L-Citrulline conversion to L-arginine in sphenopalatine ganglia and cerebral perivascular nerves in the pig


1Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, Illinois 62794; and 2Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030

Yu, J. G., T. Ishine, T. Kimura, W. E. O'Brien, and T. J. F. Lee. L-Citrulline conversion to L-arginine in sphenopalatine ganglia and cerebral perivascular nerves in the pig. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2192–H2199, 1997.—The presence of nitric oxide synthase (NOS), argininosuccinate synthetase (ASS), and argininosuccinate lyase (ASL) and their coexistence with NADPH-diaphorase (NADPHd), a marker for NOS, in the porcine sphenopalatine ganglia (SPG), pial veins, and the anterior cerebral arteries was examined using immunohistochemical and histochemical staining techniques. NOS-immunoreactive (I), ASS-I, and ASL-I fibers were found in pial veins and the anterior cerebral arteries. NOS, ASS, and ASL immunoreactivities were also found in neuronal cell bodies in the SPG. Almost all neuronal cell bodies in the SPG and nerve fibers in pial veins and the anterior cerebral arteries that were reactive to ASS, ASL, and NOS were also stained positively with NADPHd, suggesting that ASS, ASL, and NOS were colocalized in the same neurons in the SPG and perivascular nerves. With the use of in vitro tissue bath techniques, L-citrulline but not L-citrulline reversed inhibition of neurogenic vasodilation in isolated porcine pial veins produced by NOS inhibitors such as N^3-nitro-L-arginine methyl ester. In the presence of L-aspartate, L-arginine was synthesized from L-citrulline in homogenates of SPG and endothelium-denuded cerebral arteries and pial veins. These results provide evidence indicating that perivascular nerves in pial veins like cerebral arteries can convert L-citrulline to L-arginine for synthesizing nitric oxide. The conversion is most likely via an argininosuccinate pathway.

argininosuccinate lyase; argininosuccinate synthetase; reduced nicotinamide adenine dinucleotide phosphate-diaphorase; nitric oxide synthase; colocalization; cerebral arteries and veins; porcine

IT IS WELL ESTABLISHED that cerebral blood vessels from several species receive dense nitric oxidergic innervation and that neurogenic vasodilation of isolated large cerebral arteries at the base of the brain is mediated predominantly by nitric oxide (NO) (9, 12, 17). NO is synthesized from L-arginine with L-citrulline as a byproduct catalyzed by NO synthase (NOS) in various types of cells (2, 4, 31). Based on biochemical and pharmacological studies, we have proposed that L-citrulline is actively converted to L-arginine, which is then converted to L-citrulline and NO in perivascular nerves in cerebral arteries (6, 20). This active citrulline-arginine cycle in cerebral perivascular nerves provides not only a means to maintain an adequate supply of NO during neurovascular transmission, but also the first biochemical evidence indicating that NO is synthesized in perivascular nitric oxidergic nerves (5). Therefore, NO can be released from cerebral perivascular nerves and has been considered a transmitter substance.

The known pathway of L-citrulline conversion to L-arginine in the urea cycle is via the production of argininosuccinate (AS) (13, 32). In this pathway, AS synthetase (ASS) catalyzes the formation of AS from L-citrulline and aspartate. AS is then cleaved to produce L-arginine and fumarate by argininosuccinate lyase (ASL). Our preliminary studies using sequential double-labeling techniques, i.e., immunoperoxidase or immunofluorescence combined with NADPH-diaphorase (NADPHd) histochemical staining, have demonstrated that dense NOS-immunoreactive (I), ASS-I, and ASL-I fibers in porcine middle cerebral arteries, basilar arteries, and the circle of Willis are completely coincident fibers (40). These results indicate the presence of enzymes catalyzing the conversion of L-citrulline to L-arginine and that these enzymes are colocalized with NOS and NADPHd in the same perivascular neurons in cerebral arteries. Thus conversion of L-citrulline to L-arginine is likely mediated by the AS pathway.

