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PEPTIDYL DIPEPTIDASE IN RABBIT BRAIN MICROVESSELS

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Summary

The activity of peptidyl dipeptidase (peptidyldipeptide hydrolase, EC 3.4.15.1), also known as angiotensin-converting enzyme, was studied in small blood vessel preparations isolated from rabbit brain. The vascular preparation contained arterioles and capillaries and was essentially free of extravascular material. Enzymatic activity was demonstrated in microvessel homogenates using both hippuryl-histidyl-leucine and tritium-labeled angiotensin I as substrates. Activity in the microvessels was dependent on the presence of chloride ion and was sensitive to inhibition by converting enzyme inhibitors previously shown to be effective both in vivo and in vitro. Specific activity in the microvessels was approximately 20 times that in homogenates of brain, and was almost 60% of that found in rat lung homogenates. The data were consistent with an endothelial localication for peptidyl dipeptidase in the cerebral vasculature and supports the proposal that this enzyme has a physiological role in extrapulmonary vasuclar beds.

Peptidyl dipeptidase (peptidyldipeptide hydrolase, EC 3.4.15.1) acts on the decapeptide, angiotensin I, by cleaving the COOH-terminal dipeptide (His-Leu), thereby forming the potent pressor octapeptide, angiotensin II [1]. This enzyme also cleaves the COOH-terminal dipeptide from bradykinin, a nonapeptide with vasodepressor properties, resulting in bradykinin inactivation [2]. Because of these effects on the metabolism of vasoactive peptides, peptidyl dipeptidase, also known as angiotensin-converting enzyme, may contribute to the overall regulation of blood pressure.

In vivo studies on angiotensin I metabolism have suggested a major physiological role for angiotensin-converting enzyme in the pulmonary circulation [3,4]. The enzyme was shown to be localized in pulmonary vascular endothelial cells by immunohistochemical techniques [5,6] and purification from pulmonary tissue of several species has been described [7—10]. Peptidyl dipep-

tidase activity also was demonstrated in cultured endothelial cells from human umbilical vein [11]. Although activity is localized in the lung, converting enzyme also has been demonstrated in several other tissues [12,13] suggesting that generation of angiotensin II in tissues other than lung may be of physiological significance.

Recently, methods for the preparation of purified metabolically active small blood vessels (microvessels) from the brain have been described [14—16]. Since peptidyl dipeptidase activity has been demonstrated in brain homogenates [12, 13,17—19] and angiotensin II is known to have direct effects on the central nervous system [20], we utilized the isolated microvessel preparations to demonstrate the presence and some properties of this enzyme in the cerebral vasculature.

Materials and Methods

Angiotensin I and histidyl-leucine were obtained from Sigma Chemical Company. Hippuryl-histidyl-leucine (Hip-His-Leu) was purchased from Peninsula Laboratories, Inc., and fluorescamine (Fluran) from Roche Diagnostics. Two inhibitors of the converting enzyme, the nonapeptide SQ 20,881 (<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) and SQ 14,255 (2-D-methyl-3-mercaptopropanoyl-L-proline) were obtained from Squibb Pharmaceutical Co. Angiotensin I, containing [3,4,5-^{3}H]leucine at the COOH-terminal position (specific activity 75 Ci/mmol), was obtained from New England Nuclear Corporation and was purified before use by thin layer chromatography on silica gel using $CHCl_3/CH_3OH/28\%NH_3$, (16 : 16 : 1, v/v) as developing solvent.

