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## Pyridoxal Phosphate Inhibits Pituitary Cell Proliferation And Hormone Secretion

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### Abstract

Pyridoxal phosphate (PLP), a bio-active form of pyridoxine, (10-1000  $\mu$ M) dose-dependently inhibited cell proliferation in rat pituitary MMQ and GH3 cells, and in mouse AtT-20 cells. After 4 days, MMQ cell numbers were reduced by up to 81%, GH3 cell numbers were reduced by up to 64% ( $p < 0.05$ ) and AtT-20 cell numbers were reduced by up to 90 % respectively. Cell proliferation rates recovered and dose-dependently reverted to control levels after PLP withdrawal. After 4 days, PLP (400 and 1000  $\mu$ M) decreased H-3-thymidine incorporation by up to 71% ( $p < 0.05$ ). PLP (400-1000  $\mu$ M) reduced GH3 cell GH and PRL secretion, and AtT-20 cell ACTH secretion (adjusted for cell number) by  $\sim 70$  % after 2 days. 100  $\mu$ M PLP also inhibited PRL secretion (65%,  $p < 0.05$ ) in primary rat pituitary cells treated for 2 days. PLP decreased the percentage of AtT-20 and GH3 cells in S phase, and increased those in GO-G1 phase. Furthermore, PLP induced AtT-20 and GH3 cell apoptosis (28 vs 6,  $p < 0.05$ ; 26 vs 3,  $p < 0.05$ , respectively), and dose-dependently reduced content of the anti-apoptosis gene, Bcl-2. These results indicate that pharmacologic doses of PLP inhibit pituitary cell proliferation and hormone secretion, in part mediated through PLP-induced cell-cycle arrest and apoptosis. Pyridoxine may therefore be appropriate for testing as a relatively safe drug for adjuvant treatment of hormone-secreting pituitary adenomas.

### Keywords

Pyridoxal phosphate; Proliferation; GH; PRL; Pituitary; Apoptosis

### Introduction

Pyridoxal phosphate (PLP), a bio-active form of pyridoxine (vitamin B6) in the circulation and tissues, is a coenzyme for over 100 enzymatic reactions including decarboxylation and transamination (1). PLP serves multiple functions, and is a necessary nutrient for body growth, development, and overall health (1-3). Low vitamin B6 status results in impaired glucose, lipid and amino acid metabolism, and is also associated with some pathologic conditions and cancers (1).

Patients with breast (4), or colon (5,6), bladder (7), or laryngeal (8) cancers, and or Hodgkin's disease (1) have lower plasma PLP levels compared with healthy controls. In vitro studies have shown that pharmacologic doses of vitamin B6 (from 0.25 to 5 mM) inhibit cell proliferation and protein synthesis in HepG2 human hepatoma cells (9), human malignant melanoma (10),

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11), and human lymphocytes (12). Mice pretreated with pyridoxal followed by injection of B16 melanoma cells had a 62% reduction in tumor weight compared to controls (11). These results suggest that vitamin B6 may have potential use as an antineoplastic agent.

Administration of vitamin B6 (either 300 mg acute infusion or 400-600 mg orally daily for 2-3 months) to human volunteers (13-16) reduced circulating PRL levels (13,14,16), but PLP-induced GH reduction was only observed in acromegaly (14) and in infants (15). Vitamin B6-induced hormone suppression was not reproduced in other in vivo studies (17,18).

Mechanisms for the observed inhibition of cell proliferation and alteration of hormone secretion by vitamin B6 are unclear. We present results from in vitro studies showing that pharmacologic levels of PLP inhibit rodent pituitary cell growth and hormone secretion, mediated in part through apoptosis.

## Materials and Methods

**Cell cultures**—Normal rat pituitary tissues were obtained from adult Sprague-Dawley rats, following the guidelines of the Institutional Animal Care and Use Committee. Pituitary cells were prepared as described (19,20). Briefly, pituitary tissue was minced, and dissociated with 0.35% collagenase (Sigma, St. Louis, MO) and 0.15% hyaluronidase (Sigma) at 37 C for 45 min, followed by adding FBS (Gibco, Grand Island, NY) to neutralize enzymes. Rat pituitary cells were collected by centrifugation and incubated in DMEM (Gibco) containing 10% FBS. GH3 and MMQ rat pituitary cells and mouse AtT-20 pituitary cells were purchased from ATCC (Rockvill, MD). GH3 cells and MMQ cells were maintained in RPMI-1640 medium (Gibco) containing 15% house serum and 2.5% FBS. AtT-20 cells were maintained in DMEM medium with 10% FBS.

Pituitary cells were pre-incubated in maintenance medium for 48-72 h, starved in phenol-free RPMI-1640 medium with 0.3% BSA (Sigma, St, Louis, MO) for 6-12 h, followed by PLP (Sigma) treatments with varying doses for the times indicated. Stock solutions of PLP (200 mM) were prepared in 1 N HCl. The highest dose (1 mM) of PLP was prepared by dilution of stock with phenol-free RPMI-1640 medium with 2.5% FBS, in which the final concentration was 0.005 N HCl. Lower doses of PLP were prepared by further dilution of the highest dose solution with phenol-free RPMI-1640 medium with 2.5% FBS and 0.005 N HCl. Vehicle controls were treated with medium containing 0.005N HCl.

