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Thioredoxin interacting protein: A novel target for neuroprotection in experimental thromboembolic stroke in mice

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Abstract

Redox imbalance in the brain significantly contributes to ischemic stroke pathogenesis but antioxidant therapies have failed in clinical trials. Activation of endogenous defense mechanisms may provide better protection against stroke-induced oxidative injury. TXNIP (thioredoxin-interacting protein) is an endogenous inhibitor of thioredoxin (TRX), a key antioxidant system. We hypothesize that TXNIP inhibition attenuates redox imbalance and inflammation and provide protection against a clinically relevant model of embolic stroke. Male TXNIP-knockout (TKO), wild-type (WT) and WT mice treated with a pharmacological inhibitor of TXNIP, resveratrol (RES; 5mg/kg body weight) were subjected to embolic middle cerebral artery occlusion (eMCAO). Behavior outcomes were monitored using neurological deficits score and grip strength meter at 24 h after eMCAO. Expression of oxidative, inflammatory and apoptotic markers were analyzed by Western blot, immunohistochemistry and slot blot at 24h post-eMCAO. Our result showed that ischemic injury increases TXNIP in WT mice and that RES inhibits TXNIP expression and protects brain against ischemic damage. TKO and RES-treated mice exhibited 39.26% and 41.11% decrease in infarct size and improved neurological score and grip strength compared to WT mice after eMCAO. Furthermore, the levels of TRX, nitrotyrosine, NOD-like receptor protein (NLRP3), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and activations of caspase-1, caspase-3 and poly ADP ribose polymerase (PARP) were significantly ($P < 0.05$) attenuated in TKO and RES-treated mice. The present study suggests that TXNIP is contributing to acute ischemic stroke through redox-imbalance and inflammasome activation, and inhibition of TXNIP may provide a new target for therapeutic interventions. This study also affirms the importance of the antioxidant effect of RES on the TRX/TXNIP system.

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DISCLOSURES/ CONFLICT OF INTEREST

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Keywords

thioredoxin-interacting protein; antioxidant; resveratrol; embolic stroke; oxidative stress; inflammasome

INTRODUCTION

Ischemic stroke is a leading cause of death and long-term disability in the United States [1]. Reperfusion therapy is currently delivered to less than 5.0% of the 0.8 million ischemic strokes that occur annually in the US. Free radical formation and redox imbalance have been identified in the central nervous system (CNS) and is thought to be an important response to pathogens in the brain [2–4]. Current evidence suggests that redox imbalance in the brain significantly contributes to ischemic injury [5,6]. Nevertheless, large-scale clinical trials with classic antioxidants (e.g., NXY-059) failed to demonstrate any benefit for stroke patients. In parallel, attempts to treat patients with “neuroprotective” agents have been notoriously unsuccessful in clinical trials, possibly due to a failure to recognize redundant and compensatory processes. Hence, there is a great need to identify targets that are “upstream” of the terminal effectors and primarily involved in mediating “secondary damage” in order to devise new therapeutics that could be administered in a practical treatment window.

Thioredoxin-interacting protein (TXNIP) is an endogenous inhibitor of the thioredoxin (TRX) system, a major cellular thiol-reducing and antioxidant system. TRX also exerts anti-inflammatory and anti-apoptotic effects at the cellular level by binding and inhibiting the pro-apoptotic protein apoptosis signal-regulating kinase (ASK-1), which activates the pro-apoptotic signaling pathways [7–9]. We and others have demonstrated the pro-inflammatory and pro-apoptotic consequences as a result of the significant increases in TXNIP expression in models of stress-related diseases including stroke, neurotoxicity and metabolic stress [10–15]. Preventive strategies using TRX overexpressing transgenic mice or knocking down TXNIP expression via siRNA showed neuroprotective effects against ischemic brain damage [16,12]. Although promising, targeting early neuronal damage, an irreversible step is not practical in the clinic. On the other hand, growing interest links redox-signaling to sterile inflammatory response that can further aggravate neuronal damage after cerebral ischemia. Activation of the NOD-like receptor protein (NLRP3) inflammasome, a well-established multi-molecular protein complex and a pivotal mediator of sterile inflammation, has been postulated in detecting cellular damage and mediating inflammatory responses to aseptic tissue injury during ischemic stroke [17–19]. Recently, a significant body of literature has supported an essential role for TXNIP in the activation of the NLRP3 inflammasome [20–23]. Oxidative stress has been established to facilitate TRX1-TXNIP dissociation and hence increased association between TXNIP and the receptor of the inflammasome; NLRP3 [20]. The NLRP3 inflammasome is composed of oligomers of the receptor (NLRP3), apoptosis-associated speck-like (ASC) adapter protein and the downstream effector enzyme (pro-caspase-1). Following NLRP3-inflammasome activation, the pattern recognition receptor NLRP3 responds by recruiting and assembling with the ASC, which in turn recruits pro-caspase-1, causing its cleavage and activation into active cleaved

