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JNK1 is inactivated during thiamine deficiency-induced apoptosis in human neuroblastoma cells

James J.-L. Wang,* Zhaolin Hua,† Hugh M. Fentress,† and Charles K. Singleton†

**The Burnham Institute, La Jolla, CA USA and †Department of Molecular Biology, Vanderbilt University, Nashville, TN USA*

Thiamine deficiency results in selective neuronal damage. A number of mechanisms have been proposed to account for brain damage associated with thiamine deficiency and to account for the focal nature of the loss of neurons. One proposed mechanism is programmed cell death. We found efficient induction of apoptosis in human neuroblastoma cells when the cells were deprived of thiamine. Although extensive mitochondrial damage was seen, the release of cytochrome c was not the triggering mechanism for thiamine deficiency-induced apoptosis. Instead, the activity of the cJun amino terminal kinase Jnk1 was lost, and this loss correlated temporally with induction of apoptosis. The loss was specific for Jnk1; Jnk2/3 activity remained unchanged. Loss of Jnk1 activity was not found in lymphoblasts, a cell type that did not undergo apoptosis when deprived of thiamine. These findings suggest that thiamine deficiency results in a cellular stress that brings about the loss of Jnk1 activity and the loss of its function of protecting cells from programmed cell death. We postulate that focal sensitivity to thiamine deficiency results, in part, from specific neuronal cell types being susceptible to the inactivation of Jnk1 in response to depletion of cellular thiamine. (J. Nutr. Biochem. 11:208–215, 2000)

Keywords: apoptosis; thiamine deficiency; cJun amino terminal kinases; neuroblastoma

Introduction

Thiamine deficiency (TD) in humans can manifest itself in region-specific damage in brain structures such as the cerebellum, thalamus, midbrain, and brainstem.[1–3] The resulting neurologic disorder is frequently referred to as Wernicke's encephalopathy. The lesions are morphologically distinct among the damaged regions,[4] and this and subsequent findings indicate multiple mechanisms for neuronal damage induced by TD.[5] Many of the characteristics of Wernicke's encephalopathy are reproduced in rats fed a thiamine-deficient diet alone or in combination with pyrithiamine, a thiamine analog that inhibits thiamine uptake and conversion to its diphosphate derivative.[2] Thiamine diphosphate is used as a cofactor for several enzymes involved in carbohydrate catabolism. The results of many studies indicate that alterations in the activities of one or more of these enzymes represent the initial insult that ultimately leads to neuronal death.[6–9] Several mechanisms have been proposed to account for TD-induced neuronal cell death.[5] One mechanism is apoptosis.

In pyrithiamine-treated rats, apoptosis was observed in the thalamus but not in other regions affected during TD,[10] and alterations of immediate-early gene expression were observed in the thalamus.[11] Depletion of thiamine from cultured cells results in variable effects on cell growth and survival depending on cell type and culture conditions.[12–15] Rat cardiac cells in TD medium underwent morphologic and functional alterations and eventually degenerated and died.[15] Cultured neuroblastoma cells from mice also died in TD medium when further depletion of thiamine was caused by using an inhibitor of thiamine transport.[16] Mitochondrial morphology in these cells was severely altered and evidence was provided for possible alterations in the structural integrity of the outer membrane. Cell death was ascribed to impaired mitochondrial function rather than apoptosis. Conflicting results of apoptosis[17] or no effect on growth[13] have

Address correspondence to Dr. Charles K. Singleton, Department of Molecular Biology, Vanderbilt University, Box 1820 Station B, Nashville, TN 37235.
This work was supported by the NIAAA (AA12014).
Received November 12, 1999; accepted January 24, 2000.

655 Avenue of the Americas, New York, NY 10010
0955-2863/00/$–see front matter
PII S0955-2863(00)00067-X

been obtained for fibroblasts from patients with thiamine-responsive megaloblastic anemia.

Herein, we report that depletion of thiamine from cultured human neuroblastoma cells but not from lymphoblasts efficiently induces apoptosis. Although mitochondria underwent significant morphologic damage, cytochrome c release did not mediate TD-induced neuroblastoma cell death. Instead, a role for the cJun amino terminal kinase Jnk1 was suggested, where TD results in the loss of a postulated anti-apoptotic or cell protection function of Jnk1. Jnk1 has been shown to regulate region-specific apoptosis during neuronal development,[18] and Jnks are expressed in diverse neuronal subpopulations throughout the brain.[19] We postulate that focal sensitivity to TD results, in part, from specific neuronal cell types being susceptible to the inactivation of Jnk1 and the loss of its function of protecting cells from programmed cell death in response to TD-induced stress.

Methods and materials

Cell culture

Human neuroblastoma (SH-SY5Y) and lymphoblast cells were cultured as previously described.[13,20] Control medium contained 10 μM thiamine and thiamine-deficient medium (tdm) was estimated to contain 0.1 nM thiamine.[13] For cell number determinations when neuroblastoma cell growth rates were examined, several 2 mm^2 areas were marked on the bottom of the flasks. Cells were counted daily in four such areas, and the numbers were totaled. For apoptosis assays, 5×10^5 cells per well were seeded in a six-well plate and grown overnight in control medium. The cells were washed three to four times with phosphate-buffered saline (PBS), followed by the addition of 1 mL of tdm plus varying amounts of pyrithiamine. The medium was changed every 2 to 3 days. Electron microscopy of cells grown under different conditions of thiamine depletion were performed by the EM Laboratory of the Pathology Department of Vanderbilt University.

Cell death assays

To assay for cell death and apoptosis, several methods were used. Caspase 3 activity was measured using the CPP32 fluorescent kit (Clontech, Palo Alto, CA USA) according to the manufacturer's directions. An annexin V apoptosis kit (Clontech) also was used to detect apoptosis. Flow cytometry of propidium iodide stained cells was used to detect cell death. Aliquots of the cells were treated with 20 mM propidium iodide for 30 min, followed by counting of dead (stained) cells by flow cytometry. To determine the total number of cells, similar aliquots were treated with 10 mM digitonin, incubated for 10 min at 37°C, and counted by flow cytometry. Most assays were repeated four times in independent experiments starting with cells growing in control medium.

