Dietary vitamin B6 modulates the gene expression of myokines, Nrf2-related factors, myogenin and HSP60 in the skeletal muscle of rats

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Abstract. Previous studies have suggested that vitamin B6 is an ergogenic factor. However, the role of dietary vitamin B6 in skeletal muscle has not been widely researched. The aim of the present study was to investigate the effects of dietary vitamin B6 on the gene expression of 19 myokines, 14 nuclear factor erythroid 2-related factor 2 (Nrf2)-regulated factors, 8 myogenesis-related factors and 4 heat shock proteins (HSPs), which may serve important roles in skeletal muscles. Rats were fed a diet containing 1 (marginal vitamin B6 deficiency), 7 (recommended dietary level) or 35 mg/kg of pyridoxine (PN) HCl/ for 6 weeks. Gene expressions were subsequently analysed using reverse transcription-quantitative polymerase chain reaction. Food intake and growth were unaffected by this dietary treatment. The rats in the 7 and 35 mg/kg PN HCl groups exhibited a significant increase in the concentration of pyridoxal 5'-phosphate in the gastrocnemius muscle compared with the 1 mg/kg PN HCl diet ($P<0.01$). The expressions of myokines, such as IL-7, IL-8, secreted protein acidic and rich in cysteine, IL-6, growth differentiation factor 11, myonectin, leukaemia inhibitory factor, apelin and retinoic acid receptor responder (tazarotene induced) 1, the expression of Nrf2 and its regulated factors, such as heme oxygenase 1, superoxide dismutase 2, glutathione peroxidase 1 and glutathione S-transferase, and the expression of myogenin and HSP60 were significantly elevated in the 7 mg/kg PN HCl group compared with the 1 mg/kg PN HCl diet ($P<0.05$). No significant differences in levels of these genes were observed between the 35 and 1 mg/kg PN HCl, with the exception of GDF11 and myonectin, whose expressions were significantly increased in the 35 mg/kg PN HCl ($P<0.05$). Notably, the majority of gene expressions that were affected responded to dietary supplemental vitamin B6 in a similar manner. The results suggest that compared with the marginal vitamin B6 deficiency, the recommended dietary intake of vitamin B6 upregulates the gene expression of a number of factors that promote the growth and repair of skeletal muscle.

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Abbreviations: PLP, pyridoxal 5'-phosphate; PN, pyridoxine; IL, interleukin; F, forward; R, reverse; SPARC, secreted protein acidic and rich in cysteine; GDF, growth differentiation factor; LIF, leukemia inhibitory factor; RARRES1, retinoic acid receptor responder (tazarotene induced) 1; OSM, oncostatin M; FGF, fibroblast growth factor; GDNF, glial cell derived neurotrophic factor; LTBP2, latent-transforming growth factor β-binding protein 2; CHI3l1, chitinase-3-like protein 1; SERP4, septin 4; Metrn, meteorin; HO, heme oxygenase; SOD, superoxide dismutase; GPX, glutathione peroxidase; Nrf, nuclear respiratory factor; GST, glutathione S-transferase; Keap1; kelch-like ECH associated protein 1; PGC1a, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; Imp-11, importin 11; Trx2, thioredoxin reductase-2; Nqo1, NADPH quinone oxidoreductase 1; UBE2E3, ubiquitin-conjugation enzyme E2 E3; GCLC, glutamate-cysteine ligase catalytic subunit; MuRf1, muscle ring-finger protein-1; Myf, myogenic factor; MyoD1, myogenic differentiation 1; RPB3, RNA polymerase B3; Sp1, specificity protein; HSP, heat shock protein; Nrxn, neurexin; Ntn, netrin

Key words: vitamin B6, myokine, nuclear factor (erythroid-derived 2)-like 2, myogenin, heat shock protein 60, gastrocnemius muscle, rats

Introduction

Pyridoxal 5'-phosphate (PLP), the active form of vitamin B6, is known to modulate important metabolisms in the body (1). Skeletal muscle is the major source of PLP in the body (1). Some biological functions of vitamin B6 have been reported to be associated with fuel metabolism during exercise (2). It has been suggested that the exercise-related function of vitamin B6 is at least in part associated with the breakdown of muscle glycogen (2). Glycogen phosphorylase is the enzyme responsible for the release of glucose-1-phosphate from muscle glycogen, and PLP is a cofactor for this enzyme (2). Vitamin B6 deficiency has been reported to decrease the activity and amount of muscle phosphorylase (3). Rats fed high levels of vitamin B6 have previously exhibited elevated concentrations of muscle phosphorylase (4). Furthermore, it is thought that vitamin B6 may have a favourable effect on the skeletal muscle, as a previous case study demonstrated that vitamin B6 supplementation was beneficial for the treatment of McArdle

disease, a glycogenetic myopathy (5). Exercise has also been reported to increase blood PLP levels (6).

