

Angiotensin II potentiates methacholine-induced bronchoconstriction in human airway both *in vitro* and *in vivo*

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Angiotensin II potentiates methacholine-induced bronchoconstriction in human airway both in vitro and in vivo. E.A. Millar, J.E. Nally, N.C. Thomson. ©ERS Journal Ltd 1995.

ABSTRACT: Angiotensin II levels are elevated in patients with acute severe asthma. In addition, intravenous angiotensin II causes bronchoconstriction in mild asthmatic patients. In the present study, we examined the effects of this hormone on bronchi *in vitro* and its interaction with the cholinergic agonist methacholine both *in vivo* and *in vitro*.

Contractions of rings of human bronchi were measured isometrically. Concentration-response curves were obtained to angiotensin II and to methacholine in the presence and absence of angiotensin II. In addition, seven asthmatic patients with mild bronchial hyperreactivity to methacholine received placebo, angiotensin II, 1 or 2 ng·kg⁻¹·min, by infusion, followed by methacholine challenge. Forced expiratory volume in one second (FEV₁) values were measured at baseline, at the end of the infusion and during methacholine challenge.

Angiotensin II alone *in vitro* evoked small contractions of human bronchi (<0.25 g wt). Pre-incubation with low concentrations of angiotensin II significantly enhanced contractions to methacholine. In mild asthmatic patients, angiotensin II alone evoked no change in baseline FEV₁ values at the levels studied. Compared to placebo, angiotensin II 2 ng·kg⁻¹·min, but not 1 ng·kg⁻¹·min, evoked a significant increase in bronchial reactivity to methacholine.

Angiotensin II in subthreshold concentrations enhances methacholine-evoked bronchoconstrictions both in human bronchi *in vitro* and in mild asthmatic patients *in vivo*. Our findings suggest a novel role for angiotensin II as a putative mediator in asthma.

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Angiotensin II is an octapeptide hormone with potent vasoconstrictor activity [1]. In a number of tissues, pre-treatment with angiotensin II can uncover or enhance contractions evoked by other agonists, *e.g.* in rabbit saphenous artery, α_2 -adrenoceptor-mediated contractions are enhanced in the presence of low concentrations of angiotensin II [2]. In addition, angiotensin II has been shown to potentiate vagal-mediated contractions of rabbit trachea, probably by prejunctional stimulation of acetylcholine release [3].

We have recently observed elevated plasma angiotensin II levels in patients with acute severe asthma [4], and have also shown that when mild asthmatic patients receive this hormone intravenously at doses which evoke similar plasma levels of angiotensin II to that observed in acute asthma, it causes bronchoconstriction [4]. The mechanism of action of angiotensin II in causing this contraction is as yet unknown and may involve either a direct action on airway smooth muscle, or perhaps modulation of the effects of other mediators of bronchoconstriction.

In the present study, we examined the direct effects of angiotensin II *in vitro* in human bronchial smooth muscle. We also investigated the ability of angiotensin II to interact with the bronchoconstrictor, methacholine. This study was then extended to examine *in vivo* the ability of angiotensin II to modulate bronchoconstriction evoked by inhaled methacholine in patients with mild asthma, where these patients were known to exhibit hyperresponsiveness to methacholine.

Materials and methods

In vitro

Tissue collection and preparation. Macroscopically normal human bronchial tissues (3rd–6th order) were obtained from patients undergoing thoracic surgery for bronchial carcinoma. Tissues were dissected free of connective tissue and fat and stored overnight at 4°C in oxygenated

Krebs-Henseleit solution of the following composition (mM), NaCl 118.4, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 24.9, KH₂PO₄ 1.2 and glucose 11.1. Published data have shown that overnight storage of this tissue does not alter its reactivity [5].

Measurement of contractile responses. Contractile responses were measured from rings of human bronchi (3–5 mm) in vertical organ baths (10 ml) at 37±0.5°C in oxygenated (95% O₂, 5% CO₂) Krebs-Henseleit solution. Tension (2 g wt) was applied *via* two platinum wires into the lumen. One wire was anchored and the other attached to a force displacement transducer (Grass FT-03T). Tissues were allowed to equilibrate for 2 h, during which time the tension applied was readjusted to the initial level. Concentration-response curves were constructed to angiotensin II to ascertain the threshold for contraction to this hormone. In a subsequent set of experiments, concentration-response curves to methacholine were constructed in the presence and absence of angiotensin II (10⁻⁷ M). Drugs were added directly to the organ bath and angiotensin II was added 15 min before methacholine concentration-response curves.

