Long-term prednisolone treatments increase bioactive vitamin B6 synthesis in vivo

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[Running title] Prednisolone induces bioactive vitamin B6 synthesis \textit{in vivo}

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pyridoxal 5’-phosphate (PLP)

pyridoxal (PL)

4-pyridoxic acid (PA)

rheumatoid arthritis (RA)

disease-modifying antirheumatic drugs (DMARDs)

glutamic-pyruvic transaminase (GPT)

pyridoxine kinase (PDXK)

pyridoxamine-5’-phosphate (PMP)

pyridoxamine-5’-phosphate oxidase (PMPO).

pyrixoxine (PN)

adrenocorticotropic hormone (ACTH)

pyridoxamine (PM)

Pyridoxal-5’-phosphate phosphatase (PDXP)
Abstract

Objective. The etiology of vitamin B6 depletion in inflammation remains unknown. Hepatic vitamin B6 decreased in adrenalectomized rats and such reductions were restored by an acute muscle injection of very high-dose of glucocorticoids. We tested the hypothesis that long-term prednisolone treatment for treating inflammation restores vitamin B6 status by induction of tissue B6 metabolic enzymes. Design. Two independent in vivo models. Lewis rats and C57BL/6J mice received predisolone regimens that reflected clinical prednisolone uses in treating human inflammation. Novel findings. 1) Prednisolone increased circulating B6 vitamer pyridoxal 5’-phosphate (PLP, bioactive B6 vitamer), pyridoxal (PL), and 4-pyridoxic acid (PA) without altering vitamin B6 excretion. 2) Prednisolone simultaneously induced the hepatic PLP synthesizing enzyme pyridoxine kinase (PDXK) and pyridoxamine-5’-phosphate oxidase (PMPO) and suppressed PLP catabolic enzyme pyridoxal-5’-phosphate phosphatase (PDXP). 3) Elevations in circulating PL were due to its release from the liver, not due to PLP dephosphorylation (PDXP was suppressed and alkaline phosphatase was unaltered). Conclusions. Long-term prednisolone treatments promoted hepatic bioactive vitamin B6 synthesis by inducing the synthesizing enzyme PDXK, PMPO and simultaneously suppressing the catabolic enzyme PDXP. Prednisolone increased circulating B6 vitamer without altering urinary B6 excretion. As the major form of vitamin B6 across cell membrane, elevated circulating PL may facilitate the cellular uptake and
utilization of B6. The elevated plasma PLP may increase vitamin B6 supply to tissues with
a higher B6 demand during inflammation. Results from 2 independent \textit{in vivo} models
suggested potential advantage of clinical prednisolone use in treating inflammation with
respect to vitamin B6 status.
**Introduction**

While vitamin B6 depletion has long been associated with chronic inflammation, the etiology for this abnormality remains to be established. The regulation of tissue vitamin B6 status is dependent upon the intake, metabolism, transport in the blood, uptake mechanisms, binding to proteins and the activities of its metabolic enzymes (Lumeng, et al., 1974; Bosron, et al., 1978). Prednisolone is a corticosteroid commonly used to treat a wide variety of chronic disorders, including rheumatoid arthritis (RA), asthma, systemic lupus erythematosus, allergic diseases, hepatitis, and many other inflammatory conditions (Francisco, et al., 1984; Pickup, 1979). When the disease cannot be effectively controlled by other disease-modifying antirheumatic drugs (DMARDs), low-dose prednisolone (≤15 mg daily) is commonly used intermittently in patients with RA (Gotzsche & Johansen, 2004). Back in the 1950s, a few animal studies indicated that acute subcutaneous injection of high-dose adrenal corticosteroids increased the activity of liver B6-dependent enzyme glutamic-pyruvic transaminase (GPT). We speculated that corticosteroids induce GPT via interference with vitamin B6 metabolism; as such induction was not seen in vitamin B6 deficient animals (Gavosto, et al., 1957; Rosen, et al., 1959b; Rosen, et al., 1959a; Eisenstein, 1960)

Pyridoxal-5’-phosphate (PLP) is the major phosphorylated bioactive form of vitamin B6 which serves as an important cofactor for more than 100 biochemical reactions. In the liver, PLP can be synthesized from the nonphosphorylated pyridoxal (PL) by pyridoxine kinase
(PDXK) or can be converted from pyridoxamine-5'-phosphate (PMP) by pyridoxamine-5'-phosphate oxidase (PMPO). Muscle is the largest vitamin storage site for vitamin B6; and the glycogen phosphorylase-bound PLP is the major vitamin B6 present in the muscle (Krebs & Fisher, 1964). The role of erythrocyte in the metabolism and transport of vitamin B6 remains to be established. In the erythrocytes, PL and pyrixoxine (PN) are easily taken up by simple diffusion; they are then converted to PLP by kinase and oxidase (Mehansho & Henderson, 1980). The tight binding of PLP and PL to hemoglobin (Fonda and Harker, 1982; Ink, et al., 1982) makes erythrocyte a potential vitamin B6 reservoir in the circulatory system. PL is irreversibly converted to 4-pyridoxic acid (4-PA), the end product of vitamin B6 metabolism that is excreted in urine. In the 1960s, a rat study discovered that both hepatic PLP level and PMPO activity decreased in adrenalectomized rats; such reductions were restored by an acute muscle injection of very high-dose of glucocorticoids that included cortisone, hydrocortisone, deoxycorticosterone and prednisolone (2.5 mg per rat daily). On the other hand, administration of adrenocorticotropic hormone (ACTH) decreased PMP level and increased PL and pyridoxamine (PM) concentrations in the adrenal tissue in swine (Mahuren, et al., 1999). Pyridoxal-5'-phosphate phosphatase (PDXP) activity increased ten-fold in the ACTH-treated pigs (Mahuren, et al., 1999). While these earlier studies implied that high-dose glucocorticoids may interfere with vitamin B6 synthesis and catabolism, the vitamin B6 homeostasis in humans on long-term glucocorticoid therapies...
remained to be investigated. We hypothesized that long-term prednisolone treatments help restore vitamin B6 homeostasis by inducing vitamin B6 synthesizing enzyme PDXK and PMPO and/or suppressing B6 catabolic enzyme PDXP.

