Effects of B Vitamins Overload on Plasma Insulin Level and Hydrogen Peroxide Generation in Rats

Wuping Sun 1, Mingzhu Zhai 2, Qian Zhou 1, Chengrui Qian 1, and Changyu Jiang 1

1 Department of Pain Medicine, The Affiliated Nanshan People’s Hospital of Shenzhen University, Shenzhen Municipal Sixth People’s Hospital, Shenzhen 518060, People’s Republic of China

2 Institute of Science and Technology Austria, Am Campus 1, Klosterneuburg 3400, Austria

Abstract

It has been reported that nicotinamide-overload induces oxidative stress associated with insulin resistance, the key feature of type 2 diabetes mellitus (T2DM). This study aimed to investigate the effects of B vitamins in T2DM. Glucose tolerance tests (GTT) were carried out in adult Sprague-Dawley rats treated with or without cumulative doses of B vitamins. More specifically, insulin tolerance tests (ITT) were also carried out in adult Sprague-Dawley rats treated with or without cumulative doses of Vitamin B3. We found that cumulative Vitamin B1 and Vitamin B3 administration significantly increased the plasma H2O2 levels associated with high insulin levels. Only Vitamin B3 reduced muscular and hepatic glycogen contents. Cumulative administration of nicotinic acid, another form of Vitamin B3, also significantly increased plasma insulin level and H2O2 generation. Moreover, cumulative administration of nicotinic acid or nicotinamide impaired glucose metabolism. This study suggested that excess Vitamin B1 and Vitamin B3 caused oxidative stress and insulin resistance.

Key Words: B vitamins, insulin resistance, nicotinamide, nicotinic acid, oxidative stress

Introduction

Type 2 diabetes mellitus (T2DM), characterized by hyperglycemia and insulin resistance, has become a major global health concern (7). Increased plasma glucose levels and decreased insulin sensitivity, i.e., insulin resistance, are the characteristics of T2DM. It has been demonstrated that insulin resistance is caused by oxidative stress due to overproduction of reactive oxygen species (ROS) (5, 10, 11, 29). Therefore, clarification of the factors that regulate ROS production and insulin resistance is of great importance in determining the etiology of T2DM.

Increasing evidence has indicated that changes in dietary patterns may play a key role in the development of T2DM and T2DM-related metabolic diseases (2, 22, 24). In the US, the rapid nationwide increase in the prevalence of T2DM has occurred following the mandatory food fortification with vitamins, which started in the early 1940s. Similarly, sudden nationwide increase in T2DM in China also occurred following the execution of vitamin fortification started since the early 1980s. The incidence of diabetes in China increased sharply from 1% in the early 1980s before vitamin fortification to 5.5% in the early 2000s (9), and up to 11.7% in 2014, about three decades after vitamin fortification (28). In contrast, non-fortifying countries, such as Norway, has a low incidence of type 2 diabetes (4, 21). Thus, there is a possibility that the dietary risk factors may...
involve vitamin overload.

It was suspected that the simultaneous increases in obesity in almost all countries seemed to be driven mainly by changes in the global food system (26). Indeed, excessive B vitamins intake is a very common phenomenon. For example, the US per capita daily consumption of Vitamins B1, B2, B3 (nicotinic acid and nicotinamide) and B6 has been increasing from 1.4, 1.8, 16 and 1.9 mg in the latter 1930s to 3.0, 2.9, 35 and 2.5 mg in the early 2010s1. The consumption of Vitamins B1, B2, B3 and B6 in US population has far exceeded the recommended dietary allowance (RDA) by US Food and Nutrition Board2. Therefore, it seems clear that excess B vitamins mainly come from vitamin fortification. This raises the possibility that obesity and T2DM may involve excess B vitamins intake.

