Role of nitric oxide in hyperpnea-induced bronchoconstriction and airway microvascular permeability in guinea pigs

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QUERY SHEET

Q1. Au: Mehta in listing but Metha throughout text. Which is correct?
Q2. Au: Please define Ptr here and Ers below.
Q3. Au: ". . .by correcting. . ., total airway tissue weight and relating. . .." Missing word before "total airway tissue weight"?
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Q5. Au: What legend in Figure 1 are you referring to?
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ABSTRACT

The present study aimed to evaluate the role of nitric oxide (NO) on hyperpnea-induced bronchoconstriction (HIB) and airway microvascular hyperpermeability (AMP). Sixty-four guinea pigs were anesthetized, tracheotomized, cannulated, and connected to animal ventilator to obtain pulmonary baseline respiratory system resistance (Rrs). Animals were then submitted to 5 minutes hyperpnea and Rrs was evaluated during 15 minutes after hyperpnea. AMP was evaluated by Evans blue dye (25 mg/kg) extravasation in airway tissues. Constitutive and inductible NO was evaluated by pretreating animals with N^G-nitro-l-arginine methyl ester (l-NAME) (50 mg/kg), aminoguanidine (AG) (50 mg/kg), and l-arginine (100 mg/kg) and exhaled NO (NOex) was evaluated before and after drug administration and hyperpnea. The results show that l-NAME potentiated (57%) HIB and this effect was totally reversed by l-arginine pretreatment, whereas AG did not have effect on HIB. l-NAME decreased basal AMP (48%), but neither l-NAME nor AG had any effect on hyperpnea-induced AMP. NOex levels were decreased by 50% with l-NAME, effect that was reversed by l-arginine treatment. These results suggest that constitutive but not inducible NO could have a bronchoprotective effect on HIB in guinea pigs. The authors also observed that neither constitutive nor inducible NO seems to have any effect on hyperpnea-induced AMP.

KEYWORDS: aminoguanidine; bronchoconstriction; hyperpnea; l-arginine; l-NAME; nitric oxide

Exercise, hyperventilation, and exposure to frigid air cause a transient increase in pulmonary resistance in approximately 70% to 80% of patients with asthma [1] as well as in many species [2–4]. In both humans and animals for a fixed level of ventilation, colder and dryer inspired air exacerbates this response, whereas warmer and more humidified air reduces its severity [2, 5, 6].

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Nitric oxide (NO) is synthesized by a family of NO synthases (NOSs) that are found in a variety of cell types, such as epithelial, endothelial, macrophages, fibroblasts, neutrophils, and in smooth muscle [7–11]. This endogenously produced NO is thought to be an important modulator of airway function in the basal state [12–15] and modulates basal airway microvascular permeability (AMP) [16].

Previous studies have yielded conflicting results on the role of NO in modulating airway function. ~~Metha~~ and colleagues found an increase in bronchoconstriction during histamine-induced bronchoprovocation after inhibiting NO production with N^G-nitro-L-arginine methyl ester (L-NAME) in guinea pigs [17].

TABLE 1 Description of Studied Groups

		Drug treatment			Evaluated parameters
Groups	HP	L-NAME	AG	L-ARGININE	Rrs/NOEX/AMP
Control	−	−	−	−	+
Control + L-NAME	−	+	−	−	+
Control + AG	−	−	+	−	+
Control + L-arginine	−	+	−	+	+
HP	+	−	−	−	+
HP + L-NAME	+	+	−	−	+
HP + AG	+	−	+	−	+
HP + L-arginine	+	+	−	+	+

Note. Control: animals not submitted to hyperpnea; HP: animals submitted to hyperpnea; L-NAME: N^G-nitro-L-arginine methyl ester; AG: aminoguanidine; Rrs: respiratory resistance; NOex: exhaled nitric oxide; AMP: airway microvascular permeability; (+): yes; (−): no.

In addition, Yoshihara and colleagues found that L-NAME enhanced bronchoconstriction induced by cold air inhalation without hyperpnea (HP) in guinea pigs [4]. Both studies thus suggest that endogenous NO exerted a bronchoprotective effect. In contrast, Nogami and colleagues [18] and Suman and Beck [19] found no effect of NO synthase inhibitor on the increase in pulmonary resistance after a hyperpnea challenge in guines pigs.

