Doctoral Thesis

Study on the influence of dietary vitamin B6 on heart and skeletal muscles

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Abbreviations

PLP	pyridoxal 5′-phosphate
PN	pyridoxine
UPLC-MS/MS	ultra-performance liquid chromatography coupled with tandem mass spectrometry
HPLC	high performance liquid chromatography
ANOVA	analysis of variance

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Chapter 1 Introduction

I. Vitamin B6, its metabolism, and source

Vitamin B6 is an essential water-soluble vitamin required for normal growth and development in mammal (Tryfiates, 1980). Vitamin B6 is a collective term for metabolically and functionally compounds including pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM), and their phosphorylated forms pyridoxal 5′-phosphate (PLP), pyridoxine 5′-phosphate (PNP), and pyridoxamine 5′-phosphate (PMP) (Combs, 1992). PLP, the principal active coenzyme form of vitamin B6, is involved in almost 100 enzymatic reactions (Mackey et al., 2006).

After intestinal absorption, most of vitamin B6 is transported to liver and taken up by facilitated diffusion (Middleton, 1977). After being phosphorylated by pyridoxal kinase, PNP and PMP are converted to PLP by a flavin-dependent oxidase (Kazarinoff & McCormick, 1973). PLP is bound by apo-enzymes, or released into plasma and carried to tissues as a tight complex with albumin (Dempsey & Christensen, 1964). Upon hydrolysis by alkaline phosphatase, free PL is taken up by cells and re-phosphorylated (for muscle as an example, Buss et al., 1980).

Major food sources of vitamin B6 include fortified cereals, meat, fish, poultry, starchy vegetables, and some fruits (e.g., bananas and avocado, Mackey et al., 2006). Vitamin B6 is obtained from various dietary sources, although bioavailability can vary considerably (Gregory & Kirk, 1981). Foods contain varying proportions of the three vitaminic forms PN, PM, and PL. In animal-derived foods, it is also present largely as PLP and in greatest amounts in meat (including poultry and fish) because, in animals, the largest store of PLP is present in muscle, associated with glycogen phosphorylase. Smaller amounts of PMP are also present in animal-derived foods. In plant-derived foods, vitamin B6 is present mainly as PN, PNP, and PN glucoside. Although vitamin B6 is widely distributed in various foods, there is evidence that many adults are not obtaining adequate amounts of this nutrient from their diet, suggesting that the nutritional importance of vitamin B6 is under-recognized (Moshfegh et al., 2005).

In the studies on the effect of dietary vitamin B6, PN is the most used vitamer (Hansen et al., 2001). Several studies have been reported that higher consumption of vitamin B6 elevates the concentration of PLP in blood and tissues (Spinneker et al., 2007). Blood vitamin B6 status has been also substantially studied as an indicator of nutritional status of vitamin B6.

II. Vitamin B6, its physiological function, and its deficiency

1. Vitamin B6 and amino acids metabolism

One function of vitamin B6 is its role in the 1-carbon metabolic pathway, which involves the transfer of 1-carbon groups for DNA synthesis and DNA methylation. Deficiency of vitamin B6 has been associated with considerably impaired 1-carbon metabolism in animal (Martinez et al., 2000). Inadequate vitamin B6 status has been associated with cardiovascular disease risk (Cheng et al., 2008; Page et al., 2009; Rimm et al., 1998; Robinson et al., 1998), possibly due to derangement of 1-carbon metabolism. McCully (1969) first noted that patients with homocysteinemia show a high incidence of atherosclerosis at a very early age. Homocysteine is an important intermediate in 1-carbon metabolism and accumulates in vitamin B6 deficiencies (Hustad et al., 2007). However, specific metabolic processes that involved in mediating this disease risk remains unclear.

Severe vitamin B6 deficiency has substantial effects on amino acid metabolism as reflected by changes in the concentration of several amino acids in plasma, tissue, and urine (Okada & Suzuki, 1974; Park & Linkswiler, 1971; Swendseid et al., 1964). Furthermore, the involvement of PLP in tryptophan catabolism represents another important role of vitamin B6 in amino acid metabolism by regulating tryptophan turnover and providing NAD synthesis (Wolf, 1974). However, there is limited studies reported the effect of dietary vitamin B6 on amino acid profiles in the tissues.

2. Vitamin B6 and muscle phosphorylase

PLP also acts as a cofactor for glycogen phosphorylase, although the mechanism is different from that seen in reactions involving amino acids (Takagi et al., 1982). The glycogen phosphorylase activities in the gastrocnemius muscle and heart of vitamin B6-deficient rats were significantly decreased (Okada et al., 1997). In addition, rats fed high levels of vitamin B6 exhibit elevated concentration of muscle phosphorylase (Black et al., 1977).

3. Other roles of vitamin B6

Other physiological roles of vitamin B6 include modulation of steroid receptor interaction (Tully et al., 1994) and regulation of immune function. Some of the important PLP-dependent enzymes in relation to vitamin B6 deficiency are known or suspected to be involved in the metabolism of inborn errors and/or seizures (Clayton, 2006). Furthermore, metabolism of the following

neurotransmitters could be affected by PLP deficiency: dopamine, serotonin (5-hydroxytryptamine), glycine, D-serine, glutamate, γ-aminobutyrate (GABA), and histamine (Clayton, 2006).

III. Vitamin B6 and diseases

Beyond the role as a coenzyme, there is accumulating evidence for the preventive roles of vitamin B6 in certain diseases, including colon cancer (Komatsu et al., 2001; Larsson et al., 2010), heart disease (Friso et al., 2004), and brain diseases (Ohtahara et al., 2011). Vitamin B6 deficiency is also reported to be associated with stroke (Kelly et al., 2003) as well as an elevated risk of Alzheimer's disease (Miller et al., 2002). Furthermore, a case-study demonstrated that vitamin B6 supplementation is beneficial for McArdle disease, a glycogenetic myopathy (Izumi et al., 2010). In the present report, I focused on the anti-diseases effect of vitamin B6 in heart and skeletal muscles.

1. Vitamin B6 and its antioxidant effect

Apart from its function as a cofactor, antioxidant activities of vitamin B6 are reported (Ehrenshaft et al., 1999; Stocker et al., 2003; Chen & Xiong, 2005). Furthermore, vitamin B6 is reported to be functioning as an antioxidant scavenger itself (Spinneker et al., 2007). Consistently, in vitro studies demonstrate vitamin B6 itself acts as a radical scavenger and suppresses the lipopolysaccharide (LPS)-induced expressions of inflammatory mediators in macrophages (Kannan & Jain, 2004; Yanaka et al., 2005). Furthermore, there is some evidence that in some situations B6 vitamers can function as antioxidants, quenching singlet oxygen at a rate comparable to vitamins C and E (Ehrenshaft et al., 1999; Bilski et al., 2000).

Previous studies suggest such anti-disease activities of vitamin B6 are mediated via decreased oxidative stress and inflammation (Komatsu et al., 2002; Sakakeeny et al., 2012). Furthermore, Ullegaddi et al (2004) reported that vitamin B6 supplementation may have antioxidant effect in stroke disease independent of a homocysteine-lowering effect. These studies therefore provide evidence that vitamin B6 play important role as an antioxidant acts against development of lifestyle diseases.

2. Vitamin B6 and heart

Several epidemiological studies have shown the positive benefits of vitamin B6 to cardiovascular health (Martinez et al., 2000). Friso et al (2004) indicates vitamin B6 intake reduces the risks of coronary heart disease and atherosclerosis, with the anti-inflammatory effect of vitamin

B6 is considered to be at least partially responsible for its protective effect against these diseases. A cross-sectional study found that higher levels of PLP, were linked to lower levels of C-reactive protein and 8-hydroxy-2'-deoxyguanosine, both of which are related to heart disease risk (Shen et al., 2010). It is also reported that vitamin B6 exerts an anti-ischemic effect in the heart by blocking purinergic receptors (Dhalla et al., 2013).

Vitamin B6 deficiency is associated with elevation in blood homocysteine, a risk factor for cardiovascular disease, by impairing homocysteine metabolism (Friso et al., 2004 and Martinez et al., 2000). Another study suggested that the oxidative stress caused by a low level of vitamin B6 accelerates the development of homocysteine-induced atherosclerosis in rats (Endo et al., 2006). Although some studies reported that there was inverse association between vitamin B6 and cardiovascular disease, there is almost no study showing clear mechanism between vitamin B6, homocysteine, and atherosclerosis in rat experiments. Additional research is needed to further characterize the effect of vitamin B6 on cardiovascular disease.

3. Vitamin B6, skeletal muscle, and exercise

Skeletal muscle contains at least 80% of the vitamin B6 pool in the body as PLP bound to glycogen phosphorylase. This information led to the studies on the effect of vitamin B6 status on exercise. Vitamin B6 is reported to enhance the endurance of skeletal muscles (Richardson & Chenman, 1981). Vitamin B6 status and muscle performance may be linked in McArdle's disease and, there is potential for enhancement of performance by vitamin B6 supplementation (Phoenix et al., 1998). McArdle's disease is a rare hereditary metabolic myopathy caused by a deficiency of muscle glycogen phosphorylase. Patients are characterized by exercise intolerance, associated with rapid fatigue and onset of muscle pain, due to their inability to mobilize glycogen in response to sustained muscular activity. Continued muscle activity is therefore highly dependent on alternative sources of energy, such as amino acids and fatty acids (Felig & Wahren, 1975). These studies suggest ergogenic effect of vitamin B6 in skeletal muscle.

Leklem & Shultz (1983) for the first time reported that exercise in the form of long distance running dramatically alters plasma levels of PLP. Belko (1987) reported that exercise acutely affected vitamin B6 metabolism. On the other hand, there is a report showed that supplemental vitamin B6 can alter plasma fuel substrates during exercise in women (Manore & Leklem, 1988). However, studies on the relationship among vitamin B6, skeletal muscle, and exercise are still limited.

IV. Carnosine, anserine, and myokines

1. Carnosine and anserine

Carnosine (β-alanyl-L-histidine) is a cytoplasmic dipeptide synthesized from β-alanine and histidine, and is found at highest concentrations in the skeletal muscle. Along with its methylated analogue, anserine (β-alanyl-N-methyl-L-histidine), recent studies reported an understanding of their role as an intracellular pH buffer that is important for the athletic population with its potential to increase high-intensity exercise performance and capacity. Power athletes are reported to have higher carnosine levels than untrained individuals and endurance athletes (Parkhouse et al., 1985). It is reported there are numerous determinants of the muscle carnosine concentration, including species, gender, age, muscle fiber type, diet, supplementation, exercise and training (Harris et al., 2012). Furthermore, several enzymes related to the synthesis of β-alanine, carnosine precursor, is reported as PLP-dependent enzymes (Ladeuix et al., 2014; Van den Berg et al., 1984). Thus it is of interest to investigate the effect of vitamin B6 on the carnosine pathway.

2. Myokines

In line with the acceptance of adipose tissue as an endocrine organ, path-breaking work during the last decade demonstrated that skeletal muscle is an active endocrine organ releasing myokines. They are released when muscle contraction is occurred (Raschke & Eckel, 2013), and might be in part responsible for the beneficial effect of exercise (Pedersen, 2009; Pedersen, 2011a; Pedersen & Febbraio 2012). These myokines are considered to communicate with cells in an autocrine/paracrine manner, locally within the muscles, or in an endocrine fashion to distant tissues (Raschke & Eckel, 2013). It is assumed that contraction-regulated myokines play a pivotal role in the communication between muscle and other tissues such as adipose tissue, liver, and pancreatic cells (Pedersen, 2009; Pedersen, 2011b; Ellingsgaard et al., 2011).

Recently, there is accumulating evidence for the prevalence of inadequate daily intake of vitamin B6 (marginal vitamin B6 deficiency) in USA and Japan (Morris et al., 2008; Murakami et al., 2008). Little is known about the influence of vitamin B6 deficiency on the pattern of amino acids and related metabolites in rats, especially during marginal deficiency. In this study, I focused on the effect of dietary vitamin B6 on the amino acids and related metabolites in heart and skeletal muscle of rats.

Firstly, I investigated the responses of free amino acids and related metabolites in the blood and tissues of rats to recommended- and high-level vitamin B6 diets (7 and 35 mg PN HCl/kg,

respectively) compared to a marginal vitamin B6-deficient diet (1 mg PN HCl/kg). According to the preliminary study using amino acid analyzer, I found the concentrations of several free amino acids and related metabolites were modulated by supplemental dietary vitamin B6. In chapter 2, I focused on the effect of dietary vitamin B6 on the concentrations of carnosine and anserine in heart of male rats. Since it is reported that the concentrations of carnosine and anserine are highest in the skeletal muscle compared to other tissues (Harris et al., 2012), in chapter 3, I explored the effect of dietary vitamin B6 on the concentrations of carnosine, anserine, and β-alanine, the carnosine precursor, in skeletal muscles of male and female rats, and also examined in human samples. In chapter 3, the result showed that carnosine and PLP concentrations in skeletal muscles were markedly elevated by dietary supplemental vitamin B6. It is reported that both blood carnosine and PLP concentrations are elevated by exercise. Dietary carnosine and vitamin B6 are reported to have significant impact on the fatigue caused by exercise (Richardson & Chenman, 1981). Thus, in chapter 4, I hypothesized that dietary supplemental vitamin B6 has a significant impact on skeletal muscles. To examine this possibility, I investigated the effect of vitamin B6 on the expressions of genes related to exercise in gastrocnemius muscle of male rats, such as myokines and others.

