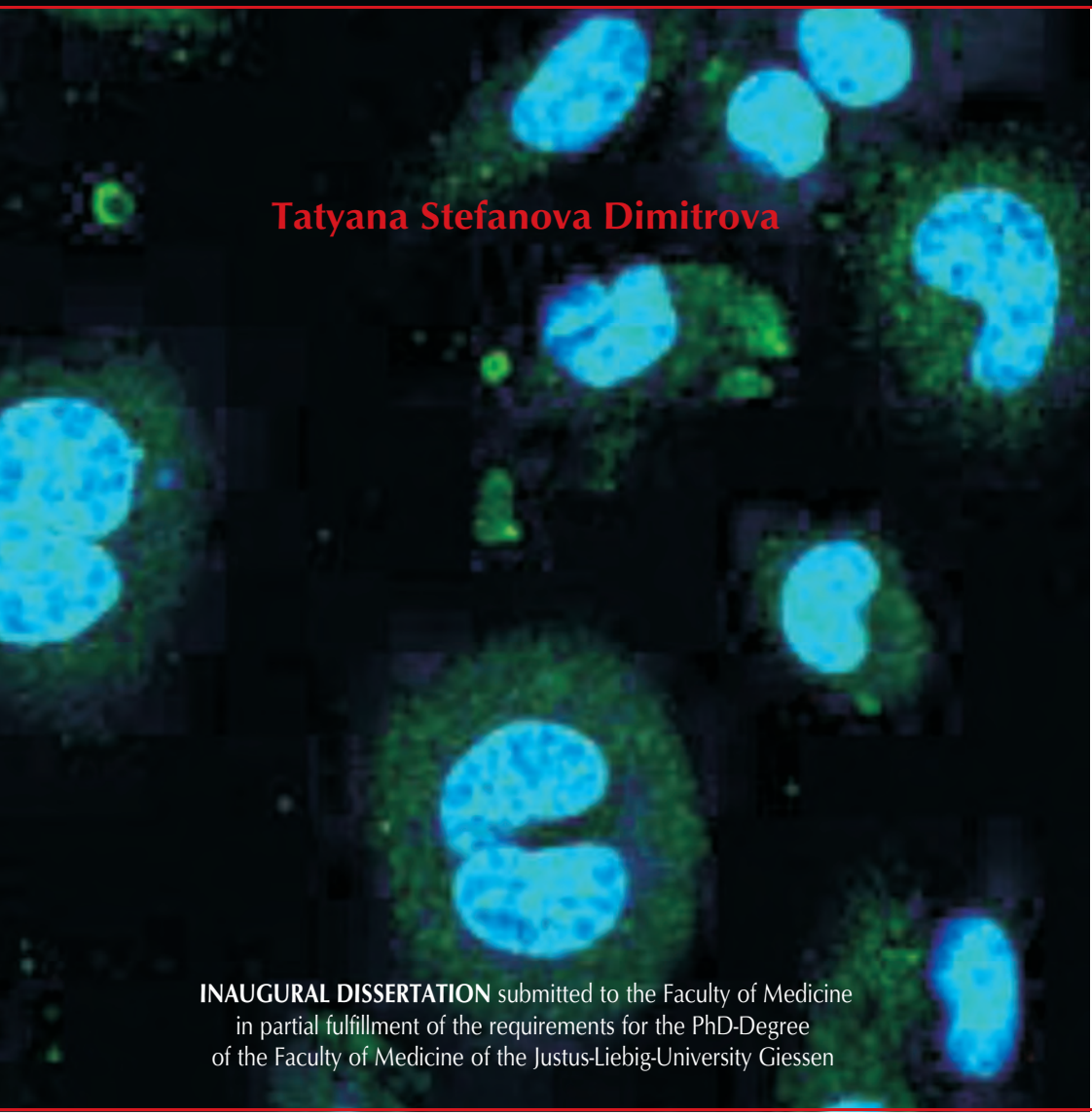
Inhibition of HIF prolyl 4-hydroxylases protect endothelial cells from apoptosis



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by

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Logical thinking is the example of a complete fiction

Friedrich Nietzsche

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ABBREVIATIONS

AA	Acrylamid
ANOVA	Analysis of variance
APAF-1	Apoptotic Protease Activating Factor-1
APS	Ammonium per sulfate
ARF	Alternate reading frame
ATP	Adenosin Tri-Phosphat
ATR	The ataxia telangiectasia rad-3-related kinase
Bax	Bcl-associated X protein
Bcl-2	B-cell lymphoma gene 2
Bcl-xL	Lange Spleissvariante des Bcl-x Proteins
BisAA	Bisacrylamid
BSA	Bovine serum albumin
°C	Degree celcius
$CaCl_2$	Calcium chloride
CBP cAMP	Response element-binding protein
CBP/p300	Interacting transactivator
CO_2	Carbon dioxide
Ctr	Control
ddH_2O	Double-distilled water
DMOG	Dimethyloxalyl glycine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Endothelial cells
ECGS	Endothelial cell growth supplement
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
e.g	For example
EGTA	Ethylene glycol-bis(2-aminoethylether)N,N,N',N'

EPO	Erythropoietin
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein-5-isothiocyanat
FIH	Factor-inhibiting HIF-1
h	Hours
H	Hypoxia
H_2O	Water
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
hEGF	Human epidermal growth factor
HEPES	4(2-hydroxyethyl)1piperazine ethane sulfon
HIF-1α	Hypoxia-inducible faktor alpha
HLH	Helix-Loop-Helix
HO-1	Heme Oxygenase-1
H/R	Hypoxia/reperfusion
HRE	Hypoxia responsive elements
HRP	Horseradishperoxidase
HSP90	Heat shock protein 90
HUVEC	Human umbilical vein endothelial cells
IgG	Immunglobulin G
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
I/R	Ischemia/reperfusion
IU	International unit
KCl	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
M	Molar
mM	Millimolar

µM	Micromolar
Mdm2	Mouse doubles minute 2
$MgCl_2$	Magnesium chloride
MG-132	Proteasome inhibitor
min	Minutes
N	Normoxia
NaCl	Sodium chloride
NaF	Sodium fluoride
Na_2HPO_4	Di-sodium hydrogen phosphate
NES	Nuclear export signals
NLS	Nuclear localization signal
nM	Nanomolar
NO	Nitric oxyde
NP-40	Nonidet P-40
NS	Nonsense
O_2	Oxygen
ODD	Oxygen-dependent degradation domain
OH	Hydroxyl
p53	Tumor suppressor protein 53 kDa
PAGE	Polyacrylamid-Gelelektrophorese
PAR-2	Protease activated receptor-2 agonist
PBS	Phosphate buffered saline
PFT-α	Pifithrin alpha
PFA	Paraformaldehyd
PHD	Prolyl hydroxylase domain protein
pH	Negative log of H+ concentration
PI	Propidium-Jodid
PMSF	Phenylmethylsulfonyl fluoride
Po_2	Oxygen partial pressure
RISK	Reperfusion injury survival kinases
RNA	Ribonucleic acid

siRNA	RNA interference
ROS	Reactive oxygen species
RT	Room temperature
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TEMED	N, N, N’, N’,-tetramethylethylenediamine
TOPRO-3	Stain
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylene-sorbitan-monolaureate
U/ml	Units/ml
VHL	von Hippel-Lindau
WB	Western blot
% vol/vol	Volume by volume percentage
% wt/vol	Weight by volume percentage

1 INTRODUCTION

Background

Hypoxia/reoxygenaion (H/R) is one of the most frequent complications leading to necrotic or apoptotic cell-death in the heart as well as in other organs. Since H/R is normally an unpredictable event, a strategy like preconditioning is of limited clinical relevance in protecting the heart against reperfusion-induced injury. Therefore, a maneuver like postconditioning, aiming to protect the heart at the onset or during reperfusion has gained much greater clinical interest. In this context, the hypoxia-inducible factor (HIF-1α) has emerged into focus. It is well established that HIF-1α is stabilized under hypoxic conditions and involved in transcriptional and non-transcriptional regulation of key pathways for hypoxic adaptation of cell metabolism and cell survival. With the onset of reoxygenation, however, HIF-1α is rapidly degraded and its effect on hypoxic adaptation is ebbing. HIF-1α stability is controlled by HIF-1α prolyl 4-hydroxylase-2 (PHD2), targeting HIF-1α for degradation in presence of oxygen. The central hypothesis of the present study is that preservation of HIF-1α beyond the period of hypoxia may enhance the protective effect of HIF-1α on endothelial cells against ongoing apoptotic cell death during H/R.

1.1 Ischemia/reperfusion injury

The cardiovascular system supplies the tissues of the body with blood. Restriction of regional blood flow results in ischemia, which causes the cellular oxygen tension to decrease and the carbon dioxide tension to rise. Clinically, the treatment for ischemia involves reestablishing blood flow to the ischemic tissue. However, reperfusion of ischemic tissue paradoxically triggers the morphological appearance of tissue injury, presumably by initiating events that contribute to the activation of cell-death pathways. The cellular events leading to ischemia/reperfusion (I/R) -induced cellular injury are complex, but the key elements include radical production, cellular disturbances of calcium homeostasis, and activation of cellular proteases. This has led to the concept of I/R injury as a sequence of events that contribute to cell death (Braunwald and Kloner

1985). It has been observed that different cell types show widely differing sensitivity to I/R.

1.2 Endothelial cell changes related to ischemia/reperfusion

Endothelium is the first line of defense between the circulation and the vessel wall. The integrity of endothelium is necessary for the function of the whole cardiovascular system. Vascular endothelium, when unperturbed, provides a surface to the blood vessel, and has the potential to regulate the passage of materials and the transit of blood cells in and out of the bloodstream. This characteristic is essential for vascular homeostasis. Endothelial cells are important in modulating the pathophysiology of I/R injury. It is known to cause changes in endothelial cells, including membrane depolarization, increased membrane fluidity, perturbations in the distribution of ions, cell swelling, cytoskeletal derangements, and recruitment and activation of inflammatory cells (Menger and Vollmar, 2007). Many changes occur during reperfusion. The importance of apoptosis in cell death following ischemia and reperfusion has been demonstrated in *in vivo* rodent models. Gottlieb and coworkers (1994) reported that apoptosis occurs in the rabbit myocardium after 30 min of ischemia and 4 h of reperfusion, but not in the permanent ischemic period. Anversa and coworkers (1998) quantified the level of apoptosis and necrosis in the myocardium, and reported much higher levels of apoptosis after 2 h of ongoing ischemia. Cell death based on apoptosis was 86% versus 14% on necrosis. Until today, there are controversial data over the extent of the apoptosis following ischemia and reperfusion. Necrosis and apoptosis appear to be ongoing during ischemia, while apoptosis is boosted by the reperfusion event (Eefting et al., 2004). Increasing evidence suggests that apoptosis of endothelial cells can be responsible for endothelial dysfunction (Werner et al., 2006). In the very early stages of reperfusion, apoptosis is first seen in the endothelial cells from small coronary vessels (Lee et al., 2005; Scarabelli T et al., 2001). The spread of apoptosis to surrounding cardiac myocytes suggests that reperfusion induces the release of soluble pro-apoptotic mediators from endothelial cells that promote cardiac myocyte apoptosis. The authors suggested that the spread of apoptosis to surrounding cardiac myocytes is due to reperfusion-induced release of soluble pro-apoptotic mediators from endothelial cells

that promote myocyte apoptosis. However, reperfusion also restores energy required for the completion of apoptosis and can accelerate the apoptotic process. It is well documented that, compared to other cells of the cardiovascular system, endothelial cells are metabolically robust and able to maintain a high phosphorylation potential (ATP/ADP) even under low oxygen content (Mertens et al., 1990). This metabolic robustness, however, makes endothelial cells rather prone to apoptotic stimuli that are generated during I/R by all type of myocardial cells (Lelli et al., 1998). In contrast to necrosis, which occurs in the absence of ATP, apoptosis is an energy dependent process.

1.3 Cell death during ischemia/reperfusion

Apoptotic cascade is regulated by a family of proteins called caspases. These apoptosis executor proteins are present as proforms in all cells. After cleavage, pro-caspases become active and initiate pathways leading to apoptosis. The signalling pathway leading to programmed cell death is fine-tuned by positive and negative regulators, and a tight balance between these factors decides whether the cell undergoes apoptosis or survives. Normally, the tumor suppressor p53 controls cellular homeostasis by affecting cell cycle progression and apoptosis. p53 induces apoptosis via transcriptional activation of pro-apoptotic genes or repression of anti-apoptotic genes. Alternatively, p53 may provoke transcriptional independent alterations in facilitating apoptosis by affecting the mitochondrial pathway of cell demise (Schmid et al., 2004; Caelles et al., 1994). p53 regulates the expression of genes involved in growth arrest (e.g $p21^{Cip/WAF-1}$, 14-3-3G) and apoptosis (Puma, Bax, APAF-1), (Vousden and Lu 2002). Proteins that can shift the balance towards survival are the anti-apoptotic Bcl-2 and Bcl-x_L, whereas the pro-apoptotic proteins Bax, Bad, Bak, and Bid induce programmed cell death. p53 has been shown to accumulate under hypoxia and is clearly involved in the pathological response within endothelial cells (Stempien-Otero et al., 1999). Mechanisms, which are induced by ischemia or triggered by I/R leading to apoptotic cell death are still not fully understood. Endothelial cell death may contribute to hypoxia as well as the reperfusion components of this injury and different routes of protection need to be considered for the pre-ischemic or post-ischemic period.

Currently, there is no treatment for the heart tissue that directly aims to preserve the heart muscle during the time of blood deprivation and protect against reperfusion injury. Protecting the heart from I/R-induced injury represents the greatest challenge of cardiology, since myocardial infarction is the major cause of mortality in industrialized countries.

1.4 Protective strategies against ischemia/reperfusion

1.4.1 Preconditioning

The known mechanisms of protection include targets in specific intracellular signalling pathways, functions of intracellular organelles such as sarcoplasmic reticulum, and mitochondria, and control of intracellular ion homeostasis. Other strategies for cytoprotection by activation of endogenous mechanisms occur in 2 mechanistically distinct phases: before or after the ischemic event occurs (Das et al., 2008). Firstly, ischemic preconditioning is the most successful form of pre-treatment to stimulate adaptive mechanisms in myocardium before the major ischemic event. The effects of the so called ‘first window’ or early preconditioning, lasts 1–2 h, after which the protection wanes. The so called ‘second window’ or late preconditioning occurs 24 h following the initial preconditioning ischemia and lasts for 48–72 h. A major distinction between the two stages is that early preconditioning has been shown to result in the modification of existing myocardial proteins, whereas late preconditioning is exerted by newly synthesized cytoprotective proteins in the heart (Downey et al., 2007). Following the initial discovery of preconditioning, it became clear that pharmacological agents could also exhibit cardioprotection when administered prior to the onset of sustained myocardial ischemia (Bolli et al., 2001). This is significant, since pharmacological agents could be more readily applied to clinical practice as a means of protecting the heart and other organs against I/R injury rather than to place ischemia directly. In ischemia, the best protection is achieved when these agents are administrated prior to ischemia, as in preconditioning (Pasupathy and Homer-Vanniasinkam, 2005). An alternative treatment opportunity to intervene at reperfusion is primary percutaneous coronary intervention and thrombolytic therapy. Apart from cardiac surgery, however, pre-ischemic treatment

of the myocardium is clinically of limited relevance, since acute myocardial infarction is normally an unforeseen event. Therefore, a maneuver like postconditioning, aiming to protect the heart at the onset or during reperfusion has gained much greater clinical interest (Vinten-Johansen et al., 2007). In reperfusion, the first seconds to minutes have the most significant impact on 'acute' reperfusion injury. In view of the acute changes during reoxygenation, protective interventions must be applied early on. Identification of such endogenous endothelial strategies may open new opportunities to protect endothelial cells from cell death. Endothelial survival mechanisms which may also be activated under the same conditions are largely unknown.

1.4.2 Postconditioning

The postconditioning, consists of repeated brief episodes of ischemia and reperfusion (paralleling the durations used in preconditioning) (Zhao et al., 2003). By several cycles of postconditioning repeated at the onset of reperfusion, infarct size is reported to be reduced by up to 50% in canines. In addition, postconditioning reduced reperfusion arrhythmias (Halkos et al., 2004; Galagudza et al., 2004; Kloner et al., 2006), neutrophil adherence to the post-ischemic coronary artery, neutrophil accumulation in the area at risk, myocardial and endothelial injury (Zhao et al., 2003). The mechanisms responsible for the postconditioning-induced cardioprotection include endogenous stimulation of adenosine receptors, activation of the so called reperfusion injury survival kinases (RISK) (Hausenloy and Yellon 2007), opening of K_{ATP} channels, attenuation of intracellular and mitochondrial calcium accumulation as well as inhibition of mitochondrial permeability transition pore opening (Zhao and Vinten-Johansen, 2006). One physiological mechanism by which postconditioning exerts its protective effects is by delaying the normalization of tissue pH in the heart during early reperfusion (Vinten-Johansen et al., 2007). Studies have also revealed that postconditioning *in vitro* reduced reactive oxygen species (ROS) generation, which was also associated with reduced cell death (Sun et al., 2005).

1.4.3 Pharmacological postconditioning

Studies have identified certain drugs, which reduce reperfusion injury, when given at reperfusion or reoxygenation. This approach is termed as 'pharmacological postconditioning' and has the advantage that they also can be used after myocardial infarction as well as in the situations of "non-scheduled" infarct. Agents such as inhalational anaesthetics (Feng et al., 2005; Weber et al., 2005), bradykinin, the chemotherapeutic agents cyclosporine A (Lim et al., 2007), erythropoietin (EPO), nitric oxide (NO) (Johnson et al., 1991), hydrogen sulfide, and adenosine (Lefer et al., 2007) have demonstrated cardioprotective effects when given at reperfusion in experimental studies. Adenosine infusion at the onset of reperfusion has demonstrated cardioprotective effects in part by attenuating neutrophil–endothelium interactions. EPO, inhalational anaesthetics, bradykinin, and other agents activate RISK pathway. Another strategy used by Jiang and coworkers (2007) to enhance the effect of postconditioning is concomitantly delivered pharmacological agents. For instance the combination of postconditioning and a protease activated receptor-2 agonist (PAR-2), both applied at the onset of reperfusion, reduced infarct size to a greater range than either intervention alone (Jiang et al., 2007). Therefore, pharmacological agents may not only perform postconditioning, but they can also enhance its effects by triggering different pathways, extending its effects beyond the early reperfusion phase.

