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Thiamine Deficiency Results in Metabolic Acidosis and Energy Failure in Cerebellar Granule Cells: An In Vitro Model for the Study of Cell Death Mechanisms in Wernicke's Encephalopathy

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Thiamine deficiency (TD) in both humans and experimental animals results in severe compromise of mitochondrial function and leads to selective neuronal cell death in diencephalic and cerebellar structures. To examine further the influence of TD on neuronal survival in relation to metabolic changes, primary cultures of rat cerebellar granule cells were exposed to thiamine-deficient medium for up to 7 days in the absence or presence of the central thiamine antagonist pyrithiamine (Py). Exposure of cells for 7 days to thiamine-deficient medium alone resulted in no detectable cell death. On the other hand, 50 μM Py treatment led to reductions of thiamine phosphate esters, decreased activities of the thiamine-dependent enzymes α-ketoglutarate dehydrogenase and transketolase, a twofold increase in lactate release ($P < 0.001$), a lowering of pH, and significant (58%, $P < 0.001$) cell death. DNA fragmentation studies did not reveal evidence of apoptotic cell death. Addition of 50 μM α-tocopherol (vitamin E) or 100 μM of butylated hydroxyanisole (BHA) to Py-treated cells resulted in significant neuroprotection. On the other hand, addition of 10 μM MK-801, an NMDA receptor antagonist, was not neuroprotective. These results suggest that reactive oxygen species (ROS) play a major role in thiamine deficiency-induced neuronal cell death. Insofar as this experimental model recapitulates the metabolic and mitochondrial changes characteristic of thiamine deficiency in the intact animal, it might be useful in the elucidation of mechanisms involved in the neuronal cell death cascade resulting from thiamine deficiency. J. Neurosci. Res. 62: 286–292, 2000.

Key words: cerebellar granule cells; pyrithiamine; thiamine deficiency; cell culture; energy metabolism; cell viability

Thiamine deficiency (TD) in humans leads to Wernicke's encephalopathy (WE), a neuropsychiatric disorder commonly encountered in chronic alcoholism and in patients with impaired nutrition associated with gastrointestinal disease or AIDS (Victor et al., 1989; Butterworth et al., 1991). Histopathologic evaluation of brain tissue from WE patients reveals neuronal loss, gliosis, and vascular damage in focal regions of the brain that include the mammillary bodies, inferior olive, thalamus, and other periventricular regions (Kril, 1996; Troncoso et al., 1981). Cerebellar degeneration associated with a loss of Purkinje cells as well as a shrinkage of the cerebellar cortex and the molecular and granule cell layers have also been reported in TD (Collins and Converse, 1970; Phillips et al., 1987, 1990). In addition, positron emission tomography (PET) studies reveal reduced cerebellar glucose utilization (Gilman et al., 1990), which may be a consequence of severe compromise of energy metabolism in thiamine deficiency (Aikawa et al., 1984).

The use of pyrithiamine (Py) to induce experimental TD in the rat provides a reliable model that reproduces both the metabolic and the pathologic characteristics of WE (Héroux and Butterworth, 1992). Py is a potent inhibitor of thiamine pyrophosphokinase (Rindi and Perry, 1961; Johnson and Gubler, 1965) and thus inhibits the synthesis of thiamine diphosphate (TDP), which acts as a cofactor for several enzymes involved in cerebral energy metabolism.

The present study was undertaken to characterize the metabolic changes responsible for the cerebellar degeneration in TD. Use was made of rat cerebellar granule cells (CGCs) in primary culture. The influence of TD on the functional integrity and energy metabolism of CGCs was investigated following exposure to thiamine-deficient media with or without added Py. The findings indicate that

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the latter treatment leads to profound metabolic changes resulting in compromised cellular energy metabolism, oxidative cell damage, and necrosis.

MATERIALS AND METHODS

Primary Cultures of Cerebellar Granule Cells

CGCs were prepared from 7-day-old Sprague-Dawley rats as previously described (Thangnipon et al., 1983; Gallo et al., 1987). Briefly, cerebella were mechanically chopped into 0.5-mm cubes, and cells were dissociated by trypsinization. The dissociated cells were plated onto 35-mm dishes precoated with poly-L-lysine at a density of approximately 1.5×10^6 cells/dish and cultured in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mM glutamine, and 25 mM KCl. The medium was changed 20 hr later with addition of cytosine arabinoside (20 μM) to arrest the growth of nonneuronal cells. The cultures were allowed to develop for 6 days before the beginning of treatment. Cells were subsequently treated with thiamine-deficient DMEM (Gibco Life Technologies, Gaithersburg, MD) containing 5% FCS, 2 mM glutamine, and 25 mM KCl. For Py-treated and control groups, 50 μM Py and 8 mg/liter thiamine were added, respectively.