In vivo

Patients. Seven mild asthmatic patients (3 females and 4 males) were recruited from the out-patient clinic mean (SD) age 27 (8) yrs, mean (SEM) forced expiratory volume in one second (FEV₁) 82 (9)% predicted. All patients demonstrated at least mild bronchial hyperreactivity to inhaled methacholine according to the method of COCKROFT *et al.* [6]. All patients were taking inhaled short-acting β₂-agonists as required in addition to regular inhaled corticosteroids. None were on long-acting inhaled β₂-agonists or oral theophylline. All patients were otherwise in good health, in particular, none had a history of hypertension or was on diuretic therapy.

For the purposes of each study visit inhaled β₂-agonists were discontinued for at least 8 h prior to attendance but inhaled corticosteroids continued unchanged. The study had the approval of the Glasgow West Ethics Committee and informed written consent was obtained from each patient.

Study design. A randomized double-blind study design was employed. At an initial screening visit, the individual concentration of methacholine required to provoke a 20% fall in FEV₁ was determined (PC₂₀). Thereafter patients attended the laboratory on three separate study days. At each visit baseline FEV₁ was measured and blood pressure recorded. An intravenous cannula (Venflon) was inserted into a forearm vein followed by a 20 min rest. A baseline blood sample was obtained for estimation of plasma angiotensin II. Patients then received an intravenous infusion of placebo (5% dextrose) or angiotensin II (1 or 2 ng·kg⁻¹·min) for a period of 40 min. These doses were chosen as being below threshold for bronchoconstriction in a previous study [4]. The drugs were administered by 50 ml syringe driver (Perfusor

Secura E, B.B. Braun Melsunger AG, Germany). A second blood sample was obtained (25 min from commencement of infusion) from the contralateral arm for estimation of plasma angiotensin II and the FEV₁ was recorded. While the intravenous infusion continued, patients received a methacholine challenge to determine the PC₂₀. Following completion of the methacholine challenge, a final blood sample was withdrawn from the contralateral arm for plasma angiotensin II measurement and the intravenous infusion was discontinued. The effect of methacholine was then rapidly reversed with inhalation of nebulized salbutamol.

Blood pressure was monitored at 15 min intervals and patients remained semirecumbent for the duration of the study.

Measurements

FEV₁. This was measured using a dry wedge spirometer (Vitalograph S, Vitalograph, Buckingham, UK), the best of three measurements being taken at each time-point.

Pulse and blood pressure. These were measured using a semiautomatic sphygmomanometer (Critikon; Dinamap Vital Signs Monitor 1846 FX, Berkshire, UK). Three readings were taken at each time-point from the non-dominant arm, the mean being recorded.

Methacholine inhalation test. After measurement of baseline FEV₁, a saline inhalation was administered and the doubling doses of nebulized methacholine were dispensed at 5 min intervals, beginning with 0.0312 mg·mL⁻¹. Each concentration was given for 2 min *via* a Wright's gas nebulizer driven by compressed air, with an output of 0.13 mL·min⁻¹. The FEV₁ was measured at 0.5, 1.5 and 3 min after each inhalation, until a fall of at least 20% was achieved. The result was then expressed as the PC₂₀.

Angiotensin II assay. Blood samples for plasma angiotensin II analysis were collected into iced glass sample tubes containing ethylenediamine tetra-acetic acid (EDTA) and o-phenanthroline inhibitor. These were kept on ice and separated by centrifugation within 2 h of collection. Thereafter, plasma was frozen at -20°C until hormone analysis. The assay for angiotensin II is a modified radioimmunoassay which uses C¹⁸ cartridges (Sep-Pak®; Waters, Milford, MA, USA) to extract angiotensin II from plasma [7]. The intra-assay coefficient of variation is 6.4% and interassay variation 10%. The reference range for our laboratory is 3–12 pg·mL⁻¹.

Drugs. The following chemicals were used: methacholine chloride (Sigma) and angiotensin II acetate (Sigma). For the *in vitro* studies, both methacholine and angiotensin II were prepared in distilled water and then serial dilutions prepared in Krebs-Henseleit solution. Where *in vivo* studies were undertaken, methacholine was diluted in phosphate buffered saline from a stock solution

containing methacholine ($64 \text{ mg}\cdot\text{mL}^{-1}$). Angiotensin II was prepared in 5% dextrose.

Statistical analysis. Statistical significance between data samples in the *in vitro* studies was tested by two-way analysis of variance (ANOVA). Statistical difference between pD_2 values ($-\log \text{EC}_{50}$; the concentration effecting 50% of control maximum contraction) was by Student's t-test. In the *in vivo* studies, differences between placebo and active days was by Student's t-test with subsequent Dunnett test performed on logarithmically transformed data. A probability level of p less than 0.05 was considered significant. Number of observations (n) refers to the number of patients tested or from which tissue was obtained.