It has also been reported that excess nicotinamide, a major form of Vitamin B3, may induce oxidative stress associated with insulin resistance, suggesting that excess nicotinamide may be involved in T2DM (29). Indeed, the data of previous study demonstrated that T2DM subjects had a slow rate of detoxification of nicotinamide (29). Both low-dose supplementation (fortification doses) (30) and high-dose nicotinic acid (lipid-lowering doses) (1, 8) are found to be associated with increased risk for T2DM. Low-dose supplementation is associated with increased prevalence of diabetes with a lag time of 26 years (30), while high-dose nicotinic acid therapy is found to be associated with a rapid increase in diabetes rates within years (1, 8). Previous reports have also shown that in animal studies excess nicotinamide causes DNA hypomethylation, an important epigenetic alteration in diseases (16, 27). Nicotinamide supplementation induces tissue-specific alterations in the mRNA expression of the genes encoding nicotinamide N-methyltransferase (NNMT), DNA methyltransferase 1, catalase and tumour protein p53 in the placenta and fetal liver of rats (27). Our previous study further demonstrated that excess nicotinamide inhibited methylation-mediated degradation of catecholamines due to competition of methyl groups (25). These data provided evidences for the first time that food fortification-induced nicotinamide overload may play a role in the development of T2DM and T2DM-related diseases. Because several other B vitamins have also been used to fortify grain products in the US and other countries, it may be of great importance to determine whether these B vitamins are involved in T2DM. Moreover, the involvement of nicotinic acid in T2DM is still unknown. Therefore, this study aimed to investigate the involvement of B vitamins overload in insulin resistance and ROS generation.

Materials and Methods

Chemicals

Vitamin B1 (Thiamin), Vitamin B2 (riboflavin), Vitamin B6 (pyridoxine) and Vitamin B3 (nicotinamide and nicotinic acid) were purchased from Sigma (St. Louis, MO, USA). N1-methylnicotinamide (NMN) was purchased from Takeda Chemical Industries (Osaka, Japan).

Animals and Experimental Designs

All animal protocols were approved by the Animal Research Committee of Nanshan People’s Hospital, Shenzhen, China, and were performed in accordance with institutional guidelines. Male Sprague-Dawley rats between 180-220 g were housed in a controlled environment with 12 h light/dark cycle, 22-24°C and 50-60% humidity, and with food and water ad libitum. The rats were fasted for 14 h before the experiments.

In the experiments comparing the effects of Vitamins B1, B2, B6 and nicotinamide, rats were randomly divided into five groups (each n = 7): the control group and four drug-treated groups, respectively. The rats in the four drug-treated groups were intraperitoneally administered Vitamin B1 (100 mg/kg), Vitamin B2 (100 mg/kg), nicotinamide (400 mg/kg) and Vitamin B6 (100 mg/kg), and the intraperitoneal administration was repeated every 2 h for 5 doses. The rats in the control group were given saline. Blood was collected into heparin tubes under urethane anesthesia (1.5 g/kg, i.p.) 2 h after glucose administration. Plasma was separated by centrifugation at 1,500 g for 10 min. Aliquots of plasma and harvested samples of liver and gastrocnemius muscle were directly plunged into liquid nitrogen and subsequently stored at -80°C until assay.

In the experiments comparing the effects of nicotinic acid and nicotinamide, rats were randomly divided into four groups: the control group and three drug-treated groups, each n = 7. The rats in the drug-treated groups were intraperitoneally administered nicotinic acid (400 mg/kg or 800 mg/kg) and nicotinamide (400 mg/kg), and the administration was repeated every 2 h for 5 doses. The rats in the control groups were given saline. Glucose (2 g/kg, i.p.) was given 2 h after the final injection. Samples of

---

2 In Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline, Washington (DC): 1998
plasma, liver and gastrocnemius muscle were collected and stored 2 h after glucose administration.

Glucose tolerance tests (GTT) were performed by intraperitoneal injection of glucose at 2 g/kg 2 h after the last injection of saline or vitamins according to a previous method (3). Insulin tolerance tests (ITT) were conducted 2 h after the last injection of saline or vitamins according to a reported method (19). Porcine insulin, at 1 unit/kg bodyweight, was injected into the intraperitoneal space. Blood glucose level was measured before and 15, 30, 45, 60, and 120 min after glucose or insulin injection.

**Assays of Glucose, Insulin, Glycogen and H$_2$O$_2$**

Blood glucose was measured using a glucometer (OneTouch Ultra, LifeScan Inc., New Jersey, USA). Within- and between-assay precision values for replicate quality control samples were within 6% and 8%, respectively, for the following three measurements.

Plasma insulin was measured by radioimmunoassay using commercial kits (Beijing North Institute of Biological Technology, Beijing, PRC). One hundred-microliter samples, including standards and quality controls, were prepared in duplicates, and 100 μl hydrated $^{125}$I-Insulin was added to all samples. Then, 100 μl rat insulin antibody was added to all samples, mixed well and incubated overnight at 4°C. Subsequently, 1.0 ml of cold precipitating reagent was added to all samples, mixed well and incubated for 20 min at 4°C. All samples were then centrifuged at 4°C for 20 min at 2,000-3,000 g. After centrifugation, the supernatants of all samples were removed, and the radioactivities were counted in a gamma counter for 1 min. Finally, the concentrations of rat plasma insulin were calculated in the unknown samples using automated data reduction procedures.

