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The Disparate Roles of Cobalt in Erythropoiesis, and Doping Relevance

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ABSTRACT

Some athletes seek to increase their red blood cells, because the aerobic capacity correlates with the mass of hemoglobin. Cobalt as part of dietary supplements is proclaimed to increase erythropoiesis and physical performance. However, the knowledge of the disparate mechanisms of cobalt´s actions in erythropoiesis is generally insufficient. First, there are cobalt-containing corrinoids, termed cobalamin or vitamin B_{12}. These are naturally present only in animal-derived foods. The active forms in the human body are methylcobalamin and deoxyadenosylcobalamin, which are essential cofactors for the methionine synthase and the L-methylmalonyl-coenzyme A mutase. Cobalamin deficiency can result in anemia. However, supplemental cobalamin does not benefit performance unless a nutritional deficit is present. Second, inorganic cobalt ions (Co^{2+}) stimulate erythropoiesis, even in non-anemic subjects. Co^{2+} stabilizes the hypoxia-inducible transcription factors (HIFs) which increase the expression of the erythropoietin gene (EPO). Cobaltous salts are orally active, easy to obtain and inexpensive. Typical side effects associated with chronic Co^{2+} exposure include nausea, vomiting, heart failure, hypothyroidism and goiter. The potential misuse of inorganic cobalt deserves attention in anti-doping efforts.

INTRODUCTION

The aerobic capacity of athletes correlates with the total mass of hemoglobin (Hb). Therefore, some athletes seek to increase their red blood cells (RBCs) by pharmacological means. Recombinant human erythropoietin (rHuEpo) and analogues are available for this intention. Yet their use is forbidden and the drugs can be detected by chemical tests [30]. In addition, the recombinant products are expensive. Alternatively, there are low-cost non-peptidic compounds capable of stimulating erythropoiesis. Of particular concernment are substances that induce Epo gene (EPO) expression, such as cobalt (II) ions (Co^{2+}). Indeed, numerous over-counter cobalt-containing products are available in the USA [18]. The suppliers advertize cobalt as part of dietary supplements for sportspeople to increase their

performance (proposed not only for endurance sports but also for athletic sports). An Internet search (*Google*; assessed 10/23/2012) for "doping, cobalt, sport" yielded >100,000 hits, including reports of the effects of cobalt on erythropoiesis. However, like in the early days of hematological research [13] the understanding of the mechanisms of the action of cobalt in erythropoiesis is generally poor, as the role of cobalt in cobalamin (vitamin B_{12}, contains cobalt-corrin complexes) is not separated from the function of inorganic cobalt ions (Co^{2+}) [29, 64]. Cobalamin plays a vital role in DNA synthesis and cell proliferation. In contrast, Co^{2+} stabilizes the hypoxia-inducible transcription factors (HIFs) that increase *EPO* expression. The present essay reviews these two disparate roles of cobalt in erythropoiesis.

BASICS OF ERYTHROPOIESIS

RBCs circulate for 100–120 days in the human organism. Over time, the RBCs undergo membrane alterations and are engulfed by macrophages. About 1% of the 25 × 10^{12} erythrocytes of an adult are removed every day; these are replaced by reticulocytes derived from erythrocytic progenitors in the red bone marrow. Hence, RBC numbers and blood Hb concentration are constant in health.

The most primitive erythrocytic progenitors are the burst-forming units-erythroid (BFU-Es). Human BFU-Es give rise to bursts of several hundreds of erythroblasts after two to three weeks in semisolid tissue culture (see [42]). The more differentiated colony-forming units-erythroid (CFU-Es) require one week to form colonies of up to 64 erythroblasts. Thus, BFU-Es, CFU-Es and their progeny have several rounds of DNA synthesis and cell division. Cobalamin is strictly needed in these processes.

