Inhibition of Activator Protein 1 by Barbiturates Is Mediated by Differential Effects on Mitogen-Activated Protein Kinases and the Small G Proteins Ras and Rac-1

Matjaž Humar, Nikolaos Andriopoulos, Soeren E. Pischke, Torsten Loop, Rene Schmidt, Alexander Hoetzel, Martin Roesslein, Heike L. Pahl, Klaus K. Geiger, and Benedikt H. J. Pannen

Department of Anesthesiology, University Hospital, Freiburg, Freiburg, Germany

ABSTRACT

Barbiturates are known to suppress protective immunity, and their therapeutic use is associated with nosocomial infections. Although barbiturates inhibit T cell proliferation, differentiation, and cytokine synthesis, only thiobarbiturates markedly reduce the activation of immune regulatory transcription factors such as nuclear factor-κB and nuclear factor of activated T cells. In this study, we investigated barbiturate-mediated effects on the regulation of the transcription factor activator protein 1 (AP-1) in primary T lymphocytes. We show that both thiobarbiturates and their oxy-analogs inhibit AP-1-dependent gene expression and AP-1 complex formation at clinically relevant doses. Furthermore, mitogen-activated protein (MAP) kinase activity, which transcriptionally and posttranslationally regulates AP-1 complex formation, is suppressed by most barbiturates. CD3/CD28- or phorbol 12-myristate 13-acetate (PMA)/ionomycin-induced p38 and extracellular signal-regulated kinase 1/2 phosphorylation or c-jun N-terminal kinase (JNK) 1/2 kinase activity was significantly diminished by pentobarbital, thiamylal, secobarbital, or methohexital treatment. These barbiturates also inhibited the initiators of the MAP kinase cascade, the small G proteins ras and rac-1, and prevented binding to their partners Raf-1 and PAK, respectively. Thiopental, unlike the other barbiturates, only reduced ras and JNK activity upon direct CD3/CD28 receptor engagement. Contrarily, upon PMA/ionomycin stimulation, thiopental blocked AP-1-dependent gene expression independently of the small G protein ras and MAP kinases, thus suggesting an additional, unknown mechanism of AP-1 regulation. In conclusion, our results contribute to the explanation of a clinically manifested immune suppression in barbiturate-treated patients and support the idea of a MAP kinase-independent regulation of AP-1 by PKC and calcium in human T cells.

Barbiturates such as thiopental are used in patients suffering from severe traumatic brain injury to control intracranial hypertension and cerebral perfusion (Tsai et al., 2000). Prolonged infusion and high-dose administration are necessary to achieve this effect, but this regimen is associated with a loss of protective immunity and an increased incidence of infectious diseases (Eberhardt et al., 1992).

Numerous pharmacological and cell biological effects of barbiturates have been described, including immunosuppressive and immunomodulatory actions on lymphocyte and leukocyte function. Barbiturates impair phagocytosis and superoxide generation in macrophages or polymorphonuclear leukocytes (Krumholz et al., 1995; Salman et al., 1998), inhibit neutrophil function (Nishina et al., 1998), and reduce natural killer cell activity (Ben-Eliyahu et al., 1999). Most important, barbiturates affect various T cell functions such as cytokine synthesis, mitogen or antigen responsiveness, and cytotoxicity (Thomas et al., 1982; Correa-Sales et al., 1997). T cells play a central role in the immune response, and their inhibition is known to affect innate as well as adaptive immunity. However, it is still not completely understood which of the molecular effects precipitate the increased rate of infections observed clinically.

Small G proteins, mitogen-activated protein (MAP) kinases, and activator protein 1 (AP-1) are among the most

ABBREVIATIONS: MAP kinase, mitogen-activated protein kinase; AP-1, activator protein 1; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift assay; PMSF, phenylmethylsulfonyl fluoride; RBD, Rho-binding domain of mouse Rhotekin; GST-PAK-CD, Cdc42/Rac interactive binding domain, corresponding to the Rac and Cdc42-binding domain of human PAK1B; GTPγS, guanosine 5′-3′-O-(thio)triphosphate.
Barbiturates Inhibit the Transcription Factor AP-1

Materials and Methods

Barbiturates were obtained as sodium salts: thiopental from Byk Gulden (Konstanz, Germany), pentobarbital from Alveta (Neumünster, Germany), thiamylal from Amershams Biosciences Inc. (Piscataway, NJ), secobarbital from Sigma-Aldrich (St. Louis, MO), and methohexital from Lilly Deutschland GmbH (Erlangen, Germany). All other reagents were obtained from Sigma (Deisenhofen, Germany) unless indicated otherwise.

