Neurogenic Vasodilatation of Canine Isolated Small Labial Arteries

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ABSTRACT

Mechanisms underlying vasodilatation to nerve stimulation by electrical pulses and nicotine were analyzed in isolated canine small labial arteries. Transmural electrical stimulation (5 and 20 Hz) produced a contraction followed by a relaxation in labial arterial strips denuded of the endothelium, partially contracted with prostaglandin F$_{2a}$. The contraction was abolished by prazosin or combined treatment with $\alpha$, $\beta$-methylene ATP. In the treated strips, neurogenic relaxation was abolished by $N^{\omega}$-nitro-L-arginine ($\omega$-NA), a nitric oxide (NO) synthase inhibitor, and restored by L-arginine. The $\alpha$-enantiomers were without effect. Nicotine ($10^{-3}$ M) also relaxed the arteries, in which the contractile response was abolished by prazosin and $\alpha$, $\beta$-methylene ATP. The relaxant response was attenuated but not abolished by $\omega$-NA; the inhibition was reversed by L-arginine. The remaining relaxation by nicotine was abolished by calcitonin gene-related peptide (CGRP)-[8 to 37], a CGRP$_1$ receptor antagonist. Relaxations elicited by a lower concentration of nicotine ($2 \times 10^{-5}$ M) sufficient to produce similar magnitudes of response to those induced by 5-Hz electrical nerve stimulation were also inhibited partially by L-NA. Histochemical study with the NADPH-diaphorase method demonstrated positively stained nerve fibers and bundles in the arterial wall, suggesting the presence of neuronal NO synthase. It is concluded that the relaxation induced by electrical nerve stimulation of small labial arteries is mediated exclusively by NO synthesized from L-arginine in nerve terminals, whereas nicotine in the concentrations used evokes relaxations by a mediation of nerve-derived NO and also CGRP, possibly from sensory nerves. The reason why nicotine but not electrical pulses stimulates sensory nerves and elicits vasorelaxation remains unsolved.

The vascular tone has widely been recognized to be regulated mainly by tonic, efferent discharges of sympathetic vasoconstrictor nerves. However, recent studies have provided evidence for vasodilator innervation that contributes to counteract the neurogenic vasoconstriction; thus, the reciprocal innervation of blood vessels, like many other autonomically innervated organs and tissues, is expected to play important regulatory roles (Toda and Okamura, 1992). The neurogenic vasodilatation in many vascular beds of a variety of mammals is mediated mainly by nitric oxide (NO) (Toda and Okamura, 1992; Toda, 1995; Toda et al., 1996). Findings supporting the hypothesis that this gaseous molecule acts as a neurotransmitter are as follows: the vasodilator response to nerve stimulation is abolished by NO synthase inhibition and restored by L- but not $\omega$-arginine; the nerve stimulation liberates NO, measured as NO$_2$; and the vascular wall has networks of nerve fibers and bundles containing NO synthase immunoreactivity (reviewed by Toda, 1995; Toda and Okamura 1996). The vasodilator nerve is called “nitroxidergic” (Toda and Okamura, 1992). In addition, there is pharmacological and morphological evidence for autonomic and sensory innervation responsible for vasodilatation that is mediated possibly by polypeptides (Morris et al., 1995).

Small arteries and arterioles distributing to s.c. tissues have functional characteristics distinct from those of vascular beds in organs and tissues in the thoracic and abdominal cavities (Fernandez et al., 1994). Cutaneous vascular resistance is strongly influenced by sympathetic tone (Hardman and Limbird, 1996). However, functioning of vasodilator nerves had not been elucidated until our recent article on skin arteries of the abdomen was published (Uchiyama et al., 1997). Nerve terminals innervating this artery were stimulated by nicotine, resulting in vasoconstriction followed by vasodilatation, but electrical stimulation was without effects under the experimental conditions used. Responsiveness to nerve stimulation seems to differ in the vasculature of various skin regions.

Therefore, we sought to determine the actions and mechanisms of action of nerve stimulation by electrical pulses and nicotine in isolated small arteries of the lips, a branch of the maxillary artery, with reference to NO and polypeptides, and to demonstrate neurons containing NO synthase in the arterial wall. Vasodilator nerve functions were compared in the skin of the face (present study) and the abdomen (Uchiyama et al., 1997).

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ABBREVIATIONS: NO, nitric oxide; CGRP, calcitonin gene-related peptide; PG, prostaglandin; $\omega$-NA, $N^{\omega}$-nitro-L-arginine.
Materials and Methods

The Animal Care and Use Committee at Shiga University of Medical Science approved the experimental protocol and the use of dog arteries in this study.

