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Original Contribution

A novel role for vitamin B_{12}: Cobalamins are intracellular antioxidants in vitro

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ABSTRACT

Oxidative stress is a feature of many chronic inflammatory diseases. Such diseases are associated with up-regulation of a vitamin B_{12} (cobalamin) blood transport protein and its membrane receptor, suggesting a link between cobalamin and the cellular response to inflammation. The ability of cobalamin to regulate inflammatory cytokines suggests that it may have antioxidative properties. Here we show that cobalamins, including the novel thiolatocobalamins *N*-acetyl-L-cysteinylcobalamin and glutathionylcobalamin, are remarkably effective antioxidants in vitro. We also show that thiolatocobalamins have superior efficacy compared with other cobalamin forms, other cobalamins in combination with *N*-acetyl-L-cysteine (NAC) or glutathione (GSH), and NAC or GSH alone. Pretreatment of Sk-Hep-1 cells with thiolatocobalamins afforded robust protection (>90% cell survival) against exposure to 30 μM concentrations of the pro-oxidants homocysteine and hydrogen peroxide. The compounds inhibited intracellular peroxide production, maintained intracellular glutathione levels, and prevented apoptotic and necrotic cell death. Moreover, thiolatocobalamins are remarkably nontoxic in vitro at supraphysiological concentrations (>2 mM). Our results demonstrate that thiolatocobalamins act as powerful but benign antioxidants at pharmacological concentrations. Because inflammatory oxidative stress is a component of many conditions, including atherosclerosis, dementia, and trauma, their utility in treating such disorders merits further investigation.

Chronic inflammation, often accompanied by oxidative stress, is a component of many age-related diseases, including cancer, atherosclerosis, neurodegenerative disease, and arthritis. As such, it is a significant cause of morbidity and mortality.

Recent observations suggest that cobalamins (vitamin B_{12} derivatives) may modulate the oxidative stress responses, including those of the inflammatory response. Inflammatory diseases are associated with elevated blood levels of transcobalamin (a cobalamin transport protein) [1], and its membrane receptor is up-regulated by TNF-α [2]. Cobalamin concentration also modulates TNF-α levels in cerebrospinal fluid [3]. TNF-α is important in inflammatory responses, so taken together, these observations suggest that elevation of cobalamin could be used to supplement the cellular response to inflammation.

In the cell, two enzymes use cobalamin as a cofactor in either the adenosylcobalamin (AdoCbl) or the methylcobalamin (MeCbl) form. In the mitochondrial AdoCbl-dependent L-methylmalonyl-CoA mutase (EC 5.4.99.2) reaction, L-methylmalonyl-CoA is converted to succinyl-CoA, which then enters the Krebs cycle. In the cytosolic MeCbl-dependent methionine synthase (EC 2.1.1.13) reaction, tetrahydrofolate and methionine are generated by a methyl group transfer from methyltetrahydrofolate to homocysteine (Hcy) [4]. Hcy is a junction metabolite that can also be catabolized by cystathionine β-synthase, ultimately leading to synthesis of the important intracellular antioxidant glutathione (GSH) [4]. Elevated Hcy is associated with endothelial cell dysfunction and promotes the formation of reactive oxygen species, primarily by a mechanism involving endothelial nitric oxide synthase, but also by autoxidation [5]. Hcy also inhibits the antioxidant enzymes superoxide dismutase and glutathione peroxidase and activates endothelial proinflammatory signaling pathways [6].

Thiol derivatives of cobalamin (thiolatocobalamins) such as glutathionylcobalamin (GSCbl) were first identified in the 1960s [7,8]. GSCbl can be isolated from mammalian cells and is a potential precursor of the cofactor forms of cobalamin, although the exact pathways leading to the incorporation of cobalamin into its dependent enzymes remain unclear [9–13]. GSCbl is more active than other cobalamins in promoting methionine synthase activity [14], suggesting that thiolatocobalamins might be more efficacious than other cobalamins in treating conditions associated with hyperhomocysteinemia and oxidative stress, such as Alzheimer disease [4,15].

