Anesthesiology 2003; 99:360–7

Thiopental Inhibits Tumor Necrosis Factor α–induced Activation of Nuclear Factor κB through Suppression of IκB Kinase Activity

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Background: **Thiopental is frequently used for the treatment of intracranial hypertension after severe head injury and is associated with immunosuppressive effects. The authors have recently reported that thiopental inhibits activation of nuclear factor (NF) κB, a transcription factor implicated in the expression of many inflammatory genes. Thus, it was the aim of the current study to examine the molecular mechanism of this inhibitory effect.**

Methods: **The authors tested γ-aminobutyric acid (GABA), the $GABA_A$ antagonist bicuculline, and the $GABA_B$ antagonist dichlorophenyl-methyl-amino-propyl-diethoxymethyl-phosphinic acid (CGP 52432) in combination with thiopental for their influence on the activation of NF-κB. In addition, they investigated the direct effect of thiopental on activated NF-κB DNA binding activity. These experiments were conducted in Jurkat T lymphocytes using electrophoretic mobility shift assays. The presence of the phosphorylated and dephosphorylated NF-κB inhibitor IκBα (Western blotting) and IκB kinase activity were studied in Jurkat T cells and human $CD3^+$ T lymphocytes. In addition, the authors tested the effect of the structural barbiturate analog pairs thiopental–pentobarbital and thiamylal–secobarbital and of thiopental in combination with the thio-group containing chemical dithiothreitol on the activation of NF-κB.**

Results: **GABA did not inhibit NF-κB activation, and the $GABA_A$ and $GABA_B$ antagonists bicuculline and CGP did not diminish the thiopental-mediated inhibitory effect on NF-κB activation. Thiopental did not inhibit activated NF-κB directly in a cell-free system. The phosphorylation of IκBα was prevented after incubation with 1,000 μg/ml thiopental. The same concentration of thiopental also inhibited IκB kinase activity in tumor necrosis factor–stimulated Jurkat T cells and human $CD3^+$ T lymphocytes (60% suppression, $P < 0.05$ *vs.* tumor necrosis factor α alone). Thiobarbiturates (4×10^{-3} M) inhibited NF-κB activity, whereas equimolar concentrations of the structural oxyanalogs did not. Preincubation of thiopental with dithiothreitol diminished the inhibitory effect.**

Conclusion: **Thiopental-mediated inhibition of NF-κB activation is due to the suppression of IκB kinase activity and depends at least in part on the thio-group of the barbiturate molecule.**

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Received from the Department of Anesthesiology and Critical Care Medicine, University Hospital, Freiburg, Germany. Submitted for publication October 17, 2002. Accepted for publication March 19, 2003. Supported by departmental funding; the European Academy of Anaesthesiology (EAA Clinical Scholar Research Award 2001), Heverlee, Belgium; and a grant (Heisenberg-Stipend DFG PA 533/3-1 and 3-2) from the Deutsche Forschungsgemeinschaft, Bonn, Germany (to Dr. Pannen).

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BARBITURATES such as thiopental are frequently used for the treatment of intracranial hypertension after severe head injury.[1] Recent evidence suggests that thiopental treatment increases the incidence of nosocomial infections, causing a higher mortality rate. The immunmodulatory properties of barbiturates could at least partially explain the impaired immune function in these patients.[2–5] However, the molecular mechanism of thiopental-induced immunosuppression remains elusive. Several studies have provided evidence that thiopental may interfere with the function of immune cells. For example, incubation of neutrophil leukocytes with thiopental inhibited the production of reactive oxygen species[6,7] and decreased chemotaxis as well as phagocytosis.[8] Moreover, T lymphocyte functions, as measured indirectly by cytokine accumulation, were decreased by exposure to thiopental.[9,10]

The nuclear transcription factor (NF) κB plays a central role in the expression of a wide range of immunmodulatory genes including proinflammatory cytokines, such as interleukin (IL)-1, IL-2, IL-6, IL-8 and tumor necrosis factor (TNF) α, as well as genes encoding immunoreceptors, cell adhesion molecules, hematopoietic growth factors, growth factor receptors, and acute-phase proteins.[11] In most unstimulated cell types, NF-κB proteins are sequestered in the cytosol as an inactive complex bound to IκB, its inhibitory subunit.[12] A large variety of stimuli induce NF-κB DNA binding activity *via* activation of an IκB kinase (IKK) complex, which in turn phosphorylates IκB at two conserved serines (S32 and S36).[13] Two catalytic subunits of the IKK complex, designated IKKα (or IKK1) and IKKβ (or IKK2), and the regulatory subunit IKKγ (NEMO) have been cloned and demonstrated to be part of this multicomponent IKK complex, called the IKK signalsome.[13–16] Activation of the IKK signalsome is followed by IκB phosphorylation, ubiquitination, and rapid proteolytic degradation of the inhibitor.[17,18] This allows translocation of free, active NF-κB into the nucleus, where it binds to its cognate DNA elements and activates gene transcription.

