PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 169, 380–385 (1982)

Free *myo*-Inositol in Canine Kidneys: Selective Concentration in the Renal Medulla[1] (41361)

MARK A. H. COHEN, KEITH A. HRUSKA, AND WILLIAM H. DAUGHADAY

Metabolism and Renal Divisions, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. The concentration of free and lipid-bound *myo*-inositol (MI) was measured in outer and inner cortical zones and in outer and inner medullary zones of the dog kidney. Lipid-bound MI was highest in the outer cortex (0.86 μmole/g wet wt) and decreased progressively to the inner medulla (0.28 μmole/g). In contrast free MI was highest in the outer medulla (18.4 ± 2.2 μmole/g wet wt) as compared to the cortical zones (3.45 ± 0.19 and 5.30 ± 0.33 μmole/g in outer and inner zones, respectively). The concentration ratio of free MI in outer medullary zone cellular water to plasma water was in excess of 1000. We cannot attribute this high MI concentration to tubular transport because this transport system is present in cortical zone proximal tubules. Water diuresis did not deplete outer medullary MI significantly which suggests that the high medullary MI is independent of the medullary countercurrent mechanism.

Free *myo*-inositol (MI) is present in most cells in concentrations that greatly exceed that found in serum (1–5) (Table I). While it would be reasonable to attribute this intracellular concentration of MI to active membrane transport, this has been difficult to establish in most tissues. Active transport of MI is known only to occur in the intestinal mucosa (6), choroid plexus (7), and kidney (8) and possibly in brain (9). Such transport has not been demonstrable in liver (10) and skeletal muscle (5).

The kidney is an organ of special importance in MI metabolism. It possesses a sodium-linked specific active transport system in the proximal tubule brush border membrane (8) which normally leads to reabsorption of nearly all the MI filtered by the glomerulus. In addition, the kidney is the only significant organ in which MI breakdown occurs within the mammalian organism (11).

Because of the unique role of the kidney in MI metabolism, the present study was originally undertaken to determine whether or not the kidney cortex, which is engaged in active transport of MI, contains higher concentrations of free MI than the renal medulla. When the zone of greatest MI concentration was found to be in the outer medullary zone rather than in the cortical zones, we examined the effect of water diuresis on the MI concentration.

Methods. *Materials.* MI and α-methyl-D-mannopyranoside were purchased from Sigma Chemical Company, St. Louis, Missouri. Nembutal was purchased from Abbott Laboratories, North Chicago, Illinois, and 3% SE-30 on 100/120 mesh Gas-Chrom Q from Applied Science, State College, Pennsylvania.

Protocol of study. Mongrel dogs (six control and four water-loaded) weighing 14–18 kg were fed standard dog chow and water *ad libitum* before the experiment. Unilateral nephrectomy was performed on anesthetized animals. In some experiments unanesthetized dogs were given an intragastric administration of water amounting to 5% of body weight. After 2 hr and 25 min the dogs were anesthetized and a kidney removed.

The kidneys were placed on ice and perfused through the renal artery with 100–150 ml of ice-cold 0.90% saline solution. With

[1] This work was supported in part by NIAMDD Program Project AMO 9976, Diabetes Research and Training Center Grant AM 20579 from the National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Md., and also by American Heart Association Grant in Aid 80-721.

0037-9727/82/030380-06$01.00/0

TABLE I. REPRESENTATIVE TISSUE LEVELS OF *myo*-INOSITOL

Tissue	Species	MI (μmole/g)	Tissue MI / Plasma MI	Ref. No.
Plasma	Dog	0.025		[a]
	Rabbit	0.042		(2)
	Rat	0.063		(2)
Reproductive organs				
Seminal vesicles	Rat	35.2	558	(1)
Epididymis	Rat	11.9	184	(1)
Testis	Rabbit	5.7	136	(3)
Nervous system				
Cerebral cortex	Rabbit	13.4	319	(2)
CSF	Rabbit	0.47	11	(3)
Endocrine glands				
Pituitary	Rabbit	23.4	557	(2)
Thyroid	Rabbit	12.6	300	(2)
Viscera				
Spleen	Rabbit	5.64	134	(3)
Pancreas	Rabbit	2.77	65	(3)
Liver	Rat	0.57	9.0	(4)
Kidney				
Whole	Rat	4.51	72	(4)
Muscle				
Skeletal	Rat	0.18	2.85	(5)

[a] Determined in this laboratory.

the kidney still on ice, the renal capsule was removed and a median longitudinal slice with a thickness no greater than 0.4 cm was cut. The inner and outer cortical and medullary section were identified visually and separated. Each isolated section was blotted, frozen in liquid nitrogen, and placed in a vial standing in powdered dry ice. The samples were subsequently stored at −70°.

