The Thiazide-Sensitive Co-Transporter Promotes the Development of Sodium Retention in Mice with Diet-Induced Obesity

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Key Words
NCC • Diet induced obesity • Obesity related hypertension • Sodium reabsorption

Abstract
Background/Aims: Intravascular volume expansion due to sodium retention is involved in the pathogenesis of obesity-related hypertension. Institution of high fat diet (HFD) feeding leads to an initial state of positive sodium balance due to enhanced tubular reabsorption of sodium, but which tubular sodium transporters are responsible for this remains undefined. Methods: C57/Bl6 mice were fed control or HFD for 3 weeks. Blood pressures were recorded by tail cuff method. Sodium transporter expression and phosphorylation were determined by Western blotting. In vivo activity of NCC was determined using natriuretic responses to hydrochlorothiazide. Expression of NCC mRNA was determined using qPCR. Results: At 3 weeks HFD mice had significant weight gains compared to control mice, but blood pressures were not yet elevated. There were no changes in expression or phosphorylation of the bumetanide-sensitive cotransporter, NKCC2, or in expression of subunits of the amiloride-sensitive ion channel, ENaC. However, there were significant increases in mRNA and protein expression of the thiazide-sensitive co-transporter, NCC, in kidneys from HFD mice. Consistent with this, HFD mice had increased in vivo activity of NCC. Conclusions: Increased expression of NCC promotes the sodium loading response to institution of HFD feeding before onset of hypertension.
Introduction

Obesity-related hypertension (ORH) is characterized by an increase in circulating volume due to renal sodium retention [1]. Animal models of diet induced obesity (DIO) have shown that initiation of high fat diet (HFD) feeding leads to a state of positive sodium balance [2]. Following this initial period of sodium-retention, a new steady state is reached in which blood pressure is elevated but sodium balance returns to neutral. The excess sodium, however, is not excreted, despite the elevation in blood pressure, due to a resetting of the pressure-natriuresis mechanism [1]. Hence, there are distinct periods where sodium overload is, firstly, established and then maintained despite the resulting hypertension. Whilst both of these processes involve enhanced tubular reabsorption of sodium [1, 3, 4], it is not known whether the same tubular mechanisms are responsible for both phases.

The bumetanide-sensitive cotransporter, NKCC2, and the thiazide-sensitive cotransporter, NCC, are members of the cation chloride co-transporter family. NKCC2 is found in the loop of Henle and NCC is found in the distal convoluted tubule. They are responsible for the reabsorption of approximately 20% and 5-10% of filtered sodium, respectively, under basal conditions [5]. The amiloride-sensitive ion channel, ENaC, is found in the collecting duct and is responsible for reabsorption of approximately 3% of filtered sodium. It exists as a heterotrimer of α, β and γ sub-units [6].

We have recently demonstrated that, after 14 weeks of HFD feeding, C57Bl/6 mice have enhanced activity of NKCC2 due to increased phosphorylation of this cotransporter [7]. At this time-point, mice have established hypertension. We hypothesized that there are likely to be different tubular mechanisms involved earlier in the establishment of ORH, during the period of positive sodium balance. In the present study, therefore, we have examined the profile of the most important distal sodium transporters in mice fed a HFD at the earlier time-point of 3 weeks, before the onset of hypertension. By this means, we hoped to identify the sodium transporters responsible for establishing sodium overload in response to a HFD.

Materials and Methods

Antibodies

Rabbit Antibodies (Ab) pNCCT58 and pNKCC2T96/T101 [8] and pNKCC2S126 [9] have previously been described. ‘T4’ Mouse Ab against NKCC1/2 was obtained from Developmental Studies Hybridoma Bank (University of Iowa, USA). Rabbit Abs against α-ENaC, β-ENaC and γ-ENaC were obtained from StressMarq Biosciences Inc. (BC, Canada). Rabbit Abs against NCC and p(383/325)SPAK/OSR1 were obtained from Merck Millipore (Darmstadt, Germany). Rabbit antibodies against Beta-Tubulin were obtained from Sigma Aldrich (MO, USA) and pAMPK from Cell Signalling (MA, USA).

