Renin, angiotensins, and angiotensin-converting enzyme in neuroblastoma cells: Evidence for intracellular formation of angiotensins

(cell culture/neuropeptide/radioimmunoassay/coexistence of renin and angiotensins/dipeptidyl carboxypeptidase)

TOMIO OKAMURA, DANIEL L. CLEMENS, AND TADASHI INAGAMI

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Communicated by William J. Darby, July 22, 1981

ABSTRACT The mechanism of formation of various peptide hormones in neuronal cells in the brain is not clear. The question of whether brain angiotensin II is formed by an extracellular mechanism as in the peripheral system or by an intracellular mechanism can be answered by using cloned cells in culture. We have screened several neuroblastoma cell lines of rat and mouse origin and found at least three cell lines that contain renin (EC 3.4.99.19), angiotensin-converting enzyme (dipeptidyl carboxypeptidase; peptidyl dipeptide hydrolase, EC 3.4.15.1), and angiotensins I and II. This finding was interpreted to indicate that in these cells angiotensin formation takes place by an intracellular mechanism, in contrast to the extracellular mechanism well known to occur in plasma. This study also demonstrates the existence of viable and cloned cell lines that produce renin.

Many polypeptide hormones originally discovered in peripheral tissues are also present in various regions of the brain. The nature and localization of the enzymes involved in the processing of prohormone to hormone in the brain have not been established. Angiotensin II, a potent vasoconstrictor octapeptide, has been found in various regions of the brain (1–4). The presence of its receptor in the brain (5, 6) and multiple physiological responses elicited by centrally administered angiotensin II (7–12) indicate a direct effect of this hormone on neuronal function. Although the extracellular pathway of angiotensin formation in the circulation is well established, the intracellular localization of angiotensin II in the brain (1–4) suggests that the pathway in the central nervous system may be different from that of the periphery.

The first step of angiotensin formation in the periphery is mediated by the circulating enzyme renin (EC 3.4.99.19). In the central system, demonstration of specific renin in brain extracts by affinity chromatography has established that the renin-like activity (1, 2) is indeed due to specific renin (13) rather than a nonspecific action of cathepsin D (14, 15). However, it has not been clear whether brain renin is synthesized endogenously or is derived from the blood-borne enzyme of renal origin. Angiotensin-converting enzyme (dipeptidyl carboxypeptidase; peptidyl dipeptide hydrolase, EC 3.4.15.1) activity has also been reported in brain tissues (16–18). However, this enzyme is present on the luminal aspect of the vascular endothelium throughout the body (19, 20), and the existence of the enzyme in brain parenchyma has not been demonstrated unequivocally. Whether angiotensin II is formed intracellularly in cells producing both renin and angiotensin-converting enzyme, in different types of cells containing these enzymes separately, or by an extracellular pathway has not been clarified. It is difficult to answer such questions by experiments using brain tissue,

which contains various cell types as well as blood and vascular tissue. In order to resolve these problems, we have screened several cloned neuroblastoma cell lines for the existence of renin, angiotensins, and angiotensin-converting enzyme.

Experimental results favoring an intracellular mechanism of angiotensin formation in these cloned cells were obtained.

MATERIALS AND METHODS

Neuroblastoma Cells. Established neuroblastoma cell lines used were B82, B103 (21), and RT4E4 (22) of rat origin, and Neuro-2a (23), NB41A3 (24), and N4TG1 (25) derived from mouse neuroblastoma C-1300. Cells, except for NB41A3 cells, were cultured in Dulbecco's modified Eagle's medium containing 12.5% horse serum and 2.5% fetal calf serum until confluency (4–10 days). NB41A3 cells were cultured in Ham's F-10 medium with additions of horse and fetal calf serum to the same concentrations. Twenty-four hours prior to harvesting the cells, media were changed to serum-free solutions. Rat neuroblastoma cells were detached from culture flasks with 1 mM EDTA, washed twice with the serum-free medium, suspended in water containing a mixture of 1 mM diisopropyl phosphorofluoridate (iPr₂P-F), 1 mM Captopril (an angiotensin-converting enzyme inhibitor; Squibb, leupeptin at 5 µg/ml, and 5 mM EDTA and lysed by five cycles of freezing and thawing. Insoluble pellet fractions, separated from lysate by centrifugation at 800 × g for 15 min, contained large proportions of renin and angiotensins. For complete solubilization, treatment with the detergent Triton X-100 (0.1%) was used in addition to the freezing and thawing.