caspase-1. Activated cleaved caspase-1 then cleaves pro-IL-1 β into the active cleaved IL-1 β form [24,25], which exerts its well-founded detrimental pro-inflammatory and pro-apoptotic response upon release into the extracellular environment [26–28]. Moreover, our group has demonstrated that TXNIP is required for NLRP3 inflammasome activation and its deletion protects against neurovascular degeneration in models of metabolic and neurotoxic stress in the retina, a highly related neurovascular tissue with shared similarities to the brain [10,15]. However, so far little is known about the role of TXNIP-mediated NLRP3 activation in the pathogenesis of ischemic stroke.

Resveratrol (trans-3, 4', 5-trihydroxystilbene, RES), a natural polyphenolic compound enriched in grape skin and red wine, has attracted wide attention lately because of its antioxidant, anti-inflammatory, vasodilatory and inhibitory effect on platelet aggregation [29–31]. Studies continue to demonstrate the variety of mechanisms and pathways by which RES provides protection in ischemic stroke [31,32]. Nivet-Antoine et al. [33], recently showed that RES downregulates TXNIP overexpression occurring during liver ischemia-reperfusion. However, the effect of RES on TRX/TXNIP system and stroke outcomes in embolic stroke model remains to be determined.

Most cases of human stroke involve thromboembolic occlusion of cerebral arteries, especially middle cerebral artery occlusion (MCAO) [34,35]. In this study we used an embolic model to mimic the human clinical condition with spontaneous reperfusion [36,37]. We hypothesized that TXNIP inhibition by both genetic (TXNIP-knock out) and pharmacological (with RES) approaches attenuate redox imbalance and inflammation leading to protection in embolic stroke. Our findings support the contribution of TXNIP in ischemic injury, and TXNIP inhibition protected the brain against ischemic injury.

EXPERIMENTAL PROCEDURES

Animals

The experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee (IACUC) of the Charlie Norwood VA Medical Center. All experiments were performed using age-matched (8–10weeks) WT C57Bl/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) and TXNIP knockout mice (TKO, generated from genetic background of C56BL/6J) provided as a kind gift from Dr. AJ Lusis and Dr. ST Hui at the BioSciences Center, San Diego State University, San Diego, CA. TKO mice have a global knockdown of the expression of functional TXNIP as characterized previously [38,39]. TKO mice are similar in weight and activity to WT or heterozygous littermates. No phenotypic differences were observed among the TKO and WT mice. All mice were maintained and housed in the vivarium under controlled conditions ($23 \pm 2^{\circ}\text{C}$; 12 h light/dark periods) with access to food and water ad libitum.

Anatomy of the MCA and Circle of Willis

Naive mice (n=3 in each type of mouse) were deeply anesthetized and transcardially perfused with blue latex. Mice were decapitated after 30 seconds, and brains were removed and scanned for imaging.

Experimental groups and treatment regimen

In order to determine the contribution of both genetic TXNIP-deletion (TKO) and the pharmacologic TXNIP inhibition with RES on outcome/recover after embolic middle cerebral artery occlusion (eMCAO) stroke, the total 64 mice (WT and TKO) were separated into following groups: WT mice subjected to sham operated control + vehicle treatment group I (sham only); WT mice subjected to eMCAO + vehicle treatment group II (WT-eMCAO only); WT mice subjected to eMCAO + RES (5mg/kg) treatment group III (WT-eMCAO + RES only) and TKO mice subjected to eMCAO group + vehicle treatment IV (TKO-eMCAO only).