Results

In vitro studies

Angiotensin II produced small ($<0.25 \text{ g wt}$ maximum), concentration-dependent contractions of human bronchi, with the threshold for contraction occurring between 3×10^{-8} and $3\times 10^{-7} \text{ M}$.

Preincubation with angiotensin II (10^{-7} M) evoked contraction in only two out of six tissues and in those cases the level of contraction was less than 0.1 g wt (compared with control maximum contraction of $2.5\text{--}4.5 \text{ g wt}$ with methacholine ($3\times 10^{-4} \text{ M}$)). This concentration of angiotensin II, however, significantly ($p<0.001$) enhanced contractions to methacholine. This did not manifest itself as a shift in pD_2 values, but rather as an increase in the magnitude of contractions evoked at concentrations of methacholine above 10^{-6} M . The maximum response was increased by $39\pm 3\%$ in human tissues ($n=6$, fig. 1).

In vivo studies

There was no significant difference between baseline FEV1 on any study day (mean (SEM) baseline FEV1 values were $2.98 (0.4)$, $2.8 (0.4)$ and $2.9 (0.3)$ prior to

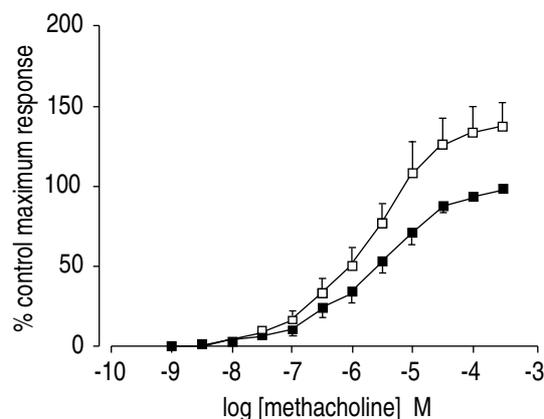


Fig. 1. — Concentration response curves evoked by methacholine (10^{-9} to $3\times 10^{-4} \text{ M}$) alone or in the presence of angiotensin II (10^{-7} M). Angiotensin II produced a significant ($p<0.001$ by two-way analysis of variance (ANOVA)) potentiation of the concentration response curves evoked by methacholine. Number of observations (n)=6. —■—: control; —□—: plus angiotensin II (10^{-7} M).

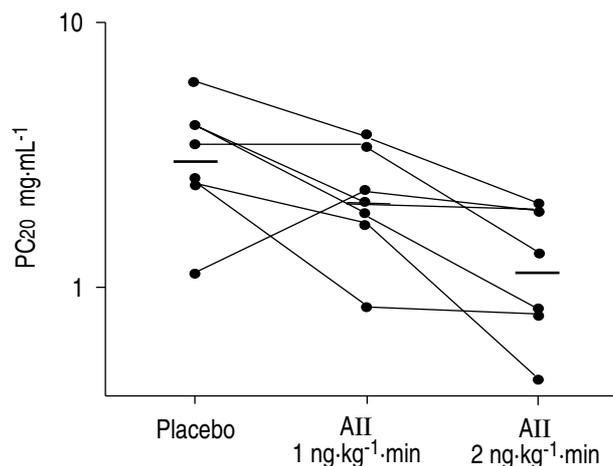


Fig. 2. — The concentration of methacholine provoking a 20% fall in forced expiratory volume in one second (PC_{20}) after placebo infusion (5% dextrose) and after *i.v.* infusion with angiotensin II $1 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$ or angiotensin II $2 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$ in mild asthmatic patients. Angiotensin II $1 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$ produced an insignificant fall in PC_{20} . Angiotensin II $2 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$, however, significantly ($p=0.006$, by Student's t-test with Dunnett's post-test) potentiated the effect of inhaled methacholine in 6 out of 7 patients, resulting in a significant decrease of PC_{20} compared to placebo (horizontal bars equal mean values). AII: angiotensin II.

placebo, angiotensin II $1 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$ and angiotensin II $2 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$ respectively).

