Research Article

The Werner syndrome gene product (WRN): a repressor of hypoxia-inducible factor-1 activity

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ABSTRACT

Werner syndrome (WS) is a rare autosomal disease characterized by the premature onset of several age-associated pathologies. The protein defective in WS patients (WRN) is a helicase/exonuclease involved in DNA repair, replication, transcription and telomere maintenance. Hypoxia-inducible factor-1 (HIF-1) is a decisive element for the transcriptional regulation of genes essential for adaptation to low oxygen conditions. HIF-1 is also implicated in the molecular mechanisms of ageing. Here, we show that the cellular depletion of WRN protein (by siRNA targeting) leads to increased HIF-1 complex stabilization and activation. HIF-1 activation in the absence of WRN involves the generation of mitochondrial reactive oxygen species (mtROS) since SkQ1, a mitochondrial-targeted antioxidant, and stigmatellin, an inhibitor of mitochondrial complex III, blocked increased HIF-1 levels. Ascorbate, an essential co-factor involved in HIF-1 stability, was decreased in WRN-depleted cells. Interestingly, expression levels of GLUT1, a known dehydroascorbic acid transporter, were also decreased in WRN-depleted cells. Ascorbate supplementation of WRN-depleted cells led to a dose-dependent inhibition of HIF-1 activation. These results indicate that WRN protein regulates HIF-1 activation by affecting mitochondrial ROS production and intracellular ascorbate levels. This work provides a novel mechanistic link between HIF-1 activity and different age-associated pathologies.

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Introduction

Patients with Werner syndrome (WS), an autosomal recessive disorder, display many clinical symptoms of normal ageing at an early age. From their second decade of life onwards, WS patients prematurely develop pathologies that resemble traits of normal ageing; such as ocular cataracts, osteoporosis, dyslipidemia, type II diabetes, atherosclerosis, and cancer [1]. Death generally occurs from heart disease or cancer during the fourth or fifth decade of life [2]. Fibroblasts isolated from WS patients characteristically senesce prematurely in culture and display increased chromosomal aberrations [3,4]. The protein defective...
in WS, WRN, is a RecQ family 3’–5’ DNA helicase possessing a 3’–5’ exonuclease activity [5–7]. WRN is involved in DNA recombination, transcription, repair and telomere maintenance [8]. The inhibition of WRN activity renders cells more sensitive to oxidative damage [9]. Additionally, plasma from WS patients or tissues from mice lacking a functional WRN helicase (WRn<sup>−/−</sup>) display pro-oxidative hallmarks [10,11]. Furthermore, WRn<sup>−/−</sup> mice also exhibit a metabolic syndrome and pro-inflammatory traits [12].

It is well established that increased levels of reactive oxygen species (ROS) are involved in a number of diseases including diabetes, obesity, atherosclerosis, and cancer [13–15]. An important source of endogenous ROS comes from the mitochondria during the process of oxidative phosphorylation [16]. Inflammation is also a major source of ROS at sites of tissue fibrosis [13,15]. Interestingly, ROS-sensitive transcription factors, such as Hypoxia-inducible factor-1 (HIF-1) and NFκB, are increased in the liver from WRn<sup>−/−</sup> mice [12].

In mammalian cells, Hypoxia-inducible factor-1 is a critical component for adaptive responses to low oxygen conditions [17]. HIF-1 regulates the expression of key genes involved in angiogenesis, erythropoiesis, glucose metabolism, cellular respiration, survival and invasion [18]. Heterodimeric HIF-1 is formed following the interaction of a constitutive HIF-1α subunit with a tightly regulated HIF-1β subunit. In normal oxygen conditions, HIF-1α is hydroxylated through the action of HIF prolyl-hydroxylases (PHD). HIF-1α hydroxylation permits the binding of the product of the von Hippel-Lindau tumor suppressor gene (pVHL). As the recognition component of an E3 ubiquitin ligase complex, pVHL allows for HIF-1α polyubiquitination and subsequent proteasomal degradation [19]. When oxygen levels are decreased, hydroxylation of HIF-1α is blocked resulting in the stabilization of the HIF-1α subunit and the formation of the HIF-1 transcriptional complex. The resulting HIF-1 heterodimer binds to hypoxic response elements (HRE) found on regulatory sequences of target genes which include glycolytic enzymes, glucose transporters, erythropoietin, and essential pro-angiogenic factors [20]. Previous work have demonstrated that HIF-1-dependent responses occur during mitochondrial dysfunction and that HIF-1 activity is linked to certain ageing responses [21–27].

As mentioned above, our previous findings showed that HIF-1α protein levels were significantly increased in the liver of WRn<sup>−/−</sup> mice [12]. However, the mechanistic details with which WRN achieved this response are unknown. With the present study, we report that WRN protein depletion in primary or established human cell lines increases the level of HIF-1α protein induction in low oxygen conditions. This increase is dependent on ROS production and the cellular oxidative status since the treatment of cells with either ascorbate or inhibitors of mitochondrial ROS generation blocked increased HIF-1 induction by WRN depletion. These results reveal a novel and interesting pathway regulated by WRN, which may have important physiological implications in age-related diseases.