Drug administration

Resveratrol was dissolved in a solution of 30% ethanol/saline. After induction of eMCAO, the animals received an initial intravenous injection of RES (5 mg/kg) or vehicle followed by intraperitoneal injection at 3 h post-eMCAO. The dose (5 mg/kg body weight) of RES used in this study was determined from previous work demonstrating neuroprotection in the suture model of stroke [40,41].

Embolic stroke in mice

Preparation of the clot—The blood donor mice were anesthetized with 1.5% isoflurane. Fresh arterial blood was withdrawn from the heart by a 1ml syringe. To increase the strength and uniformity of the fibrin rich core of the clot and stability of the occlusion, blood was supplemented with human fibrinogen (2mg/mL), and retained in 25cm of PE-10 tubing for overnight at room temperature and subsequently was stored at 4°C. The clot was taken out into sterile phosphate-buffer saline (PBS) and washed to remove most of red blood cells. The clots were left in clean sterile PBS at room temperature for retraction. Finally, clots were again washed after the retraction process and cut into 1.0 ± 0.3 cm long fibrin rich core pieces. A single clot of 10mm was transferred to a modified PE-10 catheter filled with PBS.

Surgical procedure—Briefly, prior to eMCAO, isoflurane anesthesia was induced at 5% and then maintained at 1.5–2% during surgery. Temperature was monitored and maintained ($37 \pm 2^\circ\text{C}$) during surgery by a homeothermic heating system (Fine Science Tools, Foster City, CA). Focal ischemia (eMCAO) was induced by injection of the fibrin rich portion of blood clot in the internal carotid artery as described by Zhang et al. [36] and modified by Hoda et al., [42]. Polyethylene (PE) tubing catheter containing the clot was inserted gently from the external carotid artery (ECA) into the distal part of internal carotid artery (ICA) (just proximal to the origin of MCA, approximately 8mm of catheter is inserted) after removing the clip. A 10 mm long clot was gently delivered with 25–50 μL of PBS. The catheter was left in place for 5min, and withdrawn. The incision on the ECA was closed with tight knots. Antegrade blood flow was restored by loosening and removing the knot on the common carotid artery (CCA). Successful occlusion was confirmed by laser doppler flowmetry and the presence of a neurologic deficit.

Cerebral perfusion imaging using laser doppler imaging system

To ensure relative uniformity of the ischemic insult, cerebral perfusion was measured using the Periscan PIM 3 System (Stockholm, Sweden). A skin incision was performed, and the skull was exposed and cleaned. Whole brain scan was performed using the PIM3 to measure perfusion in both hemispheres at baseline, 10min and 24h after eMCAO. A built-in photo detector assisted with LDPI win software (Perimed Inc) detected the reflected light from moving blood cells within 0.5cm depth of the cortical surface. Color-coded images were acquired 3 times continuously, and the average perfusion was calculated based on the concentration and mean velocity of the blood cells using the LDPI win software. On induction of ischemia, cerebral perfusion decreased to ~30%.

Assessment of functional outcome

Neurological deficit score—Neurological deficits were evaluated in a blinded manner at 24 h (just before animals were sacrificed) using the modified neurological deficit score. An animal with no apparent deficits obtained a 0; the presence of forelimb flexion, 1; decreased resistance to push, 2 and circling, 3. A score of 3 was consistent with eMCAO, only animals with a score of 3 after embolization (eMCAO) were included in further analysis.

Grip strength—Forelimb grip strength in mice was determined before ischemia and 24h after surgery using a grip strength meter (Columbus Instruments, OH, USA). We used an electronic digital force gauge that measured the peak force exerted by the action of the animal while gripping the sensor bar. While being drawn back along a straight line leading away from the sensor, the animal released its grip at some point and the gauge then recorded the maximum force attained at the time of release. The digital reading (in Newtons) of three successive trials were obtained for each mouse, averaged and used for data analysis.

Assessment of infarct size

At 24 h after the onset of eMCAO, anesthesia was performed with ketamine 44mg/kg and xylazine 13mg/kg administered intramuscularly. Animals were then perfused with saline, killed, and their brains were removed. The brain tissue was sliced into seven 2 mm-thick slices in the coronal plane and stained with a 2% solution of TTC (Sigma, Chemical Co., St. Louis, Missouri, USA) for 15–20 min. Images of the stained sections were scanned using ImageJ analysis software (Image J, NIH) and infarction zones were measured. The calculations and presentation of infarct sizes were corrected for hemispheric edema and expressed as a percentage of the contralateral side $\pm$ SEM. The percentage of brain edema formation was calculated with the formula [(contralateral side–ipsilateral side)/contralateral side] X100%.