To measure the dry weight of the tissue, the stored tissue was taken to a −20° cold room. There, in two cases, single, otherwise duplicate or triplicate frozen samples were cut on dry ice, weighed, placed in tared vials that had been preheated for at least 24 hr in a 110° oven, and then the vials and samples were put back in the oven until constant weight was reached; in most cases, it took 48 hr.

Tissue sodium, urea, and MI assay. Frozen sections of kidney, with an average weight of 93.4 mg, were sliced and weighed as in the dry weight assay. They were added to 1.5 ml of ice-cold 0.65 *N* perchloric acid with 3.23 ng/ml of α-methyl-D-mannopyranoside as an internal standard and immediately homogenized in a Polytron (Brinkman Instruments, Inc., Westbury, N.Y.) with a PT10ST blade at a setting of 8 for 45 sec, and then put in ice water until all samples were prepared. The homogenate was centrifuged at 20,000*g* for 12 min in a refrigerated International centrifuge. The pellets from two kidneys were stored at −70° for lipid MI assay. One milliliter of supernate was neutralized with KOH–imidazole KCl buffer (12), to a pH of 6–7 and recentrifuged.

Two hundred microliters of the neutralized supernate was analyzed for sodium and urea. Sodium levels were determined by flame photometry (Model 143, Instrumentation Laboratory, Inc., Lexington, Mass.). Urea was measured on a Beckman BUN Analyzer 2.

Calculation of the concentration of micromoles MI per gram H_2O and per gram intracellular H_2O in the basal kidneys was based on the mean percentage water content for four kidneys. Calculations were based on the sodium, urea, H_2O, and MI levels determined for that kidney.

For the calculation of the intracellular concentration of MI, the values of the inulin space in dog kidneys by deJairala *et al.* (13) were used. The level of intracellular water was considered to be the difference between the percentage composition of water in the kidney and the percentage level of inulin space. It was assumed that the levels of MI in the extracellular space were the same as serum levels. (This, indeed, is probably an overestimate because the saline perfusion most likely reduced the extracellular concentration of MI.) Since the tissue levels of MI are so much greater than the serum levels, the extracellular concentration of MI was negligible.

Duplicate samples of 400 μl of the neutralized supernate were prepared for gas–liquid chromatography as described by Molitoris *et al.* (14) except that 200 μl of silylation reagent were used instead of 100 μl. Gas chromatography of samples from two of the basal kidneys was carried out by the method of Hipps *et al.* (15) as modified by Molitoris *et al.* (14). The remaining samples were analyzed on a Hewlett–Packard 5880A gas–liquid chromatograph, with nitrogen at a flow rate of 50 ml/min as the carrier gas on 3% SE-30, on 100/120 mesh Gas-Chrom Q. The area of the MI derivative peak was determined by means of a Hewlett–Packard Model 5880A Level 4 Integrator (Hewlett–Packard Co., Palo Alto, Calif.). The recovery of α-methyl-D-mannopyranoside derivative was measured by peak height instead of area due to a slight overlap of a small preceding peak.

Lipid MI assay. The extraction was a modification of the method of Palmano *et al.* (4): the pellets after PCA precipitation and centrifugation were twice extracted at room temperature with 4 ml of a $CHCl_3$/MeOH/concentrated HCl solution (500:500:3). The phases were separated with 0.8 ml of 1 *N* HCl with 30 m*M* $CaCl_2$. The organic phases were combined and dried under N_2. The lipid was resuspended in 6 ml of $CHCl_3$/MeOH (1:1). Duplicate samples of 1 ml of this solution were placed in an ampule; the solvent was evaporated under a flow of N_2; 1 ml of 6 *M* HCl was added; and the ampules were sealed *in vacuo*. The samples were hydrolyzed for 20 hr at 120°. Following the hydrolysis the ampules were allowed to cool to room temperature and then broken. The HCl was removed by vacuum desiccation over NaOH pellets for 3 hr. Forty microliters of 0.1 mg/ml α-methyl mannoside solution were added to the ampule followed by lyophilization. The samples were transferred to silylation vials by three washings with 1 ml of H_2O. Next, the samples were lyophilized, silylated, and then assayed for MI by the method of Hipps *et al.* (15) as modified by Molitoris *et al.* (14).