**Cell proliferation assays**—Cells were treated with PLP in 48- multiple well culture plates, conditioned medium was collected and stored at -20C until measurement of hormones, and treated cells were collected with (for GH3 and AtT-20 cells) or without (for MMQ cells) trypsinization of 0.05% trypsin EDTA (Gibco). Cell number was measured using a Coulter Counter (Beckman Coulter, Miami, FL). For recovery studies, cells were pretreated with PLP for 4 days and then re-exposed to maintenance medium without PLP. At the end of the experiments, cells were collected and counted.

Alternatively, cells were treated with PLP in multi-well X6 culture plates for 80 h, then further exposed to 0.5  $\mu$ Ci [ $^3$ H]-thymidine / well for 16 h. The medium was discarded and cells washed three times with Ca and Mg-free PBS (Gibco). Incorporated [ $^3$ H]-thymidine was measured by beta-counter.

**Hormone assay**—Growth hormone (GH) and prolactin (PRL) concentrations in culture media were measured by RIA using the National Hormone and Peptide Program (Dr. Parlow, Harbor-UCLA Medical Center, Torrance, CA)-provided immuno-reagents. GH and PRL were iodinated using the iodogen method (21). ACTH concentrations in AtT-20 cell culture medium were measured using a commercial RIA kit (ICN Diagnostics Inc. Costa Mesa, California).

**Cell-cycle analysis**—Cells were treated with different doses of PLP in multi-well X6 plates or 100x20 mm dishes for 96 h. After trypsinization,  $10^6$  cells were washed with 1X PBS buffer (Gibco), fixed in 3 ml 70% methanol, washed with staining buffer and resuspended in the staining buffer with 50  $\mu\text{g}/\text{ml}$  RNase A (Sigma) and 50  $\mu\text{g}/\text{ml}$  propidium iodide. Cell-cycle analysis was performed using fluorescence-activated cell sorting.

**Apoptosis analysis**—Cells were treated with or without 1 mM PLP in multi-well X6 culture plates for 72 h. Apoptosis was assessed using the annexin V-FITC apoptosis detection kit I (BD Biosciences Pharmingen, San Diego, CA). After trypsinization, cells were washed twice with PBS, suspended in binding buffer and stained with Annexin V-FITC and propidium iodide. Cells undergoing apoptosis were detected by flow cytometry.

**Western blotting**—After 48 hour PLP treatment, cells were lysed in lysis buffer (Cell Signaling, Beverly, MA) containing 20 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM  $\text{Na}_2\text{VO}_4$ , 1  $\mu\text{g}/\text{ml}$  leupeptin and 1 mM PMSF. After centrifugation, cell protein in the supernatant was quantified by the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA), equal amount (35  $\mu\text{g}$ ) were separated on 10% SDS-polyacrylamide gel by electrophoresis, and transferred to PVDF membranes. Nonspecific binding was blocked with 5% nonfat milk and 0.1% tween-20 in PBS (Sigma). Antibody against Bcl-2 (Santa Cruz), or actin (Sigma) was incubated overnight with membranes at 4 C. Protein bands were visualized using second antibody conjugated with horseradish peroxidase and the ECL detection kit (Amersham).

**Statistical analysis**—Results are presented as mean  $\pm$  SEM. Students t-test without (for two groups) or with (for multiple groups) Bonferroni correction was used to determine differences between groups.

## Results

**PLP inhibits GH3, MMQ, and AtT-20 cell proliferation**—PLP dose-dependently inhibits cell proliferation in MMQ and GH3 rat pituitary adenoma cells, as well as in mouse pituitary AtT-20 cells. Maximal inhibition of proliferation was achieved after four days of cell exposure to PLP (Fig.1), when MMQ cell numbers were reduced by 12 - 95% ( $p < 0.05$  for all) at doses of 10-1000  $\mu\text{M}$ , respectively. GH3 cells were reduced by 10 - 64% ( $p < 0.05$  for all), and AtT-20 cells by 7 - 90 % ( $p < 0.05$ , for all), at doses of 100 - 1000  $\mu\text{M}$ , respectively. After 4 days of PLP treatment (400, and 1000  $\mu\text{M}$ ), H-3-thymidine incorporation decreased by 33% and 52% in GH3 cells, and by 57% and 71% in AtT-20 cells, respectively. ( $p < 0.05$  for all) (Fig.2).

**Recovery of cell proliferation after PLP withdrawal**—During 7 days of PLP treatments, pituitary cells continue to grow. Exposure of cells to higher doses of PLP resulted in lower cell proliferation rates (Fig. 3). Even when exposed to 1 mM PLP for 7 days, cell numbers were maintained, suggesting that this high dose of PLP is not toxic to cells. To further test this, recovery studies were performed. After 4 days of PLP (from 100 to 1000  $\mu\text{M}$ ) treatments, GH3 or AtT-20 cells were incubated in maintenance medium without added PLP for 3-11 days. After withdrawal of PLP, cells regained their growth in a dose- and time-dependent manner (Fig. 4). Cells exposed to higher doses (1000  $\mu\text{M}$ ) of PLP required a longer time (9-11 days) to totally recover to control levels; when exposed to 100  $\mu\text{M}$  PLP, they only required 3-6 days for growth recovery.

