NF-κB Signaling-Mediated Activation of WNK-SPAK-NKCC1 Cascade in Worsened Stroke Outcomes of Ang II-hypertensive mice

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ABSTRACT

BACKGROUND AND PURPOSE: Worsened stroke outcomes with hypertension (HTN) comorbidity are insensitive to blood pressure (BP) lowering therapies. In an experimental stroke model with comorbid HTN, we investigated causal roles of angiotensin II (Ang II)-mediated stimulation of the brain WNK-SPAK-NKCC1 complex in worsened outcomes.

METHODS: Saline or Ang II-infused C57BL/6J male mice underwent stroke induced by permanent occlusion of the distal branches of the middle cerebral artery (pdMCAO). Mice were randomly assigned to receive either vehicle DMSO/PBS (2 ml/kg body weight/day, i.p.), a novel SPAK inhibitor ZT-1a (5 mg/kg/day, i.p.) or a NF-κB inhibitor TAT-NBD (20 mg/kg/day, i.p.). Activation of brain NF-κB and WNK-SPAK-NKCC1 cascade as well as ischemic stroke outcomes were examined.

RESULTS: Stroke triggered a 2- to 5-fold increase of WNK (isoforms 1, 2, 4), SPAK/OSR1, and NKCC1 protein in the Ang II-infused hypertensive mouse brains at 24 hours after stroke, which was associated with increased nuclear translocation of phospho-NF-κB protein in the cortical neurons (a Pearson's correlation r of 0.77, $p < 0.005$). The upregulation of WNK-SPAK-NKCC1 cascade proteins resulted from increased NF-κB recruitment on *Wnk1, Wnk2, Wnk4*, *Spak* and *Nkcc1* gene promoters and was attenuated by NF-κB inhibitor TAT-NBD. Post-stroke administration of SPAK inhibitor ZT-1a significantly reduced WNK-SPAK-NKCC1 complex activation, brain lesion size, and neurological function deficits in the Ang II hypertensive mice without affecting BP and cerebral blood flow.

CONCLUSIONS: The Ang II-induced stimulation of NF-κB transcriptional activity upregulates brain WNK-SPAK-NKCC1 cascade and contributes to worsened ischemic stroke outcomes, illustrating the brain WNK-SPAK-NKCC1 complex as a therapeutic target for stroke with comorbid HTN.

Key Words: angiotensin II, ischemic stroke, NF-κB, NKCC1, SPAK, WNK, ZT-1a

Nonstandard Abbreviations and Acronyms:

Ang II: angiotensin II
AT1R: ang II type 1 receptors
CL: contralateral
HTN: hypertension
IKK: activated IκB kinase
IL: ischemic ipsilateral
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NKCC1: Na-K-Cl cotransporter
OSR1: oxidative stress-responsive kinase 1
pdMCAO: permanent occlusion of the distal branches of the middle cerebral artery
rCBF: regional cerebral blood flow
SPAK: STE20/SPS1-related proline/alanine-rich kinase
Veh: vehicle
WNK: with no lysine (K) kinases

INTRODUCTION

Hypertension (HTN) is the number one risk factor for stroke[1], with 70% of stroke patients having HTN.[2] Stroke patients with comorbid HTN have worsened outcome, including increased risk of mortality, dependency, and other clinical deteriorations.[3] Ang II, the central product of the renin-angiotensin system, plays a role in developing HTN[4] primarily through Ang II type 1 receptors (AT1R) expressed in the kidney, vessels, heart, and brain.[5,6] In the central nervous system (CNS), Ang II increases reactive oxygen species (ROS) and inflammation in the subfornical organ (SFO) and hypothalamic paraventricular nucleus (PVN), causing increased sympathetic nervous system activity and neurogenic HTN.[7,8] A large body of preclinical studies showed that brain AT1R plays an important role in pathogenesis of ischemic stroke[6,9] and pharmacological blockade of AT1R after ischemic stroke provides neuroprotection in normotensive mice, rats or spontaneously hypertensive rats.[9,10] However, treating acute ischemic stroke patients with AT1R blockers has failed to generate positive outcomes.[11,12] The Scandinavian Candesartan Acute Stroke Trial (SCAST), a large phase III clinical trial showed that early blood pressure (BP) lowering using AT1R blocker candesartan within 30 h post ischemic stroke was associated with worse neurological outcome (assessed by modified Rankin Scale score at 6 months), possibly due to reduction of cerebral perfusion.[12,13] These findings suggest that new strategies are needed to reduce acute ischemic stroke brain damage with comorbid HTN, such as selectively attenuating the Ang II-mediated detrimental effects in ischemic brains without acutely lowering BP or compromising cerebral perfusion.

Evolutionary conserved WNK [with no lysine (K)] kinases and the downstream SPAK (the STE20/SPS1-related proline/alanine-rich kinase)/OSR1 (oxidative stress-responsive kinase 1) kinases regulate activities of multiple ion transporters and channels via protein phosphorylation

(Moriguchi et al 2005 J Biol Chem; Richardson et al 2008 J Cell Sci). One of the major targets of WNK and SPAK/OSR1 is Na^+-K^+-$2Cl^-$ cotransporter isoform 1 (NKCC1) (Dowd et al 203 JBC). The WNK/SPAK-regulated, NKCC1-mediated ion transport has been implicated in the pathogenesis of multiple brain pathologies associated with impaired brain ion and water homeostasis (Huang et al 2019 Aging and Dis). We reported that increased phosphorylation of the catalytic T-loop of SPAK/OSR1 and of NKCC1 at Thr^{203}/Thr^{207}/Thr^{212} were detected in neurons and oligodendrocytes of mouse stroke brain or in rat choroid plexus epithelium in experimental hydrocephalus model (Begum et al 2015 Stroke; Karimy et al 2017 Nat Med). Therefore, the WNK/SPAK-NKCC1 cascade is involved in multiple neurological disorders.

