Alternate Pathways Preserve Tumor Necrosis Factor-α Production After Nuclear Factor-κB Inhibition in Neonatal Cerebral Hypoxia–Ischemia

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Background and Purpose—Nuclear factor-κB (NF-κB) is an important regulator of inflammation and apoptosis. We showed previously that NF-κB inhibition by intraperitoneal TAT-NBD treatment strongly reduced neonatal hypoxic–ischemic (HI) brain damage. Neuroprotection by TAT-NBD was not associated with inhibition of cerebral cytokine production. We investigated how tumor necrosis factor-α (TNF-α) production is maintained after NF-κB inhibition and whether TNF-α contributes to brain damage.

Methods—Postnatal Day 7 rats were subjected to unilateral carotid artery occlusion and hypoxia. Rats were treated immediately after HI with TAT-NBD, the JNK inhibitor TAT-JBD, and/or the TNF-α inhibitor etanercept. We determined brain damage, NF-κB and AP-1 activity, Gadd45β, XIAP, (P-)TAK1, TNF-α, and TNF receptor expression.

Results—Our data confirm that TAT-NBD treatment reduces brain damage without inhibiting TNF-α production. We now show that TAT-NBD treatment increased HI-induced AP-1 activation concomitantly with reduced Gadd45β, XIAP, and increased (P-)TAK1 expression. Combined inhibition of NF-κB and JNK/AP-1 abrogated HI-induced TNF-α production. However, this treatment reduced the neuroprotective effect of NF-κB inhibition alone. We show that etanercept was detectable in the HI brain after intraperitoneal administration and that etanercept treatment also reduced the neuroprotective effect of NF-κB inhibition. Finally, NF-κB inhibition decreased HI-induced upregulation of TNF-R1 and increased TNF-R2 expression.

Conclusions—When NF-κB was inhibited after neonatal cerebral HI, JNK/AP-1 activity was increased and required for increased TNF-α expression. Our data indicate that the switch to JNK/AP-1 activation preserves HI-induced TNF-α expression and thereby might contribute to the neuroprotective effect of TAT-NBD possibly through a TNF-R2 dependent mechanism. (Stroke. 2009;40:3362-3368.)

Key Words: JNK ■ neonatal ischemia ■ neuroprotection ■ NF-κB ■ TNF-α

In the human newborn, hypoxic–ischemic (HI) brain damage remains a major cause of mortality and morbidity. Insight into the interplay among excitotoxic, inflammatory, and apoptotic pathways will increase our understanding of the mechanisms of neonatal HI brain injury and is required for development of intervention strategies.1

Transcription factors activated in the brain during and after HI modulate expression of key molecules involved in the complex cerebral response to injury.2 One important transcription factor is nuclear factor-κB (NF-κB), which regulates many inflammatory target genes and is also acknowledged for its role in regulating cell death and survival.3,4

In previous studies, we investigated the contribution of NF-κB to HI brain injury in neonatal postnatal Day 7 rat pups by administration of TAT-NBD, an established NF-κB inhibitor that rapidly distributed to the brain after intraperitoneal treatment.5,6 NF-κB inhibition by TAT-NBD markedly reduced brain injury as determined at 48 hours or at 6 weeks after the insult.5 TAT-NBD treatment attenuated upregulation and mitochondrial association of the NF-κB target p53 and increased upregulation of antiapoptotic Bcl family members.5,6 Remarkably, however, HI-induced pro- and anti-inflammatory cytokine production was not affected by NF-κB inhibition.5

We address the question how HI-induced cerebral cytokine production is regulated when NF-κB is inhibited. We also investigated the contribution of the JNK/AP-1 pathway and tumor necrosis factor-α (TNF-α) to the neuroprotective effect of NF-κB inhibition.

Materials and Methods

Animals
The animal committee of Academic Biomedical Center Utrecht approved all experiments. At postnatal Day 7, Wistar rats (Charles River, Sulzfeld, Germany) underwent occlusion of the right common
carotid artery under isoflurane anesthesia following 120 minutes hypoxia (8% O2). Sham controls underwent anesthesia and incision only. Analyses were performed in a blinded setup.

