EXPERIMENTAL STUDY

A beneficial influence of Provinol on the reduction of allergen induced hyperreactivity in guinea pigs

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Abstract: *Background:* The anti-inflammatory, anti-allergic, antioxidant properties of flavonoids are known in the respiratory tract. We are interested in the role of Provinol during an allergic inflammation of the airway. *Objectives:* The aim of this study was to examine the influence of an acute administration of Provinol on tracheal smooth muscle reactivity in guinea pigs and to assess the involvement of nitric oxide in the mechanism of Provinol action.
Methods: This experiment was performed 14 days after the sensitization of animals by ovalbumin. In vivo, the specific airway conductance, as a tracheal smooth muscle reactivity parameter in response to bronchoconstrictor histamine, was evaluated after peroral administration of Provinol alone or together with L-NAME (N^{omega}-nitro-L-arginine methyl ester). In vitro, Provinol alone or in combination with L-NAME were added into an organ baths before the supplement of direct bronchoconstrictor histamine, acetylcholine and the allergen ovalbumin in rising concentrations. The amplitude of the tracheal smooth muscle contraction, as a tracheal smooth muscle reactivity parameter in response to histamine, acetylcholine and ovalbumin was evaluated.
Results: Our results showed that a Provinol has significant bronchodilatory activities both in vivo and in vitro.
Conclusion: Provinol alleviated the contraction of tracheal smooth muscle in guinea pigs sensitized by ovalbumin. Nitric oxide plays an important role in the mechanism of Provinol action *(Fig. 2, Ref. 28).* Full Text (Free, PDF) *www.bmj.sk.*
Key words: hyperreactivity of the airways, allergen, nitric oxide, polyphenols, Provinol.

Epidemiological studies have proved a favorable effect of food or beverages containing polyphenolic compounds with their anti-allergic activities, which can contribute to moderation of asthma symptoms (1).

Flavonoids as a big group of low molecular weight polyphenols have established antiinflammatory, bronchodilatory effect and elevate mucosus secretion in respiratory tract experimentally.

Flavonoids have various cellular mechanism acting on different sites of cell. Their antiinflammatory properties result from the suppression of eicosanoid generating enzymes and modulation on the expression of proinflammatory molecules via inhibition of the transcription factors activation (activator protein 1, nuclear factor κB). They have different action mechanisms depending on their chemical structures (2, 3). Butterbur acquired from Petasites hybridus has confirmed its antihistaminic effect in treating seasonal allergic rhinitis in randomised controlled trial

(4). Wu et al (2006) find out that nobiletin significantly alleviated ovalbumin (OVA) induced airway inflammation in rats by reduced increases of eosinophils, remarkably decreased level of eotaxin in blood and bronchoalveolar lavage fluid (5). Quercetin and isoquercitrin in experimental murine allergic asthma are following flavonoids to corroborate the reduction of eosinophilic inflammation (6). Ermanin and 5,3'-dihydroxy-4'-methoxy-7-methoxycarbonylflavonol isolated from Tanacetum microphyllum might be prospective antinflammatory agents via the reduction of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (7). The extract of Apium graveolens contained apiin inhibiting nitric oxide (NO) production and also iNOS expression (8).

Khellin, hispidulin and luteolin were the first flavonoids with bronchodilatory properties. Luteolin (chrysoeriol) is a bioactive flavonoid of Rooibos tea which has been attributed anti-inflammatory, anti-allergic, antioxidant, antitumor, antimicrobial, antiviral and free radical scavenging properties. It significantly affected the OVA-induced airway bronchoconstriction and bronchial hyperreactivity, notably at a dose of 0.1 mg/kg body weight (9, 10). Hispidulin isolated from Clerodendrum petasites or Inula viscosa was tested for its potential spasmolytic activity on the isolated tracheal strips of guinea pigs. It cased concentration dependend relaxation of the trachea (11, 12). Isoquercitrin from Argemone platyceras, employed in modulation of cough, bronchitis and pneumonia, inhibited both carbachol and leukotriene D4-induced contraction in guinea pigs airways (13). And finaly,

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hesperidin can increase mucine release by a direct acting on airway mucine secreting cells in respiratory tract (14).

