Aus dem Institut für Physiologie, der Universität zu Lübeck
Direktor: Prof. Dr. med. W. Jelkmann

Erythropoietin production: Molecular mechanism of the antagonistic actions of cyclic adenosine monophosphate and interleukin-1

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Chimedtseren Batmunkh
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*

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Abbreviations

ACD	Anaemia of chronic disease
APS	Ammonium persulphate
ARNT	Aryl hydrocarbon receptor nuclear translocator
BSA	Bovine serum albumin
BFU-E	Burst-forming units-erythroid
Bt_2-cAMP	Dibutyryl cyclic adenosine monophosphate
CACA	Sequence of the 3' enhancer of Epo gene
CREBP	cAMP responsive element binding protein
DEAE	Diethylaminoethanol
DFO	Deferoxamine
DH5α	Competent cells
ds DNA	Double-stranded DNA
cDNA	Complementary DNA
CNS	Central nervous system
CFU-E	Colony-forming units-erythroid
DEAE	Diethylaminoethanol
DMEM	Dulbecco's modified Eagle medium
DNA	Desoxyribonucleic acid
DR-2	Direct repeat
DTT	Dithiothreitol
dNTP	Deoxynucleotide triphosphate
ECL	Enhanced chemiluminescence substrate
EDTA	Ethyleneglycoltetraacetic acid
ELISA	Enzyme linked immuno sorbent assay
EMSA	Electrophoretic mobility shift assay
Epo	Erythropoietin
Epo wt	Erythropoietin wild type plasmid
ECL	Enhanced chemiluminescence substrate
FCS	Fetal bovine serum
Fig	Figure
GATA-2	Transcription factor

GTC	Guanidinium thiocyanate-chloroform
HeLA	Cervix carcinoma cell line (Helena Lange)
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HepG2	Human hepatoma carcinoma cell line
Hep3B	Fetal hepatoma carcinoma cell line
HIF-1	Hypoxia inducible factor-1
HNF-4	Hepatic nuclear factor-4
HRP	Horseradish peroxidase
HRG1	Stably transfected HepG2 cells
kDa	Kilo Dalton
Ig G	Immunoglobulin G
IL-1	Interleukin-1
IL-6	Interleukin-6
LPS	Lipopolysaccharide
mU/ml	Milliunits per milliliter
mRNA	Messenger ribonucleic acid
n	Number of independent experiments
NF-κB	Nuclear Factor κappa-B
NP-40	Nonylphenylpolyethylene glycol
OD	Optical density
Oligo (dT)	Primer
ONPG	o-nitrophenyl-β-D-galactopyranoside
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pCMV-IκBα	Dominant-Negative vector of IκBα
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
pGL3	Vector
PMSF	Phenylmethylsulfonyl fluoride
p50	NF-κB subunit
p65	NF-κB subunit

pRLSV40..... Renila plasmid
RBC……..... Red blood cells
Rh……. Recombinant human
RPMI 1640…….....Medium for cell culture
RT……...…….....Reverse transcriptase
RT-PCR…….....Reverse transcription polymerase chain reaction
SDS……..... Sodium dodecylsulphate
SP1……...Transcription factor
TBE…….....Tris borate-EDTA buffer solution
TAE…….. Tris acetate-EDTA buffer solution
TEMED…….....N,N,N',N'-Tetramethylethylenediamine
TNF α…….....Tumor necrosis factor alpha
TRIS…….....Tris [Hydroxymethyl] aminomethane
T-PBS…….. TEMED-phosphate buffered saline

1. Introduction

1.1. Erythropoietin production and sites

Erythropoietin (Epo) is a haematopoietic hormone required for the differentiation and proliferation of red blood cell (RBC) progenitors and for increased oxygen transfer and delivery. Before birth, Epo is primarily produced in the liver (Zanjani et al., 1977; Dame et al., 1998; Clemons et al., 1986). The primary site of Epo production switches from liver to kidneys shortly after the birth (Jelkmann et al., 1981; Maxwell et al., 1993; Maxwell et al., 1990). Localization of Epo production in kidneys was first demonstrated in 1957 by Leon Jacobsen et al., who showed that rats and rabbits, after bilateral nephrectomy, do not respond to haemorrhage with an appropriate increase in plasma Epo levels. The liver synthetises relatively little Epo. In addition, Epo is produced in the brain (Masuda et al., 1994) with Epo receptors having been detected in mouse brain (Digicaylioglu et al., 1994) and in cell lines with neuronal properties. Additional extrarenal sites of Epo synthesis like ovary, testis, uterus and intestine have been described (Yasuda et al., 1998; Magnanti et al., 2001).

The Epo synthesis is subject to a complex control circuit that links kidneys and bone marrow in a feedback loop (**Fig. 1**) (Bauer et al., 1989; Ratcliffe et al., 1993). Epo is produced by specialized cells in kidneys. These cells are sensitive to the level of the oxygen in blood, and they are able to enhance the release of Epo when the oxygen concentration is low or when the oxygen affinity of blood is increased (Jelkmann 1992; Porter et al., 1993). Since RBC carry oxygen, a low number of RBC (for example in the case of anaemia) will result in Epo release. At the bone marrow, Epo acts on both the burst forming units (BFU-E) and the colony forming units (CFU-E) for terminal differentiation. With the formation of new red cells, the red cell mass is increased, which augments oxygen delivery to the tissues, thereby restoring normal tissue oxygen tension. This translates into a relationship between plasma Epo and haematocrit.

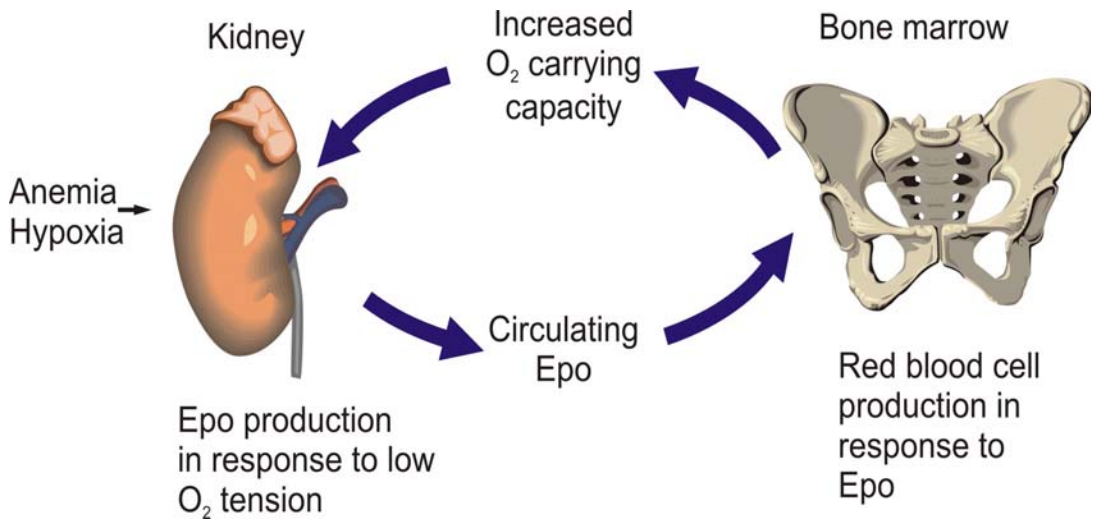

Fig. 1. Feedback of Epo synthesis

1.2. Anaemia due to a lack of erythropoietin

The hormone Epo can be detected and measured in the blood. Normal level of plasma Epo is between 11 and 34 milliunits per millilitre (mU/mL). The result of the underproduction of Epo is linked to a condition known as anaemia, or the exhaustion of RBC (Purves et al., 2001). Decreased RBC production reduces the oxygen-carring capacity of the blood, resulting in tissue hypoxia. Epo levels normally increase in anaemia in order to restore the normal tissue oxygenation conditions. Among some of the diseases associated with decreased level of Epo are cancer, autoimmune diseases like rheumatoid arthritis, HIV infection, and anaemia of prematurity. In some of these cases, like anaemia of prematurity, a problem within the translation of the Epo-coding gene into its protein is the cause of low Epo levels (Faruki and Kiss 1995). It is assumed that the switch of the synthesis of Epo from liver to kidneys that in many mammals occurs at birth may be the cause of underproduction of Epo in premature infants. In other diseases, such are chronic renal diseases, the cause is the destruction of Epo-producing cells in the kidney (Adamson et al., 1968). A resulting lack of Epo production is due to the fact the kidneys' function is impaired, and likewise, because Epo is produced mostly in the kidneys, its production is also lowered (Erslev 1991). However, in cases of anaemia associated with cancer, chronic inflammations and other chronic diseases, decreased levels of Epo are due to the inhibition of Epo and Epo mRNA production by proinflammatory cytokines such as interleukin (IL-1)

and tumour necrosis factor alpha (TNF-α). These compounds are generated as a result of these diseases (Faruki and Kiss 1995).

1.3. Proinflammatory cytokines and their role in anaemia of chronic disease

The proinflammatory cytokines IL-1 and TNF-α are central mediators of immune responses and are produced by macrophages, lymphocytes and endothelial cells (Mizel 1982). TNF-α is a 17-kDa protein mainly released by monocytes and macrophages and is a common mediator of toxic shock, cachexia and tumour necrosis. IL-1 is also a 17-kDa protein that has a wide variety of actions in inflammation and immunity and shares many of the properties of TNF. IL-1 was first described as a lymphocyte-activating factor. Later, it was discovered that IL-1 has a number of other biological activities. There are at least two major types of IL-1 (alpha and beta) that bind to the same receptor and play an important role in the pathophysiology of a variety of chronic inflammatory diseases (Dinarello 1984).

The anaemia of chronic disease (ACD) is a hypoproliferative anaemia defined by a low serum or plasma iron concentration in the presence of adequate reticuloendothelial iron stores. In the pathogenesis of ACD (**Fig. 2**), several factors act concomitantly - increased haemolysis, impaired iron turnover and reduced erythropoiesis (Means et al., 1992). IL-1 and TNF-α have the ability to induce anaemia by inhibiting the Epo mRNA and Epo production *in vitro* and *in vivo* (Jelkmann et al., 1992). The erythropoiesis-suppressing effect of inflammation is mainly due to the increased activity of the proinflammatory cytokines (Trey et al., 1995; Means 1999). *In vivo*, the cytokines IL-1 and TNF-α act in concert to affect precursor cells at different stages of erythropoiesis. In experimental animal studies and in humans, administration of both cytokines causes a hypoproliferative anaemia, either directly, by influencing erythroid progenitor cells, or indirectly, by stimulating interferon production (Feelders et al., 1998; Allen et al., 1999; Goicoechea et al., 1988). Despite the overall suppressive effect on erythropoiesis, some studies have shown that TNF-α and IL-1 stimulate the growth of early progenitors BFU-E, while suppressing the growth of later stages CFU-E (Trey et al., 1995). Administering recombinant Epo (Rh-Epo, epoetin) can over-

come the inhibitory effect of TNF-α and IL-1 on erythropoiesis in a dose-dependent fashion.

One theory about cytokines and other immune factors and their role in anaemia suggests that they change the dynamics of iron circulation, causing iron to be held back from release into the developing RBC in the marrow. In this case, iron stores are high, but the usable iron in circulation is low. Moreover, TNF-α increases in patients with cancer (Balkwill et al., 1987), rheumatoid arthritis (Vreugdenhil et al 1992, Stokenhuber et al., 1994), parasitic and bacterial infections (Tempo et al., 1987; Kern et al., 1989). Infection with pathogenic microbes often results in a significant inflammatory response. A cascade of proinflammatory cytokines including TNF-α and IL-1 initiates this response (Shin et al., 2003). In the animal model, TNF-α administration has resulted in changes of iron metabolism characteristic for the anaemia of inflammation or malignancy. Also, IL-1 and lipopolysaccharide (LPS) have been shown to suppress renal Epo gene expression in rats (Frede et al., 1997).

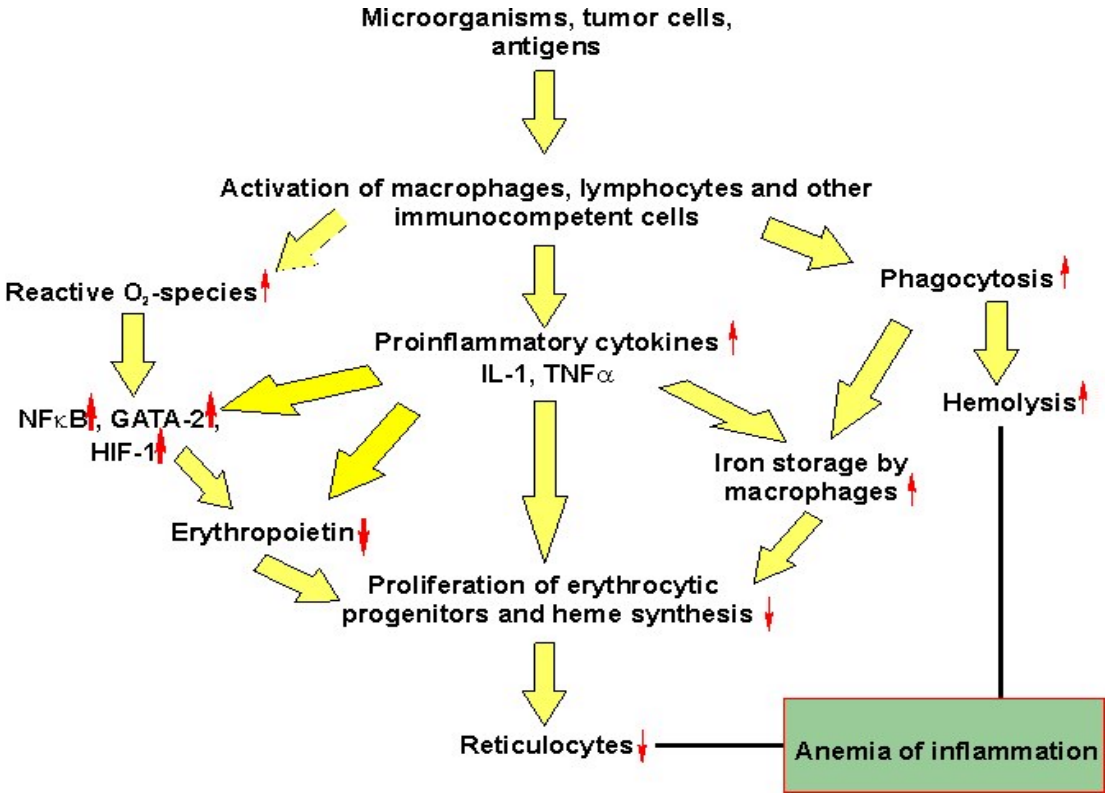

Fig. 2. Scheme representing pathogenesis of ACD (Jelkmann et. al., J. Interferon Cytokine Res. 1998)

1.4. Cyclic adenosine monophosphate

Cyclic adenosine monophosphate (cAMP) is a molecule that is important in many biological processes. It is derived from adenosine triphosphate (ATP) by adenylate cyclase. Adenylate cyclase is located at the cell membranes. cAMP is a secondary messenger, with the role in intracellular signal transduction, such as transferring the effects of hormones, and other substances which cannot get through the cell membrane. cAMP is degraded by specific phosphodiesterases. cAMP is able to influence some protein-dependent kinases, for example, protein kinase A (PKA). PKA is a heterotetrameric holoenzyme consisting of two catalytic (C) and two regulatory (R) subunits. In the presence of cAMP both regulatory subunits bind two cAMP molecules and release the catalytic C subunits. Once released, these C subunits are fully active to phosphorylate substrate proteins on threonine and serine residues. ACD results from increased macrophage activation and enhanced cytokine secretion (TNF-α, IL-1, IFN-γ), which can be regulated by cAMP-increasing agents (Manuel et al., 1995; Claessens et al., 2002). cAMP-increasing agents, like forskolin and certain prostaglandins, affect erythropoiesis by modulating the Epo-mediated signalling cascades, both in vitro and in vivo (Fisher et al., 1980; Belegi et al., 1983; Rossi et al., 1980; Pelus et al., 1988).

1.5. Structure of erythropoietin

In humans, the Epo gene is located on the long arm of chromosome 7 (7q21) (Lai et al., 1986; Watkins et al., 1986) and it comprises five exons. This gene codes for Epo - a circulating, 165 amino acids long glycoprotein with a molecular mass of approximately 30.4 kilo-Dalton (kDa) (Jacobs et al., 1985; Lin et al., 1985). Epo is made of a four-membered alpha-helical bundle with connecting loops (Wen et al., 1994). There are four terms used to identify the helices of the Epo molecule - A, B, C and D (**Fig. 3**). Four cystein residues form two intrachain disulfide bridges, one from Cys 7 to Cys161, and another from Cys 29 to Cys 33. The role of the disulfide bridge between Cys 7 and Cys 161 is to hold together helices A and D. The B and C helices are linked with a short loop. Additionally, the structure of Epo is stabilized by several

aromatic amino acids, which form the hydrophobic core of Epo. These amino acids are located along the interior side of the D-helix and are directed against the hydrophobic side chains of the A, B and C helices.

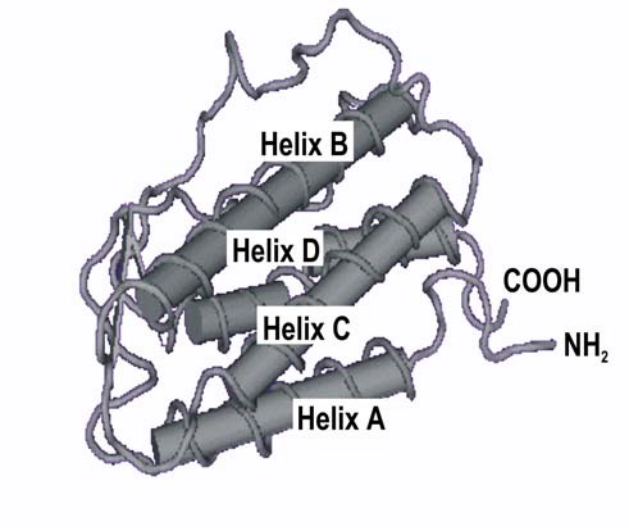

Fig. 3. Model of the three-dimensional structure of Epo. (Cheetham et al., Nat. Struct. Biol. 1998)

1.6. Transcriptional regulation of the erythropoietin gene

The rate of the transcription of Epo is regulated by several transcription factors like Hypoxia Inducible Factor-1 (HIF-1), Hepatocyte Nuclear Factor-4 (HNF-4), GATA-2 and GATA-4 and Nuclear Factor Kappa of B-cells (NF-κB). Therefore, the Epo gene possesses several regulatory DNA elements, either in the 5' promoter or in the 3' enhancer regions (**Fig. 4**).