TAT-NBD (20 mg/kg*), TAT-JBD (10 mg/kg); L-JNK1: YGRKKRRQRRR-PP-RPKRPTTLNLFPQVPRSQDT) or control peptide TAT-NBDmut (20 mg/kg; W. M. Keck facility, Yale University, New Haven, Conn) were administered intraperitoneally 0 and 3 hours after HI. 8, 9 JBD is the JNK binding domain of JNK-interacting protein-1 and acts as a specific JNK inhibitor. 7

The TNF-α inhibitor etanercept (5 mg/kg; Wyeth Pharmaceuticals Inc, Philadelphia, Pa) was administered intraperitoneally directly after HI.

Rats were euthanized by an overdose of pentobarbital and decapitated or perfused with 4% paraformaldehyde in phosphate-buffered saline.

**Histology**

Coronal paraffin sections (8 μm) were incubated with mouse-anti-MAP2 (Sigma-Aldrich, Steinheim, Germany) and biotin–horseradish peroxidase–anti-mouse antibody and revealed using Vectastain ABC kit (Vector-Labs, Burlingame, Calif) and diaminobenzamidine. MAP2 staining was outlined on full section images and the ratio of ipsi- and contralateral areas was calculated. 8

To detect etanercept, we used goat–anti-human IgG (Jackson Immuno-research, Westgrove, Pa) or goat–anti-human IgA (Molecular Probes [Invitrogen, Eugene, Ore] followed by biotin–horseradish peroxidase antibody.

**Western Blotting and Electromobility Shift Assays**

Nuclear, cytosolic and membrane fractions were prepared as described. 8, 9 Proteins were separated by SDS-PAGE, transferred to Hybond-C membranes (Amersham, Buckinghamshire, UK), and revealed using rabbit–anti-XIAP, rabbit–anti-TAK1, rabbit–anti-phospho-TAK1 antibodies (Cell Signaling, Danvers, Mass), goat–anti-Gadd45β, rabbit–anti-TNF-R1, rabbit–anti-TNF-R2, or goat–anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, Calif); donkey–anti-rabbit–peroxidase (Amersham); or donkey–anti-goat–peroxidase (Santa Cruz) followed by enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, UK), and Hybond-C membranes (Amersham, Buckinghamshire, UK) were incubated with mouse-anti-MAP2, rabbit–anti-TAK1, rabbit–anti-phospho-TAK1 antibodies (Cell Signaling, Danvers, Mass), goat–anti-Gadd45β, rabbit–anti-TNF-R1, rabbit–anti-TNF-R2, or goat–anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, Calif); donkey–anti-rabbit–peroxidase (Amersham); or donkey–anti-goat–peroxidase (Santa Cruz) followed by enhanced chemiluminescence (Amersham) and analyzed with a GS-700 Imaging Densitometer (Bio-Rad, Hercules, Calif).

Electromobility shift assays on nuclear brain extracts with 32P-labeled NF-κB probe* or AP-1 probe (Promega, Madison, Wis; sequence 5’-CGCCTTGTAGATGTCAGCGGGA-3’) were performed as described. 8

**Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction**

Total RNA was isolated with TRIzol (Invitrogen, Paisley, UK). cDNA was synthesized with SuperScript Reverse Transcriptase (Invitrogen). The polymerase chain reaction was performed with the iQ5 Real-Time PCR Detection System (Bio-Rad) using primers for TNF-α, interleukin (IL)-1β, IL-10, and IL-4; 10 TNF-R1: forward:GT-TGCCTTCGTTATCTCTC; reverse: GCTTAGTAAACTCTCCT-TCAC; TNF-R2: forward: TCAAGCAGACCCCAAAG; reverse: CATCAGCAGACCCAGAGT. Data were individually normalized to the mean of the relative expression of β-actin and GAPDH.

**Enzyme-Linked Immunosorosben Assay**

TNF-α levels in cytosolic brain fractions were analyzed by enzyme-linked immunosorosben assay (Ucytech, Utrecht, The Netherlands).

**Statistical Analysis**

Data were normally distributed, presented as mean±SEM and analyzed by one- or 2-way analysis of variance with Bonferroni posttests.

