*Original Article*

# Cilostazol's Effect on the Response to Perivascular Nerve Stimulation in Isolated Dog Cerebral and Mesenteric Arteries

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**Clinical studies have demonstrated that cilostazol (CZ), an antiplatelet agent with type 3 phosphodiesterase inhibition, reduces the risk of secondary stroke. To analyze CZ's vascular action, especially in relation to endothelial and perivascular nerve functions, we examined CZ's effects on the responses to endothelial and nerve stimulation in dog cerebral arteries, and on the response to nerve stimulation in dog mesenteric arteries. Low concentrations of CZ ($10^{-8}$ and $10^{-7}$ mol/L) failed to relax the cerebral arteries, but a higher concentration ($10^{-6}$ mol/L) relaxed them in an endothelium-independent manner. Substance P–induced relaxation was endothelium-dependent in the cerebral arteries, whereas transmural electrical stimulation (TES) and nicotine-induced relaxation were endothelium-independent. This relaxation was abolished by $N^G$-nitro-L-arginine, an NO synthase (NOS) inhibitor. A lower concentration ($10^{-7}$ mol/L) of CZ enhanced the relaxation caused by nerve-derived NO but did not affect the relaxation caused by endothelium-derived NO in the cerebral arteries; moreover, it did not affect the contractions caused by nerve-derived noradrenaline in the mesenteric arteries under treatment with the NOS inhibitor. It is concluded that CZ may selectively enhance nitrergic nerve function, possibly *via* the activation of neuronal NOS, in dog cerebral arteries, since it does not affect the function of the noradrenergic nerve or that of endothelial NOS. Therefore, this novel vasodilatory effect of CZ may explain the reduction in the risk of secondary stroke in addition to the antiplatelet action and the direct vasodilatory action on smooth muscle. (*Hypertens Res* 2008; 31: 1425–1433)**

***Key Words*: cilostazol, nitrergic nerve, neuronal nitric oxide synthase, cerebral artery, mesenteric artery**

## Introduction

Antiplatelet agents benefit patients suffering from ischemic vascular diseases such as stroke, myocardial infarction, and peripheral artery diseases (*1*). The incidence rates of stroke are significantly higher in Japan than in the United States and other countries (*2*, *3*). A recent study reported that cilostazol (CZ), a type 3 phosphodiesterase (PDE) inhibitor, reduced the incidence of secondary stroke by 41.7% compared with placebo in 1,095 Japanese patients with recent stroke, and was especially effective against lacunar stroke. Further, the patients treated with CZ were not accompanied by more adverse events compared to those with placebo (*4*). CZ's preventive effect on stroke and cerebral ischemic attacks seems to be greater than that of other antiplatelet drugs such as aspirin, ticlopidine, and clopidogrel (*5*). Further, CZ's superior efficacy compared with these drugs may be attributed not only to its inhibition of platelet aggregation but also to its vasodilatory action (*4*).

Nitric oxide (NO) is known to be an important physiological substance in the control of cardiovascular and nervous systems. The constitutive NO synthases (NOS) are classified into endothelial and neuronal forms based on their structure and location (*6*), and are called endothelial and neuronal NO synthases (eNOS and nNOS) (*7*), respectively. We first found and introduced the functional roles of the nitrergic nerve in the cerebral arteries of many species, including dogs (*8*).

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The present study aimed to examine CZ's possible preventive effects on stroke and cerebral ischemic attacks, other than its antiplatelet action and direct dilatation of cerebroarterial smooth muscle. For this purpose, we attempted to determine whether CZ affects nitrergic nerve function and/or endothelial function in the isolated dog cerebral artery. If CZ did affect both functions, we further examined whether there is a more selective effect on the response associated with nNOS or eNOS. CZ's effect on the function of noradrenergic nerves in dog mesenteric arteries was also examined in comparison with the effect on the function of nitrergic nerves in cerebral arteries.

## Methods

### Preparation

Hybrid dogs (HBD) of either sex, weighing 16 to 20 kg, were used in the present study. The Animal Care and Use Committee of our University approved the use of dog blood vessels.

Dogs anesthetized with both ketamine (40 mg/kg, i.m.) and sodium pentobarbital (30 mg/kg, i.v.) were killed by bleeding from the carotid arteries. The brain and mesentery were removed, and cerebral (middle cerebral, posterior cerebral, or basilar) and mesenteric arteries were isolated. Since the responses to transmural electrical stimulation (TES) and nicotine did not differ among these three cerebral arteries (*8*), any of them can be used as cerebral arteries. The arteries were helically cut into strips of approx. 20 mm in length, with special care being taken to preserve the endothelium. The functional integrity of the endothelium was verified by the extent of relaxation (35–45%) induced by substance P ($10^{-8}$ mol/L) in the presence of $10^{-6}$ mol/L indomethacin. In some strips, the endothelium was removed by gently rubbing the intimal surface with a cotton ball; endothelial denudation was verified by the abolishment of the response to substance P. Each specimen was vertically fixed between hooks in a muscle bath (10 mL capacity) containing modified Ringer-Locke solution of the following composition (mmol/L): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25.0, and dextrose 5.6. The bathing media were maintained at 37±0.3°C and aerated with a mixture of 95% $O_2$ and 5% $CO_2$; the pH of the solution was in the range of 7.38 to 7.43. The hook fixing the upper end of the strips was connected to the lever of a force-displacement transducer, and the resting tension was adjusted to 1.5 g, which is the optimal tension for inducing a maximal contraction (*9*). Before the start of the experiments, the arterial strips were allowed to equilibrate in the bathing media, during which time the fluids were replaced three times with 10-min interval.

