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Proliferation of peripheral blood mononuclear cells causes increased expression of the sodium-dependent multivitamin transporter gene and increased uptake of pantothenic acid ☆

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Abstract

Antigenic or mitogenic stimulation of peripheral blood mononuclear cells (PBMC) causes rapid cell proliferation. PBMC proliferation is associated with increased activities of pantothenic acid-dependent metabolic pathways, suggesting increased demand for pantothenic acid. We sought to determine whether PBMC respond to proliferation by increased cellular uptake of pantothenic acid and, if so, by what mechanism(s) the increased uptake is mediated. Uptake of pantothenic acid into PBMC was mediated by the sodium-dependent multivitamin transporter, SMVT, as judged by sodium dependency of uptake, substrate affinity and specificity, and RT-PCR of PBMC RNA. Proliferating PBMC accumulated two times more [^{3}H]pantothenic acid than quiescent PBMC. Rates of [^{3}H]pantothenic acid uptake paralleled rates of PBMC proliferation, as judged by uptake of [^{3}H]thymidine. The increased uptake of [^{3}H]pantothenic acid into proliferating PBMC was mediated by increased expression of SMVT (as judged by RT-PCR using total RNA from PBMC), leading to an increased number of transporters on the cell surface (as judged by maximal transport rates for pantothenic acid). We conclude that proliferating PBMC increase expression of the gene encoding SMVT to increase uptake of pantothenic acid.

Keywords: Human; Pantothenic acid; Peripheral blood mononuclear cells; Proliferation; Sodium-dependent multivitamin transporter; Transport

1. Introduction

Peripheral blood mononuclear cells (PBMC) represent a heterogeneous population of immune cells (B cells, T cells, and various granulocytes) that arise from pluripotent hematopoietic stem cells in the bone marrow [1]. PBMC account for cellular and humoral immune responses; some PBMC (B and T cells) proliferate rapidly after either antigenic or mitogenic stimulation.

Proliferation of PBMC is associated with increased flux through numerous metabolic pathways, e.g., synthesis of DNA, RNA, membranes, and proteins [2]. Increased metabolic activity of proliferating PBMC is paralleled by increased uptake of certain amino acids [3] and by increased activity of glucose catabolism [4–6]. These are thought to be responses to demand for amino acids for protein synthesis and for glucose to provide metabolic energy. Likewise, PBMC respond to proliferation by increased uptake of some vitamins (cobalamin and biotin) to provide coenzymes for metabolic pathways [7,8]. In contrast, increased net influx per cell of some other factors (NAD and riboflavin) parallels an increase in cellular volume of proliferating PBMC and thus does not lead to an increased intracellular vitamin concentration [9,10].

The importance of pantothenic acid for cell proliferation has been well documented. The vitamin is a portion of coenzyme A and 4′-phosphopantetheine, both essential for synthesis of fatty acids. Coenzyme A is also necessary for synthesis of membrane phospholipids, for oxidative degradation of fatty acids and amino acids, and for acetylation and acylation of proteins [11]. Pantothenic acid deficiency

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adversely affects cell proliferation. For example, pantothenic acid deficiency causes reduced hepatocellular proliferation [12]. Moreover, pantothenic acid supplementation increases rates of proliferation and protein synthesis in cultured fibroblasts [13], and pantothenic acid stimulates blastocyst production in cultures of one-cell hamster embryos [14]. Taken together, these observations provide evidence that proliferating cells may have an increased demand for pantothenic acid and raise the possibility that cells meet this increased demand by increasing rates of pantothenic acid uptake.

A sodium-dependent transporter for pantothenic acid in human placenta has been characterized, cloned, and functionally expressed [15,16]. This transporter has been named the "sodium-dependent multivitamin transporter" (SMVT) because it binds pantothenic acid, biotin, and lipoic acid with similar affinity. Northern blot analysis suggested that the SMVT is expressed in various human tissues. However, whether PBMC express SMVT is unknown. In the present study, we specifically sought (*i*) to identify the mechanism for uptake of pantothenic acid in PBMC; (*ii*) to determine whether proliferation causes an increased pantothenic acid uptake into PBMC; and, if so, (*iii*) to determine whether the increased pantothenic acid uptake is mediated by increased expression of the gene encoding SMVT.

2. Experimental procedures

2.1. Materials

D-[^{3}H]Pantothenic acid (specific radioactivity $\geq$ 0.74 TBq/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO); the radiolabel was located in position 2 and 3 of the β-alanine portion of the molecule. Radiochemical purity of [^{3}H]pantothenic acid was greater than 95% as determined by thin-layer chromatography (see below). [^{3}H]Thymidine (specific radioactivity 1.29 TBq/mmol) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). The RNeasy mini kit for isolation of total RNA was purchased from Qiagen (Valencia, CA). The Advantage® cDNA PCR kit, the Smart™ PCR cDNA synthesis kit for reverse transcription, and the AdvanTAge PCR cloning kit were purchased from Clontech (Palo Alto, CA). Reverse transcriptase Superscript II RT™ was purchased from Life Technologies (Gaithersburg, MD). Customized oligonucleotide primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA); oligonucleotide primers for the human transferrin receptor were purchased from Clontech (cat.# 5407-1). Biotin- and pantothenic acid-free RPMI-1640 was prepared by Atlanta Biologicals (Norcross, GA). All other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Subjects

This study was approved by the Human Research Advisory Committee of the University of Arkansas for Medical Sciences, Little Rock, AR. Written, informed consent was obtained from all subjects. Eleven subjects (five women) donated blood for isolation of PBMC; some subjects donated blood on more than one occasion, i.e., for discrete experimental designs. The age of the subjects ranged from 23 to 53 years; all subjects were nonsmokers. None of the subjects consumed vitamin supplements that contained more than the Recommended Dietary Allowance of pantothenic acid [17].

