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ORIGINAL ARTICLE

Role of reactive oxygen species in the regulation of HIF-1 by prolyl hydroxylase 2 under mild hypoxia

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Abstract

The function and survival of eukaryotic cells depends on a constant and sufficient oxygen supply. Cells recognize and respond to hypoxia by accumulation of the transcription factor hypoxia-inducible factor 1 (HIF-1), composed of an oxygen-sensitive HIF-1α and a constitutive HIF-1β subunit. Besides physiology, HIF-1 induction is involved in major pathological processes such as cardiovascular disease, inflammation and cancer, which are associated with the formation of reactive oxygen species (ROS). ROS have been reported to affect HIF-1 activity but the role for ROS in regulating HIF-1 has not been definitely settled. In order to shed light on the redox-regulation of HIF-1 by ROS, we studied the impact of exogenous ROS treatment (H_2O_2) on HIF-1α and HIF-1 regulatory protein prolyl hydroxylase 2 (PHD2) in the human osteosarcoma cell line U2OS. At early reaction periods, H_2O_2 induced HIF-1α but at prolonged observation phases the opposite occurred. Herein, modulation of PHD activity appeared to be the key element, because knockdown and inhibition of the PHD2 prevented reduction of HIF-1α. However, H_2O_2 treatment constantly suppressed HIF-1 transactivation at all time-points. Our data indicate a dual redox regulation of HIF-1α protein amount with a constant suppression of HIF-1 target gene expression by ROS.

Keywords: *redox regulation, H_2O_2, prolyl hydroxylation, oxygen sensing, hypoxia-inducible factor*

Introduction

Hypoxia-inducible factor 1 (HIF-1) belongs to a family of heterodimeric transcription factors, which have a bHLH-PAS (basic helix-loop-helix/Per-Arnt-Sim) domain in common. Ubiquitously expressed HIF-1 is the central player in hypoxic gene regulation and mediates the upregulation of hypoxia-inducible genes [1,2]. HIF-1 consists of an α-subunit isoform (HIF-1α, HIF-2α or HIF-3α) and a common β-subunit (HIF-1β), identical with the formerly described ARNT (aryl-hydrocarbon receptor nuclear translocator). Both, α- and β-subunits, are constitutively expressed in the cell [3] but while the β-subunit is not sensitive to changes in oxygenation, the α-subunit is oxygen-labile. For example, oxygen-dependent hydroxylation of proline residues 564 and 402 within the oxygen dependent degradation domain (ODD) [4] of HIF-1α by prolyl hydroxylases (PHDs) leads to recognition by the von Hippel–Lindau protein, part of an E3 ubiquitin ligase, and to instantaneous polyubiquitylation and proteasomal degradation of HIF-α in normoxia [5].

The PHDs are Fe^{2+} and 2-oxoglutarate dependent dioxygenases whose catalytic activity strictly depends on the availability of oxygen. Three isoforms exist (PHD1, PHD2 and PHD3), of which the PHD2 plays the most important role in HIF-1 regulation, limiting HIF-1α abundance [6–9]. In normoxia, hydroxylase activity is high and newly expressed HIF-1α is rapidly degraded. When oxygen tension

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decreases, hydroxylase activity declines and HIF-1α escapes proteasomal degradation, translocates to the nucleus and binds to HIF-1β, thereby forming the active transcription factor HIF-1. Functional HIF-1 binds to specific DNA sequences (hypoxia-responsive elements) in the promoter or enhancer region of HIF-1 target genes. Target genes of HIF-1 are involved in glucose metabolism and glucose transport (GLUT-1, glycolytic enzymes), angiogenesis (VEGF, vascular endothelial growth factor), hematopoiesis (erythropoietin) or iron transport (transferrin, transferrin receptor) and aim for the adaptation to hypoxia on systemic, local and cellular level [10].

Besides protein stabilisation, HIF-1 transcriptional activity is controlled by an additional Fe^{2+} and 2-oxoglutarate dependent dioxygenase termed factor inhibiting HIF (FIH) [11]. In normoxia FIH hydroxylates the asparagyl residue 803 (Asn803) within the C-terminal transactivation domain (C-TAD) of human HIF-1α. Hydroxylation of Asn803 causes steric inhibition of coactivator binding (p300/CREB binding protein) to the C-TAD and suppresses HIF-1 transcriptional activity in normoxia [12].

Apart from posttranslational regulation by Fe^{2+} and 2-oxoglutarate dependent oxygenases, HIF-1 stability and activity are modulated in an oxygen independent way by protein binding (binding of HSP90, RACK1, p53, ARD1, hypoxia associated factor HAF or Ca^{2+}-dependent protease, calpain) or non-hypoxic stimuli (i.e. growth factors, cytokines, hormones like angiotensin II, thrombin) [13–17]. For many of these factors it has been implicated that increased ROS levels can be involved in HIF-1 activation [18].

Intracellular ROS are formed by reduction of oxygen, which gives rise to the initial production of superoxide anions that subsequently yield hydrogen peroxide (H_2O_2) and hydroxyl radicals, respectively [19]. The main sources of cellular ROS are the mitochondrial electron chain as well as NADPH oxidases that are involved in signalling pathways of cytokines, hormones or growth factors. Both systems generate superoxide anions that are, due to their charge and short lifetime, not well suited for second messengers within the cell. Superoxide derived H_2O_2, however, conveys more stability and is uncharged, which makes H_2O_2 a likely candidate for cellular redox signalling [20]. H_2O_2 is generated from dismutation of superoxide. This reaction is catalysed by superoxide dismutases (SODs), which exist in the cytoplasm as copper/zinc and in mitochondria as manganese containing SODs in eukaryotic cells [21]. SODs are highly effective in converting superoxide anions instantaneously, which further supports the notion that H_2O_2 is the predominant cellular ROS [22].

Accumulation of ROS has consequences for the HIF pathway. Overexpression of the H_2O_2 degrading enzymes, glutathione peroxidase and catalase but not superoxide anion converting SOD, abolished ROS effects on HIF-1α [23]. However, contradictory results report HIF-1α stabilisation [24–27] as well as destabilisation [3,27,28] by ROS treatment. Additionally, even a need of ROS production for hypoxic HIF-1α stabilisation has been proposed [23,29–33]. Likewise, there is disagreement about the influence of ROS on HIF-1 target gene expression [18,27,28,34]. Further, it is still under debate, whether ROS production is increased or decreased under hypoxic conditions [35]. In order to improve our understanding of changes in the cellular redox state for the hypoxic HIF-1 response, we analysed the effects of exogenous H_2O_2 (20 μM) on HIF-1α and PHD2, and investigated its impact on HIF-1 target gene expression.

Methods

Cell culture

Human osteosarcoma cells (U2OS) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO Karlsruhe, Germany), with the addition of 10% foetal calf serum (FCS), 2 mM L-glutamine (Sigma) and 100 U/ml penicillin/100 μg/ml streptomycin (Pen-Strep, GIBCO).

For experiments, cells were grown to about 70% confluence in 35-mm cell culture dishes. Normoxic incubations were carried out at 37°C in humidified atmosphere of 5% CO_2 in air. Hypoxic incubations were carried out in humidified atmosphere of 1% O_2 and 94% N_2 (hypoxia) or 6% O_2 and 89% N_2 (mild hypoxia) at 37°C. Hypoxic incubations were performed in an incubator (BB 6220 CUO_2, Heraeus Instruments) or in a hypoxia workstation ($InVivo_2$ 400, Hypoxia Workstation, Ruskinn Technology).

