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The Protein Kinase IKKe Is a Potential Target for the Treatment of Inflammatory Hyperalgesia

Christine V. Möser,1 Katharina Kynast,1 Katharina Baatz, Otto Quintus Russe, Nerea Ferreirós, Heike Costiuk, Ruirui Lu, Achim Schmidtko, Irmgard Tegeder, Gerd Geisslinger, and Ellen Niederberger

Inhibitor-κB kinase ε (IKKe) was only recently identified as an enzyme with high homology to the classical I-κB kinase subunits, IKKα and IKKβ. Despite this similarity, it is mainly discussed as a repressor of viral infections by modulating type I IFNs. However, in vitro studies also showed that IKKe plays a role in the regulation of NF-κB activity, but the distinct mechanisms of IKKe-mediated NF-κB activation are not clear. Given the paramount role of NF-κB in inflammation, we investigated the regulation and function of IKKe in models of inflammatory hyperalgesia in mice. We found that IKKe was abundantly expressed in nociceptive neurons in the spinal cord and in dorsal root ganglia during hind paw inflammation evoked by injection of zymosan or formalin. IKKe knockout mice showed normal nociceptive responses to acute heat or mechanical stimulation. However, in inflammatory pain models, IKKe-deficient mice exhibited a significantly reduced nociceptive behavior in comparison with wild type mice, indicating that IKKe contributed to the development of inflammatory hyperalgesia. Antinociceptive effects were associated with reduced activation of NF-κB and attenuated NF-κB–dependent induction of cyclooxygenase-2, inducible NO synthase, and metalloproteinase-9. In contrast, IRF-3, which is an important IKKε target in viral infections, was not regulated after inflammatory nociceptive stimulation. Therefore, we concluded that IKKe modulates inflammatory nociceptive sensitivity by activation of NF-κB–dependent gene transcription and may be useful as a therapeutic target in the treatment of inflammatory pain. The Journal of Immunology, 2011, 187: 2617–2625.

Chronic pain is associated with a number of adaptive changes in the peripheral and CNS, which may enhance the sensitivity to painful stimuli and lead to an independent “pain disease”. The sensitization involves several adaptive and maladaptive processes that depend, in part, on the transcription factor NF-κB–activation cascade (1). During inflammation, NF-κB is activated by the so-called “classical” pathway, which depends on an inhibitor-κB kinase (IKK) complex consisting of the regulatory subunit IKKα (also known as NF-κB essential modulator) and the catalytic subunits IKKβ and IKKγ (2–4). In most unstimulated cells, NF-κB dimers are localized in the cytoplasm and sequestered by binding to the inhibitory subunit IκB. Upon activation by inflammatory stimuli, such as cytokines and bacterial LPS, IκB is phosphorylated by IκB kinases, subsequently ubiquitinated, and degraded by a proteasome complex. Degradation of IκB initiates the release of NF-κB from the trapping complex and its translocation into the nucleus, where it binds to the promoter region of various genes, including cytokines (e.g., TNF-α and IL-1β), cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS), and proteases (e.g., matrix metalloproteinases [MMPs]), thereby activating the transcription of these genes (2, 5).

In addition to the classical complex, a novel IKK complex was recently characterized that is activated by phorbol esters (PMA), LPS, and cytokines (6, 7). This pathway involves the activation of IKKe and tank-binding kinase 1 (TBK1), which are structurally similar to the classical IKKs. However, unlike classical IKKs, IKKe and TBK1 were shown to play major roles in the response to viral infections because both phosphorylate IRF-3 and -7 and, thereby, activate type I IFN (8, 9). Activation of NF-κB by phosphorylation of several proteins of the NF-κB–activation cascade, including IκBα, IκB kinase β, p65, or c-Rel, has only been assessed in vitro studies (6, 10–13). Several studies suggested that this novel IKK complex also contributes to nonviral inflammatory adaptations [e.g., in macrophages and mouse embryonic fibroblasts, IKKe expression is enhanced by proinflammatory stimulation with LPS (7) and is necessary for the regulation of various proinflammatory proteins (14)]. Furthermore, IKKe expression has been observed in fibroblast-like synoviocytes of patients suffering from rheumatoid arthritis or osteoarthritis (15, 16). However, its potential effects in inflammatory nociception in vivo are unknown. Therefore, we investigated the impact of IKKe in models of inflammatory pain by analyzing behavioral and molecular biological differences between wild type (WT) and IKKe-deficient mice.