The WNK/SPAK cascade also plays important roles in renal salt handling, maintenance of arterial tone and HTN develoment.[14,15] Ang II infusion increases BP in C57BL/6 male mice via renal salt reabsorption through increased WNK/SPAK-dependent stimulatory phosphorylation of renal Na-Cl cotransporter (NCC at $pThr^{55}$) and vessel contraction via activation of aortic NKCC1 (at $pThr^{212}$).[16] We recently reported that Ang II-mediated hypertensive mice exhibited more severe ischemic brain damage and neurological deficits.[17] However, whether Ang II-associated worsened ischemic brain damage involves in pathological stimulation of brain WNK-SPAK/OSR1-NKCC1 cascade remains unknown. In exploring pharmacological approaches to block brain WNK/SPAK-NKCC1 cascade, to date, there are no specific WNK inhibitors available, and NKCC1 inhibitors (such as bumetanide) have limitations due to its poor BBB penetration and/or off-target effects (Romermann et al. 2017 Neuropharmacol; Lemonnier et al 2017 Transl Psychiatry 7). We recently reported that a newly developed non-ATP competitive, specific inhibitor of SPAK kinase ZT-1a crossed the BBB and significantly reduced infarction and cerebral edema, and improved neurological function recovery in normotensive C57BL/6J mice in the transient large-vessel

ischemic stroke model (Zhang et al. 2020). In this study, we examined efficacy of ZT-1a in reducing the worsened stroke outcomes in the AngII-infused C57BL/6J mice. We report here that Ang II-induced stimulation of NF-κB transcriptional activity upregulates the brain WNK-SPAK-NKCC1 cascade, which is in part responsible for worsened brain damage and neurological deficits after ischemic stroke. Most importantly, post-stroke administration of SPAK inhibitor ZT-1a is neuroprotective without affecting either BP or regional cerebral blood flow (rCBF).

METHODS

This article adheres to the American Heart Association Journal implementation of the Transparency and Openness Promotion. The data that support the findings of this study are available within the article and its Data Supplement.

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. Eleven- to 16-week-old C57BL/6J mice (male and female, Jackson laboratories, Bar Harbor, ME) were used in the study.

Ang II-mediated HTN and blood pressure (BP) measurement

Hypertension triggered by Ang II infusion is a well-established rodent hypertension model (Cardinale et al. 2012). C57BL/6J mice received subcutaneous (s.c.) infusion of either saline or Ang II via osmotic minipumps (model 1002, Alzet) at a rate of 1000 ng/kg/min for 14 days. BP

was measured in awake mice by a tail-cuff method (Kent Scientific) as described previously.[17] See Data Supplement for details.

Focal cerebral ischemia with permanent middle cerebral artery occlusion

Saline-infused control and AngII-infused HTN mice subsequently underwent permanent occlusion of the distal branches of the middle cerebral artery (pdMCAO) as described previously.[17] Arterial blood gases (pCO2, tCO2), Na, K, Cl, glucose, HCO_3, pH at 24-h post-sham or pdMCAO were measured as described previously (Mehta et al 2021 Stroke) and shown in **Data Supplement.**

Drug treatment

One cohort of Ang II-mediated hypertensive mice were randomly assigned to receive either vehicle (Veh, 100% DMSO, 2 ml/kg body weight/day) or ZT-1a (5.0 mg/kg body weight/day), administered via intraperitoneal injection (i.p. **Figure 1A**) with an initial half dose at 3-h and the second half dose at 8-h post pdMCAO. In another cohort of Ang II-mediated hypertensive male mice, either sterile PBS (2 ml/kg body weight/day) or cell permeable NF-κB blocker TAT-NBD peptide (20 mg/kg body weight/day, Enzo Life Science) was administered (i.p.) with an initial half dose at 0-h and the second half dose at 3-h post pdMCAO to inhibit stroke-induced early activation of NF-kB as previously established.[18]

Cerebral blood flow measurement

Regional cerebral blood flow was measured using a two-dimensional laser speckle contrast analysis system (PeriCam PSI High Resolution with PIMSoft, Perimed) as described previously.[19] See Data Supplement for details.

Neurological Function Tests

Foot fault, cylinder test, and adhesive tape removal tests were used to assess pdMCAO-induced somatosensory and motor deficits in a blinded manner as described previously.[17] See Data Supplement for details.

Brain infarction volume and hemispheric swelling measurements

Cerebral infarction and hemisphere swelling were assessed at 24 hours reperfusion as described previously.[17] See Data Supplement for details.

Protein fraction preparation and immunoblotting

Membrane and cytosol proteins were prepared from brain homogenates as previously described.[19] Protein samples (40 µg) were boiled in sample buffer (Thermo Scientific, Rockford, IL, USA) for 5 minutes, resolved by 7.5 % sodium dodecyl sulfate polyacrylamide-gel electrophoresis and electrotransferred onto a polyvinylidene difluoride membrane as described before.[19] See Data Supplement for details.

Immunofluorescence staining

Immunofluorescent staining for NKCC1, pNF-kB, GFAP, Iba-1, MAP2 and NeuN were performed as described previously.[17] See Data Supplement for details.