We have previously shown that thiopental inhibits tumor necrosis factor (TNF)–induced activation of NF-κB in human T lymphocytes.[19] These results suggested that the NF-κB pathway is a target for the immunosuppressive effect of thiopental. The current work was performed to examine by which molecular mechanism thio-

pental-mediated inhibition of NF-κB activation is achieved.

As described in detail in the section just above, many components of the NF-κB signal transduction pathway have been identified so far. Thus, in this work, we sought to determine which components of the activation pathway may be affected by thiopental. Because many cells of the immune system express receptors for neuroactive molecules, this phenomenon creates a link between the nervous and the immune system.[20,21] γ-Aminobutyric acid (GABA), which acts through $GABA_A$ and $GABA_B$ receptor subtypes, is an important inhibitory neurotransmitter in the mammalian brain.[22] $GABA_A$, a ligand-gated ion channel, has long been regarded as a common target for all general anesthetics, including thiopental.[23] It has been shown recently that T cells express $GABA_A$ receptors that may control some of their functions.[24] For example, T-cell receptor–induced T-lymphocyte proliferation, as well as basal and stress-induced IL-6 levels, are mediated *via* a GABA receptor pathway.[25,26] Therefore, we currently investigated whether GABA receptors are involved in the thiopental-mediated inhibition of NF-κB. In a next step, using a cell-free system, we evaluated whether thiopental acts directly on activated NF-κB protein to inhibit DNA binding. Furthermore, we investigated in the current study whether thiopental affects the upstream signal transduction pathway of phosphorylation on stimulation. Thus, we treated human Jurkat T cells and primary $CD3^+$ T lymphocytes with thiopental, stimulated them with TNF-α, and examined IκB phosphorylation and IKK activity. Finally, we determined the functional role of the thio-group at the C2 position of thiobarbiturates in the inhibition of NF-κB.

Material and Methods

Reagents

The following anesthetics and substances were used: GABA (Sigma, Deisenhofen, Germany), bicuculline-methochloride (Tocris Cookson Inc., Ellisville, MO), dichlorophenyl-methyl-amino-propyl-diethoxymethyl-phosphinic acid (CGP 52432; Tocris Cookson Inc.), pentobarbital, thiamylal (Surital; Pharmacia, Erlangen, Germany), secobarbital (Sigma), and thiopental (Byk-Gulden, Konstanz, Germany). Recombinant human TNF-α was purchased from Sigma. All other reagents were purchased from Sigma unless specified otherwise.

Isolation of $CD3^+$ T Lymphocytes

Peripheral blood mononuclear cells were isolated from buffy coats donated from healthy donors by density centrifugation on Ficoll-Hypaque (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 $CD3^+$ T lymphocytes, the peripheral blood mononuclear cells ($3–4 \times 10^8$) were incubated for 15 min on ice with anti CD3-antibodies conjugated to magnetic beats (Miltenyi Biotech, Bergisch-Gladbach, Germany). Separation of $CD3^+$ cells was performed using an L/S column (Miltenyi Biotech) and confirmed by fluorescence-associated cell sorting (> 85% purity). For electrophoretic mobility shift assays (EMSAs) $> 5 \times 10^6$, T lymphocytes were analyzed per sample.

Cell Culture

Jurkat T cells (ACC 282; DSMZ, Braunschweig, Germany) and primary human T lymphocytes, which had been isolated as described previously, were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum, 1% glutamine, and 50 mg/ml penicillin and streptomycin (all from Gibco-BRL, Karlsruhe, Germany) and were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

Electrophoretic Mobility Shift Assays

Total cell extracts were prepared using a high-salt detergent buffer, Totex (20 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic) acid [HEPES], pH 7.9, 350 mM NaCl, 20% (v/v) glycerol, 1% (w/v) NP-40, 1 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid [EDTA], 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid [EGTA], 0.5 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride [PMSF], 1% aprotinin). Cells were harvested by centrifugation, washed once in ice-cold PBS, 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 13,000*g* at 4°C. EMSAs were performed using a ^{32}P-labeled NF-κB oligonucleotide as previously described.[27,28] The reaction mixture consisted of 37 μl purified water, 1 μl NF-κB oligonucleotides (25 ng/μl; Promega, Madison, WI), 5 μl kinase buffer, 5 μl γ-^{32}P-dATP (Amersham International, Braunschweig, Germany), and 1.5 μl T4 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 μg) were added to a 20-μl reaction mixture containing 20 μg bovine serum albumin, 2 μg polydesoxyiosine-desoxycytosine (dI-dC; Roche, Mannheim, Germany), 2 μl 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 μl 5× Ficoll buffer (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM dithiothreitol, 0.1% PMSF), 4 μl double-distilled water, and 1 μl NF-κB ^{32}P-labeled oligonucleotide. These samples were incubated at room temperature for 30 min and then loaded on an acrylamide gel containing 60 ml dou-

ble-distilled water, 10 ml 30% acrylamide, 3.8 ml 10× Tris-borate-EDTA buffer (TBE: 900 mM TRIS-HCl, 900 mM boric acid, 20 mM EDTA [pH 8.0]), 400 μl ammonium persulfate, and 40 μl tetramethylethylenediamine. After running the gel in 0.5× TBE 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 (Kodak, Stuttgart, Germany).

