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Long-Term Prednisolone Treatments Increase Bioactive Vitamin B_6 Synthesis In Vivo

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ABSTRACT

The etiology of vitamin B_6 depletion in inflammation remains unknown. Hepatic vitamin B_6 decreased in adrenalectomized rats, and such reductions were restored by an acute muscle injection of a very high dose of glucocorticoids. We tested the hypothesis that long-term prednisolone treatment for treating inflammation restores vitamin B_6 status by induction of tissue B6 metabolic enzymes. Two independent in vivo models were used. Lewis rats and C57BL/6J mice received prednisolone regimens that reflected clinical prednisolone uses in treating human inflammation. We found: 1) prednisolone increased circulating B6 vitamer pyridoxal 5′-phosphate (PLP; bioactive B6 vitamer), pyridoxal (PL), and 4-pyridoxic acid without altering vitamin B_6 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); and 3) elevations in circulating PL were caused by its release from the liver, not by PLP dephosphorylation (PDXP was suppressed and alkaline phosphatase was unaltered). We conclude that long-term prednisolone treatments promoted hepatic bioactive vitamin B_6 synthesis by inducing the synthesizing enzymes PDXK and PMPO and simultaneously suppressing the catabolic enzyme PDXP. Prednisolone increased circulating B6 vitamer without altering urinary B6 excretion. As the major form of vitamin B_6 across cell membrane, elevated circulating PL may facilitate the cellular uptake and utilization of B6. The elevated plasma PLP may increase vitamin B_6 supply to tissues with a higher B6 demand during inflammation. Results from two independent in vivo models suggested a potential advantage of clinical prednisolone use in treating inflammation with respect to vitamin B_6 status.

Introduction

Although vitamin B_6 depletion has long been associated with chronic inflammation, the etiology for this abnormality remains to be established. The regulation of tissue vitamin B_6 status depends on the intake, metabolism, transport in the blood, uptake mechanisms, binding to proteins, and 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 (Pickup, 1979; Francisco et al., 1984). When the disease cannot be effectively controlled by other disease-modifying antirheumatic drugs, low-dose prednisolone (≤15 mg daily) is commonly used intermittently in patients with RA (Gotzsche and Johansen, 2004). 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. We speculated that corticosteroids induce glutamic-pyruvic transaminase via interference with vitamin B_6 metabolism; because such induction was not seen in vitamin B_6-deficient animals (Gavosto et al., 1957; Rosen et al., 1959a,b; Eisenstein, 1960). Pyridoxal-5′-phosphate (PLP) is the major phosphorylated bioactive form of vitamin B_6 that 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 B_6, and the glycogen phosphorylase-bound PLP is the major vitamin B_6 present in the muscle (Krebs and Fisher, 1964). The role of erythrocyte in the metabolism and transport of vitamin B_6 remains to be established. In the erythrocytes, PL and pyrixoxine are easily

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ABBREVIATIONS: RA, rheumatoid arthritis; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PA, pyridoxic acid; DMARD, disease-modifying antirheumatic drug; PDXK, pyridoxine kinase; PMP, pyridoxamine-5′-phosphate; PMPO, pyridoxamine-5′-phosphate oxidase; PM, pyridoxamine; PDXP, pyridoxal-5′-phosphate phosphatase; HPLC, high-performance liquid chromatography.

taken up by simple diffusion; they are then converted to PLP by kinase and oxidase (Mehansho and Henderson, 1980). The tight binding of PLP and PL to hemoglobin (Fonda and Harker, 1982; Ink et al., 1982) makes erythrocyte a potential vitamin B_6 reservoir in the circulatory system. PL is irreversibly converted to 4-pyridoxic acid (4-PA), the end product of vitamin B_6 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 a 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 decreased PMP levels and increased PL and pyridoxamine (PM) concentrations in the adrenal tissue in swine (Mahuren et al., 1999). Pyridoxal-5′-phosphate phosphatase (PDXP) activity increased 10-fold in adrenocorticotropic hormone-treated pigs (Mahuren et al., 1999). Although these earlier studies implied that high-dose glucocorticoids may interfere with vitamin B_6 synthesis and catabolism, the vitamin B_6 homeostasis in humans on long-term glucocorticoid therapies remained to be investigated. We hypothesized that long-term prednisolone treatments help restore vitamin B_6 homeostasis by inducing the vitamin B_6-synthesizing enzyme PDXK and PMPO and/or suppressing the B6 catabolic enzyme PDXP.

