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The Role of Nuclear Factor κB in Tumor Necrosis Factor-Regulated Transcription of the Human μ-Opioid Receptor Gene

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ABSTRACT

Opioids and their receptors are key players in a cross-talk between the nervous and immune systems. For example, the endogenous opioid system is activated during inflammation as a physiological feedback mechanism to attenuate inflammatory pain. Herein, we report that in primary human T lymphocytes, Raji B cells, U937 monocytes, primary human polymorphonuclear leukocytes, and mature dendritic cells, the proinflammatory cytokine tumor necrosis factor induced μ-opioid receptor gene transcription. Transcriptional induction of the gene in immune cells was mediated via tumor necrosis factor receptor type 2. Using selective in vivo disruption of possibly involved transcription factors with decoy oligonucleotides, nuclear factor-κB was identified as the factor responsible for induction of the gene in immune cells, whereas activator protein-1 was found to be uninvolved. Nuclear factor-κB also mediates upregulation of μ-opioid receptors in neuronal cells stimulated with tumor necrosis factor. Among six putative nuclear factor-κB binding sites on the μ-opioid receptor gene promoter, three *cis*-active elements at nt −2174, −557, and −207 were identified using transfection experiments of reporter gene constructs, electrophoretic mobility shift assays, and in vivo binding studies with decoy oligonucleotides. An allelic variation within the −557 element significantly reduced its *trans*-activating potency, which may affect regulation of the μ-opioid receptor gene in persons carrying this mutation. This study suggests a regulatory function of tumor necrosis factor in opioid-mediated processes in neuronal and immune cells, with possible impact on the complex of inflammation-induced analgesia.

Opioids are typically associated with phenomena such as analgesia, respiratory depression, euphoria, and addiction, which are mediated by three different opioid receptors termed μ, δ, and κ (Kieffer, 1995). In connection with the immune system, endogenous opioids, such as β-endorphin, and exogenous opioids, such as morphine, are potent immunomodulators with inhibitory, and stimulatory effects on immune function. These include lymphoid organ atrophy, changes in $CD4^+$ and $CD8^+$ expression in thymocytes, reduced natural killer cell activity (Weber and Pert, 1989; Gaveriaux-Ruff et al., 1998), the balance between T helper type 1 and type 2 cells (Roy et al., 1999; Sacerdote et al., 2000), lipopolysaccharide-induced production of IL-6 and TNF, activity of the transcription factor NFκB in macrophages and peripheral blood mononuclear cell cultures (Chao et al., 1993; Roy et al., 1998a), and chemotactic responsiveness in thymocytes (McCarthy et al., 2001). These findings indicate the presence of μ-opioid receptors on various immune cells, because morphine binds preferentially this receptor subtype. The hypothesis that μ-opioid receptors are expressed on immune cells is further supported by recent observations demonstrating that endomorphins, which are extremely selective for this receptor subtype, have diverse immunomodulatory properties as well (Azuma and Ohura, 2002). However, transcription of μ-opioid receptors in immune effector cells does not proceed constitutively; rather, it is regulated by cytokines such as IL-1 (Vidal et al., 1998) and IL-4 (Kraus et al., 2001).

The cytokine TNF regulates a plethora of vital functions in the whole body, under both physiological and pathophysiological conditions (e.g., inflammation). Evidence for alterations within the opioid system in inflammation, which include changes in the levels of endogenous opioid peptides, as well as their receptors, is presented in numerous reports (Stein et al., 1990; Czlonkowski et al., 1993; Ji et al., 1995; Pol et al., 2001; Buzas et al., 2002; Cahill et al., 2003). An

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ABBREVIATIONS: IL, interleukin; NFκB, nuclear factor κB; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction; bp, base pair(s); tk, thymidine kinase; AP, activator protein; STAT, signal transducer and activator of transcription; nt, nucleotide; wt, wild type; CAT, chloramphenicol acetyl transferase.

earlier study demonstrated that TNF up-regulates μ-opioid receptors in neuronal cells via the transcription factor NFκB; however, a detailed promoter analysis identifying binding sites for this factor remains to be done (Börner et al., 2002).

Here, we addressed the question of whether TNF induces transcription of μ-opioid receptors in immune effector cells, and we investigated molecular mechanisms of this regulation. The most prominent transcription factors modulating target genes in response to TNF are NFκB and AP-1 (Baud and Karin, 2001). Using an approach in which function of selected transcription factors can be specifically disrupted in vivo by decoy oligonucleotides, we directly identified NFκB as the responsible transcription factor for TNF induction of μ-opioid receptor transcription in immune cells. Finally, NFκB binding sites were localized on the human μ-opioid receptor gene promoter to link the action of this transcription factor to transcriptional regulation of the gene in neuronal and immune cells.

Materials and Methods

Cell Culture, Reagents, Antibodies, and Cytokines. Isolation and cultivation of all primary immune cells, cultivation of Raji and U937 immune cell lines, and maturation of dendritic cells, have been described in detail (Kraus et al., 2001). All cytokines and the anti-human TNFR1 and TNFR2 antibodies (mab225 and mab226) were purchased from R&D Systems (Wiesbaden, Germany). Cycloheximide (Sigma, Taufkirchen, Germany) was used at a concentration of 10 μg/ml. Sulfasalazine (Sigma) was dissolved in cell culture medium and added at a concentration of 1 mM 30 min before TNF.

Conventional RT-PCR from Human Immune Effector Cells. Total RNA was extracted using RNeasy columns (QIAGEN, Hilden, Germany) and checked for accidental degradation on an agarose gel. Two micrograms were used for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase, RNase H− (Promega, Mannheim, Germany), and diluted to 100 μl. GAPDH control RT-PCR, and amplification of μ-opioid receptor-specific transcripts has been described in detail previously (Kraus et al., 2001).

