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ANGIOTENSIN II AND NON-ANGIOTENSIN II DISPLACEABLE BINDING SITES FOR [^{3}H]LOSARTAN IN THE RAT LIVER

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Abstract—By virtue of the more than 1000-fold selectivity of losartan (DuP 753) for the AT_1 angiotensin II (AII) receptor subtype compared with the AT_2 subtype, [^{3}H]losartan may be a useful radioligand for studies of the AT_1 receptor subtype. Comparison of B_{max} values in the liver obtained from saturation isotherms using [^{3}H]losartan (B_{max} = 194 pmol/g tissue) and [^{125}I]sarcosine^1,isoleucine^8 angiotensin II (B_{max} = 20 pmol/g tissue) indicated that the AII receptor concentration was approximately 10% that of the [^{3}H]losartan binding sites. In addition, AII at concentrations as high as 10 μM displaced less than one-third of specific [^{3}H]losartan binding in the liver and less than 80% in the whole adrenal. The presence of non-AII displaceable [^{3}H]losartan binding in the liver did not appear to result from metabolism of the radioligand since HPLC analysis of free and bound ^{3}H revealed that greater than 90% of the ^{3}H eluted at the same time as the parent [^{3}H]losartan. This suggests that [^{3}H]losartan binds with high affinity to a site(s) other than angiotensin II receptors in the rat liver.

Angiotensin II (AII)† receptors have been differentiated into two subtypes [1–5], AT_1 and AT_2, using the nomenclature of Bumpus *et al.* [6]. Radiolabeled angiotensins show little selectivity for the two AII receptor subtypes. Moreover, they are peptides and are susceptible to enzymatic degradation in the absence of peptidase inhibitors. Attempts have been made to devise incubation mixtures containing peptidase inhibitors to maintain the integrity of the peptide radioligands for AII receptors. However, these peptidase inhibitors also have the potential to inhibit the binding activity of specific AII receptors. The most notable example is the inhibition of radioligand binding to the AT_1 subtype by sulfhydryl reducing agents [1, 7, 8].

The discovery of selective, high affinity, non-peptide antagonists for the AII receptor subtypes [5, 9–14] allows for the development of radioligands that are not subject to the limitations of peptide radioligands. One such compound, losartan (DuP 753), is highly selective for the AT_1 subtype [1, 2]. [^{3}H]Losartan has been used recently to radiolabel putative AT_1 receptors in the rat adrenal cortex microsomes [15] and rat liver [16].

This study further examines [^{3}H]losartan as a radioligand for the AT_1 receptor subtype and reveals additional non-AII displaceable binding sites for [^{3}H]losartan in the rat liver.

METHODS

Adult male rats were killed by decapitation and the livers were removed. Each liver was homogenized in hypotonic, 20 mM sodium phosphate (pH 7.2) with 0.1 mM bacitracin, and the suspension was centrifuged at 48,000 *g* for 20 min. The precipitated pellet was resuspended in assay medium (50 mM sodium phosphate, pH 7.2) and recentrifuged as described above. The pelleted membrane fraction was resuspended in assay medium to a final concentration of 20–28 mg/mL initial wet weight for [^{3}H]losartan binding experiments and 5–7 mg/mL initial wet weight for [^{125}I]sarcosine^1,isoleucine^8 AII ([^{125}I]SI AII) binding experiments. For one experiment, differential centrifugation was used to obtain 1,000 *g*; 1,000–10,000 *g*; and 10,000–48,000 *g* membrane fractions.

In an additional experiment adult male rats were killed as described above and the liver and adrenals were removed. Whole adrenals from three rats were pooled. The tissue was homogenized in hypotonic solution, and the homogenates were divided into two aliquots and centrifuged as described above. The aliquots were resuspended in 50 mM $NaPO_4$ or Tris buffer (150 mM NaCl, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 50 mM Tris, pH 7.4) as described by Zelezna *et al.* [16]. The tissues were recentrifuged and resuspended a final time in their respective assay buffers at a concentration of 20–28 mg/mL initial wet weight for the liver and 12–16 mg/mL initial wet weight for the adrenal.

[^{3}H]*Losartan binding.* [^{3}H]Losartan ([^{3}H]DuP 753, NEN Research Products, Boston, MA) was incubated with the membrane fraction for 30 min at 21–23°, in the absence or presence of competing ligand with a total assay volume of 0.2 mL. The reaction was terminated by filtration through glass fiber filters (No. 32 Schleicher & Schuell, Keene, NH) rinsed three times with 3 mL of 50 mM sodium potassium phosphate buffer (pH 7.4). Bound

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† Abbreviations: AII, angiotensin II; and SI AII, sarcosine^1, isoleucine^8 AII.

