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Hydrogen Peroxide Induces p16^{INK4a} Through an AUF1-Dependent Manner

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ABSTRACT

Elevation of p16^{INK4a} has been described as an important mechanism for hydrogen peroxide (H_2O_2)-induced replicative senescence. However, the mechanisms underlying remain unknown. In this study, we demonstrate an important role of RNA-binding protein AUF1-mediated mRNA turnover in H_2O_2-induced p16^{INK4a} expression. The induction of p16 by H_2O_2 was accompanied with declined cytoplasmic AUF1 level. Accordingly, exposure of cells to H_2O_2 remarkably reduced the binding of AUF1 to p16 3′UTR and increased the half-life of an EGFP-p16-3′UTR chimeric transcript. In AUF1-silenced cells, the effect of H_2O_2 on p16 induction was abolished. Furthermore, in cells co-transfected with vectors expressing AUF1s, treatment with H_2O_2 failed to significantly reduce the expression of AUF1 and subsequently elevate the levels of p16. Moreover, HeLa cells overexpressing AUF1s were resistant to H_2O_2-induced senescence. Our results indicate that AUF1 is critical for H_2O_2-induced p16 expression and cellular senescence. J. Cell. Biochem. 109: 1000–1005, 2010.

KEY WORDS: p16^{INK4a}; AUF1; HYDROGEN PEROXIDE; mRNA TURNOVER

The expression of p16^{INK4a}, a cyclin-dependent kinase inhibitor, has been found to increase following exposure of cells to a variety of extracellular stimuli [Ramirez et al., 2001], including DNA damage [Shapiro et al., 1998], oxidative stress [Chen, 2000; Frippiat et al., 2000; Chen et al., 2004], and chemotherapeutic drugs [Schmitt et al., 2002]. Increased p16 participates in mediating the growth arrest, senescence, and affects the outcome of the stressed cells, frequently favoring cell survival [Shapiro et al., 1998; Schmitt et al., 2002].

The expression of p16 could be regulated both at transcriptional and at post-transcriptional levels. For example, a few transcription factors, including Sp1, Ets1, Ets2, and Bmi-1, are reported to be involved in p16 regulation at transcriptional level during replicative senescence [Ohtani et al., 2001; Itahana et al., 2003; Guney et al., 2006; Wu et al., 2007]. In a previous study, we also demonstrated that p16 expression during replicative senescence was regulated by altered mRNA turnover. The p16 3′-untranslated region (3′UTR) was found to be a specific target of AUF1, an RNA binding protein implicated in mediating mRNA decay [Wang et al., 2005]. Oxidative stress-induced p16 expression and replicative senescence have been intensively reported [Chen, 2000; Frippiat et al., 2000; Chen et al., 2004]. However, the contribution of either transcriptional or post-transcriptional regulation has not been elucidated. Emerging evidences suggest that the stability of ARE-containing mRNAs can change dramatically in response to a variety of extracellular stresses [Burd and Dreyfuss, 1994; Jackman et al., 1994]. These observations prompt us to examine whether AUF1-mediated mRNA decay is involved in the regulation of p16 under oxidative stress. Here, we have investigated into the mechanisms underlying p16 expression after exposure of cells to hydrogen peroxide (H_2O_2). We provide strong evidence to support that AUF1-mediated mRNA decay is a major mechanism in regulating p16 expression under oxidative stress.

Gai E Guo and Li Wei Ma contributed equally to this work.

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MATERIALS AND METHODS

CELL CULTURE, TREATMENTS, FACS ANALYSIS, AND SENESCENCE-ASSOCIATED β-GALACTOSIDASE (SA-β-GAL) ACTIVITY

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in 5% CO_2. Thirty to 50% confluent cells were treated with H_2O_2 (Sigma, St. Louis, MO) at concentrations indicated and collected 4 h later for further analysis. For FACS analysis, cells were stained with propidium iodide (50 μg/ml) and analyzed for DNA content as described [Wang et al., 1998]. For assessment of SA-β-gal activity, cells were seeded in 30 mm diameter dishes, transfected either with vectors expressing AUF1s or with empty vector. Forty-eight hours later, cells were exposed to H_2O_2 (400 μM) for 4 h and cultured in regular medium for additional 24 h, whereupon SA-β-gal activity was tested as described previously [Wang et al., 2005].

