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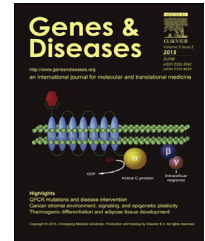


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REVIEW ARTICLE

# NKCC1 and NKCC2: The pathogenetic role of cation-chloride cotransporters in hypertension



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**Abstract** This review summarizes the data on the functional significance of ubiquitous (NKCC1) and renal-specific (NKCC2) isoforms of electroneutral sodium, potassium and chloride cotransporters. These carriers contribute to the pathogenesis of hypertension via regulation of intracellular chloride concentration in vascular smooth muscle and neuronal cells and via sensing chloride concentration in the renal tubular fluid, respectively. Both NKCC1 and NKCC2 are inhibited by furosemide and other high-ceiling diuretics widely used for attenuation of extracellular fluid volume. However, the chronic usage of these compounds for the treatment of hypertension and other volume-expanded disorders may have diverse side-effects due to suppression of myogenic response in microcirculatory beds.

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**Abbreviations:** CCC, cation-chloride cotransporters; CNS, central nervous system; EFV, extracellular fluid volume; GABA,  $\gamma$ -aminobutyric acid; GFR, glomerular filtration rate; JGA, juxtaglomerular apparatus; KCC,  $K^+, Cl^-$  cotransport; MD, macula densa; NCC,  $Na^+, Cl^-$  cotransport; NKCC,  $Na^+, K^+, 2Cl^-$  cotransport; OSR1, oxidative stress response kinase; PVN, paraventricular nucleus; SMC, smooth muscle cells; SNS, sympathetic nervous system; SPAK, Ste20-related praline-alanine-rich kinase; TAHL, thick ascending limb of Henle's loop; TGF, tubuloglomerular feedback; WNK, with no K = lysine kinase.

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$\text{Na}^+$ ,  $\text{K}^+$ ,  $2\text{Cl}^-$  cotransporters (NKCC) encoding by *SLC12A2* (NKCC1) and *SLC12A1* (NKCC2) belong to the subfamily of cation-chloride cotransporters (CCCs) that provide electroneutral transport of sodium, potassium and chloride across the plasma membrane and are inhibited by bumetanide, furosemide and several other structurally similar compounds (Table 1). Because the major target of these drugs is inhibition of ion transport in thick ascending limb of Henle's loop (TAHL), they were termed as high-ceiling diuretics. Gene structure, membrane architecture and pharmacology of CCCs were subjected to detailed analysis in several comprehensive reviews.<sup>1–3</sup> This review focuses on physiological significance of NKCCs and their involvement in the pathogenesis of hypertension via regulation of intracellular chloride concentration and sensing chloride concentration in the renal tubular fluid.

## Functional implications of NKCCs

### Cell volume regulation

Animal cells maintain their volume with an accuracy of 1%–2% by means of systems providing inwardly and outwardly directed fluxes of monovalent ions and organic osmolytes and termed as regulatory volume increase (RVI) and regulatory volume decrease (RVD), respectively.<sup>4,5</sup> In early studies with mammalian erythrocytes, we and others have shown that cell shrinkage and swelling result in activation of NKCC and  $\text{K}^+$ ,  $\text{Cl}^-$  cotransport (KCC), respectively.<sup>6–8</sup> We also demonstrated that in vascular smooth muscle cells (VSMC) subjected to hyperosmotic shrinkage, RVI is caused by activation of NKCC.<sup>9</sup> It is noteworthy that under the baseline isosmotic conditions, inhibition of NKCC by bumetanide does not significantly affect the volume of human lung fibroblasts (Table 2), suggesting that in the absence of external stimuli, NKCC has a minor impact on the generation of net osmolyte fluxes in these cells. However, under inhibition of the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase by ouabain, treatment with bumetanide resulted in ~2-fold elevation in the volume of human lung fibroblasts (Table 2). This observation suggests that dissipation of transmembrane gradient of monovalent cations evoked by

$\text{Na}^+$ ,  $\text{K}^+$ -ATPase inhibition results in generation of inwardly-directed ion flux mediated by NKCC1, the only isoform of NKCC expressed in these cells. Functional consequences of the disturbances of cell volume regulatory machinery are discussed elsewhere.<sup>4,10,11</sup>

**Table 2** Effect of ouabain and bumetanide on the volume of human lung fibroblasts.

Additions	Cell volume, pl per cell
None (control)	0.23 ± 0.04
Ouabain	0.26 ± 0.04
Bumetanide	0.22 ± 0.01
Ouabain + bumetanide	0.43 ± 0.04*

Cells were incubated in the presence of 0.1 μM ouabain and/or 10 μM bumetanide for 24 h. Cell volume was measured as <sup>14</sup>C-urea available space.<sup>9</sup> Means ± S.E. obtained in experiments performed in quadruplicate are shown. \*p < 0.005 compared to control.