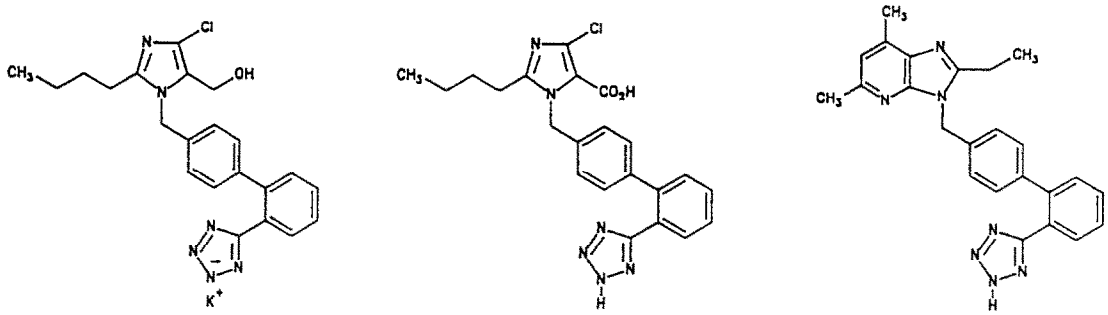

Fig. 1. Structural diagrams of losartan and its analogs.

radioactivity was determined by liquid or crystal scintillation spectrometry. For saturation isotherms, 1.5 to 150 nM [^{3}H]losartan in 50 mM $NaPO_4$ was used. Losartan (10 μM) was added to alternate tubes to define nonspecific binding. Binding constants (K_D and B_{max}) were determined by the method of Rosenthal [17].

Competition binding assays and subcellular fraction binding assays were carried out in the presence of concentrations of [^{3}H]losartan ranging from 0.3 to 90 nM in 50 mM $NaPO_4$. AII and nonpeptide competing ligands (losartan, EXP 3174, and L 158,809), shown in Fig. 1, were present in the assays at concentrations ranging from 1 nM to 10 μM. Competition binding data for specific (10 μM losartan displaceable) binding was evaluated using nonlinear regression analysis (PROC NLIN, SAS, Cary, NC) as follows:

One-site model: $Y = 100 - [100 \cdot X/(X + IC_{50})]$

Two-site model: $Y = 100 - [A \cdot X/(X + IC_{50\text{-}1}) + (100 - A) \cdot X/(X + IC_{50\text{-}2})]$ where Y is the percent of binding in the absence of competing ligand, X is the competing ligand concentration, and A is the percent of high affinity ($IC_{50\text{-}1}$) sites for the competing ligand. For AII competition, a modified two-site model was used in which the term $(100 - A)$ was replaced with a different variable D to represent the percent of low affinity ($IC_{50\text{-}2}$) binding, to enable the plotted regression line to exceed 100%.

For the second series of competition binding assays, the [^{3}H]losartan was diluted in 50 mM $NaPO_4$ or 50 mM Tris, 40 mg/mL bacitracin, and 2.5 μg/mL leupeptin. The total assay volume was 0.1 mL, with 10 μL of [^{3}H]losartan (final concentration of 6 nM), 10 μL of competitor (losartan, AII, or SI AII; all at a final concentration of 10 μM), and 80 μL of tissue.

Tissue linearity. Tissue linearity assays were done for the liver in both the $NaPO_4$ and Tris buffers described above. The liver was resuspended to a concentration of 15, 30, and 60 mg/mL initial wet weight. For tissue linearity, the assay volume was 0.4 mL (0.2 mL of tissue, 0.1 mL [^{3}H]losartan [final concentration of 6 nM], and 0.1 mL of competing ligand [final concentration of 10 μM losartan, AII, or water]). The incubation and filtration conditions were as described above.

[^{125}I]SI AII binding. The binding was carried out as described previously for liver membranes [18], except that the incubations were for 2 or 3 hr (in the presence of 0 or 3 nM losartan) in the absence or presence of 1.5 μM AII to define total and nonspecific binding, respectively. For saturation isotherms, 0.06 to 4 nM [^{125}I]SI AII was used. For comparison of the ability of losartan and AII to compete for [^{125}I]SI AII binding, the assay medium was 50 mM $NaPO_4$ and the incubation time was 1 hr.

