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EFFECTS OF OUABAIN ON THE TISSUE DISTRIBUTION AND METABOLISM OF ^{3}H-1,2-*d*-ALDOSTERONE*

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Abstract—The effect of ouabain on the uptake of ^{3}H-1,2-*d*-aldosterone was studied in rats on a controlled Na diet at various time intervals. The homogenized tissues (heart, liver, lung, muscle, spleen and small intestine) were extracted for total dichloromethane-soluble radioactivity and the extracts chromatographed to isolate authentic ^{3}H-aldosterone. ^{3}H-aldosterone levels in blood and fat were increased by ouabain at time intervals of 15, 30 and 60 min, but in general decreased in the other tissues. Ouabain also significantly decreased both urinary and biliary excretion of ^{3}H-aldosterone. Spirolactone 3(3-oxo-17-β-hydroxy-19-nor-4androsten-17-α-yl)propionic acid γ lactone (SC-8109) did not significantly alter the dichloromethane-soluble radioactivity in the tissues of rats receiving 500 μg of SC-8109 simultaneously with ^{3}H-aldosterone.

SINCE aldosterone enhances active sodium and potassium transport in a variety of tissues and ouabain inhibits active sodium and potassium transport across cell membranes,[1–3] the possibility exists that these two steroids might be mutually antagonistic insofar as their cation transport effects are concerned. Wilbrandt and Rosenberg[4] propose that the action of ouabain may be antagonized or competitively inhibited by the presence of aldosterone. Such antagonism has been shown in erythrocyte ion transport,[5] rat aortic strip contraction,[6] frog skin potential,[7] and taenia coli smooth muscle contraction.[8] However, other investigators have not been able to demonstrate an antagonistic action between ouabain and aldosterone on ion transport in red cells.[2,9] Lefer and Sayers[10] have reported that aldosterone antagonized both the positive inotropic effect of cardiotonic concentrations and the bradycardia and cardiac arrest of toxic concentrations of ouabain in the isolated cat Lagendorff and papillary muscle preparations. Since ouabain has been proposed as an antagonist of aldosterone action, we have determined the tissue distribution and excretion of ^{3}H-1, 2-*d*-aldosterone after simultaneous administration of ouabain with the isotope.

Another group of compounds, the spirolactones, are known antagonists of aldosterone action. These compounds increase urinary excretion of sodium, chloride and water and reduce the excretion of potassium, ammonia, titratable acid and phosphate

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in normal subjects and in patients with edema.[11] Therefore, in the present study we have also determined the effect of 3(3-oxo-17-β-hydroxy-19-nor-4-androsten-17α-yl) propionic acid γ lactone (SC-8109) on the tissue distribution of aldosterone in rats.

MATERIALS AND METHODS

Holtzman strain male rats weighing 150–250 g were used in all experiments. The animals were allowed tap water and a sodium-deficient diet (Nutritional Biochemicals Corp,, Cleveland, Ohio) with 0·25 g NaCl/100 g of diet added.

Each rat received 4 μc of chromatographically pure ^{3}H-1, 2-*d*-aldosterone (New England Nuclear Corp., Boston, Mass.) with a specific activity of 87 mc/mg in 1 ml of 5% glucose intravenously. Ouabain was dissolved in 20% ethanol (400 mg/100 ml) and the dosage was 0·4 mg/100 g of body weight. The ouabain was mixed with 1 ml of ^{3}H-aldosterone at the time of injection, the total volume varying slightly with the body weight of the rat. Control rats received the ^{3}H-aldosterone and an equal volume of 20% ethanol.

At least six animals composed each 5-, 15-, 30-, and 60-min post-injection group studied. After an appropriate time interval, the rat was stunned by a blow on the head and decapitated. During exsanguination, a sample of blood was collected in a cold, heparinized extraction tube. The tissues to be used were quickly dissected, chilled, blotted, weighed, minced and homogenized in 2·5 ml of 0·25 M sucrose/g of tissue. ^{14}C-4-aldosterone was added to the homogenizer tubes before homogenization of the tissues to correct for losses.