### Isometric Tension Recording

Isometric mechanical responses of cerebral and mesenteric artery strips were displayed on a pen recorder. The contractile response to 30 mmol/L $K^+$ was obtained first, and the strips were repeatedly washed with fresh medium and equilibrated. In order to obtain the relaxant responses to electrical and chemical stimulations, the strips were precontracted with prostaglandin $F_{2\alpha}$ ranging from $5\times10^{-7}$ mol/L to $2\times10^{-6}$ mol/L. At the end of each series of experiments, papaverine ($10^{-4}$ mol/L) was applied to attain the maximal relaxation.

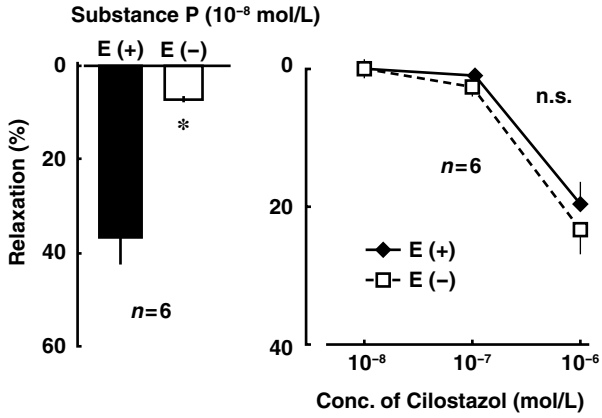


**Fig. 1.** *Relaxant responses to substance P ($10^{-8}$ mol/L, left) and cilostazol ($10^{-8}$–$10^{-6}$ mol/L, right) in dog cerebral artery strips with and without the endothelium in the presence of indomethacin ($10^{-6}$ mol/L) and partially contracted with prostaglandin $F_{2\alpha}$. Relaxation induced by $10^{-4}$ mol/L papaverine was taken as 100% on the ordinate. E (+), strips with endothelium; E (−), strips without endothelium; n, number of strips; n.s., no significance. Significant difference from E (+): *$p<0.01$ (unpaired t-test). Vertical bars represent SEM.*

Some of the strips without the endothelium were placed between stimulating electrodes. TES of 0.2-ms square-wave pulses at 2, 5, and 20 Hz was applied to the strips, then a frequency-related response was obtained (*8*). In order to analyze the effect of treating agents such as CZ ($10^{-7}$ mol/L), we used a stimulus frequency of 5 Hz for 40 s, which induced reproducible responses.

Drugs such as substance P, CZ, nicotine, nitroglycerin, NO and noradrenaline were applied directly to the bathing media singly or cumulatively to obtain the responses of the strips.

To examine the endothelium-dependency, responses to substance P ($10^{-8}$ mol/L) or CZ ($10^{-8}$, $10^{-7}$ and $10^{-6}$ mol/L) were compared in cerebral artery strips with and without the endothelium treated with indomethacin ($10^{-6}$ mol/L).

In some cerebral artery strips treated with indomethacin ($10^{-6}$ mol/L), nicotine ($10^{-4}$ mol/L), substance P ($10^{-8}$ mol/L), NO ($10^{-8}$ and $10^{-7}$ mol/L) and/or nitroglycerin ($10^{-9}$ and $10^{-8}$ mol/L) were successively applied to the bathing media, the effects of CZ ($10^{-8}$, $10^{-7}$ and/or $10^{-6}$ mol/L) on the responses to the drugs were examined.