Metabolism. Evaluation of metabolic degradation of [^{3}H]losartan by rat liver homogenates was determined in samples incubated in sodium phosphate buffer as described above. Following a 30-min incubation, two samples were centrifuged at 10,000 *g* for 5 min. The supernatant was decanted and 50 mM acetic acid was added to the pellet. After overnight extraction at 4° the pellet was recentrifuged and the

Table 1. Saturation isotherms in the rat liver

Radioligand	N	K_D (nM)	B_{max} (pmol/g initial wet wt)
[^{3}H]Losartan	6	16.1 ± 2.7	194 ± 41
[^{125}I]SI AII	6	0.303 ± 0.07	20.1 ± 3.3
[^{125}I]SI AII + losartan*	6	0.801 ± 0.30	19.1 ± 2.2

Values (mean ± SD) were derived from a one-site Rosenthal [17] analysis.
* Losartan (DuP 753) was present at a 3 nM concentration.

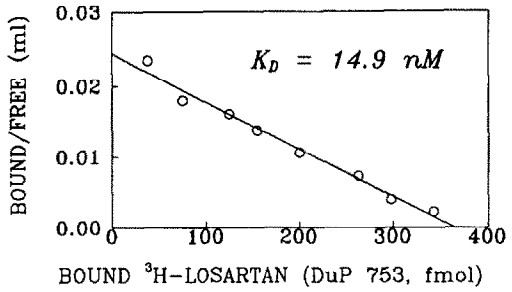

Fig. 2. Rosenthal plot of [³H]losartan binding in rat liver. A Rosenthal (Scatchard) plot of specific (10 μM losartan displaceable) [³H]losartan binding in rat liver membranes at concentrations ranging from 1.5 to 150 nM is shown. The assay volume was 0.2 mL and contained 2.4 mg initial wet weight of tissue. The incubation time was 30 min.

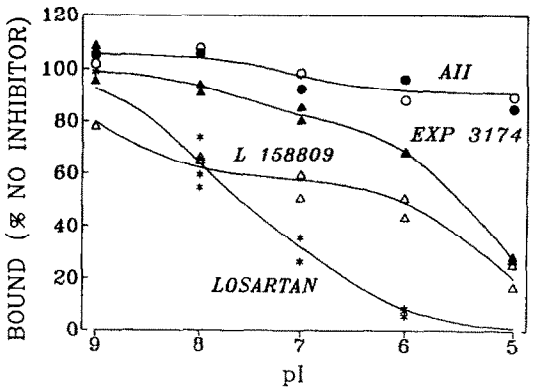

Fig. 4. Competition for [³H]losartan binding to rat liver. Competition for specific (10 μM losartan displaceable) [³H]losartan binding by AII and non-peptide antagonists of the AT_1 receptor subtype. The [³H]losartan concentration was 16 nM. Key: (○) AII in the absence of bovine serum albumin, (●) AII in the presence of 1 mg/mL bovine serum albumin, (*) losartan, (△) L 158,809, and (▲) EXP 3174. Lines drawn are for two-site model fits of the data given in Table 2.

acetic acid supernatant was decanted. Aliquots of fresh [³H]losartan and the supernatants were applied to a reverse phase C_{18} HPLC column and eluted with a mobile phase of 0.6% triethylamine acetate (6 g acetic acid/L, adjusted to pH 4.0 with triethylamine):acetonitrile (60:40) at a flow rate of 1.5 mL/min. Fractions collected at 15-sec intervals were assayed for ³H by liquid scintillation counting. Samples of unlabeled losartan and its major metabolite, EXP 3174, were also applied to the column and monitored by UV absorbance at 248 nm.

RESULTS

Specific [³H]losartan binding to liver membranes was abundant (B_{max} = 194 ± 41 pmol/g initial wet wt, mean ± SD) and displayed high affinity (K_D = 16.1 ± 2.7 nM) as depicted in Table 1 and Fig. 2. Specific [¹²⁵I]SI AII binding in these same liver homogenates displayed a significantly ($P < 0.05$) smaller population of receptors (B_{max} = 20.1 ± 3.3 pmol/g initial wet wt) with a higher binding affinity (K_D = 0.303 ± 0.07 nM) as depicted in Table 1 and Fig. 3. Saturation analysis of [¹²⁵I]SI AII binding in the presence of 3 nM losartan yielded

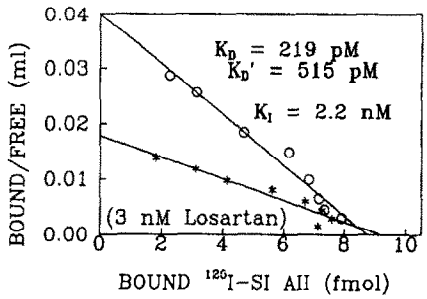

Fig. 3. Inhibition of [¹²⁵I]SI AII binding by 3 nM losartan. A Rosenthal (Scatchard) plot of specific (1.5 μM AII displaceable) [¹²⁵I]SI AII binding in rat liver membranes in the presence of 0 or 3 nM losartan, at concentrations ranging from 0.06 to 2 nM [¹²⁵I]SI AII is shown. The assay volume was 0.2 mL and contained 0.5 mg initial wet weight of tissue. The incubation time was 3 hr.

