

## Thiopental Inhibits the Activation of Nuclear Factor $\kappa$ B

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**Background:** Thiopental is frequently used for the treatment of intracranial hypertension after severe head injury. Its long-term administration increases the incidence of nosocomial infections, which contributes to the high mortality rate of these patients. However, the mechanism of its immunosuppressing effect remains unknown.

**Methods:** The effect of thiopental (200–1000  $\mu$ g/ml) on the activation of the nuclear transcription factor  $\kappa$ B (NF- $\kappa$ B; electrophoretic mobility shift assays), on NF- $\kappa$ B–driven reporter gene activity (transient transfection assays), on the expression of NF- $\kappa$ B target genes (enzyme-linked immunoassays), on T-cell activation (flow cytometric analyses of CD69 expression), and on the content of the NF- $\kappa$ B inhibitor I $\kappa$ B- $\alpha$  (Western blotting) was studied in human T lymphocytes *in vitro*.

**Results:** Thiopental inhibited the activation of the transcription factor NF- $\kappa$ B but did not alter the activity of the cyclic adenosine monophosphate response element binding protein. Other barbiturates (methohexital), anesthetics (etomidate, propofol, ketamine), or opioids (fentanyl, morphine) did not affect NF- $\kappa$ B activation. Thiopental-mediated suppression of NF- $\kappa$ B could be observed in Jurkat cells and in primary CD3<sup>+</sup> lymphocytes from healthy volunteers, was time- and concentration-dependent, occurred at concentrations that are clinically achieved, and persisted for hours after the incubation. It was associated with an inhibition of NF- $\kappa$ B–driven reporter gene activity, of the expression of interleukin-2, -6, and -8, and interferon  $\gamma$ , and of the activation of CD3<sup>+</sup> lymphocytes. Suppression of NF- $\kappa$ B appeared to involve reduced degradation of I $\kappa$ B- $\alpha$ .

**Conclusion:** The results demonstrate that thiopental inhibits the activation of NF- $\kappa$ B and may thus provide a molecular mechanism for some of the immunosuppressing effects associated with thiopental therapy.

HEAD injury remains the leading cause of death in young males in developed countries.<sup>1,2</sup> This high lethality is partly because of the fact that patients with severe traumatic brain injury frequently develop cerebral edema, leading to intracranial hypertension. Brain damage caused by elevated intracranial pressure can be minimized by applying different neuroprotective strategies, including the induction of a barbiturate coma with agents such as thiopental.<sup>3,4</sup> During these conditions,

thiopental has been shown to decrease cerebral metabolic demands and to lower intracranial pressure, thus improving cerebral oxygenation.<sup>5</sup>

Despite these favorable effects, accumulating evidence suggests that the long-term administration of high doses of thiopental is associated with a profound increase in the incidence of nosocomial infections, which may in turn contribute to the high mortality rate of these patients.<sup>6,7</sup> For example, a recently published prospective study showed that long-term infusion of thiopental in patients with elevated intracranial pressure caused bone marrow suppression with leukopenia or agranulocytosis. In addition, the total rate of infection was more than doubled in response to high-dose thiopental therapy in these patients suffering from isolated severe head injury.<sup>8</sup>

In contrast to the clinically well-documented association between thiopental therapy and immunosuppression, it is unclear if this reflects a specific effect of thiopental. Moreover, a precise molecular mechanism of immunosuppression by thiopental remains to be identified. Therefore, it is of particular interest that several studies have provided evidence that thiopental may interfere with leukocyte function. Incubation of polymorphonuclear leukocytes with thiopental inhibited the production of reactive oxygen species<sup>9,10</sup> and decreased chemotaxis as well as phagocytosis.<sup>11</sup> In addition, thiopental has been shown to inhibit the mitogen-induced blast transformation, the proliferation of T lymphocytes, and the production of cytokines by these cells.<sup>12,13</sup>

This suggests that thiopental could exert its permissive effect on nosocomial infections, in part by inhibiting the function of immune cells. The nuclear transcription factor  $\kappa$ B (NF- $\kappa$ B) is a central regulator of the immune response.<sup>14</sup> It controls the transcription of a variety of genes, including inflammatory cytokines such as interleukin (IL)-1, IL-2, IL-6, IL-8, and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), as well as genes encoding immunoreceptors, cell adhesion molecules, hematopoietic growth factors, growth factor receptors, and acute phase proteins.<sup>15,16</sup> In most cell types, NF- $\kappa$ B is sequestered in an inactive, cytoplasmic complex by binding to I $\kappa$ B, its inhibitory subunit.<sup>17</sup> A large variety of inflammatory conditions, including bacterial or viral infections, rapidly induce NF- $\kappa$ B activity.<sup>15</sup> During these conditions, I $\kappa$ B is phosphorylated, followed by ubiquitination and rapid proteolytic degradation of the inhibitor.<sup>18,19</sup> This allows translocation of free, active NF- $\kappa$ B into the nucleus, where it binds to its cognate DNA elements and activates gene transcription. Thus, the aim of the current study was to

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determine whether thiopental interferes with the activation of NF- $\kappa$ B.

## Methods

### Reagents

The following anesthetics and opioids were used: etomidate (Braun, Melsungen, Germany), fentanyl (Janssen-Cilag, Neuss, Germany), ketamine (Parke Davis, Berlin, Germany), methohexital (Lilly, Giessen, Germany), midazolam (Hoffman-La Roche, Grenzach-Wyhlen, Germany), morphine (Mundipharma, Limburg, Germany), propofol (AstraZeneca, Plankstadt, Germany), and thiopental (Byk Gulden, Konstanz, Germany). Recombinant human TNF- $\alpha$  was a gift from Karl Decker, Ph.D. (Department of Biochemistry, University of Freiburg, Germany). All other reagents were purchased from Sigma (Deisenhofen, Germany) unless specified otherwise.

### Isolation of Mononuclear Cells and T Lymphocytes from Healthy Donors

The study was approved by the ethics committee of the University Hospital of Freiburg (Freiburg, Germany) and conducted according to the principles of the Helsinki Declaration. Before inclusion into the study, informed, written consent was obtained from healthy volunteers. Blood was withdrawn after venipuncture. Peripheral blood mononuclear cells were isolated from 40 ml heparinized blood by density centrifugation on Ficoll-Hypaque<sup>®</sup> (Amersham-Pharmacia, Freiburg, Germany) according to the manufacturer's recommendations. The cells were microscopically analyzed and counted in a Neubauer chamber. For the isolation of T lymphocytes, peripheral blood mononuclear cells ( $3-4 \times 10^7$ ) were incubated for 15 min on ice with anti-CD3 antibodies conjugated to magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Separation of CD3<sup>+</sup> cells was performed using an L/S column (Miltenyi Biotec) and confirmed by fluorescence-associated cell sorting (> 85% purity). For electrophoretic mobility shift assays (EMSAs), more than  $5 \times 10^5$  T lymphocytes were analyzed per sample.

