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FAILURE OF PERIPHERAL BLOOD LYMPHOCYTES TO BE A SURROGATE MARKER OF ARTERIAL TISSUE FOR TISSUE ACE INHIBITION

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Previous studies in rats demonstrated that the inhibition of vascular tissue angiotensin-converting enzyme (ACE) was the determinant of antihypertensive action of an ACE inhibitor, whereas plasma ACE inhibition was not. In patients, however, it is not practical to perform vascular tissue-biopsy merely to estimate drug effects. Therefore, the present study examined in rats whether or not peripheral blood lymphocytes (PBL) could be used as a surrogate marker for aortic tissue in respect of tissue ACE inhibition. Rats were treated daily for 6 days with one of the three ACE inhibitors (captopril, enalapril, and trandolapril) bearing different pharmacokinetic properties from each other. At a certain time after the final dosing, PBL and various tissues were harvested to measure ACE activity *ex vivo*. The present results confirmed the previous data for ACE inhibition in aorta and other tissues. Unexpectedly, however, ACE activity *ex vivo* of PBL was not reduced at all by any of the drug treatments, suggesting some drug-efflux mechanisms involved. In conclusion, PBL could not be used as the surrogate marker to estimate the vascular tissue ACE inhibition.

Key words: angiotensin-converting enzyme, captopril, enalapril, trandolapril, P-glycoprotein (MDR-1)

INTRODUCTION

Angiotensin-converting enzyme (ACE; peptidyl dipeptide hydrolase 1; EC 3.4.15.1) converts inactive decapeptide angiotensin I into diversely-active octapeptide angiotensin II by cleaving the carboxyl terminal dipeptide His-Leu. Although little is known about regulatory mechanism of ACE gene expression, ACE is known to be upregulated in affected tissues of hypertension (1-3) and other cardiovascular diseases (4-8) and is likely to provoke and progress those diseases by overproducing angiotensin II which has a variety of pathogenic and disease-aggravating activities (9). Those diseases in which ACE and its catalytic product angiotensin II are supposedly involved include hypertension, congestive heart failure, cardiac and arterial hypertrophy, post-myocardial infarction syndrome, diabetic nephropathy and neuropathy, atherosclerosis, and others (9, 10). It is for this reason why ACE inhibitors (ACEIs) exert their beneficial effects on variety of cardiovascular diseases.

Currently, more than ten ACEIs are available for clinical use in the world and in Japan as well. These drugs differ from each other with regard to pharmacokinetic characteristics (i.e., extent of absorption, plasma half-life, tissue distribution, hepatic metabolism, and mechanism of elimination). However, it has been generally understood that there is no compelling reason to favor one ACEI over another (9). To date, there has been no comparison study that clearly demonstrated the clinical superiority of one ACEI over another. Nevertheless, ACEIs differ markedly in tissue distribution (9, 11), and such pharmacokinetic difference may yield some differences in therapeutic effectiveness which are yet to be identified by future clinical studies comparing ACEIs.

A series of studies using rats and mice have shown substantial difference between ACEIs in tissue distribution that led to difference in duration of antihypertensive action (1, 11-14), to difference in efficacy of antihypertrophic action onto left ventricle and aorta (4, 5), and to difference in antinociceptive

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action (15). As to the duration of antihypertensive action, a given ACEI that inhibited the vascular ACE activity for a longer time showed a longer duration of antihypertensive action than other ACEIs (1, 11-14). In other words, the duration of vascular ACE-inhibitory action is the major determinant of the duration of antihypertensive action of a given ACEI. On the other hand, a certain ACEI's duration of inhibitory action onto plasma ACE activity was not correlated with its antihypertensive action. This means that vascular tissue is the optimum indicator to estimate not only a short-term antihypertensive effect of an ACEI but also long-term prognosis of hypertensive patients who are treated with ACEIs. However, in humans, it is not practically allowed to conduct vascular tissue biopsy merely to monitor the drug effect. Therefore, we have examined in the present study with rats whether or not peripheral blood lymphocytes (PBL) could serve as a surrogate marker for vascular tissue with regards to tissue ACE inhibition.

