Effects of NH₄Cl intake on renal growth in rats: role of MAPK signalling pathway

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Abstract

Background. There is a surprising lack of experimental data on the mechanisms of NH₄Cl-induced chronic metabolic acidosis which causes kidney hypertrophy. The NH₄Cl treatment results in an absolute increase in kidney mass. Despite findings to indicate a close interaction between NH₄Cl-induced chronic metabolic acidosis and renal enlargement, the role of the stimulated serine kinase cascade, mediated by the stepwise activation of extracellular signal-regulated kinase (ERK) signalling, on kidney hypertrophy has not yet been investigated.

Methods. To test this hypothesis, the present study was undertaken to further explore the possible involvement of mitogen-activated protein kinase (MAPK) signalling pathway in renal growth in chronic NH₄Cl-treated rats by western blot analysis.

Results. Our major findings are as follows: (1) Urinary sodium excretion significantly increased during the early phases of NH₄Cl-induced acidosis, (2) This occurrence is associated with sustained renal hypertrophy, and (3) sustained basal phosphorylation of IRS-1, Shc, and MAPK/ERKs in acidotic kidneys.

Conclusions. The present study confirms that NH₄Cl-induced acidosis causes disturbances in renal sodium handling. In addition, these findings demonstrate a sustained pre-stimuli activation of kidney MAPK/ERKs signalling pathways in the NH₄Cl-treated rats that may correlate with an increased rate of kidney hypertrophy and a transient renal tubule inability to handle sodium. Thus, the altered renal electrolyte handling may result from a reciprocal relationship between the level of renal tubule metabolic activity and ion transport. In addition, the study shows that the appropriate regulation of tyrosine kinase protein phosphorylation, and its downstream signal transduction pathway, plays an important role on renal growth in the NH₄Cl-treated rats.

Keywords: IRS-1; kidney growth; MAPK/ERK; metabolic acidosis; NH₄Cl; Shc; sodium excretion

Introduction

There is a surprising lack of experimental data on the mechanisms of metabolic acidosis-induced kidney hypertrophy. The NH₄Cl treatment results in an absolute increase in kidney weight. A growing body of evidence suggests that chronic metabolic acidosis, due to NH₄Cl feeding, leads to nephronal hypertrophy, particularly in the proximal tubule cells of rats [1]. However, acidification in vitro has failed to produce cellular hypertrophy when not caused by ammonium chloride [1], suggesting that the hypertrophy results from the increased production of ammonia rather than from the acidosis per se [2]. An increased rate of ammoniagenesis per nephron characterizes the hypertrophy of renal ablation, protein loading, potassium depletion, and ammonium chloride loading. Since internal pH is perturbed only minimally, increased cellular ammonia availability may act as a stimulus to hypertrophy and may also increase the activity of the Na⁺-H⁺ exchanger, by acting as a substrate for the transporter on its cytoplasmic side.

In the NH₄Cl-induced acidotic rat, we and others have shown that kidney protein synthesis increases, protein degradation falls, and the kidneys enlarge [1,2–4]. These changes in renal protein turnover can be induced by ammonia that is produced in increased amounts in acidosis. It appears that ammonia can stimulate cellular protein synthesis directly [1,3] and
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by altering lysosomal pH and thus depressing cathepsin B and L activity; ammonia also inhibits protein degradation [2,3]. Another possibility is that metabolic acidosis plays a role in kidney growth by modulating hormone and/or peptide receptors and post-receptor pathways in renal tissues.

We have previously demonstrated that one of the earliest cellular responses to stimulation by growth factors is the activation of tyrosine kinase receptors which, when activated, undergo rapid autophosphorylation and phosphorylate intracellular protein substrates, including insulin receptor substrates (IRSs: IRS-1 and IRS-2 are the most important) [5,6], and other cytoplasmic phosphoproteins found in most tissues, including renal tissues [6]. Growth factors stimulate the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK). This pathway involves the tyrosine phosphorylation of IRS proteins and/or cytoplasmic tyrosine protein kinase homology and collagen domain protein (Shc), which in turn interact with the adapter growth factor receptor-bound protein 2, recruiting the Son-of-sevenless exchange protein to the plasma membrane for activation of the G-protein coupled-receptor (Ras). The activation of Ras also requires stimulation of the phosphotyrosine phosphatase 2, through its interaction with receptor substrates such as IRS1/2. Once activated, Ras operates as a molecular switch, stimulating a serine kinase cascade through the stepwise activation of cytoplasmatic serine/threonine protein kinase and ERK. Activated ERK can translocate into the nucleus, where it catalyses the phosphorylation of transcription factors such as p62TCF, initiating a transcriptional programme that leads to cellular proliferation or differentiation [7,8]. Despite findings indicating a close interaction between \( \text{NH}_4\text{Cl} \)-treatment and renal enlargement, the role of the stimulated serine kinase cascade, mediated by the stepwise activation of ERK signalling, on kidney hypertrophy has not yet been investigated. To test this hypothesis, the present study was undertaken to further explore the possible involvement of the MAPK signalling pathway in renal growth in chronic acidic rats, induced by \( \text{NH}_4\text{Cl} \) treatment.

