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Vitamin B_{12} protects against superoxide-induced cell injury in human aortic endothelial cells

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Abstract

Superoxide ($O_2^{\bullet-}$) is implicated in inflammatory states including arteriosclerosis and ischemia-reperfusion injury. Cobalamin (Cbl) supplementation is beneficial for treating many inflammatory diseases and also provides protection in oxidative-stress-associated pathologies. Reduced Cbl reacts with $O_2^{\bullet-}$ at rates approaching that of superoxide dismutase (SOD), suggesting a plausible mechanism for its anti-inflammatory properties. Elevated homocysteine (Hcy) is an independent risk factor for vascular disease and endothelial dysfunction. Hcy increases $O_2^{\bullet-}$ levels in human aortic endothelial cells (HAEC). Here, we explore protective effects of Cbl in HAEC exposed to various $O_2^{\bullet-}$ sources, including increased Hcy levels. Hcy increased $O_2^{\bullet-}$ levels (1.6-fold) in HAEC, concomitant with a 20% reduction in cell viability and a 1.5-fold increase in apoptotic death. Pre-treatment of HAEC with physiologically relevant concentrations of cyanocobalamin (CNCbl) (10 – 50 nM) prevented Hcy-induced increases in $O_2^{\bullet-}$ and cell death. CNCbl inhibited both Hcy and rotenone- induced mitochondrial $O_2^{\bullet-}$ production. Similarly, HAEC challenged with paraquat showed a 1.5-fold increase in $O_2^{\bullet-}$ levels and a 30% decrease in cell viability, both of which were prevented with CNCbl (10 nM) pre-treatment. CNCbl also attenuated elevated $O_2^{\bullet-}$ levels following exposure of cells to a Cu/Zn-SOD inhibitor. Our data suggest that Cbl acts as an efficient intracellular $O_2^{\bullet-}$ scavenger.

Keywords

Superoxide; Homocysteine; Cobalamin; Apoptosis; Oxidative stress; Vascular Endothelium; Antioxidant

Introduction

Cobalamins (Cbls, vitamin B_{12} derivatives) are micronutrients essential for the synthesis of methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl), the respective cofactors for

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cytosolic methionine synthase (MS) and mitochondrial L-methylmalonyl-CoA mutase. Reduced Cbl cofactors in these catalytic cycles are sensitive to oxidation [1, 2]; hence, both MS and L-methylmalonyl-CoA mutase are inactivated by reactive oxygen species (ROS). MS plays a key role in the metabolism of homocysteine (L-Hcy, Hcy), a byproduct of methylation reactions which is metabolized to cysteine or methionine. In endothelial cells, Hcy metabolism depends exclusively on Cbl-dependent MS [3]. Impaired MS activity due to Cbl deficiency results in elevated Hcy, a risk factor for cardiovascular disease [4]. Hcy also elevates $O_2^{\bullet -}$ levels, leading to increased oxidative stress, which further hinders Cbl's metabolism. Importantly, Cbl exhibits antioxidant effects in Hcy-independent systems [5, 6]. Birch *et al* reported that Cbl protects against hydrogen peroxide-induced oxidative stress [5], and our preliminary experiments demonstrate the potential of Cbl to attenuate elevated $O_2^{\bullet -}$ levels [6].

Cbl deficiency is a common and significant public health problem, particularly amongst the elderly [4]. Up to 40% of the elderly US population are B_{12}-deficient [7]. Following folate fortification in food, Cbl deficiency became the primary modifiable cause of hyperhomocysteinemia [8]. Vitamin supplements containing cyanocobalamin (CNCbl, vitamin B_{12}) decrease LDL oxidation in both healthy individuals and patients with coronary artery disease [9]. Cbl supplementation is also beneficial in treating many inflammatory diseases, and there is accumulating evidence that Cbl can protect against oxidative stress-associated pathologies [10-16]. Levels of the Cbl transport protein transcobalamin increase during inflammation [17, 18], concomitant with NF-κB activation induced by various stimuli, including ROS. Taken together, these studies suggest a potential role for Cbl in the regulation of inflammatory processes [15, 19].

Recently we demonstrated that the reduced form of Cbl, cob(II)alamin (Cbl(II)) reacts with $O_2^{\bullet -}$ with a second-order rate constant of 7×10^8 $M^{-1}.s^{-1}$, close to that observed for superoxide dismutase itself (Cu,Zn-SOD; $k = 2 \times 10^9$ $M^{-1}.s^{-1}$) [6]. Given that Cbl(II) is a major intracellular Cbl form [2], we hypothesized that scavenging of $O_2^{\bullet -}$ by Cbl is an important mechanism by which Cbl can protect cells against oxidative stress.