Preparation of microvessels

The method used was a modification of the procedure described by Brendel et al. [14]. Weanling New Zealand white rabbits were killed by decapitation, the brains were removed and immediately placed in aeared 18 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 118 mM NaCl, 5.4 mM KCl, 1.8 $CaCl_2$, 1.0 mM $MgSO_4$, 1 mM NaH_2PO_4, and 5.5 mM glucose. All subsequent steps were carried out at ambient temperature (23—25° C). The pial membrane was stripped from the cerebrum using fine tweezers, and most of the white matter was removed. The remaining tissue was minced briefly, weighed, resuspended in buffer (1 : 1, w/v) and then transfered to a Dounce type tissue homogenizer matched with a loose-fitting pestle having clearance of 0.09—0.16 mm. Homogenization was performed manually by 4—6 slow vertical passages. The homogenate was diluted 10-fold with buffer and filtered through a 149 μm nylon mesh cut to fit a 25 mm Millipore Swinnex adaptor mounted on a 30 ml syringe. The filtrate (filtrate 1) was collected and the material remaining on the mesh was washed with 10 ml buffer, dislodged from the mesh by a rapid flow of buffer (15 ml) delivered through a syringe fitted with a 26 gauge needle, and transferred back to the Dounce homogenizer. This material was rehomogenized using 2 vertical passages of the pestle; the homogenate was combined with filtrate 1; and the combined preparation filtered using upward flow through a 105 μm and 74 μm nylon mesh arranged serially in Swinnex adaptors mounted on a 30 ml syringe.

This filtrate (filtrate 2) was refiltered through a single 74 μm mesh and the resulting filtrate discarded. The material contained on the 3 meshes (two 74 μm and one 105 μm) were washed, dislodged from the meshes and transferred to a specially constructed Duall homogenizing apparatus fitted with a Teflon pestle (clearance 0.25 mm). Homogenization was performed by gently passing the Teflon pestle through the Duall homogenizer 12 times. Microscopic examination at this stage revealed intact microvessels with small amounts of debris separated from the vessels. The homogenate was refiltered through 105 μm and 74 μm nylon meshes arranged serially, washed with buffer and rehomogenized in the Duall apparatus. Microscopic examination at this stage showed an extremely clean preparation of small blood vessels essentially free of adhering debris. This material was collected onto a 53 μm nylon mesh.

Homogenization procedures

To obtain a concentrated suspension of microvessels suitable for use in an assay system, the 53 μm nylon mesh containing the microvessels from 1 rabbit brain was placed in an all-glass, motor-driven Duall homogenizer containing 2 ml 50 mM potassium phosphate buffer (pH 8.3). Brief homogenization (15 s) at 0° C resulted in almost complete separation of microvessels from the nylon mesh. The mesh, which was not fragmented by the procedure, could be removed with forceps after homogenization was complete. The resulting homogenized suspension of microvessels was kept at 0° C and used directly for enzymatic studies. For comparative studies, tissues other than microvessels were minced and homogenized in 10 vols 50 mM potassium phosphate buffer (pH 8.3) using all the glass motor-driven Dual homogenizer.

Peptidyl dipeptidase assays

The standard assay used was the procedure of Cushman and Cheung [21] in which hippuric acid, released from Hip-His-Leu, was determined spectrophotometrically. Incubations were carried out in duplicate at 37° C in a total volume of 0.25 ml, and individual incubation tubes contained 5 mM Hip-His-Leu/100 mM potassium phosphate buffer (pH 8.3)/300 mM NaCl and an amount of tissue protein. Control tubes lacking NaCl or tissue protein were included with all assays. Activity was expressed as the nmol hippuric acid formed per min per mg tissue protein. The method of Lowry et al. [22] was employed for all protein determinations.

When angiotensin I was used as substrate, incubations were performed in Beckman Microfuge tubes in a total volume of 20 μl. Each incubation tube contained 5 μM unlabelled angiotensin I, [^{3}H]angiotensin I (0.1 μC), 100 mM potassium phosphate buffer (pH 7.5) 150 mM NaCl and the designated amount of tissue protein. Incubations in which NaCl was omitted or SQ 14,225 was added are designated in the text. Incubations were carried out at 37° C for 15 min. A 2-μl aliquot of the reaction mixture was spotted onto plastic coated silica gel plates (Eastman Chromogram). Thin layer chromatography was performed using $CHCl_3/CH_3OH/28\%$ NH_3 (16 : 16 : 1, v/v) as the developing solvent. Unlabelled angiotensin I, His-Leu, and Leu also were applied as standards. Following development, the plates were sprayed lightly with 0.01% fluorescamine to identify the reaction components. R_F values for angiotensin I,

Leu, and His-Leu were 0.1, 0.45 and 0.65, respectively. Separation was optimum when the developing solvent was prepared shortly before use. The thin layer plate was separated into regions corresponding to the standards, the silica gel scraped into scintillation vials containing a toluene-Triton X-100 cocktail, and radioactivity determined at an efficiency of 20%.

