Mol. Nutr. Food Res. 2008, *52*, 379 – 385 DOI 10.1002/mnfr.200600190

Research Article

Preventing cell death induced by carbonyl stress, oxidative stress or mitochondrial toxins with vitamin B anti-AGE agents

Rhea Mehta, Nandita Shangari and Peter J. O'Brien

Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto, Toronto, ON, Canada

Carbonyls generated by autoxidation of carbohydrates or lipid peroxidation have been implicated in advanced glycation end product (AGE) formation in tissues adversely affected by diabetes complications. Tissue AGE and associated pathology have been decreased by vitamin B_1/B_6 in trials involving diabetic animal models. To understand the molecular cytoprotective mechanisms involved, the effects of B_1/B_6 vitamers against cytotoxicity induced by AGE/advanced lipid end product (ALE) carbonyl precursors (glyoxal/acrolein) have been compared to cytotoxicity induced by oxidative stress (hydroperoxide) or mitochondrial toxins (cyanide/copper). Thiamin was found to be best at preventing cell death induced by carbonyl stress and mitochondrial toxins but not oxidative stress cell death suggesting that thiamin pyrophosphate restored pyruvate and α-ketoglutarate dehydrogenases inhibited by mitochondrial toxicity. However, B_6 vitamers were most effective at preventing oxidative stress or lipid peroxidation cytotoxicity suggesting that pyridoxal or pyridoxal phosphate were antioxidants and/or Fe/Cu chelators. A therapeutic vitamin cocktail could provide maximal prevention against carbonyl stress toxicity associated with diabetic complications.

Keywords: Advanced glycation end products / Advanced lipid end products / Cytotoxicity / Lipid peroxidation / Reactive oxygen species

Received: October 3, 2006; revised: April 2, 2007; accepted: May 3, 2007

1 Introduction

Reactive dicarbonyls formed endogenously by the autoxidation of carbohydrates, nucleic acids, unsaturated lipids or amino acids bind covalently to proteins and undergo further oxidation to form advanced glycation end products (AGE) and advanced lipid end products (ALE). The prolonged dyslipidemia and hyperglycemia in diabetes increases AGE that contribute to the development of diabetes complications [1, 2].

High-dose vitamin B_1 (thiamin) therapy prevents dyslipidemia, incipient nephropathy, mild thiamin deficiency and the increases in plasma AGE in streptozotocin-induced diabetic rats [3]. Benfotiamine, a thiamin phosphate analogue, has also been shown to be effective at alleviating diabetic polyneuropathy in a clinical trial [4]. Furthermore, we have shown that partial thiamin deficiency increases plasma AGE levels in rats [5]. Pyridoxamine, a B_6 vitamer, prevents incipient diabetic nephropathy or retinopathy in streptozotocin-induced diabetic rats [3, 6]. Pyridoxamine is currently on the FDA "fast track" to phase III clinical trials for treatment of diabetic nephropathy [7]. Pyridoxamine also prevents the renal and vascular pathology and the increases in skin collagen AGE/Ales and hyperlipidemia in the Zucker obese non-diabetic rat model [8].

The therapeutic mechanisms of thiamin and pyridoxamine are not known. However, the following mechanisms [9, 10] have been proposed. (i) Thiamin is converted in the cell to thiamin pyrophosphate (TPP), the coenzyme for transketolase (TK), a rate-limiting step of the pentose phosphate pathway. TK activation could decrease the accumulation of glyceraldehyde-3-phosphate and fructose-6-phosphate during glycolysis and thereby prevent methylglyoxal formation [3, 11]. B_6 vitamers are also coenzymes required by transaminases for mitochondrial function, but their con-

Correspondence: Dr. Peter J. O'Brien, University of Toronto, Department of Pharmaceutical Sciences 144 College St., Rm 1004, Toronto, ON M5S 3M2 Canada
E-mail: peter.obrien@utoronto.ca
Fax: +1-416-978-8511

Abbreviations: AGE, advanced glycation end product;, **ALE**, advanced lipid end product; **Cu**, copper; **DCFD**, dichlorofluorescin diacetate; **α-KGDH**, alpha-ketoglutarate dehydrogenase; **PDH,** pyruvate dehydrogenase; **PLP**, pyridoxal phosphate; **ROS**, reactive oxygen species; **tBuOOH**, tertiary-butyl hydroperoxide; **TPP**, thiamin pyrophosphate