In this study we explored the relative effects of thiols and cobalamins, including the novel thiolatocobalamin *N*-acetyl-L-

Abbreviations: AdoCbl, adenosylcobalamin; CNCbl, cyanocobalamin; DCFH-DA, dichlorofluorescin diacetate; GSH, glutathione; GSCbl, glutathionylcobalamin; Hcy, homocysteine; HOCbl, hydroxocobalamin; MeCbl, methylcobalamin; NAC, *N*-acetyl-L-cysteine; NACCbl, *N*-acetyl-L-cysteinylcobalamin.

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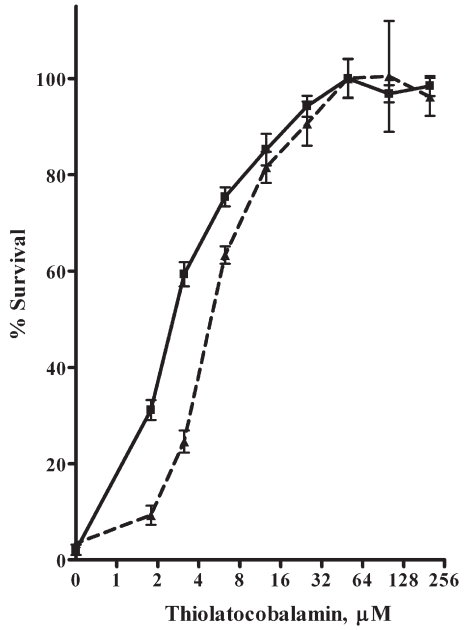

Fig. 1. Thiolatocobalamins protect endothelial cells from the effects of Hcy. Sk-Hep-1 cells were exposed to increasing concentrations of NACCbl (■) or GSCbl (▲) for 2 h before exposure to 30 μM Hcy for 24 h. Cell activity was measured by MTS assay at 490 nm. Data are shown as means ± SEM.

cysteinylcobalamin (NACCbl) [16], in protecting cells against Hcy- and hydrogen peroxide (H_2O_2)-induced oxidative stress.

Experimental procedures

Sk-Hep-1 (ECACC 91091816) cells were maintained in MegaCell MEME (Sigma; M-4067) with 3% serum and 200 mM L-glutamine at 37 °C in 5% CO_2. The Sk-Hep-1 cell line was chosen as it is highly sensitive to oxidative stress. Cells were plated into 96-well microtiter plates and cultured for 24 h. Medium was replaced with 100 μl fresh medium containing various concentrations of DL-homocysteine or H_2O_2 as oxidants for up to 24 h. Cells were preincubated with cobalamin, thiol (NAC or GSH), or cobalamin derivative, GSCbl or NACCbl, for 2 h.

Cell survival was measured using reduction of MS tetrazolium compound to formazan (490 nm) as a proxy for cell number in the CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Madison, WI, USA) [17].

Apoptosis was measured from caspase-3 activity and necrosis from propidium iodide (PI) uptake [17]. Caspase-3 activity was measured in cells resuspended in 100 μl of DMEM without phenol red after the addition of 50 μl caspase-3 substrate (EnzoLyte Rh110 caspase-3 kit; AnaSpec, San Jose, CA, USA). Plates were incubated at 37 °C for 60 min, and formation of free 7-amino-4-trifluoromethylcoumarin was acquired by fluorescence measurement at λ_{ex} 496/λ_{em} 520 nm. PI uptake was measured after the addition of 50 μl of 5 μg ml^{-1} propidium iodide solution to cells resuspended in 50 μl of phenol-red-free medium. Plates were incubated in the dark at 37 °C for 20 min and fluorescence was measured at λ_{ex} 535 nm/λ_{em} 617 nm.

Peroxide generation was measured by the addition of the redox-active probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to cells at a concentration of 10 mM for 30 min. After treatment, samples were solubilized in NaOH (0.1 N). Activity was measured at λ_{ex} 488 nm/λ_{em} 525 nm. Glutathione was measured using a kinetic assay measuring the reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) to TNB at 412 nm, after deproteinization with 5% 5-sulfosalicylic acid solution (Sigma glutathione assay kit; CS0260).

