ORIGINAL RESEARCH

In vitro binding and receptor-mediated activity of terlipressin at vasopressin receptors V_1 and V_2

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Abstract: Terlipressin, a synthetic, systemic vasoconstrictor with selective activity at vasopressin-1 (V_1) receptors, is a pro-drug for the endogenous/natural porcine hormone [Lys_8]-vasopressin (LVP). We investigated binding and receptor-mediated cellular activities of terlipressin, LVP, and endogenous human hormone [Arg_8]-vasopressin (AVP) at V_1 and vasopressin-2 (V_2) receptors. Cell membrane homogenates of Chinese hamster ovary cells expressing human V_1 and V_2 receptors were used in competitive binding assays to measure receptor-binding activity. These cells were used in functional assays to measure receptor-mediated cellular activity of terlipressin, LVP, and AVP. Binding was measured by [^{3}H]AVP counts, and the activity was measured by fluorometric detection of intracellular calcium mobilization (V_1) and cyclic adenosine monophosphate (V_2). Binding potency at V_1 and V_2 was AVP>LVP>>terlipressin. LVP and terlipressin had approximately sixfold higher affinity for V_1 than for V_2. Cellular activity potency was also AVP>LVP>>terlipressin. Terlipressin was a partial agonist at V_1 and a full agonist at V_2; LVP was a full agonist at both V_1 and V_2. The in vivo response to terlipressin is likely due to the partial V_1 agonist activity of terlipressin and full V_1 agonist activity of its metabolite, LVP. These results provide supportive evidence for previous findings and further establish terlipressin pharmacology for vasopressin receptors.

Keywords: hormones, in vitro techniques, pharmacology, hepatorenal syndrome

Introduction

Terlipressin (N^α-tryglycl-8-lysine-vasopressin), a pro-drug for the endogenous/natural porcine hormone [Lys_8]-vasopressin (LVP),[1,2] is a synthetic vasopressin analog derived from the natural/endogenous human hormone [Arg_8]-vasopressin (AVP). Terlipressin differs from AVP in the substitution of lysine for arginine at the eighth position of endogenous AVP and the addition of three glycyl residues at the amino terminus (Figure 1). Large randomized clinical studies in patients with hepatorenal syndrome type 1 have shown that intravenous administration of terlipressin improves renal function in this setting.[3,4] Terlipressin exerts its effects via its partial vasopressin-1 (V_1) agonist activity and the full V_1 agonist activity of its metabolite, LVP.[5] Findings from experimental studies conducted in rat and human tissues provide convincing evidence of the rapid, acute vasopressor function of terlipressin following intravenous bolus administration of clinically relevant doses, as well as the longer term vasoconstrictive effects achieved through terlipressin conversion to LVP.[6]

V_1 receptors (previously known as V_{1a} receptors) are located on vascular smooth muscle in the kidney, myometrium, bladder, liver, spleen, platelets, and fat tissue; V_1 receptors mediate vasoconstriction, hepatic glycogenolysis, and platelet aggregation.[7,8]

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Vasopressin-2 (V_2) receptors are located on the basolateral membrane of the distal tubule and collecting ducts in the kidney; V_2 receptors mediate the antidiuretic effects of vasopressin.[7] Cleavage of the *N*-triglycyl residue on terlipressin occurs in vivo, resulting in a prolonged duration of action for terlipressin compared with LVP.[7] The purpose of this study was to investigate the binding activities and receptor-mediated cellular activities of terlipressin, LVP, and AVP at vasopressin receptor subtypes V_1 and V_2 in vitro. Notably, vasopressin-3 receptors (previously known as V_{1b} receptors) were not investigated in this study because they are not relevant to the hepatorenal syndrome type 1 disease state.

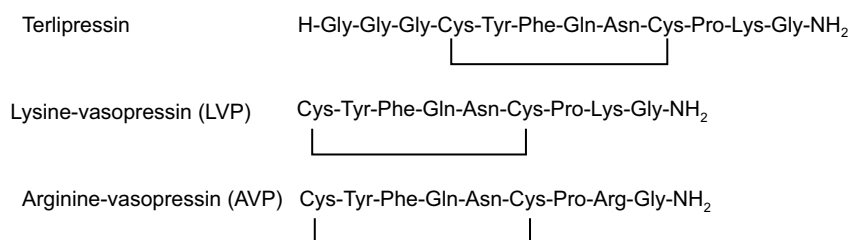## Materials and methods
Cell lines

Chinese hamster ovary (CHO) cells expressing human V_1 receptors (line CHO-S; study numbers 20404, 21104, and 100009187; Cerep, Celle l'Evescault, France) and human V_2 receptors (line CHO-K1; study numbers 20404, 21104, and 100009187; Cerep)[9] were used for the competitive binding and functional assays (Table 1).[9,10] The expression of vasopressin receptors was approximately fivefold higher in the V_2 cell line; thus, different amounts of cell membrane proteins or cells were used in the assays (Table 2).

