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WNK-Cab39-NKCC1 signaling increases the susceptibility to ischemic brain damage in hypertensive rats

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Abstract

With-no-lysine kinase (WNK) and Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) are involved in the pathogenesis of hypertension. In this study, we investigated the WNK-NKCC1 signaling pathway in spontaneously hypertensive rats (SHR) and their associated susceptibility to stroke injury. Basal NKCC1 protein levels were higher in SHR than in normotensive Wistar Kyoto (WKY) rat brains. After inducing ischemic stroke, adult male WKY and SHR received either saline or NKCC1 inhibitor bumetanide (10 mg/kg/day, i.p.) starting at 3-h post-reperfusion. NKCC1 inhibition blunted the extent of ischemic infarction in SHR and improved their neurobehavioral functions. Interestingly, ischemia led to increased NKCC1 phosphorylation in SHR but not in WKY rats. Pronounced elevation of WNK1, WNK2 and WNK4 protein and downregulation of WNK3 were detected in ischemic SHR, but not in ischemic WKY rats. Upregulation of WNK-NKCC1 complex in ischemic SHR brain was associated with increased Ca^{2+}-binding protein 39 (Cab39), without increases in Ste20-related proline alanine-rich kinase or oxidative stress-responsive kinase-1. Moreover, subacute middle cerebral artery stroke human brain autopsy exhibited increased expression of NKCC1 protein. We conclude that augmented WNK-Cab39-NKCC1 signaling in SHR is associated with an increased susceptibility to ischemic brain damage and may serve as a novel target for anti-hypertensive and anti-ischemic stroke therapy.

Keywords

Bumetanide, Cab39, hypertension, ischemic stroke, NKCC1, SHR, WNK kinase

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Introduction

WNK (with-no-lysine (K)) kinases are serine (Ser)/threonine (Thr) kinases, which regulate electroneutral SLC12 cation-Cl^- cotransporters (CCC) by phosphorylation.[1] The Ste20-related proline alanine-rich kinase (SPAK) and oxidative stress-responsive kinase-1 (OSR1) are downstream substrates of WNK kinases acting as links between WNKs and ion transporters.[1–3] The WNK signaling pathway plays an important role in renal NaCl and K^+ handling and the pathogenesis of hypertension in humans, while *SPAK* is a hypertension susceptibility gene.[4,5] *WNK1* and *WNK4* mutations lead to constitutive activation of the kidney-specific thiazide-sensitive Na^+-Cl^- cotransporter. This constitutive activation causes familial hyperkalemic hypertension (FHHt, also known as pseudohypoaldosteronism type 2), an autosomal dominant disorder characterized by hypertension, increased renal Na^+ reabsorption, and impaired excretion of K^+ and H^+.[6] However, mutations in kelch-like 3 (*KLHL3*) and cullin 3 (*Cul3*), components of an E3 ubiquitin ligase complex, can also cause FHHt via impaired regulation of WNK degradation.[7,8] On the other hand, the WNK-SPAK/OSR1 signaling pathway is involved in maintaining arterial tone and blood pressure by directly stimulating Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) activity in resistance vessels. Global heterozygous knockdown of OSR1 or global knockout of SPAK or NKCC1 can lower blood pressure by extrarenal mechanisms.[9–11]

Developmental up-regulation of NKCC1 protein and mRNA was detected in aorta, heart, and kidney in spontaneously hypertensive rats (SHRs) at postnatal ages of 10–18 weeks.[12] NKCC1 up-regulation in hypothalamus disrupts Cl^- homeostasis and impairs GABAergic inhibition, providing a neurological basis for the diminished synaptic inhibition and elevated sympathetic vasomotor tone that accompany hypertension in SHRs.[13] It is well documented that SHRs exhibit more severe ischemic brain damage than normotensive rats.[14,15] Previous studies from our group and others showed that stimulation of NKCC1 activity is involved in ischemic cell damage through NKCC1-mediated Na^+ and Cl^- overload, cytotoxic edema as well as excitotoxicity.[16–19] Interestingly, we have recently found that WNK3 and SPAK kinases are stimulated in cortical neurons and oligodendrocytes in mice after ischemic stroke.[20] Knockout of *WNK3* or *SPAK* in mice abolishes phosphoactivation of NKCC1 and reduces brain infarction and associated malignant edema after ischemic stroke.[20,21] These findings led us to investigate the possibility that the WNK-SPAK/OSR1-NKCC1 signaling pathway is stimulated in the brains of SHRs, leading to increased susceptibility to ischemic damage.

Here, we report that SHR brain expressed higher levels of total and phosphorylated (activated) NKCC1 protein and exhibited larger cerebral infarct and hemispheric swelling than did normotensive Wistar Kyoto (WKY) rats. WNK1, WNK2, and WNK4 were up-regulated in ischemic SHR brains, but not in those of WKY. Pharmacological inhibition of NKCC1 function with bumetanide (BMT) nearly abolished the SHR-associated increment in post-ischemic infarct and hemispheric swelling volume. Together, our data suggest that an alternative WNK-Cab39-NKCC1 signaling pathway may play an important role in exacerbating cerebral ischemic damage in hypertensive SHRs.

Material and methods

Animal preparation

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The manuscript adheres to the ARRIVE guidelines for reporting animal experiments.

Male SHR and WKY rats of 10–13 weeks of age were purchased from Harlan Laboratory (Indianapolis, IN) and maintained in the University Laboratory Animal Facility until experimental use. All rats were housed in group (two per case) in a controlled temperature and humidity environment. They were maintained on a 12-h light/12-h dark cycle and provided with food and water ad libitum. At the time of experimental use, rats weighed 270–320 g.

Middle cerebral artery (MCA) occlusion model

Focal cerebral ischemia was induced by transient occlusion of the left MCA for 2 h as described previously.[22] A detailed description of this method is provided in the Data Supplement.

Drug treatment

Either saline or bumetanide (10 mg/kg body weight/day in saline) was administered via intraperitoneal injection (Figure 1(a)). The initial dose of 5 mg/kg at 3 h and the second dose of 5 mg/kg at 8-h post-reperfusion were followed by two daily injections (b.i.d.).

Calculation of infarct volume and hemispheric swelling

After 24 h of reperfusion, rats were anesthetized with 5% isoflurane vaporized in N_2O and O_2 (3:2) and

decapitated. Coronal brain sections were stained with 1% 2,3,5-triphenyltetrazolium chloride monohydrate to measure infarct volume and hemispheric swelling as described before.[23,24] A detailed description is provided in the Data Supplement.

Immunofluorescence and immunohistochemistry

De-identified, formalin-fixed, paraffin-embedded human brain autopsy tissue sections were obtained from the Neurodegenerative Brain Bank at the University of Pittsburgh with approval of the local Committee for Oversight of Research and Clinical Training Involving Decedents (CORID). Tissue sections of infarct tissue and separate sections of non-ischemic cortical tissue were obtained for all cases. All strokes were located within the MCA vascular territory and were classified by morphological criteria as 'early subacute'.

In a blinded manner, immunohistochemical study was conducted on human autopsy brain samples. Brain sections were blocked for endogenous peroxidase and biotin before overnight application of primary antibodies (mouse anti-tNKCC1 1:100 or rabbit anti-MAP-2 1:200) at 4°C followed by 60-min RT incubation with secondary antibodies. Subsequent immunodetection was with the Elite Vector Stain ABC System, using DAB as the chromogen substrate. Images were acquired with a Nikon TE-2000 brightfield microscope.

The immunofluorescence staining was conducted on human autopsy brain tissue sections and SHR brain sections as previously described.[25,26] A detailed description is provided in the Data Supplement.

The online-only Data Supplement of the article is available for materials and other experimental methods used in the study.

Statistical analysis

Animal subjects were randomly assigned into different studies and surgical procedures, and data analyses were performed by investigators blinded to experimental conditions. The number of animals studied was 80% powered to detect 20% changes with α (two-sided) = 0.05. A total of 86 rats were used in the study, and no results were excluded from the analysis. Data were expressed as mean ±SD. Statistical significance was determined by Student's *t*-test, or two-way ANOVA using the Tukey's post-hoc test in case of multiple comparisons (GraphPad Prism 6.0, San Diego, CA, USA). Neurological deficit score was analyzed by the non-parametric Mann–Whitney test. The Pearson correlation coefficient was calculated using online statistics software (Office for Research Development and Education, version 1.1.23-r7). A probability value < 0.05 was considered statistically significant.

