

Role of new agents affecting NO/cGMP pathway on ovalbumin-sensitized guinea pig trachea

Ahmet Parlak,¹ Sahin Yildirim,² Ihsan Bagcivan,² and Nedim Durmus³

¹Department of Pharmacy, Okmeydani Training and Research Hospital, Istanbul, Turkey

²Department of Pharmacology, Cumhuriyet University School of Medicine, Sivas, Turkey

³General Directorate of Pharmacy and Pharmaceuticals, Ministry of Health, Ankara, Turkey

ABSTRACT

Asthma is a chronic inflammatory disease in which cell components play important roles. We aimed to evaluate the effects of NO/cGMP cleavage at trachea preparations isolated from ovalbumin-sensitized guinea pigs *in vitro*. Trachea rings were exposed to 3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC-12), (±)-(E)-4-ethyl-2-[(Z)-hydroxyimino]-5-nitro-3-hexen-1-yl-nicotinamide (NOR-4), 2-(2-methylpyridin-4-yl)methyl-4-(3,4,5-trimethoxyphenyl)-8-(pyrimidin-2-yl) methoxy-1,2-dihydro-1-oxo-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride (T-0156), and electrical field stimulation (EFS). cGMP levels in trachea tissues were also measured. The relaxation responses of NOC-12, NOR-4, T-0156, and EFS were significantly decreased at ovalbumin-sensitized group. Nitric oxide (NO) donors significantly decreased the relaxation responses in the presence of 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ). L-Nitro-Arginine Methyl Ester (L-NAME) significantly decreased the EFS relaxation responses in both groups (experimental group and control group), but this effect was reversed by L-Arginine addition. In the experimental group, cGMP levels after EFS, carbachol, NOC-12, NOR-4, and T-0156 exposure were significantly lower than control group. In both groups, cGMP levels after NO donors' exposure were significantly lower in the presence of ODQ and the cGMP levels after EFS + L-NAME were significantly lower than EFS alone. These results may show the increased formation of NO because of the increased iNOS activity in airway sensitization leading to the inhibition of cNOS resulting in the decrease of endogenous NO and decrease of activation of guanylyl cyclase.

KEYWORDS guinea pig, *in vitro*, NO/cGMP, ovalbumin, trachea

INTRODUCTION

Asthma, which is characterized by airway inflammation and hyperresponsiveness [1, 2], is a common disease that is rising in prevalence worldwide with the highest prevalence in industrialized countries. It is estimated that nearly 300 million people in the world have asthma [1, 3]. Because of the increasing prevalence of asthma over the years, the definition, insights into, and the pathophysiology and treatment modalities of this disease are important topics of studies. Different animal models are used for various types of allergic diseases such as bronchial asthma. Guinea pigs are often used as the models of allergic diseases

because their respiratory system shows similar immunological sensitivity and develops pathophysiological symptoms similar to bronchial asthma in humans [4, 5].

Nitric oxide (NO), an important immunomodulatory mediator of inflammatory responses in the lung, is enzymatically synthesized from L-Arginine through the action of NO synthase (NOS) isoforms, which are derived from different genes [1–3, 6]. Several studies have indicated that both under physiological and pathophysiological conditions, NO production and airway responsiveness are tightly controlled by the availability of L-Arginine to NOS [7–9]. L-Arginine is a semiessential amino acid, which is taken up by the diet and may be endogenously produced from L-Citrulline by the intestinal mucosa as well as by some NO-producing cells [9]. The concentration of NO in exhaled air is increased in asthmatic patients and is thought to reflect the state of airway inflammation

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Address correspondence to Nedim Durmus, Department of Risk Management, Medicines and Medical Devices Agency, Ministry of Health of Turkey, Cankaya, Ankara, Turkey. E-mail: drnedimdurmus@gmail.com

[10, 11]. The basal NO production by constitutive NOS (cNOS) shows protective effect against bronchoconstrictor stimulus whereas excessive NO produced by inflammatory NOS (iNOS) shows controversial effect [12]. Although Singh et al. showed that treatment with a selective iNOS inhibitor did not affect allergen-induced allergen airway hyperresponsiveness in asthmatic patients and levels of exhaled NO were markedly reduced [13], the benefit or harm of NO in asthma is not clear yet.

In this study, we aimed to evaluate the role of NO/cGMP pathway in trachea preparations of guinea pigs sensitized with ovalbumin, which is a model of allergic airway disease.