Table 2. Competition for [³H]losartan binding

Competing ligand	One-site model	Two-site model
Losartan* (DuP 753)	IC_{50} = 27 nM	H.A. = 8 nM (62%)
		L.A. = 264 nM (38%)
	SSe = 1261	SSe = 498
	df = 10	df = 8
EXP 3174†	IC_{50} = 2550 nM	H.A. = 19 nM (19%)
		L.A. = 5160 nM (81%)
	SSe = 696	SSe = 131
	df = 10	df = 8
L 158,809†	$-IC_{50}$ = 289 nM	H.A. = 1 nM (42%)
		L.A. = 5320 nM (58%)
	SSe = 5549	SSe = 149
	df = 10	df = 8
AII	IC_{50} = ND	H.A. = 82 nM (14%)
		L.A. = ND (86%)
		SSe = 99
		df = 6

The values in parentheses are percentages of high or low affinity sites. AII data are corrected to total 100%. The radioligand concentration was approximately 16 nM. Abbreviations: H.A., high affinity IC_{50}; L.A., low affinity IC_{50}; SSe, sum of squares of residual error; and ND, could not be determined.

*† Goodness of fit test gave a better fit for the two-site model versus the one-site model: *$P < 0.05$; †$P < 0.01$; N = 4.

Table 3. Subcellular fractionation of [^{3}H]losartan binding

Membrane fraction	mg protein/ g wet wt	[^{3}H]-Losartan binding (fmol/mg protein)	Specific [^{3}H]losartan binding (%)		
			Fraction	Displaced by: AII*	Displaced by: SI AII*
1,000 *g*	10	858	10.3	13.8	16.4
10,000 *g*	60	815	59.6	12.2	13.0
48,000 *g*	15	1359	24.9	4.2	3.8
Supernatant	76	55	5.2	9.9	16.1
TOTAL†	161	510	100.0	10.5	13.8

* AII and SI AII were added at 10 μM. [^{3}H]Losartan was present at 6 nM. Specific binding is binding displaced by 10 μM losartan. Protein was assayed by the method of Lowry *et al.* [19]; N = 2.
† Total values are sums of proportional amounts of each fraction.

a similar concentration of binding sites (B_{max} = 19.1 ± 2.2 pmol/g initial wet wt), with a significantly lower affinity ($K_{D'}$ = 0.801 ± 0.30 nM) (Table 1, Fig. 3). This indicates a competitive inhibition of [^{125}I]SI AII binding by losartan. The average K_I for losartan, derived from the equation: K_I = losartan concentration/(($K_{D'}/K_D$) − 1), was 1.8 nM. At a concentration of 10 μM, losartan inhibited greater than 90% of the 10 μM AII displaceable [^{125}I]SI AII binding in the rat liver (N = 4). In these experiments 10 μM AII displaced greater than 80% of total [^{125}I]SI AII binding at concentrations up to 2 nM.

The presence of 1 mg/mL of bovine serum albumin did not alter the ability of AII (10 μM) to inhibit [^{3}H]losartan binding at a concentration of 16 nM. The competition by AII for [^{3}H]losartan binding sites was dependent on the concentration of [^{3}H]-losartan. At 0.3 to 0.5 nM [^{3}H]losartan, 10 μM AII displaced 31.7 ± 8.0% of the losartan displaceable binding, while at 4–5 nM [^{3}H]losartan, AII displaced only 22.5 ± 5.6% of the binding ($P < 0.05$; N = 4).

