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Niacin (nicotinic acid) in non-physiological doses causes hyperhomocysteineaemia in Sprague–Dawley rats

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Niacin (nicotinic acid) in its non-physiological dose level is known to be an effective lipid-lowering agent; its potential risk as a therapeutic agent, however, has not been critically considered. Since niacin is excreted predominantly as methylated pyridones, requiring methionine as a methyl donor, the present study was undertaken to examine whether metabolism of the amino acid is altered in the presence of large doses of niacin. Male Sprague–Dawley rats were given a nutritionally adequate, semi-synthetic diet containing niacin at a level of either 400 or 1000 mg/kg diet (compared to 30 mg/kg in the control diet) for up to 3 months. Supplementation with niacin (1000 mg/kg diet) for 3 months resulted in a significant increase in plasma and urinary total homocysteine levels; this increase was further accentuated in the presence of a high methionine diet. The hyperhomocysteineaemia was accompanied by a significant decrease in plasma concentrations of vitamins B_6 and B_{12}, which are cofactors for the metabolism of homocysteine. The homocysteine-raising action of niacin, in particular, has an important toxicological implication, as hyperhomocysteineaemia is considered to be an independent risk factor for arterial occlusive disease. The niacin-associated change in homocysteine status may be an important limiting factor in the use of this vitamin as a lipid-lowering agent.

Niacin: Homocysteine: Methionine: Cysteine: B-vitamins

Niacin (nicotinic acid; vitamin B_3) in large doses (>2·0 g/d) has been known to be effective in the treatment of hyperlipidaemia (Goldberg, 1998; Guyton, 1998; Knopp, 1998). The potential risk of long-term usage of large doses of niacin has not been critically considered. Its only consistent deleterious effect in high doses has hitherto been cutaneous flushing and/or itching (McKenney *et al.* 1994; Capuzzi *et al.* 1998), which are thought to be caused by prostaglandin-mediated vasodilatation (Keenan *et al.* 1991).

Niacin is excreted as methylated pyridones (Shibata & Matsuo, 1989). By way of these metabolites, methylation occurs through a simple methyl transfer reaction, in which S-adenosylmethionine is the methyl donor. Niacin is a water-soluble vitamin and, therefore, is not stored in the body beyond its tissue saturation level. Since niacin excretion is dependent upon the availability of methionine, an intake of niacin in large amounts may affect the metabolism of this essential amino acid. Such an effect has been demonstrated in a recent study (Basu & Mann, 1997), as evidenced by niacin-associated increases in plasma and urinary levels of homocysteine in rats. This is an important observation, since homocysteine is thought to be an independent risk factor for arterial occlusive disease (Verhoef *et al.* 1997; Jacobsen, 1998; Welch & Loscalzo, 1998; Finkelstein, 2000). The study, however, measured the free homocysteine fraction, which accounts for only a small percentage of the total homocysteine (Mansoor *et al.* 1992). More than 80 % of homocysteine in blood is bound to protein by a disulfide linkage, and during storage there may be redistribution of thiols resulting in an increase in the protein-bound fraction at the expense of the free form. It is, therefore, important to determine whether niacin treatment modifies the total homocysteine status.

Using Sprague–Dawley rats the present study was undertaken to investigate the modifying effect of non-physiological dose levels of niacin on methionine metabolism and its consequence on total homocysteine status. Methionine synthesis requires 5-methyl tetrahydrofolate as a methyl donor and vitamin B_{12} as a cofactor; furthermore, its degradation to cysteine is catalysed by

Abbreviation: PLP, pyridoxal-5′phosphate.

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cystathionase, for which vitamin B_6 is the cofactor (Stipanuk, 1986). To elucidate the biochemical basis of the presumed hyperhomocysteineaemic effect of niacin, the biochemical status of vitamins B_{12} and B_6, as well as folate, was measured in niacin-treated rats.