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP assays were performed using a commercial kit (Invitrogen, Life technologies, Carlsbad, CA) according to the manufacturer's protocol and subsequent qPCR was performed as described previously.[20] See Data Supplement for details.

Flow Cytometry

Flow cytometry analyses of immune cells in the mouse spleen and brain tissues were performed as described previously (Song et al. 2018 Glia). See Data Supplement for details.

Statistical analysis

Animal subjects were randomly assigned into different studies and surgical procedures. All data analyses were performed by investigators blinded to experimental conditions. The number of animals studied was 80% powered to detect 25% changes with α (2-sided) = 0.05. A total of 130 male and 44 female mice were used in the study. All mice were included in the study except of 2 male mice which died from AngII-induced abdominal aortic aneurysm rupture prior to stroke experiments. Data were expressed as mean ± SEM. Statistical significance was determined by one-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. A probability value < 0.05 was considered statistically significant.

RESULTS

Ang II-induced hypertensive mice displayed heightened brain WNK-SPAK/OSR1-NKCC1 signaling activation after stroke

Figure 1A demonstrates that Ang II infusion for 14 days significantly elevated arterial BP in male, but not in female mice, consistent with published reports that estrogen hormone in female mice provides resistance to BP elevation.[21] Therefore, the rest of study was conducted in male mice unless indicated. Subjecting saline- and Ang II-infused mice to ischemic stroke led to activation of the WNK-SPAK-NKCC1 complex in the ischemic ipsilateral (IL) hemisphere of the

Ang II hypertensive mice at 24 h post-stroke, but not in the saline-infused normotensive mice, which is reflected with a 3.2 ± 0.5 fold increase in expression of pNKCC1 (pThr212) and 5.0 ± 0.6 fold in pSPAK/pOSR1 ($pSer^{383}$ SPAK, $pSer^{325}$ OSR1) ($p < 0.05$, **Figure 1B**). In addition, total protein amounts (tNKCC1, tSPAK/tOSR1, tWNK1, tWNK2, tWNK4, but not tWNK3) were also significantly upregulated in the Ang II hypertensive ischemic brains (**Figure 1B**). These findings show that the brain WNK-SPAK-NKCC1 signaling complex is robustly activated in the Ang II-mediated hypertensive mice in response to ischemic stroke.

Ang II-induced hypertensive mice displayed selective activation of WNK-SPAK-NKCC1 signaling in neurons and reactive astrocytes.

We then examined the location and cell types that express activated WNK-SPAK-NKCC1 proteins after ischemic stroke. **Figure 2A** illustrates immunostaining data collection in the CL and IL cortical peri-lesion tissues. The non-lesioned CL cortices from the Saline- or AngII-infused mice displayed low tNKCC1 protein expression (low anti-tNKCC1 immunoreactive signals) in the $NeuN^+$ neuronal soma or dendritic processes (**arrowhead, Figure 2B)**. The IL persi-lesion cortices of the saline-infused normotensive mice did not show any increase of tNKCC1 protein expression (**Figure 2B, C**). In contrast, the peri-lesion cortices of the AngII-mediated hypertensive mice showed increase in tNKCC1 protein expression in swollen dendrites (**arrows**), cololocalized with neurofilament 200 protein (NF) at 1-3 day after stroke (~3-fold, $p < 0.05$, **Figure 2B-D**). No elevated tNKCC1 protein was detcted in $GFAP^+$ reactive astrocytes or $Iba1^+$ microglia/macrophages (**Figure 2C, D**) at 1 day post-stroke. But tNKCC1 expression was increased in $GFAP^+$ astrocytes by ~5-fold ($p < 0.05$) as astrogliosis progressed by day 3 post-stroke, while remained at low levels in the Iba-1^+microglia/macrophages (**Figure 2C, D**). Among

WNK-SPAK protein complex, only the tWNK4 antibody is suitible for IF, with which we detected significant elevation of tWNK4 protein expression in the soma of $NeuN^+$ cells of the Ang II hypertensive stroke brains **(Figure IA-B in the Data Supplement)**. These results indicate that ischemic stroke in the Ang II-induced hypertensive mice triggers robust stimulation of WNK-SPAK-NKCC1 cascade in cortical neurons at 24-72 h after stroke, a peak period for ischemic neurodegeneration.

Ang II hypertensive mice displayed increased NF-κB p65 nuclear translocation and binding to the WNK-SPAK-NKCC1 complex gene promoters.

It is well known that Ang II induces HTN pathogenesis via NF-κB-mediated neuroinflammation in the brain.[22] To test a possible role of NF-κB activity in regulating the Ang II-induced expression of the brain WNK-SPAK-NKCC1 complex, we first quantified changes of NF-κB p65 protein in nuclear and cytosolic fractions in ischemic brains by Western blot analysis. NF-κB dimers (p65/p50) are normally sequestered in the cytoplasm by specific inhibitory factor-κB proteins (IκBα/β/ε).[23] Activated IκB kinase (IKK) can phosphorylate IκB proteins. triggering their dissociation from the NF-κB complex and translocation to the nucleus.[23] **Figure 3A** shows that the Saline-infused normotensive mouse brains displayed no changes of nuclear translocation of the NF-κB p65 protein at 24 h post-stroke ($p > 0.05$). In contrast, a 2.7-fold increase of NF-κB p65 protein was detected in the nuclear fractions of Ang II-infused hypertensive brains ($p < 0.05$), indicating increased nuclear translocation of NF-κB protein. Moreover, immunostaining in the Ang II-mediated hypertensive brains using an antibody against activate phosphorylated NF-κB protein (pNF-κB p65 at Ser^{536}) revealed a profound increase of pNF-κB p65 nuclear translocation (30%) in the peri-lesion $NeuN^+$ neurons (arrow, $p < 0.05$, **Figure 3B**), exclusively colocalized with

the NeuN signals (**merged white signals**). In contrast, minimal nuclear translocation of pNF-κB p65 was detected in the $NeuN^+$ neurons in the IL hemispheres of the saline-infused normotensive brains (**arrow, Figure 3B**).

