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c-Jun NH2-terminal kinase decreases ubiquitination and promotes stabilization of p21$^{WAF1/CIP1}$ in K562 cell

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Abstract

Proteasome-dependent degradation of regulatory proteins is a known mechanism of cell cycle control. p21$^{WAF1/CIP1}$ (p21), a negative regulator of the cell division cycle, exhibits proteasome-sensitive turnover and ubiquitination. In the present study, we analyzed the regulatory effects of JNK1 on p21 protein accumulation in p53 null K562 cells. We found that JNK1 (wild type, WT) mediated H_2O_2-induced p21 protein up-regulation. Over-expression of JNK1 (WT) could elevate endogenous p21 protein level but did not affect p21 mRNA level and also prolong the p21 half-life as well as inhibited the p21 ubiquitination. These findings indicated that JNK1 could regulate cellular p21 level via inhibiting ubiquitination of p21, which provided a new insight for analyzing the regulatory effect of JNK after stress.

Keywords: p21$^{WAF1/CIP1}$; JNK; H_2O_2; Proteasome; Ubiquitination; Stabilization

Oxidative signals are thought to be mediated by cellular signal transduction systems, which eventually result in cell cycle arrest, senescence, or apoptosis [1]. Positive regulation of the cell cycle progression is mediated by the sequential activation of members of the cyclin-dependent kinase (CDK) family. p21$^{WAF1/CIP1}$ (p21) inhibits the cell cycle through its interaction with cyclin-CDK complexes [2,3]. p21 is originally considered as a major mediator of the G1 growth arrest induced by activation of p53 in response to DNA damage [4]. Although most of the studies on p21 regulation have concentrated on its transcriptional regulation by p53-dependent and p53-independent mechanisms [3,5–7], a series of new studies have shown that p21 can be regulated post-translationally [8]. p21 is a highly unstable protein with a half-life of 20–60 min in most cells [9]. It has been reported that proteasomal turnover of p21 does not require p21 ubiquitination [10], but more recent studies demonstrate that proteasomal degradation of p21 is regulated by the ubiquitin pathway and suggest that the site of the ubiquitin chain is critical in making p21 a competent substrate for the proteasome [11].

JNK (c-Jun NH2-terminal kinases) and ERK (extracellular signal-regulated kinase), two members of MAPKs (mitogen-activated protein kinases), play important roles in regulating cell growth, differentiation, and programmed cell death [12–15]. Activation of the JNK leads to both cell cycle arrest and cell cycle progression. It has been reported that modulation of JNK1 activity occurred through protein–protein interactions between JNK1, p53, and p21 proteins [16]. p21 dynamically associated with JNK1 *in vivo* and JNK1 activation correlated with dissociation of the p21^{WAF1}/JNK1 complex in human T-lymphocytes [17]. Furthermore, JNK1 also can mediate increased stability of p21 protein by inducing phosphorylation at ser^{130} *in vivo* and *in vitro* [18]. Since p21

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degradation could be mediated by ubiquitination dependent proteasome, we are interested whether JNK1 can regulate p21 level in cells by proteasome/ubiquitin mediated degradation of p21.

We here reported for the first time that H_2O_2-induced increase of p21 protein was related with JNK activation in p53 null K562 cells. JNK1 regulated cellular p21 level by post-transcriptional mechanism and maintained p21 protein stability via inhibiting ubiquitination of p21.

Materials and methods

Plasmids constructs. Flag and Xpress tagged p21 was cloned into the pcDNA3 vector (Invitrogen) using standard PCR protocol as described previously [19]. Dominant-negative, catalytically inactive of JNK1, JNK1 (mut), and HA-ubiquitin plasmids were generous gifts from Dr Ze'ev Ronai (The Burnham Institute, USA). Human wild-type JNK1 expression plasmids pcDNA3-JNK1-HA (WT) and pcDNA3-Flag-JNK1 (WT) were sub-cloned into pcDNA3.