Luciferase Reporter Gene Assay. The human T cell leukemia cell line, Jurkat, was used for transfection with pAP-1(PMA)-TA-Luc (BD Biosciences, Heidelberg, Germany), an AP-1-dependent luciferase reporter gene construct, containing six tandem repeats of an AP-1 binding site in front of a TATA box. Cells (1.5 × 10⁶ cells/1.5 ml) were distributed into six-well plates and starved over night in RPMI 1640 medium supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM L-glutamine. Cells were transfected with 3 μg of plasmid DNA and 30 μl of lipofectin (QIAGEN GmbH, Hilden, Germany). MAP kinases are serine/threonine kinases that phosphorylate various transcription factors and thereby regulate gene expression. One major downstream target is the transcription factor AP-1. MAP kinases regulate the transcriptional activation of AP-1 and control AP-1 protein function by phosphorylation (Foletta et al., 1998).

In previous studies, we demonstrated that two important regulators of immune function, the transcription factors nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB), are significantly inhibited by thiobarbiturates but are only marginally affected by their oxy-analogs (Loop et al., 2002; Humar et al., 2004). However, cellular immunomodulatory effects such as impaired proliferation, cytokine expression, and CD69 activation marker expression are exerted by both thiobarbiturates and their oxy-analogs, indicating additional, crucial immunosuppressive mechanisms. Since such T cell responses were described to be directly or indirectly dependent on MAP kinase and AP-1 activation (Castellanos et al., 1997; Foletta et al., 1998), we provide detailed insight into barbiturate-mediated effects on these targets. Thus, our data considerably enhance the understanding of how these substances exert their immunomodulatory effects.
Results

Barbiturates Inhibit AP-1-Dependent Luciferase Reporter Gene Expression. In this study, we investigated whether AP-1, an essential transcription factor for numerous T cell functions, is affected by barbiturates. Jurkat cells were transiently transfected with an AP-1-dependent luciferase reporter gene construct, and effects of thiobarbiturates were compared with their structurally similar oxy-analogs: thiopental and pentobarbital and thiamylal and secobarbital. The oxybarbiturate methohexital has no respective thio-analog (Fig. 1). AP-1-dependent reporter gene expression was induced by incubation with PMA plus ionomycin (Fig. 2). Barbiturates inhibited AP-1-dependent reporter gene expression. Maximal inhibitory potential of the barbiturates was reached at 2 mM, and half-maximal inhibition was observed at 0.1 to 0.5 mM. These concentrations are within clinically relevant dose ranges and comparable with tissue concentrations in organs that play a central role in the development of nosocomial infections (Newell et al., 1982; Schalen et al., 1992; Yasuda et al., 1993). Previous results demonstrate that 2-thiobarbiturates are more potent inhibitors of transcription factors such as NFAT or NF-κB than their structurally identical oxy-analogs (Loop et al., 2002; Humar et al., 2004). In contrast to those results, AP-1 was affected similarly by both thiopestal and pentobarbital or thiamylal and secobarbital, suggesting that C2 thiosubstitutions to the barbiturate backbone do not noticeable influence the potency of AP-1 inhibition.

Barbiturates Inhibit the Transcription Factor AP-1 in Primary CD3+ T Cells. Effects of barbiturates on the formation of active AP-1 complexes were analyzed by EMSA shifts using nuclear cell extracts of primary human CD3+ T lymphocytes. Activation of naive T cells requires ligation of the T cell receptor conferring specificity and the provision of a second, costimulating signal (Bretscher, 1992). Therefore, AP-1 was induced by CD3/CD28 T cell receptor cross-linking. Supershift experiments using various antibodies directed against different AP-1 protein family members demonstrated the contribution of c-Jun, JunD, c-Fos, and FosB proteins (data not shown). Similar to the results of the reporter gene expression assays, thiobarbiturates and their oxy-analogs inhibited AP-1 complex formation (Fig. 3). Identical results were obtained when direct CD3/CD28 receptor activation was replaced by stimulation with PMA and ionomycin (data not shown). In contrast, a direct coincubation of barbiturates with activated nuclear extracts had no effect on AP-1 com-
complex formation at corresponding DNA-binding sites (Fig. 4).

Apparently, a direct interaction of barbiturates with AP-1 protein family members is not responsible for repression. These observations rather implicate the participation of biological processes, regulating the transcriptional and post-translational activation of AP-1.