In the mesenteric artery strips without the endothelium treated with $N^G$-nitro-L-arginine (L-NA; $10^{-5}$ mol/L) to avoid

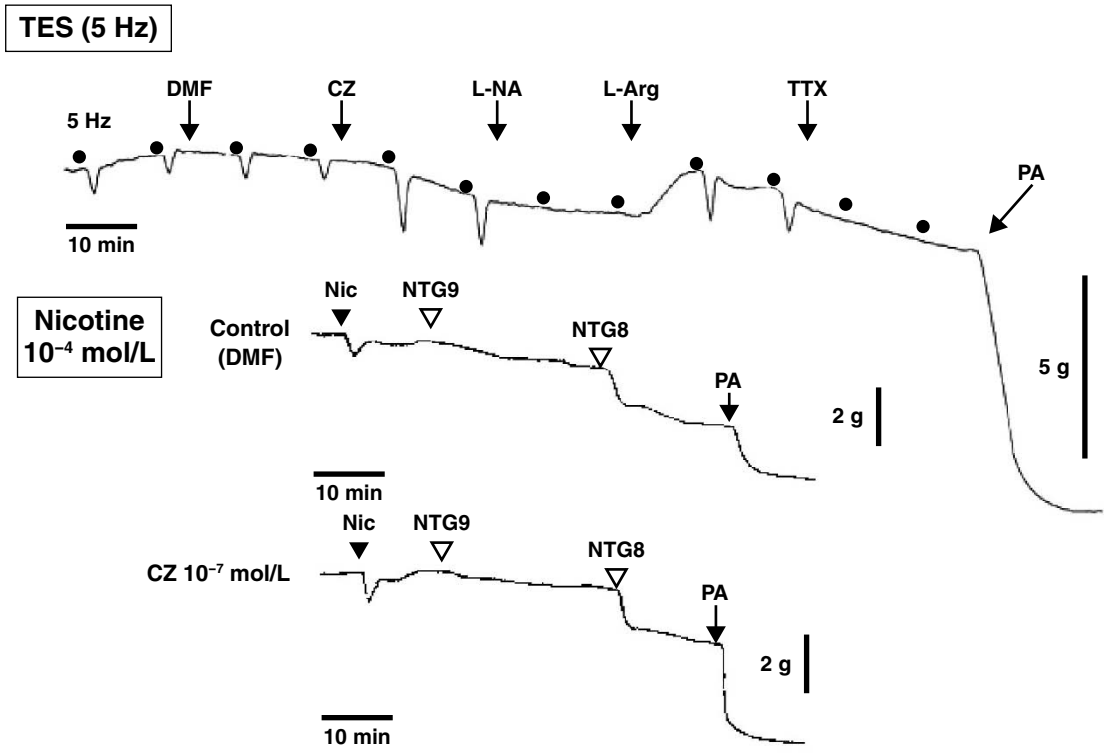


**Fig. 2.** *Top tracing represents the response of a dog cerebral artery strip to TES at 5 Hz before and after treatment with N,N-dimethylformamide (DMF, 0.01%), cilostazol (CZ, $10^{-7}$ mol/L), $N^G$-nitro-L-arginine (L-NA, $10^{-6}$ mol/L), L-arginine (L-Arg, $3\times10^{-4}$ mol/L) and tetrodotoxin (TTX, $3\times10^{-7}$ mol/L). Middle and bottom tracings represent responses to nicotine (Nic, $10^{-4}$ mol/L) and nitroglycerin (NTG9 and NTG8 are $10^{-9}$ mol/L and $10^{-8}$ mol/L, respectively) of a dog cerebral artery strip denuded of endothelium before (control, DMF) and after treatment with CZ ($10^{-7}$ mol/L). The denuded strip was partially contracted with prostaglandin $F_{2\alpha}$. PA represents $10^{-4}$ mol/L papaverine, the dose that induced maximal relaxation.*

the influences of the endothelium and endogenous NO, the responses to TES (5 Hz), nicotine ($10^{-4}$ mol/L), and noradrenaline ($2\times10^{-8}$–$10^{-5}$ mol/L) were obtained, and the effect of CZ ($10^{-8}$, $10^{-7}$ and/or $10^{-6}$ mol/L) on the responses were examined.

Relaxations induced by TES and drugs are presented as relative values to those induced by $10^{-4}$ mol/L papaverine, whereas contractions induced by TES and drugs are presented as relative values to those induced by 30 mmol/L $K^+$.

### Statistics and Drugs Used

Results shown in the text and figures are expressed as mean values±SEM. Statistical analyses were made using Student's unpaired *t*-test in the case of comparisons between two groups, or using one-way analysis of variance in the case of comparisons among three or more groups. The drugs used were L-NA, substance P (Peptide Institute, Minoh, Japan), nicotine (base [Nacalai Tesque, Kyoto, Japan], indomethacin [Sigma, St. Louis, USA], *dl*-noradrenaline hydrochloride, tetrodotoxin (Daiichi Sankyo Co., Tokyo, Japan), prazosin hydrochloride (Wako Pure Chemical Industries, Osaka, Japan), prostaglandin $F_{2\alpha}$ (Pfizer Japan, Tokyo, Japan), nitroglycerin (Nihon-Kayaku Co., Tokyo, Japan), and papaverine hydrochloride (Dainippon Sumitomo Pharma Co., Osaka, Japan). CZ was dissolved in *N,N*-dimethylformamide (DMF). Responses to NO (exogenously applied NO) were obtained by adding the $NaNO_2$ solution adjusted to pH 2 (*10*).