Mouse cells were detached from culture flasks by 5-min treatment with 0.25% trypsin containing 1 mM EDTA, washed twice with serum-free media containing 2.5% soybean trypsin inhibitor, and treated identically as rat cells.

For the assay of angiotensin-converting enzyme, rat and mouse cells were treated in a similar manner as above with 0.25% trypsin but without EDTA. Cells were lysed in 0.1 mM phenylmethanesulfonyl fluoride. The detergent Nonidet P-40 was used instead of Triton X-100. Insoluble fractions were also separated as above.

Enzyme Assay. Aliquots of cell lysate and the detergent-treated cell pellet fractions were used for renin activity assay. The solutions were incubated for 6 hr at 37°C in 0.2 M sodium maleate buffer, pH 6.0, containing 5 mM phenylmethanesulfonyl fluoride, 10 mM EDTA, and 0.1% neomycin sulfate, using the plasma of bilaterally nephrectomized rats as substrate. This reaction was stopped by heating in boiling water for 10 min, and generated angiotensin I was determined by radioimmunoassay (26). As a control for interference by nonspecific renin-like ac-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: iPr₂P-F, diisopropyl phosphorofluoridate.

Table 1. Renin activity and angiotensins I and II/III in cloned neuroblastoma cells

Cell line	n	Total renin-like activity, pg angiotensin I/hr per 10 ⁶ cells	% inhibition by specific antibodies to renin	Angiotensin I, pg/10 ⁶ cells	Angiotensin II, pg/10 ⁶ cells*
Mouse					
Neuro-2a	3	156.3 ± 29.2	92.9 ± 1.6	11.77 ± 1.37	4.26 ± 1.30
NB41A3	3	501.9 ± 182.4	93.0 ± 3.9	8.61 ± 2.65	4.49 ± 0.61
N4TG1	3	1.34 ± 0.15	58.1 ± 5.4	4.69 ± 0.53	Undetectable
Rat					
B82	1	3.66	60.6	9.01	5.17
B103	3	2.02 ± 0.24	0	Undetectable	Undetectable
RT4E4	3	1.98 ± 0.38	47.0 ± 9.1	29.26 ± 12.60	7.23 ± 0.64

Results are given ± SEM.

* Determined in the absence of EDTA and Captopril.

tivity of cathepsin D (14, 15), parallel experiments were performed with the extract preincubated with specific antibodies to pure rat (27) and mouse renin (28) at 1:10,000 dilutions for 16 hr at 4°C. At this dilution the antisera were found to inhibit 10 ng of renin completely, but not cathepsin D. The pure rat and mouse renin used for producing the antibodies were shown to satisfy multiple criteria of purity (27, 28). The antibody to mouse renin was shown not to crossreact with renin of other species (28). The antibody to rat renin was shown to give a single precipitin band by Ouchterlony immunodiffusion, and does not crossreact with human renin (unpublished results). The pH dependence of renin activity was determined in a similar manner, using the following buffers at 0.2 M: sodium acetate for pH 3.0–5.0, sodium maleate for pH 5.0–6.5, sodium phosphate for pH 6.5–7.5, and Tris-HCl for pH 7.5–8.5.

Angiotensin-converting enzyme activity was determined by a modification of the method of DePierre and Roth (29). Sample solutions were incubated with the synthetic substrate hippuryl-His-Leu for 30 min at 37°C in 0.4 ml of 50 mM phosphate/50 mM borate buffer, pH 8.0, containing 0.2 M NaCl. The extent of the hydrolysis of the hippurylhistidyl peptide bond was determined by fluorometric assay using *o*-phthalaldehyde (29). Inhibition of the reaction by 12 nM Captopril, 2.3 mM EDTA, and by omission of NaCl from the incubation medium was examined. The Captopril solutions were prepared immediately prior to use.

Angiotensin Determination. Aliquots of cell extracts were treated with 3 vol of acetone and centrifuged to obtain supernatant fractions, which were dried under nitrogen gas, redissolved in 0.1 M Tris/acetate buffer, pH 7.4, heated in a boiling water bath, and subjected to radioimmunoassay of angiotensin I (26) and angiotensin II (30). The angiotensin II antiserum kindly supplied by Robert J. Workman showed less than 1% crossreactivity with angiotensin I but 100% crossreactivity with angiotensin II-(2–8) hexapeptide, angiotensin II-(3–8) hexapeptide, and angiotensin II-(4–8) pentapeptide (purchased from Protein Research Foundation, Osaka, Japan). Greater than 90% of exogenous angiotensins I and II/III added to cell lysates or pellet fractions were recovered by the assay methods employed.