The doses used in the above mentioned animal studies are much higher than those used in human chronic inflammation; such doses would only be used in acute steroid pulse therapy in human patients. According to the established converting factor between rat (i.m.) and human (oral), a daily i.m. dose at 16.7-33.3 mg/kg/day in rats would be comparable to a human daily oral dose from 645 to 1290 mg. However, in our clinical studies, the mean daily oral dose of prednisolone used in human chronic inflammation ranged from 1.07 to 35 mg (median daily dose is ~5 mg)(Chiang, et al., 2003b, Chen, et al., 2011).

Hormonal alterations other than cortisols may also affect vitamin B6 homeostasis. Administration of follicle-stimulating hormone to normal rats increased PMPO activity in both liver and kidney, resulting in increased PLP levels in these tissues. Luteinizing hormone administration resulted in diminished PLP level in the tissues by decreasing the activity of PMPO (Chatterjee, 1980). Although indirect evidence suggests that acute and very high dose glucocorticoids may affect vitamin B6 homeostasis, it is not known if long-term clinical doses of prednisolone used in treating human inflammation impacts vitamin B6 metabolism. As a commonly used anti-inflammatory DMARD in arthritis, long-term prednisolone treatments may help restore vitamin B6 homeostasis in subjects with
chronic inflammation. Alternatively, prednisolone may interfere with normal vitamin B6 metabolism and partially account for the abnormal vitamin B6 status in patients with inflammation. This study systematically investigated the impact of long-term physiological doses of prednisolone on vitamin B6 profiles as well as the regulation of prednisone on vitamin B6 metabolic enzymes in vivo.

Material & Methods

Animal and diet

The present study was approved by the Institutional Animal Care and Use Committee of National Chung Hsing University, Taichung, Taiwan (IACUC 95-09). Two separate animal studies were performed to examine the effects of super-physiological high dose or long-term pharmacological prednisolone uses on vitamin B6 metabolism.

Three-week-old Lewis rats and C57BL/6J mice were obtained from the National Laboratory Animal Center (NLAC, Taipei, Taiwan). In the first experiment (Study I) we utilized the blood and tissue samples from an earlier unpublished rat experiment as a pilot experiment. In study I, female healthy Lewis rats were treated with a super-physiological high dose of prednisolone (10 mg/kg/day) for 35 days. This dose has been shown to affectively ameliorate the collagen induced arthritis in rodents (Joosten, et al., 1999). Rats were divided into two treatment groups according to the initial body weight after 7 days adaptation in the facility, then animals were housed individually in metal cages and each rat received the same
amount of food (AIN-93G, Dyets, Bethlehem, PA) by a group pair-feeding protocol (Chiang et al., 2005) in order to minimize variations in vitamin B₆ consumption or body weight caused by differences in dietary intake. In study II, C57BL/6J mice were treated with long-term (27 weeks) physiological doses (0.1 mg/kg/2 day or at 1 mg/kg/2 day) that comparable to human clinical use. Five mice were housed in filter top cages with water and food provided ad libitum. Some researchers advocated the use of amino acid defined diets to avoid lot-to-lot variability in the content of methionine and other amino acids in the protein source (Schwahn, et al., 2004). In study II, we used an amino acid-defined diet to carefully control dietary supplies of amino acid and vitamins. This approach is also helpful when we need to compare results among different studies on folate and vitamin metabolism (Wang, et al., 2011). In study II, mice were fed a modified Clifford/Koury amino acid-defined rodent diet containing the RDA for vitamin B6 for rodents (7 mg/Kg pyridoxine-HCl) (Dyets, Bethlehem, PA). All animals were maintained in a temperature and humidity-controlled condition (20~25 °C) with 12 h light/12 h dark cycle.

Clinical relevance of prednisolone regimens

Prednisolone (P-6004) was purchased from Sigma Chemicals (St Louis, MO, USA), and dissolved in phosphate buffered saline with 0.01% DMSO. The doses and durations were chosen based on those used in humans followed the definition by Buttgereit et al (Buttgereit et al., 2002). In study I, rats were treated once a day with normal saline (control
group, n=6) or super physiological dose of prednisolone (10 mg/kg/day, n=6) via gastric gavages for 35 days. In study II, mice received long-term physiological doses of prednisolone. Mice were evenly divided into 3 groups by body weight and treated once on alternate days with 1) PBS (control group, n=5); or 2) 0.1 mg prednisolone/kg/2days (n=5); or 3) 1 mg prednisolone/kg/2 days (n=5) i.p. with prednisolone for 27 weeks. Based on the life expectancy which would reflect >20 years of long-term clinical use of prednisolone treatment in humans. Prednisolone is generally administered to arthritis patients at a dosage of 7.5-35 mg/week (Chiang, et al., 2003b). More than one-tenth of the rheumatoid arthritis patients we recruited from the outpatient clinic have been taking low-dose prednisolone for more than 10 years (Chen, et al., 2011). Among them more than 6% have taking prednisolone for more than 20 years (Chen, et al., 2011). According to the “Guideline for the timing of non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals” (ICH International Conference, 1998), studies that continue for longer than 10% of a test subject’s life span are considered chronic. Plasma B6 profiles and B6 metabolic enzymes in tissues were determined.