2.3. Isolation of PBMC

PBMC were isolated from blood by gradient centrifugation as described previously [18,19]. The studies described below were performed using either quiescent, nonproliferating PBMC or mitogen-stimulated, proliferating PBMC.

2.4. Quantitation of [^{3}H]pantothenic acid uptake and metabolism in quiescent PBMC

For a given experiment, PBMC were isolated from approximately 120 ml of blood and suspended in approximately 30 ml of phosphate-buffered saline containing 5.5 mmol/L D-glucose. Cell number and viability were determined as described before [8]. Cell density was $5.1 \pm 1.2 \times 10^6$ cells/mL; viability was greater than 99%. The uptake rates of [^{3}H]pantothenic acid were normalized per 10^6 viable cells.

Cellular pantothenic acid uptake was measured in 1-mL aliquots of the PBMC suspension, as described previously [19], with one alteration: [^{3}H]biotin was replaced with [^{3}H]pantothenic acid. Typically, [^{3}H]pantothenic acid uptake was determined over a 15-min period at 37°C using a physiologic concentration of pantothenic acid (100 nmol/L) in the medium [20]. Immediately after incubation, extracellular [^{3}H]pantothenic acid was removed by washing with phosphate-buffered saline followed by liquid scintillation counting of the cell pellet as described before [19]. We confirmed that PBMC do not release measurable quantities of intracellular [^{3}H]pantothenic acid during washing with phosphate-buffered saline at 4°C (data not shown). Blanks were prepared by replacing PBMC suspension with cell-free medium; blank values were subtracted from sample values.

Effects of inhibitors and pantothenic acid analogs on cellular uptake of [^{3}H]pantothenic acid were studied as described previously [19]. For solubility reasons, ouabain and 2,4-dinitrophenol were dissolved in dimethyl sulfoxide as previously described [19]. To test for solvent artifacts, we measured [^{3}H]pantothenic acid uptake in the presence of the highest concentration of dimethyl sulfoxide used in any of the current studies (5% by vol); dimethyl sulfoxide did not significantly change pantothenic acid uptake [dimethyl sulfoxide treated = 49.8 ± 27.2 fmol/(10^6 cells $\times$ 15 min);

control = 52.3 ± 4.6 fmol/(10^6 cells × 15 min); n = 3; P = 0.91].

To investigate whether PBMC metabolize pantothenic acid during transport studies, we incubated cells with 100 nmol/L [^{3}H]pantothenic acid at 37°C. At timed intervals (15, 30, 90 min after addition of [^{3}H]pantothenic acid), PBMC were harvested by centrifugation at 2,260 g for 90 sec. PBMC pellets were suspended in 40 μL of phosphate-buffered saline, followed by lysis with 5 μL 0.5% Triton X-100 and 5 μL of 2 mol/L trichloroacetic acid. After vortexing, 10 μL of this lysate was spotted onto a silica gel plate for thin-layer chromatography; the plate was developed using n-butyl alcohol : pyridine : acetic acid : water (15 : 50 : 15 : 55, by vol) as solvent [21]. Lanes of the dried chromatography plate were cut into equal pieces, and radioactivity was determined by liquid scintillation counting of the pieces. An authentic standard of [^{3}H]pantothenic acid was run in a separate lane and located as described for the cell lysate.

2.5. Quantitation of [^{3}H]pantothenic acid and [^{3}H]thymidine uptake in mitogen-stimulated, proliferating PBMC

PBMC were isolated aseptically as described before [8]. After the final wash, the PBMC pellet was suspended in sterile-filtered RPMI-1640 (originally free of pantothenic acid and biotin) containing 10% autologous plasma (vol/vol), 100 I.U./mL penicillin, and 100 μg/mL streptomycin; unlabeled pantothenic acid and biotin were added to produce final concentrations of 50 and 0.5 nmol/L, respectively. The cell number per mL of medium was adjusted to 4 × 10^6 PBMC/mL by dilution with medium. Then, either the T cell mitogen concanavalin A (final concentration 20 μg/mL) or the T and B cell mitogen pokeweed lectin (2.0 μg/mL; from *Phytolacca americana*) was added to the PBMC suspension. These suspensions were then incubated at 37°C for 2 days (unless stated otherwise) in a humidified atmosphere of 95% air and 5% CO_2 to induce proliferation; nonproliferating controls were incubated without mitogen. After incubation, cell numbers per mL and cell viability were determined again [8]. The uptake rates of [^{3}H]pantothenic acid were determined as described above; the specific radioactivity of [^{3}H]pantothenic acid was adjusted for the concentration of unlabeled pantothenic acid in the medium. The uptake rates of the proliferation marker [^{3}H]thymidine were determined as described previously [8].

2.6. RNA extraction and RT-PCR

Total RNA was extracted from quiescent and mitogen-stimulated, proliferating PBMC by the RNeasy mini kit according to the manufacturer's instructions. The amount of RNA was quantitated spectrophotometrically at 260 nm. One microgram of RNA from each sample was reverse transcribed using the Smart™ PCR cDNA synthesis kit and Reverse transcriptase Superscript II RT™. The ssDNA was amplified by PCR using the following oligonucleotide primers for either SMVT or various housekeeping genes: (*i*) 5′-CAG AGC CCT GGA GCA GCA ACT GGC T-3′ and 5′-GGA GGT CTC CTG GAG GAT GCA GGT GG-3′for SMVT [16]; (*ii*) 5′-ACC ACA GTC CAT GCC ATC ACT GCC ACC-3′ and 5′-TCC ACC ACC CTG TTG CTG TAG CCA AAT-3′ for human glyceraldehyde-3-phosphate dehydrogenase [22]; (*iii*) 5′-ATG TCG GAG ACT GCT CCA CTT GCT C-3′ and 5′-TCA CTT TTT CTT CGG AGC TGC CTT CTT T-3′ for human histone H1.3 [23]; (*iv*) 5′-ATG TCT GGT AGA GGC AAA GGT GGT AAA-3′ and 5′-TCA GCC ACC AAA GCC GTA CAG AGT GCG-3′ for human histone H4 [23]; (*v*) oligonucleotide primers for the human transferrin receptor were purchased from Clontech.