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

tribution to xenobiotic cytotoxicity has not been studied. (ii) Pyridoxamine, pyridoxal phosphate (PLP) and to a lesser extent TPP were much more effective than pyridoxal or thiamin as "late"AGE inhibitors when ribose was incubated with ribonuclease A for days. This effect of vitamin B_1 and B_6 derivatives was attributed to the trapping of Amadori carbonyl intermediates or post-Amadori precursors [12]. Pyridoxamine was later shown to trap the dicarbonyl AGE precursors glyoxal and methylglyoxal and the adducts formed *in vitro* were identified [13, 14]. However, dicarbonyl trapping by thiamin or TPP has not been reported and no adducts have been identified. (iii) Pyridoxamine can also trap lipid peroxide intermediates *in vivo* to form amide adducts that are excreted in the urine [15]. (iv) Thiamin has antioxidant and reactive-oxygen species (ROS) scavenging activity that results in thiamin oxidation [16]. Pyridoxamine or PLP also prevents H_2O_2 cytotoxicity [17]. Pyridoxamine chelates copper (Cu)/iron (Fe) [18] and also inhibits Cu-catalyzed AGE formation [19]. The copper chelator trientine has also been effective at preventing diabetic neuropathy and diabetic heart disease [20]. It would be useful to prioritize these molecular cytoprotective mechanisms that have been suggested for thiamin and B_6 vitamers in order to develop second-generation AGE inhibitors.

We hypothesize that "the relative cytoprotectiveness of vitamin B_1 or B_6 vitamers against carbonyl stress, oxidative stress or mitochondrial toxicity differs widely and depends on the molecular cytotoxic mechanism involved." Therefore the agents' relative effectiveness at preventing carbonyl stress cytotoxicity induced by dicarbonyl AGE precursors or acrolein (an ALE precursor) versus oxidative stress cytotoxicity induced by hydroperoxide versus bioenergetic stress using mitochondrial toxins e. g. cyanide or copper has been determined. The cytoprotective effectiveness of these agents was also correlated using the biomarkers ROS and lipid peroxidation as further evidence to support the molecular cytoprotective mechanisms proposed.

2 Materials and methods

2.1 Chemicals

Cupric sulfate, potassium cyanide, acrolein, glyoxal, tertiary-butyl hydroperoxide, thiamin, thiamin pyrophosphate, pyridoxal 5′-phosphate, pyridoxal, pyridoxine, pyridoxamine, dichlorofluorescin diacetate (DCFD), thiobarbituric acid (TBA), trichloroacetic acid (TCA) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Type II Collagenase was purchased from Worthington (Lakewood, NJ).

2.2 Animal treatment and hepatocyte preparation

Male Sprague-Dawley rats weighing 275–300 g (Charles River Laboratories) were housed in ventilated plastic cages over PWI 8–16 hardwood bedding. There were 12 air changes per hour, 12-h light photoperiod (lights on at 08:00 h) and an environmental temperature of 21–23°C with a 50–60% relative humidity. The animals were fed with a normal standard chow diet and water ad libitum. Hepatocytes were isolated from rats by collagenase perfusion of the liver as described by Moldéus and coworkers [21]. Isolated hepatocytes (10^6 cells/mL) (10 mL) were suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating 50-mL round-bottom flasks, under an atmosphere of 95% O_2 and 5% CO_2 in a water bath of 37°C for 30 min [21]. Stock solutions of chemicals were made in H_2O, DMSO or methanol.

2.3 Cell viability

Hepatocyte viability was assessed microscopically by plasma membrane disruption as determined by the trypan blue (0.1% w/v) exclusion test [21]. Hepatocyte viability was determined every 30 min during the 3-h incubation and the cells were at least 80–90% viable before use. Cupric sulfate, cyanide, acrolein, glyoxal and tertiary-butyl hydroperoxide stock solutions were prepared immediately prior to use. The ED_{50} concentration for these toxins that caused 50% cytotoxicity in 2 h was determined. Toxins were added to the hepatocytes either directly after the addition of vitamins or after the hepatocytes had been pre-incubated with vitamins. The pre-incubation time is specified in each table and mostly reflects differences in vitamer transportation and metabolic activation [9, 10]. Various concentrations of vitamins were used in the range of 1–10 mM. At these concentrations, the vitamins alone were not cytotoxic. Stock solutions of chemicals were made in H_2O, DMSO or methanol. Lethal toxin doses were used that caused approximately 50% cytotoxic death at 2 h. The minimal concentrations of various vitamins that prevented cytotoxicity in 2 h were determined for each of the different toxic systems.