**Results**

**JNK/AP-1 Activation When NF-κB Is Inhibited**

Intraperitoneal TAT-NBD treatment after HI completely blocked HI-induced NF-κB activation determined at 3 hours post-HI (Figure 1A). HI also induced activation of the transcription factor AP-1 in the ipsilateral hemisphere at 3 hours post-HI. Importantly, when NF-κB activation was inhibited by TAT-NBD treatment, AP-1 activity was significantly higher (Figure 1B).

The NF-κB pathway can regulate JNK/AP-1 activity through multiple pathways (Figure 1C). For example, the NF-κB target genes Gadd45β and XIAP can inhibit activation of the JNK/AP-1 pathway by inhibiting MKK7 or inducing degradation of TAK1, respectively. 9, 10–11 At 3 hours post-HI, Gadd45β levels were upregulated and this was prevented by TAT-NBD treatment (Figure 1D). Ipsilateral XIAP levels were further reduced by TAT-NBD treatment after HI as compared with vehicle (Figure 1E). In addition, TAT-NBD treatment increased both total TAK1 and P-TAK1 compared with vehicle (Figure 1F–G). Treatment with the inactive NBDmut peptide as a control had no effect.

**HI-Induced Cytokine Production After Combined Inhibition of NF-κB and JNK/AP-1**

To determine the contribution of NF-κB and JNK/AP-1 to cytokine production after HI, pups were treated intraperitoneally with TAT-NBD, TAT-JBD, or both. Treatment with TAT-JBD or TAT-NBD+TAT-JBD significantly inhibited AP-1 activation at 3 hours post-HI (Figure 2A). TAT-NBD treatment had no effect on NF-κB activation at 3 hours post-HI (Figure 2B).

HI induced an increase in cerebral TNF-α mRNA starting at 3 hours and peaking at 6 hours post-HI (Figure 3A). TNF-α protein in the brain was increased from 3 hours and further increased until the last time point tested (24 hours post-HI; Figure 3B).

HI-induced TNF-α mRNA at 3 hours and protein at 6 hours post-HI were not inhibited by TAT-NBD treatment (Figure 3C–D). 5 TAT-JBD treatment slightly, but not statistically significantly, decreased TNF-α mRNA without affecting TNF-α protein (Figure 3C–D). Importantly, however, inhibition of both NF-κB and JNK/AP-1 by treatment with TAT-NBD+TAT-JBD completely prevented the HI-induced increase in TNF-α mRNA and protein (Figure 3C–D). Similar results were obtained for IL-1β, IL-4, and IL-10 (Supplemental Figure, available online at http://stroke.ahajournals.org).

**Role of JNK/AP-1 and TNF-α in HI Brain Injury**

Figure 4A confirms our earlier data that NF-κB inhibition by TAT-NBD treatment after HI has a strong neuroprotective effect. 5 Inhibition of the JNK/AP-1 pathway by TAT-JBD also significantly reduced ipsilateral MAP2 loss, although to a lesser extent than TAT-NBD. However, the protective effect of combined TAT-NBD and TAT-JBD treatment was significantly smaller than the effect of TAT-NBD alone (Figure 4A–B).

Collectively, our data indicate that maintaining HI-induced cytokines when NF-κB is inhibited may contribute to neuroprotection. To specifically address the role of TNF-α, pups were treated with the TNF-α inhibitor etanercept alone or in combination with TAT-NBD directly after HI. When TAT-NBD treatment was combined with etanercept, the strong neuroprotective effect of TAT-NBD treatment alone was...
diminished approximately 32% ($P < 0.05$; Figure 4C–D). Notably, in vehicle-treated animals, etanercept had a significant neuroprotective effect (Figure 4C–D).

To detect whether etanercept (a fusion protein of TNF-R2 and human IgG1) reached the brain after intraperitoneal administration, we stained rat brains for the presence of human IgG at 48 hours post-HI (Figure 5). Brains from vehicle- and etanercept-treated HI animals stained positively for human IgG throughout the brain. In contrast, SHAM controls treated with etanercept were negative, indicating that HI facilitated distribution of etanercept to the brain. All controls were negative (Figure 5).

