Effect of 2-(acetyloxy)-N,N,N-trimethylpropan-1-aminium on Tracheal Smooth Muscle Reactivity

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The purpose of this paper was to study the muscarinic agonist methacholine and selective M₁-antagonist pirenzepine effects on the tracheal smooth muscle in vitro in presence/absence of hydrogen peroxide.

Keywords: methacholine, hydrogen peroxide, pirenzepine, tracheal smooth muscle.

Muscarinic receptor subtypes have been identified on airways of several mammalian species, including humans. M₁-receptors are found in airway ganglia, M₂-receptors (autoreceptors) are present in cholinergic postganglionic nerves at the prejunctional level, whereas the muscarinic receptor subtypes found in airway smooth muscle are of the M₃-receptor subtype (1, 12).

Methacholine - 2-(acetyloxy)-N,N,N-trimethylpropan-1-aminium is primarily used to diagnose bronchial hyperreactivity, which appears in asthma and in chronic obstructive pulmonary disease. This is accomplished through the bronchial challenge test, or methacholine challenge, in which a subject inhales aerosolized methacholine, leading to bronchoconstriction. Methacholine has a charged quaternary amine structure, rendering it insoluble to lipid cell membranes. Methacholine has a β-methyl group which provides selectivity towards M-type receptors as compared to N-type receptors. The quaternary ammonium group is essential for activity. The ester however makes it susceptible to the enzyme acetylcholine esterase (2). It is highly active at all of the muscarinic receptors, but has little effect on the nicotinic receptors.

Methacholine utilizes the M₃ receptor for bronchoconstriction. The degree of narrowing can then be quantified by spirometry. People with pre-existing airway hyperreactivity, such as asthmatics, will react to lower doses of drug (3, 5).

Anticholinergic drugs which selectively block M₁ and M₂-receptors may have an advantage over currently used non-selective antagonists in the treatment of airway obstruction (10).

Pirenzepine (C₁₉H₂₁N₅O₂) is an antimuscarinic agent that inhibits gastric secretion at lower doses than are required to affect gastrointestinal motility, salivary, central nervous system, cardiovascular, ocular, and urinary function. Pirenzepine is a M₁-muscarinic receptor antagonist and binds to the muscarinic acetylcholine receptor. The muscarinic acetylcholine receptor mediates various cellular responses, including inhibition of adenylate cyclase, breakdown of phosphoinositides and modulation of potassium channels through the action of G proteins (9).

Experimental part

There were studied 12 spiral trachea, obtained from male Wistar rats (200 g) sacrificed under narcosis. Preparations were put in an organ bath containing 50 mL Krebs-Hensellet solution at 37 Celsius degree, continuously gassed with a mixture of 95% O₂ and 5% CO₂ in order to maintain oxygen tension and a pH of 7.4.

A force transducer for recording isometric contraction, displayed on an xy inscriptor, has been used in order to follow up tracheal smooth muscle contractility.

After an equilibrium period of 60 min with 6 intermediate changes of solution, a dose-response curve was performed using methacholine concentrations from 10⁻⁵ to 10⁻⁶ M. The maximal response was obtained at 10⁻⁵ M when a contractile plateau was recording. This was considered a maximal effect 100% E max, then was established the % values of contractile response to another doses.

After 5 doses response curves and a washing period, samples were incubated for a long term (30 min) with

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hydrogen peroxide (10⁻³ M) and a new methacholine dose-response curve was performed.

The tracheal preparations were washed for 30 min with six intermediate changes of buffer solution followed by a preincubation procedure, 10 min with 10⁻⁵ M of pirenzepine before the incubation with hydrogen peroxide.

For each study we have performed 4 to 6 experiments. Statistical analysis included calculation of mean values, standard deviation and Student’s test.

**Results and discussions**

Preincubation 10 min with pirenzepine 10⁻⁵ M decreased the methacholine constrictor effect - mean value 20.19% (p<0.001) (fig.1).

Long-term incubation with H₂O₂ decreased the methacholine effect depending on the methacholine doses, less than pirenzepine relaxing effect.

Long term incubation with H₂O₂ (30 min-10⁻³ M) decreased the methacholine effect with 13.68±0.82% at doses between 10⁻⁶ - 4X10⁻⁶ M. (p<0.001). For the dose of 8.10⁻⁶ M methacholine, incubation with hydrogen peroxide enhanced the methacholine constrictor effect with 26.36 % (p<0.0001).

Pirenzepine reduced the amplitude of contraction induced by methacholine and hydrogen peroxide (which is Eₘₐₓ 90%) with mean value 17.89%. Contractile effect decreasing was very low for a methacholine dose 4.10⁻⁶ M (only with 5.91%) (fig 2.)

Hyperreactivity is expressed by a hyperfunction of excitatory mechanisms and/or a hypofunction of inhibitory mechanisms. Inhibition of excitatory mechanism (cholinergic antagonists) or activation of inhibitory mechanism (β-adrenoreceptors agonists) is useful in therapy of tracheo-bronchial hyperreactivity (19-20). The parasympathetic system is the most important system which regulates the airway smooth muscle activity. Cholinergic agonists induce constriction but in the same time induce the modulatory effect of sympathetic reflex which determines the inhibition of methacholine release and the relaxation of the airway smooth muscle. The high cholinergic neurotransmission can be blocked by muscarinic M1 antagonists which decrease the parasympathetic ganglia M1 receptor activation (17). Muscarinic M1 receptors have a high affinity for the antagonist pirenzepine. The M1 antagonist is important for the blockade of the methacholine excess production. Another experimental studies show that in guinea pig, rats, the methacholine release is inhibited by stimulation of neuropeptide Y, opiod receptors and histamine H₁ receptors (18).

We considered that the 10⁻³ M dose for H₂O₂ is optimal for long term incubation.

Long term incubation determinated the decreased of methacholine contractile effect, observed also by another authors (15). The H₂O₂ effect depends on the methacholine dose, the decreased contractile response was observed in low doses of methacholine.

This effect is explained by a disbalance between cholinergic and beta adrenergic response receptors. This aspect is illustrated by the potential toxic effect of H₂O₂ on muscarinic receptors and a high sensibility of beta adrenoreceptors to hydrogen peroxide depending on methacholine dose used. H₂O₂ and methacholine determinated the increase of intracellular calcium concentration with a direct toxic effect on smooth muscle fibers. Both substances decreased intracellular ATP

**Fig. 1.**

**Fig. 2.**
production with the effect on the muscarinic response in rats tracheal smooth muscle.

Conclusions
The H₂O₂ effect on tracheal smooth muscle is dose- and incubation time dependent. These data confirm the literature data which indicated the decrease of methacholine contractile effect in H₂O₂ presence on rat tracheal smooth muscle.

The H₂O₂ effect depends also by the methacholine dose. Low doses of methacholine determined the destroy of tracheal muscarinic receptors with an increase of β-adrenoreceptors sensibility.

A high methacholine dose determined a synergism with H₂O₂ which in this situation potentiated the methacholine contractile effect. Pirenzepine - a M₁ selective antagonist, reduced the amplitude of contraction induced by methacholine and hydrogen peroxide.

References
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Manuscript received: 15.08.2012