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Metabolic Impairment Elicits Brain Cell Type-Selective Changes in Oxidative Stress and Cell Death in Culture

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Abstract: Abnormalities in oxidative metabolism and inflammation accompany many neurodegenerative diseases. Thiamine deficiency (TD) is an animal model in which chronic oxidative stress and inflammation lead to selective neuronal death, whereas other cell types show an inflammatory response. Therefore, the current studies determined the response of different brain cell types to TD and/or inflammation in vitro and tested whether their responses reflect inherent properties of the cells. The cells that have been implicated in TD-induced neurotoxicity, including neurons, microglia, astrocytes, and brain endothelial cells, as well as neuroblastoma and BV-2 microglial cell lines, were cultured in either thiamine-depleted media or in normal culture media with amprolium, a thiamine transport inhibitor. The activity levels of a key mitochondrial enzyme, α-ketoglutarate dehydrogenase complex (KGDHC), were uniquely distributed among different cell types: The highest activity was in the endothelial cells, and the lowest was in primary microglia and neurons. The unique distribution of the activity did not account for the selective response to TD. TD slightly inhibited general cellular dehydrogenases in all cell types, whereas it significantly reduced the activity of KGDHC exclusively in primary neurons and neuroblastoma cells. Among the cell types tested, only in neurons did TD induce apoptosis and cause the accumulation of 4-hydroxy-2-nonenal, a lipid peroxidation product. On the other hand, chronic lipopolysaccharide-induced inflammation significantly inhibited cellular dehydrogenase and KGDHC activities in microglia and astrocytes but not in neurons or endothelial cells. The results demonstrate that the selective cell changes during TD in vivo reflect inherent properties of the different brain cell types. **Key Words:** α-Ketoglutarate dehydrogenase complex—Astrocytes—Cell culture—Endothelial cells—Inflammation—Microglia—Neurons—Oxidative stress—Thiamine deficiency.
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Inflammation, glial activation, abnormalities in oxidative metabolism, and selective cell death are common in many pathological conditions, including Alzheimer's disease (Blass, 1993; Beal, 1996; Griffin et al., 1998), Parkinson's disease (Schwab et al., 1996), and Wernicke-Korsakoff's syndrome (Victor et al., 1989). Thiamine deficiency (TD) is a classic animal model of selective vulnerability, in which chronic oxidative abnormalities and inflammation lead to selective neurodegeneration (McCandless, 1985; Victor et al., 1989). In thiamine-deficient rodents, neuronal death in select brain regions is associated with inflammation, as indicated by microglial activation, induction of endothelial nitric oxide synthase, altered blood–brain barrier, microglial accumulation of iron and ferritin, and increased levels of inducible nitric oxide synthase (Calingasan et al., 1998). Astrogliosis and edematous oligodendrocytes have been reported during TD (Watanabe and Kanabe, 1978; Zhang et al., 1995). During later stages of TD, metabolic impairment leads to increased levels of free radicals and 4-hydroxy-2-nonenal (HNE), a major product of lipid peroxidation in neurons, and inflammatory responses (e.g., increased amount of $NO^{\bullet}$) in activated microglia and astrocytes (Langlais et al., 1997; Calingasan et al., 1998, 1999).

Studying selective cellular responses and vulnerability to TD in vivo is complicated by extensive cross-talk among different cell types in brain. Thus, tissue culture models of TD were developed using various CNS cell lines and peripheral tissues (Schwartz et al., 1975; Bettendorff et al., 1995*a*,*b*; Pekovich et al., 1996; Romero et al., 1997) to test whether the distinctive response of each brain cell type to TD reflects an inherent property of the cells. For example, Bettendorff et al. (1995*a*,*b*) studied in vitro TD by incubating neuroblastoma cell lines with thiamine-depleted me-

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Abbreviations used: BSA, bovine serum albumin; cTD, complete thiamine deficiency; DMEM, Dulbecco's modified Eagle's medium; GFAP, glial fibrillary acidic protein; high amp-TD, high amprolium thiamine deficiency; HNE, 4-hydroxy-2-nonenal; KGDHC, α-ketoglutarate dehydrogenase complex; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; TD, thiamine deficiency.

dium containing amprolium, a cellular thiamine transport inhibitor. The results show that TD induces mitochondrial uncoupling and reduces oxygen consumption in neuroblastoma cell lines. TD also increases the monolayer permeability and alters metabolism in an endothelial cell line (Romero et al., 1997). Although previous studies provide important observations on a TD-induced pathophysiological cascade of events, none of the groups used primary cultures of the cells involved in TD-mediated neurotoxicity, nor did they examine the cellular effects of the inflammation that accompanies TD. The present study compares purified cultures of the various cell types that have been implicated in TD-mediated neurotoxicity in vivo, including primary cultures of neurons, microglia, astrocytes, and brain endothelial cells, as well as N2a neuroblastoma and BV-2 microglial cell lines. In vitro TD in the present work was achieved by culturing the cells either in culture medium devoid of thiamine in the presence of a low concentration of amprolium (Bettendorff et al., 1995*a*,*b*) or in regular culture medium containing a high concentration of amprolium. The inflammatory response that accompanies TD-mediated neurodegenerative processes in vivo was modeled by adding lipopolysaccharide (LPS) to cultured cells (Sekut et al., 1995; Hauss-Wegrzyniak et al., 1998). Inflammation caused by LPS includes glial activation and induction of various proinflammatory cytokines and reactive oxygen species (ROS), i.e., $NO^{\bullet}$ and superoxide (for reviews, see Mukaida et al., 1996; Mayeux, 1997).

TD also interferes with the biochemical regulation of oxidative energy metabolism. The activities of key Krebs cycle enzymes, especially the α-ketoglutarate dehydrogenase complex (KGDHC), are reduced in brains of TD animals (Sheu et al., 1998). KGDHC, a multicomponent mitochondrial enzyme complex, uses thiamine as a cofactor. KGDHC is one of the most vulnerable and sensitive mitochondrial enzymes to oxidative insults. ROS induced by mitochondrial dysfunction and/or $NO^{\bullet}$ production inhibits KGDHC activity in microglial cells (Park et al., 1999). KGDHC is more sensitive than various other cellular and mitochondrial enzymes tested to the lipid peroxidation product HNE (Humphries et al., 1998). KGDHC activity declines in numerous neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and Wernicke–Korsakoff's syndrome (Gibson et al., 1988; Butterworth et al., 1993; Mizuno et al., 1994; Sheu et al., 1994; Mastrogiacomo et al., 1996).