It has also been suggested that endogenously produced pulmonary NO have a role in the regulation of airway microvascular permeability (AMP) [20–22]. Recently, ~~Metha~~ and coworkers have shown that endogenous pulmonary NO contributes to basal and antigen-stimulated levels of AMP in guinea pigs and that this NO-dependent activity does not appear to be derived from type II NOS (inducible NO) [23].

Hyperpnea-induced bronchoconstriction (HIB) in guinea pigs seems to share many common features with human exercise-induced bronchoconstricion (EIB) in terms of the time course of onset of constriction, spontaneous resolution, diminution of response with humidification of inspired gas, reproducible on consecutive identical challenge, and the relationship between the amount of hyperpnea and the degree of response elicited [2, 24].

Although there are some evidences suggesting that NO might have effects on vascular permeability and bronchoconstriction, its effect remains poorly known. Thus we tested the hypothesis that NO could modulates AMP and bronchoconstriction using HIB in guinea pigs that present both phenomena.

METHODS

Animals

Sixty-four male Hartley guinea pigs (250 to 400 g) were randomly assigned to 1 of 8 groups defined by presence of hyperpnea and type of drug administration (Table 1). The animals were housed in conditions of a constant temperature and relative humidity and were fed a standard guinea pig diet. Animals were kept from all evidences of infectious diseases. All animals received humane care in compliance with the Helsinki convention for the use and care of animals. The study was approved by the Institutional Review Board of the School of Medicine of the University of Sao Paulo.

Animal preparation

We anesthetized each animal with pentobarbital sodium (30 mg/kg) injected intraperitoneally. The maintance of anesthesia was reached through additional doses of pentobarbital sodium (3 mg/kg) each 30 minutes. Once anesthetized, animals were intubated with a midline tracheotomy and a catheter was inserted into the left jugular vein for the administration of intravascular drugs. Animals were then attached to a small-animal ventilator (Harvard 683; Harvard Apparatus, South Natick, MA, USA) via a 2-inch silicon rubber tubing (2.0 mm ID) with a Y piece at the end and ventilated with a tidal volume (V_T) of 6.0 ml/kg and respiratory rate (RR) at 70 breaths/min. Three measurements of basal lung mechanics were obtained at room temperature in 1-minute intervals and exhaled NO (Noex) was collected. After that, NO synthase inhibitor was then administered and after 10 minutes animals received propranolol (1 mg/kg) to minimize changes in bronchoconstrictor response resulting from potential changes in levels of circulating catecholamines [2]. No changes in respiratory resistance or dynamic compliance resulted from these infusions. Animals received Evans Blue dye and after 5 minutes a second sample of NOex and another mechanic was obtained. Animals were then submitted to 5 minutes

hyperpnea challenges (tidal volumes of 12 mL/kg at 150 breaths/min) using 5% CO_2–95% O_2 (from compressed gas tanks) as inspired gas. Gas temperature (13°C) was monitored using a bypass system where gas passed in a vaporization camera just before entry of the tracheal tube as we previosly described [6]. After hyperpnea, baseline ventilator settings at room temperature were reset and lung mechanics were recorded during 15 minutes at 1-min interval. At the end, animals were sacrificed by exsanguinations and airway microvascular permeability was quantified.

Measurement of respiratory system resistance

Measurement of respiratory system resistance (Rrs) was performed as we previously described [25, 26]. A pneumotachograph (Fleish-4.0; OEM Medical, Richmond, VA, USA) was connected to the tracheal tube and to a differential pressure transducer (Honeywell 142PCO5D), V' and ~~Ptr~~ signals were measured and conditioned during 10 s at 200 Hz with a 12-bit analog-to-digital converter (DT 01-EZ; Data Translation, Marlboro, MA, USA) and stored in a microcomputer. In each measurement, 10 cycles were analyzed for each data point. Lung volume changes (V) were obtained by electronic integration of the V' signal. Rrs were obtained using the equation of motion of the respiratory system:

$$Ptr = Ers \times V(t) + Rrs \times V'(t) \quad [1]$$

~~where t is time~~.