$O_2^{\bullet -}$ is a free radical product of a 1 electron reduction of oxygen. It is produced by mitochondrial and reticular membrane electron transport systems, or enzymes including NADPH oxidase (NOX) and xanthine oxidase (XO), and uncoupled nitric oxide synthase (NOS)[20]. Exposing cells to Hcy also results in elevated levels of $O_2^{\bullet -}$ [21, 22]. $O_2^{\bullet -}$ is involved in both physiological and pathological processes [23], with $O_2^{\bullet -}$ overproduction implicated in a range of inflammatory states such as rheumatoid arthritis, osteoarthritis, arteriosclerosis, and ischemia-reperfusion [24]. The toxicity of increased $O_2^{\bullet -}$ levels is evident in homozygous SOD2 knockout mice, which die within the first three weeks of age [25]. $O_2^{\bullet -}$ can inactivate a range of enzymes in addition to causing direct molecular damage by initiating lipoperoxidation, leading to the destruction of neurotransmitters and hormones, and DNA single strand damage [20]. Moreover, $O_2^{\bullet -}$ can generate peroxynitrite via its reaction with nitric oxide and hydrogen peroxide via dismutation. From these species, stronger damaging oxidants can be generated such as the carbonate, hydroxyl and nitrogen dioxide radicals and oxoferryl complexes [26]. Therefore a tight control of $O_2^{\bullet -}$ levels is paramount to prevent the formation of secondary oxidants.

In this work we describe the protective effects of physiologically relevant concentrations of CNCbl against elevated intracellular $O_2^{\bullet -}$ levels induced by paraquat and L-Hcy and the associated cell injury in primary human aortic endothelial cells (HAEC). Both paraquat and L-Hcy induced elevated $O_2^{\bullet -}$ levels which paralleled cell death and which were prevented by pre-treating the cells with CNCbl prior to the insult. Elevated $O_2^{\bullet -}$ levels were also

observed in cells treated with the Cu/Zn-SOD inhibitor diethyldithiocarbamate (DETC) [27], and were similarly attenuated by CNCbl pretreatment.

Materials and Methods

Synthesis of L- and D-Hcy

L- or D-Homocysteine thiolactone (20 mg, 130 μmol) was dissolved in NaOH (5 N, 200 μl) and incubated at 37 °C for 10 min. The solution was chilled and neutralized with HCl (5 N, 200 μl). PBS was added to a total volume of 1 ml and the solution bubbled with N_2 for 10 min [28]. The yield was typically > 95%, determined by quantifying the reduced thiol groups by the Ellman's assay [29].

Cell culture

Primary HAEC clones were a generous gift from Donald W. Jacobsen (Lerner Research Institute, The Cleveland Clinic). Each endothelial cell isolate was stored and passaged separately. HAEC were cultured in fibronectin coated flasks in M199 supplemented with LONZA bullet kit supplements for EBM-2 in a humidified 95% air, 5% CO_2 incubator at 37 °C. For experiments cells were seeded onto 96- or 6-well plates at a density of 12000-20000 cells/cm^2 and used up to passage 6.

Intracellular Cbl content and Cbl uptake

For Cbl uptake experiments, pre-confluent HAEC were incubated with 0.2 nM ^{57}(Co)-CNCbl. Adherent cells were harvested at different time points up to 24 h and thoroughly washed with PBS. The intracellular Cbl uptake was determined by counting the cell-associated radioactivity. To measure total intracellular Cbl content, pre-confluent HAEC were incubated with or without varying concentrations of CNCbl for 24 h, and intracellular Cbl content was determined by the SimulTRAC radioassay.

Assessing ROS production

Cells with or without CNCbl pre-treatment (500 pM - 10 μM) were incubated with L-Hcy (100 or 150 μM), H_2O_2 (50 - 200 μM), paraquat (1.5 mM), rotenone (5 μM), or with culture medium alone. To assess general ROS production, cells were incubated with the oxidation sensitive fluorescent probe dichlorofluorescein acetate (DCFA, 3 μM) for the duration of the L-Hcy or H_2O_2 treatment. For the assessment of $O_2^{\bullet -}$ production, cells were incubated with the oxidative fluorescent probes, dihydroethidium DHE (5 μM), or MitoSOX (5 μM) for 1 h subsequent to the L-Hcy, paraquat, or rotenone treatment. Fluorescence was quantified in a microplate reader (DCF: $\lambda_{ex/em}$ = 420/520; 2-hydroxyethidium: $\lambda_{ex/em}$ = 510/605 nm; MitoSOX: $\lambda_{ex/em}$ = 510/580 nm).

Cell viability

Cell viability was assessed with trypan blue staining or with the MTT assay. For the MTT assay cells were incubated with thiazolyl blue tetrazolium (0.4 mg/ml) in M199 for 3 h at 37°C. Mitochondrial-dependent tetrazolium reduction to formazan was measured by reading optical density at 540 nm.

