Vitamin B6 regulates mRNA expression of peroxisome proliferator-activated receptor-γ target genes

NORIYUKI YANAKA, MAYUMI KANDA, KEIGO TOYA, HARUNA SUEHIRO and NORIHISA KATO

Department of Bioresource Science and Technology, Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan

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Abstract. We previously demonstrated that vitamin B6 suppresses tumorigenesis in the colon of mice and exerts an anti-inflammatory effect through the inhibition of NF-κB activation. As these effects resemble the pharmacological properties of thiazolidinedione (TZD), a synthetic peroxisome proliferator-activated receptor-γ (PPARγ) ligand, this study was designed to examine the effect of vitamin B6 on the activation of PPARγ and adipogenesis in 3T3-L1 adipocyte cells. Pyridoxal 5'-phosphate (PLP), one of the vitamin B6 derivatives, was shown to promote adipogenesis in the 3T3-L1 adipocytes. In addition, PLP specifically induced mRNA expression of PPARγ target genes in the 3T3-L1 adipocytes and enhanced the lipid accumulation and adipocyte fatty acid-binding protein (aP2) mRNA expression in NIH3T3 cells stably expressing PPARγ. Furthermore, the administration of vitamin B6 increased the expression of aP2 mRNA in mouse adipose tissues. Collectively, these observations suggest a novel function of vitamin B6 as an activator for PPARγ, which may contribute to the anti-tumor and anti-inflammatory effects of vitamin B6.

Introduction

Vitamin B6 is well known as a water-soluble vitamin essential for normal growth, development and metabolism. Natural vitamin B6 consists of six interconvertible compounds: pyridoxine, pyridoxal, pyridoxamine and their phosphorylated forms, pyridoxine 5'-phosphate, pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate. In particular, PLP is a biologically active form of vitamin B6 and functions as a cofactor for numerous enzymes involved in amino acid and cellular metabolisms (1). Vitamin B6 has been classically classified as a coenzyme; however, recent reports have prompted us to consider other physiological functions for vitamin B6. Vitamin B6 has been reported to play an important protective role against several types of diseases (2-4). We previously demonstrated a preventive effect of dietary vitamin B6 in moderate dosage against tumorigenesis in the colon of mice which were administered azoxymethane (AOM) (5). Further studies have demonstrated that the anti-tumorigenesis effect of vitamin B6 in the colon of AOM-injected mice may be mediated, in part, via the suppression of cell hyperproliferation, inducible nitric oxide synthase (iNOS) expression and oxidative stress (5,6). Matsubara *et al* found an anti-angiogenic effect of vitamin B6 in an *ex vivo* serum-free matrix culture using rat aortic ring models (7). Furthermore, our recent study revealed an anti-inflammatory effect of vitamin B6, showing that treatment with vitamin B6 inhibits lipopolysaccharide (LPS)-induced iNOS and cyclooxygenase-2 (COX2) expression in RAW 264.7 cells through suppression of NF-κB activation, the pro-inflammatory transcription factor (8). In addition to this *in vitro* experiment, dietary vitamin B6 inhibited nitric oxide (NO) production in response to LPS administration *in vivo* (8). Since angiogenesis and inflammation have been considered to play critical roles in the pathogenesis of colon cancer, the anti-angiogenic and anti-inflammatory effects of vitamin B6 may elucidate the mechanisms underlying the anti-tumor properties of vitamin B6.

Peroxisome proliferator-activated receptor-γ (PPARγ) is one such protein that may explain, in part, the anti-tumor behavior of vitamin B6. PPARγ, one of three PPAR subtypes (PPARα, PPARγ and PPARδ/β), is a ligand-dependent transcription factor that belongs to the nuclear hormone receptor superfamily (9). PPARγ is found highly expressed in adipose tissue, and to a lesser extent in the colon and immune system (10). PPARγ enhances adipocyte-specific gene expression via the formation of a heterodimeric DNA-binding complex with the retinoid X receptor. To date, overexpression and knockout studies with mice strongly suggest that PPARγ is an essential transcription factor in adipogenesis *in vitro* and *in vivo* (11,12). Furthermore, the most extensively employed insulin-sensitizing drugs, thiazolidinedione derivatives (TZDs), such as

