The excision of N terminal two amino acids of a novel human parathyroid hormone analog with dipeptidylpeptidase

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Abstract. Pro-Pro-[Arg11]hPTH(1-34)-Pro-Pro-Asp (hPTH') is more effective than hPTH(1-34) in the treatment of osteoporosis in our previous studies. The hypothesis that the active form of hPTH' was introduced by the excision with dipeptidyl peptidase IV (DPPIV) in vivo was validated in the present study. Following the determinations of DPPIV activities in rats tissues and serum, the N-terminus dipeptides of hPTH' were excised by DPPIV in rats tissues and serum and the excision production was determined by Tris-Tricine-SDS-PAGE. The results indicated that the N-terminus dipeptides of hPTH' could be excised with DPPIV in rats tissues and serum. The molecular weight of excision production was greater than that of hPTH (1-34) and less than that of hPTH'. In addition, the excision production was injected to mice for detecting its effect on serum calcium of mice. The results suggested that the serum calcium levels of mice treated with hPTH' were similar with those of mice treated with hPTH (1-34). In conclusion, appearing to function as a prodrug, the active form of hPTH' was introduced by the excision with DPPIV in vivo.

Introduction

Serine-valine, the first two amino acids of N-terminus of hPTH(1-34) were the performers of activating the cAMP signaling pathway in the form of free amino acids. Consequently, bone formation was stimulated. The loss and extension of N-terminus amino acids of hPTH(1-34) significantly reduce their activities of bone formation [1-3]. However, Pro-Pro-[Arg11]hPTH(1-34)-Pro-Pro-Asp (hPTH') is more effective than hPTH(1-34) in stimulating bone formation and improving skeletal microarchitecture. It is potentially a more effective therapeutical agent on osteoporosis [4]. So there is a hypothesis that the active form of hPTH' should be introduced in vivo.

Dipeptidylpeptidase IV (DPPIV) is expressed in various tissues, particularly epithelial tissues of the liver, kidney and small intestine, and exists as a soluble circulating form [5]. As a serine protease, it has a specificity to remove dipeptides from the N-terminus of substrate poly-peptides by cleaving postproline or alanine residues [6-7]. The catalytic reaction has relatively strict specificity to substrate. This multifunctional enzyme is implicated in several biological processes, including the degradation of chemokines, neuropeptides, and incretin hormones, e.g. glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [7]. Accordingly, as the substrate of DPPIV, hPTH' should be excised into [Arg11]hPTH(1-34)-Pro-Pro-Asp rapidly and combined with receptors after administration.

In this study, The hypothesis that hPTH', as a potential substrate of DPPIV could be excised in vivo to be present as its active form was verified.

Materials and methods

Materials and animals. hPTH' was synthesized in Laboratory of Minigene Pharmacy, China Pharmaceutical University, with purity >96%. hPTH(1-34) was synthesized in the GL Biochem (Shanghai) CO., Ltd., China, with purity >98%. H-Ala-Pro-paranitroanilinum (H-Ala-Pro-pNA) and Ile-Pro-Ile were purchased from Sigma, USA. Gel extraction was prepared by 5% trifluoroacetic acid (TFA) and 50% acetonitrile. Serum calcium reagent kit was purchased from NanJing JianCheng Bioengineering Institute.

Ten-week old female Sprague-Dawley rats were purchased from Shanghai SIPPR-BK Lab animal Co., Ltd., China (licence no. SCXK2007-0005). Female ICR mouses were purchased from Comparative Medicine Centre of Yangzhou University (licence no. SCXK2007-0001). All animals received humane care as described in the Principles of Laboratory Animal Care formulated by China Pharmaceutical University.

Preparation of tissue homogenate of rat. Heart, liver, spleen, lung, and kidney of rat were taken and weighed. Tissue homogenates were made by mixing 1 g tissue with 5 ml normal saline. Homogenate was kept at room temperature for 3 h, followed by centrifuged at 12 000 g for 5 min. The supernatant was used for excision.

Activity determination of DPPIV. Specific excision activities of tissue homogenates and serum were determined at 37 °C with H-Ala-Pro-pNA (0.5 mmol/L) in 50 mM Tris–HCl buffer containing 2 mM EDTA (pH 8.0). The final concentration of tissue homogenates and serum was 10% (v/v). The reactions were ended with 0.2% (v/v) trifluoroacetic acid (TFA). The absorbance value was determined at 410 nm by using spectrophotometry. An active unit of DPPIV is defined as the amount of DPPIV that released 1 μmol pNA per minute. The molar absorption coefficient of pNA was 8800 L/mol·cm.