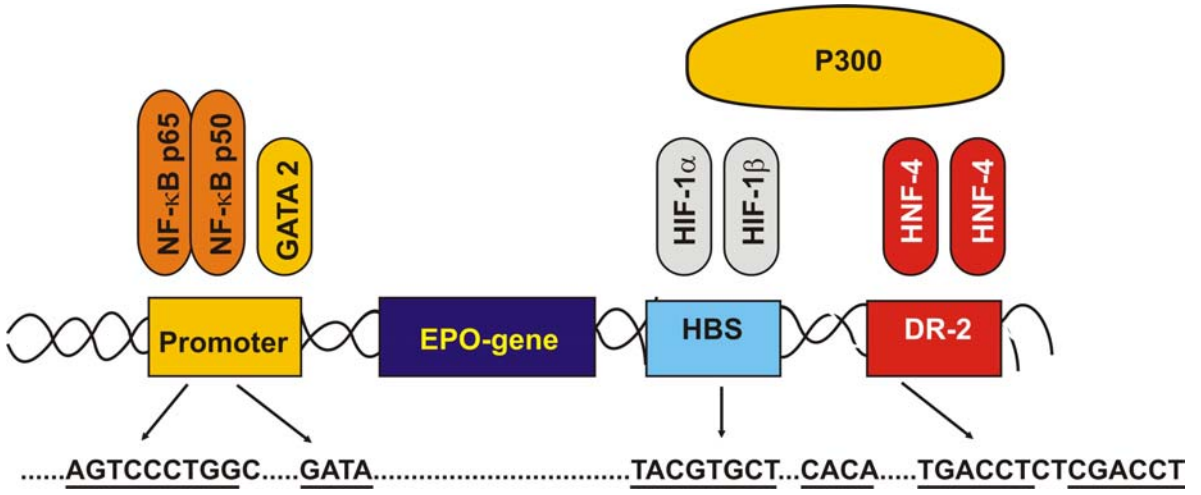

Fig. 4. Scheme of the human Epo gene

Detailed characterization of the Epo 3' enhancer led to the identification of three sites that are important for regulation by hypoxia (Semenza et al., 1992; Blanchard et al., 1992; Pugh et al., 1994). On the 3' side, the sequence TACGTGCT was the first response element to be characterized for the transcription factor, HIF-1 (Semenza et al., 1992). Binding of HIF-1 to this site is induced by hypoxia, and an intact HIF-1 binding site is necessary for hypoxically inducible function of the Epo enhancer. A second site, 7 base pairs (bp) 3' to the HIF-1 binding site, has the sequence CACA in the human Epo gene. No proteins are known to bind to this site, but mutation of this site abrogates hypoxia inducible activity of the enhancer. The sequence of the third site in the Epo enhancer is a direct repeat of 2 steroid hormone receptor half sites separated by 2 bp, termed a DR-2 site (Blanchard et al., 1992) which interacts with orphan receptor HNF-4. HNF-4 is a nuclear protein and belongs to the family of ligand-activated transcription factors. It functions as a transcriptional activator for hypoxia-specific Epo expression, and it is antagonized by EAR3/COUP-TF1 (Galson et al., 1995). Several studies have shown that mutations of DR-2 site inhibit hypoxic induction (Semenza et al., 1992; Blanchard et al., 1992; Pugh et al., 1994). The most common promoter element in eukaryotic protein genes is the TATA box, located at 35 to 20 nucleotides upstream from the transcription start site.

The Epo promoter does not have consensus TATA or GAAT motifs in either the mouse or human genes. Instead, this region contains the GATA motif, which has a binding site for GATA-2 transcription factor, a negative regulator of Epo. One report suggests that the Epo promoter contributes to the hypoxic inducibility of the Epo gene (Imagawa et al., 1991). After deletion of the 3' enhancer, expression of a stably transfected marked Epo gene was induced approximately 10-fold in response to hypoxia (Ho et al., 1995). Moreover, the 5' flanking region of human Epo promoter includes several other putative elements like cytokine consensus sequence, tissue specific and metal responsive elements and also binding sites for transcription factors like NF-κB (Barnes et al., 1997), SP1 and AP1.

1.6.1. HIF-1, a positive regulator

Low level of oxygen in the body, known as hypoxia, triggers the pathway leading to Epo production, and consequently, erythrocyte production. This process is accomplished, through the use of the transcription factor HIF-1, a positive regulator of Epo,

located on Epo 3' enhancer (Maxwell et al., 1993). Hypoxia, cobalt, and deferoxamine (DFO), stimuli that trigger Epo expression also activate HIF-1 DNA-binding (Jiang et al., 1996). The HIF-1 site in the Epo 3' enhancer was the first hypoxia response element (HRE) to be identified and was used for the affinity purification of HIF-1 (Wang et al., 1995). Moreover, HIF-1 activates transcription of several genes including HIF-1 (inducible) nitric oxide synthase (Melillo et al., 1995; Palmer et al., 1998), heme oxygenase-1 (Lee et al., 1997), transferrin (Rolfs et al., 1997), vascular endothelial growth factor (VEGF) and glycolytic enzymes.

HIF-1 is a member of the basic helix-loop-helix/PAS domain protein family that consists of HIF-1 alpha (HIF-1 α) (120 kDa) and the aryl hydrocarbon receptor nuclear translocator (ARNT) or HIF-1 beta (HIF-1 β) (94 kDa) (Wang et al., 1995; Salceda et al., 1997). There are two transcriptional activation domains in HIF-1 α, the one that is referred to as N-terminal transactivation domain (N-TAD), and the other that was named C-terminal transactivation domain (C-TAD). The transcriptional activity of the C-TAD is hypoxia inducible (Li et al., 1996). In contrast, HIF-1 β contains only one transcriptional activation domain (TAD) at the C terminus. Furthermore, HIF-1 α possesses a unique oxygen-dependent degradation domain (ODDD) that critically controls protein stability (**Fig. 5**). Deletion of this ODDD resulted in stabilization of HIF-1 α and constitutive HIF-1 DNA-binding activity independent of oxygen tension (Huang et al., 1996). Under normoxic conditions HIF-1α is rapidly hydroxylated by prolyl hydroxylases (PHDs). The PHDs are characterized as non-heme iron enzymes, whose activity requires Fe^{2+}, oxygen, ascorbate and 2-oxoglutarate as co-substrates. Under normoxia, the PHDs transfer one oxygen atom to the proline residues of HIF-1 α subunits, and another oxygen atom reacts with 2-oxoglutarate, yielding succinate and carbon dioxide as products. The modified HIF α-subunit becomes associated with the von Hippel-Lindau tumour suppressor protein (VHL), which targets it for ubiquitinylation and proteasomal degradation (Salceda et al., 1997; Huang et al., 1998). Under hypoxia the activity of PHDs is limited by the lack of oxygen and thus HIF-1α is enabled to enter to nucleus and dimerises with HIF-1 β. These heterodimers bind to specific HIF-1 DNA motifs (CCA CGT GG) (Semenza et al., 1992) and interact with adjacent p300/CBP cofactor to initiate transcription of target genes. Although signalling through the HIF-1 pathway has been studied primarily in the context of cellular adaptation to hypoxia, some of data suggest that proinflammatory mediators can also

upregulate HIF-1α protein expression and HIF-1 DNA-binding activity (Haddad et al., 2001; Thornton et al., 2001).

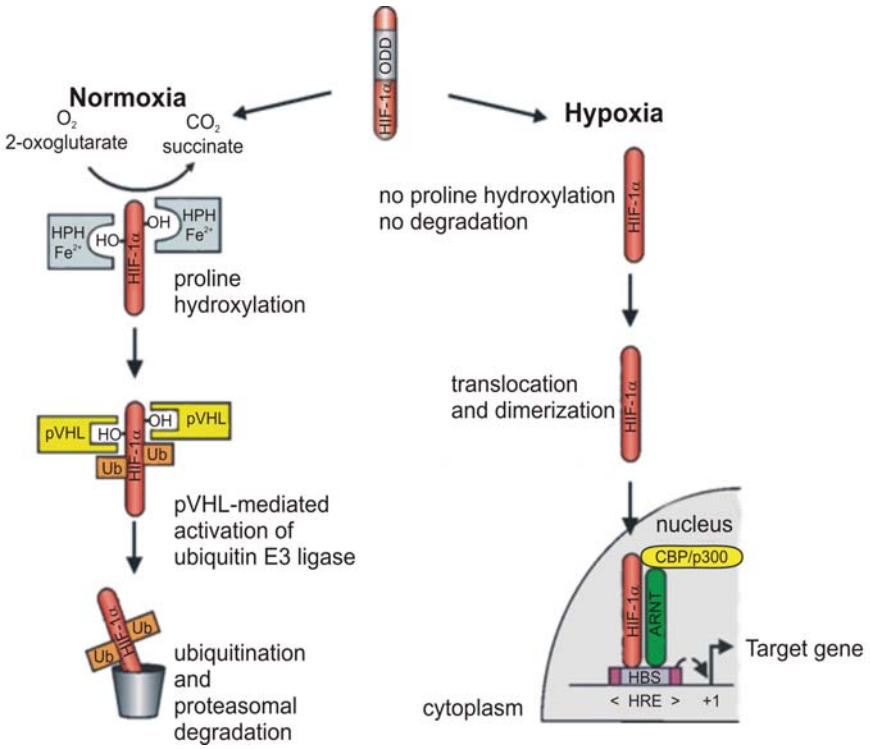

Fig. 5. HIF-1 stabilization and degradation pathway

1.6.2. GATA-2, a negative regulator

GATA transcription factors are zinc finger proteins that bind to GATA sequences in DNA. There are six GATA transcription factors (GATA-1-6) that can be subdivided into two main groups. GATA-1, GATA-2, and GATA-3 belong to the first group and they all have unique functions in the haematopoietic system. Members of the second group are GATA-4, GATA-5 and GATA-6, which are mainly expressed and involved in the formation of the extra-embryonic and embryonic endoderm, as well as in the cardiogenic mesoderm.

GATA-1 is the founding member of the GATA transcription factor family localized on chromosome X. It is expressed at high level in erythroid, megakaryocytic, Sertoli cells of the murine testis (Ito et al., 1993) mast and eosinophilic cells and it is required for megakaryocyte and erythrocyte differentiation. GATA-1 contains two zinc-fingers; the C-finger, sufficient for sequence-specific directs DNA-binding, and the N-finger, which plays a role in the stabilization of GATA-1 binding to DNA. GATA-2 especially con-

tributes to the proliferation and maintenance of haematopoietic stem cells, although the expression of GATA-2 is not limited to haematopoietic tissue and is found in various organs, such as erythroid cells, fibroblasts, embryonic brain, liver, cardiac muscle, kidney, and central nervous system (CNS) during development (Zhou et al., 1998; Minegishi et al., 1999; Yamamoto et al., 1990). GATA-3 is expressed in haematopoietic cells, most abundantly in the developing central and peripheral nervous system, liver, kidney, adrenal gland, thymus and endothelial cells (Pandolfi et al., 1995; Yamamoto et al., 1991). Within the haematopoietic lineages, expression of GATA-3 is restricted to thymocytes and T cells. In humans, the gene transcription factor GATA-4 is located in chromosome 8. GATA-4 is expressed from the onset of gonadal development and is later found in multiple cell lineages including testicular Sertoli and Leyding cells and granulosa cells of the ovary (Tremblay et al., 2003; Viger et al., 1998; Ketola et al., 1999). Furthermore, GATA-4 plays an important role in hepatic Epo gene transcription (Dame et al., 2004). GATA-5 is important for controlling the growth, morphogenesis, and differentiation of the heart and endoderm, and it expresses very early in heart development and continue to be expressed in myocardial and endocardial tissues (Morrisey EE et al., 1997). GATA-6 is mainly expressed in the testicular seminiferous tubules and ovary (Ketola et al., 1999; Laitinen et al., 2000).

Besides other, there are binding sites for the GATA-2 transcription factor in the human Epo promoter region. It has been reported that GATA-2 binds to the GATA motif located in the -30 regions relative to the transcription initiation site of the Epo promoter. In this manner GATA-2 negatively regulates Epo expression (Tarumoto et al., 2000; Iwagawa et al., 1994; 1997; 2003).

1.6.3. NF-κB, a negative regulator

Rel/NF-κB proteins represent a family of transcription factors that consist of homo- and heterodimers and are characterized by the highly conserved DNA-binding dimerization domain - the Rel homology region. This domain was initially described as an expression regulator of the kappa light-chain gene in murine B lymphocytes (Sen et al., 1986) and was later found in many different cells. The members of Rel/NF-κB proteins family include p50, p52, p65, c-Rel, and Rel B. Among these, the

best studied are p50/p65 heterodimer and p50/p50 homodimer complexes. NF-κB is mostly involved in controlling a large number of normal cellular and systemic processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. In resting cells NF-κB subunits are complexed with IκB proteins and are retained in the cytoplasm (**Fig. 6**). After stimulation of cells with variety NF-κB inducers such as proinflammatory cytokines TNF-α, IL-1, bacterial LPS, viral infection, and UV irradiation, the NF-κB-IκB complex becomes phosphorylated by IκB kinases. Following the ubiquitination, NF-κB is dissociated from the IκB proteins. Activated NF-κB subunits are further translocated to the nucleus in order to bind to specific DNA motifs (AGTCCCTGGGC) and in general, to initiate the transcription of target genes.

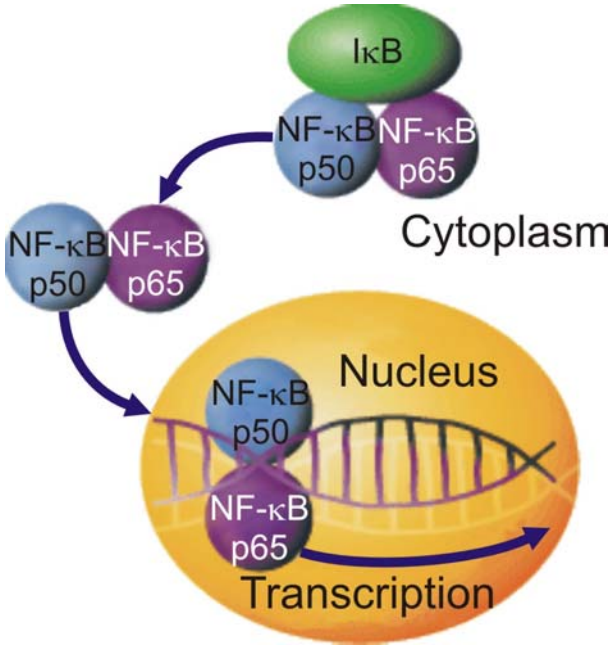

Fig. 6. Activation of NF-κB signalling pathway

NF-κB has a role in signalling pathways induced by Epo in neuronal cell (Digicaylioglu et al., 2001). In addition, NF-κB is activated by cytokines such as TNF-α and interferon-γ that regulate erythropoiesis (Claudio et al., 1996; Lee et al., 2000). Moreover, the human 5' flanking region of the Epo gene contains a binding site for transcription factor NF-κB (Barnes et al., 1997). Recently it has been shown that the proinflammatory cytokines IL-1β and TNF-α activate GATA-2 and NF-κB and that, upon activation, these two transcription factors reduce Epo expression (La Ferla et al., 2002).

2. Aim of the study

Epo, a glycoprotein produced primarily by the kidney, is the factor regulating RBC production in bone marrow. The expression rate of the Epo gene depends normally on the oxygen availability. This regulation mechanism is disrupted in patients with chronic inflammatory diseases or malignancies. These conditions may cause anaemia that directly results from Epo production repression by proinflammatory cytokines such as IL-1 and TNF-α. Both cytokines inhibit Epo formation in rat kidneys (Jelkmann et al., 1992; Frede et al., 1997) and they reduce Epo mRNA expression and Epo protein secretion in the human hepatoma cell lines HepG2 and Hep3B (Goldberg et al., 1987; Faquin et al., 1992; Fandrey et al., 1994). Protein kinase A (PKA) activity is apparently required for Epo synthesis, since cAMP partly reverses the inhibitory action of the proinflammatory cytokines (Fandrey et al., 1994). However, the molecular mechanisms by which cAMP counteracts the IL-1 suppression of Epo production are still unknown and it was the aim of this study to investigate these mechanisms.

Since Epo gene expression is balanced by the activities of either enhancing or repressing transcription factors, namely HIF-1, GATA-2, NF-κB, effects of cAMP on these transcription factors were studied in hypoxic and IL-1 treated HepG2 cultures.

