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The Protein Kinase IKKε 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 (IKKε) 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 IKKε plays a role in the regulation of NF-κB activity, but the distinct mechanisms of IKKε-mediated NF-κB activation are not clear. Given the paramount role of NF-κB in inflammation, we investigated the regulation and function of IKKε in models of inflammatory hyperalgesia in mice. We found that IKKε 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. IKKε knockout mice showed normal nociceptive responses to acute heat or mechanical stimulation. However, in inflammatory pain models, IKKε-deficient mice exhibited a significantly reduced nociceptive behavior in comparison with wild type mice, indicating that IKKε 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, IKKε–deficient mice showed normal nociceptive responses to acute heat or mechanical stimulation. However, in inflammatory pain models, IKKε-deficient mice exhibited a significantly reduced nociceptive behavior in comparison with wild type mice, indicating that IKKε 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 IKKε 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 inflammatory 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 IKKγ (also known as NF-κB essential modulator) and the catalytic subunits IKKα and IKKβ (2–4). In most unstimulated cells, NF-κB dimers are localized in the cytoplasm and sequestered by binding to the inhibitory subunit I-κB. Upon activation by inflammatory stimuli, such as cytokines and bacterial LPS, I-κB is phosphorylated by I-κB kinases, subsequently ubiquitinylated, and degraded by a proteasome complex. Degradation of I-κB 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 metalloproteases [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 IKKε and tank-binding kinase 1 (TBK1), which are structurally similar to the classical IKKs. However, unlike classical IKKs, IKKε 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 I-κBα, I-κB 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, IKKε expression is enhanced by proinflammatory stimulation with LPS (7) and is necessary for the regulation of various proinflammatory proteins (14)]. Furthermore, IKKε 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 IKKε in models of inflammatory pain by analyzing behavioral and molecular biological differences between wild type (WT) and IKKε-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 IKKe 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′-TCTTCTTCCTTACGCCGGTTGC-3′; IKKe extra, 5′-AGAAAGCGGATAATGGACTCCAGC-3′, and IKKe neo, 5′-CTCTCCAGAACCACACTCTCG-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 light/dark 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 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 HTC PAL autosampler (Chromtech, Idstein, Germany) (20) using a 1-ml 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 extract was injected into the liquid chromatography–mass spectrometry/mass spectrometry system was operated in gas 1 and 2 were 50 and 50 %.

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 mM 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 mM 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′-GAUUGAUUGAGAUAACAAAATG-3′ and antisense, 5′-UUUGUUAAUCCUCAUGAUUGTT-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, Lauen, Switzerland) immediately after preparation. To remove cellular debris, extracts were centrifuged at 14,000 rpm for 1 h at 4°C, and supernatants were stored at −80°C.

RAW 264.7 cells were seeded in six-well plates at a density of 3 × 10^5 cells/well. At 80% confluency, cells were stimulated with LPS for the indicated time points with and without transfection with IKKe siRNA. At the end of the incubation period, cells were washed with 0.1 M PBS, scraped with a rubber policeman, and collected in 1.5-ml tubes. After a short centrifugation, the pellet was resuspended 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 until further analysis.

