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Comparisons of uptake and cell surface binding among pyridoxal, pyridoxine, and pyridoxamine in RAW264.7 cells

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ABSTRACT

Objective: Vitamin B6 (B6) suppresses the expression of cyclooxygenase-2 stimulated by lipopolysaccharide in mouse macrophage RAW264.7 cells. The greatest effect is recognized for pyridoxal (PL) compared with pyridoxamine (PM), pyridoxine (PN), and pyridoxal 5′-phosphate (PLP). However, it has not been elucidated why PL has the strongest effect. We compared the uptakes and cell surface interactions among PL, PM, PN, and PLP in RAW264.7 cells.
Methods: Cyclo-oxygenase-2 mRNA expression was evaluated by real-time polymerase chain reaction. Intracellular B6 concentrations were measured by high-performance liquid chromatography. Interactions of B6s with the cell surface were analyzed using a surface plasmon resonance biosensor. B6 uptake speeds were measured using [^{3}H]-PN.
Results: The intracellular PLP levels did not change significantly when cells were cultured in medium containing PL, PM, PN, or PLP. Only PL interacted with the cell surface. Although PM and PN were associated with the cell surface, their binding was only recognized during sample loading. After the change to phosphate buffered saline after sample loading, the binding resonances of PM and PN returned to baseline, whereas that of PL did not. Uptake of [^{3}H]-PN was inhibited by non-labeled PN, PL, or PLP, but not PM, at 1 μM. The inhibition rate of PL was higher than those of PN and PLP.
Conclusion: The inhibition of cyclo-oxygenase-2 mRNA expression by PL may be related to the cell surface interaction of PL, rather than the intracellular PLP level. The uptake mechanism for PN and PL may differ from that for PM.

Introduction

Vitamin B6 (B6) is required for normal growth and development in mammals. Pyridoxal 5′-phosphate (PLP) is the cofactor form of B6. PLP generally acts as a cofactor of enzymes in various reactions involved in amino acid metabolism. Previous studies have shown new functions of B6s, such as anti-proliferation activity [1–3] and involvement in adipocyte differentiation [4]. These new functions are thought to result from direct binding of PLP to certain proteins, such as DNA polymerase [5], RNA polymerase [6], steroid hormone receptors [7], hepatocyte nuclear factor 1 [8], nuclear receptor-interacting protein 140 [4], and topoisomerase-IB [9] and/or from B6-mediated suppression of oxidative stress [1,10]. These findings suggest that the intracellular and extracellular levels of B6 may influence cell physiology.

Yanaka et al. [11] reported that pyridoxal (PL) suppresses the expression of cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthetase in mouse macrophage RAW264.7 cells stimulated with lipopolysaccharide (LPS). Nuclear factor-κB (NF-κB) is an important transcriptional regulator of many macrophage proinflammatory cytokine genes, including those for COX-2 and inducible nitric oxide synthetase. The inhibition of NF-κB activation by PL results from suppression of IκB phosphorylation [11]. However, the details of how B6s affect IκB phosphorylation have not yet been elucidated. PL has a potent anti–NF-κB activation effect compared with other B6s. However, the reason why PL has the strongest effect among B6s has not been clarified. Different effects depending on the forms of B6 were shown for the anti-proliferation activity. Specifically, PL and PLP suppressed

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the proliferation of human umbilical vein endothelial cells, whereas pyridoxine (PN) and pyridoxamine (PM) did not [2]. In feline mammary tumor cell lines, PN and PL had higher effects on proliferation than PM [3]. To understand why PL has a potent effect compared with other forms of B6, we investigated the uptakes and cell-surface interactions of B6s in RAW264.7 cells.

Materials and methods

Materials

General reagents were purchased from Wako Pure Chemicals (Tokyo, Japan). PN, PM, PL, and PLP were dissolved at 0.5 M in water and diluted to the required concentrations in Dulbecco's Modified Eagle's Medium (DMEM; Wako Pure Chemicals). CELLSTAR tissue culture dishes were obtained from Greiner Bio-One (Tokyo, Japan). [^{3}H]-PN was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA).

