Effects of darbepoetin injections on erythrocyte membrane transport protein expressions in humans

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Effects of darbepoetin injections on erythrocyte membrane transport protein expressions in humans. J Appl Physiol 101: 164–168, 2006. First published March 30, 2006; doi:10.1152/japplphysiol.01376.2005.—The present study investigated the effects of injected darbepoetin [novel erythropoiesis stimulating protein (NESP)] on the density of three erythrocyte membrane transport proteins: the lactate-\(H^+\) cotransporter (monocarboxylate transporter 1), the chloride/bicarbonate exchanger 1 (anion exchanger 1), and the water channel aquaporin 1. Thirteen subjects were injected with NESP once a week for 4 wk. Blood samples were obtained before, during, and after the injection period, and the erythrocyte transport proteins were determined by Western blotting. The NESP injections induced a transient increase in hematocrit, red cell volume, and reticulocyte fraction. The density of aquaporin 1 protein was higher (maximal increase +59%) \((P < 0.01)\) during the injection period compared with the preinjection value and lower \((P < 0.01)\) after the injection period. The density of anion exchanger 1 protein was higher (maximal increase +15%) \((P < 0.05)\) during the injection period compared with the preinjection value and tended \((P = 0.06)\) to be lower after the injection period than before the injection period. The density of the erythrocyte monocarboxylate transporter 1 protein was higher (maximal increase +43%) \((P < 0.05)\) during the injection period than in the preinjection period. Age separation experiments using self-creating Percoll gradients demonstrated a higher density of membrane transport proteins in young red blood cells. These data suggest that the NESP-induced increase in membrane transport proteins is caused by a higher fraction of newly formed erythrocytes (and reticulocytes), which have a higher density of membrane transport proteins. However, increased incorporation of membrane proteins during erythrocyte formation may also be involved. We suggest that NESP improves the quality of erythrocyte membrane transport through these mechanisms.

erythropoietin; anion exchanger 1; monocarboxylate transporter 1; aquaporin 1

A STUDY OF THE EFFECTS of chronic hypoxia in lowlanders who traveled to high altitude demonstrated that chronic hypoxia increases the concentration of proteins involved in lactate transport [monocarboxylate transporter 1 (MCT1) protein] and in anion exchange 1 (AE1) protein, both of which are important for the acid-base balance (7). That study also reported that the densities of these transporters are higher in humans living permanently at high altitude than in lowlanders and suggested two explanations for the changes in lowlanders. First, high altitude might upregulate the synthesis of transport proteins during red blood cell formation. Second, because the density of erythrocyte transport proteins declines during the life span of each cell, the increased density of transport proteins at high altitude might arise because of a greater proportion of newly formed erythrocytes. These two explanations for the increased density of erythrocyte transporter proteins are not mutually exclusive. The finding that the densities of transporter proteins are upregulated in humans living permanently at high altitude supports the suggestion that more transporters are incorporated during erythrocyte formation at altitude (7). The underlying mechanism for the effects of hypoxia is probably an increase in erythropoietin (EPO), which has strong effects on the regulation of red blood cell formation; increased plasma concentration of EPO increases blood hemoglobin (Hb) concentration and hematocrit (Hct).

Injections of EPO increase cation transport (2), and injection of recombinant human EPO increases the lactate transport capacity of red blood cells (4). EPO is thought to increase the synthesis of MCT1 protein during erythrocyte formation. We hypothesized that EPO injections cause a general increase in the concentration of membrane transport proteins. In the present study, we investigated the effects of injecting the EPO darbepoetin [novel erythropoiesis stimulating protein (NESP)] (12) on erythrocyte membrane proteins. Three erythrocyte transporters were selected: 1) the AE1, which is important for bicarbonate exchange; 2) the lactate-\(H^+\) cotransporter (MCT1), which is the only MCT isoform in erythrocytes; and 3) the water channel aquaporin 1 (AQP1), which is thought to be important for \(CO_2\) diffusion (5). The concentrations of these transport proteins were quantified in blood samples obtained before, during, and after repeated injections with NESP.

Red blood cells decrease in size with age, leading to a greater density in old red blood cells (11, 12). To evaluate whether the concentrations of the three transport proteins decreased with cell age, we fractionated red blood cells by density and subjected each fraction to Western blotting.

MATERIALS AND METHODS

Subjects and Injections

Thirteen subjects (six women and seven men) participated in the study. Their mean age was 25.2 yr (SD 3.9), body weight was 68.4 kg (SD 15.3), and height was 174 cm (SD 9). The subjects were fully informed of any risk and discomfort associated with the experiments before giving their informed, written consent to participate. The study conformed to the code of ethics of the World Medical Association.