1.5 Role of HIF-1α in cell survival during ischemia/reperfusion injury

Over the past decade, HIF-1α has emerged as a key regulator of the molecular hypoxic response by mediating a wide range of physiological and cellular mechanisms necessary to adapt to oxygen deficiency. It has been reported that an increase in the level of HIF-1α is one of the first adaptive responses, at the molecular level, of myocardium to ischemia (Lee et al., 2000). Recent research (Eckle et al., 2008) has demonstrated that HIF-1α stabilization is central to cardio protection achieved through ischemic preconditioning. Moreover, Zhao and coworkers (2009) showed that cardioprotection by postconditioning is associated with up-regulation of HIF-1α expression. Furthermore, expression of a constitutively active HIF-1α hybrid has been shown to protect cardiac myocytes against I/R injury (Date et al., 2005). In this context,

increasing evidence suggests that cell survival during I/R can be influenced by the expression of genes that promote glycolysis, suppress ROS production, limit mitochondrial metabolism, and inhibit pro-apoptotic protein expression (Loor and Schumacker, 2008). HIF-1α is a member of transcription factors that regulate the expression of nearly 200 genes that can affect the cellular adaptive responses to hypoxia (Semenza et al., 2000).

1.5.1 Regulation of HIF

Investigation on the molecular mechanisms involved in hypoxia, and the induction of hematopoietic growth hormone EPO led to identification of HIF-1 by Semenza and Wang in 1992. The HIF system is discovered as a key regulator of a broad range of cellular and systemic responses to hypoxia and acts in all mammalian cells. Three isoforms of HIF-α exist (HIF-1α, HIF-2α, and HIF-3α). All are encoded by distinct gene loci and further diversity is generated by alternative splicing in the promotor region. HIF-1α and HIF-2α share a similar domain structure and undergo similar proteolytic regulation; however, the tissue expression of HIF-2α seems to be more limited (Wiesener et al., 2003). HIF-1α-mediated pathways influence the processes of metabolic adaptation, erythropoiesis, angiogenesis, vascular tone, cell growth and differentiation, survival and apoptosis, and thus are critical factors in development, physiology, and disease (Maxwell et al., 2001). HIF-1α is a heterodimeric DNA-binding complex composed of two basic helix-loop-helix (HLH) proteins of the PER-ARNT-SIM (PAS) family, the constitutive HIF-1β and one of either hypoxia-inducible α-subunits, HIF-1α or HIF-2α (Wang et al., 1995). In hypoxia, the α/β heterodimer binds to a core sequence in the hypoxia response elements (HRE) of target genes. HIF-β subunit is a non-oxygen-responsive nuclear protein that also has other roles in transcription, for example, in the xenobiotic response. In contrast, the HIF-α subunits are highly inducible by hypoxia. Under normoxic conditions, HIF-α subunits have a very short half-lives (Jewell et al., 2001). Cells continuously synthesize and degrade HIF-1α protein. Oxygen-dependent degradation of HIF-1α is regulated by the hydroxylation of specific prolyl residues in a region of the peptide referred as the oxygen-dependent degradation (ODD). A family of 2-oxoglutarate-dependent prolyl 4-hydroxylases is responsible for this event, which

requires O_2, iron, 2-oxoglutarate, and ascorbate. The hydroxylated proline residues in the ODD domain of HIF-1α facilitate recognition by the von Hippel-Lindau (VHL) protein. VHL is the recognition component of an E3-ubiquitin ligase complex that targets HIF-1α for proteolysis by the ubiquitin–proteasome pathway (Ohh et al., 2000).

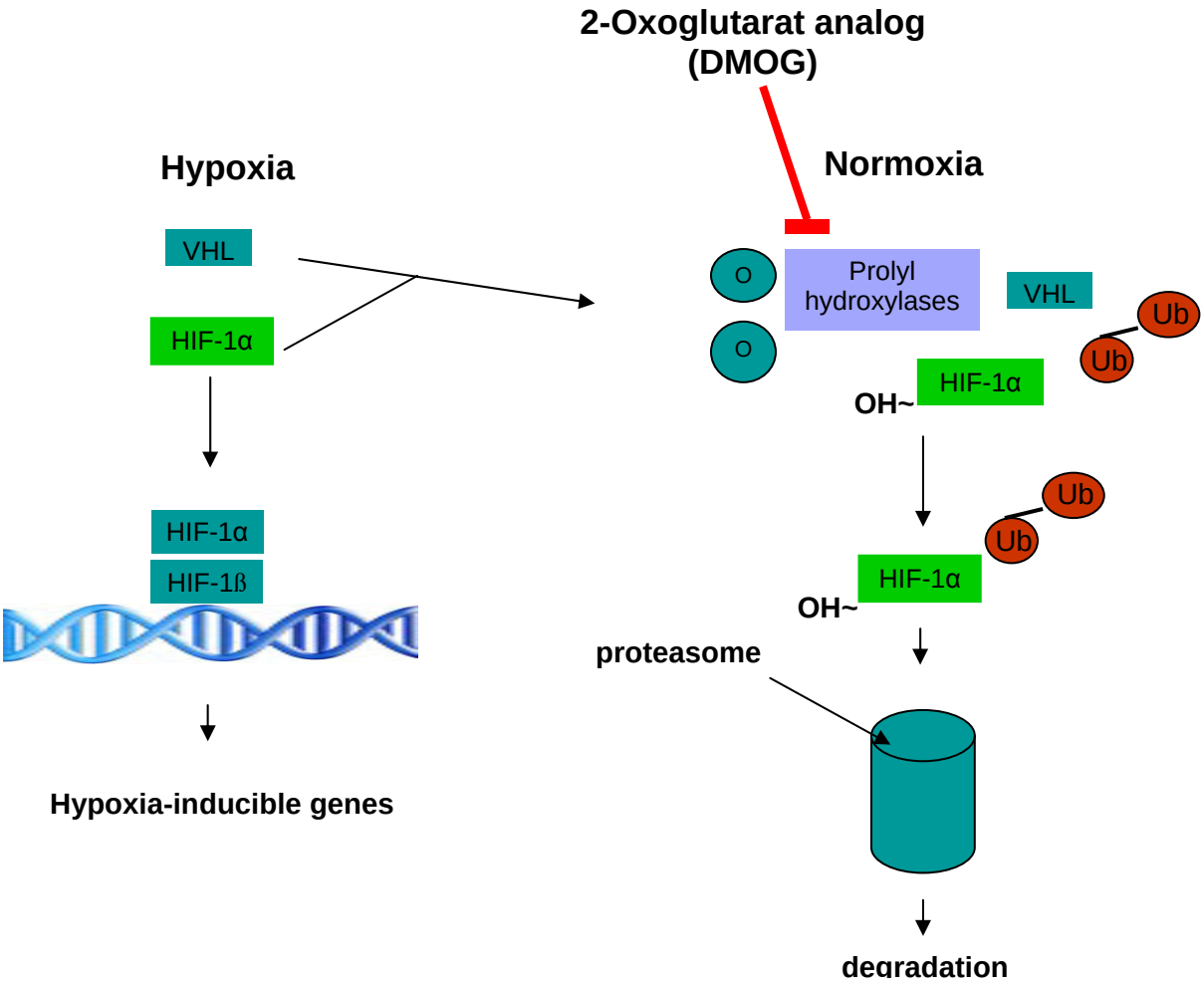


Fig. 1 Regulation of HIF-1α under normoxia and hypoxia. Hypoxia or inhibitors that prevent HIF-1α degradation promote HIF-1α nuclear translocation and heterodimerization with HIF-1β. Oxygen-dependent hydroxylation by prolyl hydroxylases regulates the interaction with VHL and proteasomal degradation.

Under hypoxic conditions, prolyl hydroxylation is suppressed, allowing HIF-1α subunit to accumulate, heterodimerize with HIF-1β, and initiate transcription. The effects of hypoxia

can be mimicked by iron chelation, by use of 2-oxoglutarate analogs such as dimethyloxalyl glycine (DMOG) or by substitution of Fe(II) by metal ions such as cobalt. In contrast to regulation of HIF-1α stability by proline modification in the ODD, transcriptional activity is regulated by the hydroxylation of asparagine residues in the C-terminal region of HIF-1α (Hewitson et al., 2002; Lando et al., 2002).

1.5.2 Alternative modifications affecting HIF-1α transcription

In addition to asparagine hydroxylation, various post-translational modifications occur on the HIF-1α C-terminal region, which can specifically modulate its activity. For example, phosphorylation of HIF-1α at Thr 796 by casein kinase II was first postulated by Gradin and coworkers (2002). Enhanced activation of mitogen-activated protein kinase p42/p44 mediates phosphorylation of serine residues 641 and 643 and increases HIF-1α -mediated transcriptional activity (Mylonis et al., 2006). Also, SUMOylation of HIF-1α has been suggested to increase HIF-1α stability and transcriptional activity (Carbia-Nagashima et al., 2007). Another option for HIF-1α regulation can be by heat shock protein 90 (HSP90), a molecular chaperone that protects its target proteins from misfolding and degradation through its ATPase activity (Neckers et al., 2003). HSP90 binds to the PAS domain of HIF-1α and increases its stability (Isaacs et al., 2004). Other stimuli for HIF-1α activation under normoxia include cytokines, growth factors, and oncogenes.

1.5.3 Pharmacological manipulation of HIF-1α

The central role of HIF-1α in physiology and pathophysiology makes it an attractive target for pharmacological manipulations. HIF-1α has an important role in triggering cellular protection and expression of genes involved in cell survival, apoptosis, and resistance to oxidative stress. For example HIF-dependent expression of glycolytic genes enhances ATP generation by anaerobic glycolysis. Similarly, HIF-dependent genes such as Heme Oxygenase-1 (HO-1) may regulate cell survival in I/R by affecting the response to oxidant stress. Other genes regulated by HIF-1α include inducible nitric oxide synthase (iNOS), which has been associated with enhanced resistance to ischemia in mice (Guo et al., 1999). Activators of HIF-1α might be useful for the

treatment of ischemic disease; however, inhibitors of HIF-1α could have some potential as anticancer therapeutics. Different strategies have been utilized to activate HIF-1α, where the best studied so far are inhibitors of the prolyl-hydroxylases (Warnecke et al., 2003; Kim et al, 2006). Downregulation of PHD by siRNA in murine microvascular endothelial caused a time- and dose-dependent HIF-1α protein stabilization, corresponding with increase in iNOS mRNA. Treatment of intact murine hearts with siRNA directed against PHD2 produced a decrease in infarct size and cardiac dysfunction following global I/R. The improved functional recovery was lost in iNOS-/- mice (Natarajan et al., 2006). This study provides evidence that activation of HIF-1α inducible iNOS contributes to the protective effect against I/R. *In vivo* studies in a rabbit model of myocardial I/R showed that systemic administration of PHD inhibitor DMOG, before I/R is associated with a significant reduction in infarct size. The attenuation of tissue injury was associated with expression of HO-1, a gene regulated by HIF-1α (Ockaili et al., 2005). In both microvascular endothelium *in vitro*, and *in vivo*, DMOG attenuated interleukin 8 (IL-8) productions and this was associated with robust HO-1 expression.These data show that HIF-1α activation induces HO-1 expression that is associated with attenuated proinflammatory chemokine production (Cai et al., 2008; Natarajan et al., 2007). Wild-type mice, or mice heterozygous to HIF-1α (HIF-1+/-) subjected to brief periods of ischemia, followed by 30 min of continuous ischemia showed improved infarct size and function after I/R in wild-type animals, but not in HIF-1+/- hearts. This suggests that HIF-1α activation should confer protection against I/R injury, although this protection might require HIF-1α activation before the onset of lethal ischemia. Moreover, two isoforms of the α subunit with high sequence homology, HIF-1α and HIF-2α, have been identified. Research (Hill et al., 2008) with mice heterozygous for a defect in either HIF-1α or HIF-2α provide direct evidence that both subunits have protective roles in the renal I/R injury. Similarly, a recently reported study showed that a HIF hydroxylase inhibitor with non-disclosure name FG-4487 (FibroGen), protected the rat from renal I/R injury and also induced accumulation of both HIF-1α and HIF-2α subunits (Bernhardt et al., 2006). Taken together, all this finding provide evidence that both HIF-1α and HIF-2α isoforms when activated predispose to protection against I/R injury. In this context, up-regulation HIF appears to play an important role in the

protection against injury. However, the molecular mechanisms responsible for this protection are still under intensive investigation.

1.6 Alternative regulators

1.6.1 Regulation of p53

HIF-1α as well as p53 are transcription factors involved in stress responses which require an immediate reaction of the affected cell (Schmid et al., 2004). Normally, p53 controls cellular homeostasis by affecting cell cycle progression and apoptosis. In unstressed cells p53 exhibits short half-life and the protein amount is maintained at a low level. Under stress such as DNA damage, nutrient deprivation, or hypoxia, p53 (Levine et al., 2006) is stabilized mainly by posttranslational modification. p53 becomes active as a transcription factor and promotes transcription of cell cycle regulating genes such as $p21^{WAF1/CIP1}$, mouse doubles minute 2 (Mdm2) as well as genes involved in apoptotic events like Bax, Fas, Bid (Vousden and Lu 2002). Each of these genes when silenced or removed induces partial resistance to p53-induced apoptosis. Mechanisms responsible for p53 inactivation include the regulation of protein activity, stability and subcellular localization. Different enzymes involved in post-translational modification of p53 such as ataxia telangiectasia, rad-3-related kinase (ATR) kinase as well as transcriptional coactivators can modulate the transcriptional activity of p53 (Boyd et al., 2000; Shieh et al.,1997; Shirangi et al., 2002). Protein stability of p53 can be regulated by ubiquitin ligases. This is illustrated by Mdm2, one of the key E3-ubiquitin ligases responsible for limiting the levels of p53 (Harris and Levine, 2005). Deletion of Mdm2 in mice results in an extremely early embryonic lethality that is the direct result of impeding p53-mediated apoptosis. Negative regulation of p53 is accomplished by Mdm2, either through ubiquitin-dependent p53 degradation in the cytoplasm (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997) or repression of p53 transcriptional activity in the nucleus (Thut et al., 1997). Both, Mdm2 and p53 are nuclear proteins that shuttle constantly through the nuclear pore complex. Mdm2 and p53 are translocated between the cytoplasm and the nucleus by their intrinsic nuclear localization signal (NLS) and nuclear export signal (NES) sequences (Chen et al., 1995; Liang and Clarke, 2001; Roth

et al.,1998). Blocking their nuclear export by mutations in the NES or by leptomycin B leads to their stabilization, indicating that both Mdm2 and p53 are degraded in the cytoplasm (Freedman and Levine 1998). A recent study demonstrating that phosphorylation at the p53 N-terminal inhibits its nuclear export, emphasizes the importance of export in controlling p53 function (Zhang et al., 2001).

All these signals affecting p53 suggest that each stress response utilizes a different pathway to induce a p53 response. For example reduced expression of alternate reading frame (ARF), a small protein that inhibits Mdm2 leads to stabilization of p53.

1.6.2 HIF-1α-p53-interplay

p53 like HIF-1α, is continuously expressed and immediately marked for degradation by specific E3-ubiquitin ligases: Mdm2 and VHL consequently. HIF-1α and p53 are kept at low levels during normoxia. HIF-1α is hydroxylated under normoxic conditions, subsequently bound by VHL and marked for proteasomal degradation by ubiquitination. Under hypoxic conditions, HIF-1α accumulates, binds to the transcriptional cofactor cAMP-response element-binding protein (CBP/p300) and induces expression of HIF-1α target genes. A similar process takes place during normoxia, where p53 is bound and targeted for degradation by Mdm2. During hypoxia, the interaction between Mdm2 and p53 can be impaired by phosphorylation of p53 by ATR kinase or through Mdm2 downregulation, which prevents binding of Mdm2 and allows accumulation of p53. Then p53 binds CBP/p300 and becomes transcriptionally active. The illustrated regulation via constant synthesis of protein becomes understandable when the function of both proteins is taken into account. Competition between p53 and HIF-1α for limiting amounts of the shared transcriptional coactivator CBP/p300 (Freedman et al., 2002) can explain how transcriptional activity of both p53 and HIF-1α might be affected, depending on the relative amount of either factor. An and coworkers (1998) demonstrated that p53 stabilization under hypoxia is dependent on HIF-1α, either directly (Hansson et al., 2002) or via Mdm2 (Chen et al., 2003). Such interactions resulted in p53 stabilization and activation, and concomitantly, HIF-1α degradation. This illustrates that p53 also seems to play a pivotal role in HIF-1α regulation. In line with these observations are reports showing that p53 inhibits HIF-1α activity by targeting HIF-1α for Mdm2-mediated

ubiquitination and proteasomal degradation (Ravi et al., 2000). In turn, the loss of p53 has been shown to enhance hypoxia-induced HIF-1α levels. Besides directly affecting HIF-1α protein level, p53 represses HIF-1α-stimulated transcription (Ravi et al., 2000). Interestingly, p53 levels required to affect HIF-1α were higher than those needed for p53-activated gene transcription (Blagosklonny et al., 1998). In endothelial cells p53 activation by hypoxia is partly independent of HIF-1α. This could explain particularly high levels of inactive p53 by the fact that regulation in endothelial cells is different (Sabapathy et al., 1997). The above observation supports the concept that similar and in many cases common regulatory mechanisms exist that regulates the transcriptional activity and protein stability of both HIF-1α and p53. Therefore, the present study aims to further elucidate the impact of HIF-1α on p53, specifically focusing on cell survival and H/R injury.

1.7 Mdm2

1.7.1 Regulation of Mdm2

Mdm2 gene encodes a protein with a predicted molecular weight of 56 kDa. This protein contains several conserved structural domains including the N-terminal p53 interaction domain. Mdm2 also contains C-terminal RING domain, which confers E3-ubiquitin ligase activity. There are several known mechanisms for Mdm2 regulation (Meek et al., 2003). One of these mechanisms is phosphorylation of the Mdm2 protein. Mdm2 is phosphorylated at multiple sites by Protein kinase B, Death-associated protein kinase, cyclin dependent kinase and ATR kinase. Following DNA damage, phosphorylation of Mdm2 leads to changes in protein function and stabilization of p53. Additionally, phosphorylation at certain residues of Mdm2 may stimulate its ability to target p53 for degradation. The induction of ARF product of the p14arf protein is also a negative regulator of p53-Mdm2 interaction. It interacts directly with Mdm2 leading to up-regulation of p53 transcriptional response. ARF captures Mdm2 in the nucleus, resulting in inhibition of nuclear export and activation of p53, since nuclear export is essential for proper p53 degradation. The Mdm2 gene is in turn transcriptionally activated by p53, constituting a feedback regulatory loop (Christophorou et al., 2006).

