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Research Article

The interaction of superoxide with nitric oxide destabilizes hypoxia-inducible factor-1α

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Abstract. In renal carcinoma cells (RCC4) hypoxia inducible factor-1 (HIF-1) is constitutively expressed due to a von Hippel Lindau protein deficiency, but can be degraded by calpain, independently of the 26S proteasome, when exposed to hypoxia/nitric oxide (NO). In this study we examined molecular mechanisms to explain calpain activation. The inability of hypoxia/NO to degrade HIF-1α in respiratory-deficient RCC4-ρ0 cells pointed to the requirement for mitochondria-derived reactive oxygen species. A prerequisite for O_2^- in combination with NO to destabilize HIF-1α was corroborated in RCC4-p0 cells, when the redox cycler 2,3-dimethoxy-1,4-naphthoquinone was used as a source of superoxide. Degradation of HIF-1α required intracellular calcium transients and calpain activation. Using uric acid to interfere with signal transmission elicited by NO/O_2^- blocked HIF-1α degradation and attenuated a calcium increase. We conclude that an oxidative signal as a result of NO/O_2^- coformation triggers a calcium increase that activates calpain to degrade HIF-1α, independently of the proteasome.

Keywords. HIF-1α, nitric oxide, oxygen radicals, calcium, calpain, mitochondria.

Introduction

Hypoxia inducible factor-1 (HIF-1) is a key component for sensing and coordinating adaptation to low oxygen availability. It is composed of a 120-kDa HIF-1α and a 91–94-kDa HIF-1β subunit [1, 2]. While HIF-1β is constitutively expressed, the α subunit is affected by oxygen availability. Under normoxia (21 % O_2), HIF-1α is hydroxylated by prolyl hydroxylases (PHDs), polyubiquitinated by an E3-ubiquitin ligase complex containing the von Hippel Lindau protein (pVHL) and concomitantly degraded by the 26S proteasome [3–7]. Oxygen also regulates the transcriptional activity of HIF-1. Under normoxia, factor inhibiting HIF (FIH) [8, 9] hydroxylates a critical Asn803 residue within the C-terminal domain of HIF-1α to prevent p300/CBP binding, thereby suppressing HIF-1 activity.

Although oxygen is of major importance in affecting HIF, there are other regulators such as superoxide and/or nitric oxide (NO). A model that was proposed some time ago, but which is still controversially discussed, involves the generation of reactive oxygen species (ROS) by mitochondria under hypoxic conditions. Chandel and coworkers [10] proposed that mitochondria produced a burst of ROS under hypoxia, which was both necessary and sufficient to

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stabilize HIF-1α. This approach was questioned because other groups reported that cells lacking a functional respiratory chain (ρ0 cells) still stabilized HIF-1α in response to hypoxia [11]. More recently, genetic and pharmacological inhibition of the electron transport precluded ROS formation, which impaired HIF induction by hypoxia but not anoxia, arguing that ROS produced by mitochondria altered the shape of the PHD dose-response curve [12, 13]. Mechanistically, inactivation of PHD *via* conversion of Fe(II) to Fe(III) by ROS is seen in junD-deficient cells [14]. Moreover, transcriptional regulation by ROS could explain stabilization of HIF-1α by oxidants such as O_2^- or H_2O_2 [15, 16].

In addition, nitric oxide stabilizes HIF-1α under normoxia due to inhibition of PHD activity [17, 18] or based on S-nitrosylation of a critical cysteine in the oxygen-dependent degradation (ODD) domain [19]. However, cotreatment of NO together with O_2^- reduced HIF-1α due to the reaction of NO with O_2^-, which lowered NO bioavailability, thus reversing PHD inhibition [20, 21]. Interestingly, NO supplied under hypoxia also attenuated HIF-1α accumulation and HIF-1 transactivation [22, 23]. An additional layer of complexity is added considering the near diffusion controlled interaction between NO and O_2^- [24]. The NO/O_2^- reaction product is strongly oxidizing, often believed to be identical with peroxynitrite ($ONOO^-$). Although $ONOO^-$ has been proposed to destabilize HIF-1α under hypoxia, mechanisms have not jet been defined [25, 26]. Intermediates formed from the reaction of NO with O_2^- did not show 3-nitrotyrosine formation, which is considered to be a marker for $ONOO^-$, although a compensatory flux of O_2^- during NO generation increased oxidative intermediates and caused a proportional decrease in the level of HIF-1α [21].

We approached expression of HIF-1α in renal carcinoma (RCC4) cells, which are pVHL deficient and therefore permanently express HIF-1α. RCC4 cells not only express HIF-1α under normoxia but also allow its transcriptional activity, although FIH should interfere with binding of p300/CBP and thus, attenuate HIF-1 activity under 21% oxygen. Datta und Li [27, 28] resolved this discrepancy by showing that FIH mRNA is down-regulated by protein kinase C zeta (PKC ζ). Furthermore, PKC ζ provokes binding of the Cut-like homeodomain protein (CDP/Cut) to FIH, thereby decreasing the repressor activity of FIH. Previously, we noticed that DETA-NO [2,2′-(hydroxynitroso-hydrazono) bis-ethanimine] supplied under hypoxia decreased HIF-1α protein. The reduction of HIF-1α protein is solely due to calpain-mediated degradation, while transcriptional down-regulation of HIF-1α was precluded [29]. Using RCC4-ρ0 cells, we now present evidence that hypoxia provokes mitochondrial ROS production that is needed for NO to destabilize HIF-1α. Under normoxia, the combination of DETA-NO together with O_2^-, supplied by the redox cycler DMNQ (2,3-dimethoxy-1,4-naphthoquinone), were able to reduce HIF-1α in RCC4-ρ0 as well as RCC4 cells. Uric acid, a proposed peroxynitrite scavenger, not only blocked destabilization of HIF-1α but also calcium transients and calpain activation under normoxia/NO/O_2^-. We assume that oxidative reactions between NO and O_2^- are required to lower the protein amount of HIF-1α in RCC4 cells, independently of the classical proteasomal destruction pathway.