Western blot analysis

To isolate cellular proteins, medium was removed and the cells were washed with phosphate buffered saline (PBS). Cell lysis was performed on ice with cell lysis buffer (300 mM NaCl, 10 mM Tris pH 7.9, 1 mM EDTA, 0.1% Igepal, 1x Protease-Inhibitor-Cocktail; Roche, Mannheim, Germany). Cell debris was removed by centrifugation (3600 rpm, 4°C, 5 minutes) and cellular proteins in the supernatant were used. Protein concentration of cell lysates was quantified by the Bio-Rad protein assay reagent (Bio-Rad, Munich, Germany). Equal amounts (70 μg) of cellular protein were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane (0.2 μM pore size; Schleicher and Schuell, Dassel, Germany). The membrane was blocked with 5% semi-skimmed milk overnight, and then washed with PBS three times for 2 minutes. The membrane was incubated with the following primary antibodies: mouse

monoclonal anti-HIF-1α antibody (BD Transduction Laboratories), diluted 1:750 in 5% skimmed milk overnight for HIF-1α detection; rabbit polyclonal anti-PHD2 antibody (Novus Biologicals), diluted 1:2000 in 5% skimmed milk overnight for PHD2 detection; mouse anti-α-tubulin antibody (Santa Cruz Biotechnology) diluted 1:750 in 5% skimmed milk for 2 hours for α-tubulin detection. After incubation, the membrane was washed in PBS three times and the second antibody was added: For HIF-1α and α-tubulin detection, horseradish bound anti-mouse IgG and for PHD2 detection, horseradish bound anti-rabbit IgG were applied, both diluted 1:10000 in 5% skimmed milk for an incubation time of 2 hours at room temperature. Afterwards, the membrane was washed with PBS for five times and antibody binding was detected by addition of HRP detection solution (10 ml of 100 mM Tris buffer pH 8.5, 3 µl of 30% H_2O_2, 25 µl of 90 mM coumaric acid, 50 µl of 250 mM Luminol) for 1 minute or by addition of enhanced chemiluminescence solution (ECL, Amersham Biosciences) for 5 minutes.

RNA extraction and real-time PCR

Total cellular RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method [36]. U2OS cells were grown in 6-well plates (Greiner) and incubated under the respective conditions. Medium was removed and the cells were lysed in 700 µl of 4 M GTC (guanidinium thiocyanate) per well. The plates were stored in 20°C overnight until the next day. RNA was extracted as described [36]. One microgram of RNA were reverse transcribed into cDNA using the M-MLV (Moloney murine leukaemia virus) reverse transcriptase (M-MLV-RT, Promega) according to the manufacturer's instructions. cDNA was quantified by real-time PCR in each sample using the MESA Green qPCR Mastermix for SYBR Assay (Eurogentec).

Luciferase reporter gene assay

U2OS cells were grown in 6-well plates (Greiner) for 24 hours to a confluence of 60% and then transfected with luciferase reporter gene plasmids using transfection reagent FuGENE (Roche), following the manufacturer's instructions. Luciferase reporter gene plasmids were kindly provided by R. Wenger, Zürich (pH3SVL-plasmid) and E. Metzen, Essen (pGL3-prom3E-P2P-wt and pGL3-prom3E-P2P-mt). The pH3SVL plasmid contains the luciferase reporter gene under control of a SV40 promoter and three hypoxia-responsive elements (HRE) of the HIF-1 target gene transferrin. Both pGL3-prom3E-P2P-wt (wildtype) and pGL3-prom3E-P2P-mt (mutant) drive the luciferase reporter gene under control of a HIF-binding site (HBS) and the PHD2 promoter. The wildtype plasmid contains a functional HBS; the HBS of the mutant plasmid is mutated and thus dysfunctional. After transfection the cells were incubated for additional 24 hours and then taken into experiment. For luciferase reporter gene assay, the cells were harvested and total protein concentration (Lowry protein assay) was determined. To quantify HIF-1 activity, the luciferase activity of the whole cell lysates was measured using the *Firefly* Luciferase assay kit (Biotium), according to manufacturer's instructions.

Transient transfection of cells with siRNA

U2OS cells were grown in 6-well plates (Greiner) over 24 hours before transfection under normoxia and to a confluence of about 60%. Cells were then transfected once with siRNA directed against HIF-1α (20 µM) or twice with siRNA directed against the PHD2 (20 µM) every 24 hours, using the transfection reagent oligofectamine (Invitrogen) following manufacturer's instructions. After transfection, the cells were grown for additional 24 hours in normoxia and then incubated for the indicated time in normoxia or hypoxia.

VBC binding assay

Prolyl-hydroxylase activity was measured using the VBC binding assay [37]. A biotinylated, synthesized HIF-1α peptide, bound to a streptavidin covered plate, was incubated for 1 hour with either recombinant PHD2 or whole cell lysates and PHD cosubstrates (Fe^{2+}, ascorbate, 2-oxoglutarate) in excess. To determine hydroxylase activity of recombinant PHD2 in the presence of H_2O_2, 20 µM H_2O_2 were added to the assay. To determine the influence of H_2O_2 on cellular PHD activity, cell lysates of H_2O_2 treated U2OS cells were used in the assay. Normoxic measurements were performed in air; hypoxic measurements were carried out in an incubator at 1% O_2. After incubation, hydroxylated HIF-1α peptide was detected by binding to a thioredoxin-bound VBC complex. Binding of the VBC was detected by a primary anti-thioredoxin rabbit antibody and a secondary, peroxidase bound anti-rabbit antibody. PHD activity was visualised after addition of the peroxidase substrate tetramethyl-benzidine and stopping solution H_2SO_4 by photometric measurement at a wavelength of 450 nm.

H_2O_2 measurement

H_2O_2 concentration was fluorometrically determined by employing the AmplexRed®/peroxidase method. Horseradish peroxidase catalyses the oxidation of Amplex Red® reagent to fluorescent resorufin by

H_2O_2 in a 1:1 stoichiometry. Resorufin has an excitation maximum of 571 nm and an emission maximum of 585 nm. Using Amplex Red® solution (2 mM in DMSO) and H_2O_2 solutions in a concentration of 0 to 1.5 μM, a H_2O_2 standard curve was established.

U2OS cells were grown in 35-mm culture dishes (Falcon) to a confluence of about 70% in normoxia. For medium controls, 35-mm culture dishes were filled with medium only and incubated in normoxia as well. After 24 hours, the medium was removed and replaced by 1 ml fresh medium to which distilled water or 20 μM H_2O_2 were added. For measurement, 100 μl samples of the medium were taken at 0, 2.5, 5, 7.5, 10, 15, 20, 25 and 30 minutes after the addition of H_2O_2. As the H_2O_2 concentration of the samples was expected to be in the micromolar range, samples were diluted 1:10 in 900 μl of 50-mM phosphate buffer (pH 7.4) before measurement. Then, 1 μl of 2-mM Amplex Red® solution and 1 μl peroxidase solution (3,800 U/ml) were added, mixed and incubated for 5 minutes at room temperature. The reaction was stopped by addition of 1 μl catalase (1,300,000 U/L). After addition of 100 μl NaOH (140 nM), the samples were measured fluorometrically at 585 nm after excitation at 571 nm.

Measuring oxygen concentrations in the culture dish (PreSens)

For non-invasive oxygen measurements the SensorDish® Reader system (SDR, PreSens) including 6-well Oxodishes® (PreSens) and the SensorDish® Reader (PreSens) was used. The 6-well Oxodishes® contain a sensor with a luminescent dye at the bottom of each well. The luminescence lifetime of the dye is dependent on the surrounding oxygen concentration. U2OS cells were grown in 6-well Oxodishes® (PreSens) overnight to a confluence of about 60%. For medium control, the wells were filled with medium only. At the following day, the 6-well Oxodishes® were placed on the SDR and incubated at normoxia (21% O_2) or mild hypoxia (6% O_2), and the oxygen concentration of the medium was measured at regular intervals. After formation of a steady state oxygen concentration in the medium, the medium was replaced by preheated (37°C), pre-equilibrated fresh medium with or without 100 μM H_2O_2, continuing oxygen measurement during and after H_2O_2 addition. Oxygen measurement was continued for at least 6 hours after H_2O_2 addition.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) applying the Student's t-test. *P*-values below 0.05 were considered significant.