### NKCC as a regulator of intracellular $\text{Cl}^-$ concentration

In all types of cells studied so far, CCCs generate both inwardly and outwardly-directed ion movements and the direction of net flux depends on carrier's stoichiometry and transmembrane gradients of cations created by  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase. Thus, the stoichiometry 1:1 predicts that the value of ion flux is in direct proportion to concentration of co-transporting ions. Because  $[\text{Na}^+]_o \gg [\text{Na}^+]_i$ ,  $[\text{K}^+]_i \gg [\text{K}^+]_o$  and  $[\text{Cl}^-]_o > [\text{Cl}^-]_i$ , net fluxes generated by  $\text{Na}^+$ ,  $\text{Cl}^-$  cotransport (NCC) and KCC exhibit inward and outward directions, respectively. In the majority of cells  $[\text{Cl}^-]_o \gg [\text{Cl}^-]_i$ , and net flux mediated by electroneutral NKCC functioning with the stoichiometry  $1\text{Na}^+:1\text{K}^+:2\text{Cl}^-$  has inward direction.

The above consideration suggests that CCCs play a key role in the regulation of  $[\text{Cl}^-]_i$  whereas their contribution to the adjustment of intracellular concentration of monovalent cations is negligible because of the highly active  $\text{Na}^+$ ,  $\text{K}^+$ -pump. Indeed, it was shown that inhibition of NCC

**Table 1** Major characteristics of  $\text{Cl}^-$ -coupled cation cotransporters.<sup>1,3</sup>

Gene	Human chromosome localization	Protein	Alternative splicing	Tissue localization	Inhibitors, $\text{IC}_{50}$ (μM)
SLC12A2	5	NKCC1	NA	Ubiquitous	Bumetanide, 0.05–0.60 Furosemide, 10–50
SLC12A1	15	NKCC2	Isoforms A, B and F	Kidney	Bumetanide, 0.10–0.50 Furosemide 15–60
SLC12A3	16	NCC	NA	Kidney	Polythiazide, 0.5
SLC12A4	16	KCC1	NA	Ubiquitous	Bumetanide, 60
SLC12A5	20	KCC2	NA	Neurones	Bumetanide, 55 Furosemide, 10
SLC12A6	15	KCC3	Isoforms A and A	Neurones	Bumetanide, 40 Furosemide, 25
SLC12A7	3	KCC4	NA	Ubiquitous	Bumetanide, 900 Furosemide, 900

NA, information not available.

and NKCC results in attenuation of  $[Cl^-]_i$ ; whereas suppression of KCC activity increases this parameter.<sup>12</sup> Importantly, the alteration of CCC activity may lead to adjustment of  $[Cl^-]_i$  above or below the values corresponding to Nernst's equilibrium potential. This means that in cells abundant with anion channels, CCCs contribute to maintenance of electrical potential thus having an impact on the whole spectrum of cellular functions controlled by potential-sensitive proteins localized within the plasma membrane. This conclusion is supported by data discussed in the next sections.

### VSMC contraction

In skeletal and cardiac muscle, plasma membrane permeability for  $K^+$  ( $P_K$ ) plays a major role in the maintenance of electrical resistance and resting potential ( $E_m$ ). In contrast, the values of  $P_K$  and  $P_{Cl}$  in smooth muscles are about the same,<sup>13</sup> suggesting that in VSMC CCCs may be involved in regulation of the  $[Cl^-]_i/[Cl^-]_o$  ratio and therefore of  $E_m$  and excitation-contraction coupling. Indeed, NKCC inhibition by furosemide or bumetanide resulted in a decreased  $[Cl^-]_i$ <sup>14,15</sup> and led to hyperpolarization of rat VSMC.<sup>14</sup> These data suggest that decreased baseline tone seen in smooth muscles treated with Henle's loop diuretics,<sup>16–18</sup> as well as attenuation by these compounds of contraction of smooth muscle strips evoked by modest increment of  $[K^+]_o$ ,<sup>15</sup> by electrical stimulation,<sup>19</sup> by histamine,<sup>20</sup> angiotensin II,<sup>21</sup> thromboxane  $A_2$ ,<sup>22,23</sup> oxytocin,<sup>24,25</sup>  $\alpha$ -adrenergic<sup>15,26,27,28</sup> or purinergic<sup>29</sup> agonists is caused by  $Cl^-$ -dependent hyperpolarization and suppression of the activity of voltage-gated L-type  $Ca^{2+}$  channels.

### Myogenic response

Myogenic tone (response) is a unique property of small (<100–200  $\mu m$ ) blood vessels to decrease rather than increase their inner diameter in response to elevated intraluminal pressure. Both kinetic and the amplitude of myogenic response are different in blood vessels of different origins. Importantly, myogenic response in blood vessels from the brain, skeletal muscle and renal afferent arteriole provides constant blood supply of these tissues independently of the changes in the systemic blood pressure.<sup>30–32</sup>

It was reported that bumetanide decreased the myogenic tone of mesenteric arteries<sup>33</sup> and completely abolished myogenic response of renal afferent arteriole.<sup>23</sup> We demonstrated that inhibitory action of bumetanide (but not of L-type  $Ca^{2+}$  channel blocker nifedipine) on the myogenic response as well as on contraction triggered by  $\alpha$ -adrenergic stimulation is absent in mesenteric arteries from  $NKCC1^{-/-}$  mice.<sup>33</sup> Because NKCC2 is not expressed in SMC, these data suggest that bumetanide and other loop's diuretics inhibit contraction and myogenic response of vascular SMC via their interaction with NKCC1, i.e., an ubiquitous isoform  $Na^+$ ,  $K^+$ ,  $2Cl^-$  cotransporter.