Excision of hPTH' by DPPIV in homogenates and serum. Specific excision activities of tissue homogenates and serum were determined at 37 °C with hPTH' (2 mg/ml) in 50 mM Tris–HCl buffer containing 2 mM EDTA (pH 8.0). The final concentration of tissue homogenates and serum was 10% (v/v). Samples were collected regularly. The reactions were ended with 0.2% (v/v) TFA. The release of [Arg11]hPTH(1-34)-Pro-Pro-Asp was monitored by Tricine-SDS-PAGE.

Excision of hPTH' by DPPIV in homogenates and serum with inhibitor. Ile-Pro-Ile, used as a DPPIV inhibitor, was added into the reaction system (5 mg/ml) as described. The reactions were ended with 0.2% (v/v) TFA. The release of [Arg11]hPTH(1-34)-Pro-Pro-Asp was monitored by Tricine-SDS-PAGE.

Results.

Activity determination of DPPIV. The activities of DPPIV of rats kidney, spleen, lung, liver and heart were 12650, 4826, 3753, 2579 and 696 nmol/min· g, respectively. The activity of DPPIV of rats serum was 231 nmol/min· mL.

Excision of hPTH' by DPPIV in homogenates and serum. Tris-Tricine-SDS-PAGE analyses of the excision of hPTH' by kidney, spleen, lung, liver, heart and serum are shown in Figure 1~6. The results indicated that hPTH' was excised within 10 min or even less by kidney, spleen, lung and liver, while 20 min by liver and heart and 5 h by serum. In addition, we observed complete excision within 1 h in kidney and spleen, while 1.5 h in lung and liver, 4 h in heart, 9 h in serum. As the same of hPTH(1-34), hPTH' could be eliminated completely in liver and kidney homogenate [8].

Excision of hPTH' by DPPIV in homogenates and serum with inhibitor. As shown in Fig. 7~9, with the addition of excess Ile-Pro-Ile, hPTH' was not excised within 45 min in kidney, 1 h in spleen, while 13 h in serum. These results indicated that Ile-Pro-Ile was able to inhibit the maximum excision activities of DPPIV in tissues and the minimum excision activities of serum. It suggested that DPPIV was indeed the performer of excising the dipeptide from hPTH'. Moreover, it is implied that the medium excision activities of DPPIV in tissues could also be inhibited by Ile-Pro-Ile (Fig. 10). Consequently, the determination of inhibition by tissues homogenate could be simplified. In addition,

Ile-Pro-Ile, an inhibitor of DPPIV, was able to completely inhibit the excision of hPTH' within the time that hPTH' was completely excised in tissues and serum.

Discussion.

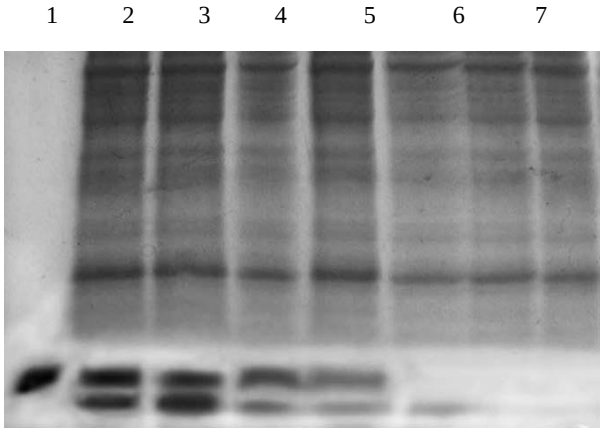

Fig. 1. Tris-Tricine-SDS-PAGE analysis of the excision of hPTH' by kidney. Lane 1: hPTH'; Lane 2 to 8: hPTH' excised by kidney for 10 min, 20 min, 30 min, 40 min, 50 min, 1 h and 1.5 h.

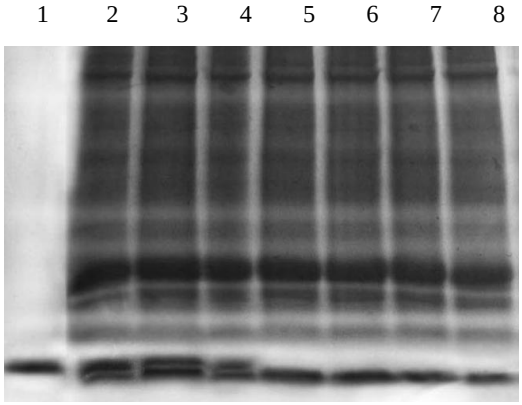

Fig. 2. Tris-Tricine-SDS-PAGE analysis of the excision of hPTH' by spleen. Lane 1: hPTH'; Lane 2 to 8: hPTH' excised by spleen for 10 min, 20 min, 30 min, 45 min, 1 h, 1.5 h and 2 h.