Provinol, as a dry extract of red wine polyphenolic compounds (RWPC), became our interest. RWPC are components of diet flavonoids. There is some evidence suggested their benefit under pathological conditions. The protective effect of RWPC on cardiovascular diseases involves particularly their antithrombotic, antioxidant, antiischaemic, vasorelaxant and antihypertensive properties (15). They have dose depending properties such as the protection against cardiac and cerebral ischemia but the suppression of angiogenesis in vitro. RWPC show either proangiogenic (low dose) or antiangiogenic (high dose) features on postischemic neovascularisation in vivo. This dual effect can be profitable for the treatment and prevention of ischemic diseases or cancer growth (16). Low doses of RWPC and pure resveratrol positively modify the circulating endothelial progenitor cells playing an important role during neovascularization of ischaemic tissues and reendothelization of injured blood vessels (17).

There are some known action mechanisms of Provinol, which included the stimulation of constitutive forms of nitric oxide synthase (18) or the inhibition of the inductive nitric oxide synthase through the reduction of nuclear factor-kappaB (NF-kappaB) expression (19). On the other side, Provinol is the free radical scavenger. Pecháňová et al (2004) demonstrated a significant reduction of L-NAME (N^{omega}-nitro-L-arginine methyl ester) induced hypertension, myocardial fibrosis and vascular dysfunction via the increase of nitric oxide synthase (NOS) activity and prevention of oxidative stress after the Provinol treatment. In the next study, Buffoli et al (2005) showed that the administration of Provinol gave rise to the diminution of the step-up of iNOS expression in the kidney of cyclosporine A treated rats. It was probably resulting from the reduction of NF-kappaB expression. Under physiological conditions, constitutive forms of NOS (neuronal NOS, endothelial NOS) contribute to the production of low levels of NO in organism by the inhibition of NF-kappaB expression. It may contribute to limitation of processes such as immune inflammation. An important step to the enzymatic activity of constitutive forms of nitric oxide synthase (cNOS) is their thyrosine residuum phosphorylation. Under pathological circumstances, for example immune inflammation, the decrease of the intracellular concentration of NO below putative threshold is ongoing due to the phosphorylation of cNOS by bacterial lipopolysaccharides and (or) proinflammatory cytokines. The consequence of this process is a decrease in NOS activation associated with an increase in iNOS expression (20). This disequilibrium of NO can contribute to the lack of physiological NO in the airways and cause hyperreactivity and intensify the inflammation (21, 22). Thus activators of cNOS as well known as donors of NO might increase intracellular levels of NO together with the ability to keep a suppression of NF-kappaB activation (20).

For the mechanism action of Provinol, which is connected to NO, we determined to investigate the acute effect of Provinol on tracheal smooth muscle reactivity in the inflammation. The second part of our experiment included a confirmation of NO participation in the bronchodilatory mechanism action of Provinol.

Materials and methods

Materials

Red wine polyphenolic compounds (Provinol) consists of proanthocyanins, total anthocyanins, free anthocyanins, catechin, hydroxycinnamic acid and flavonols and were obtained from D.Ageron (Société Francaise de Distillerie, Vallont Pont d Arc, France). Another used chemicals such as ovalbumin (OVA), histamine hydrochloride (Hi), acetylcholine (ACh) were bought from the Sigma Aldrich (Taufkirchen, Germany).

Experimental protocol

All procedures were carried out according to EU directives and reviewed by the Ethical Committee of the Comenius University.

Guinea pigs (Trik) were acclimatized for one week in an animal house before the start of experiments.

