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EFFECT OF ANTIDIURESIS ON RENAL CREATINE METABOLISM

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The current work investigates whether creatine metabolism is involved in renal adaptation to dehydration. Wistar rats were either deprived of water or induced to drink water abundantly during 60 h. Cortical and medullar mRNA levels of Na^+/Cl^-/creatine transporter (CRT), l-arginine: glycine amidinotransferase (AGAT), guanidinoacetate methyltransferase (GAMT) and of the tonicity sensitive genes coding for aquaporin 2, Na^+/Cl^-/betaine transporter and glucocorticoid-inducible kinase were measured by real-time PCR assays. The activity of the CRT and that of Na^+/α-methyl-glucose transporter were evaluated in renal brush-border membrane vesicles. In water loaded animals, the mRNA levels of AGAT and CRT, and the activity of the CRT were greater in the cortex than in the medulla. GAMT mRNA levels were of similar magnitude and lower than those of AGAT mRNA. Dehydration decreased cortical and medullar AGAT and CRT mRNA levels and CRT activity and it did no affect GAMT mRNA abundance. These decreases were creatine specific because dehydration increased Na^+/α-methyl-glucose transporter activity and the mRNA abundance of aquaporin 2, Na^+/Cl^-/betaine transporter and glucocorticoid-inducible kinase. In conclusion, this is the first report showing that: i) the kidneys express significant amounts of GAMT mRNA, ii) dehydration down-regulates the expression of AGAT gene and iii) dehydration down-regulates CRT gene expression and activity.

Key words: *creatine transporter, l-arginine: glycine amidinotransferase, guanidinoacetate methyltransferase, dehydration, kidney*

INTRODUCTION

The cells of the renal medulla are exposed to variably high levels of NaCl and urea, especially during antidiuresis. Cells regulate their volume by adjusting cytosolic solute content to produce an osmotically driven gain of water. Cell solute content is increased by a variety of mechanisms, including ion transport and accumulation of organic osmolytes. Organic osmolytes accumulation does not perturb normal cell function, allowing cell survival during severe hypertonic stress (1-5). Osmolytes accumulation originates from increased metabolic production and/or up-regulation of membrane transport systems, such Na^+- and HCl^--dependent organic solute transporters (*e.g.* Na^+/Cl^-/betaine transporter) (4).

The best known function of creatine is its role in energy metabolism. Recently, Alfieri *et al*. (6) reported that following hypertonic stress C2C12 muscle cells accumulate creatine as a compatible osmolyte, by increasing both the activity and the mRNA levels of the Na^+/Cl^-/creatine transporter (CRT). Levillain *et al*. (7) found that 48 h water deprivation increased the content of creatine and other guanidino compounds in rat kidneys, but they concluded that the increases would hardly contribute to cell osmolality.

The kidneys effectively reabsorb the filtered creatine *via* the CRT located at the apical membrane of the renal epithelia (8) and they synthesize creatine (9).

De novo creatine biosynthesis involves two enzymes. The first enzyme is l-arginine: glycine amidinotransferase (AGAT) and the second guanidinoacetate methyltransferase (GAMT). The regulation of creatine biosynthesis occurs at the level of AGAT (10-13), and creatine represses renal AGAT mRNA expression and activity regulating in this manner its own biosynthesis (10-14). The kidneys of mammals express high activities of AGAT but low (or undetectable) activities of GAMT (9).

This work investigates whether body hydration status affects renal creatine metabolism, by measuring the mRNA levels of AGAT, GAMT and CRT, and the activity of CRT in the renal cortex and medulla of Wistar rats in two hydration states, namely, after 2.5 days of either dehydration or water overloading. The mRNA levels of the well-established tonicity sensitive genes coding for aquaporin 2 (15, 16), Na^+/Cl^-/betaine transporter (4) and glucocorticoid-inducible kinase (17) were determined for comparison. To test whether another apical Na^+-dependent organic solute transporter behaves as CRT in response to dehydration, Na^+/α-methyl-glucose transporter activity was also evaluated.