Data are presented as the means ± SEM of six separate experiments. Multiple comparisons of means were carried out by ANOVA using the Bonferroni test post hoc.

Results

Preliminary experiments established that a Hcy concentration of 30 μM achieved >90% cell death in Sk-Hep-1 cells. Dose-dependent protection from cell death was observed when Sk-Hep-1 cells were pretreated with increasing concentrations of either GSCbl or NACCbl (2–64 μM) for 2 h before exposure to Hcy (30 μM) (Fig. 1). Cell survival (>90%) from Hcy toxicity was achieved with GSCbl (30 μM) and NACCbl (30 μM) (Fig. 1). Cobalamin and thiol (NAC and GSH) concentrations were optimized in terms of protection against Hcy-induced (30 μM) cell death (Supplementary Figs. 1–7). The non-thiolatocobalamins cyanocobalamin (CNCbl), hydroxocobalamin (HOCbl), and MeCbl were least effective at protecting against Hcy-induced death; their optimized concentrations of 12.5–17.5 μM provided ~30% protection ($P<0.001$; Fig. 2). At these concentrations, thiolatocobalamins afforded greater than 80% protection (Fig. 1). Cells pretreated with a greater concentration of thiolatocobalamin (30 μM) were not significantly different from unexposed control cells (Fig. 2). Although cell survival was moderately enhanced (~55%) by pre-incubating with NAC (45 μM) or GSH (100 μM), either alone or with any nonthiolatocobalamin, the protection provided by the thiolatocobalamins was significantly superior to that of their corresponding thiol ($P<0.001$; Fig. 2). Caspase-3 is a key enzyme in apoptotic cell death pathways and a suitable marker for quantifying apoptosis after

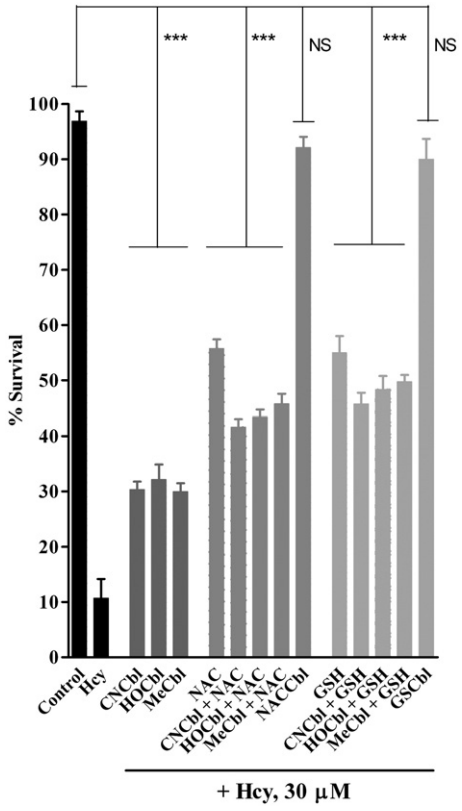

Fig. 2. The protective effects of cobalamins and thiols against Hcy toxicity. Sk-Hep-1 cells were pretreated for 2 h with CNCbl (15.0 μM), HOCbl (17.5 μM), MeCbl (12.5 μM), NAC (45 μM), GSH (100 μM), NACCbl (30 μM), or GSCbl (30 μM) before exposure to Hcy (30 μM) for 24 h. Cell activity was measured by MTS assay at 490 nm. Data are shown as means ± SEM, ***$P<0.001$; NS, not significant. All results are significantly different from cells treated with Hcy only ($P<0.001$); these and other obvious differences are not annotated on the graph for simplicity.