Correspondence to: Dr Noriyuki Yanaka, Department of Bioresource Science and Technology, Graduate School of Biosphere Science, Hiroshima University, 4-4 Kagamiyama 1-chome, Higashi-Hiroshima 739-8528, Japan
E-mail: yanaka@hiroshima-u.ac.jp

Abbreviations: PPARγ, peroxisome proliferator-activated receptor-γ; TZD, thiazolidinedione; PLP, pyridoxal 5'-phosphate; PBS, phosphate-buffered saline

Key words: vitamin B6, 3T3-L1, peroxisome proliferator-activated receptor-γ, adipocytes

troglitazone, pioglitazone and rosiglitazone, have been found to possess a high affinity for PPARγ (13), suggesting that the pharmacological actions of TZDs are mediated through PPARγ activation in adipocytes. It has been reported that improvement in insulin resistance by PPARγ activation is due to an increase in the number of differentiating adipocytes, which promotes adipose-secreted hormone expression and, eventually, glucose homeostasis (9). Furthermore, several substances including the prostaglandin J2 derivative are potent natural ligands of PPARγ (14) which control a variety of physiological functions through the PPARγ signaling pathway.

Activation of PPARγ via TZDs has been found to elicit both anti-neoplastic and anti-inflammatory effects. Therefore, PPARγ is expected to be a pharmacological target for several types of cancers and cardiovascular diseases (15,16). In colon carcinogenesis, troglitazone, one of the TZDs, has been shown to inhibit tumor growth (17) and to reduce the number of aberrant crypt foci, which are precursor lesions of colon cancer in the colon of mice administered AOM (18,19). In addition, recent reports have demonstrated that the anti-atherosclerotic effect of TZDs is partially explained by the anti-inflammatory functions of PPARγ, and that TZDs inhibit the expression of various inflammatory proteins including iNOS and COX2 in macrophages. To date, several mechanisms underlying the anti-inflammatory effects of PPARγ ligands have been proposed, including the inhibition of NF-κB activity (20).

Kawada *et al* previously tested the effects of vitamins and their analogues on the terminal differentiation of 3T3-L1 cells and found that vitamin B6 derivatives regulated adipogenesis of 3T3-L1 adipocyte cells (21). As the pharmacological properties of TZDs appear to be similar to the biological effects of vitamin B6, we hypothesized that vitamin B6 regulates PPARγ activation similar to TZDs. To examine this hypothesis, we investigated whether vitamin B6 affects adipogenesis in 3T3-L1 adipocytes, which are able to be efficiently differentiated in the presence of TZDs and support PPARγ target gene mRNA expression. The present study shows that treatment with PLP, one of the vitamin B6 derivatives, accelerates adipogenesis in 3T3-L1 adipocytes and that PLP up-regulates the expression of PPARγ target genes in NIH3T3 cells transfected with PPARγ. Furthermore, an acute administration of vitamin B6 was shown to enhance the expression of a PPARγ target gene *in vivo*. These observations suggest that vitamin B6 plays an important role as an activator of PPARγ *in vitro* and *in vivo*.

Materials and methods

Chemical reagents. Pyridoxal hydrochloride, PLP and pyridoxine hydrochloride were obtained from Nacalai Tesque (Osaka, Japan). T-174 [5-[2-(naphthalenylmethyl)-5-benzoxazolyl]-methyl]-2,4-thiazolidinedione), a specific ligand for PPARγ, was kindly provided by Tanabe Mitsubishi Pharma Co. (Osaka, Japan).

Cell culture. Mouse 3T3-L1 preadipocytes, NIH3T3 cells and Phoenix 293 cells were cultured in a maintenance medium (10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in Dulbecco's modified Eagle's medium at 37˚C in 5% CO_2/95% air under a humidified condition. High titer retroviruses harboring PPARγ were produced in Phoenix 293 cells and used to infect NIH3T3 cells, as reported previously (22).