Cytochrome c detection

Cytochrome c was detected by Western analysis on cell extracts using mouse anti-cytochrome c antibody from Pharmingen (San Diego, CA USA). Cells were harvested after varying times of culture in the desired medium. Dounce homogenization in 50 mM piperazine diethanesulfonic acid (PIPES), pH 7.4, 50 mM KCl, 5 mM ethyleneglycotetraacetic acid (EGTA), 2 mM Mg Cl_2, 10 μM cytochalasin, 1 mM dithiothreitol, 220 mM mannitol, and 68 mM sucrose was used to disrupt 2×10^6 cells. Nuclei and unbroken cells were removed with a 5-min spin at 600 × g. Mitochondria were pelleted by spinning at 10,000 × g for 10 min. All of the above manipulations were carried out on ice or at 4°C. The supernatant from the final spin was used as a source for potentially released cytochrome c, and the pellet was resuspended in 10 mM Tris, pH 8.0, 50 mM NaCl, 0.5% βME, 1% NP-40, 1% sodium dodecyl sulfate (SDS), and 0.5% deoxycorticosterone (DOC) and used as the source of mitochondrial cytochrome c. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on the samples, followed by transfer to nitrocellulose. A positive control for cytochrome c release was generated by adding staurosporine (1 μM final concentration) to a culture of cells under each condition 6 hr prior to harvesting and work-up.[21]

Gel mobility shift assays

Cells were cultured for varying times in the desired medium. The cells were washed once with cold PBS, followed by harvesting in 1 mL of cold hypotonic buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride]. The cells were lysed for 10 min on ice in hypotonic buffer containing 0.1% NP-40, and the lysate was centrifuged at 14,000 × g for 10 min. The resulting pellet was resuspended in 3 mL of nuclear lysis buffer [20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 1.0 mM DTT, 0.5 mM PMSF, 20% glycerol, and 1% NP-40], and incubated for 15 min on ice. After spinning for 14,000 × g for 10 min, the supernatant was collected and used as a nuclear extract in mobility shift assays. Two micrograms of nuclear extract was incubated with 10,000 cpm of a ^{32}P-labeled (by polynucleotide kinase) oligonucleotide probe in the presence of 1 μg of poly(dI-dC) for 20 min at room temperature. Reactions were then fractionated by nondenaturing gel electrophoresis. Unlabeled competitor oligonucleotides were added at a 100-fold molar excess. The nuclear factor-κB (NF-κB) oligonucleotide and the competitor oligonucleotide were from Promega. The conditions for the mobility shift gel were as recommended by the oligonucleotide supplier (Promega, Madison, WI USA).

Jnk activity assay

Cells were cultured for varying times in the desired medium. The cells were washed once with cold PBS, followed by harvesting in 1 mL of cold buffer (50 mM HEPES, pH 7.6, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 1 μM Na_3VO_4, 10% glycerol). The cells were lysed on ice for 30 min by adding triton X-100 to 1% and NP-40 to 0.5%. The lysates were spun for 10 min at 14,000 × g, and the supernatants were collected for immunocomplex kinase assay and Western analysis. Jnk1 protein was precipitated by adding protein A agarose beads to the cleared lysates, which had been incubated for 2 hr at 4°C with anti-Jnk1 antibody (gift of Dr. L. Chang, University of California, San Diego, CA USA). The beads were washed two times with lysis buffer and once with kinase buffer (20 mM HEPES, pH 7.6, 1 mM $MgCl_2$, 10% glycerol). The immunoprecipitates were then subjected to kinase assays in 35 μL kinase buffer containing glutathione-S-transferase (GST)-cJun (see below) and 0.05 μM γ-^{32}P-adenosine triphosphate (ATP) and 20 μM cold ATP. After 20 min at 30°C, the reactions were terminated by the addition of SDS-PAGE loading buffer and boiling for 5 min. The samples were fractionated by SDS-PAGE. The gel was then subjected to autoradiography or Western analysis using the anti-Jnk1 antibody. GST-cJun was obtained using the pGEX-KG expression vector (gift of Dr. L. Chang, University of California) and *Escherichia coli* strain BL21. The protein was expressed and purified on GSH-sepharose as described.[22] The GST-cJun protein contains amino acids 1-79 of human cJun fused in frame at its amino terminus with GST.

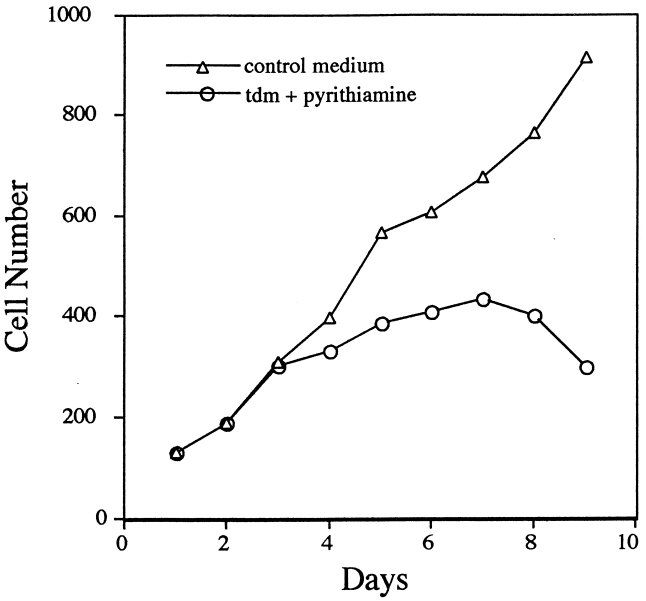

Figure 1 Effect of thiamine deficiency on neuroblastoma cell growth. Neuroblastoma cells were cultured in control medium or thiamine-deficient medium (tdm) containing 300 nM pyrithiamine. Attached cells were counted daily by microscopic examination. Similar growth rates were observed in several repeat experiments.