### Cell Culture

Jurkat T cells and primary human peripheral blood mononuclear cells and T lymphocytes, which had been isolated as described above, were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% glutamine, and 50 mg/ml penicillin-streptomycin (all from Gibco-BRL, Karlsruhe, Germany) and were grown in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

### Electrophoretic Mobility Shift Assays

Total cell extracts were prepared using a high-salt detergent buffer (20 mM Hepes, pH 7.9, 350 mM NaCl, 20% [vol/vol] glycerol, 1% [wt/vol] NP-40, 1 mM MgCl<sub>2</sub>,

0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.1% PMSF, 1% aprotinin). Cells were harvested by centrifugation, washed once in ice-cold phosphate-buffered saline, and resuspended in four cell volumes of the detergent buffer. The cell lysate was incubated for 30 min on ice and then centrifuged for 5 min at 13,000g at 4°C. EMSAs were performed with <sup>32</sup>P-labeled oligonucleotides of either the inducible NF- $\kappa$ B or the constitutively active cyclic adenosine monophosphate response element binding protein (CREB) as previously described.<sup>20</sup> The reaction mixture consisted of 37  $\mu$ l purified water, 1  $\mu$ l NF- $\kappa$ B or CREB oligonucleotides (25 ng/ $\mu$ l; Promega, Madison, WI), 5  $\mu$ l kinase buffer, 5  $\mu$ l  $\gamma$ -<sup>32</sup>P-dATP (Amersham International, Braunschweig, Germany), and 1.5  $\mu$ l T4 kinase (polynucleotide kinase [PNK] buffer and PNK T4 kinase; New England Biolabs, Schwalbach, Germany) and was incubated for 30 min at 37°C. The protein content of the cell lysates was determined using a Bradford-Assay system (Bio-Rad Laboratories, München, Germany), and equal amounts of protein (30  $\mu$ g) were added to a 20- $\mu$ l reaction mixture containing 20  $\mu$ g bovine serum albumin, 2  $\mu$ g poly (dI-dC) (Roche, Mannheim, Germany), 2  $\mu$ l buffer D+ (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, 2 mM dithiothreitol, 0.1% PMSF), 4  $\mu$ l 5x Ficoll buffer (20% Ficoll 400, 100 mM Hepes, 300 mM KCl, 10 mM dithiothreitol, 0.1% PMSF), 4  $\mu$ l ddH<sub>2</sub>O, and 1  $\mu$ l NF- $\kappa$ B or CREB <sup>32</sup>P-labeled oligonucleotides. These samples were incubated at room temperature for 30 min and then loaded on an acrylamide gel containing 60 ml ddH<sub>2</sub>O, 10 ml 30% acrylamide, 3.8 ml 10x Tris-borate-EDTA buffer (TBE: 900 mM TRIS-HCl, 900 mM Boric acid, 20 mM EDTA [pH 8.0]), 400  $\mu$ l ammonium persulfate, and 40  $\mu$ l tetramethylethylenediamine. After running the gel in 0.5x Tris-borate-EDTA running buffer, gels were vacuum dried (Gel dryer 543; Biorad, Hercules, CA) for 30 min on a 3 MM chromatography filter (Whatman, Maidstone, United Kingdom) and exposed to x-ray film.

### Experimental Protocols for Electrophoretic Mobility Shift Assay Studies

To determine whether thiopental interferes with the activation of NF- $\kappa$ B in human T lymphocytes, Jurkat T lymphoma cells were incubated with thiopental (400 or 1,000  $\mu$ g/ml) for 2 h. One hour before harvesting, the cells were stimulated with TNF- $\alpha$  (5 U/ml) for 1 h, after which total cell extracts were prepared. The cell extracts were analyzed for the DNA binding activity of NF- $\kappa$ B or CREB by EMSA.

An additional series of experiments was performed to test if the effect of different concentrations of thiopental (200, 400, or 1,000  $\mu$ g/ml) on the activation of NF- $\kappa$ B DNA binding may vary, depending on the time of thiopental exposure. In these experiments, Jurkat T cells were incubated with the various concentrations of thio-

pental for 2, 4, or 8 h and stimulated with TNF- $\alpha$  (5 U/ml) 1 h before harvest.

To determine whether the effect of thiopental on the activation of NF- $\kappa$ B is limited to the actual time of exposure, Jurkat T cells were incubated with thiopental for 2 h. Subsequently, the cells were extensively washed and cultured in medium free of thiopental for another 2, 4, 6, 8, or 10 h. One hour before harvesting, the cells were stimulated with TNF- $\alpha$  (5 U/ml).

To evaluate whether the action of thiopental on the activation of NF- $\kappa$ B is confined to Jurkat cells or may also affect the response of primary T cells from healthy humans to stimulation with TNF- $\alpha$ , CD3<sup>+</sup> cells were isolated from the peripheral blood of healthy donors and incubated with thiopental (400 or 1,000  $\mu$ g/ml) for 2 h. One hour before harvesting, the cells were stimulated with TNF- $\alpha$  as described above.

To test if inhibition of NF- $\kappa$ B activation may also occur in the presence of an oxybarbiturate, Jurkat T cells were incubated with methohexital (400 or 1,000  $\mu$ g/ml), and its effect on the activation of NF- $\kappa$ B was studied during similar conditions as described for thiopental. To determine whether other anesthetics or opioids may affect the DNA binding of NF- $\kappa$ B, Jurkat cells were incubated with either etomidate, fentanyl, ketamine, midazolam, morphine, or propofol during the same experimental conditions. The minimal effective plasma concentration of these substances as well as concentrations 10- and 100-fold lower or higher were evaluated.

#### *Characterization of the Experimental Conditions*

To determine whether the anesthetics used may cause significant changes in the pH of the culture medium, the following control experiments were performed. The different agents were added to the medium to achieve the respective final concentrations, and pH was determined using a standard electrode (Schott, Hofheim, Germany) after equilibration in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. However, the pH remained always within the physiologic range, even in the presence of the highest concentrations of the anesthetic agents used. Thiopental was dissolved in ultrapure distilled water and was added to the culture medium without any solvents or additives. Additional experiments did not reveal any effects of benzethium chloride or intralipid (Pharmacia, Erlangen, Germany), which are contained in ketamine, etomidate, and propofol solutions, on the activation of NF- $\kappa$ B. To exclude potential toxic effects of the agents tested, the cells were stained with trypan blue at the end of the experimental period, and positive cells were counted. These experiments did not reveal any differences in cell counts between samples from control experiments and from those performed in the presence of the various anesthetic agents.

#### *Nuclear Factor $\kappa$ B-driven Reporter Gene Activity*

Jurkat T cells were plated 12–16 h before transfection at a density of  $3 \times 10^5$  cells per well in a six-well plate. Cells were transiently transfected with a luciferase reporter gene driven either by the minimal thymidine kinase (tk) promoter alone or by the tk promoter preceded by six NF- $\kappa$ B binding sites. The vectors were gifts from Markus Meyer, Ph.D. (European Molecular Biology Laboratory, Heidelberg, Germany), and have been previously described.<sup>21</sup> Transfections were performed using the reagent superfect (Qiagen, Hilden, Germany) according to the manufacturer's description. To exclude the possibility that differences in transfection efficiency could influence the results, all cells were pooled at 6 h after transfection, gently mixed, and then equally distributed into individual wells for all further determinations. Moreover, the entire experiment had been repeated six times. Cells were pretreated with thiopental (100, 400, or 1,000  $\mu$ g/ml) for 1 h before stimulation with 100 U/ml TNF- $\alpha$  and harvested *in situ* after 17 h using a commercial lysis buffer and luciferase assay system (Promega Corp., Heidelberg, Germany). Luciferase activity was determined using a microplate luminometer (Eg & G-Berthold, Bad Wilbach, Germany) measuring light emission over an interval of 30 s, and the results are expressed as percent of the arbitrary light units of the respective positive control.