Materials and methods

Animals and diets

Male Sprague–Dawley rats (weighing 150–200 g) were used throughout the study. They were individually housed in stainless steel metabolic cages in a well-ventilated room maintained at approximately 21°C and were on a 12 h light–dark cycle. All animals were fed a pellet diet (Laboratory Rodent Diet no. 5001; PMI Feeds Inc., Richmond, Ontario) for 1 week before being fed an experimental semi-synthetic diet (Table 1). The study protocol was approved by the Animal Welfare Committee of the University of Alberta.

After a run-in period (1 week) the rats were randomly divided into five groups of six each. Group 1 animals were fed a semi-synthetic diet containing physiological levels of niacin (Table 1) and were used as controls (diet A). Groups 2 and 3 were fed the control diet containing niacin at two additional dose levels: 400 mg (diet B) and 1000 mg (diet C)/kg diet. Groups 4 and 5 were fed the semi-synthetic diet supplemented with 10 g methionine/kg diet, either alone (diet D) or in combination with 1000 mg niacin/kg diet (diet E). The dose levels of niacin are close to those consumed by humans on a body weight basis. This calculation is based upon the fact that the niacin dose level, necessary to achieve its lipid-lowering effect in humans, is usually in the range of 2–6 g/d (Guyton, 1998). The rats used in the present study weighed 150–200 g and they ate approximately 20 g food/d. All animals had free access to water and their respective diets for 1–3 months.

Table 1. Composition of the semi-synthetic diet fed to Sprague–Dawley rats

Ingredient*	g/kg
Casein	200
Maize starch	648
Maize oil	60
Vitamin mix†	10
Mineral mix‡	30
Cellulose powder	50
DL-Methionine	2

* Ingredients were from ICN Biomedicals, Cleveland, OH, USA.
† AIN vitamin mix providing (mg/kg diet): thiamin hydrochloride 6, riboflavin 6, pyridoxine hydrochloride 7, nicotinic acid 30, D-calcium pantothenate 16, folic acid 2, D-biotin 0·2, cyanocobalamin 0·01, retinyl palmitate 16, DL-α-tocopherol acetate 200, ergocalciferol 2·5, menaquinone 0·05.
‡ AIN mineral mix providing (g/kg diet): calcium phosphate dibasic 15, sodium chloride 2·22, potassium citrate monohydrate 6·6, potassium sulfate 1·56, magnesium oxide 0·72, manganese carbonate 0·105, ferric citrate 0·18, zinc carbonate 0·048, cupric carbonate 0·009, potassium iodate 0·0003, sodium selenite 0·0003, chromium potassium sulfate 0·0165.

Body weight and daily food intake of all rats were recorded at weekly intervals throughout the study period. Urine samples (24 h) were collected twice from each rat towards the end of each experiment; their pooled volumes were recorded. Rats were killed in a CO_2 chamber following overnight food deprivation. Blood samples were collected through cardiac puncture in heparinized tubes, and plasma was separated by centrifugation (2800 ***g*** for 10 min at −4°C) within half an hour of collection. The livers were removed and weighed. The separated plasma and aliquots of pooled urine samples were stored at −40°C for a maximum period of 3 weeks before analyses were performed.

Amino acid analysis

Total methionine, homocysteine and cysteine levels in plasma and urine were determined using the method of Sedgwick *et al.* (1991), but following a modification. To 100 μl plasma or urine or standard, 50 μl of 100 nmol/ml internal standard comprising L-α-amino-β-guadinopropionic acid and 25 μl 10 % (v/v) tributylphosphine in dimethylformamide were added. A volume of 100 μl cold 10 % TCA was added to all tubes, which were then vortexed and centrifuged at 3000 rpm for 15 min. To 150 μl supernatant, 100 μl performic acid (formic acid:hydrogen peroxide, 9:1) was added and tubes were left overnight at 4°C. On the following day, 50 μl 0·3362 g/ml sodium metasulfite was added to all tubes and mixed vigorously. Samples were vacuum dried and the precipitate was reconstituted with 600 μl saturated potassium tetraborate. In chromatographic vials, 300 μl sample and 100 μl water were added (pH 9·5). Samples were analysed using *o*-phthaldialdehyde as a pre-column derivatizing agent. The quantification of amino acids was accomplished by the use of a Varian 5000 HPLC and a Varian fluorichrome detector (Columbia, MD, USA) at excitation 340 nm and emission 450 nm. Urine and plasma samples were mixed 1:1 with the fluraldehyde reagent prior to injection. The mixed sample (25 μl) was injected onto a supelcosil 3 μm LC 18 reverse phase column (4·6 × 150 mm; supelco). Chromatographic peaks were recorded and integrated using the Shimadzu Ezchrom Chromatography Data System (Shimadzu, Kyoto, Japan).

