Effects of dietary salt intake on plasma arginine

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Effects of dietary salt intake on plasma arginine. Am J Physiol Regulatory Integrative Comp Physiol 280: R1069–R1075, 2001.—Because L-arginine is degraded by hepatic arginase to ornithine and urea and is transported by the regulated 2A cationic amino acid y+ transporter (CAT2A), hepatic transport may regulate plasma arginine concentration. Groups of rats (n = 6) were fed a diet of either low salt (LS) or high salt (HS) for 7 days to test the hypothesis that dietary salt intake regulates plasma arginine concentration and renal nitric oxide (NO) generation by measuring plasma arginine and ornithine concentrations, renal NO excretion, and expression of hepatic CAT2A, and arginase. LS rats had lower excretion of NO metabolites and cGMP, lower plasma arginine concentration (LS: 83 ± 7 vs. HS: 165 ± 10 μmol/l, P < 0.001), but higher plasma ornithine concentration (LS: 82 ± 6 vs. HS: 66 ± 4 μmol/l, P < 0.05) and urea excretion. However, neither the in vitro hepatic arginase activity nor the mRNA for hepatic arginase 1 was different between groups. In contrast, LS rats had twice the abundance of mRNA for hepatic CAT2A (LS: 3.4 ± 0.4 vs. HS: 1.6 ± 0.5, P < 0.05). The reduced plasma arginine concentration with increased plasma ornithine concentration and urea excretion during LS indicates increased arginine metabolism by arginase. This cannot be ascribed to changes in hepatic arginase expression but may be a consequence of increased hepatic arginine uptake via CAT2A.

NITRIC OXIDE (NO) is generated from its substrate L-arginine in the kidney by distinct isoforms of NO synthase (NOS) (20, 26, 27, 36). Several investigators have concluded that NO generation and/or action decreases with dietary salt restriction. This may serve to adapt renal function to salt intake (8, 19, 25, 34, 36). How- ever, the reduction in NO generation with salt restriction cannot be ascribed to changes in hepatic arginase activity or CAT2A expression.

METHODS

Experiments were performed on male Sprague-Dawley rats weighing 210–300 g. Rats were maintained on a standard rat chow (NaCl content 0.3 g/100 g) until 7 days before study, when they were housed in individual metabolic cages with 12-h cycles of light and dark.

Animal preparation. Groups (n = 6 each) of rats were fed a low-salt (LS) diet or a high-salt (HS) diet for the 7 days before the study. Controlled diets were obtained from Harlan Teklad (Madison, WI). They were identical apart from salt and sucrose contents. They contained the following: casein (high protein 200 g/kg), sucrose (661 g/kg), nonnutritive fiber (cellulose 20 g/kg), corn oil (70 g/kg), mineral mix (40 g/kg), and vitamin mix (9.08 g/kg). The LS diet provided 0.03 g/100 g of NaCl. This is a minimal requirement for normal growth over a 1- to 2-wk period. For the HS diet, NaCl was added to give 6 g/100 g of NaCl, and sucrose was decreased to 655 g/kg. The NO2− + NO3− (NOx) content of the salt added to the high-salt diet was undetectable and was less than one part in

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8 x 10^4. The casein protein source contained ~3.3% protein. The 20% casein in the diet supplied ~0.66% arginine, which is above the amount required to maintain normal growth (11). Rats were pair-fed but had unrestricted water intake. The body weight and food consumed were recorded daily.

On the last day of equilibration, the cages were cleaned, and a 24-h urine was collected for analysis of NO and GMP. Urine was collected in containers with streptomycin (2000 IU), penicillin G (2000 IU), and amphotericin B (5 μg) to prevent microbial overgrowth. Total urine volume was recorded. The collected urine was centrifuged, separated from the sediments, and stored at −70°C until analysis. On day 7, between 9 AM and 11 AM, rats were anesthetized with thiobutabarbital (Inactin, 100 mg/kg, Research Biochemicals International, Nantuck, MA). A cannula was inserted into the abdominal aorta for blood sampling (500 μl). The plasma was separated and frozen at −70°C for subsequent amino acid analysis. The liver was removed immediately, frozen in crushed dry ice, and stored at −70°C.

