Brief report

False-positive detection of recombinant human erythropoietin in urine following strenuous physical exercise

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Abstract

Erythropoietin (Epo) is a glycoprotein hormone that promotes the production of red blood cells. Recombinant human Epo (rhEpo) is illicitly used to improve performance in endurance sports. Doping in sports is discouraged by the screening of athletes for rhEPO in urine. The adopted test is based on a combination of isoelectric focusing and double immunoblotting, and distinguishes between endogenous and recombinant human Epo. We show here that this widely-used test can occasionally lead to the false-positive detection of rhEpo (epoetin-β) in post-exercise, protein-rich urine, probably because the adopted monoclonal anti-Epo antibodies are not monospecific.

Introduction

Erythropoietin (Epo) is a glycoprotein hormone that is mainly produced by the kidney. It boosts the production of red blood cells by promoting the proliferation, differentiation and survival of progenitor cells of the erythroid lineage. Recombinant human Epo (rhEpo) is widely used for the treatment of various forms of anaemia. Since rhEpo increases the body's maximum oxygen consumption capacity and endurance by increasing red cell mass, it has also been embraced as an aid in endurance sports. However, this use of Epo was prohibited by the International Olympic Committee, which has led to the screening of athletes for rhEpo abuse.

Endogenous and recombinant human Epo isoforms have a somewhat different glycosylation pattern and the resulting charge differences have been exploited to distinguish endogenous and recombinant isoforms by isoelectric focusing. Subsequently, the Epo isoforms can be visualized by a double-immunoblotting technique. This two-step procedure forms the basis for a test that has been adopted by the World Anti-Doping Agency (WADA) to screen for rhEpo in urine samples.

As a result of a disputed case of alleged rhEpo-abuse by an endurance athlete with post-exercise proteinuria, we wondered whether the test for rhEpo can in such cases lead to false-positive results, perhaps as a result of cross-reactivity of the Epo-antibodies with unrelated proteins. A similar problem has recently been reported for the Epo receptor. We report here that anti-Epo antibodies are not
monospecific and that their use can result in the false-positive detection of epoetin-β, a recombinant form of human Epo.5

Materials and Methods

NeoRecormon/Epoetin-β, Aranesp/Darbepoetin-α and mouse monoclonal anti-human Epo antibodies (clone AE7A5) were obtained from Roche, Amgen and R&D, respectively. The endurance athlete RB participated to this study voluntarily. In accordance with the Declaration of Helsinki, an informed consent was obtained from the athlete that participated in this study. Urine samples were collected after a 4-km jogging followed by four running periods of 1000m separated by a short resting period. The samples were immediately supplemented with a protease inhibitor cocktail (Complete, Roche) and stored at -20°C. Details of sample treatment, isoelectric focusing (CleanGel IEF polyacrylamide gels from Amersham Biosciences) and double immunoblotting were as described by the group of Lasne.9,11 These papers also form the basis for the currently adopted WADA Epo-test. Deglycosylations were performed for 16 h at 37°C with N-glycosidase F from Roche (2 units in 40 μl). The reaction was stopped by boiling in NuPAGE-SDS sample buffer and the proteins were separated on NuPAGE gels (10% Bis-Tris) with NuPage MOPS running buffer (Invitrogen). Standard tests were used to quantify total13 and specific14 urinary proteins, and to perform flow cytometric analysis.15

Results and discussion

The endurance athlete showed a normal creatinine clearance (127 ml/min) and no proteinuria at rest. After an overnight period without fluid intake, urinary osmolality reached 619 mOsm/kg (reference >800 mOsm/kg). This mildly reduced urinary concentration capacity suggests a pre-existing tubulopathy. Following strenuous physical exercise, proteinuria varied between 0.3 and 1.2 g/l. Flow cytometry revealed a marked hyaline cast count post-exercise (9 casts/μL versus a reference value of <0.3/μL).

Urine samples from this athlete, obtained immediately after a strenuous interval training session (0h) and 1 hour later (1h), were analysed for the presence of Epo. Neither of the samples was positive for endogenous Epo or Darbepoetin-α, but the 0h sample clearly contained bands that migrated like epoetin-β isoforms.
during isoelectric focusing (Fig. 1A). In contrast, the 1h sample did not show any signal at these positions. Since recombinant human Epo has a half-life of more than 8 hours, this was a first indication that the bands detected at 0h could not be explained by the presence of epoetin-β.