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Abbreviations used in this article: COX-2, cyclooxygenase-2; DRG, dorsal root ganglia; IKK, inhibitor-κB kinase; iNOS, inducible NO synthase; i.t., intrathecal (ly); KO, knockout; MMP, matrix metalloprotease; siRNA, small interfering RNA; TBK1, tank-binding kinase 1; WT, wild type.
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Materials and Methods

Animals
Male C57BL/6 mice were obtained from Harlan Winkelmann (Borchen, Germany) at the age of 6–8 wk. Homozygous IKKe−/− mice with a C57BL/6 background were kindly provided by Prof. Shizuo Akira (WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan). In these mice, exons 7 and 8 of the IKKε gene were replaced, resulting in an inactive protein that contains a truncated kinase domain and is still detectable at mRNA and protein levels. IKKe−/− mice are viable, fertile, and healthy (17). IKKe−/− mice were backcrossed with C57BL/6 WT mice. Heterozygous offspring were mated to get WT and IKKe−/− littermates. Genotyping was performed using the following primers, as described: IKKe wild, 5′-TGTGGTTCTCTGGAGGCGATTG-3′; IKKe extra, 5′-AGAAACCGGAAATGAGACCTCAGC-3′, and IKKe neo, 5′-CTTTGCTGAAAACACACTCTGC-3′.

Animals had free access to food and water and were maintained in climate- and light-controlled rooms (24 ± 0.5°C, 12/12-h dark/light cycle). In all experiments, the European ethical guidelines for investigations in conscious animals were followed, and the procedures were approved by the local Ethics Committee for Animal Research. All efforts were made to minimize animal suffering and to reduce the number of animals used. All behavioral experiments were performed by an observer blinded for the genotype in a dedicated room with restriction on sound level and activity.

Drugs
BX795 used as IKKe inhibitor (18) was purchased from Axon Medchem (Groningen, The Netherlands). The drug was dissolved in 50% DMSO/H2O at a concentration of 10 mM. Five microliters of this solution was injected intrathecally (i.t.) into mice (20 g), which corresponded to a dose of 1.5 mg/kg body weight, 30 min prior to the formalin test. Control mice received an injection of the same volume of vehicle.

Determination of BX795 concentrations in the spinal cord
After finishing the formalin test (2–4 h after i.t. injection of BX795), mice were sacrificed, and the spinal cord was dissected, rapidly frozen in liquid nitrogen, and kept at −80°C until further analysis. Quantification of BX795 was done by liquid chromatography (Agilent 1200 Series (Waldbronn, Germany) connected to an HPLC autosampler (Chromtech, Idstein, Germany), coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer AB Sciex 5500 Qtrap (AB Sciex, Darmstadt, Germany). Spinal cord samples were homogenized with PBS to achieve a concentration of 0.1 mg tissue/µl. Twenty microliters of this solution was mixed with 80 µl H2O and 450 µl methanol and centrifuged at 15,000 rpm for 5 min. The supernatant was transferred to a chromatographic vial, evaporated under N2 at 45°C, and reconstituted in 50 µl acetonitrile. Ten microliters of the extract was injected into the liquid chromatography–mass spectrometry system. Desmethylcelecoxib was used as internal standard.

Western blot analysis
For Western blot analysis, mice were injected with either formalin or zymosan in the hind paws. Lumbar spinal cords and DRG (L4-L6) were dissected at the indicated time points. Tissues were homogenized in PhosphoSafe Extraction Buffer (Merck, Darmstadt, Germany) and submitted to cytokine-stimulation experiments.

Preparation of primary cells
Neuronal cultures. For the generation of primary neuronal cell cultures, dorsal root ganglia (DRG) neurons from adult mice were prepared. After dissection, DRG were directly transferred to ice-cold HBSS containing Ca2+/Mg2+free HBSS and treated with 0.25% trypsin (5 mg/ml) and collagenase (5 mg/ml; Biochrom, Berlin, Germany), followed by mechanical separation using a 1-mm Gilson pipette. After two additional digestion steps with trypsin and DNase 1, the suspension was filtered through a 30% BSA gradient to remove cellular waste. To obtain neuron-enriched cultures, the cell suspension was plated onto poly-L-lysine–coated 3.5-cm dishes and incubated for 2 h in neurobasal medium containing B-27 supplement (Invitrogen), 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine at 37°C and 5% CO2. After the cells became adherent, fresh medium containing 0.01 µg/ml murine nerve growth factor (Invitrogen) was added.