**Arginase activity**. This was measured in disrupted hepatic cells using a modification of the Schimke method that is based on urea production from excess l-arginine (7). Liver tissue was homogenized with ice-cold phosphate-buffered saline containing 0.1% Triton X-100 and the protease inhibitors aprotinin (5 μg/ml), antipain (5 μg/ml), pepstatin (5 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). The homogenate was centrifuged at 10,000 g for 10 min to remove unbroken cells. The quantity of hepatic material was selected to fall in the linear range for arginase activity, as established in a preliminary study. The supernatant was diluted with the sediment, and stored at −70°C until analysis. Incubation was performed at 37°C for 60 min. The reaction was bated at 55°C for 10 min. Arginine hydrolysis was initiated by the addition of MnCl2 (10 mM) in Tris-HCl (50 mM, pH 7.5). The resultant mixture was incubated at 55°C for 10 min. Arginine hydrolysis was initiated by the addition of excess arginine (25 μl of 0.5 M arginine, pH 9.7) to a 50-μl aliquot of the activated supernatant mixture. Incubation was performed at 37°C for 60 min. The reaction was stopped by the addition of 400 μl of an acid mixture containing H2SO4, H3PO4, and H2O (1:3:7). The urea formed was quantified colorimetrically after the addition of 25 μl of 9% 1-phenyl-1,2-propanediol-2-oxide in ethanol and incubation at 95°C for 45 min. The color was assessed in 200-μl aliquots in triplicate at 540 nm in a microplate reader (Bio-Rad, Hercules, CA). A calibration curve was constructed for urea concentration of 2.5–10 mM. The protein content of the homogenate was quantified using the Bradford method (Bio-Rad, Hercules, CA). The plasma was separated and frozen at −70°C for subsequent amino acid analysis. The liver was removed immediately, frozen in crushed dry ice, and stored at −70°C.

**Quantification of mRNA for CAT and arginase I**. Total RNA was isolated from liver using the guanidinium isothiocyanate method (QIAGEN, Valencia, CA). Briefly, the liver was lysed and homogenized under highly denaturing conditions (guanidinium isothiocyanate and β-mercaptoethanol) to inactivate RNAses. After addition of ethanol (70%), the sample was applied to an RNAse spin column to extract total RNA. RNA was eluted in water and quantified spectrophotometrically by measuring the absorbency at 260 nm. The RNA integrity was assessed by comparing the ethidium bromide-stained 18S and 28S ribosomal RNA bands.

RT-PCR was used to assess the expression of mRNA for gene products of the four isoforms of CAT genes and arginase I. Two-step RT-PCR reactions were performed using the SuperScript Preamplification System for the first strand of cDNA synthesis (GIBCO BRL, Rockville, MD) and AmpliTaq DNA Polymerase (Perkin-Elmer, Foster City, CA). The first strand of cDNA was prepared from 1.5 μg of total RNA with a random hexomers primer and reverse transcribed by incubating at 42°C for 50 min. The reaction was terminated after 15 min at 70°C. The resulting single-stranded cDNA was amplified using synthetic oligonucleotide primers (Table 1). For the CAT2A and CAT2B (gene products, primers were designed using sequences specific to each of the splice variants.