MATERIALS AND METHODS

Substances

^{14}[C]-creatine was purchased from American Radiolabel Chemicals, Inc. and α^{14}[C]-methyl-glucose from GE Healthcare Europe GmbH. Other reagents were obtained from Sigma Chemical Co., Madrid, Spain.

Animals

One month-old male Wistar rats (90-100 g) were kept in metabolic cages for 2.5 days for animals to become acclimated before the start of treatments. The rats were fed with a rat chow diet (Panlab 04) *ad libitum* and they were either deprived of water or induced to drink water abundantly by adding sucrose (20 g/100 ml) to their drinking water during a further 2.5 days. The duration of the treatment was chosen based in our previous report (16), which used 2.5 days treatment, a time period between the 2 days used by Terashima *et al*. (18) and the 3 days used by Swenson *et al*. (19) and Cha *et al*. (20).

Food and water intake were measured as the difference between the food and water given at the beginning of the treatment and those left at the end, respectively.

Rats were sacrificed with a lethal intra-peritoneal injection of pentobarbital (50 mg/kg). The animal experimentation was conducted in accordance with national/local ethical guidelines.

Plasma and urine parameters

Urine and blood were collected at the end of the treatment and its volume and osmolality monitored. Blood samples were taken from the heart and collected on EDTA tubes, which were centrifuged immediately at 4°C. Urine and plasma osmolality were measured using a freezing point depression micro-osmometer (Osmometer Gonotec, Osmomat 030).

Preparation of the tissue

The kidneys were removed and rinsed with ice-cold saline solution. The cortex and medulla were dissected and immediately dropped into liquid nitrogen and kept frozen at -80°C until use.

Solute uptake studies into brush-border membrane vesicles (BBMV)

BBMV were isolated from the kidney cortex and medulla by $MgCl_2$ precipitation, following the method of Biber *et al.* (21) as described (8). Unless otherwise stated, the BBMV were loaded with a pH 7.5 buffer consisting of, in mM, 200 mannitol, 50 Kgluconate and 50 HEPES-Tris. Protein was measured by the method of Bradford (22), using γ-globulin as the standard.

^{14}[C]-creatine and α-^{14}[C]-methyl-glucose uptake were measured at 25°C by a rapid filtration technique, as described (23). Except where indicated otherwise, the uptake buffer consisted of (in mM): 100 mannitol, 100 NaCl, 50 HEPES-Tris (pH 7.5), 0.025 valinomycin and 5 µM of either [^{14}C]-creatine or α-^{14}[C]- methyl-glucose. Valinomycin was dissolved in DMSO (0.5% v/v) and the vehicle alone had no effect on substrate transport measurements. The amount of protein in the assay tube ranged from 100 to 150 µg/100 µl of uptake buffer. All experiments were done in triplicate.

Relative quantification of real-time PCR

Total RNA was extracted from the renal cortex and medulla of rats either deprived of water or induced to drink water abundantly during 60 h, using RNeasy® kit (Qiagen). Real-time PCR was performed as described (23). Analysis confirmed a single PCR product at the predicted melting temperature. Primers for the genes tested are given in *Table 1*. β-actin was used as reference gene for samples normalization.

Statistical analyses

Data are presented as means±SEM. for *n* separate animals. In the *Figures*, the vertical bars representing the SEM are omitted when smaller than symbol size. Comparison between different experimental groups was evaluated by the two-tailed Student's t-test. Differences were set to be significant for $p<0.05$.

RESULTS

Effect of water intake on several body parameters

Table 2 shows that the addition of sucrose to the drinking water increased water intake. Water deprivation significantly reduced food intake and body weight, whereas these parameters were not significantly affected by water overloading.

Urine osmolality and volume were inversely related (*Table 2*) and could be used as good indicators of the effectiveness of the treatments. The values indicate that the control animals have a hydration state intermediate between water deprived and water overloaded animals. Compared with control condition, the osmolality of the urine either increased or decreased depending on whether the animal was water deprived or overloaded, respectively.