### Synaptic transmission

Neuron–neuron interactions are controlled by neurotransmitters via regulation of transduction of electrical signals.

Excitatory and inhibitory neurotransmitters lead to depolarization and hyperpolarization of postsynaptic membrane, respectively. For example, ionotropic glutamate receptor and acetylcholine receptors cause depolarization of postsynaptic membrane via increment of ion current mediated by ion channels permeable for  $Na^+$  and  $Ca^{2+}$ . In contrast, hyperpolarization results from the increment of the permeability of  $K^+$  channels triggered by activation of metabotropic acetylcholine receptors. Unlike above-listed neurotransmitters, gamma-aminobutyric acid (GABA) increases permeability for  $Cl^-$  and other low molecular weight anions via its interaction with ionotropic  $GABA_A$  receptors. Direction of net flux mediated by these receptors is determined by transmembrane chloride gradient and electrical potential of postsynaptic membrane. In case  $RT/F \times \ln([Cl^-]_o/[Cl^-]_i) < E_m$ , the net chloride flux will be directed into the cells which will lead to hyperpolarization and attenuation of neuronal activity. An elevation of  $[Cl^-]_i$  changes the direction of net chloride flux and in this case activation of  $GABA_A$  receptors leads to elevation of neuronal activity.

Keeping this in mind, one may assume that the ratio of activity of NKCC1 and KCC2, providing inwardly and outwardly directed  $Cl^-$  fluxes in neuronal cells, respectively, plays a key role in the function of  $GABA_A$  receptors. Indeed, the attenuation of NKCC1 activity on the background of elevated KCC2 is the major mechanism of the alteration of the functional properties of  $GABA_A$  receptors in central neuronal system (CNS) of mammals during ontogenesis:  $GABA_A$  receptors function as activatory receptors in prenatal stage but became inhibitory ones few days after birth (for review see<sup>34–36</sup>).

### Salt reabsorption by NKCC2

A major impact of NKCC in salt reabsorption and regulation of extracellular fluid volume (EFV) is well-established by the clinical use of furosemide and other inhibitors of these carriers as potent diuretics,<sup>37</sup> as well as by salt-lasting characteristic of the loss-of-function mutations of NKCC2 that underpin type I Bartter syndrome.<sup>38</sup> Importantly, in addition to salt reabsorption in the TAHL, NKCC2 contributes to the regulation of salt excretion via tubuloglomerular feedback (TGF) in the juxtaglomerular apparatus (JGA) neighbouring to the distal segment of TAHL and consisting of epithelial cells of macula densa (MD), mesangial cells and VSMC. Such location is unique along the nephron and plays a key role in the JGA function. Indeed,  $[NaCl]$  in renal fluid delivered to the MD is in the range of 20–60 mM. This is in sharp contrast to the proximal tubule, where concentrations of most solutes deviate modestly from those in plasma (for more details, see<sup>39–41</sup>). TGF is triggered immediately after elevation of salt concentration in the tubular fluid delivered to the JGA and results in the contraction of VSMC of afferent arterioles, thus causing increases in the exposure of proximal tubules to high-salt fluid via the attenuation of glomerular capillary pressure and the glomerular filtration rate (GFR). As a consequence of this negative feedback loop, salt delivery to the distal nephron is kept within a narrow range. This process facilitates the fine adjustment of salt handling in the distal

tubules by corticosteroids and peptide hormones, such as aldosterone and arginine vasopressin (AVP).<sup>42,43</sup>

Similar to TAHL, the apical membrane of the MD cells is abundant in NKCC2, which provides up to 80% of apical NaCl entry in these cells.<sup>44–46</sup> Early studies of Wilcox and co-workers demonstrated that changes in the plasma [NaCl] affect renal blood flow in dogs mainly via modulation of plasma chloride concentration.<sup>47,48</sup> It was also shown that regulation of renal blood flow is mediated by activation of  $[Cl^-]_o$ -sensitive, osmolality-independent TGF.<sup>49–51</sup> Because of its stoichiometry ( $1Na^+:1K^+:2Cl^-$ ), the  $[Cl^-]_o$  sensing by NKCC2 has an advantage compared to monovalent cations. Indeed, unlike Michael–Menten's pattern for  $Na^+$  and  $K^+$  dependencies, binding of  $Cl^-$  to NKCC is a cooperative process with a Hill coefficient of 2,<sup>52</sup> providing high-efficiency regulation of carrier activity in the range of  $[Cl^-]_o$  existing in the JGA and close to the  $EC_{50}$  value of the transporter.

