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SPECIAL REPORT

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Human brain contains a metalloprotease that converts big endothelin-1 to endothelin-1 and is inhibited by phosphoramidon and EDTA

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Incubation of big endothelin-1 (bET-1) with protein derived from the detergent-extracted 100,000 *g* pellet prepared from human brain tissue resulted in the formation of endothelin-1 (ET-1) at a rate of 90 fmol mg^{-1} protein min^{-1}. This formation was inhibited in a concentration-dependent manner by either phosphoramidon or EDTA, with half-maximal inhibitory concentrations of 2 and 20 μM, respectively. No conversion of big endothelin-3 (bET-3) to endothelin-3 (ET-3) was detected under the same conditions. These results show the presence in the human brain of a metalloprotease-like enzymatic activity which selectively converts bET-1 and ET-1. Together with earlier reports of mRNA for ET-1 this suggests the presence of the entire synthetic pathway for ET-1 in human brain.

Keywords: Endothelin-1; big endothelin-1; endothelin-3; big endothelin-3; buman brain; endothelin-converting enzyme; phosphoramidon; EDTA.

Introduction Endothelin-1 (ET-1) was first discovered as a potent vasoconstrictor peptide synthesized and released from porcine endothelial cells (Yanagisawa *et al.*, 1988). It is believed that the synthesis of ET-1 involves the cleavage of its precursor big ET-1 (bET-1) by a specific endothelin-converting enzyme (ECE) (Ohnaka *et al.*, 1990). This enzyme is most probably a metalloprotease which is inhibited by phosphoramidon, or the metal ion chelator, EDTA. It is present in endothelial (Ohnaka *et al.*, 1990; Okada *et al.*, 1990; Warner *et al.*, 1992) and smooth muscle cells (Matsumura *et al.*, 1991), and we have found it also in rat brain (Warner *et al.*, 1992). Here we show that human brain contains ECE activity.

Methods *Preparation of subcellular fractions* Human cerebellum was obtained during a normal autopsy procedure, and frozen and stored at − 80°C. After thawing the tissue was homogenized in buffer A (HEPES 50 mM; NaCl 100 mM; pH 7.4) and centrifuged (100,000 *g*, 1 h). The particulate fraction was resuspended in buffer A and treated with the detergent CHAPS (20 mM, for 30 min) to remove membrane-associated and solubilizable particulate proteins. After centrifugation (100,000 *g*, 1 h) the remaining pellet containing the cytoskeletal fraction was resuspended in buffer A and assayed for ECE activity. This fraction was used because the cytoskeleton from rat brain contains the most clearly distinguishable metalloprotease-ECE (Warner *et al.*, 1992).

Determination of ECE activity Protein (22.5 μg) from the cytoskeletal fraction was incubated (37°C, 60 min), at pH 7.4 with bET-1 (human) or bET-3 (human) (135 pmol) and sometimes test inhibitors, in a total volume of 52.5 μl. All samples were incubated in duplicate in the absence and presence of phosphoramidon (100 μM). The reaction was stopped by the addition of a mixture of protease/peptidase inhibitors (phenylmethylsulphonyl fluoride, 1 mM; leupeptin, 2 μM; pepstatin A, 1 μM) and EDTA (1 mM), to a total volume of 62.5 μl. The incubates were then rapidly frozen in liquid N_2 and stored (max. 2 h) at − 20°C. After rapid thawing the amounts of ET-1/3 in each sample were assessed by bioassay and ELISA (enzyme-linked immunosorbent assay). Each ECE/bET-1/3 combination was assayed in triplicate in each experiment. For ET-1/3 bioassay aliquots (12.5 μl) of each sample were added to single wells of porcine kidney epithelial (PK_1) cells, grown to confluency in 12-well plates, bathed in Locke buffer. After 4 min the incubation buffer was removed and the reactions stopped by addition of 1 ml ice-cold sodium acetate buffer (50 mM, pH 4.0). The samples were quickly frozen with liquid N_2 (Ishii *et al.*, 1991). The amount of ET-1 or ET-3 present in the sample was then calculated from the levels of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) produced in the PK_1 cells by known amounts of ET-1 or ET-3 (Warner *et al.*, 1992). In these cells bET-1, at concentrations up to 100 nM, does not produce significant elevations in cyclic GMP levels (Warner *et al.*, 1992). The ELISA for ET-1/3 used antibody directed against the C-terminal fragment of ET-1/3 ($ET\text{-}1/3_{(17-21)}$) with approximately 0.01% cross reactivity with bET-1 or bET-3. Binding was detected by peroxidase-labelled goat anti-rabbit IgG antibody using *o*-phenylenediamine-2HCl as a substrate. The optical density of each well was measured at 490 nm with a microplate reader (Bio-Tek model EL 311). The amount of ET-1 or ET-3 formed in each incubation mixture by metalloprotease enzyme-activity was calculated as the difference between paired samples incubated with and without phosphoramidon (100 μM).