WESTERN BLOT ANALYSIS

The total cell lysates and cytoplasmic fractions were prepared as previously described [Wang et al., 2000]. Whole-cell (20 μg) or cytoplasmic (40 μg) lysates were size fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride membranes. p16 protein levels were detected with a monoclonal antibody (Santa Cruz Biotechnology), AUF1 was detected with a polyclonal antibody (Upstate Biotechnology), and GAPDH and α-tubulin were detected with monoclonal antibodies (Abcam Biotechnology). After secondary antibody incubation, signals were detected by SuperSignal WestPico Chemiluminescent Substrate (Pierce) following the manufacturer's instruction and quantitated by densitometric analysis with ImageMaster VDS software.

RT-PCR AND mRNA HALF-LIFE MEASUREMENT

Total RNA was isolated and converted to cDNA using ThermoScript™ RT-PCR System (Invitrogen). PCR was carried out with primers 5′-CGATTGAAAGAACCAGAGAG-3′ and 5′-GTTCTGCCATTTGCTAGCAG-3′ for p16 (product of 440 bp), and primers 5′-CGAGTCAACGGATTTGGTGGTAT-3′ and 5′-AGCCTTCTCCATGGTGAAGAC-3′ for GAPDH (product of 320 bp), an endogenous normalization control.

The half-life of p16 mRNA was determined as previously described [Wang et al., 2005]. Briefly, HeLa cells stably expressing tTA were transiently transfected with the reporter gene vector expressing either EGFP (V) or EGFP-p16-3′UTR (FL) chimeric transcript for 48 h, then cells were exposed to H_2O_2 (400 μM) for 4 h. Doxycyclin (Dox, 1 μg/ml) was added to shut off the tTA-driven transcription, and the rates of clearance of the transcripts were monitored by real-time PCR.

PULL-DOWN ASSAY

To prepare the p16 3′UTR, primers 5′ (T7) CGATTGAAAGAACCAGAGAG-3′ and 5′GTTCTGCCATTTGCTAGCAG-3′ were used for PCR amplification of p16 3′UTR with a cDNA template. The 5′ primers contained the T7 promoter sequence 5′-CCAAGCTTCTAATACGACTCACTATAG GGAGA-3′ (T7). For biotin pull-down assays, PCR-amplified DNA was used as template to transcribe biotinylated RNA by using T7 RNA polymerase in the presence of biotin-UTP, and purified as described [Wang et al., 2005]. Six micrograms of biotinylated transcripts were incubated with 120 μg of cytoplasmic extracts for 30 min at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dynal, Oslo), and pull-down material was analyzed by Western blotting.

RNA INTERFERENCE AND TRANSFECTIONS

To transiently silence AUF1, AUF1 siRNA (GAUCCUAUCACAGGGCGAU), and a control (Ctrl) siRNA (AAGTGTAGTAGATCACCAGGC) were transfected at a concentration of 20 nM by oligofectamine (Invitrogen) following the manufacturer's recommendations. To overexpress AUF1, HeLa cells were co-transfected with pcDNA 3.1-AUF1p45, p42, p40, and p37 vectors by lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations. Cells were collected 48–72 h after transfection for further analysis.

RESULTS

H_2O_2 INDUCES P16 BUT REDUCES AUF1 LEVELS IN A DOSE-DEPENDENT MANNER

To explore the possible link of AUF1 to p16 regulation under oxidative stress, we examined the p16 and AUF1 levels after exposure of cells to different doses of H_2O_2. Shown in Figure 1A are Western blots of p16 and AUF1 levels following treatment of HeLa cells with different doses of H_2O_2 for 4 h. In agreement with previous observations [Frippiat et al., 2000; Chen et al., 2004], treatment of HeLa cells with 100, 200, or 400 μM H_2O_2 induced p16 expression ~2.5-, 3.0-, or 4.8-fold, respectively. Conversely, exposure of cells to the same doses of H_2O_2 reduced AUF1 levels ~1.3-, 1.7-, or 5-fold relative to that observed in untreated cells, respectively. These results suggest that AUF1 may be implicated in the regulation of H_2O_2-induced p16 expression.

