

Thioredoxin 1 and Thioredoxin 2 Have Opposed Regulatory Functions on Hypoxia-inducible Factor-1 α *

Received for publication, August 30, 2006, and in revised form, January 8, 2007. Published, JBC Papers in Press, January 12, 2007, DOI 10.1074/jbc.M608289200

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Hypoxia inducible factor 1 (HIF-1), a key regulator for adaptation to hypoxia, is composed of HIF-1 α and HIF-1 β . In this study, we present evidence that overexpression of mitochondria-located thioredoxin 2 (Trx2) attenuated hypoxia-evoked HIF-1 α accumulation, whereas cytosolic thioredoxin 1 (Trx1) enhanced HIF-1 α protein amount. Transactivation of HIF-1 is decreased by overexpression of Trx2 but stimulated by Trx1. Inhibition of proteasomal degradation of HIF-1 α in Trx2-overexpressing cells did not fully restore HIF-1 α protein levels, while HIF-1 α accumulation was enhanced in Trx1-overexpressing cells. Reporter assays showed that cap-dependent translation is increased by Trx1 and decreased by Trx2, whereas HIF-1 α mRNA levels remained unaltered. These data suggest that thioredoxins affect the synthesis of HIF-1 α . Trx1 facilitated synthesis of HIF-1 α by activating Akt, p70S6K, and eIF-4E, known to control cap-dependent translation. In contrast, Trx2 attenuated activities of Akt, p70S6K, and eIF-4E and provoked an increase in mitochondrial reactive oxygen species production. MitoQ, a mitochondria specific antioxidant, reversed HIF-1 α accumulation as well as Akt activation under hypoxia in Trx2 cells, supporting the notion of translation control mechanisms in affecting HIF-1 α protein accumulation.

Hypoxia inducible factor-1 (HIF-1)² is composed of HIF-1 α and HIF-1 β and senses low oxygen availability to coordinate patho-physiological responses (1, 2). Under normoxia, HIF-1 α protein is continuously degraded via the 26S proteasome, whereas HIF-1 β is constitutively present. Hypoxia attenuates hydroxylation of HIF-1 α at proline 564 and/or 402. Subsequently, this blocks binding of the von Hippel-Lindau protein

(pVHL) and ubiquitination. As a result, proteasomal destruction is impaired and HIF-1 α accumulates. Alternatively, increased translation (3) and possibly transcription (4) emerged as regulatory mechanisms of HIF-1 α . Phosphatidylinositol 3 kinase (PI3K)/Akt-dependent and/or mitogen-activated protein kinase (MAPK) signaling pathways phosphorylate components required for cap-dependent translation such as p70S6 kinase (p70S6K) or eukaryotic initiation factor-4E (eIF-4E) and thus provoke accumulation of HIF-1 α in response to growth factors, hormones, or cytokines (1).

Thioredoxins (Trx) compose a class of small multifunctional 12-kDa proteins containing two cysteine residues in the Trp-Cys-Gly-Pro-Cys motif that are reduced from the oxidized (inactive) form by the flavoenzyme thioredoxin reductase (TrxR) and NADPH. Cells are equipped with two thioredoxin species; Trx1 is present in the cytosol, while Trx2 is located in mitochondria (5). Trx1 regulates apoptosis by interacting with signal-regulating kinase 1 (6), protects against oxidative stress (7), and modulates the transcriptional and DNA binding activity of the glucocorticoid (8) and estrogen receptors (9), AP-1 (10, 11), or p53 (12, 13).

Overexpression of Trx1 stimulated HIF-1 activity (14, 15) and provoked an HIF-1 α increase under normoxia as well as hypoxia (16). In addition, attenuating thioredoxin reductase activity blocked HIF-1 transactivation (17). Moreover, Trx1 may be involved in HIF-1 α degradation during reoxygenation (18), perhaps pVHL-mediated (19) but molecular details for understanding the interplay between Trx1 and HIF remain unclear.

Incomplete reduction of dioxygen in mitochondria during respiration results in the formation of reactive oxygen species (ROS). Increased levels of ROS may provoke lipid peroxidation, inactivation of proteins and DNA strand breakage. However, ROS also contribute to signaling by affecting protein kinases and phosphatases, thus regulating gene expression and controlling cell proliferation and death (20). Considerable evidence suggests that mitochondria-derived ROS during hypoxia might control activation of HIF-1 α (21, 22). The mitochondrial redox environment depends on both total cellular redox environment and compartmentalized mitochondrial reduction capacity (23). The reductive capacity depends on the concentration of electron donors such as NADPH, NADH, and glutathione that may act as an antioxidant buffers as well as specific ROS detoxification enzymes including mitochondrial thioredoxin-2. Little is known on the function(s) of the mitochondrial thioredoxin. Recent studies show that Trx2 is essential for mitochondria-dependent apoptosis (24) but also for cell growth and mammalian

* This work was supported by grants from Deutsche Forschungsgemeinschaft (BR999), Sander Foundation, Deutsche Krebshilfe, European Union (PROLIGEN), and the Swedish Research Council (13X-10370). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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² The abbreviations used are: HIF-1, hypoxia inducible factor 1; pVHL, von Hippel-Lindau protein; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; MAPK, mitogen-activated protein kinase; p70S6K, p70 S6 kinase; eIF-4E, eukaryotic initiation factor-4E; Trx, thioredoxin; ROS, reactive oxygen species; MG132, Z-Leu-Leu-Leu-al; HEK, human embryonic kidney cell; COXI, cytochrome c oxidase subunit I; HRE, hypoxia-responsive element; PBS, phosphate-buffered saline.

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development (25). Overexpression of Trx2 enhanced mitochondrial membrane potential and protected HEK-293 cells against etoposide-mediated cytotoxicity (26).

In an attempt to study whether or not Trx2 affects hypoxic signaling we used cells that stably express either Trx1 or Trx2. Herein, we found that mitochondria-located Trx2, in contrast to cytosolic located Trx1, decreased HIF-1 α protein levels and HIF-1 transactivation. The contrasting actions of Trx1 *versus* Trx2 do not result from altered transcription or degradation of HIF-1 α but rather affect cap-dependent translational control mechanisms in association with altered phosphorylation and thus activities of Akt, p70S6K, eIF-4E, and 4E-BP1, as well as ROS production.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of the highest grade of purity and commercially available. Medium and supplements were purchased from PAA (Linz, Austria). FCS was from Biochrom (Berlin, Germany). Z-Leu-Leu-Leu-al (MG132) and anti-actin antibody were ordered from Sigma (Schnellendorf, Germany). Nitrocellulose membrane, ECLTM detection system and horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies were delivered by GE Healthcare (Munich, Germany). The mitochondrial specific antioxidant MitoQ was a gift from Dr. Michael Murphy (Medical Research Council Dunn Human Nutrition Unit, Cambridge, UK). Trx1 antibodies were from IMCO (Stockholm, Sweden). Trx2 antibodies were prepared as described (27). Antibodies to phospho-p70S6K (Thr-421/Ser-424), p70S6k, phospho-eIF-4E and eIF-4E came from Cell Signaling Technology (Beverly, MA). Anti-HIF-1 α antibody was purchased from BD Biosciences (Heidelberg, Germany). Anti-Bcl-2 anti-phospho-4E-BP1, anti-4E-BP1, and anti-luciferase antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cytochrome *c* oxidase subunit I (COXI) antibody came from Invitrogen (Carlsbad, CA). Primers were ordered from MWG-Biotech (Ebersberg, Germany). The plasmid pGLEPOHRE harboring three erythropoietin hypoxia-responsive elements (HREs) was provided by Dr. T. Kietzmann (Technical University of Kaiserslautern, Kaiserslautern, Germany). Reporter plasmid cap-Luc and luciferase activity assay kit were supplied by Promega (Mannheim, Germany). Plasmids pCMV5, pCMV5.-m/p-PKB α (pAkt), and pCMV5.-m/p-PKB α K179A (pAkt^{dn}) were given by Dr. B. Hemmings (F. Miescher Institute, Basel, Switzerland).