Results

ROS treatment influences HIF-1α protein levels in a biphasic manner

To test for effects of low ROS concentrations on HIF-1α protein levels, U2OS cells were incubated at three oxygen concentrations (21% O_2, 6% O_2 or 1% O_2) in the absence or presence of 20 μM H_2O_2. Since short and long term effects of ROS treatment on HIF-1α and PHD2 had been reported, U2OS cells were lysed after 1 or 6 hours of incubation and whole cell lysates were analysed by Western blot. As expected, hypoxia induced HIF-1α protein levels. ROS treatment induced HIF-1α after 1 hour of incubation (short term), but led to HIF-1α decrease in the later phase of hypoxic exposure (long term; 6 hours). Prolyl hydroxylase domain protein 2 (PHD2), a typical HIF-1 target gene, was induced by hypoxia but reduced by ROS treatment at all time points (Figure 1A).

PHD2 is the main regulator of HIF-1α abundance in the cell under oxygenated conditions [6]. Because PHD2 activity is half maximal at 6% oxygen [9], this oxygen fraction was selected for further experiments. To specify the exact point of ROS mediated HIF-1α induction and decrease, U2OS cells were incubated for up to 1 hour or up to 8 hours in 6% oxygen with or without 20 μM H_2O_2. Western blot analysis revealed that HIF-1α protein levels rose after about 5–15 minutes in H_2O_2 treated cells but declined at about 4–6 hours after ROS addition compared to hypoxic controls (Figure 1B and C). Thus, for further experiments, 1 and 6 hours of incubation were chosen to investigate the early induction and the late phase decrease of HIF-1α by H_2O_2, respectively.

To investigate whether ROS act in a dose-dependent manner, U2OS cells were grown to the usual density of 70% and increased density of 90% confluence. Cells were then treated with 20 or 40 μM H_2O_2. At 1 and 6 hours, higher H_2O_2 concentrations enhanced the ROS effects on HIF-1α protein levels. At higher cell density, the effects of H_2O_2 were less pronounced at 1 hour but stronger at 6 hours (Figure 1D). H_2O_2 lowered hypoxia-induced expression of PHD2, which became most prominent at moderate hypoxia over the 8-hour time course (Figure 1C). Higher doses of H_2O_2 led to a stronger decrease in PHD2 protein levels (Figure 1D).

U2OS cells degrade H_2O_2 within 30 minutes by activity of catalase

Cells usually degrade H_2O_2 within minutes [34]. Since we observed opposing effects on HIF-1α protein at earlier and later time points, we decided to determine the rate of H_2O_2 degradation in U2OS cells. Four experiments were prepared: medium without H_2O_2, medium containing 20 μM H_2O_2, U2OS cells without H_2O_2 and U2OS cells treated

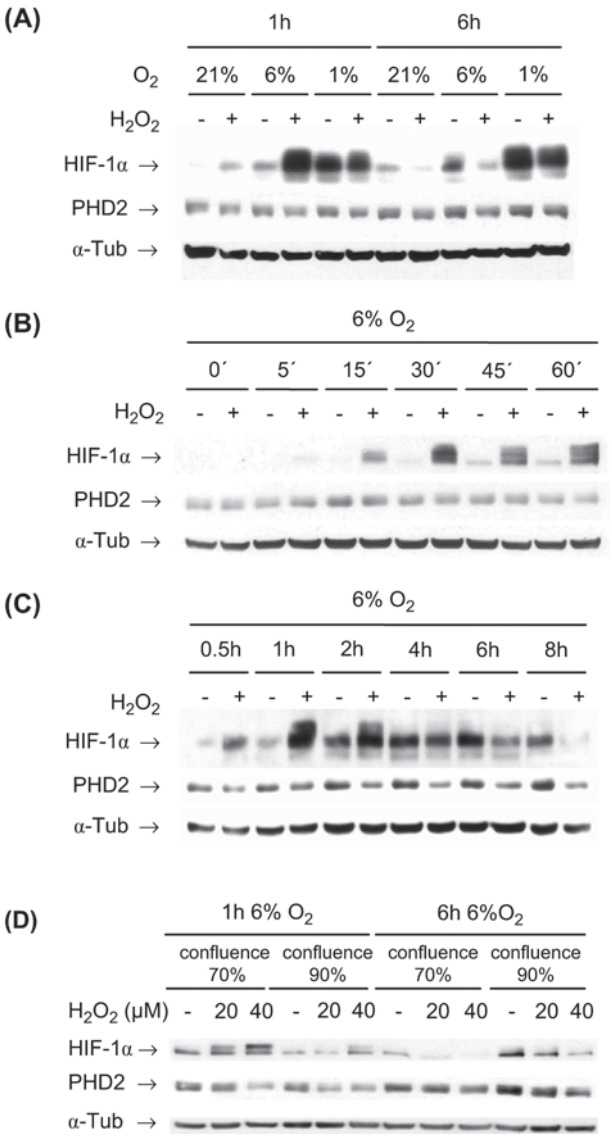

Figure 1. H_2O_2 influences HIF-1α and PHD2 in a biphasic manner. U2OS cells were incubated with or without 20 μM H_2O_2 under different oxygen fractions and incubation periods. Afterwards cells were lysed and 70 μg protein of the whole cell lysate were subjected to Western Blot analysis. HIF-1α and PHD2 were detected, α-tubulin served as a loading control. Immunoblots shown are representative of at least two independent experiments. (A) The impact of H_2O_2 treatment is time-dependent. Cells were incubated under normoxia, mild hypoxia or hypoxia (21%, 6% or 1% O_2) for 1 and 6 hours. (B) Cells were incubated under mild hypoxia (6% O_2) for up to 60 minutes. (C) Cells were incubated under mild hypoxia (6% O_2) up to 8 hours. (D) The impact of H_2O_2 treatment is dose-dependent. Cells were grown to a confluence of 70% or 90% and treated with 20 or 40 μM H_2O_2 under normoxia and mild hypoxia (21% or 6% O_2) for 1 and 6 hours.

with 20 μM H_2O_2. Every 2.5 minutes of H_2O_2 dependent oxidation of AmplexRed® to Resorufin was monitored.

AmplexRed® signals as a measure of H_2O_2 concentration were stable in medium alone, medium containing H_2O_2 only or medium covering cells only. In contrast, H_2O_2 (20 μM) added to U2OS cells was rapidly degraded with a half-life of about 10 minutes (Figure 2A).

In most cells, intracellular H_2O_2 is primarily degraded by catalase, yielding oxygen and water. To prove catalase mediated H_2O_2 reduction in U2OS cells, we measured the cellular generation of oxygen upon H_2O_2. Steady state oxygen concentrations in the medium were achieved after 10 minutes (Figure 2B and C), the medium was then replaced by warmed (37°C), pre-equilibrated fresh medium with or without 100 μM H_2O_2. Upon medium exchange, we observed a transient increase in the oxygen concentration of H_2O_2 treated cells which lasted for approximately 30 minutes. Oxygen concentrations in the media of non-treated cells and in medium alone (data not shown) were stable throughout the entire experiment (Figure 2B). The generation of oxygen from H_2O_2 by catalase was most prominent under hypoxic conditions (Figure 2C).

H_2O_2 interferes with HIF-1α degradation

HIF-1α abundance in the cell is primarily regulated on the posttranslational level by hydroxylation and subsequent proteasomal degradation. In addition, increased expression of mRNA or mRNA translation can contribute to cellular HIF-1α levels. In order to find the regulatory steps at which ROS interfere with HIF-1α accumulation, PHD activity (DMOG), the 26S proteasome (MG132), transcription (Act. D) or translation (CHX) were inhibited in U2OS cells. Cells were treated with the respective inhibitor and 20 μM H_2O_2 and were incubated for 1 or 6 hours in 6% oxygen (Figure 3A, B). In the early phase (1 hour of incubation), neither DMOG nor MG132 nor Act. D had any effect on H_2O_2 induced HIF-1α induction. CHX treatment led to a complete loss of HIF-1α protein (Figure 3A). In the late phase (6 hours of incubation) inhibition of PHD activity and of 26S-proteasome, both antagonised the H_2O_2 mediated decrease in HIF-1α (Figure 3B). Again, neither inhibition of transcription nor inhibition of translation showed an effect (Figure 3A, B).