2.4 Determination of reactive-oxygen species

Hepatocyte reactive-oxygen species (ROS) generation induced by the toxins was determined by adding dichlorofluorescin diacetate (DCFD) to the hepatocyte incubate. DCFD penetrates hepatocytes and is hydrolyzed to form non-fluorescent dichlorofluorescin. Dichlorofluorescin then reacts with 'ROS' to form the highly fluorescent dichlorofluorescein and effluxes the cell. The 1-mL samples were withdrawn at 30 and 90 min after incubation with copper, cyanide and glyoxal. These samples were then centrifuged for 1 min at $50 \times g$. The cells were resuspended in H_2O and 1.6 μM DCFD was added [22]. The cells were allowed to incubate at 37°C for 10 min. The fluorescence intensity of ROS product was measured at $\lambda_{excitation} = 490$ nm and $\lambda_{emission} = 520$ nm.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

2.5 Lipid peroxidation assay

Acrolein and tertiary-butyl hydroperoxide reacted with thiobarbituric acid (TBA) (0.8% w/v) to form pink products that absorbed at 532 nm. Lipid peroxidation was therefore assayed by measuring thiobarbituric acid reactive substances (TBARS) mostly formed from malondialdehyde, a lipid peroxide decomposition product, but can also be formed from the oxidation of DNA deoxyribose by ROS. The 1-mL samples were withdrawn at 30 and 90 min from the hepatocyte flasks. Trichloroacetic acid (TCA, 250 μL, 70% w/v) was added to the samples to stop the reaction and lyse the cells. The formation of lipid peroxidation products was determined by adding 1 mL of TBA (0.8% w/v) to the samples. The samples were then incubated in a boiling water bath for 20 min. The samples were cooled on ice for 5 min, and then centrifuged at high speed for 5 min. The supernatant was measured at 532 nm to detect the amount of TBARS formed during the decomposition of lipid hydroperoxides, using a Pharmacia Biotech Ultrospec 1000 [23]. Results were expressed as μM concentration of malondialdehyde ($e = 1.56 \times 10^5\ mol^{-1} \cdot cm^{-1}$). None of the vitamins at the concentrations used reacted with TBA or absorbed at 532 nm.

2.6 Microsomal preparation

Adult male Sprague-Dawley rats (250–300 g) were anesthetized by sodium pentobarbital (60 mg/kg body). Livers were removed under sterile conditions and perfused with KCl solution (1.18% w/v, 4°C). Hepatic microsomes were prepared by differential centrifugation as described by Dallner *et al.* [24]. The microsomal pellet was suspended and homogenized in sterile potassium phosphate buffer with KCl solution (50 mM KH_2PO_4 and 0.23% w/v KCl, pH 7.4) before storage at −70°C. Microsomal protein was determined by method of Joly *et al.* [25].

2.7 Determination of microsomal lipid peroxidation

Microsomal lipid peroxidation was determined by measuring the amount of thiobarbituric reactive substances (TBARS) at 532 nm, formed during the decomposition of lipid hydroperoxides using a Pharmacia Biotech Ultrospec 1000. Briefly, the reaction mixture contained 1 mL of 0.1 M potassium phosphate buffer (pH 7.4), microsomes (1 mg/mL protein), 500 μM tertiary-butyl hydroperoxide, and B vitamins. All of the test tubes containing the reactions mixture were incubated at 37°C in a water bath with agitation. At 90 min, each test tube was treated with TCA (70% w/v) and TBA (0.8% w/v). The suspension was then boiled for 20 min and read at 532 nm [23]. Results were expressed as μM concentration of malondialdehyde ($e = 1.56 \times 10^5\ mol^{-1} \cdot cm^{-1}$).

2.8 Statistical analysis

Statistical analysis was performed by one-way ANOVA. A probability of less than 0.05 was considered significant.

3 Results and discussion

3.1 Carbonyl stress-induced cytotoxicity

3.1.1 Glyoxal cytotoxicity and ROS formation

Previously it has been shown that glyoxal added to hepatocytes caused cell death after lipid peroxidation, ROS formation and mitochondrial toxicity. Inhibiting lipid peroxida-

Table 1. Prevention of glyoxal cytotoxicity and ROS formation[a)]

Toxin and B vitamin treatment	Percent cytotoxicity (trypan blue uptake)			ROS formation (FI units)
	60 min	120 min	180 min	90 min
Control	18 ± 3	22 ± 3	25 ± 4	98 ± 5
+ Glyoxal 5mM	42 ± 4[b)]	58 ± 7[b)]	100[b)]	240 ± 12[b)]
+ Thiamin 3mM	23 ± 2[c)]	31 ± 3[c)]	83 ± 7[c)]	126 ± 10[c)]
+ Thiamin Pyrophosphate 3mM	18 ± 1[c)]	41 ± 3[c)]	91 ± 8[c)]	133 ± 13[c)]
+ Pyridoxal Phosphate 3mM[d)]	19 ± 2[c)]	31 ± 3[c)]	66 ± 6[c)]	140 ± 11[c)]
+ Pyridoxal 3mM	22 ± 2[c)]	26 ± 2[c)]	73 ± 4[c)]	131 ± 12[c)]
+ Pyridoxine 3mM	33 ± 3[c)]	41 ± 5[c)]	92 ± 9[c)]	145 ± 7[c)]
+ Pyridoxamine 3mM	29 ± 2[c)]	36 ± 3[c)]	90 ± 8[c)]	111 ± 11[c)]

a) Note: The results shown represent the average of three separate experiments ± SD.
Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37°C in rotating round-bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH = 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue uptake assay. ROS was determined by measuring DCFD oxidation, which was expressed as fluorescence intensity (FI) units. $\lambda_{ex} = 490$, $\lambda_{em} = 520$.
b) Significant as compared to control ($p < 0.05$).
c) Significant as compared to Glyoxal 5 mM ($p < 0.05$).
d) Pre-incubation for 1 h.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 2. Prevention of acrolein cytotoxicity and lipid peroxidation[a)]