Animals

C57Bl/6 mice were maintained under specific pathogen-free conditions with a 12-hour light/12-hour dark cycle and all procedures were carried out in accordance with the regulations set by the Austin Health Animal Ethics Committee. Animals were given free access to food and water. Mouse diets were obtained from Specialty Feeds, Western Australia. Mice were fed control (5% fat modified AIN93G) or high fat (23% fat modified AIN93G diet (43% energy from fat), with 15% increase in NaCl) diet for 3 weeks. The sodium chloride content of the high fat diet was 15% higher than that of the control diet to match sodium chloride intake between groups (we have previously found the mice eat approximately 15% less food by weight of the HFD).

Body weights were measured weekly and at termination. Kidneys were removed from anaesthetized animals and snap frozen in liquid nitrogen.
Blood Pressures

Systolic blood pressure was measured non-invasively in 11-week old mice that had been on a control (n=8) or high fat diet (n=8) for 3 weeks, using Life Science (Woodlands, CA, USA) tail-cuff plethysmography equipment and software. Non-anaesthetized mice were acclimatized to the technique for 2 days prior to recordings used for analysis. Three consecutive readings were taken for each mouse. Mice used for blood pressure recordings were not used in tissue analysis experiments.

Western blot analysis

Kidneys were excised rapidly and snap frozen in liquid nitrogen. Kidneys (n=8 per group) were sliced in half transversely. Tissue was sliced from the superior pole for preparation of cortical preparations. The inferior half of the kidney was used for ‘whole kidney’ (cortex and medulla) preparations. Lysates were prepared using a glass on glass Dounce homogenizer in lysis buffer (50mM Tris/HCl, pH 7.5, 1mM EGTA, 1mM EDTA, 50mM sodium fluoride, 5mM sodium pyrophosphate, 1mM sodium orthovanadate, 1% (w/v) NP-40, 0.27M sucrose, 0.1% (v/v) 2-mercaptoethanol, and protease inhibitor cocktail (1 tablet per 10 ml; Roche Diagnostics, Basil, Switzerland). Homogenates were centrifuged at 10,000 g for 15 min at 4°C and protein concentration in the supernatants measured using the Bradford method (Bio-Rad protein assay kit). Homogenates were stored at -80°C until required. Immunoprecipitations for NKCC2 were performed using the T4 antibody and equal protein concentrations of whole kidney lysates from HFD and control diet mice. Anti-mouse IgG-agarose was used to immunoprecipitate immune complexes (Sigma Aldrich, MO, USA).

Samples were separated by SDS-PAGE and electrically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) using Bio-Rad Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked in 10% BSA in Tris-buffered saline (TBS) for 1 h and then incubated in primary antibody. The optimal antibody concentration and duration of incubation were determined for each antibody. After washing in TBS-0.05% Tween 20, the membrane was incubated for 30 min in FITC conjugated secondary antibody (Dako, Glostrup, Denmark). Antibody complexes were detected with anti-FITC POD (Roche Diagnostics, Basil, Switzerland). Immunoreactive proteins were detected by enhanced chemiluminescence with the Western Lightning system (PerkinElmer, MA, USA). If the membrane was to be probed with another primary antibody, existing antibody bound to the membrane was stripped by incubation in Reblot stripping solution (Chemicon, MA USA) for 15 min. Quantification of Western blots was performed by densitometry with analysis performed using Image J software (NIH, Bethesda, MD, USA).

Natriuretic Studies

To measure activity of NCC in vivo, 11 week old mice that had been on either a HFD (n=7) or control diet (n=5) were administered intraperitoneal injections of 50mg/kg of Hydrochlorothiazide (Sigma Aldrich, MO, USA) in 20ml/kg of saline. Mice were placed in metabolic cages and urine collected for 3 hours. 3 days prior, urine was collected after IP injections of an equal volume of vehicle (saline). Sodium, potassium and creatinine concentrations were measured using a Roche Hitachi Cobas c-series autoanalyser. Sodium/creatinine ratios after Hydrochlorothiazide were compared with baseline levels to give a marker of the relative in vivo activity of NCC.