**Materials and methods**

**Chemicals and antibodies**

Stigmasteralin, MG132 and CoCl<sub>2</sub> were from Sigma-Aldrich (St-Louis, MO). The mitochondrial antioxidant, SkQ1 was from Dr. Vladimir Skulachev and Dr. Oleg Fedorkin at the Institute of Mitoengineering, Moscow State University [28]. Anti-HIF-1α antibody was raised in rabbits immunized against the last 20 amino acids of the C terminal of human HIF-1α [29]. Anti-α-tubulin antibody was from Sigma-Aldrich. The polyclonal antibody against human WRN protein (H-300) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GST antibody was from Novus Biologicals (Littleton, CO). Monoclonal HA.11 antibody was from Covance (Emeryville, CA). Horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies were from Promega (Madison, WI). A GST-HIF-1α fusion protein, comprised of amino acids 344–582 from human HIF-1α, and pVHL-HA constructs were kind gifts from Dr. Jacques Pouysségur and Dr. Peter Ratcliffe, respectively.

**Cell culture**

Normal human diploid fibroblasts (GM08402) were obtained from the Coriell Cell Repository (Camden, NJ). HeLa cells were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, 50 units/mL streptomycin (Invitrogen, Carlsbad, CA) and 2 mM L-glutamine in a humid atmosphere (5% CO<sub>2</sub>–95% air). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, 50 units/mL streptomycin (Invitrogen, Carlsbad, CA) and 2 mM L-glutamine in a humid atmosphere (5% CO<sub>2</sub>–95% air). Cells were intentionally passaged once reaching confluence. Low oxygen conditions were obtained by placing cells in a sealed hypoxic workstation (Ruskinn Technology Ltd, Bridgend, United Kingdom). The oxygen level in this workstation was maintained at 3%, with the residual gas mixture containing 92% nitrogen and 5% carbon dioxide.

**Western blot analysis**

Confluent cells were lysed in a 2 x Laemmli buffer. Protein concentration was determined by Lowry assay. Samples were resolved on SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF, Immobilon-P, Millipore, Danvers, MA) or Hybrid-C Extra membranes (GE Healthcare, Piscataway, NJ). Proteins were analyzed using indicated antibodies and visualized with an enhanced chemiluminescence (ECL) system (GE Healthcare) for imaging or with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) for quantitation. α-tubulin was used as a control for protein loading. Western blots were quantitated using LI-COR Image Studio software 2.0 (LI-COR Biosciences). Protein bands for quantitation were identified using specific siRNAs. The background signal for each band was determined using an identical area to the target band covering a region in the same lane where no protein signal was observed. Results were determined by calculating a ratio of target protein signal (minus background) over α-tubulin signal (minus background).

**RNA silencing**

Cells were seeded in 6-well plates at a density of 6 x 10<sup>5</sup> cells/well. 24 h after plating, siRNA oligonucleotides (Applied Biosystems/Life Technologies, Carlsbad, CA) were transfected by calcium phosphate precipitation. Thirty-six hours post-transfection, cells were then deprived of FBS for 16 h prior to treatment. Two different siRNAs specific to separate regions of human WRN mRNA (accession no.
NM_000553.4) were used: siWRN HSS111385 siRNA: 5'-UUAA-CCAGACGUGAAGGCCUCAGC-3'; and siWRN HSS111387 siRNA: 5'-AUUAACAAUGCCAUUGGUCGCC-3'. Knockdown efficiency was confirmed by Western blot analysis. HIF-1α siRNAs (siHIF-1α) were previously described [30]. The Silencer Negative Control #2 siRNA (siCtrl) was used as a control siRNA (Applied Biosystems).

**Mitochondrial ROS assay**

Mitochondrial ROS production was determined using MitoSOX Red mitochondrial superoxide indicator (Invitrogen), which is selectively targeted to the mitochondria and fluorescent upon ROS oxidation. MitoSOX was used according to the manufacturer's protocol and published literature [31]. Cells were seeded on glass bottom cell culture dishes and serum deprived overnight in phenol red-free DMEM. Cells were then incubated with MitoSOX (1 μM) for a total of 1 h prior to analysis. Cells were pretreated or not with inhibitors as indicated 20 min after MitoSOX addition. Cells were then incubated in low oxygen conditions or not during the final 20 min. Cell imaging was performed with a FV1000 confocal microscope equipped with a live cell apparatus (60 x oil, 1.4 numerical aperture (NA)) driven by FluoView software (Olympus, Tokyo, Japan). Fluorescence quantification was performed using the Measure Integrated Density function of the ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/).