**PLP inhibits hormone secretion**—PLP dose-dependently inhibited hormone secretion in MMQ, GH3, and AtT-20 cells, which was also confirmed after adjusting for cell number (Fig. 5). After 2 days of treatment, PLP (10 - 1000  $\mu\text{M}$ ) reduced PRL secretion per MMQ cell to 67

- 26 % ( $p < 0.05$ ) of vehicle- treated controls respectively. PRL levels per GH3 cell were reduced to 75 - 40 % ( $p < 0.05$ ) of controls, respectively, within 2 days of PLP treatment (100 -1000  $\mu\text{M}$ ). GH levels were suppressed to 53 - 31% ( $p < 0.05$ ), respectively, by the same treatments. ACTH levels per AtT-20 cell were reduced to  $56 - 32 \pm 14\%$  ( $p < 0.05$ ) of controls, respectively, after 2 days of PLP treatment (200 - 1000  $\mu\text{M}$ ).

PLP also dose-dependently inhibited PRL secretion from rat primary pituitary cultures. As shown in Fig. 6, 2 day PLP treatment (1-1000  $\mu\text{M}$ ) reduced PRL concentrations to 66 %, 48%, 35 % and 37% of controls ( $p < 0.05$  for all), respectively. In contrast, GH levels were not effected by PLP treatment in this system (Fig. 6). Cell numbers in rat primary pituitary cultures were reduced to  $82 \pm 9\%$  and  $59 \pm 6\%$  ( $p < 0.05$ ) of controls.

**PLP arrests the G1/S phase cell cycle transition.**—Cell cycle analysis from three independent experiments (Fig. 7) showed that 4 days of treatment with 1 mM PLP decreased the percentage of AtT-20 cells in S and G2-M phases (4.98 vs control 12.50,  $p = 0.0025$ ; and 0.65 vs 3.00,  $p = 0.0025$ , respectively). Similar results were observed in GH3 cells (13.90 vs control 30.00,  $p = 0.0025$ ; and 3.5 vs 5.33,  $p > 0.05$  respectively). The reductions in S-phase and G2-M fractions were associated with corresponding accumulations of cells in G1 (93.50 vs control 85.00,  $p = 0.001$ , for AtT-20 cells, 82.70 vs 68.90,  $p = 0.012$ , for GH3 cells).

The tendency of cell cycle arrest was also observed in two experiments of primary rat pituitary cell culture. PLP (10  $\mu\text{M}$ ) treatment for 48 h decreased the cell population in S phase (from 15.52 % for control to 8.34 %), and increased cells in G0-G1 (from 84.06% for control to 91.66 %).

**PLP induces apoptosis in pituitary cells.**—Three days treatment with 1 mM PLP increased the number of AtT-20 cells undergoing apoptosis (27.7% vs 6.0% for control,  $p = 0.05$ ), as well as in GH3 cells (25.5% vs 3.2 % for control,  $p = 0.005$ ). As shown in Fig. 8, increased apoptosis fraction was associated with decreased viable cell population after PLP treatment, suggesting that higher doses of PLP cause pituitary cell apoptosis.

**PLP reduces Bcl-2 content in pituitary cells**—Western blot showed that PLP reduced content of the anti-apoptosis gene Bcl-2 in GH3 cells after 2 days treatment. Bcl-2 content was reduced from 100% (control) to 52 % (10 $\mu\text{M}$ ), 50% (100  $\mu\text{M}$ ), and 22 % (1000  $\mu\text{M}$ ) ( $p < 0.05$  for all), respectively (Fig. 9).

## Discussion

This study demonstrates that pharmacological doses of pyridoxal phosphate, the biologically active form of vitamin B6, inhibits rodent pituitary tumor cell proliferation, consistent with literature reports of pyridoxine-induced anti-proliferative effects on other tumor cells (4-12). This effect was not caused by necrosis or potential toxic action of pyridoxine at high doses, because cell numbers were not significantly changed at the end of 7 days of exposure to 1 mM PLP, and cell growth recovered to normal levels after PLP withdrawal. PLP-induced inhibitory effects may be mediated through cell-cycle arrest and apoptosis, as suggested by our observations that (1) PLP treatment decreases the fraction of cells in S and G2-M phases, and increases G0-G1; (2) PLP increases the apoptotic population; and (3) PLP reduced content of the anti-apoptosis gene, Bcl-2.