Materials and methods

Experimental design

The experiments were performed in male Wistar-Hannover rats (200–250 g) allowed free access to water and normal rat chow. The general guidelines established by the Brazilian College of Animal Experimentation (COBEA) were followed throughout the investigation. Metabolic acidosis was produced by substituting 0.25 M \( \text{NH}_4\text{Cl} \) for the drinking water. All animals drank \textit{ad libitum} and the volumes ingested were recorded. Only those rats which drank nearly equivalent amounts of water or \( \text{NH}_4\text{Cl} \) were used. \( \text{NH}_4\text{Cl} \)-treated rats were maintained on their respective regimens for 10 days and studied at intervals of 1.5, 3 and 12 h and then again after 120 and 240 h. To correct for the hypophagia induced by \( \text{NH}_4\text{Cl} \) metabolic acidosis, renal function was examined in pair-fed animals maintained on a level of food intake similar to that observed in the acidic rats. Fourteen hours before the renal function evaluation, the rats were housed individually in metabolic cages with free access to tap water but no food. The experiments were performed in parallel for each group of pair-fed and acidic rats. At 8:00 a.m., each animal received a tap water load by gavage (5% of body weight), followed by a second load of the same volume 1 h later. Twenty minutes after the second load, spontaneously voided urine was collected over 120 min into a graduated centrifuge tube. At the end of the experiment, blood samples were drawn through the tail vein or cardiac puncture in anaesthetized rats and the kidneys were immediately removed, decapsulated and weighed. Plasma and urine sodium and potassium concentrations were measured by flame photometry (Micronal, B262, São Paulo, Brazil), while creatinine concentration was determined spectrophotometrically (Instruments Laboratory, Genesys V, USA) [4].

Western blot analysis

The western blot analysis was performed in a particular group of anaesthetized pair-fed or acidic animals, induced as described above. After 10 days of water or \( \text{NH}_4\text{Cl} \) administration, the rats were anaesthetized with pentobarbital sodium (50 mg kg\(^{-1}\) body weight), intraperitoneally, and used as soon as anaesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the vena cava exposed and 0.5 ml of vehicle (0.15 M NaCl) or 0.15 M NaCl containing 10 mM insulin was injected. After 5 min, fragments of kidney were removed, minced coarsely and homogenized immediately in the solubilization buffer containing 100 mM Tris (pH 7.6), 1% Triton X-100, 150 mM NaCl, 0.1 mg aprotinin, 35 mg PMSF/ml, 10 mM Na\(_2\)VO\(_4\), 100 mM NaF, 10 mM Na\(_2\)P\(_2\)O\(_7\), and 4 mM EDTA, using a politron PTA 205 generator operated at maximum speed for 30 s and clarified by centrifugation. Equal amounts of protein were used for immunoprecipitation followed by western blot analysis with the indicated antibodies and 125I-Protein A [6]. The antibodies to IRS-1 (SC-559), phosphotyrosine (SC-508), p-ERK (sc-7383), and Shc (sc-288), were obtained from Santa Cruz.