The total homogenate was extracted and washed according to the method of Kliman and Peterson.[12] Each extract was diluted to a volume that could be conveniently divided and one-half was transferred to a counting vial to determine total dichloromethane-soluble radioactivity. The other portion was subjected to chromatography to determine the percentage of unmetabolized aldosterone.

The thin-layer chromatography method of Gerdes and Staib[13] was used to isolate aldosterone. After the third chromatographic system, the labeled aldosterone was eluted from the silica gel, transferred to counting vials, 10 ml of toluene phosphor was added, and the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. Quenching was measured by adding an internal standard of ^{3}H-toluene to each sample.

In the study on the effects of SC-8109, each rat received 500 μg of SC-8109 (G. D. Searle and Co., Des Plaines, Ill.). Singer[14] has demonstrated that this amount of SC-8109 is effective in altering the urinary Na:K ratio in rats. The spirolactone was dissolved in 1:4 ethanol–propylene glycol (200 mg/100 ml) and 0·25 ml was mixed with 1 ml of the glucose solution containing 4 μc ^{3}H-aldosterone. The same calibrated syringe was used for all injections in this group. Control rats received the labeled hormone plus 0·25 ml of 1:4 ethanol–propylene glycol.

To determine the effect of ouabain on biliary excretion of aldosterone, each rat was first anesthetized with sodium pentobarbital (5 mg/100 g body weight) and attached to a rodent respirator (Harvard Apparatus Co., Dover, Mass.). After biliary duct cannulation, a 30-min collection of bile was obtained before intravenous injection of ^{3}H-aldosterone into the tail vein. Continuous collection of bile was accomplished with samples being removed at 30-min intervals for 5·5 hr after injection. McCaa and

Sulya[15] have reported that a major portion of injected aldosterone is excreted in bile within 5 hr in normal rats.

The volume of bile for each 30-min period was recorded and 100 μl of each 30-min sample was dissolved in 0·5 ml hyamine hydroxide by allowing the mixture to stand at 25° for 24 hr. One ml ethanol and 10 ml phosphor were added to each sample, and the radioactivity and quenching were determined as described previously.

Urinary excretion of aldosterone was measured by placing a group of rats in individual stainless steel metabolism cages after i.v. injection of either ^{3}H-aldosterone plus diluent or ^{3}H-aldosterone plus ouabain and collecting the urines at 24, 48 and 72 hr. The volume of each 24 hr sample was measured, 100 μl was digested in 1 ml hyamine hydroxide for 24 hr, and 10 ml of a dioxane phosphor[15] was added. Counting was accomplished as described previously.

RESULTS

The effects of ouabain on the tissue content of ^{3}H-aldosterone and its dichloromethane-soluble metabolites are shown in Tables 1 and 2. When ouabain was injected simultaneously with ^{3}H-aldosterone, the blood levels of both the labeled hormone

TABLE 1. ^{3}H-ALDOSTERONE IN RAT TISSUES AFTER A SINGLE I.V. DOSE OF ^{3}H-ALDOSTERONE OR ^{3}H-ALDOSTERONE PLUS OUABAIN

Tissue	Treatment	Radioactivity (cpm/g × 10^{-3}*)			
		5 min	15 min	30 min	60 min
Blood	control	1251 ± 168	1052 ± 181	320 ± 32	161 ± 15
	ouabain	1898 ± 96	1494 ± 167	457 ± 40	223 ± 35
Brain	control	361 ± 80	509 ± 47	435 ± 81	160 ± 36
	ouabain	491 ± 40	354 ± 57	215 ± 34	63 ± 11
Heart	control	1506 ± 263	810 ± 124	794 ± 100	296 ± 65
	ouabain	1120 ± 95	716 ± 89	299 ± 43	220 ± 49
Kidney	control	3657 ± 458	3293 ± 524	1705 ± 210	794 ± 154
	ouabain	3372 ± 379	2058 ± 114	1223 ± 108	296 ± 68
Liver	control	954 ± 231	1333 ± 317	1143 ± 275	337 ± 48
	ouabain	50 ± 17	475 ± 151	12 ± 4	10 ± 4
Lung	control	618 ± 231	709 ± 150	349 ± 117	184 ± 37
	ouabain	451 ± 87	174 ± 59	322 ± 32	20 ± 20
Muscle	control	1416 ± 154	1343 ± 93	502 ± 57	358 ± 45
	ouabain	1423 ± 200	939 ± 59	564 ± 64	169 ± 23
Intestine	control	1038 ± 86	1410 ± 166	1338 ± 76	861 ± 97
	ouabain	1039 ± 67	570 ± 109	353 ± 44	46 ± 8
Spleen	control	1162 ± 127	1248 ± 116	749 ± 163	150 ± 30
	ouabain	1185 ± 107	639 ± 67	325 ± 30	302 ± 73

