Nutlin-3 induces HO-1 expression by activating JNK in a transcription-independent manner of p53

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Abstract. A recent study reported that p53 can induce HO-1 by directly binding to the putative p53 responsive element in the HO-1 promoter. In this study, we report that nutlin-3, a small molecule antagonist of HDM2, induces the transcription of HO-1 in a transcription-independent manner of p53. Nutlin-3 induced HO-1 expression at the level of transcription in human cancer cells such as U2OS and RKO cells. This induction of HO-1 did not occur in SAOS cells in which p53 was mutated and was prevented by knocking down the p53 protein using p53 siRNA transfection, but not by PFT-α, an inhibitor of the transcriptional activity of p53. Accompanying HO-1 expression, nutlin-3 stimulated the accumulation of ROS and the phosphorylation of MAPKs such as JNK, p38 MAPK and ERK1/2. Nutlin-3-induced HO-1 expression was suppressed by TEMPO, a ROS scavenger, and chemical inhibitors of JNK and p38 MAPK but not ERK1/2. In addition, nutlin-3-induced phosphorylation of JNK but not p38 MAPK was inhibited by TEMPO. Notably, the levels of nutlin-3-induced ROS were correlated with the mitochondrial translocation of p53 and this induction was prevented by PFT-μ, an inhibitor of the mitochondrial translocation of p53. Consistent with the effect of the ROS scavenger and MAPK inhibitors, PFT-μ reduced HO-1 expression and the phosphorylation of JNK induced by nutlin-3. In the experiments of analyzing cell death, the knockdown of HO-1 augmented nutlin-3-induced apoptosis. Collectively, these results suggest that nutlin-3 induces HO-1 expression via the activation of both JNK which is dependent on ROS generated by p53 translocated to the mitochondria and p38 MAPK which appears to be stimulated by a ROS-independent mechanism, and this HO-1 induction may inhibit nutlin-3-induced apoptosis, constituting a negative feedback loop of p53-induced apoptosis.

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Abbreviations: DDR1, discoidin domain receptor 1; H_2DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; ERK, extracellular signal-regulated kinases; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HO-1, heme oxygenase-1; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MDM2, murine double minute 2; MnSOD, manganese superoxide dismutase; PFT, pifithrin; ROS, reactive oxygen species; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy

Key words: heme oxygenase 1, c-jun N-terminal kinase, murine double minute 2 protein, nutlin-3, tumor suppressor protein p53

Introduction

The tumor suppressor protein p53 is a transcription factor that orchestrates anti-carcinogenesis programs such as cell cycle arrest, apoptosis, DNA repair and senescence in response to genotoxic and non-genotoxic cellular injuries (1,2). Although various transcriptional target genes of p53 contribute to the suppression of tumor development, they are not always compatible in terms of cell survival. For example, several p53 target genes that are involved in cell cycle arrest and DNA repair such as p21WAF1 and 14-3-3σ, an inhibitor of G1-S and G2-M transition, respectively, and Ku70, a non-homologous end joining repair gene, inhibit DNA damage-induced p53-dependent apoptosis (3-5). In addition, transcriptional target genes of p53 that protect various cells from apoptosis have been identified. In γ-irradiated hematopoietic progenitors, p53 induces SLUG, which functions to repress PUMA, thereby inhibiting p53-induced apoptosis (6). PIDD, which is induced by p53 upon double strand DNA breaks, can activate NF-κB, thus contributing to the inhibition of apoptosis and cancer cell survival (7). p53 also activates cell survival signaling such as the Ras-Raf-MEK1/2-ERK1/2 pathway and PI3K/AKT via the transcriptional induction of HB-EGF and DDR1, the blocking of which results in the augmentation of genotoxic stress-induced apoptosis (8,9). These survival target genes of p53 are simultaneously induced with the induction of apoptotic target genes of p53, resulting in fine-tuning or constituting the negative feedback loop of p53-induced apoptosis. Therefore, it is possible that p53 may induce cell survival signaling as well as apoptosis. Nutlin-3, a cis-imidazoline analog, was initially developed as an antagonist of MDM2, an E3 ubiquitin ligase of proteins of the p53 family, that ubiquitinates and directs them to proteasomal degradation (10).

Nutlin-3 interferes with the binding between MDM2 and p53 by preoccupying the p53 binding pocket of MDM2. *In vitro* and *in vivo* treatments of nutlin-3 induce p53 functions, such as cell cycle arrest and apoptosis (11,12). Nutlin-3 does not

induce damage to genomic DNA to activate the p53-dependent pathway so that the adverse effect on non-transformed cells and the risk of tumor development secondary to nutlin-3 treatment would be expected to be much less than conventional chemotherapeutic agents which induce DNA damage (13). However, it has been reported that nutlin-3 predominantly induces cell cycle arrest in cancer cells particularly derived from solid tumors rather than apoptosis (14). Nutlin-3 induces prominent p21WAF1 expression by upregulating hnRNP K, a coactivator of the transcriptional activity of p53, and downregulating HIPK2, an activator of p53-induced apoptosis, that phosphorylates Ser 46 on p53 (15,16). In addition to these genetic interactions, it is likely that nutlin-3 activates other cell survival pathways as well as apoptosis and such cell survival pathways may inhibit the nutlin-3-induced apoptotic signaling pathway. During our studies directed at cell survival pathways that are modulated by nutlin-3, we found that, along with inducing apoptosis, nutlin-3 activates ERK1/2 (17). It should be noted that nutlin-3-induced ERK1/2 activation was independent of the transcriptional activity of p53. Instead, nutlin-3 induces the mitochondrial translocation of p53 and subsequently ROS accumulation, which activates the MEK1/2-ERK1/2 pathway to confer survival characteristics to cancer cells.

