# EFFECTS OF ERYTHROPOIETIN ON GLUCOSE METABOLISM AND THE ERYTHROPOIETIN RESISTANCE

Ph.D. theses summary

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# 1. Introduction

The glycoprotein hormone erythropoietin (EPO) was first identified as the key regulator of red blood cell differentiation and maturation. EPO is synthesized by the kidney peritubular interstitial cells; typically, the lack of EPO production associated with chronic kidney disease results in hypoproliferative anemia. While much of our understanding of the role of EPO is related to promoting erythropoiesis, accumulating evidence demonstrates that EPO is more ubiquitous possessing non-erythroid biological actions, thus affecting different cellular functions in multiple cell systems. EPO-receptor (EPO-R) is a member of the cytokine-receptor superfamily involving the activation of downstream effectors via PKB/Akt, p44/42 ERK, and JAK/STAT pathways. Expression of EPO-R has been demonstrated in numerous cell types in the nervous system e.g., brain capillary endothelial cells, hippocampal and cortical neurons, human neurons, astrocytes, and microglia as well as in the pancreatic islets [1][2][3][4][5]. It has been reported that EPO inhibited apoptosis in breast cancer cells via the PKB/Akt pathway [6]. Administration of EPO protected neurons against cell death and promoted post-stroke recovery in experimental models of stroke and cerebral ischemia. It has been shown that EPO reduced the accumulation of lipids in foam cells and suppressed the progression of atherosclerosis [7]. Hojman et al. showed that EPO led to an increased fat oxidation in striated muscles and prevented diet-induced obesity [8].

Based on unpublished data, we found that introduction of r-hu-EPO therapy markedly decreased fructosamine levels in two patients with type 2 diabetes mellitus within the 2-4 months after post-injection, indicative of better glycemic-status [fructosamine (µmol/l) 372 to 321 and 464 to 428]. We concluded, that introduction of EPO-therapy ameliorates glycaemic state. Although consequences of sustained EPO treatment are well described, to date, results have been lacking on the acute glucose lowering effect of EPO. The major aim of present study was to investigate the acute changes in blood glucose levels after EPO administration in vivo. Moreover, we purposed to determine in adipocytes the effect of EPO on cellular glucose uptake and intracellular signaling events under normal and high glucose conditions, as well as the effect of EPO on glucose transporter 4 (GLUT4) trafficking to the plasma membrane using 3T3-L1 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 (r-hu-EPO) receiving subjects are hyporesponsive [9]. Several possible pathomechanisms are discussed, such as iron deficiency, use of angiotensin converting enzyme inhibitors, uremic toxins, insufficient dialysis, hyperparathyroidism or malignancy.

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. 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 [10][11]. Tyrosine is a non-essential amino-acid, physiologically formed from phenylalanin by enzimatic hydroxylation. 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*. According to our present knowledge, beside the acute, cytotoxic effect of the two tyrosine isomers, a long-term effect is possible. Specifically, the incorporation of ortho- and meta-tyrosine into cellular proteins can lead to the disturbance of cellular signaling.

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.

# 2. Aims

2.1. We aimed to perform *in vivo* and *in vitro* experiments to study the effects of EPO on glucose metabolism

- To prove that EPO has acute glocose lowering effect (*in vivo*)
- To investigate the EPO-induced alterations in signal transduction and consequent glucose uptake of adipose tissue
- To identify the EPO-R in 3T3-L1 cell line

2.2. We aimed to investigate the possible role of ortho- and meta-tyrosine in the development of EPO-resistance.

- To determine the *in vitro* effect of tyrosine isomers on EPO-induced erythroblast cell proliferation
- To prove the incorporation of tyrosine isomers into cellular proteins with high performance liquid chromatography (HPLC)
- To clarify the intracellular signal pathway alteration with Western blot method

# 3. Materials and Methods

## 3.1. Effects of EPO on glucose metabolism

**Interstitial glucose monitoring:** CGMS (Continuous Glucose Monitoring System) was used in our human investigations. The monitor records the interstitial glucose levels 24 hours a day. CGMS is well tolerated and routinely used for a better glucose control.