Materials and methods

Materials. Medium and supplements were purchased from PAA (Linz, Austria). Fetal calf serum (FCS) was from Biochrom (Berlin, Germany). 2,2′-(Hydroxynitroso-hydrazono) bis-ethanimine (DETA-NO), 3-morpholinosydnonimine hydrochloride (SIN-1), 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), ionomycin, Fura-2AM, carbonyl-cyanide-*p*-trifluoro-methoxy-phenylhydrazone (FCCP), potassium cyanide (KCN), oligomycin and anti-actin antibody were from Sigma (Schnelldorf, Germany). Dihydrorhodamin 123 (DHR) was purchased from Alexis Corporation (Lausen, Switzerland); calpastatin peptide from Calbiochem (Bad Soden, Germany); hydroethidine (HE) from Molecular Probes (Eugene, USA); MitoSOX Red from Invitrogen (Karlsruhe, Germany), and ethidium bromide from Roth (Karlsruhe, Germany). Other reagents were more recently described [29].

Cell culture. Human RCC4 were cultured as described before [29]. To generate RCC4-ρ^0 cells, 50 ng/ml ethidium bromide and 50 μg/ml uridine were added to RCC4 medium containing 2 mM sodium pyruvate [30]. Cells were kept at 37 °C in a humidified atmosphere with 5% CO_2. For hypoxic exposure, cells were incubated at 0.5% O_2 in a hypoxia workstation (Ruskinn Technology, Leeds, UK).

Western blotting. Western analysis was performed as described before [29]; 100 μg protein was used on 10% SDS-polyacrylamide gels.

ROS determinations. ROS were measured by flow cytometry using the redox-sensitive dye HE or DHR; 5×10^5 cells were used. For HE measurements, cells were treated as indicated and 2 μM HE was loaded for 30 min before the end of the incubations. For DHR measurements, cells were loaded prior to incubations

with 50 μM DHR. For measurement of mitochondrial ROS, cells were stained with 5 μM MitoSOX Red for 10 min and washed with media three times for 5 min each. Cells were harvested and resuspended in PBS. Hypoxic samples were collected under hypoxic conditions and immediately analyzed. Fluorescence was recorded on the PE channel (excitation 488 nm, emission 578 nm) of a FACS Canto (BD Biosciences, Heidelberg, Germany). For each analysis 1×10^4 cells were counted. Mean values of the log fluorescence in individual samples were recorded and normalized to control cells.

High-resolution respirometry. Cellular respiration was measured at 37 °C using an Oxygraph-*2k* (Oroboros, Innsbruck, Austria) with chamber volumes of 2 ml. DatLab 4.0 software was used acquire data (2-s time interval) and further analysis. This includes online calculations of the cell-dependent oxygen flux, calibration and background oxygen flux corrections according to the manufacturer's instructions [31]. For each oxygen consumption measurement, cells were freshly trypsinized and centrifuged; 6×10^6 cells were then resuspended in media. After recording the steady-state oxygen flux of the cells, ATP synthase was inhibited with 2 μg/ml oligomycin, followed by uncoupling of the oxidative phosphorylation by stepwise addition of FCCP up to the optimum concentration of 1 μM. Finally, the respiration was inhibited by the addition of 2 mM KCN. Respiratory rates are expressed as pmol O_2/s per 10^6 cells.

Calpain measurements. For calpain activity assays, 1×10^4 cells were used and assays were performed with the fluorogenic calpain activity assay kit (Calbiochem). Following incubations, media was removed from cells and assay buffer provided with the kit was added. When inhibitors were used they were present throughout the experiment. Calpain substrate was added according the manufacturer's instructions and fluorescence was monitored using a Mithras fluorescence reader for 45 min (Berthold Technologies, Bad Wildbad, Germany). Excitation was at 355 nm and emission was recorded at 460 nm.

Calcium measurements. RCC4 cells (1×10^5) were seeded in WillCo-dish glass bottom dishes (WillCo Wells BV, Amsterdam, The Netherlands). Calcium measurements were performed essentially as described [32]. Briefly, cells were stained with 1 μM Fura-2AM for 30 min in HEPES buffer (145 mM NaCl, 10 mM HEPES, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$) at 37 °C. Cells were then washed and incubated in media at 37 °C for 15–20 min. Calcium fluctuations were recorded using an Axiowert 200 M fluorescent microscope (Zeiss, Göttingen, Germany). Fluorescence intensities at λ380 nm and λ340 nm are measured and the ratio λ380/λ340 was used to calculate calcium concentrations. For calibration, 5 μM ionomycin was added at the end of each experiment to measure the maximal ratio (Rmax), followed by 5 mM EGTA to obtain the minimal ratio (Rmin). Calcium concentrations were calculated from ratio data using SlideBook software (Intelligent Imaging Innovation, Göttingen, Germany).

Statistical analysis. Each experiment was performed at least three times. Representative data are shown. Data in bar graphs are given as mean values ± SD. Means were checked for statistical differences using the Student's *t*-test with error probabilities of $p < 0.05$ (*).

Results

ROS in the presence of NO destabilize HIF-1α in RCC4 cells. RCC4 cells contain a defective pVHL and, thus, show constitutive expression of HIF-1α under normoxia (Fig. 1a). Exposing RCC4 cells to 0.5 % oxygen or 0.5 mM DETA-NO for 4 h left expression of HIF-1α unaltered. However, when the NO donor was supplied under hypoxia, the protein amount of HIF-1α was lowered, while HIF-1α expression was restored by the calpain inhibitor calpastatin (1 μM). Even though DETA-NO has a very long half-life (~24 h) the effect on HIF-1α is obvious from 2 h onwards (Fig. 1b). The amount of HIF-1α was more drastically reduced at 4 h, with no further alterations after incubation for 8 or 16 h. We therefore chose 4-h incubations, which allowed DETA-NO to be highly effective and to avoid cytotoxic side affects of NO that might emerge with longer incubation periods. Moreover, 0.5 mM DETA-NO produces steady-state concentrations of NO that are roughly 300-fold lower [33, 34]. Therefore, effective NO concentrations in our experiments are around 1 μM, which is a level of NO probably reached under inflammatory conditions.