The aim of the present study was to elucidate the mechanism responsible for selective cellular changes in TD that produces selective neuronal loss and the relation of those alterations to diminished KGDHC activity. These experiments test whether the distinctive response of each brain cell type to TD reflects an inherent property of the cells.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University.

Cell culture

As described above, the goal of the experiments was to test the differential sensitivity or vulnerability of the individual brain cell types to TD. Thus, for each cell type we used what we regarded as a well-standardized preparation. This decision also dictated our choice of brain regions for each preparation. Culture of brain cells depends on developmental profiles of when a particular cell type can survive the transition to culture and when other cell types are less able to survive. For example, hippocampal neurons at embryonic day 15–16 are well established, and few glial cells are present. Therefore, the brain region and age of donor animals varied for each cell type.

Hippocampal neuronal culture. Cultures of hippocampal neurons from embryonic day 15–16 C57BL/6 mice (Harlan Sprague–Dawley Co., Indianapolis, IN, U.S.A.) were prepared by modification of published procedures (Brewer et al., 1993). Twelve hippocampi in 2 ml of modified Hanks' balanced salt solution [without Ca^{2+} and Mg^{2+} (GIBCO, Grand Island, NY, U.S.A.)] containing 0.035% sodium bicarbonate, 1 m*M* pyruvate, and 10 m*M* HEPES (pH 7.4) were disrupted mechanically by triturating with siliconized (Sigma Chemical Co., St. Louis, MO, U.S.A.) Pasteur pipettes that had three different bore openings (~1.0, 0.5, and 0.25 mm in diameter). The tissue was centrifuged at 200 *g* for 2 min, and the supernatant was discarded. The pellet was resuspended in Neurobasal/B27 medium [Neurobasal medium (GIBCO), B27 supplement (1×; GIBCO), 0.5 m*M* L-glutamine, 0.025 m*M* L-glutamate, and 0.5× antibiotic–antimycotic solution (GIBCO; catalogue no. 15245–012)] and further dissociated by trituration with siliconized Pasteur pipettes. The cells were either plated on poly-D-lysine (10 μg/ml; Sigma)-coated MatTek glass bottom microwells (diameter, 14 mm; MatTek, Ashland, MA, U.S.A.) or in 35-mm-diameter Petri dishes with 2 ml of Neurobasal/B27 medium. After 4 days, cells were replenished with new Neurobasal/B27 medium containing Neurobasal medium, 1× B27 supplement, and 0.5 m*M* L-glutamine. TD was induced after 7 days by incubating neurons in Neurobasal/B27 medium in the presence of 1 m*M* amprolium (Sigma) for 3 or 7 days. The neuron-specific nuclear protein (NeuN; Chemicon, Temecula, CA, U.S.A.) and neurofilament 200 (NF200; Sigma) immunocytochemical staining showed that the purity of primary neurons was >97%. Microglia [positive for MAC-1 (Vector Laboratory, Burlingame, CA, U.S.A.) and ferritin (DAKO, Carpinteria, CA, U.S.A.)] or astrocytes [positive for glial fibrillary acidic acid (GFAP); DAKO] constituted <2% of the cell population. The method for immunochemical staining was described previously (Calingasan et al., 1995). Furthermore, all the cells showed increases in cytosolic free calcium levels in response to KCl-mediated depolarization (data not shown).

Primary cultures of microglia and astrocytes. Cultures of microglia or astrocytes from forebrains of newborn (postnatal 1–2 days) C57BL/6 mice (Harlan Sprague–Dawley) were prepared according to previously published methods (Levison and McCarthy, 1991; Park et al., 1999). Primary microglial and astrocytic cultures were used without passaging to maximize consistency and to minimize the possibility of alterations in enzyme activity and oxidative damage. Ferritin and MAC-1 (microglia) and GFAP (astrocyte) immunocytochemical stain-

ing showed that the respective purity of microglia or astrocytes was >98%.

Brain endothelial cell culture. Cultures of microvascular cerebrovascular endothelial cultures from 6–8-week-old C57BL/6 mice (Harlan Sprague–Dawley) were prepared by modification of published procedures (Hsu et al., 1993; Beetsch et al., 1998). In brief, the forebrains of 12 mice were removed and placed into M199 isolation solution containing 15 m*M* HEPES (pH 7.4), sodium heparin (1 U/ml), 0.1 mg/ml L-glutamine, and 0.35 mg/ml sodium bicarbonate. After removal of the meninges and large vessels, the tissue was minced, homogenized, and centrifuged at 800 *g* for 15 min. The pellet was digested in 15 ml of M199 containing collagenase/dispase (1 mg/ml; Sigma) for 1 h at 37°C on a gyratory shaker. The resultant pellets were washed with fresh M199, centrifuged (800 *g*, 15 min), and resuspended in 30 ml of M199 containing 25% bovine serum albumin (BSA). The microvessels were separated from gray matter tissue by centrifugation (1,000 *g*, 20 min) and redigested in 10 ml of M199 containing collagenase/dispase for 2.5–3 h at 37°C. Cells were washed, centrifuged, and resuspended in 6 ml of M199 containing 1% BSA. The suspended cells were carefully layered over an established Percoll gradient. The Percoll gradient was made by centrifuging the Percoll mixture (1:1 mixture of Percoll and M199 containing 1% BSA) at 25,000 *g* for 1 h. The cell band (located at a density between 1.052 and 1.055 g/ml) was collected, centrifuged (1,000 *g*, 15 min), and resuspended in 12 ml of endothelial medium containing Dulbecco's modified Eagle's medium (DMEM; GIBCO), 10% fetal bovine serum, 10% horse serum (HyClone, Logan, UT, U.S.A.), 100 μg/ml endothelial cell growth supplement (Sigma), and sodium heparin (1 U/ml). The suspended cells were seeded onto 100-mm-diameter collagen [5 μg/cm^2 Vitrogen 100 (Collagen, Palo Alto, CA, U.S.A.)]-coated culture plates and cultured in 12 ml of endothelial medium. Cells at the second passage were used for all experiments. Von Willebrand factor (Sigma) immunocytochemical staining showed that the purity of cultured brain endothelial cells (at second passage) was >90%. Most of the contaminating cells were smooth muscle cells.

