Eur. J. Clin. Chem. Clin. Biochem.
Vol. 32, 1994, pp. 61–64

Effect of Bacitracin on the Degradation of a Vasopressin Receptor Ligand with High Affinity for the V_1 and V_2 Vasopressin Isoreceptors

By *B. Hocher* [1,2], *J. A. Dürr* [3*)] and *J. Hensen*[1**)]

[1] *Abteilung für Innere Medizin, Klinikum Steglitz, Freie Universität Berlin, Berlin, Germany*
[2] *Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Berlin, Germany*
[3] *Department of Medicine, University of Colorado, Denver, Colorado, USA*

(Received September 8/November 24, 1993)

Summary: We previously described a new iodinated vasopressin analogue (N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin) with high affinity for the vasopressin V_1 and V_2 isoreceptors. The aim of the present study was:

i) to analyse the degradation pathway of N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin and
ii) to look for an effective inhibitor of radioligand degradation.

N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin was processed in a temperature-dependent manner by crude cell membranes from LLC-PK$_1$ cells. Only one degradation product was seen using RP-HPLC. The degradation product co-eluted with monoiodotyrosine. The stereoisomer, N^{ε}-[^{125}I]*D*-Tyr-[Lys^8]-vasopressin, underwent the same degradation process. Bacitracin prevented degradation at doses as low as 40 mg/l without alterating the binding affinity.

Introduction

Vasopressin stimulates the kidney to concentrate the urine by increasing the water permeability of renal collecting duct epithelial cells (1). Vasopressin, like other peptide hormones, exerts its physiological function by binding to cell-surface receptors in association with a specific guanine nucleotide-binding protein (2). These actions in the kidney are mediated by the adenylate cyclase-coupled V_2-receptor subtype. The lack of highly specific vasopressin radioligands has hampered further progress in vasopressin receptor research (e. g. internalization studies on vasopressin, autoradiography and mapping of the renal vasopressin receptor along the nephron). We previously described a new series of iodinated vasopressin analogues with high specific radioactivity and high affinity for the vasopressin V_1 and V_2 isoreceptors (1). But little is known about the degradation of these new receptor ligands. It was therefore the aim of this study

i) to analyse the degradation pathway of N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin and

ii) to look for an effective inhibitor of N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin degradation.

Materials and Methods

Materials

N-*tert*-Butoxycarbonyl-*L*-tyrosine-hydroxysuccinimidyl ester, N-*t*Boc-*D*-tyrosine, N-hydroxysuccinimidyl ester, and, N',N', dicyclocarbodiimide were from Bachem, Heidelberg. Unless otherwise stated, the other reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) and Sigma (Munich, Germany). We used LLC-PK$_1$ cells (a porcine renal tubular cell line, ATCC CRL 1392). N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin (740 TBq/mmol) and N^{ε}-[^{125}I]*D*-Tyr-[Lys^8]-vasopressin (740 TBq/

*) Present address:
Department of Medicine, Baypines VA Medical Center, Baypines, USA
**) Present address:
Medizinische Klinik I, Friedrich-Alexander-Universität Erlangen, Erlangen, Germany.

mmol) were prepared in our laboratory (1); [Lys^8]-vasopressin was obtained from Peninsula Laboratories, Inc. (Belmont, USA).

Cell culture

LLC-PK$_1$ cells were grown in monolayers in 75 cm^2 culture flasks containing HAM's F12 medium (Seromed, Germany), fetal calf serum, volume fraction 0.06 (Seromed, Germany), 2 mmol/l *L*-glutamine and antibiotics (100 mg/l streptomycin and 10^5 U/l penicillin) as described previously (3, 4). The cells were cultured at 37 °C in a humidified CO_2 incubator (95% air and 5% CO_2). Twenty thousand cells were sown per cm^2 on the first day of cultivation. The medium was changed every 48 hours. Viability was estimated by the trypan blue method.