Detection of IκB-α by Western Blotting

The activation and translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of the inhibitory IκB-α proteins. This process is readily detectable in Western blots.[18] To determine whether thiopental may interfere with the degradation of IκB-α, Jurkat T cells were pretreated with different doses of thiopental (200, 400, or 1,000 μg/ml) for 105 min and subsequently stimulated with TNF-α (20 U/ml) for 15 min. These time points were chosen on the basis of the previously published time course of TNF-α–mediated degradation of IκB-α.[29] Total cell extracts of Jurkat T cells (30 μg) were boiled in Laemmli sample buffer and subjected to 10% SDS-PAGE. Prior to transfer, gels were equilibrated for 15 min in cathode buffer (25 mM Tris, 40 mM glycin, 10% methanol). Proteins were transferred at 0.8 mA/cm^2 for 1 h onto Immobilon P membranes (Millipore Corp., Eschborn, Germany) preequilibrated in methanol (15 s), double-distilled water (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 (v/v)] containing 2% bovine serum albumin) overnight at 4°C. Membranes were washed in TBST and incubated in a 1:1,000 dilution of anti-IκBα antibody or antiphosphorylated-IκBα antibody (IκBα^P) (cat. No. 9241; Cell Signaling Technology Inc., Beverly, MA) 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) and 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 was performed for 15 s to 1 min.

Immunoprecipitation and In Vitro *Kinase Assay*

Lymphocytes were treated with vehicle, with TNF-α alone, or with TNF-α and thiopental at specified concentrations and harvested by centrifugation in ice-cold phosphate-buffered saline containing phosphatase inhibitor cocktail set II (Calbiochem, La Jolla, CA). Equal amounts (500 μg) of whole cell protein extracts were obtained in immunoprecipitation buffer (50 mM HEPES [pH 7.6], 250 mM NaCl, 10% glycerol, 1 mM EDTA) containing 0.1% NP40 with protease and phosphatase inhibitors. The cell lysate was cleared and incubated for 2 h at 4°C with an antibody against IKKα (cat. No. sc-7606 AC; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The immunoprecipitates were washed extensively with immunoprecipitation buffer. One portion of the immunoprecipitated IKKα complexes was run on a separate 10% SDS-PAGE and was Western blotted with the IKKα antibody to check for equal loading. The remaining portion was used to perform the *in vitro* kinase assay by incubating the immunoprecipitates with 4 μg IκBα (cat. No. sc-4094; Santa Cruz Biotechnology Inc.) in 20 μl kinase buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 μM ATP, and 5 μCi [γ-^{32}P]ATP for 30 min. The immunoprecipitates were subjected to SDS-PAGE, dried, and visualized by autoradiography.

Experimental Protocols

To determine whether the inhibitory effect of thiopental on NF-κB activation is mediated through a GABA receptor–dependent agonistic mechanism, we performed EMSAs using total cell extracts from Jurkat T cells after a 2-h incubation with thiopental (400 or 1,000 μg/ml) or GABA (3 and 10 mM). To evaluate whether $GABA_A$ antagonism with bicuculline (10 and 100 μM) or $GABA_B$ antagonism with CGP 52432 (100 and 500 μM) would be able to prevent the thiopental-mediated inhibitory effect, cells were pretreated with these antagonists for 1 h before thiopental incubation. One hour before harvesting, the cells were stimulated with TNF-α (1 ng/ml) for 1 h, after which total cell protein extracts were prepared and analyzed for the DNA binding activity of NF-κB by EMSA.

To determine whether the inhibitory effect of thiopental could be explained by direct targeting of the NF-κB, cell extracts from TNF-stimulated Jurkat T cells were pooled to achieve equality of activation and were than subsequently fractionated. Extracts were incubated with thiopental (100, 200, 400, or 1,000 μg/ml) for 1 or 2 h in a humidified atmosphere containing 5% CO_2 at 37°C in a cell-free system. The cell extracts were analyzed for the DNA binding activity of NF-κB by EMSA.

Because the phosphorylated form of IκBα (IκBα^P) is highly transient and is therefore difficult to capture, the proteasome inhibitor MG 132 (Calbiochem Corp.) was used to inhibit the IκBα degradation and to visualize the phosphorylated form of IκBα. Consequently, cells were treated with 10 or 20 μM MG 132 for 1 h before the

addition of thiopental or vehicle, and after TNF-α stimulation the whole cell lysates were then collected as described previously.