* Mean of tissues from at least six rats ± S.E.M. corrected for blood content.

and its metabolites were increased. Fifteen min after injection, all tissues, except fat, exhibited a decreased content of total CH_2Cl_2-soluble radioactivity when ouabain was administered. Levels of ^{3}H-aldosterone in all tissues examined, except heart, were significantly decreased at 15 min after injection. However, a significant decrease was observed in heart at 30 min after injection. Isolation of ^{3}H-aldosterone from fat was not feasible.

Results of the biliary excretion studies are shown in Fig. 1. Ouabain significantly reduced the biliary excretion of radiometabolites of ^{3}H-aldosterone at all time periods.

TABLE 2. TOTAL CH_2Cl_2-SOLUBLE RADIOACTIVITY IN RAT TISSUES AFTER A SINGLE I.V. DOSE OF ^3H-ALDOSTERONE OR ^3H-ALDOSTERONE PLUS OUABAIN

Tissue	Treatment	Radioactivity (cpm/g × 10^{-3})* 5 min	15 min	30 min	60 min
Blood	control	1526 ± 204	1268 ± 218	572 ± 53	360 ± 32
	ouabain	2315 ± 117	1660 ± 190	761 ± 34	506 ± 37
Brain	control	361 ± 81	579 ± 52	481 ± 92	365 ± 81
	ouabain	483 ± 43	357 ± 60	236 ± 38	167 ± 40
Heart	control	1836 ± 321	1478 ± 207	1053 ± 261	710 ± 154
	ouabain	1132 ± 102	755 ± 96	367 ± 58	223 ± 63
Kidney	control	4637 ± 471	4070 ± 647	2699 ± 675	1553 ± 302
	ouabain	3859 ± 436	3196 ± 173	1589 ± 142	789 ± 179
Liver	control	1419 ± 331	1873 ± 434	1828 ± 445	789 ± 112
	ouabain	30 ± 25	539 ± 170	77 ± 5	171 ± 60
Lung	control	899 ± 322	1303 ± 234	823 ± 254	482 ± 74
	ouabain	713 ± 116	296 ± 72	213 ± 35	94 ± 60
Muscle	control	1501 ± 163	1489 ± 104	965 ± 110	628 ± 79
	ouabain	1443 ± 204	1135 ± 72	562 ± 65	352 ± 48
Intestine	control	1640 ± 136	1884 ± 222	1881 ± 108	1657 ± 184
	ouabain	1793 ± 114	1290 ± 237	559 ± 70	301 ± 61
Spleen	control	1252 ± 140	1303 ± 124	919 ± 206	707 ± 131
	ouabain	1118 ± 109	834 ± 84	408 ± 40	262 ± 81
Fat	control	243 ± 40	241 ± 18	339 ± 16	154 ± 25
	ouabain	354 ± 38	435 ± 38	617 ± 58	708 ± 38

*Mean of tissues from at least six rats ± S.E.M. corrected for blood content.