**NF-κB Inhibition Differentially Regulates Expression of TNF-R1 and 2**

Accumulating evidence suggests that TNF-α signaling through TNF-R1 promotes cell death, whereas TNF-R2 signaling is associated with protective mechanisms.12,13 HI induced an increase in both TNF-R1 and TNF-R2 mRNA and protein expression in the ipsilateral hemisphere (Figure 6). TAT-NBD treatment reduced TNF-R1 mRNA and tended to decrease TNF-R1 protein, whereas TNF-R2 mRNA and protein were increased compared to vehicle treatment (Figure 6). TAT-JBD did not influence HI-induced changes in both receptors. Combined treatment with TAT-NBD and TAT-JBD completely abolished the HI-induced effect on TNF-R1 and TNF-R2 expression. TAT-NBDmut treatment had no effect (data not shown).

**Discussion**

Our recent studies in a neonatal rat model of HI brain damage showed that the strong neuroprotection after NF-κB inhibition with the TAT-NBD peptide was not associated with
inhibition of pro- or anti-inflammatory cytokine production. This is in contrast to the general view that NF-κB inhibition contributes to neuroprotection by preventing pro-inflammatory cytokine production.14

In the present study, we demonstrate that inhibition of NF-κB by TAT-NBD increased activation of the JNK/AP-1 pathway. Moreover, combined JNK/AP-1 and NF-κB inhibition prevented HI-induced TNF-α production, indicating that switching to the JNK/AP-1 pathway contributes to maintaining HI-induced TNF-α expression when NF-κB is inhibited. Moreover, our data indicate that JNK/AP-1 activity and TNF-α production contribute to the neuroprotective effects of NF-κB inhibition after HI.

Data from in vitro studies using embryonic fibroblasts or Ewing sarcoma cells and in vivo studies using models of liver injury have shown that preventing NF-κB activation induced prolonged and increased JNK activation.9,15–18 In vitro, there are multiple pathways through which JNK/AP-1 activation can be facilitated after NF-κB inhibition (Figure 1C). For example, the NF-κB target genes Gadd45 and XIAP can inhibit the JNK/AP-1 pathway by inhibiting MKK7 or facilitating degradation of TAK1. MKK7 and TAK1 are both upstream activators of JNK.11,15 We show here for the first time that Gadd45 and XIAP may also contribute to regulation of the JNK/AP1 pathway by changes in NF-κB activity in the brain in vivo. When neonatal HI-induced activation of cerebral NF-κB was inhibited by TAT-NBD treatment, up-regulation of Gadd45 and XIAP was prevented, the reduction in XIAP was more pronounced, and TAK1 and P-TAK1 were maintained at high levels. Concomitantly, cerebral JNK/AP-1 activity was increased. It should be noted, however, that other factors, including A20, a regulator of TAK1 activity, and changes in superoxide dismutase and ferritin heavy chain that can modulate formation of reactive oxygen species may also contribute to enhanced JNK/AP-1 activation when NF-κB is inhibited after HI in the neonatal brain.9,10 (Figure 1C). It is

Figure 2. TAT-JBD inhibits cerebral AP-1 activation. Rats were treated directly after HI with TAT-JBD alone or in combination with TAT-NBD. AP-1 (A) or NF-κB (B) activity was determined by electromobility shift assays on brain nuclear extracts of both hemispheres obtained at 3 hours post-HI. **P<0.01 versus vehicle-treated animals. Inset, Representative examples. Sham n=5, other groups n=9.