BV-2 microglial cell line and N2a neuroblastoma cells. The murine BV-2 cell line was developed by Dr. V. Bocchni (University of Perugia, Perugia, Italy) and was obtained from Dr. Kelvin W. Gee (University of California, Irvine, CA, U.S.A.) as a gift. N2a neuroblastoma cells were purchased from American Type Culture Collection (Manassas, VA, U.S.A.). Both of the cell lines were maintained at 37°C in a humidified incubator under 5% CO_2 and 95% air in DMEM (GIBCO) supplemented with 10% fetal bovine serum.

TD in the cells. Two slightly different approaches were required to achieve TD in culture. Both used amprolium, a competitive inhibitor of thiamine transport in cells (Bettendorff et al., 1995*a*). The goal of using amprolium was to induce a more complete TD (cTD) than is possible by just reducing the thiamine content in the media. Another common inhibitor that is used to induce thiamine deprivation in vivo is pyrithiamine. Pyrithiamine blocks the formation of thiamine diphosphate (Gubler, 1976). This has effects other than just diminishing thiamine availability (Pannunzio et al., 1999). Thus, amprolium has several advantages over pyrithiamine.

The first approach used thiamine-free DMEM (GIBCO) in combination with 20 μ*M* amprolium. In thiamine-depleted culture medium, the only source of thiamine is the fetal bovine serum. Bettendorff et al. (1995*a*) determined that the concentration of thiamine in thiamine-depleted media with 10% fetal bovine serum is ~12 n*M*, a value well below the K_m for the high-affinity thiamine transport. Most cultured cells take up thiamine by high- (K_m = 35 n*M*) and low-affinity (K_m > 1 m*M*) transporters. Thus, addition of 20 μ*M* amprolium to thiamine-depleted medium would make the cells severely thiamine-deficient (this approach is referred to as cTD). Induction of in vitro TD experiments with neurons required a second approach. Neuronal cultures require Neurobasal/B27 medium that contains 10 μ*M* thiamine, and thiamine-free Neurobasal/B27 medium was not available. Therefore, to achieve TD in Neurobasal/B27 medium, a higher concentration of amprolium (1 m*M*) was added. To compare the effect of TD on neurons and other cell types, 1 m*M* amprolium was also added to those cells cultured in regular DMEM. The latter approach is referred to as high amprolium-TD (high amp-TD). The aim of the study using high amp-TD was to test whether neurons were more sensitive to TD than other cell types.

Immunocytochemistry

The immunocytochemical staining of the cells was done according to previously published methods (Calingasan et al., 1995).

Detection and quantification of apoptosis

Detection of apoptotic cells in situ was done with the commercially available ApopTag kit (Oncor, Gaithersburg, MD, U.S.A.), which detects apoptotic cells using terminal deoxynucleotidyl transferase on DNA fragments. Apoptosis was quantified by counting ApopTag-stained cells on the grid of the microscope field (40× objective). The same cells were counterstained with methyl green. Methyl green stained normal nuclei a pale to medium green. The nuclei that contain DNA fragments or condensation were positively stained dark brown (by the ApopTag detection procedure) and were not stained with the methyl green. Because a positive terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay reaction can also occur in necrotic cells (Charriaut-Marlangue and Ben-Ari, 1995), chromatin condensation and apoptotic bodies were confirmed with high-magnification light microscopy. Five random fields per culture dish were counted with three dishes per culture, and counts were made with at least three separate cultures per treatment condition.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion

The conversion of the dye MTT (Sigma) to formazan crystals in cells has been shown to be related to total cellular dehydrogenase activity and mitochondrial redox state (Mosmann, 1983; Shearman et al., 1995). Cultures of various cell types (8,000–10,000 cells/0.28-cm^2 well) were plated in a 96-well culture plate for 3 days. The cells were then treated in various ways for varying time, as indicated in the legend to Fig. 1. Ten microliters of MTT (final concentration, 0.1 mg per well) was added to each well 4.5 h before the end of the treatment. At the end of the incubation, the media containing unreduced MTT were removed, and the reduced MTT formazan in the cells was solubilized in 100 μl of mineral oil for 24 h at 37°C. The optical density of formazan was measured in a microtiter plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The difference between the optical densities measured at 570 nm (for absorbance of MTT formazan) and 650 nm (the reference wavelength) was calculated.

KGDHC activities

The cells in a 35-mm-diameter Petri dish were washed and harvested in scraping buffer [10 m*M* NaH_2PO_4 (pH 7.2), 150

m*M* NaCl, and 1 m*M* EGTA], and these harvested cells were centrifuged at 300 *g* for 5 min. The cells in the pellet were solubilized with lysate buffer containing 50 m*M* Tris-HCl (pH 7.2), 1 m*M* dithiothreitol, 50 μM leupeptin, 0.2 m*M* EGTA, and 0.4% Triton X-100. Samples were assayed for KGDHC activities immediately as described previously (Gibson et al., 1988). The apparent activity of KGDHC was calculated after correction for NADH oxidase, which oxidizes the product of KGDHC activity, β-NADH. For the protein content, cells were solubilized with the lysate buffer and digested in 1 *M* NaOH at 37°C for 1 h. Protein concentration was determined with Coomassie Brilliant Blue G-250 dye (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