Adipocyte differentiation and Oil-Red-O staining. For differentiation assays, confluent 3T3-L1 cells were treated with differentiation medium [maintenance medium plus 0.5 mM 3-isobutyl-1-methylxanthine (IBMX)], 5 μg/ml insulin, and 1 μM dexamethasone (DEX) and incubated for 2 days. Differentiation medium was then replaced with adipocyte growth medium (maintenance medium supplemented with 5 μg/ml insulin) with or without PLP, which was refreshed every 2 days. Differentiated 3T3-L1 cells were fixed with 4% buffered paraformaldehyde for 15 min. A stock solution of 0.3% Oil-Red-O (Sigma) in isopropanol (w/v) was diluted 6:4 prepared in water, filtered and added to the fixed cells. Cells were washed twice in phosphate-buffered saline (PBS) and photographed.

Glycerol-3-phosphate dehydrogenase activity. Differentiated 3T3-L1 cells were washed twice with ice-cold PBS and suspended in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin, and 1 mg/ml leupeptin. Membranes were disrupted by sonication, and supernatants were collected following centrifugation at 13,000 x g for 10 min. The reaction was initiated by the addition of supernatants to a standard mixture containing 100 mM triethanolamine/HCl buffer (pH 7.5), 2.5 mM EDTA, 0.12 mM β-nicotinamide adenine dinucleotide disodium salt (NADH), 0.2 mM dihydroxyacetone-phosphate and 0.1 mM β-mercaptoethanol. The change in absorbance at 340 nm was measured using a spectrophotometer at 25˚C. One unit of enzyme activity was determined to correspond to the oxidation of 1 nmol of NADH/min.

Experimental animals. Male ICR mice (5-weeks old) (Charles River Japan Inc., Japan) were housed in groups of 6 animals in plastic cages in a room with controlled temperature (24˚C) and a 12-h light/dark cycle. The animals were provided with free access to the diet and water and were maintained according to the 'Guide for the Care and Use of Laboratory Animals' established by Hiroshima University. The experimental diet (vitamin B6-free) consisted of the following components (in g/kg diet): α-corn starch, 402; casein, 200; sucrose, 200; corn oil, 100; cellulose, 50; AIN-93G salt mixture, 35; AIN-93 vitamin mixture (pyridoxine-free), 10; L-cystine, 3. The experimental feeding period was 2 weeks, and the mice were divided into 3 groups of 8 mice each. The animals received an oral administration of pyridoxine hydrochloride (200 or 500 mg/kg body weight) or physiological saline. Six hours after oral administration, epididymal fat pads were removed.

Northern blot hybridization. Total RNA from differentiated 3T3-L1 cells was isolated using Isogen (Nippon Gene). Total RNA from mouse adipose tissues was isolated using RNeasy lipid tissue kit (Qiagen). Total RNA (10 μg) was subjected to Northern blot hybridization. Briefly, RNA was fractionated in 1% agarose gel containing 0.66 M formaldehyde and 0.02 M MOPS (pH 7.0). Fractionated RNAs were transferred onto a Hybond-N^+ nylon filter (GE Healthcare) by capillary blotting