The resistance (Rrs) was obtained in 3 time points: (1) 5 minutes after the animals were connected to the ventilator (baseline); (2) after 15 minutes of administration of drugs (L-NAME, or aminoguanidine, or L-NAME + L-arginine), i.e., immediately before the induction of hyperpnea; and (3) after 5 minutes of induction of hyperpnea. The Rrs was recorded each 1 minute, during 15 minutes.

Experimental design

Animals were randomly assigned to 2 groups ($n = 32$ for each group) that were submitted or not to hyperpnea. Each group was further divided into 4 subgroups receiving either drug solvent (saline) or treatment with N^G-nitro-L-arginine methyl ester (L-NAME; 50 mg/kg intravenously), or aminoguanidine (AG; 50 mg/kg intravenously), or L-arginine and L-NAME (respectively, 100 and 50 mg/kg intravenously). L-NAME or AG was administered 15 minutes before the hyperpnea challenge and L-arginine was administered 5 minutes after L-NAME administration. Evans blue (20 mg/kg intravenously) was administered 5 minutes before hyperpnea or basal ventilation (animals not submitted to hyperpnea). Exhaled nitric oxide (NOex) was collected 15 minutes and immediately before hyperpnea.

Quantitative analysis of airway microvascular permeability

Vascular permeability was quantified by the extravasation of Evans blue dye as previously described [27], which correlates well with the extravasation of radiolabeled albumin [28]. The tissue content of Evans blue dye after experimental intervention was determined by perfusing the systemic circulation with saline to remove intravascular dye. Fifteen minutes after hyperpnea, the thorax was opened and a blunt-ended, 13-gauge needle was passed through a left ventriculotomy into the aorta. The ventricles were cross-clamped and blood atrium with about 20 mL of saline at 50 mm Hg pressure until the perfusate was clear. The lungs were then removed. The connective tissues, vasculatures, and parenchyma were gently scrapped off, and the airways were divided into 3 components: lower part of the trachea (Tr), main bronchi (MB), and intrapulmonary airways (IPA). The tissues were divided in 2 parts: one part weighed and reweighed 24 hours after drying in 37°C, the other part was used to extract Evans blue dye after 24 hours in formamide (4 mL/g of tissue) at room temperature. The dye concentration was quantified from light absorbance at 620 nm using Labsystems Multiskan Bichromatic (Titertek Multiskan, USA), and was calculated from a standard curve dye in the range of 0.125 to 2.5 μg/mL. Total Evans blue dye was calculated by correcting formamide dilution. ~~Increase in airway microvascular permeability was obtained by correcting Evans blue dye (obtained by spectrophotometry), total airway tissue weight, and relating dry/wet tissue.~~

Exhaled nitric oxide (NOex)

Mixed expired gas was collected before collecting basal lung mechanic (basal NOex) and immediately before hyperpnea (after 15 minutes administration the drugs). Gas was collected during 5 minutes from the expiratory port of the ventilator into a Mylar bag for measurement of expired NO (see below). The intake air for the ventilator was essentially NO free (<1 parts/billion [ppb]) and had an oxygen fraction (FiO_2) of 0.21. The expired gas was vigorously mixed manually for 5 to 10 seconds before determination of the NO concentration by chemiluminescence (NO Analyzer; Sievers, Boulder, CO, USA). The NO

analyzer itself was "zeroed" using the NO absorbent chamber supplied by Sievers and calibrated daily using a reference gas of known concentration.

Statistics

The software Sigma Stat 2.03 (San Juan, CA, USA) was used for statistical analysis and the homogeneity of variance and normality were tested using Kolmogorov-Smirnov test. Descriptive data are reported as mean ± SE. An analysis of variance (ANOVA) was used for comparisons of expired NO concentrations, respiratory system resistance, and airway microvascular permeability. Tukey's *t* test was conducted to compare all measurements and drug groups. To be considered significant, the P value was set as.05.

