Vol. 184, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1992 Pages 323-329

PEPTIDE AMIDATING ENZYMES ARE PRESENT IN CULTURED ENDOTHELIAL CELLS

Charlie D. Oldham[1], Cuizhen Li[1], Peggy R. Girard[2], Robert M. Nerem[2] and Sheldon W. May[1]*

[1]School of Chemistry & Biochemistry and [2]School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA 30332

Received March 6, 1992

SUMMARY: Carboxy-terminal amidation is a prevalent post-translational modification necessary for the bioactivity of many peptides. We now report that the two enzymes essential for amidation, peptidylglycine α-monooxygenase (PAM) and peptidylamidoglycolate lyase (PGL), are present in both the cytosol and membrane fractions of cultured bovine aortic endothelial cells. Endothelial PAM exhibits ascorbate-dependent turnover and is inactivated by the mechanism-based inactivator, 4-phenyl-3-butenoic acid (PBA), whereas PGL activity is independent of ascorbate and is not affected by PBA. These enzymological characteristics correspond to those of amidating enzymes from other tissues. These results suggest a heretofore unrecognized role for α-amidated peptides in cardiovascular function.

Peptide hormones are synthesized as biologically inactive precursors which are then subjected to a series of modifications in order to produce the bioactive forms (1). One of the most common post-translational modifications is carboxy-terminal amidation, which arises from enzymatic processing of the glycine-extended prohormone (2). Previous work has established that carboxy terminal amidation arises from the sequential action of two enzymatic activities -- peptidylglycine α-monooxygenase (PAM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PGL, EC 4.3.2.5). PAM, a copper and L-ascorbate dependent monooxygenase, catalyzes formation of an α-hydroxyglycine peptide from the glycine-extended precursor (3-6) and PGL converts the α-hydroxyglycine peptide to the corresponding amidated peptide and glyoxylate (5).

* To whom correspondence should be addressed.

Abbreviations used: MES, 2-(N-morpholino)ethanesulfonic acid; PAM, peptidylglycine α-monooxygenase (EC 1.14.17.3); PBA, 4-phenyl-3-butenoic acid; PGL, peptidylamidoglycolate lyase (EC 4.3.2.5); TNP, 2,4,6-trinitrophenyl.

0006-291X/92 $1.50

Endothelial cells, which play a critical role in cardiovascular circulation, are known to produce and release a variety of vasoactive substances which are not amidated (7). Among these are the potent vasorelaxants, prostacyclin and endothelium-derived relaxing factor, and the vasoconstricting peptides, angiotensin II and endothelin. The enzymes responsible for carboxy-terminal amidation have not been reported to exist in endothelial cells. Substance P, an amidated peptide, has been detected in endothelial cells (8,9) and a variable release of substance P has been reported from columns of immobilized endothelial cells in response to shear stress (10). However, this might reflect accumulation from other sources as opposed to amidative processing within endothelial cells (9).

We now report, for the first time, that PAM and PGL are both present in both the cytosol and membrane fractions of cultured endothelial cells. Moreover, endothelial PAM exhibits the cofactor dependence and sensitivity toward mechanism-based inactivation characteristic of PAM from other tissues. These findings, taken together with the known presence of amidating enzyme activity in heart atrial granules (11), suggest a heretofore unrecognized role for α-amidated peptides in cardiovascular function.

METHODS

Cell culture. Bovine aortic endothelial cells (passages 8-12) were obtained from Dr. E. A. Sprague, University of Texas Health Science Center, San Antonio. For the experiments, the cells were seeded onto 100 mm tissue culture plates and grown to a confluent monolayer in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Cell extract preparation. The medium was decanted and the cell layer was washed 4 times with 0.9% saline. Cells from ten plates were harvested by scraping with a rubber policeman and separated from liquid by low speed centrifugation. The cell pellet was suspended in 500 µL of 50 mM MES, pH 6.6, and frozen at -70 °C. Cell extracts were thawed, centrifuged at 16,000 x g relative centrifugal force, and the supernatant was used for the soluble enzyme experiments. The pellet was suspended in 500 µL of 50 mM MES, pH 6.6, containing 1% Triton X-100 and allowed to stand for 15-30 min. before centrifuging to remove cell debris. The supernatant was used for the membrane enzyme experiments. The thawed extracts were kept at 4 °C until assayed.