Cell Culture—Human embryonic kidney (HEK-293) cells are transfected with empty plasmid (HEKi) or plasmids encoding Trx1 or Trx2 as described (26). Cells were cultured in Dulbecco's modified eagle medium with 4.5 g/liter D-glucose, 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin and 500 μ g/ml G418. Cells were kept in a humidified atmosphere of 5% CO₂ in air at 37 °C or exposed to hypoxia in a Ruskinn work station. Cells were maintained in medium without G418 for 24 h prior to experiments.

Western Blot Analysis—1 \times 10⁶ cells were seeded in 6-cm dishes 1 day prior to experiments. After treatments, cells were scraped off, lysed in 150 μ l of WB-lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, protease inhibitor mixture, pH 7.5), and sonicated. Following centrifugation

(15,000 \times g, 15 min) the protein content was determined in the supernatants by a protein assay kit (Bio-Rad, Munich, Germany), and 100 μ g protein was added to the same volume of 2 \times SDS-PAGE sample buffer (125 mM Tris/HCl, 2% SDS, 10% glycerol, 1 mM dithiothreitol, 0.002% bromophenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Non-specific binding sites were blocked with 5% (w/v) defatted milk powder in TTBS (50 mM Tris/HCl, 140 mM NaCl, 0.05% Tween 20, pH 7.2) for 1 h. Primary antibodies (1:1000 in 1% milk/TTBS or 5% bovine serum albumin/TTBS) were added and incubated overnight at 4 °C. Afterward, nitrocellulose membranes were washed three times for 5 min each with TTBS. Blots were then incubated with goat anti-mouse or goat anti-rabbit secondary antibodies conjugated with peroxidase (1:2000 in 1% milk/TTBS) for 1 h, washed three times for 5 min each with TTBS, followed by ECLTM detection (GE Healthcare).

Cell Transfection—Cells were transfected with reporter plasmids using calcium-phosphate precipitation. Briefly, plasmids in the presence of 125 mM CaCl₂ and HBS buffer (25 mM Hepes, 140 mM NaCl, 0.75 mM Na₂HPO₄, pH 7.05) were incubated for 15 min at room temperature and added dropwise to cells. 16 h later medium was changed and incubations continued for another 8-h period.

Mitochondria Fractionation and Proteinase K Sensitivity Assays—Cells were homogenized and subjected to subfractionation according to the procedure from Psarra *et al.* (28). Briefly, cells grown on 150-mm dishes were washed twice with ice-cold PBS, harvested in homogenization buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose) with addition of a protease inhibitors mixture (Roche Diagnostics, Mannheim, Germany). Homogenization was performed at 4°C with 20 strokes of a glass Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged for 5 min, at 1000 \times g in a Sorvall centrifuge using a SS-34 rotor. The supernatant (S1) was used for the preparation of the mitochondria. The S1 fraction was further centrifuged at 10,000 \times g for 20 min to give the crude mitochondrial pellet. The resulting supernatant was centrifuged at 100,000 \times g for 1 h to obtain the cytosol. The crude mitochondrial pellet was washed twice with mitochondrial wash buffer (20 mM Tris, pH 7.5, 0.07 M sucrose, 0.21 M mannitol, 2.5 mM EDTA, 2.5 mM EGTA) and resuspended in the same buffer. The mitochondrial fraction was treated for 20 min with proteinase K (0.2 or 1 mg/ml), followed by centrifugation at 12,000 \times g. Pellets were washed three times in mitochondrial wash buffer and boiled in 1 \times SDS-PAGE sample buffer.

Immunofluorescence—Cells were seeded on coverslips treated with poly-L-lysine. After 2 days, cells were fixed in 3.7% paraformaldehyde for 30 min at room temperature. Cells were permeabilized in PBST (PBS with 0.1% Tween 20) and then blocked for 1 h in PBST, 5% goat serum. Subsequently, slides were incubated with either anti-Trx1 or anti-Trx2 for 1 h in PBST. After washing 3 \times 10 min with PBST, slides were incubated with anti-rabbit Alexa 488 (Invitrogen, Karlsruhe, Germany) for 1 h, washed 3 \times 10 min, and mounted onto glass slides using Fluorosave (Merck Biosciences, Darmstadt, Ger-

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many). Images were acquired using a Leica ASMDW microscope equipped with a 63 \times objective. Captured z stacks were deconvolved using Leica Deblur.

Quantitative Real-time Reverse Transcription PCR— 2×10^6 cells were seeded in 10-cm dishes 1 day prior to experiments. The following day medium was changed and cells were treated as indicated. Total RNA was isolated using the peqGOLD RNA-Pure kit (Peqlab, Erlangen, Germany). The reverse transcription was completed with a iScript cDNA synthesis kit (Bio-Rad). The following primer pairs were selected for quantitative real time PCR: human HIF-1 α forward, 5'-CTC AAA GTC GGA CAG CCT CA-3'; human HIF-1 α backward, 5'-CCC TGC AGT AGG TTT CTG CT-3'; human Trx1 forward, 5'-CCT TTC TTT CAT TCC CTC TCT GA-3'; human Trx1 backward, 5'-GCA ACA TCC TGA CAG TCA TCC A-3'; human Trx2 forward, 5'-CTG GTG GCC TGA CTG TAA CAC-3'; human Trx2 backward, 5'-GTT GAC CAC TCG GTC TTG AAA-3'; human actin forward, 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'; human actin backward, 5'-CTA GAA GCA TTT GCG GTC GAC GAT GGA GGG-3'.

The quantitative real-time PCR was performed by MyiQ (Bio-Rad). Reaction mixtures containing SYBR Green (Abgene, Hamburg, Germany) were composed according to the manufacturer's protocol. The cycling program was: 50 $^{\circ}$ C, 2 min; 95 $^{\circ}$ C, 15 min; followed by 35 cycles at 95 $^{\circ}$ C, 15 s; 55 $^{\circ}$ C, 30 s; 72 $^{\circ}$ C, 30 s. Values of HIF-1 α were then normalized to the relative amounts of actin.

Reporter Assay—The reporter plasmid-transfected cells were treated as indicated for an additional 16-h period. Then, cells were harvested and lysed. Luciferase activities were measured using commercial kits (Promega, Mannheim, Germany).