Real time quantitative RT-PCR

Total RNA was purified from whole mouse kidney samples (n=8 per group) using TRizol reagent (Invitrogen) in accordance with the manufacturer’s instructions. RNA quality and quantity were determined using spectrophotometry and reverse transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR used the following primers: NCC: 5′-CGAGAGTAATCCAGCAGTA-3′ and 5′-ATGAAGAGATTAACAAGAA-3′ and 18S (housekeeping gene): 5′-AGTCCCTGCCCTTTTGAC-3′ and 5′-GATCCAGGGGCTCCTAACA-3′. Real-time PCR was performed on a Stratagene MX-3000 with the Solis Biodyne Evagreen master mix (Tartu, Estonia) according to the manufacturer’s instructions. Primer efficiency was measured using standard dilution, and the Pfaffl method [10] was used to calculate relative expression. Results are expressed as fold expression relative to wild type mice that received the control diet.
Statistics
Statistics were performed using Instat version 3.05 (GraphPad Software, San Diego, CA). Data are presented as means ± SD. RT-PCR is presented as standard error of the mean. Comparison of means from two groups was performed by an unpaired t-test. P values of <0.05 were considered significant.

Results

Characteristics of C57BL/6 Mice fed a high fat diet (HFD mice)
HFD mice demonstrated a significantly increased percentage weight gain compared with controls (Table 1). At 3 weeks, the percentage weight gains in the two groups were 3.8% and 10.6% for control and HFD mice, respectively (p<0.001). Kidney weights were significantly higher in the HFD mice (252mg vs 219mg, p<0.001). At 3 weeks, there was no difference in blood pressures between the two groups of mice.

Expression and Phosphorylation of Sodium Transporters in Kidneys from HFD Mice

NKCC2. HFD did not alter the expression of NKCC2 in cortex or whole kidney. There were no changes in T96/T101-phosphorylation of NKCC2 in cortex or whole kidney preparations. S126-phosphorylation of NKCC2 could only be detected after immunoprecipitation of NKCC2 from whole kidney preparations. There were no differences in S126-phosphorylation between groups (Figure 1(a)-(c)).

NCC. A marked and significant increase in the abundance of NCC was detected in the renal cortex of mice fed HFD (~2-fold increase, p<0.001). Abundance of pT58-NCC was also significantly increased in HFD mice (70% increase, p<0.005), while the ratio of phosphorylated to total NCC was unchanged between groups (Figure 1(d)).

ENaC. There was no difference in expression of any of the ENaC subunits in cortex or whole kidney of HFD mice (Figure 2(a)-(b)). Proteolysis of γ ENaC subunits has been identified as a possible mechanism of regulation of ENaC [6]. A 70kDa molecular weight isoform of γ ENaC subunit was detectable in renal cortex and whole kidney samples. There was also no difference between control and HFD fed animals in abundance of this lower molecular weight isoform of γ ENaC when expressed relative to either the abundance of full length γ ENaC or β-tubulin (Figure 2 (c)-(d)).

Protein Kinases
It has previously been shown that 14 weeks of a HFD led to phosphorylation changes in the kinases SPAK/OSR1 and AMPK [7], which are known to be involved in the regulation of NKCC2 [11, 12] and NCC [13]. In contrast to the findings at 14 weeks, no differences were detectable in pAMPK (Figure 3(a)) or pSPAK/OSR1 (Figure 3(b)) in the cortex or whole kidney specimens after 3 weeks of HFD.

Table 1. Characteristics of mice fed control and high fat diet for 3 weeks. Data is presented as mean ± standard deviation.

<table>
<thead>
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<th>Control Diet</th>
<th>High Fat Diet</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Final Mouse Weight (n=12)</td>
<td>25.7±1.6g</td>
<td>28.6±2.2g</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>%age Weight change (n=12)</td>
<td>3.8±5.6%</td>
<td>10.6±7.6%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Kidney Weight (n=20)</td>
<td>214±25mg</td>
<td>252±32mg</td>
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<tr>
<td>Systolic Blood Pressure (n=8)</td>
<td>111mm±10Hg</td>
<td>112±13mmHg</td>
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Increased expression of NCC mRNA in HFD mice

To explore the mechanism of increased protein expression of NCC in mice fed HFD, qPCR for NCC mRNA was performed on cortical samples from HFD and control fed mice. There was a dramatic and highly significant increase in NCC mRNA in renal cortex of HFD mice (18-fold increase, p<0.001) (Figure 4(a)).
Natriuretic response to Hydrochlorothiazide