Using isolated rabbit cortical TAHL with attached glomeruli, Laamerti and co-workers determined that NKCC in MD cells was activated by  $Na^+_o$  and  $Cl^-_o$  with the  $EC_{50}$  of 1.0 and 17 mM, respectively.<sup>45</sup> Importantly, three alternatively-spliced NKCC2 isoforms (A, B and F) cloned from rabbit<sup>53</sup> and mouse<sup>54</sup> cDNA libraries. These splice variants, are differently distributed along the nephron: the NKCC2B and the NKCC2A are co-expressed in the MD, whereas the NKCC2F is prevalent in the medullary TAHL.<sup>55–57</sup> In *Xenopus* oocytes, the  $EC_{50}$  values of mouse NKCC2B for  $K^+_o$ ,  $Na^+_o$  and  $Cl^-_o$  are 0.8, 3.0 and 12 mM, respectively.<sup>58</sup> Very similar  $EC_{50}$  values for  $Na^+$  and  $Cl^-$  were obtained in a study of the rabbit NKCC2B.<sup>59</sup> In *Xenopus* oocytes injected with NKCC2A, the  $EC_{50}$  values for  $Na^+$  are very close to the those for NKCC2B, whereas the affinity for  $Cl^-$  is 2–5 folds lower.<sup>58,59</sup> It was also shown that the NKCC2F-transfected oocytes are more energy-efficient in  $Na^+$  uptake thus indicating a key role of this splice-variant in overall salt reabsorption in TAHL.<sup>60</sup> Indeed, experiments performed in gene knock-out animals demonstrated that NKCC2B and NKCC2A contribute to salt absorption and MD function in the low and high NaCl concentration ranges, respectively.<sup>61,62</sup> Importantly, the range of NKCC activation by  $Cl^-_o$  was consistent with the range of modulation of TGF and renin production by the apical NaCl and was similar to the  $Cl^-$  concentration in tubular fluid delivered to rat distal tubules.<sup>63</sup> Downstream signaling events triggered by acute changes in apical  $[Cl^-]$  were discussed in several comprehensive reviews.<sup>2,44,64</sup>

### Salt secretion by NKCC1

Unlike apical location of NKCC2 in the absorptive epithelia, NKCC1 was found in the basolateral membrane of the secretory epithelia.<sup>65</sup> Several laboratories including our team reported that activity of NKCC is decreased by 2–3 folds in erythrocytes from Blacks compared to Caucasians.<sup>66–70</sup> Keeping in mind that NKCC1 is the only isoforms expressed in erythrocytes, we proposed that attenuated activity of this carrier evokes salt retention seen in African-Americans via its manifestation in the basolateral membrane of sweat glands.<sup>71</sup> Several observations favour this hypothesis. *First*, both bumetanide-

sensitive  $^{86}Rb$  uptake and chloride fluxes across secretory epithelium are suppressed in *NKCC1*<sup>-/-</sup> knock-out mice.<sup>72</sup> *Second*, in humans, sweat glands contribute to up to 50% of total salt and water excretion during extensive exercise and psychoemotional activity.<sup>73</sup> *Third*, in a hot environment, the function of sweat glands as a potent regulator of EFV is suppressed in Blacks compared to Caucasians.<sup>74</sup> *Fourth*, salt retention by sweat glands results in augmented salt loading of renal distal tubule, which, in turn, leads to apical membrane depolarization and enhanced  $K^+$  secretion.<sup>75</sup> The latter is consistent with more than a 5-fold prevalence of unprovoked hypokalemia detected in African-Americans compared to Caucasians.<sup>76</sup>

In contrast to human, sweat glands have a negligible impact on EFV regulation in rodents and other small mammals.<sup>77</sup> Because of this, *NKCC1*<sup>-/-</sup> knockout mice possessing slightly attenuated blood pressure than wild-type animals<sup>72</sup> cannot be used to examine the sweat gland-mediated mechanism of NKCC1 involvement in salt handling and hypertension. Keeping this comment in mind, the comparative analysis of  $Na^+, K^+, 2Cl^-$  cotransport and transcellular ion transport in secretory epithelial cells from Blacks and Whites is probably the best approach to examine the relative impact of NKCC1 in blood pressure regulation via its implication in the EFV adjustment.

### Role of NKCC in the pathogenesis of hypertension

Elevation of systemic blood pressure has been documented in 25% of adults and is a major risk factor for stroke, heart failure, renal disease resulting in premature invalidization and death.<sup>78</sup> A priori, elevation of the systemic blood pressure may be a consequence of the increase of peripheral resistance of blood flow, heart rate and EFV. In turn, above-listed parameters are under the control of dozen of hormones, neurotransmitters and sympathetic nerve system (SNS) affecting heart, blood vessels and renal function.<sup>79</sup> In several forms of systematic hypertension, servomechanisms underlying long-term elevation of blood pressure are well-documented. This is the case of hypertension caused by adrenal tumors and renal insufficiency as well as by a set of single gene mutations in monogenous hypertension and hypotension. These forms of disease found in less than 5% of patients with elevated blood pressure are combined by common name of a secondary hypertension. In the rest of patients with primary or essential hypertension, the mechanisms of blood pressure elevation remain poorly understood.