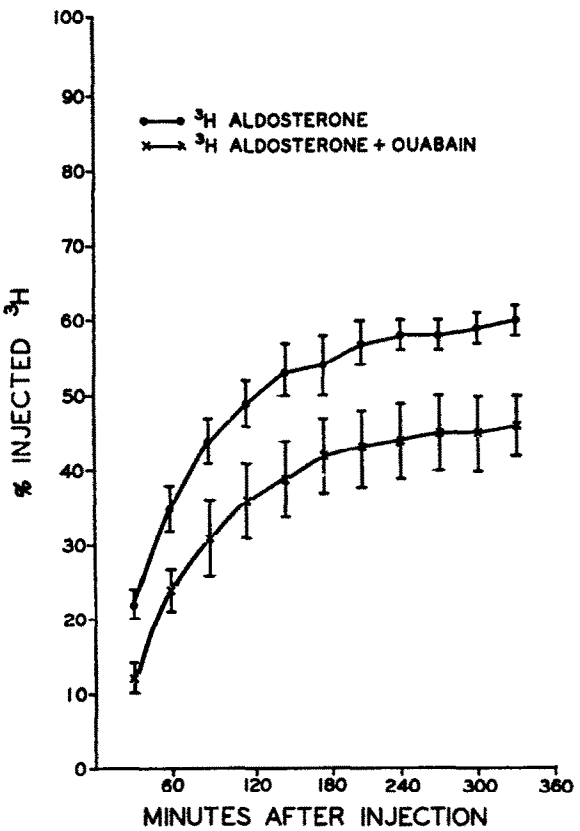

FIG. 1. Biliary excretion of ^3H-aldosterone by control and ouabain-treated rats.

TABLE 3. URINARY EXCRETION OF TRITIUM AFTER I.V. INJECTION OF ^{3}H-1,2-*d*-ALDOSTERONE OR ^{3}H-1,2-*d*-ALDOSTERONE PLUS 0·4 mg OUABAIN/100 g OF BODY WEIGHT INTO RATS

Group	Percentage of injected dose ± S.E.M. excreted			
	24 hr	48 hr	72 hr	Total ^{3}H excreted in 72 hr
Control (7)*	8·64 ± 0·55	1·03 ± 0·13	0·27 ± 0·03	9·94 ± 0·48
Ouabain (7)*	7·64 ± 0·25†	0·80 ± 0·03†	0·23 ± 0·03‡	8·70 ± 0·23§

*Number of rats per group.
†$P<0·2$.
‡$P>0·2$.
§$P<0·05$.

TABLE 4. TOTAL CH_2Cl_2-SOLUBLE RADIOACTIVITY IN RAT TISSUES AFTER A SINGLE I.V. DOSE OF ^{3}H-ALDOSTERONE OR ^{3}H-ALDOSTERONE PLUS SC-8109

Tissue	Treatment	Time after injection*		
		15 min	30 min	60 min
Blood	Control	1286 ± 172	772 ± 98	508 ± 90
	SC-8109	1339 ± 192	865 ± 123	597 ± 87
Brain	Control	536 ± 53	405 ± 62	340 ± 95
	SC-8109	547 ± 27	328 ± 97	369 ± 53
Heart	Control	1573 ± 253	1061 ± 169	691 ± 93
	SC-8109	1471 ± 246	832 ± 88	684 ± 178
Kidney	Control	3767 ± 548	2428 ± 292	1330 ± 369
	CS-8109	3613 ± 370	2004 ± 426	1199 ± 470
Liver	Control	1704 ± 530	1620 ± 434	731 ± 123
	SC-8109	1173 ± 362	1268 ± 257	595 ± 109
Lung	Control	1149 ± 286	619 ± 325	432 ± 106
	SC-8109	955 ± 269	642 ± 221	615 ± 150
Muscle	Control	1619 ± 122	968 ± 189	663 ± 129
	SC-8109	1709 ± 346	973 ± 57	753 ± 144
Small intestine	Control	1828 ± 218	1852 ± 123	1669 ± 242
	SC-8109	1425 ± 124	1743 ± 116	1615 ± 95
Spleen	Control	1379 ± 197	830 ± 225	657 ± 101
	SC-8109	1004 ± 195	544 ± 84	556 ± 69

*Values represent the mean and standard error of the mean. Each value represents the average from the tissues of at least four animals (cpm/g × 10^{-3}).