Toxin and B vitamin treatment	Percent cytotoxicity (trypan blue uptake)			Lipid peroxidation
	60 min	120 min	180 min	90 min
Control	18 ± 2	19 ± 2	20 ± 2	0.24 ± 0.01
+ Acrolein 150μM	24 ± 1[b)]	51 ± 1[b)]	100[b)]	2.71 ± 0.24[b)]
+ Thiamin 3mM[d)]	14 ± 2[c)]	32 ± 2[c)]	34 ± 2[c)]	0.35 ± 0.03[c)]
+ Thiamin Pyrophosphate 3mM	23 ± 2[c)]	46 ± 2[c)]	100[c)]	2.78 ± 0.22[c)]
+ Pyridoxal Phosphate 3mM	23 ± 2[c)]	43 ± 2[c)]	71 ± 6[c)]	1.22 ± 0.08[c)]
+ Pyridoxal 3mM	17 ± 1[c)]	32 ± 1[c)]	58 ± 5[c)]	0.77 ± 0.06[c)]
+ Pyridoxine 3mM	16 ± 1[c)]	32 ± 1[c)]	96 ± 7[c)]	2.47 ± 0.12[c)]
+ Pyridoxamine 3mM	16 ± 1[c)]	41 ± 2[c)]	79 ± 6[c)]	1.53 ± 0.09[c)]

a) Note: The results shown represent the average of three separate experiments ± SD. Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37°C in rotating round-bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH = 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue uptake assay. Lipid peroxidation was determined by measuring thiobarbituric acid reactive metabolites as μM concentration of malondialdehyde ($\varepsilon = 1.56 \times 10^5$ $mol^{-1} \cdot cm^{-1}$).
b) Significant as compared to control ($p < 0.05$).
c) Significant as compared to Acrolein 150 μM ($p < 0.05$).
d) Pre-incubation for 1 h.

tion with the antioxidant butylated hydroxyanisole also prevented glyoxal-induced cytotoxicity [22]. As shown in Table 1, the cytoprotective effectiveness of the vitamers against glyoxal cytotoxicity observed was PLP > thiamin > pyridoxal whereas TPP, pyridoxamine and pyridoxine were less effective at preventing cytotoxicity. The effectiveness of vitamers at preventing ROS formation induced by glyoxal however was pyridoxamine > thiamin > pyridoxal > TPP > PLP > pyridoxine which for PLP and pyridoxamine was more similar to that found for preventing copper-induced ROS formation than glyoxal-induced cytotoxicity. This suggests that the ROS scavenging activity of the vitamers does not fully explain their cytoprotectiveness. The ROS scavenging activity of pyridoxamine has been attributed to its 3-hydroxyl ring substituent [26]. Thiamin protection has been attributed to its intracellular conversion to TPP, which is an essential coenzyme for transketolase, a rate-limiting enzyme of the pentose phosphate pathway that supplies NADPH. Glyoxal is reductively detoxified by aldehyde reductase and NADPH [27, 28]. Furthermore, TPP is a coenzyme for alpha-ketoglutarate dehydrogenase (α-KGDH) that is inactivated by mitochondrial toxins including glyoxals [29, 30] or ROS formed by glyoxal [22]. The cytoprotection by thiamin but not TPP is likely because hepatocytes take up thiamin (but not TPP) via a thiamin transporter THTR [10, 31].

Pyridoxal diffuses rapidly into hepatocytes whereas PLP is first converted to pyridoxal by a plasma membrane phosphatase when it enters the cell [10]. The cytoprotection by PLP could be related to the prevention of AGE formation by PLP when ribonuclease A was incubated with ribose for days [12]. This was attributed to PLP forming Schiff base adducts with protein amino groups that limited the amount of amino groups available for AGE formation by ribose glycation products [12]. Pyridoxamine was less effective than PLP or pyridoxal in preventing glyoxal cytotoxicity even though its primary amine group can slowly form a hemiaminal adduct with glyoxal ($t_{1/2}$ = 0.94 h) at high concentrations (10 mM) [14]. Pyridoxamine therefore may not trap glyoxal.

3.1.2 Acrolein cytotoxicity and lipid peroxidation

The addition of acrolein to hepatocytes caused iron release and lipid peroxidation. Cytotoxicity was prevented by the iron chelator desferrioxamine but was only partly decreased by antioxidants [32]. The lipid peroxidation inhibition and cytoprotective order of effectiveness of the vitamers was thiamin > pyridoxal > PLP > pyridoxamine > pyridoxine whereas TPP was ineffective (Table 2).

