Nitric Oxide Is the Predominant Mediator for Neurogenic Vasodilation in Porcine Pial Veins

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ABSTRACT
The innervation pattern and the vasomotor response of the potential transmitters in the porcine pial veins were investigated morphologically and pharmacologically. The porcine pial veins were more densely innervated by vasoactive intestinal polypeptide (VIP)- and neuropeptide Y-immunoreactive (I) fibers than were calcitonin gene-related peptide (CGRP)-I, choline acetyltransferase-I, Substance P (SP)-I, and NADPH diaphorase fibers. Serotonin (5-HT)-I fibers, which were not detected in normal control pial veins, were observed in isolated pial veins after incubation with 5-HT (1 μM). 5-HT-I fibers, however, were not observed when incubation with 5-HT was performed in the presence of guanethidine (1 μM), suggesting that 5-HT was taken up into the sympathetic nerves. In vitro tissue bath studies demonstrated that porcine pial veins in the presence of active muscle tone relaxed on applications of exogenous 5-HT, CGRP, SP, VIP, and sodium nitroprusside, whereas exogenous norepinephrine and neuropeptide Y induced only contractions.

Transmural nerve stimulation (TNS) did not elicit any response in pial veins in the absence of active muscle tone. However, in the presence of active muscle tone, pial veins relaxed exclusively on TNS. This tetrodotoxin-sensitive relaxation was not affected by receptor antagonists for VIP, CGRP, 5-HT, or SP but was blocked by L-glutamine (1 mM) and abolished by Nω-nitro-L-arginine (10 μM) and Nω-nitro-L-arginine methyl ester (10 μM). The inhibition by L-glutamine, Nω-nitro-L-arginine, and Nω-nitro-L-arginine methyl ester was reversed by L-arginine and L-citrulline but not by their d-enantiomers. These results demonstrate that the vasomotor effect of all potential transmitters except 5-HT in the pial veins examined resembles that in cerebral arteries. Although porcine pial veins receive vasodilator and constrictor nerves, a lack of constriction on TNS suggests that the dilator nerves that release nitric oxide may play a predominant role in regulating porcine pial venous tone.

It is well established that cerebral arteries receive dense vasoconstrictor and vasodilator nerves and that multiple transmitters may mediate these dual vasomotor responses (Lee, 1994). In general, neuropeptide Y (NPy), serotonin (5-HT), acetylcholine (ACh), and Substance P (SP) released from adventitial perivascular nerves are vasoconstrictor messengers in large arteries at the base of the brain (Lee, 1994). Norepinephrine (NE) is either a vasoconstrictor or a vasodilator transmitter (Lee et al., 1982; Winquist et al., 1982), and calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and nitric oxide (NO) mediate vasodilation (Lee, 1994). Similarly, pial veins from several species have been shown to receive various types of innervation (Itakura, 1983; Markina et al., 1990; Asada and Lee, 1992; Cuevas et al., 1994; Edvinsson et al., 1994; Branst, 1995). The vasomotor effects (vasodilation or constriction) of the exogenously applied vasoactive messengers, except NE and 5-HT, which are found in the perivascular nerves in the pial veins, qualitatively resemble that found in the pial arteries (Hardebo et al., 1987; Asada and Lee, 1992; Lee et al., 1994). Direct demonstration of neuromediated vasomotor responses mediated by these vasoactive messengers on stimulation of the perivascular nerves in pial veins, however, has not been presented.

Evidence has been presented to indicate that pial veins play an important role in regulating cerebral blood volume and i.c. pressure (Schmidek et al., 1985). Sympathetic nerves may exhibit a greater influence on pial venous diameter than that of pial arteries in some species, implying that the autonomic nervous system is functional in regulating pial venous tone and brain circulation (Auer and Johansson, 1980). The pial pial venous walls have been shown to contain two layers of smooth muscle endowed with various types of re-

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); ACh, acetylcholine; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; i, immunoreactive; l-NAME, Nω-nitro-L-arginine methyl ester; NE, norepinephrine; NDA, Nω-nitro-o-arginine; NLA, Nω-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; NPY, neuropeptide Y; PLP, periodate-aldehyde; SNP, sodium nitroprusside; SP, neuropeptide Y; VIP, vasoactive intestinal polypeptide.
ceptors (Lee et al., 1994; Ueno et al., 1995; Ishine and Lee, 1996). Our preliminary results also indicated that porcine pial veins were innervated by various autonomic nerves like those found in the cerebral arteries. The isolated porcine pial veins are therefore excellent preparations for investigating the transmitter mechanisms in regulating pial venous tone.