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Each subject received 0.78 μg/kg darbepoetin (NESP, Amgen Europe BV) (12) once a week for ~4 wk. The weekly dose of 30–60 μg is equivalent to 4,000–12,000 IU HuEpo. We aimed to increase Hct to 47 and 50% in women and men, respectively. Therefore, the Hct was used as stop criteria for the NESP injection. Because the starting level and rate of Hct increase differed, the number of injections varied between subjects. Nine subjects received four injections, two received only three injections, and two subjects received extra injections. The indicated last day of injection (day 39), therefore, represents a mean value with a SD of 3 days. The female and male subjects were supplemented with 200 and 100 mg iron, respectively, starting 1 wk before the investigation began and continuing until 2 wk after the end of the study.

Blood Samples

Three blood samples were obtained before the injection period (days 1–14). Blood samples were obtained twice a week during the injection period (days 14–39) and for 3 wk after the injection period (days 39–60). In the last part of the experiment, blood samples were obtained once a week for 4 wk (days 60–85) and on day 113.

Hb, Hct, reticulocyte count (RCV), and mean fraction of reticulocytes (Ret%) were measured using an ADVIA 120 (Bayer).

Western Blotting

One hundred microliters of erythrocytes were spun down (14,000 g for 5 min), the supernatant was removed, and the sample was diluted with 8 mM phosphate buffer. The procedure was repeated four times to completely remove Hb. The membranes were dissolved in Tris-SDS (10 mM Tris, 4% SDS, 1 mM EDTA, 2 mM PMSF, pH 7.4), and the protein content was determined with a BSA standard (DC protein assay, Bio-Rad). Samples were mixed 1:1 with sample buffer (10% SDS, 5% glycerol, 10 mM Tris- HCl, 1 mM EDTA, 10 mM dithiothreitol) and subjected to SDS-PAGE (8–18% gradient gel). The amount of protein loaded per lane was 11 μg. The separated proteins were electroblotted onto a Millipore Immobilon-P polyvinylidene difluoride membrane. The membrane was blocked with 2% BSA and 1% low-fat dry milk, and 0.1% Tween 20, and incubated overnight with the primary antibody diluted in a BSA-containing buffer. After treatment with horseradish peroxidase-coupled secondary antibodies for 90 min, the membrane was washed repeatedly with distilled water, 0.05% Tween 20, and 1 M NaCl; incubated with electrochemiluminescence ECL reagent; and visualized on a film. The film was scanned, and the band intensities were analyzed with UnScanIt software to quantify the protein densities (arbitrary density units). The 21 blood samples obtained from each subject were run on the same gel, and the protein content was calculated relative to the mean of the three samples obtained before the injections (Fig. 2).

AQP1 was detected with anti-AQP1 antibodies (no. AQPI-s, Alpha Diagnostic International) diluted 1:10,000. The inorganic anion exchanger (Cl–/HCO3– exchanger) AE1 was detected with anti-AE1 antibodies (no. AE1-M, Alpha Diagnostic International) diluted 1:10,000. The lactate-H+ cotransporter isofrom 1 (MCT1) was detected with anti-MCT1 antibodies (no. sc-14916, Santa Cruz) diluted 1:1,000. The anti-mouse secondary antibody used for AE1 measurements was diluted 1:5,000, and the anti-rabbit antibodies for the AQP1 measurements and the anti-goat antibodies for the MCT1 experiments were diluted 1:3,333.

Separation of Red Blood Cells

Erythrocytes were obtained from subjects who were not receiving the NESP injections. Erythrocytes were density separated using self-forming Percoll gradients (11, 13). Briefly, 1.5 ml of blood were mixed with 30 ml of 55% Percoll solution in saline and spun for 30 min at 19,000 g in a Sorwall SS34 rotor. Density Marker Beads (Amersham) (density 1.051, 1.064, 1.074, 1.002, and 1.120) were treated similarly. Five fractions of blood cells were harvested, and the density was evaluated from the layers of the density beads. The fractions were spun down in 10-ml saline (860 g), the pellet resuspended, and the procedure repeated four times to remove the Percoll. The pellet was hemolysed, and the membranes sedimented at 15,000 g in phosphate buffer. The procedure was repeated to remove Hb. The membranes were then treated with SDS and used for Western blotting, as described above.

Statistical Analysis

The changes in protein density during and after the NESP injections were analyzed using individual data and one-way ANOVA for repeated measurements (SigmaStat software). The significance level was set at P < 0.05.

RESULTS

Hematological Parameters

The mean Hct was 39.2 ± 0.4% (means ± SE) before the injections began. Hct increased during the injection period and for the following 2 wk; the highest value (46.4 ± 0.8%) was obtained 2 wk after the last injection, after which the Hct decreased slowly but was still higher than preinjection values 8 wk after the injection period (Fig. 1A).

