The Blockade of NF-κB Activation by a Specific Inhibitory Peptide Has a Strong Neuroprotective Role in a Sprague-Dawley Rat Kernicterus Model*

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Kernicterus, the permanent nerve damage occurring as a result of bilirubin precipitation, still occurs worldwide and may lead to death or permanent neurological impairments. However, the underlying mechanisms remain unclear, and effective therapeutic strategies are lacking. The present study aims to investigate the activation of NF-κB and to identify the effect of NF-κB inhibition on the newborn rat kernicterus model. The NF-κB essential modifier-binding domain peptide (NBD), coupled with the HIV trans-activator of transcription peptide (TAT) was used to inhibit NF-κB. NF-κB was significantly activated in the cerebrum at 1 and 3 h (p < 0.05) after the model was established, as measured by EMSA. NF-κB activation was inhibited by intraperitoneal administration of TAT-NBD. The general conditions of the TAT-NBD-treated rats were improved; meanwhile, these rats performed much better on the neurological evaluation, the rotarod test, and the Morris water maze test (p < 0.05) than the vehicle-treated rats at 28 days. Furthermore, the morphology of the nerve cells was better preserved in the TAT-NBD group, and these cells displayed less neurodegeneration and astrocytosis. Simultaneously, apoptosis in the brain was attenuated, and the levels of the TNF-α and IL-1β proteins were decreased (p < 0.01). These results suggested that NF-κB was activated, and inhibition of NF-κB activation by TAT-NBD not only attenuated the acute neurotoxicity, apoptosis, and inflammation, but also improved the long term neurobehavioral impairments in the kernicterus model rats in vivo. Thus, inhibiting NF-κB activation might be a potential therapeutic approach for kernicterus.

Hyperbilirubinemia is a common condition in newborns, and the outcome for the majority is benign. However, neonates with severe unconjugated hyperbilirubinemia may develop kernicterus, particularly those who are premature or have suffered sepsis, hypoxia, etc. Although the incidence rate of kernicterus has been dramatically reduced due to advancements in perinatal medicine, it still occurs throughout the world, particularly in developing countries (1, 2). Kernicterus is considered a chronic sequela of acute bilirubin encephalopathy, which may lead to death or lifelong neurological sequelae, such as mental retardation, a developmental motor delay, and epilepsy (3, 4). Unfortunately, although the level of unconjugated bilirubin (UCB)3 in the serum can be rapidly reduced by phototheraphy and/or exchange transfusion, the UCB that has already entered the nervous system cannot currently be replaced, and the irreversible neurologic damage cannot be prevented. Meanwhile, the limited availability of exchange transfusion and its severe complications remind us that it is critical to explore other effective strategies (2).

The current studies suggest that UCB-induced oxidative stress, intracellular calcium overload, immunostimulation, and overactivated NMDA receptor might be involved in the pathogenesis of bilirubin encephalopathy. However, the effects of some specific interventions, such as the NMDA channel antagonist MK-801, are controversial, and the underlying mechanisms of bilirubin neurotoxicity remain unclear (5). Thus, further investigations are required. Nuclear factor κB (NF-κB) is a transcription factor that regulates the expression of numerous genes involved in cell survival, apoptosis, and inflammation. In the resting state, the inactivated NF-κB is retained in the cytoplasm by an inhibitory subunit called IκB. The phosphorylation of IκB by Iκ-kinase, containing Iκ-kinase α and β, and the regulatory protein NEMO (NF-κB essential modifer) is a key step in NF-κB activation in response to various stimuli. Then the activated, free NF-κB translocates into the nucleus and regulates target gene expression (6). It is confirmed that NF-κB plays a critical role in the development and function of the nervous system and in the pathological process of many nervous system diseases, such as ischemic stroke, brain trauma, and neurodegenerative disease (7, 8).

Recently, several studies have demonstrated that UCB activates NF-κB in vitro, and inhibitors that block this activation...
exhibit an important influence on UCB-induced cellular inviability and cytokine secretion (9–11). Nevertheless, there have been no in vivo studies conducted to identify the role of NF-κB in kernicterus, given that it has both protective and damaging roles in response to nervous system damage (7). It is unclear whether the inhibition of NF-κB activation results in protection or damage in rat models of kernicterus.

In previous studies, we have successfully established a rat kernicterus model that is stable and suitable for exploring bilirubin-mediated neurotoxicity in vivo (12). In this study, we used the NEMO binding domain peptide (NBD), a specific inhibitor of the IKK complex that does not affect basal NF-κB activity (13), coupled to the HIV trans-activator of transcription peptide (TAT), to facilitate the penetration of TAT-NBD though the blood-brain barrier and allow it to enter the cells (14). This study aimed to investigate whether NF-κB activation was involved in the pathogenesis of kernicterus in vivo. Furthermore, the effects of TAT-NBD in the kernicterus rats were observed, including the morphologic changes of the nerve cells, cerebral apoptosis, cytokine production, general conditions, long term motor abilities, sensation function, and learning and memory.

