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Abstracts

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1
Mechanism of Action of Erythropoiesis Stimulating Proteins (ESPs)

S. Elliott, B. Agoram, S. Doshi, G. Molineux. Amgen Inc, Thousand Oaks CA, USA

Erythropoietin (Epo) is the primary regulator of red blood cell (RBC) formation. When Epo levels decline below normal (typically 10–20 mU/ml) reduced formation of RBCs (and Hb) leads to anemia. Recombinant human Epo (rHuEpo) and other erythropoiesis stimulating proteins (ESPs) have the same mechanism of action, they activate Epo receptors (EpoR), though they can have differences in serum half-life and/or receptor affinity. Aranesp (darbepoetin alfa) for example, has 2 additional N-linked carbohydrate chains and a 3-fold longer half-life compared to rHuEpo. AMG114 has 2 additional carbohydrate chains and 2-fold longer half-life compared to Aranesp. Polyethylene glycol-linked Aranesp (Peg-Aranesp) has similar half-life compared to AMG114. Aranesp, AMG114 and Peg-Aranesp all had reduced EpoR affinities but increased in vivo activity compared to rHuEpo due to their longer half-lives and consequently extended time above a minimal effective concentration. Thus altered EpoR affinity was not predictive of in vivo activity. Furthermore, an Epo analog unable to bind EpoR had clearance that was similar to rHuEpo. The terminal half-life was extended somewhat (1.5–2-fold) compared to rHuEpo in rodents, however the extension was considerably less than observed for Aranesp (3-fold) and AMG114 or Peg-Aranesp (6-fold). The decreased receptor binding of Aranesp, AMG 114 and Peg-Aranesp taken together with half-life results of the EpoR analog, suggest that their longer half-lives are primarily the result of decreased susceptibility to receptor-independent elimination mechanisms.

2
Novel strategies for stimulating erythropoiesis, as potential new treatments for anaemia

Iain C Macdougall, Department of Renal Medicine, Kings College Hospital, London, UK

Recombinant human erythropoietin (epoetin) is an established therapeutic agent for the treatment of anaemia. A second-generation erythropoiesis stimulating agent (ESA) with a longer half-life (darbepoetin alfa) was introduced several years ago. Several new products are in clinical development.

CERA (Continuous Erythropoietin Receptor Activator) contains a large polymer chain which results in a long-living molecule of twice the molecular weight of epoetin (at around 60 kDa). Phase II studies have demonstrated that CERA administered SC or IV allows stable haemoglobin control with dosing intervals of up to once-monthly in CKD patients.

Hematide is a modified erythropoietin-mimetic peptide, which is also investigated in once-every-four-weeks dosing schedules. The main advantage of Hematide is that it is not a biologic, and can thus be synthesised by techniques not requiring a cell line. Furthermore, it may be possible to "rescue" patients who have developed antibody-mediated PRCA with another ESA, since the antibodies do not cross-react with Hematide.

HIF stabilisers are in Phase II studies, orally active, being administered three times weekly. By preventing HIF degradation, these compounds upregulate erythropoietin gene expression, even in CKD patients, although there may be upregulation of other HIF-sensitive genes. Some of these may be beneficial, e.g. genes associated with hepcidin, while others such as VEGF may be a concern. Thus, the selectivity and the safety of the HIF stabilisers require further elucidation.

3
What caused Eprex associated PRCA?

H. Schellekens, Utrecht University, Utrecht, The Netherlands

The peak of erythropoietin-associated pure red cell aplasia (PRCA) incidents occurred over fours years ago. The association with the recombinant human erythropoietin (epoetin, rhEPO) alpha branded Eprex®, makes PRCA of interest to medical and scientific communities as well as the biotechnology industry. The mechanism by which products like epoetin induce antibodies is based on breaking immune tolerance existing normally to self-antigens. An important way to break tolerance is to present the self-antigens in a repetitive way. A periodicity of these antigens as present in aggregates of proteins is apparently very efficient in activating ignorant or anergetic B cells that are responsible for tolerance. The most common biological effect is the loss of efficacy. The most dramatic effect of antibodies occurs if a natural protein with an essential biological activity is neutralised. Such a consequence has been described for Megakaryocyte Derived Growth Factor (MDGF). This thrombopoietin like protein induced antibodies neutralising endogenous TPO leading to severe thrombocytopenia in volunteers and cancer patients. This effect is comparable with the EPO-associated PRCA. The upsurge of PRCA is associated with a formulation change introduced in 1998 when human serum albumin (HSA) as protein stabiliser was exchanged with polysorbate 80. Several explanations have been offered to explain how this change led to Eprex associated PRCA. Leachates from uncoated rubber stoppers acting as adjuvant are blamed by the manufacturer of Eprex®, but the experimental data substantiating this claim

are poor. The only explanation which is consistent with all data is a higher tendency for aggregate formation due to the exchange of HSA by polysorbate 80 as stabiliser.

4

An optimized supravalent EPO mimetic peptide with unprecedented efficacy

A Pötgens, U Haberl, A Rybka, K Knorr, M Emgenbroich, M Casaretto, B Breuer, A Greindl, C Kessler, A Kromminga*, H-G Frank. Aplagen GmbH, Baesweiler, Germany, and *IPM, Hamburg, Germany

Recombinant human erythropoietin (recEPO) therapy is partly hampered by the short in vivo half-life of this drug. In addition, some cases of EPO induced PRCA after long-term treatment with recEPO have been described. EPO mimetic synthetic peptides may circumvent some of the disadvantages of recEPO. Having optimized a 20 residue peptide sequence for its efficacy to stimulate growth of human cell line TF1, we produced a bivalent version of this peptide called AGEM40, being 100-fold more efficient than the monovalent peptide in the TF1 assay. By conjugating AGEM40 to a macromolecule (MM) a highly efficient supravalent peptide was produced. The EC50 of MM conjugated AGEM40 was around 100 pM in in vitro assays, with EC50 values of recEPO being 20 pM. We expect that MM conjugation of AGEM40 will also improve its biological half-life. AGEM40 and its MM conjugates also efficiently stimulated development of erythroid colonies from human bone marrow cells in methylcellulose. Subcutaneous application of MM conjugated AGEM40 into normocytic mice resulted in increased numbers of reticulocytes. No immunological cross-reactivity was observed between AGEM40 and rabbit and human anti-EPO antisera by radio-immunoprecipitation, ELISA, and Western blotting. This immunogenic divergence predisposes AGEM40 and its conjugates for the treatment of PRCA.

5

Cellular Trafficking and Degradation of Erythropoietin and Novel Erythropoiesis Stimulating Protein (NESP)

Alec W. Gross and Harvey F. Lodish. Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA

How Erythropoietin (Epo) is degraded and cleared from the body is not understood. Glycosylation of Epo is required for its in vivo bioactivity, though not for in vitro receptor binding or stimulation of Epo-dependent cell lines. Interestingly, a hyperglycosylated analog of Epo, called novel erythropoiesis stimulating protein (NESP), has a lower affinity than Epo for the Epo receptor (EpoR), but has greater in vivo activity and a longer serum half-life than Epo. We hypothesize that a major mechanism for degradation of Epo in the body occurs in cells expressing the Epo receptor, through receptor mediated endocytosis of Epo followed by degradation in lysosomes, and therefore investigated the trafficking and degradation of Epo and NESP by EpoR-expressing cells. We show that Epo and NESP are degraded only by cultured cells that express the EpoR, and their receptor binding, dissociation, and trafficking properties determine their rates of intracellular degradation. Epo binds surface EpoR faster than NESP (k_{on}=5.0×10^8 M^{-1}min^{-1} vs. 1.1×10^8 M^{-1}min^{-1}) but dissociates slower (k_{off}=0.029 min^{-1} vs. 0.042 min^{-1}). Surface-bound Epo and NESP are internalized at the same rate (k_{in}=0.06 min^{-1}), and after internalization 60% of each ligand is re-secreted intact and 40% degraded. Our kinetic model of Epo and NESP receptor binding, intracellular trafficking, and degradation explains why Epo is degraded faster than NESP at the cellular level.

6

Erythropoietin treatment elevates haemoglobin concentration by increasing red cell volume and depressing plasma volume.

Carsten Lundby[1,2], Jonas Juhl Thomsen[1], José AL Calbet[1,3], Paul Robach[4]. [1]Copenhagen Muscle Research Center, Rigshospitalet section 7652, 2100 - Copenhagen, Denmark, [2]Department of Sport Science, University of Århus, 8200 - Århus, Denmark, [3]Department of Physical Education, University of Las Palmas de Gran Canaria, Spain, and [4]Ecole Nationale de Ski et d'Alpinisme, 74401 - Chamonix, France.

Erythropoietin (Epo) has been suggested to affect plasma volume. This was tested in 8 healthy subjects receiving 5000 IU recombinant human Epo for 14 weeks at a frequency aimed to increase and maintain haematocrit at, or close to, 50%. Red blood cell volume was increased from 2933±402 ml before rHuEpo treatment to 3210±356, 3117±554, and 3172±561 ml after 5, 11, and 13 weeks, respectively. This was accompanied by a decrease in plasma volume from 3645±538 ml before rHuEpo treatment to 3267±333, 3119±499, and 3323±521 ml after 5, 11, and 13 weeks, respectively. Concomitantly, plasma renin activity and aldosterone concentration were reduced. This maintained blood volume relatively unchanged. We conclude that Epo treatment in healthy humans induces an elevation in Hb concentration by two mechanisms i) an increase in red cell volume and ii) a decrease in plasma volume which is likely mediated by a downregulation of the renin-angiotensin-aldosterone axis.

7

Blood volume measurement in awake volunteer athletes under EPO stimulation

A. Krafft[1], M. Jacob[2] and C. Breymann [1]; *[1]Obstetric Research Unit, Dept. of Obstetrics and Gynaecology, University Hospital Zurich; [2]Dept. of Anaesthesiology, Grosshadern University Hospital Munich*

Purpose: To provide a valid and simple method for blood volume measurement in awake patients.
Methods: 4 groups (10 each) of healthy volunteer athletes (amateur). 10 injections over 4 weeks (group 1 Iron sucrose 100 mg + placebo; group 2 Iron sucrose 100 mg + 2,500 E rhEPO; group 3 Iron sucrose 100 mg + 5,000 E rhEPO; group 4 Iron sucrose 100 mg + 10,000 E rhEPO). Blood volume measurement and physical performance test (Insitute of Sports Physiology, University Zurich) before and after treatment.
Results: So far 16 athletes were treated (4 in each group). Athletes treated with rhEPO only remained constant with red blood cell and plasma volume over the study period. If treated with different dosing of rhEPO red blood cell volume (corrected for body surface area) increased (group 2 from 1229±140 ml to 1356±103 ml ; group 3 from 1165±89 ml to 1325±108 ml; group 4 from 1147± 149 ml to 1507±285 ml; data given as mean±standard error). Plasma volume showed the following changes: group 2 from 2048±43 ml to 1880±279 ml; group 3 from 1921±276 ml to 1750±167 ml; group 4 from 1782± 221 ml to 1681±194 ml (means±standard error).
Conclusions: This ongoing study shows a trend that rhEPO application leads to decreased plasma volume and increased red cell volume. We have set up a valid method for blood volume measurement in awake volunteers/ patients. Plasma volume measurement might be a useful tool in obstetrics to distinguish patients at risk for pre-eclamsia (associated with low plasma volume).

8

Enhancement of endurance performance by hypoxia inducible gene manipulation

Shigehiko Imagawa, Doctoral Program of Sports Medicine, GSCHS, University of Tsukuba, Tsukuba, Ibaraki, JAPAN

Hypoxia resulting from training at high altitude stabilizes HIF-1α and induces it to bind to HIF-1β to form a heterodimer that then binds to the hypoxia-responsive element of the Epo 3′-enhancer. This event stimulates expression of Epo protein in the peritubular capillary interstitial cells of the kidney, which increases Hb production. Increased concentrations of Hb increase the amount of oxygen transported to the muscles, where it is transferred to myoglobin. Elevated arterial oxygen content increases maximal oxygen uptake in well-trained athletes. In oxygenated cells, HIF-1α subunits are rapidly destroyed by a mechanism that involves ubiquitination by the pVHL E3 ligase complex, using 2-oxoglutarate as substrate. In this situation, the interaction between human pVHL and a specific domain of the HIF-1α subunit is regulated through hydroxylation of proline residues 402 and 564 by HIF-PHD. Therefore, inhibitors of HIF-PHD stimulate Epo gene expression even under normoxic conditions. On the other hand, Epo gene is negatively regulated by GATA. We have previously reported that K-11706 (a GATA inhibitor and HIF-1 activator) increases Epo and Hb production, and enhances the endurance performance of mice. FibroGen has recently developed FG-2216 (a HIF-PHD inhibitor), which increases Epo and red blood cell production in humans. In this presentation, the mechanisms of enhancement of endurance performance by hypoxia inducible gene manipulation, such as K-11706 or FG-2216 will be discussed.

9

The Regulation of HIF-α-Hydroxylases

Eric Metzen, Institute of Physiology, University of Luebeck, Luebeck, Germany

The transcription factor hypoxia-inducible factor 1 (HIF-1) is central to the cellular adaptation of gene expression in a situation of reduced oxygen availability. HIF-1 promotes the transcription of an array of target genes, most prominent of which are erythropoietin (EPO) and the vascular endothelial growth factor (VEGF). HIF-1 is a heterodimer composed of an oxygen-sensitve α-subunit and a constitutive β-subunit. HIF-1α is constantly produced but almost immediately undergoes degradation in normoxia. As HIF-1α is stable in hypoxia it confers activity of HIF-1 selectively in hypoxia. Normoxic inactivation is achieved by oxygen-dependent enzymatic hydroxylation of two distinct proline residues in the oxygen-dependent degradation domain and one asparagine in the carboxy-terminus of the protein. Hydroxylated HIF-1α binds to the von Hippel Lindau protein which leads to polyubiquitination and rapid proteasomal destruction. Three prolyl hydroxylases (PHD1, PHD2, and PHD3) and one asparaginyl hydroxylase (FIH-1) have been identified. The hydroxylases have been characterized as 2-oxoglutarate dependent dioxygenases which contain ferrous iron in a non-heme configuration. Lack of iron and of ascorbate can inactivate the enzymes and thus lead to atypical, normoxic activation of HIF-1. Recent studies have provided evidence that metabolites produced in the citric acid cycle can compete with 2-oxoglutarate and thus contribute to tuning the cellular response to hypoxia.

10

Regulated oxygen sensing by PHDs

Daniel P. Stiehl, Renato Wirthner, Sandra Barth, Gieri Camenisch, Roland H. Wenger; Institute of Physiology, University of Zürich, Switzerland.

