Is Renal β-Adrenergic-WNK4-NCC Pathway Important in Salt Hypertension of Dahl Rats?

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Summary
In 2011 Fujita and coworkers proposed that β-adrenergic stimulation causes decreased serine/threonine-protein kinase WNK4 transcription leading to the activation of Na-Cl cotransporter (NCC) which participates in salt sensitivity and salt hypertension development in rodents. The aim of our study was to investigate whether the above hypothesis is also valid for salt hypertension of Dahl rats, which are characterized by high sympathetic tone and abnormal renal sodium handling. Male 8-week-old salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) Dahl rats were fed either low-salt diet (LS, 0.4% NaCl) or high-salt diet (HS, 4% NaCl) for 6 weeks. Half of the animals on either diet were chronically treated with non-selective β-blocker propranolol (100 mg/kg/day). At the end of the experiment diuresis and sodium excretion were measured prior and after hydrochlorothiazide injection (HCTZ, 10 mg/kg i.p.). Furthermore, blood pressure (BP), heart rate (HR), sympathetic (pentolinium 5 mg/kg i.v.) and NO-dependent (L-NAME 30 mg/kg i.v.) BP components were determined. Chronic HS diet feeding increased BP through sympathoexcitation in SS/Jr but not in SR/Jr rats. Concomitant propranolol treatment did not lower BP in either experimental group. Under the conditions of low salt intake HCTZ increased diuresis, natriuresis and fractional sodium excretion in SR/Jr but not in SS/Jr rats. HS diet feeding attenuated renal response to HCT in SR/Jr rats, whereas no HCTZ effect was observed in SS/Jr rats fed HS diet. Propranolol treatment did not modify diuresis or natriuresis in any experimental group. In conclusions, our present data do not support the idea on the essential importance of renal β-adrenergic-WNK4-NCC pathway in pathogenesis and/or maintenance of salt hypertension in Dahl rats.

Key words
Sympathetic nervous system • β-adrenergic blockade • Renal sodium excretion • NCC cotransporter • Hydrochlorothiazide • Serine/threonine-protein kinase WNK4

Introduction
High salt intake is considered to be one of the major factors in the pathogenesis and maintenance of human hypertension. Salt sensitive subjects are characterized by enhanced increase of blood pressure (BP) occurring during the exposure to salt loading (Fujita et al. 1980). The mechanism of increased salt sensitivity is still unclear, but there is an attractive hypothesis on the participation of renal β-adrenergic-WNK4 pathway in salt sensitivity and salt hypertension in rodents (Mu et al. 2011). It was suggested that β2-adrenergic stimulation causes decreased transcription of the gene encoding WNK4, which is a regulator of sodium reabsorption. This pathway involves cAMP-dependent inhibition of histone deacetylase-8 and increased histone acetylation, leading to binding of the glucocorticoid receptor to a negative glucocorticoid-responsive element in the promoter region. Mu et al. (2011) suggested that in rat models of salt hypertension with sympathetic hyperactivity, salt loading suppressed the renal WNK4 expression and
activated thiazide-sensitive sodium-chloride cotransporter (NCC), leading to sodium retention and the induction of salt-sensitive forms of hypertension.