To further determine whether NF-κB p65 nuclear translocation regulates transcriptional upregulation of the WNK-SPAK-NKCC1 complex genes, we took a bioinformatics analysis approach using Match,[24] a search tool for transcription factor binding sites, and Transfac, a database of transcription factor binding sites (geneXplain GmbH). As a result, we identified multiple putative NF-κB binding sites (5′-GGGRNYYYCC-3′) in human and mouse *Wnk1/2/4*, *Spak*, and *Nkcc1* gene promoters **(Figure 3C**). We performed ChIP-qPCR assays using a ChIP grade anti-NF-κB (p65) antibody to detect NF-kB binding to *Wnk1/2/4*, *Spak* and *Nkcc1* gene promoters in cortical brain tissues of naive or pdMCAO mice. **Figure 3D** shows no changes in the Ctrl IgG fractions in naive or pdMCAO brains. Moderate levels of NF-κB recruitment in the *Wnk1*, *Wnk2*, *Wnk4*, *Spak*, or *Nkcc1* gene promoters were detected in the naïve brains. However, a 3-7 fold increase in NF-kB recruitment on these gene promoters were detected in the IL hemispheres of the Ang II-infused hypertensive brains ($p < 0.05$). These data further support our hypothesis that NF-κB is likely a transcription factor for the increased expression of WNK-SPAK-NKCC1 genes in the ischemic Ang II hypertensive brains.

NF-κB signaling blocker TAT-NBD abolished the Ang II-mediated stimulation of WNK-SPAK-NKCC1 cascade after pdMCAO

We performed Pearson's correlation analyses to examine relationships between stroke-induced increased expression and nuclear translocation of pNF-kB and subsequent expression of neuronal WNK-SPAK-NKCC1 cascade proteins in hypertensive mouse brains following ischemic

stroke. We found a positive correlation between increased pNF-kB expression and tNKCC1 expression (Pearson's correlation r of 0.77, $p < 0.01$) and pNF-kB and tWNK4 expression (Pearson's correlation r of 0.80, $p < 0.01$; **Figure II in the Data Supplement)**. To confirm that NF-κB activity is indeed involved in regulation of WNK-SPAK-NKCC1 cascade protein expression, we investigated effects of NF-κB pathway blocker TAT-NBD on attenuating Ang II-mediated upregulation of the WNK-SPAK-NKCC1 cascade protein expression. Compared to the profound increase in expression and nuclear translocation of pNF-κB p65 in the peri-lesion $NeuN^+$ neurons in the Veh-treated HTN brains (arrow, $p < 0.05$, **Figure 4B**), TAT-NBD treatment significantly reduced the expression and nuclear translocation of pNF-κB p65 in the $NeuN^+$ neurons of the ischemic HTN brains (**arrow, Figure 4B**). Moreover, Western blot analysis of the Veh-treated Ang II hypertensive mouse brain tissues showed significantly increased expression of pNKCC1 (1.7 ± 0.2 fold), tNKCC1 (1.9 ± 0.2 fold), pSPAK/pOSR1 (1.9 ± 0.2 fold), tSPAK/tOSR1 (1.7 ± 0.2 fold), tWNK1 (2.1 ± 0.3 fold), tWNK2 (1.9 ± 0.1 fold), and tWNK4 (1.8 ± 0.1 fold) proteins in the ischemic IL hemisphere (**Figure 4C)**. TAT-NBD treatment in the Ang II-infused hypertensive mice attenuated expression of WNK-SPAK-NKCC1 cascade proteins (**Figure 4C**), consistent with the immunofluorescence staining results. Together, we conclude that NF-κB transcriptional activity is responsible for the upregulation of WNK-SPAK-NKCC1 genes and proteins in the Ang II hypertensive brains after stroke.

Post-stroke administration of SPAK inhibitor ZT-1a reduced neurodegeneration in Ang II hypertensive mice.

We then tested the efficacy of post-stroke administration of the newly developed SPAK inhibitor ZT-1a in the Ang II hypertensive mice. To assess whether ZT-1a affects physiological

parameters, we measured arterial blood gases (pCO2, tCO2), Na, K, Cl, glucose, HCO_3, pH in four different treatment groups (Sham+Veh, Sham+ZT-1a, pdMCAO+Veh, pdMCAO+ZT-1a) at 24-h post-procedures. As shown in **Supplemental Table II**, there were no significant differences in pCO2, tCO2, Na, K, Cl, HCO3, pH levels in the Veh- or ZT-1a-treated sham or stroke mice. pdMCAO+Veh and pdMCAO+ZT-1a mice displayed significantly decreased glucose level, compared to the sham+Veh group, which is consistent with a previous report that blood glucose levels decreased in mice at 3-7-day after MCAO (Lourbopoulos et al 2017 JCBFM), likely due to stroke-induced reduction of food and water intake and metabolism changes resemble fasting (Lourbopoulos et al 2017 JCBFM).