Furthermore, H_2O_2 treatment had no impact on HIF-1α mRNA expression in U2OS cells. Total RNA of U2OS cells incubated in mild hypoxia (6% O_2) for 6 hours and treated with 20 μM H_2O_2 was obtained and HIF-1α expression in relation to the expression of β-actin was determined by real-time PCR. There was no significant difference in the relative HIF-1α expression between H_2O_2 treated and non-treated cells (Figure 3C).

H_2O_2 reduces PHD activity in the early phase

Hydroxylase activity of recombinant PHD2 in the presence of 20, 40 and 200 μM H_2O_2 was determined in the VBC binding assay. Recombinant PHD2 was

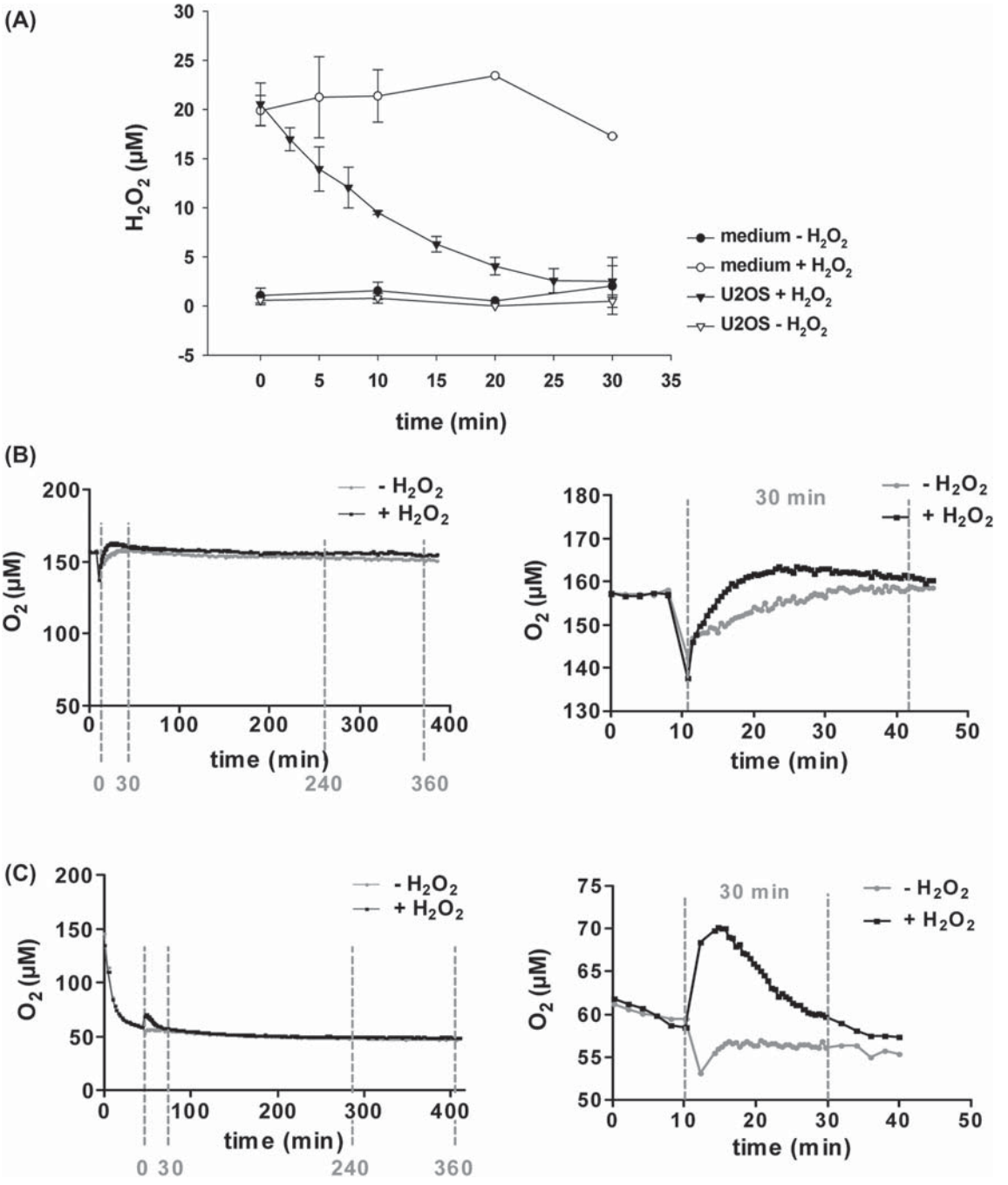

Figure 2. U2OS cells abolish H_2O_2 within 30 minutes. (A) H_2O_2 is decomposed within 30 minutes. U2OS cells were incubated with or without 20 µM H_2O_2 under normoxic conditions. The H_2O_2 concentration was measured by fluorometric measurement of H_2O_2 dependent oxidation of AmplexRed® to Resorufin over a time course of 30 minutes. To exclude that H_2O_2 was decomposed by the medium, the H_2O_2 concentration was measured in medium without cells. Shown are means and standard deviation (S.D.) of two independent experiments in triplicate. (B, C) Parallel to the H_2O_2 degradation, U2OS cells produce oxygen. U2OS cells were incubated under normoxic (B) or mild hypoxic conditions (C) for a time course of 6 hours. Oxygen concentration (µM O_2) was measured at the bottom of a cell culture plate before, during and after addition of 100 µM H_2O_2. Upon incubation the formation of a steady state oxygen concentration was awaited before replacing medium by fresh medium with or without 100 µM H_2O_2. Shown are experiments performed under normoxia (21% O_2, B) or mild hypoxia (6% O_2, C), aside each the critical time interval around the H_2O_2 addition is shown in detail. The data shown are representative for at least two independent experiments performed in duplicate. The O_2 concentration of the medium control (without cells) with and without 100 µM H_2O_2 remained constant throughout the entire experiment (data not shown).

incubated with a synthetic HIF-1α peptide and cosubstrates (ascorbate, Fe^{2+} and α-ketoglutarate in excess) under normoxic and hypoxic conditions. H_2O_2 reduced PHD2 activity under normoxic and hypoxic conditions dose-dependently (Figure 4A and B). Surprisingly, extracts of U2OS cells treated with H_2O_2 in mild hypoxia (6% O_2) for 1 hour, in turn, displayed an increased hydroxylase activity,