Heme oxygenase-1 (HO-1, EC1:14.99.3) is a microsomal enzyme that catalyzes the degradation of heme derived from heme-containing proteins (18). The degradation of heme results in the production of biliverdin, ferrous iron and carbon monoxide (CO). Because these metabolites have anti-inflammatory and anti-apoptotic effects, it is possible that HO-1 has the potential for anti-inflammatory activity, which could protect cells against cellular insults, including oxidative stress. Chronic inflammation and oxidative stress are frequently associated with the initiation and progression of cancer and it can therefore be assumed that HO-1 would be elevated in cancer tissues and if so, HO-1 could endow cancer cells with growth-enhancing characteristics by inhibiting apoptosis (19). The expression of HO-1 was reported to be higher in various cancer tissues than in normal tissues (20). Moreover, the knockdown of HO-1 results in cancer cells being more susceptible to anticancer drug treatment (21,22). The expression of HO-1 is usually regulated at the transcriptional level, which occurs via a three-layered pathway i.e. challenging of the stimuli-activation of MAPK-activation of transcription factors. The transcription of HO-1 can be induced by a wide range of stimuli including oxidative and pro-inflammatory stress, various chemicals and a change in extracellular pH (18,23). These stimuli activate one or more of the MAPKs such p38 MAPK, JNK, and ERK1/2 and PI3K/AKT, which, in turn, direct various transcription factors to bind to their cognate sites in the promoter of HO-1. Among the transcription factors, Nrf2, AP-1, NF-κB and HSF are the most responsible for activating HO-1 transcription (24). Interestingly, a recent report showed that p53 induces the expression of HO-1 and thus protects cells against oxidative stress-induced apoptosis, suggesting that p53 itself contributes to the survival of cancer cells, but not cell death (25).

This report prompted us to speculate that nutlin-3 may also stimulate the expression of HO-1, and the possibility exists that this induction could inhibit the nutlin-3-induced apoptosis, resulting in predominant cell cycle arrest. To address this hypothesis, we examined the induction of HO-1 in nutlin-3-treated cells and analyzed the underlying mechanism of HO-1 responsible for induction by nutlin-3.

Materials and methods

Cell culture. Human osteosarcoma U2OS cells and human colon cancer RKO cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin/streptomycin (HyClone) and glutamate (Invitrogen, Calsbad, CA, USA) at 37˚C under 5% CO_2. Cells were sub-cultured or refreshed with media every 3 days.

Chemicals. Nutlin-3 was purchased from Selleck Chemicals (Houston, TX, USA). Pifithrin (PFT)-α, PFT-μ and TEMPO were obtained from Sigma-Aldrich (St. Louis, MO, USA). Inhibitors of MAPK including SB203580, SP600125 and U0126 were obtained from TOCRIS Bioscience (Bristol, UK) or AdooQ Bioscience (Irvine, CA, USA). Other chemicals were obtained from Sigma-Aldrich, unless otherwise specified.

Immunoblot analysis. For immunoblot analysis, U2OS cells were treated as described in the figure legends and were lysed with RIPA buffer. Following a protein assay, equal amounts of proteins of each sample were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were submerged in TBS-T (Tris-buffered saline with 0.025% Tween-20) containing 5% skim-milk for 30 min. The NC membranes were then processed sequentially for incubation with primary antibodies against proteins of interest, washing with TBS-T, incubation with secondary antibodies, and washing with TBS-T. Finally, the protein that reacted with the primary antibody of interest was visualized by an enhanced chemiluminescence detection method (ECL, GE Healthcare, Buckinghamshire, UK).

Real-time quantitative reverse-transcription PCR (QRT-PCR). Transcripts of HO-1 and p21WAF1 were measured by QRT-PCR using GAPDH as the reference gene following a previous report (17). Briefly, first strand cDNA synthesis from total RNA and subsequent QRT-PCR were performed using a PrimeScript™ RT reagent Kit (Takara Bio Inc., Shiga, Japan) and SYBR Premix Ex Taq (KAPA), respectively. All reactions were performed in triplicate in an ABI 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Relative changes of transcripts level were calculated by the ΔΔCt method (26).

Luciferase reporter assay. A human HO-1 promoter (hHO-1) cloned into a basic pGL3 plasmid was obtained from Professor J. Alam at the Ochsner Medical Center, New Orleans, LA, USA. Cells were transfected with 0.3 μg of hHO-1 promoter-luciferase and 0.03 μg of *Renilla* luciferase (Promega, Madison, WI, USA) using FuGENE HD (Roche Applied Science, Indianapolis, IN, USA) for 24 h, and cells were treated with nutlin-3. At the indicated times after the treatments, the activities of firefly and *Renilla* luciferase were determined using the Dual Luciferase kit (Promega) and the data are expressed as relative luciferase activity (RLA) of three independent experiments performed in triplicate. *Renilla* luciferase activity was for the normalization of transfection efficiency.