Materials and Methods

Fresh heads of adult pigs of either sex were collected from a local slaughterhouse. The entire brain was removed, within 2 h after sacrifice of the animals, and placed in a modified Krebs’ solution equilibrated with 95% O₂/5% CO₂ at room temperature. The pial veins were then excised under a dissection microscope. The composition of the Krebs’ solution was 122.0 mM NaCl, 5.2 mM KCl, 1.33 mM CaCl₂, 1.2 mM MgSO₄, 25.0 mM NaHCO₃, 0.03 mM EDTA, 0.01 mM L-ascorbic acid, and 11.0 mM glucose, pH 7.4.

Immunohistochemistry. The isolated pial veins (250–400 μm O.D.) were fixed by immersion fixation in 4% paraformaldehyde for demonstrating 5-HT immunoreactivities (Saito and Lee, 1987), periodate-lysine-paraformaldehyde (PLP) fixative for choline acetyltransferase (ChAT) immunoreactivities, and Zamboni’s fixative for NPY, VIP, CGRP, and SP immunoreactivities (Miao and Lee, 1991; Morris, 1991). After fixation, 4 h in paraformaldehyde, or overnight in PLP or Zamboni’s fixative, the specimens were washed with PBS and incubated with 0.5% H₂O₂ solution for 30 min at room temperature. The tissues were incubated in 1% normal goat serum containing 0.3% Triton X-100 for 30 min and then incubated with the primary antibody against ChAT, 5-HT, NPY, VIP, CGRP, and SP (1:1000 dilution) for 24 h. After a brief wash with PBS, the specimens were incubated with biotinylated goat anti-rabbit IgG (1:200 dilution) with 1% normal goat serum for 1 h and subsequently incubated with avidin D and biotinylated horseradish peroxidase complex in 1:400 dilution with 1% normal goat serum. The tissues were then washed with 0.05 M Tris-HCl buffer (pH 7.5) and placed in 0.02% 3,3-diaminobenzidine (dissolved in Tris-HCl with 0.03% hydrogen peroxide). The peroxidase reaction as evidenced by color changes was monitored closely under a light microscope. After the brownish fibers were visualized, the tissues were removed from 3,3-diaminobenzidine solution, washed with PBS, mounted on glass slides, air dried, dehydrated with a graded alcohol series (50%, 70%, 80%, 90%, and 100%), cleared in xylene, and mounted with Permount (Fisher Scientific). To approximate the density of nerve fibers, a point count method was used (Asada and Lee, 1992). The densities (count/cm) were expressed as the number of nerve fibers crossing an imaginary 1-cm line drawn longitudinally along the middle of each vessel on the photomicrographs with the same final magnification of 240×.

NADPH Diaphorase Histochemical Staining. NADPH diaphorase (NADPHd) activity in perivascular nerves of porcine pial veins was examined histochemically (Chen and Lee, 1995). Briefly, after fixation in PLP overnight, the tissues were incubated in 0.1 M PBS (pH 8.0) containing 0.3% Triton X-100, 0.5 mg/ml NADPH, and 0.1 mg/ml nitroblue tetrazolium at 37°C for 40 min. The tissues were then rinsed with PBS and examined under a Zeiss light microscope after mounting procedures as described for immunohistochemistry. As a negative control, NADPH is excluded from the incubation medium.

Measurement of Vascular Tone by an In Vitro Tissue Bath Technique. The in vitro tissue bath techniques were used to measure changes in the venous wall tension (Lee et al., 1994). The pial venous ring (4 mm long; 250–400 μm O.D.) was cannulated with a stainless steel rod and a platinum wire and was mounted horizontally in a plastic bath containing 5 ml of Krebs’ solution at 37°C and gassed with 95% O₂ and 5% CO₂. Changes in isometric tension were measured with Gould Statham UC-2 transducers and recorded on a Grass Polygraph (Lee et al., 1994). A resting tension of 75 mg was applied, and the tissues were equilibrated for an additional 60 min. An active muscle tone of approximately 0.6g in each ring segment was then elicited by U-46619 (a thromboxane A₂ analog, 0.3–1 μM). Relaxation induced by cumulative applications of each vasodilating agonist [VIP, SP, CGRP, sodium nitroprusside (SNP), 5-HT, or ACh] was obtained in the presence of guanethidine (1 μM). The relaxation was expressed as a percentage of the maximum relaxation induced by 300 μM papaverine, which was administered at the end of the experiment in the presence of the vasodilating agonist (Lee et al., 1994). On the other hand, NE- and NPY-induced constrictions in the presence of guanethidine (1 μM) were expressed as a percentage of the maximum constriction induced by U-46619 (0.3 μM), which was administered at the end of the experiment while NE or NPY was still present (Lee et al., 1982). One ring preparation was used to generate one concentration-response curve for each agonist.