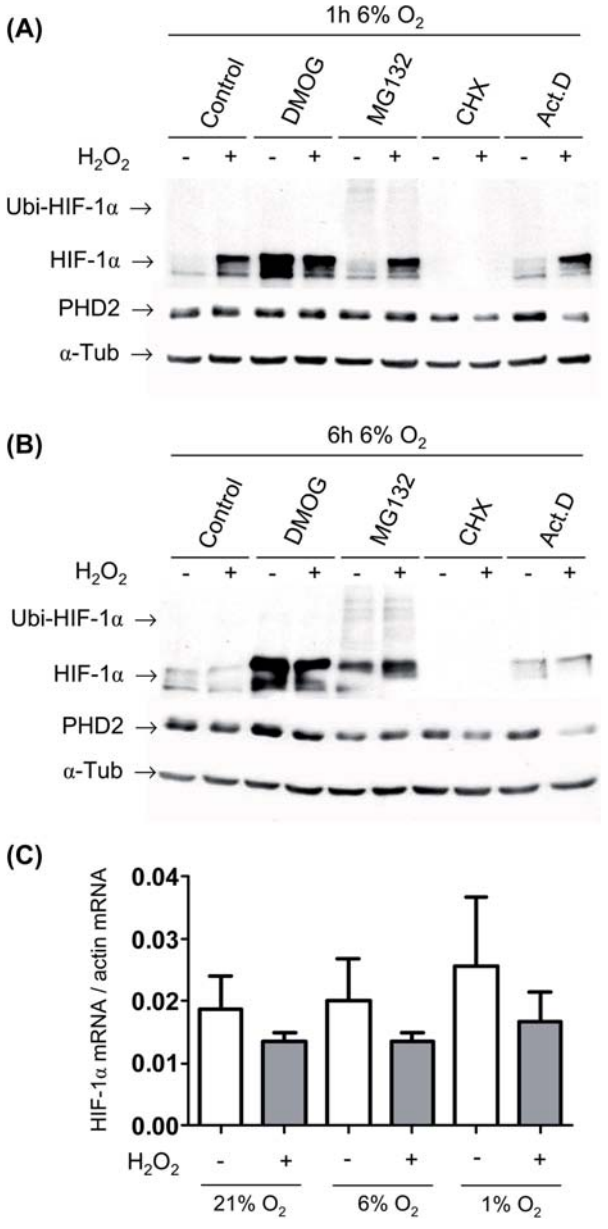

Figure 3. H_2O_2 modulates HIF-1α by affecting the PHD2. U2OS cells were incubated with or without 20 μM H_2O_2 under normoxic or hypoxic (6% or 1% O_2) conditions for 1 or 6 hours. (A, B). Cells were treated with or without an inhibitor of PHD activity (2 mM DMOG), an inhibitor of the 26S proteasome (5 μM MG132) or inhibitors of translation (10 μM cycloheximide, CHX) and transcription (1 μg/ml ActinomycinD, Act.D). Cells were lysed and 70 μg protein of whole cell lysates was subjected to Western Blot analysis. HIF-1α, PHD2 and α-tubulin were detected. MG132 treatment lead to accumulation of ubiquitinated HIF-1α (Ubi-HIF-1α). Immunoblots are representative for at least two independent experiments. (C) U2OS cells were incubated with or without 20 μM H_2O_2 under 21% O_2, 6% O_2 or 1% O_2. Total RNA was isolated and mRNA expression of the HIF-1α and β-actin was quantified by real-time PCR. HIF-1a cDNA was normalised to β-actin cDNA as an internal control. The data shown are the means of three independent experiments. The error bars represent standard error. Data were analysed with Student's t-test. There was no significant difference in the relative HIF-1α mRNA expression between H_2O_2 treated and non-treated cells detectable.

twice as high as the hydroxylase activity of cell extracts drawn from non-treated cells (data not shown).

Subsequent ubiquitination of HIF-1α in U2OS cells, however, was attenuated by H_2O_2 treatment. U2OS cells treated with MG132 and incubated in 6% oxygen in the absence or presence of 20 μM H_2O_2 showed a H_2O_2-derived diminution of ubiquitinated HIF-1α which accompanied the induction of overall HIF-1α (Figure 4C).

H_2O_2 increases PHD activity in the late phase

We next investigated whether HIF-1α decrease of the late phase was also due to H_2O_2 mediated modification of cellular PHD activity. U2OS cells were incubated in 6% oxygen for 6 hours in the absence or presence of 20 μM H_2O_2 and PHD activity was determined with the VBC assay. H_2O_2 treated cells showed a significantly increased hydroxylase activity (Figure 5A).

All three isoforms – PHD1, 2 and 3 – may contribute to overall PHD activity, while the PHD2 has been described to have the highest importance for HIF-1α hydroxylation. To test whether the H_2O_2 mediated increase in cellular hydroxylase activity was due to enhanced PHD2 activity, PHD2 expression was lowered by siRNA and cells were incubated in the absence or presence of 40 μM H_2O_2 in mild hypoxia for 6 hours. In the control cells (mock transfected cells), H_2O_2 lowered hypoxia induced HIF-1α, whereas this effect was lost after the knockdown of PHD2 (Figure 5B). Interestingly, 6 hours after H_2O_2 treatment, ubiquitination of HIF-1α had recovered and almost reached the level of non-treated cells (Figure 5C).

H_2O_2 treatment attenuates hypoxic HIF-1 activity

To study the influence of H_2O_2 on HIF-1 transcriptional activity and HIF-1 target gene expression, U2OS cells were transfected with the pH3SVL luciferase reporter gene plasmid and exposed to 21%, 6% or 1% O_2. Cells treated with 20 μM H_2O_2 displayed significantly reduced HIF-1 activity under hypoxic conditions (Figure 6A). In a second series of experiments, cells were transfected with a luciferase reporter gene construct encompassing the PHD2 promoter and the respective HIF-1 binding site (HBS) or a mutant control in which the HBS had been inactivated by a point mutation. Again, cells were exposed to 21%, 6% or 1% O_2 and treated with 20 μM H_2O_2. H_2O_2 reduced hypoxia-induced activity of the wildtype reporter plasmids but not of the controls indicating that the inhibition of reporter gene activity was attributable to HIF-1 activity (Figure 6B). Finally, the hypoxic expression of the HIF-1 target genes carboanhydrase 9 (CA9) and

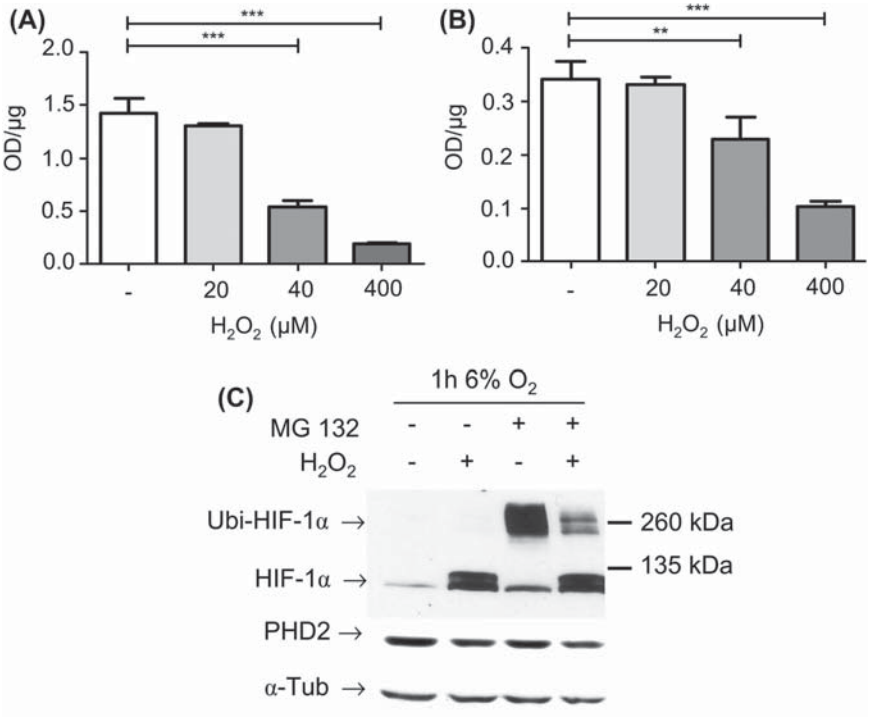

Figure 4. H_2O_2 reduces HIF-1α degradation by attenuating PHD2 activity. (A, B) Increasing H_2O_2 concentrations attenuated PHD2 activity significantly under normoxia and hypoxia. PHD2 activity was measured using a VBC binding assay (see methods). Recombinant PHD2 was incubated with a synthetic HIF-1α-peptide (556–574) under (A) normoxia (21% O_2) or (B) hypoxia (1% O_2) and 20, 40 or 400 µM H_2O_2 was added. Shown are means and standard error of the mean. Data were analysed with Student's t-test (** for $P < 0.05$, *** for $P < 0.001$ vs. control (–)) Data shown are representative for two separate experiments in triplicate. (C) H_2O_2 reduces ubiquitination of HIF-1α. U2OS cells were incubated with or without 20 µM H_2O_2 under mild hypoxic conditions for 1 hour. For detection of polyubiquitinated HIF-1α cells were treated with 5 µM MG132. After lysis, 70 µg protein of the whole cell lysate was analysed by Western Blotting. HIF-1α, PHD2 and α-tubulin were detected. MG132 treatment led to accumulation of ubiquitinated HIF-1α (Ubi-HIF-1α). The immunoblot is representative for three independent experiments. Apparent molecular weight markers (kDa) are provided.