Studies on Mechanical Response. Twenty-five mongrel dogs of either sex, weighing 6 to 14 kg, were anesthetized with i.v. injections of sodium thiopental (30 mg/kg) and sacrificed by bleeding from the common carotid arteries. The labial artery (0.2–0.4 mm internal diameter) was obtained by tracing branches of the maxillary artery close to the skin of upper lip and was helically cut into strips of approximately 20 mm long. The endothelium was removed by gently rubbing of the intimal surface with a cotton ball. Endothelial denudation of the strips was confirmed by the abolishment of relaxations caused by 10^{-6} M acetylcholine. The specimens were fixed vertically between hooks in a muscle bath (20-mL capacity) containing the modified Ringer-Locke solution that was maintained at 37 ± 0.3°C and aerated with a mixture of 95% O2 and 5% CO2. The composition of the solution was as follows: 120 mM NaCl, 5.4 mM KCl, 2.2 mM CaCl_2, 1.0 mM MgCl_2, 25.0 mM NaHCO_3, and 5.6 mM dextrose. The pH of the solution was 7.38 to 7.44. The hook anchoring the upper end of the strips was connected to the lever of a force-displacement transducer (Nihon-Kohden Kogyo Co., Tokyo, Japan). The resting tension was adjusted to 0.7 g, which was optimal for inducing the maximal contraction. Before the start of experiments, the strips were allowed to equilibrate for 60 to 90 min in the bathing medium, during which time the fluid was replaced every 10 to 15 min.

Isometric mechanical responses were displayed on an ink-writing oscillograph. The contractile response to 30 mM KCl was first obtained, and the arterial strips were repeatedly washed with fresh fluids and equilibrated. The strips were partially contracted with prostaglandin F_2α (PGF_2α) (5–20 × 10^{-7} M), the contraction being in a range between 35 and 50% of KCl (30 mM)-induced contraction. Nicotine, calcitonin gene-related peptide (CGRP), and NO (acidified NaNO_2) were applied directly to the bathing media. Some of the strips were placed between stimulating electrodes (Toda, 1982). Electrical pulses (0.2-ms duration, supramaximal intensity, and frequencies of 5 and 20 Hz for 40 and 10 s, respectively) were transmurally applied to stimulate nerve terminals in the artery strips. After the responses to vasodilator agonists or electrical stimulation were found to be stabilized, the preparations were treated for 20 to 30 min with blocking agents. At the end of each series of experiment, papaverine (10^{-4} M) was applied to attain the maximal relaxation. Relaxations induced by agonists or electrical stimulation were expressed as relative values to papaverine (10^{-4} M)-induced relaxations.

Histochemical Study. Tissue blocks containing the labial arteries were fixed for 3 h in ice-cold PBS (0.1 M, pH 7.4) containing 0.3% glutaraldehyde and 4% paraformaldehyde, and then postfixed overnight in PBS with 4% papaoformaldehyde, followed by cryoprotection in 15% sucore. Thin sections (20-μm thick) were cut on a cryostat (−18°C) and mounted onto gelatin-chrome-alum-coated glass slides. The slide-mounted tissue sections were stained with the NADPH-diaphorase staining method (Vincent and Kimura, 1992). Briefly, the sections were incubated with 0.1 M PBS at pH 8.0, containing 1 mM β-NADPH (reduced form; Kohjin, Tokyo, Japan), 2 mM nitro blue tetrazolium (Sigma Chemical Co., St. Louis, MO), and 0.3% (v/v) Triton X-100 at 37°C. The period of incubation (10–20 min) was based on staining intensity. The reaction was terminated by washing the sections in 0.1 M PBS. The section was dried and coverslipped with Entellan (Merck, Darmstadt, Germany). A histochemical control experiment in which NADPH was excluded from the reaction mixture gave no positive staining.

Results

Responses to Transmural Electrical Stimulation of Labial Artery Strips. In the artery strips denuded of the endothelium and partially contracted with PGF_2α, transmural electrical stimulation at 5 Hz for 40 s produced a transient contraction followed by a relaxation (Fig. 1), with the responses being abolished by treatment with 3 × 10^{-7} M tetrodotoxin. In three of five strips, the contraction (167 ± 20 mg) was inhibited by 65.7 ± 5.2% by prazosin (10^{-6} M) and was abolished in the remaining two strips. The stimulation-induced contraction in these proazosin-treated strips was abolished by α,β-methylene ATP (10^{-6} M). In the strips in which the contractile response was abolished, relaxations by nerve stimulation were potentiated from 6.8 ± 2.5 to 15.6 ± 2.3% (n = 5). Therefore, mechanisms underlying the relaxant response were analyzed in the strips treated with prazosin (10^{-6} M) alone or in combination with α,β- methylene ATP (10^{-6} M).

Relaxations induced by electrical nerve stimulation (5 Hz for 40 s) were abolished by treatment with L-NA (10^{-6} M) and restored by L-arginine (3 × 10^{-4} M) (Fig. 1). Quantitative data are shown in Fig.