35 S-Radioisotopic Labeling—Cells were starved for 1 h in serum- and methionine-free medium, followed by replacement with methionine-free medium containing 10% FCS and 100 μ Ci/ml [35 S]methionine. Cells were then incubated under hypoxia for 4 h. Afterward, cells were washed with PBS and lysed with IP lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture, pH 7.5). Following centrifugation (15,000 \times g for 30 min) supernatants were transferred to fresh tubes. Supernatants, containing 1 mg of protein each, were supplied with 1 μ g of anti-luciferase antibody and incubated at 4 $^{\circ}$ C for 1 h. Thereafter, 50 μ l of protein A microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added and incubations continued at 4 $^{\circ}$ C overnight. Beads were magnetically collected, following the instructions provided by the manufacturer and washed three times with 100 μ l of IP lysis buffer. Co-precipitated proteins were finally eluted by 95 $^{\circ}$ C preheated 1 \times SDS-PAGE sample buffer and separated by 10% SDS-PAGE. Gels were dried and exposed to x-ray films.

Measurement of Mitochondrial ROS—Mitochondrial ROS were measured by flow cytometry in cells loaded with the dye MitoSOX. 1×10^6 cells were seeded in 6-cm dishes 1 day prior to experiments. Hypoxic incubations were carried out in the Ruskinn working station. Cells cultured under normoxia or exposed to hypoxia were loaded with 5 μ M MitoSOX 30 min before completing incubations. Subsequently, cells were washed three times with medium, harvested, and resuspended

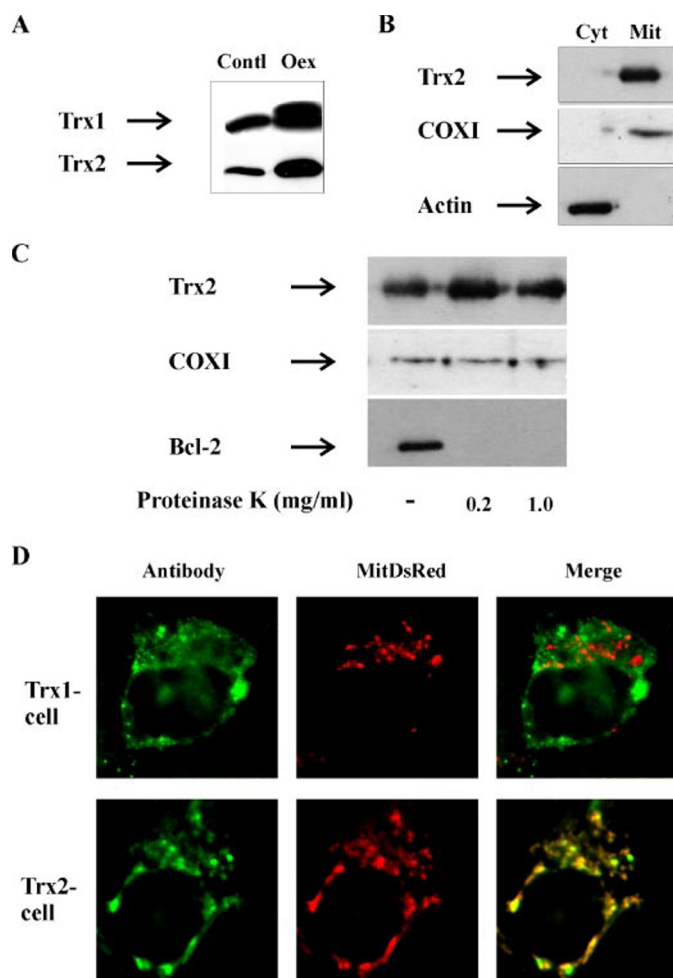


FIGURE 1. Characterization of Trx1- and Trx2-overexpressing cells. Overexpression (Oex) of Trx1 and Trx2 in stably transfected HEK cells was verified by Western analysis compared with control (Contl) cells (A). HEK cell fractionation toward the cytosol versus mitochondria (10 μ g of each fraction) was analyzed by Western blotting using Trx2, COX1, and actin antibodies (B). The mitochondrial fraction with or without prior proteinase K (0.2 or 1 mg/ml) treatment was analyzed by Western blotting for Trx2, COX1, and Bcl-2 (C). Cells were further characterized by immunofluorescence and thus localization of Trx1 and Trx2 (D). Cells are stained with Trx1 or Trx2 antibodies, respectively; mitochondria are highlighted with MitDsRed. Data are representative for three similar experiments.

in PBS under normoxic or hypoxic conditions. Fluorescence was recorded on the PE channel of a BD FACSCanto flow cytometer (BD Biosciences). 1×10^4 cells were counted for analysis. The mean values of the fluorescence in the samples were recorded and normalized to control cells.

Statistical Analysis—Each experiment was performed at least three times and representative data are shown. Data in bar graphs are given as means \pm S.E. Means were checked for statistical differences by using the Student's *t* test with an error probability of $p < 0.05$ (* on Figs. 2, 4, and 7).

RESULTS

HIF-1 α Accumulation and HIF-1 Activity under the Control of Trx2 Versus Trx1—Considering that cytosolic Trx1 enhanced HIF-1 α stabilization, we determined the impact of mitochondrial Trx2. We used HEK cells, stably transfected with Trx1, Trx2, or with a control vector (HEKi). Characterization of cells by Western blotting confirmed overexpression of Trx1 or

