

Effect of Chronic Salt Loading on Adenosine Metabolism and Receptor Expression in Renal Cortex and Medulla in Rats

Ai-Ping Zou, Feng Wu, Pin-Lan Li, Allen W. Cowley, Jr

Abstract—Previous studies have shown that chronic salt loading increased renal interstitial adenosine concentrations and desensitized renal effects of adenosine, a phenomenon that could facilitate sodium excretion. However, the mechanisms responsible for the increased adenosine production and decreased adenosine response are poorly understood. This study examined the effects of the dietary high salt intake on adenosine metabolism and receptor expression in the renal cortex and medulla in Sprague Dawley rats. Fluorescent high-performance liquid chromatography analyses were performed to determine adenosine levels in snap-frozen kidney tissues. Comparing rats fed a normal (1% NaCl) versus high salt (4% NaCl) diet, renal adenosine concentrations in rats fed a high salt diet were significantly higher (cortex: 43 ± 3 versus 85 ± 4 , $P < 0.05$; medulla: 183 ± 4 versus 302 ± 8 nmol/g wet tissue, $P < 0.05$). Increased adenosine concentrations were not associated with changes in the 5'-nucleotidase or adenosine deaminase activity, as determined by quantitative isoelectric focusing and gel electrophoresis. Western blot analyses showed that a high salt diet (4% NaCl for 3 weeks) downregulated A_1 receptors (antinatriuretic type), did not alter A_{2A} and A_{2B} receptors (natriuretic type), and upregulated A_3 receptors (function unknown) in both renal cortex and medulla. The data show that stimulation of adenosine production and downregulation of A_1 receptors with salt loading may play an important role in adaptation in the kidney to promote sodium excretion. (*Hypertension*. 1999;33[part II]:511-516.)

Key Words: adenosine ■ 5'-nucleotidase ■ adenosine deaminase ■ salt intake ■ kidney ■ isoelectric focusing ■ gel electrophoresis

The role of adenosine in the regulation of renal vascular tone and tubular function has been studied extensively. Adenosine produces a prompt, transient fall in renal blood flow and a decrease in glomerular filtration rate (GFR) when infused into the renal artery. It has been reported that the adenosine-induced decrease in GFR is associated with a fall in glomerular hydrostatic pressure resulting from preglomerular vasoconstriction and postglomerular vasodilation.^{1,2} Adenosine A_1 receptor mediates preglomerular vasoconstriction, and A_2 receptor mediates postglomerular vasodilation.¹⁻⁴ Infusion of adenosine into the renal artery also produces diuresis and natriuresis in rats.⁵ Micropuncture studies have indicated that adenosine inhibits sodium reabsorption in the loop of Henle.⁶ With the use of isolated, perfused tubules and cultured tubular cell lines, adenosine has been reported to alter ion transport in the collecting duct^{7,8} or the thick ascending limb,⁹ indicating that adenosine may have a direct effect on tubules. Moreover, adenosine increases medullary blood flow, which also plays an important role in mediating the diuresis and natriuresis.¹⁰

The role of endogenous adenosine in the signal transmission of the tubuloglomerular feedback (TGF) response may

be of substantial importance.¹¹ Adenosine is produced in response to increased metabolic activity associated with tubular active transport and induces vasoconstriction in the afferent arterioles, which reduces GFR. Decrease in GFR results in the energy-sparing effect on tubular transport, because the reduction of the solute delivered to the tubular epithelium may decrease the tubular transport activity. Therefore, adenosine production generally mediates changes in vascular resistance that maintain a constant GFR. This hypothesis was extended to the single-nephron level and led many investigators to propose that adenosine may be the mediator of preglomerular vasoconstriction in the TGF.^{4,11}

Adenosine is produced primarily by a 5'-nucleotidase (5'-ND)-catalyzed dephosphorylation of 5'-AMP in response to tubular metabolic activity in the kidney. The expression of 5'-ND activity has been reported in cytosol and membrane throughout the renal nephron,⁴ indicating that 5'-ND-mediated production of adenosine may be an intrarenal adaptive mechanism to the tubular activity. Adenosine is mainly catabolized by deamination to inosine by intracellular and extracellular adenosine deaminase (ADA).⁴ Despite the physiological importance of adenosine in the control of renal

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function, little is known regarding changes in adenosine metabolism in the kidney under different circumstances. Given that renal adenosine participates in the regulation of sodium excretion, a high salt intake may alter the adenosine metabolism to adapt to high salt loading. Recent studies have indicated that renal interstitial adenosine levels increase during high salt intake.¹² However, the mechanism underlying changes in renal adenosine levels has not yet been defined.