**Luciferase assay**

HeLa cells were seeded in 6-well plates at a density of 6 x 10⁵ cells/well. 24 h after plating, cells were transfected, by calcium phosphate precipitation, with siRNA oligonucleotides along with 1 μg/well CMV-luc-HIF-1α-ODDD or pGL3(R2.2) 3HR-euk-LUC luciferase reporter vectors [30,32]. Renilla reniformis luciferase expression vector (250 ng/well) was also used as a control for transfection efficiency. 36 h post-transfection, cells were treated as indicated for 6 h. Cells were washed with cold phosphate-buffered saline, and luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega). Results were quantified with a Luminoskan Ascent microplate reader with integrated injectors (Thermo Fisher Scientific, Waltham, MA). Results are expressed as a ratio of firefly luciferase activity over *R. reniformis* luciferase activity. Experiments are an average ± SEM of triplicate data representative of 3 independent experiments.

**HIF-1α half-life analysis**

HeLa cells transfected with siWRN or siCtrl were incubated under normoxic or low oxygen conditions. After 3 h, cycloheximide (50 μg/mL) was added for different periods of time (up to 30 min) followed by rapid lysis with 2 x Laemmli buffer. HIF-1α and β-tubulin protein levels were evaluated by Western blotting followed by quantification and the ratio of HIF-1α to β-tubulin determined. HIF-1α half-life under experimental conditions was estimated by plotting data as HIF-1α/β-tubulin ratio versus time under cycloheximide treatment.

**pVHL capture assay**

pVHL capture assay was performed as previously described [33]. Briefly, HeLa cells transfected with siWRN or siCtrl were incubated with or without CoCl₂. Cells were then washed once in PBS and twice in ice-cold HEB buffer (20 mM Tris pH7.5, 5 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol). Cells were then lysed using a Dounce homogenizer and cytoplasmic extracts were isolated by centrifugation (20,000 g). Cytoplasmic extracts (250 μg) were incubated with sepharose-bound GST-HIF-1α (30 μg) for 1 h at room temperature. The sepharose-bound GST-HIF-1α was then washed with NETN buffer (150 mM NaCl, 0.5 mM EDTA, 20 mM Tris pH 8.0, 0.5% (g/L) Igepal, 100 μM deferoxamine) and incubated overnight with in vitro translated pVHL-HA in NETN at 4 °C. Samples were then washed with NETN, denatured with 2 x Laemmli buffer, resolved in SDS-polyacrylamide gels (12%) and revealed using anti-HA and anti-GST antibodies.

**Intracellular ascorbate assay**

HeLa cells were grown to confluence on 100 mm plates in culture media supplemented with ascorbate (250 μM). Cells were serum-deprived for 16 h in ascorbate-supplemented DMEM and fresh DMEM without ascorbate was added 1 h before ascorbate measurements. Cells were harvested and ascorbate levels were then analyzed using the Ferric Reducing Ascorbate (FRASC) assay kit (BioVision, Mountain View, CA) [34]. A Lowry protein assay was used for the normalization of samples.

**Glucose uptake**

HeLa cells were seeded in 6-well plates at a density of 6 x 10⁵ cells/well. Twenty-four hours after plating, cells were transfected, by calcium phosphate precipitation, with siRNA oligonucleotides. Forty-eight hours post-transfection, cells were washed with PBS and incubated in 1 mL of KRH buffer (25 mM HEPES–NaOH (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.3 mM CaCl₂, and 1.3 mM KH₂PO₄) with or without insulin (100 nM) for 10 min at 37 °C (5% CO₂). 2-deoxy-D-[2, 6-3H]-glucose (1 μCi) was added to each well for an additional 10 min. Cells were then washed with the KRH buffer and lysed in 1 mL of 0.5 mM NaOH. 300 μL of lysate was counted in a ScintiVerse cocktail (Fisher Scientific, Fair Lawn, NJ). A Lowry protein assay was used for the normalization of samples.

**Real-Time qRT-PCR**

Real-Time qRT-PCR was performed with the FastStart SYBR Green Master kit according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN). Real-Time qRT-PCR primers used in this study are outlined in Table 1.

**Statistical analysis**

For Western blot and imaging studies, results are representative of three independent experiments. For quantifications, results are expressed as means ± SEM. Statistical analyses of different experiments were performed using the program R 2.11.1 (http://cran.r-project.org/). Unless otherwise noted, a Student t-test was
Table 1 – Primers used for real time RT-PCR.

<table>
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<tr>
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<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>CA9 fwd</td>
<td>5'-CACAGCTCATTGGGCGCTAT-3'</td>
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<tr>
<td>CA9 rev</td>
<td>5'-ACACCTGCGGCGTATT-3'</td>
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<tr>
<td>GLUT1 fwd</td>
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<tr>
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<tr>
<td>GLUT3 fwd</td>
<td>5'-GCCTAACACTCGGGGCTAT-3'</td>
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<tr>
<td>GLUT3 rev</td>
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<tr>
<td>WRN rev</td>
<td>5'-GCACCGAACACTGACACAGA-3'</td>
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PCR conditions on cDNA
10 min 95 °C 1 cycle
15 s 95 °C, 30 s 59 °C, 30 s 72 °C 50 cycles.