PHD2 under H_2O_2 treatment was examined. U2OS cells treated with 20 µM H_2O_2 showed reduced mRNA expression of CA9 (Figure 6C) and PHD2 (Figure 6D) compared with hypoxic controls under 1% O_2. Both genes were significantly upregulated under hypoxic conditions, whereas only the inhibition of CA9 expression by H_2O_2 reached statistical significance. Early HIF-1α induction did neither yield a significant increase in DNA binding nor a significantly increased expression of the HIF-1 target gene PHD2 (data not shown).

Discussion

The effects of ROS on HIF-1α protein are matter of a lively scientific debate. Destabilisation [3] and stabilisation of HIF-1α by ROS [24,32,33,38] have been reported. Accordingly, under ROS treatment, controversial findings showing either induction [18,24,25] or reduction of HIF-1 target gene expression [3,28,34] were found.

Our data herein may provide a solution of the contradiction by showing a biphasic regulation of HIF-1α abundance in the presence of H_2O_2. At short reaction periods ($t < 1$ hour), a stabilisation of HIF-1α was observed, whereas a destabilisation of HIF-1α was evident at prolonged observation intervals. Both stabilisation and destabilisation of HIF-1α occurred irrespective of the oxygenation status in unfailing chronological sequence, depicting a solid mechanism of ROS interference, valid and effective at high as well as at low pO_2 (Figure 1A). Physiologically, ROS are produced constantly within the cell. A bolus addition imitates the situation of cells exposed to an oxidative burst. Continuous addition/production within the cell may produce different results, considering that the HIF-1α stabilization/destabilization is not a rigid system with fixed set points but a balanced regulation that adapts to changes in oxygen availability and is then able to level off at novel set points [8]. All H_2O_2 dependent effects increased with rising H_2O_2 concentrations and were less pronounced when cell number and the corresponding H_2O_2 depleting defence were increased, indicating the dose-dependent fine tuning functions of ROS in HIF regulation (Figure 1D).

Closer examination over the time revealed, that HIF-1α accumulation appeared quite rapidly within 5 minutes (Figure 1B). The promptness and extent of this accumulation points towards a direct interference of H_2O_2 with the cellular HIF degradation machinery, first of all the key regulator PHD2. Indeed,

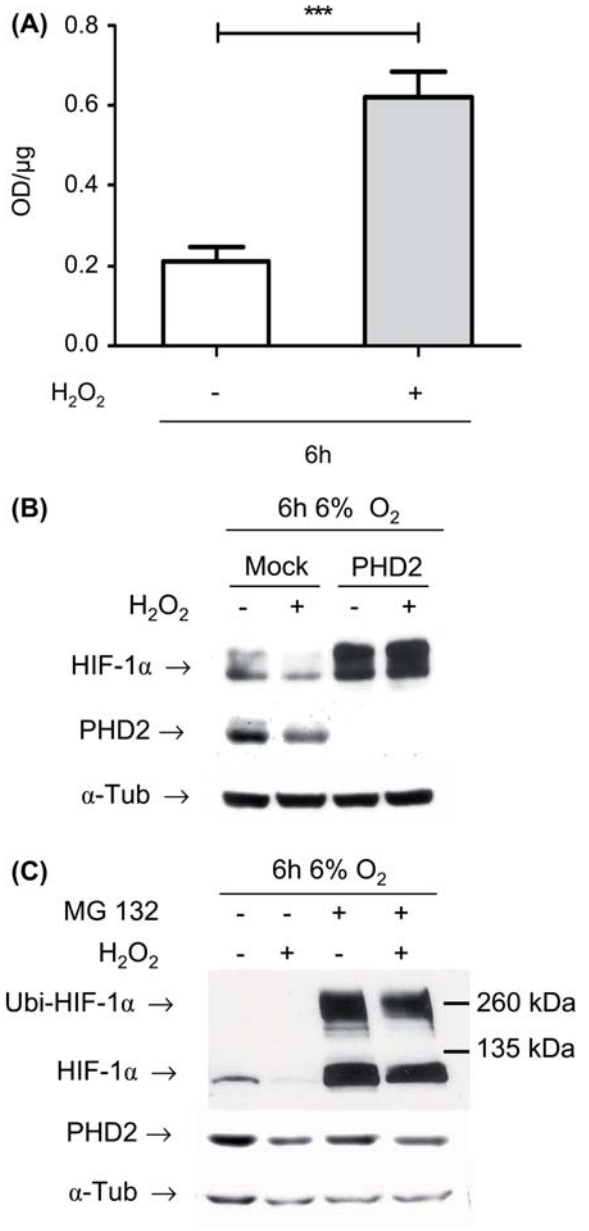

Figure 5. HIF-1α is reduced by H_2O_2 mediated increase in PHD2 activity. (A) H_2O_2 increased hydroxylation capacity of U2OS cells. Cells were incubated with or without 20 µM H_2O_2 in mild hypoxia (6% O_2) for 6 hours. To analyse hydroxylation capacity of the cells 100 µg protein of whole cell lysates were subjected to the VBC binding assay which was performed under normoxic conditions, as described in the method section. Data were analysed with Student's t-test (** for $P < 0.05$, *** for $P < 0.001$ vs. control (–)). The data are the means of four independent experiments. The error bars represent standard error. (B) Knockdown of the PHD2 reversed H_2O_2 mediated HIF-1α degradation. U2OS cells were transfected with PHD2 siRNA and incubated for 6 hours under mild hypoxia (6% O_2) with or without 40 µM H_2O_2. After incubation the cells were lysed and 70 µg protein were applied to Western Blot analysis. The immunoblot is representative for two independent experiments. (C) Inhibition of the proteasome lead to stabilisation and repression of H_2O_2 mediated HIF-1α degradation. The proteasomal degradation of HIF-1α in U2OS cells was blocked by addition of 5 µM MG132 and the cells were incubated under mild hypoxia (6% O_2) with or without 20 µM H_2O_2 for 6 hours. For Western blot analysis, 70 µg of protein was used and HIF-1α, PHD2 and α-tubulin were detected. The immunoblot is representative for three independent experiments. Apparent molecular weight markers (kDa) are provided.

the data obtained draw a picture of inhibited hydroxylase activity as the underlying mechanism of early H_2O_2 action.

Examining the various regulatory steps of HIF-1α abundance, merely the PHD inhibitor DMOG but not inhibitors of transcription/translation or even proteasomal degradation achieved a prompt HIF-1α induction, comparable to H_2O_2 treatment (Figure 3A). Further, *in vitro* tested activity of recombinant PHD2 declined dose-dependently in the presence of H_2O_2 (Figure 4A and B). The applied *in vitro* hydroxylase assay solely demonstrates the direct impact of H_2O_2 treatment on the activity of a recombinant PHD2 in a cell-free environment. The assay contains ascorbate but no catalase activity and may therefore, free of interference, reflect the immediate action of H_2O_2 treatment on PHD activity within the first minutes of treatment, when H_2O_2 leads to HIF-1α accumulation. For the assay, a first significant decrease in hydroxylase activity was achieved by 40 µM H_2O_2 but not by 20 µM H_2O_2. Considering that the *in vitro* hydroxylase assay employs a far larger amount of recombinant PHD2 than would be expected endogenously, 20 µM H_2O_2 may well be perfectly sufficient to inhibit cellular *in vivo* PHD2 activity. Consistent with the findings that H_2O_2 dose-dependently induced HIF-1α in normoxia as well as in hypoxia, hydroxylase activity declined dose-dependently in normoxia and hypoxia (Figure 4A and B). Accordingly, 20 µM H_2O_2 attenuated polyubiquitination of HIF-1α in U2OS cells (Figure 4C).