Glycogen contents of the muscle and liver were determined with the glycogen assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, PRC). In brief, fresh liver or muscle samples were collected and rinsed with physiological saline, dried by filter paper and weighed to a sample weight of about 100 mg. An adjusted volume of alkaline solution was added to each sample for hydrolysis, mixed well and incubated at room temperature for 30 min. The mixture was then put into a boiling water bath for 3 min, followed by cooling down on ice. After that, 1 ml liver glycogen determination solution or 0.2 ml muscle glycogen determination solution was added for hepatic and muscular glycogen concentration measurement, respectively. Optical density (OD) values of all samples were measured at 620 nm for glycogen level measurement.

H$_2$O$_2$ concentrations in rat plasma were measured using an H$_2$O$_2$ assay kit (Beyotime Biotechnology, Jiangsu, PRC). In brief, 5 μl plasma from each sample or standard was diluted 50 times with 50 mM phosphate buffer, pH 6.0, and 50 μl diluted samples or standards were transferred into a 96 well plate. Then, 100 μl H$_2$O$_2$ assay reagent were added, mixed well and incubated at room temperature for 30 min. The H$_2$O$_2$ assay reagent generates ferric ion by oxidation of ferrous iron in each sample to form a purple product with xylene orange in a specific solution. OD values of all samples were measured at 560 nm for H$_2$O$_2$ measurement.

**Determination of NMN**

NMN is a major nicotinamide metabolite degraded from NNMT catalyzation. NMN was analyzed using a high-performance liquid chromatography (HPLC) system consisting of a LC-9A pump (Shimadzu, Kyoto, Japan), a Rheodyne 7725i sample injector with a 20 μl-sample loop (Rheodyne LLC, Rohnert Park, CA, USA), a Hypersil ODS C18 column (Thermo, Bellefonte, PA, USA) and a Waters 470 fluorescence detector (Milford, MA, USA). NMN concentration was determined by detecting of its fluorescent 1,6-naphthyridine derivatives according to the method of Musfeldt C et al. (20) using 366 nm excitation and 418 nm emission wavelength, with the mobile phase, which was composed of 10 mM sodium heptanesulfonate, 0.5% triethylamine and 22% acetonitrile in water, pH 3.2, with 85% H$_3$PO$_4$, at a flow rate 1 ml/min. The experiments were performed at room temperature. Within- and between-assay precision values for replicate quality control samples were within 7% and 10% for all the analyses.

**Statistical Analysis**

The data are presented as mean ± standard error of the mean (SEM). Statistical differences in the data were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc tests or repeated measures ANOVA followed by Dunnett’s post hoc tests as appropriate. Results were considered significant at $P < 0.05$.

**Results**

**Effects of B Vitamins on Blood Glucose Concentrations and Tissue Glycogen Contents**

The effects of B vitamins on the blood glucose concentrations and the hepatic and muscular glycogen contents are shown in Fig. 1. No significant differences were observed in the blood glucose either before or 1 h after glucose administration among the groups treated with or without different B vitamins (Fig. 1A).
Vitamin B6-treated group showed a statistically significant increase in hepatic glycogen content ($P < 0.01$), but there were no significant changes in the muscular glycogen content, compared with control ($P > 0.05$) (Figs. 1B and 1C). Treatment of nicotinamide led to a significant decrease in the hepatic and muscular glycogen contents ($P < 0.05$), whereas Vitamins B1 and B2 treatments were without significant effects on either the muscular or hepatic glycogen contents (all $P > 0.05$ vs. control) (Figs. 1B and 1C).

**Effects of B Vitamins on the Plasma Insulin and $H_2O_2$ Levels**

The plasma insulin levels in Vitamin B1- and nicotinamide-treated groups were significantly elevated compared with the control group ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 2A). Associated with the increase in plasma insulin levels, there was a significant increase in the plasma $H_2O_2$, the major ROS responsible for oxidative stress, in Vitamin B1-, nicotinamide- and Vitamin B6-treated groups, compared with control ($P < 0.05$ or 0.01 vs. control) (Fig. 2B).