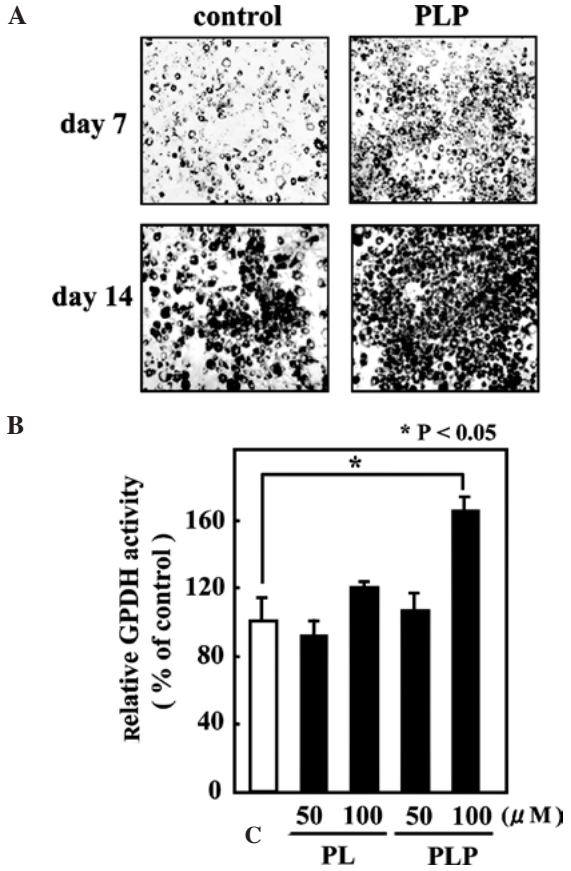

Figure 1. The effect of vitamin B6 on adipogenesis in 3T3-L1 adipocytes. (A) 3T3-L1 adipocytes were treated with 100 μM PLP for 12 days (from day 2-14). Intracellular lipid droplets were visualized by Oil-Red-O staining. (B) GPDH activity was determined after a 12-day cultivation with vitamin B6 at the indicated concentrations. The GPDH activity of untreated cells was set at 100%, and the relative activities were presented as fold induction compared to that of the untreated cells (C). Values are the mean ± SE (n=5). *P<0.05 vs. control.

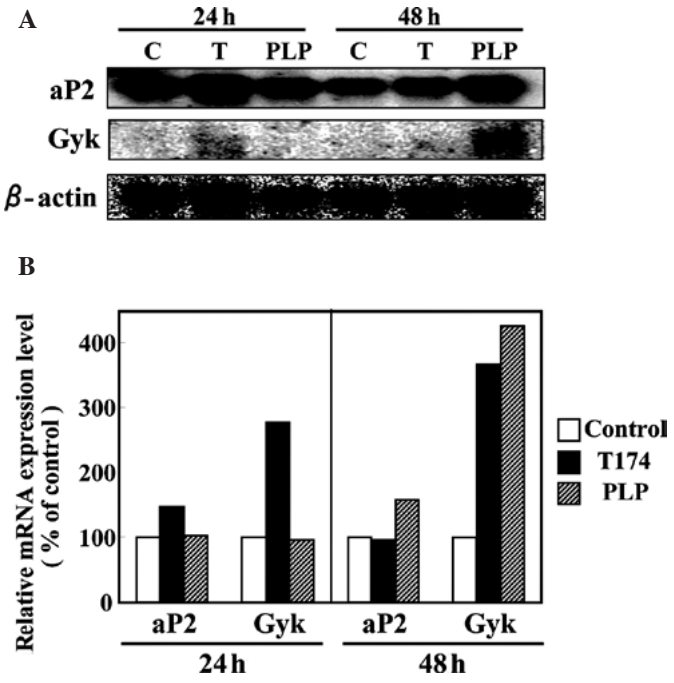

Figure 2. PLP induces the mRNA expression of PPARγ target genes in 3T3-L1 adipocytes. On day 6 after induction of differentiation, 3T3-L1 adipocytes were treated with 10 μM T-174 (T) or 100 μM PLP for 24 or 48 h. (A) Total RNA (10 μg) from 3T3-L1 cells was prepared and subjected to Northern blot analyses. (B) The radioactivity in each band was quantified using an image analyzer and normalized to that of β-actin mRNA.

and then cross-linked by ultraviolet irradiation. cDNA fragments of mouse adipocyte fatty acid-binding protein (aP2) and mouse glycerol kinase (GyK) were amplified using specific primers. Primers were as follows: aP2 forward primer, 5'-GAAGACAGCTCCTCCTCGAAGGTT-3'; aP2 reverse primer, 5'-GGAAGTCACGCCTTTCATAACA-3'; Gyk forward primer, 5'-TGGTGTCAGCAACCAGAGGGA-3'; Gyk reverse primer, 5'-GGCCATAGATCTCAGAAGAAC-3'. ^{32}P-labeled cDNA fragments encoding aP2, GyK, human β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for Northern blot hybridization as probes. Hybridization was performed in PerfectHyb (Toyobo) at 65˚C for 20 h. The membrane was finally washed with 0.2X SSC and 0.1% SDS at 65˚C for 30 min, and the hybridization signals were analyzed using the BAS system (Fuji Film).