Angiotensins were identified by thin-layer chromatography on cellulose plates (Analtech, Newark, DE) by a modification of the method of Semple *et al.* (31) using *sec*-butyl alcohol/3% ammonia (5:1, vol/vol). After the plate had been developed, areas containing angiotensins, as determined in reference to standard angiotensins developed in adjacent channels, were scraped, extracted in methanol/ammonia (2:1, vol/vol), and treated in the same manner as above for the radioimmunoassay of angiotensins I and II.

RESULTS

Renin Activity. Results of determination of renin activity and angiotensins are summarized in Table 1. These results were obtained with extract from cells treated with the combination of freezing and thawing and with 0.1% Triton X-100. Two mouse neuroblastoma cell lines (NB41A3 and Neuro-2a) showed high levels of renin activity that could be almost completely inhibited by specific antibodies to mouse renin. N4TG1 cells, which were also derived from C-1300, showed much lower but measurable renin activity than did NB41A3 and Neuro-2a. Low but demonstrable levels of renin activity were present in rat neuroblastoma cell line B82. A lower renin level was observed in RT4E4. Large parts of the renin activity (30–60%) were inhibited by specific antibodies to rat renin, indicating that the activity is not due to nonspecific action of cathepsin. B103 cells grown, harvested, and lysed under identical conditions did not show detectable levels of the antibody-sensitive renin activity. Experiments using separated cell lysate and pellet fractions, as prepared by five cycles of freezing and thawing, showed that the enzyme activity was present not only in the cell lysate but also in the pellet fraction (40–75% of the total activities in whole cells), from which it could be released only after the treatment with 0.1% Triton X-100. Repeated washing of the pellet with the buffer did not release this form of renin, indicating the possibility of the presence of a membrane-bound form of renin.

FIG. 1. The pH profile of renin activity of mouse neuroblastoma cell Neuro-2a. Angiotensin I generating activities of cell extracts (expressed per 10⁶ cells) were determined after preincubation of the extracts with and without anti-mouse renin antibody at 1:10,000 dilution. The differences of the two values were plotted as antibody-sensitive renin activities that do not include contribution of nonspecific renin-like activity of cathepsin.

In contrast to the intracellular localization of renin, the serum-free culture media showed little renin activity.

The pH profiles of renin activity in the extracts of mouse neuroblastoma NB41A3 and Neuro-2a showed optima between pH 6.5 and 7.5 (Fig. 1), in agreement with that of hog brain renin (32), again demonstrating the characteristic renin pH profile rather than acid pH optima attributable to the nonspecific renin-like action of cathepsin D (14, 15).

Angiotensins. Renin-producing cells showed low but readily immunoassayable levels of angiotensin I (Table 1), which could be identified by thin-layer chromatography as shown by an example in Fig. 2. Angiotensin I was detectable only when cells were lysed in the presence of EDTA, iPr₂P-F, Captopril, and leupeptin. When EDTA and Captopril were omitted, this peptide disappeared, while angiotensin II/III immunoreactivity increased. As shown in Fig. 2, approximately 60% of the angiotensin II immunoreactivity was attributable to the heptapeptide angiotensin III, and most of the remainder to the octapeptide angiotensin II. Again a large proportion of angiotensin II/III (50–90% of the total) became measurable after treatment

Table 2. Converting enzyme activity and its inhibition in cloned neuroblastoma cells

Cell line	Total angiotensin- converting enzyme-like activity, pmol His-Leu/min per 10 ⁶ cells	% inhibition of angiotensin- converting enzyme activity		
		2.3 mM EDTA	12 nM Captopril	Omission of chloride*
Mouse				
Neuro-2a	351	98	91	96
NB41A3	762	97	83	96
Rat				
B82	202	98	91	100
B103	<4 [†]	—	—	—
RT4E4	849	97	85	100

* The 0.2 M NaCl was omitted.
† Below detection limit.

with Triton X-100. These observations suggest that much of angiotensin II may be stored in a membrane-bound or vesicular form.

Angiotensin-Converting Enzyme. Angiotensin-converting enzyme-like activities determined by using a synthetic substrate are shown in Table 2. The total cell activities represent the sum of those in the detergent-treated cell pellet and cell lysate. Greater portions (60–80%) of the total activity were detected in the pellet fractions after treatment with Nonidet P-40, consistent with a membrane-bound form of the enzyme. Repeated washing without the detergent was not effective in releasing the enzyme from the pellet. RT4E4, Neuro-2a, and NB41A3 showed detectable levels of the converting enzyme activity, whereas the activity levels of other cell lines were very low. Potent inhibition of the hippuryl-His-Leu hydrolyzing activity was observed in the presence of 2.3 mM EDTA or with 12 nM Captopril, a specific inhibitor of angiotensin-converting enzyme. A strong dependence of the enzyme activity on chloride ion concentration was observed (Table 2).