Salt hypertension in salt-sensitive Dahl rats (Dahl et al. 1962) has been a subject of our research for more than 30 years (for review see Zicha et al. 2012). This was a reason why we decided to test the above hypothesis proposed by Fujita (2014) in this hypertensive model. Salt hypertensive Dahl rats are characterized by sympathoexcitation of central origin (Mark 1991, Huang and Leenen 1998, Zicha et al. 2001, Dobešová et al. 2002) as well as by numerous renal abnormalities (Alvarez-Guerra et al. 2002, Aoi et al. 2004, Amin et al. 2011, Nishimoto and Fujita 2015, Vokurková et al. 2015, Pavlov and Starushenko 2017, Kítikulsuth et al. 2018). Thus, we could expect increased β-adrenergic suppression of renal WNK4 pathway which could be prevented by chronic β-adrenergic blockade with non-selective β-blocker propranolol. It was demonstrated in late 1970s that chronic administration of thiazide diuretics in high-salt diet can almost prevent hypertension development in salt-sensitive Dahl rats (Iwai et al. 1977, Tobian et al. 1979). This finding has been confirmed by several other labs (Sharma et al. 1984, Yamada et al. 2011, Wei et al. 2017). Chronic treatment of salt-sensitive animals with hydrochlorothiazide (HCTZ) also attenuates salt-induced renal damage consisting of podocyte injury, peritubular capillary loss, tubular atrophy, macrophage infiltration and interstitial fibrosis (Wei et al. 2017). The activation of NCC through β-adrenergic-WNK4 pathway could cause the augmented natriuretic response to HCTZ in salt hypertensive animals. However, the information on the acute natriuretic response to HCTZ in Dahl rats is still missing.

The aim of our study in salt-sensitive and salt-resistant Dahl rats was to evaluate BP effects of chronic β-adrenergic blockade by propranolol and the impact of this pharmacological intervention on natriuretic response to acute hydrochlorothiazide administration. The obtained results might help us to evaluate the importance of renal β-adrenergic-WNK4 pathway in salt sensitivity and salt hypertension of Dahl rats.

Material and Methods

Inbred salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) Dahl rats aged 8 weeks (young adult rats) were used in the experiments. The animals were obtained from the breeding colony of the Institute of Physiology CAS in Prague and housed under the controlled conditions (23±1 °C, 24h L/D cycle) with tap water to drink and fed a low-salt (LS, 0.4 % NaCl) or high-salt (HS, 4 % NaCl) diets during the experiment lasting 6 weeks. In each experimental group 50 % animals were treated with non-selective β-blocker propranolol (100 mg/kg/day) in the drinking fluid throughout the whole experiment.

All the procedures and experimental protocols were performed in accordance with guidelines and practice established by the Ethical Committees of the Institute of Physiology CAS, and conformed to the European Convention on Animal Protection and Guidelines on Research Animal Use.

Natriuretic response to acute hydrochlorothiazide administration

Before the end of the experiment the rats were placed individually in metabolic cages and urine was collected prior and after hydrochlorothiazide administration (10 mg/kg, i.p.). In the first series of experiments, which started at 8 a.m., the urine was collected for 4 h before and 4 h after HCTZ injection in all experimental groups. Further experiments were carried out in animals of both genotypes fed either LS or HS diet that were not treated with propranolol. In this series, which began at 4 p.m., the urine was collected for 16 h overnight without HCTZ application. Two days later HCTZ was injected just before the repeated urine collection. Separate groups of rats (not treated with propranolol) received furosemide injection (10 mg/kg i.p.) and urine was collected overnight. All experiments were repeated twice. Urinary volume as well as urinary and plasma concentrations of sodium and creatinine were determined. Sodium excretion as well as fractional sodium excretion were calculated.

Participation of major vasoactive systems in BP maintenance (vasoactive balance)

One week later, the changes of mean arterial pressure (MAP) elicited by the sequential blockade of major vasoconstrictor and vasodilator systems (renin-angiotensin system, sympathetic nervous system and NO) were determined in conscious cannulated rats. One day prior to the experiment two polyethylene cannulas, which were implanted under 2.5 % isoflurane anesthesia (PE 50 for BP measurement in the left carotid artery, PE 10 for the infusion of drugs to the jugular vein), were exteriorized in the interscapular region. Blood pressure

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and heart rate (HR) were recorded using a pressure transducer and a multichannel recorder (ADInstruments, Bella Vista, Australia). We used a modified protocol of Minami et al. (1995) which was adapted in our laboratory (Kuneš et al. 2002, Vaněčková et al. 2012). Briefly, baseline MAP levels were recorded after a 30-min adaptation period in transparent measuring plastic cages. Then, we started with RAS blockade (captopril 10 mg/kg) which was followed 15 min later by ganglionic blockade of SNS (pentolinium 5 mg/kg). Finally, NO synthase inhibitor (30 mg/kg L-NAME) was injected and BP was monitored for further 20 min. BP levels before and after particular blockades were determined and the respective BP changes were calculated. All drugs were dissolved in saline and administered as intravenous bolus injections in a volume of 1 ml/kg of body weight.