RESULTS

Effect of nitric oxide (NO) on hyperpnea-induced bronchoconstriction

The time course of the hyperpnea-induced bronchoconstriction (HP) in all groups is presented in Figure 1. Four groups that were not submitted to hyperpnea were used as control groups to observe drug effect and they received saline (drug solvent), aminoguanidine (AG), L-NAME, or L-arginine + L-NAME, and there was no significant difference in basal respiratory resistance among these groups. HP group (animals receiving saline and submitted to hyperpnea) presented an increase of 145% in Rrs compared with all groups that did not receive hyperpnea (Figure 1). When animals received L-NAME pretreatment before hyperpnea, an increase of 100% in respiratory resistance 2 minutes after hyperpnea was observed and this increase was present until the 15th minute after hyperpnea. The increase in respiratory resistance observed by L-NAME treatment was inhibited when a group of animals received L-arginine. AG treatment did not have any effect on hyperpnea-induced bronchoconstriction and respiratory resistance presented similar results as the HP group (respectively, 0.473 and 0.490 cm H_2O/mL/s).

Effect of ventilation and nos inhibitors on airway microvascular permeability

Airway microvascular permeability (AMP) was evaluated in 3 distinct airways tissues: lower trachea and extrapulmonary and intrapulmonary bronchi (respectively, Figure 2A, B, and C). In animals that were not submitted to hyperpnea, it was observed that L-NAME treatment presented a decrease of AMP in trachea (53%), extrapulmonary bronchi (62%) and intrapulmonary bronchi (55%) when compared with animals receiving drug solvent (respectively, Figure 2A, B, and C). Hyperpnea caused substantial increase in AMP all airway tissues when compared to that obtained in animals receiving basal ventilation (Figure 2A, B, and C). Neither L-NAME nor aminoguanidine treatment modified hyperpnea-induced airway microvascular extravasation.

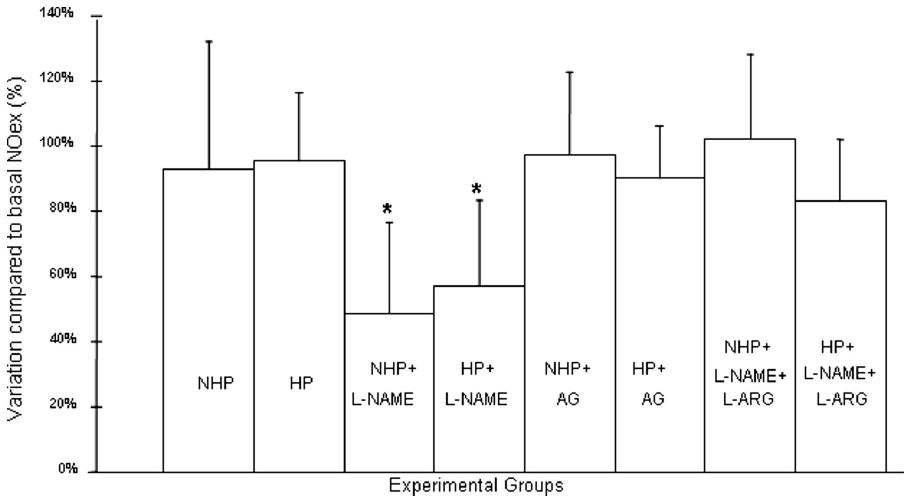

FIGURE 1 Effect of nitric oxide synthase inhibitors NO levels on ~~exhaled~~ air of guinea pigs. First sample was collected before treatment and second sample was collected 15 minutes after treatment. For further details see legends in the ~~figure~~. $^*P < .05$ compared with control and HP groups. Q4

