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Research Article

myo-inositol, a major compatible osmolyte in renal medulla, is accumulated in several kinds of cells under hypertonic conditions via Na+/myo-inositol cotransporter (SMIT). To investigate the physiological role of the SMIT, we sought to determine its localization by in situ hybridization and its acute regulation by NaCl and furosemide administration. Northern analysis demonstrated that SMIT is strongly expressed in the medulla and at low levels in the cortex of kidney. Intraperitoneal injection of NaCl rapidly induced SMIT mRNA in both the cortex and medulla, and furosemide completely abolished this induction. In situ hybridization revealed that SMIT it predominantly present in the medullary and cortical thick ascending limbs of Henle's loop (TALH) and macula densa cells. Less intense signals were seen in the inner medullary collecting ducts (IMCD). NaCl loading increased the signals throughout the TALH, and furosemide reduced the signals. SMIT in the IMCD is less sensitive to these kinds of acute regulation. Thus, the distribution pattern of SMIT does not correspond to the corticomedullary osmotic gradient, and SMIT in the TALH and macula densa cells is regulated very rapidly. These results suggest that SMIT expression in TALH may be regulated by intracellular and/or peritubular tonicity close to the basolateral membrane, which is supposed to be proportional to the magnitude of NaCl reabsorption.

Localization and Rapid Regulation of Na^+/*myo*-Inositol Cotransporter in Rat Kidney

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Abstract

***myo*-Inositol, a major compatible osmolyte in renal medulla, is accumulated in several kinds of cells under hypertonic conditions via Na^+/*myo*-inositol cotransporter (SMIT). To investigate the physiological role of the SMIT, we sought to determine its localization by in situ hybridization and its acute regulation by NaCl and furosemide administration. Northern analysis demonstrated that SMIT is strongly expressed in the medulla and at low levels in the cortex of kidney. Intraperitoneal injection of NaCl rapidly induced SMIT mRNA in both the cortex and medulla, and furosemide completely abolished this induction. In situ hybridization revealed that SMIT is predominantly present in the medullary and cortical thick ascending limbs of Henle's loop (TALH) and macula densa cells. Less intense signals were seen in the inner medullary collecting ducts (IMCD). NaCl loading increased the signals throughout the TALH, and furosemide reduced the signals. SMIT in the IMCD is less sensitive to these kinds of acute regulation. Thus, the distribution pattern of SMIT does not correspond to the corticomedullary osmotic gradient, and SMIT in the TALH and macula densa cells is regulated very rapidly. These results suggest that SMIT expression in TALH may be regulated by intracellular and/or peritubular tonicity close to the basolateral membrane, which is supposed to be proportional to the magnitude of NaCl reabsorption. (*J. Clin. Invest.* 1995. 96:1195–1201.) Key words: osmolyte • hypertonicity • macula densa • furosemide • thick ascending limbs of Henle's loop**

Introduction

The cells of diverse organisms respond to extracellular hypertonicity by the accumulation of high concentrations of small organic solutes that protect cells from the perturbing effects of high intracellular concentrations of electrolytes (1, 2). These solutes are referred to as "compatible osmolytes," since they are compatible with enzyme function (1). The general classes of organic osmolytes are polyols, sugars, amino acids, methylamines, and urea (3). In mammals organic osmolytes have been most widely investigated in renal medullary cells, which normally undergo wide swings in tonicity related to the renal concentrating mechanism.

myo-Inositol has been identified as one of major osmolytes in the renal medulla, some kidney-derived cell lines (4) and brain cells (5). Madin-Darby canine kidney (MDCK)[1] cells accumulate *myo*-inositol through Na^+-coupled transporter when cultured in hypertonic medium (6). A cDNA encoding Na^+/*myo*-inositol cotransporter (SMIT) was isolated from MDCK cells (7). The abundance of SMIT mRNA and the transcription rate of SMIT gene increased when the cells were cultured in hypertonic medium, suggesting that transcription is the primary step in regulation of *myo*-inositol transport by hypertonicity (8). Similar results were obtained from brain glial cells (9) and lens epithelial cells (10).

The other predominant osmolytes in renal medulla are sorbitol, betaine, and glycerophosphorylcholine. The distribution of these osmolytes in the kidney corresponds largely to the corticomedullary osmotic gradient, with the highest concentration found in the inner medulla and papillary segments (11). *myo*-inositol is the only osmolyte found in substantial amounts in the cortex and outer medulla in addition to the inner medulla (12). Our previous report (13) has shown that SMIT mRNA abundance was highest in the outer medulla, and a little but significant amount of SMIT mRNA was present in the cortex. SMIT mRNA in the cortex as well as that in the medulla was significantly induced by dehydration. We have recently demonstrated that cultured rat mesangial cells respond to extracellular hypertonicity by increasing *myo*-inositol transport activity and accumulating *myo*-inositol into cells (14). It is probable that the part of SMIT we observed in the cortex is derived from mesangial cells since extraglomerular mesangial cells are considered to be faced with hypertonic stress (15).