Thiamin was the best at preventing death induced by acrolein likely because it is a coenzyme for pyruvate dehydrogenase (PDH) and α-KGDH, which were inhibited when acrolein was incubated with mitochondria [33, 34]. TPP, on the other hand, was not effective, suggesting that TPP was taken up poorly by hepatocytes. PLP was effective at protecting against acrolein toxicity likely because of its iron chelating effectiveness. Acrolein causes the release of iron from hemoproteins in intact cells which results in ROS formation and lipid peroxidation [32, 35]. Pyridoxal complexes iron *in vivo* as ^{59}Fe excretion in the rat increased when pyridoxal was given intravenously at a dose of 150 mg/kg at 6 h after the i.v. administration of ^{59}Fe-ferritin [36]. Pyridoxal was more effective than PLP in preventing acrolein cytotoxicity. Pyridoxamine was a poor acrolein scavenger [37].

3.2 Oxidative stress cytotoxicity: tertiary-butyl hydroperoxide induced cytotoxicity and lipid peroxidation

A common model for studying molecular mechanisms of oxidative stress cytotoxicity involves treating cells with

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 3. Prevention of tertiary-butyl hydroperoxide cytotoxicity and lipid peroxidation[a)]

Toxin and B vitamin treatment	Percent cytotoxicity (trypan blue uptake)			Hepatocyte lipid peroxidation	Microsome lipid peroxidation
	60 min	120 min	180 min	90 min	90 min
Control	16 ± 1	22 ± 2	24 ± 3	0.35 ± 0.03	0.34 ± 0.02
+ tert-butyl hydroperoxide 500μM	43 ± 5[b)]	63 ± 8[b)]	79 ± 7[b)]	2.58 ± 0.15[b)]	4.15 ± 0.25[b)]
+ Thiamin 3mM[d)]	50 ± 7[c)]	72 ± 7[c)]	82 ± 8[c)]	2.69 ± 0.16[c)]	3.50 ± 0.21[c)]
+ Thiamin 3mM	45 ± 3[c)]	73 ± 4[c)]	95 ± 6[c)]	2.06 ± 0.18[c)]	3.24 ± 0.19[c)]
+ Thiamin Pyrophosphate 3mM	61 ± 7[c)]	76 ± 7[c)]	84 ± 9[c)]	2.91 ± 0.17[c)]	4.18 ± 0.33[c)]
+ Pyridoxal Phosphate 3mM	27 ± 2[c)]	44 ± 3[c)]	46 ± 3[c)]	0.96 ± 0.09[c)]	2.34 ± 0.14[c)]
+ Pyridoxal 3mM	20 ± 2[c)]	36 ± 3[c)]	39 ± 3[c)]	0.38 ± 0.03[c)]	1.99 ± 0.12[c)]
+ Pyridoxine 3mM[d)]	14 ± 2[c)]	21 ± 2[c)]	29 ± 3[c)]	0.92 ± 0.07[c)]	2.86 ± 0.17[c)]
+ Pyridoxine 3mM	24 ± 2[c)]	47 ± 4[c)]	54 ± 4[c)]	1.64 ± 0.09[c)]	3.01 ± 0.18[c)]
+ Pyridoxamine 3mM[e)]	31 ± 3[c)]	55 ± 6[c)]	70 ± 6[c)]	0.92 ± 0.04[c)]	3.28 ± 0.20[c)]

a) Note: The results shown represent the average of three separate experiments ± SD. Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37°C in rotating round bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH = 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue uptake assay. Lipid peroxidation was determined for hepatocytes and microsomes by measuring thiobarbituric acid reactive metabolites as μM concentration of malondialdehyde ($\varepsilon = 1.56 \times 10^5 mol^{-1} \cdot cm^{-1}$).
b) Significant as compared to control (P < 0.05).
c) Significant as compared to tBuOOH 500 μM ($p < 0.05$).
d) Pre-incubation for 30 min.
e) Pre-incubation for 1 h.

hydroperoxides. Tertiary-butyl hydroperoxide (tBuOOH) also induces hepatic lipid peroxidation, DNA damage and toxicity *in vivo* that can be prevented by antioxidants or desferrioxamine, a ferric chelator. Similar results were obtained with isolated hepatocytes [38]. As shown in Table 3, the cytoprotective order of effectiveness of the vitamers found in this study was pyridoxine > pyridoxal > PLP, pyridoxamine. A similar order of effectiveness was found for hydroperoxide induced microsomal lipid peroxidation. Cytotoxicity and lipid peroxidation were not prevented by TPP or thiamin. The slight increase by TPP of hydroperoxide-induced cytotoxicity is a trend but was not statistically significant.

