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Heterocyclic Thioureylenes Protect from Calcium-Dependent Neuronal Cell Death

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ABSTRACT

Calcium-dependent cell death occurs in neurodegenerative diseases and ischemic or traumatic brain injury. We analyzed whether thioureylenes can act in a neuroprotective manner by pharmacological suppression of calcium-dependent pathological pathways. In human neuroblastoma (SK-N-SH) cells, thioureylenes (thiopental, carbimazole) inhibited the calcium-dependent neuronal protein phosphatase (PP)-2B, the activation of the proapoptotic transcription factor nuclear factor of activated T-cells, BAD-induced initiation of caspase-3, and poly-(ADP-ribose)-polymerase cleavage. Caspase-3-independent cell death was attenuated by carbimazole and the protein kinase C (PKC) δ inhibitor rottlerin by a PP-2B-independent mechanism. Neuroprotective effects were mediated by the redox-active sulfur of thioureylenes. Furthermore, we observed that the route of calcium mobilization was differentially linked to caspase-dependent or independent cell death and that BAD dephosphorylation did not necessarily induce intrinsic caspase activation. In addition, a new 30- to 35-kDa caspase-3 fragment with an unknown function was identified. In organotypic hippocampal slice cultures, thioureylenes inhibited caspase-3 activation or reduced *N*-methyl-D-aspartate and kainic acid receptor-mediated cell death that was independent of caspase-3. Because prolonged inhibition of caspase-3 resulted in caspase-independent cellular damage, different types of cell death must be taken under therapeutic consideration. Here we show that thioureylenes in combination with PKCδ inhibitors might represent a promising therapeutic approach to attenuate neuronal damage.

Excitotoxic neuronal cell death occurs in brain ischemia, traumatic injury, epilepsy, hypoglycemia, and neurodegenerative disorders such as Alzheimer's, Parkinson's, or Huntington's disease (Choi, 1995). Dysfunction and subsequent death of neurons is initiated by excessive exposure to the excitatory neurotransmitter glutamate and stimulation of the NMDA-type glutamate receptors. NMDA receptor-mediated calcium entry and the consequent disruption of cellular calcium homeostasis is an obligatory phase preceding neuronal death (Hara and Snyder, 2007). Cytoplasmic calcium ($[Ca^{2+}]c$) modulates the activity of diverse proteins that are involved in the execution of the intrinsic apoptotic cascade. The calcium-dependent serine/threonine phosphatase 2B (PP-2B; calcineurin) dephosphorylates Bad, a proapoptotic member of the Bcl-2 family (Wang et al., 1999). In consequence, Bad heterodimerizes with Bcl-2 and Bcl-X_L and initiates the disruption of the outer mitochondrial membrane (Yang et al., 1995). Proapoptotic factors such as cytochrome *c*, apoptosis-inducing factor, and Smac/Diablo are released from the mitochondria and facilitate the caspase cascade, resulting in cell death (Jiang and Wang, 2004). Proteins of the Bcl-2 family also reside in the endoplasmic reticulum (ER) that regulates intracellular calcium homeostasis and cell death (Pinton and Rizzuto, 2006). Massive $[Ca^{2+}]c$ overload induced by the ER is apoptogenic, both by sensitizing mitochondria to death stimuli and by initiating ER-mediated death signals (Rizzuto et al., 2003). Mitochondrial calcium loading by the ER is a prerequisite for glutamate neurotoxicity (Stout et al., 1998).

B.H. and B.H.J.P. contributed equally to this work.

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CaM, calmodulin; CsA, cyclosporin A; LDH, lactate dehydrogenase; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; PP, protein phosphatase; Z-VAD, benzyloxycarbonyl-Val-Ala-Asp; DEVD, Asp-Glu-Val-Asp; NFAT, nuclear factor of activated T cells; ER, endoplasmic reticulum; DTT, dithiothreitol; TOTEX buffer, HEPES/NaCl/Nonidet P-40/$MgCl_2$/EDTA/EGTA/DTT/phenylmethylsulfonyl fluoride/aprotinin; MK-801, 5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (dizocilpine maleate); Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; A23187, calcimycin.

It has been suggested that high $[Ca^{2+}]c$ levels promote cell death through necrosis, whereas low $[Ca^{2+}]c$ increases promote cell death through apoptosis (Choi, 1995; Nicotera and Orrenius, 1998). Pathological $[Ca^{2+}]c$ flux also induces de novo protein synthesis (e.g., of proapoptotic Fas and Fas ligand) (Holtz-Heppelmann et al., 1998). De novo synthesis of Fas seems to be transcriptionally regulated by NFAT, which is dephosphorylated and activated by the PP-2B (Holtz-Heppelmann et al., 1998).

Because most neuronal death occurs over a period of days or months after the initial insult, clinical therapy is implementable. Promising therapeutic strategies exist that target calcium-regulated processes. NMDA receptor antagonists are protective in neurodegenerative disorders such as Alzheimer's disease, in which neurotoxicity is elicited by the amyloid-β (1–42) peptide that augments NMDA receptor transmission and $[Ca^{2+}]c$ (Tanovic and Alfaro, 2006). In vascular occlusion, the selective NMDA receptor antagonists MK-801, memantine, and kynurenic acid derivatives reduce NMDA receptor transmission and stroke damage (Hara and Snyder, 2007). However, these drugs elicit psychotomimetic effects and hypertensive actions and are heavily sedating, limiting their success in clinical trials (Hara and Snyder, 2007). PP-2B is an intracellular target in experimental sciatic nerve injury treatment or traumatic and ischemic brain injury models (Kaminska et al., 2004). Increased activity of PP-2B has been described as sufficient to predispose neuronal cells to apoptosis (Asai et al., 1999), and inhibitors of PP2B have been shown to decrease ischemic and traumatic brain damage by reducing axonal damage, infarct size, and edema (Kaminska et al., 2004). PP-2B suppressants also induce neuronal sprouting and regeneration of peripheral nerve fibers (Kaminska et al., 2004).

We demonstrated previously that heterocyclic thioureylenes impair central immune functions by modulating the PP-2B activity in T lymphocytes (Humar et al., 2004, 2007). Neuroprotection by the thioureylene thiopental is clinically established and associated with the modulation of intracranial pressure by reducing cerebral blood flow and the cerebral metabolic rate of oxygen consumption (Turner et al., 2005). These observations indicate that certain thioureylenes might be useful neuroprotective agents. Our study demonstrates the neuroprotective effects of thioureylenes as modulators of neuronal PP-2B and their ability to prevent the initiation and progression of the neuronal apoptotic cascade.

Materials and Methods

Culture and Treatment of SK-N-SH Neuroblastoma Cells. Human neuroblastoma (SK-N-SH) cells were cultured in Eagle's minimum essential medium supplemented with 10% calf serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 2.0 mM L-glutamine in a humidified, CO_2-enriched atmosphere at 37°C. Cells were treated in fresh medium with or without 50 μM cyclosporin A (CsA; Sigma, St. Louis, MO), 0.1 to 5 mM thiopental (Altana, Wesel, Germany), 1.5 mM pentobarbital (Merial, Hallbergmoos, Germany), 0.1 to 5 mM carbimazole (LKT Laboratories, St. Paul, MN), 1 μM chelerythrine (Calbiochem, San Diego, CA), 1 μM rottlerin (Calbiochem), 5 μM Gö6976 (Calbiochem), or 20 μM benzyloxycarbonyl-Val-Ala-Asp (Z-VAD; Alexis, Lausen, Switzerland). Cell death was induced by 1 μg/ml ionomycin, 40 μM A23187, or 40 μM thapsigargin (all from Calbiochem) as indicated.