Quantitative Real-Time PCR. Real-time RT-PCR with Raji and U937 cDNAs was performed according to the supplier's manual in a total volume of 20 μl on a LightCycler instrument using the LightCycler fast-start DNA master SYBR green I kit (both from Roche, Mannheim, Germany). To calculate the amounts of μ-opioid receptor transcripts relative to the housekeeping gene GAPDH, a 163-bp fragment of this gene was amplified (forward primer 5′-CAACTACATGGTTTACATGTTC-3′, reverse primer 5′-GCCAGTGGACTCCACGAC-3′, located on different exons) under the following conditions: 1) preincubation for 7 min at 95°C; 2) 50 cycles for 5 s at 95°C, 5 s at 64°C, and 10 s at 72°C. μ-Opioid receptor transcripts were amplified as follows: 1) preincubation for 8 min at 95°C. 2) 50 cycles for 5 s at 95°C, 10 s at 65°C, and 18 s at 72°C. The sequences of μ-opioid receptor primers were 5′-GATCATGGCCCTCTACTCCA-3′ (located at position 216 in exon 1) and 5′-GCATTTCGGGGAGTACGGAA-3′ (located at position 557 in exon 2, to avoid amplification of genomic DNA).

Reporter Gene Plasmids. Construction of the human μ-opioid receptor promoter reporter constructs −2624, −2229, −1372, −779, −2478/−2287, and −1854/−1224 has been described earlier (Kraus et al., 2001; Börner et al., 2002). The 5′-end deletion constructs −1854, −254, and −174 were made by cutting the −2624 plasmid with *Hin*dIII and *Acc*I, *Pst*I, and *Blp*I, respectively, and religation of the blunted ends. For the −2229/−1854 construct, a *Sau*I-*Acc*I fragment of the human μ-opioid receptor upstream region was inserted in front of the Herpes simplex virus tk promoter of pBLCAT2 (Luckow and Schutz, 1987). Plasmids −1372/−910, −910/−714, −714/−590, and −590/−363 were generated by digestion of the μ-opioid receptor promoter with Sau3A and shotgun cloning of the fragments into pBLCAT2's *Bam*HI site. Insertion of oligonucleotides into pBLCAT2 was performed according to a described method (Kang and Inouye, 1993). All plasmids were sequenced from both sides to ensure correct orientations and sequences of the inserts.

Transfection Experiments. Plasmid DNA was isolated using QIAGEN plasmid kits. Before transfection, cells received fresh medium with 1% fetal calf serum. Raji and U937 cells were transfected as follows: 5×10^6 cells were pelleted, resuspended in 5 ml of medium, and transfected by drop-wise addition of 10 μg of plasmid DNA in 500 μl of a buffer containing 125 mM $CaCl_2$, 140 mM NaCl, 25 mM HEPES, and 0.5 mM Na_2HPO_4, pH 7.05. After a 17-h incubation at 3% CO_2 and 35°C, cells were pelleted again and given fresh medium. SH SY5Y monolayer cells were transfected by the same procedure, but without centrifugation steps. Transient expression of CAT reporter gene products was allowed for a further 48 h with or without 100 U/ml TNF. The CAT enzyme-linked immunosorbent assay (Roche) was performed according to the supplier's manual.

Decoy Oligonucleotide Approach in Immune Cells. Generally, in the decoy approach, double-stranded oligonucleotides with specific binding sequences for transcription factors are brought into living cells to selectively disrupt the function of these factors. In the cells, transcription factors then interact with an excess of decoy oligonucleotides instead of binding to the natural regulatory motifs of genes (Bielinska et al., 1990; Kraus et al., 2003). Decoy oligonucleotides (100 nM) were directly cotransfected either with reporter gene plasmid DNA (for the experiments shown in Figs. 4 and 7) or carrier DNA (the promoterless cloning vector pBLCAT3, for the experiments shown in Fig. 3). The oligonucleotides had no chemical modifications. To avoid any effect on transfection efficiency caused by different amounts of transfected DNA, the total amounts of both plasmid DNA and oligonucleotides were kept constant in the individual experiments by addition of oligonucleotides containing an AP-2 binding site, if necessary. To exclude unspecific effects of decoy oligonucleotides that might be a result solely of their presence, the following controls were performed: 100 nM oligonucleotides containing motifs for transcription factors AP-1, AP-2, or STAT6 did not change inducibility of a NFκB-responsive reporter construct; single-stranded NFκB oligonucleotides were not active as decoys (100 nM sense or antisense strand, or a mixture of 100 nM each of nonannealed sense and antisense strand). Oligonucleotides used are shown in Table 1.

Extraction of Nuclear Proteins and Electrophoretic Mobility Shift Assay. Both procedures have been described recently (Börner et al., 2002). For immunoshift experiments, the p65 NFκB antibody C-20 (Santa Cruz Biotechnology, Heidelberg, Germany) was used.

Results

TNF Induces Transcription of the μ-Opioid Receptor Gene in Various Human Immune Effector Cells. Primary T cells and polymorphonuclear leukocytes, the cell lines

TABLE 1
Oligonucleotides

M1	5′-ATAAGAAGAAAGTTTCCGTAATCA-3′
M2	5′-AAAAAAGGGACTTTCATTGTACTGGT-3′
M3	5′-AGTCTCTAGGAAATCTCTGTAACA-3′
M4	5′-CTATCGAGGAAGTCTTCAGATAAA-3′
M5 wild-type	5′-GAGCATTGGGGTTTTAGGGCTGTT-3′
M5 polymorphic	5′-GAGCATTGGGATTTTAGGGCTGTT-3′
M6	5′-GTGGGAGGGGGCTATACGCAGAGG-3′
classic NFκB	5′-AAAGTTGAGGGGACTTTCCCAGGCCT-3′
AP-1	5′-CGCTTGATGACTCAGCCGGAA-3′
AP-2	5′-TGCGGGCTCCCCGGGCTTGGGCGAGC-3′
STAT6	5′-CAACCTTCTTCTCAGAAGCATATGT-3′
AP-1*mu1*	5′-ACTGAAAGGACTCAGAACTAC-3′
AP-1*mu2*	5′-GGAAAATTGAGTGATGTTAGC-3′