DISCUSSION

The present study shows that some of the cloned neuroblastoma cell lines contain both renin and angiotensin-converting enzyme and continue to produce these enzymes even after many passages. Furthermore, intracellular presence of angiotensins I, II, and III was also demonstrated in these cells. These findings indicate intracellular formation of angiotensin II/III in the neuroblastoma cells. This is in contrast to the peripheral system, in which angiotensins are formed by an extracellular mechanism in plasma. Thus, the present study demonstrates a hitherto unrecognized mechanism of angiotensin formation.

Intraneuronal localization of angiotensin II/III was demonstrated in various regions of the brain (3, 4). Many other peptide hormones have also been found in neuronal cells. The mechanism of intraneuronal synthesis of these peptide hormones originally discovered in various peripheral tissues was not clear. Although neuroblastoma cells are not normal neuronal cells, mouse cell lines and rat cell lines used in this study possess characteristics of differentiated neuronal cells (20–23) and may be considered as models of renin-containing neuronal cells. Thus the present study suggests that similar mechanisms involving highly specific proteases or peptidases for the conversion of prohormones to active hormones may be functional in various neuronal cells containing these peptide hormones. Failure to observe extracellular renin in appreciable quantity in

FIG. 2. Chromatographic separation of angiotensins I, II, and III on a cellulose thin-layer plate by *sec*-butyl alcohol/3% ammonia (5:1, vol/vol). After samples had been developed, cellulose was scraped off the plate. Angiotensins were extracted in methanol/3% ammonia (2:1, vol/vol). After the solvent had been evaporated under a nitrogen stream, angiotensins were determined by radioimmunoassay. (A) RT4E4 cell extract assayed for angiotensins II and III; (B) same extract assayed for angiotensin I; (C) separation of standard angiotensins visualized by ninhydrin.

culture media is compatible with the intracellular mechanism.

That some of the cell lines such as B103 did not contain renin or angiotensin-converting enzyme can be taken as a control to show that the cellular renin activity is not due to entrapment of small amounts of renin from calf and horse sera used in culture media. This view is further strengthened by the finding that renin activities in mouse neuroblastoma cell lines are inhibited almost completely by the antibody, whose immunoreactivity has been shown to be species specific to mouse renin. A large variation in renin activity noted among the various cell lines presumably reflects a difference in the capacity for endogenous synthesis of this enzyme in different types of neuronal cells.

The strong requirement for chloride ion of the angiotensin-converting enzyme-like activity and its potent inhibition by 12 nM Captopril confirm that the activity measured was indeed angiotensin-converting enzyme. Enkephalinase A (33), another dicarboxypeptidase, would not be inhibited by this concentration of Captopril (34) and is inhibited by chloride ion (33). Furthermore, the presence of 50 mM potassium phosphate in the incubation buffer would have inhibited any enkephalinase that was present (33).

The observations that large proportions of renin, angiotensin II, and angiotensin-converting enzyme were present in the pellet fractions and were not released by repeated washing without detergent suggest their localization in particulate organelles or bound to plasma membranes. In this preliminary study we have not attempted to identify the type of organelles or membranes containing these components of the renin-angiotensin system. However, because all the components are located in the same cell, it is reasonable that these enzymes and peptides are compartmentalized for the reaction to proceed in a regulated manner.

The question may be asked whether angiotensinogen is present in these cells in measurable quantities. The presence of renin makes the measurement difficult without technical refinement.

These neuroblastoma cell lines are the only cloned cell lines identified to date as containing or producing renin even after numerous passages. Further studies on these cells will yield valuable information on production and processing of renin and angiotensins because these lines may be considered as models of neuronal cells.

We gratefully acknowledge the kindness of Drs. N. Sueoka, Y. Tomozawa, and J. D. Puett for their gifts of neuroblastoma cell lines, the generous supply of antibodies to angiotensins by Dr. R. J. Workman, and technical assistance of Mrs. Trinita Fitzgerald. This research was supported by Research Grants HL-24112 and HL-14192 from the National Institutes of Health.