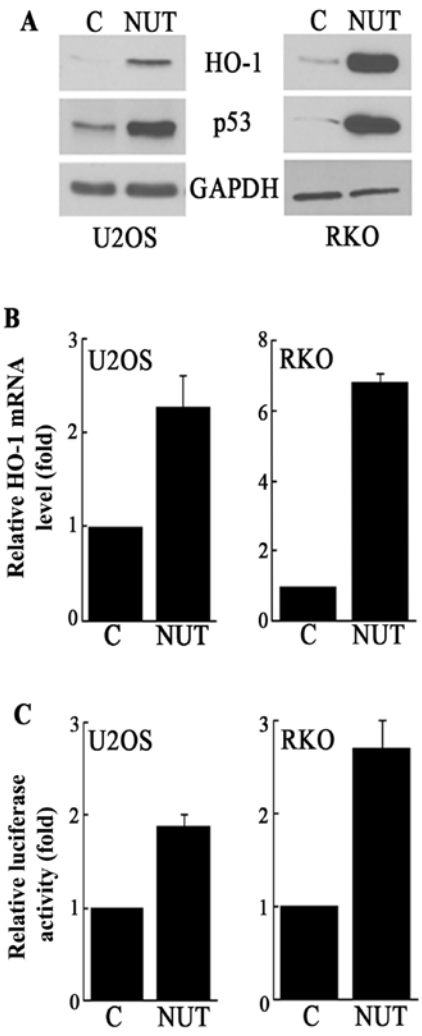

Figure 1. The induction of HO-1 by nutlin-3 at the transcriptional level. (A) Whole cell lysates of U2OS and RKO cells treated with vehicle (C) or 20 μM nutlin-3 (NUT) for 24 h were subjected to immunoblot analysis against HO-1, p53 and GAPDH used as a protein loading control. (B) Total cellular RNA from U2OS and RKO cells treated with vehicle (C) or 20 μM nutlin-3 (NUT) for 24 h were subjected to QRT-PCR against HO-1 and GAPDH as an endogenous reference. (C) U2OS and RKO cells were transfected with human HO-1 promoter-firefly luciferase contruct (pGL3-hHO-1, 0.03 μg) along with *Renilla* luciferase (0.003 μg) to normalize transfection efficiency, and 24 h later, were treated with vehicle (C) or 20 μM nutlin-3 (NUT). At 24 h post-treatment of nutlin-3, firefly and *Renilla* luciferase activities were measured and are presented as relative luciferase activity. The value presented is the mean ± SD of three independent experiments performed in triplicate.

Transfection of siRNA. Small interfering RNA against p53 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and siRNA against HO-1 and scrambled siRNA were from Sigma-Aldrich. They were dissolved in RNase-free H_2O and diluted with siRNA diluent. SiRNAs were transfected into cells using Lipofectamine RNAiMAX™ (Invitrogen) following the manufacturer's instruction.

Measurement of ROS. Intracellular ROS levels were measured using H_2DCF-DA dye (Invitrogen). After cells were incubated in the presence of 20 μM H_2DCF-DA, the intensity of the fluorescence in cells was observed by means of a fluorescence inverted microscope (Olympus IX71, Tokyo, Japan) and was quantified by flow cytometry.

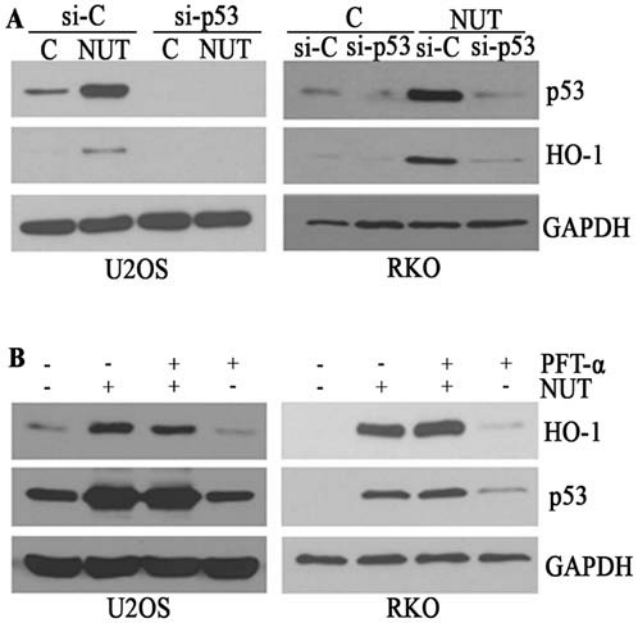

Figure 2. The transcriptional activity of p53 is not required for nutlin-3-induced HO-1 expression. (A) U2OS and RKO cells transfected with siRNA against p53 (si-p53) or scrambled siRNA (si-C) used as negative control for 48 h were treated with 20 μM nutlin-3 (NUT) for an additional 24 h. The whole cell lysates were then subjected to immunoblot analysis. (B) Cells were incubated in the absence or presence of 20 μM PFT-α for 1 h and treated with vehicle (C) or 20 μM nutlin-3 (NUT). At 24 h post-treatment of nutlin-3, cells were subjected to immunoblot analysis. GAPDH was used to normalize the protein loading.

Assessment of cell death. To measure the translocation of phosphatidylserine in the cytoplasmic membranes, treated cells were stained with propidium iodide (PI) and FITC-labeled Annexin V using ApoScan Kit (BioBud, Gyunggido, Korea), followed by flow cytometry analysis as described previously (27). Emissions of Annexin V-FITC and PI were measured in the FL1 and FL3 channels with emission filters of 488 and 635 nm, respectively.