Raji (B cells), U937 (monocytes), and HMEC-1 (microvascular endothelial cells), and, in addition, mature CD83$^+$ dendritic cells were used to study the effect of TNF on the expression of the μ-opioid receptor gene in immune effector cells (Fig. 1A). The human cell line SH SY5Y (neuroblastoma), which is known to express μ-opioid receptors constitutively, served as control. No μ-opioid receptor-specific transcripts could be detected in any of the unstimulated immune cells, even though the highly sensitive nested PCR technique was used. This technique allows detection of as few as approximately five copies of a template, as revealed in control experiments (data not shown). Contrasting with this, μ-opioid receptor gene transcription was strongly induced in all immune cells tested after stimulation of the cells with TNF for 16 h. The amounts of μ-opioid receptor transcripts in the stimulated immune cells were 5 to 20 times less than in the SH SY5Y cells, as revealed by quantitative real-time PCR (data not shown). The following experiments were designed to investigate in greater detail the mechanisms underlying the TNF regulation in Raji cells. Studying the time course, real-time PCR revealed that transcription of the gene initiates between 2 and 6 h after TNF-stimulation (Fig. 1B). Cycloheximide had no effect on TNF-induced transcription of the μ-opioid receptor gene (Fig. 1B).

TNFR2 Mediates Induction of μ-Opioid Receptor Transcription in Raji Cells. Neutralizing antibodies against TNFR1 (p55, TNFRSF1A) and TNFR2 (p75, TNFRSF1B) were used to determine the TNFR by which the effect on μ-opioid receptor gene transcription is mediated (Fig. 2). These antibodies lack agonistic properties, which has been observed for some TNFR antibodies, and are suitable to neutralize effects of TNF (Matthews and Neale, 1987). Addition of TNFR1 antibodies before stimulation of Raji cells with TNF had no inhibitory effect. In contrast, μ-opioid receptor transcription was blocked in a concentration-dependent manner by TNFR2 antibodies, suggesting that the TNF effect is mediated solely via the TNFR2.

Induction of μ-Opioid Receptor Transcription in Immune Cells Depends on NFκB, but Not on AP-1. Next, we addressed the question of whether the transcription factor AP-1 or NFκB, or possibly both, mediates TNF-induction of μ-opioid receptor transcription. We used an approach that combined selective disruption of transcription factor function using AP-1 and NFκB decoy oligonucleotides and quantitative real-time PCR to monitor μ-opioid receptor-specific transcripts (Fig. 3). Consistent with the results shown in Fig. 1, no μ-opioid receptor transcripts were observed in unstimulated Raji and U937 cells (data not shown). TNF-induced μ-opioid receptor transcription in these cells is displayed in curves 1 and 5 (Fig. 3). This TNF-induced transcription was negligibly affected by decoy oligonucleotides containing an AP-1 binding site (curves 2 and 6), suggesting that AP-1 is not involved in mediating the TNF effect. In contrast, loss of NFκB function by transfection of NFκB decoy oligonucleotides completely inhibited transcriptional induction of the μ-opioid receptor gene in TNF-stimulated immune cells (Fig. 3, curves 3, 4, 7, and 8), which directly demonstrates that the

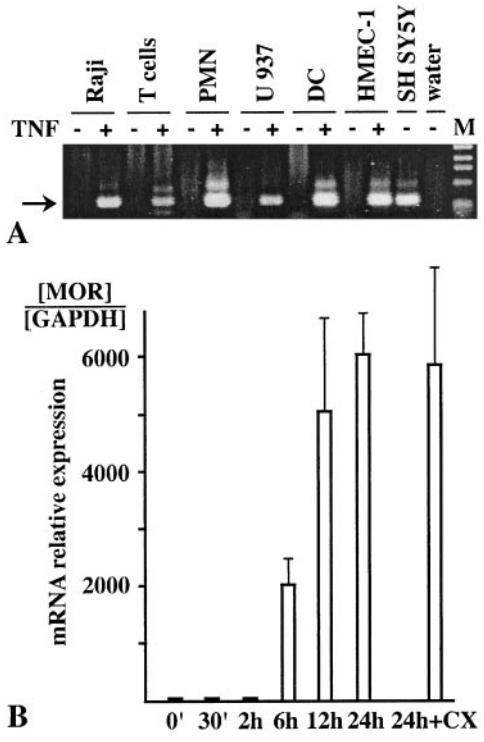


Fig. 1. TNF induces μ-opioid receptor transcription in human immune effector cells. A, detection of transcripts in immune cells. cDNAs from TNF-stimulated (100 U/ml for 16 h; +) and unstimulated (−) Raji B cells, primary T cells, and polymorphonuclear leukocytes (PMN), U937 monocytes, mature dendritic cells (DC), microvascular endothelial cells (HMEC-1), and neuroblastoma cells (SH SY5Y, controls) were subjected to RT-PCR. The arrow indicates μ-opioid receptor-specific 342-bp amplification products using amounts of cDNAs that produced comparable amounts of GAPDH amplificates. A typical gel is shown. M, *Hae*III-digested ϕX174 size marker (band-sizes from top to bottom: 1353 bp, 1078 bp, 872 bp, 603 bp, and 281/217 bp double band). B, time kinetic of μ-opioid receptor transcription initiation in Raji cells and effect of cycloheximide. Raji cells were stimulated with 100 U/ml TNF for different times as indicated. The 24-h incubations were tested with and without cycloheximide (CX). RNA and cDNA were prepared and subjected to real-time RT-PCR. The relative amounts of μ-opioid receptor mRNA at the different time points are plotted after normalization to equal GAPDH mRNA levels. The results are from two independent experiments performed in triplicate.