## Results

### Relaxations Caused by TES and Drugs in Cerebral Artery

In the artery strips with the endothelium, CZ ($10^{-8}$–$10^{-6}$ mol/L) induced relaxations at $10^{-6}$ mol/L, which were not significantly different from those induced in strips without the endothelium (Fig. 1, right). In the endothelium-denuded cerebral artery strips partially contracted with prostaglandin $F_{2\alpha}$, TES at 5 Hz induced transient relaxation endothelium-independently, which was abolished by treatment with L-NA ($10^{-6}$ mol/L) or tetrodotoxin ($3\times10^{-7}$ mol/L). This suggested that the stimulated nerve was nitrergic (Figs. 2 and 3). The abolished response to TES by treatment with L-NA ($10^{-6}$ mol/L)

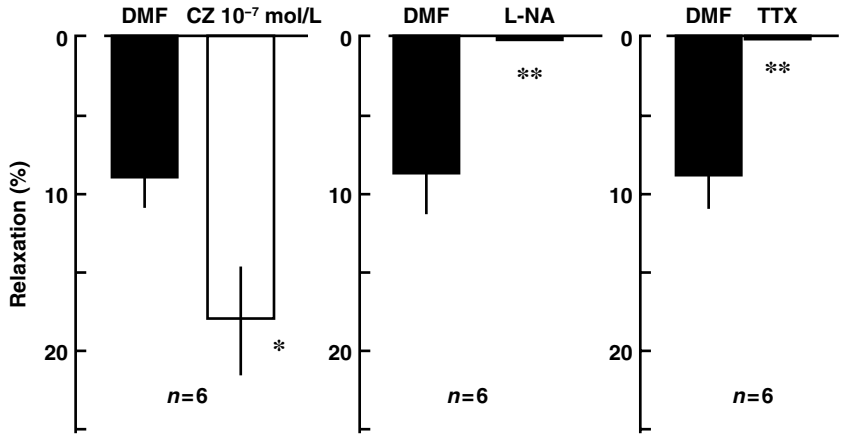


**Fig. 3.** *Modification by N,N-dimethylformamide (DMF, 0.01%), cilostazol (CZ, $10^{-7}$ mol/L), $N^G$-nitro-L-arginine (L-NA, $10^{-6}$ mol/L), and tetrodotoxin (TTX, $3\times10^{-7}$ mol/L) of the responses to transmural electrical stimulation (5 Hz) in dog cerebral artery strips denuded of the endothelium and partially contracted with prostaglandin $F_{2\alpha}$. Relaxation induced by $10^{-4}$ mol/L papaverine was taken as 100% on the ordinate. n, number of strips. Significant difference from control (DMF): *$p<0.05$, **$p<0.01$ (unpaired t-test). Vertical bars represent SEM.*

was restored by the addition of L-arginine ($3\times10^{-4}$ mol/L), (Fig. 2) (10.3±2.1% relaxation of L-NA + L-arginine, $n=6$). Nicotine ($10^{-4}$ mol/L) induced a transient relaxation endothelium-independently, which was abolished by $10^{-5}$ mol/L hexamethonium ($n=7$) in the endothelium-intact cerebral artery strips (data not shown). In the artery strips with intact endothelium treated with indomethacin ($10^{-6}$ mol/L) and partially contracted with prostaglandin $F_{2\alpha}$, substance P ($10^{-8}$ mol/L) induced relaxation, which was abolished by removal of the endothelium. This suggested that the substance P–induced relaxation was endothelium-dependent (Fig. 1, left). Nitroglycerin ($10^{-9}$ and $10^{-8}$ mol/L) and exogenously applied NO ($10^{-8}$ and $10^{-7}$ mol/L) relaxed the arteries in a concentration-dependent manner. Since the responses to TES at 5 Hz, nicotine ($10^{-4}$ mol/L), substance P ($10^{-8}$ mol/L), nitroglycerin ($10^{-9}$ and $10^{-8}$ mol/L), and exogenously applied NO ($10^{-8}$ and $10^{-7}$ mol/L) were consistent and reproducible, these concentrations and the frequency were used to quantitatively analyze the action of CZ.

## CZ-Induced Modification of the Responses to TES and Drugs in Cerebral Arteries

Treatment with CZ ($10^{-7}$ mol/L) potentiated TES-induced relaxation at 5 Hz (Fig. 3) in endothelium-denuded cerebral arteries, although the agent at concentrations of $10^{-8}$–$10^{-6}$ mol/L failed to significantly affect the relaxation induced by substance P ($10^{-8}$ mol/L), nitroglycerin ($10^{-8}$ mol/L), or exogenously applied NO ($10^{-8}$ and $10^{-7}$ mol/L) in the endothelium-intact cerebral artery (Figs. 4 and 5). This suggested that CZ potentiated the relaxation mediated by nerve-derived NO but not by endothelium-derived NO. Further, the relaxation induced by nicotine ($10^{-4}$ mol/L) was also potentiated

