High Dose of Pyridoxine Induces IGFBP-3 mRNA Expression in MCF-7 Cells and Its Induction Is Inhibited by the p53-Specific Inhibitor Pifithrin-α

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Summary It has been reported that supplementation with high-dose vitamin B₆ (B₆) exerts antitumor effects in rodent models of cancer. However, the mechanism of these effects remains poorly understood. High-dose B₆ also suppresses cell proliferation and induces apoptosis of human breast adenocarcinoma MCF-7 cells. Based on preliminary experiments using DNA microarray analyses, we hypothesized that high-dose pyridoxine (PN) might induce IGF-binding protein-3 (IGFBP-3) expression in MCF-7 cells. In this study, we investigated IGFBP-3 induction by 3 or 10 μM PN using a quantitative real-time PCR method. We found that the induction reached a maximum of 24-fold with 10 μM PN for 72 h compared with non-treated cells. The induction of IGFBP-3 by PN was inhibited by a p53-specific inhibitor, pifithrin-α, in a dose-dependent manner, but was not affected by PD169316 (MAPK inhibitor), AS601245 (c-Jun N-terminal kinase inhibitor) or SL327 (MEK1/2 inhibitor). High-dose PN did not induce p53 mRNA expression. The IGFBP-3 induction by PN seemed to be related to p53 activation.

Key Words vitamin B₆, pyridoxine, MCF-7, IGFBP-3, p53

Vitamin B₆ (B₆) is an essential coenzyme for many enzymes involved in amino acid metabolism. The bioactive form of B₆ is pyridoxal-5′-phosphate (PLP) converted from other B₆ forms, such as pyridoxine (PN), pyridoxine-5′-phosphate (PNP), pyridoxal (PL), pyridoxamine (PM) and pyridoximamine-5′-phosphate (PMP), in vivo. Recently, a novel function of B₆ has been revealed, namely that it can regulate transcription (1). It is known that PLP can bind to DNA and RNA polymerases (2, 3) and inhibit their activities (4). PL or PLP also binds to some transcription factors such as glucocorticoid receptor (5), HNF-1 and C/EBP (6), and inhibits their transcriptional activities by binding to a lysine residue in the DNA-binding domain of these proteins through a Schiff base linkage (7, 8). These transcriptional activities are enhanced in B₆-deficient mice and rats (9). Huq et al. (10) reported that the transcriptional activity of the nuclear corepressor RIP140 is downregulated by PLP conjugation, resulting in the suppression of adipocyte differentiation. These findings may suggest that B₆ has physiological roles through translational regulation (1).

There appears to be a correlation between B₆ and cancer, since the serum B₆ concentration was reported to be lower in cancer patients than in normal subjects (11, 12). A lower risk for lung cancer in patients with elevated serum levels of B₆ (5.7–28.4 nm vs. 57.8–629 nm of B₆; odds ratio, 0.44; 95% confidence interval, 0.33–0.60; p value for the trend, <0.000001) was found in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study (13). Analyses of PN supplementation in cancer animal models (azoxymethane-induced colon tumorigenesis and 7,12-dimethylbenzanthracene-induced mammary tumorigenesis) revealed that high-dose PN prevented tumor progression (14, 15). In human hepatoma HepG2 cells (16) or feline mammary tumor FRM cells (17), high-dose B₆ was found to inhibit cell growth and induce apoptosis. However, the mechanism of the antitumor effects of B₆ has not yet been elucidated.

In preliminary experiments using DNA microarray analyses, we found that some genes were upregulated by PN in human breast adenocarcinoma MCF-7 cells. IGF-binding protein-3 (IGFBP-3) was one of these genes. IGFBP-3 is a multifunctional protein that has not only IGF-dependent effects but also IGF-independent effects (18, 19). Regarding the IGF-independent IGFBP-3 effects, IGFBP-3 inhibits cell proliferation and induces apoptosis (19). In this study, we clarified whether IGFBP-3 was induced by PN in MCF-7 cells.