**Animal experiments:** Animal experiments were approved by the local Hungarian Animal Experiment Committee at the University of Pécs. Male, 3-5 months-old Sprague-Dawley CFY rats were employed weighing at 300 ± 30 gram. Animals were randomized to receive either a single intraperitoneal streptozotocin (STZ, 60 mg/kg body wt; vehicle: 0.05 mol/l citrate buffer, pH 4.0) injection to induce diabetes mellitus [Diabetic, n=5] or to be healthy controls [Control, n=5]. Post-injection glucose levels were randomly measured in whole blood from the tail vein using Accu-Check Active glucometer. Diabetes was defined as hyperglycemia reached > 13 mmol/l within two consecutive days. Animals were then closely monitored for further 2-3 weeks and insulin (NPH, 2.0 IU/kg body wt) injections were given to avoid ketoacidosis, which were stopped 48 hours prior to r-hu-EPO treatments. Healthy and diabetic rats were fasted overnight, sedated with chloral hydrate (10 mg/kg body wt, per os) and then r-hu-EPO (epoetin-beta; 35, 50 and 100 IU) or vehicle control (physiological saline; 0.2, 0.5 and 1.0 ml) was intravenously administered 15 min apart through a tail vein canule. Blood samples were taken from the tail tip for glucose measurements at baseline and 5 min intervals for 90 min. Additionally, we also monitored interstitial glucose levels with glucose-oxidase sensor of an inactive insulin pump in selected diabetic animals (n=2) via a sensor placed into the interscapular subcutaneous space.

**Cell culture and treatments:** Cell line of 3T3-L1 adipocytes was used, when majority of cells (> 90%) exhibited adipocyte phenotype showing accumulated lipid droplets. Briefly, pre-adipocytes were grown for 48 hours in DMEM (Dulbecco's Modified Eagle Medium) containing 10% calf serum and a mixture of penicillin-streptomycin. Differentiation was induced by isobuthylmethylxantine (0.5 mM), dexamethasone (0.25 μM) and insulin (1.0 μg/ml) in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotic mixture for 48 hours. Cells were then cultured in DMEM containing 10% FBS, antibiotic mixture and 1.0 μg/ml insulin for the next 6 days. Indicative of normal and high glucose levels, cells were

cultured in glucose-containing DMEM throughout at either 5 mmol/l or 25 mmol/l concentration using 60 mm collagen-coated plates. Confluent monolayers (~90-100%) of adipocytes were serum-deprived for 12 hours prior to experiments and were subsequently used as described below. Treatments (5 min for phosphorylation studies; 30 min for GLUT4 trafficking measurements) involved the addition of 2, 20, 200 or 400 nmol/l insulin, as well as increasing concentrations of r-mo-EPO (2.5, 5, 10, 20, and 40 ng/ml). The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (50 μmol/l, for 30 min) was used for blocking Akt activation.

**Glucose uptake:** Adipocytes were incubated in glucose-free medium for 30 min. Briefly, it was started by addition of 1.0 μCi/ml of Deoxy-D-[$^3$H]Glucose in 2 ml of glucose-free medium in the absence or presence of treatments with increasing concentrations of insulin and r-mo-EPO with or without incubation with the JAK2 inhibitor AG490 (10 μmol/l; for 6 hours), as indicated. Uptake was stopped after 100 min, when we have previously found the highest kinetics of glucose uptake in this model. After being scraped off into the medium, cells were collected by centrifugation and then were lyzed (30 min) in 70 μL of Tris-Triton extraction buffer (see in western blot section) before aliquots (30 μL) were taken for scintillation counting and protein measurement using a Bio-Rad kit and spectrophotometer. Glucose uptake thus is expressed as the ratio of an average radioactivity during 5 min of measurements and protein concentration [counts per minute (CPM)/μg) protein].

**Immunoblot analysis**: Following insulin or r-mo-EPO treatments, cells were lyzed in Tris-Triton extraction buffer on ice for 30 min. Lyzed proteins were harvested, then protein concentration of the supernatant was assessed using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard. Solubilized proteins were mixed with Laemmli buffer (2X), denaturated, then were separated on 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane. Equal protein loading (75-100 μg) was confirmed 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) against phospho-Ser(473) Akt and total Akt, phospho-Thr(202)/Tyr(204) ERK and total ERK (p44/42 MAPK) for overnight at 4$^o$C. After being washed, the membranes were incubated with appropriate, horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000; anti-rabbit IgG) for 60 min at room temperature. After further washing, immunoblots were visualized with enhanced chemiluminescence and developed on X-ray

films. Total protein levels of Akt and ERK were immunostained after stripping the blots. Densitometric analyses were performed using Scion Image software.