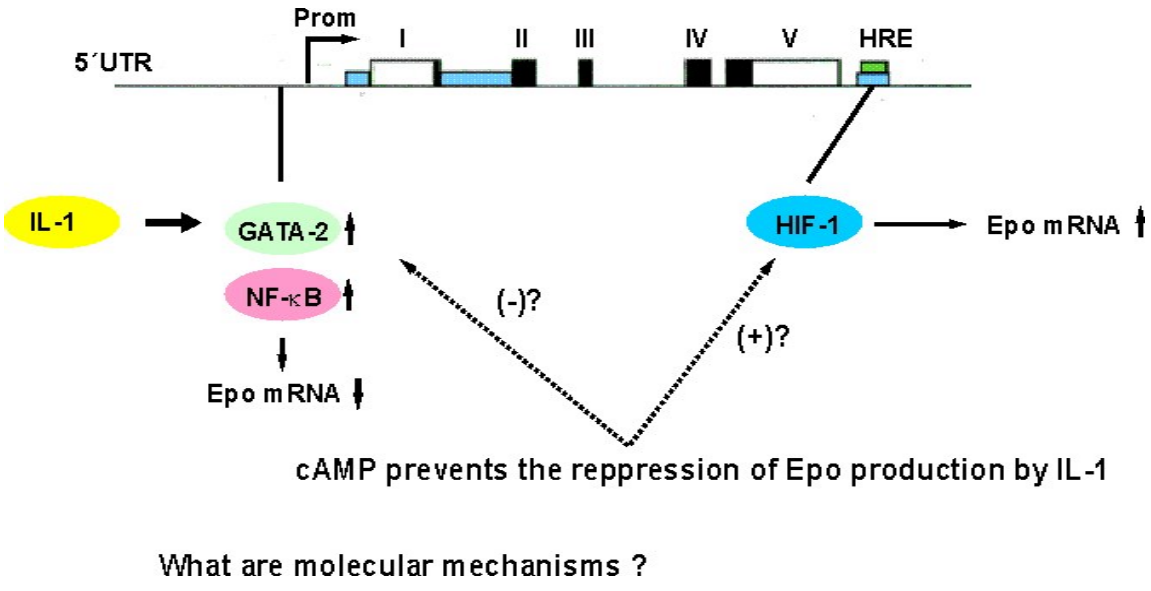

Fig. 7. Scheme representing the study

3. Materials

3.1. Chemicals and other reagents

Acrylamide……..……. Rotiphorese, Roth
Agarose.....…… Invitrogen Corporation
Ampicillin.....……...Biomol GmbH, Hamburg
Ammoniumpersulfate (APS)…….....……Merck, Darmstadt
Bacto agar.....……Becton, France
Boric acid.....…… Fluka GmbH, Seelze
Bovine serum albumin..... Sigma-Aldrich GmbH, Taufkirchen
Bromphenol blue.....……..Sigma-Aldrich GmbH, Taufkirchen
Calcium chloride.....……Fluka GmbH, Seelze
Chloroform.....…….. Merck, Darmstadt
Coomassie blue.....…….. Biomol Feinchemikalien, Hamburg
Dibutyryl cyclic adenosine.....……..Sigma-Aldrich GmbH, Taufkirchen
monophosphate (Bt_2-cAMP)
Dithiothreitol (DTT) .....……..Fluka GmbH, Seelze
DMSO..... Sigma-Aldrich GmbH, Taufkirchen
DNA molecular weight marker.....……...Biolab, New England
(100bp Ladder DNA)
(1 kb Ladder DNA)
dNTP…….....Peqlaboratories Biotechnologie GmbH
ECL…….. Amersham Pharmacia Biotech, UK
Ethanol 96%.....……...Carl Roth, Karlsruhe
Ethidium bromide.....……Invitrogen Corporation
EDTA..... Merck, Darmstadt
Fetal calf serum..... Gibco, Karlsruhe
FuGENE6…….....Roche, Ort
G418..... PAA Laboratories, Austria
L-Glutamine .....…… PAA Laboratories, Austria
Glycerol..... Sigma, Deisenhofen
Glycine.....……Biomol GmbH, Hamburg

Guanidine thiocyanate.....Roth, Karlsruhe
N'-2-Hydroxyethylpiperazine-
N'-2-ethanesulfonic acid (HEPES).....Sigma-Aldrich GmbH, Taufkirchen
NP-40.....Fluka GmbH, Seelze
Isopropanol.....Merck, Darmstadt
Magnesium chloride .....Merck, Darmstadt
Methanol .....Carl Roth, Karlsruhe
β-Mercaptoethanol.....Serva, Feinbiochemica, Heidelberg
Non-fat dry milk.....Töpfer GmbH, Dietmannsried
Oligonucleotides (EMSA).....MWG, Ebersberg
O-nitrophenyl-β-D-galactopyranoside
(ONPG).....Sigma-Aldrich GmbH, Taufkirchen
Penicilline/streptomycine.....PAA Laboratories, Austria
Phenol ....Sigma-Aldrich GmbH, Taufkirchen
Phosphoric acid.....Merck, Darmstadt
Phosphate buffered saline (PBS).....PAA Laboratories, Austria
Poly-dIdC (ICN).....Fluka GmbH, Seelze
Salmon testes DNA.....Sigma-Aldrich GmbH, Taufkirchen
Sephadex G-50.....Amersham Biosciences, UK
SDS.....Sigma-Aldrich GmbH, Taufkirchen
Sodium pyruvate and pyrodoxine.....Gibco, Karlsruhe
N, N, N', N' – Tetramethylethyl-
Enediamine (TEMED).....Fluka, GmbH, Seelze
Tris base.....Biomol GmbH, Hamburg
Triton X-100.....Serva, Feinbiochemica, Heidelberg
Tween-20.....Merck, Darmstadt

3.2. Radiochemicals

γ [^{32}]-ATP, 10 µCi/µL.....NEN, Köln

3.3. Assay Kits

Assays-on Demand for Epo.....Applied Biosystems, Darmstadt

Epo ELISA Kit.....Medac GmbH, Wedel
5 x lysis buffer.....Promega, Mannheim
Nucleospin DNA Extraction Kit.....Macherey-Nagel, Düren
Plasmid Mini Kit .....Promega, Mannheim
Plasmid Maxi Kit.....Qiagen GmbH, Hilden
QIAquick Gel Exraction Kit.....Qiagen GmbH, Hilden
SYBR Green PCR Kit.....Eurogenic, Seraining

3.4. Cytokines, enzymes and vectors

Oligo (dT) primer.....Promega, Mannheim
BgI II.....Biolab, New England
Hind III.....Biolab, New England
Kpn I.....Biolab, New England
Nhe I.....Biolab, New England
Interleukin-1.....Ciba-Geigy, Basel
Superscript II Reverse Transcripts.....Promega, Mannheim
T-4 polynucleotide kinase.....MBI Fermentas GmbH, St Leon-Rot
T-4 DNA ligase.....Biolab, New England
Taq-DNA Polymerase.....Invitrogen Corporation
Thermal Ace.....Invitrogen Corporation
Trypsin.....Invitrogen Corporation
pGL3 prom.....Promega, Mannheim
pCMV-IκBα and pCMV-IκBαM .....Clontech Labs, CA

3.5. Cell lines, media and antibodies

Human hepatoma cell line (HepG2).....DSMZ, Braunschweig
HRG-1 cells-stably transfected HepG2.....Institute of Physiology, Lübeck
IκBα cells-stably transfected HepG2.....Institute of Physiology, Lübeck
IκBαM cells-stably transfected HepG2.....Institute of Physiology, Lübeck
DMEM medium.....Gibco, Karlsruhe
RPMI 1640 medium.....Gibco, Karlsruhe
Monoclonal Anti-β-actin.....Sigma-Aldrich, Steinheim

Monoclonal Anti- HIF-1.....Transduction Laboratories
Polyclonal Anti-SP1.....Santa Cruz, Heidelberg
Polyclonal Anti- GATA-2.....Santa Cruz, Heidelberg
Polyclonal Anti- NF-κB p50 subunit.....Santa Cruz, Heidelberg
Polyclonal Anti- NF-κB p65 subunit.....Santa Cruz, Heidelberg
Polyclonal Anti-IκBα phosphorylated.....Santa Cruz, Heidelberg
Polyclonal Anti-IκBα not phosphorylated.....Santa Cruz, Heidelberg
Anti-Mouse IgG-HRP Conjugate.....DAKO, Hamburg
Anti-Rabbit IgG-HRP Conjugate.....DAKO, Hamburg
Anti-Goat IgG-HRP Conjugate.....DAKO, Hamburg

3.6. Protease inhibitors

Aprotinine.....Fluka, GmbH, Seelze
Benzamidine.....Fluka, GmbH, Seelze
Leupeptine.....Calbiochem, Darmstadt
Levamisole..... Sigma, Deisenhofen
Pepstatine.....Calbiochem, Schwalbach
Phenylmethylsulfonyl fluoride (PMSF).....Sigma, Deisenhofen

3.7. Phosphatase inhibitors

β-Glycerophosphate.....Sigma-Aldrich GmbH, Taufkirchen
Sodium-ortho-vanadate.....Calbiochem, Darmstadt

3.8. Solutions and buffers

Cell culture

PBS (phosphate-buffered saline)

Na_2HPO_4	10.9 g
NaH_2PO_4	3.2 g
NaCl	90 g
Dist. water	1000 mL

Adjust pH to 7.2

Nuclear protein extracts

Triton-Lysis buffer	100 mL
25 mM Tris-Phosphate pH 7.8	5 mL 0.5 M
1 mM EDTA	200 µL 0.5 M
1 mM DTT	100 µL 0.5 M
15 % (v/v) Glycerol	17.2 mL (87 %)
1 % (v/v) Triton X-10	1 mL

Buffer A-Hypotonic (for NF-κB and GATA-2)	100 mL
10 mM Hepes-OH pH 7.9	2 mL 0.5M
1.5 mM $MgCl_2$	751 µL 200 mM $MgCl_2$
10 mM KCl	333 µL 3 M
Dist. water	96.9 mL
Inhibitors/DTT	6.2 mL

Buffer B-Hypertonic (for NF-κB and GATA-2)	100 mL
20 mM Hepes-OH pH 7.9	4 mL 0.5 M
420 mM NaCl	14 mL 420 mM
1.5 mM $MgCl_2$	751 µL 200 mM $MgCl_2$
0.5 mM EDTA	40 µL 0.5 M
25 % Glycerol	25 µL 85 % glycerol
Dist. water	55.8 mL
Inhibitors/DTT	6.2 mL

Buffer A-Hypotonic (for HIF)	100 mL
10 mM Tris pH 7.8	1 mL 1M Tris pH 7.8
1.5 mM $MgCl_2$	750 µL 200 mM $MgCl_2$
10 mM KCl	333 µL 3M KCl
Dist. water	91.72 mL
Inhibitors/DTT	6.2 mL

Buffer C-Hypertonic (for HIF)	10 mL
20 mM Tris pH 7.8	200 µL 1M Tris pH 7.8

1.5 mM $MgCl_2$	54 µL 200 mM $MgCl_2$
420 mM KCl	1.4 mL 3 M KCl
20 % Glycerol	2.3 mL 85 % glycerol
Dist. water	6.1 mL
Inhibitors/DTT	620 µL

EMSA

5 x binding buffer (for NF-κB and GATA-2)	10 mL
5 mM $MgCl_2$	250 µL 200 mM $MgCl_2$
2.5 mM EDTA	50 µL 0.5 M EDTA
2.5 mM DTT	0.5 mL 50 mM DTT
250 mM NaCl	833 µL 3 M NaCl
50 mM Tris-HCl pH 7.5	0.5 mL 1 M Tris-HCl
20 % Glycerol	2.4 mL 85 % glycerol
Dist. water	add to 10 mL

5 x binding buffer (for HIF)	10 mL
25 mM Tris, pH 7.5	250 µL 1 M Tris
125 mM KCl	416 µL 3 M KCl
5 mM $MgCl_2$	25 µL 2 M $MgCl_2$
4.75 mM EDTA	95 µL 0.5 M EDTA
25 mM DTT	250 µL 1 M DTT
0.15 % NP40	150 µL NP40 1:10
Dist. water	add to 10 mL

10 x TBE buffer	1 L
Tris base	108.9 g
Na_4EDTA	9.3 g
Boric acid	55 g
Dist. water	1000 mL
adjust pH to 8.3	

Western blot

5 x running buffer	1L

Tris base	15.0 g
Glycine	72.0 g
5 % SDS	5.0 g
Dist. water	1000 mL
Do not adjust pH	

Blotting buffer	1L
25 mM Tris base	3.03 g
192 mM glycine	14.41 g
20 % methanol	200 mL
Dist. water	980 mL
Do not adjust pH	

4-fold loading buffer	8 mL
Dist. water	3.0 mL
0.5 M Tris-HCl, pH 6.8	1.0 mL
Glycerol	1.6 mL
10 % SDS	1.6 mL
0.5 % (w/v) bromophenol blue (in water)	0.4 mL

β-mercaptoethanol: add fresh at 1:20 to necessary volume of loading buffer

5 % milk solution	100 mL
Non-fat dry milk	5 g
PBS	100 mL

T-PBS (Tween-PBS)	1L
PBS	1L
0.05 % Tween-20	500 µL

Transformation

LB (Luria Bertani) medium	1L
1 % Tryptone	10 g
0.5 % NaCl	5 g
0.5 % Yeast extract	5 g

Dist. water	1000 mL
Autoclave	

SOB	1L
Bacto Tryptone	20 g
Bacto Yeast etxract	5 g
5 M NaCl	2 mL
1 M KCl	2.5 mL
1 M $MgCl_2$	10 mL
Dist. water	1000 mL
Autoclave and add 10 mL of 1 M sterile $MgSO_4$ solution	

TB solution for competent cells

10 mM PIPES

15 mM $CaCl_2$

250 mM KCl

pH to 6.7 with KOH. Sterilize by filtration

TE buffer (Tris-EDTA)

10 mM Tris-HCl

1 mM Na_2EDTA pH 8.0

Luciferase assay

5x lysis buffer

Substrate

Agarose gel electrophoresis

TAE buffer 50 x:	1L
Tris base	60.56 g
EDTA	100 mL 0.5 M
Acetic acid	57.1 mL
Dist. water	1000 mL
Adjust pH to 8.5	

DNA loading buffer	10 mL
0.25 % Bromphenol blue	25 mg
0.25 % Xylencyanid	25 mg
30 % Glycerine	3 mL
Dist. water	10 mL

Real time PCR

DEPC water:	1L
0.1 % (v/v)	1 mL DEPC in distilled water mix overnight, autoclave

β-galactosidase assay

Z buffer	500 mL
Na_2HPO_4	4.26 g
KCl	0.37 g
$MgSO_4$	0.123 g
Dist. water	500 mL

Adjust pH to 7.0 with NaOH

Just before add β-mercaptoethanol (2.7 µL per mL)

3.9. Laboratory supply

Agfa-Film…….....Gevaert N.V., Belgium
Automatic pipette……...Gibson, France
Automatic pipette……Roth, Germany
Cell culture flasks……...Nunc, Wiesbaden
Cell culture plates (6, 12, 24 wells)……..Greiner, Frickenhausen
Tubes (0.2mL, 0.5mL, 1.5mL, 2mL).....Sarstedt, Nürmbrecht
PC Microtiter plates (96 well).....Greiner, Frickenhausen
Multichannel pipette.....Eppendorf, Hamburg
Nitrocellulose membranes…….Hybond, Amersham Biosciences
Pipette tips with barrier
(1-10µL, 10-100µL, 100-1000µL).....Greiner, Frickenhausen

Pipette tips without barrier
(1-10µL, 10-100µL, 100-1000µL).....Greiner, Frickenhausen
Phosphoimager BAS 1000.....Fuji Photo Film Co., Dusseldorf
Phosphoimaging plate……...Raytest, Straubenhardt
Plastic tubes (15 mL, 50 mL)……Sarstedt, Nürmbrecht
Serological pipette (10mL, 25mL, 50mL).....Sarstedt, Nürmbrecht
Whatman Paper……..Schleicher & Schuell, Dassel

3.10. Instruments and equipments

ABI Prism detection system…….....Applied Biosystems; Darmstadt
Centrifuge 5417R……...Eppendorf, Hamburg
Centrifuge 5804R……...Werner Hassa GmbH, Lübeck
Cell incubator……. Heraus, incubators, Kendro
3 % O2 cell incubator…….Heraus, incubators, Kendro
Ice machine…….Scotsman
Luminator LB 96P ……..Berthold Technologies GmbH, Badwilsbad
Liquid scintillation counter LS 6500……....Beckman GmbH, Munich
Microscope (Axiovert 25)……..Carl Zeiss, Jena
Precision microplate reader……..Greiner, Bio-one
PCR-Thermocycler…….Bio-Rad Laboratories, Hercules
pH-meter 765 Calimatic…….Werner Hassa GmbH, Lübeck
Semidry protein transfer cell…….Bio-Rad Laboratories, Hercules
Shaking thermostat……Sanyo-Laborgeräte
Slab Gel Dryer SGD 2000. …….....Werner Hassa GmbH, Lübeck
Spectrophotometer…….Bio-Rad Laboratories, Hercules
Thermoblock……...Werner Hassa GmbH, Lübeck
Thermomixer……...Eppendorf, Hamburg
Vigorous shaking rotator……..Werner Hassa GmbH, Lübeck
Vortex……...Werner Hassa GmbH, Lübeck
Water bath……...Werner Hassa GmbH, Lübeck

4. Methods

4.1. Cell cultures

HepG2 cells are adherent hepatocellular carcinoma cells with an epithelial morphology, obtained originally from a Caucasian male. The cells are an established model for Epo production studies, because they produce Epo in an oxygen pressure-dependent manner and react to the relevant cytokines.

The human hepatoma cell line HepG2 was purchased from the German Collection of microorganisms and Cell Cultures. HepG2 cells were stably transfected (named HRG1) with a firefly luciferase reporter gene plasmid pH3SLV with six HIF-1 binding sites derived from the transferrin gene (Stiehl et al., 2002). NF-kB responsive and non-responsive HepG2 cells were generated by stable transfection with either pCMV-IkBα or pCMV-IkBαM expression plasmids (La Ferla et al., 2002).

The HepG2 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 50 U/mL penicillin and 50 µg/mL streptomycin sulphate. Medium for HepG2 derivation HRG-1, HepG2-IκBα and HepG2-IκBαM cells were additionally supplemented with 0.2 mg/mL G418. For subcultivation medium was removed, cells were trypsinized with 3 mL of 0.05 % trypsin solution for 5 min at 37° C. Trypsinization was stopped by adding 12 mL fresh medium supplemented with FCS. Cell cultures were maintained in a humidified atmosphere containing 5 % CO_2 at 37° C and were subcultivated 2-3 times a week. The medium was changed the day before each experiment. For hypoxia condition, the cells were placed in humidified atmosphere containing 3 % O_2, 5 % CO_2 and balanced N_2.