Results

Depletion of WRN gene product increases HIF-1α protein accumulation

A siRNA-based approach was used to deplete the expression of the WRN gene product in order to determine the direct consequence of WRN protein on HIF-1 activity. The HeLa cervical cancer cell line was transfected with a siRNA specific for WRN mRNA (siWRN; HSS111385) or a non-targeting control siRNA sequence (siCtrl). Forty-eight hours post-transfection, cells were incubated under normoxic (20.9% O2) or low oxygen (3% O2) conditions. As expected, HIF-1α levels could be seen in normoxic conditions for up to 4 h. HIF-1α levels were markedly increased in siWRN-transfected cells over time in siCtrl-transfected cells under low oxygen conditions. Interestingly, HIF-1α protein induction was more potent in WRN-depleted cells. HIF-1α levels were markedly increased in siWRN-transfected cells at early periods of oxygen deprivation (within 2 h) as compared to siCtrl-transfected cells. Similar results were obtained when HeLa cells were treated with CoCl2, another potent inhibitor of HIF-1α hydroxylation and degradation (Fig. 1B). It is important to note that no significant increase in HIF-1α levels could be seen in normoxic conditions in siWRN-transfected cells. Additionally, comparable results were obtained with a second siWRN (HSS111387 siRNA; results not shown) or a different cell line, diploid human fibroblasts (GM08402; Supplemental Fig. 1). Finally, when cells were treated in low oxygen for longer time periods of up to 72 h, HIF-1α protein levels gradually returned to those seen in untreated cells. Interestingly, HIF-1α levels were markedly higher in siWRN-transfected cells...
(between 12 and 48 h) as compared to siCtrl-transfected cells (Supplemental Fig. 2). Taken together, these results indicate that WRN-depletion increases the potential for HIF-1α protein induction and accumulation.

**HIF-1α protein stability is increased in WRN-depleted cells**

Since under low oxygen conditions HIF-1 is primarily regulated by increased protein stability, we next attempted to determine the effect of WRN depletion on HIF-1α stabilization. To insure that HIF-1α gene expression levels are constant between conditions, we first examined HIF-1α mRNA levels. As seen in Supplemental Fig. 3, HIF-1α mRNA is not significantly different between siCtrl- and siWRN-transfected cells either in control or low oxygen conditions. We then determined HIF-1α half-life by using the general protein synthesis inhibitor, cycloheximide [35–37]. Incubating cells with cycloheximide following HIF-1α induction for 3 h under low oxygen conditions blocks further de novo protein synthesis. Cellular HIF-1α protein levels are thus dependent on the maintenance of protein stabilization mechanisms. In these experimental conditions, changes in HIF-1α half-life therefore reflect changes in HIF-1α protein stability. Here, HIF-1α half-life was determined by Western blotting and by quantification of HIF-1α levels over time in the presence of cycloheximide. As shown in Fig. 2, both siCtrl- and siWRN-transfected cells exhibited similarly increased HIF-1α levels under low oxygen conditions for 3 h. After the addition of cycloheximide to cells transfected with siCtrl, HIF-1α half-life under low oxygen was 16±5 min. In comparison, HIF-1α half-life under the same conditions in cells transfected with siWRN was greater than 30 min (Fig. 2B). Alternatively, we also investigated HIF-1α half-life under re-oxygenation. In this assay, cells were treated as above except that cycloheximide was added during the return of cells to ambient oxygen conditions (re-oxygenation). Under these conditions of more rapid degradation, HIF-1α half-life in siCtrl-transfected cells was 8.7±0.4 min. More importantly, HIF-1α half-life in siWRN-transfected cells was strikingly increased to 18.6±1.1 min (Western blot data not shown). Throughout these assays and in our different experimental conditions, we did not observe any significant variations of either WRN or α-tubulin protein levels. Taken together, these two assays indicate that HIF-1α protein stabilization is significantly increased in WRN-depleted cells.

We next attempted to determine the effect of WRN on the mechanisms of HIF-1α stability/degradation. Regulation of HIF-1α stability is mediated through its hydroxylated and highly unstable oxygen-dependent degradation domain (ODDD). To determine whether WRN changes the stability of the ODDD, we utilized a chimeric protein construct comprising the amino acids 401–602 of HIF-1α’s ODDD fused in-frame to the c-terminal end of the firefly luciferase protein. This construct generates an unstable form of luciferase when transfected into cells. The half-life of this luciferase construct is increased by oxygen deprivation and can be quantified by traditional luciferase assays [32]. HeLa cells were transiently transfected with the CMV-luc-HIF-1α-ODDD vector along with siRNAs. As expected, treatment of siCtrl-transfected cells with MG132, a proteasome inhibitor, or low oxygen increased luciferase activity by 4.1- and 1.8-fold over basal levels, respectively (Fig. 3). More interestingly, siWRN-transfected cells showed a significant increase in luciferase activity under low oxygen (2.2-fold over siCtrl-transfected cells in low oxygen). This result demonstrates that WRN can target mechanisms, which modulate HIF-1α ODDD stability/degradation.