#### *Cytokine Assays*

Primary CD3<sup>+</sup> T lymphocytes were pretreated with different concentrations of thiopental (100, 400, or 1,000  $\mu$ g/ml) for 1 h. The cells were subsequently stimulated by addition of TNF- $\alpha$  (100 U/ml), and 24 h later the cell culture supernatant was analyzed for IL-8, a typical target gene of NF- $\kappa$ B in T lymphocytes.<sup>15</sup> Because the expression of IL-2, IL-6, and interferon  $\gamma$  is also regulated by NF- $\kappa$ B, an additional series of experiments was performed to determine the effect of thiopental on the release of these cytokines. However, the amount of immunoreactive protein secreted by CD3<sup>+</sup> T lymphocytes was below the detection limit of the respective assays (data not shown). Therefore, peripheral blood mononuclear cells were used for these experiments. The cells were pretreated with thiopental (400 or 1,000  $\mu$ g/ml) for 1 h, subsequently stimulated by addition of phorbol-12-myristate-13-acetate (125 ng/ml), and 24 h later the concentration of IL-2, IL-6, and interferon  $\gamma$  was determined in the supernatant. All measurements were performed using enzyme-linked immunosorbent assay kits purchased from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

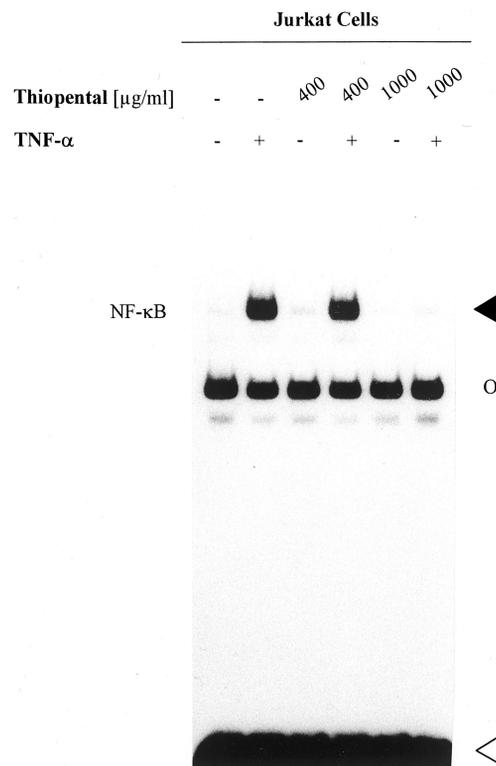
#### *T-cell Activation Assays*

The effect of thiopental on the function of primary CD3<sup>+</sup> T lymphocytes was assessed by fluorescence-associated cell sorting analysis using the Fastimmune Assay System (BD Biosciences, Heidelberg, Germany) accord-

ing to the manufacturer's recommendations. Briefly, 50  $\mu$ l whole blood was incubated with thiopental (400 or 1,000  $\mu$ g/ml) 1 h before the activation of the cells by cross-linking of the CD2 receptor (20  $\mu$ l CD2-CD2-receptor-ligand-antibody mix per ml of blood; Cat. No. 340366, BD Biosciences). After 12 h, CD3-CD69-CD4 Fastimmune Assay reagent was added to the reaction for 45 min. Erythrocytes were lysed by addition of 450  $\mu$ l fluorescence-associated cell sorting lysis solution (BD Biosciences), and whole blood cells were fixed in 500  $\mu$ l of cell fix solution (BD Biosciences). The cells were analyzed using a Calibur fluorescence-associated cell sorter (BD Biosciences).

#### Detection of I $\kappa$ B- $\alpha$ by Western Blotting

The activation and translocation of NF- $\kappa$ B to the nucleus is preceded by the phosphorylation and proteolytic degradation of the inhibitory I $\kappa$ B- $\alpha$  proteins. This process is readily detectable in Western blots.<sup>19</sup> To determine whether thiopental may interfere with the degradation of I $\kappa$ B- $\alpha$ , Jurkat T cells were pretreated with different doses of thiopental (200, 400, or 1,000  $\mu$ g/ml) for 105 min and subsequently stimulated with TNF- $\alpha$  (20 U/ml) for 15 min. These time points were chosen on the basis of the previously published time course of TNF- $\alpha$ -mediated degradation of I $\kappa$ B- $\alpha$ .<sup>22</sup> Total cell extracts of Jurkat T cells (30  $\mu$ g) were boiled in Laemmli sample buffer and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Before transfer, gels were equilibrated for 15 min in cathode buffer, (25 mM Tris, 40 mM glycine, 10% methanol). Proteins were transferred at 0.8 mA/cm<sup>2</sup> for 1 h onto Immobilon P membranes (Millipore Corp., Eschborn, Germany), pre-equilibrated in methanol (15 s), ddH<sub>2</sub>O (2 min each side), and anode buffer II (25 mM Tris-10% methanol), using a semidry blotting apparatus (Bio-Rad Laboratories). Equal loading and transfer were monitored by Ponceau S staining of the membranes. Nonspecific binding sites were blocked by immersing the membrane into blocking solution (TBST: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20 [vol/vol], containing 2% bovine serum albumin) overnight at 4°C. Membranes were washed in TBST and incubated in a 1:1,000 dilution of anti-I $\kappa$ B- $\alpha$  antibody (New England Biolabs) in blocking solution for 1 h at room temperature, followed by extensive washing with TBST. Bound antibody was decorated with goat-antirabbit-horseradish peroxidase conjugate (Amersham Pharmacia), diluted 1:5,000 in blocking solution, for 30 min at room temperature. After washing four times (5 min each), the immunocomplexes were detected using ECL Western blotting reagents (Amersham-Pharmacia) according to the manufacturer's instructions. Exposure to Kodak XAR-5 films (Stuttgart, Germany) was performed for 15 s to 1 min.



**Fig. 1.** The effect of thiopental on nuclear factor  $\kappa$ B (NF- $\kappa$ B) DNA binding after tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) stimulation. Jurkat T cells were treated for 1 h with thiopental at the concentrations indicated (lanes 3–6) and subsequently stimulated with 5 U/ml TNF- $\alpha$  for 1 h (lanes 2, 4, and 6) or the respective volumes of ppH<sub>2</sub>O as vehicle control. Filled arrowhead = position of NF- $\kappa$ B DNA complexes; open circle = a nonspecific activity binding to the probe; open arrowhead = unbound oligonucleotide. The data shown are representative of six independent experiments.

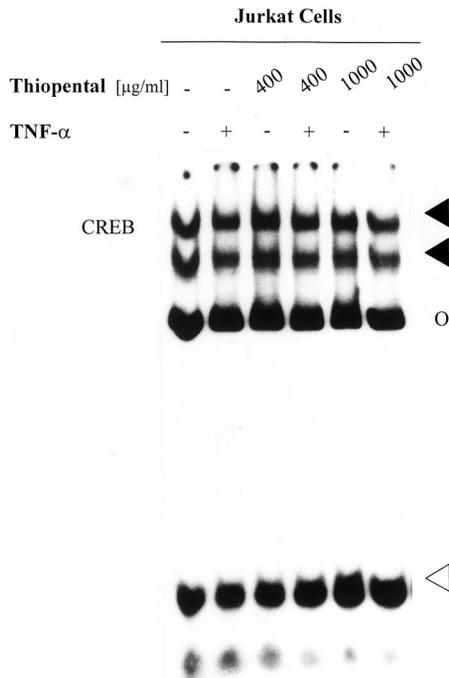
#### Statistical Analysis

Cytokine and reporter gene activity data are depicted as box plots (median, 25–75%, and 95% confidence interval). Differences in measured variables between the various experimental conditions were assessed using an one-way analysis of variance on ranks followed by a nonparametric Student-Newman-Keuls test for multiple comparisons. Results were considered statistically significant at  $P < 0.05$ . All statistical tests were performed using the SigmaStat software package (Jandel Scientific, San Rafael, CA).

## Results

#### Electrophoretic Mobility Shift Assays

Stimulation of Jurkat cells with TNF- $\alpha$  induced one novel DNA binding activity (fig. 1, lane 2). Antibody reactivity and competition assays identified this complex as an NF- $\kappa$ B p50-p65 heterodimer (data not shown; see Pahl and Baeuerle<sup>23</sup>). Although activation of NF- $\kappa$ B DNA binding was not affected by pretreatment with 400  $\mu$ g/ml (1.5 mM) thiopental, 1,000  $\mu$ g/ml (4 mM)



**Fig. 2.** The effect of thiopental on the DNA binding activity of the cyclic adenosine monophosphate response element binding protein (CREB). Jurkat cells were treated for 1 h with thiopental at the concentrations indicated (lanes 3–6), and the cells analyzed in lanes 2, 4, and 6 were subsequently incubated with 5 U/ml of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) for 1 h or the respective volumes of ppH<sub>2</sub>O as vehicle control. Filled arrowhead = position of CREB DNA complexes; open circle = a nonspecific activity binding to the probe; open arrowhead = unbound oligonucleotide. The results shown are representative of three independent experiments.