Preparation of Hippocampal Slice Cultures. Newborn mouse pups (P0–P2) were decapitated, the brains were aseptically removed, the hippocampi were dissected out, sliced (400 μm) perpendicular to their longitudinal axis with a McIlwain tissue chopper, and stored in Petri dishes filled with cold (4°C) preparation solution (minimal essential medium and 2 mM glutamine). Four intact slices were then placed onto translucent porous membranes (0.4-μm Culture Plate Insert, Millicell-CM; Millipore, Schwalbach, Germany), which were inserted in a six-multiwell plate. Each well was filled with 1.2 ml of medium [minimal essential medium (50%), basal medium Eagle (25%), heat-inactivated horse serum (25%); Invitrogen, Karlsruhe, Germany] supplemented with glutamine (2 mM; Invitrogen) and glucose (0.65%; Sigma). The pH was adjusted to 7.3. Hippocampal sections were maintained in a humidified, CO_2-enriched atmosphere at 37°C. The culture medium was changed every 48 h.

Labeling of RII Peptides and Serine/Threonine Protein Phosphatase Activity Assay. The RII peptide DLDVPIPGRFDRRVSVAAE was phosphorylated at the serine residue with unlabeled dATP tk;4by the catalytic subunit of the cAMP-dependent protein kinase as described previously (Humar et al., 2004). The resultant RII phosphopeptide was loaded onto Sephadex G-25 spin columns (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) to remove free phosphates.

PP-2B activity was measured by a serine/threonine phosphatase assay system, according to the instructions of the manufacturer with minor modifications (Calbiochem). Cells were homogenized by an Ultra Turrax T25 (IKA Labortechnik, Staufen, Germany) at 4°C for 30 s using 1 g of cells in 3 ml of lysis buffer (25 mM Tris pH 7.5, 0.5 mM DTT, 50 μM EDTA, 50 μM EGTA, and 0.1% Nonidet P-40). The homogenate was centrifuged at 100,000*g* at 4°C for 1 h. The supernatant was subjected to Sephadex G-25 spin columns to remove free phosphates. Dephosphorylation of the synthetic RII-peptide by the eluate or 50 U of recombinant PP-2B (Calbiochem) was determined at 30°C for 20 min in reaction buffer (12.5 mM Tris, pH 7.2, 0.5 mg/ml acetylated bovine serum albumin, and 10 μg of RII peptide) supplemented with or without 5 mM EDTA, 2.5 μM okadaic acid, 1 mM $NiCl_2$, and 10 μg/ml calmodulin (CaM). The formation of molybdate-malachite green-phosphate complexes was photospectrometrically quantified at 600 nm (SPECTRAmax PLUS^{384}; Molecular Devices, Sunnyvale, CA).

NFAT-Dependent Luciferase Reporter Gene Expression. The human neuroblastoma cell line, SK-N-SH, was used for transfection with pNFAT-TA-Luc (BD Biosciences, Heidelberg, Germany), an NFAT-dependent luciferase reporter gene construct, containing three tandem repeats of the NFAT consensus sequence, located upstream of the minimal TA promoter, the TATA box from the herpes simplex virus thymidine kinase promoter. Cells (1.5×10^6 cells/1.5 ml) were distributed into six-multiwell plates and transfected with 3 μg of plasmid DNA and 30 μl of Superfect (QIAGEN, Hilden, Germany). SK-N-SH cells were treated with 0.1 to 5 mM thionamides for 15 h and activated by 1 μg/ml ionomycin for the last 13 h. Harvested cells were washed with phosphate-buffered saline and lysed in 100 μl of luciferase reporter lysis buffer (Promega, Madison, WI). Luciferase reporter gene expression was measured in lysates using a luminometer (Microluminat Plus LB 96P; Berthold Technologies, Bad Wildbad, Germany) and normalized to protein levels.

Transfection of SK-N-SH. SK-N-SH cells were nucleofected by the Nucleofector Kit V according to the instructions of the manufacturer (Amaxa Biosystems, Cologne, Germany). In brief, 1.2×10^6 cells were suspended in 100 μl of supplemented Nucleofector Solution containing 2 μg of endo-free plasmid pEGB-mBAD (Cell Signaling Technology, Danvers, MA) before they were electroporated by using Amaxa's Nucleofector II (program X-005). Nucleofected cells were plated in 1 ml of prewarmed medium using six-multiwell plates. The phosphorylation status of BAD proteins was analyzed after 24 h.

Cytotoxicity Assay. Supernatants containing the released lactate dehydrogenase (LDH) from SK-N-SH cells (3.5×10^4 cells/well of a 96-well plate) and hippocampal slice cultures were analyzed by

the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany). In brief, 50 μl of the reaction mixture containing LDH assay catalyst and dye solution (1:46) were added to 50 μl of cell culture supernatant and incubated for 30 min at 25°C. The absorbance at 490 nm was measured on a plate reader (SPECTRAmax PLUS^{384}) with the reference wavelength of 690 nm. Total LDH content of cells was determined upon the addition of 2% Triton X-100 to control cells. Results were expressed as the percentage compared with total cell lysis (100% LDH; SK-N-SH cells) or as OD_{490} to OD_{690} values (slice cultures).

Immunoblot Analysis. SK-N-SH cells were directly lysed in 5× SDS electrophoresis sample buffer in tissue culture plates. Alternatively, hippocampal slice cultures were solubilized in 100 μl of TOTEX buffer (10 mM HEPES, pH 7.9, 350 mM NaCl, 1% Nonidet P-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 5 mM DTT, 2.5 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin) before the addition of 5× SDS electrophoresis sample buffer. Lysates were sonicated and boiled for 3 min. Equal amounts of protein were used for immunoblot analysis. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, blocked with 5% dry milk in Tris-buffered saline, and then reacted overnight with antibodies raised against Bad, phospho-Bad(Ser136), caspase-3, cleaved caspase-3, cleaved poly(ADP-ribose) polymerase (PARP), or β-actin (Cell Signaling). After repeated washing, specific bands were visualized using horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence reagents (GE Healthcare).

Caspase Activity Assay. SK-N-SH cells (10^7) were lysed in 200 μl of TOTEX buffer (10 mM HEPES pH 7.9, 350 mM NaCl, 1% Nonidet P-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 5 mM DTT, 2.5 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin). Alternatively, four hippocampal slices were lysed in 100 μl of TOTEX buffer. Extracts were diluted 1:10 in 100 mM HEPES-KOH, pH 7.5, containing 2 mM DTT and a 60 μM concentration of the fluorogenic caspase-3 substrate acetyl-DEVD-7-amino-4-methylcoumarin (Alexis). Caspase-3-like activity was determined by a time kinetic in a SpectraMax GeminiXS plate reader at 380/460 nm (Molecular Devices) for 30 min at 27°C.

Statistical Analysis. Data are shown as mean ± S.E.M. Statistical analysis was performed by the SigmaStat 3.1 Software (Systat Software, Inc., San Jose, CA) using a one-way analysis of variance on ranks followed by a multiple comparison versus a control group (Holm-Sidak post hoc test). *P* values less than 0.001 were considered significant.

