NF-κB signalling in cerebral ischaemia

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Abstract

In acute stroke, neuronal apoptosis and inflammation are considered to be important mechanisms on the road to tissue loss and neurological deficit. Both apoptosis and inflammation depend on gene transcription. We have identified a signalling pathway that regulates transcription of genes involved in apoptosis and inflammation. In a mouse model of focal cerebral ischaemia, there is an induction of the cytokine TWEAK (tumour necrosis factor-like weak inducer of apoptosis) and its membrane receptor Fn14. TWEAK promotes neuronal cell death and activates the transcription factor NF-κB (nuclear factor κB) through the upstream kinase IKK [IκB (inhibitory κB) kinase]. In vivo, IKK is activated in neurons. Neuron-specific deletion of the subunit IκK2 or inhibition of IKK activity reduced the infarct size and neuronal cell loss. A pharmacological inhibitor of IKK also showed neuroprotective properties. IKK-dependent ischaemic brain damage is likely to be mediated by NF-κB, because neuron-specific inhibition of NF-κB through transgenic expression of the NF-κB superrepressor was found to reduce the infarct size. In summary, there is evidence that IKK/NF-κB signalling contributes to ischaemic brain damage and may provide suitable drug targets for the treatment of stroke.

Introduction

Stroke is a frequent clinical disorder. The WHO (World Health Organization) estimates that worldwide 5–6 million people die from strokes every year. Thrombolysis has provided proof of principle that stroke can be treated. However, the practical benefit of thrombolysis is limited because, even in Western countries, only a minority of stroke patients are eligible for this treatment mainly due to the short therapeutic time window of 3 h in which thrombolysis is safe. Therefore new treatments are needed that are still effective when administered with some delay after onset of stroke.

The analysis of gene expression may reveal delayed mechanisms of ischaemic brain damage. Genes that are induced by cerebral ischaemia may be targets of drugs with a prolonged efficacy. This reasoning has led many researchers to profile gene expression after stroke [1–4].

TWEAK [TNF (tumour necrosis factor)-like weak inducer of apoptosis]: a cytokine induced in cerebral ischaemia

By massively parallel signature sequencing, we detected an increase in the cytokine TWEAK at the mRNA level in a mouse model of focal cerebral ischaemia [5]. TWEAK belongs to the TNF family of cytokines. It is an angiogenic factor [6,7]. The name reflects the induction of apoptosis by TWEAK that was observed in tumour cells in vitro [8].

In vivo, TWEAK has been shown to attenuate the transition from innate to adaptive immunity and to stimulate liver regeneration [9,10]. TWEAK acts through Fn14, a member of the TNFR (TNF receptor) superfamily. Interestingly, Fn14 was also induced by cerebral ischaemia [5]. Fn14 was expressed on primary cortical neurons. Recombinant TWEAK induced apoptosis in a moderate number of cortical neurons in vitro. Interestingly, a neutralizing anti-TWEAK antibody reduced the infarct size, demonstrating an in vivo role of TWEAK in ischaemic brain damage [5]. This finding was confirmed by others using a soluble form of Fn14 [11]. In addition to the effect on the infarct size, TWEAK increases the permeability of the blood–brain barrier in cerebral ischaemia [12].

Members of the TNFR superfamily, such as Fas or TNFRI, induce apoptosis through their death domain. However, Fn14 is the smallest member of the TNFR family and does not contain a death domain. How then does Fn14 induce apoptosis? An answer may lie in the binding site for TRAFs (TNFR-associated factors) 1, 2, 3, and 5 in the intracellular domain of Fn14 [13,14]. TRAFs link Fn14 to intracellular signal transduction cascades that stimulate the expression of other pro-apoptotic factors [15]. Indeed, TWEAK stimulates the transcription factor NF-κB (nuclear factor κB) in primary cortical neurons through the IKK [IκB (inhibitory κB) kinase] complex [5].

However, NF-κB is well known to prevent apoptosis in various experimental paradigms. Therefore it seems unlikely that NF-κB activation could underlie the pro-apoptotic function of TWEAK. Nevertheless, there is also clear evidence that NF-κB is able to exert a pro-apoptotic action [16,17]. Inhibition of NF-κB in neurons reduced TWEAK-induced apoptosis, suggesting that NF-κB is, indeed, pro-apoptotic in the context of TWEAK stimulation [5].
NF-κB signalling

In cerebral ischaemia, there is ample evidence for activation of NF-κB [18–20]. Figure 1 shows the signalling pathways involved. NF-κB is a preformed transcription factor consisting of dimers of the five subunits p50, p52, c-Rel, RelA and RelB. In an inactive state, the NF-κB dimers are complexed to IκB proteins in the cytosol. IκB proteins cover the nuclear translocation sequence in NF-κB subunits and thereby retain the NF-κB complex in the cytosol. A key step in activation of NF-κB is the degradation of IκB proteins, releasing NF-κB to the nucleus. The degradation of IκBα, IκBβ and IκBε is tightly regulated by the kinase complex IKK. Upon phosphorylation by IKK, the IκB proteins are ubiquitinated and degraded by the 26 S proteasome. Thus IKK is a key player for the activation of NF-κB by most stimuli. Diverse stimuli such as genotoxic stress, cytokines or bacterial components enhance NF-κB transcriptional activity through IKK.

IKK is a multisubunit complex. Its main components are the catalytic subunits IKK1 (IKKa) and IKK2 (IKKβ), and the regulatory subunit NEMO (NF-κB essential modulator) (IKKγ). In addition, other proteins such as hsp90 (heat-shock protein 90) and cdc37 are part of the complex [21]. Although IKK1 and IKK2 have similar catalytic activity in vitro, their function in vivo is quite different. This is evident from the different phenotype of mice with a deficiency of IKK1 or IKK2 in the germline. IKK2−/− mice succumb to liver failure at E12–E13 (embryonic day 12–13), whereas IKK1−/− mice die shortly after birth and display defective epidermal keratinocyte differentiation and skeletal abnormalities [22]. Indeed, IKK2 and NEMO but not IKK1 are required for the classical pathway of NF-κB activation that is triggered by TNF, IL-1β (interleukin 1β) and many other cytokines. IKK1 mediates an alternative pathway of NF-κB activation in response to stimulation by the cytokines CD40L, BAFF (B-cell activating factor) and TWEAK [23]. In the alternative pathway of NF-κB activation, the kinases NIK (Nck-interacting kinase) and IKK1 lead to activation of RelB/p52 heterodimers [24].

IKK signalling in cerebral ischaemia

Although a key role of IKK in NF-κB signalling has been established, NF-κB may be activated independent of IKK by stimuli that are relevant for cerebral ischaemia, such as glutamate and hypoxia [24,25]. Therefore it is interesting that IKK is activated in cerebral ischaemia [26]. We confirmed this finding using a kinase pull-down assay [27]. IKK was activated for 5 h after onset of MCAO (middle cerebral artery occlusion), a mouse model of stroke. The activation was confined to the periphery of the ischaemic territory. In the centre of the ischaemia, IKK may be degraded as has been reported recently [28]. It is known that IKK phosphorylates not only IκB proteins but also RelA at Ser536. This phosphorylation of RelA is considered to modify the transcriptional activity [29,30]. A specific antibody allowed us to detect phospho-RelA by immunohistochemistry after cerebral ischaemia. The staining co-localized with the neuronal marker NeuN, indicating that RelA is phosphorylated by IKK in neurons.

To analyse the function of IKK in cerebral ischaemia, we performed a conditional deletion of IKK2, an approach that had been pioneered by Pasparakis et al. [31]. In cooperation with Rossana di Lorenzi and Manolis Pasparakis, Cologne, Germany, we performed MCAO in mice deficient for IKK2 in neurons or in neurons and glial cells. Surgery was performed and ischaemic damage was measured without knowledge of the genotype of the mice. Deficiency for IKK2 significantly reduced the infarct size. In addition, the number of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling)-positive cells in the ischaemic tissue was reduced in mice deficient in IKK2 in neurons. In models of focal cerebral ischaemia, TUNEL-positive cells predominantly correspond to neurons [19,32]. Thus deletion of IKK2 in neurons had a neuroprotective effect.

These experiments did not address IKK1. Because there is evidence that IKK1 and IKK2 may have partially redundant [33] or opposing functions [34], we were interested in the effect of IKK1/2 inhibition selectively in neurons. This issue could be addressed with a double transgenic technique in cooperation with Bernd Baumann and Thomas Wirth, Ulm, Germany [27]. Under the control of the tTA (tetracycline-controlled transactivator), a dominant-negative mutant of IKK2 (D145N) was expressed in neurons. In the brains of these mice, basal IKK activity was markedly reduced. Interestingly, also IKK activation in response to cerebral ischaemia was diminished. Furthermore, mice expressing the inhibitor in neurons had a significantly smaller infarct volume after MCAO. To put the concept that IKK contributes to ischaemic brain damage to the test, we investigated the effect of a constitutive activation of IKK on cerebral ischaemia.

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Using the same double transgenic approach as described above, a constitutively active mutant of IKK2 was expressed in neurons (S177E, S181E). As predicted by the results of deletion or inhibition of IKK, mice with constitutively active IKK2 in neurons had larger infarcts although they did not show obvious signs of neuronal cell death before cerebral ischaemia. Thus IKK activity contributes to ischaemic neuronal cell death but is not able to kill neurons on its own.

Recently, several small-molecule inhibitors of IKK have been developed [35,36]. Up to now, most research has focused on their use in inflammatory diseases such as rheumatoid arthritis and in cancer. Little has been published on the effects of selective small-molecule IKK inhibitors in neurological disorders. To test the pharmacological implications of the role of IKK in cerebral ischaemia, we used BMS-345541, a selective and potent inhibitor of IKK1 and IKK2 [37]. BMS-345541 is rather selective for IKK2, with IC50 values of 0.3 and 4 µM for IKK2 and IKK1 respectively [37]. Although BMS-345541 had no effect after intraperitoneal injection, it reduced the infarct size when injected intracerebroventricularly in a dose-dependent manner. The dependence of the effect on the route of administration may indicate that the compound is not freely permeable through the blood–brain barrier. BMS-345541 also decreased the number of TUNEL-positive neurons, PARP [poly(ADP-ribose) polymerase] cleavage reflecting caspase 3 activity, and DNA fragmentation. These findings indicate that IKK inhibition reduces apoptotic cell death. BMS-345541 improved ischaemic brain damage when administered within 4.5 h after onset of cerebral ischaemia, although its efficacy decreased with time. A time window of 4.5 h in the rather stringent stroke model we have used compares favourably with what has been found for other neuroprotective compounds. In the mean time, another selective small-molecule inhibitor of IKK has been reported to be neuroprotective [38].

NF-κB mediates the effect of IKK
IKK was identified as a pivotal kinase upstream of NF-κB. However, emerging evidence suggests that IKK may have substrates other than IkB and RelA. For example, IKK has been shown to phosphorylate IRS-1 (insulin receptor substrate) at Ser307, with the consequence of a reduced signalling to the neuroprotective kinase Akt [39]. Other substrates of IKK are β-catenin, Forkhead transcription factors and histone H3 [40–43]. Their significance for cerebral ischaemia is unclear so far. However, there is clear evidence that NF-κB has a detrimental role in cerebral ischaemia. Expression of the NF-κB superrepressor in neurons reduced the infarct size. In contrast, expression in astrocytes had no effect on the infarct volume [44]. Adenoviral expression of the NF-κB superrepressor also diminished the infarct size [45]. The concept that NF-κB mediates IKK-induced neuronal injury in cerebral ischaemia is further supported by pharmacological data showing a correlation between NF-κB inhibition and neuroprotection [29,46–48]. Thus, it seems likely that NF-κB mediates the detrimental effect of IKK in cerebral ischaemia, but other pathways may also be involved.

NF-κB is well known for its anti-apoptotic effects. So far, the mechanism that turns this anti-apoptotic effect into neurodegeneration is enigmatic. However, pro-apoptotic effector genes of NF-κB have been identified in the literature. Interestingly, we noted that inhibition of IKK in cerebral ischaemia reduced the induction of three genes involved in eicosanoid metabolism cytosolic phospholipase A2 (Pla2g4a), cyclo-oxygenase 2 (Ptge2) and microsomal prostaglandin E synthase 1 (Ptges) [27]. All three genes have been shown to be under transcriptional control of NF-κB. Pla2g4a and Ptge2 are known to promote ischaemic brain damage [49,50]. This suggests that prostaglandin E2 signalling may be involved in IKK-mediated neurodegeneration.

Conclusion
Neuroinflammation is generally thought to be maintained by leucocytes, microglia and astrocytes. In cerebral ischaemia, IKK, a key player in inflammation, is activated in neurons. We have identified the cytokine TWEAK as a potential stimulus of IKK in cerebral ischaemia, but other stimuli are possible. IKK contributes to ischaemic neurodegeneration probably through activation of the transcription factor NF-κB. IKK and NF-κB signalling promote neuronal apoptosis and the induction of inflammatory genes involved in eicosanoid metabolism. Thus inhibition of NF-κB signalling is a promising strategy for stroke treatment. The signalling cascade provides numerous steps that are suitable as drug targets, particularly IKK. In the future, it will be important to search for small-molecule inhibitors of IKK, which are effective when administered orally or intravenously. Furthermore, the future development of NF-κB inhibitors for neurodegenerative disorders will require a better understanding of the molecular mechanisms that make NF-κB a pro-apoptotic factor in cerebral ischaemia.

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