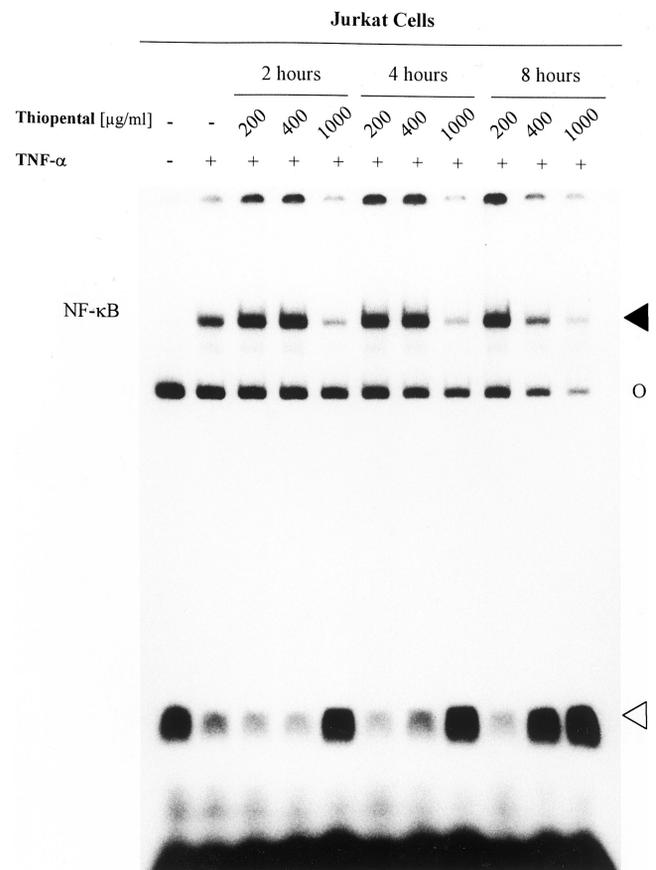
thiopental completely abolished the TNF- $\alpha$ -stimulated NF- $\kappa$ B activation (fig. 1, lanes 4 and 6). The results obtained with thiopental alone did not differ from control conditions (fig. 1, lanes 1, 3, and 5). Moreover, a similar inhibition of the activation of NF- $\kappa$ B by thiopental could also be observed after stimulation with bacterial lipopolysaccharide or phorbol-12-myristate-13-acetate (data not shown).

To determine whether the inhibitory effect of thiopental is confined to the inducible transcription factor NF- $\kappa$ B or may also alter the DNA binding activity of a constitutively active transcription factor, we performed additional EMSAs using a probe containing the consensus motif for CREB. However, treatment of Jurkat T cells with thiopental at concentrations up to 1,000  $\mu$ g/ml with or without subsequent administration of TNF- $\alpha$  did not affect DNA binding of CREB to its DNA probe (fig. 2).

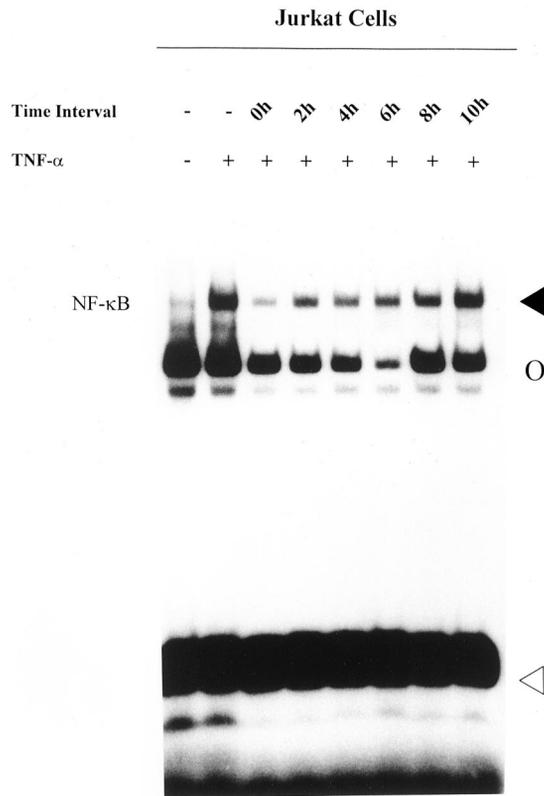
In an additional series of experiments, we evaluated whether the concentration of thiopental needed to suppress the activation of NF- $\kappa$ B DNA binding may vary depending on the time of thiopental exposure. In agreement with the results described above, activation of NF- $\kappa$ B was inhibited after exposure to 1,000  $\mu$ g/ml thiopental for 2 h (fig. 3, lanes 1–5; compare with fig. 1) and remained suppressed at all later time points studied (fig.

3, lanes 8 and 11). However, NF- $\kappa$ B activation was also reduced by much lower concentrations of thiopental after longer times of exposure. For example, although NF- $\kappa$ B DNA binding was not affected by pretreatment with 400  $\mu$ g/ml thiopental for 2 or 4 h (fig. 3, lanes 4 and 7), the same concentration significantly attenuated the activation of NF- $\kappa$ B by TNF- $\alpha$  after 8 h of thiopental pretreatment (fig. 3, lane 10).

To investigate whether the inhibitory effect of thiopental is confined to the actual time of exposure to thiopental, we analyzed the effect of TNF- $\alpha$  on NF- $\kappa$ B activation at different intervals after the end of thiopental pretreatment. These experiments revealed that the suppressing action of thiopental on the TNF- $\alpha$ -mediated activation of NF- $\kappa$ B persisted well beyond the time of thiopental exposure and showed a gradual time-dependent recovery (fig. 4, lanes 3–6). Full restoration of the ability of Jurkat T cells to activate NF- $\kappa$ B on stimulation with TNF- $\alpha$  could only be observed as late as 8–10 h after discontin-



**Fig. 3.** The effect of the time of incubation with different thiopental concentrations on the tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )-mediated activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B). Jurkat T cells were pretreated for 2, 4, and 8 h with thiopental at the concentrations indicated and subsequently stimulated with 5 U/ml TNF- $\alpha$  1 h before harvest (lanes 2–11) or the respective volumes of ppH<sub>2</sub>O as vehicle control. Filled arrowhead = position of NF- $\kappa$ B DNA complexes; open circle = a nonspecific activity binding to the probe; open arrowhead = unbound oligonucleotide. The results are representative of six independent experiments.



**Fig. 4.** The effect of the time interval between thiopental pretreatment and the subsequent stimulation with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) on nuclear factor  $\kappa$ B (NF- $\kappa$ B) DNA binding. Jurkat cells were incubated with thiopental (1,000  $\mu$ g/ml) for 2 h (lanes 3–8). Cells that were stimulated with TNF- $\alpha$  (5 U/ml) during the second hour of thiopental treatment (no time interval between thiopental exposure and stimulation; similar experimental design as in figure 1) were included for comparison (lane 3). One hour before harvest, all other cells were also stimulated with TNF- $\alpha$  (5 U/ml) for 1 h. Filled arrowhead = position of NF- $\kappa$ B DNA complexes; open circle = a nonspecific activity binding to the probe; open arrowhead = unbound oligonucleotide. The results are representative of three independent experiments.

uation of thiopental exposure (fig. 4, lanes 7 and 8). Moreover, additional experiments using primary CD3<sup>+</sup> T cells obtained from healthy donors revealed that the suppressing action of thiopental on NF- $\kappa$ B activation is not confined to Jurkat cells but may rather affect normal human T cells in a similar fashion (fig. 5).