Results

Thioureylenes Inhibit Neuronal PP-2B. In this study, we searched for inhibitors of pathological, calcium-dependent neuronal cell death. We described previously thioureylenes as immunosuppressants by reducing CD3/CD28 receptor-mediated PP-2B activity (Humar et al., 2004, 2007). Here, we investigated whether thioureylenes also inhibit calcium-dependent neuronal phosphatases that mediate neuronal cell death. The structure of the examined thioureylenes and the corresponding oxy-analogs is described in Fig. 1.

First, SK-N-SH neuroblastoma cells were pretreated with thioureylenes before extracts were assayed for serine/threonine protein phosphatase activity. Maximal activity, defined as 100%, was obtained in the presence of 1 mM $NiCl_2$ and 10 μg/ml CaM, reflecting the combined activities of PP1, PP-2A, PP-2B, and PP-2C (Fig. 2A). Pretreatment of SK-N-SH cells with the pyrimidine-derived thioureylene thiopental or the imidazole-derived thionamide carbimazole significantly reduced total serine/threonine phosphatase activity (Fig. 2A).

Four classes of cytoplasmic serine/threonine phosphatases have been characterized in mammalian tissue (Stemmer and Klee, 1991). Therefore, different phosphatase inhibitors were used to estimate the specific calcium-dependent phosphatase activity of PP-2B within cellular extracts (Fig. 2B). The majority of the measured phosphatase activity was attributed to a combined action of PP-1 and PP-2A because okadaic acid, a selective inhibitor of PP-1 and PP-2A but not PP-2B or PP-2C, reduced phosphatase activity to approximately 27.3 ± 7.2% (Fig. 2B, bar 2). This was supported by the observation that 68.9 ± 10.9% of phosphatase activity remained when nickel and magnesium were substituted with EDTA (Fig. 2B, bar 4), which inhibits PP-2B and magnesium-dependent PP-2C but leaves PP-1 and PP-2A activity unaffected. Next, the contribution of PP-2B and PP-2C to the okadaic-resistant activity was discerned by depletion of nickel and CaM from the reaction buffer (Fig. 2B, compare bars 2 and 3). In the presence of okadaic acid, approximately 92% of the residual activity was dependent on the presence of the PP-2B cofactors nickel and CaM, indicating that PP-2C activity is negligible. This residual phosphatase activity was comparable with the loss of total phosphatase activity in the absence of nickel and CaM from the assay buffer (27.5 ± 14.3%) (Fig. 2B, bar 5).

In Fig. 2C, cellular lysates were analyzed in the presence of 2.5 μM okadaic acid to assay the specific phosphatase activity of PP-2B in cells pretreated with thioureylenes. Treatment of SK-N-SH cells with thioureylenes resulted in a significant and dose-dependent decrease in the okadaic acid-resistant serine/threonine dephosphorylation of the synthetic phospho-RII-peptide substrate. Carbimazole was more potent in inhibiting PP-2B activity than thiopental.

Because thioureylenes were able to block the okadaic acid-resistant, PP-2B-dependent serine/threonine dephosphorylation of RII-peptides in cellular lysates, we analyzed whether thioureylenes were capable of directly inhibiting the catalytic activity of the PP-2B/CaM complex. Incubation of thiopental with recombinant PP-2B and CaM in a cell-free system resulted in a dose-dependent inhibition of the PP-2B-dependent serine/threonine dephosphorylation of RII peptides (Fig. 2D). This observation was comparable with the reduced PP-2B activity in cellular lysates (Fig. 2C) and suggests a direct inhibition of the PP-2B/CaM complex by thiopental. In contrast, carbimazole failed to efficiently repress the recombinant PP-2B activity (Fig. 2D), thus affecting PP-2B in cellular lysates by a different mechanism.

Thioureylenes Inhibit NFAT-Dependent Gene Expression. In calcium-mediated apoptosis, de novo protein synthesis of proapoptotic proteins can be observed (Holtz-

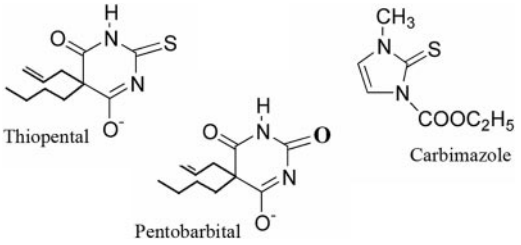

Fig. 1. Structure of mononuclear heterocyclic thioureylenes in clinical use. The thio-barbiturate thiopental is used for the induction of anesthesia and treatment of elevated intracranial pressure. Pentobarbital is an oxy-derivative of thiopental. Carbimazole is an imidazole-derived antithyroid drug containing a thionamide group.