PCR amplification was performed on a Thermal Cycler (Perkin-Elmer, model no. 2400) with 2'-deoxynucleoside 5'-triphosphates and Taq DNA polymerase. The conditions were 94°C for 30 s for denaturing, 58°C for 30 s for annealing, and 72°C for 30 s for extension. Positive control cDNAs for CAT isoforms were obtained from RNA extracted from kidney (CAT1), brain [CAT2B], and brain (CAT3) (10, 22). PCR was also performed without reverse transcriptase treatment to exclude contamination by genomic DNA. Reamplified PCR products for CAT isoforms and arginase I were sequenced and compared with published data. Direct sequencing of PCR products was performed after gel purification according to the Dye Primer and Dye Terminator systems (Applied BioSystems) using fluorescent-labeled dideoxynucleotides and primers. The labeled extension products were analyzed on an Applied BioSystems (model no. 373A) DNA sequencer.

The mRNA abundance for CAT2A and arginase I was quantified using a multiplex RT-PCR. Multiplex RT-PCR uses two primer sets in a single tube in a single reaction, one set to amplify the cDNA of interest and a second set to amplify an invariant endogenous control. This method minimizes differences caused by variations in quantity or quality of the sample RNA as well as PCR conditions. The selected test mRNAs are related to 18S ribosomal RNA (Ambion, Austin, TX). 18S RNA competitors were added with 18S primers in the reaction. The 18S competitors are modified at their 3' ends to block extension by DNA polymerase. By

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide Sequences for Forward (F) and Reverse (R) Primers (5'-3')</th>
<th>Location of Product, nt</th>
<th>Size, bp</th>
<th>GeneBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT1</td>
<td>F CCAAGAACCGCCAGTGAGCT R TGATGGGCTGCTACACAGCAT</td>
<td>1395-1413</td>
<td>306</td>
<td>U70476</td>
</tr>
<tr>
<td>CAT2A</td>
<td>F GGATGGCTTACTGTTTAGAT R AGAATCAGCAAAAAGTAGCC</td>
<td>1116-1135</td>
<td>556</td>
<td>AF158025</td>
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<tr>
<td>CAT2B</td>
<td>F TAATCTATGCTATGGCGGAG R AGAATCAGCAAAAAGTAGCC</td>
<td>1700-1682</td>
<td>578</td>
<td>M62838</td>
</tr>
<tr>
<td>CAT3</td>
<td>F GACCAAGAAATGAGAGAGGTT R CAGAAGCCACGAGCGAGCAT</td>
<td>1316-1337</td>
<td>318</td>
<td>AB000113</td>
</tr>
<tr>
<td>Arginase I</td>
<td>R ATCGGAAAGCCAGCGGTTG R ATCTGCAAGCGGATGTAAC</td>
<td>1613-1633</td>
<td>329</td>
<td>JO2720</td>
</tr>
</tbody>
</table>

CAT, cationic amino acid transporters; nt, nucleotides; bp, base pairs.

Table 1. Primers used for RT-PCR of cationic amino acid transporters and arginase I
mixing 18S primers with increasing amounts of 18S competimers, one can reduce the overall PCR amplification efficiency of 18S cDNA without the primers becoming limiting. The use of competimers ensures that the endogenous control is in the same linear range as the RNA under study when amplified under the same conditions. The template was 1 µl of cDNA, amplified from 1.5 µg total RNA. Pilot experiments were undertaken to optimize all the reaction conditions. Quantitative measurements were made only during the exponential phase of extension. There were 28 cycles of amplification for CAT2A and 24 cycles for arginase I. Products were separated on a 1.5% agarose gel containing ethidium bromide and visualized by ultraviolet transillumination. Band intensities were assessed using an AlphaImager 2200 (Alpha Innotech, San Leonardo, CA).

Chemical methods. Urine for NOx was analyzed as described previously (14). The NO3 was converted to NO2 by incubation overnight with nitrate reductase, and the NO2 quantitated by the Greiss reaction. cGMP in urine was analyzed by an ELISA method (Cayman Chemical, Ann Arbor, MI). Plasma amino acids were analyzed with an automated amino acid analyzer (Beckman Instruments, Brea, CA). Urea was analyzed with a blood urea nitrogen analyzer (Beckman Instruments).

Statistical methods. All reagents were from Sigma (St. Louis, MO) unless otherwise stated.

