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Vitamin B_{12} and Redox Homeostasis: Cob(II)alamin Reacts with Superoxide at Rates Approaching Superoxide Dismutase (SOD)

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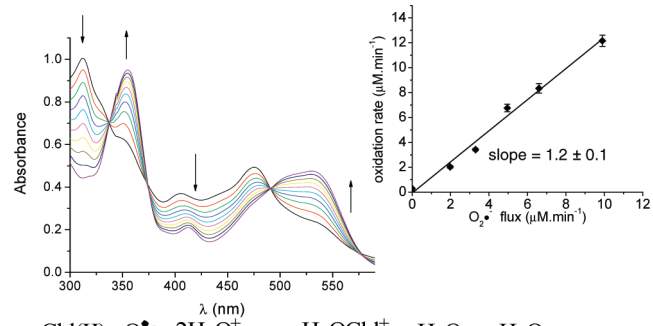
Superoxide ($O_2^{\bullet-}$), a byproduct of cellular metabolism, is produced by mitochondrial and reticular membrane electron transport systems, or enzymes such as NADPH oxidase and xanthine oxidase (XO).[1] $O_2^{\bullet-}$ is an important Reactive Oxygen Species (ROS), levels of which are augmented in acute or chronic inflammation.[2] ROS can damage lipids, nucleic acids, and proteins but also act as signaling molecules by modulating cellular responses to various stressors.[3] If $O_2^{\bullet-}$ overwhelms its primary enzymatic defenses, superoxide dismutases (SOD), elevated levels of pro-inflammatory cytokines, chemotactic factors, and adhesion molecules ensue, and nitric oxide is depleted with concomitant peroxynitrite formation.[2b] Low SOD levels are associated with oxidative stress,[2] and there is considerable interest in developing therapeutics that mimic SOD.[2b]

Cobalamins (Cbl, vitamin B_{12} derivatives) are essential cobalt-containing cofactors for two mammalian enzymes.[4a] Cbl deficiency is common in the elderly (10−40%),[5] and Cbl supplementation can be beneficial in several inflammatory diseases including sepsis, arthritis, Alzheimer's disease, multiple sclerosis, and chronic fatigue syndrome. There is evidence that Cbl protects against oxidative stress associated pathologies.[4] Cbl modulates the immune response and influences cytokine and growth factor production.[4a,6] Cbl therapy normalizes levels of TNF-α (which inhibits SOD[7]) and epidermal growth factor in Cbl deficient patients.[6] Cbl also suppresses production of inducible transcription factor NF-κB.[6] The exact mechanism(s) by which Cbl achieves these effects is unclear and difficult to ascribe solely to its known coenzyme activities.[4b]

We recently showed that Cbl protects against oxidative stress in a cellular model.[4e] Given that an important intracellular Cbl form cob(II)alamin (Cbl(II))[8] reacts rapidly with the radical nitric oxide ($^{\bullet}$NO) to form nitrosylcobalamin ($k = 7.4 \times 10^8\ M^{-1}\ s^{-1}$, $K_{eq} \approx 1 \times 10^8\ M^{-1}$, 25 °C[9]) and that $O_2^{\bullet-}$ reacts rapidly with other Co(II) macrocycles,[10] we hypothesized that $O_2^{\bullet-}$ scavenging by Cbl(II) is an important mechanism by which Cbl modulates intracellular signal transduction and protects against chronic inflammation.

We wish to report a kinetic study of the reaction between $O_2^{\bullet-}$ and Cbl(II). Upon addition of $O_2^{\bullet-}$ produced by the XO/acetaldehyde system, Cbl(II) is cleanly converted to aquacobalamin (H_2OCbl^+/HOCbl, $pK_a \approx 7.8$; λ_{max} = 353, 413, 501, 530 nm[11]), with sharp isosbestics at 337, 375, 490, and 578 nm (Figure 1). The absorbance change observed is that expected when Cbl(II) is completely oxidized to H_2OCbl^+/HOCbl (see Supporting Information (SI)). Furthermore, the initial rate of Cbl(II) oxidation equals the $O_2^{\bullet-}$ flux, as expected if Cbl(II) reacts directly with $O_2^{\bullet-}$ (Figure 1, Inset (slope ∼1); see SI for further details). $O_2^{\bullet-}$ is reduced to H_2O_2, which can also oxidize



$$Cbl(II) + O_2^{\bullet-} + 2H_3O^+ \longrightarrow H_2OCbl^+ + H_2O_2 + H_2O$$

Figure 1. Cbl(II) oxidation by superoxide. UV−vis spectra recorded every 1.00 min for the reaction between Cbl(II) (33 μM) and $O_2^{\bullet-}$ ($O_2^{\bullet-}$ flux = 2.7 μM/min) in phosphate buffer, 25.0 °C. Inset: Initial rate of Cbl(II) oxidation versus $O_2^{\bullet-}$ flux (1000 U/mL catalase, phosphate buffer, 25.0 °C).