Astrocytes. Primary astrocytes were prepared from embryonic mouse brain (embryonic day 15). Brains were dissected, washed in HBSS, and digested for 2 h in 10% trypsin solution at 37°C. After two washes with DMEM containing 10% FCS/1% penicillin/streptomycin, the brains were homogenized and plated onto culture dishes in DMEM with the addition of 10% FCS/1% penicillin/streptomycin and 1% 200 µm glutamine. The cultures were used for two or three passages.

Microglia. For primary microglia cultures, embryonic mouse brains (embryonic day 15) were dissected, washed in HBSS, and digested for 2 h in 10% trypsin solution at 37°C. After two washes with DMEM containing 10% FCS, the brains were homogenized and plated onto culture dishes in DMEM with the addition of 10% FCS and 1% 200 µm glutamine. The culture was then incubated for 10–14 d until microglia could be shaken off and separated from the astrocytes. Then cells were plated onto new dishes and submitted to cytokine-stimulation experiments.

Cell culture
RAW 264.7 mouse macrophages were cultured and incubated in RPMI 1640 medium containing 10% FCS and 1% penicillin/streptomycin. Cells were stimulated with 10 µg/ml LPS for the indicated times, with and without transfection with IKKe–small interfering RNA (siRNA). The following oligonucleotide sequences were used as IKKe-siRNA: sense, 5′-GAUUGAUUGAAUAACAAAT-3′ and antisense, 5′-UUUGUUAGUUCCUCAUGAAUACTG-3′ (Ambion, Applied Biosystems, Darmstadt, Germany). Transfection of 50 nM siRNA was performed using siPORT Amine (Ambion) on two consecutive days. After incubation for 48 h, cells were treated with LPS for the indicated times. siRNA treatment resulted in 60–80% reduction in the IKKe protein level, which was stable throughout the entire incubation period. Transfection efficiency was monitored with a Cy3–labeled siRNA (Silencer Cy3 negative control siRNA #1; Ambion). Fluorescence microscopy and subsequent analysis with Image J software revealed transfection of ~80% of cells in the culture.

Primary cells were incubated with a cytokine mix consisting of 5 ng/ml TNF-α (PeproTech EC, London, U.K.), 1 ng/ml IL-1β (PeproTech EC), and 1 µg/ml LPS (Sigma) for 8 h (neuronal culture) or 24 h (astrocytes and microglia).

Western blot analysis
For Western blot analysis, mice were injected with either formalin or zymosan in the hind paws. Lumbar spinal cords and DRG (L4-L6) were dissected at the indicated time points. Tissues were homogenized in PhosphoSafe Extraction Buffer (Merck, Darmstadt, Germany) containing protease inhibitor (1 mM Pefabloc SC; Alexis Biochemicals) and kept on ice for 30 min. After sonication for 10 s, the suspension was centrifuged at 14,000 rpm for 10 min at 4°C in an Eppendorf centrifuge; the supernatant was stored at −80°C, then thawed, and the proteins were separated in a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred onto nitrocellulose membranes by wet-blotting. To remove non-specific binding, membranes were blocked with 2% BSA in Tris-buffered saline (TBS) and incubated with the antibodies for specific proteins of interest, as detailed in the figure legends, for 1 h in TBS containing 5% milk. After several washes in TBS containing 0.1% Tween 20, the blots were incubated for 60 min with an IRDye 800-conjugated secondary Ab (1:10,000 in blocking buffer; Rockland Immunochemicals) followed by rinsing in 0.1% Tween 20 in PBS. The blots were incubated with the Odyssey Infrared Imaging System (LI-COR Biosciences) and exposed to film.

Proteins (30 µg) were separated electrophoretically by 10% SDS-PAGE and transferred onto nitrocellulose membranes by wet-blotting. To confirm equal loading, all blots were stained with Ponceau red solution. Membranes were blocked for 1 h at room temperature in Odyssey blocking reagent (LI-COR Biosciences) diluted 1:2 in 0.1 M PBS (pH 7.4). The blots were incubated overnight at 4°C with primary Ab against IKKe (80 kDa) or phospho-p65 (Ser536) (1:250; Cell Signaling Technology, Boston, MA) in blocking buffer. After washing three times with 0.1% Tween 20 in PBS, the blots were incubated for 60 min with an IRDye 800- or IRDye 700-conjugated secondary Ab (1:10,000 in blocking buffer; Molecular Probes). After rinsing in 0.1% Tween 20 in PBS, protein–Ab complexes were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences). The Odyssey Infrared Imaging System (LI-COR Biosciences) was used for densitometric analysis. The bands were quantified with Quantity One Software (Bio-Rad, Munich, Germany).