Competitive binding assays

Cell membrane homogenates were prepared from cells of the CHO-S and CHO-K1 cell lines (study numbers 20404, 21104, and 100009187; Cerep). [³H]AVP at a concentration of 0.3 nM was added to each cell membrane homogenate (V_1 assay: 40-μg protein; V_2 assay: 16-μg protein), which was then incubated (V_1 assay: 60 minutes; V_2 assay: 120 minutes) at 22°C with various concentrations of terlipressin, LVP, and AVP in assay buffer containing Tris–HCl 50 mM (pH 7.4), $MgCl_2$ 5 mM, and 0.1% bovine serum albumin (Table 2). The following terlipressin concentrations were tested (molar): 1.0×10^{-3}, 3.0×10^{-4}, 1.0×10^{-4}, 3.0×10^{-5}, 1.0×10^{-5}, 3.0×10^{-6}, 1.0×10^{-6}, 3.0×10^{-7}, 1.0×10^{-7}, and 1.0×10^{-8} M. The following LVP and AVP concentrations were tested (molar): 1.0×10^{-6}, 1.0×10^{-7}, 3.0×10^{-8}, 1.0×10^{-8}, 3.0×10^{-9}, 1.0×10^{-9}, 3.0×10^{-10}, 1.0×10^{-10}, 3.0×10^{-11}, 1.0×10^{-11}, and 1.0×10^{-12} M. Nonspecific binding was determined in the presence of AVP 1 μM. All assays were performed in triplicate.

After incubation, samples were filtered rapidly under vacuum through glass fiber filters (GF/B; Packard Bioscience Company, Meriden, CT, USA [now PerkinElmer, Inc., Waltham, MA, USA]) presoaked with 0.3% polyethylenimine and rinsed several times with ice-cold Tris–HCl 50 mM buffer in a 96-sample cell harvester (UniFilter; PerkinElmer, Inc.). The filters were air dried, and the radioactivity retained in the filters was counted in a scintillation counter (TopCount; PerkinElmer, Inc.) in a scintillation cocktail (MicroScint 0; PerkinElmer, Inc.). The standard reference agonist for V_1 was [d(CH₂)₅¹,Tyr(Me)₂]-AVP, and the standard reference agonist for V_2 was AVP. These reference compounds were tested at

Terlipressin	H-Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH₂
Lysine-vasopressin (LVP)	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH₂
Arginine-vasopressin (AVP)	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH₂

Figure 1 Molecular formulas for terlipressin, lysine-vasopressin, and arginine-vasopressin.
Abbreviations: AVP, [Arg₈]-vasopressin; LVP, [Lys₈]-vasopressin.

Table 1 Cells and reference compounds

Assays	Cells	Receptor density (B_{max}, fmol/mg protein)	Reference compound
Binding			
Human V_1 (agonist radioligand)[9]	Human recombinant (CHO-S cells)	880	[d(CH₂)₅¹,Tyr(Me)₂]-AVP
Human V_2 (agonist radioligand)[9]	Human recombinant (CHO-K1 cells)	4000	AVP
Functional			
Human V_1 (agonist effect)[9]	Human recombinant (CHO-S cells)	880	AVP
Human V_2 (agonist effect)[10]	Human recombinant (CHO-K1 cells)	4000	AVP

Abbreviations: AVP, [Arg₈]-vasopressin; B_{max}, maximum number of receptor-binding sites; CHO, Chinese hamster ovary; fmol, fentomole; V_1, vasopressin-1; V_2, vasopressin-2.