There is some evidence that *myo*-inositol in the outer medulla is mainly present in the thick ascending limbs of Henle's loop (TALH). Microdissection study has revealed that cells in the medullary and cortical TALH and the medullary collecting ducts contain the highest concentration of *myo*-inositol (~ 15–30 mM), whereas proximal tubule cells contain only ~ 3–5 mM (16). Considering its unique distribution, something about the control or function of *myo*-inositol must differ from the other renal compatible osmolytes.

To gain insight into the potential function and physiological roles of *myo*-inositol and its transporter, we determined regional and cellular distribution of Na^+/*myo*-inositol cotransporter in rat kidney, using in situ hybridization technique. Moreover, we investigated the effects of acute loading of NaCl as well as furosemide administration on the expression of SMIT.

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1. *Abbreviations used in this paper:* MDCK, Madin-Darby canine kidney; RT, reverse transcribed; SMIT, Na^+/*myo*-inositol cotransporter; TALH, thick ascending limbs of Henle's loop.

Methods

Animals. Male Wistar rats, weighed ~ 150 grams, received water and standard rat chow for several days before the experiments. To load NaCl, 1.5 ml of 1.5 M NaCl per 100 grams body weight was administered by intraperitoneal injection. After 1, 2, 3, 5, or 8 h animals were deeply anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg). For in situ hybridization, rats were transcardially perfused with a solution of 4% paraformaldehyde. For RNA extraction, rats were killed by decapitation and blood samples were taken. The cortex and medulla were dissected from the kidney slices and were subjected to RNA extraction.

To see the effects of furosemide, intraperitoneal injection of 20 mg/kg of furosemide was performed simultaneously with NaCl loading. After 2 or 5 h, the rats were treated by the same procedures described above.

Sodium concentrations were measured using an electrolyte autoanalyzer (TYPE-710; Hitachi, Tokyo, Japan). Osmolality was measured with a freezing point osmometer (OM-6010; Kyoto-Daiichi-Kagaku, Kyoto, Japan).

Probes. To prepare the probe specific for rat SMIT, rat kidney RNA was reverse transcribed (RT) and subjected to polymerase chain reaction (PCR) amplification with specific 5′ and 3′ primers for SMIT cDNA from MDCK cells. The primer sequences were as follows: CCAGCTTCAGTATGGTACTG (sense primer, corresponding to nucleotides 808 to 827) and GCATCTCCACGATGATTGGC (antisense primer, corresponding to nucleotides 1297 to 1278),

The PCR fragment with the expected size (490 bp) was subcloned into pT7 Blue (R) T-vector plasmid (Novagen, Madison, WI). Sequencing of double stranded DNA was done using fluorescence labeled M13 forward or M13 reverse primer on an automated sequencer (Applied Biosystems Inc., Foster City, CA). There is 91% identity of the DNA sequence and 97% identity of amino acid sequences compared to canine SMIT cDNA. When used this probe for Northern blot analysis, the pattern of the blot was the same as canine probe but the signal was more intense than canine probe even after high stringency wash. These results confirmed us that the probe was partial clone of rat SMIT cDNA.

Genomic DNA for rat ribosomal RNA was a generous gift from Japanese Cancer Research Resources Bank. The BamHI–EcoRI fragment of the coding region (17) was used as a probe for Northern blot analysis.

For in situ hybridization, the 760-bp $EcoRI_{724}$ to $SphI_{1484}$ fragment of the canine SMIT cDNA was subcloned into pSPORT (GIBCO BRL, Gaithersburg, MD). The partial clone of rat SMIT cDNA (490 bp) was also subcloned into pSPORT. To make ^{35}S-labeled sense or antisense cRNA probe, in vitro transcription was performed using T7 RNA polymerase or SP6 RNA polymerase after linearizing by cutting with appropriate restriction enzyme. The signals using rat probe was more intense than canine probe with similar pattern. So we showed only the result of rat probe in this report.

Northern blot and slot blot analysis. Total RNA was isolated from the cortex and medulla of kidney tissue by acid guanidium thiocyanate-phenol-chloroform extraction as described (18). For Northern analysis, 20 μg of total RNA was separated on a 1% agarose formaldehyde gel and was transferred to nylon membrane (Amersham, Hybond™-N). For slot blot analysis, 5 μg of RNA was spotted on to a nylon membrane using a Slot-blot filtration manifold (Schleicher & Schuell, Dasel, W. Germany). Full-length canine SMIT cDNA (7), partial clone of rat SMIT cDNA and cDNA for rat ribosomal RNA were labeled by random priming (Amersham) using [α-^{32}P] dATP (3,000 Ci/mmol, Amersham). Hybridization was carried out at 42°C overnight in 50% formaldehyde, 5× SSC, 0.1% SDS, 50 mM Na phosphate, 5× Denhadt's solution and 100 μg/ml salmon sperm DNA. The blots were washed three times at 45°C for 30 min in 1× SSC and 0.1% SDS. To remove the hybridized probe, a boiling solution of 0.1% SDS was poured on the membrane and allow to cool to room temperature. After removal of the SMIT probe, hybridization for the ribosomal RNA probe was performed. The hybridized probe was quantified by a bioimaging analyzer BAS2000 (FUJIX, Tokyo, Japan).