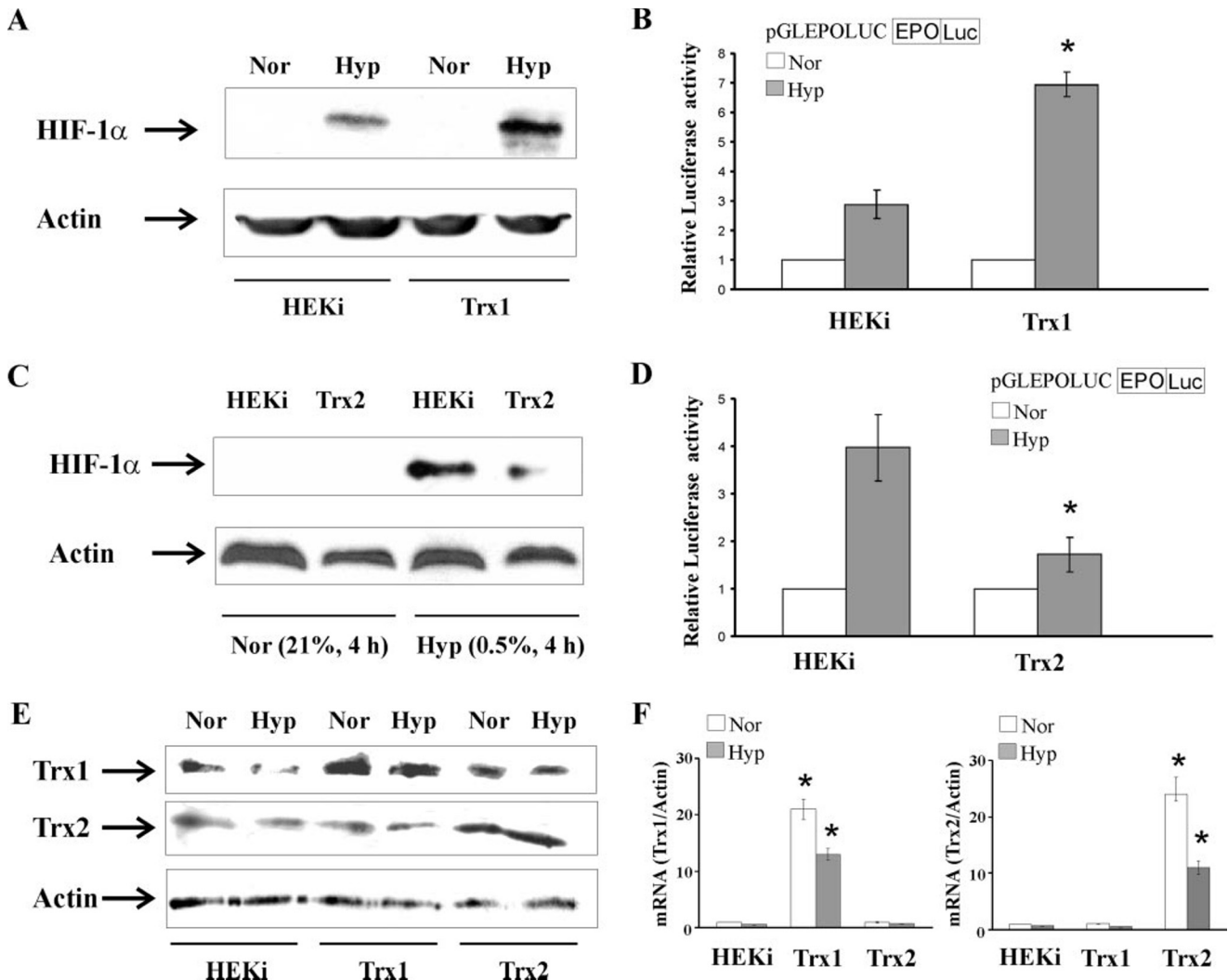


FIGURE 2. Expression of HIF-1 α in Trx1- or Trx2-overexpressing cells. HEK cells transfected with empty plasmid (*HEKi*) and cells overexpressing Trx1 (*Trx1*) or Trx2 (*Trx2*) were exposed to 0.5% hypoxia for 4 h followed by detection of HIF-1 α , Trx1, Trx2, and actin by Western analysis (A, C, and E). Cells were transfected with pGLEPOLUC reporter plasmid and then incubated under normoxia (*empty columns*) or 0.5% hypoxia (*filled columns*) for 16 h. Following cell lysis luciferase activities were measured and normalized to controls (B and D). The mRNA content of Trx1 or Trx2 are detected by real-time quantitative PCR (F). Blots are representative of three independent experiments. Luciferase activity data and relative mRNA levels are the mean \pm S.E. of at least three independent experiments. *, $p < 0.05$.

Trx2 (Fig. 1A). To demonstrate that Trx2 in HEK-Trx2 cells is indeed mitochondrial located and not simply associated with the organelle, mitochondria were separated from the cytosol followed by detection of Trx2. Trx2 only appeared in the mitochondrial fraction similarly to COXI (Fig. 1B). The mitochondrial fraction was then treated with proteinase K, a nonspecific serine protease that cannot penetrate the mitochondrial membrane. Both Trx2 and COXI were detected in the proteinase K-resistant compartment of mitochondria (Fig. 1C). In contrast, Bcl-2, which is a surface mitochondrial protein, was completely proteolyzed. The resistance of Trx2 to proteinase K digestion indicates that it is a genuine resident of mitochondria in HEK-Trx2-overexpressing cells. Furthermore, immunofluorescence proved cytosolic localization of Trx1 and mitochondrial localization of Trx2 (Fig. 1D). Antibody staining in control cells gave much weaker signals for both Trx1 and Trx2 but showed a similar compartmentalization of endogenous proteins compared with overexpressed proteins.

Following their exposure to hypoxia (0.5% O₂) for 4 h, HIF-1 α accumulated in HEKi cells (Fig. 2A) but showed a stronger response in Trx1 transfectants. To investigate the transcriptional activity of HIF-1, we employed a HRE-based reporter system. HER reporter activity in control cells (HEKi) was further enhanced in Trx1 expressing cells (Fig. 2B), while basal activity was not significantly changed. Unexpectedly, in Trx2-overexpressing cells, HIF-1 α accumulation under hypoxia was significantly reduced compared with controls, although minor amounts were still detectable (Fig. 2C). Moreover, the Epo-Luc reporter was stimulated by hypoxia in control cells but showed reduced activity in Trx2-overexpressing cells (Fig. 2D). To confirm the expression of Trx1 and Trx2 in these cells under normoxic *versus* hypoxic conditions, we performed Western analysis to detect Trx1 and Trx2 protein and went on to quantify Trx1/Trx2 mRNA amount by real-time PCR. The protein amount and mRNA content of Trx1 and Trx2 remained elevated in Trx1/Trx2-

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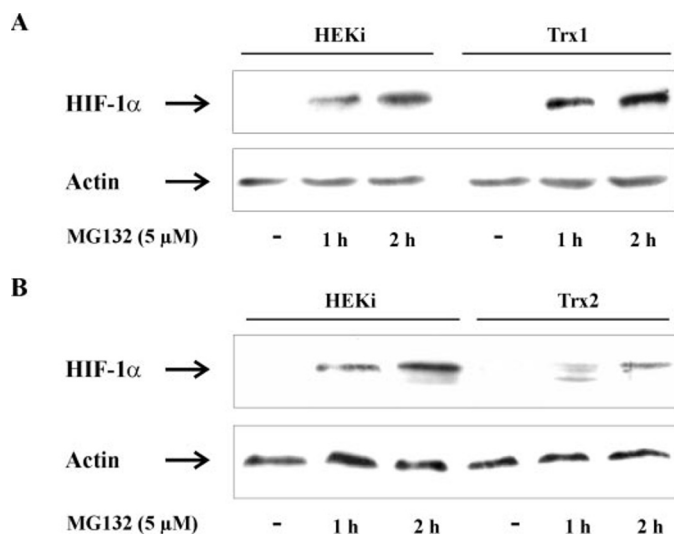


FIGURE 3. HIF-1 α degradation under the control of Trx1/Trx2. HIF-1 α was determined in HEK cells transfected with empty plasmid (HEKi, A and B) and cells overexpressing Trx1 (Trx1, A) or Trx2 (Trx2, B). Cells were incubated with 5 μ M MG132 for 1 and 2 h. HIF-1 α and actin were determined by Western analysis. Blots are representative of three independent experiments.

overexpressing cells under both normoxia and hypoxia (Fig. 2, E and F). Although Trx1/Trx2 protein amount was comparable under normoxia and hypoxic conditions, the mRNA content of Trx1 as well as Trx2 were equally down-regulated under hypoxic conditions. We conclude that Trx2 attenuates the expression of HIF-1 α protein as well as HIF-1 activity. The next step aimed at explaining the contrasting behavior of Trx1 versus Trx2 on HIF regulation.

Trx1 and Trx2 Affect HIF-1 α Protein Synthesis Rather than Degradation—Regulation of HIF-1 α protein may be achieved by enhancing protein synthesis and/or blocking degradation. We assumed that blocking proteasomal degradation with MG132 should result in equal protein amounts of HIF-1 α irrespective of Trx1 or Trx2 expression. As expected, MG132 supplied for 1 or 2 h evoked HIF-1 α accumulation in control cells. Interestingly, Trx1 expressing cells showed stronger HIF-1 α protein accumulation (Fig. 3A), whereas Trx2 cells revealed lower amounts of HIF-1 α compared with controls (Fig. 3B). From these results one can predict that different amounts of HIF-1 α seen as a response to hypoxia in Trx1 versus Trx2-overexpressing cells do not simply result from differences in protein degradation. Rather, HIF-1 α protein synthesis may be regulated by transcription and/or translation.