Further, the CFU-Es and their progeny, to the basophilic erythroblasts, depend on the glycoprotein hormone erythropoietin (Epo; see [42]). Epo prevents the cells from apoptotic die-off. Under normal conditions, the concentration of circulating Epo is only ~10^{-11} mol/L (6–32 International Units [IU] per L blood plasma). This value can increase by to up to three orders of magnitude under hypoxic conditions (see [28]). Even in healthy, non-anemic persons, an increase in Epo concentration will lead to a stimulation of erythropoiesis.

FUNCTION OF COBALAMIN

Chemistry

Cobalamin, or vitamin B_{12}, is the largest (1.4 kDa) and most complex ($C_{63}H_{88}CoN_{14}O_{14}P$) out of all vitamins [5]. Cobalamin comprises a class of chemically related water-soluble compounds that possess a corrin (tetrapyrrole) ring with a central cobalt atom, positioned by four coordinated bonds of nitrogen from the pyrroles. The four pyrroles are also connected to each other. The fifth ligand of the cobalt atom is a nitrogen from 5, 6-dimethylbenzimidazolyl ribonucleotide. The sixth coordination site is variable, yielding either one of the precursor molecules cyano-(CN)-cobalamin or hydroxyl-(OH)-cobalamin, or one of the active coenzymes methyl-(CH_3)-cobalamin or 5-deoxyadenosylcobalamin.

Metal ions play a variety of roles in natural proteins, including nucleophilic catalysis, electron transfer, and the stabilization of protein structure. Here, the functions of cobalt have rarely been studied. Although far less common than other metalloproteins (*e.g.* those of zinc and iron), cobaltoproteins are known aside from cobalamin. These proteins include methionine aminopeptidase 2, an enzyme that does not use the corrin ring, but binds cobalt directly [33]. Methionine aminopeptidase is a ubiquitous enzyme that cleaves the N-terminal methionine from many newly translated polypeptide chains.

Nutritional uptake

Cobalamin can only be synthesized by bacteria or archaea [21]. Since these microorganisms grow in faunal intestinum, cobalamin is found in fish and shellfish, meat (especially liver), eggs, milk, and milk products [46]. The daily nutritional requirement of an adult amounts to 2–3 µg cobalamin. While lacto-ovo vegetarians may get cobalamin through dairy products, vegans lack cobalamin unless they consume vitamin B_{12} containing dietary supplements. Of note, the cobalamin-producing bacteria that are present in the lower parts of the human gut do not provide enough of the vitamin.

Degradation of ingested cobalamin is prevented by haptocorrin (HC), a glycoprotein secreted by the salivary glands. Cobalamin in

dietary protein is released by gastric acid and pepsin for HC binding. In the less acidic environment of the duodenum cobalamin separates from HC and combines with intrinsic factor (IF), a 50 kDa glycoprotein of gastric origin. The cobalamin-IF complex is resistant to enzymes en route to the terminal ileum. There, the cobalamin-IF complex binds to cubilin, a specific 460 kDa luminal membrane receptor. On endocytotic uptake the cobalamin is released from the IF and combines with transcobalamin II (TC II), a 43 kDa secretory protein [53]. The cobalamin-TC II complex (holotranscobalamin) enters the blood for transfer to the other cells of the organism. The holotranscobalamin is taken up by cells via the TC II receptor, a 62 kDa protein that is expressed as a functional dimer in cell membranes [53]. On endocytotic uptake the holotranscobalamin is degraded in a lysosome and the free cobalamin activated to methylcobalamin in the cytosol or to deoxyadenosylcobalamin in a mitochondrium.