Thiamine and Thiamine Esters Analysis by HPLC

Thiamine and its phosphates were measured essentially as described by Bettendorf et al. (1986), with minor modifications. CGCs were harvested in 400 μl ice-cold potassium phosphate buffer 50 mM, pH 7.4, and sonicated. Protein content was determined by the method of Lowry et al. (1951). Homogenates were then deproteinized with 75 μl of cold TCA (50%) and centrifuged at 12,500 rpm for 20 min at 4°C. The supernatant was extracted twice with five volumes of water-saturated diethyl ether. The solvent delivery system for HPLC (Waters Co. model 501) was equipped with an automatic sampler (Varian 9090). Eighty microliters of the supernatant were derivatized by mixing 30 μl of alkaline ferricyanide solution (4.3 mM potassium ferricyanide in 15% sodium hydroxyde) prior to injection on the column PRP-1 (150 mm × 4.1 mm I.D., 5 μm; Hamilton, Reno, NV). Standard curves for thiamine and its esters were generated using external standards prepared in the same extraction solutions as the cell extracts. Peak area measurements were computed using the Baseline 810 program (Héroux and Butterworth, 1995).

Measurement of α-Ketoglutarate Dehydrogenase

The activity of α-ketoglutarate dehydrogenase (KGDH) was measured spectrophotometrically at 25°C by measuring the rate of increase of absorbance resulting from NADH at 340 nm (Lai and Cooper, 1986). Cells were harvested in homogenizing buffer: 20 mM MOPS containing 0.2 mM mercaptoethanol, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 g/liter Triton X-100, and 1 g/liter Lubrol-Px, and the pH was adjusted to 7.2 with Tris. The reaction mixture contained 0.2 mM TDP, 2 mM NAD, 0.2 mM CoA, 1 mM $MgCl_2$, 0.3 mM DTT, 0.1% Triton X-100, 10 mM α-ketoglutarate, and 130 mM HEPES-Tris, pH 7.4. The reaction was initiated by the addition of CoA, and the rate of increase of absorbance was monitored for 6 min.

Measurement of Transketolase

Transketolase (TK) activity was measured according to the method of Giguère and Butterworth (1987), with minor modifications. Cultures were harvested in 100 μl cold potassium phosphate buffer (50 mM), pH 7.4, and were sonicated. To 25 μl of the homogenate was added 15 μl of ribose-5-phosphate (10 mM), and the mixture was incubated at 37°C for 30 min. The reaction was then stopped by adding 20 μl of cold TCA (20%), and samples were centrifuged at 2,500 rpm for 15 min. To 50 μl of the supernatant was added 0.5 ml H_2SO_4 (50%) and the mixture boiled for 4 min. Addition of 15 μl of cysteine (3%) was followed 15 hr later by measurement of sedoheptulose 7-phosphate levels, obtained from the difference in absorbance at 540 nm and 510 nm using a standard curve. Protein content was measured by the method of Lowry et al. (1951).

Measurement of Pyruvate Dehydrogenase

The activity of pyruvate dehydrogenase (PDH) was assayed spectrophotometrically at 25°C by measuring the rate of reduction of iodonitrotetrazolium violet (INT) by NADH at 500 nm (Elnageh and Gaintonde, 1988). Cells were harvested in homogenizing buffer: 20 mM MOPS containing 0.2 mM mercaptoethanol, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 g/liter Triton X-100, and 1 g/liter Lubrol-Px, and the pH was adjusted to 7.2 with Tris. The reaction mixture contained 0.2 mM TDP, 2.5 mM NAD, 0.1 mM CoA, 1 mM $MgCl_2$, 0.1 mM oxalate, 1 mg/ml BSA, 0.6 mM INT, 5 mM pyruvate, 7 U lipoamide dehydrogenase, 0.2% Triton X-100, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4. The reaction was initiated by the addition of pyruvate, and the rate of increase in absorbance was monitored for 10 min.