4.2. Electrophoretic mobility shift assay

The gel shift or electrophoretic mobility shift assay (EMSA) facilitates the detection of sequence-specific DNA-binding proteins. The technique was originally developed for DNA-binding proteins, but has been extended to allow detection of RNA binding. The assay is based on the observation that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than unbound DNA frag-

ments or double-stranded oligonucleotides. The assay is performed by incubating a purified protein or nuclear extract with a ^{32}P end-labelled DNA fragment containing the putative protein-binding site. The reaction products are then analyzed on a non-denaturing polyacrylamide gel. The specifity of the DNA-binding protein is established by competition experiments using DNA fragments or oligonucleotides containing a binding site for the protein of interest (specific), or unrelated DNA sequences (non-specific). Another test for specifity is the supershift. Antibodies to the putative DNA-binding protein can be incubated with the purified protein or nuclear extract before incubation with labelled target DNA. The antibody may prevent protein-DNA interactions by blocking regions of the protein, which bind DNA, and as a result, eliminate its ability to induce a mobility shift.

4.2.1. Nuclear protein extraction

Nuclear proteins were extracted according to protocols (Hellwig-Bürgel et al., 1999; La Ferla et al., 2000) with minor modifications. After incubation, the medium was removed and HepG2 cells were washed once with ice cold phosphate-buffered saline (PBS) and then scraped off. For the analysis of NF-κB and GATA-2 the cell suspensions were centrifuged at 4000 x g for 5 min at 4° C and pellets were resuspended in 350 µL of a low salt buffer-A (see Materials 3.8) by flicking the tube. Then they were placed on ice for 15 min. For cell lysis 25 µL 10% of NP-40 solution was added and vigorously vortexed for 30 s. Nuclei were collected by centrifugation, resuspended in 60 µL of a high salt buffer-B (see Materials 3.8) and placed on ice with gentle agitation on a shaking platform for 20 min. Nuclei were centrifuged at 12500 x g for 5 min at 4° C and supernatants were stored at -80° C.

For the analysis of HIF-1, cell suspensions were centrifuged at 800 x g for 5 min at 4° C and cell pellets were washed with 2 mL low salt buffer-A (see Materials 3.8) and subsequently resuspended in 1 mL buffer-A and placed on ice for 30 min. Nuclei were collected by centrifugation and resuspended in 100 µL of high salt buffer-C (see Materials 3.8) by gentle pipetting up and down. Extracts were assembled by centrifugation at 13000 x g for 30 min at 4° C. Buffers were freshly supplemented with 2 µg/mL aprotinine, 10 µg/mL leupeptine, 20 µg/mL pepstatine, 1 mM sodium orthovanadate, 0.5 mM benzamidine, 2 mM levamisole, 10 mM β-glycerophosphate, 0.5 mM DDT and 0.4 mM PMSF. Extracts were shock-frozen and stored at -80° C.

4.2.2. Determination of the protein concentrations

For protein measurement, the Bradford method was applied, as it involves few mixing steps, does not require heating, and results in a stable colorimetric response (Bradford, 1976). Coomassie Brilliant Blue G interacts with protein and becomes blue under acidic conditions. The dye reagent reacts primarily with amino acids such as arginine residues and to a lesser extent with histidine, lysine, tyrosine, tryptophane or phenylalanine residues. The assay is rather sensitive to bovine serum albumin (BSA).

Six different volumes of 0.5 mg/mL BSA (3 µL to 18 µL) were used for the standard curve determination. 1 µL of protein samples were mixed with 20 µL of NaCl in 96 well plates. To this mixture 200 µL of Bradford reagent were added. The mixture was placed on a shaking rotator at room temperature for 2 min. The absorbance was measured at 595 nm.

4.2.3. [^{32}P] 5' end labelling

^{32}P is the most common isotope used to radioactively label nucleic acids. It emits energetic β particles, it has the highest specific activity (9200 Ci/mmoL in pure form), and it has a fairly short half-life (14 days). ^{32}P is preferred for the preparation of radioactive probes and for most autoradiographic procedures.

Oligonucleotides were labelled with T4 polynucleotide kinase in the presence of γ [32]-ATP. T4 polynucleotide kinase, the product of the phage T4 pseT gene, was originally purified from T4-infected E. coli cells (Richardson, 1981). The forward reaction of T4 polynucleotide kinase catalyses the transfer of the terminal (γ) phosphate of ATP to the 5'-hydroxyl termini of DNA. This reaction is very efficient and hence is the general method for labelling 5' ends or for phosphorylating oligonucleotides. The end labelled DNA separated from incorporated nucleotides by column chromatography through Sephadex G-50 in TE buffer.

Two pmoL of single strand oligonucleotides were used as specific probes for EMSAs and were labelled by adding 10 U of T4 polynucleotide kinase, 1.1 MBq of γ [32]-ATP in a final volume of 10 µL containing 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine. Reactions were incubated in water bath for 30 min at 37 ° C. Two pmoL antisense oligonucleotides were annealed by heating the reaction at 90 ° C for 5 min and slowly cooling down overnight. Reactions were purified on

Sephadex columns by centrifugation at 1200 rpm for 3 min. One µL of eluted labelled oligonucleotide was used for measurement of the incorporated radioactivity in a liquid scintillation counter.

4.2.4. Preparation of the binding reactions

For EMSA, nuclear protein extracts were incubated with radioactive oligonucleotide probes that contained the specific recognition sequence.

For NF-κB,

sense: 5' - AGT TGA GGG GAC TTT CCC - 3'

antisense: 5' - GCC TGG GAA AGT CCC CTC - 3'

For GATA-2,

sense: 5' - CAC ACA TGC AGA TAA CAG CCC CGA CC - 3'

antisense: 5' - GGT CGG GGC TGT TAT CTG GAT GTG TG - 3'

For HIF-1,

sense: 5 '- TTC CTG CAC GTA CAC ACA AAG CGC ACG TAT TTC - 3'

antisense: 5' - GAA ATA CGT GCG CTT TGT GTG TAC GTG CAG GAA - 3'

Binding reactions were performed in a final volume of 20 µL containing 5 µg of nuclear extracts, 400 ng of salmon testes DNA (HIF-1, NF-κB), or 1 µg Poly (di:dC) (GATA-2), 2 pmoL labelled oligonucleotides (10.000 cpm), 4 µL 5 x binding buffer (see Material 3.8). Poly-dIdC and salmon testes DNA were added to binding reactions in order to prevent unspecific binding of proteins to the NF-κB oligonucleotide probe.

Binding reactions were incubated on ice for 30 min for study of NF-κB and GATA-2. For study of HIF-1, binding reactions were incubated overnight at 4° C. EMSA signals were supershifted by adding different specific antibodies to the binding reactions. For supershift analyses anti NF-κB p50 antibody (H-119), anti NF-κB p65 antibody (SC-109) or anti CATA-2 antibody (H-116) were added just prior to the ^{32}P-labeled oligonucleotides. For HIF-1 supershift anti HIF-1α antibody was added to the reaction an hour before the gel was run.

4.2.5. PAA gel electrophoresis and detection of the bands

Protein DNA complexes were resolved by electrophoresis in non-denaturing 6 % PAA gels using 0.3 x TBE buffer as a running buffer. For drying, one side of the gel was covered with foil and of other side with 3 sheets of Whatman paper and put in a vacuum dryer. Gels were dried at 80° C for an hour and analyzed by exposure to a phosphoimaging plate overnight in order to determine which NF-κB subunits, GATA-2 and HIF-1 specific bands were responsible for the observed shifted bands.

4.3. Western blot

Western blotting is an electrophoretic technique that allows one to test the cross reactivity of individual protein bands on sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE), with an antibody rose against a specific antigen. Proteins in a crude lysate are first resolved by SDS-PAGE. SDS is an anionic detergent that denatures proteins by wrapping the hydrophobic tail around the polypeptide backbone. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions, and partially unfolds the protein molecules, minimizing differences in molecular form by eliminating the tertiary and secondary structures. There are two types of buffer systems, used in protein gel electrophoresis: continuous and discontinuous. The discontinuous system is the most widely used method, in which the proteins are concentrated in a stacking zone before they enter the separating gel. After separation, the proteins are transferred onto membranes electrophoretically from the gel to a sheet of nitrocellulose. The membrane is blocked with non-antigenic protein, usually non-fat dry milk, to prevent non-specific immunoglobulin binding to the nitrocellulose. The nitrocellulose is then treated with a primary antibody raised against the target protein, and next with a labelled secondary antibody, which is conjugated to a detection reagent. The horseradish peroxidase catalyzed oxidation of luminol which produces light, on which ECL detection system is based has revolutionized the field of non-radioactive protein detection.

4.3.1. Nuclear protein extraction

For Western blot analysis nuclear protein were extracted as described in Method 4.2.1.

4.3.2. Whole cell protein extract

After incubation, the medium was removed and HepG2, IκBα-HepG2, IκBαM-HepG2 were washed once with ice-cold PBS and scraped off. The cell suspensions were centrifuged at 2500 rpm for 5 min at 4° C and cell pellets were resuspended in 300 µL NP-40 lysis buffer using ultra-schall for 30 s. The NP-40 lysis buffer freshly supplemented with protease-inhibitors coctail and 0.4 mM PMSF. Whole cell proteins were stored at -80 until use.

4.3.3. SDS-PAGE

20-25 µg of nuclear and whole cell protein extracts were diluted 1:4 in loading buffer and placed in a boiling water bath for 5 min followed by a short spin.

Separating Gel	10 %	7.5 %
30 % Acrylamide/bisacrylmide	6.66 mL	5 mL
Dist. water	8.04 mL	9.7 mL
1.5 M Tris-HCl pH 8.8	200 µL	200 µL
10 % APS	100 µL	100 µL
TEMED	10 µL	10 µL
Total	20 mL	20 mL
Stacking gel	4 %	
30 % Acrylamide/bisacrylmide	1.3 mL	.
Dist. water	8.04 mL	
1.5 M Tris-HCl pH 6.8	2.5 mL	
10 % SDS	100 µL	
10 % APS	50 µL	
TEMED	10 µL	
Total	10 mL	

Samples were resolved by electrophoresis in denaturing 7.5 % (HIF-1), 10 % (NF-κB) and 14% (IκBα) SDS-PAGE. The electrophoresis was performed at 60 V constant voltages for the stacking gel and at 100 V for the separating gel for two hours.

4.3.4. Immunoblot

Proteins were transferred onto nitrocellulose membranes electrophoretically. Equal amount of proteins and transfer efficiency were checked by staining with 2 % Ponceau and polyclonal anti SP1 antibody. Membranes were blocked overnight at 4° C in 5 % skim milk in PBS and then incubated with polyclonal anti NF-kB p65 (1:10000), polyclonal anti NF-κB p50 antibody (1:200), monoclonal anti HIF-1α antibody (1:1000), and polyclonal anti SP1 (1:1000) antibodies for 2 h at room temperature. Membranes were washed three times with T-PBS buffer for 10 min and incubated with a secondary HRP-conjugated anti rabbit IgG antibody (1:2000) and anti mouse IgG antibody (1:2000) for 1 h. For subsequent detection of signals by ECL the film was exposed for 5 to 20 min.

4.4. Enzyme-linked immunoassay (ELISA)

The HepG2, IκBα-HepG2 and IκBαM-HepG2 cells were seeded in 24 well plates and were grown for 5 to 7 days up to 90-95 % confluence. The cells were washed once with FCS free medium and the medium was renewed. The cells were stimulated and incubated at 3 % oxygen for 24 h. Epo concentration was measured in cell culture supernatants by an enzyme linked immuno-sorbent assay (ELISA) using a commercial kit (Medac, Wedel). The OD measured at 405 nm is proportional to the Epo concentration.

A monoclonal antibody specific for Epo has been pre-coated onto a micro plate. Standards and samples are pipetted into the wells and the immobilized antibody binds any Epo present. After removal of unbound substances, an enzyme-linked polyclonal antibody specific for Epo is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of Epo bound in the initial step. The colour development is stopped and the intensity of the colour is measured.

4.5. Real time RT-PCR

Quantitative real-time PCR is based on the detection of a fluorescent signal that increases linearly with accumulating amplification product during the PCR reaction. A dual-labelled fluorogenic oligonucleotide probe (Taq Man probe) consists of a short

20-25 base oligodeoxynucleotide, which anneals to the target sequence between the forward and reverse primers. The probe is labelled with different fluorescent dyes, a reporter dye and quencher dye which suppresses the reporter fluorescence activity by energy transfer within the intact probe. During the extension phase of PCR the probe is cleaved by the endogenous 5' nuclease activity of Taq DNA polymerase, which separates the reporter chromophor from the Taq Man probe, leading to an increase in the intensity of reporter fluorescence.

4.5.1. Isolation of total RNA

For RNA extraction, HepG2 cells were seeded in 24 well plates, were stimulated with IL-1 and cAMP and were incubated for 24 h under hypoxic conditions. The total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform (GTC) method according to protocol (Chomczynski et al., 1989). Medium was removed and cells were lysed with 150 µL of 4 M GTC, the lysate was mixed by pipetting up and down to homogeneity. To this lysate were added 15 µL of 2 M NaOAc (pH 4.0), 120 µL phenol, and 60 µL phenol:chloroform solution and were vortexed. The mixed final suspension was placed on ice for 15 min. After centrifugation for 20 min at 12000 rpm at 4° C the upper phase was taken. The aqueous phases were precipitated with 500 µL isopropanol, were vortexed and were placed at -20° C for 1 h. After centrifugation at 12000 rpm at 4° C for 20 min pellets were resuspended in 64 µL of 4 M GTC and RNA was again precipitated with 64 µL isopropanol, vortexed and placed at -20° C for 1 h. After centrifugation at 12000 rpm at 4° C for 15 min pellets were washed with ice-cold 75 % ethanol, were vortexed and were incubated at room temperature for 15 min. After centrifugation at 12000 rpm at 4° C for 15 min, supernatants were removed and pellets were dried for 10-15 min at room temperature. Pellets were dissolved in 15-20 µL of DEPC-treated water by heating at 56° C for 15 min. Samples were vortexed and mixed by pipetting up and down. RNA was stored at -80° C until use.

4.5.2. Determination of the RNA concentrations

The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260 nm (A_{260}). RNA was diluted 1:100 with Na_2HPO_4. The RNA concentrations were determined by measuring the optical density (OD_{260})

with a spectrophotometer. Ratio of reading at 260 nm and 280 nm provides an estimate of purity of the nucleic acid. The A260:A280 ratio was 1.80 indicating highly purified RNA.

4.5.3. RT-PCR

The cDNA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) of RNA from 90-95 % confluent hypoxic HepG2 cells. 1 µg RNA was mixed with 500-pmoL oligo (dT) primers in a final volume 14.5 µL in DEPC-treated water, reactions were incubated at 68° C for 15 min and on ice for 15 min. 5 µL RT buffer and 5 µL dNTP mix (2.5 mM each of dGTP, dATP, dCTP, dTTP) and 0.5 µL reverse transcriptase were added to above reactions. The reaction mixes were briefly vortexed and followed by spin down.

Cyclic conditions:

45 min at 42° C

45 min at 52° C

10 min at 100° C.

The cDNAs were stored at -80° C until use. Target cDNAs were quantified by a fluorescence-based real time RT-PCR on an ABI PRISM 7700 Sequence detection system using either a commercially available SYBR Green PCR Kit (for detection of L28) or Assays-on-Demand Kit (for detection of Epo, part number 4331182). 5 µL of the target cDNA was used as a template in a real time RT-PCR under the following conditions:

5 min at 95° C for 1 cycle

30 s at 95° C

30 s at 60° C

30 s at 72° C for 40 cycles.

4.5.4. L28 probe

L28 served as an endogenous control. For L28 the primers and sequences were used as follows:

Reverse: 5 '- TTG TAG CGG AAG GAA TTG CG - 3'
Forward: 5 '- ATG GTC GTG CGG AAC TGC T - 3 '

4.5.5. Calculation

The point characterizes reactions in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. Relative expression levels were calculated using the $\Delta\Delta C_T$-method. The amount of target gene was normalized to the endogenous control (L28). $\Delta Ct = Ct_{(target\ gene)} - Ct_{(L28)}$. To compare gene expression from cells exposed to different conditions to control cells, the ΔCt for the control cells: $\Delta\Delta Ct = \Delta Ct_{(test\ condition)} - \Delta Ct_{(control\text{-}hypoxia)}$ was calculated. Hypoxia samples were taken as a calibrator.

4.6. Directional cloning

4.6.1. Plasmid construction

Plasmids are circular double-stranded DNA (dsDNA) molecules that are separate from the chromosomal DNA. They usually occur in bacteria, sometimes in eukaryotic organisms. Uncut plasmid DNA can be in any of five forms: nicked, circular, linear, covalently closed, supercoiled or circular single-stranded. Their size varies from 1 to 250 kilo base pairs. A plasmid vector contains a replication origin, which enables it to replicate in the bacterial cell independently from the bacterial chromosome. The plasmid also usually contains a gene for some selectable property such as antibiotic resistance, which enables bacteria that take up the recombinant plasmid to be identified. It also needs a cutting site for a restriction nuclease that the plasmid can be linerized and a foreign DNA fragment can be inserted. To insert the DNA to be cloned, the purified plasmid DNA is exposed to a restriction nuclease that cleaves it in one place, and the DNA fragment covalently inserted into it using T4-DNA ligase. This recombinant DNA molecule is then introduced into bacterium by transformation. The pGL3-prom vector is a luciferase reporter vector including a SV-40 promoter upstream of the luciferase gene. This pGL3-prom vector was used for the construction

of Epo-wt plasmid (**Fig. 8A**). For Epo-wt, Epo-enhancer binding site (HRE-CACA-DR_2) was subcloned into pGL3 prom vector using Hind III and Kpn I restriction sites, which were located downstream of the luciferase gene. (Krajewski et al., submitted).

Fig. 8A. Construct of the Epo-wt plasmid

The minimal Epo-promoter (**Fig. 8B**), which contains a binding site for GATA-2, was subcloned instead of SV-40 promoter using Bgl II and Nhe I restriction sites.