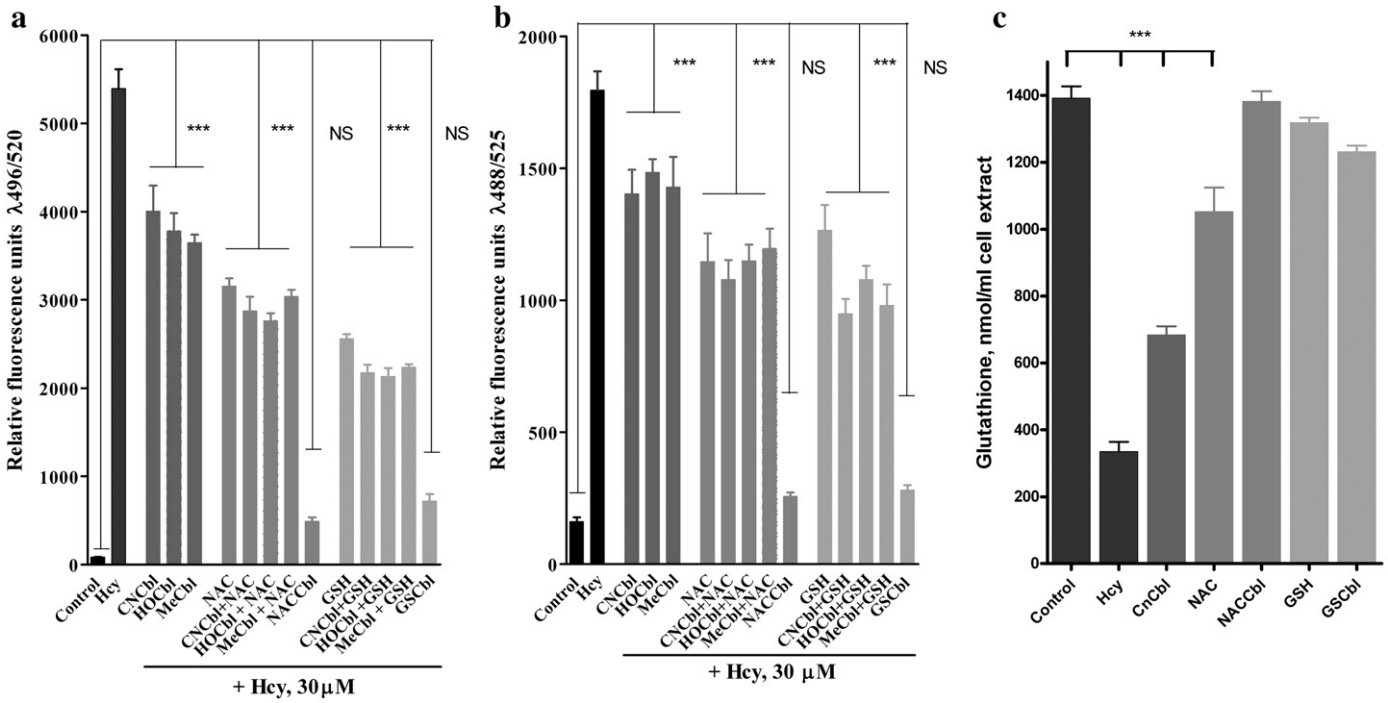

Fig. 3. The effects of cobalamins and thiols on Hcy-induced (a) caspase-3 activation, (b) peroxide production, and (c) glutathione levels. Sk-Hep-1 cells were pretreated for 2 h with CNCbl (15.0 μM), HOCbl (17.5 μM), MeCbl (12.5 μM), NAC (45 μM), GSH (100 μM), NACCbl (30 μM), or GSCbl (30 μM) before exposure to Hcy (30 μM) for 24 h. (a) Caspase 3 activity was measured at 520 nm. (b) DCFH-DA uptake was measured at 488/525 nm. (c) Glutathione was measured from the reduction of DTNB to TNB at 412 nm. Data are shown as means ± SEM. ***$P<0.001$; NS, not significant. All results are significantly different from cells treated with Hcy only ($P<0.001$), these and other obvious differences are not annotated on the graph for simplicity.