To determine whether thiopental inhibits signaling pathways leading to IκB phosphorylation, we measured IKK activity in TNF-stimulated Jurkat T cells and $CD3^+$ T lymphocytes *in vitro*. To detect IKK activity, Jurkat T cells and $CD3^+$ T lymphocytes were incubated with TNF-α (1 ng/ml) for 15 min (described by Rossi *et al.*[30]; data not shown) after incubation with various concentrations of thiopental (200, 400, 1,000 μg/ml) for 2 h. Equal loading of IKK was determined by Western blotting for IKKα.

To investigate whether the inhibitory effect of thiopental is confined to the thio-group at the C2 position of the pyrimidine ring, we analyzed the effect of different pairs of structural barbiturate analogs (thiopental–pentobarbital and thiamylal–secobarbital) differing exclusively in this substituent on TNF-induced NF-κB activation. Therefore, Jurkat T cells were incubated with equimolar concentrations of thiopental or pentobarbital as well as thiamylal or secobarbital for 2 h. One hour before harvesting, the cells were stimulated with TNF-α (1 ng/ml) for 1 h, after which total cell extracts were prepared. The cell extracts were analyzed for the DNA binding activity of NF-κB by EMSA.

To test whether NF-κB inhibition is due to the thio-group of thiopental, we precoincubated thiopental with dithiothreitol for 1 h at room temperature in double-distilled water, since thiopental would react with the large quantities of free sulfhydryls in dithiothreitol to chemically modify the sulfur atom by developing disulfide bonds. Jurkat T cells were incubated with dithiothreitol alone (0.01, 0.1, 1, or 5 mM), with thiopental alone (1,000 μg/ml), or with dithiothreitol (0.1, 1, or 5 mM) together with thiopental (1,000 μg/ml) for 2 h. One hour before harvesting, the cells were stimulated with TNF-α (5 U/ml) for 1 h, after which total cell extracts were prepared and analyzed for NF-κB DNA binding activity by EMSA.

Quantitative and Statistical Analysis

Autoradiographs of the kinase assay experiments were evaluated by volume quantification and local median background correction using two-dimensional scanning (Personal Densitometer; Amersham-Pharmacia). Differences in measured variables between the experimental conditions were assessed using one-way analysis of variance on ranks followed by a nonparametric Student-Newman-Keuls test for multiple comparisons. Results were considered statistically significant at $P < 0.05$. The tests were performed using the SigmaStat software package (Jandel Scientific, San Rafael, CA).

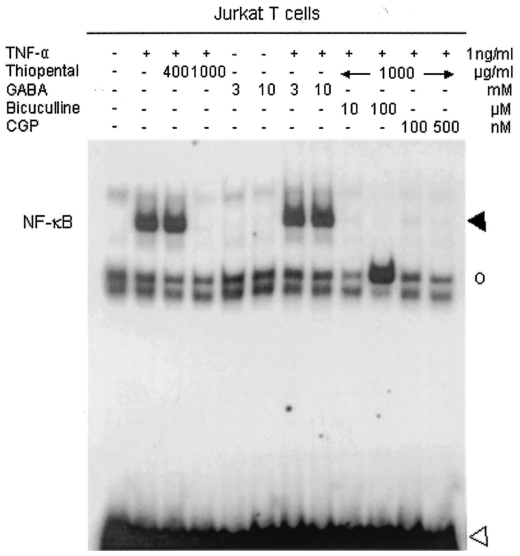

Fig. 1. The effect of γ-aminobutyric acid (GABA), of the $GABA_A$ antagonist bicuculline, and of the $GABA_B$ antagonist CGP on nuclear factor (NF) κB DNA binding after tumor necrosis factor (TNF) α stimulation. Jurkat T cells were treated for 1 h with either thiopental (*lanes 3* and *4*) or GABA (*lanes 5–8*) at the concentrations indicated and subsequently stimulated with 1 ng/ml TNF-α for 1 h (*lanes 2–4* and *7–12*) or with the respective volumes of ppH_2O as vehicle control. Alternatively, Jurkat T cells were pretreated for 1 h with either bicuculline or CGP at the concentrations indicated, subsequently incubated with thiopental (1,000 μg/ml), and stimulated with TNF-α as described previously. DNA binding activity was analyzed by electrophoretic mobility shift assays. *Filled arrow* = position of NF-κB DNA complexes; *circle* = nonspecific activity binding to the probe; *hollow arrow* = unbound oligonucleotide. The data shown are representative of six independent experiments.

Results

Effects of GABA Receptor Agonists and Antagonists on NF-κB Activation

Treatment of Jurkat T cells with TNF-α induced NF-κB DNA binding activity (fig. 1, *lanes 1* and *2*), which was inhibited by pretreatment of cells with 1,000 μg/ml thiopental as previously reported (fig. 1, *lane 4*).[19] In contrast, incubation of Jurkat cells with GABA alone (3 and 10 mM) had no effect on the activation of NF-κB (fig. 1, *lanes 5* and *6*). The addition of TNF-α to GABA-treated cells resulted in NF-κB activation similar to that observed in control cells (fig. 1, *lanes 7* and *8 vs. lane 2*). Pretreatment of Jurkat T cells with either the $GABA_A$-antagonist bicuculline (10 and 100 μM) or the $GABA_B$-antagonist CGP 52432 (100 and 500 μM) did not prevent the inhibition of NF-κB by thiopental (fig. 1, *lanes 9–12*).

