RESPIRATORY
MEDICINE

The ACE gene polymorphism and cough threshold for capsaicin after cilazapril usage

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Persistent dry cough is an occasional but clinically important adverse reaction to angiotensin I-converting enzyme (ACE) inhibitors (ACEI). Its reported incidence is variable, and why cough occurs in only certain individuals has been unclear. An insertion/deletion (I/D) polymorphism of the ACE gene is associated with serum ACE activity. We have previously shown that susceptibility to cough induced by ACEI is associated with this polymorphism such that patients with genotype II are more susceptible to cough than patients with other genotypes. In order to confirm and extend our previous observation, we conducted a randomized, placebo-controlled, double-blind, cross-over study in 10 healthy volunteers with genotype II and 10 with genotype DD. The cough threshold was determined by the concentration of inhaled capsaicin causing two or more coughs. After the usage of an ACEI, cilazapril, for 4 weeks, changes in the cough threshold in subjects with genotype II [before: $6 \cdot 6 \pm 3 \cdot 7$ nM (mean $\pm$ SD); after: $5 \cdot 0 \pm 4 \cdot 6$ nM] significantly differed from those in subjects with genotype DD (before: $9 \cdot 0 \pm 9 \cdot 4$ nM; after: $9 \cdot 3 \pm 9 \cdot 1$ nM). Skin responses to intradermal bradykinin, which is a substrate of ACE and tussigenic, were significantly increased in subjects with genotype II (before: $1 \cdot 6 \pm 0 \cdot 6$ *vs.* after: $2 \cdot 6 \pm 0 \cdot 5$ cm^2, $P < 0 \cdot 05$) but not in subjects with genotype DD (before: $1 \cdot 4 \pm 0 \cdot 5$ *vs.* after: $1 \cdot 6 \pm 0 \cdot 6$ cm^2, n.s.) after usage of cilazapril. By contrast, skin responses to intradermal substance P did not change in subjects with either genotype. These findings provide further evidence of a link between ACEI-induced cough and I/D polymorphism of the ACE gene and suggest that ACEIs induce cough by modulating the tissue level of bradykinin.

Key words: ACE gene polymorphism; ACE inhibitor; cough; bradykinin; substance P.

Introduction

Cough accompanied by an increased sensitivity of the cough reflex is the most common symptom of inflammatory airway diseases. Susceptibility to cough is also frequently reported in patients receiving angiotensin I-converting enzyme (ACE) inhibitors (ACEI) for treatment of hypertension and/or heart failure (1–3). Because cough is usually unresponsive to treatment, ACEI treatment can become intolerable for some patients. Why only certain patients are affected by this unique side-effect remains unclear.

Several mechanisms have been postulated for ACEI-induced cough, but none adequately explains the phenomenon. Bradykinin and substance P, both of which are degraded by ACE, have been suggested to cause sensitization of airway sensory nerves and enhance the occurrence

of cough (4–10). The inflammatory skin and mucous membrane reactions elicited by ACEIs, such as rash and angioedema, are rare but potentially life-threatening. These adverse tissue reactions may also be mediated by kinins, since the wheal responses to intra-dermal bradykinin and substance P have been demonstrated to be potentiated by treatment with ACEIs (11,12). Thus, cough and angioedema are likely to have common causative mediators whose levels are regulated by tissue activity of ACE.

Rigat *et al.* have described an insertion/deletion (I/D) polymorphism of the human ACE gene, which consists of the presence or absence of a 287 base pair (bp) insert in intron 16 on chromosome 17q23 (13). The most notable and clinically relevant phenomenon is that this polymorphism of the ACE gene is associated with inter-individual variations in serum ACE activity, which is high in genotypes II, ID and DD, in increasing order (13,14). This fact indicates that catalytic rates of mediators responsible for cough are also high in this order, and thus that I/D polymorphism of ACE may affect the occurrence rate of ACEI-induced cough. Indeed, we have previously reported that the genetic susceptibility to cough induced by ACEIs in patients with hypertension and/or heart failure may be

related to I/D polymorphism, with subjects having genotype II being most susceptible to cough (15).