Cell culture

Mouse macrophage RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum. When the cells reached 80–90% confluence, they were detached from the dishes with trypsin/ethylenediaminetetra-acetic acid solution and subsequently cultured in fresh medium. Viable and dead cells were counted after the addition of 10% Trypan blue. To confirm the toxicity of each B6, cells (1×10^5) were inoculated into six-well plates, and the numbers of viable and dead cells cultured in medium containing 0.5 mM PL, PM, PN, or PLP were counted each day until 7 d. To activate NF-κB signaling, the cells were stimulated with LPS (1 μg/mL) for 4 h.

Quantitative real-time polymerase chain reaction

RAW264.7 cells (2×10^6) were inoculated into six-well plates. After the required culture period, total RNA was prepared from the cells using TRIzol reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's protocol. Complementary DNA was synthesized with a PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). Quantitative real-time polymerase chain reaction analyses were performed using a Lightcycler 1.5 (Roche Diagnostics, Tokyo, Japan) and SYBR Premix Ex Taq (Takara). Designed primer sets for COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPD) were purchased from Takara (COX-2, 5′-GCCAGGCTGAACTTCGAAACA-3′ and 5′-GCTCACGAGGCCACTGATACCTA-3′; GAPD, 5′-AAATGGTGAAGGTCGGTGTG-3′ and 5′-TGAAGGGGTCGTTGATGG-3′). The cycling parameters were an initial step at 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 10 s. The COX-2 mRNA expression levels were corrected by the GAPD mRNA expression levels. Data were representative of three separate mRNA isolations.

Quantification of B6s by high-performance liquid chromatography

RAW264.7 cells (6×10^6) were inoculated into 10-cm dishes. After 24 h, the cells were cultured in medium containing 0.1 mM PL, PM, PN, or PLP for 1, 3, and 7 d. Each medium was changed every 2 d. Determinations of the PM, pyridoxamine 5′-phosphate (PMP), PL, and PLP concentrations were carried out as previously described [12,13]. These previous reports showed that the recovery of added B6 from biological samples was around 90%. Briefly, the cells were rinsed three times with 10 mL of phosphate buffered saline (PBS; pH 7.4) and then scraped with a rubber scraper. Cell pellets obtained by centrifugation at $300 \times g$ for 3 min were immediately homogenized for 5 s in 0.5 mL of cold PBS using a PT-2100 Polytron (Kinematica AG, Lucerne, Switzerland) at maximum speed. The protein concentrations of the cell homogenates were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). After addition of perchloric acid (final concentration 0.25 M), the cell homogenates were centrifuged at $16\,000 \times g$, and the resulting supernatants were analyzed for their B6 concentrations. For determination of the B6 concentrations, supernatants were prepared from three dishes. The B6 concentrations were determined by high-performance liquid chromatography. An RF-535 fluorescence monitor (Shimadzu, Tokyo, Japan) was used to detect the B6 fluorescence (excitation 325 nm, emission 420 nm). Data were analyzed using peak areas of standards of known concentrations. The data were expressed as mean ± standard deviation (picomoles per milligram of protein) of three independent experiments.

Binding analysis using a surface plasmon resonance biosensor

Analyses of the interactions between B6s and RAW264.7 cells were performed using a surface plasmon resonance (SPR) biosensor (SPR670; Nippon Laser and Electronics Laboratories, Nagoya, Japan). The SPR biosensor assays were carried out as previously described [14]. Briefly, RAW264.7 cells (1.5×10^4) were immobilized on the sensor chip and the chip was then equilibrated in SPR running buffer (PBS; 30 μL/min). PL, PM, and PN were diluted to each concentration in SPR running buffer in 60-μL injection volumes. The flow rate was 30 μL/min at 25 °C. Binding was measured for 2 min, after which the loading buffer containing each B6 was changed to SPR running buffer for 2 min. The volumes of the angles shown in the figure corresponded to the binding strengths.