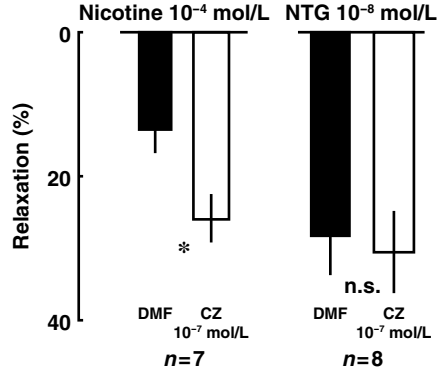


**Fig. 4.** *Modification by N,N-dimethylformamide (DMF, 0.01%) and cilostazol (CZ, $10^{-7}$ mol/L) of the responses to nicotine ($10^{-4}$ mol/L), and nitroglycerin (NTG, $10^{-8}$ mol/L) in dog cerebral artery strips with the intact endothelium in the presence of indomethacin ($10^{-6}$ mol/L) and partially contracted with prostaglandin $F_{2\alpha}$. Relaxation induced by $10^{-4}$ mol/L papaverine was taken as 100% on the ordinate. n, number of strips; n.s., no significance. Significant difference from control (DMF): *$p<0.05$ (unpaired t-test). Vertical bars represent SEM.*

by CZ ($10^{-7}$ mol/L) in the endothelium-intact cerebral arteries (Fig. 4). Typical tracings of the responses are illustrated in Fig. 2.

## Contractions in Response to TES and Drugs in Mesenteric Arteries

In endothelium-denuded mesenteric artery strips treated with

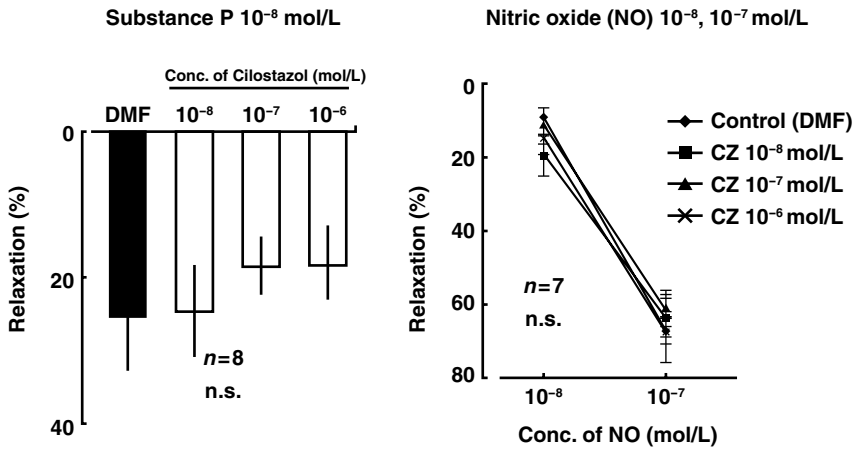


**Fig. 5.** *Modification by N,N-dimethylformamide (DMF, 0.01%) and cilostazol (CZ, $10^{-8}$, $10^{-7}$, $10^{-6}$ mol/L) of the responses to substance P ($10^{-8}$ mol/L), and exogenously applied NO ($10^{-8}$, $10^{-7}$ mol/L) in dog cerebral artery strips with the intact endothelium in the presence of indomethacin ($10^{-6}$ mol/L) and partially contracted with prostaglandin $F_{2\alpha}$. Relaxation induced by $10^{-4}$ mol/L papaverine was taken as 100% on the ordinate. n, number of strips; n.s., no significance. Vertical bars represent SEM.*

