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Inhaled fluoride, magnesium salt and l-arginine reverse bronchospasma

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***In vitro* studies showed that fluoride and magnesium salts relax bronchial smooth muscle cells. Their combined administration could have potential interest. Magnesium fluoride salt (MgF_2) is nearly insoluble. A soluble derivate can be obtained by introducing L-arginineinine (L-arginine) between the ions. L-arginine, being the substrate leading to the release of NO, might add another relaxing effect to this derivate. Relaxing effects of NaF, $MgSO_4$, L-arginine, NaF+$MgSO_4$ and MgF_2+L-arginine given via the inhaled route were studied on rats challenged with acetylmethylcholine (ACMCH) following eight successive doses. Tested salts were given at the fourth dose of ACMCH. Changes in bronchial resistances (R) were measured and compared to results obtained in a control group, receiving ACMCH alone. NaF, $MgSO_4$, and L-arginine led to significant bronchorelaxing effects ($p<0.05$). The association NaF+$MgSO_4$ gave a greater decrease in bronchial resistance compared to that obtained with each salt (fluoride and magnesium) separately. (MgF_2, 2L-arginine) induced a significant fall in R at the fourth dose of ACMCH just after its inhalation. R values obtained with (MgF_2, 2L-arginine) were significantly lower than L-arginine alone at the sixth dose of ACMCH ($p<0.05$). (MgF_2, 2L-arginine) is a triple combination able to induce a significant and constant bronchodilating effect through three different pathways. The effect looked partly additive.** Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: magnesium; NaF; L-arginineinine; acetycholine challenge; rat; bronchodilator

Introduction

Several reports[1,2] have suggested that magnesium sulfate ($MgSO_4$) may be a therapeutic agent for patients suffering from severe asthma. *In vitro* studies have shown that magnesium ion (Mg^{2+}) modulates smooth muscle contractility and mediator release by its antagonism with calcium ion (Ca^{++}).[3]

The fluoride anion (F^-) can have contractile or relaxing effect on smooth muscle. F^- can induce contraction when given at a high concentration or in the form of aluminium fluoride salt (AlF_3).[4,5] The contractile effect is due to the mobilization of cellular calcium (Ca^{++}) and/or enhancement of calcium sensitivity. Cushing *et al*.[4] found that sodium fluoride (NaF) relaxed arteries by releasing an endothelium derived relaxing factor and one or more prostanoid. Zhao *et al*.[6,7] proved that NaF induced bronchial relaxation on precontracted bovine bronchi *in vitro*[7] and rats *in vivo*.[6] In fact, F^- is an inhibitor of *enolase*,[6] an enzyme of the glycolysis pathway leading to phosphoenolpyruvate.

L-arginine is an amino acid which led to the formation of nitric oxide (NO) by the action of NO-synthase (NOS)[8] and so could have a relaxing effect. Therefore $MgSO_4$, NaF and L-arginine are potent bronchodilators through different pathways.

The purpose of this study was to estimate the bronchorelaxing effect of inhaled magnesium salt, fluoride salt and L-arginine isolated or in a combined administration, making the hypothesis that the association should have a cumulative effect.

Materials and Methods

The study protocol was approved by our university Animal Care Committee. We assessed the relaxant effect of the bronchodilators and their associations by the measure of total lung resistance (R) during acetylmethylcholine (ACMCH) challenges. R was measured with Pneumomultitest equipment (EREMS, Toulouse, France). Sixty-four male Wistar adult rats were included in the study. They were anesthetized intraperitoneally with ketamine (150 mg/Kg). Rats were randomly assigned to six groups: ACMCH alone (n=12), NaF 0.5M (n=8), $MgSO_4$ 0.5M concentration (n=11), NaF 0.5M + $MgSO_4$ (n=12), L-arginine (n=10) and magnesium fluoride (MgF_2, 2L-arginine) group (n=11).