Table I. Serum Sodium Concentration and Osmolality in NaCl Infused Rats

Time after injection	Control	1	2	3	5	8
Na	142±2	153±5*	156±2*	151±4‡	156±10*	150±4‡
Osmolality	290±10	318±13*	316±9*	308±13‡	308±8‡	310±13*

Intraperitoneal injection of 1.5 ml of 1.5 M NaCl per 100 grams body weight was performed into rats. After 1, 2, 3, 5, or 8 h blood samples were taken and sodium concentration (mEq/liter) and osmolality (mosmol/kg) were measured. Values are means±SD for three different animals. * $P < 0.01$, and ‡ $P < 0.05$ vs control values.

In situ hybridization. The kidneys after the perfusion of paraformaldehyde were placed in a solution of 4% paraformaldehyde for 2 h and 15% sucrose overnight. Cryostat sections (5 μm) were mounted on siliconized slides. The slides were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min. After washing with 0.1 M phosphate buffer, the slides were treated with a solution of 10 μg/ml proteinase K in 50 mM Tris-HCl, pH 7.5, and 5 mM EDTA, pH 8.0, for 1 min at room temperature. They were postfixed in 4% paraformaldehyde and then treated with 0.25% acetic anhydride for 10 min. They were rinsed in phosphate buffer (28 mM NaH_2PO_4, 72 mM Na_2HPO_4) and dehydrated in increasing concentrations of ethanol.

Tissue sections were hybridized for 24 h at 55°C in a buffer (50% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, 1× Denhardt's solution, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 0.2% sarcosyl, 200 μg/ml salmon sperm DNA, and 500 μg/ml yeast tRNA) containing one of ^{35}S-labeled RNA probes. The probe concentration was 1×10^6 cpm/200 μl per slide. After hybridization, the sections were immersed in 5× SSC at 55°C, rinsed in 50% deionized formamide, 2× SSC at 65°C for 30 min. After rinsing with RNase buffer (0.5 M NaCl, 10 mM Tris-HCl [pH 8.0], 5 mM EDTA [pH 8.0]) three times for 10 min each at 37°C, the sections were incubated with 1 μg/ml RNase A in RNase buffer for 10 min at 37°C. After rinsing in RNase buffer for 10 min, the sections were washed in 50% formamide, 2× SSC at 65°C for 30 min, rinsed with 2× SSC and 0.1× SSC for 10 min each at room temperature, dehydrated in alcohol, and air-dried.

Slides were initially exposed to x-ray film for 3 d to provide an indication of the intensity of the hybridization signal. They were then coated with Kodak NTB-2 emulsion diluted 1:1 with water. The sections were exposed at 4°C for 2–3 wk in tightly sealed dark boxes. After developed in Kodak D-19, the sections were fixed with photographic fixer, and washed with water. They were then counterstained with hematoxylin and eosin to allow morphological identification.

Data analysis. The results shown are means±SD. When no error bar is shown in a figure, the SD is smaller than the symbol. The data were statistically analyzed by the one-way analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$. All experiments were performed more than twice with similar results.

Results

Blood chemistry in NaCl infused rats. To examine acute regulation of SMIT in kidney, NaCl was administered by intraperitoneal injection. Table I shows the data of blood chemistry in control and NaCl injected rats. Serum Na concentration and osmolality were rapidly increased one hour after the infusion of NaCl. The significant elevation of serum Na and osmolality persisted for at least 8 h.

Northern and slot blot of SMIT mRNA. To see if SMIT mRNA is in vivo regulated by acute administration of NaCl, total RNA was isolated from the renal cortex and medulla of

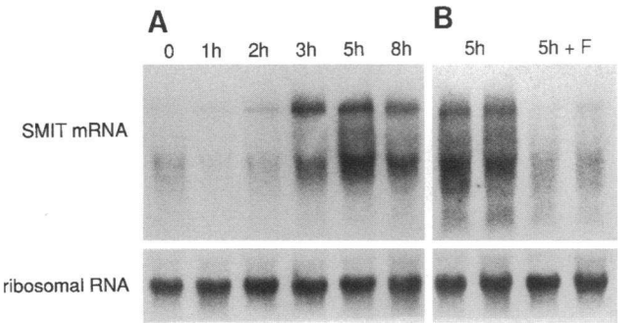

Figure 1. (*A*) Northern blot analysis of the time course of increase in renal medullary SMIT mRNA after NaCl infusion. After 1, 2, 3, 5, or 8 h after intraperitoneal injection of NaCl, RNA was extracted from the cortex and medulla of kidney separately. Samples of 20 μg of total RNA were separated by electrophoresis on 1% agarose formaldehyde gel and was transferred to nylon membrane. After the experiments for SMIT mRNA, the filter was reused for the determination of ribosomal RNA. The levels of ribosomal RNA were almost the same, indicating equal loading. Numbers are hours after NaCl infusion. SMIT, Na^+/*myo*-inositol cotransporter. (*B*) The effects of furosemide administration on SMIT mRNA abundance. Intraperitoneal injection of 20 mg/kg of furosemide was performed simultaneously with NaCl loading. *5h*, 5 h after NaCl administration. *5h + F*, 5 h after NaCl and furosemide administration.