MATERIALS AND METHODS

Chemicals

Captopril and enalapril maleate were purchased from Sigma Chemical (St. Louis, MO, USA). Trandolapril was a gift from Rousell-Uclaf (Romainesville, France). Lympholyte-Rat™ was from Cedarane Laboratories, Ontario, Canada. Carboxymethyl cellulose sodium salt (CMC-Na) was from Maruishi Pharmaceuticals, Osaka. Dimethylsulfoxide (DMSO), *o*-phthaldialdehyde (OPA), and metaphosphoric acid were from Nacalai Tesque, Kyoto. Dithiothreitol (DTT) from Wako Pure Chemical Industries, Osaka. A synthetic substrate for ACE, Hip-His-Leu (BzGly-His-Leu), and the assay standard, His-Leu, were from Peptide Institute, Minoh, Osaka. Polyoxyethylene octylphenyl ether (Wako) was used as a non-ionic detergent to solubilize membrane-bound ACE and other lipophilic substances. Methanol (MeOH) for HPLC mobile phase was of HPLC-grade. All other chemicals were of reagent grade.

Experimental Animals

Male Wistar rats aged 24 weeks and weighing 410-480 g (SLC, Shizuoka) were used. We used heavy-weight rats because it was easier to collect whole blood as much as possible for lymphocyte preparations. Rats were fed regular rat chow, given tap water *ad libitum*, and housed at constant temperature and constant light/dark cycle. The present study was performed in line with the Guiding Principles for the Care and Use of Laboratory Animals approved by Japanese Pharmacological Society and by the Committee of Shimane Medical University.

Allocation of rats into drug-treatment groups

The rats were randomly allocated to the following 5 groups and they received daily dosage for 6 days by gavage using polypropylene stomach catheters. Group T received 0.5 mg/kg of trandolapril once daily. Group E received 20 mg/kg of enalapril maleate once daily. Group C6 was treated with 25 mg/kg of captopril twice daily for 6.5 days, and sample collection was done 6 h after the final dosing. Group C12 was treated with the same dose of captopril for the same period as above then followed by the sample collection 12 h after the final dosage. Group V was given 0.4 ml/kg of the vehicle once daily. For the groups T, E, and V, samples were harvested 24 h after the final dosing. Each drug was dissolved at first with 5% DMSO in distilled water, and then diluted 20-fold with 0.25% CMC-Na aqueous solution. Thus, the vehicle used here was aqueous solution containing 0.25% DMSO and 0.25% CMC-Na.

Preparation of peripheral blood lymphocytes (PBLs)

Under anesthesia with intraperitoneal pentobarbital (40 mg/kg), abdomen was opened by midline incision and whole blood was taken from the abdominal aorta into a syringe which contained 0.25 ml of heparin sodium solution (1,000 U/ml). The collected blood (approx. 12 ml per rat) was diluted two-fold with phosphate-buffered saline (PBS: 8 mM sodium phosphate, 140 mM NaCl, pH 7.4), and then added to the lymphocyte-separation solution, Lympholyte-Rat™. Lymphocytes were separated according to the manufacturer's instructions. The separated PBLs were washed twice with PBS, counted with a hemocytometer (ranging $2.6\text{-}7.0 \times 10^7$ cells/ml PBS), added with an appropriate volume (see RESULTS

AND DISCUSSION for detail) of homogenizing medium [20 mM Tris-HCl, 5 mM Mg$(CH_3COO)_2$, 30 mM KCl, 0.25 M sucrose, and 0.5 % polyoxyethylene octylphenyl ether; pH 8.3], and then disrupted by sonication (160 W, 1 sec-intervals for 60 sec; repeated twice) using Bioruptor UCD-200™ (Cosmo Bio, Tokyo). The cell lysate was kept on ice overnight, and then centrifuged at 3,000 rpm for 10 min at 4℃ to obtain supernatant for ACE assay.

Tissue harvesting

Immediately after the whole blood collection as described above, thoracic aorta (wet weight 60-100 mg), whole heart, lung (right lower lobe), whole brain, and the right kidney were harvested. After being rinsed in PBS to wash off residual blood, the tissues were minced and homogenized with 10-volume (v/w) of the homogenizing medium using a glass/glass Potter-type homogenizer while cooled in ice-water. After standing still overnight, the homogenates were centrifuged at 20,000×g for 20 min to obtain the supernatant. For all samples from the groups C6 and C12 both of which received captopril, homogenizing buffer added with 10^{-7}M DTT was used. DTT at this concentration did not interfere with the ACE assay and yet protected captopril from inactivation due to S-S dimerization (Shimoura, unpublished data). Thus, DTT was used to keep captopril, which might remain as an SH-monomer in lymphocytes and various tissue compartments, as active *ex vivo* as had been *in vivo*.