Total lung resistances measurement

After dissecting the neck, a tracheal cannula was inserted into a mid-line incision of the trachea. A catheter was inserted into the esophagus and connected to a pressure transducer to measure the intra-esophageal pressure. A small pneumotachograph (PTG, 8431B, Hans Rudolph, Kansas, USA) was connected to tracheal cannula. The period of measurement of the flow rate with the PTG was set at 10 s to avoid change in ventilation due to the PTG dead volume. The PTG was connected to a differential pressure transducer. Both pressure and flow transducers were assembled together with connecting valves to ease the calibration. Calibration in volume was done daily with a 10 ml syringe. Total lung resistance was calculated by using a first order mechanical model of the lung. Aerosolizations were made through a DeVilbiss nebulizer (Ref. 123 016 Marquette Medical products, Englewood, CO, USA)

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connected to a compressor (flow rate 100 ml/s). Aerosols were delivered at a flow rate of 0,1 ml/s in a rigid plastic chamber placed over the rat body.

Bronchoconstriction was induced by gradually increasing concentrations of ACMCH: 0,5 mg/l, 1 mg/l, 2,12 mg/l, 4,25 mg/l, 8,5 mg/l, 17 mg/l, 34 mg/l and 68 mg/l. ACMCH solutions were aerosolized within the chamber for 1 min with 3-min intervals between doses.

NaF, $MgSO_4$, L-arginine and (MgF_2, 2L-arginine) were inhaled at a concentration of 0.5M. Aerosols of theses salts were delivered for 1 min after each dose of ACMCH from the fourth dose of ACMCH. R were measured before the challenge, after an aerosol of isotonic saline and 2 min after each dose of ACMCH.

(MgF_2, 2L-arginine) synthesis

All fluoride solutions were prepared and stored in polyethylene or polypropylene bottles in order to prevent attack on glass surfaces.

Dissolution experiments were made in nitrogen atmosphere using a double-walled polyethylene-lined cell of 300 ml capacity. The cell contents were maintained at 25 °C by circulated thermostatted water through the outer jacket.

L-arginine was purchased from Sigma and used as received. L-arginine (8.71g, 0.05 mol) was dissolved in 100 ml of NaCL at 0.9%. To this solution, MgF_2 (1.557g, 0.025 mol) was added and the resulting mixture was then stirred at 25 °C for 5 h.

Chemicals

NaF, Ketamine, $MgSO_4$, L-arginine and MgF_2 were purchased from Sigma (St Louis, MO, USA) and ACMCH from Allerbio (Lavarene, France).

Data analysis

All data are reported as mean ± SEM. A p value <0.05 was considered significant. Mean values of R between control and other groups were compared using the Mann-Withney U test. Comparison of rats' resistances (R) values among the same group of rats at different concentrations of ACMCH was made using the paired Student's test. Changes in R during the metacholine challenge in different groups were analyzed with a two-way ANOVA.

Results

Wistar rats had a mean weight of 180 ± 30 g. Basal resistances were not different for all groups. R value in the control group receiving ACMCH increased significantly with the cumulative doses of ACMCH. Compared to the basal value of R, at the start of the challenge, the increase was significant at the third dose ($p<0.05$).

NaF 0.5M, $MgSO_4$ 0.5M and their association decreased R values at the fifth dose of ACMCH ($p<0.05$) (Figure 1) when they were compared to control group (ACMCH alone). R values at the two highest concentrations of ACMCH obtained with NaF group were not significantly different from basal values. R values, at the last four doses of ACMCH of $MgSO_4$ group and $MgSO_4$+NaF group, were not different from basal R values. R was smaller with the association ($MgSO_4$+NaF) than with salts alone for the highest three doses (Figure 1).