Results

Fig. 1 shows phase contrast photomicrographs of the isolated microvessel preparation before and after homogenization. Prior to homogenization, the vessels were intact and consisted largely of capillaries associated with larger vessels. The preparation was free of extravascular debris and was similar to that described by Brendel et al. [14]. The final homogenization procedure effectively disrupted over 95% of the vessels, resulting in a particulate suspension.

The characteristics of the assay system used to measure peptidyl dipeptidase activity in homogenates of brain microvessels are shown in Fig. 2. Hydrolysis of Hip-His-Leu was proportional both to incubation time and protein concentration. A relatively broad pH optima between pH 8 and 9 was observed, and conditions were standardized at pH 8.3 to be consistent with previously published data [21]. Under standard conditions activity could be measured conveniently using 25—50 μg tissue protein, representing about 2.5—5% of the total yield of microvessels from 1 rabbit brain, and by incubating for 30 min at 37°C.

Table I compares enzyme activity in microvessels with that in the original homogenates of brain tissue from which the microvessel preparation was obtained. Activity both in the microvessels and brain homogenate was almost completely dependent on chloride ion. Specific activity expressed on the basis of tissue protein was approximately 20 times greater in the microvessels (21.7 $nmol \cdot min^{-1} \cdot mg^{-1}$) than in brain homogenates (1.1 $nmol \cdot min^{-1} \cdot mg^{-1}$). Activity in the microvessels was reproducible between preparations, with values for specific activity ranging between 16 and 28 $nmol \cdot min^{-1} \cdot mg^{-1}$ in 12 separate preparations. The microvessels routinely contained 10—20% of the total activity originally present in the brain homogenate.

Previous studies have compared converting enzyme activity in different tissues of the rat using dialyzed low-speed supernatant fractions obtained from tissue homogenates at the enzyme source [13]. Those studies indicated a relatively high specific activity for the enzyme in the lung as compared to most other tissues. Table II compares enzymatic activity on the basis of tissue protein in homogenate and low speed supernatant fractions of rabbit brain microvessels with similar fractions obtained from lung and aortic tissue of the rat. Specific activity in the supernatant fraction of rat lung was 85% of that in the homogenate with approximately 40% of the total protein being recovered in supernatant fraction. Specific activity in the microvessel homogenate was amost twice that in the corresponding supernatant fraction, and approximately 40% of the total protein was recovered in the supernatant fraction. Aortic tissue, which contains a high content of extracellular connective tissue protein relative to most other tissues, had a greater specific activity in the supernatant fraction than in the homogenate and only 10% of the total protein was

