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Short communication

Ambroxol as an inhibitor of nitric oxide-dependent activation of soluble guanylate cyclase

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Abstract

The influence of ambroxol on the activity of human platelet soluble guanylate cyclase and rat lung soluble guanylate cyclase was investigated. Ambroxol in the concentration range from 0.1 to10 μM had no effect on the basal activity of both enzymes and slightly enhanced it at 50 and 100 μM . Ambroxol inhibited in a concentration-dependent manner the sodium nitroprusside-induced activation of both enzymes. The IC_{50} values for inhibition by ambroxol of sodium nitroprusside-stimulated human platelet soluble guanylate cyclase and rat lung soluble guanylate cyclase were 3.9 and 2.1 μM, respectively. Ambroxol did not influence the stimulation of soluble guanylate cyclase by protoporphyrin 1X. Thus, it is possible that the molecular mechanism of the therapeutic action of ambroxol involves the inhibition of nitric oxide (NO)-dependent activation of soluble guanylate cyclase.

Keywords: Guanylate cyclase; Nitric oxide (NO); Ambroxol

1. Introduction

Endogenous nitric oxide (NO) formed from L-arginine by L-arginine-nitric oxide-synthase (NOS) (Palmer et al., 1988) plays an important role in many physiological processes in the airways (Barnes and Belvisi, 1993). NO is the dominant transmitter of airway bronchodilator nerves (Belvisi et al., 1995); it prevents cholinergic mediated bronchoconstriction. NO is also involved in the regulation of pulmonary blood flow (Barnes and Lui, 1995), bronchial plasma exudation (Erjefalt et al., 1994) and airway ciliary beat frequency (Jain et al., 1993). NO and NO-donors were shown to relax airway smooth muscles in vitro owing to the activation of soluble guanylate cyclase and cyclic 3′,5′-guanosine monophosphate (cGMP) accumulation (Ward et al., 1995). A high concentration of NO in exhaled air leads to bronchodilatation (Dupuy et al., 1992) but does not affect the lung function of normal subjects and only insignificantly influences the bronchodilatation of asthmatic patients (Hogman et al., 1993).

The regulation of physiological functions in the airways is commonly mediated via NO formation catalyzed by constitutive nitric oxide synthase (cNOS) (Kharitonov et al., 1994b), whereas NO derived from inducible nitric oxide synthase (iNOS) is involved in inflammatory disturbances of the airways. In asthma, the exhaled NO concentration is increased abruptly (Kharitonov et al., 1994a) as a result of increased iNOS activity; the latter is in agreement with iNOS expression in the epithelial cells of asthmatic patients. iNOS induction occurs in response to the release of cytokines from macrophages and other inflammation-inducing cells. Therefore, the NO elevation in exhaled air owing to an increase in iNOS activity is observed not only in asthmatic patients but also in normal subjects with upper respiratory tract viral infection, in patients with bronchoextasis and upon chronic inflammation of airways caused by the formation of cytokines such as a tumor necrosis factor-α, interleukin-1β, interferon-γ (Kharitonov et al., 1995; Heiss et al., 1994; Barnes, 1994).

Normal functioning of the bronchial tract requires the formation of bronchial secretions. The formation of mucus and its mucociliar transport by ciliated epithelium of the bronchi and trachea is an important mechanism of normal airway function. The role of NO in the release of bronchial

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secretions has not however been investigated in detail. It is believed by some researchers that endogenous NO inhibits airway mucus secretion (in particular in ferret trachea) while NO synthase inhibitors increase mucus secretion (Rammarine et al., 1996). At the same time, NO formed in great amounts owing to iNOS expression (e.g. upon various inflammatory processes in the respiratory tract) was shown to increase mucus secretion (Adler et al., 1995) and to enhance phlegm thickness. It has also been reported that enhanced phlegm secretion is stimulated by increased levels of cGMP (Brown et al., 1993), consistent with the data on intensive NO formation, increased activity of the intracellular (NO-soluble guanylate cyclase-cGMP) signaling system and cGMP accumulation in asthmatic patients (Hobbs, 1997).

The inflammatory processes in the airways arising as a result of excessive NO formation are accompanied by increased mucus thickness. A need exists for new pharmaceutical drugs that lower the enhanced thickness of mucus and improve the mucociliary transport of bronchial secretions. Ambroxol is related to mucolytic drugs that increase phlegm secretion, which is conducive to phlegm dilution and normalisation of impaired airway function. Ambroxol (lasolvan) is a derivative of the alkaloid visicyn and is an active metabolite of bromgexin (bisolvan). Ambroxol has been used to increase surfactant secretion in the lungs, and has been reported to be effective in reducing the exacerbation of chronic bronchitis. The drug has a cough-suppressing effect (Nemcekova et al., 1998). It is frequently used in the treatment of bronchial asthma and chronic bronchitis and has been reported, following clinical and in vivo studies, to exhibit an anti-inflammatory action (Pfeifer et al., 1997). The therapeutic action of ambroxol has been observed in asthmatic patients, in patients with bronchitis and in patients with chronic viral infection, that is, in diseases accompanied by intensive NO formation, and hence, possibly increased soluble guanylate cyclase activity.