Antibodies and reagents. Polyclonal antibodies to JNK, phospho-JNK (p-JNK), ERK, and phospho-ERK (p-ERK) were obtained from Cell Signaling Technology. Antibodies to p21, ubiquitin, and protein A/G were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against HA-tag and Flag M2 were from Sigma, monoclonal antibody against Xpress-tag was from Invitrogen. Horseradish peroxidase-conjugated secondary antibodies, cycloheximide (CHX), SP600125, and PD98059 were purchased from Calbiochem. Arbobenzoxyl-leucinyl-leucinyl-leucinal-H (MG-132) was from Sigma.

Cell culture and transfection. Human leukemic K562 cells purchased from the CBCAS (Cell Bank of the Chinese Academic of Sciences, Shanghai, China) were maintained in RPMI1640 (Gibco) supplemented with 10% (v/v) fetal bovine serum (HyClone), sodium bicarbonate, 100 μg/ml streptomycin, and 100 U/ml penicillin (HyClone) at 37 °C with 5% CO_2. Transient transfection was performed by the LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions as described previously [20]. In all cases, the total amount of DNA was normalized by the empty control plasmids.

Immunoprecipitation and immunoblotting analysis. Immunoprecipitation was performed as described previously [21] with indicated antibodies. The beads were washed three times using the lysis buffer and then the immunoprecipitates were subjected to SDS–PAGE followed by transferring onto polyvinylidene difluoride (PVDF) Western membranes. The antibody–antigen complexes were visualized by chemiluminescence method using Lumi-Light Western Blotting Substrate (Roche Applied Science) or using TMB immunoblotting system (Promega). The total density of the protein bands was calculated using Gel Doc2000 (Bio-Rad).

RT-PCR analysis. Total RNA was extracted with Trazol reagent (Gibco) as described by the manufacturer. RT-PCR was performed by Access RT-PCR System kit (Promega) according to the protocol with indicated primers (p21: sense CTGGGGATGTCCGTCAGAAC, antisense GAGTCTCCAGGTCCACCTGG; β-actin: sense TCATGAGG TAGTCAGTCAGG, antisense TGACCCAGATCATGTTTGAG). PCR was performed for 30 cycles in 25 μl of reaction mixture. PCR products were visualized in 1.2% agarose gels stained with EtBr. β-Actin was utilized as a housekeeping gene where indicated.

Analysis of p21 half-life in cells. K562 cells were treated with 20 μM cycloheximide (CHX) before harvested at different time points as indicated for the preparation of cell lysates. Equal amounts of proteins were analyzed by SDS–PAGE followed by immunoblot using the polyclonal anti-p21 antibody. These experiments were repeated three times.

Results

JNK mediates H_2O_2-induced up-regulation of p21 protein level

Previous study showed that JNK was activated by oxidative stresses [22] and p21 was induced by H_2O_2 [23]. Western blot analysis results of this study showed that p21 expression peaked at 4 h after cells being treated with 500 μM H_2O_2 (Fig. 1A) and in contrast, JNK as well as ERK was activated dramatically 1 h after H_2O_2 exposure (Fig. 1B). Thus, we next observed the relationship between JNK or ERK activation and p21 up-regulation. We found that SP600125 (a JNK specific inhibitor), but not PD98059 (an inhibitor of ERK signal pathway) decreased H_2O_2-induced p21 protein up-regulation (Fig. 1C), suggesting that H_2O_2-stimulated p21 protein expression was related with JNK. To confirm this result, K562 cells were transfected with plasmids encoding Flag-JNK1 (mut), a dominate negative constructs of JNK1, or empty vector and 36 h after transfection cells were stimulated with 500 μM H_2O_2 for 4 h. Western blot analysis showed that JNK1 (mut) exerted the inhibitory effect on H_2O_2-induced increase of p21 protein level (Fig. 1D). All these data indicated that JNK mediates H_2O_2-induced p21 up-regulation.