Chapter 2 Effect of dietary vitamin B6 on the level of histidine dipeptides in heart

I. Brief Introduction

Increasing evidence indicates vitamin B6 intake reduces the risks of coronary heart disease and atherosclerosis (Friso et al., 2004). Furthermore, vitamin B6 exerts an anti-ischemic effect in the heart by blocking purinergic receptors (Dhalla et al., 2013). However, the role of vitamin B6 in the heart per se remains still obscure.

PLP, the active form of vitamin B6, acts as a cofactor for several enzymes involved in amino acid metabolisms. Some reports indicate vitamin B6 deficiency modulates free amino acids levels in blood and tissues (Swendseid et al., 1964; Park & Linkswiler, 1971; Lamers et al., 2009). Furthermore, functional amino acids such as citrulline and carnosine are very promising for the prevention and treatment of cardiovascular disorders (Wu, 2013). However, there is no information about the effects of dietary vitamin B6 intake on amino acid metabolisms in the heart.

My preliminary study using amino acid analyzer showed that dietary vitamin B6 supplementation (7 and 35 mg PN HCl/kg) compared to a marginal vitamin B6-deficient diet (1 mg PN HCl/kg) caused significantly higher concentration of carnosine in the heart of male rats. To confirm these results, ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) was used to analyze this dipeptide. Carnosine is known to act as antioxidant, pH-buffering, anti-glycation, and ergogenic factors (Boldyrev et al., 2013; Caruso et al., 2012). Furthermore, carnosine is reported to exert anti-inflammatory and anti-ischemic effects on the heart (Fleisher-Berkovich et al., 2009; Stvolinsky & Dobrota, 2000). The objective of this study was to explore the effect of dietary vitamin B6 on the concentrations of carnosine and its methylated analogue, anserine, in heart of male rats.

II. Materials and methods

1. Animal and diets

Male Sprague–Dawley rats (4 wk old, Charles River Japan, Hino, Japan) were maintained in accordance with the Guide for the Care and Use of Laboratory Animals established by Hiroshima University. Rats were housed in metal cages in a temperature controlled room (24 ± 1°C) and a 12-h light/dark cycle (lights on, 0800–2000 h). The basal diet was composed of the following components (g/kg diet): α-cornstarch, 302; casein, 200; sucrose, 200; corn oil, 200; cellulose, 50; AIN-93G mineral mixture (Reeves et al., 1993), 35; AIN-93 vitamin mixture (PN free) (Reeves et al., 1993), 10; and L-cystine, 3. Pyridoxine (PN) HCl (Nacalai Tesque, Kyoto, Japan) was supplemented to the basal diet

at the levels of 1, 7, or 35 mg/kg diets. The level of PN HCl/kg diet recommended in the AIN-93 diet is 7 mg/kg (Reeves et al., 1993). Meanwhile, 1 mg PN HCl/kg diet is reported to be the minimum level required for preventing growth depression caused by vitamin B6 deficiency (Coburn, 1994). After being fed a commercial non-purified diet (MF, Oriental Yeast, Tokyo, Japan) for one week, 24 rats (average, 117 g) were divided randomly into 3 groups receiving 1, 7, or 35 mg PN HCl/kg diet (n = 8/group) for 6 weeks. The animals were sacrificed by decapitation under diethyl ether anesthesia. Serum was collected and stored at −60°C. Heart was quickly dissected, frozen in liquid nitrogen, and immediately stored at −80°C.

2. Analysis of PLP in serum and heart by HPLC

The analysis of PLP was conducted by high performance liquid chromatography (HPLC) described elsewhere (Tsuge, 1997). Vitamin B6 from serum and heart were extracted using 3 M perchloric acid (PCA) and measured by HPLC with a fluorometric detector. PLP was converted to pyridoxic acid 5′-phosphate and measured. A 4.6mm (ID) × 250 mm (L) column packed with 10μm pore size TSK-gel ODS-120A (Tosoh) was used. The optimum mobile phases consist of the appropriate concentration of mixtures of 1%, v/v acetonitrile (CH_3CN), 0.1 M sodium perchlorate ($NaClO_4$), and 0.1 M monopotassium phosphate (KH_2PO_4). A fluorescence measurement was set at an emission wavelength of 420 nm with an excitation wavelength of 320 nm. The flow rate for the analysis was set to 0.5 ml/min. Prior to injection into an HPLC line, all of the solutions were passed through a 0.45μm pore size membrane filter, and 200μl was analyzed by HPLC.

3. Analysis of free amino acids and related metabolites in heart by amino acid analyzer

To measure the concentrations of amino acids and related metabolites, heart was treated with cold 3% sulfosalicylic acid to precipitate the proteins. After centrifugation at 3,000 × rpm at 4°C for 20 min, the supernatants were collected and filtered through a 0.22μm pore size membrane filter and immediately stored at −80°C until analysis. Amino acids and related metabolites were quantified by an amino acid analyzer (JLC-500; JEOL, Tokyo, Japan). Amino acids mixture standard solution type AN-2 and type B were used as standard solution (Wako, Osaka, Japan).

4. Analysis of carnosine and anserine in heart by UPLC-MS/MS method

Protein precipitation was prepared as above. The supernatant samples of each two rats from the same group were combined to obtain the pooled samples for the analysis (n = 4/group). Liquid chromatography was performed at 30 °C using Intrada Amino Acid column (3.0mmI.D. x 50mm, 3um, Imtakt Corporation, Kyoto, Japan) and a gradient system with the mobile phase consisting of buffer A; acetonitrile / formic acid =100/0.1 solution and buffer B; 100mM ammonium formate. The ratio of buffer B was changed as follows: 0% from 0 to 3 min, then increased to 17% from 3 to 9 min, maintained at 100% for 7 min, and returned back to 0% over 16 min, followed by a 2 min re-equilibration prior to the next sample injection. The flow rate was 0.6 mL/min for all stages and the sample injection volume was 0.5 μL.

Detection was performed using an ACQUITY TQD tandem mass spectrometer (Waters, USA) operated in the electrospray ionization (ESI) positive-ion mode. The condition for carnosine and anserine measurement as follows: carnosine: m/z 227.3→109.9 (25v→20v), anserine: m/z 241.3→ 109.2 (25v→20v).

5. Analysis of serum carnosine by ELISA

Serum samples were analyzed using a commercial carnosine ELISA kit (USCN Life Science, Inc., Wuhan, China).

6. Statistical analysis

Data are expressed as means ± SE. Tukey's multiple-range test was used to compare means after one-way ANOVA. Statistical significance of the difference among means was estimated at $P<0.05$. Data analysis was performed using Excel Statistics 2010 for Windows (Social Survey Research Information Co., Ltd, Tokyo, Japan).

III. Results

1. Food intake, body weight, and heart weight

Dietary manipulation did not affect food intake (1, 7, and 35 mg PN HCl/kg groups: 731 ± 10, 787 ± 15, and 772 ± 24 g/6 weeks, respectively, $P>0.05$) or final body weight (412 ± 9, 444 ± 9, and 437 ± 13 g, respectively, $P>0.05$). The weight of heart was also unaffected (1, 7, and 35 mg PN HCl/kg groups: 1.14 ± 0.05, 1.20 ± 0.03, and 1.16 ± 0.03 g, respectively, $P>0.05$).

2. PLP concentrations in serum and heart

PLP concentrations in the heart and serum were significantly higher in the 7 and 35 mg PN HCl/kg groups than the 1 mg PN HCl/kg group ($P<0.05$) (Fig.2-1A and 2-2A, respectively).

3. Concentrations of free amino acids and related metabolite in heart determined by amino acid analyzer

Vitamin B6 supplementation increased heart citrulline concentration up to 25% in a dose-dependent manner (ANOVA, $P<0.05$, Table 2-1). The concentration of ornithine was 48% lower in the 35 mg PN HCl/kg group than the 1 mg PN HCl/kg group ($P<0.05$, Table 2-1). The concentrations of carnosine in the 7 and 35 mg PN HCl/kg groups were higher 123% and 121%, respectively, than the 1 mg PN HCl/kg group ($P<0.05$, Table 2-1). Since heart carnosine was found to markedly affected, the carnosine and its methylated analogue, anserine, were determined by UPLC-MS/MS.

4. Concentrations of carnosine, anserine, and β-alanine in heart determined by UPLC-MS/MS

In the heart, the 7 and 35 mg PN HCl/kg groups had significantly higher concentrations of carnosine (114% and 162%, respectively, $P<0.01$, Figure 2-1B) and anserine (89% and 101%, respectively, $P<0.05$, Figure 2-1C) than that in the 1 mg PN HCl/kg group. Carnosine and anserine concentrations were strongly correlated ($r=0.97$, $P<0.01$). β-Alanine, the precursor of carnosine, was not detected in any group.

5. Serum carnosine concentration

Vitamin B6 supplementation increased serum carnosine concentration in a dose-dependent manner (ANOVA, $P<0.05$, Figure 2-2B). The concentration was 34% higher in the 35 mg PN HCl/kg group than the 1 mg PN HCl/kg group ($P<0.05$).

IV. Discussion

By using an amino acid analyzer and UPLC-MS/MS method, this study demonstrated that dietary vitamin B6 supplementation (7 and 35 mg PN HCl/kg) to a marginal vitamin B6-deficient diet (1 mg PN HCl/kg) markedly increased the concentrations of carnosine and anserine in the heart

of male rats. Vitamin B6 is known to exert an anti-ischemic effect in the heart (Dhalla et al., 2013). On the other hand, carnosine is reported to exert anti-inflammatory and anti-ischemic effects on the heart (Fleisher-Berkovich et al., 2009; Stvolinsky & Dobrota, 2000). Furthermore, carnosine and anserine act as antioxidant, pH-buffering, anti-glycation, and ergogenic factors (Boldyrev et al., 2013; Caruso et al., 2012). Thus, the present results imply the possibility that dietary vitamin B6 supplementation improves the functions of heart by elevating the concentrations carnosine and anserine.

Vitamin B6 supplementation increased serum carnosine concentration in the present study. Physical exercise is recently suggested to release carnosine from the skeletal muscles into the bloodstream (Nagai et al., 2012). The higher concentration of carnosine caused by exercise is considered to decrease blood pressure in rats and humans (Nagai et al., 2012). On the other hand, vitamin B6 supplementation is reported to attenuate blood pressure in rat models of hypertension (Lal et al., 1996). Thus, the elevated serum carnosine concentration caused by dietary vitamin B6 supplementation may at least be partially related to the anti-hypertensive effect of vitamin B6. Nevertheless, further study is required to examine this possibility.

The present results also indicated dietary vitamin B6 supplementation affects heart citrulline concentration in a dose-dependent manner. Citrulline is suggested to be a putative radical scavenger (Akashi et al., 2001). Citrulline has been reported to be useful for the prevention and treatment of cardiovascular disorders. Thus, the modulation of citrulline concentration due to dietary vitamin B6 supplementation may also positively affect heart functions. The present study further showed dietary vitamin B6 supplementation reduced ornithine concentration in heart of male rats. One of the ornithine catabolism pathway is converting ornithine to citrulline (Ladeuix et al., 2014). However, ornithine was not significantly correlated with citrulline in these data (data not shown), suggesting another possible pathway might be underlying these results. The reason why ornithine concentration was reduced by dietary vitamin B6 supplementation is unknown at present.

V. Table and figures

Table 2-1. Effect of dietary vitamin B6 levels on the concentrations of free amino acids and related metabolites in heart of male rats.