H_2O_2-induced apoptosis has been intensively reported [Hiraoka et al., 1997; Wang et al., 1998]. To analyze cell viability, HeLa cells were exposed to H_2O_2 at indicated doses for 4 h and subjected to FACS analysis. As shown in Figure 1B, exposure of cells to 200 or 400 μM H_2O_2 exhibited 0.90% or 2.39% cell death (sub-G_1 compartments) relative to that observed in untreated cells (0.46%). In other words, no significant decrease of cell viability (M2) was observed (99.54% for untreated, 99.10% for 200 μM, and 97.61% for 400 μM H_2O_2-treated cells, respectively).

H_2O_2 INDUCES P16 MRNA LEVELS AND REDUCES BINDING OF AUF1 TO P16 3′UTR

Given the inverse expression patterns of AUF1 and p16 in cellular response to H_2O_2 and based on our previous finding that AUF1 is an important regulator for p16 mRNA instability, we hypothesize that AUF1-mediated mRNA decay may be a critical mechanism for the regulation of p16 under oxidative stress. To verify this hypothesis, the mRNA levels of p16 after H_2O_2 treatment were measured by RT-PCR. As shown in Figure 2A, treatment with 400 μM H_2O_2 increased p16 mRNA ~4.5-fold, suggesting that mRNA turnover mechanism may be involved in this process. We previously reported that the