Fluorometric determination of ACE activity

Lymphocyte ACE activity was determined by fluorometric assay according to the method by Santos *et al*. (16) with slight modifications. In this assay method, the enzymatic reaction product, His-Leu, was labeled with OPA to form a fluorescent adduct which was to be quantitatively determined. Although rat lymphocyte extracts may contain significant levels of dipeptidase activity which cleaves His-Leu and thereby potentially interferes with the assay, the method by Santos et al has supposedly solved the problem by using sodium borate buffer that contains high concentration of NaCl (16). Practically, an aliquot (25 μl) of the lymphocyte extracts, which were prepared from lymphocyte lysate as already mentioned, was added into 225 μl of the substrate-containing medium [0.55 M sodium borate buffer (pH 8.3) containing 5 mM Hip-His-Leu and 900 mM NaCl] to start incubation at 37℃ for 15 min. Enzyme reaction to produce the dipeptide His-Leu was stopped by addition of 0.6 ml of 0.34 M NaOH. In this alkalized condition, 50 μl of 2% (w/v) OPA in MeOH was added to form a fluorescent adduct with His-Leu. After exactly 10 min of OPA-labeling reaction at room temperature, 100 μl of 3 M HCl was added to terminate the reaction and then centrifuged at 800×g for 5 min. Fluorescence intensity of the supernatant was measured at excitation wavelength 365 nm and emission wavelength 495 nm. A series of calibration standard (0.125-10.0 μM) was prepared with authentic His-Leu solution, which was processed for OPA-labeling in the same way as were unknown samples. Blank was taken by incubating each sample in the presence of 10^{-6}M lisinopril. Lisinopril is a water-soluble, dicarboxylic acid-type ACEI and is active as it is (not a prodrug-type ACEI). Our pilot study confirmed that lisinopril did not interfere with fluorescence at the given condition as above. Lymphocyte ACE activity was represented as μU/10^6 cells, i.e., pmol/min/10^6 cells. One unit of the ACE activity was defined as amount of the enzyme that generated 1 μmol His-Leu every minute at 37℃.

HPLC determination of ACE activity

HPLC determination method (1, 4, 5) was applied to plasma and tissue samples other than lymphocyte samples. Because this method is based on the measurement of UV absorbance of hippuric acid (BzGly), the other product from Hip-His-Leu substrate by ACE cleavage, care for dipeptidase interference as stated earlier is not necessary. Aliquots (50 μl) of samples were added to 200 μl of substrate solution which consisted of (final concentrations during incubation) 5 mM Hip-His-Leu, 300 mM NaCl, and 100 mM KH_2PO_4 (pH 8.3) and then were incubated at 37℃ for 30 min. Reaction was stopped by addition of 750 μl of 3% metaphosphoric acid, followed by centrifugation at 3,000 rpm for 10 min to remove denatured proteins. An aliquot (50 μl) of the supernatant was injected into and quantitatively analyzed for its hippuric acid content by a reversed-phase HPLC system (LC10AD, Shimadzu, Kyoto).

Conditions for HPLC analysis were as follows: a Puresil™ C18 column (4 mm i.d.×180 mm, Waters, Milford, MA, USA); column temperature precisely kept at 40°C with a column oven; mobile phase was a 1:1 mixture of 10 mM KH_2PO_4 (pH 3.0) and MeOH; flow rate 0.7 ml/min. UV absorbance at 228 nm was monitored with in-line UV detector with a flow-cell volume of 8 μl (SPD-10AD, Shimadzu). Chromatograhic peak area of hippuric acid was data-processed by Chromatopac™ (Shimadzu) to convert to ACE activity. Protein concentrations of tissue extracts were determined by the method of Smith et al (17) using BCA™ Protein Assay Reagent (Pierce, Rockford, IL, USA).

Statistics

Data were presented as mean±S.E.M unless otherwise stated. Statistical analyses were done with Stat View™ software (Abacus Concepts Inc., Berkeley, CA, USA). To evaluate the difference between treatment groups, multiple comparison was done by ANOVA and *post-hoc* Bonferroni/Dunn's test. Difference was considered significant if $P<0.05$.

