Chapter 8

Role of the L-citrulline/L-arginine cycle in iNANC nerve-mediated NO production and airway smooth muscle relaxation in allergic asthma

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Summary
Nitric oxide synthase (NOS) converts L-arginine into nitric oxide (NO) and L-citrulline. In NO-producing cells, L-citrulline can be recycled to L-arginine in a two-step reaction involving argininosuccinate synthase (ASS) and -lyase (ASL). In guinea pig trachea, L-arginine is a limiting factor in neuronal nNOS-mediated airway smooth muscle relaxation upon inhibitory nonadrenergic noncholinergic (iNANC) nerve stimulation. Moreover, in a guinea pig model of asthma iNANC nerve-induced NO production and airway smooth muscle relaxation are impaired after the allergen-induced early asthmatic reaction (EAR), due to limitation of L-arginine. Using guinea pig tracheal preparations, we now investigated whether (i) the L-citrulline/L-arginine cycle is active in airway iNANC nerves and (ii) the NO deficiency after the EAR involves impaired L-citrulline recycling. Electrical field stimulation (EFS)-induced relaxation was measured in tracheal open-rings precontracted with histamine. L-Citrulline as well as the ASL inhibitor succinate did not affect EFS-induced relaxation under basal conditions. However, reduced relaxation induced by a submaximal concentration of the NOS inhibitor \( \text{N}^\omega \)-nitro-L-arginine was restored by L-citrulline, which was prevented by the additional presence of succinate or the ASS inhibitor \( \alpha \)-methyl-D,L-aspartate. Remarkably, the impaired iNANC relaxation after the EAR was restored by L-citrulline. In conclusion, the L-citrulline/L-arginine cycle is operative in guinea pig iNANC nerves in the airways and may be effective under conditions of low L-arginine utilization by nNOS (caused by NOS inhibitors), and during reduced L-arginine availability after allergen challenge. Enzymatic dysfunction in the L-citrulline/L-arginine cycle appears not to be involved in the L-arginine limitation and reduced iNANC activity after the EAR.

Introduction
L-Arginine, a semi-essential amino acid, is the precursor of nitric oxide (NO), which is generated by NO synthase (NOS) enzymes that utilize L-arginine, oxygen and NADPH to produce NO and L-citrulline [1]. Three NOS isoforms have been identified: the constitutively expressed isoforms neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III) and the inducible isoform (iNOS or NOS II) [2]. In the airways, the constitutive isoforms (cNOS) are mainly expressed in inhibitory nonadrenergic noncholinergic (iNANC) neurons (nNOS), the endothelium (eNOS) and epithelium (nNOS and eNOS), whereas iNOS is mainly expressed in macrophages, neutrophils and epithelial cells during airway inflammation [2]. NO is the major bronchodilating neurotransmitter of the iNANC nervous system, which is a most effective bronchodilating neural pathway of the airways. Thus, inhibition of NOS markedly reduces iNANC nerve-mediated relaxation of both guinea pig [3,4][Chapter 6] and human airways [5]. Vasoactive intestinal polypeptide is another neurotransmitter of the iNANC system and colocalization of NOS and vasoactive...
The availability of L-arginine to NOS determines the production of NO to an important extent [7]. In guinea pig tracheal preparations, we recently demonstrated that L-arginine is a limiting factor in the production of iNANC nerve-derived as well as agonist-induced epithelium-derived NO and airway relaxation under basal conditions [4,8][Chapter 6]. In most cells, L-arginine requirements are met primarily by uptake of extracellular L-arginine via specific cationic amino acid transporters (system y\(^{+}\)) [9]. However, in NO producing cells, L-arginine may also be generated by the L-citrulline/L-arginine cycle [10]. In this cycle L-citrulline, the co-product of NO biosynthesis catalyzed by NOS, can be recycled to the NOS substrate L-arginine, presumably to maintain NO production. The conversion of L-citrulline to L-arginine is a two-step reaction involving argininosuccinate synthase (ASS), which converts L-citrulline and L-aspartate into L-argininosuccinate, and argininosuccinate lyase (ASL), which hydrolyses L-argininosuccinate into L-arginine and fumarate [10-12]. In some tissues, including the opossum internal anal sphincter and rat gastric fundus, it has been shown that the L-citrulline/L-arginine cycle is involved in iNANC nerve-mediated smooth muscle relaxation, particularly at relatively low L-arginine availability to nNOS induced by competitive inhibitors of the enzyme [7,13-15]. Furthermore, it was demonstrated that ASS and ASL are colocalized with nNOS in canine enteric neurons [16,17], as well as in neurons of the myenteric plexus and in nerve fibers of circular and longitudinal smooth muscles of the rat gastric fundus [15]. Although not conclusively, some indication for a role of citrulline recycling in the airways was also found in the iNANC nerves of guinea pig tracheal and human bronchial preparations [18].