Fig. 1. Responses to transmural electrical stimulation (5 Hz) of a labial artery strip denuded of the endothelium and contracted with PGF_2α before and after treatment with prazosin (10^{-6} M), L-NA (10^{-6} M), L-arginine (3 × 10^{-4} M), and tetrodotoxin (TTX, 3 × 10^{-7} M). PA represents 10^{-4} M papaverine that produced maximal relaxation. Dots denote application of electrical stimulation.
2. Tetrodotoxin abolished the restored response. On the other hand, N\textsuperscript{\textdagger}O-nitro-L-arginine (10\textsuperscript{-6} M) did not alter the response (15.6 ± 4.8 versus 15.8 ± 5.3%, n = 4), and L-arginine (3 × 10\textsuperscript{-4} M) failed to restore the abolished response by L-NA (n = 4).

The frequency of stimulation was raised to 20 Hz (for a period of 10 s) to determine whether the other nerves innervating the arterial wall were stimulated additionally. Greater relaxations induced at 20 Hz than at 5 Hz (21.5 ± 3.8 versus 13.7 ± 3.1%, n = 4) were also abolished by treatment with L-NA (10\textsuperscript{-6} M), and the inhibitory effect was reversed by L-arginine (3 × 10\textsuperscript{-4} M). Mean values of the relaxation induced by 20 Hz stimulation before and after L-NA and L-arginine plus L-NA in four strips from individual dogs were 21.5 ± 3.9%, 0% (P < .01 versus control and L-NA + L-arginine, Tukey’s test) and 23.0 ± 3.5%, respectively. CGRP-[8 to 37] (10\textsuperscript{-7} M) did not influence the response to nerve stimulation in the strips soaked in control media and those containing L-NA (n = 3). Representative responses are illustrated in Fig. 3.

**Responses to Nicotine of Labial Artery Strips.** In the endothelium-denuded artery strips precontracted with PGF\textsubscript{2\alpha}, the addition of nicotine (10\textsuperscript{-4} M) produced a contraction followed by a relaxation in 4 of 20 strips from individual dogs, only a contraction in 1 of 20 strips and only a relaxation in the remaining fifteen. The contraction was reversed to a relaxation in three of the five strips by treatment with propranolol (10\textsuperscript{-6} M), and was partially inhibited by prazosin and reversed to a relaxation by additional treatment with α,β-methylene ATP (10\textsuperscript{-6} M) in the remaining two. Mechanisms of the nicotine-induced relaxation were thus analyzed in the strips treated with propranolol or combined treatment with α,β-methylene ATP. The relaxation was inhibited partially in 15 of 20 strips from individual dogs by L-NA (10\textsuperscript{-6} M) and was abolished in the remaining five. Typical recordings of the nicotine-induced relaxation of two strips responding differentially to L-NA are illustrated in Fig. 4. In 15 artery strips obtained from different dogs, including 5 strips in which the response was abolished by the NO synthase inhibitor and 10 strips in which the response was partially inhibited, nicotine-induced relaxations were reversed by L-arginine (3 × 10\textsuperscript{-5} M) (Figs. 4 and 5). The inhibition by L-NA of the response averaged 73.7 ± 5.9% (n = 15, P < .001, paired t test). Relaxations in response to NO (10\textsuperscript{-6} M) were not influenced by L-NA (n = 7, 68.8 ± 4.5 versus 69.1 ± 3.6%). Raising the concentration of L-NA to 10\textsuperscript{-4} M did not produce additional attenuation; mean values of the response in control and L-NA (10\textsuperscript{-5} and 10\textsuperscript{-4} M)-treated strips (n = 4) were 54.6 ± 4.1, 23.6 ± 3.3, and 24.2 ± 4.3%, respectively. In the strips treated with 10\textsuperscript{-5} M L-NA, neurogenic responses were inhibited by CGRP-[8 to 37] (10\textsuperscript{-7} M) from 21.5 ± 4.8 to 8.3 ± 3.4% (n = 7, P < .05, unpaired t test; 71.3 ± 8.3%) and were abolished by 3 × 10\textsuperscript{-7} M CGRP-[8 to 37] (n = 5). Typical tracings of the response to nicotine depressed by the CGRP receptor antagonist in a L-NA-treated strip are illustrated in Fig. 4, lower. The artery strips denuded of the endothelium responded to CGRP with dose-related relaxations; mean values at 3 × 10\textsuperscript{-10}, 10\textsuperscript{-9}, and 3 × 10\textsuperscript{-8} M were 14.2 ± 3.0, 59.0 ± 6.4, and 83.5 ± 6.4% (n = 4), respectively. The relaxation by 3 × 10\textsuperscript{-10} M CGRP was abolished (n = 4) and the response at 10\textsuperscript{-9} M was moderately inhibited by 53.5 ± 8.2% (n = 4, P < .01, paired t test) in the strips treated with 10\textsuperscript{-7} M CGRP-[8 to 37]. CGRP (10\textsuperscript{-9} M)-induced relaxations were markedly suppressed by 3 × 10\textsuperscript{-7} M CGRP-[8 to 37] (87.5 ± 3.0%, n = 7).

