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Research article

Cyclooxygenase-2 is a neuronal target gene of NF-κB

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Abstract

Background: NF-κB is implicated in gene regulation involved in neuronal survival, inflammmatory response and cancer. There are relatively few neuronal target genes of NF-κB characterized.

Results: We have identified the neuronal cyclooxygenase-2 (COX-2) as a NF-κB target gene. In organotypic hippocampal slice cultures constitutive NF-κB activity was detected, which was correlated with high anti-COX-2 immunoreactivity. Aspirin a frequently used painkiller inhibits neuronal NF-κB activity in organotypic cultures resulting in a strong inhibition of the NF-κB target gene COX-2. Based on these findings, the transcriptional regulation of COX-2 by NF-κB was investigated. Transient transfections showed a significant increase of COX-2 promoter activity upon stimulation with PMA, an effect which could be obtained also by cotransfection of the NF-κB subunits p65 and p50. In the murine neuroblastoma cell line NB-4, which is characterized by constitutive NF-κB activity, COX-2 promoter activity could not be further increased with PMA or TNF. Constitutive promoter activity could be repressed upon cotransfection of the inhibitory subunit IκB-α. EMSA and mutational analysis conferred the regulatory NF-κB activity to the promoter distal κB-site in the human COX-2 promoter.

Conclusions: NF-κB regulates neuronal COX-2 gene expression, and acts as an upstream target of Aspirin. This extends Aspirin's mode of action from a covalent modification of COX-2 to the upstream regulation of COX-2 gene expression in neurons.

Background

NF-κB a transcription factor with inducible activity, present in most cell types. This factor is crucially involved in regulation of genes relevant in neuronal survival, inflammmatory response, cancer and innate immunity [1,2]. The activation of NF-κB is mainly controlled at the posttranscriptional level by complex formation with the inhibitory subunit IκB in the cytoplasm [3]. Phosphorylation of IκB prior to degradation is catalyzed by the activation of a complex consisting of two kinases (IKK-α and IKK-β) [4] together with a modifying subunit called NEMO [5] or IKK-γ [6]. Binding of NEMO is important to mediate the cytokine response in a aktivation of the kinases [7]. Recently it was shown that mutations of NEMO/IKK-γ were linked to human genetic diseases (for review see [8]).

NF-κB is also frequently found in different cells of the nervous system (for review see [9]. Many neurons of the central nervous system contain NF-κB as a heterodimer of the DNA-binding subunits p50 and p65, complexed with IκB [10]. Constitutive activity of NF-κB is present in fields of the hippocampus and in the cerebral cortex [10]. These data suggest an endogenous, physiological stimulus, which controls the activity of NF-κB. One candidate is the neurotransmitter glutamate, which can activate NF-κB in cerebellar granule cells and hippocampal neurons [11–15]. Furthermore the presence of inducible NF-κB in synaptosomes [16,17] and the transport of GFP-tagged p65 from neurites to the nucleus [15] suggest that NF-κB could be involved in connecting synaptic activity with gene expression. This notion is also supported by the ultrastructural localization of activated NF-κB in dendrites [18]. A gene induced by synaptic activity is the inducible cyclooxygenase or prostaglandin H (PGH) synthase-2 (COX-2). In contrast to peripheral tissues the cyclooxygenase-2 activity and expression is high in normal brain, where it is restricted to neurons [19,20]. We investigated wether COX-2 is regulated by NF-κB. COX-2 and activated NF-κB immunoreactivity colocalized in hippocampal and cortical neurons. Aspirin, a described inhibitor of NF-κB [21] inhibited neuronal NF-κB, leading to a robust inhibition of COX-2 protein expression. These data were further corroborated by an analysis of the COX-2 promoter. A promoter distal κB element was identified as the only functional κB-site in NB-4 neuroblastoma cells. In addition this element is also responsible for the constitutive promoter activity. Thus the previously described constitutive COX-2 activity in neurons [22] is dependent on constitutive NF-κB activity.

Results

NF-κB and cyclooxygenase-2 colocalize in subsets of cortical and hippocampal neurons

COX-2 was identified as a gene induced after seizures [23]. Basal expression of this enzyme is high in brain, in comparison to other organs were COX-1 is the major isoenzyme. COX-2 expression in brain is dependent on normal neuronal activity, as demonstrated with intra-ocular tetrodotoxin injection which blocks COX-2 expression in the visual cortex. Moreover COX-2 expression in the CNS is obligate neuronal [20]. Here we tested if COX-2, as a marker of neuronal activity, is present in the same neurons, that show activated NF-κB. Double labeling immunofluorescence was used to correlate the activation of NF-κB with COX-2 protein amount at single cell level. Previously we developed a monoclonal antibody specific for the activated form of p65 [24]. This antibody is directed against an epitope of the nuclear localization signal (NLS) of p65. In the non-activated cytoplasmic form of NF-κB the NLS is predominantly covered by the inhibitory subunit IκB, making binding of the antibody impossible. Upon stimulation active NF-κB is generated after IκB degradation. This active NF-κB can be visualized with the activity specific antibody.

Using this antibody binding predominantly to the activated form of p65 and a polyclonal antibody to COX-2, a distinct staining pattern for both proteins in rat cortex (Fig. 1) and hippocampus (Fig. 2a,2b,2c dentate gyrus; d-f CA3) was detected. Immediately evident is the overlap of both stainings in many, but not all neurons (arrow heads in Fig. 1 and Fig. 2), where the activated p65 subunit is localized in the nucleus and the COX-2 protein in the corresponding cytoplasm of the same neuron. In accordance with previous findings [20,25] we detect also a perinuclear localization of COX-2.

In addition to the abundant colocalization of both stainings, cells with distinct staining for both COX-2 and p65 could be detected in the cortex and hippocampus. The specificity of both antibodies was analyzed by incubation without primary antibody, which showed no significant staining (data not shown).

Inhibition of NF-κB leads to down regulation of COX-2 expression

To examine a causal link between nuclear NF-κB and COX-2 expression, we investigated in hippocampal slice cultures a possible correlation between NF-κB activation and COX-2 expression. All cultures are treated with bicuculline and picrotoxine to interfere with GABA-ergic input. Under these conditions NF-κB activity and COX-2 expression is similar to the *in vivo* situation (compare to Fig. 2). A high level of activated NF-κB was observed in hippocampal principal cells (Fig. 3, Con). However, a co-treatment with the anti-inflammatory pain killer aspirin resulted in a robust inhibition of both, activated NF-κB and COX-2 (Fig. 3 +aspirin). The results of a previous study has shown that NF-κB is inhibited by aspirin [21] via specific inhibition of the IκB kinase IKKβ [26].