Free amino acids	PN HCl (mg/kg diet)		
	1	7	35
Val (nmol/g tissue)	124 ± 11	125 ± 8	114 ± 8
Leu (nmol/g tissue)	115 ± 9	112 ± 4	111 ± 8
Ile (nmol/g tissue)	70.1 ± 4.1	66.1 ± 2.5	66.8 ± 5.2
Met (nmol/g tissue)	73.0 ± 2.2	78.9 ± 4.9	77.1 ± 7.6
Arg (nmol/g tissue)	283 ± 10	305 ± 18	305 ± 8
Lys (nmol/g tissue)	910 ± 56	941 ± 41	915 ± 63
Thr (nmol/g tissue)	577 ± 41	533 ± 37	562 ± 42
Ala (μmol/g tissue)	1.84 ± 0.13	2.19 ± 0.09	1.89 ± 0.12
Gly (nmol/g tissue)	632 ± 19	571 ± 23	561 ± 27
Ser (nmol/g tissue)	504 ± 37	566 ± 47	609 ± 59
Asn (nmol/g tissue)	405 ± 27	394 ± 34	375 ± 16
Gln (μmol/g tissue)	7.83 ± 0.33	7.73 ± 0.35	6.80 ± 0.32
Asp (μmol/g tissue)	2.92 ± 0.18	3.12 ± 0.06	2.67 ± 0.30
Glu (μmol/g tissue)	5.84 ± 0.07	5.43 ± 0.05	5.33 ± 0.28
Phe (nmol/g tissue)	63.3 ± 4.2	67.3 ± 3.0	65.1 ± 4.8
Tyr (nmol/g tissue)	122 ± 10	108 ± 6	101 ± 8
His (nmol/g tissue)	218 ± 9	198 ± 5	195 ± 16
Phosphoserine (nmol/g tissue)	21.8 ± 5.1	26.2 ± 4.1	14.1 ± 3.8
Taurine (μmol/g tissue)	22.2 ± 0.1	22.1 ± 0.6	21.6 ± 0.6
Urea (μmol/g tissue)	27.5 ± 1.3	30.3 ± 1.4	31.5 ± 2.0
Citrulline (nmol/g tissue)	208 ± 4^{b}	231 ± 5^{ab}	260 ± 15^{a}
Ornitine (nmol/g tissue)	19.5 ± 2.9 ^{a}	14.1 ± 1.7 ^{ab}	10.2 ± 0.9 ^{b}
NH3 (μmol/g tissue)	1.21 ± 0.13	1.28 ± 0.11	1.36 ± 0.12
Carnosine (nmol/g tissue)	32.0 ± 3.3^{b}	71.3 ± 7.9^{a}	70.6 ± 5.4^{a}

Mean ± SE (n = 4-6). Values with different superscript are significantly different by Tukey's multiple-range test (P<0.05).

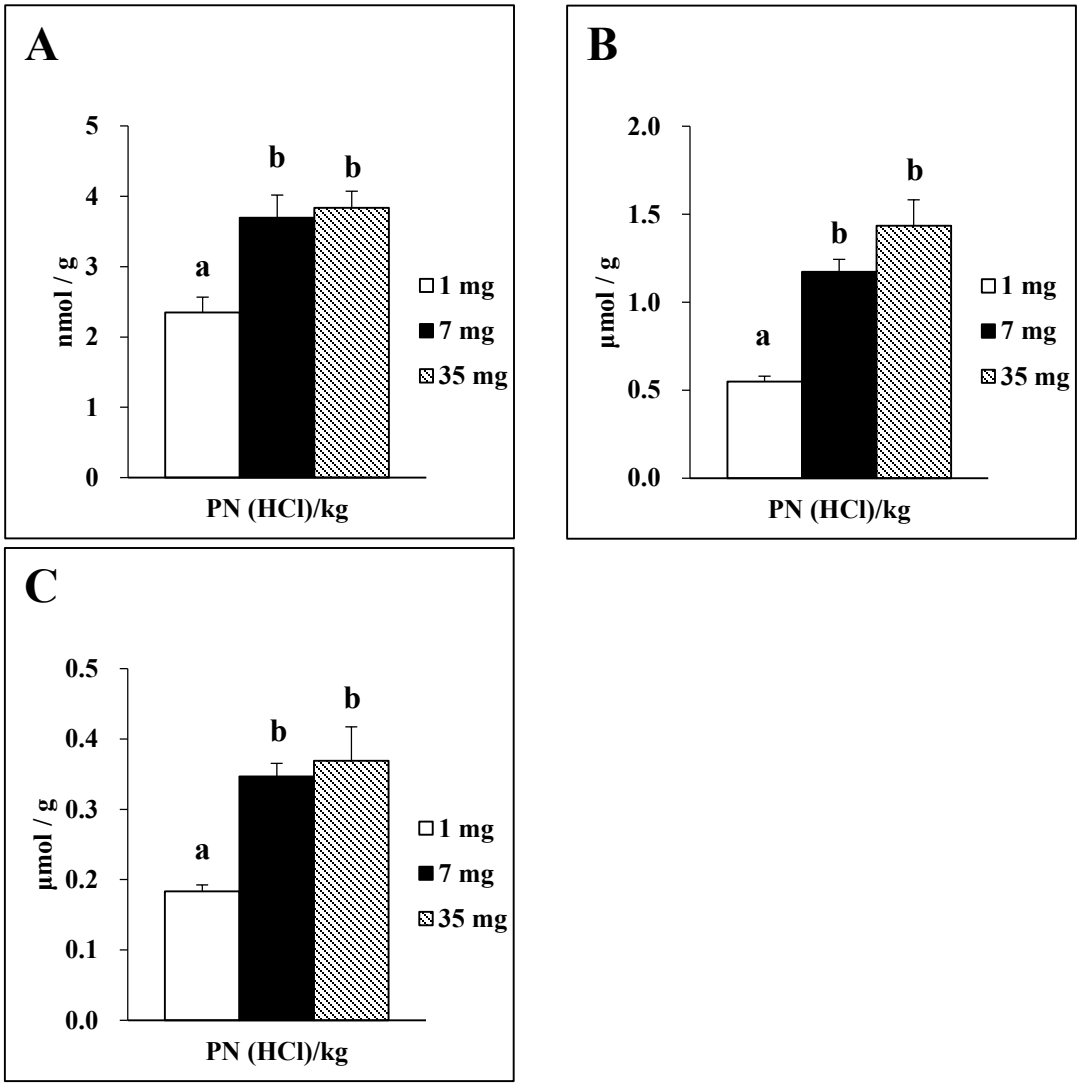

Fig. 2-1. Effect of dietary vitamin B6 levels on the concentrations of PLP (A), carnosine (B), and anserine (C) in the heart of male rats. Means ± SE (n = 4). Values with different superscript are significantly different by Tukey's multiple-range test (P<0.05).

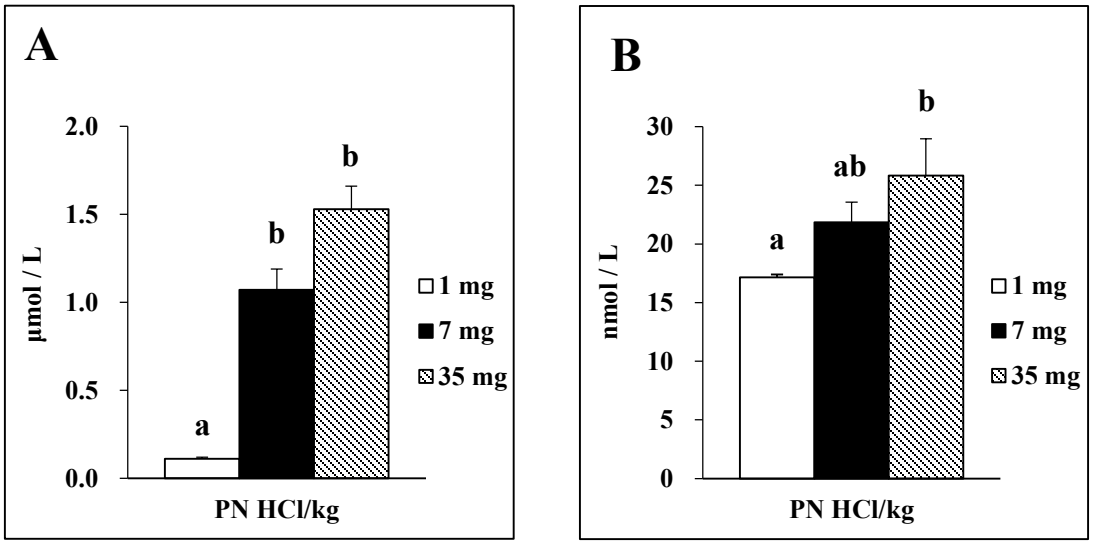

Fig. 2-2. Effect of dietary vitamin B6 levels on the concentrations of PLP (n = 5) (A) and carnosine (n = 8) (B) in the serum of male rats. Means ± SE. Values with different superscript are significantly different by Tukey's multiple-range test (P<0.05).

VI. Summary

Although previous studies reported that there was inverse association between vitamin B6 and cardiovascular disease, and suggested anti-heart diseases effect of vitamin B6, the mechanism of the anti-heart disease effect is still unclear. The results of this study indicated dietary vitamin B6 supplementation elevated carnosine and anserine concentrations in the heart and serum of male rats. These metabolites are reported to have antioxidant, pH-buffering, anti-glycation functions that considered to be beneficial for heart disease, and also suggested to have anti-inflammatory and anti-ischemic effects on the heart. The present study suggested a novel insight into possible mechanism of beneficial function of vitamin B6 in heart via the carnosine and anserine elevation. However further investigation are necessary to prove this hypothesis, and further study is required to determine the underlying mechanisms by which vitamin B6 alters these dipeptides in the heart.

Chapter 3 Effect of dietary vitamin B6 on the level of histidine dipeptides in skeletal muscle

I. Brief Introduction

Vitamin B6 is suggested to have ergogenic function in skeletal muscle. A study by Richardson & Chenman (1981) suggested vitamin B6 intake increased stamina as a result of prolonging muscle contraction. A case-study demonstrated that vitamin B6 supplementation is beneficial for McArdle disease, a glycogenetic myopathy, especially for alleviating fatigue (Izumi et al., 2010). A clinical trial also showed that a combined restricted intake of thiamine, riboflavin, and vitamins B6 and C causes a decrease in physical performance in humans (Van der Beek et al., 1988). On the other hand, plasma PLP concentration is reported to be increased after exercise (Crozier et al., 1994). However, the role of vitamin B6 in skeletal muscle still remains unclear.

In chapter 2, I found that insufficient dietary vitamin B6 lowered the concentrations of carnosine and anserine in the heart of male rats. Carnosine and its methylated analogue, anserine, are abundant dipeptides in skeletal muscle. They constitute an integral part of skeletal muscle contractility and homeostasis, presumably through their role as antioxidant, pH-buffering, anti-glycation and/or calcium regulator (Boldyrev et al., 2013; Caruso et al., 2012). I hypothesized that dietary vitamin B6 also plays an important role in maintaining muscle carnosine and anserine. Accordingly, in this chapter, I investigated the effects of vitamin B6 supplementation diets compared to a marginal vitamin B6-deficient diet, on the levels of carnosine and anserine in the skeletal muscles of rats. The objective of this chapter study was to explore the effect of dietary vitamin B6 on the concentrations of carnosine and anserine in skeletal muscles of male and female rats. In order to explore the relevance of our findings to humans, I additionally investigated the possible relationship between vitamin B6 status and skeletal muscle carnosine in human volunteers.

II. Materials and methods

Animal study

1. Animal and diets

Male and female Sprague–Dawley rats (3 weeks old, Charles River Japan, Hino, Japan) were maintained in accordance with the Guide for the Care and Use of Laboratory Animals established by Hiroshima University. Rats were housed in metal cages in a temperature controlled room (24±1°C) and a 12-h light/dark cycle (lights on, 0800–2000 h). Rats had free access to food and deionized water. Rats were fed a commercial non-purified diet (MF, Oriental Yeast, Tokyo, Japan) for one week. In experiment 1, 24 male rats (average, 113 g) were divided randomly into 3 groups receiving 1, 7, or

35 mg PN HCl/kg diet (n = 8/group) for 6 weeks. In experiment 2, 24 female rats were treated in the same way (average, 109 g). The experimental diets were prepared in the same way with chapter 2 experiment diets. The animals were sacrificed by decapitation under diethyl ether anesthesia. Serum was collected and stored at −60°C. Gastrocnemius and soleus muscles were quickly dissected, frozen in liquid nitrogen, and immediately stored at −80°C until analysis.

2. Analysis of PLP in serum and skeletal muscle by HPLC

The experimental methods were done as described in chapter 2. Briefly, serum and muscles supernatant of each two rats from the same group were combined to obtain pooled samples for the analysis (n = 4 /group). Vitamin B6 from serum and skeletal muscles were extracted using 3 M perchloric acid. PLP was converted to pyridoxic acid 5′-phosphate and measured by HPLC with a fluorometric detector (Tsuge, 1997).

3. Analysis of carnosine, anserine, and β-alanine in skeletal muscles by UPLC-MS/MS method

The experimental methods were done as described in chapter 2. Briefly, gastrocnemius and soleus muscles were treated with cold 3% sulfosalicylic acid to precipitate the proteins. The supernatant liquid of each two rats from the same group were combined to obtain pooled samples for the analysis (n = 4/group). The samples from male and female rats are represented in experiment 1 and 2, respectively.