The nicotine-induced relaxation was not affected by treatment with indomethacin (10\textsuperscript{-6} M), timolol (10\textsuperscript{-7} M), atropine (10\textsuperscript{-7} M), and tetrodotoxin (3 × 10\textsuperscript{-7} M), but was abolished by hexamethonium (10\textsuperscript{-6} M). The data are summarized in Table 1.

To determine the reason why the mechanisms of action of electrical stimulation and nicotine differed, the susceptibility to L-NA was evaluated in the response to a lower concentration (2 × 10\textsuperscript{-5} M) of nicotine, which produced the similar magnitude of relaxation to that associated with electrical stimulation. Mean values of the response before and after treatment with L-NA (10\textsuperscript{-5} M) were 20.4 ± 3.9 and 4.9 ± 2.2% (n = 4, 74.5 ± 9.6% inhibition, P < .001 paired t test).

**Histochemical Study.** There were nerve fibers and bundles containing NADPH-diaphorase in the adventitia of the canine small labial artery (Fig. 6). Similar data were also obtained in two additional arteries obtained from individual dogs.

**Discussion**

Transmural electrical stimulation at 5 Hz induced a contraction of canine isolated small labial arteries denuded of the endothelium and partially contracted with PGF\textsubscript{2\alpha}. The contraction was reversed to a relaxation by a lower concentration (2 × 10\textsuperscript{-5} M) of nicotine, which produced the similar magnitude of relaxation to that associated with electrical stimulation. Mean values of the response before and after treatment with L-NA (10\textsuperscript{-5} M) were 20.4 ± 3.9 and 4.9 ± 2.2% (n = 4, 74.5 ± 9.6% inhibition, P < .001 paired t test).

**Histochemical Study.** There were nerve fibers and bundles containing NADPH-diaphorase in the adventitia of the canine small labial artery (Fig. 6). Similar data were also obtained in two additional arteries obtained from individual dogs.

**Fig. 2.** Modifications by L-NA (10\textsuperscript{-6} M), L-NA plus L-arginine (L-Arg., 3 × 10\textsuperscript{-4} M), and tetrodotoxin (TTX, 3 × 10\textsuperscript{-7} M) of relaxation induced by transmural electrical stimulation at 5 Hz in labial artery strips denuded of endothelium and contracted with PGF\textsubscript{2\alpha}. Strips were treated with prazosin alone or in combination with α,β-methylene ATP. Ordinate represents relaxations induced by electrical stimulation relative to those by 10\textsuperscript{-4} M papaverine. Significantly different from control, \(*P < .01; \text{ significantly different from value with L-NA + L-arginine, } bP < .01\) (Tukey’s test). Numbers in parentheses indicate number of strips from individual dogs. Vertical bars represent S.E.M.
lip (Kerezoudis et al., 1993), rabbit ear (Khan et al., 1993), and the human forearm and finger skin (Coffman, 1994), although whether NO is derived from the vasodilator nerve, endothelium, or both has not been determined. In contrast to the labial artery, canine abdominal skin artery strips did not respond to transmural electrical stimulation in the same way.

The reason for the different responsiveness to the physical stimulus could not be elucidated.

Relaxations induced by nicotine (10^{-4} M) of labial artery strips were greater than those by electrical nerve stimulation at 5 and 20 Hz. The nicotine-induced relaxation was abolished by hexamethonium but not influenced by tetrodotoxin.
suggesting that the release of neurotransmitters is derived from stimulation of nicotinic receptors in nerve terminals, which do not generate nerve action potentials. The relaxant response was reduced but not abolished by L-NA even in high concentrations, and the inhibitory effect of L-NA was reversed by L-arginine. NO would be involved in the response; from stimulation of nicotinic receptors in nerve terminals, acetylcholine coexist (Yoshida and Toda, 1997). It is particularly interesting for us to note that NO is the neurotransmitter in humans in response to heat stress.

Our present study revealed that canine isolated small labial arteries responded to electrical nerve stimulation and nicotine with relaxations. It appears that NO synthesized from L-arginine in nitrorenergic, efferent nerves is involved in the response to the electrical stimulation, whereas CGRP from sensory nerves, together with NO, mediates the response to the chemical stimulation. It is intriguing to determine how the neurogenic vasodilatation participates in the axon reflex and the inflammatory and immune reactions in the skin of various regions.

References
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