Transmural Nerve Stimulation (TNS). Tissues were electrically, transmurally stimulated with a pair of platinum electrodes through which 100 biphasic square-wave pulses of 0.2 ms in duration and 180 mA in intensity were applied at various frequencies (Lee et al., 1982). The stimulating parameters have been used to stimulate all perivascular nerves (Lee et al., 1982; Liu and Lee, 1999; Zhang et al., 1998). The neurogenic origin of this TNS-induced response was verified by its complete blockade by tetrodotoxin (TTX) (0.9 μM). The magnitude of a vasodilator response was expressed as a percentage of the maximum response induced by 300 μM papaverine (Lee et al., 1982). The duration of a vasodilator response was determined by measuring distance between half of the relaxation response and half of the recovery response.

Incubation with 5-HT Before Fixation. Freshly dissected pial veins were incubated in Krebs’ solution (37°C) containing 5-HT (1 μM) for 30 min according to our previous reports (Saito and Lee, 1987).

Statistical Methods and Drugs. The data were computed as mean ± S.E.M. and evaluated statistically with Student’s t-test for paired or unpaired samples, as appropriate. N⁴⁵-Nitro-L-arginine (NLA), N⁴⁵-nitro-L-arginine methyl ester (L-NAME), d-arginine, L-arginine, carbamylcholine chloride, L-citrulline, L-glutamine, imipramine hydrochloride, sodium nitroprusside, NADPH, NE bitartrate, TTX, U-46619, 5-HT, and L-732,138 (acetyl-L-tryptophan-3,5-bistri氟omethyl benzyl ester) were from Sigma Chemical Co. (St. Louis, MO). d-Citrulline was from Research Plus Inc. (Bayonne, NJ). CGRP, SP, VIP, NPY, [Ac-Tyr¹, d-Phe²]GRF(1–29) amide, (8–37)hCGRP, rabbit NPY antiserum, and rabbit CGRP antiserum were from Peptides International (Louisville, KY). Atropine sulfate monohydrate was from Calbiochem (San Diego, CA). Guanethidine sulfate was from CIBA GEIGY Corp. (Summit, NJ). Methiothepin mesylate and N⁴⁵-nitro-L-arginine (NDA) were from Research Biochemicals International (Natick, MA). Rabbit VIP antiserum, rabbit SP antiserum, and rabbit 5-HT antiserum were from Immunonuclear Corp. (Stillwater, MN). Biotinylated affinity-purified goat anti-rabbit IgG, avidin D, biotinylated horseradish peroxidase H, and normal goat serum were from Vector Laboratories (Burlingame, CA). Rabbit polyclonal ChAT antiserum was a gift from Dr. Jang-Yen Wu (University of Kansas).

Results

Morphological Studies. Porcine pial veins were found to receive NPY-I, VIP-I, CGRP-I, SP-I, ChAT-I, and NADPHd fibers (Fig. 1 and Table 1). The NPY-I and VIP-I fibers were found to be denser than other types of innervation (Fig. 1, Table 1). In negative controls, no I or NADPHd fibers were observed.

5-HT-I fibers were never detected in normal pial veins. Dense 5-HT-I fibers, however, were observed in veins incu-
bated with 5-HT (1 μM) for 30 min before fixation (Fig. 1). The density of 5-HT fibers is similar to that of NPY fibers (Table 1). When incubation with 5-HT (1 μM) was carried out in the presence of guanethidine (1 μM) or imipramine (10 μM), no 5-HT-I fibers were detected (n = 3, data not shown).