Water intake and renal gene expression

In water loaded animals, the mRNA levels of the tonicity sensitive genes coding for Na^+/Cl^-/betaine transporter and aquaporin 2 were greater in the medulla than in the cortex (*Fig. 1*) and, as expected, they were significantly increased following 60 h water deprivation. Water deprivation significantly increased

Table 1. Oligonucleotides used in this study

Gene	Accesion number	Antisense (5' …3')	Sense (5' …3')
CRT	NM017348	TTCTATTACCTGGTCAAGTCCT	GCCTCAAGACTTTGTTCTCC
AGAT	NM031031	CCTGTCTCCTCTTACAACGA	TTCATATGTATTGGCCTTCACC
GAMT	NM012793	GCCTATGATACGTCTGACAC	CCATCGTTGCATTCAATAATCC
BGT1	NM017335	CAGTCCATGAAGATGGGTGTC	GGAAGAACACTGGGATACCAC
AQP2	NM012909	CCATCCTCCATGAGATTACTCC	CCAGGGTAACAGAGAAACCA
SGK1	NM019232	CAGAAGCTTGCCAACAACTC	TTGTGCCTTGCTAGAAGAACC
β-Actin	NM031144	ACCCACACTGTGCCCATCTA	CGGAACCGCTCATTGCC

Oligonucleotides were chosen according to the rat cDNA sequences entered in Genbank and designed using PerlPrimer program. BGT1, Na^+/Cl^-/betaine transporter; AQP2, aquaporin 2 and SGK1, glucocorticoid-inducible kinase .

Table 2. Food and water intake, body weight, plasma and urine osmolality and volume of urine of rats maintained under different water intake conditions.

Animal Groups	Water intake	Food intake	Body weight	Urine		Plasma
	mL/24h	g/60h	g	mOsm/Kg	mL/24h	mOsm/Kg
Control (n=6)	28.8 ± 1.1	30.3 ± 0.8	106.3 ± 0.5	1,440 ± 15	6.3 ± 0.3	300 ± 4
H_2O + 20% sucrose (n=12)	39.0* ± 1.0	33.3 ± 0.5	110.5 ± 0.5	780* ± 30	15* ± 0.9	300* ± 4
Water deprived (n=12)		11.6* ± 0.4	78* ± 0.8	2,790* ± 80	1.2 * ± 0.13	340* ± 8

The weight of the rats before the initiation of the treatments was 92.33±2.1. Values are means±SEM of the number of animals between brackets. *p<0.001 as compared with control animals.