To obtain additional information on the nature of the detected signals in the 0h sample, we performed an immunoblotting after SDS-PAGE (Fig. 1B), using the same anti-Epo antibodies. This immunoblotting visualized a major band of 42 kDa which had, however, a higher mass than that detected for the epoetin-β isoforms (32-39 kDa). The distinct migration was confirmed in a mixing experiment whereby epoetin-β was added to the 0h sample, resulting in the visualization of two distinct bands. Furthermore, the removal of N-linked carbohydrates by a preincubation with N-glycosidase F decreased the apparent mass of epoetin-β isoforms from 32-39 kDa to 18 kDa, as detected by immunoblotting, but such a treatment did not cause a shift in the mass of the detected band in a post-exercise urine sample (Fig. 1C). The lack of an effect of N-glycosidase F in the latter case can not be due to an inhibition of N-glycosidase F by urine components since a 18-kDa band was generated when epoetin-β was added to this urine sample before the glycosidase treatment. Thus, immunoblotting before and after a glycosidase treatment confirmed that the major urinary protein that was visualized with the anti-Epo antibodies was not Epo.

Immunoblotting on more concentrated urine samples yielded, in addition to a band of 42 kDa, also bands of 48, 93 and 125 kDa, although the latter two were only detected in three out of four tested post-exercise urine samples from the athlete (Fig. 2). Importantly, none of these bands were detected in the absence of the primary antibodies, showing that they did not result from the interaction of urine proteins with the secondary antibodies. It should also be pointed out that the immunoblots shown in Figs. 1B, 1C and 2 were obtained with urine samples that were less concentrated than those that are routinely used for Epo-tests which leads, if anything, to an underestimation of the problem of non-specificity of the anti-Epo antibodies. In any case, our data clearly show that the monoclonal anti-Epo antibodies (clone AE7A5) visualize multiple polypeptides during immunoblotting of protein-rich urine samples. Some of these proteins may have a similar isoelectric point as the epoetin-β isoforms, which possibly accounts for the false-positive detection of epoetin-β. Recently, Kahn et al. also reported the non-specific binding
of these anti-Epo antibodies to several proteins in the urine of a non-athletic volunteer.\textsuperscript{16}

The athlete that we tested was only false-positive for epoetin-\(\beta\) in two out of seven post-exercise urine samples (not illustrated). We also want to point out that the false-positive detection of epoetin-\(\beta\) may be restricted to (very) few athletes, as it may be linked to the extent and type of proteinuria. The extent of proteinuria correlates more with the intensity than the duration of exercise and has a half-time decay of about 1h. The athlete that we tested showed a mixed glomerular-tubular proteinuria, which is characterized by a broad spectrum of urinary proteins. Some of these proteins show some structural homology with epoetin-\(\beta\),\textsuperscript{16} which possibly accounts for their cross-reactivity with the anti-Epo antibodies. In a WADA report, the possible existence of such analytical interferences was already predicted.\textsuperscript{17} The false-positive detection of epoetin-\(\beta\) may be prevented by sampling before or at least one hour after exercise, which is particularly important for athletes that present with pronounced exercise-induced proteinuria. Additional tests can be performed to identify false-positive test results, such as two-dimensional electrophoresis,\textsuperscript{16} deglycosylation assays, as described in this study, or indirect assays.\textsuperscript{18}

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**References**


Legends to the figures

Figure 1. False positive detection of epoetin-β in urine.
(A). Urine samples collected from the athlete immediately after a 4 x 1000 m sprint (0h) and 1 hour later (1h) contained 1.2 g and 0.3 g proteins/l, respectively. The samples were concentrated 200-fold and 800-fold, respectively, and processed for the detection of Epo by double immunoblotting after isoelectric focusing, as detailed in the Materials and Methods section. Also shown are the reference samples Darbepoetin-α (1 ng) and epoetin-β (0.6 ng) (B). Immunoblotting of a post-exercise urine sample (0h, 10-fold concentrated) after SDS-PAGE with anti-Epo antibodies. Also illustrated are epoetin-β (0.9 ng) and a mixture of the urine sample and 0.9 ng epoetin-β. C. Immunoblotting of the same samples before and after a treatment with N-glycosidase F, as indicated.

Figure 2. Lack of specificity of the AE7A5 anti-Epo antibodies.
Four urine samples (lanes 1-4), obtained from the same athlete on four different days immediately after a 4 x 1000 m sprint, were concentrated 20-fold by ultrafiltration and subjected to immunoblotting after SDS-PAGE with monoclonal (clone AE7A5) anti-Epo antibodies (left blot). The right blot was treated identically except for the absence of anti-Epo antibodies. The protein concentration in the non-concentrated samples amounted to 1.2 (lane 1), 1.1 (lane 2), 0.47 (lane 3) and 0.9 (lane 4) g/l.
A. Double-immunoblotting after IEF

B. Immunoblotting after SDS-PAGE

C. Treatment with N-glycosidase F
Fig. 2

[Image of a Western blot analysis with the following details:
- Lanes 1, 2, 3, and 4 labeled with + + + +.
- MW kDa markers: 97, 64, 51, 39, 28, 14.
- Anti-Epo (AE7A5) and Secondary antibody labeled separately.]
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