Creatinine clearance was used to estimate the glomerular filtration rate. Fractional sodium (\( \text{FE}_{\text{Na}} \)) excretion was calculated as \( \text{C}_{\text{Na}}/\text{C}_\text{Cr} \), where \( \text{C}_{\text{Na}} \) is ion clearance and \( \text{C}_\text{Cr} \) is creatinine clearance [4]. Changes in fractional excretion were estimated using the pair-fed values. All data are reported as means ± SEM for 10 rats per group. Data obtained over time were analysed using appropriate ANOVA. Post hoc comparisons between selected means were done with Bonferroni’s contrast test when initial ANOVA indicated statistical differences between experimental groups. Western blot results are presented as comparisons of bands present in the autoradiographs. Results involving only two means within or between groups were compared using a Student’s \( t \)-test. Band intensities were quantified by optical densitometry using Scion Image software (Frederick, MD, USA). A \( P \) value < 0.05 was considered to indicate significance.
Results

Animal characteristics

On day 10, metabolic acidosis was confirmed by a blood pH of 7.16±0.13 and plasma bicarbonate values of 11±3 mM (vs pair-fed pH and bicarbonate values, respectively, of 7.38±0.07 and 23±2 mM). As shown in Table 1, body mass gain in the NH4Cl-treated rats was similar to that observed in pair-fed animals after ten days of follow-up. There was a significant increase in kidney mass but not in oral liquid intake in acidotic animals compared to the other experimental group (Figure 1A). Similarly, the serum potassium levels were significantly higher in the NH4Cl-treated compared to the pair-fed rats (Table 1). The renal function test results for the pair-fed (control) and NH4Cl-treated rats maintained on their respective regimens for 10 days and studied at intervals of 1.5, 3, and 12 h and then again after 120 and 240 h are shown in Figure 1B and 1C. Metabolic acidosis caused a sustained and significant increase in renal fractional sodium excretion compared to the pair-fed rats (Figure 1C). These differences occurred despite an unchanged creatinine clearance and sodium filtered load.

In vivo effect of insulin on tyrosine phosphorylation of IRS-1, Shc, and ERK in the kidney of acidotic rats

As shown in Figure 2, the basal levels of phosphorylation of IRS-1 were higher (69±5%) in the kidneys of NH4Cl-treated rats compared to pair-fed animals (P<0.01). Insulin infusion into the vena cava induced increases in IRS-1 tyrosine phosphorylation levels in the kidney of pair-fed rats, but not in the kidneys of NH4Cl-treated animals. In the pair-fed animals, insulin increased IRS-1 tyrosine phosphorylation by 72±5%. The protein expression of IRS-1 in the kidney from pair-fed and acidicotic rats was quantitated by immunoblotting with zIRS-1 antibodies and no differences were detected between the kidneys of NH4Cl-treated animals and pair-fed rats (data not shown).

The Shc phosphorylation paralleled the changes in phosphorylation of IRS-1 (Figure 3). A Shc striking phosphorylation band appears after insulin administration in pair-fed rats (61±7% higher than control, P<0.001). In addition, the basal levels of phosphorylation of this Shc band were increased by 53±8% (P<0.05) and Shc phosphorylation was unchanged in

Table 1. Body and renal weight, liquid intake, and serum sodium and potassium levels in 10-day NH4CL-treated compared to pair-fed (PF) rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body mass (g)</th>
<th>Renal mass (g)</th>
<th>Liquid intake (ml)</th>
<th>Na+ (mM)</th>
<th>K+ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF</td>
<td>225±3.6</td>
<td>1.40±0.05</td>
<td>34.8±0.5</td>
<td>142±1.2</td>
<td>3.5±0.1</td>
</tr>
<tr>
<td>NH4</td>
<td>232±4.3*a</td>
<td>1.73±0.05*a</td>
<td>35.7±0.7</td>
<td>143±0.4</td>
<td>4.4±0.1*a</td>
</tr>
</tbody>
</table>

Data are reported as means±SEM for 10 rats per group. *P<0.01 compared to pair-fed (PF) rats (ANOVA).
acidotic rats after insulin injection in the kidney of NH$_4$Cl-treated rats. There was no change in Shc protein expression (data not shown).

We next examined the expression and phosphorylation of MAPKs. The levels of ERK1 and ERK2 expression were similar at all times (Figure 4). The ERK-1 and ERK-2 basal phosphorylation increases significantly (82±10%) after 10 days of NH$_4$Cl treatment in rats. Sustained activation of ERKs is observed in pair-fed and acidotic rats after insulin administration.