(MgF_2, 2L-arginine) induced a significant fall in R at the fourth dose of ACMCH just after its inhalation. R values were significantly

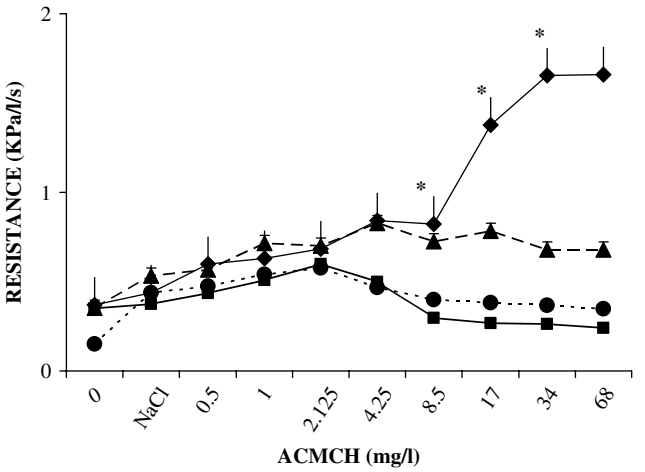

Figure 1. Bronchial resistances variation with NaF alone or associated to MgSO4 ♦ ACMCH ● MgSO4 ▲ NaF 0.5 ■ MgSO4+NaF $^{*}p < 0.05$, Comparisons with Mann-Withney U test between ACMCH group and NaF, MgSO4 and NaF+MgSO4.

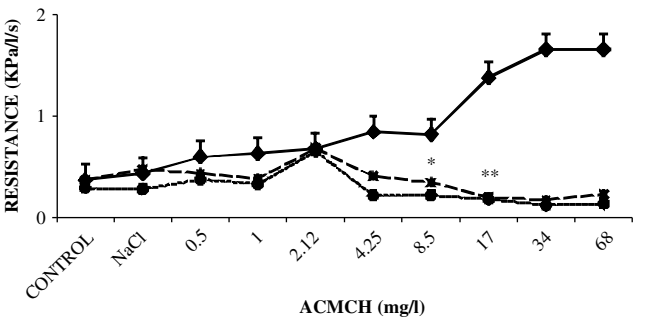

Figure 2. Effects of L-arginine alone or associated to MgF2 on bronchial resistances ♦ ACMCH ● MgF2+L-arginine ■ L-arginine $^{*}p < 0.05$, significant decrease of R with MgF2+L-arginine (ANOVA of Freidman). $^{**}p < 0.05$, significant decrease of R with L-arginine alone (ANOVA of Freidman).

lower than L-arginine alone at the sixth dose of ACMCH. R values obtained with (MgF_2, 2L-arginine) remained at the level of the basal values since the fourth dose of ACMCH (Figure 2).

Discussion

The main findings of this study are: 1/NaF, $MgSO_4$ and their combined administration, inhaled during ACMCH challenge, decreased significantly R as compared with the control group 2/Inhalation of L-arginine alone and associated to MgF_2 during metacholine challenge induced a significant, fast and maintained decrease of R values.

The mechanism of the relaxant effect of fluoride is poorly documented. NaF has been reported to stimulate adenylyl cyclase activity on smooth muscles[9] and induce NO synthesis[4] which would relax bronchi. NaF inhibits the glycolytic enzyme, enolase, wich converts 2-phospho-glycerate to phospho-enol-pyruvate.[6] The inhibition of glycolysis induced by NaF is illustrated by the sharp decrease in lactate production.[6] In this last study, a 0.5M concentration of NaF was used. This concentration of NaF was chosen as it gave a greater bronchorelaxant effect than a 0.25M concentration. The association $MgSO_4$+NaF gave a greater

decrease in R than $MgSO_4$ and NaF alone. $MgSO_4$ had a well known bronchodilator effect due to the magnesium salt (Mg^{++}).