4. Analysis of ornithine in skeletal muscles by amino acid analyzer

The same pooled samples for the UPLC-MS/MS analysis were used for ornithine analysis (n = 4/group). Ornithine concentration was quantified by an amino acid analyzer as described in chapter 2.

5. Human study

Human data were obtained from 24 male (age 20.6±0.12 years) and 58 female (age 24.0±0.12 years) healthy individuals. The study protocols were approved by the local ethical committee (Ghent University Hospital, Belgium) and written informed consent was obtained from all participants prior to the study.

Muscle carnosine concentration was determined non-invasively by proton magnetic resonance spectroscopy (1H-MRS) by means of a 3-T whole body MRI (magnetic resonance imaging) scanner (Siemens Trio, Erlangen, Germany) as described by Baguet et al (2012). Muscle carnosine was determined in the soleus and gastrocnemius muscles of the right leg.

Venous blood samples were collected from an antecubital vein in EDTA (ethylenediamine tetraacetic acid) tubes, immediately centrifuged after which the plasma was immediately stored at −80 °C before analysis. Pre-analytical handling was performed in light-protected vials as described in the Chromsystem manual. PLP concentration was determined using a commercial HPLC-fluorescence method (52000/Premix, Chromsystems instruments & chemicals GmbH, Germany) (Panton et al., 2013).

6. Statistical analysis

Data are expressed as means ± SE. Tukey's multiple-range test was used to compare means after one-way ANOVA. Statistical significance of the difference among means was estimated at $P<0.05$. Data analysis was performed using Excel Statistics 2010 for Windows (Social Survey Research Information Co., Ltd, Tokyo, Japan). Some data underwent regression analysis and the correlation coefficient was calculated.

The relationship between human plasma PLP levels and muscle carnosine levels was analyzed separately for men and women, given the known sex difference in muscle carnosine (Baguet et al., 2012). Within each sex, subjects were divided into tertiles based on plasma PLP levels. Differences between tertiles were analyzed by ANOVA or Kruskal Wallis depending on the results of the Levene test. Post-hoc analyses were performed using Tukey or Wilcoxon tests. Relationships between some variables for both human and rodent studies were investigated by regression analysis and the correlation coefficient was determined (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

III. Results

1. Animal study experiment 1 (male rats)

a) Food intake, body weight, and skeletal muscles weight of male rats

Dietary manipulation did not affect food intake, final body weight, and weight of skeletal muscles ($P>0.05$, Table 3-1).

b) PLP concentrations in serum and skeletal muscles of male rats

Serum PLP concentration increased in dose-dependent manner by vitamin B6 supplementation (0.17±10, 0.55±0.03, and 1.08±0.07 μmol/L in the 1, 7, and 35 mg PN HCl/kg groups, $P<0.01$ by ANOVA analysis). In gastrocnemius muscle, both the 7 and 35 mg PN HCl/kg groups showed +41% higher concentration of PLP ($P<0.05$, Table 3-1) when compared to the 1 mg PN HCl/kg group. In soleus muscle, compared to the 1 mg PN HCl/kg group, the 7 and 35 mg PN HCl/kg groups had +81% and +100% respectively higher concentration of PLP ($P<0.05$, Table 3-1), without any differences between these groups ($P>0.05$).

c) Concentrations of carnosine, anserine, β-alanine, and ornithine in skeletal muscles of male rats

In the gastrocnemius muscle, the 7 and 35 mg PN HCl/kg groups had higher concentrations of carnosine (+70% and +61%, respectively, $P<0.05$, Fig. 3-1A) than that in the 1 mg PN HCl/kg group. The concentration of anserine was also higher (+58%) in 35 mg PN HCl/kg group when compared to 1 mg PN HCl/kg group ($P<0.05$, Fig. 3-1B). Carnosine and anserine concentrations were strongly correlated ($r=0.80$, $P<0.01$). The concentration of β-alanine was markedly higher in the 7 and 35 mg PN HCl/kg groups than in the 1 mg PN HCl/kg group (+153% and +148%, respectively, $P<0.05$, Fig. 3-1C). There was a strong correlation between carnosine and β-alanine concentrations ($r=0.89$, $P<0.01$). Concentrations of carnosine, anserine, and β-alanine in the gastrocnemius muscle, did not differ between the 7 and 35 mg PN HCl/kg groups.

Analysis using amino acid analyzer indicated that in the gastrocnemius muscle, the concentration of ornithine was significantly lower in the 7 and 35 mg PN HCl/kg groups than the 1 mg PN HCl/kg group (−46% and −54%, respectively, $P<0.05$, Fig. 3-1D). Ornithine concentration was inversely correlated with β-alanine concentration ($r=-0.84$, $P<0.01$).

In the soleus muscle, the 7 mg PN HCl/kg group had higher concentration of carnosine (+43%) than that in the 1 mg PN HCl/kg group ($P<0.05$, Fig. 3-1E), but the 35 mg PN HCl/kg group did not. There was no difference in the concentration of anserine among these groups ($P>0.05$, Fig. 3-1F). However, carnosine and anserine concentrations showed a significant correlation ($r=0.70$, $P<0.05$). The concentration of β-alanine did not differ between 1 and 35 mg PN HCl/kg groups ($P>0.05$, Fig. 3-1G), but lower in 35 mg PN HCl/kg group when compared to 7 mg PN HCl/kg group

(−34 %, P<0.05, Fig. 3-1G). There was no significant correlation between carnosine and β-alanine concentrations (r=0.45, P>0.05).

In the soleus muscle, the concentration of ornithine was significantly lower in the 7 and 35 mg PN HCl/kg groups than the 1 mg PN HCl/kg group (−19% and −25%, respectively, P<0.05, Fig. 3-1H). There was no significant correlation between ornithine and β-alanine concentrations (r=0.32, P>0.05).

2. Animal study experiment 2 (female rats)

a) Food intake, body weight, and skeletal muscles weight of female rats

Dietary manipulation did not affect food intake and skeletal muscles weight (P>0.05, Table 3-1). Compared to the 1 mg group, 35 mg PN HCl/kg group showed slightly higher final body weight in female rats (P<0.05, Table 3-1).

b) PLP concentrations in serum and skeletal muscles of female rats

Serum PLP concentration increased in dose-dependent manner by vitamin B6 supplementation (0.05±0.01, 0.45±0.03, and 0.73±0.07 μmol/L in the 1, 7, and 35 mg PN HCl/kg groups, P<0.01 by ANOVA analysis). In gastrocnemius muscle, both 7 and 35 mg PN HCl/kg groups showed +91% higher concentration of PLP (P<0.05, Table 3-1) when compared to 1 mg PN HCl/kg group. In soleus muscle, compared to the 1 mg group, the 7 and 35 mg PN HCl/kg groups had higher concentration of PLP (+156% and +172%, respectively, P<0.05, Table 3-1).

c) Concentrations of carnosine, anserine, β-alanine, and ornithine in skeletal muscles of female rats

In the gastrocnemius muscle, the 7 and 35 mg PN HCl/kg groups had markedly higher concentrations of carnosine (+211% and +226%, respectively, P<0.001, Fig 3-2A) and anserine (+100% and +128%, respectively, P<0.01, Fig. 3-2B), than those in the 1 mg PN HCl/kg group. Carnosine and anserine concentrations were strongly correlated (r=0.89, P<0.01). The concentration of β-alanine was remarkably higher in the 7 and 35 mg PN HCl/kg groups than in the 1 mg PN HCl/kg group (+381% and +437%, respectively, P<0.001, Fig. 3-2C). There was a strong correlation between carnosine and β-alanine concentrations (r=0.96, P<0.01). Concentrations of carnosine, anserine, and β-alanine in the gastrocnemius muscle, did not differ between the 7 and 35 mg PN HCl/kg groups.

In the gastrocnemius muscle, the concentration of ornithine tended to be lower in the 7 and 35 mg PN HCl/kg groups, −64% and −54% respectively, than the 1 mg PN HCl/kg group (P=0.066 by ANOVA analysis, Fig. 3-2D). Ornithine concentration was inversely correlated with β-alanine concentration (r=−0.65, P<0.05)

In the soleus muscle, the 7 and 35 mg PN HCl/kg groups had significantly higher concentrations of carnosine (+156% and +172%, respectively, P<0.001, Fig. 3-2E), when compared to the 1 mg PN HCl/kg group. There were no significant differences in the concentrations of anserine and β-alanine among the three groups (P>0.05, Fig. 3-2F and Fig. 3-2G). There was no significant correlation between carnosine and β-alanine concentrations (r=0.56, P>0.05).

In the soleus muscle, the concentration of ornithine tended to be lower in the 7 and 35 mg PN HCl/kg groups, −64% and −54% respectively, than the 1 mg PN HCl/kg group (P=0.069 by ANOVA analysis, Fig. 3-2H). There was no significant correlation between ornithine and β-alanine concentrations (r=−0.01, P>0.05).

3. Human study

a) Concentrations of plasma PLP and muscle carnosine in humans

In the human cohort, plasma PLP concentrations ranged between 25.2 and 203.9 nmol/L in women, and between 52.2 and 571.3 nmol/L in men. No individuals could be termed deficient (<20nmol/L) and only one has insufficient (<30nmol/L) plasma PLP (25.2 nmol/L). In the latter subject, the carnosine concentrations in soleus and gastrocnemius muscle were respectively 2.72 and 3.21 µmol/g tissue, which is −0.19 and −0.63 z-score below the average of our reference database containing 90 women.

The known difference in carnosine concentrations between men and women (Blancquaert et al., 2015) was confirmed in this cohort (P<0.05). Therefore, tertiles for PLP were calculated separately for both sexes. Carnosine concentrations were 19.5 (P<0.05) and 18.8% (P<0.05) lower in the lowest tertile of the soleus of women compared to mid and high tertile (Fig. 3-3A). No other differences (women gastrocnemius, or men soleus and gastrocnemius) were found (Figs. 3-3A and 3-3B). In women a positive correlation of r=0.31 and r=0.29 was established between plasma PLP and respectively soleus and gastrocnemius muscles (P<0.05 and P<0.05). In men, a correlation in the soleus (r=0.41, P<0.05), but not in the gastrocnemius (r=0.04, P>0.05) muscle was found. The male cohort contained one outlier (plasma PLP = 570 nmol/L). After deletion of this outlier no correlation was found in either muscle (respectively for soleus and gastrocnemius r=−0.23 and −0.10; P>0.05).

IV. Discussion

This study demonstrated that recommended- and high-level vitamin B6 diets (7 and 35 mg PN HCl/kg, respectively) compared to a marginal vitamin B6-deficient diet (1 mg PN HCl/kg) significantly increased the concentrations of carnosine in the skeletal muscles of male and female rats. Meanwhile, dietary supplemental vitamin B6 appeared to affect the anserine concentrations to a lesser extent. An elevated muscle carnosine and anserine content exerts beneficial effects on the skeletal muscle contractility, as shown in isolated human muscle fibers (Dutka et al., 2012), isolated rodent muscles (Everaert et al., 2013), and *in vivo* human muscles (Hannah et al., 2014). The underlying ergogenic mechanism probably relates to the dipeptides' capacity as an antioxidant, pH-buffering, and/or calcium regulator (Boldyrev et al., 2013; Blancquaert et al., 2015). Furthermore, these dipeptides are also reported to activate muscle phosphorylase (Johnson et al., 1982). Thus, the present results imply that maintaining recommended amounts of dietary vitamin B6 might be favorable for skeletal muscles function by elevating the histidine-dipeptides, especially carnosine.

The present study shows that the dependency of carnosine concentration on dietary vitamin B6 intake was more remarkable in the gastrocnemius than the soleus muscle in both male and female rats. Oppositely, in humans the link between vitamin B6 status and muscle carnosine was only found in (female) soleus, not gastrocnemius muscle. The gastrocnemius muscle comprises a high proportion of type II myofibers, which are "fast-twitch" fibers; meanwhile, the soleus muscle comprises predominantly type I myofibers, which are characteristically "slow-twitch" fibers. On the other hand, compared to the male rats, the female rats appeared to show more remarkable increase in carnosine concentration by dietary vitamin B6 supplementation in gastrocnemius muscle. Thus, the effects of vitamin B6 status on carnosine concentrations in skeletal muscles appear to differ with respect to muscle fiber type, gender, species, and the interaction of these factors. However, the reason for this is unknown.