Real-time PCR (TaqMan)
RNA was prepared from the lumbar spinal cords and DRG (L4-L6) using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), according to
the manufacturer’s instructions. RNA from primary cells was prepared with a mirVana miRNA Preparation Kit (Ambion). Two hundred nanograms of total RNA was used for the reverse transcription, which was performed with Random Primers in a Superscript III First-Strand Synthesis System (Invi-
trogen, Karlsruhe, Germany) (tissue) or a Verso cDNA Kit (ABgene) (cells). Twenty nanograms of RNA equivalent was subjected to real-time PCR in an Applied Biosystems sequence detection system ABI7500 using a FastStart Universal Master Mix Kit (Roche Diagnostics, Mannheim, Germany), with SYBR Green fluorescence staining. Expression of IKKe, COX-2, iNOS, MMP-9, c-fos, and TLR4 mRNA was determined and normalized to 18S mRNA, which was detected with VIC-labeled predesigned 18S probe (Applied Biosystems, Weiterstadt, Germany). The following gen-specific primers were used: IKKe: forward, 5′-GTACAAGGCGAGCCGACTGCTCT-3′; reverse, 5′-TCTCCTACTGCAATGATCTT-3′; COX-2: forward, 5′- AGCTGATGATTTCGTTGCTCTT-3′; reverse, 5′-GGGACCACTTCAATCAGGTT-3′; iNOS: forward, 5′-CAAAGGAGCTACACCTTCC-3′; reverse, 5′-CTCTGAGGCTGTACACAGG-3′; MMP-9: forward, 5′-GAAGGACCAACCCTCGTGTGTT-3′; reverse, 5′-AGAAGGTGCCTGTTCGCA-3′; c-fos: forward, 5′-ACCATGATGTTCTCGGTTTCAA-3′; reverse, 5′-GCTGGTGAGAGTGCCTGTCAC-3′; TLR4: forward, 5′- ATGGCTAGCTGCTACACCC-3′; reverse, 5′-GAGGCCATTTTGTCTC- TCCACA-3′.

The cycle number at which the fluorescence signals cross a defined threshold (Ct-value) is proportional to the number of RNA copies present at the start of the PCR. The threshold cycle number for the specific mRNA was determined by subtracting the Ct value of 18S from the Ct value of IKKe.

Results

IKKe mRNA and protein are constitutively expressed in the spinal cord of C57BL/6 mice, as assessed by TaqMan real-time PCR and Western blot analysis. IKKe mRNA and protein levels at all indicated time points after zymosan injection showed a constitutive distribution of IKKe mRNA throughout the gray matter of the spinal cord and an inflammation-induced increase in IKKe mRNA, which was most intense in the dorsal horn where the primary afferent neurons synaptically transmit the nociceptive signal to secondary neurons (Fig. 1C). In DRG, we also observed a constitutive IKKe mRNA and protein expression that was enhanced after peripheral noxious stimulation (Fig. 1D, 1E).

To clarify in which cells basal IKKe is expressed, we performed in situ hybridization with subsequent immunostainings to establish cell markers. Abundant IKKe staining was observed in laminae I and II in the spinal cord. IKKe was also expressed in neurons with NF200 immunoreactivity but not in astrocytes of the spinal cord (Supplemental Fig. 1A, 1B). In DRG, IKKe also colocalized with NF200 and isolectin B4, indicating its expression in neuronal cells (Supplemental Fig. 1C, 1D).

IKKe KO mice show normal motor function and acute nociception

The Rota Rod test was performed to rule out motor function deficits that may interfere with nociceptive testing. WT and IKKe−/− mice were able to balance on the rotating rod up to the cut-off time of 60 s. Twenty microliters of a 5% formaldehyde solution (formalin) was injected s.c. into the dorsal surface of the left hind paw. The time spent licking the formalin-injected paw was recorded in 5-min intervals up to 45 min, beginning immediately after formalin injection.
90 s, demonstrating that the genetic modification does not impair their motor functions. We used Hot Plate and Dynamic Plantar tests to assess the impact of the IKKε deletion on acute thermal and mechanical nociception. The results of both tests revealed no differences between WT and IKKε2/2 mice (Hot Plate: 14.82 ± 0.55 s and 14.60 ± 0.95 s, respectively; Dynamic Plantar: 8.57 ± 0.28 s and 8.38 ± 0.55 s, respectively), indicating that the physiologically important immediate response to acute noxious thermal and mechanical stimulation was intact in IKKε2/2 mice.