For determining Jnk2/3 activity, the lysates described above and immunodepleted of Jnk1 were used in conjunction with the JNK (FL) antibody from Santa Cruz Biotechnology (Santa Cruz, CA USA) that primarily binds Jnk2 and to a lessor extent Jnk1 and Jnk3.

Results

TD-induced apoptosis

We previously demonstrated that for three types of human cells in culture, different degrees of thiamine depletion were required to alter their growth rate.[13] Lymphoblasts demonstrated an intermediate sensitivity, with cell proliferation being dramatically slowed but not blocked completely, even with severe thiamine depletion. Neuroblastoma cells were most sensitive to TD with respect to slowing or inhibiting growth and fibroblasts were least sensitive with very little effect on their growth rate. Not only was less depletion of thiamine required to slow growth of neuroblastoma cells, but as shown in *Figure 1*, we now report that cell numbers decrease after 7 to 8 days in tdm plus 300 nM pyrithiamine. Seven days in tdm represents the required time for maximum loss of thiamine requiring enzyme activity.[13,20] Thus, at least for neuroblastoma cells, it appears that cell death occurs simultaneously with reaching maximal loss of enzyme activity or very shortly thereafter. As demonstrated previously, the growth rate of lymphoblasts could be dramatically inhibited, albeit by a more severe thiamine depletion, but no loss of cell numbers was observed[13] as we find herein for neuroblastoma cells.

Several assays for cell death and apoptosis were performed to determine if the loss of neuroblastoma cells under TD is due to apoptosis. Propidium iodide staining followed by flow cytometry[23] was used on cells cultured for either 3 or 7 days in tdm containing different concentrations of pyrithiamine. As seen in *Figure 2A*, cell death was induced efficiently (>50% stained cells) by 7 days of culture in tdm plus 10 nM or higher concentrations of pyrithiamine. No significant cell death above background was found by day 3, a time when thiamine-requiring enzymes have not reached their maximum loss of activity.[13] Cell death could be detected by day 6 (not shown) in tdm plus pyrithiamine. Cell death was induced by depletion of thiamine availability to the cells and was not an unrelated response to added pyrithiamine. This was demonstrated by culturing cells with 300 nM pyrithiamine in control medium; no cell death above background (control medium alone) was observed. As expected, no differences in cell death were found between thiamine-sufficient and thiamine-deficient conditions (7 days in tdm plus 3 μM pyrithiamine) for lymphoblasts (not shown). These culture conditions slowed the growth of lymphoblasts by at least 90%.[13]

Interestingly, the percent of cell death closely paralleled the loss of α-ketoglutarate dehydrogenase (αKGDH) activity (*Figure 2A*, data from Pekovich et al.[13]). αKGDH is a mitochondrial enzyme that participates in the tricarboxylic acid cycle (TCA), and the loss of its activity in TD rats results in decreases in neuronal levels of glutamate, aspartate, and GABA.[24]

To examine if the cell death revealed by propidium iodide staining was apoptotic, caspase 3 activity was determined in neuroblastoma cells cultured for 3 or 7 days in tdm containing various concentrations of pyrithiamine. *Figure 2B* indicates that TD induction of caspase 3 activity was similar to and paralleled propidium iodide staining of TD cells. Similar experiments were carried out with apoptosis detection performed by an annexin V assay. The results (not shown) were essentially identical to the data presented in *Figure 2*. Thus, depletion of thiamine from neuroblastoma cells resulted in the efficient induction of apoptosis.

TD-induced mitochondrial damage

Although they did not find evidence for apoptosis, Bettendorff and coworkers[12] found substantial mitochondrial damage in mouse neuroblastoma cells under TD. Alterations in mitochondria also have been found for TD rats in degenerating thalamic neurons.[25] These findings, the parallel between apoptosis induction and loss of αKGDH activity described above, and the involvement of mitochondria in apoptosis[26] led us to examine possible mitochondrial damage in human neuroblastoma cells depleted of thiamine. *Figure 3* indicates significant alterations in mitochondrial morphology in human neuroblastoma cells cultured 7 days in tdm containing 300 nM pyrithiamine, conditions that resulted in greater than 80% apoptotic cells. The alterations observed are similar to those found for mouse neuroblastoma cells,[12] with enlargement and disruption of cristae being the most prominent features in the vast majority of the mitochondria. Lymphoblasts cultured with tdm plus 3 μM pyrithiamine showed partial disruption of cristae and a few enlarged mitochondria even though no apoptosis is induced in this cell type under these conditions.

One mechanism for initiation of apoptosis is the release of cytochrome c, a caspase activator, from damaged mito-

