Strong Neuroprotection by Inhibition of NF-κB After Neonatal Hypoxia-Ischemia Involves Apoptotic Mechanisms but Is Independent of Cytokines

Cora H.A. Nijboer, MSc; Cobi J. Heijnen, PhD; Floris Groenendaal, MD, PhD; Michael J. May, PhD; Frank van Bel, MD, PhD; Annemieke Kavelaars, PhD

Background and Purpose—Interactions between excitotoxic, inflammatory, and apoptotic pathways determine outcome in hypoxic-ischemic brain damage. The transcription factor NF-κB has been suggested to enhance brain damage via stimulation of cytokine production. There is also evidence that NF-κB activity is required for neuronal survival. We used the NF-κB inhibitor NBD, coupled to TAT to facilitate cerebral uptake, to determine the neuroprotective capacity of NF-κB inhibition in neonatal hypoxia-ischemia (HI) and to identify its contribution to cerebral inflammation and damage.

Methods—Brain damage was induced in neonatal rats by unilateral carotid artery occlusion and hypoxia and analyzed immunohistochemically; NF-κB activity was analyzed by EMSA. We analyzed cytokine mRNA levels and activation of apoptotic pathways by Western blotting. In vitro effects of TAT-NBD were determined in a neuronal cell line.

Results—Inhibition of cerebral NF-κB activity by TAT-NBD had a significant neuroprotective effect; brain damage was reduced by more than 80% with a therapeutic window of at least 6 hours. In contrast to earlier suggestions, the protective effect of TAT-NBD did not involve suppression of early cytokine upregulation after HI. Moreover, NF-κB inhibition prevented HI-induced upregulation and nuclear as well as mitochondrial accumulation of p53, prevented mitochondrial cytochrome-c release and activation of caspase-3. Finally, TAT-NBD could directly increase neuronal survival because TAT-NBD was sufficient to inhibit death in a neuronal cell line. A nonactive mutant peptide did not have any effect.

Conclusions—Inhibition of NF-κB has strong neuroprotective effects that involve downregulation of apoptotic molecules but are independent of inhibition of cytokine production. (Stroke. 2008;39:2129-2137.)

Key Words: nuclear factor–kappa-B ■ inflammation ■ neonatal ■ neuroprotection

Perinatal cerebral hypoxia-ischemia (HI) is a major cause of neonatal morbidity and mortality. The mechanisms underlying HI brain damage are only partially understood and involve excitotoxicity, apoptosis, and inflammation. To date, effective therapeutic strategies to combat HI brain injury are lacking.

Nuclear factor kappa B (NF-κB) is a ubiquitously expressed transcription factor that regulates expression of genes involved in inflammation, cell survival, and apoptosis. In resting cells, NF-κB is sequestered in the cytoplasm by binding to inhibitory IκB proteins typified by IκBα. Signal-induced phosphorylation of IκBα by a high molecular-weight complex of proteins named the IκB-kinase (IKK)-complex is a key step in NF-κB activation. The IKK complex consists of 2 kinases, IKKα and IKKβ, and the regulatory protein NEMO (NF-κB essential modulator). Phosphorylated IκBα becomes ubiquitinated and is proteasome-degraded after which free NF-κB enters the nucleus to regulate transcription.

NF-κB activation has been described in various in vivo and in vitro models of brain injury (reviewed in ), but its role in cerebral damage is complex as it functions in both protective and damaging pathways. In neurons, NF-κB supports survival by increasing the expression of antioxidants, growth factors, and antiapoptotic molecules. However, NF-κB also upregulates expression of proapoptotic factors such as p53. Reciprocal interaction between proapoptotic activity of p53 and antiapoptotic signals provided by NF-κB further complicates the prediction of the effect of NF-κB activity on brain damage. Finally, glial NF-κB activation induces production of proinflammatory cytokines, a pathway proposed to promote neuronal death in vivo.

Previous studies investigating the role of NF-κB in cerebral damage have used either nonselective pharmacological NF-κB inhibitors or mice harboring targeted deletions of elements of the IKK/NF-κB pathway (eg, ). The results of the latter studies are conflicting, and the precise effects of...
selectively targeting the NF-κB pathway on HI brain injury remains to be determined. In our previous study of neonatal cerebral HI using 12-day-old rats, we explored the effect of inhibiting IKK/NF-κB by peripheral administration of a peptide inhibitor of the IKK complex, the NEMO Binding Domain (NBD)-peptide at 0, 6, and 12 hours after HI. This treatment schedule did not reduce HI-induced brain damage.

