

Effect of Barbiturates on Hydroxyl Radicals, Lipid Peroxidation, and Hypoxic Cell Death in Human NT2-N Neurons

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Background: Barbiturates have been shown to be neuroprotective in several animal models, but the underlying mechanisms are unknown. In this study, the authors investigated the effect of barbiturates on free radical scavenging and attempted to correlate this with their neuroprotective effects in a model of hypoxic cell death in human NT2-N neurons.

Methods: Hydroxyl radicals were generated by ascorbic acid and iron and were measured by conversion of salicylate to 2,3-dihydroxybenzoic acid. The effect of barbiturates on lipid peroxidation measured as malondialdehyde and 4-hydroxynon-2-enal was also investigated. Hypoxia studies were then performed on human NT2-N neurons. The cells were exposed to 10 h of hypoxia or combined oxygen and glucose deprivation for 3 or 5 h in the presence of thiopental (50–600 μM), methohexital (50–400 μM), phenobarbital (10–400 μM), or pentobarbital (10–400 μM), and cell death was evaluated after 24 h by lactate dehydrogenase release.

Results: Pentobarbital, phenobarbital, methohexital, and thiopental dose-dependently inhibited formation of 2,3-dihydroxybenzoic acid and iron-stimulated lipid peroxidation. There were significant but moderate differences in antioxidant action between the barbiturates. While phenobarbital (10–400 μM) and pentobarbital (10–50 μM) increased lactate dehydrogenase release after combined oxygen and glucose deprivation, thiopental and methohexital protected the neurons at all tested concentrations. At a higher concentration (400 μM), pentobar-

bital also significantly protected the neurons. At both 50 and 400 μM , thiopental and methohexital protected the NT2-N neurons significantly better than phenobarbital and pentobarbital.

Conclusions: Barbiturates differ markedly in their neuroprotective effects against combined oxygen and glucose deprivation in human NT2-N neurons. The variation in neuroprotective effects could only partly be explained by differences in antioxidant action. (Key words: Antioxidants; dizocilpine; free radicals; *in vitro*.)

BARBITURATES¹ and several other anesthetics, such as ketamine,² propofol,³ and diazepam,⁴ have been shown to have neuroprotective effects. The mechanism of neuroprotection provided by barbiturates has not been determined but has mainly been attributed to a reduction in cerebral metabolic rate. However, Warner *et al.* recently demonstrated that maximal protection by pentobarbital was achieved with doses much lower than those needed to obtain an isoelectrical electroencephalogram.⁵ Furthermore, other anesthetics can also provide isoelectrical electroencephalogram⁶ but offer less protective effects than those obtained with barbiturates.

Other possible protective mechanisms are reduction of cerebral blood flow with less edema, inhibition of excitotoxicity,⁷ attenuation of hypoxia-induced increase in blood-brain barrier permeability,⁸ and scavenging of free radicals.^{9,10} Free radicals are presumed to take part in multiple pathophysiologic processes. During reoxygenation after cerebral ischemia, oxygen free radicals are produced,^{11,12} and inhibition of free radical production attenuates ischemic damage.^{13,14} Earlier reports on the antioxidant effects of barbiturates have mainly measured lipid peroxidation as thiobarbituric acid-reactive substances.^{9,10} We wanted to explore further the antioxidative effects of barbiturates and correlate these with their effect on hypoxic cell death.

To test the latter we used human NT2-N neurons derived from a human teratocarcinoma cell line. After treatment with retinoic acid for 5 weeks and mitotic inhibitors for 3 weeks, these cells develop into stable

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polarized neuron-like postmitotic cells.¹⁵ They express several central nervous system neuronal marker proteins,¹⁶ express functional non-*N*-methyl-D-aspartate (NMDA) and NMDA receptors,^{17,18} and develop neuritic processes that can be identified as axons or dendrites. We have previously established a model with hypoxic hypoxia in these cells and showed that hypoxic neuronal cell death could be reduced by hypothermia or by treatment with the NMDA-receptor antagonist dizocilpine (MK-801) or the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) antagonist CNQX.¹⁹ Our hypothesis was that barbiturates differed in their antioxidant potential and that this would translate into different degrees of neuroprotection after hypoxia.

Materials and Methods

Materials

Salicylic acid (sodium salt), L-ascorbic acid, 2,3-dihydroxybenzoic acid (2,3-DHBA), *N*-methyl-2-phenylindole, methanesulfonic acid, and 3-morpholinopyridone (SIN-1) were purchased from Sigma Chemical Co. (St. Louis, MO). Ascorbic acid was dissolved immediately before use and protected from light. Storage of dissolved ascorbic acid for 3 h in room temperature did not affect the results. Deionized water (Millipore, Bedford, MA) was used in buffers or as solute. We tested intravenous injection preparations of diazepam (Apothekernes Laboratorium A/S, Oslo, Norway) and phenobarbital (Pharmacy of the National Hospital, Oslo, Norway). Pentobarbital sodium (Norsk Medisinaldepot, Oslo, Norway), methohexital sodium (Eli Lilly & Co, Indianapolis, IN), and thiopental sodium (Abbott Laboratories, North Chicago, IL) were dissolved in deionized water. Methohexital and thiopental contain 30 mg sodium carbonate per 500 mg barbiturate. Diazepam (pure drug) and the drug-free solution of the intravenous preparation were provided by Apothekernes Laboratorium. Dizocilpine (Research Biochemicals Internationals, Natick, MA) was prepared as 1 mM stock in deionized water and stored at -20°C .