Metabolic functions

There are two enzymatic reactions in mammalian cells that require either one of the two activated cobalamin forms as cofactor. (i) Methylcobalamin acts as a methyl group donor for the conversion of homocysteine to methionine, which is pivotal for normal synthesis of purines and pyrimidines, and therefore of DNA. The methyl groups of methylcobalamin are provided by methyltetrahydrofolate. This folate-cobalamin interaction accounts for the requirement of both vitamins in normal erythropoiesis [34]. (ii) Deoxyadenosylcobalamin is a cofactor for the mitochondrial mutase that catalyzes the isomerization of L-methylmalonyl coenzyme A (CoA) to succinyl CoA, an important reaction in the catabolism of some branched-chain amino acids and odd-chain fatty acids. Succinyl CoA is also required for porphyrin and, thus, Hb synthesis, which constitutes another link with erythropoiesis.

Cobalamin deficiency

Cobalamin deficiency is prevalent worldwide [21]. It affects at least 15% of the elderly population, with prevalence rates depending on the decision limits used [23]. Decreased secretion of IF and hydrochloric acid due to gastric atrophy is a common cause of cobalamin deficiency. Intestinal diseases can interfere with absorption, including pancreatic disorders, intestinal parasites, sprue, and immune processes. Most cases take years to produce obvious deficiency signs [12], because the hepatic cobalamin stores (2–3 mg) normally suffice to supply daily needs for 2 to 3 years.

The common view is that cobalamin deficiency is more likely to occur when IF-driven absorption fails (pernicious anemia) than when diet is poor (vegetable diet). However, attention has been restricted to elderly people, while there is a lack of information on vitamin B_{12} deficiency in young populations [24]. One study has shown that 40% of vegetarians presented with lower blood Hb concentration, hematocrit, mean corpuscular Hb mass and serum cobalamin levels than the control subjects [48]. Apart from vegans and strict vegetarians [25], adolescent athletes on energy-restrictive diets can be at risk for cobalamin depletion [38]. The problem has arisen that many young female athletes are concerned about nutrition yet choose diets that reflect inadequate knowledge or poor judgement [38].

Manifest cobalamin deficiency is characterized by megaloblastic anemia and weakness. Erythropoiesis under these deficiency conditions is termed ineffective because the erythrocytic progenitors are present in the hematopoietic tissue, but cannot mature to the late stages of differentiation [4, 34]. Of note, cobalamin deficiency causes primarily a neurological disease with demyelination of corticospinal tracts and posterior columns of the spinal cord. This can produce a wide range of neurological signs and symptoms, including paresthesias of the hands and feet, decreased deep tendon reflexes, and in the later stages, loss of cognitive functions [36]. Given its high prevalence, cobalamin deficiency should be included in the differential diagnosis of patients with progressive neuropsychiatric findings and/or hematologic derangements as rapid diagnosis and supplementation may prevent permanent complications [41].

Cobalamin therapy

Cobalamin is available for oral administration or injection [8]. The cyanocobalamin form is typically used for food additives and pills because it is easy to crystallize and is not sensitive to air-oxidation.

Cyanocobalamin is converted to its active forms, methylcobalamin and adenosylcobalamin, in the liver. Doses in excess of 100 µg are cleared rapidly from blood plasma and excreted with the urine. The sublingual route of intake has no proven advantages, even though lozenges and pills are designed for this effect [55]. For treatment of a true deficiency state cobalamin should be administered by intramuscular or subcutaneous injection. Apart from cyanocobalamin, hydroxocobalamin and methylcobalamin are other formulations encountered in pharmacy.

Cobalamin has been used in the therapy of a number of conditions, including trigeminal neuralgia and other neuropathies, various psychiatric disorders, poor growth, and as a "tonic" for persons suffering from easy fatigue. It has been argued that the current recommended daily intake (2-3 µg) may underestimate cobalamin requirements in normal subjects [59]. It has been further proposed that high-dose cobalamin therapy may have salutary pharmacological effects on the nervous system [59]. Evidence supports a role for cobalamin (and folic acid) supplements in lowering homocysteine levels, but results from several large prospective studies have not shown that these supplements decrease the risk of cardiovascular disease [3]. *E.g.*, in the Women's Antioxidant and Folic Acid Cardiovascular Study (WAFACS) trial, a combination pill of 1 mg cobalamin, 2.5 mg folic acid and 50 mg vitamin B_6 had no effects on a combined outcome of total major cardiovascular events in a high-risk population of women over 7.3 years of follow-up [1]. In summary, the administration of cobalamin without documented cobalamin deficiency is of dubious value.