The recent discovery of several myokines has established a new paradigm of muscle as an endocrine organ (7). Myokines are released during muscle contraction, for example during exercise (7). Some known contraction-regulated myokines are interleukin (IL)-6, IL-7, IL-15, brain-derived neuro factor, fibroblast growth factor 21 (FGF21), follistatin-like 1, leukaemia inhibitory factor (LIF), vascular endothelial growth factor, irisin, myostatin and myonectin (7). Myokines have been reported to serve important roles in biological homeostasis, being involved in energy metabolism, angiogenesis and myogenesis (8). They are also considered to be putative preventive factors against chronic diseases such as type II diabetes (9,10). Some studies have suggested that myokines serve pivotal roles in the communication between skeletal muscles and other tissues, such as adipose tissues and liver and pancreatic cells (10-12).

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) regulates oxidative defences by inducing the expression of cytoprotective genes (13). Nrf2 induces these genes primarily by binding the antioxidant response element (ARE) in the promoter of the genes that code for anti-oxidative enzymes (13). Nrf2/ARE signalling is activated by acute exercise stress in the myocardium (14). Several previous studies have suggested that the role of Nrf2 goes beyond antioxidant mechanisms (15,16). Al-Sawaf *et al* (16) reported that Nrf2 regulated myogenic regulatory factor (Mrf) expression and that Nrf2-knockout retarded skeletal muscle regeneration after injury. Heat shock proteins (HSPs) have also been reported to have a cytoprotective role in skeletal muscles (17). Furthermore, it has been reported that exercise induces the expression of HSPs in skeletal muscle, heart and liver (18).

In the present study, the mRNA of myokines, Nrf2-related factors, myogenic factors and HSPs in the gastrocnemius muscles of rats that were fed a low vitamin B6 diet with and without vitamin B6 supplementation were analysed. Further analyses using DNA microarrays were also performed.

Materials and methods

Animals and diets. A total of 24 male 3-week-old Sprague-Dawley rats (Charles River Laboratories Japan, Inc., Tokyo, Japan) were purchased and maintained in accordance with the Guide for the Care and Use of Laboratory Animals established by Hiroshima University (Higashi-Hiroshima, Japan). The rats were housed in metal cages in a temperature-controlled room (24±1˚C) with a 12 h light/dark cycle (lights on from 08:00-20:00) at a humidity of 40-50%. The rats had free access to food and deionized water. The basal diet comprised the components as described previously (19). Pyridoxine (PN) HCl (Nacalai Tesque, Inc., Kyoto, Japan) was supplemented to the basal diet at 1, 7, or 35 mg PN HCl per kg of food. The level of PN HCl diet recommended in the AIN-93 diet is 7 mg/kg PN HCl (20). Meanwhile, 1 mg/kg PN HCl diet is reported to be the minimum level required to prevent growth depression caused by vitamin B6 deficiency (21). Following one week of commercial nonpurified diet (MF basic food; Oriental Yeast Co., Ltd., Tokyo, Japan) 24 rats (mean weight, 70 g for all three groups) were divided randomly into 3 groups (n=8) receiving 1, 7 or 35 mg PN HCl/kg diet for 6 weeks. At the end of this period, rats were sacrificed by decapitation following anaesthesia with inhalation exposure of diethyl ether (>99.5%, Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 20-30 sec in total in the desiccator to reduce the suffering. The gastrocnemius muscle was quickly dissected, and immediately homogenized in Qiagen lysis buffer for RNA isolation (Qiagen AB, Sollentuna, Sweden). Ethical approval was granted (approval no. C10-20) by the Ethics Committee of Hiroshima University (Higashi-Hiroshima, Japan).

Gastrocnemius muscle PLP. PLP levels in the gastrocnemius muscle were measured as previously described (19). Briefly, PLP from skeletal muscle was extracted using ice-cold 1 M perchloric acid. The samples were adjusted to pH 7.5 with 5 M potassium hydroxide. Samples were then, centrifuged at 12,000 x g at 4˚C for 10 min and the supernatant was collected. For the extraction of PLP, 30 μl of 0.1 M potassium cyanide was added to 400 μl supernatant and agitated at 50˚C for 3 h. The pH was readjusted to 3.5 with 1 M HCl. Then the samples were left to stand at 25˚C for 24 h. The resulting solution of PLP was mixed with deionized water. Prior to injection into an HPLC line, all of the solutions were passed through a 0.45 μm pore size membrane filter, and analyzed by a HPLC device with a fluorometric detector as previously described (22). For the determination of PLP, a fluorescence measurement was set at emission wavelength of 420 nm with an excitation wavelength of 320 nm.

DNA microarray analysis. Pooled RNAs from the gastrocnemius muscle of each group rats (n=8) were subjected to complementary RNA synthesis for a DNA microarray analysis. Cyanine-3 labelled cRNA was prepared from 100 ng RNA using the One-Color Low Input Quick Amp labelling kit (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. All procedures of hybridization, slide, and scanning were carried out according to the manufacturer's protocol (Agilent Technologies, Inc.). The data were analysed by GeneSpring software version 12.6.1 (Agilent Technologies, Inc.).