The aim of the present study was to further explore the possible neuroprotective effect of IKK/NF-κB inhibition. In addition, we analyzed the contribution of IKK/NF-κB activity to cytokine production and regulation of apoptotic cell death to delineate the role of NF-κB in the pathophysiology of HI brain damage. To inhibit IKK/NF-κB, we used a modified NBD peptide, coupled to the protein transduction sequence of HIV-TAT (TAT-NBD), to facilitate cerebral uptake so that it can be administered intraperitoneally.

**Materials and Methods**

**Animals**

The local animal committee approved all experiments. Timed-pregnant Wistar rats (Charles River, Sulzfeld, Germany) delivered at the Utrecht Central Animal Laboratory. In postnatal day 7 (P7) pups the right common carotid artery was occluded under anesthesia (isoflurane, 5% induction, 1.5% maintenance in O2:N2O; 1:1). Pups recovered for 1 to 3 hours, followed by 120 minutes O2 in N2. Sham controls underwent anesthesia and incision only. All analyses were performed in a blinded set-up.

**Histology**

Coronal paraffin sections (8 μm) were cut at 3.30 mm from bregma and stained with hematoxylin-eosin. Both hemispheres were outlined on full section images and the ratio of ipsi- and contralateral areas was calculated.

**Neuronal Cultures**

SK-N-MC human neuronal cells (ATCC; Manassas, Va.) were plated in 96-well plates in DMEM/F12 (1:1) medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100U/mL penicillin, 100 μg/mL streptomycin, 0.5 μg/mL Fungizone, and 0.1 mmol/L nonessential amino acids (Gibco) for 48 hours before 24-hour incubation with 1 mmol/L NMDA (Sigma-Aldrich) and different concentrations of TAT-NBD or TAT-NBDmut peptide (10 to 100 μmol/L) for 6 hours. 0.5 mg/mL MTT (Sigma-Aldrich) was added for 4 hours. MTT crystals were dissolved in DMSO and absorbance was measured at 550 nm.

**Data Analysis**

Statistical Analysis

Data are presented as mean ± SEM and were analyzed by 1-way ANOVA with Bonferroni post tests.

**Results**

**Neuroprotection by Intraperitoneal TAT-NBD**

HI brain damage was induced in P7 rat pups by unilateral occlusion of the carotid artery and 120 minutes hypoxia. This procedure resulted in severe brain damage with 79.3 ± 1.6% MAP2 loss at 48 hours after HI and a 66.6 ± 4.8% reduction in ipsilateral hemisphere size at 6 weeks after the insult (Figure 1A and 1B) without detectable damage in the contralateral hemisphere. I.p. administration of TAT-NBD at 0 and 3 hours after HI had a significant neuroprotective effect; MAP2 loss at 6 weeks after HI when lesion size was reduced by 79.3 ± 1.6% and 34.6 ± 12.1% in TAT-NBD-treated animals (P<0.01; Figure 1C). TAT-NBD treatment did not simply delay damage as the effect of treatment was even more pronounced at 6 weeks after HI when lesion size was reduced by >80% (P<0.001; Figure 1D). In fact, the size of the ipsilateral hemisphere of TAT-NBD-treated animals did not differ significantly from that of sham-control animals (Figure 1D). White matter loss as determined at 6 weeks after HI was also reduced by more than 80% (Figure 1E and 1F). The protec-
tive effect was specific for the NBD sequence because treatment with a mutated NBD peptide (TAT-NBDmut), which does not inhibit IKK,18 did not have any effect on cerebral damage (Figure 1C, 1D, and 1F).

**Therapeutic Window**

To determine the therapeutic window for the neuroprotective effect of TAT-NBD, we administered TAT-NBD i.p. as a single injection at various time points after HI. Significant neuroprotection was obtained with only a single i.p. administration of TAT-NBD immediately (0 hours) or 3 hours after the insult (Figure 1G). Importantly, the therapeutic window of peripheral administration of TAT-NBD was at least 6 hours. MAP2 loss was still significantly reduced by administration of TAT-NBD at 6 hours after HI (P<0.05; Figure 1G). Treatment at only 9 or 12 hours after HI did not have a significant neuroprotective effect (Figure 1G).