These data are consistent with previous findings by Nytko et al. [39], and correspond to an elegant model describing the direct chemical action of ROS on the activity of prolyl hydroxylases proposed by Gerald et al. [25]. As prolyl hydroxylases are ferrous iron (Fe^{2+}) and α-ketoglutarate dependent enzymes that are strictly dependent on oxygen for hydroxylation function, PHD activity is primarily controlled by the given oxygen concentration and secondarily by the availability of cofactors, first of all, Fe^{2+}. Changes in the Fe^{2+} pool therefore directly lead to impaired hydroxylation. ROS utilise this sensitivity towards Fe^{2+} availability. By depletion of cellular Fe^{2+} via Fenton's reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH$), H_2O_2 represses the PHD hydroxylation function [25,40]. Thus, following this model, direct H_2O_2 action results in PHD inhibition and thereby prevents HIF-1α degradation. However, for direct action and Fe^{2+} oxidation, the presence of H_2O_2 inside the cell is necessary. Our data support the notion of H_2O_2 presence in the cell at least during 30 minutes following addition: In line with previous findings a 20 µM bolus of exogenous H_2O_2 was eliminated by U2OS cells within 30 minutes after addition (Figure 2A) [29,34]. As cell-free medium failed to degrade H_2O_2, this elimination showed absolute dependency on cellular activity (Figure 2A). Further data corroborating the intracellular presence of H_2O_2 were provided by oxygen measurements.

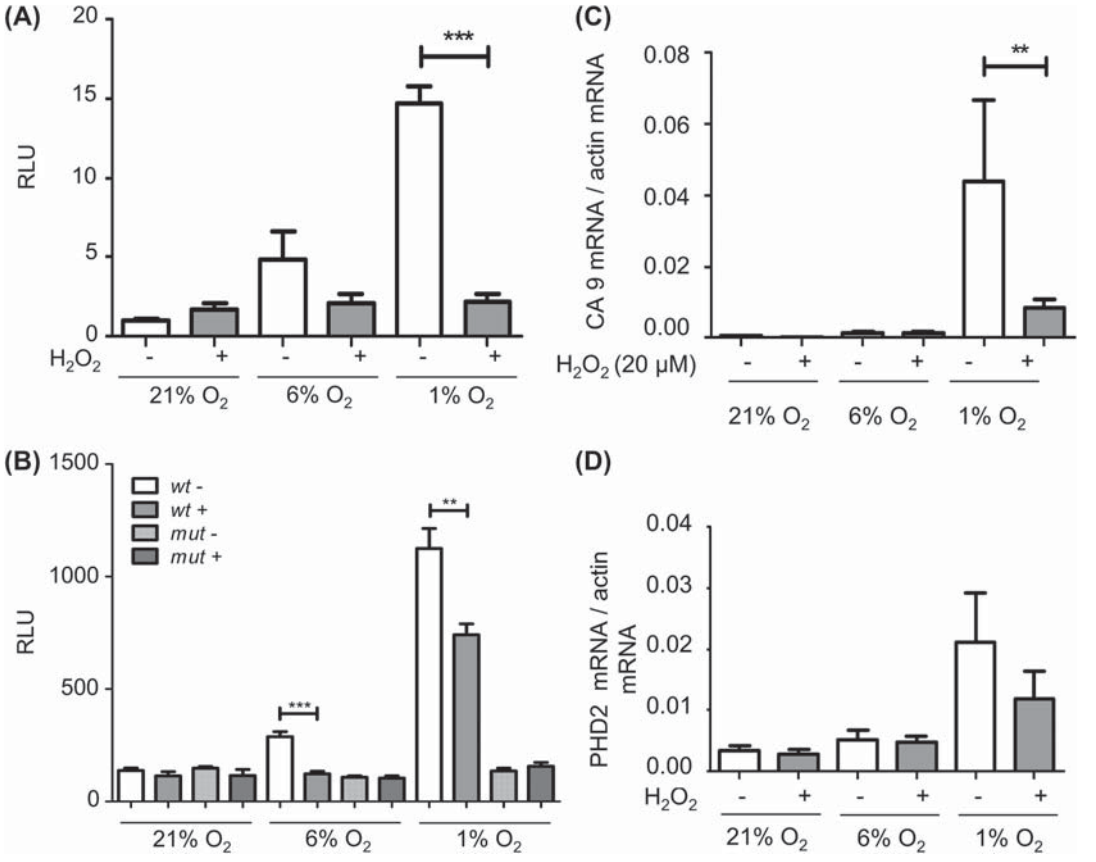

Figure 6. HIF-1 transcriptional activity and transcription of HIF-1 target genes is reduced by H_2O_2. (A) U2OS cells were transfected with the pH3SVL plasmid, which encodes the luciferase reporter gene under control of a SV40 promoter and three hypoxia-responsive elements of the human transferrin gene. The cells were incubated for 6 hours under 21% O_2, 6% O_2 or 1% O_2 with or without 20 µM H_2O_2, afterwards lysed and HIF-1 transcriptional activity was determined by measuring luciferase activity. Measured luciferase activity was related to the cellular protein concentration and is shown as n-fold induction of the normoxic control. Data shown are representative for three independent experiments performed in triplicate (B) U2OS cells were transfected with pGL3-prom-wt (wildtype) or pGL3-prom-mt (mutant) plasmid respectively. The wildtype plasmid encodes for the luciferase enzyme under control of the PHD2 promoter and a functional HBS (wildtype). The HBS of the mutant plasmid is mutated, so that HIF-1 cannot bind. Transfected cells were incubated for 16 hours in 21% O_2, 6% O_2 or 1% O_2 with or without 20 µM H_2O_2 and luciferase activity was measured. The data are the means of three independent experiments. The error bars represent standard error. (C) U2OS cells were incubated for 6 hours under 21% O_2, 6% O_2 or 1% O_2 with or without 20 µM H_2O_2. Total RNA was isolated and mRNA expression of the HIF-1 target gene carboanhydrase 9 and of the housekeeping gene β-actin was quantified by real-time PCR. Carboanhydrase 9 mRNA expression was normalised to β-actin mRNA expression. Data were analysed with Student's t-test: ** for $P < 0.05$, *** for $P < 0.001$ versus control (−). (D) U2OS cells were incubated with or without 20 µM H_2O_2 in 21% O_2, 6% O_2 or 1% O_2 for 6 hours and total RNA was extracted. By employing real-time PCR expression of PHD2 mRNA normalised to mRNA expression of the housekeeping gene β-actin was determined as above. The data are the means of three independent experiments. The error bars represent standard error.

The major eliminator of H_2O_2 in the cell is catalases that convert H_2O_2 into water and oxygen [34,41,42]. Measurement of the oxygen concentration in the media of H_2O_2 treated U2OS cells before, during and after addition of 100 µM H_2O_2 revealed a transient, about 30 minutes lasting increase in the oxygen concentration of the medium, indicating intracellular, catalase mediated H_2O_2 elimination over this time (Figure 2B and C). H_2O_2 addition to cell-free medium left the oxygen concentration unchanged, demonstrating that oxygen production strictly depends on cellular catalase activity (data not shown). Besides, the steady state oxygen consumption of the cells was unchanged after treatment, additionally indicating that 100 µM H_2O_2 did not have any toxic effect on the cells. Regarding the temporal overlap of H_2O_2 degradation and transient cellular O_2 production, we conclude that up to 30 minutes after application, H_2O_2 is present within the cell and can directly interact with the HIF regulation through PHD inhibition.

