L-Arginine Deficiency Causes Suppression of Nonadrenergic Noncholinergic Nerve-Mediated Smooth Muscle Relaxation: Role of L-Citrulline Recycling

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ABSTRACT

Studies were performed on the internal anal sphincter (IAS) smooth muscle strips obtained from opossums (Didelphis virginiana). Isometric tension and L-arginine levels of the tissues were measured under basal conditions, in the presence of electrical field stimulation (EFS) and after treatment with different concentrations of arginase. For the nonadrenergic noncholinergic nerve stimulation, short trains (4 sec) as well as continuous EFS were used. During continuous EFS, after the initial IAS relaxation, the response began to fade within several min to ~80% recovery of the basal tone. We also examined the influence of L-arginine and L-citrulline on these responses. For some studies, the tissues were pretreated with L-glutamine (an inhibitor of L-citrulline uptake), L-glutamate or N^G-hydroxy-L-arginine (an inhibitor of arginase). Interestingly, the basal levels of L-arginine were found to be significantly higher in the IAS (tonic smooth muscle) than in the rectal (phasic smooth muscle) muscle. Arginase caused a concentration-dependent attenuation of the IAS relaxation caused by EFS. L-Citrulline and L-arginine were equipotent in reversing the attenuation. Both arginase (60 min pretreatment) and continuous EFS (tissues collected at the time of maximal recovery of the basal IAS tone after the initial relaxation) caused significant decreases in L-arginine levels. The decreases in the levels of L-arginine were restored by the exogenous administration of either L-arginine or L-citrulline. The restoration of L-arginine levels by L-citrulline but not by L-arginine was selectively blocked by L-glutamine. Furthermore, the IAS relaxation, attenuated by arginase was unaffected by L-glutamine but was restored by N^3-hydroxy-L-arginine pretreatment. The studies suggest that L-citrulline-L-arginine recycling may play a significant role in the maintenance of IAS relaxation in response to nonadrenergic noncholinergic nerve stimulation.

The IAS relaxation in response to NANC nerve stimulation with short train EFS can be maintained for several hours without a significant fade. Furthermore, we have recently reported that the IAS relaxation in response to continuous EFS can be maintained for several min that is followed by a gradual recovery of the basal tone toward the prestimulus level (Rattan et al., 1996). The exact mechanism responsible for the maintenance of the IAS relaxation and for the fade is not known.

Different laboratories have identified the role of multiple inhibitory neurotransmitters such as vasoactive intestinal polypeptide (Biancani et al., 1985; Nurko and Rattan, 1988), NO (Rattan and Chakder, 1992; Tottrup et al., 1992) and possibly heme oxygenase pathway (Rattan and Chakder, 1993; Tottrup et al., 1995) in the relaxation of IAS. The inhibitory substances appear to work either independently or in concert with other inhibitory neurotransmitters. For example, a part of the inhibitory action of vasoactive intestinal polypeptide in the IAS is mediated via L-arginine-NO pathway (Rattan and Chakder, 1992). The role of NO as an inhibitory neurotransmitter in the opossum IAS is similar to that of humans (Burleigh, 1992; O’Kelly et al., 1993). In addition, adenosine triphosphate has also been suggested to play a role in the inhibitory neurotransmission in the IAS of the rabbit (Tottrup et al., 1995; Knudsen et al., 1995) and the guinea pig (Rae and Muir, 1996).

It is well known that in the central nervous system as well as in the peripheral autonomic nervous system, NO and L-citrulline are produced stoichiometrically (1:1) from L-arginine by NOS (Moncada et al., 1991; Goyal and Hirano, 1996; Makhlouf and Grid, 1993; Moncada and Higgs, 1993; Bush et al., 1992; Jaffrey and Snyder, 1995; Stark and Szurszewski, 1992; Sanders and Ward, 1992). Although, the role of L-citrulline recycling to L-arginine in a number of systems is