Pharmacological Studies. In the presence of active muscle tone induced by U-46619 (0.3 μM), the pial veins relaxed concentration-dependently on the application of 5-HT (0.1 nM to 10 μM), CGRP (0.1 pM to 10 μM), SP (0.1 pM to 10 μM), VIP (1 pM to 30 nM), and SNP (0.1 nM to 10 μM) (Fig. 2). Based on the EC50 values, VIP and CGRP were found to be equally potent and were significantly more potent than the other three dilator substances (Table 2). The maximum relaxations (efficacy) induced by these vasodilators also varied, being VIP = 5-HT = SNP > CGRP = SP. Only the relaxation induced by SP was blocked by NLA (n = 6, data not shown).

TNS did not elicit any response in pial venous ring segments in the absence of active muscle tone. However, TNS elicited an exclusive relaxation in 60% of the control venous ring preparations preconstricted with U-46619 (0.3 μM). The relaxation was frequency dependent and TTX (0.9 μM) sensitive (Fig. 4). The remaining ring preparations did not respond with either constriction or relaxation on TNS. The

**TABLE 1**

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>n</th>
<th>Density (counts/cm)</th>
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<tbody>
<tr>
<td>VIP-I</td>
<td>4</td>
<td>57.6 ± 2.1</td>
</tr>
<tr>
<td>5-HT-I (5-HT incubation)</td>
<td>4</td>
<td>47.5 ± 1.9</td>
</tr>
<tr>
<td>NPY-I</td>
<td>4</td>
<td>44.0 ± 8.7</td>
</tr>
<tr>
<td>NADPHd</td>
<td>4</td>
<td>26.0 ± 5.8</td>
</tr>
<tr>
<td>CGRP-I</td>
<td>4</td>
<td>23.5 ± 7.1</td>
</tr>
<tr>
<td>SP-I</td>
<td>4</td>
<td>16.3 ± 6.5</td>
</tr>
<tr>
<td>ChAT-I</td>
<td>4</td>
<td>15.8 ± 5.9</td>
</tr>
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**TABLE 2**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>n</th>
<th>EC50</th>
<th>Maximum Response</th>
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<tr>
<td>NE</td>
<td>3</td>
<td>6.68 (3.39–13.18) × 10⁻⁸</td>
<td>73.3 ± 5.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NPY</td>
<td>7</td>
<td>2.33 (1.65–3.29) × 10⁻⁶</td>
<td>81.9 ± 8.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SNP</td>
<td>7</td>
<td>2.03 (0.16–25.95) × 10⁻⁸&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SP</td>
<td>6</td>
<td>0.83 (0.23–3.04) × 10⁻⁸&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.9 ± 9.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5-HT</td>
<td>7</td>
<td>4.54 (1.96–10.52) × 10⁻⁸&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98.2 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CGRP</td>
<td>6</td>
<td>0.96 (0.31–2.97) × 10⁻⁹</td>
<td>59.7 ± 7.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VIP</td>
<td>9</td>
<td>0.93 (0.48–1.77) × 10⁻⁹</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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<sup>a</sup> Percentage of 3 × 10⁻⁷ M U-46619-induced constriction.

<sup>b</sup> Percentage of papaverine-induced maximum relaxation.

<sup>*</sup> P < .01 vs norepinephrine and ** P < .05 vs VIP, indicate significant differences (unpaired Student’s t test).
magnitude and duration of relaxation elicited by TNS at 8 and 16 Hz were not affected by the CGRP receptor antagonist (8–37)hCGRP (0.3 mM; n = 4, Fig. 5), VIP receptor antagonist [Ac-Tyr1,D-Phe2]GRF(1–29) amide (0.3 mM; n = 4), SP receptor antagonist L-732138 (0.3 mM; n = 4), timolol (0.1 mM; n = 5), or methiothepin (0.3 mM; n = 4, data not shown) in preparations preincubated with or without 5-HT (1 mM). These receptor antagonists at the concentrations used have been shown to significantly block the specific receptors or relaxation induced by their respective receptor agonists (Lee, 1987; Lee et al., 1982, 1984, 1993, 1994; Cascieri et al., 1994). The TNS-elicited relaxation, however, was abolished by NLA (10 μM; n = 5) and l-NAME (10 μM; n = 4) but was not affected by NDA (10 μM; n = 5, Figs. 5 and 6). The l-NAME- and NiA-induced inhibitions were reversed by l-arginine (100 μM) and l-citrulline (100 μM) but not by d-arginine (100 μM; n = 4, Figs. 5 and 6) or d-citrulline (100 μM, data not shown). Furthermore, TNS-induced relaxation was inhibited by L-glutamine (100 μM), and the inhibition was reversed by L-citrulline (0.1–1 mM) (Fig. 7) but not by d-citrulline (0.1–1 mM, data not shown). Again, in the absence of active muscle tone, even after the addition of NiA or l-NAME, TNS at 8 or 16 Hz did not elicit any response.
Discussion