### Monogenous forms of secondary hypertension and hypotension

Monogenous forms of hypertension and hypotension identified so far are caused by mutations of genes involved in regulation of EFV by renal epithelial cells.<sup>80,81</sup> This is consistent with a key role of the kidney in long-term maintenance of elevated blood pressure as described by Artur Guyton.<sup>82</sup> Among monogenous forms of hypertension and hypotension, three types of mutations result in altered function of CCCs. The loss-of-function mutations of NKCC2 and NCC detected in patients with Barter type I and

Gitelman syndrome, respectively, lead to attenuated salt reabsorption in thick ascending limb of Henle's loop and distal nephron, which, in turn, results in a decreased EFV and systemic blood pressure.<sup>38,83</sup> In both diseases inherited in accordance with classic Mendel's genetics, hypotension is accompanied by hypokalemia and alkaloidosis – universal markers of decreased reabsorption of salt in distal nephron. In contrast, in patients with pseudohypoaldosteronism type II (PHAII) also known as Gordon's syndrome, hypertension is caused by mutations in with-no-K(lysine) kinases WNK1 and WNK4 resulting in activation of NCC, increased sodium reabsorption and hyperkalemia.<sup>84</sup>

## Primary hypertension

Unlike monogenous forms of secondary hypertension, elevation of blood pressure in primary hypertension is a consequence of a complex combination of inheriting traits and several environmental factors including limited physical activity, obesity, smoking, the augmented consumption of salt and alcohol. Inherited traits are probably caused by altered function of 4–5 genes whose combination may be different even within the same human population,<sup>85</sup> underlining mosaic origin of the pathogenesis of essential hypertension as noted for the first time by Pickering.<sup>86</sup> Up-to-now, there are no reports on mutations of NKCC2 and other renal-specific CCCs in patients with essential hypertension. Schiebl and co-workers found that in mice differential splicing of NKCC2 pre-mRNA is modulated by dietary salt intake,<sup>87</sup> which may be triggered by recently discovered  $[Na^+]_i/[K^+]_i$ -mediated excitation-transcription coupling (for review, see<sup>88</sup>). Enhanced tubular reabsorption of salt and osmotically-obliged water is important determinant of obesity-related hypertension. Davies and co-workers reported that increased activity of NKCC2 in high-fat diet mice is caused by STE20/SPS1-related proline/alanine-rich kinase SPAK/OSR1-mediated phosphorylation of this carrier at serine-126.<sup>89</sup> Additional studies should be performed to examine the role of this signaling pathway in the regulation of renal-specific CCCs in the pathogenesis of essential hypertension.

In the early studies, it was shown that elevated permeability for monovalent cations of VSMC<sup>90</sup> as well as erythrocytes from spontaneously hypertensive rats (SHR)<sup>91</sup> and patients with essential hypertension<sup>92</sup> is at least partially caused by augmented activity of NKCC (for review, see<sup>93–97</sup>). Because NKCC1 is the only isoform of  $Na^+$ ,  $K^+$ ,  $2Cl^-$  cotransporters identified in erythrocytes, these data indicate that at least in these experimental models of human primary hypertension, augmented activity of this carrier contributes to activation of servomechanisms for long-term elevation of systemic blood pressure. This conclusion can be supported by several observations. *First*, in erythrocytes of F1 hybrids obtained by crossing of Milan hypertensive strain (MHS) and Milan normotensive strain (MNS) rats and subjected to X-ray irradiation, NKCC activity was increased after transplantation of bone marrow from MHS but not from MNS.<sup>98</sup> *Second*, in erythrocytes of F<sub>2</sub> MHS × MNS hybrids as well as F<sub>2</sub> hybrids obtained by crossing of SHR and normotensive Wistar-Kyoto (WKY) rats, NKCC activity positively correlated with blood

pressure.<sup>98,99</sup> *Third*, several researchers demonstrated decreased blood pressure in  $NKCC1^{-/-}$  knock out mice.<sup>72,100,101</sup> *Fourth*, administration of bumetanide, a potent inhibitor of  $Na^+$ ,  $K^+$ ,  $2Cl^-$  cotransport, decreased blood pressure in wild-type but not in  $NKCC1^{-/-}$  mice.<sup>27</sup>

These findings raise a question on the mechanisms for the involvement of NKCC1 in the pathogenesis of hypertension. Data considered above suggest that these mechanisms may involve NKCC1-mediated regulation of  $[Cl^-]_i$  which, in turn, affects VSMC contraction and SNS activity. Indeed, it was shown that inhibitory action of bumetanide on the contraction of mesenteric arteries evoked by activation of  $\alpha$ -adrenergic receptor is increased in SHR compared to normotensive controls.<sup>102,103</sup> Due to the methodological problems, data on the activity of NKCC in freshly isolated VSMC in primary hypertension are limited to few publications.<sup>94,95</sup> It was shown, however, that the content of NKCC1 mRNA and immunoreactive protein is increased in aorta and heart from SHR.<sup>102</sup> Elevation of sympathetic tone may lead to elevation of systemic blood pressure via its impact on cardiovascular system and the kidney.<sup>104–107</sup> This hypothesis is consistent with numerous reports on activation of SNS in patients with essential hypertension<sup>104</sup> and SHR.<sup>108</sup>