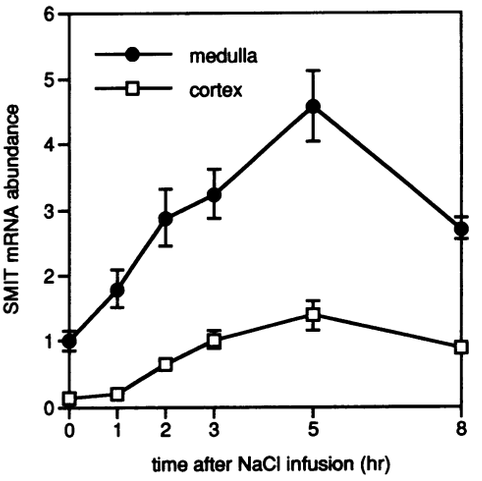

Figure 2. Time course of abundance of SMIT mRNA after NaCl administration. To load NaCl, 1.5 ml of 1.5 M NaCl per 100 grams body weight was administered by intraperitoneal injection. After 1, 2, 3, 5, or 8 h, total RNA was isolated from the cortex and medulla of kidneys. Samples of 5 μg of RNA was spotted on to a nylon membrane using a Slot-blot filtration manifold. Data were abundance relative to abundance of SMIT mRNA in the medulla of control rats. Each point is mean of three experiments.

NaCl loaded rats, and hybridized with the rat SMIT cDNA probe, which has been originally cloned using RT-PCR method. When using canine probe, the results were essentially the same with less intense signals. SMIT mRNA was present in the cortex although the quantity of the mRNA was much less than that in the medulla of kidney. With 20 μg of total RNA/lane, detection of SMIT mRNA in the cortex took about a week, whereas the mRNA was detected in the medulla with one or two overnight exposure. These results were consistent with our previous results (13). Fig. 1 shows the Northern blot of the time course of increase in renal medullary SMIT mRNA after NaCl loading. Similar results were obtained for the RNAs from the cortex of kidney although the quantity of the mRNA was much less (data not shown). Slot blot assay was used to quantify the abundance of mRNA for the cotransporter (Fig. 2). SMIT mRNA rapidly increased after NaCl injection, reaching a maximum 5 h after the injection. The relative increase in the mRNA abundance was about 9.3 times control levels in the cortex. In the medulla of kidney, the mRNA abundance in control rats was much higher (6.7-fold) than that in the cortex, and the relative increase in the mRNA abundance after NaCl loading was about 4.6 times control levels. Thus, SMIT mRNA was rapidly induced by peritoneal injection of NaCl in vivo in both the cortex and medulla of kidney. In contrast, there were no significant differences in the abundance of ribosomal RNA (Fig. 1), indicating that the same amount of RNA was loaded to each lane.

We next examined the effect of loop diuretics. Intraperitoneal administration of 20 mg/kg of furosemide was performed simultaneously with NaCl injection. Serum Na concentration and osmolality were not changed by furosemide (data not shown), so that those values were significantly higher than controls. Fig. 1 and 3 showed the results of Northern and slot blot analyses. Furosemide dramatically reduced the increase of SMIT mRNA caused by NaCl loading either 2 or 5 h after the injection. When furosemide was injected without NaCl, SMIT mRNA significantly decreased compared to controls either 2 or 5 h after the injection (~ 30–40% of control values). The pattern of the results of the cortical mRNA was similar with less quantity (data not shown).

In situ hybridization of the SMIT probe. Film autoradiograms generated from sections hybridized with the SMIT probe revealed most intense hybridization signal in outer medulla (Fig. 4). There were also significant signals in the inner medulla and papilla of the kidney although these signals were less intense

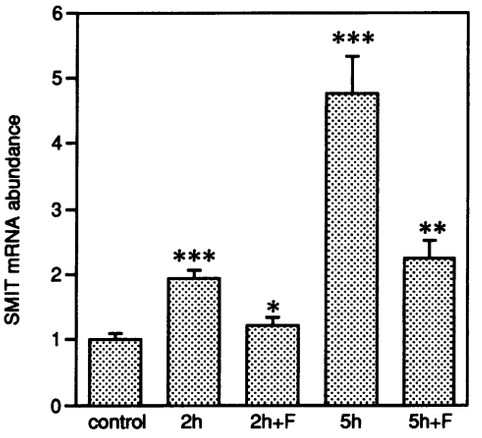

Figure 3. The effects of furosemide on abundance of SMIT mRNA in the medulla of kidney. Intraperitoneal injection of 20 mg/kg of furosemide was performed simultaneously with NaCl loading. Total RNA was extracted after 2 or 5 h. Slot blot assay was done to quantify abundance of SMIT mRNA. Data were abundance relative to abundance of SMIT mRNA in control rats. Each point is mean of three experiments. *2h* or *5h*, 2 or 5 h after NaCl administration. *2h + F* or *5h + F*, 2 or 5 h after NaCl and furosemide administration. * $P < 0.01$ vs. 2 h after NaCl administration. ** $P < 0.05$ vs. 5 h after NaCl administration. *** $P < 0.01$ vs. controls.