**Intraperitoneal TAT-NBD Treatment Prevented Cerebral IKK/NF-κB Activation**

Next we analyzed the effect of TAT-NBD on NF-κB activity in cerebral extracts by EMSA. I.p. administration of TAT-NBD directly after HI completely prevented the HI-induced increase in NF-κB activity at 3 hours after HI (P<0.001 versus vehicle). TAT-NBDmut did not inhibit NF-κB (Figure 2A).

NBD acts as an inhibitor of the IKK complex, thereby preventing phosphorylation of IκBα and subsequent degradation of this molecule.18 In line with this mechanism of action, treatment with TAT-NBD completely prevented the HI-induced decrease in cerebral IκBα at 3 hours after HI (Figure 2B).

The data in Figure 2C demonstrate that HI induces nuclear translocation of NF-κB in neurons and that nuclear translo-
ication of NF-κB was completely blocked by i.p. treatment with TAT-NBD and not by treatment with TAT-NBDmut.

**TAT-NBD Distributes to the Brain After i.p. Administration**

To determine whether inhibition of cerebral NF-κB activity after i.p. TAT-NBD was associated with distribution of the peptide to the brain, we administered biotin-TAT-NBD i.p. at 0 and 3 hours after HI. At 1 hour after HI, clear biotin staining throughout the brain parenchyma was observed (Figure 3A and 3B). At 3 hours after HI, more intense biotin staining was detected throughout the entire brain and appeared to be intracellularly located (Figure 3C and 3D). At 12 hours after HI, we no longer detected biotin-TAT-NBD in the brain (Figure 3E and 3F). The staining was specific because no staining was detected in brains of animals that received unlabeled TAT-NBD (Figure 3G and 3H).

**Effect of TAT-NBD on Cytokines**

Inhibition of NF-κB activity may contribute to prevention of brain damage via inhibition of the cytokine response. A previous study in this model of HI brain damage suggested that increases in cytokine mRNA do not occur until 12 hours after HI. However, we already observed a HI-induced increase in the mRNA encoding the proinflammatory cytokines TNF-α and IL-1β at 3 hours after HI (Figure 4A and 4B) and the antinflammatory cytokines IL-4 and IL-10 (Figure 4C and 4D). We could not detect changes in IL-1RA expression at this time point (Figure 4E). Interestingly, TAT-NBD treatment did not induce any changes in the HI-induced increase in cytokine expression, despite complete inhibition of NF-κB activity at this time point (Figures 4A through 4D and 2A).

**Effect of TAT-NBD on Proapoptotic Molecules**

Kinetic analysis of the HI-induced increase in cytosolic cytochrome-c and cleaved (active) caspase-3 revealed that both were significantly increased starting at 6 hours after HI and further increased at 12 and 24 hours after HI (figure 5B and 5C). TAT-NBD treatment significantly prevented the HI-induced increase in cytosolic cytochrome-c and the increase in active caspase-3 determined at 24 hours after HI (Figure 5D and 5E; \(P<0.01\) versus vehicle-treated).

Based on in vitro studies, it has been suggested that changes in expression and cellular localization of the tumor suppressor p53 contribute to initiation of apoptosis. Mitochondrial association of p53 was increased at 30 minutes after the insult and remained elevated up to 6 hours after HI (Figure 5F). TAT-NBD treatment almost completely prevented the HI-induced increase in mitochondrial association of p53 at 3 hours after HI, and TAT-NBDmut did not have any effect (Figure 5G; \(P<0.01\) versus vehicle-treated). In addition, TAT-NBD treatment completely prevented the HI-induced increase in the levels of nuclear (\(P<0.01\) versus vehicle-treated) and total cytosolic (\(P<0.05\) versus vehicle-treated) p53 as determined at 3 hours after HI (Figure 5H and 5I).

**TAT-NBD Protects Neuronal Cells In Vitro**

To determine whether inhibition of NF-κB by TAT-NBD can have direct protective effects on neuronal cells, we treated SK-N-MC cells with NMDA, hydrogen peroxide (H2O2), etoposide, or staurosporine in the presence or absence of TAT-NBD or TAT-NBDmut. TAT-NBD but not TAT-NBDmut dose-dependently inhibited neuronal death in vitro in response to all stimuli used (Figure 6A through 6D; \(P<0.01\) versus stimulated cells without TAT-NBD).