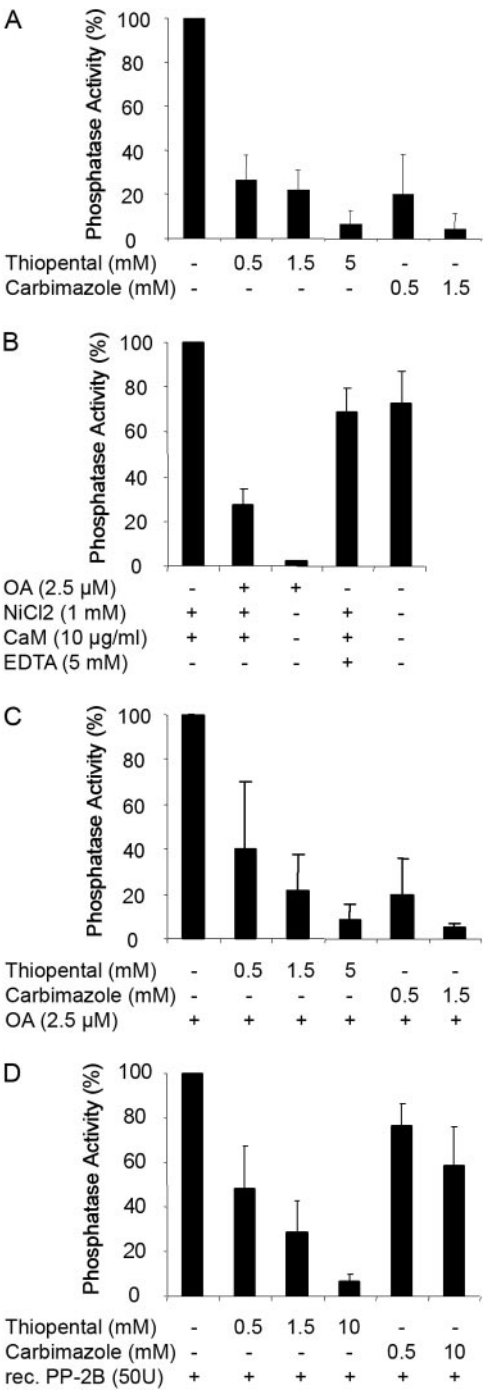

Fig. 2. The thioureylenes thiopental and carbimazole inhibit neuronal phosphatase activity. SK-N-SH cells were pretreated with thiopental or carbimazole for 2 h. Cellular lysates were prepared and fractionized. The serine/threonine dephosphorylation of a synthetic RII phosphopeptide substrate (10 μg/ml) was determined at 30°C for 20 min in a reaction buffer containing 12.5 mM Tris, pH 7.2, 1 mM $NiCl_2$, 0.5 μg/ml acetylated bovine serum albumin, 5 μl of cytoplasmic cell eluate, and 10 μg/ml CaM. Reactions were terminated by the addition of Malachite Green reagent. Released phosphates reacted to molybdate-malachite phosphate complexes and were spectrophotometrically quantified at 600 nm. Color development was expressed as the percentage of induction by absorbance compared with phosphatase activity in the absence of thioureylenes (100%). A, total phosphatase activity was determined in the presence of thioureylenes. B, different phosphatase inhibitors were used: 2.5 μM okadaic acid (OA) inhibited PP1 and PP-2A; depletion of $NiCl_2$ and CaM inhibited PP-2B; and 5 mM EDTA inhibited PP-2B and PP2C. C, cellular PP-2B activity was determined in 12.5 mM Tris, pH 7.2, 0.5 μg/ml acetylated bovine serum albumin, 10 μg of synthetic RII phosphopeptide substrate, 2.5 μM okadaic acid, 1 mM $NiCl_2$, 5 μl of cytoplasmic eluate, and 10 μg/ml CaM. D, 50 U of recombinant PP2-B was coincubated with thioureylenes before RII-peptide dephosphorylation was determined. Error bars indicate ± S.E.M. of four independent experiments.

Heppelmann et al., 1998). De novo synthesis of proapoptotic Fas is transcriptionally regulated by NFAT, which is dephosphorylated and activated by PP-2B (Holtz-Heppelmann et al., 1998). We explored the possibility that inhibition of PP-2B by thioureylenes might influence the induction of the transcription factor NFAT and the subsequent transactivation of target genes. Thioureylene treatment of cells transfected with an NFAT-dependent luciferase reporter gene expression plasmid resulted in a dose-dependent decrease of luciferase reporter gene expression upon calcium mobilization by low levels of ionomycin (1.25 μM; Fig. 3), demonstrating that thioureylenes inhibit NFAT transcription factor activation. However, when neuronal cells were treated with high levels of ionomycin (40 μM) or other calcium-mobilizing agents, including the calcium ionophore A23187 (40 μM) and the endoplasmic reticulum [Ca^{2+}]-adenosine triphosphatase inhibitor thapsigargin (40 μM), rapid cell death was observed without detectable luciferase gene expression (M. Humar, unpublished data). Repression of protein synthesis by actinomycin D and cycloheximide did not prevent calcium-induced LDH release from SK-N-SH cells (M. Humar, unpublished data). Therefore, we believe that NFAT-dependent gene expression is not the dominant signal cascade to induce short term neuronal cell death by pathological [Ca^{2+}]c flux.

PP-2B-Dependent Dephosphorylation of Bad Is Inhibited by Thioureylenes. Bad is a proapoptotic member of the Bcl-2 family that heterodimerizes with antiapoptotic proteins such as Bcl-2 and BcL-XL and thereby promotes cell death (Yang et al., 1995). PP-2B is the only known serine/threonine phosphatase, dephosphorylating and thus activating Bad by a calcium-dependent mechanism (Wang et al., 1999). Changes in Bad dephosphorylation were therefore examined in neuronal cells treated with A23187 or thapsigargin. SK-N-SH cells contain low levels of phospho-Bad-(Ser136). Therefore, these cells were nucleoporated with the Bad expression vector pEGB-mBAD to obtain an increased expression of phospho-Bad when cultured in growth medium (Fig. 4A). As determined by immunoblot analysis with an antibody specific to phospho-Bad(Ser136), treatment of these cells with the [Ca^{2+}]-mobilizing agents thapsigargin and A23187 induced dephosphorylation of Bad without altering the amount of Bad protein (Fig. 4B). Pretreatment of these cells with the PP-2B inhibitors CsA, thiopental, or carbimazole prevented the thapsigargin (Fig. 4C) or A23187 (Fig. 4D) induced Bad dephosphorylation. When A23187 was replaced by 40 μM ionomycin, similar results were observed (M. Humar, unpublished data). Pentobarbital, the oxy-analog of thiopental, did not affect the dephosphorylation of Bad (Fig. 4, C and D), demonstrating that the sulfur side chain of thioureylenes bears functional relevance.

The PP-2B Inhibitors CsA, Thiopental, and Carbimazole Protect from Calcium-Dependent Cellular Damage. The functional relevance of the thioureylene-mediated suppression of PP2B and impairment of Bad dephosphorylation was explored by accessing the effects on neuronal cell integrity. Initial time and dose kinetics, as measured by an LDH-release assay, demonstrated that calcium-dependent

cellular damage was noticeably induced after 1 h by 40 μM thapsigargin, A23187, or ionomycin (M. Humar, unpublished data). Preincubation of cells with the PP-2B inhibitors CsA and the thioureylenes thiopental and carbimazole significantly reduced the LDH release upon thapsigargin treatment (Fig. 5A). The protective effect of thioureylenes was mediated by redox-active sulfur, because pentobarbital, the oxy-analog of thiopental, could not diminish LDH leakage of SK-N-SH cells. The use of the PKC inhibitors chelerythrine (1 μM), rottlerin (1 μM), or Gö6976 (5 μM) had no impact on thapsigargin-induced neuronal damage. When cellular damage was initiated by A23187, the cytoprotective effects of CsA and thiopental were abolished (Fig. 5B). In contrast, carbimazole and the selective PKCδ inhibitor rottlerin significantly reduced cellular damage upon A23187 treatment (Fig. 5B). When A23187 was replaced with 40 μM ionomycin, similar results were observed (M. Humar, unpublished data).

The PP-2B Inhibitors CsA, Thiopental, and Carbimazole Inhibit Caspase-3 Activation. To investigate whether calcium-induced neuronal cell damage is due to necrosis or is accompanied by apoptosis, the proteolysis of caspase-specific substrates was analyzed. In SK-N-SH cells, apoptosis was induced only by the depletion of intracellular calcium stores (thapsigargin treatment) but not by extracellular calcium influx mediated by ionophores. Thapsigargin (40 μM) induced proteolysis of specific caspase substrates such as acetyl-DEVD-7-amino-4-methylcoumarin (Fig. 5C) and the DNA repair protein PARP (Fig. 5D). PARP cleavage was accompanied by processing of procaspase-3 to its active subunits of 17 and 19 kDa (Fig. 5D). Accordingly, the caspase inhibitor Z-VAD inhibited LDH release upon thapsigargin treatment but not in the presence of A23187 or ionomycin (M. Humar, unpublished data). A23187 (Fig. 5, C and D) or ionomycin (M. Humar, unpublished data) were not able to induce caspase-3-like activity.

Next, the effects of CsA, thioureylenes, and PKC inhibitors on caspase activation were determined. PP-2B inhibitors such as CsA, thiopental, and carbimazole inhibited caspase activity by repression of procaspase-3 processing (Fig. 5D). A low concentration of thiopental (500 μM) was not able to significantly suppress procaspase-3 or PARP cleavage when preincubated for 4 h (Fig. 5D), demonstrating a dose-specific effect. The use of the oxy-derivative pentobarbital had no impact on caspase-mediated proteolytic activity, indicating that the redox-active sulfur of thioureylenes bears functional relevance. The selective PKC inhibitors rottlerin and Gö6976 did not influence thapsigargin-mediated caspase activity, procaspase-3 processing, or PARP cleavage (Fig. 5, C and D). Our observations demonstrate that thioureylene-mediated inhibition of PP2-B and Bad dephosphorylation result in the suppression of caspase-mediated neuronal apoptosis.