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ABBREVIATIONS: IAS, internal anal sphincter; EFS, electrical field stimulation; NANC, nonadrenergic noncholinergic; NO, nitric oxide; NOS, nitric oxide synthase; HOArg, N^3-hydroxy-L-arginine.
well known, its role in the gastrointestinal tract has only been recognized recently (Shuttleworth et al., 1995; Rattan et al., 1996). In the IAS and colon, the inhibition of NOS has been shown to cause significant attenuation of the smooth muscle relaxation that can be overcome enantiomer-specifically not only by L-arginine but also by L-citrulline. The data suggest the presence of enzymatic machinery responsible for the recycling of L-citrulline into L-arginine (Shuttleworth et al., 1995; Yu et al., 1995). The studies showed that L-citrulline can substitute for L-arginine to restore the inhibitory neurotransmission inhibited by the NOS inhibitor. However, direct studies to demonstrate changes in L-arginine levels to test the hypothesis have not been performed.

The purpose of our investigation was to further examine the role of L-citrulline recycling in the IAS smooth muscle relaxation by the simultaneous determinations of tissue levels of L-arginine. Arginase pretreatment and continuous EFS were used to determine the influence of L-arginine deficiency on the IAS relaxation, and the role of L-citrulline-L-arginine recycling in the maintenance of IAS relaxation.

Materials and Methods

Preparation of smooth muscle strips. Adult opossums (Didelphis virginiana) of either sex weighing from 2.3 to 3.6 kg, were killed after a high dose of pentobarbital sodium (50 mg/kg; i.p.). The anal canal along with a section of the rectum was removed and transferred to oxygenated Krebs physiological solution of the following composition (mM): NaCl, 118.07; KCl, 4.69; CaCl₂, 2.52; MgSO₄, 1.16; NaH₂PO₄, 1.01; NaHCO₃, 25 and glucose, 11.10. The anal canal was freed of all extraneous tissues including the external anal sphincter by sharp dissection, opened and pinned flat on a dissecting tray containing oxygenated Krebs solution. The mucosal and submucosal layers were carefully removed using sharp dissection and IAS circular smooth muscle strips (~1 × 10 mm) were prepared as described previously (Moumni and Rattan, 1988).

Measurement of isometric tension. The muscle strips were secured at both ends with silk sutures and transferred to 2-mL muscle baths containing oxygenated (95% oxygen plus 5% carbon dioxide) Krebs’ solution (34°C). One end of the muscle strip was anchored at the bottom of the muscle bath and the other end was attached to a force transducer (model PT03; Grass Instruments Co., Quincy, MA) for the measurement of isometric tension on a Dyonograph recorder (model R411; Beckman Instruments, Schiller Park, IL). The muscle strips were stretched initially at 1 g of tension and allowed to equilibrate for at least 1 hr by replacement of bath solution with fresh oxygenated Krebs solution at 20-min intervals. The IAS smooth muscle strips developed spontaneous and steady tension during this equilibration period. Only the strips that developed spontaneous tension and relaxed in response to EFS were used for the study. The optimal length (Lₒ) and the baseline of the smooth muscle strips were determined as described previously (Moumni and Rattan, 1988).

NANC nerve stimulation with EFS. EFS was delivered from a Grass stimulator (model S88; Grass Instruments Co.) via platinum electrodes consisting of a pair of platinum wires fixed at both sides of the smooth muscle preparation. Neuromediately mediated relaxation of the IAS smooth muscle strips was measured in response to different frequencies (0.5–20 Hz) of EFS (20–30 V, 0.5-msec pulse duration for 4 sec). These parameters of EFS are known to cause IAS relaxation by selective activation of NANC myenteric neurons. Two EFS protocols were used for the study.

In the first protocol, the NANC nerves were stimulated with short train EFS (4-sec train; 0.5-msec pulse duration; 20–30 V) at varying frequencies (0.5–20 Hz) before and after treatment with different drugs. In these experiments, the smooth muscle strips showed transient relaxation. This protocol was used for the functional studies dealing with the influence of arginase treatment alone and arginase plus either L-arginine or L-citrulline on EFS-induced IAS relaxation.

In the second protocol, long trains of EFS were used for the study. The optimal length (Lₒ) and the baseline of the smooth muscle strips were used for the study. The optimal length (Lₒ) of the smooth muscle strips was measured in response to different concentrations (15, 30 or 60 μM in the muscle baths) for 60 min. The influence of arginase on the basal IAS tone, NANC nerve-mediated IAS relaxation and on the basal L-arginine levels were determined. In the second type, continuous EFS was used as explained above. To determine the effects of L-citrulline and L-arginine on the influence of L-arginine deficiency by arginase pretreatment or continuous EFS, the tissues were treated with L-arginine or L-citrulline for 30 min.