Direct Effect of Thiopental on Activated NF-κB

In vitro incubation of cell extracts containing activated NF-κB with different concentrations of thiopental (100, 200, 400, 1,000 μg/ml as indicated) for 1 h (fig. 2, *lanes 2–6*) and 2 h (fig. 2, *lanes 8–12*) had no effect on NF-κB DNA binding activity as detected by EMSA.

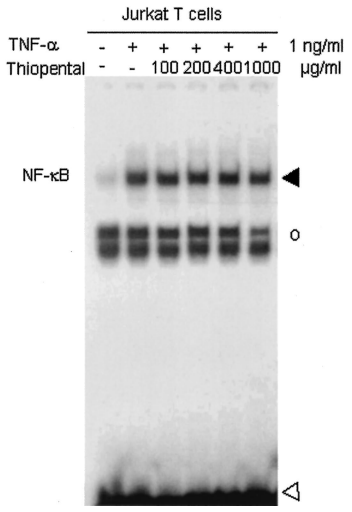

Fig. 2. The effect of thiopental on activated nuclear factor (NF) κB. Cell extracts were obtained from tumor necrosis factor (TNF) α–stimulated Jurkat T cells and incubated with thiopental (100, 200, 400, or 1,000 μg/ml) *in vitro* for 1 h or with the respective volumes of ppH_2O as vehicle control. DNA binding activity was analyzed by electrophoretic mobility shift assays. *Filled arrow* = position of NF-κB DNA complexes; *circle* = nonspecific activity binding to the probe; *hollow arrow* = unbound oligonucleotide. The results are representative of three independent experiments.

Effects of Thiopental on IκBα Phosphorylation

We have previously demonstrated that thiopental (1,000 μg/ml) prevents the degradation of IκBα.[19] As shown in the Western blot depicted in figure 3, Jurkat T cells that were treated with the proteasome inhibitor MG 132 (10 or 20 μM) and subsequently stimulated with TNF contained $I\kappa B\alpha^P$ in much larger amounts (fig. 3, *lanes 3* and *4, top*), had a higher IκBα content (fig. 3,

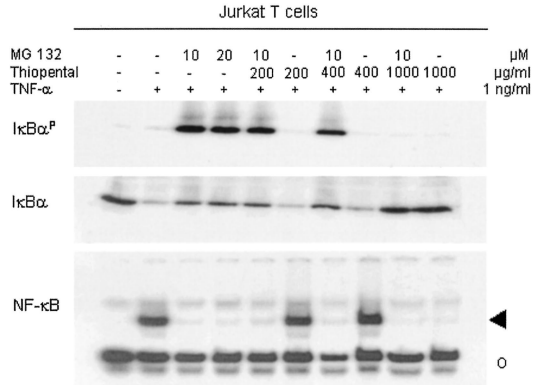

Fig. 3. The effect of thiopental on the phosphorylation of IκBα. Incubation with thiopental, tumor necrosis factor (TNF) α stimulation or administration of the respective volumes of ppH_2O as vehicle control was performed as described previously. IκBα was detected in Western blots using specific antibodies for the unphosphorylated or phosphorylated form. To visualize the highly transient phosphorylated form of IκBα, the cells were treated with the proteasome inhibitor MG 132 for 1 h before the addition of thiopental. *Filled arrow* = position of NF-κB DNA complexes; *circle* = nonspecific activity binding to the probe. The results are representative of six independent experiments.