MATERIALS AND METHODS

Sensitization of Guinea Pigs

Male guinea pigs, weighing 280–330 g, were randomly allocated to two experimental groups (experimental group and control group) each consisting of 10 animals. The animal's weights were recorded and individually placed in plastic cages in a temperature-controlled room ($22^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$) in which a 12–12-hour light–dark cycle was maintained (07:00–19:00-hour light). The animals were provided food and water ad libitum. Guinea pigs ($n = 10$) in experimental groups were sensitized by i.m. injections of 0.30 mL of a 5% (w/v) ovalbumin/saline solution into each thigh (0.6 mL total) on days 1 and 4. Guinea pigs ($n = 10$) in control group received i.m. injections of 0.30 mL of saline solution into each thigh (0.6 mL total) on days 1 and 4 as placebo. The guinea pigs were ready for use after day 25 [14]. The local ethical committee for animal experimentation of the Cumhuriyet University Medical Faculty approved all protocols described in this study.

Experimental Procedures

Guinea pigs were killed by decapitation, the trachea was removed rapidly, and transverse rings (3 mm long) were cut and then mounted in thermostatically controlled (37°C) 10 mL organ baths. The organ baths contained Krebs–Henseleit solution in composition of (in mmol/L): NaCl, 120; KCl, 4.6; MgSO_4 , 1.2; NaHCO_3 , 22; NaH_2PO_4 , 1.2; CaCl_2 , 2.5; and glucose, 11.5. The pH of the solution was 7.4 by bubbling with 5% CO_2 and 95% O_2 . Isometric tension was continuously measured with a force transducer (Grass FT03, Quincy, MA, USA). The tissues were stretched initially to a tension of 1 g for 30 minutes and thereafter maintained for 60 minutes under

a resting tension of 0.5 g, which was found to be optimal for measuring the changes in tension. The preparations were washed with Krebs–Henseleit solution every 15 minutes during the equilibration period.

Isometric Measurements on Isolated Tracheal Rings

At the beginning of each experiment, 80 mM KCl was added to the bath, and the contraction was considered as reference response. At the end of the experiment, the contraction response to 80 mM KCl was measured again. The contraction responses to carbachol (10^{-6} M) were calculated as a percentage of the contraction caused by KCl (80 mM) on the isolated trachea preparations from control and ovalbumin-sensitized guinea pigs.

We tested the effects of NO donors, NOC-12, which releases NO under physiological conditions without a cofactor with a half-life of 327 minutes, at 10^{-8} – 10^{-3} M concentrations and NOR-4, which releases NO spontaneously in a rate-controlled manner with a half-life of 60 minutes, at 10^{-8} – 10^{-3} M concentrations, on the isolated trachea preparations from control and ovalbumin-sensitized guinea pigs precontracted with submaximal dose of carbachol (10^{-6} M) in the presence or absence of a guanylate cyclase inhibitor 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) (10^{-5} M). Additionally, we also tested the effects of a phosphodiesterase-5 inhibitor, T-0156 (10^{-9} – 10^{-4} M) on the trachea preparations, precontracted with 10^{-6} M carbachol. After addition of each dose, we waited until a plateau response was obtained before adding the next one. All agents were added in a cumulative manner into the organ bath. At the end of the experiment, papaverine (10^{-4} M), a nonspecific phosphodiesterase inhibitor, was added to the organ bath to induce a maximal relaxation as an internal reference to show the unimpaired tissue [15].

Electrical Field Stimulation-Induced Relaxation Experiments

Two parallel platinum electrodes provided stimulation for tracheal preparations from both control and ovalbumin-sensitized guinea pigs. Sequential frequencies of 1–64 Hz, as square-wave pulses of 60 V (0.5 ms), were delivered at 10-second intervals by a current amplifier and a stimulator (S88; Grass Instruments, MA, USA) in the presence or absence of L-Nitro-Arginine Methyl Ester (L-NAME) (3×10^{-5} M) and L-Arginine (3×10^{-5} M). The preparations were allowed to return to their baseline tension between the tests at each frequency. We waited for 30 minutes in order to see the effect of L-NAME and

L-Arginine. The relaxation responses were calculated as a percentage of the contraction caused by 10^{-6} M carbachol. All measurements were performed in triplicate.