Vitamin analysis

Plasma vitamin B_{12} and folate were determined by using the commercially available 'dualcount solid phase boil assay kit' (Inter Medico, Markham, Ontario). The vitamins present in the sample were released from the carrier proteins by incubation at 100°C in the presence of dithiothreitol and potassium cyanide. Addition of purified hog intrinsic factor and purified β-lactoglobulin served as the binders for vitamin B_{12} and folate, respectively. The unlabelled vitamin B_{12} or folate competes with its labelled species for the limited number of available binding sites on its specific binder, thus reducing the amount of labelled vitamin B_{12} or folate bound. After an adequate incubation period, isolation of the bound fraction is achieved by centrifuging and decanting. Counts in the precipitate are then converted, by comparison with the calibration curve,

into vitamin B_{12} and folate concentrations. The level of radioactivity bound is inversely related to concentration in the sample or standard.

Plasma pyridoxal-5′-phosphate (PLP) was determined by using a commercially available radioassay kit (Buhlmann Laboratories AG, Switzerland), as a modification of the method described by Shin-Buckring *et al.* (1981). The principle of the assay involves the decarboxylation of H-tyrosine by the vitamin B_6-dependent tyrosine apo-decarboxylase to H-tyramine. The excess of H-tyramine remains in the aqueous phase and is measured by means of a liquid scintillation counter.

Statistical analysis

Means and standard error of the means were determined for all groups of rats. Data were analysed by using one-way ANOVA. Significant effects were determined by using the Student *t* and multiple comparison tests (Steel & Torrie 1953). In the present study the level of significance considered was set at 0·05.

Results

Feeding rats a semi-synthetic diet containing 400 mg niacin/kg diet (diet B) for 1 month did not affect food intake, growth rate or liver weight (data not shown). They remained unaffected even when the diet was fed for 3 months (Table 2). However, feeding for this period of time a diet containing 1000 mg niacin/kg diet (diet C) resulted in a significant increase in both body weight gain and liver weight, but not the daily food intake. Similarly, the plasma and urinary levels of total methionine, homocysteine and cysteine remained unchanged when diet B was fed for 1 month (results not shown). Feeding this diet for 3 months, on the other hand, resulted in significant increases in plasma levels of the amino acids; their levels in urine were also elevated but a significant level was attained only for methionine (Table 3). The magnitude of increase in both plasma and urinary levels of homocysteine and cysteine, in particular, was exacerbated when the supplemental amount of niacin was increased to 1000 mg/kg diet.

Rats fed for 3 months with a semi-synthetic diet loaded with methionine (10 g/kg) plus niacin (1000 mg/kg) showed a significant decrease in homocysteine and no changes in cysteine and methionine levels in the plasma, compared with those fed a diet loaded with only methionine (Table 4). However, urinary concentrations of these amino acids were all significantly higher in the presence of niacin.