**Blood and tissues collection**

In study I, blood was collected from sinus orbital vein with anticoagulant for analyzing plasma B6 profiles at baseline and 35 days after the prednisolone treatment started. Animals were fasted overnight and sacrificed on day 36. In study II, blood samples were
collected 10 and 22 weeks after the treatment started for determining the effects of long-term prednisolone treatments on vitamin B6 profiles. Animals were sacrificed 27 weeks after prednisolone treatment started. Plasma was collected after over-night fasting and stored at -80°C for analysis of B6 vitamers and alkaline phosphatase activity. Plasma was precipitated with 5% trichloroacetic acid for deproteinization before HPLC analysis (Chiang, et al., 2003a).

The freshly packed red blood cells were washed twice with phosphate buffered saline, followed by addition of equal volume of 0.67 M perchloric acid for protein precipitation (Chiang, et al., 2005a). The supernatants were stored at -80°C until analysis. Liver, brain, kidney, heart and gastrocnemius muscle were immediately excised and weighed after animals were sacrificed by cardiac puncture under anesthesia. All tissue samples were stored in the liquid nitrogen until analysis.

**Western Blotting**

Approximately 0.03g of tissue was homogenized in ten volumes of RIPA buffer containing 0.33% (v/v) phosphatase inhibitor cocktail 1, 2 (Sigma, St. Louis, MO, USA) and 0.1% (v/v) protease inhibitor cocktail Set 1 (Calbiochem, La Jolla, CA, USA). The protein content was quantified by BCA Protein Assay (Pierce, Rockford, IL, USA). Protein lysates from each tissue were denatured in and then separated on a 12% SDS-PAGE gel using a Minigel apparatus, and transferred onto a PVDF membrane using a transfer cell (Bio-Rad, Hercules, CA, USA). After blocking with TBS containing with 10% skim milk, the
membranes were incubated with the primary antibody including anti-PDXP (1:1000) antibody (Cell Signaling, Danvers, MA), anti-PDXK (1:1000) and anti-PMPO (1:1000) antibody (Abnova, Taipei, Taiwan). Membranes were washed three times with TBS containing 0.1% Tween 20 (TBST) and then covered with HRP-linked anti-mouse or rabbit IgG (1:5000) at room temperature for 2 h. The immunoblots were visualized by enhanced chemiluminescence kit (New England Biolabs, Beverly, MA, USA). To ensure equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody.

**RNA isolation and Real-time polymerase chain reaction**

Total RNA was isolated and the integrity was checked by electrophoresis. Two μg of liver whole-cell RNA was reverse-transcribed using oligo dT as primer and M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Gene expression was determined by using SYBR Green I on the quantitative real-time PCR ABI7000 (Applied Biosystem Inc., Foster City, CA, USA). The expression of each gene was calculated by normalizing the threshold cycle value of target gene to that of the control housekeeping gene.

**Determinations of vitamin B6 profiles in plasma, red blood cells and tissues**

We have modified the pre-column semicarbazide derivatisation HPLC method from Talwar et al (Talwar et al., 2003) with additional detection of PMP and optimized this procedure in plasma, erythrocytes and tissues (Chiang et al unpublished data). To measure the tissue vitamin B6 concentrations, animal tissue (~0.03 g) was homogenized in nine
volumes of 0.4 M ice-cold perchloric acid (Chiang, et al., 2007). The extracts were kept on ice for 30 minutes then centrifuged at 12000 rpm for 10 minutes at 4°C, and the supernatants were frozen in -80°C until analyses (Chiang, et al., 2009). The detailed HPLC procedure will be described elsewhere.

**Analyses of tissue PL kinase, PMP (PNP) oxidase and PLP phosphatase activities**

The tissue samples were prepared following the procedure previously described (Wada & Snell, 1961) for enzyme activity analyses. The activities of PDXK, PMPO, and PDXP were determined at pH 7.4 according to the procedure of Ubbink and Schnell (Ubbink & Schnell, 1988).

**Determination of urinary vitamin B6 concentrations**

Urinary 4-PA levels were analyzed by isocratic reversed-phased HPLC described by Gregory *et al* (Gregory, III & Kirk, 1979). The mobile phase consisted of 60 mmol/L disodium hydrogen phosphate and 400 mg/L EDTA disodium salt (9.5% methanol, v/v), pH 5.5. The wavelengths for fluorometric detection were 320 nm for excitation and 420 nm for emission.

**Determination of plasma alkaline phosphatase activity**

The activity of plasma alkaline phosphatase was measured using a Randox reagent kit and a Spectronic Genesys5 spectrophotometer (Thermo, Madison, WI, USA).
**Statistical analysis**

All data were reported as means ± SD. Comparisons of means between the control and the Prednisolone treatment groups were determined using the Student’s t-test. Results are expressed as mean ± SD. A Pearson correlation matrix was performed to examine correlations between continuous variables (B6 vitamers). A significant correlation was defined as P <0.05. All statistical analyses were performed using Systat 11.0 for Windows (Systat Software Inc., Richmond, CA, USA).