OVA-induced allergic inflammatory in guinea pigs

Guinea pigs (200–250 g) of both sexes were randomly divided into 3 experimental groups. There were 12 animals in each group. The active sensitization of guinea pigs was realized by a subcutaneous injection of 5 mg OVA and by an intraperitoneal injection of 5 mg OVA on day 1.

5 mg OVA administered intraperitoneally was again utilized in the sensitization of guinea pigs on day 4. 14 days later, after the first dose of OVA, the sensitized animals were used for our experiments.

Experiments

The experiments were performed during in vivo and in vitro conditions. The guinea pigs in the first group received no treatment. The animals in the second group received Provinol in the peroral dose of 20 mg/kg during in vivo experiments, 10^{-4} mol.l^{-1} added into organ bath during in vitro experiments. Provinol and a non selective inhibitor NOS L-NAME, in the intraperitoneal dose 40 mg/kg during in vivo conditions, 10^{-6} mol.l^{-1} added into organ bath during in vitro conditions, were administrated to the third group in order to confirm the participation of NO in the bronchodilatory effect of Provinol.

Monitoring changes of tracheal smooth muscle reactivity was determined by the whole body plethysmography as the changes in specific airway conductance after an acute administration of Provinol during in vivo experiments. It was realized 14 days after the sensitization of guinea pigs by OVA. The specific airway conductance is a good predictor of tracheal smooth muscle reactivity in response to bronchoconstriction mediator Hi 10^{-6} mol.l^{-1} in vivo. Bronchodilatory effect of Provinol was studied 30 minutes and 5 hours after its peroral administration within the second group. L-NAME simultaneously added to Provinol helped to us to prove that NO plays the role in the mechanism of action of Provinol in the third group.

The second phase of our experiment continued during in vitro conditions. A tracheal smooth muscle reactivity parameter was studied by the insertion of tracheal strips into an organ baths
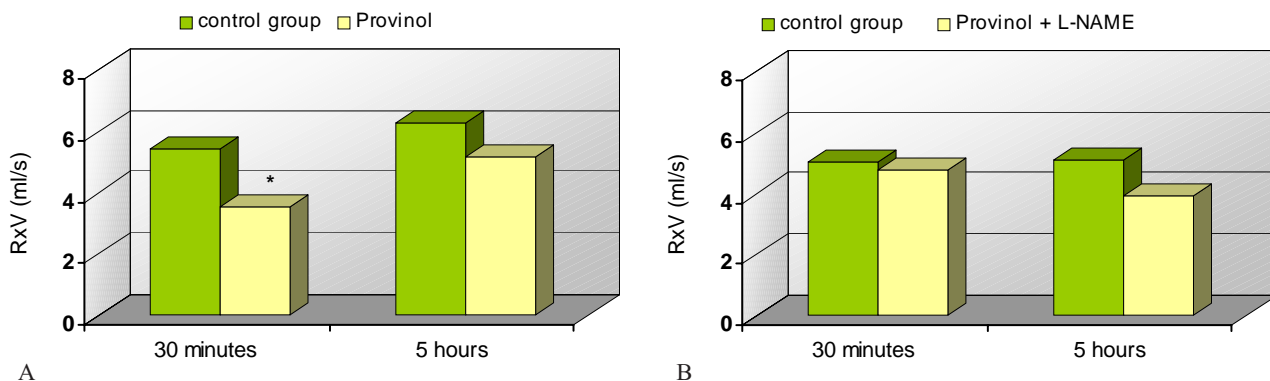
Fig. 1. The specific airways conductance (RxV/ml/s) of guinea pigs 14 days after sensitization by ovalbumin, 30 minutes and 5 hours after peroral administration of Provinol alone (A) or Provinol plus L-NAME (B) during in vivo conditions. Control group – ovalbumin sensitized guinea pigs without pretreatment, Provinol – second group, ovalbumin sensitized guinea pigs pretreated with Provinol alone, Provinol + L-NAME – third group, ovalbumin sensitized guinea pigs pretreated with Provinol plus L-NAME. Data are mean ±S.E.M. n=12; *p<0.05
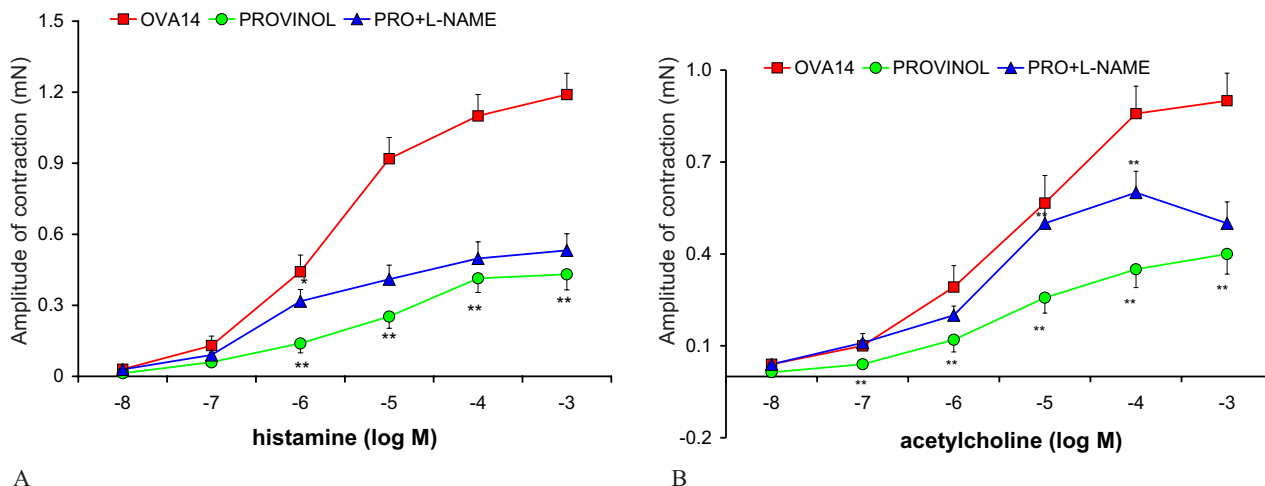