A crucial event leading to HIF-1α protein degradation is its binding to the product of the von Hippel-Lindau tumor suppressor protein, pVHL. Since WRN depletion increases HIF-1α protein levels, we investigated whether WRN changes the stability of the ODDD. To determine whether WRN changes the stability of the ODDD, we utilized a chimeric protein construct comprising the amino acids 401–602 of HIF-1α’s ODDD fused in-frame to the c-terminal end of the firefly luciferase protein. This construct generates an unstable form of luciferase when transfected into cells. The half-life of this luciferase construct is increased by oxygen deprivation and can be quantified by traditional luciferase assays [32]. HeLa cells were transiently transfected with the CMV-luc-HIF-1α-ODDD vector along with siRNAs. As expected, treatment of siCtrl-transfected cells with MG132, a proteasome inhibitor, or low oxygen increased luciferase activity by 4.1- and 1.8-fold over basal levels, respectively (Fig. 3). More interestingly, siWRN-transfected cells showed a significant increase in luciferase activity under low oxygen (2.2-fold over siCtrl-transfected cells in low oxygen). This result demonstrates that WRN can target mechanisms, which modulate HIF-1α ODDD stability/degradation.

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![Fig. 4 - Cellular WRN depletion decreases pVHL-HIF-1α interaction. HeLa cells were transfected with a siRNA against WRN (siWRN) or a control sequence (siCtrl). Forty-eight hours post-transfection, cells were exposed or not to 100 μM CoCl₂ for 2 h when indicated. Cytoplasmic extracts were incubated with GST-HIF-1α coupled to sepharose beads for 1 h. Samples were then incubated in the presence of in vitro-translated pVHL and resolved by SDS-PAGE (12%). Immunoblotting was performed using anti-HA (pVHL) and anti-GST antibodies (upper panel). Western blots were quantified with the Odyssey Infrared Imageing System using GST-HIF-1α as a loading control (lower panel). Results are expressed as a percentage of pVHL binding normalized to GST-HIF-1α protein levels compared to untreated siCtrl cells and are an average ± SEM of three independent experiments: *P<0.01 and **P<0.001 as compared to untreated siCtrl cells.](image-url)
suppressor gene, pVHL [38]. Since pVHL binding is a direct end result of total HIF-1α hydroxylation, we performed a pVHL capture assay in order to determine total levels of HIF-1α hydroxylation during WRN depletion. A GST-HIF-1α fusion protein, comprised of amino acids 344–582 from human HIF-1α, was subjected to modification with cellular extracts from HeLa cells and interaction with in vitro translated pVHL. As expected, the treatment of cells with CoCl2 completely blocked HIF-1α binding to pVHL (Fig. 4). Interestingly, siWRN-transfected cells showed a significant decrease in pVHL binding to HIF-1α as compared to siCtrl-transfected cells. These results indicate that cellular WRN depletion leads to decreased HIF-1α prolyl hydroxylase activity, reduced pVHL binding and increased HIF-1α stability.

Mitochondrial ROS are essential to increased HIF-1α protein accumulation in WRN-depleted cells

Reactive oxygen species are major contributors for HIF-1α protein stabilization and induction in a number of systems [24,33,39–42]. Different studies, including our own, have pointed to mitochondrial ROS (mtROS) as essential intermediates in this response [24,37,43,44]. We first examined whether mtROS generation is increased in WRN-depleted cells. siCtrl- and siWRN-transfected cells were treated with MitoSOX, a mitochondriatargeted probe for detecting ROS. As shown in Fig. 5A and B, mtROS generation is significantly increased in siWRN-transfected cells as compared to siCtrl-transfected cells. We next determined the importance of mtROS in increasing HIF-1α induction in WRN-depleted cells by treatment with a mitochondrial-targeted antioxidant, SkQ1. Developed to investigate the role of mitochondrial ROS in ageing, this Skulachev (Sk) ion is strongly localized to the mitochondria. SkQ1 is a “rechargeable” antioxidant since it is reduced by the mitochondrial electron transport chain (ETC) [28]. While mtROS inhibitors can block the induction of HIF-1α during hypoxia [39,41,45], the induction of HIF-1α by CoCl2 is insensitive to mtROS inhibitors [37,44]. In order to evaluate the role of mtROS in the regulation of HIF-1α levels by WRN, we used short-term treatment with 100 μM CoCl2 to induce HIF-1α. Under these sub-maximal treatment conditions with CoCl2, further increases of HIF-1α protein levels are still possible (data not shown). As shown in Fig. 6, CoCl2 increased HIF-1α levels by 3.5-fold in siCtrl-transfected cells. HIF-1α levels were further increased in siWRN-transfected cells (7.5-fold as compared to siCtrl-transfected cells without CoCl2). When siWRN-transfected cells were pretreated with SkQ1 prior to CoCl2 treatment, the mtROS inhibitor blocked the additional increase of HIF-1α levels in WRN-depleted cells (only a 4.1-fold increase as compared to siCtrl-transfected cells without CoCl2) (Fig. 6A).