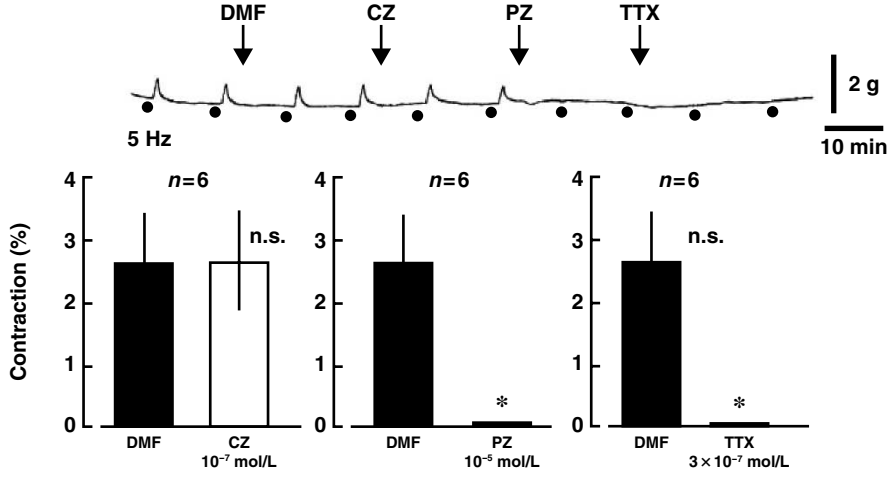


**Fig. 6.** *Modification by N,N-dimethylformamide (DMF, 0.01%), cilostazol (CZ, $10^{-7}$ mol/L), prazosin (PZ, $10^{-5}$ mol/L), and tetrodotoxin (TTX, $3\times10^{-7}$ mol/L) of the responses to transmural electrical stimulation (5 Hz) in dog mesenteric artery strips denuded of the endothelium and treated with indomethacin ($10^{-6}$ mol/L) + $N^G$-nitro-L-arginine (L-NA, $10^{-5}$ mol/L). Contractions induced by 30 mmol/L $K^+$ were taken as 100% on the ordinate of the summary data (bottom). n, number of strips; n.s., no significance. Significant difference from control (DMF): *$p<0.01$ (unpaired t-test). Vertical bars represent SEM.*

L-NA ($10^{-5}$ mol/L) in order to abolish the nitrergic nerve function, TES at 5 Hz induced a transient contraction, which was abolished by treatment with prazosin ($10^{-5}$ mol/L) or tetrodotoxin ($3\times10^{-7}$ mol/L), suggesting that the contraction is due to noradrenaline released from sympathetic nerves (Fig. 6). Nicotine ($10^{-4}$ mol/L) induced a transient contraction, which was abolished by hexamethonium ($10^{-5}$ mol/L, $n=7$) (data not shown). Noradrenaline ($2\times10^{-8}$–$10^{-5}$ mol/L) induced contractions in a concentration-dependent manner ($n=6$), (Fig. 7).

## CZ Modification of Contractions in Response to TES and Drugs in Mesenteric Arteries

The contractions induced by TES at 5 Hz were not affected by treatment with CZ ($10^{-7}$ mol/L) in the mesenteric artery strips without the endothelium treated with L-NA ($10^{-5}$ mol/L). CZ ($10^{-8}$–$10^{-6}$ mol/L) also failed to significantly affect the contractions induced by nicotine ($10^{-4}$ mol/L) and those by noradrenaline in the endothelium-denuded mesenteric artery strips treated with $10^{-5}$ mol/L L-NA (Fig. 7).

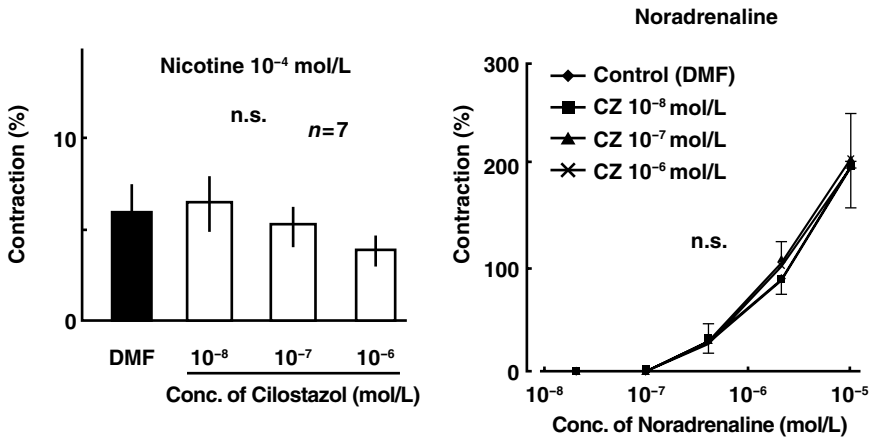


**Fig. 7.** *Modification by N,N-dimethylformamide (DMF, 0.01%) and cilostazol (CZ, $10^{-8}$, $10^{-7}$, $10^{-6}$ mol/L) of the responses to nicotine ($10^{-4}$ mol/L, left), and exogenously applied noradrenaline ($5\times10^{-8}$–$10^{-5}$ mol/L, right) in dog mesenteric artery strips denuded of the endothelium treated with indomethacin ($10^{-6}$ mol/L) + $N^G$-nitro-L-arginine ($10^{-5}$ mol/L). Contractions induced by 30 mmol/L $K^+$ were taken as 100% on the ordinate. n, number of strips; n.s., no significance. Vertical bars represent SEM.*