Measurement of cGMP Levels in Trachea Preparations

The isolated trachea preparations were mounted in tissue baths, equilibrated in Krebs' solution (composition as above), and continuously gassed with a mixture of 95% O_2 and 5% CO_2 at $37^\circ C$ (pH 7.4) for 60 minutes. Fourteen sets of experimental studies were performed. In the first and second sets, control experiments were performed without adding any of the study drugs. Tissues were exposed to 10^{-6} M carbachol for 15 minutes in the third and the fourth sets. In the fifth, sixth, seventh, and eighth sets, tissues were separately exposed to 10^{-4} M NOC-12 (control and ovalbumin-sensitized) and 10^{-5} M ODQ + 10^{-4} M NOC-12 (control and ovalbumin-sensitized) after incubation with 10^{-6} M carbachol for 15 minutes in a tissue bath. In the 9th, 10th, 11th, and 12th sets, tissues were separately exposed to 10^{-4} M NOR-4 (control and ovalbumin-sensitized) and 10^{-5} M ODQ + 10^{-4} M NOR-4 (control and ovalbumin-sensitized) after incubation with 10^{-6} M carbachol for 15 minutes in a tissue bath. In the thirteenth and fourteenth sets, tissues were separately exposed to 10^{-4} M T-0156 (control and ovalbumin-sensitized) after incubation with 10^{-6} M carbachol for 15 minutes in a tissue bath. At the end of a total incubation period, the samples were immediately transferred into liquid nitrogen, homogenized with ethanol, and incubated for 30 minutes at $0^\circ C$ – $4^\circ C$. The homogenate was then centrifuged at $4^\circ C$ for 10 minutes at 12,000 g. The supernatants were poured into a clean test tube and dried in vacuum at $50^\circ C$ to remove ethanol. Aliquots of the supernatant were tested for cyclic GMP by ELISA (ParameterTM, Cyclic GMP Assay, Catalog Number KGE003). The samples were then processed according to the instructions provided with the kits for determination of cGMP levels. Sampling data were divided according to the weight of the isolated trachea preparations, and the results were expressed in fmol/mg trachea preparations.

Drugs

The following drugs were obtained from Sigma Chemical Co. (St. Louis, MO, USA): carbachol chloride, 3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC-12), ($\pm$)-(E)-4-ethyl-2-[(Z)-hydroxyimino]-5-nitro-3-hexen-1-yl-nicotinamide (NOR-4), 2-(2-methylpyridin-4-yl)methyl-4-(3,4,5-

trimethoxyphenyl)-8-(pyrimidin-2-yl) methoxy-1,2-dihydro-1-oxo-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride (T-0156), ODQ, L-NAME, and L-Arginine. All drugs were dissolved in distilled water except ODQ, which was dissolved in dimethylsulfoxide and then diluted with distilled water for preparation of decreasing concentrations of this agent. The amount of dimethylsulfoxide used to dissolve the agent had no effect on tracheal preparations. Drug-containing solutions were freshly prepared on the day of the experiments.

Data Analysis

The effects of cumulative concentrations of agents on carbachol-induced contractions in the absence or presence of antagonists were measured, and values for $-\log_{10}$ EC₅₀ (pD₂) and mean maximal inhibition (E_{max}) were compared. Maximal inhibitor effects were calculated for each concentration–response curve. The EC₅₀ value represents 50% of the maximal inhibitor effect. The EC₅₀ values were calculated by linear regression of the probit of response versus $\log_{10}$ molar concentration for agents. Experimental values were presented as means $\pm$ SEM and analyzed by Mann–Whitney U-test and Wilcoxon test and a *t*-test when appropriate. A *P* value of $<.05$ was considered to indicate significance. All statistical analyses were performed by using SPSS ver. 14.0.

RESULTS

Isometric Studies

The contractions elicited by carbachol (10^{-8} – 10^{-4} M) were not significantly different in the ovalbumin-sensitized group from that in the control group (Figure 1) ($n = 10$, for each group).

In the control group, NOC-12 and NOR-4 produced concentration-dependent relaxation at only 10^{-6} – 10^{-3} and 10^{-5} – 10^{-3} concentrations, respectively, on isolated trachea preparations precontracted by carbachol (10^{-6} M) and in the presence of ODQ (10^{-5} M), this relaxation was significantly decreased ($P < .05$). The relaxation response to NOC-12 and NOR-4 significantly decreased in ovalbumin-sensitized group compared with control group and in the presence of ODQ, these responses were almost abolished (Figure 2A and B).