The doses used in the above-mentioned animal studies were 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 (intramuscular) and human (oral), a daily intramuscular dose at 16.7 to 33.3 mg/kg/day in rats would be comparable with 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 of ~5 mg) (Chiang et al., 2003b, 2005b; Chen et al., 2011).

Hormonal alterations other than cortisols may also affect vitamin B_6 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 levels 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 B_6 homeostasis, it is not known whether long-term clinical doses of prednisolone used in treating human inflammation affects vitamin B_6 metabolism. As a commonly used anti-inflammatory disease-modifying antirheumatic drug in arthritis, long-term prednisolone treatments may help restore vitamin B_6 homeostasis in subjects with chronic inflammation. Alternatively, prednisolone may interfere with normal vitamin B_6 metabolism and partially account for the abnormal vitamin B_6 status in patients with inflammation. This study systematically investigated the impact of long-term physiological doses of prednisolone on vitamin B_6 profiles as well as the regulation of prednisone on vitamin B_6 metabolic enzymes in vivo.

Materials and Methods

Animal and Diet. The present study was approved by the Institutional Animal Care and Use Committee of National Chung Hsing University, Taichung, Taiwan. Two separate animal studies were performed to examine the effects of superphysiological high-dose or long-term pharmacological prednisolone uses on vitamin B_6 metabolism. Three-week-old Lewis rats and C57BL/6J mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). In the first experiment (study I) we used 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 superphysiological high dose of prednisolone (10 mg/kg/day) for 35 days. This dose has been shown to affectively ameliorate 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 of 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., 2005a) to minimize variations in vitamin B_6 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 or 1 mg/kg/2 days) comparable with human clinical use. Five mice were housed in filter top cages with water and food provided ad libitum. Some researchers have 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 recommended daily allowance for vitamin B_6 for rodents (7 mg/Kg pyriodoxine-HCl) (Dyets). 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-Aldrich (St Louis, MO), and dissolved in phosphate-buffered saline with 0.01% dimethyl sulfoxide. The doses and durations were chosen based on those used in humans following the definition by Buttgereit et al. (2002). In study I, rats were treated once a day with normal saline (control group; $n = 6$) or super physiological doses 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 three groups by body weight and treated once on alternate days with 1) phosphate-buffered saline (control group; $n = 5$); 2) 0.1 mg prednisolone/kg/2 days ($n = 5$); or 3) 1 mg prednisolone/kg/2 days ($n = 5$) intraperitoneally with prednisolone for 27 weeks (based on the life expectancy that would reflect >20 years of long-term clinical use of prednisolone treatment in humans). Prednisolone is generally administered to patients with arthritis at a dosage of 7.5 to 35 mg/week (Chiang et al., 2003b, 2005b). More than one-tenth of patients with RA we recruited from an outpatient clinic have been taking low-dose prednisolone for more than 10 years (Chen et al., 2011). Among them more than 6% have been taking prednisolone for more than 20 years (Chen et al., 2011). According to the *Guidelines for the Timing of Nonclinical 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 Tissue Collection. In study I, blood was collected from the 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 pred-

nisolone treatments on vitamin B_6 profiles. Animals were sacrificed 27 weeks after prednisolone treatment started. Plasma was collected after overnight 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 the 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 liquid nitrogen until analysis.

Western Blotting. Approximately 0.03 g of tissue was homogenized in 10 volumes of radioimmunoprecipitation assay (25 mM Tris·HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) buffer containing 0.33% (v/v) phosphatase inhibitor cocktail 1, 2 (Sigma-Aldrich) and 0.1% (v/v) protease inhibitor cocktail set 1 (Calbiochem, San Diego, CA). The protein content was quantified by BCA Protein Assay (Pierce, Rockford, IL). Protein lysates from each tissue was denatured and then separated on a 12% SDS-polyacrylamide gel electrophoresis using a Minigel apparatus and transferred onto a polyvinylidene difluoride membrane using a transfer cell (Bio-Rad Laboratories, Hercules, CA). After blocking with Tris-buffered saline containing with 10% skim milk, the membranes were incubated with the primary antibody including anti-PDXP (1:1000) antibody (Cell Signaling Technology, Danvers, MA), anti-PDXK (1:1000) and anti-PMPO (1:1000) antibody (Abnova, Taipei, Taiwan). Membranes were washed three times with Tris-buffered saline containing 0.1% Tween 20 and then covered with horseradish peroxidase-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, Ipswich, MA). 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 micrograms of liver whole-cell RNA was reverse-transcribed using oligo(dT) as primer and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Gene expression was determined by using SYBR Green I on the quantitative real-time polymerase chain reaction ABI7000 (Applied Biosystems Inc., Foster City, CA). 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 B_6 Profiles in Plasma, Red Blood Cells, and Tissues. We modified the precolumn semicarbazide derivatization HPLC method from Talwar et al. (2003) with additional detection of PMP and optimized this procedure in plasma, erythrocytes, and tissues (E.-P.I. Chiang, H.-Y. Chang, and S.-J. Lin, unpublished data). To measure tissue vitamin B_6 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 min then centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were frozen in −80°C until analyzed (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 described previously (Wada and 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 (1988).