RESULTS AND DISCUSSION

Precision assay of lymphocyte ACE activity

We at first tried to quantify lymphocyte ACE activity by reversed-phase HPLC method as usually for other tissues (1, 4, 5, 11, 13-15). However, levels of the lymphocyte ACE activity were not sufficiently high to be monitored by nonspecific UV absorbance of hippuric acid, a product liberated by ACE from the substrate Hip-His-Leu. Therefore, we chose the fluorometric assay which detected His-Leu, the other product liberated from Hip-His-Leu by ACE action. It is reported that the dipeptide His-Leu, when conjugated with OPA, shows approx. 12 times more intense fluorescence than does His on the molar basis, and still more intense than do other amino-compounds such as amines, amino acids, and peptides (18). In addition, we employed a fluorometric method that was specifically improved for higher sensitivity and less interference than conventional fluorometry for rat serum and plasma ACE (16), as already described in METHODS section. Taking these advantages, we succeeded to precisely determine the ACE activity of rat PBLs as shown in Table 1 (the discussion on the results is given in the next section). Table 1 also shows that there was no significant difference among the treatment-groups in respect of PBL counts, nor in the final dilution factor at the time of enzyme reaction. This indicates that the assay procedure we did in the present study was reasonably reproducible and the ACE activity values for PBL preparations were sufficiently reliable.

Unlike PBLs, rat peripheral blood erythrocytes showed no detectable ACE activity by our present fluorometry (data not shown).

Table.1 Determination of ACE activity of peripheral blood lymphocytes(PBLs)

Treatment group	Capt-6 [a]	Capt-12 [b]	Trandolapril [c]	Enalapril [c]	Vehicle [c]
Number of rats / group	5	5	5	5	5
PBL count [d] (× 10^6 cells / 25 μl)	0.90 ± 0.19	1.02 ± 0.22	1.03 ± 0.37	0.79 ± 0.12	0.68 ± 0.19
Total PBL volume [e] (mm^3 / 25 μl)	0.129	0.147	0.148	0.114	0.098
Final dilution factor [f]	1938	1712	1689	2193	2251
PBL ACE activity (μU/10^6 cells) [g]	6.42 ± 0.45	5.11 ± 0.86	6.00 ± 0.74	6.26 ± 0.60	6.19 ± 0.46

[a], PBL was collected 6 h after the final dose of captopril. [b], PBL was collected 12 h after the final dose of captopril. [c], PBL was collected 24 h after the final dose of trandolapril, enalapril, or vehicle. [d], Cell count in 25 μl aliquot of PBL preparations (mean ± SD). [e], Total cell volume in 25 μl aliquot of PBL preparations, which is calculated with an assumption that the average diameter of PBLs is 6.5 μm. [f], The final dilution factor by which PBLs are supposedly diluted (v/v) in the reaction mixture (250 μl) for ACE activity measurement. [g], The dimension corresponds to [pmol of His-Leu generated]/min/10^6 cells (mean ± SEM).