Results

Pharmacological inhibition of NKCC1 activity reduces infarction and severity of swelling and improves neurological function of SHR

NKCC1 activation during cerebral ischemia has been documented to disrupt cerebral ion homeostasis, leading to cellular damage in rat and mouse.[16,18] We first tested the ability of pharmacological inhibition of NKCC1 with bumetanide (BMT) to attenuate the increased infarction severity in SHR. Regional cerebral blood flow was indistinguishable in WKY and SHR prior to, during, and after MCAO (Figure 1(b)). After 24-h reperfusion post-MCAO, cortical infarct volume in SHR brains ($299.3 \pm 35.7\,mm^3$) was markedly larger than in WKY brains ($220.0 \pm 30.6\,mm^3$, $p < 0.05$, Figure 1(c) and (d)). BMT administration significantly attenuated infarction size in SHR, reducing infarct volume to $214.5 \pm 17.3\,mm^3$ ($p < 0.05$). BMT treatment only moderately reduced infarct volume in WKY rats to $165.2 \pm 14.2\,mm^3$, a difference that did not reach statistical significance ($p = 0.16$). Figure 1(e) illustrates brain swelling in saline-treated WKY and SHR brains (17.0 ± 2.1 and $21.9 \pm 2.5\%$, respectively). BMT treatment blunted brain swelling to indistinguishable lower levels in both SHR and WKY brains ($12.3 \pm 2.0\%$ and $10.4 \pm 1.8\%$, respectively).

BMT treatment also improved sensorimotor deficits in WKY and SHR. As shown in Figure 1(f), latency times in the turn-alley test ($79.2 \pm 21.5\,s$) were longer in saline-treated SHR than in WKY ($46.8 \pm 15.6\,s$, $p < 0.05$), reflecting the worsened sensory motor function deficits in SHR. BMT treatment significantly improved the sensory motor function deficits in SHR, with latency values indistinguishable from those of WKY. Faster improvement of neurological deficit scores was also detected in the BMT-treated WKY and SHR groups (Figure 1(g)). Taken together, these data strongly suggest that augmented NKCC1 expression and activation in ischemic SHR brains are associated with the latter's greater severity of ischemic injury.

Ischemic stroke-induced NKCC1 phosphorylation is elevated in SHR brain

Basal, non-ischemic levels of total NKCC1 protein (tNKCC1, 130 kDa and 170 kDa) in SHR brain exceeded those in WKY brain by $52.8 \pm 9.1\%$ in membrane fractions and by $39.1 \pm 2.8\%$ in whole brain lysates (Figure 2(a)). tNKCC1 protein levels in cytosol fractions of WKY and SHR brains did not differ. These data suggest that the increased tNKCC1 protein of SHR brain is enriched in membrane

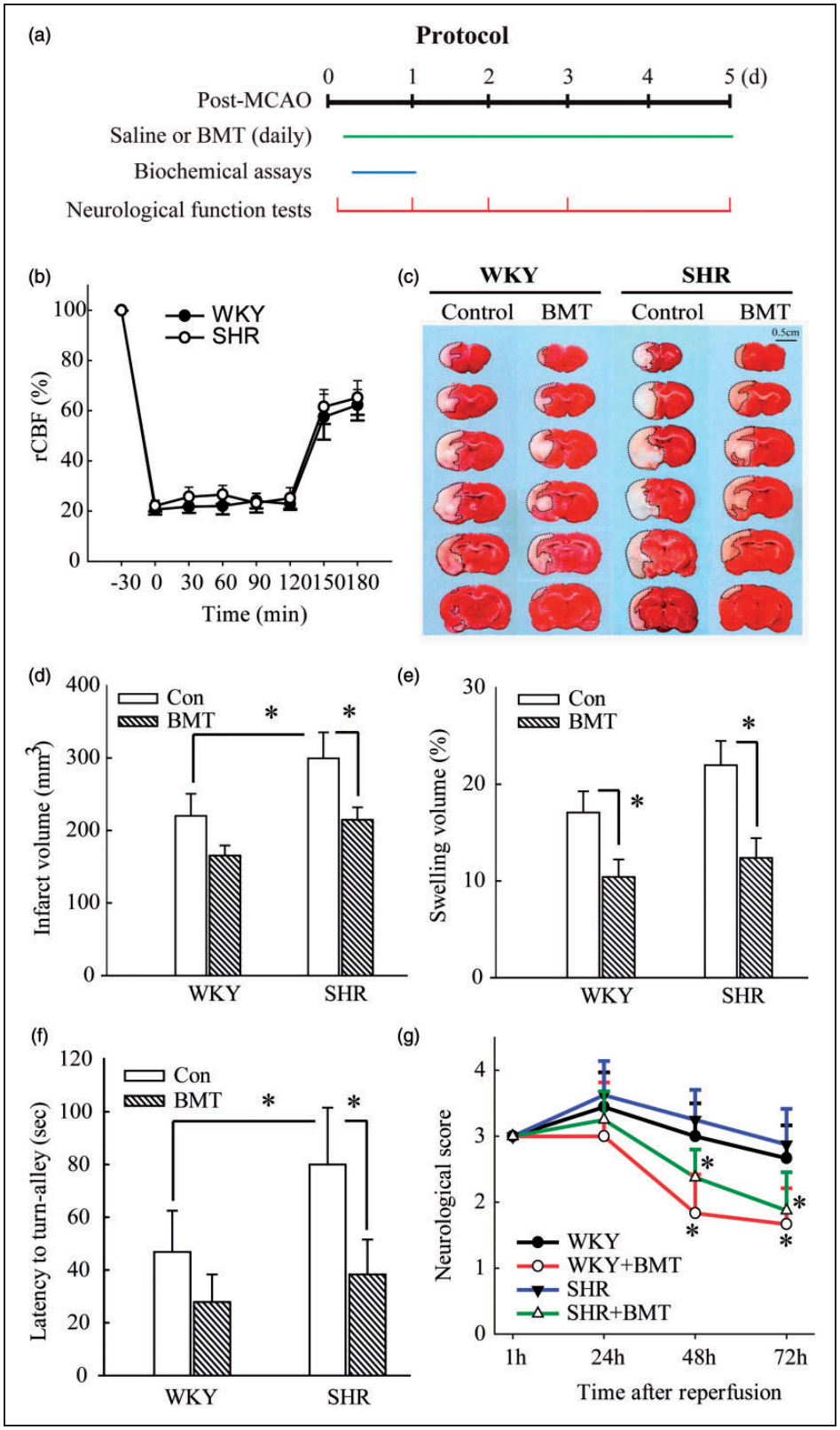

Figure 1. NKCC1 inhibitor BMT attenuates ischemic stroke-mediated exacerbation of infarction and improves sensorimotor deficits in SHR. (a) Experimental protocol and data collection. BMT: bumetanide; tMCAO: transient middle cerebral artery occlusion. (b) rCBF measurements during and after 2-h tMCAO were indistinguishable in SHR and WKY rats. (c) Representative TTC staining images in WKY and SHRs at 24-h reperfusion, with quantitative analysis of infarct volume (d) and percent hemisphere swelling (e). Saline or bumetanide (10 mg/kg body weight/day in saline) was administered via intra-peritoneal injection, with an initial BMT dose (5 mg/kg) at 3 h and the second dose (5 mg/kg) at 8-h reperfusion and followed by two daily injections (bid). Data are mean ± SD, n = 4, $^*p < 0.05$. (f) Turning-in-alley test latency. Data are mean ± SD, n = 8, $^*p < 0.05$. (g) Neurological deficit scores in WKY and SHRs. Data are mean ± SD, n = 8, $^*p < 0.05$ vs. respective control.