T-0156 produced concentration-dependent relaxation at only 10^{-8} – 10^{-4} concentrations on isolated trachea preparations precontracted by carbachol (10^{-6} M) in the control group and this

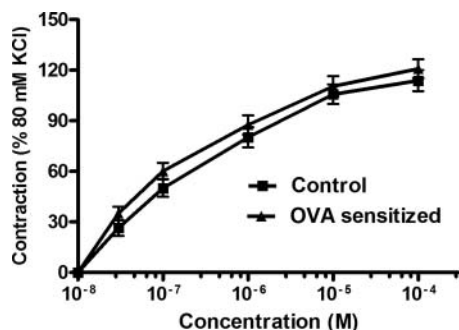


FIGURE 1. The effects of carbachol (10^{-8} – 10^{-4} M) in the isolated trachea preparations from control and ovalbumin-sensitized guinea pigs. Contraction responses are expressed as a percentage of 80 mM KCl-induced contraction and shown as means $\pm$ SEM ($n = 10$).

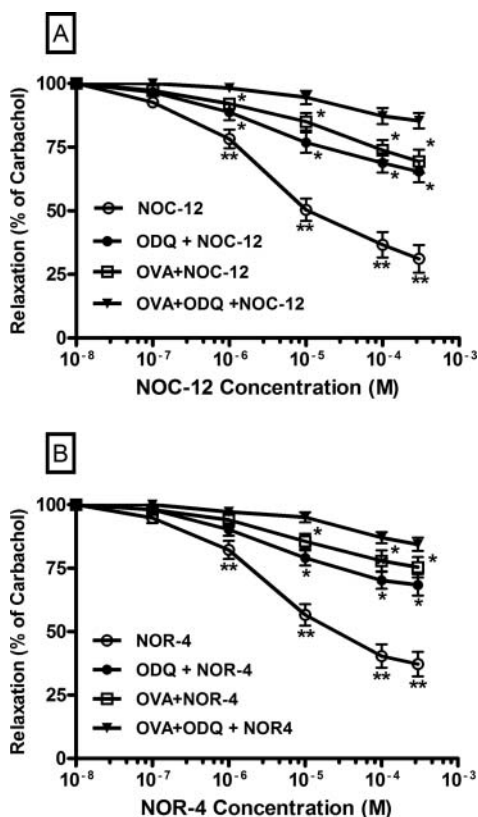


FIGURE 2. The effects of NOC-12 (10^{-8} – 10^{-3}) and NOR-4 (10^{-8} – 10^{-3}) in the isolated trachea preparations from control and ovalbumin-sensitized guinea pigs in the presence and absence of ODQ (10^{-5} M). Relaxation responses are expressed as a percentage of carbachol-induced contraction and shown as means $\pm$ SEM ($n = 10$ in each drug). * $P < .05$, statistically different from ODQ + NOC-12 (A) or ODQ + NOR-4 (B) in ovalbumin-sensitized group. ** $P < .05$, statistically different from all groups.

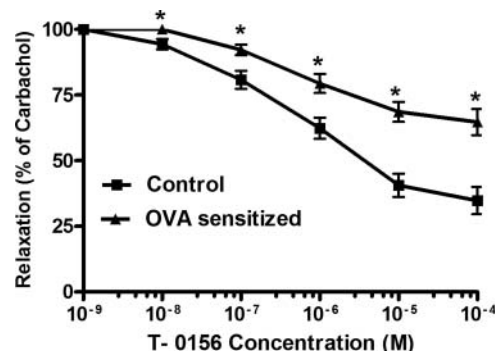


FIGURE 3. The effects of T-0156 (10^{-9} – 10^{-4} M) in the isolated trachea preparations from control and ovalbumin-sensitized guinea pigs. Relaxation responses are expressed as a percentage of carbachol-induced contraction and shown as means $\pm$ SEM ($n = 10$ in each drug). * $P < .05$, statistically different from control group.

relaxation was significantly decreased in the ovalbumin-sensitized group (Figure 3).

Table 1 presents values for $E_{\max}$ (% of 10^{-6} M carbachol) and pD2 values ($-\log EC_{50}$) for

TABLE 1 $E_{\max}$ (% of 10^{-6} M Carbachol) and pD2 Values ($-\log EC_{50}$) for NOC-12 and NOR-4 in the Absence or Presence of ODQ, T-0156, and EFS in the Presence of L-NAME or L-NAME + L-Arginine in Trachea Preparations from Control and Ovalbumin-Sensitized Guinea Pigs.