oxidative stress [17]. Remarkably, Hcy-induced caspase activity was almost completely prevented in cells pretreated with either NACCbl (30 μM) or GSCbl (30 μM) (Fig. 3a). Although cells pretreated with optimized concentrations of nonthiolatocobalamins and thiols (singly or combined) all showed protection varying from ~25 to ~60%, they were all significantly less protected than those treated with the thiolatocobalamins ($P<0.001$; Fig. 3a). The thiolatocobalamins also prevented Hcy-induced increases in intracellular peroxide (Fig. 3b) and decreases in intracellular glutathione (Fig. 3c) concentrations. Again, the optimized concentrations of nonthiolatocobalamins and thiols were less effective—ranging from ~20 to ~50% reduction in Hcy-induced peroxide production ($P<0.001$; Fig. 3b).

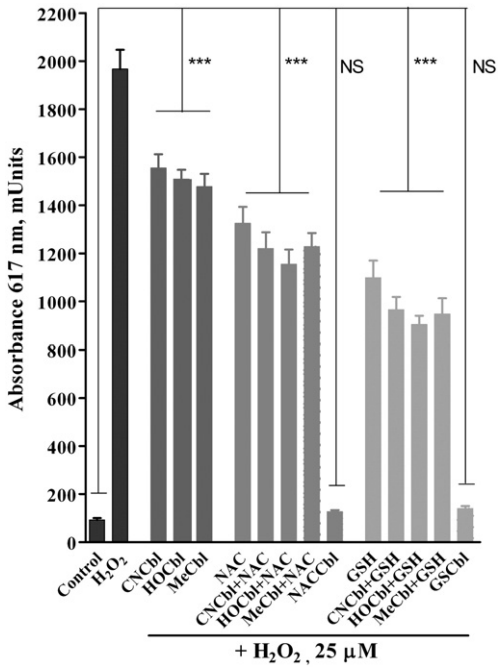

Fig. 4. The effects of cobalamins and thiols on H_2O_2-induced necrosis. Sk-Hep-1 cells were pretreated for 24 h with CNCbl (15.0 μM), HOCbl (17.5 μM), MeCbl (12.5 μM), NAC (45 μM), GSH (100 μM), NACCbl (30 μM), or GSCbl (30 μM) before exposure to H_2O_2 (25 μM) for 2 h. PI uptake was measured at 617 nm. Data are shown as means ± SEM. ***$P<0.001$; NS, not significant. All results are significantly different from cells treated with Hcy only ($P<0.001$); these and other obvious differences are not annotated on the graph for simplicity.

H_2O_2 is one of the key reactive oxygen species involved in oxidative stress-induced necrotic cell damage, as measured by PI uptake [17]. Preliminary experiments established that 25 μM H_2O_2 resulted in >90% cell death of Sk-Hep-1 cells. Pretreatment with NAC and then exposure to 25 μM H_2O_2 reduced PI uptake by ~35% compared with a positive control of 25 μM H_2O_2 ($P<0.01$; Fig. 4). Similarly, pretreatment with GSH followed by exposure to 25 μM H_2O_2 resulted in an ~45% decrease in PI uptake ($P<0.01$; Fig. 4). When NAC or GSH was combined with nonthiolatocobalamins as a pretreatment, PI uptake was reduced by ~50% compared with positive control ($P<0.001$; Fig. 4). The thiolatocobalamins provided total protection against necrosis induced by 25 μM H_2O_2 (Fig. 4), demonstrating their superior antioxidant properties. At high concentrations (>250 μM) NACCbl or GSCbl significantly reduced cell viability, but even at 2.5 mM both compounds reduced cell viability only to 75% that of controls (Supplementary Fig. 8).

To demonstrate that the results were not exclusive to Sk-Hep-1 cells we repeated some of this work with human umbilical vein endothelial cells (HUVECs). HUVECs required higher concentrations (50 μM) of Hcy and H_2O_2 to achieve >90% death. However, thiolatocobalamins were again superior to nonthiolatocobalamins in protecting against cell death (Fig. 5a), apoptosis (Fig. 5b), and necrosis (Fig. 5c).