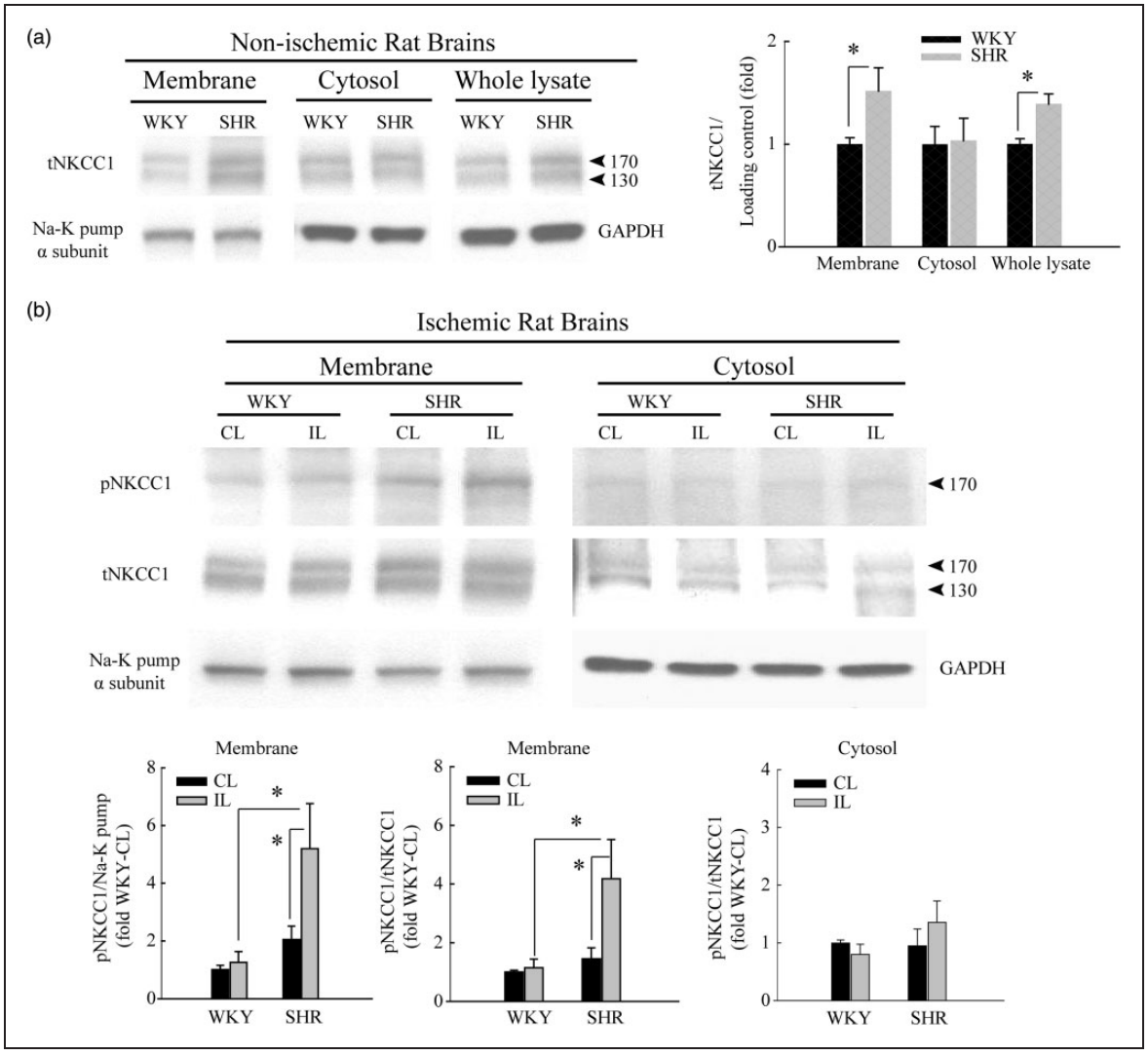

Figure 2. Selective elevation of NKCC1 protein expression in the cerebral cortex of SHR brain after ischemic stroke. (a) Representative immunoblots showing higher baseline expression of total NKCC1 (tNKCC1) in whole homogenate and crude membrane fractions of brain cortices from normal control SHR than in those from WKY rats. α-subunit of Na^+-K^+ pump and GAPDH served as loading controls for membrane and cytosol fractions, respectively. Data are mean ± SD, n = 4, *$p < 0.05$ WKY vs. SHR. (b) Ischemic stroke selectively stimulates phosphorylated NKCC1 (pNKCC1) expression at 6-h post-tMCAO in SHR but not WKY rats. Cytosol and crude membrane protein fractions were prepared from contralateral (CL) and ipsilateral (IL) cortices of WKY and SHR. Data are mean ± SD, n = 4, *$p < 0.05$ WKY vs. SHR.

fractions, consistent with recent reports of elevated levels of glycosylated NKCC1 protein in SHR hypothalamus.[13]

We investigated the possible contribution of elevated NKCC1 expression and function to the increased susceptibility of SHR brains to ischemic damage.[15] The contralateral (CL) hemispheres of SHR brains revealed basal levels of phosphorylated NKCC1 (pNKCC1, 170 kDa band) 103.0 ± 19.5% higher than in WKY brains (Figure 2(b)). At 6-h post-reperfusion following tMCAO, no significant differences in expression of either tNKCC1 or pNKCC1 were detected in the membrane-enriched fraction from ipsilateral hemispheres of (IL) WKY brains. In contrast, ischemic stroke increased pNKCC1 expression by 2.5 ± 0.5 fold in the IL membrane-enriched fraction of SHR brains compared with CL membrane fractions of SHR. No differences in pNKCC1 expression were detected in cytosol fractions of either WKY or SHR brains. These findings further indicate that ischemic stroke triggers an earlier onset of stimulatory phosphorylation of NKCC1 in SHR brains than WKY brains. We hypothesized that the WNK kinase signaling pathway may be involved in this stimulation in SHR brains.

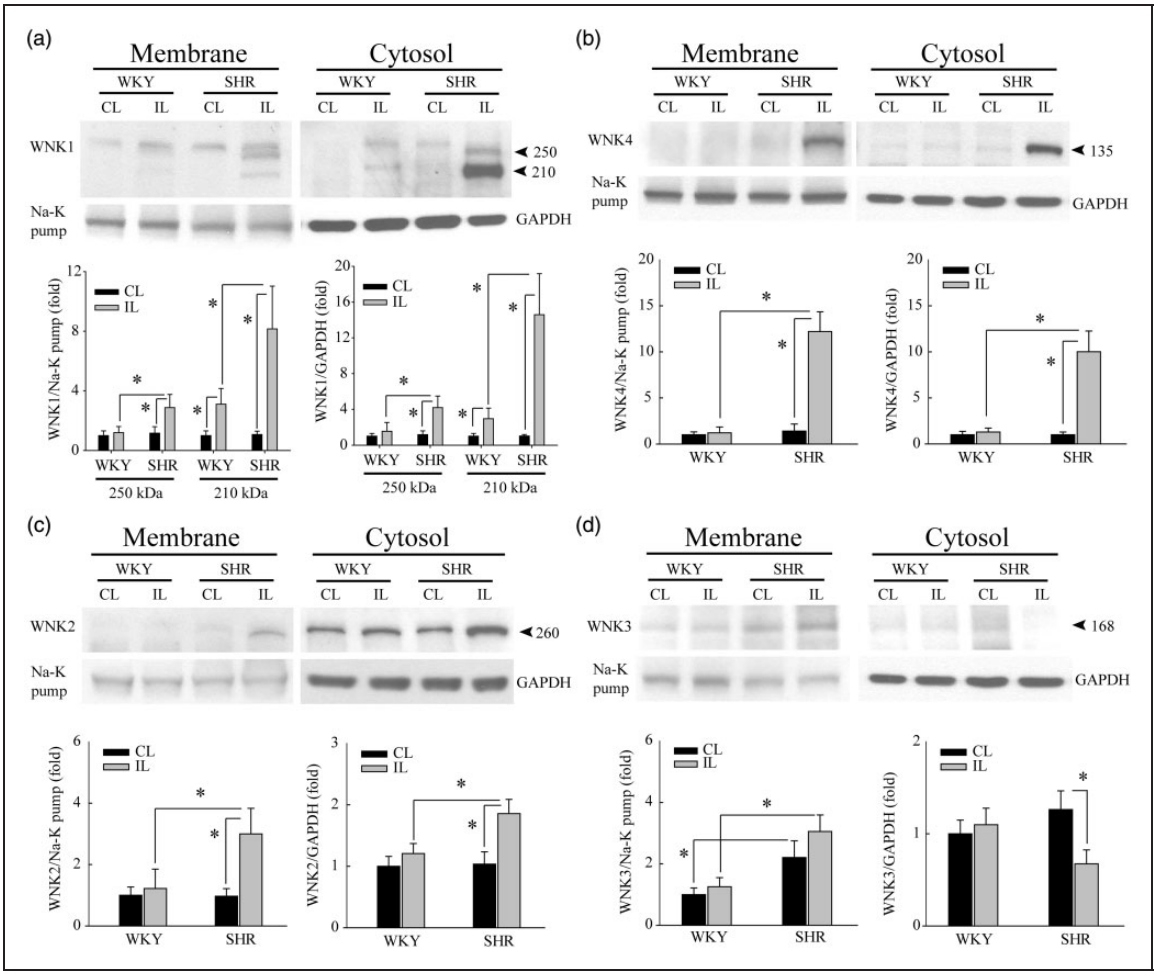

Figure 3. SHR brains exhibit pronounced upregulation of WNK1, WNK2, and WNK4 proteins after ischemic stroke. (a) Upregulation of full length WNK1 protein (250 kDa) was detected at 6-h reperfusion post-tMCAO only in IL cortices of SHR but not WKY rats. Cytosol and crude membrane protein fractions were prepared from contralateral (CL) and ipsilateral (IL) cortices of WKY and SHR. Data are mean ± SD, n = 4, $*p < 0.05$. (b) Upregulation of WNK4 protein in the same samples as in (a). Data are mean ± SD, n = 4, $*p < 0.05$. (c) Upregulation of WNK2 protein in the same samples as in (a). Data are mean ± SD, n = 4, $*p < 0.05$. (d) Ischemic stroke did not cause significant changes of WNK3 protein expression in either WKY or SHR brains but reduced WNK3 expression in the IL cytosol fraction of SHR brains. Data are mean ± SD, n = 4, $*p < 0.05$.