## Discussion

Endothelium-denuded dog cerebral artery strips responded to TES and nicotine by relaxation, which was abolished by tetrodotoxin and hexmethonium, respectively (*8*). We previously reported that NO synthesized by nNOS in perivascular nerve terminals acts as a neurotransmitter in cerebral arteries (*11*). NOS in the perivascular nerve is also activated by nicotine in the mediation of an increased influx of $Ca^{2+}$, relaxing the cerebral artery (*12*, *13*). CZ, introduced as an antiplatelet agent *via* type 3 PDE inhibition (*14*), relaxes the arteries *via* the increment of cyclic AMP in arterial smooth muscle cells (*15*). The present study revealed for the first time that CZ, at a dose lower than that causing direct vasorelaxation, effectively enhanced the cerebroarterial response to nitrergic nerve stimulation (Fig. 8). There are several reasons for this conclusion. CZ potentiated nicotine-induced cerebral artery relaxation, which was abolished by hexamethonium (*16*) and L-NA, a NOS inhibitor (*17*). The degree to which CZ potentiated nicotine-induced relaxation was similar to that of TES-induced relaxation. CZ's inability to potentiate the response to nitroglycerin or to exogenous NO indicates that this agent does not interfere with NO's action during intercellular transport or in vascular smooth muscle cells.

It was reported that ebselen, an antioxidant, protects against cerebral injury in stroke-prone spontaneously hypertensive rats (*18*). Choi *et al*. (*19*) reported that CZ rescued the brain from enlargement of cerebral infarction in an ischemic rat model through scavenging hydroxyl and peroxyl radicals. However, this is not the case in the present study because CZ failed to enhance relaxation caused by endogenous NO from the endothelium induced by substance P or exogenously applied NO. Therefore, CZ's action as a free radical scavenger is negligible in the non-ischemic dog cerebral arteries.

CZ did not affect the endothelium-dependent relaxation induced by substance P in the arteries treated with indomethacin at a concentration ($10^{-7}$ mol/L) sufficient to enhance the response to TES or nicotine. Even at the higher concentration of $10^{-6}$ mol/L, CZ failed to enhance the relaxation induced by substance P. In dog cerebral arteries with intact endothelium, substance P–induced relaxation in the presence of indomethacin is abolished by denudation of the endothelium or by treatment with L-NA or methylene blue, a soluble guanylyl cyclase inhibitor (*20*, *21*). This suggests that substance P acts on the endothelium and activates eNOS, resulting in the release of NO. These findings led us to understand that CZ enhances the function of nNOS, but not that of eNOS, in dog cerebral arteries. According to Nakamura *et al*. (*22*), CZ at concentrations above $3\times10^{-7}$ mol/L stimulated eNOS in rat thoracic aorta, resulting in aortic relaxation. CZ at concentrations higher than $10^{-5}$ mol/L is postulated to release NO from the endothelium *via* cyclic AMP/PKA- and PI3K/Akt-dependent mechanisms in human aortic endothelial cells (*23*). Therefore, CZ may increase NO release from the endothelium by activation of protein kinase A and PI3K/Akt *via* increased cyclic AMP in rat aorta. However, this is not the case in dog cerebral arteries, because $10^{-6}$ mol/L CZ failed to induce endothelium-dependent relaxation in dog cerebral arteries. In addition, β agonists are reported to produce both endothelium-dependent and -independent relaxation in rat aorta, whereas isoproterenol did not produce endothelium-dependent relaxation in dog coronary arteries (*24*). Thus, CZ-induced relaxation may not involve eNOS activation *via* a cyclic AMP/PKA- or PI3K/Akt-dependent mechanism in dog blood vessels.

The agent, a specific inhibitor of type 3 PDE, increases cyclic AMP in platelets and arterial smooth muscle, and thus

