COMMENTS ON SPECTROFLUOROMETRIC ASSAY OF ANGIOTENSIN-CONVERTING ENZYME ACTIVITY IN BLOOD PLASMA OF DIFFERENT SPECIES

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The activity of the angiotension-converting enzyme (ACE) in human, dog, rabbit, and rat blood plasma was assayed by spectrofluorometric determination of the product liberated by enzymatic cleavage (L-His-L-Leu). In parallel experiments the hydrolysis of L-His-L-Leu by blood plasma was examined. The hydrolytic activity of rat blood plasma was high and therefore lower values of ACE activity were obtained; the use of the spectrofluorometric assay with rat blood plasma is therefore problematic. By contrast, L-His-L-Leu was not degraded by human, dog, and rabbit blood plasma and the spectrofluorometric determination of this peptide can thus be used to advantage to assay the ACE activity of blood plasma samples of these species.

During the past few years considerable attention has been directed toward the angiotension-converting enzyme (ACE, peptidyl dipeptide hydrolase, E.C. 3.4.15.1) which is responsible for the conversion of angiotensin *I* into angiotensin *II* (ref.[1]) in the organism. There are several reasons for increased interest in this enzyme, namely (*a*) its identity with kinase *II* (ref.[2]) indicating a relationship between the renin–angiotensin system and the kallikrein–kinin system, (*b*) its relevance to the measurement of the degree of sarcoidosis[3], and (*c*) the antihypertensive effect of ACE inhibitors[4].

Even though differences in the level of ACE activity of blood sera of different species have been reported[5–7] little attention only has been paid to the peptidase activity of the serum which is responsible for the hydrolysis of peptide L-His-L-Leu formed during the ACE assay. This peptidase activity represents a serious problem especially if fluorescence methods are used for the determination of L-His-L-Leu liberated from various synthetic substrates[8–14] by ACE to measure the activity of the enzyme. Since the fluorescence of L-His is only 8% of that of L-His-L-Leu (ref.[12]) the values of ACE activity obtained by such measurements are lower because of the hydrolysis of L-His-L-Leu during the assay.

It has been observed, however, that the dipeptide is cleaved to an insignificant degree only by human blood serum[8,12]. Since the fluorometric assay described has been also used for the testing of ACE activity of blood serum obtained from other

species it was necessary to find out the degree of hydrolysis of the dipeptide by these sera.

For these reasons the activity of ACE and the peptidase activity of human, dog, rabbit, and rat blood plasma was examined in parallel experiments. We have been able to show that the blood plasma from different species varies both in ACE activity and in the plasma peptidase activity.

EXPERIMENTAL

Materials. Z-L-Phe-L-His-L-Leu and L-His-L-Leu were purchased from Bachem, A.G. *o*-phthaldialdehyde and urethane were from Merck. All the remaining chemicals were of analytical purity grade.

Plasma samples. Blood collected in ice-cold heparinized plastic tubes was obtained from the cubital vein of 11 healthy volunteers, from the hind limb vein of 6 unanesthesized female mongrel dogs, from the jugular or marginal ear vein of 10 anesthesized and 6 unanesthesized rabbits and from 7 decapitated male rats. Blood plasma was separated by centrifugation at 4°C and was stored at −20°C until used.

Enzyme assays. ACE activity was measured by the method of Piquilloud and coworkers[8] with several modifications (buffer composition, substrate concentration, volume of compounds added, final volume, and incubation time). In brief, the ACE activity was monitored at 37°C on duplicate samples prepared in 0·07 mol l^{-1} Na_2HPO_4/NaOH buffer (pH 8·0 at 25°C). The final concentration of the substrate, Z-L-Phe-L-His-L-Leu was 2·5 mmol l^{-1} and 25 μl of plasma was used. The final volume was 0·25 ml. The time profile of product formation was measured for periods of up to 1 h with samples of blood plasma from each species. The optimal incubation period over which the rate of product formation was linear is 15 min for rat and rabbit blood plasma and 30 min for human and dog plasma. The reaction was discontinued by mixing duplicate 0·1 ml aliquots of the reaction mixture with 1 ml of 0·1 mol l^{-1} NaOH. *o*-Phthaldialdehyde (1·5 mmol l^{-1}) was added in the dark, followed by 1 ml of 0·8 mol l^{-1} HCl 30 min later. The samples were centrifuged and their fluorescence measured within one hour at λ_{exc} = 365 nm and λ_j = 500 nm (Turner Model 430 Spectrofluorometer, CA). The peptide assay was carried out by the same procedure except that Z-L-Phe-L-His-L-Leu was replaced by L-His-L-Leu as substrate in the incubation mixture. The concentration of the dipeptide in the peptidase activity assay mixture (first value) corresponded to the average concentration of L-His-L-Leu which is formed in the incubation mixtures during the ACE activity assay (value in parentheses) and was for human plasma 0·100 mmol l^{-1} (0·086 mmol l^{-1} . 30 min^{-1}), dog plasma 0·050 mmol l^{-1} (0·045 mmol l^{-1} . . 30 min^{-1}), rabbit plasma 0·200 mmol l^{-1} (0·299 mmol l^{-1} . 15 min^{-1}), rat plasma 0·186 mmol . . l^{-1} (0·178 mmol l^{-1} . 51 min^{-1}). In both assays the concentration of L-His-L-Leu either formed or degraded was read off from a standard curve prepared by adding increasing concentrations of L-His-L-Leu to tubes containing 1 ml of 0·1 mol l^{-1} NaOH and the sample of plasma. Control samples (zero time incubation, endogeneous L-His-L-Leu formation during incubation without the substrate, nonenzymatic hydrolysis of substrate in sodium hydroxide) were treated in the same manner.