Plasma vitamin B_{12}, folate or PLP levels were unaffected (results not shown) by feeding rats a semi-synthetic diet containing 400 mg niacin/kg diet for 1 month. The same level of niacin supplementation for 3 months, however, caused a significant reduction in plasma vitamin B_{12} but not PLP or folate (Table 5). The plasma levels of both vitamin B_{12} and PLP were significantly decreased when dietary niacin level was increased to 1000 mg/kg diet (diet C). Unlike these vitamins the plasma folate levels remained unchanged in rats fed niacin at all dose levels. The responses of the B-vitamins to niacin were similar in rats fed a methionine-rich diet (Table 5).

Table 2. Effect of feeding a diet* supplemented with niacin for 3 months on food intake and growth of Sprague–Dawley rats

(Mean values with their standard errors for six rats)

	Control*		Niacin (400 mg/kg)		Niacin (1000 mg/kg)	
	Mean	SE	Mean	SE	Mean	SE
Food intake (g/d)	19·9[a]	0·5	20·02[a]	0·4	22·6[a]	1·0
Body wt gain (g)	326·8[a]	7·8	330·0[a]	5·7	416·0[b]	21·1
Liver wt (g)	12·7[a]	0·3	13·9[a]	0·2	18·0[b]	1·3

[a,b] Values within a row not sharing a common superscript letter were significantly different ($P<0·05$).

* For details of the diets, see Table 1.

Discussion

Supplementing a diet containing 1000 mg niacin/kg diet to male Sprague–Dawley rats for 3 months resulted in a significant increase in both plasma and urinary total homocysteine levels. The increase, especially, in the urine, was further accentuated by loading the diet with methionine. These results are in agreement with a previous study (Basu & Mann, 1997), which demonstrated a niacin-associated increase in plasma free homocysteine level. This agreement is of particular interest since the earlier study measured only free homocysteine, which comprises only a small portion of total homocysteine. The fact that the present study measured total homocysteine status, and that the results obtained are in parallel with those of the previous study, suggests that niacin at pharmacological doses is potentially hyperhomocysteineaemic.

In the present study the addition of supplemental niacin to a diet resulted in a marked decrease in the levels of plasma PLP. The niacin-associated decline in plasma PLP was in parallel with an increase in plasma homocysteine. These results imply that the breakdown of homocysteine to cysteine is affected by the presence of supplemental niacin. As vitamin B_6 in its coenzyme form of PLP is involved in the *trans*-sulfuration of homocysteine, niacin at pharmacological doses may affect the biochemical status of this cofactor. In the presence of excess methionine the *trans*-sulfuration pathway is generally favoured by up-regulation of its rate-limiting enzyme, cystathionine β-synthase (Finkelstein & Martin, 1986). The cysteine that is formed from homocysteine is either oxidized to taurine and inorganic sulfates or excreted in the urine. This means that dietary methionine load stresses the metabolic pathway responsible for the irreversible degradation of homocysteine to cysteine. It is noteworthy that niacin in the presence of excess dietary methionine lowered the level of plasma homocysteine, while its urinary concentration along with cysteine was markedly increased. This further suggests that excess niacin may affect the *trans*-sulfuration of homocysteine to cysteine.

Homocysteine can be recycled to methionine. Methionine synthase is a rate-limiting enzyme for this remethylation reaction for which vitamin B_{12} and folate serve as cofactor or co-substrate, respectively. The plasma vitamin B_{12}, but not folate, was markedly reduced in the presence of niacin.

Table 3. Effect of a diet* supplemented with niacin for 3 months on plasma and urinary total methionine, homocysteine and cysteine levels in Sprague–Dawley rats

(Mean values with their standard errors for six rats)

	Control*		Niacin (400 mg/kg)		Niacin (1000 mg/kg)	
	Mean	SE	Mean	SE	Mean	SE
Plasma (nmol/ml)						
Methionine	27·25[a]	0·24	42·17[b]	1·35	38·61[b]	0·74
Homocysteine	3·58[a]	0·17	7·39[b]	0·44	9·52[c]	0·56
Cysteine	247·00[a]	10·8	300·90[b]	7·6	415·6[c]	11·0
Urine (nmol/24 h)						
Methionine	1993[a]	433	2077[b]	410	3869[c]	421
Homocysteine	242[a]	15	275[a]	25	451[c]	64
Cysteine	950[a]	78	1137[a]	103	1791[c]	229

[a,b] Values within a row not sharing a common superscript letter were significantly different ($P<0·05$).
* For details of the diets, see Table 1.