Pial veins have also been shown to receive NADPHd fibers (7). Our preliminary results from in vitro pharmacological studies indicated that transmural nerve stimulation (TNS) elicited an exclusive relaxation in isolated porcine pial veins. The relaxation was blocked by inhibition of NO synthesis, and the inhibition was reversed by L-citrulline, suggesting that L-citrulline is also converted to L-arginine in perivascular nerves in porcine pial veins. It is possible that NOS, ASS, and ASL are colocalized in perivascular nerves in pial veins.

The sphenopalatine ganglia (SPG) have been shown to be one major source of NOS-I fibers innervating cerebral arteries of the rat and cat (15, 26, 28). Although this has not been shown in the pig, if neurons in the porcine SPG are also found to contain NOS, ASS, ASL, and NADPHd, it will provide evidence supporting the colocalization of NOS, ASS, ASL, and NADPHd in perivascular nerves in pial veins and arteries. The present study was designed to determine by immunohistochemistry, histochemistry, in vitro tissue bath techniques, and thin-layer chromatography (TLC) whether conversion of L-citrulline to L-arginine in porcine SPG and perivascular nerves in pial veins like those in pial arteries is via the AS pathway.

EXPERIMENTAL PROCEDURES

Fresh heads of adult pigs (n = 12) of either sex were obtained at a local slaughterhouse. The entire brain was removed, and cerebral arteries (anterior cerebral, middle cerebral and basilar arteries, and the circle of Willis) and pial veins were dissected under a dissecting microscope in oxygenated (95% O_2-5% CO_2) Krebs bicarbonate solution (pH 7.4)
containing (in mM) 196.87 NaCl, 5.16 KCl, 1.33 CaCl2, 25.56 NaHCO3, 1.22 MgSO4·7H2O, 1.01 dextrose, 0.34 EDTA, and 0.28 ascorbic acid (18) at room temperature. The SPG were also removed under a dissecting microscope.

Immunohistochemistry

The SPG, anterior cerebral arteries, and pial veins were fixed in either periodate-lysine-paraformaldehyde fixative (24) or periodate-parafomaldehyde-picroc acid-formaldehyde-lysine fixative (25) for 4 h. The anterior cerebral arteries and pial veins were stored in phosphate-buffered saline (PBS) and the SPG in PBS containing 30% sucrose at 4°C for 24 h. The SPG were sectioned at 8-µm thickness with a cryostat microscope and placed on Vectabond (Vector Labs)-coated slides and air-dried for 2 h. Immunohistochemistry for whole-mount specimens (arteries and veins) and sections of SPG was carried out using indirect immunofluorescence method (38, 39). Specific antiser to purified ASS and ASL were raised in the rabbits. The procedures of enzyme purification, characterization, and specificity of antisera were described elsewhere (1, 27, 29, 30). The fixed arteries, veins, and SPG sections were washed in 0.01 M PBS and blocked with 1% normal goat serum (1:200 diluted in PBS, Vector Labs) for 4 h at 4°C. After being washed in PBS, the specimens were incubated in rabbit antiserum against ASS in a dilution of 1:2,000 (27, 30), ASL at 1:2,000 (27, 30), or brain NOS at 1:1,000 (Affinity BioReagents) for 24-48 h at 4°C. After another wash in PBS, specimens were incubated in affinity-purified biotinylated goat anti-rabbit immunoglobulin G antibody (1:200 diluted in PBS, Vector Labs) for 4 h at 4°C, washed with PBS, and incubated for 4 h at 4°C with fluorescein avidin D (Vector Labs). The specimens were then rinsed and coverslipped with Vectashield mounting medium (Vector Labs) for photography under a fluorescence microscope fitted with proper filters. After the immunofluorescence fibers and ganglionic cells were photographed, the specimens were washed and processed for NADPHd histochemistry as described below.

NADPHd Histochemical Staining

After fixation, incubation with specific antibodies in demonstrating immunofluorescence labeling, and photographing as described above, the PBS-washed specimens were incubated in 0.1 M phosphate buffer (pH 8.0) containing 0.5 mg/ml NADPH (reduced form), 0.1 mg/ml nitro blue tetrazolium, and 0.3% Triton X-100 at 37°C for 1 h (5). The specimens were rinsed with PBS and mounted with Gel Mount (Biomeda, Foster City, CA) and examined under a Zeiss light microscope.