Western blotting

For WB analysis, we used peri-infarct (penumbra) cortical regions. By using a brain matrix, the brains were rapidly dissected into 4.0 mm coronal sections (approximately 0.5 mm and –3.5 mm from bregma). Brain tissue was homogenized and processed for western blotting as previously described [43]. Fifty-microgram of proteins were loaded in each lane and separated followed by transfer to nitrocellulose membranes. The membranes were blocked

in phosphate-buffered saline, 0.01% Tween 20, (PBS-T) containing 5% nonfat milk. Antibodies for TXNIP (Invitrogen, Grand Island, NY), TRX (Santa Cruz Biotechnology, Santa Cruz, CA), NLRP3 (Enzo Life Sciences, Farmingdale, NY), IL-1 β (Cell signaling, Boston, MA), tumor necrosis factor- α (TNF- α) cleaved caspase-1 (Enzo Life Sciences, Farmingdale NY), cleaved PARP (BD Bioscience Pharmingen, San Diego, CA) and cleaved caspase-3 (cell signaling, Boston, MA) were used in 1:500 dilutions in PBS-T containing 5% nonfat milk overnight at 4°C. Membranes were re-probed with 1:2000 β -actin/ tubulin (Sigma-Aldrich, St. Louis, MO) in PBS-T containing 5% nonfat milk for 2-hours to confirm equal amounts of protein were loaded. Primary antibody was detected by 1:5000 dilutions of horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit antibodies in PBS-T and enhanced-chemiluminescence (GE Healthcare, Piscataway, NJ). The band intensity was quantified using densitometry software (Alpha Innotech, Santa Clara, CA) and expressed as relative optical density (ROD).

Immunofluorescence staining

At 24 h after eMCAO (n=3), mice were anaesthetized with ketamine/xylazine and transcardially perfused with 30 mL of PBS followed by 50 mL of 10% formalin (Fischer Scientific). Brains were removed and postfixed in the same fixative overnight at 4°C and then with 30% sucrose in PBS for 72 h. The brains were sectioned in the coronal plane at a thickness of 10 μ m. Sections were blocked with 3% horse serum and then incubated with primary antibodies: rabbit anti-NLRP3 (1:100; Lifespan BioSciences, Inc) and caspase-1 (1:50; Enzo Life Sciences, Inc), overnight at 4°C. After washing, slides were incubated with fluorescent secondary antibodies, cover slipped with Vectashield mounting medium (Vector Laboratories) and viewed using Zeis Axio Observer.Z1 fluorescent microscope. Negative controls were prepared by omitting the primary antibodies.

Slot blot for nitrotyrosine

Nitrotyrosine (NT) immunoreactivity was measured as an indicator of superoxide – dependent peroxynitrite formation by slot blot analysis. Brain homogenates (20 μ g) prepared for immunoblotting experiments were immobilized onto a nitrocellulose membrane using a slot blot microfiltration unit. After blocking with 5% nonfat milk, membrane was incubated with an anti-nitrotyrosine antibody from Calbiochem and visualized with Pierce Super Signal Kit. The optical density of various samples was quantified using densitometry software (Alpha Innotech, CA).

Statistical analysis

The results were expressed as mean $\pm$ SEM. Differences among experimental groups were evaluated by ANOVA or student's t-test, followed by Tukey's test. Significance was defined as $P < 0.05$.

RESULTS

Cerebral vasculature and perfusion in WT and TKO mice

To evaluate whether globally knocking out TXNIP caused a phenotypic change in the cerebral vasculature, we transcardially injected latex blue and imaged the cerebral blood

vessels (Figure 1A). Both wild-type and TKO mice showed intact and correct alignment of the Circle of Willis, anterior cerebral arteries (ACAs), MCAs, and posterior cerebral arteries (PCAs) with no remarkable difference in the vasculature.