In this in vitro study, however, we could not determine if our observed affects were due to direct or indirect actions of PLP. Vitamin B6 actions may be mediated indirectly through regulation of steroid hormone action (22,23). Estrogen-induced gene expression was reduced by 30% under conditions of elevated intracellular vitamin B6 concentrations, and was enhanced by 85% in vitamin deficiency (3). Estrogen induced increased incorporation of [3H]-thymidine

into estrogen receptor-positive breast cancer cells (24); pyridoxal (300  $\mu\text{M}$ ), however, prevented estrogen-induced cell proliferation activity. Anti-estrogens effectively inhibit cell growth and induce apoptosis in rat pituitary GH4 cells (25). Anti-proliferation activation by pyridoxal was, however, also observed in estrogen receptor-negative breast cancer cells, and expression of the estrogen-sensitive gene pS2 was not affected by pyridoxal treatment (24), suggesting that vitamin B6-mediated cell growth may also occur through a mechanism that appears to be steroid independent.

PLP-induced effects may also be indirectly mediated through one-carbon metabolism pathways. Low levels of vitamin B6, B12, and folate can impair one-carbon metabolism pathways, resulting in homocysteine accumulation, insufficient methyl groups for DNA methylation, and depletion of DNA synthesis and repair, which potentially promote carcinogenesis (26-30). Moreover, PLP is thought to be a potential precursor of sulfane sulfur, a highly reactive sulfur atom with a reduced oxidation state and anti-proliferation activity (31). PLP-induced anti carcinogenesis may also be mediated in vivo through improving immune-function (1,2,32), as well as anti-angiogenic effects (33,34).

This study also demonstrates that PLP inhibits GH and PRL secretion, which resulted not only from PLP-induced reduction of cell numbers, but also reduction of hormone secretion from individual cells treated with PLP (Fig. 5). In primary rat pituitary cells, PLP treatment inhibited PRL, but not GH secretion (Fig.6), likely because of reduced negative-feedback by IGF-I derived from fibroblasts. In primary rat pituitary cultures, proliferation of somatotrope and lactotrope cells ceases, as fibroblast cells grow with culture time. Therefore, PLP appears to inhibit fibrocyte proliferation and reduced IGF-1 secretion (our no-presented data showed that IGF-1 concentration in the culture media were reduced to 79 -70 % of control by 0.5-10 $\mu\text{M}$  PLP treatment for 6 days, and cell number and IGF-1 levels were significantly correlated), thus decreasing local negative feedback effects on GH secretion (35), resulting in maintenance of GH levels from primary somatotroph cells exposed to PLP. PLP may also inhibit IGF-1 secretion from other sources such as somatotrope and GH3 cells (36), but block of the autocrine-negative feedback seems to not effect PLP-induced inhibition of GH secretion in GH3 cells. The observed inhibition of hormone secretion might also be attributed to PLP-induced pituitary cell apoptosis.

Reports on effects of PLP on PRL and GH secretion in vivo have been contradictory (13-18). Vitamin B6 may act on neural function in vivo through its involvement as a cofactor for dopamine. The effect of vitamin B6 on circulating PRL and GH concentrations in human subjects were thought due to increased dopaminergic activity (37,38). L-dopa or levodopa-induced alteration of GH and PRL secretion in vivo were affected by pyridoxine infusion (39,40), and the effect of intravenous administration of vitamin B6 on circulating levels of human pituitary hormones was abolished by pretreatment with sulpiride, a dopamine receptor antagonist (41). The reason for the diverse responses to PLP observed in vivo are not clear, but may be due to different study designs and small sample sizes.

Pharmacologic doses of PLP used to inhibit cell proliferation in our study in vitro (0.01-1mM) and in reported references (0.25-5 mM) (9-12) are likely not achieved in vivo (42,43). Human plasma PLP levels increased 6 fold (0.5  $\mu\text{M}$ ) after administration of 100 mg pyridoxine-HCL per day (50-100 times recommended dose) orally for 1-3 weeks (42), but not significantly further elevated by excess dietary pyridoxine (43). Plasma PLP represents the major vitamin B6 available to tissues, because the inactive pyridoxine form can convert to PLP in some tissues (1). Reports of several subjects who received 2-4 g pyridoxine per day (1-4 thousand-fold recommended supplement dose) for 2-48 months developed severe sensory-nervous dysfunction (44,45), and was considered a selective dorsal root ganglion toxicity (45). PLP (0.5  $\mu\text{M}$ ) did not show anti-proliferation effects on human and murine cells in vitro (46). But,

4-10 fold the recommended pyridoxine diets did play roles in anti cell proliferation in vivo (47,48). Dietary supplemental pyridoxine (7 or > 7 mg/kg body weight) significantly reduced colon tumorigenesis and cell proliferation in mice receiving azoxymethane (47,48), and high-fat diet markedly enhances pyridoxine-induced inhibitory effect (49). In human studies, there is an association between cancer incidence and lower plasma PLP levels (4-8). These results suggest that pyridoxine doses required to inhibit tumor cell growth in vivo are much lower than those required in vitro. Inhibitory effects of pyridoxine on cell proliferation in vivo may be mediated through multiple pathways as discussed above; therefore, smaller doses of pyridoxine in vivo may achieve similar effects as do large doses in vitro. In vivo experiments are required to test the effect of lower doses of PLP on pituitary tumor growth and hormone secretion, which may provide further information about applying vitamin B6 in clinic settings. Thus, PLP is a potential anti tumor growth reagent, and may serve as an adjunct drug in treatment of GH- and PRL secreting adenoma growth and hormone secretion. PLP should also be considered as an added “cocktail” compound to potentiate available treatments for resistant pituitary adenomas. Optimal doses of pyridoxine applied clinically, however, remain to be determined.