JNK post-transcriptionally induces accumulation of p21 protein

To further test whether JNK1 plays a key role in regulating p21 protein levels, K562 cells were transfected with plasmids encoding Flag-JNK1 (WT), Flag-JNK1 (mut) or empty plasmids, followed by detection of endogenous p21 protein level with Western blotting. As shown in Fig. 2A, over-expression of JNK1 (WT) but not JNK1 (mut) increased endogenous p21 protein level. On the other hand, exogenous p21 protein level was also elevated by over-expression of JNK1 (WT) when K562 cells were co-transfected with Flag-p21 along with JNK1 (WT) or JNK1 (mut), respectively (Fig. 2B). Fig. 2C and D showed that SP600125 not only inhibited the p21 protein accumulation induced by over-expression of JNK1 (WT) but also decreased endogenous basic cellular p21 protein level. Therefore above results indicated that JNK physiologically increased the endogenous p21 level.

We next determine whether JNK regulated p21 protein accumulation at transcription level or not. K562 cells were transfected with Flag-JNK1 or empty plasmids and RT-PCR was performed to test p21 transcription. Results showed that over-expression of JNK1 (WT) did not increase p21 mRNA levels in K562 cells (Fig. 2E). Correlatively, if K562 cells were treated with SP600125, their basic p21 mRNA level would not decrease (Fig. 2F). Moreover, H_2O_2 stimulation significantly increased p21 mRNA in K562 cells, but such enhancement of p21 transcription was not affected by JNK1 (mut) transfection or SP600125 treatment (Fig. 2F). These results together with the finding