Results are presented as mean ± SE. The significance of differences between means was evaluated by Student's *t* test for paired data. A *P* value less than 0.05 was considered significant.

Results. The mean sodium and urea concentrations were similar in the outer and inner cortex and rose successively in the outer and inner medulla (Table II). Little change in water content was observed. In the basal kidneys the outer medullary sodium concentration was 42% greater and the inner medullary sodium concentration was 115% greater than mean cortical concentrations. These results confirm an earlier report by others (16). The increase in urea concentrations in the medullary zones was greater than that observed for sodium. As a manifestation of the medullary countercurrent exchange, outer medullary concentration of urea increased to 91 mmole/kg H_2O, over 5-fold that of the cortex, and inner levels over 18-fold to 330 mmole/kg H_2O.

The mean concentration of free MI was lowest in the outer cortical zone and rose 50% in the inner cortical zone. The outer medullary zone had a mean MI concentration of 18.4 μmole MI/g wet wt, which was 430% greater than in the outer cortical level. The mean concentration of MI in the

TABLE II. SODIUM, UREA, AND FREE AND LIPID-*myo*-INOSITOL LEVELS IN DOG KIDNEY ZONES BEFORE AND AFTER WATER DIURESIS

	Na (meq/liter)	Urea (m*M*)	H_2O (%)	Free MI (μmole/g wet wt)	Free MI tissue / Free Mi plasma[b]	Lipid MI (μmole/g wet wt) (n = 2)
			Basal			
1. Outer cortex	131 ± 13[a]	14 ± 3	80.6 ± 0.3	3.45 ± 0.19	138	0.861
2. Inner cortex	117 ± 11	17 ± 3	80.2 ± 0.5	5.30 ± 0.33	212	0.843
3. Outer medulla	176 ± 7	91 ± 7	86.1 ± 1.0	18.4 ± 2.2	732	0.386
4. Inner medulla	267 ± 20	330 ± 50	87.7 ± 1.5	14.1 ± 0.4	564	0.277
			Water diuresis			
5. Outer cortex	114 ± 6	8.2 ± 3.1	80.4 ± 0.7	3.38 ± 0.18	135	
6. Inner cortex	123 ± 10	6.2 ± 2.5	82.2 ± 2.5	5.20 ± 0.43	208	
7. Outer medulla	142 ± 9	26 ± 5	85.5 ± 1.3	16.2 ± 1.2	648	
8. Inner medulla	165 ± 16	50 ± 10	87.3 ± 0.5	9.12 ± 0.80	366	
			Statistical comparisons (*P < 0.05; **P < 0.01)			
	3 vs 7 *	3 vs 7 **		1 < 2 *		
	4 vs 8 **	4 vs 8 **		2 < 3 **		
				2 < 4 **		
				8 < 4 **		
				5 < 6 **		
				6 < 7 **		
				6 < 8 **		
				8 < 7 **		

[a] Values given are mean ± SE; n = 4, except where indicated.
[b] Calculated on basis of normal dog plasma determined in our laboratory as 0.025 m*M*.

inner medullary zone was lower than in the outer medullary zone but the difference was not significant.

In order to determine whether the medullary concentration of MI was linked to the renal medullary countercurrent exchange mechanism, four dogs were studied after water diuresis. Water loading resulted in a marked decrease in medullary sodium and urea concentrations. The sodium concentration dropped to 81 and 62% of basal levels in the outer and inner medulla (P < 0.05 and P < 0.005). The outer and inner medullary urea concentrations in the kidney after water diuresis were, respectively, 28% (P < 0.005) and 15% (P < 0.01) of the basal levels. Using 2[Na] + [urea] as an estimate of osmolality, water loading lowered the mean inner medullary osmolality from 864 to 380 osm/kg H_2O. The inner medullary levels of Na and urea after water loading are close to those found by Levitin *et al.* (17) in dog kidneys 2 hr after water loading.

Water diuresis produced no significant change in MI concentrations in most of the kidney. Water diuresis lowered outer medullary concentration MI concentration by 12% but this change was not significant. Water diuresis lowered mean MI concentration in the inner medullary zone by 36%. This decrease was significant (P < 0.005). As in the basal kidneys, the difference between the MI levels of the two cortical zones after diuresis remained significant (P < 0.005).