Recently, adenosine receptors have been cloned and designated A₁, A_{2a}, A_{2b}, and A₃ receptors.^{13,14} However, the role of different adenosine receptor subtypes, especially two recently characterized subtypes, A_{2b} and A₃ receptors, in mediating the renal effects of adenosine is far less clear, and the expression of adenosine receptor subtypes in response to various stimulations has yet to be determined. Previous studies have indicated that the renal effects of adenosine are modulated or desensitized by chronic salt loading to facilitate water and sodium excretion.^{15,16} However, the mechanism leading to desensitization of renal adenosine responses remains unknown. The purpose of the present study was to examine whether chronic salt loading alters the adenosine production and results in downregulation or upregulation of different adenosine receptor subtypes. We also explored the possible mechanism responsible for increased adenosine production in the kidney during chronic salt loading.

Methods

Animals

Male Sprague Dawley rats were purchased from Harlan (Madison, WI) and housed in the Animal Resource Center at the Medical College of Wisconsin. The rats were fed pellet diets with normal salt (1% NaCl) and high salt (4% NaCl) for 3 weeks, and water was provided *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

Adenosine Extraction and HPLC Assay

The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg). A midline incision was made, and the kidneys were exposed, snap frozen *in situ* with aluminum tongs precooled to the temperature of liquid N₂, and rapidly removed. The frozen kidneys were thawed, and the renal cortex and medulla were dissected at 0°C to 4°C. Given the difficulty of separating the renal outer and inner medulla in snap-frozen kidneys, the whole renal medulla including outer and inner medulla was used in these studies, referred to in this paper as the renal medulla. The dissected cortical and medullary tissues were frozen and powdered under liquid N₂. The tissue powder (1 mg) was mixed with 1 mL of 0.6 mol/L perchloric acid by vigorous vortexing and sonicated for 10 seconds at 45 W with use of a microsonicator. Then, the tissue mixture was centrifuged at 8000 rpm for 20 minutes at 4°C. The supernatant was neutralized with 5 mol/L K₂CO₃ to pH 7.2 to 7.4, centrifuged at 3000 rpm to remove precipitates, and stored at -80°C until HPLC analysis. HPLC analysis of adenosine was performed as described previously.¹⁷

Assay of 5'-ND Activity

Renal tissue homogenate was prepared from snap-frozen kidneys as we described previously.¹⁸ Renal cortical and medullary homogenates (20 µg protein) were mixed with 10 µL of 0.15 mol/L sodium phosphate buffer (pH 6.8), containing 0.75% Zwittergent 3-14 (Calbiochem) and sonicated 3 times for 15 seconds at 45 W with use of a microsonicator. The reaction mixtures were incubated in the

presence of 0.75% Zwittergent 3-14 overnight at 4°C to release 5'-ND from bound membrane lipid. A positive reaction with 1 mU purified 5'-ND protein (Sigma) was performed to serve as reference. Then, 5'-ND in the reaction mixtures was separated electrophoretically in 0.5-mm-thick layers of agarose gel (electroendosmosis=0) with a horizontal electrophoresis apparatus (Multi Temp III, Pharmacia Biotech) as described previously.¹⁹ Electrophoresis was performed at 20 V/cm across the gel for 2.5 hours at 4°C. After electrophoresis, the gel was incubated in the reaction solution containing Tris-maleate buffer (pH 7.0, 50 mmol/L), 5'-AMP (1 mmol/L), lead nitrate (2 mmol/L), manganous nitrate (50 mmol/L) at 37°C for 3 hours. After incubation, the gel was rinsed with distilled water, and the reaction bands of the enzyme were made visible with a 2% sodium sulfate solution. Then, the intensity of the enzyme reaction bands on the gel was measured with a densitometer. The activity of 5'-ND was estimated by comparing the densitometric units of unknown samples with that of purified 5'-ND (1 mU) on the same gel. The specificity of 5'-ND assay was confirmed in our previous study²⁰ by omitting the substrate, 5'-AMP, and adding a selective inhibitor of 5'-ND, α,β-methyleneadenosine 5'-diphosphate (5 µmol/L).