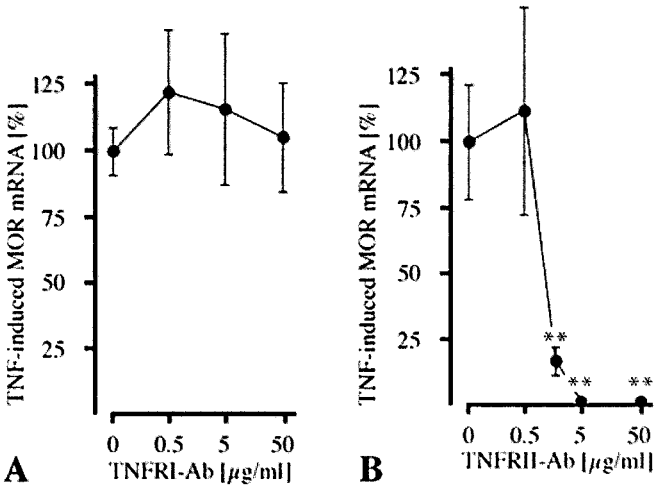
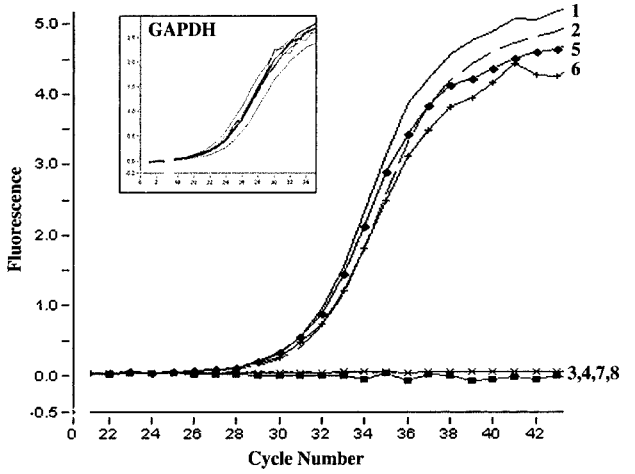


Fig. 2. TNF-induction of μ-opioid receptor transcription in Raji cells is mediated by TNFR2. A, TNFR1 antibodies have no effect on TNF-induced μ-opioid receptor transcription. Raji cells were preincubated for 1 h with the indicated amounts of TNFR antibodies, then stimulated with TNF and further incubated for 24 h before RNA extraction and RT-PCR. The results of three independent experiments performed in duplicate are plotted, after normalization, to equal amounts of GAPDH mRNA. B, TNFR2 antibodies block TNF-induced μ-opioid receptor transcription in a dose-dependent manner. (**, $p < 0.01$).

TNF effect is dependent on NFκB. Controls demonstrating the efficacy and specificity of the decoy oligonucleotides are displayed in Fig. 4. Therefore, a reporter gene construct containing a classic NFκB binding motif was cotransfected in Raji cells together with various decoy oligonucleotides (Fig. 4A). Then, the decoy oligonucleotide-provoked inhibition of the TNF-/NFκB-mediated reporter gene activity was measured. Homologous NFκB decoys resulted in a dose-dependent inhibition of reporter gene activity (IC_{50}, 47 nM). The specificity of NFκB decoy oligonucleotides is shown in Fig. 7 with NFκB-like motifs with mutations within the binding sequence. AP-1, AP-2, and STAT6 decoys did not inhibit reporter gene activation. Figure 4B shows similar controls for an AP-1–responsive reporter gene construct with wild type (IC_{50}, 45 nM) and mutant AP-1 and NFκB decoy oligonucleotides. The finding that the TNF-induced transcription of the μ-opioid receptor gene is entirely blocked by NFκB decoy oligonucleotides implies that almost all cells were transfected with the decoys, which is unlikely, because the transfection rate of these cells in our experiments was lower than 50%. However, we observed that the decoy oligonucleotides were entering the cells also without transfection, by simple addition into the medium. Thus, addition of NFκB decoys to culture medium immediately before TNF stimulation resulted in a decrease in TNF-induced μ-opioid receptor mRNA in Raji cells in a dose-dependent manner, as revealed by quantitative real time PCR, 24 h after stimulation: TNF-stimulated control cells, 100 ± 9% μ-opioid receptor transcripts; TNF-stimulated cells grown in medium containing 25 nM decoy oligonucleotides, 145 ± 39%; 50 nM decoys, 111 ± 15%; 75 nM decoys, 75 ± 14%; 100 nM decoys, 9 ± 3%**. Thus, both passive uptake of the decoy oligonucleotides and transfection of them contributes to the highly efficient inhibition of TNF-induced μ-opioid receptor transcription in our experiments.

The Human μ-Opioid Receptor Gene Promoter Contains Three NFκB Binding Sites. Transient transfection experiments with reporter gene constructs containing sequences of the μ-opioid receptor promoter were performed to identify NFκB elements. SH SY5Y cells were used for these experiments, because stimulation of these cells with TNF results only in activation of NFκB, not, for example, AP-1, for which the promoter is responsive as well (Börner et al., 2002). As suggested by sequence homology, the μ-opioid receptor promoter contains six motifs to which NFκB may bind. These putative elements, termed M1 through M6, are shown in Fig. 5A and their sequences are shown in Fig. 7A. Transfection of the longest promoter construct, −2624, revealed significant inducibility with TNF (Fig. 5A, lane 1). Consecutive 5′-end deletion of the promoter up to −254 did not abolish inducibility (lanes 2 to 6). However, the −174 construct showed no responsiveness to TNF (Fig. 5A, lane 7), indicating that M6 at position −207 may be an NFκB site. To identify additional NFκB elements and to determine whether these elements would be functional in front of a heterologous promoter, the μ-opioid receptor upstream sequences were dissected and tested in front of the Herpes simplex virus tk promoter. These experiments showed that sequences containing M2 (at nt −2174) and M5 (at nt −557) also conferred



1: U 937 + TNF
2: U 937 + TNF + AP-1-decoy
3: U 937 + TNF + NFκB-decoy
4: U 937 + TNF + AP-1- + NFκB-decoy
5: Raji + TNF
6: Raji + TNF + AP-1-decoy
7: Raji + TNF + NFκB-decoy
8: Raji + TNF + AP-1- + NFκB-decoy

Fig. 3. TNF-induction of μ-opioid receptor transcription in immune cells depends on NFκB but not AP-1. Real-time RT-PCR monitoring amplification of μ-opioid receptor transcripts in Raji and U937 cells after stimulation with 100 U/ml TNF for 16 h (curves 1 and 5, as indicated below). The other samples contained cDNAs from TNF-stimulated cells that were transfected with decoy oligonucleotides specific for AP-1 and/or NFκB transcription factors (100 nM each) as indicated below. NFκB decoy oligonucleotides completely inhibited TNF-induced transcription of the gene. All samples were transfected with equal amounts of oligonucleotides (an unrelated AP-2 oligonucleotide was used to adjust the amount). Inset, amplification of mRNA of the *GAPDH* gene. A typical experiment (of three) is shown.