Preparation of N$^\varepsilon$-[^{125}I]*D*-Tyr-[Lys^8]-vasopressin

The N-succinimidyl ester of N-*t*Boc-*D*-tyrosine was synthesized according to *Rudinger & Ruegg* (10) using N',N',-dicyclocarbodiimide. *t*Boc-*D*-Tyr-N-hydroxysuccinimidyl ester was iodinated as described previously (1). Briefly, [Lys^8]-vasopressin was labelled by conjugation on the eighth amino acid after blocking the free α-amino group of [Lys^8]-vasopressin by incubation in acetone, volume fraction 0.68, for 48 hours (1). The conjugation procedure is identical for all radiolabelled N-succinimidyl esters (i. e. of [^{125}I]3-(p-hydroxyphenyl)propionic acid, *t*Boc-*L*-[^{35}S]methionine, *t*Boc-*D*-[^{125}I]tyrosine, and *t*Boc-*L*-[^{125}I]tyrosine. For example, *t*Boc-[^{125}I]*D*-tyrosine N-succinimidyl ester was reacted with 10 µg acetone-[Lys^8]-vasopressin in 20 µl N,N-dimethylformamide and triethylamine (1 + 1000 by vol.). Conjugation was allowed to proceed for 8 h at 4 °C. The conjugated tracer, diluted in 150 µl of 1 g/l trifluoroacetic acid, was then purified by C-18 reverse-phase high performance liquid chromatography (RP-HPLC) (1).

Preparation of crude plasma membrane fractions

Partially purified membranes from LLC-PK$_1$ cells were prepared as described (4). Briefly, cells were detached by incubation for 30 min at 37 °C in 50 mmol/l Tris, 0.15 mol/l NaCl, 1 g/l Na_2EDTA, pH 7.4. They were then lysed using a hypotonic buffer (5 mmol/l Tris-HCl, 1 mmol/l EDTA and 3 mmol/l $MgCl_2$, pH 7.4), and finally the lysate was homogenized. The homogenate was centrifuged at 2000 *g* for 10 min and the pellet thus obtained was washed twice in assay buffer and processed immediately.

Binding studies

Plasma membranes of LLC-PK$_1$ cells (150 µg/tube) were incubated with N$^\varepsilon$-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin (5×10^3 – 2×10^6 cpm/tube, which is equivalent to 0.012 nmol/l – 4 nmol/l; the incubation volume was 150 µl) for "hot saturation" studies. Unless otherwise stated, binding studies were performed at 22 °C for 90 min as described previously (3, 4). Assay buffer for binding studies contained 100 mmol/l Tris, 5 mmol/l $MgCl_2$, and 1 g/l bovine serum albumin, pH 7.8. Non-specific binding was determined by addition of 3.3 µmol/l [Lys^8]-vasopressin. Cold binding buffer (2 ml) was added. After the separation of free and bound radioactivity by centrifugation at 3000 *g* for 10 min at 4 °C, washing was performed twice in cold binding buffer.

Degradation studies

Radioligands were incubated with membrane preparations for 60 to 90 min at room temperature in 12 × 75 mm polystyrene tubes. Approximately 10^5 counts/min of radioligand were incubated with the biological membrane at a final volume of 150 µl assay buffer. After centrifugation the incubation mixture at 2000 *g*, the supernatant was directly applied to a RP-HPLC column. In some experiments, 1 ml of 1 g/l trifluoroacetic acid was added to the pellet. The pellet was resuspended and homogenized, and the suspension was applied to a methanol-activated Sep-Pak C-18 cartridge. The cartridge was then washed with 2 ml 1 g/l trifluoroacetic acid. No radioactivity eluted from the Sep-Pak C-18 cartridge during this washing procedure. Radioactivity was then eluted with 0.5 ml methanol. Methanol was evaporated, 150 µl 1 g/l trifluoroacetic acid was added and the solution was applied to the RP-HPLC-column, as described above.