Previously, we have shown that destabilization of HIF-1α under hypoxia/NO in RCC4 cells was calpain mediated [29]. However, molecular details to explain calpain activation remained elusive. Considering that hypoxia produces ROS, we determined HE oxidation as a marker for ROS production (Fig. 1c). Under hypoxia, HE oxidation increased roughly twofold, was not affected by the presence of NO, but was lowered to some extent by the combination of hypoxia/NO compared to hypoxia alone. Taking into account that potential ROS scavengers might be unspecific, might account for side effects and do not address the source

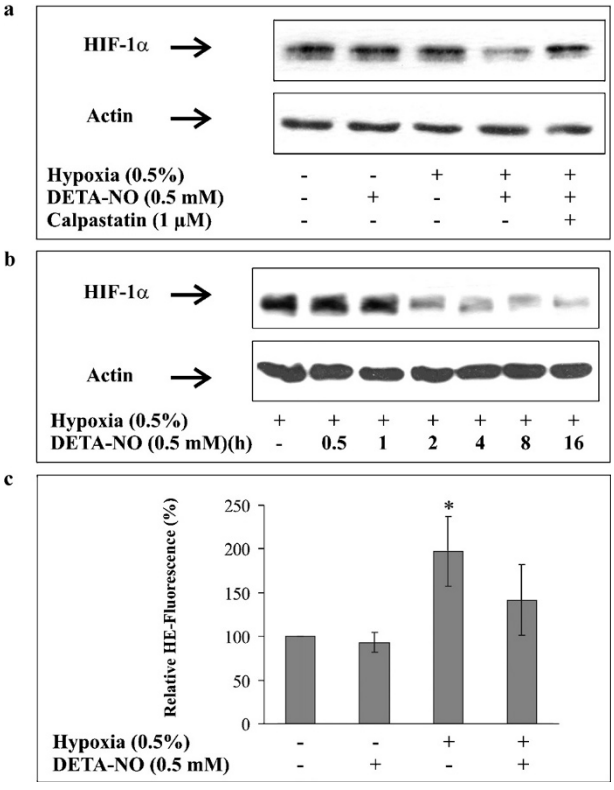

Figure 1. Hypoxia inducible factor-1α (HIF-1α) accumulation and reactive oxygen species (ROS) formation in renal carcinoma cells (RCC4) cells. (*a*) RCC4 cells were exposed to hypoxia (0.5%), treated with 0.5 mM 2,2′-(hydroxynitroso-hydrazono) bis-ethanimine (DETA-NO), a combination of hypoxia/DETA-NO and additionally received 1 μM calpastatin for 4 h, or remained as controls. Calpastatin was preincubated for 30 min. Relative expression of HIF-1α and actin was followed by Western analysis. Results are representative for three individual experiments. (*b*) RCC4 cells were exposed to hypoxia (0.5%) and treated with 0.5 mM DETA-NO for 0.5–16 h. Hypoxic controls were incubated for 4 h. Expression of HIF-1α and actin was followed by Western analysis. Results are representative for three individual experiments. (*c*) Hydroethidine (HE) oxidation was followed by FACS analysis as described in Material and methods. RCC4 cells were treated as specified for (*a*) except the use of calpastatin. Results are presented as mean values ± SD from at least three individual experiments.

of ROS formation, we decided to circumvent potential drawbacks by generating RCC4-ρ0 cells.

Checking the ρ0 status of cells, mRNA expression of a fragment of cytochrome c oxidase subunit II was analyzed by RT-PCR. Cytochrome c oxidase was absent in cells treated with ethidium bromide, which is reminiscent for cells lacking a functional respiratory chain (data not shown). In addition, we followed cellular oxygen consumption (Fig. 2a). RCC4-ρ0 cells had a low respiratory rate compared to wild-type RCC4 cells. Additionally, RCC4-ρ0 cells were not affected by either oligomycin, FCCP or KCN. In contrast, RCC4 cells reacted as expected for cells with a functional mitochondrial chain. Routine state 3-like respiration was measured before addition of 2 μg/ml oligomycin, which arrests mitochondrial respiration at a state 4-like minimal level by inhibiting ATP synthase. After oxygen flux had stabilized, 1 μM FCCP was applied. FCCP, by uncoupling oxidative phosphorylation, elevated respiratory rates. Finally, we blocked respiration with 2 mM KCN.

To confirm that ρ0 cells produced less ROS under hypoxia, we followed MitoSox oxidation to specifically monitor mitochondrial ROS (Fig. 2b). Hypoxia induced relative amounts of MitoSox oxidation, compared to a normoxic control. As expected, ρ0 cells revealed little MitoSox oxidation under hypoxia, which indicates reduced ROS formation. Measurements of HE oxidation corroborated reduced ROS formation in ρ0 cells under hypoxia (data not shown). Functional consequences of mitochondrial ROS production in affecting expression of HIF-1α under hypoxia/NO were then shown by Western analysis (Fig. 2c). Importantly, the decrease of HIF-1α under the influence of hypoxia/NO that was evident in control cells (see Fig. 1a, lane 4) was eliminated in ρ0 cells. To further prove specificity, we used the redox cycling compound DMNQ, which is known to penetrate the plasma membrane and to generate intracellular O_2^- at stoichiometric amounts [20]. Using DETA-NO in combination with DMNQ lowered the amount of HIF-1α in RCC4-ρ0 cells under hypoxia as well as under normoxia (Fig. 2c, lanes 4 and 8), which supports the idea that both ROS and NO and/or NO-derived species are required to lower the protein amount of HIF-1α in RCC4 cells. Neither DETA-NO nor DMNQ alone affected HIF-1α protein amount, under hypoxia or normoxia. This hold for RCC4-ρ0 (Fig. 2c) as well as RCC4 cells (Fig. 1a and below). Treatments of RCC4 with DMNQ under hypoxia also left HIF-1α protein expression unaltered (data not shown). Therefore, the use of RCC4-ρ0 cells argues for mitochondrial generated ROS in achieving HIF-1α destruction under hypoxia in combination with NO.