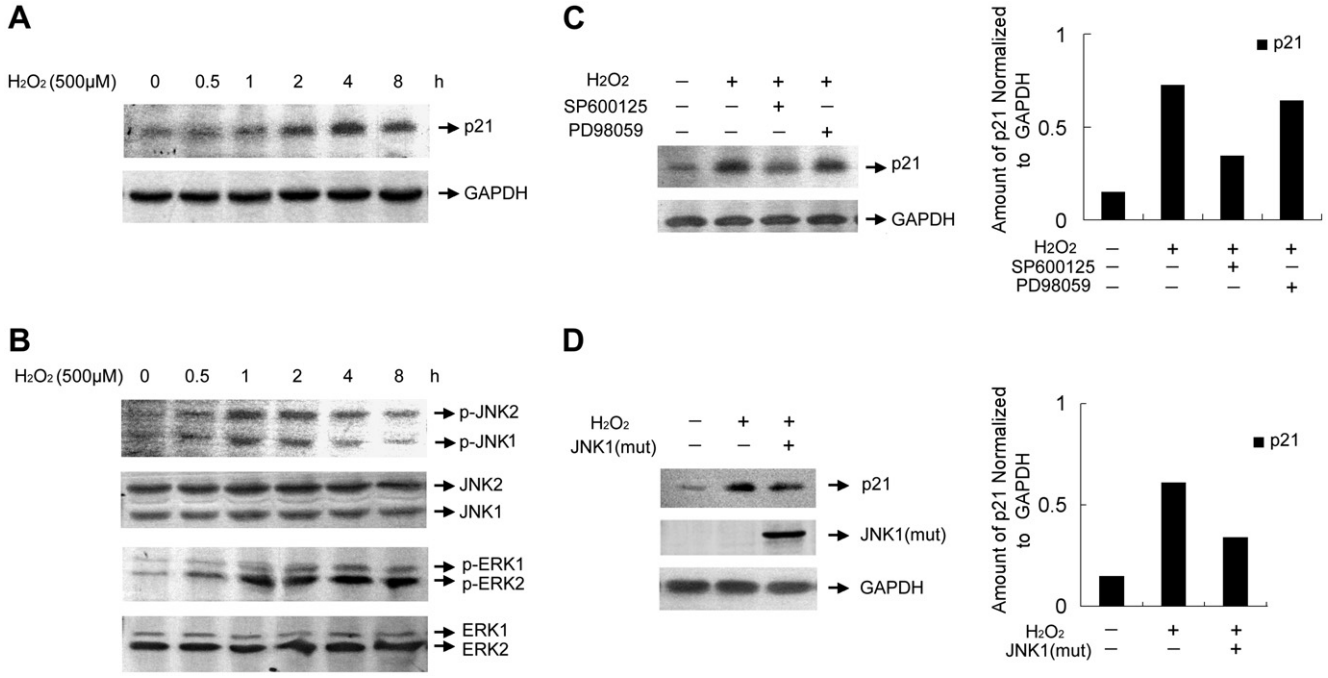

Fig. 1. JNK mediated H_2O_2-induced up-regulation of p21protein level. (A,B) K562 cells were treated with 500 μM H_2O_2 for 8 h. p21 protein level, JNK, and ERK phosphorylation were determined by Western blot analysis using indicated antibodies. (C) K562 cells were pretreated with 10 μM SP600125 or PD98059 as indicated for 30 min and then were exposed to 500 μM H_2O_2 for 4 h. p21 protein level was detected by Western blotting. (D) K562 cells were transfected with a dominate-negative constructs of JNK1, Flag-JNK1 (mut), or corresponding empty vectors. Thirty-six hours after transfection, the cells were treated with 500 μM H_2O_2 for 4 h. Target proteins were determined by Western blotting. All the results were representative of three independent experiments. GAPDH expression was used as control. All the results were representative of three independent experiments.

that JNK1 (mut) or SP600125 inhibited H_2O_2 stimulated p21 protein increase suggested that JNK post-transcriptionally mediated endogenous p21 protein accumulation.

JNK prolongs the half-life of p21

Since the p21 protein is unstable in most cells, further experiments were performed to test whether JNK post-transcriptionally induced p21 protein accumulation throng stabilizing p21 protein. Results showed that H_2O_2 prolonged the half-life of p21 and this effect was blocked by SP600125 (Fig. 3A). Over-expression of JNK1 (WT) stabilized the p21 protein, whereas over-expression of JNK1 (mut) did not show the same effect (Fig. 3B). These data indicated that JNK prolonged the half-life of p21 by stabilizing p21protein in K562 cells.

JNK decreases the ubiquitination of p21

It has been reported that proteasomal degradation of p21 is regulated via the ubiquitin pathway [11]. We thus test the effect of JNK on ubiquitination of p21 *in vivo*. As shown in Fig. 4A, SP600125 increased the ubiquitination level of endogenous p21, suggesting the inhibitory effect of JNK on p21 ubiquitination. Over-expression of JNK1 (WT) but not JNK1 (mut) markedly decreased the ubiquitination of p21 *in vivo* (Fig. 4B). To confirm this result, expression plasmids of Xpress-p21, HA-ubiquitin, and Flag-JNK1 (WT) or Flag-JNK1 (mut) were co-transfected in various combinations into K562 cells. Results showed that over-expression of JNK1 (WT) but not JNK1 (mut) inhibited transfected p21 ubiquitination. All these data demonstrated that JNK decreased p21 ubiquitination *in vivo*.

Discussion

Although $p21^{WAF1/CIP1}$ (p21) was originally identified as a gene regulated by the tumor suppressor protein p53 [3], induction of p21 in response to mitogenic stimulation and to other stresses occurs via mechanisms that are independent of p53 [24].

JNK is a family of stress kinase activated by change in redox potential, H_2O_2, osmotic shock, UV irradiation, and inflammatory cytokines [25–27]. Though it has been showed that JNK1 could stabilize p21 in TGF-β treated HD3 cells [18], the mechanism is unclear. While, in the present research we observed the regulatory effects of JNK on p21 in p53 null K562 cell. Here we found that H_2O_2 stimulated JNK activation dramatically and sequentially up-regulated p21. Moreover, if JNK was inhibited by SP600125 (data not shown), H_2O_2-induced p21 up-regulation was suppressed. In contrast, JNK1 (mut), a JNK1 dominate negative constructs, suppressed p21 up-regulation induced by H_2O_2. Over-expressed JNK1 (WT), but not JNK1 (mut) increased p21 protein level *in vivo*. SP600125 decreased basic p21 protein level as well as