Measurement of Lactate and pH

Lactate was measured using a commercial lactate determination kit (Sigma, St. Louis, MO). Medium (200 μl) from different cell treatment groups was deproteinized with 400 μl trichloroacetic acid (10%), and the samples were centrifuged at 1,500*g* for 10 min. The supernatant was then assayed for lactate content by measuring spectrophotometrically the change in absorbance at 340 nm resulting from the conversion of lactate and NAD to pyruvate and NADH by lactate dehydrogenase (LDH). The remainder of the media was used to determine pH.

Measurement of ATP Levels

CGCs exposed to TD or control media were initially frozen in liquid nitrogen and stored at −80°C. Cells were immediately transferred to tubes containing a final concentration of 7% perchloric acid following harvesting in ice-cold 0.1 M NaOH containing 1 mM EDTA. Samples were centrifuged at 5,000*g*, 4°C for 15 min. The supernatants were neutralized with 3 M K_2CO_3 and centrifuged again. Resulting supernatants were assayed for ATP content by spectrofluorometry at 340 nm as described by Lowry and Passonneau (1972).

DNA Fragmentation Assay

Total genomic DNA was isolated from cultured cells, and the extent of DNA fragmentation was analyzed by agarose gel electrophoresis as previously described (Hockenbery et al.,

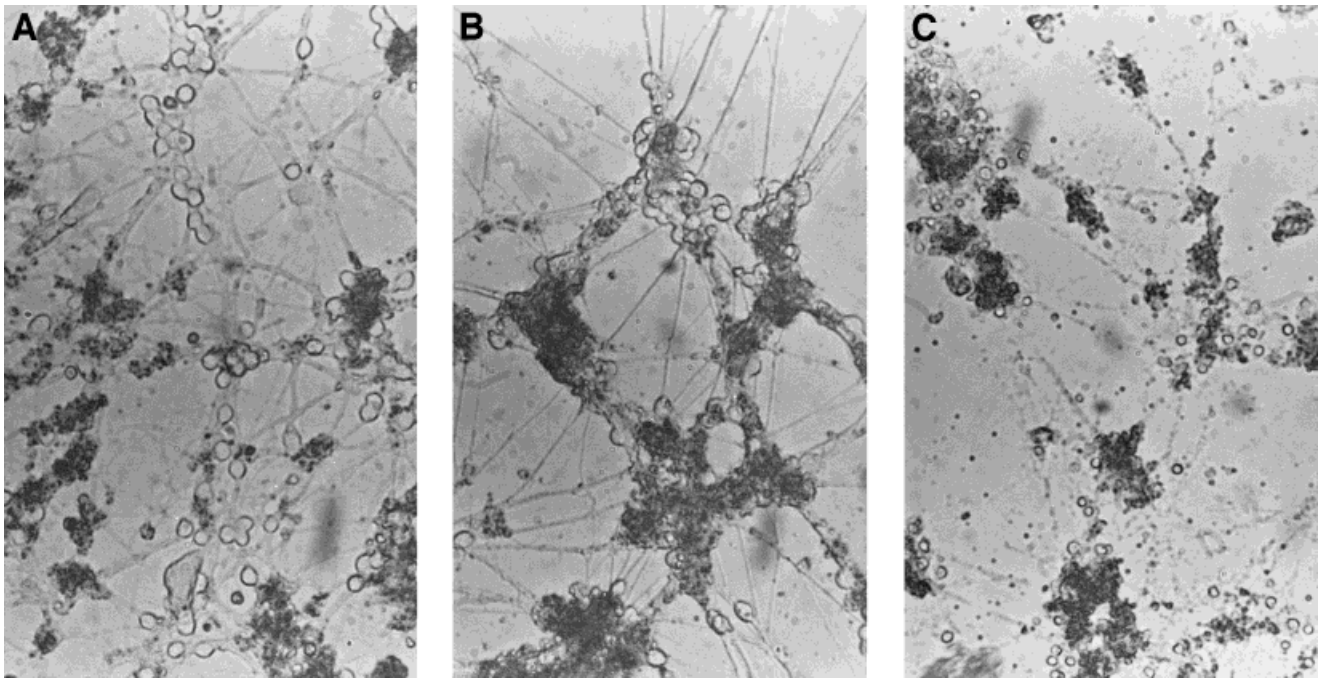

Fig. 1. Light micrographs of rat cerebellar granule neurons cultured for 7 days in control media containing thiamine (**A**), thiamine-deficient media (**B**), or thiamine-deficient media containing 50 μM Py (**C**). Note the loss of neurites and shrinkage of cells in C compared to A or B. Representative seeding. ×400.

