TRACHEAL RESPONSIVENESS TO METHACHOLINE AND MUSCARINIC RECEPTOR BLOCKADE BY ATROPINE IN ANIMAL MODEL OF COPD

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Abstract

Airway hyperresponsiveness (AHR) is the main feature of asthma which also exists in cigarette smokers. However the mechanism of AHR is uncertain due to the complexity of stimuli action used for measuring AHR. The mechanism of competitive antagonist blockade which is measured as concentration ratio-1 (CR-1) is far simpler than that of agonists and depends only on drug delivery to the receptor sites and receptor affinity. Therefore, in this study we have examined the muscarinic receptor blockade by atropine on isolated tracheal chains of a model of COPD compared to control guinea pigs. An experimental model of COPD was induced in guinea pigs by exposure to cigarette smoke for three months. The responses of tracheal chains of COPD and control animals (for each group n=7) to cumulative concentrations of methacholine (M) in the absence and presence of 10 nM atropine were measured, and the effective concentrations of M causing 50% of maximum response (EC_{50} M) were obtained. The atropine blockade (CR-1) was calculated by: (post atropine EC_{50} M/EC_{50} M)-1. The tracheal responses of COPD guinea pigs were significantly higher than those of control animals to methacholine (EC_{50} M for COPD and control animals were 0.80±0.22 and 4.19±1.08μmol, respectively, $p<0.001$). The muscarinic receptor blockade by atropine (CR-1) was also significantly higher in trachea of COPD compared to that of control animals (19.94±3.79 *vs* 2.73±3.19, $p<0.005$). There was a significant correlation between EC_{50} M and (CR-1) ($r=-0.639$, $p<0.05$). This enhanced muscarinic receptor blockade, increased tracheal response to methacholine in tracheal chains of COPD animals and significant correlation between these two phenomena may indicate that increased drug delivery to the receptors could also be a determinant factor for bronchial responsiveness to stimuli in COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a global health problem, reaching almost epidemic proportions in the developing world [1]. The research into understanding of basic mechanisms of the disease and development of new treatment to prevent the progression of this condition presents a major challenge. It is thus important to develop tools that can study different mechanisms involved in the development of COPD and characteristics of this condition. By leading to a clearer understanding of the key events in the pathophysiology of COPD and enabling short term studies to develop appropriate strategies, animal models can provide a framework for the rational and safe design of expensive and long term clinical studies.

In cigarette smokers it is believed that airflow obstruction is caused by parenchyma disease (emphysema) and/or by smoke-induced distortion of the structure of the small airways [2-4]. Investigations of the mechanisms of smoke-induced disease would be aided by an animal model recapitulating the lesions seen in human smokers, but such models have been difficult to create. In fact a model of cigarette smoke-induced lung disease in which guinea pigs develop airflow obstruction and emphysematous lung destruction has been described [5].

There are reports regarding airway hyperresponsiveness to different stimuli in animals exposed to cigarette smoke [6-10], but little is known regarding the mechanism(s) of increased airways responsiveness in animal exposed to cigarette smoke as well as asthmatic patients. This is mainly due to complexity of action of stimuli use in measuring AHR.

The mechanism(s) of action of a competitive antagonist measured as the degree of rightward shift or dose ratio/concentration ratio (DR or CR) is far simpler than that of an agonist and depends only on concentration of antagonist at the receptor sites ([I]) and receptor affinity (Ka) [11]. Thus, receptor blockade by a competitive antagonist could inside into the mechanism (s) of airway responsiveness. In previous studies we demonstrated enhanced blockade of different receptors by their antagonists in both asthmatic patients [12-14] and COPD animals [15, 16].

Therefore, in the present study tracheal responsiveness of guinea pigs exposed to cigarette smoke (an animal model of COPD) to methacholine and muscarinic receptor blocked by atropine was studied.