	Control	Ovalbumin-sensitized
NOC-12		
$E_{\max}$	68.9 ± 6.4	30.6 ± 2.8^a
pD2	5.54 ± 0.06	5.20 ± 0.08
ODQ + NOC-12		
$E_{\max}$	34.6 ± 3.6^b	14.6 ± 2.7^b
pD2	5.44 ± 0.04	4.95 ± 0.03
NOR-4		
$E_{\max}$	62.8 ± 5.8	24.6 ± 3.9^a
pD2	5.50 ± 0.06	5.10 ± 0.08
ODQ + NOR-4		
$E_{\max}$	31.6 ± 4.1^b	15.6 ± 2.8^b
pD2	5.52 ± 0.09	5.12 ± 0.07
T-0156		
$E_{\max}$	65.2 ± 3.9	35.3 ± 4.7^a
pD2	6.14 ± 0.08	6.20 ± 0.06
EFS		
$E_{\max}$	42.8 ± 4.5	26.2 ± 4.2^a
L-NAME + EFS		
$E_{\max}$	26.6 ± 3.9^c	12.2 ± 2.1^c
L-NAME + EFS + L-Arginine		
$E_{\max}$	38.4 ± 3.4	24.0 ± 2.6

Notes. Data were expressed as means $\pm$ SEM ($n = 10$, in each group). ^a $P < .05$, statistically different from $E_{\max}$ values of control group. ^b $P < .05$, statistically different from $E_{\max}$ values of without ODQ in both groups. ^c $P < .05$, statistically different from $E_{\max}$ values of without L-NAME in both groups.

NOC-12 and NOR-4 in the absence or presence of ODF and T-0156 and electrical field stimulation (EFS) in the presence of L-NAME or L-NAME + L-Arginine in trachea preparations from control and ovalbumin-sensitized guinea pigs.

The relaxation response elicited by electrical field stimulation was significantly decreased in ovalbumin-sensitized group compared with the control group ($P < .05$) (Figure 4A). In both groups, L-NAME significantly decreased relaxation responses to EFS but addition of L-Arginine after L-NAME reversed this decrease. (Figure 4B and C).

At the end of all the experiments, papaverine (10^{-4} M) induced a maximal relaxation for all preparations showing the normal relaxation function of the tissues.

cGMP Levels in Trachea Preparation

Table 2 presents values of significant decrease in cGMP levels in isolated trachea preparations from ovalbumin-sensitized guinea pigs in the presence of none of the agents, NOC-12, NOR-4, and T-0156 compared with control group ($P < .05$). ODF significantly decreased the cGMP levels compared with the absence of this inhibitor in both of the control and ovalbumin-sensitized groups ($P < .05$). In EFS group, the cGMP level was significantly decreased in ovalbumin-sensitized group, but in the presence of L-NAME, the amount of cGMP was significantly decreased in both control and ovalbumin-sensitized groups. On the other hand, the addition of L-Arginine reversed the effect of L-NAME and there was only a significant difference between control and ovalbumin-sensitized groups ($P < .05$).

DISCUSSION

Bronchial asthma is a chronic airway inflammatory disorder, characterized by airway infiltration of inflammatory cells such as eosinophil and T-lymphocyte, which are involved in a network of various cytokines [16] and contribute to bronchial hyperresponsiveness and structural changes [17] like goblet cell hyperplasia/hypertrophy [18], subepithelial collagen deposition and smooth muscle hyperplasia/hypertrophy, and progressive airflow obstruction. Airway hyperresponsiveness is a well-established characteristic of allergic asthma and is believed to be the result of airway inflammation as well as epithelial damage [19, 20].