1. Ganten, D., Marquez-Julio, A., Granger, P., Hayduk, K., Kar-sunky, K. P., Boucher, R. & Genest, I. (1971) *Am. J. Physiol.* **221**, 1733–1737.
2. Fischer-Ferrario, C., Nahmod, V. E., Goldstein, D. J. & Fin-kielman, S. (1971) *J. Exp. Med.* **133**, 353–361.
3. Changaris, D., Severs, W. & Kiel, L. (1978) *J. Histochem. Cy-tochem.* **26**, 593–609.
4. Fuxe, K., Ganten, D., Mokfelt, T. & Bolme, P. (1976) *Neurosci. Lett.* **2**, 229–234.
5. Bennett, J. & Snyder, S. (1976) *J. Biol. Chem.* **251**, 7423–7430.
6. Sirett, N., McLean, A., Bray, J. & Hubbard, J. (1977) *Brain Res.* **122**, 299–312.
7. Bickerton, R. R. & Buckley, J. P. (1961) *Proc. Soc. Exp. Biol. Med.* **106**, 834–836.
8. Ferrario, C. M., Gildenberg, P. L. & McCubbin, J. W. (1972) *Circ. Res.* **30**, 257–262.
9. Fitzsimons, J. T. & Simons, B. J. (1968) *J. Physiol. (London)* **196**, 39p–41p.
10. Severs, W. B. & Daniels-Severs, A. E. (1973) *Pharmacol. Rev.* **25**, 415–499.
11. Epstein, A. N., Fitzsimons, J. T. & Rolls, B. J. (1970) *J. Physiol. (London)* **210**, 457–474.
12. Hawtley, T. & Felix, D. (1980) *Brain Res.* **195**, 187–195.
13. Hirose, S., Yokosawa, H. & Inagami, T. (1978) *Nature (London)* **274**, 392–393.
14. Day, R. P. & Reid, I. A. (1976) *Endocrinology* **99**, 93–100.
15. Hackenthal, E., Hackenthal, R. & Hilgenfeld, U. (1978) *Biochim. Biophys. Acta* **522**, 561–573.
16. Yang, H. & Neff, N. H. (1972) *J. Neurochem.* **19**, 2443–2450.
17. Poth, M. M., Heath, R. G. & Ward, M. (1975) *J. Neurochem.* **25**, 83–85.
18. Benuck, M. & Marks, N. (1978) *J. Neurochem.* **30**, 729–734.
19. Wigger, H. J. & Stalcup, S. A. (1978) *Lab. Invest.* **38**, 581–585.
20. Caldwell, P. R. B., Seegal, B. C., Hsu, K. C., Das, M. & Soffer, R. L. (1976) *Science* **191**, 1050–1051.
21. Schubert, D., Heineman, S., Carlisle, W., Tarikas, A., Kines, B., Patrick, J., Steinback, J. H., Culp, W. & Brandt, B. L. (1974) *Nature (London)* **249**, 224–227.
22. Tomozawa, Y. & Sueoka, N. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 6305–6309.
23. Kleb, R. & Ruddle, F. (1969) *J. Cell Biol.* **43**, 69A.
24. Augusti-Tocco, G. & Sato, G. (1969) *Proc. Natl. Acad. Sci. USA* **64**, 311–315.
25. Minna, J., Nelson, P., Peacock, J., Glazer, D. & Nirenberg, M. (1969) *Proc. Natl. Acad. Sci. USA* **68**, 234–239.
26. Haber, E., Koerner, T., Page, L., Kliman, B. & Purnode, A. (1969) *J. Clin. Endocrinol. Metab.* **29**, 1349–1355.
27. Matoba, T., Murakami, K. & Inagami, T. (1978) *Biochim. Biophys. Acta* **526**, 560–571.
28. Michelakis, A. M., Yoshida, H., Menzie, J., Murakami, K. & In-agami, T. (1974) *Endocrinology* **94**, 1101–1105.
29. DePierre, D. & Roth, M. (1975) *Enzyme* **19**, 65–70.
30. Gocke, D. J., Sherwood, L. M., Oppenhoff, I., Gerten, J. & Lar-agh, J. H. (1968) *J. Clin. Endocrinol.* **28**, 1675–1678.
31. Semple, P. F., Macrae, W. A. & Morton, J. J. (1980) *Clin. Sci.* **59**, 61s–64s.
32. Hirose, S., Yokosawa, H., Inagami, T. & Workman, R. J. (1980) *Brain Res.* **191**, 489–499.
33. Gorenstein, C. & Snyder, S. H. (1979) *Life Sci.* **25**, 2065–2070.
34. Swerts, J. P., Perdrisot, R., Patey, G., Delabaume, S. & Schwartz, J. (1979) *Eur. J. Pharmacol.* **57**, 279–281.