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HUMAN PARATHYROID HORMONE RELATED PROTEIN FRAGMENT-(1-34) HAD GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY ON DISTAL CONVOLUTED TUBULES IN CYTOCHEMICAL BIOASSAY

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Summary: In the present study, the action of parathyroid hormone related protein (PTHrP) on glucose-6-phosphate dehydrogenase (G6PD) activity of the distal convoluted tubules was examined utilizing cytochemical bioassay (CBA). Recently full amino acid residues of human PTHrP (hPTHrP), one of the causative agents of HHM, was identified based on the cDNA clone using BEN cells. We synthesized hPTHrP-(1-34) and examined the effect of this protein on G6PD activities on the distal convoluted tubules, and compared its bioactivity to that of human parathyroid hormone (hPTH)-(1-84). hPTHrP-(1-34) stimulated G6PD activity in a log linear fashion with equivalent activity to that of hPTH-(1-84) on a molar basis. Conclusively, we found that PTHrP act on distal convoluted tubules similar to hPTH.

Hypercalcemia frequently occurs in malignant disorders. Two general mechanisms seems to be responsible for this type of hypercalcemia. One is local factor(s) and the other is caused by circulating bone resorbing factors produced by malignant cells, and is called humoral hypercalcemia of malignancy (HHM). HHM has several characteristics corresponding to the features of primary hyperparathyroidism (including hypercalcemia, hypophosphatemia, hyperphosphaturia and accelerated bone resorption) (1-4). However, plasma immunoreactive PTH showed low normal or lower level

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Abbreviations used are: PTHrP: parathyroid hormone related protein, G6PD: glucose-6-phosphate dehydrogenase, CBA: cytochemical bioassay, HHM: humoral hypercalcemia of malignancy, hPTH: human parathyroid hormone, hPTHrP: human parathyroid hormone related protein, M.I.E.: mean integrated extinction.

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(3), and PTH messenger RNA was not detected in tumor cells (5). Several lines of investigations revealed that substances responsible for HHM were PTHrP (7,8), possibly transforming growth factor-α (9.10), and interleukin-1 α (11). In 1987, Suva et al. determined the amino acid sequence of PTHrP based on the cDNA clones from BEN cells (12). hPTHrP consists of 141 amino acid residues and shows homology with NH_2-terminal of hPTH particularly first 13 residues. PTHrP has been reported to stimulate bone resorption, have adenylate cyclase stimulating activity in bone and kidney cells, and to promote the nephrogenous cAMP excretion in thyroparathyroidectomized rats (13-16). However the bioactivity of PTHrP on the distal covoluted tubules have not been reported so far.

CBA, although technical difficulties prevented this from general availability, has been used as a most sensitive method for the measurement of PTH *in vitro*, allowing 100-1000 times greater sensitivity than radioimmunoassay (17,18,19), and is based on the stimulation of G6PD activity in the distal convolutcd tubules of guinea pig kidney. This assay also has been employed to detect PTH-like bioactivities from peripheral plasma (6) and tumor extracts (20).

Therefore, utilizing this excellent method CBA, we examined the action of our synthetic hPTHrP-(1-34) on G6PD stimulating activity of the distal convoluted tubules of guinea pig kidney, and we compared the potency of this peptide to that of hPTH-(1-84).

Materials and Methods

Chemicals Trowell's T8 medium was purchased from Flow Laboratories Co. (Scotland, UK). Glucose-6-phosphate, NADP and phenazine methosulfate were purchased from Sigma Chemicals Co. (St. Louis, MO), and neotetrazolium Chloride from Serva (Heidelberg, FRG). Human PTH-(1-84) was purchased from Peptide Institute Inc. (Osaka, Japan). hPTHrP-(1-34) was synthesized according to the amino acid sequence of Suva et al.(12) by a solid-phase method (21) and prepared by Toyo Jozo Co. Ltd., where the final product was identified to be pure by the method of thin layer electrophoresis and high pressure liquid chromatography. Other reagents were of the highest quality available.

Parathyroid hormone and parathyroid hormone related protein preparations PTH and PTHrP were dissolved in 0.01N HCl solution with 0.2% bovine serum albumin.

Animals Female guinea pig of the Hartley strain after weaning (Shizuoka Experimental Animals Inc., Shizuoka, Japan) were used. They were placed on a vitamin D-depleted diet containing 1.44% calcium and 0.64% phosphorous

(Oriental Yeast CO., Tokyo, Japan) for at least 6 weeks prior to experiments.