Because IKKε shows homology to other I-kB kinases, and a systemic KO of genes is frequently associated with compensatory regulation of similar proteins, we assessed the protein levels of IKKa, IKKβ, and TBK1 in spinal cord protein extracts of IKKε KO and WT mice. The results of the Western blot analysis showed equal protein expression in both genotypes for all kinases tested, suggesting that these proteins were not compensatory regulated in IKKε KO mice (data not shown).

IKKε deficiency attenuates the nociceptive response in inflammatory pain models

The zymosan-induced paw inflammation model was used to assess inflammatory hyperalgesia. In behavioral experiments, WT mice showed a strong decrease in paw-withdrawal latencies upon mechanical stimulation after injection of zymosan, which lasted until the end of the observation period at 48 h, indicating inflammatory hyperalgesia. In the first 5 h after zymosan injection, there were no differences between WT and IKKε2/2 mice. However, in accordance with the IKKε mRNA and protein regulations, the nociceptive response curves started to diverge at 5 h, and latencies returned almost to baseline in IKKε-deficient mice at 48 h after zymosan injection (*p < 0.05, repeated-measures ANOVA) (Fig. 3A). To assess whether the behavioral differences between WT and IKKε KO mice after zymosan injection were due to differences in the release of different proinflammatory cytokines, we analyzed TNF-α and IL-1β concentrations 5 h after zymosan injection. TNF-α levels in the spinal cord were decreased in IKKε−/− mice compared with IKKε+/+ mice (KO, 212.1 ± 26.2 pg/mg protein; WT, 311.7 ± 47.2 pg/mg protein), indicating that TNF-α might be involved in the decreased nociceptive response in KO mice. IL-1β concentrations in spinal cord protein extracts were below the quantification limit. Therefore, IL-1β levels were additionally measured in the serum, where they did not differ between the genotypes (WT, 66.5 ± 16.2 pg/ml; KO, 62.8 ± 8.9 pg/ml).
The zymosan-evoked paw edema, which was analyzed by weighing the inflamed paw compared with the untreated paw at the end of the observation period, did not differ between genotypes (WT, 0.372 ± 0.099 g; IKKε2/2, 0.318 ± 0.093 g), suggesting that the antinociceptive effects are not solely mediated by the anti-inflammatory effects of NF-κB inhibition.

We applied the formalin test as a second model of inflammatory pain. IKKε mRNA, as well as protein levels, were also upregulated in the spinal cord and DRG after peripheral injection of formalin, supporting the results obtained in the zymosan model (Supplemental Fig. 2). Injection of formalin into one hind paw of WT mice induced the typical biphasic paw-licking behavior. Notably, the nociceptive behavior was reduced in IKKε2/2 mice (Fig. 3B).

Statistical analysis revealed that IKKε2/2 mice showed a similar response in the first phase but spent significantly less time licking the formalin-injected hind paw in the second phase of the formalin assay compared with WT mice, suggesting that IKKε contributes to formalin-evoked C-fiber sensitization of pain pathways. Local injection of the IKKε inhibitor BX795 into the spinal cord of C57BL/6 mice (1.5 mg/kg body weight, i.t.) attenuated nociceptive behavior in the formalin test and, hence, provided similar pain protection as did the deletion of IKKε (Fig. 3C). BX 795 concentrations in the lumbar spinal cord were assessed by liquid chromatography–mass spectrometry/mass spectrometry analysis and reached 3.8 ± 1.0 ng/mg tissue, which is in a concentration range that selectively inhibits the IKKe/TBK1 complex (18).
Regulation of NF-κB and IRF-3 activity

NF-κB p65 and IRF-3 transcription factor activity have been analyzed in nuclear extracts from spinal cord tissue. In WT mice, we observed a significant increase in p65 DNA-binding activity in the spinal cord 2 h after formalin injection. This increase was completely abolished in IKKe KO mice. The basal level of NF-κB activity in IKKe−/− mice was similar to that of WT mice (Fig. 4A). We found only very low basal p65 activity in DRG tissue, which was slightly higher in IKKe KO animals. After formalin treatment, we observed similar effects as shown in the spinal cord extracts (Fig. 4B). Based on these data, we performed cell-culture experiments in RAW 264.7 macrophages and downregulated IKKe expression by means of RNA interference. IKKe siRNA transfection led to a stable 60–80% downregulation of IKKe protein levels that lasted for ≥48 h. Western blot analysis of LPS-stimulated control cells revealed an immediate upregulation of phosphorylated p65-Ser536 after 2 and 5 min. Phosphorylation of p65 did not occur in macrophages transfected with IKKe siRNA.