The finding of increased expression of NCC and pT58-NCC would be predicted to increase the activity of this transporter. To verify that NCC activity was increased in vivo, natriuretic responses to hydrochlorothiazide (HCT) were measured in mice fed control and HFD for 3 weeks. Baseline urinary sodium to creatinine (Na/Cr) ratios after saline injection were lower in HFD mice, suggesting lower sodium excretion in these mice (Figure 4(b)(i)). However, creatinine excretion—which could not be accurately determined - may be different between groups, which could affect this result. For this reason the fold change in urinary Na/Cr after hydrochlorothiazide was calculated as a measure of activity of NCC. HFD mice had a significantly greater increase in the urinary Na/Cr ratio from baseline in response to HCT (4.0-fold vs 1.6-fold increase from baseline, n=5-7, p<0.05) (Figure 4(b)(ii)). There were no differences in urinary potassium to creatinine ratios between groups after either vehicle or hydrochlorothiazide.

5-Day High Fat Diet

To help differentiate between the effects of HFD and those of obesity, and to investigate further the time-point at which NCC expression is upregulated, a study of the effect of 5 days of HFD feeding on NCC expression was performed.
Fig. 3. Renal kinases involved in the regulation of NKCC2 and NCC are unchanged after 3 weeks of High Fat Diet. Representative western blots and densitometry of (a) pAMPK and (b) pSPAK/OSR1 in cortex and whole kidney preparations (n=8). Protein abundances are corrected for tubulin expression and expressed relative to the mean of the controls. Error bars represent standard deviations.

Fig. 4. Increased NCC mRNA and in vivo Activity of NCC after 3 weeks of High Fat Diet. (a) Quantitative PCR of NCC mRNA from renal cortex of control (white bars) and high fat diet (black bars) fed mice. Data is presented as mean fold expression relative to 18s ribosomal RNA and normalized to controls (n=8). Error
There were no differences in body weight, percentage weight change or kidney weight between groups. No differences in expression or phosphorylation of NCC were found between the two groups (Figure 5(a)), and quantitative PCR revealed no increase in expression of mRNA after 5 days of HFD (Figure 5(b)).

Discussion

This study has shown that 3 weeks of HFD feeding leads to enhanced activity of NCC due to upregulation of expression of this transporter. No changes in the NKCC2 or ENaC, were detectable, suggesting that NCC is the most important distal sodium transporter involved in mediating enhanced tubular sodium reabsorption at this time-point. Interestingly, these results are in contrast to the changes reported after 14-weeks of HFD, where enhanced activity of NKCC2 due to increased activating phosphorylation (primarily at S126) was the key change identified [7].

A change in the profile of renal sodium transporters with time in this HFD model is perhaps not surprising as the effect of obesity on sodium balance changes with time. Obesity is associated with sodium retention [1]. In HFD models of obesity there is an initial active-phase of sodium accumulation due to enhanced tubular reabsorption of sodium after institution of high fat feeding [3, 4, 14]. Subsequently, however, sodium balance returns to neutral, though excess sodium is retained despite elevated blood pressure due to disturbed pressure natriuresis [1]. Renal plasma flow and glomerular filtration rate are elevated at
In the current study, at 3 weeks the HFD mice had not yet developed hypertension, indicating that they were still in the phase of active sodium retention. This is consistent with experiments in HFD fed dogs, which found sodium is actively retained for at least 5 weeks after institution of the HFD [2, 3]. In contrast to these observations at earlier time points, after 14 weeks of HFD mice are hypertensive [7] consistent with blunted pressure-natriuresis perpetuating the already established sodium excess. This is supported by the findings of a study by Deji and colleagues who examined the effects of acute salt loading in C57BL/6 mice fed control or HFD for 12 weeks. They found that 6 hours after an intraperitoneal injection of saline there was no difference in total sodium excreted between groups, suggesting that at 12 weeks, the HFD mice were no longer actively accumulating sodium [17]. Taken together, these data indicate that differences in sodium transporter regulation identified at 3 and 14 weeks reflect distinct tubular mechanisms underlying two distinct phases in the sodium overload associated with establishing and maintaining obesity-related hypertension. These data implicate NCC as important in driving the earlier active phase of sodium retention, and NKCC2 as the key transporter responsible for maintaining sodium overload in the presence of ORH. That different transporters mediate the increased sodium reabsorption response to HFD at different time-points is surprising. Further work will be needed to understand the mechanisms leading to these changes.