Thus the inhibition by aspirin suggests that COX-2 is a neuronal NF-κB target gene. To further corroborate this notion we performed a promoter analysis of the COX-2 gene.

Two conserved κB-binding sites are present in the human cyclooxygenase-2 promoter

We noted the presence of two NF-κB consensus motifs and additional binding sites for Sp-1, AP-2 and NF-IL6 (see Fig. 4A) in the human COX-2 promoter sequence. For a further analysis of the 5'-upstream region of the COX-2 promoter the sequence from position -495 to +15 was cloned from human genomic DNA. In order to test if the cloned sequences have promoter activity, two different constructs driving a luciferase reporter vector (Clone A

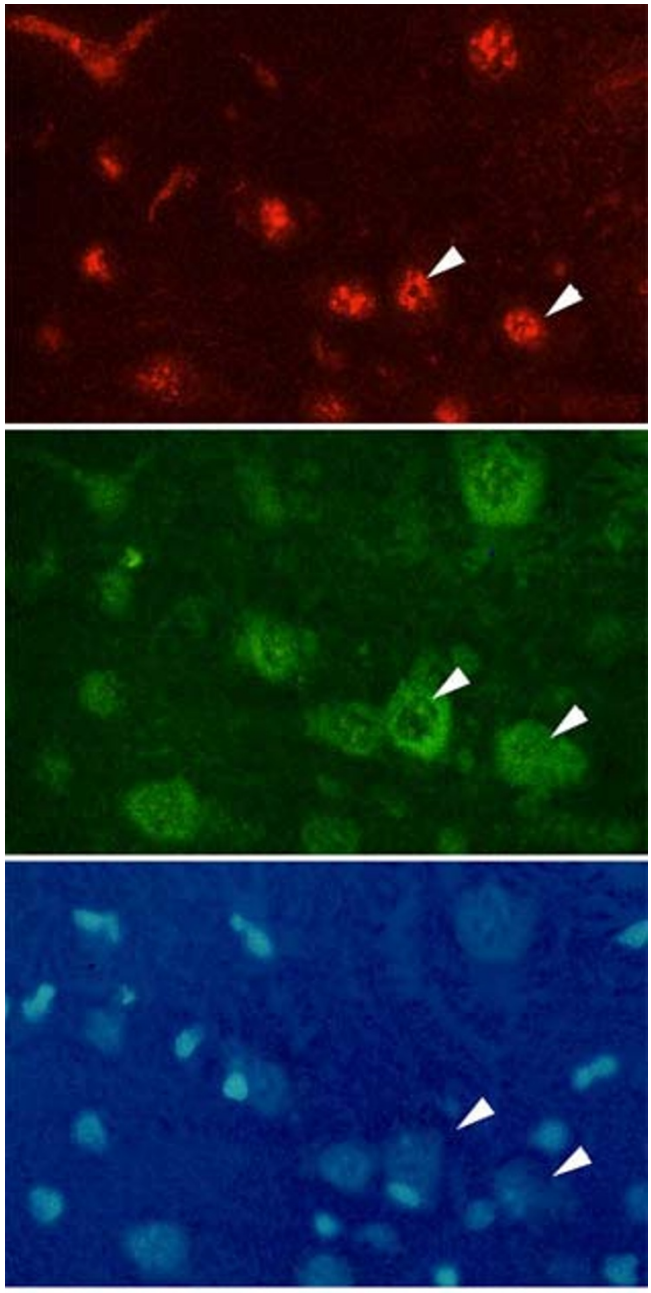

Figure 1
Co-localization of NF-κB and COX-2 in the cerebral cortex. Upper panel showing activated NF-κB (anti-p65 monoclonal antibody) in red; middle panel showing COX-2 in green; lower panel showing cellular architecture visualized with DAPI staining. Note double immunopositive cells (arrowheads) with cytoplasmic COX-2 and nuclear NF-κB staining (× 400).

and B in Fig. 4B) were transfected in human embryonic kidney cells 293, a well established cell line for the analysis of NF-κB activity. As expected both constructs show a high basal-level promoter activity in comparison to the promoter-less pGL-2 vector (Fig. 4B).

The cyclooxygenase-2 promoter is strongly induced by NF-κB activating stimuli

A prerequisite of a NF-κB target gene would be its inducibility in cells which respond to known NF-κB activating stimuli. The phorbol ester phorbol 12-myristate 13-acetate (PMA) is a strong inducer of NF-κB activity [27]. Non-neuronal cells were used to characterize the mechanisms of promoter-induction, since neuronal cells support already a full blown constitutive COX-2 promoter activity (see below). Therefore we analyzed the COX-2 promoter for response to activated NF-κB, in comparison to the exclusively NF-κB driven tk(NF-κB)6-luc reporter (Fig. 5). Both PMA and TNF induced the tk(NF-κB)6-luc reporter significantly (more than 50-fold for PMA and bout 10-fold for TNF). A strong increase of luciferase activity in response to PMA-stimulation was detected for the COX-2 promoter construct. In contrast, TNFα, a strong activator of NF-κB did not induce measurable COX-2 promoter activity (Fig. 5). The TNF induction of the COX-2 promoter might not be detectable, since TNF in HeLa cells is a weaker activator of NF-κB-dependent reporter genes than PMA (see induction of the tk(NF-κB)6-luc reporter). These data also lend crescence to the idea, that TNF is a short time stimulus which can potently activate NF-κB binding activity (see Fig. 8) as does PMA. In contrast, the longer lasting stimulus PMA is also a potent activator of reporter gene expression due to the short half life of luciferase. PMA also activates the formation of a transcriptionally active AP-1 complex [28]. But induction of the COX-2 promoter via AP-1 is very unlikely since there are no binding sites for AP-1 within the used COX-2 promoter fragment. Another clue for the involvement of NF-κB in COX-2 gene expression is derived from cotransfection experiments of the COX-2 reporter and expression vectors for p65 and p50. This cotransfection leads to a significant increase of reporter gene activity (Fig. 6) demonstrating the strong transactivational potential of p65 [29]. On the other hand, cotransfection of p50 shows a similar effect (Fig. 6). This might be due to the interaction of p50 with Bcl-3 and is frequently observed also in other promoters [30]. These data suggest a crucial role of NF-κB as a regulator of COX-2 promoter activity.