Discussion

In this study, preincubation of Sk-Hep-1 cells with GSH or NAC only partially protected against Hcy- or H_2O_2-induced damage. Their respective thiolatocobalamin derivatives (GSCbl and NACCbl)

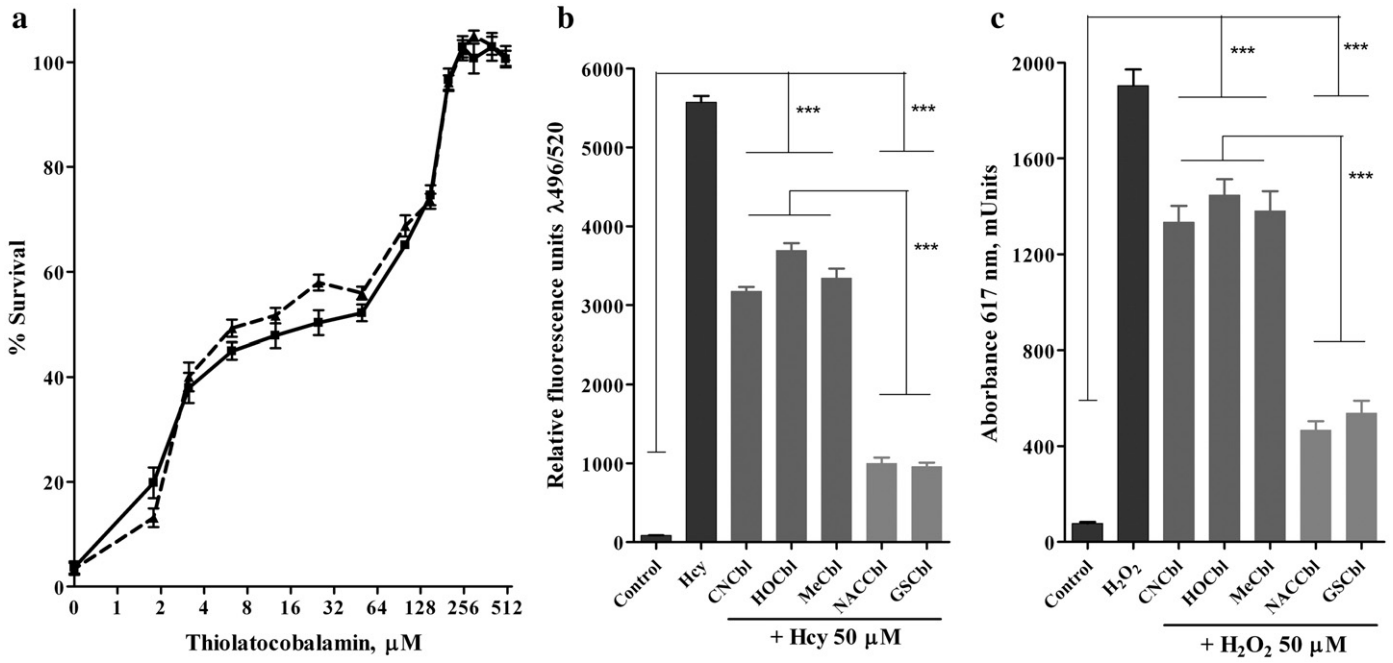

Fig. 5. Thiolatocobalamins protect HUVECs from the effects of Hcy. (a) HUVECs were exposed to increasing concentrations of NACCbl (■) and GSCbl (▲) for 2 h before exposure to 50 μM Hcy for 24 h. (b and c) HUVECs were pretreated for 2 h with CNCbl (15.0 μM), HOCbl (17.5 μM), MeCbl (12.5 μM), NACCbl (30 μM), or GSCbl (30 μM) before exposure to (b) Hcy (50 μM) or (c) H_2O_2 for 24 h and measurement of (b) caspase 3 activity or (c) propidium iodide uptake. Data are shown as means ± SEM. ***$P<0.001$. All results are significantly different from cells treated with Hcy or H_2O_2 only ($P<0.001$); these differences are not annotated on the graph for simplicity.

demonstrated significantly greater protective capabilities than the thiol alone or the thiol combined with standard cobalamin derivatives.