Cbl(II) to H_2OCbl^+/HOCbl.[12] Indeed, adding 0.5 mol equiv of H_2O_2 to a solution of Cbl(II) results in formation of H_2OCbl^+/HOCbl (Figure S1). Catalase (1000 U/mL) rapidly disproportionates H_2O_2 to O_2 and H_2O, and the rate of Cbl(II) oxidation is decreased by the addition of catalase as expected (Figure S2). The addition of 2000 U/mL catalase did not cause any further decrease in the oxidation rate. All kinetic experiments were therefore carried out in the presence of 1000 U/mL catalase. H_2O_2 also reacts with H_2OCbl^+/HOCbl, albeit at a much slower rate,[12] and the addition of up to 5 mol equiv of H_2O_2 to H_2OCbl^+/HOCbl does not induce any change in the UV−vis spectrum in the experimental time frame ($\Delta Abs_{351\ nm} < 0.001$); hence this reaction is unimportant.

The second-order rate constant for oxidation of Cbl(II) to H_2OCbl^+/HOCbl by $O_2^{\bullet-}$ was determined by measuring the reaction rate in the presence of varying concentrations of a competitor, Cu,Zn-SOD. (A Mn^{III} SOD mimetic and ferricytochrome c were also tested as possible competitors but found to be unsuitable (see SI)). The initial rate of oxidation of Cbl(II) by $O_2^{\bullet-}$ was inhibited by Cu,Zn-SOD in a concentration-dependent fashion (Figure 2A). The rate constant for the reaction between Cbl(II) and $O_2^{\bullet-}$ was calculated using eq 1 (see SI for derivation), assuming a steady state concentration of $O_2^{\bullet-}$:[13]

$$\frac{V_0}{V_{SOD}} - 1 = \frac{k_{SOD}}{k_{Cbl}[Cbl]}[SOD] \qquad (1)$$

V_0 and V_{SOD} are the observed rates of Cbl(II) oxidation in the absence and presence of SOD, respectively, k_{SOD} is the rate constant for enzymatic dismutation of $O_2^{\bullet-}$ to $H_2O_2 + H_2O$ for Cu,Zn-SOD ($2 \times 10^9\ M^{-1}s^{-1}$),[14] and k_{Cbl} is the rate constant for oxidation of Cbl(II) by $O_2^{\bullet-}$. This indirect competition kinetic method is widely used and well validated.[13] The scatter of the rate (ΔAbs/min) data was similar for all experiments (∼0.01 min^{-1}). However, from Figure 2B it is clear

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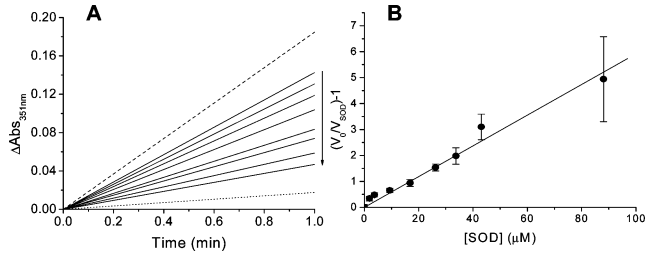

Figure 2. Competition kinetics with Cu,Zn-SOD. (A) Plot of change of absorbance at 351 nm (dashed line) versus time for the oxidation of Cbl(II) (50 μM) by $O_2^{\bullet-}$ ($O_2^{\bullet-}$ flux = 10 μM/min; 1000 U/ml catalase, 1 mM EDTA, 50 mM NaCl, 10 mM phosphate buffer, pH 7.4, 25.0 °C). The experiment was repeated with increasing SOD (2.0−88 μM, solid lines; arrow indicates increasing SOD concentration). The rate of Cbl(II) autoxidation is also shown (dotted line). (B) Plot of $V_0/V_{SOD} - 1$ vs [SOD]. The best fit to eq 1 gave $k_{Cbl} = (6.8 \pm 0.8) \times 10^8$ M^{-1} s^{-1}.

that increased scatter in the data occurs at high SOD concentrations as expected, given that the plots use the ratio V_0/V_{SOD}, which has a larger relative error at smaller V_{SOD} values (i.e., high [SOD]). The best fit of the data at 351 nm gave $k_{Cbl} = (6.8 \pm 0.8) \times 10^8$ M^{-1} s^{-1} (Figure 2B). Similar experiments at 474 nm gave $k_{Cbl} = (8 \pm 1) \times 10^8$ M^{-1} s^{-1} (Figure S4). The smaller ΔAbs at 474 nm results in a less reliable value of k_{Cbl}.

