Original Paper

Incorporation of Ortho- and Meta-Tyrosine Into Cellular Proteins Leads to Erythropoietin-Resistance in an Erythroid Cell Line

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Key Words

Erythropoietin-resistance • Cell signaling • Oxidative stress • Tyrosine isomers

Abstract

Background/Aims: Erythropoietin-resistance is an unsolved concern in the treatment of renal anaemia. We aimed to investigate the possible role of ortho- and meta-tyrosine – the hydroxyl free radical products of L-phenylalanine – in the development of erythropoietin-resistance. **Methods:** TF-1 erythroblast cell line was used. Cell concentration was determined on day 1; 2 and 3 by two independent observers simultaneously in Bürker cell counting chambers. Protein concentration was determined with colorimetric method. Para-, ortho- and meta-tyrosine levels were measured using reverse phase-HPLC with fluorescence detection. Using Western blot method activating phosphorylation of STAT5 and ERK1/2 were investigated. **Results:** We found a time- and concentration-dependent decrease of erythropoietin-induced proliferative activity in case of ortho- and meta-tyrosine treated TF-1 erythroblasts, compared to the para-tyrosine cultured cells. Decreased erythropoietin-response could be regained with a competitive dose of para-tyrosine. Proteins of erythroblasts treated by ortho- or meta-tyrosine had lower para-tyrosine and higher ortho- or meta-tyrosine content. Activating phosphorylation of ERK and STAT5 due to erythropoietin was practically prevented by ortho- or meta-tyrosine treatment. **Conclusion:** According to this study elevated ortho- and meta-tyrosine content of erythroblasts may lead to the dysfunction of intracellular signaling, resulting in erythropoietin-hyporesponsiveness.

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Introduction

Erythropoietin (EPO) is a hematopoietic growth factor playing major role in the proliferation and differentiation of erythroid cells. Renal anaemia is present in patients suffering in chronic kidney disease (CKD), resulting in the impairment of quality of life. Approximately 15 % of the recombinant-human-EPO (rh-EPO) receiving subjects are hyporesponsive [1]. Several possible pathomechanisms are discussed, such as iron deficiency, inflammation, use of angiotensin converting enzyme inhibitors, uremic toxins, insufficient dialysis, hyperparathyroidism or malignancy [2]. In a hemoglobin target-based study, performed by Solomon et al., the decreased response to rh-EPO was associated with higher risk of mortality [3, 4].

Erythropoietin-receptor (EPO-R) is a member of type I cytokine receptor superfamily, present in a variety of tissues and has been characterized in a wide range of cell lines [5-12]. EPO-binding results in EPO-R dimerization, JAK2 tyrosine-kinase phosphorylation, and activation of signal transducer and activator of transcription-5 (STAT5) and nuclear factor κ B [13]. The stimulus also results in the activation of the Ras/mitogen-activated protein kinase (MAPK) pathway. Erythroid cell proliferation and differentiation is dependent on both the MAPK p42/44 and the JAK-STAT axis [14].

The imbalance between pro- and antioxidant processes leads to the formation of reactive oxygen species (ROS). When excessive amount of hydroxyl radical is present, L-phenylalanine is converted into *meta*-tyrosine and *ortho*-tyrosine, besides the enzymatic formation of the physiological isomer, *para*-tyrosine [15]. Gurer-Orhan et al. proved that the concentration-dependent integration of meta-tyrosine into cellular proteins may be a mechanism of cytotoxicity [16]. Ehrlich observed in 1906 the phenomenon, known as concomitant tumor resistance, where a tumor-bearing host inhibits the growth of secondary tumor implants or metastasis. Ruggiero et al. identified the active serum fraction responsible for this phenomenon containing a mixture of the three isoforms of tyrosine [17]. According to their in vitro and in vivo studies ortho- and meta-tyrosine inhibits tumor growth in a dose dependent manner. Using immunoblot analysis, they found impaired ERK and STAT3 activation in the presence of meta-tyrosine [18]. Molnar et al. found significantly lower para-tyrosine level, and also a non-significant, but obviously higher plasma ortho-tyrosine level in patients with CKD [19]. According to the recent work of our group, on the one hand para-tyrosine level was significantly lower, on the other hand meta- and ortho-tyrosine levels were significantly higher in dialyzed patients compared to control groups (data under publication).