Uptake speed of [^{3}H]-PN

RAW264.7 cells (5×10^5) were inoculated into 24-well plates. After 1 d, the cells were rinsed three times with warmed PBS. [^{3}H]-PN dissolved in PN-free DMEM (Cell Science & Technology Institute, Miyagi, Japan) was added to each well with or without each concentration of unlabeled PL, PM, PN, or PLP for 30 min. After three rinses with cold PBS, the cells were lysed in 0.5 mL of 0.5 M NaOH. The cell lysates were neutralized by the same volume of 0.5 M HCl. After addition of Cytoscint (MP Biomedicals, Irvine, CA, USA), the radioactivities (disintegrations per minute) were counted in a Tri-Carb 2100TR liquid scintillation counter (Packard, Meriden, CT, USA). The protein concentrations were measured under the same conditions, and the data were corrected to disintegrations per minute per microgram of protein.

Results

RAW264.7 cells were cultured in DMEM containing PL, PM, PN, or PLP for 7 d. None of the B6s altered the pH of the medium or cell proliferation and viability (data not shown). The cells were cultured in each B6-containing medium for 3 d and then stimulated with LPS for 4 h. The rates of induction of COX-2 mRNA expression by LPS were compared among the B6s. All the B6s significantly suppressed COX-2 mRNA induction at 0.5 mM and showed tendencies toward dose-dependent effects (Fig. 1). PL showed a marked suppressive effect compared with the other B6s. PN, PM, and PLP showed approximately the same inhibition rates for suppression of COX-2 mRNA expression.

We doubted that the differences in the suppressive effects depending on the forms of B6 were caused by the accumulation of intracellular PLP, because PLP is an active form. To clarify this, we measured the intracellular concentrations of PLP after incubation with the individual B6s. Cells were cultured in medium containing 0.1 mM PN, PM, PL, or PLP for 1, 3, and 7 d. Each B6 was separated and its concentration was measured by high-performance liquid chromatography (Fig. 2). The initial intracellular concentrations of PM, PMP, PL, and PLP in RAW264.7 cells were 0.5 ± 0.2, 37 ± 5, 12.6 ± 1.6, and 107.7 ± 46 pmol/mg of

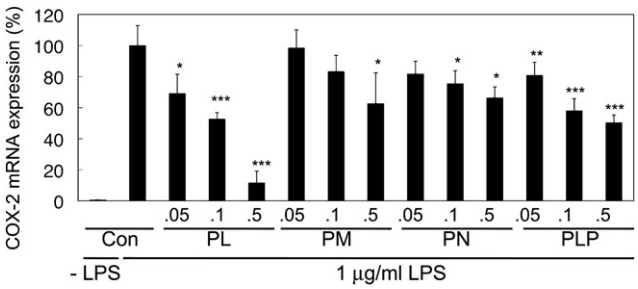

Fig. 1. Inhibition of LPS-stimulated COX-2 expression by individual vitamin B6s. RAW264.7 cells (2×10^6) were inoculated into six-well plates. After 24 h, the culture medium was changed to medium containing the indicated concentrations (millimolar) of PL, PM, PN, or PLP. After 3 d, the cells were stimulated with LPS (1 μg/mL) in medium containing each B6 for 4 h. Total RNA was extracted from these cells and analyzed for COX-2 mRNA expression. The COX-2/glyceraldehyde-3-phosphate dehydrogenase expression ratios were determined by real-time polymerase chain reaction. The data shown are means ± SDs of at least three independent experiments. *P* values were calculated by Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control cells with added LPS. Con, control; COX, cyclooxygenase-2; LPS, lipopolysaccharide; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PN, pyridoxine.