Cytochemical Bioassay The cytochemical bioassay was performed according to the method of Sakaguchi and co-workers (17). Briefly after decapitation, kidneys were removed, decapsulated, and halved in saggital plane and then divided into two equal parts from both sides of the respective half kidneys. The larger planar division of each segment did not exceed 8mm. The segments were cultured in Trowell's T8 medium at 37°C in an atmosphere of 95% O_2 5% CO_2 for 5 hours, after which the medium was then replaced by fresh medium for washing. Then the medium was replaced by fresh medium containing a known concentrations of test agents for 6 minutes. Then the segments were immediately chilled in *n*-hexane and stored at -80°C. Within two days they were sectioned in thickness of 16μm, and tested for G6PD activity using the cytochemical method (17,18,19). The sections were incubated at 37°C for 10.5 minutes in a medium containing 5mM glucose-6-phosphate as substrate, 3mM NADP, 0.67mM phenazine methosulfate, 5mM neotetrazolium chloride, 10mM potassium cyanide, and 30%(w/v) polyvinyl alcohol (G04/140 polyvinyl from Wacker-Chemie GMBH, West Germany) in 50mM glycylglycine buffer, pH 8.0. The reaction medium was deoxygenated by bubbling with nitrogen gas. The amount of formazan produced in the distal convoluted tubules was determined with a Nikon-Vickers M85 scanning and integrating microdensitometer (Nikon, Tokyo, Japan) at 585nm. The density per cell in each of 10 different tubules was measured in the duplicated sections from each kidney segment. G6PD activity was expressed as mean integrated extinction (MIE) x 100.

Statistical analysis Results are expressed as mean ± S.E. Significant differences among the groups were determined by Duncan new multiple range test (22).

Results and Discussion

Figure 1 shows the dose-response curves of hPTH-(1-84) and hPTHrP-(1-34) on G6PD stimulating activity of the distal convoluted tubules of guinea pig kidney in CBA. hPTHrP-(1-34) exhibited potent G6PD activity in a log-linear fashion with increasing concentrations over the range 10^{-16} - 10^{-14}M. Furthermore, the activity of hPThrP-(1-34) was equipotent to that of hPTH-(1-84) on a molar basis. Reproducibility was made sure by repeating study four times. Recent advances of hPTHrP were focussed on proximal tubules and combined renal segments but not solely on the distal tubules. Our result firstly demonstrated that hPTHrP, like hPTH, acts on distal convoluted tubules of guinea pig kidney *in vitro*.

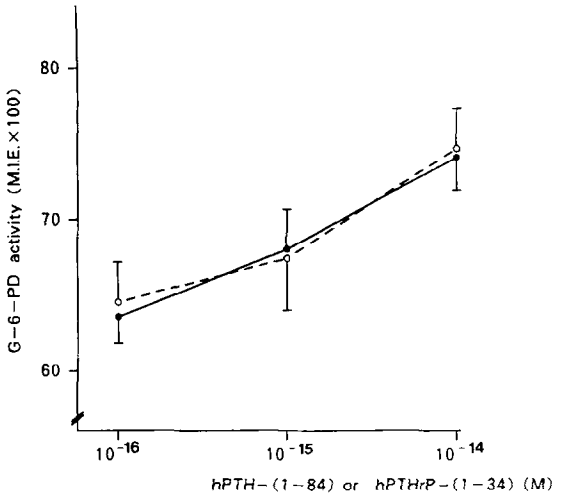

Figure.1 Comparison of the effects of dilutions of hPTH-(1-84) and hPThrP-(1-84) on G6PD activity in distal convoluted tubules of guinea pig. Each points represents the mean ± S.E. of 20 measurements from duplicated sections. No significant difference exists between hPTH-(1-84) and hPThrP-(1-34) at the same concentrations.
(•——•) hPTH-(1-84)
(∘----∘) hPThrP-(1-34)

Accumulative data of hPTHrP are that, hPTHrP-(1-34) stimulated adenylate cyclase stimulating activity in rat osteosarcoma cells (UMR-106), in rabbit kidney cortex homogenates and in rabbit proximal straight tubules (15), and hPThrP-(1-34)NH_2 showed binding affinity to PTH receptors and had adenylate cyclase activity in bovine renal cortical membrane (13). These findings suggested that PTHrP act through cAMP-coupled PTH receptor. In CBA, PTH is known to stimulate G6PD activity probably through cAMP-coupled PTH receptor in the distal tubules (6). Therefore, it is likely that PTHrP-(1-34) also stimulate G6PD activity through PTH receptor.

Our investigation also suggested that CBA could be a highly sensitive method for the detection of plasma PTHrP. Because of the non-selectivity of CBA to PTH and PTHrP, plasma G6PD activity appears not to reflect plasma PTHrP levels. However, once combined with the result of plasma immunoreactive PTH, this assay might be useful for the early detection and treatment of PTHrP producing tumor, in which plasma immunoreactive PTH is usually low. This hypothesis is supported by the fact that G6PD activity was reported to be normalized after tumor resection in peripheral plasma from a HHM patient (6). It is indeed, not all the plasma from HHM showed high G6PD activity, suggesting other substances responsible for HHM might be exist. Further studies will be required to elucidate these issues.