Amidation assays. Assays were performed as previously described (12) in 100 mM MES, pH 6.6, containing 2 mM TNP-D-Tyr-Val-Gly synthesized as described (12,13), 4 µM $CuSO_4$, 4.5 mM L-ascorbate, and 1 mg/mL catalase (Boehringer Mannheim) and various amounts of PAM in a total volume of 0.5 mL at 37 °C. Aliquots of 100 µL were

withdrawn from the incubation mixture at the appropriate time, quenched with 10 µL of 2 M H_2ClO_4, centrifuged, and analyzed quantitatively for TNP-D-Tyr-Val-NH_2 product using HPLC (C8 reverse phase column; detection at 344 nm). The elution buffer was 50% (V/V) acetonitrile and 50% 50 mM ammonium acetate, pH 5.5.

PGL assays. Assays were performed as described previously (5) in 100 mM MES, pH 6.6, containing 2 mM α-hydroxybenzoylglycine (Aldrich Chemical) as a substrate and various amounts of PGL in a total volume of 0.5 mL, at 37 °C. Aliquots of 100 µL were withdrawn from the incubation mixture at the appropriate time, quenched with 10 µL of 2 M H_2ClO_4, centrifuged, and analyzed quantitatively for benzamide product using HPLC (C8 reverse phase column; detection at 225 nm). The elution buffer was 20% (V/V) acetonitrile and 80% water containing 0.1% trifluoroacetic acid.

Protein measurements Protein was measured by the dye binding assay of Bradford (14) for extracts without detergent and by the Bicinchoninic acid method (15) for extracts containing detergent.

Enzyme inactivation. The assay mixture for the inactivation experiments contained enzyme, 4 µM $CuSO_4$, 4.5 mM L-ascorbate, and 1 mg/mL catalase in 100 mM MES, pH 6.6, with or without PBA (Aldrich Chemical) and was allowed to incubate for 10 minutes before the reaction was initiated by the addition of substrate. The inhibitor concentration, when present, was 25 µM in the final assay mixture.

RESULTS AND DISCUSSION

Figure 1 shows the results of amidation assays carried out on extracts of both the soluble and membrane fractions of cultured endothelial cells. Two types of assays were used in these experiments. In the first assay, conversion of the glycine-extended peptide TNP-D-Tyr-Val-Gly to the corresponding amide, TNP-D-Tyr-Val-NH_2 was monitored. We have previously established that this conversion involves sequential action of PAM and PGL; initial PAM-catalyzed formation of TNP-D-Tyr-Val-α-hydroxyGly is followed by PGL-catalyzed formation of TNP-D-Tyr-Val-NH_2 plus glyoxylate (5,13,16). In the second assay procedure, the lyase reaction catalyzed by PGL was monitored independently by following conversion of α-hydroxybenzoylglycine to benzamide (5). From the results in Figure 1 it is clearly evident that both PAM and PGL are present in both the soluble and membrane fractions from cultured bovine aortic endothelial cells. In crude endothelial cell extracts, PAM and PGL specific activities were ca 2-7 nmol/mg/h and 10-40 nmol/mg/h, respectively; these are quite comparable to the amidating specific activity of ca 6 nmol/mg/h which we have previously reported for crude chromaffin cell extracts (12).

