Plasma choline concentration varies with different dietary levels of vitamins B6, B12 and folic acid in rats maintained on choline-adequate diets.
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Abstract
Choline is an important component of the human diet and is required for the endogenous synthesis of choline-containing phospholipids, acetylcholine and betaine. Choline can also be synthesized de novo by the sequential methylation of phosphatidylethanolamine to phosphatidylcholine. Vitamins B_6, B_{12} and folate can enhance methylation capacity and therefore could influence choline availability not only by increasing endogenous choline synthesis but also by reducing choline utilization. In the present experiment, we determined whether combined supplementation of these B vitamins affects plasma choline concentration in a rat model of mild B vitamin deficiency which shows moderate increases in plasma homocysteine. To this end, we measured plasma choline and homocysteine concentrations in rats that had consumed a B vitamin-poor diet for 4 weeks after which they were either continued on the B vitamin-poor diet or switched to a B vitamin-enriched diet for another 4 weeks. Both diets contained recommended amounts of choline. Rats receiving the B vitamin-enriched diet showed higher plasma choline and lower plasma homocysteine concentrations as compared to rats that were continued on the B vitamin-poor diet. These data underline the interdependence between dietary B vitamins and plasma choline concentration, possibly via the combined effects of the three B vitamins on methylation capacity.
Chapter 3

Introduction

Choline is an important nutritional component for humans (Zeisel et al. 1991) and is required for the endogenous synthesis of phosphatidylcholine (PC), lysophosphatidylcholine, choline plasmalogen and sphingomyelin, which are all essential components of cell membranes. Choline is also a precursor for the biosynthesis of the neurotransmitter acetylcholine and can be metabolized in the liver to betaine, which under certain conditions serves as a source of methyl groups. Choline can be derived not only from the diet, but also from de novo synthesis, principally in the liver. Endogenous choline is produced by the sequential methylation of phosphatidylethanolamine (PE) to PC. Choline is then liberated from the newly formed PC and is released into the bloodstream. This is the only known endogenous pathway for choline biosynthesis in animals (Zeisel and Blusztajn 1994).

The methylation of PE to PC is catalyzed by PE-N-methyltransferase (PEMT), which requires the methyl donor S-adenosylmethionine (SAM). This reaction is influenced not only by the availability of SAM, but is also inhibited by S-adenosylhomocysteine (SAH), and the ratio of SAM:SAH (an indicator of methylation capacity) therefore affects the activity of PEMT (Vance et al. 1997). Moreover, SAH is hydrolyzed to homocysteine via a reversible reaction: thus excess homocysteine will result in increased SAH, thereby inhibiting PEMT (Yi et al. 2000). Vitamins B_6, B_12 and folate can support PEMT activity both by reducing homocysteine levels and by increasing methionine levels, resulting in an increased SAM:SAH ratio, and thus an increased methylation capacity. An alternative pathway for the regeneration of methionine from homocysteine in the liver is provided by the betaine-homocysteine methyltransferase pathway which involves the conversion of choline in the methyl donor betaine. This pathway thus utilizes choline (Zeisel and Blusztajn 1994) and is assumed to be less significant for maintaining methylation capacity when endogenous concentrations of B vitamins are sufficient (Holm et al. 2004, Jacob et al. 1999, Park and Garrow 1999, Yan et al. 2011). B vitamins may therefore affect choline availability not only by increasing endogenous choline synthesis through the PEMT pathway, but also by reducing choline utilization by the betaine-homocysteine methyltransferase pathway.