Proteins (30 μg) were separated electrophoretically by 10% SDS-PAGE and then transferred onto nitrocellulose membranes by wet-blotting. To confirm equal loading, all blots were stained with Poncova red solution. Membranes were blocked for 60 min at room temperature in Odyssey blocking reagent (LI-COR Biosciences) diluted 1:2 in 0.1 M PBS (pH 7.4). Then 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 ringing in 0.1% Tween 20 in PBS, protein–Ab complexes were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences). IRDye 800 (70 kDa) and IRDye 700 (68 kDa) were imaged separately. IB2 mouse anti-phospho-ERK-2 (42 kDa) and β-actin (37 kDa) (1:1200; Sigma, Germany) were used as loading controls. Densitometric analysis of the blots was performed 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 (Invitrogen, Karlsruhe, Germany) (cDNA) 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 AB7500 using a FastStart Universal Master Mix Kit (Roche Diagnostics, Mannheim, Germany), with SYBR Green fluorescent 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 predeveloped 18S probe (Applied Biosystems, Weiterstadt, Germany). The following gen-specific primers were used: IkKeF: forward, 5′-GTCAAGCCCGGAACAG-A3′; reverse, 5′-TCCCTACTGCAATGTGGT-3′; COX-2: forward, 5′-AGAAGATGTGCTGATTC-3′; reverse, 5′-GGATCCACACAACAAAGATCC-3′; iNOS: forward, 5′-CCAAGCCCTACTAC-3′; reverse, 5′-CTCTAGGACGTGACACAAGG-3′; MMP-9: forward, 5′-GAAGGCACCAACTCCTGCTGTT-3′; reverse, 5′-AGATGACTGGTTGCCAGGA-3′; c-fos: forward, 5′-ACCATGATGTTCCTGGGTTTCA-3′; reverse, 5′-GGCTGGTTGAGATTGTCCTAC-3′; TLR4: forward, 5′-ATGGCATGGCTTACACCACC-3′; reverse, 5′-GGGCCATTTTGTTCTC-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 standardized by subtracting the Ct value of 18S from the Ct value of IkKe, COX-2, iNOS, MMP-9, c-fos, or TLR4 of the same sample. Relative threshold (Ct-value) is proportional to the number of RNA copies present at the beginning of the reaction. Relative expression and fold change were calculated and expressed as fold change of a reference control sample (untreated control mice of either genotype or untreated cells).

**Transcription-factor analysis**

Nuclear extracts from spinal cord and DRG were prepared using a commercially available Nuclear Extract Kit, according to the manufacturer’s instructions (Active Motif, Riexensart, Belgium). These nuclear extracts were then subjected to TransAM NF-κB-p65 and IRF-3 transcription factor ELISAs, according to the manufacturer’s instructions (Active Motif). Briefly, 10 μg of protein from spinal cord or DRG extracts was used. The colorimetric read-out was measured photometrically and is proportional to transcription factor activity.

**Cytokine ELISA**

ELISA kits for the analysis of TNF-α or IL-1β were purchased from Biotrend (Cologne, Germany; assay designs, mouse enzyme immune assays). For the assessment of TNF-α concentrations, 30 μg spinal cord protein extracts from IkKe knockout (KO) and WT mice dissected 5 h after injection of zymosan into one hind paw were used. Because no measurable results could be obtained for IL-1β from spinal cord protein extracts, we used serum from the respective mice to analyze this cytokine. ELISAs were performed, according to the manufacturer’s instructions.

**Behavioral testing**

IkKe−/− and IkKe+/− littermates were used in all behavioral tests. Animals were habituated to the experimental room and were investigated by an observer blinded to the genotypy.

**Rota Rod test.** Motor coordination was assessed with a Rota Rod Treadmill (Ugo Basile, Comerio, Italy) at a constant rotating speed of 32 rpm. All mice had five training sessions before the day of the experiment. The fall-off latency was averaged from five tests. The upper cut-off time was 90 s.

**Mechanical sensitivity.** Paw-withdrawal latency to mechanical stimulation was assessed with an automated testing device consisting of a steel rod that is pushed against the plantar surface of the paw with increasing force until the paw is withdrawn (Dynamic Plantar Aesthesiometer; Ugo Basile, Varese, Italy). The maximum force was set at 5 × g to prevent tissue damage, and the ramp speed was 0.5 g/s (cut-off, 20 s). Mice were placed in test cages with a metal grid bottom. They were kept in the test cages for 1 h to allow accommodation. The paw-withdrawal latency was obtained as the mean of four to six consecutive trials at each time point.

**Hot-plate test.** Animals were placed into a Plexiglas cylinder on a heated plate maintained at 52 ± 0.2°C (Ugo Basile), and the latency to jump or shake/flutter of a hind paw was recorded. Each animal was tested only once, because repeated testing in this assay can lead to latency changes (19). The cut-off time was 30 s.