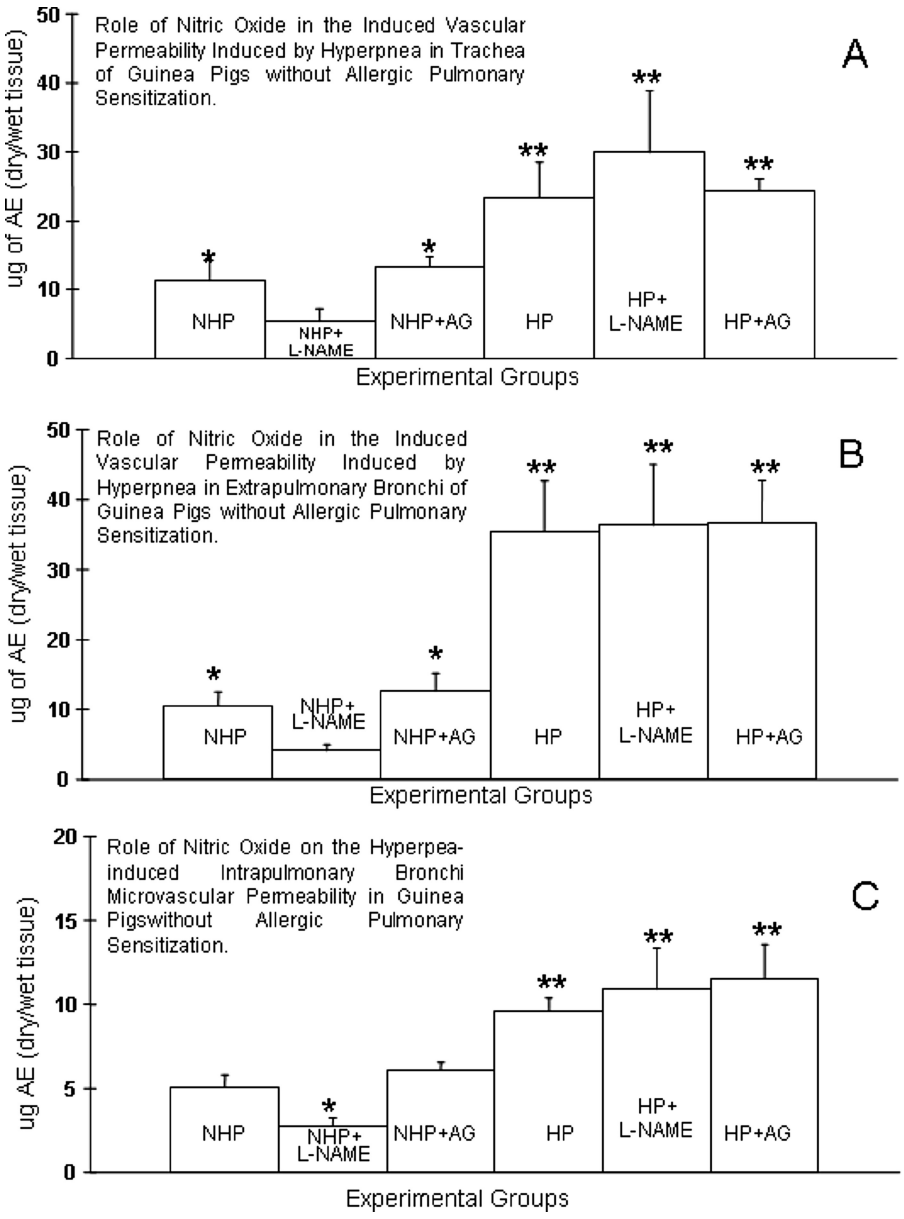

FIGURE 2 Effect of NO on airway-induced microvascular permeability leakage in guinea pigs. (**A**) Lower trachea, (**B**) upper bronchi, and (**C**) lower bronchi. For further details see legends in ~~Figure 1~~. $^{*}P < .05$ compared with control group; $^{**}P < .05$ compared with control groups.

Q5

Exhaled nitric oxide (NOex) levels in guinea pigs

NOex was collected before collecting basal lung mechanic (basal NOex) and immediately before hyperpnea (after 15 minutes administration the drugs). These data are presented in Figure 3. It can be observed that L-NAME, but not aminoguanidine, treatment decreased NOex either in animals submitted or not to hyperpnea and these effect was reversed by L-arginine treatment. In contrast, aminoguanidine did not have any effect on NOex levels.

DISCUSSION

In the present study, we showed that L-NAME, a non-selective nitric oxide synthase (NOS) inhibitor (inhibitor constitutive and inducible NOSs), potentiated hyperpnea-induced bronchoconstriction (HIB) effect that was reversed by L-arginine pretreatment. Treatment with aminoguanidine (AG), an inducible selective NOS inhibitor, did not have any effect on HIB. In addition, we observed that neither L-NAME nor AG have any effect on hyperpnea-induced airway microvascular permeability (AMP).