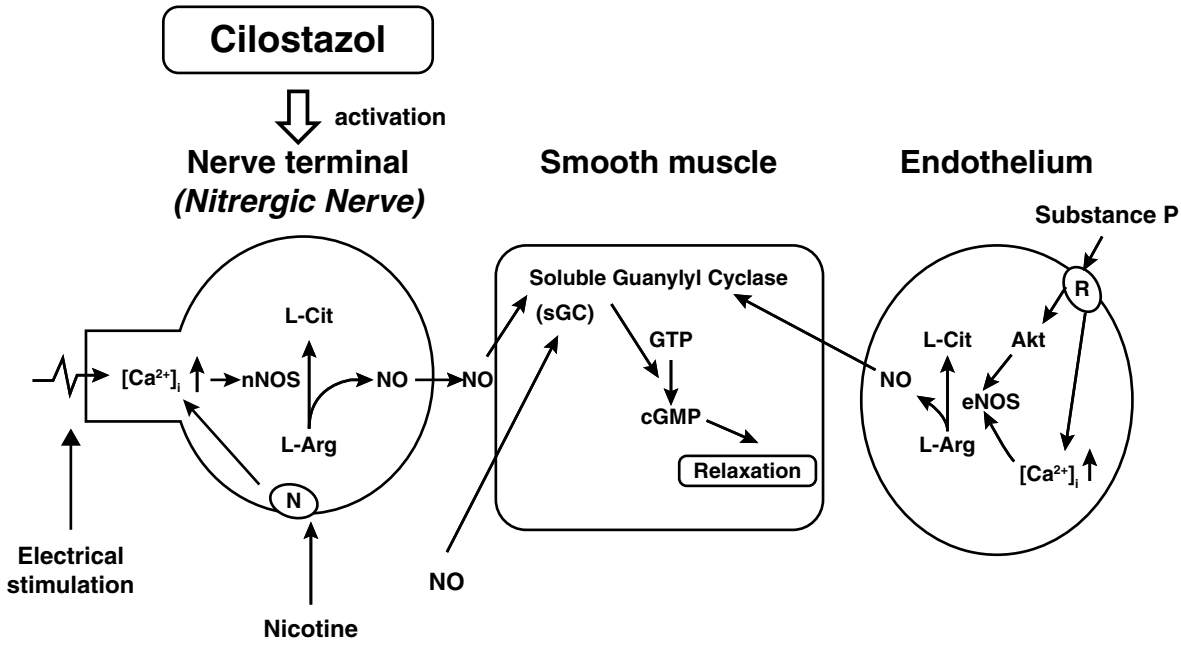


**Fig. 8.** *Hypothetical scheme of the action of cilostazol in the dog cerebral artery. $[Ca^{2+}]_i$, intracellular calcium; nNOS, neuronal nitric oxide synthase; L-Arg, L-arginine; L-Cit, L-citrulline; N, nicotinic receptor; R, receptor on the endothelium of substance P; Akt, serine/threonine kinase; eNOS, endothelial nitric oxide synthase.*

inducing antiplatelet and vasodilator actions (*5*). However, higher concentrations of CZ may non-specifically inhibit type 5 PDE and enhance the action of NO mediated by the increment of cyclic GMP in cerebral arteries. However, this was not the case in the present study because a higher concentration of CZ failed to enhance nitroglycerin-induced and exogenous NO-induced relaxation in the cerebral arteries.

One may think that CZ simply enhances the function of peripheral nerves. It has been reported that CZ improves impaired sciatic nerve motor and saphenous nerve sensory conduction velocity in diabetic rats (*25*) and prevents impairment of slow axonal transport in streptozocin-diabetic rats (*26*). Thus, CZ improves damaged neuronal function. However, these neuroprotective effects of CZ are explained by its improvement of diabetes-induced hemodynamic abnormality in the nerve's feeding artery (*25*, *26*).

In the present study, CZ did not potentiate contractions induced by TES or nicotine in dog mesenteric arteries, which contraction is caused by noradrenergic nerve stimulation (*27*, *28*). However, the same concentration of the agent enhanced the relaxation induced by nitrergic nerve stimulation with TES or nicotine in the cerebral arteries, suggesting that CZ selectively potentiated nitrergic nerve function without affecting noradrenergic nerve function. Further, the increment of the 10-times-higher concentration of CZ failed to potentiate neurogenic contraction caused by nicotine in mesenteric arteries. These findings led us to speculate that the potential effect of CZ is selective for nitrergic nerve function. It has been reported that forskolin and prostacyclin, which increase intracellular cyclic AMP, enhance neuronal NO release caused by electrical field stimulation through cyclic AMP/PKA activation in rat mesenteric arteries (*29*). Therefore, CZ may selectively activate nNOS in the nitrergic nerve but not eNOS in the endothelium of dog cerebral arteries.

We reported previously that an intracisternal application of NOS inhibitor contracts cerebral arteries by suppressing NO synthesis in the nitrergic nerve rather than in the endothelium (*30*), and that tonic discharge of postganglionic nitrergic neurons from the pterygopalatine ganglion plays crucial roles in dog cerebral vasodilation (*31*). Further, the present study revealed that CZ enhances NO production and/or release from the nitrergic nerve but not from the endothelium. Therefore, vasodilation in cerebral arteries caused by orally administered CZ may be mediated *via* the enhanced release of NO from the nitrergic nerve, but not from the endothelium of cerebral arteries, together with cerebroarterial smooth muscle relaxation by the increment of cAMP by type 3 PDE inhibition.

CZ induces vascular headache as an adverse effect (*32*). We have reported that sumatriptan, an anti-migraine drug, may resolve the headaches by counteracting nNOS-activation–induced neurogenic vasodilation (*33*). Thus, the present study may explain the headache induced by CZ together with its preventative effect against recurrent strokes.

It has been reported that chronic treatment with milrinone, a type 3 PDE–selective inhibitor, was associated with an increased risk of mortality (*34*). However, the present study has shown that a low concentration of CZ, which failed to relax the cerebral arteries directly, potentiated the nitrergic

nerve function innervating those arteries. Therefore, CZ's adverse effect on the heart muscle might be minimal if a low concentration is used.

The present study provides evidence that CZ is a possibly selective nNOS activator in dog cerebral arteries. This novel effect of the agent may explain the reduction in the risk of secondary stroke and the provocation of headache reported in many clinical studies, in addition to the antiplatelet action and the direct vasodilation of cerebrovascular smooth muscle.

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