**Formalin test.** The formalin test was performed, as described (20). Briefly, mice were placed in a Plexiglas cage and were allowed to habituate for 30 min. 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.

**Zymosan-induced paw inflammation, mechanical hyperalgesia.** Paw-withdrawal latency was assessed using a Dynamic Plantar Aesthesiometer, as described above. After baseline measurements, hind paw inflammation was induced by s.c. injection of 20 μl a 1% mg/ml zymosan A (Sigma-Aldrich, Munich, Germany) suspension in PBS (0.1 M [pH 7.4]) into the mid-plantar region of the left hind paw (21). The mean of four consecutive trials, with 10-s intervals, was determined hourly up to 8 h, as well as at 24 and 48 h after zymosan injection.

At the end of the observation period (48 h after zymosan injection), animals were deeply anesthetized with isoflurane and killed by cardiac puncture. Both hind paws were cut and weighed, and the difference between the inflamed and the noninflamed control paw was used to assess the inflammatory paw edema.

**Data analysis**

Statistical evaluation was done with SPSS 17.0 for Windows. Data are presented as mean ± SEM. Data were either compared by univariate ANOVA, with subsequent t tests using a Bonferroni α-correction for multiple comparisons, or by the Student t test. For analysis of inflammatory hyperalgesia in the zymosan-induced paw inflammation and the formalin test, repeated-measures ANOVA was performed. For all tests, p < 0.05 was considered statistically significant.

**Results**

**IkKe expression and regulation**

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. Inflammatory noxious stimulation induced a significant upregulation of mRNA and protein levels at all indicated time points after zymosan injection into the hind paws (Fig. 1A, 1B). In situ hybridization of the spinal cord 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 with established 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). Immunofluorescence experiments with markers for microglia and leukocytes (CD45) revealed no expression of IkKes in these cell types (data not shown). To further define the expression of IkKE and its regulation after inflammatory stimulation in different cell types, we investigated primary neurons, astrocytes, and microglia. Western blot analysis revealed only low protein levels of IkKe in all cell types. However, after stimulation with proinflammatory agents, the protein expression significantly increased in neurons, astrocytes, and microglia, indicating that neurons and glia contribute to IkKe up-regulation in the nervous system upon peripheral inflammatory stimulation (Fig. 2).

**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...
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 IKKe deletion on acute thermal and mechanical nociception. The results of both tests revealed no differences between WT and IKKe<sup>−/−</sup> 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 IKKe<sup>−/−</sup> mice.

Because IKKe 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 IKKe 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 IKKe KO mice (data not shown).

**IKKe 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 IKKe<sup>−/−</sup> mice. However, in accordance with the IKKe mRNA and protein regulations, the nociceptive response curves started to diverge at 5 h, and latencies returned almost to baseline in IKKe-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 IKKe 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 IKKe<sup>−/−</sup> mice compared with IKKe<sup>+/+</sup> mice (*p < 0.05, repeated-measures ANOVA) (Fig. 3B). 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). Time course of the licking behavior in WT and IKKε−/− mice (n = 9/group) after injection of formalin (5%, 20 μl) into the left hind paw. Formalin was injected at time 0, and the time spent licking the injected paw was measured in 5-min intervals for 45 min. C, Time course of the licking behavior (n = 8/group) after formalin injection into the left hind paw of vehicle-treated control mice or after i.t. injection with BX795 (1.5 mg/kg body weight). *p < 0.05, **p < 0.01. Black square, WT; black diamond, IKKε−/−; dark gray circle, control; light gray triangle, BX795.

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).

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FIGURE 2. Regulation of IKKe in primary cell cultures. Protein expression of IKKe with and without stimulation with a cytokine mix (CM) for 8 or 24 h in primary astrocytes (A), primary neurons (B), or primary microglia (C). Blots show representative results from three to five independent incubations. Bar graphs show the densitometric analysis of all blots. *p < 0.05.