Cbl protection against $O_2^{\bullet -}$-generating insults- L-Hcy, paraquat or rotenone

Pre-confluent HAEC were incubated for 24 h in the absence or presence of CNCbl (500 pM – 10 μM) prior to adding L-Hcy (100 or 150 μM), paraquat (1.5 mM), or rotenone (5 μM). For some experiments SOD (3 μM) was added at the time of paraquat addition or apocynin (0.1 mM) was added 30 min before adding L-Hcy or paraquat. Cells were incubated for 24 h

with L-Hcy, H_2O_2, or paraquat, or for 1 h with rotenone. ROS and cell viability were assessed as described above.

Diethyldithiocarbamate (DETC)

Pre-confluent HAEC were incubated for 24 h in the absence or presence of CNCbl, then subjected to 10 mM DETC for 2 hr. This concentration has been previously shown to inhibit Cu/ZnSOD activity by up to 50% and to increase vascular $O_2^{\bullet -}$ [30].

DNA and Cbl quantification

Confluent cells were harvested in lysis buffer (50 mM Tris pH 7.4, 0.5 % Triton X-100) and DNA quantified using the CyQuant cell proliferation kit (Roche). Cbl was quantified using the SimulTRAC Radioassay for vitamin B_{12} and folate by MP Biomedicals (Orangeburg, NY) according to the manufacturer's specifications.

Apoptosis measurement

To detect apoptotic cell death, cells were seeded onto 6-well plates and pre-treated with or without CNCbl (10 – 100 nM) for 24 h. Cells were washed then incubated in the absence or presence of L-Hcy for 18 h. Apoptosis was assessed using the Cell Death Detection ELISA (Roche) according to the manufacturer's specifications.

General solution preparation

Thiol solutions were prepared immediately before use and the concentrations were determined by the Ellman's method [29]. A fresh solution of H_2O_2 was prepared before experiments and the concentration determined spectrophotometrically (ε_{240nm} = 43.6 $M^{-1}cm^{-1}$ [31]). The concentration of the stock solution of CNCbl was determined by the dicyanocobalamin test (ε_{368nm}=30.4 mM^{-1} cm^{-1} [32]).

Statistics

All experiments were carried out using at least three separate cell clones. Results are expressed as mean ± SEM. Statistical comparisons were carried out using ANOVA with the Bonferroni *post hoc* test.

Online Supplemental Material

Figures S1-S4 are included as supplemental figures.

Results

Cbl protects against elevated $O_2^{\bullet -}$ levels induced by Hcy exposure

Treatment of HAEC with varying concentrations of L-Hcy induced a concentration-dependent increase in ROS detected by increasing dichlorofluorescein (DCF) fluorescence, a general probe for ROS. L-Hcy (100 μM) elicited a significant increase in ROS compared to control (Figure 1). To verify that the ROS increase is a L-Hcy-specific effect, HAEC were incubated for 48 h with 0.1 mM of a range of thiols (glutathione, L-cysteine, D-Hcy, L-Hcy, and β-mercaptoethanol (βME)). Only L-Hcy elicited a significant increase in DCF fluorescence (Figure S1A), concomitant with a significant decrease in cell viability as measured using the MTT assay (Figure S1B).

Prior to determining the effect of vitamin B_{12} (CNCbl) on L-Hcy-dependent ROS levels, the concentration- and time-dependent uptake of CNCbl by HAEC was assessed. Incubating HAEC with ^{57}Co-labeled CNCbl (0.2 nM) induced a time dependent increase in intracellular CNCbl (Figure 2A). Increasing the CNCbl in the medium (0.1 – 10 μM non-

labeled Cbl) lead to higher intracellular Cbl levels after 24 h (Figure 2B). A 24 h incubation time with CNCbl was selected as an appropriate time for all subsequent experiments.

Exposing HAEC to L-Hcy (150 μM) over 48 h induced a 1.25-fold increase in DCF fluorescence (Figure 3A) that correlated with a ~ 25% decrease in cell viability (Figure 3B).To assess the effects of CNCbl on the L-Hcy-dependent ROS production and the L-Hcy-dependent decrease in cell viability, HAEC were pre-incubated with increasing concentrations of CNCbl for 24 h prior to treating the cells with L-Hcy (150 μM). To ensure that extracellular Cbl was not responsible for Cbl effects on ROS, cells were washed after Cbl treatment and medium replaced prior to further treatments. Pre-incubation of HAEC with CNCbl prevented the L-Hcy-dependent increase in ROS and decrease in cell viability in a concentration-dependent fashion (Figure 3). 10 nM CNCbl completely inhibited both the L-Hcy-dependent ROS increase ($p < 0.05$) and the L-Hcy-dependent decrease in cell viability ($p < 0.05$). L-Hcy (150 μM) treatment of cells over a 24 h period resulted in a ~20% decrease in cell viability; hence, subsequent experiments were conducted using a 24 h L-Hcy treatment protocol, unless otherwise stated.