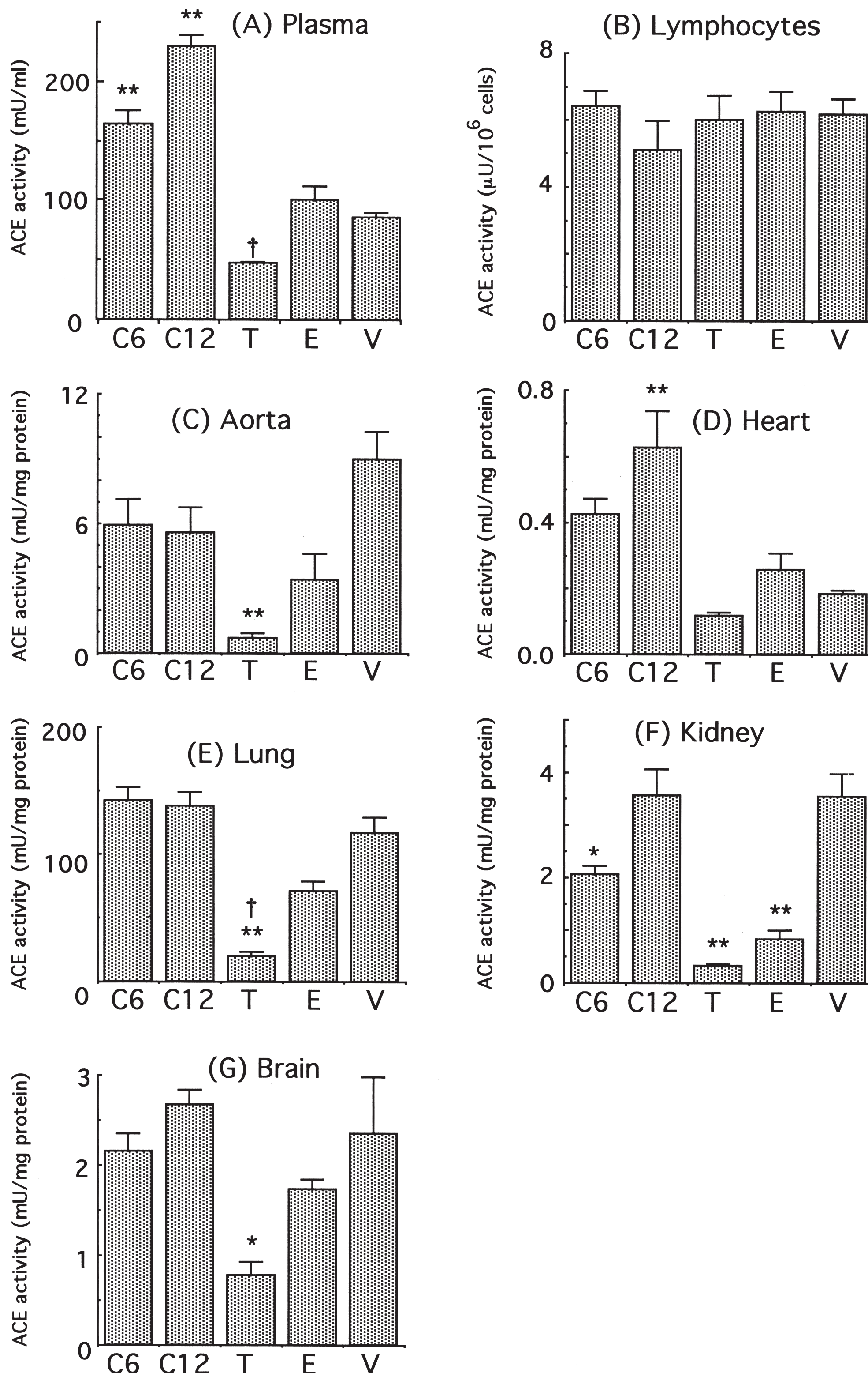

Fig. 1. Comparison of ACE activities between the treatment-groups in respective rat tissue compartments: (A) plasma, (B) lymphocytes, (C) aorta, (D) heart, (E) lung, (F) kidney, and (G) brain. ACE activity is expressed as mU/ml for plasma (panel A), as μU/10^6 cells for lymphocytes (panel B), and as mU/mg of protein for all other tissues (panels C through G). Means ± S.E.M. for 5 rats of each group are shown throughout the Figure. C6: captopril-treated rats from which tissue samples were harvested 6 h after the final dose. C12: captopril-treated rats from which samples were harvested 12 h after the final dose. T: trandolapril-treated rats, E: enalapril-treated rats, and V: vehicle-treated rats from which tissue samples were harvested 24 h after the final dose. *$P<0.05$ and **$P<0.01$ vs. vehicle. †, $P<0.05$ between T (trandolapril)-group and E (enalapril)-group.

Effects of repeatedly given ACEIs on lymphocyte ACE activity

The bottom row of Table 1 compares PBL-ACE activities between different treatment groups. According to the statistical analysis, any of the drug-treated groups did not show a significant difference (Table 1 and Fig. 1: panel B) from the control (vehicle-treated) group. This fact indicated that a significant amount of ACEI did no longer remain in the PBL compartment at 24 h after the final dose of trandolapril (a long-acting ACEI) and enalapril, neither did at 6 h or 12 h after the final dose of captopril (a short-acting ACEI). This is a unique property to PBLs, and contrasting with the inhibition profile of other tissue compartments as discussed later.

Instead of interpreting as above, one may argue that the absence of significant ACE inhibition in the PBL extracts from the rats receiving repeated doses of ACEIs may be an artifact. For example, one may consider that the apparent absence of ACE inhibition was due to the unintended washout of ACEIs, which might have remained within the PBL cells, during the sample preparation procedure or due to a substantial dilution of residual ACEIs upon incubation process. However, we consider these are unlikely, because the lung ACE extracts obtained from the trandolapril-treated rats yet showed a potent inhibition (Fig. 1: panel E) despite that the lung preparation was diluted as high as 500 times during the final incubation procedure.

Does the PBL-ACE molecule differ from that of other somatic tissues?