Table 3 shows the urinary excretion of radiometabolites of ^{3}H-aldosterone by control and ouabain-treated rats. Ouabain decreased the urinary excretion during 24 and 48 hr after injection. No significant difference was observed on the third day after injection. Ouabain-treated rats excreted significantly less tritium during the total 72-hr period.

Results of the study with spirolactone are shown in Table 4. Since SC-8109 did not significantly alter the total CH_2Cl_2-soluble radioactivity in tissues, no further studies were conducted.

DISCUSSION

It is evident from these studies that ouabain altered the tissue distribution of aldosterone. With a decreased tissue uptake or retention of ^{3}H-aldosterone, one

might expect to see an increase in blood radioactivity in comparison with control rats. The values for blood radioactivity in the ouabain-treated group were higher at all time periods than in the control group. The greatly increased accumulation of labeled aldosterone in the fat of ouabain-treated animals is interesting, especially since this occurs while the uptake by other tissues is significantly decreased. Parra and Reddy[16] suggested that the mechanism by which fat accumulates cortisol is a solubility phenomenon rather than the essentially physical phenomenon of protein binding. Earlier studies conducted in our laboratory[17] also indicate that the mechanism by which fat accumulates aldosterone is different from that occuring in other tissues. It has been observed that obese animals retain proportionally more steroid hormones than lean animals.[18] This retention appears to be a function of the increased fat content in the obese state. There was no difference in the nutritional status or weight of the two groups of rats used in the present study. The increased radioactivity found in the fat of the ouabain-treated group may simply represent a solubility phenomenon, assuming that the ^{3}H-aldosterone was unable to enter other tissues or was not retained by other tissues as it was in control rats.

There are several ways in which ouabain may have lowered tissue levels and increased blood levels. One or more of the following factors may be implicated: ouabain could alter the metabolism or excretion of ^{3}H-aldosterone; it may decrease tissue levels by blocking entry of the circulating hormone into the tissues, possibly by preventing binding of aldosterone at some receptor site; or release of bound aldosterone or an increase in synthesis could be induced by ouabain.

McCaa and Sulya[15] have shown that bile is the primary route of excretion of aldosterone in the rat. Ouabain significantly decreased the biliary excretion of aldosterone and its metabolites as well as the urinary excretion of these substances in 72 hr. This decrease in biliary excretion is probably related to the fact that the bile is also the primary route for elimination of ouabain in the rat.[19] Farah's[19] work also demonstrated that rat liver does not metabolize ouabain, but excretes it via bile primarily unchanged. He showed that 1 g of rat liver combined with 0·24 mg ouabain. We did not observe any consistent effect of ouabain in the percentage of the total CH_2Cl_2-soluble radioactivity which was present as ^{3}H-aldosterone.

Since ouabain decreased the excretion of ^{3}H-aldosterone and its metabolites in bile and urine, one would expect to see an increase in the radioactivity present in the tissues and blood. Instead, we found a decreased amount of tritium in the tissue other than fat along with an elevated level of tritium in blood after 15 min. We interpret this to indicate that, in addition to altering the excretion of ^{3}H-aldosterone, ouabain in some manner prevents binding of aldosterone at the tissue level. A study of the uptake of ^{3}H-ouabain by tissues of the rat has been recently reported.[20] It is interesting to note that the tissues showing the highest uptake of ^{3}H-ouabain at 15 min (heart, liver, kidney and muscle) are among the tissues in our experiment which showed a statistically significant decrease in the level of ^{3}H-aldosterone (heart, kidney, liver, lung, muscle, small intestine and spleen). The uptake of ^{3}H-ouabain by lung and spleen was not remarkable and that present in the small intestine was not reported. The uptake of ^{3}H-ouabain by fat was among the lowest at 15 min, but was exceeded only by pituitary in percentage dose per 100 g at the end of 6 hr.