1.8 HIF-1α and Mdm2 interplay

Recent reports confirmed an association between endogenous Mdm2 and HIF-1α under conditions of oxygen deprivation that results in increased physiologically-regulated levels of HIF-1α (Nieminen et al., 2005). Furthemore, under hypoxia, HIF-1α directly binds to Mdm2 both *in vitro* and *in vivo,* thereby stabilising p53 (Chen et al., 2003). Thus, Mdm2 expression significantly induces the indirect interaction between p53 and HIF-1α in cells, indicating that Mdm2 may act as a bridge, mediating the p53-HIF-1α interaction.

1.9 Working hypothesis

Hypoxia, H/R, and chronic hypoxia are all capable of engaging cellular death pathways leading to tissue injury and organ dysfunction. In the present study we focus on ongoing apoptosis during H/R in endothelial cells. It is well established that HIF-1α may play a role in H/R-induced injury of endothelial cells. HIF-1α is the principal regulator of cellular transcriptional responses to hypoxia. However, HIF-1α is degraded during reoxygenation, which may limit its effect on cell survival during the hypoxic period only.

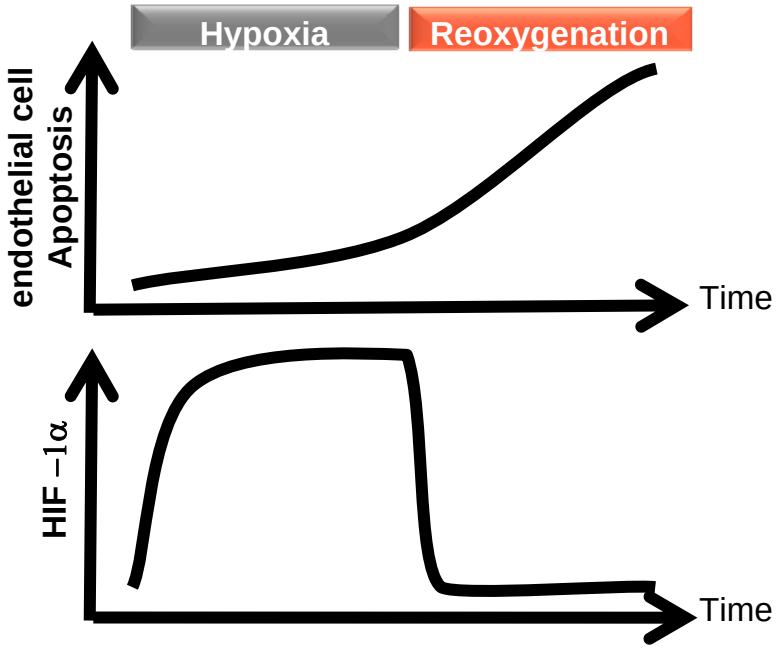


Fig. 2 These schematic diagrams illustrate the progression of apoptosis (upper panel) in relation to HIF-1α expression (lower panel) during hypoxia and reoxygenation.

It was hypothesized that a maneuver that stabilizes HIF-1α beyond the period of hypoxia may protect endothelial cell against ongoing apoptotic cell death during H/R. To further elucidate the function of HIF-1α, DMOG, 2-oxoglutarate analog, an inhibitor of PHD, was given at the onset of reoxygenation, an approach termed 'pharmacological postconditioning' (Hausenloy et al., 2005).

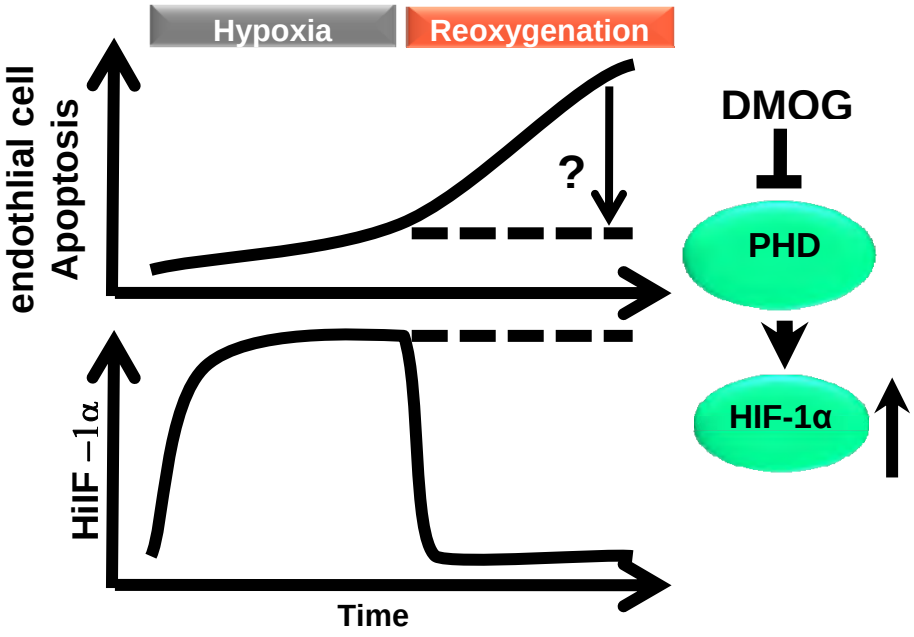


Fig. 3 Effect of the hypothesized pharmacological postconditioning mediated through prolyl-hydroxylase inhibition applied at the onset of reoxygenation leading to HIF-1α stabilization (dotted line lower panel). As a result endothelial cell apoptosis during reoxygenation is inhibited (dotted line upper panel).

1.10 Aims of the study

The present study was conducted to elucidate the molecular mechanism by which inhibition of PHD pathway leads to protection of endothelial cells against ongoing apoptotic cell death during H/R. Since previous studies using preservation of HIF-1α suggest its beneficial therapeutic potential in the treatment or prevention of ischemic injury, the present study was focused to analyse the molecular mechanism by which stabilization of HIF-1α exerts anti-apoptotic effects during H/R. The study was performed using an established model of cultured monolayers of human umbilical vein endothelial cells (HUVEC). The following questions were addressed:

Does inhibition of PHD protect endothelial cells against ongoing apoptotic cell death during H/R?

Does inhibition of PHD cause pro-apoptotic p53 protein degradation?

Is this p53 downregulation dependent on HIF-1α stabilization?

Does stabilization of HIF-1α regulate Mdm2, a specific E3-ubiquitin ligase?

Is this ligase responsible for p53 destabilization?

Does HIF-1α induce p53 degradation mediated by Mdm2?

As a pro-apoptotic challenge endothelial cells were cultured in serum free medium for 12 h and subjected to hypoxia for 1 h followed by reoxygenation. Under this condition, the effect of HIF-1α was analysed.

2 MATERIALS

2.1 Chemicals and consumables

Acrylamide solution (40%; wt/vol)	Amersham Pharmacia, Bucks
Acrylamide	Carl Roth, Karlsruhe
Ammonium persulfate	SERVA, Heidelberg
Annexin V FITC-Apoptosis	BD-Pharmingen, Heidelberg
Benzonase	Merck, Darmstadt
bFGF	PromoCell, Heidelberg
Bisacrylamide solution (2%; wt/vol)	Amersham Biosciences, Buckinghamshire, UK
Bisacrylamide	Carl Roth, Karlsruhe
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim
Bromophenol blue	Sigma-Aldrich, Steinheim
Calcium chloride	Merck, Darmstadt
Collagenase II	PAA, Pasching
Complete® protease inhibitor cocktail	Roche, Mannheim
Culture dishes	BD-Pharmingen, Heidelberg
Dimethyloxallyl Glycine (DMOG)	Cayman, Michigan, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim
Di-sodium hydrogen phosphate	Carl Roth, Karlsruhe
Dithiothreitol (DTT)	Amersham Biosciences, Buckinghamshire, UK
Chemiluminescent Substrate	Pierce Biotechnology, Rockford, USA
EDTA	Carl Roth, Karlsruhe
EGTA	Boehringer, Mannheim
Endothelial cell basal medium®	PromoCell, Heidelberg
Endothelial cell growth supplement	PromoCell, Heidelberg
Eppendorf tubes (0.5, 1.5, 2 ml)	Eppendorf, Hamburg
FACS tubes	BD-Pharmingen, Heidelberg
FACS Flow	BD-Pharmingen, Heidelberg
Falcon tubes (50 ml, 12 ml)	BD, Heidelberg
Fetal Calf Serum (FCS)	PAA, Pasching

Filter papers	Biotech-Fischer, Reiskirchen
FuGENE® 6	Roche, Mannheim
Glass coverslips	Menzel, Braunschweig
Glycerol (100%)	Sigma-Aldrich, Steinheim
Glycine	Carl Roth, Karlsruhe
HBSS	PAA, Pasching
hEGF	PromoCell, Heidelberg
HEPES	Sigma-Aldrich, Steinheim
Hydrocortisone	PromoCell, Heidelberg
Magnesium chloride	Fluka, Buchs, Switzerland
Magnesium sulfate	Merck, Darmstadt
ß-mercaptoethanol	Merck, Darmstadt
Methanol	Merck, Darmstadt
Millipore water	Millipore, Eschborn
MG-132	Biomol, Plymouth Meeting, USA
Molecular weight marker	Sigma-Aldrich, Steinheim
Nitrocellulose membrane	Schleicher & Schuell, Dassel
Nitrogen (100%)	Liquid, Krefeld
Non-fat milk powder	Applichem, Darmstadt
Nonidet P-40	Sigma-Aldrich, Steinheim
Paraformaldehyde	Sigma-Aldrich, Steinheim
Parafilm®	Pechiney Plastic Pack, Menasha, USA
Penicillin/streptomycin	Gibco BRL, Eggenstein
Phosphate buffered saline (PBS)	Gibco BRL, Eggenstein
Pifithrin-α (PFT-α)	Sigma-Aldrich, Steinheim
Pipette tips	Eppendorf, Hamburg
Pipettes	Eppendorf, Hamburg
PMSF	Sigma-Aldrich, Steinheim
Ponceau S solution	SERVA, Heidelberg
Potassium chloride	Merck, Darmstadt
Potassium dihydrogen phosphate	Merck, Darmstadt

Protein G-coated magnetic beads	Dynal, Oslo, Norway
Primary cell culture dishes	BD Falcon, Heidelberg
Rubber policeman	BD, Heidelberg
Scalpel (disposble)	Feather, Japan
See Blue® (pre-stained marker)	Invitrogen GmbH, Karlsruhe
Sodium azide	Merck, Darmstadt
Sodium chloride	Carl Roth, Karlsruhe
Sodium orthovanadate	Sigma-Aldrich, Steinheim
Sodium di-hydrogen phosphate	Carl Roth, Karlsruhe
Sodium dodecyl sulfate (SDS)	SERVA, Heidelberg
Sodium fluoride	Sigma-Aldrich, Steinheim
Sodium hydroxide	Carl Roth, Karlsruhe
Sodium orthovanadate	Sigma-Aldrich, Steinheim
Sterile filters (0.22 µm)	Sartorius, Goettingen
Sterile pipettes	BD, Heidelberg
Super signal-west®	Pierce biotech, Bonn
Syringes (20 ml, 2 ml)	BD, Heidelberg
TEMED	Sigma-Aldrich, Steinheim
TOPRO-3	Invitrogen GmbH, Karlsruhe
Trypsin-EDTA	Gibco-BRL, Eggenstein
Tris base	Carl Roth, Karlsruhe
Tritone X-100	Gibco-BRL, Eggenstein
Tween 20	Amersham Biosciences, Buckinghamshire, UK
Whatman® 3 MM filter paper	Millipore, Eschborn

2.2. siRNAs

1. Control siRNA duplex negative Eurogentec, Seraing, Belgium

2. HIF-1α sc-44225 Santa Cruz Biotechnology, Heidelberg, Germany
target sequence (target sequence: CGAGGAAGAACUAUGAACA)
target sequence (GAAUCAGAAGAUACAAGUA)
target sequence (CGAUGGAAGCACUAGACAA)

3. PHD2 (EGLN 1) ON-Target plus SMARTpool, Fischer Scientific, Germany
target sequence (GCGAUAAGAUCACCUGGAU)
target sequence (GACCUGAUACGCCACUGUA)
target sequence (GCUCAUCGCUGUUCCAGGA)
target sequence (GAACAAGCACGGCAUCUGU)

4. Mdm2 sc-29394 Santa Cruz Biotechnology, Heidelberg, Germany
target sequence (GCUUCGGAACAAGAGACCC)

2.3 Antibodies

Primary antibodies:	**Manufacture**	**Dilution**
HIF-1α (Mouse)	BD Bioscience, Heidelberg	1:250
Mdm2 (N-20) (Rabbit)	Santa Cruz Biotechnology, USA	1:1000
p53 (DO-1) (Mouse)	Santa Cruz Biotechnology, USA	1:1000
PHD2 (Rabbit)	Novus Biologicals, Hiddenhausen	1:1000
Vinculin (hVIN-1) (Mouse)	Sigma, Steinheim	1:2000

Secondary antibodies:		
Mouse IgG-HRP	BD Bioscience, Heidelberg	1:2000
Rabbit IgG-HRP	Upstate, Charlottesville, USA	1:2000
Mouse IgG (H+L) Alexa Fluor 488	Invitrogen, Karlsruhe	1:400

2.4 Laboratory equipment

Beckman Allegra 64R centrifuge	Beckman Coulter, Fullerton
Beckman TL 100 ultracentrifuge	Beckman Coulter, Fullerton
Blotting chambers	Biotech-Fischer, Reiskirchen
Confocal Microscope LSM 510	Carl Zeiss AG, Oberkochen
counter	Packard Instrument Company CT, USA
Electrophoresis apparatus	Biometra, Goettingen
FACS Calibur®	BD Bioscience, Heidelberg
Gel documentation system	Quantity One series Bio-Rad, Munich
Glass ware	Schott, Mainz
Hamilton syringe	Hamilton, Bonaduz
Heatblock	Techne, Burkhardtsdorf
Hypoxia-Chambers	workshop Institute of Physiology of JLU
Incubators	Heraeus, Hanau
Laminar flow hood	Heraeus, Hanau
Magnet stirrer	Jahnke und Kunkel, Staufen
Magnetic rack	DYNAL, Oslo, Norway
Neubauer chamber	Superior, Marienfeld
Phase contrast microscope	Olympus, Japan
pH-Meter	WTW-Weinheim
Photometer	Carl Zeiss, Jena
Power supply	Biometra, Goettingen
Rocker	Biometra, Goettingen
Table top (centrifuge)	Eppendorf, Hamburg
Vortexer	Heidolph, Kelheim
Water bath	Julabo, Seelbach

3 METHODS

3.1 Isolation and cultivation of human umbilical vein endothelial cells

HUVEC were isolated from freshly collected umbilical cords (from Gynecology Department, University Hospital Giessen). To isolate HUVEC the vein in the umbilical cord was canulated and rinsed with warm HBSS supplemented with 1.3 mM $CaCl_2$ and 1.2 mM $MgCl_2$. To detach the endothelial cells from the vessel wall, the umbilical cord vein was incubated with collagenase II A solution in a humidified incubator for 20 min. The primary endothelial cells were then washed out of the vessel with HBSS containing 3% (vol/vol) FCS, added to inactivate collagenase activity. Following collection of the cells, they were pelleted by centrifugation at 250 x g for 5 min at RT. The supernatant was discarded and cells were resuspended in endothelial cell culture medium and seeded into 3-4 primary cell culture dishes. After incubation for 2 h at 37 °C and 5% CO_2, cells were extensively washed with HBSS to remove unattached non–endothelial cells and cell debris. Adherent cells were incubated in 15-20 ml of cell culture medium at 37 °C and 5% CO_2 in a humidified incubator. After 24 h the medium was replaced with fresh cell culture medium. The cells were passaged and maintained in HUVEC growth medium with 20% (vol/vol) FCS at 37 °C with 5% CO_2 in a humidified incubator.

Endothelial cell culture medium

Endothelial cell basal medium (PromoCell®) supplemented with FCS (vol/vol)	10%
Endothelial cell growth supplement/Heparin (wt/vol)	0.4%
Hydrocortisone (wt/vol)	0.1%
bFGF	1 ng/ml
hEGF	0.1 ng/ml
Penicillin/streptomycin (vol/vol)	2%

Collagenase solution

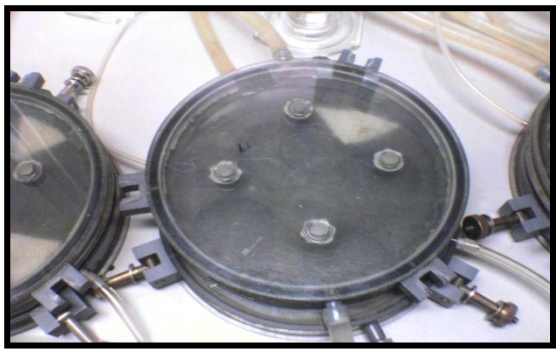
HBSS	x ml
Collagenase II, 293 IU/mg (wt/vol)	0.025%
$MgCl_2$	0.5 mM
$CaCl_2$	1.5 mM

3.2 Subcultivation of endothelial cells

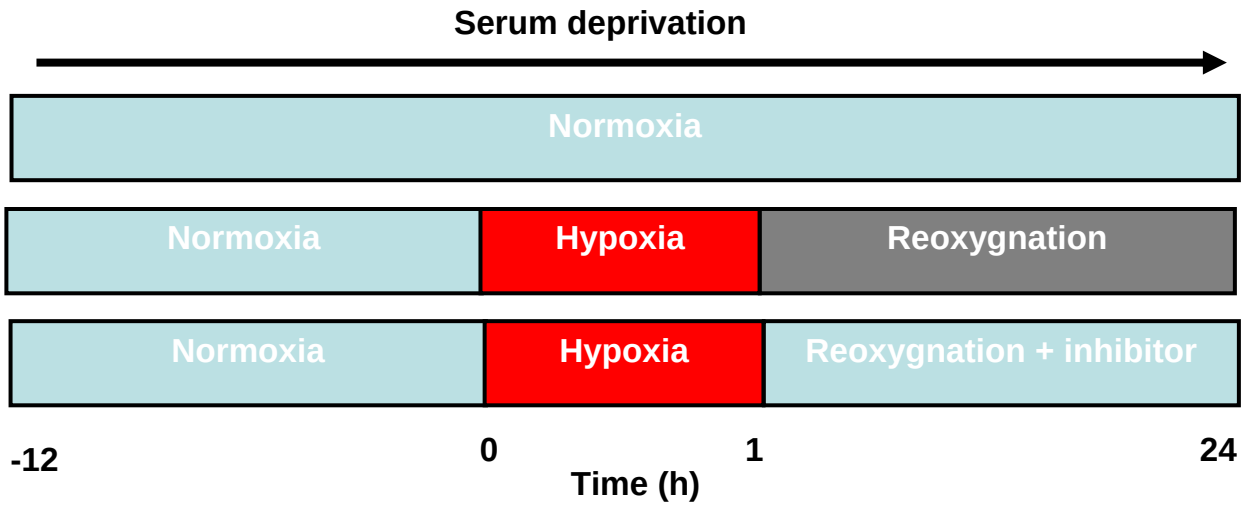
Confluent monolayer of primary endothelial cells were trypsinized 5-7 days after seeding. Cells were washed once with warm HBSS followed by incubation with trypsin-EDTA (Composition in mM: 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 8.0 Na_2HPO_4; pH 7.4, 0.05% (wt/vol) trypsin and 0.02% (wt/vol) EDTA) for approximately 2 min to allow the majority of the cells to detach from the cell culture dish. Trypsinized cells were collected into cell culture medium and seeded at a density of 5.5 x 10^4 cells/cm^2 on 35 mm dishes, (according to the experiment being performed). For immunostaining and confocal microscopy cells were seeded onto 25 mm glass coverslips. Experiments were performed with confluent endothelial monolayers of passage 1, 3-4 days after seeding.