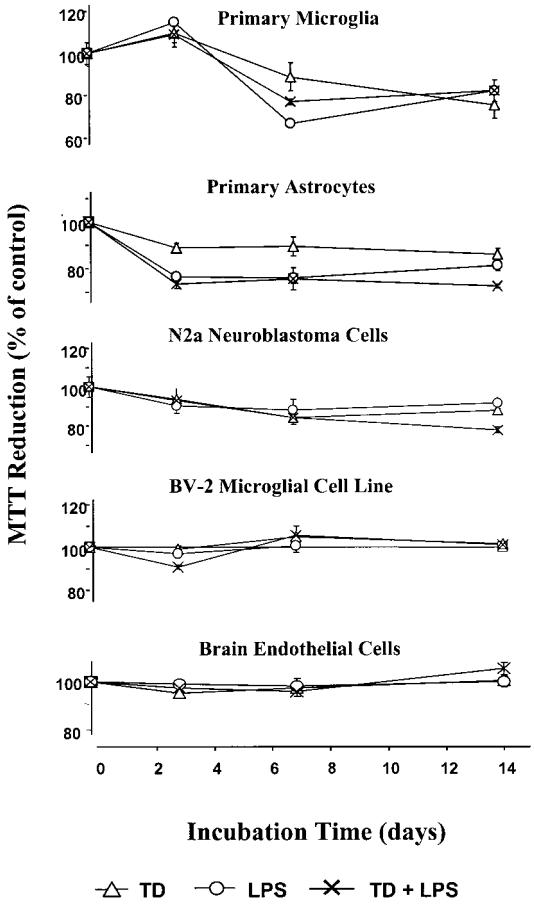
RESULTS

Cellular dehydrogenase activity by MTT measurement

Reduction of MTT was used as a general measure of cellular dehydrogenase activity (Shearman et al., 1995) to determine whether cTD and/or LPS influence cellular energy metabolism differently in selected cell types. cTD and/or LPS had different effects on MTT reduction depending on the cell types (Fig. 1). To clarify the data comparison in Fig. 1, the statistical analysis of the data is presented in the legend to Fig. 1. The largest effect of cTD was observed in primary glial cells, and the effect was biphasic in primary microglia. During the first 3 days, cTD increased MTT conversion by ~10% and then gradually reduced the conversion. By 14 days of treatment, cTD decreased MTT conversion to 75% of the basal levels. cTD slightly reduced MTT reduction in neuroblastoma cells (−15%). cTD did not exert any effect on primary astrocytes or brain endothelial cells. It is surprising that LPS (0.1 μg/ml) alone altered MTT reduction in primary microglia (15%) and astrocytes (−24%) as early as day 3 of treatment, and it maximally reduced cellular dehydrogenase activity in primary microglia by ~35% and primary astrocytes by ~25% after 7 days of treatment (Fig. 1). LPS did not affect the MTT conversion by BV-2 or neuroblastoma cell lines. The combination of cTD and LPS only had an additive effect in astrocytes. cTD and LPS had a slight synergistic effect on MTT conversion in astrocytes at day 14 of treatment, at which time they decreased the conversion to ~72% of the basal levels.

KGDHC inhibition in different cell types by cTD/LPS

In vivo TD reduces the activity of KGDHC, a key Krebs cycle enzyme (Sheu et al., 1998). To test whether the inhibition of the activities is ubiquitous to any CNS cell types, the effects of cTD and/or LPS on KGDHC activity of various cultured cells from the brain were determined (Fig. 2). The inhibitory effects of cTD and/or LPS on KGDHC activities varied between cell types. The neuronal-type, neuroblastoma cells were the most vulnerable to TD. TD inhibited KGDHC activity of neuroblastoma cells by 40% after 3 days and by 70% after 14 days of treatment. The KGDHC in BV-2 cells was also inhibited ~30% by cTD as early as 3 days of



MTT conversion

Cell types	Days of treatment	Control	TD	LPS	LPS + TD
Microglia	3	a	b;1	b;1	b;1
	7	a	a;1,2	b;1	b;1
	14	a	b;1	b;1	b;1
Astrocytes	3	a	b;1	c;2	c;2
	7	a	b;1,2	c;2	c;1
	14	a	b;2	b;1	c;2
N2a neuroblastoma	3	a	a;1	a;3	a;3
	7	a	b;1	b;3	b;1
	14	a	b;2,3	a,b;1,2	c;1,2
BV-2	3	a	a;1	a;4	b;3
	7	a	a;2	a;4	a;2
	14	a	a;4	a;2	a;3
Endothelial cells	3	a	a;1	a;4	a;3
	7	a	a;1,2	a;4	a;3
	14	a	a;4	a;2	a;3

FIG. 1. Effects of TD and/or LPS on total cellular dehydrogenase activities of various cultured cells. Cultures of microglia, astrocytes, the BV-2 microglial cell line, brain endothelial cells, and N2a neuroblastoma cells were incubated in either regular DMEM or cTD medium in the presence or absence of LPS (0.1 μg/ml) for 3, 7, or 14 days, and their ability to reduce MTT was measured. Data are mean ± SEM (bars) percentages of controls of at least four independent experiments (at least quadruplicate per group) on two separate days. Statistical significance was tested by one-way ANOVA with post hoc Student–Newman–Keuls comparison. Different letters within the same day (i.e., a, b, and/or c in the same row) represent a statistically significant difference ($p < 0.05$) among different treatments of the same cell type. Different numbers within the same day, i.e., 1, 2, and/or 3 in the same column, represent a statistically significant difference ($p < 0.05$) among different cell types within the same treatment group.

treatment but was not reduced any further. cTD began to reduce KGDHC activity of brain endothelial cells at 7 days of treatment and significantly inhibited the activity after 14 days by ~35%. However, KGDHC of primary glial cells was more resistant to cTD than other cell types, such that a slight (~24% in astrocytes and ~10% in microglia) reduction was observed after 14 days of the treatment.

LPS reduced KGDHC activity in glial cells as early as 3 days of treatment. LPS inhibited KGDHC in primary microglia at 7 days and maximally inhibited the activity by 35% at 14 days. Unlike in microglia, the greatest inhibition of LPS-mediated KGDHC activities in BV-2 microglial cells (17%) and astrocytes (24%) by LPS occurred within 3 days, and the inhibition was maintained for up to 14 days of treatment. LPS had little or no inhibitory effect on KGDHC activity in neuroblastoma or endothelial cells at any time.

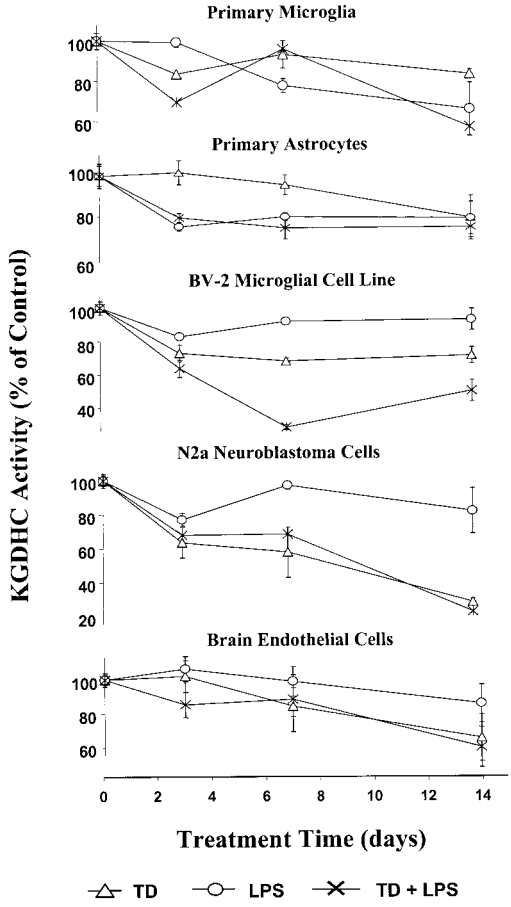
To test whether cTD and LPS have a synergistic effect on KGDHC activity, cells were treated with cTD and LPS for up to 14 days. Synergistic effects were only apparent in BV-2 cells. The additive effects of cTD and LPS on BV-2 cells started to appear at 7 days of treatment and inhibited the KGDHC activity by 70%.