---

**Figure 2.** Intraperitoneal TAT-NBD treatment inhibits HI-induced cerebral NF-κB activation. A, NF-κB activity was determined by EMSA on nuclear extracts obtained at 3 hours after HI. Inset, Representative example of n=7 animals per group. NBD indicates TAT-NBD treated; VEH, vehicle-treated, mut, TAT-NBDmut-treated. **P<0.001 vs ipsilateral levels of vehicle- or TAT-NBDmut-treated animals. B, TAT-NBD treatment prevented ipsilateral degradation of IκBα; Western blot analysis at 3 hours after HI. n=7. **P<0.01 vs vehicle- or TAT-NBDmut-treated littermates. C, HI-induced nuclear translocation of NF-κB (p65 subunit; red) in neuronal cells (NeuN positive [neuronal nuclei], green) in the hippocampus at 3 hours after HI in vehicle- and TAT-NBDmut-treated animals. Scale bar=10 μm.
features the addition of TAT-NBD or TAT-NBDmut alone had no effect on neuronal survival (data not shown).

**Discussion**

Here we demonstrated for the first time that inhibition of cerebral activation of the IKK/NF-κB pathway by i.p. administration of TAT-NBD early after the insult reduced HI-induced neuronal and white matter damage by more than 80%. Thus, inhibition of the formation of a functional IKK complex, which is a specific and critical step in NF-κB activation, might represent one of the most effective treatments for neonatal HI brain damage to date.

By using biotinylated TAT-NBD we demonstrated that after i.p. administration, the peptide was present in the brain at 1 hour after HI and was no longer detectable at 12 hours, indicating that in vivo delivery of TAT-NBD to the brain was rapid and transient. Comparable kinetics of cerebral delivery of TAT-fused peptides after i.p. administration have been described by Yin et al.\(^{21}\) and Cai et al.\(^{25}\) We conclude that intraperitoneally administered TAT-NBD inhibited the IKK/NF-κB pathway in the brain after HI as we did no longer observe the HI-induced nuclear translocation of neuronal NF-κB (p65), the activation of cerebral NF-κB as determined by EMSA, or the decrease in cytosolic IkB. It should be noted that by using TAT-NBD only HI-induced activation of the IKK/NF-κB pathway was inhibited without inhibiting basal NF-κB activity, which might play a positive role in cell survival\(^{6}\) (Figure 2A; sham versus TAT-NBD).

In our previous study using NBD\(^{19}\) and not TAT-NBD, we did not observe neuroprotection by treatment with NBD at 0, 6, and 12 hours after HI. The most important difference with the present study is that we now administered the peptide at 0 and 3 hours after HI, suggesting that 0-, 6-, and 12-hour treatment may lead to adverse (aspecific) effects. In addition, we have preliminary evidence suggesting that prolonged (0, 6, and 12 hours) treatment with TAT-NBD may not be protective, because it may interfere with the HI-induced upregulation of antiapoptotic molecules like Bcl-2 at later time points.

Our data provide new insights into the pathophysiologic mechanisms involved in neonatal HI brain damage. First of all, it is commonly accepted that inhibition of proinflammatory cytokine production mediates the protective effect of inhibition of cerebral NF-κB activity in models of brain damage.\(^{10}\) Our data, however, do not support this concept as TAT-NBD treatment did not inhibit pro- (IL-1β and TNF-α)

---

**Figure 3. Cerebral distribution of TAT-NBD after i.p. administration.** Biotin-labeled TAT-NBD was administered i.p. at 0/3 hours after HI, and sections were stained for biotin. Biotin-TAT-NBD (small black precipitates) was detectable in the brain parenchyma at 1 hour after HI (A and B) and was more pronounced at 3 hours after HI (C and D). Biotin-TAT-NBD was no longer detectable at 12 hours after HI (E and F). No staining in brains of control animals that received unlabeled TAT-NBD (G and H). Counterstaining with hematoxylin. Scale bar (A)=25 μm. Same magnification for all photographs. hc indicates hippocampus; cx, parietal cortex.

<table>
<thead>
<tr>
<th>1h</th>
<th>3h</th>
<th>12h</th>
<th>unlab NBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>E</td>
<td>F</td>
<td>G</td>
<td>H</td>
</tr>
</tbody>
</table>
and antiinflammatory (IL-4, and IL-10) cytokine expression early after HI, although NF-κB activation was clearly inhibited (Figure 2A). These data suggest that HI-induced early production of pro- and antiinflammatory cytokines is not wholly dependent on cerebral NF-κB activity and suggest that HI-induced cytokine production in the brain can be maintained by other transcription factors, eg, AP-1. Moreover, despite the HI-induced upregulation of cytokines, we observed very strong neuroprotection, suggesting that early cytokine production is not detrimental in the context of cerebral NF-κB inhibition. It should be noted, however, that inhibition of cytokine production (eg, deletion of IL-18) or activity (eg, IL-1RA treatment) can at least partially protect against brain damage.26,27