Results

Nutlin-3 induces the expression of HO-1 at the transcriptional level. It was recently reported that p53 directly activates the transcription of HO-1 in response to treatment with H_2O_2 (25). We therefore attempted to observe whether the activation of p53 by nutlin-3, an antagonist of MDM2, stimulates the expression of HO-1. As shown in Fig. 1A, the nutlin-3 treatment resulted in increased levels of the HO-1 protein as well as the p53 protein in both U2OS (human osteosarcoma) and RKO (human colon cancer) cells. This increase in HO-1 protein levels was accompanied by an increase in HO-1 mRNA and HO-1 promoter activity (Fig. 1B and C). Therefore, these data demonstrate that nutlin-3 induces HO-1 expression at the level of transcription.

Nutlin-3-induced HO-1 is dependent on p53 but not the transcriptional activity of p53. Next, since nutlin-3 is an antagonist of MDM-2, a ubiquitin ligase of proteins of the p53 family, and in fact, nutlin-3 was also reported to enhance the function of p73 (28), it became necessary to determine the role of p53 in this HO-1 induction. In experiments using siRNA against p53, nutlin-3 failed to induce HO-1 expression in p53-knocked down cells (Fig. 2A) as well as SAOS cells in which p53 was mutated

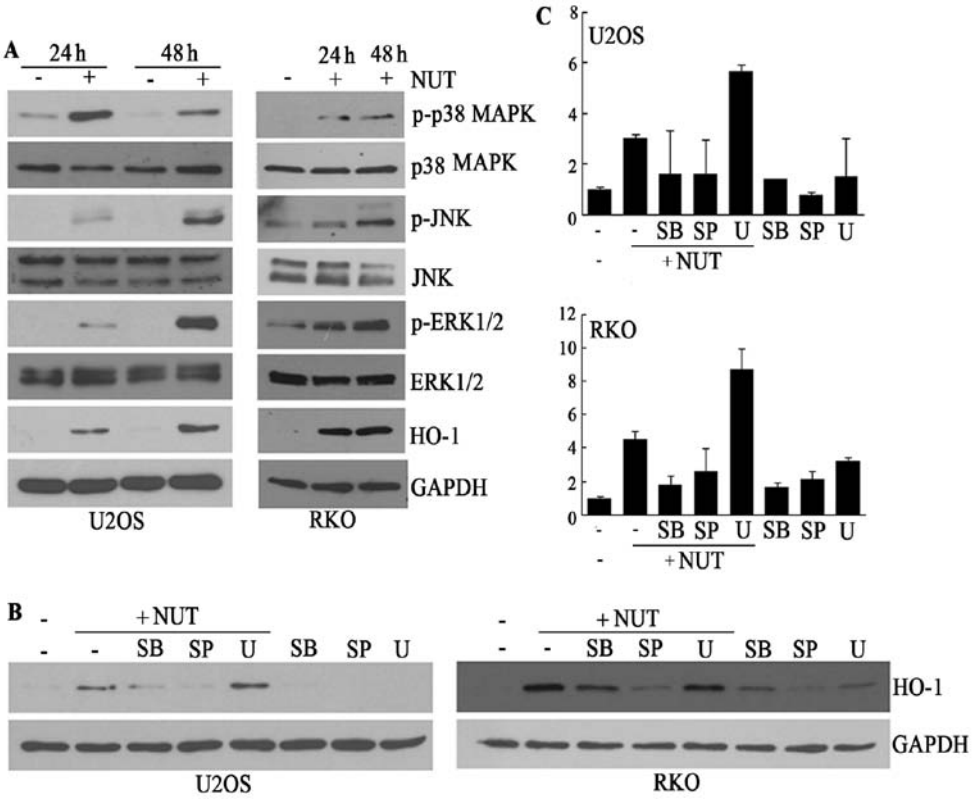

Figure 3. The involvement of JNK and p38 MAPK in nutlin-3-induced HO-1. (A) U2OS and RKO cells were treated with vehicle (C) or 20 μM nutlin-3 (NUT). At 24 and 48 h post-treatment, cells were subjected to immunoblot analysis against the indicated proteins. (B) U2OS and RKO cells were incubated in the absence or presence of SB203580 (SB), SP600125 (SP) or U0126 (U) for 1 h, and treated with vehicle (C) or 20 μM nutlin-3 (NUT) for another 24 h. Cell lysates were then subjected to immunoblot analysis against HO-1. (C) Total cellular RNAs of U2OS and RKO cells treated the same as above (B) were subjected to QRT-PCR against HO-1 mRNA and GAPDH mRNA as an endogenous normalizer. The normalized HO-1 mRNA levels were compared to that of vehicle-treated cells (C) and expressed as relative HO-1 mRNA level. The data are presented as mean ± SD of three independent experiments performed in triplicate. p-p38 MAPK, phospho-p38 MAPK (T180/Y182); p-JNK, phospho-JNK (T183/Y185); p-ERK1/2, phospho-ERK1/2 (T202/Y204).

(data not shown), indicating that p53 is indispensible for this nutlin-3-induced HO-1 expression. However, PFT-α, an inhibitor of the transcriptional activity of p53 did not interfere with the increase in HO-1 levels in nutlin-3-treated cells (Fig. 2B). Collectively, these data suggest that the nutlin-3-induced HO-1 expression is dependent on functions other than the transcriptional activity of the p53 protein and that HO-1 may not be a direct transcriptional target of p53 in nutlin-3-treated cells.