CsA and Thioureylenes Inhibit Caspase-3 Activity in Hippocampal Slice Cultures. To support our findings in a complex system of brain tissue, we performed experiments in organotypic hippocampal slice cultures. In hippocampal tissue, thapsigargin induced cleavage of the fluorogenic caspase-3 substrate acetyl-DEVD-7-amino-4-methylcoumarin (Fig. 6A), processing of procaspase-3 (Fig. 6B), and proteolytic degradation of the cellular caspase substrate PARP (Fig. 6B). The proteolytic activity was abolished by the caspase inhibitor Z-VAD, demonstrating a caspase-specific effect. Both thiopental and carbimazole inhibited thapsigargin-mediated caspase activity (Fig. 6A) and repressed procaspase-3 processing to an active 17/19-kDa fragment (Fig. 6B). Likewise, the cleavage of PARP was significantly reduced by these thioureylenes (Fig. 6B). Pentobarbital

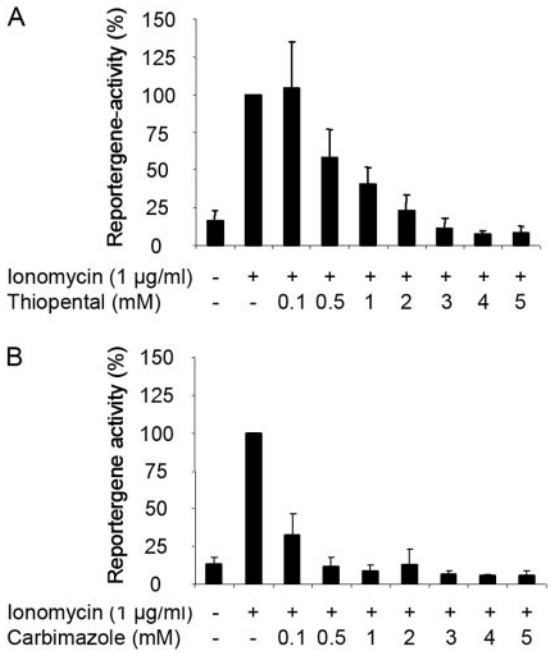

Fig. 3. Thioureylenes inhibit NFAT-dependent reporter gene expression. Reporter gene assays are shown. SK-N-SH cells were transfected with pNFAT-TA-Luc and treated with 0.1 to 5 mM thiopental (A) or carbimazole (B) for 2 h before luciferase reporter gene transcription was induced by 1.25 μM ionomycin for an additional 13 h. Cellular lysates were analyzed for luciferase protein levels and normalized to protein content. Reporter gene expression in the absence of thioureylenes was 100% (positive control). Values are expressed as the means of four independent experiments ± S.E.M.

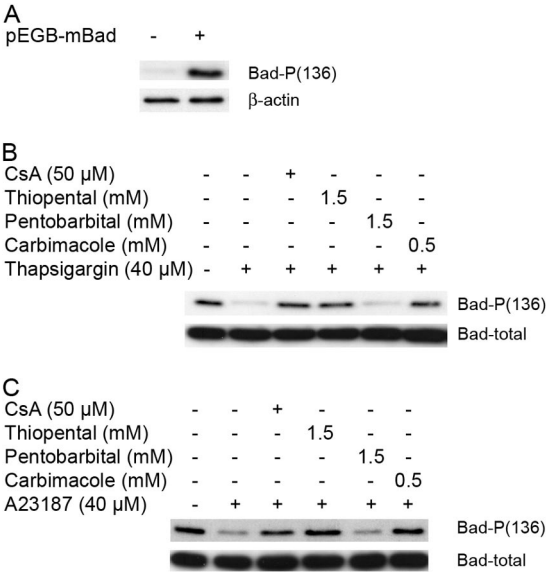

Fig. 4. Thioureylenes inhibit the calcium-dependent dephosphorylation of phospho-Bad(Ser136). Immunoblot experiments analyzing the phosphorylation of Bad at serine 136 are shown. SK-N-SH cells were nucleofected with 2 μg of pEGB-mBad (A–C). Dephosphorylation of Bad was induced for 1 h by 40 μM thapsigargin or 40 μM A23187. Pretreatment of SK-N-SH cells for 4 h with 50 μM CsA, 1.5 mM thiopental, or 0.5 mM carbimazole protected from thapsigargin (B) or A23187 (C) induced Bad-dephosphorylation. Pentobarbital had no effect. Representatives of three independent experiments are shown.

had no impact on caspase function, again supporting the biological relevance of redox-active sulfur. In addition, in the presence of thiopental, carbimazole, and pentobarbital, we observed processing of procaspase-3 to a large, unknown 30- to 35-kDa fragment that was not associated with a caspase-typical proteolytic activity (Fig. 6B, top blot).

NMDA Receptor Agonists and Kainic Acid Induce Caspase-Independent Cell Death in Hippocampal Slice Cultures that Is Inhibited by Thioureylenes. Different pharmacological calcium inducers were coincubated with hippocampal slice cultures. As observed before, release of calcium from intracellular ER stores after thapsigargin treatment induced caspase activity and cleavage of the fluorogenic caspase-3 substrate acetyl-DEVD-7-amino-4-methylcoumarin (Fig. 7A). This observation was accompanied by proteolytic processing of procaspase-3 to an active 17/19-kDa fragment and cleavage of the cellular caspase substrate PARP (Fig. 7B). On the other hand, A23187 treatment resulted in the generation of a high-molecular caspase-3 cleavage product of approximately 30 to 35 kDa (Fig. 7B, top blot). The presence of this high molecular caspase-3 cleavage product was accompanied by neither caspase-3-like protease activity (Fig. 7A) nor PARP-cleavage (Fig. 7B), indicating an unknown function of this caspase variant.

In contrast to neuroblastoma cell lines, hippocampal slice cultures express functional NMDA receptors (Bahr et al., 1995). Therefore, the effect of NMDA receptor agonists and kainic acid on caspase activation was analyzed in hippocampal tissue. Receptor agonists were used at concentrations published previously (Kristensen et al., 2001; Matthews et al., 2003). Similar to A23187, no significant caspase-3-like activity was detectable at 6, 12, 24, or 48 h (Fig. 7, A and B; data shown only for 48 h) after application of L-glutamate, NMDA, or kainic acid. However, despite the absence of cel-