The interdependence between B vitamin intake and choline status is suggested by several animal (Akesson et al. 1979, Akesson et al. 1982, Chan et al. 2008, Kennedy et al. 1992, Kim et al. 1994, Loo and Smith 1986, She et al. 1995, Troen et al. 2008) and human
Dietary B-vitamins increase plasma choline levels (Abatte et al. 2009, Abatte et al. 2008, Hung et al. 2008, Jacob et al. 1999) studies. However, to date, research has concentrated on the effects of single B vitamins and on conditions of inadequate choline intake, but none of these reports has shown direct effects of B vitamins on plasma choline. The aim of the present experiment was to investigate the interdependence between dietary vitamins B<sub>6</sub>, B<sub>12</sub> and folate and plasma choline concentration in a dietary background of choline adequacy and mild B vitamin deficiency. The mild B vitamin deficiency was induced with a B vitamin-poor diet which was previously shown in our laboratory to induce a moderate increase in plasma homocysteine concentration (N. van Wijk and L. M. Broersen, unpublished results) and is thought to be relevant for conditions and/or diseases which are associated with mild B vitamin deficiencies and/or moderate increases in plasma homocysteine, such as ageing or Alzheimer’s Disease (AD) (Selhub et al. 2000, van Dam and van Gool 2009). To this end, we measured plasma choline and homocysteine concentrations in rats that had consumed supplemental amounts of vitamins B<sub>6</sub>, B<sub>12</sub>, and folic acid with recommended amounts of dietary choline after a mild B vitamin deficiency was induced.

**Methods**

**Animals**

A total of thirty-two male Sprague-Dawley rats (Charles River, Wilmington, MA, USA), aged 6 weeks at arrival, were housed in pairs at room temperature, under 12 h light – 12 h dark cycles. Animals had free access to food and water. Body weight and food intake were registered once a week. Experiments were carried out in accordance with the 1996 Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and Massachusetts Institute of Technology policies and were approved by the Committee on Animal Care at Massachusetts Institute of Technology (Cambridge, MA, USA).

**Diets**

Two different diets with varied vitamins B<sub>6</sub>, B<sub>12</sub> and folic acid contents were used: (1) B vitamin-poor; (2) B vitamin-enriched. Diets were AIN-93M based (Reeves et al. 1993), isoenergetic and identical with respect to their protein, carbohydrate, fat, fiber, mineral and choline contents. The vitamin mix (AIN-93-VX) was prepared without vitamins B<sub>6</sub>, B<sub>12</sub> and folic acid and these vitamins were subsequently supplemented accordingly. Choline was added at AIN-93M levels, i.e. 1·0 g/kg (Reeves et al. 1993), which meets the minimal requirements for rats (National Research Council 1995). Diets were formulated with
vitamin-free, ethanol-precipitated casein (Harlan Teklad, Madison, WI, USA) and were manufactured by Research Diet Services (Wijk bij Duurstede, The Netherlands; reference no. 1652B).

The B vitamin-poor diet contained low amounts of vitamin $B_6$ (<0.6 mg/kg), $B_{12}$ (<1.0 µg/kg) and folic acid (<0.1 mg/kg). No sulfathiazole drugs were added to the diet and therefore a limited amount of folate was still expected to be provided by the gut flora. Induction of vitamin $B_{12}$ deficiency in the rat is difficult to achieve because of significant endogenous storage of vitamin $B_{12}$. To attain a moderate reduction of endogenous vitamin $B_{12}$, the diets were supplemented with 50 g/kg pectin (polygalacturonic acid, high methoxyl, Obipektin®, NF/USP Citrus; TEFCO FoodIngredients b.v., Bodegraven, The Netherlands), which binds vitamin $B_{12}$ in the intestine, making it less bioavailable (Cullen and Oace 1989). Pectin consequently promotes depletion of endogenous vitamin $B_{12}$ through the enterohepatic circulation of vitamin $B_{12}$. However, since pectin could affect food intake (Hove and King 1979), the B vitamin-enriched diet also contained pectin to maintain uniform intakes of the diets. Pectin has minimal effects on vitamin $B_{12}$ status when the diet contains adequate amounts of this vitamin (Cullen and Oace 1989).

The B vitamin-enriched diet was supplemented with 20.0 mg/kg vitamin $B_6$, 0.2 mg/kg vitamin $B_{12}$ and 4.0 mg/kg folic acid. For each, the diet provided 400% of the recommended daily intake according to the National Research Council report on the nutrient requirements of laboratory animals (National Research Council 1995).