In summary, our study provided new evidence that hPTHrP-(1-34) stimulated G6PD activity on the distal convoluted tubules of a guinea pig kidney *in vitro* and its activity is equal to that of hPTH-(1-84) on a molar basis.

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References

1. Sherwood, L. M., O'Riordan J. L. H., Aurbach, G. D., and Potts, J. T., Jr. (1967) J. Clin. Endocrinol. Metab. 27, 140-146
2. Powell, D., Singer, F. R., Murray, T. M., Minkin, C., and Potts, J. T., Jr. (1973) N. Engl. J. Med. 289, 176-181.
3. Mundy, G. R., and Martin, T. J., (1982) Metabolism 31, 1274-1277.
4. Mundy, G. R., Ibbotson, K. j., D'Souza, S. M., Simpson, E. L., Jacobs, J. W., and Martin, T. J., (1984) N. Engl. J. Med. 310, 1718-1727.
5. Simpson, E. L., Mundy, G. R., D'Souza, S. M., Ibbotson, K. G., Beckman, R., Kronenberg, H., and Jakobs, . W. (1983) N. Engl. J. Med. 309, 325-332.
6. Goltzman, D., Stewart, A.F., and Broadus, A.E. (1981) J. Clin. Endocrinol Metab. 53,899-904.
7. Rodan, S. B., Insogna, K. L., Vigney, A. M. C., Stewart, A. F., Broadus, A. E., D'Souza, S. M., Bertolini, D. R., Mundy, G. R., and Rodan, G. A. (1983) J. Clin. Invest. 72, 1511-1515.
8. Strewler, G. J., Williams, R. D., and Nissenson, R. A. (1983) J. Clin. Invest. 71, 769-774.
9. Ibbotson, K. J., Twardzik, D. R., D'Souza, S. M., Hargreaves, W. R., Todaro, G. J., and Mundy, G. R. (1985) Science 228, 1007-1009.
10. Ibbotson, K. J., Harrod, K. J., Gowen, M., D'souza, S., Smith, D. D., Wrinkler, M. E., Derynck, R., and Mundy, G. R. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 2228-2232.
11. Sato, K., Fujii, Y., Kasono, K., Saji, M. Tsushima, T., and Shizume, K. (1986) Biochem. Biophys. Res. Commun. 138, 618-624.
12. Suva, L. J., Winslow, G. A., Wettenhall, R. E. H., Hammonds, R. J., Moseley, J. M., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodoriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J., and Wood, W. I. (1987) Science 237, 893-896.
13. Horiuchi, N., Caufield, M.P., Fisher, J.E., Goldman, M.E., McKee, R.L., Reagan, J.E., Levy, J.J., Nutt, R.F., Rodan, S.B., Schfield, T.L., Clemens, T.L., and Rosenblatt, M. (1987) Science 238, 1566-1568.
14. Kemp, B.E., Moseley, J.M., Rodda, C.P.,Ebeling, P.R., Wettenhall, R.E.H., Stapleton, D., Diefenbach-J.H., Ure, F., Michelangeli, V.P., Simmons, H.A., Raisz, L.G., and Martin, T.J. (1987) Science 238,1568-1570.
15. Rodan, S.B., Noda, M., Wesolowski, G.,Rosenblatt, M., and Rodan, G.A. (1988) J. Clin. Invest. 81, 924-927.
16. Yates, A.J.P., Gutierrez, G.E., Smolens, P., Travis, P.S., Katz, M.S., Aufdemorte, T.B., Boyce, B.F., Hymer, T.K., Poser, J.W., and Mundy, G.R. (1988) J. Clin. Invest. 81, 932-938.
17. Sakaguchi, K., Fukase, M., Kobayashi, I., and Fujita, T. (1987) J. Bone Mineral Res. 1, 259-265.
18. Chayen, J. (1980) The cytochemical bioassays of parathyroid hormones. Springer, New York.
19 Chayen, J., Bitensky, L., and Butcher, R.G. (1973) Practical histochemistry. Willey, New York.
20. Rabbani, S.A., Mitchell, J., Roy, D.R., Kremer, R., Bennett, H.P., and Goltzman, D. (1986) Endocrinology 118, 1200-1210.
21. Merrifield, R. B., (1969) Adv. Enzymol. Relat. Subj. Biochem. 32, 221-296.
22. Duncan, D.B. (1955) Biometrics 11,1.