While the effect of ambroxol on soluble guanylate cyclase function has not yet been studied, it has been demonstrated that ambroxol attenuates excessive nitric oxide production (Lee et al., 1999). Moreover, Kotova and Yalkut (1981) have shown a sharp increase in cGMP level in platelets of asthmatic patients. This paper, therefore, investigates the possible biochemical mechanism of the therapeutic action of ambroxol, in particular, its influence on human platelet soluble guanylate cyclase and rat lung soluble guanylate cyclase, and its effect on the NO-dependent activation of the two enzymes by sodium nitroprusside.

2. Materials and methods

In this study, human platelets and rat lungs were used as sources of soluble guanylate cyclase. Platelets were isolated from the blood of donors in accordance with Chirkov et al. (1987). A suspension of washed platelets in 50 mM Tris–HCl buffer (pH 7.6) containing 0.2 mM dithiothreitol was sonicated in an MSE 5-78 ultrasonic sonicator (UK) for 20 s at 2°C and centrifuged at 105,000 g. The supernatant was used as human platelet soluble guanylate cyclase. Rat lung was homogenised in 50 mM Tris–HCl buffer (pH 7.6) containing 0.5 mM dithiothreitol. The activity of rat lung soluble guanylate cyclase was measured in the supernatant obtained after centrifugation of 10% lung homogenate at 105,000 g for 1 h.

Guanylate cyclase activity was assayed as described by Garbers and Murad (1979). Briefly, the samples (final volume 150 μl) contained 50 mM Tris–HCl buffer (pH 7.6), 1 mM guanosine triphosphate, 4 mM $MgCl_2$, 4 mM creatine phosphate, 20 μg (120–160 units) creatine phosphokinase, 10 mM theophylline, and 20 μg of human platelet or 50 μg of rat lung 105,000 g supernatants, respectively. The effect of ambroxol was studied in the concentration range from 0.1 to 50 μM. The amount of cGMP formed (15 min, 37°C) was estimated by Enzyme-Linked Immuno-Sorbent Assay (ELISA) method using Bioimmunogen kits (Russia). Protein was determined by the method of Bradford (1976). The following reagents were used: guanosine triphosphate sodium salt (Fluka, Switzerland) and ambroxol (Boehringer Ingelheim, Germany). Other reagents were from Sigma (USA).

Statistical differences were evaluated using the Student's *t*-test.

3. Results

It was found that ambroxol in the concentration range from 0.1 to10 μM had no influence on the basal activity of human platelet soluble guanylate cyclase and rat lung soluble guanylate cyclase, but slightly enhanced it (about 1.5 fold) at 50 μM. Fig. 1 shows the dose–response curves of the effects of ambroxol on human platelet soluble guanylate cyclase (curve 1) and rat lung soluble guanylate cyclase (curve 2) activation by sodium nitroprusside (100 μM). In both cases, ambroxol attenuated sodium nitroprusside-stimulated guanylate cyclase activity with an IC_{50} value of 3.9 and 2.1 μM for human platelet guanylate cyclase and rat lung guanylate cyclase, respectively. Inhibition of NO-dependent guanylate cyclase activation was also confirmed in a series of independent experiments with protoporphyrin 1X-induced soluble guanylate cyclase activation.

Protoporphyrin 1X, an immediate heme precursor, is an endogenous stimulator of guanylate cyclase activity (Ignarro et al., 1982). However, in contrast with NO, which requires the guanylate cyclase heme for activation, the latter is not involved in the protoporphyrin 1X-induced stimulation of the enzyme (Ignarro, 1992). The addition of 10 μM ambroxol (final concentration) did not influence