Hydroxylation of Salicylate

Hydroxyl radicals were quantified by their ability to convert salicylate to 2,3-DHBA. Measurement of 2,3-DHBA was performed as previously described.²⁰ The hydroxyl radical-producing system consisted of (final concentrations): 250 μM ascorbic acid, 25 μM FeCl_3 , 500 μM salicylic acid, and 25 mM K_2HPO_4 , pH 7.4. After preincubation for 10 min in a water-heated shaker, the

reaction was started with the addition of ascorbic acid. Total volume was 2.0 ml. The samples were incubated for 60 min at 37°C . The reaction was terminated by the addition of 1 ml 1 N HCl, and the samples were analyzed immediately. Mean and SDs for four to eight samples are given.

Analysis of Lipid Peroxidation Products

Formation of malondialdehyde (MDA) and 4-hydroxynon-2-enal (HNE) was tested in a lipid emulsion with fatty acids and phospholipids (Intralipid, Pharmacia AB, Stockholm, Sweden). Lipid emulsion (600 μl ; 200 mg/ml) was preincubated with the drug examined or with water as control. The reaction was started by addition of 312.5 μM FeCl_3 (final concentration). The samples were incubated at 37°C in a water-heated shaker for 60 min. Formation of lipid peroxidation products was linear during this period. Lipid peroxidation was measured immediately with a kit (LPO-586; R&D Systems Europe, Abingdon, United Kingdom). The reaction of 10.3 mM *N*-methyl-2-phenylindole in acetonitrile with MDA and 4-hydroxyalkenals produces a stable chromophore with maximal absorbance at 586 nm. A 360- μl sample was mixed with 1,170 μl reaction solution (75%, 10.3 mM *N*-methyl-2-phenylindole and 25% methanol). Reaction was started by the addition of 270 μl of either 15.4 M methanesulfonic acid (analysis of MDA and HNE) or 37% aqueous HCl (MDA only) and incubated at 45°C for 40 and 60 min, respectively. None of the tested drugs interfered with the assay. Blanks with the drug, without iron, were subtracted. Mean and SD for at least four samples are given.

We also tested the effects of barbiturates on peroxy-nitrite- and hydrogen peroxide-stimulated formation of MDA and HNE. The lipid emulsion was incubated with 4 mM SIN-1 or 4 mM hydrogen peroxide for 60 min in a water-heated shaker, and MDA and HNE were analyzed immediately. SIN-1 spontaneously releases superoxide and nitric oxide, combining to form peroxy-nitrite.

Culture of NT2-N Neurons

NT2 cells were prepared as previously described,¹⁹ with some modifications. NT2 cells, 2.3×10^6 , were plated in a T75 flask and fed twice weekly with Dulbecco's Modified Eagle Medium (DMEM)-high glucose (Gibco, Grand Island, NY) with 10% fetal bovine serum (Bio-Whittaker, Walkersville, MD), 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco), and 10 μM retinoic acid (Sigma) for 5 weeks. The cells were split 1:4.3 and grown for 2 more days with identical medium without

retinoic acid in T162 flasks. To separate an upper layer of neuronal cells from a bottom nonneuronal layer, the cells were washed with Hank's balanced salt solution twice and treated with trypsin. Under microscopic inspection, the flasks were shaken gently until most of the neuronal cells were dislodged. The cells were then plated in 12-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) with 2 million cells per well. The wells had been pretreated with polylysine and Matrigel (Becton Dickinson, Bedford, MA). Polylysine treatment was performed 2 days before final plating, and the wells were dried at 37°C overnight. Matrigel coating was performed 2–6 h before use. The cells were fed weekly for 3 weeks with DMEM-high glucose, 5% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and mitotic inhibitors (10 µM uridine, 10 µM 5-fluoro-2'-deoxyuridine, and 1 µM cytarabine; all from Sigma). After 3 weeks, cells were fed with identical medium without mitotic inhibitors. At the first feeding after the final plating, all of the old medium was removed and replaced by 1 ml new medium. Subsequently, only 50% of the medium was changed with every feeding. When the cells were used for experiments between 4 and 5 weeks, they contained approximately 5–15% nonneuronal cells. Passage numbers 54–62 were used for hypoxia studies.

Hypoxia-Reoxygenation of NT2-N Neurons

The hypoxia experiments were started 3–7 days after last feeding. At the day of the experiment, each well was examined microscopically at least 30 min before use and then returned to the incubator. DMEM with or without glucose, with 100 IU/ml penicillin and 100 µg/ml streptomycin, was bubbled for 10 min with 5% CO₂ and 95% N₂ and heated to 37°C. Immediately before the experiment, the cells were washed twice with 0.5 ml phosphate-buffered saline, and 0.5 ml deoxygenated medium was added to each well. DMEM with or without glucose was used for hypoxia or for combined glucose and oxygen deprivation, respectively. Concentrated drug solution, 5 µl, was added as indicated to each well, and the cells were placed in a preheated, humid anaerobic chamber (GasPak 150; Becton Dickinson, Cockeysville, MD). Vacuum was applied to the chamber for 20–25 s (510–635 mmHg), and the air was replaced with 5% CO₂ and 95% N₂. This procedure was performed four times. Inside the chamber, hydrogen was generated with a GasPak Plus envelope containing palladium catalyst to remove trace amounts of oxygen (Becton Dickinson). Separate experiments with an MI-730 Micro-Oxygen electrode (Microelectrodes Inc., Bedford, NH) inside the

chamber connected to OM-4 Oxygen Meter (Microelectrodes Inc.) showed that the oxygen concentration was 0.1% after the fourth gas exchange, decreased to < 0.1% within 30–60 min, and remained < 0.1% for at least 6 h. The chamber was placed in an incubator for 3–5 h (combined glucose and oxygen deprivation) or 10 h (hypoxia). Temperature was monitored continuously inside the anaerobic chamber and in the incubator, and the cells were not exposed to temperatures > 38°C. Afterward, the cells were reoxygenated and returned to the normoxic incubator until 24 h (combined glucose and oxygen deprivation) or 48 h (hypoxia) after the start of the hypoxic exposure. At reoxygenation, 5 µl glucose (final concentration, 5.5 mM) was added to each well exposed to combined glucose and oxygen deprivation.