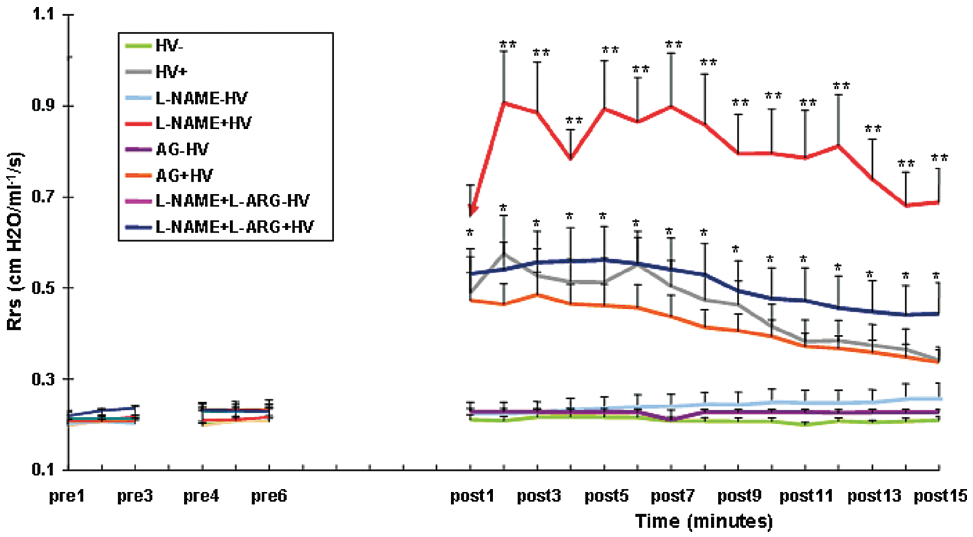

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FIGURE 3 Effect of nitric oxide (NO) on hyperpnea-induced bronchoconstriction in guinea pigs. Data are expressed as mean ± SD. NHP groups were composed of animals not submitted to hyperpnea. HP groups were composed of animals submitted to 5 minutes of hyperpnea. AG = aminoguanidine. Compounds were given 15 minutes before hyperpnea or basal mechanical ventilation. $^{*}P < .05$ compared with control group; $^{**}P < .05$ compared with HP group.

HIB in guinea pigs seems to share many common features with human exercise-induced bronchoconstriction (EIB) in terms of the time course of onset of constriction, spontaneous resolution, diminution of response with humidification of inspired gas, reproducible on consecutive identical challenge, and the relationship between the amount of hyperpnea and the degree of response elicited [2, 24]. Although all these common features between HIB and EIB, we are aware that results obtained in this animal model cannot be transposed to what occurs in human beings.

Endogenously produced NO is thought to have a modulator effect on airway function in the basal state [12, 13, 29] and during the past few years there is accumulating evidence about its role in asthma pathophysiology [30]. NO has also been designated one of the possible candidates to exert bronchodilation during exercise in healthy subjects by inducing smooth muscle relaxation [31]. Functionally, NO can oppose other mediators, such as histamine and leukotrienes, that contribute to the subsequent airway narrowing during and after exercise in asthma [32, 33]. Several studies have suggested that NO have a protective effect on histamine, allergen and cold induced bronchoconstriction [9, 18, 34].

The effect of NO on HIB has been poorly studied. Nogami et al. [18] and Suman and Beck [19] have observed that NO did not have any effect on HIB and our results are contrary with their findings. Looking closely, we observed that our study presented some methodological differences comparing with Nogami's and Suman and Beck's studies in terms of hyperpnea protocol and NOS inhibitors, which could perhaps explain discrepancies among these studies. Nogami and colleagues performed hyperpnea with carbogenic (95% O_2 and 5% CO_2) dry air and animals were pretreated with a smaller dose of L-NAME (8 mg/kg of L-NAME given 1 minute before hyperpnea supplemented with a 2 mg/kg/min dose during the hyperpnea period) [18]. Interesting to note that in this study hyperpnea did not induce a significant change in basal lung resistance, suggesting that hyperpena did not really induced bronchoconstriction. Suman and Beck performed hyperpnea with compressed dry air ($FiO_2 = 0.21$) using a lower respiratory frequency (100 breaths per minute) and tidal volume (8 mL/kg) [19]. Besides, they inhibited nonselective NOS by treating the animals with ~~L-NMMA (50 mg/kg)~~. In our study, we performed hyperpnea with carbogenic cold (13°C) and dry air using a higher respiratory frequency (150 breaths per minute) and tidal volume (12 mL/kg). In our study, nonselective NOS were inhibited by pretreating the animals with L-NAME (50mg/kg). The HIB protocol used in our study has been described by others [2, 24, 35] as well as the dose of L-NAME and AG [4, 23].