The prolyl-4-hydroxylase domain enzymes PHD1, PHD2 and PHD3 function as molecular oxygen sensors, regulating hypoxia-inducible factor (HIF)α subunit degradation by oxygen-dependent proline hydroxylation. Due to their complex reaction mechanism - involving the substrate protein, oxygen, 2-oxoglutarate, ferrous iron and vitamin C - the PHD oxygen sensors can also be regulated by changes in transition metal concentrations, Krebs cycle intermediates, reactive oxygen species and redox conditions. Paradoxically, PHD2 and PHD3 expression is induced by hypoxia despite the lack of oxygen co-substrate, and it has been suggested that they become functionally relevant only following re-oxygenation to rapidly terminate the HIF response. As demonstrated under chronically hypoxic conditions, PHD2 and PHD3 show a transient maximum but remain upregulated over more than 10 days, suggesting a feedback downregulation of HIFα which then levels off at a novel setpoint. Increased exogenous PHD levels compensated for a wide range of hypoxic conditions in vivo as well as in vitro. Interestingly, the novel PHD2 interactor FKBP38 decreases the protein stability of PHD2, providing another mechanism of PHD regulation. Our results suggest that regulated PHD protein levels play a functional role even in hypoxia, allowing the HIF system to adapt to a novel oxygen threshold and to respond to another hypoxic insult. Such an autoregulatory oxygen-sensing system would also explain how a single mechanism works in a wide variety of differently oxygenated tissues.

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Novel PHD oxygen sensor targets

Jens Köditz, Jutta Nesper, Daniel P. Stiehl, Corinna Franke, Johanna Myllyharju, Roland H. Wenger and Dörthe M. Katschinski. Heart and Circulatory Physiology, Georg August University Göttingen, Germany.

In a yeast two-hybrid screen besides HIF-1α various transcription factors were identified as novel interactors of the oxygen sensor prolyl-4-hydroxylase domain 3 (PHD3). One of the newly identified interacting transcription factors is the activating transription factor-4 (ATF-4). ATF-4 is part of the unfolded protein response and a critical regulator of cell fate following ER stress. The zipper II domain (amino acids 89–123) of ATF-4 was found to interact with PHD3. Previously it has been described that ATF-4 is translationally induced under anoxic conditions. However, the PHD inhibitors DMOG, CoCl2 and hypoxia, or a proteasomal inhibitor, also induced ATF-4. Hypoxic induction of ATF-4 was due to increased protein stability but independent of pVHL. ATF-4 amino acid stretch 154 to 183 adjacent to the zipper II was identified as a novel oxygen-dependent degradation domain. Alanine mutations of all five proline residues within this sequence resulted in normoxically stabilized ATF-4. Treatment of HeLa cells with siRNA targeting PHD3 but not PHD2 resulted in increased expression of ATF-4. These data demonstrate that oxygen-regulated ATF-4 protein stability is specifically mediated by PHD3 in a proline cluster-dependent manner. PHD-dependent oxygen sensing thus recruits both the HIF and ATF-4 systems and hence not only confers adaptive responses, but also cell fate decisions.

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Stability regulation of HIF-1α under the impact of calcium

B. Brüne, B. Herr, C. Werno and J. Zhou, Biochemistry I, Frankfurt University Medical School, Frankfurt, Germany

The protein amount of hypoxia inducible factor 1 (HIF-1) is controlled through stability regulation of HIF-1α or is under transcriptional/translational influence. Normally, degradation of HIF-1α requires prolyl hydroxylation, association of the von Hippel Lindau protein and 26S-proteasomal digestion. Besides hypoxia, stability regulation of HIF-1α is affected by nitric oxide (NO), which stabilizes HIF-1α under normoxia but destabilizes the protein under hypoxia. Exposing RCC4 (pVHL-deficient renal carcinoma cells that show high steady-state HIF-1α expression under normoxia) cells to hypoxia in combination with e.g. the NO donor DETA-NO (2,2′-(hydroxynitroso-hydrazono) bis-ethanimine), but not hypoxia or DETA-NO alone, decreased HIF-1α protein and attenuated HIF-1 transactivation. Mechanistically, we propose the contribution of calpain as calpain inhibitors reversed HIF-1α degradation and chelating intracellular calcium attenuated HIF-1α destruction by hypoxia/DETA-NO. We propose that the diffusion-controlled interaction of NO with O_2^- provokes formation of peroxynitrite ($ONOO^-$) which subsequently releases Ca^{2+} to activate calpain. In line, $ONOO^-$ scavengers (methionine or uric acid) preserved HIF-1α protein amount, while SIN-1 (an $ONOO^-$ generating compound) decreased it. Furthermore, our data question the established concept that a Ca^{2+} increase stabilizes HIF-1α under normoxia. We will present data to show that the calcium ionophore A23187 uses Ca^{2+}-independent pathways to stabilize HIF-1α and discuss the hypothesis that ER-stress pathways might be involved. We conclude that calcium contributes to HIF-1α destruction via the calpain system when NO and O_2^- are cogenerated, while established calcium liberating agents may use alternative signaling pathways.

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Nitric oxide modulates oxygen sensing by HIF-1 dependent induction of prolyl hydroxylase 2

U. Berchner-Pfannschmidt, H. Yamac, B. Trinidad and J. Fandrey, Institut für Physiologie, Universität Duisburg-Essen, Germany

The transcription factor complex hypoxia-inducible factor 1 (HIF-1) composed of an α- and a β-subunit plays a crucial role in cellular adaptation to low oxygen availability. O_2-dependent HIF prolyl hydroxylases (PHDs) modify HIF-1α, which is sent to proteasomal degradation under normoxia. Reduced activity of PHDs under hypoxia allows stabilization of HIF-1α and induction of HIF-1 target gene expression. Like hypoxia, nitric oxide (NO) was found to inhibit normoxic PHD activity leading to HIF-1α accumulation while under hypoxia, NO reduced HIF-1α levels. Herein, we report a biphasic response of HIF-1α to NO treatment. In the early phase NO inhibited PHDs leading to HIF-1α accumulation whereas in the late phase increased PHD levels reduced HIF-1α. NO induced expression of PHD2 and 3 mRNA and protein under normoxia and hypoxia in a strictly HIF-1 dependent manner. NO treated cells with elevated PHD levels displayed delayed HIF-1α accumulation and accelerated degradation of HIF-1α upon reoxygenation. Subsequent suppression of PHD2 and 3 expression using siRNA revealed that PHD2 was exclusively responsible for regulating HIF-1α degradation under NO treatment. In conclusion, NO acutely inhibited PHDs but HIF-1 dependent induction of PHD2 hydroxylation capacity induced reduction of HIF-1α at later time points.

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The asparagine hydroxylase FIH-1: Not(ch) only a regulator of HIF activity

Rebecca Bilton, Sarah Linke, Rachel Hampton-Smith, Sarah Wilkins and Daniel Peet. Molecular Biosciences and the ARC Special Research Centre for the Molecular Genetics of Development, Adelaide University, South Australia, Australia 5005.

The asparagine hydroxylase, factor inhibiting hypoxia-inducible factor 1 (FIH-1), is now well characterised as a key oxygen sensor involved in the regulation of the transcriptional activity of the hypoxia-inducible factors (HIFs). Unlike the family of prolyl-hydroxylase enzymes, there is only one FIH-1 gene product identified to date. One of the primary interests of our laboratory is in characterising and understanding the physiological role of FIH-1. Oxygen dependent hydroxylation at the conserved asparagine 803 for HIF-1 prevents transcriptional cofactor CPB/P300 binding, and hence transcription of target genes. However it appears other roles for FIH-1 may exist. Our lab and others have identified putative substrates of FIH-1; these include ankyrin-repeat-domain containing proteins such as Notch-1. We show that Notch-1 is hydroxylated in vitro on a single asparagine residue in a FIH-1 dependant manner. With the aid of in vitro hydroxylation assays and mass spectrophotometry we have compared these data to those obtained for the characterised HIF substrates. Additionally, preliminary data indicates that hydroxylation of Notch-1 also occurs within mammalian cells. Notch-1 is involved in regulation of cell fate and the effect of hydroxylation by FIH-1 upon Notch-1 activity is being investigated.

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Protective effects of HIF in acute renal failure

W.M. Bernhardt[1], V. Câmpean[2], S. Kany[3], J.-S. Jürgensen[3], A. Weidemann[3], C. Warnecke[3], C. Willam[3], V. Günzler[4], S. Klaus[4], K. Amann[2], M.S. Wiesener[5], K.-U. Eckardt[3]
[1]*Med4, Dept. of Nephrology and Hypertension Erlangen-Nuremberg, Germany;*[2]*Department of Pathology Erlangen-Nuremberg, Germany;*[3]*Department of Nephrology and Medical Intensive Care, Charité-CVK, Berlin, Germany;* [4]*FibroGen Inc., South San Francisco, California, USA;* [5]*Nikolaus-Fiebiger-Zentrum for Molecular Medicine, Erlangen, Germany*

Normoxic inactivation of HIF is regulated by oxygen dependent hydroxylation of specific proline residues by prolyl hydroxylases (PHDs). Hypoxia or pharmacological inhibition of PHDs results in HIF accumulation with subsequent activation of renoprotective genes. Therefore, preconditional inhibition of the PHDs may protect the kidney against hypoxic injury. We induced ischemic acute renal failure (iARF) in rats. Animals were pre-treated with carbon monoxide 0.1% (CO) or a PHD-inhibitor (PHD-I) prior to iARF. Vehicle treated (Veh), untreated (UnT) animals and sham operated (sham) rats served as controls (n=10). Serum creatinine (S-crea) and urea were determined at 0 h, 24 h and 72 h (final). Renal damage was quantified by different histological scorings (blinded analysis).
CO and PHD-I led to HIF-accumulation in the kidney including the proximal tubules (immunohistochemistry). This was paralleled by upregulation of target genes (RNase protection). At 24 h and 72 h S-crea and urea were significantly lower in the CO and PHD-I group compared to Veh and UnT. Morphological changes were less severe in the CO and PHD-I group. These data provide a proof of principle that preconditional activation of the HIF pathway by CO or PHD-I protects against iARF.

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A novel mutation in the prolyl hydroxylase PHD2 which hydroxylates HIF-1 and its role in erythrocytosis

MJ Percy[1], Q Zhao[2], A Flores[2], C Harrison[3], TR.J Lappin[4], PH Maxwell[5], MF McMullin[5], and FS Lee[2].
[1]Haematology, Belfast City Hospital, Belfast, UK; [2]Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, USA; [3]Haematology, St Thomas' Hospital, London, UK; [4]Haematology, Queen's University, Belfast, UK; [5]Renal Section, Imperial College, London, UK.

Red cell production is tightly controlled by erythropoietin (Epo), which is transcriptionally regulated by the hypoxia-inducible factor (HIF). In normoxia, key prolines in the oxygen degradation domain of the alpha subunit of HIF-1 are hydroxylated by the prolyl hydroxylase enzyme, PHD2, permitting HIF-1 alpha to associate with the von Hippel Lindau protein (pVHL). Subsequently, ubquitination occurs and HIF-1 alpha is degraded by the proteasome. Although mutations in the VHL gene are the most commonly identified cause of erythrocytosis, a significant cohort of patients remain in whom the molecular defect has not been defined. Thus we screened other components of the oxygen sensing pathway and detected a heterozygous C to G change at base 950 in PHD2 in 3 members of a family with erythrocytosis. This base change was absent in normal controls and resulted in loss of proline 317, located 2 amino acids away from the iron chelating residue in the active site of PHD2. By performing in vitro binding and enzymatic assays this mutation was found to affect the function of PHD2 and therefore to play a role in the development of familial erythocytosis.

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PDH E1A PHOSPHORYLATION UNDERLIES AEROBIC GLYCOLYSIS IN HUMAN CANCERS

T. McFate, A. Mohyeldin, N. Halim, H. Wu; M. Schell, H. Lu, A. Verma; Uniformed Services University of the Health Sciences, Bethesda, MD 20814.

Malignant cancers exhibit high rates of lactate and pyruvate production in the presence of normal oxygen conditions. This metabolic phenomenon, is highly correlated with malignant features such as invasiveness, metastasis, and treatment resistance. Products of aerobic glycolysis provoke angiogenesis, induce extracellular matrix turnover, and activate gene expression through the hypoxia-inducible transcription factor HIF-1. These observations suggest that aerobic glycolysis directly impacts the malignant progression. We hypothesize that aerobic glycolysis results from inhibition of the pyruvate dehydrogenase complex (PDC), a crucial enzyme at the crossroads between anaerobic and aerobic energy metabolism. PDC activity is regulated by a set of specific phosphatases and kinases, which govern the phosphorylation status of the pyruvate dehydrogenase (PDH) E1a subunit. PDH E1a phosphorylation reduces overall PDC activity. Using a novel antibody generated against phosphorylated E1a we demonstrate a high level of PDHE1α phosphorylation in many human cancer cells. Moreover, PDHE1α phosphorylation is highly correlated with increased rate of lactate production, malignant phenotype, and expression of the PDK1 isoform of PDH kinases. Although PDK1 is a HIF-1 regulated gene and its expression can be enhanced under hypoxia, we observe a high expression of PDK1 in many human cancers under normoxia. Enhancement of PDC activity may be a novel therapeutic strategy for treating human cancers.

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IGF-1-dependent regulation of PAI-1 gene - role of HIF-1

Elitsa Y. Dimova and Thomas Kietzmann; Department of Biochemistry, Faculty of Chemistry, University of Kaiserslautern, D-67663 Kaiserslautern, Germany

The insulin-like growth factor (IGF) system regulates proliferation and differentiation of hematopoietic and various other cells among them hepatocytes. In addition to IGF, the plasminogen activator inhibitor-1 (PAI-1) system also stimulates cellular proliferation and differentiation whereas it inhibits fibrinolysis. However, the link between IGF and PAI-1 is not yet fully understood. Therefore, we investigated the regulation of PAI-1 expression by IGF-1 in HepG2 cells and demonstrated that: First, PAI-1 mRNA and protein levels were increased by IGF-1. Second, IGF-1 triggered transcription from the PAI-1 promoter and using reporter gene assays we demonstrated that the IGF-1 responsive elements overlapped with the HIF-1 -binding hypoxia response elements. Third, IGF-1 enhanced HIF-1α protein levels and HIF-1 DNA-binding to each of the response elements. Fourth, we showed that this occurred through activation of the PI3-kinase and MAPK but not the PKB signaling cascade, although inhibition of the PI3K and ERK1/2 completely abolished the IGF-1 effect on HIF-1α. These findings suggest a mechanism by which IGF-1 might induce a more prolonged and sustained increase of PAI-1 even in the presence of low amounts or short peaks of IGF-1. This may contribute not only to differentiation but also to inhibition of fibrinolysis in a number of patients.

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Is there a link between proinflammatory cytokines, hepcidin and anemia in pregnancy?