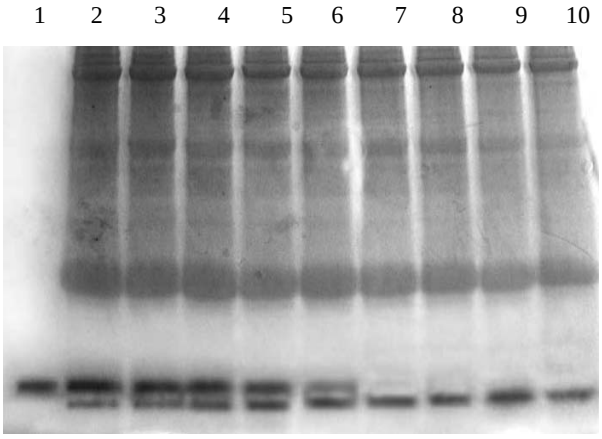

Fig. 3. Tris-Tricine-SDS-PAGE analysis of the excision of hPTH' by lung. Lane 1: hPTH'; Lane 2 to 10: hPTH' excised by lung for 10 min, 20 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 2.5 h and 3 h.

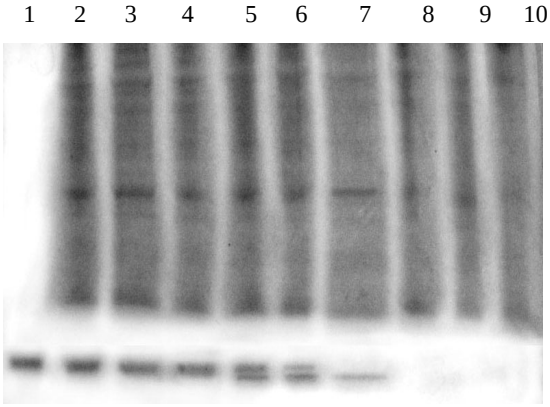

Fig. 4. Tris-Tricine-SDS-PAGE analysis of the excision of hPTH' by liver. Lane 1: hPTH'; Lane 2 to 10: hPTH' excised by liver for 10 min, 20 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 2.5 h and 3 h.

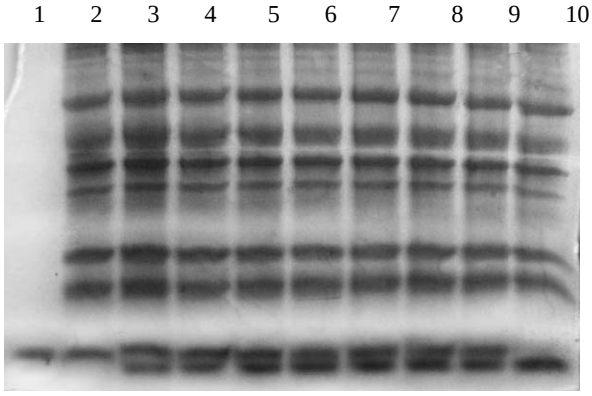

Fig. 5. Tris-Tricine-SDS-PAGE analysis of the excision of hPTH' by heart. Lane 1: hPTH'; Lane 2 to 10: hPTH' excised by heart for 20 min, 40 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h and 4 h.

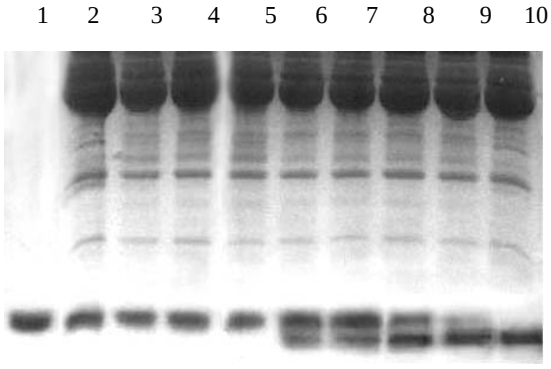

Fig. 6. Tris-Tricine-SDS-PAGE analysis of the excision of hPTH' by serum. Lane 1: hPTH'; Lane 2 to 10: hPTH' excised by serum for 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h and 9 h.