Table 4. Effect of feeding a diet* supplemented with methionine (10 g/kg) and niacin (1000 mg/kg) for 3 months on plasma and urinary total methionine, homocysteine and cysteine levels in Sprague–Dawley rats

(Mean values with their standard errors for six rats)

	Methione		Methionine plus niacin		Student's *t* test (*P* value)
	Mean	SE	Mean	SE	
Plasma (nmol/ml)					
Methionine	40·6	1·3	43·6	2·3	NS
Homocysteine	7·5	0·4	5·6	0·5	<0·05
Cysteine	394	8	360	12	NS
Urine (nmol/24 h)					
Methionine	3632	1344	16 483	4273	<0·001
Homocysteine	443	55	806	127	<0·001
Cysteine	1030	146	2015	195	<0·001

* For details of the diets, see Table 1.

Table 5. Effect of niacin supplementation for 3 months on the plasma levels of B-vitamins in Sprague–Dawley rats

(Mean values with their standard errors for six rats)

	Vitamin B_{12} (pmol/l)		Folate (nmol/l)		PLP (nmol/l)	
	Mean	SE	Mean	SE	Mean	SE
Control	450·8[a]	21·1	150·6[a]	4·2	425·6[a]	9·2
Niacin (400 mg)*	375·9[b]	28·6	148·9[a]	6·1	418·7[a]	11·1
Niacin (1000 mg)*	370·4[b]	17·1	148·2[a]	4·6	370·1[b]	10·2
Methionine (10 g)*	409·4[a]	31·6	149·8[a]	4·1	441·4[a]	11·8
Methionine (10 g) + niacin (1000 mg)*	327·6[b]	10·8	142·6[a]	6·6	389·6[b]	14·1

[a,b] Mean values within a column not sharing a common superscript letter were significantly different ($P<0·05$).
PLP, pyridoxal-5′-phosphate.
* Per kg diet.

Adequate vitamin B_{12} must be present for the activity of methionine synthase, the enzyme that removes the methyl group from 5-methyl tetrahydrofolate and delivers it to homocysteine (Finkelstein, 1998). The latter is then converted to methionine and consequently the free tetrahydrofolate is regenerated. Since 5-methyl tetrahydrofolate may return to the body's folate only via a vitamin B_{12}-dependent step, a deficiency of vitamin B_{12} traps body folate in its methylated form. It is plausible that the niacin-induced deficiency of vitamin B_{12} may have resulted in the 'trapping' of folate, which would maintain normal plasma folate levels. This may explain why folate levels were not affected by niacin.

The metabolic fate of homocysteine is linked to vitamin

B_{12}, folate and vitamin B_6. Results from the present study suggest that niacin in pharmacological doses may affect the status of vitamins B_{12} and B_6. Hyperhomocysteineaemia may be a consequence of their metabolic interactions.

In recent years, there has been a substantial amount of research leading to the development of the 'homocysteine theory of atherosclerosis'. Both retrospective and prospective studies have shown that elevated plasma total homocysteine is an independent risk factor for occlusive disease in the coronary, cerebrovascular and peripheral vessels and for arterial and venous thrombosis (Boushey *et al.* 1995; DenHeijer *et al.* 1996; D'Angelo *et al.* 1997; Refsum *et al.* 1998).

It appears from the present study that the hypolipidaemic action of niacin could be potentially negated by its hyperhomocysteineaemic effect. It is important that clinical studies are carried out in order to determine whether these results could be extrapolated to humans. Future studies should also examine whether concurrent supplementation of niacin with vitamins B_6 and B_{12} counteract the niacin-induced hyperhomocysteineaemia without affecting its hypolipidaemic action.

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