Importantly, since XO requires oxygen for its activity, the experiments were conducted in an aerobic buffer. However, Cbl(II) undergoes slow disproportionation to cob(I)alamin (Cbl(I)) and Cbl(III) (= H_2OCbl^+/HOCbl) in air, followed by rapid oxidation of Cbl(I) to Cbl(III).[15] The rate of Cbl(II) autoxidation in the absence of XO was therefore determined and subtracted from the V_0 and V_{SOD} data (dotted line in Figure 2A). The reaction between H_2OCbl^+/HOCbl and $O_2^{\bullet-}$ to form superoxocobalamin (this species has been characterized at low temperature) is unimportant on the time scale of our experiments.[16]

There is considerable interest in compounds that efficiently scavenge $O_2^{\bullet-}$, given their effectiveness in ameliorating conditions associated with oxidative stress.[2b] To our knowledge, only one known SOD mimetic, M40401,[2b] reacts faster with $O_2^{\bullet-}$ than Cbl(II) (Table S1). Cbl has an advantage of being nontoxic even at high doses.[17a] In our in vitro studies, Cbl(II) is a stoichiometric scavenger of $O_2^{\bullet-}$. However, cells also have the ability to re-reduce cob(III)alamins (including H_2OCbl^+/HOCbl) to Cbl(II),[17b] making the reaction catalytic.

It was of interest to assess the ability of the common vitamin B_{12} form in vitamin pills, cyanocobalamin (CNCbl), to prevent elevated intracellular $O_2^{\bullet-}$ levels in a cell model. We therefore tested the effect of pretreating human aortic endothelial cells (HAEC) with CNCbl prior to exposure to paraquat, a well established $O_2^{\bullet-}$ source.[18] A 2-fold increase in $O_2^{\bullet-}$ levels was observed upon exposing HAEC to paraquat (1.5 mM), measured by hydroxylethidium fluorescence (Figure 3). Preincubating the cells in a medium containing CNCbl (100 nM, 24 h) and subsequently washing the cells to remove extracellular Cbl prior to paraquat exposure resulted in intracellular $O_2^{\bullet-}$ levels returning to normal. The same effect was observed with SOD itself (Figure 3). Further details are given in the SI.

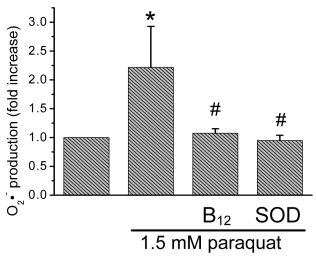

Figure 3. CNCbl inhibits paraquat-dependent $O_2^{\bullet-}$ levels in HAEC. Preconfluent HAEC were incubated in the absence or presence of CNCbl (100 nM) for 24 h. The cells were washed with PBS and fresh medium in the absence or presence of 1.5 mM paraquat with or without SOD (3 μM) added. Data expressed as mean ± SEM; $N = 3$, $^*p < 0.05$ compared to control, $^{\#}p < 0.05$ compared to paraquat alone.

To summarize, we have shown that cob(II)alamin, an important intracellular Cbl form, reacts rapidly with $O_2^{\bullet-}$ at rates approaching those of SOD (7×10^8 versus 2×10^9 M^{-1} s^{-1}, respectively). This suggests that direct scavenging of $O_2^{\bullet-}$ is an important molecular mechanism by which Cbl modulates intracellular signal transduction and protects against chronic inflammation. Cbl likely acts as a second line of defense when $O_2^{\bullet-}$ levels overwhelm the SOD protection system, perhaps accounting for the significantly increased oxidative damage markers in patients with inherited disorders of intracellular Cbl metabolism.[19] Given that B_{12} is nontoxic even at high doses and that a significant proportion of the elderly are B_{12} deficient, our results provide a compelling argument for clinical trials studying the pharmacological effects of B_{12} in the treatment and prevention of diseases associated with chronic inflammation and aging.