About 2–4 hours after H_2O_2 addition, H_2O_2 induced HIF-1α accumulation switches into a H_2O_2-dependent decrease of HIF-1α. This course closely resembles the reported feedback mechanism for HIF-1α abundance in prolonged hypoxia: Hypoxic induction of HIF-1α is within hours (4–8) followed by an increased expression of the HIF-1 target gene PHD2, that in turn leads to degradation of HIF-1α even under hypoxic conditions [7,8]. Crucial for increased HIF degradation is the gain in PHD2 amount [6,8]. Surprisingly, H_2O_2-treated U2OS cells not only lacked the elevation in PHD2 protein, H_2O_2 even

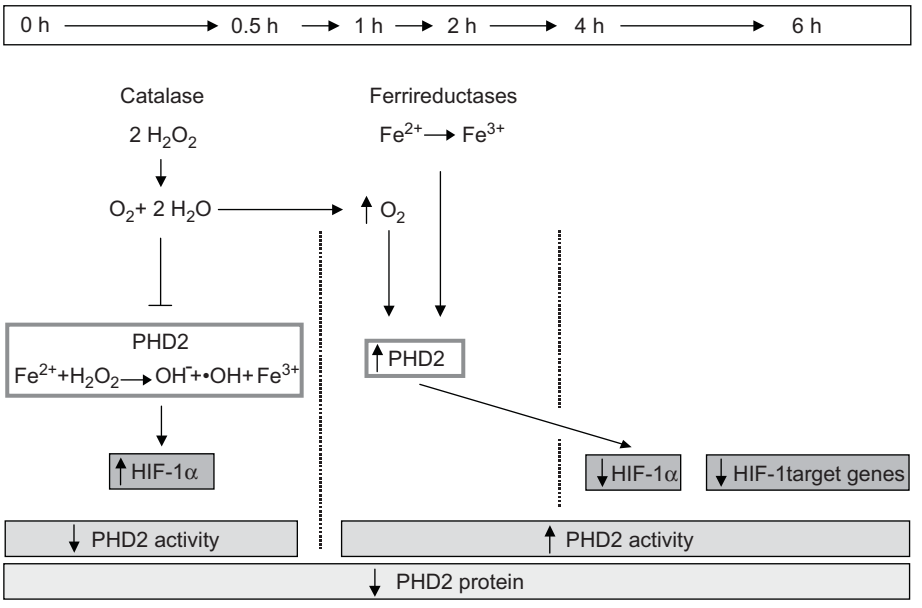

Figure 7. Schematic model of the ROS effect on the HIF pathway in U2OS cells. In U2OS cells, H_2O_2 exerts influence on HIF-1α via iron-mediated changes in PHD activity. Initially, at time point 0–0.5 hours, H_2O_2 attenuates PHD2 activity by oxidation of Fe^{2+} via Fenton reaction. Depletion of Fe^{2+} leads to HIF-1α accumulation. Within 30 minutes, H_2O_2 is eliminated by catalase activity under production of oxygen. In the late phase, from 0.5 hours onwards, oxygen production and reduction of Fe^{3+} to Fe^{2+} by activity of H_2O_2 activated ferrireductases lead to increased PHD activity which results in HIF-1α destabilisation and suppressed HIF-1 target gene expression. Throughout the whole experiment, H_2O_2 reduces the PHD2 protein amount. Nevertheless, increased cofactor availability (O_2, Fe^{2+}) compensates for this loss in protein amount.

decreased the PHD2 protein amount despite elevated HIF-1α levels (Figure 1C). Furthermore, although PHD2 levels stayed low in H_2O_2 treated cells throughout the whole experiment, HIF-1α was subsequently decreased (Figure 1B–D).

Out of transcription, translation, PHD activity and the 26S proteasome, it was the inhibition of the HIF degradation machinery, PHD and proteasome that reversed late HIF-1α decline, indicating that an increased degradation accounts for the late HIF-1α diminution (Figure 3A). Furthermore, hypoxic cell extracts of H_2O_2 treated U2OS cells displayed increased prolyl hydroxylase activity (Figure 5A). By applying cell lysates instead of recombinant enzyme to the assay, possible cellular reactions to the redox stimulus were included and influenced the resulting *in vitro* hydroxylation. Involvement of reactive cellular processes apparently turned the mere inhibition of PHD activity by H_2O_2 as seen with the recombinant PHD2, to a strong activation of PHD activity by H_2O_2. Likely candidates for this switch in PHD activity have been reported: a family of ferrireductases has been identified, that reduce Fe^{3+} to Fe^{2+} and are activated upon redox stimuli (H_2O_2) [43]. As outlined above, modulation of cellular Fe^{2+} content is a potent and effective way to mediate instantaneous changes in PHD activity. A raise of Fe^{2+} content may very well account for this substantially enhanced PHD activity.

The prominent role of the PHD2 in this increased cellular prolyl hydroxylase activity and in the decrease of HIF-1α by H_2O_2 was clearly demonstrated by the fact that siRNA knockdown of the PHD2 completely abrogated late HIF-1α diminution (Figure 5B). Besides, ubiquitination of HIF-1α had recovered, hence indicating enhanced hydroxylation of HIF-1α (Figure 5C). Although due to the strong exposure of the film minor changes in the HIF-1α amount in siRNA treated cells might not be noted, the immunoblot in Figure 5B supports the notion of PHD2 as the main regulator of HIF-1α in U2OS cells and thus that the H_2O_2 mediated HIF-1α induction is dependent on the effects on PHD2. Interestingly, for U2OS cells an increase in PHD2 hydroxylase activity beyond the increase due to induction of PHD2 protein amount has been reported upon NO treatment, suggesting an additional mechanism raising the activity level [9]. NO is an inhibitor of the mitochondrial electron chain. This may result in redistribution of oxygen in the cell with induction of PHD activity, when the oxygen sink mitochondrion takes up less O_2 [44]. Analogously, the transient O_2 production associated with the elimination of H_2O_2 might provide increased PHD activity.

Taken together, late HIF-1α decline seems to result from increased hydroxylase activity, ubiquitination and proteasomal degradation. Hence, both H_2O_2-induced accumulation and diminution of HIF-1α appear to be a consequence of respective changes in PHD2 activity. In support of these findings, HIF-1α mRNA expression showed no significant changes within 6 hours of H_2O_2 treatment. Though H_2O_2 has

been reported to induce HIF-1α transcription via activation of nuclear factor-kappa B (NF-κB), H_2O_2 treatment even slightly decreased HIF-1α mRNA [45,46].

What is the impact of H_2O_2 treatment regarding HIF-target genes? In consent with previous studies, we found that ROS suppress the hypoxic induction of HIF-1 target gene expression [27,28,34]. This was true for reporter gene expression under control of the transferrin hypoxia-responsive element or under control of the PHD2 promoter and for mRNA expression of carboanhydrase 9 (Figure 6A–C). There was also a moderate but not significant reduction of PHD2 mRNA expression (Figure 6D).

PHD2 protein decline under H_2O_2 seems to be due to increased degradation of PHD2 as well, as MG132 treatment restored PHD2 (Figures 4C and 5C) [47,48]. Besides, although H_2O_2 suppresses hypoxic induction of HIF-1 target gene expression, a detectable decrease in PHD2 protein due to lowered mRNA would not be expected to occur before 2 hours. The drop in PHD2 protein, however, occurred within minutes of mild hypoxia and even in normoxia with H_2O_2 (Figure 1A and C) [8].

From our findings, we propose a model of time-dependent ROS effects on the HIF pathway (Figure 7). ROS can influence the HIF pathway via modulation of PHD activity. The character of the regulation changes with time from initial inhibition of PHD activity to activation of PHD activity. The underlying mechanism of this reverse regulation seems to be the modulation of the cellular ferrous iron pool. H_2O_2 as a direct effector initially oxidizes ferrous iron, probably via Fenton's reaction (Figure 7) and thus deprives the PHD catalytic site of the necessary cofactor. This leads to a decreased hydroxylase activity and a transient accumulation of HIF-1α. The direct H_2O_2 effect is of brief nature, as H_2O_2 is rapidly degraded by catalase activity. Oxygen produced thereby might enhance the persisting indirect H_2O_2 effect that renders the increase in PHD activity of the late phase, leading to enhanced degradation of HIF-1α and suppression of HIF-1 target gene expression.

In conclusion, our data indicate that the PHD2 is not only the main regulator of oxic HIF regulation but also the centre of redox regulation of hypoxia-inducible factor-1. The here presented dual influence of ROS on PHD activity in U2OS cells, reflected in the reverse regulation of HIF-1α, may shed light on the putative mechanisms in redox regulation of the hypoxia inducible factor-1.

Acknowledgments

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Declaration of interest

The authors have no financial conflict of interest to disclose. The authors are responsible for the content and writing of the paper. The work was supported by IFORES Grant D/107-21200 to H. Niecknig and DFG Grant FA225/22 to J. Fandrey, U. Berchner-Pfannschmidt and M. Kirsch.

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