Table 2. Plasma amino acid concentrations and renal urea excretion in rats adapted to high salt or low salt

<table>
<thead>
<tr>
<th></th>
<th>HS (n=6)</th>
<th>LS (n=6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine, µmol/l</td>
<td>164.9±10.7</td>
<td>65.7±4.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ornithine, µmol/l</td>
<td>65.7±4.3</td>
<td>81.6±6.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Citrulline, µmol/l</td>
<td>50.0±3.0</td>
<td>46.9±1.3</td>
<td>NS</td>
</tr>
<tr>
<td>Total amino acids, mmol/l</td>
<td>4.3±0.2</td>
<td>4.2±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Urea excretion, mmol·kg⁻¹·24 h⁻¹</td>
<td>28.5±1.2</td>
<td>34.4±2.2</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Mean ± SE values. HS, high salt; LS, low salt; NS, not significant.

RESULTS

The HS and LS rats had similar body weights [HS: 255 ± 4 g vs. LS 257 ± 6 g, not significant (NS)], daily weight gain (HS: 3.1 ± 0.6 vs. LS: 3.6 ± 0.5 g/24 h, NS), and food intake (HS: 17.1 ± 1.3 vs. LS: 16.8 ± 1.6 g/24 h, NS).

Figure 1 depicts the 24-h excretion of NOx and cGMP. Compared with HS rats, LS rats had a diminished excretion of NOx (HS: 1,267 ± 206 vs. LS: 492 ± 75 nmol/24 h, P < 0.01) and cGMP (HS: 10.8 ± 4.1 vs. LS: 2.4 ± 0.9 nmol/24 h, P < 0.05).

As shown in Table 2, the plasma arginine concentration of LS rats was less than half that of HS rats (P < 0.001), whereas the plasma ornithine concentration was increased modestly (P < 0.05). By contrast, the plasma concentrations of citrulline and total amino acids were similar in the two groups. The generation of urea was estimated from urea excretion during controlled protein intake. It was increased in LS rats (P < 0.05).

As shown in Fig. 2, the hepatic arginase activity, measured in disrupted hepatic cells in the presence of excess L-arginine, was similar in the two groups (HS: 240 ± 15 vs. LS: 264 ± 21 µmol urea·mg protein⁻¹·h⁻¹, NS). The arginase activity of the kidney was significantly less than the liver but was similar in the two groups (HS: 6.5 ± 0.9 vs. LS: 5.5 ± 0.8 µmol urea·mg protein⁻¹·h⁻¹, NS).

PCR products for CAT1, CAT2A, CAT2(B), and CAT3 are shown in Fig. 3. The liver was confirmed to express only the CAT2A isoform, which was also confirmed to be expressed in skeletal muscle but not in...
kidney or brain (not shown) (5, 6, 10, 22). Dietary salt restriction did not induce the expression of CAT1, CAT2(B), or CAT3 in the liver (data not shown). Figure 3 also shows positive controls taken from different rat tissues for CAT1 (kidney), CAT2(B) (brain), and CAT3 (brain). The PCR products conformed to the predicted sequences.

Figure 4A shows that rats adapted to a low-salt diet had a significantly enhanced mRNA abundance for CAT2A in the liver (CAT2A/18S ratio: HS: 1.6 ± 0.5 vs. LS: 3.4 ± 0.4, P < 0.05). In contrast, Fig. 4B shows that the mRNA abundance for arginase in the liver is similar in the two groups (arginase I/18S: HS: 1.4 ± 0.2 vs. LS: 1.6 ± 0.3, NS).

**DISCUSSION**

The major new findings of this study are that dietary salt restriction decreases the plasma arginine concentration but increases the plasma ornithine concentration and the rate of urea generation. Salt restriction does not modify the intrinsic hepatic arginase activity but doubles the abundance of mRNA for CAT2A in the liver. These findings suggest that salt restriction may increase hepatic arginine uptake via the CAT2A isoform of the system y+ transporter. Its subsequent metabolism by the hepatic arginase-urea pathway could account for the decrease in the plasma arginine concentration and increase in urea appearance seen during dietary salt restriction. The stable plasma citrulline concentration suggests that arginine synthesis was not greatly modified.