3.3 Experimental protocol for hypoxia/reoxygenation

Confluent monolayers of HUVEC were cultivated in serum free culture medium 12 h before onset of experiments. After an initial equilibration period of 15 min in HBSS supplemented with 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 0.05% (wt/vol) bovine serum albumin (BSA), cells were subjected to 1 h of hypoxia (Po_2 < 10 mmHg) in an air tight chamber flushed with 100% humidified N_2, followed by 24 h of reoxygenation. Normoxic controls were exposed to humidified air (Po_2 = 140 mmHg).



To investigate the effect on cell signaling and ongoing apoptotic cell death, pharmacological inhibitors were added with the onset of reoxygenation. Stock solutions of inhibitors were prepared immediately in DMSO. Appropriate volumes of these solutions were added to the cells yielding in a final concentration < 0.1% (vol/vol). In a set of pilot experiments concentration-response relationships were determined to find the optimum effective concentration of the inhibitors used in this study. They were applied in their optimum effective concentrations.



Starvation media

Endothelial cell basal medium (PromoCell®)
supplemented with Penicillin/streptomycin (vol/vol) 2%

Basal media for hypoxia/normoxia (H/N)

HBSS supplemented with 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 0.05% (wt/vol) BSA

List of pharmacological inhibitors

Inhibitor	Inhibiting protein concentration used	Solvent
DMOG	**1 mM**	**DMSO**
MG-132	**10 µM**	**DMSO**
PFT-α	**100 µM**	**DMSO**

3.4 siRNA interference

Knockdown of endogenous HIF-α, PHD2, and Mdm2 was achieved by transfection of specific siRNA and transfection agent Fugene 6 according to manufacture's instructions. **Protocol:** 24 h prior to experiments 70-80% confluent cell monolayers were transfected with 2 µl of Fugene 6 to 97 µl of OPTI-MEM using 1-2 µg of siRNA. After 6 h 10% (vol/vol) FCS was added. Control samples were treated with identical concentration of non-specific siRNA following the same protocol.

3.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Sample preparation: Endothelial cells were washed with HBSS and subsequently lysed in 150 µl 1 x SDS sample buffer and 10 mM DTT added freshly before use. Subsequently, 50 IU/ml Benzonase® and 2 mM $MgCl_2$ was added and the lysate was collected in a 1.5 ml Eppendorf tube. Samples were denatured for 3 min at 95 °C and used immediately or stored at –20 °C.

Procedure: Discontinuous SDS polyacrylamide gel electrophoresis (Laemmli 1970) was performed to analyse protein content in cell lysates. The resolving gel solution, composition is given below, was poured into the assembled gel mold between two glass plates separated by 1 mm thick spacers leaving about 1 cm space for the stacking gel solution and layered with water. After polymerization the water was removed, the

stacking gel solution was poured on top and the comb was inserted. After polymerization of the stacking gel, the comb was removed and the gel mounted in the electrophoresis chamber. Electrode reservoirs were filled with 1 x SDS running buffer, the wells were cleaned and samples loaded. Electrophoresis was run overnight at 45 V.

1x-SDS sample buffer

Tris/HCl (pH 6.8)	2.5 mM
Glycerol	5% (vol/vol)
SDS	2% (wt/vol)
DTT	1 mM
ß-mercaptoethanol	0.9% (vol/vol)
Bromophenol Blue	0.002% (wt/vol)

Resolving gel buffer: Tris/HCl; pH 8.8	1.5 M
Stacking gel buffer: Tris/HCl; pH 6.8	0.5 M

10x Gel running buffer

Tris	250 mM
Glycine	2.0 M
SDS (wt/vol)	10%

Gel	Resolving gels				Stacking gel
Solutions	7.5 %	10 %	12.5 %	15 %	6 %
Acryl. 40% (wt/vol)	7.7 ml	10.2 ml	12.7 ml	15.3 ml	3.8 ml
Bisacryl.2%(wt/vol)	4.2 ml	5.6 ml	7.0 ml	8.4 ml	2.0 ml
Millipore water	17.7 ml	13.8 ml	9.8 ml	5.8 ml	17.5 ml
Resolving gel buff.	9.5 ml	9.5 ml	9.5 ml	9.5 ml	------
Stacking gel buff.	------	------	------	------	6.0 ml
SDS 10%(wt/vol)	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.25 ml
TEMED	30 µl	30 µl	30 µl	30 µl	20 µl
APS 10% (wt/vol)	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.25 ml

3.6 Western blot

Proteins were separated by 7,5-15% SDS-PAGE and electro-transferred onto a nitrocellulose membrane at 0.8-0.9 mA/cm^2 for 120 min using a semi-dry blot system. For this, the nitrocellulose membranes together with filter papers (Whatman® 3 MM filter) were soaked in transfer buffer (25 mM Tris, 150 mM Glycin, 10% Methanol, pH 8.3). The membrane and filters were stacked as a “sandwich” in the following order: filter paper, membrane, gel, and filter paper. To check for equal amounts of protein and successful blotting, the membrane was incubated with Ponceau S, a reversible protein staining solution. The membrane was washed in millipore water to the desired contrast and photographed. To remove the stain completely the membrane was washed with 1 x TBST. To prevent unspecific binding of the antibodies the membrane was blocked with a blocking buffer depending on the antibody to be used for 1 h at RT. After blocking, the membrane was incubated with the primary antibodies diluted in their respective blocking buffer BSA or Milk for 1 h at RT or at 4 °C overnight. Unbound antibody was removed by washing 3 x 5 min with TBST. The membrane was then incubated for 1 h at RT with a secondary antibody conjugated with horseradish peroxidase. After washing 3 times as described above, the enzyme activity was detected by use of enhanced

chemiluminescence (ECL) according to the supplier's protocol with a bioluminescence detection system and recorded with Bio-Rad Quantity One gel documentation system.

10x Tris-buffered saline (TBS)

Tris/HCl (pH 7.4)	100 mM
NaCl	1.6 M

TBS Tween (TBST)

1 x TBS	
0.1% (vol/vol)	Tween 20

Blocking-buffer and antibody-dilution buffer

3% (wt/vol) BSA in 1 x TBST or
5% (wt/vol) nonfat dry milk powder in 1 x TBST (Milk)

3.6.1 Stripping membranes

For removing primary and secondary antibodies from the membrane, blots were incubated in stripping buffer shaking for 2 min at 60 °C. After six washing steps (5 min in TBST at RT), the membrane was blocked for 1 h with 5% nonfat dry milk (in TBST) before detection with another primary antibody.

Stripping buffer

Tris/HCl (pH 6.8)	62.5 mM
SDS	2% (wt/vol)
β-mercaptoethanol	0.8% (vol/vol)

3.7 Immunoprecipitation

Preparation of beads: Protein G-coated magnetic beads (6 µl beads suspension for approximately 1 mg of total cell lysate) were washed 3-4 times with 0.1 M 1 x PBS (Composition in mM: 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 8.0 Na_2HPO_4; pH 7.4) and incubated with the respective antibody (4-5 µg for 1 mg total cell lysate) overnight at 4 °C with end-over-end rotation. Afterwards the beads were washed 3-4 times with 0.1 M sodium phosphate buffer containing 0.1 % (vol/vol) Tween 20 and stored in 50 µl of 1 x PBS.

Protocol: Confluent endothelial monolayers cultured on 10 cm dishes, subjected to 1 h hypoxia and reoxygenated in absence or presence of different inhibitors as indicated in the text, were incubated in 500 µl lysis buffer for 10 min on ice and subsequently harvested by scraping with a rubber policeman. Cells were further lysed by using a 27 G needle and 1 ml syringe, 4-6 times. The lysate was centrifuged at 1000 x g for 5 min at 4 ° C. The supernatant was transferred to another tube and incubated with the respective antibodies pre-immobilized on protein G-coated magnetic beads for 1.5 h at 4 °C on a permanent rotator. After incubation, beads were washed 3 times with 1 x PBS containing 0.1 % (vol/vol) Tween 20. Finally, the precipitates were detached from the G-coated magnetic beads by application of hot SDS-sample buffer and heated for 5 min at 90 °C. Proteins were detected and analysed by SDS-PAGE and Western blot.

10x PBS

Na_2HPO_4	0.1 M
KH_2PO_4	17 mM
NaCl	1.37 M
KCl	27 mM

Lysis buffer Immunoprecipitation

Tris/HCl (pH 7.4)	50 mM
NaCl	150 mM
Triton X-100	1% (vol/vol)
Nonidet P 40	0.5% (vol/vol)
EDTA	1 mM
EGTA	1 mM
NaF	20 mM
Na-orthovanadate	1.5 mM
DTT	10 mM
PMSF	0.5 mM
Complete ®	10 ml

3.8 Nuclear and cytoplasmic protein extraction

Endothelial cell monolayers (2-4 10^6 cells per 10 cm dish) were washed with 1 x PBS and then resuspended in 500 µl hypotonic buffer A (containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, 0.5 mM PMSF, and 1 tablet of proteinase inhibitor) and incubated for 10 min on ice before adding NP-40 to a final concentration of 0.6% (vol/vol) vortexed, and centrifugated at 13.000 x g for 15 min. The supernatant contained the cytoplasmic fraction. The pellet was washed with buffer A and resuspended in 150 µl high salt buffer B (containing 20 mM HEPES pH 7.9, 400 mM NaCl, 0.1 mM DTT, 1 mM EDTA, 10% glycerol (vol/vol), 0.5 mM PMSF, and 1 tablet of proteinase inhibitor). The pellet was incubated at 4 °C on a full speed shaker for 2 h. After incubation, the suspension was centrifugated at 13.000 x g at 4 °C for 5 min. The supernatant contained the nuclear fraction. Protein concentration of the nuclear and cytoplasmic extracts was determined by Bradford.

3.9 Determination of protein concentration by Bradford

The Bradford assay was used to determine the concentration of proteins in a solution. The assay reagent was prepared by diluting 1 volume of the dye stock with 4 volumes of distilled H_2O. BSA was used a standard. Both, the standard and the samples were prepared in PBS and 1 ml assay reagent was mixed with 20 µl sample or standard. After 5 min incubation time they were measured at 595 nm absorbance in an ELISA reader (spectrophotometer).

3.10 FACS analysis

The effect of hypoxia, reoxygenation, and different pharmacological treatments on endothelial cell apoptosis was determined by Fluorescence-activated cell sorting (FACS) by analysing of Annexin V-FITC and propidium (PI) staining according to manufacture's instructions. Annexin V and PI were added to trypsinized and suspended cells. Samples of 10.000 cells were analysed by flow cytometry on FACS-Calibur using CELLQuest Pro software. Cells that were Annexin V-FITC positive were identified as apoptotic, while cells that were PI positive and Annexin V negative were identified as necrotic. Nonstained cells were identified as vital.

3.11 Immunoflorescence

After stimulation on of endothelial monolayers on glass coverslips, cells were washed 3 times with 1 x PBS. Thereafter, cells were fixed in 4% (wt/vol) paraformaldehyde (PFA) for 20 min at 37 °C and washed 3 times with 1 x PBS. Cells were then permeabilized with 0.1% (vol/vol) Triton X-100 for 7 min and additionally washed another 3 times with 1 x PBS. Subsequently, fixed and permeabilized HUVEC were blocked with blocking solution 5% (wt/vol) GFCFS, 5% (wt/vol) BSA, 1 x PBS for 60 min at RT. Samples were incubated for 3 h at 37 °C or 4 °C overnight with primary antibody (in blocking solution containing 0.1% (vol/vol) Tween 20, dilution 1:200). Afterwards, samples were washed 4 times with 1 x PBS and incubated for 60 min at RT with 1:400 Alexa-Fluor 488 tagged secondary antibody in blocking solution and kept in dark.

Nuclear staining was performed by incubating cells with TOPRO-3 (1:200) dilution in 1 x PBS for 15 min. Following final washing for 3 times with 1 x PBS in dark, coverslips were taken from dishes and were fixed with mounting medium (1 x PBS/100% glycerol (vol/vol) 1:1) on a microscopic slide. After storing overnight, the cells were analysed with a Zeiss LSM 510 confocal laser scanning microscope.

3.12 Statistical analysis

All statistical analysis were performed using Sigma Stat version 8.0. For comparison of two normally distributed groups of data, the Student's t-test was used. For multiple comparisons of normal distributed data the one-way analysis of variance (one-way ANOVA) was used. For descriptive purposes all data are presented as means ± the standard error of means. Results were considered significant at an error probability level of $P < 0.05$.

4 RESULTS

4.1 Effect of DMOG on endothelial apoptosis during reoxygenation

There is evidence that infarct size and cardiac dysfunction following global I/R is decreased after inhibition of PHD either by treatment with the inhibitor DMOG (Ockaili et al., 2005) or silencing with siRNA (Natarajan et al., 2006). In adition, it has been shown that HIF-1α is a key factor in ischemic preconditioning-induced cardioprotection (Cai et al., 2008).

First it was proven whether inhibition of PHD with DMOG during reoxygenation protects endothelial cells against apoptosis induced by serum starvation. HUVEC were cultured in serum-free medium for 12 h and exposed to 1 h of hypoxia. Endothelial cells were analysed for apoptotic cell death by FACS analysis after 24 h of reoxygenation in absence of serum.

As shown in Fig. 1 serum deprivation induced apoptotic cell death in 60% of the total cell population that was not significantly reduced in those cells exposed to 1 h of hypoxia. However in presence of DMOG during reoxygenation reduced apoptosis by 50%. Correspondingly, cell number of vital cells was increased by inhibition of PHD. These data indicate that DMOG reduces serum starvation-induced apoptotic cell death when added at the onset of reoxygenation.

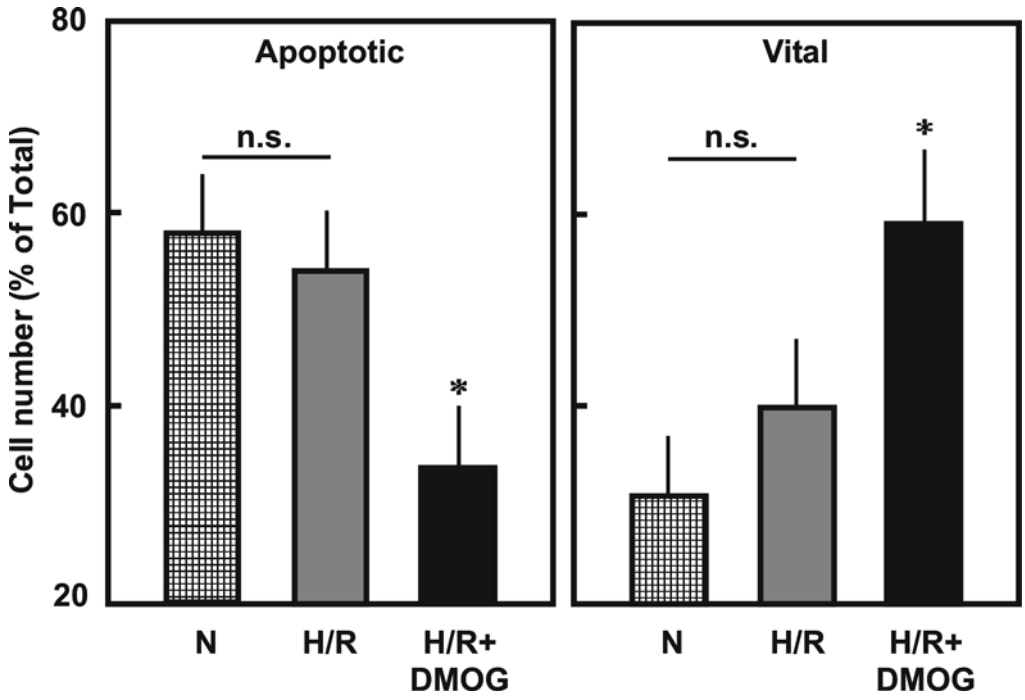


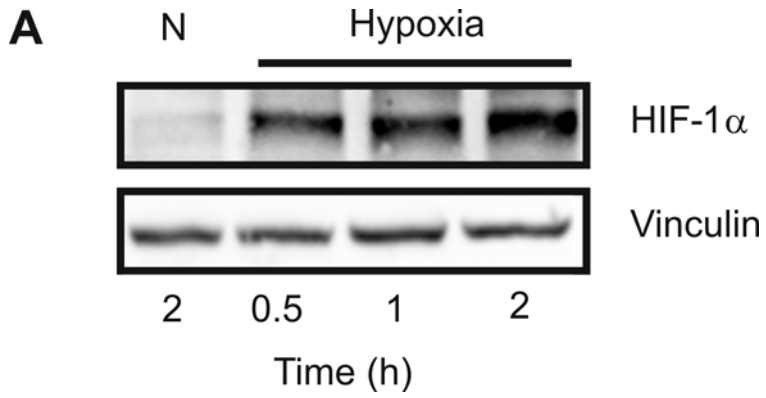
Fig. 1 Effect of DMOG during reoxygenation on serum starvation-induced apoptosis. HUVEC were serum deprived for 12 h. Afterwards, cells were exposed to 1 h of hypoxia followed by 24 h of reoxygenation (H/R) in serum-free medium. Control cells were simultaneously incubated under normoxic conditions (N). DMOG (1 mM) was applied at the onset of reoxygenation (H/R + DMOG). The distribution of vital and apoptotic cells was analysed after 24 h by FACS analysis. Data are means ± SD of n=3 separate experiments of independent cell preparations. *P< 0.05 vs normoxia (N).