To demonstrate the reproducibility of the model, we measured perfusion before, 10 min and 24 h after eMCAO in a subset of both WT and TKO animals (n=3). When cerebral perfusion in the ischemic hemisphere was expressed as a percentage of the contralateral hemisphere (Figure 1B and 1C), there was a dramatic drop at 10min after eMCAO in WT and TKO mice. Deletion of TXNIP resulted in a better improvement in cerebral perfusion at 24 h compared to WT.

TXNIP inhibition reduces infarct size and improves neurological outcome after embolic stroke

TXNIP expression was analyzed by Western blot at 24 h after eMCAO (Figure 2A). The expression of TXNIP was significantly ($P<0.05$) increased in WT-eMCAO mice compared to shams, suggesting the contribution of TXNIP enhanced expression in ischemic injury. Further, RES administration significantly ($P<0.05$) inhibited the expression of TXNIP compared to vehicle-treated WT-eMCAO mice. We next examined the effect of pharmacologic and genetic inhibition of TXNIP (TKO) on infarct size at 24 h after eMCAO. As shown in Figure 2B, WT mice treated with RES and TKO mice showed significantly ($P<0.05$) smaller infarct size (21.85 ± 2.51 and 22.92 ± 3.12 respectively) than that of the WT mice (45.02 ± 3.25).

To examine whether the inhibition of TXNIP improves the neurological outcome, we used neurological deficit scores and grip strength meter at 24 h after eMCAO (Figure 2C and 2D). The WT animals exhibited prominent neurological deficits. RES-treated WT and TKO mice showed significantly ($P<0.05$) better neurological outcome after eMCAO compared to WT mice. In addition, RES-treated WT and TKO mice improved grip-strength score (in Newtons) compared to WT mice at 24 h after eMCAO. These data indicate that mice subjected to TXNIP inhibition not only had less ischemic infarct but also maintained better neurological outcome. Furthermore, there was no significant difference in the post-eMCAO mortality rates between WT and TKO (24.50% and 21.25% respectively, data not shown). Animals that died were excluded from further evaluation.

TXNIP inhibition increases thioredoxin (TRX) expression and reduces tyrosine nitration after embolic stroke

To study the effect of TXNIP inhibition on oxidative damage, we examined TRX and NT after eMCAO (Figure 3A and 3B). NT, a product of free radical oxidation of nitric oxide damage is increased under a variety of disease conditions including stroke [44]. TRX expression did change after eMCAO compared to shams, but RES-treated and TKO mice showed significantly ($P<0.05$) higher expression of TRX compared to WT-eMCAO and shams. Further, expression of NT was also inhibited in RES-treated and TKO mice after eMCAO.

TXNIP inhibition prevents inflammasome activation (NLRP-3, IL-1 β , and caspase-1) after embolic stroke

To investigate the effect of TXNIP on inflammasome activation, we examined the expression of regulatory molecules in the NLRP3 inflammasome activation pathway. The NLRP3 inflammasome can mediate neuronal death in ischemic stroke via a number of mechanisms by increasing the production and secretion of pro-inflammatory cytokines (e.g. IL-1, IL-18), and through pleiotropic effects of cleaved caspase-1 in mediating apoptosis. At 24 h after eMCAO, WT-eMCAO mice showed significantly ($P<0.05$) elevated levels of NLRP3 compared to shams (Figure 4A). The increase in NLRP3 protein expression was accompanied by an increase in cleavage of caspase-1, and upregulation of IL-1 β release (Figure 4C and 4E). These findings were further confirmed with increased immunopositive signals of NLRP3 and caspase-1 in peri-infarct area of brain sections in WT-eMCAO group (Figure 4B and 4D). Both pharmacologic and genetic inhibition of TXNIP prevented the activation of NLRP3, cleavage of caspase-1 and the subsequent release of IL-1 β after eMCAO.

TXNIP inhibition mitigates TNF- α expression after embolic stroke

We further elucidate the effect of TXNIP inhibition on TNF- α , a pleiotropic cytokine that rapidly upregulates in the brain after injury. Western blotting revealed that TNF- α was expressed at very low levels in the sham group, highly expressed after injury in the WT-eMCAO group (Figure 5), and reduced following eMCAO in the TKO and RES-treated groups. Densitometry analysis showed that TNF- α was significantly ($P<0.05$) expressed in WT-eMCAO mice compared to the shams, and significantly reduced ($P<0.05$) in the TKO- and RES-treated groups.