The fact that the ACE activity of PBLs was not inhibited significantly at a certain time even after the repeated doses of ACEIs has raised a question if ACE molecule of PBLs may not be the same as that of other tissues. However, this possibility seems very unlikely, because there has been no report to demonstrate more than one ACE gene in any species (2, 7) and also because both the ACE activity of PBLs and that of other tissues were readily and completely inhibited by 10^{-6} M lisinopril in vitro in the present study (blank assay). Therefore, when compared in vitro, the PBL-ACE and the somatic ACE are probably the same

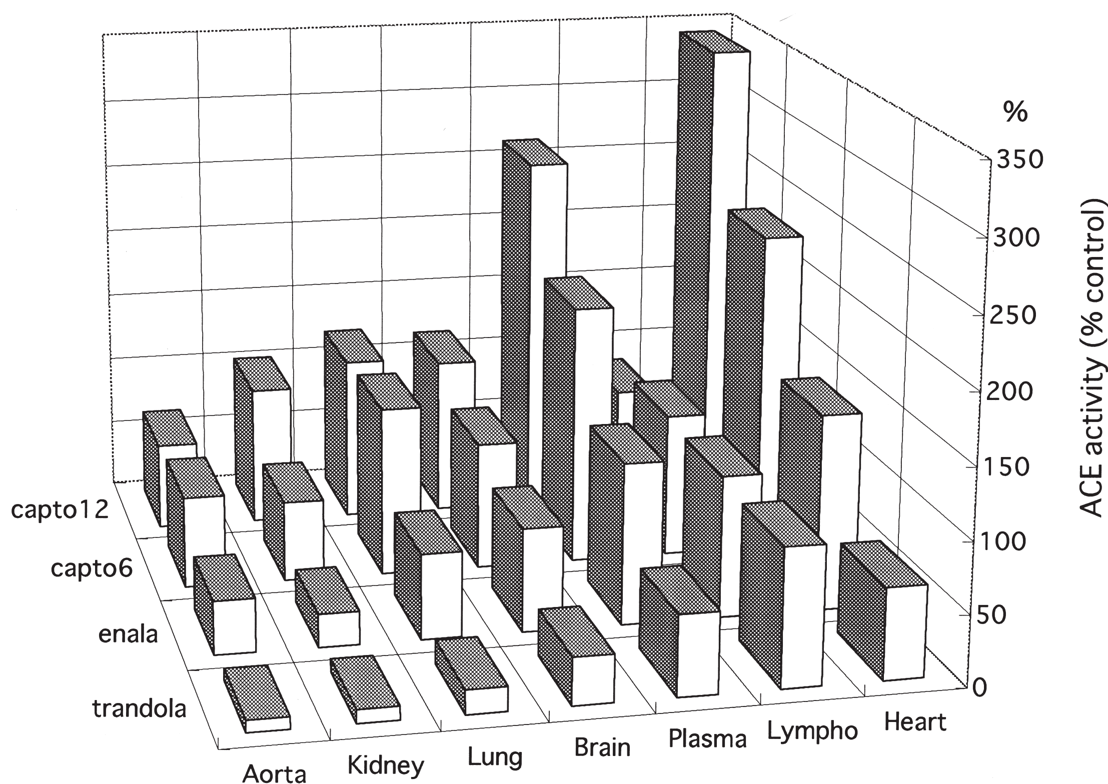

Fig. 2. ACE activity relative to respective control data. Corresponding data of the vehicle-treated group were taken as 100%. Marked ACE induction in the heart and plasma of captopril-treated group should be noted. Profile of the "Lympho" row is completely different from those of others, indicating no ACE inhibition and no ACE induction (see text for detail).

or quite similar in respect of susceptibility to ACEIs. Thus, the fact that PBL-ACE activity was no longer inhibited at all 24 h after the final dose of trandolapril (the most potent and longest-acting ACEI as evidently shown in the next section) strongly indicates that any significant amount of the drug did not remain in the PBL compartment at the time of blood sampling.

Comparison of drug effect among different tissues and among different ACEIs

Figure 1 compares the ACE activities between treatment-groups for respective rat tissue compartments: plasma, lymphocytes, aorta, heart, lung, kidney, and brain. Caution should be taken for that the y-axis ranges are not the same for all tissue compartments. ACE activity of blood plasma is expressed in mU/ml, that of lymphocytes is in μU/10^6 cells (pmol/min/10^6 cells), and those of somatic tissues are expressed as mU/mg protein.

Figures 1 and 2 have evidently shown that trandolapril was the most potent and long-acting among the ACEIs used here, throughout all the tissue compartments except lymphocytes. On the other hand, captopril (D-2-methyl-3-mercaptopropanoyl-L-proline) is well known to be short-acting because it is rapidly oxidized to form S-S dimer that is inactive to ACE at all, in the absence of reducing agents. To prevent this oxidation, all the tissue extracts from captopril-treated groups were treated with DTT in the present study (as already mentioned in METHODS). As a result, any potentially remaining captopril in tissue extracts should have been kept as the active form during the assay procedure. Accordingly, in Fig. 1, if the ACE activity of the captopril-treated group was lower than that of the vehicle-treated group, then it indicated that captopril still remained within that tissue compartment. On the other hand, if the ACE activity of captopril groups was not lower than that of the vehicle group, it indicated that captopril no longer remained there. It should be noted in Figs. 1 and 2 that even captopril (an ACEI bearing short plasma half-life) showed a trend to inhibit the aorta ACE activity 6 and 12 h after the final dosage although the extent of inhibition was much smaller than that by trandolapril- and enalapril-treatment. The property that the ACE inhibition lasts longer than in other tissue compartments appears intrinsic to vascular tissue (13).