Dietary supplementation of carnosine or β-alanine is reported to increase carnosine concentration in the skeletal muscles (Artioli et al., 2010; Maynard et al., 2001), whereas a histidine-deficient diet can reduce carnosine concentration (Tamaki et al., 1977). However, those studies involve chronic dietary intervention of high doses of carnosine or β-alanine, or severe histidine-deficiency conditions (Artioli et al., 2010; Maynard et al., 2001; Tamaki et al., 1977). In contrast, the present study used diets ranging from a low-vitamin B6 diet (marginal vitamin B6-deficient diet) that did not induce severe vitamin B6 deficiency to somewhat higher vitamin B6 diets far below the

supraphysiological dose (acute toxic dose). To my knowledge, this is the first evidence indicating dietary intake of a nutrient close to the range of regular daily intake plays a pivotal role in maintaining carnosine concentration in skeletal muscles.

In order to find out whether the rodent data could be replicated in humans, I measured carnosine and PLP in volunteers. I divided the female and male participants separately into tertiles in order to try to mimic the three conditions in the rats. However, the lowest tertile was not as insufficient as the rats in the 1 mg PN HCl/kg group were. Still, I found that women have significantly reduced muscle carnosine content in the lowest PLP tertile compared to the higher tertiles. Moreover this was somewhat confirmed by the low, but significant correlation. This means that women having PLP concentrations close to the insufficient range have lower concentrations of carnosine. In men this could not be confirmed. This is probably due to the fact that the lowest tertile in men was not as close to the insufficient range as the women were. I can conclude from the human data in this study that to some extent a similar pattern could be recognized as in the rats, albeit only in women. Yet, it is expected that the vitamin B6 dependency would probably be more pronounced if the study group would have included truly vitamin B6 deficient humans. Obviously, further study is necessary to explore the relationship between muscle carnosine concentration and vitamin B6 status in truly vitamin B6 deficient volunteers.

In the rat study, it is noteworthy that the concentration of β-alanine, a precursor of carnosine, in gastrocnemius muscles was markedly elevated by supplemental vitamin B6. Likewise, I consistently found a strong correlation between β-alanine and carnosine concentrations in this muscle. It remains to be determined which PLP dependent enzymes are responsible for the changes in carnosine concentrations. β-Alanine is the rate-limiting precursor of carnosine (Harris et al., 2006). The most important source of β-alanine is considered to be uracil degradation in the liver, but the enzymes involved are not PLP dependent. Enzymes that might be responsible are PLP dependent transaminases ‘β-alanine – 2-oxoglutarate transaminase’ (ABAT) and/or ‘β-alanine – pyruvate transaminase’ (AGXT2). Furthermore, animal study suggested β-alanine as one of the metabolites resulted from the injection of spermidine or spermine, which are produced by PLP dependent ornithine decarboxylase from ornithine (Ladeuix et al., 2014; Van den Berg et al., 1984). Interestingly, this study indicated significant inverse correlation between ornithine and β-alanine concentrations in gastrocnemius muscle. This raises the possibility that the elevation in the level of β-alanine in the gastrocnemius muscle by dietary supplemental vitamin B6 is at least in part mediated by enhancing the conversion from ornithine to β-alanine in skeletal muscles. In my preliminary experiment,

concentration of β-alanine in the liver was unaffected by dietary level of vitamin B6 (data not shown). Thus, it seems unlikely that the elevated carnosine in the skeletal muscles by dietary vitamin B6 is associated with the altered synthesis of β-alanine in the liver.

V. Table and figures

Table 3-1. Body weight, food intake, muscles weight, and muscles PLP

PN HCl/kg	1 mg	7 mg	35 mg
Experiment 1 (Male rats)			
Initial body wt (g)	113±2	113±1	113±2
Final body wt (g)	434±23	452±12	461±12
Food intake (g/6 wk)	738±40	793±29	768±18
Gastrocnemius muscle wt (g)	2.57±0.08	2.47±0.06	2.58±0.14
Gastrocnemius PLP (nmol/g)	9.1±0.7[a]	12.8±0.6[b]	12.8±0.8[b]
Soleus muscle wt (g)	0.18±0.01	0.17±0.01	0.19±0.01
Soleus PLP (nmol/g)	2.7±0.3[a]	4.8±0.2[b]	5.3±0.1[b]
Experiment 2 (Female rats)			
Initial body wt (g)	108±2	109±2	109±2
Final body wt (g)	253±5a	276±9ab	288±8b
Food intake (g/6 wk)	583±13	630±18	638±16
Gastrocnemius muscle wt (g)	1.58±0.04	1.73±0.06	1.75±0.07
Gastrocnemius PLP (nmol/g)	7.6±0.8[a]	14.5±1.4[b]	14.5±0.6[b]
Soleus muscle wt (g)	0.11±0.00	0.12±0.01	0.13±0.01
Soleus PLP (nmol/g)	2.0±0.1[a]	5.1±0.4[b]	5.4±0.2[b]

Mean ± SE (n = 7-8). Means with different superscript are significantly different by Tukey's multiple-range test (P<0.05)

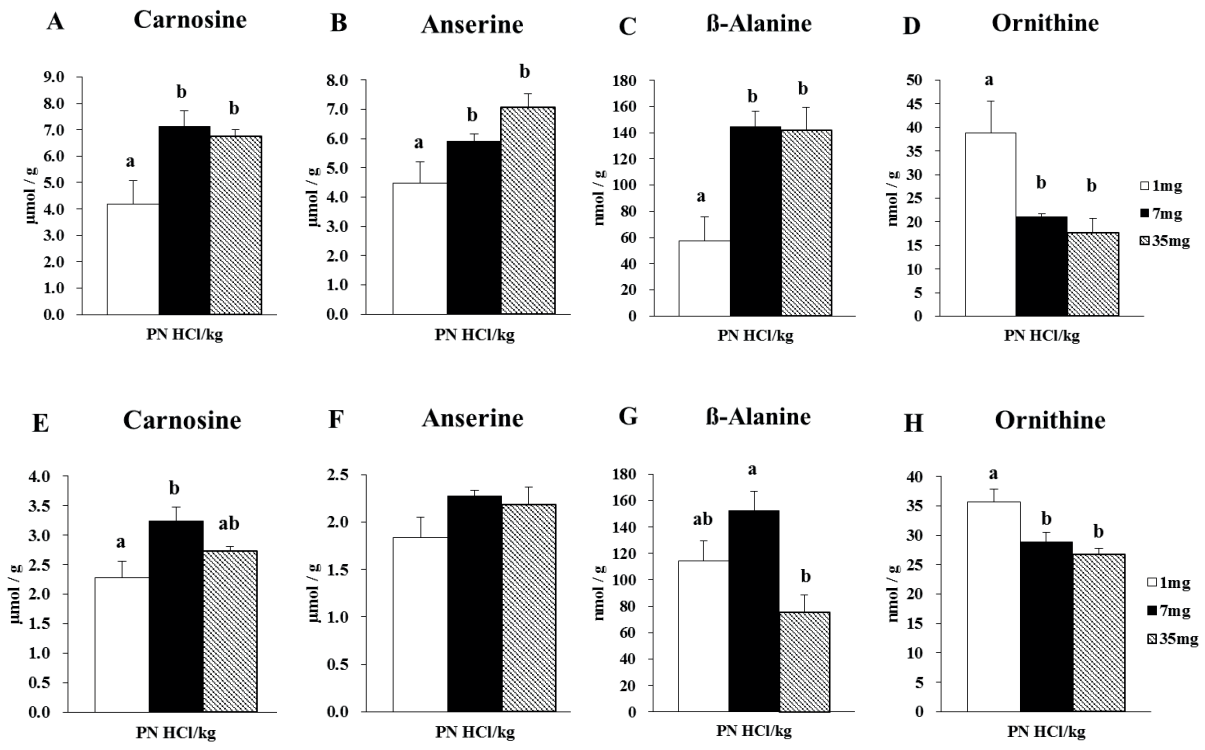

Fig. 3-1. Effect of dietary vitamin B6 levels on the concentrations of carnosine, anserine, β-alanine, and ornithine in gastrocnemius and soleus muscles of male rats. Gastrocnemius muscle samples from male rats (n = 4) were analyzed for its concentrations of carnosine (A), anserine (B), β-alanine (C), and ornithine (D). Soleus muscle samples from male rats (n = 4) were analyzed for its concentrations of carnosine (E), anserine (F), β-alanine (G), and ornithine (H). Values are means ± SE. Groups with different letters are significantly different from each other, P<0.05 (Tukey's multiple-range test).

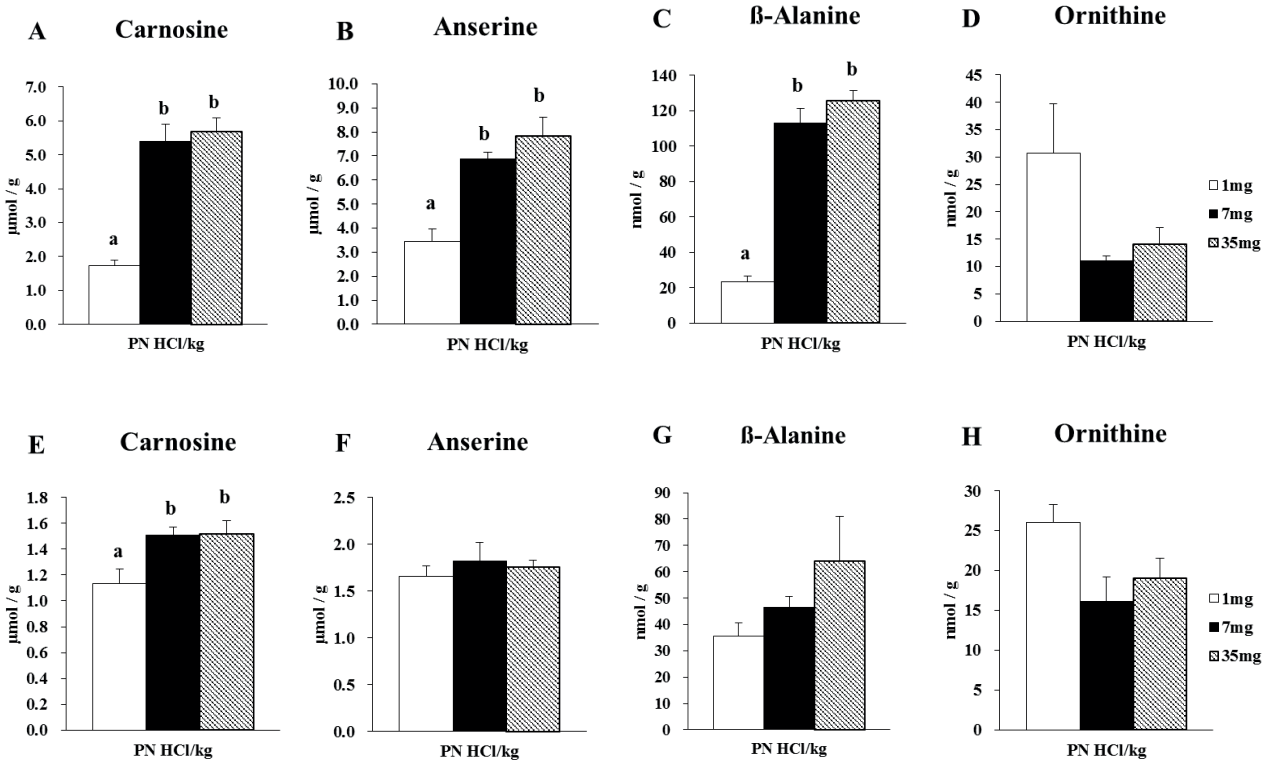

Fig. 3-2. Effect of dietary vitamin B6 levels on the concentrations of carnosine, anserine, β-alanine, and ornithine in gastrocnemius and soleus muscles of female rats. Gastrocnemius muscle samples from female rats (n=4) were analyzed for its concentrations of carnosine (A), anserine (B), β-alanine (C), and ornithine (D). Soleus muscle samples from female rats (n=4) were analyzed for its concentrations of carnosine (E), anserine (F), β-alanine (G), and ornithine (H). Values are means ± SE. Groups with different letters are significantly different from each other, P<0.05 (Tukey's multiple-range test).

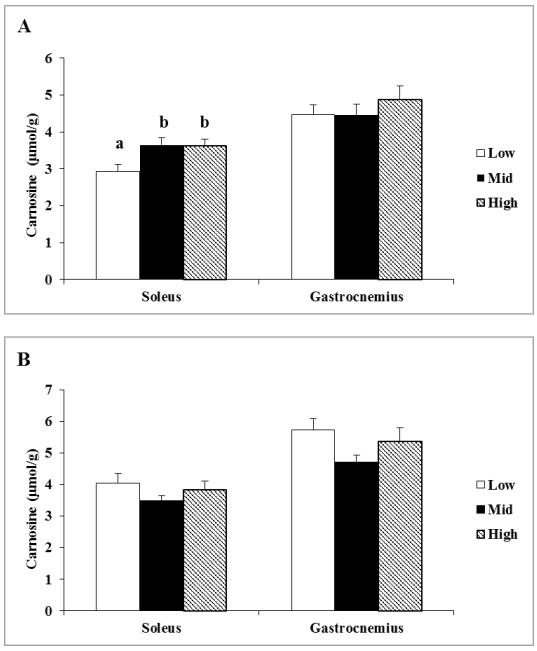

Fig. 3-3. Association of plasma PLP with carnosine concentrations in different tertiles of women (A) and men (B). Values are means ± SE. Groups with different letters are significantly different from each other, $P<0.05$ (Tukey's multiple-range test).