Statistical analyses. Values are presented as the means ± SE. Statistical significance among the means was estimated at P<0.05 according to the Student's t-test.

Results

The effect of vitamin B6 on adipogenesis in 3T3-L1 adipocytes. To investigate the effect of vitamin B6 on adipogenesis, 3T3-L1 preadipocytes, which are well characterized as an *in vitro* model of adipocyte differentiation were used. 3T3-L1 cells differentiated into mature adipocytes upon exposure to a hormonal stimulus (0.5 mM IBMX, 5 μg/ml insulin and 1 μM DEX). After a 2-day incubation with the hormonal mixture, 3T3-L1 cells were cultured in a medium containing vitamin B6 for 12 days and then subjected to Oil-Red-O staining for lipid droplet visualization. As shown in Fig. 1A, 3T3-L1 cells treated with PLP accumulated larger and a greater number of lipid droplets than the control cells. Since glycerol-3-phosphate dehydrogenase (GPDH) occupies a central position in the pathway of triglyceride synthesis, GPDH enzyme activity was measured as an adipocyte differentiation marker. In agreement with the Oil-Red-O staining, treatment with 100 μM PLP resulted in a significant increase (by 60%) in GPDH enzyme activity compared to untreated cells (Fig. 1B).

PLP induces the expression of PPARγ target genes in 3T3-L1 adipocytes. As described above, PLP promoted adipogenesis in 3T3-L1 cells. Subsequently, mRNA expression of PPARγ target genes was investigated in the differentiated 3T3-L1 adipocytes. On day 6 after differentiation was induced with the hormonal stimulus, 3T3-L1 cells were treated with either a vehicle control (DMSO), 10 μM T-174 or 100 μM PLP for 24 or 48 h. After 24 h, the addition of 10 μM T-174 resulted in an increase in the mRNA expression levels of PPARγ target genes, *aP2* and *Gyk* (Fig. 2). Although the addition of 100 μM PLP for 24 h had no influence on the mRNA expression levels of PPARγ target genes, PLP treatment for 48 h demonstrated 1.6- and 4.3-fold increases in the mRNA expression levels of *aP2* and *Gyk* genes, respectively (Fig. 2).