Demikhov VG, Morshchakova EF, Pavlov AD, Demikhova EV; Federal Clin Research Center for Pediatric Hematology, Oncology and Immunology, Ryazan Branch, Russia

Objective: To determine an influence of some cytokines and hepcidin in anemia of pregnancy pathogenesis. Patients and methods: A total 62 pregnant women were divided into 3 groups: group1 - iron deficiency anemia (IDA) −18, group 2 - anemia with normal iron status and inadequately low production of EPO for the degree of the anemia −20 and group 3 - pregnant women with normal Hb levels −24. Control group consisted of 11 non-pregnant healthy women. We determined serum levels of IL-6, IL-8, INF-γ, TNF-α and pro-hepcidin by using commercial ELISA kits. Results: The significant elevated serum levels of IL-8 and INF-γ were observed at all pregnant women groups versus control. Serum INF-γ concentration in IDA pregnant women (group 1) was significant higher than in group 2: 414.7±131.6 ng/L and 95.9±30.0 ng/L respectively ($p<0.05$). Increased IL-6 serum level was in group 3 only: 78.4±35.1 ng/L vs 3.13±3.13 ng/L in control ($p<0.05$). Group 3 pregnants have had significant decreased serum level of hepcidin: 9.6±2.34 μg/L vs 30.2±8.62 μg/L in control ($p<0.05$). A considerable inverse correlations (r) between EPO and hepcidin, EPO and IL-8, EPO and INF-γ serum levels were found in IDA pregnant women. Conclusions: Disruption of the delicate balance of cytokines by bacteria or other factors increases production of proinflammatory cytokines and leads to blunt erythropoiesis and anemia. Hepcidin serum levels indicate that hepcidin may be one of inhibiting erythropoiesis factors during pregnancy.

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High thrombopoietin concentrations in the cerebrospinal fluid of neonates with sepsis and intraventricular hemorrhage may contribute to brain damage

Juan Zhang, Anke Reinhold, Reinhard Gessner, Ursula Felderhoff-Mueser, Michael Obladen, Christof Dame
Department of Neonatology, CVK; Department of Clinical Chemistry, Charité - Universitätsmedizin Berlin, Germany

Thrombopoietin (Tpo) and its receptor (Tpo-R) are expressed in the developing and adult central nervous system (CNS). Recent data indicate a pro-apoptotic function of Tpo in the CNS. Herein, we analyzed Tpo concentrations in the cerebrospinal fluid (CSF) of neonates. Human neuroblastoma-derived SH-SY5Y cells served as model to elucidate Tpo expression under inflammation and hypoxia. Tpo was detectable in the CSF of 6/16 neonates with sepsis (median 140, range 2–613 pg/ml), 5/9 neonates with posthemorrhagic hydrocephalus (PHC; median 31, range 1.4–469 pg/ml), 3/4 neonates with PHC plus sepsis (median 97, range 6–397 pg/ml), but not in controls. Neither the presence of Tpo nor its level in the CSF did significantly correlate with any clinical or laboratory parameter, including IL-6. In SH-SY5Y cells, Tpo mRNA levels decreased significantly (22–43%, after 6–24 hrs) under hypoxia (1% O_2) in a time-dependent manner while Epo mRNA increased. In contrast to human hepatoma cells, which serve as model for hepatic Tpo production, IL-6 did not significantly stimulate Tpo expression in neurons. Combined data indicate that in neurons Tpo production is regulated by specific mechanisms. Understanding of these mechanisms is important to judge on Tpo's relevance in brain development and function.

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Human Fetal Serum Erythropoietin Levels in Patients with Fetal Alloimmune Thrombocytopenia

F. Wenzel[1,2], Ch. Schmülling[1], S. Hasberg[1], H. Kroll[3], R. Bald[4], R. Görlich[5], G. Giers[1]
[1]Institut für Hämostaseologie und Transfusionsmedizin, Universitätsklinik Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany, [2]ab 01.06.2006 Institut für Experimentelle Hämatologie und Transfusionsmedizin, Universität Bonn, [3]Institut für Transfusionsmedizin, Universität Giessen, [4]Klinik für Geburtshilfe und Gynäkologie, Universität Köln, [5]Institut für Biologie VII, RWTH Aachen

Purpose: To evaluate the intrauterine time course of serum erythropoietin (sEPO) levels in fetuses with fetal alloimmune thrombocytopenia (FAITP). **Methods:** Serum samples from 6 fetuses with FAITP were obtained at different gestational ages. Umbilical blood sampling (cordocentesis) under continuous ultrasound guidance was necessary to evaluate platelet count sEPO levels were determined by a commercially available ELISA-Kit (R&D Systems). **Results:** From the 22 to 24th, 25 to 28th, 30 to 34th and 37 to 40th week of gestation sEPO mean values were 8.7± 1.2 U/l, 9.5±2.2 U/l, 16.7±0.6 U/l and 24.6±6.6 U/l, respectively. The corresponding mean values of the body weight determined by ultrasound parameters were 743± 130 g, 973±218 g, 2135±516 g and 3550±342 g. sEPO levels and body weight of the fetuses showed a linear regression (r=0.7).
Conclusions: In advanced gestational ages, elevated serum erythropoietin levels were observed. In addition, serum erythropoietin levels correlated with body weight of the fetuses. These changes of sEPO levels may be caused by

the dynamic adjustment from paracrine to endocrine functions of the hormone.

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Serum erythropoietin concentration $[EPO]_s$ following prolonged physical exercise after administering a diet with varied sodium content

Z. Szygula, J. Wnorowski, W. Pilch, M. Wiecek, *A. Zembron-Lacny; University School of Physical Education, Krakow, *Faculty of Physical Culture, Gorzow Wlkp., Poland

Objective: To examine EPO and plasma volume (PV) following prolonged physical exercise performed after administering a diet with varied NaCl content. **Methods:** Ten healthy untrained male subjects exercised at 55% VO_2max until exhaustion on the cycloergometer: after an unchanged diet (Test I), high sodium diet (Test II), and low sodium diet (Test III). Venous blood samples were taken prior to the modification of diet (T-1), before (T0), 3 min (T1), 3 hr (T2), 9 hr (T3) after exercise, and on the 2nd (T4) and 3rd day (T5) of the experiment.

Table 1. Serum EPO concentration [mU/l] after exercise and after unchanged-Test I, high sodium-Test II and low sodium diet-Test III

	Mean ±SD	T-1	T0	T1	T2	T3	T4	T5
t1.1	Test I	8.39 ±3.37	7.9 ±4.62	9.01 ±4.18	7.72 ±4.26	13.48* ±4.72†	12.50* ±6.49†	12.25a ±5.62†
t1.2	Test II	9.17 ±2.67	9.25 ±3.18	9.49 ±2.05	9.15 ±3.74	12.04 ±5.27	9.93 ±1.91	12.50* ±4.68†
t1.3	Test III	8.9 ±4.65	11.06 ±5.52	9.57 ±3.91	8.74 ±4.66†	12.14 ±6.93	12.11* ±6.15	12.23 ±8.58
t1.4								

*p<0.05; [a]p=0.07-T0, T1, T2, T3, T4, T5 vs. T-1; †p<0.05-T1, T2, T3, T4, T5 vs. T0

Conclusion: No statistical differences were observed in the patterns of change in $[EPO]_s$ after prolonged exercise.

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Hematide™, a Synthetic Peptide-based Erythropoiesis Stimulating Agent (ESA), Achieves Correction of Anaemia in Patients with Chronic Kidney Disease (CKD)

Iain C. Macdougall, King's College Hospital, London, UK; A. Wiecek, Slaska Akademia Medyczna, Katowice, Poland; A. Mikhail, Morriston Hospital, Swansea, UK; B. Tucker, Mayday University Hospital, Croydon, UK; I. MacPhee, St George's Hospital, London, UK; M. Yaqoob, Royal London Hospital, London, UK; M. Mysliwiec, Wojewodzki Szpital, Bialystok, Poland; M. Nowicki, Medical University, Lodz, Poland; W. Sulowicz, Szpital Universytecki, Krakow, Poland; A. Zamauskaite, Parexel, Uxbridge, UK; R. Leong, J. Iwashita and AM. Duliege, Affymax Inc., Palo Alto, US.

Hematide, a novel synthetic PEGylated peptide that binds to and activates the erythropoietin receptor, is being developed for the treatment of anaemia associated with CKD and cancer. A Phase 2 open-label dose-finding study is being conducted in ESA-naïve CKD patients not on dialysis. Patients with baseline haemoglobin (Hgb) values between 9 and 11 g/dL receive subcutaneous Hematide injections every 4 weeks for a total of 3–6 doses. The first 2 cohorts tested a Hematide dose of 0.05 mg/kg. Mean baseline Hgb was 10.3 g/dL. After 4, 8, and 12 weeks of dosing, preliminary data show a mean Hgb change from baseline of 1.3, 1.6, and 2.1 g/dL, and correction of anaemia (Hgb≥11 g/dL) in 23 of 29 (79%), 24 of 27 (89%), and 25 of 26 (96%) patients, respectively.

Multiple doses of Hematide appear well tolerated and result in a sustained increase in Hgb when injected once-monthly in CKD patients. The study is ongoing, and data from further cohorts will be presented.

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Validation of the normocythaemic mice bioassay for the potency assessment of rhEPO in pharmaceutical formulations

Thiago Barth, Diogo Paim Leal, Ricardo Machado Ferretto and Sergio Luiz Dalmora. Department of Industrial Pharmacy, Postgraduate Program in Pharmaceutical Sciences, Federal University of Santa Maria, Santa Maria - RS, Brazil.

Erythropoietin is a glycoprotein hormone which stimulates erythropoiesis. The normocythaemic mice bioassay was validated for the potency evaluation of the recombinant human erythropoietin (rhEPO) against the Ph. Eur. BRP for erythropoietin. Single and multiple daily injections protocols were evaluated. The bioassays were carried out in eight-week old female BALB/c mice, which received a multiple daily injections of standard and sample solutions (3+3), during four days. The blood sampling was performed 24 hours after the last injection and the reticulocytes counted by automated flow cytometry. Method validation investigated parameters such as the linearity, precision, accuracy, specificity and robustness giving results within the acceptable range. The dose-response curve was linear in the concentration range of 1.0 to 64.0 IU/mL and the value of the determination coefficient (r^2) was 0.9708. The bioassay was applied for the potency evaluation of rhEPO pharmaceutical products containing alfa or beta forms expressed in different cell lines, giving potencies between 82.8 and 119.7%, with the mean weight of 368, for the independent assays. The bioassay validated improves the

potency assessment of pharmaceutical formulations assuring the therapeutic efficacy of the biological medicine. *Financial support: CNPq and FAPERGS.*

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Detection of anti-EPO antibodies in human sera by a bridging ELISA is much more sensitive by coating of biotinylated rhEPO to streptavidin than direct coating of rhEPO

Wolfgang Hoesel[a], Johann Gross[b], Renate Moller[b], Wolfgang Henke[b]; [a]Roche Diagnostics GmbH, 82377 Penzberg, Germany; [b]Mol. Biol. Res. Lab., Dept. Otorhinolaryngology, Humboldt University, Charité, Spandauer Damm 130, 14050 Berlin, Germany;

ELISA is an attractive alternative to labour-intensive radioimmunoprecipitation assays for detecting anti-erythropoietin (anti-EPO) antibodies in human sera, but apparently conflicting reports question its sensitivity. We sought to resolve this issue by directly comparing different reported ELISA approaches to determine if rhEPO-coating methods affect detection of anti-EPO antibodies. Investigators reporting low sensitivity used ELISAs in which rhEPO was directly coated to microtiter plates while the high sensitivity ELISA used plate-bound streptavidin to bind biotinylated rhEPO. Using anti-EPO positive human sera, our results confirmed a large (100 to 300-fold) difference in sensitivity between the ELISAs and suggested that the inferiority of the low sensitivity ELISA was caused by the direct coating procedure of rhEPO which may disrupt epitopes by masking recognition sites or introducing conformational changes. Thus, a bridging ELISA can be an appropriate and effective system for antibody analysis and screening of human sera with high sensitivity and specificity but only if performed with streptavidin binding of biotinylated antigen. This finding may also be more generally applied to detection of antibodies towards other proteins in addition to anti-EPO antibodies.

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Erythropoietin receptor: expression in the human heart and upregulation after cardio-pulmonary bypass: role of hypoxia and possible mechanism of myocardial protection)

KF Wagner, MD, PhD, H-H Sievers, MD, PhD, R Depping, PhD. Depts. of Anesthesiology, Physiology, Clinic of Cardiac Surgery, University of Luebeck, UKSH-CL, Germany

We investigated adult human cardiac tissue for the presence to identify cells expressing the EPO receptor.
Methods. After approval by the Institutional Review Board, ventricular tissue from the muscular septum obstructing the left ventricular out flow track (Morrow procedure) or right atrial tissue (venous canulation) before and after cardiopulmonary bypass (CPB) was obtained (n=4 per group). Samples were investigated for the EPO receptor and the transcription factor HIF-1alpha by RT-PCR, Western blot and/or immunohistochemistry.
Results and Discussion. We show for the first time that EPO receptor mRNA and protein is indeed expressed in adult human atrial and ventricular tissue. Prior to CPB, HIF-1alpha was strongly upregulated consistent with preexisting myocardial ischemia/hypoxia. After CPB HIF-1alpha was decreased due to the improved perfusion following CABG, and the EPO-receptor was markedly upregulated.
Conclusion. EPO receptor is expressed in the human heart. These findings encourage to investigate the potential of EPO-induced myocardial protection in humans. The EPO receptor in the heart appears not to be regulated by HIF-1alpha.
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R. Depping et al. 2005 J Thorac Cardiov Sur 130(3): 877e1–e4

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The use of rHuEPO in pediatric patients with solid tumors

Pavlov AD, Morshchakova EF, Beznoshchenko AG, Skobin VB; Federal Clinical Research Centre for Pediatric Hematology, Oncology and Immunology (Ryazan branch), Russia.

Objective: To assess the effectiveness of rHuEPO in pediatric oncology. **Patients and methods**: Fifty two children with solid tumors were enrolled into the non-randomized controlled study. All the patients received chemotherapy. 28 patients received rHuEPO in the doses ranging from 150 to 600 U/kg three times a week. In 17 patients the weekly dose was delivered by three subcutaneous injections. In 11 patients the weekly dose of 600 U/kg was delivered by one intravenous injection. Twenty four patients were controls. The level of endogenous EPO and iron status were studied in all the patients before the start of rHuEPO treatment. During the course of rHuEPO treatment, the patients received iron supplementation orally. The patients were transfused when Hb levels felt below 7.0 g/dl. The transfusion requirements were assessed as the RBC volume per kg of body mass per patient. **Results**: RBC transfusions were necessary in 21% of patients on rHuEPO treatment and in 54% of controls ($p<0.05$). The control patients received 12.4±3.08 ml of RBC concentrate per kg of body mass while the EPO treated patients needed only 2.4±0.97 ml/kg ($p<0.05$). No side effect of rHuEPO was observed except for moderate pain in the place of subcutaneous injections. **Conclusions**: The treatment of tumor associated anemia with rHuEPO is safe and

considerably reduces the requirements for RBC transfusions in pediatric patients with solid tumors.