PCR was performed using the following temperatures and times per cycle: 94°C for 1 min (denaturating), 55°C for 1 min (annealing), and 72°C for 2 min (extending); for glyceraldehyde-3-phosphate dehydrogenase the annealing temperature was 60°C. The PCR-amplified ssDNA was collected at timed intervals after 15, 18, 21, 25, 30, 35, 40, 50, and 60 cycles. Equal aliquots (10 μL) of each sample were loaded onto a 1.5% agarose gel, stained with ethidium bromide, visualized and digitized using FOTO/Analyst® PC image, version 2.0 (Fotodyne, Inc.; Hartland, WI), and quantitated using the Molecular Analyst software package, version 1.5 (Bio-Rad Laboratories; Hercules, CA). cDNA that was produced using the SMVT oligonucleotide primers was inserted into the pT-Adv vector. A 649-basepair fragment was sequenced at the Molecular Resource Laboratory (University of Arkansas for Medical Sciences, Little Rock, AR) to confirm identity; 99% identity with the published sequence was found.

2.7. Statistics

Significance of differences among groups (e.g., among the two different mitogens and the control) were tested by one-way ANOVA. Dunnett's posthoc procedure was used for posthoc testing; Dunnett's compares the mean of each treatment group (e.g., mitogen-stimulated cells) to that of a designated control (e.g., unstimulated cells) [24,25]. Effects of incubation time and temperature were tested by two-way ANOVA (time by temperature). Paired comparisons were made using the paired, two-tailed t-test. SuperANOVA 1.11 and StatView 4.5 (Abacus Concepts, Berkeley, CA) were used to perform all calculations. Differences were considered significant if $P \leq 0.05$. Data are expressed as mean ± 1 SD.

3. Results

We initially determined an interval over which [^{3}H]pantothenic acid uptake increased linearly with time. When

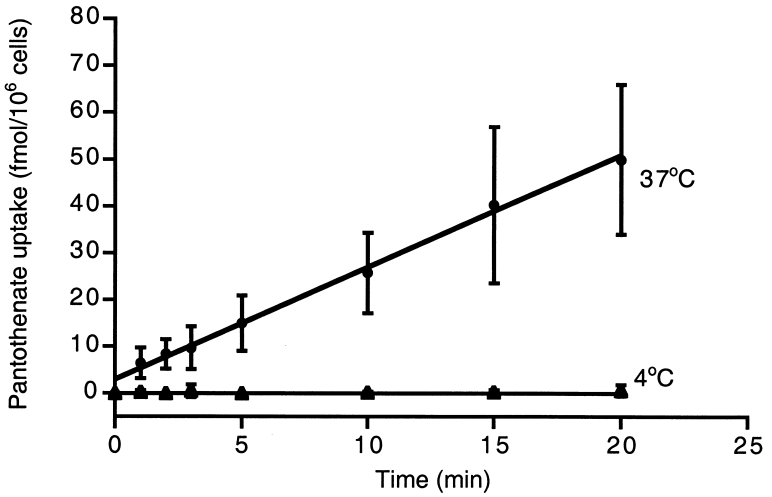

Fig. 1. Effects of incubation time and temperature on the uptake of [^{3}H]pantothenic acid (100 nmol/L) into human PBMC. Values are means ± SD of 6 determinations. Effects of time and temperature were both significant ($P < 0.01$ by two-way ANOVA). At 4°C: uptake = 0.02 × time + 0.1 (r = 0.526); at 37°C: uptake = 2.4 × time + 2.5 (r = 0.997).

quiescent PBMC were incubated at 37°C with a physiologic concentration of pantothenic acid (100 nmol/L), pantothenic acid uptake increased linearly with incubation time for at least 20 min (Figure 1). An incubation time of 15 min was used for all subsequent experiments. Pantothenic acid uptake into PBMC also increased linearly with time (r = 0.992) when PBMC were incubated with a pharmacologic concentration of pantothenic acid (i.e., 20,000 nmol/L) at 37°C. Thus, the studies of saturation kinetics (see below) were not confounded by saturating transport prematurely (i.e., before termination of the 15-min incubations) when pharmacologic concentrations of [^{3}H]pantothenic acid were used.

We next determined whether uptake of [^{3}H]pantothenic acid into PBMC was temperature dependent. Pantothenic acid uptake into quiescent PBMC was significantly greater at 37°C than at 4°C (Figure 1).

To assess whether pantothenic acid uptake into quiescent PBMC exhibits saturation at high concentrations of pantothenic acid, PBMC were incubated with [^{3}H]pantothenic acid at concentrations between 0.05 to 20 μmol/L. At a physiologic concentration of [^{3}H]pantothenic acid (0.1 μmol/L) in the medium, cellular uptake was 61 ± 10 fmol/(10^6 cells × 15 min) at 37°C. Uptake approached a plateau at high pantothenic acid concentrations consistent with saturation kinetics (Figure 2). Data sets from individual experiments were accurately fit (r = 0.997 ± 0.002) by nonlinear regression using the Michaelis-Menten equation. Mean Michaelis-Menten constant (K_m) among six experiments was 17.7 ± 5.3 μmol/L; mean maximal transport rate (V_{max}) was 10.4 ± 1.6 pmol/(10^6 cells × 15 min). Both the observations concerning temperature and those demonstrating saturation are consistent with carrier-mediated uptake.