**Results**

*Super-physiological high dose prednisolone increased plasma PLP and PA and up-regulated vitamin B6 metabolic enzymes in rats*

In study I, prednisolone at the dose of 10 mg/kg/day significantly increased plasma PLP and PA concentrations by 37% and 59%, respectively (Table 1A). Hepatic PDXP and PDXK gene expressions were drastically induced by 18 folds and 2 folds, and PDXK enzyme activity was increased by 73% (Table 1B). Results from this experiment suggested that high dose prednisolone induced vitamin B6 enzyme expression and activity, leading to elevated plasma PLP and PA levels.
Long-term pharmacological prednisolone use altered circulating B6 vitamers and urinary excretion of vitamin B6

To further investigate the effects of long-term pharmacological prednisolone use on vitamin B6 metabolism, B6 profiles and vitamin B6 metabolic enzymes were investigated in mice received 0.1 or 1 mg/kg/2d prednisolone for 27 weeks. Body weight and food intake did not differ between control mice and prednisolone treated mice throughout the study period (data not shown). At week 10, prednisolone (1 mg/kg/2d) increased plasma PLP and PA concentrations by 36 and 107%, respectively (Table 2A). At week 22, prednisolone at the dose of 0.1 mg/kg/2d tended to increase plasma PLP concentration (by ~30%, p=0.075). At the dose of 1 mg/kg/2d, prednisolone drastically increased plasma PLP, PL, and PA by 73%, 45%, and 207%, respectively (p<0.05) (Table 2B). Furthermore, plasma concentrations of PLP, PL or PA closely correlated with each other in prednisolone treated mice (PLP vs. PL, r= 0.81 p=0.005; PLP vs. PA, r=0.68 p=0.031 PL vs. PA, r=0.79 p=0.015, n=10), yet these B6 vitamers did not correlate in the untreated control mice. At week 22, plasma alkaline phosphatase levels were similar among control mice and mice received 0.1 mg/kg/2d or 1 mg/kg/2d of prednisolone (Table 2C), suggesting that the increased plasma PLP level observed was independent of alkaline phosphatase status, a known significant determinant for phosphorylated form for B6 vitamers. Long-term pharmacological prednisolone treatments did not alter steady state B6 vitamers in erythrocyte (Table 3A) or urinary excretion of the
vitamin B6 end product (Table 3B). We conclude that long-term prednisolone use increased plasma B6 levels without altering B6 intake or excretion, and the effects of prednisolone could be tissue specific.

*Long-term pharmacological prednisolone uses selectively alter vitamin B6 status in extra hepatic tissues*

In the muscle, concentrations of PMP and PLP did not differ between control and prednisolone treated mice, but PL concentrations were dose-dependently increased by prednisolone (Table 4A). There was also a trend of increased PL in the heart (Table 4B), but B6 vitamers was unchanged in the brain or kidney (Table 4C-D). These data indicated that at the dose of 0.1 to 1 mg/kg/2d, long-term administration of prednisolone did not alter B6 profiles in erythrocyte, kidney, or brain. On the other hand, prednisolone dose-dependently increased PL in the muscle and tended to increased PL in the heart. We further investigated B6 metabolic enzyme activities and found that low-dose prednisolone significantly decreased PDXP activity by 23% in the muscle (Table 4E). These data suggested that prednisolone may selectively alter vitamin B6 metabolic enzymes and result in B6 mobilization among tissues.
Long-term pharmacological prednisolone use reduced hepatic pyridoxamine 5’-phosphate concentration

Long-term administration of prednisolone significantly decreased hepatic PMP levels by 15% (Table 5A) without altering hepatic PLP, PL or PA concentrations. The distinguished B6 profiles among different tissues suggested that prednisolone alters B6 metabolic enzymes in a tissue specific manner. The effects of long-term pharmacological prednisolone use on mRNA, protein and activity hepatic of B6 metabolic enzyme PDXP, PDXK, and PMPO were then examined.

Long-term pharmacological prednisolone use significantly induced hepatic PDXK protein and enzyme activity

Long-term pharmacological prednisolone use did not alter PDXP or PMPO protein expression, but PDXK protein levels were dose-dependently increased (Table 5B).

Furthermore, prednisolone (1 mg/kg BW/2days) significantly induced hepatic PDXK and PMPO activities and tended to decrease PDXP activity (Table 5C). Prednisolone tended to increase hepatic PMPO mRNA expression but not PDXK (Table 5D). In summary, long-term administration of prednisolone reduced hepatic PMP concentration, increased hepatic PDXK protein and enzyme activity in a dose-dependent manner.
Discussions

In the present study we demonstrated novel findings on the effects of long-term prednisolone treatment in increasing circulating vitamin B6 levels through direct induction of hepatic PLP synthesizing enzymes. The effects of prednisolone on vitamin B6 metabolism are summarized in Figure 1. Abnormal vitamin B6 metabolism is commonly present in subjects with chronic inflammation. Previously we have shown that the severity of abnormal vitamin B6 status is associated with severity of symptoms in patients with rheumatoid arthritis (Chiang et al., 2003b), and that inflammation causes tissue-specific depletion of vitamin B6 (Chiang et al., 2005). Plasma PLP concentration is correlated with functional vitamin B6 indices in patients with rheumatoid arthritis and marginal vitamin B6 status (Chiang et al., 2003a). In the present study, based on results from 2 independent in vivo models, we demonstrated that prednisolone’s effect on vitamin B6 is a potential advantage additional to its anti-inflammatory function in treating RA patients. Several novel findings are addressed more specifically below. First, the increase in circulating B6 vitamers PLP, PL, and 4-PA in prednisolone-treated mice implies that prednisolone may significantly increase hepatic B6 concentrations, because the liver is the primary organ that supplies the active form of vitamin B6 to the circulation (Lumeng et al., 1980). Second, because PLP can be synthesized from PMP (Wada & Snell, 1961) or PL, we postulated that prednisolone may induce the hepatic PLP synthesizing enzymes PMPO and PDXK. Our
data showing that prednisolone increases PLP production not only by inducing PDXK and
PMPO activity but also by suppressing PDXP activity in the liver supported our hypothesis.
Third, we suggest that the elevated plasma PL observed in prednisolone-treated mice is likely
to be due to its release from the liver, because ALP levels were found to be unchanged in
prednisolone-treated mice.  Fourth, we suggest that these elevated levels of plasma vitamers
do not result in accelerated excretion of vitamin B6.  Although the conversion of PL to 4-PA
is an irreversible reaction that generates the end product of vitamin B6 metabolism, neither
the daily dietary intake nor the urinary excretion of 4-PA differed between
prednisolone-treated mice and control mice.  In summary, our observations have
demonstrated that at clinically relevant doses, prednisolone does not alter vitamin B6
ingestion or excretion but may accelerate the inter-conversions and mobilizations of B6
vitamers in a tissue-specific manner.