Assay of ADA Activity

Renal cortical and medullary homogenates (20 µg protein) were mixed with 10 µL Tris-HCl buffer (10 mmol/L Tris-Cl, 1 mmol/L EDTA, 1 mmol/L mercaptoethanol, pH 7.4) and sonicated for 15 seconds twice at 45 W before loaded on the gel. ADA in the reaction mixture was separated by isoelectric focusing performed in 0.5-mm gels containing 4.85% acrylamide, 0.15% bis-acrylamide, 2% (v/v) preblended ampholine, pH 3.5 to 9.5, 300 mmol/L sucrose, and 2 µmol/L riboflavin. The samples were electrically focused at 150 V/cm at 4°C for 3 hours with use of electrode solutions composed of 150 mmol/L acetic acid for the anode and 150 mmol/L ethanolamine for the cathode. Immediately after isoelectric focusing, the gel was overlaid with 1% agar-Noble gel mixture containing 1.5 mmol/L adenosine, 0.2 mmol/L tetrazolium salt MTT, 0.3 mmol/L phenazine methosulfate, 0.3 U xanthine oxidase, and 3 U nucleoside phosphorylase in 0.1 mol/L sodium phosphate buffer (pH 7.5).²¹ After incubation for 2 hours at 37°C, a blue band representing the ADA activity was exhibited. The activity of ADA was estimated, and the specificity of ADA assay was confirmed as described in the section on the assay of the 5'-ND activity.

Western Blot

Western blot was performed as we described previously.¹⁸ Forty micrograms of protein of the homogenate were subjected to 12% SDS-PAGE and transferred onto nitrocellulose membrane. Then, the membrane was washed and probed with 1:1000 polyclonal antibody against adenosine A₁, A_{2a}, A_{2b}, or A₃ receptors (Alpha Diagnostics, Inc.) and 1:1000 horseradish peroxidase-labeled goat anti-rabbit IgG. All antibodies against adenosine receptors were purified by affinity chromatography. Finally, 10 mL of enhanced chemiluminescence (ECL) detection solution (Amersham) were added, and the membrane was wrapped and exposed to Kodak Omat film.¹⁸ Each membrane was stripped of bound antibodies and reprobed with an anti-β-actin antibody. The intensity (densitometric units) ratio of adenosine receptors to β-actin on the same membrane was calculated and used for quantitative comparison. Protein concentration of the tissue homogenate was measured with a Bio-Rad protein assay kit according to the procedures described by the manufacturer.

Statistics

Data are presented as mean ± SEM. The significance of differences within and between groups was evaluated by use of a two-way ANOVA and Duncan's post hoc test for multiple groups and Student's *t* test for two groups. *P* < 0.05 was considered statistically significant.

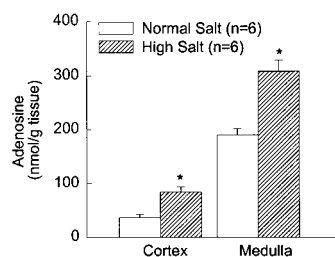


Figure 1. Tissue adenosine concentrations in the renal cortex and medulla from rats fed normal and high salt diets. * $P < 0.05$ compared with rats fed a normal salt diet.

Results

Effects of Chronic Salt Loading on Adenosine Concentrations in the Renal Cortex and Medulla

The results of these experiments are presented in Figure 1. Adenosine concentrations were higher in the renal medulla than in the renal cortex in rats receiving a normal salt diet. In rats fed a high salt diet, adenosine concentrations increased significantly in both renal cortex and medulla.

Effects of Chronic Salt Loading on the 5'-ND Activity in the Renal Cortex and Medulla

Typical gel documents depicting the 5'-ND activity in the renal cortex and medulla are presented in Figure 2A. A reaction band with a molecular size of about 134 kD represented the 5'-ND activity. The intensity of 5'-ND bands was lower in the renal medulla than in the renal cortex, and chronic salt loading had no effect on the 5'-ND activity in both renal cortex and medulla. Figure 2B summarizes the results of these experiments. The 5'-ND activities in the renal cortex and medulla were not statistically different between rats fed normal and high salt diets.