Fig. 8B. Construct with minimal Epo-promoter

4.6.2. Restriction enzyme digestion

1 µg plasmid was digested with 10 U Nhe I and Hind III restriction enzymes for 2 h at 37° C in a final volume of 20 µL supplemented with 250 mM NaCI, 10 mM Tris-HCI (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 0.15 % Triton X-100, 200 µg/mL BSA and 50 % glycerol. Enzyme activity was stopped by incubation at 65° C for 15 min. Reactions were run on a 1.2 % agarose gel at 60 V for 2 h.

4.6.3. Genomic DNA extraction

HepG2 cells were subcultivated in 75 mm Petri dishes and were grown to 80-85 % confluence. Genomic DNA was extracted from HepG2 cells using a commercial kit NucleoSpin Tissue according to the manufacturer' s protocol. With the NucleoSpin Tissue method, genomic DNA can be prepared from tissues and cells. Lysis was achieved by incubation of the sample in a proteinase K/SDS solution. Appropriate conditions for binding of DNA to the silica membrane in the NucleoSpin columns are created by addition of chaotropic salts and ethanol to the lysate. After a washing pro-

cedure pure genomic DNA was finally eluted under low ionic strength conditions in a slightly alkaline elution buffer and DNA was diluted 1:100 with Na_2HPO_4 and the DNA concentrations were determined by measuring the OD_{260} with a spectrophotometer.

4.6.4. PCR

The sequence coordinates of the human Epo gene are based on the GenBank accession number M-11919. A 240-bp fragment of the 5' promoter region of the human Epo gene was produced by the polymerase chain reaction (PCR) with the following primers:

F (-1830) to -1850: 5'- ATC GCT AGC CGG GAG CAG CCC CCA TGA CCC -3'

R (-2055) to -2075: 5'- CCC AAG CTT GGA CAG CGC GGT GCG GCG CAG - 3'

For PCR Thermal Ace instead of Taq Polymerase was used. Thermal Ace DNA Polymerase is an extremely thermostable enzyme from archaebacterium that is specifically designed for high-yield PCR amplification of GC-rich templates (> 65 % GC content).

PCR were performed on ice in 25 µL volumes containing 1.5 mM $MgCl_2$, 10 mM each of dATP, dCTP, dGTP, and dTTP, 2 U/µL Thermal Ace, 100 ng of each primer and 0.8 µL of genomic DNA (200 ng), 2.5 µL 10 x PCR buffer (600 mM Tris-HCl (pH 9.25), 15 mM $MgSO_4$, 300 mM NaCl, 0.1 mg/mL BSA, 0.1 % Triton X-100). PCR was performed by heating the samples at 98° C for 3 min, followed by 98° C for 30 s, 52° C for 45 s, and 72° C for 1 min for 32 cycles, and then 72° C for 10 min as a final extension.

15 µL of PCR products were analysed by 1.5 % agarose gel electrophoresis.

4.6.5. Agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate DNA strands by size, and to determine the size of the separated strands by comparison to strands of known length. An electric field is used to drag the negatively charged DNA molecules through a gel matrix. The shorter DNA species move faster than the longer ones since they are able to slip through the gel easily. Most commonly, ethidium bromide is added to the gel at this point to facilitate visualization of DNA after electrophoresis. 15 µL of PCR products were mixed with 5 µL gel tracking dye and loaded into the

sample wells of agarose gel. Gel was run at 60 V for 1.5 h in 1 x TAE as the running buffer. After electrophoresis, the gel was placed on a UV light box and a picture of the fluorescent ethidium bromide-stained DNA separation pattern was taken with a Polaroid camera.

4.6.6. Ligation of DNA to the vector

DNA ligases catalyze the formation of a phosphodiester bond between the 5' phosphate of the one strand of DNA and 3' hydroxyl of another DNA. This enzyme is used to covalently link or ligate fragments of DNA together. Most commonly, the reaction involves ligating a fragment of DNA into a plasmid vector. The most widely used DNA ligase is derived from the T4 bacteriophage.

Linearized vector and DNA fragment (insert) were taken in a molar ratio of 1:5 and were ligated with 10 U of T4 ligase in a final volume of 10 µL supplemented with 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1mM ATP and 25 µg/mL BSA. Reactions were incubated overnight at 16° C.

4.6.7. Preparation of competent E. coli

Competent cells are bacteria, which can accept extra-chromosomal DNA. There are two main methods for preparation of competent bacterial cells for transformation, the calcium chloride and elecroporation method. DNA uptake of plasmids is performed by treatment of bacterial cells with calcium chloride.

Frozen DH-5 α E. coli cultures were plated on agar surface and were incubated in a thermostat overnight at 37° C. Next day, 10-12 large colonies were cultured in 250 mL SOB medium and were placed at 19° C on the vigorous shaking rotator. Bacterial culture was grown until OD_{600} nm reached about 0.5. After OD measurement cells were chilled on ice for 10 min and were centrifuged at 2500 x g for 10 min at 4° C. Pellets were dissolved in 80 mL of ice-cold TB solution and were placed on ice for 10 min. The solutions were centrifuged at 2500 x g for 10 min at 4° C and new pellets were gently resuspended in 20 mL of ice-cold TB solution supplemented with 1.4 mL DMSO. Cells were stored at -80° C until use.

4.6.8. Transformation of bacteria

200 µL of competent (DH5 α) cells were mixed with 7 µL ligation product and placed on ice for 30 min. Samples were incubated in a water bath at 42° C for 90 s and placed on ice for 3 min. 800 µL LB special medium was added to above reaction and cultures were incubated in a shacking rotator at 37° C for 1 h. Samples were transferred to fresh vials and were centrifuged at 13000 rpm for 30 s. Supernatant was discarded and pellets were resuspended in 150 µL of LB special medium. Cell suspensions were spread on agar plates containing 100 µg/µL ampicilin and were incubated at 37° C overnight.

4.6.9. Colony PCR

Several single colonies were picked up and were dissolved in distilled water. Bacterial suspensions were taken as a template. PCR reactions were performed on ice in 25 µL volumes consisting of 1.5 mM $MgCl_2$, 10 mM each of dATP, dCTP, dGTP, and dTTP, 2 U/µL Thermal Ace, 100 ng of each primer and 0.8 µL of genomic DNA (200 ng), 2.5 µL 10 x PCR buffer (600 mM Tris-HCl (pH 9.25), 15 mM $MgSO_4$, 300 mM NaCl, 0.1 mg/mL BSA, 0.1 % Triton X-100).

PCR was performed by heating the samples at 98° C for 3 min, followed by 98° C for 30 s, 52° C for 45 s, and 72° C for 1 min for 32 cycles, and then 72° C for 10 min as a final extension.

15 µL of PCR products were analysed by 1.5 % agarose gel electrophoresis.

4.6.10. Plasmid DNA isolation, Mini preparation

Small-scale rapid isolation of plasmid DNA is a routine procedure used for screening and analysis of recombinant plasmid DNAs in cloning and subcloning experiments. Single bacterial colonies were incubated in 2 mL of LB medium supplemented with 100 µg/mL ampicillin and placed overnight at 37° C on a shaking rotator. Plasmid DNA was isolated using a commercial kit from Promega, according to the manufacturer's protocol.

4.6.11. Plasmid digest test

After isolation of plasmid DNA by Miniprep, the plasmid was checked by restriction analysis. 1 µg plasmid was digested with 10 U Nhe I and Hind III restriction enzymes at 37° C for 2 h in a final volume of 20 µL supplemented with 250 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 0.15 % Triton X-100, 200 µg/mL BSA and 50 % glycerol. Enzyme activity was stopped by incubating at 65° C for 15 min. Reactions were run on a 1.2 % agarose gel at 60 V for 2 h. The size of plasmids was compared to a TriDye 1 kb DNA ladder.

4.6.12. Maxi preparation

After the plasmid sizes were assessed by agarose gel electrophoresis, plasmid DNA purification was carried out using the commercial kit, according to the manufacturer's protocol. The kit is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low salt and pH conditions. RNA, proteins, dyes, and low-molecular weight impurities were removed by a medium-salt wash procedure. Plasmid DNA is eluted in high-salt buffer and then concentrated and desalted by isopropanol precipitation. Finally, DNA was resuspended in TE buffer, pH 8.0. The yield of plasmid DNA was determined by a spectrophometer.

4.6.13. Sequence analysis

Fidelity of plasmid constructs was verified by sequense analysis of the junction region, which was performed by SEQLAB (Sequence Laboratories GmbH, Göttingen)

4.7. Transfection

Transfection is the general process of bringing foreign DNA into a cultured mammalian cell. Such experiments are usually performed using cloned DNA containing coding sequences and control regions (promoter etc) in order to test whether the DNA will be expressed. Also, this procedure is often used to test whether a particular modification affects the function of a gene. For transfection, several techniques can be used:

- Calcium phosphate coprecipitation
- Electroporation
- Application of cationic liposomal reagents

FuGENE 6 is a lipid-based transfection reagent that complexes with and transports DNA into the cells during transfection. The advantages of FuGENE 6 reagent include:
- It provides high transfection efficiency in many cell types.
- It gives low cytotoxicity.
- It functions exceptionally well in the presence or absence of serum.

4.7.1. Transient transfection

With transient transfection, the DNA is introduced into the nucleus of the cell, but does not integrate into the chromosome. Transcription of the transfected gene can be analyzed within 24-72 h. The optimal harvest time depends on the specific cell type and the growth conditions. This method is generally used when reporter genes like luciferase, CAT, beta-galactosidase or GFP are quantitated.

HepG2 cells were seeded in 24 well plates on the day before the transfection. The medium was renewed and the transfection of plasmid DNA into cells was performed by using FuGENE-6 according to the manufacturer's protocol. The transfected cells were allowed to grow for 24 h. After renewal of the medium the cells were stimulated for 24 h and incubated either in normoxia or in hypoxia. β-Galactosidase assay was performed to control transfection efficiency.

4.7.2. Stable transfection

The purpose of stable transfection is to generate a clone that contains permanently the transfected DNA in the genome. Usually this process is employed to generate clones expressing a particular protein of interest for overexpression and purification. After transfection of the DNA, cells are subjected to selective pressure and colonies that are resistant to the selective chemical are isolated. Obtaining stable clones may take from 6 to 8 weeks. The advantage of stable transfection is that the clones can be used for long-term functional studies.

NF-κB responsive and non-responsive HepG2 cells were generated by stable transfection with either pCMV-IκBα or pCMV-IκBαM expression plasmids. These plasmids

are convenient tools for examining NF-κB regulation by manipulating its inhibitor, IκBα. NF-κB responsive cells (IκBα-HepG2) express a native IκBα isoform, which can be phosphorylated by Ik-kinases. IκBαM-HepG2 cells express a mutated form of IκBα that cannot be phosphorylated as it contains two mutations at residues 32 to 36 that prevent NF-κB activation.

30 µg of plasmid DNA was mixed with FuGENE 6 transfection reagent. Cells were transfected by using FuGENE-6 according to the manufacturer's protocol, and were incubated in a humidified incubator at 37 ° C for 48 h. Thereafter, cells were seeded in 96-well plates in the presence of G418 (0.2 mg/mL). G418 containing medium was changed every 3 days, and G418-resistant clones were selected over 3 weeks.

4.8. Luciferase assay

Luciferase assay is a convenient, rapid, and sensitive procedure for quantifying gene expression. In general, firefly luciferase is used as a reporter because one only needs a few seconds to assay each sample. Converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin, produces light, which can be measured by a luminometer.

For luciferase assay cells were washed once with ice-cold PBS. Thereafter, cells were lysed with 100 µL 1 x passive lysis buffer at room temperature for 15-20 min. Lysates were collected and were placed on ice for 15 min. Samples were centrifuged at 12000 rpm for 5 min. 10 µL of supernatants were mixed with 20 µL of luciferase assay reagent and luminescence was measured using a Micro Lumate, LB 96P for 15 s per well.

4.9. β-Galactosidase assay

β-Galactosidase is an enzyme that catalyzes the hydrolysis of β-galactosides, including lactose and the galactoside analogue o-nitrophenyl-β-D-galactopyranoside (ONPG). The β-Galactosidase gene functions as a reporter gene for two major reasons: its protein product is extremely stable and resistant to proteolytic degradation in cellular lysates of transfected cells, and most importantly, the enzyme activity is easily assayed. Cell lysates are incubated with a reaction buffer (Z-buffer) and ONPG substrate. β-Galactosidase converts the colourless ONPG substrate into galactose

and the chromophore o-nitrophenol, yielding a bright yellow solution. β-Galactosidase activity of the solution can be quantitated using a spectrophotometer or a microplate reader to determine the amount of substrate at 420 nm.

35 µL of cell lysates were pipetted into a 96 sample wells. To each well 140 µL Z-buffer and 35 µL ONPG were added. The reaction mixtures were incubated at room temperature for 90 min and β-galactosidase activity was measured by spectrophotometer at 420 nm.

4.10. Statistics

Data were analysed by ANOVA followed by Tukey-Kramer’s test or Student' s t-test as appropriate. A significant difference was assumed at P value less than 0.05. All values are expressed as means + standard deviations (SD) of separate experiments.

5. Results

5.1. cAMP partially rescues IL-1 impaired Epo synthesis in native HepG2 cells

As previously described, IL-1 reduced Epo protein levels which was determined by ELISA. cAMP alone had no apparent effect on Epo production, hence simultaneous administration partly rescued the IL-1 induced Epo suppression (**Fig. 9A**).

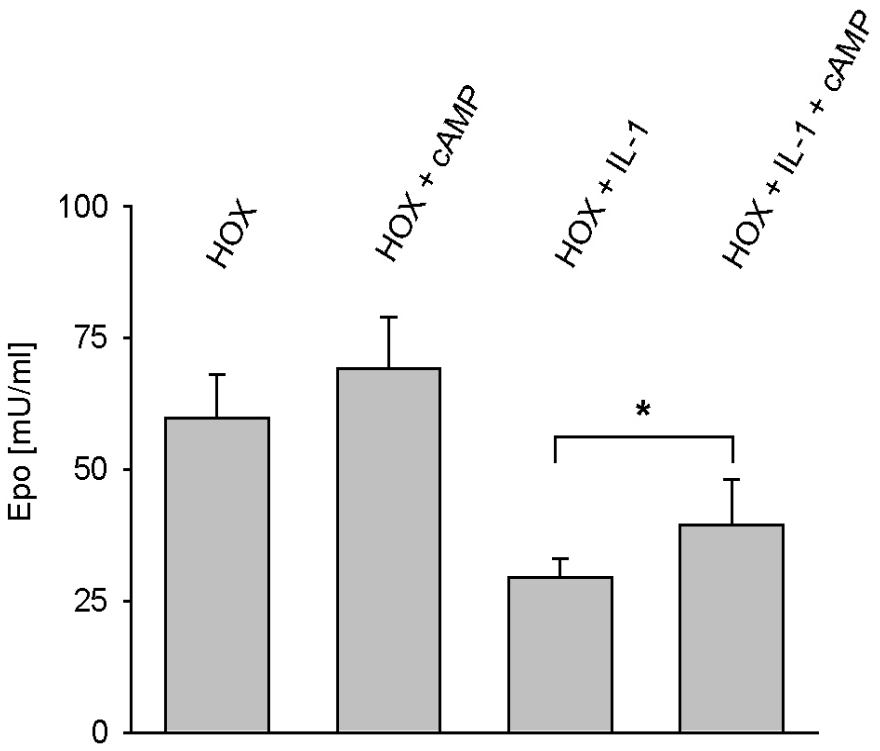

Fig. 9A. Influence of cAMP on Epo protein level

Epo was assayed by ELISA in cell culture supernatants. Hypoxic (HOX-3 % O_2) native HepG2 cells were incubated with IL-1 (300 pg/mL), with cAMP (50 µM), or with the combination of IL-1 and cAMP for 24 h. Statistics were computed using Student's t-test *$p \leq 0.05$ compared to non-stimulated control samples. Each bar graph represents the mean + SD of five separate experiments.

Real time RT-PCR measurements revealed that IL-1 treated cells expressed only 50 % of the amount of Epo messenger mRNA compared to hypoxic control cells after 8 h of incubation. Epo mRNA levels were significantly greater in cells treated with cAMP. Combined treatment with IL-1 and cAMP led to moderately higher Epo mRNA levels than those measured in cells treated with IL-1 alone. (**Fig. 9B**).

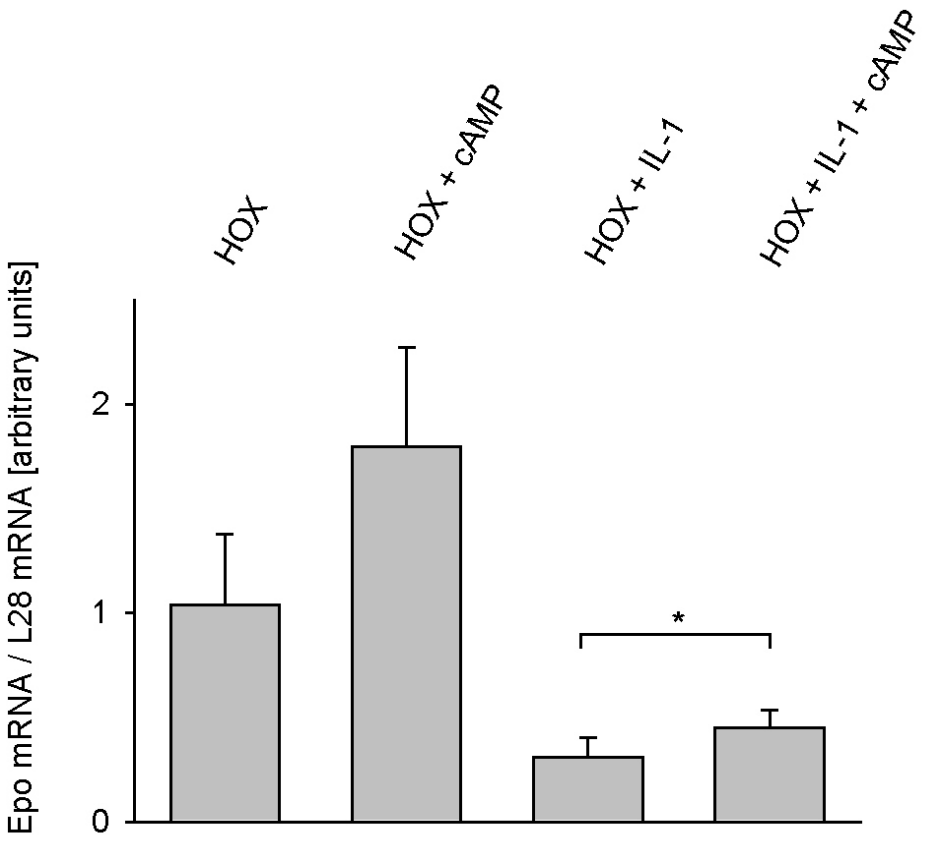

Fig. 9B. Epo mRNA

Epo mRNA was quantified by real-time RT-PCR. Hypoxic (HOX-3 % O_2) native HepG2 cells were incubated for 8 h. Relative expression levels of 60S ribosomal protein L28 were calculated with the $\Delta\Delta C_T$-method. The hypoxic non-stimulated samples (HOX) were taken as a calibrator. Statistics were computed using Student's t-test $^{*}p \leq 0.05$ compared to non-stimulated control samples. Each bar graph represents the mean + SD of six separate experiments.

