RESEARCH ARTICLE

Vitamin B6 suppresses serine protease inhibitor 3 expression in the colon of rats and in TNF-α-stimulated HT-29 cells

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Scope: Previous reports in the areas of animal studies and, recently epidemiology, have linked anti-tumorigenic and anti-inflammatory effects to dietary vitamin B6. This study investigated the molecular mechanism of these effects of vitamin B6.

Methods and results: DNA microarray analysis was used to obtain information on changes in colon gene expression from vitamin B6 (pyridoxine) repletion in vitamin B6-deficient rats. Pyridoxine supplementation down-regulated the inflammatory molecule, serine protease inhibitor clade A member 3 (SPI-3) mRNA expression in the colon. This study also showed that tumor necrosis factor α (TNF-α) induced SPI-3 mRNA expression in HT-29 human colon cancer cells, and vitamin B6 (pyridoxal hydrochloride) pretreatment of HT-29 cells inhibited TNF -induced mRNA expression of SPI-3. Vitamin B6 inhibited TNF-α-induced NF-κB activation via suppression of IκBα degradation in HT-29 cells. HT-29 cells stably expressing epitope-tagged ubiquitin were generated and vitamin B6 pretreatment was shown to inhibit ubiquitination of the IkB protein in response to TNF-α-i.

Conclusion: Vitamin B6 suppressed SPI-3 expression in the colon of rats and in TNF-α-stimulated HT-29 cells. Further, this study showed a possible role of vitamin B6 in the regulation of protein ubiquitination.

Keywords:
Colon / NF-κB / Serine protease inhibitor / Ubiquitin / Vitamin B6

1 Introduction

Vitamin B6 is recognized as a water-soluble vitamin essential for normal growth, development and metabolism. The metabolically active form of the vitamin, pyridoxal 5′-phosphate (PLP), acts as a cofactor for numerous enzymes that are involved in amino acid metabolism [1, 2]. Although vitamin B6 is widely distributed in various foods, there is evidence that many adults are not obtaining adequate amounts of this nutrient from their diet, suggesting that the nutritional importance of vitamin B6 is under-recognized [3]. Subnormal vitamin B6 status leading to functional deficiency has been demonstrated in inflammatory diseases, including rheumatoid arthritis, asthma and cardiovascular diseases [4–7]. Possible causes of decreased levels of vitamin B6 seen in inflammation are discussed. Chiang *et al.* have reported that the low plasma PLP levels are not due to insufficient intake or excessive vitamin B6 excretion [8] and that pyridoxine (PN) supplementation corrects vitamin B6 status, quantitatively and functionally, in patients with rheumatoid arthritis [9, 10]. Vitamin B6 deficiency has been discussed from the points of view of nutritional status and pathogenesis. Adequate dietary vitamin B6 intake has been demonstrated to have protective roles against several

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; **IKK,** Iκ-B kinase; **NF-κB,** nuclear factor-κB; **PL,** pyridoxal; **PLP,** pyridoxal 5′-phosphate; **PN,** pyridoxine; **SPI-3,** serine protease inhibitor clade A member 3; **TNF-α,** tumor necrosis factor-α; **RIP140,** receptor interacting protein 140

diseases. We have shown that vitamin B6 supplementation reduced cell proliferation and significantly suppressed the incidence and number of colon tumors in mice receiving azoxymethane [11]. Recent case–control studies have also indicated an inverse association between vitamin B6 intake and colorectal cancer risk, strongly supporting the anti-tumorigenic effect of dietary vitamin B6 [12, 13]. We have shown that vitamin B6 suppresses the protein expression of the proliferation-related genes, c-*myc* and c-*fos*, and reduces oxidative stress in azoxymethane-treated mice. Furthermore, a report has demonstrated that vitamin B6 exerts an anti-angiogenic effect in an *ex vivo* serum-free matrix culture model [14, 15]. However, the molecular mechanisms by which vitamin B6 carries out anti-tumorigenic effects are not yet fully understood.

Previous reports on the existence of transcription factors that interact with vitamin B6, such as hepatocyte nuclear factor 1 [16], steroid hormone receptors [17, 18] and receptor interacting protein 140 (RIP140) [19], prompt us to consider that vitamin B6 may exert an anti-tumorigenic effect *via* a genomic mechanism. In this study, we examined the regulatory effects of dietary vitamin B6 on gene expression in the colon. Using DNA microarray analysis, we obtained information on colon gene expression in vitamin B6-deficient rats with vitamin B6 repletion. We showed that PN supplementation down-regulates the mRNA expression of the inflammatory molecule, serine protease inhibitor clade A member 3 (SPI-3) in the colon. As chronic inflammation and colorectal carcinogenesis have been considered to be mechanistically linked [20], in this study we focused on the suppressive effect of vitamin B6 on SPI-3 mRNA expression. We showed that vitamin B6 inhibited SPI-3 mRNA expression in HT-29 human colon cancer cells in response to tumor necrosis factor-α (TNF-α) and determined the molecular mechanism by which its inhibitory function is carried out. This study provides a novel insight into the biological roles of vitamin B6 which may include taking part in the regulation of protein ubiquitination.