mRNA analysis. The Qiagen Midi kit (Qiagen AB) was used to isolate total RNA (500 and 1,200 ng/μl) from rat gastrocnemius muscle, which was subsequently prepared according to the manufacturers protocol. In the purification of total RNA with this kit, silica-gel membrane, spin-column technology was used to efficiently remove the genomic DNA. The reverse transcriptase reaction was carried out with 1 μg total RNA as a template to synthesize cDNA using ReverTra Ace (Toyobo, Co., Ltd., Osaka, Japan) and random hexamers (TaKaRa Bio, Inc., Otsu, Japan) in a final reaction volume of 20 μl, according to the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was performed with a StepOne™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the Thunderbird SYBR qPCR Mix (Toyobo, Co., Ltd.). For qPCR analysis, 10 μl SYBR-Green (containing DNA polymerase), forward and reverse primer (0.8 μM each), and 1,000 ng cDNA were

contained in a final reaction volume of 20 μl. All primers were purchased from Greiner Bio-One, Ltd. (Kremsmünster, Austria) and are displayed in Table I. The cycling parameters were as follows: Initial step at 90˚C for 10 sec, followed by 40 cycles at 90˚C for 5 sec, 60˚C for 10 sec and 72˚C for 10 sec. The mRNA level was normalized to that of GAPDH, and all reactions were performed at least in duplicate. Analysis of relative gene expression levels was performed using the following formula: $2^{-\Delta\Delta Cq}$ with $\Delta\Delta CT$ = Cq (target gene) - Cq (control) (23).

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Tukey-Kramer HSD test was used to compare means following one way analysis of variance. Data analysis was performed using Excel Statistics 2010 for Windows (Microsoft Corporation, Redmond, WA, USA). Some data underwent regression analysis and the correlation coefficient was calculated. P<0.05 was considered to indicate a statistically significant difference.

Results

Food intake, body weight, muscle weight and muscle PLP. Dietary manipulation had no significant effect on food intake, final body weight and weight of the gastrocnemius muscle from one leg (Table II). Muscle PLP concentrations in the 7 and 35 mg/kg PN HCl groups were significantly higher than those in the 1 mg/kg group (Table II). No significant difference was observed in muscle PLP concentrations between the 7 and 35 mg/kg PN HCl groups.

Effect of dietary vitamin B6 on the gene expressions in the gastrocnemius muscle of rats. Among the 19 myokines genes that were examined, 9 genes were significantly affected by dietary vitamin B6 (Table III; P<0.05). Among these genes, the expressions of IL-7, IL-8, protein acidic and rich in cysteine (SPARC), IL-6, growth differentiation factor (GDF)11, myonectin, LIF, apelin and retinoic acid receptor responder (tazarotene induced) 1 (RARRES1) were significantly higher in the 7 mg/kg PN HCl group (+107, +105, +92, +77, +75, +65, +62, +61 and +53%, respectively; P<0.05) compared with the 1 mg/kg PN HCl group. No significant differences were observed in levels of these genes between the 1 and 35 mg/kg PN HCl groups, with the exception of GDF11 and myonectin, whose expressions were significantly increased in the 35 mg/kg PN HCl group compared with the 1 mg/kg PN HCl group (Table III; P<0.05). The expressions of other myokines were not significantly affected by the dietary vitamin B6 levels (Table III).

Dietary vitamin B6 also had a significant effect on the expression of some genes associated with mitochondrial function and genes associated with Nrf2 in the gastrocnemius muscle of rats; among the 14 genes evaluated, the expressions of heme oxygenase (HO)-1, superoxide dismutase (SOD)2, glutathione peroxidase (GPX)1, Nrf2 and glutathione S-transferase (GST) were significantly elevated in the 7 mg/kg PN HCl group compared with the 1 mg/kg PN HCl group (Table IV; P<0.05). No significant differences in expressions of these genes were observed between the 1 and 35 mg/kg PN HCl groups. The expressions of all other genes evaluated were unaffected (Table IV).