Fig. 2. Amplitude of tracheal smooth muscle contraction (mN) in ovalbumin sensitized guinea pigs in response to bronchoconstrictor Hi 10^{-8}–10^{-3} mol.l^{-1} (A) and ACh 10^{-8}–10^{-3} mol.l^{-1} (B) in vitro. OVA14 – control group with ovalbumin sensitized guinea pigs without pretreatment, PROVINOL – second group, ovalbumin sensitized guinea pigs pretreated with Provinol alone, PRO+L-NAME – third group, ovalbumin sensitized guinea pigs pretreated with Provinol plus L-NAME. Data are mean ±S.E.M. n=12; *p<0.05, **p<0.01

containing Krebs-Henseleit solution. The constant conditions were maintained (temperature, pH). Provinol alone or together with L-NAME were supplemented 30 minutes before the administration of bronchoconstrictor. The amplitude of tracheal smooth muscle contraction as a tracheal smooth muscle reactivity parameter was used in response to direct bronchoconstrictors Hi, ACh and the allergen OVA added into organ baths in rising concentrationes (Hi 10^{-8}–10^{-3} mol.l^{-1}, ACh 10^{-8}–10^{-3} mol.l^{-1}, OVA 10^{-7}–10^{-3} g.ml^{-1}).

Statistical data analysis

Statistical analysis was performed by a one-way analysis of variance. Differences were statistically significant when the p value was below 0.05. All results are expressed as mean ±S.E.M.

Results

A significant decrease in the specific airways conductance compared to the control group was observed 30 minutes after a peroral administration of Provinol during in vivo experiments. The decrease of the specific airways conductance was observed 5 hours after the administration, but was not significant (Fig. 1 A). The significant decrease of the specific airways conductance compared to the control group after an addition of the non-selective inhibitor NOS L-NAME became on no account (Fig. 1 B).

