Pyridoxal Phosphate Inhibits Pituitary Cell Proliferation and Hormone Secretion

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Pyridoxal phosphate (PLP), a bioactive form of pyridoxine, dose-dependently (10–1000 μM) inhibited cell proliferation in rat pituitary MMQ and GH3 cells and in mouse AtT-20 cells. After 4 d, MMQ cell numbers were reduced by up to 81%, GH3 cell numbers were reduced by up to 50% (P < 0.05), and AtT-20 cell numbers were reduced by up to 90%. Cell proliferation rates recovered and dose-dependently reverted to control levels after PLP withdrawal. After 4 d, PLP (400 and 1000 μM) decreased [%H]thymidine incorporation by up to 71% (P < 0.05). PLP (400–1000 μM) reduced GH3 cell GH and prolactin secretion and AtT-20 cell ACTH secretion (adjusted for cell number) by approximately 70% after 2 d. The 100 μM PLP also inhibited prolactin secretion (65%, P < 0.05) in primary rat pituitary cells treated for 2 d. PLP decreased the percentage of AtT-20 and GH3 cells in S phase and increased those in G0–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 antiapoptosis gene Bcl-2. These results indicate that pharmacological 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. (Endocrinology 147: 3936–3942, 2006)

PYRIDOXAL PHOSPHATE (PLP), a bioactive 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 pathological conditions and cancers (1). Patients with breast (4), 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 pharmacological doses of vitamin B6 (from 0.25–5 mm) inhibit cell proliferation and protein synthesis in HepG2 human hepatoma cells (9), human melanoma cells had a 62% reduction in tumor weight compared with 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 prolactin (PRL) levels (13, 14, 16), but PLP-induced GH reduction was observed only 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 prolifera-

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Abbreviations: FBS, Fetal bovine serum; FITC, fluorescein isothiocyanate; PLP, pyridoxal phosphate; PRL, prolactin.

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Technologies). Cell number was measured using a Coulter Counter (Beckman Coulter, Miami, FL). For recovery studies, cells were pretreated with PLP for 4 d and then reexposed to maintenance medium without PLP. At the end of the experiments, cells were collected and counted.

Alternatively, cells were treated with PLP in six-well culture plates for 80 h and then exposed to 0.5 µCi [3H]thymidine per well for 16 h. The medium was discarded and cells washed three times with Ca- and Mg-free PBS (Life Technologies). Incorporated [3H]thymidine was measured by β-counter.

Hormone assay

GH and PRL concentrations in culture media were measured by RIA using immunoreagents provided by the National Hormone and Peptide Program (Dr. Parlow, Harbor-UCLA Medical Center, Torrance, CA). 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, CA).

Cell-cycle analysis

Cells were treated with different doses of PLP in six-well plates or 100 × 20-mm dishes for 96 h. After trypsinization, 106 cells were washed with 1× PBS buffer (Life Technologies), fixed in 3 ml 70% methanol, washed with staining buffer, and resuspended in the staining buffer with 50 µg/ml RNase A (Sigma) and 50 µg/ml propidium iodide. Cell-cycle analysis was performed using fluorescence-activated cell sorting.

Apoptosis analysis

Cells were treated with or without 1 µM PLP in six-well culture plates for 72 h. Apoptosis was assessed using the annexin V-fluorescein isothiocyanate (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 h PLP treatment, cells were lysed in lysis buffer (Cell Signaling, Beverly, MA) containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation, cell protein in the supernatant was quantified by the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA), and equal amounts (35 µg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Nonspecific binding was blocked with 5% nonfat milk and 0.1% Tween 20 in PBS (Sigma). Antibody against Bcl-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 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 Biosciences, part of GE Healthcare, Piscataway, NJ).

Statistical analysis

Results are presented as mean ± SEM. Student’s 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 4 d 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 µM, 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 µM (for GH3 cells) or 100, 200, 400, and 1000 µM (for GH3 and AtT-20 cells) (bars from left to right) of PLP for 1–7 d. At the end of each incubation time, cells were counted. Results are presented as percent inhibition of cell proliferation compared with control groups for the same incubation time. Each bar is mean ± SEM of 12 wells in three independent experiments. *, P < 0.05 vs. control groups. 0.05 for all) at doses of 100–1000 µM, respectively. After 4 d of PLP treatment (400 and 1000 µM), [3H]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 d of PLP treatments, pituitary cells continued 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 d, 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 d of PLP (from 100–1000 μM) treatments, GH3 or AtT-20 cells were incubated in maintenance medium without added PLP for 3–11 d. After withdrawal of PLP, cells regained their growth in a dose- and time-dependent manner (Fig. 4). Cells exposed to higher doses (1000 μM) of PLP required a longer time (9–11 d) to totally recover to control levels; when exposed to 100 μM PLP, they required only 3–6 d 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 d of treatment, PLP (10–1000 μM) 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 53–31% (P < 0.05), by the same treatments. ACTH levels per AtT-20 cell were reduced to 56–32% (P < 0.05) of controls, respectively, after 2 d of PLP treatment (200–1000 μM).