Table I. Sequences of primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Direction	Sequence (5'-3')
GAPDH	F	TGACAACTCCCTCAAGATTGTC
	R	GGCATGGACTGTGGTCATGA
IL-7	F	TTTGGAATTCCTCCCCTGAT
	R	TCATCAGCACACTCCCAAAG
IL-8	F	GAAGATAGATTGCACCGA
	R	CATAGCCTCTCACACATTTC
SPARC	F	GGGCAGACCAATACCTCACTA
	R	CCGACCATTCCTTCCGTTG
IL-6	F	CCCAACTTCCAATGCTCTCCTA
	R	GCACACTGAGTTTGCCGAATA GACC
GDF11	F	AGCATCAAGTCGCAGATCCT
	R	CTTATGACCGTCTCGGTGGT
Myonectin	F	TGTTGTTGAAAGGTGCGGTA
	R	TCTCAAGCTCCTGGGTGACT
LIF	F	AAGTACCATGTGGGCCATGT
	R	GGACCACCGCACTAATGACT
Apelin	F	GGCTAGAAGAAGGCAACATGC
	R	CCGCTGTCTGCGAAATTTCCT
RARRES1	F	GCTGCACTTCCTCAACTTCC
	R	TGTGCTAAACACCAGGTCCA
OSM	F	GAACATCCAAGGGATCAGGA
	R	AACCCATGAAGCGATGGTAG
FGF21	F	GCCAACAACCAGATGGAACTC
	R	CTGGTACACATTGTATCCGTC CTT
GDNF	F	CAAAAGACTGAAAAGGTCACC AGAT
	R	GCTTGCCGGTTCCTCTCTCT
IL-15	F	CTTCTTAACTGAGGCTGG
	R	GCAACTGGGATGAAAGTC
LTBP2	F	AAGAACACTGCGCTCCTCAT
	R	TGGAAGCCAGTCTCACACAG
Decorin	F	TCGGATACATCCGCATCTCAGA
	R	GGCACTCTGAGGAGTTTGTTGT
CHI3L1	F	GGGCAGTGGATTTGGATG
	R	TGCAAGTGACCAGACTCCTG
Irisin	F	GACCTGGAGGAGGACACAGA
	R	CCCATCTCCTTCATGGTCAC
SERP4	F	CGCAGGATGAGTCAAGTCAG
	R	TGACCAGATGTCCACAAGGA
Metrn 1	F	TCGAGGATGTCACCCATGTA
	R	CCCTGGTCGTACTCCACACT
HO-1	F	CGACAGCATGTCCCAGGATT
	R	TCGCTCTATCTCCTCTTCCAGG
SOD2	F	GGCCAAGGGAGATGTTACAA
	R	GCTTGATAGCCTCCAGCAAC
GPX1	F	GCTGCTCATTGAGAATGTCG
	R	GAATCTCTTCATTCTTGCCATT
Nrf2	F	CAAGAGCAACAGATGAATGAG
	R	ACTTTAATCGTAGTCGGTGTAG
GST	F	CCCTGAGAACCAGAGTCAGC
	R	CTGCGGATTCCCTACACATT

Table I. Continued.

Gene	Direction	Sequence (5'-3')
Catalase	F	GAGGTCACCCACGATATTACCAGA
	R	AGAATTTCACTGCAAACCCACG
Keap1	F	GTGCATCGACTGGGTCAAATAC
	R	CTGGAAGATCTGCACCAGGTAG
Nrf1	F	TTACTCTGCTGTGGCTGATGG
	R	CCTCTGATGCTTGCGTCGTCT
PGC1a	F	CCTTTCTGAACTTGATGTGA
	R	ATGCTCTTTGCTTTATTGCT
Imp-11	F	AGACGAAGAACCACCCACAG
	R	GTCTCCATGAGGGACTGGAA
Trx2	F	CAGTTATGTGGCCCTGGAGT
	R	TCGGGAGTTTTCTGATGAGG
Nqo1	F	TCCGCCCCCAACTTCTG
	R	TCTGCGTGGGCCAATACA
UBE2E3	F	GGGAGTCATCTGCCTGGATA
	R	TCCTGTCGTGTTCTGCTCTG
GCLC	F	CTCTGCCTATGTGGTATTTG
	R	TTGCTTGTAGTCAGGATGG
Myogenin	F	GACCCTACAGGTGCCCACAA
	R	ACATATCCTCCACCGTGATGCT
MuRF1	F	GTGAAGTTGCCCCCTTACAA
	R	TGGAGATGCAATTGCTCAGT
Myf-6	F	CCGGGAGCGACAGCAGTGG
	R	AGCCGGTGCAGCAGGTCCT
MyoD1	F	GCGACAAGCCGATGACTTCTAT
	R	GGTCCAGGTCCTCAAAAAAGC
Myf-5	F	GAGCCAAGAGTAGCAGCCTTCG
	R	GTTCTTTCGGGACCAGACAGGG
Atrogin-1	F	CCTTCTCCAGGCCAGTAGGTG
	R	CGCGTCCGCGCTCTGTA
RPB3	F	CGATCCAGACAACGCACTTA
	R	AGACAGGGCTGAGAGGACAA
Sp1	F	TGAATGCTGCTCAACTGTCC
	R	CTCCACCTGCTGTCTCATCA
HSP60	F	AAATCCGGAGAGGTGTGATG
	R	CTTCAGGGGTTGTCACAGGT
HSP70	F	GTTGCATGTTCTTTGCGTTT
	R	TACACAGGGTGGCAGTGCT
HSP90	F	GGTGTCGGCTTCTACTCTGC
	R	CTGCTCATCATCGTTGTGCT
HSP27	F	CCTGGTGTCCTCTTCCCTGT
	R	TGGTGATCTCCGCTGATTGTG
Nrxn2	F	TCAACCTGTCCCTCAAGTCC
	R	GGTGTAATCCTCCTGCGTGT
Ntn4	F	GGCCTGGAAGATGATGTTGT
	R	TCTCTGACAAGGGCAGGACT