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Serum Thrombopoietin Levels in Patients with Chemotherapy-Induced Aplasia

I. Kafka[1], F. Fleschutz[1], R. Görlich[3], G. Giers[1], F. Wenzel[1,2]
[1]Institut für Hämostaseologie und Transfusions-medizin, Moorenstr. 5, 40225 Düsseldorf, Germany, [2]ab 01.06.2006 Institut für Experimentelle Hämatologie und Transfusionsmedizin, Universität Bonn, [3]Institut für Biologie VII, RWTH Aachen

Purpose: Serum thrombopoietin (sTPO) levels were determined in patients with chemotherapy-induced aplasia but maintained protein synthesis by the liver. **Methods:** Serum blood samples from 8 patients (mean age 46±18 years) were obtained before and during chemotherapy-induced aplasia (3 cases of acute myelocytic leukaemia, 3 cases of multiple myeloma, 1 case of malignant granuloma, 1 case of ovarian teratoma). Serum thrombopoietin (sTPO) levels were determined by a commercially available ELISA-Kit (R&D Systems). In addition, leukocyte count (LC) and platelet count (PC) were measured. **Results:** In patients, LC, PC and sTPO were within a normal range before start of the chemotherapy. LC was found to be 6.1±2.9/nl, and PC was found to be 255±138/nl. During chemotherapy-induced aplasia (after 12±3 days) LC and PC decreased to <0.1/nl and to 23±16/nl corresponding to the myelosuppressive effect. In contrast, sTPO showed a clear increase from 244±201 pg/ml to 3038±459 pg/ml ($p<0.05$, Mann-Whitney-Test). **Conclusions:** In patients with chemotherapy-induced aplasia decrease in platelet count was accompanied by a clear increase in sTPO levels. In aplasia, increase in serum thrombopoietin concentrations may be due to low binding capacity of peripheral thrombocytes and due to suppressed megakaryocytic thrombopoiesis.

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Erythroid progenitors express TNFα and its receptors in vitro

I. Dorn, P. Lazar, P. Koritke, S. Malchow, J. Luhm, H. Kirchner, P. Schlenke, Institut für Immunologie und Transfusionsmedizin, UK S-H, Campus Lübeck, Lübeck, Germany

Subject: We established an in vitro model of human erythropoiesis. Using gene arrays, TNFα and its receptors were identified as possible candidates with an influence on erythopoiesis. **Methods:** Human CD34+ cells were isolated and cultured over 21 days in a three-phase liquid assay (d1-7: SCF, TPO, FLT3-L; d8-14: SCF, EPO, IGF-1; d15-21: EPO). Cell growth and differentiaton was evaluated by flow cytometry and cytospin preparations. An RT-PCR for TNFα, TNF-RSF1a (TNF-R1) and TNF-RSF1b (TNF-R2) was performed. Furthermore, RT-PCR was performed after incubation of cells in the presence of antibodies against TNF-R1 and 2. **Results:** TNFα RNA increased continuously during in vitro erythropoiesis, with a maximum reached on d15 (58fold compared to d1). On this day about 95% cells expressed CD36 and GPA and were mainly at the stage of polychromatic erythroblasts and normoblasts. TNF-R1 mRNA was mainly detected between d1-d7, where only stem cells and non-erythroid cells could be observed. TNF-R2 could be detected during the entire time with a clear increase after addition of EPO on d7. By inhibition of TNF-R1, TNFα mRNA increased 150fold and also an increase in TNFα protein concentration was observed. This was associated with a decreased proliferation and increased differentiation. **Conclusions:** Although TNFα is discussed as being a negative regulator of erythropoiesis, erythroid progenitors themselves seem to produce TNFα and differ in TNF receptor expression. Furthermore, we could show that inhibition of TNF-R1 resulted in a higher TNFα production, associated with a reduced proliferation and enhanced differentiation.

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In vitro generation of human red blood cells

I.Dorn, J.Ribbat, S.Boie, B.Driller, H.Kirchner, P.Schlenke Institut für Immunologie und Transfusionsmedizin, UK S-H, Campus Lübeck, Lübeck, Germany

Subject: An in vitro erythropoiesis model might be very useful in order to investigate the regulation of human red blood cell development. Methods: Human CD34+ stem cells were isolated and cultured over 16 days in a two-phase liquid assay (d1-d8: SCF, EPO, IGF-1; d9-d16: EPO and insulin). To enhance proliferation, a modification into a three-phase model was made and cells were first incubated in the presence of SCF, thrombopoetin (TPO) and Flt3-L. Furthermore, the influence of the oxygen content was investigated by incubating cells under mild (5% O_2) and severe (1% O_2) hypoxia. Differentiation was evaluated by flow cytometry (CD34, CD71, glycophorin A (GPA) and CD36) and cytospin preparations. Results: In the two-phase model, cell numbers increased up to 50fold and on d16 >95% expressed GPA and were strongly hemoglobin positive. Cells showed morphological characteristics of normoblasts (55%) and reticulocytes (35%). In the three-phase model, a higher proliferation up to 250fold could be generated, accompanied by a higher degree of mature reticulocytes (55%). Compared to normoxia (20% O_2), proliferation was 2.8fold higher under mild and 3.8fold reduced under severe hypoxia. Conclusion: The established assay was able to show different stages of erythopoiesis, including terminal

enucleation. By preincubation with SCF, TPO and Flt3-L a higher proliferation of erythroid progenitors as well as terminal maturation was generated.

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A Study of the Novel Protein JUNE-1

Vivien M. Hodges[1], Susan Rainey[1], Victoria Smyth[1], Terry R.J. Lappin[1] and A. Peter Maxwell[2]. [1]Department of Haematology, Centre for Cancer Research and Cell Biology, University Floor, [2]Department of Nephrology, Belfast City Hospital, Lisburn Road, Belfast, BT9 7AB, Northern Ireland.

JUNE-1 was originally identified as a gene upregulated in response to erythropoietin in a murine model of terminal erythroid differentiation using differential display PCR. JUNE-1 is a novel protein with only 1 other related human protein PHF13, also of unknown function. The gene is widely expressed suggesting a role beyond erythropoiesis and although highly conserved across vertebrate species the function of JUNE-1 remains to be elucidated. A splice variant, missing a 201 bp section of exon 4, has also been identified. *In silico* sequence analysis reveals a PHD motif (plant homeodomain) and a nuclear localization signal suggesting JUNE-1 may function as a transcription factor. Cellular localisation studies using GFP tagged JUNE-1 protein indicates that the protein is, as expected, predominantly located in the nucleus. Western blot analysis of immunoprecipitated JUNE-1 from transient over-expression in 293T cells reveals both tyrosine and serine phosphorylation as well as sumoylation suggesting additional regulation at the post-translational level. The protein is being analysed by mass spectrometry to reveal the exact residues that are modified. Chromatin immunoprecipitation and pull-down assays will be performed to assess DNA binding and identify potential JUNE-1 binding partners to further assess the function of this novel protein.

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The Wilms' tumor suppressor Wt1 activates transcription of the erythropoietin receptor gene

Karin Kirschner, Patricia Hagen, Christiane Hussels, Christof Dame, Holger Scholz, Charité, Berlin, Germany

The aim of this study was to establish a role for the Wilms' tumor transcription factor Wt1 in murine hematopoiesis and to identify potential downstream targets. We show here that CD117-positive hematopoietic progenitor cells from the liver of Wt1-deficient murine embryos ($Wt1^{-/-}$) exhibited an approx. 10-fold lower proliferative responsiveness to recombinant erythropoietin (Epo) than CD117+ cells from wild-type embryos ($Wt1^{+/+}$). Benzidine staining revealed a reduced fraction of hemoglobin expressing cells in Wt1-deficient vs. normal progenitor assays. Consistently, expression of the erythropoietin receptor (EpoR) was reduced significantly in hematopoietic precursor cells with lack of Wt1 compared to $Wt1^{+/+}$ cells. Transient co-transfection of Wt1 into hepatoma-derived HepG2 cells increased EpoR transcripts more than 2-fold. A luciferase reporter plasmid carrying 308 bp of the proximal human EpoR promoter was stimulated more than 8-fold by co-transfection of a Wt1 expression construct. In contrast, transient co-transfection of a Wt1 form that lacks transcriptional activity did not significantly change endogenous EpoR mRNA levels and transcription from the EpoR promoter. The responsible cis-element in the EpoR promoter was identified by mutation analysis, electrophoretic mobility shift assay and chromatin immunoprecipitation (ChIP) assay. These findings indicate that the Wt1 transcription factor is required for normal murine hematopoiesis and activates EpoR gene expression.

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Mature erythrocytes as targets for Epo action

D.Mihov, J.Vogel, M.Gassmann, A.Bogdanova; Institute of Veterinary Physiology, Vetsuisse Faculty and Center of Integrative Human Physiology, University of Zurich, Zurich Switzerland

When released into the circulation in response to hypoxic stimulus or administered iv erythropoietin comes in contact with mature erythrocyte membranes. So far it was suggested that Epo is unable to interact with erythrocytes due to the lack of Epo receptors (EpoR) on the membrane of the latter. Our observations revealed that erythrocytes of mice with chronic overexpression of Epo red cell life span, osmotic resistance, cell surface-to-volume ratio, and ion and water balance is significantly different from the wild type controls. We have shown that the observed changes in cellular rheological properties at least partially result from the Epo-induced changes in activity of Na/K ATPase and Na-K-2Cl cotransporter. The effect could be observed in transgenic mice, wild type animals exposed to 8% O_2 for 4 hours and in red cell suspensions treated with human recombinant Epo at concentrations of 1–10 U/ml. As a result erythrocytes treated with Epo had higher K^+ and water content. In addition, Epo treatment affected metabolism and redox state of erythrocytes. The involvement of EpoR in the observed changes, possible mechanisms of Epo action on mature red cells and consequences at the systemic level will be discussed.

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Erythropoietin Receptor of the *Spalax* Mole Rat Displays Enhanced Maturation Conferred by its Extracellular Domain

Ravid Orly[1], Shams Imad[2], Ben-Califa Nathalie[1], Nevo Eviatar[2], Avivi Aaron[2] and Neumann Drorit[1]
[1]Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel-Aviv University; [2]Institute of Evolution, University of Haifa, Israel

The high tolerance of *Spalax* to hypoxia is attributed to a wide array of respiratory and cardiovascular adaptations. Since in mice, most EPO receptors (mEPO-R) are retained in the endoplasmic reticulum and less than 1% are present on the cell surface, our hypothesis was that enhanced cell-surface expression of *Spalax* EPO-R (sEPO-R) is a key factor in the adaptation to hypoxia.

sEPO-R and mEPO-R were stably transfected into BaF/3 cells. Glycan maturation of sEPO-R was enhanced as compared to mEPO-R. To assess the contribution of the extracellular region of EPO-R to its maturation, truncated *Spalax* and mouse EPO-Rs, lacking the intracellular region, were transiently-transfected into BOSC cells. Maturation of truncated sEPO-R was more pronounced than that of the corresponding mEPO-R. To map extracellular domains that augment receptor maturation we generated chimeric receptors in which parts of the extracellular region of the mEPO-R were replaced with the corresponding fragments of sEPO-R. This analysis demonstrated that an extracellular domain of the sEPO-R containing the N-glycosylation site conferred enhanced kinetics and increased extent of glycan maturation to the respective chimera. Our study introduces sEPO-R as a unique model of adaptation to hypoxia underground, for determining structure-function relationship of this protein.

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Effects of Epo on iNOS in a model of left renal ischemia in rats: immunoistochemical analysis of ischemic and non-ischemic kidney

Buemi M.*, Sturiale A.*, Rastaldi M.P.**, D'Amico G**.
* Chair of Nephrology, University of Messina, Italy
** Renal Immunopathology Laboratory, Fondazione D'Amico, Milan, Italy.

We evaluated the effect of Epo administration on the expression of inducible nitric oxide synthase (iNOS) in both kidneys of rats subjected to a left renal ischemia-reperfusion. 66 rats were divided in four groups. The first group underwent only surgical procedure without ischemia (sham). The second was only treated with Epo. The third underwent I/R and received Epo. The fourth was the I/R control group and received only the vehicle. The remainder animals were used to assess normal iNOS expression. iNOS expression was evaluated by immunofluorescence and immunoperoxidase 4 and 7 days after reperfusion. The left kidneys of control and treated groups showed the same level of iNOS. So Epo did not influence iNOS expression in the reperfused kidney. The right kidney of the control I/R group showed an increased level of iNOS but less than the ischemic kidney: this underlines the involvement of the non-ischemic kidney in the stress induced by left renal I/R.

In the treated group iNOS expression in the right kidney appeared statistically lower than in the control group. Our data demonstrate a strong influence of Epo on iNOS expression in the right kidney, affected by functional overload during left ischemia. This effect can be considered as a "pre-conditioning" which prevents some of the consequences of the functional overload.

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Erythropoietin enhances the regeneration of the liver in partially hepatectomized rats

Klemm K, Eipel C, Abshagen K, Vollmar B, Department of Experimental Surgery, University of Rostock, Rostock, Germany

We analyzed the effect of recombinant EPO on hepatic regeneration in rats after partial hepatectomy. Male Sprague-Dawley rats underwent a 70% hepatectomy. EPO (Erypo®) was administered to rats either locally (1500 IU/kg) or intravenously (5000 IU/kg, every 24 h for 4 days). Intravital fluorescence microscopy of the liver was performed after 48 h with quantitative analysis of nutritive perfusion, leukocyte-endothelial-interaction, as well as hepatocellular apoptosis. PCNA- (proliferating cell nuclear antigen) expression in hepatocytes was determined by means of immunohistochemistry. After intravenous, but not local EPO administration significantly higher values of reticulocytes (10^{10}/l; 74±3 vs. control: 34±6; $p<0.05$), hematocrit (%; 55± 1 vs. control: 46±2; $p<0.05$) and serum EPO concentrations (mU/ml; 841±254 vs. control: 1.5±0.3; $p<0.05$) were found when compared to the control. Topical EPO application led to a 13-fold increase of serum EPO concentration (mU/ml; 19±4 vs. control; $p<0.05$), however, without substantial stimulation of erythropoiesis. Both local and systemic EPO administration caused a marked reduction of hepatocellular apoptosis (cells/mm^2; 9±3 and 0.7± 0.3 vs. control: 18± 11; $p<0.05$) with a concomitant rise of cell proliferation (PCNA-positive hepatocytes/hpf; 95±7 and 123±3 vs. control: 72± 11; $p<0.05$). Topical but in particular systemic EPO administration leads to detectable serum EPO concentrations including the induction of erythropoiesis. Systemic EPO application stimulates the regeneration of the liver after partial hepatectomy. Thus,

EPO could represent an attractive approach supporting compromised liver regeneration.