Materials and Methods

Experimental Animals—All animal procedures were approved by the Ethics Committee of Chongqing Medical University (Permit SYXK2007-0016). All experimental Sprague-Dawley rats (SPF grade) were obtained from the Animal Experiment Center of Chongqing Medical University. The animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Efforts were made to minimize animal suffering and to reduce the number of animals used. All rats were housed on a 12-h/12-h light/dark cycle with a moderate temperature (23 ± 2 °C).

Animal Model Establishment and Treatment—The rat kernicterus model was established as described previously (12). Briefly, bilirubin (Sigma-Aldrich) was solubilized in a 0.5 M NaOH solution (100 mg/ml) and diluted in double-distilled H2O (10 mg/ml), and the pH was adjusted to 8.5 with HCl (0.5 M). At postnatal day 5, the Sprague-Dawley rat pups (10–15 g) were anesthetized with diethyl ether, and then ∼10–15 μl of the cerebrospinal fluid was released from the cisterna magna using a microsyringe (measuring range: 25 μl) to prevent intracranial hypertension. Finally, the bilirubin solution or double-distilled H2O (pH 8.5) was then injected into the cisterna magna at 10 μg/g (body weight).

To explore the activation of NF-κB and the effect of TAT-NBD in the rat kernicterus model, the rat pups were divided into four groups (n ≥20 in each group): control group, vehicle group, TAT-NBD group, and TAT-NBDmut group. The TAT-NBD (YGRK-KRRQRRR-TALDWSWLQTE) and TAT-NBDmut (YGRK-KRRQRRR-TALDASALQTE) peptides (Science Peptide, Shanghai, China) were each dissolved in DMSO (40 mg/ml), diluted in PBS to a concentration of 1 mg/ml, and administered intraperitoneally at 10 μl/g (body weight) at 0 and 2 h after the model was established. The controls and vehicles were intraperitoneally administered equal volumes of DMSO and PBS.

Dynamic Assessment of the Clinical Manifestations—The clinical manifestation scores were assessed every 2 h a total of 3 times after the model was established by a double-blind method. As described previously (12), clenched fists, opisthotonus, latericubent positioning, and rolling were included in the clinical manifestation scores. One point was recorded if the rat showed any of these manifestations.

Recording Body Weights—The body weights were recorded daily for 3 consecutive days after the model was established.

Mortality—The number of rats that died after the model was established to postnatal day 28 was recorded to calculate the mortality.

Histology—Based on a preliminary study (data not shown), four randomly selected rat pups in each group were sacrificed 24 h after the model was established, and the brain was fixed in 4% paraformaldehyde and embedded in paraffin. Coronal paraffin sections (5 mm) were cut from the bregma at the optic chiasm through the dorsal and ventral hippocampus to reach the hippocampal formation, and three consecutive sections that contained hippocampus (cornu ammonis 1 (CA1), CA2, and CA3) and dentate gyrus were cut. Hematoxylin-eosin (H&E) and terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) staining (Roche Applied Science) were performed. The morphological changes in the hippocampus and the cerebral cortex were observed by light microscopy (Nikon, Japan). The total number of cells and the number of TUNEL-positive cells in the pyramidal neuron layers of the CA1, CA2, CA3, and dentate gyrus regions of the hippocampus or in the cerebral cortex were counted in eight randomly selected high magnification fields (×400) per section in a blinded fashion.

Immunofluorescence Staining—The brain tissues fixed in 4% paraformaldehyde (described above) were then stored in a 30% sucrose, PBS (pH 7.4) solution for 48 h, sectioned (40 μm) coronally for a 1:6 section series on a freezing microtome, and stored in 0.1% sodium azide in PBS at 4 °C until further use (15). The brain sections were washed with PBS containing 0.3% Triton X-100 (Bio-Rad) (PBST; 3 × 10 min) and then blocked with 5% nonfat milk diluted in PBST (room temperature, 1 h). After washing with PBST (3 × 10 min), the brain sections were incubated with the following primary antibodies: neurofilaments (NF) for neuron (1:50) or glial fibrillary acidic protein (GFAP) for astrocytes (1:100; Cell Signaling Technology, Danvers, MA) overnight at 4 °C. After washing with PBST (3 × 10 min), the brain sections were incubated for 1 h at 37 °C with an Alexa Fluor 488-conjugated goat anti-mouse IgG. After washing, the sections were co-stained with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min to stain the nuclei and then mounted on slides. The slides were imaged using a fluorescent microscope (Nikon, Japan). For image acquisition, the exposure time, detector gain, and amplifier offset were standardized across the compared sections. The percentage of the areas occupied by NF and GFAP were measured by ImageJ. At least seven random and separate microscopic fields (×200) of the hippocampus or the cerebral cortex were selected from each section, and the results were obtained from the averaged values (15, 16).