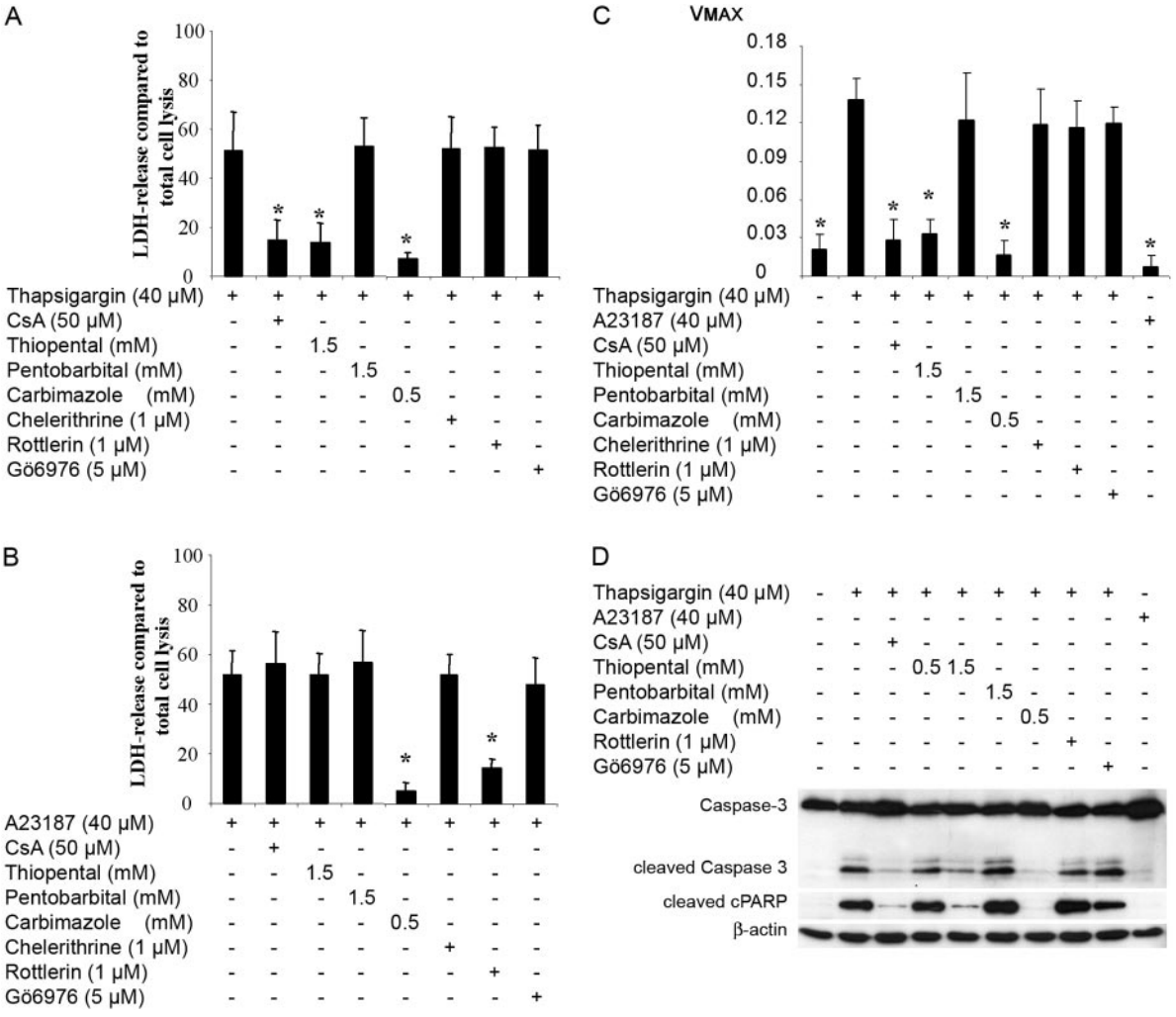

Fig. 5. The PP-2B inhibitors CsA, thiopental, and carbimazole protect from thapsigargin-induced cell death and caspase-3 activation. SK-N-SH cells were pretreated with 50 μM CsA, 0.5 or 1.5 mM thiopental, 1.5 mM pentobarbital, 0.5 mM carbimazole, 1 μM chelerythrine, 1 μM rottlerin, or 5 μM Gö6976 for 4 h. Subsequently, cell death was induced by 40 μM thapsigargin or 40 μM A23187 for 1 h. Cell death was determined by a cytotoxicity LDH-release assay (A and B). Values of LDH release are presented as a percentage of total cellular LDH content (100%). Error bars indicate ± S.E.M. of four independent experiments. *, $P < 0.001$ versus thapsigargin- or A23187-induced cells (positive controls) was considered as significant. C, caspase-3-like activity was determined by a time kinetic for 30 min in cellular lysates using a 60 μM concentration of the fluorogenic caspase-3 substrate acetyl-DEVD-7-amino-4-methylcoumarin. Caspase-3-like activity was depicted as V_{max}, the turnover rate of the caspase substrate. Error bars indicate ± S.E.M. of four independent experiments. *, $P < 0.001$ versus thapsigargin-induced caspase-3-like activity was considered significant. Immunoblot analysis demonstrated inhibition of procaspase-3 cleavage by CsA, thiopental, and carbimazole (D, top blot). PARP cleavage was also inhibited by CsA, thiopental, and carbimazole (D, middle blot). Detection of β-actin served as a loading control (D, bottom blot). Representative immunoblots of three independent experiments are shown.

lular caspase activation, NMDA and kainate receptor agonists induced severe cellular damage, as demonstrated by a cytotoxicity assay (Fig. 8, A and B). To determine early and late events of cell death, the release of intracellular LDH into the extracellular lumen upon calcium induction was measured by a time kinetic. Hippocampal LDH release was significantly reduced by coincubation with thiopental or carbimazole (Fig. 8, A and B). This demonstrates that thioureylenes also reduce caspase-independent cell death in slice cultures after NMDA and kainate receptor activation.

Discussion

Neuronal cell death after cerebral hemorrhage or ischemia is characterized by a core region of imminent necrotic cell death and an extensive surrounding area of moderate ischemia, in which secondary injury progresses over days and months, resulting in significant neurological dysfunction (Kaminska et al., 2004). This secondary injury is induced by a disruption of the calcium homeostasis and is believed to be amenable to therapeutic manipulation (Pulsinelli et al., 1997; Kaminska et al., 2004). Different pathways initiate a neuropathological calcium overload, including excitatory amino acid receptor stimulation, ATP deprivation, inactivation of active carrier proteins, the loss of membrane integrity, radical formation, and ER stress (White et al., 2000). In SK-N-SH cells, kainic acid, NMDA, and L-glutamate failed to induce calcium-dependent neuronal cell death due to a down-regulation of functional glutamate and NMDA receptors (Yoshioka et al., 1996). We used ionophores or pharmacologically induced the release of calcium from internal stores to analyze thioureylene-mediated effects on calcium-mediated neuronal cell death. A differential analysis of defined routes of calcium flux was possible by the choice of the calcium-releasing molecules.