The mean RCV was 83.4 ± 0.6 fl before the injections. The highest value (87.6 ± 0.9 fl) was observed ~2 wk after the last injection. RCV followed a similar pattern to that of Hct (Fig. 1B). The Ret% was 1.5 ± 0.1% before the injections. The time course of changes in Ret% differed from that of Hct and RCV. Ret% increased rapidly in the first phase of the injection period, reached the maximal value (3.3 ± 2%) 2 wk after the first injection, and remained high during the rest of the injection period. After the injection period, Ret% decreased rapidly and reached the lowest value (0.62 ± 0.01%) (which was lower than before the injection) 3 wk after the last injection, after which Ret% slowly increased toward the initial value (Fig. 1C).

Membrane Proteins

AQP1 protein. During the injection period (days 14–39), the density (pmg of total membrane protein) of AQP1 protein was higher (P < 0.01) than the preinjection value. After the injection period, the AQP1 density was significantly lower (P < 0.01) than the preinjection value and lower than during the injection period (Fig. 2A).

AE1 protein. During the injection period, the density of AE1 protein was higher (P < 0.05) than the preinjection value. After the injection period, the AE1 protein density tended (P = 0.06) to be lower than the preinjection value (Fig. 2B).

MCT1 protein. During the injection period, the density of the erythrocyte MCT1 protein was higher (P < 0.05) than the preinjection value. The MCT1 content in the postinjection period was not different from the preinjection value (Fig. 2C).

Density Separation

Five fractions of blood cells were harvested from different layers of the Percoll gradient. The middle part of the gradient contained the main fraction of the blood cells. A low-density fraction, which contained cells with a high degree of hemolysis, was not included in the analysis.
The protein contents of the three membrane proteins (AQP1, AE1, and MCT1) were determined in each fraction (Fig. 3). The three transport proteins were present at a higher content per milligram of total protein in the low-density samples than in the high-density samples. Since the low-density fractions are expected to represent young erythrocytes (see DISCUSSION), Fig. 3 demonstrates that the contents of transport protein decrease with age.

### DISCUSSION

The main finding of the present study is that NESP injections increased the density of membrane transporter proteins in red blood cells.

Hct and RCV increased gradually during the injection period, and these increases continued after the injection period. The highest values were observed ~2 wk after the last NESP injection, after which Hct and RCV gradually declined toward the preinjection value, although Hct and RCV did not return to their preinjection levels.

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**Protein Changes**

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**Fig. 1.** Erythropoietin-induced changes in the hematological parameters hematocrit (Hct; %) (A), red cell volume (RCV; fl) (B), and reticulocyte fraction (Ret%; C). The bar represents the novel erythropoiesis stimulating protein (NESP) injection period. Values are means ± SE from 13 subjects.

**Fig. 2.** Erythrocyte membrane content of aquaporin 1 (AQP1; A), anion exchanger 1 (AE1; B), and monocarboxylate transporter 1 (MCT1; C) proteins before, during, and after the injections with NESP. For each time point, the protein content was calculated relative to the protein content before the injection period. The protein densities at each time point were determined in duplicate in the same blood sample for each of the subjects. The values are means ± SE from 13 subjects. The bar represents the NESP injection period. **Insets:** examples of Western blots of sample 1 (control), sample 6 in the NESP-injection period, sample 3 after injection, and the last sample.
protein. Values are means calculated relative to the mean protein density, which was set to 100% for each placement of density beads with a known density. The protein values are because the increase in Ret% accounted for erythrocyte age.

proteins and that the densities of these proteins decline with new erythrocytes, these data suggest that new erythrocytes the reticulocyte fraction is an index of the rate of formation of mechanism underlying the changes in protein. Assuming that changes in membrane protein and Ret% may help elucidate the course of changes in the membrane protein densities was closer to that of Hct and RCV. The similar patterns of change in Ret% increased rapidly during the first part of the injection period and reached the maximal value during the injection period. After the injection stopped, Ret% declined below the preinjection value; the lowest value was observed ~2 wk after the last injection, after which Ret% gradually increased toward the preinjection level. Thus the pattern of change in Ret% differed from that of Hct and RCV. The changes in Hct and RCV indicate that the NESP injections were sufficient to stimulate red cell formation.

The densities of the membrane transport proteins increased during the injection period and declined to less than the preinjection levels after the injections (Fig. 2). Thus the time course of changes in the membrane protein densities was closer to that of Ret% than to that of Hct. The similar patterns of changes in membrane protein and Ret% may help elucidate the mechanism underlying the changes in protein. Assuming that the reticulocyte fraction is an index of the rate of formation of new erythrocytes, these data suggest that new erythrocytes exhibit a higher density of the studied membrane transport proteins and that the densities of these proteins decline with erythrocyte age.