As shown in **Figure 5A-B**, we observed that administration of ZT-1a in the Ang II-mediated hypertensive mice blocked the stroke-induced increase in expression and phosphorylation of WNK-SPAK/OSR1-NKCC1 cascade proteins compared to the Veh-treated group. Immunofluorescence analysis revealed significantly more $NeuN^+$ neuron count in the ZT-1a treated group at 1 day after pdMCAO than the Veh-treated brains (arrow, $p < 0.05$, **Figure 5C**). However, the ZT-1a treatment did not reduce activation of $GFAP^+$ astrocytes or $Iba1^+$ microglia/macrophages. Moreover, ZT-1a treatment blunted the increased expression of NKCC1 in $NeuN^+$ neurons (arrowhead, $p < 0.05$, **Figure 5C**) but not in $GFAP^+$ astrocytes or $Iba1^+$ microglia cells (**Figure IV in the Data Supplement**). These data suggest that ZT1a effects are largely mediated by protecting neurons in acute stage of ischemic stroke.

To explore whether ZT-1a-mediated neuroprotective effects involves altering peripheral or brain immune cells, we performed flow cytometry experiments to assess changes of immune cells in spleen and brain (**Figure V in the Data Supplement**). Compared to sham, the Veh-treated stroke mice displayed ~25% reduction of $CD11b^+CD45^{hi}$ macrophages and ~20% increase in

$CD11b^{+}CD45^{hi}Ly6G^{+}$ neutrophils in the spleen tissue at 1-day after pdMCAO while the $CD3^{+}$ T cells were unchanged (**Figure VB in the Data Supplement**). The spleen of the ZT1a-treated stroke mice showed similar immune cell profiles (**Figure VB in the Data Supplement**). In contrast, consistent with other reports (Liu 2021 Front Immunol 12; Dotson et al 2016 Transl Stroke Res 7), stroke led to increased brain infiltration of macrophages, neutrophils, and T cells in the Veh-treated mice, but with no changes in the $CD11b^{+}CD45^{lo}P2Ry12^{+}$ microglia population (**Figure VC in the Data Supplement**). Interestingly, the ZT-1a-treated stroke brains displayed an increase in brain T cell infiltration. Whether changes in T cell infiltration in these brains are enriched protective CD3+ T cells but not pro-inflammatory Th1 and Th17 cells (Liu et al. 2021) remains to be determined in future studies. Taken together, these new results suggest that ZT-1a-mediated neuroprotection in acute phase of ischemic stroke may not involve peripheral immune cells.

SPAK inhibitor ZT-1a reduced neurological deficits in Ang II hypertensive mice without changing rCBF or BP.

Using TTC staining, we examined whether Ang II-mediated HTN caused worsened ischemic brain damage compared to their normotensive counterparts. Saline-infused normotensive mice displayed 14.3 ± 4.3 mm^3 infarct at 24 h post-stroke (**Figure 6A, B).** In comparison, the Ang II hypertensive mice exhibited significantly larger infarct volume (26.3 ± 8.3 mm^3, $p < 0.05$, **Figure 6B**). Moreover, severe hemisphere swelling was detected in the Ang II-infused mice, compared to that of Saline-controls (**Figure 6B**, $p < 0.05$). We then assessed the efficacy of the post-stroke administration of SPAK inhibitor ZT-1a on reducing infarction and improving neurological deficits. **Figure 6C** shows that the Ang II hypertensive mice (Ang II alone, or Ang II + Veh groups) exhibited worse neurological function following pdMCAO (foot faults, increased

asymmetry in limb usage and longer time in adhesive tape removal), compared to the Saline-controls ($p < 0.05$, **Figure 6C**). In contrast, ZT-1a-treated Ang II hypertensive mice displayed significantly less infarction and functional neurological deficits, indistinguishable from the normotensive saline-controls ($p < 0.05$, **Figure 6C**), suggesting a causative role for the brain WNK-SPAK-NKCC1 cascade in the worsened post-stroke outcomes. To support this conclusion, we measured changes of the systemic BP in saline-infused normotensive mice and Ang II-infused hypertensive mice prior to stroke induction, 1-day post stroke, and 14-day post stroke (**Figure 6D**). Compared with normotensive mice, Ang II-infused mice displayed significant higher BP prior to stroke induction (**Figure 6D**). pdMCAO caused transient reduction of BP in both normotensive and Ang II-infused hypertensive mice at 1-day post stroke, which was consistent with the report about pdMCAO-induced transient decrease in mean arterial BP in C57BL/6 background wild type mice (Zhang et al 2005 JCBFM 25 30-40). The possible causes for transient decrease of BP could be stroke-induced reduction of food and water intake (Lourbopoulos et al 2017 JCBFM). Importantly, systolic BP in the Ang II-infused mice was restored at 14-day after pdMCAO and remained significantly higher than that of saline-infused normotensive mice (**Figure 6D**). These findings demonstrated that despite the stroke-induced transient BP reduction, systolic BP in the Ang II-infused mice at 14-day post stroke (in the absence of Ang II minipump) remains higher than that of saline-infused normotensive mice. However, ZT-1a treatment in the Ang II mice did not lower BP, suggesting that ZT-1a directly inhibited the Ang II-mediated detrimental effects in ischemic brain without affecting BP. This view is further supported by **Figure VI in the Data Supplement** that ZT-1a treatment effectively reduced infarct volume and hemisphere swelling after stroke ($p < 0.05$) in the Ang II-infused female mice, failing to trigger systemic BP elevation (consistent with previous reports; Xue et al. 2005 AJP). Lastly,

the Veh- and ZT-1a-treated Ang II-infused hypertensive mice displayed similar changes of rCBF during 0-24 h post-pdMCAO (**Figure 6E; Figure VII in the Data Supplement**). Taken together, these data clearly suggest that the neuroprotective efficacy of ZT-1a is largely mediated by blocking of the brain WNK-SPAK-NKCC1 cascade activity in the Ang II hypertensive mice, but not through changing systemic BP or rCBF.