These results raised the question of whether the suppression of NF- $\kappa$ B activation by thiopental is a unique characteristic of this agent or may be a common biologic effect of anesthetics and opioids. However, in contrast to the results obtained with thiopental, pretreatment of Jurkat cells with either the oxybarbiturate methohexital (fig. 6) or etomidate (fig. 7A), fentanyl (fig. 7B), ketamine (fig. 7C), midazolam (fig. 7D), morphine (fig. 7E), or propofol (fig. 7F) had no effect on the activation of NF- $\kappa$ B.

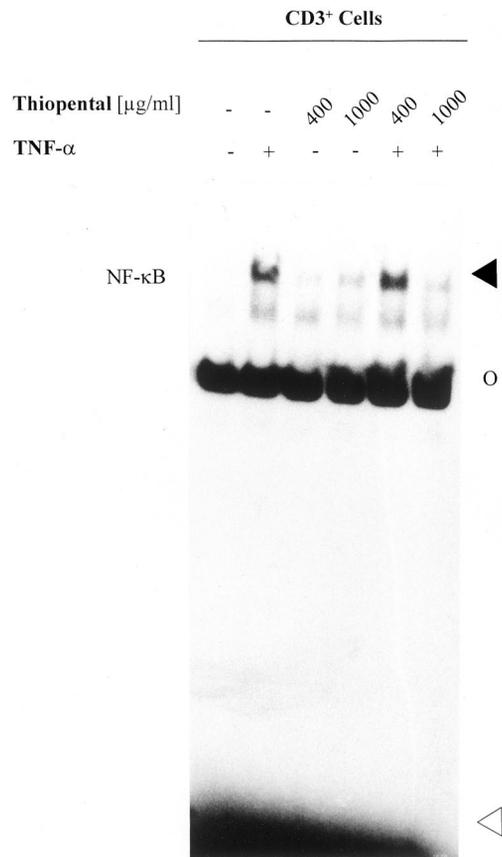
*Nuclear Factor  $\kappa$ B-driven Reporter Gene Activity*

The results described above raised the question of whether the inhibition of NF- $\kappa$ B DNA binding by thio-

pental is associated with a respective inhibition of NF- $\kappa$ B-dependent gene expression. To answer this question, transient transfection assays were performed. Although TNF- $\alpha$  did not cause any changes in luciferase activity in cells transfected with the minimal tk promoter (data not shown), it resulted in a profound increase in reporter gene activity in 6 $\times$  $\kappa$ B-tk-Luc cells (fig. 8). This activation was suppressed by thiopental in a dose-dependent fashion. Although incubation of the cells with thiopental in concentrations of 400  $\mu$ g/ml or less for 17 h attenuated the TNF- $\alpha$ -induced increase in reporter gene activity by 50–80%, higher concentrations of thiopental (> 400  $\mu$ g/ml) completely suppressed the activation of the  $\kappa$ B-driven promoter during these experimental conditions (fig. 8).

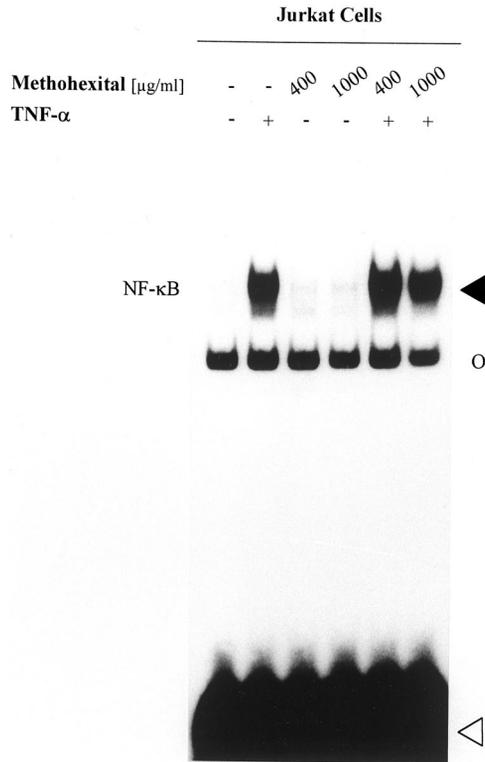
*Cytokine Assays*

These experiments were performed to determine whether inhibition of NF- $\kappa$ B transcriptional activity by



**Fig. 5.** The effect of thiopental on nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation in primary CD3<sup>+</sup> T lymphocytes, isolated from the blood of healthy donors, after stimulation with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ). The cells were incubated with thiopental *in vitro* for 1 h at the concentrations indicated (lanes 3–6) and subsequently stimulated with 20 U/ml TNF- $\alpha$  for 1 h (lanes 2, 5, and 6) or the respective volumes of ppH<sub>2</sub>O as vehicle control. Filled arrowhead = position of NF- $\kappa$ B DNA complexes; open circle = a nonspecific activity binding to the probe; open arrowhead = unbound oligonucleotide. The results are representative of six independent experiments.

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**Fig. 6.** The effect of methohexital on nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) DNA binding after tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) stimulation. Jurkat cells were treated for 1 h with methohexital at concentrations indicated (lanes 3–6) and subsequently stimulated with 5 U/ml of TNF- $\alpha$  for 1 h (lanes 2, 5, and 6) or the respective volumes of  $\text{ppH}_2\text{O}$  as vehicle control. Filled arrowhead = position of NF- $\kappa\text{B}$  DNA complexes; open circle = a nonspecific activity binding to the probe; open arrowhead = unbound oligonucleotide. The results are representative of six independent experiments.

thiopental is associated with altered cytokine release by immune cells. Pretreatment of primary  $\text{CD}3^+$  cells with thiopental prevented the increase in IL-8 within the supernatant that could otherwise be observed after stimulation (fig. 9). Similarly, thiopental reduced the amount of IL-6 released by peripheral mononuclear cells into the culture supernatant to approximately 50% of the maximal response that could be observed on activation in the absence of the agent (fig. 10). Moreover, the release of IL-2 and interferon  $\gamma$  after stimulation in the presence of thiopental was reduced to the level of untreated controls (fig. 10).

#### T-cell Activation Assays

To test if the inhibition of NF- $\kappa\text{B}$  by thiopental is associated with impairments of lymphocyte activation, we performed flow cytometric analysis using CD69 expression as a marker of lymphocyte activation. Cross-linking of the CD2 receptor resulted in an expression of CD69 by 53% of the cells (figs. 11A and B). After preincubation with 400  $\mu\text{g/ml}$  of thiopental, CD69 expression on stimulation was reduced to almost 27% (fig. 11C). Treatment with 1,000  $\mu\text{g/ml}$  thiopental decreased the

number of cells expressing CD69 to less than 6% (fig. 11D), almost the value obtained with unstimulated T cells.