Cobalamin and physical performance

Cobalamin is frequently promoted as an energy and performance enhancer. This reputation is likely based on the fact that correcting the megaloblastic anemia caused by cobalamin deficiency alleviates the associated fatigue and weakness. However, cobalamin supplementation has no proven beneficial effect on performance in the absence of a nutritional deficit [39]. When adolescent boys (age 12–17 years) received daily doses of 50 µg cyanocobalamin no improvement in the half-mile run or Harvard step test scores were detected after 7 weeks as compared with a non-supplemented control group [43]. Similarly, parenteral cobalamin administered to non-anemic men failed to elicit improvements in muscle strength and endurance [62]. Thus, supplemental cobalamin does not benefit performance unless a nutritional deficit is present. On the other side, cobalamin has very low toxicity and even taking it in extensive doses appears not to be harmful to healthy individuals.

EFFECTS OF Co^{2+} ON EPO PRODUCTION

Molecular biology of Epo synthesis

The human *EPO* gene (2.9 kb; 7q11-q22) contains 5 exons and 4 introns. The *EPO* enhancer possesses hypoxia-response elements (HREs) that are binding sites for hypoxia-inducible transcription factors (HIF-1 and HIF-2). The HIFs are heterodimeric proteins composed of α- and ß-subunits. The C-termini of the HIF-α subunits comprise domains in which proline residues are hydroxylated in the presence of O_2. This reaction requires HIF-α-specific prolyl hydroxylases (PHDs). Prolyl hydroxylated HIF-α undergoes immediate proteasomal degradation, as it is bound by the *von Hippel-Lindau tumor suppressor protein* (pVHL) in complex with an ubiquitin-protein E3-ligase. HIF-2 is the main transcription factor inducing EPO. Under hypoxic conditions, the HIF-2α subunits dimerize with HIF-1ß in the nucleus and the resulting complex activates *EPO* expression.

The PHDs are Fe^{2+} containing enzymes requiring α-ketoglutarate as cofactor. Competitors of α-ketoglutarate (clinical term: "HIF stabilizers") prevent HIF-α degradation and stimulate Epo production (see [11]). A Phase I clinical trial has shown that single injections of the α-ketoglutarate competitor FG-2216 (FibroGen Inc., San Francisco, CA, USA) produces a 13-fold increase in the plasma Epo concentration in healthy volunteers [6].

Effects of inorganic Co^{2+} on *EPO*

It was earlier believed that cobalt ions would stimulate erythropoiesis by causing tissue hypoxia [61]. However, this concept was disproven with the discovery of the HIF-α prolyl hydroxylases functioning as cellular O_2 sensors. Initially it was thought that Co^{2+} could replace the Fe^{2+} in the HIF-α prolyl hydroxylases (see [22]).

Subsequent studies indicated that Co^{2+} may bind to HIF-1α and -2α thereby preventing the interaction with pVHL [32, 68, 69]. In fact, Co^{2+} can interact with various proteins and alter their function [63]. In addition, Co^{2+} lowers the availability of ascorbate, the latter being necessary to reduce Fe^{3+} to Fe^{2+} within the catalytic site of the HIF-α hydroxylases. Ascorbate *per se* failed to stimulate Epo production in pre-clinical studies [31].

The potential of inorganic cobalt to stimulate Epo production was discovered in the late 1950s [20]. *Per definition*, one International Epo Unit (IU) elicits the same erythropoiesis-stimulating activity as 5 µmol Co^{2+} [27]. The seminal studies of the effects of inorganic cobalt administrations in healthy men revealed that the daily intake of 150 mg cobaltous chloride produced an increase in RBC numbers by about 1 Mio. cells per µL blood within 7 to 22 days. The high RBC counts returned to normal within 9 to 15 days after cessation of cobalt administration [15]. It is assumed that the erythrocytosis of miners working at altitude is partially caused by inorganic cobalt inhalation [26].

Therapeutic use of Co^{2+} and tolerability

According to reports from the 1950s to 1980s inorganic cobalt salt is an effective therapeutic for anemic patients [9, 14, 17, 19, 35, 49, 52, 65, 67]. For anemia treatment Co^{2+} salt (mostly cobalt chloride) was administered as tablets and at daily doses of 10-300 mg. Diseases treated included chronic renal failure [9, 14, 17, 19, 52, 65], rheumatoid arthritis [65], chronic suppurative infection [49] and sickle-cell disease [67]. In clinical practice cobalt salt is no longer used as an anti-anemia treatment due to its potential unwanted effects [18, 58, 61, 64].

Inorganic cobalt is a dietary mineral required in low amounts in health (recommendation ~0.1 µg daily). It can be absorbed following ingestion, inhalation and dermal exposure [10]. The normal body burden of inorganic cobalt is about 1.1 mg, ~43% of this being in muscle, ~14% in bone and the remainder in other soft tissues [61]. Inorganic cobalt is only moderately toxic on single exposure [10]. However, gastrointestinal sensations are likely after substantial ingestions (>100 mg) [10]. Mucklow *et al.* [44] reported on a 6 year-old boy who developed abdominal pain and vomited after taking a drink containing 2.5 g cobalt chloride. The cobalt concentration in his blood plasma was 7.23 µmol/L (normal value <0.02 µmol/L) seven hours post ingestion and 0.09 µmol/L one month later [44].

Typical side effects associated with chronic cobalt exposure include nausea, vomiting, neuropathies, thyroid dysfunction, and heart failure. One of 12 renal failure patients on hemodialysis treated with cobalt chloride (25-50 mg daily) had to discontinue therapy after ten days due to nausea and constipation. Symptoms resolved on withdrawal of cobalt supplements [17]. A 35 year-old woman with chronic nephritis and anemia developed severe neurological symptoms (vestibular damage and peripheral neuritis) in addition to nausea, vomiting and weight loss on treatment with cobalt chloride (100 mg daily) [51]. Co^{2+} was further shown to inhibit thyroidal iodide uptake [47], and chronic cobalt chloride ingestion can cause hypothyroidism and goiter [35].

Curtis *et al.* [14] described a hemodialysis patient who died three months after treatment with cobalt chloride (50 mg daily). At *post mortem* the myocardial cobalt concentration was 1.65 µg/g wet tissue, some 25–80 times greater than the concentration in control samples. In another report [40] a 17 year-old woman on maintenance hemodialysis died from rapidly progressive dilated cardiomyopathy after nine months cobalt chloride therapy (50 mg daily). At necropsy her myocardial cobalt concentration was ~45 times greater than normal [40]. Thus, cobalt-induced cardiotoxicity is a critical issue. Note that cobalt-beer cardiomyopathy is a special syndrome, differing from alcoholic cardiomyopathy by its rather abrupt onset of left ventricular failure, cardiogenic shock and acidosis [2]. Cobalt salts were earlier added to beer to act as a foam stabilizer. The cardiotoxic effect of cobalt is incompletely understood because the amount ingested in the beer (up to 10 mg/day) is far less than the amount earlier used in the treatment of anemias.

In experimental animals the administration of cobalt salt promoted the development of carcinomas [16]. Cobalt was also shown to induce DNA damage [7]. Finley *et al.* [18] have recently determined a chronic oral reference dose (RfD) for

inorganic cobalt, employing the standard US EPA risk assessment methodology. This approach has yielded a chronic oral RfD of 0.03 mg cobalt per kg body weight and day, a value considered to be protective of non-cancer health effects in the general population for a lifetime of daily exposure to cobalt [18].

Potential misuse of Co^{2+} in sports

Soluble cobalt supplements with recommended daily doses up to 1 mg cobalt per day are increasingly being marketed to consumers interested in healthy living practices [18]. Hence, suspicion has been raised that Co^{2+} salts could be misused in sports as an alternative to rHuEpo doping [29, 37]. Indeed, athletes may take cobalt salt tablets to enhance the production of their own, endogenous, Epo. Cobalt salt is readily available, inexpensive and very potent.

Apart from *EPO*, cobalt may activate several other HIF-dependent genes [45]. These include genes encoding proteins involved in iron metabolism (transferrin and transferrin-receptor), angiogenesis (VEGF [vascular endothelial growth factor] and VEGF-receptor), vascular tone (iNOS [inducible NO-synthase]), transmembrane glucose transport (GLUT-1 and GLUT-3), and glycolytic enzymes [66]. Thus, Co^{2+} intake may improve physical performance by mechanisms other than stimulation of erythropoiesis. For example, a recent rat study has shown that hypoxia preconditioning by cobalt chloride supplementation increases mitochondrial biogenesis, glucose uptake and metabolism by aerobic respiration in skeletal muscle, which leads to increased physical performance [50]. Studies in hypoxia exposed rats have shown that the preconditioning with cobalt (12.5 mg/kg body weight) has protective effects against high altitude pulmonary edema (HAPE) [56]. In contrast, cobalt administration failed to increase capillarization in skeletal muscle of experimental animals [60].

First attempts have been made to develop methods for detection of cobalt doping in athletes. One group measured the intra-erythrocytic cobalt content, which reflects irreversible uptake of free Co^{2+} [57]. Others used a cobalt-specific biokinetic model to estimate whole blood and urine cobalt levels resulting from oral exposure or ingestion of cobalt in amounts exceeding typical dietary intake rates [64].

CONCLUSION

The interest in cobalt among sportspeople stems from hearings about its effects on RBC production. Cobalt is recommended as part of dietary supplements to increase well-being and performance. However, one has to differentiate between the role of cobalt as part of cobalamin (vitamin B_{12}) and the potential of cobalt ions (Co^{2+}) to stabilize HIF-α thereby inducing HIF-dependent *EPO* expression.

Animal foods are the only natural source of cobalamin in human diet. The recommended daily intake for a healthy adult is 2-3 µg cobalamin, corresponding to about 0.01 µg cobalt. There are two enzymatic reactions that require activated cobalamin forms, methionine synthase and methylmalonyl-CoA-mutase. The prevalence of cobalamin deficiency is relatively high, with incidence increasing with age [21]. Cobalamin supplementation has no proven beneficial effect on performance in the absence of a nutritional deficit.

Inorganic cobalt is a dietary mineral required in low amounts (~0.1 µg daily) in health. Doses of – at least – 10 to 25 mg cobalt chloride are required to stimulate Epo production. In earlier days, cobalt salt tablets were administered to anemic patients, but this therapy is now considered obsolete [14]. The toxicity of ingested cobalt containing food resides primarily with ionized cobalt [58]. On regular intake of high cobalt salt doses (>25 mg/day) there is clear danger of organ injury. The HIFs can stimulate the expression of >300 genes apart from *EPO* [45]. A major concern is the tumor growth-promoting potential, because the HIFs affect several genes encoding proteins that are involved in tumor growth, such as *VEGF* [54]. On the other hand, it is likely that some of the HIF-activated genes encode proteins which may increase physical performance (e.g. glycolytic enzymes, glucose transporters, angiogenic peptides) independent from the stimulation of erythropoiesis. Detailed information on the health hazards of cobalt salt is demandable to discourage athletes from potentially deleterious doping practices.

CONFLICT OF INTEREST

The author has no conflict of interest.

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