DISCUSSION

NF-κB transcriptionally regulates brain WNK-SPAK/OSR1-NKCC1 cascade in the Ang II-mediated hypertensive mice.

It is well documented that Ang II-mediated HTN is associated with augmented production of ROS, increased NF-κB p65 binding activity[8,22] and NF-κB-mediated inflammation in the SFO and PVN in rat and mouse brains.[7,8] Ang II-infusion and activation of AT1R can stimulate IKK via several mechanisms, including Toll-like receptor 4 (TLR4)-, TNFR1-, and NADP(H) oxidase (NOX2/4)-mediated ROS signaling in cerebral vessels, brain stem and PVN neurons.[8,25] Ang II-mediated activation of AT1R leads to IKK activation and phosphorylation of IκB proteins, their dissociation from the sequestered NF-κB complex, translocation of NF-κB complex to the nucleus in order to activate transcription.[23] In this study, we detected enhanced nuclear translocation of NF-κB in the peri-lesioned cortical neurons of the Ang II-mediated hypertensive mice after stroke, concurrent with NKCC1 protein upregulation. In addition, except in the peri-lesioned cortex and striatum tissues, we did not detect any changes of NF-κB activation or upregulation of tNKCC1 protein in the SFO, PVN, and RVLM neurons in the Ang II-induced hypertensive brains after stroke (d**ata not shown**).

We detected increased NF-κB binding to *Wnk1/2/4*, *Spak* and *Nkcc1* gene promoters in the Ang II-mediated hypertensive stroke brains. These ChIP-qPCR data are corroborated with significant upregulation of brain tWNK (1, 2, 4), tSPAK/OSR1, and tNKCC1 protein expression in the Ang II-induced hypertensive mice. We speculated that the AT1R-NF-κB signaling pathway is involved in transcriptional regulation of these proteins. A recent study has suggested that expression of the WNK-SPAK-NKCC1 cascade is regulated by numerous inflammatory cytokines.[26] For example, TNF-α induces SPAK mRNA upregulation by increased binding of NF-κB to the *Spak* gene promoter in the Caco2-BBE cells in vitro.[27,28] Importantly, the NF-κB-mediated increase in SPAK expression was involved in the pathogenesis of intestinal epithelial inflammation and inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease.[27,29] Our findings are consistent with these reports on NF-κB-mediated upregulation of WNK-SPAK-NKCC1 complex expression. Blocking of stroke-induced increase in expression of WNK-SPAK-NKCC1 cascade proteins by an IKK/NF-κB pathway blocker NBD. A positive correlation between activation of brain NF-κB and increased expression of WNK4/NKCC1 in hypertensive stroke brains (**Figure II in the Data Supplement**) further supports our conclusion. Taken together, our study demonstrates that NF-κB-mediated upregulation of WNK-SPAK/OSR1-NKCC1 cascade in response to ischemic stroke contributes to the worsened neurodegeneration in the Ang II HTN mice.

Increasing evidence demonstrates that NF-kB plays a crucial role in post-ischemic gene expression and ischemic brain injury via upregulation of inflammatory and proapoptotic factors (Ridder et al 2009 Neurosci). Inhibition of iNOS or COX-2 activity resulted in significant neuroprotection at 24h after permanent or transient MCAO (Zhang et al 1996; Sugimoto et al 2003), suggesting inflammation-mediated ischemic brain damage. Moreover, acute ischemic

stroke activated neuronal NF-kB and inhibition of neuronal NF-kB by pharmacological inhibitors attenuated ischemia-induced expression of TNF-α, IL-1β, and COX-2 and neuronal cell death (Nurmi et al 2004 J Neurochem 91). Our results are in agreement with the notion that stroke-induced neuronal NF-κB activation is detrimental as we observed pdMCAO in the Ang II-infused mice caused increased expression and activation of NF-kB and NKCC1 in neurons, causing neuronal cell death. However, we cannot exclude the possibility that other NF-kB-dependent inflammatory mediators are also activated in hypertensive brains after stroke and play a role in brain damage, which remains to be elucidated in the future studies.

SPAK inhibitor ZT-1a abolishes the Ang II-induced excessive brain damage and neurological deficits after stroke without changing BP or rCBF.

A large body of preclinical research literature indicates that Ang II-mediated activation of brain AT1R plays an important role in pathogenesis of ischemic stroke.[6,9] AT1R blockers are effective in reducing infarct volume and improving neurological functions in experimental ischemic stroke.[9,10] However, treating acute ischemic stroke patients with AT1R blockers failed to generate positive outcomes[11,12] and early BP lowering with candesartan within 30 h from ischemic stroke onset is associated with worse neurological outcome, possibly due to reduction of cerebral perfusion.[12,13] These findings suggest that new strategies are needed to reduce acute ischemic stroke brain damage with comorbid HTN. One potential mechanism could be to selectively attenuate the Ang II-mediated detrimental effects in ischemic brain without acutely lowering BP or compromising cerebral perfusion.