TXNIP inhibition attenuates PARP and caspase-3 activation after embolic stroke

To examine the effect of TXNIP inhibition on neuronal death pathways, we next examined the activation of PARP and caspase-3 at 24 h after eMCAO (Figure 6A and 6B). In the ischemic condition, the activation of PARP induced by DNA damage is also responsible for caspase-3 activation. The expression of cleaved PARP and caspase-3 were significantly ($P<0.05$) increased in WT-eMCAO mice compared to shams. RES-treated WT and TKO mice demonstrated reduced activation of PARP and cleaved caspase-3 expression compared to vehicle-treated WT-eMCAO.

DISCUSSION

The present study demonstrates that pharmacological inhibition or genetic deletion of TXNIP attenuated brain infarction and neurological outcome after embolic stroke. We also found that these effects were associated with restoring redox-balance and inhibition of TXNIP-NLRP3 inflammasome activation. Together, our results suggest for the first time that TXNIP is a novel target for neuroprotection.

Because of its high sensitivity to stress-induced degenerative conditions, the brain is targeted by different stressors. Oxidative or nitrosative stress is the result of an imbalance in pro-oxidant/ antioxidant homeostasis and both have been well documented in the etiology of

ischemic brain injury [44,45]. Our results showed that TKO and RES-treated WT mice increased TRX expression and decreased tyrosine nitration. The beneficial effects of RES are corroborated with previous studies demonstrated that RES, as a potent antioxidant protects brain in several experimental models of neurodegenerative diseases including stroke [31,46]. A recent study showed that RES antagonized TXNIP upregulation after hepatic ischemic injury [33]. Further, we found that ischemic injury increases TXNIP in WT mice and that RES inhibits TXNIP expression and protect brain against ischemic damage. Studies have demonstrated that TXNIP deletion in mice increases antioxidant status compared to WT [38,15,39]. In addition, transgenic overexpression TRX is protective against ischemic brain damage [16]. Moreover, we have shown that overexpression of TXNIP mediates inflammation and neurotoxicity in N-methyl-D-aspartate (NMDA)-induced retinal injury in rats [13]. In the present study, TKO or pharmacologic (RES treatment) inhibition of TXNIP significantly reduced the infarct size and improved neurological outcome following eMCAO. These data corroborate a previous finding that verapamil, a calcium channel blocker, inhibits TXNIP expression and attenuates ischemic damage in a transient suture mice model of stroke [12].

The mechanism (s) of action by which TXNIP inhibition provides neuroprotection after eMCAO are not completely elucidated. The TRX/TXNIP system has been identified in the central nervous system and is thought to be an important response to pathogens in the brain [3,4,2]. The TRX/TXNIP system is involved in multiple signaling pathways, which are involved in a variety of cellular functions [38,47,10]. TXNIP acts as a pro-apoptotic protein by interacting with ASK-1 leading to activation of the p38 MAPK pathway and cell death [13,48,8].

Recent findings have provided insight into new inflammatory mechanisms that may contribute to neuronal death during cerebral ischemia [19]. It is thought that the NOD-like receptor protein (NLRP3) inflammasome in neurons and glial cells may play an important role in detecting cellular damage and mediating inflammatory responses to aseptic tissue injury during ischemic stroke [17,18]. Formation of NLRP3 inflammasome can convert pro-caspase-1 into cleaved caspase-1 [24,25]. Following activation, cleaved caspase-1 will cleave pro-IL-1 β into the biologically active pro-inflammatory cytokine, mature IL-1 β , which are then released into the extracellular environment [49], in addition to its well established role in apoptosis [26,27,50].