Trx1 and Trx2 Modulate HIF-1 α Protein Synthesis via cap-dependent Translation—We then determined HIF-1 α mRNA by real-time PCR in Trx1 and Trx2 overexpressing cells (Fig. 4A). The HIF-1 α mRNA content was unaltered by hypoxic conditions and similar in controls and Trx1- and/or Trx2-overexpressing cells. Therefore, changes in HIF-1 α mRNA do not account for modulation of protein amounts as seen under the impact of Trx1 versus Trx2. Next, we determined HIF-1 α translation. We used a reporter assay with a plasmid carrying a permanent SV40 promoter to follow cap-dependent protein translation. Trx1 overexpression increased cap-dependent luciferase activity, whereas Trx2 reduced the reporter activity below control values (Fig. 4B). In corroborating experiments,

we confirmed luciferase synthesis by [³⁵S]methionine pulse labeling. Compared with controls, luciferase production was enhanced in Trx1-overexpressing cells but reduced in Trx2-overexpressing cells (Fig. 4C). These results point to a cap-dependent translation control mechanism to be differently affected by Trx1 versus Trx2.

Trx1 and Trx2 Affect Phosphorylation of p70S6K, eIF-4E, and Akt—To gain a better insight in Trx1- versus Trx2-mediated modulation of HIF-1 α translation, we analyzed phosphorylation of p70S6K and eIF-4E, which participate in regulating HIF-1 α translation. Under normoxic as well as hypoxic conditions, p70S6K, eIF-4E, and 4E-BP1 were more heavily phosphorylated in Trx1-overexpressing cells compared with control cells. In contrast, phosphorylation of p70S6K, eIF-4E, and 4E-BP1 was reduced in Trx2-overexpressing cells compared with controls (Fig. 5). These results confirm that Trx1 positively affects protein translation components, whereas Trx2 shows a negative impact.

It is known that the PI3K/Akt pathway is upstream of p70S6K and eIF-4E. Therefore, activation of Akt might be helpful to better understand HIF-1 α regulation by Trx1 and Trx2. Akt activity was followed by determining the level of phospho-Akt versus total Akt. Compared with a basal kinase activity in control (HEKi) cells, Trx2- expressing cells showed decreased phospho-Akt levels under hypoxia, whereas the amount of phospho-Akt was increased in Trx1 expressing cells (Fig. 6A). Importantly, the expression of total Akt remained unaltered. In addition, we transfected cells with a dominant negative Akt plasmid (pAkt_{dn}) as well as a permanent active Akt plasmid (pAkt) to study HIF-1 α expression. As expected, the dominant negative Akt reduced the HIF-1 α protein under all conditions. Importantly, the increased accumulation of HIF-1 α as a result of Trx1-overexpression was reduced (Fig. 6B). However, transfection of the permanent active Akt provoked increased expression of HIF-1 α in Trx2-overexpressing cells (Fig. 6C) when exposed to hypoxia. Decreased expression of HIF-1 α under the impact of Trx2 is at least in part reversed by overexpression of permanently active Akt.

Trx1 and Trx2 Differently Affect Production of ROS in Mitochondria—As reducing compounds, Trx1 and Trx2 are expected to influence the cellular redox system. We therefore explored ROS production in mitochondria of Trx1- versus Trx2-overexpressing cells. The dye MitoSOX was employed to measure and quantify mitochondrial ROS production by FACS. While Trx1 reduced mitochondrial ROS levels, Trx2 increased ROS production (Fig. 7A). However, hypoxia did not affect ROS formation in these cells.

To reverse ROS production in Trx2-overexpressing cells, we applied the specifically mitochondria-targeting antioxidant MitoQ, which is reported to influence HIF-1 α stabilization (29). MitoQ (1 μ M) slightly reduced hypoxia-induced HIF-1 α accumulation in control and Trx1-overexpressing cells but allowed to recover HIF-1 α accumulation in Trx2-overexpressing cells under hypoxia (Fig. 7B). Moreover, as MitoQ allowed recovering HIF-1 α expression in hypoxic Trx2-overexpressing cells, phosphorylation and thus activity of Akt was regained as well (Fig. 7C).