Different levels of KGDHC activities among CNS cell types

To test whether the levels of KGDHC activities are uniquely distributed among CNS cell types, the absolute values of the KGDHC activities among different brain cell types were quantified and compared. KGDHC activities varied considerably among the different cell types (Fig. 3). KGDHC activities in primary astrocytes, the BV-2 microglial cell line, and N2a neuroblastoma cells ranged from 9 to 12 mU/mg of protein. KGDHC activities of primary neurons and microglia were the lowest among the cell types tested, about one-third of the value in the former cells (~3 mU/mg of protein). Endothelial cells showed the highest level of activity, about two times more than any other cell type (~20 mU/mg of protein).

Apoptotic death in neurons

To test whether the selective neuronal death that occurs in vivo also occurs in vitro, the effects of TD on nuclear condensation and fragmentation of the cells were determined. Exposure of neurons to high amp-TD increased the number of cells exhibiting nuclear condensation and fragmentation (Fig. 4). The change occurred as early as day 3 and increased even more by day 7 (Fig. 5). A time course analysis showed that 40% of neurons exhibited apoptosis during a 7-day exposure to high amp-TD compared with those exposed to vehicle (control; 5–10% apoptosis). In contrast, no microglia, astrocytes, endothelial cells, or N2a neuroblastoma cells showed nuclear signs of DNA fragmentation. In addition, no change in the total number of attached neurons, microglia, astrocytes, or endothelial cells occurred during exposure to vehicle or high amp-TD. Routine examination of the cells did not reveal morphological



KGDHC activities

	Days of treatment	Control	TD	LPS	LPS + TD
Microglia	3	a	a,b;1,2	a,b;1	b;1
	7	a	a;1	a;1	a;1
	14	a	a,b;1	b;1	b;1
Astrocytes	3	a	a;1	b;2	b;1
	7	a	a;1	a;1	a;1
	14	a	b;1	b;1	b;1
BV-2	3	a	b;2	c;2	b;1
	7	a	b;1,2	c;2	d;2
	14	a	b;1	a;1	c;1
N2a neuroblastoma	3	a	a;2	a;2	a;1
	7	a	b;2	a;2	b;1
	14	a	b;2	c;1	b;2
Endothelial cells	3	a	a;1	a;1	a;1
	7	a	a;1,2	a;2	a;1
	14	a	a,b;1	a;1	b;1

FIG. 2. Time-dependent effects of TD and/or LPS on KGDHC activities of cultured cells. Cultures of microglia, astrocytes, the BV-2 microglial cell line, brain endothelial cells, and N2a neuroblastoma cells were incubated in either regular DMEM or cTD medium in the presence or absence of LPS (0.1 μg/ml) for 3, 7, and 14 days, and their KGDHC activities were measured. Data are mean ± SEM (bars) percentages of controls of at least six independent measurements, i.e., duplicate assays, on at least three separate days. Statistical significance was tested by one-way ANOVA with post hoc Student–Newman–Keuls comparison. Different letters within the same day, i.e., a, b, and/or c in the same row, represent a statistically significant difference ($p < 0.05$) among different treatments of the same cell type. Different numbers within the same day, i.e., 1, 2, and/or 3 in the same column, represent a statistically significant difference ($p < 0.05$) among different cell types within the same treatment group.

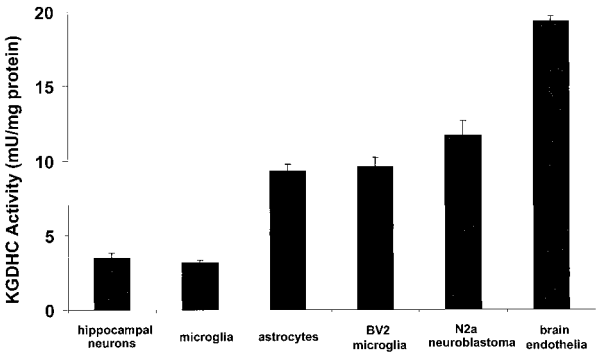


FIG. 3. Levels of KGDHC activities of cultured brain cells. Cultures of primary hippocampal neurons, microglia, astrocytes, the BV-2 microglial cell line, N2a neuroblastoma cells, and brain endothelial cells were established, and their KGDHC activities (in mU/mg of protein) were measured. Data are mean ± SEM (bars) values of at least six independent measurements (i.e., duplicate assays) on at least three separate days.

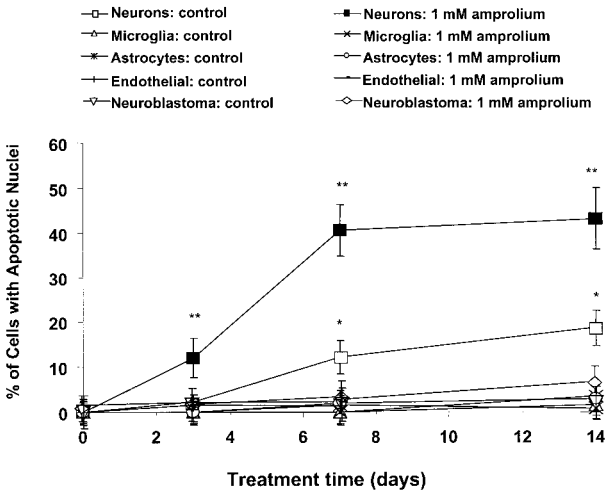


FIG. 5. Effect of TD on cell viability of neurons, microglia, astrocytes, and brain endothelial cells. Cultures of hippocampal neurons, microglia, astrocytes, and brain endothelial and N2a neuroblastoma cells were maintained in regular culture medium or high amp-TD. The cells were stained for apoptosis using ApopTag, and the percentage of cells with apoptotic nuclei was determined (an average of 100 cells per field for five fields) with three dishes per culture. $^{*}p < 0.05$, neurons without amprolium differ from all other cell types without amprolium. $^{**}p < 0.01$, neurons with amprolium differ from all other cell types with amprolium and from neurons without amprolium. Data are mean ± SEM (bars) values of at least six independent measurements, i.e., duplicates, on three separate cultures.

indicators of cell death, e.g., cell swelling. Moreover, the methyl green counterstaining indicated that the cells that did not show condensed DNA were still alive (data not shown).