The major role of CNS in activation of paraventricular nucleus (PVN) in hypothalamus possessing hyperactivity in primary hypertension is well documented.<sup>109–111</sup> It is also known that presynaptic neurons in PVN are activated by excitatory glutamatergic neurones and suppressed by inhibitory GABAergic neurones, respectively.<sup>112</sup> Importantly, activity of GABAergic neurones is decreased in PVN of SHR.<sup>112,113</sup> As mentioned above, the relationship between the inhibitory and stimulatory actions of GABA<sub>A</sub> receptors is determined by intracellular chloride concentration that is under the control of the ratio of NKCC1 vs. KCC2 activities. Recent studies demonstrated that the value of the equilibrium potential for GABA<sub>A</sub> receptors ( $E_{GABA}$ ) is shifted to positive values in PVN of SHR by 15 mV, which corresponds to a 2-fold elevation of  $[Cl^-]_i$  compared to normotensive control.<sup>114</sup> This difference as well as decreased inhibitory activity of GABAergic neurones in SHR was abolished by addition of low doses of bumetanide but not furosemide. These results allowed authors to propose that augmented  $[Cl^-]_i$  in SHR neurons is caused by activation of NKCC1 rather than inhibition of KCC2. This conclusion is in agreement with elevated of NKCC1 mRNA and immunoreactive protein levels with no changes in KCC2 content in PVN of SHR.<sup>114</sup>

Mechanisms of elevation of NKCC1 activity in primary hypertension remain poorly investigated, which probably reflects the polygenic and mosaic origin of this disease as well as diverse mechanisms of regulation of the activity and expression of CCCs. For example, elevation of  $[Ca^{2+}]_i$  activates whereas production of cAMP inhibits this carrier in VSMC.<sup>26,115,116</sup> Numerous investigations documented abnormal activity of both signaling systems in primary hypertension.<sup>117,118</sup> Recent studies demonstrated a key role of WNK, SPAK and OSR1 kinases in regulation of several CCCs including NKCC1 and NKCC2 (for reviews, see<sup>119–122</sup>). Bergaya and co-workers reported that both phosphorylation of NKCC1 and increment of blood pressure evoked by activation of  $\alpha$ -adrenergic receptors is decreased in  $Wnk^{+/-}$

mice.<sup>123</sup> Bumetanide-sensitive component of vessel contraction was also attenuated in SPAK knock-out mice.<sup>124</sup> To further examine the role of this signalling pathway, Rafiqi and co-workers generated knock-in mice in which SPAK cannot be activated by WNKs. These animals display reduced salt-dependent increment of blood pressure as well as decreased expression of NKCC2 and NCC proteins with no changes in mRNA level.<sup>125</sup> It is noteworthy, however, that there is no evidence for mutations of genes encoding CCCs or WNK/SPAK/OSR1 regulatory pathway in primary hypertension in contrast to monogenic hypertension.

Epigenetic phenomena include DNA methylation, post-translation histone modification and expression of non-coding RNAs. Epigenetic mechanisms are under complex control of diverse environmental factors, which make them crucial for their involvement in the pathogenesis of hypertension and other complex and multifactorial disorders.<sup>126</sup> Recent studies suggest that NKCC1-mediated abnormalities of ion transport have epigenetic origin.<sup>127</sup> It has been shown that the contents of NKCC1 mRNA and protein are increased in aorta, heart and PVN neurones from rats with spontaneous hypertension.<sup>102,114</sup> In SHR aorta and heart, increased expression of NKCC1 is accompanied by attenuated methylation of its promoter.<sup>102</sup> Importantly, methylation of *NKCC1* promoter exhibited age-dependent increase in normotensive rats but not in SHR. It was also shown that the activity of DNA methyltransferase 3B (DNMT3B) is 3-fold higher in normotensive rats as compared to age-matched SHR.<sup>103</sup> These results suggest that in this experimental model of primary hypertension, decreased activity of DNMT3B results in hypomethylation of *NKCC1* promoter, which in turn leads to augmented NKCC1 expression, increment of  $[Cl^-]_i$ , depolarization and contraction of SMC, increased vascular resistance and elevation of blood pressure.

The role of epigenetic factors in augmented expression of NKCC1 in neurones of PVN of SHR, controlling activity of SNS, remains unknown. However, it was shown that in PVN from SHR, NKCC1 is highly glycosylated, which may contribute to increased content of membrane-bound protein, i.e., the fraction of the carrier providing  $Na^+$ ,  $K^+$ ,  $2Cl^-$  cotransport.<sup>114</sup> Thus, glycosylation of NKCC could represent another level of its regulation.