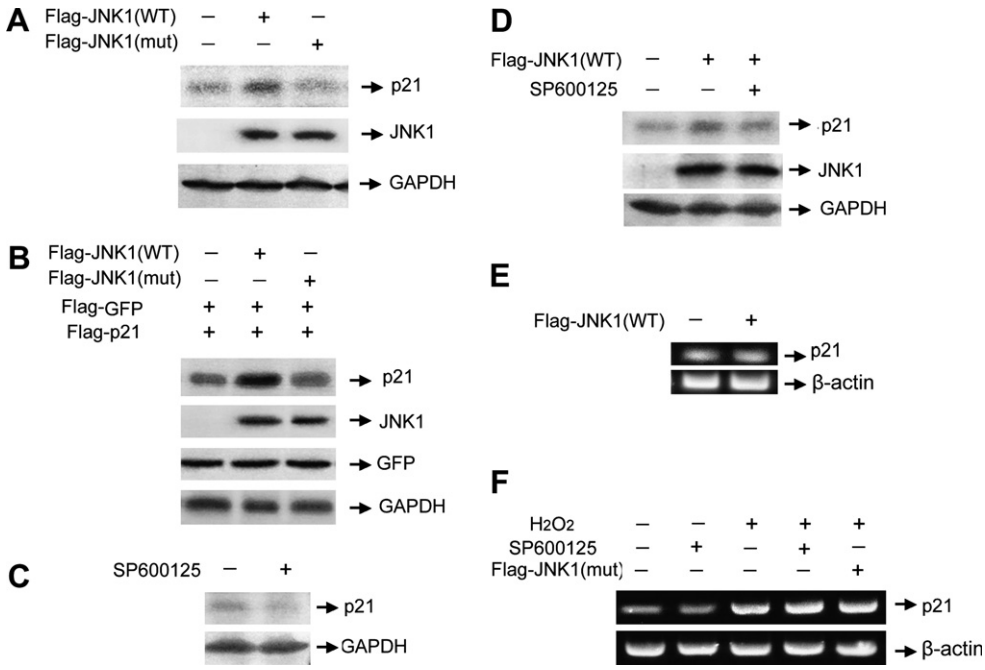

Fig. 2. JNK post-transcriptionally induced accumulation of p21 protein. (A) K562 cells were transiently transfected with expression plasmids for Flag-JNK1 (WT) or Flag-JNK1 (mut), respectively. After 36 h, targeted proteins were examined by Western blotting. GAPDH was used as the control. (B) K562 cells were transiently co-transfected with either of Flag-JNK1 (WT) or Flag-JNK1 (mut) and Flag-p21. GFP used as an expression internal control. After 36 h of expression, cells were lysed and Western blotting was performed with indicated antibodies. (C) K562 cells incubated with 10 μM SP600125 for 4 h and p21 protein level was determined by Western blotting. (D) K562 cells were transfected with Flag-JNK1 (WT), and after 36 h, cells were treated with 10 μM SP600125 or not for 4 h. Endogenous p21 protein level was determined by Western blot analysis. The total DNA level in each experiment was adjusted with empty vector pcNDA3 in each transfection. (E) K562 cells were transfected with Flag-JNK1 or empty plasmids. Total RNA was isolated and p21 mRNA level was detected by RT-PCR. β-Actin mRNA was used as control. (F) K562 cells were transfected with Flag-JNK1 (mut) or empty vector, and after 36 h of expression, cells were treated with 10 μM SP600125, 500 μM H_2O_2 or 10 μM SP600125 plus 500 μM H_2O_2 for 4 h as indicated. For SP600125 and H_2O_2 co-treated cells, SP600125 was added into culture medium 30 min before H_2O_2 stimulation. Total RNA was isolated and p21 mRNA level was detected by RT-PCR. β-Actin mRNA was used as control. All the results were representative of three independent experiments.

