**Responses to the reviewers' comments**

**Reviewer #1 (**#00503929**)**

1. There is a conceptual gap in the discussion, concerning what the authors think is the explanation for their findings. There are many possible alternatives, but the essential issue is whether release of acetylcholine from parasympathetic fibers accounts for the effects of LES, (LES is stimulatory, so yes) and blockade of this release by HES-induced reversible impairment of vagal conduction accounts for the benefit observed. A review that addresses the concern would likely do much to increase the interest of the manuscript to the Journal's readership.

**Response:** We have emphasized in the revised Discussion that "Actually, vagal LES-induced bronchoconstriction [22-24] and reduce $C_{dyn}$ [27] have been observed in rats, rabbits, and guinea pigs. This excitatory response is achievable mainly via promoting release of acetylcholine from parasympathetic fibers innervating airway smooth muscle." We have also described neural pathways regulating the airway smooth muscle tone and further interpreted our data related to these neural pathways (see the second paragraph in Discussion section).

2. This model may be supported by a number of researchers, but there is also evidence from several groups that immunological processes, through inflammatory mechanisms, modify methacholine responses in the target organ, leading to what can be termed AHR, not necessarily depending on the integrity of parasympathetic innervation. Regardless of the relative importance of nerves versus target organ in the development of AHR, the authors did not address the status of cholinergic transmission in their paper, but the responses to nonspecific electrical stimulation in a nerve that contains a variety of afferent and efferent fibers.

**Response:** According to the reviewer's suggestion, we have pointed out (at the beginning of the first paragraph of Discussion section) that "Multiple mechanisms (parasympathetic nerve-dependent or -independent) are involved in generating airway hyperreactivity. In the present study, we addressed an essential issue as to whether HES reversibly impairs vagal conduction via blocking acetylcholine release from parasympathetic fibers, and thereby alleviates the MCh-produced bronchoconstriction." In the revised Discussion section, we menioned that "....the cervical vagal trunk consists of both afferent

(myelinated and unmyelinated) and efferent (myelinated) fibers [34]. Our data are unable to delineate which type(s) of vagal fibers are responsible for the HES-induced change."

3. It also discusses extensively the possibility of translating HES into clinical medicine. I think this discussion should be more concise, as it is far from the immediate concerns of the experimental work.

**Response:** This paragraph has been omitted to prevent extensively discussion of the possibility of translating HES into clinical medicine.

4. Not enough attention has been given to the fact that asthma is a chronic condition.

**Response:** - We have described that "Asthma is a chronic disease; however, severe asthmatic attack is acute. In our study, the airway responses to MCh mimic the asthmatic attack, while HES-induced bronchodilation during the acute bronchoconstriction may provide a clue of alleviating the asthmatic attack." (See last portion of the first paragraph of Discussion).

5. Repeated HES may have undesirable consequences, even though HES seems not to damage nervous fibers in a short round of treatment. Furthermore, no one would consider such a complex approach to relieving asthma symptoms, unless more conventional approaches have failed. It is therefore important to examine whether preexposure to the conventional treatments, including topical corticosteroids and bronchodilators, does not interfere with the effectiveness of HES.

**Response:** We have specifically addressed the concern about possible nerve damage by HES in the response to Q7 (see below). As mentioned above, we have omitted paragraph related to translating HES into clinical medicine (Please see the response to Q3). It is true that no one would consider the electric stimulation to relieving asthmatic attack unless more conventional approaches have failed. We agree that pre-exposure to the conventional treatments may inerter with the effectiveness of HES, which has been reflected in our revised discussion.

**6.** It is clear from the text that measurements were carried out in a limited number of animals, and that each animal was therefore stabilized under anesthesia/paralysis before a series of repeated stimuli/measurements were carried out. The text

describes the evaluation of effects that generated the means and standard errors for $R_L$ and $C_{dyn}$, as consisting of a standard methacholine exposure of 2 minutes, which reproducibly induces increases in $R_L$ and decreases in $C_{dyn}$, and reaches a peak, from which LES can induce an aggravation while HES has the desired beneficial effects. All of this only makes sense if: a) the timing of methacholine exposure relative to electrical stimulation is fixed, and the effects of both, in duration and magnitude, are highly reproducible, no matter how many cycles have already been carried out; b) the effects of LES and HES, tested in the individual animal, are independent of the order in which they have been carried out (stated to be random), as well as of the ratio of LES/HES rounds for the individual animal; c) HES, stated to consist of a variety of treatments differing in their characteristics, can legitimately be shown to be equivalent for the purposes of the study. Perhaps this is the case, but it should be made clear in the methods section, since this paper is not intended for specialists alone.

**Response:** In the revised Experimental procedures, we have made following clarifications to address the concerns from the reviewer. First, the electrical stimulations were randomly applied with the ratio of LES vs. HES equal to 1:3 in each MCh exposure, and each stimulus lasted for a 10-s with an approximately 30 s interveal or occationally for 2-min period (only HES). Second, the same patterns of electrical stimulations applied before MCh exposure were repeated with the first electrical stimulation applied at $30^{th}$ s of the plateau $R_L$ response to MCh. After recovery from the MCh exposure, the animal received the same MCh exposure and electrical stimulations again once or twice to ensure the reproducibility of the $R_L$ responses. Third, both monophonic and biphasic square-waveform of HES have been reported to block nerve conduction in previous studies [11, 19-21]. Therefore, the similar patterns of HES were also applied in the present study. Correspondingly, we have added "The $R_L$ responses to the $1^{st}$, $2^{nd}$, and $3^{rd}$ MCh exposure were compared to determine the reproducibility" in the revised Data Acquisition and Statistical Analysis. With respect to the relevant results, we have stated that "The amplitudes and durations of evoked RL responses to the $1^{st}$, $2^{nd}$, and $3^{rd}$ MCh exposure in Sal-treated and Ova-treated animals are listed in Table 2. The lack of significant difference among the $R_L$ amplitude and duration in response to the three MCh exposures strongly indicates that these evoked responses are reproducible."