Material and Methods

Animals and cigarette smoke exposure

Fourteen adult Dunkin-Hartley guinea pigs (600 to 650 g) of both sexes were divided into two groups of 7 experimental and 7 controls. Experimental animals were exposed to cigarette smoke as previously described [17, 18]. The animals were exposed to cigarette smoke in a waking, restrained state and spontaneously breathing in a smoking chamber which was a modification of that described by Simani and co-workers [17]. Animals were placed in a Plexiglas box with their heads secured in a compartment (15x12x7cm). Twenty-millilitre puffs of cigarette smoke was drawn out of cigarettes with syringes and then exhausted, at a rate of two puffs per minute into the animals' head chamber. Exposure of animals to each cigarette lasted for 8-9 minutes, with a10 minute resting period between cigarettes. The animals were initially exposed to one commercial non-filter cigarette per day, and this dose was increased to a maximum of 5 cigarettes per day over a 2-week period. In a pilot study it was observed that animals could not tolerate the exposure of cigarette smoke of more than 5 cigarettes per day. The exposure to the smoke of 5 cigarettes per day, 6 days per week, continued for 3 months. The guinea pigs exposed to cigarette smoke were called COPD animals. The control animals were not exposed to cigarette smoke, and they were kept in an animal house under normal conditions for the same period of time. The study was approved by the ethical committee of Mashhad University of Medical Sciences.

Tissue preparations

Guinea pigs were killed by a blow on the neck, and trachea were removed. Each trachea was cut into 10 rings (each containing 2-3 cartilaginous rings). All the rings were then cut open opposite the trachealis muscle, and sutured together to form a tracheal chain [19, 20]. Tissue was then suspended in a 10 ml organ bath (organ bath 61300, BioScience Palmer-Washington, Sheerness, Kent, U.K.) containing Krebs-Henseleit solution of the following composition (mmol/l): NaCl 120, $NaHCO_3$ 25, $MgSO_4$ 0.5, KH_2PO_4 1.2, KCl 4.72, $CaCl_2$ 2.5 and dextrose 11.

The Krebs solution was maintained at 37^oC and gassed with 95% O_2 and 5% CO_2. Tissue was suspended under isotonic tension of 1 g and allowed to equilibrate for at least 1 h while it was washed with Krebs solution every 15 min.

Measurement of tracheal response to methacholine and muscarinic receptor blockade

In each experiment two cumulative log concentration-response curves (LCRC) of methacholine-induced contraction of tracheal chain were obtained, one 10 min after adding 10 nM atropine sulphate (Sigma Chemical Ltd UK) to the organ bath (post atropine methacholine response curve), and the other 10 min after adding the same volume of saline (baseline methacholine response curve).

A cumulative log concentration-response curve of tracheal chain to increasing concentrations of methacholine (10 nM to 5 mM) was obtained with the addition of consecutive concentrations every 2 min. To obtain the curve the percentage of contraction of the tracheal smooth muscle due to each concentration of methacholine in proportion to the maximum contraction obtained by the final concentration of methacholine (5 mM), in baseline methacholine response curve, was calculated and plotted against log concentration of methacholine. The effective concentration of methacholine causing 50% of maximum response (EC_{50} M) of baseline and post atropine methacholine response curve in each experiment was measured (expressed as EC_{50} M and post atropine EC_{50} M respectively). The EC_{50} M was considered as tracheal response to methacholine.

The muscarinic receptor blockade by atropine was assessed as concentration ratio minus one (CR-1) which was calculated by: (post atropine EC_{50} M/EC_{50} M)-1.

The experiments for measuring post atropine methacholine response curve and baseline methacholine response curve in each tracheal chain were performed randomly with an 1 h resting period between each of the two experiments while washing the tissues every 10 min. In all experiments responses were recorded on a kymograph (ET8 G-Boulitt, Paris) and measured after fixation.

Pathological evaluation

The lungs of all animals exposed to cigarette smoke underwent normal pathological evaluation using hematoxin-eosin dye by a pathologist.

Statistical analysis

The data of tracheal response to methacholine (EC_{50} M), and muscarinic receptor blockade (CR-

1) were quoted as both arithmetic mean±SEM and geometric mean because it has been shown previously that these values in *in vivo* studies are non-normally distributed [21]. In comparing these values between COPD and control guinea pigs both unpaired "t" test and Mann Whitney "U" test were employed. Tracheal response to methacholine (EC_{50} M), was related to (CR-1), using the least square regression and also using Spearman rank correlation to avoid any assumption of normal distribution of the data. Significance was accepted at $p<0.05$.

Results

Histology

The following pathological changes were observed in the lungs of animals exposed to cigarette smoke (Figure 1):

1) Increased inter-alveolar septum in all specimens.
2) Increased lymphatic tissue in the lung parenchyma of all specimens.
3) The destruction of alveolar wall and existence of emphysema in the lungs of most animals.
4) Intra-alveolar bleeding in most animals' lungs.