Plasma levels of amino acids and the rate of urea excretion are closely dependent on protein intake. Therefore, rats were pair-fed a diet that differed only in the salt and sucrose contents. Moreover, the HS and LS diets led to similar rates of weight gain. Therefore, we conclude that the salt-induced changes in the plasma concentrations of arginine and ornithine and in urea excretion reflect specific effects of salt on arginine metabolism.

Many of the physiological actions of NO are mediated by guanylate cyclase. The excretion of cGMP has been used to provide an indirect measure of total NO action (29, 31). The excretion of NOx and cGMP may be modified by distinct factors in addition to changes in NO generation (2). In this study, excretion of both NOx and cGMP was decreased by dietary salt restriction, which suggests that the total body NO generation and action in the tissues was decreased, confirming previous conclusions (9, 10, 25, 32, 34).

A low-salt diet decreases neuronal NOS (nNOS) activity in the inner medulla of the rat (19). On the other hand, the expression of endothelial NOS and nNOS in the renal cortex is increased during salt restriction (27, 28). Salt-induced changes in the expression of NOS in the renal cortex do not correlate with the results of functional studies that have concluded that salt re-
striction decreases the role of NO in blunting the tu-
buloglomerular feedback (TGF) response (34, 36) or in
maintaining whole kidney blood flow (8). Therefore, we
have sought factors other than NOS expression to
explain the apparent decrease in NO action in the
renal cortex during dietary salt restriction.

The plasma arginine concentration in rats fed a
high-salt diet was similar to those reported previously
in rats fed a normal salt diet (12). Plasma levels of
arginine are more than 10-fold above the in vitro
Michaelis constant ($K_m$) for constitutive NOS (1).
However, several studies have shown that the apparent $K_m$
for arginine stimulation of NO production in vivo is
similar to the plasma concentrations (9, 32). This could
be explained by the presence of endogenous inhibitors
such as asymmetric dimethyl arginine (30) or glu-
tamine (1), or the association of NOS and y$^+$ trans-
porter in caveolae (24). The $K_m$s for the y$^+$ transporters
are similar to the plasma L-arginine concentrations
(10, 22). Indeed, infusions of L-arginine into rats cause
renal vasodilation that is dependent on NO generation
since it is stereospecific and is blocked by inhibition of
NOS (9). Dietary salt restriction increases the renal
vascular sensitivity to physiological concentrations of
infused L-arginine. This effect is specific for L-arginine
(9). Moreover, a limited availability of L-arginine dur-
ding LS apparently restricts NO-dependent blunting of
TGF (32). The transport of NaCl by the thick ascending
limbs of the loop of Henle is enhanced by L-arginine
(23). These results indicate that the plasma concen-
tration of L-arginine and its uptake into cells may adapt
renal NO production to changes in dietary salt intake.

Citrulline is formed from amino acid metabolism
primarily in the gut wall (37). Arginine is synthesized
from citrulline predominantly in the proximal tubule
cells of the kidney (18). Since arginine synthesis de-
pends on plasma citrulline concentrations (12), the
decrease in plasma arginine concentrations in LS rats
cannot be ascribed to a decrease in renal arginine
synthesis, because the plasma citrulline concentrations
were not altered. Furthermore, dietary salt re-
striction increased the plasma ornithine concentration
and the rate of urea generation. This implies an in-
creased traffic of arginine through arginase during
extracellular fluid volume depletion, as shown previ-
ously (17).