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Supporting Information Available: Experimental section; derivation of eq 1; Figures S1−S5, Table S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) McCord, J. M.; Omar, B. A. *Toxicol. Ind. Health* **1993**, *9* (1−2), 23–37.
(2) (a) McCord, J. M.; Edeas, M. A. *Biomed. Pharmacother.* **2005**, *59* (4), 139–42. (b) Muscoli, C.; Cuzzocrea, S.; Riley, D. P.; Zweier, J. L.; Thiemermann, C.; Wang, Z. Q.; Salvemini, D. *Br. J. Pharmacol.* **2003**, *140* (3), 445–60.
(3) Droge, W. *Physiol. Rev.* **2002**, *82* (1), 47–95.
(4) (a) Solomon, L. R. *Blood Rev* **2007**, *21* (3), 113–30. (b) Miller, J. W. *Nutr. Rev.* **2002**, *60* (5 Pt 1), 142–4. (c) Greenberg, S. S.; Xie, J.; Zatarain, J. M.; Kapusta, D. R.; Miller, M. J. *J. Pharmacol. Exp. Ther.* **1995**, *273* (1), 257–65. (d) Wheatley, C. *Med. Hypotheses* **2006**, *67* (1), 124–42. (e) Birch, C. S.; Brasch, N. E.; McCaddon, A.; Williams, J. H. *Free Radical Biol. Med.* **2009**, *47* (2), 184–188.
(5) Wolters, M.; Strohle, A.; Hahn, A. *Prev. Med.* **2004**, *39* (6), 1256–66.
(6) Scalabrino, G.; Veber, D.; Mutti, E. *Brain Res. Rev.* **2008**, *59* (1), 42–54.
(7) Afonso, V.; Santos, G.; Collin, P.; Khatib, A. M.; Mitrovic, D. R.; Lomri, N.; Leitman, D. C.; Lomri, A. *Free Radical Biol. Med.* **2006**, *41* (5), 709–21.
(8) Padovani, D.; Banerjee, R. *Biochemistry* **2006**, *45* (30), 9300–6.
(9) (a) Wolak, M.; Zahl, A.; Schneppensieper, T.; Stochel, G.; van Eldik, R. *J. Am. Chem. Soc.* **2001**, *123* (40), 9780–91. (b) Zheng, D.; Birke, R. L. *J. Am. Chem. Soc.* **2001**, *123* (19), 4637–8.
(10) Simic, M. G.; Hoffman, M. Z. *J. Am. Chem. Soc.* **1977**, *99* (7), 2370–1.
(11) (a) Xia, L.; Cregan, A. G.; Berben, L. A.; Brasch, N. E. *Inorg. Chem.* **2004**, *43*, 6848–57. (b) Schneider, Z.; Stroinski, A. *Comprehensive B_{12}*; Walter de Gruyter: Berlin, New York, 1987.
(12) Nazhat, N. B.; Golding, B. T.; Johnson, G. R.; Jones, P. *J. Inorg. Biochem.* **1989**, *36* (2), 75–81.
(13) Spasojevic, I.; Batinic-Haberle, I.; Stevens, R. D.; Hambright, P.; Thorpe, A. N.; Grodkowski, J.; Neta, P.; Fridovich, I. *Inorg. Chem.* **2001**, *40* (4), 726–39.
(14) Fridovich, I. *J. Biol. Chem.* **1989**, *264* (14), 7761–4.
(15) (a) Yamada, R.; Shimizu, S.; Fukui, S. *Biochemistry* **1968**, *7* (5), 1713–9. (b) Abel, E. W.; Pratt, J. M.; Whelan, R.; Wilkinson, P. J. *S. Afr. J. Chem.* **1977**, *30*, 1–12.
(16) (a) Hohenester, E.; Kratky, C.; Kräutler, B. *J. Am. Chem. Soc.* **1991**, *113*, 4523–30. (b) Prigoda, S. V.; Afanas'ev, I. B. *Koordinatsionnaya Khimiya* **1978**, *4* (9), 1386–90.
(17) (a) Mangiarotti, G.; Canavese, C.; Salomone, M.; Thea, A.; Pacitti, A.; Gaido, M.; Calitri, V.; Pelizza, D.; Canavero, W.; Vercellone, A. *Int. J. Artif. Organs* **1986**, *9* (6), 417–20. (b) Kim, J.; Gherasim, C.; Banerjee, R. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (38), 14551–4.
(18) Day, B. J.; Patel, M.; Calavetta, L.; Chang, L. Y.; Stamler, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96* (22), 12760–5.
(19) McGuire, P. J.; Parikh, A.; Diaz, G. A. *Mol. Genet. Metab.* **2009**, *98*, 173–80.

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