To prospectively examine the association between the occurrence of ACEI-induced cough and I/D polymorphism of the ACE gene, we measured cough threshold as determined by inhalation of capsaicin, a relatively specific C-fibre stimulant, before and after administration of an ACEI, cilazapril, and investigated the relation between changes in the threshold and the gene polymorphism in healthy volunteers. We also examined dermal response to bradykinin and substance P in an attempt to estimate ACE activity in the tissue.

Subjects and methods

SUBJECTS

Twenty healthy female subjects without any lung and airway disease were selected so as to provide equal numbers of the two genotypes, i.e. $n=10$ for each of the genotypes DD [age, $19\cdot3\pm0\cdot9$ (SD) years] and II ($19\cdot6\pm0\cdot7$ years). Since women have been reported to be more susceptible to cough induced by ACEI (4), we selected only female volunteers, expecting more occurrence of cough in the present study. All were non-smokers and normotensive. They gave written informed consent to participate in the study, which was approved by the Medical Ethics Committee of the School of Medicine, Hokkaido University.

DETERMINATION OF ACE GENOTYPE

Genomic DNA was purified from peripheral blood leukocytes by the use of an extraction kit (SepaGene; Sanko Junyaku Co. Ltd., Tokyo, Japan). A 287-bp I/D polymorphism in intron 16 of the ACE gene was identified by polymerase chain reaction (PCR) as described previously (16). Briefly, two primers, sense oligo 5′-CTGGA-GACCACTCCCATCCTTTCT-3′ and anti-sense oligo 5′-GATGTGGCCATCACATTCGTCAGAT-3′, were synthesized to amplify the polymorphic fragment. Reactions were performed with 10 pmol of each primer in a final volume of 50 μl containing 100 ng of genomic DNA, 3 mM MgCl$_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8·4, 0·1 mg ml^{-1} gelatin, 0·5 mM of each dNTP, and 1 unit of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT, U.S.A.). The DNA was amplified for 30 cycles. The PCR products were electrophoresed in 4% agarose gel and visualized by ethidium bromide staining.

STUDY PROTOCOL

The present study was conducted in a randomized, placebo-controlled, double-blind, cross-over fashion. Randomization was performed by one of the investigators, and capsaicin challenge tests and skin tests were performed by the others. Placebo containing exact exipients of cilazapril tablets (Inhibace^{TM}) were prepared at the Pharmaceutical

Division of Hokkaido University Hospital. Each subject received oral doses of 1 mg cilazapril and matched placebo daily for 4 weeks in a random order on two separate occasions with a washout period of 2 weeks between each administration period. The capsaicin challenges were performed before and 2 h after dosing on the first and the last day of each administration period. Study subjects took no other medication for the duration of the study.

CAPSAICIN CHALLENGE TESTS

Cough challenge testing with inhaled capsaicin was performed as previously described (2). Capsaicin (Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany) was dissolved in ethanol and diluted in saline in doses of 0·0, 0·1, 0·2, 0·4, 0·8, 1·6, 3·2, 6·4, 12·8, 25·6, and 51·2 nM. The subjects wore a nose clip and inhaled single breaths of aerosol generated by a nebulizer (Model 646; DeVilbiss Health Care Inc., Somerset, PA, U.S.A.) in incremental doses starting from pure saline (0.0 nM of capsaicin) with a 5-min interval between doses. The number of coughs occurring during a 5-min interval after each dose was recorded. The concentration of capsaicin causing two or more coughs was taken as the cough threshold (17).

SKIN REACTION

Skin responses were examined in nine subjects with genotype II and nine with DD. Two injection sites were marked on the volar aspect of the dominant forearm, and 0·1 ml of saline containing 10 mg ml^{-1} bradykinin (BACHEM Feinchemikalien AG, Bubendorf, Switzerland) or 0·1 mg ml^{-1} substance P (BACHEM Feinchemikalien AG) was administered intradermally. Skin responses were photographed after 15 min and quantified as the wheal area outlined in the photos. A digital computer and NIH image (Wayne Rasband Analytics, NIH, MD, U.S.A.) were used to calculate the wheal area.

MEASUREMENT OF ACE ACTIVITY, BRADYKININ AND SUBSTANCE P

Serum ACE activities were measured by colorimetric assay (ACE Color; Fujirebio Inc., Tokyo, Japan) (18). Plasma bradykinin (19) and substance P (20) were determined by radioimmunoassay. All samples were stored at $-70°$C until the assay was performed.