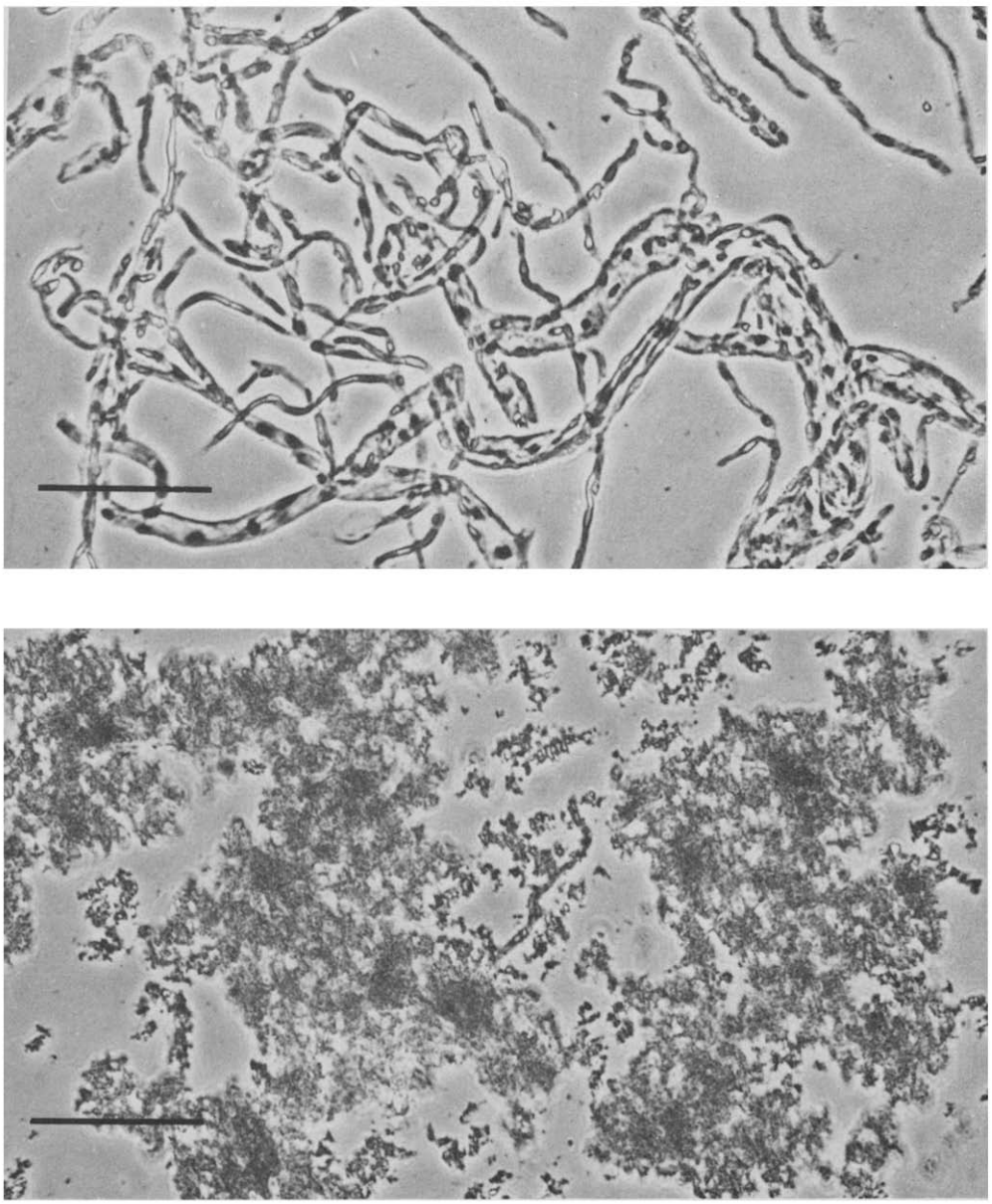

Fig. 1. Phase contrast photomicrographs of intact microvessels (upper figure) and the microvessel homogenate used for enzymatic studies (lower figure). Scale marker = 100 μm (X230).

recovered in the supernatant fraction. Enzymatic assays performed on the low speed pellet fractions accounted for the activity not recovered in the supernatant fractions (unpublished data).