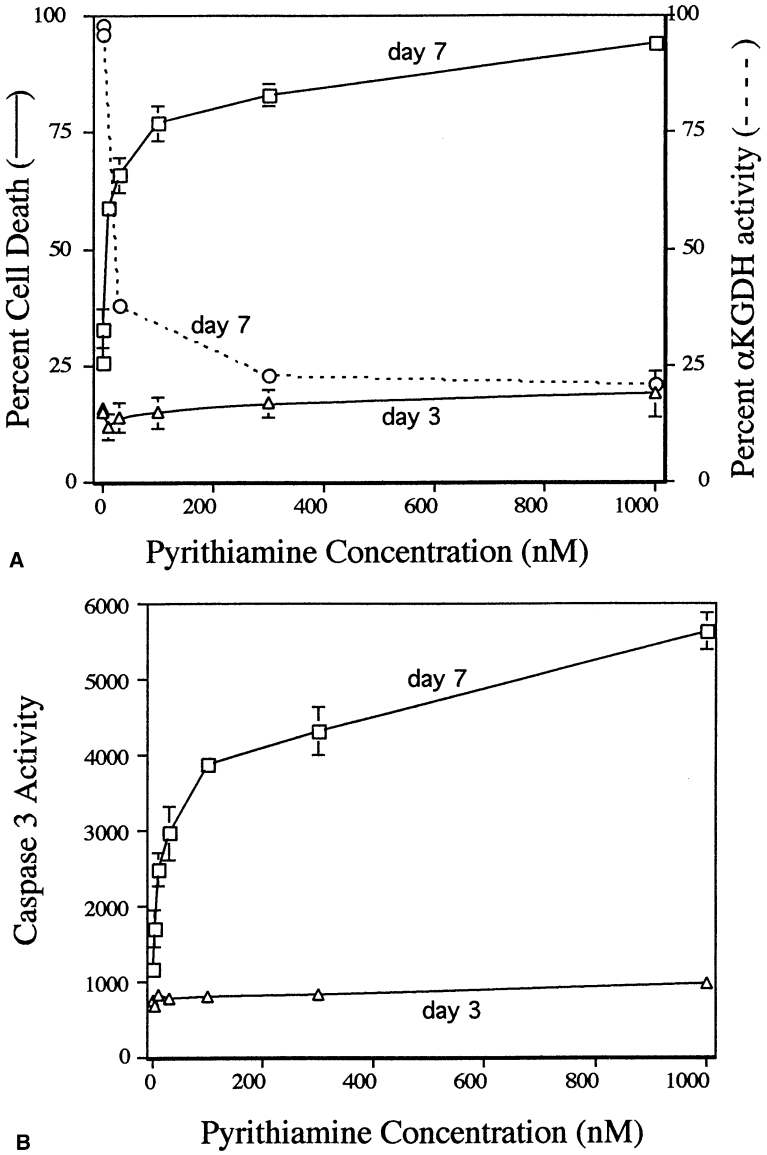

Figure 2 Cell death and apoptosis of neuroblastoma cells during thiamine deficiency. Neuroblastoma cells were cultured in thiamine-deficient medium containing varying amounts of pyrithiamine. On days 3 and 7, aliquots were removed and the cells were assayed for cell death and caspase activity. (A) Cells were stained with propidium iodide and the percent of stained cells (indicative of dead cells) was determined by flow cytometry. The dotted line indicates the loss of activity of α-ketoglutarate dehydrogenase (αKGDH) in neuroblastoma cells and was taken from Pekovich et al.[13] (B) Cells were lysed and assayed for caspase 3 activity as described in Materials and methods. The activity is given in relative fluorescence. Four independent experiments were performed for propidium iodide staining and for assaying caspase 3 activity; the data are expressed as mean values ±SEM.

chondria.[21,27] Mitochondria and cytosol were isolated from neuroblastoma cells cultured for 7 days in control medium or tdm containing 300 nM pyrithiamine. The samples were fractionated by gel electrophoresis and subjected to Western analysis using anti-cytochrome c antisera. Although mitochondria were visibly altered by TD, no significant release of cytochrome c was detected (*Figure 4*). Cytochrome c was found in the cytosol fraction when the known cytochrome c releasing factor staurosporine[21] was added to either culture 6 hr prior to harvesting and work-up. Therefore, it seems unlikely that cytochrome c is involved in TD-induced apoptosis.

A role for Jnk kinases in TD-induced apoptosis

Because cytochrome c release from the damaged mitochondria appeared not to be a factor, we examined several other potential mechanisms of establishing apoptosis. The transcription factor NF-κB has been implicated in apoptosis in response to N-methyl-D aspartate (NMDA) receptor mediated neuronal cell death.[28–30] Because NMDA receptor mediated excitotoxicity is a proposed mechanism for neuronal cell death during TD,[5,31] we examined NF-κB activity in neuroblastoma cells depleted of thiamine. Several independent experiments were performed in which neuroblastoma cells were cultured for various times in tdm containing 1 μM pyrithiamine, conditions that resulted in more than 90% apoptotic cells. Nuclear extracts were prepared, and a gel mobility shift assay for specific NF-κB binding to DNA was performed. A representative example is shown in *Figure 5*. No consistent and reproducible variation or enhanced NF-κB DNA-binding activity was found out to 7 days of TD.

We next examined the possibility of a role for cJun amino-terminal kinases [Jnk or stress activated protein kinases (SAPK)] in TD-induced apoptosis. Neuroblastoma cells were cultured for various times in tdm containing 1 μM pyrithiamine. Cell lysates were prepared and used for immunoprecipitation of Jnk1 with anti-Jnk1 antisera. The