Because dizocilpine offers almost complete protection in this hypoxia model,¹⁹ at least one well was treated with dizocilpine in all experiments as a positive treatment control.

Lactate Dehydrogenase Assay

Cell viability was assessed by measuring lactate dehydrogenase (LDH) activity in the supernatant. The remaining cells were lysed with deionized water, and LDH was analyzed to ensure that total LDH activity was in the similar range in all wells. LDH was assayed with a kit (No 1,644,793; Boehringer Mannheim GmbH, Mannheim, Germany). The measurements were performed at 492 nm in a Titertek Multiskan Plus MK11 enzyme-linked immunosorbent assay reader (Labsystems and Life Sciences International Ltd., UK) connected to a computer with the program Genesis (Labsystems and Life Sciences International Ltd.). None of the barbiturates nor dizocilpine interfered with the assay. We have previously shown that LDH release to the supernatant correlates well with a fluorescent cytotoxicity assay in this model.¹⁹

Hypoxanthine

To assess the degree of energy failure after combined oxygen and glucose deprivation, 50 µl supernatant was removed immediately after 3 h of oxygen and glucose deprivation (before glucose was added) and after 24 h. Samples were frozen immediately at –20°C for later analysis. High-performance liquid chromatography was performed on a reversed-phase C18 column (Pecosphere-5C C18, 0.46 × 15.0 cm; Perkin Elmer Company, Norwalk, CT). KH₂PO₄ 0.01 M (pH 4.2) was used as mobile phase with a flow rate of 0.8 ml/min. The eluting compounds were detected at 254 nm.

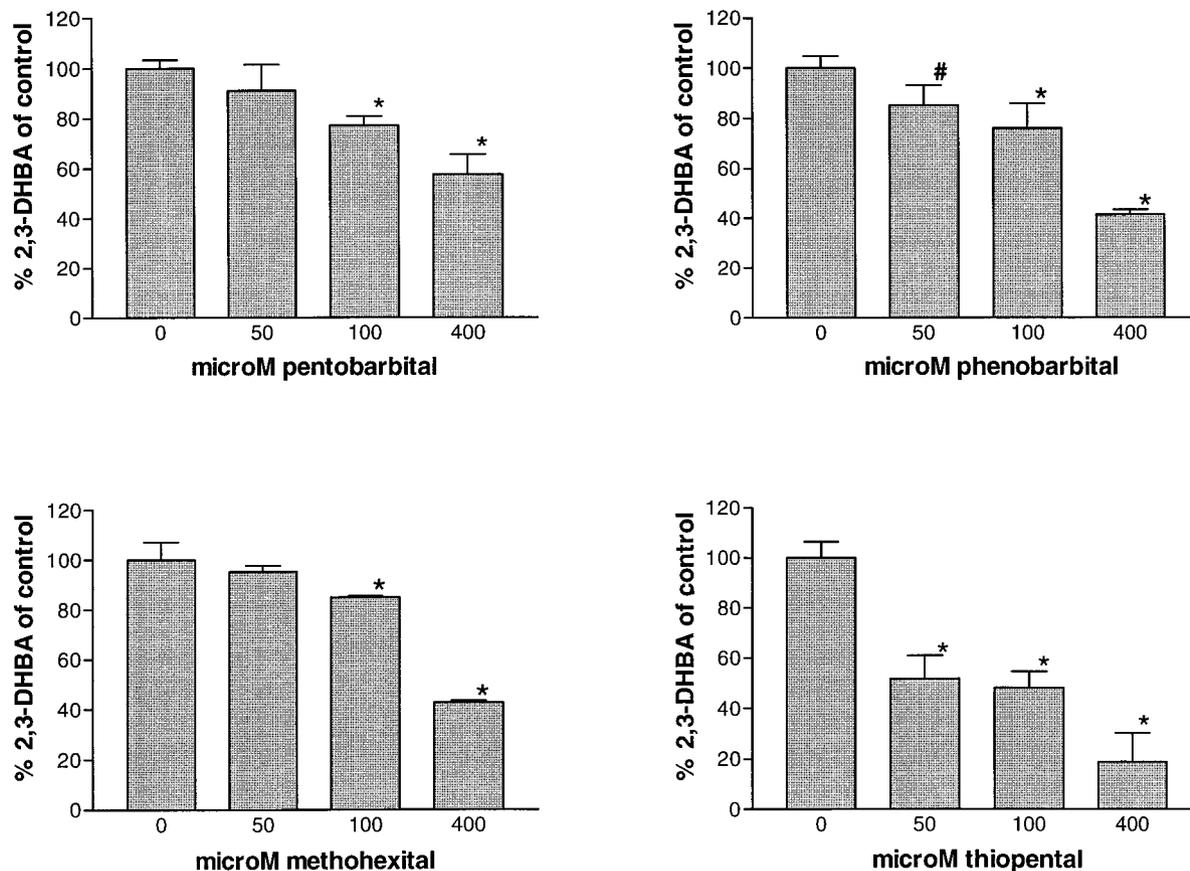


Fig. 1. Effect of barbiturates on hydroxyl radicals. Hydroxyl radicals were measured as conversion of salicylate to 2,3-dihydroxybenzoic acid (2,3-DHBA). FeCl_3 ($25 \mu\text{M}$) was incubated with ascorbic acid ($250 \mu\text{M}$) and salicylate ($500 \mu\text{M}$) and produced $5.4 \pm 2.3 \text{ nM}$ 2,3-DHBA. Mean and SD from four to eight samples are given. * $P < 0.01$ versus control. # $P < 0.05$ versus control.