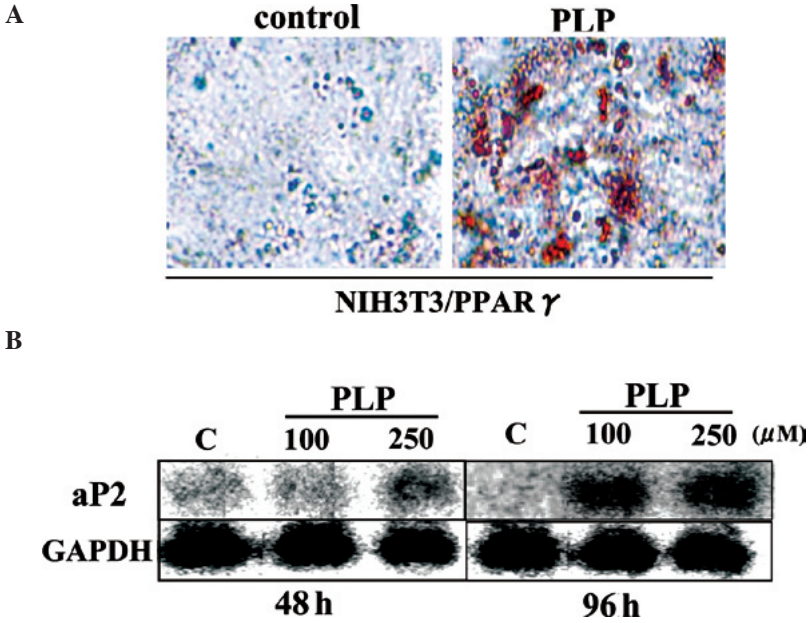

Figure 3. PLP enhances lipid accumulation and *aP2* mRNA expression in NIH3T3 cells stably trasnfected with PPARγ. Retroviruses harboring PPARγ were used to infect NIH3T3 cells. (A) NIH3T3 cells stably expressing PPARγ (NIH3T3/PPARγ) were treated with 250 μM PLP for 96 h. Intracellular lipid droplets were visualized by Oil-Red-O staining. (B) NIH3T3/PPARγ cells were treated with 100 μM or 250 μM PLP for 48 or 96 h. Total RNA (10 μg) from NIH3T3 cells was prepared and subjected to Northern blot analyses.

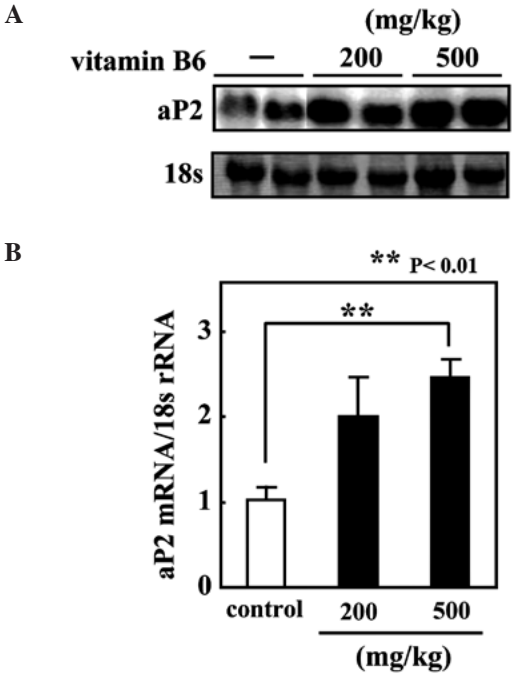

Figure 4. The effect of the acute administration of vitamin B6 on *aP2* mRNA expression in mouse adipose tissues. (A) Mice were fed a vitamin B6-free diet for 2 weeks and then received an oral administration of pyridoxine hydrochloride (200 or 500 mg/kg body weight) or physiological saline as control. After a 6-h administration of pyridoxine hydrochloride, 10 μg of total RNA from epididymal fat pads was isolated and subjected to Northern blot analysis. (B) The radioactivity in each band was quantified using an image analyzer and normalized to that of 18S. Each value relative to the mRNA level of the control mice represents the mean ± SE. (n=4). **P<0.01 vs. control.

PLP induces the expression of a PPARγ target gene in NIH3T3 cells transfected with PPARγ. PLP was shown to up-regulate mRNA expression in PPARγ target genes in differentiated 3T3-L1 adipocytes. To examine whether PLP activates PPARγ-dependent transcription in cultured cells, retroviruses harboring PPARγ were used to infect NIH3T3 cells. In PPARγ-transfected NIH3T3 cells, PLP enhanced *aP2* mRNA expression and lipid accumulation (Fig. 3). Taken together, these results showed that PLP activates PPARγ-dependent transcription in cultured cells.

The effect of acute administration of vitamin B6 on expression of a PPARγ target gene in mouse adipose tissues. The present study demonstrated that PLP promotes adipogenesis in 3T3-L1 cells and the mRNA expression of PPARγ target genes in differentiated 3T3-L1 adipocytes. Therefore, we aimed to ascertain whether vitamin B6 regulates the expression of a PPARγ target gene *in vivo* and to examine the effect of vitamin B6 on the transactivation activity of PPARγ *in vivo*. ICR mice were fed a vitamin B6-free diet for 2 weeks, and the animals subsequently received an oral administration of pyridoxine hydrochloride (either 200 or 500 mg/kg body weight). Six hours after oral administration, total RNA was isolated from the epididymal fat pads. The administration of vitamin B6 significantly increased *aP2* mRNA level in the adipose tissues in a dose-dependent manner (Fig. 4).