**Comparison of the Effects of Nicotinic Acid and Nicotinamide on Plasma Insulin and $H_2O_2$ Levels**

Vitamin B3 comes in two forms, nicotinic acid and nicotinamide. Therefore, we next compared the effects of nicotinic acid and nicotinamide. NMN is a methylated intermediate metabolite of nicotinamide. It has been reported that plasma NMN is positively correlated with obesity and diabetes in humans (18). Administration of nicotinic acid, either 400 mg/kg or 800 mg/kg, and nicotinamide at 400 mg/kg both significantly increased the levels of plasma NMN (Fig. 3A). The effect was more profound in nicotinamide-treated rats. The level of plasma NMN in nicotinamide-
Fig. 3. Comparison of the effects of nicotinic acid and nicotinamide. Changes in the plasma levels of NMN (A), H$_2$O$_2$ (B) and insulin (C) in rats treated with or without cumulative nicotinic acid (NA) (400 or 800 mg/kg) or nicotinamide (NM) (400 mg/kg) after glucose loading. *$P < 0.05$ vs. control, **$P < 0.01$ vs. control.

Fig. 4. Effects of nicotinic acid and nicotinamide on glucose metabolism. (A & B) Changes in plasma glucose levels in rats treated with or without cumulative nicotinic acid (800 mg/kg) (A) or nicotinamide (400 mg/kg) (B) after glucose loading, or insulin administration (C & D). IPGTT, intraperitoneal GTT. *$P < 0.05$ or **$P < 0.01$ vs. control.
treated rats was almost five times higher than that in nicotinic acid-treated rats under similar exposure conditions. The results also showed that excessive nicotinic acid, like nicotinamide, enhanced the plasma H$_2$O$_2$ levels associated with high plasma insulin levels in a dose-dependent manner (Figs. 3B and 3C). It should be noted that the effects of nicotinic acid on plasma H$_2$O$_2$ and insulin levels were accompanied with the increase in plasma NMN, indicating that metabolism of nicotinic acid was partially through nicotinamide to NMN pathway (Fig. 3).

The Effects of Nicotinic Acid and Nicotinamide on Glucose Metabolism

To further examine the effects of nicotinic acid and nicotinamide on glucose metabolism, we carried out intraperitoneal GTT (IPGTT) and ITT in rats after accumulative nicotinic acid (800 mg/kg) and nicotinamide (400 mg/kg) administration. In the IPGTT experiments, the results showed that the blood glucose levels were significantly higher at 15 min ($P < 0.05$) and 30 min ($P < 0.05$ or $P < 0.01$) after glucose administration in both the accumulative nicotinic acid and nicotinamide administration groups, compared with the control group (Figs. 4A and 4B). Moreover, the results in the ITT experiments showed that the blood glucose levels were significantly higher at 60 min ($P < 0.05$) after insulin administration in both the accumulative nicotinic acid and nicotinamide administration groups (Figs. 4C and 4D).

Comparison of the Effects of Nicotinic Acid and Nicotinamide on Tissue Glycogen Contents

Insulin resistance is a reduced response capacity of peripheral tissues to physiological levels of circulating insulin, and results in hampered muscular and hepatic glycogen synthesis (12, 17). Therefore, we next compared glucose-induced glycogen synthesis in rat livers and skeletal muscles after nicotinic acid or nicotinamide administration. Glycogen contents in both the muscle and liver decreased significantly after either nicotinic acid or nicotinamide accumulative administration (Figs. 5A and 5B). Moreover, nicotinic acid administration-induced decrease of glycogen contents exhibited a dose-dependent manner. These results further suggested that both nicotinic acid and nicotinamide administration caused an insulin resistance.

Discussion

In T2DM, there are two distinct and separate phenomena: one is that dietary patterns play a key role in the development of T2DM (2, 22, 24); the other is the persistent oxidative stress-mediating insulin resistance (7). It may be argued that there should be some risk factors in food to trigger oxidative stress and insulin resistance. Therefore, identifying the food risk factors may be helpful in gaining insight into the pathogenesis of T2DA as well as to facilitate the development of new therapeutic approaches.
So far as food risk factors for T2DM are concerned, whether excess B vitamins play a role has largely been neglected, although these vitamins are well-known to be closely related to energy metabolism in the body. A previous study has found that nicotinamide overload may enhance H₂O₂ generation associated with insulin resistance (29), the key feature of T2DM. These findings suggest that nicotinamide overload may be involved in T2DM, thus raising another possibility of involvement of other B vitamins in the development of T2DM.