For the detection of EPO-R lysates of untreated 3T3-L1 cells and K-562 cells serving as positive control were used. Proteins were separated, immunoblotted and probed with anti-EPO-R antibody (1:200) overnight at 4 $^{o}$C. After incubation with appropriate secondary antibody (1:2,000, anti-rabbit IgG) for 60 min at room temperature, EPO-R was visualized as described above.

**Plasma membrane fractination for GLUT4:** Plates were biotinylated as described earlier. After lysis cells were incubated with Pierce Streptavidin Magnetic Beads overnight at 4 $^{o}$C. Biotinylated proteins were pulled down, washed three times with Tris-Triton extraction buffer and then were resolved by 7,5% SDS-PAGE for blotting followed by immunostaining with primary anti-GLUT4 antibody (1:1,000; overnight at 4$^{o}$C) and secondary antibody (1:2,000; anti-rabbit IgG) for the visualization described above.

**Immunofluorescence of GLUT4:** After tryptic digestion, cultured adipocytes were plated onto poly-L-lysine-coated cover slips (20,000 cells/slip), then were incubated in DMEM containing 0.5% FBS for 24 hours. Following 60 min of serum deprivation, the cell monolayers were subjected to insulin and r-mo-EPO treatments for 30 min. At the end of treatment, cells were fixed with 4% para-formaldehyde for 60 min at room temperature. After a blocking step with high-saline phosphate buffer (PBS; 23.38 g NaCl additionally) containing 10 (wt/vol)% BSA and 0.1 % Triton-X 100, the permeabilized cells were exposed to the GLUT4 primary antibody at 1:1.000 in 3 (wt/vol)% BSA/PBS for overnight at 4$^{o}$C. After washing with high-saline PBS, cells were incubated with Cy3-conjugated anti-rabbit IgG (Jackson Immuno Research Europe, Suffolk, UK) at 1:4.000 in 3 (wt/vol)% BSA/PBS for overnight at 4$^{o}$C. After further washing, cells were stained with 0.5 mg/ml of Hoechst 33342 (Calbiochem, Gibbstown, NJ, US). Cover slips were mounted (Slow Fade, Invitrogen, Carlsbad, CA, USA) and cells visualized using a laser confocal microscope (Olympus FV-100, photon count mode).

**Statistical analysis:** Data are expressed as means ± SE. Statistical analyses were performed using one-sample t-test, paired- and unpaired t-test or Mann-Whitney U-test for data as appropriate using SPSS 17.0. Statistically significant differences were defined as $P < 0.05$ and are indicated in the figure legends.

## 3.2. In vitro study of EPO-resistance

**Cell culture and treatments:** TF-1 (CRL-2003) erythroblasts were used. 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 stabile; no considerable alteration of para-, ortho- or meta-tyrosine concentration could be detected in the absence of cells, during 3 days.

For proliferation studies 3 IU/ml rh-EPO was added. 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 was planted onto 60 mm plates in culture medium containing additional 20 mg/ml para-, ortho-, or meta-tyrosine, lacking GM-CSF, with or without addition of 3 IU/ml rh-EPO for 3 days. Cell concentration (cells/µl) 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 $^{o}$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/ml 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, then cells were washed three times by addition of 1 ml physiological NaCl-solution and centrifugation. *Para*-, *ortho*- and *meta*-tyrosine levels were determined using reverse phase-HPLC ($C_{18}$ silica column, 250x4 mm) with fluorescence detection ($\lambda_{EX}$=275 nm; $\lambda_{EM}$=305 nm) as described earlier. 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** was performed as previously described. Specifc antibodies were used against phospho-Thr(202)/Tyr(204) ERK and total ERK (p44/42 MAPK); phospho-Tyr (694) STAT5 and total STAT5 or ß-actin. Data are expressed as the ratio of phosphorylated and total ERK1/2 or STAT5, corrected to total cellular ß-actin level.

**Statistical analysis:** Data are expressed as means±SE or means±SD, as indicated. Analyses were performed as appropriate using SPSS 17.0. 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. To compare the means between the groups of relative phosphorylations ANOVA with Bonferroni's post-hoc test was used.