Efficient treatment of traumatic brain injury by thiopental requires high doses and a long-termed application of the barbiturate (Russo et al., 1997). In brain tissue, thiopental concentrations may be increased as much as 20-fold compared with plasma concentrations (Yasuda et al., 1993). This corresponds with our observation that high concentrations of thioureylenes are necessary to prevent neuronal cell damage. We show that thiopental and other thioureylenes inhibit the enzymatic activity of the calcium-dependent neuronal PP-2B at clinically relevant doses and thus protect neurons from apoptosis. In addition, thioureylenes inhibit PP1 or PP-2A, because repression of total neuronal phosphatase activity was extensive, but PP-2B activity accounted only for 27.5 ± 14.3% of total phosphatase activity. The consequences of PP1 and PP-2A inhibition are still unclear. Thioureylenes inhibit the activity of PP-2B by two mechanisms. First, the pyrimidine-based thioureylene thiopental directly repressed the en-

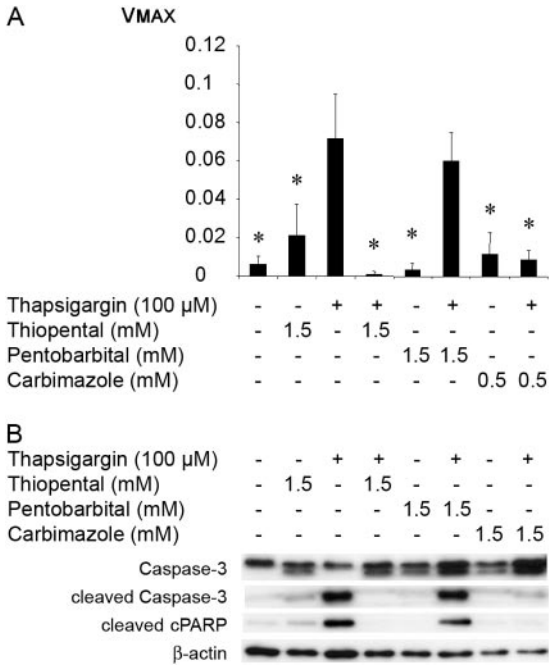

Fig. 6. CsA and thioureylenes inhibit caspase-3-like protease activity, procaspase-3 processing, and PARP cleavage in hippocampal slice cultures. After 10 days of culture, hippocampal slice cultures were pretreated with 50 μM CsA, 1.5 mM thiopental, 1.5 mM pentobarbital, or 1.5 mM carbimazole for 4 h before 100 μM thapsigargin was added for 48 h. Tissue lysates were prepared, and the V_{MAX} of caspase-3-like activity was determined for 30 min in hippocampal lysates by the addition of a 60 μM concentration of the fluorogenic caspase-3 substrate acetyl-DEVD-7-amino-4-methylcoumarin (A). Error bars indicate ± S.E.M. of four independent experiments. *, $P < 0.001$ versus thapsigargin-induced caspase-3-like activity was considered significant. In addition, tissue lysates were analyzed for procaspase-3 and PARP cleavage by immunoblotting (B). Detection of β-actin served as a loading control. Representative blots of three independent experiments are shown.

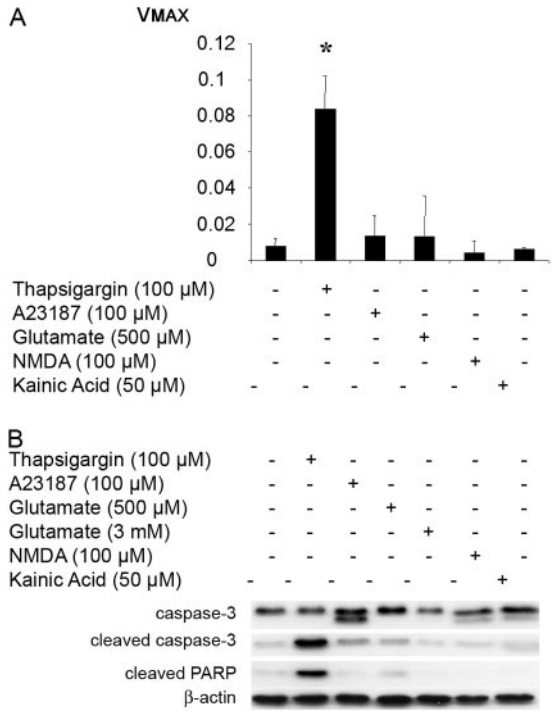

Fig. 7. Thapsigargin but not A23187, L-glutamate, NMDA, or kainic acid induces caspase-3-like activity in hippocampal slice cultures. After 10 days of culture, hippocampal slice cultures were induced by 100 μM thapsigargin, 100 μM A23187, 0.5 or 3 mM L-glutamate, 100 μM NMDA, or 50 μM kainic acid for 48 h. The V_{MAX} of caspase-3-like activity was determined for 30 min in hippocampal lysates by addition of a 60 μM concentration of the fluorogenic caspase-3 substrate acetyl-DEVD-7-amino-4-methylcoumarin (A). Error bars indicate ± S.E.M. of three independent experiments. *, $P < 0.001$ versus caspase-3-like activity in noninduced hippocampal slice cultures was considered significant. In addition, tissue lysates from hippocampal slice cultures were analyzed by immunoblotting for procaspase-3 processing and PARP cleavage (B). Detection of β-actin served as a loading control. Representative blots of three independent experiments are shown.

zymatic function of the PP-2B/CaM complex, as demonstrated by phosphatase activity assays using recombinant PP-2B and CaM in the absence of further cellular components. Most likely, CaM is a direct target for thiopental, because competition experiments revealed that increasing amounts of CaM restore the activity of recombinant PP-2B in the presence of thiopental (Humar et al., 2007). In contrast, the imidazole-derived thioureylene carbimazole blocked PP-2B activity only in cellular lysates and had marginal effects on the enzymatic function of isolated recombinant PP-2B/calmodulin. This argues for an indirect inhibition of PP-2B and a participation of biochemical processes that depend on the functional integrity of the cell. Carbimazole is probably enzymatically converted to an active metabolite, or the PP-2B/calmodulin complex is inactivated by a still-unidentified cellular component during carbimazole treatment.

PP-2B inhibition by thioureylenes resulted in a repression of NFAT-dependent gene transcription and thus might influence long-term apoptotic events by limiting the expression of proapoptotic genes (Holtz-Heppelmann et al., 1998). However, NMDA receptor activation, disruption of calcium homeostasis, and PP-2B activation is most probably associated with rapid cell death initiated by proapoptotic Bad proteins (Wang et al., 1999). Thioureylenes inhibited PP-2B-dependent Bad dephosphorylation and the subsequent initiation of the intrinsic pathway of apoptosis (Yang et al., 1995). Thus, caspase-3 activity, cleavage of the cellular caspase-3 substrate PARP, and caspase-3-dependent cell death were inhibited in SK-N-SH cells and organotypic hippocampal slice cultures. These events depended on active PP-2B activation, as demonstrated by coincubation with CsA, a specific PP-2B inhibitor, and included the repression of procaspase-3 processing.