A major site of mtROS production is the mitochondrial complex III [43]. We therefore pretreated siCtrl- and siWRN-transfected cells with stigmatellin, a compound that decreases mtROS generation by inhibiting the ETC at complex III. Stigmatellin produced similar results as SkQ1 and inhibited increased HIF-1α accumulation in WRN-depleted cells (Fig. 6B). Finally, as expected [37,44], these two compounds did not affect HIF-1α accumulation by CoCl2 in siCtrl-transfected cells (results not shown). Taken together, our results indicate that mtROS generation is implicated in increasing HIF-1α protein accumulation induced by WRN depletion.

Increased HIF-1α protein accumulation in WRN-depleted cells is dependent on ascorbate levels

It was previously shown that ascorbate (vitamin C) delays cellular senescence in a continuous culture of Werner syndrome (WS) fibroblasts [46]. Intracellular ascorbate is an essential cofactor for the activity of prolyl hydroxylases [47,48]. Changes in the cellular levels of ascorbate have been linked to increased HIF-1 levels in certain cell systems [32,33,49]. Our previous studies showed that increased mtROS led to decreased cellular ascorbate levels, decreased hydroxylase activity and increased HIF-1α stability and accumulation [37]. We therefore determined the levels of ascorbate in WRN-depleted cells to assess its relation with HIF-1α accumulation. Forty-eight hours post-transfection, cells were loaded with 1 μM MitoSOX for 1 h prior to imaging as described in “Materials and Methods”. Differential interference contrast microscopy (DIC) was used for whole cell imaging. (B) Mean global fluorescence intensity of HeLa cells transfected with siCtrl and siWRN molecules. Mean fluorescence was calculated from 90 fields for each sample.

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in hypoxic conditions. As shown in Fig. 7 B and C, WRN-depleted cells increased HIF-1α accumulation by 2.0-fold over siCtrl-transfected cells in low oxygen. Interestingly, pretreating cells with ascorbate abolished this increase in HIF-1α accumulation (Fig. 7 C). Taken together, these results indicate that lower intracellular ascorbate levels may contribute, at least in part, to increased HIF-1α accumulation in WRN-depleted cells.

To determine the possible cause for lower intracellular ascorbate levels in WRN-depleted cells, we measured GLUT1, GLUT3, SVCT1, and SVCT2 mRNA levels by real time quantitative PCR. GLUT1 and GLUT3 import dehydroascorbic acid in cells, while the SVCT family of sodium-coupled transporters transport ascorbic acid [50–52]. As indicated in Fig. 7 D, both GLUT1 and GLUT3 mRNA levels were lower in siWRN-transfected cells as compared to siCtrl-transfected cells (37±5% and 46±21%, respectively). However, no significant changes were observed for mRNA expression of SVCT1 and SVCT2 (Fig. 7 E). These results suggest that decreased intracellular ascorbate may be caused by decreased GLUT1 and GLUT3 expression in WRN-depleted cells.

Lower levels of GLUT transporters in WRN-depleted cells could cause a decrease in glucose uptake and changes in the NADP+ /NADPH ratio. Such changes could, in return, affect ascorbate levels and modify rates of cell proliferation. Therefore, we first estimated the rate of cell proliferation in siWRN- and siCtrl-transfected cells. As indicated in Fig. 8, siWRN-transfected cells showed a reduced rate of growth than siCtrl-transfected cells (estimated doubling time of 25 and 39 h for siCtrl and siWRN-transfected HeLa cells, respectively). Additionally, as shown by FACS analyses, siWRN-transfected cells exhibited a reduced number of cells in S phase of the cell cycle (Fig. 8B) as compared to siCtrl-transfected cells (Fig. 8C and D). We next determined the levels of glucose uptake in siWRN- and siCtrl-transfected cells. As shown in Fig. 8E, WRN-depleted cells exhibited a significant 25% decrease in glucose uptake as compared to siCtrl-transfected cells. As expected, insulin increased glucose uptake by 45% in siCtrl-transfected cells. WRN-depleted cells did not significantly respond to insulin with regards to glucose uptake. Taken together, these results indicate that the decrease in cell proliferation in WRN-depleted cells is correlated with lower glucose uptake. Finally, we examined NADP+/NADPH ratio in WRN-depleted cells. As indicated in Fig. 8F, the NADP+/NADPH ratio was similar between siCtrl- and siWRN-transfected cells. These results indicate that the changes in ascorbate levels in WRN-depleted cells were not due to a significant modification of the NADP+/NADPH ratio in our experimental conditions.

HIF-1α is present in WRN-depleted cells

The presence of HIF-1α protein in the nucleus is essential for HIF-1 complex activation. siRNA-transfected cells, which were incubated in normoxic or low oxygen conditions, were fractionated into nuclear and cytoplasmic fractions followed by Western blot analysis to determine which fractions contained HIF-1α protein. As shown in Fig. 9A, HIF-1α levels were increased in the nuclear fraction of siWRN-transfected cells. HIF-1α protein was undetectable in the cytoplasmic fraction. WRN protein was also only detected in the nuclear fractions. PARP1 was used as a nuclear protein marker while α-tubulin was used as a cytoplasmic protein marker. These results indicate that HIF-1α is indeed localized to the nucleus when increased by WRN repression.