The activators of the inflammasome can induce the dissociation of TXNIP from TRX in a reactive oxygen species-sensitive manner, allowing it to bind to NLRP3. NLRP3 then oligomerizes with the ASC that recruits procaspase-1, allowing its auto-cleavage and activation. Activated caspase-1 enzyme in turn cleaves up-regulated premature proinflammatory cytokines (IL-1 β) before their release [51,52]. Our recent work demonstrated that TXNIP triggers NLRP3 expression, activation of caspase-1 and release of IL-1 β in a cellular model of NMDA-induced retinal neurotoxicity [15]. We also have shown that TXNIP is required for high-fat diet-mediated activation of the NLRP3 inflammasome [10]. Moreover, enhanced TXNIP expression sustain neuro- and vascular degeneration through inflammasome activation in primary Muller cells in response to NMDA [15]. In the present study, we examined whether TXNIP deletion/inhibition protects the brain against

ischemic injury in part through the inhibition of NLRP3 inflammasome. The expression of NLRP3, cleaved IL-1 β and caspase-1 was significantly increased in WT mice subjected to eMCAO. Further, we found that the expression of NLRP3 inflammasome proteins was significantly less in the TKO and RES-treated mice than in the WT mice after eMCAO. These results lend further support to previous report showing that TXNIP is integral to inflammasome assembly and release of IL-1 β [53]. We and others have demonstrated that the role of enhanced TXNIP in induction of inflammation and proinflammatory cytokine expression in models of diabetic retinopathy and retinal neurotoxicity in vivo and in cells [54,55,13,56–58]. The contribution of TXNIP to inflammatory cytokine production was first demonstrated at the transcription level. Forced expression of TXNIP in isolated microvascular endothelial cells resulted in nuclear translocation and direct activation of the canonical NF B pathway [54]. Moreover, studies have showed that TXNIP induces the expression of other proinflammatory cytokines including IL-1 β and TNF- α [54,13,53]. TNF- α is a key pro-inflammatory cytokine, which commonly manifests synergistic effects with IL-1 β to produce inflammatory processes [59]. However, more elaborate work demonstrated that TNF- α can induce caspase-1 activation and IL-1 β release in mouse macrophages [60]. TNF- α is known to promote cell survival and death in the CNS [61] and is expressed in ischemic brain [62]. Our results show that TNF- α expression was increased in WT-eMCAO mice and mitigated in TKO and RES-treated mice after eMCAO. Taken together, our data suggest that in addition to reducing oxidant stress, TXNIP inhibition can also protect against cerebral damage by alleviating inflammatory processes. The limitations of this report are those associated with the use of a single endpoint at 24 h. This investigation was designed as a proof of concept study, however, and longer term studies will be needed to assess the actual impact of the manipulation of this promising target. One might expect the impact to be even greater at later time points, where inflammation and apoptosis are even more important contributors to the ultimate damage after stroke. We have, however, confirmed our results in a clinically relevant model of embolic stroke. Further studies are already planned to explore the mechanism by which TXNIP inhibition/ablation protects the brain from ischemia and tPA-initiated reperfusion injury in embolic stroke.

Thus, our data suggest that the TXNIP inhibition protects brain from infarction and improves neurological outcome by inhibiting oxidative stress and inflammasome activation (Figure 7). Our findings further suggest that TXNIP, an endogenous redox regulator, may represent an important future target to develop newer therapeutics for stroke.

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Abbreviations

PARP	poly-ADP-ribose polymerase
ROD	relative optical density
TRX	thioredoxin

TXNIP	thioredoxin interacting protein
eMCAO	embolic middle cerebral artery occlusion
NLRP3	NOD-like receptor pyrin domain containing-3
IL-1β	Interleukin-1 β
TNF-α	tumor necrosis factor- α
RES	resveratrol
ROS	reactive oxygen species
NT	nitrotyrosine
WT	wild type
TKO	TXNIP knock out
TTC	2, 3, 5-triphenyltetrazolium chloride

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Figure 1.

Cerebral vasculature and perfusion in WT and TKO mice. (A). Cerebrovascular anatomy in wild type and knockout mice. Brains from blue latex perfused wild type and TKO animals are depicted. Anterior cerebral artery (ACA), middle cerebral artery (MCA) and posterior cerebral artery (PCA) are pointed out in the figure. (B). Representative Images of cerebral perfusion detected with laser scanner post-eMCAO. (C). The cerebral blood flow (CBF) measured at 0, 10min and 24 after eMCAO with Laser Doppler flowmetry (n=3). Values are expressed as mean $\pm$ SEM (n=3).

Figure 2.