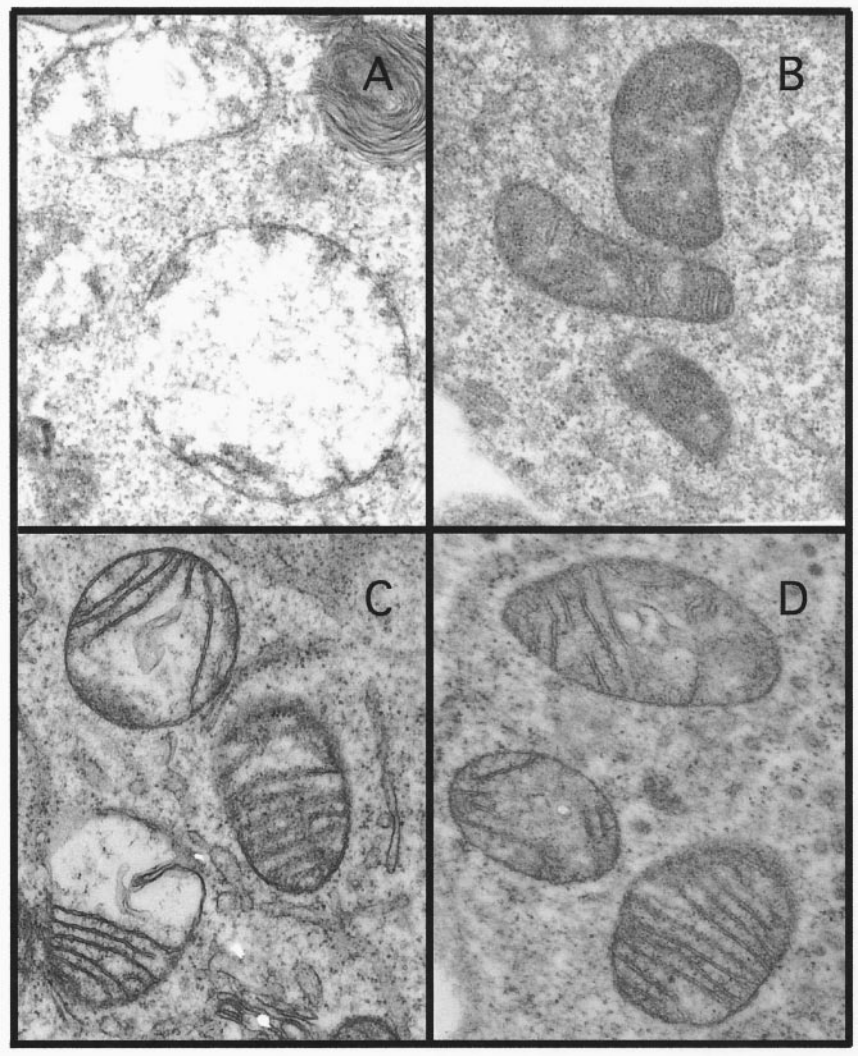

Figure 3 The effect of thiamine deficiency on mitochondrial morphology in neuroblastoma and lymphoblast cells. Cells were cultured for 7 days in either control medium or thiamine-deficient medium (tdm) containing pyrithiamine. Cells were harvested, fixed, and sectioned for electron microscopy. Magnification is 40,000×. (A) Neuroblastoma cells in tdm containing 300 nM pyrithiamine. (B) Neuroblastoma cells in control medium. (C) Lymphoblast cells in tdm containing 3 μM pyrithiamine. (D) Lymphoblast cells in control medium.

immunoprecipitates were used in kinase assays using the amino terminal 79 amino acids of cJun (fused to GST) as substrate. *Figure 6A* shows that Jnk1 activity was progressively lost with time of culture in tdm, and it was virtually undetectable by 6 to 7 days. Jnk1 protein levels remained relatively constant, with a modest decrease occurring between days 3 and 4 (*Figure 6B*). The results shown in *Figs. 6A,B* are representative of several independent experiments.

In contrast to the loss of Jnk1 activity in neuroblastoma cells undergoing TD-induced apoptosis, no loss of Jnk1 activity was found in lymphoblasts under severe TD (*Figure 6C*). As described above, lymphoblasts were found not to undergo TD-induced apoptosis. Thus, the loss of Jnk1 activity during TD was found only in cells that were undergoing apoptosis. Finally, the loss of cJun amino terminal kinase activity in neuroblastoma cells under TD was specific for Jnk1 as little or no change was found for Jnk2/3 activity (*Figure 6D*).

Discussion

We have found that TD can induce efficient apoptosis in human neuroblastoma cells. Previously, neuroblastoma cells were found to be more sensitive than other human cell types to effects of TD on cell growth and proliferation. Apoptosis was not caused by depleting thiamine from lymphoblasts, indicating a selectivity of TD-induced apoptosis. Apoptosis has been found in vivo within the thalamus of TD rats but not in other regions that are susceptible to damage during TD.[10] It seems that sensitivity to TD-

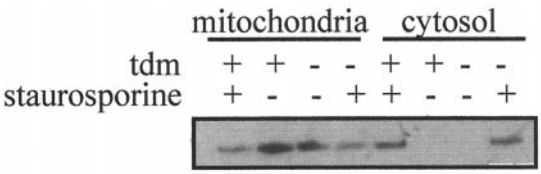

Figure 4 Localization of cytochrome c in neuroblastoma cells during thiamine deficiency. Neuroblastoma cells were cultured in either control medium [minus signs in thiamine-deficient medium (tdm) row] or tdm containing 300 nM pyrithiamine (plus sign in tdm row). After 7 days, cells were lysed, and the cytosol (supernatant) and mitochondria (pellet) were isolated. Staurosporine, which was used as a positive control for cytochrome c release, was added to a culture of cells under each medium condition 6 hr prior to harvesting and work-up. Samples were subjected to Western analysis using anti-cytochrome c antisera.

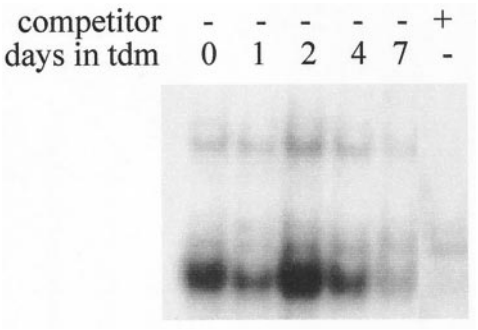

Figure 5 Nuclear factor-κB (NF-κB) activity in neuroblastoma cells during thiamine deficiency. Neuroblastoma cells were cultured in thiamine-deficient medium containing 300 nM pyrithiamine. Nuclear extracts were prepared at the indicated times and used in gel mobility shift assays. The labeled probe was an oligonucleotide containing the specific binding site for NF-κB. Nonradioactive oligonucleotide was used at 100× excess over the labeled probe. Several independent experiments, a representative example of which is shown, revealed no reproducible alteration in NF-κB DNA-binding activity as revealed by the shifted bands.

induced apoptosis may underlie the selective vulnerability of at least some brain regions to TD. The culture system developed here should prove valuable in determining why a given cell type is more prone than other cells types to undergo apoptosis when deprived of thiamine. Having two cell types, one of which is highly sensitive to TD-induced apoptosis (human neuroblastoma cells) and one that is resistant (human lymphoblasts), will facilitate elucidating such an understanding.

A number of studies have implicated increased extracellular glutamate and subsequent NMDA receptor mediated excitotoxicity as a mechanism of neuronal damage during TD.[31–35] Because apoptosis occurs during NMDA excitotoxicity via activation of NF-κB,[28–30] we examined the possibility that NF-κB was being activated in TD-induced apoptosis. No activation was found, indicating that such a mechanism does not underlie apoptosis in neuroblastoma cells depleted of thiamine.