Table 2 Experimental conditions

Binding assays	Cell membrane homogenates	Ligand	Concentration	Nonspecific	Incubation	Method of detection
Human V_1 (agonist radioligand)	40 µg protein	[^{3}H]AVP	0.3 nM	AVP (1 µM)	60 minutes at room temperature	Scintillation counting
Human V_2 (agonist radioligand)	16 µg protein	[^{3}H]AVP	0.3 nM	AVP (1 µM)	120 minutes at room temperature	Scintillation counting
Functional assays	**Cell density/well**	**Stimulus**		**Measured component**	**Incubation**	**Method of detection**
Human V_1 agonist effect)	45,000	None (1 µM AVP for control)		Intracellular (calcium)	60 minutes at room temperature	Fluorometry
Human V_2 (agonist effect)	3000	None (1 nM AVP for control)		cAMP	30 minutes at room temperature	HTRF

Abbreviations: AVP, [Arg$_8$]-vasopressin; cAMP, cyclic adenosine monophosphate; HTRF, homogeneous time-resolved fluorescence; V_1, vasopressin-1; V_2, vasopressin-2.

several concentrations to obtain competition curves from which the concentration achieving half-maximal inhibition concentration (IC$_{50}$) was calculated.

Receptor-mediated cellular activity assays

Cell cultures were established from the same CHO cell lines used in the binding assays described earlier (Table 1).[9,10] Cells expressing human V_1 receptors were suspended in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA, USA), complemented with 0.1% fetal calf serum, and then distributed in microplates at a density of 45,000 cells per well (Table 2). The fluorescent probe (Fluo4 NW; Thermo Fisher Scientific) was mixed with probenecid in Hank's balanced salt solution buffer (Thermo Fisher Scientific) complemented with N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) 20 mM buffer (pH 7.4; Thermo Fisher Scientific) and added into each well and equilibrated with the cells for 60 minutes at 37°C, and then for 15 minutes at 22°C. Assay plates were then positioned in a microplate reader (CellLux; PerkinElmer, Inc.) for the addition of various concentrations of terlipressin, LVP, and AVP and for subsequent measurements of activity. The following terlipressin concentrations were tested (molar): 1.0×10^{-3}, 1.0×10^{-4}, 1.0×10^{-5}, 3.0×10^{-6}, 1.0×10^{-6}, 3.0×10^{-7}, 1.0×10^{-7}, 3.0×10^{-8}, 1.0×10^{-8}, 3.0×10^{-9}, 1.0×10^{-9}, 1.0×10^{-10}, and 1.0×10^{-11} M. The following LVP and AVP concentrations were tested (molar): 1.0×10^{-4}, 1.0×10^{-5}, 1.0×10^{-6}, 1.0×10^{-7}, 1.0×10^{-8}, 3.0×10^{-9}, 1.0×10^{-9}, 3.0×10^{-10}, 1.0×10^{-10}, 3.0×10^{-11}, 1.0×10^{-11}, 3.0×10^{-12}, 1.0×10^{-12}, 1.0×10^{-13}, and 1.0×10^{-14} M. All assays were performed in triplicate. Changes in fluorescence intensity reflected changes in the concentration of free cytosolic calcium. The standard reference agonist was AVP.

The activity at V_2 receptors was measured based on cyclic adenosine monophosphate (cAMP) production using the homogeneous time-resolved fluorescence method. Cells expressing human V_2 receptors were suspended in Hank's balanced salt solution buffer complemented with HEPES 20 mM buffer (pH 7.4), 0.01% bovine serum albumin, and isobutyl-methylxanthine 0.5 mM, and then distributed in microplates at a density of 3000 cells per well and preincubated for 5 minutes at room temperature in the absence (control) or in the presence of the various concentrations of terlipressin, LVP, AVP, and the reference antagonist ([adamantaneacetyl^1, O-Et-D-Tyr^2, Val^4, aminobutyryl^6]-AVP). Next, the reference agonist, AVP, was added to a final concentration of 0.03 nM. After 30 minutes of incubation at room temperature, the cells were lysed, and the fluorescence acceptor (D2-labeled cAMP) and the fluorescence donor (anti-cAMP antibody labeled with europium cryptate) were added. After 60 minutes of incubation at room temperature, the plates were positioned in a microplate reader (RUBYstar; BMG Labtech, Cary, NC, USA), and fluorescence transfer was measured at λex = 337 nM and λem = 620 and 665 nM. The following terlipressin concentrations were tested (molar): 1.0×10^{-3}, 1.0×10^{-4}, 1.0×10^{-5}, 3.0×10^{-6}, 1.0×10^{-6}, 3.0×10^{-7}, 1.0×10^{-7}, 3.0×10^{-8}, 1.0×10^{-8}, 3.0×10^{-9}, 1.0×10^{-9}, 1.0×10^{-10}, and 1.0×10^{-11} M. The LVP and AVP concentrations were tested (molar): 1.0×10^{-4}, 1.0×10^{-5}, 1.0×10^{-6}, 1.0×10^{-7}, 1.0×10^{-8}, 3.0×10^{-9}, 1.0×10^{-9}, 3.0×10^{-10}, 1.0×10^{-10}, 3.0×10^{-11}, 1.0×10^{-11}, 3.0×10^{-12}, 1.0×10^{-12}, 1.0×10^{-13}, and 1.0×10^{-14} M. All assays were performed in triplicate.