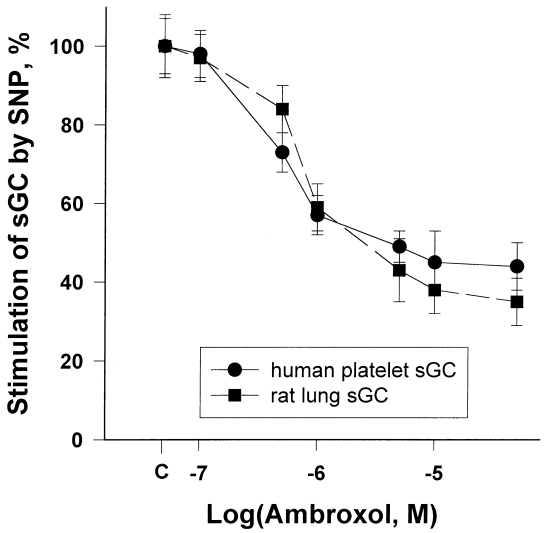

Fig. 1. Effect of ambroxol on the sodium nitroprusside-stimulated soluble guanylate cyclase activity from human platelets (1) and rat lung (2). Soluble guanylate cyclase (GC) activity was determined in the presence of 100 μM sodium nitroprusside (SNP) and in the absence (con) and presence of ambroxol (0.1–50 μM). Abscissa: ambroxol concentration in the sample ($\log M$). Ordinate: sodium nitroprusside-stimulated guanylate cyclase activity in the absence of ambroxol (con) was taken as 100%. Basal guanylate cyclase activity from human platelets and rat lung was 152 ± 16 and 18 ± 1.8 pmol cGMP/min/mg of protein, respectively. Guanylate cyclase activity from human platelets and rat lung in the presence of 100 μM sodium nitroprusside was 1733 ± 140 and 413 ± 28 pmol cGMP/min/mg of protein, respectively. Data represent means ± S.D. of three independent experiments.

human platelet guanylate cyclase activation by protoporphyrin 1X (5 μM) (guanylate cyclase activity with and without ambroxol was 428 ± 25 and 403 ± 20 pmol cGMP/min/mg of protein, respectively; the basal guanylate cyclase activity was 84 ± 3.8).

The molecular mechanism of the inhibition by ambroxol of NO-dependent guanylate cyclase activation is not clear. In order to investigate a possible inhibitory mechanism, we compared the effect of ambroxol on sodium nitroprusside-induced human platelet guanylate cyclase activation with that of ionol (Table 1). Ionol, like ambroxol, in final concentration 10 μM inhibited sodium nitroprusside-induced guanylate cyclase activation. The inhibitory effect was more pronounced for ambroxol (Table 1). Ionol has antioxidative properties and inhibits the activation of soluble guanylate cyclase by sodium nitroprusside (Craven and De Rubertis, 1977) in the course of its chemical interaction with nitric oxide (Janzen et al., 1993). Ambroxol in final concentrations from 1 to 100 μM (comparable with those used in our study) also exhibited antioxidative activity (Gillissen et al., 1997; Nowak et al., 1994). An increase in basal human platelet guanylate cyclase activity from 70 ± 3.5 pmol cGMP/min/mg of protein in the absence of ambroxol to 105 ± 3.8 and 133 ± 5.7 pmol cGMP/min/mg of protein in the presence of ambroxol 50 and 100 μM, respectively, is consistent with the antioxidative properties of this drug.

4. Discussion

Ambroxol is a drug used in the treatment of inflammatory and other disturbances of airway function. These disturbances are associated with excessive nitric oxide formation within inflamed airways, enhancing the activation of soluble guanylate cyclase and cGMP accumulation. The data presented here demonstrate that ambroxol is an inhibitor of NO-dependent activation of soluble guanylate cyclase and thus reveal, for the first time, the possible biochemical mechanism of the therapeutic action of ambroxol. It is also possible that the inhibition of sodium nitroprusside-induced soluble guanylate cyclase activation by ambroxol results in suppression of excessive mucus secretion, thus lowering phlegm viscosity and improving the mucociliary transport of bronchial secretions. Our data on the close similarity of the inhibitory effect of ambroxol on sodium nitroprusside-induced activation of human platelet guanylate cyclase and rat lung guanylate cyclase (Fig. 1) suggest that the study of NO-dependent human platelet guanylate cyclase activation and inhibition of this stimulation by ambroxol would be useful for evaluating the intensity of airway inflammatory disturbances mediated by excessive NO formation and the effectiveness of treatment with ambroxol. Since the inflammatory processes in the airways of both normal subjects and asthmatic patients are accompanied by iNOS expression with excessive NO formation (Morris and Billar, 1994) and, hence, by activation of soluble guanylate cyclase, we believe that the molecular mechanism of the therapeutic

Table 1
Effect of ionol and ambroxol on human platelet guanylate cyclase activity and sodium nitroprusside-induced enzyme activation

Additives	Guanylate cyclase activity (pmol cGMP/min/mg of protein)	
	Without sodium nitroprusside	With sodium nitroprusside (100 μM)
None	70 ± 3.5	952 ± 47
Ionol (10 μM)	102 ± 4.4[a]	686 ± 33[a]
Ambroxol (10 μM)	73 ± 5.3[b]	470 ± 25[b]

Data represent means ± S.D. of guanylate cyclase activity of four independent experiments.
[a] $P < 0.01$ compared with control.
[b] $P < 0.05$ effect of ambroxol vs. ionol.

action of ambroxol may involve the inhibition of NO-dependent activation of this enzyme. The demonstration of iNOS expression and excessive NO production upon inflammation in normal and asthmatic airways leads us to suggest that iNOS inhibitors may be efficient therapeutic agents in these pathological states (Barnes, 1995). Studies are currently in progress in this direction. The data on ambroxol inhibition presented here demonstrate for the first time that a therapeutic effect analogous to the one elicited by an iNOS inhibitor may also occur with inhibitors of NO-dependent soluble guanylate cyclase activation.

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