**Discussion**

This study was designed to further our understanding of the changes that occur in the kidney in response to metabolic acidosis and that cause the kidney to enlarge. In the first part of the study, we set out to confirm the metabolic acidosis-induced disturbances upon renal sodium handling. The present findings are in agreement with our, and other, previous studies [4,9,10] demonstrating a transient decreased sodium transport in the presence of a reduced pH. Fractional sodium excretion was increased in acidic animals, although the filtered sodium load was similar to that of pair-fed rats. Sartorius et al. [10] reported the occurrence of natriuresis in the early phases of NH$_4$Cl-induced acidosis in humans. In rats and dogs undergoing metabolic acidosis, there is a decrease in the renal tubular reabsorption of salt and water [9,10]. Our previous and micropuncture studies have localized the site of this depressed sodium reabsorption to the proximal tubule and post-proximal tubule segments in the absence of any change in creatinine clearance [7,8,20]. A reduction in the proximal tubular reabsorption of salt and fluid in rats has been correlated with a reduced bicarbonate concentration in the peritubular capillaries [9,10].

*In vivo*, the nephron mechanism and the site of the renal sodium handling abnormalities have not been identified. In addition, the metabolic acidosis induced by NH$_4$Cl resulted in an absolute increase in kidney weight. These data are consistent with *in vitro* studies of proximal tubule cells [3], which showed that cellular hypertrophy was accompanied by a marked decline in protein degradation, but not in cell protein synthesis, after the administration of NH$_4$Cl. Acidification *in vitro* has failed to produce cellular hypertrophy when not caused by ammonium chloride [1,2], suggesting that the...
hypertrophy results from the increased production of ammonia rather than from the acidosis per se [2]. An increased rate of ammoniagenesis per nephron characterizes the hypertrophy of renal ablation, protein loading, potassium depletion, and ammonium chloride loading. Since internal pH is perturbed only minimally, progressively increased cellular ammonia availability, after the administration of NH₄Cl, may act as a stimulus to hypertrophy and cell transporter expression and/or may also increase the activity of the ion exchanger by acting as a substrate for the transporter on its cytoplasmic side. However, a causal relationship remains to be established. Studies have demonstrated that thyroid hormone stimulates this antiporter without causing hypertrophy, indicating that activation of Na⁺-H⁺ exchange activity does not, per se, initiate the growth response [11]. Previous studies have shown that alterations in the acid–base balance modify renal gluconeogenesis. Metabolic acidosis stimulates gluconeogenesis in a variety of preparations by increasing the level of phosphoenolpyruvate carboxykinase mRNA and hence enzyme activity [12]. There is considerable evidence that gluconeogenesis and the reabsorption of Na⁺ are reciprocally related. Thus, American opossum kidney (OK) cells respond to acidosis with increased glutamine metabolism and ammonium formation [13].

In these cells, acidosis decreases the activity of the Na⁺-H⁺ exchanger, thereby increasing intracellular H⁺ [14]. Studies using isolated proximal tubules have shown that enhanced glutamine metabolism and ammonia production are linked to increased gluconeogenesis [13]. On the other hand, maneuvers that inhibit Na⁺-K⁺-ATPase, and hence sodium tubule transport by the kidney, stimulate gluconeogenesis [15]. Since metabolic acidosis results in a lower filtered load of bicarbonate and consequently in less bicarbonate being reabsorbed, and since decreased Na⁺-H⁺ antiporter activity is associated with stimulated gluconeogenesis, the overall effect should be dissipation of the Na⁺ electrochemical gradient, leading to a decreased reabsorption of Na⁺ as observed in the present study. As a preliminary, the present findings suggest that in energy-requiring processes, renal growth, sodium transport, and possibly gluconeogenesis may compete for the available energy in nephron tubules and could explain the striking natriuresis observed in our rats.

In the second part of the study, we addressed the question of the possible involvement of the modulation of the MAPK signalling pathway by hormone and/or peptide receptors and by post-receptor protein expression on renal hypertrophy, induced by chronic NH₄Cl-treatment.