Results The cytoskeletal fraction converted bET-1 to ET-1 as detected by either PK_1-bioassay or ELISA. The rate of conversion was 90 fmol mg^{-1} protein min^{-1} (mean of 3 ELISA and 2 bioassay determinations, each in triplicate). This conversion was inhibited in a concentration-dependent manner by either the metalloprotease inhibitor, phosphoramidon (10 nM - 1 mM, Figure 1a), or the metal ion chelator, EDTA (300 nM - 10 mM, Figure 1b), with half-maximal inhibitory concentrations of 2 and 20 μM, respectively. The maximum inhibition caused by phosphoramidon was 88 ± 2%, at a concentration of 1 mM, and by EDTA 60 ± 4% at a concentration of 10 mM. In experiments with bET-3 as a substrate in the same assay conditions no formation of ET-3 was detected by PK_1-bioassay or ELISA ($n = 3$ for each).

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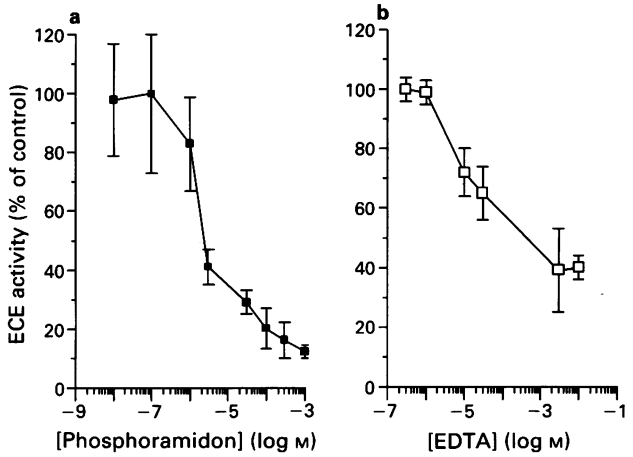

Figure 1 Phosphoramidon and EDTA inhibit in a concentration-dependent manner the conversion of big endothelin-1 (bET-1) to endothelin-1 (ET-1) by the cytoskeletal fraction prepared from human brain. When incubated with protein (22.5 μg) from the cytoskeletal fraction of human brain and bET-1 (135 pmol) in a total volume of 52.5 μl the metalloprotease-inhibitor, phosphoramidon (10 nM–1 mM, a), or the metal ion-chelator, EDTA (300 nM–10 mM, b) inhibited the formation of ET-1 with half-maximal inhibitory concentrations of 2 and 20 μM, respectively. Data shown were derived by bioassay (a) and by ELISA (b). Similar results were obtained when samples were tested in the alternate assay.

Discussion Our results show the presence of an enzyme in human brain which selectively converts bET-1 to ET-1, and which resembles other mammalian ECE activities previously described in rat, bovine and porcine cells. The formation of ET-1 was detected by both bioassay and ELISA, demonstrating that our biochemical assay was not detecting inactive ET-1 fragments, but biologically active ET-1. The ET-1 production was due to the activity of a metalloprotease similar to that present in endothelial cells, for it was inhibited in a concentration-dependent manner by both phosphoramidon and EDTA. This is the first report of metalloprotease-like ECE activity in human nervous tissue.