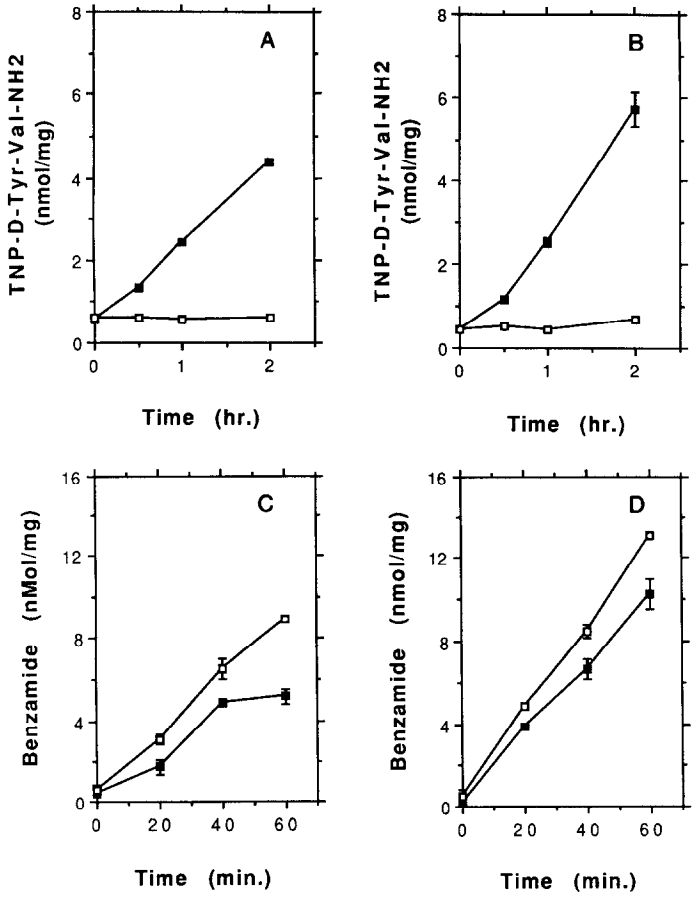

Figure 1. Effect of L-ascorbate on PAM and PGL activity. Soluble (**A**) and membrane (**B**) PAM activity and soluble (**C**) and membrane (**D**) PGL activity. Filled symbols denote presence and open symbols denote absence of 4 mM L-ascorbate. Data shown are the mean values for duplicate assays of the same sample with vertical bars indicating the range. All data have been corrected for the nonenzymatic background rate. These experiments were repeated several times and typical results are shown.

Also shown in Figure 1 are the ascorbate dependencies of both assays. Both soluble and membrane extracts showed the expected dependence of TNP-D-Tyr-Val-NH_2 formation on the presence of ascorbate, reflecting the ascorbate requirement of the PAM-catalyzed monooxygenation step (3-6). In contrast, benzamide formation occurs in both fractions even in the absence of ascorbate.

We have previously reported that the olefinic substrate analog 4-phenyl-3-butenoic acid (PBA) is a potent turnover-dependent inactivator of PAM, with inactivation exhibiting the characteristics expected for mechanism-based inactivation by a reactive species along the olefin oxygenation pathway (13). In contrast, we have shown that PBA is not an inactivator of PGL (5).

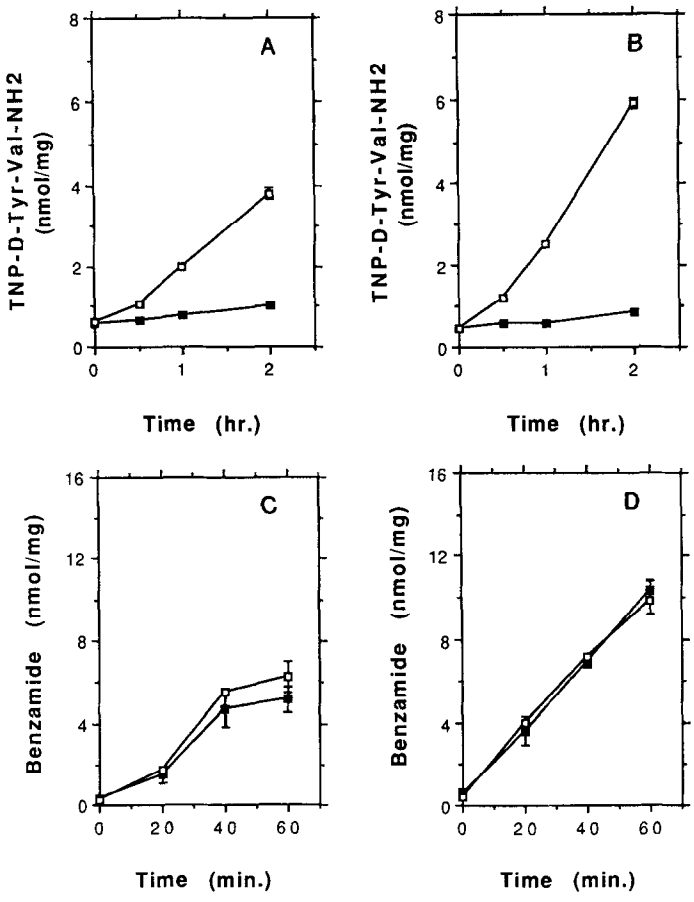

Figure 2. Effect of 4-phenyl-3-butenoic acid on PAM and PGL activity. Soluble (**A**) and membrane (**B**) PAM activity and soluble (**C**) and membrane (**D**) PGL activity assayed after preincubation with or without PBA. Filled symbols denote presence and open symbols denote absence of PBA. Data shown are the mean values for duplicate assays of the same sample with vertical bars indicating the range. All data have been corrected for the nonenzymatic background rate. These experiments were repeated several times and typical results are shown.