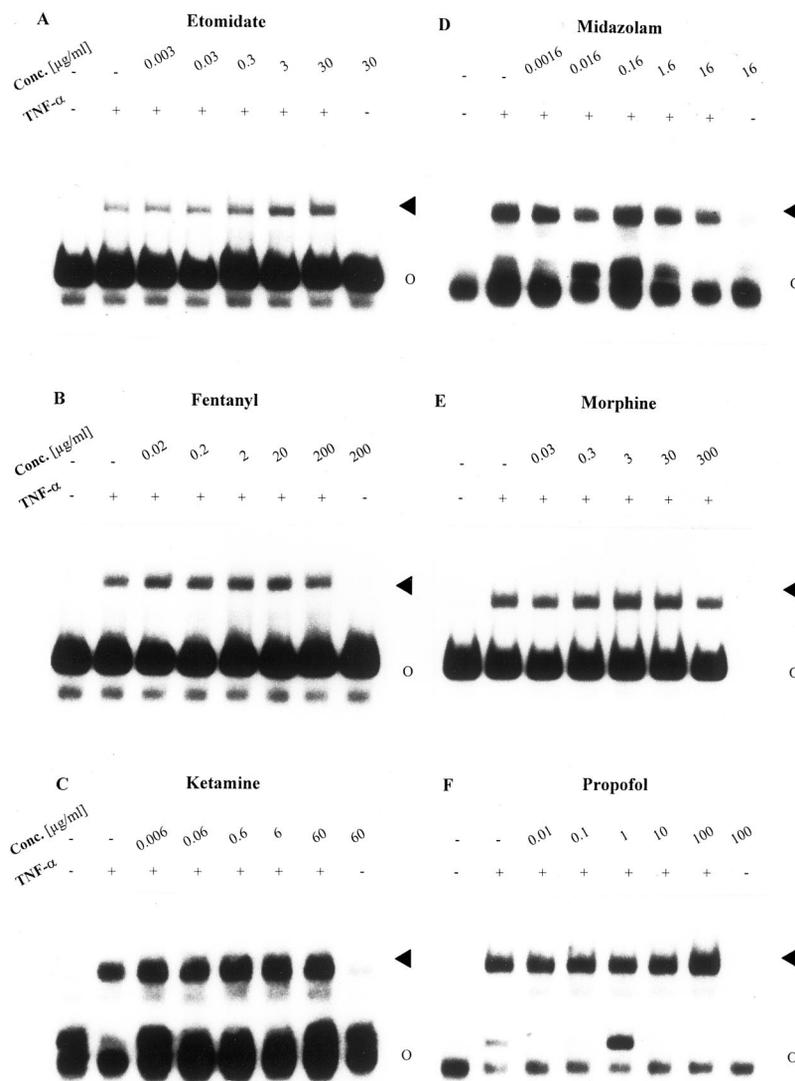
#### Detection of I $\kappa\text{B}$ - $\alpha$ by Western Blotting

Pretreatment of Jurkat T cells with thiopental alone did not change I $\kappa\text{B}$ - $\alpha$  content as compared with control conditions (fig. 12, lanes 1 and 3–5, top). Incubation of the cells with TNF- $\alpha$  in the absence of thiopental led to a reduction in I $\kappa\text{B}$ - $\alpha$  steady state protein concentrations (fig. 12, lane 2, top), which coincided with NF- $\kappa\text{B}$  activation in EMSAs (fig. 12, lane 2, bottom). Although this pattern remained basically unchanged if thiopental was present in low (200 or 400  $\mu\text{g/ml}$ ) concentrations (fig. 12, lanes 6 and 7, top and bottom), more I $\kappa\text{B}$ - $\alpha$  was present and, consequently, less NF- $\kappa\text{B}$  was activated after pretreatment of the cells with 1,000  $\mu\text{g/ml}$  thiopental (fig. 12, lane 8, top and bottom) than after incubation with TNF- $\alpha$  alone.

#### Discussion

Barbiturates such as thiopental are frequently used for the treatment of intracranial hypertension after severe head injury.<sup>24</sup> Despite the potent neuroprotective effects of thiopental, long-term administration of the high doses necessary to achieve this effect is associated with a profound increase in the incidence of nosocomial infections, which may contribute to the high mortality rate of these patients.<sup>8,25–27</sup> Separating the neuroprotective from the immunosuppressing effects would thus represent a major progress in the therapy of patients with severe intracranial hypertension. This challenge is compounded by the lack of knowledge about the molecular mechanism of thiopental-induced immunosuppression.

We show here by different criteria that thiopental is an inhibitor of the transcription factor NF- $\kappa\text{B}$ , a central regulator of the immune response.<sup>28–30</sup> The effect of thiopental had the following characteristics: (1) it was confined to the inducible transcription factor NF- $\kappa\text{B}$  and did not extend to the constitutively active transcription factor CREB; (2) it appeared to be a special pharmacologic characteristic of thiopental because it occurred in a dose-dependent manner and all other barbiturates, anesthetics, or opioids tested did not exert any inhibitory effect on NF- $\kappa\text{B}$  activation; (3) it could be important for the function of immune cells because it was associated with an inhibition of NF- $\kappa\text{B}$ -driven reporter gene activity, of the expression of the NF- $\kappa\text{B}$  target genes IL-2, IL-6, IL-8, and interferon  $\gamma$ , and of the activation of T lymphocytes, as revealed by decreased CD69 expression; (4) it appeared to involve the inhibition of I $\kappa\text{B}$ - $\alpha$  degradation because the disappearance of immunoreactive I $\kappa\text{B}$ - $\alpha$  that could otherwise be observed on stimulation was prevented by thiopental; and (5) it may be of



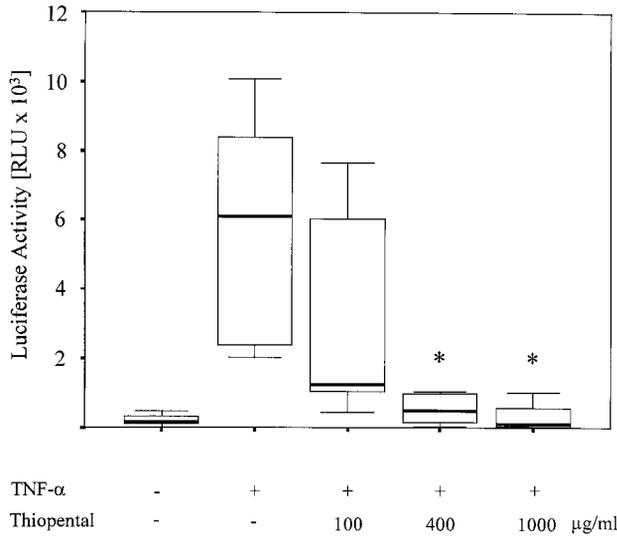
**Fig. 7.** Effect of etomidate (A), fentanyl (B), ketamine (C), midazolam (D), morphine (E), and propofol (F) on nuclear factor  $\kappa$ B (NF- $\kappa$ B) DNA binding after tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) stimulation. Jurkat cells were treated for 1 h with the different agents at the concentrations indicated (lanes 3–8) and were subsequently stimulated with 5 U/ml TNF- $\alpha$  for 1 h (lanes 2–7) or the respective volumes of ppH<sub>2</sub>O as vehicle control. The first lane shows unstimulated control cells. Cells treated with the drugs without TNF- $\alpha$  stimulation showed no NF- $\kappa$ B DNA binding activity (last lanes). Filled arrowhead = position of NF- $\kappa$ B DNA complexes; open circle = a nonspecific activity binding to the probe; open arrowhead = unbound oligonucleotide. The results are representative of three independent experiments.

clinical relevance because thiopental-mediated inhibition of NF- $\kappa$ B activation was time-dependent, persisted beyond the exposure, could be observed not only in Jurkat lymphoma cells but also in human primary CD3<sup>+</sup> T lymphocytes from healthy donors, and occurred at *in vitro* concentrations comparable to those attained in the plasma of patients during therapeutic coma. We thus propose that thiopental may exert some of its immunosuppressing effects through inhibition of the activation of NF- $\kappa$ B.