4.2 Effect of DMOG on HIF-1α content during reoxygenation in endothelial cells

To analyse the effect of hypoxia/reoxygenation, and DMOG during reoxygenation on HIF-1α, endothelial cells were incubated under the same experimental conditions as described above and HIF-1α protein content was analysed by Western blot analysis.

Consistent with literature, exposure of endothelial cells to hypoxia induced an increase of HIF-1α protein content over time (Fig. 2 A). After 1 h of hypoxia HIF-1α content almost reached protein concentrations obtained with 2 h of exposure. Because of these finding cells were exposed to 1 h of hypoxia in all further experiments.

Next the effect of reoxygenation of HIF-1α content in absence and presence of DMOG was analysed. As depicted in Fig. 2 B hypoxia-induced increase of HIF-1α vanished with onset of reoxygenation and stayed low for the following 24 h of reoxygenation. However, addition of DMOG at the onset of reoxygenation not only maintained HIF-1α content at the end-hypoxic level but rather induced a further increase during the reoxygenation period, indicating that decrease in HIF-1α is mediated in a PHD dependent manner. A similar increase was observed when endothelial cells were exposed to 1 mM of DMOG under normoxic conditions. A concentration dependent stabilization of HIF-1α by DMOG is depicted in Fig. 2 C showing that 1 mM of the PHD inhibitor led to a maximum increase in HIF-1α protein content. Therefore, this concentration was applied in all further experiments.



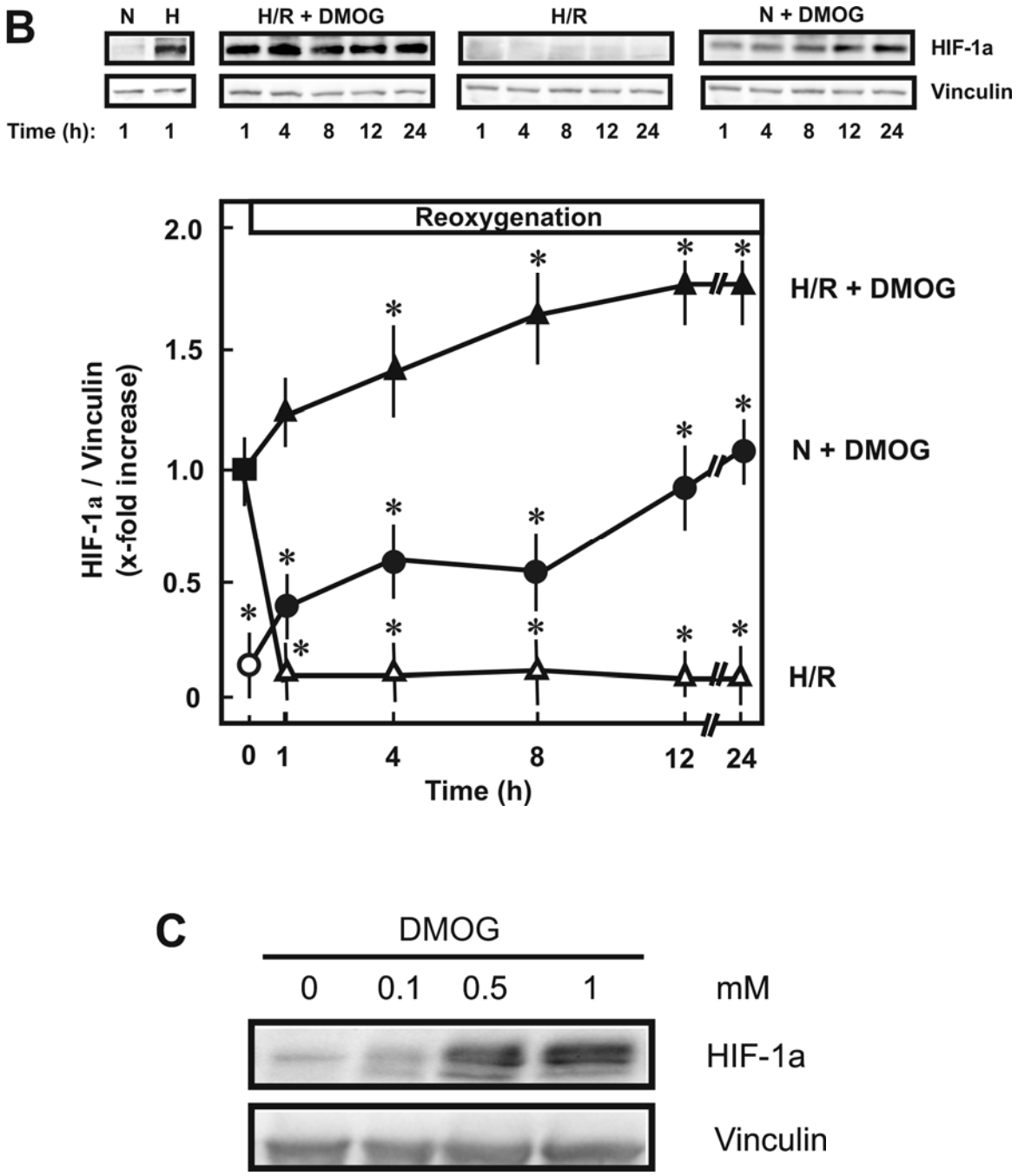


Fig. 2 Effect of hypoxia/reoxygenation on HIF-1α protein content in presence and absence of DMOG. **A)** HUVEC were exposed to 0.5, 1, and 2 h of hypoxia while control cells were exposed to 2 h of normoxia. Representative Western blot analysis shows HIF-1α protein content relative to vinculin, taken as internal loading control. **B)** Cells were exposed to 1 h of hypoxia (H, ■) followed by 24 h of reoxygenation (H/R, Δ). Dimethyloxalyl glycine (DMOG; 1 mM), a pan-specific inhibitor of prolyl 4-hydroxylases (PHD), added at the onset of reoxygenation (H/R+DMOG, ▲) or applied to normoxic control cells (N + DMOG, ●). Cells exposed to 1 h normoxia in presence of vehicle, was

taken as a control (N, ○). Representative Western blot (upper panel) depicts HIF-1α and vinculin, taken as internal loading control. Densitometric analysis of HIF-1α relative to vinculin is depicted below. The mean of HIF-1α/vinculin ratio of HUVEC exposed to 1 h hypoxia was set to 1. Data are means ± SD of 3 separate experiments with independent cell preparations. *P< 0.05 vs H. **C)** HUVEC were exposed to 1 h of hypoxia followed by 24 h of reoxygenation. Different concentrations of DMOG 0, 0.1, 0.5, and 1 mM were applied at the onset of reoxygenation. Representative Western blot depicts HIF-1α protein content relative to vinculin, taken as internal loading control.

4.3 Effect of DMOG on p53 protein content during reoxygenation

Cell cycle arrest and apoptotic cell death are regulated by the tumor suppressor p53 (Vousden and Lu 2002). Under hypoxia p53 is stabilized and there is evidence that p53 and HIF-1α interact with each other and influence one another concerning stabilization, activation, and proteasomal degradation. In order to analyse whether p53 is involved in endothelial cell death and can be influenced by stabilization of HIF-1α, p53 protein content was determined by Western blot analysis during reoxygenation in absence and presence of 1 mM DMOG.

As shown in Fig. 3, the protein level of p53 increased in HUVEC exposed to 1 h of hypoxia. During reoxygenation, unlike HIF-1α, p53 protein levels maintain throughout 24 h of reoxygenation. However, in presence of DMOG p53 protein levels declined over time.

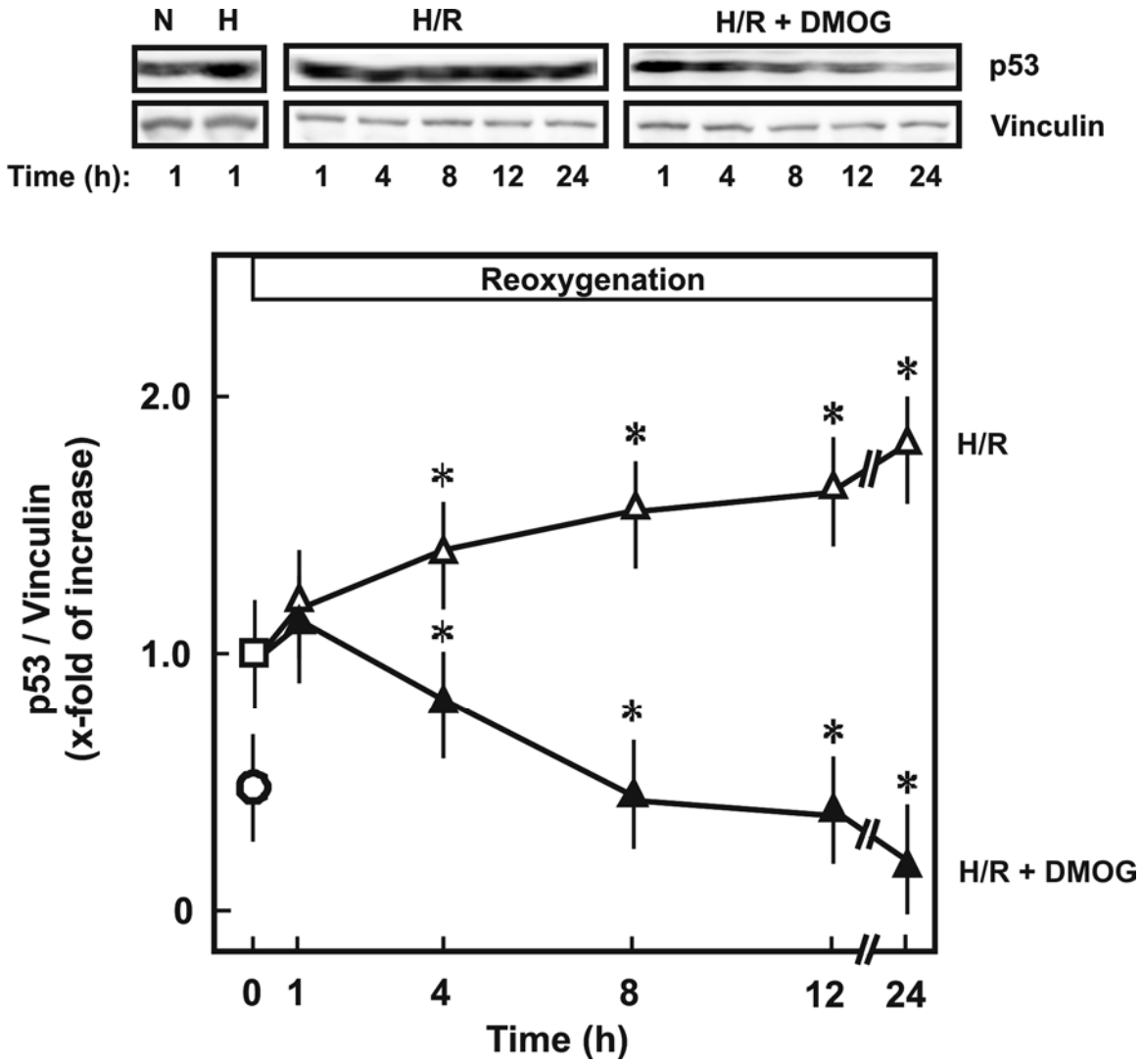


Fig. 3 Effect of DMOG on p53 protein content in HUVEC during reoxygenation in comparison to normoxic control cells. Cells were exposed to 1 h of hypoxia (H, □) followed by 24 h of reoxygenation (H/R, Δ). DMOG (1 mM) was added at the onset of reoxygenation (H/R+DMOG, ▲). Cells exposed to 1 h normoxia in presence of vehicle, was taken as a control (N, ○). Representative Western blot (upper panel) depicts p53 and vinculin, taken as internal loading control. Densitometric analysis of p53 relative to vinculin is depicted below. The mean of p53/vinculin ratio of HUVEC exposed to 1 h hypoxia was set to 1. Data are means ± SD of 3 separate experiments with independent cell preparations. *P< 0.05 vs H.

4.4 Effect of PHD2 silencing on HIF-1α and p53 protein content in serum starvation-induced apoptosis during reoxygenation

To analyse whether the effect of DMOG during reoxygenation on HIF-1α and p53 content as well as cell survival is due to inhibition of PHD2 by DMOG, a key PHD regulating HIF-1α (Berra et al., 2003), PHD2 was silenced by siRNA. Therefore, HUVEC were transfected 24 h prior to experiments with siRNA specific for PHD2, while control cells were transfected with non-specific siRNA. Afterwards cells were serum starved for 12 h and exposed to hypoxia and reoxygenation as described above.

As depicted in Fig. 4 A, PHD2 protein content was significantly reduced in a concentration dependent manner by specific siRNA. Here PHD2 silencing also induced an increase of HIF-1α and a reduction in p53 protein content as observed before in presence of the pharmacologic PHD inhibitor DMOG. Under the same conditions (Fig. 4 B) apoptotic cells were reduced by 67% in PHD2 silenced cells compared to the corresponding cells transfect with non-specific siRNA.

These data suggest that HIF-1α stabilization and reduction of p53 plays a fundamental role for the observed anti-apoptotic effect of PHD inhibition during reoxygenation.

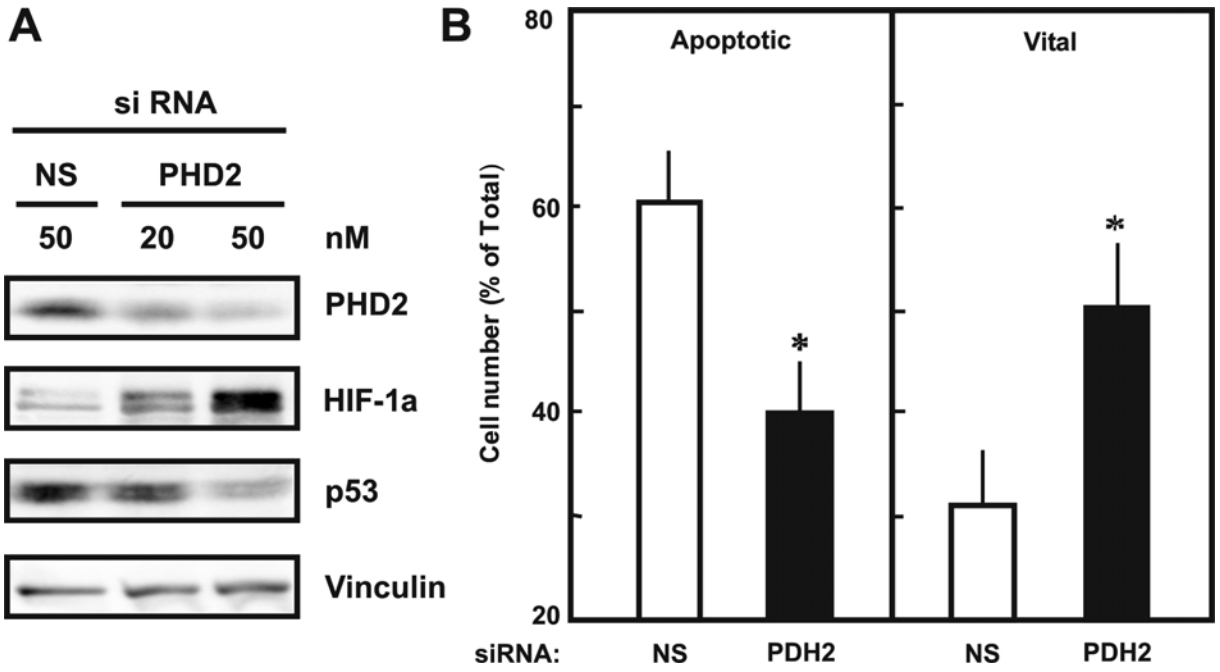


Fig. 4 Effect of PHD2 silencing on HIF-1α and p53 protein content and serum starvation-induced apoptosis during reoxygenation. Cells were transfected with 20 or 50 nM PHD2 siRNA (PHD2) or 50 nM non-specific control siRNA (NS) 24 h prior to 1 h hypoxia followed by 24 h of reoxygenation. **A)** Representative Western blot depicts PHD2, HIF-1α, and p53 content relative to vinculin, taken as internal loading control. **B)** The percentage distribution of vital and apoptotic cells determined by FACS analysis are given. Data are means ± SD of n=3 separate experiments with independent cell preparations. *P< 0.05 vs NS.

4.5 Effect of HIF-1α silencing on p53 protein levels and serum starvation-induced apoptosis during reoxygenation

Pharmacological inhibition as well as silencing of endogenous PHD2 by siRNA led to stabilization of HIF-1α and reduction of apoptosis. So the question was raised whether HIF-1α is directly involved in endothelial cell survival. To prove its functional role, HUVEC were transfected with siRNA specific for HIF-1α, serum starved, and exposed to H/R in presence of DMOG as described before. As shown in Fig. 5 A, HIF-1α content was significantly reduced in a concentration dependent manner, compared to non-specific control siRNA transfected cells. Downregulation of HIF-1α was accompanied by an increase of p53 content to a similar extend as observed 24 h after hypoxia and

reoxygenation (H/R). Analysis of apoptotic cell death (Fig. 5 B) revealed that under this condition apoptosis was increased from 40% to 60%.