Numerous studies have implicated alterations in αKGDH activity as the initial biochemical lesion leading to cell damage during TD.[5] Loss of αKGDH activity results in reduced pyruvate entry into the TCA cycle[36] and altered oxidative metabolism and neurotransmitter synthesis (GABA, glutamate, aspartate).[24,37] These alterations, in turn, are thought to generate the mitochondrial damage observed during TD.[38] The induction of apoptosis that we observed in human neuroblastoma cells paralleled the inactivation of αKGDH and seems unrelated to the loss of transketolase activity, which occurred at a more modest depletion of thiamine.[13] Although the close parallel between inactivation of αKGDH and TD-induced apoptosis may be coincidental, it is possible that the loss of activity is directly related to induction of apoptosis by some as yet unidentified mechanism. The mitochondrial damage we observed was severe, yet cytochrome c did not become cytosolic. This would seem to rule out a role for cytochrome c in TD-induced apoptosis. Similarly, Bettendorff and coworkers[38] found extensive mitochondrial damage in rat neuroblastoma cells deprived of thiamine,

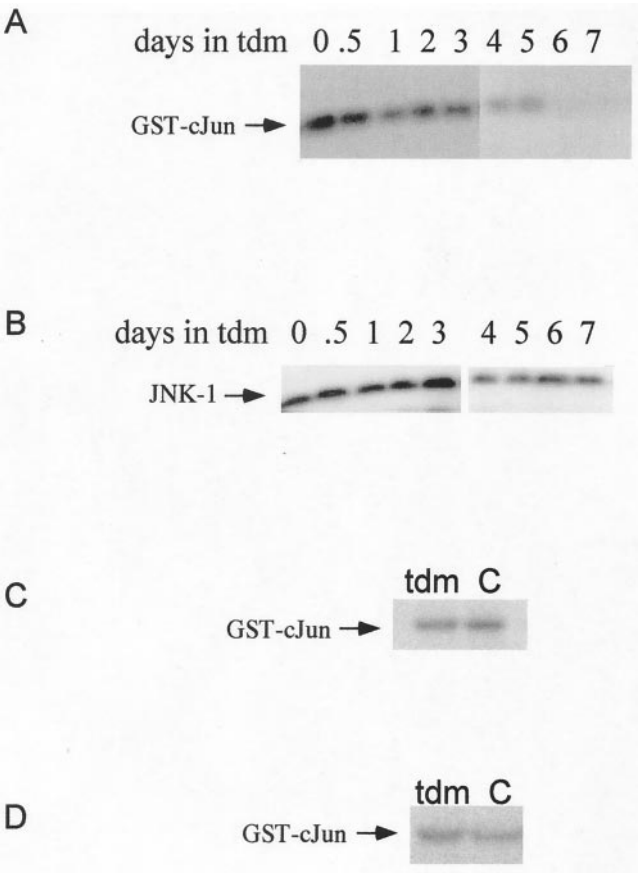

Figure 6 Loss of Jnk1 activity in neuroblastoma cells during thiamine deficiency. Neuroblastoma cells were cultured in thiamine-deficient medium (tdm) containing 300 nM pyrithiamine. At the indicated times, aliquots were removed and cell lysates were prepared. (A) Jnk1 was immunoprecipitated using anti-Jnk1 antisera. The immunoprecipitates were used in assays for Jnk1 activity, with cJun-glutathione-S-transferase (GST) fusion protein as a substrate. Reactions were fractionated by gel electrophoresis, and the gel was subjected to autoradiography to detect labeled (phosphorylated) cJun-GST protein. (B) To determine the amount of Jnk1 protein, the gel was also subjected to Western analysis using anti-Jnk1 antisera. (C) No loss of Jnk1 activity was found in lymphoblasts cultured for 7 days in tdm plus 3 μM pyrithiamine (conditions that slow growth by 90% without causing cell death) when compared with lymphoblasts grown in control (C) medium. (D) Jnk2/3 were immunoprecipitated after immunodepletion of Jnk1 from lysates derived from neuroblastoma cells cultured for 7 days in either control medium (C) or in tdm plus 300 nM pyrithiamine. The immunoprecipitates were used in assays for Jnk2/3 activity, with cJun-GST fusion protein as a substrate.

although they found that part of the mitochondria remained functional.

There are other apoptosis mediators that can be released from mitochondria of some cell types, including procaspase 3[39] and apoptosis-induced factor (AIF).[40] Even though we observed a significant increase of caspase 3 activity, we have not examined the possibility that some of the increase is accounted for by procaspase release from mitochondria. It seems unlikely, however, that procaspase release is a major contributor to TD-induced apoptosis, because this would preclude a role for the loss of Jnk1 activity. AIF also would not probably be involved because cytosolic AIF causes the subsequent release of cytochrome c,[40] which was not

observed. Nonetheless, direct examination will be required to rule out an involvement of these and other mitochondrial apoptosis mediators.

The loss of Jnk1 activity was concurrent with the induction of apoptosis in neuroblastoma cells deprived of thiamine, and loss of activity was not found in non-apoptotic lymphoblasts under severe TD. A number of early studies on Jnk kinases indicated that this class of kinases function to induce apoptosis in response to a variety of cellular stresses.[41,42] More recently, anti-apoptotic functions for Jnk kinases have been found. Jnks provide cell survival signals for thymocytes,[43] hepatocytes,[44] neurons,[18,19,45] and other cell types[46] and are required for anti-apoptotic activity of hILP.[47] Recently, Kuan et al.[18] demonstrated both pro-apoptotic and anti-apoptotic roles for Jnk1 and Jnk2 during neuronal development, and their findings indicated that these kinases regulate regional specific apoptosis during brain development.