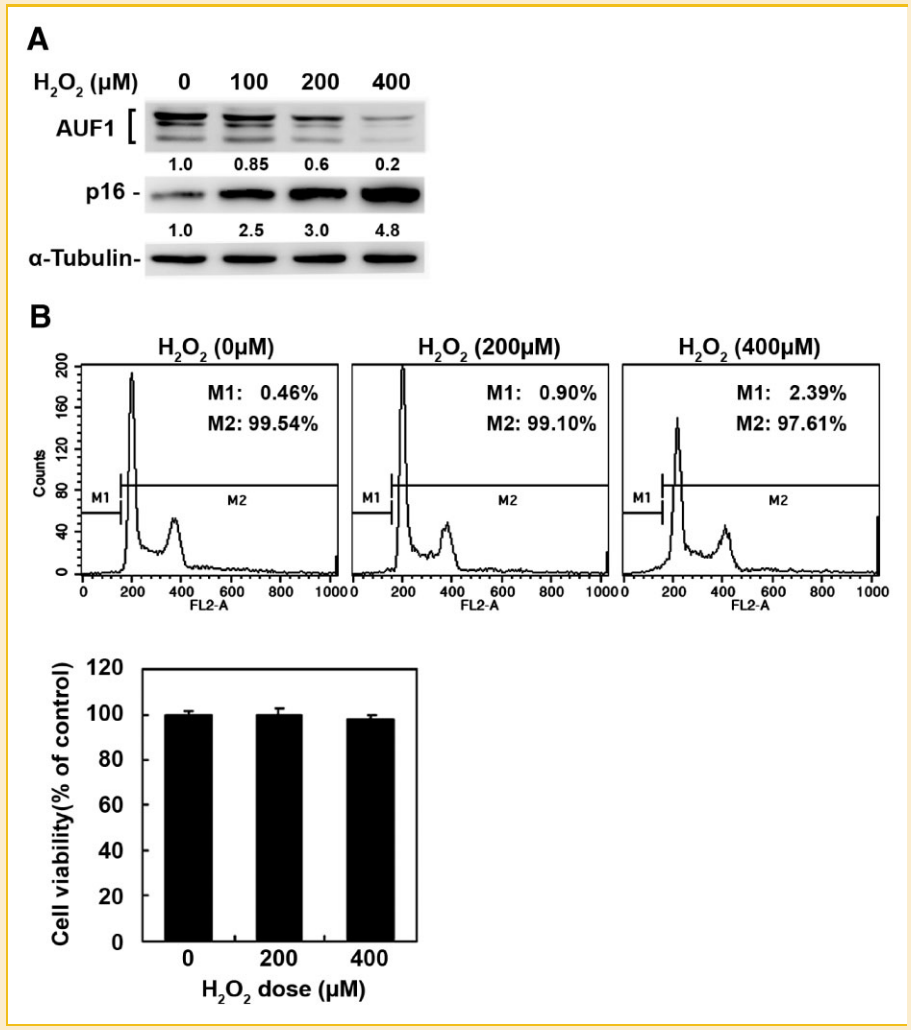

Fig. 1. H_2O_2 induces p16 but reduces AUF1 in a dose-dependent manner. A: Western blot analysis of AUF1 and p16 expression in untreated or H_2O_2-treated HeLa cells as indicated. α-tubulin was served as a loading control. The abundance of AUF1 and p16 was measured by densitometry and expressed as fold increase of control (untreated cells). B: Analysis of cell viability following H_2O_2 treatment. HeLa cells (1×10^7) were treated as in panel A. Apoptotic (sub-G_1 phase (M1)) and survival cells (M2) were determined by FACS analysis (upper panels). The cell viability was represented as percent of mean ± SEM from three independent experiments (bottom panel).

cytoplasmic presence is important for AUF1 to regulate the mRNA decay of cyclin D1 and p16 [Lin et al., 2000; Wang et al., 2005]. Therefore, the cytoplasmic fractions were prepared following H_2O_2 treatment and subjected to Western blot to analyze the cytoplasmic AUF1 levels. As shown in Figure 2B (upper and middle panels), consistent with the reduction of total AUF1, cytoplasmic AUF1 abundance was substantially decreased by H_2O_2 ~3.3-fold relative to that observed in untreated cells. The alteration in the cytoplasmic AUF1 was specific, as the cytoplasmic GAPDH was essentially unchanged after H_2O_2 treatment.

We next examined the association of cytoplasmic AUF1 with p16 mRNA after H_2O_2 treatment. For this purpose, biotinylated transcripts spanning the 3′UTR of p16 mRNA were prepared and incubated with cytoplasmic lysates in pull-down assays. As shown by Western blotting of AUF1 in the pull-down materials (Fig. 2B, bottom panel), AUF1 in H_2O_2-treated cells exhibited substantially reduced binding (~5-fold) to the 3′UTR of p16 mRNA, and this decrease was apparent in respect of all isoforms of AUF1 protein. After normalized to AUF1 levels in the respective lysates (middle panel), the binding decrease was amplified ~1.6-fold. Therefore, it remains possible that the post-translational modification may also be involved in influencing AUF1 binding affinity. However, we favor the view that the decrease in AUF1 binding by H_2O_2 treatment is mainly due to the reduction of AUF1 levels.

To further test whether the half-life of p16 mRNA was altered with H_2O_2 treatment, a transcriptional pulse strategy based on the Tet-regulatory system [Lin et al., 2000; Wang et al., 2005] was used. As shown in Figure 2C, the half-life of control transcript (EGFP (V)) was longer (longer than 8 h) and comparable in both the untreated (untr.) and H_2O_2-treated cells (H_2O_2) (Fig. 2C, left), while the half-life of EGFP-p16-3′UTR (FL) chimeric transcript was shorter (half-life ≈ 4.8 h) in untreated population (untr.) and significantly extended (longer than 8 h) by H_2O_2 treatment (Fig. 2C, right).