Determination of Urinary Vitamin B_6 Concentrations. Urinary 4-PA levels were analyzed by isocratic reversed-phased HPLC as described by Gregory and Kirk, 1979. The mobile phase consisted of 60 mM disodium hydrogen phosphate and 400 mg/liter 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 Fisher Scientific, Waltham, MA).

Statistical Analysis. All data are reported as means ± S.D. Comparisons of means between the control and the prednisolone treatment groups were determined using Student's t test. 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., San Jose, CA).

Results

Superphysiological High-Dose Prednisolone Increased Plasma PLP and PA and Up-Regulated Vitamin B_6 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 1). Hepatic PDXP and PDXK gene expressions were drastically induced

TABLE 1

Effect of superphysiological high-dose prednisolone on hepatic vitamin B6 profile and B6-metabolizing enzyme activity and gene expression

Prednisolone dose was 10 mg/kg/day via gastric gavage for 35 days. Data are presented as means ± S.D. Percentage changes and *P* values were calculated compared with controls. Bold values indicate data significantly different when compared to controls ($P<0.05$) in Table 1.

	Control (n = 6)	Prednisolone (n = 6)	*P* Value	% Change
Plasma profile (nmol/l)				
PLP	742.8 ± 164.1	**1016.2 ± 189.2**	**0.01**	**+36.8 ± 25.5**
PL	201.8 ± 61.1	172.6 ± 40.0	0.36	−14.5 ± 19.8
4-PA	42.3 ± 11.3	**68.9 ± 24.7**	**0.05**	**+59.0 ± 57.0**
Liver B6 status				
B6 vitamer (nmol/g liver)				
PMP	22.0 ± 3.0	24.2 ± 3.9	0.47	10.0 ± 17.6
PLP	19.0 ± 4.7	16.5 ± 3.2	0.47	−13.2 ± 16.7
PL	1.1 ± 0.9	0.6 ± 0.3	1.00	−50.2 ± 23.1
Gene expression				
PDXP/18S	0.22 ± 0.14	**4.14 ± 2.82**	**0.01**	**+1799 ± 1293**
PDXK/18S	0.32 ± 0.11	**0.94 ± 0.40**	**0.01**	**+196.2 ± 126.4**
PMPO/18S	0.38 ± 0.31	0.74 ± 0.53	0.29	93.6 ± 138.3
Enzyme activity (nmol/h · mg)				
PDXP	325.0 ± 25.7	299.0 ± 25.8	0.06	−8.1 ± 7.9
PDXK	9.2 ± 2.2	**15.9 ± 4.3**	**0.02**	**+72.7 ± 46.1**
PMPO	1.4 ± 0.2	1.5 ± 0.1	0.58	6.1 ± 6.8

TABLE 2

Long-term pharmacological prednisolone use altered plasma vitamin B6 profile and alkaline phosphatase (ALP) levels

Data are presented as means ± S.D. Data in a row with different superscripts are statistically different. Percentage changes were calculated compared with the controls. *P* values were calculated by Student's *t* test. Bold values indicate data significantly different when compared prednisolone (1mg/kg/2 days) group to controls ($P<0.05$) in Table 2.