Tracheal response to methacholine

The mean value of EC_{50} M in tracheal chains of COPD animals (0.80±0.22 μM, range 0.12-2 μM) was significantly lower than in control animals (4.18 ±1.08μμM, range 0.8-9 μM, $p<0.001$), (Table I, Figure 2a). The most responsive trachea of COPD animals was 75 times more sensitive to methacholine than the least responsive trachea from control animals.

Atropine blockade (CR-1)

The rightward shift of the post atropine methacholine response curve compared to the baseline methacholine response curve in tracheal chains of COPD animals was greater than that of control animals (Figure 2 c and d). Mean CR-1 in tracheal chains of COPD animals (19.94±3.79, range 7-36.5) was 7.3 times greater than in control animals (2.73±0.93, range 0.125-6, $p<0.001$), (Table I, Figure 2b). The value of CR-1 of the most sensitive trachea of COPD animals was 292 times greater than that for the least sensitive trachea from control animals.

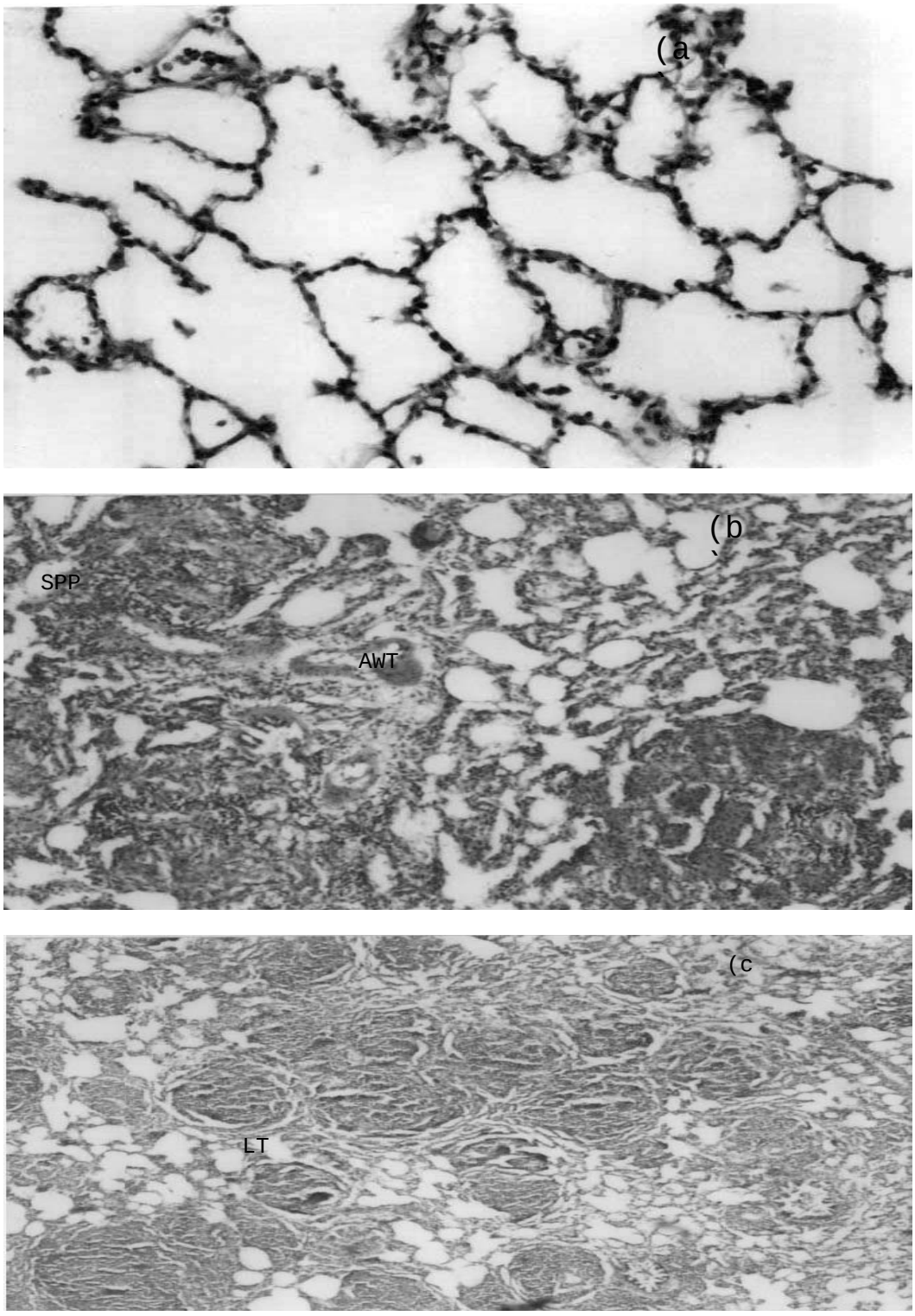

Figure 1. Photograph of a lung specimen of control guinea pig (a), and two lung specimens of animals exposed to cigarette smoke (b and c) with participation of smoke particles (SPP), alveolar wall thickening (AWT), and increased lymphoid tissues (LT), (magnification 40x10).