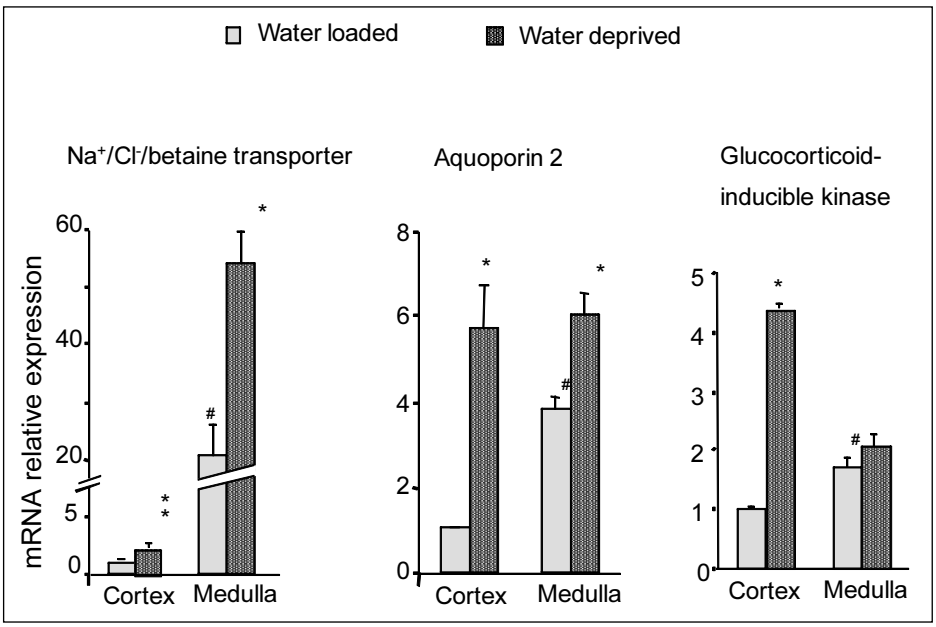

Fig. 1. Cortical and medullar mRNA levels of Na^+/Cl^-/betaine transporter, aquaporin 2 and glucocorticoid-inducible kinase. Total RNA was obtained from rats either deprived of water or induced to drink water abundantly during 60 h. The mRNA relative expression values of the histograms were normalized in each sample by using the β-actin as reference gene. For each gene, the mRNA levels measured in the cortex of water loaded animals were set at 1. Means±SEM of 6 animals in each experimental condition. #p<0.001, comparisons made between cortex and medulla of water loaded animals. *p<0.001, **p<0.01, comparisons made between water loaded and deprived animals.

cortical but not medullar glucocorticoid-inducible kinase mRNA levels.

In water loaded animals, AGAT mRNA levels were significantly higher in the cortex than in the medulla (*Fig. 2*) and in both renal regions significantly higher than those of GAMT, which did not show intranephron differences. In the cortex AGAT mRNA/GAMT mRNA ratio was 10 and in the medulla the ratio was close to 2. Antidiuresis significantly decreased cortical and medullar AGAT mRNA levels, without modifying those of GAMT (*Fig. 2*).

The abundance of CRT mRNA was significantly higher in the cortex than in the medulla. Antidiuresis significantly decreased CRT mRNA levels in both, cortex and medulla (*Fig. 3A*).

Water intake and renal transport of creatine and α-methyl-glucose

Brush-border membrane vesicles (BBMV) were isolated from the renal cortex and medulla of well hydrated and water deprived rats. Uptake of either creatine or α-methyl-glucose into the BBMV was measured in the presence and absence of electrochemical NaCl gradients. The activity of the CRT and that

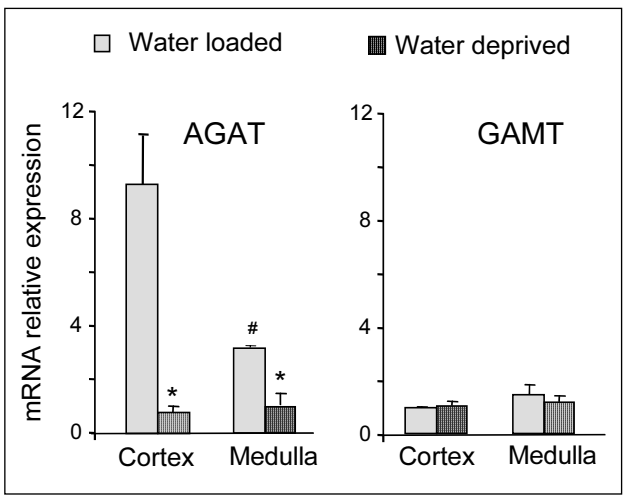

Fig. 2. Cortical and medullar L-arginine: glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT) mRNA levels. The GAMT mRNA levels measured in the cortex of water loaded animals were set at 1. Other details as in *Fig. 1*.