Discussion

In the present study, PLP, one of the vitamin B6 derivatives, was found to promote adipogenesis in 3T3-L1 cells and up-regulate PPARγ-dependent gene expression *in vitro* and *in vivo*. To address whether the effect of PLP on adipogenesis is dependent on the transactivation of PPARγ, NIH3T3 cells stably infected with retroviruses encoding PPARγ were constructed. NIH3T3 cells, which are not committed to adipocytes, have been well studied and show a similar phenotype to 3T3-L1 cells when PPARγ is expressed. In fact,

adipocyte differentiation of PPARγ-infected cells was induced in response to T-174 treatment (data not shown), indicating that NIH3T3 cells stably expressing PPARγ are well suited for monitoring adipogenesis simply for PPARγ activation capabilities. In this stable cell line, PLP was shown to promote lipid accumulation, strongly suggesting that PLP stimulates PPARγ transactivation.

Two molecular mechanisms by which PLP affects the expression of PPARγ target genes in a direct or indirect manner were considered for study. First, as it was previously reported that thiazolidine compounds are produced by condensation of aminothiols, such as cysteine, with PLP under a physiological condition (23), PLP may act as a PPARγ ligand by conversion to thiazolidine compounds. Indeed, PLP treatment alone was shown to require more time to induce mRNA expression of PPARγ target genes compared to T-174 treatment. In general, PPARγ ligands are well characterized as lipophilic substances, which contain long-chain polyunsaturated fatty acids and fatty acid metabolites, such as 15-deoxy-Δ12,14-prostaglandin J2. PPARγ is thought to have a larger ligand-binding pocket compared to that of other nuclear receptors (24), which may allow PLP-derived compounds to act as ligands. However, observations in this study raise a critical question concerning the selectivity of PLP for PPARγ target genes. As shown in Fig. 2, PLP was shown to up-regulate *aP2* and *Gyk* mRNA expression, but not lipoprotein lipase (LPL) (data not shown), which is also a well-known PPARγ target gene (25), in 3T3-L1 cells. There is increasing evidence that transcriptional regulation varies even among the PPARγ-responsive genes via distinct mechanisms (26-28), and, in fact, several isoprenols were found to induce the expression of *aP2* mRNA, but not LPL mRNA in 3T3-L1 cells (29), suggesting that PLP may act as a partial (or weak) agonist similar to the isoprenols.

Second, PLP may influence the interaction of cofactors with PPARγ, as it has been proposed that ligand-induced transcriptions of PPARγ target genes are mediated via the recruitment of distinct cofactors (26-28). Recently, Huq *et al* found an important role for vitamin B6 in gene regulation by direct PLP conjugation to receptor interacting protein 140 (RIP140) (30). RIP140 was originally identified as a ligand-dependent co-repressor which binds to estrogen receptors, and, to date, has been shown to interact with and repress a number of other nuclear receptors, including thyroid hormone receptors and estrogen-related receptors (31). Leonardsson *et al* reported that RIP140-null mice show defects in fat accumulation and energy expenditure in adipose tissues, suggesting an important role of RIP140 in regulating the balance between energy storage and energy expenditure (32). Furthermore, in RIP140-null adipocytes, energy expenditure was reportedly elevated with high expression levels of uncoupling protein 1 (Ucp1) and carnitine palmitoyltransferase 1b mRNAs (33). Debevec *et al* proposed that the absence of RIP140 leads to the recruitment of PPARγ, together with PPARα and estrogen receptor α, to the UCP1 enhancer to allow activation of *Ucp1* gene transcription (34). These observations prompted us to further consider the physiological significance of the conjugation between PLP and RIP140 for PPARγ activation in adipocytes.

In summary, these observations presented here show that vitamin B6 up-regulates the expression of PPARγ target genes *in vitro* and *in vivo*. This study indicates a novel physiological function of vitamin B6 which may be, in part, mediated by PPARγ activation. The anti-tumor and anti-inflammatory effects of vitamin B6, furthermore, may relate to PPARγ activation. Further study is necessary to elucidate the molecular mechanism underlying the action of vitamin B6 on PPARγ.

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