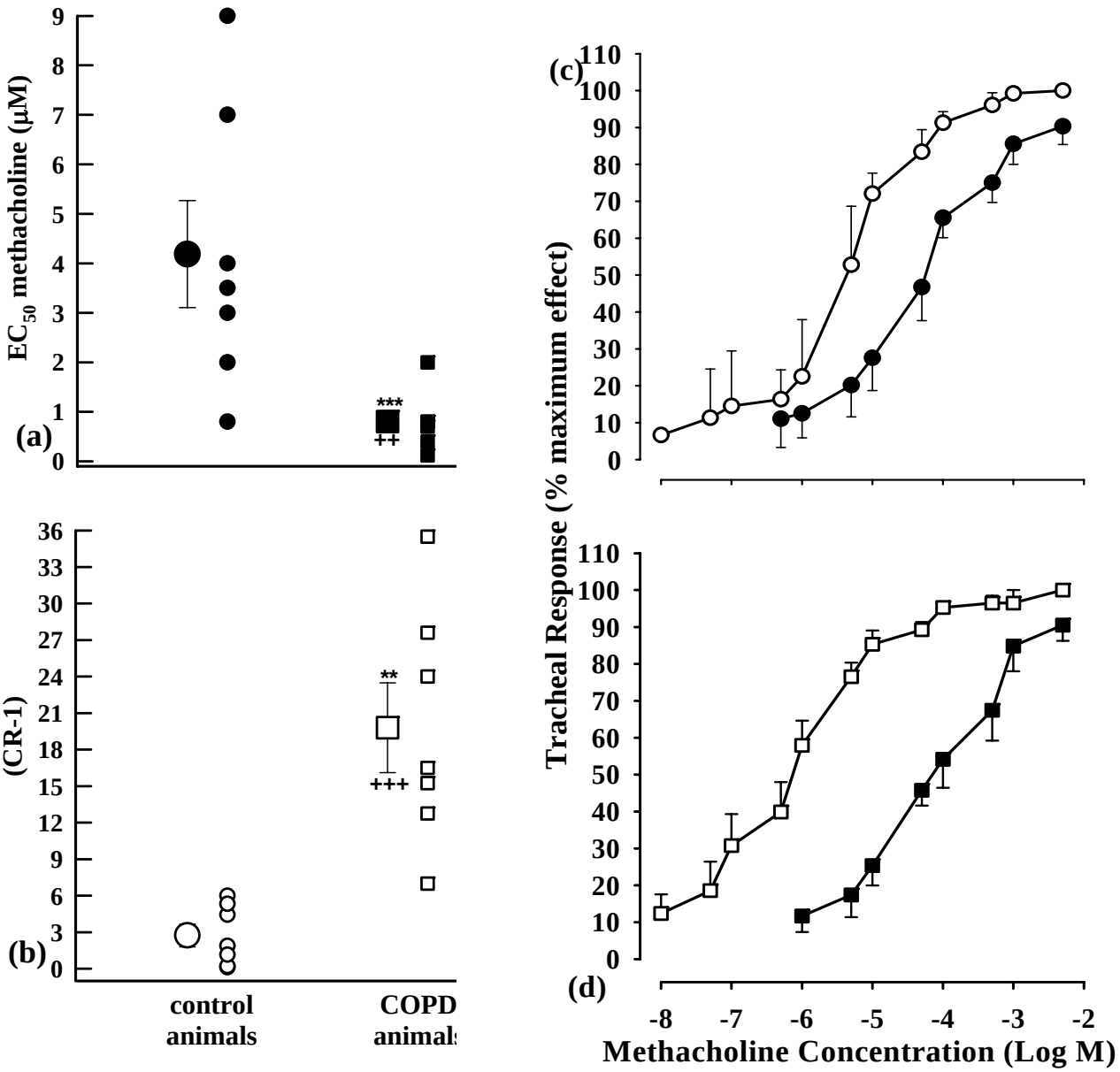

Figure 2. Individual values and mean±SEM (big symbols with bars) of tracheal response to methacholine (EC_{50} M) (a), and muscarinc receptor blockade by atropine (CR-1) (b), in tracheal chains of control (open symbols) and COPD animals (filled symbols), (for each group, n=7). Statistical differences between values in COPD with those of control animals: **; $p<0.005$, ***; $p<0.001$. Cumulative log concentration-response curves of methacholine induced contraction of isolated trachea in the presence