	Control	Prednisolone (0.1 mg/kg/2 days)	Prednisolone (1 mg/kg/2 days)	*P* Value
Week 10				
PLP (nmol/l)	116.0 ± 13.4[a]	103.0 ± 42.2[a]	**157.4 ± 18.2[b]**	**0.009**
% Change		−11.3 ± 36.4	**+35.7 ± 15.7**	
PL (nmol/l)	523.8 ± 124.4	537.8 ± 96.9	599.7 ± 105.1	N.S.
% Change		2.7 ± 18.5	14.5 ± 20.1	
PA (nmol/l)	18.7 ± 5.2[a]	21.9 ± 13.2[a]	**38.7 ± 13.3[b]**	**0.028**
% Change		17.1 ± 70.7	**+107.3 ± 71.0**	
Week 22				
PLP (nmol/l)	140.1 ± 26.7[a]	181.7 ± 45.8[a]	**242.6 ± 75.9[b]**	**0.047**
% Change		29.7 ± 32.7	**+73.1 ± 54.2**	
PL (nmol/l)	414.2 ± 35.8[a]	416.3 ± 56.1[a]	**600.2 ± 50.1[b]**	**0.009**
% Change		0.5 ± 13.5	**+44.9 ± 12.1**	
PA (nmol/l)	10.3 ± 4.6[a]	10.0 ± 7.8[a]	**31.6 ± 8.8[b]**	**0.009**
% Change		23.9 ± 70.7	**+206.7 ± 85.2**	
ALP	116.1 ± 13.6	112.4 ± 10.4	108.3 ± 5.4	N.S.
% Change		−3.2 ± 9.0	−5.9 ± 4.5	

N.S., not significantly different from the controls.

by 18- and 2-fold, respectively, and PDXK enzyme activity was increased by 73% (Table 1). Results from this experiment suggested that high doses of prednisolone induced vitamin B_6 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 B_6. To further investigate the effects of long-term pharmacological prednisolone use on vitamin B_6 metabolism, B6 profiles and vitamin B_6 metabolic enzymes were investigated in mice that received 0.1 or 1 mg/kg/2 days 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/2 days) increased plasma PLP and PA concentrations by 36 and 107%, respectively (Table 2). At week 22, prednisolone at the dose of 0.1 mg/kg/2 days tended to increase plasma PLP concentration (by ~30%; $p = 0.075$). At the dose of 1 mg/kg/2 days, prednisolone drastically increased plasma PLP, PL, and PA by 73, 45, and 207%, respectively ($p < 0.05$) (Table 2). Furthermore, plasma concentrations of PLP, PL, or PA closely correlated with each other in prednisolone-treated mice (PLP versus PL, $r = 0.81$, $p = 0.005$; PLP versus PA, $r = 0.68$, $p = 0.031$; PL versus PA, $r = 0.79$, $p = 0.015$; $n = 10$), but 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 that received 0.1 or 1 mg/kg/2 days of prednisolone (Table 2), suggesting that the increased plasma PLP level observed was independent of alkaline phosphatase status, a known significant determinant for the phosphorylated form for B6 vitamers. Long-term pharmacological prednisolone treatments did not alter steady-state B6 vitamers in erythrocyte (Table 3) or urinary excretion of the vitamin B_6 end product (Table 3). 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 B_6 Status in Extrahepatic 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 4). There was also a trend of increased PL in the heart (Table 4), but B6 vitamers were unchanged in the brain or kidney (Table 4). These data indicated that at the dose of 0.1 to 1 mg/kg/2 days, 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 increase PL in the

TABLE 3

Effect of prednisolone treatment on erythrocyte vitamin B6 status and urinary vitamin B6 excretion

Data are presented as means ± S.D.

	Control	Prednisolone (0.1 mg/kg/2 days)	Prednisolone (1 mg/kg/2 days)	*P* Value
Erythrocyte B6 (nmol/l packed cells)				
PMP	27.5 ± 5.2	31.8 ± 10.2	21.9 ± 8.9	N.S.
% Change		15.7 ± 36.9	−20.2 ± 32.5	
PLP	732.4 ± 96.9	787.4 ± 200.3	800.5 ± 256.3	N.S.
% Change		7.5 ± 27.4	9.3 ± 35.0	
PL	135.7 ± 47.7	114.9 ± 51.9	106.5 ± 46.5	N.S.
% Change		−15.3 ± 38.3	−21.5 ± 34.3	
B6 excretion				
4-PA μg/24 h	0.94 ± 0.20	0.94 ± 0.34	0.86 ± 0.26	N.S.
% Change		−0.53 ± 36.2	−8.87 ± 27.2	
Creatnine mg/24 h	0.43 ± 0.05	0.38 ± 0.14	0.42 ± 0.13	N.S.
% Change		−12.3 ± 31.6	−2.99 ± 31.5	
4-PA μg/mg Creatnine	2.22 ± 0.56	2.49 ± 0.34	2.08 ± 0.34	N.S.
% Change		12.1 ± 2.8	−6.15 ± 15.4	

N.S., no significant difference found among groups.