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Darbepoetin-α reduces endothelial activation and platelet reactivity, but has no influence on microvascular thrombosis

Lindenblatt N[1,2], Menger MD[3], Klar E[2], Vollmar B[1]
[1]Department of Experimental Surgery and [2]Department of General Surgery, University of Rostock, [3]Department of Clinical and Experimental Surgery, University of Saarland, Homburg-Saar, Germany

Elevation of hematocrit is generally assumed to be associated with thrombosis, while a bleeding disorder in EPO overexpressing transgenic mice has been observed. Thus we intended to study the effect of Darbepoetin-α on microvascular thrombosis. Mice were pretreated with Darbepoetin-α for 4 weeks. In microvessels of mouse cremaster muscle preparations ferric chloride-induced thrombus formation was analyzed. Pretreatment with Darbepoetin-α caused a marked increase of reticulocytosis, hematocrit and hemoglobin concentration. Immunohistochemistry revealed a significant increase of eNOS expression and a decrease of endothelial ICAM-1 and P-selectin expression following chronic Darbepoetin-α application. ELISA showed a significant reduction in cE-selectin and cICAM-1. FACS analysis revealed a reduction of P-selectin expression upon stimulation with thrombin. However no significant differences in microvascular thrombus formation and subaquatic tail bleeding time were detected. These data indicate a reduction of endothelial and platelet activation. In contrast to this no significant influence of Darbepoetin-α on microvascular thrombus formation and tail bleeding time was seen. Erythropoietin and its derivatives should further be studied in this setting.

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Epo in protection of the heart against cold global ischemia-reperfusion injury

R.Tavakoli, D.Mihov, B.Grenacher, M.Genoni,
M.Gassmann, A.Bogdanova,
D.Mihov, J.Vogel, M.Gassmann, A.Bogdanova*
Institute of Veterinary Physiology, Vetsuisse Faculty and *Center of Integrative Human Physiology, University of Zurich

Erythropoietin (Epo) has been shown to effectively reduce infarct size and post-ischemic contractile function in animal models of coronary artery occlusion (warm ischemia-reperfusion) and in Tyrode-perfused isolated heart models. Using heterotopic rat heart transplantation as a model of cold global ischemia-reperfusion (surgical insult with cardiac arrest) we were able to show that treatment of the recipient animal with Epo (final concentration in plasma ~150 U/ml) 20 min before the onset of reperfusion of the graft results in protection of the heart tissue from the oxidative stress, oedema and Na^+ accumulation as well as from the tissue ATP deprivation. Myocardial tissue damage is significantly reduced in Epo-treated animals compared to the control group. The cardioprotective effect of Epo is complex including its action on redox-controlling enzymes, ion transporters, anti-apoptotic signalling cascades and efficiency of oxygen delivery to the tissue. Mechanisms of Epo-induced cardioprotection will be discussed.

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Human skin and hair follicles are targets and sources of erythropoietin

Arno Kromminga[1]*, Enikő Bodó[2]*, Magdalena Laugsch[3], Wolfgang Funk[4], Ute Duske[1], Wolfgang Jelkmann[3] and Paus R[2]
**contributed equally*
[1]Institute for Immunology, Clinical Pathology, Molecular Medicine, Hamburg, Germany; [2]Dept. of Dermatology, University of Lübeck, Lübeck, Germany; [3]Dept. of Physiology, University of Lübeck, Lübeck, Germany; [4]Klinik Dr. Kozlowski, Munich, Germany

The expression and function of the EPO/EPO-R signaling system are not limited to the hematopoietic sytem. This system has recently been found to be expressed in multiple non-hematopoietic tissues, but the expression of EPO/EPO-R has not yet been reported in normal mammalian skin. Our aims were to investigate the expression of EPO/EPO-R in human skin. By real time RT-PCR, we detected specific PCR products for both EPO (132 bp) and EPOR (315 bp) in human scalp skin and isolated hair follicles. By immunohistology (EnVision-AP method), specific EPO immunoreactivity was confined to the skin epithelium. EPO immunoreactivity was most prominent in the central outer root sheath of anagen VI hair follicles. Using ELISA we were able to detect EPO in the culture medium. Under hypoxic conditions cultured hair follicles up-regulate EPO mRNA, while the expression of the EPOR mRNA remains unaltered. Our preliminary data suggest furthermore that EPO is able to diminish experimentally induced apoptosis in hair follicles. These data provide the first evidence that normal human skin expresses EPO and EPO-R, and suggest that hair follicles are the predominant sources and targets of EPO/EPO-R signaling in human skin.

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The necessity of oxygen channels between interlobular arteries and veins to facilitate oxygen shunt diffusion in the renal cortex. *A prerequisite to select the kidney for the effective regulation of EPO production*.

Hans-Joachim Schurek and Harald Bertram.
Department of Nephrology, Medizinische Hochschule Hannover

Measurements of oxygen pressure at the renal cortex led to the concept of a highly effective shunt diffusion of blood gases in the renal cortex between interlobular arteries and veins. These data stimulated us to construct a mathematical model of gas exchange by shunt diffusion between these anatomical structures. The result fits well for the exchange of carbondioxide, but not as well for oxygen. There was a discrepancy of a factor of 3–4 dependent upon the assumptions made for the calculation for oxygen, when common diffusion coefficients were used to calculate gas diffusion. The hypothesis of Boron that aquaporins are used as gas channels through cell membranes was established for CO_2, not for O_2 due to methodological reasons. In our mathematical model, extra gas channels are not necessary for CO_2 (the diffusion coefficient is 20times higher than for oxygen), but for O_2 it would explain some of the experimentally obtained data using different methods. These data led us to formulate the hypothesis that gas pores for oxygen must be present between interlobular arteries and interlobular veins. This would overcome the discrepancy mentioned and to facilitate oxygen shunt diffusion and the regulation of the production of erythropoietin in the kidney cortex.

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Tissue-specific activation of the erythropoietin gene expression by the Wilms tumor suppressor 1

Christof Dame[1], Karin M. Kirschner[2], Katharina V. Bartz[1], Thomas Wallach[1], Christiane S. Hussels[1], Holger Scholz[2]
Department of Neonatology, Campus Virchow-Klinikum; Johannes-Mueller-Institute of Physiology, Charité - Universitaetsmedizin Berlin.

The molecular mechanisms for the developmental-stage and tissue-specific regulation of the erythropoietin (*Epo*) gene are poorly understood. Herein, we tested the hypothesis that the Wilms tumor suppressor 1 (Wt1) is a transcriptional regulator of the *Epo* gene. Electrophoretic mobility shift assay and chromatin immunoprecipitation showed the binding of the transcriptionally competent Wt1(-KTS) isoform to the minimal *Epo* promoter. Under normoxia, overexpression of Wt1(-KTS) in HEK 293 and HepG2 cells resulted in a significantly increased Epo expression. A reporter construct harboring the 117-bp minimal human *Epo* promoter was activated up to 20-fold by transient co-transfection of Wt1(-KTS) in different cell lines. Mutation of the Wt1 binding site in the *Epo* promoter abrogated this stimulatory effect of the Wt1(-KTS) protein. In embryonic mice with homozygous *Wt1* deletion hepatic *Epo* mRNA expression was significantly reduced. Using double-fluorescent immunohistochemistry, Wt1 and Epo were co-localized in hepatocytes of the liver and in neuronal cells of the dorsal root ganglia in developing mice. Both proteins were also detected in Sertoli cells of the adult murine testis. In conclusion, we identified Wt1(-KTS) as a novel transcriptional activator for the tissue-specific expression of the *Epo* gene.

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Distinct roles for HIF-1 and HIF-2 in liver EPO production

Erinn B. Rankin, Mangatt P. Biju, Qingdu Liu, Jennifer Rha, M. Celeste Simon, Brian Keith and Volker H. Haase, University of Pennsylvania, Philadelphia, U.S.A.

We have previously shown that genetic inactivation of the von Hippel-Lindau tumor suppressor pVHL in mouse hepatocytes results in the development of severe polycythemia. Under normoxic conditions pVHL acts as the substrate recognition component of an E3-ubiquitin ligase that targets hydroxylated HIF-α for proteasomal degradation. Inactivation of pVHL results in constitutively active HIF and increased expression of HIF target genes, including EPO. In order to examine the contribution of HIF-1 and HIF-2 to liver EPO production, we generated mice that in addition to being VHL-deficient, lacked HIF-1α or HIF-2α in hepatocytes. Mutant mice that lacked HIF-1α developed polycythemia, whereas mice that lacked HIF-2α had normal red blood cell counts, suggesting that liver EPO transcription in this model is dependent on HIF-2 and not HIF-1. In order to examine the role of HIF-1 and HIF-2 in the hypoxic induction of liver EPO (mice were wild-type for pVHL), we determined liver EPO mRNA levels in control, HIF-1α- or HIF-2α-deficient mice that were made anemic by either treatment with phenylhydrazine or by phlebotomy. We found a comparable up-regulation of liver EPO mRNA in control and HIF-1α-deficient mice, whereas HIF-2α-deficient mice lacked the ability to significantly increase liver EPO in response to severe anemia. In summary, our findings provide genetic evidence that HIF-1 and HIF-2 have different roles in the regulation of hypoxic liver EPO induction.

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Regulation and essential role of the *erythropoietin* gene in the liver

Norio Suzuki, Naoshi Obara, Kim Ki-Bom, Xiaoqing Pan, Ko-ichi Jishage, Shigehiko Imagawa, Masayuki Yamamoto. JST-ERATO and Center for TARA, University of Tsukuba, Tsukuba, Japan. Chugai Research Institute for Medical Science, Gotemba, Japan

There is a *cis*-regulatory sequence on the 3′ flanking region of the erythropoietin (Epo) gene, which is first described as a hypoxia-responsible enhancer (3′HRE). To clarify function of 3′HRE in vivo, we performed transgenic mouse reporter assays using GFP transgene under the control of 180-kb Epo gene flanking region. In the anemic adult mouse kidney, expression of the transgene was observed regardless of a point mutation on 3′HRE. In the liver, expression of the wild-type transgene was detected from embryonic day 9 (E9) through adulthood, but expression of the mutant transgene disappeared beyond E15. These data demonstrate that 3′HRE is dispensable for the Epo gene expression in the early embryonic hepatocytes and in the kidney. Additional GFP transgenic assays demonstrated that a 0.5-kb mouse genomic fragment (referred to as BS region) including 3′HRE is sufficient for the inducible gene expression in the adult liver. We also showed that Epo production from the hepatocytes after E15 was abolished in BS-null mouse line generated by gene targeting strategy, and BS-deletion resulted severe anemia in mice in the neonatal stage. The mutant mice was recovered from the anemia within 2 weeks after birth. In the kidney, BS-deletion did not affect the Epo gene expression. In conclusion, 3′ flanking region of the mouse Epo gene is essential and sufficient for liver-specific and hypoxia-inducible gene expression after E15, and the hepatic Epo production is required for normal erythropoiesis in neonatal mice.

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Does liver arterialization can contribute to erythropoietin (EPO) secretion following CO exposure in rats?

H. Zhang[1], A. Grenz[1], C. Gebhard[1], C. Köhle[1], M. Wehrmann[2], R. Lehmann[3], CH. Gleiter[1], H. Osswald[1] Depts. of [1]Pharmacology and Toxicology, [2]Pathology, [3]Internal Medicine IV, University Hospital Tuebingen, Germany

During fetal life EPO is mainly produced by the liver, whereas in adults EPO is predominantly synthesized in the kidneys. We hypothesize that this switch of EPO production is due to the change of oxygen supply to the liver following birth from mainly arterial blood to mainly venous blood supply. Therefore, we arterialised liver blood flow supply by renal portal shunt to assess the extent to which hepatic EPO production can be stimulated by carbon monoxide (CO) exposure. Seven days after liver arterialization rats (LA) were exposed to CO for four hours. Unilateral nephrectomy and ligation of the right gastric vein served as sham-controls (S-LA). Two hours before exposure to CO the left kidney was extirpated in both groups to eliminate renal EPO production. Renal and liver EPO mRNA content in control rats were significantly elevated from 1.2- to 221-fold and 2- to 33.3 fold, respectively, following CO exposure to 100 up to 900 ppm. Under normoxic conditions EPO serum concentrations and hepatic EPO mRNA in LA rats were similar compared to S-LA. However, in LA, exposure to 600 ppm CO exhibited a 1.6 fold increase in EPO serum concentrations and a 2-fold increase in hepatic mRNA content compared to S-LA ($p<0.05$). We conclude, that increased oxygen supply to the liver does not sufficiently compensate the absence of renal EPO secretion following hypoxia.

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GATA factors constitutively repress the erythropoietin gene expression in the epithelial cells

Naoshi Obara, Norio Suzuki, Kim Ki-Bom, Shigehiko Imagawa, Toshiro Nagasawa and Masayuki Yamamoto

GATA transcription factors bind to the promoter proximal region of the Erythropoietin (*Epo*) gene, and repress production of Epo in the human hepatoma cell lines. In order to investigate whether the *Epo* gene expression is negatively regulated by GATA factors *in vivo*, in this study we established transgenic lines of mice expressing green fluorescent protein (GFP) under the control of 180-kb mouse *Epo* gene locus with or without mutation on the GATA-binding motif. GFP expression of the wild-type transgene was induced by anemia or hypoxia specifically in peritubular interstitial cells of renal cortex and hepatocytes surrounding the central vein. Surprisingly, a single nucleotide mutation in the GATA motif (to TATA) resulted in constitutive ectopic expression of GFP in the renal distal tubules, collecting ducts, and certain epithelial cells of other tissues. Since GATA-2 and GATA-3 were both expressed in the renal distal tubules and collecting ducts, we examined whether these GATA factors bind to the GATA motif on the *Epo* gene in these cells using the chromatin immunoprecipitation assay. The results showed that both GATA factors constitutively bound to the promoter region of the *Epo* gene. Taken together, GATA-2 and GATA-3 are likely to constitutively repress ectopic *Epo* gene expression in these cells. Thus, GATA-based repression is essential for the inducible and cell-type specific expression of the *Epo* gene *in vivo*.