of saline (open symbols) and atropine (filled symbols) of control (c) and COPD guinea pigs (d).

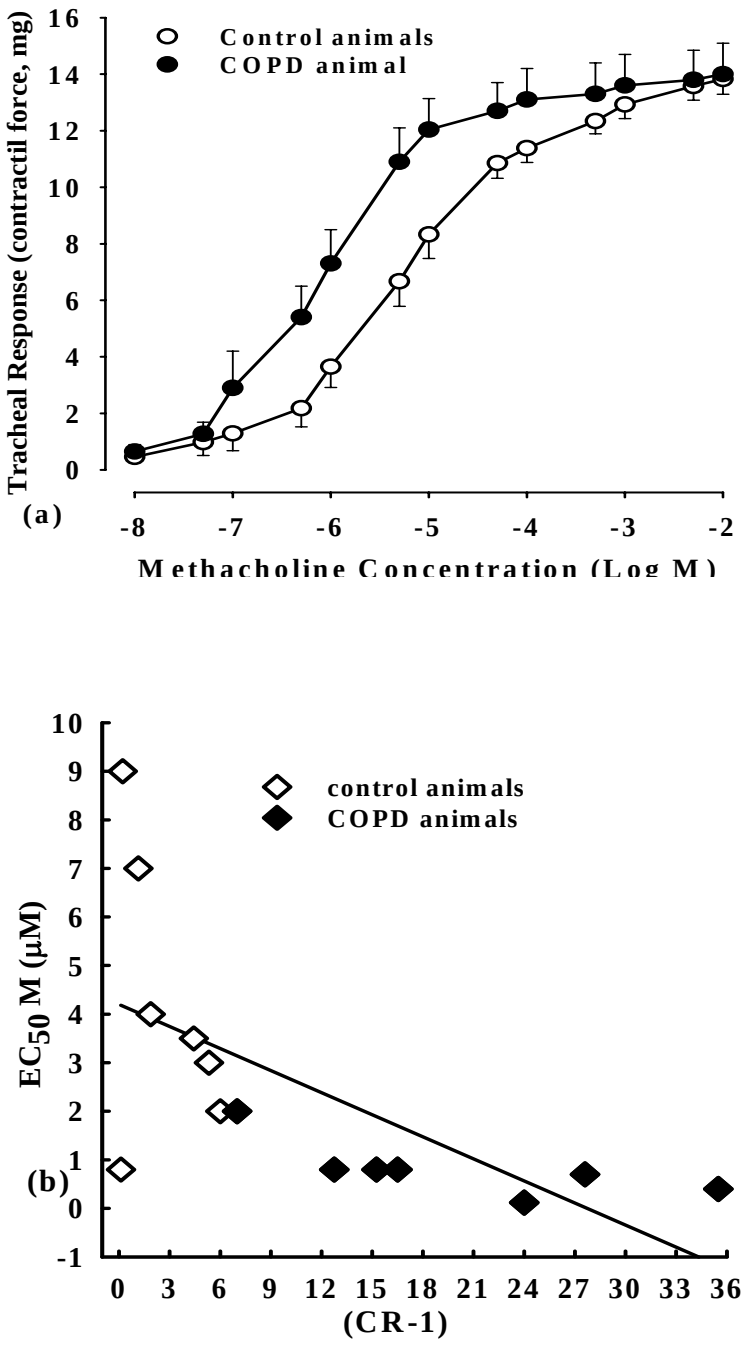

Figure 3. (a) Correlation between tracheal response to methacholine (EC_{50} M): and muscarinic receptor blockade by atropine (CR-1) in all control and COPD guinea pigs (n=14): r=-0.639, $p<0.05$. (b) Cumulative log concentration-contractile response curves of methacholine of isolated trachea in the presence of saline of control (open symbols) and COPD (filled symbols) guinea pigs (for each group, n=7).