Based on these observations we hypothesized, that integration of meta- and ortho-tyrosine into cellular proteins may result in the alteration of signal transduction, leading to EPO-hyporesponsiveness.

Materials and Methods

Materials

Unless otherwise noted, chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Culture medium products were purchased from Life Technologies (Carlsbad, CA, USA).

Cell culture and treatments

TF-1 (CRL-2003) erythroblasts (American Type Culture Collection, Rockville, MD, USA) were used. Cells were grown in RPMI-1640 medium, containing 2 ng/ml of GM-CSF, 10% fetal calf serum and mixture of antibiotics. Prior to experiments, cells were cultured in medium containing indicated amount of para-, ortho-, or meta-tyrosine for 3 days. Tyrosine content of the culture media was stable; no considerable alteration of para-, ortho- or meta-tyrosine concentration could be detected in the absence of cells, during 3 days (data not shown).

For proliferation studies instead of GM-CSF, 3 IU/ml rh-EPO was added under similar conditions. For Western blotting experiments, after 12 hours of serum and factor deprivation, treatments were performed by addition of 3 IU/ml of rh-EPO for 10 minutes.

Analysis of cell proliferation

Standard number of cells (2×10^5 /ml) was planted onto 60 mm plates in culture medium containing additional 20 mg/l para-, ortho-, or meta-tyrosine, lacking GM-CSF, with or without addition of 3 IU/ml rh-EPO for 3 days. Cell concentration (cells/ μ l) was determined on day 1; 2 and 3 by two independent observers simultaneously, with the application of Bürker cell counting chambers. We calculated the mathematical mean of the two observed cell counts. Eventually samples were lyzed, then after storage in -80 °C for one night protein concentration was determined as described below. For concentration-dependence experiments cells were cultured in medium containing para-, ortho-, or meta-tyrosine with the addition of 0; 20; 40 or 80 mg/l para-tyrosine for 3-days. RPMI-1640 medium contains 20 mg/l para-tyrosine originally.

HPLC (Investigating tyrosine incorporation into cellular proteins)

To prove incorporation of the different tyrosine isoforms into cellular proteins we used a fluorescence HPLC-method. After three-day incubation cell culture was terminated. Medium was removed by centrifugation (1000 rpm; 10 min), then cells were washed three times by addition of 1 ml physiological NaCl-solution and centrifugation (1000 rpm; 5 min). Subsequently 200 μ l distilled water was added to the cells, followed by resuspension and ultrasound treatment. Thereafter 100 μ l 60% trichloro-acetic acid (TCA) was added. Mixture was then vortexed and centrifuged (4000 rpm; 10 min). After removing supernatant, 200 μ l 1% TCA was added, followed by resuspension, vortexing and addition of 100 μ l 60% TCA. Samples were then centrifuged (4000 rpm; 10 min), supernatant was removed and 200 μ l 1% TCA was added. Samples were then resuspended, treated by ultrasound and added by 100 μ l 60% TCA, followed by vortexing. Subsequently precipitate was separated by centrifugation (4000 rpm; 10 min) and 40 μ l buthyhydroxyltoluol (BHT; 500mM), 4 μ l desferal (400mM) and 400 μ l 12N HCl was added to it. Thereafter samples were undergone an overnight hydrolysis on 120°C. Samples were then centrifuged (5000 rpm; 15 min). The supernatant was filtered by a syringe filter (0.2 μ m) before analysis. Finally para-, ortho- and meta-tyrosine levels were determined using reverse phase-HPLC (C_{18} silica column, 250x4 mm) with fluorescence detection (λ_{ex} =275 nm; λ_{em} =305 nm) as described earlier [19]. Concentrations were calculated using an external standard. We calculated the ratios of para-tyrosine and total-tyrosine, ortho-tyrosine and total-tyrosine and meta-tyrosine and total-tyrosine.