The exact consequences of prednisolone induction of elevated circulating PLP
remain to be determined. However, since vitamin B6 depletion is commonly present in
patients with RA (Chiang 2003a), we suggest that the prednisolone treatment-caused elevated
plasma PLP can increase the supply of vitamin B6 to tissues that may have a higher B6
requirement during inflammation.  Furthermore, because PL is the major form of vitamin B6
crossing the cell membrane, elevated circulating PL can facilitate the cellular uptake and
utilization of vitamin B6 in tissues with a higher demand or during B6 depletion.  Both of
the hepatic PLP synthesizing enzymes (PMPO and PDXK) were increased and the PLP
degradation enzyme reduced by prednisolone treatment, yet hepatic PLP concentrations
remained unaltered and plasma PLP was elevated. These findings suggesting that the
increased PLP is readily released into the circulation. The elevated plasma PLP in humans
receiving prednisolone could be a potential advantage because the bioactive form of vitamin
B6 is involved in over 100 biochemical reactions, including the syntheses of serotonin,
dopamine, and histamine, and the degradation of homocysteine in the body. By promoting
the availability of B6 vitamers, prednisolone may potentially impact the pathogenesis and
outcomes of those diseases involving numerous vitamin B6 dependent reactions. One of the
key vitamin B6-dependent pathways is the degradation of homocysteine. The commonly
seen hyperhomocysteinemia in RA is believed to account at least in part, for the number of
increased cardiovascular events seen in these patients. The common daily dosage of
prednisolone for treating RA is between 1.0 to 7.5 mg/day. Although no evidence to date
shows that clinical use of low-dose prednisolone directly reduces homocysteine levels, pulsed
glucocorticoid treatment has been found to reduce plasma homocysteine levels by 27% in
patients with RA. And in rats, a two-week period of subcutaneous cortisol injections
(5mg/kg BW) was seen to lower plasma homocysteine levels by 50% (Kim et al., 1997),
presumably due to the induction of BHMT, one of the 2 enzymes that catalyze homocysteine
remethylation for methionine synthesis (Schwahn et al., 2004). Here we provide another
potential homocysteine-lowering mechanism as a consequence of prednisolone treatment, the induction of vitamin B6-dependent transsulfuration. The possible systemic regulation by prednisolone of homocysteine transsulfuration is currently under investigation.

Other potential health benefits can result from improved vitamin B6 status. Accumulated evidences suggest that vitamin B6 is protective against heart diseases. Vitamin B6 deficiency can induce renal arteriosclerotic lesions in swine (Smolin et al., 1983). In humans, decreased PLP has been observed in patients suffering from myocardial infarction (Serfontein et al., 1985), and a low plasma PLP level was found to be an independent risk factor for cardiovascular disease (Robinson et al., 1998; Friso et al., 2004). Furthermore, patients receiving vitamin B6 in the treatment of carpal tunnel syndrome and other degenerative diseases had a lower risk of developing acute cardiac chest pain or myocardial infarction (Ellis & McCully, 1995). And naturally occurring PLP and other synthesized pyridoxine 5'-phosphonates have direct anti-ischemic effects in a rat model of myocardial ischemia (Pham et al., 2003).

Glucocorticoids have been associated with numerous side effects including skin atrophy, defective wound healing, osteoporosis, myopathy, depression, fatigue, adrenal insufficiency, peptic ulcer, hypertension, and the induction of diabetes mellitus (Schacke et al., 2002). Considering the significantly high prevalence of long-term vitamin B6 depletion in RA, we suggest that the action of prednisolone in increasing circulating vitamin B6 should be
taken into account when considering the potential advantages and disadvantages of its use in treatment. Taken together, it may be beneficial for patient to receive long-term pharmacological prednisolone use in order to facilitate vitamin B6-dependent biochemical reactions. Future studies on the effects of long-term clinical prednisolone use on homocysteine metabolism are warranted.

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Authorship contributions

EPC: conceived of the study, created the original hypothesis, acquired fundings, study designs, statistical analysis, data interpretation, drafted, revised, and corresponded the manuscript.