The results of the present study indicate that porcine pial veins are innervated by CGRP-I, VIP-I, NPY-I, SP-I, ChAT-I, and NADPHd fibers. Although it is not universally agreeable (Sanater and Symons, 1993; Kishimoto et al., 1994), NADPHd has been used as a marker for nitric oxide synthase (NOS) (Hope et al., 1991). Our results from morphological studies have indicated that NOS-I fibers and NADPHd fibers in the porcine cerebral arteries and veins are completely coincident fibers (Yu et al., 1997), suggesting that NOS-I and NADPHd fibers are identical fibers in the porcine cerebral blood vessels. In the present study, NADPHd fibers were therefore used to estimate the density of NOS-I fibers. Pore pig pial veins have already been shown to receive dense adrenergic, sympathetic innervation (Asada and Lee, 1992). Thus, porcine pial veins, like pial arteries (Lee, 1994), are equipped with adrenergic, cholinergic, peptidergic, and nitric oxidergic innervation.

The presence of 5-HT-I fibers in cerebral arteries has been shown to be due to uptake of 5-HT into perivascular sympathetic nerves (Saito and Lee, 1987). This appears to be true also in the porcine pial veins. There were no 5-HT-I fibers in normal pial veins. Only after incubation with 5-HT were 5-HT-I fibers observed. The density of 5-HT-I fibers was similar to that of NPY-I fibers and noradrenergic fibers (Asada and Lee, 1992). In the presence of guanethidine (or imipramine), which is known to block 5-HT uptake into sympathetic nerves (Fukuda et al., 1986), 5-HT-I fibers were not observed after incubation with 5-HT. These results suggest that 5-HT was taken up into sympathetic adrenergic nerves in pial veins, like that found in pial arteries (Saito and Lee, 1987). Thus, pial pial veins do not receive authentic serotonergic innervation.

The isolated pial veins from several species have been shown to constrict on application of NE, NPY, and 5-HT, whereas venous relaxation is induced by VIP, CGRP, and SP (Edvinsson et al., 1982; Hardebo et al., 1987). Similar results, except that of 5-HT, were observed in porcine pial veins from the present pharmacological studies. 5-HT induced an exclusive relaxation of porcine pial veins. This unique effect of 5-HT has been shown to be mediated by an as-yet-unidentified vascular 5-HT receptor subtype on the smooth muscle cells, which is coupled to an increased cAMP synthesis (Lee et al., 1994; Ueno et al., 1995; Ishine and Lee, 1996). A dilatatory effect of 5-HT in the cat pial veins smaller than 200 μm in diameter was also reported (Leber et al., 1983). Accordingly, if it is present in the sympathetic nerves via neuronal uptake and is released on nerve stimulation, 5-HT is potentially an alternative vasodilator transmitter in porcine pial veins. It is interesting to note that in the rabbit basilar arteries, 5-HT taken up into the sympathetic nerves is released on TNS to induce an exclusive constriction that is mediated by the postsynaptic 5-HT receptor (Saito and Lee, 1987). 5-HT, as an alternative transmitter, appears to have different effects on porcine cerebral arteries and veins.