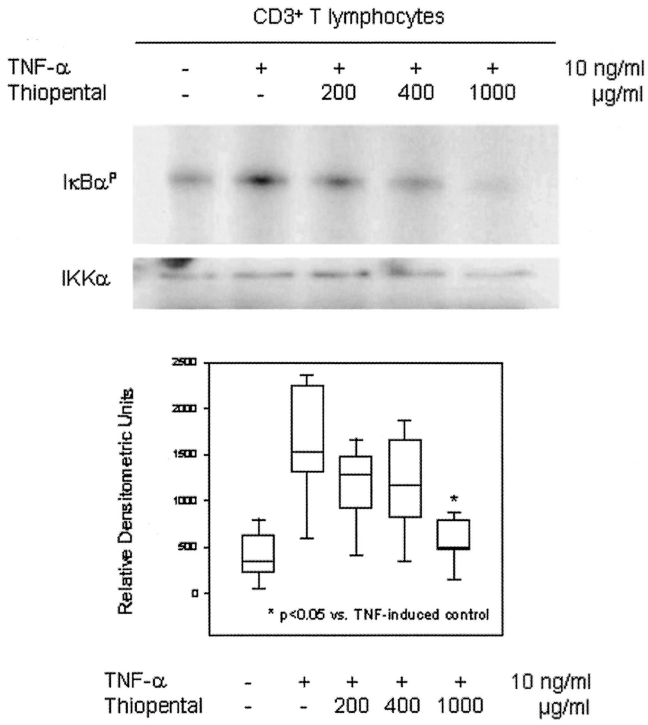

Fig. 4. The effect of thiopental on IκB-kinase (IKK) activity in $CD3^+$ cells. IKKα complexes were immunoprecipitated from cells that had been treated with tumor necrosis factor (TNF) α, thiopental, or the respective volumes of ppH_2O as vehicle control, using an antibody against IKKα to perform an *in vitro* kinase assay. (*Top*) Autoradiograph of a typical experiment. (*Bottom*) Results of the quantitative densitometric analysis of all individual experiments. Data represent the median and 25–75% and 95% confidence intervals of six independent experiments. *$P < 0.05$ compared with TNF-α alone.

lanes 3 and *4, middle*), and showed a reduced activation of NF-κB (fig. 3, EMSA, *lanes 3* and *4, bottom*) as compared to TNF alone (fig. 3, *lane 2*). This pattern remained unchanged if Jurkat T cells were pretreated with 10 μM MG 132, followed by incubation with thiopental at concentrations of 200 or 400 μg/ml (fig. 3, *lanes 5* and *7, top, middle,* and *bottom*), *i.e.*, $I\kappa B\alpha^P$ accumulated, IκBα was degraded, and consequently, no NF-κB was activated. In contrast, after preincubation with 10 μM MG 132, followed by incubation with 1,000 μg/ml thiopental and subsequent TNF stimulation, no $I\kappa B\alpha^P$ could be detected (fig. 3, *lane 9, top*), much more IκBα was present (fig. 3, *lane 9, middle*), and consequently, much less NF-κB was activated (fig. 3, *lane 9, bottom*).

Thiopental-mediated Effects on IKK Activity

Basal IKK activity was low in unstimulated $CD3^+$ T cells (fig. 4, *lane 1, top*), whereas the subsequent incubation with TNF-α caused an increase in IKK activity (fig. 4, *lane 2, top*). Lower concentrations of thiopental (200 and 400 μg/ml) had no major effect on the TNF-induced increase in IKK activity (fig. 4, *lanes 3* and *4, top*). In contrast, IKK activity remained at the level of unstimulated controls if the cells were treated with TNF in

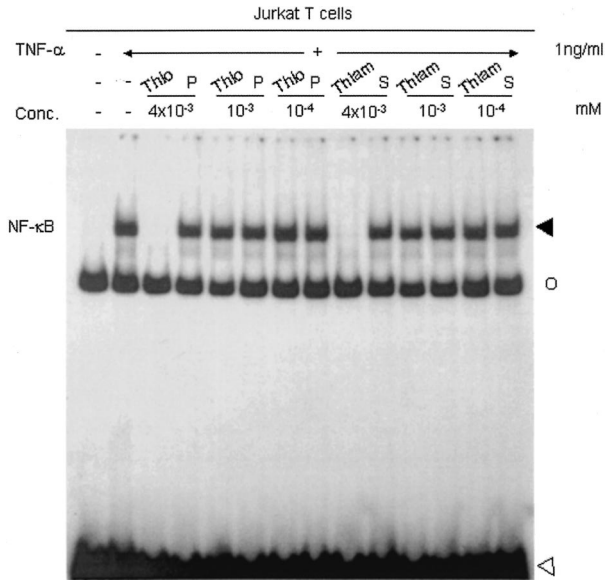

Fig. 5. The effect of the thiobarbiturate–oxybarbiturate analogs thiopental–pentobarbital, and thiamylal–secobarbital on the nuclear factor (NF) κB DNA binding activity. Jurkat cells were treated for 1 h with either thiopental, pentobarbital, thiamylal, and secobarbital at equimolar concentrations as indicated (*lanes 3–14*) and subsequently stimulated with tumor necrosis factor (TNF) α or incubated with the respective volumes of ppH_2O as vehicle control. DNA binding activity was analyzed by electrophoretic mobility shift assays. *Filled arrow* = position of NF-κB DNA complexes; *circle* = nonspecific activity binding to the probe; *hollow arrow* = unbound oligonucleotide. The results shown are representative of six independent experiments.