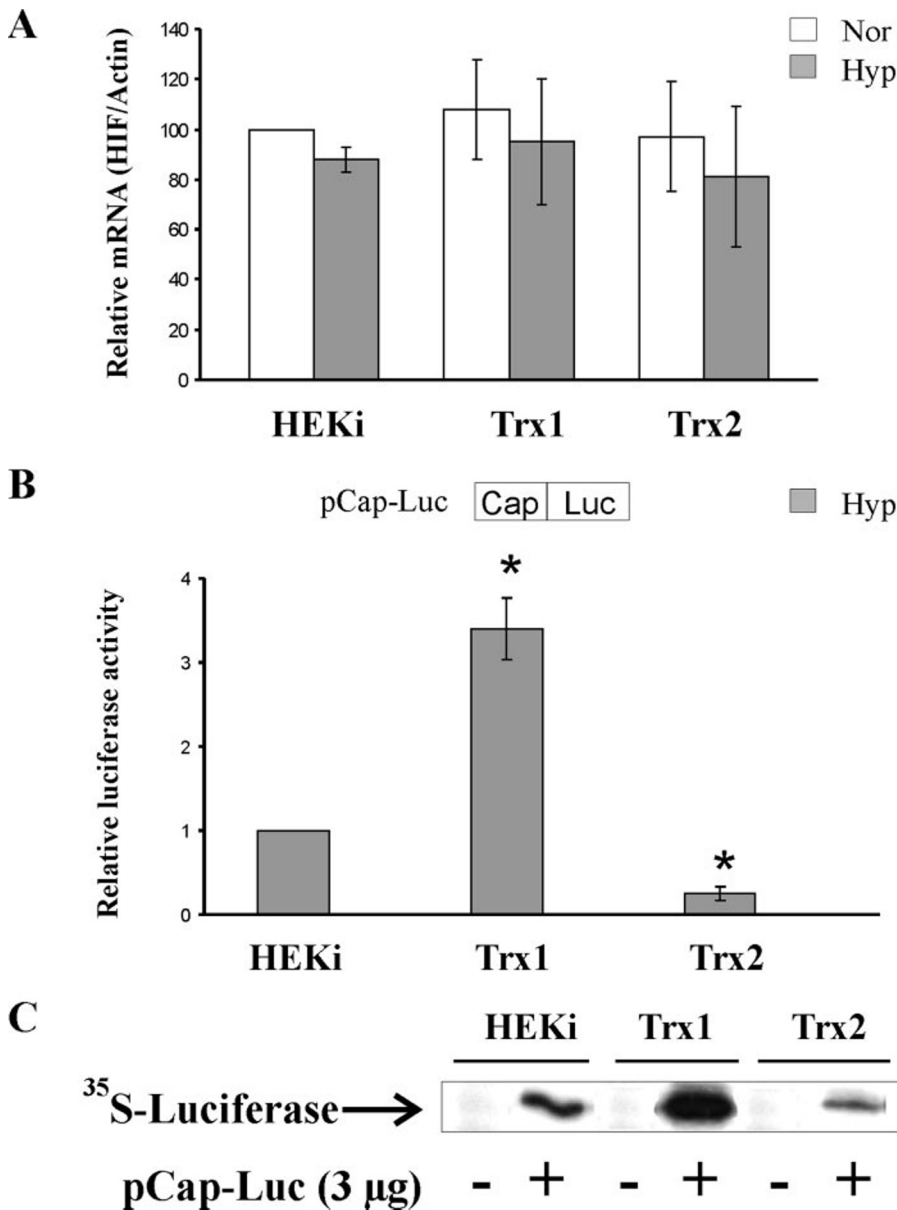


FIGURE 4. HIF-1 α mRNA content and translational control under the impact of Trx1 versus Trx2. HEK cells overexpressing Trx1 or Trx2 or control (HEKi) cells under normoxia (empty columns) or 0.5% hypoxia (filled columns) were incubated for 4 h followed by quantification of HIF-1 α mRNA by real-time quantitative PCR (A). Cells were transfected with pCap-Luc reporter plasmid and then incubated under 0.5% oxygen for 16 h. Following cell lysis luciferase activities were measured and normalized to controls (B). Cells transfected with pCap-Luc reporter plasmid were pulse-labeled with [³⁵S]methionine under hypoxic (0.5% oxygen) condition (C). [³⁵S]Methionine-labeled luciferase was immunoprecipitated with an anti-luciferase antibody and visualized by autoradiography as described under "Experimental Procedures." The image is representative of three independent experiments. Data represent the mean \pm S.E. of at least three independent experiments. *, $p < 0.05$.

DISCUSSION

We reestablished that cytosolic Trx1 enhanced accumulation of HIF-1 α protein during hypoxia. Unexpectedly, overexpression of mitochondrial Trx2 attenuated this response by slowing down protein synthesis of HIF-1 α . While Trx1 enhanced cap-dependent translation of HIF-1 α , Trx2 had the opposite effect. In addition, factors known to modulate protein translation, such as Akt, p70S6K, and eIF-4E, were affected. While Akt, p70S6K, and eIF-4E activities increased in Trx1-overexpressing cells, their activities were lowered in Trx2-overexpressing cells. Decreased translation of HIF-1 α in Trx2-over-

expressing cells was correlated to increased mitochondrial ROS production and blocking ROS formation with MitoQ allowed to recover a functional HIF-1 response in Trx2 overexpressing cells.

It is believed that reactive oxygen species may act as second messengers in transduction pathways such as the PI3K/Akt pathway and may interfere with the activity of transcription factors such as NF- κ B or p53 (30). Major sources of ROS are mitochondria, and manipulating the redox status of the mitochondria may affect signaling pathways that occur in other cell organelles. For example, overexpression of human mitochondrial superoxide dismutase (MnSOD) provoked induction of mouse endogenous SOD2 and Trx2 mRNA levels (31). In the presence of the mitochondria specific antioxidant MitoQ, effects were greatly diminished, suggesting that the redox status of mitochondria orchestrates events that occur in the nucleus. It is also speculated that manipulation of the mitochondrial redox state provokes post-transcriptional events, as well as alterations in protein stability and turnover (31). There is evidence that mitochondrial ROS may leave the organelle, thereby altering ROS in the cytosol (reviewed in Ref. 32). Thus, cytosolic Trx1 and mitochondrial Trx2 may influence ROS levels not only in the cytosol but also in mitochondria.

HIF-1 α is a redox-sensitive transcription factor whose ability to activate target genes via HIF-1 is enhanced by Trx1. It is assumed that Trx1 keeps the cysteine 800 of HIF-1 α reduced, which is critical for CBP/p300 recruitment and thus HIF-1 transactivation (15). Moreover, HIF-1 α protein levels are influenced by Trx1 (16), and it is proposed that Trx1 causes dissociation of HIF-1 α and pVHL, thus blocking HIF-1 α degradation (19). In our work, blocking degradation of HIF-1 α still did not equalize HIF-1 α amount, rather keeping increased levels in Trx1-overexpressing, but decreased levels in Trx2-overexpressing, cells. Results imply that translation may be involved and Trx1 enhances indeed cap-dependent translation. Moreover, increased activities of Akt, p70S6K, eIF-4E, and 4E-BP1 support the idea of Trx1 in stimulating translation. Actually, HIF-1 α accumulation resulting

HIF-1 α Regulation by Trx1 Versus Trx2

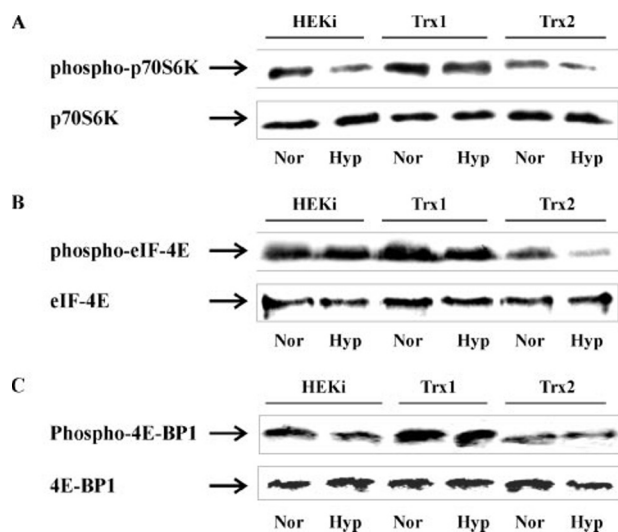


FIGURE 5. Trx1 versus Trx2 differently affect phosphorylation of p70S6K, eIF-4E, and 4E-BP1. Western blotting was performed in HEK cells overexpressing Trx1 or Trx2 compared with control cells (*HEKi*) to follow the occurrence of phospho-p70S6K (A), phospho-eIF-4E (B), or phospho-4E-BP1 (C) relative to protein expression of p70S6K, eIF-4E, or 4E-BP1 under normoxic and hypoxic (0.5% oxygen, 4 h) conditions. Data are representative of three independent experiments.

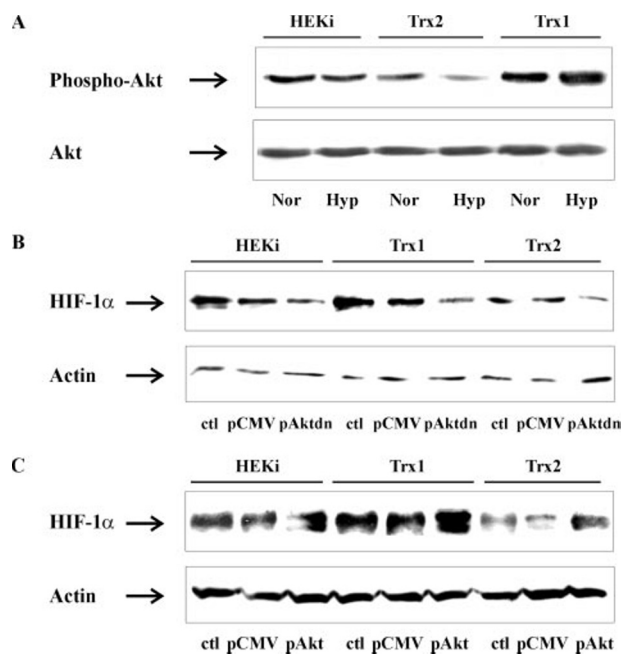


FIGURE 6. Trx1 versus Trx2 differently affect kinase signaling. Phosphorylation of Akt was examined under normoxic versus hypoxic (0.5% oxygen, 4 h) conditions by following the occurrence of phospho-Akt relative to total protein expression of Akt in HEK control cells or cells overexpressing Trx1 or Trx2 (A). Cells were transfected with the dominant negative Akt (*pAktdn*) plasmid (B) or the permanently active Akt (*pAkt*) plasmid (C), the empty plasmid (*pCMV*), or left as untransfected controls (*ctl*). Following transfection, cells were incubated under hypoxia (0.5% oxygen) for 4 h. HIF-1 α and actin were detected by Western blotting. Data are representative of three independent experiments.