NO is synthesized by NOS enzyme during the formation of L-Citrulline from L-Arginine. There are two isoforms of NOS as cNOS (endothelial NOS and

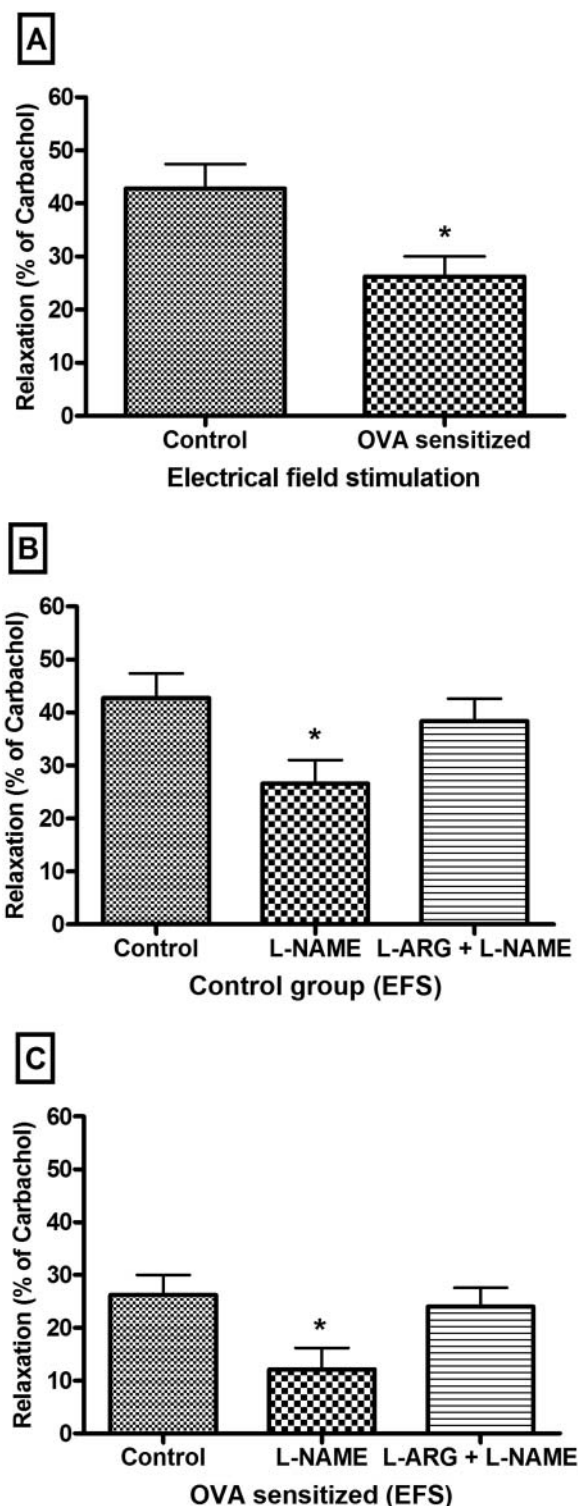


FIGURE 4. The relaxation response elicited by electrical field stimulation in both control and ovalbumin-sensitized group in the presence of L-NAME and L-Arginine + L-NAME. * $P < .05$, statistically different from all other groups.

TABLE 2 The cGMP Levels in Absence or Presence of Different Agents or Antagonists in Isolated Trachea Preparations from Control and Ovalbumin-Sensitized Guinea Pigs.

	Control (<i>n</i> = 10)	Ovalbumin- sensitized (<i>n</i> = 10)
No agent	103.36 ± 29.26	73.09 ± 7.33 ^a
Carbachol	109.53 ± 25.50	86.97 ± 23.12 ^a
NOC-12	217.11 ± 30.41	154.04 ± 21.08 ^a
ODQ + NOC-12	121.43 ± 7.39 ^b	73.08 ± 13.89 ^b
NOR-4	208.28 ± 34.90	121.17 ± 8.01 ^a
ODQ + NOR-4	123.38 ± 10.03 ^b	63.30 ± 15.95 ^b
T-0156	160.20 ± 44.33	84.69 ± 22.53 ^a
EFS	132.75 ± 24.73	82.96 ± 6.22 ^a
L-NAME + EFS	97.37 ± 17.28 ^c	41.26 ± 3.58 ^c
L-NAME + EFS + L-Arginine	126.11 ± 12.43	75.67 ± 8.34 ^a

Notes. cGMP level is expressed in fmol/mg protein. Data were expressed as means + SEM (*n* = 10 in each group). ^a*P* < .05, statistically different from control group. ^b*P* < .05, statistically different from without ODQ in both groups. ^c*P* < .05, statistically different from without L-NAME in both groups.

neuronal NOS) and iNOS. The synthesis duration of cNOS is short, the amount of produced NO is low, and it is calcium-dependent. However, iNOS produces simultaneously more NO independent of calcium from macrophages and other inflammatory cells in response to the endotoxin or different cytokines. NO produce relaxations via cGMP, which is abolished by phosphodiesterase enzyme systems. While the amount of peroxynitrite formed from the NO produced by cNOS is negligible, it has been shown that the excessive amount of NOS produced by iNOS may lead to tissue damage in lungs [21, 22].