Statistical Analysis

Malondialdehyde, HNE, and 2,3-DHBA values are presented as percent of control without drug. Mean values and SDs are given. Comparisons of treatment groups with control were performed with one-way analysis of variance with a *post hoc* Dunnett's test. For comparison of selected concentrations between different treatment groups, analysis of variance with Tukey-Kramer *post hoc* test was used.

Lactate dehydrogenase release to the supernatant during hypoxia/reoxygenation in the treatment groups was calculated as percent of the mean LDH release of untreated hypoxic wells in the same experiment. Mean and SD from at least six to eight samples from at least two independent experiments are given.

All treatments were compared with untreated hypoxic cells by analysis of variance and Dunnett's *post hoc* test. For comparison of selected concentrations between different treatment groups, analysis of variance with Tukey-Kramer

post hoc test was used. For comparison of antioxidative abilities with LDH release and hypoxanthine and LDH release, Pearson's correlation coefficient was used. A two-tailed P value < 0.05 was considered statistically significant. Analyses were performed with a GraphPad INSTAT tm/PC statistical package (San Diego, CA).

Results

Antioxidative Properties

Hydroxyl Radicals. Pentobarbital, phenobarbital, methohexital, and thiopental dose-dependently inhibited formation of 2,3-DHBA (fig. 1). Thiopental was significantly more effective than the other barbiturates at all the tested concentrations ($P < 0.01$). Sodium carbonate was added to commercial thiopental and methohexital but did not affect production of 2,3-DHBA at relevant concentrations (data not shown). The intravenous prep-

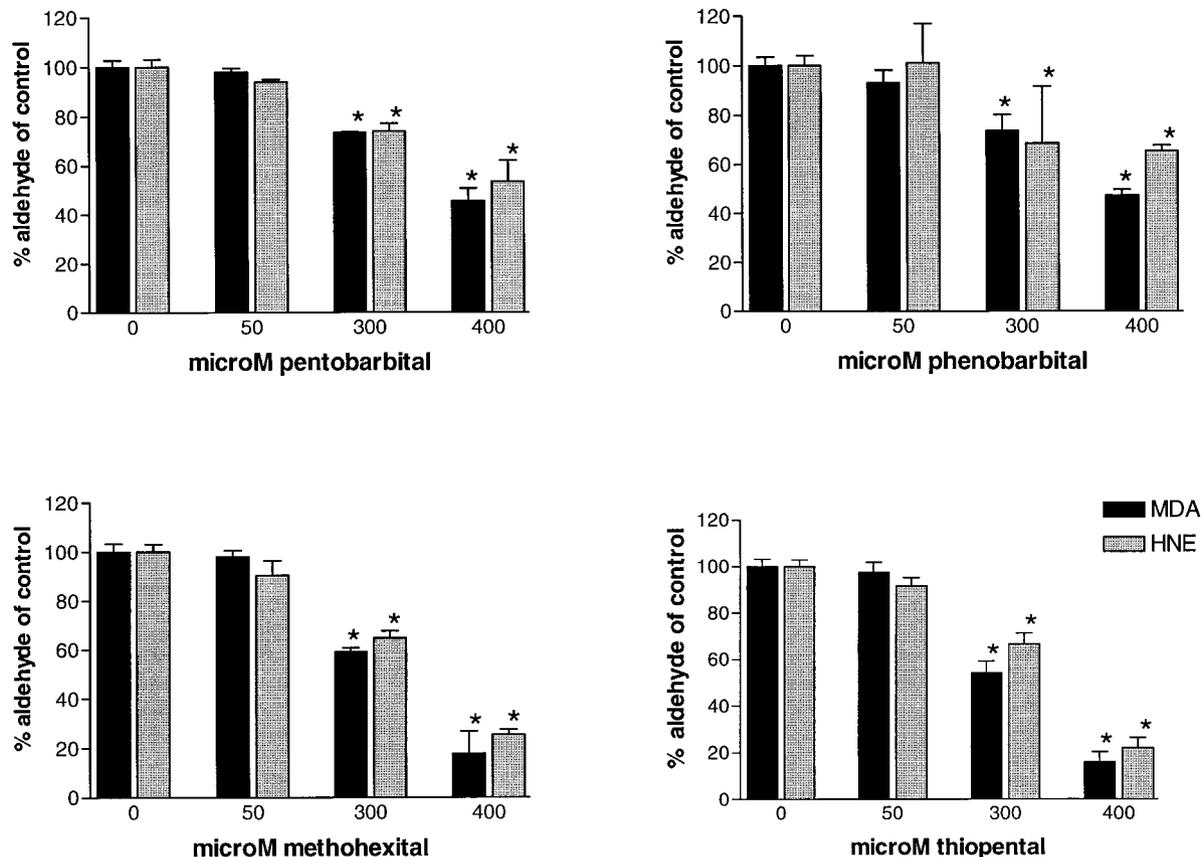


Fig. 2. Effect of barbiturates on formation of malondialdehyde (MDA) and 4-hydroxynon-2-enal (HNE). Incubation of $312.5 \mu\text{M}$ FeCl_3 in Intralipid for 60 min produced $55 \pm 15 \mu\text{M}$ HNE and $22 \pm 5 \mu\text{M}$ MDA. Mean and SD from four to six samples are given. * $P < 0.01$ versus control.

aration of diazepam dose-dependently inhibited formation of 2,3-DHBA (data not shown). However, this was a result of the vehicle rather than the drug *per se*, because solutions of the pure drug did not inhibit 2,3-DHBA formation. We also tested the drug-free solution, prepared by the manufacturer, and the pattern of inhibition followed that made by the intravenous preparation closely (data not shown).