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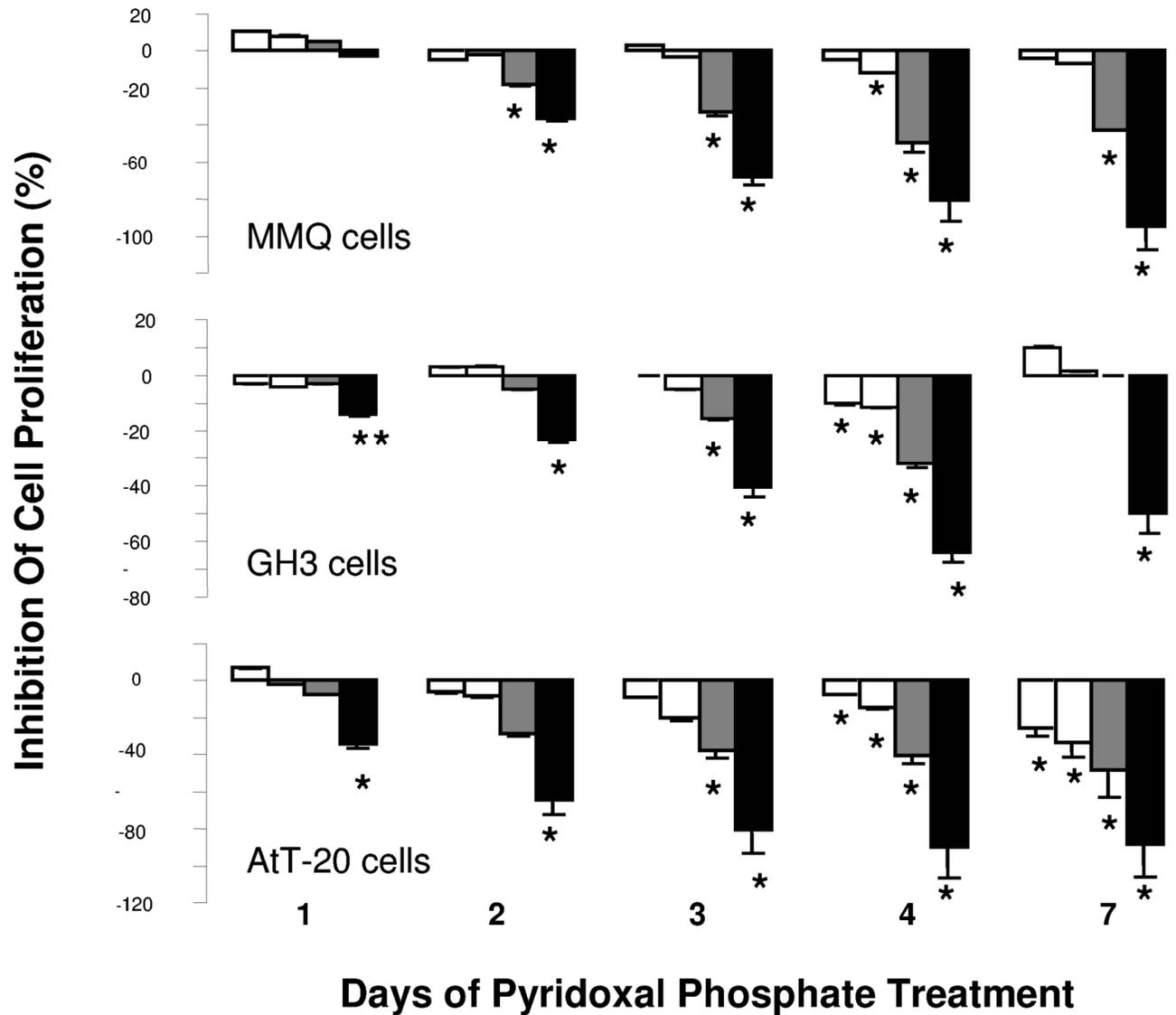
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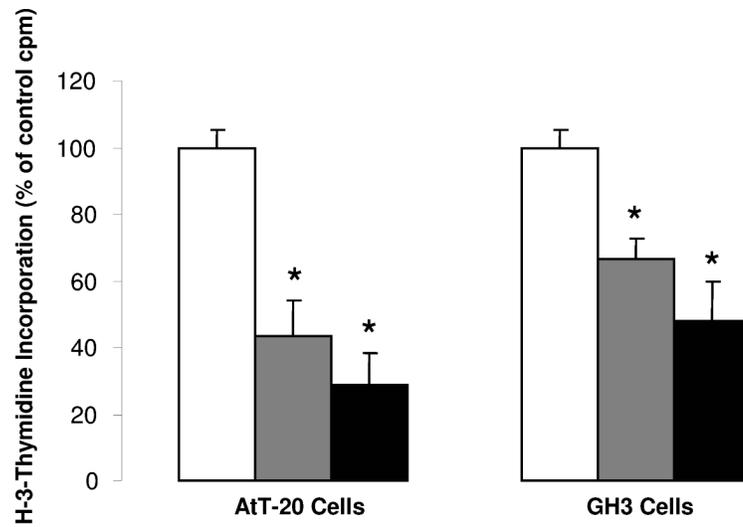
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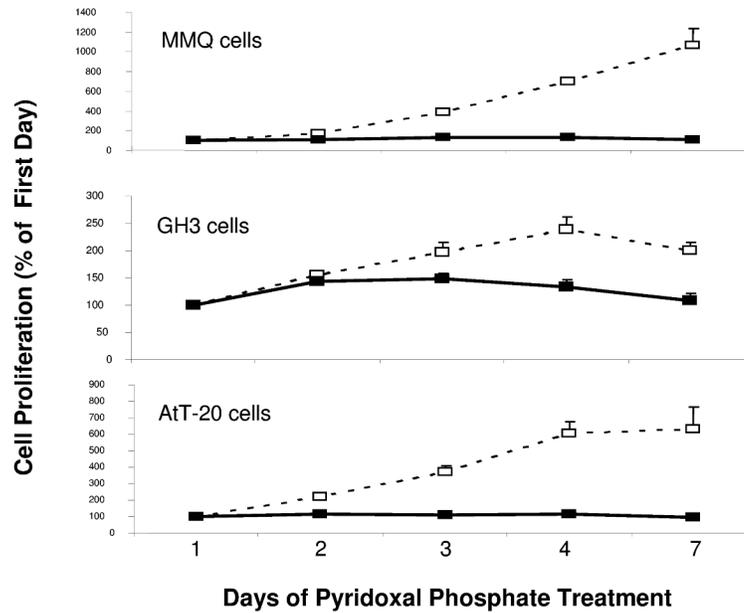
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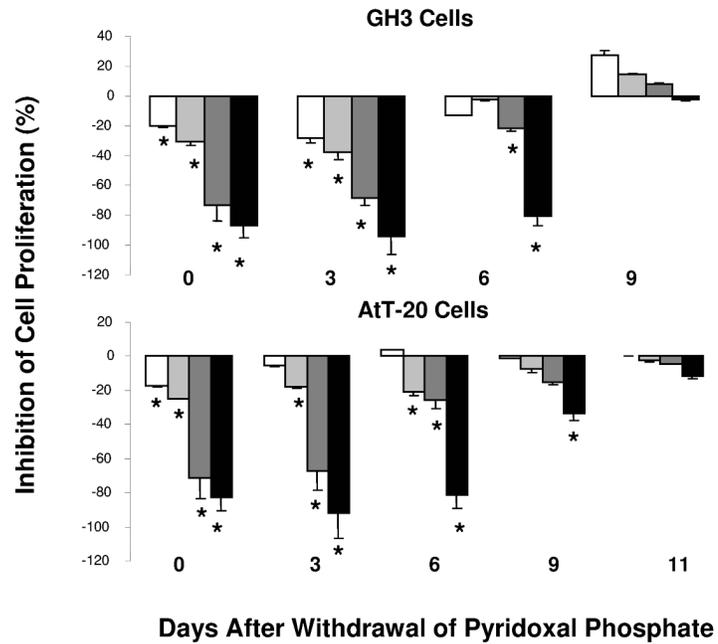
**Fig.1.** Time course and dose-response effects of PLP on proliferation of MMQ, GH3, and AtT-20 cells. Cells were treated with 1, 10, 100, and 1000  $\mu$ M (for MMQ cells) or 100, 200, 400 and 1000  $\mu$ M (for GH3 and AtT-20 cells) (bars from left to right) of PLP for 1-7 days. At the end of each incubation time, cells were counted. Results are presented as percentage inhibition of cell proliferation compared to control groups for the same incubation time. Each bar is mean  $\pm$  SEM of 12 wells in three independent experiments. \*  $p < 0.05$  vs control groups.