Nutlin-3-induced HO-1 is dependent on JNK and P38 MAPK activation. Since MAPK has been shown to be a critical mediator of HO-1 induction in many models and moreover, nutlin-3 can activate ERK1/2, independent of the transcriptional activity of p53 (17), we speculated that nutlin-3-activated MAPKs, including ERK1/2, may mediate the induction of HO-1 transcription. Based on this speculation, we analyzed the activation of MAPK by nutlin-3. As shown in Fig. 3A, nutlin-3 clearly induced the phosphorylation of p38 MAPK, JNK, and ERK1/2 accompanied by the induction of HO-1 and p53. The phosphorylation of MAPK, except for p38 MAPK, was dependent on incubation time up to 48 h, which was also the case for HO-1 and p53, implying that JNK and ERK1/2 might be mediators of HO-1 transcription. However, contrary to this expectation, the inhibition of JNK and p38 MAPK, but not ERK1/2, by chemical inhibitors prior to the nutlin-3 treatment suppressed the elevation of both HO-1 protein and mRNA (Fig. 3B and C). These findings, therefore, suggest that the p53 protein levels that were elevated as the result of the nutlin-3 treatment induced the transcription of HO-1 via the activation of JNK and p38 MAPK.

Nutlin-3-induced HO-1 and JNK activation but not p38 MAPK activation is dependent on ROS generation. In a previous report, we showed that nutlin-3-induced ERK1/2 activation was due to ROS, which prompted us to analyze the effect of ROS on HO-1 induction. As reported previously, nutlin-3 was found to induce the accumulation of ROS in both U2OS and RKO cells (Fig. 4A). In addition, TEMPO, a ROS scavenger, suppressed the induction of HO-1 protein expression as well as ROS accumulation (Fig. 4B). Under these conditions, the phosphorylation of JNK but not p38 MAPK was inhibited (Fig. 4B). These findings suggest that the nutlin-3-upregulated p53 may induce ROS generation, which, in turn, would activate JNK, which mediates the transcriptional induction of HO-1, and that the activation of p38 MAPK occur via a different mechanism than that for JNK activation, irrespective of whether ROS is present or not.

Nutlin-3-induced HO-1 is dependent on mitochondrial translocation of p53. The above data showing that the nutlin-3-induced formation of HO-1 is dependent on p53-induced ROS genera-

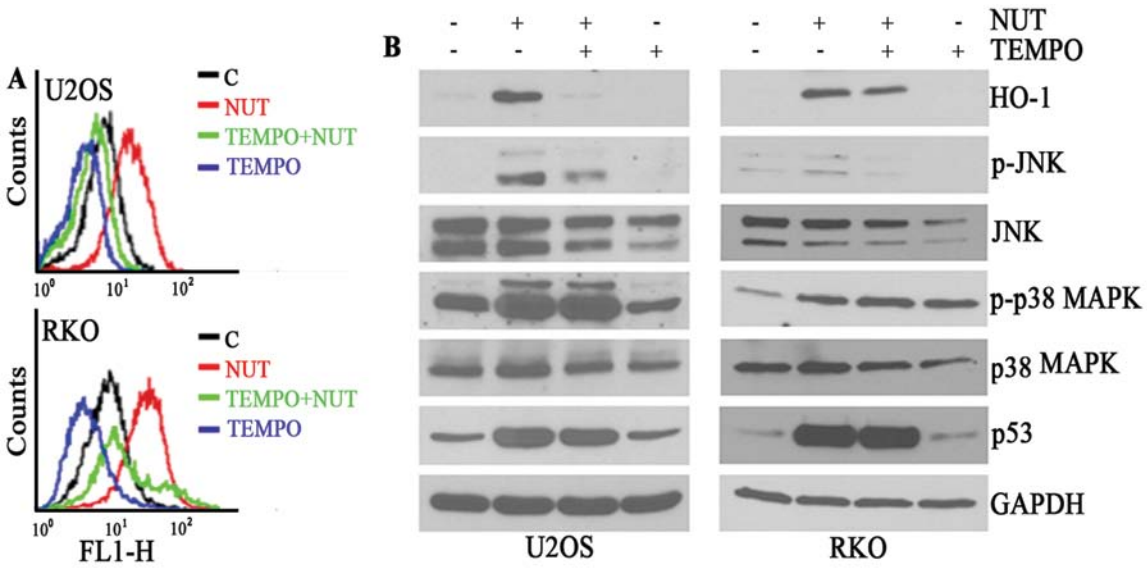

Figure 4. The role of ROS in nutlin-3-induced HO-1 expression. (A) U2OS and RKO cells were incubated with vehicle (C) or TEMPO for 1 h, and then treated with 20 μM nutlin-3 (NUT) for a further 24 h. Cells were then stained with H2DCF-DA and the intensity of cellular H2DCF-DA was measured by flow cytometry. (B) Whole cell lysates treated the same as above (A) were subjected to immunoblot analysis against indicated proteins using GAPDH as a loading control. p-p38 MAPK, phospho-p38 MAPK (T180/Y182); p-JNK, phospho-JNK (T183/Y185).