Maximum contractile response to methacholine

There was no significant difference in maximum tracheal response to methacholine between COPD and control animals (14.08±1.10 and 13.83±0.54 in COPD and control group respectively), (Table I, Figure 3a).

Relationship between bronchial response to methacholine and atropine blockade

There was a significant negative correlation between tracheal response to methacholine (EC_{50} M) and muscarinic receptor blockade by atropine (r=-0.639, p<0.05, *Figure 3b*). The correlation between tracheal response to methacholine and muscarinic receptor blockade by atropine in copd group was also significant (r=-0.735, p<0.05), but in control group this correlation was not significant (r=-0.446, p=0.316).

Table I. Values of tracheal response to methacholine (EC_{50} M), and muscarinic receptor blockade by atropine (CR-1) in control and COPD guinea pigs (for each group, n=7) and statistical differences between the two groups.

Tracheal Response		Control	COPD	p value "t" test	p value "U" test
EC_{50} (µmol)	Arithmetic 0±SD	4.19±1.08	0.80±0.22	p<0.001	
	Geometric 0	3.30	0.86		p<0.005
(CR-1)	Arithmetic 0±SD	2.73±0.93	19.94±3.79	p<0.005	
	Geometric 0	1.36	17.62		p<0.001
Maximum Response (mg)		13.83±0.54	14.08±1.10	NS	

Values are quoted as both arithmetic mean±SEM (Arithmetic 0±SD) and geometric mean (Geometric 0). In comparing values between the two groups both unpaired "t" test and Mann Whitney "U" test were employed. NS: non significant difference

Discussion

This study showed increased tracheal response to methacholine in guinea pigs exposed to cigarette smoke compared to control animals. The histological findings in animals exposed to cigarette smoke showed increased intra-alveolar septum, increased lymphatic tissue, destruction of alveolar walls and inter-alveolar bleeding which indicated pathological changes in the lungs of these animals are similar to those of COPD patients [22-24]. The pathological changes in the lungs of animals exposed to cigarette smoke were also similar to those of previous studies [5, 25-26]. Therefore the pathological changes in the lung of the guinea pigs exposed to cigarette smoke confirmed the induction of COPD in the experimental group of animals. Tracheal responsiveness to methacholine seen in guinea pigs exposed to cigarette smoke was similar to the results of sensitized guinea pigs [15] and asthmatic patients [12].

In addition several other studies showed AHR in guinea pigs exposed to cigarette smoke to different stimuli [6-10]. However the results of the present study also demonstrated an increased muscarinic receptor blockade by atropine (CR-1) in the animals exposed to cigarette smoke. The increased receptor blockade by a competitive antagonist was similar to the results of our previous *in vitro* results indicating increased muscarinic and histamine (H_1) receptors by atropine [15] and chlorpheniramine [16] in sensitized guinea pigs respectively. The consistent increase in antagonist blockade in the present study and our previous studies in sensitized animals [15, 16], indicated that the cause of enhanced receptor blocked is increase of either receptor affinity (Ka) or drug delivery to the receptors ([I]) or both of these factors [11]. The fact that two receptor systems showed enhanced competitive antagonist blockade in sensitized animals [15, 16] and one receptor system in cigarette exposed animals in *in vitro* studies suggests that the abnormality lies with [I] rather than Ka. This conclusion is also supported by *in vitro* experiments, which predict that receptor affinity for a given antagonist shows little variation between species and tissues [27]. We therefore suggest that this enhanced antagonist blockade may be caused by epithelial damage leading to increased epithelial permeability and accessibility of ligands to the receptor sites.

In fact, the increased airway epithelial permeability to different agents has been demonstrated in animals exposed to cigarette smoke [17, 28, 29] and in smokers [30, 31]. The existence of airway inflammation in animal exposed to cigarette smoke [18, 32, 33], smokers [30, 31, 34] and

COPD [35, 36] is well documented. Thus airway inflammation can cause epithelial damage; and this, in turn, can result in better access of ligands to the active sites in the airways, causing increased receptor blockade by competitive antagonists. In addition Simani et al [17] showed that one of the pathological change of guinea pigs exposed to cigarette smoke is epithelial damage of trachea which can lead to better access of ligand to receptor sites and increased tracheal responsiveness. The increased tracheal responsiveness to methacholine in epithelium denuded tracheal chains was also demonstrated previously [37].