Q7

There are evidences suggesting that L-NAME, but not L-NMMA, could have an antimuscarininc effect because it decreases in vitro cholinergic-induced contractility of rabbit coronary artery and canine proximal colon effects that were not reversed by L-arginine treatment [36]. In our study, L-NAME amplified HIB response and this effect was totally reversed by L-arginine treatment. The potentiation of HIB

observed in our study is in agreement with previous results showing that NO could have a bronchoprotective effect on histamine-induced bronchoconstriction [23], antigen-induced hyperresponsiveness [34], and cold-induced bronchoconstriction [4]. Although we cannot explain the difference between our and Suman and Beck's results, we could discard unspecific effects of L-NAME because this effect was by reversed L-arginine. We then suggest that the difference between these two studies could be explained by hyperpnea protocol, mainly to the air temperature during hyperpnea. Yoshihara and coworkers showed a protective effect of NO on cold-induced bronchoconstriction [4]; however, they did not hyperventilate their animals, which makes difficult the comparison between both studies. It has been shown that NO also have a weak bronchodilator effect in asthmatic but not in nonasthmatic individuals [37]. However, this effect has not been observed on exercise-induced bronchoconstriction in mild to moderate asthmatics subjects [38].

The pattern of Rrs response after HP presented in our present study is similar to that reported by other investigators [39, 40]: i.e., an increase of Rrs that decreases around 50% in the first 15 minutes after hyperpnea. Ray and colleagues suggest that the decrease in the respiratory resistance after hyperpena is a bronchoprotective counter-effect; however, they do not suggest any specific dilator mediator or mechanism [24]. Nonadrenergic/noncholinergic (NANC) nervous control of airway smooth muscle has been extensively studied in human health and disease; however, its importance remains no established and controversial [40]. NANC system may have an important participation in airway tone either by excitatory pathway (through substance P and ~~NKA~~) or inhibitory pathway (through vasointestinal peptide, neuropeptide Y, and NO). Belvisi and Bai suggest that neural co-transmission represents a fundamental mechanism employed by autonomic nerve to achieve efficient and precise control over airway tone [42]. Although the regulation of NANC system on airway tone is not well understood, there are evidences that i-NANC system could be activated reflexively by the pulmonary C-fiber reflex [43]. Because neurokinins (NKs) has been shown to be involved in hyperpnea-induced bronchoconstriction [44], our results may suggest that NO could have a bronchodilatator effect to minimize NK response; however, this hypothesis still have to be proved and other studies are necessary.

HIB increases airway microvascular leakage and this response involves NK [45]. The same study also showed that, although significant, there is a loose correlation ($r^2 = .126$) between the intensity of airway obstruction and Evans blue extravasations, which may suggest that these 2 phenomena could not be related. It has been suggested that endogenously produced pulmonary NO may have a role in the regulation of AMP; however, there are conflicting data about its actual importance and NOS isoform involvement [20–23]. Recently, Mehta and colleagues have shown that L-NAME decreases basal and antigen-induced AMP in guinea pigs and this effect was not observed with AG treatment [23]. These results support our data because we also show that basal AMP was decreased by L-NAME but not by AG. Interestingly, as observed by ~~Metha~~ and coworkers, our results also show that the inhibition obtained with L-NAME treatment on extrapulmonary airway tissues were more important than in intrapulmonary airways. However, we observed a smaller effect of L-NAME on AMP that could be, at least partially, explained by the type of treatment, since we evaluated the acute instead of chronic effect of L-NAME.

In conclusion, our results show that constitutive NO seems to have a bronchodilatator effect on HIB in guinea pigs. Constitutive NO also appears to regulate basal AMP but not hyperpnea-induced AMP. Inducible NO seems not be involved neither in HIB nor in hyperpnea-induced hyperpermeability in guinea pigs.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. Q10

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