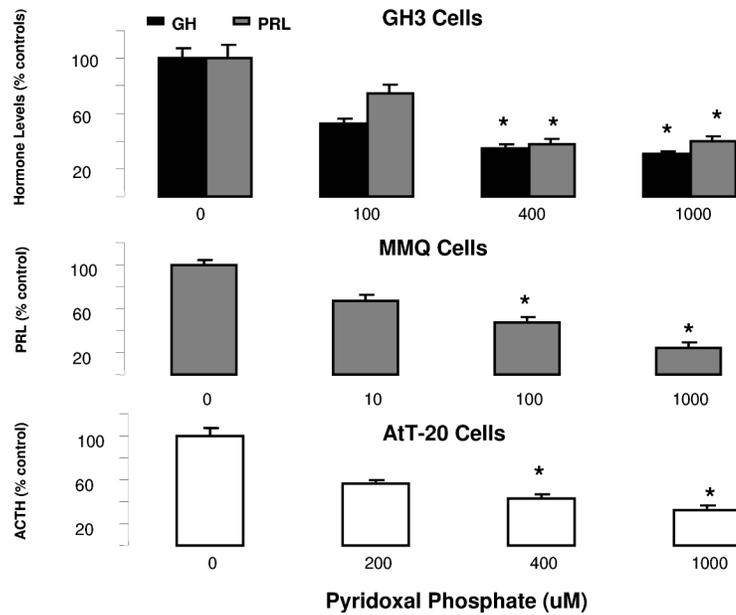
**Fig. 2.** Effects of PLP on 3-H-thymidine incorporation in AtT-20 and GH3 cells. AtT-20 or GH3 cells were treated for 96 h with PLP at doses of zero (control, open bar), 400  $\mu$ M (grey bar), and 1000  $\mu$ M (black bar) in multi-well X6 culture plates. 1  $\mu$ Ci H-3-thymidine was added to each well for final 16 h of treatment. H-3-thymidine incorporation was measured as described in “Materials and Methods”, and results presented as percent incorporation of control cpm. Each bar is mean  $\pm$  SEM of 8 wells in two experiments \*;  $p < 0.05$  vs controls..