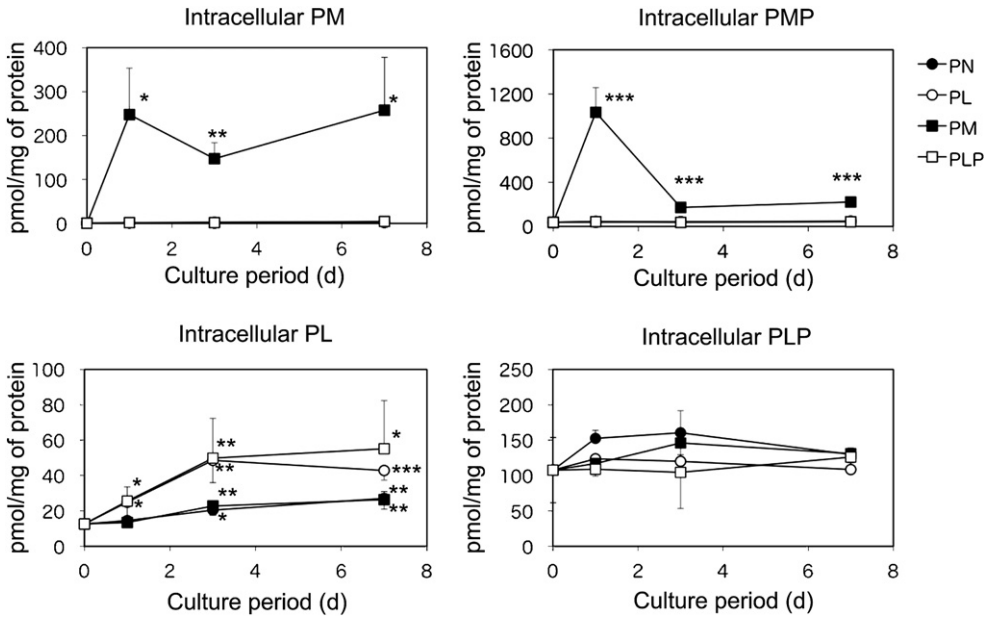

Fig. 2. Intracellular concentrations of vitamin B6s. RAW264.7 cells (2×10^6) were inoculated into 10-cm dishes. After 24 h, the medium was changed to medium containing 0.1 mM PL, PM, PN, or PLP. Cells were collected at 1, 3, and 7 d. The concentrations of intracellular PM, PMP, PL, and PLP were determined by high-performance liquid chromatography. The data shown are means ± SDs of at least three independent experiments. *P* values were calculated by Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the initial concentration of each B6. PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5′-phosphate; PN, pyridoxine.

protein, respectively. Previously, the individual B6 concentrations were measured in certain tissues of Wister rats and reported as picomoles per milligram of wet tissue weight [15]. Specifically, the PM, PMP, PL, and PLP concentrations were 0.1–14.0, 13.0–23.0, 1.0–3.0, and 6.8–21.0 pmol/mg of wet tissue weight, respectively, among the liver, kidney, and cerebral cortex [15]. In the present study, the individual B6 concentrations in each homogenate were corrected by the milligrams of protein in the homogenate. Therefore, the present B6 concentrations cannot simply be compared with the previous in vivo data. Addition of PM led to drastic increases in the intracellular PM and PMP levels at 1 d. The increase in intracellular PM was sustained for 7 d, whereas the increase in intracellular PMP was transient. The concentration of PM was about 200 pmol/mg of protein at 3 and 7 d. Addition of PN, PL, and PLP did not affect the intracellular PM level. The intracellular PL level was increased by addition of PL or PLP. The intracellular PL level reached its maximum at 3 d. Treatment with PL or PLP resulted in approximately four-fold increases in the intracellular PL level at 3 and 7 d. Approximately 1.7-fold increases in PL appeared in PN- and PM-treated cells at 3 and 7 d. There were no significant differences in the intracellular PLP levels in any of the B6-treated cells. Approximately 1.5- to 1.6-fold increases in intracellular PLP appeared in PN- and PM-treated cells. In these experiments, we could not measure the intracellular PN and pyridoxine 5′-phosphate levels.