Effects of Chronic Salt Loading on the ADA Activity in the Renal Cortex and Medulla

Typical isoelectric focusing gel documents depicting the ADA activity in the renal cortex and medulla are presented in

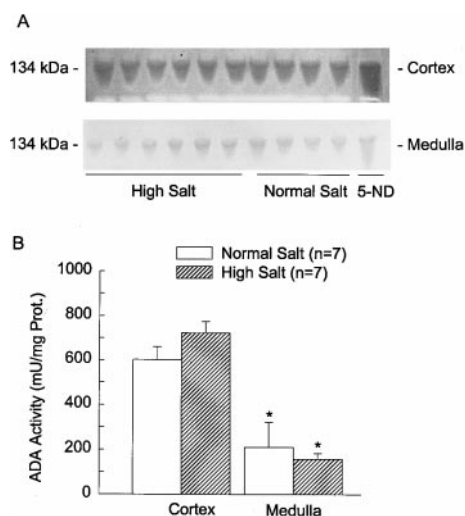


Figure 2. 5'-Nucleotidase (5'-ND) activity in the renal cortex and medulla from rats fed high and normal salt diets. A, Photographs presenting the 5'-ND activity in the renal cortex and medulla. B, A summary of the 5'-ND activity in the renal cortex and medulla. * $P < 0.05$ compared with cortex.

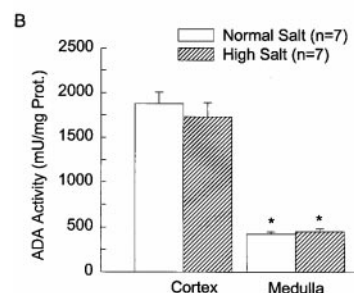
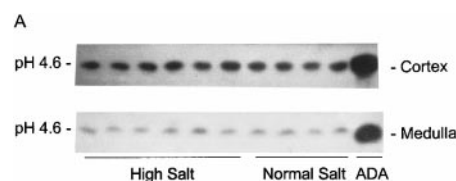


Figure 3. Adenosine deaminase (ADA) activity in the renal cortex and medulla from rats fed high and normal salt diets. A, Photographs presenting the ADA activity in the renal cortex and medulla. B, A summary of the ADA activity in the renal cortex and medulla. * $P < 0.05$ compared with cortex.

Figure 3A. An isoelectric focusing band with pH 4.6 represented the ADA activity. Chronic salt loading did not alter the ADA activity in the renal cortex and medulla. Figure 3B summarizes the effects of the high salt intake on the ADA activity in both renal cortex and medulla. No significant difference in both cortical and medullary ADA activity was found between rats fed normal and high salt diets.

Effects of Chronic Salt Loading on the Expression of Adenosine Receptor Subtypes in the Renal Cortex

Figure 4A presents typical ECL blots of nitrocellulose membrane carrying renal cortical proteins probed with different antibodies against adenosine A_1 , A_{2a} , A_{2b} , and A_3 receptors. An immunoreactive band with 39 kD was identified when the membrane was probed with anti- A_1 receptor antibody. One band of 45 kD ($A_{2a}R$) and 50 kD ($A_{2b}R$) was detected when the membranes were probed with anti- A_{2a} and A_{2b} receptor antibodies, respectively. Specific anti- A_3 receptor antibody recognized a 52-kD protein in the renal cortex. All membranes exhibited a 42-kD immunoreactive band when probed with anti- β -actin antibody (data not shown). The expression of A_1 receptors was decreased, whereas A_3 receptors increased in renal cortical tissue of rats fed a high salt diet, compared with rats fed a normal salt diet. However, the expression of two A_2 receptor subtypes was not different in the renal cortex in rats fed normal and high salt diets. Changes in adenosine receptor expression in renal cortical homogenate in rats fed normal ($n=7$) and high salt ($n=7$) diets are summarized in Figure 4B. The blot intensity ratio of A_1 receptor to β -actin was significantly lower in rats fed a high salt diet than those fed a normal salt diet, and the ratios of A_{2a} and A_{2b} receptors to β -actin were similar. However, the blot intensity ratio of A_3 receptor to β -actin was markedly increased in rats fed a high salt diet compared with those fed a normal salt diet.

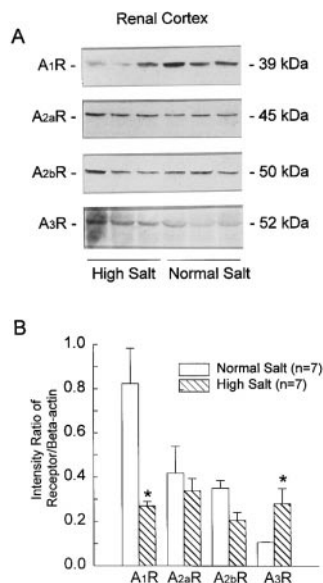


Figure 4. Western blot analyses of adenosine receptor subtypes in the renal cortex from rats fed high (4% NaCl) and normal (1% NaCl) salt diets. A, Typical gel documents of blots. B, A summary of densitometric analyses of blot intensity ratio of adenosine receptors to β -actin. A₁R, A_{2a}R, A_{2b}R, and A₃R represent A₁, A_{2a}, A_{2b}, and A₃ receptors. * $P < 0.05$ compared with rats fed a normal salt diet.