In the present study, we demonstrated a sustained pre-stimuli activation of MAPK/ERKs pathways in NH₄Cl-induced metabolic acidotic kidney of rats that may correlate with an increased rate of kidney hypertrophy and a transient renal tubule inability to handle sodium. Our major findings are as follows: (1) A significant but transient increase in urinary sodium excretion occurs during the early phases of NH₄Cl-treatment, (2) this occurrence is associated with sustained renal hypertrophy, and (3) sustained basal phosphorylation of IRS-1, Shc, and MAPK/ERKs in acidotic kidneys. In this series of experiments, further activation of IRS-1 tyrosine phosphorylation was demonstrated to be reduced in kidneys from acidotic rats (Figure 2). Up to this step of the signalling cascade studied (Shc and ERK expressions), the data are similar to those of the control study. Although the rationale for the induction of increased IRS-1 phosphorylation in NH₄Cl-treated animals before insulin administration remains unknown, speculatively, we raise the possibility that, under acidic conditions, a specific non-insulin receptor activation may occur, promoting rapid and parallel tyrosine phosphorylation or downstream serine/threonine phosphorylation, decreasing the responsiveness of the insulin receptor β-subunit tyrosine kinase. Our data indicate that the under-stimulation of IRS-1 phosphorylation, after insulin injection, may be explained by the basal phosphorylation state of this protein. In addition, basal phosphorylation of IRS-1 by NH₄Cl administration may initiate downstream signalling of transcription factors involved in increased gene expression and renal hypertrophy. Tyrosine kinase proteins play a pivotal role in integrin and growth factor signalling pathways. Protein tyrosine phosphorylation is a dynamic reversible process in
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which the level of phosphorylation, at any time, is the result of kinases and/or phosphatases activity. Altered regulation of interaction between kinases and phosphatases induced by NH₄Cl treatment, may lead to organ growth and/or differentiation. MAPKs, including extracellular signal-regulated kinases (ERKs) play important roles in the cell by transmitting extracellular signals from the cell membrane to the nucleus [8]. MAPKs are activated by various stimuli, influencing cell proliferation, differentiation, and apoptosis. The present study provides the first evidence that regulation of MAPKs expression may be profoundly altered by metabolic acidosis.

The role of peptides, hormones, and their molecular signal transduction pathways in renal nephrogenesis and renal hypertrophy have not been elucidated. Previous studies have demonstrated that chronic metabolic acidosis causes severe growth retardation, muscle wasting, decreased albumin synthesis, and a negative nitrogen balance [16]. These manifestations are partly caused by reduced food intake and by changes in hormonal balance. Especially important are changes in the growth hormone–IGF-I axis, a system that promotes body and organ growth and anabolism [17,18]. The main source of circulating IGF-I is the liver, but IGF-I is also produced in tissues throughout the body. Production of IGF-I is nutrient-sensitive and is largely regulated by growth hormone (GH).

In chronic metabolic acidosis, pulsatile GH secretion is attenuated, and hepatic IGF-I gene expression and serum IGF-I levels fall [16–18]. Since IGF-I has been implicated as a mediator of renal hypertrophy in other conditions such as diabetes and hypokalaemia, where growth retardation and muscle wasting are present, we may suppose that IGF-I might be playing a role in the renal hypertrophy of acidosis. Indeed, Bereket et al. [17] reported that 4 h after the onset of acute acidosis, renal IGF-I levels increase. However, it is not known whether this increase is sustained long enough for IGF-I to serve as a mediator of the renal hypertrophy that is first apparent after 3 days of acidosis [18].

In addition, there is a surprising lack of experimental data on the mechanisms of metabolic acidosis-induced disturbances in renal sodium handling and how this may be related to kidney hypertrophy. Tong and Stockand, demonstrated that growth factors decrease sodium reabsorption across nephron epithelia, mediated by tyrosine kinase receptors [19]. Activity of the epithelial Na⁺ channel (ENaC) is limiting for Na⁺ transport in different portions of nephron. In this case, abnormal ENaC opens probably by decreasing membrane phosphatidylinositol 4,5-bisphosphate levels. Additionally, Na-K ATPase inhibition has been associated with signal transduction, triggering growth, and proliferation of cultured cells [20]. These effects on Na-K ATPase seem to be related to molecular mechanisms involved in cellular hypertrophy and activation of ERK1/2 [19].

In summary, chronic NH₄Cl treatment of rats leads to an increased renal mass and ion excretion. This altered renal Na⁺ and K⁺ handling may result from a reciprocal relationship between the level of metabolism of renal tubules and ion transport. Furthermore, the present study shows that the appropriate regulation of tyrosine kinases protein phosphorylation and its downstream signal transduction pathway play an important role in renal growth during chronic metabolic acidosis in rats. Further studies are required to establish the influence of NH₄Cl treatment on renal growth and on renal function.

References


*Received for publication: 10.2.05*

*Accepted in revised form: 12.8.05*