**FIGURE 4.** Regulation of NF-κB and IRF-3 activation. A and B, p65 transcription factor activity, as assessed by TransAM transcription factor ELISA. Nuclear extracts of the spinal cord (A) and the DRG (B) of WT and IKKe KO mice were prepared from control mice and mice 2 h after injection of formalin into the hind paws (n = three mice/group); darker columns, control; lighter columns, 2-h formalin. The insets show the basal NF-κB activity in spinal cord and DRG of WT and IKKe KO mice. C, Western blot showing serine 536 phosphorylation of p65 in LPS-stimulated RAW 264.7 macrophages with (gray columns) and without (black columns) treatment with IKKe-siRNA. D, Western blot showing IKKe protein levels after different treatments of RAW 264.7 cells. Cells were transfected with siRNA for 48 h and then treated with LPS for 24 h. The blots show a representative result; the diagram shows the densitometric analysis of three independent experiments. E, IRF-3 transcription factor activity, as assessed by TransAM transcription factor ELISA. Nuclear extracts of the spinal cord of naive WT mice, as well as 30 min and 2 h after injection of formalin in the hind paws (n = 3 mice/group). *p < 0.05, **p < 0.01, ***p < 0.001. #, scrambled siRNA; C, control; TR, transfection reagent.
and MMP-9, this increase was not disturbed in IKKε after formalin injection in WT mice. In contrast to COX-2, iNOS, and TLR4, the induction of c-fos and MMP-9 mRNA was completely abolished in IKKε−/− mice. To analyze whether these effects were limited to NF-κB–dependent genes, we further investigated the regulation of c-fos and TLR4, which are NF-κB–independent genes, in the spinal cord extracts, the cellular source remained elusive. Therefore, we investigated mRNA expression of COX-2 and iNOS, and MMP-9 in the spinal cord (Fig. 5) after formalin injection by real-time PCR. Formalin injection evoked a significant upregulation of all three transcripts 2 h after formalin injection in WT animals. Interestingly, the induction of COX-2, iNOS, and MMP-9 mRNA was completely abolished in IKKε−/− mice. To analyze whether these effects were limited to NF-κB–dependent genes, we further investigated the regulation of c-fos and TLR4, which are NF-κB independent but also upregulated in inflammatory processes. Accordingly, their expression increased 2 h after formalin injection in WT mice. In contrast to COX-2, iNOS, and MMP-9, this increase was not disturbed in IKKε−/− mice, indicating that a KO of IKKε affected only NF-κB–regulated genes (Fig. 5). Baseline levels of all mRNAs investigated did not differ between the genotypes (data not shown).

Because mRNA analyses were performed in complete spinal cord extracts, the cellular source remained elusive. Therefore, we investigated mRNA expression of COX-2 and iNOS in primary neuron, astrocyte, and microglia cell culture by quantitative RT-PCR and performed communofluorescence studies for iNOS in the spinal cord. We found low levels of COX-2 and iNOS mRNA in untreated primary cells. Treatment with a cytokine mix for 1 and 6 h led to a time-dependent increase of these genes in all three cell types, which was most pronounced for iNOS in astrocytes and microglia 6 h after stimulation (Supplemental Fig. 3). In accordance with these cell-culture results, we found low levels of iNOS in spinal cord slices of untreated control mice, which increased in astrocytes and microglia 24 h after formalin injection into the hind paw (Supplemental Fig. 4). In summary, these results indicated that, similarly to IKKε, its downstream targets are regulated in a number of different cells in the nervous system that cooperate in the processing of the painful inflammatory response.

**Discussion**

The present study was designed to clarify whether the recently detected IKKε is involved in inflammatory nociception and, if yes, whether this process is mediated by modulation of NF-κB activity. We showed that IKKε mRNA and protein are expressed in DRG and spinal cord and are upregulated during paw inflammation. Neurons and glia apparently contribute to this adaptation because we observed an upregulation of IKKε protein in primary cultured neurons, astrocytes, and microglia upon inflammatory stimulation. IKKε-deficient mice show attenuated nociceptive behavior in inflammatory models. This effect could be mimicked pharmacologically in C57BL/6 mice by treatment with the IKKε inhibitor BX795. From these results, we inferred that IKKε at basal levels is involved in early inflammatory nociception, as shown in the formalin test, whereas its upregulation contributes to the sensitization of the nervous system during inflammation and the manifestation of hyperalgesia, as observed in the late phase of zymosan-induced paw inflammation. We further showed that IKKε deficiency abolishes inflammation-evoked NF-κB activation and DNA-binding activity and prevents upregulation of NF-κB–dependent proinflammatory genes. Therefore, in contrast to former studies that mainly focused on IKKε as an antiviral IFN-regulating kinase, our results suggested that the in vivo effects of IKKε in inflammatory pain are mediated through NF-κB–signaling cascades.