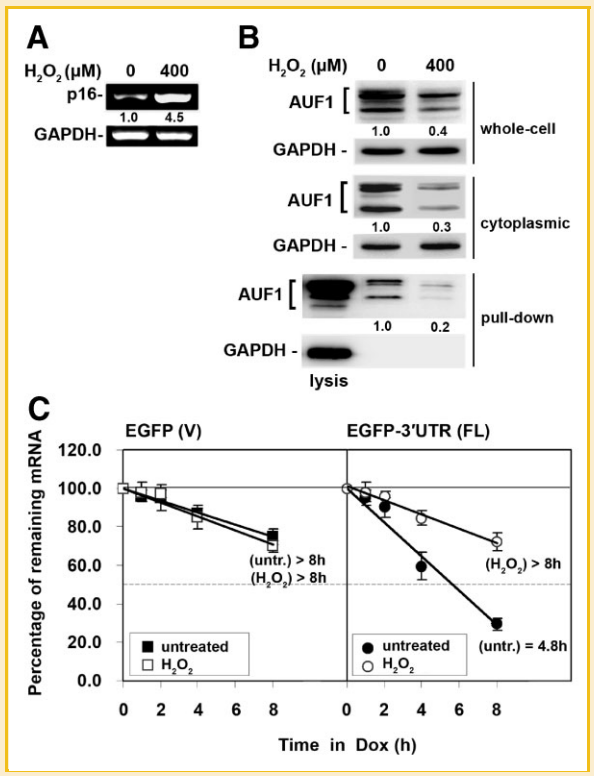

Fig. 2. H_2O_2 induces p16 mRNA levels and reduces the binding of AUF1 to p16 3′UTR. A: RT-PCR analysis of p16 mRNA levels in untreated or H_2O_2-treated HeLa cells. B: Western blot analysis of the total and cytoplasmic AUF1 following H_2O_2 treatment (400 μM, 4 h) (upper and middle panels). GAPDH was served as a loading control. Bottom panel, pull-down assay using biotinylated p16 3′UTR to detect bound cytoplasmic AUF1 in untreated or H_2O_2-treated samples by Western blotting. A 10 μg portion of cytoplasmic lysates (lysis) and binding of GAPDH to p16 mRNA (negative control) was included. C: mRNA half-life assay after exposure of the Tet-off cells to H_2O_2 (see RT-PCR and mRNA Half-Life Measurement Section).

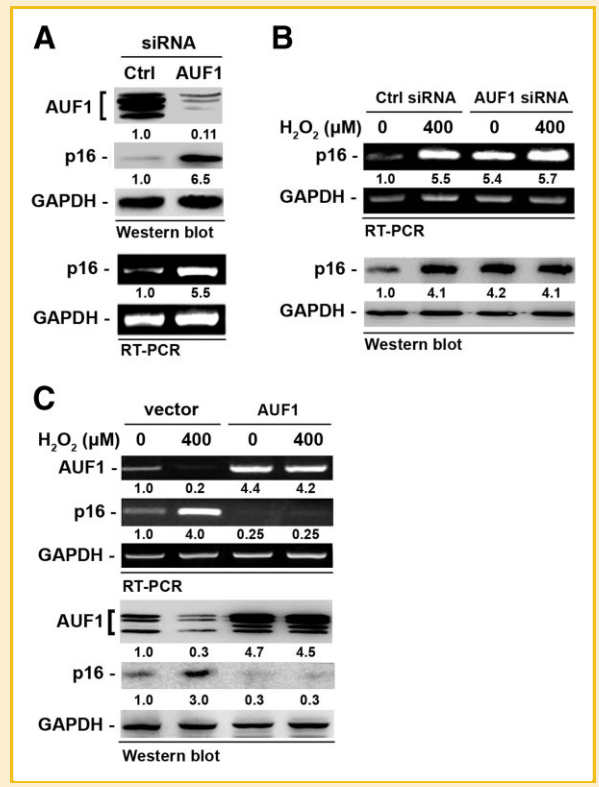

Fig. 3. AUF1 is critical for H_2O_2-induced p16 expression. A: Western blot or RT-PCR analysis of AUF1 and p16 expression in AUF1 siRNA (20 nM) or control siRNA transfected HeLa cells. B: RT-PCR and Western blot analysis of p16 expression in siRNA-transfected cells following H_2O_2 treatment (400 μM, 4 h). GAPDH was served as a loading control. The relative abundance of p16 was evaluated as described in Figure 1. C: RT-PCR and Western blot analysis of p16 and AUF1 levels in pcDNA-AUF1 plasmids (p45, p42, p40, and p37) transfected cells following H_2O_2 treatment (400 μM, 4 h). GAPDH was served as a loading control. The relative abundance of p16 was evaluated as described in Figure 1.

Together, our results suggest that AUF1-regulated p16 mRNA decay is involved in the up-regulation of p16 under H_2O_2 treatment.