Different inhibition profiles between panels B and C in Fig.1 suggest that the principal question of the present study, i.e. whether or not the PBL-ACE activity reflects the ACEI effect on vascular tissue ACE activity, is quite unlikely. This is more clearly illustrated in Fig. 3 which has examined the potential correlation between PBL-ACE activity and the aorta ACE activity. No correlation was observed at all between these two parameters either in all treatment groups as a whole or in each treatment group.

ACE-induction in response to repeated doses of ACE inhibitors

Figs. 1 and 2 also implicate the ACEI-induced upregulation of ACE molecules. If an ACE activity of a drug-treated group is higher (>100% in Fig. 2) than that of the vehicle-group, it generally indicates that *de novo* ACE is induced in response to repeated administration of ACEIs. Such ACEI-induced ACE induction (AIAI) has been confirmed with captopril (19), lisinopril (14, 20), enalapril and trandolapril (11, 13). Indeed, in the plasma and the heart, it should be noted that remarkable (200-350%) ACE induction occurred after the repeated doses of captopril. This AIAI should deserve further studies for mechanisms of cardiac-tissue-specific regulation of ACE gene expression, and also to know whether or not such marked AIAI in the heart has some clinical relevance.

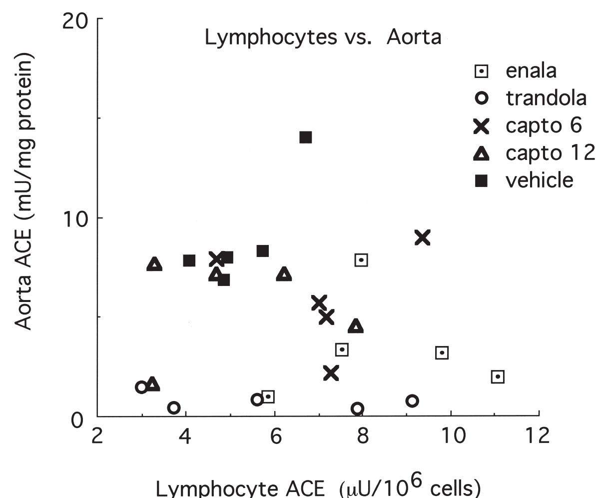

Fig. 3. Correlation analysis between aorta ACE activity and lymphocyte ACE activity. It is evident that no correlation is found at all between these two compartments. Each plot represents the data set obtained from individual rat. enala: enalapril-treated rats. trandola: trandolapril-treated rats. capto 6: same as C6 in Fig. 1. capto 12: same as C12 in Fig.1. vehicle: vehicle-treated control rats.

Significance of the present study from a technical aspect to quantify PBL-ACE activity

To the best of our knowledge, this paper is the first to describe the rat lymphocyte ACE activity which was precisely quantified by fluorometric assay. As stated earlier, we at first tried to quantify PBL-ACE activity with reversed-phase HPLC as usually for other tissues, because a report by Costerousse *et al.* which had been the only one report available as to human peripheral blood lymphocyte ACE activity, employed HPLC analysis to measure hippuric acid on the basis of its UV absorption at 228 nm (21). However, the level of lymphocyte ACE activity was not sufficiently high to be determined on the basis of nonspecific UV absorbance of hippuric acid. Thus, we changed the assay method to the fluorometric technique. Fluorometric assay is, in general, more sensitive and more specific than quantification method based on the UV absorption at 228 nm (nonspecific absorption and thereby yielding low S/N ratio).

In addition, the fluorometric assay technique we employed had been further improved for higher sensitivity and less interference due to contaminating dipeptidase activity (16). As a result, we succeeded to precisely determine ACE activity of PBLs. Accuracy of the measures can be seen from the relatively narrow range of standard deviation shown in Table 1.