Constitutive NF-κB activity in NB-4 neuroblastoma cells is essential for cyclooxygenase-2 promoter activity

We and others have previously shown that many neurons contain constitutive NF-κB activity both, in primary cultures and *in vivo* [10,31,24]. For our investigations we have chosen the murine neuroblastoma cell line NB-4

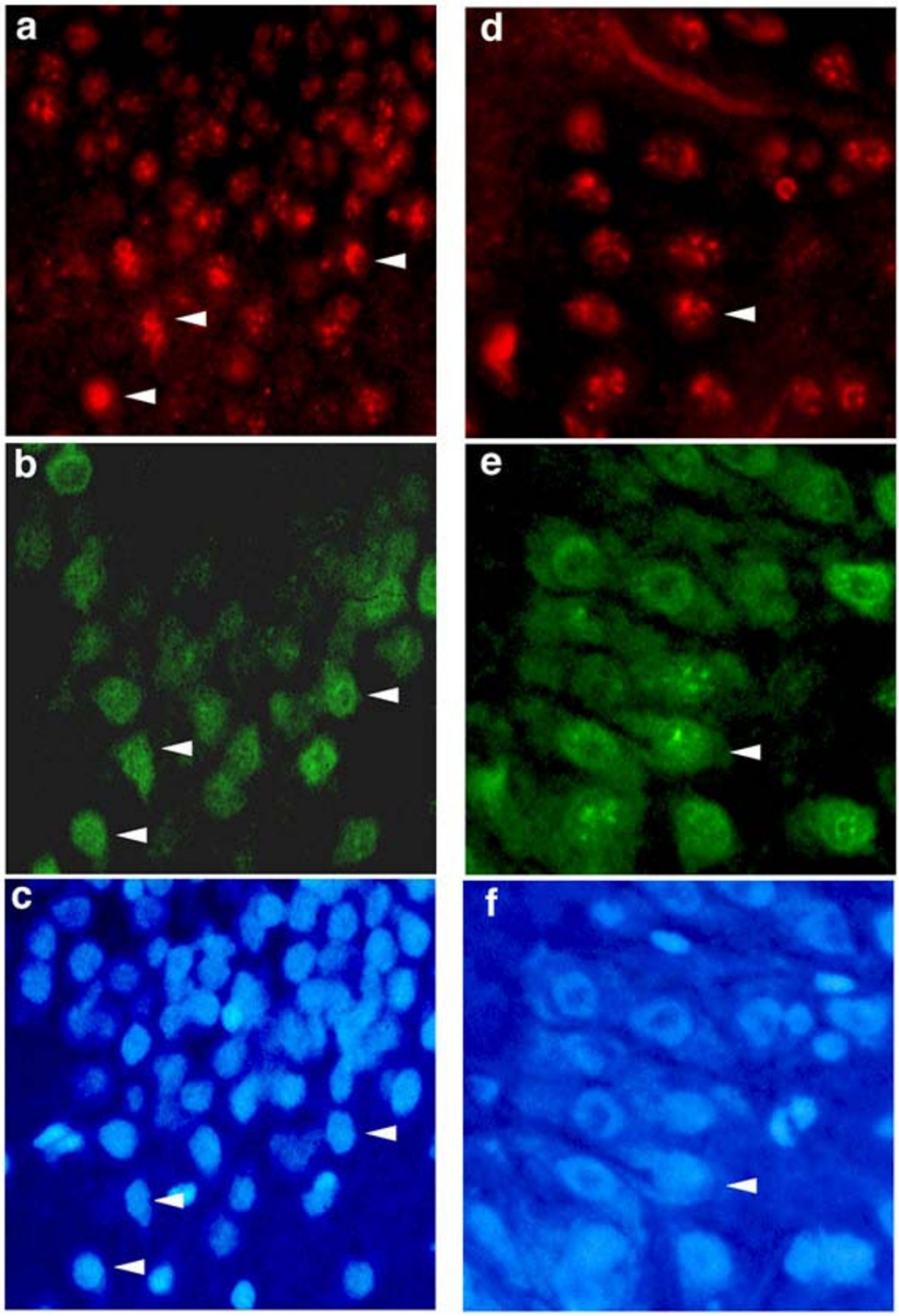

Figure 2
Co-localization of NF-κB and COX-2 in the hippocampus. a-c) A region of the dentate gyrus is shown. Nuclei of granule cells are visualized by DAPI staining (c). a) anti-p65 staining (red), b) anti-COX-2 staining (green); colocalisation of p65 and COX-2 in some neurons is marked by arrow heads. d-f) A region of the CA3 field is shown. d) anti-p65 staining (red), e) anti-COX-2 staining (green); colocalisation of p65 and COX-2 in a pyramidal neuron is marked by an arrow head; f) nuclear staining of the same blow-up with DAPI. (× 400)

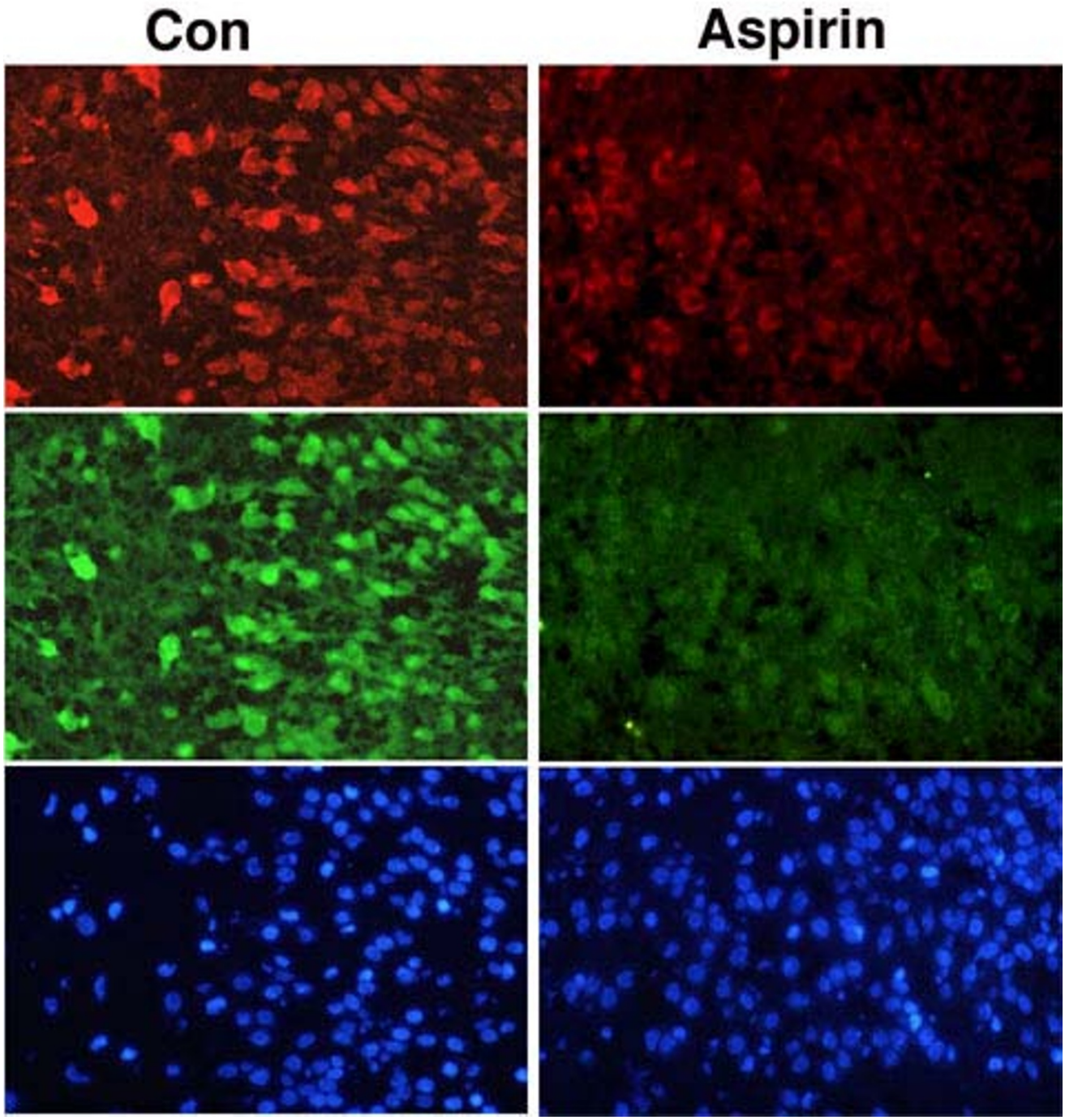

Figure 3
NF-κB and COX-2 are repressed by aspirin in cultivated hippocampal slices. Left panel: Control (Con) cultures are treated with bicuculline and picrotoxine to influence GABAergic input. Under these conditions NF-κB activity and COX-2 expression is similar to the *in vivo* situation (see Fig. 2). Right panel: cultures were treated with bicuculline and picrotoxine as control cultures and co-treated with aspirin for3 15 min and analyzed 6 h post treatment.3 Note the strong inhibition of nuclear NF-κB immunoreactivity and the correlated down regulation of COX-2 expression. (× 400)

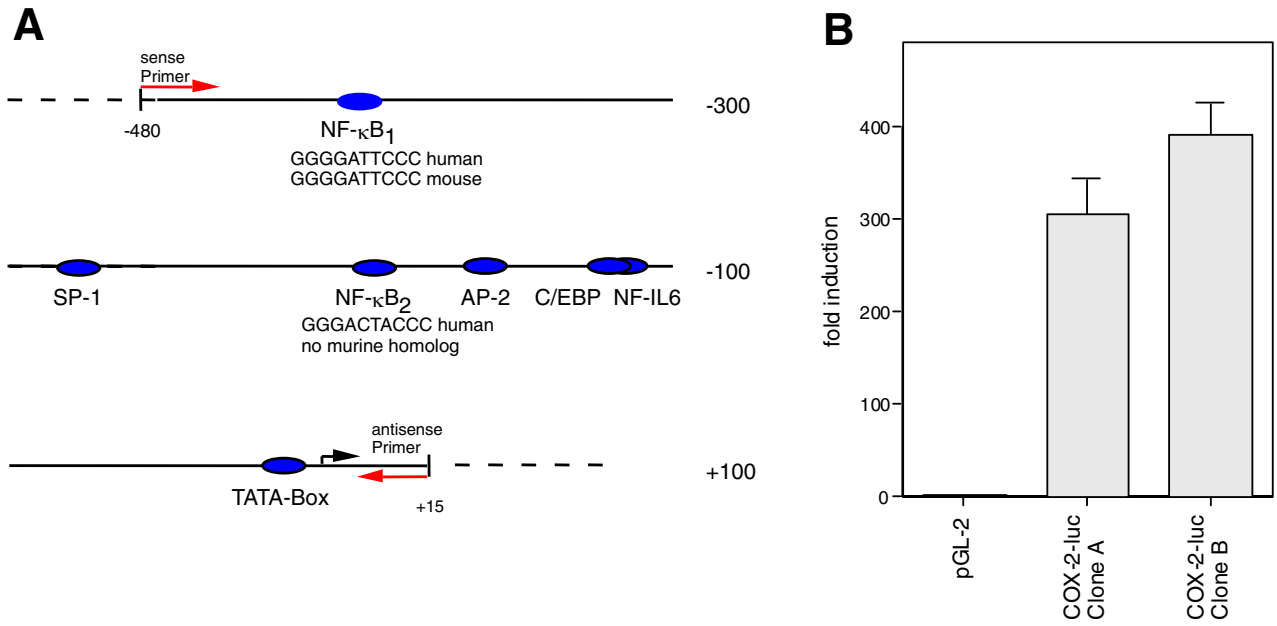

Figure 4
Analysis of a human COX-2 promoter region. (A) Scheme of the promoter region. Transcription factor binding sites were identified using the computer program Factor (HUSAR program package, DKFZ Heidelberg). Note the presence of two NF-κB binding sites NF-κB1 (promoter-distal) and NF-κB2 (promoter-proximal). (B) Analysis of COX-2 promoter activity. Two PCR-generated COX-2 promoter constructs in the promoter-less luciferase vector pGL-2 (Promega, Heidelberg) were choosen for transfection in HEK 293 cells. The luciferase activity of the empty vector pGL-2 was set to one. The fold induction of luciferase activity is depicted in a linear scale on the ordinate. Both COX-2 promoter constructs show a more than 300-fold activity over the promoter-less vector pGL-2. The standard deviation of triplicate transfection assays is depicted as arrow bars.

which displays characteristics of mature cholinergic neurons. In contrast to the non-neuronal cell lines used in the above described experiments, transfection of a tk(NF-κB)6-luc reporter revealed a constitutive activation of NF-κB (Fig. 7A). The activity of the NF-κB-luc reporter in NB-4 cells was essentially dependent on NF-κB, since it was totally abrogated after cotransfection of the inhibitory subunit IκB-α. This constitutive NF-κB activity could also not be further augmented by stimulation with TNF or PMA. But a downregulation of NF-κB activity was observed after treatment with PMA and TNF, as described earlier for PMA-treated HeLa cells [32]. Constitutive NF-κB activity was described previously also for neurons in the cortex and the hippocampus [10]. The analysis of the COX-2 promoter led to a similar result (Fig. 7B), showing high activity of the promoter without stimulation and displaying a missing responsiveness to the otherwise potent stimulus PMA. These results provide evidence to the idea that the constitutive activity of NF-κB found here is already the full blown activity, which can no longer be affected by activating agents.

The activity of the cyclooxygenase-2 promoter is only dependent on the promoter-distal NF-κB binding-site

For a more detailed analysis of the regulatory involvement of NF-κB, we tested the two κB-binding sites in the COX-2 promoter (see Fig. 4A) for their potential to bind NF-κB-proteins and to stimulate transcription. For the first purpose, electrophoretic mobility shift assays using oligonucleotides corresponding to the two κB-binding sites were performed (Fig. 8). The promoter-distal NF-κB1-binding site showed a clear binding activity for nuclear proteins (Fig. 8, lanes 1–3), whereas the promoter-proximal NF-κB2-site reproducibly failed in binding (Fig. 8, lanes 4–6).

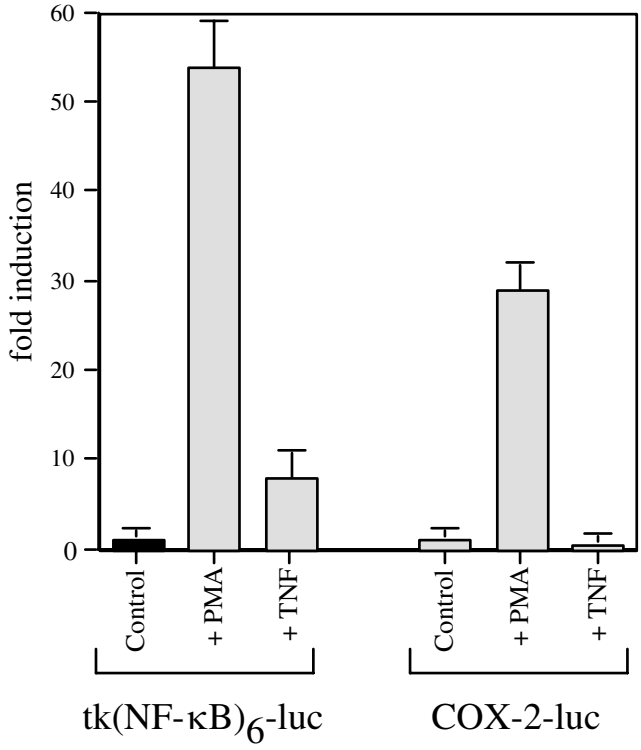

Figure 5
Stimulation of COX-2 promoter activity in HeLa cells. As control, a luciferase construct driven by the synthetic NF-κB dependent promoter tk(NF-κB)6-luc was transfected in HeLa cells. The basal level of the unstimulated promoter was set to one. Treatment of transfected cells with 50 ng PMA per ml medium for 6 h resulted in a more than 50-fold increase in luciferase activity, whereas treatment of transfected cells with TNF α (200 U/ml) resulted in a moderate increase (< 10-fold). Within the same assay the COX-2 promoter driven luciferase construct was tested. The activity of the promoter without treatment was set to one. PMA induced the promoter activity about 30-fold, whereas TNF had no measurable effect. Standard deviations of triplicate transfection assays are depicted as arrow bars.

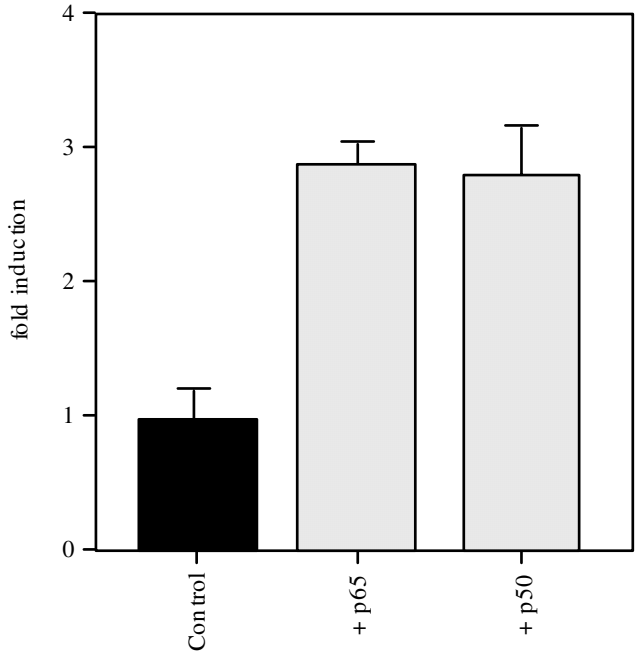

Figure 6
Induction of COX-2 promoter activity by NF-κB subunits. Expression vectors for the NF-κB subunits p50 and p65 were cotransfected with the COX-2 promoter in HeLa cells. Both subunits can activate COX-2 promoter activity. Standard deviations of triplicate transfection assays are depicted as arrow bars. Fold induction was normalized to control, that is the COX-2 promoter reporter vector alone (1 fold induction).

The NF-κB binding-site from the κ light chain enhancer was used as control (Fig. 8, lanes 7–9). These results can be explained by a nonconsensus nucleotide within the putative binding site (Fig. 3A). In addition the sequence of the NF-κB2-site could not be selected as optimal κB/Rel DNA-binding motif [33]. Therefore the regulation of the COX-2 promoter via NF-κB is solely dependent on the promoter-distal NF-κB1-site. To verify this hypothesis, a mutant of the NF-κB1-site in the COX-2 promoter was constructed. Mutant and wildtype COX-2 promoter constructs were transfected in NB-4 cells and analyzed for luciferase activity (Fig. 9). In accordance with the EMSA data mutation of the NF-κB1-site leads to a complete loss of COX-2 promoter activity. These results underscore the central role of NF-κB in regulating COX-2 promoter in neuronal cells: in NB-4 cells the COX-2 promoter is constitutively active and this activation is dependent on the integrity of the NF-κB1 binding-site in this promoter.

Discussion

Here the regulation of the human COX-2 promoter was analyzed. Immunocytochemistry was used to colocalize COX-2 immunoreactivity and activated NF-κB in neurons *in vivo*. This was investigated in the rat hippocampus and cortex cerebri, using an antibody specific for the activated form of NF-κB. In cultured hippocampal slices the specific NF-κB inhibitor aspirin, inhibited both, NF-κB activation and COX-2 expression. The promoter region of the human COX-2 gene contains, in contrast to the mouse promoter region, two putative NF-κB binding sites. It was

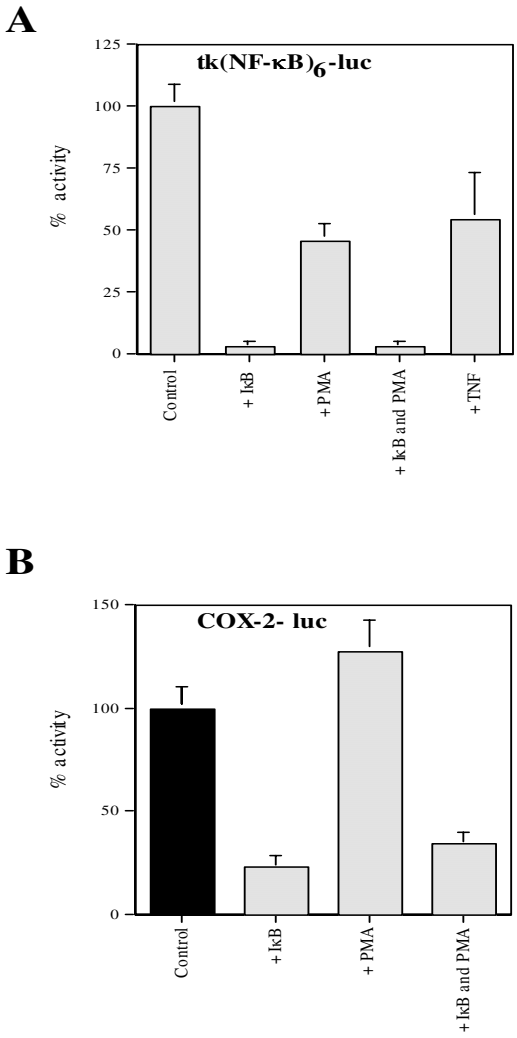

Figure 7
Analysis of NF-κB dependent promoter activation in NB-4 neuroblastoma cells. (A) Analysis of a synthetic NF-κB-dependent reporter. The synthetic NF-κB-dependent reporter-gene tk(NF-κB)6-luc was transfected in NB-4 neuroblastoma cells (1). Luciferase activity of tk(NF-κB)6-luc set to 100 %. Cotransfection of tk(NF-κB)6-luc with an expression vector for IκBα totally abrogated the activity of this luciferase reporter (2). Treatment of cells transfected with tk(NF-κB)6-luc with PMA (3) or TNFα (5) resulted in a reduction of reporter gene activity. This residual activity could be further inhibited after expression of IκBα (4). (B) Analysis of the human COX-2 promoter. The COX-2-promoter-driven luciferase reporter was transfected in NB-4 neuroblastoma cells (1). Luciferase activity of COX-2-luc was set to 100 %. Cotransfection of COX-2-luc with an expression vector for IκBα significantly inhibited the activity of this luciferase reporter (2). Treatment of cells transfected with COX-2-luc with PMA (3) resulted in a slight increase of reporter gene activity. This residual activity also could be inhibited after expression of IκBα (4). Standard deviations of triplicate transfection assays are depicted as arrow bars.

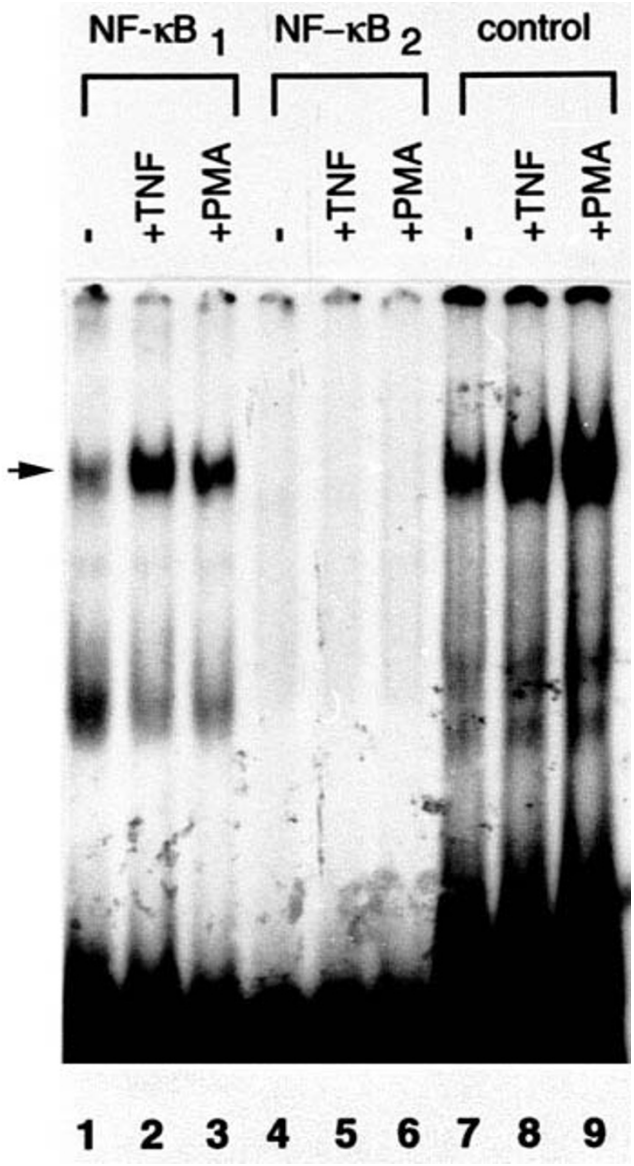

Figure 8
Electrophoretic mobility shift analysis (EMSA) of NF-κB binding sites derived from the human COX-2 promoter. Nuclear extracts derived form HeLa cells without treatment (-, lanes 1, 4, 7), with TNFα treatment (lanes 2, 5, 8) and with PMA treatment (lanes 3, 6, 9) were used for EMSA with different probes. EMSA with a probe spanning the promoter element NF-κB1 from the human COX-2 promoter is shown in lane 1–3. NF-κB2 was used as probe in lane 4–6 and a κB-element from the κ light chain enhancer was used as control in lanes 7–9. The specific NF-κB complex is marked with an arrowhead.