GSH and NAC, unlike the cobalamins, are well-characterized antioxidants. GSH is a thiol-containing tripeptide and a major antioxidant defense molecule. Reduced GSH levels correlate with increased oxidative stress, mitochondrial damage, and apoptosis [18]. A proportion of the protection afforded by the cobalamins against Hcy-induced cell death might be attributable to its increased intracellular clearance due to enhanced methionine synthase activity [19]. However, the protection shown against H_2O_2 suggests that cobalamins also act as antioxidants via other mechanisms.

Consistent with our results, GSH and NAC reduce apoptosis in cultured endothelial-derived hepatocytes by down-regulating oxidative stress-related mechanisms such as caspase-3 activation [20]. Caspase-3 cleavage is reduced by folic acid and CNCbl in a mouse model of amyotrophic lateral sclerosis [21]. Similarly, we show that thiolatocobalamins efficiently prevent peroxide-induced oxidative stress, maintain intracellular glutathione levels, and inhibit caspase-3-mediated cell death. However, there are no previous reports of a direct antioxidant effect of cobalamin alone. Indirect copper-mediated protection against LDL oxidation is reported for CNCbl and attributed to an altered in vitro equilibrium between oxidized and reduced cobalamin forms [22]. Indeed, cob(II)alamin—a reduced form of cobalamin—is an efficient radical trap [23], and the superior protective effect of thiolatocobalamins compared with nonthiolatocobalamins might relate to their more facile reduction to cob(II)alamin or cob(I)alamin and direct scavenging-type reactions between these reduced forms and reactive oxygen and/or nitrogen species [24–27].

Cobalamin's protective effects may also relate to other recently described novel non-coenzymatic functions [28–30]. Cobalamin-deficient rats exhibit increased cerebrospinal fluid levels of some neurotoxic molecules, including TNF-α, and decreased levels of neurotrophic molecules, including epidermal growth factor (EGF) [30]. Similarly, patients with severe cobalamin deficiency have high TNF-α levels and low EGF levels in cerebrospinal fluid and serum, which are correctable by cobalamin replacement [3]. These observations suggest that cobalamin modulates the expression of certain cytokines and growth factors. It is possible that this occurs as a consequence of cobalamin modifying the activity of signaling molecules such as NF-κB [30].

The antioxidant properties of cobalamin probably result from a combination of direct and indirect effects: stimulation of methionine synthase activity [4,23], direct reaction with reactive oxygen and nitrogen species, a glutathione sparing effect [31], and modification of signaling molecules [30], leading to induction of stress responses. The remarkably superior protection of thiolatocobalamins in vitro presumably relates to their enhanced function in one or more of these potential mechanisms, the balance of which may differ between the two compounds. In conclusion, cobalamins, and in particular the thiolatocobalamins, exhibit a marked antioxidant activity at pharmacological concentrations and afford significant cellular protection against oxidative stress. Thiolatocobalamins might have potential in treating a number of pathological conditions in which oxidative stress is a clinically important component.

Acknowledgments

GSCbl and NACCbl are currently the subjects of U.S. Patent applications by N.E.B., C.S.B., and J.H.H.W. (U.S. Application 20080113900, "Pharmaceutical compositions and therapeutic applications for the use of a synthetic vitamin B_{12} derivative, glutathionylcobalamin," and U.S. Application 20080076733, "Pharmaceutical compositions and therapeutic applications for the use of a novel vitamin B_{12} derivative, *N*-acetyl-L-cysteinylcobalamin") and by A.M. (U.S. Application 20040157783, "Method for treating or preventing a functional vitamin B_{12} deficiency in an individual and medical compositions for use in said method"). A.M. is a Scientific Advisor and shareholder of COBALZ Ltd—a private limited company developing novel B vitamin and antioxidant supplements.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2009.04.023.

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