B vitamin paucity was first induced in all rats by feeding them the B vitamin-poor diet for 4 weeks. Subsequently, animals were either continued on the B vitamin-poor diet or switched to the B vitamin-enriched diet for another 4 weeks. Previously, it was shown in our laboratory (N. van Wijk and L. M. Broersen, unpublished results) that the B vitamin-poor diet increased plasma homocysteine concentration from 7.3 (SEM 0.4) µM (control levels) up to 10.4 (SEM 0.8) µM and 9.2 (SEM 0.8) µM after the diet was consumed for 4 and 8 weeks, respectively.

**Tissue preparation**
After the supplementation period, animals that were fasted for 3-4 h were killed by CO$_2$ gas inhalation and subsequent decapitation by guillotine. Trunk blood was collected
through a funnel into EDTA-containing tubes. After centrifugation at 1750 g for 10 min, plasma was aspirated and analyzed for plasma homocysteine and choline.

**Plasma free choline and plasma total homocysteine assay**

High performance liquid chromatography (HPLC)-electrochemical detection of plasma free choline was performed according to a method adapted from Fossati (Fossati et al. 1994). After protein precipitation, samples were centrifuged to remove proteins. The supernatant was injected into the HPLC using a post-column immobilized enzyme reactor, in an on-line enzyme reaction to produce H$_2$O$_2$, which was detected electrochemically.

Plasma total homocysteine was determined by fluorometric HPLC as previously described (Krijt et al. 2001). Briefly, thiol amino acids (free and protein-bound) were reduced with tri-$n$-butylphosphine. After protein precipitation and centrifugation to remove the proteins, thiol groups were derivatized with 7-fluoro-2-oxa-1,3-diazole-4-sulfonamide reagent. The content of the derivatized thiol amino acids was determined by fluorescence detection with excitation at 385 nm and emission at 515 nm.

**Statistical analyses**

All statistical analyses were performed using SPSS (version 15.0; SPSS Inc., Chicago, IL, USA). Data were expressed as means with their standard errors (SEM). P-values $<$0.05 were considered significant. Variables were checked for normal distribution with Shapiro-Wilk’s test. Effects of dietary B vitamins on body weight and food intake were analyzed using repeated-measures analysis of variance (ANOVA) with dietary B vitamins as between-subject factor and week as within-subject factor. Plasma choline and homocysteine concentrations were compared between rats fed the B vitamin-enriched and B vitamin-poor diet using ANOVA. Standard Pearson correlation coefficients were calculated for plasma choline and homocysteine.

**Results**

At the start of the 4-week supplementation period, animals were randomized into the experimental groups according to their body weights. During the entire experimental period, body weight ($F_{(1,30)}=0.02$, $P=0.89$) and food intake ($F_{(1,14)}=2.92$, $P=0.11$) were unaffected by dietary B vitamins.
After the supplementation period, analyses of plasma samples revealed that plasma free choline concentration was up to 10% higher in rats fed the B vitamin-enriched diet than in rats that were continued on the B vitamin-poor diet ($F_{(1,30)}=9.52$, $P<0.005$; Fig. 1(a)). In addition, plasma total homocysteine concentration was lower in animals receiving the B vitamin-enriched diet as compared to the B vitamin-poor group ($F_{(1,30)}=56.95$, $P<0.001$; Fig. 1(b)). A significant negative correlation between plasma concentrations of choline and homocysteine was observed within animals fed the B vitamin-poor diet ($r=-0.545$, $P=0.029$, inset) but not in the B vitamin-enriched group ($r=-0.006$, $P=0.98$, inset).

**Fig. 1**  Plasma free choline and plasma total homocysteine concentrations and their correlation in rats that either received a B-vitamin-poor diet for 8 weeks (B-vitamin-poor) or a B-vitamin-poor for 4 weeks diet followed by a B-vitamin-enriched diet for 4 weeks (B-vitamin-enriched). Values are means, with their standard errors represented by vertical bars. * Mean values were significantly different ($P<0.005$).
Discussion