Exposing cells to L-Hcy is reported to increase intracellular $O_2^{\bullet-}$levels [21, 22]. To determine if $O_2^{\bullet-}$ is indeed generated in our system, $O_2^{\bullet-}$ levels following exposure to L-Hcy were assessed using the $O_2^{\bullet-}$ specific probe hydroethidine (DHE), which upon reacting with $O_2^{\bullet-}$ yields the fluorescent product 2-hydroxyethidium. Incubation of HAEC with L-Hcy (150 μM) for 24 h induced a 1.6-fold increase in hydroxyethidium fluorescence (Figures 4 and S2). This increase was completely inhibited by pre-incubation of the cells with 10 nM CNCbl ($p < 0.05$), the antioxidant apocynin (0.1 mM), or SOD itself (3 μM) (Figures 4, S2 and S3).

Cbl protects against elevated mitochondrial $O_2^{\bullet-}$ levels

To investigate the subcellular localization of the L-Hcy-induced increase in $O_2^{\bullet-}$, cells were assayed with mitoSOX, a mitochondrial specific $O_2^{\bullet-}$ probe. Incubation of HAEC with L-Hcy (150 μM) for 24 h elicited a moderate but significant increase in mitoSOX fluorescence, which was completely inhibited by preincubation of the cells with CNCbl (50 nM) (Figure 5). Moreover, treatment of HAEC with the mitochondrial electron transport chain inhibitor rotenone (5 μM) induced an increase in mitochondrial $O_2^{\bullet-}$ which was also significantly inhibited by pre-incubation with CNCbl (100 nM) (Figures 5 and S4).

Cbl protects against elevated $O_2^{\bullet-}$ levels induced by paraquat

To assess the ability of CNCbl to protect against a direct source of $O_2^{\bullet-}$, HAEC were exposed to paraquat (1.5 mM, 24 h), a well established $O_2^{\bullet-}$ source [33]. Paraquat induced a 1.5-fold increase in $O_2^{\bullet-}$ production measured by hydroxyethidium fluorescence, which was prevented by CNCbl pre-treatment (10 nM), apocynin (0.1 mM), and SOD (3 μM) (Figure 6A). The paraquat-induced increase in oxidative stress correlated with a 30% decrease in cell viability (Figure 6B). Pre-incubation with CNCbl for 24 h, treatment with apocynin (0.1 mM), or SOD (3 μM) protected HAEC against the $O_2^{\bullet-}$-dependent decrease in cell viability (Figure 6B).

Cbl attenuates elevated $O_2^{\bullet-}$ levels resulting from Cu/Zn-SOD inhibition

To explore the possibility that Cbl can act as a second line of defense when $O_2^{\bullet-}$ production overwhelms the SOD capacity, we inhibited Cu/Zn-SOD by treating HAEC with diethyldithiocarbamate (DETC, 10 mM) for 2 h with or without CNCbl pre-treatment. Incubation with DETC elicited an increase in DHE fluorescence indicative of higher intracellular $O_2^{\bullet-}$ levels. Pre-treatment with CNCbl (100 nM) significantly reduced the

DETC-induced DHE fluorescence (Figure 7), providing support for CNCbl's ability to scavenge $O_2^{\bullet -}$ in SOD-compromised cells.

Cbl protects against Hcy-dependent increase in apoptotic cell death

Exposing HAEC to L-Hcy (150 μM) for 24 h caused a significant decrease in cell viability as measured by the MTT assay (Figure 8A). Since the MTT assay is a measure of mitochondrial function and metabolic activity, cell death was directly assessed by trypan blue staining, which corresponded with the MTT results (Figure 8B). Finally, to characterize the L-Hcy induced cell death, apoptosis was assessed by measuring cytosolic fragmented DNA with an ELISA-based cell death assay (Roche). HAEC showed a significant increase in apoptotic cell death in response to L-Hcy (150 μM) for 18 h (Figure 9). Since both CNCbl and apocynin prevented the L-Hcy-induced decrease in cell viability as measured by the MTT assay (Figure 8A), and the increase in cell death, as measured by trypan blue staining (Figure 8B) or ELISA (Figure 9), this is indicative of CNCbl providing protection against apoptosis.

