioned basilar arteries. (A) Only endothelin applied group; deeply folded lamina elastica interna, thickened vessel wall and narrowed lumen are seen (Toluidine blue, LM, X10). (B) Endothelin applied group; convoluted LEI and plumped endothelial cells (E) which are caused by vessel contraction are viewed. Intercellular spaces between smooth muscle cells are widened. Some structural degeneration is seen in smooth muscle cells which were located between and under the convolution of LEI. (Inset) Another view of deeply convoluted IEL and plumped endothelial cells (E). (Uranyl acetate-lead citrate; TEM, bX7000; inset X3000). (C) Endothelin and NPPB applied group; 1 $\mu$  thick cross sectioned basilar artery. Morphological features are similar with control group. (Toluidine blue, LM, X10). (D) Endothelin and NPPB applied group; LEI collagens (Co), endothelial cells (E), smooth muscle cells (SMC) and their (nuclei) represent the similar structural appearance with control group samples. (Uranyl acetate-Lead citrate; TEM X3000). (E) NPPB applied group; 1 $\mu$  thick cross sectioned basilar artery. Similar with control ones. (Toluidine blue, LM, X10). (F) NPPB applied group. LEI, smooth muscle cells (SMC), endothelial cells (E), nuclei of endothelia and collagen (Co) distribution represent normal ultrastructural appearance. (Uranyl acetate-Lead citrate, TEM X4000). (G) Control group of basilar arteries; Endotel (E), nucleus (N),

IEL, smooth muscle (SMC) and collagen fibers (Co) represent normal ultrastructural appearance. (Uranyl acetate-lead citrate, TEM X4400)

### DISCUSSION

A considerable body of evidence suggests that oxyhemoglobin is the primary spasmogenic agent, followed by arachidonic acid metabolites, endothelins, free radicals, serotonin, adenosine (1,3,12,16,24,27). Experimental data suggest that Ca<sup>2+</sup>-dependent and independent vasoconstriction play role in cerebral vasospasm. However, the extent to which each mechanism is responsible is still unknown. Various agents including blood products and ET-1 that



**Figure 4:** Light and electron microscopic comparative findings of brainstem and its vessels in various calibers. (A) Only endothelin applied group; thick section of brainstem was shown construction in vessels (Bv) and widened perivascular space (edema?) (\*). Neurons (Ne) and axons were seemed to be normal. (Toluidine blue, LM, X400). (B) Only endothelin applied group; widened perivascular space (edema?) (+) and vasoconstriction were prominent in medulla oblongata. Endothelium (E) and its nucleus were seen. (Inset) Neuron (Ne) cytoplasm was shown vacuoles in different size and mitochondria with crystalalysis. (Uranyl- acetate-lead citrate, TEM, X3000; inset X4400). (C) Endothelin + NPPB applied group; 1 $\mu$  thick sections of medulla oblongata. Neurons (Ne), neuron nucleus (N), blood vessels (Bv), and myelinated axons (A), show the similar findings with control groups. All these structures show normal appearance (Toluidine blue, LM, X100). (D) Endothelin + NPPB applied group; endothelial cells (E), neurons (Ne) and myelinated axons (A) represent normal ultrastructural appearance. (Inset) Another normal myelinated axon (A), and its nucleus (N), (Uranyl acetate-Lead citrate, TEM X400; inset X3000). (E) NPPB applied group; 1 $\mu$  thick section of the brainstem. Normal structure of vessels (Bv) and myelinated axon structures (A). (Inset) Another neuron and its nucleus (N) were normal (Toluidine blue, LM, X400; inset X100). (F) NPPB applied group; endothelial cell (E) and its nucleus (N), axon (A) and edema in vessel wall\* were seen. (Inset) Neuron (Ne) and its nucleus (N) were seen in their normal structure (Uranyl acetate-Lead citrate, TEM, X4400; inset X3000).

cause depolarization and contraction in arteries, induce the release of Ca<sup>2+</sup> from intracellular stores, leading to Cl<sup>-</sup> efflux through plasma membrane by Ca<sup>2+</sup>-activated-Cl<sup>-</sup> channels (1,22,23,25,33).