Inhibition of MDA and HNE. Pentobarbital, phenobarbital, methohexital, and thiopental dose-dependently inhibited iron-stimulated formation of HNE and MDA (fig. 2). Sodium carbonate did not inhibit HNE or MDA formation at relevant concentrations (data not shown). At $400 \mu\text{M}$, thiopental and methohexital were significantly more efficient than pentobarbital and phenobarbital in inhibiting iron-stimulated MDA and HNE formation. Diazepam (pure drug) caused limited inhibition of MDA and HNE formation at a high concentration ($20 \mu\text{M}$).

We also investigated whether barbiturates could inhibit peroxynitrite-stimulated formation of HNE and MDA. Incubating the lipid emulsion with SIN-1 resulted in formation of $12 \pm 0.3 \mu\text{M}$ HNE and $1.5 \pm 0.1 \mu\text{M}$ MDA. At $400 \mu\text{M}$, thiopental, pentobarbital, or phenobarbital did not significantly inhibit SIN-1 stimulated HNE or MDA formation.

Incubation of the lipid emulsion with 4 mM hydrogen peroxide produced $30 \pm 3 \mu\text{M}$ HNE and $1.1 \pm 0.3 \mu\text{M}$ MDA. Thiopental, pentobarbital, or phenobarbital at $400 \mu\text{M}$ did not affect HNE formation. However, the small levels of MDA were significantly inhibited by thiopental ($18.5 \pm 8.6\%$ of untreated controls), pentobarbital ($53.8 \pm 9.2\%$), and phenobarbital ($66.2 \pm 12.9\%$).

Neuroprotective Effects in Cell Culture

Hypoxic Damage of NT2-N Neurons. With 10 h of anoxia and 38 h of reoxygenation, 15% of the cells died (LDH supernatant/LDH total). Treatment with $10 \mu\text{M}$

FREE RADICALS, HYPOXIA, AND BARBITURATES

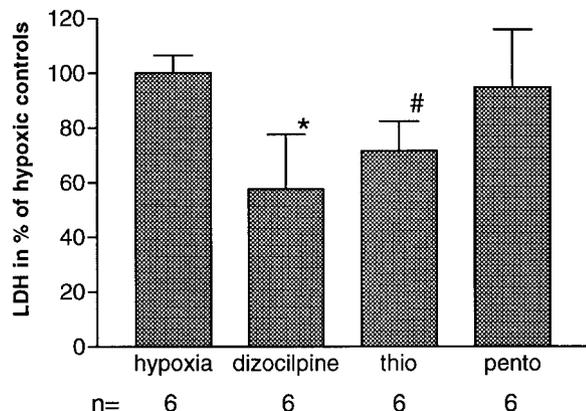


Fig. 3. Neuroprotective effects of treatment with barbiturates in human NT2-N neurons exposed to 10 h of hypoxia. Cell death was evaluated by measuring lactate dehydrogenase release to the supernatant in percent of hypoxic controls 48 h after the start of the insult. Mean and SD from six samples are given. * $P < 0.01$ versus hypoxic control. # $P < 0.05$ versus hypoxic control.

dizocilpine or 200 μM thiopental significantly protected the neurons from hypoxia, whereas 200 μM pentobarbital did not offer significant protection (fig. 3). Because this hypoxia model caused little cell death, we proceeded with another model. The NT2-N neurons were exposed to combined oxygen and glucose deprivation for 3 h and then reoxygenated in the presence of glucose until 24 h after start of the insult (figs. 4 and 5). After 24 h, $39 \pm 6.0\%$ of total LDH was released to the supernatant, compared with $8.6 \pm 2.1\%$ in normoxic controls. Treatment with 10 μM of the NMDA antagonist

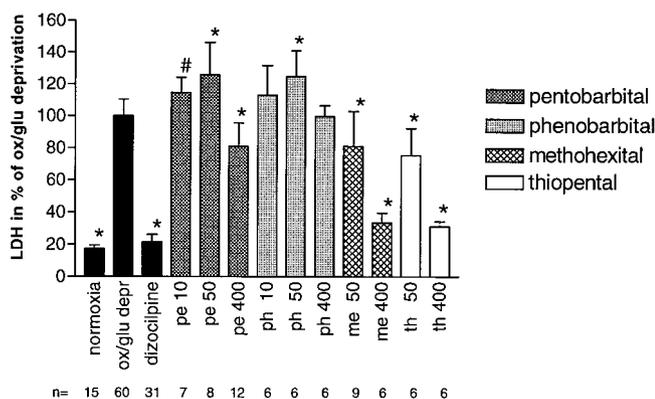


Fig. 4. Neuroprotective effects of treatment with barbiturates on combined oxygen and glucose deprivation for 3 h in human NT2-N neurons. Concentrations of the barbiturates are given in micromolars. Cell death was evaluated by measuring lactate dehydrogenase release to the supernatant in percent of untreated oxygen/glucose-deprived controls 24 h after the start of the insult. * $P < 0.01$ versus oxygen/glucose-deprived controls. # $P < 0.05$ versus oxygen/glucose-deprived controls.