To test whether amprolium is indeed a competitive inhibitor of thiamine transport in cellular system, thiamine (500 μM) was added to neurons that were treated with high amp-TD. Most of neurons were protected from apoptotic cell death (data not shown).

High amp-TD inhibits neuronal KGDHC activity

To determine whether TD-mediated apoptotic cell death is linked to mitochondrial dysfunction, KGDHC activities of cells that have varying degree of vulnerability to high amp-TD were measured (Fig. 6). KGDHC activities of primary astrocytes and N2a neuroblastoma were unaffected by high amp-TD. The enzyme activities of astrocytes or N2a cells were unchanged even up to 7 days of high amp-TD treatment (data not shown). On the other hand, KGDHC activity in neurons was significantly reduced by 50% even as early as day 3 of the treatment. It is interesting that the levels of KGDHC activity in neurons were similar to those in primary microglia, which were about one-third the activities of the other two cell types.

High amp-TD induces HNE immunostaining in neurons

Immunocytochemical analysis showed that high amp-TD increased HNE immunoreactivity in hippocampal

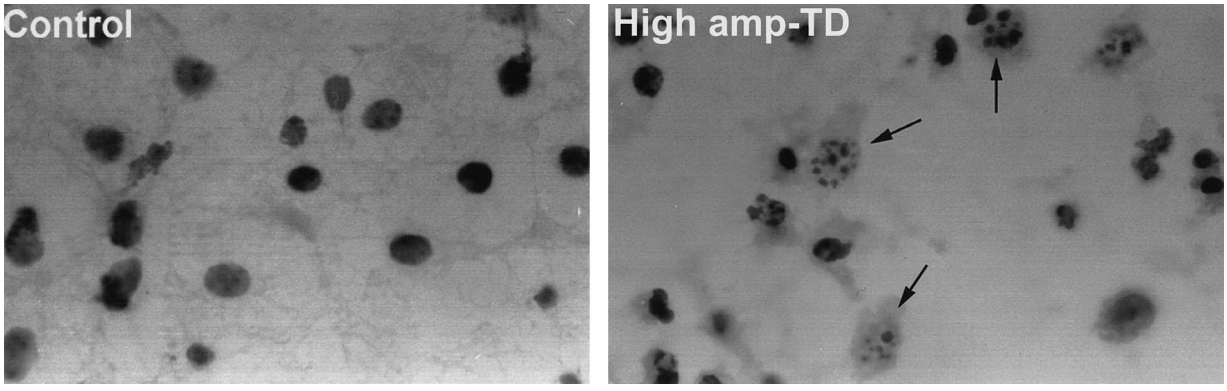


FIG. 4. TD causes apoptosis in hippocampal neurons. Hippocampal neurons were treated with vehicle (Control) or 1 m*M* amprolium (High amp-TD) for 7 days. Apoptotic cells were detected in situ using ApopTag. DNA fragments in many amprolium-treated neurons were stained with ApopTag, which appeared to be condensed and fragmented, e.g., arrows. Cells are shown here in a 100× objective with oil immersion. Similar results were seen in duplicates of the samples from three independent cultures. Quantification of the results is presented in Fig. 5.

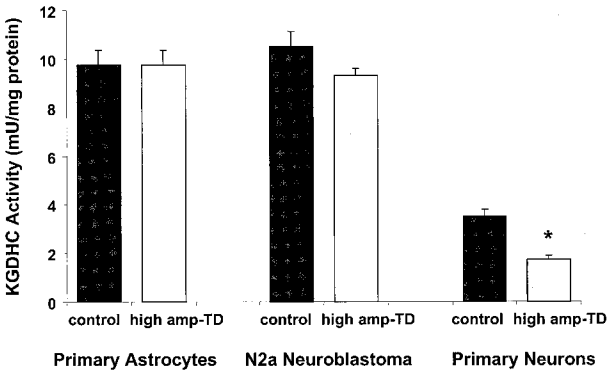


FIG. 6. High amp-TD selectively reduces KGDHC activity of primary neurons. Astrocytes, N2a neuroblastoma cells, and hippocampal neurons were incubated in regular DMEM or high amp-TD for 3 days, and their KGDHC activities were measured. $^{*}p < 0.01$, significantly different from control by Student's *t* test. Data are mean ± SEM (bars) values of six to 10 experiments.

neurons. HNE immunoreactivity was increased in essentially all neurons as early as 3 days of posttreatment [Fig. 7, High amp-TD (3d)], and the intensity of HNE immunoreactivity in cells exposed to high amp-TD was even greater at 7 days [Fig. 7, High amp-TD (7d)]. The increase of HNE immunoreactivity appeared to be concentrated in perinuclear regions and at the cell periphery. In contrast, levels of HNE immunoreactivity in microglial cells, astrocytes, and endothelial cells exposed to high amp-TD were not affected (data not shown).

To test whether elevated levels of the oxidative markers ferritin and heme oxygenase-1 in microglia in the brain of TD mice (Calingasan et al., 1998, 1999) are the direct results of increased iron levels from blood–brain barrier infiltration or TD-induced cellular metabolic and oxidative impairment, immunocytochemical staining of ferritin and heme oxygenase-1 was done on TD and/or LPS-treated microglia and astrocytes in culture. Neither ferritin nor heme oxygenase-1 immunostaining was increased in any cell type (data not shown).

DISCUSSION

The present in vitro study demonstrates that the selective cell changes that accompany metabolic impairment and chronic inflammation in vivo reflect inherent properties of individual brain cell types. As in vivo, TD killed neurons but not nonneuronal cells (e.g., microglia, astrocytes, or endothelial cells) in culture. TD impaired neuronal energy metabolism as shown by a marked reduction in KGDHC activity and diminished cellular dehydrogenase activities, which contributed to an increase in oxidative stress and apoptotic DNA fragmentation. On the other hand, the chronic inflammation, mimicked by LPS, affected the energy metabolism of microglia and astrocytes but not neurons. This finding implies that neurotoxicity during inflammation may be, in part, related to altered glial energy metabolism.