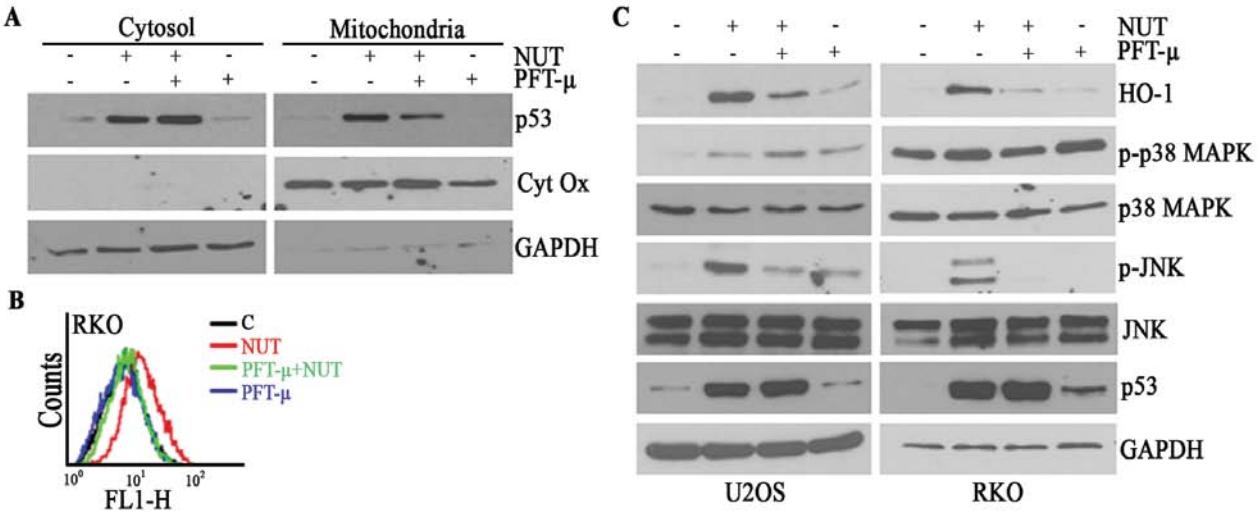

Figure 5. Dependency of nutlin-3-induced HO-1 on the mitochondrial translocation of p53. (A) RKO cells incubated with vehicle (C) or 10 μM PFT-μ for 1 h, and successively treated with 20 μM nutlin-3 (NUT) for another 24 h were fractionated into cytosol and mitochondria fractions. Each fraction was subjected to immunoblot analysis. (B) RKO cells were treated the same as above (A). At 24 h post-treatment of nutlin-3, cells were stained with H2DCF-DA and the cellular intensity of H2DCF-DA was measured by flow cytometry. (C) U2OS and RKO cells were treated with vehicle or 10 μM PFT-μ for 1 h, and incubated with vehicle (C) or 20 μM nutlin-3 (NUT) for another 24 h. Cells were then subjected to immunoblot analysis against indicated proteins using GAPDH as a loading control. Cyt Ox, cytochrome *c* oxidase; p-p38 MAPK, phospho-p38 MAPK (T180/Y182); p-JNK, phospho-JNK (T183/Y185).

tion regardless of the transcriptional activity of p53 led us to examine the generation of ROS by mitochondrial p53. We and others recently reported that p53 moves to mitochondria, where it stimulates the generation of ROS. Also in this model, nutlin-3 induced the mitochondrial translocation of p53, which was prevented by PFT-μ pretreatment in U2OS (data not shown) and RKO (Fig. 5A) cells. PFT-μ also prevented the accumulation of ROS in these cells (Fig. 5B), implying that the mitochondrial translocation of p53 plays a pivotal role in ROS generation. Consistent with the suppressive effect of TEMPO on the phosphorylation of JNK and the resulting HO-1 expression, PFT-μ reduced the nutlin-3-induced phosphorylation of JNK as well as the level of HO-1 expression (Fig. 5C). These findings suggest that both nutlin-3-induced HO-1 expression and the phosphorylation of JNK can be attributed to the ROS generated subsequent to the mitochondrial translocation of p53.

The effect of HO-1 on the nutlin-3-induced apoptosis. Because HO-1 is an anti-apoptotic protein, we examined the effect of HO-1 induction on apoptosis in this model. As expected, the knockdown of HO-1 using siRNA against HO-1 significantly increased Annexin V-positive cells in nutlin-3-treated U2OS and