It is interesting to note that exogenous VIP, 5-HT, and NO induced a full relaxation, whereas SP and CGRP, which are candidate transmitters in sensory nerves (Franco-Cereceda et al., 1987; Moskowitz, 1989), induced a partial relaxation. The reason for this difference is not known. These results, however, indicate that porcine pial veins like pial arteries receive different types of nerves that contain multiple messengers for vasodilation and constriction. Carbachol, a cholinoreceptor agonist, which has been shown to induce an endothelium-dependent relaxation in cerebral arteries (Lee, 1982), did not affect the pial venous tone. This is consistent with our previous reports that muscarinic receptors, if present, on the smooth muscle and/or endothelial cells do not play a significant role in regulating porcine pial venous tone (Lee et al., 1994). Failure of cholinergic agonists in inducing an endothelium-dependent relaxation in pial veins from other species has also been shown (Dora and Kovach, 1983; Hardebo et al., 1987). These findings suggest that endogenous ACh, released from the cholinergic nerves, plays a negligible role in direct regulation of pial venous tone. It should be noted that cholinergic innervation was not found in pial veins in some species (Itakura, 1983; Nakakita et al., 1983), suggesting that species variation in cholinergic innervation in pial veins may exist. Our preliminary results further indicate that ChAT-I and NOS-I are colocalized in the same perivascular neurons in the porcine pial veins, like those found in the porcine (Yu et al., 1998) and feline (Kimura et al., 1997) cerebral arteries. Thus, the perivascular “cholinergic” nerves in pore pial veins may release ACh and NO as cotransmitters. Because NO is a potent vasodilator (Lee, 1994; present results), the perivascular “cholinergic” nerves, which have been suggested to be cholinergic-nitric oxidergic nerves (Kimura et al., 1997), are logically considered vasodilator nerves in the pial pial veins. Thus, ACh may act as a presynaptic transmitter in modulating NO release in pial veins like that found in the pial arteries (Toda et al., 1997; Liu and Lee, 1999).

The failure of carbachol in inducing endothelium-dependent relaxation in pial veins does not seem to be due to a lack of NOS activity in the endothelial cells. Among the vasodilators examined, SP-induced relaxation was blocked by inhibitors of NO synthesis, suggesting that the relaxation induced by SP is dependent on NO that is most likely derived from the
endothelium (Lee et al., 1984). The effect of denuding endothelium on SP-induced pial venous relaxation was not demonstrated due to technical difficulty.

Whether SP released from the adventitial nerves can induce a relaxation via the indirect, endothelium-dependent mechanism in vivo is not known. If so, SP released in the adventitia will have to diffuse across the vessel wall and act in sufficient concentrations on the endothelial cells to release NO from these cells and subsequently dilate the smooth muscle cells. This indirect neurogenic vasodilation in isolated bovine cerebral arteries has been suggested (Gonzalez and Estrada, 1991). However, this indirect neurogenic vasodilation in large cerebral arteries from several species, which contain several muscle layers, was questioned due to the long distance between the adventitial nerves and the endothelial cells (Lee, 1982; Lee et al., 1982). In fact, denuding the endothelium has been shown to enhance the TNS-elicited neurogenic vasodilation in cerebral arteries from the cat and pig (Lee, 1982; Lee et al., 1982). Because the porcine pial venous walls contain no more than two muscle layers (Asada and Lee, 1992), the “short” distance between the adventitial nerves and the endothelial cells may make it more likely to allow this indirect neurogenic vasodilation to occur. This possibility, however, is not likely because the TNS-elicited relaxation was not affected by SP receptor antagonist.

In the presence of U-46619-induced active muscle tone, pial veins relaxed exclusively on TNS. The relaxation was frequent and TTX sensitive, suggesting that the TNS-elicited vasodilation was neurogenic. The TNS-elicited relaxation, however, was not affected by guanethidine, which is known to inhibit NE and NPY release from sympathetic nerves (Morris, 1991). This result is similar to that found in porcine cerebral arteries (Lee, 1994). In these arteries, NE induces exclusive relaxation, which is predominantly mediated by postsynaptic α1-adrenoceptors (Winquist et al., 1982; Wang and Lee, 1986). Propranolol, however, did not affect the TNS-elicited vasodilation (Lee et al., 1982), suggesting that endogenous NE is not mediating TNS-elicited vasodilator response. NE, on the other hand, is a potent constrictor in the porcine pial veins by acting on α2-adrenoceptors (Asada and Lee, 1992; present results), although β-adrenoceptors are also present in these vessels (Lee et al., 1994). In the absence of active muscle tone, pial vein pial veins, with or without pretreatment with NLA (10 μM), did not respond on TNS. These results suggest that NE and NPY released from sympathetic nerves on TNS are minimally involved in direct regulation of pial venous tone. The TNS-elicited relaxation in the porcine pial venous ring segments in the presence of active muscle tone appeared to be predominantly mediated by NO. This is based on the findings that TNS-elicited relaxation was abolished by L-NAME and NLA but not by NDA. The blockade was reversed by L-arginine but not by D-arginine. Furthermore, L-citrulline (the byproduct of NO synthesis) has been shown to be recycled to form L-arginine, which is the precursor for NO synthesis in perivascular nerves in porcine cerebral arteries and veins (Yu et al., 1997). Thus, in the present study, the finding that NLA inhibition of TNS-elicited relaxation was reversed by L-citrulline but not by D-citrulline suggests that L-citrulline was converted to L-arginine, which was then catalyzed in the presence of NOS to generate NO and L-citrulline in the perivascular nerves (Lee et al., 1996). Our results from morphological studies indeed have indicated that the necessary enzymes for converting L-citrulline to L-arginine, argininosuccinate synthetase and argininosuccinate lyase (Ratner, 1973), are found to localize with NOS immunoreactivities in the same perivascular nerves in porcine pial arteries and veins (Yu et al., 1997). The presence of arginine-citrulline cycle in the perivascular nerves suggests that NO can be synthesized in the perivascular nerves in porcine pial veins, providing strong evidence for the neuronal source of NO.