Controls

The specificity of the immunolabeling was determined by omitting ASS, ASL, or NOS antiserum as well as by using nonimmunized normal rabbit serum or nonimmunized normal goat serum. No labeling was found in these control specimens. For control of NADPHd activity, NADPH was omitted from the incubation medium. This resulted in elimination of NADPHd reaction product in perivascular nerves and ganglionic cells (6).

Measurement of Vascular Tone

The dissected pial venous rings (4 mm long; 250-400 µm OD) were cleaned of surrounding tissue under a dissecting microscope. The pial venous ring was cannulated with a stainless steel rod and a platinum wire and was mounted horizontally in a plastic bath containing 5 ml Krebs solution at 37°C and gassed with 95% O2-5% CO2 (21). Changes in isometric tension were measured by Gould Statham UC-2 transducers and recorded on a Grass polygraph. A resting tension of 75 mg was applied, and the tissues were equilibrated for an additional 60 min. An active muscle tone of 0.6 mg in each ring segment was then elicited by U-46619 (a thromboxane A2 analog, 0.3–1 µM), TNS at various frequencies (19) with a pair of platinum electrodes through which 100 biphasic square-wave pulses of 0.2 ms in duration and 180 mA in intensity was applied. The neurogenic origin of the TNS-induced response was verified by its complete blockade by tetrodotoxin (TTX; 0.9 µM). Guanethidine (1 µM) was present in the bath throughout the entire experiment to eliminate potential contribution from adrenergic component (18). The magnitude of a vasodilator response was expressed as a percentage of the maximum response induced by 300 µM papaverine (18).

TLC Analysis of Conversion of L-[14C]citrulline to L-Arginine

Radiolabeled L-[ureido-14C]citrulline (55.9 mCi/mM) was used to assay the conversion of L-citrulline to L-arginine (6, 35). Porcine cerebral arteries (endothelium intact or denuded), pial veins (endothelium denuded), and SPG (3 pairs) were homogenized in 500 µl of ice-cold 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4) containing (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1.0 MgCl2 and 5 glucose. The homogenate (0.5 mg/ml protein) was incubated for 3 h at 37°C in a solution containing 10 µM L-[ureido-14C]citrulline (55.9 mCi/mM), 1 mM ATP and 1 mM L-aspartate. Incubations were terminated by adding ice-cold methanol. Sample solution (25 µl) was spotted onto TLC plates (Whatman silica gel 60 A). The plates were developed in the solvent system chloroform-methanol-ammonium hydroxide-water, 0.5: 4.5:2.0:1.0 (vol/vol), over a distance of 16 cm. After drying, the plates were scanned with Bio-Rad model GS-250 molecular imager system. The Rf values for L-[14C]arginine (0.6) and L-[14C]citrulline (0.3) were identical to those of authentic standards.

Drugs and Solutions

L-Arginine, L-citrulline, N6-nitro-L-arginine(L-NNa), N6-nitro-L-arginine methyl ester (L-NAME), HEPES, tetrodotoxin, NADPH, nitroblue tetrazolium (all from Sigma Chemical, St. Louis, MO), d-citrulline (from Research Plus), Triton X-100 (from Amersham), L-[ureido-14C]citrulline (55.9 mCi/mM; from New England Nuclear, Charlestown, NC), U-46619 (from Upjohn, Kalamazoo, MI), and guanethidine (from CIBA, Summit, NJ) were used.

RESULTS

Colocalization of NOS, ASS, and ASL Immunoreactivities With NADPHd in Porcine SPG

Numerous neuronal cell bodies in the SPG (n = 10 sections from each of 8 ganglia) expressed NOS, ASS, and ASL immunoreactivities (Fig. 1, A–C). All NOS-I, ASS-I, and ASL-I neurons in the SPG were also stained positively for NADPHd (Fig. 1, a–c).