We do not expect that the increase in blood transport proteins occurred entirely within the reticulocytes themselves, because the increase in Ret% accounted for <2% of the increase in total cell number. On the other hand, the increase in new red blood cells at the end of the injection period did not exceed ~11% (the Hct increased ~5%). In addition, it has been reported that a selective removal of some proteins takes place at the transition from the reticulocyte stage to the final erythrocyte (8). Furthermore, the present study found a remarkably similar time course of changes in reticulocyte counts and membrane proteins. These data suggest that the increase in reticulocyte numbers could have contributed to the increase in the densities of membrane transport proteins. This is supported by the observed decrease in membrane proteins in the 2 wk after the last injection, when Hct increased and Ret% decreased.

The rapid decline in membrane protein densities (Fig. 2A) after the injection period suggests that only very new erythrocytes (and reticulocytes) possess extra transport proteins. The decrease in membrane transport protein densities with cell age seems to occur faster than the changes in RCV, which slowly decreased after the injection period (Fig. 1B). These data suggest that the transport protein densities seem to decrease faster with age than the structural proteins.

The age separation experiments supported the idea that new blood cells have a greater density of transporter proteins (Fig. 3). We found greater transport protein density (per mg of total protein) in the low-density fractions than in the high-density fractions. We assume that the low-density fractions were enriched with young erythrocytes and reticulocytes, whereas the denser fractions contained mainly older cells (6, 11, 13); however, it has also been suggested that red blood cell size is the important factor for the age separation (9). Labeling experiments have shown that some young cells are also found in the dense fractions (10), and, although reticulocytes are enriched in the light fractions, these cells may also be present in the denser fractions (6). The age separation may, therefore, not have been complete in our experiments, which is consistent with our observation that most of the erythrocytes were found in the middle fractions. Therefore, although the light fraction contained the greatest amount of transport proteins, this method cannot be used quantitatively to compare young and old erythrocytes, but gives only an indication of changes in transport proteins with cell age.

The finding that RCV decreased with cell age indicates that structural proteins are gradually lost as the erythrocyte ages. Because changes in membrane protein densities were calculated relative to the total protein content, we conclude that the decrease in transport protein densities with age occurs faster than the reduction in structural proteins.

**Underlying Mechanism**

MCT1 and AE1 proteins increased in erythrocytes from Danish lowlanders, who spend 8 wk at altitude (4,100 m) (7). It was suggested that hypoxia induces an increased incorporation of these membrane proteins during the formation of erythrocytes. Alternatively, young erythrocytes have a higher density of proteins (11, 13); a higher proportion of young erythrocytes might explain the effect of hypoxia (7). The erythrocyte lactate transport (mediated by MCT1) increased in trained subjects exposed to exercise-induced hypoxia (3). EPO, which is induced by hypoxia, is a likely underlying factor responsible for these changes. This is supported by a report showing that injection of human EPO increases the erythrocyte lactate transport capacity (4) and that EPO induces a number of membrane proteins, among these the band 3 protein AE1 (8).

In the present study, NESP injections increased the protein densities of MCT1, AE1, and AQP1. The finding that the three investigated transport proteins had higher densities in young
erythrocytes (Fig. 3) supports the idea that the NESP-induced changes are the result of a greater proportion of young erythrocytes, as argued above.

However, a comparison of protein densities in lowlanders and Bolivian natives (7) demonstrated that proteins may be incorporated differently into erythrocytes during the formation. It is unclear whether our data reflect an increased incorporation of membrane proteins.

Although the age separation experiments demonstrated greater transport protein densities in young erythrocytes, the incomplete age separation makes it impossible to evaluate whether the changes in age distribution can fully explain the effect of NESP in the present study. It is also possible that NESP increases protein incorporation during red blood cell maturation.

The reversible nature of the changes speaks against this possibility. For example, if newly formed erythrocytes contain more transport protein, one would expect to find an elevated level a few weeks later, which was not observed. However, the two explanations are not mutually exclusive.

Functional Importance

A rat study showed that training increases erythrocyte MCT concentration and that the increase in MCT1 content is the underlying mechanism for the improved ability to regulate plasma pH (1). Human studies demonstrated that recombinant EPO increases the lactate influx rate in erythrocytes, and that the effect is probably mediated by increased MCT1 content (4).

In another human study, it has been demonstrated that EPO improves the erythrocyte cation transport (2). It is, therefore, expected that the protein changes reported in the present study are functionally important.

In conclusion, NESP injection induced greater protein densities of the three membrane transport proteins studied. It is, therefore, concluded that NESP increases both the quantity of blood (Hct) and the quality of the blood cells by enhancing membrane transport properties.

GRANTS

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REFERENCES