Peptidyl dipeptidase activity in the microvessels was inhibited by agents previously shown to be effective antagonists for the enzyme both in vivo and in vitro [23,24]. Figure 3 shows the inhibitor effects of the drugs SQ 20,881 and SQ 14,225 in the assay system. Both drugs inhibited the hydrolysis of Hip-His-

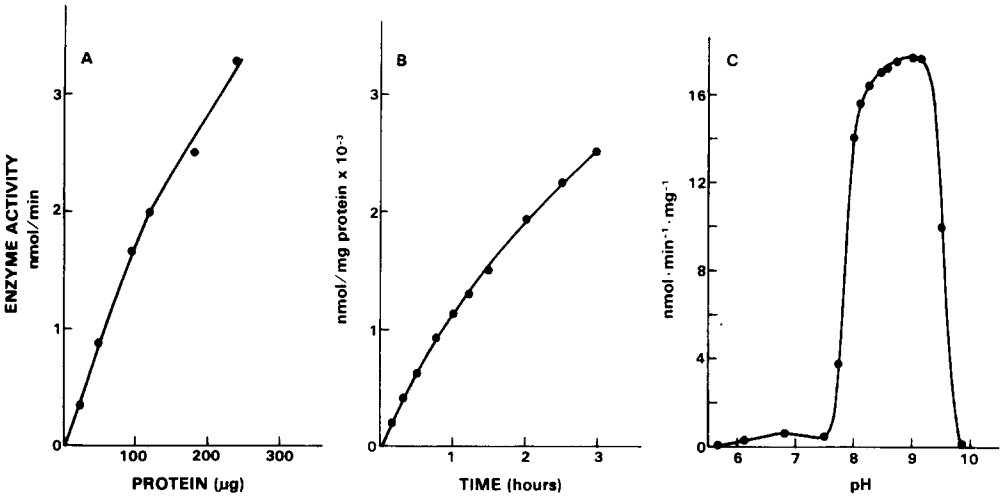

Fig. 2. The effect of tissue protein concentration, incubation time, and pH on the hydrolysis of Hip-His-Leu by microvessel homogenates. Unless indicated otherwise, incubation tubes contained 40–60 μg tissue protein and were incubated at 37°C for 30 min at pH 8.3. pH was adjusted in the presence of 0.3 M NaCl, using phosphate buffer. Experimental point represent the average of duplicate determinations.

Leu in a dose-dependent manner, however the SQ 14,225 compound was about 100 times as effective as an inhibitor, producing a 50% inhibition at a concentration of $5 \cdot 10^{-9}$ M, whereas SQ 20,881 produced 50% inhibition at a concentration of $6 \cdot 10^{-7}$ M.

To confirm further the presence of peptidyl dipeptidase activity in brain microvessels, assays were performed using as the substrate angiotensin I, labeled with tritium in the terminal leucine position. Comparative studies were also performed using aliquots of rat lung homogenates. Reaction conditions were established so that formation of His-[3,4,5-^{3}H]Leu was linear with incubation time and directly proportional to tissue protein concentration. Separate studies also showed that formation of labeled His-Leu was maximum between pH 7 and 8 with an optimum at about 7.5. The data in Table III show the effects of added chloride and SQ 14,225 on the formation of labeled His-Leu from angiotensin I by homogenates of microvessel in lung. Activity was observed in the absence of chloride with both tissue fractions, and addition of

TABLE I

COMPARISON OF PEPTIDYL DIPEPTIDASE ACTIVITY IN MICROVESSEL AND BRAIN HOMOGENATES

Data are expressed as mean ± S.E. Values in parenthesis refer to the number of preparations tested.

	Enzyme activity (nmol · min^{-1} · mg^{-1})		Total protein (mg)	Total activity (nmol/min)
	Without NaCl	With NaCl		
Brain (4)	0.08 ± 0.01	1.1 ± 0.2	162 ± 5.1	178.2 ± 5.4
Microvessels (12)	0.82 ± 0.11	21.7 ± 1.3	1.2 ± 0.1	26.0 ± 1.5

TABLE II

PEPTIDYL DIPEPTIDASE ACTIVITY IN SUBCELLULAR FRACTIONS FROM DIFFERENT TISSUES

Lung and aortic tissue were homogenized in 10 vol. (w/v) of 50 mM potassium phosphate buffer, pH 8.3. Microvessels obtained from 2 rabbit brains were homogenized in 2.0 ml of buffer as described in the methods section. Homogenates were centrifuged at 9000 × *g* for 10 min at 4°C. Data are expressed as mean ± S.E. for 3 separate preparations of each tissue.