The present study investigated the effects of dietary B vitamin supplementation on plasma parameters in a rat model of mild B vitamin deficiency and choline adequacy. The results demonstrate for the first time that both plasma homocysteine and plasma choline concentrations are dependent on dietary intake of vitamins B₆, B₁₂ and folic acid in rats. Rats receiving the B vitamin-enriched diet showed higher plasma choline and lower plasma homocysteine concentrations as compared to rats that were continued on the B vitamin-poor diet. These findings add to previous findings in animals (Akesson et al. 1979, Akesson et al. 1982, Chan et al. 2008, Kennedy et al. 1992, Kim et al. 1994, Loo and Smith 1986, She et al. 1995, Troen et al. 2008) and humans (Abratte et al. 2009, Abratte et al. 2008, Hung et al. 2008, Jacob et al. 1999) and underline an interdependence between dietary B vitamins and plasma choline concentration. A negative correlation between plasma choline and plasma homocysteine was observed solely in the B vitamin-poor group, indicating their mutual dependency on B vitamins. Thus, even at moderately increased plasma homocysteine levels choline status can be compromised, even in a situation where dietary choline intake is considered adequate. This observation is relevant for elderly and AD patients who frequently show mild B vitamin deficiencies and/or moderate plasma homocysteine increases (Selhub et al. 2000, van Dam and van Gool 2009) and could therefore be at risk of an affected choline status.

The effects of dietary B vitamin levels on plasma choline concentration may have been mediated by enhancing methylation capacity. An enhanced methylation capacity could influence choline availability not only by increasing endogenous choline synthesis through the PEMT pathway but also by reducing choline utilization by the betaine-homocysteine methyltransferase pathway. It is important to note that vitamins B₆, B₁₂ and folate all play a crucial role in enhancing methylation capacity. Folate (as 5-methyl-tetrahydrofolate) and vitamin B₁₂ are cofactors in the remethylation reaction catalyzed by the enzyme methionine synthase which transforms homocysteine to methionine, from which SAM is subsequently regenerated. Vitamin B₆ is involved in facilitating the reversible conversion of serine to glycine which generates 5,10-methylene-tetrahydrofolate, which can be reduced to 5-methyl-tetrahydrofolate, i.e. the methyl donor for the remethylation of homocysteine to methionine by methionine synthase. Vitamin B₆ is also the cofactor for the transsulfuration reaction responsible for the irreversible conversion of homocysteine to cysteine, i.e. the clearance of homocysteine. It can be speculated that the effects of the three B vitamins on methylation capacity are additive and therefore have a greater impact.
on choline metabolism than each B vitamin individually, presumably explaining the observed efficacy in the present experiment encompassing combined supplementation of the three B vitamins.

Poor B vitamin status has been associated with poor cognitive functioning, dementia and AD (Selhub et al. 2000), while high B vitamin intake has been associated with a reduced risk of AD (Luchsinger et al. 2007). Several hypotheses have been proposed to explain the link between B vitamins and cognitive function, generally invoking effects of B vitamins to reduce presumed neurotoxic homocysteine levels and to increase methylation capacity (Miller 2003, Selhub et al. 2000). The present data suggest that this association may at least in part be due to the effects of B vitamins on methylation capacity and subsequent effects on choline availability. Small increases in plasma choline can exert significant effects on brain choline levels (Klein et al. 1990), which in turn control the rates at which it is utilized to form acetylcholine and phospholipids (Wurtman et al. 2009, Wurtman et al. 1980).

In the present experiment we showed, for the first time, a direct effect of varying dietary B vitamin levels on plasma choline concentration in rats. The fact that B vitamins influence choline availability might be one of the mechanisms by which B vitamins influence cognition.
Disclosure statement
This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors. NvW, RJH, PJGHK and LMB are all employees of Danone Research, Centre for Specialised Nutrition. RJW is a scientific consultant of Danone Research, Centre for Specialised Nutrition. CJW, MB and TJM have no conflicts of interest to declare.

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List of abbreviations
AD, Alzheimer’s disease
ANOVA, analysis of variance
HPLC, high performance liquid chromatography
PC, phosphatidylcholine
PE, phosphatidylethanolamine
PEMT, phosphatidylethanolamine-N-methyltransferase
SAH, S-adenosylhomocysteine
SAM, S-adenosylmethionine.
SEM, standard error of means
References


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