Reversed phase high performance liquid chromatography

RP-HPLC was performed with a system from LKB-Pharmacia (Uppsala, Sweden) (HPLC Gradient Pump 2249, Solvent Conditioner 2156, Fraction Collector Frac-100) coupled to a UV-Vis Detector (Variable Wavelength Monitor 2141) and a radioactivity flow through monitor (Ramona 90) from RAYTEST Isotopenmeßgeräte GmbH (Straubenhardt, Germany). Analysis and integration were performed on an Intel 80386 processor-based computer using the Nelson- (LKB Pharmacia) or Raytest Software. Peptides were applied to a spherisorb S 5 ODS II column (5 µm, 250 × 4 mm) from Knauer (Berlin, Germany) and eluted by a gradient of 1 g/l trifluoroacetic acid in water (solvent A) and 1 g/l trifluoroacetic acid in acetonitrile (solvent B, 5–60%) within 60 min (or in 20 min, as specified). The flow rate was 1 ml/min, and 0.5 ml fractions were collected.

Results and Discussion

Binding of N$^\varepsilon$-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin to LLC-PK$_1$ cell membranes is a temperature-dependent process. At 4 °C, non-specific binding is 31% of total binding; at 37 °C, total binding is nearly identical with non-specific binding, i. e., specific binding of N$^\varepsilon$-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin to LLC-PK$_1$ cell membranes is thus no longer detectable at 37 °C (fig. 1). These findings suggest a temperature-dependent degradation of the radioligand N$^\varepsilon$-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin by LLC-PK$_1$ cell membranes. RP-HPLC analysis of N$^\varepsilon$-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin after binding to LLC-PK$_1$ cell membranes at 22 °C shows that a large amount of the tracer is already degraded within 15 min. In the 60-min run, a new peak appears at 16.5 min besides the N$^\varepsilon$-[^{125}I]*L*-

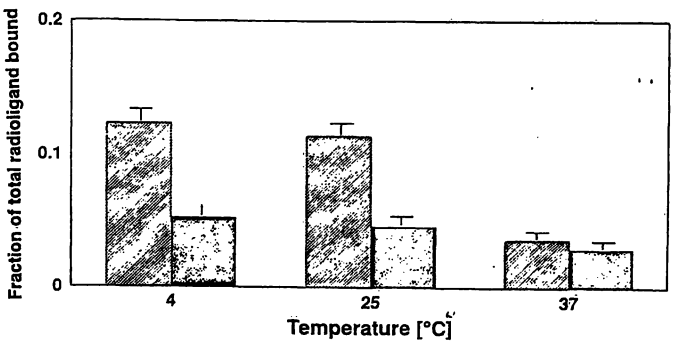

Fig. 1 Total and non-specific binding of N$^\varepsilon$-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin to LLC-PK$_1$ cell membranes at 4, 25 and 37 °C. Binding assays were carried out as described under Material and Methods. Values are means ± s from 5 individual determinations.

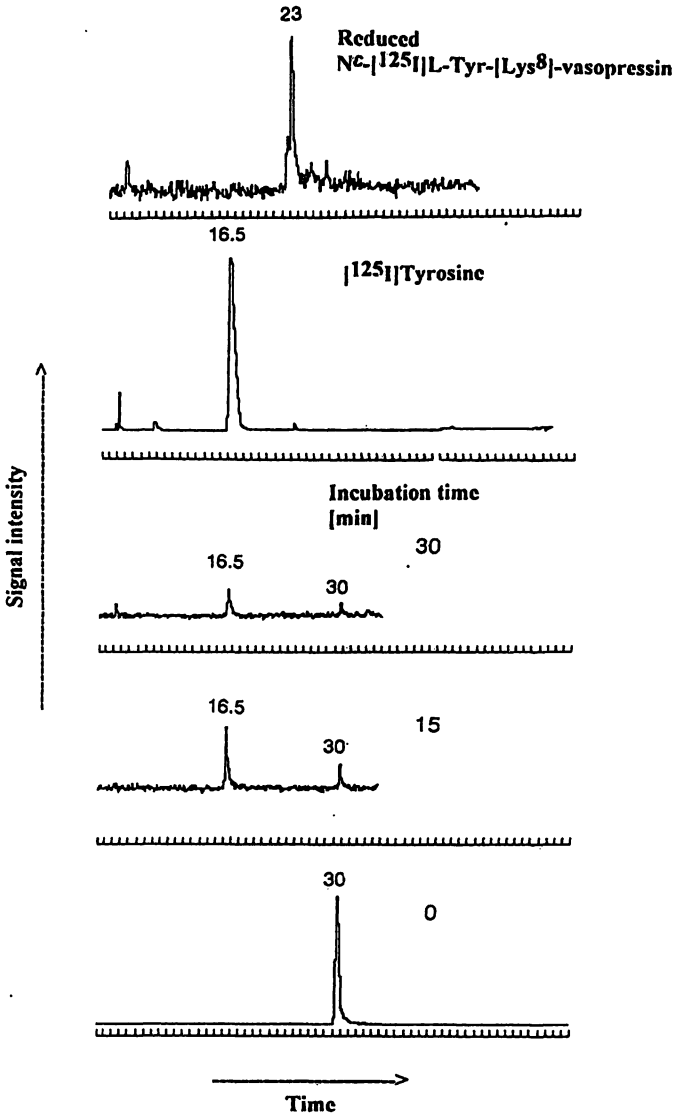

Fig. 2 RP-HPLC-analysis (60 min run) of N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin and its degradation after binding to LLC-PK$_1$ cell membranes for 0, 15 and 30 min Binding assays and degradation studies were carried out as described under Material and Methods at 22 °C. The RP-HPLC analysis (60 min run) of [^{125}I[tyrosine and reduced N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin is also shown. The figures on the peaks are the retention times in minutes.

Tyr-[Lys^8]-vasopressin peak at 30 min (fig. 2). Further prolongation of incubation did not yield any additional degradation products. Without addition of LLC-PK$_1$ cell membranes, N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin was not degraded at 4, 25, and 37 °C (RP-HPLC analysis, data not shown). The same degradation mechanism occurred when tracer was incubated with plasma membranes from A 10 cells (vascular smooth muscle cells), or with rat liver or kidney plasma membranes (data not shown).

The main degradation pathways are known for vasopressin and vasopressin derivatives (5):

(i) reduction of the disulphide bond by thiol : protein disulphide oxidoreductases;

(ii) cleavage of the cysteine-tyrosine peptide bond by aminopeptidases;

(iii) postproline cleavage between positions 7 and 8 with release of a C-terminal dipeptide;

(iv) separation between positions 8 and 9 with release of glycinamide.

The degradation product of N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin co-elutes with monoiodotyrosine but not with the reduced radioligand (cleavage of the disulphide bond of N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin by mercaptoethanol, fig. 2), suggesting that the rapid radioligand degradation is mainly due to cleavage of the neighbouring radiolabelled acid at the N^{ε}-group of [Lys^8]-vasopressin.

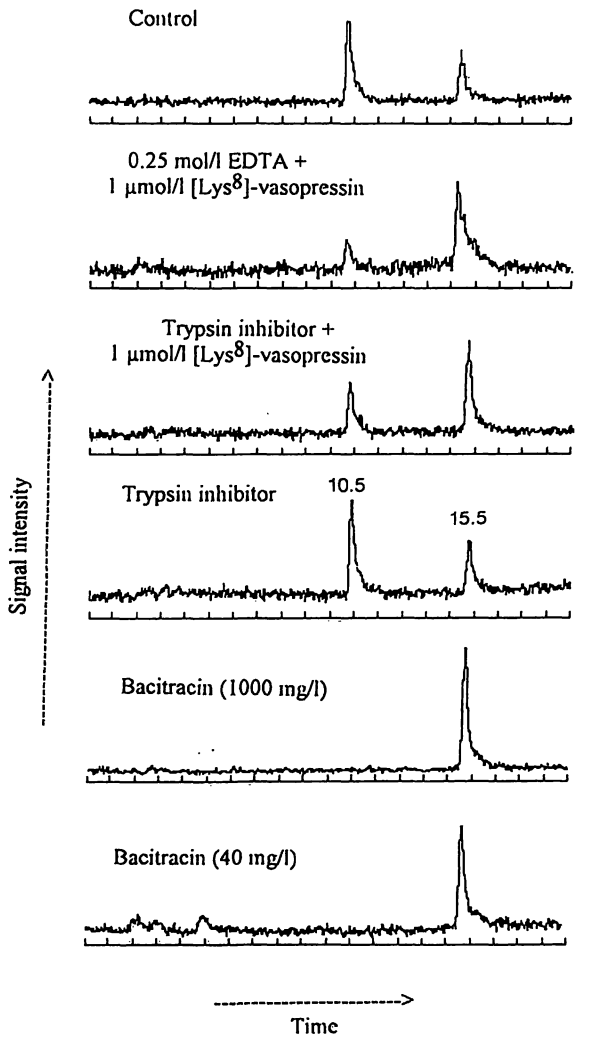

Fig. 3 RP-HPLC analysis (20 min run) of N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin and its degradation after binding to LLC-PK$_1$ cell membranes. Binding assays were carried out with the enzyme inhibitors aprotinin, bestatin, soybean trypsin inhibitor, EDTA, 1,10-phenanthroline, 8-hydroxyquinoline, and bacitracin for 90 min at 22 °C (see also Materials and Methods). Only bacitacin prevented degradation of N^{ε}-[^{125}I]*L*-Tyr-[Lys^8]-vasopressin. The figures on the peaks are the retention times in minutes.

The stereoisomer N^{ε}-[^{125}I]*D*-Tyr-[Lys^{8}]-vasopressin undergoes the same degradation process as N^{ε}-[^{125}I]*L*-Tyr-[Lys^{8}]-vasopressin. Monoiodotyrosine is also formed, as suggested by RP-HPLC (data not shown). The (probably enzymatic) degradation of the radioligand by the LLC-PK_1 cell membrane is obviously not affected by the chirality of the amino-acid bound to the N^{ε}-amino group.

To determine whether the degradation of N^{ε}-[^{125}I]*L*-Tyr-[Lys^{8}]-vasopressin can be blocked by enzyme inhibitors, we investigated the influence of bacitracin. After binding of the radioligand to LLC-PK_1 cell membranes, the supernatants were purified in a Sep-Pak column and analysed by RP-HPLC. Bacitracin completely inhibited degradation at dosages as low as 40 mg/l (fig. 3). Degradation was not prevented completely at concentrations below 40 mg/l; furthermore degradation was not prevented at all by any of the common enzyme inhibitors, such as aprotinin, bestatin, soybean trypsin inhibitor, EDTA, 1,10-phenanthroline, and 8-hydroxyquinoline. Sulph-hydryl-specific reagents such as ethylmaleimide and p-toluenesulphonyl fluoride did prevent tracer degradation but also abolished binding of the radioligand (data not shown).

Bacitacin is a well-known broad-spectrum proteinase inhibitor preventing degradation of insulin, endogenous opioids, etc. (6–8). The inhibitory effect of bacitracin on degradation was also confirmed in other systems (A 10 cells as well as kidney and liver plasma membranes, data not shown). Specific binding increased up to a bacitracin concentration of 1 g/l (fig. 4) and was inhibited only at concentrations of 10 g/l or more. The