We investigated the cell-surface binding of each B6 using the SPR biosensor. As shown in Fig. 3 (left), PN, PM, and PL showed

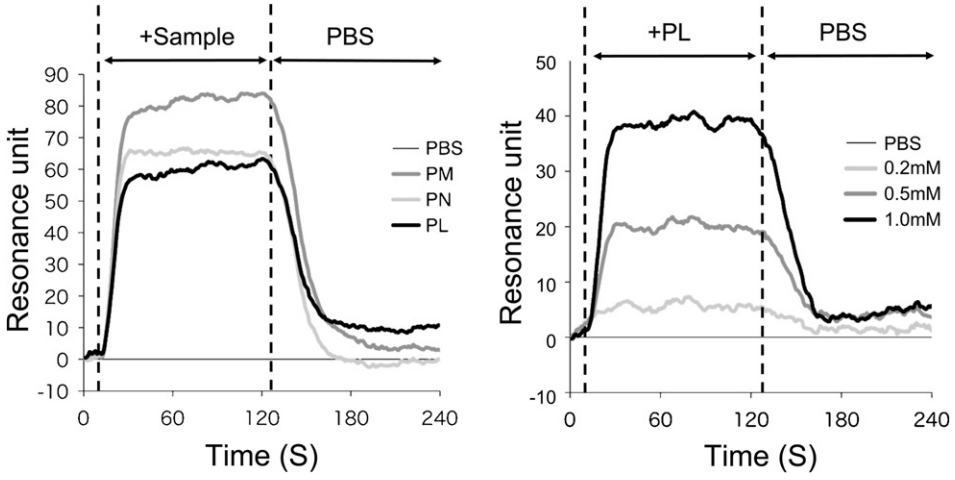

Fig. 3. Cell-surface binding of various B6s to RAW264.7 cells. The resonance units in the figure correspond with the binding strengths. (Left) PL, pyridoxamine, or pyridoxine was injected at a concentration of 1 mM for the indicated interval (+Sample), and the overlapping sensor gram of each sample is shown. (Right) Different concentrations of PL were injected. All sensor graphic data shown are the averages of three independent experiments. PBS, phosphate buffered saline; PL, pyridoxal.

rapid binding curves. The highest binding was recognized for PM during sample loading. The resonance units quickly declined after changing to PBS from sample loading for PN, PM, and PL. For PN and PM, the resonance units returned to the basal level. In contrast, the resonance units of PL were sustained from 170 to 240 s. Furthermore, the binding curve of PL showed dose-dependency (Fig. 3, right). During PBS flow, the resonance units of 0.5 and 1.0 mM PL were almost the same.

Next, we determined whether uptake of [^{3}H]-PN was inhibited by other unlabeled B6s. To this end, the time courses of [^{3}H]-PN uptake were evaluated. The uptake speed for 50 μM [^{3}H]-PN was 68.5 pmol/min and did not change until 60 min (data not shown). Although the dose-dependency of the uptake speed was also determined, a saturable concentration was not recognized up to 100 μM [^{3}H]-PN (data not shown). The 50% inhibitory concentrations were nearly equal between labeled and unlabeled PN (data not shown). We then investigated whether each unlabeled B6 affected the uptake of [^{3}H]-PN. The results were calculated as the relative [^{3}H]-PN uptake speed with each unlabeled B6 compared with the uptake speed without the unlabeled B6. PL, PN, and PLP inhibited the uptake of [^{3}H]-PN, and PL showed the strongest inhibitory effect. In contrast, PM did not inhibit the uptake of [^{3}H]-PN (Fig. 4).