However, our previous studies [12-14] also showed enhanced blockade when pharmacological antagonists were administered by i.v. Injection in asthmatic patients; and this cannot be due to increased epithelial permeability. The strongest possibility is increased tissue permeability due to airway inflammation which could increase diffusion of antagonist ligands administered by either way and would explain the variation in (CR-1) produced by both routes of administration. In addition, the results of the present *in vitro* study cannot be fully explained by increased epithelial permeability because the barrier role of epithelium against ligands diffusion [38] may be appreciated only when perfused tracheal or bronchial tubes are exposed to ligands from the mucosal sides but not in the model used in our study, using tracheal rings. This mostly excludes the barrier function of the epithelium. In addition destruction of lung parenchyma is also documented in animals exposed to cigarette smoke [18]. This can support an increased tissue permeability and better accessibility of ligands to the receptor sites.

The results of this study also showed significant correlations between muscarinic receptor blockade by atropine (CR-1) and tracheal response to methacholine in COPD animals and in both groups of guinea pigs. The significant correlation between (CR-1) and tracheal response to methacholine in the present study, as well as blockade by other antagonists and agonist responsiveness in previous studies [12-16], indicates that bronchial hyperresponsiveness to different stimuli in COPD and asthma, at least in part, is due to increased bronchial epithelial and tissue permeability which is perhaps due to airway inflammation in these diseases. If this is true, the treatment of these diseases should be focused on prevention this increased permeability. The absence of a significant correlation between muscarinic receptor blockade by atropine and tracheal response to methacholine in control group is perhaps due to less variation of tracheal response to methacholine and muscarinic receptor blockade among animals of this group.

The present *in vitro* study indicated higher atropine blockade at muscarinic receptors than tracheal response to methacholine in tracheal chains of exposed guinea pigs to cigarette smoke compared to control animals (13.0 times for atropine blockade and 3.9 times for methacholine response), which was very similar to the results of our *in vitro* study in sensitized guinea pigs compared to control animal [15]. These similarities may indicate that similar mechanism(s) e.g. increased tissue and epithelial permeability is responsible for increased tracheal response to methacholine and muscarinic receptor blockade in both sensitized and exposed animals to cigarette smoke. The reason of more increased atropine blockade compared to responsiveness to methacholine seems to lie in differences between the molecular size of atropine (MW = 695) and methacholine (MW = 196) because normal tracheal tissues may have a little barrier function to diffusion of smaller molecules.

Although the major pathogenesis of COPD occurs in lower airways, most studies examining airway responsiveness in animals exposed to cigarette smoke have used a parameter relating to mainly central airways. For example Lee et al [9], Hulbert et al [10], Matsumoto et al [39] and Zhong et al [40] all used lung resistance (R_L) for measuring airway responsiveness in guinea pig exposed to cigarette smoke which is mainly dependent on central airways (generation 5-7). Verhoeven et al [41] demonstrated that in patients with COPD, both prejunctional mechanisms (ie, epithelial damage, neural control, and inflammation) and postjunctional mechanisms (ie, loss of lung elasticity, swelling of the airway wall, and intraluminal secretions) can be responsible for the occurrence of airway hyperresponsiveness.

The other possible explanation for the finding of the present study is upregulation and increased expression of the receptors due to cigarette smoke exposure. However, upregulation and increased expression of the receptors would result to increased tracheal responsiveness to methacholine without affecting muscarinc receptor blockade. Therefore, based on the results of the study this possibility is excluded. In addition there was no significant difference in maximum response in tracheal contractility between two groups. Cigarette smoke exposure may also lead to dysfunction of M_2 receptors which can enhanced both tracheal responsiveness to methacoline and muscarinic receptor blockade. However this hypothesis should examined in further studies.

In conclusion, this study demonstrated enhanced muscarinic receptor blockade by atropine in tracheal chains of guinea pigs exposed to cigarette smoke. The cause of this enhanced antagonist

blockade at the trachea bronchial tree is perhaps increased epithelial and tissue permeability due to airway inflammation. The increased epithelial and tissue permeability of the airways appears, at least in part, is responsible for airway hyper responsiveness.

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