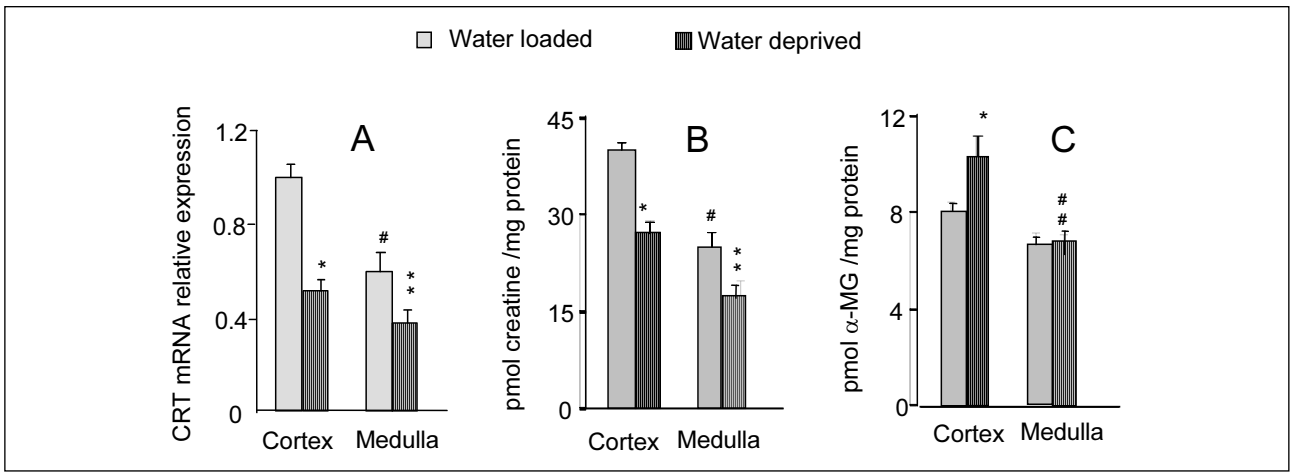

Fig. 3. Na^+/Cl^-/creatine transporter (CRT) mRNA levels (A), creatine uptake (B) and α-methyl-glucose (α-MG) uptake (C). 15s ^{14}[C]-creatine and 10s α-^{14}[C]-methyl-glucose uptake were measured in BBMV isolated from the cortex and medulla of rats, either deprived of water or water loaded animals. Uptake was measured in the presence and absence of electrochemical NaCl gradients. NaCl was isosmotically replaced by 100 mM mannitol and 50 mM Kgluconate. Data represent the difference between total uptake and that measured under nominally NaCl-free conditions. Means±SEM of 6 different BBMV preparations. $^{\#}p<0.001$, $^{\#\#}p<0.01$, comparisons made between cortex and medulla of water loaded animals. *p<0.001, **p<0.01, comparisons made between water loaded and deprived animals.

of Na^+/α-methyl-glucose transporter were evaluated as total uptake minus that measured in nominally NaCl-free conditions.

In water loaded animals, CRT activity was significantly higher in the cortex than in the medulla, and water deprivation significantly reduced both, cortical and medullar CRT activity (*Fig. 3B*).

In water loaded animals, the cortical Na^+/α-methyl-glucose transporter activity was significantly higher than that in the medulla. Water deprivation significantly increased the cortical, but not the medullar, Na^+/α-methyl-glucose transporter activity (*Fig. 3C*).

The Na^+-independent component of either creatine or α-methyl-glucose uptake was not affected by the water status of the animals (results not shown).

Since α-methyl-glucose and creatine transport rates were differently affected by antidiuresis, the observed antidiuresis-induced changes in transport activities might be specific for each substrate.

DISCUSSION

Aniso-osmotic environments do not normally represent a challenge to most mammalian cells because the osmolality of virtually all the extracellular body fluids is closely controlled. An exception is the renal medullar cells, which are exposed to variable high levels of NaCl and urea, especially during antidiuresis. The adaptation mechanisms to hypertonicity include changes in the expression of genes, which leads to cell accumulation of organic osmolytes. This maintains osmotic equilibrium with the hyperosmotic environment and normal intracellular concentrations of inorganic electrolytes. Accumulation of compatible organic osmolytes originates from up-regulation of their synthesis and/or membrane transporters (1-5).

The current work investigates whether the kidneys use creatine as a compatible organic osmolyte in response to dehydration. To achieve this, we measured the mRNA levels of CRT, AGAT and GAMT, and the activity of CRT in the cortex and medulla of rat kidney.

The changes in the rat body weight and urine osmolality induced by either dehydration or water overloading were similar to those previously reported (18-20). The urine osmolality values of water overloaded rats were higher than those previously reported by us (16). These differences might be related with the age of the rats. The rats used in our previous study were older that those of the current study. To check this we repeated the treatment using 1- and 2.5-month-old rats and found that the urine osmolality values of young rats were similar to those shown in *Table 2*. However, in 2.5 month-old rats, the urine osmolality of the control rats was 500 mOsmol/Kg and it was decreased to 200 mOsmol/Kg following 60 h water overloading.