The reference compounds for V_1 and V_2 were tested at several concentrations to obtain concentration–response curves from which the concentration producing half-maximal effective concentration (EC$_{50}$) or IC$_{50}$ was calculated.

Data analysis

For the binding assays, specific ligand binding to receptors was defined as the difference between the total binding and

the nonspecific binding determined in the presence of an excess of unlabeled ligand. Results were expressed as a percentage of control-specific binding (measured specific binding/control-specific binding $\times$ 100) and as percent inhibition of control radioligand-specific binding (100 − [measured specific binding/control-specific binding] $\times$ 100) obtained in the presence of terlipressin, LVP, or AVP. Selectivity of terlipressin and LVP for V_1 and V_2 receptors was calculated as compared with AVP.

Values for the IC_{50} and the Hill coefficient (nH) were determined by nonlinear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting ($Y = D + [A − D]/[1 + (C/C_{50})^{nH}]$, where Y = specific binding, D = minimum specific binding, A = maximum specific binding, C = test article concentration, $C_{50} = IC_{50}$, and nH = slope factor). Inhibition constants (K_i) were calculated from the Cheng Prusoff equation ($K_i = IC_{50}/1+[L/KD]$, where L = concentration of radioligand in the assay and KD = affinity of the radioligand for the receptor).

For the functional assays, results were expressed as a percentage of control agonist response (measured response/control response $\times$ 100) in the presence of the test article. The EC_{50} values were determined by nonlinear regression analysis of the functional assay curves generated with mean replicate values using the Hill equation curve fitting ($Y = D + [A − D]/[1 + (C/C_{50})^{nH}]$, where Y = response, D = right asymptote of the curve, A = left asymptote of the curve, C = test article concentration, $C_{50} = EC_{50}$, and nH = slope factor). The results for V_1 were expressed as a percentage of the control response to AVP 1 µM, and the results for V_2 were expressed as percent inhibition of the control response to AVP 0.03 nM.

Results
Binding affinity

Results of the binding affinity assays are summarized in Table 3 and illustrated in Figure 2. The potency of binding affinity of the tested compounds to both V_1 and V_2 receptors was AVP>LVP>>terlipressin. For V_1 receptors, the binding affinity of LVP was about twofold less than that of AVP, and the binding affinity of terlipressin was about 600-fold less than that of LVP. For V_2 receptors, the binding affinity of LVP

Table 3 Binding affinity assay results

Compound tested	IC_{50} (moles)		K_i (moles)		Selectivity for V_1 (K_i−V_2/K_i−V_1)
	Human V_1	Human V_2	Human V_1	Human V_2	
Terlipressin	1.7×10^{-6}	9.6×10^{-6}	1.1×10^{-6}	6.9×10^{-6}	6.3
LVP	2.9×10^{-9}	1.4×10^{-8}	1.8×10^{-9}	1.0×10^{-8}	5.6
AVP	1.3×10^{-9}	1.2×10^{-9}	8.0×10^{-10}	8.5×10^{-10}	1.1