A number of genes listed in this table are not mentioned in the text, this is primarily because the discussion focused on the genes of interest with regard to the present study. IL, interleukin; F, forward; R, reverse; SPARC, secreted protein acidic and rich in cysteine; GDF, growth differentiation factor; LIF, leukemia inhibitory factor; RARRES1, retinoic acid receptor responder (tazarotene induced) 1; OSM, oncostatin M; FGF, fibroblast growth factor; GDNF, glial cell derived neurotrophic factor; LTBP2, latent-transforming growth factor beta-binding protein 2; CHI3l1, chitinase-3-like protein 1; SERP4, septin 4; Metrn, meteorin; HO, heme oxygenase; SOD, superoxide dismutase; GPX, glutathione peroxidase; Nrf, nuclear respiratory factor; GST, glutathione S-transferase; Keap1; kelch-like ECH associated protein 1; PGC1a, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; Imp-11, importin 11; Trx2, thioredoxin reductase-2; Nqo1, NADPH quinone oxidoreductase 1; UBE2E3, ubiquitin-conjugation enzyme E2 E3; GCLC, glutamate-cysteine ligase catalytic subunit; MuRf1, muscle ring-finger protein-1; Myf, myogenic factor; MyoD1, myogenic differentiation 1; RPB3, RNA polymerase B3; Sp1, specificity protein; HSP, heat shock protein; Nrxn, neurexin; Ntn, netrin.

Table I. Continued.

Table II. Food intake, final body weight, and gastrocnemius weight.

Variable	1 mg/kg PN HCl	7 mg/kg PN HCl	35 mg/kg PN HCl
Food intake (g/6 wk)	731±10	787±15	772±24
Final body wt (g)	412±9	444±9	437±13
Gastrocnemious muscle (g)	2.4±0.1	2.6±0.1	2.7±0.1
PLP in gastrocnemius muscle (nmol/g)	4.5±0.4	10.8±1.5[a]	10.2±1.0[a]

Values are presented as the mean ± standard error of the mean. n=8, [a]P<0.05 vs. 1 mg/kg group. PLP, pyridoxal 5'-phosphate; PM, pyridoxine.

The effect of dietary vitamin B6 on the expression of genes associated with myogenesis and sarcopenia was also evaluated. Among the 8 genes analysed, only myogenin expression was significantly affected by dietary vitamin B6. Compared with the 1 mg/kg PN HCl group, there was a 52% elevation in myogenin expression in the 7 mg/kg PN HCl. Group (Table V; P<0.05); however, there was no significant difference between the 1 and 35 mg/kg PN HCl groups (Table V).

Of the 4 HSP genes evaluated, a significant increase was observed in the gene expression of HSP60 in the 7 mg/kg PN HCl group compared with the 1 mg/kg PN HCl group (+66%; P<0.05; Table VI). However, no significant differences were observed between levels of HSP70, HSP90 or HSP27 among any of the groups (Table VI). Furthermore, there was no significant difference in HSP60 expression between the 1 and 35 mg/kg PN HCl groups.

The results of the microarray demonstrated that the expressions of neurexin (Nrxn)2 and netrin (Ntn)4 were also altered by dietary supplemental vitamin B6 (data not shown). RT-qPCR results showed that the 7 mg/kg PN HCl diet elevated the expression of Nrxn2 and Ntn4 by 85 and 82%, respectively (Table VII; P<0.05) compared with the 1 mg/kg PN HCl diet. No significant differences were observed in Nrxn2 or Ntn4 levels between the 1 and 35 mg/kg PN HCl groups.