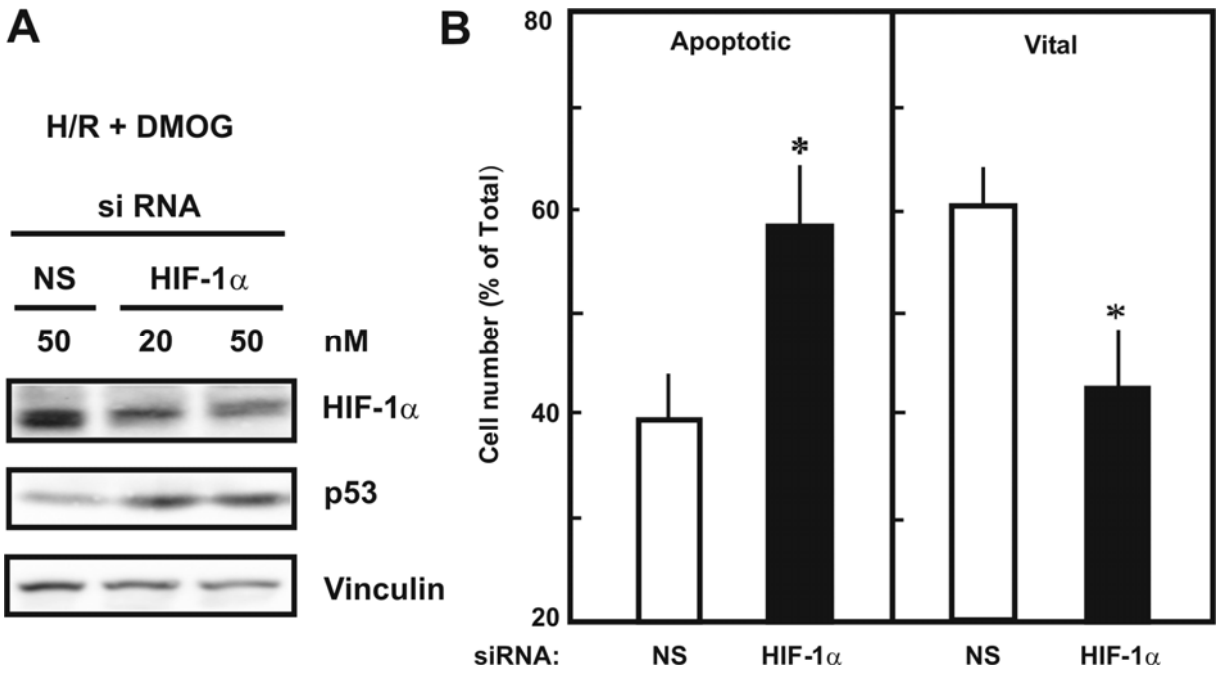


Fig. 5 Effect of HIF-1α silencing on p53 protein content and serum starvation-induced apoptosis during reoxygenation in presence of DMOG. Cells were transfected with 25 or 50 nM HIF-1α siRNA (HIF-1α) or 50 nM non-specific control siRNA (NS) 24 h prior to 1 h hypoxia followed by 24 h of reoxygenation in presence of DMOG (1 mM). **A)** Representative Western blot depicts HIF-1α and p53 relative to vinculin, taken as internal loading control. **B)** The percentage distribution of vital and apoptotic cells determined by FACS analysis are given. Data are means ± SD of n=3 separate experiments with independent cell preparations. *P< 0.05 vs NS.

4.6 Effect of pharmacological inhibition of p53 on serum starvation-induced apoptosis during reoxygenation

To analyse whether p53 is responsible for the observed apoptotic cell death, 100 µM Pifitrin-α (PFT-α), a specific inhibitor of p53, was applied with onset of reoxygenation and apoptotic cell death as well as cell survival was analysed 24 h later. PFT-α caused a decrease in apoptosis from 58% to 43% versus cells treated with vehicle only (Fig. 6). This indicates that p53 is involved in the observed apoptotic cell death of HUVEC.

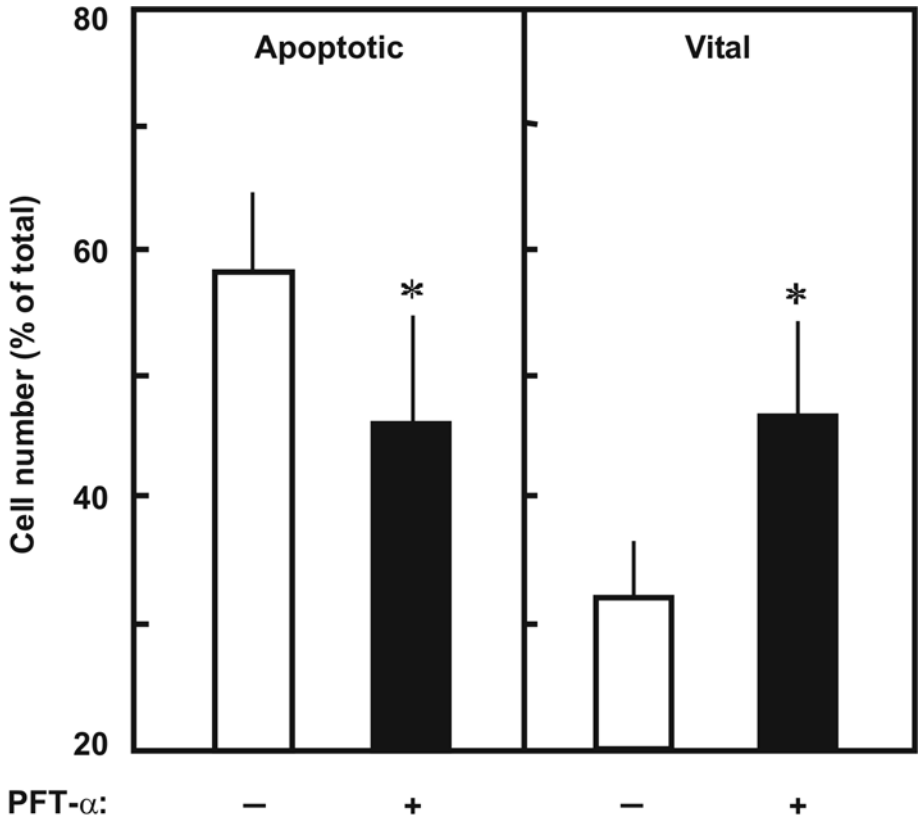


Fig. 6 Effect of pharmacological inhibition of p53 during reoxygenation in HUVEC. Serum starved cells were exposed to 1 h of hypoxia followed by 24 h of reoxygenation. PFT-α (100 µM), a specific inhibitor of p53, was applied at the onset of reoxygenation (+). Control cells were treated alike in presence of vehicle (-). The percentage distribution of vital and apoptotic cells determined by FACS analysis are given. Data are means ± SD of n=3 separate experiments with independent cell preparations. *P< 0.05 vs control (-).

4.7 Effect of DMOG on HIF-1α and p53 localization

Immunofluorescence staining was performed to determine the abundance and subcellular localization of HIF-1α and p53. Apoptosis in endothelial cells caused by serum deprivation is characterized by presence of p53 primarily found in the nuclei under normoxic conditions (Fig. 7). Exposure to hypoxia induced an increase of p53 accumulation in cell nuclei accompanied by a concurrent increase of HIF-1α in nuclei. During reoxygenation p53 levels were preserved in the nuclei while HIF-1α disappeared. In contrast, addition of DMOG at the onset of reoxygenation preserved HIF-1α protein levels in the nuclei while p53 vanished below normoxic levels.

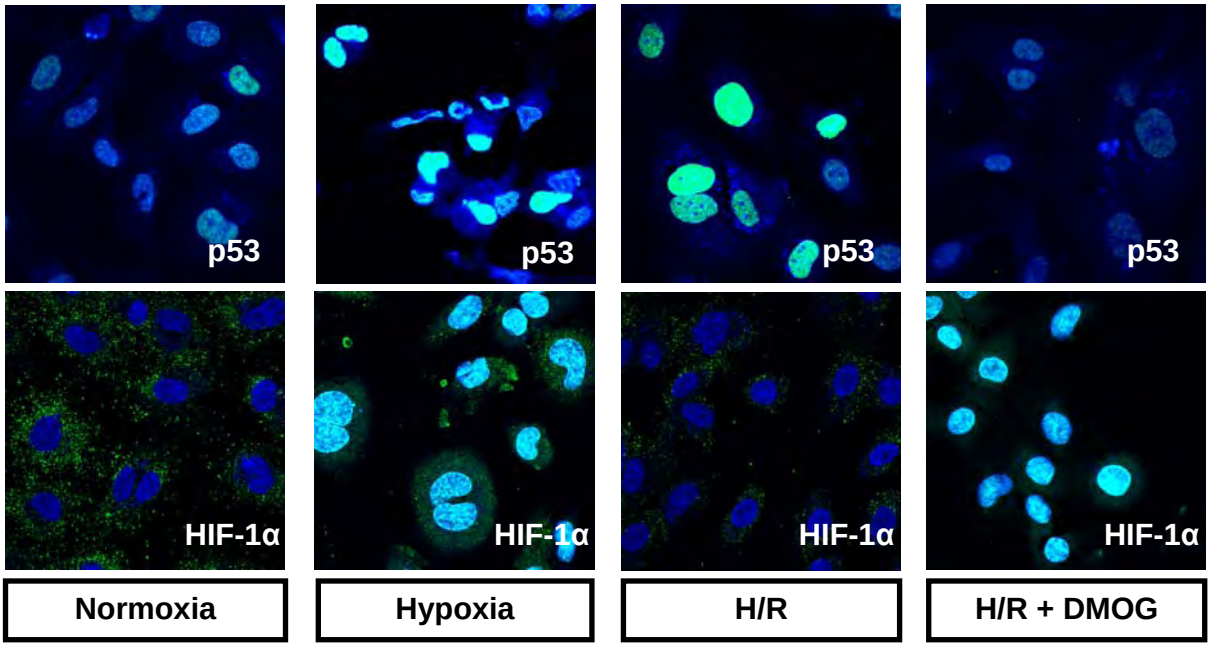


Fig. 7 Localization of HIF-1α and p53 in nuclei of HUVEC. Cells were grown on coverslips, serum starved for 12 h and exposed to 1 h of hypoxia followed by 24 h of reoxygenation (H/R). DMOG (1 mM) was applied at the onset of reoxygenation (H/R + DMOG). Control cells were exposed to normoxia only. Endogenous p53 (top row) and HIF-1α (bottom row) were both detected with alexa 488-labeled secondary antibody (green). Cell nuclei were co-stained with TOPRO-3 (blue).

4.8 Interaction between HIF-1α and p53

Earlier reports have shown that HIF-1α can bind to p53. To examine whether HIF-1α directly binds to p53 under the experimental conditions described here, co-immunoprecipitation was performed with anti-p53 antibody. Subsequently, precipitated proteins were analysed by Western blot analysis. As shown in Fig. 8, HIF-1α was co-immunoprecipitated with p53 in apoptotic cells exposed to 1 h of hypoxia (Fig. 8), indicating complex formation between both proteins. As expected, after normoxia as well as 24 h after H/R no interaction could be detected, since HIF-1α is subjected to rapid degradation in presence of O_2. On the other hand, no interaction between HIF-1α and p53 was observed in presence of DMOG added with onset of reoxygenation. Under this condition HIF-1α was preserved while p53 was significantly reduced. Beside p53 the heavy light chain of the immunoprecitation antibody (IgG) was detected too. These co-immunoprecipitation data indicate that during hypoxia HIF-1α/p53 complexes are formed while no direct interaction can be observed in presence of DMOG during reoxygenation suggesting another mechanism of p53 degradation.

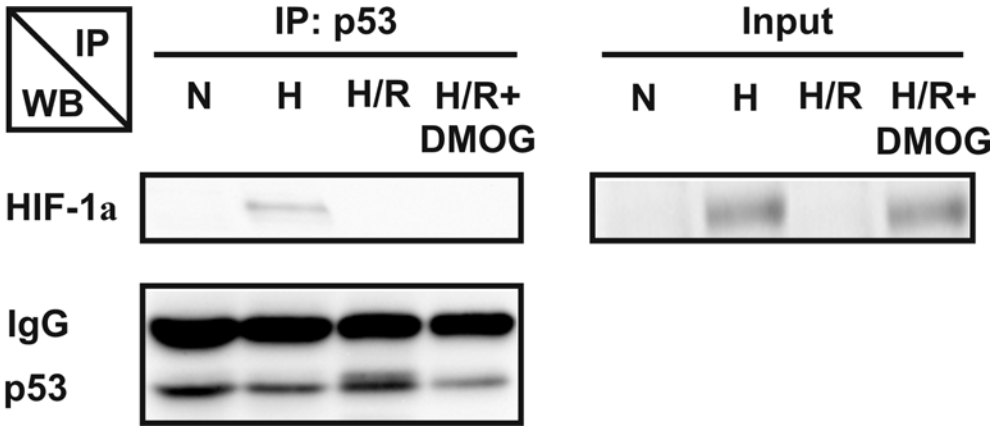


Fig. 8 Detection of protein interaction and complex formation between HIF-1α and p53. Serum starved HUVEC were either exposed to 1 h of hypoxia (H) or normoxia (N) alone or to 1 h hypoxia followed by 24 h of reoxygenation (H/R) in absence or presence of DMOG (1 mM) added at the onset of reoxygenation (H/R + DMOG). Equal amounts of whole cell lysates were prepared (input) and p53 was immunoprecipitated (IP) with anti-p53 antibody coupled to protein G-coated magnetic beads. Co-immunoprecipitation of

HIF-1α with p53 was analysed by Western blot analysis. The antibody used for immunoprecipitation of p53 (IgG) was detected too.

4.9 Effect of PHD inhibition by DMOG on Mdm2 and p53

The results so far suggest that not the direct interaction between HIF-1α and p53 but rather another mechanism might be responsible for DMOG-induced p53 degradation during reoxygenation. There is evidence that the ubiquitin ligase Mdm2 is a key regulator of p53, capable to inhibit p53 transcriptional activity and to target it for protein degradation.

To elucidate the molecular mechanism by which HIF-1α destabilizes p53, the effect of HIF-1α stabilization, p53 and Mdm2 protein content was analysed. As shown in Fig. 9, addition of DMOG at the onset of reoxygenation caused a reduction of p53 content, as shown before. In contrast, the Mdm2 content increased in a time-dependent manner under the same conditions.

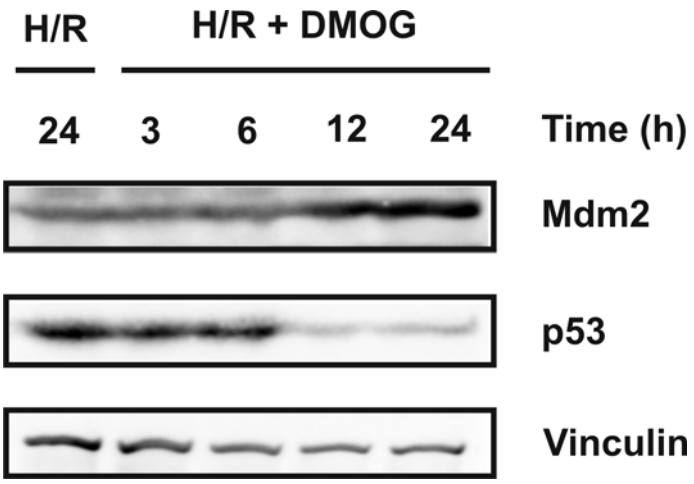


Fig. 9 Effect of DMOG on Mdm2 and p53 protein content. Serum starved HUVEC were exposed to 1 h of hypoxia followed by 24 h of reoxygenation in presence of DMOG (1 mM), applied at the onset of reoxygenation (H/R + DMOG). Samples were analysed after 3, 6, 12, and 24 h. Control cells were exposed to 1 h of hypoxia and 24 h of reoxygenation only (H/R). Representative Western blot depicts Mdm2 and p53 protein content relative to vinculin, taken as an internal loading control.

4.10 Interaction between HIF-1α and Mdm2

To analyse whether DMOG-induced HIF-1α stabilization is responsible for the observed Mdm2 accumulation, endogenous HIF-1α was downregulated by siRNA. In comparison to cells transfected with non-specific siRNA, HIF-1α silencing abrogated the DMOG-induced increase in Mdm2 protein content during reoxygenation (Fig. 10 A). In contrast, silencing of Mdm2 had no effect on HIF-1α content (Fig. 10 B), suggesting that HIF-1α is upstream of Mdm2 expression.

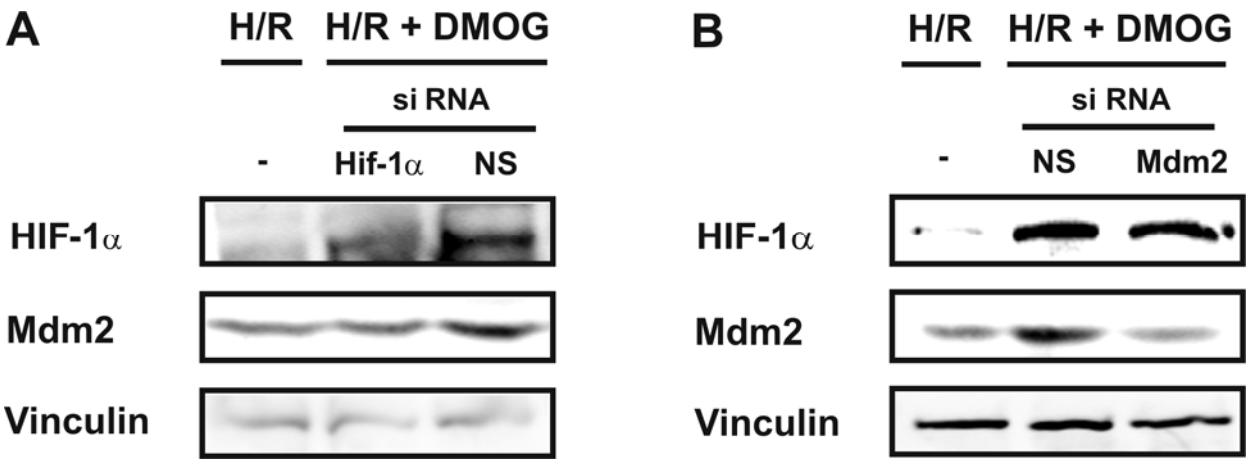


Fig. 10 Effect of HIF-1α and Mdm2 silencing on each other. Serum starved HUVEC were transfected with 50 nM HIF-1α siRNA (HIF-1α), 50 nM Mdm2 siRNA (Mdm2) or 50 nM non-specific control siRNA (NS) for 24 h. Afterwards cells were exposed to 1 h hypoxia followed by 24 h of reoxygenation in presence of DMOG (1 mM) applied at the onset of reoxygenation (H/R + DMOG). As a control, cells were exposed to 1 h of hypoxia followed by 24 h of reoxygenation (H/R). Representative Western blots depict HIF-1α and Mdm2 content relative to vinculin, taken as a loading control.