A previous report showed that there was a time-lag of about 26 years after niacin fortification until diabetes prevalence (30). Moreover, long-term niacin fortification could cause the accumulative effects of niacin and its metabolites in the human body. In addition, several previous reports demonstrated that daily intake of niacin up to 6.0 g or more was applied for the prevention of human diseases, such as diabetes (14), skin cancers (6) and cardiovascular disease (1, 8).

Importantly, it has been demonstrated that higher level of plasma NMN, a key metabolite of niacin, has been found in obesity and T2DM patients (13, 18). Therefore, we have applied relatively high doses of B vitamins in rats to examine the effects of B vitamins on insulin release and hydrogen peroxide generation in the current study. Our results indicated that there was an increase in the plasma insulin levels in the rats treated with B vitamins. More significantly, like the effects of nicotinamide overload, excess Vitamins B1 and B6 metabolism also significantly increased plasma H₂O₂ levels. Moreover, the present data demonstrated that nicotinic acid, another form of Vitamin B3, induced similar changes in plasma H₂O₂ and insulin levels as nicotinamide did. In addition, nicotinic acid and nicotinamide administration impaired glucose metabolism. These results suggest that excess Vitamins B1, B3 and B6 may be important sources of excess ROS, and thus trigger oxidative stress and insulin resistance and subsequent T2DM. Therefore, our current findings may help to explain firstly why vitamin fortification in the US has been followed by a significant increase in the prevalence of T2DM after about 20 years of fortification. Secondly, why abrupt nationwide increase in T2DM prevalence occurred in PRC (9), not only in the urban but also in the rural areas about 20 years after vitamin fortification started in the early 1980s, and thirdly, why Norway, a western country prohibiting food inhibition with vitamin, has a low incidence of T2DM compared with the US (4, 21). Thus, the relationship between the global prevalence of T2DM in the last few decades and the global increase in mandatory flour fortification with B vitamins may need to be reconsidered.

It is well known that insulin resistance is a reduced capacity of peripheral tissues to respond to physiological levels of circulating insulin, resulting in decreased synthesis of muscular and hepatic glycogen (12, 17). Our results indicated that Vitamin B3 caused an insulin resistance. Thus, it is possible that the reduction of glycogen contents in the liver and muscle in rats could be due to insulin resistance, which occurred upon treatment with Vitamin B3. On the other hand, Vitamin B1 treatment-induced insulin resistance and oxidative stress could not be as severe as the Vitamin B3 treatment, because the plasma hydrogen peroxide level in the Vitamin B1-treated rats was not as high as in the Vitamin B3-treated rats. This might be the reason why we failed to observe the decreased synthesis of glycogen in the muscle and liver of the Vitamin B1-treated rats. NNMT has been reported to be increased in the adipose tissue and liver in obese mice. NNMT knockdown also improved insulin sensitivity and prevented high fat diet-induced obesity in mice (15). Moreover, our previous data in animal studies indicated that excess nicotinamide caused DNA hypomethylation, which is an important epigenetic alteration in diseases (16, 27). Nicotinamide supplementation also induced tissue-specific alterations in the mRNA expression of the genes encoding NNMT, namely DNA methyltransferase 1, catalase and tumour protein p53, in the placenta and fetal liver of rats (27). Based on the previous studies and our data, Vitamin B3 overdose might cause an alteration in the expression level of NNMT, which could lead to oxidative stress and insulin resistance. Insulin resistance may further reduce the contents of muscular and hepatic glycogen, and subsequently causes T2DM. In addition, it has been reported that long-term nicotinamide intake impairs mitochondrial respiration capacity and energy production in skeletal muscle (23), which may be an alternative mechanism for Vitamin B3-induced insulin resistance and decreased glycogen synthesis in the liver and muscle.

In summary, we concluded that both Vitamin B1 and Vitamin B3 (nicotinic acid and nicotinamide) are risk factors in causing insulin resistance and oxidative stress. Therefore, B vitamins fortification and abuse may need to be reappraised.

**Acknowledgments**

This work was supported by grants from Shenzhen Municipal Science, Technology and Innovation Commission (No. JCYJ20160429182122843, No. JCYJ20160429181451546), Municipal Key Laboratory Program of Shenzhen (No. ZD-SYS20140509150415945).

**Conflict of Interest**

The authors declared no conflicts of interest.
References