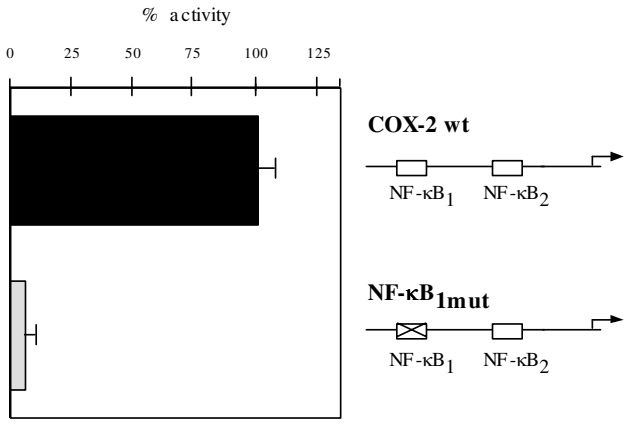

Figure 9
Mutational analysis of the COX-2 promoter in NB-4 neuroblastoma cells. The activity of a COX-2 promoter construct containing both NF-κB binding sites was set to 100 %. A construct containing the same promoter context but a mutation of the promoter distal NF-κB1 binding site (κB1mut) had only one tenth of wild-type COX-2 promoter activity. These data show that the constitutive NF-κB activity of the COX-2 promoter in NB-4 cells is merely dependent on the functional integrity of the evolutionary conserved NF-κB1 element. Standard deviations of triplicate transfection assays are depicted as arrow bars.

found, that only the conserved NF-κB binding site, present in the mouse and human COX-2 promoter region (see Fig. 4A), is of functional relevance in neuronal cells.

Evidence for COX-2 as a neuronal NF-κB target gene

Non-neuronal cells were used to characterize the mechanisms of promoter-induction, since neuronal cells support already a full blown constitutive COX-2 promoter activity (see below). In HeLa cells the human COX-2 promoter is fully inducible with PMA, but only to a low amount with TNF, whereas a synthetic NF-κB-dependent promoter is readily activated with TNF. This difference might be exaggerated through the lower COX-2 promoter activity, which is the result of only one functional NF-κB binding site. During the analysis of NF-κB-subunits mediating an induction of the COX-2 promoter, we found that the NF-κB subunits p50 and p65 were active. In contrast to p65, which contains a transactivating domain, the p50 subunit does not contain its own transactivation domain. This effect might be mediated by interaction of p50 with Bcl-3, which can provide the transactivating function. This transactivating effect of the NF-κB p50 subunit is not a characteristic of the COX-2 promoter but is now frequently observed also in other promoters [30].

In accordance with the inter-species conservation of the promoter-distal κB1 element (Fig. 4A), DNA-binding of NF-κB proteins to this element was detected. The promoter proximal κB2 element is different in one nucleotide from the NF-κB consensus binding site and could not be bound by NF-κB subunits. In a recent approach recombinant NF-κB subunits were used to select target sequences bound by the DNA-binding domains (target detection assay). In accordance with our data the κB2 element was not selected as a binding site [33].

It was reported earlier that many neurons of the cortex cerebri and the hippocampus contain constitutive NF-κB activity [10]. Using an antibody specific for activated p65 [24], here a colocalization of constitutive NF-κB activity and basal level COX-2 expression was detected. In accordance to the constitutive activity detected *in vivo* we also found that NB-4 cells contain constitutive activity based on the following criteria: 1.) A high-level basic activity of a promoter containing 6 κB elements could not be further augmented after treatment with PMA or TNF. 2.) Constitutive promoter activity could be repressed with cotransfection of IκB. The COX-2 promoter in NB-4 cells showed the same level of constitutive activity that could not be augmented strongly with PMA or TNF, but is repressed after cotransfection of IκB. Therefore we conclude that NB-4 neuroblastoma cells are a suitable model for the constitutive NF-κB activity found *in vivo* in neurons of the cortex and hippocampus. Here we show that a mutation of the COX-2 promoter κB1 element entirely abolishes the constitutive activity of this promoter in NB-4 cells.

Recent studies have shown, that NF-κB can be activated in cerebellar granule cells via stimulation of glutamate receptors [11–13,34]. In addition it was shown that the transcription of NF-κB subunits p50 and p65 was increased during seizure activity [17]. Taken together NF-κB is one of the transcription factors regulated via neuronal activity (see [9]). One of the important physiological consequences of NF-κB activation might be the induction of a compensatory neuroprotective gene expression program [14,35–38].

COX-2 mediated inflammatory pathways may play important roles in pathogenesis of neurodegenrative diseases such as Alzheimer disease [22]. In Alzheimer disease patients early plaque stages are surrounded by neurons with activated NF-κB [37,39]. Moreover high levels of NF-κB binding activity could be strongly correlated to high levels of COX-2 transcription in Alzheimer disease and age matched control brains [40]. There is evidence reviewed by [41] that induction of high level of COX-2 epression might be responsible for patho-physiological changes which are also seen in a COX-2 overexpressing mouse model [42]. But the activation of COX-2 might

also serve protective functions as shown in seizure paradigms. Seizures can activate NF-κB [43], which in turn may lead to prostaglandin production after COX-2 gene induction. These newly produced prostaglandins might exert a protective effect against new seizure attacks [44].