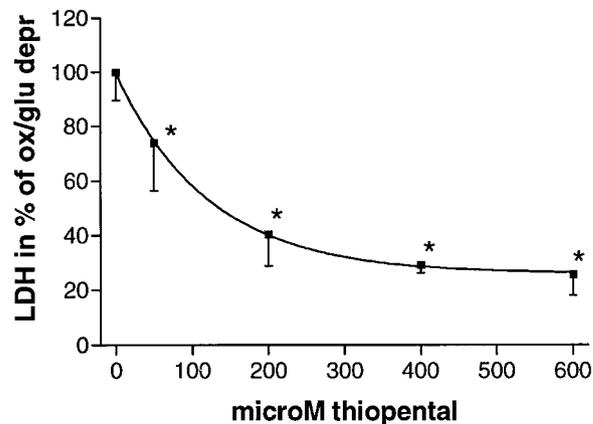


Fig. 5. Dose-response relationship of treatment with thiopental in combined oxygen and glucose deprivation for 3 h in human NT2-N neurons. Cell death was evaluated by measuring lactate dehydrogenase release to the supernatant in percent of oxygen/glucose-deprived controls 24 h after the start of the insult. * $P < 0.01$ versus oxygen/glucose-deprived controls.

dizocilpine strongly inhibited LDH release ($22 \pm 4.5\%$ of untreated oxygen- and glucose-deprived controls). Treatment with 2 μM ($93 \pm 8\%$) or 5 μM ($97 \pm 19\%$) of diazepam afforded no significant protection. Thiopental (fig. 5) and methohexital dose-dependently inhibited LDH release. Surprisingly, phenobarbital (10, 50, and 400 μM) and pentobarbital (10 and 50 μM) did not protect the neurons from combined oxygen and glucose deprivation; rather, they aggravated the damage (fig. 4). However, at a higher concentration (400 μM), pentobarbital offered significant protection. At both 50 and 400 μM , wells treated with pentobarbital and phenobarbital released significantly ($P < 0.01$) more LDH to the supernatant than those treated with methohexital and thiopental. There were no significant differences between methohexital and thiopental at any tested concentration. Significant correlations between neuroprotective effects and inhibition of 2,3-DHBA formation ($r^2 = 0.54$; $P = 0.04$), inhibition of MDA ($r^2 = 0.62$; $P = 0.007$), and inhibition of HNE ($r^2 = 0.76$; $P = 0.001$) were found.

To investigate whether dizocilpine and thiopental could have additive effects, the severity of the insult was increased, as both dizocilpine and thiopental almost fully protected the neurons in the 3-h model. The duration of the combined oxygen/glucose deprivation was extended to 5 h, and this caused release of $49 \pm 2\%$ LDH after 24 h. In this model, 10 μM dizocilpine was not protective, thiopental (200 and 400 μM) and 20 μM dizocilpine afforded moderate protection, whereas the combination of 10 μM dizocilpine and 200 μM thiopental caused a synergistic protective effect (fig. 6).

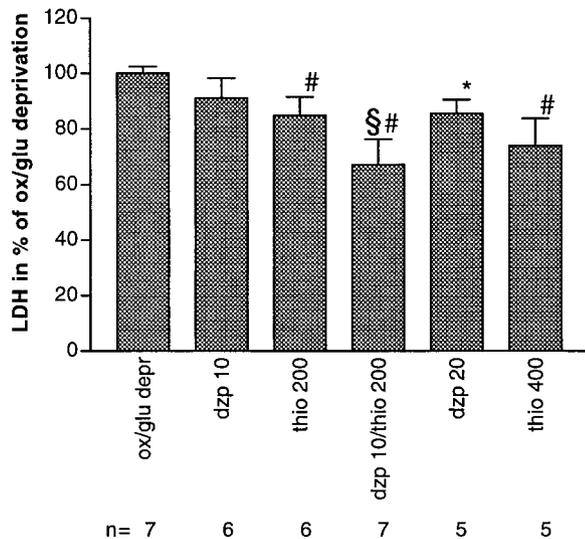


Fig. 6. Neuroprotective effects of treatment with thiopental and dizocilpine on combined oxygen and glucose deprivation for 5 h in human NT2-N neurons. Concentrations of the drugs are given in micromolars. Cell death was evaluated by measuring lactate dehydrogenase release to the supernatant in percent of oxygen/glucose-deprived controls 24 h after the start of the insult. dzp = dizocilpine; thio = thiopental. * $P < 0.01$ versus oxygen/glucose-deprived controls. # $P < 0.05$ versus oxygen/glucose-deprived controls. § $P < 0.01$ versus 20 μM dizocilpine.

Hypoxanthine. Hypoxanthine measured in supernatant immediately after 3 h of combined oxygen and glucose deprivation was significantly lower in wells treated with dizocilpine compared with untreated hypoxic controls (table 1). There were no significant differences between wells treated with dizocilpine, 400 μM thiopental, or 400 μM pentobarbital. The correlation between hypoxanthine immediately after hypoxia and LDH after 24 h was weak ($r^2 = 0.28$; $P = 0.034$; $n = 23$). Twenty-four hours after the start of hypoxia, hypoxanthine in the supernatant was significantly lower in wells

Table 1. Hypoxanthine after Oxygen and Glucose Deprivation

	Hypoxanthine (μM)	
	3 h	24 h
Oxygen/glucose deprivation (n = 5)	5.1 \pm 0.6	16.6 \pm 0.9
Dizocilpine (n = 6)	4.2 \pm 0.6*	6.1 \pm 1.2†
Thiopental 400 μM (n = 6)	4.8 \pm 0.3	8.5 \pm 0.9†
Pentobarbital 400 μM (n = 6)	5.1 \pm 0.5	13.9 \pm 1.6†

Hypoxanthine in supernatant immediately after combined oxygen and glucose deprivation (3 h) and 24 h after the start of the insult.

* $P < 0.05$ versus oxygen/glucose-deprived controls.

† $P < 0.01$ versus oxygen/glucose-deprived controls.