STATISTICAL ANALYSIS

Data are presented as mean$\pm$SD. The changes between values before and after the usage of cilazapril or placebo in different genotypes were compared by a two-way analysis of variance (ANOVA) with one between-subject factor (i.e. factor 'genotype') and one within-subject factor (i.e. factor 'time': repeated measurements before and after the usage of cilazapril or placebo in the same study subjects) or with two within-subject factors (i.e. factors 'time' and 'period'). We

compared serum ACE activities and skin responses before and after the administration of cilazapril or placebo by the Student's paired *t*-test. The Student's unpaired *t*-test was used to assess difference in baseline serum ACE levels or cough thresholds between subjects with the genotype II and those with DD. Statistical analyses were performed using the SPSS statistical package (SPSS, Chicago, IL, U.S.A.). Differences with a *P*-value less than 0·05 were considered significant.

Results

All subjects completed the study. Each subject recorded symptoms in a 'symptom diary'. None developed cough throughout the study period. After the administration of cilazapril, serum ACE activities were effectively reduced in all subjects, and an interaction between genotypes and changes in ACE activities during the cilazapril period was significant by a two-way ANOVA (Fig. 1). ACE activities after the use of cilazapril were significantly decreased compared with those before the use of cilazapril by the Student's paired *t*-test both in subjects with genotype II (before: $11\cdot1\pm3\cdot7$ *vs*. after: $3\cdot8\pm4\cdot1$ $IU\,l^{-1}$, $P<0\cdot01$) and those with DD (before: $17\cdot0\pm2\cdot3$ *vs*. after: $3\cdot2\pm3\cdot5$ $IU\,l^{-1}$, $P<0\cdot01$). An interaction between genotypes and changes in ACE activities during the placebo period was not significant by a two-way ANOVA (genotype II, before: $12\cdot0\pm4\cdot3$ *vs*. after: $11\cdot9\pm4\cdot7$ $IU\,l^{-1}$; genotype DD, before: $18\cdot5\pm3\cdot2$ *vs*. after: $16\cdot8\pm2\cdot1$ $IU\,l^{-1}$).

Cough could be induced by the inhalation of increasing concentrations of capsaicin in all subjects. There was no significant difference in changes of the cough threshold during the placebo period between subjects with genotype II and those with genotype DD by a two-way ANOVA (genotype II, before: $6\cdot2\pm3\cdot9$ *vs*. after: $6\cdot0\pm4\cdot2$ nM; genotype DD, $5\cdot3\pm2\cdot4$ *vs*. $6\cdot6\pm3\cdot7$ nM). After the administration of cilazapril for 4 weeks, changes in the cough threshold in subjects with genotype II significantly differed from those in subjects with genotype DD (genotype II, before: $6\cdot6\pm3\cdot7$ *vs*. after: $5\cdot0\pm4\cdot6$ nM; genotype DD, before: $9\cdot0\pm9\cdot4$ *vs*. after: $11\cdot5\pm10\cdot1$ nM, $P<0\cdot05$) by the same statistical method (Fig. 2). However, when time (i.e. before or after the use of either cilazapril or placebo) and periods (i.e. placebo or cilazapril period) were included as two within-subject factors, interaction between these two factors was not significant in subjects with either genotype II or genotype DD. Thus, changes in the cough threshold during cilazapril period did not significantly differ from those in the placebo period for either genotype.

Changes in skin responses to intra-dermal bradykinin during cilazapril period in subjects with genotype II significantly differed from those in subjects with genotype DD by a two-way ANOVA (Fig. 3). Skin responses to bradykinin in subjects with genotype II were significantly increased after the use of cilazapril (before: $1\cdot6\pm0\cdot6$ *vs*. after: $2\cdot6\pm0\cdot5$ cm^2, $P<0\cdot05$) by the Student's paired *t*-test, whereas they did not change in subjects with genotype DD (before: $1\cdot4\pm0\cdot5$ *vs*. after: $1\cdot6\pm0\cdot6$ cm^2, n.s.) (Fig.3). Skin responses to intra-dermal substance P during cilazapril period did not significantly change in subjects with either genotype II (before: $1\cdot3\pm0\cdot5$ *vs*. after: $1\cdot6\pm0\cdot8$ cm^2, n.s.) or genotype DD (before: $1\cdot0\pm0\cdot5$ *vs*. after: $1\cdot1\pm0\cdot5$ cm^2, n.s.) (data not shown). We found no significant difference in plasma levels of bradykinin and substance P before and after the administration of cilazapril in subjects with either genotype (data not shown).