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Evidence for the regulation of the renal erythropoietin synthesis by a brainstem factor

U von Wussow, U Frackowski, S Dürkop, H Pagel
Institut für Physiologie, Universität zu Lübeck, Germany

As recently shown (Pflügers Archive 499 Suppl.1 P15-07) an elevation of the intracranial pressure (ICP) leads to significant increases in plasma Epo concentrations. During our efforts to prove that a brainstem factor is involved in renal erythropoietin production, we took a closer look at cerebral Epo gene expression in the brain of the rat by real time RT PCR. We investigated different brain areas (cortex, stem, cerebellum, hypothalamus and hypophysis) under hypobaric hypoxia (increased ICP: 100 mmHg for 10 min) and under anaemic hypoxia (lowering of the hematocrit from 40 to 30%). Both, local hypoxia achieved by raised ICP and anaemic hypoxia led to a two- to fivefold induction of Epo mRNA in the cerebral cortex, stem, cerebellum and hypothalamus. In the hypophysis increased ICP led to a 100 fold induction of the Epo mRNA, whereas anaemic hypoxia led to a downregulation of the basic Epo gen expression; hence, the hypophysis seems to differ from the rest of the brainstem.

These findings support our hypothesis that the hypopysis plays a decisive role in the regulation of the renal Epo production. Under hypoxic conditions the hypophysis possibly releases some yet unknown humoral factors, which trigger Epo synthesis in the kidney.

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Role of CD73 and adenosine signaling for erythropoietin (EPO) secretion during hypoxia

A. Grenz[1], H. Zhang[1], J. Weingart[1], S. von Wietersheim[1], C. Köhle[1], V. Vallon[2], J. Schnermann[3], C. Ledent[4], M. Jacobson[5], HK. Eltzschig[6], LF. Thompson[7], CH. Gleiter[1], H. Osswald[1]
[1]Dept. of Pharmacology and Toxicology, University Tübingen, Germany; [2]University of California, San Diego, USA; [3]NIH, Bethesda, USA; [4]Université Libre de Bruxelles, Begium; [5]Merck, West Point, USA; [6]Dept. of Anesthesiology, University Tübingen, Germany; [7]Immunobiology and Cancer Programm, Oklahoma, USA

The role of adenosine (ADO) and ecto 5`nucleotidase (CD73) in renal EPO production and secretion is still controverse. Therefore, we studied EPO secretion in A_1, A_{2a} and A_3 adenosine receptor (AR) and CD73 knock-out (KO) and their respective wild typ (WT) mice following exposure to 400 and 750 ppm CO and 8% O_2 for 4 h and at 24 h after hemorrhage. To assess possible compensatory mechanisms, we blocked the A_1, $A_{2a/b}$, A_3 AR with DPCPX, DMPX and MRS1191, respectively, and CD73 activity with AOPCP. The data shown that (i) WT and KO mice have approximately the same basal EPO levels under normoxia, (ii) exposure to 400 and 740 ppm CO induced a dose-dependent increase in EPO levels in KO and WT mice, (iii) EPO serum levels and renal EPO mRNA content were similar increased in KO and their respective WT mice to the applied stimuli (CO, 8% O_2, hemorrhage), (iiii) neither AOPCP nor the selective AR antagonists influenced EPO serum levels in KO or WT mice. These experiments clearly demonstrate that neither A_1, A_{2a} or A_3 AR nor CD73 activity are required for stimulation of EPO secretion in response to different hypoxic stimuli.

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Attenuation of HIF by mTOR inhibition is cell-type, serum and oxygen tension dependent and leads to a restricted repression of erythropoietin in the kidney

K. Knaup, K.-U. Eckardt, M. Wiesener. IZKF and Med. Kl. 4; Uni-Klinikum Erlangen, Germany

It has been shown recently that mTOR activates HIF, yet the mechanism and the biological relevance of this interaction are not understood.

mTOR inhibition led to a pronounced decrease of HIF activation in the majority, yet not all cells. The inhibitory effect was dependent on the oxygen tension, concentration and type of serum. In all cases of HIF-insensitivity, there was still a complete blockage of the mTOR/p70S6K/rpS6 pathway by everolimus. Maximum activation of this pathway by insulin treatment or overexpression of the p70S6K did not lead to HIF activation, which implicates that the influence of mTOR on HIF might be (at least partially) independent of the insulin-PI3K-mTOR-p70S6K axis. To test whether the mTOR-HIF regulation is also traceable in vivo we treated rats with everolimus. RNAse protection assays from kidneys showed only moderate effects for VEGF, GLUT-1, and HO-1. On the other hand erythropoietin (EPO) induction showed a strong attenuation by mTOR inhibition.

The regulation of HIF by mTOR is a broad and seemingly potent mechanism, which occurs at pathophysiologically relevant oxygen tensions. This interaction could be clinically relevant in situations such as tumour biology, solid organ transplantation and wound healing, when patients are treated with mTOR inhibitors. The pronounced effects on EPO gene induction could partially explain the frequently observed development of anemia under mTOR inhibition.

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The influence of thyroid hormones on hypoxic gene expression - nuclear or non-nuclear?

T. Otto, Y. Ma, J. Fandrey, Institut für Physiologie, Universitaet Duisburg-Essen

Thyroid hormones are required for proper function of nearly all tissues and have major effects on oxygen consumption and metabolic rate. Especially in the liver many genes, necessary for gluconeogenesis, proliferation and protein glycosylation are regulated by 3,5,3′-tri-iodo-L-thyronine (T_3). In addition, thyroid hormone has recently been found to promote angiogenesis, which is part of the hypoxic adaptational response controlled by the transcription factor complex hypoxia inducible factor 1 (HIF-1). HIF-1 is composed of an O_2-labile α- and a constitutive β-subunit. We have recently reported that HIF-1α is increased by T_3, but whether this is mediated by nuclear or non-nuclear effects of T_3 remains to be resolved. In addition to nuclear receptors (e.g. thyroid hormone receptors, TR) binding of T_3 to several cell surface receptors (e.g. Integrin $\alpha_V ß_3$) \has been described. In this study we tried to clarify to what extend HIF-1α accumulation is due to increased HIF-1α mRNA levels and protein synthesis. HIF-1 target genes involved in angiogenesis like ADM or EPO are regulated by T_3 in hepatocellular carcinoma cells (HepG2). We found that TRß plays an important role in regulating HIF-1α transcription and its target genes. In addition, analysis of several intracellular signal transduction pathways revealed a significant contribution of non-nuclear signalling to elicit T_3 effects of HIF-1 and HIF-1 dependent genes.

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Intracellular allocation of human HIF-α hydroxylases.

Amrei Hilz[1], Antje Kettelhake[1], Matthias Köhler[2], Eric Metzen[1], Reinhard Depping[1]
[1]University of Lübeck, Department of Physiology, Lübeck, Germany; [2]Reha Clinic Damp GmbH, Dialysis Center, Damp, Germany

HIF (hypoxia inducible factor) family members act as transcriptional master regulators of genes involved in cellular and systemic oxygen homeostasis. HIF-1 is the prototypical member of the family and the most important factor in the cellular response to hypoxia. HIF-1 consists of two subunits, HIF-1α and HIF-1β. In normoxia the oxygen-sensitive α-subunit of human HIF-1 is hydroxylated on Pro564 and Pro402 by three distinct oxygen-dependent HIF-1α prolyl hydroxylases (PHD1, PHD2, and PHD3) and thus targeted for proteasomal degradation. The PHDs act as cellular oxygen sensors. 3D 2-photon confocal fluorescence microscopy has shown previously that PHD1 is exclusively present in the nucleus, PHD2 is mainly located in the cytoplasm and PHD3 is homogeneously distributed in cytoplasm and nucleus. Nuclear import occurs through the nuclear pore complex (NPC). Import of macromolecules into the cell nucleus is mediated by soluble import factors (Importins), which bind their import substrates at specific nuclear localization signal sequences (NLS). So far, six human α Importins have been described. Here we report on the intracellular allocation of the human prolyl hydroxylases −1, −2, −3, and the involvement of the human importins in this process.

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A new cell permeable HIF hydroxylase inhibitor stimulates HIF target gene expression *in vitro*

Kathrin Doege, Eric Metzen
Department of Physiology, University of Luebeck, Germany

The adaptation to low oxygen concentrations evoked by chronic or acute hypoxia is mediated by the hypoxia-inducible factor HIF-1. HIF-α levels are affected by changes in the intracellular oxygen concentration. In normoxia the α-subunit is subject to posttranslational modification by oxoglutarate dependent dioxygenases (prolyl hydroxylases PHD1-3 and the asparaginyl hydroxylase FIH-1) and rapidly degraded. In hypoxia, HIF-1α is stabilised, dimerises with HIF-1β leading to the transcription of an array of target genes. HIF-1 is also involved in many pathological processes as ischemic diseases and tumour growth. It is thus an attractive target for pharmacological manipulation.
We synthesised a new cell permeable HIF hydroxylase inhibitor to upregulate HIF-1 in normoxia. 2,4-pyridine dicarboxylic acid (2,4-PDC), an oxoglurarate analogue known from collagen hydroxylases, was esterified and tested for its properties in cell culture studies. ^{t}Bu-2,4-PDC induced HIF-1α accumulation and target gene expression in the low micro molar range in normoxia on mRNA and protein level. Compared to the widely used oxoglutarate analogue dimethyl oxalylglycine (DMOG), our new hydroxylase inhibitor ^{t}Bu-2,4-PDC can be used in an up to 250fold reduced concentration. Thus, we are able to provide an alternative substance to DMOG to analyse HIF-1 induction and target gene expression in vitro under normoxic conditions.

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Identification of hypoxia-inducible factor (HIF)-2α target genes by the use of RNA interference (RNAi) and gene expression profiling

C. Warnecke, A. Weidemann, M. Volke, K.-U. Eckardt, M. S. Wiesener. Department of Nephrology and Hypertension, and IZFK, University of Erlangen-Nuremberg, Erlangen, Germany.

The contribution of HIF-2α to the hypoxic gene regulation is not well understood. Recently we demonstrated by the use of RNAi in different cell lines that EPO is a HIF-2α

target gene, whereas all other known HIF targets investigated were HIF-1α-dependent.
To further define the specific role of HIF-2α we therefore applied microarrray analysis to HIF-1α and HIF-2α-siRNA-treated and 2,2′dipyridyl (DP)-stimulated Hep3B cells. Results were confirmed by RNase protection.
We identified 91 genes which were upregulated more than 3-fold by DP, among them 74 whose induction was decreased by at least 33% by HIF-1α knock-down. Seven genes responded to HIF-2α knock-down and further 10 to both siRNAs. HIF-2α targets were e.g. CITED2, IGFBP1, IL1RAP and PTPRM. These genes were also induced by exposure to 1% O_2 and confirmed to be HIF-2α targets in Hep3B cells. Some of the novel HIF target genes were also upregulated in renal clear cell carcinomas. Target gene specificity, in particular for HIF-2α, was not completely conserved across different cell types, e.g. EPO was HIF-1α-regulated in HepG2 and IGFBP1 in HeLa cells. In HeLa cells no HIF-2α target gene was found.
Thus, we identified and confirmed a large number of novel HIF-1α and several potential HIF-2α target genes. HIF-α isoform-dependency, in particular of HIF-2α, appears to be subject to modulation by cell type-specific factors.

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Role of Erythropoietin in Endurance and Regulation of Respiration

Tyagi MG, R Hema and D Mukherjee
Department of Pharmacology, Christian Medical College, Vellore-632002, India

The gene transcription of haemopoietic factor erythropoietin (Epo) is controlled by hypoxia inducuble factor. The Epo receptor belongs to the cytokine receptor family. The tyrosine kinase JAK2 phosphorylates the Epo receptor. Another enzyme called PI-3 kinase binds to the tyrosine phosphorylation site of the receptor and could be crucial for its actions in the nervous system. Epo release and plasma levels has been shown to be enhanced during physical exercise. A central role for the release of erythropoietin from the kidney has been suggested by recent studies. This study was conducted on Swiss albino mice weighing between 25–35 g. The animals were divided into control (n=7) and Epo treated groups (n=7). The animals were subjected to a 3 minute swim test in normal water. The respiratory rate was counted before and after the swim test. In control animals pretreated with saline only the respiratory rate was reduced by 30.5%. In test group of mice pretreated 20 minutes before with increasing doses of Epo (300–900 IU/kg i.p) attenuated this reduction in respiratory rate thus implying the development of endurance in respiratory system. A possible central nervous role of Epo in this development is suggested.

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Role of HIF signalling in mouse skeletal muscle cells during ischemia

Nathalie Dehne*, Uta Kerkweg#, and Joachim Fandrey*; *Institut für Physiologie, # Institut für Physiologische Chemie, Universität Duisburg-Essen, Hufelandstr. 55, D-45122 Essen, Germany

Hypoxia-inducible factor (HIF) is thought to play an important role in regulating gene-expression in response to ischemia/reperfusion (I/R) in muscle tissue *in vivo*. The meaning and mechanisms in muscle cells is still unclear. Here we studied activation of HIF in skeletal muscle cell cultures. Dimethyl oxalyl glycine (DMOG) was used to identify HIF target genes. Adrenomedulin (ADM), VEGF, lactate dehydrogenase and pyruvate kinase were significantly upregulated in myoblasts after addition of DMOG but GLUT-4 was not HIF-dependent regulated in muscle cells.
By Western blot analysis increased HIF-1 was found in myotubes after hypoglycemia, hypoxia and ischemia whereas myoblasts did not respond to hypoglycemia. Induction of ADM mRNA correlated with the induction of HIF-1α protein in both cell types. VEGF was upregulated in a similar pattern but to a lower degree, whereas lactate dehydrogenase and pyruvate kinase were upregulated only under hypoxic condition but not during ischemia. Though HIF was activated during ischemia in myotubes cells viability tests revealed no protective effect of DMOG against ischemia induced cell death and ATP loss. Myoblasts showed no loss of viability and no loss of ATP. This indicates that though HIF is activated in myotubes it did not protect during ischemia. In contrast, myoblasts showed less signs of injury and seem to play an important role during regeneration and HIF-induced revascularization.

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Human Ovarian Cancer Cells Treated With Erythropoietin Exhibit Paclitaxel-Resistance

Peter Solar, Laurie Feldman, Arthur J Sytkowski, Laboratory for Cell and Molecular Biology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA

Recombinant human erythropoietin (epoetin; Epo) is effective in the treatment of cancer-related anemia and the anemia of chemotherapy. The growth-promoting, anti-apoptotic action of Epo is well established. In addition, several studies - including our own - have shown that Epo may directly stimulate the growth of some tumor cells. We now report that long-term Epo treatment (>2 months) of

A2780 human ovarian cancer cells resulted in a paclitaxel resistant phenotype. This effect was specific for paclitaxel, since cisplatin and carboplatin sensitivity was not affected by Epo treatment. The degree of paclitaxel-induced apoptosis (mono- and oligonucleosome production) was lower in Epo-treated than in non-Epo-treated cells. Flow cytometry demonstrated reduced numbers of Epo-treated cells in G2 phase and increased numbers of cells in S phase of the cell cycle in comparison to control cells following paclitaxel treatment. Interestingly, there was down-regulation of Bcl-2 and Bcl-10 protein in long-term Epo-treated cells. GeneChip expression analysis revealed increased expression of several genes in Epo-treated cells that may be involved in the chemoresistance phenotype. Several other genes were underexpressed in Epo-treated cells. These results may have important clinical implications.