MATERIALS AND METHODS

Cell culture. MCF-7 cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in MEM supplemented with 50 μg/mL streptomycin, 50 U/mL penicillin and 10% fetal bovine serum. The cells were replated before they reached confluency, and the medium was changed every 3 d. Viable and dead cells were quantified using a
hemocytometer following the addition of 10% Trypan Blue (Gibco Invitrogen, Tokyo, Japan). Pyridoxine hydrochloride (Nacalai Tesque, Inc., Kyoto, Japan) was dissolved directly in medium adjusted to pH 7.4, and filtered through 0.2 μm membrane (Minisart, Sartorius Stedim Biotech GmbH, Goettingen, Germany). Cells were seeded on Tissue Culture Treated 96-well Plates (Iwaki, Tokyo, Japan) at 1×10^5 cells/well for 1 d, and the culture medium was changed to medium containing 0, 1, 3, 5, or 10 mM PN. After 24, 48, and 72 h, WST-1 (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was added into culture medium and cultured for just 2 h, and then absorbance at 560 nm was measured by a microplate reader (Sunrise Thermo, Tecan, Luzern, Switzerland). Cells were seeded on Tissue Culture Treated 12-well Plates (Iwaki) at 1×10^5 cells/well for 1 d, and the culture medium was changed to medium containing 1 or 10 mM PN and each inhibitor. After 24 h, total RNA was extracted from the cells. PD169316 (p38 MAPK inhibitor), AS601245 (c-Jun N-terminal kinase (JNK) inhibitor), SL327 (MEK1/2 inhibitor) and pifithrin-α (p53 inhibitor) were all purchased from Merck-Calbiochem (Tokyo, Japan). Cells were seeded on 12-well plates for 1 d, and the culture medium was changed to medium containing 10 mM PN and each inhibitor. After 24 h, total RNA was extracted from the cells.

Real-time PCR. The expression levels of IGFBP-3, p53, GAPD and β-actin mRNAs were analyzed by real-time PCR. Briefly, total RNA was extracted from MCF-7 cells using TRIzol (Invitrogen, Tokyo, Japan). The total RNA was reverse-transcribed using a PrimeScript® RT Reagent Kit (TaKaRa, Tokyo, Japan) according to the manufacturer’s instructions. Quantitative real-time PCR analyses were performed using a Lightcycler 1.5 (Roche Diagnostics, Tokyo, Japan) and SYBR® Premix Ex Taq™ (TaKaRa). The primer sets for IGFBP-3, p53, GAPD and β-actin were purchased from TaKaRa (IGFBP-3: 5′-GCAGCCCTGTGCTATCTAGA-3′ and 5′-GATCCACGCCCTTGTTTCAGA-3′; p53: 5′-ACTAAGCCGACGAGACTGCCAACC-3′ and 5′-CCTATTGCCTGCTCAGGAACAATC-3′; GAPD: 5′-GCACCGTCAAGCTGAGAAC-3′ and 5′-ATGGTGGTGAAGACGCCAGT-3′; β-actin: 5′-TGGACCCACACAATGGAA-3′ and 5′-CTAAGTCATAGTCCGCTAGAACGCA-3′). The cycling parameters were an initial step at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 60°C for 10 s and 72°C for 10 s. Melting curves were analyzed for all PCR experiments to evaluate the specific amplifications.

Immunoblotting. MCF-7 cells were seeded at 2×10^6 cells/10 cm dish and treated with 3 mM PN for 12, 24, and 72 h. At each time period, the cells were washed with PBS and lysed in 0.5 mL Lysis buffer (50 mM Tris-HCL, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue). The lysed cells were sonicated using a BioRaptor (Cosmo Bio, Tokyo, Japan) according to the manufacturer’s instruction. They were subjected to SDS-polyacrylamide gel electrophoresis (12.5%) and transferred to PVDF membranes (Millipore, Tokyo, Japan). The membranes were blocked for 30 min in Block Ace (DS Pharma Biomedical, Osaka, Japan), and washed once with 0.5% Tween-X100 containing Tris-buffered saline (T-TBS, pH 7.4). The membranes were incubated with 1:1,000 diluted monoclonal anti-IGFBP-3 monoclonal antibody (MAB305, R&D Systems, Inc., Minneapolis, MN) or 1:10,000 diluted monoclonal anti-β-actin (Sigma, St. Louis, MO) at 37°C for 1 h. The membranes were then washed with T-TBS, and incubated with 1:3,000 diluted horseradish peroxide-conjugated anti-mouse IgG (GE Healthcare, Tokyo, Japan) at room temperature for 1 h. Bound antibody was detected using the ECLplus (GE Healthcare) with a Fuji LAS-1000 Luminescent Image Analyzer (Fuji Film, Tokyo, Japan).

Statistical analysis. Data were analyzed by Student’s t-test or one-way ANOVA with Tukey’s post hoc analysis (Statistical Package for the Social Sciences, v. 17; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Pyridoxine induces IGFBP-3 mRNA expression