Previous studies have suggested that uptake of pantothenic acid into human placenta is mediated by a transporter (SMVT) that binds pantothenic acid, biotin, and lipoic acid [15,16]. We examined whether biotin and other organic acids (hexanoic acid, L-alanine, L-leucine) compete with [^{3}H]pantothenic acid for uptake into PBMC. We incubated quiescent PBMC with 100 nmol/L [^{3}H]pantothenic acid and 150 μmol/L competitor. Uptake of [^{3}H]pantothenic acid decreased significantly in the presence of either biotin (20.5 ± 12.5% of control) or hexanoic acid (76.8 ± 15.7%) (Figure 3). In contrast, uptake did not decrease significantly in the presence of either L-alanine or L-leucine. These data provide evidence that the pantothenic acid transporter in PBMC also binds biotin and hexanoic acid, suggesting that the transporter has specificity for carboxyl groups. Compounds that are present in zwitterionic form (L-alanine, L-leucine) do not appear to bind to the transporter.

To assess further the importance of various regions of the pantothenic acid molecule for binding to the transporter, we conducted a series of competition experiments with pantothenic acid analogs. Quiescent PBMC were incubated with

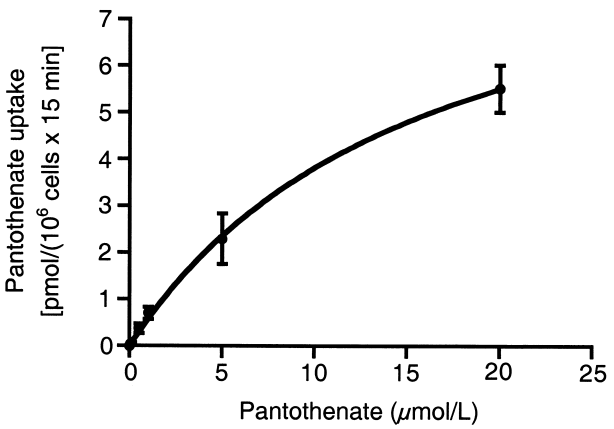

Fig. 2. Saturation kinetics of [^{3}H]pantothenic acid uptake into human PBMC. PBMC were incubated with 0.05 to 20 μmol/L [^{3}H]pantothenic acid at 37°C for 15 min. Values are means ± SD of 6 determinations.

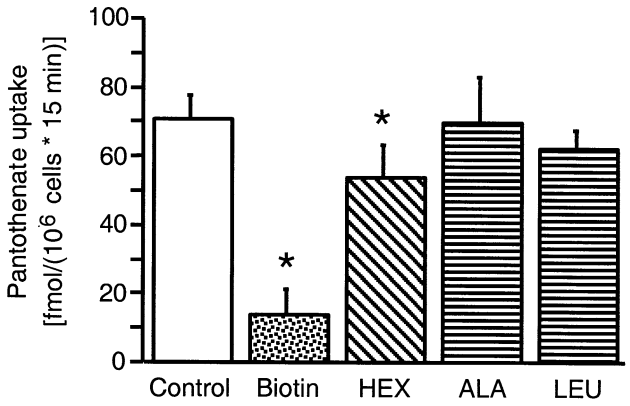

Fig. 3. The effects of biotin, hexanoic acid, L-alanine, or L-leucine (150 μmol/L) on the uptake of [^{3}H]pantothenic acid (100 nmol/L) into human PBMC. Controls were incubated without competitors. Values are means ± SD of 6 determinations. *$P < 0.01$ versus control (by one-way ANOVA and Dunnett's posthoc test). HEX = hexanoic acid; ALA = L-alanine; LEU = L-leucine.

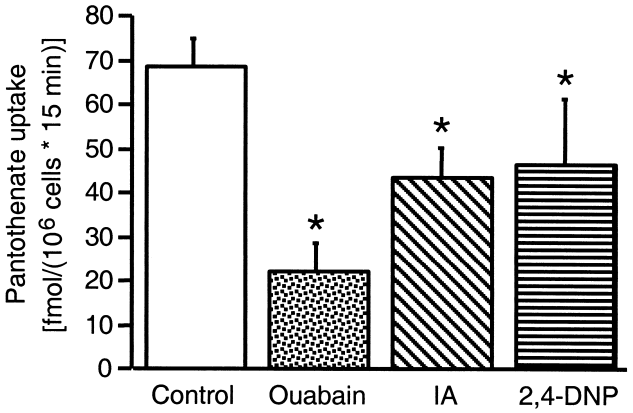

Fig. 5. The effects of ouabain, iodoacetate, and 2,4-dinitrophenol (all at 1 mmol/L) on the uptake of [^{3}H]pantothenic acid (100 nmol/L) into human PBMC. Controls were incubated without transport inhibitors. Values are means ± SD of 6 determinations. *$P < 0.01$ versus control (by one-way ANOVA and Dunnett's posthoc test). IA = iodoacetate; 2,4-DNP = 2,4-dinitrophenol.

100 nmol/L [^{3}H]pantothenic acid in the presence (150 μmol/L) of either unlabeled D-pantothenic acid, D-pantethine (a disulfide dimer of pantetheine, which is an intermediate in the biosynthesis of coenzyme A), D-pantothenyl alcohol (the carboxyl group of pantothenic acid is replaced by an alcohol group), β-alanine (an integral part of the pantothenic acid molecule), or coenzyme A (the biologically active form of pantothenic acid); the control was incubated without unlabeled pantothenic acid or any analog. The uptake of [^{3}H]pantothenic acid was reduced significantly by unlabeled pantothenic acid to 9.6 ± 1.7% of control (Figure 4), providing further evidence of carrier-mediated transport. Pantothenic acid analogs also decreased the cellular uptake of [^{3}H]pantothenic acid, but effects were smaller than for unlabeled pantothenic acid (53.1 ± 23.4% to 74.1 ± 14.7% of control). These findings suggest that removal of the carboxyl group (D-pantethine, D-pantothenyl alcohol, and coenzyme A) or presence as zwitterion (β-alanine) reduces binding affinity to the pantothenic acid transporter.