VI. Summary

Here I report evidence that insufficient vitamin B6 intake lowered concentration of carnosine, which is a putative ergogenic factor, in the gastrocnemius and soleus muscles of male and female rats, and partly confirmed in human sample. The results of this study suggest dietary vitamin B6 status is a determinant of carnosine concentration in mammalian skeletal muscles. The present study also suggests vitamin B6 intake close to the range of regular daily intake is able to affect carnosine concentration in skeletal muscles. To my knowledge, this is the first study to suggest a specific determinant up-regulating muscle carnosine concentration.

As histidine-containing dipeptides are critical for muscle function, this is a novel mechanism why adequate vitamin B6 status is essential to safeguard optimal muscle contractility and exercise performance. The results further suggest that the elevation in the level of β-alanine and carnosine in the gastrocnemius muscle by dietary supplemental vitamin B6 is at least in part mediated by enhancing the conversion from ornithine to β-alanine in the skeletal muscle. This

study shows the first evidence of the regulation of β-alanine concentration by dietary factor. However, these results need further investigation.

Chapter 4 Effect of dietary vitamin B6 on gene expression in skeletal muscle

I. Brief Introduction

Skeletal muscle is the major pool of PLP in the body. Some biological functions of vitamin B6 have been related to fuel metabolism during exercise (Manore, 1994). The exercise-related function of vitamin B6 is at least in part related to the breakdown of muscle glycogen. Glycogen phosphorylase is the enzyme responsible for the release of glucose-1-phosphate from muscle glycogen; PLP is a cofactor for this enzyme (Manore, 1994). Vitamin B6 deficiency is reported to decrease the activity and amount of muscle phosphorylase (Okada et al., 1997). Rats fed high levels of vitamin B6 have exhibited elevated concentrations of muscle phosphorylase (Black et al., 1977). In addition, vitamin B6 may have a favorable effect on the skeletal muscle, as a case study demonstrated that vitamin B6 supplementation was beneficial for McArdle disease, a glycogenetic myopathy (Izumi et al., 2010). On the other hand, exercise has been reported to increase blood PLP levels (Crozier et al., 1994).

The recent discovery of several myokines has established a new paradigm of muscle as an endocrine organ. Myokines are released during muscle contraction, such as during exercise (Raschke & Eckel, 2013). 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, leukemia inhibitory factor (LIF), vascular endothelial growth factor, irisin, myostatin and myonectin (Raschke & Eckel, 2013). Myokines play important roles in biological homeostasis, such as energy metabolism, angiogenesis and myogenesis (Yoon et al., 2012). They are also considered to be putative preventive factors against chronic diseases such as type 2 diabetes (Pedersen & Febbraio, 2012). Some studies have suggested that myokines play pivotal roles in the communication between skeletal muscles and other tissues, such as adipose tissues, as well as liver and pancreatic cells (Pedersen, 2011b; Ellingsgaard et al., 2011; Pedersen & Febbraio, 2008).

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) regulates the oxidative defenses by inducing expression of cytoprotective genes. Nrf2 induces these genes mainly by binding the antioxidant response element (ARE) in the promoter of the genes that code for anti-oxidative enzymes (Wakabayashi et al., 2010). Nrf2/ARE signaling is activated by acute exercise stress in the myocardium (Muthusamy et al., 2012). Recently, several studies have suggested a role of Nrf2 that goes far beyond antioxidant mechanisms and underlines its possible impact on the muscle tissue formation. Al-Sawaf et al (2014) reported that Nrf2 regulated myogenic regulatory factor (Mrf) expression and that Nrf2-knockout retarded skeletal muscle regeneration after injury. Heat shock proteins (HSPs) have been also reported to have a cytoprotective role in skeletal muscles (Bayer et

al., 2008). Furthermore, it has been reported that exercise induces the expression of HSPs in skeletal muscle, heart and liver (Salo et al., 1991).

In chapter 3, I found that carnosine and PLP concentrations in skeletal muscles were markedly higher in dietary supplemental vitamin B6 groups. On the other hand, carnosine and PLP are reported to be stimulated by exercise. Thus, in this chapter, I postulated that dietary supplemental vitamin B6 has a significant impact on skeletal muscles. To examine this possibility, I investigated the effect of dietary vitamin B6 on the expressions of genes related to exercise, such as myokines, Nrf2-related factors, myogenic factors and HSPs in the gastrocnemius muscles of rats that were fed a low vitamin B6 diet (1 mg PN HCl/kg) with and without vitamin B6 supplementation. Further analyses using DNA microarrays were also conducted.

II. Materials and methods

1. Animal and diets

Male Sprague–Dawley rats (3 wk old, Charles River Japan, Hino, Japan) were maintained in accordance with the Guide for the Care and Use of Laboratory Animals established by Hiroshima University. The rats were housed in metal cages in a temperature controlled room (24 ± 1°C) and a 12-h light/dark cycle (lights on, 0800–2000 h). The rats had free access to food and deionized water. The experimental diets were prepared in the same way with chapter 2 experiment diets. After being fed a commercial nonpurified diet (MF, Oriental Yeast, Tokyo, Japan) for one week, 24 rats (average, 117 g) were divided randomly into 3 groups receiving 1, 7, or 35mg PN HCl/kg diet (n = 8/group) for 6 weeks. The animals were sacrificed by decapitation under diethyl ether anesthesia. Gastrocnemius muscle was quickly dissected, and immediately homogenized in Qiagen lysis buffer for RNA isolation.

2. Gastrocnemius muscle PLP

The experimental method for PLP measurement was done as describe before (Masisi et al., 2012). Briefly, vitamin B6 from gastrocnemius muscle was extracted using 3 N perchloric acid. Meanwhile, PLP was converted to pyridoxic acid 5′-phosphate and measured by HPLC with a fluorometric detector (Tsuge, 1997).

3. DNA microarray analysis

Pooled RNAs were subjected to cRNA synthesis for a DNA microarray analysis. Cyanine-3 (Cy3) labeled cRNA was prepared from 100 ng RNA using the One-Color Low Input Quick Amp labeling kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. All procedures of hybridization, slide, and scanning were carried out according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). The data were analyzed by GeneSpring software version 12.6.1 (Agilent Technologies, Santa Clara, CA).

4. mRNA analysis

The Qiagen Midi kit was used to isolate total RNA from rat gastrocnemius muscle, which was subsequently prepared according to the standard protocol. Total RNA (1 μg) was reverse-transcribed using the First Strand cDNA Synthesis kit (Toyobo, Japan) according to the manufacturer's instructions. Real-time PCR was performed with a StepOne^{TM} Real-Time PCR System (Applied Biosystems) using Thunderbird SYBR qPCR Mix -Plus- (TOYOBO, Japan). All of the primer sets were purchased from Greiner bio-one (Japan) (Table 4-1). The cycling parameters were: initial step at 90°C for 10 sec, followed by 40 cycles of 90°C for 5 sec, 60°C for 10 sec and 72°C for 10 sec. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method normalizing to GAPDH expression levels, and fold differences in expression were calculated relative to control samples.

5. Statistical analysis

Data are expressed as means ± SE. Tukey's multiple-range test was used to compare means after one-way ANOVA. Statistical significance of the difference among means was estimated at $P<0.05$. Data analysis was performed using Excel Statistics 2010 for Windows (Social Survey Research Information Co., Ltd, Tokyo, Japan). Some data underwent regression analysis and the correlation coefficient was calculated.

III. Results

1. Food intake, body weight, muscle weight, and muscle PLP

Dietary manipulation did not affect food intake (1, 7, and 35 mg PN HCl/kg groups: 706 ± 24, 784 ± 25, and 769 ± 13 g/6 weeks, respectively, $P>0.05$) or final body weight (402 ± 10, 445 ± 13, and 441 ± 8 g, respectively, $P>0.05$). The weight of the gastrocnemius muscle from one leg was also

unaffected by dietary vitamin B6 treatment (1, 7, and 35 mg PN HCl/kg groups: 2.4 ± 0.1, 2.6 ± 0.1, and 2.7 ± 0.1 g, respectively, $P>0.05$). Gastrocnemius muscle PLP concentrations in the 7 and 35 mg PN HCl/kg groups (10.8 ± 1.5 and 10.2 ± 1.0 nmol/g tissue, respectively) were markedly higher than that in the 1 mg PN HCl/kg group (4.5 ± 0.4 nmol/g tissue, $P<0.01$). There was no difference in muscle PLP concentrations between the 7 and 35 mg PN HCl/kg groups ($P>0.05$).

2. Effect of dietary vitamin B6 on the gene expressions in the gastrocnemius muscle of rats

Among the 19 myokines genes that were examined, nine genes were significantly affected by dietary vitamin B6 (Table 4-2, ANOVA $P<0.05$). Among these genes, the expression of IL-7, IL-8, SPARC, IL-6, GDF11, myonectin, LIF, apelin, and RARRES1 were significantly higher in the 7 mg PN HCl/kg group (+107%, +105%, +92%, +77%, +75%, +65%, +62%, +61%, and +53%, respectively, $P<0.05$) than in the 1 mg PN HCl/kg group. There were no significant differences in these gene expressions between the 1 and 35 mg PN HCl/kg groups, except for GDF11 and myonectin whose expressions were significantly increased in the 35 mg PN HCl/kg group compared with the 1 mg PN HCl/kg group (Table 4-2, $P<0.05$). The expression of other myokines was not affected by the dietary vitamin B6 levels (ANOVA, $P>0.05$).

Dietary vitamin B6 also significantly affected the expression of some genes that were related to mitochondrial function and genes that were related to Nrf2 in the gastrocnemius muscle of rats (Table 4-3, ANOVA, $P<0.05$). Among the 14 genes evaluated, the 7 mg PN HCl/kg diet resulted in higher expressions of HO-1, SOD2, GPX1, Nrf2 and GST than the 1 mg PN HCl/kg diet (+71%, +56%, +56%, +45% and +28%, respectively, $P<0.05$). There were no significant differences between the 1 and 35 mg PN HCl/kg groups ($P>0.05$). The expression of other genes was unaffected ($P>0.05$).

The effect of dietary vitamin B6 on gene expression that was related to myogenesis and sarcopenia was also evaluated. Among the 8 genes analyzed, only myogenin expression was significantly affected by dietary vitamin B6 (Table 4-4, ANOVA, $P<0.05$). Compared with the 1 mg PN HCl/kg group, the 7 mg PN HCl/kg group showed +52% increase in myogenin expression; however, there was no significant difference between the 1 and 35 mg PN HCl/kg groups ($P<0.05$).

Among the 4 HSP genes evaluated, the 7 mg PN HCl/kg diet increased the gene expression of HSP60 (+66%, $P<0.05$, Table 4-5) compared with the 1 mg PN HCl/kg diet. There were no significant differences in these expressions between the 1 and 35 mg PN HCl/kg diets ($P>0.05$). The expressions of the other three genes were not affected by dietary vitamin B6 (ANOVA, $P>0.05$).

Our microarray data showed that the gene expressions of Nrxn2 and Ntn4 were also altered by dietary supplemental vitamin B6 (data not shown). Real-time PCR results showed that the 7 mg PN HCl/kg diet elevated the gene expression of Nrxn2 and Ntn4 by 85% and 82%, respectively (P<0.05, Table 4-6) compared with the 1 mg PN HCl/kg diet. There were no significant differences in these expressions between the 1 and 35 mg PN HCl/kg groups (P>0.05).

The correlations among the genes affected by the dietary level of vitamin B6 were evaluated. As a result, significant correlations were found between the expression of Nrf2 and other genes affected by dietary vitamin B6 (r=0.43~0.93 for IL-7, SPARC, GDF11, myonectin, apelin, RARRES1, HO-1, SOD2, GPX1, GST, myogenin, HSP60, Nrxn2, and Ntn4, respectively, P<0.05, Fig.4-1) and between the expression of HSP60 and other genes affected by dietary vitamin B6 (r=0.49~0.92 for IL-7, SPARC, GDF11, myonectin, apelin, RARRES1, HO-1, SOD2, GPX1, Nrf2, GST, myogenin, HSP60, Nrxn2, and Ntn4, respectively, P<0.05, Fig.4-1). Significant correlations were also found between the expression of myogenin and other genes affected by dietary vitamin B6 (r=0.43~0.93 for IL-7, SPARC, GDF11, myonectin, apelin, HO-1, SOD2, GPX1, GST, myogenin, HSP60, Nrxn2, and Ntn4, respectively, P<0.05, Fig.4-1).