We found expression of IKKε in fibers in the superficial dorsal horn of the spinal cord and the isolecitin B4+ primary C-fiber afferent neurons of the DRG. This suggested a participation of IKKε in nociceptive processing, because isolecitin B4 defines a population of nociceptive small-diameter, nonmyelinated DRG neurons that essentially contribute to peripheral sensitization and the development of inflammatory hyperalgesia (22). In contrast, IKKε also colocalized with NF200+ large nonnociceptive neurons and seems to be pan-neuronal in the DRG, indicating that, in addition to its potential role in nociception, it might fulfill other functions in the nervous system. The abundant distribution of IKKε in nociceptive and nonnociceptive neurons of the DRG and the spinal cord is in line with the pan-neuronal expression of NF-κB at these sites and suggested that baseline IKKε is involved in the physiological constitutive NF-κB activation state in DRG and spinal cord neurons (23). Irrespective of these physiological functions, IKKε activation upon noxious stimulation of the hind paws and subsequent NF-κB activation apparently contribute to inflammatory nociception.
hyperalgesia supported by results in two models of inflammatory nociception: the formalin assay, which involves a sensitization of C-fiber neurons and synapses in the second phase (24), and the zymosan-induced paw inflammation model, which represents persistent inflammatory hyperalgesia (25, 26). Our data indicated that IKKe is a modulator of inflammatory sensitization but does not affect acute physiological pain, because IKKe deficiency did not affect responses to acute heat or mechanical stimuli or the first phase of the formalin assay, which is due to chemical stimulation of A-fiber nociceptive neurons (27). The inhibitory effects of IKKe deletion on inflammatory nociception is in accordance with studies that showed that IKKe is upregulated in inflamed joint tissue in patients with rheumatoid arthritis and osteoarthritis (15, 16), modulated by proinflammatory stimuli, and responsible for the regulation of several proinflammatory genes (14). It might be suggested that the depletion of IKKe is compensated by upregulation of other IKKs. However, we found that protein levels of IKKα, IKKβ, and TBK1 were similar in WT and IKKe KO mice. Therefore, compensatory protein regulations are unlikely. Furthermore, the antinociceptive effect in two inflammatory models is apparently existent, and the results from KO mice could be confirmed in experiments using the IKKe inhibitor BX795, which leads to similar effects in the formalin assay. Therefore, a compensatory regulation of other IKKs can be excluded.

The role of IKKe as an NF-κB–activating kinase has been controversial in recent years. Several reports indicated that IKKe is not involved in NF-κB signaling and plays its major role in the regulation of IFN-β, whereas others described coordinated regulation of both IFN regulatory factors and NF-κB (reviewed in Refs. 3, 28). On the one hand, our data indicated that IRF3 is not activated during inflammatory hyperalgesia and, therefore, is unlikely to substantially contribute to nociceptive sensitization in these models. On the other hand, we showed that formalin-induced NF-κB activation in the spinal cord is almost completely abrogated in IKKe KO mice, which were also devoid of an upregulation of the proinflammatory factors COX-2, iNOS, and MMP-9. These enzymes are typically NF-κB–regulated genes that contribute to inflammatory nociception (29–32). Moreover, COX-2, MMP-9, and iNOS have no binding sites for IRF-3 or -7 in their promoter regions. Hence, they are probably not controlled by the IRF pathway. These results strongly suggested that the decreased inflammatory hyperalgesia in IKKe KO mice is independent of type I IFN responses and is more likely dependent on disturbed NF-κB binding to the promoter regions of these genes. Upregulation of NF-κB–independent inflammation-associated genes was not affected in IKKe−/− mice. Further cell-culture experiments with IKKe siRNA indicated that an impaired phosphorylation of p65 serine 536 might be the underlying molecular mechanism for the NF-κB inhibition.