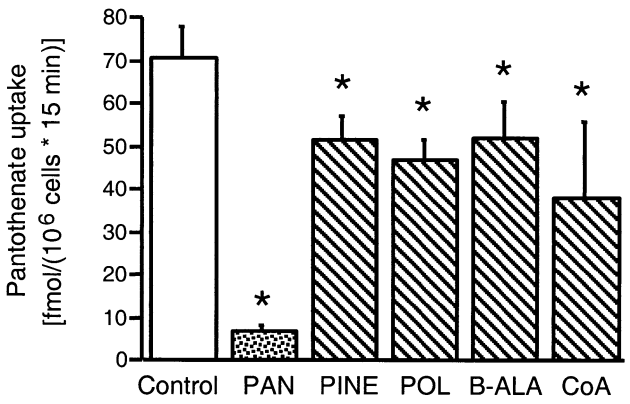

Fig. 4. The effects of D-pantothenic acid (PAN), D-pantethine (PINE), D-pantothenyl alcohol (POL), β-alanine (B-ALA), or coenzyme A (CoA; all at 150 μmol/L) on the uptake of [^{3}H]pantothenic acid (100 nmol/L) into human PBMC. Controls were incubated without pantothenic acid or analogs. Values are means ± SD of 6 determinations. *$P < 0.01$ versus control (by one-way ANOVA and Dunnett's posthoc test).

We further characterized the transporter as follows. PBMC were incubated with [^{3}H]pantothenic acid in the presence of one of the following transport inhibitors (all at 1 mmol/L): ouabain (an inhibitor of Na-K-ATPase), iodoacetate (a compound that covalently modifies sulfhydryl groups), or 2,4-dinitrophenol (an inhibitor of the respiratory chain which reduces intracellular ATP levels); controls were incubated without inhibitors (Figure 5). Ouabain decreased pantothenic acid uptake to 31.9 ± 8.9% of control values, suggesting that pantothenic acid uptake is sodium dependent. Iodoacetate decreased pantothenic acid uptake to 63.8 ± 11.7% of control values, suggesting that sulfhydryl groups of the transport protein are involved in uptake. 2,4-Dinitrophenol decreased pantothenic acid uptake to 67.4 ± 16.7% of control values, providing evidence that pantothenic acid uptake is energy dependent.

The cation requirement was further examined in experiments in which sodium in the medium was replaced by equimolar concentrations of either choline, lithium, or ammonium (Figure 6). Replacement of sodium by one of the three reduced pantothenic acid uptake to 8.5 ± 5.0% to 11.6 ± 5.1% of control, providing additional evidence that cellular pantothenic acid uptake requires cotransport with sodium.

Next, we determined whether quiescent and mitogen-stimulated, proliferating PBMC metabolize pantothenic acid during the 15-min incubation times used in these transport studies. We incubated PBMC with 100 nmol/L [^{3}H]pantothenic acid and collected cell pellets after 15, 30, and 90 min. Thin-layer chromatography followed by liquid scintillation counting of tritium was used to track metabolism (see Experimental Procedures). By 90 min, no metab-

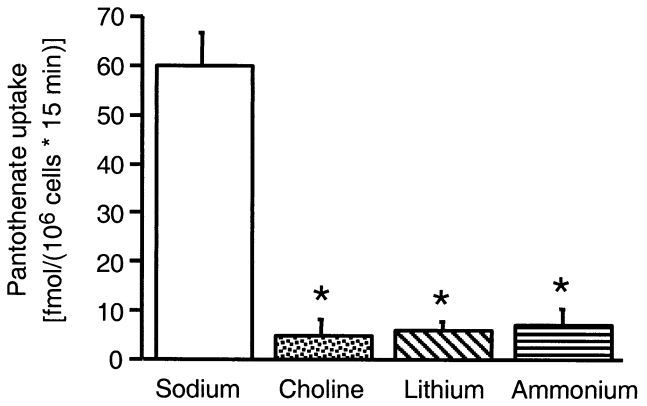

Fig. 6. The effects of sodium-free medium on the uptake of [^{3}H]pantothenic acid (100 nmol/L) into human PBMC. Sodium in the medium was replaced by equimolar concentrations of either choline, lithium, or ammonium; controls were incubated in sodium-containing medium. Values are means ± SD of 6 determinations. *$P < 0.01$ versus control (by one-way ANOVA and Dunnett's posthoc test).

olism of pantothenic acid was detectable in quiescent PBMC (Figure 7); in contrast, proliferating PBMC metabolized approximately 10–15% of the intracellular pantothenic acid (see peak in TLC fraction 10). Similar observations were made for 15-min and 30-min incubations (data not shown). The arrow in Figure 7 denotes the chromatographic position expected for pantothenic acid.

The effect of PBMC proliferation on the cellular uptake of pantothenic acid was assessed at a physiologic concentration of [^{3}H]pantothenic acid (100 nmol/L). Proliferating PBMC accumulated [^{3}H]pantothenic acid at a greater rate than unstimulated controls: quiescent controls = 61 ± 34 fmol/(10^6 cells × 15 min); concanavalin A-stimulated PBMC = 114 ± 40 fmol/(10^6 cells × 15 min); pokeweed lectin-stimulated PBMC = 95 ± 44 fmol/(10^6 cells × 15 min). The difference among groups reached statistical significance only at $P = 0.1$ (by one-way ANOVA). In a separate experiment we measured cellular uptake of [^{3}H]pantothenic acid at a different physiologic concentration (500 nmol/L). Uptake increased significantly in concanavalin A-stimulated PBMC ($P < 0.05$ versus control): quiescent controls = 250 ± 39 fmol/(10^6 cells × 15 min); concanavalin A-stimulated PBMC = 458 ± 156 fmol/(10^6 cells × 15 min); pokeweed lectin-stimulated PBMC = 393 ± 142 fmol/(10^6 cells × 15 min).