TABLE 4

Effects of prednisolone on vitamin B6 status in extra hepatic tissues

Data are presented as means ± S.D. Data in a row with different superscripts are statistically different. Percentage changes were calculated compared with the controls. *P* values were calculated by Student's *t* test. Bold values indicate data significantly different when compared to controls ($P<0.05$) in Table 4.

	Control	Prednisolone (0.1 mg/kg/2 days)	Prednisolone (1 mg/kg/2 days)	*P* Value
Muscle (nmol/g tissue)				
PMP	4.1 ± 3.4	5.5 ± 2.2	4.4 ± 2.6	N.S.
% Change		31.4 ± 52.7	6.5 ± 62.8	
PLP	20.6 ± 2.8	21.1 ± 4.1	19.0 ± 4.4	N.S.
% Change		2.3 ± 20.1	−7.8 ± 21.5	
PL	0.05 ± 0.01[a]	0.08 ± 0.02[b]	0.11 ± 0.08[b]	**0.028***
% Change		**+44.4 ± 29.5**	**+99.1 ± 150.4**	**0.047****
Heart (nmol/g tissue)				
PMP	30.2 ± 1.8	31.9 ± 2.2	32.1 ± 2.3	N.S.
% Change		5.8 ± 7.3	6.2 ± 7.7	
PLP	10.6 ± 0.6	10.9 ± 0.7	9.8 ± 1.6	N.S.
% Change		2.9 ± 6.2	−8.0 ± 14.7	
PL	0.24 ± 0.07	0.34 ± 0.13	0.40 ± 0.20	N.S.
% Change		44.0 ± 53.0	70.3 ± 86.0	
Brain (nmol/g tissue)				
PMP	13.7 ± 1.7	14.8 ± 1.7	13.7 ± 1.7	N.S.
% Change		8.3 ± 12.7	0.2 ± 12.7	
PLP	6.9 ± 0.7	7.9 ± 0.9	6.5 ± 1.1	N.S.
% Change		14.4 ± 13.2	−5.2 ± 16.2	
PL	0.95 ± 0.12	1.12 ± 0.35	1.18 ± 0.56	N.S.
% Change		18.5 ± 37.2	24.6 ± 59.1	
Kidney (nmol/g tissue)				
PMP	8.3 ± 0.7	8.1 ± 0.4	7.8 ± 0.3	N.S.
% Change		−2.6 ± 4.8	−5.9 ± 3.1	
PLP	11.6 ± 0.8	12.1 ± 1.3	11.7 ± 1.1	N.S.
% Change		4.2 ± 11.1	0.8 ± 9.6	
PL	0.65 ± 0.10	0.69 ± 0.11	0.80 ± 0.17	N.S.
% Change		6.1 ± 16.9	23.3 ± 26.6	
PA	0.07 ± 0.03	0.07 ± 0.01	0.07 ± 0.01	N.S.
% Change		8.4 ± 11.7	1.2 ± 21.4	
Enzyme activity in the muscle (nmol/h · mg protein)				
PDXP	9.2 ± 1.6[a]	11.3 ± 1.3[b]	9.0 ± 0.9[a]	**0.028****
% Change		**+22.9 ± 13.8**	−1.4 ± 9.6	
PDXK	Undetectable	Undetectable	Undetectable	
PMPO	0.20 ± 0.04	0.22 ± 0.03	0.18 ± 0.05	N.S.
% Change		+10.4 ± 17.5	-9.1 ± 23.6	

N.S., no significant difference found among groups.
* Predinisolone (1 mg /kg/2 days) vs. controls.
** Predinisolone (0.1 mg/kg/2 days) vs. controls.

heart. We further investigated B6 metabolic enzyme activities and found that low-dose prednisolone significantly decreased PDXP activity by 23% in the muscle (Table 4). These data suggested that prednisolone may selectively alter vitamin B_6 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 5) 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 5). Furthermore, prednisolone (1 mg/kg body weight/2 days) significantly induced hepatic PDXK and PMPO activities and tended to decrease PDXP activity (Table 5). Prednisolone tended to increase hepatic *PMPO* mRNA expression but not *PDXK* (Table 5)**.** In summary, long-term administration of prednisolone reduced hepatic PMP concentration and increased hepatic PDXK protein and enzyme activity in a dose-dependent manner.