The present study used cultured cells to determine the inherent vulnerability of various brain cells to TD (Gib-

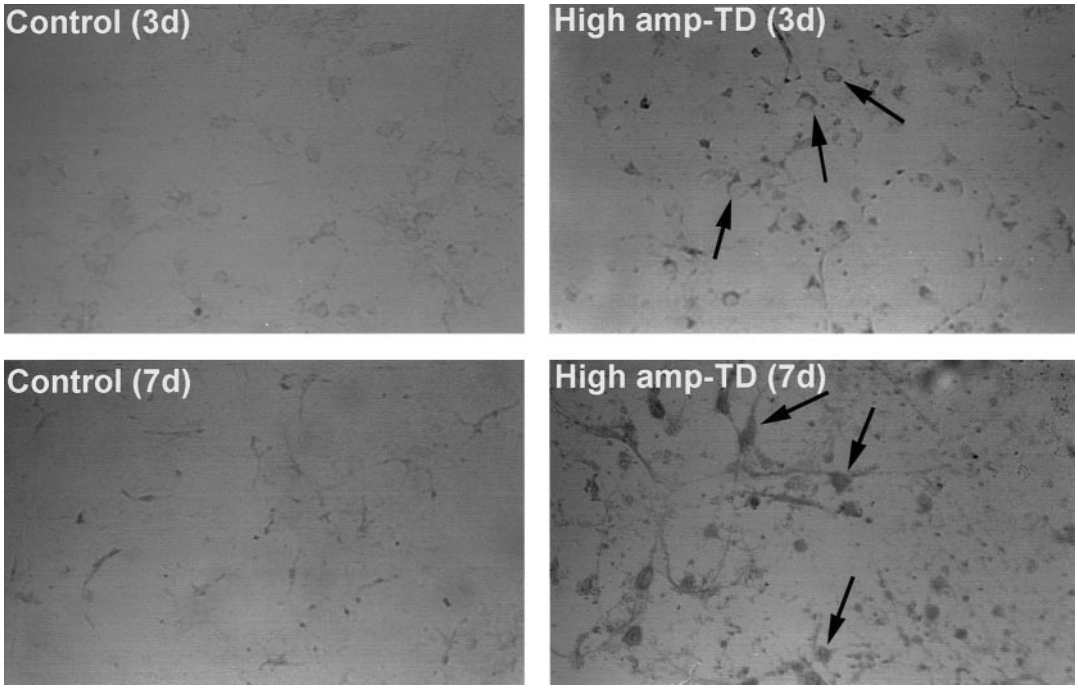


FIG. 7. TD increases levels of HNE–protein conjugates in cultured neurons. Cultures of hippocampal neurons were treated with vehicle (Control; 3 or 7 days) or 1 m*M* amprolium (high amp-TD; 3 or 7 days). Then, cells were fixed and immunostained with anti-HNE antibody. Highly stained neurons with HNE antibodies are clearly observed in high amp-TD groups (arrows). Similar results were seen in duplicates of the samples from three independent cultures.

son et al., 1989; Butterworth, 1993). Thiamine in the form of thiamine pyrophosphate is a cofactor for transketolase, a key enzyme of the pentose shunt, and the tricarboxylic cycle enzymes, pyruvate dehydrogenase complex and KGDHC. In the present study, high amp-TD significantly inhibited KGDHC in neurons, whereas activities in other cell types were largely unaffected. It is interesting that cTD markedly inhibited KGDHC in neuroblastoma cells, although high amp-TD did not. The results are consistent with the suggestion that the neurons are the most sensitive cell type and that high amp-TD is a milder treatment than cTD. This conclusion is supported by the observation that measures of metabolism in glial cells such as astrocytes were less affected by high amp-TD than those in neurons and were only marginally affected by cTD. Although no TD-induced cell death occurs in N2a neuroblastoma cells, TD in neuroblastoma cells causes mitochondrial swelling, disorganization of the mitochondrial cristae (Bettendorff et al., 1995*b*), and reduced KGDHC activity (present study). These findings suggest that primary neurons and neuron-like cells are the most sensitive to TD and inherently prone to metabolic damage. One possible mechanism that could underlie the selective vulnerability is a different ability of the cells to retain thiamine even in media containing a low level of thiamine. Although various cells have similar K_m values for the thiamine transporter (Bettendorff and Wins, 1994; Stagg et al., 1999), they are unknown for the cell types that we have tested.

TD increased HNE accumulation, a major indication of oxidative stress in neurons. HNE, an α,β-unsaturated aldehyde produced by lipid peroxidation, is a prominent cytotoxic aldehyde. HNE conjugates to lysine, cysteine, and histidine residues of the proteins via Michael addition (Uchida et al., 1994). The present result agrees with our previous in vivo study that showed a marked increase of neuronal HNE and selective neuronal death during late stages of TD (Calingasan et al., 1999). Oxidative stress, rather than energy depletion, likely underlies many effects of TD (Langlais et al., 1997), because little or no reduction of ATP content occurred during TD in vivo (Aikawa et al., 1984; McCandless, 1985). Reduced glutathione levels do accompany in vivo TD (McCandless and Schenker, 1968; Parkhomenko et al., 1990), and the reduced antioxidant capacity may exacerbate oxidative stress. If oxidative stress is the major factor that leads to cell death during TD, glial cells would be more protected from damage because glial cells possess a greater antioxidant capacity than neurons (Sagara et al., 1993). For example, the glutathione concentration in astrocytes is double that of neurons (Makar et al., 1994; Bolaños et al., 1995). As shown in the present study, oxidative stress or cell death was not observed in microglia or astrocytes.

HNE inhibits mitochondrial function in synaptosomes (Keller et al., 1997) and directly affects KGDHC. The treatment of rat heart mitochondria with HNE selectively inhibited KGDHC and pyruvate dehydrogenase complex, whereas other NADH-linked dehydrogenases and electron chain complexes were unaffected (Humphries and Szweda, 1998). Furthermore, in our preliminary study, HNE inhibited isolated KGDHC activity in a dose-dependent manner and completely abolished the activity at 3 m*M* (data not shown). A prime target of HNE is reduced lipoic acid, a strong nucleophile at physiological pH (Esterbauer et al., 1991), which scavenges oxygen radicals (Packer et al., 1997) and is located on the surface of the subunit of KGDHC and pyruvate dehydrogenase complex (Perham, 1991). KGDHC may function as an antioxidant under certain conditions because of its ability to cycle lipoic acid between its oxidized and reduced states (Bakker et al., 1997). Thus, the reduction of KGDHC activity may stimulate the production of ROS, leading to additional increases of HNE in TD-treated neurons. Therefore, the increased HNE could potentiate the TD-mediated reduction of KGDHC activities, and the resulting enzyme inhibition would further increase HNE accumulation. Such a spiral of deteriorating energy metabolism and increased oxidative stress would promote neurodegeneration.