# 4. Results

## 4.1. Effects of EPO on glucose metabolism

**EPO-treatment decreases interstitial glucose level based on human studies**

CGMS monitoring was necessary in case of two diabetic patients. Subjects recieved EPO-treatment for renal anaemia. Data were evaluated 2 days before and after EPO-administration (epoetin beta). Postprandial interstitial glucose levels recorded by the monitor were significantly lower after EPO-administration.

**Administration of r-hu-EPO reduced blood glucose levels in a dose-dependent manner *in vivo* in rats.**

The average value of baseline blood glucose levels was 24±10 mmol/l in the diabetic and 5±3 mmol/l in the healthy littermates. Addition of r-hu-EPO significantly reduced the blood glucose levels in rats at all concentrations (i.e. 35, 50, and 100 IU) compared to the vehicle controls (*P<0.05*), with a maximal decrease (1.32 mmol/l) at 100 IU of EPO. The decreases of blood glucose levels showed a clear dose-dependent response (*P<0.05).* There were no significant, dose dependent effect in the reductions of blood glucose of healthy control animals. Adverse effects of EPO injections or hypoglycemia (i.e. blood glucose < 4 mmol/l) were not seen in either of the groups.

**Administration of r-mo-EPO increased the glucose uptake in 3T3-L1 adipocytes *in vitro*.**

In normal glucose (5 mmol/l) cultured adipocytes, insulin treatments significantly increased the rate of Deoxy-D-[$^3$H]Glucose uptake at all concentrations (i.e. 2, 20, 200 and 400 nmol/l) compared to both untreated controls (*P<0.05*) and those cultured with high glucose (25 mmol/l) medium (*P<0.05*). In contrast, the rate of glucose uptake in high glucose cultured adipocytes was unchanged compared to untreated controls at any concentrations, indicating that these cells were insulin resistant. On the contrary, in normal glucose cultured adipocytes, EPO treatments did not alter glucose uptake at any concentrations (i.e. 0.15, 0.3, 0.625, 1.25 and 2.5 ng/ml) compared to untreated controls. While in high glucose cultured adipocytes, as little as 0.15 ng/ml EPO, markedly increased

the rate of [$^3$H]Deoxy-D-Glucose uptake (*P<0.05*), which was further increased (up to 65%) with higher concentrations of EPO compared to untreated controls.

Under high glucose conditions co-treatment with EPO (0.625 ng/ml) and insulin resulted in significantly higher rates of glucose uptake compared to untreated controls at the concentrations of 20; 200 and 400 nmol/l; in addition co-treatment with insulin and EPO resulted in significantly higher rate of glucose uptake at 400 nmol/l compared to insulin treatment alone (P<0.05).

**Acute effects of EPO treatment on the activation of Akt, and ERK pathways in 3T3-L1 adipocytes.**

Immunoblot analyses showed that EPO treatment had no effect on Akt activation in normal glucose cultured cells at any concentrations; however, in high glucose cells EPO considerably increased phospho-Ser(473) Akt levels compared to untreated control cells, with a maximal effect at 10 ng/ml (*P<0.05)*. The levels of phospho-Ser(473) Akt were not increased further with higher concentrations of EPO but remained elevated. We demonstrated that PI3K inhibitor (LY294002) significantly blunted the insulin-, and more importantly, the EPO-induced activation of Akt, indicating that downstream effects of EPO are mediated via the PI3K/Akt-pathway.

Co-treatment with EPO and insulin resulted in greater Akt activation, indicating that the effect of insulin in high-glucose cultured adipocytes can be increased by additional EPO treatment.

Addition of EPO had no effect on either ERK1 or ERK2 activation in normal glucose cultured adipocytes at any concentration compared to untreated control cells; not even at higher concentrations (i.e.>10 ng/ml) of EPO (data not shown). In high glucose cultured cells, in turn, there were significant increases in phospho-ERK1/2 levels at all concentrations compared to untreated control cells (*P<0.05)*.

Here we demonstrated for the first time that EPO-R is indeed expressed in 3T3-L1 adipocytes. We proved the presence of EPO-R even in unstimulated adipocytes, using K-562 cell lysates as the positive control.