Assuming a two-site model for AII competition, AII had an IC_{50} of 82 nM for 14% of the [^{3}H]losartan binding sites (Table 2, Fig. 4). Initial analyses of the competition curves for the non-peptide antagonists indicated Hill slopes of less than one for the non-peptide compounds. Further analysis yielded a significantly better fit ($P < 0.05$) to the two-site competition model, compared with the one-site model, for all three non-peptide antagonists (losartan, EXP 3174, and L 158,809) (Table 2, Fig. 4). The specific AT_2 receptor antagonist PD 123177 (10 μM) inhibited less than 5% of the [^{3}H]losartan binding in the liver. Working estimates of K_D values for 10 μM losartan displaceable [^{3}H]losartan binding sites derived from binding observed using two concentrations (0.4 and 4–5 nM) of radioligand was 6.0 ± 0.8 nM. In contrast, the comparable estimate of the K_D for 10 μM AII displaceable [^{3}H]losartan binding was 2.8 ± 1.2 nM ($P < 0.01$; N = 4).

As shown in Table 3, differential centrifugation did not reveal a membrane fraction that contained a considerable enrichment of specific [^{3}H]losartan binding sites, expressed as fmol/mg protein. Nor did any individual membrane fraction display a substantially larger portion of 10 μM AII or SI AII

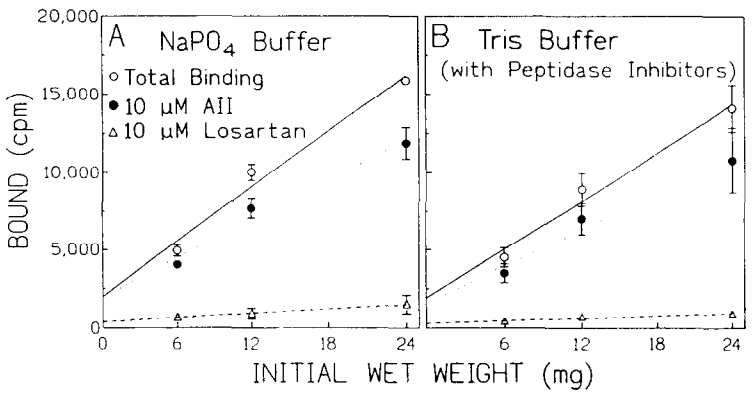

Fig. 5. Tissue linearity of [^{3}H]losartan binding to rat liver. The radioligand concentration was 6 nM. Key: (○) total binding, (●) AII (10 μM) inhibition of total binding, and (△) binding in the presence of losartan (10 μM). The lines through the symbols is a best fit linear regression. Values are means ± SD, N = 4.

Table 4. Competition assay comparing sodium phosphate buffer to Tris buffer with peptidase inhibitors

Tissue/buffer	N	Specific binding (fmol/mg initial wet wt)	AII (% Inhibition)	SI AII (% Inhibition)
Adrenal/$NaPO_4$	3	14 ± 5	56 ± 16	56 ± 34
Adrenal/Tris	3	9 ± 2	77 ± 10*	85 ± 11
Liver/$NaPO_4$	4	73 ± 21†	15 ± 9	15 ± 14
Liver/Tris	4	55 ± 7†	26 ± 3	18 ± 8

Values are means ± SD. AII and SI AII were present at 10 μM, and values are expressed as percent inhibition of specific (10 μM losartan displaceable) binding.

* Significantly greater ($P < 0.05$) inhibition of [3H]losartan binding in the Tris buffer compared to the $NaPO_4$ buffer.

† Significantly greater concentration of [3H]losartan binding than in the whole adrenal ($P < 0.05$).

displaceable binding sites than seen with whole membranes.

Analysis of the tissue linearity of [3H]losartan binding in the liver is shown in Fig. 5. Both total and non-AII displaceable [3H]losartan binding was pseudolinear in both the $NaPO_4$ and Tris buffers.

In both buffers, AII and SI AII competition for [3H]losartan binding sites was consistently less than that of losartan (Table 4). There was a significant ($P < 0.05$) enhancement of AII inhibition of [3H]-losartan binding in the whole adrenal, but not the liver, in the Tris buffer (in the presence of peptidase inhibitors) compared with the phosphate buffer. AII and SI AII also inhibited a greater percentage of [3H]losartan binding in the adrenal than in the liver in both the phosphate and Tris buffers (Table 4). The amount of [3H]losartan binding in the liver was 3.5 to 4 times greater than that in the whole adrenal on a per mg initial wet weight basis in both buffers.