Discussion

Vitamin B_{12} is an essential micronutrient required for one-carbon metabolism and branched amino acid catabolism. Recent studies in our laboratories showed that Cbl(II) can directly scavenge $O_2^{\bullet -}$ to form aquacobalamin (and hydrogen peroxide) extremely rapidly, at a rate approaching that of SOD-catalyzed dismutation (7×10^8 vs 2×10^9 $M^{-1}.s^{-1}$) [6]. Substantial free (non-protein bound) intracellular Cbl can be achieved with supplementation [36-39]. Upon entering cells the cobalt(III) center of Cbl is reduced to cobalt(II) (= Cbl(II)) prior to binding to the B_{12}-dependent enzymes [40]. The ability of cells to re-reduce free aquacobalamin to Cbl(II) ("aquacobalamin reductase activity") is well established [41-46]; therefore, providing the theoretical basis for Cbl-mediated catalytic $O_2^{\bullet -}$ scavenging. This led us to speculate that Cbl might protect cells from oxidative stress by efficient $O_2^{\bullet -}$ scavenging.

In our studies, intracellular $O_2^{\bullet -}$ was generated by treating HAEC with paraquat , and the ability of CNCbl to protect cells against damage was assessed. CNCbl at nanomolar concentrations could prevent the increase in$O_2^{\bullet -}$ levels and the associated reduction in cell viability (Figure 6). The inhibitory effects of CNCbl were comparable to those observed with apocynin or SOD itself (Figure 6).

The inhibition of Cu/Zn-SOD with DETC treatment also elevated cytosolic $O_2^{\bullet -}$ levels. DETC-treated cells were round and loosely attached compared to spindle-shaped control cells, demonstrating the dramatic effect of inhibiting Cu/Zn-SOD in HAEC. Pre-treating HAEC with CNCbl (100 nM) attenuated the DETC-induced increase in $O_2^{\bullet -}$ and also partially reverted the altered cell morphology, which further supports the direct effect of Cbl on intracellular $O_2^{\bullet -}$.

Additionally, CNCbl could blunt the generation of $O_2^{\bullet -}$ by Hcy. Hcy has been shown to induce an increase in intracellular $O_2^{\bullet -}$ levels [21, 22]. Moreover, elevated Hcy is associated with vascular oxidative stress. Normally, plasma Hcy levels are maintained below 12 μM; however, in severe clinical hyperhomocysteinemia, Hcy levels can exceed 100 μM in severe instances [47]. To ensure a universal response among our individual HAEC clones, our experiments used a high but still pathophysiological range of Hcy concentrations (100 – 150 μM), which increased $O_2^{\bullet -}$ levels and resulted in associated loss of cell viability. As with paraquat, these effects were completely inhibited by pre-treating HAEC with CNCbl (10 nM) or by treating the cells with apocynin or SOD itself.

The increase in L-Hcy-induced cell death correlated with increased apoptotic cell death. L-Hcy induces apoptosis in human bone marrow stromal cells [52], human umbilical vein endothelial cells [51, 53], and endothelial progenitor cells [54]. It also inhibits growth [55] and reduces cell viability in HAEC [56]. Our results are consistent with previous studies in other endothelial cell lines; however to our knowledge, we are the first to show L-Hcy-induced apoptotic cell death in primary cultures of HAEC. L-Hcy-induced apoptosis was prevented by apocynin or by pretreating HAEC with CNCbl (50 nM).

The protective effects of Cbl against L-Hcy-induced oxidative stress are perhaps not suprising, given the cofactor role of Cbl in Hcy metabolism. However, our experiments indicate that Cbl shows effects against $O_2^{\bullet-}$ generated in response to a variety of insults apart from Hcy (ie paraquat, rotenone, DETC). Such results indicate that Cbl protection against Hcy-mediated oxidative stress may not be due to increasing Hcy metabolism alone, and that Cbl can act to protect against oxidative stress in a general manner. These data, combined with our previous *in vitro* studies showing a direct and fast reaction between Cbl(II) and $O_2^{\bullet-}$ strongly implicate Cbl as an intracellular $O_2^{\bullet-}$ scavenger. However, in the case of Hcy, we cannot rule out the involvement of other mechanisms independent of $O_2^{\bullet-}$ scavenging in the CNCbl-mediated protective effects.

An important finding from our studies is the effectiveness of CNCbl against mitochondrial oxidative stress. Oxidative stress-associated mitochondrial dysfunction is a common feature in cardiovascular pathologies [57, 58] and there is considerable interest in developing mitochondrial-specific antioxidants [58, 59]. Hcy increases mitochondrial oxidative stress in brain [60] and cardiac myocytes [61]. Cbl is present in the mitochondria (Cbl-dependent L-methylmalonyl-CoA mutase is a mitochondrial enzyme) and ~80% of Cbl is in its Cbl(II) form [2]. In endothelial cells the mitochondrial AdoCbl concentration is 4-fold higher than cytosolic MeCbl concentration [62] and a substantial fraction of mitochondrial Cbl is not protein-bound [63]. In our studies, CNCbl treatment effectively inhibited the generation of mitochondrial $O_2^{\bullet-}$ by Hcy or rotenone treatment (Figure 5).