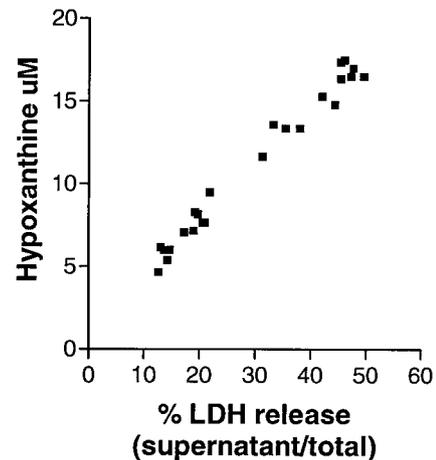


Fig. 7. Correlation between hypoxanthine in supernatant (micromolar) and percent lactate dehydrogenase release (supernatant/total) after 3 h of oxygen and glucose deprivation and 21 h reoxygenation. Untreated oxygen/glucose-deprived controls and dizocilpine-, thiopental-, and pentobarbital-treated wells are analyzed together. $r^2 = 0.98$; $P < 0.0001$; $n = 24$.

pretreated with dizocilpine, thiopental, and pentobarbital compared with untreated hypoxic controls. At this time, percent LDH release (supernatant/total) and hypoxanthine in supernatant correlated well ($r^2 = 0.98$; $P < 0.0001$; $n = 24$; fig. 7).

Discussion

The main finding of this study was the profound variation in neuroprotective effects between different barbiturates. Both the variation between the barbiturates and the fact that a major part of the protective effect of thiopental was obtained at low concentrations support the conclusion that reduction in cerebral metabolic rate is not the only mechanism of neuroprotective effects of barbiturates.

In addition to reduction in cerebral metabolic rate, alteration in blood flow distribution, enhancement of γ -aminobutyric acid (GABA)ergic inhibitory activity, suppression of sodium channels,²¹ modulation of glutamate receptors, and antioxidative effects have been suggested as possible mechanisms behind neuroprotection exerted by barbiturates. Our *in vitro* hypoxic model circumvents indirect effects such as alterations in cerebral blood flow. Because the treated and untreated cells were kept in the same normothermic incubator, hypothermia is also unlikely to be the cause of protection in this model.

Table 2. Therapeutic Concentrations

Diazepam ³⁹	> 600 ng/ml	> 2.1 μM
Methohexital ⁴⁰	3.5–11 $\mu\text{g/ml}$	12–39 μM
Pentobarbital ⁴¹	33–74 $\mu\text{g/ml}$	144–323 μM
Phenobarbital ³⁹	10–25 $\mu\text{g/ml}$	43–108 μM
Thiopental ³⁹	40 $\mu\text{g/ml}$	150 μM

Previously, thiopental has been shown to act as an antioxidant by inhibiting MDA formation *in vitro*.^{9,10} Smith *et al.* found that high concentrations of thiopental (1 mM), but not pentobarbital (1 mM), inhibited formation of iron/ascorbic acid stimulated formation of MDA (measured as thiobarbituric acid reactive substances) in rat brain homogenate.¹⁰ Pentobarbital anesthesia has been shown to reduce mitochondrial lipid peroxidation *in vivo*.²² However, this is not necessarily the result of a direct effect of pentobarbital on radicals *per se*, but may also be a result of indirect effects such as reduction in metabolic rate and reduction of influx of calcium. The effect of barbiturates on lipid peroxidation and free radicals *in vivo* may thus be a result of additional mechanisms than those suggested by *in vitro* experiments. In our experiments, we found direct effects of thiopental, pentobarbital, methohexital, and phenobarbital on hydroxyl radicals and on formation of both HNE and MDA *in vitro*. HNE is an aldehydic breakdown product of lipid hydroperoxide and is reported to mediate oxidative-stress-induced neuronal apoptosis²³ and impair glutamate transport in cortical astrocytes.²⁴

Although central nervous system levels of barbiturates during therapy are largely unknown, common therapeutic plasma concentrations are given in table 2. However, the clinical indications and use of these barbiturates differ markedly, and direct comparisons of these therapeutic concentrations are difficult. In this discussion, we have compared the barbiturates on a molar-to-molar basis, but it should be kept in mind that the relevant clinical concentrations in the microenvironment surrounding neurons *in vivo* are unknown.

Although the differences in antioxidant action between the barbiturates were moderate, we found thiopental and methohexital to be significantly better antioxidants than pentobarbital and phenobarbital with respect to inhibition of iron-stimulated MDA formation. This effect paralleled the better effect of thiopental and methohexital on neuronal survival than those of pentobarbital and phenobarbital. Furthermore, thiopental reduced formation of 2,3-DHBA significantly better than the other barbiturates, possibly because of its sulfhydryl group. The antioxidant profile of the barbiturates paral-

leled their neuroprotective abilities fairly well, and significant correlations were found between neuroprotective effects and inhibition of 2,3-DHBA formation, inhibition of MDA, and inhibition of HNE. However, even if 50 μM thiopental and methohexital had very modest effects on MDA and HNE, both exerted significant protection at this concentration. Furthermore, 50 μM methohexital also had minimal effect on hydroxyl radicals and was not superior to phenobarbital at any tested concentration despite its much better protective effects. Thus, the variation in neuroprotective effects could only be partly explained by differences in antioxidant action, and other mechanisms must also be sought.

Thiopental attenuates both NMDA- and AMPA-mediated glutamate excitotoxicity,²⁵ and an alternative explanation for the different neuronal protection afforded by the barbiturates is their effect against glutamate receptors, as barbiturates vary in potency against different glutamate receptors. Previously, blocking of AMPA receptors with CNQX has been shown to protect NT2-N cells from hypoxia.¹⁹ The ranking of barbiturates in descending order of potency against AMPA receptors (thiopental [IC_{50} 103 μM], methohexital [IC_{50} 172 μM], pentobarbital [IC_{50} 213 μM], and phenobarbital [IC_{50} 883 μM])²⁶ parallels the ranking of these barbiturates with respect to protective effect against combined oxygen and glucose deprivation in our study.