Discussion

We have previously reported that the genetic susceptibility to cough induced by ACEI in patients with hypertension
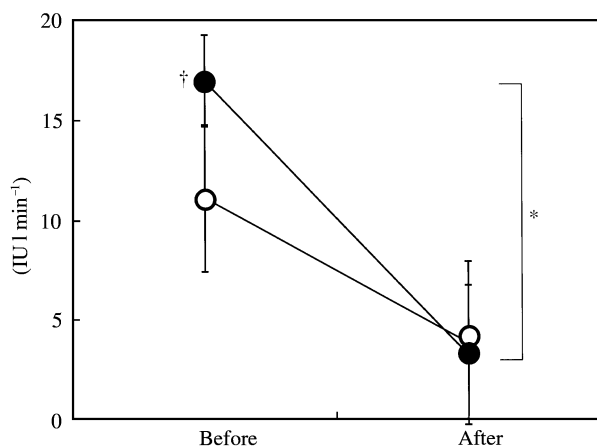

FIG. 1. Serum ACE activity before and after the administration of cilazapril according to genotypes of the ACE gene. Results are reported as mean $\pm$ SD for 10 subjects of each genotype. A significant difference between before and after administration of cilazapril is indicated by *$P<0\cdot01$. Significant difference between genotype DD and II before administration of cilazapril is indicated by †$P<0\cdot01$.
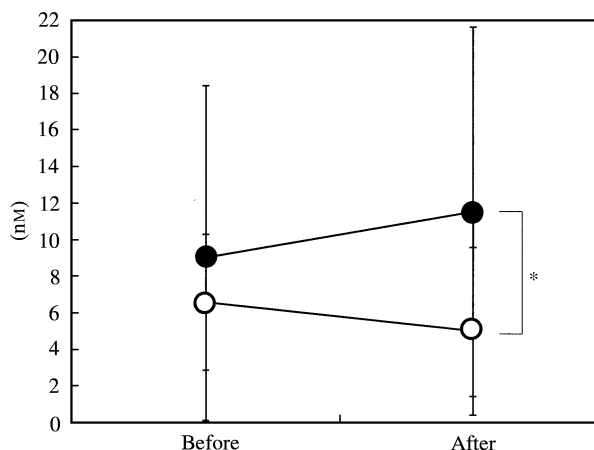

FIG. 2. Changes in cough threshold before and after the usage of cilazapril for 4 weeks. Data are mean $\pm$ SD. Open symbols and closed symbols denote subjects with the genotypes II and DD, respectively. *$P<0\cdot05$ by a two-way ANOVA.
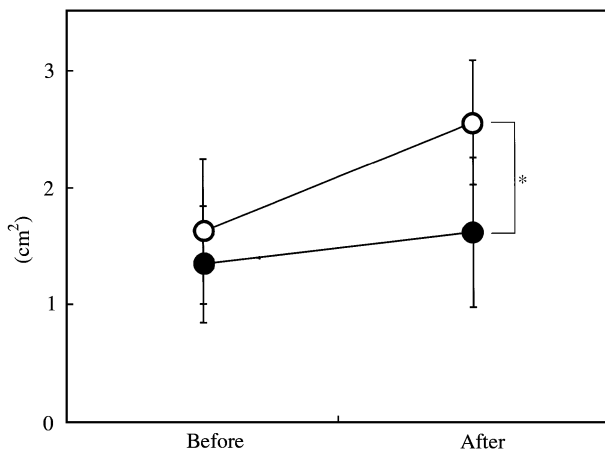
FIG. 3. Changes in skin responses to intra-dermal bradykinin as expressed in wheal area, before and after the usage of cilazapril for 4 weeks. Data are mean $\pm$ SD. Open symbols and closed symbols denote subjects with the genotypes II and DD, respectively. *$P < 0.05$ by a two-way ANOVA.