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin are the major therapy for inflammatory pain. In addition to its action as an inhibitor of prostaglandin synthesis aspirin also acts as an inhibitor of NF-κB. A recent study has shown that inflammatory pain is induced via COX-2, expressed in neurons within the CNS[25]. Here we have shown that neuronal COX-2 expression is essentially dependent on NF-κB activity. In addition the NSAID aspirin inhibits neuronal NF-κB, which results in strongly reduced COX-2 activity. Similarly an enantiomer of the NSAID flurbiprofen which could not repress COX-2 enzyme activity is still acting anti-phlogistically as an inhibitor of NF-κB [45].

Conclusions

We found that COX-2 is a neuronal target gene of NF-κB. Aspirin inhibited both, NF-κB activation and COX-2 expression in neurons. Thus preventing COX-2 gene transcription via NF-κB inhibition might provide novel means of normalizing pain sensitivity.

Methods

Materials

Human genomic DNA was from Promega, Heidelberg, Germany; Taq-Polymerase and PCR-reagents from Stratagene, Heidelberg, Germany. Sequencing was performed using reagents and equipment from Applied Biosystems (Weiterstadt, Germany). D-Luciferin, PMA and TNF were obtained from Sigma, Deisenhofen, Germany; Lipofectin from Gibco, Karlsruhe, Germany.

Vector construction

The human COX-2 promoter was cloned by PCR using the following primers: tgcagctcttgactcatcgg and cccaagcttgacaattggtcgctaaccga according to a published sequence [46]. The obtained sequences were cloned in front of the luciferase gene into the promoter-less luciferases reporter vector pGL-2 (Promega, Heidelberg, Germany) and verfied by sequencing. For mutational analysis an EcoRI site was introduced in the κB1 element (κB1mut:gagagaattctccctgcgc) with a PCR-mediated strategy.

Organotypic hippocampal slice cultures

Hippocampal slice cultures (N= 30) were prepared from slices 350 μm thick taken from five-day-old pubs of Wistar rats. After 6 days *in vitro* [47,48] all cultures were treated with GABA antagonists to mimick the glutamatergic input present *in vivo*. All used drugs were from Sigma, Deisenhofen, Germany. Treatment with the GABA antagonists bicuculline (200 μM) and picrotoxine (1000 μM) of all slice cultures was done as described [49] for 3 days. Cultures were co-treated with aspirin (5 mM for 15 min as described [34] and assayed for immunoreactivity after 6 h post treatment. Cultures were fixed with 4% formaldehyd (pH 7.2) for 12–24 h at room temperature, kryoprotected in 30% sucrose solution (overnight at 4°C) and sectioned with a kryostat (20 μm).

Immunocytochemistry was done as detailed below.

Culture of cell lines

Cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). HeLa and 293 cells were grown in DMEM (Gibco) containing 10% fetal calf serum. NB-4 cells were cultivated in Ham's F10 medium (Sigma, Deisenhofen, Germany) with addition of 15% horse serum, 2.5% fetal calf serum, antibiotics and glutamine. For luciferase assays, cells were plated in six-well plates (25.000 cells per well with 2 ml of culture medium).

Transfection of cells and luciferase reporter assays

The Rc/CMV derived expression vectors for p50 and p65 were described earlier [29]. The tk(NF-κB)6 luciferase reporter construct contains 6 reiterated copies of the HIV-1 κB-site in front of the truncated Herpes simplex thymidine kinase (tk) promoter spanning position -105 to +51 [50]. HeLa and 293 cells were transfected according to a modified calcium phosphate protocol [51]. NB-4 cultures were transfected using lipofectin according to the instructions of the manufacturer (Gibco, Karlsruhe, Germany). Twenty hours after transfection, cells were lysed and assayed for luciferase activity [52]. At least three independent transfection experiments were performed in triplicates using different cell and DNA preparations. All experiments gave the same qualitative result. One representative experiment is shown. In several experiments cells were stimulated for 6 h with either 50 ng/ml PMA (Sigma, Deisenhofen, Germany) or 2 ng/ml (200 U/ml) human TNF-α (Roche, Mannheim, Germany).

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were prepared as previously described [32]. Briefly, cells were lysed in buffer A containing 20 mM HEPES, 0.35 M NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM MgCl2, 0.5 M EDTA, 0.1 mM EGTA, 5 mM dithiothreitol, phenylmethylsulfonyl fluoride and aprotinin. Nuclei were extracted with buffer C and stored at -80°C for EMSA. EMSAs were performed using 3.5 μg nuclear proteins to bind to ^{32}P-labeled oligonucleotides, encompassing the κB-site from the murine κ light chain enhancer (Promega, Heidelberg, Germany), κB1: gagaggggattccctgcg and κB2:agtgggactacccccctc from the human COX-2 promoter.

Immunocytochemistry

Brains were dissected from adult Wistar rats and embedded in OTC-compound (Miles-Bayer, Leverkusen, Germany). 8 μm cryo-sections were cut from snap frozen material with a Leica cryostat (Leica Instruments, Heidelberg, Germany). The sections were collected on gelatine coated slides and dried. After fixation in methanol at -20°C for 5 min, the sections were blocked in 5% goat serum. For double-label immunofluorescence the sections were incubated with the two primary antibodies (diluted 1:50): a monoclonal antibody against p65 (Roche, Germany, see [24] and a rabbit polyclonal antibody against murine COX-2 (cyclooxygenase-2, Cayman Chemical Company, Ann Arbor, USA). Bound antibodies were detected with an anti-mouse IgG antibody coupled with Cy3 (1:1000, Dianova, Hamburg, Germany) and an anti-rabbit IgG coupled with DTAF (1:100, Dianova, Hamburg). Nuclei were stained with DAPI (4',6-Diamidine-2'phenylindole dihydrochloride, Roche, Germany). Microphotographs were taken with a Zeiss Axioskop equipped with epifluorescence. Mounting of colour plates was done on an Apple PowerPC with Adobe Photoshop.

List of abbreviations

AP, activator protein; C/EBP, CCAAT/enhancer binding protein; COX-2, cyclooxygenase-2; DAPI, 4',6-Diamidine-2'phenylindole dihydrochloride; EMSA, electrophoretic mobility shift assay; IκB-α, Inhibitor of kappaB-alpha; IKK, IkappaB kinase; NEMO, NF-kappaB Essential MOdulator; NF-κB, nuclear factor kappa B; PMA, phorbol ester; TNF-α, tumor necrosis factor alpha.

Authors' contributions

BK, RL and JD performed the experiments. BK supervised the cell biology work. CK conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

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