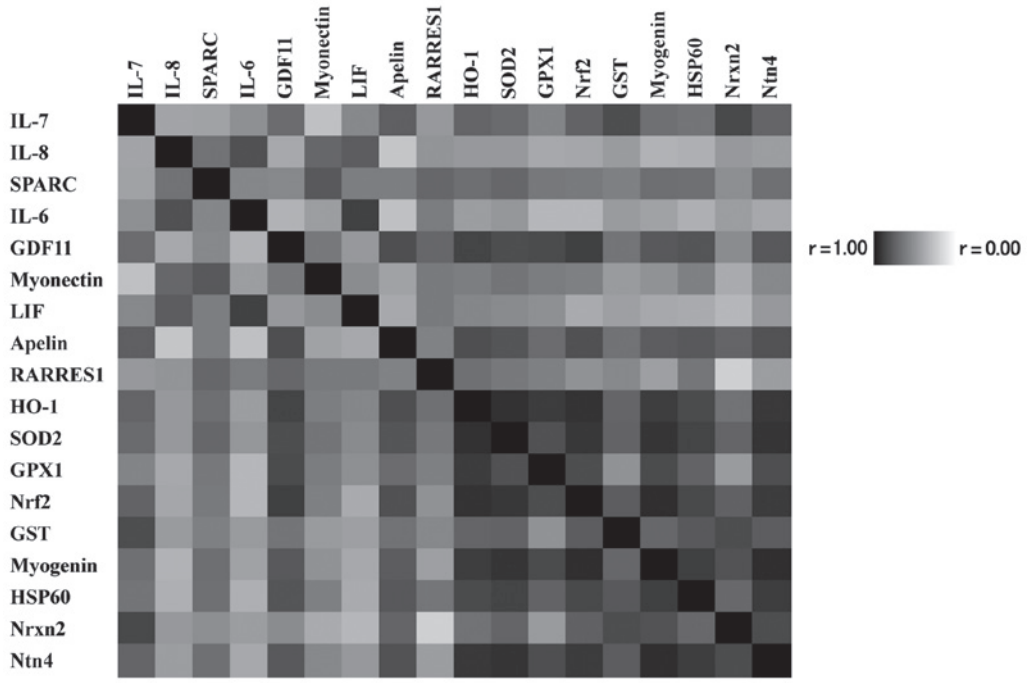

Figure 1. Correlation matrix among the gene expressions altered by dietary level of vitamin B6. The dark colour scheme corresponds to correlation strength as shown by the deep colour bar. IL, interleukin; SPARC, secreted protein acidic and rich in cysteine; GDF, growth differentiation factor; LIF, leukemia inhibitory factor; RARRES1, retinoic acid receptor responder (tazarotene induced) 1; HO, heme oxygenase; SOD, superoxide dismutase; GPX, glutathione peroxidase; Nrf, nuclear respiratory factor; GST, glutathione S-transferase; HSP, heat shock protein; Nrxn, neurexin; Ntn, netrin.

In the present study, it was investigated whether there was any correlation between the genes that were affected by the dietary levels of vitamin B6. Significant correlations were observed between the expression of Nrf2 and the following genes (all of which were significantly affected by dietary vitamin B6): IL-7, SPARC, GDF11, myonectin, apelin, RARRES1, HO-1, SOD2, GPX1, GST, myogenin, HSP60, Nrxn2 and Ntn4 ($P<0.05$; Fig. 1). Furthermore, HSP60 expression was found to be significantly correlated with the expressions of IL-7, SPARC, GDF11, myonectin, apelin, RARRES1, HO-1, SOD2, GPX1, Nrf2, GST, myogenin, HSP60, Nrxn2 and Ntn4 ($P<0.05$; Fig. 1). Myogenin expression was demonstrated to be significantly correlated with IL-7, SPARC, GDF11, myonectin, apelin, HO-1, SOD2, GPX1, GST, myogenin, HSP60, Nrxn2 and Ntn4 expressions ($P<0.05$; Fig. 1).

Discussion

To the best of our knowledge, the present study is the first to evaluate the effect of dietary vitamin B6 level on the expression of genes in the gastrocnemius muscle of rats. The results indicated that the recommended levels of dietary vitamin B6 (7 mg/kg PN HCl) significantly elevated the mRNA of several myokines, such as IL-7, IL-8, SPARC, IL-6, GDF11, myonectin, LIF, apelin and RARRES1, compared with a low vitamin B6 diet (1 mg/kg PN HCl; marginal vitamin B6 deficiency level). Compared with the 1 mg/kg group, the 7 mg/kg PN HCl group also exhibited higher expressions of antioxidant genes, such as HO-1, SOD2, GPX1 and GST, and their master transcription factor, Nrf2. Furthermore, compared with the low vitamin B6 diet, the recommended level of dietary vitamin B6 increased the expression of myogenin, which is essential for the development of functional skeletal muscle, and HSP60, a mitochondrial protein which is a key factor in muscle cytoprotection (24). Similar results were observed in the expressions of Nxrn2, a membrane protein important for motor neuron neurotransmitters, and Ntn4, a secreted molecule involved in angiogenesis. Notably, in the majority of genes that were affected by the dietary level of vitamin B6, there were no significant differences in gene expressions between the 1 and 35 mg/kg PN HCl groups, whereas there was a significant difference in the levels of PLP between the two groups. These results suggest that the recommended level of vitamin B6 (7 mg/kg PN HCl) may be important for the expression of genes that are beneficial for skeletal muscles, whereas excessive levels of vitamin B6 (35 mg PN HCl/kg) may not facilitate this expression.

In the present study, it is notable that almost all of the genes whose expressions were elevated by the recommended level of dietary vitamin B6 were exercise-induced genes. Myokines are released during muscle contraction (7) and may be partly responsible for the beneficial effects of exercise on the reduction of risk for chronic diseases including diabetes, cardiovascular disease and cancers (7-9,25,26). A previous study reported that exercise upregulated both antioxidant enzymes and the antioxidant defence system that is associated with Nrf2 (27). Exercise induces the expression of myogenin in human skeletal muscle (28), and HSP60 is upregulated in cardiac and skeletal muscles following endurance training (29). Furthermore, Nxrn2 may be associated with motor neuron function, as the knockdown of Nrxn2 results in a significant decrease in motor axon excitability (30). Ntn4 is a putative angiogenic factor, and exercise has previously been demonstrated to induce angiogenesis (31). Further

Table III. Effect of dietary level of vitamin B6 on expression of myokine genes in gastrocnemius muscle of rats.