**Acute effects of EPO treatment on GLUT4 translocation in 3T3-L1 adipocytes.**

It is well-documented that translocation of GLUT4 is an insulin-dependent and Akt-mediated process in adipocytes. As shown, EPO treatment of adipocytes activated Akt and augmented glucose uptake only in high glucose cultured cells, thus we next determined whether EPO also led to alterations in intracellular GLUT4 localization using immunocytochemistry and immunoblot experiments. We found that translocation of GLUT4 was strikingly evident in high glucose cultured adipocytes after EPO treatment, similarly to that seen with insulin; while it was not present in untreated control cells. While there was hardly detectable amount of GLUT4 in the membrane fraction of the lysates of untreated control cells, intense bands could be detected in the membrane fraction of EPO- or insulin-treated cell lyzates in two independent cases.

**Ortho-tyrosine decreases EPO-induced glucose uptake**

Culturing 3T3-L1 cells in ortho-tyrosine containing medium resulted in the decrease of EPO-induced glucose uptake, while in para-tyrosine cultured cells significant glucose uptake was found.

## 4.2. Incorporation of ortho- and meta-tyrosine leads to EPO-resistance

**Cell proliferation**

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 (*P<0.05)*. Maximal difference between cell counts was observed at day 3 (time curve).

We investigated 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 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.

Relative protein content (EPO/non-EPO) of meta- or ortho-tyrosine treated cultures were significantly lower, than that grown on para-tyrosine (*P<0.05)*.

**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 *(P=0.003)*.

Cells grown in medium containing ortho- or meta-tyrosine and EPO showed less para-tyrosine compared to the para-tyrosine supplemented cells *(P<0.001)*.

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) and EPO cells, as well *(P<0.001)*. 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 *(P<0.001)*.

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. *(P<0.001)*

**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. The same inhibition by ortho- and meta-tyrosine was seen in case of phosphorylation of ERK 1 and 2 *(P<0.05)*.

**Stability of the tyrosine-isomers**

Original RPMI-1640 media were modified by adding 20-20 mg/l (110.4 µmol/l) of meta- or ortho-tyrosine, cells were cultured in the indicated media for 3 days. Original RPMI-1640 medium - according to our measurements – is contaminated with ortho- and meta-tyrosine in a very low level. We detected 0.03 µmol/l of ortho- and meta-tyrosine each on day 0 (0.02% of the total para-tyrosine concentration). No significant change has been observed in ortho- or meta-tyrosine concentration during the 3-day storage.

# 5. Discussion

Since r-hu-EPO became available, a growing body of reports has demonstrated that EPO possesses several biological actions beyond stimulating erythropoiesis, including altered glucose metabolism. Rigalleau et al. found that glycemic status dramatically worsened in patients with chronic anemia when r-hu-EPO therapy was started [12]. On the other hand, we showed that EPO treatment decreased the blood glucose levels in STZ-induced diabetic rats. Although Jen M Ng et al. reported a significant $HbA_{1c}$ level reduction after EPO therapy, while mean blood glucose levels were unaltered [13], we found significant decrease of postpradial glucose levels in human studies with CGMS. Regarding the widespread use of EPO-treatment in renal anaemia of CKD patients, additional effects of EPO – just like effects on glucose metabolism – are highly relevant.

We demonstrated that r-hu-EPO dose-dependently reduced the blood glucose levels in rats compared to control littermates after as short as 5 min, which supports the notion that EPO has an acute glucose lowering effect *in vivo*. This suggests the role of rapid molecular mechanisms in mediating this metabolic effect of EPO. In subsequent *in vitro* studies, using 3T3-L1 adipocytes, we determined whether reduced blood glucose levels seen with EPO treatment could be as a result of increased cellular glucose uptake, and if so, whether the effect of EPO was mediated via those intracellular signaling pathways that are related to the actions of insulin (i.e. PKB/Akt and ERK1/2 branches) or the JAK/STAT pathway.

Given that our glucose uptake studies showed Akt-dependent increases with EPO, we also examined whether EPO could promote the shift of GLUT4 to the cell membrane in 3T3-L1 adipocytes as well. We showed that intense GLUT4 translocation as well as increased plasma membrane-bound GLUT4 levels occurred in EPO-treated 3T3-L1 cells, which were not present in untreated, high glucose cultured controls. Thus, the EPO-induced increases in glucose uptake could be, at least in part, a result of increased GLUT4 trafficking toward the plasma membrane in 3T3-L1 cells grown in high glucose.