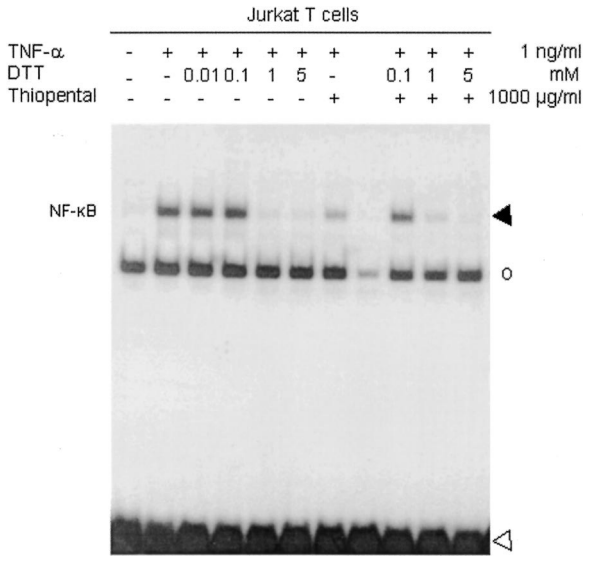

Fig. 6. The effect of thiopental and dithiothreitol (DTT) on the tumor necrosis factor (TNF) α–mediated activation of nuclear factor (NF) κB. Jurkat cells were incubated with thiopental and/or dithiothreitol and were stimulated with TNF-α at the concentrations indicated or incubated with the respective volumes of ppH_2O as vehicle control. Combined treatment of cells with thiopental and dithiothreitol was performed after *in vitro* precoincubation of thiopental with dithiothreitol for 1 h. DNA binding activity was analyzed by electrophoretic mobility shift assays. *Filled arrow* = position of NF-κB DNA complexes; *circle* = nonspecific activity binding to the probe; *hollow arrow* = unbound oligonucleotide. The results are representative of six independent experiments.

the presence of high concentrations of thiopental (1,000 μg/ml; fig. 4, *lane 5, top*). Under these conditions, densitometric analysis revealed a 60% decrease of IKK activity (fig. 4, *bottom*). IKK recovery was comparable under all conditions as determined by immunoblotting for IKKα (fig. 4, *top*). Similar results could also be obtained using Jurkat T cells instead of $CD3^+$ T cells (data not shown).

Effects of Structurally Different Barbiturate Analogs on NF-κB Activation

Pretreatment of Jurkat T cells with 4 mM thiopental inhibited the activation of NF-κB, whereas an equimolar concentration of pentobarbital did not (fig. 5, *lanes 3* and *4*). In addition, 4 mM thiamylal suppressed the DNA binding of NF-κB to its DNA probe, whereas the equimolar concentration of secobarbital had no detectable effect (fig. 5, *lanes 9* and *10*). Lower equimolar concentrations of thiopental–pentobarbital and thiamylal–secobarbital did not affect DNA binding of NF-κB to its DNA probe in a detectable manner (fig. 5, *lanes 5–8* and *11–14*).

Effects of Dithiothreitol on NF-κB Activation

Incubation of Jurkat T cells with dithiothreitol followed by subsequent stimulation with TNF-α showed no detectable inhibition at 0.01 mM and 0.1 mM dithiothreitol (fig. 6, *lanes 3* and *4*). In contrast, NF-κB activation was suppressed at 1 and 5 mM dithiothreitol alone (fig. 6, *lanes 5* and *6*) and, as previously shown, by thiopental alone (1,000 μg/ml; fig. 6, *lane 7*). Precoincubation of thiopental (1,000 μg/ml) with dithiothreitol (0.1 mM) attenuated the inhibitory effect of thiopental on the DNA binding activity of NF-κB (fig. 6, *lane 8*). Because of the intrinsic inhibitory properties of higher concentrations of dithiothreitol on NF-κB activation, an attenuation of the thiopental-mediated suppressing effect could not be demonstrated if dithiothreitol was present at concentrations of 1 or 5 mM (fig. 6, *lanes 9* and *10*).

Discussion

Barbiturates may be beneficial in patients with severe head injury and refractory intracranial hypertension. This conclusion is based on a series of clinical studies showing that administration of barbiturates can reduce intracranial pressure and increase cerebral perfusion pressure following brain trauma, as long as systemic hemodynamic stability is maintained.[31,32] Despite these favorable effects, accumulating evidence suggests that the long-term administration of high doses of thiopental is associated with a suppression of immune functions. For example, it has been shown that thiopental treat-

ment causes a profound increase in the incidence of nosocomial infections, which may in turn contribute to the high mortality rate of these patients.[2,33] Evidence from our previous work suggested that inhibition of the activation of NF-κB by thiopental may be involved in mediating the immunosuppressive effects of this agent.[19] However, the molecular mechanism by which thiopental exerts these effects remains unknown. Thus, it was the aim of the current study to identify the molecular mechanism by which thiopental inhibits the activation of NF-κB.

Barbiturates are potent agonists of the receptors for the ubiquitous inhibitory neurotransmitter GABA.[34] Interestingly, immunomodulatory actions through the $GABA_A$ receptor complex have been reported.[26,35,36] Furthermore, Tian *et al.*[25] recently demonstrated the presence of functional $GABA_A$ receptors on T cells, which mediate the lymphoproliferating effects of GABA and can be pharmacologically manipulated in a manner similar to neuronal $GABA_A$ receptors. Thus, it would be tempting to speculate that the inhibitory effect of thiopental on NF-κB is mediated through GABA receptor stimulation. However, suppression of the NF-κB DNA binding was not mimicked by incubation of the cells with GABA, nor could pretreatment of T cells with $GABA_A$ (bicuculline) or $GABA_B$ (CGP) antagonists attenuate the thiopental-mediated inhibition of NF-κB. These findings strongly argue against a key role of the GABA receptor complex in mediating the inhibition of NF-κB by thiopental.