Our data show that upregulation of brain tWNK (1, 2, 4), tSPAK/OSR1, and tNKCC1 protein expression in the Ang II-induced hypertensive mice is associated with worsened stroke brain damage and neurological deficits, therefore suggesting the complex to be a potential

therapeutic target for stroke with Ang II HTN comorbidity. In our study, post-stroke administration of the novel, non-ATP competitive, selective SPAK inhibitor ZT-1a in the Ang II hypertensive mice significantly reduced infarct size, reduced edema, and improved neurological function recovery without affecting either BP or rCBF. ZT-1a-treatment blocked stroke-induced brain WNK-SPAK-NKCC1 cascade activation in the Ang II hypertensive mice. We found that ZT-1a exhibited dual mechanism of action that involves (1) inhibiting SPAK activity as shown by reduced phosphorylation of its substrate NKCC1 as well as (2) preventing its binding to upstream kinases as shown by reduced level of pSPAK/pOSR1 (**Figure 5**), consistent with our previous report.[19] A similar dual mechanism of action has also been reported for the WNK-SPAK binding disruptor STOCK1S-50669 in cultured cells.[30,31] The lack of influence ZT-1a on BP and rCBF implies that the underlying mechanisms are largely CNS-dependent. Indeed, this view is supported by our findings that despite the absence of Ang II-mediated systemic BP elevation in the Ang II-infused female mice, ZT-1a treatment effectively reduced their infarct volume and hemisphere swelling ($p < 0.05$, **Figure VI in the Data Supplement)**. However, future study is required to examine whether ZT-1a is neuroprotective against stroke brain injury with other types of hypertension comorbidity, including genetic or salt sensitive hypertension.

We unexpectedly found that post-stroke ZT-1a treatment not only prevented increased expression of phosphorylated WNK, SPAK and NKCC1 proteins, but also total protein expression in the Ang II HTN mouse brains. One of the possible mechanisms for this phenomenon could be that ZT-1a reduced brain inflammation. In fact, it has been reported that the WNK-SPAK-NKCC1 cascade not only regulates fluid regulation, but also inflammatory responses in mouse isolated lungs.[32] Using a specific inhibitor of NKCC1 bumetanide in acute lung ischemia/reperfusion (IR) injury model, Lan et al. showed that bumetanide-treated lungs of WT and $WNK4^{D561A/+}$ knock-in

mice exhibited less edema as well as less inflammation i.e. less expression of NF-kB p65, p-IKKβ and TNF-α after IR injury.[32] They also showed that lung inflammation was attenuated in SPAK$^{-/-}$ mice after acute IR injury.[32] Therefore, it is likely that along with its proven anti-edema property, ZT-1a has anti-inflammatory effect like bumetanide.

In this study, we have employed two pharmacological inhibitors, a peptide inhibitor of the IKK/NF-kB cascade NBD (NEMO Binding Domain-peptide) coupled to the protein transduction sequence of HIV-TAT (Nijboer et al 2008) and a small molecule SPAK kinase inhibitor ZT-1a. TAT-NBD is a cell permeable and selective inhibitor of the IKK complex (May et al 2000 Science). TAT-NBD has been shown to penetrate CNS and block NF-kB-mediated inflammatory responses in multiple models including ischemic stroke (Nijboer et al 2008; Yang et al 2013). TAT-NBD-mediated decrease in NF-kB activity was demonstrated in neonatal mouse brains by electrophoretic mobility shift assay (Nijboer et al 2008). On the other hand, the kinase selectivity of ZT-1a has been established in our recent study (Zhang et al 2020) using standard radioisotopic enzymatic assays against a panel of 140 recombinant kinases. ZT-1a exhibited high kinase selectivity for SPAK, and 98% of the 140 kinases were not inhibited by ZT-1a. Moreover, phosphorylation of GSK-3β Ser9, p38 MAPK, ERK44/42 or MAPKAPK2 kinases were not inhibited by ZT1a in either HEK-293 cells or in ZT-1a-treated ischemic mouse brains (Zhang et al 2021). Taken together, we speculate that the effects of TAT-NBD and ZT-1a observed in our study are mainly through targeting NF-kB and SPAK signaling. However, future studies using specific deletion of SPAK or specific deletion of NF-kB p65 or NF-kB phospho-p65 mutation in mice are needed to validate our findings.

CONCLUSIONS

We report that Ang II-mediated HTN triggers upregulation of brain WNK-SPAK-NKCC1 complex via NF-κB transcriptional activity, resulting in worsened outcomes after ischemic stroke. SPAK inhibitor ZT-1a is effective in blocking brain WNK-SPAK-NKCC1 cascade activation and improving outcomes for Ang II-infused mice (both male and female) without affecting BP and cerebral perfusion. Together, the Brain WNK-SPAK-NKCC1 complex emerges as a novel therapeutic target for stroke with comorbid HTN.

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FIGURE LEGENDS

Figure 1. Ang II hypertensive ischemic brains exhibit upregulation of WNK-SPAK/OSR1-NKCC1 cascade after permanent ischemic stroke.

(A) Experimental outline. Saline- or Ang II-infusion in adult male and female C57BL/6J mice was via osmotic minipump and significant elevation of arterial BP was only detected in male mice but not in female mice. Data are mean ± SEM. n=7. *p < 0.05. **(B)** Representative immunoblots and quantitative analyses of brain WNK-SPAK/OSR1-NKCC1 cascade proteins in Saline- or Ang II-infused mice (male) at 24 h after pdMCAO. Na-K pump (α subunit) was used as crude membrane protein loading control. Data are mean ± SEM. n = 6. *p < 0.05.

Figure 2. Ang II-induced hypertensive mice exhibit selective elevation of NKCC1 protein expression in peri-infarct neurons and astrocytes after ischemic stroke.