To directly determine the effects of WRN on HIF-1 complex activation, we used a HRE—controlled reporter assay. HeLa cells

Fig. 6 – mtROS are essential for increased HIF-1α accumulation during cellular WRN depletion. HeLa cells were transfected with a siRNA against WRN (siWRN) or a control sequence (siCtrl). 48 h post-transfection, cells were exposed or not to 100 μM CoCl2 for 1 h in the presence of a mitochondrial-targeted antioxidant, 250 nM SKQ1 (A) or an inhibitor of mitochondrial complex III, 1 μM stigmatellin. HIF-1α, WRN and α-tubulin levels were analyzed by Western blot. Western blots were quantified with the Odyssey Infrared Imaging System using α-tubulin as a loading control (lower panels). Results expressed as HIF-1α protein levels normalized to α-tubulin protein levels compared to untreated siCtrl cells are an average±SEM of three independent experiments. *P<0.1 as compared to untreated siCtrl cells. **P<0.01 as compared to CoCl2 treated siCtrl cells and ***P<0.001 as compared to CoCl2 treated siWRN cells (unpaired student t-test).
were transiently transfected with a 3HRE-tk-LUC reporter vector along with siRNAs followed by the treatment of cells in low oxygen conditions. Under these conditions, low oxygen led to a 3.8-fold induction in luciferase activity in siCtrl-transfected cells (Fig. 9B). More interestingly, reporter activity was significantly increased in siWRN-transfected cells (1.7-fold over siCtrl-transfected cells in low oxygen). Similar results were obtained when cells were treated with CoCl2. Finally, we examined the expression of VEGF mRNA, a well-known HIF-1 target gene in HeLa cells [30]. As shown in Fig. 9C, VEGF mRNA expression, assessed by Real-Time qRT-PCR analysis, was increased by 1.6-fold in siWRN-transfected cells compared to untreated siCtrl cells are an average ± SEM of three independent experiments. 

To determine whether other HIF-1 targets were affected in a similar manner, we measured the mRNA expression of phosphofructokinase (PFK), phosphoglycerate kinase 1 (PGK1), hypoxia inducible gene 2 (HIG2), and carbonic anhydrase IX (CA9) in WRN-depleted cells. As indicated in Supplemental Fig. 5, the depletion of WRN in HeLa cells caused a decrease in CA9 and PFK expression. To confirm the role of HIF-1 in increased VEGF mRNA expression in these conditions, a siRNA against HIF-1α (siHIF-1α) was co-transfected along with siWRN. Repression of HIF-1α levels significantly decreased VEGF mRNA expression to levels similar to those found in siCtrl-treated cells (Fig. 9C). Taken together, these results indicate that WRN represses HIF-1 activation and that WRN-depletion leads to increased HIF-1-mediated transcriptional activity and target gene expression.
mRNA expression. PGK1 was not affected. There was a tendency for HIG2 to increase in siWRN-transfected cells as compared to siCtrl-transfected cells. These results indicate that in HeLa cells, not all HIF-1 target genes behave in a similar manner upon WRN depletion.

Discussion

We recently observed that tissue from mice lacking part of the helicase domain of the WRN gene product homolog showed significant increases in HIF-1α protein levels [12]. Concomitantly with the increase in HIF-1α levels, these mice exhibit augmented oxidative stress and pro-inflammatory markers. Although such results need to be confirmed in Wrn null mice, not all HIF-1 target genes behave in a similar manner upon WRN depletion.

Fig. 8 – Growth rate, glucose uptake, and NADPH status of WRN-depleted HeLa cells. (A) Growth curves of siCtl- and siWRN-transfected cells. (B) Graph representing the percentage of siRNA-transfected cells in each phase of the cell cycle. (C) Example of cell cycle by FACS analyses with siCtl-transfected cells. (D) Example of cell cycle by FACS analyses with siWRN-transfected cells. (E) Intracellular uptake of radioactive glucose in siCtl- and siWRN-transfected cells with or without 100 nM insulin. (F) NADP/NADPH ratio in siCtl and siWRN-transfected cells. Results are an average ± SEM of three independent experiments. P-values were calculated using unpaired student t-test.
used in this study instead of shRNAs or strategies using retroviral vectors, to specifically target WRN mRNA directly without the requirement for cellular RNA synthesis or selection with resistance markers that could potentially affect the outcome of the expression profile. In our experience, transfection efficiency with siRNA molecules is more than 90% [55].