Immunoblot analysis

Following rh-EPO treatments, cells were lyzed in Tris-Triton extraction buffer (1 M Tris-HCl, pH 7.4, 1.15 (vol/vol)% Triton X-100, 500 mM EDTA, 200 mM EGTA, 100 mM dithiothreitol containing the protease inhibitors of 100 mM and 0.5 (wt/vol)% phenylmethylsulfonyl fluoride, leupeptin, aprotinin and phosphatase inhibitor cocktail 2/3) on ice for 30 min. Lyzed proteins were harvested, centrifuged (14 000 g, for 15 min, at 4°C), then protein concentration of the supernatant was assessed using the Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin (BSA) as standard. Solubilized proteins were mixed with Laemmli buffer (2X), denaturated (for 5 min, at 90°C), then were separated on 10% SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Equal protein loading (50-75 μ g) was confirmed by staining the membrane with Ponceau-S. The blots were blocked in tris base saline containing 5 (wt/vol)% BSA and 0.1 (vol/vol) % Tween, then were incubated with primary antibodies (1:1 000; Cell Signaling, Beverly, MA, USA) against phospho-Thr(202)/Tyr(204) ERK and total ERK (p44/42 MAPK); phospho-Tyr (694) STAT5 and total STAT5 or β -actin for overnight at 4°C. After being washed, the membranes were incubated with appropriate, horseradish peroxidase-conjugated secondary antibody (1:2 000; anti-rabbit IgG, Cell Signaling) for 60 min at room temperature. After further washing, immunoblots were visualized with enhanced chemiluminescence (Super-Signal West Pico, Thermo Fisher Scientific, MA, USA) and developed on X-ray films (Kodak XAR). Total protein levels of STAT5 and ERK were immunostained after stripping the blots. Densitometric analyses were performed using Scion Image software (Frederick, MD, USA). Data are expressed as the ratio of phosphorylated and total ERK1/2 or STAT5, corrected to total cellular β -actin level.

Fig. 1. Effect of para-, ortho-, or meta-tyrosine supplementation on the proliferation of cells cultured in medium with or without EPO. *Panel A* shows the time dependent rise of cell count in para-tyrosine (open bars), ortho-tyrosine (black bars) or meta-tyrosine (grey bars) supplemented medium. (*, $P < 0.05$ vs. para-tyrosine cultured cells on the same day; $n = 10$). *Panel B* shows the alterations of cell counts after incubation for 3-days in media containing ortho- or meta-tyrosine and the indicated additional amount of para-tyrosine (*, $P < 0.001$ vs. para-tyrosine containing medium; $n = 5$). *Panel C* shows the effect of supplementation by ortho- or meta-tyrosine on the increment of cellular protein induced by EPO. Results are expressed as the ratio of protein content of EPO and non-EPO (control) cells. (*, $P < 0.05$ vs. para-tyrosine cultured cells; $n = 10$).

Statistical analysis

Data are expressed as means $\pm$ SE or means $\pm$ SD, as indicated. Analyses were performed as appropriate using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Statistically significant differences were defined as signed and are indicated in the figure legends. Normal distribution was verified with the application of Kolmogorov-Smirnov test. In case of cell proliferation studies, HPLC and protein concentration measurements ANOVA with Bonferroni's post-hoc test was employed. In case of Western-blot results, phosphorylation of controls was taken as 100% and one sample t-test was performed for the comparison versus controls. To compare the means between the groups of relative phosphorylations ANOVA with Bonferroni's post-hoc test was used.

Results

Cell proliferation

Panel A of Figure 1 shows the EPO-induced, time-dependent proliferation of TF-1 erythroblasts grown in para-, ortho- or meta-tyrosine containing medium. Culturing TF-1 cells in the presence of ortho- and meta-tyrosine EPO-induced proliferative activity was found to be decreased compared with para-tyrosine cultured cells. Maximal difference between cell counts was observed at day 3 (time curve).