Tissue	Activity (nmol · min^{-1} · mg^{-1})		Protein concentration (mg/ml)	
	Homogenate	Supernatant	Homogenate	Supernatant
Lung	39.1 ± 2.9	33.8 ± 2.9	11.6 ± 0.3	5.5 ± 0.1
Microvessels	25.6 ± 2.2	14.1 ± 1.7	1.1 ± 0.1	0.42 ± 0.06
Aorta	0.44 ± 0.1	5.0 ± 0.9	18.1 ± 1.2	1.8 ± 0.2

chloride resulted in the increased formation of His-Leu. This chloride-sensitive activity was almost completely inhibited when SQ 14,225 was present during the incubation with microvessels. In the lung preparation, addition of SQ 14,225 reduced activity to levels below that observed in the absence of chloride, suggesting the presence of endogenous chloride in lung homogenates. In separate experiments employing a 9000 × *g* supernatant fraction from lung homogenate which was dialyzed overnight at 2° C against 100 mM phosphate buffer (pH 7.5), formation of labeled His-Leu again was observed in the absence of chloride, but no inhibition occurred when SQ 14,225 was added. It

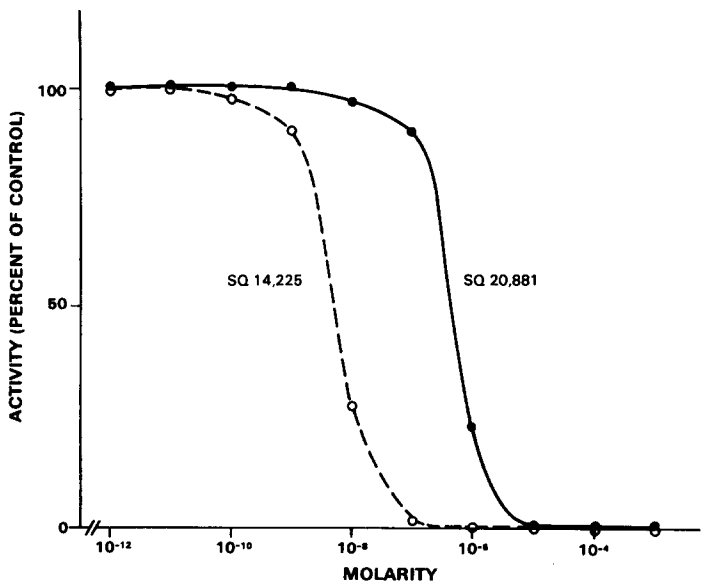

Fig. 3. Inhibition of peptidyl dipeptidase activity in microvessel preparations by SQ 20,881 and SQ 14,225. Varying amounts of the drugs were added to the incubation tubes containing 80—100 μg tissue protein and incubated for 30 min under standard assay conditions. The data were obtained from 3 separate experiments and are expressed as a percentage of the chloride sensitive activity found in the absence of inhibitor, which was 20.5, 18.8, and 19.5 nmol · min^{-1} · mg^{-1} for the 3 experiments. All experimental points represent the average of duplicate determinations.

TABLE III

EFFECT OF CHLORIDE AND SQ 14,225 ON THE HYDROLYSIS OF ANGIOTENSION I BY MICROVESSEL AND LUNG HOMOGENATES

Assays were performed as described in Methods, using tritiated angiotensin I as substrate. For the assays containing SQ 14,225, tissue aliquots were preincubated at 25°C for 10 min with the drug prior to adding labeled angiotensin I. Data are expressed as mean ± S.E. for 3 separate determination. Asterisks indicate significant differences ($P < 0.05$) from the incubations performed in the absence of chloride and SQ 14,225.