NCC mRNA was found to be dramatically increased after 3-weeks of HFD, suggesting increased transcription and/or stability of NCC mRNA as an important factor increasing NCC protein expression. The underlying mechanism leading to upregulation of NCC expression has not been determined by the present study, but could relate to changes in hormone/adipokine profiles induced by HFD/obesity. Further studies are required to determine the mechanism of increased NCC expression with a HFD.

A possible explanation for the difference in sodium transporter profiles identified at the different time points could be that the changes at 3 weeks occur in response to HFD, while those at 14 weeks require the presence of obesity. To explore this possibility, and better delineate the timepoint of upregulation of NCC expression, the expression of NCC was determined after 5-days of HFD, before significant weight gain had occurred. There was no difference in NCC protein expression or abundance of NCC mRNA detectable at this time-point. This suggests that the changes seen at 3 weeks require the onset of obesity and are not purely due to the effects of HFD feeding, per se. Further evidence to support the upregulation of NCC as an effect of obesity rather than a response to HFD comes from the finding of upregulation of NCC expression in another animal model of obesity - obese Zucker (ZO) rats [18-20], which have not been exposed to HFD. In contrast to the HFD model, upregulation of NCC appears to be a persisting change in ZO rats. This difference is likely due to differences between models and perhaps suggests that leptin signaling (which is dysregulated in ZO-rats) may play a role in the changes of sodium transporter profiles seen in the HFD model. ZO rats have also been reported to have enhanced phosphorylation of NCC [21]. This latter finding was felt to be related to the profound hyperinsulinaemia seen in these animals, and was not seen in the current HFD model of obesity.

In the current study mice were 14 weeks old at termination, while in the 14 week study, mice were 22 weeks old at termination. It is possible that the differences in sodium transporter profiles identified at the different time points could be due to mice of different ages responding differently to HFD. However, while this can’t be excluded from the available data, it is thought to be unlikely for several reasons. Though not age matched, mice were young mature adults in both studies. Changes in renal sodium transporter status with age...
has not been previously reported. Similarly, we are unaware of any reports of differing hormonal response to HFD when commenced at different ages within this range.

When activated, the protein kinases AMPK and SPAK/OSR1 regulate the cotransporter activity of NKCC2 by phosphorylation [12, 22]. SPAK/OSR1 also regulates NCC activity by phosphorylation [13]. Fourteen weeks of HFD has been shown to be associated with an increase in pSPAK/OSR1 and a reduction of pAMPK in the renal cortex [7]. Consistent with the absence of changes in phosphorylation of NCC or NKCC2 after 3 weeks of HFD, there were no changes in the abundance of pAMPK or pSPAK/OSR1 at this time-point. In contrast, one previous study has reported reduced pAMPK, measured by ELISA, in the renal cortex of mice fed HFD for 1 week [23]. In that model, mice were fed a 60% fat diet and had greater weight gains at 7 days than the mice in the current study had at 3 weeks, potentially accounting for the differences in pAMPK between these studies.

Conclusion

This study shows that expression and activity of NCC increases in mice fed a HFD, and that this phenomenon is established at 3 weeks but has not occurred at 5 days. The upregulation of NCC appears to be dependent upon the initiation of obesity, rather than HFD feeding, and is associated with a marked increase in NCC mRNA. The current study complements our earlier study demonstrating that activation of NKCC2 was the most important mechanism for the maintenance of sodium overload in established ORH [7]. If it is considered desirable to target the sodium cotransporters responsible for sodium overload in ORH, then the current study suggests that the target molecule may change during the course of the disease. The effect of obesity on tubular sodium transport appears to be a complex and dynamic process, with different areas of the nephron playing different roles as ORH develops.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

References

519

Davies/Gleich/Katerelos/Lee/Mount/Power: NCC Promotes Sodium Retention in DIO Mice