2 Materials and methods

2.1 Materials

Pyridoxal hydrochloride, PLP and PN hydrochloride were obtained from Nacalai Tesque (Osaka, Japan). Pyridoxamine dihydrochloride was obtained from Calbiochem (Gibbstown, NJ, USA). Antibodies specific for nuclear factor-κB (NF-κB) p65 and Iκ-Bα were products of Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific for phospho-NF-κB p65 (Ser536) was from Cell Signaling Technology (Beverly, MA, USA). Recombinant human TNF-α was obtained from GT Laboratories (Minneapolis, MN, USA). MG-132 was from Peptide Institute (Osaka, Japan). Mouse monoclonal FLAG (M5) antibody was purchased from Sigma (St. Louis, MO, USA). HT-29 human colon cancer cells were obtained from Health Science Research Resources Bank (Sennan, Japan).

2.2 Animals and diets

Male Wistar rats (4 wk old, Charles River Japan, Japan) were maintained according to the 'Guide for the Care and Use of Laboratory Animals' established by Hiroshima University. After being fed a PN-deficient basal diet for 3 wk, the rats (average 293.0 g) were divided into two groups of seven rats. The two groups were fed PN-deficient basal diet or basal diet [21] containing 35 mg/kg vitamin B_6 (PN hydrochloride) for 4 days, respectively. Plasma PLP was measured according to Tsuge [22].

2.3 DNA microarray

After removing the rectum from large intestine, the colon was opened longitudinally with fine scissors, and mucus and feces were removed in ice-cold phosphate-buffered saline. Total RNAs derived from two groups were isolated using RNeasy kit and RNase-Free DNase set (Qiagen Sciences, Germantown, MD), and subjected to cRNA synthesis for a DNA microarray analysis according to the manufacturer's instructions (44K whole rat genome 60-mer oligo microarray, Agilent Technologies, Palo Alto, CA, USA). All procedures of fluorescence labeling, hybridization, slide and image processing were carried out according to the manufacturer's instructions. In this experiment, DyeSwap method was carried out in order to eliminate the bias between dyes because the difference between Cyanine 3-CTP and Cyanine 5-CTP altered the efficiency of hybridization in case of competitive DyeCoupling assay. Gene expression data were obtained using Agilent Feature Extraction software, using defaults for all parameters except ratio terms, which were changed according to the Agilent protocol to fit the direct labeling procedure. Files and images, including error values and *p*-values, were exported from the Agilent Feature Extraction Program (version 9.5). The reverse transcriptase reaction was carried out with 1 μg of total RNA as a template to synthesize cDNA using Super-Script II reverse transcriptase and random hexamers (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. cDNA and primers were added to the GoTaq Master Mix (Promega, Madison, WI, USA) to give a total reaction volume of 20 μL. The primers are as follows: rat SPI-3, forward, 5′-ACCCCTGAGACAGAAATCCACCG-3′ and reverse, 5′-TTCAGCTCCTCATCCCGGACGTA-3′.

2.4 Cell cultures and stable transfection

HT-29 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum,

100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2. HT-29 cells were transfected with a reporter plasmid encoding 5xNF-κB-luc with pCMV-β-gal together using LipofectAMINE 2000 (Invitrogen), according to the manufacturer's instructions. Twenty hours later, the transfected HT-29 cells were cultured in the presence or absence of 500 μM pyridoxal (PL) for 1 h, and either untreated or stimulated with human TNF-α (50 ng/mL) for 6 h. Cell lysates were prepared, and luciferase activities were measured with luciferase assay kit (Toyo Inki, Japan). NF-κB activities were determined by normalization of NF-κB-dependent luciferase activity to β-galactosidase activity. pcDNA-FLAG-ubiquitin was provided by Dr Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science, Japan). DNA transfection was performed using LipofectAMINE 2000. After replating, the HT-29 cells were treated with 500 μg/mL G418 (Sigma) for 14 days. G418-resistant colonies were identified, and independent colonies were re-seeded and subjected to immunoblotting with anti-FLAG antibody.