Oxidizing components down-regulate HIF-1α. It is generally accepted that the coformation of NO and O_2^- produces strong oxidizing components when delivered at appropriate flux rates. The formation of oxidative species when superoxide reacts with NO is highly dependent on the stoichiometry. To demonstrate this effect, we systematically varied the concentrations of both DMNQ (Fig. 3a) or DETA-NO (Fig. 3b) and observed bell-shaped curves that showed degradation of HIF-1α only at defined combinations of DETA-NO/DMNQ.

Using DETA-NO and DMNQ in RCC4 cells revealed identical results compared to their use in RCC4-ρ0

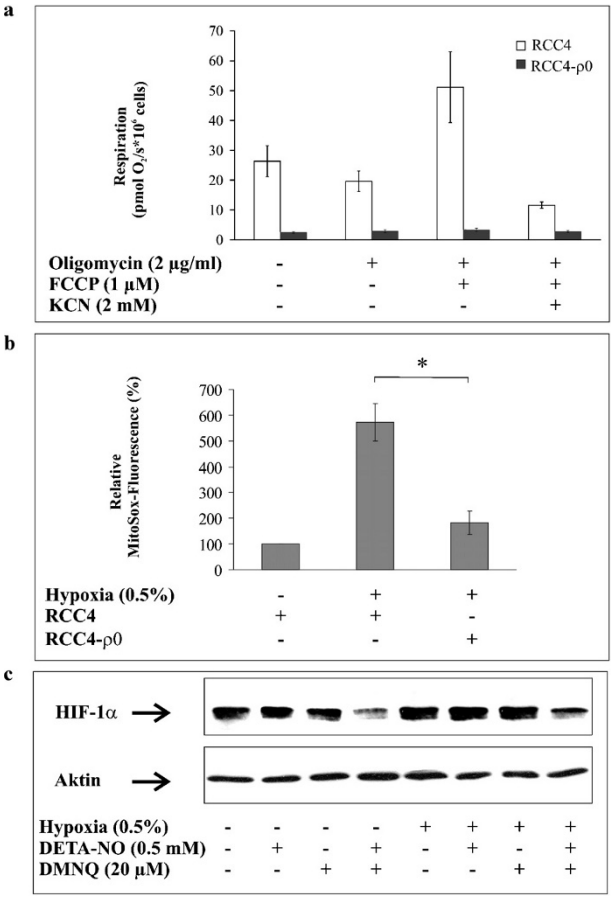

Figure 2. Respiration, ROS formation and HIF-1α accumulation in RCC4-ρ0 cells. (*a*) Cellular respiration of RCC4 and RCC4-ρ0 cells was monitored using an Oxygraph-*2k*. Routine state 3-like respiration was measured before addition of 2 µg/ml oligomycin, which arrests mitochondrial respiration at a state 4-like minimal level. After oxygen flux had stabilized, 1 µM carbonyl-cyanide-*p*-trifluoro-methoxy-phenylhydrazone (FCCP) was supplied. Finally, the respiratory chain was blocked by 2 mM KCN. Results represent mean values ± SD from at least three individual experiments. (*b*) Mitochondrial ROS production was followed by FACS analysis using MitoSox as described under Material and methods. RCC4 *versus* RCC4-ρ0 cells were exposed to 0.5 % oxygen or normoxia for 4 h. Results represent mean values ± SD from at least three individual experiments. Significant alterations are marked with asterisks. (*c*) RCC4-ρ0 cells were exposed to normoxia, treated with 0.5 mM DETA-NO, 20 µM 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), or with a combination of both. Cells exposed to hypoxia, additionally received 0.5 mM DETA-NO, 20 µM DMNQ or DETA-NO/DMNQ for 4 h. Expression of HIF-1α *versus* actin was followed by Western analysis. Results are representative for at least three individual experiments.

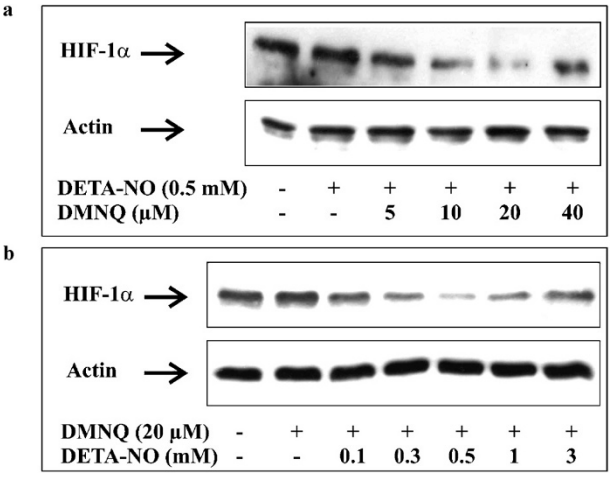

Figure 3. Dose-dependent responses of NO/O_2^- on HIF-1α degradation. (*a*) RCC4 cells were exposed to 0.5 mM DETA-NO and increasing concentrations of DMNQ or (*b*) to 20 µM DMNQ and increasing doses of DETA-NO for 4 h. Protein expression was followed by Western analysis. Results are representative for three individual experiments.

cells in destructing HIF-1α (Fig. 4a). Degradation of HIF-1α by DETA-NO/DMNQ was attenuated by calpastatin, as shown before for hypoxia/DETA-NO (Fig. 1a). In addition, SIN-1, a compound known to deliver NO and O_2^- during its breakdown, dose-dependently decreased HIF-1α expression in RCC4 cells (Fig. 4b). Uric acid, a commonly used antioxidant and putative peroxynitrite scavenger dose-dependently reversed expression of HIF-1α in the presence of SIN-1 (Fig. 4b) or DETA-NO/DMNQ (Fig. 5b). Besides uric acid we also used methionine to inhibit the HIF-1α breakdown and obtained identical results (data not shown).