	NFκB – tk – CAT	4.9 ± .3 ↓
	+ 25 nM NFκB decoy	5.1 ± .5
	+ 50 nM NFκB decoy	3.2 ± .3
	+ 75 nM NFκB decoy	1.0 ± .3**
	+ 100 nM NFκB decoy	0.9 ± .1***
	+ 100 nM AP-1 decoy	4.7 ± .4
	+ 100 nM AP-2 decoy	5.0 ± .8
A	+ 100 nM STAT6 decoy	4.4 ± .9
	AP-1 – tk – CAT	6.1 ± .4 ↓
	+ 25 nM AP-1 decoy	6.0 ± .7
	+ 50 nM AP-1 decoy	3.5 ± .8*
	+ 75 nM AP-1 decoy	1.5 ± .2**
	+ 100 nM AP-1 decoy	1.0 ± .2**
	+ 100 nM AP-1 *mut1* decoy	5.6 ± .5
	+ 100 nM AP-1 *mut2* decoy	5.8 ± .4
B	+ 100 nM NFκB decoy	5.3 ± .5

Fig. 4. Efficacy and specificity of the decoy oligonucleotides. A, NFκB decoy oligonucleotides. Top, TNF inducibility (right, reported as -fold induction) of a reporter gene construct containing a classic NFκB response element (left) in Raji cells. Bottom, cotransfection of this construct together with various decoy oligonucleotides in various amounts as indicated (left) and their effect on TNF inducibility of the reporter construct (right). B, AP-1 decoy oligonucleotides. Similar experiments showing the effect of various decoy oligonucleotides on *O*-tetradecanoylphorbol 13-acetate-inducible expression of an AP-1 site containing reporter gene in Raji cells. The results of at least two independent transfection experiments performed in triplicate plus S.E.M. are shown (*, $p < 0.5$; **, $p < 0.01$; ***, $p < 0.001$).

a response to TNF, whereas other sequences and the other motifs did not (Fig. 5A, lanes 8 to 14). The reporter gene vector pBLCAT2 was not responsive to TNF (Fig. 5A, lane 15). When oligonucleotides containing M2, M5, and M6 sequences were cloned in front of the tk promoter, they were sufficient to mediate the TNF response independent of their orientation (Fig. 5B). Treatment of transfected cells with the specific NFκB inhibitor sulfasalazine (Wahl et al., 1998) before TNF stimulation abolished the effect of the cytokine (data not shown), which further supports the conclusion that these elements bind NFκB. In a previous study, polymorphisms of the human μ-opioid receptor gene had been localized (Hoehe et al., 2000). Interestingly, one of these naturally occurring allelic variation alters the sequence of the −557 M5 motif from 5′-GGGGTTTTAG-3′ to 5′-GGGATTTTAG-3′. To test whether this variation may have any impact on its *trans*-activating potency, a construct containing the mutated allele was also tested. The mutated motif also conferred a significant response to TNF but with a significantly reduced potency compared with the wild-type sequence (Fig. 5B).