2.5 RNA analyses

Total RNAs from HT-29 cells were isolated using TRIzol™ (Invitrogen). Ten micrograms of total RNA was transferred to Hybond N^+ nylon membranes (GE Healthcare, Piscataway, NJ, USA). To isolate cDNA encoding human SPI-3, we performed PCR using cDNA isolated from HT-29 cells as a template and a set of PCR primers (5′-CAGTCTGCTG-GACAGGTTCACG-3′ and 5′-ACAGGTCAGCCTTGCTGGT-GAA-3′). ^{32}P-labeled cDNA fragments encoding rat SPI-3, human SPI-3, rat β-actin and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were generated using a Random Prime Labeling Kit (Takara Bio, Kyoto, Japan) according to the manufacturer's protocol. Hybridization was performed with each probe in hybridization buffer (Perfect Hyb, Toyobo, Osaka, Japan) at 65°C for 16 h. The membranes were exposed to a Fuji imaging plate (FUJI Photo Film, Japan) overnight. Expression of IL-8 mRNA was examined using RT-PCR with primers 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ and 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′.

2.6 Immunoblotting

HT-29 cells were washed with ice-cold phosphate-buffered saline and scraped in an ice-cold lysis buffer (10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μg/mL aprotinin and 10 μg/mL leupeptin). Total cell extracts were centrifuged at 16 000 × *g* for 15 min at 4°C to remove cellular debris. The supernatants were resolved by SDS-PAGE and transferred to Immobilon P membrane (Millipore, Bedford, MA, USA). The membrane was probed with primary antibodies as described above. Antibodies were detected using horseradish peroxidase-conjugated anti-rabbit (GE Healthcare) or anti-mouse (Sigma) IgG and visualized with the ECL system (GE Healthcare). Stably transfected HT-29 cells were scraped in an ice-cold lysis buffer with 20 μM MG-132. Total cell extracts were centrifuged at 16 000 × *g* for 15 min at 4°C to remove cellular debris. The supernatants were immunoprecipitated with anti-FLAG M5 monoclonal antibody with protein G-Sepharose (GE Healthcare) for 3 h at 4°C by rotation. The beads were washed five times with ice-cold lysis buffer, and immune complexes were eluted by heating at 95°C in 2 × SDS sample buffer, subjected to SDS-PAGE and analyzed by immunoblotting as described above.

2.7 Statistical analyses

Values are presented as means ± SE. Statistical significance among means was estimated at $p < 0.05$ according to one-way ANOVA and Duncan's multiple-range test.

3 Results

Both laboratory animal testing and epidemiological studies have demonstrated that supplemented vitamin B6 plays a role in suppressing colon tumorigenesis. The purpose of this study was to investigate the potential anti-tumorigenic properties of vitamin B6 at the molecular level with particular emphasis on the biological significance of vitamin B6 dietary supplementation.

The rats, after being fed a supplemental PN-deficient basal diet for 3 wk, were divided into two groups ($n = 7$) and fed either a PN-deficient basal diet (–PN) or a basal diet containing 35 mg/kg vitamin B6 (PN hydrochloride, +PN) for 4 days. The changes in body weight and concentration of the metabolically active PLP in the plasma, in response to PN repletion, are shown in Table 1. Supplemental vitamin B6 caused increased body weight as compared with the control group fed on a PN-deficient basal diet (–PN). The concentration of PLP in the plasma was significantly increased in response to PN repletion (Table 1). In this study, we isolated total RNA from colon tissues from each

Table 1. Body weight and plasma pyridoxal 5′-phosphate concentration

Rats	Body weight (g)	Plasma PLP (nmol/L)
Deficient	321.6 ± 4.3	118.6 ± 50.8
PN	339.6 ± 4.5*	1684.5 ± 300.2*

Body weight and plasma pyridoxal 5′-phosphate (PLP) concentration were increased in response to PN supplementation. Values represent means ± SE ($n = 7$). *$p < 0.05$ compared with those of PN-deficient group (*deficient*).