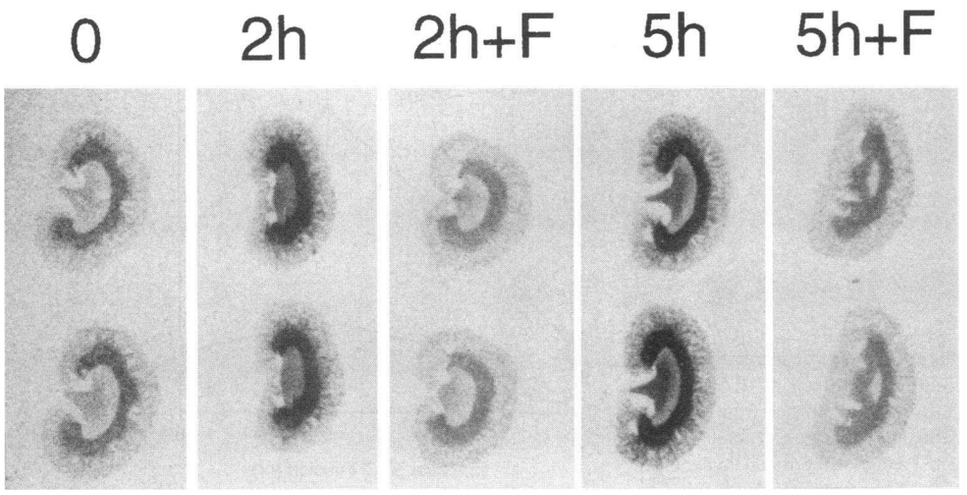

Figure 4. Film autoradiograms illustrating SMIT mRNA distribution in rat kidney. *2h* or *5h*, 2 or 5 h after NaCl administration. *2h + F* or *5h + F*, 2 or 5 h after NaCl and furosemide administration.

than those in the outer medulla. A punctate pattern of the signal was apparent in the cortex of kidney.

As shown in Figs. 5 and 6, microscopic examination of emulsion-coated kidney sections confirmed a higher grain density in the outer medulla than in the inner medulla or papilla (Fig. 5 *A*). The lack of hybridization with control "sense" probe apparently demonstrated that hybridization signal detected was highly specific (Fig. 5 *B*). The SMIT hybridization signals were most intense in the medullary TALH (Fig. 6 *C*). The radial striations running outward from the outer medulla, which were mainly cortical TALH, were apparent in the cortex (Figs. 5 *A* and 6 *A*). Macula densa cells were also labeled in the cortex of kidney (Fig. 6 *A*). Interestingly, the signals of the cortical TALH and macula densa cells seemed to be as intense as those of the medullary TALH. Thus, the distribution of SMIT mRNA in this segment does not correspond to the corticomedullary osmotic gradient.

NaCl loading dramatically induced the expression of SMIT as expected from the results of Northern blot (Figs. 4 and 5 *C*). The medullary TALH had strong signals after NaCl injection (Figs. 5 *C* and 6 *D*). The signals in the cortical TALH were also increased. The magnitude of the increase in the SMIT signal appeared to be similar from the medullary to cortical TALH (Fig. 5 *C*). In the control glomeruli, specific hybridization of the SMIT probe appeared to be localized in the macula densa cells (Fig. 6 *A*). NaCl loading markedly increased SMIT signals in the macula densa cells, with a decreasing gradient of expression to the juxtaglomerular interstitium (Fig. 6 *B*).

The significant SMIT signals in the inner medulla, which were less intense than in the outer medulla, seemed to be localized to the inner medullary collecting ducts (Fig. 6 *E*). These results were consistent with the previous results of *myo*-inositol content using microdissection technique (16). In contrast to SMIT in the TALH, the signals in the inner medulla were not induced by NaCl infusion very much (Fig. 6 *F*), and furosemide was not so effective as TALH (Fig. 5 *D*).

Furosemide administration (20 mg/kg i.p.) abolished the increase in SMIT mRNA by NaCl injection (Figs. 4 and 5 *D*). The attenuation of the increased mRNA was universally seen in the medullary and cortical TALH and macula densa cells, either 2 or 5 h after the treatment.