We used a very high dose of PN, since a high concentration of PN is needed to inhibit MCF-7 and FRM cell proliferation (16, 17). The proliferation of MCF-7 cells was also inhibited by almost the same concentration of PN in this study (Fig. 1). At 10 mM, PN completely suppressed cell growth. At 3 mM, PN suppressed the cell growth by one-half compared with control cells. To elucidate how PN suppressed the cell growth, we compared the gene expression levels in the presence of 0, 1 and 10 mM PN for 1 d using DNA microarray analyses (data not shown). We found that some genes, including IGFBP-3, were upregulated by more than 2-fold in the
presence of both 1 and 10 mM PN compared with the control. To confirm the IGFBP-3 induction by PN, we assayed its mRNA expression using a quantitative RT-PCR method (Fig. 2 left). Total RNA samples were extracted from cells treated with 0, 3 or 10 mM PN for 0, 12, 24, 48 or 72 h. The data were expressed as IGFBP-3/GAPD ratios vs. 0 mM PN in each period. At 10 mM, PN-induced IGFBP-3 expression increased by nearly 5-fold compared with the control at 24 h, followed by gradual induction in a time-dependent manner until 72 h. At 3 mM, PN also induced IGFBP-3 expression, but the induction rates were low compared with 10 mM PN. These findings were confirmed in three independent experiments and there were significant differences (p<0.05) in each period, except for 0 mM vs. 3 mM at 12 h. The induction of IGFBP-3 protein was also detected when cells were treated with 3 mM PN (Fig. 2 right). The mature form of IGFBP-3 is 45,000 Da. We assessed intracellular IGFBP-3 protein expression, because there was a correlation between the intracellular and the conditioned medium of the expression level of IGFBP-3 protein (20).

Pifithrin-α inhibits IGFBP-3 induction by PN

To identify the molecules related to IGFBP-3 induction by PN, we investigated the effects of several kinase inhibitors on the expression of IGFBP-3. PD169316 is a cell-permeable and potent selective inhibitor of p38 MAPK (IC50=89 nM) (21). AS601245 is a cell-permeable selective inhibitor of c-Jun N-terminal kinases JNK1, 2 and 3 (IC50=150, 220 and 70 nM, respectively), and has no effect on other kinases at 10 µM (22). SL327 is a cell-permeable and selective inhibitor of MEK1 and 2 (IC50=180 and 220 nM, respectively) (23). At higher concentrations (IC50>10 µM), SL327...
also inhibits ERK1, p38MKK, MKK4, JNK and PKC activities. The data were expressed as percentiles of the IGFBP-3/GAPD ratio compared with the ratio without each inhibitor (Fig. 3). Among the PN non-treated cells, SL327 induced an increase of IGFBP-3 by about 2-fold, while the other inhibitors had no effect. The induction of IGFBP-3 by PN was not markedly suppressed by the addition of PD169316, AS601245 or SL327. In this experiment, the working concentrations of PD169316, AS601245 and SL327 were 10, 20 and 33 μM, respectively, representing sufficiently high concentrations of the inhibitors. Leibowitz and Cohick (24) reported that SB203580, a p38 MAPK inhibitor, suppressed the expression of IGFBP-3 induced by TNF-α stimulation. However, our results indicated that the IGFBP-3 induction by PN was not related to p38 MAPK activity. On the other hand, Buckbinder et al. (25) identified IGFBP-3 as one of the p53-inducible genes after ultraviolet radiation or addition of doxorubicin. Therefore, we evaluated the effect of pifithrin-α (26), a specific p53 inhibitor, on the IGFBP-3 induction by PN. We found that pifithrin-α remarkably inhibited the IGFBP-3 induction in a dose-dependent manner (Fig. 4). To clarify the relationship between activation of p53 and cell growth, we have investigated whether the suppression of cell growth by PN is recovered by pifithrin-α. Unfortunately, the cell growth suppression was caused by the addition of only pifithrin-α. The p53 mRNA expression level was not changed by PN, as evaluated by RT-PCR (data not shown), indicating that high-dose PN treatment may stabilize p53 protein for its activation. The protein level of p53 needs to be examined in a further study. Although it has not been revealed how PN activates p53 activity, there are some possibilities. It is known that topoisomerase inhibitors activate p53 (27). PLP also inhibits topoisomerase activity (28, 29). Therefore, p53 activation by PN may be related to suppression of topoisomerase activity. Another hypothesis regarding p53 activation by PN is a relationship with NF-κB. It has been reported that NF-κB induces the expression of the p53 E3 ubiquitin ligase Mdm2, thereby negatively regulating p53 stability (30). Yanaka et al. (31) reported that PL inhibits NF-κB activation through PL-mediated suppression of IKK activation. Therefore, it is possible that p53 activation by PN is mediated by downregulation of NF-κB. However, we note that the LPS-activated NF-κB pathway is usually activated during immune responses. Moreover, it is possible that there are other mechanisms of p53 activation by PN.

We have shown that high-dose PN can induce IGFBP-3 expression and this induction was inhibited by pifithrin-α, a specific p53 inhibitor. Usually B6 is required as a coenzyme. Recently, it has been found that PN also downregulates gene expression by directly binding to RNA polymerase or transcription factors. We report the upregulation of gene expression by PN for the first time.

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