(A) Illustration of data collection in the contralateral (CL) and ipsilateral peri-lesion (IL) areas in a TTC-stained brain section at 24h after pdMCAO (white box). (**B**) Increased NKCC1 immunofluorescence in AngII-mediated hypertensive brains (arrow) than in saline-control brains (arrowhead). (**C**) Colocalization of NKCC1 immunofluorescence with neuronal process marker neurofilament (NF) (arrowhead), astrocyte marker GFAP (arrowhead) and microglia/macrophage marker Iba-1at day 1 and 3 post pdMCAO. (**D**) Quantitative analysis. Data are mean ± SEM. n = 3 (male mice). *p < 0.05.

Figure 3. Elevation of NF-κB p65 nuclear translocation and binding to *Wnk1, Wnk2, Wnk4, Spak* and *Nkcc1* gene promoters in the Ang II-induced hypertensive brains after stroke.

(**A**) Representative immunoblots of nuclear translocation of NF-kB p65 subunit in the saline- and Ang II-infused brains at 24h after pdMCAO. Quantitative analyses of NF-kB p65 protein expressions were performed in nuclear and cytosolic protein fractions. Data are mean ± SEM. n = 5 (male mice only). *p < 0.05. (**B**) Compared to saline controls, Ang II-mediated hypertensive brains exhibited increased nuclear pNF-κB expression (pSer536, arrowhead), which colocalized with $NeuN^+$ neurons (arrow) at 24h after pdMCAO. Data are mean ± SEM. n=3 (male mice only). *p < 0.05, #p=0.068. (**C**) Analysis of MATCH/TRANSFAC database predicts the presence of multiple NF-κB binding consensus sites (5′-GGGRNYYYCC-3′) in human (Hu) and mouse (Ms) *Wnk1, Wnk2, Wnk4, Spak* and *Nkcc1* gene promoters. (**D**) ChIP-qPCR analyses of NF-κB recruitment on *Wnk1, Wnk2, Wnk4, Spak* and *Nkcc1* gene promoters in the naïve and Ang II-induced hypertensive brains at 24h after pdMCAO. Data are mean ± SEM. n = 5 (male mice). *p < 0.05.

Figure 4. Effect of NF-κB blocker TAT-NBD on regulation of WNK-SPAK-NKCC1 cascade in the Ang II-induced hypertensive stroke brains.

(A) Experimental outline of saline- or Ang II-infusion, pdMCAO, drug administration, western blotting (WB) and immunofluorescence (IF). (**B**) Compared to Veh (PBS)-treated controls, NBD-treated Ang II HTN mouse brains exhibited decreased nuclear pNF-κB expression (pSer536, arrowhead) colocalized with $NeuN^+$ neurons (arrow) at 24h after pdMCAO. Data are mean ± SEM. n = 3 (veh) and 4 (NBD) (male mice only). *p < 0.01. (C) Representative immunoblots and quantitative analyses of WNK-SPAK/OSR1-NKCC1 cascade proteins in Ang II-induced hypertensive mouse (male) brains treated with Veh (PBS) or NF-kB inhibitor TAT-NBD peptide.

Na-K pump (α subunit) was used as membrane protein loading control. Data are mean ± SEM. n = 5. *p<0.05.

Figure 5. ZT-1a attenuated stimulation of WNK-SPAK/OSR1-NKCC1 cascade in the Ang II-induced hypertensive brains after pdMCAO

(**A**) Representative immunoblots and (B) quantitative analyses of WNK-SPAK/OSR1-NKCC1 cascade protein expression in the Veh- or ZT-1a-treated Ang II-induced hypertensive brains at 24 h after pdMCAO. Na-K pump (α subunit) was used as protein loading control for membrane protein fractions. Data are mean ± SEM. n = 5. *p < 0.05. (**C**) Increased expression of NKCC1 (arrowhead) colocalized with $NeuN^+$ neurons (arrow) in Veh (DMSO)-treated mouse brains were decreased in ZT-1a-treated mouse at 24h after pdMCAO. Right Panels showed quantitative analyses. Compared to Veh-treated controls, ZT-1a-treated Ang II HTN mouse brains exhibited significantly reduced NKCC1 expression and higher number of $NeuN^+$ neurons at 24h after pdMCAO. Data are mean ± SEM. n = 4 mice (male only). *p < 0.01.

Figure 6. Post-stroke administration of SPAK inhibitor ZT-1a in the Ang II-mediated hypertensive mice reduces ischemic infarct and neurological deficits after stroke.

(**A**) Experimental outline of ZT-1a administration in the AngII-infused mice. (**B**) Representative TTC staining images at 24 h after pdMCAO were shown and infarct volume and hemisphere swelling were analyzed. Data are mean ± SEM. n = 6 -8 (male mice). *p < 0.05. (**C**) AngII-induced hypertensive mice exhibited severe neurological deficits, whereas, ZT-1a treatment blunted the

worsened outcomes. Data are mean ± SEM. n = 6 (male mice). *p < 0.05. (**D**) Changes of systemic blood pressure in the saline-infused normotensive mice, and Veh- and ZT-1a-treated Ang II-infused hypertensive mice prior to and following pdMCAO. Data are mean ± SEM. n = 5 (male mice only). *p < 0.05. (**E**) Effect of ZT-1a on regional cerebral blood flow (rCBF) in ipsilateral side of Ang II-infused hypertensive mice following pdMCAO. Data are mean ± SEM. n = 6 (Veh) and 4 (ZT-1a). *p < 0.01.