DHE remains one of the most widely used probes for detection of $O_2^{\bullet-}$ in live cells, although 2-hydroxyethidium, the specific reaction product between DHE and $O_2^{\bullet-}$, is not the only DHE fluorescent oxidation product. Ethidium, a 1 e^- oxidation product of DHE has an emission spectrum with a 45% overlap with that of 2-hydroxyethidium [64]. The same applies for the mitochondria-targeted ethidium derivative, mitoSOX and its oxidation products [64]. Therefore, the specificity of DHE for detecting $O_2^{\bullet-}$levels has been questioned. However, our conclusions are not solely based on the effect of Cbl on DHE or mitoSOX oxidation. The oxidative-stress-inducing insults used in our studies have been shown to increase $O_2^{\bullet-}$ levels in previous studies using lucigenin chemiluminescence or EPR [21, 22, 30, 65, 66], and these data correlate well with an increase in DHE-derived fluorescence. Thus, because $O_2^{\bullet-}$ is the predominant ROS produced in these systems, the Cbl effect was mimicked by SOD, and our previous studies show that Cbl reacts very rapidly with $O_2^{\bullet-}$, we conclude that the Cbl-dependent decrease in DHE fluorescence is due to a direct Cbl-mediated $O_2^{\bullet-}$-scavenging mechanism.

It is likely that Cbl has biological roles beyond its ability to act as a cofactor for the two mammalian B_{12}-dependent enzyme reactions (reviewed by Solomon) [19]. Cbl supplementation can be beneficial in treating a range of inflammatory and viral based diseases associated with oxidative stress [10-16] and also modulates the immune response [68, 69] . Moreover, high doses of Cbl have been used to treat pernicious anaemia for decades with no apparent toxicity [67]. Cbl therapy normalizes levels of TNF-α and epidermal growth factor in Cbl deficient patients [68], by mechanism(s) which are currently unclear. Thus, there are intriguing clinical implications for our observed association between

Cbl and intracellular $O_2^{\bullet-}$ levels. Our results support the hypothesis that Cbl can act as a second line of defense when $O_2^{\bullet-}$ production overwhelms the SOD protection system. This perhaps accounts for significantly increased oxidative damage markers in patients with inherited disorders of intracellular Cbl metabolism [70].

Our data show that physiologically relevant concentrations (up to 10^{-7} M are achievable in plasma [34, 35]) of CNCbl (the common form of Cbl in vitamin supplements) effectively protect against increased intracellular levels of $O_2^{\bullet-}$, both in the cytosol and in the mitochondria, resulting in a concomitant reduction in cell death. Importantly, these effects were found to be independent of Hcy metabolism. These results combined with the in vitro kinetic data demonstrating that Cbl(II) efficiently scavenges $O_2^{\bullet-}$ [6], suggest that direct scavenging of $O_2^{\bullet-}$ by Cbl is an important mechanism by which Cbl protects against intracellular oxidative stress. Our results have important implications both in regard to the high percentage of the elderly who are B_{12}-deficient and in the treatment of chronic inflammatory diseases associated with oxidative stress. These results encourage further studies with animal models to test the efficacy of Cbl as a $O_2^{\bullet-}$ scavenger *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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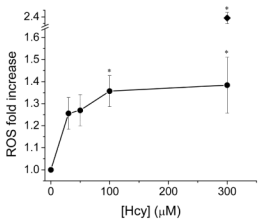

Figure 1. L-Hcy-induced ROS increase

Pre-confluent HAEC were incubated with increasing concentrations of L-Hcy in the presence of 3 μM DCFA. After 48 h ROS production was assessed fluorometrically. *$p < 0.05$ compared to control cells not exposed to L-Hcy. Positive control exposed to 200 μM H_2O_2 (◆). Data are expressed as mean ± SEM; N = 3.

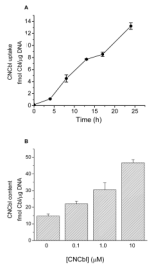

Figure 2. Effect of exogenous CNCbl on intracellular Cbl content

A. Pre-confluent HAEC were incubated with 0.2 nM ^{57}(Co)-CNCbl for up to 24 h, then harvested at different time points. The intracellular Cbl uptake was determined by counting the cell-associated radioactivity. Data represents mean ± SEM; N = 3. **B.** Pre-confluent HAEC were incubated with or without varying concentrations of CNCbl for 24 h, and intracellular Cbl content was determined by the SimulTRAC radioassay. Data represents mean ± SEM; N = 3.