Our results are consistent with the anti-apoptotic function of Jnk1 and suggest that Jnk1 mediates cell survival signals in human neuroblastoma cells and perhaps in neurons of some regions selectively damaged by TD. We envision that depletion of thiamine places undue stress on the cell due to the loss of activity of αKGDH and perhaps other thiamine-requiring enzymes. The stress could be directly or indirectly related to the subsequent mitochondrial damage that is suffered, or it could be due to a reduction in critical neuroactive amino acids,[24,37] reduced cellular energy production,[1] or some other as yet unidentified induced stress of TD. In select cell types, TD-induced stress may lead to the inactivation of Jnk1 and the loss of its cellular protective function from programmed cell death. It is of interest to note that TD-induced apoptosis was observed in the thalamus but not in other regions affected during TD[10] and that Jnk1 is abundant in the thalamus relative to other brain regions (L. Chang, personal communication). We postulate that the TD-induced stress results in the loss of an activating signal for Jnk1. Interestingly, this signal seems specific for Jnk1 because there was no change in Jnk2/3 activity in TD versus thiamine sufficient culture conditions. The nature of the activating signal, its specificity for Jnk1, and its link to TD and/or to the loss of activity of αKGDH remain to be elucidated. Apoptosis is prominent in many neurodegenerative disorders[48] and during neuronal development.[18] Our culture system and the study of apoptosis in neuronal damage associated with TD provide new approaches for understanding the function of and the mechanisms underlying apoptosis in neurons.

Acknowledgment

We thank Peter Martin for critical comments on the manuscript.

References

1 Aikawa, H., Watanabe, I.S., and Furuse, T. (1984). Low energy levels in thiamine deficient encephalopathy. *J. Neuropath. Exp. Neur.* **43,** 276–287

2 Tronscoso, J.C., Johnston, M.V., and Hess, K.M. (1981). Model of Wernicke's encephalopathy. *Arch. Neurol.* **38,** 350–354

3 Victor, M., Davis, R.D., and Collins, G.H. (1989). *The Wernicke-Korsakoff syndrome and related neurological disorders due to alcoholism and malnutrition*. F.A. Davis Company, Philadelphia, PA USA

4 Torvik, A. (1987). Topographic distribution and severity of brain lesions in Wernike's encephalopathy. *Clin. Neuropathol.* **6,** 25–29

5 Hazell, A.S., Todd, K.G., and Butterworth, R.F. (1998). Mechanisms of neuronal cell death in Wernicke's encephalopathy. *Metab. Brain Dis.* **13,** 97–122

6 Gibson, G.E., Ksiezak-Reding, H., Sheu, K.-F.R., Mykytyn, V., and Blass, J.P. (1984). Correlation of enzymatic, metabolic, and behavioural deficits in thiamine deficiency and its reversal. *Neurochem. Res.* **9,** 803–814

7 Sheu, K.-F.R., Calingasan, N.Y., Lindsay, J.G., and Gibson, G.E. (1998). Immunochemical characterization of the deficiency of the alpha-ketoglutarate dehydrogenase complex in thiamine-deficient rat brain. *J. Neurochem.* **70,** 1143–1150

8 Butterworth, R.F., Kril, J., and Harper, C. (1993). Thiamine-dependent enzyme changes in the brains of alcoholics: Relationship to the Wernicke-Korsakoff syndrome. *Alcohol. Clin. Exp. Res.* **16,** 634–639

9 Butterworth, R.F. and Heroux, M. (1989). Effect of pyrithiamine treatment and subsequent thiamine rehabilitation on regional cerebral amino acids and thiamine dependent enzymes. *J. Neurochem.* **52,** 1079–1084

10 Matsushima, K., MacManus, J.P., and Hakim, A.M. (1997). Apoptosis is restricted to the thalamus in thiamine-deficient rats. *NeuroReport* **8,** 867–870

11 Hazell, A.S., McGahan, L., Tetzlaff, W., Bedard, A.M., Robertson, G.S., Nakabeppu, Y., and Hakim, A.M. (1998). Immediate-early gene expression in the brain of the thiamine-deficient rat. *J. Mol. Neurosci.* **10,** 1–15

12 Bettendorff, L., Goessens, G., Sluse, F., Wins, P., Bureau, M., Laschet, J., and Griasar, T. (1995). Thiamine deficiency in cultured neuroblastoma cells: Effects on mitochondrial function and peripheral benzodiazepine receptors. *J. Neurochem.* **64,** 2013–2021

13 Pekovich, S.R., Martin, P.R., Poggi, V., and Singleton, C.K. (1998). Sensitivity to thiamine deficiency in cultured human cells is dependent on cell type and is greatly increased in cells from thiamine-responsive megaloblastic anemia patients. *J. Nutr. Biochem.* **9,** 215–222

14 Schwartz, J.P. and McCandless, D.W. (1976). Glycolytic metabolism in cultured cells of the nervous system. The effects of thiamine deficiency on thiamine levels, metabolites, and thiamine-dependent enzymes of the C-6 glioma and C-1300 neuroblastoma cell lines. *Mol. Cell. Biochem.* **13,** 49–53

15 Zangen, A. and Shainberg, A. (1997). Thiamine deficiency in cardiac cells in culture. *Biochem. Pharmacol.* **54,** 575–582

16 Bettendorff, L. (1995). Thiamine homeostasis in neuroblastoma cells. *Neurochem. Int.* **26,** 295–302

17 Stagg, A.R., Fleming, J.C., Baker, M.A., Sakamoto, M., Cohen, N., and Neufeld, E.J. (1999). Defective high-affinity thiamine transporter leads to cell death in thiamine-responsive megaloblastic anemia syndrome fibroblasts. *J. Clin. Invest.* **103,** 723–729

18 Kuan, C.-Y., Ynag, D.D., Samata Roy, D.R., Daive, R.J., Rakic, P., and Flavell, R.A. (1999). The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* **22,** 667–676

19 Xu, X., Rabor, J., Yang, D., Su, B., and Mucke, L. (1997). Dynamic regulation of cJun amino terminal kinase activity in mouse brain by environmental stimuli. *Proc. Natl. Acad. Sci. USA* **94,** 12655–12660

20 Pekovich, S.R., Martin, P.R., and Singleton, C.K. (1996). Thiamine pyrophosphate-requiring enzymes are altered during pyrithiamine-induced thiamine deficiency in cultured human lymphoblasts. *J. Nutr.* **126,** 1791–1798

21 Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. *Cell* **86,** 147–157

22 Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Gene. Dev.* **7,** 2135–2148