5.2. HIF-1

5.2.1. Nuclear translocation of HIF-1α is not affected by cAMP

HIF-1 is the most important transcription factor mediating hypoxic Epo production. To study whether cAMP influences in the stabilization of HIF-1α, nuclear extracts of HepG2 cells were immunoblotted with an anti-HIF-1α antibody. In normoxia the HIF-1α protein was not detectable, whereas IL-1 treatment resulted in moderately increased HIF-1α protein amounts in the nuclei (**Fig. 10**). Hypoxia led to a strong accumulation of nuclear HIF-1α, which was further increased following IL-1 treatment. The addition of cAMP had no effect on HIF-1α protein amounts in untreated or IL-1 treated cells either in normoxia or hypoxia.

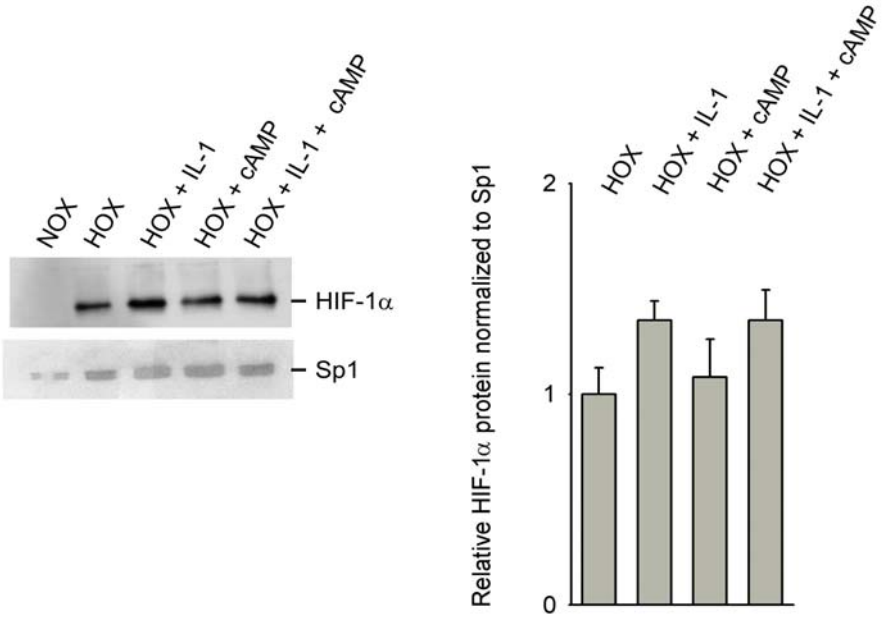

Fig. 10. Accumulation of HIF-1α protein in the nucleus

HepG2 cells were exposed to the experimental conditions for 4 h. HIF-1α was detected by Western blot analysis using the anti HIF-1α mouse monoclonal antibody. 25 µg of nuclear extract from non-stimulated normoxic (NOX-20 % O_2) cells (lane 1), hypoxic (HOX-3 % O_2) cells (lane 2), hypoxic cells in the presence of IL-1 (300 pg/mL) or cAMP (10 µM) (lanes 3 and 4) and the combination of IL-1 and cAMP (lane 5) were used for Western blot. Proteins were separated on 7.5 % SDS-PAGE described in Methods. The membranes were preincubated with a polyclonal anti SP1 antibody. Relative HIF-1α amounts were determined by densitometry (HIF-1α-specific signal/SP1-specific signal; hypoxia alone was set to 1). Each bar graph represents the mean + SD of three separate experiments.

5.2.2. cAMP does not alter HIF-1 DNA-binding activity in IL-1 treated cells

The DNA-binding capability of nuclear HIF-1 was investigated by EMSAs. HIF-1 DNA-binding complexes were not detectable in nuclear extracts from normoxic cells (**Fig. 11**). In cells maintained in hypoxia for 4 h, strong HIF-1 DNA-binding was induced. HIF-1 DNA-binding was further increased by IL-1. In contrast, cAMP did not alter HIF-1 DNA-binding.

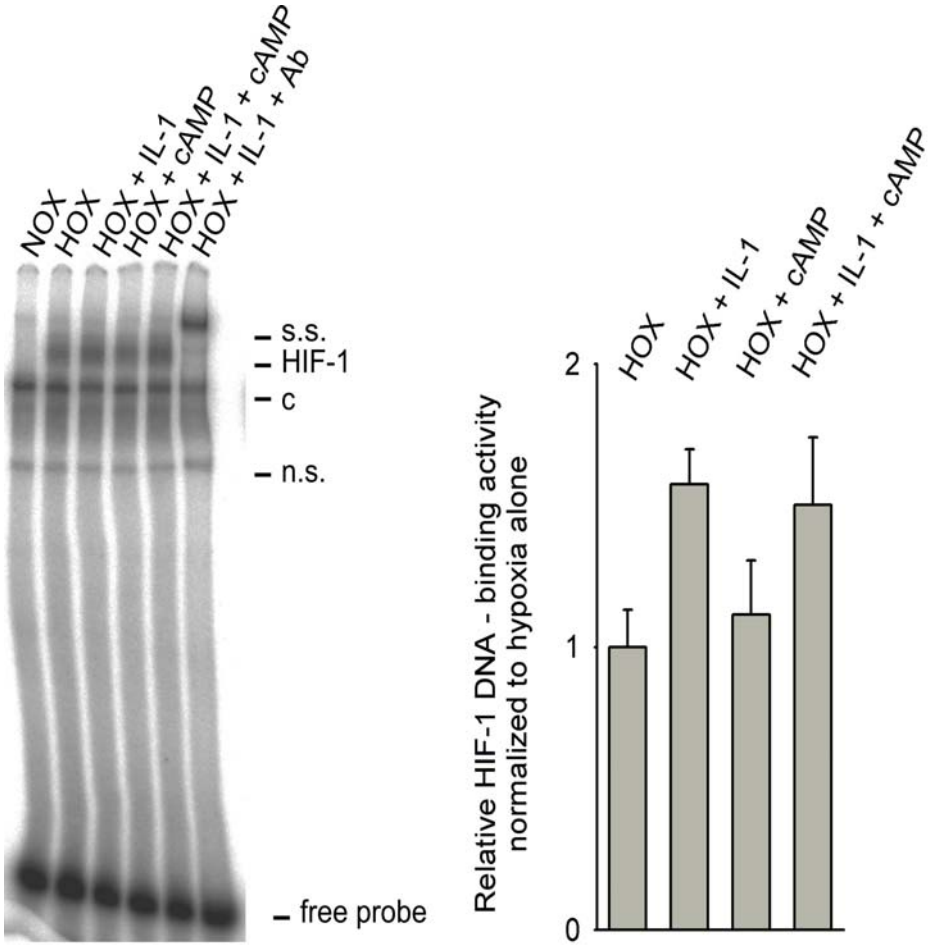

Fig. 11. HIF-1 DNA-binding activity in HepG2 cells

EMSAs were performed with 5 µg of nuclear extracts from non-stimulated normoxic (NOX-20 % O_2) cells (lane 1), hypoxic (HOX-3 % O_2) cells (lane 2), hypoxic cells stimulated with IL-1 (300 pg/mL) or cAMP (10 µM) (lanes 3 and 4) and with the combination of IL-1 and cAMP (lane 5). Cells were incubated for 4 h. Specificity of signals was verified by supershift analysis (HOX + IL-1+Antibody) by add-

ing anti HIF-1 antibody to the binding reactions an hour before the gel was run (lane 6). Binding reactions were run on a 6 % non-denaturating PAA gel as described in Methods. Abbreviations: HIF: HIF-1 specific DNA-binding, C: constitutive DNA-binding, n.s.: Non specific DNA-binding, s.s.: Supershift, free probe: unbound oligonucleotides. The bars represent the densitometrical evaluation of HIF-1 specific signals related to corresponding constitutive signals. The hypoxic control was set to 1). Each bar graph represents the mean + SD of three separate experiments.

5.2.3. cAMP has no effect on the expression of a hypoxia-dependent reporter gene

HIF-1 dependent reporter gene assays were performed using HRG-1 cells, which are stably transfected HepG2 cells with a HIF-1 dependent hypoxia responsive luciferase reporter plasmid. (**Fig. 12A**).

Fig. 12A. Construct of HIF-1 reporter gene

IL-1 induced HIF-1 and subsequent by expression of the luciferase gene (**Fig. 12B**). In harmony with the Western blot and EMSA results, cAMP did not alter HIF-1-dependent luciferase activity.

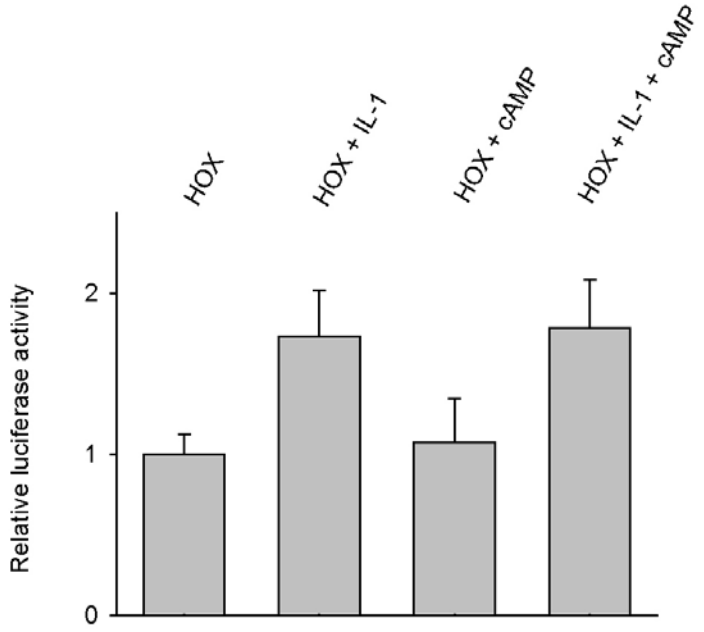

Fig. 12B. Hypoxia dependent reporter gene assay

Non-stimulated hypoxic (HOX-3 % O_2) cells were taken as a control (lane 1). Hypoxic HRG-1 cells were stimulated with IL-1 (300 pg/mL) or cAMP (10 µM) or with the combination of IL-1 and cAMP (lanes 2, 3 and 4). The cells were exposed to the experimental conditions for 22 h. Luciferase activity in cultures exposed to hypoxia alone was set to 1). Each bar graph represents the mean + SD of six separate experiments.

5.3. GATA-2

5.3.1. IL-1 induced GATA-2 DNA-binding activity is not attenuated by cAMP

GATA-2 is a negatively regulating factor for the Epo gene. GATA-2 DNA-binding activity was observed in normoxia (**Fig. 13**). In hypoxia, DNA-binding ability was reduced compared to normoxia. IL-1 restored GATA-2 DNA-binding under hypoxic conditions. cAMP administration increased hypoxic GATA-2 DNA-binding to the normoxic level. Combined treatment with IL-1 and cAMP did not exert additive effects, implying that cAMP effects on Epo expression are not mediated via GATA-2.

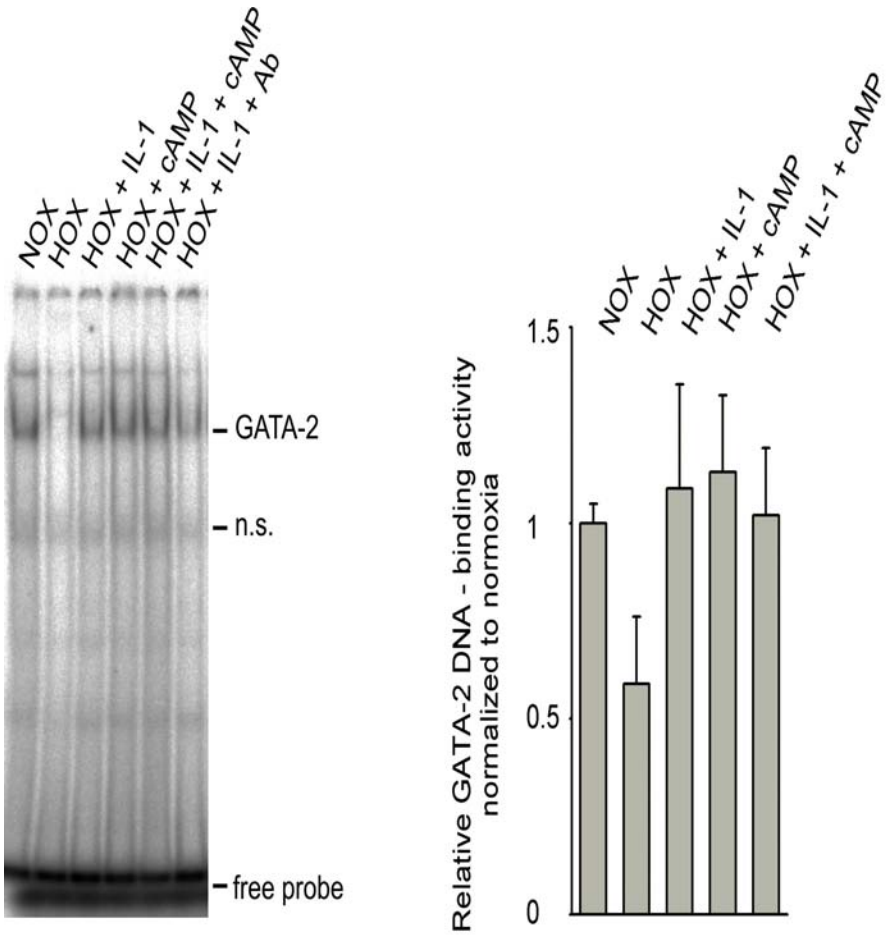

Fig. 13. **GATA-2 DNA-binding activity in HepG2 cells**

HepG2 cells were treated for 4 h. EMSAs were performed with 5 µg of nuclear extracts from non-stimulated normoxic (NOX-20 % O_2) cells (lane 1), hypoxic (HOX-3 % O_2) cells (lane 2), hypoxic cells stimulated with IL-1 (300 pg/mL) or cAMP (10 µM) (lanes 3 and 4) and with the combination of IL-1 and cAMP (lane 5). Specificity of signals was verified by supershift analysis (HOX + IL-1+Antibody)

using polyclonal anti GATA-2 antibody before adding the labelled oligonucleotides to binding mixtures. Binding reactions were run on a 6 % non-denaturating PAA gel as described in Methods. GATA-2: GATA-2 specific DNA-binding, n.s.: Non-specific DNA-binding, free probe: unbound oligonucleotides. Relative GATA-2 DNA-binding was determined by densitometric evaluation of the signals, with signal intensities of the normoxic samples being set to 1). Each bar graph represents the mean + SD of five separate experiments.

5.4. NF-κB

5.4.1. cAMP decreases the IL-1 induced expression of NF-κB protein in the nuclei

Immunoblotting of nuclear extracts revealed a moderate translocation of p50 and p65 subunits during hypoxia (**Fig. 14**). More appreciably, p50 and p65 subunits accumulated in the nuclei of IL-1 treated cells. cAMP treatment did not change the amount of hypoxic induced p50 subunits, whereas the p65 subunits were slightly reduced. However, the induction of NF-κB by IL-1 was attenuated in the presence of cAMP, which becomes obvious from the reduced p50 subunits. The changes in p65 subunits were subtle, but were basically parallel to those of p50.