The profound effects of the NMDA-receptor antagonist dizocilpine in the 3-h combined oxygen- and glucose-deprivation model suggest that stimulation of NMDA receptors is important in this NT2-N model. Phenobarbital is reported to have minor effects on NMDA-mediated cytotoxicity on cultured cerebral cortex neurons.²⁷ Pentobarbital inhibits NMDA receptors with higher potency than phenobarbital.²⁸ As far as we know, no direct comparison on the NMDA-blocking effect of thiopental and pentobarbital has been reported. However, the IC_{50} of pentobarbital for depression of NMDA-evoked currents is 250 μM ,²⁹ whereas 200 μM thiopental suppresses the increase in intracellular calcium induced by NMDA,³⁰ and 100 μM thiopental completely abolishes NMDA-stimulated formation of cyclic guanosine monophosphate in rat cerebellar slices.³¹ Taken together, this suggests that thiopental blocks NMDA receptors more effectively than does pentobarbital. The better neuroprotective effect of thiopental than pentobarbital in our NT2-N model may therefore also be explained by higher affinity for NMDA receptors. In addition, improved neuronal survival at a high dose of pentobarbital is in accor-

dance with the effect of pentobarbital on NMDA receptors at high concentrations.

Although Kass *et al.* (anoxia)⁷ and we found protective effects of thiopental, Giffard *et al.*³² reported adverse effects of thiopental during combined glucose and oxygen deprivation. This may be a result of differences in distribution of receptors in the models and severity of the insults. Thiopental is more effective in depressing NMDA-induced increase in intracellular calcium in cortical slices than in hippocampal slices. The number of NMDA receptors in the NT2-N neurons is 1/10 of that found in rat brains, but approximately at the same level as those numbers found in fetal human neurons.^{17,33}

In the model with 5 h of combined oxygen and glucose deprivation, no significant protective effect was obtained with 10 μM dizocilpine. In contrast, 200 μM thiopental, which was less effective than 10 μM dizocilpine in the 3-h model, offered significant protection. The combination of 10 μM dizocilpine and 200 μM thiopental afforded synergistic protection, which was significantly greater than that obtained with a double dose of dizocilpine. This suggests that thiopental has neuroprotective effects other than or in addition to NMDA antagonism.

Treatment with phenobarbital and pentobarbital (10 and 50 μM) aggravated neuronal cell damage after combined oxygen and glucose deprivation. However, higher doses tended to preserve neuronal viability, suggesting dose-dependent counteracting mechanisms qualitatively or quantitatively specific to each barbiturate. Previously, thiopental has been demonstrated to enhance recovery of evoked population spike recordings from rat hippocampal slices subjected to anoxia.⁷ However, rather than preserving energy stores, this treatment enhanced the decrease of adenosine triphosphate levels. The investigators suggested that the effect of thiopental on adenosine triphosphate might counteract other beneficial effects. Similar results were obtained by Giffard *et al.*, who found that although both secobarbital and thiopental protected against NMDA, AMPA, and kainate excitotoxicity, they afforded little or no protection against anoxia and aggravated combined oxygen and glucose deprivation.³² During combined glucose and oxygen deprivation, reduction in adenosine triphosphate levels were more pronounced in samples treated with secobarbital compared with controls. Barbiturates may inhibit oxidative phosphorylation and decrease glycolysis through inhibition of hexokinase activity,³⁴ and this may account for the detrimental effects of pentobarbital and phenobarbital. Accumulation of hypoxanthine is an in-

indicator of hypoxia,³⁵ and it has been shown to react abruptly to changes in oxygenation status³⁶ and to be negatively correlated ($r = -0.87$) with energy charge.³⁷ We have previously found that hypoxanthine is elevated before LDH is released, proving that cell membrane disruption is not a prerequisite for increase in hypoxanthine.¹⁹ Immediately after oxygen and glucose deprivation in our study, hypoxanthine was not significantly higher in wells treated with pentobarbital compared with oxygen and glucose deprivation alone. This does not provide any support for such a speculation of detrimental effects of pentobarbital on energy charge.

Barbiturates enhance GABA_A-mediated neuronal inhibition, and this has been suggested as one possible mechanism behind its neuroprotective effects. The NT2-N neurons express functional human GABA_A receptors, and diazepam (EC₅₀ 74 nM), pentobarbital (EC₅₀ 41 μM), and phenobarbital (EC₅₀ 412 μM) enhance GABA-receptor currents evoked by application of GABA in NT2-N cells.³⁸ However, because we tested diazepam at concentrations well above those necessary to enhance GABA-receptor currents without any profound effect on neuronal survival, enhancement of GABA-receptor currents seems unlikely to account for the neuroprotective effects of barbiturates in this model.

In this model with combined oxygen and glucose deprivation in human NT2-N neurons, the tested barbiturates differed markedly in neuroprotective effects. The detrimental effect of low-dose pentobarbital compared with the protective effects at high concentrations also indicates counteracting effects depending on dose and type of barbiturate. The effect of relatively low doses of thiopental and methohexital and the pronounced differences between the barbiturates indicate that reduction in cerebral metabolic rate is not the only protective mechanism. Differences in inhibitory effect of free oxygen radical formation paralleled variation in neuroprotection fairly well, but the antioxidant action could only partly account for the observed differences in protective abilities. We speculate that at least a part of the variation in neuroprotection is the result of different effects of the barbiturates on glutamate receptors, but the synergistic effect of thiopental and dizocilpine in the 5-h combined glucose- and oxygen-deprivation model suggests that blocking of NMDA receptors is not the only protective mechanism either. Barbiturates have a number of different metabolic effects, some with opposite actions on neuronal survival. The relative importance of these effects depends on concentration and type of barbiturate. The net effect also depends on the character and severity

of the insult. In our experiments, thiopental and methohexital turned out to be more robust than pentobarbital and phenobarbital in protecting human NT2-N neurons from combined oxygen and glucose deprivation at the tested doses.

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