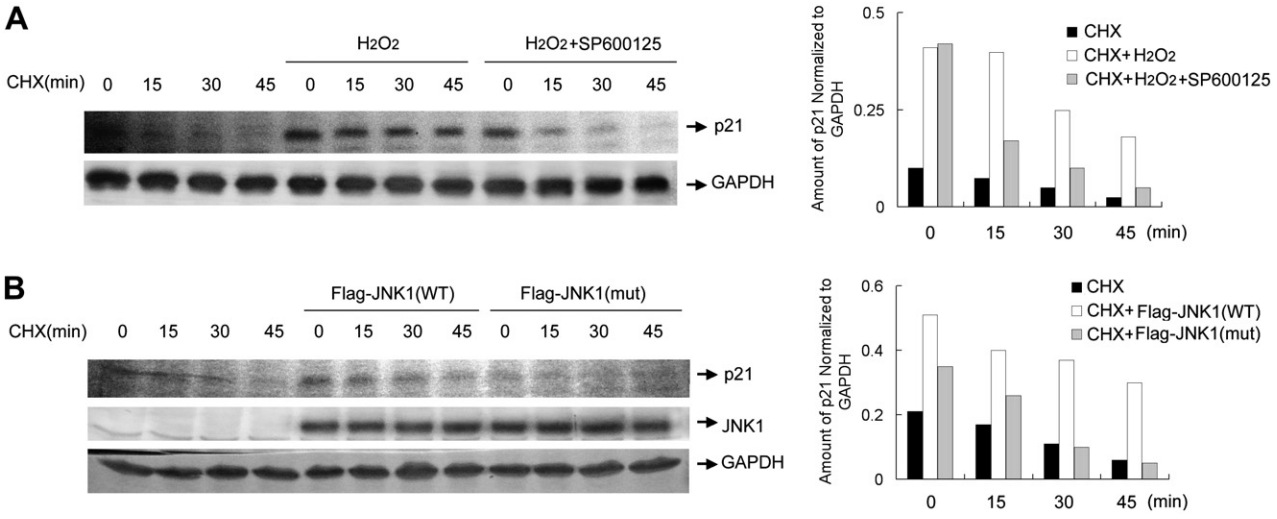

Fig. 3. JNK increases the half-life of p21. (A) K562 cells were stimulated with 500 μM H_2O_2 or not for 4 h and half of H_2O_2 treated cell cultures were incubated with 10 μM SP600125 for 30 min. All cultures were then treated with 20 μM cycloheximide (CHX) and p21 level was determined at the indicated times by Western blotting. (B) Flag tagged JNK1 (WT), JNK1 (mut) or empty vector was transfected into K562 cells. After 36 h of expression, all cultures were treated with 20 μM CHX and cells were harvested at indicated time points. p21 protein level was detected by Western blot analysis. GAPDH was used as the control. All the results were representative of three independent experiments.

JNK1-induced p21 up-regulation *in vivo*. These findings demonstrated that JNK regulated p21 level. As similar as the previous study which reported that p21 associated with JNK1 *in vivo* [17], the present study also found that JNK1 existed as a complex with p21 in K562 cells (data not shown).