The B_6 vitamers were effective at preventing tBuOOH-induced hepatocyte cytotoxicity and microsomal lipid peroxidation suggesting they are effective antioxidants and/or iron chelators. Thiamin did not prevent tBuOOH-induced toxicity suggesting α-KGDH was not inhibited and thiamin was a poorer lipid antioxidant than what has been suggested [16].

3.3 Mitochondrial toxin-induced cytotoxicity

3.3.1 Cyanide cytotoxicity and ROS formation

Cyanide is a cytochrome oxidase inhibitor which when added to hepatocytes prevents mitochondrial NADH oxidation thereby causing reductive stress and iron release as the hepatocyte $NADH:NAD^+$ ratio is increased [39]. Cyanide added to hepatocytes caused rapid ROS formation [40] and ROS scavengers or desferrioxamine prevented cytotoxicity [39]. The following order of cytoprotection and inhibition of ROS formation by the vitamers was observed: thiamin > pyridoxamine > pyridoxine. However, cytotoxicity and ROS formation were not prevented by TPP, pyridoxal and PLP (Table 4).

Thiamin was likely the most effective agent at preventing cyanide-induced toxicity probably due to its α-KGDH coenzyme activity that partly restored α-KGDH activity in hepatocytes or mixed neuronal cells that was inhibited by ROS. This restoration of α-KGDH activity by thiamin also partly normalized cellular ATP levels [30]. ROS generated by xanthine and xanthine oxidase has also been shown to inhibit α-KGDH [41] and pyruvate dehydrogenase [42], another thiamin-requiring enzyme.

3.3.2 Copper cytotoxicity and ROS formation

Copper overload in rats induces hepatotoxicity, lipid peroxidation and mitochondrial dysfunction [43]. The addition of cupric sulfate to hepatocytes caused ROS formation before cytotoxicity ensued [28]. As shown in Table 5, the following order of vitamer cytoprotection was observed: Thiamin, TPP > PLP or pyridoxamine > pyridoxal > pyridoxine.

Thiamin protected against copper-induced toxicity likely by restoring mitochondrial α-KGDH and PDH enzyme activity. Thiamin was also shown to restore α-KGDH activity and prevent cytotoxicity towards mixed neuronal/glial cells incubated with copper [30].

The estimated effectiveness reported for the B_6 vitamer inhibition of copper-catalyzed ascorbic acid (expressed as IC_{50}s) was 1.0 mM pyridoxamine >3.6 mM pyridoxine > 5.0 mM pyridoxal [19]. Pyridoxamine also complexes copper more readily than pyridoxal [44]. The low cytoprotectiveness of pyridoxine could result from the inhibition by copper of pyridoxine phosphate oxidase required for PLP

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 4. Cyanide cytotoxicity and ROS formation[a)]

Toxin and B vitamin treatment	Percent cytotoxicity (trypan blue uptake)			ROS formation (FI units)
	60 min	120 min	180 min	90 min
Control	19 ± 2	23 ± 2	25 ± 2	103 ± 7
+ Cyanide 1.5mM	45 ± 4[b)]	65 ± 8[b)]	100[b)]	161 ± 10[b)]
+ Thiamin 2mM[d)]	34 ± 2[c)]	36 ± 3[c)]	51 ± 4[c)]	132 ± 7[c)]
+ Thiamin 2mM	30 ± 3[c)]	55 ± 4[c)]	100[c)]	154 ± 9[c)]
+ Thiamin Pyrophosphate 3mM	37 ± 3[c)]	55 ± 5[c)]	93 ± 8[c)]	155 ± 13[c)]
+ Pyridoxal Phosphate 3mM	46 ± 6[c)]	61 ± 6[c)]	100[c)]	170 ± 12[c)]
+ Pyridoxal 3mM	40 ± 3[c)]	51 ± 5[c)]	100[c)]	151 ± 12[c)]
+ Pyridoxine 3mM[d)]	37 ± 3[c)]	49 ± 4[c)]	69 ± 5[c)]	138 ± 12[c)]
+ Pyridoxamine 3mM[d)]	32 ± 2[c)]	44 ± 4[c)]	62 ± 5[c)]	130 ± 8[c)]

a) Note: The results shown represent the average of three separate experiments ± SD. Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37°C in rotating round bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH = 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue uptake assay. ROS was determined by measuring DCFD oxidation, which was expressed as fluorescence intensity (FI) units. λ_{ex} = 490, λ_{em} = 520.
b) Significant as compared to control ($p < 0.05$).
c) Significant as compared to Cyanide 1.5 mM ($p < 0.05$).
d) Pre-incubation for 5 min.