Table 2. Response of mRNA expressions in the colon to repletion in the pyridoxine-deficient rat

Gene ID	Gene symbol	Gene description	Fold	*p* - Value
MHC and related proteins				
NM_001004084	RT1-Bb	RT1 class II, locus Bb (RT1-Bb)	0.05	0.000
X14879	RT1-Ba	RT1.B-1 (alpha) chain of integral membrane protein	0.10	0.000
BX883043	BX883043	Chromosome 20, major histocompatibility complex	0.10	0.000
NM_001008843	RT1-CE5	RT1 class I, CE5 (RT1-CE5), transcript variant 1	0.38	0.000
AF387339	AF387339	Vacuolar ATPase NG38, Bat1, and MHC class I antigen	0.36	0.000
NM_001008839	RT1-CE16	RT1 class I, CE16	0.56	0.000
NM_001008841	RT1-CE3	RT1 class I, CE3	0.58	0.000
Y13890	RT1-Aw2	Mature MHC class Ib alpha chain	0.59	0.000
NM_152848	Ly49i2	Ly49 inhibitory receptor 2	0.60	0.018
Antibodies				
AF217588	AF217588	Clone 122.4 immunoglobulin kappa light chain variable region	0.52	0.000
L22655	L22655	Anti-acetylcholine receptor antibody gene, kappa-chain, VJC region	0.60	0.001
Z93359	Z93359	Immunoglobulin variable region (clone ERF2.13)	2.15	0.002
M61884	M61884	Ig rearranged light chain V-region	1.87	0.005
AF217590	AF217590	Clone 122.77 immunoglobulin kappa light chain variable region	1.71	0.000
AF217587	AF217587	Clone 122.33 immunoglobulin kappa light chain variable region	1.53	0.006
M87784	M87784	Hybridoma YTH906, immunoglobulin kappa chain variable region	1.96	0.003
Secreted proteins				
NM_053289	Pap	Pancreatitis-associated protein	0.16	0.000
NM_173097	Reg3g	Regenerating islet-derived 3 gamma	0.17	0.000
NM_053791	Prlpm	Prolactin-like protein M	0.33	0.021
NM_031531	Serpina3n	Serine (or cysteine) peptidase inhibitor, clade A, member 3N	0.47	0.000
NM_181625	Retnlg	Resistin-like gamma	0.49	0.000
NM_199082	Sectm1	Secreted and transmembrane 1	0.64	0.003
NM_033233	Csh1l1	Chorionic somatomammotropin hormone 1-like 1	0.66	0.027
XM_342575	Svs6_predicted	Seminal vesicle secretion 6 (predicted)	3.43	0.030
NM_012662	Svp4	Seminal vesicle protein 4	3.45	0.000
NM 001033076	Defa6	Defensin alpha 6	1.56	0.003
Channel and transporter				
NM_017046	Scnn1g	Sodium channel, nonvoltage-gated 1 gamma	2.10	0.000
NM_031736	Slc27a2	Solute carrier family 27 (fatty acid transporter), member 2	1.53	0.012
Other (Signal transduction and metabolism)				
NM_134403	Abtb2	Ankyrin repeat and BTB (POZ) domain containing 2	0.61	0.001
TC541487	TC541487	Ankyrin repeat and SOCS box containing protein 3	0.51	0.000
NM_212488	Btnl7	Butyrophilin-like 7	0.46	0.001
NM_001002827	Notch4	Notch homolog 4	0.58	0.001
NM_139192	Scd1	Stearoyl-Coenzyme A desaturase 1	1.83	0.000
XM_342395	Adamts13_predicted	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 13	1.87	0.000
NM_199381	NAPE-PLD	*N*-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D	1.95	0.036
XM_001078237	Trim16_predicted	Tripartite motif protein 16 (predicted)	5.88	0.000
NM_133547	Sult1c2	Sulfotransferase family, cytosolic, 1C, member 2	1.99	0.001
XM_227485	Msr2_predicted	Macrophage scavenger receptor 2 (predicted)	1.80	0.000
NM_012923	Ccng1	Cyclin G1	1.73	0.000
NM_173136	Akr1b8	Aldo-keto reductase family 1, member B8	1.76	0.000
NM_012912	Atf3	Activating transcription factor 3	1.68	0.001
XM_235296	Ddef1_predicted	Development and differentiation enhancing (predicted)	1.73	0.003
NM_133581	Wfdc1	WAP four-disulfide core domain 1	1.69	0.000
NM_017004	Es2	Esterase2	1.56	0.016

List of differentially expressed genes grouped into functional categories. DNA microarray analysis was repeated with the Cy3 and Cy5 dyes reversed (a dye swap), and fold change (*Fold*) represents the average of mRNA expression level in +PN group relative to –PN group (deficient).

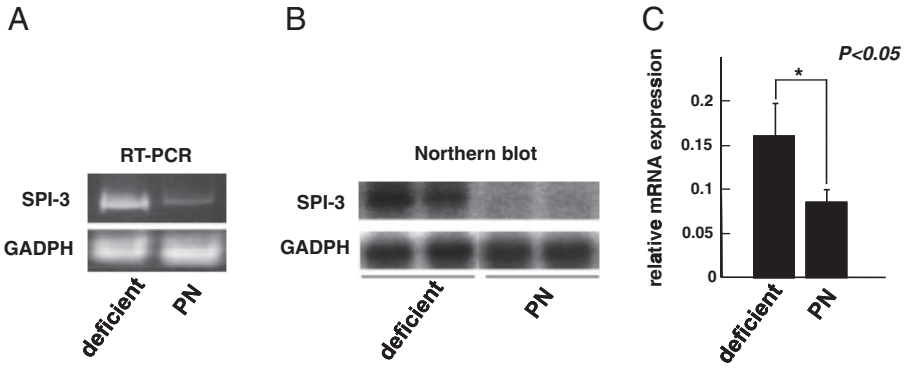

Figure 1. Response of SPI-3 mRNA expression in the colon to repletion in the pyridoxine-deficient rat. (A) After being fed a PN-deficient basal diet for 3 wk, the rats were divided into two groups ($n = 7$) and fed PN-deficient basal diet (–PN group, *deficient*) or basal diet containing 35 mg/kg PN (+PN group, *PN*) for 4 days. Ten micrograms of pooled total RNAs from colons of two groups was subjected to semi-quantitative RT-PCR. (B) Ten micrograms of total RNAs was subjected to Northern blot analysis. In the autoradiograms, each lane represents a sample from an individual rat. (C) The radioactivity in each band was quantified using an image analyzer and normalized to that of GAPDH mRNA. Each value is expressed relative to the mRNA level of the –PN group (*deficient*) and represents the mean ± SE ($n = 6$, $^*p < 0.05$ *vs deficient*).

dietary group and used DNA microarray analysis (whole rat genome microarray) to search for colon genes with altered expression from PN repletion. Microarray data analysis indicated 132 transcripts in the colon of the +PN group compared with the –PN group (p-value < 0.05, Table 2). To confirm the differential expression of these genes, the total RNA from individual rats in the –PN and +PN groups was subjected to Northern blot analysis (Fig. 1B). The SPI-3 mRNA level was significantly down-regulated in the +PN group compared with the –PN group (with a 42% decrease, $p < 0.05$, Fig. 1C).

The next aim of this study was to investigate the potential properties of vitamin B6 by elucidating the mechanism by which the SPI-3 mRNA expression was down-regulated. We used human colon cancer HT-29 cells and characterized SPI-3 mRNA expression in these cancer cells. In this study, we found that interleukin-8 and SPI-3 mRNA expression is up-regulated by stimulation with TNF-α (Fig. 2A and B), which indicates that SPI-3 mRNA expression can be considered an inflammatory response in HT-29 cells. Treatment of the HT-29 cells with 500 μM of PL for 1 h prior to TNF-α stimulation resulted in significant decreases in SPI-3 mRNA expression (with 53% inhibition; Fig. 2C and D). Pretreatment of HT-29 cells with PL inhibited TNF-α-induced SPI-3 mRNA expression in a dose-dependent manner, at concentrations of 100–500 μM (Fig. 2D). Previous studies by us and Oka *et al.* reported that PL inhibited the expression of cyclooxygenase-2 in RAW264.7 cells stimulated by lipopolysaccharide, and that the inhibitory effect of PL was the highest among those reported for PL, pyridoxamine, PN and PLP [23, 24]. The uptake mechanism for exogenously added PL may differ from other vitamin B6 derivative mechanisms. We focused this study on elucidating the molecular mechanisms underlying the suppressive effect of PL treatment on the inflammatory responses. Activation of NF-κB is thought to play a critical role in increased transcription activity of the SPI-3 gene in response to TNF-α stimulation, so we examined whether PL influences NF-κB activation by TNF-α using the HT-29 cells. As shown in Fig. 3A, the treatment of HT-29 cells with PL resulted in a significant inhibition of the NF-κB reporter activity, suggesting the possibility that PL inhibits TNF-α-induced NF-κB activation. It is well documented that the degradation of Iκ-B proteins results in the translocation of the NF-κB p65 subunit to the nucleus and inhibits transcription by binding consensus DNA sequences [25]. In this study, we showed that pretreatment with PL prolonged Iκ-Bα degradation in response to TNF-α (Fig. 4B). Previous reports show that phosphorylation of cellular Iκ-Bα by Iκ-B kinase (IKK), in response to TNF-α, results in the degradation of Iκ-Bs and subsequent activation of NF-κB; IKK, activated in the TNF signaling pathway, phosphorylates the NF-κB p65 subunit [25, 26]. We examined the inhibitory effect of PL treatment on IKK activation in HT-29 cells, which was determined by the state of NF-κB p65 subunit phosphorylation. Figure 3B shows that pretreatment with PL did not affect the phosphorylation of NF-κB p65 after TNF-α stimulation, compared with the control HT-29 cells. These observations indicate that the inhibitory effect of PL on Iκ-Bα degradation and subsequent NF-κB activation in response to TNF-α stimulation is not due to the suppression of IKK activation. In TNF-α-stimulated HT-29 cells, Iκ-Bα is reportedly phosphorylated by IKK, ubiquitinated and then rapidly degraded *via* the 26S proteasome [27, 28]. To examine the effect of vitamin B6 on Iκ-Bα ubiquitination, in response to TNF-α stimulation, we used HT-29 cells stably expressing FLAG-tagged ubiquitin (HT-29/FLAG-Ub). To detect Iκ-Bα protein ubiquitination, the HT-29/FLAG-Ub cells were precultured with proteasome inhibitor MG-132 and stimulated by TNF-α. The cell lysates were immunoprecipitated using anti-FLAG antibody to concentrate the ubiquitinated proteins, which were then detected by

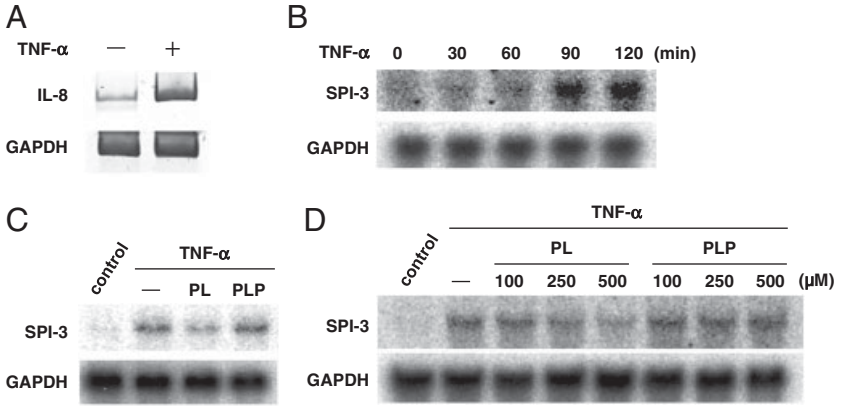

Figure 2. Effect of PL on SPI-3 mRNA expression in TNF-α-stimulated HT-29 cells. (A) HT-29 cells were stimulated with 50 ng/mL of TNF-α for 12 h. Ten micrograms of total RNAs was subjected to semi-quantitative RT-PCR. (B) HT-29 cells were stimulated with 50 ng/mL of TNF-α for the indicated time. Expression of SPI-3 and GAPDH mRNA was examined by Northern blot analysis. Each lane contained 10 μg of total RNA. (C) After treatment with 500 μM of PL or PLP for 1 h, HT-29 cells were stimulated with 50 ng/mL of TNF-α for 2 h. Expression of SPI-3 and GAPDH mRNA was examined by Northern blot analyses. Each lane contained 10 μg of total RNA. (D) After treatment with PL or PLP at the indicated concentration for 1 h, HT-29 cells were stimulated with 50 ng/mL of TNF-α for 2 h. Expression of SPI-3 and GAPDH mRNA was examined by Northern blot analyses. Each lane contained 10 μg of total RNA.