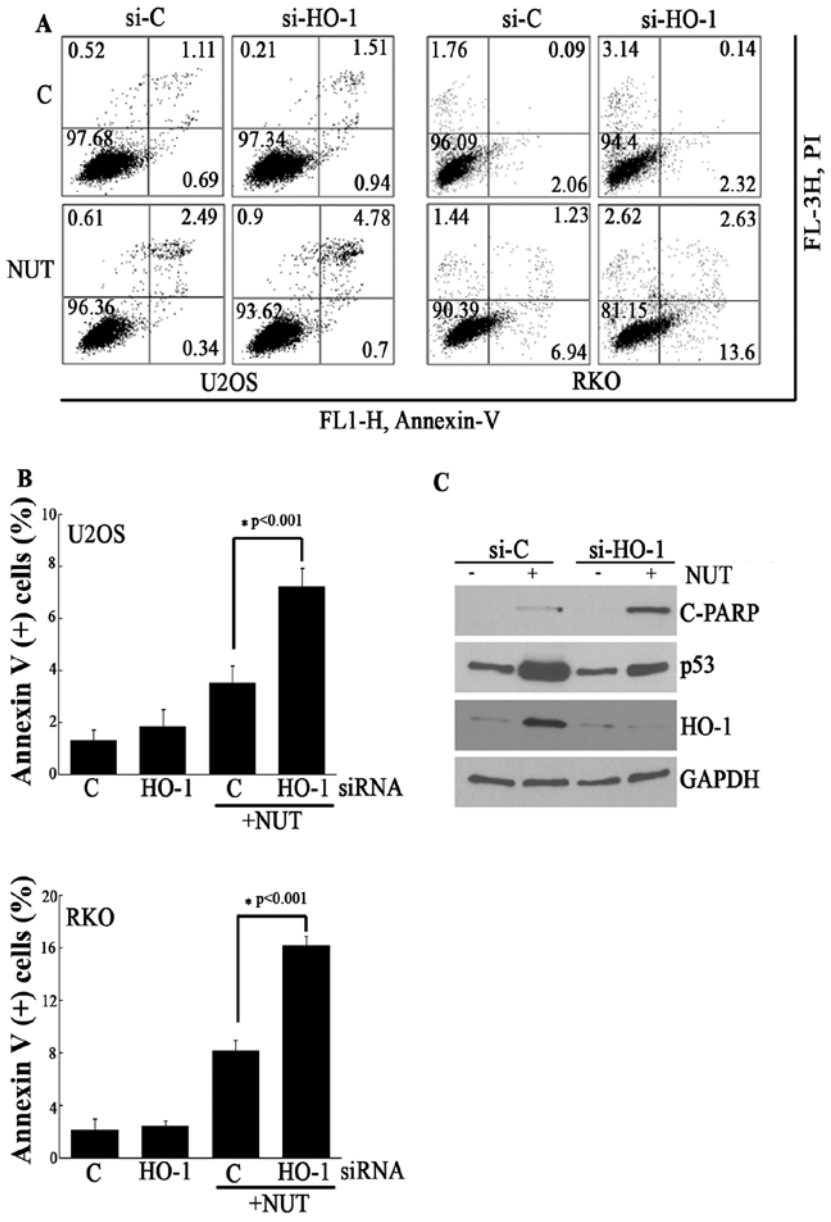

Figure 6. The effect of HO-1 siRNA on nutlin-3-induced apoptosis. (A) U2OS and RKO cells were transfected with siRNA against HO-1 (si-HO-1) or scrambled RNA (si-C) as a negative control for 48 h, and treated with vehicle or 20 μM nutlin-3 (NUT). At 24 h post-treatment of nutlin-3, cells were stained with Annexin V and PI, and the intensities of stained cells were measured by flow cytometry. (B) Total Annexin V positive cells are presented as percentage of population. The value represents the mean ± SD of three independent experiments performed in triplicate. Statistical significance was decided by one-way ANOVA test. P-values were <0.001. (C) U2OS cells transfected with si-HO-1 or si-C for 48 h, were treated with vehicle (C) or 20 μM nutlin-3 for 24 h. Cells were then subjected to immunoblot analysis against the indicated proteins using GAPDH as a loading control. C-PARP, cleaved poly(ADP-ribose) polymerase (p25).

RKO cells (Fig. 6A and B). Furthermore, HO-1 siRNA-transfected U2OS cells increased the nutlin-3-induced cleavage of poly (ADP-ribose) polymerase-1 (PARP) to a greater extent than control siRNA-transfected U2OS cells (Fig. 6C). Based on these findings, it can be suggested that HO-1 induced by nutlin-3 plays a role in protecting cancer cells from p53-induced apoptosis.

Discussion

In addition to its cell death-inducing activity, p53 has the potential to increase cell survival as well. The cell survival effect of p53 is mediated by target genes of p53 such as EGFR ligands (HB-EGF), and anti-apoptotic transcription factors (SLUG), thus being dependent on its transcriptional activity (6-9). Recently, HO-1, an anti-apoptotic gene was added to the target gene list of p53 (25). Based on this report, it would be expected that nutlin-3 could induce the expression of HO-1 in a transcription-dependent manner of p53. As expected, nutlin-3 induced the expression of HO-1 at the transcriptional level in cancer cells such as U2OS and RKO cells. However, the transcriptional activity of p53 was not involved and instead, the activities of JNK and p38 MAPK played critical roles in nutlin-3-induced HO-1 expression. As summarized in Fig. 7, the results reported herein demonstrate that the nutlin-3 treatment induced both p53 protein levels and the mitochondrial translocation of p53 in cancer cells. Mitochondrial p53 induces the generation

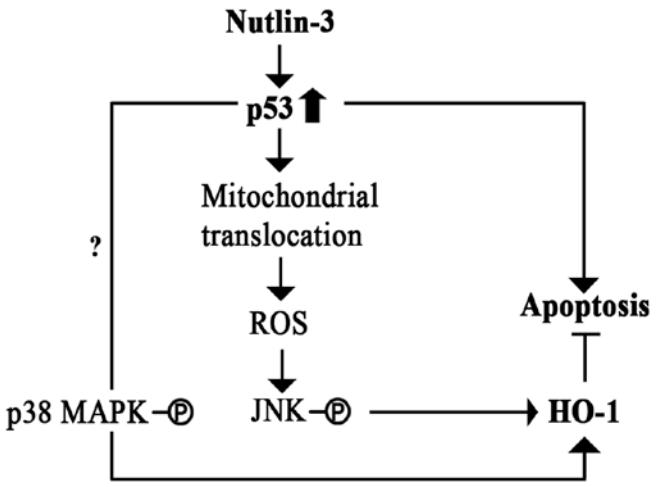

Figure 7. Schematic diagram of nutlin-3-induced HO-1 expression.

of ROS, which, in turn, activates JNK, which mediates HO-1 transcription. In the meanwhile, nutlin-3-induced p38 MAPK activation was not prevented by the presence of either TEMPO, a ROS scavenger, or PFT-μ, a blocker of the translocation of p53 to mitochondria, suggesting that ROS and mitochondrial p53 is not involved in p38 MAPK activation and that this process is controlled by an alternative mechanism (Figs. 4 and 5). However, since the respective treatment of TEMPO or the PFT-μ stimulated activation of p38 MAPK, the involvement of ROS and mitochondrial p53 in p38 MAPK activation cannot be confirmed, yet.