After infusion of angiotensin II (1 or $2 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$) and prior to methacholine challenge, there was no significant change in baseline FEV1 values ($2.8 (0.4)$, $2.74 (0.4)$ and $2.96 (0.4)$ for placebo, angiotensin II $1 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$ and angiotensin II $2 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$, respectively). The PC_{20} for methacholine (expressed as geometric mean with range) after placebo infusion was $3.09 (1.15\text{--}6.0) \text{ mg}\cdot\text{mL}^{-1}$. After infusion with angiotensin II $1 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$, this decreased to $2.14 (0.85\text{--}3.8) \text{ mg}\cdot\text{mL}^{-1}$, although this did not reach statistical significance. In 6 of the 7 patients, angiotensin II $2 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$ potentiated the effect of inhaled methacholine. There was a significant ($p=0.006$) decrease in PC_{20} compared to placebo (geometric mean $1.2 (0.45\text{--}2.08) \text{ mg}\cdot\text{mL}^{-1}$) (fig. 2). Plasma angiotensin II concentrations (at baseline, prior to and on completion of each infusion) were mean (SEM) $9.2 (1.8)$, $9.5 (2.0)$ and $9.9 (1.8) \text{ pg}\cdot\text{mL}^{-1}$ after placebo, $5.1 (1.2)$, $16.2 (0.9)$ and $27.3 (5.2) \text{ pg}\cdot\text{mL}^{-1}$ after angiotensin II $1 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$ and $6.0 (1.5)$, $32.9 (5.1)$ and $37.7 (9.5) \text{ pg}\cdot\text{mL}^{-1}$ after angiotensin II $2 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}$, respectively. Blood pressure did not alter significantly during angiotensin II infusion (data not shown), and patients did not report any adverse symptoms.

Discussion

The presence of angiotensin II in subthreshold concentrations markedly enhanced contractions evoked by the cholinergic agonist methacholine both *in vitro* and in mild asthmatic patients *in vivo*. Furthermore, the potentiating effect of angiotensin II occurred at doses which did not themselves cause bronchoconstriction. These *in vitro* results are in keeping with our previous findings [8], where subthreshold concentrations of angiotensin II likewise potentiated methacholine-evoked contraction

in bovine bronchi. In addition, we have shown that angiotensin II potentiates the effect of endothelin-1 in bovine bronchi *in vitro* [9] suggesting that this interaction with angiotensin II is not unique to cholinergic agents. The levels of angiotensin II used in the present study *in vitro* were higher than occurs in the plasma of normal subjects. However, local levels in the airway may not match the general plasma level; and, in addition, in conditions such as acute severe asthma or severe congestive cardiac failure, angiotensin II levels can be markedly elevated [4, 10]. *In vivo*, the circulating levels of angiotensin II produced were similar to those seen during exercise [11]. Thus, the potentiating action observed with angiotensin II on airway responsiveness is likely to have important effects on airway function both at physiological and pathological circulating concentrations of this hormone.

The means by which angiotensin II may potentiate bronchoconstriction is unclear, although a number of possibilities exist. YAMAWAKI *et al.* [3] demonstrated in rabbit trachea that angiotensin II potentiated contractions evoked by electrical field stimulation, by increasing acetylcholine release. This occurred in the absence of any change in baseline tone evoked by angiotensin II alone. It is unlikely that reflex changes in vagally-mediated tone account for this action of angiotensin II in the present study, as blood pressure and heart rate were unaltered in the subjects during angiotensin II infusion. In addition, in the present study, *in vitro*, we have demonstrated a postjunctional effect of angiotensin II in potentiating methacholine-evoked contractions. The possibility, therefore, exists that angiotensin II may act both pre- and postjunctionally to facilitate bronchoconstriction.

Other means by which angiotensin II may enhance bronchoconstriction include the release of potential spasmogens, such as endothelins [12], platelet-activating factor [13], or arachidonic acid metabolites [14].

It has previously been reported that angiotensin II evokes bronchoconstriction when present in higher concentrations, such as occurs in acute severe asthma [4]. It now seems likely that the presence of relatively low concentrations of this hormone may result in paradoxically large constrictions by potentiating other mediators of bronchoconstriction. This may be of clinical importance not only during acute attacks of asthma, but also in patients with exercise-induced asthma where angiotensin II levels are elevated [11]. In addition, our findings may be of relevance to patients receiving high dose nebulized β_2 -agonists since plasma angiotensin II levels comparable to those in the present study have been observed after nebulized salbutamol [15].

In conclusion, angiotensin II potentiates methacholine-evoked bronchoconstrictions, both in isolated human bronchi and in mild asthmatic patients. This may represent an important mechanism by which levels of angiotensin II which have little direct activity may produce substantial changes in airway tone. The mechanism for this interaction has yet to be elucidated. These findings suggest a novel role for angiotensin II as a putative mediator in asthma, and further studies to examine the effects

of angiotensin II receptor antagonists in different forms of experimental asthma are now indicated.

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