Second, we show here for the first time the important role of HI-induced NF-κB activity in expression and localization of p53 in the neonatal brain in vivo. In vitro studies have provided evidence that increased mitochondrial p53 association occurs in conjunction with a rapid first episode of cell death independently of p53 target gene activation.28,29 Here we show that in vivo increased mitochondrial association of p53 occurred early after HI (0.5 to 6 hours). TAT-NBD treatment almost completely prevented mitochondrial translocation of p53. At the mitochondria, p53 is thought to inhibit the activity of antiapoptotic Bcl-2 members and to activate Bax leading to cytochrome-c release and apoptosis.30,31 In line with this model, we also show that HI-induced cytosolic translocation of cytochrome-c as well as the increase in active caspase-3 occur after the increase in mitochondrial p53 and are inhibited by TAT-NBD treatment. Finally, we demonstrated that the HI-induced increase in total p53 was prevented by TAT-NBD. Collectively, our data suggest that TAT-NBD treatment protects against neuronal death via preventing p53 upregulation and mitochondrial and nuclear localization, thereby inhibiting mitochondrial damage and preventing upregulation of proapoptotic p53 target genes, ultimately leading to reduced apoptosis-like cell death. Our in vitro study using a neuronal cell line further confirmed the hypothesis that inhibition of neuronal NF-κB activity can suffice to prevent neuronal death. However, we cannot exclude the possibility that other NF-κB–dependent pathways are ultimately responsible for the effect of TAT-NBD treatment on neuronal survival.

In exploring clinical applicability of specific NF-κB inhibitors like NBD to prevent cerebral damage, one should keep in mind that apoptosis-like cell death is known to play a more pronounced role in neonatal than in adult cerebral cell death.10,32,33 It might therefore be that the immature brain can
benefit more from the protective effect of NF-κB inhibition than the adult brain. However, the neuroprotective effects observed by Herrmann et al.\textsuperscript{13} in adult IKK\textsuperscript{−/−} mice or after intracerebral administration of the IKK inhibitor BMS-34541 after MCAO suggest that the adult brain will also profit from NF-κB inhibition after cerebral HI.

In conclusion, we demonstrated that inhibition of the cerebral IKK/NF-κB pathway by i.p. administration of TAT-NBD has significant neuroprotective effects in a model of severe HI-induced neonatal brain injury. The therapeutic window for neuroprotective effects was at least 6 hours, and the neuroprotective effect was associated with inhibition of...
apoptosis-like neuronal death but did not involve abrogation of early cytokine expression. This finding sheds a novel light on the role of cytokines in cerebral damage and suggests that at least the early NF-κB–independent production of cytokines is not detrimental. The possibility that early cytokine production may contribute to neuroprotection or repair, eg, via TNF-R2 signaling, will be focus of future research.

Acknowledgments
We thank S. van Neerven, H. Willemen, and J. Zijlstra for excellent technical assistance.

Sources of Funding
This study was funded in part by the Wilhelmina Children’s Hospital Research Fund.

Disclosures
None.

References

Figure 6. TAT-NBD protects neuronal cells against apoptosis in vitro. The human neuronal cell line (SK-N-MC) was exposed to 1 mmol/L NMDA (A), 100 μmol/L H2O2 (B), 250 nmol/L etoposide (C), or 100 nmol/L staurosporine (D) for 24 hours, and cell survival was determined using MTT. Addition of TAT-NBD for 6 hours dose-dependently prevented neuronal death. **P<0.01 vs control without TAT-NBD. TAT-NBD or TAT-NBDm treatment as such had no effect on neuronal survival. Data are from 3 independent experiments performed in quadruplicate. mut indicates 100 μmol/L TAT-NBDm.


Strong Neuroprotection by Inhibition of NF-κB After Neonatal Hypoxia-Ischemia Involves Apoptotic Mechanisms but Is Independent of Cytokines
Cora H.A. Nijboer, Cobi J. Heijnen, Floris Groenendaal, Michael J. May, Frank van Bel and Annemieke Kavelaars

Stroke. 2008;39:2129-2137; originally published online April 17, 2008;
doi: 10.1161/STROKEAHA.107.504175
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/39/7/2129

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/