SHR brains exhibit pronounced upregulation of WNK1, WNK2, and WNK4 proteins after ischemic stroke

We investigated changes in the abundance of ion transporter kinases WNK1, WNK2, WNK3, and WNK4 in WKY and SHR brains after ischemic stroke. Figure 3(a) shows that WKY brains had low basal WNK1 protein expression (250 kDa) and did not exhibit significant changes after ischemic stroke. In contrast, ischemic SHR brains showed 4.2 ± 1.2 fold increase and 2.8 ± 0.8 fold increase of WNK1 protein (250 kDa) in membrane and cytosol fractions, respectively. A lower molecular weight WNK1 band (~210 kDa) was detected in WKY and SHR brains, which was not detected in kidney tissue (Supplemental Figure 1). Ischemic stroke increased WKY brain abundance of the 210 kDa WNK1 protein by 2.9 ± 1.1 fold (cytosol) and 3.1 ± 1.0 fold (membrane fractions), respectively. Ischemic SHR cortical tissues exhibited far more dramatic increases of the 210 kDa WNK1 protein in the membrane fraction (8.1 ± 2.5 fold) and cytosol fraction (14.5 ± 4.5 fold).

WNK4 protein upregulation (135 kDa) was detected in ischemic SHR brains, with a 12.2 ± 2.1 fold increase in the IL membrane fraction and a 10.0 ± 2.2 fold increase in the IL cytosol fraction (Figure 3(b)). In the case of WNK2 protein (260 kDa), SHR brains exhibited only a moderate upregulation of WNK2 (2–3 fold) in the IL cortex in response to ischemic stroke (Figure 3(c)). No such change was detected in

ischemic WKY brains. Last, higher basal expression of WNK3 protein (168 kDa) was detected in the membrane fractions of SHR brains than of WKY brains under both control and ischemic conditions. Interestingly, ischemic stroke did not further elevate WNK3 levels in the membrane fraction, but instead selectively reduced expression of WNK3 in the cytosol fraction of SHR brains (Figure 3(d)). Taken together, these data demonstrate differential regulation of WNK kinase proteins in WKY and SHR brains in response to ischemic stroke.

Ischemic stroke triggers inhibition of SPAK/OSR1 phosphorylation and degradation of SPAK in SHR brains

We hypothesized that up-regulation of WNK1, WNK2, and WNK4 may stimulate the intermediate kinases SPAK/OSR1.[2,3] High basal levels of pSPAK protein (~68 kDa) were detected in WKY and SHR brains. Ischemic stroke did not significantly alter pSPAK protein expression in WKY brains; however, we unexpectedly observed decreased levels of pSPAK protein (~68 kDa) in both membrane and cytosol fractions of SHR brains (Figure 4(a) and (b)). tSPAK/tOSR1 (68 kDa, 55 kDa) were also drastically reduced in the cytosol fraction of IL cortex of SHR. This decrease was accompanied by emergence in both membrane and cytosol fractions of IL cortex from SHR brains of a novel 45 kDa polypeptide, not recognized by specific antibodies to OSR1 (Supplemental Figure 2) or to pSPAK (pSer383) (Figure 4(a) and (b)). Therefore, the 45 kDa band may be a truncated form of SPAK protein. On the other hand, pOSR1 levels in IL cortices of ischemic SHR and WKY brains did not change. Therefore, increased NKCC1 phosphorylation in ischemic SHR brains may be independent of SPAK/OSR1 complex activation.

The ischemia-induced expression of the lower molecular weight 45 kDa SPAK-immunoreactive polypeptide in ischemic SHR brain prompted us to assess the expression of a SPAK-specific protease, DNPEP (aspartyl aminopeptidase).[27] DNPEP levels in ischemic SHR brains were 1.9 ± 0.2 fold higher in the membrane fraction and 2.1 ± 0.4 fold higher in the cytosol fraction than those of ischemic WKY brain (Figure 4(c)). These results strongly suggest that the 45 kDa SPAK-immunoreactive band of ischemic SHR brain is a proteolytic product of SPAK produced by DNPEP protease activation.

We also examined expression of KLHL2/3 and CUL3 proteins, critical components of the canonical E3 ubiquitin ligase complex, which regulates WNK abundance (Supplemental Figure 3(A) and (B)).[28,29] KLHL2/3 was down-regulated in the membrane fraction of ischemic SHR brains, but increased in the cytosol fraction (Supplemental Figure 3(A)). These results indicate that ischemic stroke-induced net increases of WNK proteins are unlikely due to compromised function of CUL3 and KLHL2/3 proteins. However, changes of other CUL and KLHL proteins in ischemic brains remain to be determined.

Ischemic stroke-mediated up-regulation of scaffolding protein Cab39 in SHR brains

The lack of SPAK/OSR1 activation in ischemic SHR brains led us to hypothesize that stimulation of the WNK-NKCC1 complex may involve intermediaries that do not require SPAK or OSR1. One candidate is the scaffolding protein Cab39 (or MO25 for mouse protein 25), which facilitates SPAK/OSR1-independent phosphorylation of NKCC1.[30] Figure 5(a) shows that trends of Cab39 (~39 kDa) levels were higher (although not statistically significant) in both CL and IL membrane fractions from SHR than from WKY brains. Ischemic stroke further increased Cab39 protein levels in the cytosol fraction of SHR brains (2.7 ± 0.5 fold) at 6-h post-tMCAO, in contrast to a moderate Cab39 increase (1.5 ± 0.3 fold) in the cytosol fraction of ischemic WKY brains. By 24-h post-tMCAO, SHR brains showed significantly increased Cab39 protein expression in the membrane fraction (1.8 ± 0.2 fold) and sustained elevation in the cytosolic fractions (2.0 ± 0.4 fold). We speculate that the apparent lack of Cab39 protein upregulation at 6-h post-ischemia as detected by immunoblotting of membrane fractions reflects a regional increase of limited magnitude. This speculation is supported by immunofluorescence staining (Figure 5(b)). The low basal level of Cab39 protein expression observed in the non-ischemic SHR cortex contrasted with clearly elevated Cab39 in neuronal processes of SHR cortical tissues 6-h post-ischemia. Increased Cab39 expression in neuronal processes co-localized with neuronal dendrite marker protein MAP-2 in SHR brains (Supplemental Figure 4). These results suggest that upregulation of Cab39 expression may facilitate WNK-NKCC1 interactions. To test this possibility, we conducted a co-immunoprecipitation study. As shown in Figure 5(c), WNK1, WNK4, and Cab39 proteins were co-immunoprecipitated along with NKCC1 protein using the anti-tNKCC1 T4 monoclonal antibody. WNK1, WNK4, and Cab39 proteins were specifically enriched in tNKCC1 immunoprecipitations from ischemic SHR brains. Taken together, these findings suggest that WNK1/4, Cab39, and NKCC1 protein contribute to a multiprotein complex.