23 Mehlen, P., Rabizadeh, S., Snipas, S.J., Assa-Munt, N., Salvesen, G.S., and Bredesen, D.E. (1998). The DCC gene product induces

apoptosis by a mechanism requiring receptor proteolysis. *Nature* **395,** 801–804
24 Heroux, M. and Butterworth, R.F. (1988). Reversible alterations of cerebral gamma-aminobutyric acid in pyrithiamine treated rats: Implications for the pathogenesis of Wernicke's encephalopathy. *J. Neurochem.* **51,** 1221–1226
25 Zhang, S.X., Weilersbacher, G.S., Henderson, S.W., Corso, T., Olney, J.W., and Langlais, P.J. (1995). Excitotoxic cytopathology, progression, and reversibility of thiamine deficiency-induced diencephalic lesions. *J. Neuropath. Exp. Neur.* **54,** 255–267
26 Green, G.R. and Reed, J.C. (1998). Mitochondria and apoptosis. *Science* **281,** 1309–1312
27 Newmeyer, D.D., Farschon, D.M., and Reed, J.C. (1994). Cell-free apoptosis in Xenopus egg extracts: Inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* **79,** 353–364
28 Qin, Z.-H., Wang, Y., and Chase, T.N. (1998). Nuclear factor-κB contributes to excitotoxin-induced aoptosis in rat striatum. *Mol. Pharmacol.* **53,** 33–42
29 Nakai, M., Qin, Z.-H., Wang, Y., and Chase, T.N. (1999). Free radical scavenger OPC-1417 attenuates quinolinic acid-induced NF-κB acitivation and apoptosis in rat striatum. *Mol. Brain Res.* **64,** 59–68
30 Savolainen, K.M., Loikkanen, J., Eerikainen, S., and Naarala, J. (1998). Interaction of excitatory neurotransmitters and xenobiotics in excitotoxicity and oxidative stress: Glutamate and lead. *Toxicol. Lett.* **102–103,** 363–367
31 Armstrong-James, M., Ross, D.T., Chen, F., and Ebner, F.F. (1988). The effect of thiamine deficiency on the structure and physiology of the rat forebrain. *Metab. Brain Dis.* **3,** 91–124
32 McEntee, W.J. (1997). Wernicke's encephalopathy: An excitotoxicity hypothesis. *Metab. Brain Disease* **12,** 183–192
33 Hazell, A.S., Butterworth, R.F., and Hakim, A.M. (1993). Cerebral vulnerability is associated with selective increase in extracellular glutamate concentration in experimental thiamine deficiency. *J. Neurochem.* **61,** 1155–1158
34 Langlais, P.J. and Zhang, S.X. (1993). Extracellular glutamate is increased in thalamus during thiamine deficiency-induced lesions and is blocked by MK-801. *J. Neurochem.* **61,** 2175–2182
35 Todd, K.G. and Butterworth, R.F. (1998). Evaluation of the role of NMDA-mediated excitotoxicity in the selective neuronal loss in experimental Wernike encephalopathy. *Exp. Neurol.* **149,** 130–138
36 Hakim, A.M. (1984). The induction and reversability of cerebral acidosis in thiamine deficiency. *Ann. Neurol.* **16,** 673–679
37 Gaitonde, M.D., Fayein, N.A., and Johnson, A.L. (1975). Decreased metabolism in vivo of glucose into amino acids of the brain of thiamine-deficient rats after treatment with pyrithiamine. *J. Neurochem.* **24,** 1215–1223
38 Bettendorff, L., Goessens, G., and Sluse, F.E. (1997). Reversibility of thiamine deficiency-induced partial necrosis and mitochondrial uncoupling by addition of thiamine to neuroblastoma cell suspensions. *Mol. Cell. Biochem.* **174,** 121–124
39 Mancini, M., Nicholson, D.W., Roy, S., Thornberry, N.A., Peterson, E.P., Casciola-Rosen, L.A., and Rosen, A. (1998). The caspase-3 precursor has a cytosolic and mitochondrial distribution: Implications for apoptotic signaling. *J. Cell Biol.* **140,** 1485–1495
40 Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M., and Kroemer, G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397,** 441–446
41 Miden, A. and Karin, M. (1997). Relation and function of the JNK subgroup of MAP kinases. *Biochim. Biophys. Acta* **F85–F104,** 1333–1347
42 Cosulich, S. and Clarke, P. (1996). Apoptosis: Does stress kill? *Curr. Biol.* **6,** 1586–1588
43 Nishina, H., Fischer, K.D., Radvanyi, L., Shahinian, A., Hakem, R., Rubie, E.A., Bernstein, A., Mak, T.W., Woodgett, J.R., and Penninger, J.M. (1997). Stress-signalling kinase Sek1 protects thymocytes from apoptosis mediated by CD95 and CD3. *Nature* **385,** 350–353
44 Nishina, H., Vaz, C., Billia, P., Nghiem, M., Sasaki, T., De la Pompa, J.L., Furlonger, K., Paige, C., Hui, C., Fischer, K.D., Kishimoto, H., Iwatsubo, T., Katada, T., Woodgett, J.R., and Penninger, J.M. (1999). Defective liver formation and liver cell apoptosis in mice lacking the stress signalling kinase SEK1/MKK4. *Development* **126,** 505–516
45 Luo, Y., Umegaki, H., Wang, X., Abe, R., and Roth, G.S. (1998). Dopamine induces apoptosis through an oxidation-involved SAPK/JNK activation pathway. *J. Biol. Chem.* **273,** 3756–3764
46 Yujiri, T., Sather, S., Fanger, G.R., and Johnson, G.L. (1998). Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. *Science* **282,** 1911–1914
47 Sanna, M.G., Duckett, C.S., Richter, B.W.M., Thompson, C.B., and Ulvitch, R.J. (1998). Selective activation of JNK1 is necessary for the anti-apoptotic activity of hILP. *Proc. Natl. Acad. Sci. USA* **95,** 6015–6020
48 Shoulson, I. (1998). Experimental therapeutics of neurodegenerative disorders: Unmet needs. *Science* **282,** 1072–1074