	Relative gene expression		
Gene	1 mg/kg PN HCl	7 mg/kg PN HCl	35 mg/kg PN HCl
IL-7	1.00±0.09	2.07±0.28[b]	1.33±0.16[c]
IL-8	1.00±0.04[a]	2.05±0.28[b]	1.61±0.16
SPARC	1.00±0.11	1.92±0.20[b]	1.39±0.07[c,d]
IL-6	1.00±0.10	1.77±0.22[b]	1.32±0.20
GDF11	1.00±0.04	1.75±0.15[b]	1.56±0.18[b]
Myonectin	1.00±0.08	1.65±0.18[b]	1.46±0.13[b]
LIF	1.00±0.09	1.62±0.13[b]	1.23±0.03
Apelin	1.00±0.09	1.61±0.17[b]	1.33±0.11
RARRES1	1.00±0.11	1.53±0.10[b]	1.33±0.08
OSM	1.00±0.12	1.66±0.33	1.07±0.16
FGF21	1.00±0.13	1.54±0.27	1.16±0.17
GDNF	1.00±0.10	1.53±0.24	1.18±0.18
IL-15	1.00±0.07	1.22±0.15	1.30±0.20
LTBP2	1.00±0.11	1.19±0.10	0.89±0.08
Decorin	1.00±0.07	1.17±0.09	1.02±0.04
CHI3L1	1.00±0.09	1.14±0.10	0.94±0.08
Irisin	1.00±0.10	1.10±0.11	0.94±0.08
SERP4	1.00±0.11	1.02±0.09	0.91±0.08
Metrn1	1.00±0.10	1.00±0.06	0.96±0.07

Values are presented as the mean ± standard error of the mean. n=6-8. [a]Indicate studies using 6 animals and others are the data of 8 animals. [b]P<0.05 vs. 1 mg/kg group, [c]P<0.05 vs. 7 mg/kg group. [d]Indicate studies using 7 animals and others are the data of 8 animals. PN, pyridoxine; IL, interleukin; SPARC, secreted protein acidic and rich in cysteine; GDF, growth differentiation factor; LIF, leukemia inhibitory factor; RARRES1, retinoic acid receptor responder (tazarotene induced) 1; OSM, oncostatin M; FGF, fibroblast growth factor; GDNF, glial cell derived neurotrophic factor; LTBP2, latent-transforming growth factor beta-binding protein 2; CHI3L1, chitinase-3-like protein 1; SERP4, septin 4; Metrn, meteorin.

investigation is required to determine whether the beneficial effects of dietary vitamin B6 in skeletal muscle are due to the altered expression of factors similar to that observed during physical exercise.

The expressions of HO-1, SOD2, GPX1 and GST (antioxidant genes that are also Nrf2-regulated factors) were increased in the 7 mg/kg PN HCl group compared with the 1 mg/kg PN HCl group. It has previously been reported that vitamin B6 may have antioxidant functions in the body (32), and it is therefore important to investigate the possibility that the antioxidant function of vitamin B6 may occur in part via the modulation of the Nrf2 signalling pathway. The results of the present study suggested a possible physiological role of vitamin B6 as a regulator of the Nrf2 signalling pathway. However, further investigations are necessary to investigate this possibility.

It has been reported that myokines may possess some anti-disease functions. RARRES1 may serve a role in the

Table IV. Effect of dietary level of vitamin B6 on expression of genes related to mitochondrial function and Nrf2 in gastrocnemius muscle of rats.

	Relative gene expression		
Gene	1 mg/kg PN HCl	7 mg/kg PN HCl	35 mg/kg PN HCl
HO-1	1.00±0.05[a]	1.71±0.24[b]	1.18±0.13
SOD2	1.00±0.06	1.56±0.17[b]	1.18±0.14
GPX1	1.00±0.12	1.56±0.20[b]	0.96±0.14
Nrf2	1.00±0.06	1.45±0.11[b]	1.19±0.14
GST	1.00±0.06	1.28±0.07[b]	1.09±0.06[a]
Catalase	1.00±0.05	1.45±0.18	1.17±0.18[a]
Keap1	1.00±0.03	1.42±0.13	1.23±0.15
Nrf1	1.00±0.06	1.38±0.19	1.09±0.19
PGC1a	1.00±0.05	1.12±0.11	0.97±0.04
Imp-11	1.00±0.06	1.10±0.01	1.05±0.06
Trx	1.00±0.09	1.10±0.09	1.04±0.05
Nqo1	1.00±0.10[a]	1.07±0.12	0.85±0.07
UBE2E3	1.00±0.09	1.03±0.04	1.10±0.12
GCLC	1.00±0.09[a]	1.03±0.07	0.90±0.04[a]

Values are presented as the mean ± standard error of the mean. n=7-8; [a]indicates studies using 7 animals and others are the data of 8 animals. [b]P<0.05 vs. 1 mg/kg group. Nrf, nuclear respiratory factor; PN, pyridoxine; HO, heme oxygenase; SOD, superoxide dismutase; GPX, glutathione peroxidase; GST, glutathione S-transferase; Keap1; kelch-like ECH associated protein 1; Nrf1, nuclear respiratory factor 1; PGC1a, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; Imp-11, importin 11; Trx, thioredoxin reductase; Nqo1, NADPH quinone oxidoreductase 1; UBE2E3, ubiquitin-conjugation enzyme E2 E3; GCLC, glutamate-cysteine ligase catalytic subunit.

Table V. Effect of dietary level of vitamin B6 on expression of genes related to myogenesis and sarcopenia in gastrocnemius muscle of rats.