In two kidneys of control dogs, the concentration of lipid-MI levels were two to three times higher in cortical than medullary zones. There was little difference between levels of the inner and outer cortex, and inner and outer medulla.

Discussion. Previous workers have measured MI either in renal cortex (18) or in whole kidney (4) and so have not recognized the high concentration we report to be present in medullary zones. We found the mean concentration in the outer

medullary zone to be 18.4 μmole/g wet wt which was five times that in the outer cortical zone. Most of the MI in the outer medulla must be intracellular because the plasma concentration of MI is less than 1/500th that found in the outer medullary zone. In addition, the kidneys were perfused with saline which removed most of the contained plasma.

We have calculated the molar concentrations of MI per gram of H_2O and per gram of intracellular H_2O in the kidney. Before water loading values ranged from 4.28 ± 0.24 μmole per gram of H_2O and 5.89 ± 0.33 μmole per intracellular gram of H_2O in the outer cortex to 21.2 ± 2.7 μmole per gram of H_2O and 30.1 ± 3.9 μmole per intracellular gram of H_2O in the outer medullary zone. The latter value indicates that the cellular concentration of MI may be over 1000-fold that of plasma if intracellular MI existed in free solution. In the water-loaded kidneys mean MI concentrations varied from 4.24 ± 0.23 μmole MI per gram of H_2O and 5.79 ± 0.34 μmole MI per gram of intracellular H_2O in the outer cortex to 19.0 μmole MI per gram of H_2O and 27.0 ± 2.0 μmole MI per gram of intracellular H_2O in the outer medulla.

The concentration of MI in the outer medullary zone of the kidney is among the highest reported levels of MI for any tissue. Only rat seminal vesicle (1) and the rabbit pituitary (2) have greater MI concentrations (see Table I). However, due to the relatively low plasma MI level in dogs, the outer renal medullary zone has the largest known tissue-to-plasma MI gradient. The kidney before water loading has tissue-to-plasma MI values 31% greater than that of rat seminal vesicle, the tissue with the second highest gradient.

The discovery of this very high free MI concentration in the outer medullary zone was unexpected. We had anticipated that the greatest concentration would be in the cortex where the proximal tubular segments are located because that portion of the tubule contains the MI transport mechanism (8). Glucose which also is transported in the proximal tubule has its highest concentration in the renal cortex (19).

What other explanations can be offered for the remarkable concentration of MI in the outer medullary zone? One possibility that we have considered is that the concentration of MI in this zone is linked to the medullary osmotic gradient in a similar manner to that which leads to the medullary concentration of urea. Against this possibility is the fact that the greatest mean concentration was not observed in the inner medullary zone but in the outer medullary zone. Much more importantly, water diuresis greatly reduced medullary urea concentration without significant change in outer medullary MI concentration. Water diuresis did decrease inner medullary MI significantly so that countercurrent concentration mechanisms may have been partially responsible for the MI concentration in this zone.

A third possibility that we have considered is that MI was being synthesized in the renal medulla and that the cells were relatively impermeable to MI. While such a mechanism may explain the high concentration of MI in seminal fluid (20), no evidence exists for such permeability barriers. All previous cell studies show a relatively rapid exchange of intra- and extracellular MI and increased synthesis would not be expected to sustain a concentration gradient of this magnitude (5, 9, 10).

Last, we have considered that MI may enter renal medullary cells by a process not involving active transport. Maintenance of increased intracellular concentration of MI would in this model require binding by some intracellular component. Precedent for such a mechanism of maintaining the intracellular concentration of MI appears to exist in epitrochlearis muscle of the rat. Molitoris *et al.* (5) could find no evidence of active transport of MI, although MI rapidly entered and left the cell. In this tissue, the intracellular to extracellular MI gradient of fourfold was attributed to intracellular binding of MI by an undetermined cellular component. If such a mechanism is responsible for the MI concentration in the outer medulla, it should be relatively easy to determine the existence of the putative MI binding component in the outer medulla.

Measurements of MI present in phospholipids in the perchloric acid precipitate do not parallel those of free MI. Although this fraction may not have contained all the lipid inositol, the highest concentration was noted in the outer cortex with much lower concentrations in the medulla. As lipid-associated MI is a relatively constant constituent of plasma membrane, the lower lipid MI in the medullary zones can probably be attributed to decreased cellularity and increased extracellular fluid of the medulla as compared to the cortex.