The liver contains high concentrations of arginase I
and the urea cycle enzymes (37). Although arginase I is
compartmentalized in the liver, the conversion of in-
fused $^{15}$Narginine to $^{15}$Nornithine is dependent on
arginine intake (4). Moreover, patients with a genetic
defect in hepatic arginase I have elevated plasma ar-
ginine concentrations despite upregulation of renal ar-
ginase II (15). Thus hepatic arginase I is critical in
regulating plasma arginine concentrations. We confirm
that the arginase activity in the liver is over 50-fold

Fig. 4. Results of multiplex RT-PCR for rat liver. A: data for CAT2A
mRNA expression in the liver. B: data for arginase I mRNA expres-
sion in the liver. Both A and B compare results in rats adapted to HS
($n = 6$) or LS ($n = 6$).
higher than of the kidney (16). The liver normally contains only arginase I (37). We found no effect of salt intake on the arginase activity in disrupted liver cells in the presence of excess substrate nor any effect of salt intake on the mRNA abundance for arginase I in the liver. Therefore, changes in hepatic arginase expression are unlikely to account for the effects of dietary salt on plasma arginine concentration.

Arginine transport into mammalian cells depends largely on the four CAT proteins that subserve for system $y^+$ transport (10). Each CAT has specific tissue distribution and transport characteristics. The CAT2 gene contains two mutually exclusive splice variants (10, 22). The CAT2A is the only isoform expressed by the normal liver (5, 6) and is the major transporter for arginine uptake in the liver (37). The transport of arginine, lysine, and ornithine by a cloned CAT2A expressed in oocytes displays similar kinetic properties and substrate specificity to the transport of cationic amino acids in the hepatocyte (5, 33). We confirm that the mRNA for CAT2A, but not for any of the other cloned CAT isoforms, is expressed in the liver. Its low affinity for $L$-arginine limits the hepatic uptake and clearance of $L$-arginine, thereby protecting the plasma arginine levels (21, 33). The low affinity of CAT2A predicts that hepatic uptake of arginine is closely dependent on the expression of the transporter. Therefore, doubling the abundance of the mRNA for CAT2A in the liver during dietary salt restriction, if accompanied by increased CAT2A protein, could result in increased hepatic uptake of plasma arginine into the liver. Although the transport of cationic amino acids of CAT2A is bidirectional (5), there is likely to be a net uptake of arginine into the liver because of a low intracellular arginine concentration that is maintained by the highly active arginase (33). The metabolism of arginine by arginase generates ornithine. Because ornithine is also a substrate for the $y^+$ transporter and cationic amino acids are normally counter-transported (22), an enhanced CAT2A expression in the liver could account for the increased plasma concentration of ornithine that we observed during salt restriction. The extensive metabolism of ornithine (37) may account for the smaller changes in its plasma concentration compared with arginine.

The CAT2 gene contains multiple functional promoters that produce isoform-specific regulation in different tissues (13). The signal that increases hepatic transcription of CAT2A during salt restriction is unknown. Mild metabolic alkalosis reduces the plasma concentration of arginine and increases urea generation because of increased hepatic arginine metabolism (3). Therefore, a contraction alkalosis that normally accompanies salt depletion (35) may have contributed to increased arginine metabolism.

**Perspectives**

Dietary salt restriction normally results in decreased total body and renal cortical NO generation (8, 9, 25, 32, 34) and enhanced urea appearance (17). A reduced total body and renal cortical NO generation may contribute to blood pressure and NaCl homeostasis during salt depletion by enhancing vasoconstriction (8, 25, 31), TGF (32), and tubular NaCl reabsorption. An increased urea generation may contribute to body fluid homeostasis by enhancing the tubular fluid concentrating capacity. An increase in hepatic arginine uptake and metabolism provides a mechanism whereby salt restriction could decrease plasma arginine concentration and thereby limit the generation of NO yet increase the generation of urea. This conclusion suggests that the circulating levels of $L$-arginine are tightly controlled and may regulate the function of target organs during changes in salt intake. This would elevate the status of arginine from that of a semiessential amino acid to the level of a hormone.

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