We found that EPO treatment of 3T3-L1 cells markedly increased the Deoxy-D-[$^3$H]Glucose uptake rates under high, but not normal glucose conditions, which were opposite to changes seen with insulin treatment. Moreover, in 3T3-L1 cells grown on high glucose - where insulin alone was ineffective - the addition of EPO enhanced more the glucose uptake rates. With the presence of a specific JAK2-inhibitor (AG490) the glucose-uptake of adipocytes remained uneffected. Our findings proved, that the metabolic effect of EPO is not only mediated by the JAK/STAT pathway, but other subcellular processes can be involved.

On the other hand, the presence of PI3K-inhibitor LY294002 blocked the Akt-phosphorylation, suggesting that insulin-like processes can be responsible. Here we showed that EPO treatment stimulated both Akt and ERK1/2 activation, the two major branches of the insulin/IRS pathway in 3T3-L1 cells under high, but not normal glucose conditions. Moreover, the addition of EPO potentiated the effect of insulin on Akt phosphorylation under high glucose conditions.

Since the majority of diabetic kidney patients receive EPO therapy, and the fact that EPO afforded greater response on glucose uptake under high glucose than insulin alone, it could have clinical implications representing a promising strategy to reduce hyperglycemia. Further studies are warranted to examine other metabolic effects of EPO and for developing analogues as potential anti-diabetic agents with less adverse effects.

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 [14]. Vanesse and Berliner suggested shorter erythrocyte life span as a possible explanation [15]. 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 reactive oxygen species (ROS) [16]. Their findings indicate – similarly to our observations – that aging related ROS overproduction may play a role in the decrease of EPO-sensitivity. 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 [17]. 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 [18].

Our observations altered the way of our thinking about the etiology of EPO-hyporesponsiveness. Drug side affects, iron- or vitamin $B_{12}$- deficiency or concomittant diseases cause decreased response to EPO-therapy, but not the real resistance. In these cases EPO-hyporesponsiveness can be succesfully treated by the elimination of the responsible factor. We consider real EPO-resistance as a complex subcellular pathomechanism, where the signal transduction is directly blocked by subclinical inflammation or oxidative stress.

Para-tyrosine levels of CKD patients are proved to be decreased, compared to healthy controls, in a previous study of our workgroup [19]. On the other hand, ortho-tyrosine levels were significantly higher in CKD group compared to the healthy subjects. Ortho-tyrosine plasma level was even higher in patients suffering in CKD and diabetes mellitus either [20]. Furthermore, our workgroup found that ortho- and meta-tyrosine accumulates in crystalline lenses with cataract [21].

Although the two non-physiological isomers of phenylalanine – ortho- and meta-tyrosine – were considered as biomarker molecules of oxidative stress, 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. Beyond creating a model of EPO-hyporesponsiveness, our long term goal is to work out a possible future treatment with para-tyrosine supplementation, that is the basis of a European/US patent application (reference number: 01319EP-13773E/). 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.

# 6. Thesis

## 6.1. The effect of EPO on carbohydrate metabolism

- EPO has an *in vivo* acute blood glucose lowering effect
- EPO induced the trafficking of GLUT4 into the plasma membrane, increasing the glucose uptake of adipocytes
- EPO causes the increase of ERK- and Akt- phosphorylation
- Co-administration of EPO and insulin results in higher phosphorylation of Akt and [$^3$H]Deoxy-D-Glucose uptake in adipocytes than insulin treatment alone
- Ortho-tyrosine inhibits EPO-induced glucose uptake of adipocytes *in vitro*

## 6.2. Incorporation of ortho- and meta-tyrosine into cellular proteins leads to erythropoietin-resistance in an erythroid cell line

- Ortho- and meta-tyrosine treatment results in time-dependent decrease of erythropoietin-induced proliferative activity in TF-1 erythroblasts; response could be regained with a competitive dose of para-tyrosine
- Proteins of erythroblasts treated with ortho- or meta-tyrosine had lower para-tyrosine and higher ortho- or meta-tyrosine content
- EPO-induced activation of ERK and STAT5 was practically prevented by ortho- or meta-tyrosine treatment

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