1990). After treatment with RNAse A (50 μg/ml) and proteinase K (0.1 mg/ml) at 37°C for 30 min, soluble DNA was subjected to electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining.

Determination of Cell Viability

Cytotoxicity and neuroprotection were quantified by measuring LDH release. Media were collected, and cells were lysed in 0.1 M potassium phosphate buffer containing 0.5% Triton X-100. LDH activity was measured by monitoring the decrease in absorbance at 340 nm resulting from the conversion of pyruvate and NADH to lactate and NAD^+ (Koh and Choi, 1987). For the neuroprotection study, 50 μM α-tocopherol (vitamin E; ICN, Costa Mesa, CA), 100 μM butylated hydroxyanisole (BHA; Sigma), or 10 μM MK-801 (RBI, Natick, MA) was added at the start of the Py treatment, as previously described (Ciani et al., 1986), and LDH release was measured 7 days later. Cells were also examined for morphology at the light microscopic level.

Determination of Media Glucose Levels

Measurement of media glucose levels was carried out using a routine technique in the Diagnostic Laboratory of Clinical Biochemistry, Hôpital Saint-Luc.

Statistical Analysis

Data are expressed as mean ± SEM values. Differences between TD- or Py-treated groups and controls were compared using one-way ANOVA (Student-Newman-Keuls multiple comparisons test). A P value of <0.05 was considered significant.

RESULTS

General Observations

Under the culture conditions employed in this study, at least 95% of cells were determined to be CGCs based on morphological criteria, as was previously reported (Thangnipon et al., 1983). Treatment of cultures with TD media with or without addition of Py resulted in a differential response; Py treatment led to altered cellular morphology (Fig. 1), including shrinkage of the cell body and a severe loss of neurites, compared to cells exposed to control or TD media in the absence of Py.

Effects of TD on Thiamine and Its Esters

Progression of TD resulted in changes in the levels of intracellular thiamine and thiamine esters, as shown in Figure 2. However, there were notable differences in the rate of decrease of thiamine and its esters during TD. Exposure of CGCs to TD medium resulted in a 97% decrease of thiamine within 1 day (Fig. 2A). CGCs treated with Py resulted in a less dramatic decrease in thiamine content (63%). Intracellular content of TDP declined significantly after 1 day (43%) following Py treatment (Fig. 2B). Treatment with TD and Py for up to 7 days caused significant further decreases in TDP levels. When CGCs were exposed to Py, thiamine monophosphate (TMP)

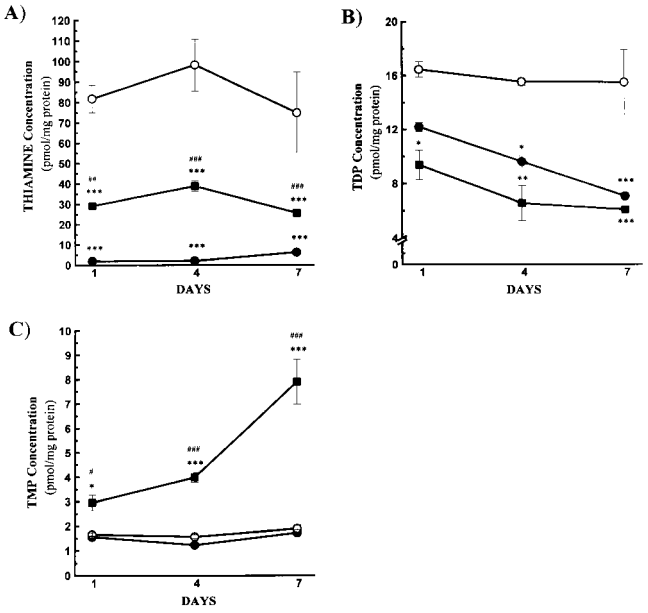

Fig. 2. Effects of thiamine deficiency on concentrations of thiamine and its phosphate esters in cultured CGCs. Intracellular concentrations of thiamine (**A**), TDP (**B**), and TMP (**C**) in CGCs in relation to treatment conditions and days of exposure. Results represent the mean ± SEM of three separate experiments each performed in duplicate. Open circles, control (CTL); solid circles, TD; squares, TD + 50 μM Py. $\star P < 0.05$, $\star\star P < 0.01$, and $\star\star\star P < 0.001$ vs. control for the same time point. $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.001$ vs. TD for the same time point (ANOVA followed by Student-Newman-Keuls test).