To check for the potential involvement of oxidizing compounds we used DHR to monitor formation of reaction products derived from NO and O_2^-. A significant increase of DHR oxidation in the presence of SIN-1 or DETA-NO/DMNQ was measured compared to controls. DETA-NO/DMNQ showed a fourfold, and SIN-1 a fivefold induction of DHR oxidation that was blocked by uric acid (Fig. 4c). Controls, using DMNQ or DETA-NO individually, showed no or only minor DHR oxidation. These data suggest that an oxidative process that is sensitive to uric acid accounts for destruction of HIF-1α under conditions in which NO and O_2^- coexist in RCC4 cells.

In other studies it has been noticed that NO under normoxic conditions stabilized HIF-1α, while NO-evoked protein accumulation was antagonized by O_2^-. This can be reproduced in RCC4-pVHL cells, which, based on the RCC4 background, contain a reintroduced and thus functional pVHL (Fig. 5a). Consequently, RCC4-pVHL cells show no HIF-1α under control conditions but express HIF-1α when exposed to DETA-NO. A cotreatment of DETA-NO/DMNQ eliminated the HIF-1α signal. However, in RCC4-pVHL cells, the signal was insensitive to uric acid, while in RCC4 cells degradation of HIF-1α by DETA-NO/DMNQ was reversed by uric acid (Fig. 5b).

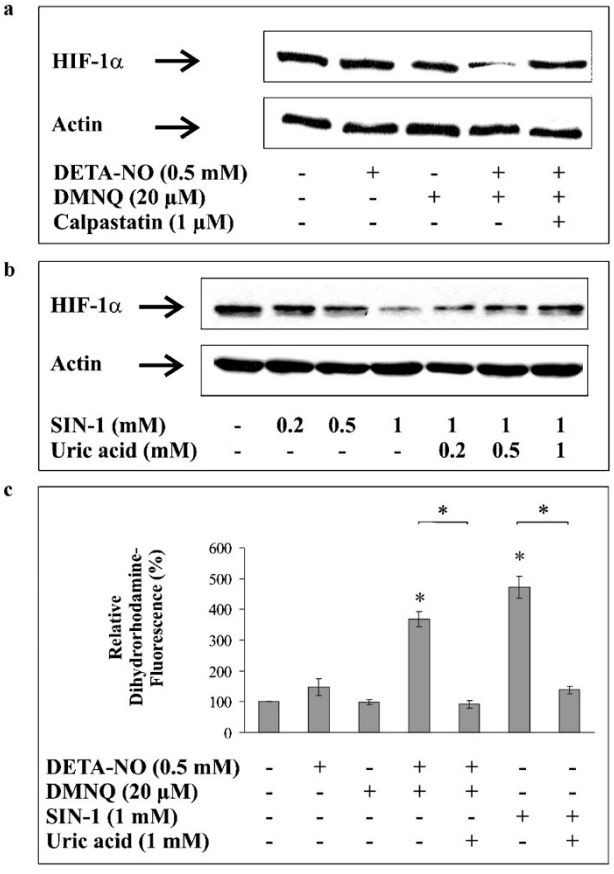

Figure 4. Degradation of HIF-1α in RCC4 cells is facilitated by NO/O_2^--coformation. (*a*) RCC4 cells were exposed to 0.5 mM DETA-NO, 20 μM DMNQ, a combination of DETA-NO/DMNQ or additionally received 1 μM calpastatin. (*b*) Cells are dose-dependently exposed to 3-morpholinosydnonimine hydrochloride (SIN-1) in combination with increasing concentrations of uric acid or remained as normoxic controls. Incubations went for 4 h and protein expression was followed by Western analysis. Uric acid and calpastatin were preincubated for 30 min. Results are representative for three individual experiments. (*c*) DHR oxidation was followed by FACS analysis as described under Material and methods. Cells were exposed to either DETA-NO or DMNQ as well as SIN-1, alone or in combination with uric acid. Uric acid was preincubated for 30 min. Results represent mean values ± SD from at least three individual experiments. Significant alterations are marked with asterisks and refer to normoxic controls, if not indicated differently.

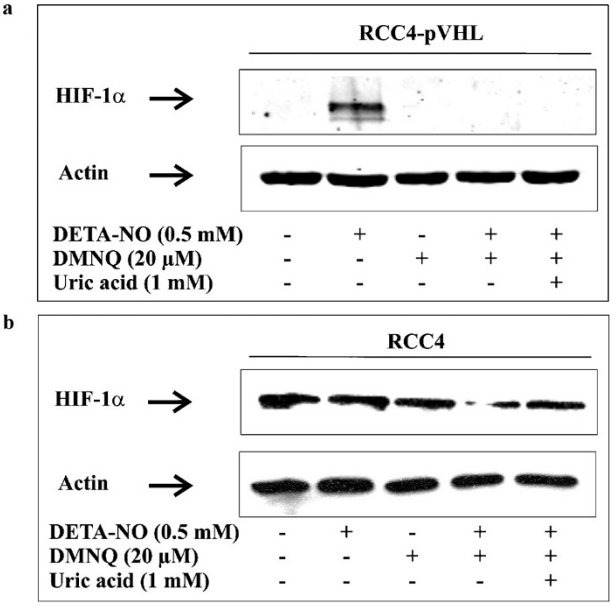

Figure 5. Uric acid has opposing effects in RCC4 *versus* RCC4-pVHL cells. (*a*) RCC4-pVHL cells and (*b*) RCC4 cells were exposed to 0.5 mM DETA-NO, 20 μM DMNQ, combinations of DETA-NO/DMNQ in the presence or absence of 1 mM uric acid, or remained as normoxic controls for 4 h. Uric acid was preincubated for 30 min. Protein expression was followed by Western analysis. Results are representative for three individual experiments.