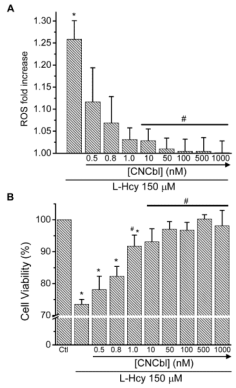

Figure 3. Cbl protection against L-Hcy-induced oxidative stress

Pre-confluent HAEC were incubated with increasing concentrations of CNCbl for 24 h prior to the addition of 150 μM L-Hcy. ROS was measured by detecting oxidation of 3 μM DCFA. **A.** ROS fold-increase after 48 h. **B.** Cell viability after 48 h as measured with MTT assay. Data are expressed as mean ± SEM; N = 7; *$p<0.05$ with respect to control cells not exposed to L-Hcy; $^{\#}p<0.05$ with respect to L-Hcy-treated cells not exposed to CNCbl

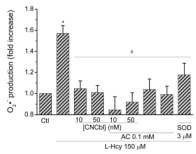

Figure 4. Cbl protection against L-Hcy induced superoxide production

Pre-confluent HAEC were incubated in the absence or in the presence of 10 or 50 nM CNCbl for 24 h. Cells were washed with PBS, prior to incubation with 150 μM L-Hcy with or without 0.1 mM apocynin or 3 μM SOD. Hydroxyethidium fluorescence was measured with a fluorescent plate reader after 24 hr. * $p < 0.05$ with respect to untreated HAEC; # $p < 0.05$ with respect to L-Hcy-treated cells. Data are expressed as mean ± SEM; N = 6 except for the SOD experiment where N = 3.

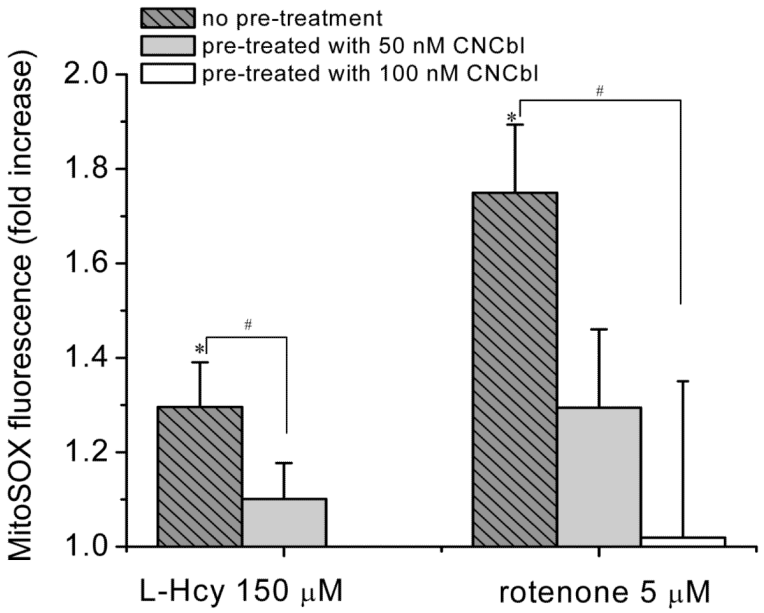

Figure 5. Subcellular localization of Hcy-induced oxidative stress

HAEC were incubated in the absence or in the presence of CNCbl (50 nM or 100 nM) for 24 h. HAEC were washed and exposed to L-Hcy (150 μM, 24 h) or rotenone (5 μM; 1 h). Mitochondrial $O_2^{\bullet -}$ was detected by quantifying mitoSOX fluorescence in a microplate reader ($\lambda_{ex/em}$ = 510/580 nm). Data are expressed as mean ± SEM; N ≥ 4; * $p < 0.05$ compared to control, $^{\#}$ $p < 0.05$.

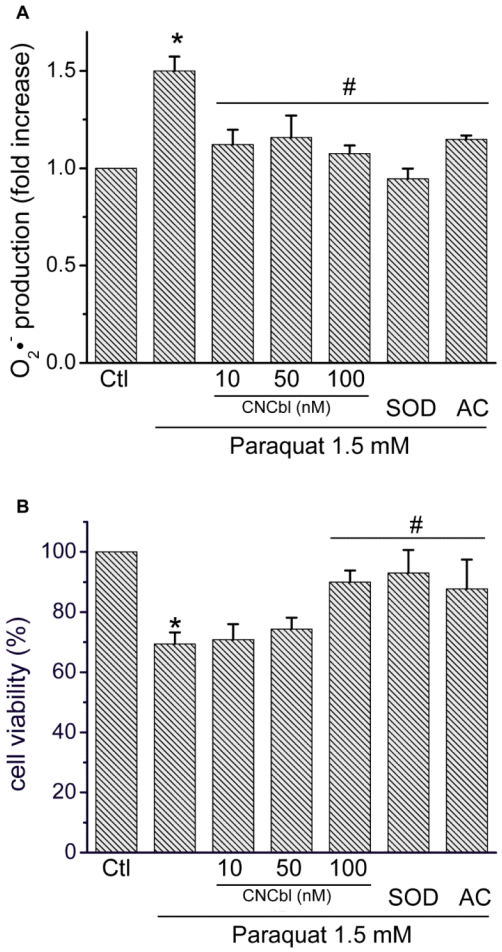

Figure 6. Effect of Cbl on paraquat-induced $O_2^{\bullet-}$ levels and cell death