L-Arginine availability in the airways is also under control of arginase, which hydrolyzes L-arginine into L-ornithine and urea [4,19]. Arginase (type I) is classically considered to be an enzyme of the urea cycle, but is also expressed in extrahepatic tissues (arginase type I and type II), that do not express a complete urea cycle [20]. We have previously established that arginase is importantly involved in both the neural and nonneural regulation of airway smooth muscle tone by competition with cNOS for the common substrate [4,19,20]. Moreover, increased arginase activity in the airways contributes to airway hyperresponsiveness after the allergen-induced early asthmatic reaction (EAR) by inducing reduced L-arginine availability to cNOS [20-22][Chapters 2&7]. However, another mechanism causing L-arginine limitation and NO-deficiency could be impaired recycling of L-citrulline, due to dysfunction of the L-citrulline/L-arginine cycle.

In the present study, we investigated (i) to what extent the L-citrulline/L-arginine cycle is active in iNANC nerves in guinea pig airways and (ii) whether the observed NO deficiency and L-arginine limitation after the EAR may involve impaired L-citrulline recycling. To this aim, we investigated the effects of exogenous L-citrulline and inhibitors ASS and ASL on electrical field stimulation (EFS)-induced, NO-
mediated iNANC relaxation of guinea pig tracheal preparations under basal conditions and at 6 h after allergen challenge (i.e. after the EAR).

**Methods**

**Animals**

Male specified pathogen-free Dunkin Hartley guinea pigs (Harlan Hillcrest, UK), weighing 500 – 800 g, were used in this study. The animals were IgE-sensitized to ovalbumin with Al(OH)₃ as adjuvant as described previously [23] and used experimentally 4 to 6 weeks later. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

**Allergen provocation**

Allergen provocations were performed by inhalation of an aerosolized solution of 0.5 mg/ml ovalbumin in saline as described previously [24]. Allergen inhalations were discontinued when the first signs of respiratory distress were observed. The animals were sacrificed 6 h after allergen provocation, i.e. after the EAR [25]. Nonchallenged animals were used as controls.

**Tissue preparation**

Guinea pigs were sacrificed by a sharp blow on the head followed by rapid exsanguination. The trachea was removed and prepared free of serosal connective tissue in a Krebs-Henseleit buffer solution of 37°C, gassed with 95% O₂ and 5% CO₂. The composition of the KH-solution in mM was: NaCl 117.50; KCl 5.60; MgSO₄ 1.18; CaCl₂ 2.50; NaH₂PO₄ 1.28; NaHCO₃ 25.0 and d-glucose 5.50; pH 7.4. Twelve single proximal tracheal open-ring preparations were mounted for isotonic recording (0.3 g preload) between two parallel platinum point-electrodes in 20 ml organ baths containing gassed Krebs-Henseleit solution and indomethacin (3 µM) to eliminate any influence of prostanoids.

**Electrical field stimulation-induced relaxation experiments**

After 30 min equilibration, tracheal preparations were relaxed with isoprenaline (0.1 µM) to establish basal tone. After thorough washout, maximal contraction to histamine was determined with cumulative additions of the agonist (0.1, 1, 10 and 100 µM). After washout, the preparations were precontracted with histamine to 30% of maximal histamine-induced tone, in the presence of atropine (1 µM) to prevent EFS-induced cholinergic airway contraction. Biphasic electrical field stimulation (EFS; 150 mA, 4 ms, 4s, 0.5 – 16 Hz) was applied and frequency-response curves (0.5 – 16 Hz in doubling steps) were recorded. Per preparation, one frequency response curve was performed. When used, L-NNA (30 µM) and L-citrulline (5.0 mM) were applied to the organ bath 30 min prior to histamine addition, while the ASL inhibitor succinate (5 mM) and the ASS inhibitor α-methyl-D,L-aspartate (αMDLA; 3 mM) were applied 40 min before histamine. None of these agents influenced basal
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or histamine-induced tone. After the final EFS-induced relaxation, followed by washout, isoprenaline (10 µM) was added to re-establish basal tone. All measurements were performed in triplicate.

Data analysis
All individual relaxations elicited by EFS were estimated as peak height of the EFS-induced response, and were expressed as a percentage of maximal relaxation, as established in the presence of isoprenaline. All data are expressed as means ± SEM of n experiments. Statistical significance of differences was evaluated using one-way analysis of variance (ANOVA) and significance was accepted when P<0.05.

Chemicals
Ovalbumin (grade III), aluminium hydroxide, histamine dihydrochloride, indomethacin, atropine sulphate, L-citrulline, succinate, α-methyl-D,L-aspartate, Nω-nitro-L-arginine and (-)-isoprenaline hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Results
Role of L-citrulline recycling on EFS-induced airway relaxation under basal conditions
In line with our previous studies [4,21][Chapters 6&7], EFS (0.5 – 16 Hz) induced a frequency-dependent relaxation of histamine-induced tone of tracheal open-ring preparations from unchallenged guinea pigs, ranging from 8.7 ± 1.9% at 0.5 Hz to 68.6 ± 3.0% at 16 Hz. To study the role of recycling of endogenous L-citrulline to L-arginine, preparations were incubated with the ASL-inhibitor succinate (5.0 mM). Succinate did not affect iNANC relaxation at any frequency, indicating that the recycling of endogenous L-citrulline is not importantly involved under basal conditions (Figure 1). Furthermore, incubation with exogenous L-citrulline (5.0 mM) alone or in combination with succinate did not affect iNANC relaxations either (Figure 1). Like succinate, incubation with the ASS-inhibitor αMDLA (3 mM) did not affect basal iNANC relaxations either (data not shown).
Figure 1: EFS-induced relaxation of tracheal open-rings obtained from unchallenged guinea pigs in the absence and presence of the ASL inhibitor succinate (5 mM), L-citrulline (5 mM) or a combination of both. Results are means ± SEM of 6 experiments.

L-Citrulline recycling restores L-NNA-induced inhibition of iNANC nerve-mediated airway smooth muscle relaxation

Subsequently, we studied the effect of L-citrulline recycling under conditions of reduced substrate utilization by nNOS in the presence of a submaximal concentration of the NOS inhibitor L-NNA (30 µM). Similar to our previous studies [4,21][Chapters 6&7], incubation with L-NNA resulted in reduced iNANC nerve-mediated airway smooth muscle relaxation, particularly at the lower frequencies (P<0.05 all; Figures 2A and B). Interestingly, incubation with exogenous L-citrulline completely reversed the decreased iNANC relaxation in the presence of L-NNA at 0.5 to 8 Hz (P<0.05 all; Figures 2A and B). Coincubation with the ASS inhibitor αMDLA (3 mM; Figure 2A) or the ASS inhibitor succinate (5 mM; Figure 2B) prevented the effect of L-citrulline on decreased iNANC relaxation induced by L-NNA at these frequencies (P<0.05 all), indicating that recycling to L-arginine underlies the normalizing effect of L-citrulline.
Figure 2: EFS-induced relaxation of tracheal open-rings obtained from unchallenged guinea pigs in the absence and presence of a submaximal concentration of the NOS inhibitor L-NNA (30 µm) alone or with L-citrulline (5 mm) without and with the ASS inhibitor αMDLA (3 mm; panel A) or the ASL inhibitor succinate (5 mm; panel B) present. Results are means ± SEM of 6 (panel A) and 8 (panel B) experiments. †P<0.05 compared to control, *P<0.05 compared to L-NNA treated and #P<0.05 compared to L-NNA plus L-citrulline treated.
Effect of L-citrulline on allergen-induced impaired iNANC relaxations

Having established that the L-citrulline/L-arginine cycle is operative in the guinea pig airways, we further investigated whether impaired recycling underlies the observed L-arginine limitation and reduced iNANC relaxation after the allergen-induced EAR. According with our previous study [21][Chapter 7] iNANC relaxations were markedly impaired at 6 h after OA-challenge as compared to unchallenged controls (P<0.05 all; Figure 3). Remarkably, incubation with exogenous L-citrulline (5 mM) fully restored the impaired iNANC relaxation after allergen challenge at 0.5 to 8 Hz to the normoreactive levels of unchallenged controls (P<0.05, Figure 3).

Figure 3: EFS-induced relaxation of tracheal open-ring preparations obtained from unchallenged and OA-challenged guinea pigs in the absence and presence of L-citrulline (5.0 mM). Results are means ± SEM of 4-6 experiments. *P<0.05 compared to unchallenged control and †P<0.05 compared to OA-challenged control.

Discussion

Recently, we demonstrated that the availability of L-arginine to nNOS importantly regulates iNANC relaxation of guinea pig tracheal preparations [4][Chapter 6]. Thus, increasing the L-arginine availability to nNOS, by addition of exogenous L-arginine or by inhibition of endogenous arginase activity, significantly increased EFS-induced NO-mediated airway smooth muscle relaxation [4][Chapter 6]. Since recycling of L-citrulline to L-arginine has been demonstrated in nitrergic neurons, we now investigated the role of this recycling mechanism in the iNANC relaxation of guinea pig trachea. Inhibition of ASS by αMDLA, resulting in reduced conversion of L-citrulline to L-argininosuccinate, as well as inhibition of ASL by succinate, resulting in
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reduced conversion of L-argininosuccinate to L-arginine, did not affect iNANC relaxations under basal conditions. Furthermore, incubation with exogenous L-citrulline did not affect iNANC relaxations either, indicating that L-citrulline is not a limiting factor. Taken together, these data suggest that L-citrulline recycling to L-arginine does not play a significant role in maintaining NO generation under basal conditions.

By contrast, L-citrulline fully reversed the inhibitory effect of the NOS inhibitor L-NNA, particularly at the lower frequencies, i.e. the frequencies at which NO is the most predominant neurotransmitter. This finding is in line with a previous study in guinea pig tracheal preparations using a single frequency of 8 Hz [18]. Moreover, similar results were found in human bronchus [18]. In the present study, the normalizing effect by L-citrulline was significantly reversed by the ASL inhibitor succinate as well as the ASS inhibitor αMDLA, indicating that the L-citrulline/L-arginine cycle is present in iNANC neurons of the airways indeed and comes into play under conditions of limited L-arginine availability to nNOS.

Outside the airways, L-citrulline has been found to revert the inhibitory effect of L-NNA on EFS-induced relaxation in canine [16] and murine proximal colon [26], opossum internal anal sphincter [13,14] and in rat gastric fundus [15]. In these preparations, basal EFS-induced relaxations were not affected by exogenous L-citrulline either, while the inhibitory effect of the NOS inhibitors were reverted by L-citrulline. The tight relationship between NOS activity and ASS was supported by the finding that ASS (and ASL) are colocalized with nNOS in canine enteric neurons [16,17], as well as in neurons of the myenteric plexus and nerve fibers of the circular and longitudinal smooth muscle of the rat gastric fundus [15]. The hypothesis that the L-citrulline/L-arginine cycle may be important to support NO synthesis in maintaining cellular function is also supported by findings that iNOS and ASS are coinduced by LPS or cytokines in various cell types, including neuronal cells [27], macrophages [28] and whole lung [28,29]. In addition, transfection of bovine pulmonary endothelial cells with iNOS causes increased ASS activity and NO production [30]. Furthermore, in rat vascular smooth muscle cells transfected with ASS, NO production was markedly increased as compared to untransfected cells after treatment with a combination of LPS and interferon-γ [31]. In rat aortic rings contracted with phenylephrine, L-citrulline concentration-dependently induced relaxation due to increased NO production, which effect was increased after treatment with LPS [32]. Taken together, these data indicate that ASS is importantly involved in regulating the substrate availability to iNOS.

Since after the EAR a deficiency of L-arginine underlies the reduced iNANC relaxation induced by nNOS [21][Chapter 7], we also investigated the role of L-citrulline recycling in the iNANC response at 6 h after allergen challenge of OA-sensitized guinea pigs. Remarkably, the impaired iNANC relaxations after allergen challenge were fully reversed by exogenous L-citrulline, as was previously also demonstrated with exogenous L-arginine [21][Chapter 7]. The full reversal of the
attenuated iNANC response by L-citrulline indicates that the L-arginine deficiency of
the neurones does not involve an enzymatic dysfunction in the citrulline/arginine
cycle, but is rather due to arginase activity, which is strongly increased after the EAR
already [21][Chapter 7]. In our guinea pig model of asthma, we have also
established that increased arginase activity contributes to airway
hyperresponsiveness after the EAR by causing a deficiency of contractile agonist-
induced, cNOS-derived NO [22][Chapter 2]. A deficiency of cNOS-derived NO
contributing to airway hyperresponsiveness has also been observed in patients with
severe asthma [33], and may also be induced by allergen exposure [34].
Interestingly, decreased plasma levels of L-arginine have been observed in
asthmatic patients during exacerbations, while serum arginase activity was
increased [35], which supports the hypothesis that a disturbed L-arginine
homeostasis due to increased arginase activity underlies airway
hyperresponsiveness in allergic asthma [20].

Since limitation of L-arginine may be importantly involved in the pathogenesis of
allergic asthma, the effects of oral L-arginine treatment have been studied. However,
oral administration of L-arginine failed to reduce airway hyperresponsiveness in a
murine model of allergic asthma [36] or in asthmatic patients [37]. This might at least
partially be explained by the fact that orally administered L-arginine is largely taken
up by the liver and metabolized to urea. Since L-citrulline is not taken up by the liver
from the portal circulation but is metabolized in the kidney to L-arginine,
administration of L-citrulline could be considered as a masked form of L-arginine
bypassing the liver [12]. Indeed, in a pilot phase II clinical trial in patients suffering
from sickle cell disease, oral administration of L-citrulline raised the decreased
plasma levels of L-arginine, without side effects or toxicity [38].

In conclusion, the L-citrulline/L-arginine cycle is active in guinea pig tracheal
smooth muscle and may particularly be effective under conditions of low L-arginine
availability to NOS, e.g. after allergen challenge or in the presence of NOS inhibitors,
thus acting as a rescue mechanism to maintain cellular function. However, recycling
of L-citrulline to L-arginine does not appear to play a significant role in iNANC nerve-
induced airway relaxation under basal conditions. Moreover, enzymatic dysfunction
in the L-citrulline/L-arginine cycle does not appear to be involved in the L-arginine
limitation after the EAR.

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