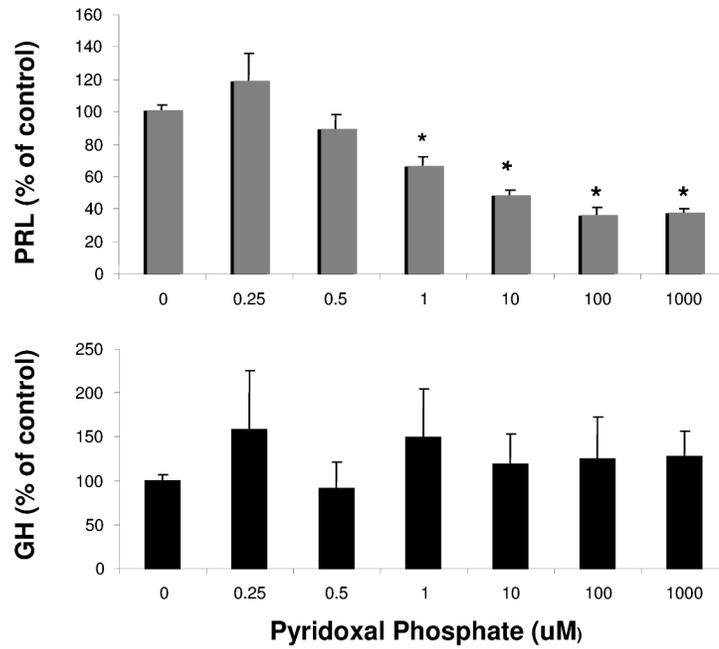
**Fig. 3.** Cell growth rates. Data depicted in this figure was obtained from experiments indicated in Figure 1. Results are presented as percent of cell numbers on the incubation day compared to values at the first day. Dash line for control and solid line for treated group with 1000  $\mu$ M PLP. Each point represents mean  $\pm$  SEM of 12 wells in three experiments.



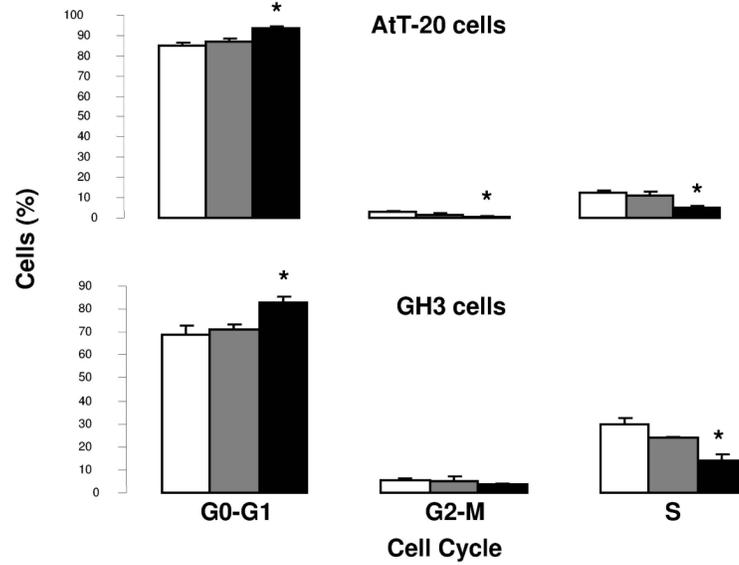
**Fig. 4.** Recovery of cell proliferation after withdrawal of PLP is dose-dependent. GH3 or AtT-20 cells were treated with 100, 200, 400 and 1000  $\mu$ M (bars from left to right) PLP for 4 days, then exposed to maintenance medium without PLP for the indicated times, and cells then counted. Results are presented as percent inhibition of cell proliferation compared to control groups at the same treatment times, respectively. Each bar is mean  $\pm$  SEM of 8 wells of two experiments. \*  $p < 0.05$  vs control groups.