Statistical calculations. Student's test and linear regression analysis were used. Intra-assay and inter-assay variation coefficients calculated according to Rodbard[15] were 1·3 and 12·6%, respectively.

RESULTS AND DISCUSSION

The ACE activities and blood plasma peptidase activities are summarized in Table I. The former values are in good agreement with the data on human plasma serum[3,7,12,16,17] and dog serum[5,7], obtained by other authors, and are roughly two times higher than the values obtained for rabbit serum[5]. No statistically significant difference between ACE plasma activity of unanesthesized and anesthesized rabbits was found. This finding corresponds to earlier data on the lack of effect of anesthetics on the activity of ACE in rat blood serum[14]. The peptidase activity of

TABLE I

ACE activity and peptidase activity of blood plasma of four different species[a]

Source of plasma	ACE activity His-Leu nmol min^{-1} ml^{-1}	*n*	Peptidase activity His-Leu recovery in % of initial amount	*n*
Human	28·6 ± 1·3	22	103·6 ± 1·2	5
Dog	15·0 ± 1·1	19	109·2 ± 1·4	12
Rabbit	anesthesized			
	165·2 ± 7·8	10	not determined	
	unanesthesized			
	199·1 ± 15·4	6	95·7 ± 3·2	6
Rat	119·0 ± 5·3 (362·7 ± 16·1)[b]	7	32·8 ± 3·0	7

[a] The values are means ± SEM; [b] value corrected for peptidase activity.

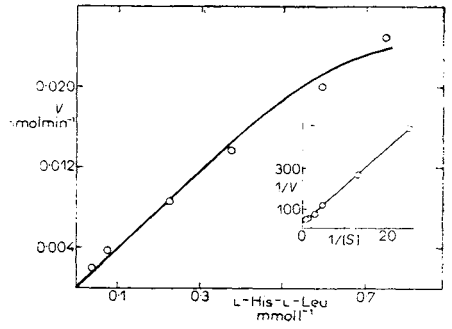

FIG. 1

The rate of L-His-L-Leu degradation by rat blood plasma peptidases as function of substrate concentration. The K_m and V_{max} values were calculated from the Lineweaver–Burke plot (inset) by linear regression analysis. The straight line equation representing this example is $y = 17{\cdot}4801x + 25{\cdot}1055$; $K_m = 0{\cdot}6963$ mmol l^{-1} and $V_{max} = 0{\cdot}0398$ mmol l^{-1}

human, dog, and rabbit blood plasma was negligible when the appropriate amount of L-His-L-Leu was used. By contrast, a high hydrolytic activity was observed with rat blood plasma. The K_m and V_{max} values for rat blood plasma peptidase were $0{\cdot}819 \pm 0{\cdot}1831$ mmol l^{-1} and $0{\cdot}0385 \pm 0{\cdot}052$ mmol His-Leu min^{-1}, respectively ($n = 3$, Fig. 1). If the values of ACE activity of rat blood plasma are corrected with respect to the degradation of L-His-L-Leu by plasma peptidase the figures obtained are much higher than the values measured in the assay. Therefore approximate values of ACE activity only can be calculated.

The finding that rat blood plasma has a high peptidase activity may explain why its values of ACE activity as determined by fluorometric measurement of L-His-L-Leu formation are lower than the values determined by the radiometric assay[14,21]. Since the correction of the ACE activity values according to the quantity of the product recovered yields approximate values only and requires a parallel measurement of the peptidase activity to be carried out, the spectrofluorometric assay of L-His-L-Leu formed during the ACE activity assay is complicated when samples of rat blood plasma are examined. On the other hand, our finding of the lack of degradation by human blood serum of the peptide already formed in assays of ACE activity confirmed earlier reports[8,12]; we have been able to show that the same holds true also for dog and rabbit blood plasma. In view of these data the spectrofluorometric determination of L-His-L-Leu can be used to advantage for the measurement of the ACE activity of blood plasma of these animals.

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