Effects of Chronic Salt Loading on the Expression of Adenosine Receptor Subtypes in the Renal Medulla

Figure 5A presents typical ECL blots of nitrocellulose membrane carrying renal medullary proteins probed with different antibodies against adenosine A₁, A_{2a}, A_{2b}, and A₃ receptors. Four types of antibodies recognized corresponding receptors with molecular sizes similar to those in the renal cortex, but

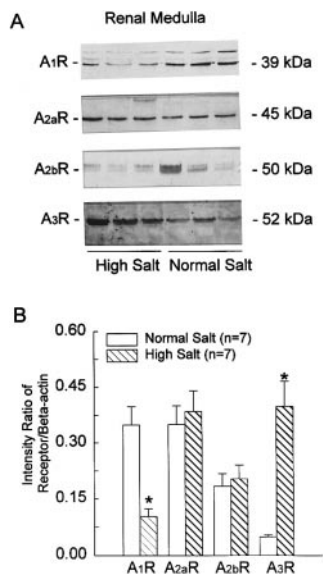


Figure 5. Western blot analyses of adenosine receptor subtypes in the renal medulla from rats fed high salt (4% NaCl) and normal (1% NaCl) diet. A, Typical gel documents of blots. B, A summary of densitometric analyses of blot intensity ratio of adenosine receptor and β -actin. * $P < 0.05$ compared with rats fed a normal salt diet.

an extra immunoreactive band with 31 or 48 kd was detected in the renal medulla when the membrane was probed with anti-A₁ or A_{2b} antibodies, respectively. The identity of these bands was unknown. Similar to the renal cortex, the expression of A₁ receptors was decreased, two subtypes of A₂ receptors unaltered, and A₃ receptors increased in the renal medulla from rats fed a high salt diet, compared with rats fed a normal salt diet. Changes in the blot intensity ratio of adenosine receptors to β -actin between rats fed normal (n=7) and high salt diets (n=7) are summarized in Figure 5B.

Discussion

The present study demonstrated that both renal cortical and medullary adenosine concentrations were higher in salt-loaded rats than in normal rats. These results are consistent with previous findings that adenosine levels in renal interstitial fluid dialyzed from salt-loaded rats were increased.¹² To determine the mechanism by which high salt intake increased renal tissue adenosine levels, we analyzed the enzyme activity of 5'-ND and ADA, which are the major enzymes in the kidney responsible for the adenosine production and metabolism, respectively.⁴ We found no difference in both 5'-ND and ADA activity between salt-loaded and normal rats, suggesting that increase in adenosine concentrations during chronic salt loading is not associated with induction or inhibition of the enzymes. As indicated by a previous study, the high metabolic activity in the kidney during chronic salt loading may increase the substrate of 5'-ND and consequently enhance adenosine production.¹²

It is well known that mammalian kidneys are capable of adaptive responses to high salt intake, which is essential to maintain the constancy of body fluid volume and arterial pressure.^{1,4,22} We propose that increased renal adenosine levels may be implicated in this adaptive mechanism to high salt loading, because adenosine plays an important role in the control of sodium excretion under physiological conditions. However, whether increased adenosine produces antidiuretic or natriuretic effects largely depends on its actions on different receptor subtypes. Previous studies using ligand binding, autoradiography, and measurement of adenylyl cyclase activity have shown that two classical adenosine receptors, A₁ and A_{2a} receptors, are present in the renal cortex, outer medulla, and papilla.^{23,24} These two subtypes of adenosine receptors have also been functionally localized to specific nephron segments including glomeruli, thick ascending limb, and papillary collecting duct.^{24,25} Recent studies with reverse-transcriptase polymerase chain reaction have detected these two subtypes of adenosine receptors in most segments along the nephron and in outer medullary descending vasa recta.^{26,27} It is generally concluded that A₁ and A_{2a} receptors are widely distributed throughout the nephron and renal vasculature.³ It has been demonstrated that stimulation of A₁ receptors produced preglomerular vasoconstriction, activation of tubuloglomerular feedback response, and consequently reduction of GFR and sodium excretion.^{4,28} Recently, works in our laboratory and by others have also shown that A₁ receptor activation may increase cortical and medullary tubular sodium reabsorption.^{10,29} In contrast, A₂ receptor activation dilates pre- and postglomerular vessels and inhibits

tubular sodium reabsorption.^{4,10,29} Therefore, A₁ receptors are considered as an antidiuretic and antinatriuretic adenosine receptor, and A₂ receptors as a diuretic and natriuretic adenosine receptor. To further define the functional significance of increased adenosine during chronic salt loading, we have examined the changes in the expression of adenosine receptor subtypes under these circumstances.