Our study provides evidence for an increased HIF-1α accumulation and activation in cells depleted for WRN. HIF-1α half-life evaluations along with pVHL interaction studies indicate that HIF-1α protein stability is increased in WRN-depleted cells. HIF-1α protein degradation is dependent on the activity of prolyl hydroxylase enzymes, which hydroxylate HIF-1α on conserved proline residues. In the process, hydroxylated HIF-1α becomes the target of pVHL, a E3 ubiquitin ligase component, and is marked for proteasomal degradation. Ascorbate is an important co-factor for prolyl hydroxylases, as it maintains the non-heme ferrous iron active site in its reduced form [56,57]. Indeed, supplementation with ascorbate in the culture media of WRN-depleted cells decreased intracellular HIF-1α levels. GLUT1 (and to some extent GLUT3) is a known transporter of oxidized ascorbate. Interestingly, despite the fact that GLUT1 is a known transporter of oxidized ascorbate, our study provides evidence for an increased HIF-1α accumulation and activation in cells depleted for WRN. GLUT1 protein stability is increased in WRN-depleted cells. HIF-1α accumulation and activation in cells depleted for WRN, GLUT1, and CA9 were also down-regulated in WRN-depleted cells. These data suggest that increased HIF-1α protein levels observed in WRN-depleted cells may also come from other intracellular sources [9].

We also show for the first time that WRN depletion specifically promotes the production of mitochondrial reactive oxygen species. Although increases in ROS observed previously in WRN-depleted cells may also come from other intracellular sources [9], the augmentation of mtROS involves the mitochondrial electron chain transfer reactions. During oxidative phosphorylation, ROS leak out from mitochondria. Complex III of the mitochondrial electron transfer chain is responsible for the production of cytoplasmic mtROS, which, in turn, affects several proteins. It has been shown that the mitochondrial complex III regulates the production of mitochondrial reactive oxygen species. Although increases in ROS observed previously in WRN-depleted cells may also come from other intracellular sources [9], the augmentation of mtROS involves the mitochondrial electron chain transfer reactions. During oxidative phosphorylation, ROS leak out from mitochondria. Complex III of the mitochondrial electron transfer chain is responsible for the production of cytoplasmic mtROS, which, in turn, affects several proteins. It has been shown that the mitochondrial complex III regulates both the non-hypoxic and hypoxic activation of HIF-1α in different cell models [37,43]. Our results using a complex III inhibitor, stigmatellin, or a specific inhibitor of mtROS indicate that these oxidative molecules are essential for generation of increased HIF-1α levels observed in WRN-depleted cells. From these observations, we suggest that increased HIF-1α protein levels seen in the liver of Wrn mutant mice is possibly also caused by increased mtROS generation in these animals [12]. Presently, the link between WRN and mtROS is unclear. However, our studies indicate that several nuclear encoded mitochondrial proteins

Fig. 9 – Cellular WRN depletion increases HIF-1-dependent transcriptional activity. (A) HeLa cells were transfected with a siRNA against WRN (siWRN) or a control sequence (siCtrl). Cells were then fractionated into cytoplasmic and nuclear fractions and WRN, HIF-1α, PARP1, and α-tubulin protein levels were analyzed by Western blot. (B) HeLa cells were transfected with a control siRNA (siCtrl; black bars) or a siRNA against WRN mRNA (siWRN; gray bars) along with 1 µg of pGL3(R2.2) 3HRE-tk-LUC and 250 ng of an expression vector coding for Renilla reniformis luciferase. Thirty-six hours post-transfection, cells were maintained under control conditions (normoxia), under 3% oxygen, or in the presence of 100 µM CoCl2 for 6 h. Cells were lysed and luciferase activity was measured. Results are expressed as a ratio of firefly luciferase activity to $R. reniformis$ luciferase activity and are an average ± SEM of at least 3 independent experiments performed in triplicate. ** $P<0.01$ and *** $P<0.001$ as compared to untreated (control) siCtrl cells. †† $P<0.01$ as compared to siCtrl cells under respective treatments. (C) HeLa cells were transfected with indicated siRNAs. Thirty-six hours post-transfection, cells incubated under 3% oxygen for 3 h followed by RNA extraction and cDNA synthesis. Real-time PCR was performed using VEGF and HPRT (internal control) oligonucleotides. Results are an average ± SEM of three independent experiments. $P$-values were calculated using unpaired student $t$-test.
are differentially expressed in WRN-depleted fibroblasts, which may affect mitochondrial activity [58]. In addition, WRN depletion also affects lipidogenesis in cells. Alteration in lipid metabolism is known to affect mitochondrial activity and intracellular redox status [59]. These results are consistent with the mitochondrial morphological changes that we detect in the liver of Wrn helicase mutant mice [60,61]. Finally, WRN depletion activates several protein kinase C isoforms that can affect mitochondrial function and ROS production in cells [62,63]. Future mass spectrometry analyses of mitochondrial protein in WRN-depleted cells will give a better picture of the status of the mitochondria in such cells.

To conclude, the present study provides an interesting link between age-related pathologies and the HIF system and identifies mitochondrial ROS as a common denominator in this regulation. We believe that the HIF-1 system may play an important role in certain WRN syndrome-associated pathologies.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2012.04.010.

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