HAEC were incubated in the absence or in the presence of varying concentrations of CNCbl for 24 h prior to subjection to paraquat (1.5 mM). DHE (5 μM) was added for the final 1 h of treatment. Also shown are the effects of SOD (3 μM) and apocynin (AC, 0.1 mM) on the paraquat-induced increase in $O_2^{\bullet-}$ and cell death. **A.** $O_2^{\bullet-}$ measured as hydroxyethidium fluorescence ($\lambda_{ex/em}$ = 520/605 nm) compared to untreated cells. **B.** Cell viability was assessed with the MTT assay. Data expressed as ± SEM; N = 3; * $p < 0.05$ compared to control, # $p < 0.05$ compared to paraquat alone.

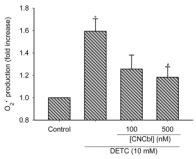

Figure 7. Cbl attenuates the DETC-induced increase in superoxide levels

Pre-confluent HAEC were incubated in the absence or in the presence of 100 or 500 nM CNCbl for 24 h. Cells were washed with PBS, prior to incubation with 10 mM DETC. After 2 h, cells were incubated with 5 μM DHE for 1 h. Hydroxyethidium fluorescence was measured with a fluorescent plate reader. * $p < 0.05$ with respect to untreated HAEC; # $p < 0.05$ with respect to DETC alone. Data are expressed as mean ± SEM; N = 5.

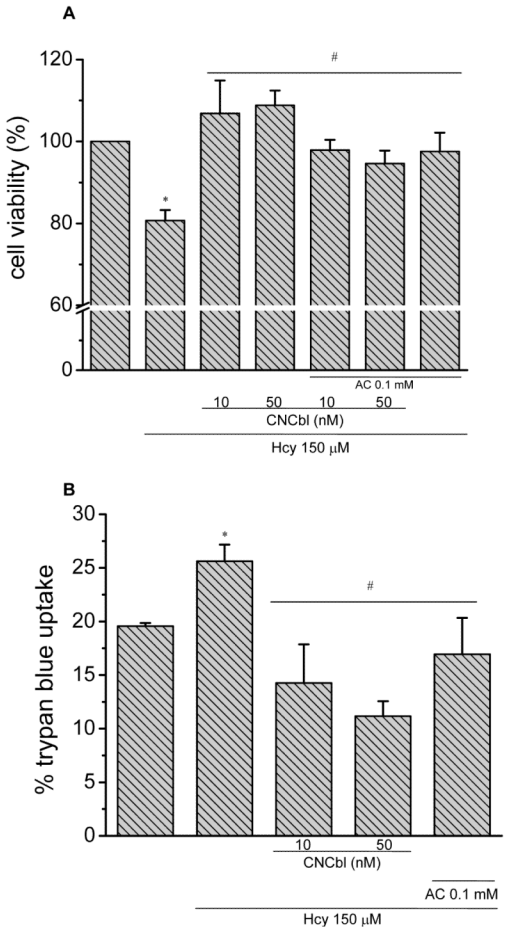

Figure 8. Cbl protection against L-Hcy-induced cell death

Pre-confluent HAEC were incubated in the presence or absence of 10 or 50 nM CNCbl for 24 h prior to adding 150 μM L-Hcy for 24 h in the presence or absence of apocynin (0.1 mM). *$p < 0.05$ with respect to control cells not exposed to L-Hcy; $^{\#}p < 0.05$ with respect to L-Hcy-treated HAEC. **A.** Cell viability by the MTT assay. Data are expressed as mean ± SEM; N = 4. **B.** Trypan blue uptake. Data are expressed as mean ± SEM; N = 3.

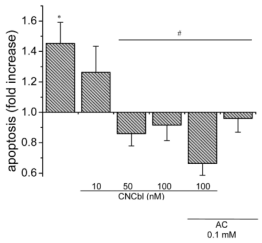

Figure 9. Cbl protection against L-Hcy-induced apoptosis

Pre-confluent HAEC were incubated in the presence of absence of varying concentrations of CNCbl for 24 h prior to exposing the cells to 150 μM L-Hcy in the presence or absence of apocynin (0.1 mM). Shown also are corresponding data for apocynin and apocynin + CNCbl. Apoptotic DNA fragmentation was measured by ELISA. Data are expressed as mean ± SEM; N = 4; *$p < 0.05$ with respect to control; $^{\#}p < 0.05$ with respect to L-Hcy-treated HAEC.