Figure 3. TNF-α expression. A, Kinetics of HI-induced brain TNF-α mRNA expression in contra- and ipsilateral hemispheres. B, Kinetics of HI-induced ipsilateral TNF-α protein determined by enzyme-linked immunosorbent assay. n=5 animals/time point. **P<0.01, ***P<0.001 versus sham controls. C, TNF-α mRNA at 3 hours post-HI after treatment at 0/3 hours with vehicle, TAT-NBD, TAT-JBD, or TAT-NBD+TAT-JBD. A difference of 0.29 (relative units) could be detected with a power of 80%. n=9 animals/group. Two-way analysis of variance: F(1,32)=9.5; ***P<0.001. D, Ipsilateral TNF-α protein at 6 hours post-HI after the different treatments under C were determined. A difference of 15 pg/mg could be detected with a power of 80%. n=6 animals/group. Two-way analysis of variance: F(1,20)=12.8; ***P<0.001.
also possible that NF-κB and JNK/AP-1 activation occurs in different cell types and that soluble mediators produced under control of NF-κB regulate JNK/AP-1 in other cells.

HI-induced TNF-α mRNA and protein expression were only inhibited when we blocked both NF-κB and AP-1 activation by combining TAT-NBD with TAT-JBD treatment. These data indicate that when NF-κB is inhibited, activity of the JNK/AP-1 pathway is required for preservation of HI-induced TNF-α production. It remains to be determined whether direct interactions between the 2 pathways in the cells producing pro- and anti-inflammatory cytokines are responsible for this effect.

In murine adult stroke models, exogenous administration of TNF-α exacerbates ischemic brain injury, whereas treatment with TNF inhibitors reduces brain damage. In line with these data, we demonstrate here that inhibition of TNF-α by etanercept is neuroprotective after neonatal HI. However, a neuroprotective role for TNF-α in brain injury has also been described. For example, in vitro studies showed that TNF-α may protect neurons against excitotoxic insults and in vivo ischemic brain damage is exacerbated in mice genetically deficient for TNF receptors. Similarly, we show here that etanercept treatment reduced the protective effect of TAT-NBD, indicating that under these conditions, TNF-α contributes to neuroprotection. In addition, abrogation of TNF-α production by simultaneous JNK/AP-1 and NF-κB inhibition was associated with a reduction of the neuroprotective effect of NF-κB inhibition. We suggest that the reduction in neuroprotection when NF-κB is inhibited together with JNK/AP-1 or when NF-κB inhibition is combined with etanercept treatment is due to a protective effect of TNF-α on neurons. A concomitant reduction in production of anti-inflammatory cytokines may have a negative effect on neuronal survival as well and may thereby contribute to the observed effects. In addition, putative effects of TAT-NBD and/or TAT-JBD treatment on pro- and antiapoptotic molecules might also play an additional role in the observed effect on brain damage.

TNF-α signals through TNF receptor 1 (TNF-R1), an important death receptor and TNF receptor 2 (TNF-R2), a receptor that can promote survival. Both receptors are expressed on neurons. Data obtained in a model of retinal ischemia in TNF-R1−/− or TNF-R2−/− mice showed that...
TNF-R1 signaling augmented neuronal death, whereas TNF-R2 promoted neuroprotection. An essential role of TNF-R2 in neuronal survival has also been described in an in vitro model of glutamate-induced excitotoxicity in TNF-R1−/− or TNF-R2−/− primary cortical neurons. Interestingly, we observed that TAT-NBD treatment after HI differentially regulated expression of TNF-R1 and TNF-R2; HI-induced TNF-R1 expression was decreased, whereas TNF-R2 expression was increased after TAT-NBD. We therefore suggest that after NF-κB inhibition, TNF-α might contribute to neuroprotection via enhanced signaling through TNF-R2. In the brain, TNF-R1 and TNF-R2 are also expressed on glia and endothelial cells. Little is known on the functional consequences of TNF-R1 and TNF-R2 activation on these cells. It may well be possible, however, that the protective and damaging effects of TNF-α we describe here are mediated (in part) by indirect effects mediated through glia or endothelial cells.

In conclusion, the present study shows that switching to the use of the JNK/AP-1 pathway after NF-κB inhibition preserves upregulation of cerebral TNF-α production after neonatal HI in vivo. This study also provides novel insights into the role of TNF-α during HI brain injury; when NF-κB is inhibited, TNF-α contributes to neuroprotection possibly through signaling through TNF-R2. To better understand the contribution of inflammation to cerebral injury and for definition of anti-inflammatory therapeutic strategies, it will become important to take the role of specific receptor subtypes into account.

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Disclosures

None.

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