Human subacute MCA stroke brain autopsy exhibited elevated NKCC1 protein expression

To test the possible role of NKCC1 in human ischemic stroke, we examined expression of tNKCC1 protein in

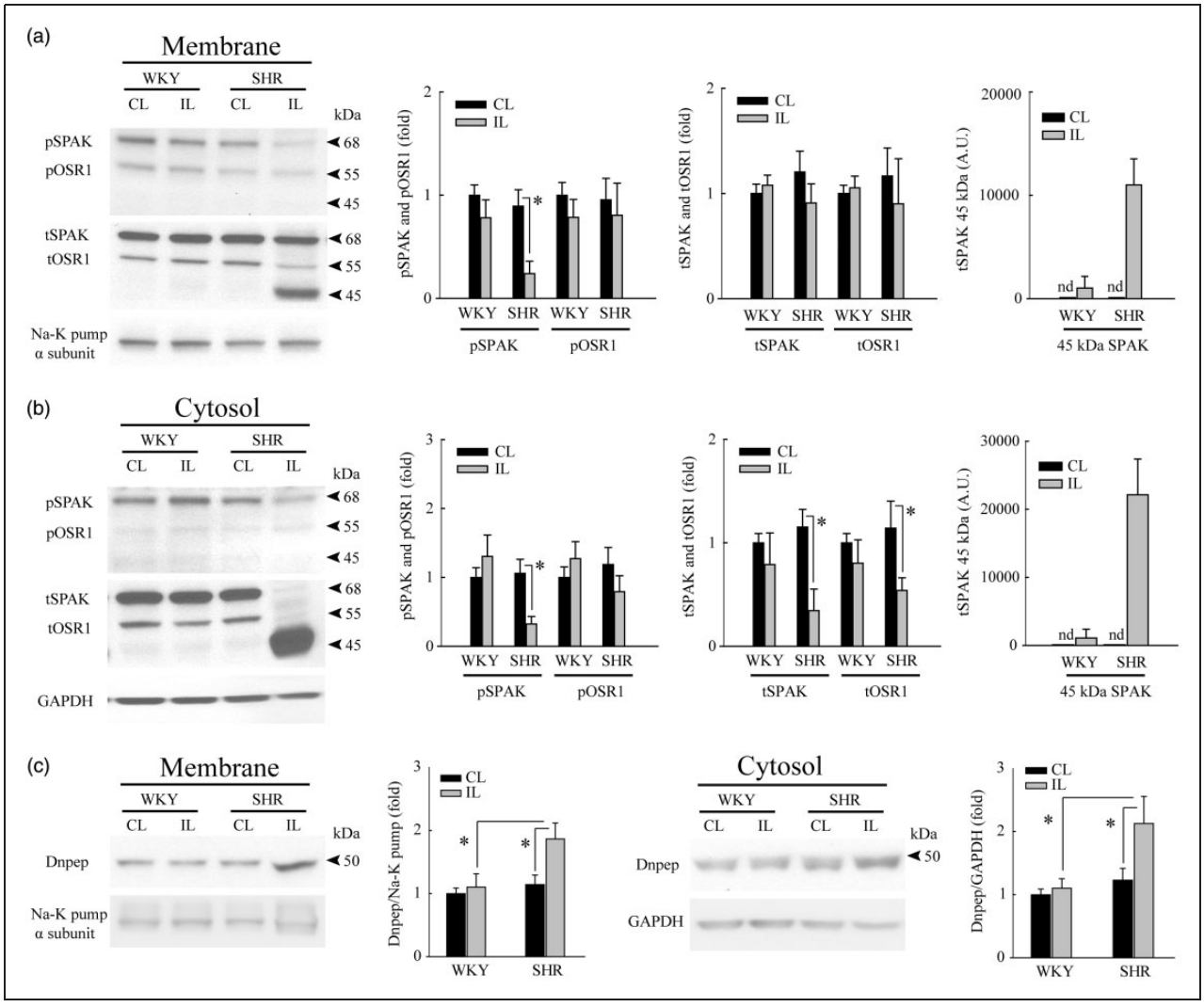

Figure 4. Ischemic stroke selectively triggers inhibition of SPAK/OSR1 phosphorylation and degradation of SPAK in SHR brains. (a) Plasma membrane fraction of the IL cerebral cortex of SHR brains exhibits selective reduction of pSPAK (68 kDa) expression at 6-h reperfusion post-tMCAO. A protein band of ~45 kDa was detected in the membrane fraction of SHR brains by anti-tSPAK antibody but not by the anti-pSPAK/pOSR1 antibody (arrowhead). The band was interpreted as a putative, proteolytic cleavage product of pSPAK. Data are mean ± SD, n = 4, $^*p < 0.05$. (b) Cytosol fractions from IL cerebral cortex of SHR brains show reduced expression of pSPAK, tSPAK, tOSR1, and increased accumulation of the putative cleaved SPAK band (~45 kDa) undetected by the anti-pSPAK/pOSR1 antibody (arrowhead). Data are mean ± SD, n = 4, $^*p < 0.05$. (c) Upregulation of Dnpep protease (aspartyl aminopeptidase, ~50 kDa) was detected only in IL cortices of SHR but not in WKY rats at 6-h reperfusion post-tMCAO. Cytosol and crude plasma membrane protein fractions were prepared from the contralateral (CL) and ipsilateral (IL) cortices of WKY and SHR. Data are mean ± SD, n = 4, $^*p < 0.05$.

brain autopsy specimens from three subacute MCA ischemic stroke subjects (Supplemental table I). Non-lesion and peri-lesion penumbra regions were identified with H & E staining (Figure 6(a), upper panel). In the non-lesion region, neurons exhibited abundant MAP-2 expression, in contrast to the low intensity of tNKCC1 immunoreactivity (arrow). The latter represents non-specific signals because a similar low immunoreactive background of tNKCC1 staining was detected in the brain sections of $NKCC1^{-/-}$ global knockout mice (data not shown). However, in the peri-lesion penumbra region, many neurons with larger soma showed significantly increased expression of tNKCC1 protein (arrowhead, Figure 6(a), middle panel) as compared with the non-lesion regions (Figure 6(b)). Most tNKCC1-positive cells exhibited neuron-like morphology, similar to that of MAP-2-positive cells. Increased expression of pNKCC1 protein was also detected in the peri-lesion penumbra region (Figure 6(a), the bottom panel). However, positive immunoreactive signal of pNKCC1 was detected in three out of six subjects. Taken together, these data demonstrate