Several previously reported effects of thiopental on the function of immune cells could be explained by its inhibitory effect on NF- $\kappa$ B. NF- $\kappa$ B plays a crucial role in the control of the activation, proliferation, and differentiation of neutrophils, macrophages, and T and B lymphocytes.<sup>29</sup> Therefore, the reduced chemotaxis, phagocytotic activity, and oxidative burst of human neutrophils,<sup>9,11,31</sup> the attenuation of TNF- $\alpha$  production and CD14 expression of peripheral blood mononuclear cells in response to bacterial endotoxin,<sup>32</sup> as well as the reduction of the mitogen-activated lymphocyte prolifer-

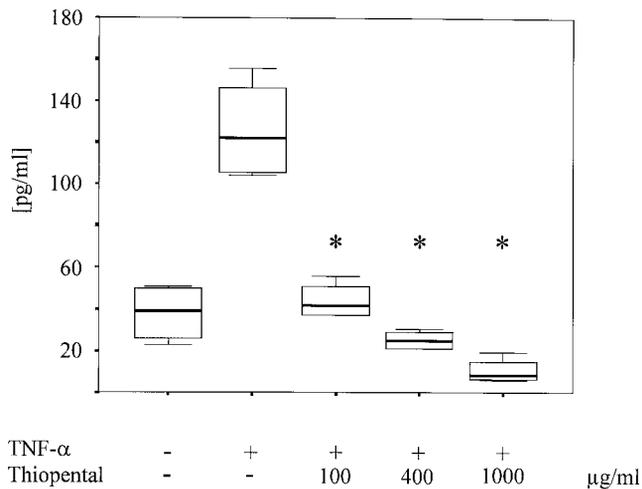
ation after thiopental exposure *in vitro*<sup>12</sup> would be consistent with an inhibition of NF- $\kappa$ B by thiopental. Likewise, the reduced natural killer cell activity,<sup>33</sup> and the impaired phagocytotic activity and superoxide generation of rat macrophages<sup>34</sup> in response to thiopental exposure *in vivo*, as well as the inhibition of the antigen-specific lymphocyte proliferation in patients after anesthesia with thiopental,<sup>35</sup> could be explained in the same manner. Moreover, NF- $\kappa$ B controls the transcription of genes for the adhesion molecules intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 and endothelial cell adhesion molecule 1.<sup>36</sup> Therefore, the fact that pretreatment with thiopental reduces the ability of human polymorphonuclear leukocytes to penetrate through an endothelial cell layer *in vitro*<sup>37</sup> may reflect inhibition of NF- $\kappa$ B by thiopental.

Our observation raises the question of how thiopental may inhibit the activation of NF- $\kappa$ B. Most anesthetic agents are potent agonists of the receptors for the ubiquitous inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (GABA).<sup>38</sup> In this regard, it is of interest that Tian *et al.*<sup>39</sup>

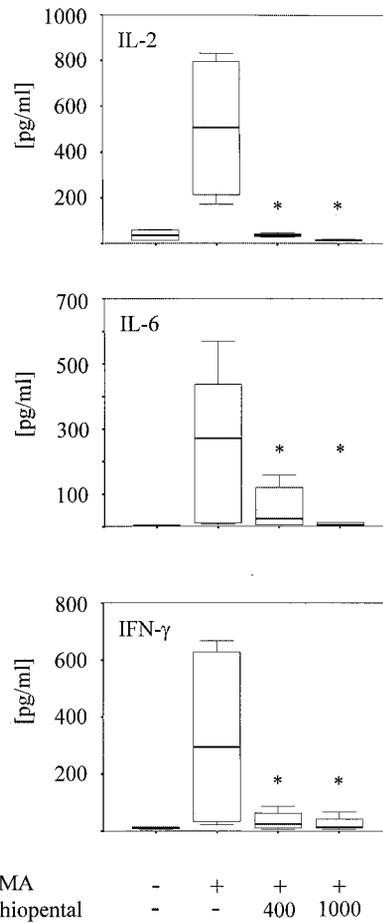


**Fig. 8.** The effect of thiopental on nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) promoter-driven reporter gene activity. Jurkat cells were transiently transfected with a six-copy  $\kappa\text{B}$  reporter construct (6 $\times$ - $\kappa\text{B}$ -tk-luciferase). After pretreatment with thiopental at the concentrations indicated for 1 h, cells were stimulated with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ; 100 U/ml). After 17 h, the cells were harvested, and luciferase activity was determined. The results are given as percentage of the arbitrary light units of control cells stimulated with TNF- $\alpha$  in the absence of thiopental. \* $P < 0.05$  compared with TNF- $\alpha$  stimulation alone. Data represent the median, 25–75%, and 95% confidence interval of six to eight experiments in each group.

recently provided evidence for the presence of functional GABA receptors on T cells. In this study, GABA receptor stimulation inhibited anti-CD3 and antigen-specific T-cell proliferation, which both require appropriate



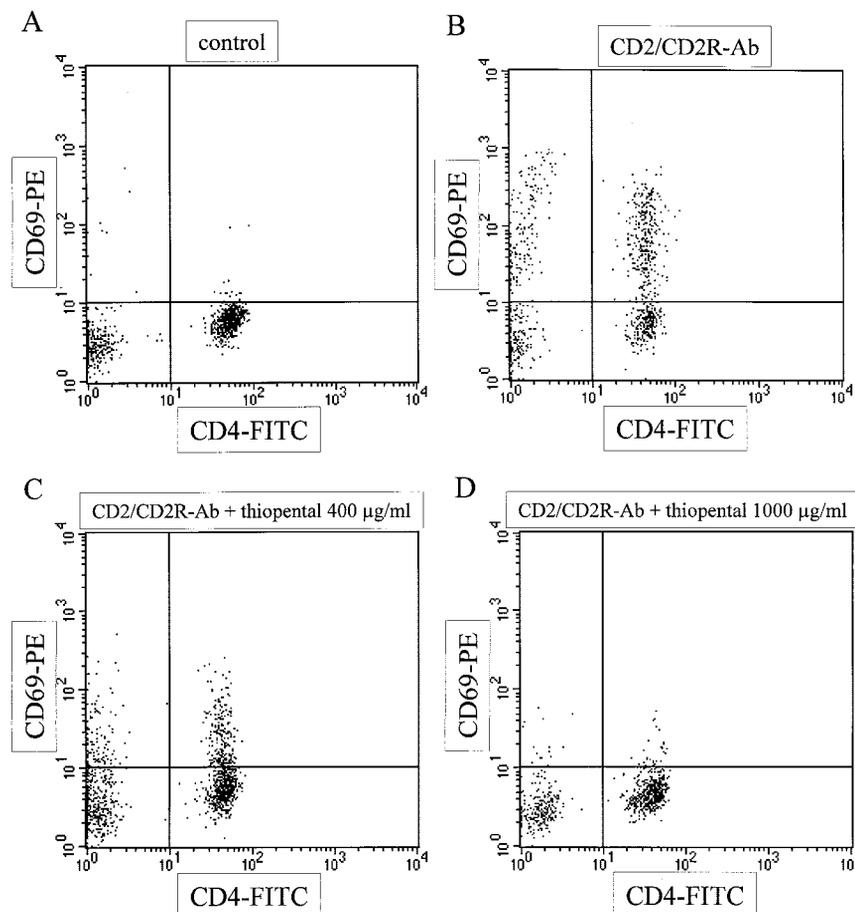
**Fig. 9.** The effect of thiopental on tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )-induced interleukin-8 production in primary  $\text{CD3}^+$  T lymphocytes, isolated from the blood of healthy donors and pretreated with different concentrations of thiopental (100, 400, or 1,000  $\mu\text{g/ml}$ ) 1 h before stimulation with TNF- $\alpha$  (100 U/ml) for 24 h. The cell culture supernatant was collected and analyzed for the concentration of interleukin-8 by enzyme-linked immunosorbent assay. \* $P < 0.05$  compared with TNF- $\alpha$  stimulation alone. Data represent the median, 25–75%, and 95% confidence interval of six to eight experiments in each group.