NOS-I, ASS-I, ASL-I, and NADPHd Fibers Are Coincident Fibers in Pial Veins and Anterior Cerebral Arteries

Anterior cerebral arteries. The whole-mount anterior cerebral arteries (n = 8 from 4 animals) were densely...
innervated by NOS-I, ASS-I, ASL-I, and NADPHd fibers (Fig. 2). They were composed of bundles of various sizes and fine fibers, which ran longitudinally and spirally along the walls of the arteries. The density and distribution pattern of NOS-I, ASS-I, and ASL-I fibers were very similar (Fig. 2, A–C). NOS-I, ASS-I, or ASL-I fibers were not observed after omitting primary antibody or incubating tissues with normal serum from nonimmunized rabbits or goats (data not shown). Results from sequential "double labeling," i.e., immunofluorescence labeling combined with NADPH diaphorase histochemical staining, showed that all NOS-I, ASS-I, and ASL-I fibers were coincident with NADPHd fibers (Fig. 2, a–c). These results are consistent with those found in the basilar and middle cerebral arteries and the circle of Willis of the pig (40).

Pial veins. The whole-mount pial veins (n = 12 from 4 animals) were found to receive NOS-I, ASS-I, ASL-I, and NADPHd fibers. They were composed of predominantly fine fibers and some thin bundles. Large bundles, like those found in the anterior cerebral arteries, were not observed. The distribution pattern of NOS-I, ASS-I, and ASL-I fibers was very similar (Fig. 3, A–C). Results from sequential double labeling, i.e., immunofluorescence labeling combined with NADPHd histochemical staining, indicated that all NOS-I, ASS-I, and ASL-I fibers were coincident with NADPHd fibers (Fig. 3, a–c). ASS-I, ASL-I, and NOS-I fibers were not observed after omitting primary antibody or incubating tissues with normal serum from nonimmunized rabbits or goats (data not shown).

L-Citrulline Reversal of Inhibition of Neurogenic Vasodilation Produced by NOS Inhibition in Porcine Pial Veins

In the presence of active muscle tone induced by U-46619 (0.1–1 µM), porcine pial veins relaxed on TNS at 8 and 16 Hz (Fig. 4). The TNS-induced relaxation, which was sensitive to TTX (0.9 µM), was abolished by L-NAME (10 µM). The inhibition was completely reversed by L-citrulline (0.1 mM) (Figs. 4 and 5) but not by D-citrulline (1 mM, n = 3, data not shown).

L-Arginine Synthesis From L-Citrulline in Homogenates of Cerebral Arteries, Pial Veins, and the SPG

In the presence of 1 mM L-aspartate, significant amounts of [14C]arginine were formed from [14C]citrulline in homogenates of SPG (Fig. 6A, lane 1), cerebral arteries with or without endothelium (Fig. 6B, lanes 1 and 2), and pial veins without endothelium (Fig. 6B, lane 3). In the absence of L-aspartate, however, a negligible amount of [14C]arginine was found or not detected in the SPG homogenates (Fig. 6A, lane 2). Application of L-citrulline alone (without incubation as negative control) did not exhibit any trace of L-arginine (Fig. 6A, lane 3, and Fig. 6B, lane 4).
Results of the present studies indicated that ASS, ASL, and NOS were colocalized in ganglion cells in the SPG and perivascular nerves in pial veins and the anterior cerebral arteries. Blockade of NO-mediated neurogenic vasodilation in isolated pial veins was completely reversed by L-citrulline. This L-citrulline reversal of inhibition by NOS inhibitors of neurogenic vasodilation was accompanied by conversion of L-citrulline to L-arginine, suggesting that L-citrulline conversion to L-arginine in cerebral perivascular nerves is via the AS pathway.

We have reported that NOS-I, ASS-I, and ASL-I fibers innervate porcine middle cerebral and basilar arteries and the circle of Willis, and the immunoreactivities of these three enzymes were found to be localized in the same neurons (40). Similar results were obtained in the anterior cerebral arteries in the present studies, suggesting that conversion of L-citrulline to L-arginine for further synthesis of NO in perivascular nerves in cerebral arteries (6) is mediated by the AS pathway (40). This suggestion is further supported by results of the present study that ASS, ASL, and NOS are colocalized in almost all neurons in the SPG. This ganglion has been shown to be one major origin of NOergic nerves to cerebral arteries in several species (15, 26, 28).