In a next step, we systematically evaluated if and how thiopental may interfere with different components of the signal transduction pathway leading to the activation of NF-κB. Both the physicochemical properties of thiobarbiturates and the fact that other agents have been shown to directly modulate the DNA binding activity of NF-κB prompted us to examine whether thiopental may act in a similar fashion.[37] However, our experiments using a cell-free system strongly suggest that the inhibitory effect of this agent is not due to a direct molecular targeting of the p50/p60 heterodimer protein and must therefore occur further upstream. The translocation of free, active NF-κB into the nucleus is preceded by the ubiquitination and degradation of its inhibitor IκB.[18] This requires the phosphorylation of IκB.[13] Therefore, our observation that less phosphorylated IκB is detectable after TNF stimulation in the presence of thiopental provided a first hint toward a potential target of its inhibitory action. Theoretically, this finding could be due to an increased degradation of phosphorylated IκB. However, this can be excluded because the experiments were performed in the presence of the proteasome inhibitor MG 132 at concentrations proven to effectively prevent its breakdown, and the amount of unphosphorylated IκB remained unchanged as compared to TNF stimulation alone. Alternatively, suppression of the accumulation of phosphorylated IκB on TNF stimulation in the presence of thiopental could be the result of an interference with its formation. Therefore, we evaluated whether thiopental may abrogate the activity of IKK, the enzyme complex responsible for the phosphorylation of IκB. The observation that thiopental suppresses the increase in IKK activity, which can otherwise be observed on TNF stimulation, identifies this enzyme complex as a target of thiobarbiturates.

This raises the question of how thiopental alters IKK activity. Previous studies have demonstrated that thiogroup reactive agents may block IKK activity and prevent the subsequent activation of NF-κB.[38] Structural analyses of IKK subunits suggest that cysteine residues are present in the activation loop and within the kinase domain of IKKα and IKKβ at sites critical for enzymatic activity[16,39,40] and could therefore serve as molecular targets for these agents. Thus, we hypothesized that thiopental acts in a similar fashion, *i.e.*, the inhibitory action depends on its thio-group. To test this hypothesis, we compared the effects of the thiobarbiturates thiopental and thiamylal on the activation of NF-κB with those of their structural oxyanalogs pentobarbital and secobarbital. Interestingly, in contrast to the former agents, the latter two oxybarbiturates failed to inhibit NF-κB, if present in equimolar amounts. These results have two important implications. First, they support the hypothesis that the thio-group is of functional importance for the inhibitory action of barbiturates. Second, the difference between thiobarbiturates and oxybarbiturates in their ability to inhibit the activation of NF-κB described herein would provide an explanation for the results of previous reports showing that thiobarbiturates are more potent suppressors of immune responses that depend on the appropriate activation of NF-κB as compared to their oxyanalogs.[7,41]

In another attempt to define the functional role of the thio-group within the barbiturate molecule for the suppression of NF-κB, we performed a series of experiments with dithiothreitol, which contains large quantities of free sulfhydryls. As could be expected from this molecular structure and in agreement with the results of previous studies,[27] dithiothreitol inhibited the activation of NF-κB at concentrations of 1 mM or higher. However, precoincubation of thiopental with dithiothreitol at lower concentrations that did not exert any intrinsic inhibitory effect, attenuated the inhibition of NF-κB by thiopental. These findings strongly suggest that interaction of the thiol-group containing thiopental with the sulfhydryl groups within the dithiothreitol molecule may have limited the availability of this functional group for interactions with the IKK complex, and in turn attenuated the inhibitory action of thiopental. Therefore, these results suggest that the sulfur atom at the C2 position within the thiobarbiturates is a structural requirement

for the inhibitory action of these agents on the activation of NF-κB.

Identification of the mechanism and the molecular structure responsible for thiopental-mediated immunosuppression could form a basis for the development of new strategies for the therapy of intracranial hypertension. For example, separation of the neuroprotective from the immunomodulating effects may be beneficial in patients whose survival depends on lowering the intracranial pressure but who are simultaneously at a high risk for the development of nosocomial infections. Thus, the results of the current study provide a molecular rationale for future investigations that systematically examine the comparative clinical efficacy of oxybarbiturates *versus* thiobarbiturates in improving the outcome after severe head injury.

In conclusion, inhibition of the NF-κB DNA binding activity by thiopental is not due to GABA receptor stimulation and does not involve direct targeting of activated NF-κB. Our results rather indicate that thiopental suppresses the NF-κB activating signaling cascade by altering IKK activity and that the thio-group at the C2 position within the barbiturate molecule plays a key role in mediating this effect.

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