AUF1 IS CRITICAL FOR H_2O_2-INDUCED P16 EXPRESSION AND CELLULAR SENESCENCE

The evidence presented thus far indicated that exposure to H_2O_2 decreased the formation of cytoplasmic AUF1-p16 mRNA complex and increased the stability of p16 3′UTR associated reporter transcript (EGFP-p16-3′UTR). To assess the contribution of AUF1-regulated mRNA decay in H_2O_2-induced p16 expression, we developed AUF1 knockdown cells through transfection with siRNA targeting all the AUF1 isoforms. The effect of the transfection was monitored by Western blot analysis. As shown in Figure 3A, AUF1 siRNA transfection reduced AUF1 expression ~89% relative to that seen in control siRNA transfected cells (Fig. 3A). In keeping with our previous study [Wang et al., 2005], knockdown of AUF1 substantially induced p16 expression in both protein (~6.5-fold, upper panels) and mRNA levels (~5.5-fold, down panels) (Fig. 3A). Using these transfectants, we investigated whether AUF1 is critical for H_2O_2-induced p16 expression. As shown in Figure 3B, in cells transiently transfected with the control siRNA, both mRNA (~5.5-fold) and protein levels (~4.1-fold) of p16 were induced by H_2O_2 treatment (400 μM). By contrast, H_2O_2 treatment failed to induce both mRNA (5.7-fold vs. 5.4-fold) and protein levels (4.1-fold vs. 4.2-fold) of p16 in AUF1-silenced cells. As a control, neither knockdown of AUF1 nor H_2O_2 treatment could influence GAPDH expression. Apparently, introduction of AUF1 siRNA greatly reduced endogenous AUF1 level (~11% of control) such that H_2O_2 could not bring it down much further, which led to p16 expression resistance to H_2O_2-triggered up-regulation.

To further investigate the contribution of AUF1-regulated mRNA decay in H_2O_2-induced p16 expression, AUF1 were overexpressed by co-transfecting HeLa cells with pcDNA-AUF1 p45, p42, p40, and p37 vectors for 48 h. Cells then were exposed to H_2O_2, and the expression of p16 was evaluated by RT-PCR and Western blot analysis. As shown in Figure 3C, transfection of cells with the pcDNA-AUF1 vectors increased AUF1 ~4.4-fold at mRNA and ~4.7-fold at protein levels. Consistently, overexpression of AUF1 reduced p16 ~4.0-fold at mRNA and ~3.3-fold at protein levels. However, neither the protein nor the mRNA level of AUF1 or p16

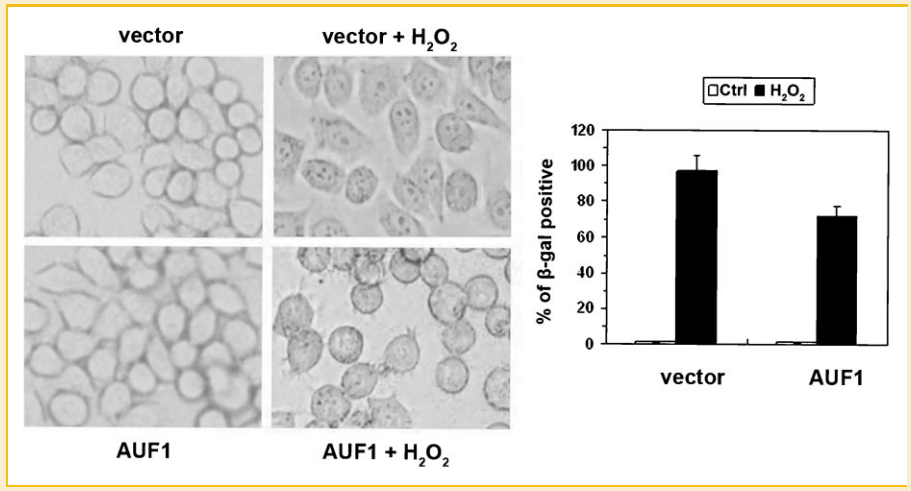

Fig. 4. HeLa cells overexpressing AUF1 were resistant to H_2O_2-induced senescence. HeLa cells were transfected either with plasmids expressing AUF1s (p45, p42, p40, and p37) or with empty vector. Forty-eight hours later, cells were exposed to H_2O_2 or left untreated for 4 h and cultured in regular medium for additional 24 h, whereupon SA-β-gal activity was assessed as described in the Materials and Methods Section (left, representative fields; right, quantification of SA-β-gal-positive cells).