from enhanced protein synthesis is often seen in cells treated with growth factors, cytokines, or in cells carrying gain-of-function mutated oncogenes (1). The PI3K/Akt and MAPK pathways are shown to be involved in these processes, and p70S6K, eIF-4E, and 4E-BP1 are responsible for enhancing HIF-1 α translation (33, 34). We also studied activation/in-

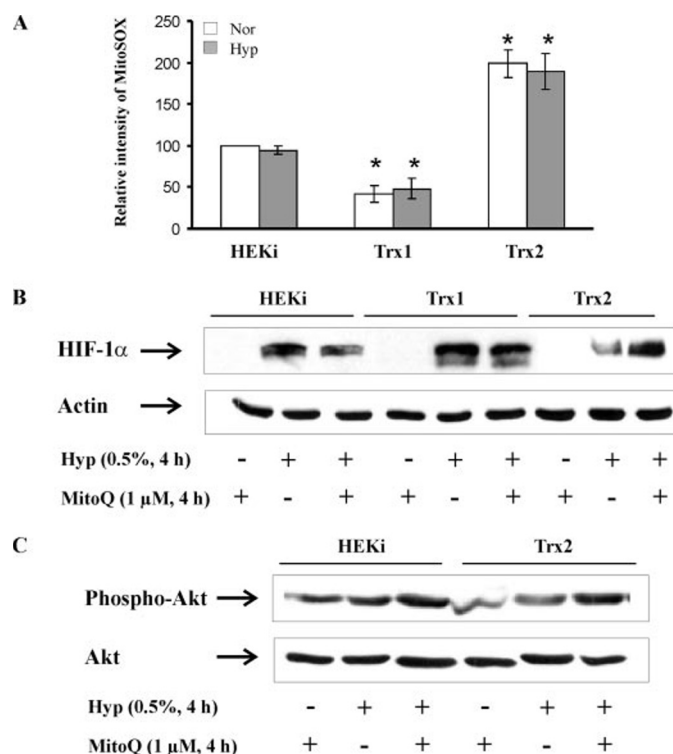


FIGURE 7. Trx1 versus Trx2 differently affect mitochondrial ROS production. HEK cells overexpressing Trx1, Trx2, or control cells (*HEKi*) were kept under normoxia (*Nor*) or exposed to 0.5% hypoxia (*Hyp*) for 4 h. Mitochondrial ROS production was measured using MitoSOX as described under "Experimental Procedures" and normalized to controls. Data represent the mean \pm S.E. of at least three independent experiments (A). HEK cells overexpressing Trx1, Trx2, or control cells (*HEKi*) were kept under normoxia or exposed to 0.5% hypoxia (*Hyp*) in the presence or absence of 1 μ M MitoQ for 4 h. HIF-1 α and actin (B) as well as phospho-Akt versus total Akt (C) were determined by Western analysis. Blots are representative of three independent experiments. *, $p < 0.05$.

activation of p42/44MAPK (Erk) in Trx1- and Trx2-overexpressing cells. Similar to Akt, Erk phosphorylation was increased by Trx1 but reduced by Trx2 under both hypoxic and normoxic conditions.³ Additionally, it is shown that Akt gain-of-function could be facilitated through inhibition of PTEN activity by Trx1 (35). This may explain how Trx1 enhanced the kinase cascade and thereby speeds up the rate of HIF-1 α translation.

Expression of Trx2 attenuated the response to hypoxia and cap-dependent translation was reduced. Impaired phosphorylation of Akt and transfection of permanent active Akt, which allowed regaining HIF-1 α accumulation in Trx2-overexpressing cells, indicated that Trx2 negatively interferes with Akt-dependent translation of HIF-1 α . Although this may explain how HIF-1 α protein levels were affected, the overall mechanism by which mitochondrial ROS may regulate translation remains an open issue. We previously noticed that oligomycin inhibits rotenone-induced cytotoxicity in Trx2-overexpressing cells (26), which may point to an interference of Trx2 with ATP synthase. If indeed, ATP synthase works as an ATPase under hypoxia in Trx2 cells alterations in the energy charge and thus the AMP/ATP ratio may follow. A decrease in ATP may provoke activation of the

³ J. Zhou, A. E. Damdimopoulos, G. Spyrou, and B. Brüne, unpublished data.

AMP-activated protein kinase, which is known to affect mammalian target of rapamycin (mTOR) and its effectors 4E-BP1 and p70S6K (36, 37), thereby decreasing HIF-1 α expression (38, 39).

There is increasing evidence that translational regulation of HIF-1 α contributes to control HIF-1 expression and activity. Whereas degradation of HIF-1 α predominantly is affected by oxygen availability, translation regulation of HIF-1 α is associated with the action of growth factors, cytokines, tumor suppressors, or other signaling molecules (1, 40–42). HIF-1 α induction is dependent on PI3K/Akt, MAPK, and ROS. Kinase inhibitors impair the induction of HIF-1 α and removing ROS by the antioxidant *N*-acetylcysteine, superoxide dismutase, as well as pharmacological or molecular interference with mitochondrial ROS production diminished HIF-1 α induction (4, 21, 43). Most of these studies have used acute interventions, often with the idea to induce a burst of ROS. The situation in Trx2-overexpressing cells might be different because cells have to adapt to long lasting changes in mitochondrial functionality. This is reflected at the level of Akt activity. A burst of ROS is reported to activate Akt, whereas others including this study showed that increased ROS production can attenuate Akt activity with the further notion that reducing the ROS amount reactivates Akt signaling (44–46). One might speculate on an opposed redox status in cells based on different localizations of Trx1 versus Trx2, which may affect the rate of HIF-1 α translation. On one side, cytosolic Trx1 may keep cells optimized to speed up HIF-1 α synthesis, while Trx2 may interact with specific components of the mitochondrial respiratory chain (26), thereby allowing mitochondrial-mediated redox alteration to deactivate a kinase cascade and decline HIF-1 α translation. Additionally, we need to consider other possibilities, e.g. transcription factors such as NF- κ B, which are regulated by thioredoxin (47) and are closely related to HIF-1 α regulation (3).

Acknowledgments—The technical assistance of Sandra Christmann and Bettina Wenzel is highly appreciated.