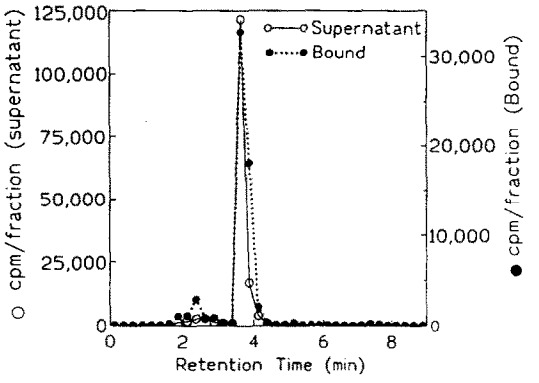

Fig. 6. [3H]Losartan metabolism by rat liver homogenates. [3H]Losartan was incubated with liver homogenate as described in Methods. The supernatant of a 10,000 *g* pellet (○) and an acetic acid extract of the pelleted tissue (●) were applied to a C_{18} reverse phase HPLC column and eluted. Greater than 98% of the 3H applied to the column was recovered within the time frame shown. In the supernatant samples, 92.3% of the eluted 3H was present in the major peak at 3.5 min, while 5.3% of the eluted 3H was in the minor peak occurring at 2.5 min. In the "bound" sample 91% of the eluted 3H was present in the 3.5 min peak, while 6% of the 3H was present in the 2.25 min peak. In this system, the retention time for [3H]losartan and unlabeled losartan was approximately 3.5 min, while that for EXP 3174 was approximately 2.1 min.

HPLC analysis of [3H]losartan, losartan and EXP 3174 indicated that the [3H]losartan used for these studies was extremely pure. Greater than 96% of the 3H eluted at the same retention time as unlabeled losartan. The HPLC system was capable of resolving losartan from its major metabolite, EXP 3174, with a baseline separation (data not shown).

HPLC analysis of 3H in the supernatants of two liver homogenates following a 30-min incubation at room temperature revealed little degradation of free [3H]losartan; 91 and 92% of the 3H eluted from the column migrated in the same fractions as [3H]-losartan and unlabeled losartan. A small proportion of the 3H, 5 and 7%, eluted at about 2 min, corresponding to the retention time of EXP 3174 (Fig. 6).

HPLC analysis of the acetic acid extract of the two liver pellets also indicated that the majority of the bound 3H was also intact [3H]losartan: 85 and 91% of the 3H migrated identically with [3H]losartan and unlabeled losartan, while 11 and 6% of the 3H migrated identically with EXP 3174 (Fig. 6).

Table 5 lists numerous agents that were used in an attempt to characterize the alternative [3H] losartan binding site. Various catecholaminergic transmitter receptor ligands, such as propranolol, phenoxybenzamine, clonidine, and prazosin, inhibited a portion of the [3H]losartan binding, but only at high concentrations; however, other transmitter ligands, such as naloxone, pimozide, carbachol, and serotonin, showed negligible inhibition. Structural analogs, devoid of the biphenyl tetrazole moiety (see Fig. 1) were completely ineffective at inhibiting [3H]losartan binding even at concentrations as high as 1 mM. Relatively nonselective agents that bind to ion channels, such as propranolol, verapamil, lidocaine, and flunarizine, were also capable of inhibiting 30–50% of the [3H] losartan binding at concentrations of 10 μM (data not shown). However, more selective ion channel

Table 5. Agents that inhibited less than 50% of [^{3}H]losartan binding in the liver at a concentration of 10 μM or greater

Agents	Function	Agents	Function
Verapamil*	L-Ca^{2+} channel, adrenergic antgst.	Phenoxybenzamine*	α-Adrenergic antgst.
Nifedipine	L-Ca^{2+} channel antgst.	Clonidine*	α_2-Adrenergic, 5-HT_1 agst.
Nimodipine	Ca^{2+} channel angst.	Idazoxan*	α_2-Adrenergic antgst.
Diltiazem	L-Ca^{2+} channel antgst.	Prazosin*	α_1-Adrenergic antgst.
Pimozide	L-Ca^{2+} channel/D-2 dopamine antgst.	Pindolol	β-Adrenergic antgst.
Flunarizine*	Ca^{2+}/Na^+ channel antgst.	L-Propranolol*	β-Adrenergic antgst., Na^+ channel antgst.
Tetrodotoxin	Na^+ channel antgst.	D-Propranolol*	Na^+ channel antgst.
Amiloride	Na^+ channel antgst.	Isoproterenol*	β-Adrenergic agst.
Benzamil*	Na^+ channel antgst.	Butoxamine*	β_2-Adrenergic antgst.
Lidocaine*	Na^+ channel antgst.	Bretylium tosylate*	Norephinephrine uptake inhibitor
Veratridine	Na^+ channel agst.	Carbachol†	Muscarinic agst.
Veratrine	Na^+ channel agst.	Naloxone	Opioid antgst.
Quinidine*	Na^+ channel antgst.	Serotonin*	5-HT receptor agst.
Minoxidil	K^+ channel agst.	L-Histidine*	Histamine precursor
Glibenclamide*	K^+ channel antgst.	Aminopyridine†	Activates GABA neurons
Endothilan	Endothilan receptor agst.	ATP†	Cellular metabolism, adenosine precursor
PD 123177	AT_2 antgst.	Adenosine†	Adenosine receptor agst.
Triazole†	Structural analog	Me-CH_3-tetrazole†	Structural analog
Imidazole†	Structural analog	Cl-CH_3-imidazole†	Structural analog
Tetrazole†	Structural analog	OH-CH_3-imidazole†	Structural analog
Me-CH_3-imidazole*†	Structural analog	Cl-Et-CH_3-imidazole†	Structural analog