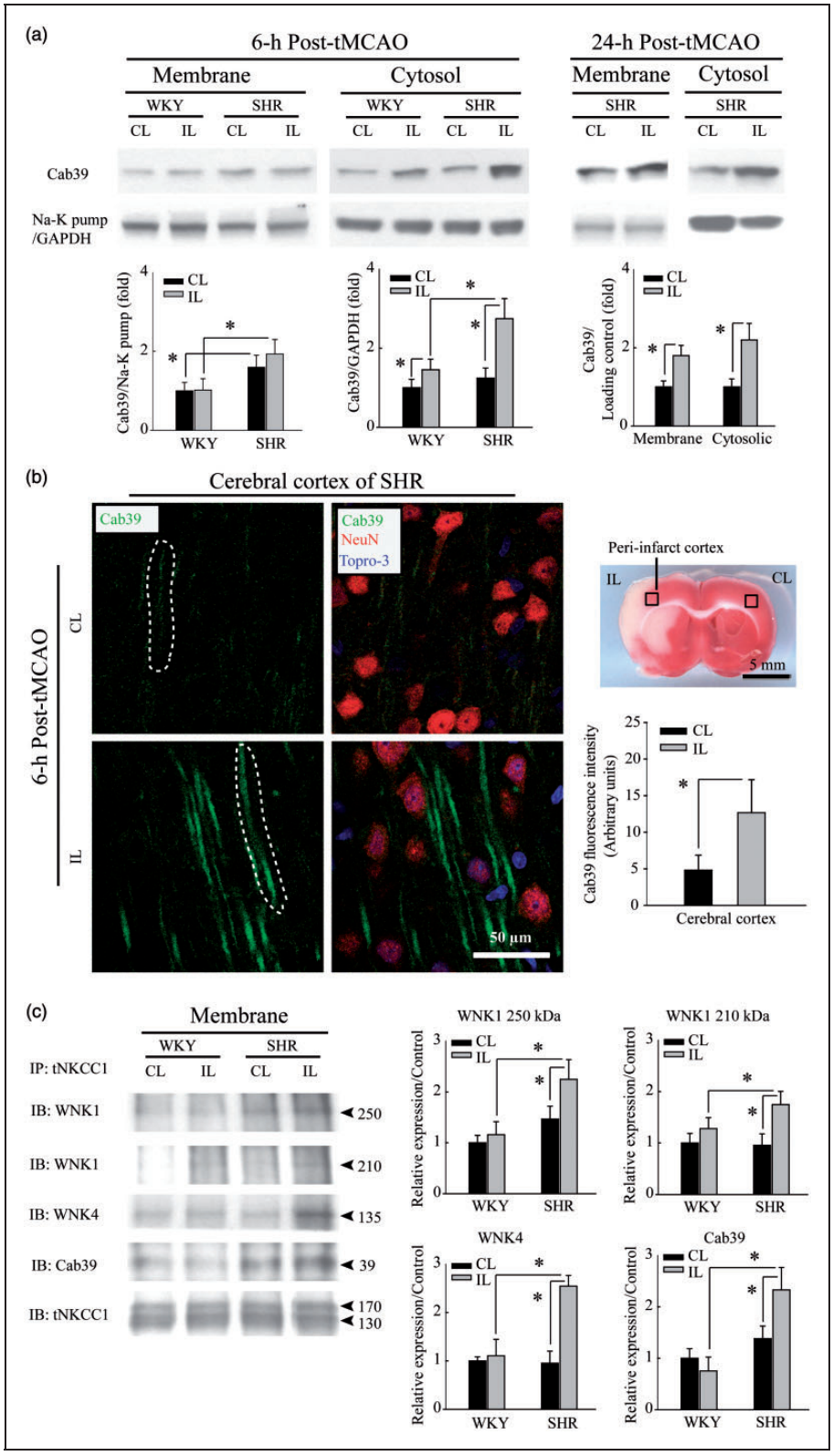

Figure 5. Ischemic stroke selectively triggers upregulation of Cab39 protein expression in SHR brains. (a) SHR brain membrane fractions exhibit greater Cab39 protein abundance than do WKY brain membrane fractions. Ischemic stroke increases Cab39 levels in SHR cytosol fractions to a greater degree than in WKY brain cytosol fractions at 6-h reperfusion post-tMCAO. At 24-h reperfusion post-tMCAO, Cab39 expression was further increased in both membrane and cytosolic fractions in SHR brain. Data are mean ± SD, n = 4, $^*p < 0.05$. (b) Immunofluorescence analysis showing Cab39 upregulation in peri-infarct cortex of SHR brain at 6-h post-tMCAO. Data are mean ± SD, n = 3, $^*p < 0.05$. (c) Increased levels of WNK1, WNK4, and Cab39 proteins were detected in anti-tNKCC1 immunoprecipitates from ischemic IL cerebral cortices of SHR at 6-h reperfusion post-tMCAO compared with CL cortex of WKY. Data are mean ± SD, n = 4, $^*p < 0.05$.

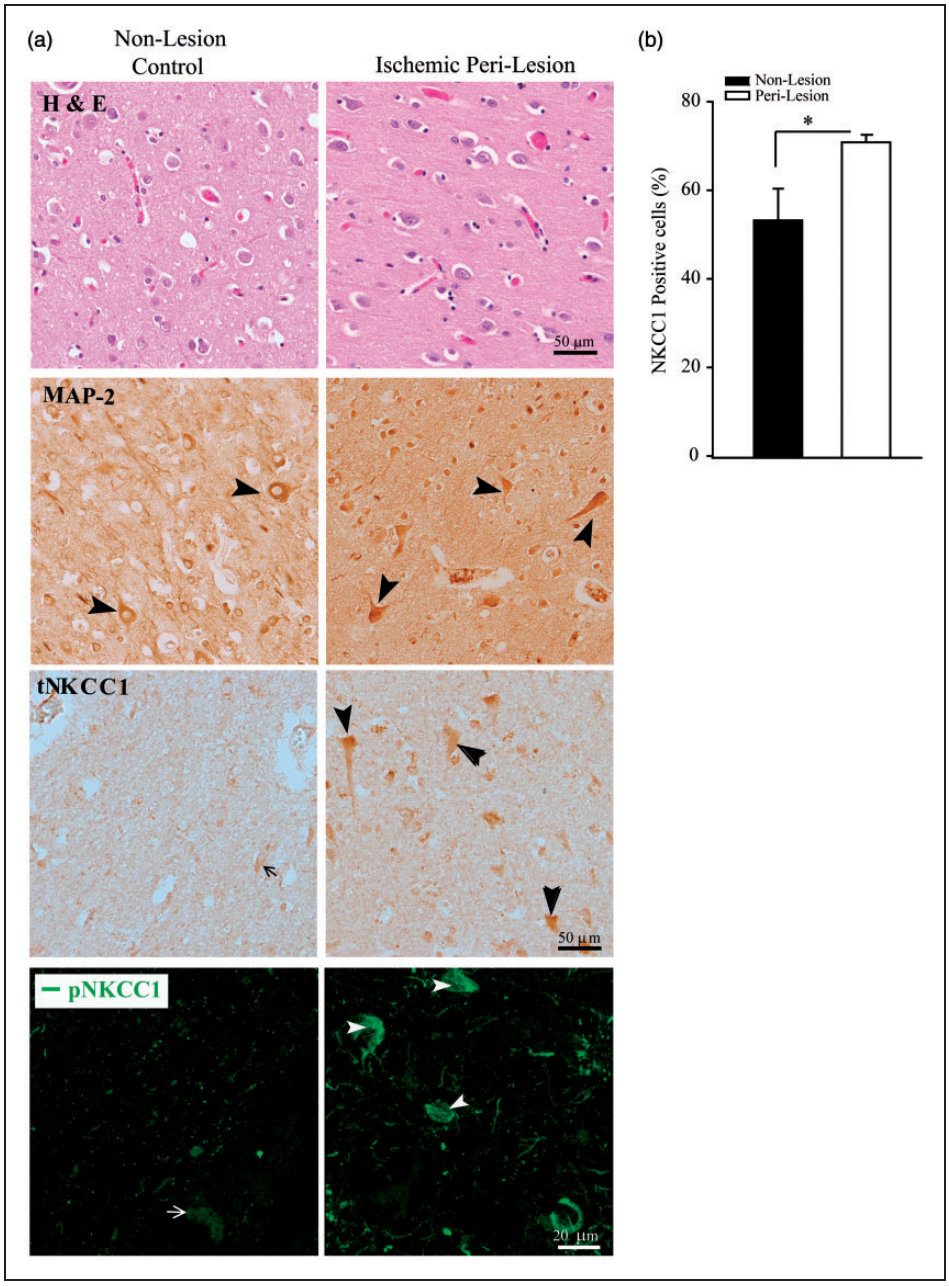

Figure 6. Elevated tNKCC1 and pNKCC1 protein expression in subacute human middle cerebral artery ischemic stroke brain autopsy. (a) Non-lesion and peri-lesion regions in human ischemic stroke brain autopsy samples were identified with H & E staining. tNKCC1 expression in neuronal cells was shown in representative immunohistochemical images. Arrow: low NKCC1 expression. Arrowhead: high NKCC1 expression. Scale bar: 50 μm. Representative immunofluorescence images of pNKCC1 expression in brain cells (bottom panel). Arrow: low pNKCC1 expression. Arrowhead: high pNKCC1 expression. Scale bar: 20 μm. (b) Percentage of tNKCC1-positive cells among total To-pro3-positive cells. Numerical data are mean ± SD, n = 3 (one male, two female), $*p < 0.05$.

that human ischemic stroke upregulated tNKCC1 and pNKCC1 protein expression in neurons in the ischemic penumbra, possibly contributing to ischemic neuronal damage. The data suggest that targeting the WNK-NKCC1 signaling pathway may help rescue at risk peri-infarct neurons and so improve therapy of ischemic stroke.

Discussion

Elevated NKCC1 protein expression and activation in SHR brains

SHR exhibit developmental upregulation of NKCC1 protein and mRNA between 10 and 18 weeks of age[12]

and excessive NKCC1 activity underlies in large part the diminished synaptic inhibition and elevated sympathetic vasomotor tone contributing to the hypertension of SHR.[13] In agreement with these reports, we detected elevated basal expression of tNKCC1 and pNKCC1 protein in cerebral cortex of SHR brains, as compared with normotensive WKY brains. We found that ischemic stroke triggered a significant upregulation of pNKCC1 in the membrane fraction of ischemic SHR brains but not in WKY brains at 6-h post-ischemia. This suggests NKCC1 phosphoactivation is differentially regulated in SHR and WKY in response to ischemic stroke. This finding led us to investigate changes in the WNK-SPAK/OSR1 signaling pathway, a key regulator of NKCC1 activity, in SHR and WKY rats.