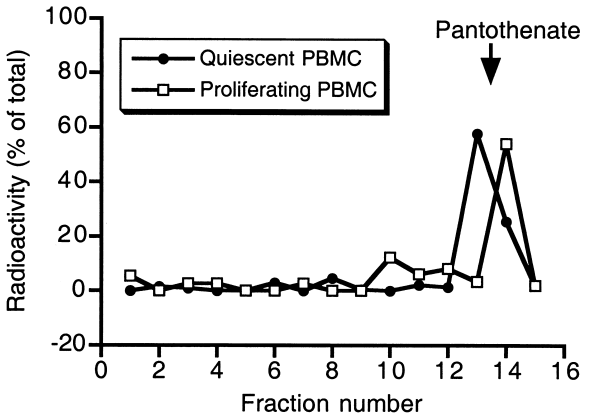

Fig. 7. Pantothenic acid metabolism in human PBMC. Quiescent and mitogen-stimulated, proliferating cells (20 μg/ml concanavalin A for 48 h) were incubated with [^{3}H]pantothenic acid (100 nmol/L) for 90 min. Contents of lysed cells were chromatographed using thin-layer chromatography. The TLC lanes were cut into 15 equal pieces and quantitated by liquid scintillation counting. The arrow indicates the chromatographic mobility of an authentic standard of pantothenic acid.

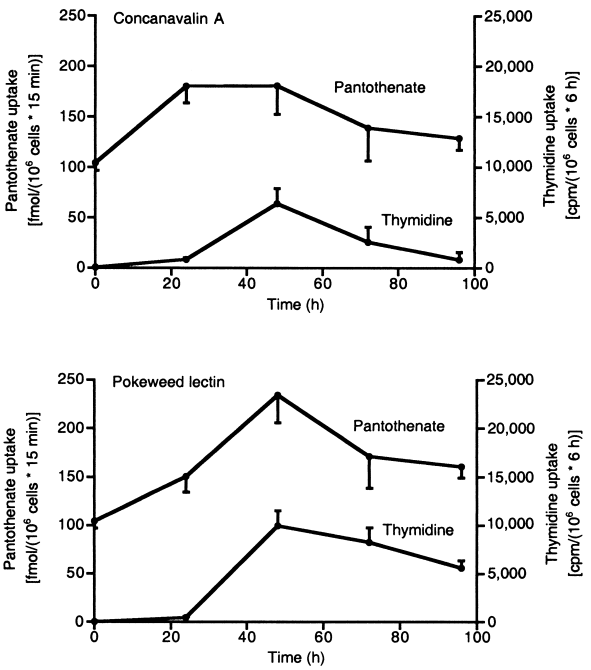

Fig. 8. Time course of [^{3}H]pantothenic acid and [^{3}H]thymidine uptake in mitogen-stimulated human PBMC. Cells were incubated with either 20 μg/ml concanavalin A (upper panel) or 2.0 μg/ml pokeweed lectin (lower panel) for up to 96 h. At timed intervals, aliquots were collected and cellular uptake of [^{3}H]pantothenic acid and [^{3}H]thymidine was measured. Zero-time values were measured before addition of mitogen to the medium. Values are means ± SD of 6 determinations.

In these studies, proliferation of mitogen-stimulated PBMC was confirmed by cellular [^{3}H]thymidine uptake. Mitogen-stimulated PBMC accumulated greater than 500 times more thymidine than quiescent control PBMC ($P <$ 0.01 versus control): quiescent controls = 237 ± 209 cpm/(10^6 cells × 6 h); concanavalin A-stimulated PBMC = 131,122 ± 4,818 cpm/(10^6 cells × 6 h); pokeweed lectin-stimulated PBMC = 181,133 ± 9,667 cpm/(10^6 cells × 6 h).

To further assess whether the increased pantothenic acid uptake in mitogen-stimulated PBMC results from PBMC proliferation, we investigated the time course of stimulation of pantothenic acid uptake into proliferating PBMC. In these experiments, we added either concanavalin A (final concentration 20 μg/mL) or pokeweed lectin (final concentration 2.0 μg/mL) to a suspension of PBMC (4 × 10^6 cells/mL). Stimulation of pantothenic acid uptake reached maximal values 48 h after addition of mitogen (Figure 8).

Table 1
Transport kinetics of pantothenic acid in mitogen-stimulated PBMC. PBMC were incubated with either concanavalin A (20 μg/mL) or pokeweed lectin (2.0 μg/mL) to stimulate proliferation; controls were incubated without mitogen. On day 2, cells were harvested and cellular [^{3}H]pantothenic acid uptake was measured using [^{3}H]pantothenic acid concentrations of 0.05 to 20 μmol/L in the medium. K_m (Michaelis-Menten constant) and V_{max} (maximal transport rate) were determined by nonlinear regression. Values are means ± SD of 6 to 12 determinations. NS = values within the same column are not significantly different by one-way ANOVA.

Treatment	K_m μmol/L	V_{max} pmol/ (10^6 cells × 15 min)
Concanavalin A	19.2 ± 6.0	42.9 ± 10.6
Pokeweed lectin	19.0 ± 7.6	36.6 ± 11.0
Control (no mitogen)	13.2 ± 6.7	7.5 ± 3.3
P value	NS	0.01

The increase of pantothenic acid uptake paralleled the increase of [^{3}H]thymidine uptake.

To explore the mechanism of increased uptake, quiescent and mitogen-stimulated PBMC were incubated with [^{3}H]pantothenic acid at concentrations from 50 to 20,000 nmol/L. As judged by the transport kinetics (Table 1), proliferation caused an increase of maximal transport rates by approximately five fold but did not affect affinity of the transporter for pantothenic acid. These observations are consistent with increased number of pantothenic acid transporters on the PBMC surface due to increased SMVT synthesis.