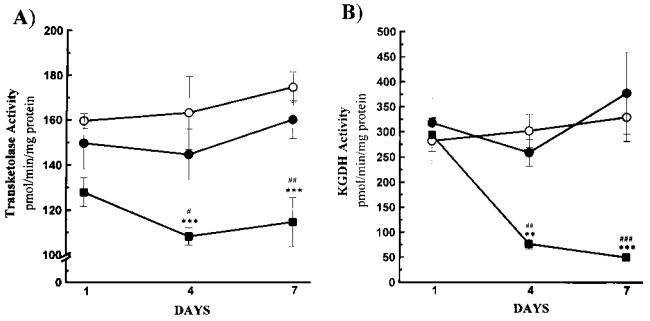

Fig. 3. Effects of thiamine deficiency on thiamine-dependent enzyme activities in cultured CGCs. TK (**A**) and KGDH (**B**) activities in CGCs in relation to treatment conditions and days of exposure. Data points represent mean ± SEM from three separate experiments each performed in triplicate. Open circles, CTL; solid circles, TD; squares, TD + 50 μM Py. $\star P < 0.05$, $\star\star P < 0.01$, $\star\star\star P < 0.001$ vs. control for the same time point. $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.001$ vs. TD for the same time point (ANOVA followed by Student-Newman-Keuls test).

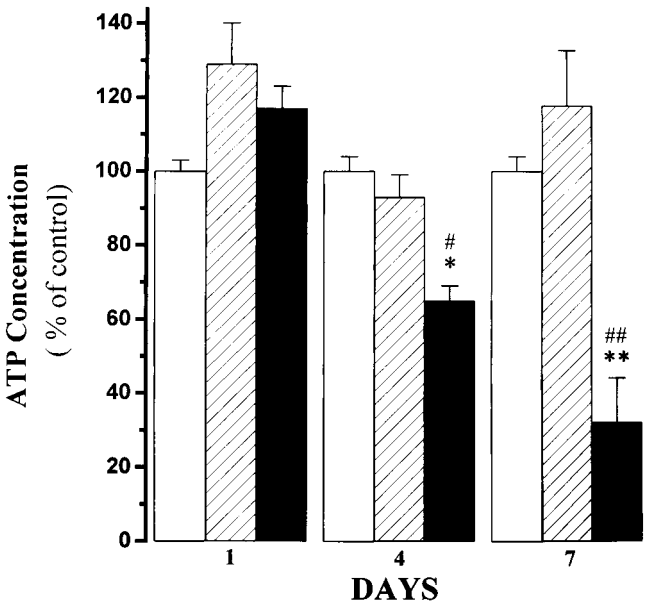

Fig. 4. Effects of thiamine deficiency or Py treatment on ATP content in CGCs. Cells were exposed to TD medium with or without the addition of 50 μM Py for 7 days. Results show the means ± SEM of three separate experiments each performed in duplicate. Open bars, CTL; striped bars, TD; solid bars, TD + 50 μM Py. $\star P < 0.05$, $\star\star P < 0.001$ vs. control for the same time point. $\#P < 0.05$, $\#\#P < 0.001$ vs. TD for the same time point (ANOVA followed by Student-Newman-Keuls test).

levels were increased up to fourfold after 7 days (Fig. 2C), whereas TMP levels in the other treatment groups remained unchanged.

Effects of TD on Thiamine-Dependent Enzyme Activities

Exposure of cultured CGCs to TD conditions supplemented with Py resulted in a significant decrease in TK activity at days 4 and 7 ($p < 0.001$; Fig. 3A). Exposure to TD media alone did not lead to reductions in TK activity compared to controls. On the other hand, treatment of CGCs with Py resulted in an 80% reduction in KGDH activity after 7 days of Py treatment (Fig. 3B). In cells treated with TD media alone, no reduction in KGDH activity was observed at day 7. No reductions in PDH activity were observed in TD or Py treatment groups over the 7-day period of exposure (results not shown).

Effects of TD on ATP Levels

Treatment with TD media alone did not affect ATP levels. However, exposure of CGCs to TD medium and Py resulted in reduced ATP levels (to 65% and 32% of controls at days 4 and day 7, respectively; Fig. 4).