The N-terminus X-Ala- of Glucagon-like peptide-1 (GLP-1) was able to be degraded by DPPIV. The half life of GLP-1 was less than 2 min in vivo [7,9-11]. Accordingly, as a hypothesis, hPTH', as a potential substrate of DPPIV could also be excised in vivo to present as its active form. In the present study, tissues and serum of rats were used to excise hPTH' in vitro. The N-terminus Pro-Pro- of hPTH' could be excised within a short time by tissues and serum. The distribution of DPPIV showed the differences with species, sex and organ. DPPIV distributed among kidney, alimentary canal (small intestine, salivary glands, pancreas and liver), immune system (spleen, thymus), blood cell, cardiovascular system, respiratory system and skin in human body. The DPPIV activity of human serum was about 24 U/L [12]. Twenty-four μmol/L pNA could be released from H-Ala-Pro-pNA with the catalysis by DPPIV of human serum. As the same of H-Ala-Pro-pNA, the second amino acid of N-terminus of hPTH' was Proline. It is implied that the excision efficiency of hPTH' was similar to that of H-Ala-Pro-pNA by DPPIV. In the treatment of osteoporosis with hPTH (1-34), the dosage is generally 20~40 μg/d, at most 50~100 μg/d [13].

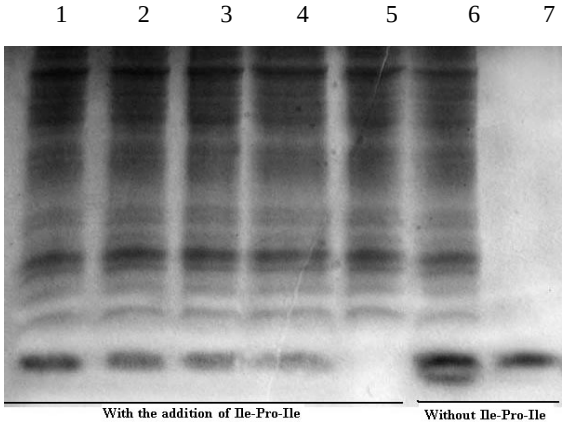

Fig. 7. Tris-Tricine-SDS-PAGE analysis of the excision of hPTH' by kidney with or without the addition of Ile-Pro-Ile. Lane 1 to 5: hPTH' excised by kidney for 10 min, 20 min, 30 min, 45 min and 1 h with the addition of Ile-Pro-Ile. Lane 6: hPTH' excised by kidney for 30 min without the addition of Ile-Pro-Ile. Lane 7: hPTH'.

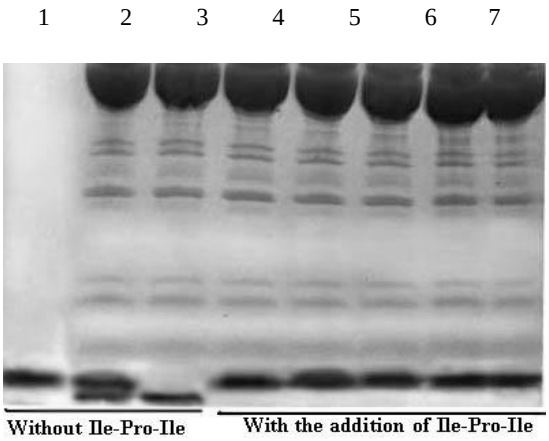

Fig. 9. Tris-Tricine-SDS-PAGE analysis of the excision of hPTH' by serum with or without the addition of Ile-Pro-Ile. Lane 1: hPTH'. Lane 2 to 3: hPTH' excised by serum for 5 h and 9 h without the addition of Ile-Pro-Ile. Lane 4 to 8: hPTH' excised by serum for 5 h, 7 h, 9 h, 11 h and 13 h with the addition of Ile-Pro-Ile.