To test this hypothesis, we determined SMVT gene expression by RT-PCR of total RNA that was isolated from quiescent and mitogen-stimulated, proliferating PBMC; certain housekeeping genes were quantitated for comparison. Expression was quantitated during the exponential phase of PCR amplification, i.e., before PCR amplification reached a plateau. The concentration of RNA encoding SMVT in proliferating PBMC was 1064 ± 158% compared to quiescent controls (Figure 9). The increased expression of SMVT in proliferating PBMC exceeded the increased expression of the housekeeping genes encoding transferrin receptor (558 ± 644% of control), glyceraldehyde-3-phosphate dehydrogenase (100 ± 9.4% of control), histone H1.3 (175 ± 10% of control), and histone H4 (113 ± 9.5% of control); typical examples are depicted in Figure 9. Together, these data suggest that the increased expression of the pantothenic acid transporter gene is sufficient to explain increased pantothenic acid transport and that the increase is fairly specific relative to the increased expression of other genes.

Are these results an artifact of normalizing per mass of RNA? The values in Figure 9 are normalized per μg of RNA used for reverse transcription. However, the difference between proliferating and quiescent PBMC actually increases if values of RT-PCR analysis are normalized per 10^6 PBMC. This conclusion is based on the following line of reasoning: We extracted 88 μg of total RNA from 4×10^7 proliferating PBMC but only 32 μg from 4×10^7 quiescent PBMC. One microgram of RNA was used for RT-PCR as shown in Figure 9; 1 μg of RNA equals only 0.45×10^6 proliferating PBMC compared to 1.3×10^6 quiescent PBMC. If the data from Figure 9 instead are normalized per 10^6 PBMC then the difference between proliferating and quiescent PBMC increased by a factor of 2.9 to about 30-fold increase.

4. Discussion

This study provides evidence that pantothenic acid uptake into human PBMC is mediated by the SMVT that has been identified previously in human placenta [15,16]. This conclusion is based on the following observations: (*i*) pantothenic acid affinity (as judged by the Michaelis-Menten

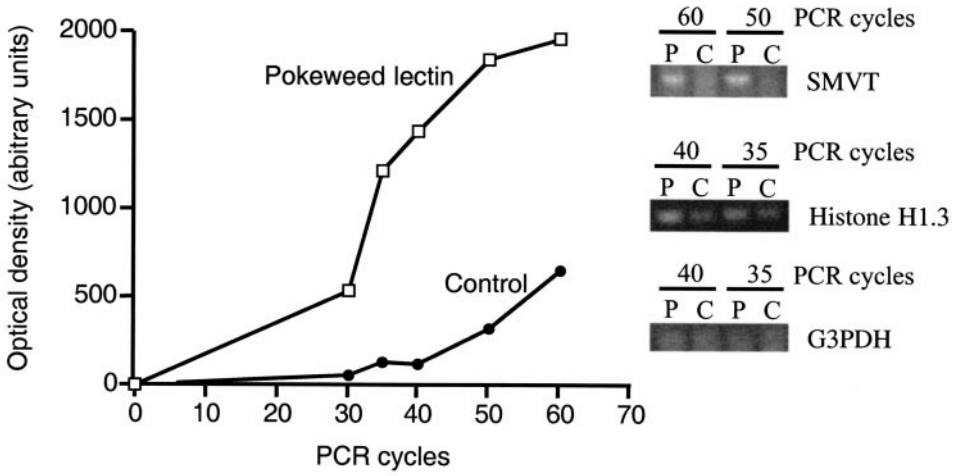

Fig. 9. Expression of the sodium-dependent multivitamin transporter (SMVT) gene in PBMC. Cells were incubated with 2.0 μg/mL pokeweed lectin (P) for 48 h; controls (C) were incubated without mitogen. Total RNA was extracted and assayed by RT-PCR using primers that were specific for SMVT; histone H1.3 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were quantitated by RT-PCR as controls (see text for additional controls).

constant) is similar for the transporters in human placenta and PBMC; (*ii*) excess biotin significantly reduced pantothenic acid uptake in both human placenta and PBMC; (*iii*) uptake of pantothenic acid is sodium-dependent in both human placenta and PBMC; and (*iv*) oligonucleotide primers based on the nucleotide sequence of the placental SMVT were used in RT-PCR and demonstrated an increase in SMVT mRNA in mitogen-stimulated PBMC that coincided with the increase in pantothenic acid transport.

Uptake of pantothenic acid at 15 min was approximately two times greater in proliferating PBMC compared to quiescent controls. The increased uptake of pantothenic acid approximately paralleled proliferation rates of PBMC as judged by thymidine uptake. What is the mechanism that mediates the increased uptake of pantothenic acid into proliferating PBMC? This study suggests that increased uptake of pantothenic acid is mediated by an increased number of transporters on the cell surface, based on the following three observations: (*i*) Maximal transport rates in proliferating PBMC were approximately five times greater than in quiescent controls. This is consistent with an increased number of pantothenic acid transporters on the cell surface. In contrast, affinity for pantothenic acid (as judged by the Michaelis-Menten constant) was not significantly different in proliferating and in quiescent PBMC providing evidence against the synthesis of a different transporter in response to mitogens. (*ii*) The five fold increase of transport rates of pantothenic acid substantially exceeds the approximately two fold increase of PBMC volume that occurs during cell proliferation [9]. Hence, increased transport rates likely are an effect of increased transport sites rather than an effect of increased cell volume. (*iii*) Intracellular levels of RNA encoding SMVT were approximately 10 times greater in proliferating than in quiescent PBMC. Not surprisingly, levels of RNA encoding some housekeeping genes also increased with proliferation, but the increase was typically much smaller than that for SMVT. Taken together, these data are consistent with the hypothesis that proliferating PBMC specifically increase expression of the SMVT gene in response to increased cellular demand for pantothenic acid.