Differential Effects of Barbiturates on the Phosphorylation-Dependent Activation of p38 and ERK1/2 MAP Kinases. In nonstimulated cells, AP-1 expression is low or undetectable, whereas there is a rapid induction of AP-1 activity by MAP kinases upon T cell receptor activation (Foletta et al., 1998). The activity of these MAP kinases is critically regulated by their phosphorylation status. Effects of barbiturates on the phosphorylation of p38 MAP kinase (Fig. 5) and ERK1/2 (Fig. 6) were analyzed. Maximal MAP kinase phosphorylation was induced within 10 min following CD3/CD28 T cell receptor activation. The barbiturates pentobarbital, thiamylal, secobarbital, and methohexital inhibited the phosphorylation of p38 MAP kinase and ERK1/2 MAP kinase in a dose-dependent manner (Figs. 5 and 6). Surprisingly, thiopental had no influence on p38 or ERK1/2 phosphorylation, suggesting a different mechanism of AP-1 inhibition. The structural similarities of thiopental with thia-
mylal or pentobarbital implicate that a distinctive combination of side chains at the C2 and C5 of the barbiturate backbone exerts differential effects on the phosphorylation of MAP kinases, whereas such substitutions per se are not a prerequisite for this kind of inhibition. Structural components of the barbiturate backbone such as malonic acid, barbituric acid, and thiobarbituric acid had no effect on MAP kinase phosphorylation (data not shown).

**Differential Effects of Barbiturates on the Phosphorylation of c-Jun by JNK1/2.** In contrast to p38 and ERK1/2, phosphorylation of JNK1/2 upon CD3/CD28 or PMA plus ionomycin stimulation could not be detected in total cellular lysates (data not shown). This might be due to the fact that JNK is expressed at low levels in naive T cells (Weiss et al., 2000). For this reason, we determined the c-Jun NH2-terminal kinase activity. JNK1/2 were immunoprecipitated from total T cell lysates, and immunoprecipitates were used to phosphorylate c-jun in vitro. Results are shown in Fig. 7A. Pentobarbital, thiamylal, secobarbital, and methohexitol inhibited the kinase activity of JNK1/2 when stimulated by CD3/CD28 T cell receptor activation. Similar results were obtained upon activation with PMA plus ionomycin (Fig. 7B, data shown only for thiopental and pentobarbital). Thio- pental showed differential effects on JNK1/2, depending on the type of activation. Although it inhibited CD3/CD28-induced JNK1/2 activity and c-jun phosphorylation, thiopental did not affect JNK1/2 when stimulated with PMA plus ionomycin (Fig. 7B).

**Differential Effects of Barbiturates on p21ras Activation.** The p21ras pathway has been assigned a crucial role in T cell activation (Izquierdo Pastor et al., 1995). Moreover, p38, ERK1/2, and JNK1/2 are jointly regulated by p21ras. Therefore, we investigated whether barbiturate-dependent MAP kinase inhibition is associated with an inactivation of the ras protein. The GTP-bound, activated form of ras was selectively precipitated with glutathione-Sepharose beads coupled to a GST-fusion protein containing the RAF-1 binding domain for ras. Activated, barbiturate-treated T cells showed a dose-dependent decrease of the GTP-bound ras. Effects were most pronounced with thiamylal and methohexitol, followed by secobarbital and pentobarbital (Fig. 8A). Similar results were obtained upon activation with PMA plus ionomycin. Results are shown for pentobarbital, the oxygen analog of thiopental (Fig. 8B). Likewise, inhibition of ras by thiopental was pronounced upon CD3/CD28 receptor activation but again, following PMA and ionomycin stimulation, not further detectable (Fig. 8B). These findings are consistent with the observations described above (Figs. 4 and 5).
and thus substantially support the assumption that barbiturate-mediated impairment of MAP kinase activity is regulated on the level of the small G proteins. 