Activation of calpain and calcium transients in response to NO/O_2^-. In a previous report, we have shown that the ability of hypoxia/DETA-NO to lower HIF-1α protein amount in RCC4 cells was sensitive to inhibition by ALLM and calpastatin, two established calpain inhibitors [29]. We now demonstrated that calpain activity increased twofold in RCC4 cells when exposed to DETA-NO in combination with DMNQ (Fig. 6a). Neither DETA-NO nor DMNQ alone, at concentrations used in this experiment, activated calpain. However, calpain activity was suppressed below controls by calpastatin as well as uric acid.

In analogy, SIN-1 dose-dependently provoked calpain activation. Concentrations of SIN-1 that activated calpain also degraded HIF-1α (see Fig. 4b). Calpain activity was again reduced by calpastatin and uric acid (Fig. 6b). These results suggest that calpain activity is a consequence of NO/O_2^- coformation.

Since activation of calpain is coupled to an intracellular calcium increase, we predicted that DEAT-NO/DMNQ or SIN-1 should evoke calcium transients. Using Fura-2AM, we determined an intracellular resting calcium level of 200–300 nM (Fig. 7a). DETA-NO (0.5 mM) or DMNQ (20 μM) alone did not increase intracellular calcium. A rise in calcium up to 500 nM only occurred using a concentration of 80 μM DMNQ (data not shown). When RCC4 cells were exposed to the combination of DETA-NO/DMNQ for up to 1 h, intracellular calcium increased to 750 nM. SIN-1 caused a more pronounced elevation of calcium to values around 1.5 μM.

Following time-response curves, we noticed that DETA-NO/DMNQ elicited a slow but steadily growing intracellular calcium increase during the first 60 min of incubation (Fig. 7b). Repeating experiments in the presence of uric acid suppressed the calcium increase. SIN-1 produced faster calcium transients compared to DETA-NO/DMNQ towards higher intracellular calcium levels, with slightly declining levels from 30 min onwards (Fig. 7c). However, a SIN-

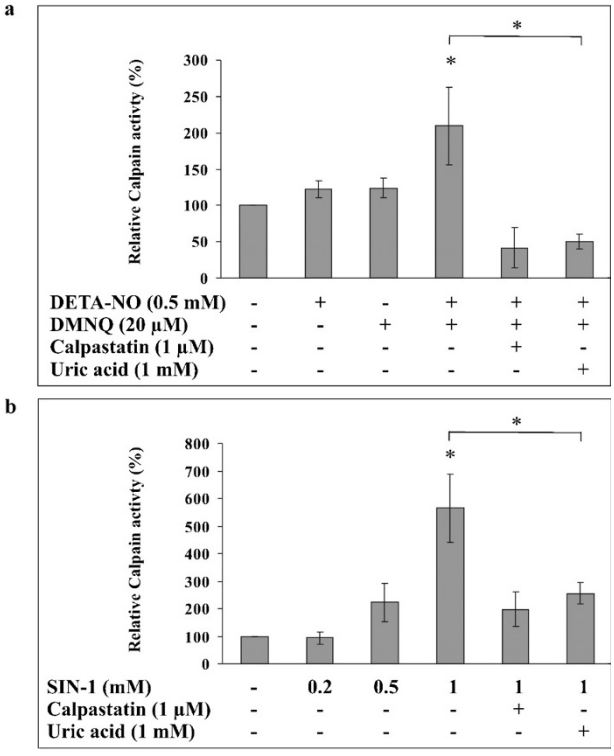

Figure 6. Calpain activity in response to NO/O_2^- or SIN-1. Calpain activity was measured as described under Material and methods. Cells were incubated for 4 h (*a*) with 0.5 mM DETA-NO or 20 µM DMNQ or with the combination of DETA-NO/DMNQ in the presence or absence of 1 µM calpastatin or 1 mM uric acid, or (*b*) with increasing concentrations of SIN-1 in the presence or absence of 1 µM calpastatin or 1 mM uric acid. Controls were cultured under normoxia for 4 h. Calpastatin and uric acid were preincubated for 30 min. Results represent mean values ± SD from at least three individual experiments. Significant alterations are marked with asterisks and refer to normoxic controls, if not indicated differently.