Discussion

In the present study we demonstrated novel findings on the effects of long-term prednisolone treatment in increasing circulating vitamin B_6 levels through direct induction of hepatic PLP-synthesizing enzymes. The effects of prednisolone on vitamin B_6 metabolism are summarized in Fig. 1. Abnormal vitamin B_6 metabolism is commonly present in subjects with chronic inflammation. Previously, we have shown that the severity of abnormal vitamin B_6 status is associated with severity of symptoms in patients with rheumatoid arthritis (Chiang et al., 2003b), and inflammation causes tissue-specific depletion of vitamin B_6 (Chiang et al., 2005a,b). Plasma PLP concentration is correlated with functional vitamin B_6 indices in patients with rheumatoid arthritis and marginal vitamin B_6 status (Chiang et al., 2003a). In the present study, based on results from two independent in vivo models,

TABLE 5

Effect of prednisolone treatment on vitamin B6 status in the liver

Data are presented as means ± S.D. Data in a row with different superscripts are statistically different. Percentage changes were calculated compared with the controls. *P* values were calculated by Student's *t* test. Bold values indicate data significantly different different when compared to controls ($P<0.05$) in Table 5.

	Control	Prednisolone (0.1 mg/kg/2 days)	Prednisolone (1 mg/kg/2 days)	*P* Value
B6 vitamer (nmol/g tissue)				
PMP	2.9 ± 0.3[a]	**2.5 ± 0.3[b]**	**2.4 ± 0.3[b]**	**0.028***
% Change		**−14.7 ± 10.1**	**−15.1 ± 9.4**	**0.047****
PLP	5.0 ± 0.3	4.6 ± 0.7	4.5 ± 0.9	N.S.
% Change		−8.4 ± 13.1	−24.9 ± 25.1	
PL	0.40 ± 0.17	0.42 ± 0.13	0.33 ± 0.10	N.S.
% Change		5.7 ± 33.5	−17.8 ± 24.2	
PA	0.04 ± 0.06	0.01 ± 0.00	0.04 ± 0.06	N.S.
% Change		−72.0 ± 7.6	−3.7 ± 160.3	
B6 metabolic enzyme protein expression				
PDXP/β-actin	0.98 ± 0.26	0.94 ± 0.29	1.14 ± 0.30	N.S.
% Change		−3.6 ± 29.6	+16.8 ± 30.9	
PDXK/β-actin	0.83 ± 0.11[a]	**1.04 ± 0.10[b]**	**1.12 ± 0.20[b]**	**0.016***
% Change		**+24.8 ± 11.7**	**+34.6 ± 23.7**	**0.028****
PMPO/β-actin	1.05 ± 0.13	1.02 ± 0.07	0.96 ± 0.11	N.S.
% Change		-2.5 ± 6.5	-8.5 ± 10.6	
B6 metabolic enzyme activity (nmol/h · mg protein)				
PDXP	25.3 ± 1.6	24.2 ± 2.1	23.6 ± 1.2	0.08**
% Change		−4.6 ± 8.2	−6.8 ± 4.6	
PDXK	2.7 ± 0.1[a]	2.9 ± 0.2[ab]	**3.0 ± 0.3[b]**	**0.042***
% Change		7.1 ± 8.3	**+13.0 ± 11.1**	
PMPO	3.1 ± 0.1[a]	3.0 ± 0.6[ab]	**3.7 ± 0.5[b]**	**0.026***
% Change		-4.8 ± 19.3	**+18.2 ± 14.5**	
B6 metabolic gene expression				
PMPO/18S	92.4 ± 31.9	31.8 ± 10.2	192.3 ± 113.9	0.08*
% Change		−15.5 ± 63.0	108.2 ± 123.2	
PDXK/18S	105.1 ± 108.9	128.1 ± 50.7	97.3 ± 111.7	N.S.
% Change		21.9 ± 48.2	−7.5 ± 106.3	

N.S., no significant difference among groups.
* Predinisolone (1 mg /kg/2 days) vs. controls.
** Predinisolone (0.1 mg/kg/2 days) vs. controls.