REFERENCES

- Semenza, G. L. (2003) *Nat. Rev. Cancer* **3**, 721–732
- Pugh, C. W., and Ratcliffe, P. J. (2003) *Nat. Med.* **9**, 677–684
- Zhou, J., Callapina, M., Goodall, G. J., and Brune, B. (2004) *Cancer Res.* **64**, 9041–9048
- Page, E. L., Robitaille, G. A., Pouyssegur, J., and Richard, D. E. (2002) *J. Biol. Chem.* **277**, 48403–48409
- Vlami-Gardikas, A., and Holmgren, A. (2002) *Methods Enzymol.* **347**, 286–296
- Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) *EMBO J.* **17**, 2596–2606
- Nakamura, H., Masutani, H., Tagaya, Y., Yamauchi, A., Inamoto, T., Nanbu, Y., Fujii, S., Ozawa, K., and Yodoi, J. (1992) *Cancer* **69**, 2091–2097
- Makino, Y., Okamoto, K., Yoshikawa, N., Aoshima, M., Hirota, K., Yodoi, J., Umesono, K., Makino, I., and Tanaka, H. (1996) *J. Clin. Invest.* **98**, 2469–2477
- Hayashi, S., Hajiro-Nakanishi, K., Makino, Y., Eguchi, H., Yodoi, J., and Tanaka, H. (1997) *Nucleic Acids Res.* **25**, 4035–4040
- Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3633–3638
- Wei, S. J., Botero, A., Hirota, K., Bradbury, C. M., Markovina, S., Laszlo, A., Spitz, D. R., Goswami, P. C., Yodoi, J., and Gius, D. (2000) *Cancer Res.* **60**, 6688–6695
- Ueno, M., Masutani, H., Arai, R. J., Yamauchi, A., Hirota, K., Sakai, T., Inamoto, T., Yamaoka, Y., Yodoi, J., and Nikaido, T. (1999) *J. Biol. Chem.* **274**, 35809–35815
- Jayaraman, L., Murthy, K. G., Zhu, C., Curran, T., Xanthoudakis, S., and Prives, C. (1997) *Genes Dev.* **11**, 558–570
- Huang, L. E., Arany, Z., Livingston, D. M., and Bunn, H. F. (1996) *J. Biol. Chem.* **271**, 32253–32259
- Ena, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J., Sogawa, K., Poellinger, L., and Fujii-Kuriyama, Y. (1999) *EMBO J.* **18**, 1905–1914
- Welsh, S. J., Bellamy, W. T., Briehl, M. M., and Powis, G. (2002) *Cancer Res.* **62**, 5089–5095
- Moos, P. J., Edes, K., Cassidy, P., Massuda, E., and Fitzpatrick, F. A. (2003) *J. Biol. Chem.* **278**, 745–750
- Jewell, U. R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R. H., and Gas-smann, M. (2001) *FASEB J.* **15**, 1312–1314
- Kim, W. J., Cho, H., Lee, S. W., Kim, Y. J., and Kim, K. W. (2005) *Int. J. Oncol.* **26**, 1049–1052
- Cadenas, E. (2004) *Mol. Aspects Med.* **25**, 17–26
- Chandel, N. S., McClintock, D. S., Feliciano, C. E., Wood, T. M., Melendez, J. A., Rodriguez, A. M., and Schumacker, P. T. (2000) *J. Biol. Chem.* **275**, 25130–25138
- Schroedl, C., McClintock, D. S., Budinger, G. R., and Chandel, N. S. (2002) *Am. J. Physiol.* **283**, L922–L931
- Schafer, F. Q., and Buettner, G. R. (2001) *Free Radic. Biol. Med.* **30**, 1191–1212
- Tanaka, T., Hosoi, F., Yamaguchi-Iwai, Y., Nakamura, H., Masutani, H., Ueda, S., Nishiyama, A., Takeda, S., Wada, H., Spyrou, G., and Yodoi, J. (2002) *EMBO J.* **21**, 1695–1703
- Nonn, L., Williams, R. R., Erickson, R. P., and Powis, G. (2003) *Mol. Cell Biol.* **23**, 916–922
- Damdimopoulos, A. E., Miranda-Vizuete, A., Pelto-Huikko, M., Gustafsson, J. A., and Spyrou, G. (2002) *J. Biol. Chem.* **277**, 33249–33257
- Spyrou, G., Enmark, E., Miranda-Vizuete, A., and Gustafsson, J. (1997) *J. Biol. Chem.* **272**, 2936–2941
- Psarra, A. M., Solakidi, S., Trougakos, I. P., Margaritis, L. H., Spyrou, G., and Sekeris, C. E. (2005) *Int. J. Biochem. Cell Biol.* **37**, 2544–2558
- Sanjuan-Pla, A., Cervera, A. M., Apostolova, N., Garcia-Bou, R., Victor, V. M., Murphy, M. P., and McCreath, K. J. (2005) *FEBS Lett.* **579**, 2669–2674
- Martindale, J. L., and Holbrook, N. J. (2002) *J. Cell Physiol.* **192**, 1–15
- Kim, A., Murphy, M. P., and Oberley, T. D. (2005) *Free Radic. Biol. Med.* **38**, 644–654
- Jezek, P., and Hlavata, L. (2005) *Int. J. Biochem. Cell Biol.* **37**, 2478–2503
- Fukuda, R., Hirota, K., Fan, F., Jung, Y. D., Ellis, L. M., and Semenza, G. L. (2002) *J. Biol. Chem.* **277**, 38205–38211
- Laughner, E., Taghavi, P., Chiles, K., Mahon, P. C., and Semenza, G. L. (2001) *Mol. Cell Biol.* **21**, 3995–4004
- Meuillet, E. J., Mahadevan, D., Berggren, M., Coon, A., and Powis, G. (2004) *Arch. Biochem. Biophys.* **429**, 123–133
- Brugarolas, J., Lei, K., Hurley, R. L., Manning, B. D., Reiling, J. H., Hafen, E., Witters, L. A., Ellisen, L. W., and Kaelin, W. G., Jr. (2004) *Genes Dev.* **18**, 2893–2904
- Liu, L., Cash, T. P., Jones, R. G., Keith, B., Thompson, C. B., and Simon, M. C. (2006) *Mol. Cell* **21**, 521–531
- Hudson, C. C., Liu, M., Chiang, G. G., Otterness, D. M., Loomis, D. C., Kaper, F., Giaccia, A. J., and Abraham, R. T. (2002) *Mol. Cell Biol.* **22**, 7004–7014
- Brugarolas, J. B., Vazquez, F., Reddy, A., Sellers, W. R., and Kaelin, W. G., Jr. (2003) *Cancer Cell* **4**, 147–158
- Brugarolas, J., and Kaelin, W. G., Jr. (2004) *Cancer Cell* **6**, 7–10
- Zhou, J., and Brune, B. (2006) *Cardiovasc. Hematol. Agents Med. Chem.* **4**, 189–197
- Bernardi, R., Guernah, I., Jin, D., Grisendi, S., Alimonti, A., Teruya-Feld-

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- stein, J., Cordon-Cardo, C., Simon, M. C., Rafii, S., and Pandolfi, P. P. (2006) *Nature* **442**, 779–785
43. Gorlach, A., Diebold, I., Schini-Kerth, V. B., Berchner-Pfannschmidt, U., Roth, U., Brandes, R. P., Kietzmann, T., and Busse, R. (2001) *Circ. Res.* **89**, 47–54
44. Zhuang, S., Ouedraogo, G. D., and Kochevar, I. E. (2003) *Oncogene* **22**, 4413–4424
45. Rahmani, M., Reese, E., Dai, Y., Bauer, C., Payne, S. G., Dent, P., Spiegel, S., and Grant, S. (2005) *Cancer Res.* **65**, 2422–2432
46. Xu, D., Guthrie, J. R., Mabry, S., Sack, T. M., and Truong, W. E. (2006) *Am. J. Physiol.* **291**, L966–L975
47. Arner, E. S., and Holmgren, A. (2000) *Eur. J. Biochem.* **267**, 6102–6109