The results are expressed as the mean ± SEM. The statistical differences were evaluated by one-way analysis of variance (ANOVA) (Instat, La Jolla, California, USA) followed by the Fisher LSD post-hoc test. P<0.05 values were considered to be statistically significant.

Results

Blood pressure was significantly higher in SS/Jr than in SR/Jr rats fed LS diet and it was further elevated by high salt intake only in SS/Jr rats (Fig. 1). BP elevation was due to enhanced sympathetic tone (reflected by augmented pentolinium-induced MAP change) which was further enhanced by high salt intake. Moderate compensatory increase of NO-dependent vasodilation (L-NAME-induced MAP change) was observed in SS/Jr rats fed a HS diet (Fig. 1). Chronic treatment with propranolol did not lower BP in any experimental group, while heart rate tended to be lower in all groups, the effect being significant in salt hypertensive animals (Fig. 1). There were no significant differences in the contribution of RAS to BP maintenance between particular experimental groups (Table 1).

Propranolol treatment significantly lowered diuresis only in SR/Jr animals fed a LS diet but chronic β-blockade had no significant effect on sodium excretion in any experimental group. This was true not only before (Fig. 2, left panels) but also after HCTZ injection (Fig. 2, right panels).

![Mean arterial pressure, heart rate, sympathetic BP component (pentolinium-induced BP change) and NO-dependent BP component (L-NAME-induced BP change) in conscious salt-resistant (SR/Jr, blue columns) and salt-sensitive (SS/Jr, red columns) Dahl rats that were fed a low-salt (LS) or high-salt (HS) diet and were drinking either water (columns with white background) or propranolol solution (columns with yellow background) for 6 weeks. Data are means ± SEM, n=9. Significantly different (p<0.05): ● SS/Jr vs. SR/Jr rats, + HS vs. LS animals, * water drinking vs. propranolol-treated rats.](image-url)
Table 1. MAP changes elicited by acute captopril administration in conscious salt-resistant (SR/Jr) and salt-sensitive (SS/Jr) Dahl rats that were fed a low-salt (LS) or high-salt (HS) diet and were drinking either water or propranolol solution for 6 weeks.

<table>
<thead>
<tr>
<th></th>
<th>SR-LS</th>
<th>SS-LS</th>
<th>SR-HS</th>
<th>SS-HS</th>
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<tr>
<td><strong>Water drinking</strong></td>
<td>-6.5 ± 1.8</td>
<td>-2.3 ± 1.2</td>
<td>-12.2 ± 3.4</td>
<td>-9.3 ± 3.4</td>
</tr>
<tr>
<td><strong>Propranolol-treated</strong></td>
<td>-5.6 ± 1.1</td>
<td>-3.9 ± 1.7</td>
<td>-6.3 ± 2.2</td>
<td>-4.3 ± 1.8</td>
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Data are means ± SEM, n=9.

Fig. 2. The influence of chronic β-blockade with propranolol on urinary water and sodium excreted 4 h before and 4 h after hydrochlorothiazide injection (10 mg/kg, HCTZ, applied at 12 a.m.) by conscious salt-resistant (SR/Jr, blue columns) and salt-sensitive (SS/Jr, red columns) Dahl rats that were fed a low-salt (LS) or high-salt (HS) diet and were drinking either water (columns with white background) or propranolol solution (columns with yellow background) for 6 weeks. Data are means ± SEM, n=7. Significantly different (p<0.05): * HS vs. LS animals, + water drinking vs. propranolol-treated rats.