There are several *in vivo* and *in vitro* studies investigating the effects of NO in respiratory system disorders. It has been shown that the amount of iNOS enzyme and NO levels have been increased in the airways of asthmatic patients [23]. Koarai et al. showed that the hypersensitivity of airways completely disappeared and the eosinophil infiltration significantly decreased by 1400W, a specific inhibitor of iNOS enzyme. They concluded that the NO produced by iNOS enzyme was responsible from the airway hypersensitivity and eosinophil infiltration in asthmatic patients and specific inhibition of this enzyme may be useful in asthma [24]. The clear relationship between NO and airway hypersensitivity and the benefit/harm balance has not been clearly understood. The basal amount of NO produced by cNOS enzyme has a protective effect against bronchoconstriction, but the excessive amount of NO produced by iNOS seems to have contrary effects [12].

Immunohistochemical studies showed that nNOS has been localized in the airway nerves of the guinea

pigs and human. These nerves are localized in the vessels, smooth muscles, and lamina propria of respiratory system [25]. Maarsingh et al. showed that the decreased NO produced by nNOS enzyme after early asthmatic reaction leads to the significant decrease of iNANC (nonadrenergic, noncholinergic) relaxation responses in a guinea pig airway sensitization model [26]. Vaali et al. showed the inhibition of relaxation responses of NO donors by ODQ, a soluble guanylate cyclase inhibitor, in guinea pig trachea preparations *in vitro* [27]. In another study by Tuncel et al., zaprinast, a cGMP-specific type 5 phosphodiesterase inhibitor, increased the NANC relaxations responses of by EFS and NO donors, SIN-1 and sodium nitroprussiate. They concluded that NO may be a mediator in NANC responses and phosphodiesterase-5 inhibition may increase the NO-mediated relaxation responses [28].

In the present study, the relaxation responses of NOC-12 and NOR-4, T-0156, and EFS were significantly decreased at ovalbumin-sensitized group than control group. The relaxation responses of NO donors were significantly decreased in the presence of ODQ. L-NAME significantly decreased the EFS relaxation responses in both groups but this effect was reversed by L-Arginine addition. In ovalbumin-sensitized group, cGMP levels after EFS, carbachol, NOC-12, NOR-4, and T-0156 exposure were significantly less than control group. In both groups, cGMP levels after NO donors' exposure were significantly lower in the presence of ODQ than its absence. Separately, in both groups, the cGMP levels after EFS + LNAME were significantly less than EFS alone.

The decrease of relaxation responses in the ovalbumin-sensitized group shows the deterioration of NO/cGMP pathway. The decrease of relaxation responses and cGMP levels by NO donors in the presence of ODQ supports the knowledge of their relaxation mechanism by cGMP increasing effect through guanylyl cyclase activation. The decrease of both relaxation responses and cGMP levels in the ovalbumin-sensitized group may be due to the guanylyl cyclase insensitivity and inhibition of cNOS by the negative feedback effect of increased activation of iNOS in the inflammatory asthma. Similarly, the relaxation responses of T-0156 in both groups and the decreased effects of T-0156 in the experimental group shows the relaxation mechanism of T-0156 by cGMP and the deterioration of this mechanism in the experimental group. As per our knowledge, EFS induces the nNOS and shows the effects of this NOS isoform, the decrease of EFS relaxation responses in both groups by L-NAME and the reversal of this effect by L-Arginine addition support the possible guanylyl cyclase insensitivity and inhibition of nNOS by the

negative feedback of increased activation of iNOS in the inflammatory asthma.

CONCLUSIONS

Agents acting through NO/cGMP pathway may be adjunctive therapy to the bronchodilators in current use by allowing lower doses of each individual agent to be used. Furthermore, NO may also protect airway smooth muscle against airway remodeling, which is an important component of airway thickening in asthma by inhibiting its proliferation besides its relaxant effect. The further studies evaluating the effects of new iNOS enzyme inhibitors and measuring the guanylate cyclase enzyme activity and level may be useful for clarifying the pathophysiology of asthma and discovering the clinical benefits of new anti-asthma therapies that could be used in conjunction with existing drugs.

Declaration of interest: The authors report no conflicts of interest.

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