Table 5. Copper cytotoxicity and ROS formation[a)]

Toxin and B vitamin treatment	Percent cytotoxicity (trypan blue uptake)			ROS formation (FI units)
	60 min	120 min	180 min	90 min
Control	19 ± 1	23 ± 2	25 ± 3	96 ± 8
+ Copper 35μM	52 ± 3[b)]	54 ± 3[b)]	68 ± 3[b)]	149 ± 7[b)]
+ Thiamin Pyrophosphate 1mM	34 ± 2[c)]	38 ± 3[c)]	42 ± 3[c)]	129 ± 12[c)]
+ Thiamin 1mM[d)]	33 ± 2[c)]	38 ± 2[c)]	39 ± 2[c)]	122 ± 4[c)]
+ Pyridoxal Phosphate 3mM	21 ± 1[c)]	25 ± 1[c)]	29 ± 1[c)]	111 ± 7[c)]
+ Pyridoxal 3mM	39 ± 4[c)]	48 ± 4[c)]	65 ± 6[c)]	132 ± 7[c)]
+ Pyridoxine 3mM	44 ± 3[c)]	51 ± 4[c)]	65 ± 5[c)]	137 ± 9[c)]
+ Pyridoxamine 3mM	25 ± 2[c)]	25 ± 2[c)]	29 ± 2[c)]	109 ± 7[c)]

a) Note: The results shown represent the average of three separate experiments ± SD. Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37°C in rotating round bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH = 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue uptake assay. ROS was determined by measuring DCFD oxidation, which was expressed as fluorescence intensity (FI) units. λ_{ex} = 490, λ_{em} = 520.
b) Significant as compared to control ($p < 0.05$).
c) Significant as compared to CuSO4 35μM ($p < 0.05$).
d) Pre-incubation for 30 min.

formation from pyridoxine phosphate [10]. PLP was also more effective than pyridoxal in preventing iron-induced hepatocyte lipid peroxidation and cytotoxicity [45].

4 Concluding remarks

The B vitamin cytoprotective ranking differs considerably and depends upon the intracellular target and whether carbonyl stress, oxidative stress or mitochondrial toxicity initiates the cytotoxicity mechanism. Thiamin was the most effective B vitamin at preventing cell death induced by acrolein/glyoxal intermediates or mitochondrial toxins, but was not effective against hydroperoxide oxidative stress cytotoxicity. Few cellular metabolic pathways are dependent on a single B coenzyme. Thus, vitamin combination treatment or supplementation should be more beneficial for increasing cellular resistance to oxidative stress or preventing oxidative stress diseases such as diabetes. This screening technique could prove useful for determining which agent combination provides maximal protection against each toxin and may also assist in target enzyme identification.

This study was funded by the National Cancer Institute of Canada, grant #015066 and the Natural Sciences and Engineering Research Council of Canada, grant #3783-03.

The authors have declared no conflict of interest.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

5 References

[1] O'Brien, P. J., Siraki, A. G., Shangari, N., *Crit Rev. Toxicol.* 2005, *35*, 609–662.

[2] Thomas, M. C., Baynes, J. W., Thorpe, S. R., Cooper, M. E., *Curr. Drug Targets* 2005, *6*, 453–474.

[3] Babaei-Jadidi, R., Karachalias, N., Ahmed, N., Battah, S., *et al*., *Diabetes* 2003, *52*, 2110–2120.

[4] Haupt, E., Ledermann, H., Kopcke, W., *Int. J. Clin. Pharmacol. Ther.* 2005, *43*, 71–77.

[5] Shangari, N., Depeint, F., Furrer, R., Bruce, W. R., *et al*., *FEBS Lett.* 2005, *579*, 5596–5602.

[6] Stitt, A., Gardiner, T. A., Alderson, N. L., Canning, P., *et al*., *Diabetes* 2002, *51*, 2826–2832.

[7] Khalifah, R. G., Chen, Y., Wassenberg, J. J., *Ann. N Y Acad. Sci.* 2005, *1043*, 793–806.

[8] Alderson, N. L., Chachich, M. E., Youssef, N. N., Beattie, R. J. *et al*., *Kidney Int.* 2003, *63*, 2123–2133.

[9] Depeint, F., Bruce, W. R., Shangari, N., Mehta, R., *et al*., *Chem. Biol. Interact.* 2006, *163*, 94–112.

[10] Depeint, F., Bruce, W. R., Shangari, N., Mehta, R., *et al*., *Chem. Biol. Interact.* 2006, *163*, 113–132.

[11] Karachalias, N., Babaei-Jadidi, R., Kupich, C., Ahmed, N., *et al*., *Ann. N. Y. Acad. Sci.* 2005, *1043*, 777–783.

[12] Booth, A. A., Khalifah, R. G., Todd, P., Hudson, B. G., *J. Biol. Chem.* 1997, *272*, 5430–5437.

[13] Voziyan, P. A., Metz, T. O., Baynes, J. W., Hudson, B. G., *J. Biol. Chem.* 2002, *277*, 3397–3403.