### Complications caused by elevated blood pressure

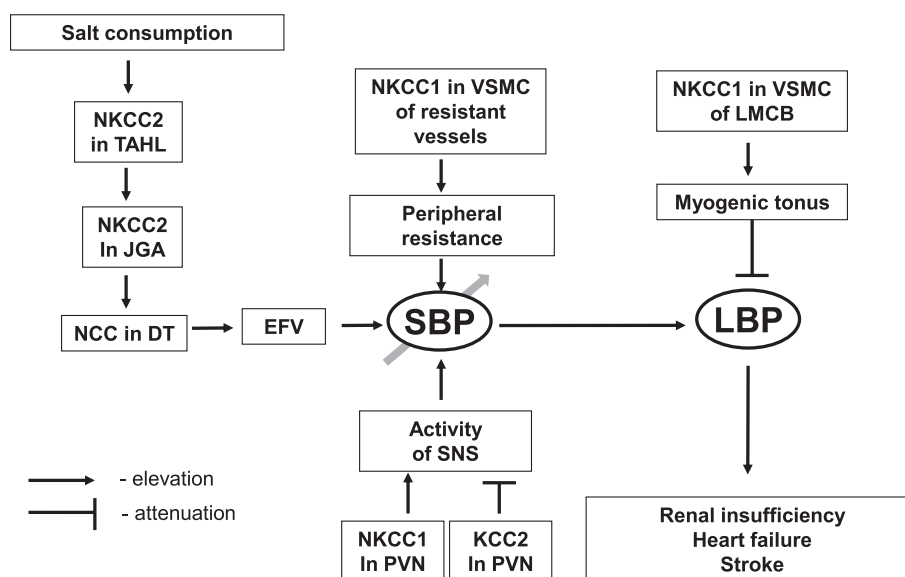
The major cause of the premature death of patients with essential hypertension is a damage of the target organs such as brain vessels and the kidney due to chronic elevation of local blood pressure.<sup>128</sup> Blood pressure elevation in the brain microcirculation increases the probability of nonreversible damage of the blood flow that results in stroke, whereas in the kidney high blood pressure leads to structural alterations in the nephron, changes of salt-water homeostasis and proteinuria.<sup>78</sup>

Keeping in mind that  $R_{bf} \sim 1/d^4$  (where  $R_{bf}$  is the blood flow resistance and  $d$  is the inner vessel's diameter),<sup>129</sup> myogenic response plays a key role in the protection of target organs from elevation of systemic arterial pressure. It was shown that chronic suppression of myogenic

response in patients with essential hypertension as a consequence of hypertrophy of vessel's wall leads to attenuation of its sensitivity to the changes of intraluminal pressure. As a result of these changes, an increment of systemic blood pressure is transferred to microcirculation beds incorporated into the brain, heart, retina, kidney, which causes irreversible changes in the structure and function of these and other target-organs of hypertension.<sup>130,131</sup>

To investigate the role of myogenic response in the kidney function, Loutzenhiser and co-workers employed isolated perfused kidney, which allowed them to study renal microcirculation in the absence of its modulation by JGA.<sup>132</sup> Using this model, they have shown that bumetanide completely suppresses myogenic response of afferent arteriole in the rat kidney.<sup>23</sup> Together with the absence of myogenic response in *Nkcc1*<sup>-/-</sup> mice<sup>33</sup>, these results may suggest that augmented activity of NKCC1 in SHR, MHS rats and in patients with essential hypertension protects kidney from the damage by prolonged elevation of the systemic blood pressure whereas chronic administration of furosemide and other NKCC inhibitors accelerates the renal insufficiency and proteinuria.<sup>71,96,97</sup> In other words, high activity of NKCC1 in VSMC of the afferent arteriole maintains the constant renal blood flow even after elevation of systemic blood pressure caused by activation of this carrier in mesenteric arteries and other vessels contributing to regulation of peripheral resistance (Fig. 1). This hypothesis is consistent with 4-fold increase of renal complications in African-Americans with hypertension possessing up to 3-fold attenuation of NKCC activity in erythrocytes as compared to age-matched hypertensive Caucasians.<sup>70,71</sup> The relative contribution of  $Ca^{2+}$  channels and NKCC1 in the myogenic response of coronary and SNS microcirculatory beds remains unexplored.

Ischemia is the major consequence of even a brief disturbance of blood circulation in the brain vessels that leads to irreversible damage of the neuronal function. It was shown that attenuation of the oxygen partial pressure results in astrocyte swelling, which in turn leads to a release of glutamate and other neurotransmitters triggering massive  $Ca^{2+}$  influx in neurones and their death.<sup>133,134</sup> It was shown that bumetanide suppresses astrocyte swelling, neurotransmitter's release<sup>135</sup> and the death of neurones in the hippocampus subjected to hypoxia and hypoglycemia.<sup>136</sup> Moreover, both  $[K^+]_o$ -induced swelling and release of neurotransmitters evoked by ischemia are sharply decreased in astrocytes from *NKCC1*<sup>-/-</sup> mice,<sup>137</sup> thus indicating that activation of NKCC1 is a mechanism for astrocyte swelling. We have reported that elevation of  $[HCO_3^-]_o$  decreases NKCC activity in VSMC from rat aorta.<sup>138</sup> Thus, activation of NKCC1 under hypoxic conditions might be caused by acidosis-mediated attenuation of  $[HCO_3^-]$  in cerebrospinal fluid. Hypoxia is also accompanied by sharp attenuation of intracellular ATP content and inhibition of the  $Na^+$ ,  $K^+$ -ATPase.<sup>139</sup> As shown in Table 2, inhibition of the  $Na^+$ ,  $K^+$ -ATPase changes the direction of NKCC1-mediated net ion fluxes and evokes cell swelling. Additional experiments should be performed for evaluation of the relative impact of  $Na^+$ ,  $K^+$ -pump and NKCC1 in regulation of astrocyte volume in ischemic conditions.