IV. Discussion

This is the first study 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 PN HCl/kg) 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 PN HCl/kg, marginal vitamin B6-deficiency level). Compared with the 1 mg PN HCl/kg group, the 7 mg PN HCl/kg group also showed 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. Similar results were found in the expression of Nxrn2, a membrane protein important for motor neuron neurotransmitters, and the expression of Ntn4, a secreted molecule involved in angiogenesis. Interestingly, in almost all genes affected by the dietary level of vitamin B6, there were no significant differences in the gene expressions between the 1 and 35 mg PN HCl/kg (excessive vitamin B6 diet without any toxic symptoms) groups, whereas there was a marked difference in the levels of PLP between the two

groups. Thus, the recommended level of vitamin B6 (7 mg PN HCl/kg) 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 be appropriate for this expression.

In the present study, it was noteworthy that almost all genes affected by the recommended level of vitamin B6 were exercise-induced genes. Myokines are released during muscle contraction (Raschke & Eckel, 2013) and may be partly responsible for the beneficial effects of exercise (Pedersen, 2011a; Pedersen, 2011b; Pedersen, 2009). A study reported that exercise upregulated both antioxidant enzymes and the antioxidant defense system that is related to Nrf2 (Powers et al., 1999). Myogenin expression is induced by acute exercise in the human skeletal muscle (Yang et al., 2005). HSP60 is also increased in cardiac and skeletal muscles after endurance training (Samelman, 2000). Furthermore, Nxrn2 may be important for motor neurons because the knockdown of Nrxn2 results in a significant decrease in motor axon excitability (See et al., 2014). Ntn4 is a putative angiogenic factor, and exercise has been known to induce angiogenesis (Egginton, 2009). Further investigation will be required to determine if the beneficial effects of dietary vitamin B6 on the skeletal muscle are ascribed to the altered expression of these factors, similar to the mechanism of beneficial effects of exercise on the skeletal muscle.

The expression of HO-1, SOD2, GPX1, and GST, the antioxidant genes that were also Nrf2-regulated factors, were increased in the 7 mg PN HCl/kg group compared with the 1 mg PN HCl/kg group. Vitamin B6 may have antioxidant functions in the body (Endo et al., 2006). Thus, it will be important to test the possibility that the antioxidant function of vitamin B6 may be partly via the modulation of Nrf2 signaling pathway. The present study suggested a possible physiological role of vitamin B6 as a regulator of the Nrf2 signaling pathway. However, further investigations will be required to test this possibility.

Recently, there has been growing evidence that myokines have some anti-disease functions. RARRES1 may play a role in colorectal adenocarcinoma because the downregulation of RARRES1 has been related to disease progression (Wu et al., 2006). Another study reported that colon tumor growth was increased in SPARC-null mice, suggesting an anti-tumor function of SPARC (Brekken et al., 2003). Previous study indicated that dietary supplemental vitamin B6 to a low vitamin B6 diet lowered colon tumorigenesis in mice (Komatsu et al., 2001). Further investigations will be required to examine the involvement of RARRES1 and SPARC in the mechanisms of the anti-tumor effects of dietary vitamin B6.

I further evaluated the correlations among the gene expressions affected by dietary vitamin B6. Although Nrf2 has been reported as a potent activator of IL-6 gene transcription *in vivo* (Wruck et al., 2011), our results did not show any significant correlation between Nrf2 and IL-6 (r=0.27). Intriguingly, IL-7, SPARC, GDF11, myonectin, apelin, and RARRESS1 showed a strong correlation with Nrf2. Thus, it would be important to examine if the alterations in these myokines by dietary vitamin B6 is related to the altered Nrf2 expression by dietary vitamin B6. Because myogenin is the major regulator of muscle regeneration, the correlation of gene expressions between myokines and myogenin was also evaluated. As a result, the expression of myogenin was significantly correlated with the expression of IL-7, SPARC, GDF11, myonectin, and apelin. Further, our results showed the significant correlation of HSP60 with IL-7, SPARC, GDF11, myonectin, apelin, and RARRES1. It will be required to evaluate the effect of Nrf2, HSP60, and myogenin on the expression of other myokines in muscle cell cultures.

V. Table and figures

Table 4-1. Sequences of primers used for real-time PCR

Name			Sequence (5'-3')
GAPDH	Glyceraldehyde3-Phosphate Dehydrogenase	F	TGACAACTCCCTCAAGATTGTC
		R	GGCATGGACTGTGGTCATGA
IL-7	Interleukin 7	F	TTTGGAATTCCTCCCCTGAT
		R	TCATCAGCACACTCCCAAAG
IL-8	Interleukin 8	F	GAAGATAGATTGCACCGA
		R	CATAGCCTCTCACACATTTC
SPARC	Secreted Protein Acidic and Rich in Cysteine	F	GGGCAGACCAATACCTCACTA
		R	CCGACCATTCCTTCCGTTG
IL-6	Interleukin 6	F	CCCAACTTCCAATGCTCTCCTA
		R	GCACACTGAGTTTGCCGAATAGACC
GDF11	Growth Differentiation Factor 11	F	AGCATCAAGTCGCAGATCCT
		R	CTTATGACCGTCTCGGTGGT
Myonectin	Myonectin	F	TGTTGTTGAAAGGTGCGGTA
		R	TCTCAAGCTCCTGGGTGACT
LIF	Leukemia Inhibitory Factor	F	AAGTACCATGTGGGCCATGT
		R	GGACCACCGCACTAATGACT
Apelin	Apelin	F	GGCTAGAAGAAGGCAACATGC
		R	CCGCTGTCTGCGAAATTTCCT
RARRES1	Tumor suppressor Retinoic Acid	F	GCTGCACTTCCTCAACTTCC
	Receptor Responder Protein 1	R	TGTGCTAAACACCAGGTCCA
OSM	Oncostatin M	F	GAACATCCAAGGGATCAGGA
		R	AACCCATGAAGCGATGGTAG
FGF21	Fibroblast Growth Factor 21	F	GCCAACAACCAGATGGAACTC
		R	CTGGTACACATTGTATCCGTCCTT
GDNF	Glial Cell Derived Neurotrophic Factor	F	CAAAAGACTGAAAAGGTCACCAGAT
		R	GCTTGCCGGTTCCTCTCTCT
IL-15	Interleukin 15	F	CTTCTTAACTGAGGCTGG
		R	GCAACTGGGATGAAAGTC
LTBP2	Latent-Transforming Growth	F	AAGAACACTGCGCTCCTCAT
	Factor Beta-Binding Protein 2	R	TGGAAGCCAGTCTCACACAG
Decorin	Decorin	F	TCGGATACATCCGCATCTCAGA
		R	GGCACTCTGAGGAGTTTGTTGT
CHI3L1	Chitinase-3-Like Protein 1	F	GGGCAGTGGATTTGGATG
		R	TGCAAGTGACCAGACTCCTG
Irisin	Irisin	F	GACCTGGAGGAGGACACAGA
		R	CCCATCTCCTTCATGGTCAC
SERP4	Septin 4	F	CGCAGGATGAGTCAAGTCAG
		R	TGACCAGATGTCCACAAGGA
Metrn 1	Meteorin 1	F	TCGAGGATGTCACCCATGTA
		R	CCCTGGTCGTACTCCACACT
HO-1	Heme Oxygenase 1	F	CGACAGCATGTCCCAGGATT
		R	TCGCTCTATCTCCTCTTCCAGG
SOD2	Superoxide Dismutase 2	F	GGCCAAGGGAGATGTTACAA
		R	GCTTGATAGCCTCCAGCAAC
GPX1	Glutathione Peroxidase 1	F	GCTGCTCATTGAGAATGTCG
		R	GAATCTCTTCATTCTTGCCATT
Nrf2	Nuclear respiratory factor 2	F	CAAGAGCAACAGATGAATGAG
		R	ACTTTAATCGTAGTCGGTGTAG
GST	Glutathione S-Transferases	F	CCCTGAGAACCAGAGTCAGC

		R	CTGCGGATTCCCTACACATT
Catalase	Catalase	F	GAGGTCACCCACGATATTACCAGA
		R	AGAATTTCACTGCAAACCCACG
Keap1	Kelch-like ECH-Associated Protein 1	F	GTGCATCGACTGGGTCAAATAC
		R	CTGGAAGATCTGCACCAGGTAG
Nrf1	Nuclear Respiratory Factor 1	F	TTACTCTGCTGTGGCTGATGG
		R	CCTCTGATGCTTGCGTCGTCT
PGC1a	Peroxisome Proliferator-Activated	F	CCTTTCTGAACTTGATGTGA
	Receptor Gamma, Coactivator 1 Alpha	R	ATGCTCTTTGCTTTATTGCT
Imp-11	Importin 11	F	AGACGAAGAACCACCCACAG
		R	GTCTCCATGAGGGACTGGAA
Trx2	Thioredoxin Reductase-2	F	CAGTTATGTGGCCCTGGAGT
		R	TCGGGAGTTTTCTGATGAGG
Nqo1	NAD(P)H:Quinone Oxidoreductase 1	F	TCCGCCCCCAACTTCTG
		R	TCTGCGTGGGCCAATACA
UBE2E3	Ubiquitin-Conjugating Enzyme E2 E3	F	GGGAGTCATCTGCCTGGATA
		R	TCCTGTCGTGTTCTGCTCTG
GCLC	Glutamate-Cysteine Ligase, Catalytic Subunit	F	CTCTGCCTATGTGGTATTTG
		R	TTGCTTGTAGTCAGGATGG
Myogenin	Myogenin	F	GACCCTACAGGTGCCCACAA
		R	ACATATCCTCCACCGTGATGCT
MuRF1	Muscle RING-Finger Protein-1	F	GTGAAGTTGCCCCCTTACAA
		R	TGGAGATGCAATTGCTCAGT
Myf-6	Myogenic Factor 6	F	CCGGGAGCGACAGCAGTGG
		R	AGCCGGTGCAGCAGGTCCT
MyoD1	Myogenic Differentiation 1	F	GCGACAAGCCGATGACTTCTAT
		R	GGTCCAGGTCCTCAAAAAAGC
Myf-5	Myogenic Factor 5	F	GAGCCAAGAGTAGCAGCCTTCG
		R	GTTCTTTCGGGACCAGACAGGG
Atrogin-1	Atrogin-1	F	CCTTCTCCAGGCCAGTAGGTG
		R	CGCGTCCGCGCTCTGTA
RPB3	RNA Polymerase B 3	F	CGATCCAGACAACGCACTTA
		R	AGACAGGGCTGAGAGGACAA
Sp1	Specificity Protein 1	F	TGAATGCTGCTCAACTGTCC
		R	CTCCACCTGCTGTCTCATCA
HSP60	Heat Shock Protein 60	F	AAATCCGGAGAGGTGTGATG
		R	CTTCAGGGGTTGTCACAGGT
HSP70	Heat Shock Protein 70	F	GTTGCATGTTCTTTGCGTTT
		R	TACACAGGGTGGCAGTGCT
HSP90	Heat Shock Protein 90	F	GGTGTCGGCTTCTACTCTGC
		R	CTGCTCATCATCGTTGTGCT
HSP27	Heat Shock Protein 27	F	CCTGGTGTCCTCTTCCCTGT
		R	TGGTGATCTCCGCTGATTGTG
Nrxn2	Neurexin 2	F	TCAACCTGTCCCTCAAGTCC
		R	GGTGTAATCCTCCTGCGTGT
Ntn4	Netrin 4	F	GGCCTGGAAGATGATGTTGT
		R	TCTCTGACAAGGGCAGGACT

Table 4-2. Effect of dietary level of vitamin B6 on expression of myokine genes in gastrocnemius muscle of rats

Gene	PN HCl (mg/kg diet)		
	1	7	35
IL-7	1.00±0.09[b]	2.07±0.28[a]	1.33±0.16[b]
IL-8	1.00±0.04[b]	2.05±0.28[a]	1.61±0.16[ab]
SPARC	1.00±0.11[b]	1.92±0.20[a]	1.39±0.07[b]
IL-6	1.00±0.10 [b]	1.77±0.22 [a]	1.32±0.20 [ab]
GDF11	1.00±0.04[b]	1.75±0.15[a]	1.56±0.18[a]
Myonectin	1.00±0.08[b]	1.65±0.18[a]	1.46±0.13[a]
LIF	1.00±0.09[b]	1.62±0.13[a]	1.23±0.03[ab]
Apelin	1.00±0.09[b]	1.61±0.17[a]	1.33±0.11[ab]
RARRES1	1.00±0.11[b]	1.53±0.10[a]	1.33±0.08[ab]
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
SFRP4	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 means ± SE, n = 6-8. Groups with different letters are significantly different from each other, $P<0.05$ (Tukey's multiple-range test).