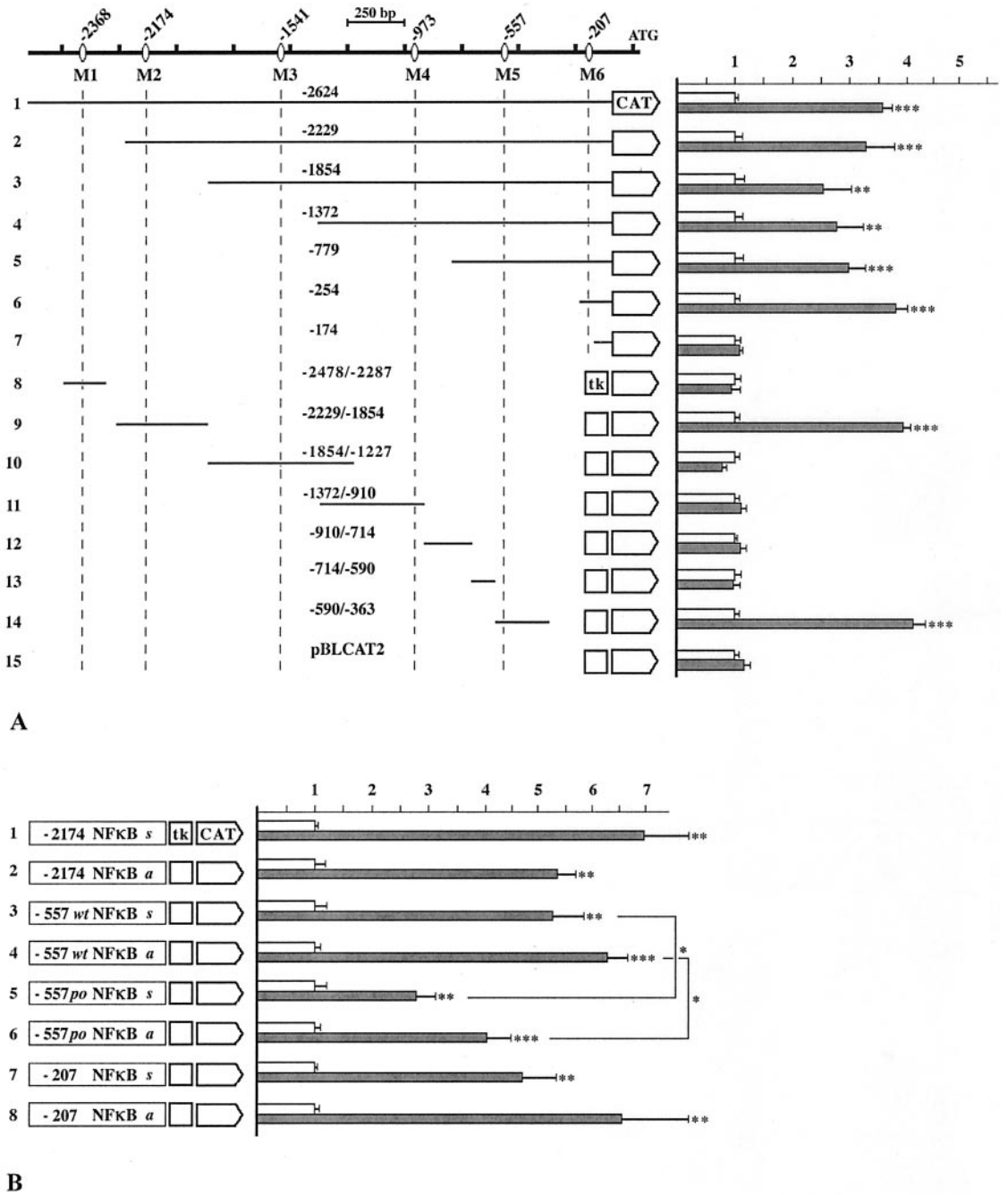


Fig. 5. The μ-opioid receptor gene promoter contains three NFκB binding elements. A, CAT reporter gene activities for constructs containing various parts of the human μ-opioid receptor promoter obtained after transfection of SH SY5Y cells (open bars, unstimulated controls; gray bars, TNF-treated transfectants, 100 U/ml). The locations of six putative NFκB binding sites (M1-M6, oval symbols) are indicated by vertical dashed lines. B, transfection of constructs containing oligonucleotides with NFκB elements M2 (nt −2174), M5 [wt and polymorphic (po) nt −557], and M6 (nt −207) in sense (s) and antisense (a) orientation in front of the tk promoter. The results of at least three independent transfection experiments performed in triplicate plus S.E.M. are plotted (*, $p < 0.5$; **, $p < 0.01$; ***, $p < 0.001$).

The μ-Opioid Receptor Promoter Elements Bind NFκB in Vitro. Electrophoretic mobility shift assays were performed to investigate binding of NFκB to the putative sites within the μ-opioid receptor gene promoter (Fig. 6). First, oligonucleotides containing M1 through M6 were tested to determine whether they were able to compete for NFκB binding to a classic NFκB element. In addition to homologous NFκB oligonucleotides, M2, M5 (wild type), and M6 were efficient competitors (Fig. 6A), indicating that they are indeed NFκB sites. This is in good accordance with the transfection experiments described above. To compare the wild-type M5 and the polymorphic M5 motif, the same experiments were performed using multiple amounts of competitor DNA (Fig. 6B). Clearly, the ability to compete for NFκB binding was more pronounced for the wild-type motif, with a complete competition using 3.3 pmol of oligonucleotides. In contrast, using the mutated M5 motif, 10 pmol of oligonucleotides was needed for a complete competition.

Then, M2, M5 wild type, M5 variant, and M6 oligonucleotides were used as probes and tested for NFκB binding (Fig. 6C). The band-shift patterns were identical for the three μ-opioid receptor motifs as well as for the classic NFκB motif, yielding a typical double band. The μ-opioid receptor elements were confirmed as NFκB binding sites by competition experiments with classic NFκB oligonucleotides as well as by supershift experiments with a specific NFκB antibody (Fig. 6D).

The μ-Opioid Receptor Promoter Elements Bind NFκB in Intact Raji Cells. The decoy oligonucleotide approach was used to demonstrate binding of the μ-opioid receptor elements to NFκB in intact cells. For these experiments, a reporter gene construct containing a classic NFκB binding motif was cotransfected in Raji cells together with decoy oligonucleotides containing the μ-opioid receptor NFκB sequences (Fig. 7A). In full accordance with the transfection and gel-shift experiments, the M2, M5 (wt), and M6 sequences were targets for NFκB in the immune cells and inhibited TNF-induced stimulation of the reporter gene construct by capturing NFκB in vivo. The effect of the polymorphism within element M5 could also be demonstrated with the decoy approach in Raji cells (Fig. 7B). Thus, wild-type M5 decoy oligonucleotides were significantly more effective competitors than mutated M5 decoys, with 44 and 55 nM concentrations of oligonucleotides, respectively, needed for a 50% inhibition of TNF-induced reporter gene activation.

Discussion

Multiple immunomodulatory effects of opioids are transduced to immune effector cells mainly via three mechanisms: either indirectly via the central nervous system [e.g., by activation of the hypothalamus-pituitary-adrenal axis (Cabot et al., 2001; Roy et al., 2001)], directly via atypical opioid receptors [which were postulated because some opioid effects cannot be blocked by the classic opioid receptor antagonist naloxone (Roy et al., 1998b)], or directly via the classic opioid receptor subtypes μ, δ, and κ present on immune cells. It has been shown previously that classic μ-opioid receptors are induced by IL-4 in various immune cells (Kraus et al., 2001) and by IL-1 in endothelial cells (Vidal et al., 1998). Here, we demonstrated that TNF also induces μ-opioid receptor transcription in immune cells. Considerable evidence indicates that transcription of μ-opioid receptors is correlated with expression of functional receptors on immune cells, demonstrated by the capacity of μ-opioid receptor ligands to affect