Mitochondrial p53 has been reported to induce ROS generation by virtue of its interaction with MnSOD (SOD2), thereby inhibiting its activity (29). In this model, however, whereas nutlin-3-induced HO-1 expression was augmented by MnSOD siRNA, an MnSOD mimetic such as Mn(III)TMPyP [Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride, $C_{44}H_{36}MnN_8$•5HCl] failed to prevent the induction of HO-1 (data not shown). It can therefore be suggested that the ROS-scavenging effect of MnSOD modulates the nutlin-3-induced HO-1 but MnSOD itself may not be a critical regulator of nutlin-3-induced HO-1 expression in these cells. In support of this, no evidence was found for an interaction between p53 and MnSOD at the endogenous level (data not shown). The potentiating effect of MnSOD siRNA on HO-1 induction can be considered to constitute additional evidence for the contribution of ROS to the induction of HO-1 expression.

It has been reported that mitochondrial p53 induces apoptosis in many models, and that this process occurs via a transcription-independent apoptotic mechanism (30,31). P53 interacts with the anti-apoptotic proteins, BCL-2 and BCL-xL in the mitochondria, relieving their inhibitory effects on the apoptotic protein BAK (30). Mitochondrial p53 can directly interact with BAK (32). Mitochondrial p53 stimulates BAK oligomerization via these pathways, leading to mitochondrial outermembrane permeabilization, a rate-limiting step in intrinsic apoptosis. The interaction of p53 with Mn-SOD in mitochondria also activates apoptosis by initiating a ROS-dependent pathway (29). It was recently reported that p53, after being translocated to mitochondria as the result of oxidative stress, interacts with cyclophilin D and thus induces mitochondrial permeability transition, resulting in the necrosis of neuronal cells (33). Taken together, mitochondrial p53 can be solely regarded as an inducer of cell death such as apoptosis and necrosis. To be consistent with the effects of the genetic expression of p53 and DNA damage-induced p53, the non-genotoxic activation of p53 by nutlin-3 was also reported to induce apoptosis by the mitochondrial translocation of p53 in cancer cells including leukemia and lymphoma cells (34). However, p53, when translocated to mitochondria in response to γ-irradiation, was not found to induce apoptosis in various cancer cells (35), and moreover, as reported in our previous study, the nutlin-3-induced mitochondrial translocation of p53 induced a cell survival pathway consisting of ROS and ERK1/2 (17). Therefore, it appears that mitochondrial p53 has the ability to induce different pathways such as apoptosis, necrosis and cell survival, depending on the type of toxic stress and cellular context. These different effects may be due to the interaction of different proteins with p53 in mitochondria. In this model, although we were not able to identify the protein that binds to mitochondrial p53 and is responsible for ROS accumulation, MnSOD, BCL-xL and cyclophilin D were not detected in the protein complexes that were immunoprecipitated with p53 in mitochondria (data not shown), implying the presence of unidentified proteins being involved in the accumulation of ROS to induce MAPK activation.

The mitochondrial translocation of p53 appears to occur by nutlin-3 treatment in all cancer cells tested in our experiments including leukemia, colon cancer and glioma cells. However, ROS generation was observed in subsets of these cells such as U2OS, RKO, A172 and U87 cells but not in leukemic cells and HCT116 colon cancer cells (data not shown), suggesting that various proteins that interact with p53 responsible for ROS generation are expressed by different cell types. Although ROS generation by nutlin-3 differs with according to cell types, the location of mitochondrial p53 could be a contributing factor. For example, BCL-xL and cyclophilin D reside in the outer mitochondrial membranes and the mitochondrial matrix, respectively, implying the precise location of mitochondrial p53 could explain the dependency of the different effects of p53 according to cell type, and the mechanism underlying the different location of p53 could help to dissect the biological functions of mitochondrial p53.

Nutlin-3 is known to predominantly induce cell cycle arrest in some solid cancer cell lines including U2OS and RKO cells, as shown in this study (14). Although the induction of cell cycle arrest can inhibit the growth of cancer cells, it has the potential to suppress apoptosis initiated by chemotherapeutic agents and thus to confer cancer cells with resistance to chemotherapeutic agents (36). This preference of nutlin-3 for growth arrest has been explained by hnRNPK expression and HIPK2 activation (15,16). In our previous report, we proposed a mitochondrial p53-ROS-MEK1/2-ERK1/2 activation pathway as being responsible for the inhibition of nutlin-3-induced apoptosis (17). In addition to this pathway, we proposed an alternate pathway involving mitochondrial p53-ROS-JNK-HO-1 expression, which would inhibit the nutlin-3-induced apoptosis found in this study. These two pathways may constitute a negative feedback loop for nutlin-3-induced apoptosis, implying modulators of these

two pathways may be therapeutic targets capable of enhancing the anticancer effect of nutlin-3. It can also be speculated that ROS generation during nutlin-3 treatment may be a critical mechanism for inducing the cell survival pathway through diverse mechanisms that counteract nutlin-3-induced apoptosis. Therefore, the mechanism responsible for ROS generation by mitochondrial p53 needs to be clarified to increase the apoptosis-inducing activity of nutlin-3 and thus for the use of nutlin-3 in future anticancer treatments.

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