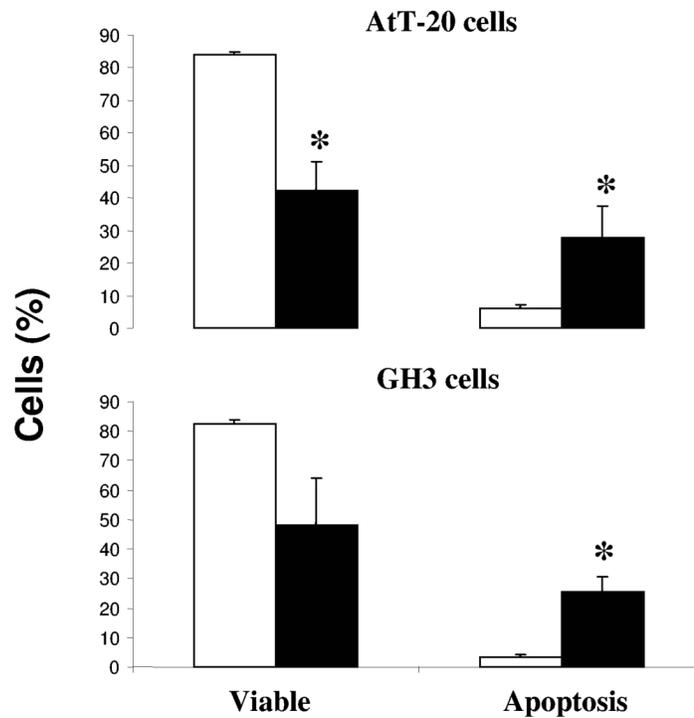
**Fig. 5.** Effects of PLP on hormone secretion in GH3, MMQ, and AtT-20 cells. Cells were treated with varying doses of PLP for 48 h. At the end of each experiment, medium concentrations of PRL, GH, or ACTH were measured by RIA, and cell number determined. Results are presented as percent of cell number-adjusted hormone levels of control groups. Each bar is mean  $\pm$  SEM of 12 wells in three experiments. \*  $p < 0.05$  vs controls.



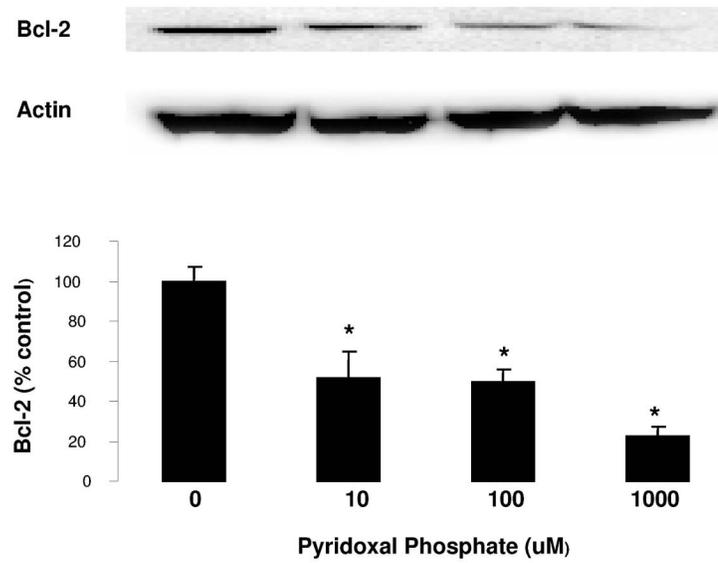
**Fig. 6.** Effects of PLP on hormone secretion in primary rat pituitary cells. Cells were treated with varying doses of PLP for two days, and hormone levels in the medium measured. Data are presented as percent of control levels. Each bar is mean  $\pm$  SEM of 12 wells from three experiments. \*  $p < 0.05$  vs controls.



**Fig. 7.** Effect of PLP on AtT-20 and GH3 cell cycle. AtT-20 and GH3 cells were treated for 4 days with PLP at doses of zero (open bar), 400  $\mu$ M (grey bar), and 1000  $\mu$ M (black bar). At the end of each experiment, the cell cycle was analyzed as described in “Materials and Methods”. Each bar depicts mean  $\pm$  SEM of 4-6 tests in three independent experiments. \*  $p < 0.05$  vs zero dose.



**Fig. 8.** PLP causes apoptosis in AtT-20 and GH3 cells. AtT-20 and GH3 cells were treated for 72 h without (control, open bar) or with 1000  $\mu$ M PLP (black bar). At the end of each experiment, cells were trypsinized, washed with PBS, resuspended in binding buffer, and stained with Annexin V-FITC and Propidium Iodide (BD Biosciences). Cells undergoing apoptosis were analyzed by flow cytometry. Each bar is mean  $\pm$  SEM of 2-3 tests in two independent experiments. \*  $p < 0.05$  vs controls.



**Fig. 9.** PLP reduces Bcl-2 content in GH3 cells. GH3 cells were treated with varying doses of PLP for 48 h. Equal amounts of lysed cell protein samples were applied for Western blotting. Each bar is mean  $\pm$  SEM of 4-5 blotting results (adjusted for actin) from five independent experiments. \*:  $p < 0.05$  vs control (dose zero). A representative Western blot is shown.