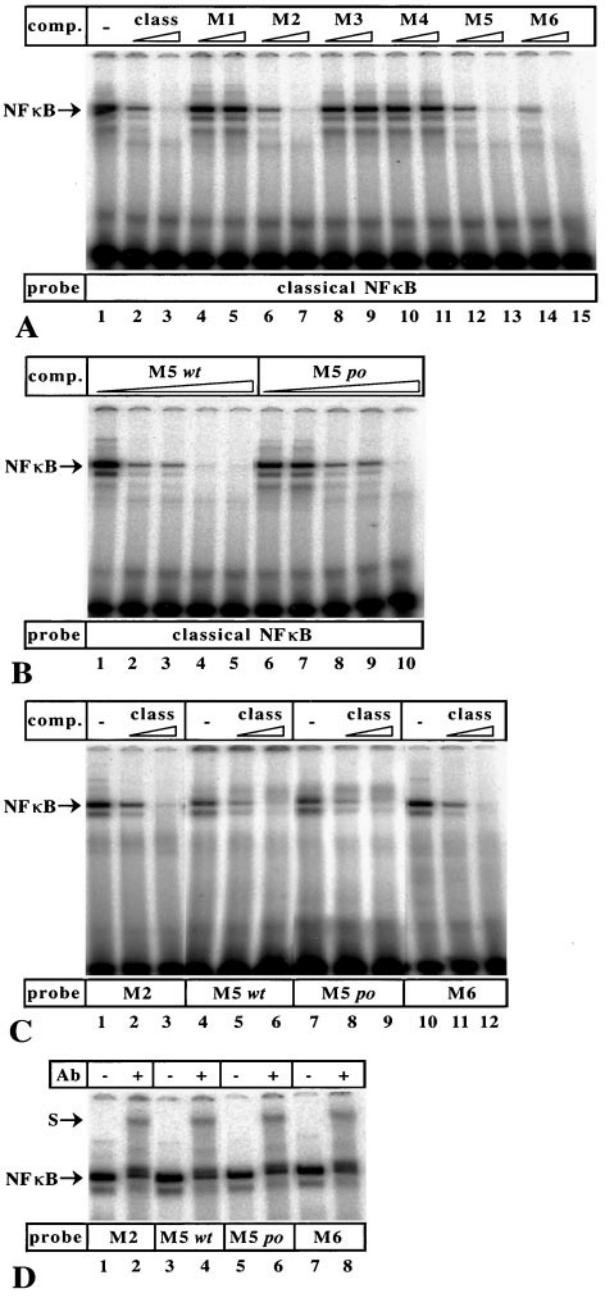


Fig. 6. The μ-opioid receptor promoter elements bind NFκB in vitro. Electrophoretic mobility shift assays were performed using various probes (indicated below the gels) and competitor DNAs or p65 NFκB antibody (indicated above the gels) with nuclear extracts of TNF-stimulated SH SY5Y cells. A, like homologous NFκB oligonucleotides, the putative μ-opioid receptor motifs M2, M5 (wild type), and M6 compete for binding to NFκB. Molar excesses of approximately 5- and 50-fold (1 and 10 pmol) of competitor DNA are indicated by wedges. B, the wild type (wt) M5 motif binds NFκB with higher affinity than the polymorphic (po) M5 motif (lanes 1 and 6, 100 fmol competitor DNA; lanes 2 and 7, 333 fmol; lanes 3 and 8, 1 pmol; lanes 4 and 9, 3.3 pmol; lanes 5 and 10, 10 pmol) C, NFκB binding to the μ-opioid receptor motifs M2, M5 wt and po, and M6 is competed by classic NFκB oligonucleotides. D, immunoshift experiments demonstrate that the μ-opioid receptor motifs M2, M5 wt and po, and M6 are NFκB elements. S, supershifted band. Typical gels representing at least three experiments are shown.

immune cell functions described in numerous reports (Weber and Pert, 1989; Chao et al., 1993; Roy et al., 1998a; Gaveriaux-Ruff et al., 1998; Roy et al., 1999; Sacerdote et al., 2000; McCarthy et al., 2001; Azuma and Ohura, 2002). In addition to cells derived from the immune system, cytokines regulate μ-opioid receptor expression also in neuronal cells. Thus, induction of μ-opioid receptor expression is observed in primary astrocyte-enriched cultures of various brain regions by IL-1 (Ruzicka et al., 1996) and in primary fetal cortical neurons and SH SY5Y cells by IL-4 (Kraus et al., 2001) and TNF (Börner et al., 2002). Studies with IL-6 knockout mice revealed that levels of μ-opioid receptors are decreased in certain brain regions compared with wild-type animals, suggesting also a positive regulation of the gene by IL-6 (Bianchi et al., 1999). From the current point of view, cytokines thus represent potent physiological modulators of μ-opioid receptor transcription.

Transcriptional activation of μ-opioid receptors in response to TNF in Raji cells is dependent on TNFR2 and independent of de novo protein synthesis, although transcripts appear somewhat delayed after 2 to 6 h. To date, the physiological role of TNFR2 is not completely understood. It is clear, however, that TNFR2 can trigger inflammation (Tartaglia et al., 1991; Akassoglou et al., 2003). The involvement of μ-opioid receptors in inflammation is discussed below. By and large, TNF may lead to activation of two major transcription factors, AP-1 and NFκB (Baud and Karin, 2001). Both factors could also be involved in TNF regulation of μ-opioid receptor transcription because the gene promoters of various species contain functional AP-1 elements as well (Kraus et al., 1995; Börner et al., 2002). Interestingly, an involvement of AP-1 could be clearly ruled out in the immune cell lines used, whereas loss of NFκB function completely inhibited induction of the gene, demonstrating that transcriptional induction of the gene in response to TNF depends solely on NFκB. The decoy oligonucleotide strategy to selectively disrupt function of a transcription factor in intact cells, which was used here, allows direct identification of the relevant factor(s) involved in a particular regulatory event. It is thus very useful to close the common gap between measuring an effect of a stimulus on a certain gene's transcription on the one hand and the identification of "possibly involved" transcription factor binding sites on that gene's promoter on the other hand.