Panel B demonstrates the effect of adding extra 0, 20, 40, or 80 mg/l para-tyrosine into the ortho- or meta-tyrosine supplemented medium on the cell counts at the 3rd day

Fig. 2. Relative para-, ortho- and meta-tyrosine content (i.e. ratios of para-, ortho-, and meta-tyrosine/total tyrosine) of cellular proteins of TF-1 cells (Panel A, B and C respectively). *, $P=0.003$ vs para-tyrosine cultured erythroblasts; §, $P<0.001$ vs para-tyrosine cultured cells; †, $P<0.001$ vs. para- and meta-tyrosine cultured cells; ‡, $P<0.001$ vs. ortho-tyrosine cultured control cells; +, $P<0.001$ vs. para- and ortho-tyrosine cultured erythroblasts; $n=10$.

of growing with or without rh-EPO (concentration dependence). The ortho-tyrosine induced impairment of EPO-response could be competed by 40 mg/l of para-tyrosine, while in the case of meta-tyrosine at least 60 mg/l of para-tyrosine concentration was necessary.

As shown in Panel C of Figure 1 relative protein content (EPO/non-EPO) of meta- or ortho-tyrosine treated cultures were significantly lower, than that grown on para-tyrosine.

Tyrosine incorporation into cellular proteins

Treatment of the cells with meta-tyrosine – compared to the culture containing para-tyrosine – decreased the para-tyrosine content of cellular proteins in non-EPO (control) experiments. No significant difference was detected in para-tyrosine in case of ortho-tyrosine cultured control cells (Panel A of Figure 2, left part).

Cells grown in medium containing ortho- or meta-tyrosine and EPO showed less para-tyrosine compared to the para-tyrosine supplemented cells (Panel A of Figure 2, right part).

Panel B of Figure 2 demonstrates, that incorporation of ortho-tyrosine was higher into the proteins of ortho-tyrosine cultured erythroblasts compared to the cells cultured with meta- or para-tyrosine, in case of non-EPO (control, left part of figure) and EPO (right part of figure) cells, as well. Moreover, ortho-tyrosine content was significantly higher in EPO-cultured cells than that of non-EPO-treated (control) cells, when both were grown in ortho-tyrosine supplemented medium (comparing left to right part of figure).

Meta-tyrosine incorporation was significantly higher in meta-tyrosine cultured erythroblasts both in non-EPO (control) and in EPO groups compared to cells cultured in para- or ortho-tyrosine supplemented medium (Panel C of Figure 2, left and right part).

Analyses of STAT5 and ERK activation

In our Western blot experiments treatment with ortho- and meta-tyrosine prevented the increase of STAT5-phosphorylation induced by EPO in para-tyrosine cultured cells (Panel A and B of Figure 3). The same inhibition by ortho- and meta-tyrosine was seen in case of phosphorylation of ERK 1 and 2 (Panel C-E of Figure 3).

Fig. 3. Representative immunoblots (Panel A) and densitometric analyses of phosphorylation of STAT5 (Panel B; n=3); immunoblots of ERK1 and 2 (Panel C) and densitometric analysis of ERK1 (Panel D; n=4) and ERK 2 (Panel E; n=4). Data are expressed as percent of untreated (control) cells. *, $P < 0.05$; EPO vs control (one-sample T-test); †, $P < 0.05$ vs para EPO (one-way ANOVA).

Discussion

In this study, we proved that ortho- and meta-tyrosine incorporate into cellular proteins in a detectable, but – compared to the total cellular protein-bound tyrosine – very low ratio (0.10-0.15%), with a consequent decrease in activation of antiapoptotic and mitogenic signaling pathways. Inadequate phosphorylation of ERK and STAT5 results in deficient proliferative response to EPO, leading to EPO-hyporesponsiveness of erythroid progenitor cells (Figure 4).

Oxidative stress is proven to play a major role in the pathogenesis of several morbidities, such as diabetic nephropathy, atherosclerosis or ischemic heart disease [20-22]. Higher plasma level or urinary excretion of ortho- and meta-tyrosine, as hydroxylated phenylalanine derivatives, is associated with altered oxidative state, thus they serve as markers of oxidative stress. On the other hand, *Ruggiero et al.* proved, that ortho- and meta-tyrosine decreases tumor proliferation due to the inhibition of ERK and STAT3 activation. They used meta- and ortho-tyrosine concentrations up to 827 $\mu\text{mol/l}$. Similarly, we detected decreased ERK and STAT5 phosphorylation in the presence of ortho- and meta-tyrosine.