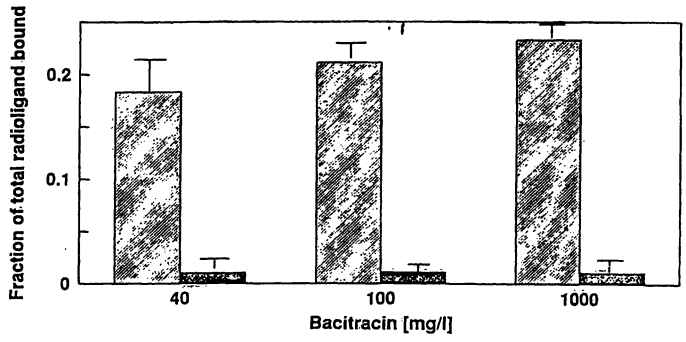

Fig. 4 Total and non-specific binding of N^{ε}-[^{125}I]*L*-Tyr-[Lys^{8}]-vasopressin to LLC-PK_1 cell membranes at 22 °C in the presence of bacitracin. Binding assays were carried out as described under Material and Methods for 90 min at 22 °C. Values are means ± s from 5 individual determinations.

binding affinity of N^{ε}-[^{125}I]*L*-Tyr-[Lys^{8}]-vasopressin to LLC-PK_1 cell membranes – or other radioligands (1, 3, 4, 9) – was not affected by bacitracin (data not shown).

Acknowledgement

We wish to thank Prof. Dr. *Ch. Bauer*, Berlin-Dahlem, Germany for valuable discussions. This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG He 1472/3-1).

References

1. Dürr, J. A., Hensen, J. & Schrier, R. W. (1992) High specific activity ^{125}I- and ^{35}S-labeled vasopressin analogues with high affinity for the V_1 and V_2 vasopressin isoreceptors. J. Biol. Chem. *267*, 18453–18458.
2. Orloff, J. & Handler, J. S. (1967) The role of adenosine 3',5'-phosphate in the action of antidiuretic hormone. Am. J. Med. *42*, 757–768.
3. Hocher, B., Merker, H.-J., Dürr, J. A., Schiller, S., Gross, P. & Hensen, J. (1992) Internalization of V_2-vasopressin receptors in LLC-PK_1-cells; Evidence for receptor-mediated endocytosis. Biochem. Biophys. Res. Commun. *186*, 1376–1383.
4. Ehnis, T., Hocher, B., Abou-Rebyeh, H., Oelkers, W. & Hensen, J. (1993) Expression of vasopressin-receptors (V2-subtype) on LLC-PK_1 cells during cell culture. Eur. J. Clin. Chem. Clin. Biochem. *31*, 273–276.
5. Walter, R. & Simmons, H. (1977) *Metabolism of Neurohypophyseals Hormones: Considerations from a Molecular Viewpoint*; Neurohypophysis: Moses, A. M. & Share, L. (eds.), pp. 167–188.
6. Dahl, D. A., Tsao, T., Duckworth, W. C., Frank, B. H. & Rabkin, R. (1990) Effect of bacitracin on retroendocytosis and degradation of insulin in cultured kidney epithelial cell line. Diabetes *39*, 339–1346.
7. Rabkin, S. W. & Redston, M. (1989) The effect of endogenous opioid degradation, bacitracin, bestatin, captopril, and D-phenylalanine, on digoxin-induced arrhythmias in guinea pigs. Can. J. Physiol. *67*, 857–863.
8. Sladek, C. D. & Armstrong, W. E. (1987) Gamma-aminobutyric acid antagonists stimulate vasopressin release from organ-cultured hypothalamo-neurohypophysial explants. Endocrinology *120*, 1576–1580.
9. Smith, J. D. & Bolger, G. T. (1989) Selective inhibition of [^{3}H]nitendipine binding to brain and cardiac membranes by bacitracin. Can. J. Physiol. *67*, 1591–1595.
10. Rudinger, J. & Ruegg, U. (1973) Preparation of N-succinimidyl-3-(4-hydroxyphenyl)-propianate. Biochem. J. *133*, 538–539.

Dr. Berthold Hocher
Klinikum Steglitz der Freien Universität Berlin
Abteilung für Innere Medizin
Hindenburgdamm 30
D-12200 Berlin
Germany