Agents not marked with a symbol were used at a concentration of 10 μM. Abbreviations: Me-CH_3-tetrazole, 5-mercapto-1-methyltetrazole; Cl-CH_3-imidazole, 5-chloro-1-methylimidazole; OH-CH_3-imidazole, 4-hydroxy-1-methylimidazole; Me-CH_3-imidazole, 2-mercapto-1-methylimidazole; Cl-Et-CH_3-imidazole, 5-chloro-1-ethyl-2-methylimidazole; agst., agonist; and antgst., antagonist.

* Agent used at a 0.1 mM concentration.

† Agent used at a 1 mM concentration.

such as nifedipine, tetrodotoxin, and veratridine, had no effect on [^{3}H]losartan binding at 10 μM.

DISCUSSION

In the rat liver [^{3}H]losartan binds with high affinity and high specificity as defined by competition with non-radiolabeled losartan. However, competition with AII at concentrations as high as 10 μM displaced less than one-third of the specific [^{3}H]losartan binding in the rat liver. Comparison of the concentrations of binding sites (B_{max}) for [^{3}H]-losartan and [^{125}I]SI AII were consistent with the interpretation that [^{3}H]losartan binds to a site(s) other than the AT_1 binding site. The B_{max} for [^{125}I]SI AII binding was only 11% of the B_{max} for [^{3}H]losartan in the rat liver in the phosphate buffer. This suggests that the majority of specific (10 μM losartan displaceable) [^{3}H]losartan binding in the rat liver is to a tissue constituent other than the AT_1 receptor binding site.

Inhibition of specific [^{3}H]losartan binding by AII was increased significantly ($P < 0.05$) in the adrenal when the competition assays were performed in a Tris buffer (with peptidase inhibitors) rather than the $NaPO_4$ buffer. However, the increased competition by AII was accompanied by a small decrease in specific binding (Table 4), suggesting that one of the components of the Tris buffer inhibited some of the non-angiotensin displaceable binding sites.

The biphasic competition curves of losartan, EXP 3174 and L 158,809 seen in Fig. 4 further support the concept that [^{3}H]losartan binds to two or more different binding sites in the liver. The low K_I of losartan (1.8 nM) for inhibition of [^{125}I]SI AII binding suggests that the affinity of losartan for the AT_1 receptor subtype is higher than its affinity for the non-AII receptor binding site(s) (Fig. 3). The significantly lower working estimate of the K_D value for AII displaceable [^{3}H]losartan binding (2.8 nM) versus that for losartan displaceable binding (6.0 nM), derived from binding at two low concentrations of [^{3}H]losartan, is also consistent with [^{3}H]losartan having a higher affinity for the AT_1 receptor than for the non-AII binding site(s). The proportion of the two different sites estimated with losartan and L 158,809 (approximately 40/60) and the proportion estimated with EXP 3174 (approximately 20/80) are different. This suggests that [^{3}H]losartan may bind to three—or more—different sites. Competition assays were also done in the presence of 30 mM β-mercaptoethanol (β-ME), which has been shown to inhibit binding at the AT_1 receptor subtype [8]. Angiotensin displaceable [^{3}H]losartan binding was reduced by 65% in the presence of β-ME. However, β-ME did not alter significantly the IC_{50} of losartan for [^{3}H]-

activators or blockers, such as nifedipine, tetrodotoxin, and veratridine, had no effect on [^{3}H] losartan binding at 10 μM.