Provinol induced a high significant drop of the amplitude of tracheal smooth muscle contraction caused by Hi (Fig. 2 A) and ACh (Fig. 2 B) compared to the control group. L-NAME resulted in the partial inhibition of the bronchodilatory effect of

Provinol in both cases, more markedly after using ACh as bronchoconstrictor. The similar effect was noticed using a nonspecific bronchoconstrictor OVA. L-NAME inhibited even rescinded effect of Provinol, particular in low concentrations of OVA (10^{-6}–10^{-5} g.ml^{-1}).

This evidence supports our theory on the acute effect of Provinol on the relaxation of trachea smooth muscle in guinea pigs in allergic inflammation. NO plays an important role in the mechanism of this polyphenolic compounds.

Discussion

Allergic asthma is a chronic respiratory disease characterized by allergen induced early and late asthmatic reactions, airway hyperresponsiveness, airway inflammation and remodelling of the airways (23). An allergic inflammation (cellular infiltration, release of mediators, increased microvascular leakage, proinflammatory cytokines, chemokines, mucosal swelling, mucus hypersecretion) results in a damage of bronchial epithelium, where NO is produced by cNOS (24). Decrease of physiological low concentrations of NO leads to the induction of iNOS expression via activation of NF-kappaB. Synthesis of high levels of NO is the result (20). Experiments using a guinea pig model of an allergic asthma showed that tracheal hyperreactivity to metacholine after the early and late asthmatic reaction indicated a shortage of cNOS (25, 26). Experimental studies prove the fact that the relaxation of tracheal smooth muscle stimulated by endogenous NO in antigen induced contraction model of trachea smooth muscle was ongoing, but NO has not a significant direct relaxed effect on the airway smooth muscle in peripheral lung. The mechanism of NO effect involves the modulation of the release of cysteinyl- leukotrienes and prostaglandin E2 after the inhibition of NOS (27).

We studied bronchodilatory effect of Provinol during in vivo conditions in our experiments after an acute administration. We found out a significant decrease of the specific airways conductance 30 minutes after a peroral administration of Provinol, which corresponded to our hypothesis. It emerged that a relaxed effect of the tracheal smooth muscle was not significant 5 hours after the administration of Provinol. We suppose an association with subsequent adaptive mechanisms, which could enfeeble participation of NO in the mechanism action of Provinol.

We confirmed that Provinol has high significant bronchodilatory properties either by using specific mediators Hi, ACh or by using the non-specific constriction mediator OVA during in vitro conditions. We repeatedly demonstrated a partial participation of NO within an acute bronchodilatory action of Provinol. We ascertained a more evident inhibition of the relaxing effect of Provinol induced by L-NAME by using ACh as a bronchoconstrictor compared to Hi. It served as a confirmation of a marked suppression of cholinergic constriction response by endogenous NO in vitro. We assume a functional antagonism of ACh mediated by stimulation of cyclic guanosine monophosphate on the level of airway smooth muscle (28). L-NAME was inhibited even in annulled bronchodilatory influence of Provinol when using

bronchoconstrictor OVA, notably after addition of the low concentrations of OVA (10^{-6}–10^{-5} g.ml^{-1}) into organ bath. It only proves the mechanism action of Provinol via an important role of NO. After addition of the higher concentrations of OVA (10^{-4}–10^{-3} g.ml^{-1}) into organ bath, the fact that L-NAME did not prevent bronchodilatory effect of Provinol in airway smooth muscle, can be attributed to unknown global impact of Provinol. It will be necessary to study other mechanisms, which may participate in the relaxation of the trachea smooth muscle evoked by Provinol in future.

Because Provinol is a polyphenolic compounds, this raises a question, which of its components is a potential source of bronchodilatory effect in airway smooth muscle.

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