We performed Western blot analyses to quantitatively determine the expression of 4 adenosine receptors including two recently characterized receptors, A_{2b} and A₃, in the renal cortex and medulla of rats. Receptor expression was compared between rats receiving either a normal salt (1% NaCl) or a high salt (4% NaCl) diet for 3 weeks. Four subtypes of adenosine receptors were found to be present in both renal cortex and medulla of all rats. Renal cortical and medullary A₁ receptors were markedly downregulated in rats fed a high salt diet, compared with rats receiving a normal salt diet. However, 2 subtypes of A₂ receptors were not altered, and A₃ receptors were substantially upregulated. These results support the view that adenosine receptors not only initiate the regulation of physiological and biological function, but also are themselves subject to regulatory and homeostatic functions.^{3,4} Alterations of adenosine receptor expression and corresponding functions may be an important intrarenal adaptive mechanism to chronic salt loading. The data indicate that downregulation of A₁ receptors without changes in A₂ receptors may dominate diuretic and natriuretic effects of endogenous adenosine in the kidney. Therefore, downregulation of antinatriuretic A₁ receptors during chronic salt loading may lead to reduction of the response of renal arterioles or tubules to adenosine, thereby facilitating renal sodium and water excretion and maintaining the constancy of body fluid volume and arterial pressure. Both adenosine and A₁ agonist, N⁶-cyclohexyladenosine, failed to produce renal preglomerular vasoconstriction and reduction of GFR in salt-loaded rats. Decline in urine flow and sodium excretion induced by A₁ activation was also absent in these animals.^{15,30} Similarly, micropuncture studies showed that TGF was blunted by chronic salt loading.³¹ Given the importance of adenosine in TGF, the absence of A₁ receptor activation by adenosine may contribute to the resetting of TGF in salt-loaded rats. Taken together, these results indicated that A₁ receptors in the kidney are no longer responsive to stimulation in salt-loaded animals. The present study provides the first direct evidence indicating the possibility that the lack of the response of renal vessels during chronic salt loading is associated with downregulation of A₁ receptors.

We found that A₃ receptors were substantially upregulated in both renal cortex and medulla in salt-loaded rats. The physiological significance of the upregulation of A₃ receptors remains unknown. Recently, A₃ receptors have been identified in different animal tissues, including rat and pig kidneys.^{32,33} This novel adenosine receptor subtype is involved in the release of autocooids or paracrines such as histamine, cytokines, leukotrienes, thromboxanes, and proteases from mast cells and other interstitial cells in response to inflammatory or noninflammatory stimulations. Activation of A₃ receptors has been reported to contribute to the development of asthma and ischemic preconditioning.³³ However, few

physiological functions of A₃ adenosine receptors are known so far. It remains to be determined whether upregulation of A₃ receptors is related to the renal adaptation to chronic salt loading. We assume that A₃ receptor-mediated release of autocooids or paracrines from renal cells is somehow involved in the intrarenal adaptation to chronic salt loading.

The mechanism of differential expression of adenosine receptor subtypes is unknown. Previous studies indicated that acute or chronic pretreatment of adipocytes, smooth muscle cells, and renal tubular cells with A₁ and A₂ receptor agonists decreased their responsiveness to these agonists, suggesting a desensitization of the receptors.³⁴ This agonist-induced receptor desensitization or downregulation of the receptors may be one of the mechanisms by which the high salt intake reduced the expression of A₁ receptors. The finding that renal tissue adenosine levels increased during high salt intake supports this view. It appears, therefore, that agonist-induced receptor downregulation or upregulation is an important mechanism in the regulation of the expression of adenosine receptors, as shown with other receptors such as Ang II and adrenergic receptors.^{35,36}

In summary, we have demonstrated an increase in tissue adenosine levels and a differential expression of adenosine receptor subtypes in the renal cortex and medulla during chronic salt loading. The results indicate that increased adenosine production, downregulation of A₁ receptors, and upregulation of A₃ receptors may be an important intrarenal adaptive mechanism to chronic salt loading.

Acknowledgments

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