NF-κB has been implicated in pathophysiological pain control in many studies (reviewed in Ref. 1). For example, NF-κB p50 KO mice exhibit reduced nociceptive responses due to a decreased expression of NF-κB–dependent proinflammatory genes (33). An inhibitor of the IKK subunit of the classical IκB kinase complex provided similar anti-inflammatory properties as observed in the current study in IKKe KO mice or after pharmacological inhibition in WT mice (34, 35). Furthermore, mice with a conditional KO of IKKβ in sensory afferent neurons demonstrated somewhat decreased hyperalgesia in the second phase of the formalin assay (36). Because it was assumed that the classical complex is inflammation specific and almost free from substrates, apart from the NF-κB–activation pathway (5), it was hypothesized that it constitutes an ideal target for anti-inflammatory drugs. However, in the meantime, several other target proteins for the different kinase subunits have been identified, and the fact that KO mice for all three “classical” IKKs die embryonically or shortly after birth (37–40) suggests that therapy in humans might be hampered by several risk factors when inhibiting the classical complex. Because IKKe mice are viable and fertile (17), and we and other investigators showed that IKKe is involved in inflammatory processes, this kinase might be the more promising target for anti-inflammatory therapy. However, much additional work is needed to confirm its potential usefulness as a drug target. In summary, our data suggest that IKKe is involved in the development and maintenance of inflammatory hyperalgesia by activation of the NF-κB–signaling pathway.

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Disclosures
The authors have no financial conflicts of interest.

References
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Supplemental Figure 1

A  IKKε sense probe  IKKε antisense probe

B

IKKε/IB4  
IKKε/NF200  
IKKε/GFAP

C  IKKε sense probe  IKKε antisense probe

D

IKKε/IB4  
IKKε/NF200
Supplemental Figure 3

A astrocytes

B microglia

C neurons
Supplemental figure 4

- naive
- 24h formalin

- iNOS/GFAP
- iNOS/NeuN
- iNOS/CD45
- iNOS/Iba1
Supplement

Supplemental Figures

**Supplemental Figure 1: Localisation of IKKε in the spinal cord and the dorsal root ganglia**

(A) Representative in situ hybridizations (ISH) showing IKKε mRNA expression in the spinal cord (one of 3 independent experiment, n = 3 mice/group). A sense probe has been used as negative control. Scale Bar: 250 µm. The dotted line shows the region which is enlarged in (B). (B) ISH combined with immunohistochemistry in the spinal cord using cell markers of central terminals of non-myelinated nociceptive afferents (IB4), large myelinated non-nociceptive neurons (NF200) and astrocytes (GFAP), respectively. IKKε has been stained with HnPP (red), cell markers with Alexa Fluor 488 (green). The images show (from left to right side): IKKε alone, cell marker alone and merged (representative result from 3 independent experiments). (C) Representative in situ hybridizations (ISH) showing IKKε mRNA expression in the DRGs (one of 3 independent experiment, n = 3 mice/group). (D) ISH combined with immunofluorescence in the DRGs. Scale Bar: 10µm

**Supplemental Figure 2: Regulation of IKKε in the spinal cord and the DRGs after formalin-induced inflammatory nociception**

(A) Quantitative Taqman PCR using spinal cord mRNA from animals with and without formalin-treatment for 2 and 8 h, respectively (n = 3). (B) Time course of the IKKε protein expression in the lumbar spinal cord after injection of formalin. The Western Blot shows one representative Blot out of 5 independent experiments (n = 4-5 mice/group), the diagram depicts the densitometric analysis of all Blots. (C) In situ hybridization showing IKKε mRNA distribution in the spinal cord with and without peripheral formalin injection. (D)
Quantitative Taqman PCR using DRG mRNA from animals with and without formalin-treatment for 2 and 8 h, respectively (n = 3). (E) Time course of the IKKε protein expression in the DRGs cord after injection of formalin. The Western Blot shows one representative Blot out of 5 independent experiments (n = 4-5 mice/group), the diagram depicts the densitometric analysis of all Blots. * P < 0.05, ** P<0.01, *** P < 0.001.

**Supplemental Figure 3:** Regulation of NF-κB dependent gene expression in different cell types after inflammatory stimulation

Regulation of iNOS and COX-2 mRNA in primary astrocytes (A), microglia (B) and neurons (C) after stimulation with a cytokine mixture for 1 and 6 h, respectively, analyzed by quantitative RT-PCR. n = 3 independent incubations/cell type. * P < 0.05, ** P<0.01, *** P < 0.001.

**Supplemental Figure 4:** Regulation of iNOS in the spinal cord after inflammatory stimulation

Upper Panel: iNOS protein expression in the dorsal horn of the spinal cord with and without peripheral treatment with formalin for 24 h. Lower panels: Co-immunofluorescence of iNOS (red) and different cell markers (green) of astrocytes (GFAP), neurons (NeuN), leukocytes (CD45) and microglia (Iba1) in the spinal cord. Scale Bar: 10µm