The TNS-elicited NO-mediated pial venous relaxation was further supported by the finding that the TNS-elicited relaxation was blocked by L-glutamine, a result similar to that found in cerebral arteries (Lee et al., 1996). Although the exact mechanism of action of L-glutamine in blocking NO-mediated neurogenic vasodilation remains unclear, it appears that glutamine interferes with synthesis and/or release of NO (Lee et al., 1996). Thus, the finding of L-citrulline reversal of inhibition induced by L-glutamine in pial veins provides further evidence that NO can be synthesized in the perivascular nerves in the pial veins and that NO is a major messenger for neurogenic vasodilation in the pial veins like that in cerebral arteries (Chen and Lee, 1995; Lee et al., 1996; Toda et al., 1997).

It has been reported in sheep cerebral arteries that relaxation induced by exogenously applied VIP was inhibited by L-Nω-monomethyl arginine (Gaw et al., 1991). Each VIP molecule contains two arginine amino acids (Moncada et al., 1991). It is possible that neuronal VIP, on release into the synapses, may initiate release of NO from arginine at its site of action. Similar results may be expected for CGRP because this peptide also contains two molecules of arginine (Moncada et al., 1991). In the present study, however, pial venous relaxation induced by VIP and CGRP was not affected by NLA at the concentrations that abolished the TNS-elicited relaxation in the same preparations. Similar results were found in porcine and feline cerebral arteries with or without endothelial cells (Lee et al., 1993). These arteries without endothelial cells also did not relax or constrict on application of L-arginine or NLA (Lee and Sarwinski, 1991; Ueno et al., 1995; Lee et al., 1996). These findings suggest that NO is not released from VIP or CGRP in the extraneuronal origin, such as in the synapses and muscle or in cerebral arteries or pial veins. These results are consistent with the hypothesis that neurogenic vasodilation in porcine pial veins, like that in cerebral arteries, is mediated by NO of neuronal origin (Chen and Lee, 1995; Gonzalez et al., 1997).

The finding that neurogenic vasodilation in pial veins on TNS is solely due to NO release is consistent to that found in cerebral arteries from porcine and several other species (Toda and Okamura, 1990; Lee, 1994; Lee et al., 1996). Indeed, the TNS-elicited relaxation (both magnitude and duration) was not affected by receptor antagonists for VIP, CGRP, 5-HT, SP, or β-adrenoceptors, whereas these receptor antagonists have been shown to block the relaxation induced by their respective agonists (Lee, 1987; Lee et al., 1982, 1984, 1993, 1994; Cascieri et al., 1994). It is possible that release of these vasodilator messengers into synaptic regions (Lee et al., 1982) and/or the corresponding receptor populations on the postsynaptic regions on smooth muscle cells are too low to elicit a response. The exact role of these putative transmitters in regulating pial venous tone remains to be clarified. Several recent findings, however, have suggested that endog-
enous NE (Zhang et al., 1998), ACh (Lee, 1985; Toda et al., 1997; Liu and Lee, 1999), and VIP (Gonzalez et al., 1997) act as presynaptic transmitters in modulating NO-mediated neurogenic vasodilation in cerebral arteries. This is consistent with the present findings that NO released from the perivascular nerves plays a predominant role in mediating neurogenic vasodilation in porcine pial veins.

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