Interestingly, although brain tissue from other species contains more ET-3 than ET-1 (Takahashi *et al.*, 1991) we were not able to detect the conversion of bET-3 to ET-3, as has been reported previously in bovine endothelial cells (Okada *et al.*, 1990). This may be because the fraction we employed does not contain the appropriate enzyme activity, or alternatively that it is not active under our assay conditions. It is not due to a lack of sensitivity of our assays as these are equally sensitive to ET-3 and ET-1 (Ishii *et al.*, 1991; Warner *et al.*, 1992). This result does suggest however, that the metalloprotease within human brain that converts bET-1 to ET-1 has a highly selective substrate requirement. The activity we detected in human brain tissue is considerably lower than that present in rat whole brain (Warner *et al.*, 1992). This may be explained by the time delay between the excision of the tissue and its use in the assay, and also by the variability of ECE activity in different brain regions (unpublished observations). Thus the tissue that was available may well be from a brain area which does not contain a high ECE activity.

Together with the previous observations of mRNA for ET-1 in human brain (Lee *et al.*, 1990) it is clear that this tissue contains the synthetic pathway for ET-1. ET-1 may have roles in brain functions from neurosecretion (Yoshizawa *et al.*, 1990) to control of motor activity (Lecci *et al.*, 1990). Our data support the conclusion that endogenous ET-1, and perhaps other members of the endothelin family of peptides, are produced in the human central nervous system and presumably have important central and peripheral effects.

References

ISHII, K., WARNER, T.D., SHENG, H. & MURAD, F. (1991). Endothelin-1 stimulates cyclic GMP formation in porcine kidney epithelial cells via activation of the L-arginine-dependent soluble guanylate cyclase pathway. *J. Cardiovasc. Pharmacol.*, **17**, Suppl. 7, S246–S250.

LECCI, A., MAGGI, C.-A., ROVERO, P., GIACHETTI, A. & MELI, A. (1990). Effect of endothelin-1, endothelin-3 and C-terminal hexapeptide, endothelin (16-21) on motor activity in rats. *Neuropeptides*, **16**, 21–24.

LEE, M.-E., DE LA MONTE, S., NG, S.-C., BLOCK, K.D. & QUERTERMOUS, T. (1990). Expression of the potent vasoconstrictor endothelin in the human central nervous system. *J. Clin. Invest.*, **86**, 141–147.

MATSUMARA, Y., IKEGAWA, R., TSUKAHARA, Y., TAKAOKA, M. & MORIMOTO, S. (1991). Conversion of big endothelin-1 to endothelin-1 by two-types of metalloproteinases of cultured porcine vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **178**, 899–905.

OHNAKA, K., TAKAYANAGI, R., YAMAUCHI, T., OKAZAKI, H., OHASHI, M., UMEDA, F. & NAWATA, H. (1990). Identification and characterization of endothelin converting activity in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.*, **168**, 1128–1136.

OKADA, K., MIYAZAKI, Y., TAKADA, J., MATSUYAMA, K., YAMAKI, T. & YANO, M. (1990). Conversion of big endothelin-1 by membrane-bound metalloendopeptidase in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.*, **171**, 1192–1198.

TAKAHASHI, K., GHATEI, M.A., JONES, P.M., MURPHY, J.K., LAM, H.-C., O'HALLORAN, D.J. & BLOOM, S.R. (1991). Endothelin in human brain and pituitary gland: comparison with rat. *J. Cardiovasc. Pharmacol.*, **17** Suppl. 7, S101–S103.

WARNER, T.D., MITCHELL, J.A., D'ORLEANS-JUSTE, P., ISHII, K., FÖRSTERMANN, U. & MURAD, F. (1992). Characterization of endothelin-converting enzyme from endothelial cells and rat brain: detection of the formation of biologically active endothelin-1 by rapid bioassay. *Mol. Pharmacol.*, **41**, 399–403.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYISHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

YOSHIZAWA, T., SHINMI, O., GAID, A., YANAGISAWA, M., GIBSON, S.J., KIMURA, S., UCHIYAMA, Y., POLAK, J.M., MASAKI, M. & KANAZAWA, I. (1990). Endothelin: a novel peptide in the posterior pituitary system. *Science*, **247**, 462–464.

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