In the present study, NOS, ASS, and ASL were also found colocalized in the same perivascular neurons in all pial veins examined. It is clear that the necessary enzymes involved in conversion of L-citrulline to L-arginine and conversion of L-arginine to L-citrulline and NO are present in the same perivascular nerves in all cerebral blood vessels examined. It should be noted that the complete colocalization of NADPHd and NOS immunoreactivities in neuronal cell bodies in the SPG and perivascular nerves of the pial veins and the anterior cerebral arteries support the previous reports that NADPHd is a specific histochemical marker for NOS (8, 11, 34), although this is not universally agreeable (16, 33). Because antisera against purified human liver ASS and ASL were both raised in the rabbit, simultaneous localization of ASS and ASL in the same specimen was not possible at the present time. The almost complete coincidence of both enzymes with NADPHd, therefore, was taken as an evidence that these enzymes are colocalized with NOS in the same neurons.

The presence of dense nitric (NOergic) nerves innervating porcine pial veins is consistent with those reported in other species (7). On TNS, a frequency-dependent, L-NAME-, L-NNA-, and TTX-sensitive relaxation in isolated pial veins was elicited. The inhibition by L-NAME or L-NNA was reversed by L-arginine but not by D-arginine (T. Ishine, J.G. Yu, Y. Asada, and T.J.F. Lee, unpublished data), suggesting that NO is the major substance mediating the TNS-elicited neuro-
genic vasodilation in pial veins, a result similar to that found in the cerebral arteries (19, 20). In the present study, inhibition by L-NAME or L-NNA of the TNS-elicited neurogenic vasodilation in pial veins was completely reversed by L-citrulline. This result from pharmacological studies suggests that there is a conversion of L-citrulline to L-arginine for synthesizing NO in perivascular nerves in pial veins like that found in cerebral arteries (20).

According to the urea cycle, L-citrulline is converted to L-arginine via AS pathway involving ASS and ASL (32). Thus reversal of L-arginine analog-induced inhibition of NO-mediated neurogenic vasodilation in the porcine basilar arteries by L-citrulline (20) was suggested to be due to conversion of L-citrulline to L-arginine. The increased concentration of L-arginine then reversed competitively the inhibition of NOS induced by L-arginine analogs (20).

The conversion of L-citrulline to L-arginine in cerebral arterial perivascular nerves was previously demonstrated indirectly by measuring CO₂ formation from urea (6). In the present study using the TLC method, a significant amount of L-arginine was formed from L-citrulline in the SPG, cerebral arteries with or without endothelial cells, and pial veins without endothelial cells. The synthesis of L-arginine from L-citrulline,
however, occurred only in the presence of L-aspartate. Without adding L-aspartate to the incubation media, no appreciable amount of L-arginine was observed. Because the known pathway involving L-citrulline and L-aspartate is for the formation of AD catalyzed by ASS (32), the present finding, therefore, provides strong evidence that conversion of L-citrulline to L-arginine is mediated by the AS pathway.

In endothelium-denuded arteries, a significant amount of L-arginine synthesis from L-citrulline was still observed. Although quantitative analysis was not performed in the present study, results of our previous biochemical studies using the CO2 method (6) did not detect any difference in L-arginine synthesis between endothelium-intact and endothelium-denuded arteries. It was suggested that the synthesis of L-arginine, although present (20), in the monolayer endothelial cells is too small, compared with the perivascular nerves, to be detected by biochemical measurements (6). This result further indicates that a significant conversion of L-citrulline to L-arginine occurs in the perivascular nerves. It appears that volume of perivascular NOergic neuronal cells is significantly greater than that of NO-generating endothelial cells in large cerebral arteries and veins.