Normotensive WKY rats were less sensitive to ischemic stroke and exhibited a smaller infarct size (<20% hemispheric volume) after 24–48 h following permanent MCA occlusion.[14,15] In our study, similar basal levels of pNKCC1 protein were detected in WKY and SHR brains. No ischemia-induced elevation of pNKCC1 was detected in ischemic WKY brains at 6-h post-ischemia. However, BMT treatment effectively decreased brain swelling at 24-h post-ischemia. We speculate that the onset of ischemic stroke-mediated stimulation of pNKCC1 in WKY rats is slower than in SHRs, explaining the lack of elevation in pNKCC1 expression in WKY rats at 6-h post-stroke. pNKCC1 levels at 6-h post-ischemia also failed to increase in normotensive WNK3 wild-type (WNK3 WT) mice, but increased significantly between 6- and 24-h post-ischemia.[20] Therefore, the moderate protective effects of BMT at 24-h post-ischemia in WKY rats may result from blocking stimulation of NKCC1 during the 24-h post-tMCAO period. This view is further supported by previous reports of BMT-mediated protection of ischemic brain injury in normotensive Sprague Dawley rats and C57BL/6 mice.[20,31] Alternatively, regulatory mechanisms other than protein phosphorylation are involved in regulation of NKCC1 activity. N-linked glycosylation of NKCC1 represents another important post-translational mechanism that governs the functional expression of NKCC1 by affecting its intrinsic activity and trafficking into the plasma membrane.[13] The possible differences in changes of N-linked glycosylation of NKCC1 in WKY and SHR brains after ischemic stroke remain to be examined in the future studies.

Increased Wnk1, Wnk2, and Wnk4 are likely responsible for NKCC1 activation in ischemic SHR brains

WNK kinases and SPAK/OSR1 regulate the phosphorylation state of ion transporter proteins and play important roles in renal salt handling and in the pathogenesis of hypertension,[2,4] the most important modifiable risk factor for stroke.[32] However, despite the importance of WNK-SPAK/OSR1 signaling in hypertension, our understanding of the function and regulation of these kinases in the ischemic brain injury remains limited. Here, we found that upregulation of WNK protein expression in the IL cortex of ischemic SHR brains (in the order WNK1 > WNK4 > WNK2) was associated with increased NKCC1 phosphorylation (Figure 7). The co-immunoprecipitation of WNK1 and WNK4 with NKCC1 suggests that WNK kinases might directly phosphorylate NKCC1 in ischemic SHR brains.

We recently reported that WNK3, an isoform highly expressed in the normotensive mouse and rat brains,[33] acts as a dominant upstream kinase of SPAK/OSR1-NKCC1 phospho-activation in ischemic stroke.[20] Knockout of *WNK3* in normotensive mice abolished phosphorylation of both SPAK/OSR1 and NKCC1 in ischemic brains and reduced infarction and cerebral edema.[20] However, the ischemic stroke-associated WNK3 protein expression in SHR brains was not as pronounced as that of WNK1 and WNK4 in the membrane fraction, and was decreased ~50% in the cytosol fraction. These findings suggest that the four mammalian WNK kinase orthologs are differentially regulated in hypertensive and normotensive brains in response to ischemic stroke. Hypertension-dependent transcriptional or post-translational changes in the expression of specific WNK kinases might account for the observed differences between the two models, a hypothesis that warrants further testing in future studies.

Supplemental Figure 5 indicates that NKCC1 activation is not up-stream of the WNK-Cab39 function. The figure shows that cerebral ischemia in SHR triggered irreversible changes of WNK1, WNK4, and Cab39 protein that were insensitive to NKCC1 inhibition by BMT.

We detected a large increase of a 210 kDa WNK1 protein in the cytosolic fraction of ischemic cortex of SHR, but pNKCC1 in the respective cytosolic fraction did not change. This implies that the 210 kDa WNK1 variant is either inactive or NKCC1 may need to be at the plasma membrane to undergo phosphorylation.[34] As shown in Supplemental Figure 1, 24-h post-ischemic brain tissue of SHRs expressed both 250 kDa and 210 kDa bands of WNK1, the latter more prominent in the cytosolic fraction. Kidney tissues, in contrast, displayed a different complement of WNK1 proteins, suggesting that the WNK1 expression pattern in brain was tissue-specific and a consequence of cerebral ischemia. Since downstream SPAK/OSR1 phosphorylation was decreased in the subcellular fraction where the 210 kDa brain WNK1 protein was expressed, we speculate that this shorter WNK1 species may be itself

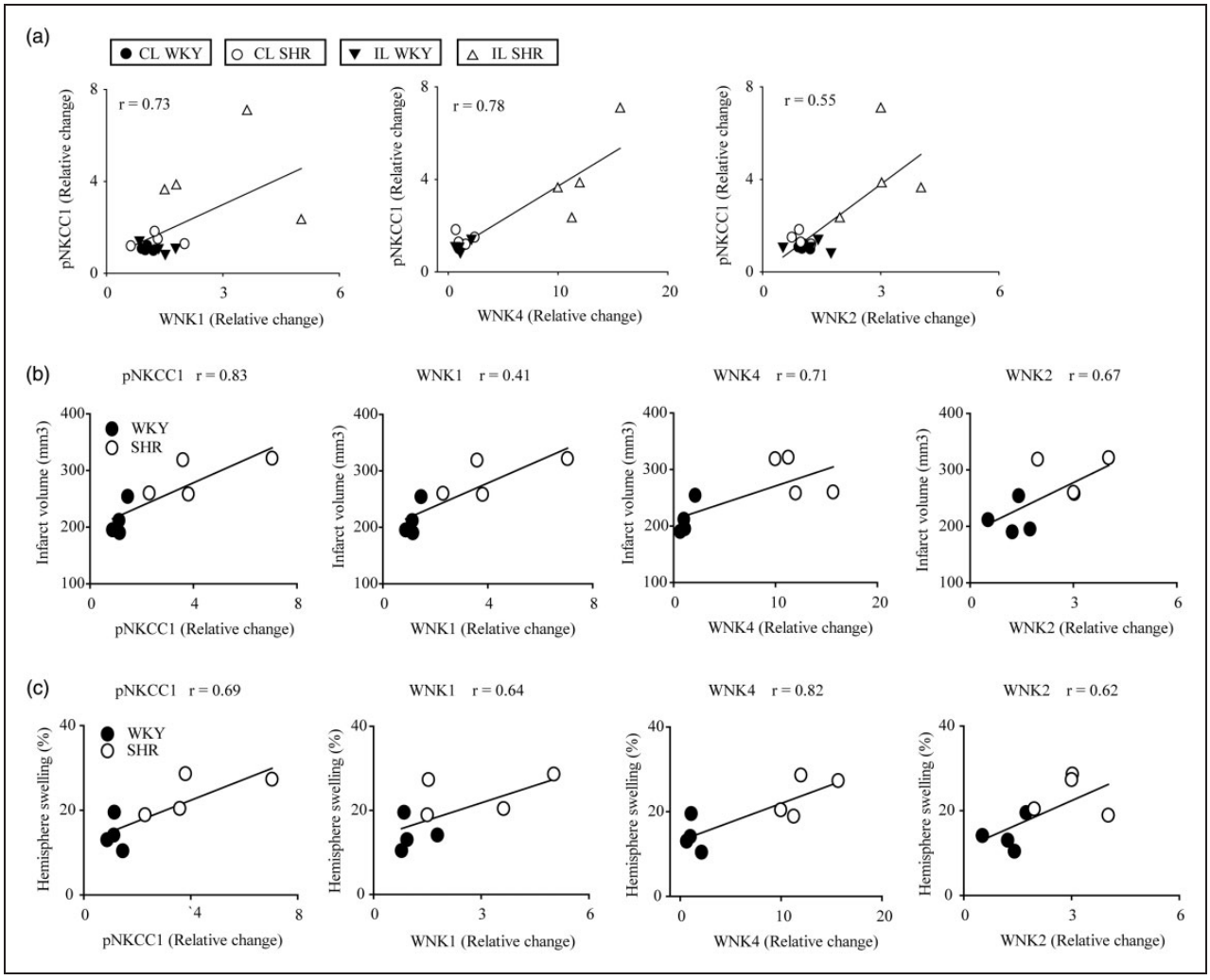

Figure 7. Correlation of expression and activation of NKCC1/WNK pathway signaling proteins, infarct volume and hemisphere swelling in ischemic SHR and WKY brains. (a) Correlation between phosphorylation of NKCC1 and expression of WNK kinases at 6 h after MCAO in WKY and SHRs ($p < 0.05$, Pearson's correlation). (b) Correlation between phosphorylation of NKCC1 and expression of WNK kinases versus infarct volume ($p < 0.05$, Pearson's correlation). (c) Correlation between NKCC1 phosphorylation, and expression of WNK1/2/4 kinases versus hemispheric swelling ($p < 0.05$, Pearson correlation). These correlation analyses used the same cohort of data from Figures 1–3.