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Aberrant Erythropoietin Receptor Downregulation in Non-Small Cell Lung Carcinoma

E.A. Dunlop[1], A.P. Maxwell[2], T.R.J. Lappin[1]
[1]Haematology Research Group, Centre for Cancer Research & Cell Biology and [2]Nephrology Research Group, Queen's University, Belfast, BT9 7AB, United Kingdom

Erythropoietin receptors have been demonstrated on a variety of cancer cell lines and have also been found in solid tumours, such as breast and prostate. This has raised concerns about the use of erythropoiesis stimulating agents in the treatment of cancer-related anaemia. We have recently identified expression of functional erythropoietin receptors in a non-small cell lung carcinoma cell line (H838) which activated key signalling pathways in response to erythropoietin stimulation. We have now demonstrated impaired downregulation of the erythropoietin receptor in these tumour cells. The erythropoietin receptor is not ubiquitinated following erythropoietin stimulation in H838 cells and there is no turnover of the receptor in either unstimulated or stimulated cells. Compounding this blunted response is impaired SOCS3 induction downstream of erythropoietin stimulation and an extremely delayed SOCS1 response. If a widespread phenomenon, impaired erythropoietin receptor downregulation and degradation in tumour cells has clinical implications for those patients receiving erythropoiesis stimulating agents for cancer-related anaemia.

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Analysis of Erythropoietin Receptor Expression in Human Tumors

Sinclair A, Busse L, Rogers N, Kassner P, Watson V, Arnold G, Sarosi I, Elliott S. Amgen, Inc, 1 Amgen Ctr Dr, Thousand Oaks, CA 91362, USA

Erythropoietin receptor (EpoR) is reportedly expressed in tumors. To investigate the validity of these findings, we evaluated the anti-EpoR antibodies and found they detected proteins significantly larger than the predicted or actual size of EpoR protein (59 kDa). Commonly used antibody C-20 was unable to detect EpoR protein differences in human control vs. cells with *EPOR* knocked down by siRNA. Protein sequencing identified the cross-reactive proteins had sequence homology to EpoR including a 66-kDa heat shock protein (HSP70). Antibody M-20 identified the 59-kDa EpoR, and reduced binding correlated with *EPOR* knockdown. Neither M-20 nor C-20 were suitable for EpoR detection by immunohistochemistry. These data question the findings of studies that used these antibodies.

We subsequently evaluated human tumors for amplification of the *EPOR* locus, levels of *EPOR* transcripts, and expression of EpoR protein. Analysis of 1084 tumors identified amplification of the *EPOR* locus was rare, equivalent to other non-oncogenes. Moreover, levels of *EPOR* transcripts in tumors and tumor cell lines were equivalent to, or lower than, levels in normal tissues of tumor origin. Although EpoR protein was produced in some tumor lines, there were no quantitative associations between protein and transcript levels or presence on the cell-surface. Expression of Janus kinase 2, required for trafficking EpoR to the cell surface, varied between tumor lines, potentially accounting for lack of surface EpoR. These data demonstrate that EpoR is not elevated in tumors compared with normal tissues.

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Evidence against effects of erythropoietin on tumor cells

Laugsch M, Metzen E, Jelkmann W.
Institute of Physiology, University of Luebeck, Germany

In cancer patients on chemotherapy Epo treatment does not only improve the quality of life but also increases tumor oxygenation. However, a concern is that Epo might stimulate tumor cell proliferation and metastasis.

In a panel of human cancer cell lines (MCF7, HepG2, U2-OS, colon carcinoma cell lines) we investigated by RT-PCR, immunofluorescence and Western blot analysis whether the Epo receptor (EpoR) is expressed and transmits a proliferative signal. We detected EpoR mRNA in all cell lines. Neither hypoxia nor Epo stimulated the expression. Three commercial antibodies were tested in immunoblot procedures. All antibodies detected multiple proteins. Accordingly, attempts to visualize endogenous EpoR by immunofluorescence led to multiple staining which was localized to the plasma membrane, but also to the cytoplasm and even the nucleus. Hence, we focused on components of the Epo signal transduction pathway. Only in U2-OS cells moderate activation of MAP-kinases and

Akt1 was occasionally detectable. However, in none of the tumor cell lines Epo induced phosphorylation of JAK2 or STAT5. Consistently, Epo failed to stimulate cell proliferation as assessed by MTT-assays. In the megakaryoblastic cell line UT7/Epo which is known to express functional EpoR phospho-JAK, phospho-STAT5 and stimulation of proliferation were easily detectable.
We conclude that none of the tumor cell lines expresses functional EpoRs. Previous reports of the presence of EpoR in tumor samples appear unreliable and cannot predict adverse effects of EPO treatment.

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Molecular analysis of the EPO receptor gene in patients with tumor associated anemia

Axel A. Fauser, Klinik für Knochenmarktransplantation und Hämatologie/Onkologie, 55743 Idar-Oberstein, Germany

Mutations in the EPO receptor (EPO-R) gene might be a reason for different response rates to EPO treatment. In a pilot study we performed sequence analysis of the EPO-R gene of 49 patients with hematological and oncological malignancies and analysed our results with regard to EPO response. 25 patients received EPO treatment, while 24 other patients served as control group. Among the 25 EPO patients, 18 were considered as EPO responders. Sequence analysis revealed defined repeat allel combinations occurring exclusively in the group of non-responder. Tetranucleotide repeats 12/15, 13/13 and 15/16 in the promoter region were observed neither in responder group nor in control group. The combination 12/15 occurred two times in the non-responder group. Tetranucleotide repeat 14/16 in combination with dinucleotide repeat 18/20 likewise was unique in the group of non-responder (n=7). Only 2 of the non-responders showed an allele combination also occurring in control and responder group, while 5 non-responders had an allele combination which did not appear in the responder group. The cause of non-response to EPO treatment may be ascribed to basic diseases (PRCA, OMF) in these cases. The results demonstrate that predictive discrimination between responder and non-responder should be possible with high probability on the basis of repeat region analysis. Among seven patients of the non-responder group there are five patients clearly discriminable to the responder group due to repeat polymorphism.

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Chronic excessive erythrocytosis increases susceptibility to vascular damage

Ogunshola, O.O.
University of Zurich, Institute of animal physiology and Zurich Center for human integrative physiology

Excessive erythrocytosis results in severely increased blood viscosity, which may have significant detrimental effects on endothelial cells and, ultimately, function of the vascular endothelium. Although our previously characterized erythropoietin-overexpressing transgenic (tg6) mouse line (which has a hematocrit of 0.8–0.9) has no significant increases in brain vascular permeability at 4–5 months of age under normoxic or acute hypoxic conditions brain vascular endothelial cells appeared to be activated, with increased luminal protrusions reminiscent of ongoing inflammatory processes. Consistent with this observation, we detected increased levels of intercellular adhesion molecule-1 and von Willebrand factor, markers of endothelial activation and damage, in brain tissue. Current data now clearly shows that by 9–11 months of age these mice have significant increase in serum levels of IL-6 and vascular VCAM-1 expression. Nuclear NF_kB levels are further augmented in these mice and the JNK and ERK/MAPK pathways are also upregulated indicating an ongoing stress response. In addition P53 levels are induced and Bcl-X_L levels decreased indicating that cell death pathways are activated in these mice. As these mice die prematurely we propose that chronic excessive erythrocytosis and sustained high hematocrit results in inflammation and endothelial damage, and ultimately, increases susceptibility to vascular disease.

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Erythropoïetin and mucle cells

Beaudry M., Launay T., Lottin-Divoux S., Quidu P., Favret F.,Pichon A., Richalet J.P., Duvallet A. Université Paris 13, UFR-SMBH, Laboratoire de Physiologie EA 2363 74, rue Marcel Cachin 93017-Boibigny cedex France

Erythropoietin (Epo) and its receptor (EpoR) are known for their role in the stimulation of proliferation and differentiation of erythroid progenitor cells. The use of recombinant erythropoietin and the studies of EpoR expression have demonstrated that Epo can act as a growth factor on many different tissues such as central nervous system, kidney, gut, (smooth, cardiac and skeletal) muscles.
Our recent data and those of Ogilvie et al. (2000) report that the EpoR is expressed on primary murine satellite cells and myoblast C2C12 cells. These cells exhibit a proliferative response to Epo and a marked decrease in terminal differentiation.
Our aim is to evaluate the functions of erythropoietin receptor (EpoR) for in vitro proliferation and differentiation of muscle cells. The working hypothesis is that in presence of Epo, EpoR induces a decrease in terminal myoblast differentiation.Experiments are carried out on rat myoblasts (L6 line) and primary muscle cell obtained from human biopsies.The presence of transcripts and proteins for EPOR

are monitored by RT-PCR, indirect immunofluorescence and confocal microscopy. Results are correlated to myoblasts differentiation using specific probes for Myf-5, MyoD, myogenin monocarboxylate transporteurs and myosine. Myf-5, MyoD and myogenin are used for early differentiation, monocarboxylate transporters and myosine for terminal differentiation.

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Erythropoietin stimulation of erythropoietin receptor in lung microvascular endothelial cells

Bojana Beleslin-Cokic, Vladan Cokic, Constance Tom Noguchi*; Institute for Endocrinology, Diabetes and Metabolic Diseases, Belgrade, Serbia and Montenegro; *National Institutes of Health, Bethesda, MD, USA.

Low oxygen tension in lung induces pulmonary vascular remodeling. Acute exposure to hypoxia results in pulmonary vasoconstriction and chronic hypoxia can cause pulmonary hypertension. Erythropoietin (EPO) is a hypoxia responsive gene and EPO induction can contribute to pulmonary hypertension via increased erythropoiesis, polycythemia and blood viscosity. In endothelial cells EPO stimulates proliferation, chemotaxis, angiogenesis and nitric oxide (NO) production, while in neuronal cells EPO effect is protective by activating bcl-xl and decreasing NO synthase 1 and 2. We used human microvascular endothelial cells from lung (HMVEC-L) to study acute response to hypoxic stress. EPO stimulates erythropoietin receptor (EPOR) expression at 21% and 2% O_2 by several folds. EPOR protein was also induced, particularly with EPO and low pO_2. We previously observed that eNOS expression followed EPOR expression in other endothelial cells. In contrast, eNOS decreased at 2% O_2 and NO production was very low in HMVEC-L, which contributes to the overall response of HMVEC-L under hypoxia and EPO stimulation and may promote vasoconstriction. A protective effect of EPO at low oxygen, is suggested by Western blotting, showing induction of the antiapoptotic protein bcl-xl after EPO stimulation. Since EPO does not increase NO production at low pO_2, an alternate mechanism must be involved in HMVEC-L adaptation at low pO_2.

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Effect of erythropoietin on human isolated blood vessel and vascular smooth muscle cells

Bojana Beleslin-Cokic, Vladan Cokic, Ljiljana Gojkovic, Constance Noguchi*; Institute for Endocrinology, Diabetes and Metabolic Diseases, Belgrade, Serbia and Montenegro; *National Institutes of Health, Bethesda, MD, USA.

Erythropoietin (EPO) and erythropoietin receptor (EPOR) regulate proliferation, differentiation and viability of erythroid progenitor cells. We have observed that human vascular endothelial cells respond to EPO stimulation by inducing EPOR and endothelial nitric oxide synthase (eNOS) expression, increasing NO production and cGMP, particularly under low pO_2. We now investigate EPO stimulation of vascular smooth muscle (VSM) and the contribution of blood vessel to relaxation/contraction. Human internal mammary artery (HIMA) and human saphenous vein (HSV) were obtained from patients undergoing coronary artery bypass. We observed that EPO (5 U/ml) potentiates noradrenalin-induced contraction by up to 2 fold in HSV and HIMA with the endothelium layer removed, suggesting that EPO may act to modulate or balance the vasodilatory effects of increased NO production by EPO stimulated endothelium. At the molecular level, we found that PI-3 kinase was involved in EPO stimulation with no change in MAP kinase. EPO (5 U/ml) increased cell proliferation more than two fold at 2% O_2, providing some protection to hypoxia. This increased EPO responsiveness at low pO_2 is accompanied by induction of EPOR mRNA by 2 fold. Unlike endothelial cells, VSM cells didn't exhibit EPO induced eNOS or NO production. These results provide evidence for the differential effects of EPO in intact blood vessels affecting VSM and endothelial cells.

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Identification of Differentially Expressed Genes in Human Non-Small Cell Lung Carcinoma by Suppression Subtractive Hybridization

Z Shi, EA Dunlop, WM Brown, TR Lappin
Haematology, Centre for Cancer Research & Cell Biology, Queen's University, Belfast, UK.

In haematopoietic cells the expression of the erythropoietin receptor (EPOR) is largely restricted to erythroid cells but functional interactions between EPO and EPO-R have also been found in numerous cells and tissues. We have shown that EPO at pharmacological concentrations can activate three major signalling cascades viz Jak2/STAT5, Ras/ERK and PI3K/Akt pathways in the non-small cell lung carcinoma (NSCLC) cell line H838. Suppressive subtractive hybridization was used to identify differentially expressed genes in EPO-treated UT-7 erythroleukaemia and H838 cells. Two cDNA libraries containing up- and down-regulated genes in the H838 cells were constructed: SSH-F, using H838 cell cDNA as tester and UT-7 cell cDNA as driver; and SSH-R, using UT-7 cell cDNA as tester and H838 cell cDNA as driver. The SSH-F and SSH-R libraries comprised 321 and 240 clones respectively, of which 34 and 32 genes were characterised by DNA sequencing. Quantitative RT-PCR was used to confirm differential expression of a subset of genes, UBE1, UCHL1, HSPA8, USP32, GATA-6 and SHC1. UCHL1, an ubiquitin

carboxy terminal hydroxylase normally found in neurons and lung neuroendocrine cells, was highly expressed in H838 but not detectable in UT-7 cells. Further characterisation of these genes may permit a better definition of EPO-EPOR signalling in NSCLC cells and help to explain the significance of cytokine receptor overexpression in neoplasia.