Discussion

Our results indicate that SMIT mRNA highly expressed throughout the TALH and is induced by acute administration of NaCl. The induction of SMIT was universally seen in the medullary and cortical TALH, and macula densa cells. Since SMIT mRNA is regulated in an osmolality-dependent manner (8), there seems to be a discrepancy between the expression pattern of SMIT and the putative regional osmolality in vivo. It is generally accepted that there is a longitudinal osmotic gradient from medulla to cortex. The SMIT expression, however, does not correspond the corticomedullary osmotic gradient. Our main issue is how to explain this discrepancy.

The TALH actively reabsorbs sodium chloride from the tubular fluid. Since this epithelium is virtually completely impermeable to osmotic flow of water, the tubular fluid emerging from the end of this segment in the cortex is very dilute. This hypotonicity of tubular fluid at the end of the thick ascending limb is achieved regardless of whether the kidney produce a dilute or a concentrated urine. Thus, it is quite unlikely that the expression of SMIT reflect the luminal (apical) osmolality. The results that furosemide administration reduced the expression of SMIT further supports this notion because furosemide increases osmolality of tubular fluid (19).

It is likely that SMIT expression changes with the magnitude of NaCl reabsorption from the TALH or macula densa cells. The $Na^+/K^+/2Cl^-$ transporter is mainly responsible for the uptake in this segment. There is evidence showing that intracellular electrolyte concentrations is increased in the TALH cells in the outer medulla by acute administration of hypertonic NaCl (20). In the case of sorbitol, another organic osmolyte, the change of the intracellular ionic strength ($Na^+ + K^+$) is thought to be the trigger for stimulation of aldose reductase gene because the increase in aldose reductase was well correlated with the change of ionic strength (21). Although the concentration is not greatly increased, the small change of intracellular Na^+ concentration or ionic strength may be a signal to induce SMIT expression.

The mechanism of luminal NaCl uptake are energized by the action of the ($Na^+ + K^+$)-pump located in the basolateral membrane. We assume that the osmolality close to basolateral plasma membrane of the tubular cells, that is peritubular osmol-