FIGURE 3. Inflammatory hyperalgesia. A, Time course of mechanical hyperalgesia in WT and IKKε−/− mice after injection of 10 mg/ml (20 μl) zymosan into a hind paw. The diagram shows the Δ paw withdrawal latencies (ΔPWL) in response to mechanical stimulation, as assessed with a Dynamic plantar aesthesiometer (n = 8 mice/group). B, Time course of the licking behavior in WT and IKKε−/− mice (n = 9/group) after injection of formalin (5%, 20 μl) into the left hind paw. Formalin was injected at time 0, and the time spent licking the injected paw was measured in 5-min intervals for 45 min. C, Time course of the licking behavior (n = 8/group) after formalin injection into the left hind paw of vehicle-treated control mice or after i.t. injection with BX795 (1.5 mg/kg body weight). *p < 0.05, **p < 0.01. Black square, WT; black diamond, IKKε−/−; dark gray circle, control; light gray triangle, BX795.
**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 IKKε KO mice. The basal level of NF-κB activity in IKKε<sup>−/−</sup> 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 IKKε 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 IKKε expression by means of RNA interference. IKKε siRNA transfection led to a stable 60–80% downregulation of IKKε 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 IKKε 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 IKKε 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 IKKε 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 IKKε-siRNA. D, Western blot showing IKKε 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.
These results indicated that IKKe is involved in quick stimulus-evoked NF-κB activation and, therefore, might also contribute to early effects in the animal models.

IRF-3 transcription factor activity in the spinal cord was not affected 30 and 120 min after formalin injection into the hind paws (Fig. 4D), suggesting that IFN signaling and IRF-3 were not involved in formalin-induced nociception.

**Regulation of NF-κB–dependent and -independent genes**

In addition to p65 DNA-binding activity, we analyzed the regulation of the NF-κB–dependent "pain-relevant" genes COX-2, 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 IKKe−/− 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 IKKe−/− mice, indicating that a KO of IKKe 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 immunofluorescence 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 IKKe, 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 IKKe is involved in inflammatory nociception and, if yes, whether this process is mediated by modulation of NF-κB activity. We showed that IKKe 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 IKKe protein in primary cultured neurons, astrocytes, and microglia upon inflammatory stimulation. IKKe-deficient mice show attenuated nociceptive behavior in inflammatory models. This effect could be mimicked pharmacologically in C57BL/6 mice by treatment with the IKKe inhibitor BX795. From these results, we inferred that IKKe 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 IKKe 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 mostly focused on IKKe as an antiviral IFN-regulating kinase, our results suggested that the in vivo effects of IKKe in inflammatory pain are mediated through NF-κB–signaling cascades.

We found expression of IKKe in fibers in the superficial dorsal horn of the spinal cord and the isoleucin B4+ primary C-fiber afferent neurons of the DRG. This suggested a participation of IKKe in nociceptive processing, because isoleucin 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, IKKe 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 IKKe 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 IKKe is involved in the physiological constitutive NF-κB activation state in DRG and spinal cord neurons (23). Irrespective of these physiological functions, IKKe activation upon noxious stimulation of the hind paws and subsequent NF-κB activation apparently contribute to inflammatory...
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 IKKε is a modulator of inflammatory sensitization but does not affect acute physiological pain, because IKKε 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 IKKε 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 IKKε is compensated by upregulation of other IKKs. However, we found that protein levels of IKKα, IKKβ, and TBK1 were similar in WT and IKKε 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 IKKε inhibitor BX795, which leads to similar effects in the formalin assay. Therefore, a compensatory regulation of other IKKs can be excluded.

The role of IKKε as an NF-κB-activating kinase has been controversial in recent years. Several reports indicated that IKKε 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 IRF-3 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 IKKε 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 IKKε KO mice is independent of type I IFN responses and is more likely dependent on NF-κB binding to the promoter regions of these genes. Upregulation of NF-κB-independent inflammation-associated genes was not affected in IKKε−/− mice. Further cell-culture experiments with IKKε 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 IKKε 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 IKKε mice are viable and fertile (17), and we and other investigators showed that IKKε 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 IKKε 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.

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