In water loaded animals CRT activity and mRNA levels were higher in the cortex than in medulla and they were significantly decreased following 60 h water restriction suggesting that dehydration regulates CRT at the pre-transcriptional level. These results contrast with those of Alfieri *et al*. (6), showing that hypertonic stress up-regulates CRT mRNA levels and activity in C2C12 muscle cells.

In water loaded rats, the AGAT /GAMT mRNA levels ratio was 10 in the cortex and 2 in the medulla. These observations contrast with previous reports showing that the kidneys of mammals express fairly high activity of AGAT but relatively low or undetectable activity of GAMT (9). The reason for the discrepancy with our results might come from differences in the post-transcriptional regulation of both genes and/or in the sensitivity of the methods used for the measurement of mRNA levels and enzyme activities. These arguments may also explain why AGAT protein (24) and activity (25) had been previously detected in cortex, but not in renal medulla.

Several studies have shown that creatine biosynthesis is regulated at the level of AGAT (10-13). The body hydration status also affects creatine biosynthesis at the level of AGAT. Thus, dehydration decreased cortical and medullar AGAT mRNA levels without affecting those of GAMT.

The results discussed so far suggest that dehydration decreases renal creatine content by reducing cell creatine synthesis and uptake. *A priori*, these observations do not agree with those of Levillain *et al.* (7), showing that water deprivation increased the renal content of creatine and other guanidine

compounds. However, in the rat creatine regulates its own biosynthesis by reducing AGAT activity and mRNA levels (10-13). It could be speculated that, as reported by Levillain *et al*. (7), dehydration initially increases renal creatine content and this in turn inhibits AGAT expression. Therefore, the reduction in AGAT mRNA levels reported here would be secondary to a dehydration-induced increase in creatine synthesis and concentration.

The down-regulation of AGAT and CRT genes expression induced by dehydration might also be secondary to changes in hormone levels, such as those of growth hormone and thyroxine, which are depressed by acute and chronic water restriction (26). Both hormones affect creatine metabolism. Growth hormone up-regulates expression of AGAT and CRT genes (27-29) and thyroxine maintains AGAT protein levels in rat kidney (27, 28, 30).

The already discussed dehydration-induced decreases in renal creatine metabolism were no due to a general down-regulation in renal function induced by dehydration. If this were the case, all the renal genes and transport systems would show a similar response to antidiuresis. However, the renal expression of the genes coding for Na^+/Cl^-/betaine transporter, aquaporin 2 and glucocorticoid-inducible kinase was significantly increased following 60 h water deprivation. In addition, the response of Na^+/α-methyl-glucose transporter to dehydration was opposite to that of CRT. Dehydration significantly increased cortical Na^+/α-methyl-glucose transporter activity without modifying that in the medulla.

The dehydration-induced changes in Na^+/α-methyl-glucose transporter activity paralleled those on glucocorticoid-inducible kinase mRNA levels: dehydration increases cortical, but not medullar, mRNA levels of the kinase. Since this kinase increases the activity of several transporters, including that of Na^+/glucose transporter (31), it could be postulated that dehydration up-regulates cortical Na^+/α-methyl-glucose transporter activity *via* increased levels of glucocorticoid-inducible kinase.

Shojaiefard *et al*. (32) reported that glucocorticoid-inducible kinase increased CRT maximal transport rate in *Xenopus* oocytes. Here we report that the mRNA levels of glucocorticoid-inducible kinase and the CRT mRNA levels and activity were differently affected by dehydration, suggesting that the kinase does not regulate CRT activity in rat kidney.

In conclusion, this is the first report showing that: i) at the mRNA levels, the kidneys express significant amounts of GAMT as compared with those of AGAT, ii) dehydration down-regulates creatine synthesis at the level of AGAT gene expression and iii) dehydration down-regulates CRT gene expression and activity.

A preliminary report of some of these results has been published as an abstract (33).

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