Diuretic and natriuretic effects of acute HCTZ administration were strongly dependent on genotype of rats and their salt intake. In animals, which were not treated with propranolol, HCTZ injection increased urinary water excretion in SR/Jr but not in SS/Jr rats. Sodium excretion was enhanced by HCTZ only in SR/Jr rats fed a LS diet. Surprisingly, acute HCTZ administration reduced water and sodium excretion in salt hypertensive SS/Jr rats (Fig. 3, left panels). There were similar but less pronounced changes of diuresis and natriuresis in propranolol-treated animals (Fig. 3, right panels).
Untreated rats

Propranolol-treated rats

**Fig. 3.** The effect of hydrochlorothiazide (10 mg/kg, HCTZ) on water and sodium excretion in untreated (left panels) and propranolol-treated (right panels) salt-resistant (SR/Jr, blue columns) and salt-sensitive (SS/Jr, red columns) Dahl rats fed a low-salt (LS) or high-salt (HS) diet. Data are means ± SEM, n=7. Significantly different (p<0.05): # HCTZ vs. saline injection.

**Fig. 4.** The effect of hydrochlorothiazide (10 mg/kg, HCTZ, applied at 4 p.m.) on overnight urinary water excretion, sodium excretion and fractional sodium excretion in untreated salt-resistant (SR/Jr, blue columns) and salt-sensitive (SS/Jr, red columns) Dahl rats fed a low-salt (LS) or high-salt (HS) diet. Data are means ± SEM, n=8. Significantly different (p<0.05): ● SS/Jr vs. SR/Jr rats, # HCTZ vs. saline injection.

To analyze the above genotype- and/or salt intake-dependent differences in HCTZ action on water and sodium excretion, that were obtained in shorter experiments carried out during the daytime, we have performed a further series of longer experiments with overnight collection of urine in Dahl rats untreated with propranolol. Figure 4 shows that under the conditions of low salt intake diuresis and natriuresis were moderately decreased in SS/Jr rats compared to SR/Jr ones, whereas both parameters were greatly enhanced in SS/Jr rats fed a HS diet. The calculation of fractional sodium excretion revealed significantly lower values in SS/Jr than SR/Jr animals fed a LS diet, but significant HCTZ effect was seen only in SR/Jr animals. As expected, high salt intake greatly increased fractional sodium excretion in both rat
strains (slightly more in SS/Jr animals) so that HCTZ can hardly elevate sodium excretion under these conditions (Fig. 4). Within this series of experiments, we have also studied the effect of loop diuretic furosemide and we have observed that SS/Jr rats fed a LS diet responded to this diuretic better than SR/Jr animals (Fig. 5). There was no significant effect of furosemide in Dahl rats of either genotype when fed high-salt diet (data not shown).

**Discussion**

Our study was designed to test the importance of renal β-adrenergic-WNK4-NCC pathway for the development of salt-induced hypertension in Dahl rats, which are characterized by enhanced sympathetic tone. We investigated the initial and the final steps of this interesting pathway proposed by Mu *et al.* (2011) in conscious animals. Therefore, we subjected our rats to chronic β-adrenergic blockade throughout the entire experiment and a non-selective β-blocker propranolol was used according to the study of Mu *et al.* (2011). Chronic propranolol treatment did not lower BP in any group studied, although the same treatment decreased BP of salt-loaded spontaneously hypertensive rats by 25 mm Hg (Zicha, unpublished data). The only effect of chronic β-blockade was a significant reduction of heart rate in salt hypertensive Dahl rats treated with propranolol (Fig. 1). In fact, there are only few positive reports on BP-lowering effects of selective β₁-blockers such as atenolol, bisoprolol or nebivolol in salt hypertensive Dahl rats (Cosentino *et al.* 2002, Ye *et al.* 2013, Watanabe *et al.* 2015), but no information on BP effects of chronic β₂-adrenergic blockade is available in this model. Non-selective β-adrenergic blockade failed to attenuate salt hypertension in salt-sensitive Dahl rats (Puleo *et al.* 2019).