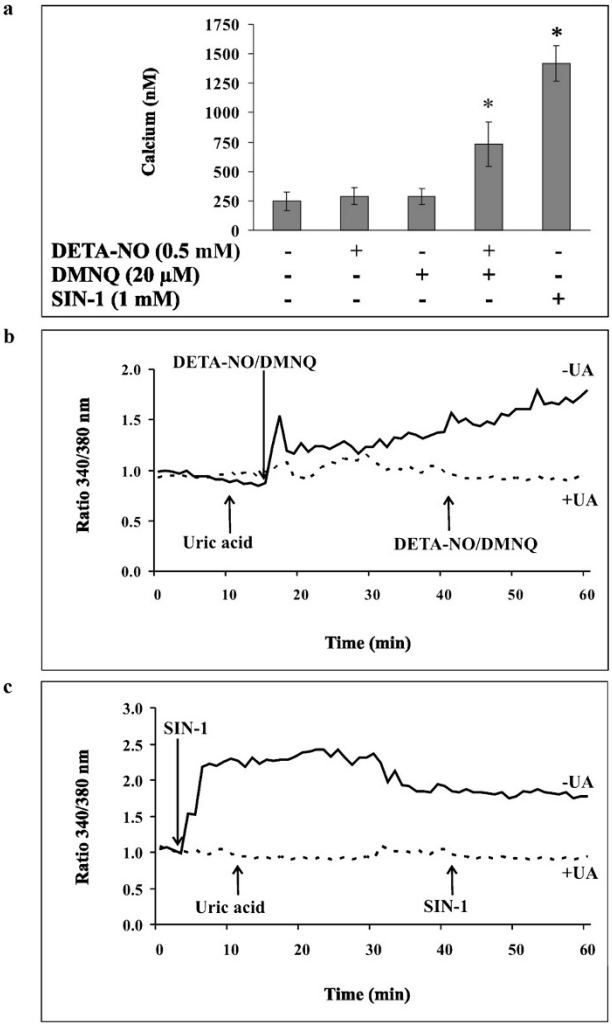

Figure 7. Calcium transients in RCC4 cells in response to NO/O_2^- or SIN-1. Intracellular calcium was measured in RCC4 cells by Fura-2AM as described under Material and methods. (*a*) Absolute calcium values were calculated for RCC4 cells at the point of maximal calcium rise, evoked either by 0.5 mM DETA-NO or 20 µM DMNQ alone or in combination (approximately after 60 min) or 1 mM SIN-1 (approximately after 30 min). Results represent mean values ± SD from at least three individual experiments. Significant alterations are marked with asterisks and refer to normoxic controls. Calcium ratios, representing single cell calcium alteration, were recorded in RCC4 cells over time (*b*) when exposed to 0.5 mM DETA-NO/20 µM DMNQ in the presence or absence of 1 mM uric acid (UA), or (*c*) when exposed to 1 mM SIN-1 in the presence or absence of 1 mM uric acid (UA). Arrows mark times for reagent applications. Data in (*a*) represent mean values ± SD from at least three individual experiments. Graphs in (*b*) and (*c*) are representative for at least three individual experiments.

1-evoked calcium increase was completely antagonized by uric acid. These results imply that NO/O_2^- as well as SIN-1 not only elicited calpain activation but also calcium transients that were sensitive to uric acid.

Discussion

In RCC4 cells, which show constitutive HIF-1α expression due to a deficiency of pVHL, activation of calpain under hypoxia/NO as well as supplementation of DETA-NO/DMNQ under normoxia degraded HIF-1α independently of the proteasome. The use of mitochondrial defective RCC4-ρ0 cells pointed to the formation and reaction of superoxide or superoxide-derived species with NO. As schematically outlined in Figure 8, concomitant to the formation of NO and O_2^- we noticed a uric acid-sensitive oxidative response and an intracellular calcium increase that were required for calpain activation.

Oxygen deficiency attenuates PHD activity, which in turn stabilizes HIF-1α by blocking 26S proteasomal degradation [35, 36]. In some analogy, NO stabilizes HIF-1α by interfering with its degradation [18, 19]. However, under hypoxia NO attenuates hypoxic signaling [22, 23]. Presumably, NO blocks mitochondrial respiration, thus leaving more oxygen available

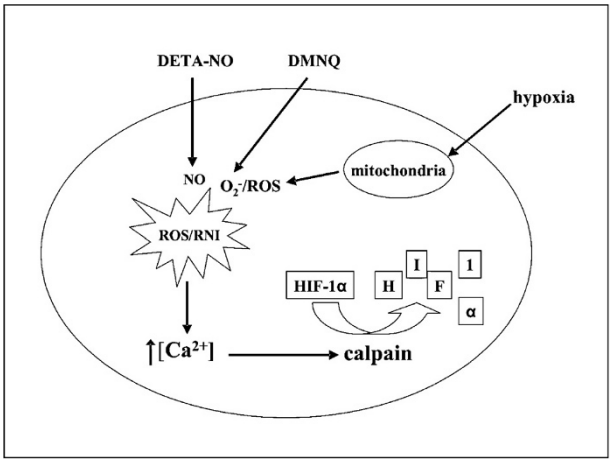

Figure 8. Overview on HIF-1α degradation by NO and O_2^- in RCC4 cells. In von Hippel Lindau protein (pVHL)-deficient cells O_2^-, delivered either by DMNQ or produced by mitochondria under hypoxia, reacts with NO to increase calcium that in turn stimulates calpain to destruct HIF-1α independently of the proteasome. Details are given in the text. In contrast, in cells containing a functional pVHL protein, the reaction of NO with O_2^- also attenuates accumulation of HIF-1α, which requires lowering of steady-state NO concentrations (for references see [20, 21]).

for non-respiratory reactions such as PHD catalysis [37]. Alternatively, NO might directly stimulate PHD activity or enhance enzyme activity by mobilizing cofactors such as iron to support substrate hydroxylation [38]. Irrespective of mechanistic details, these concepts require a functional PHD enzyme to degrade HIF-1α *via* the proteasome. The situation is different in RCC4 cells because a functional proteasome destruction pathway for HIF-1α is missing. Recently, we suggested that HIF-1α is degraded in RCC4 cells under hypoxia/NO *via* the calpain system [29]. Evidence was provided using calpain inhibitors or chelating intracellular calcium and by coprecipitating calpain and HIF-1α. Here, we explored molecular details to understand calpain activation.