7. I am especially concerned that fatigue eventually sets out, and therefore the last round of HES in a long series may not really be comparable with the first one. If

this possibility has been excluded, the criteria that lead to this exclusion should be stated. I think the authors should address the possibility that a large number of HES cycles might actually result in long-term impairment of nerve function, or selective damage to more sensitive fiber types.

**Response:** We have stated that "In this study, 4 electrical stimulations were applied during each $R_L$ plateau response to MCh aerosol. The bronchodilation responses ($R_L$) evoked by the first and last HES (usually with ~1.5 min apart) were not significantly different in amplitude (in Sal-treated GPs: -37 ± 5% vs. -33 ± 6% and in Ova-treated GPs: -51 ± 6% vs. -48 ± 7%; $P > 0.05$). In addition, no remarkable fatigue of the bronchodilation in response HES was observed during continuously stimulating vagi for 2 min (Fig. 6). These data support the notion that detectable fatigue and/or nerve damage is minimal in the experimental timeframe of this study." (see Airway Responses to Vagal Electrical Stimulations after Exposure to Aerosolized MCh in Results). We have also stated in the revised Discussion that " ... though our data failed to show a detectable fatigue and/or nerve damage induced by our HES applied within 2 min, it remains unknown whether HES applied in a prolonged period (> 2 min) would produce such pathophysiological changes."

8. A further methodological consideration is whether measurements of repeated HES cycles performed in a given animal were treated as part of a pool of comparable measurements derived from all animals in a treatment group or, alternatively, were used to calculate an average for the animal, and these averages were used to calculate the data shown.

**Response:** In revised Data Acquisition and Statistical Analysis. We have mentioned that "The $R_L$ responses to the $1^{st}$, $2^{nd}$, and $3^{rd}$ MCh exposure were compared to determine the reproducibility. In our study, multiple trials of a given electrical stimulation in the individual animal were averaged to calculate our group data unless mentioned otherwise."

9. The last, but not least, methodological concern is the so-called asthma model used. This is the first asthma model I have seen to rely entirely on very short aerosol exposures over a relatively short sensitization period. It is remarkable that, by the second week, the animals already have been sensitized to the extent that they require coverage with diphenhydramine to cope with the OVA exposure. I would

feel more comfortable if the paper had included data from their own or other groups, demonstrating that enough OVA-specific cytotropic Ig can be generated in a guinea pig by as little as 5-minute exposures over a 5 day-period to account for the functional abnormalities in the lungs.

**Response:** The same [14, 15] or even shorter Ova-exposure (see below) have been applied to induce AHR in guinea pigs. It was reported that injection of serum samples from the guinea pigs treated with two occasions of Ova-exposures (1% in saline, 10 min) separated by 7 days (only twice exposures) into the recipients significantly increased IgG and IgE-like activity and proliferation of eosinophils (*Matsumoto T and Ashida, Y 1994, J Pharmacology and experimental therapeutic, 269:1236-1244*). Interestingly, similar sensitization was also observed with a shorter Ova-exposure duration (1 or 3 min) separated by 7 or 14 days (again only twice) (*Hutson, PA, Church MK, Am Rev Respir Dis 1988, 137:540-557; Ingenito, EP, Pliss LB; Am Rev Respir Dis 1991, 143: 572-577*). The validity of the approaches used by these labs and our lab in inducing allergy/airway sensitization is also supported by positive allergy skin reaction and release of substance P in the lungs (*Wu, AJRCCM, 1999; and Matsumoto T and Ashida, Y 1994, J Pharmacology and experimental therapeutic, 269:1236-1244*); and AHR to acetylcholine or cigarette smoke stimulation [14]. In fact, we previously found that in a case, the guinea pig died immediately after the first Ova exposure in the second week exposure accidently without diphenhydramine-pretreatment.

10. The model is unclear, because AHR is supposed to be a chronic change in sensitized animals and asthmatic subjects, not an abnormal response transiently induced by the interaction between methacholine exposure and ongoing (tonic) vagal activity.

**Response:** Please see the response to Q4.

11. It is unclear to me how methacholine and acetylcholine, which are supposed to act through similar receptor-dependent mechanisms, would make qualitatively distinct contributions to the final AHR.

**Response:** Previous studies have shown that bivagotomy or vagal blockade by cooling profoundly decreases and even eliminates bronchoconstriction induced by application of

cholinergic agonists (methacholine and acetylcholine) into the respiratory tract in animals. Thus, basic vagal tone per se must play a critical role in generating the airway smooth muscle responses to acetylcholine. Our results showed that HES, likely via inhibiting or blocking the parathmpathic nerve conductance, attenuated the bronchoconstrictive response to methacholine, consistent with these previous results. All these data did not suggest that methacholine and acetylcholine make qualitatively distinct contributions to the final AHR.

**Reviewer #2 (**#01235072**) and Reviewer #3 (**#02416403**)**

Bpth reviewers have no concerns about the manuscript and believe that this paper is acceptable for publication.