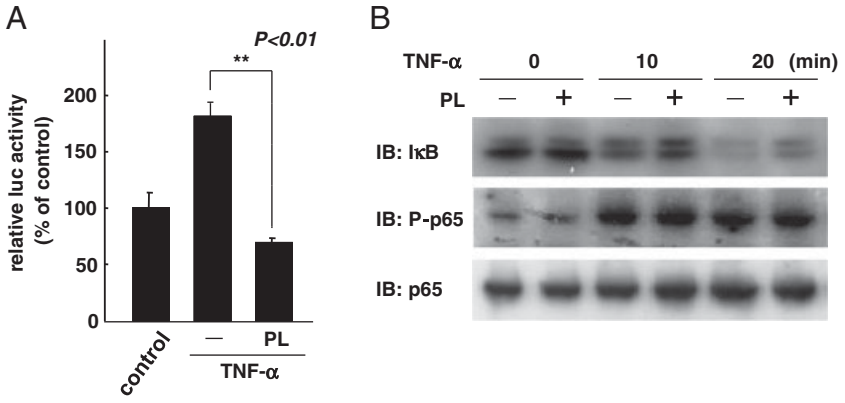

Figure 3. Effect of PL on TNF-α-induced NF-κB activation and Iκ-Bα degradation in HT-29 cells. (A) A plasmid carrying 5xNF-κB-luciferase gene and pCMV-β-gal was transfected into HT-29 cells. Twenty-four hours after the transfection, the HT-29 cells were treated with PL for 1 h and stimulated with 50 ng/mL of TNF-α. The luciferase activity of a vehicle control was set at 100% and relative activities were presented as fold induction to that of the vehicle control. Values are mean ± SE for triplicated cultures and $^{**}p<0.01$ *versus* control. (B) HT-29 cells were treated with or without PL (500 μM) for 1 h before stimulation with 50 ng/mL of TNF-α for the indicated time. Cell lysates prepared from HT-29 cells were subjected to Western blot analyses using anti-Iκ-Bα antibody (upper panel), anti-phospho-NF-κB p65 subunit antibody (middle panel) or anti-NF-κB p65 subunit antibody (lower panel).

immunoblot analysis using anti-Iκ-Bα antibody. Figure 4 shows that polyubiquitinated Iκ-Bα proteins were observed in the presence of TNF-α and MG-132, and we demonstrated that PL inhibits ubiquitination of Iκ-Bα after TNF-α treatment (Fig. 4B).

4 Discussion

In the present study, we analyzed colon gene expression in vitamin B6-deficient rats with vitamin B6 repletion and showed that vitamin B6 supplementation down-regulates SPI clade A mRNA expression in the colon. The SPI family includes genes with diverse functions in gene regulation. Three members of this family, SPI-1, SPI-2 and SPI-3, have been previously isolated and characterized at the molecular level [29–31]. Interestingly, SPI-1 and SPI-2 mRNAs are constitutively present in normal rat liver, but SPI-3 mRNA expression is not seen under normal conditions. SPI-3 mRNA is up-regulated during acute inflammation in the rat liver and following transient ischemia in the rat brain, which suggests that the physiological functions of SPI-3, during inflammation, is *via* inhibition of some proteolytic activity [32–34]. In this study, we focused on SPI-3 as an

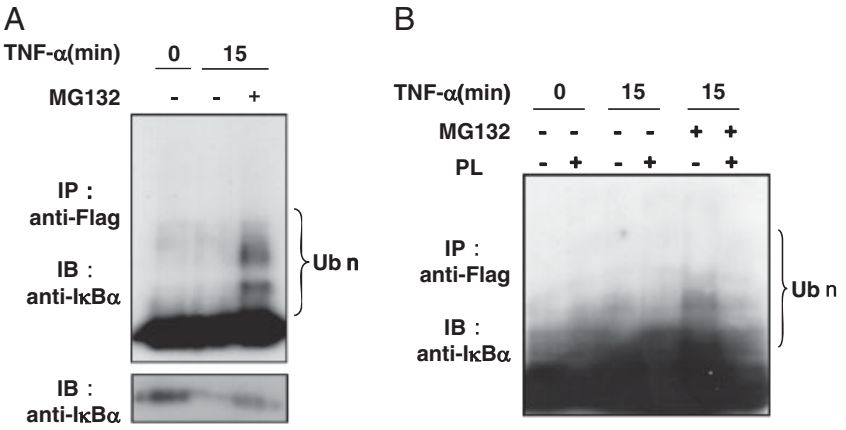

Figure 4. Effect of PL on polyubiquitination of Iκ-Bα protein in HT-29 cells. (A) HT-29 cells were stably transfected with FLAG-tagged ubiquitin (HT-29/FLAG-Ub). To detect polyubiquitination of Iκ-Bα, the HT-29/FLAG-Ub cells were pretreated in the presence or absence with proteasome inhibitor MG-132 (50 μM) for 1 h, and the HT-29/FLAG-Ub cells were stimulated by 50 ng/mL of TNF-α for 15 min. Cell lysates were immunoprecipitated using anti-FLAG antibody. Immunoprecipitated samples (IP) were immunoblotted with anti-Iκ-Bα antibody. Polyubiquitinated Iκ-Bα proteins (Ubn) were observed in the presence of both TNF-α and MG-132. (B) The HT-29/FLAG-Ub cells were pretreated in the presence or absence of 50 μM MG-132 with or without 500 μM PL for 1 h, and then the cells were stimulated with 50 ng/mL of TNF-α for 15 min. Cell lysates were immunoprecipitated using anti-FLAG antibody and subjected to immunoblotting using anti-Iκ-Bα antibody. The data are representative of two independent experiments.

inflammatory molecule in the colon and investigated the molecular mechanism underlying the suppressive effect of vitamin B6 on SPI-3 mRNA expression.