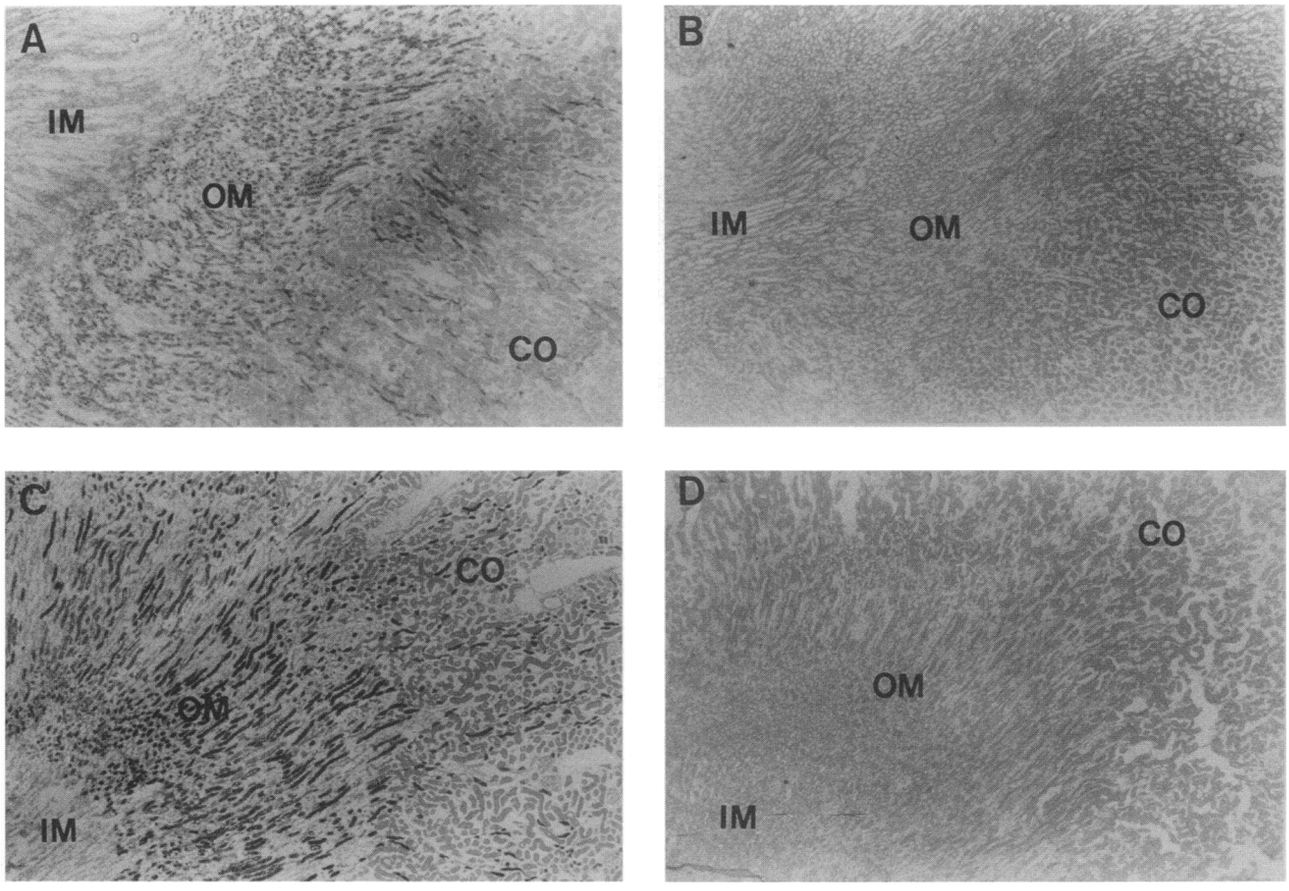

Figure 5. Bright field photomicrographs demonstrating the pattern of in situ hybridization of ^{35}S-labeled rat SMIT antisense or sense cRNA probe to a cryosection of paraformaldehyde-fixed rat kidney (×10). (*A*) Control rat with antisense cRNA probe; (*B*) sense probe control; (*C*) 5 h after NaCl administration (antisense cRNA probe); (*D*) 5 h after NaCl and furosemide administration (antisense cRNA probe). The hybridization signal of SMIT is predominantly present in the outer medulla and at a lower level in the inner medulla. The radial striations running outward from the outer medulla were seen in the cortex.

ality (22), is very high throughout the TALH since this segment is essentially impermeable to water. The peritubular osmolality is supposed to change with the magnitude of NaCl reabsorption. Our results can be easily explained by this hypothesis. When NaCl is loaded, reabsorption of the salts increases. The increased reabsorption of NaCl results in the elevation of intracellular and/or peritubular osmolality, which induces the SMIT expression throughout this segment.

There is direct evidence of peritubular hypertonicity in case of macula densa cells. Basolateral side of the cells is contact with juxtaglomerular interstitium, which is wedge-shaped compartment formed by the afferent and efferent arterioles. It has been shown in Amphiuma that the chloride concentration or osmolality of juxtaglomerular interstitium is very high and changed with the tubular flow rate (15). They found that Cl^- concentration of the juxtaglomerular interstitium exceeded plasma levels (~ 100 mM) at all tubular flow rates and increased to over 600 mM as flow rate increased. Thus, NaCl transport into the interstitium at the glomerular vascular pole proceeds through the water impermeable tubular epithelium, which results in basolateral hypertonicity during elevated flow rates. Because the mechanism of NaCl absorption across macula densa cells are at least qualitatively similar to that of TALH cells, we assume that the osmolality close to the TALH cells are also hypertonic.

A group of the juxtaglomerular interstitial cells are referred to as extraglomerular mesangial cells (23) because these cells are morphologically indistinguishable from mesangial cells and actually extend into the stalk of mesangium. We have recently reported that cultured rat mesangial cells accumulate *myo*-inositol in response to extracellular hyperosmolality and that they have Na^+/*myo*-inositol cotransporter that is regulated by tonicity (14). It is probable that the increased *myo*-inositol and SMIT mRNA observed in dehydrated renal cortex was at least partly derived from mesangial cells. As shown in Fig. 6, SMIT expression was clearly seen in the juxtaglomerular region and was induced by NaCl injection. The expression seems to be relatively high in the macula densa, with a decreasing gradient of expression to the juxtaglomerular interstitium. Although tubular cells (macula densa cells) have strong signals for SMIT, extraglomerular mesangial cells, which are just close to the tubular cells, appear to express SMIT that is induced by NaCl injection. Thus, the results of in situ hybridization are consistent with our previous in vitro studies using mesangial cells.

Furosemide acts primarily in the loop of Henle. The diuretic effect of furosemide is mainly due to its ability to bind and