Using such an improved fluorometric method as above, our precise estimation of rat PBL-ACE activity was 6.19 ± 0.46 $\mu U/10^6$ cells (n=5). Costerousse *et al.* (21) described the ACE activity in human T ($CD2^+$) lymphocytes was 47 ± 29 $\mu U/10^6$ cells, while that of B ($CD19^+$) lymphocytes was below detection limit. According to their data, ACE activity of total mononuclear cells in human blood can be assumed as approx. 15 $\mu U/10^6$ cells. Therefore, our present estimation of rat lymphocyte ACE activity is 1/2-2/5 that of human lymphocyte by their estimation. At the moment, it is unknown whether or not the observed difference between our data and their data can be ascribed to species-difference.

By the improved method, we could also estimate the extent of ACEI-effects on lymphocyte ACE activity with sufficiently high accuracy and reproducibility. Unexpectedly, we could demonstrate that there was no sign of lymphocyte ACE inhibition at all in any of the treatment groups. Even trandolapril, which is the most penetrable ACEI into various tissues including the brain, failed to yield any significant inhibition on PBL-ACE activity after 6-day repeated dosing. This was the most interesting and intriguing result of the present study, although our expectation to use PBLs as a surrogate marker for tissue ACE inhibition was not supported.

Presumable mechanisms for no appreciable ACE inhibition in the PBLs as contrasted with vascular tissues

There are supposedly several mechanisms that may explain why we could not detect any significant inhibition of PBL-ACE activity with any of ACEIs including trandolapril.

1) Possibility of unintended removal of residual ACEI from PBL extract

Firstly, ACEIs might have been unintentionally removed by repeated washing of the lymphocytes during the process of PBL preparations. However, this seems very unlikely because trandolapril can be hardly removed if it exists in a bound form to membrane-anchored ACE or intracellular ACE of the lymphocyte. The binding affinity (the inverse of K_{app}) of trandolapril is much higher than that of other ACEIs and the dissociation rate of trandolapril from the enzyme-inhibitor complex seems much slower than that of other ACEIs (22). This has been supported by our experience that we had an extreme difficulty to remove trandolapril by dialysis from the tissue extract which was obtained from the animals that had been repeatedly given trandolapril; it took more than 4 days of exhaustive dialysis with frequent changes of dialysate which contained a potent chelating agent to remove Zn that composed the catalytic center of the ACE molecule, the center to which trandolapril was supposed to be bound (13, 14; Kawamoto et al., unpublished observations). Therefore, so far as trandolapril is concerned, the possibility that ACEI was unintentionally washed away can be ruled out.

2) Potential for ACEI-induced upregulation of ACE which may cancel the effect of ACEIs

Secondly, another possible explanation is that lymphocytes may be far more capable of inducing *de novo* ACE molecules than do other tissue cells in response to a long-term ACE inhibition; an increased amount of ACE molecules may overcome or at least cancel the

inhibitory effect of ACEI which remains in PBLs at moderate or low concentrations. ACE induction was evident in the heart of the rats which were treated with captopril (Figs. 1 and 2). However, ACE induction occurs only if local tissue ACE activity is inhibited for at least a certain length of period. On the contrary, we could not detect appreciable ACE inhibition nor ACE induction. Thus, we consider it the most possible that ACEI was not present in the lymphocytes in a significant concentration.

3) Potential mechanism of drug-exclusion by PBLs

The most possible and attracting explanation is that the lymphocyte might exclude ACEIs out of the cell using an energy-dependent transport system. In this regard, the lymphocyte is well-known for its high expression of multidrug resistance (MDR) P-glycoprotein (P-gp) pump (reviewed in 23; references 24, 25). P-gp (MDR-1) is a well-known transporter that mediates efflux of chemotherapeutic agents (anti-cancer drugs, immunosuppressant agents, etc) from the intracellular milieu and thereby contributes to drug resistance (26). Indeed, PBLs are demonstrated to have high activity of P-gp (24, 25, 27). Interestingly, P-gp is proven to be able to efflux organic free anion out of lymphocytes (25). Because ACEIs are unexceptionally free anions (captopril is a monocarboxylic acid, whereas the active forms of prodrug-type ACEIs are dicarboxylic acids), all the ACEIs currently available are potential substrates of efflux transportation by P-gp (MDR protein). Whether or not ACEIs are really extruded by lymphocytes is an interesting issue that may have some clinical relevance and thus warrants extensive studies.

Conclusion

In conclusion, the present study revealed that the ACE activity of PBLs was not inhibited at all by the repeated treatment with ACEIs for 6 days and thereby indicating that lymphocytes failed to serve as a marker for vascular tissue with regards to tissue ACE inhibition. Instead, the present study has provided highly suggestive data which propose the presence of drug-extruding mechanism in the PBLs.

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