Deletion/inhibition of TXNIP reduces infarct size and improve neurological outcome at 24 h after embolic stroke. (A). Western blots analysis of TXNIP at 24 h after eMCAO. TXNIP expression was significantly increased after eMCAO compared to shams and TKO. RES administration significantly inhibited the expression of TXNIP compared to vehicle-treated WT mice. (B). Representative photographs of TTC staining at 24 h after eMCAO in WT, RES-WT and TKO mice. Percent corrected hemispheric infarct size was significantly less in RES-WT and TKO mice than in WT mice. (C). Neurological scores assessed at 24h after ischemia were significantly lower in RES-WT and TKO mice than in WT mice, indicating improved neurological deficits. D. RES-WT and TKO mice showed significantly higher average scores of grip strength compared to WT mice at 24 h post-eMCAO. Values are expressed as mean $\pm$ SEM (n=6–8, *P<0.05 WT-shams vs WT-eMCAO, *P<0.05 WT-eMCAO vs. RES-WT eMCAO or TKO-eMCAO).

Figure 3.

TXNIP inhibition increases TRX expression and decreases NT levels at 24 h post-eMCAO. (A). Representative and quantitative analysis of Western and Slot blots showing that TRX expression did not change significantly after eMCAO, but increased significantly in RES-treated WT and TKO mice compared to vehicle-treated WT and Shams. (B). TXNIP inhibition decreases NT levels at 24 h post-eMCAO. Representative and quantitative analysis of Slot blot showing that NT levels was increased significantly after eMCAO, and inhibited significantly in RES-treated WT and TKO mice compared to vehicle-treated WT. Values are expressed as mean $\pm$ SEM (n=4–6, *P<0.05 WT-shams vs WT-eMCAO, *P<0.05 WT-eMCAO vs. RES-WT eMCAO or TKO-eMCAO).

Figure 4.

TXNIP inhibition attenuates NLRP3 inflammasome proteins activation at 24 h post-eMCAO. Representative and quantitative analysis of Western blots (A) and Immunohistochemical representation around the penumbra (B) showing that NLRP3 expression and immunopositive signals increased after eMCAO, and mitigated in RES-treated WT and TKO mice. Representative and quantitative analysis of Western blots of and cleaved caspase-1 (C) and cleaved IL-1 β (E), and increased and immunohistochemical representation of caspase-1 (D) after eMCAO. The expression of these proteins were attenuated in RES-treated WT and TKO mice compared to vehicle-treated WT. Values are expressed as mean $\pm$ SEM (n=4–6, *P<0.05 WT-shams vs WT-eMCAO, *P<0.05 WT-eMCAO vs. RES-WT eMCAO or TKO-eMCAO).

Figure 5.

TXNIP inhibition attenuates the expression of TNF-α at 24 h post-eMCAO. Representative and quantitative analysis of Western blots showing that TNF-α increased significantly in WT-eMCAO compared to Shams. WT mice treated with RES and TKO mice decreased TNF-α expression compared to vehicle-treated WT-eMCAO. Values are expressed as mean $\pm$ SEM (n=4–6, *P<0.05 WT-shams vs WT-eMCAO, *P<0.05 WT-eMCAO vs. RES-WT eMCAO or TKO-eMCAO).

Figure 6.

TXNIP inhibition mitigates the expression of cleaved caspase-3 and PARP at 24 h post-eMCAO. Representative and quantitative analysis of Western blots showing that cleaved caspase-3 (A) and cleaved PARP (B) increased significantly in WT-eMCAO compared to Shams. WT mice treated with RES and TKO mice decreased cleaved caspase-3 and PARP expression compared to vehicle-treated WT-eMCAO. Values are expressed as mean $\pm$ SEM (n=4–6, *P<0.05 WT-shams vs vehicle-eMCAO, *P<0.05 WT-eMCAO vs. RES-WT eMCAO or TKO-eMCAO).

Figure 7.

Schematic representation of the proposed hypothesis of events by which inhibition/deletion of TXNIP provides protection and recovery in embolic stroke. NT= nitrotyrosine; TXNIP= thioredoxin interacting protein; TRX= thioredoxin; TXNIP^{-/-} = TXNIP knockout (TKO); PARP= poly-ADP-ribose polymerase; NLRP3= NOD-like receptor pyrin domain containing-3; IL-1 β = Interlukin-1 β ; TNF- α = tumor necrosis factor- α .