HYC: literature review, biochemical and statistical analyses

JTT: manuscript discussion

SJL: rat model and sample harvest, analytical method optimization, biochemical analyses, data acquisition

WYT: mice model and sample harvest
References


Footnotes

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Figure legends

Figure 1. Summary of the of long-term prednisolone effects on vitamin B6 metabolism.
Table 1. Effect super-physiological high dose\(^1\) prednisolone on hepatic vitamin B6 profile and B6 metabolizing enzyme activity and gene expressions\(^2\)

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>Pred (10 mg/kg/2d) (n=6)</th>
<th>P-value</th>
<th>%change</th>
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<tbody>
<tr>
<td>A) Plasma profile (nmol/L)</td>
<td></td>
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<tr>
<td>PLP(^3)</td>
<td>742.8 ± 164.1</td>
<td>1016.2 ± 189.2</td>
<td>0.01</td>
<td>+36.8 ± 25.5</td>
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<td>PL(^3)</td>
<td>201.8 ± 61.1</td>
<td>172.6 ± 40.0</td>
<td>0.36</td>
<td>-14.5 ± 19.8</td>
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<tr>
<td>4-PA(^3)</td>
<td>42.3 ± 11.3</td>
<td>68.9 ± 24.7</td>
<td>0.05</td>
<td>+59.0 ± 57.0</td>
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<td>B) Liver B6 status</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>B6 vitamer (nmol/g liver)</td>
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<td>PMP(^3)</td>
<td>22.0 ± 3.0</td>
<td>24.2 ± 3.9</td>
<td>0.47</td>
<td>10.0 ± 17.6</td>
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<td>PLP(^3)</td>
<td>19.0 ± 4.7</td>
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<td>0.47</td>
<td>-13.2 ± 16.7</td>
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<tr>
<td>PL(^3)</td>
<td>1.1 ± 0.9</td>
<td>0.6 ± 0.3</td>
<td>1.00</td>
<td>-50.2 ± 23.1</td>
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<td>Gene expression</td>
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<td>PDXP/18S(^3)</td>
<td>0.22 ± 0.14</td>
<td>4.14 ± 2.82</td>
<td>0.01</td>
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<td>PDXK/18S(^3)</td>
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<td>PMPO/18S(^3)</td>
<td>0.38 ± 0.31</td>
<td>0.74 ± 0.53</td>
<td>0.29</td>
<td>93.6 ± 138.3</td>
</tr>
<tr>
<td>Enzyme activity (nmol/hr*mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDXP(^3)</td>
<td>325.0 ± 25.7</td>
<td>299.0 ± 25.8</td>
<td>0.06</td>
<td>-8.1 ± 7.9</td>
</tr>
<tr>
<td>PDXK(^3)</td>
<td>9.2 ± 2.2</td>
<td>15.9 ± 4.3</td>
<td>0.02</td>
<td>+72.7 ± 46.1</td>
</tr>
<tr>
<td>PMPO(^3)</td>
<td>1.4 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>0.58</td>
<td>6.1 ± 6.8</td>
</tr>
</tbody>
</table>

\(^1\) Prednisolone dose: 10 mg/kg/day via gastric gavages for 35 days. \(^2\) Data are presented as means ± SD. Percent changes and P-values were calculated compared to controls.
3 Abbreviations. PLP: pyridoxal 5’-phosphate; PL: pyridoxal; 4-PA: 4-pyridoxic acid; PDXP: pyridoxal 5’-phosphate phosphatase; PDXK: pyridoxal kinase; PMPO: pyridoxamine-5’-phosphate oxidase; PMP: pyridoxamine 5’-phosphate.
Table 2. Long-term pharmacological prednisolone use altered plasma vitamin B6 profile and alkaline phosphatase (ALP) levels\(^1,\,2\)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pred (0.1 mg/kg/2d)</th>
<th>Pred (1 mg/kg/2d)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Week 10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLP (nmol/L)</td>
<td>116.0 ± 13.4(^a)</td>
<td>103.0 ± 42.2(^a)</td>
<td>157.4 ± 18.2(^b)</td>
<td>(p=0.009^3)</td>
</tr>
<tr>
<td>% change</td>
<td>-11.3 ± 36.4</td>
<td>+35.7 ± 15.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL (nmol/L)</td>
<td>523.8± 124.4</td>
<td>537.8 ± 96.9</td>
<td>599.7 ± 105.1</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>2.7 ± 18.5</td>
<td>14.5 ± 20.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA (nmol/L)</td>
<td>18.7 ± 5.2(^a)</td>
<td>21.9 ± 13.2(^a)</td>
<td>38.7 ± 13.3(^b)</td>
<td>(p=0.028^3)</td>
</tr>
<tr>
<td>% change</td>
<td>17.1 ± 70.7</td>
<td>+107.3 ± 71.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(B) Week 22</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLP (nmol/L)</td>
<td>140.1 ±26.7(^a)</td>
<td>181.7 ± 45.8(^a)</td>
<td>242.6 ± 75.9(^b)</td>
<td>(p=0.047^3)</td>
</tr>
<tr>
<td>% change</td>
<td>29.7 ± 32.7</td>
<td>+73.1 ± 54.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL (nmol/L)</td>
<td>414.2± 35.8(^a)</td>
<td>416.3 ± 56.1(^a)</td>
<td>600.2 ± 50.1(^b)</td>
<td>(p=0.009^3)</td>
</tr>
<tr>
<td>% change</td>
<td>0.5 ± 13.5</td>
<td>+44.9 ± 12.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA (nmol/L)</td>
<td>10.3 ± 4.6(^a)</td>
<td>10.0 ± 7.8(^a)</td>
<td>31.6 ± 8.8(^b)</td>
<td>(p=0.009^3)</td>
</tr>
<tr>
<td>% change</td>
<td>23.9 ± 70.7</td>
<td>+206.7 ± 85.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(C) ALP</strong></td>
<td>116.1 ± 13.6</td>
<td>112.4 ± 10.4</td>
<td>108.3 ± 5.4</td>
<td>NS</td>
</tr>
</tbody>
</table>
\begin{center}
\begin{tabular}{ccc}
\textbf{\% change} & -3.2 \pm 9.0 & -5.9 \pm 4.5 \\
\end{tabular}
\end{center}