Effects of TD on Lactate Release, pH, and Media Glucose Levels

CGCs exposed to TD media alone for 7 days showed no changes in lactate release compared to cells exposed to control media (Fig. 5A). On the other hand, CGCs exposed to Py exhibited a 100% and a 112% increase in

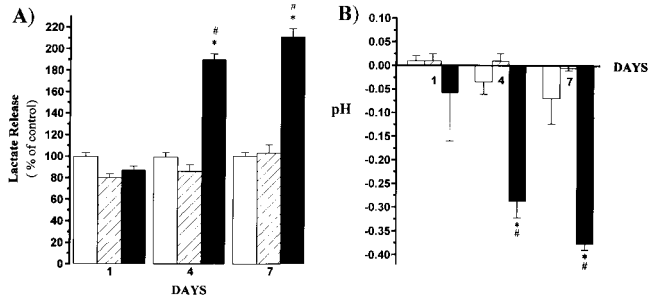

Fig. 5. Effects of thiamine deficiency or Py treatment on lactate release (**A**) and changes in pH (**B**) in cultured CGCs. Cells were exposed to TD medium with or without the addition of 50 μM Py and were treated for 7 days. Results show the means ± SEM of three separate experiments each performed in triplicate. Open bars, CTL; striped bars, TD; solid bars, TD + 50 μM Py. $\star P < 0.001$ vs. control for the same time point, $\#P < 0.001$ vs. TD for the same time point (ANOVA followed by Student-Newman-Keuls test).

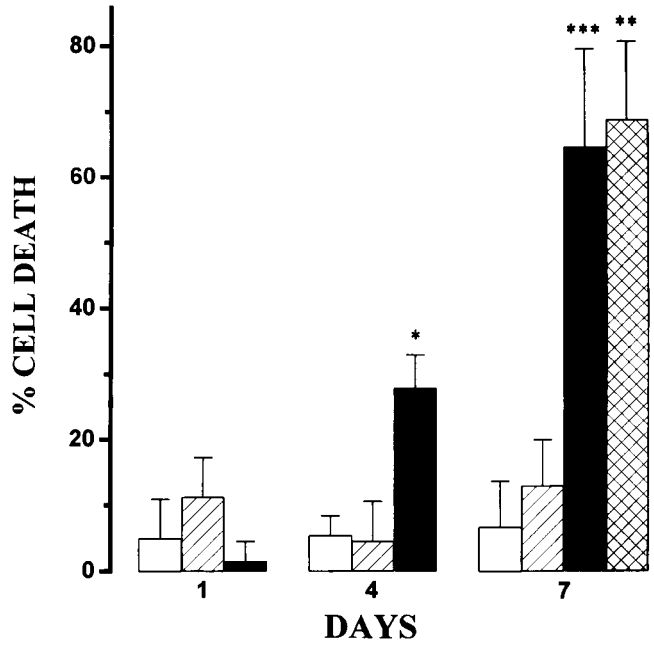

Fig. 6. Effects of thiamine deficiency or Py treatment on cell death in cultured CGCs. Cells were exposed to thiamine-deficient conditions in the absence (TD) or presence of 50 μM Py for 1, 4, and 7 days. Results are expressed in percentage cell death reflecting the percentage LDH release. Results show the means ± SEM of four separate experiments each performed in triplicate. Open bars, CTL; striped bars, TD; solid bars, TD + 50 μM Py; cross-hatched bars, without K^+. $\star P < 0.05$, $\star\star P < 0.01$, $\star\star\star P < 0.001$ vs. CTL for the same time point (ANOVA followed by Student-Newman-Keuls test).

lactate efflux relative to control values at days 4 and 7, respectively. Furthermore, pH decreased steadily in the medium of CGCs treated with Py in parallel with the increased lactate release (Fig. 5B). Levels of glucose in the TD media showed no change in the absence of Py during 7 days of thiamine deficiency (10.4 ± 0.3 vs 9.7 ± 1.0 mmol/liter in the controls). In the presence of Py, media glucose levels were decreased after 7 days of thiamine deficiency (5.8 ± 0.4 mmol/liter).

Effects of Py Treatment on Cell Viability

After 4 days of Py treatment, cell death was increased to 27% compared to controls (5%; Fig. 6). Cell death further increased after 7 days of exposure to Py (64%), i.e., to an extent similar to that of cells exposed to low (5 mM) potassium concentrations. Exposure to TD medium alone did not result in significant cell death compared to controls. An examination of the DNA fragmentation profile using agarose gel electrophoresis showed no evidence of DNA laddering, which would have been suggestive of apoptotic cell death mechanisms (data not shown).