and/or heart failure may be related to I/D polymorphism, with subjects having genotype II being most susceptible to cough (15). In the present study, we attempted to re-examine the idea that the ACE gene polymorphism may represent a susceptibility mutation for ACEI-induced cough by measuring cough threshold in healthy subjects. Though a small number of subjects with either genotype failed to develop an observable cough, we found that changes in cough threshold were significantly different between subjects with genotype II and those with genotype DD after the administration of cilazapril. However, our results only partly support our previous observation in a clinic-based study, since changes in cough threshold during the use of cilazapril in genotype II were not significantly different from those during the placebo period. This was probably caused by a weak statistical power.

The distinctly different effects of ACEI on the cough threshold between the two genotypes may result from the more intense suppression of ACE activities in genotype II. However, serum ACE activities did not differ between genotypes II and DD after the administration of cilazapril. This was apparently due to high levels of residual ACEI in the serum when ACE activities were assayed *in vitro*. The airway tissue ACE activities are of critical importance in determining a catabolic rate of mediators that can induce cough by stimulating C fibres. In this respect, serum ACE activities may not properly reflect ACE activities in the perineuronal tissue. This may be the case specifically following the administration of ACEI in as much as tissue transition of ACEI is difficult to estimate. As a surrogate measure of tissue ACE activities, wheal responses to intra-dermal bradykinin and substance P, both of which are substrates of ACE, have been used (11,12,21). Our results showed that skin responses to bradykinin were increased in

subjects with genotype II but not in those with genotype DD after the administration of cilazapril. This result indicates a significant decrease in tissue ACE activities in subjects with a low basal ACE activity after the administration of cilazapril and may explain why cough threshold was decreased in those subjects. Meanwhile, the absence of a significant change in skin responses to intra-dermal substance P may be due to a partial dependency of this substrate on ACE as a catalytic enzyme, since this substrate is also degraded by neutral endopeptidase (6).

Several mechanisms have been proposed to explain ACEI-induced cough (9,22–25). Among candidate mediators, bradykinin has been strongly implicated in the mechanism for the development of cough (21,26). However, we were unable to detect any significant changes in plasma levels of bradykinin following cilazapril usage, even in subjects with genotype II. This observation seems to be incompatible with our results on skin responses; however, data on the effect of ACEI administration on blood bradykinin levels are generally conflicting and inconclusive (27). At therapeutic doses, plasma levels of ACEI are usually not high enough to cause accumulation of circulating bradykinin (27,28), and thus our results are consistent with several previous reports (28–30). An inability to observe reduced circulating levels of a mediator by the use of ACEI does not exclude the possibility that such a mediator is actually involved in the mechanism of cough, since it is the local rather than the circulating levels, which have primary importance. Because tissue ACE activity is most likely to be reduced after the administration of cilazapril specifically in subjects with genotype II, the tissue level of endogenous bradykinin may be conversely increased, leading to bronchial irritability and cough in susceptible subjects. Though direct evidence for such a mechanism is lacking in the present study, the results of skin responses support a possible role of bradykinin in neurogenic inflammation in ACEI-induced cough.

There is evidence to suggest that the I/D polymorphism is in strong linkage disequilibrium with a major gene effect at the ACE gene locus, which controls up to 44% of the variability in ACE levels (14). Over 70 polymorphisms have recently been identified in the ACE gene (31,32) and some of these polymorphisms remained significantly associated with ACE levels (32). Other studies have suggested the existence of a second functional locus possibly unlinked to the ACE gene (33). The inability to replicate our observation (34,35) and the lack of significant linkage between I/D polymorphism and ACEI-associated cough (36) may be attributable to the polygenic nature of regulating ACE levels or to racial differences.

In conclusion, this study has provided further evidence for the involvement of ACE gene polymorphism and ACEI-induced cough. It is likely that the polymorphism affects the ACEI-induced cough via modulation of levels of bradykinin. However, we cannot exclude possible roles of other tussigenic mediators in view of the wide substrate specificity of ACE, nor can we preclude the possibility that a variant of other genes closely linked to the ACE gene causes the observed association.

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