The observation of non-AII displaceable [^{3}H]-losartan binding in the liver differed from the findings of Chiu *et al.* [15], which show an equivalent amount of competition for [^{3}H]losartan binding by AII and non-radiolabeled losartan in rat adrenal cortex microsomes. Possible explanations for this discrepancy are: (1) the adrenal cortex contains the highest concentration of AII receptors in the rat, and its microsomal fraction may be devoid of the non-AII binding site that is present in the rat liver. However, our observations revealed that the whole adrenal contains a residual non-AII displaceable [^{3}H]losartan binding site accounting for 25–50% of the losartan displaceable binding. (2) Binding in the liver may reflect binding of a radiolabeled metabolite(s) of losartan in addition to [^{3}H]losartan. HPLC analysis of the free and bound ^{3}H following incubation with liver homogenates suggests that there is only a small amount of degradation of [^{3}H]-losartan in the assay conditions used in these experiments. Moreover, approximately 90% of the bound radioligand appears to be the parent compound, [^{3}H]losartan. Thus, while losartan is metabolized to an active metabolite, EXP 3174, which binds to the AT_1 receptor with even greater affinity than losartan [20], the majority of the binding reported here cannot be attributed to formation of EXP 3174. While other metabolites of losartan have been reported, they are more polar than EXP 3174 and should not elute with losartan [21]. Moreover, the proportion of non-AII displaceable [^{3}H]-losartan binding sites did not vary with different concentrations of tissue (Fig. 5) and was only mildly affected by the presence of peptidase inhibitors (Table 4), suggesting that metabolic degradation of [^{3}H]losartan cannot account for the presence of the non-AII displaceable [^{3}H]losartan binding. Finally, metabolism of AII does not appear to explain the failure of AII to block [^{3}H]losartan binding, since AII incubated with the rat liver in the phosphate buffer for 3 hr was able to block more than 80% of total [^{125}I]SI AII binding.

The ratio of [^{3}H]losartan binding in the liver versus the adrenal, 3.5 to 1, is much larger than the ratio of AT_1 binding sites in rat liver and adrenal: 284 fmol/mg protein in liver [18] versus 246 fmol/mg protein (difference between [^{125}I]SI AII binding sites and [^{125}I]CGP 42112 binding sites) in the adrenal [22]. This again is consistent with the hypothesis that [^{3}H]losartan binds to a non-AII binding site in the rat liver. Similar observations of a greater density of [^{3}H]losartan binding in rat liver versus adrenal were reported by Zelezna *et al.* [16]. In view of its large size and high density of AT_1 binding sites [18], the liver probably contains the greatest number of AT_1 receptors in the body. Several possible physiological functions of AII in the liver have been proposed [23]. Thus, any consideration of the actions of losartan should take into account its ability to block liver AT_1 receptors.

The fact that such a large proportion of [^{3}H]-losartan binding in the rat liver is to a non-AII binding site also has several implications. The liver is a large organ that may sequester a large proportion of administered losartan. Losartan may have actions in the liver and other tissues in addition to its antihypertensive effects reported by Wong *et al.* [24, 25], Chiu *et al.* [26] and Carini *et al.* [27]. Experiments with brain homogenates and brain and adrenal sections for autoradiography (data not shown) confirmed the abundance of non-AII displaceable binding sites for [^{3}H]losartan in other tissues. It is also possible that the antihypertensive actions of losartan may derive from its ability to bind to these non-AII displaceable binding sites as well as act as a competitive antagonist of the AT_1 receptor subtype. Consistent with this proposition, Ohlstein *et al.* [28] suggested that the antihypertensive actions of losartan could not be accounted for solely by its ability to antagonize the actions of AII.

Identification of this non-AII binding site for [^{3}H]losartan has not yet been accomplished. Aside from close structural analogs of losartan, such as EXP 3174 and L 158,809, which contain the biphenyl tetrazole moiety, no substance tested thus far is a potent competitor for [^{3}H]losartan binding sites. Substances that do not compete, or are weak competitors for [^{3}H]losartan binding, include several neurotransmitter receptor ligands, ion channel ligands, various imidazoles and tetrazoles, and bovine serum albumin. It is possible that the [^{3}H]-losartan binding site is located on a transmitter receptor but that it binds to a part of the protein other than the transmitter ligand binding site. If allosteric effects of losartan binding do not affect the binding of the endogenous ligand, then competition binding assays may not reveal the nature of this binding protein. It is possible that this binding protein could be the AT_1 receptor, but it is unlikely that it will represent all of the non-AII binding sites, because that would mean that in addition to the AII displaceable binding site there are approximately 4 to 9 more binding sites for [^{3}H]losartan on the AT_1 receptor.

The findings in this study indicate that the suitability of [^{3}H]losartan as a radioligand for studying the AT_1 receptor is diminished by the large proportion of binding that cannot be displaced by AII.

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