Mg^{++} activates enzymes involved in the transfer of a phosphate group. Also, it is an indispensable element for the control of cellular permeability.[10] Mg^{++} inhibits neuromuscular transmission by reducing the release and effects of acetylcholine. Hypomagnesaemia might induce tetanus symptoms.[10,11] Mg^{++} inhibits cationic channels, sodium and especially calcium receptor and voltage dependent calcium channels.[3] The latter effect explains the use of magnesium sulphate ($MgSO_4$) as a bronchodilator in the treatment of asthma.[12] However, this bronchodilating effect shows a great variability from one patient to another and from one study to another.[1,2,13] Inhaled $MgSO_4$ as an adjuvant to beta2 agonists in the treatment of acute asthma attacks has been investigated by many authors. Mangat *et al*.,[14] Bessmertny *et al*.[15] and Hughes *et al*.[16] found a significant attenuation of bronchoconstriction. Some authors[12,17] have found that $MgSO_4$ is especially efficient in the case of severe asthma, while other researchers have been unable to demonstrate this efficiency.[1,2] In nearly all these studies, $MgSO_4$ is given as an adjuvant to beta2 agonists but not alone. In this study, $MgSO_4$ was used alone and led to a significant decrease of R in rats. Gourgoulianis[3] found that $MgSO_4$ causes partial relaxation of tracheal muscle strips when the constrictor is acetylcholine. This result had been explained by the interference of $MgSO_4$ with the cholinergic parasympathetic system nerve.[18] Mg^{++} and F^-, have different mechanisms on smooth muscles relaxation. Mg^{++} acting indirectly on the entry of Ca^{++} in the cell as F^- acts as an inhibitor of glucose metabolism. Thus, association of NaF + $MgSO_4$ have led to a significant decrease of R than that obtained with each product. This result can be explained by the cumulative effects of the two salts. The reversibility of the bronchoconstriction to L-arginine can be explained by an increase in the synthesis of NO under the control of NOS. Various observations have indicated the involvement of endogenous NO in the regulation of airway tone. *In vivo,* administration of NOS inhibitors enhanced the bronchoconstriction in response to allergen,[19,20] histamine,[21] and bradykinin in mild asthmatics.[22] *In vitro* studies have demonstrated that non-selective NOS inhibitors enhanced muscarinic agonist- and histamine-induced constriction of intact perfused guinea pig tracheal tube preparation.[21,23,24] De Boer *et al*.[25] found as in the present study that L-arginine had no effect on basal airway tone. This response can be explained by the reduced bioavailability of L-arginine after the metacholine challenge in rats by enhanced arginase activity in the airways. Arginase, which hydrolyzes L-arginineinine to L-ornithine and urea, is constitutively expressed in many cells throughout the body, including the lung.[26] It has been shown that inhibition of arginase causes a shift of L-arginineinine utilization to the NOS pathway.[27] Chakder *et al*.[28] explained this response by the reduction of the L-citrulline-L-arginine recycling as well as the reduced sensitivity of NOS for the substrate. The latter idea would be in line with reduced sensitivity to exogenous L-arginine as indicated by the partial effect of 1 mM L-arginine in the study of De Boer *et al*.[25] Sapienza *et al*.[29] showed that inhaled L-arginine induce a significant increase in exhaled NO in asthmatic subjects related to the amplification of the inflammatory response in the airways. Chambers *et al*.[30] approved this hypothesis and suggest that the increase in exhaled NO is not entirely mediated through an increase in NOS enzyme activity but also, through a reaction of L-arginine with reactive oxygen species. The discrepancy between these studies and the present study could be due to differences in species as the study of De Gouw *et al*.[31] was conducted on man and the present study was carried out on rats. However, other factors such as the experimental protocol, rats versus human, administration way, inhalation versus oral way, and differences in concentration of L-arginine cannot be ruled out. De Boer *et al*.[25] have examined the effect of various concentrations of L-arginine on metacholine-induced constriction of perfused tracheal preparations. They found that 1 and 5 mM L-arginine caused a significant decrease of the bronchial degree of constriction by 33% and 65% respectively. 0.3 mM of L-arginine had no effect on the responsiveness to metacholine.

In conclusion, as the decrease in R induced by each salt led to values close to the basal value we can say that each variety of salt (F^-, Mg^{++} and L-arginine) led to a bronchorelaxing effect by its own mechanism. Association of these salts had a cumulative bronchorelaxing effect by the more effective effect in reducing R and mainly by extending the duration of this relaxing effect on bronchials. A perspective of this work is to study the duration of the effect of each tested salt, by challenging repeatedly a rat having received a given dose of bronchodilating salt, at greater intervals than those used in the present study. This triple association could be an alternative solution to surpass resistances to traditional bronchodilating products in case of acute bronchospasma.

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