Despite a crucial role as a general housekeeping enzyme in energy metabolism and oxidation, KGDHC activities vary among neuronal and nonneuronal brain cells. KGDHC activity was highest in endothelial cells and lowest in microglial and neuronal cells. Varying levels of KGDHC activities do not appear to define the selective vulnerability of cells to metabolic and inflammatory insults. Our data showed that a lower intrinsic activity of KGDHC activity did not necessarily correlate with the cellular response. For example, TD did not produce oxidative stress or signs of cell death in microglial cells, which have the lowest intrinsic level of KGDHC among various brain cell types. Our previous in vivo data also show that intrinsic levels of KGDHC and their response to TD were not different in vulnerable or nonvulnerable regions to TD (Gibson et al., 1989; Sheu et al., 1998). One possible explanation for different activities of KGDHC in different cell types is that each cell type may contain different numbers of mitochondria. For instance, endothelial cells, especially the CNS capillary endothelial cells, have a high mitochondrial content [~10% of the cytoplasmic volume (Oldendorf et al., 1977)]. Our data showing that the brain capillary endothelial cells contain the highest KGDHC activities among various brain cell types tested support that suggestion.

TD induced apoptotic processes in neurons. High amp-TD induced apoptotic markers in neurons within 3 days of treatment, whereas other brain cell types were not affected. Although there is no direct evidence that HNE accumulation initiated apoptosis in neurons, HNE directly induces apoptosis in primary rat hippocampal neurons and PC12 cells (Kruman et al., 1997). The present in vitro finding supports the in vivo TD study in which selective neuronal death was partly apoptotic in the thalamus, a vulnerable region of TD brains (Matsushima et al., 1997). Following in vivo TD, immediate-early gene expression, which mediates programmed cell

death, also occurs in vulnerable regions (Hazell et al., 1998). Apoptosis is believed to occur in several prominent neurodegenerative conditions associated with mitochondrial dysfunction, including Alzheimer's disease (Cotman and Anderson, 1995) and cerebral ischemia (Linnik et al., 1993).

The selective vulnerability of neurons to TD is an inherent property of neurons that may be modified by glial cells in vivo. The current findings that chronic TD induced oxidative stress and the DNA fragmentation in neurons as early as the third day of exposure suggest that neurons are particularly sensitive to abnormalities in oxidation. Microglia are able to maintain their cell integrity even when their energy metabolism is severely impaired (Park et al., 1999). Neuronal survival depends on neuronal–glial interactions, and this is likely important in our understanding of TD. For example, long-term survival of cultured neurons is improved when they are cocultured with glial cells (Banker, 1980), and cultured hippocampal neurons are less vulnerable to excitotoxic insults if they are in the immediate vicinity of astrocytes (Mattson and Rychlik, 1990; Blanc et al., 1998). In addition, the present finding of spontaneous apoptotic process in control neurons indicates that neurons in the absence of glial cells may be more vulnerable to various insults, including metabolic impairment. Glial cells express and secrete various neurotrophic factors and cytokines that have been shown to stimulate neuronal survival and protect neurons against excitotoxic, metabolic, and oxidative insults (Mattson et al., 1997). Glial cells also provide neurons with energy substrates, thus maintaining metabolic homeostasis in the brain (Tsacopoulos and Magistretti, 1996).

One finding that differs between the present in vitro and previous in vivo studies is that signs of oxidative stress occur in nonneuronal cells during in vivo TD. Reactive iron, ferritin, and heme oxygenase-1 accumulate in microglia during TD in vivo (Calingasan et al., 1998, 1999). However, the increase of these oxidative markers in glial cells during in vivo TD is likely due to the direct glial response to increased iron, which enters through an abnormal blood–brain barrier during late stages of TD. The present study indicated that neither ferritin nor heme oxygenase-1 immunostaining was increased in any culture cells during in vitro TD, and no additional iron was available with which the cells could react. Alternatively, the increase of heme oxygenase-1 or ferritin activity in TD in vivo may occur as a consequence of altered cell–cell interaction/signals or a secondary response to alterations in the blood–brain barrier during TD-mediated metabolic impairment. Thus, future studies must elucidate how metabolically challenged glial cells alter neuronal homeostasis and neuronal–glial interactions and how compromised neurons affect glial function and oxidation during TD.

Chronic LPS treatment generally inhibited cellular dehydrogenase and KGDHC activities of glial cells but not neurons. One would expect glial cells to increase their energy metabolism when they are exposed to LPS because LPS activates microglia and astrocytes. However, except primary microglia, in which a transient, partial increase of cellular dehydrogenase activity was observed during the first 3 days of treatment, LPS generally caused a time-dependent inhibition of KGDHC and general cellular dehydrogenase activity in glial cells (Figs. 1 and 2). Our previous study showed that LPS not only reduced KGDHC activity, but also inhibited a general measure of cellular dehydrogenase activity in microglial cells (Park et al., 1999). Whether inhibition of KGDHC and cellular dehydrogenase affects LPS-induced activation of certain signal pathways or influences homeostasis of the glial cells is unknown. Nevertheless, the present study is consistent with our previous study, which demonstrated that exposure of glial cells to LPS can lead to oxidative stress and potentiate metabolic impairment, without producing cell death (Park et al., 1999). Although LPS can activate glial cells by inducing nitric oxide synthase expression and proinflammatory cytokines, the consequent altered energy metabolism in microglia can further diminish their physiological function and have profound effects on their cell–cell interactions.

Conclusion

The current study shows that neurons are the cell type most vulnerable to TD-induced metabolic insults. In neurons, TD induced a marked reduction in a key mitochondrial enzyme, HNE accumulation, and apoptotic cell death. Thus, just as in vivo, TD induces a cell type-specific response in vitro: Neurons are under oxidative stress and die, but other brain cells, e.g., astrocytes, are not affected. Further studies are required to address whether the signals from the dying neurons or signals from other cell types during TD alter the inherent properties of the different CNS cells. In addition, the present study uses brain cells that are cultured from different regions of the brain and from the animals at different ages. Different vulnerability and responses of the brain cell types from same regions, e.g., thalamus, and same age of the animals to TD may occur. Nevertheless, characterization of the response of various cell types to in vitro TD will help elucidate the impact of oxidative and inflammatory challenges on glia, endothelial cells, and neurons and their role in the pathogenesis of selective neurodegeneration.

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