\footnotesize

1 Abbreviations are the same as Table 1. ALP: Alkaline phosphatase. NS: not significantly different from the controls.  
2 Data are presented as means \pm SD. Data in a row with different superscripts are statistically different. Percent changes were calculated compared to the controls.  
3 Prednisolone (1 mg/kg/2d) vs. Controls. P-values were calculated by student’s t-test.
Table 3. Effect of prednisolone treatment on erythrocyte vitamin B6 status and urinary vitamin B6 excretion\textsuperscript{1, 2}

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pred (0.1 mg/kg/2d)</th>
<th>Pred (1 mg/kg/2d)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Erythrocyte B6 (nmol/L packed cells)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMP</td>
<td>27.5 ± 5.2</td>
<td>31.8 ± 10.2</td>
<td>21.9 ± 8.9</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>15.7 ± 36.9</td>
<td>-20.2 ± 32.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLP</td>
<td>732.4 ± 96.9</td>
<td>787.4 ± 200.3</td>
<td>800.5 ± 256.3</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>7.5 ± 27.4</td>
<td>9.3 ± 35.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>135.7 ± 47.7</td>
<td>114.9 ± 51.9</td>
<td>106.5 ± 46.5</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>-15.3 ± 38.3</td>
<td>-21.5 ± 34.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(B) B6 excretion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-PA µg/24 h</td>
<td>0.94 ± 0.20</td>
<td>0.94 ± 0.34</td>
<td>0.86 ± 0.26</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>-0.53 ± 36.2</td>
<td>-8.87 ± 27.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creat mg/24h</td>
<td>0.43 ± 0.05</td>
<td>0.38 ± 0.14</td>
<td>0.42 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>-12.3 ± 31.6</td>
<td>-2.99 ± 31.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-PA µg/mg Creat</td>
<td>2.22 ± 0.56</td>
<td>2.49 ± 0.34</td>
<td>2.08 ± 0.34</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>12.1 ± 2.8</td>
<td>-6.15 ± 15.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} Abbreviations are the same as Table 1. Creat: creatinine. \textsuperscript{2} Data are presented as means ± SD.

NS: no significant difference found among groups.
Table 4. Effects of prednisolone on vitamin B6 status in extra hepatic tissues \(^1,2\)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pred (0.1 mg/kg/2d)</th>
<th>Pred (1 mg/kg/2d)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Muscle (nmol/g tissue)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMP</td>
<td>4.1 ± 3.4</td>
<td>5.5 ± 2.2</td>
<td>4.4 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>31.4 ± 52.7</td>
<td>6.5 ± 62.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLP</td>
<td>20.6 ± 2.8</td>
<td>21.1 ± 4.1</td>
<td>19.0 ± 4.4</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>2.3 ± 20.1</td>
<td>-7.8 ± 21.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>0.05 ± 0.01(^a)</td>
<td>0.08 ± 0.02(^b)</td>
<td>0.11 ± 0.08(^b)</td>
<td>(p=0.028)</td>
</tr>
<tr>
<td>% change</td>
<td>+44.4 ± 29.5</td>
<td>+99.1 ± 150.4</td>
<td></td>
<td>(p=0.047)</td>
</tr>
<tr>
<td><strong>(B) Heart (nmol/g tissue)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMP</td>
<td>30.2 ± 1.8</td>
<td>31.9 ± 2.2</td>
<td>32.1 ± 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>5.8 ± 7.3</td>
<td>6.2 ± 7.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLP</td>
<td>10.6 ± 0.6</td>
<td>10.9 ± 0.7</td>
<td>9.8 ± 1.6</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>2.9 ± 6.2</td>
<td>-8.0 ± 14.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>0.24 ± 0.07</td>
<td>0.34 ± 0.13</td>
<td>0.40 ± 0.20</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>44.0 ± 53.0</td>
<td>70.3 ± 86.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(C) Brain (nmol/g tissue)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMP</td>
<td>13.7 ± 1.7</td>
<td>14.8 ± 1.7</td>
<td>13.7 ± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>8.3 ± 12.7</td>
<td>0.2 ± 12.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### (D) Kidney (nmol/g tissue)

<table>
<thead>
<tr>
<th></th>
<th>PLP</th>
<th>PLP</th>
<th>PLP</th>
<th>% change</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.9 ± 0.7</td>
<td>7.9 ± 0.9</td>
<td>6.5 ± 1.1</td>
<td>14.4 ± 13.2</td>
<td>-5.2 ± 16.2</td>
</tr>
<tr>
<td></td>
<td>0.95 ± 0.12</td>
<td>1.12 ± 0.35</td>
<td>1.18 ± 0.56</td>
<td>18.5 ± 37.2</td>
<td>24.6 ± 59.1</td>
</tr>
</tbody>
</table>

### (E) enzyme activity in the muscle (nmol/hr*mg protein)

<table>
<thead>
<tr>
<th></th>
<th>PDXP</th>
<th>PDXP</th>
<th>PMPO</th>
<th>PMPO</th>
<th>PMPO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9.2 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.3 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.0 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20 ± 0.04</td>
<td>0.22 ± 0.03</td>
</tr>
</tbody>
</table>

| % change | +22.9 ± 13.8 | -1.4 ± 9.6 | -10.4 ± 17.5 | -9.1 ± 23.6 |

---

<sup>a</sup> p=0.0284

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This article has not been copyedited and formatted. The final version may differ from this version.
1 Abbreviations are the same as Table 1. NS: no significant difference found among groups.