There is also scarce information on renal excretory effects of chronic β-blockade in Dahl rats. To our knowledge, Watanabe *et al.* (2015) did not observe any significant impact of chronic bisoprolol treatment on water and sodium excretion in salt hypertensive Dahl rats, which is in line with our present results (Fig. 2).

Finally, we were highly interested in the influence of chronic β-blockade with propranolol on diuretic and natriuretic response to acute administration of hydrochlorothiazide (HCTZ), which is an inhibitor of sodium-chloride cotransporter (NCC). Mu *et al.* (2011) reported that β₂- but not β₁-adrenergic blockade attenuated natriuretic response to HCTZ injection in two models in which augmented response to HCTZ was observed – isoproterenol-treated Sprague Dawley rats fed a HS diet or DOCA-salt treated Sprague Dawley rats. Unfortunately, these authors did not provide any information on BP, sodium excretion or renal response to HCTZ in Dahl rats which they also included in their study (Mu *et al.* 2011).

In our study, we observed diuretic effects of HCTZ only in salt-resistant (SR/Jr) but not in salt-sensitive (SS/Jr) Dahl rats and natriuretic effect of HCTZ was significant only in SR/Jr animals fed a LS diet. Under the conditions of high salt intake, HCTZ did not increase sodium excretion in SR/Jr rats and surprisingly lowered water and sodium excretion in SS/Jr animals. Chronic propranolol treatment did not influence natriuretic response to HCTZ in any experimental group including salt hypertensive SS/Jr rats (Fig. 3). Similar finding was also reported by Puleo *et al.* (2019) who did not observe significant reduction of NCC activity measured as peak natriuresis following i.v. HCTZ.
administration. Thus, our data suggest that β2-adrenergic activation of NCC cannot be easily disclosed in salt hypertensive Dahl rats with sympathetic overactivity, although Mu et al. (2011) demonstrated that high salt intake increased WNK4 expression only in salt-resistant but not in salt-sensitive Dahl rats. On the basis of this observation they suggested a “relative suppression” of renal WNK4 in salt hypertensive rats and they expected (but not demonstrated) the activation of NCC in these animals.

Let us to discuss the above findings in the light of the available information on NCC in Dahl rats. Thiazide diuretics, which effectively lower BP in Dahl rats (Iwai et al. 1977, Yamada et al. 2011, Wei et al. 2017), target sodium-chloride cotransporter (NCC, earlier known as thiazide diuretic receptor). Surprisingly, the renal density of this cotransporter was not influenced by the excess dietary salt intake in either normotensive Sprague Dawley rats or salt-sensitive Dahl rats (Fanestil et al. 1997, 1999).

NCC has been studied in Dahl rats as a component of renal sodium transport and salt handling, but the interest was paid to the consequence of the knockout of either renin gene (Pavlov et al. 2016) or Kcnj16 gene for inward rectifying K+ channel (Palygin et al. 2017) in salt-sensitive Dahl rats. This was a reason why a comparison of SS/Jr with SR/Jr rats was not included in these studies. Renin deficiency was associated with significantly lower protein expression of Na+/H+ exchanger (NH3) and Na-Cl cotransporter (NCC), whereas the expression of Na-K-2Cl cotransporter (NKCC2) was unchanged (Pavlov et al. 2016). Since salt-sensitive Dahl rats are characterized by a lower RAS activity as compared to salt-resistant animals and this activity is further suppressed by high salt intake (Iwai et al. 1973), the higher activation of NCC in SS/Jr than in SR/Jr is rather improbable. The second study revealed salt wasting in Kcnj16 knockout SS/Jr animals which was accompanied by the upregulation of both NCC and NKCC2 including their phosphorylated forms. Nevertheless, these knockout animals are characterized by decreased BP compared to intact SS/Jr rats (Palygin et al. 2017). It should be mentioned that the earlier genetic studies in F2 generation of salt-resistant and salt-sensitive Dahl rats indicated that Na-K-2Cl cotransport but not thiazide-sensitive sodium-chloride cotransport (NCC) is associated with BP of these salt-loaded F2 hybrids (Herrera et al. 2001, Song et al. 2001).