The conversion of L-citrulline to L-arginine did not seem to occur in the muscle cells in the present study. First, our previous biochemical studies demonstrated that L-citrulline conversion to L-arginine in the muscle cells was negligible (6). Second, L-citrulline has never been found to induce a relaxation in endothelium-denuded cerebral arteries in the presence of active muscle tone and L-NAME, whereas L-citrulline consistently induces a relaxation in endothelium-intact cerebral arteries in the presence of active muscle tone and L-NAME (20). This finding suggests that there is an adequate conversion of L-citrulline to L-arginine in the endothelial cells to account for the synthesis of NO and vasodilation (20), but that no conversion of L-citrulline to L-arginine occurs in the smooth muscle. This latter finding is not surprising, since vascular smooth muscle does not contain constitutive ASS (10). The possibility of the induction of ASS in smooth muscle is not likely either (36). In both arterial preparations with and without endothelial cells, L-NAME inhibition of TNS-induced relaxation, however, was fully reversed by L-citrulline, indicating the presence of L-citrulline conversion to L-arginine and L-arginine conversion to L-citrulline for synthesizing NO in perivascular nerves (20). Therefore, the synthesis of L-arginine from L-citrulline in cerebral arteries and pial veins without endothelial cells in the present study most likely oc-

![Graph showing %Relaxation/PPV against Frequency [Hz]](image)

**Fig. 5.** Summary of L-citrulline reversal of L-NAME inhibition of transmural nerve stimulation (TNS)-induced vasodilation (calculated as percentage of maximum relaxation induced by PPV) in porcine pial veins. Relaxations elicited by TNS at 8 and 16 Hz were abolished by L-NAME (10 µM) and were completely reversed by L-citrulline (0.1 mM); n, number of experiments.

![Thin-layer chromatography showing conversion of L-[14C]citrulline to L-[14C]arginine](image)

**Fig. 6.** An example of thin-layer chromatography showing conversion of L-[14C]citrulline to L-[14C]arginine in sphenopalatine ganglia (SPG; A) and cerebral arteries and pial veins (B). Samples were spotted 1.5 cm above bottom of plate and migrated over a distance of 16 cm. L-[14C]arginine was observed in homogenates of SPG containing 1 mM aspartate (A, lane 1), cerebral arteries with endothelium (B, lane 1), or without endothelium (B, lane 2), and pial veins without endothelium (B, lane 3). L-[14C]arginine was insignificant or not observed in homogenates without addition of aspartate (A, lane 2). Application of L-[14C]citrulline alone (no incubation) did not result in L-arginine formation (A, lane 3, and B, lane 4).
curred in the perivascular neurons. This is further supported by the presence of L-aspartate-dependent conversion of L-citrulline to L-arginine in the SPG, a major source of the cerebral perivascular NOergic nerves.

The presence of a functional NOergic innervation in pial veins therefore provides a means for an active vasodilation to be elicited. It has been shown that disruption of the blood-brain barrier (BBB) is associated with an increase in pial venous pressure, for example, during acute hypertension (23). Thus an active vasodilation elicited by NOergic neurons under this condition may be beneficial in maintaining normal function of BBB. However, NO has also been shown to play a role in increasing permeability of BBB and disruption of BBB during acute hypertension (14, 22). These effects of NO on BBB may be attributed mostly to NO released from the endothelium, since BBB exists at the level of endothelial cells (3). The effect of NO of perivascular origin on the function of BBB remains to be clarified.

In summary, the present immunohistochemical, histochemical, biochemical, and pharmacological studies demonstrated that in the SPG and neurons innervating pial veins and cerebral arteries, NO-containing neurons are capable of recycling L-citrulline to L-arginine via the AS pathway, and L-arginine to L-citrulline for synthesizing NO. It appears that cerebral arterial and venous perivascular nerves can maintain adequate concentrations of L-arginine for synthesizing NO in regulating cerebral vascular tone. This L-citrulline-L-arginine recycling, which has also been shown to take place in nonneuronal cells and some central nervous system neurons (1, 10, 35, 37), may play an important role in regulating cerebral circulation in health and disease.

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Address for reprint requests: T. J. F. Lee, Dept. of Pharmacology, SIU School of Medicine, PO Box 19230, Springfield, IL 62794-1222.

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