**Differential Effects of Barbiturates on Rac-1 Activation.** Rac-1 is another GTPase that is also involved in early T cell receptor signaling (Arrieumerlou et al., 2000), regulates the actin organization and cytoskeletal rearrangements (Zaffran et al., 2001), and participates in signals triggering cytotoxicity via the MAP kinase pathway (Djeu et al., 2002). For these reasons, we investigated whether barbiturates also affect rac-1 (Fig. 9). Our results demonstrate that, except for thiopental, all other barbiturates completely suppressed GTPγS-induced rac-1 activation. Once again, these differential effects of thiopental indicate that a predetermined combination of side chains at the barbiturate backbone modulates distinctive cellular effects. Thiopental and its structural analog pentobarbital only differ in the replacement of the 2-thio group by oxygen (Fig. 1). In this case, C2 oxygenation is necessary for an efficient inhibition of the GDP-GTP exchange on rac-1. In contrast, thiamylal, another thiobarbiturate (Fig. 1), also effectively blocked rac-1 activation without bound oxygen at the C2 atom of the barbiturate backbone. Therefore, C2 oxygenation is an efficient but not absolutely required component for inhibition. A structural comparison of thiopental with thiamylal argues for the 5-allyl-(1-methyl-buthyl)-side chain at the C5 as an additional component for rac-1 inhibition. In conclusion, we propose that both side chains at the C2 and C5 equally modulate differential effects on rac-1.

**Discussion**

Brain-injured patients are susceptible to secondary brain damage by decreased cerebral perfusion through edema formation and increased intracranial pressure (Tsai et al., 2000). Induction of barbiturate coma is used to control intracranial pressure, but its application is impeded by an increased incidence of infections (Eberhardt et al., 1992). In vitro, numerous immunomodulatory and immunosuppressive effects of barbiturates on leukocyte and lymphocyte function have been described (Correa-Sales et al., 1997; Salaman et al., 1998). However, to avoid these side affects and to improve barbiturate therapy, the molecular mechanisms and structural requirements for barbiturate-mediated immunosuppression need to be identified.

Here, we present evidence that barbiturates inhibit the activation of AP-1, a central transcription factor of T cell function. We show by different criteria that barbiturates are effective inhibitors of AP-1 in Jurkat T cells and primary human T lymphocytes. Barbiturates suppress AP-1-dependent gene transcription, barbiturates inhibit PMA/ionomycin- or CD3/CD28-induced AP-1 complex formation, and effects are dose-dependent and occur at clinically relevant concentrations, attained in the plasma of patients during therapeutic coma (Neuwelt et al., 1982).

Previously, we described that two transcription factors, NF-κB and NFAT, were inhibited by thiobarbiturates but only marginally by their oxybarbiturate analogs (Loop et al., 2002; Humar et al., 2004). Surprisingly, at the same time, certain biological effects such as proliferation, cytokine production, or CD69 activation marker expression were repressed by all barbiturates, suggesting further mechanisms of barbiturate-mediated immunosuppression. The results depicted here explain this discrepancy. AP-1, which cooperatively binds to NFAT, is inhibited by thiobarbiturates and their oxy-analogs at comparable concentrations. Consequently, T cell function, which is regulated by numerous interacting transcription factors, is dependent on the activity of the most affected link in the chain. In T cells, AP-1 induction requires MAP kinase-dependent c-fos and c-jun gene transcription and posttranslational protein modification (Poletta et al., 1998). Here, we have analyzed the impact of barbiturates on regulatory protein kinases. Pentobarbital, thiamylal, secobarbital, and methohexital blocked the
phosphorylation of p38, ERK1/2, and JNK1/2 kinase activity, thus explaining AP-1 inhibition. The inhibition of kinase activity by barbiturates has already been described for the IKK-kinase and might involve a common mechanism (Loop et al., 2003). However, here we provide evidence that the GDP/GTP exchange and thus G protein function are additionally impaired by these barbiturates. Although CD3/CD28- or PMA plus ionomycin-induced activation of p21ras necessitates a sophisticated presequence of cellular events, GTP\(^\gamma\)/S directly induces Rac-1 and thus excludes the participation of further biological processes. Therefore, we postulate that barbiturates can directly affect small G proteins. Indeed, our data are supported by reports demonstrating altered activity of GTP binding proteins upon phenobarbital treatment in rat brain or in a rat basophilic leukemia cell line (Dan’ura et al., 1987; Robinson-White et al., 1990).

Barbiturates are derived from barbituric acid, a cyclic compound obtained by the combination of urea and malonic acid forming a pyrimidine nucleus. Different substitutions at carbon atoms 2 and 5 of barbituric acid confer sedative-hypnotic and anticonvulsant activities and influence the pharmacokinetic properties. The latter is of particular importance due to the observed divergence of thiopental-mediated effects. Unlike the other barbiturates, thiopental has no effect on MAP kinases and the small G proteins upon stimulation by PMA and ionomycin. A structural comparison of thiopental with the other barbiturates suggests that depending on the stimulus for cell activation, both C2 oxygenation and the molecular structure of the side chain at the C5 determine the biological effects. These substitutions seem to be equipotent and interchangeable because differences between thiopental and pentobarbital (or thiamylal) affect only a single molecular modification.