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Erythropoietin (EPO) is the stimulus of nonspecific immunity (Experimental Evaluation)

T.V. Parkhomenko, *G.I. Morozova, P.S. Kalinina, V.V. Tomson*; SRC of St.-Petersburg State I.P. Pavlov Medical University, Russia

We investigated the effect of EPO ("Eprex", Cilag) on the activity of leucocytes (LC). To the rats blood we added the solution of citrate Na, fluorescent probe DSM and EPO. "Contr" and "Exp" refer to data before and after blood exposition with EPO, correspondingly. In Contr specimens about 1000 cells were investigated, whereas in Exp 1160 cells. The analysis of the distribution histogram of Contr LC,where their fluorescence intensity (ΔF_{DSM}) is plotted, allowed to reveal 5 clusters of cells; the interval between two neighboring clusters was 50 mV.1:200–250 mV and higher; 2:150–200 mV; 3:100–150 mV; 4:50–100 mV; 5: <50 mV. It was calculate the part of LC in Contr specimens for each cluster with various ΔF_{DSM}. The incubation with EPO gives the unevenness increase of ΔF_{DSM} for different clusters of cells. It was found reliable increase of part of LC with bright fluorescence (1 cluster) and decrease of part of cells with faint fluorescence (5 cluster) in Exp data in comparison with Contr data (Table).

Table (mean±SD)	Part of LC, % Contr	Exp
1	13.6±1.3	25.0±2.0*
2	15.8±2.1	16.6±1.9
3	19.1±1.8	22.1±22.2
4	26.2±2.3	22.1±2.1
5	25.6±2.1	14.2±1.6**

*) $0.001 < P_u < 0.005$; **) $P_u < 0.005$ (Wilcoxons criterion)

In conclusion, EPO exerts indirect effects on the nonspecific immunity.

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Erythropoietin improves behavioral readouts of cognition: Influence on neuroplasticity and neuronal survival

D. Sargin, K. Radyushkin, I. Hassouna, S. Sperling, N. Govindarajan, K. Hannke, A.-L. Sirén*, and H. Ehrenreich Division of Clinical Neuroscience, Max-Planck-Institute of Experimental Medicine, Göttingen, and Experimental Neurosurgery, Julius-Maximilians-University, Würzburg, Germany

Several studies of ourselves and others have shown that erythropoietin (EPO) is capable of improving cognitive function in rodents as well as in man under physiological and pathological conditions, including neurotrauma, schizophrenia, or multiple sclerosis. We are presently exploring the molecular/cellular mechanisms of EPO-induced effects on cognition. Using a standardized cryo-lesion model of the right parietal cortex of juvenile (4 week-old) mice, we found global neurodegenerative changes occurring many months after the lesion. Interestingly, EPO prevented behavioral abnormalities, cognitive dysfunction and brain atrophy, when given every other day for 2 weeks after this acute brain lesion. Work will be presented to explain mechanisms of the observed atrophy and the EPO action, e.g. effect on neuronal survival, synaptogenesis, neurogenesis and expression pattern of various markers involved in pathogenesis of neurological disorders, e.g. GABAergic genes. In addition, we are investigating how EPO influences functional synapse formation and network activity *in vitro*. The prominent EPO effects on neuroprotection and, in particular, on neuroplasticity will open new avenues for treatment of neurological and psychiatric diseases.

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Effects of thrombopoietin (TPO) and granulocyte colony-stimulating factor (GCSF) on cell viability and intracellular signaling pathways in neurons and astrocytes

N. Byts, A. Samoylenko, H. Woldt, H. Ehrenreich and A.-L. Sirén Experimental Neurosurgery, Julius-Maximilians-University, Würzburg, and Division of Clinical Neuroscience, Max-Planck-Institute of Experimental Medicine, Göttingen, Germany

Thrombopoietin (TPO) and granulocyte colony-stimulating factor (GCSF) act via cytokine single chain receptors to induce neuronal apoptosis (TPO) or protect neurons from glutamate-toxicity (GCSF). We characterised activation of intracellular signaling upon proapoptotic (TPO) or anti-apoptotic (GCSF) cytokine receptor activation by investigating the effects of TPO and GSCF on cell survival, proliferation and phosphorylation of Akt, ERK1/2 and STAT3 in primary cultures of rat hippocampal neurons and cortical astrocytes. TPO induced neuronal death and selectively activated ERK1/2. GCSF protected neurons from TPO- and hypoxia-induced cell death via Akt1. In astrocytes, TPO and GCSF inhibited proliferation and induced ERK1/2 with no effect on cell viability or Akt1 phosphorylation. TPO inhibited STAT3 activity in astrocytes. Our data demonstrate a cell-type specific pattern of activities for TPO and GCSF in brain cells. A balance

between these growth factors, their receptors and signalling proteins may play an important role in regulation of neural cell survival.

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Erythropoietin Promotes Survival and Invasiveness of Astrocytomas

A. Mohyeldin, CL Dalgard, H Lu, T Mcfate, AS Tait, VC Patel, K Wong, E Rushing, S Roy, G Acs, and A Verma. Uniformed Services University of the Health Sciences, Bethesda, MD 20814.

The hypoxia-inducible hormone erythropoietin can promote the development and survival of neurons and astrocytes. Since hypoxia has also been implicated in the malignant progression of human cancers, we determined whether erythropoietin signaling influenced malignant properties of human astrocytoma cells. RT-PCR, western blotting, and immunohistochemistry were used to measure erythropoietin and its receptor. Cell viability, Matrigel invasion assays, metalloprotease assays, erythropoietin neutralizing antibodies and erythropoietin receptor verexpression were used to study biological actions of erythropoietin. Expression of erythropoietin and its receptor was observed in the hypoxic regions and invasive margins of glioma biopsies. Erythropoietin receptor expression correlated with tumor stage. Erythropoietin receptor was also functionally up-regulated by hypoxia in cultured glioblastoma cells. Both hypoxia and erythropoietin protected cultured glioblastoma cells from cisplatin cytotoxicity and also promoted the invasiveness of glioblastoma cells through Matrigel by potentiating metalloprotease activity. Hypoxia-enhanced cell invasion was attenuated in cells over-expressing a non-functional erythropoietin receptor. Hypoxia-inducible autocrine and paracrine erythropoietin signaling participates in the malignant progression of glioblastomas.

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Signaling by hematopoietic growth factors in neural cells

A.-L. Sirén, Experimental Neurosurgery, Department of Neurosurgery, Julius-Maximilians-University, Würzburg, Germany

Hematopoietic growth factors have great potential for future therapeutic applications in brain disease but their intricate interactions in brain cells need to be uncovered. We show that three hematopoietic growth factors, erythropoietin (EPO), granulocyte-colony-stimulating factor (G-CSF) and thrombopoietin (TPO) exert cell type specific effects on survival, proliferation and differentiation of neural cells which reflect on their ability to modulate the phosphorylation of Akt1, ERK1/2 and STAT3/5 in cultures of rodent neurons and astrocytes. In neurons, TPO induces cell death and inhibits differentiation in a neuronal cell line by selectively modulating ERK1/2 activity. EPO and GCSF antagonize TPO-effects and protect neurons from hypoxia-induced cell death via activation of Akt1. In astrocytes an activation of ERK1/2 and inhibition of STAT3 activity is accompanied by an antiproliferative response to TPO. The differential response of neurons and astrocytes to hematopoietic growth factors in respect to activation of intracellular signalling molecules implies a highly specific role of these factors in mature cells of the neural lineage. A balance between growth factors, their receptors and signaling proteins may play an important role in regulation of cell survival in the brain during development and repair after injury.

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Mechanisms of *Erythropoietin-Receptor* Gene Regulation in Neuronal Cells

Iwona Palaszewki, Thomas Wallach, Anika Hartmann, Christiane Hussels, Christof Dame. Department of Neonatology, CVK, Charité - Universitätsmedizin Berlin, Germany

Erythropoietin (Epo) and its receptor (EpoR) are expressed in the nervous system. Since Epo has significant neuroprotective effects current studies aim to use recombinant Epo after brain injury.

The aim of this study was to investigate the role of GATA transcription factors in regulating *EpoR* gene expression in neuronal precursor cells. The question was approached by using molecular biological techniques.

Binding of GATA-2, -3 and -4 to the *EpoR* 5′ untranslated region (5′ UTR) was found by EMSA, but could not be confirmed by ChIP assay that indicated binding of basal transcription factors in neuronal cells. Reporter gene assays showed that the *EpoR* 5′ UTR from nt −316 to −301 in the vicinity of the −271 GATA binding site is sufficient to drive reporter gene expression in these cells. This region, which obviously binds RNA polymerase, contains a cluster of partially overlapping binding sites for several transcription factors. While overexpression of GATA factors alone activated reporter gene expression, there was no significant stimulation of *EpoR* mRNA expression.

In conclusion, our data suggest a complex mechanism of *EpoR* regulation in neuronal cells. Expression seems to be driven by a domain of the 5'UTR other than that described in hematopoietic cells. This domain contains binding sites for several transcription factors, which are currently under investigation.

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Erythropoietin and Neural Development

Constance T. Noguchi and Zhi-Yong Chen. Molecular Medicine Branch, NIDDK, National Institutes of Health, Bethesda, MD USA

Erythropoietin (Epo) signaling is critical for erythropoiesis and EpoR null mice die in utero from lack of mature erythrocyte production. Nonetheless, Epo and EpoR are expressed in the nervous system during development and adulthood. In the embryo EpoR is expressed at high levels in brain on neuronal cells, and Epo signaling prevents apoptosis. To study endogenous Epo function in the late developing and adult brain, we selectively rescued the EpoR null genotype with EpoR expression driven by its own promoter in hematopoietic tissue but not in brain. These mice survive through adulthood and exhibit normal hematopoiesis and erythrocyte production with no gross organ defects. Loss of EpoR in brain results in increased apoptosis during fetal development and neurons from these mice are more susceptible to hypoxia in culture. Adult brain EpoR-null mice perform suboptimally in the Morris Water Maze test that may relate to new neuron production in the hippocampus. Neural progenitor cell proliferation in the hippocampus and subventricular zone are reduced in these animals. These observations demonstrate that endogenous Epo/EpoR signaling in brain provides protection to the pool of proliferating neural progenitor cells during development and contributes to neurogeneisis in the adult. Therefore, Epo is neuroprotective independent of insult, injury or ischemic events, functions beyond stimulation of erythropoiesis, and contributes to neurogenesis.

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Improvement of cognitive functions in chronic schizophrenic patients by recombinant human EPO

Hannelore Ehrenreich
Division of Clinical Neuroscience, Max-Planck-Institute of Experimental Medicine, Göttingen, Germany

Schizophrenia is characterized by cognitive decline and progressive loss of cortical gray matter. We hypothesized that a neuroprotective/neurotrophic add-on strategy, recombinant human erythropoietin (rhEPO) in addition to stable antipsychotic medication, would improve cognitive function even in chronic schizophrenic patients. We designed a double-blind, placebo-controlled, randomized, multicenter, *proof-of-principle* (phase II) study, with a total duration of 2 years and an individual duration of 12 weeks. Chronic schizophrenic men (N=39) with defined cognitive deficit, stable medication and disease state were treated for 3 months with weekly intravenous infusion of 40,000 IU rhEPO (N=20) or placebo (N=19). Main outcome measure was cognitive function (RBANS subtests delayed memory, language-semantic fluency, attention, and WCST-64 - perseverative errors) tested over 2 days at baseline, 2 weeks, 4 weeks, and 12 weeks of study participation. Both, placebo and rhEPO patients improved in all evaluated categories. Patients receiving rhEPO showed a significant gradual improvement over placebo patients in schizophrenia-related cognitive functions, without yet reaching a plateau at 12 weeks, but no effects on psychopathology or social functioning. Also, a significant decline in serum levels of S100B, a glial damage marker, occured upon rhEPO. The fact that rhEPO is the first compound to exert a selective and lasting beneficial effect on cognition should encourage new treatment strategies for schizophrenia.

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Expression and action of erythropoietin in the developing mouse retina

N. Scheerer, N. Dünker* and J. Fandrey
Institute für Physiologie und *Anatomie - Neuroanatomie, Universität Duisburg-Essen

The hematopoietic hormone erythropoietin (Epo) has also been found to protect neurons from various apoptosis. To examine whether Epo plays a role in cell death decisions during neurogenesis, we made use of the developing mouse retina as an excellent model system for the CNS. The retina is easily accessible and less complex than other neuronal tissues. Complete retinas were explanted and homogenised for RNA-extraction or cultured as organotypic explants in DMEM for subsequent factor treatment. Epo and Epo receptor (EpoR) expression in the retina was determined by RT-PCR on postnatal days 0 (P0) to 20 (P20). We detected a continuous expression of Epo-mRNA during postnatal retinal development. Epo expression in the retina was compared with Epo mRNA levels in the developing liver and kidneys. EpoR expression peaked on P15, exactly within a physiological phase of retinal apoptosis. Treatment of P15 retinal whole-mount cultures with rhEpo resulted in a significant decrease of apoptosis. Moreover, transforming growth factor beta (TGF-ß)-induced apoptosis was completely blocked by Epo when both factors were applied simultaneously. These data indicate that Epo antagonises physiological neuronal cell death as well as TGF-ß mediated retinal apoptosis.
Our data indicate that a balance between pro-apoptotic TGF-ß and anti-apoptotic Epo in the developing mouse retina may contribute to proper development in the CNS.

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Recombinant human erythropoietin prevents ischemia-induced apoptosis and necrosis in explant cultures of the rat organ of Corti

Johann Gross[a], Nadeshda Andreeva[b], Nyamaa Amarjargal[a], Heidemarie Haupt[a], Renate Moller[a], Birgit Mazurek[a]
[a]Molecular-Biological Research Laboratory, Department of Otorhinolaryngology, Charité - University Medicine Berlin, Spandauer Damm 130, Bld. 31, 14050 Berlin, Germany

[b]Brain Research Institute, Academy of Medical Sciences, Obucha 5, 103064 Moscow, Russia

This study was designed to evaluate the effect of recombinant human erythropoietin (rhEPO), insulin-like growth factor-1 (rhIGF-1) and epidermal growth factor (rhEGF) on ischemia-induced hair cell loss in an organotypic cochlea culture. The apical, middle and basal parts of the organs of Corti (newborn rat, PD 3–5) were exposed to ischemia (3.5 h) in glucose-free artificial perilymph (pO_2 10–20 mm Hg) without or with growth factors. Controls were exposed to normoxia. 24 h after onset of ischemia, the cultures were stained using TRITC phalloidin (intact hair cells), propidium iodide (necrotic nuclei) and in situ DNA End Labeling Assay (ISOL; apoptotic nuclei).

Ischemia (3.5 h) induced a hair cell loss of 20 and 40% in the middle and basal cochlear parts and an increase of the numbers of apoptotic and necrotic nuclei (controls 0–1, ischemia 4–7 nuclei/100 μm). The basal part was more affected than the apical one. rhEPO and rhIGF-1 significantly attenuated the ischemia-induced hair cell loss by reducing apoptosis and necrosis.

RhEPO has been in clinical use for more than a decade and found to be well tolerated. Therefore, rhEPO could be an effective drug for the prevention of hearing loss via a hair cell protective mechanism.

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