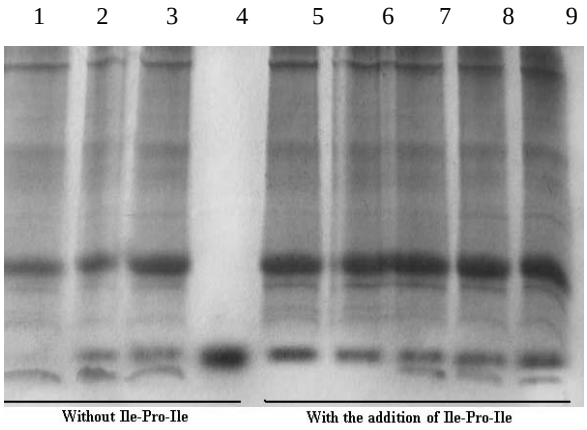

Fig. 8. Tris-Tricine-SDS-PAGE analysis of the excision of hPTH' by spleen with or without the addition of Ile-Pro-Ile. Lane 1 to 3: hPTH' excised by spleen for 1 h, 30 min and 10 min without the addition of Ile-Pro-Ile. Lane 4: hPTH'. Lane 5 to 9: hPTH' excised by spleen for 30 min,1 h, 1.5 h, 2 h and 3 h with the addition of Ile-Pro-Ile.

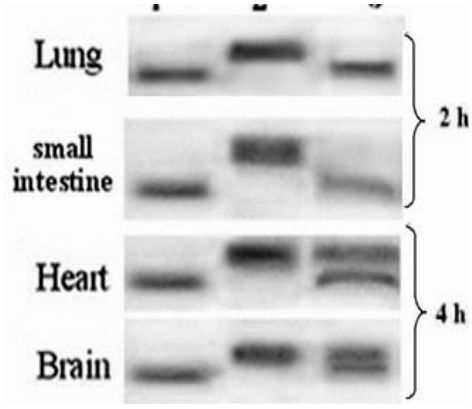

Fig. 10. Tris-Tricine-SDS-PAGE analysis of the excision of hPTH' by different tissues with or without the addition of Ile-Pro-Ile. Lane 1: control peptide hPTH(1-34); Lane 2: the cleavage products of hPTH' excised by different tissues with the addition of DPPIV inhibitor Ile-Pro-Ile; Lane 3: the cleavage products of hPTH' excised by different tissues without the addition of DPPIV inhibitor Ile-Pro-Ile.

Conclusions

The hypothesis that hPTH', as a potential substrate of DPPIV could be excised in vivo to be present as its active form, was verified in the present study. It suggested that DPPIV was indeed the performer of excising the dipeptide from hPTH'.

References

[1] A.B. Hodsman, D.A. Hanley, M.P. Ettinger, M.A. Bolognese, J. Fox, A.J. Metcalfe et al.: J. Clin. Endocrinol. Metab. Vol. 88 (2003), p. 5212.

[2] H. Takasu, J. Guo and F.R. Bringhurst: J Bone Miner. Res. Vol. 14 (1999), p. 11.

[3] A. Hogset, O.R. Blingsmo, V.T. Gautvik, O. Saether, P.B. Jacobsen, J.O. Gordeladze et al.: Biochem. and Biophys. Res. Commun.Vol. 166 (1990), p. 50.

[4] F. Jiao, L. Yanhua, X. Yun, W. Huaqian, L. Taiming, L. Jingjing et al.: Peptides, Vol. 30 (2009), p. 1173.

[5] D. Darmoul, T. Voisin, A. Couvineau, C. Rouyer-Fessard, R. Salomon and Y.X. Wang: Biochem. and Biophys. Res. Commun., Vol. 203 (1994), p. 1224–1229.

[6] A.M. Lambeir, P. Proost, C. Durinx, G. Bal, K. Senten and K.: J. Biol. Chem., Vol. 276 (2001), p. 29839.

[7] C.L. Jao, C.C. Hung, Y.S. Tung, P.Y. Lin, M.C. Chen and K.C. Hsu: Biomedicine (Taipei), Vol. 5 (2015), p. 14.

[8] E. Quattrocchi and H. Kourlas: Clin. Ther., Vol. 26 (2004), p. 841.

[9] S. Tiziana, M. Sara, F. S. Simona, D. P. Rocco and A.S. Maria: Front Pharmacol., Vol. 6 (2015), p. 126-35

[10] M. MingJie, H. Yu, K. Nobutaka, T. Kensuke, U. Ken, N. Takashi, et al.: Cardiovasc. Diabetol., Vol. 14 (2015), p. 54.

[11] L.L. Nielsen, A.A. Young and D.G. Parkes: Regul. Pept., Vol. 117 (2004), p. 77.

[12] S. Wrenger, T. Kahne, C. Bohuon, W. Weglohner, S. Ansorge and D. Reinhold: FEBS Lett., Vol. 466 (2000), p. 155.

[13] A.B. Hodsman, D.C. Bauer, D.W. Dempster, L. Dian, D.A. Hanley, S.T. Harris et al.: Endocrine Reviews, Vol. 26 (2005), p. 688.