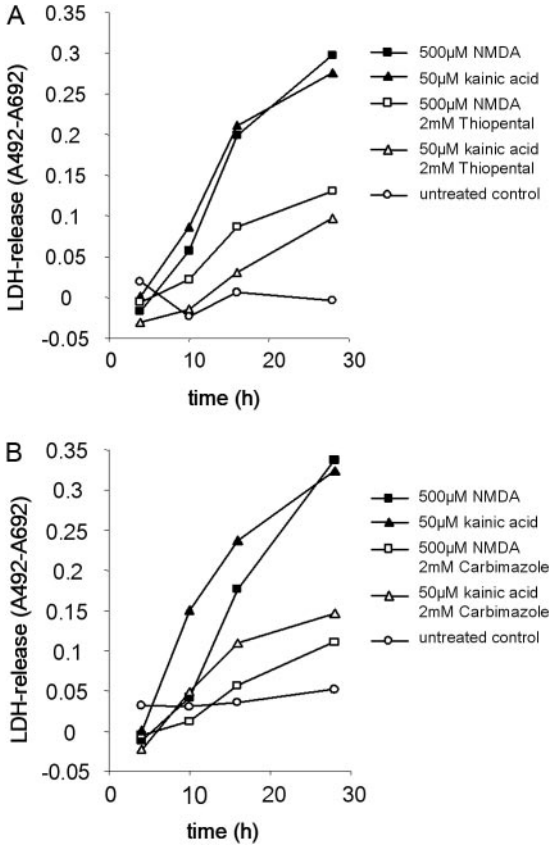

Fig. 8. Thiopental reduces the LDH release after NMDA or kainate receptor stimulation of hippocampal slice cultures. After 10 days of culture, hippocampal slice cultures were left untreated (○) or were pretreated with 2 mM thiopental (□, △; A) or 2 mM carbimazole (□, △; B) for 4 h. NMDA (100 μM; □, ■) or 50 μM kainic acid (△, ▲) was added for an additional 24 h. Cell culture supernatants of hippocampal slice cultures were analyzed for LDH content after 0, 6, 12, and 24 h after the addition of the $[Ca^{2+}]c$ inducers. The magnitude of the LDH release was presented as A_{492} to A_{690} values. A representative result is shown from three independent experiments.

Our results suggest that thioureylenes also attenuate caspase-independent cellular damage. Thioureylenes reduced caspase-independent cell damage in NMDA or kainate receptor-stimulated hippocampal slice cultures. However, only the imidazole-derived thioureylene carbimazole suppressed A23187-dependent necrosis in SK-N-SH cells, demonstrating that pyrimidine-derived thioureylenes affected cell death differently. The reason for this is unclear, but carbimazole acted independently of PP-2B because CsA was unable to prevent A23187-induced LDH leakage of cells.

Previous studies have demonstrated that the thioureylenes thiamylal, secobarbital, and propylthiouracil inhibit cellular functions by similar mechanisms (Humar et al., 2004, 2007). Therefore, these agents might also affect neuronal cell death. It is now obvious that redox-active sulfur coupled to the imidazole or pyrimidine nucleus has neuroprotective capacity because the oxy-derivative pentobarbital could not prevent neuronal apoptosis. Several reports confirm that molecular targets of thioureylenes are susceptible to an interaction with sulfur (King, 1986; Tan et al., 1996). The structural analysis of CaM suggests that cysteines participate in the formation of an EF-hand motif and thus regulate the calcium-induced conformational transition of CaM. Their inactivation leads to a decrease in affinity for calcium and a loss of ability to activate target enzymes (King, 1986). In addition, it has been shown that sulfhydryl reagents inactivate calcineurin and that a cysteine residue in the catalytic subunit of calcineurin is important for establishing the activated form of calcineurin (Tan et al., 1996). Sulfur of thioureylenes might directly interact with redox-active cysteines and inactivate the PP-2B/CaM complex.

Neuroprotection by thioureylenes is probably not limited to traumatic injury or stroke but might also include treatment of some neurodegenerative disorders. Disturbance of calcium homeostasis, dysfunction of the ER, and apoptotic biochemical cascades contribute to the progressive neurodegeneration in the pathogenic processes of Alzheimer's, Parkinson's, Huntington's, and prion-associated disorders (Rodnitzky, 1999; Ferreiro et al., 2006; Mattson, 2006). The use of pharmacological PP-2B/CaM inhibitors has led to beneficial effects in some of these diseases (Kaminska et al., 2004). Furthermore, disruption of calcium homeostasis and activation of CaM, PP-2B, and NFAT-dependent gene transcription may lead to disorders in other tissues (e.g., cardiac hypertrophy and progressive heart failure) (Molkentin, 2000; Wilkins and Molkentin, 2004). Interestingly, hyperthyroid patients with active myocardial damage or cardiomyopathy showed a complete remission upon antithyroid treatment with thioureylenes (Marti et al., 1995; Hardiman et al., 1997). In agreement with our data, a general protective effect of heterocyclic thioureylenes is plausible.

It is now clear that neuronal cell death is a complex event, and inhibition of one dominant pathway does not

necessarily result in cellular recovery (White et al., 2000). Actually, inhibition of apoptosis may result in necrosis (Scheller et al., 2006). Therefore, an effective pharmacological treatment of neuronal calcium-dependent disorders must target multiple independently acting fatal pathways (White et al., 2000). Here we demonstrate that thioureylenes inhibit caspase-dependent and caspase-independent cell death. In previous studies, these drugs exhibited further beneficial properties, such as repression of inflammation (Loop et al., 2002; Humar et al., 2008), induction of a protective heat-shock response (Roesslein et al., 2008), repression of stress-induced kinases (Humar et al., 2007), modulation of intracranial pressure (Turner et al., 2005), and reduction of the cerebral metabolic demand of oxygen consumption (Turner et al., 2005). These properties may act in a coordinated fashion and reduce neurological injury. Thus, thioureylenes may represent an ideal multifunctional pharmaceutical tool to treat neuronal damage.

Excitotoxicity and the resulting mechanisms coupling the increase of $[Ca^{2+}]c$ to cell death are still poorly understood. Extracellular calcium influx induced by the ionophore A23187 or direct NMDA receptor stimulation resulted in marked necrosis but not apoptosis, whereas thapsigargin treatment induced apoptosis via calcium release from internal stores by inhibition of the sarcoendoplasmic reticulum $[Ca^{2+}]$-adenosine triphosphatase. Obviously, specific routes of calcium influx are differentially linked to cellular fate. This observation is confirmed by reports that indicate that a large proportion of neurotoxic calcium after NMDA receptor activation originates from an intracellular calcium pool (Mody and MacDonald, 1995) and that mitochondrial calcium loading by the ER is a prerequisite for glutamate neurotoxicity (Stout et al., 1998).

Induction of BAD initiates the intrinsic pathway of apoptosis (Yang et al., 1995). However, A23187-induced BAD dephosphorylation was not accompanied by caspase activation and PARP cleavage, indicating neuronal cell death by a necrotic pathway. Induction of necrosis probably prevents the terminal execution of the apoptotic program.

Cell culture experiments revealed that thapsigargin-induced apoptosis was followed by necrosis because neuronal cell death was irresponsive to the caspase-inhibitor Z-VAD or PP-2B inhibitors in a late phase (M. Humar, unpublished data). Consequently, both apoptosis and necrosis must be repressed in an effective neuroprotective therapy. However, inhibition of necrosis was believed to be impossible because it is not regulated by a cellular program. Here, we describe for the first time that inhibition of PKCδ and the use of carbimazole affect the progression of caspase-independent cell death. For efficient prevention of neuronal cell death, we suggest a combined antiapoptotic and antinecrotic therapy (e.g., by using PP-2B inhibitors and PKCδ inhibitors).

In summary, our study describes a pharmacological approach to protect neurons from cellular damage by drugs of the thioureylene class that are currently used for treatment of hyperthyroidism or induction of anesthesia (Russo et al., 1997; Bartalena et al., 2005). Furthermore, we suggest that neuroprotective therapy must combine both the inhibition of apoptosis and necrosis. The observed cellular mechanisms that affect neuronal cell death might lead to novel preventive and therapeutic approaches to treat neurological disorders in which the progression of cell death is prominent.

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