**Fig. 10.** The effect of thiopental on phorbol-12-myristate-13-acetate (PMA)-induced cytokine production in primary peripheral blood mononuclear cells *in vitro*. Peripheral blood mononuclear cells were pretreated with different concentrations of thiopental (400 or 1,000  $\mu\text{g/ml}$ ) for 1 h before stimulation with PMA (125 ng/ml) for 24 h. The cell culture supernatant was collected and analyzed for the concentrations of interleukin-2, interleukin-6, and interferon  $\gamma$  by enzyme-linked immunosorbent assay. \* $P < 0.05$  compared with PMA stimulation alone. Data represent the median, 25–75%, and 95% confidence interval of six to eight experiments in each group.

activation of NF- $\kappa\text{B}$ . Thus, it would be tempting to speculate that the inhibitory effect of thiopental on NF- $\kappa\text{B}$  described in the current study could involve GABA receptor stimulation. However, the fact that all other anesthetics tested are also potent GABA agonists but did not exert any inhibitory effect on NF- $\kappa\text{B}$  activation strongly argues against a key role of GABA receptors in this process.

Another hint toward the signal transduction pathway involved can be derived from the fact that the oxybarbiturate methohexital did not affect NF- $\kappa\text{B}$  activation, while the thiobarbiturate thiopental did. This is in agreement with the results of previous studies showing that the suppressive effect of the sulfated analogs on neutrophil function is 10- to 100-fold stronger than that of oxybarbiturates.<sup>10,31</sup> These findings point to a potential role of the sulfur atom at the C2 position of the barbiturate

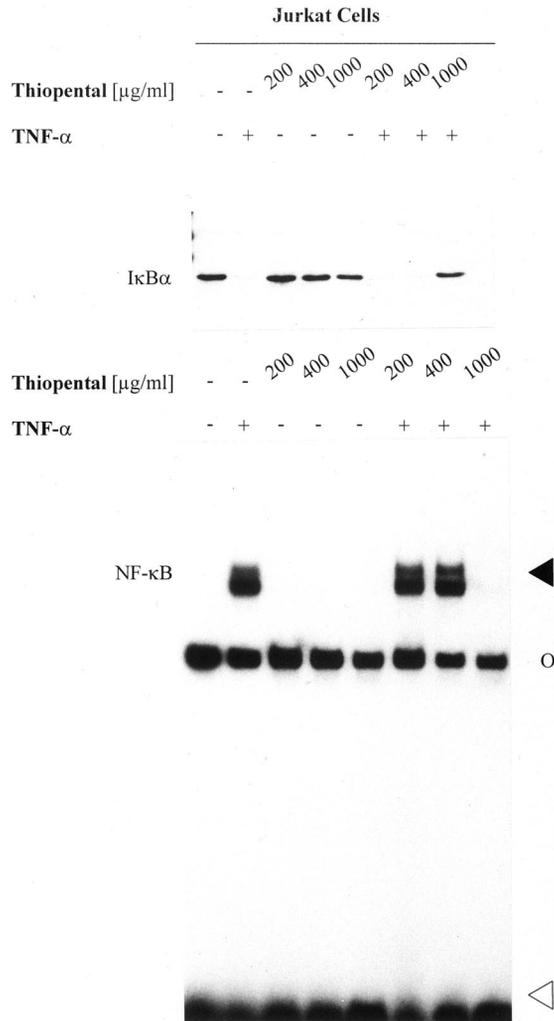


**Fig. 11. (A–D)** The effect of different concentrations of thiopental on T-cell activation. CD3<sup>+</sup> T lymphocytes were isolated from the blood of healthy donors, incubated with thiopental *in vitro* for 1 h at the concentrations indicated, and subsequently stimulated by cross-linking of the CD2 receptor using a CD2–CD2-receptor-ligand-antibody mix. Twelve hours later, the cells were assessed for CD69 expression by fluorescence-associated cell sorting analysis. Similar results were obtained in three additional independent experiments.

rate molecule as the structural requirement for the inhibition of NF- $\kappa$ B. Most agents activate NF- $\kappa$ B through a common pathway based on phosphorylation-induced, proteasome-mediated degradation of I $\kappa$ B. The key regulatory step in this pathway involves activation of an I $\kappa$ B kinase.<sup>40</sup> Recent evidence suggests that cysteine residues are present in the kinase domain of I $\kappa$ B kinases and are located at functionally important sites.<sup>41,42</sup> Oxidation of these sulfhydryl groups to disulfide inactivates the enzymes and prevents the subsequent activation of NF- $\kappa$ B.<sup>43</sup> Whether thiopental can directly interfere with any of these processes is not known. However, as a thiol-reactive agent it could also modify cysteine residues in I $\kappa$ B kinase molecules, inhibiting their activity and preventing I $\kappa$ B degradation. Because Western blot analyses revealed that thiopental prevented the disappearance of immunoreactive I $\kappa$ B- $\alpha$ , the sum of these findings suggests that the suppression of NF- $\kappa$ B activation by thiopental may involve the inhibition of I $\kappa$ B degradation.

The following findings suggest that the thiopental-mediated inhibition of NF- $\kappa$ B described here could be responsible for some of the immunosuppressing effects associated with thiopental therapy. First, the suppressing effect of thiopental could be observed not only in Jurkat lymphoma cells but also in primary T lymphocytes from healthy donors. Second, the inhibitory action oc-

curred at *in vitro* concentrations comparable to those attained in the plasma of patients during long-term administration of thiopental for the treatment of intracranial hypertension.<sup>12</sup> Moreover, tissue concentrations of thiopental may even exceed the plasma concentration. For example, it has been reported that the concentrations of thiopental were three times higher in the lungs and more than 10 times higher in the thymus than in the plasma after intravenous injection.<sup>44</sup> This is of particular importance because both organs seem to play a central role in the development of nosocomial infections during thiopental therapy.<sup>6,44</sup> Third, inhibition of the activation of NF- $\kappa$ B by thiopental persisted for several hours beyond the actual time of exposure, which would suggest that the negative immunomodulatory effects could still be present even after the discontinuation of thiopental therapy. Fourth, Adib-Conquy *et al.*<sup>45</sup> have recently reported that failure of peripheral blood mononuclear cells from patients with sepsis to respond to *in vitro* stimulation with an activation of NF- $\kappa$ B was associated with a poor outcome. Finally, identification of the molecular mechanism of thiopental-induced immunosuppression together with the evidence that the sulfur atom within the thiopental molecule may be a structural requirement for the inhibition of NF- $\kappa$ B could form a basis for the development of new approaches in the therapy of se-



**Fig. 12.** The effect of thiopental on IκB-α phosphorylation and proteolytic degradation. Jurkat T cells were incubated with different concentrations of thiopental (200, 400, or 1,000 μg/ml; lanes 3–8) for 105 min before stimulation with tumor necrosis factor α (TNF-α; 20 U/ml) for 15 min (lanes 2 and 6–8). Cell extracts were analyzed by Western blotting with an anti-IκB-α polyclonal antibody (*top*). In addition, the same extracts were analyzed for nuclear factor κB (NF-κB) DNA binding activity by electrophoretic mobility shift assays (*bottom*). The data are representative of six independent experiments.

vere intracranial hypertension, aimed at separating neuroprotection from immunosuppression.

However, it is important to point out that coma, whether induced by thiopental, other anesthetics, or neurologic injury, is associated with a high degree of infectious complications. Moreover, all other anesthetic agents tested in the current study did not inhibit NF-κB activation, although most of them have been shown to negatively affect immune functions.<sup>9–11,13</sup> This strongly suggests that, in addition to NF-κB inhibition, other signal transduction pathways must be involved in the immunosuppression associated with different types of coma that need to be identified in future experimental and clinical studies.

In conclusion, our data suggest that thiopental is an inhibitor of NF-κB, of its *trans*-acting potency, and of its downstream effects on immune cell function. Therefore, these results may provide a molecular mechanism for some of the immunosuppressing effects associated with thiopental treatment.

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