Although ROS formation under hypoxia is controversial, more recent evidence indicates that a burst of ROS under hypoxia stabilized HIF-1α [12, 39]. Considering that the presence of NO alters O_2^- signaling capabilities due to their diffusion-controlled reaction prompted us to consider O_2^- formation under hypoxia. A role for O_2^- in altering the amount of HIF-1α in combination with NO was supported by several findings. First, O_2^- formation was detected by following HE and MitoSox oxidation under hypoxia; second, MitoSox oxidation under hypoxia was eliminated in ρ0 cells; third, HIF-1α degradation under hypoxia/NO was not seen in ρ0 cells; and fourth, O_2^- generated by the redox-cycler DMNQ restored the ability of NO to degrade HIF-1α in ρ0 cells under normoxia. The ability of NO/O_2^- to reduce the HIF-1α protein amount was not only seen in RCC4-ρ0 but also occurred in RCC4 cells. Formation of oxidative species derived from DETA-NO/DMNQ, which provoked degradation of HIF-1α in RCC4 cells, occurred only at defined ratios of NO *versus* O_2^-. We and others have shown in pVHL-containing cells that NO and O_2^- work in concert, each coregulating the concentration of the other [20, 21]. The formation of O_2^- during NO generation resulted in a concomitant increase in oxidative intermediates, with a decrease in steady-state NO concentrations and a proportional reduction in the level of HIF-1α, due to a regained PHD activity [21]. Thomas et al. [21] noticed that the intermediates formed from the reaction of NO with O_2^- were nontoxic and did not form 3-nitrotyrosine, a commonly appreciated footprint of peroxynitrite. They assumed that the combination of NO and O_2^- at various flux rates alters the cellular phenotype by attenuating bioactive NO or O_2^-, with no signal transduction responses due to their interaction. In our experimental setting, HIF-1α was stabilized in RCC4-pVHL cells by DETA-NO. As expected, the combination of NO/O_2^- abrogated NO-mediated HIF-1α stabilization. Elimination of the HIF-1α signal was insensitive to uric acid, because this degradation was not due to oxidative intermediates formed during the interaction of NO with O_2^-, rather reflecting the loss of bioavailable NO due to the reaction with O_2^-. In contrast, degradation of HIF-1α *via* calpain, which would be activated by NO/O_2^- reaction products, becomes obvious only under conditions where classical, *i.e.*, proteasomal, degradation of HIF-1α was attenuated. Since HIF-1α degradation *via* the proteasome normally keeps the protein half-life below 5 min [2, 40], it is difficult to study the involvement of other destructive systems unless the proteasome is impaired, as occurs in some tumors. Downstream signaling as a consequence to the NO/O_2^- interaction in RCC4 is antagonized by uric acid or methionine. These data, in combination with the notion that SIN-1 degrades HIF-1α, imply that oxidative intermediates provide the signal that is needed to increase calcium. Supporting experiments showed that uric acid successfully prevented a calcium increase in response to SIN-1 or DETA-NO/DMNQ and therefore attenuated calpain activation. Even though degradation of HIF-1α by hypoxia/NO was also reversible by uric acid (data not shown), we could not see a calcium increase, probably due to experimental difficulties to follow calcium transients under hypoxia. The use of SIN-1, a proposed $ONOO^-$ generating compound, and the interference by uric acid, a defined $ONOO^-$ scavenger, may imply that indeed peroxynitrite is involved in signal transmission. However, as in the study of Thomas et al. [21] we were unable to detect 3-nitrotyrosine by

Western analysis. Quantitatively, the effect of $ONOO^-$ on its footprint 3-nitrotyrosine may be negligible compared to the bioregulatory and/or oxidative aspects. Although 3-nitrotyrosine remained undetectable, we followed oxidation of DHR, which is used to monitor oxidative intermediates, among others $ONOO^-$ [41]. Only DETA-NO/DMNQ and SIN-1 elevated DHR oxidation signals, but neither DETA-NO nor DMNQ alone. It is known that $ONOO^-$ and/or oxidative modifications stimulate Ca^{2+} release from mitochondria and that the combination of NO with ROS provokes calpain activation [42, 43]. Although NO itself affects calcium signals [44, 45], the concentration of the NO donor used in our experiments did not elevate intracellular calcium by itself. Without exact identification of the oxidative species involved, we know that DHR oxidation depends on the stoichiometry of NO *versus* O_2^- and that its oxidation can be scavenged using uric acid and methionine.

Despite the fact that calpain degrades HIF-1α under hypoxia/NO [29], we only see a rise of calcium when using DETA-NO/DMNQ or SIN-1. Activation of calpain may take place under hypoxia/NO, even if a calcium rise is not detectable. Calpains may bind to phospholipids at the plasma membrane thereby lowering the calcium requirement for their activation [46], although the binding of calpain to lipid bilayers itself is calcium dependent [47]. Other studies show that m-calpain expression and activity is up-regulated *via* the ERK/MAPK pathway [48]. Therefore, one of our future tasks will be to elucidate molecular mechanisms of calpain activation under hypoxia/NO. Stabilization of HIF-1α under normoxia by NO requires a constant NO exposure and, therefore, is sensitive to temporal O_2^- scavenging effects [17, 18, 21]. This suggests that in pVHL-containing cells PHD activity will immediately be affected by acute changes in the redox environment, which allows recovering hydroxylation of HIF-1α and its degradation. In pVHL-deficient RCC4 cells redox alterations result in calpain activation to destruct HIF-1α independently of the proteasome. It is known that cells differ in their sensitivity towards strong oxidizing conditions resulting from NO/O_2^- coformation, which largely is based on variations in the glutathione redox systems [49]. Although some of our experiments were reproduced in HEK293 cells (data not shown), it is too early to generalize our results. It seems that under conditions when proteasomal degradation is blocked, oxidative changes as a result of NO/O_2^- formation degrade HIF-1α. It remains open whether NO/O_2^- coformation consistently results in calpain activation or whether RCC4 cancer cells are particularly sensitive to oxidative stress and a subsequent calcium release. Our results raise the intriguing possibility to use NO under hypoxia to degrade HIF-1α in cancer cells. Expression of HIF-1α in tumors is often associated with resistance to radiotherapy, *e.g.*, in renal clear carcinomas. Destruction of HIF-1α by NO under hypoxia *via* the calpain system might help to understand how NO increases chemosensitivity [50, 51].

Furthermore, our studies demonstrate profound effects of O_2^- on NO signaling. This appears of relevance not only when NO is generated under hypoxia, but also when NO/O_2^- are cogenerated under normoxia under, for example, inflammatory conditions. These considerations help to increase our knowledge on the fine tuning of HIF-1 responses under physiological and/or pathological conditions associated with radical, i.e., NO/O_2^-, formation.

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