	Relative gene expression		
Gene	1 mg/kg PN HCl	7 mg/kg PN HCl	35 mg/kg PN HCl
Myogenin	1.00±0.06	1.52±0.20[a]	0.82±0.05[b]
MuRF1	1.00±0.07	1.35±0.17	1.21±0.16
Myf-6	1.00±0.09	1.33±0.32	1.06±0.33
MyoD1	1.00±0.12	1.27±0.18	1.09±0.19
Myf-5	1.00±0.01	1.24±0.11	1.06±0.06
Atrogin-1	1.00±0.15	1.24±0.18	1.46±0.10
RPB3	1.00±0.06	1.08±0.03	0.95±0.02
Sp1	1.00±0.10	1.08±0.06	0.93±0.07

Values are presented as the mean ± standard error of the mean. n=6 or 8. [a]P<0.05 vs. 1 mg/kg group. [b]Indicates studies using 6 animals and others are the data of 8 animals. PN, pyridoxine; MuRF1, muscle ring-finger protein-1; Myf, myogenic factor; MyoD1, myogenic differentiation 1; RPB3, RNA polymerase B3; Sp1, specificity protein.

Table VI. Effect of dietary level of vitamin B6 on gene expression of HSPs in gastrocnemius muscle of rats.

	Relative gene expression		
Gene	1 mg/kg PN HCl	7 mg/kg PN HCl	35 mg/kg PN HCl
HSP60	1.00±0.09	1.66±0.19[b]	1.20±0.11
HSP70	1.00±0.07[a]	2.00±0.48	1.23±0.28[a]
HSP90	1.00±0.07	1.43±0.22	1.37±0.21
HSP27	1.00±0.07	1.07±0.11	0.83±0.16

Values are presented as the mean ± standard error of the mean. n=7-8; [a]indicates studies using 7 animals and others are the data of 8 animals. [b]P<0.05 vs. 1 mg/kg group, HSP, heat shock protein; PN, pyridoxine.

Table VII. Effect of dietary level of vitamin B6 on gene expression of Nrxn2 and Ntn4 in gastrocnemius muscle of rats.

	Relative gene expression		
Gene	1 mg/kg PN HCl	7 mg/kg PN HCl	35 mg/kg PN HCl
Nrxn2	1.00±0.08	1.85±0.23[a]	1.02±0.09[b,c]
Ntn4	1.00±0.08	1.82±0.24[a]	1.14±0.18[c]

Values are presented as the mean ± standard error of the mean. n=7-8. [a]P<0.05 vs. 1 mg/kg group. [b]Indicates studies using 7 animals and others are the data of 8 animals. [c]P<0.05 vs. 7 mg/kg group. Nrxn, neurexin; Ntn, netrin; PN, pyridoxine.

pathogenesis of colorectal adenocarcinoma, as it has been reported that the downregulation of RARRES1 is associated with disease progression (33). Another study reported that tumour growth in the colon was increased in SPARC-null mice, suggesting that SPARC may possess anti-tumour properties (34). A previous study by the present authors indicated that supplementing a low vitamin B6 diet with dietary vitamin B6 lowered colon tumorigenesis in mice (35). In the present study, the expressions of both RARRES1 and SPARC were increased by dietary supplemental vitamin B6 (7 mg/kg PN HC) added to a low vitamin B6 diet. However, further investigations are necessary to examine the involvement of these myokines and the underlying mechanisms responsible for the anti-tumour effects of dietary vitamin B6.

In the present study, correlations among the gene expressions affected by dietary vitamin B6 were also investigated. Although Nrf2 has previously been reported as a potent activator of IL-6 gene transcription *in vivo* (36), the results of the present study did not find any significant correlation between Nrf2 and IL-6. Intriguingly, IL-7, SPARC, GDF11, myonectin, apelin and RARRESS1 expressions were positively correlated with Nrf2. It is therefore necessary to investigate whether the alterations in myokine expressions induced by dietary vitamin B6 is associated with altered Nrf2 expression. As myogenin is the major regulator of muscle regeneration, the correlation of gene expressions between myokines and myogenin was also evaluated. The results demonstrated that the expression of myogenin was significantly correlated with the expression of IL-7, SPARC, GDF11, myonectin and apelin. Furthermore a significant correlation was found between HSP60 and IL-7, SPARC, GDF11, myonectin, apelin and RARRES1. Further study is required to evaluate the effects of Nrf2, HSP60 and myogenin on the expressions of other myokines in muscle cell cultures.

In conclusion, the present study demonstrated that the recommended level of dietary vitamin B6 elevated the expression of several myokine genes, Nrf2-regulated genes, myogenin, HSP60, Nrxn2 and Ntn4 compared with a low vitamin B6 diet. However, excessive levels of dietary vitamin B6 did not induce the same response as 7 mg/kg PN HCl. This suggests that the recommended level of dietary vitamin B6 is critical for the expression of these genes in the gastrocnemius muscle of rats, and these factors may be beneficial for muscle health. Notably, the elevations in gene expression induced by supplemental vitamin B6 were similar to responses to exercise. Accordingly, the findings of the present study provide a novel insight into the physiological role of vitamin B6 in skeletal muscles as an exercise-like factor. Further study is required to fully understand the role of vitamin B6 in skeletal muscles.

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