Evaluation of Neurological Function—The extent of the neurological deficit was performed on 28-day-old rats by two
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blinded observers using the neurological evaluation system described by Garcia et al. (29). This neurological evaluation system consisted of the following tests: spontaneous activity, symmetry in the movement of the four limbs, forepaw out-stretching, climbing, body proprioception, and response to vibrissae touch. The minimum neurological score is 3 (severe neurological function deficit), and the maximum is 18 (normal). The lower the score, the more severe the neurological function deficit.

Rotarod Test—The rotarod test was performed to assess the rats’ balance and motor coordination at the age of 28 days. The experimental rats were placed onto a horizontal rotating rod with the rotation speed increasing from 10 to 80 rpm over 3 min. A single test lasted from the time the rat was able to stay on the rotating rod until it fell off or until 3 min had elapsed. The time at which the rat fell off the rotating rod was recorded. Each rat was tested in four trials. The average time spent on the rotating rod was calculated.

Open Field Test—An open field test was conducted to evaluate the locomotor activity of 28-day-old rats by a blinded observer. In brief, the open field apparatus consists of a square arena (60 × 60 × 20 cm), with a digital camera mounted above the open field arena. The video and date were recorded using the ANY-maze software. The animals were allowed to acclimate to the testing room for 30 min before testing. A single rat was placed in the center of the arena and observed for 30 min. The arena was thoroughly cleaned with 75% alcohol after every session. The distance traveled in 30 min was measured to reflect the animals’ locomotor activity (17).

Morris Water Maze—The Morris water maze was performed as described previously (12) to evaluate the rats’ learning and memory at the age of 28 days.

Electrophoretic Mobility Shift Assays—An electrophoretic mobility shift assay (EMSA) was performed to investigate NF-κB activation using a nonradioactive infrared NF-κB EMSA kit (Viagen Biotech, Inc., Wuxi, China). The nuclear proteins (Viagen Biotech) were prepared (10 μg) and mixed with 10X binding buffer and 1 μg of poly(dI-dC) for 30 min at room temperature, and then incubated with IR700-labeled oligonucleotide bio-NF-κB probe (5’-AGTTGAGGGACTTTC-CCAGGC-3’) for 30 min at room temperature, according to the manufacturer’s instructions. The mixture was subjected to 6.5% native polyacrylamide gel electrophoresis at 180 V in 0.5X TBE buffer for 40 min at 4 °C. The gels were visualized using a chemidoc system (Syngene, Cambridge, UK) using an ECL assay kit (Pierce).

Enzyme-linked Immunosorbent Assay (ELISA)—The levels of the TNF-α and IL-1β proteins were measured by ELISA (USCN Life Science Inc., Wuhan, China). The rat brain tissues were homogenized on ice in Nonidet P-40 lysis buffer (Beyotime Biotechnology), and the homogenates were quantified using the BCA assay (Beyotime Biotechnology). The absorbance of the samples (450 nm) was measured using a microplate (BioTek) according to the manufacturer’s instructions. The results were normalized to the total protein concentration. All samples were tested in duplicate.

Statistical Analysis—The statistical analyses were performed with SPSS version 17.0. The data are presented as the means ± S.D. A normality test and a homogeneity test for variance were performed first. If the data were in compliance with a normal distribution and homogeneity of variance, an ANOVA with Bonferroni’s post-test or a Student’s t test was performed; otherwise, a rank sum test was used. The categorical data were analyzed using the χ² test. p < 0.05 was considered to be statistically significant.

Results

TAT-NBD Inhibited NF-κB Activation in the Newborn Rat Kernicterus Model

Bilirubin Induced Cerebral NF-κB Activation in Vivo

We first analyzed whether NF-κB was activated in the cerebrum in the newborn rat kernicterus model. The EMSA results showed that NF-κB activity was markedly increased in the kernicterus model at 1 and 3 h (p = 0.012 and 0.044) and returned to basal levels at 6 h compared with the control group (Fig. 1A). Meanwhile, the specificity of the EMSA results was confirmed because the DNA binding was abolished after adding excess cold DNA probe, suggesting that the observed DNA binding activity is due to NF-κB (Fig. 1B).

Effect of TAT-NBD on NF-κB Activation

Subsequently, we investigated whether intraperitoneally administered TAT-NBD could inhibit NF-κB activation in the cerebrum of the rat kernicterus model. Based on the half-life of TAT-NBD and the peak time of NF-κB activity in the cerebrum, TAT-NBD was intraperitoneally administered at 0 and 2 h after the model was established. NF-κB activity was examined at 3 h after model establishment by EMSA. The NF-κB activity at 3 h was significantly inhibited in the TAT-NBD group compared with the vehicle group (p = 0.042) and the TAT-NBDmut group (p = 0.026; Fig. 1C).

Effect of TAT-NBD on the Rats’ General Condition

Clinical Manifestation Scores

The clinical manifestations were scored dynamically every 2 h within 6 h after the insult, using a double-blind method. The results showed that abnormal neurological manifestations (such as clamped fists, opisthotonus, etc.) were observed in all bilirubin-treated rats, including the vehicle, TAT-NBD, and TAT-NBDmut groups, and the clinical manifestation scores in these three groups were higher than those of the control group.
Interestingly, the scores of the TAT-NBD group were significantly lower \((p < 0.001)\). Interestingly, the scores of the TAT-NBD group were significantly lower \((p = 0.004\) and 0.005) than those of the vehicle and TAT-NBDmut groups (Fig. 2A).

**Body Weight**

There were no significant differences in the body weights of the control and TAT-NBD groups on postmodeling days 1, 2, and 3. However, the body weights in the vehicle and TAT-NBDmut groups were dramatically lower \((p = 0.008\) and 0.018) than those of the control group and showed a body weight loss on postmodeling day 1. Although all rats showed increased body weights on postmodeling days 2 and 3, the body weights in the vehicle and TAT-NBDmut groups were still significantly lower (postmodeling day 2, \(p = 0.003\) and 0.006; postmodeling day 3, \(p = 0.003\) and 0.014) than those of the control group (Fig. 2B).

**Mortality**

The mortality in each group was monitored after the model was established to postnatal day 28. The mortality of the TAT-NBD-treated rats was 11.7%, which was significantly lower than that of the vehicle (34.2%) and the TAT-NBDmut (37.1%) groups \((p = 0.027\) and 0.014, \(\chi^2\) test, \(n = 30–35\) animals/group).

**Neuroprotective Effects of TAT-NBD Using Histology**

**H&E Staining**

H&E staining 24 h after insult showed that the nerve cells in both the hippocampus and cerebral cortex were morphologically normal in the control group. Changes in the cellular morphology, including cytoplasmic condensation and endolysis, nuclear pyknosis, karyorrhexis, and karyolysis, were observed in the vehicle, TAT-NBD, and TAT-NBDmut groups. Nevertheless, the severity of these changes in the TAT-NBD group was relatively mild, compared with the vehicle and TAT-NBDmut groups (Fig. 3A).

**TUNEL Staining**

TUNEL staining was performed to assess the effect of TAT-NBD on the bilirubin-induced neuronal apoptosis. Compared with the control group, a large number of TUNEL-positive cells were observed in the hippocampus and cerebral cortex of both the vehicle and TAT-NBDmut groups, whereas only a few cells were positively stained in the TAT-NBD group (Fig. 3B). The apoptosis rates in the hippocampus and cerebral cortex were calculated based on the average total number of cells and the number of TUNEL-positive cells in the pyramidal neuron layers of the CA1, CA2, CA3, and dentate gyrus regions of the hippocampus or in the cerebral cortex in eight randomly selected high magnification fields (\(\times 400\)) from three consecutive sections. The results showed that the apoptosis rate in the hippocampus was significantly reduced (both \(p < 0.001\)) in the TAT-NBD-treated rats (7.54 ± 0.45%) compared with the vehicle-treated (22.27 ± 1.92%) or TAT-NBDmut-treated rats (21.63 ± 1.22%); the apoptosis rate in the cerebral cortex was also significantly reduced in the TAT-NBD group (9.77 ± 2.46%) compared with the vehicle (14.00 ± 3.58%; \(p = 0.013\)) and TAT-NBDmut groups (14.30 ± 4.00%; \(p = 0.011\); Fig. 3C).
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**Immunofluorescence Staining**

*TAT-NBD Treatment Attenuated the Loss of NF Expression—* Changes in the expression of NF, a major component of the neuronal cytoskeleton, were examined to investigate UCB-induced neuronal injury. The control group exhibited a normal, organized structure of the neuronal cell body and neuronal arborization in both the hippocampus and cerebral cortex, whereas the vehicle, TAT-NBD, and TAT-NBDmut groups exhibited a loss of neuronal cells and a disordered neuron structure, accompanied by the loss of NF. However, these neuronal injuries were attenuated in the TAT-NBD group (Fig. 3D). Additionally, the areas (percentage of total) occupied by NF staining in the hippocampus (p = 0.023 and 0.014) and cerebral cortex (p = 0.005 and 0.002) in the TAT-NBD group were markedly increased compared with those in the vehicle and TAT-NBDmut groups (Fig. 3F).

*TAT-NBD Treatment Inhibited Astrocytosis—* Many studies demonstrated that UCB could induce astrocytosis both in vitro and in vivo (5, 16, 18). Therefore, we subsequently used GFAP staining to investigate whether UCB-induced astrocytosis could be inhibited by TAT-NBD. The results showed that compared with the control group, the number of reactive astrocytes was apparently increased in the vehicle, TAT-NBD, and TAT-NBDmut groups. However, the TAT-NBD group exhibited a relatively lower number of GFAP-positive cells in the hippocampus and cerebral cortex (Fig. 3E), and the areas (percentage of total) occupied by the GFAP-positive staining were significantly decreased in the TAT-NBD group compared with the vehicle (p = 0.04 and 0.035) and TAT-NBDmut groups (p = 0.044 and 0.027; Fig. 3F).

**Effect of TAT-NBD on Apoptosis-related Molecules**

The Western blot results demonstrated that the TAT-NBD treatment markedly prevented the bilirubin-induced increase in the cytosolic cytochrome c levels compared with the vehicle and TAT-NBDmut groups (p = 0.002 and 0.013) 24 h after insult (Fig. 4A).

The expression of Bax (the proapoptotic protein) and Bcl-2 (the antiapoptotic protein) in the mitochondria was also examined. The expression of Bax was dramatically decreased in the TAT-NBD group (p = 0.046 and 0.019; Fig. 4B), whereas the expression of Bcl-2 was significantly increased (p < 0.001) compared with the vehicle and TAT-NBDmut group (Fig. 4C).

**TAT-NBD Treatment Prevented Cytokine Release**

To evaluate whether the neuroprotective effects of TAT-NBD were mediated via the inhibition of cytokine release, the temporal secretion profiles of TNF-α and IL-1β were first investigated in this newborn rat kernicterus model. It can be shown that the cytokines were released in a time-dependent manner. TNF-α secretion began to increase at 1 h after model establishment, reached a peak level at 3 h (p = 0.029 versus the control group), and then decreased. IL-1β seemed to be up-regulated and reached a peak level at 6 h (p = 0.014 versus the control group) after model establishment and then declined gradually (Fig. 5A). Therefore, we chose 3 and 6 h as the representative time points to measure the level of these two cytokines and investigate whether the intraperitoneal administration of TAT-NBD could inhibit cytokine release in the rat kernicterus model. The ELISA results showed that the secretion of TNF-α (3 h) and IL-1β (6 h) was significantly decreased in the TAT-NBD-treated rats compared with the vehicle-treated rats (p = 0.003 and 0.005) or TAT-NBDmut-treated rats (p = 0.008 and 0.010; Fig. 5, B and C).

**TAT-NBD Treatment Greatly Improved the Long Term Neurological Outcomes**

**Neurological Evaluation, as Described by Garcia et al. (29)**

To observe the effect of TAT-NBD on the neurological outcomes in the rat kernicterus model, the neurological function of the 28-day-old rats was evaluated using the neurological evaluation system described by Garcia et al. (29). This evaluation system assesses the motor ability and sensation function of the experimental animals. The TAT-NBD-treated rats exhibited significantly improved neurological outcomes (scores: 16.44 ± 1.59; p < 0.001) compared with those of the vehicle-treated rats (scores: 11.60 ± 2.41) and TAT-NBDmut-treated rats (scores: 11.60 ± 2.41).
10.88 \pm 2.53). Strikingly, there were no significant differences between the TAT-NBD-treated rats and the controls, indicating a prominently neuroprotective role of TAT-NBD in the rat kernicterus model (Fig. 6A).

**Rotarod Test**

The rotarod test was performed to assess whether the balance and motor coordination abilities were different in the four groups. The TAT-NBD group (79.43 \pm 25.07 s) spent significantly more time on the accelerating rotarod (p = 0.037 and 0.013) than the vehicle (61.79 \pm 23.00 s) and TAT-NBDmut groups (58.14 \pm 22.90 s; Fig. 6B). This result suggested that TAT-NBD could evidently improve the balance and motor coordination of the rats with kernicterus.

**Open Field Test**

The open field test showed that all of the tested animals traveled similar distances, and there were no significant differences among these groups in locomotion (control group, 2554.35 \pm 292.22 cm/30 min; vehicle group, 2467.92 \pm 484.03 cm/30 min; TAT-NBD group, 2530.94 \pm 617.74 cm/30 min; and TAT-NBDmut group, 2506.27 \pm 495.90 cm/30 min; Fig. 6C). The data
Intraperitoneal TAT-NBD administration inhibits bilirubin-induced apoptosis in the brain. At 24 h after establishing the kernicterus model, the expression of cytosolic cytochrome c (A) was prevented by TAT-NBD treatment (**, p < 0.01 versus the control group; *, p < 0.05 versus the TAT-NBDmut group; #, p < 0.01 versus the vehicle group using one-way ANOVA with Bonferroni’s post hoc test; n = 5–6 animals/group). Bax protein was simultaneously up-regulated (C) in these animals compared with the vehicle- and TAT-NBDmut-treated groups (#, p < 0.05 versus the control group; **, p < 0.01 versus the control, vehicle, or TAT-NBDmut groups using one-way ANOVA with Bonferroni’s post hoc test; n = 5–6 animals/group). The intensity of the bands was quantitated by scanning densitometry, standardized with respect to β-tubulin or COX-IV protein, and normalized to the values of the control group. Three independent experiments were performed in duplicate. 1, control group; 2, vehicle group; 3, TAT-NBD group; 4, TAT-NBDmut group. Error bars, S.D.

Discussion

Kernicterus, the most severe complication of neonatal hyperbilirubinemia, still occurs throughout the world. It results in death or irreversible neurologic damage due to a lack of specific therapies (1–4). NF-κB, a critical transcription factor, plays an important role in the various physiological and pathological processes of the nervous system (7, 8). Current studies suggest that activation of NF-κB signaling is a key mediator in UCB-induced inflammatory response and cell death in both neurons and glia in vitro (9–11). However, to date, few studies have been conducted to demonstrate NF-κB activation in vivo. It was only indirectly demonstrated to be activated in a humanized Ugt-1 mouse (a gene knock-out hyperbilirubinemia mouse) (18), and the possible role of NF-κB signaling in vivo was not further elaborated. There was also no evidence of NF-κB activation in other bilirubin encephalopathy animal models. To the best of our knowledge, this is the first study demonstrating that NF-κB is activated in the novel Sprague-Dawley rat kernicterus model using EMSA. Compared with the Gunn rat and Ugt-1-null or humanized Ugt-1 mice, this model shows not only the acute clinical manifestations but also the chronic sequelae of bilirubin encephalopathy, which would be more suitable for studies on bilirubin neurotoxicity in vivo (12).

This study demonstrated that NF-κB was activated in the cerebrum of the kernicterus model, reaching peaks at 1 and 3 h after the model was established. NF-κB activity has been described as being involved in inflammation, cell survival, apoptosis, neurite outgrowth, neuronal differentiation, and plasticity in nervous system pathologies. However, the exact effect of NF-κB activation on neurological diseases is complex because it either promotes or mitigates the insults (7, 8). For example, NF-κB activation enhances ischemic neuronal death in ischemic brain injury (19), whereas other studies demonstrate that NF-κB serves a protective function by enhancing the expression of neuronal apoptosis inhibitory protein-1 in neurons, which protects against ischemic brain injury (20). In vitro stud-
ies have revealed the participation of NF-κB in UCB-induced cytotoxicity, and the blockade of NF-κB signaling improved cell viability and cytokine release (9–11). Therefore, we further investigated whether inhibiting NF-κB activation in the cerebrum of the rat kernicterus model was neuroprotective.

In this study, we applied the NBD (a specific NF-κB inhibitor that does not interfere with basal expression) described by May et al. (13), coupled with the TAT, which could facilitate NBD crossing through the blood-brain barrier and entering the cells (14). Several studies have shown that TAT-NBD could effectively inhibit NF-κB activation in vivo (21, 22). Based on the half-life of TAT-NBD (23) and the kinetics of NF-κB activation in this kernicterus model, it is postulated that intraperitoneal administration of TAT-NBD (10 μl/g) at 0 h and 2 h could prevent UCB-induced NF-κB activation in the cerebrum without interfering with basal expression. Meanwhile, the TAT-NBDmut peptide was used to show that NF-κB activation is specifically inhibited by NBD instead of TAT.

As previously demonstrated in the kernicterus model (12, 24), the first 3-day period after model establishment represents clinical phases 1, 2, and 3 of acute bilirubin encephalopathy. Hence, the characteristic neurological manifestations were observed and graded in a double-blind manner, and the body weight changes were recorded. The TAT-NBD-treated rats exhibited fewer abnormal neurological manifestations and gained more body weight than the vehicle group, indicating that TAT-NBD attenuated the neurological manifestations and improved the feeding of the model rats in clinical phases 1, 2, and 3 of acute bilirubin encephalopathy. Furthermore, the morphology of the neurons was better preserved in the TAT-NBD group, as observed by H&E staining. NF is a major component of the neuronal cytoskeleton, which is believed to provide structural support for the axon (25). The TAT-NBD-treated rats showed decreased neurodegeneration and up-regulated NF expression compared with the vehicle group. This finding suggested that TAT-NBD attenuated UCB-induced neuronal injury. With regard to the glial cells, an abundance of data highlights that glia are also sensitive to UCB, which induces glial activation and dysfunction (26). In this study, we observed UCB-induced astrogliosis. As a potent source of inflammatory cytokines, astrocytes play a pivotal role in the inflammatory response of the nervous system; in addition, astrocytes are the most abundant type of glial cell in the nervous system (27). The TAT-NBD-treated rats exhibited apparently lower GFAP expression in both the hippocampus and cerebral cortex, suggesting that the number of reactive astrocytes was reduced. Taken together, we believe that TAT-NBD could alleviate acute phase neurological injury in rats with kernicterus.

According to the American Academy of Pediatrics, “acute bilirubin encephalopathy” refers to the neurological features of the acute phase, whereas “kernicterus” is used to describe the chronic and permanent clinical sequelae of bilirubin encephalopathy. The life-long neurological impairments resulting from bilirubin encephalopathy include difficulties in learning, developmental motor delays, sensorineural hearing loss, epilepsy, cerebral palsy, and mental retardation (3). In many nervous system diseases, neurological evaluation plays an important role in the diagnosis and rehabilitation (28). In animal experiments, many tests are also designed to assess the extent of the functional neurological deficits and recovery based on the animals’ clinical characteristics. In this study, we used the neurological evaluation system designed by Garcia et al. (29) to assess the animals’ motor ability and sensation functions (29). Consequently, animals with kernicterus exhibited apparent neurological function disorders, whereas the intraperitoneal administration of TAT-NBD relieved the long term neurological deficits. In addition, the TAT-NBD-treated rats with kernicterus also exhibited improved balance and motor coordination in the rotarod test. We also conducted the Morris water maze test.
which is a behavioral task to test spatial learning and memory. The results showed that the TAT-NBD-treated rats performed better in the Morris water maze test than the vehicle- and TAT-NBDmut-treated rats, both in the place navigation ability test and the spatial probe trial. Meanwhile, the swimming speeds of the tested rats were not significantly different between groups, indicating that the gross motor function was not influenced in these rat models. The open field test also showed that the spontaneous locomotor activity was also not affected. Therefore, we can conclude that the altered behavior in the Morris water test reflects learning and memory deficits and that TAT-NBD can improve the learning and memory function of rats with kernicterus. TAT-NBD can also significantly increase the survival rates. Collectively, it could be speculated that TAT-NBD exhibits a strong neuroprotective effect by inhibiting NF-κB in rats with kernicterus, both in the acute and chronic phases. Nevertheless, the underlying mechanisms need to be clarified.