Assay conditions		Microvessel activity	Lung activity
Chloride (150 mM)	SQ 14,225 ($1 \cdot 10^{-5}$ M)	(pmol · min^{-1} · mg^{-1})	
—	—	174 ± 18	362 ± 24
+	—	329 ± 30 *	538 ± 34 *
—	+	171 ± 15	244 ± 18 *
+	+	179 ± 12	241 ± 17 *

is likely that the activity shown in Table III in the absence of chloride was due to the combined effort of endogenous chloride and proteolytic enzymes other than peptidyl dipeptidase.

Discussion

These studies established the presence of peptidyl dipeptidase (angiotensin-converting enzyme) in small blood vessels obtained from rabbit brain. Using Hip-His-Leu as substrate, the activity measured was similar to that previously described in lung tissue with respect to dependency on chloride ion and inhibition by the drugs SQ 20,881 and SQ 14,225 [24]. Furthermore, the specific activity of the enzyme in the microvessel homogenates was approximately 60% of the activity in lung homogenates and considerably greater than that previously reported for most other tissues except epididymis and testis [13].

Comparative studies between tissue fractions should be interpreted with caution not only because of variations in homogenization techniques but also because differences in the subcellular distribution of enzymatic activity may occur following tissue homogenization and centrifugation. For example, our procedure for isolating brain microvessels is based in part on the selective homogenization and disruption of extravascular tissue while maintaining the structural integrity of the microvessels. Vigorous homogenization procedures were effective in fragmenting the isolated microvessel preparation, as indicated by microscopic examination, yet effective solubilization of converting enzyme activity was not achieved. A particulate fraction from lung homogenates has been used as a source for the isolation of peptidyl dipeptidase [25] and our procedure for homogenizing lung resulted in a predominantly particular localization for the activity. Activity in aortic tissue was greater in the supernatant fraction than in the whole homogenate, possibly reflecting the effective disruption of aortic endothelial cells, solubilization of enzymatic activity during the homogenization procedure, and sedimentation of the large amount of aortic connective tissue protein during centrifugation.

Pulmonary tissue is enriched in endothelial cells in comparison to most other tissues, a fact thay may explain the high content of enzyme in lung homogenates. The relatively high specific activity in microvessels is probably also due to the abundance of endothelial cells relative to total tissue mass in these preparations. Because both lung and microvessel preparations represent a mixed cell population, it is not possible to compare enzymatic activity in pulmonary and cerebral endothelial cells, nor can one exclude the possibility that other cell types in both preparations also contain the enzyme.

The recovery of almost 15% of the total brain activity in the isolated microvessels and the 20-fold increase in specific activity compared to the brain homogenate suggested that much of the activity in brain was localized in the vessels. The isolation procedure does not result in complete recovery of all blood vessels, and it is difficult by morphological techniques to assess the effectiveness of the procedure with respect to recovery. Since converting enzyme is believed to be localized predominantly in endothelial cells, it would appear to be an excellent marker enzyme for microvessels. Interestingly, Goldstein et al. [15], found that alkaline phosphatase and glutamyl transpeptidase activity, both of which are thought to be localized in blood vessels of the central nervous system, were enriched 15—20-fold in isolated rat brain capillaries, when compared to a cerebral cortex homogenate. Thus, our studies are consistent with an endothelial localization for peptidyl dipeptidase and raise the possibility that angiotensin I can be converted to angiotensin II in the cerebral circulation.

The presence of a renin-angiotensin system within the brain and the possibility that angiotensin II is actually formed within the central nervous system remains controversial [26]. However, recent evidence demonstrated that in vivo administration of the converting enzyme inhibitor SQ 20,881 to sodium-depleted dogs produced changes in blood flow to several organs, including an increase in cerebral blood flow [27]. This observation is consistent with the proposal that local production of angiotensin II in the vessel wall may play an important role in regulating regional blood flow in various tissue [28]. Our studies showed a high concentration of peptidyl dipeptidase localized in the cerebral vasculature which was sensitive to specific inhibition by pharmacological agents. It is possible that this finding may prove to have therapeutic implications in clinical situations where it is desirable to improve cerebral blood flow.

Acknowledgments

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