The role of enhanced Na-K-2Cl cotransport in Dahl rats has already been suggested by old studies of erythrocyte ion transport (Knorr et al. 1985, Zicha and Duhm 1990). Later Garay and coworkers focused their attention on both NKCC1 (erythrocytes) and NKCC2 (kidney) in Dahl rats, demonstrating a circulating inhibitor of Na-K-2Cl cotransporter in plasma of salt hypertensive Dahl rats (Alvarez-Guerra and Garay 1997, Garay et al. 1998, Alvarez-Guerra et al. 2002). In fact, our SS/Jr rats fed a low-salt diet were insensitive to HCTZ (Fig. 4), but they displayed greater natriuretic response to Na-K-2Cl cotransport inhibitor furosemide, whereas such response was almost absent in SR/Jr rats (Fig. 5).

Actually there is a series of papers focusing the attention to NKCC2 transporter in thick ascending limb of Henle loop (TALH) in Dahl rats. Hong and Garvin (2012) found that the reduction of NKCC2 activity through angiotensin II (via AT2 receptors leading to NO release) is blunted in TALH of SS/Jr rats. Haque et al. (2011) reported that high salt intake moderately decreased the activity of surface NKCC2 and sodium transport in TALH of SR/Jr rats, whereas these two parameters were substantially increased in TALH of SS/Jr rats with high salt intake. The further study of this group (Ares et al. 2012) revealed hyperphosphorylation of NKCC2 and its enhanced trafficking into the apical membrane in TALH of salt-sensitive Dahl rats, leading to increased NKCC2 activity and abnormal sodium reabsorption compared to salt-resistant animals. Recently, Haque and Ortiz (2019) demonstrated in TALH of Sprague Dawley rats that the expression of surface NKCC2 is increased by superoxide anions and blunted by endogenous NO. Superoxide-stimulated apical trafficking of NKCC2 may be involved in the enhanced surface NKCC2 expression observed in salt-sensitive Dahl rats. This also seems to be in accordance with our earlier findings on the interaction of superoxide with NO in salt hypertensive Dahl rats (Zicha et al. 2001).

Nevertheless, Fujita and coworkers also pointed out that renal WNK4 deficiency due its suppressed expression might activate epithelial sodium channels (ENaC) in the distal tubule (Mu et al. 2011, Nishimoto and Fujita 2015). The importance of ENaC in salt-induced hypertension was also considered by several other labs (Aoi et al. 2004, Kakizoe et al. 2009, Amin et al. 2011, Pavlov and Staruschenko 2017) and this mechanism may need further investigation in the future.

We can conclude that our present data do not support the essential importance of renal β-adrenergic-
WNK4-NCC pathway in the maintenance of high blood pressure in salt hypertensive Dahl rats. It seems that ENaC channels and/or NKCC2 cotransporter in the kidney of salt-sensitive Dahl rats might play a more important role in the pathogenesis of salt hypertension than NCC cotransporter.

It would be desirable to study the above mechanisms also in spontaneously hypertensive rats (SHR) in which various renal abnormalities (Persson and Boberg 1988, Cowley et al. 1992, José et al. 1996, Moreno et al. 2001, Ahmeda et al. 2018) as well as sympathetic hyperactivity (Zicha et al. 2014, Török et al. 2016, Vavřínová et al. 2016) including altered β-adrenergic vasodilatation (Pintérová et al. 2014) were reported. The comparison of both models of experimental hypertension could be highly interesting.

**Note added in proof**

Recent research on the sympathetic regulation of NCC activity in rats with various forms of salt-sensitive hypertension indicated that norepinephrine modulates NCC activity through its action on α1- rather than β-adrenergic receptors (Frame et al. 2019, Puleo et al. 2019, R.D. Wainford – personal communication).

**Conflict of Interest**

There is no conflict of interest.

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