Effects of Neuroprotective Agents on Cell Viability

Exposure of CGCs to Py in the presence of the antioxidants vitamin E and BHA (a synthetic antioxidant which counteracts lipid peroxidation) resulted in a significant 75% neuroprotection compared to cells treated with Py alone (Fig. 7). On the other hand, MK-801, an NMDA receptor antagonist, offered no neuroprotective effect for Py-treated CGCs.

DISCUSSION

Results of the present study show that primary cultures of CGCs provide a simple and effective model for investigation of the biochemical and pathophysiological processes involved in cell death resulting from compromised oxidative metabolism. This is the first report of the effects of TD on the function and viability of cultured cerebellar neurons; previous studies had been restricted to the effects of TD on neuroblastoma cells and hippocampal neurons (Bettendorff et al., 1995; Park et al., 1999).

Exposure of CGCs to 50 μM Py for 7 days resulted in significant cell death. Py treatment of this magnitude is as lethal to CGCs as a low potassium concentration (5 mM). Indeed, a previous study demonstrated that 25 mM concentrations of potassium, together with thiamine, are essential for long-term survival of CGCs (Thangnipon et al., 1983).

Py is a central thiamine antagonist that inhibits the activity of thiamine pyrophosphokinase (Johnson and Gubler, 1965), the enzyme responsible for the synthesis of TDP, the biologically active cofactor form of thiamine. Consistent with this mechanism, the present findings revealed a large decrease in intracellular thiamine and TDP content as early as 1 day after exposure of CGCs to Py. These findings are also consistent with those of Rindi and Perry (1961), who reported that an accumulation of Py in rat tissues resulted in decreased brain concentrations of TDP. Levels of thiamine remained higher in Py-treated CGCs than in cells exposed to a TD media alone. This effect is likely due to the inhibition of thiamine pyrophosphokinase by Py, resulting in less phosphorylation of thi-

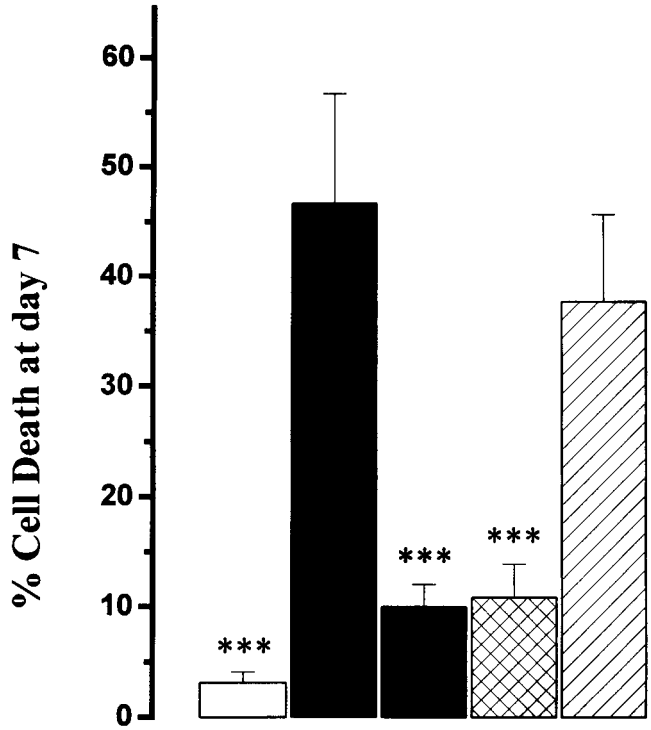

Fig. 7. Effects of the antioxidants α-tocopherol and BHA and the NMDA receptor antagonist MK-801 on 50 μM Py-mediated cell death. Antioxidants were added at the start of the Py treatment. NMDA treatment served as a positive control for excitotoxic cell death. Results are expressed as % LDH release. Results show the means ± SEM of two separate experiments, each performed in triplicate. Open bar, CTL; first solid bar, TD + 50 μM Py; TD + 50 μM Py and either 50 μM α-tocopherol (second solid bar), or 100 μM BHA (cross-hatched bar), or 10 μM MK-801 (striped bar). ***$P < 0.001$ vs. TD + Py 50 μM (ANOVA followed by Student-Newman-Keuls test).

amine to TDP in the cells. These considerations could also explain the significantly lower levels of TDP observed in Py-treated cells compared to those exposed to TD medium alone. The gradual and sustained increase of TMP in CGCs treated with Py could, in turn, be explained by the dephosphorylation of TTP and TDP by the enzymes TTPase and TDPase, on which Py has no effect. On the other hand, the absence of early changes in TMP levels in CGCs exposed to TD media alone, despite the large decreases in intracellular TDP content, suggests that the initial large decrease in TDP levels is due to decreased activity of thiamine pyrophosphokinase rather than a dephosphorylation event.