4.11 Effect of Mdm2 silencing on p53 and analysis of Mdm2 and p53 interaction for subsequent p53 degradation

To prove whether DMOG-induced increase of the ubiquitin ligase Mdm2 during reoxygenation is responsible for the observed p53 degradation, Mdm2 was downregulated by specific siRNA. Silencing of Mdm2 abrogated DMOG-induced reduction of p53 (Fig. 11). In a next step it was analysed whether Mdm2 and p53 interact with each other and whether Mdm2 directs p53 to proteasomal degradation. Therefore, cytosolic and nuclear protein extracts were prepared and Mdm2 was immunoprecipitated. Afterwards immunoprecipitated proteins were analysed by Western blot analysis. As seen in Fig. 11 B, Mdm2 was found in both the cytosolic and nuclear fraction, whereas p53 was predominantly found in the cytosolic fraction when proteasomal degradation was inhibited by the selective and reversible inhibitor MG-132. In contrast, p53 was barely detectable in absence of MG-132 or in the nuclear protein fraction. These data demonstrate that either silencing of Mdm2 or inhibition of proteasomal degradation by MG-132 led to a stabilization of p53, allowing the visualization of Mdm2/p53 complex formation and translocation from nucleus to cytosol. This indicates that the interaction of both proteins is prerequisite for Mdm2-induced translocation and degradation of p53. Taken together, these results show that stabilization of HIF-1α by DMOG-induced PHD inhibition elicits Mdm2-mediated proteasomal degradation of p53.

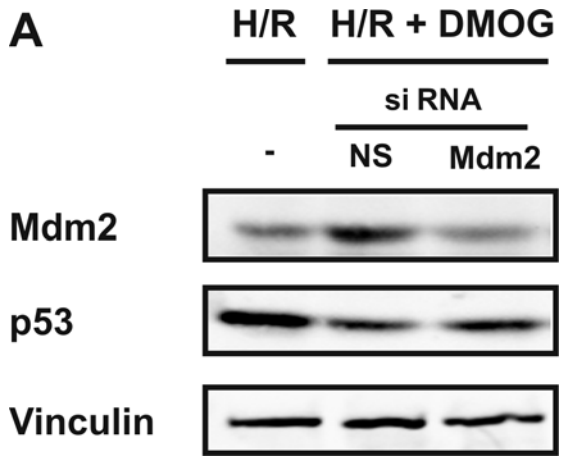


Fig. 11 A Effect of Mdm2 silencing on p53 during reoxygenation in presence of DMOG. HUVEC were transfected with 50 nM Mdm2 siRNA (Mdm2), or 50 nM non-specific control siRNA (NS) for 24 h. Afterwards cells were exposed to 1 h hypoxia followed by 24 h of reoxygenation. DMOG (1 mM) was applied at the onset of reoxygenation (H/R + DMOG). As a control, cells were exposed to 1 h of hypoxia followed by 24 h of reoxygenation (H/R). Representative Western blot analysis shows Mdm2 and p53 protein content relative to vinculin, taken as a loading control.

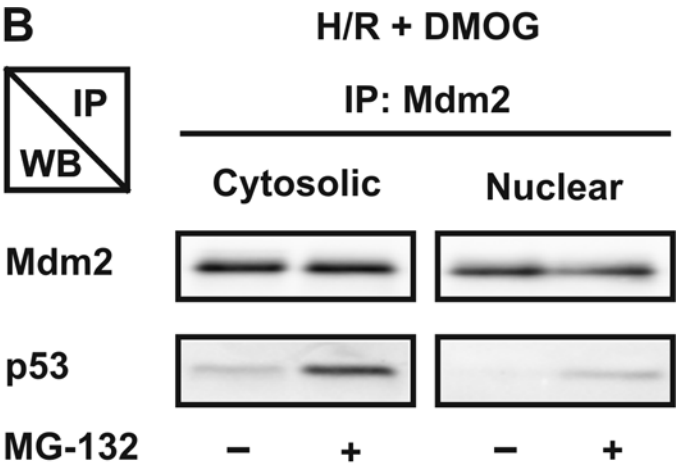


Fig. 11 B Effect of MG-132, a selective inhibitor of proteasomal degradation, on Mdm2 and p53, co-immunoprecipitated from cytosolic and nuclear protein fractions. Cells were exposed to 1 h of hypoxia followed by 24 h of reoxygenation in presence of DMOG (1 mM), applied at the onset of reoxygenation (H/R + DMOG). MG-132 (20 µM) was added during the last 6 h of reoxygenation (+). Cytosolic and nuclear fractions were prepared from equal amounts of cell homogenates and Mdm2 was immunoprecipitated with anti-Mdm2 antibody coupled to protein A-coated magnetic beads. A representative Western blot depicts immunoprecipitated Mdm2 and p53.

4.12 Effect of Mdm2 silencing on DMOG-induced cell survival

To investigate the potential effect of HIF-1α dependent Mdm2 protein accumulation on DMOG-induced cell survival, Mdm2 was silenced by siRNA. As described before HUVEC were transfected with Mdm2 specific or non-specific siRNA as a control, and were serum starved 12 h before experiments. Afterwards cells were exposed to 1 h hypoxia followed by 24 h of reoxygenation in presence of DMOG. Control cells were treated alike under normoxic conditions. Apoptotic cell death and vital cells were determined 24 h after experiments by FACS analysis. As shown in Fig. 12, Mdm2 silencing abolished the DMOG-mediated reduction of apoptosis. Accordingly, the number of vital cells was significantly reduced. This indicates that Mdm2 is required for the protection of endothelial cells against apoptotic cell death induced by PHD inhibition.

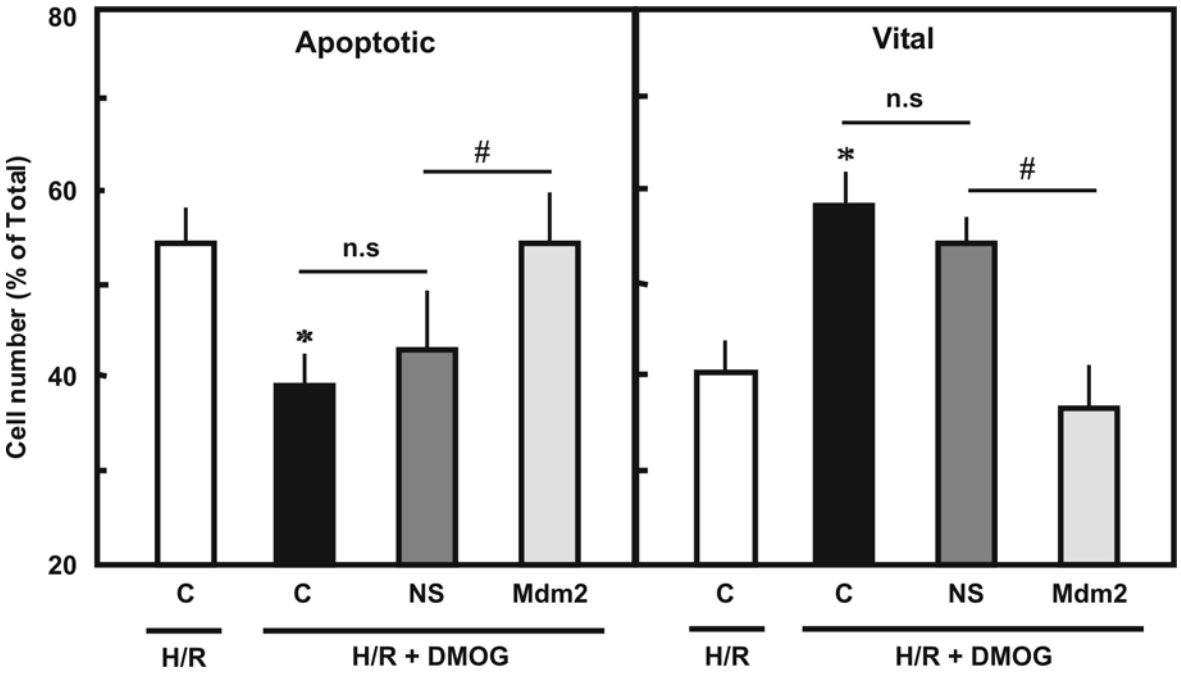


Fig. 12 Effect of Mdm2 silencing on DMOG-induced cell survival during reoxygenation. Cells were transfected with 50 nM Mdm2 siRNA (Mdm2), 50 nM non-specific control siRNA (NS), or were non-treated as a control (C), 24 h prior to 1 h hypoxia and 24 h of reoxygenation. DMOG (1 mM) was applied at the onset of reoxygenation (H/R + DMOG). As a control, non-treated cells were exposed to 1 h of hypoxia followed by 24 h of reoxygenation (H/R). The percentage distribution of vital and apoptotic cells determined by FACS analysis are given. Data are means ± SD of n=2 separate experiments *P< 0.05 vs NS.

5. DISCUSSION

5.1 Main findings

The present study was conducted to elucidate the molecular mechanism by which inhibition of PHD leads to protection of endothelial cells against ongoing apoptotic cell death during H/R. As a pro-apoptotic challenge endothelial cells were cultured in serum free medium for 12 h and subjected to hypoxia for 1 h followed by reoxygenation.

The major findings of the present study are: (1) HIF-1α is up-regulated during hypoxia. Its degration during reoxygenation can be prevented by application of PHD inhibitor DMOG, targeting HIF prolyl 4-hydroxylases. The same effect is achieved by downregulation of the specific PHD2 with siRNA. (2) The increased level of p53 protein under hypoxia did not alter during reoxygenation, while addition of DMOG significantly reduced p53. (3) DMOG, applied at the onset of reoxygenation, caused a reduction of apoptosis. (4) Downregulation of HIF-1α under these conditions aggravated ongoing apoptosis. (5) Pharmacological inhibition of p53 reduced apoptosis. (6) Stabilization of HIF-1α was accompanied by enhanced Mdm2 expression. (7) The detailed analysis by gene silencing displayed that Mdm2 is downstream of HIF-1α. (8) HIF-1α stabilization enhanced the binding between p53 and Mdm2 and subsequent degradation of p53. (9) Downregulation of Mdm2 by siRNA abolished DMOG-mediated reduction of apoptosis. The present study shows that inhibition of PHD at the onset of reperfusion, a maneuver called *pharmacological postconditioning*, reduces ongoing apoptosis in endothelial cells during H/R. This anti-apoptotic effect is mediated via HIF-1α and involves Mdm2-induced degradation of the proapoptotic molecule p53.

5.2 Inhibition of PHD protects endothelial cells from ongoing apoptosis

H/R-induced apoptosis of endothelial cells may contribute to tissue injury, organ failure and rejection. However, little is known about survival mechanisms capable of counteracting endothelial apoptosis. Different strategies aiming at protection of the heart

at the onset or during reperfusion have gained much greater interest. Postconditioning, introduced by Zhao and coworkers (2003), has now been demonstrated as a novel strategy to protect against myocardial ischemia/reperfusion injury. Recently, studies proved that the HIF prolyl 4-hydroxylases inhibitor, DMOG significantly increases nuclear HIF levels and HIF-1α transcription activity in normoxic microvascular endothelium *in vitro*. Hearts preconditioned by prior DMOG administration exhibited significantly reduced infarct size following H/R in rabbits (Ockaili et al., 2005). Another study from Natarajan and coworkers (2006) showed that the PHD2 siRNA treatment produced significant cardioprotection against H/R stress showed a reduction in infarct size by 69% in mouse heart. In this context, HIF-1α has emerged to focus.

Previous data from our laboratory (Haertel et al., 2010) has shown that transient hypoxia protects endothelial cells against apoptosis. Consistent with these previous reports, present study confirmes that incubation with DMOG given at the onset of reoxygenation resulted in 50% reduction of apoptosis. Therefore, the question whether DMOG mediates its effect on apoptosis via stabilization of HIF-1α was analysed. For that reason as a pro-apoptotic challenge, endothelial cells were cultured in serum-free medium for 12 h and subjected to hypoxia for 1 h followed by reoxygenation. Under these conditions, the effect of HIF-1α was examined. Under normoxic conditions, HIF-1α protein is rapidly degraded by HIF prolyl 4-hydroxylases. Under hypoxic conditions, however, the enzyme fails to hydroxylate HIF-1α due to the low oxygen content. It was also found that hypoxia caused a significant increase in HIF-1α content, which was undetectable in normoxic control. Inhibition of PDH by DMOG with onset of reoxygenation maintains HIF-1α at high level. Downregulation of PHD2 by siRNA revealed that HIF-1α can be preserved during reoxygenation. The results indicate that HIF-1α stabilization induced by inhibition of PHD leads to reduction of the ongoing apoptosis during H/R. However, the question remains, which approach is effective in providing protection against apoptosis in endothelial.

5.3 Inhibition of PHD affects pro-apoptotic p53

This led to the question about the possible downstream targets participating in induction of apoptosis. In previous studies it has been shown that hypoxia is a critical factor for

cell death or survival, but the combined effects of hypoxia and reperfusion caused pathological consequences that induced endothelial cell apoptosis. An increasing number of studies suggest that p53 plays a critical role in hypoxia-induced apoptosis. Graeber and coworkers (1996) found that tumor cells containing wild-type p53 were more sensitive to hypoxia-induced apoptosis when compared with tumor cells lacking functional p53. Long and coworkers (1997) showed the role of p53 in cardiac myocyte apoptosis in response to hypoxia. In 1999 Stempien-Otero and coworkers found that endogenous p53 protein levels correlated with the initiation of cell death in hypoxic HUVEC and showed the elevation of endogenous p53 protein levels via inhibition of the proteasome potentiated apoptosis in hypoxic HUVEC. p53 is a transcriptional factor that activates a variety of genes involved in DNA repair, cell cycle arrest, and apoptosis. All these reports suggest that enhanced stabilization of p53 ultimately leads to apoptosis. This raise the question whether p53 is involved in endothelial cell apoptosis. Under short hypoxia followed by reoxygenation, an increase in p53 protein levels concomitant with endothelial cell death was found. By applying of DMOG at the onset of reoxygenation, the level of p53 protein gradually decreased with time and was almost undetectable after 24 h of reoxygenation. To analyse whether p53 is responsible for the observed apoptosis, PFT-α, a p53 specific inhibitor, was applied at the onset of reoxygenation, and the amount of surviving cells was analysed 24 h later. Apoptosis was decreased from 58% in absence of PFT-α to 43% in presence of PFT-α. This indicates that p53 is involved in the ongoing apoptosis. These first findings lead to the assumption that stabilizatioin of HIF-1α induces p53 downregulation and the reduction of apoptosis. Controversial results have been reported regarding HIF-1α mediated regulation of p53. It has been demonstrated in cultured cortical neurons that HIF-1α promotes p53-dependent apoptosis (Halterman et al., 1999). HIF-1α binds to p53, and the complex formation likely plays an important role in the hypoxia-induced stabilization of p53. The concept is supported by results from Suzuki and coworkers (2001) showing an increased dephosphorylation of HIF-1α, which plays a pivotal role in the stabilization of p53 and subsequent activation of the p53-dependent apoptotic pathway during hypoxia. An and coworkers (1998), demonstrated that the induction of p53 under severe hypoxia is HIF-1α dependent and that it may be achieved by p53 stabilization along with its

association with HIF-1α. Results of Ravi and coworkers (2000) indicate that inactivation of p53 in tumor cells contributes to activation of the angiogenic switch via amplification of normal HIF-1α-dependent responses to hypoxia. To this point, the results left the question open whether the effect of p53 is HIF-1α dependent?

5.4 p53 downregulation is dependent of HIF-1α stabilization

The data of the present study show that p53 is up-regulated after hypoxia and contributes to H/R-induced injury by promoting apoptosis. Because stabilization of HIF-1α protects endothelial cell from ongoing apoptosis during H/R, it was analysed whether endogenous HIF-1α is required for endothelial survival. From these results, it was hypothesized that HIF-1α accumulation under pharmacological postconditioning may induce downregulation of elevated p53 levels observed during H/R. To show this functional role of HIF-1α, gene expression was silenced by using a specific siRNA HIF-1α following administration of DMOG. Downregulation of HIF-1α increased apoptosis from 40% to 60%. This was accompanied by an increase in p53 levels, similar to that observed under H/R without silencing of HIF-1α. This assumption is supported by reports from Sutton and coworkers (2008) showing that an acute inhibition of p53 significantly increases HIF-1α expression after H/R renal injury. Additionally, PFT-α prevents I/R injury and I/R decrease in HIF-1α expression and has opposing effect to those promoted by p53 (Sutton et al., 2008). The balance between the HIF-1α and p53 responses can determine the outcome of H/R injury. Consequently, when both pathways are simultaneously activated under appropriate stress conditions they can counteract through complex interactions at various levels. In this context it is of particular relevance- that p53 has been shown to directly repress the transcriptional activity of HIF-1α (Schmid et al., 2004) by competing with the common coactivator p300. Importantly, results from the present study indicate that p53 is a direct target for degradation, what suggests that p300 could not be the limiting factor. Further studies on the subject of the complex interaction between these two pathways can ultimately determine the degree of injury in I/R.

5.5 Stabilization of HIF-1α regulates Mdm2, a specific E3-ubiquitin ligase responsible for limiting the levels of p53

While analysing the mechanism by which HIF-1α modulates p53 response, it was observed that addition of DMOG caused degradation of p53 along with HIF-1α stabilization, which obviously makes impossible to detect HIF-1α/p53 complex formation. The results so far indicate that PHD inhibition virtually exclude a direct interaction between HIF-1α/p53. The known roles of Mdm2 in regulating p53 function suggest that the effects of hypoxia on Mdm2 and p53 may be interrelated (Lohrum and Vousden, 1999). Binding to Mdm2 promotes the proteasomal degradation of p53 through Mdm2's action as E3-ubiquitin ligase (Honda et al., 1997). Reduction of Mdm2 expression by H/R observed in the experiments could account for corresponding increase in p53 levels in endothelium. This is supported by the finding in which the interaction between p53 and Mdm2 in presence of DMOG, was examined. Immunoprecipitation of both cytosolic and nuclear Mdm2 was performed using anti-Mdm2 antibody. Mdm2 was found in both cytosol and nucleus, where p53 was almost undetectable. The reduction of p53 content was restored when the proteasome inhibitor MG-132 was added. Mdm2/p53 complex formation was established in presence of MG-132. Experiments using siRNA directed against Mdm2 illustrated a functional role of Mdm2 on p53 accumulation. Silencing of Mdm2 abrogated DMOG-induced reduction of p53. These data demonstrate that either silencing of Mdm2 or proteasomal inhibition by MG-132 could stabilize p53 by abrogating Mdm2-mediated degradation. This indicates that the interaction of both proteins is the prerequisite for p53 degradation and is Mdm2 dependent. Taken together, these results show that stabilization of HIF-1α by PHD inhibition causes Mdm2-mediated proteasomal degradation of p53. This is in line with the evidence suggesting that Mdm2 may be involved in the nucleo-cytoplasmic shuttling of p53 that translocates from its site of action, as a transcription factor, to its site of proteolysis breakdown (Tao and Levine, 1999). Different stress signals produce distinct effects on Mdm2 expression. For example, the observed reduction in Mdm2 levels after H/R in comparison with hypoxia (data not shown) is consistent with the level during apoptosis. In this respect, reduced levels of Mdm2 during H/R are predicted to permit the enhanced

nuclear accumulation of p53 that was observed by us in H/R cells. In contrast, the application of DMOG leads to accumulation of Mdm2 protein. Therefore, Mdm2 like p53 appears to be capable of promoting either apoptosis or protection depending on the stimulus.