[14] Nagaraj, R. H., Sarkar, P., Mally, A., Biemel, K. M., *et al*., *Arch. Biochem. Biophys.* 2002, *402*, 110–119.

[15] Metz, T. O., Alderson, N. L., Chachich, M. E., Thorpe, S. R. *et al*., *J. Biol. Chem.* 2003, *278*, 42012–42019.

[16] Lukienko, P. I., Mel'nichenko, N. G., Zverinskii, I. V., Zabrodskaya, S. V., *Bull. Exp. Biol. Med.* 2000, *130*, 874–876.

[17] Kannan, K., Jain, S. K., *Free Radic. Biol. Med.* 2004, *36*, 423–428.

[18] Gustafson, R. L., Martell, A. E., *Arch. Biochem. Biophys.* 1957, *68*, 485–498.

[19] Price, D. L., Rhett, P. M., Thorpe, S. R., Baynes, J. W., *J. Biol. Chem.* 2001, *276*, 48967–48972.

[20] Brewer, G. J., *Drug Discov. Today.* 2005, *10*, 1103–1109.

[21] Moldeus, P., Hogberg, J., Orrenius, S., *Methods Enzymol.* 1978, *52*, 60–71.

[22] Shangari, N., O'Brien, P. J., *Biochem. Pharmacol.* 2004, *68*, 1433–1442.

[23] Smith, M. T., Thor, H., Hartizell, P., Orrenius, S., *Biochem. Pharmacol.* 1982, *31*, 19–26.

[24] Dallner, G., *Methods Enzymol.* 1978, *52*, 71–82.

[25] Joly, J. G., Doyon, C., Peasant, Y., *Drug Metab Dispos.* 1975, *3*, 577–586.

[26] Voziyan, P. A., Hudson, B. G., *Cell Mol. Life Sci.* 2005, *62*, 1671–1681.

[27] Babaei-Jadidi, R., Karachalias, N., Kupich, C., Ahmed, N., *et al*., *Diabetologia* 2004, *47*, 2235–2246.

[28] Pourahmad, J., Ross, S., O'Brien, P. J., *Free Radic. Biol. Med.* 2001, *30*, 89–97.

[29] Ostrovtsova, S. A., *Acta Biochim. Pol.* 1998, *45*, 1031–1036.

[30] Sheline, C. T., Choi, D. W., *Ann. Neurol.* 2004, *55*, 645–653.

[31] Said, H. M., Reidling, J. C., Ortiz, A., *Biochim. Biophys. Acta* 2002, *1567*, 106–112.

[32] Silva, J. M., O'Brien, P. J., *Arch. Biochem. Biophys.* 1989, *275*, 551–558.

[33] Pocernich, C. B., Butterfield, D. A., *Neurotox. Res.* 2003, *5*, 515–520.

[34] Sun, L., Luo, C., Long, J., Wei, D., *et al*., *Mitochondrion* 2006, *6*, 136–142.

[35] Ciccoli, L., Signorini, C., Alessandrini, C., Ferrali, M. *et al*., *Exp. Mol. Pathol.* 1994, *60*, 108–118.

[36] Johnson, D. K., Pippard, M. J., Murphy, T. B., Rose, N. J., *J. Pharmacol. Exp. Ther.* 1982, *221*, 399–403.

[37] Burcham, P. C., Kaminskas, L. M., Fontaine, F. R., Petersen, D. R. *et al*., *Toxicolog*. 2002, *181–182*, 229–236.

[38] Hwang, J. M., Wang, C. J., Chou, F. P., Tseng, T. H., *et al*., *Arch. Toxicol.* 2005, *79*, 102–109.

[39] Niknahad, H., Khan, S., O'Brien, P. J., *Chem. Biol. Interact.* 1995, *98*, 27–44.

[40] Siraki, A. G., Pourahmad, J., Chan, T. S., Khan, S., *et al*., *Free Radic. Biol. Med.* 2002, *32*, 2–10.

[41] Tretter, L., Adam-Vizi, V., *Philos. Trans. R. Soc. Lond B Biol. Sci.* 2005, *360*, 2335–2345.

[42] Tabatabaie, T., Potts, J. D., Floyd, R. A., *Arch. Biochem. Biophys.* 1996, *336*, 290–296.

[43] Mehta, R., Templeton, D. M., O'Brien, P. J., *Chem. Biol. Interact.* 2006, *163*, 77–85.

[44] El-Ezaby, M., El-Shatti, N., *J. Inorg. Biochem.* 1979, *10*, 169–177.

[45] Mehta, R., O'Brien, P. J., in: Weiner, H., Maser, E., Lindahl, R., Plapp, B. (Eds.), *Enzymology and Molecular Biology of Carbonyl Metabolism, 13th Ed.*, Purdue University Press, 2007, pp. 113–120.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim