Eritropoetin između terapije i dopinga – dve strane medalje

Erythropoietin between therapy and doping – two sides of the same coin

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Sažetak

Eritropoetin je hormon koji se kod odraslih stvara pretežno u bubrezima, a u manjoj meri i u jetri, kao odgovor na hipoksiju. Primarno stimuliše eritropoezu u koštanoj srži i deluje antiapoptozno, antiinflamatorno i citoprotektivno. Stvara se lokalno i deluje i u mnogim drugim, nehematopoetskim tkivima, ispoljavajući u njima i svoj proangiogeni potencijal. Eritropoetin se vezuje za citokinski receptor koji eksprimiraju ciljne ćelije, prekursori eritrocita, ali i neuroni, ćelije glije, endotelne ćelije, kardiomiociti, miociti i dr. Pronalaskom sintetskih formi eritropoetina, krajem osamdesetih godina prošlog veka, značajno je unapređeno lečenje bolesnika sa anemijom usled hroničnih bolesti, najčešće renalne insuficijencije. Anemija u hroničnoj bubrežnoj insuficijenciji je multifaktorska, ali u najvećoj meri posledica manjka eritropoetina. Do sada su razvijene tri generacije stimulatora eritropoeze, koji se razlikuju u ugljenohidratnom delu, veličini, polu-životu u cirkulaciji, načinu primene i dozi. I pored značajno poboljšanog kvaliteta života anemičnih bolesnika, postoje ozbiljni, potencijalno ugrožavajući efekti ove terapije. Sinteza rekombinovanog humanog eritropoetina, s druge strane, otvorila je nove mogućnosti i za zloupotrebu u sportu, u domenu krvnog dopinga. Davanje eritropoetina zdravim sportistima podiže njihov aerobni kapacitet i poboljšava izdržljivost i rezultate, posebno u aerobnim disciplinama. Time se ozbiljno narušava sportski duh, ali i ozbiljno ugrožava zdravlje sportista. U upotrebi su različiti testovi za otkrivanje nedozvoljenih supstanci, ali je uspeh ovih metoda još uvek ograničen. Uporedo se stalno razvijaju i pronalaze drugi načini zloupotrebe u sportu, poput novijih, različitih modela genetskog dopinga. Zbog svega navedenog razvoj novih modela antidoping testova i bolja zdravstvena kontrola sportista i dalje predstavljaju veliki izazov.

Ključne reči: eritropoetin, eritropoeza, anemija, sportisti, doping

Abstract

Erythropoietin is a hormone that promotes the formation of red blood cells by the bone marrow. In adults it is mainly produced by the kidneys as a response to hypoxia. Besides its main role, it also acts as antiapoptotic, anti-inflammatory and cytoprotective agent. Furthermore, it is produced in many non-hematopoietic tissues where it acts locally, stimulating angiogenesis. Erythropoietin binds cytokine receptors on target cells, such as erythrocyte precursor cells, neurons, glial and endothelial cells, cardiomyocytes, myocytes etc. The discovery of synthetic erythropoietin forms, in the late eighties of the last century, has significantly improved treatment outcome of patients with anaemia related to chronic diseases, especially chronic renal failure. Renal anaemia is multifactorial, but predominantly a consequence of erythropoietin deficiency. Today, three generations of erythropoiesis stimulating agents are available, differing in glycosylation pattern, molecular size, half-life and modes of administration and dosage. In anaemic patients this therapy significantly improves their quality of life, but may also have serious, potentially dangerous adverse effects. Synthesis of recombinant human erythropoietin, on the other hand, has improved possibilities for manipulations in sport, in the field of blood doping. Erythropoietin administration in athletes increases their maximum oxygen consumption capacity, improves endurance and performance, especially in aerobic exercise. This seriously undermines the spirit of sport, and also endangers athletes` health. Different anti-doping tests have been developed and used, still with limited success. At the same time, new illicit ways of malpractice are developing, such as variuos models of gene doping. Therefore, providing new models of anti-doping tests and strategies, together with better health control of athletes, still remains a considerable challenge.

Key words: erythropoietin, erythropoiesis, anaemia, athletes, doping

Introduction

Erythropoietin (EPO) is a cytokine for erythrocyte precursors in the bone marrow. It is also a 30400 daltons glycoprotein hormone, with peptide core of 165 amino-acids for receptor binding, and carbohydrate portion (40% of the total molecule) necessary for *in vivo* survival of the hormone (1, 2). In adults EPO is mainly produced by the kidneys and, to a much lesser extent of up to 10% by the liver (3). Erythropoietin is produced mostly by interstitial, intertubular fibroblasts in renal cortex and outer medulla, but also in peritubular capillary cells (1, 4). Some studies suggest possible role of proximal tubular cells in EPO production (5). In liver, EPO producing cells are hepatocytes and perisinusoidal (Ito) cells (1, 6). Synthesis of EPO is hypoxia dependent, and physiological hypoxia appears during high altitude exposure, or strenuous exercise. Important role here play hypoxia inducible factors 1 and 2 (HIF 1 and 2) which induce erythropoietin gene transcription (1, 6). These are ubiquitary enzymes, but HIF 2 is more present in kidney and liver cells, and hence more specific for EPO gene transcription (7, 8). Non-HIF pathways that can alter EPO production include kinase C, GATA-2, nuclear factor kappa B (NFB) pathways (7, 9). Baseline EPO concentration can raise up to 1000 fold in hypoxic conditions (10). In healthy individuals, reference EPO range is usually between 1 and 27 mU/ml, while patients with chronic kidney disease (CKD) can have much higher values, but inappropriately low related to the degree of anaemia, and with inadequate response to it because of the resistance (9).

The major effect of EPO is stimulation of erythropoiesis (3). Erythropoietin induces hematopoiesis and boosts the production of erythrocytes, rising their count after 3-4 weeks. Molecules of EPO bind to the EPO receptors, type 1 transmembrane proteins belonging to hematopoietic cytokine receptor superfamily, which are Janus tyrosine kinase 2 (JAK2) dependent, and undergo dimerization in response to EPO binding (1, 2). This is followed by activation of several signaling pathways, mostly JAK2/STAT5 pathway, and PI3K/Akt, but also by other signal molecules like MAP kinase, ERK1/2, protein kinase C and heat shock proteins (11, 12). Cytoplasmic protein hematopoietic cell phosphatase (HCP) terminates signal transduction (9).

First erythroid progenitor cell expressing EPO receptor is colony forming unit erythroid cell (CFU-E), which further proliferates and differentiates, until 16 mature erythrocytes occur (1, 10, 13). In the state of intensive erythropoiesis the need of iron for haemoglobin (Hb) synthesis is elevated. This is regulated by coordination between EPO and hepcidin, a hormone involved in iron homeostasis (14). Erythropoietin indirectly inhibits synthesis of hepcidin and its action, leading to iron transfer from intracellular storage into circulation, and increase of iron intestinal absorption (9, 14).

Beside its direct hematopoietic and antiapoptotic effects, EPO also acts as anti-inflammatory and cytoprotective agent (11). There are many non-hematopoietic (non-erythroid) tissues and organs, such as brain, retina, muscles, heart, lungs, spleen, uterus etc., where EPO is locally produced and where EPO receptors have been expressed as well (1, 8, 11, 15, 16). In these organs EPO exhibits all its effects except erythropoietic, reciprocated by proangiogenic stimulation (17, 18).

During the late eighties of the last century, synthetic forms of erythropoietin have been discovered. This revelation has significantly improved treatment options for patients with anaemia due to chronic disease, especially those with CKD and certain malignancies, but also in cases of peri-operative care and prematurity (19). Unfortunately, this was also recognized by some athletes and their practitians, as much easier way to increase erythrocyte number and therefore tissue oxygenation and endurance.

Erythropoietin in renal anaemia treatment

Anaemia in renal insufficiency is multifactorial, but mainly a consequence of decreased erythropoietin production, due to EPO-producing fibroblasts transdifferentiation into myofibroblasts, predominantly collagen-secreting cells (7, 20). This occurs under the influence of proinflammatory cytokines, TNFα and TGFβ. The amount of erythropoietin produced by the liver is inadequate for organism needs (20). Uremic toxins, deficiency of vitamin D, B_{12}, folate and iron, inflammation, shorter erythrocytes lifespan, gastrointestinal bleedings, also contribute to anaemia development (19, 21). Anaemia can occur already with decline of glomerular filtration rate to 40-50 ml/min (20). The severity of anaemia does not correlate directly with CKD stages, and usually does not depend upon the nature of renal disorder. Anaemia in CKD is normochromic, normocytic, defined as Hb concentration below 13g/dl for adult males and post-menopausal women, or as Hb below 12g/dl for pre-menopausal women (20, 22).

Synthesis of recombinant human erythropoietin (rHuEPO), enabled novel therapeutic options in clinical practice. Various glycosylation patterns of rHuEPO gave rise to alpha, beta, delta, and omega forms. So far, there are three generations of erythropoietin stimulating agents (ESA), administrated by subcutaneous or intravenous route (19, 23). In 1989 epoetin alfa was synthesized, followed by epoetin beta, and these were the first generation agents, with shorter half-lives, and smaller molecular size in comparison with the following ESAs. Darbepoetin alfa is considered the second generation, with longer terminal half-life, while continuous EPO receptor activator (CERA) is the third

generation, with much longer half-life, which significantly increases the administration interval (23). An important issue, however, is resistance to ESA, developing in 10 to 20% of CKD patients, mainly because of proinflammatory cytokines that disrupt iron metabolism, inhibit erythroid progenitor cells, and reduce number and effect of EPO receptors, which altogether increases mortality risk (9, 24, 25). The major adverse effects of ESA therapy include hypertension, increase in blood viscosity, hypercoagulability and greater risk of thrombotic events, flu-like syndrome, hyperkalemia, erythroblastosis, iron deficiency, headache (11, 20). In patients with uncontrolled hypertension, or in patients with malignancies, ESA therapy is contraindicated, even though there are still controversies about possibility that rHuEPO enhances cancer progression (26, 27).

Physical activity and blood doping

Exercise has many beneficial effects on human organism. In aerobic disciplines, such as long-distance or treadmill running, cycling, cross-country skiing, the main factors determining performance are oxygen (O_2) delivery to exercising skeletal muscles and its use (28-30). Endurance training induces physiological changes that can improve exercise performance in terms of haematopoietic induction, which increases O_2 transport to muscles. This type of training and EPO both stimulate a shift in skeletal muscle, from a fast glycolytic to a slower oxidative phenotype, by inducing mitochondrial biogenesis, which also improves aerobic exercise capacity (28, 31, 32). Haemoglobin concentration rises because of increased erythrocytes mass, and decreased plasma volume during exercise (10). Altitude exposure is a well known legal stimulus of erythropoiesis.

Doping presents the use of drugs or methods to improve athletic performance. Erythropoietin improves performance in athletes primary by intensifying of erythropoiesis and raise of maximal O_2 uptake, usually between 6 and 12% (33). To improve endurance, some athletes went for blood manipulations, so called blood doping, first with blood transfusions in the 1970s and 1980s (although earliest reports in literature date back to 1945), and then rHuEPO abuse started, soon after its introduction to clinical practice (26, 29, 32, 34). Therefore, in 1990 the International Olympic Committee prohibited rHuEPO use in athletes (26, 35). The World Anti-Doping Agency (WADA) annually updates its List of Prohibited Substancies and Methods, containing different categories, such as stimulants, steroids, gene doping, etc. According to some estimates, 3 to 7% of best endurance sport athletes use rHuEPO (36). Blood doping is defined by WADA as "*the misuse of certain techniques and/or substances to increase one's red blood cell mass, which allows the body to transport more O_2 to muscles and therefore increase stamina and performance*" (33). Apart from EPO, other agents are also misused to stimulate erythropoiesis, such as HIFs, even though they induce expression of more than 200 genes, beside EPO gene, or cobalt, although it has serious toxic effects, or prolyl hydroxylase inhibitors, HCP inhibitors, EPO mimetic peptides and recombinant fusion proteins of EPO (26, 31, 33). Several hormones may also stimulate EPO production, like thyroid hormone, growth hormone, testosterone, angiotensin II, prostanoids (29, 33). In 2002 Repoxygen, an agent consisting of viral vector that transfers the modified human EPO gene under the control of genes encoding HIF 1 and 2 was developed, and its use in athletes was prohibited in 2009 (36). In 2003 WADA defined gene doping as "*nontherapeutic use of cells, genes, genetic elements of modulation of gene expression, having the capacity to enhance athletic performance*", and added it into List of prohibited practices (26, 36). In gene doping genetic material is incorporated into cells by viral or non-viral carriers and techniques, where the first ones are more efficient but also more cytotoxic. Beside all side effects of EPO overexpression, the main risk of gene doping is that gene transfer vectors can integrate at the "wrong site" in DNA, and lead to insertional mutagenesis, which may cause cancer or death (36).

Several tests to determine blood doping are used, direct and indirect, with limited success, using biological materials (blood and urine). Allogenic blood transfusion test is direct method, implemented in 2004, that uses blood group antisera to recognize different erythrocytes populations by flow cytometry (33, 37). Manipulations with autologous transfusions can still be detected only indirectly, like problematic technique of carbon-monoxide rebreathing, analysis of plastic residues following storage of some transfusions bags, or gene expression changes and proteomics, which are ongoing (29, 33). Methods for detecting rHuEPO misuse are direct and indirect. Direct method relies on different carbohydrate components - glycosylation patterns of endogenous and rHuEPO, detected by isoelectric focusing (IEF) or electrophoresis in urine (26, 38). This test, known as Lasne's method, is adopted by WADA as a screen for rHuEPO in athletes. False-positive results can occur due to strenuous exercise proteinuria and cross-reactivity with anti-EPO antibodies, while false-negative results may arise especially with CERA, which exhibit low urinary excretion (35, 39, 40). Indirect methods are focused on measuring haematological parameters, and developing athlete biological passports (ABP), a software system based on monitoring doping biomarkers in athletes (26, 29, 33, 35). The main idea with ABP is that any kind of blood doping will change haematological and other parameters. It was first implemented in cycling and athletics, and then football, triathlon, etc. Markers of hematologic model considered

in ABP are hematocrit, Hb concentration, erythrocyte count, percentage and number of reticulocyte, mean corpuscular Hb concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular Hb mass (MCH), and some additional parameters referred to reticulocytes (33). Blood profiles are then evaluated by experts (29). For gene expression profiling direct methods, like DNA microarrays, proteomic profiling, and searching for transgenic proteins are used, and still developing, while potential indirect methods are also haematological and molecular "passports" of athletes (26, 36). Different ways to falsify doping test results are continuously developing, such as altitude training to conceal EPO misuse, or adding proteases to decompose erythropoietic proteins in urine samples, or simply covering up positive doping tests (33).

Conclusion

Erythropoietin is the main hormone that induces erythrocyte production. This was the guide and basis for synthesis of recombinant human erythropoietin forms, resulting in significant improvement in anaemia treatment. On the other hand, this achievement attracted the attention of some athletes who have tried, and still try to find easier, illegal and unsporting ways to enhance their performance. While in cases of indicated medical treatment serious side effects of rHuEPO must be taken into account, there is no excuse for its abuse in sport, and potential health endangering. Simultaneously with development of anaemia-treating agents, better anti-doping tests and controls, and strategies to prevent cheating in sport have to be established. We hope that the upcoming Olympic Games in Rio will promote setting new records of athletes who respect basic Olympic motto - "Faster, Higher, Stronger", but who also respect the principles of fair play and their own health. There is no doubt that pleiotropic actions of erythropoietin, and its misuse in sport are fields of medicine that still have to be, and will be investigated, but at the same time we all have responsibility to use those findings in a proper way, ensuring further progress of medical treatment, and disabling all potential attempts for its illegal use in sport.

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