In agreement with data of Kitamura et al. [12], also in our experiments culturing cells with EPO for 3 days resulted in an approximate 1.7 fold increase in cell counts. Addition of meta- or ortho-tyrosine into the culture medium caused a markedly decreased proliferative activity, leading to lower cell counts. Since, according to the source, duplication time of this cell line is about 22 hour, differences could be already observed even after 2 days of culturing (Panel A of Figure 1).

In the study of *Ruggiero et al.*, inhibition by meta-tyrosine could be reversed with excessive phenylalanine, glutamine, aspartic acid, histidine and glutamate, but not with para-tyrosine itself. On the contrary, we could break through the EPO-resistance by para-tyrosine in a dose dependent manner (Panel B of Figure 1). These results may suggest that para-tyrosine competitively inhibits the integration of ortho- and meta-tyrosine into cellular proteins. In case of ortho-tyrosine treatments, 1:1.8 ortho- to para-tyrosine ratio was necessary to regain 50 % of EPO-sensitivity. In case of meta-tyrosine, the ratio was 1:2.6.

RPMI-1640 cell culture medium contains para-tyrosine at twice as high concentration than the normal human serum does. Consequently, the same concentration had to be used in case of ortho- and meta-tyrosine, in order to block EPO-response. We performed a model experiment, demonstrating a total block of the effect of EPO, therefore high doses of tyrosine isomers were applied. Bertin et al used similar concentrations of meta-tyrosine (20-260 $\mu\text{mol/l}$) to study its effect on plant root growth [23]. Furthermore, Rodgers et al. examined the role of meta-tyrosine incorporation into cellular proteins and their role in the protein degradation. In their experiments, concentrations of meta-tyrosine reached up to 2000 $\mu\text{mol/l}$ [24].

As EPO-treatment is widely used among patients suffering in renal anaemia, EPO-hyporesponsiveness is a common concern. The Baltimore Longitudinal Study on Aging investigated healthy, non-anemic persons and found that EPO levels rise with age [25]. *Vanesse and Berliner* suggested shorter erythrocyte life span as a possible explanation [26]. On the other hand, Dai et al. hypothesized, that aging results in impairment of the mitochondrial electron transport chain and through the leakage of electrons, leads to increased production of ROS [27]. Their findings indicate – similarly to our observations – that aging related ROS overproduction may play a role in the decrease of EPO-sensitivity.

Furthermore, we confirmed that besides its well-known role in numerous cellular signal transduction processes [28], hydroxyl free radical induced oxidative stress may be involved in hormone resistances via incorporation of ortho- and meta-tyrosine into the proteins.

In addition, *McCullogh et al.* observed increased risk for cardiovascular events related to the use of higher ESA-dose, independent of the hemoglobin level achieved [29]. Although higher efficient EPO-dose results in more prominent unwanted side effects, we hypothesize, that this is an epiphenomenon, and higher cardiovascular risk is not triggered by higher plasma EPO-level itself, but rather by the incorporation of the ortho- and meta-tyrosine into the cellular proteins leading to abnormal cellular functions. These findings show similarities to the role of insulin resistance in mortality instead of role of the necessarily high insulin dosage [30].

Fig. 4. Flowchart of the scheme of the possible subcellular process, how ortho- and meta-tyrosine incorporation leads to the dysfunction of signal pathway mechanisms, resulting in the hyporesponsiveness of erythroid progenitor cells.

Conclusion

Our findings point out that ortho- and meta-tyrosine may be responsible for EPO-resistance. Furthermore, inhibiting their cellular integration with the physiological isomer – para-tyrosine – the resistance can be broken through, preserving the adequate mitogenic activity. Nevertheless, further in vitro and animal experiments – and in case of their positivity – human examinations are necessary to determine the role of para-tyrosine treatment in the management of EPO-resistance.

Disclosure Statement

No competing financial interests exist.

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