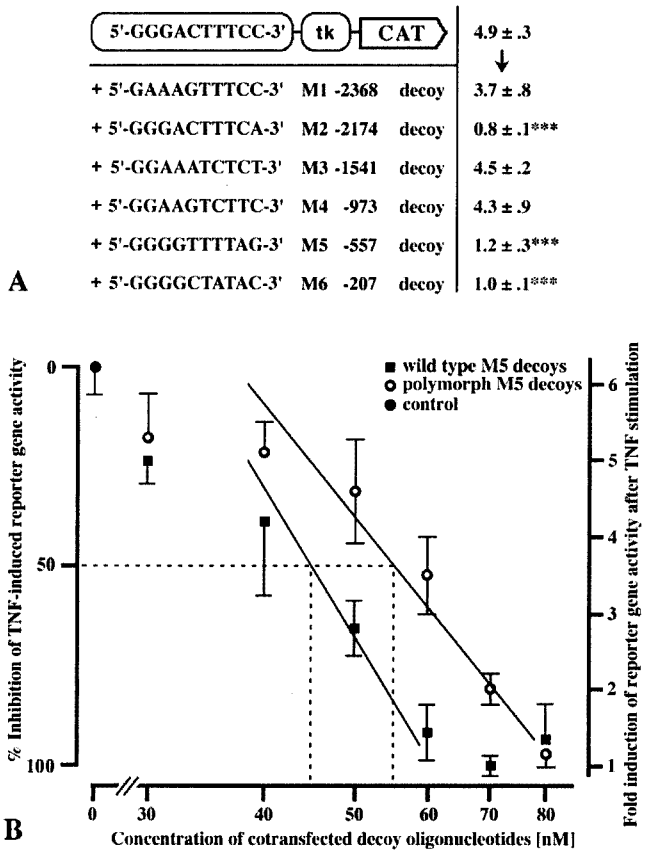


Fig. 7. The μ-opioid receptor promoter elements bind NFκB in immune cells. A, M2, M5 (wt), and M6 but not M1, M3, and M4 decoy oligonucleotides bind NFκB in Raji cells and attenuate TNF inducibility. Top, TNF inducibility (right, reported as -fold induction) of a reporter gene construct containing a classic NFκB response element (left). Bottom, cotransfection of this construct together with decoy oligonucleotides (100 nM each) containing the putative μ-opioid receptor NFκB sites (left) and their effect on TNF inducibility (right). B, wild-type M5 decoy oligonucleotides attenuate TNF inducibility more efficiently than polymorphic M5 decoys. The experimental design is similar to that shown in Fig. 7A. Here, increasing amounts of cotransfected wild type and polymorphic M5 decoy oligonucleotides inhibit TNF induction of the classic NFκB reporter gene construct (control) in Raji cells. Fold induction of the reporter construct (6.2-fold equals 0% inhibition, 1.0-fold equals 100% inhibition) is plotted against the concentration of cotransfected decoy oligonucleotides. The results of at least three independent transfection experiments performed in triplicate plus S.E.M. are displayed. (***, $p < 0.001$)

Multiple NFκB elements on the μ-opioid receptor gene most probably indicate the importance for regulation via this factor. Interestingly, the *trans*-activating potency of one of the NFκB regulatory elements is markedly reduced because of an allelic variation. Currently, two mechanisms are being discussed to explain the well known heterogeneity of patients' responses to opioid analgetics: multiple protein variants of opioid receptors and opioid receptor gene polymorphisms. The μ-opioid receptor gene is expressed in a considerable number of protein variants (Pan et al., 2001) and has plenty of polymorphisms (Hoehe et al., 2000). Notably, 28 allelic variations, more than half of the polymorphisms identified so far on this gene, are located within the promoter region. Promoter polymorphisms may have considerable consequences for regulation of the gene if they affect binding sites for transcription factors. Interestingly, apart from the polymorphism within the NFκB site described here, another allelic variation is located within a STAT6 element, which impairs regulation of the gene by cytokines like IL-4 (Kraus et al., 2001). Another polymorphism is located at nt -1699, immediately adjacent to a binding site for a still unknown nuclear protein (Börner et al., 2002).

It should be noted that transcription of the μ-opioid receptor gene in mice is under control of two distinct promoters. In addition to the main promoter flanking exon 1, there is another promoter flanking exon 11, which is located roughly 10 kilobases upstream of exon 1 (Pan et al., 2001). For the human gene, only the promoter upstream of exon 1 is known so far. Nevertheless, even if there is no evidence to date that the human gene is alternatively spliced in a similar way, the existence of an upstream promoter of the human gene cannot be excluded.

Regulation of μ-opioid receptors by TNF may be important within the complex of inflammation-induced analgesia. It is now established that immunocyte-derived endogenous opioids induce analgesia by activating peripheral opioid recep-

tors at later stages during inflammation, which can be regarded as a physiological mechanism to counteract and reduce inflammatory pain (Stein et al., 1990; Cabot et al., 1997). Most probably, the relevant opioid receptors are of the μ subtype, because these are up-regulated in dorsal root ganglia in inflammation, whereas the δ subtype is down-regulated (Ji et al., 1995). Moreover, it was shown that cytokines such as TNF, IL-1β, IL-6, and corticotropin-releasing factor contribute to inflammation-induced analgesia (Czlonkowski et al., 1993; Schafer et al., 1994). In this scenario, TNF could contribute to maximizing the analgesic effect of endogenous opioids by increasing the concentration of their receptors in the neuronal cells. In the immunocytes, TNF-induction of μ-opioid receptors could allow a feedback regulation in which the endogenous opioids could modulate either their own secretion or secretion of other cytokines.

A strategy to relieve pain in patients with rheumatoid arthritis is to inhibit TNF and/or NFκB, which are recognized as key mediators for this disease. Interestingly, use of decoy oligonucleotides to disrupt NFκB activity has also been discussed for gene therapy of rheumatoid arthritis (Tomita et al., 2000). We demonstrated that chemically nonmodified decoy oligonucleotides could enter blood cells without being transfected or transduced via viruses, which could be promising toward certain decoy gene therapies. Often, NFκB inhibitors, such as sulfasalazine (Wahl et al., 1998), or preparations of antibodies against TNF, such as infliximab (Kalden, 2002), are applied. In the end, the strategy to block TNF/NFκB may bear considerable side effects. These could include impaired antinociception as a result of the lack of natural activation of the opioid system via TNF and NFκB. Especially in the case of rheumatoid arthritis, it may be not beneficial to impair the opioid system, because a recent study demonstrated that endogenous opioids ameliorate excessive synovial cell functions via μ-opioid receptors (Takeba et al., 2001). The authors speculated that endogenous opioids act in an antinociceptive as well as an antiinflammatory manner.

In conclusion, our study contributes to the understanding of the multiple interactions between the immune system and the nervous system and suggests additional functions of TNF in physiological and pathophysiological regulatory circuits.

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