The effects of L-arginine on L-citrulline on the depletion of arginine levels were examined in the IAS smooth muscle strips attempted either by arginase pretreatment (60 U/ml at 37°C for 60 min) or continuous EFS (10 Hz). The tissues were frozen quickly as described previously (Rattan et al., 1991). To examine the effects of L-arginine, or L-citrulline, the tissues were treated with these agents for another 30 min and the tissues were frozen quickly at this time.

When the effects of L-arginine or L-citrulline on the depletion of arginine levels were examined, these agents were added after recovery of the fall in tension during continuous EFS. At this stage, the addition of L-arginine or L-citrulline was found to cause a fall in the IAS tension and the tissues were frozen by clamping at the point of maximal fall in the tension. L-Arginine or L-citrulline otherwise in the normal or L-arginine sufficient preparations was found to cause no fall in the IAS tone. In a separate series of experiments, the effect of L-glutamine for 30 min for examining the effects of EFS before and after different maneuvers.

The tissues were stored at -80°C until used for L-arginine determination. The tissues were homogenized in 1 ml of 6% trichloroacetic acid on an ice bath using a tissue homogenizer (Tekmar Tissue Disruptor; Tekmar Company Cincinnati, OH). The homogenate was allowed to stand for 20 min and then centrifuged at 3000 × g for 15 min at 4°C. The supernatant was extracted five times with 5 ml of water-saturated diethyl ether.

Arginine was separated from the extracts by a chromatographic procedure using Dowex AG 50W × 8 resin columns (sodium form; 100–200 mesh). Briefly, the extracts were diluted in 3 ml of 0.1 M citrate buffer (pH 5.3) and applied onto the columns that were preequilibrated with the same buffer. The columns were washed with 15 ml of citrate buffer and then with 4 ml of 0.2 N sodium hydroxide which quantitatively eluted the arginine from the columns.

Arginine levels of the elutes were determined by a colorimetric method using L-arginine as the standard (modified Sakaguchi reaction). The details of the method have been described elsewhere (Gold et al., 1989). Briefly, samples (3 ml) were taken into different tubes on an ice bath and treated with 0.5 ml of alkaline α-naphthol-thymine mixture and mixed well. The samples were then treated with 0.2 ml of 1% sodium hypochloride (NaOCl) solution and mixed immediately. Exactly, 1 min later 0.2 ml of 2% sodium thiosulphate was added and mixed well. The absorbance of the samples was measured at 515 nm using a spectrophotometer (model DU 64; Beckman Instruments Inc., Fullerton, CA). Recovery of L-arginine from the tissues was determined by adding known amounts of L-arginine.
arginine to some smooth muscle strips and measurements of arginine levels colorimetrically after the extraction process. The recovery of added arginine through the extraction procedure was 84.3 ± 3.5%. No correction factor for the percentage of recovery was applied for the calculation of tissue arginine levels. The protein content of the tissues was determined by the method of Lowry et al. (1951).

**Drug Responses.** Responses to different agents were examined using either single dose or cumulative concentration responses. Once the concentration-response curve to an agent was determined, the smooth muscle strips were washed for 45 min, and the basal tension was allowed to recover. The effects of agents on the NANC nerve-mediated IAS relaxation was determined using both short train and continuous EFS.

L-Arginine deficiency was monitored by the determination of L-arginine levels before and after arginase and at appropriate times of the continuous EFS as explained above. The influence of these maneuvers was further ascertained by the effect of L-arginine deficiency on EFS-induced IAS relaxation. Furthermore, the possible restoration of these parameters (L-arginine levels and NANC nerve-mediated IAS relaxation) was examined after the administration of L-arginine and L-citrulline.