could be significantly altered under H_2O_2 treatment, in contrast to that observed in empty vector transfected cells. A reasonable explanation is that the exogenous AUF1s do not contain the 5′ or 3′UTR and therefore are unable to respond to regulation either at transcriptional or mRNA turnover level following H_2O_2 treatment. In summary, our results suggest that the mRNA destabilization by AUF1 is a major mechanism for the induction of p16 under H_2O_2 treatment.

Because p16 is an important regulator of cellular senescence, we further tested whether the AUF1-p16 pathway is involved in the process of H_2O_2-induced cell senescence. To this end, HeLa cells were transfected either with vectors expressing AUF1s (p45, p42, p40, and p37) or with an empty vector. Forty-eight hours later, cells were exposed to H_2O_2 (400 μM) for 4 h and cultured in regular medium for additional 24 h. The cellular senescence was evaluated by assessment of SA-β-gal activity. As shown in Figure 4, the SA-β-gal activity that is largely absent in untreated (either AUF1s or empty vector transfected cells) cells was greatly elevated by H_2O_2 treatment. However, under H_2O_2 treatment, population where AUF1s were overexpressed displayed lower proportion of SA-β-gal-positive cells (~72%) than that observed in empty vector transfected cells (~97%). These results indicate that AUF1 could, at least partly, represses the effect of H_2O_2 in inducing cell senescence.

DISCUSSION

In this study, we have provided evidence that RNA binding protein AUF1-mediated p16 mRNA decay plays an essential role in the regulation of p16 under oxidative stress. We arrive at this conclusion based on the following observations. First, H_2O_2 induces p16 at both protein and mRNA levels but reduces the total and cytoplasmic AUF1 levels as well as the association of AUF1 with p16 3′UTR (Fig. 1A, Fig. 2A,B). Second, H_2O_2 increases the half-life of the EGFP-p16-3′UTR chimeric transcript (Fig. 2C). Third, p16 expression is not up-regulated in response to H_2O_2 treatment in AUF1-silenced cells (Fig. 3B). Finally, in cells co-transfected with vectors expressing AUF1 p45, p42, p40, and p37, which do not contain the 5′- or 3′UTR, exposure of cells to H_2O_2 failed to reduce the expression of AUF1 and subsequently elevate the expression of p16 (Fig. 3C); HeLa cells overexpressing AUF1s were resistant to H_2O_2-induced cell senescence (Fig. 4). These results suggest that AUF1 acts as an indispensable regulator for p16 expression and cell senescence under H_2O_2 treatment.

To date, the inducible effect of H_2O_2 on p16 expression and replicative senescence has been intensively reported [Chen, 2000; Frippiat et al., 2000; Chen et al., 2004]. However, the underlying mechanisms serving to regulate p16 expression have not been fully elucidated. We previously described that AUF1-regulated mRNA decay is an important mechanism for the up-regulation of p16 in replicative senescence [Wang et al., 2005]. The present study suggests that AUF1-mediated mRNA decay acts as a major mechanism for H_2O_2-induced p16 expression and cell senescence. The reduction of the cytoplasmic AUF1 level and its association with p16 mRNA during replicative senescence [Wang et al., 2005] indicates that AUF1-regulated p16 expression may not be limited to oxidative stress. Although both transcriptional and post-transcriptional regulations are important for p16 expression, the relative contribution is likely to vary in different conditions. Our findings support an essential role for AUF1 in regulating p16 expression under oxidative stress. Given that the large number of stress-regulated genes containing AU-rich elements in the 3′UTR of their mRNAs, it is likely that AUF1 plays a broad role in regulating gene expression during stresses, permitting the cell to respond to environmental changes.

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