Differential effects of barbiturates deliver new insight into T cell signaling. Thiopental, unlike the other barbiturates, can inhibit AP-1 without directly affecting the p21ras/MAP kinase pathway. Therefore, for the first time, we provide evidence for a new AP-1-modulating pathway by PMA and ionomycin, bypassing p21ras and MAP kinases. A direct inhibition of the formation of active AP-1 complexes was excluded by coincubation experiments with thiopental and activated nuclear extracts. Furthermore, thiopental exerts opposing effects on JNK and p21ras upon PMA/ionomycin stimulation as compared with direct CD3/CD28 receptor activation. PMA and ionomycin substitute for CD3 T cell recep-
tor activation. Therefore, opposing effects are CD28 dependent, thus linking JNK and p21ras with CD28 signaling. Indeed, CD28 signaling has been found to converge with signals triggered by the CD3 receptor at the level of JNK (Su et al., 1994).

Our present data might explain the impairment of many immune functions upon barbiturate therapy. Growing evidence suggests that the p21ras/MAP kinase/AP-1 pathway participates in all aspects of immune responses and thus regulates T cell activation and differentiation (Castellanos et al., 1997; Foletta et al., 1998; Dong et al., 2002). Therefore, it is not surprising that barbiturates compromise various T cell functions, such as cytokine synthesis, antigen or mitogen reactivity, delayed-type hypersensitivity responsiveness, CD69 activation marker expression, and cytotoxicity (Thomas et al., 1982; Correa-Sales et al., 1997; Loop et al., 2002). In other cell types, reduced superoxide generation, phagocytosis, mitogen responsiveness, cytokine expression, and cytotoxicity have been described upon barbiturate treatment (Krumholz et al., 1995; Nishina et al., 1998; Salman et al., 1998; Ben-Eliyahu et al., 1999). Again, accumulating evidence suggests that these pathways require efficient MAP kinase/AP-1 signaling (Raeder et al., 1999; Xaus et al., 2001; Djeu et al., 2002; Dong et al., 2002). Indeed, TNFα production and MAP kinases are also suppressed by barbiturates in primary human monocytes (M. Humar, unpublished data).

Moreover, our results demonstrate that Rac-1, a small GTPase of the Rho family of proteins, may be a central target for barbiturates.
for mediating immunosuppressive properties of barbiturates. Rac-1 is involved in early T cell signaling (Arrieumerlou et al., 2000), participates in signals triggering cytotoxicity (Djeu et al., 2002), and regulates the actin organization and cytoskeletal rearrangements (Hall, 1998). Morphological changes are crucial to T cell activation, allowing the cell to migrate through blood vessels, home into lymphoid organs, adhere to target cells, or to interact with antigen-presenting cells (Penninger and Crabtree, 1999). During T cell activation, the actin cytoskeleton permits the formation of immune synapses and scaffolds for signal transduction molecules (Grakoui et al., 1999; Dustin and Cooper, 2000). Indeed, barbiturate application affects the microtubular assembly (Ventilla and Brown, 1976), and multiple reports describe reduced neutrophil chemotaxis or leukocyte migration through human endothelial cell monolayers following barbiturate exposure (Hofbauer et al., 1998; Nishina et al., 1998). Our results suggest that barbiturates directly affect these processes on the level of rac-1, whereas thiolipot interacts differently. How thiopental inhibits such fundamental immune functions is highly speculative and cannot be substantiated by present data in the literature. We suspect that inhibition of inducible transcription factors such as AP-1, NFAT, or NF-κB may generally render immune cells nonreactive to extracellular signals.

In summary, our data suggest that barbiturates are potent inhibitors of AP-1 and act differentially on regulatory signal transduction molecules such as MAP kinases and the GTPases p21ras and rac-1. Thus, our results serve to explain the clinically manifested immunosuppression in barbiturate-treated patients. Since AP-1, MAP kinases, and small G proteins are involved in numerous aspects of cellular responses and are ubiquitously expressed in most tissues, extensive consequences of high-dose barbiturate treatment must be considered.

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References


Address correspondence to: Dr. Benedikt H. J. Pannen, Anaesthesiologie- gische Universitätsklinik, Hugetetterstrasse 55, D-79106 Freiburg, Germany. E-mail: pannen@rz.ukl.uni-freiburg.de