We also performed studies to examine the influence of L-glutamine (putative blocker of L-citrulline uptake) (Lee et al., 1996; Sessa et al., 1999) on the L-arginine levels before and after L-arginine and L-citrulline in the presence of continuous EFS. To determine the specificity of action of L-arginine, we examined the effects of L-glutamate. The tissues were pretreated with L-glutamine or L-glutamate for 30 min before testing the effects of EFS and L-citrulline or L-arginine. The effects of different concentrations of L-citrulline or L-arginine on the basal tone, NANC nerve-mediated IAS relaxation and recovered IAS tone in the presence of ongoing EFS were also determined before and after L-glutamine.

To determine the specificity of action of arginase, in a separate series of experiments, we also examined the effects of Nω-hydroxy-L-arginine (HOArg) an inhibitor of arginase (Hecker et al., 1995), on the effects of arginase on L-arginine levels and IAS relaxation.

**Drugs and chemicals.** The following chemicals were used in the study: arginine, L-arginine hydrochloride, D-arginine, L-citrulline (L-2-amino-5-ureidovaleric acid), L-glutamine (L-2-aminoglutaric acid), L-glutamic acid monosodium salt (L-glutamate), Nω-hydroxy-L-arginine (HOArg) (Sigma Chemical Co., St. Louis, MO); and ethylenediaminetetraacetic acid. Statistical significance between different groups were determined using t test (paired or unpaired) or analysis of variance and p < .05 was considered statistically significant.

**Results**

**Effect of different concentrations of arginase on IAS relaxation by NANC nerve stimulation: influence of L-arginine and L-citrulline.** Short train (4 sec) EFS was used in these experiments before and after arginase pretreatment. As shown in figure 1, in control experiments, EFS caused a frequency-dependent fall in the basal tension of the IAS. Arginase pretreatment caused attenuation in the IAS relaxation caused by different frequencies of EFS (Fig. 1).

The attenuation of IAS relaxation by arginase was restored by L-arginine pretreatment (Fig. 2). A concentration of 1 × 10⁻³ M caused a complete reversal of the IAS relaxation. The reversal of the IAS relaxation by L-arginine was stereoselective since D-arginine 1 × 10⁻³ M had no effect on the attenuated IAS relaxation. Interestingly, L-citrulline had similar effects in reversing the attenuation of IAS relaxation as L-arginine (fig. 3) in arginase-treated preparations. However, neither L-arginine nor L-citrulline had any effect on the EFS-induced IAS relaxations in arginase untreated tissues (L-arginine sufficient). The data suggest that arginase was selective in suppressing the IAS relaxation caused by EFS.

The other method to cause L-arginine deficiency was by continuous EFS. We have shown previously that this maneuver also causes an attenuation of the IAS relaxation that was reversed by L-citrulline as well as by L-arginine (Rattan et al., 1996).
To substantiate that the attenuation of the IAS relaxation and its reversal by L-arginine and L-citrulline are directly associated with L-arginine depletion and repletion, respectively, we performed L-arginine determinations in the basal state, after arginase pretreatment and arginase plus L-arginine or L-citrulline. Arginase pretreatment was found to cause no significant effect on the basal tone of the IAS. The basal IAS tone before and after arginase pretreatment (60 U/ml) were 2.12 ± 0.18 and 2.19 ± 0.19 g, respectively (P > .05; n = 5).

Interestingly, the basal levels of L-arginine were found to be significantly higher in the IAS (6.15 ± 0.57 pmol/µg protein) than in the rectum (3.91 ± 0.52 pmol/µg protein) (P < .05; n = 4). In three of the four animals investigated, the basal levels of L-arginine were distinctly lower in the rectum.

Effect of arginase treatment on L-arginine levels: influence of L-citrulline and L-arginine. In these tissues used for the isometric tension studies, the basal level of L-arginine in the IAS was found to be 6.2 ± 0.7 pmol/µg...
protein. After the treatment with arginase, L-arginine levels were 4.3 ± 0.9 pmoles/µg protein (P < .05; n = 4; fig. 4). L-Arginine and L-citrulline after arginine pretreatment in these tissues caused a significant reversal of the decrease in L-arginine levels to 8.1 ± 1.2 and 6.1 ± 0.3 pmol/µg protein respectively (P < .05; n = 4; fig. 4).

Effect of continuous EFS on the IAS relaxation and L-arginine levels: influence of L-citrulline and L-arginine. In these series of experiments, continuous EFS caused a significant fall in the basal levels of L-arginine from 8.2 ± 0.7 to 6.5 ± 0.7 pmol/µg protein (P < .05; n = 7; fig. 5). The treatment of the tissues with L-arginine or L-citrulline in the presence of continuous EFS caused a restoration of the L-arginine levels. The concentrations of L-arginine and L-citrulline were selected for these experiments because they caused maximal reversal of the IAS relaxations.

Influence of L-glutamine on the restoration of L-arginine levels by L-citrulline. To determine the role of L-citrulline recycling into L-arginine, we examined the effect of L-glutamine on L-arginine levels of the IAS tissues during continuous EFS before and after L-arginine or L-citrulline. L-Arginine blocked the reversal of the EFS-induced decrease in the L-arginine levels by L-citrulline (fig. 5). L-Arginine levels in these experiments during continuous EFS after L-citrulline were 7.8 ± 0.5 pmoles/µg protein and after L-citrulline plus L-glutamine the levels were 6.7 ± 0.5 pmoles/µg protein (P < .05; n = 5; Fig. 5). However, the reversal of the decrease in L-arginine levels by L-arginine were not significantly affected by L-glutamine (10.8 ± 1.5 pmoles/µg protein after L-arginine alone vs. 10.2 ± 0.7 pmoles/µg protein after L-arginine plus L-glutamine) (P > .05; n = 5). Furthermore, our preliminary studies showed that L-glutamate had no effect on the restoration of L-arginine levels by either L-arginine or L-citrulline.

Influence of L-glutamine and NG-hydroxy-L-arginine (HOArg) on the fall in IAS tension by NANC nerve stimulation. The effects of L-glutamine and HOArg on the attenuation of EFS-induced IAS relaxation by arginase are shown in figure 6, A and B. The data show that the attenuation of EFS-induced IAS relaxation by arginase (30 U/ml) (fig. 6A) was not affected by L-glutamine (1 × 10⁻³ M) but the attenuation was reversed by pretreatment of the tissues with HOArg (1 × 10⁻⁴ M) (fig. 6B). The data suggest the specificity of action of arginase and L-glutamine. Effects of the NO-donor SNP on the basal tension of the IAS before and after continuous EFS and arginase treatment

SNP caused a concentration-dependent fall in the basal tone of IAS. The data show that the fall in IAS tension by SNP was not affected significantly by either continuous EFS or arginase (60 U/ml) treatment (P > .05; analysis of variance) (fig. 7).

Discussion

The studies suggest that the critical levels of L-arginine and recycling of L-citrulline to L-arginine are important in the maintenance of IAS relaxation by NANC nerve stimulation. In our study, two types of experimental protocols were used to investigate the role of L-arginine deficiency: continuous EFS and arginase treatment. Continuous EFS causes an immediate fall in the IAS tension followed by a gradual recovery of the tone to ~80% of the original tone. The recovery of the tone in the presence of continuous EFS was speculated to be due to the depletion of L-arginine (Rattan et al., 1996). Our studies with the measurement of L-arginine levels provide direct evidence in that regard. There was a significant decrease in the levels of L-arginine from the basal state to the time when the IAS relaxation had recovered. Furthermore, at this time, the administration of L-citrulline leads to the restoration of L-arginine levels. The restoration of L-arginine levels by L-citrulline was similar to that by L-arginine. Furthermore, our previous studies have shown that L-citrulline as well as L-arginine cause concentration-dependent fall in the recovered basal tone of the IAS in the L-arginine deficiency state caused by the continuous EFS. Arginase pretreatment causes a state similar to that of continuous EFS because it causes an attenuation of neurally mediated IAS relaxation and decrease in the tissue levels of L-arginine. The decrease in the levels of L-arginine and the IAS relaxation can be resorted by the exogenous administration of L-arginine or L-citrulline. The data provide further evidence in support of the role of L-arginine-NO pathway and L-citrulline-L-arginine recycling in the IAS relaxation by NANC nerve stimulation (Rattan and Chakder, 1992; Rattan et al., 1992).

It is interesting that complete depletion of L-arginine levels is not a requirement for the failure or significant suppression of the IAS relaxation by NANC nerve stimulation. The data suggest that there is a critical level of L-arginine that must be maintained for the normal relaxation during the NANC nerve stimulation. As a matter of fact, the studies clearly show that only a small decrease in the L-arginine levels in the tissues is all that is required to cause a dramatic impairment of the NANC nerve-mediated relaxation of the IAS. This is supported by an immediate and complete reversal of the IAS relaxation as well as the tissue levels of L-arginine by small concentrations of exogenous L-arginine. The concept of critical levels of basal L-arginine being responsible for the IAS relaxation is supported by the findings that exogenous L-arginine in the normal tissues (L-arginine adequate or sufficient tissues) usually causes no increase in the tissue levels of L-arginine. Furthermore, exogenous L-arginine in the L-argi-
argininosuccinate lyase) responsible for the synthesis of L-arginine. In our study we show the restoration of the IAS relaxation as well as the L-arginine levels by both exogenous L-arginine and L-citrulline during prolonged EFS. The specificity of the action of L-citrulline was further examined by the pretreatment of tissues with L-glutamine, a putative inhibitor of L-citrulline uptake (Sessa et al., 1990; Lee et al., 1996). Interestingly, although L-glutamine blocked the recovery of L-arginine levels as well as the IAS relaxation by L-citrulline in the L-arginine-deficient preparations, it had no effect on the recovery by L-arginine. The data provide further evidence in favor of the proposed mechanism of action of L-glutamine. This further suggests the role of recycling of L-citrulline into L-arginine being responsible for the restoration of the IAS relaxation during continuous EFS. Furthermore, L-glutamate an amino acid that otherwise resembles L-glutamine had no significant effect on the restoration of L-arginine levels and the suppressed IAS relaxation.

The data also suggest the presence of transport systems for extracellular L-arginine and L-citrulline in the myenteric neurons of the IAS. The exact nature of affinity carriers responsible for the transcellular transport of two amino acids in the myenteric neurons has not been characterized. Furthermore, the relative contribution of extracellular vs. intracellular sites of action of exogenous arginase in causing the suppression of IAS relaxation and decrease in L-arginine levels in the IAS also remains to be determined.

The suppression of the NANC nerve-mediated IAS relaxation by L-arginine deficiency was found to be specific since the IAS relaxation by the direct acting agent sodium nitroprusside was not affected by the maneuvers causing L-arginine deficiency. Furthermore, the arginase-induced attenuation of the IAS relaxation was specifically blocked by NG-hydroxy-L-arginine (arginase inhibitor) but not by L-glutamine.

Another interesting outcome of the studies was that in the basal state, significantly higher levels of L-arginine were found in the smooth muscle of internal anal sphincter than in the rectum. The observations suggest an active uptake and efficient handling of L-arginine in the IAS tissues. The significance of these observations and the cellular site(s) responsible for the contribution to the differences remains to be determined. It is possible that differences in the uptake of L-arginine, presence of membrane transporter, intracellular...
production of l-arginine by protein degradation and the l-citrulline-l-arginine recycling may be partly responsible for the efficient inhibitory neurotransmission and the difficulty in causing a complete depletion of l-arginine levels in the IAS tissues by any of the approaches used.

l-Arginine-NO pathway has been shown to play a significant role in the appropriate peristalsis in the esophagus (Yamato et al., 1992; Murray et al., 1991; Anand and Paterson, 1994). Furthermore, it has been shown that the exogenous administration of NO donor, glyceryl trinitrate but not l-arginine (NO precursor) produces beneficial effect in such patients (Konturek et al., 1995). The improvement in the esophageal motility picture in these patients with the NO donor and the failure with l-arginine suggest that the underlying etiology of diffuse esophageal spasm may not be l-arginine deficiency, but it may be related either to the loss of or damage to the myenteric nitricergic neurons. Under these circumstances, the tissues may not be expected to utilize the available or exogenously administered l-arginine for the synthesis of NO.

In summary, the data show the importance of l-arginine-NOS pathway and a role of l-citrulline-l-arginine recycling in the maintenance of the gastrointestinal smooth muscle relaxation in response to NANC nerve stimulation.

References