Histologically, the outer medulla is distinguished from the inner medulla by the presence of thick ascending limbs of Loops of Henle. Considered part of the distal tubule, the thick ascending limbs have low water permeability and are the site of active transtubular chloride transport, the energy input for countercurrent multiplication in the renal medulla. The thick ascending limb extends from the medulla into the cortex; if the thick ascending limb were the source of marked MI concentration in the outer medullary zone, then its extension into the cortex could explain the higher levels of MI found in the inner cortex compared to the outer cortex.

1. Eisenberg Jr F, Bolden AH. Reproductive tract as site of synthesis and secretion of inositol in the male rat. Nature (London) 202:599–600, 1964.
2. Dawson RMC, Freinkel N. The distribution of free meso-inositol in mammalian tissues, including some observations on the lactating rat. Biochem J 78:606–610, 1961.
3. Sherman WR, Stewart MA, Kurien MM, Goodwin SL. The measurement of *myo*-inosose-2 and *scyllo*-inositol in mammalian tissues. Biochim Biophys Acta 158:197–205, 1968.
4. Palmano KP, Whiting PH, Hawthorne JN. Free and lipid *myo*-inositol in tissues from rats with acute and less severe streptozotocin-induced diabetes. Biochem J 167:229–235, 1977.
5. Molitoris BA, Karl IE, Daughaday WH. Concentration of *myo*-inositol in skeletal muscle of the rat occurs without active transport. J Clin Invest 65:783–788, 1980.
6. Caspary WF, Crane RK. Active transport of *myo*-inositol and its relation to the sugar transport system in hamster small intestine. Biochim Biophys Acta 203:308–316, 1970.
7. Spector R, Lorenzo AV. *Myo*-inositol transport in the central nervous system. Amer J Physiol 228:1510–1518, 1975.
8. Hammerman MR, Sacktor B, Daughaday WH. *Myo*-inositol transport in renal brush border vesicles and its inhibition by D-glucose. Amer J Physiol 239:F113–F120, 1980.
9. Warfield A, Hwang SM, Segal S. On the uptake of inositol by rat brain synaptosomes. J Neurochem 31:957–960, 1978.
10. Chen CP, Vu VT. *Myo*-inositol uptake by rat hepatocytes *in vitro*. J Pharm Sci 68:1022–1024, 1979.
11. Howard Jr CF, Anderson L. Metabolism of *myo*-inositol in animals. II. Complete catabolism of *myo*-inositol-^{14}C by rat kidney slices. Arch Biochem Biophys 118:332–339, 1967.
12. Lowry OH, Passonneau JV. A Flexible System of Enzymatic Analysis. New York, Academic Press, p124, 1972.
13. de Jairala S, Vieyra A, Garcia AP, Rasia ML. Kinetics of insulin penetration in cortex and medullary kidney slices. Canad J Physiol Pharmacol 51:511–515, 1973.
14. Molitoris BA, Hruska KA, Fishman N, Daughaday WH. Effects of glucose and parathyroid hormone on the renal handling of myoinositol by isolated perfused dog kidneys. J Clin Invest 63:1110–1118, 1979.
15. Hipps PO, Sehgal RK, Holland WH, Sherman WR. Identification and partial characterization of inositol:NAD$^+$ epimerase and inosose:NAD(P)H reductase from the fat body of the American cockroach, *periplaneta americana* L. Biochemistry 12:4705–4712, 1973.
16. Malvin RL, Wilde WS. Washout of renal countercurrent Na gradient by osmotic diuresis. Amer J Physiol 197:177–180, 1959.
17. Levitin H, Goodman A, Pigeon G, Epstein FH. Composition of the renal medulla during water diuresis. J Clin Invest 41:1145–1151, 1962.
18. Hauser G, Finelli VN. The biosynthesis of free and phosphatide *myo*-inositol from glucose by mammalian tissue slices. Biochim Biophys Acta 238:3224–3228, 1963.
19. Needleman P, Passonneau JV, Lowry OH. Distribution of glucose and related metabolites in rat kidney. Amer J Physiol 215:655–659, 1968.
20. Robinson R, Fritz IB. *Myo*-inositol biosynthesis by Sertoli cells, and levels of *myo*-inositol biosynthetic enzymes in testis and epididymis. Canad J Biochem 57:962–967, 1979.

Received July 22, 1981. P.S.E.B.M. 1982, Vol. 169.