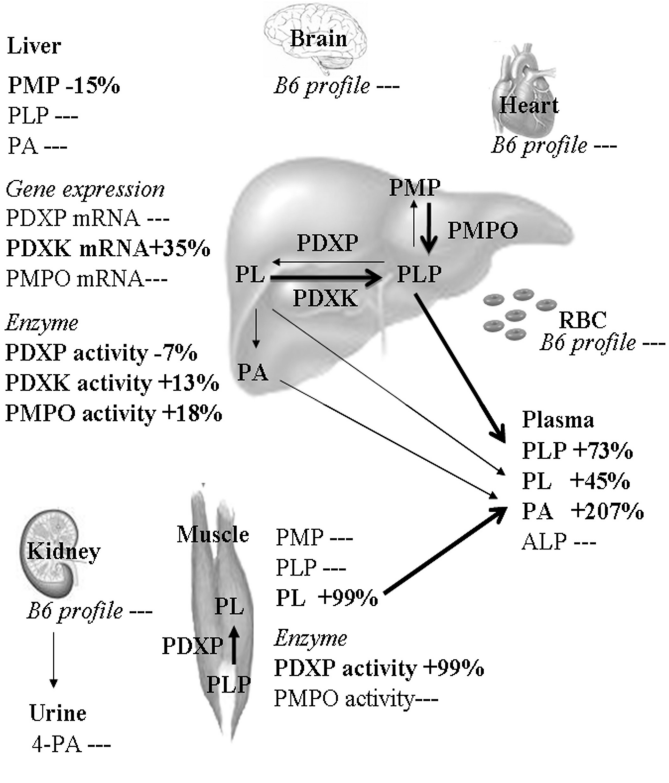

Fig. 1. Summary of long-term prednisolone effects on vitamin B_6 metabolism.

we demonstrated that prednisolone's effect on vitamin B_6 is a potential advantage in addition to its anti-inflammatory function in treating patients with RA. 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 B_6 to the circulation (Lumeng et al., 1980). Second, because PLP can be synthesized from PMP (Wada and 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 caused by 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 B_6. Although the conversion of PL to 4-PA is an irreversible reaction that generates the end product of vitamin B_6 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 B_6 ingestion or excretion but may accelerate the interconversions 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, because vitamin B_6 depletion is commonly present in pa-

tients with RA (Chiang et al., 2003a), we suggest that the prednisolone treatment-caused elevated plasma PLP can increase the supply of vitamin B_6 to tissues that may have a higher B6 requirement during inflammation. Furthermore, because PL is the major form of vitamin B_6 crossing the cell membrane, elevated circulating PL can facilitate the cellular uptake and utilization of vitamin B_6 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 was reduced by prednisolone treatment, yet hepatic PLP concentrations remained unaltered and plasma PLP was elevated. These findings suggest 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 B_6 is involved in more than 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 affect the pathogenesis and outcomes of those diseases involving numerous vitamin B_6-dependent reactions. One of the key vitamin B_6-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 and 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 (Lazzerini et al., 2003). And in rats, a 2-week period of subcutaneous cortisol injections (5 mg/kg body weight) was seen to lower plasma homocysteine levels by 50% (Kim et al., 1997), presumably because of the induction of betaine-homocysteine methyltransferase, one of the two 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 B_6-dependent transsulfuration. The possible systemic regulation by prednisolone of homocysteine transsulfuration is currently under investigation.

Other potential health benefits can result from improved vitamin B_6 status. Accumulated evidence suggests that vitamin B_6 is protective against heart diseases. Vitamin B_6 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 B_6 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 and 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 (Schäcke et al., 2002). Considering the significantly high prevalence of long-term vitamin B_6 depletion in RA, we suggest that the action of prednisolone in increasing circulating vitamin B_6 should be taken into account when considering the potential advantages and disadvantages of its use in treatment. Taken together, it may be beneficial for patients to receive long-term pharmacological prednisolone use to facilitate vitamin B_6-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

Participated in research design: Chiang.

Conducted experiments: Chang, Lin, Wu, and Chiang.

Performed data analysis: Chang, Tzen, Lin, and Chiang.

Wrote or contributed to the writing of the manuscript: Chang, Lin, and Chiang.

Other: Chiang acquired funding for this study.

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