Previous reports suggest that signal transducers and activators of transcription 3 (STAT3) mediated the up-regulation of SPI-3 mRNA expression in response to inflammatory stimuli in the rat peneal grand and ocular tissues. We could not detect phosphorylated signal transducers and activators of transcription 3 in HT-29 cells after TNF-α treatment by Western blot analysis (data not shown). Activation of NF-κB is well documented to be involved in the TNF-α-induced inflammatory response in HT-29 cells. NF-κB is a transcription factor that induces the mRNA expression of inflammatory factors including cytokines, chemokines and adhesion molecules. NF-κB is normally restricted from entering into the nucleus by Iκ-Bs; however, in response to TNF-α stimulation, activated IKK phosphorylates Iκ-B (and NF-κB p65) in the cytoplasm and phosphorylated Iκ-B is then degraded by the proteasome system. This study showed that vitamin B6 prolonged Iκ-B degradation, but did not affect phosphorylation of NF-κB p65, suggesting that the effect of vitamin B6 is not mediated by the inhibition of IKK activity. As polyubiquitination of Iκ-B protein is a critical step for degradation in the proteasome system, we generated HT-29 cells stably expressing tagged-ubiquitin and tested whether vitamin B6 actually affects polyubiquitinylation of the Iκ-B protein. We concluded that vitamin B6 inhibits Iκ-B polyubiquitination in response to TNF-α stimulation and suppresses TNF-α-induced SPI-3 mRNA expression through the inhibition of Iκ-B degradation. Protein ubiquitination requires the sequential reactions of three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin–protein ligase (E3), which is then followed by proteasome-mediated protein degradation [35]. E3 proteins are well documented to be a large family of enzymes that recognize numerous target proteins and play an essential role in their degradation. In the NF-κB signaling pathway, several polyubiquitination processes are reportedly involved in NF-κB activation in response to TNF-α stimulation. Binding of TNF-α to its receptor subsequently leads to polyubiquitination of TRAF2 and RIP1 which are included in the receptor-associated complex. The Lys^{63}-linked polyubiquitination of RIP1 and TRAF6 enable the recruitment and activation of transforming growth factor-β-activated kinase 1 (TAK1) through the binding of the TAK1 regulatory subunits, TAB2 and TAB3, to Lys^{63}-polyubiquitinated RIP1 and TRAF6 [36, 37]. The activated TAK1 then triggers the activation of the IKK, ERK1/2 and p38 MAPK, which leads to the activation of transcription factors, NF-κB and AP-1, and the up-regulation of a variety of mRNAs encoding pro-inflammatory cytokines, chemokines and adhesion molecules. Thus, in the NF-κB signaling pathway, in response to TNF-α, polyubiquitination reactions are critical for NF-κB activation. The observation of phosphorylated forms of p65 NF-κB and ERK1/2 (data not shown) in the presence of vitamin B6 can exclude the possibility that vitamin B6 may interact with ubiquitin and universally inhibit the polyubiquitination process. As the first reaction, essential for Iκ-Bα degradation in the ubiquitin/proteasome system, is ubiquitination at Lys-21 and Lys-22 in the Iκ-Bα protein [38]; it is of particular interest to test whether vitamin B6 directly interacts with the Iκ-Bα protein and can inhibit the initial ubiquitinylation step.

To date, the ubiquitin/proteasome mechanism is well studied to be involved in human colorectal carcinogenesis [39]. In particular, Wnt/β-catenin/APC/TCF4 signaling is

shown to regulate the proliferation of colorectal epithelial cells in the crypts. In most colorectal cancers, *Adenomatous polyposis coli* gene mutations reportedly interfere with the ability to degrade β-catenin in the proteasome, causing uncontrollable epithelial cell proliferation [40]. Furthermore, proteins such as p53, Smad4 and components in the k-ras pathways are also reportedly regulated by the ubiquitin/proteasome mechanism during colorectal carcinogenesis [34]. In addition, Gross-Mesilaty *et al.* reported that tyrosine aminotransferase, a PLP-enzyme involved in amino acid metabolism, is conjugated and degraded in an ubiquitin-dependent manner *in vitro* and *in vivo* and also showed that this enzyme could be protected from degradation by association with its specific cofactor, PLP [41]. Taken together, this study provides a new insight into the anti-inflammatory effects of vitamin B6 and also prompts us to further consider the biological significances of vitamin B6 in the regulation of the ubiquitin/proteasome system not only for colorectal carcinogenesis but also, most likely, under other physiological conditions.

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