FIGURE 6. TAT-NBD treatment greatly improved the long term neurological outcomes. A, neurological function scores assessed by the evaluation system developed by Garcia et al. (29). The scores of the TAT-NBD group were significantly higher than those of the vehicle and TAT-NBDmut groups. *, p < 0.01 versus the control group. **, p < 0.01 versus the vehicle or TAT-NBDmut groups using one-way ANOVA with Bonferroni’s post hoc test. n = 11 animals/group. B, rotarod test. The TAT-NBD group spent significantly more time on the accelerating rotarod than the vehicle and TAT-NBDmut groups. *, p < 0.05 versus the control or TAT-NBD groups. #, p < 0.01 versus the control group using one-way ANOVA with Bonferroni’s post hoc test. n = 10–15 animals/group. C, locomotion in the open field test. There were no significant differences in the total distance traveled in 30 min between the four groups. p > 0.05 using one-way ANOVA with Bonferroni’s post hoc test. D–I, the rats’ learning and memory were tested using the Morris water maze. D, escape latency to find the platform during days 2–7 of testing. Compared with the vehicle- and TAT-NBDmut-treated rats, the TAT-NBD-treated rats exhibited a shorter latency to escape onto the platform on the 6th and 7th day (navigation trial). *, p < 0.01 versus the control group. #, p < 0.05 versus the vehicle or TAT-NBDmut groups using two-way repeated measures ANOVA. n = 11–15 animals/group. E, number of times the rats crossed over the platform location on day 8 (probe trial). The number of times the TAT-NBD-treated rats crossed over the platform location was significantly higher compared with the vehicle- and TAT-NBDmut-treated rats. *, p < 0.01 versus control or vehicle groups. #, p < 0.05 versus the TAT-NBDmut group using one-way ANOVA with Bonferroni’s post hoc test. n = 11–15 animals/group. F, percentage of time spent in the safety quadrant during the probe trial. Compared with the vehicle- and TAT-NBDmut-treated rats, the TAT-NBD-treated rats spent more time in the safety quadrant containing the platform. *, p < 0.01 versus the control group; #, p < 0.05 versus the vehicle or TAT-NBDmut groups using one-way ANOVA with Bonferroni’s post hoc test. n = 11–15 animals/group. G and H, swimming speed. There were no significant differences in either the daily swimming speeds (G) or the average swimming speeds (H) between groups over the 7 days of testing. p > 0.05 using two-way repeated measures ANOVA. n = 11–15 animals/group. I, representative swimming paths during the probe trial (day 8). Three independent experiments were performed in duplicate. Error bars, S.D.
The mechanisms of bilirubin neurotoxicity may involve neuronal excitotoxicity, immunostimulation, intracellular calcium overload, mitochondrial energy failure, the release of excessive NO, impaired long term synaptic plasticity, etc., to trigger downstream events, such as activation of apoptotic pathways and neuroinflammation (5, 26, 30–32). It is believed that apoptosis is much more important in neonatal than in adult brain injury (33). Abundant evidence suggests that apoptosis plays a fundamental role during the progression of kernicterus (1, 4, 5). In this study, TUNEL staining was performed to observe cell apoptosis in the hippocampus and cerebral cortex, which were chosen as the representative regions affected by bilirubin. The results showed that the TAT-NBD treatment obviously reduced cell apoptosis in the hippocampus and the cerebral cortex at 24 h after model establishment. The hippocampus was believed to play a significant role in learning and memory (34, 35), which may explain why the TAT-NBD-treated rats performed better in the Morris water maze test. Furthermore, this study demonstrated that bilirubin induced apoptosis via the mitochondrial pathway, consistent with other in vitro studies (1, 36–38). The Western blot results showed that TAT-NBD prevented cytochrome c release from the mitochondria to the cytoplasm; meanwhile, expression of the mitochondrial Bax protein was down-regulated, whereas the mitochondrial Bcl-2 protein was up-regulated in the TAT-NBD-treated rats at 24 h after model establishment. The ratio of the proapoptotic (Bax)/antiapoptotic (Bcl-2) protein is a crucial factor that determines whether cells undergo apoptosis (39). Therefore, the results suggested that the protective role of TAT-NBD in the rat kernicterus model may be mediated by interfering with the mitochondrial apoptotic pathway. There is growing evidence that inflammation plays a detrimental role in nervous system diseases, given that the secretion of the cytokines, such as TNF-α and IL-1β, may be dysregulated (40). In addition, pro-inflammatory cytokines could further promote apoptosis. For example, there are studies certifying that TNF-α or IL-1β activates the apoptosis pathway (10, 26, 27). The current study demonstrated that UCB might up-regulate or directly interact with the cell surface TNF receptor 1 and IL-1 receptor 1 as a ligand, similar to TNF-α and IL-1β. Intriguingly, this could induce NF-κB activation and subsequently result in cell death and cytokine release; in turn, the increased secretion of TNF-α and IL-1β will again bind to TNF receptor 1 and IL-1 receptor 1, respectively, thus forming a vicious pathogenic cycle created by inflammation, which would exacerbate the inflammation and cell death (10, 27, 41). Therefore, many researchers have attempted to inhibit cytokine production as a therapeutic target for preventing bilirubin-mediated neurotoxicity. For example, numerous studies have proven that anti-inflammatory treatments, such as minocycline, could prevent UCB-induced neurological dysfunction both in vitro and in vivo (42, 43). However, it may cause permanent adverse effects on the developing bone and dentition of newborns (1). In addition, its exact cellular target remains unclear. Previous studies demonstrated that blockade of TNF-α or IL-1β prevented UCB-induced cell death in vitro by inhibiting membrane permeabilization or apoptosis (10, 26, 27). In this study, the ELISA results suggested that production of the pro-inflammatory cytokines TNF-α and IL-1β in the brain were inhibited by TAT-NBD, the specific inhibitor of NF-κB activation. According to the above results, it is proposed that the neuroprotective effect of TAT-NBD-mediated NF-κB inhibition in the rats with kernicterus was associated with alleviating the pro-inflammatory cytokine production, which might partially explain why apoptosis was attenuated in the TAT-NBD-treated rats.

In summary, this study certifies that NF-κB is activated in the cerebrum of rats with kernicterus. More importantly, we show here, for the first time, that the inhibition of the NF-κB pathway by intraperitoneal administration of TAT-NBD has strong neuroprotective effects in the rat kernicterus model, which might be mediated by preventing apoptosis and alleviating inflammation in the brain. Therefore, inhibition of the NF-κB pathway may be a new therapeutic target for kernicterus prophylaxis. However, further studies are needed to provide solid evidence for the clinical application of TAT-NBD, including drug safety and the therapeutic window.

Author Contributions—Z. H., M. L., and S. S. designed the study and wrote the paper. M. L. was responsible for establishment of the kernicterus rat model. S. S. synthesized the peptides described. J. F. and S. L. observed and recorded the general conditions of the model rats and performed behavioral experiments. M. L. and S. S. analyzed the experiments shown in Figs. 2–6. M. L., S. S., and S. L. performed and analyzed the experiments shown in Figs. 1 and 3–5. All authors reviewed the results and approved the final version of the paper.

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