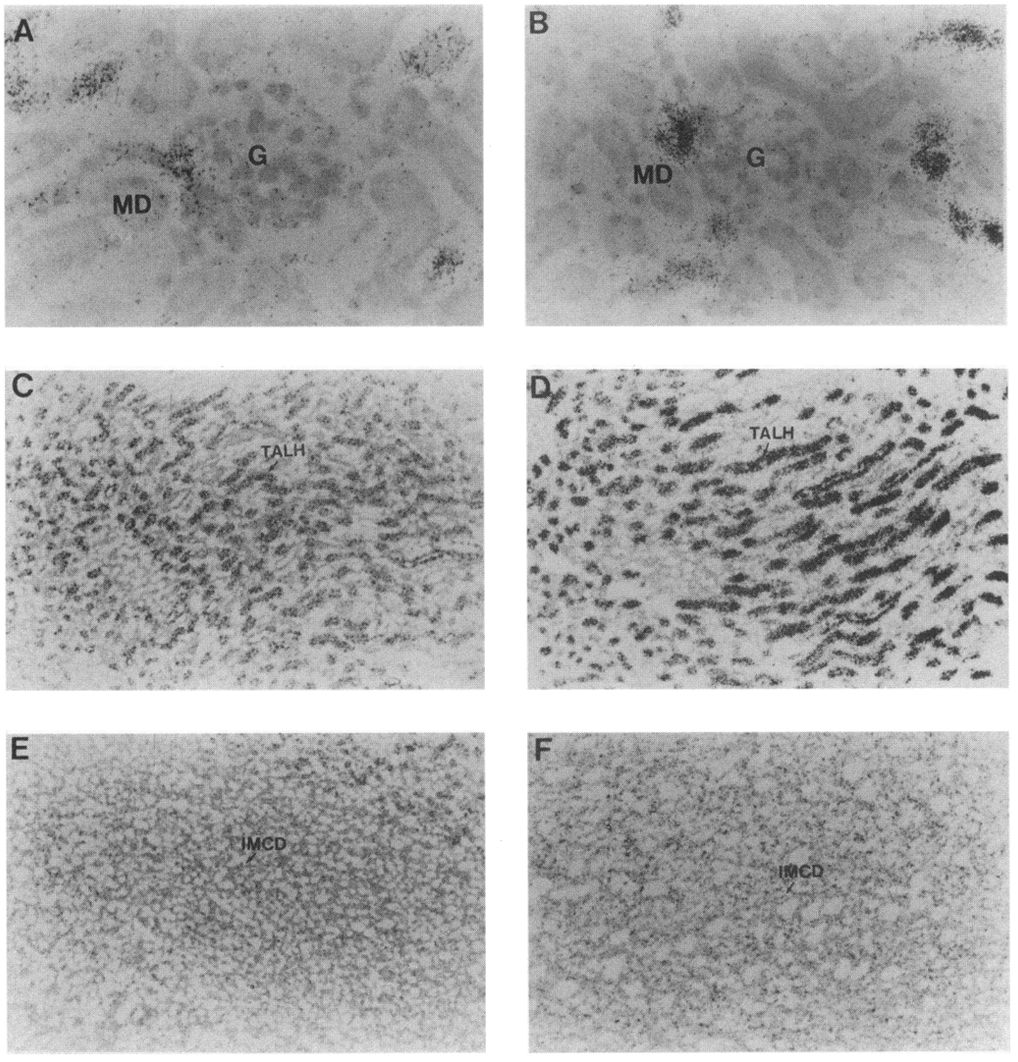

Figure 6. Localization of SMIT mRNA in the kidneys of control (*A, C,* and *E*) and NaCl loaded (*B, D,* and *F*) rats. (*A* and *B*) Cortex of kidney including glomerulus (*G*) and macula densa cells (*MD*). Intense signals were observed in the juxtaglomerular region and cortical thick ascending limbs of Henle's loop but not in the glomerulus (×400). (*C* and *D*) Outer medulla of kidney. Intense hybridization signals were seen in the medullary thick ascending limbs of Henle's loop (TALH) and were markedly induced by NaCl injection (×100). (*E* and *F*) Inner medulla of kidney. Significant but less intense signals were seen in the inner medullary collecting ducts (IMCD). SMIT in this area was not so sensitive to NaCl loading as TALH (×100).

inhibit the $Na^+/K^+/2Cl^-$ transporter, which located at the luminal plasma membrane of the TALH and macula densa cells. Micropuncture of the early distal convoluted tubule reveals that furosemide increases luminal osmolality as well as Na, Cl, and K concentrations, and inhibit water absorption (19). Because SMIT expression was diminished by furosemide, it is apparently independent on the luminal osmolality. The decreased expression by furosemide suggested that the intracellular and/or basolateral osmolality was reduced by means of inhibition of NaCl reabsorption from the lumen.

SMIT in the inner medulla or papilla, which is probably localized in collecting duct cells, is apparently less intense than outer medulla. We are not sure that the tonicity in the vicinity of TALH is really higher than interstitial NaCl concentration in the papilla. It is possible that TALH cells may express SMIT higher than collecting duct cells when the extracellular osmolality is similar. Thus, the difference in the expression level between inner and outer medulla may be simply due to the difference in the type of cells.

SMIT in IMCD is less sensitive to NaCl or furosemide administration, compared to that in TALH. It has been demonstrated that administration of hypertonic NaCl increases loop flow, which results in the increased reabsorption of NaCl from the TALH (23). The increased NaCl reabsorption is supposed to elevate intracellular and peritubular osmolality very rapidly. To increase SMIT expression in the inner medulla, in contrast, the tonicity of the inner medullary interstitium should be elevated. It will take time to change the tonicity of the interstitium and single injection of NaCl may not be enough to increase the tonicity.

In summary, we examined localization of SMIT mRNA and its rapid regulation by NaCl and furosemide administration. SMIT is predominantly present in the TALH and macula densa cells. Less intense signals were seen in the IMCD. NaCl loading

increased the signals throughout the TALH, and furosemide reduced the signals. The unique distribution and regulation pattern suggests that SMIT expression is regulated by intracellular and/or basolateral tonicity, which is supposed to be high throughout the TALH and change with NaCl reabsorption.

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