Table 4-3. Effect of dietary level of vitamin B6 on expressions of genes related to mitochondrial function and Nrf2 in gastrocnemius muscle of rats

Gene	PN HCl (mg/kg diet)		
	1	7	35
HO-1	1.00±0.05[b]	1.71±0.24[a]	1.18±0.13[ab]
SOD2	1.00±0.06[b]	1.56±0.17[a]	1.18±0.14[ab]
GPX1	1.00±0.12[b]	1.56±0.20[a]	0.96±0.14[ab]
Nrf2	1.00±0.06[b]	1.45±0.11[a]	1.19±0.14[ab]
GST	1.00±0.06[b]	1.28±0.07[a]	1.09±0.06[ab]
Catalase	1.00±0.05	1.45±0.18	1.17±0.18
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	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	1.03±0.07	0.90±0.04

Values are means ± SE, n = 7-8. Groups with different letters are significantly different from each other, $P<0.05$ (Tukey's multiple-range test).

Table 4-4. Effect of dietary level of vitamin B6 on expressions of genes related to myogenesis and sarcopenia in gastrocnemius muscle of rats

Gene	PN HCl (mg/kg diet)		
	1	7	35
Myogenin	1.00±0.06[b]	1.52±0.20[a]	0.82±0.05[ab]
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 means ± SE, n = 6-8. Groups with different letters are significantly different from each other, $P<0.05$ (Tukey's multiple-range test).

Table 4-5. Effect of dietary level of vitamin B6 on expression of HSPs in gastrocnemius muscle of rats

Gene	PN HCl (mg/kg diet)		
	1	7	35
HSP60	1.00±0.09 [b]	1.66±0.19[a]	1.20±0.11[ab]
HSP70	1.00±0.07	2.00±0.48	1.23±0.28
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 means ± SE, n = 6-8. Groups with different letters are significantly different from each other, $P<0.05$ (Tukey's multiple-range test).

Table 4-6. Effect of dietary level of vitamin B6 on expression of microarray-genes in gastrocnemius muscle of rats

Gene	PN HCl (mg/kg diet)		
	1	7	35
Nrxn2	1.00±0.08[b]	1.85±0.23[a]	1.02±0.09[b]
Ntn4	1.00±0.08[b]	1.82±0.24[a]	1.14±0.18[b]

Values are means ± SE, n = 7-8. Groups with different letters are significantly different from each other, $P<0.05$ (Turkey's multiple-range test).

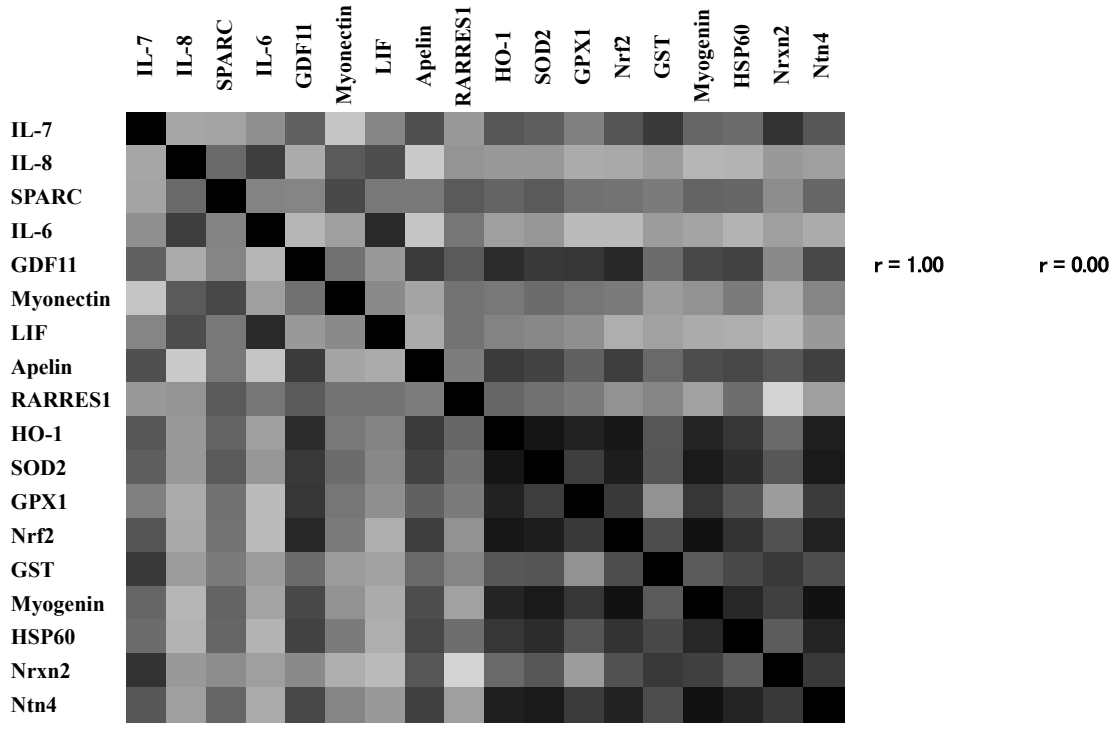

Fig. 4-1. Correlation matrix among the gene expressions altered by dietary vitamin B6. The color scheme corresponds to correlation strength as shown by the deep dark color bar.

VI. Summary

In conclusion, this chapter demonstrated that the recommended level of dietary vitamin B6 (7 mg PN HCl/kg) elevated the expression of several myokine genes, Nrf2-regulated genes, myogenin, HSP60, Nrxn2, and Ntn4 compared with a low vitamin B6 diet (1 mg PN HCl/kg). However, there was a less effect with excessive dietary levels of vitamin B6 (35 mg PN HCl/kg). This implies that the recommended level of dietary vitamin B6 is critical for the expression of these genes in the gastrocnemius muscle of rats. These factors whose gene expressions were affected by dietary vitamin B6 may be beneficial for muscle health. Intriguingly, the elevation in the expression of such genes by supplemental vitamin B6 (7 mg PN HCl/kg) to a low vitamin B6 diet (1 mg PN HCl/kg) were similar to the responses to exercise. Accordingly, these findings provide novel insights into the physiological role of vitamin B6 in skeletal muscles as an exercise-like factor. To our knowledge, this is the first evidence indicating dietary factor plays a pivotal role in gene expression of myokines in skeletal muscle. Further study will be required to understand the role of vitamin B6 in skeletal muscles.

Chapter 5 Conclusion

Pyridoxal 5′-phosphate (PLP), the active coenzyme form of vitamin B6, is involved in almost enzymatic reaction of amino acid metabolism as a cofactor. One of the important function of vitamin B6 is its role in the 1-carbon metabolic pathway, modulating the concentration of homocysteine, a non-protein amino acid. This function is linking vitamin B6 to its function as anti-disease factor, since lower vitamin B6 is known to higher the level of homocysteine considered as the risk of atherosclerosis. Increasing evidence reported vitamin B6 prevents several certain diseases, including heart disease. A case-study also demonstrated that vitamin B6 supplementation is beneficial for McArdle disease, a glycogenetic myopathy.

Accumulating evidence suggests several amino acids and their metabolites play pivotal roles in the development of several diseases such as cancers, atherosclerosis, and brain diseases. Some amino acids and their metabolites are also reported to positively affect muscle performance. Furthermore, some reports indicate the modulation of concentrations of free amino acids in blood and tissues as a result of vitamin B6 deficiency. However, information on the role of dietary vitamin B6 in the regulation of the blood and tissue concentrations of amino acids is insufficient. Recent evidence suggests marginal vitamin B6 deficiency is common in the US and Japan, and is associated an elevated risk of chronic diseases. My study aimed to elucidate the responses of amino acids and related metabolites to marginal vitamin B6 deficiency in the heart and skeletal muscle of rats.

The preliminary experiment using amino acid analyzer was conducted to examine the response of amino acids in the heart of rats fed supplemental dietary vitamin B6 compared to a marginal vitamin B6-deficient diet. The concentrations of carnosine in the 7 and 35 mg PN HCl/kg groups were higher 123% and 121%, respectively, than the 1 mg PN HCl/kg group. Carnosine is known to act as antioxidant, pH-buffering, anti-glycation, and ergogenic factors. Hence, I was interested at carnosine response to dietary vitamin B6 since there is no nutrient reported as a determinant of carnosine yet.

In the first study, I mainly focused on the effect of dietary vitamin B6 on the response of carnosine and its methylated analogue, anserine, in the heart of rats. Male rats were fed with dietary vitamin B6 supplementation (7 and 35 mg PN HCl/kg) or a marginal vitamin B6-deficient diet (1 mg PN HCl/kg) for 6 weeks. By using UPLC-MS/MS method, the results in this chapter demonstrated that dietary vitamin B6 supplementation to a marginal vitamin B6-deficient diet markedly increases the concentrations of carnosine and anserine in the heart of rats. Carnosine and anserine are reported

to exert anti-inflammatory and anti-ischemic effects on the heart. The present results suggest a possibility that dietary vitamin B6 supplementation improves the functions of heart by elevating the concentrations carnosine and anserine.

Blood PLP concentration is reported to be increased by exercise. In this study, I found that serum PLP concentration was markedly increased by dietary vitamin B6 supplementation (Suidasari et al., 2015a). On the other hand, exercise is suggested to release carnosine from the skeletal muscles into the bloodstream that is considered to decrease blood pressure in rats and humans. In this study I also measured serum carnosine concentration by ELISA, and found that vitamin B6 supplementation increased serum carnosine concentration. Vitamin B6 supplementation is reported to attenuate blood pressure in rat models of hypertension. Thus, the elevated serum carnosine concentration caused by dietary vitamin B6 supplementation may at least be partially related to the anti-hypertensive effect of vitamin B6.

The concentrations of carnosine and anserine are reported to be highest in the skeletal muscle compared to other tissues. Hence, in the second study, I was interested to explore the effect of dietary vitamin B6 on the concentrations of carnosine, anserine, and β-alanine, the precursor of carnosine, in skeletal muscles of male and female rats. I found that dietary vitamin B6 supplementation compared to a marginal vitamin B6-deficient diet significantly increased the concentrations of carnosine in the skeletal muscles of male and female rats (Suidasari et al., 2015b). Carnosine is known as an ergogenic factor. Its mechanism probably relates to the dipeptides' capacity as an antioxidant, pH-buffering, and/or calcium regulator Thus, the present results imply that maintaining recommended amounts of dietary vitamin B6 (7 mg PN HCl/kg) and/or higher amounts might be favorable for skeletal muscles function by elevating carnosine. This result was partly confirmed in human samples. I found that women have significantly reduced muscle carnosine content in the lowest PLP tertile compared to the higher tertiles (Suidasari et al., 2015b). The results of this study suggest dietary vitamin B6 is a determinant of carnosine concentration in mammalian skeletal muscles. To my knowledge, this is the first evidence suggesting a specific determinant up-regulating muscle carnosine and β-alanine concentration. This study also suggests the importance of the biosynthesis of β-alanine from ornithine in skeletal muscle rather than that from uracil in the liver.

The results in the second study showed that carnosine and PLP concentrations were markedly elevated by dietary supplemental vitamin B6. Both blood carnosine and PLP concentrations are also reported to be elevated by exercise. Thus, in the third study, I investigated the effect of dietary vitamin B6 on the expressions of genes related to exercise. The results demonstrated that the recommended

level of dietary vitamin B6 (7 mg PN HCl/kg) elevated the expression of several myokine genes, Nrf2-regulated genes, myogenin, HSP60, Nrxn2, and Ntn4 compared with a low vitamin B6 diet (1 mg PN HCl/kg) (Suidasari et al., 2015c). However, there was a less effect with excessive dietary levels of vitamin B6 (35 mg PN HCl/kg). This implies that the recommended level of dietary vitamin B6 is critical for the expression of these genes in the gastrocnemius muscle of rats. These factors whose gene expressions were affected by dietary vitamin B6 may be beneficial for muscle health. This findings provide novel insights into the physiological role of vitamin B6 in skeletal muscles as an exercise-like factor.

In conclusion, these studies indicated dietary vitamin B6 supplementation is a determinant of carnosine concentration in the heart, skeletal muscles, and serum of rats. Although brain also contains high concentration of carnosine, the level of carnosine in the brain was unaffected by dietary vitamin B6 (data not shown). Thus, the effects of dietary vitamin B6 appear to be specific for the heart, skeletal muscles and blood. Some of the findings in skeletal muscle could only be partly confirmed in human volunteers. Carnosine has been considered to have ergogenic effects and to be beneficial for heart disease. My study further indicated dietary vitamin B6 is a determinant of gene expressions of several factors playing important roles in skeletal muscle. Taken together, my studies provide a novel insight into the role of vitamin B6 in the heart and skeletal muscles.

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