Abbreviations: AVP, [Arg$_8$]-vasopressin; IC_{50}, half-maximal inhibition concentration; K_i, inhibition constant; LVP, [Lys$_8$]-vasopressin; V_1, vasopressin-1; V_2, vasopressin-2.
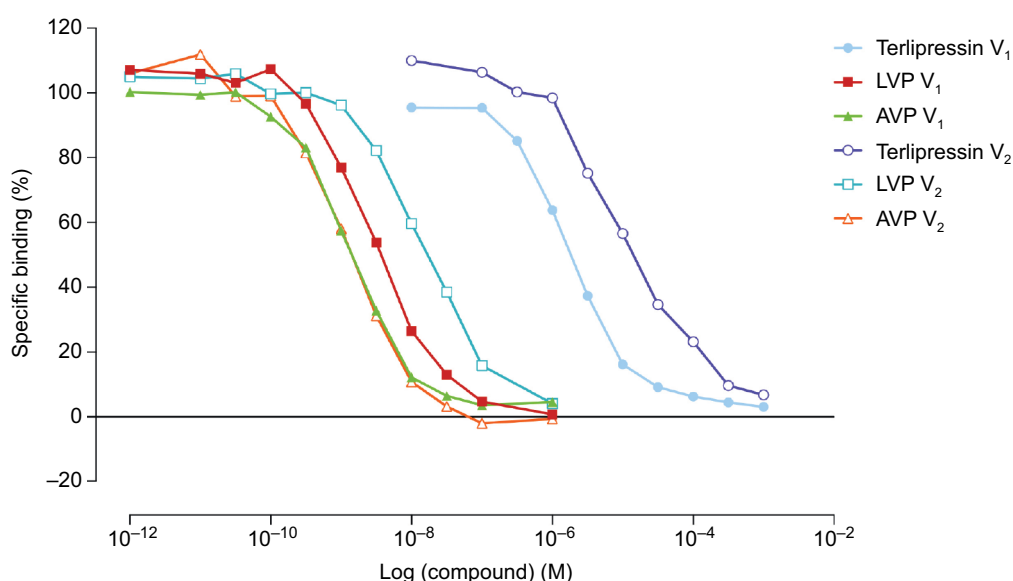
Figure 2 Binding affinity as a function of concentration for the tested compounds.
Abbreviations: AVP, [Arg$_8$]-vasopressin; LVP, [Lys$_8$]-vasopressin; V_1, vasopressin-1; V_2, vasopressin-2.

was about 12-fold less than that of AVP, and the binding affinity of terlipressin was about 700-fold less than that of LVP.

AVP bound V_1 and V_2 receptors with similar high affinities (8.0×10^{-10} and 8.5×10^{-10}, respectively). LVP also bound V_1 and V_2 receptors with high affinity, but affinity for V_1 was about sixfold higher than that for V_2 (1.8×10^{-9} and 1.0×10^{-8}, respectively). Terlipressin bound V_1 and V_2 receptors with low affinity, but affinity for V_1 was about sixfold higher than that for V_2 (1.1×10^{-6} and 6.9×10^{-6}, respectively).

Cellular activity

Results of the vasopressin-mediated functional activity assays are summarized in Table 4 and illustrated in Figure 3. The order of potency for cell activation was the same as for the binding assay: AVP>LVP>>terlipressin. LVP was a full agonist at both V_1 and V_2 receptors, producing maximal cell activation about equal to that of AVP. Terlipressin produced maximal cell activation about equal to that of AVP at V_2 receptors, and thus was a full agonist at V_2 receptors, but it produced only 41% of the maximal effect observed with

AVP at V_1 receptors, indicating that it is a partial agonist at V_1 receptors.

Discussion

Terlipressin is a pro-drug for the endogenous/natural porcine hormone LVP.[1,2] In the current study, the results of radioligand competitive binding assays in cell membrane homogenates of CHO cells expressing human V_1 and V_2 receptors demonstrated that terlipressin binds to V_1 and V_2 receptors with much lower affinities than LVP or AVP. Terlipressin was selective for V_1 receptors at a V_1:V_2 ratio of 6:1. Results of the functional assays indicated that terlipressin behaves as a partial agonist at V_1 receptors and a full agonist at V_2 receptors.

Our results support findings from previously reported studies and provide additional evidence regarding the pharmacologic properties of terlipressin.[2,11,12] Colson et al,[11] using vascular smooth muscle cells isolated from rat aorta and human uterine artery, found that the relative binding affinity (K_i [nM]) of LVP, AVP, and terlipressin for human and rat V_1 receptors was AVP>LVP>>terlipressin. The mean binding

Table 4 Functional activity assay results

Compound tested	EC$_{50}$ (moles)		Intrinsic activity (maximal activity of test compound/maximal activity of AVP)	
	Human V_1	Human V_2	Human V_1 (%)	Human V_2 (%)
Terlipressin	5.0×10^{-8}	2.8×10^{-8}	41	≈100
LVP	2.9×10^{-10}	2.4×10^{-11}	≈100	≈100
AVP	1.8×10^{-10}	4.4×10^{-12}	100	100