Discussion

A previous study reported that PL inhibited the NF-κB activation stimulated by LPS [11]. In the present study, the inhibitory effect of PL was the highest among those of PL, PM, PN, and PLP. Significant inhibition of COX-2 expression was recognized at 50 μM PL. The normal concentration of PL in human serum is about 3.5 nM [16]. However, its concentration in human serum reached almost 1 μM after oral B6 treatment (40 mg of B6 per day for 3 d), but did not exceed 1 μM (40 mg of B6 per day for 80 d) [16]. Therefore, 50 μM PL seems to be a supraphysiologic concentration. In the present study, the treatment period of B6s was prolonged to 3 d, whereas a previous study used 1 d of PL treatment [1]. As a result, the inhibition rate of COX-2 expression by PL was enhanced in the present study. After 7 d of PL treatment, COX-2 expression was significantly suppressed by 20 μM PL (data not shown). Therefore, the effects of the treatment period and concentration of PL on the inhibition of COX-2 expression need to be elucidated in more detail in future studies. However, there are no previous explanations for why PL has the greatest effect among the B6s. We doubted that PLP, as the active form, was prone to accumulation in cells cultured in medium containing PL. In fact, the intracellular PLP level was maintained at nearly the basal level. We were unable to conclude how the intracellular PLP level was maintained because the production of 4-pyridoxic acid and enzyme activities related to B6 metabolism were not determined. In any case, the intracellular PLP level did not appear to be related to the suppressive effect of PL on NF-κB activation because increases in intracellular PL were recognized after the addition of PL and PLP.

The interactions between B6s and the RAW264.7 cell surface were investigated using an SPR biosensor. The results revealed differences in the cell-surface interactions among PL, PM, and PN. Only PL appeared to maintain an interaction with the cell surface at a concentration of 1 mM. This interaction may be related to the anti–NF-κB activation effect of PL. In addition, the anti–cell proliferation effects are dependent on the forms of B6, and PL was the common effective form in all previous studies [2,3]. We propose that there are certain molecules related to PL on the RAW264.7 cell surface. However, there are no previous reports of interactions between B6s and cell-surface molecules in mammals. Further investigations are required to identify and clarify the molecules related to the anti–NF-κB activation and anti-proliferation effects of PL.

Carrier-mediated uptake of PN has been reported in a human colonic carcinoma cell line (Caco-2) [17], an opossum renal tubule epithelial cell line (OK) [18], young adult mouse colonic epithelial cells [19], and human colonic apical membrane vesicles [19]. However, the carrier proteins involved have not yet been identified. Although *TPN1* and *bsu1*$^+$ have been identified as B6 transporters in the plasma membrane of *Schizosaccharomyces* [20,21], there are no homologous genes in mammals. In the present study, the uptake of [^{3}H]-PN was inhibited by PL, PN, and PLP, but not PM. The inhibitory action of PL was the highest among those of PL, PN, and PLP. These results suggest that the B6 carriers may have high affinities for PL. Furthermore, the uptake mechanism for PN and PL may differ from that for PM.

Conclusions

The COX-2 expression was most strongly suppressed by PL compared with PM, PN, and PLP in RAW264.7 cells. Although the intracellular PLP level did not change significantly after the addition of B6s, we found that PL interacted with the cell surface. The interaction of PL with the cell surface may be related to the anti–NF-κB activation effect of PL.

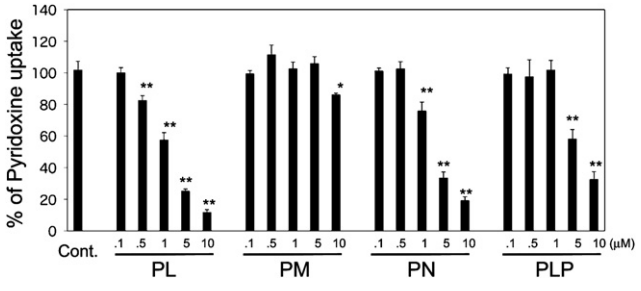

Fig. 4. Effects of unlabeled PN, PM, PL, and PLP on [^{3}H]-PN uptake by RAW264.7 cells. Confluent monolayers of RAW264.7 cells were cultured in PN-free medium containing [^{3}H]-PN in the absence or presence of various concentrations of PN, PM, PL, or PLP. [^{3}H]-PN uptake was analyzed after 30 min of incubation. The data shown are means ± SDs ($n = 4$). *P* values were calculated by Student's *t* test. * $P < 0.01$, ** $P < 0.001$ versus control. Cont., control; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PN, pyridoxine.

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