2 Data are presented as means ± SD. Data in a row with different superscripts are statistically different. Percent changes were calculated compared to the controls. 3 Predinisolone (1 mg/kg/2d) vs. Controls. 4 Predinisolone (0.1 mg/kg/2d) vs. Controls. P-values were calculated by student’s t-test.
### Table 5. Effect of prednisolone treatment on vitamin B6 status in the liver

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pred (0.1 mg/kg/2d)</th>
<th>Pred (1 mg/kg/2d)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) B6 vitamer (nmol/g tissue)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMP</td>
<td>2.9 ± 0.3</td>
<td>2.5 ± 0.3 b</td>
<td>2.4 ±0.3 b</td>
<td>p=0.0283</td>
</tr>
<tr>
<td>% change</td>
<td>-14.7 ± 10.1</td>
<td>-15.1 ± 9.4</td>
<td></td>
<td>p=0.0474</td>
</tr>
<tr>
<td>PLP</td>
<td>5.0 ± 0.3</td>
<td>4.6 ± 0.7</td>
<td>4.5 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>-8.4 ± 13.1</td>
<td>-24.9 ±25.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>0.40 ± 0.17</td>
<td>0.42 ± 0.13</td>
<td>0.33 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>5.7 ± 33.5</td>
<td>-17.8 ± 24.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>0.04 ± 0.06</td>
<td>0.01 ±0.00</td>
<td>0.04 ±0.06</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>-72.0 ± 7.6</td>
<td>-3.7 ± 160.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(B) B6 metabolic enzyme protein expression</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDXP/β-actin</td>
<td>0.98 ± 0.26</td>
<td>0.94± 0.29</td>
<td>1.14± 0.30</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>-3.6 ± 29.6</td>
<td>+16.8 ± 30.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDXK/β-actin</td>
<td>0.83 ± 0.11</td>
<td>1.04 ± 0.10 b</td>
<td>1.12 ± 0.20 b</td>
<td>p=0.0163</td>
</tr>
<tr>
<td>% change</td>
<td>+24.8 ± 11.7</td>
<td>+34.6 ± 23.7</td>
<td></td>
<td>p=0.0284</td>
</tr>
<tr>
<td>PMPO/β-actin</td>
<td>1.05 ± 0.13</td>
<td>1.02 ± 0.07</td>
<td>0.96 ± 0.11</td>
<td>NS</td>
</tr>
<tr>
<td>% change</td>
<td>-2.5 ± 6.5</td>
<td>-8.5 ± 10.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### (C) B6 metabolic enzyme activity (nmol/hr*mg protein)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control Mean ± SD</th>
<th>Prednisolone (0.1 mg/kg/2d) Mean ± SD</th>
<th>Prednisolone (1 mg/kg/2d) Mean ± SD</th>
<th>% change</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PDXP</strong></td>
<td>25.3 ± 1.6</td>
<td>24.2 ± 2.1</td>
<td>23.6 ± 1.2</td>
<td>-4.6 ± 8.2</td>
<td>-6.8 ± 4.6</td>
</tr>
<tr>
<td><strong>PDXK</strong></td>
<td>2.7 ± 0.1</td>
<td>2.9 ± 0.2</td>
<td>3.0 ± 0.3</td>
<td>7.1 ± 8.3</td>
<td>+13.0 ± 11.1</td>
</tr>
<tr>
<td><strong>PMPO</strong></td>
<td>3.1 ± 0.1</td>
<td>3.0 ± 0.6</td>
<td>3.7 ± 0.5</td>
<td>-4.8 ± 19.3</td>
<td>+18.2 ± 14.5</td>
</tr>
</tbody>
</table>

### (D) B6 metabolic gene expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control Mean ± SD</th>
<th>Prednisolone (0.1 mg/kg/2d) Mean ± SD</th>
<th>Prednisolone (1 mg/kg/2d) Mean ± SD</th>
<th>% change</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PMPO/18S</strong></td>
<td>92.4 ± 31.9</td>
<td>31.8 ± 10.2</td>
<td>192.3 ± 113.9</td>
<td>-15.5 ± 63.0</td>
<td>108.2 ± 123.2</td>
</tr>
<tr>
<td><strong>PDXK/18S</strong></td>
<td>105.1 ± 108.9</td>
<td>128.1 ± 50.7</td>
<td>97.3 ± 111.7</td>
<td>21.9 ± 48.2</td>
<td>-7.5 ± 106.3</td>
</tr>
</tbody>
</table>

---

1 Abbreviations see Table 1. NS: no significant difference among groups. 2 Data are presented as means ± SD. Data in a row with different superscripts are statistically different. Percent changes were calculated compared to the controls. 3 Prednisolone (1 mg/kg/2d) vs. Controls. 4 Prednisolone (0.1 mg/kg/2d) vs. Controls. P-values were calculated by student’s t-test.
**Figure 1. Effects of prednisolone on vitamin B6 metabolism**

Liver

- PMP -15%
- PLP ---
- PA ---

**Gene expression**
- PDXP mRNA ---
- PDXK mRNA +35%
- PMPO mRNA ---

**Enzyme**
- PDXP activity -7%
- PDXK activity +13%
- PMPO activity +18%

Kidney

- B6 profile ---

Urine

- 4-PAs ---

Brain

- B6 profile ---

Heart

- B6 profile ---

Muscle

- PL +99%
- PLP ---

Plasma

- PLP +73%
- PL +45%
- PA +207%
- ALP ---

RBC

- B6 profile ---

*---: means not significantly differ from control mice