In the present studies we assessed intracellular metabolism of pantothenic acid in order to monitor for metabolic trapping. In quiescent PBMC, we did not observe any metabolism of intracellular [^{3}H]pantothenic acid during incubations of up to 90 min. Thus, metabolic trapping is not important for uptake of pantothenic acid into quiescent PBMC. In contrast, proliferating PBMC metabolized approximately 10–15% of the intracellular [^{3}H]pantothenic acid. Whether this metabolic trapping is important for the increased uptake of pantothenic acid into proliferating PBMC remains uncertain. However, it appears to be unlikely that a 15% conversion of pantothenic acid over 90 min can account for the five fold increase of maximal transport rates observed over as little as 15 min observation time used for the transport studies.

In summary, this study provides evidence that PBMC respond to proliferation by an increased synthesis of pantothenic acid transporters. We speculate that the increased uptake is a response to increased demand for pantothenic acid caused by increased coenzyme A intermediates in various metabolic pathways that are important in cell proliferation.

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References

[1] Janeway, C.A., Travers, P., Walport, M., and Capra, J.D. (1999), Immuno Biology. 4 ed. Garland Publishing/Elsevier, London.
[2] A. Murray, T. Hunt, The Cell Cycle. Oxford University Press, New York.
[3] G.B. Segel, M.A. Lichtman, Amino acid transport in human lymphocytes: distinctions in the enhanced uptake with PHA treatment or amino acid deprivation, J. Cell Physiol. 106 (1981) 303–306.
[4] Roos, D., De Boer, J.E.G., Huismans, L, and Boom, A.J, Dose-response of lymphocyte carbohydrate metabolism to phytohemagglutinin, Exp Cell Res, 75, 185–190 (1972).
[5] Roos, D. and Loos, J.A, Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral lymphocytes. II. Relative importane of glycolysis and oxidative phosphorylation on phytohaemagglutinin stimulation, Exp Cell Res, 77, 127–135 (1973).
[6] J.A. Loos, D. Roos, Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral lymphocytes. III. Stimulation by tuberculin and allogeneic cells, Exp. Cell Res. 79 (1973) 136–142.
[7] C.E. Hall, The uptake of vitamin B12 by human lymphocytes and the relationships to the cell cycle, J. Lab. Clin. Med. 103 (1984) 70–81.
[8] Zempleni, J. and Mock D.M, Mitogen-induced proliferation increases biotin uptake into human peripheral blood mononuclear cells, Am J Physiol Cell Physiol, 276, C (1999) 1079–1084.
[9] Williams, G.T., Lau, K.M.K., Coote, J.M., Johnstone, A.P, NAD metabolism and mitogen stimulation of human lymphocytes, Exp Cell Res, 160, 419–426 (1985).
[10] Zempleni, J. and Mock, D.M, Proliferation of peripheral blood mononuclear cells increases riboflavin influx, Proc Soc Exp Biol Med, 225, 72–79 (2000).
[11] Plesofsky-Vig, N. (1996), Pantothenic acid. In: Present Knowledge in Nutrition (Ziegler, E.E. and Filer Jr., L.J., eds.), pp. 236–244, ILSI Press, Washington, D.C.
[12] Youssef, J.A., Cunningham M., Song,W.O., and Badr, M.Z, Metabolic requirements for the hepatocellular proliferation induced by peroxisome proliferators: role of coenzyme A, Ann NY Acad Sci USA, 804, 725–727 (1996).
[13] Weimann, B.J. and Herrmann D, Studies on wound healing: effects of calcium D-pantothenate on the migration, proliferation and protein synthesis of human dermal fibroblasts in culture, Internat J Vitam Nutr Res, 69, 113–119 (1999).
[14] McKiernan, S.H. and Bavister, B.D, Culture of one-cell hamster embryos with water soluble vitamins: pantothenate stimulates blastocyst production, Hum Reprod, 15, 157–164 (2000).
[15] Prasad, P.D., Ramamoorthy, S., Leibach, F.H., and Ganapathy, V, Characterization of a sodium-dependent vitamin transporter mediating the uptake of pantothenate, biotin and lipoate in human placental choriocarcinoma cells, Placenta, 18, 527–533 (1997).
[16] Wang, H., Huang, W., Fei, Y.-J., Xia, H., Fang-Yeng, T.-L., Leibach, F.H., Devore L.D., Ganapathy, V., and Prasad, P.D, Human placental Na^+-dependent multivitamin transporter, J Biol Chem, 274, 14875–14883 (1999).

[17] Yates, A.A., Schlicker, S.A., and Suitor C.W, Dietary reference intakes: the new basis for recommendations for calcium and related nutrients, B vitamins, and choline. J. Am. Diet. Assoc. 98, 699–706, 1998.
[18] Boyum, A, Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g, Scand J Clin Lab Invest, 21, 77–89 (1968).
[19] Zempleni, J. and Mock, D.M, Uptake and metabolism of biotin by human peripheral blood mononuclear cells, Am J Physiol Cell Physiol, 275, C382–C388 (1998).
[20] Banno, K., Matsuoka, M., Horimoto, S., and Kato, J, Simultaneous determination of pantothenic acid and hopantenic acid in biological samples and natural products by gas chromatography-mass fragmentography, J Chromatogr, 525, 255–264 (1990).
[21] Barbarat, B. and Podevin, R.A, Pantothenate-sodium cotransport in renal brush-border membranes, J Biol Chem, 261, 14455–14460 (1986).
[22] Arcari, P., Martinelli, R., and Salvatore, F, The complete sequence of a full length cDNA for liver glyceraldehyde-3-phosphate dehydrogenase: evidence for multiple mRNA species, Nucleic Acids Res, 12, 9179–9189 (1984).
[23] Albig, W., Kardalinou, E., Drabent, B., Zimmer, A., and Doenecke, D, Isolation and characterization of two human H1 genes within clusters of core histone genes, Genomics, 10, 940–948 (1991).
[24] Zar, J.H. (1974), Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, N.J.
[25] Abacus Concepts. (1989), SuperANOVA. Abacus Concepts, Inc., Berkeley, CA.