inactive, perhaps functioning in brain as kinase-inactive kidney-specific WNK1 isoforms in kidney.[35]

Scaffolding protein Cab39, but not SPAK/OSR1, may serve as a link between WNK-NKCC1 interactions

Phosphorylation of SPAK (68 kDa) was decreased but phosphorylation of OSR1 (55 kDa) was unchanged in ischemic SHR brains. Moreover, expression of a truncated 45 kDa SPAK-immunoreactive polypeptide emerged only in the ischemic SHR brains. Since the anti-tSPAK antibody[36] is directed against C-terminal SPAK amino acid residues 362–374, the truncated form of SPAK is likely missing an N-terminal fragment. The antibody to pSPAK directed at pS383 of SPAK[9] revealed no increase in phosphorylation of the 45 kDa SPAK band. The lack of S383 phosphorylation in the 45 kDa truncated SPAK may indicate that it is a poor substrate for WNK-mediated phosphorylation. This speculation warrants further investigation.

The concurrent increase in Dnpep expression and the 45 kDa truncated SPAK in ischemic SHR brains suggests that this truncated form of SPAK might be a previously recognized cleavage product of Dnpep.[27] Previous studies indicate that truncated SPAK isoforms can exert inhibitory effects on the kinase activity of full length SPAK and OSR1.[37,38] We propose that Dnpep-mediated proteolytic processing of SPAK into its inhibitory form might dampen stimulatory effects of WNK1, WNK2, or WNK4 on NKCC1 activity

following ischemic stroke. However, we cannot rule out a possibility that the 45 kDa protein may represent a short form of SPAK as previously identified in kidney, such as SPAK2, which is inactive and generated from an alternative translation start in the full-length transcript.[37–39] Interestingly, in contrast to a dramatic decrease of tSPAK in the IL cytosolic fraction of SHR cortex, tSPAK in the membrane fraction remains largely unchanged. This suggests that membrane-bound tSPAK may be protected from proteolytic cleavage by DNPEP, a predominantly cytosolic protease/peptidase.[40] Moreover, though tOSR1 abundance was decreased in cytosolic fractions of IL cortex of SHR brains, the 45 kDa band is not an OSR1-cleavage product, as it was not recognized by an OSR1 specific antibody. This suggests that changes in OSR1 protein abundance in the cytosolic fraction of SHR ischemic cortex may reflect transcriptional downregulation of OSR1 upon ischemic stroke.

Taken together, our data suggest that SHR brains may utilize a SPAK/OSR1-independent pathway in regulating the WNK-NKCC1 signaling complex, as summarized in the schematic of Supplemental Figure 6. WNK kinases can activate NKCC1 through phosphorylation via a SPAK/OSR1-independent pathway.[30] The scaffolding protein Cab39 was recently demonstrated to enhance WNK4-mediated phosphorylation of NCC and NKCC1 overexpressed in *Xenopus laevis* oocytes.[30] The mechanism requires the PASK/FRAY homology 2(PF2) domain of WNK4, which is homologous to a domain in SPAK and OSR1 that binds RFX[V/I] motifs present in Na-coupled cation chloride cotransporters.[30,41] Thus, the PF2 domain facilitates anchoring of WNK4 to an N-terminal RFX[V/I] motif in NKCC1. This interaction is likely quiescent, but in the presence of the Ste20 kinase activator Cab39, WNK4 can stimulate NKCC1 phosphoactivation via a kinase-dependent mechanism.[30] In the current study, increased Cab39 protein expression in ischemic SHR brains was co-localized with NKCC1, WNK1, and WNK4 in the same co-immunoprecipitation complex. These findings further suggest that elevated expression of Cab39 may serve as an intermediate link to facilitate WNK-NKCC1 signaling in ischemic SHR brains (Supplemental Figure 6). Cab39 activation depends on elevation of intracellular Ca^{2+}.[42] Excessive Ca^{2+} influx and/or Ca^{2+} release from intracellular Ca^{2+} stores are the hallmark of ischemic brain damage.[43,44] In fact, increased intracellular Ca^{2+} secondary to activation of ionotropic or metabotropic glutamate receptors stimulated NKCC1 activity in nasal epithelia[45] and neurons.[46,47] Moreover, striatal levels of phospho-GluR1 and of calcium/calmodulin kinase-IIα were higher in SHR than in WKY rats.[48] Therefore, excessive Ca^{2+} signaling in ischemic SHR brains may favor activation of the WNK-Cab39-NKCC1 complex.

It is likely that WNK-Cab39 signaling could also affect other cation chloride cotransporters. K^+-Cl^- cotransporter 2 (KCC2) and KCC3 are expressed in the brain and are required along with NKCC1 for physiological regulation of cell volume and ionic homeostasis in the central nervous system.[49] Therefore, the roles of WNK-Cab39-KCC signaling in the context of ischemic stroke merit further study.

Pharmacological inhibition of NKCC1 reduces infarction severity and improves the sensorimotor deficits in SHR after ischemic stroke

Multiple in vivo studies have shown that NKCC1 plays a role in ischemic and hypoxic cell damage, and that its inhibition is neuroprotective.[16,17,31] We detected a positive correlation between elevated NKCC1 protein expression, NKCC1 phosphorylation, ischemic infarct size, and the magnitude of brain swelling in WKY and SHR brains (Figure 7, Pearson's correlation, $p < 0.05$). Interestingly, we found that the neuroprotective effect of NKCC1 inhibition by BMT was greater in SHR brains than in normotensive WKY brains. Daily BMT administration begun 3-h post-MCAO nearly abolished the incremental severity of ischemia-induced infarction and the increased brain swelling observed in SHR. We previously showed that BMT administration either prior to ischemia induction or during ischemia significantly reduced brain edema and infarction in SHR.[16,22] The current study shows that post-ischemic administration of BMT is equally effective in reducing ischemic damage and neurological deficits in SHR. This finding further supports targeting of WNK-NKCC1 signaling pathways as novel strategies for ischemic stroke therapy.

To date, there are no specific WNK inhibitors available to block WNK kinase activity or interrupt the WNK-Cab39-NKCC1 cascade. Therefore, development of a blood–brain barrier-permeable WNK kinase inhibitor will be beneficial for the development of anti-hypertension and anti-ischemic stroke therapies.

Conclusions

In summary, we report here that unilateral ischemic stroke triggers more robust stimulation of the WNK-NKCC1 signaling pathway in SHRs than in normotensive WKY rats. Ischemic SHR brains show concurrent upregulation of WNK1, WNK4, and WNK2 protein expression and elevated NKCC1 phosphorylation, associated with increased infarction size and increased ipsilateral hemispheric swelling in ischemic SHR brains. The conventional SPAK/OSR1 effector kinases were

not stimulated in ischemic SHR brains. In contrast, the kinase scaffolding protein Cab39 was upregulated and associated with NKCC1 and WNK1/WNK4 proteins in the same immunoprecipitation complex. Moreover, inhibition of NKCC1 with BMT attenuated the increased severity of infarction and brain swelling in SHR and improved post-ischemic neurological function. These findings suggest that increased WNK-Cab39-NKCC1 signaling is involved in the pathogenesis of ischemic brain damage in SHR. Our study reveals a pathophysiological role for non-canonical WNK-Cab39 signaling in a disease model of ischemic stroke, and identifies the WNK-Cab39-NKCC1 signaling complex as a novel target for treatment of hypertension and ischemic stroke.

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Declaration of conflicting interests

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Authors' contributions

MIHB and HY performed surgery procedures, TTC staining, and data analysis. MIHB, SS, and HY performed behavioral tests and data analysis. MIHB and SS conducted and quantified immunoblotting and immunoprecipitation studies. MIHB, SS, GB, and JK performed immunohistology and immunofluorescent staining studies. MIHB, KTK, SSY, SHL, SLA, ARS, and DS designed the experiments. MIHB, JK, KTK, SLA, ARS, and DS completed the manuscript writing.

Supplementary material

Supplementary material for this paper can be found at http://jcb.sagepub.com/content/by/supplemental-data

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