Exposure of cultured CGCs to TD medium in the presence of Py resulted in decreased activities of the TDP-dependent enzymes TK and KGDH. The decrease in TK activity (35%) was not as great as the decrease in KGDH (80%) after 7 days of Py treatment. PDH activity in different treatment groups remained unchanged throughout the time course, which is consistent with previous reports (Elnageh and Gaitonde, 1988; Butterworth, 1986). TD media alone had no effect on KGDH and TK activities as well as ATP levels despite causing a decrease in the levels of TDP, a cofactor for these enzymes. Treatment with Py (in TD media) resulted in a greater lowering of TDP levels than TD media alone, so it is possible that a threshold exists for normal activities of the two enzymes that is dependent on the level of TDP. In the presence of TD media only, TDP levels may be insufficiently reduced to cause an effect on the activities of the two enzymes. The selective reductions in thiamine-dependent enzyme activities in the presence of Py, as well as the absence of changes in enzyme activity in TD media only, may also involve differences in cofactor-apoenzyme binding characteristics and/or differences in enzyme turnover times. Although levels of TMP increased greatly with Py treatment, and because the monophosphate ester has no known role in cell metabolism or function, it is unlikely that changes in TMP contribute to the effects of Py on the enzyme activities and thus ATP levels. Recently, it has been shown by Park et al. (1999) that reactive oxygen species (ROS) inhibit KGDH. In the present study, reduced KGDH activity following exposure to Py resulted in increased lactate production. Lactate is known to induce ROS production (Tombaugh and Sapolski, 1993) and, as such, could have a potential inhibitory effect on KGDH.

In that KGDH is a rate-limiting enzyme of the tricarboxylic acid cycle, a sustained reduction of its activity owing to TD would be expected to lead to decreased ATP synthesis, with a consequent depletion of cellular energy metabolism, ultimately leading to cell death (Parker et al., 1984). Results of the present study support this mechanism by demonstrating a marked decrease of ATP levels in Py-treated CGCs, concomitant with the decreased KGDH activities. These findings are consistent with those of Aikawa et al. (1984); in their paper, a loss of high-energy phosphates was reported in brain tissue from the Py-treated rat.

As has been consistently reported in previous studies in the rat, another indication of impending energy failure in TD is the accumulation of lactate (Kinnersley and Peters, 1930; McCandless and Schenker, 1968). Treatment with Py led to a decrease in media glucose levels consistent with increased glucose utilization and reflected in the observed increase in lactate production. However, insofar as glucose levels were still within physiological range after 7 days, it is unlikely that glucose depletion was a cause of the observed cell death. In the present study, the accumulation of lactate was apparent after 4 days of Py treatment (i.e., coincident with decreased activity of KGDH). Consistent with the rise of lactate in these cells, extracellular pH was decreased. It was previously suggested that lactic acidosis could be the cause of neuronal cell death in TD in the Py-treated rat (Hakim, 1984).

Addition of α-tocopherol or BHA to Py-treated CGCs led to a 75% reduction in cell death. These compounds are established potent antioxidants, having an ability to sequester ROS. As was demonstrated by Ciani et al. (1996), α-tocopherol together with BHA and MK-801

protected cultured CGCs against glutamate excitotoxicity, which gives rise to ROS. However, in the present study, MK-801 did not protect against Py-induced cell death, indicating that NMDA receptor-mediated excitotoxicity is not involved in the pathogenesis of CGC death from exposure to TD.

In conclusion, cultured CGCs afford an effective preparation with which to study the effects of TD on neuronal cell metabolism and survival. Treatment of these cells with Py recapitulates the metabolic brain dysfunction characteristics of the effects of TD in the intact animal. The CGC preparation may therefore be useful in the further elucidation of the mechanisms involved in the neuronal cell death cascade resulting from TD.

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