Abbreviations: AVP, [Arg$_8$]-vasopressin; EC$_{50}$, half-maximal effective concentration; K$_i$, inhibition constant; LVP, [Lys$_8$]-vasopressin; V_1, vasopressin-1; V_2, vasopressin-2.
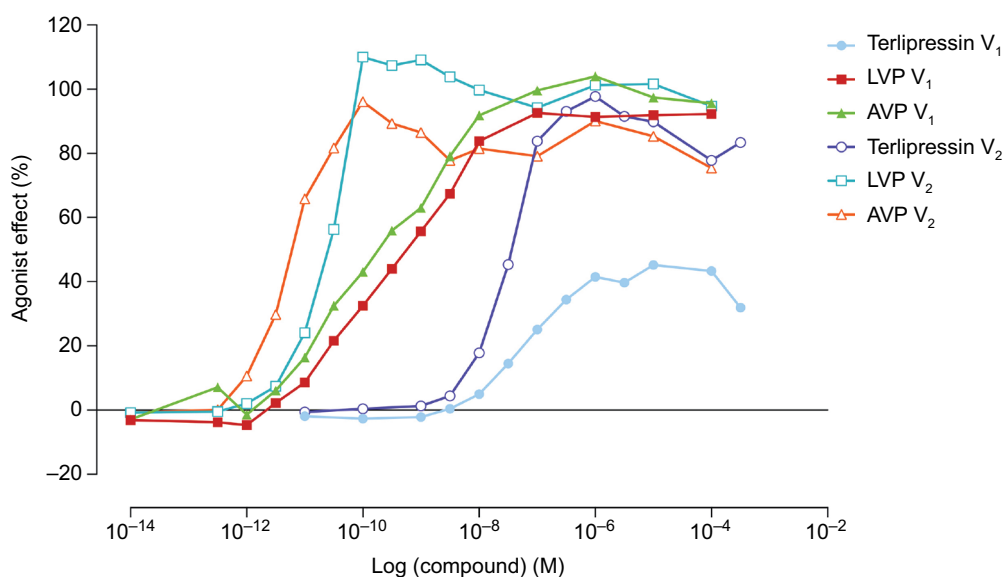
Figure 3 Vasopressin-mediated cell activation as a function of concentration for the tested compounds.
Abbreviations: AVP, [Arg$_8$]-vasopressin; LVP, [Lys$_8$]-vasopressin; V_1, vasopressin-1; V_2, vasopressin-2.

affinity of terlipressin for human V_1 receptors (1229 nM) was about 120-fold less than the binding affinity of LVP (10 nM); about a 700-fold difference in terlipressin (852 nM) versus LVP binding (1.2 nM) was observed at rat V_1 receptors.[11] Mean maximal cell activation with terlipressin, as measured by intracellular calcium mobilization, was 48% at human V_1 receptors and 66% at rat V_1 receptors versus 100% with LVP.[11] The selectivity of terlipressin for V_1 receptors was 1.9-fold higher than that for V_2 receptors compared with the 6.3-fold higher selectivity observed in our study. This difference may be a reflection of the different cell lines utilized for competitive binding assays (rat and human vascular smooth muscle cells versus CHO cells, respectively) in the two studies. Bernadich et al[12] found that the mean potency of terlipressin for V_1 activation in vasopressor tests in a rat model of portal hypertension (9 IU×µmol^{-1}) was about 66-fold less than the mean potency of vasopressin (614 IU×µmol^{-1}); they reported that terlipressin was selective for V_1 receptors, with a V_1:V_2 ratio of 2.2:1.0 compared with 1:1 for vasopressin. Wisniewski et al[2] investigated the potency of terlipressin in vitro for V_1 activation in a recombinant system with a functional assay and found that the EC_{50} (148 nM) was about 150-fold higher than that for LVP (0.93 nM). In an in vivo model in rats, the investigators showed that LVP and AVP were similar in time to effect and magnitude of effect in raising arterial blood pressure when given at identical doses; in contrast, a 100-fold higher dose of terlipressin was required to achieve a similar effect with a slower onset of action.[2]

Because terlipressin is metabolized to LVP, which is a potent, full V_1 receptor agonist in vivo, our data support the theory that the principal pharmacologic response to terlipressin administration is most likely attributable to the LVP metabolite.[1]

Conclusion

These data suggest that the in vivo pharmacologic response to the administration of terlipressin primarily reflects LVP-mediated activation of V_1 receptors. However, terlipressin demonstrated agonist activity at both V_1 and V_2 receptors. Results from the current study provide additional, supportive evidence for previous findings and further establish the binding affinity, potency, and selectivity of terlipressin at vasopressin receptors.

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