It is tempting to speculate that the effects of ouabain in aldosterone binding are, at least in part, due to antagonistic action. No work has ever been presented which

demonstrates that ouabain enters the cell. There is evidence that ouabain binds to the cell membrane. Gardos[21] has reported that ouabain is strongly bound to erythrocyte ghosts and cannot be removed by washing. Csaky[22] has presented evidence that ouabain alters the cell membrane. He showed that it inhibits the intestinal transport not only of electrolytes but also of some nonelectrolytes such as 3-methyl glucose, phenylalanine, tyrosine and uracil. Csaky proposed that ouabain combines with the cell membrane, altering its structure, permeability and enzymatic properties. Willmer[23] suggests that pharmacological action of ouabain may depend on binding or displacing other steroids rather than on inserting itself physiologically into the membrane. If ouabain does alter the cell membrane, as suggested by Willmer, then it may inhibit the normal distribution of aldosterone in the membrane and tissues. This mechanism of action may explain the unilateral natriuretic action of 0·5 mg ouabain when injected directly into the left renal artery of the dog.[24] In these experiments maximum unilateral diuresis occurred approximately 1 hr after injection and the diuresis continued for more than 3 hr. Ouabain produced no change in glomerular filtration rate, effective renal plasma flow or renal blood flow. In dogs hydrated with isotonic saline, these workers[24] demonstrated that desoxycorticosterone, injected into the same renal artery as ouabain, suppressed the natriuretic effect of ouabain to some extent. If the dogs were pretreated with the aldosterone antagonist, SC-9420, then ouabain exerted its full effect on the kidney. A further possible indication of antagonism between ouabain and naturally occurring steroids is the report of Kunz and Wilbrandt[25] that the strophanthin-induced potassium loss in the perfused guinea pig heart was antagonized by the adrenal cortical steroids, cortisol, corticosterone, aldosterone and desoxycorticosterone, and also by progesterone and testosterone.

In contrast to the firm binding of ouabain, available data indicate a weak binding of aldosterone to membranes and subcellular fractions.[26] These studies by equilibrium fractionation and equilibrium dialysis showed that significant binding of aldosterone occurred with nuclear, microsomal or supernatant fractions of kidney. Not only the subcellular fractions already noted, but also cell membranes of liver and kidney tissues, bound aldosterone with less affinity than either cortisol or progesterone. However, aldosterone bound to the kidney cell membrane was not displaced by the aldosterone antagonist, SC-9420. These investigators had previously shown[27] that the same concentration of this antagonist would displace aldosterone from serum proteins.

If an antagonism occurs at the membrane between aldosterone and ouabain, the most likely locus of this action is on sodium transport and Na^+, K^+-ATPase. This enzyme is the only one known that is sensitive to cardiac glycosides.[28] Sharp and Leaf[29] have reported that ouabain will inhibit the stimulation of sodium transport induced by aldosterone in toad bladder without interfering with the basal rate of sodium transport. The Na^+, K^+-ATPase level of rat kidney is reduced after adrenalectomy,[30,31] but administration of aldosterone in concentrations that restore salt retention does not restore ATPase activity. However, corticosterone in physiological doses does return the enzyme level to normal in 2–3 days. Aldactone, an aldosterone antagonist, will also lower the activity of this enzyme in dialyzed endoplasmic membranes.[32] Landon *et al.*[32] reported that the enzyme activity in this instance was restored by addition of EDTA, indicating that an inhibitory effect is exerted by Ca^{++}. They found that the enzyme activity in adrenalectomized rats was restored to control levels by treatment with aldosterone, desoxycorticosterone or triamcinolone.

Another possible explanation may in part account for our observed results. Tsao and King[33] have found that ouabain exerts a great stimulatory effect on the 11-hydroxylation system in adrenocortical mitochondria. They studied the hydroxylation of desoxycortisol to hydrocortisone and found that ouabain was the only cardiac glycoside of several studied which was stimulatory. In the rat the principal glucocorticoid is corticosterone rather than hydrocortisone, so such stimulatory activity would be expected to increase corticosterone and possibly aldosterone biosynthesis. A greatly increased level of unlabeled aldosterone in blood could displace the labeled material.

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