**Figure 1** The scheme showing implication of cation-chloride cotransporters in the pathogenesis of essential hypertension and its cardiovascular and renal complications. Augmented salt consumption leads to elevation of extracellular fluid volume (EFV) via salt reabsorption by NKCC2 in the thick ascending limb of Henle's loop (TAHL), NCC in distal tubule (DT) and sensing of  $\text{Cl}^-$  concentration in tubular fluid delivered to juxtaglomerular apparatus (JGA) by NKCC2. Augmented activity of NKCC1 in vascular smooth muscle cells (VSMC) resulted in elevation of peripheral resistance and systemic blood pressure (SBP). Augmented activity of NKCC1 and attenuated of KCC2 in neuronal cells of paraventricular nucleus (PVN) also contributes to SBP elevation via activation of sympathetic nervous system (SNS). On the other hand, augmented NKCC1 activity in VSMC of local microcirculatory bed (LMCB) resulted in suppression of myogenic tonus, elevation of local blood pressure (LBP) that, in turn, contributes to the pathogenesis of renal insufficiency, heart failure and stroke.

## Conclusion

The data discussed in this review leads to several conclusions.

*First*, the transport of monovalent ions across the cells as well as the regulation of intracellular chloride concentration and of cell volume are major functions of NKCCs. In renal epithelial cells, reabsorption of salt and osmotically-obliged water is provided by NKCC2, whereas NKCC1 has a major impact on  $[\text{Cl}^-]_i$  in VSMC and neuronal cells.

*Second*, in primary hypertension, NKCC1 activity is increased in VSMC and neurons of PVN leading to elevation of peripheral resistance in systemic circulation and activation of SNS, respectively. In both cases, these abnormalities are mediated by elevation of  $[\text{Cl}^-]_i$  and plasma membrane depolarization.

*Third*, furosemide and other loop's diuretics decrease systemic blood pressure via inhibition of NKCC2 in TAHL and of NKCC1 in smooth muscles of resistant vessels. However, the same compounds suppress the myogenic response in microcirculatory beds of the kidney and the brain thus increasing the risk of renal and cerebral complications.

In spite of the progress in the understanding the role of NKCC in the regulation of cellular functions at physiological and pathophysiological conditions, several questions remain unanswered. In cultured VSMC as well as in isolated blood vessels, NKCC1 is activated by phenylephrine, angiotensin II and other vasoconstrictors and is inhibited by vasodilators whose action is mediated by cAMP.<sup>26,115</sup> Recently, Danielsson and co-workers have reported that

the combination of Cl-channel blockers and bumetanide potentiates relaxation of airway smooth muscle rings by  $\beta$ -adrenergic agonists.<sup>140</sup> Hence, the question is whether NKCC1 contributes to the regulation of vascular tone by these compounds. A key role of NKCC1 in regulation of myogenic response of renal afferent arteriole is well-documented. The question is what is the relative contribution of NKCC1 to the regulation of myogenic response of microcirculatory beds of the brain and other target-organs of hypertension.

*In vitro* experiments have demonstrated that loop's diuretics might be used as a pharmacological tools augmenting inhibitory function of GABA<sub>A</sub> receptors. It was shown that at least furosemide fluently penetrates human blood-brain barrier.<sup>141</sup> This finding together with the data discussed in the previous sections suggests that high-ceiling diuretics may be used for the regulation of sympathetic tone via inhibition of NKCC1 in neuronal cells. Indeed, it was shown that orally administrated furosemide at doses commonly used in clinic reduced spinal inhibitory interneuronal activity.<sup>142</sup> It is important to point out that currently used high-ceiling diuretics exhibit the same affinity for NKCC1 and NKCC2. Because an apparent affinity for furosemide and bumetanide is proportional to carrier's activity,<sup>143</sup> inhibition of highly active NKCC2 and diuretic action of these compounds is much greater than their vasodilatory and neuronal effects. It is also important to mention another side effect of these drugs, i.e., their prolonged administration may result in the development of deafness due to inhibition of NKCC1 in epithelial cells of the

inner ear.<sup>144,145</sup> These issues become important for the development of novel antihypertensive drugs lacking side-effects caused by NKCC inhibition in epithelial cells and myogenic tonus in microcirculatory beds.

## Conflicts of interest

All authors have none to declare.

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