5.6 HIF-1α induced p53 degradation is mediated via Mdm2

The results of the present study, together with several other studies (An et al., 1998; Koumenis et al., 2001; Ravi et al., 2000), strongly support the role of HIF-1α in the regulation of the p53-Mdm2 pathway. Recent studies also indicate that Mdm2 is involved in modulating HIF-1α stability under hypoxic conditions (Ravi et al., 2000) further supporting the notion that HIF-1α directly interacts with Mdm2 but not with p53. Therefore, in context of the study, the question of effect of HIF-1α stabilization on Mdm2 protein expression and its stability was analysed. Present data show that stabilization of HIF-1α led to increased expression levels of Mdm2. To prove whether HIF-1α stabilization is responsible for Mdm2 accumulation, the effect of HIF-1α downregulation on Mdm2 was determined. Downregulation of HIF-1α abrogated the increase in Mdm2 level, similarly to that induced by H/R, indicating that HIF-1α stabilization is necessary for Mdm2 accumulation. To prove that Mdm2 has no impact on HIF-1α content, Mdm2 was downregulated by siRNA. Downregulation of Mdm2 did not affect DMOG-induced HIF-1α accumulation. These data indicate that Mdm2 is downstream of HIF-1α. Finally, the potential effect of Mdm2 accumulation on apoptosis was tested. Downregulation of Mdm2 by siRNA showed that DMOG-mediated reduction of apoptosis was abolished. The results indicate that Mdm2 is required for the protection against cell death.

In conclusion, results of the present study define a novel mechanism that modulates p53 to protect endothelial cells against ongoing apoptotic cell death during H/R. This indicates that pharmacological inhibition of PHD by DMOG negatively regulates H/R-induced expression of p53 by facilitating its degradation most probably via ubiquitination. This mechanism is distinct from the proposal that p53 inhibits HIF-1α-mediated transactivation by competing for the p300 coactivator (Blagosklonny et al., 1998). Based on all these finding, an important role of HIF-1α stabilization in modulating p53-Mdm2

system under H/R is illustrated here for the first time. The results demonstrate that application of PHD inhibitor given at the onset of reoxygenation, stabilizes HIF-1α, and that HIF-1α might act as a novel regulator of p53 by controlling the stability and localization of p53 and its regulatory protein Mdm2. The detailed regulatory mechanisms of this system need further investigation. Finally, this work indicates that strategies targeting PHD activity may provide a useful molecular approach to prevent endothelial cells from apoptotic cell death and identifies PHD2 as a new molecular target for therapeutic intervention. The approach termed *pharmacological postconditioning* is a more practical solution and could form the basis of much needed and important reperfusion strategies.

Based on these observations I proposed the model shown in the figure below.

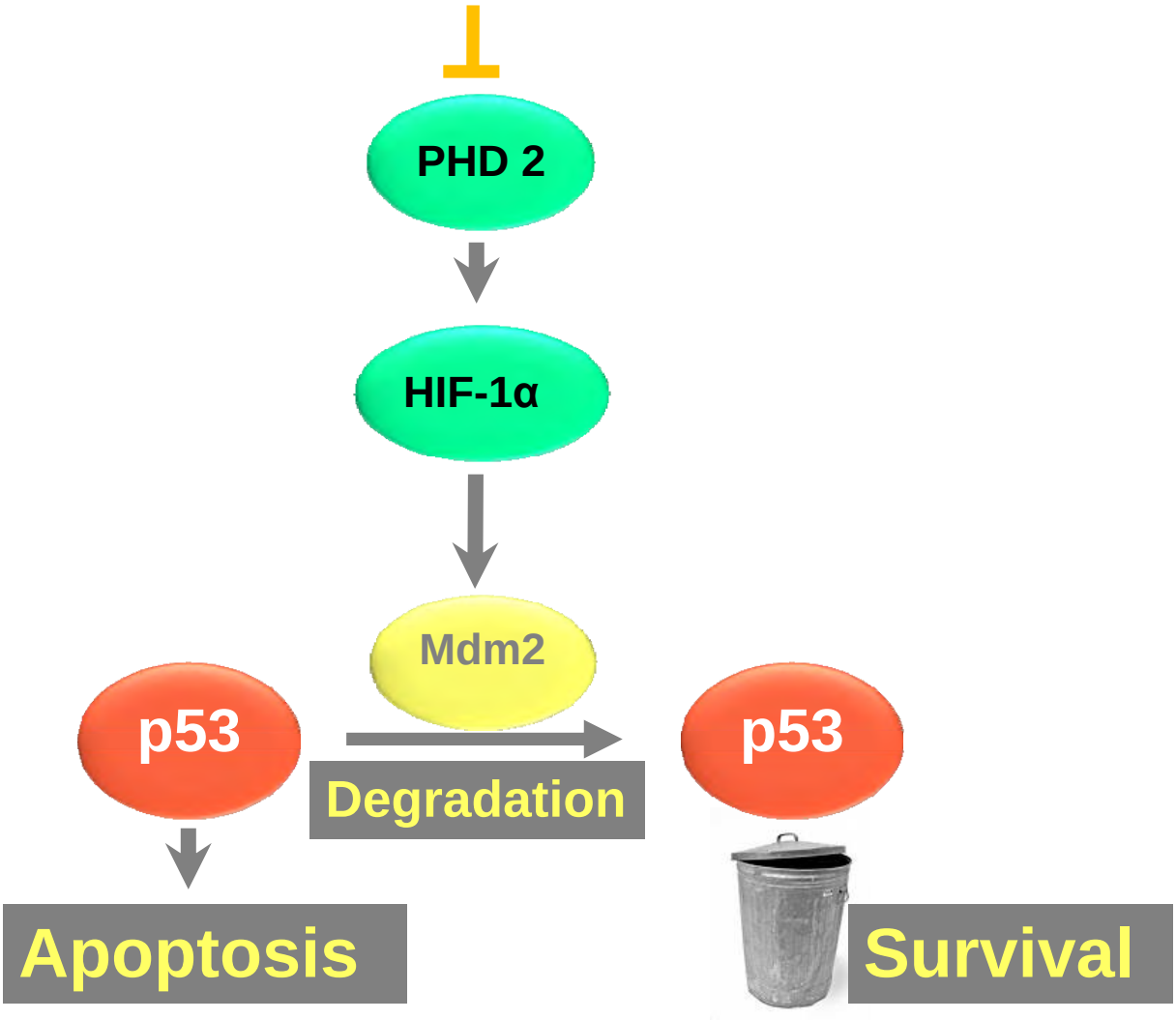


Fig. 13 Protective mechanism against ongoing apoptotic cell death during H/R

6 SUMMARY

In the present study a new approach against the ongoing apoptotic cell death during hypoxia/reoxygenation (H/R) in endothelial cells was established. Particularly, the activation of hypoxia-inducible factor (HIF-1α), plays an essential role in triggering cellular protection during hypoxia, but it's rapidly degradation during reoxygenation, may limit its effect on cell survival to the hypoxic period alone. Regulation of HIF-1α expression is controlled by repression of oxygen-dependent prolyl 4-hydroxylases (PHD) during hypoxia. The present study was conducted to elucidate the molecular mechanism by which inhibition of PHD pathway leads to protection of endothelial cells against ongoing apoptotic cell death during H/R. The study was performed using an established model of cultured monolayers of human umbilical vein endothelial cells to test the hypothesis that stabilization of HIF-1α beyond hypoxia exerts anti-apoptotic effects during H/R by preventing p53-mediated apoptosis.

Cells were serum starved for 12 h, then exposed to 1 h of hypoxia (Po_2 < 5mmHg) followed by 24 h of reoxygenation (Po_2=140mmHg). Exposure to hypoxia caused an increase in HIF-1α and p53 content. During reoxygenation HIF-1α declined towards basal level, while p53 remained unaltered. Under the same conditions, endothelial apoptosis was increased to 58% (annexin V staining). Silencing of PHD2, led to an increase of the HIF-1α content during hypoxia and maintained it at that level during reoxygenation. HIF-1α stabilization was associated by an increase in the Mdm2 content, whereas expression of p53 was reduced. PHD2 silencing reduced apoptosis to half. Additon of DMOG (1mM, dimethyloxalyl glycine), a PHD inhibitor, at the onset of reoxygenation had the same effect. Reduction of p53 content was restored when the proteosome inhibitor MG-132 was added. Interaction of Mdm2 and p53 (co-immunoprecipitation) was increased compared to reoxygenation. Downregulation of HIF-1α by siRNA increased apoptosis to 60% and abrogated Mdm2-p53 complex formation. Downregulation of Mdm2 by siRNA had no effect on HIF-1α but increased p53 level. Stabilization of HIF-1α due to PHD inhibition beyond the period of hypoxia defines a novel mechanism that exerts anti-apoptotic effects during H/R injury by preventing p53-

mediated apoptosis and identifies PHD2 as a new molecular target for therapeutic intervention.

7 ZUSAMMENFASSUNG

In der vorliegenden Studie wurde eine neue Methode gegen die anhaltende Apoptose während der Hypoxie/Reoxygenierung (H/R) in Endothelzellen (EC) etabliert. Die Aktivierung des Hypoxie-induzierten Faktors (HIF-1α) spielt eine besondere Rolle in der Auslösung zellulärer Schutzmechanismen bei Hypoxie, welcher bei Reoxygenierung jedoch schnell zersetzt wird, so dass diese Rolle vermutlich auf die hypoxische Phase beschränkt ist. Die HIF-1α-Expression wird durch die Hemmung der sauerstoffabhängigen Prolyl 4-Hydroxylasen (PHD) während der Hypoxie reguliert. Diese Studie wurde durchgeführt, um den molekularen Mechanismus zu finden, der durch die Hemmung des PHD-Weges zum Schutz der EC vor Apoptose nach H/R führt. Diese Studie nutzte ein etabliertes Modell in humanen Endothelzellen der Nabelschnurvene, um die Hypothese zu testen, ob die Stabilisierung von HIF-1α nach der Hypoxiephase anti-apoptotische Effekte während der H/R Schädigung aufzeigt, indem die p53-vermittelte Apoptose gehemmt wird.

Hierfür wurde den Zellen zunächst für 12 Stunden das Serum im Kulturmedium entzogen. Anschließend wurden die EC für eine Stunde einer Hypoxie gefolgt von 24 Stunden Reoxygenierung ausgesetzt. Die Hypoxie verursachte einen Anstieg von HIF-1α und p53. Während der Reoxygenierung fiel HIF-1α auf niedrige Konzentrationen ab, doch die p53 Konzentration blieb unverändert. Unter den gleichen Bedingungen stieg die Apoptoserate der EC auf 58% (Annexin V Färbung). Das Ausschalten des PHD2-Gens führte zu einem Anstieg an HIF-1α während der Hypoxie und gleich hohem HIF-1α-Gehalt bei der Reoxygenierung. Die Stabilisierung des HIF-1α war assoziiert mit einem Anstieg an Mdm2 und einer verringerten Exprimierung von p53. Das Ausschalten des PHD2-Gens führte zu einer Halbierung der Apoptoserate. Der Zusatz von DMOG (1mM, Dimethyloxalyl glycin), einem PHD Inhibitor, zu Beginn der Reoxygenierung zeigte den gleichen Effekt. Die Reduktion des p53 wurde durch den Proteosomen inhibitor MG-132 aufgehoben. Die Interaktion zwischen Mdm2 and p53 (Co-immunopräzipation) war im Vergleich mit der Reoxygenierung erhöht. Downregulation von HIF-1α siRNA erhöhte die Apoptoserate auf 60% und hob die Mdm2-p53-Komplexbildung auf. Downregulation von Mdm2 durch siRNA zeigte keinen Effekt auf HIF-1α, erhöhte jedoch die Konzentration an p53. Die Stabilisierung von HIF-1α über die

Hypoxiephase hinaus zeigt einen neuen Schutzmechanismus vor einer endothelialen H/R-Schädigung auf. Dieser Protektionsmechanismus beruht auf einer Inhibition der p53-vermittelten Apoptose und identifiziert PHD2 als ein neues molekulares Ziel für die therapeutische Intervention.

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11 PUBLISHED ABSTRACTS

POSTER PRESENTATIONS

1.Tatyana Dimitrova, Frauke V Härtel, Anna Reis, Muhammad Aslam, H Michael Piper, Thomas Noll (2009) Inhibition of prolyl-hydroxylase attenuates hypoxia/reoxygenation-induced injury in endothelial cells. **Acta Physiologica;** 195:Supp. 669:P274 (Annual meeting of the German Physiological Society), March 2009, Giessen

2.Tatyana Dimitrova, Frauke V Härtel, Muhammad Aslam, H Michael Piper, Thomas Noll (2009) Inhibition of prolyl-hydroxylase attenuates hypoxia/reoxygenation-induced injury in endothelial cells. **ATVB;** 29(7) p446 (Arteriosclerosis, Thrombosis, and Vascular Biology), April 2009, Washington, D.C. USA

3.Tatyana Dimitrova, Frauke V Härtel, Anna Reis, Muhammad Aslam, H Michael Piper, Thomas Noll (2009) Inhibition of HIF prolyl 4-hydroxylases protect against reoxygenation injury in endothelial cells. **ECCPS** Symposium, July 2009, Bad Nauheim

MODERATED POSTER PRESENTATION

Tatyana Dimitrova, Frauke V Härtel, Anna Reis, Muhammad Aslam, HM Piper, Thomas Noll (2009) Inhibition of prolyl-hydroxylase attenuates hypoxia/reoxygenation-induced injury in endothelial cells**. Eur Heart J;** 4475 (Annual meeting of European Society of Cardiaology), September 2009, Barcelona**, travel grant**

ORAL PRESENTATION

Tatyana Dimitrova, Frauke V Härtel, Anna Reis, Muhammad Aslam, H Michael Piper, Thomas Noll (2009) Inhibition of HIF prolyl 4-hydroxylases protect against reoxygenation injury in endothelial cells. **Circulation;** 116(16):II-204 (American Heart Association-Scientific Session), November 2009, Orlando, FL USA

12 PhD Publication

Tatyana Dimitrova, Frauke V Härtel, Aslam M, Anna Reis, Krishnaveni Gadiraju, H Michael Piper, Thomas Noll (2010) Inhibition of HIF prolyl 4-hydroxylases protect against ongoing apoptosis during H/R in endothelial cells..... (Manuscript in preparation)

13 ERKLÄRUNG

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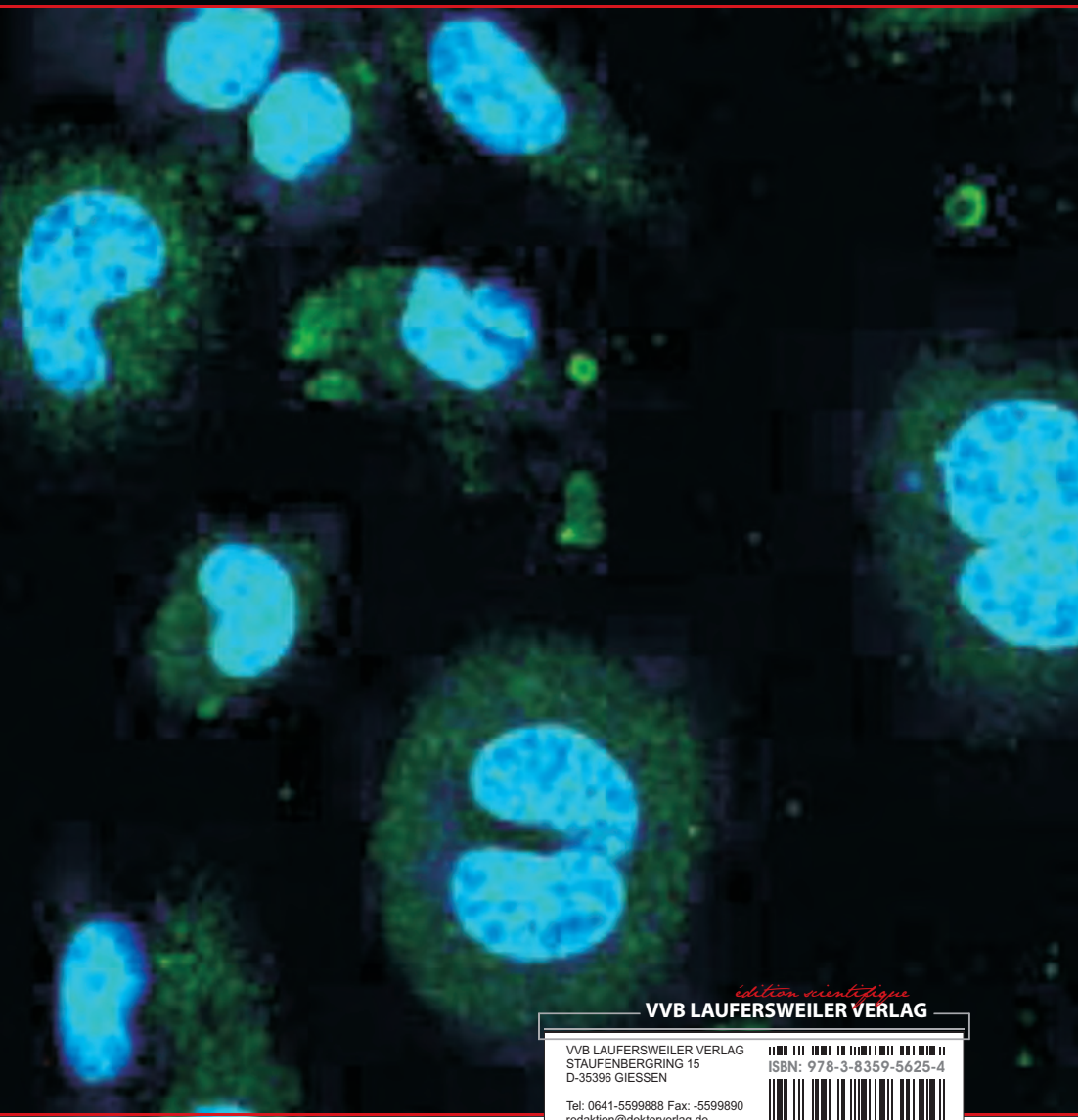

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