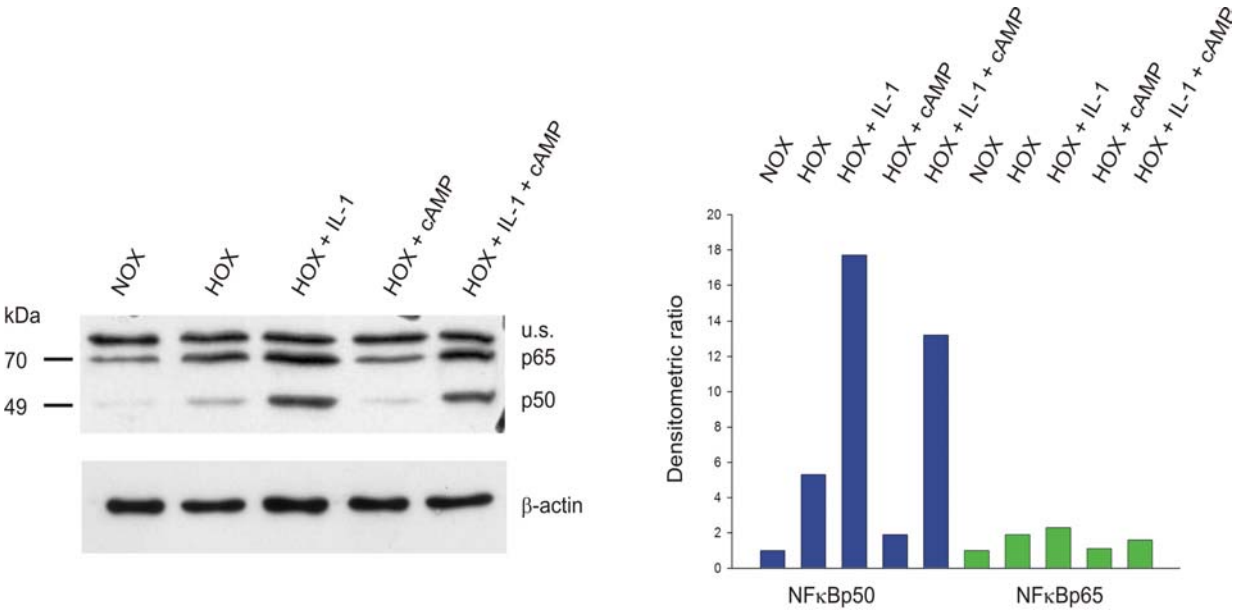

Fig. 14. Detection of p50 and p65 subunits by Western blot analysis

p50 and p65 NF-κB subunits were quantified by Western blot analysis HepG2 cells were exposed to the experimental conditions for 1 h. 25 µg nuclear extracts from non-stimulated normoxic (NOX-20 % O_2) cells (lane 1), hypoxic (HOX-3 % O_2) cells (lane 2), hypoxic cells in the presence of IL-1 (300 pg/mL) or cAMP (10 µM) (lanes 3 and 4) and the combination of IL-1 and cAMP (lane 5) were subjected to SDS-PAGE. Equal loading was verified by detection of β-actin. Relative p50 and p65 amounts were determined by densitometry, NF-κB-specific signal/β-actin-specific signal; normoxia was set to 1).

5.4.2. Binding activity of NF-κB is reduced by cAMP

NF-κB DNA-binding activity did not differ in hypoxia compared to normoxia (**Fig. 15**). Stimulation of the cells with IL-1 led to a strong induction of NF-κB DNA-binding of homo- and heterodimers. cAMP alone did not alter the pattern of NF-κB DNA-binding complex. Importantly, IL-1 in combination with cAMP strongly blocked IL-1 induced NF-κB DNA-binding compared to the effect of IL-1 alone.

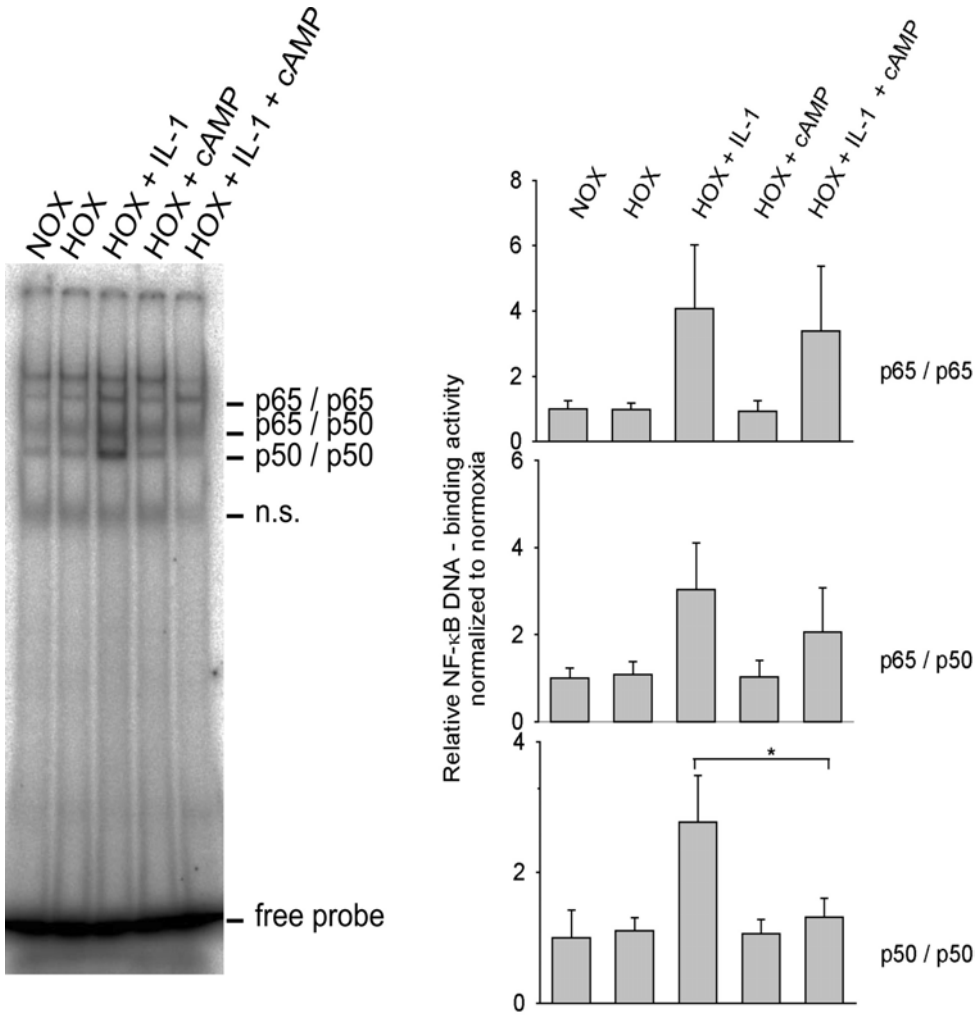

Fig. 15. DNA-binding activity of NF-κB in HepG2 cells

EMSAs were performed with 5 µg of nuclear extracts from non-stimulated normoxic (NOX-20 % O_2) cells (lane 1), hypoxic (HOX-3 % O_2) cells (lane 2), hypoxic cells stimulated with IL-1 (300 pg/mL) or

cAMP (10 µM) (lanes 3 and 4) and with the combination of IL-1 and cAMP (lane 5). Binding reactions were run on a 6 % non-denaturating PAA gel as described in Methods. Abbreviations: p65 / p65: Homodimer of p65 subunits, p65 / p50: Heterodimer of p65 and p50 subunits, p50 / p50: Homodimer of p50 subunits, free probe: unbound oligonucleotides. NF-κB fold induction was determined on p65 / p65; p65 / p50; p50 / p50 homo- and heterodimers separately and related to non-stimulated normoxic control samples. Each bar graph represents the mean + SD of five separate experiments.

5.4.3. Overexpression of mutated IκBα decreases NF-κB binding activity

HepG2, NF-κB responsive IκBα-HepG2 and non-responsive IκBαM-HepG2 cells were used for EMSAs (See Method 4.7.2).

IL-1 treated HepG2 and cells expressing the functional form of IκBα showed strong induction NF-κB DNA-binding compared to cells expressing the mutant form of IκBα (**Fig. 16**).

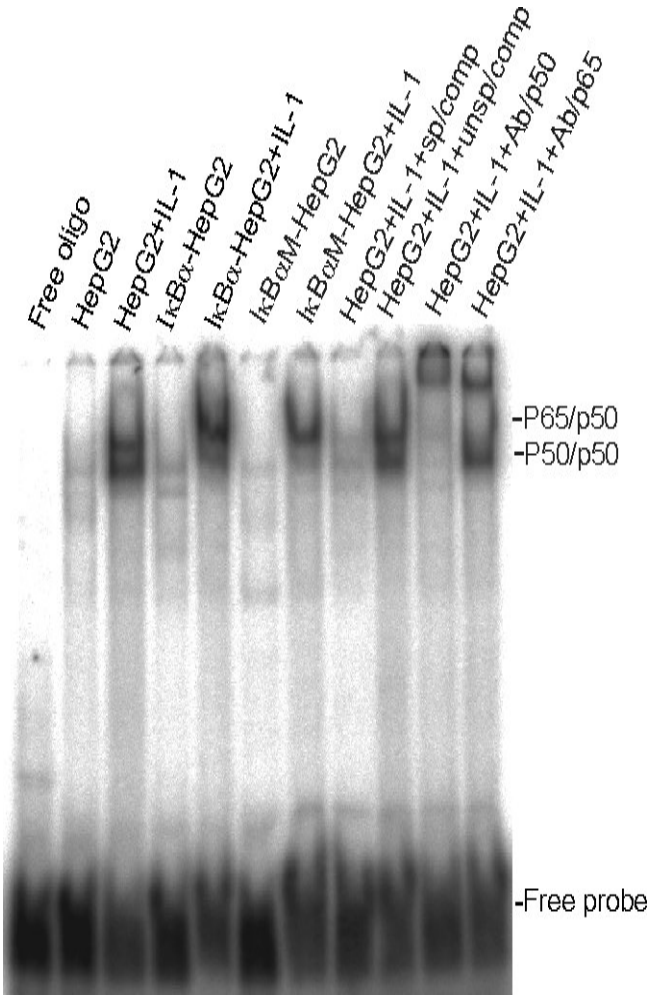

Fig. 16. NF-κB DNA-binding activity in IκBα-HepG2 and IκBαM-HepG2 cells in response IL-1

5 µg of nuclear extracts from non-stimulated normoxic (NOX-20 % O_2) cells and normoxic cells stimulated with IL-1 (300 pg/mL) for an hour and were used for EMSAs. Binding reactions were run on a 6 % non-denaturating PAA gel as described in Methods. Abbreviations: p65 / p50: Heterodimer of p65 and p50 subunits, sp/comp: specific competitor, unsp/comp: unspecific competitor, free probe: unbound oligonucleotides. Specificity of signals was verified by supershift analysis (NOX+IL-1+Antibody) using polyclonal anti p50 and p65 antibodies before adding the labelled oligonucleotides to binding mixtures.

5.4.1. Phosphorylated IκBα protein expression decreases in NF-κB non-responsive cells IκBαM-HepG2.

Expression of phosphorylated IκBα proteins were quantified in HepG2, NF-κB responsive HepG2-IκBα and non-responsive HepG2-IκBαM cells. (See Method 4.7.2). Immunoblotting of whole cell extracts in response to IL-1 revealed strong expression of phosphorylated IkBα proteins in HepG2 and also in cells expressing the functional form of IkBα compared to cells expressing the mutated form of IkBα (**Fig. 17**).

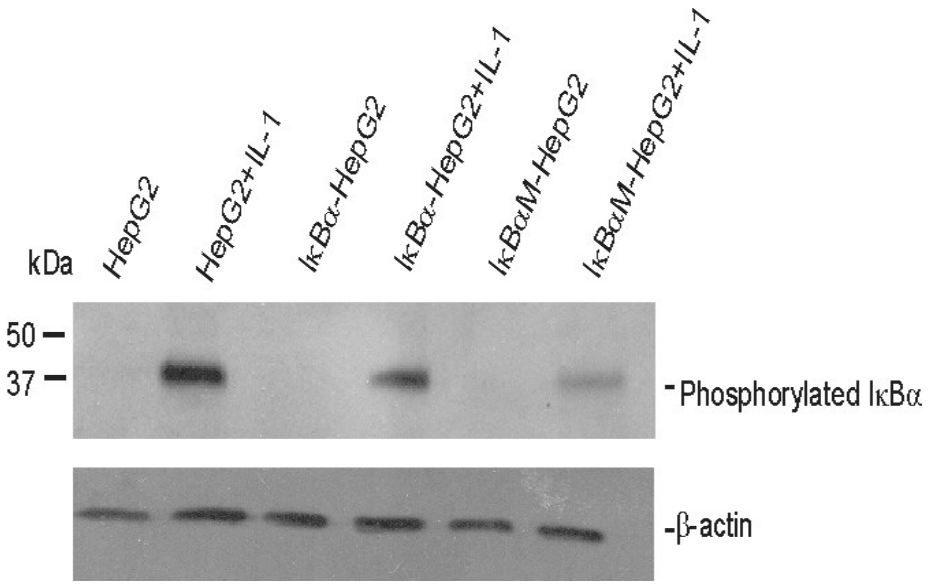

Fig. 17. Detection of phosphorylated IkBα proteins by Western blot analysis

HepG2, IκBα-HepG2 and IκBαM-HepG2 cells were exposed to the experimental conditions for 45 min. 25 µg whole cell extracts from nonstimulated normoxic (NOX-20 % O2) cells, normoxic cells in the present of IL-1 (300 pg/mL) were subjected to SDS-PAGE. Equal loading was verified by detection of β-actin.

5.4.4. Native IκBα is required for cAMP-mediated partial rescue of IL-1 induced Epo suppression

In a genetically approach with either IκBα overproducing HepG2 cells (IκBα-HepG2) or HepG2 cells expressing an isoform of IκBα with two Ser/Ala point-mutations (Ser-32 and Ala-36; IκBαM-HepG2) the effect of cAMP on Epo protein secretion was investigated. Epo protein levels in supernatants of hypoxic cells were equal in IκBα-HepG2 and IκBαM-HepG2 cells (**Fig. 18**). IL-1 reduced secreted Epo only in IκBα-HepG2 and cAMP partly rescued Epo secretion in IL-1 treated cells. In mutated cells, IL-1 had only a minor effect on Epo secretion and cAMP did not change Epo secretion while the activation of NF-κB was blocked.

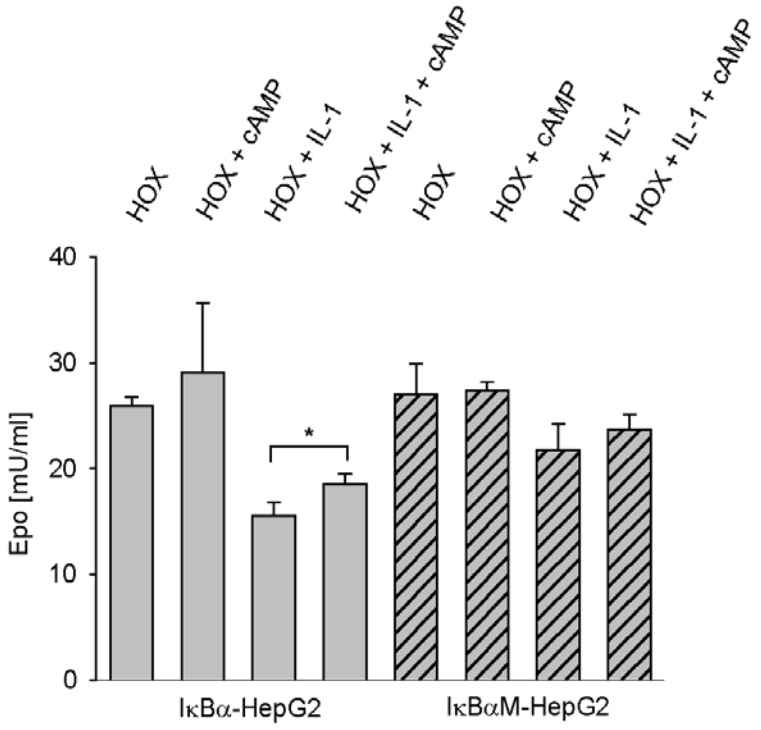

Fig. 18. Epo protein secretion in IκBα-HepG2 and IκBαM-HepG2 cells

IκBα-HepG2 and IκBαM-HepG2 cells stimulated with IL-1 (300 pg/mL), cAMP (50 µM), and the combination of IL-1 and cAMP were exposed to hypoxia (HOX-3% O_2) for 24 h. Epo protein concentrations were measured in cell culture supernatants. Statistics were computed using unpaired Student's t-test *$p<0.05$ compared to non-stimulated hypoxic control samples. Each bar graph represents the mean + SD of five separate experiments.

6. Discussion

Epo is the most important hormone for erythropoiesis. Insufficient Epo production is not only common in end-stage kidney disease but also in chronic and acute infections, autoimmune diseases, cancer and malignancies, thereby contributing to the ACD. Several mechanisms for cytokine-induced anaemia have been proposed, including intestinal bleeding, impaired iron metabolism, suppression of bone marrow erythropoiesis and dysregulation of Epo production. ACD is a cytokine-mediated disorder. In this condition, the proinflammatory cytokines IL-1 and TNF-α play a major role. IL-1 and TNF-α production rates are increased in patients with ACD-related anaemia. The proinflammatory cytokines IL-1 and TNF-α are important mediators of immune responses and are produced from macrophages, lymphocytes and endothelial cells. These cytokines have been postulated to play a role in diminishing Epo expression in these disorders. In human hepatoma cell lines and isolated perfused rat kidneys, IL-1 and TNF-α have the ability to inhibit Epo mRNA and Epo production (Jelkmann et al., 1992; Goldberg et al., 1987). IL-1 has also been shown to inhibit murine erythropoiesis in vitro and in vivo (Schooley et. al., 1997; Johnson et. al., 1989). The role of cAMP as a second messenger in erythropoiesis has been suggested earlier. While a renal cell culture model for study of the physiological mechanisms in control of Epo expression is not available, cAMP has been reported to stimulate Epo synthesis in a transformed human renal carcinoma cell line (Sherwood et al., 1987; Hagiwara et al., 1985). However, renal carcinomas derive from renal tubular cells, whereas renal interstitial cells are the physiologic sites of the renal Epo synthesis. Adding cAMP analogs to IL-1 and TNF-α treated HepG2 cells can restore cytokine-inhibited Epo synthesis (Fandrey et al., 1994). HepG2 cells produce a huge amount of biologically active Epo as well as increased levels of Epo mRNA in response to hypoxia (Goldberg et al., 1987). Also, PKA activity has been concerned in the control of Epo synthesis (Fisher 2003). Moreover, chronic treatment of normal mice with dibutyryl cyclic AMP increased haemoglobin concentration and red cell mass by 46 % compared to untreated mice (Rodgers et al., 1975).

The present results support the previous studies. Real time RT-PCR experiments with HepG2 cells revealed lowered Epo mRNA and protein amounts after IL-1 stimu-

lation. cAMP treatment partially rescued Epo production as well as on the mRNA and protein level.