As shown in Figure 2, preincubation of either the soluble or membrane extracts with PBA completely abolishes formation of TNP-D-Tyr-Val-NH_2 from TNP-D-Tyr-Val-Gly, but has little effect on conversion of α-hydroxybenzoylglycine to benzamide. Thus, these results confirm that the enzymological characteristics of endothelial PAM and PGL correspond to those of amidating enzymes from tissues where amidation is known to be an essential processing step in the biosynthesis of peptide hormones.

Elucidation of the biochemical basis for hormonal regulation of the cardiovascular system continues to attract much attention due to the prevalence of cardiovascular disease in modern society (17). Two major sources of these regulating factors are cardiac

atrial cells which produce a family of polypeptide hormones (18) and endothelial cells which produce a variety of both peptide and nonpeptide vasoactive substances (7). Atrial natriuretic peptide is the prototype for the family of polypeptide hormones produced in cardiac atrial cells. It is striking that none of the atrial natriuretic peptides or endothelial-derived substances studied to date are amidated.

The results presented here establish, for the first time, the presence of both enzymatic activities necessary for peptide amidation in endothelial cells. This finding, together with the earlier finding of amidating enzymes in atrial granules (11) suggests that heretofore unidentified amidated peptides may play a significant role in cardiovascular function.

ACKNOWLEDGMENTS

Partial support of this work by a seed grant from the Georgia Tech Research Corporation is gratefully acknowledged. We thank Dr. Eugene A. Sprague, University of Texas Health Science Center, San Antonio, for providing us with cultures of bovine aortic endothelial cells.

REFERENCES

1. Douglass, J., Civelli, O., and Herbert, E. (1984) Ann. Rev. Biochem. 53, 665-715.
2. Bradbury, A. F., and Smyth, D. G. (1991) Trends Biochem. Sci. 16, 112-115.
3. Bradbury, A. F., Finnie, M. D. A., and D. G. Smyth, D. G. (1982) Nature 298, 686-688.
4. Eipper, B. A., Mains, R. E. and Glembotskii, C. C. (1983) Proc. Natl. Acad. Sci. USA 80, 5144-5148.
5. Katopodis, A. G., Ping, D., and May, S. W. (1990) Biochemistry 29, 6115-6120.
6. Tajima, M., Iida, T., Yoshida, S., Komatsu, K., Namba, R., Yanagi, M., Noguchi, M., and Okamoto, H. (1990) J. Biol. Chem. 265, 9602-9605.
7. Furchgott, R. F., and Vanhoutte, P. M. (1989) FASEB J. 3, 2007-2018.
8. Loesch, A., and Burnstock, G. (1988) Anat. Embryol. 178, 137-142.
9. Linnik, M. D., and Moskowitz, M. A. (1989) Peptides 10, 957-962.
10. Milner, P., Kirkpatrick, K. A., Ralevic, V., Toothill, V., Pearson, J., and Burnstock, G. (1990) Proc. Royal Soc. London B 241, 245-248.
11. Eipper, B. A., May, V., and Braas, K. M. (1988) J. Biol. Chem. 263, 8371-8379 .
12. Katopodis, A. G., and May, S. W. (1988) Biochem. Biophys. Res. Commun. 151, 499-505.
13. Katopodis, A. G., and May, S. W. (1990) Biochemistry 29, 4541-4548.

14. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
15. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76-85.
16. Katopodis, A. G., Ping, D.and May, S. W. (1991) Biochemistry 30, 6189-6194.
17. Gerber, J. G., and Nies, A. S. (1990) in Goodman and Gilman's Pharmacological Basis of Therapeutics, 8th edition. (Gilman, A. G., Rall, T. W., Nies, A. S., and Taylor, P., eds.) Pergamon Press, New York, pp. 784-785; Petrillo, E. W., Jr., Trippodo, N. C., and DeForrest, J. M. (1990) Ann. Reports Med. Chem. 25, 51-60; Doherty, A. M. (1991) Ann. Reports Med. Chem. 26, 83-92.
18. Rosenzweig, A. and Seidman, C. E. (1991) Ann. Rev. Biochem. 60, 229-255.