PLP also dose-dependently inhibited PRL secretion from rat primary pituitary cultures. As shown in Fig. 6, 2 d of PLP treatment (1–1000 μM) reduced PRL concentrations to 66, 48, 35, and 37% of controls (P < 0.05 for all), respectively. In contrast, GH levels were not affected by PLP treatment in this system (Fig. 6). Cell numbers in rat primary pituitary cultures were reduced to 82 ± 9 and 59 ± 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 d 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 μM) 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 of treatment with 1 mM PLP increased the number of AtT-20 cells undergoing apoptosis (27.7 vs. 6.0% for control, P = 0.005) 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 d of treatment. Bcl-2

![Fig. 3. Cell growth rates. Data depicted in this figure were obtained from experiments indicated in Fig. 1. Results are presented as percentage of cell numbers on the incubation day compared with values at the first day. Dashed line, control; solid line, treated group (1000 μM PLP). Each point represents mean ± SEM of 12 wells in three experiments.](image-url)
content was reduced from 100% (control) to 52% (1000 μM (bars from left to right) PLP for 4 days and then exposed to maintenance medium without PLP for the indicated times, and cells were then counted. Results are presented as percent inhibition of cell proliferation compared with control groups at the same treatment times, respectively. Each bar is mean ± SEM of eight wells of two experiments. *, P < 0.05 vs. control groups.

**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 antiproliferative 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 antiapoptosis gene Bcl-2.

In this *in vitro* study, however, we could not determine whether our observed effects were a result of 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 μM), however, prevented estrogen-induced cell proliferation activity. Antiestrogens effectively inhibit cell growth and induce apoptosis in rat pituitary GH4 cells (25).

**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 μM (bars from left to right) PLP for 4 days and then exposed to maintenance medium without PLP for the indicated times, and cells were then counted. Results are presented as percent inhibition of cell proliferation compared with control groups at the same treatment times, respectively. Each bar is mean ± SEM of eight 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 hours. At the end of each experiment, medium concentrations of PRL, GH, or ACTH were measured by RIA, and cell number was determined. Results are presented as percentage of cell-number-adjusted hormone levels of control groups. Each bar is mean ± SEM of 12 wells in three experiments. *, P < 0.05 vs. controls.

Proliferative 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

**Fig. 6.** Effects of PLP on hormone secretion in primary rat pituitary cells. Cells were treated with varying doses of PLP for 2 days, and hormone levels in the medium were measured. Data are presented as percentage of control levels. Each bar is mean ± SEM of 12 wells from three experiments. *, P < 0.05 vs. controls.
vitamin B_6, B_12, 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 antiproliferation activity (31). PLP-induced anti-carcinogenesis may also be mediated in vivo through improving immune-function (1, 2, 32) as well as antiangiogenic 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 reduce IGF-I secretion (our data, which is not presented), showed that IGF-I concentration in the culture media was reduced to 79–70% of control by 0.5–10 μM PLP treatment for 6 d, and cell number and IGF-I levels were significantly correlated), thus decreasing local negative-feedback effects on GH secretion (35), resulting in maintenance of GH levels from primary somatotrope cells exposed to PLP. PLP may also inhibit IGF-I 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 B_6 may act on neuronal function in vivo through its involvement as a cofactor for dopamine. The effect of vitamin B_6 on circulating PRL and GH concentrations in human subjects were thought to be a
result of 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 iv administration of vitamin B6 on circulating levels of human pituitary hormones was abolished by pretreatment with sulpiride, a dopamine receptor antagonist (41). The reasons for the diverse responses to PLP observed in vivo are not clear but may be a result of different study designs and small sample sizes.

Pharmacological doses of PLP used to inhibit cell proliferation in our study in vitro (0.01–1 mm) 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 μM) after administration of 100 mg pyridoxine-HCl per day (50–100 times recommended dose) orally for 1–3 wk (42) but were not significantly additionally 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). There are reports of several subjects who received 2–4 g pyridoxine per day above; therefore, smaller doses of pyridoxine 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 additional 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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