Following its release from endothelium, ET-1 produces contraction in vascular smooth muscle cells via activation of ET-1 receptor type A or type B (ETA and ETB) (32). Both receptor types increase intracellular concentrations of Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) through either mobilization of Ca<sup>2+</sup> from intracellular stores or stimulation of Ca<sup>2+</sup> influx through plasmalemma (2). In smooth muscle cells, increase in [Ca<sup>2+</sup>]<sub>i</sub> lead to Cl<sup>-</sup> ion efflux which causes depolarization and contraction

(26). Tani and Matsumoto suggest that vasospasm is characterized by a continuous elevation of  $[Ca^{2+}]_i$  and 20-Kd light chain of myosin (MLC20) phosphorylation (34). Shaw et al reported that the non-selective ET receptor antagonist TAK-044 showed a trend toward a lower incidence of delayed ischemic neurological deficit (DIND) in double blinded randomized clinical trial in humans (31).

Previously, it has been shown that  $Ca^{2+}$  activated  $Cl^-$  channels exist also in rabbit cerebral artery (20). However, their contribution to contraction in cerebral arteries by agonists such as ET-1 remains undetermined.

It has been demonstrated previously that the magnitude of agonist-induced contractions was reduced by  $Cl^-$  channel blockers in the rabbit middle cerebral arteries (18). Activation of  $Cl^-$  channels is involved also in ET-1-induced contraction in endothelially intact rings obtained from rabbit basilar artery (14).

Consistent with these studies, NPPB inhibited the contractile effect of ET-1 in rabbit basilar artery in the present study. However, it is difficult to define the role of  $Cl^-$  channels precisely in cell physiology since many of these blockers have poor selectivity. For instance,  $Cl^-$  channel blockers might block L-type- $Ca^{2+}$  channels, non-selective cation channels and also activate  $Ca^{2+}$ -activated  $K^+$  channels (15, 17, 19, 21, 35). A blockade of  $Ca^{2+}$  channels could indirectly cause the depression of  $Ca^{2+}$ -activated- $Cl^-$  channels. Furthermore, it has been suggested that NPPB may block  $Ca^{2+}$ -dependent contractile processes independent of channel blockade (21). Therefore interpretation of the data is not completely adequate to clarify the exact mechanisms.

Ischemia-induced neuronal damage has been suggested to be linked to the toxicity of excitatory amino acids, particularly glutamate (7,10). Glutamate concentration has been reported to increase gradually after middle cerebral artery (MCA) occlusion in rat. Various  $Cl^-$  channel blockers including NPPB have been shown to depress the ischemia/reperfusion-evoked release of glutamate and other amino acids (36). Therefore, inhibitory effect of NPPB on glutamate release may have contributed to the reversal of ET-1-induced histological alterations in this study. The authors think that NPPB reduces the neuronal damage indirectly by preventing the vasospasm thus the ischemia, rather than passing the BBB and showing a

direct neuroprotective effect. As a principle, naturally drug treatment should be administered after the onset of vasospasm. In this study the authors firstly aim to test the role of  $Cl^-$  channels in cerebral vasospasm, that is why NBPP was administered before ET-1. NBPP could have been ineffective and we could not conclude that  $Cl^-$  channels play a role in cerebral vasospasm if ET-1 was administered before NBPP. Further studies should be done to investigate the efficacy of NPPB after the onset of vasospasm. Only in this way can we decide whether NPPB is effective in the reversal of ET-1 induced vasospasm.

Depolarization of neurons by glutamate increases intracellular  $Cl^-$  concentration and a reduction in  $Cl^-$  entry may produce a protective effect (30). It has been suggested that  $Na^+-K^+-Cl^-$  cotransporter isoform 1 (NKCC1) is involved in the acute-glutamate-mediated excitotoxicity as a result of excessive  $Na^+$  and  $Cl^-$  entry (6). Moreover, NKCC1 expression has been reported to increase in the brain in rabbits after neonatal hypoxia-ischemia and the blockade of  $Cl^-$  channels with NPPB led to improvement in the brain injury (13). This mechanism may be another explanation for the beneficial effect of NPPB on the ET-1-induced histological changes.

## CONCLUSIONS

In conclusion, NPPB prevented the ET-1-induced vasospasm in rabbit basilar artery and exhibited beneficial effect on the histological alterations in brain stem. Although the exact mechanisms are not known, these data might be useful providing new treatment strategies in drug design for cerebrovascular spasm.

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