Nothing has been reported about the molecular mechanism by which cAMP analogs restore the cytokine-inhibited Epo synthesis. Epo expression occurs mainly at the level of transcription. Therefore, we studied the influence of cAMP on three transcription factors, which control the Epo expression, namely HIF-1, GATA-2 and NF-κB. GATA-2 and NF-κB were influenced by cAMP, while HIF-1 was not affected.

In mammalian cells, adaptation to reduced oxygen availability includes the upregulation of specific genes involved in angiogenesis, erythropoiesis, glucose transport, and glycolysis. A number of different transcription factors and signalling molecules have been implicated as being important in this adaptive response, including HIF-1. HIF-1 is composed of two subunits, HIF-1α and HIF-1 β. Under hypoxic conditions, active HIF-1 heterodimers accumulate in the nucleus where they bind to cis-acting elements in the regulatory regions of HIF-1 responsive genes, so to enhance the rate of their transcription. HIF-1 is the most important transcription factor in the hypoxic induction of Epo gene expression. A number of studies have documented increased HIF-1 DNA-binding in various cell types after incubation with certain proinflammatory cytokines such as IL-1β, TNF-α, and IFN-γ. It has been shown that proinflammatory cytokines increase the HIF-1 DNA-binding activity in human hepatoma cells (Hellwig-Buergel et al., 1999), human renal tubular epithelial cells (El Awad et al., 2000), and transformed human intestinal epithelial cells (Bertges et al., 1999). In any case, the cytokines inhibit Epo mRNA expression, although they stimulate HIF-1 DNA-binding and increase nuclear HIF-1α protein levels. In addition to the hypoxically inducible DNA-binding activity, HIF-1, the Epo enhancer also binds another complex constitutively. The activating transcription factor-1 (ATF-1) and cAMP-responsive element binding-1 (CREB-1) are the major constituve transcription factors binding to the HIF-1 DNA recognition site. Binding of ATF-1/CREB-1 to the HIF-1 site suggests that the HIF-1 recognition site is cAMP responsive. CREB-1 has been shown to be target of cAMP-inducible PKA mediated phosphorylation, which is required for transcriptional activator function. Compared with CREB-1, PKA -dependent phosphorylation of ATF-1 does not seem to be major inducers of transcriptional activator function (Hurst et

al., 1991). However, it is not clear whether the transcription factors ATF-1 and CREB-1 play a functional role in the Epo enhancer.

First we investigated the influence of cAMP on the positive regulator of Epo, HIF-1. Our results showed that cAMP did not affect on HIF-1 DNA-binding activity, protein accumulation and hypoxia-dependent reporter gene assay. Therefore, cAMP is not likely to stimulate Epo production through the HIF-1 DNA recognition site.

GATA-2 is 50-kDa protein which binds to the GATA motif, located in the -30 regions relative to the transcriptional initiation site of the Epo promoter. GATA-2 negatively regulates Epo expression. In many genes, the consensus motif in the -30 regions is a TATA box. After mutation the GATA motif of the Epo promoter by TATA the Epo transcriptional activity is enhanced even in normoxia (Tsuchiya et al., 1997; La Ferla et al., 2002). The present and previous studies have shown that Epo production increases in hypoxia, because the GATA-2 DNA-binding is decreased at low oxygen tension. Also, nitric oxide stimulated Epo gene expression by reducing GATA-2 ability (Tarumoto et al., 2000). Recently it has been shown that the proinflammatory cytokines IL-1 and TNF-α activate GATA-2, thereby reducing Epo expression (La Ferla et al., 2002). One common pathogenesis of ACD and of renal disease appears to be stimulation of GATA-2 binding activity by IL-1, TNF-α or N-monomethyl L-arginine, which inhibits the Epo promoter activity. Significantly, the impaired Epo production was rescued by K-7174 (a GATA-specific inhibitor) in vitro and in vivo (Imagawa et al., 2003).

The Epo promoter clearly contributes to the hypoxic inducibility of the Epo expression (Imagawa et al., 1991). To prove the influence of cAMP on Epo promoter, the reporter construct, described under Methods 4.6 was used**.** Transient transfection of HepG2 cells with Epo-wt plasmid, which contains SV-40 promoter showed eight-fold induction of luciferase activity in hypoxia, whereas the plasmid with minimal Epo promoter showed about fourteen -fold induction of luciferase activity in hypoxia. Combined treatment with IL-1 and cAMP had no additional effect on Epo promoter induction. The influence of cAMP on GATA-2 has not been investigated previously with respect to Epo production. In considering GATA-2 a repressive transcription factor for the Epo gene, the present results indicate that IL-1 induced GATA-2 activity contributed to Epo suppression. EMSAs showed that cAMP in combination with IL-1 did not affect the cytokine induced GATA-2 activity. However, cAMP administration

followed by a hypoxic incubation induced GATA-2 activity. Thus, the cAMP transmitted rescue of IL-1 suppressed Epo production was not mediated by GATA-2.

NF-κB is a transcription factor that is major regulator of immune and inflammatory responses. In respect to the Epo gene, the role of NF-κB is controversially discussed. According to previously published data, NF-κB acts as a negatively regulating transcription factor for Epo gene. The present findings suggest that NF-κB is the most important transcription factor with respect to the antagonistic effect of cAMP on the IL-1 repressed production of Epo. EMSAs showed that IL-1 alone markedly induced NF-κB DNA-binding, which was rescued by cAMP. This effect was most obvious when looking at the NF-kB p50/p50 homodimer. Western blot analyses confirmed the EMSAs results. In IL-1 treated cells p50 and p65 subunits strongly accumulated in the nucleus. However, the induction of NF-κB by IL-1 was attenuated in the presence of cAMP, as judjed from the reduced p50 subunits.
Additionally, we repressed NF-κB function by stably transfecting HepG2 cells with a dominant-negative form of the inhibitory protein IκBα. In a genetic approach with either IκBα overproducing HepG2 cells (IκBα-HepG2) or HepG2 cells expressing an isoform of IκBα with two Ser/Ala point mutations (Ser-32 and Ala-36) (IκBαM-HepG2) the effect of cAMP on Epo protein secretion was investigated. Epo protein levels in supernatants of hypoxic cells were equal in IκBα-HepG2 and IκBαM-HepG2 cells. IL-1 reduced Epo secretion only in IκBα-HepG2 and cAMP partly rescued Epo secretion in IL-1 treated cells. In mutated cells, IL-1 had only a minor effect on Epo protein secretion and cAMP had no antagonistic effect on cytokine-inhibited Epo secretion while the activation of NF-κB was blocked. In addition, NF-κB DNA-binding and expression of phosphorylated IκBα proteins were markedly decreased in response to IL-1 in mutated cells. Likewise, impairment of NF-κB function by the oligo-decoy technique prevents the inhibition of Epo production by IL-1 (La Ferla et al., 2002). Moreover, previous studies from our laboratory have shown that NF-κB is activated in HepG2 cells in response to TNF-α, while Epo gene expression is suppressed. Thesaction of TNF-α is mediated by the p55 receptor (Hellwig-Buergel et al., 1998). This finding indicates a negative role of NF-κB in control of Epo production. In contrast to our results, there is one report stating the opposite (Figueroa et al., 2002). Gene suppression by NF-κB has been explained by competition between NF-κB and other transcription factors for the cofactor p300/CBP (Ke et al., 2001). The transcriptional

complex p300/CBP is critically involved in hypoxia-induced Epo expression (Ebert et al., 1999). In addition, the transcriptional activity of NF-κB is partially dependent on phosphorylation of NF-κB p65 by PKA. The transcriptional co-activator p300/CBP is thought to associate with NF-κB p65 in response to PKA mediated phosphorylation (Zhong et al., 1997). In harmony with the present findings in HepG2 cells, earlier studies have shown that the activation of NF-κB by IL-1 is also suppressed by cAMP elevation in human pancreatic cancer cells PaCa-2 (Kamthong et al., 2000).

Drugs activating the adenylate cyclase/cAMP/PKA pathway fail to produce a major Epo mRNA increase in rat kidneys during short term (2 h) perfusion expreriments (Tan et al., 1992). In a study on human volunteers the β_2-adrenergic receptor agonist fenoterol, which leads to an endogenous cAMP increase, proved to stimulate Epo production in vivo (Gleiter et al., 1997). It remains to be investigated, whether a beneficial value can be assigned to adenylate cyclase activating drugs in patients suffering from impaired Epo synthesis due to high levels of proinflammatory cytokines. The present study suggests an important influence of cAMP on cytokine-inhibited Epo synthesis. In view of central role for transcription in biological processes, it represents an obvious target for therapeutic drugs which could be either by stimulating the transcription of specific genes required for a desired benefical effect or by inhibiting the transcription of genes involved in an undesirable event. Our findings demonstrate that cAMP did not restore the cytokine-inhibited Epo production through the positive regulator HIF-1, but through the negative regulator NF-κB. The elevation of intracellular cAMP and thus, the activation of PKA inhibited NF-κB activity, thereby partially restoring cytokine-repressed Epo gene expression. A better understanding of the molecular mechanism of the counteraction between cAMP and IL-1 on Epo production will be important to design new therapeutic options to treat patients with ACD

7. Summary

Erythropoietin (Epo) is a 30.4 kDa glycoprotein hormone that primarily regulates the formation of red blood cells in the bone marrow. A decreased level of oxygen is the main stimulus for the synthesis of the hormone in the kidneys and the liver. Patients suffering from chronic inflammatory and malignant diseases show impaired Epo synthesis, often leading to anaemia. In blood samples from these patients the levels of proinflammatory cytokines like IL-1 and TNF-α are elevated. IL-1 and TNF-α are known to suppress Epo production in vivo and in vitro, which can be antagonized by cAMP. However, the molecular mechanisms by which cAMP counteracts the IL-1 suppression of Epo production are still unknown.

Since Epo gene expression is balanced by the activities of either enhancing or repressing transcription factors, studies were therefore performed on three transcription factors, which control the Epo gene, namely HIF-1, GATA-2 and NF-κB. The human hepatoma cell line HepG2 was used as a model for hypoxia induced, IL-1 repressed and cAMP rescued Epo production.

EMSAs, Western blots and luciferase assays showed that HIF-1 (a positively acting transcription factor) was not affected by cAMP. On the other hand, GATA-2 DNA-binding was enhanced by cAMP. Since GATA-2 acts as a negative regulator of Epo synthesis, its activity was unlikely to contribute to the IL-1 antagonizing effect of cAMP. However, EMSAs revealed that IL-1 also strongly activated DNA-binding of NF-κB, which is negatively acting transcription factor in respect to Epo expression. This NF-κB activation was antagonized by cAMP. Western blot analyses revealed that IL-1 stimulated the translocation of p50 and p65 proteins to the nucleus, which was inhibited by cAMP. Additionally, in order to determine an involment of the NF-κB in Epo gene expression we repressed NF-κB function by stably transfecting HepG2 cells with dominant-negative forms of the inhibitory protein IκBα. NF-κB DNA-binding was decreased in cells expressing the mutant form of IκBα compared to the cells expressing the normal form of IκBα. Also, Epo protein concentrations were higher in these cells after stimulation with IL-1, and cAMP had no antagonistic effect in the mutated cells. Accordingly NF-κB activation inhibits Epo expression. Furthermore, Epo mRNA expression was significantly reduced by IL-1 and reversed by cAMP in HepG2 cells.

On the basis of the present findings I suggest that the activation of NF-κB reduces Epo synthesis and that the decreased NF-κB activity on cAMP treatment explains the cAMP effect on Epo production. Thus, NF-κB is probably the primary transcription factor by which cAMP exerts a reverse effect on cytokine-repressed Epo expression.

8. Zusammenfassung

Erythropoietin (Epo) ist ein Glykoprotein-Hormon mit einer molekularen Masse von 30,4 kDa. Epo ist der wichtigste Faktor für die Produktion roter Blutkörperchen im Knochenmark. Eine veringerte Sauerstoffkonzentration im Gewebe, ist der Hauptstimulus für die Produktion dieses Hormons, die Hauptbildungsorte sind die Niere und die Leber. Bei Patienten mit chronisch entzündlichen und/oder malignen Erkrankungen ist die Epo Produktion gestört, was in vielen Fällen zu einer Anämie führt. Im Blut dieser Patienten finden sich erhöhte Zytokinspiegel, so z. B. für Interleukin 1β (IL-1) und Tumor Nekrose Faktor α. Für beide Zytokine wurden sowohl in *in vitro* als auch in *in vivo* Experimenten belegt, dass sie die Epo Produktion hemmen, und dass zyklisches Adenosin-Monophosphat (cAMP) diesen Effekt antagonisieren kann. Die molekularen Mechanismen, die zu der Antagonisierung des Zytokineffektes führen, sind nicht bekannt.

Da die Expression des Epo-Gens durch ein komplexes Zusammenspiel verschiedener, sowohl fördernder als auch hemmender, Transkriptionsfaktoren reguliert wird, wurden in dieser Arbeit drei Transkriptionsfaktoren näher untersucht. Dabei handelt es sich um den Hypoxie-induzierbaren Faktor 1 (HIF-1), den GATA-2 Transkriptionsfaktor und den nukleären Faktor α exprimiert in B-Zellen (NF-κB). Die humane Hepatom-Zellinie HepG2 wurde als Modell gewählt, da sie hypoxieabhängig Epo produziert, zytokinabhängig die Epo Produktion reduziert und durch cAMP Behandlung sich der Zytokineffekt antagonisieren lässt.

Gel-Retardationsversuche (EMSAs), Western Blots und HIF-1 abhängige Luziferase Reportergenstudien zeigten, dass HIF-1, ein aktivierender Transkriptionsfaktor für das Epo Gen, durch cAMP in seiner Aktivität nicht beeinflusst wurde. Demgegenüber stellte sich für GATA-2 heraus, dass die DNA-Bindung von GATA-2 an seine Zielsequenz durch cAMP verstärkt wurde. Da GATA-2 ein inhibitorischer Transkriptionsfaktor für das Epo-Gen ist, konnte ausgeschlossen werden, dass der IL-1 antagonisierende Effekt von cAMP durch GATA-2 vermittelt wird. NF-κB ist bezüglich der Epo-Produktion auch ein negativ regulierender Transkriptionsfaktor und IL-1 ist ein starker Induktor von NF-κB. Es zeigte sich, dass cAMP die IL-1 induzierte NF-κB Aktivierung abschwächt. In Western Blot Versuchen wurde durch eine Vorinkubation mit cAMP eine verringerte Kerntranslokation der p50 und p65 Untereinheiten von NF-κB nach

Stimulation mit IL-1 gefunden. Um die Bedeutung von NF-κB in der Vermittlung des cAMP-Effektes näher zu untersuchen, wurden stabile Zellinien hergestellt, bei denen die natürliche NF-κB Funktion reprimiert ist. Dazu wurden HepG2 Zellen mit Vektoren transfiziert, die zu einer Überexpression von dominant-negativen Formen des NF-κB inhibitorischen Proteins IκBα führten. Die Überexpression von dominant negativem IκBα führte zu reduzierter NF-κB DNA-Bindung im Vergleich zu Zellen die natives IκBα überexprimierten. Dementsprechend waren die Epo-Proteinkonzentrationen im Zellkulturüberstand nach IL-1 Stimulation in den Zellen höher, die das dominant-negative IkBα exprimierten. Schließlich zeigte sich auch, dass cAMP in diesen Zellen keinen antagonistischen Effekt mehr hatte. Des weiteren führte IL-1 Stimulation von HepG2 Zellen zu einer deutlichen Abnahme der Epo-mRNA und eine Vorbehandlung mit cAMP konnte diesen Effekt reduzieren.

Auf der Grundlage dieser Ergebnisse erscheint es plausibel anzunehmen, dass cAMP die Aktivierung von NF-κB abschwächt und damit die Zytokineffekte auf die Epo-Produktion teilweise aufhebt.

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11. Curriculum vitae

Personal information:

Surname: Batmunkh

Name: Chimedtseren

Date of birth: September 8, 1974

Nationality: Mongolian

Address: Bonus Strasse 38, 21079 Hamburg

E-mail: juujgai@yahoo.com

Phone number: 0177-3011318

Education and working experience:

2005.3-present: Reseacher, Department of Medical Molecular Biology University of Lübeck, Lübeck Germany

2002.6-2005.3: Graduate student, Department of Physiology University of Lübeck, Lübeck Germany

2001.7-2002.5: Reseacher at the Science-Technology and Production „Traditional Medicine" Corporation of Mongolia, MAS

2000-2001: Residency Training for Family Medicine at the Health Sciences University of Mongolia

1999-2001: Master of Medical Science at the Health Sciences University of Mongolia

Thema: Pharmacological study of the „Juniperius Sibirica"

1991-1998: Bachelor of Medicine at the Health Sciences University of Mongolia

Publications:

Batmunkh C, Krajewski J, Jelkmann W, Hellwig-Buergel T. Erythropoietin production: Molecular mechanisms of the antagonistic actions of cyclic adenosine monophosphate and interleukin-1 (manuscript under preparation)

Krajewski J, **Batmunkh C,** Jelkmann W, Hellwig-Buergel T. Interleukin-1β inhibits the hypoxic inducibility of the erythropoietin enhancer by suppressing hepatocyte nuclear factor 4α (submitted)

Presentation:

Batmunkh C, Jelkmann W, Hellwig-Buergel T. Mechanism of counteraction between cAMP and IL-1 on erythropoietin synthesis. "GRK-288 Mini Symposium" (2004)

Poster presentations:

Batmunkh C, Jelkmann W, Hellwig-Buergel T. cAMP prevents the negative action of interleukin-1 and NF-kB on erythropoietin expression

Ann. Hematol. 80, S109 (2003)

6th Int. Lübeck Conference on the Pathophysiology and Pharmacology of Erythropoietin and other Hemopoietic Growth Factors

Batmunkh C, Jelkmann W, Hellwig-Buergel T. Mechanism of the antagonism between IL-1 and cAMP on erythropoietin expression

Bone marrow transplant. Vol. 35, p455, S104 (2005)

EBMT 2005 / Annual Meeting of European Group of Blood and Marrow Transplantation Prague, Czech Republic

Language proficiency:

Russian: Fluent

Deutsch: Good

Englisch: Good

Polisch: Good