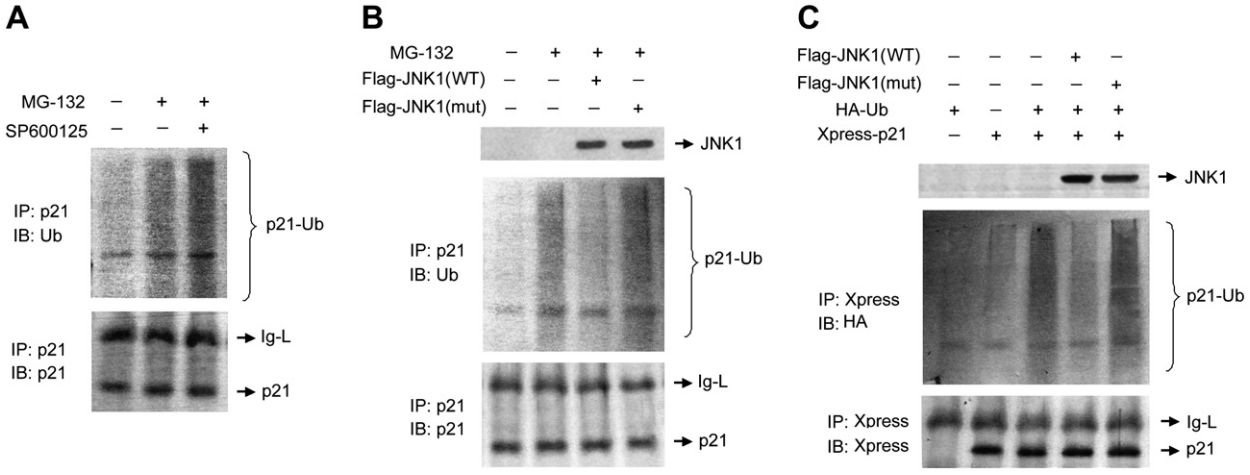

Fig. 4. JNK decreases the ubiquitination of p21. (A) K562 cells were pretreated or not with 10 μM SP600125 for 30 min and subsequently treated with the proteasome inhibitor MG-132 (20 μM) for 4 h as indicated. Cell lysates were immunoprecopitated by p21 antibody and then immunoblotted with ubiquitin antibody or p21 antibody. (B) K562 cells were transiently transfected with expression vectors for JNK1 (WT) or JNK1 (mut) and 36 h after transfection the cells were incubated with MG-132 (20 μM) for additional 4 h. Cell lysates were immunoprecipitated by p21 antibody and Western blotting was performed using antibodies to p21 and ubiquitin. (C) K562 cells were transfected with expression vectors for Xpress-p21, HA-ubiquitin, Flag-JNK1 (WT) or Flag-JNK1 (mut) or with various combinations of these vectors. Thirty-six hours after transfection, cells were incubated with MG-132 (20 μM) for additional 4 h. Cell lysates were immunoprecipitated with Xpress antibody and Western blotting was performed using antibodies to p21 and HA. All the results were representative of three independent experiments.

p21 level appears to be regulated by both transcriptional and post-transcriptional mechanisms. Although the transcriptional control of p21 has been well established [3], questions remain about the mechanism of p21 protein stabilization. Furthermore, as p21 is an unstable protein, it appears more important about the regulation of the protein stability. The function of the JNK pathway has mostly been investigated within the context of cellular stress, where its activation causes both stabilization and elevated activity of targeted transcription factors [28]. JNK signaling was thought to contribute to p21 stabilization, which was demonstrated by the evidence that senses decreased F-actin, or increased G-actin, which signals through the JNK pathway to regulate p21 stability [29]. Besides, it has been showed that JNK1 could stabilize p21 in TGF-β treated HD3 cells [18]. In our study, RT-PCR analysis showed that over-expressed JNK1 (WT) did not increase p21 mRNA level in K562 cells. SP600125 as well as JNK1 (mut) showed no effect on H_2O_2-increased p21 mRNA level. Moreover, JNK1 (WT) but not JNK1 (mut) increased half-life of p21 protein and SP600125 decreased p21 half-life increased by H_2O_2. These findings indicated that the up-regulation of p21 including H_2O_2-induced protein increase was mediated post-transcriptionally by JNK1 (WT).

It has been showed that p21 was stabilized through phosphorylation by JNK1 and AKT/PKB [18,30], but the detail mechanism was still unknown. p21 exhibited proteasome-sensitive turnover and ubiquitination *in vivo* and degradation of p21 is proteasome-mediated through its N-terminal ubiquitinylation [11]. Low doses UV irradiation triggers ubiquitin-dependent degradation of p21 to promote DNA repair [31]. The results in the present study showed that over-expression of JNK1 (WT) decreased ubiquitination of endogenous p21 whereas SP600125 enhanced the ubiquitination of it. These data indicated that JNK induced p21 protein accumulation and increased its half-life by inhibiting ubiquitination of p21.

p21 plays an important role in cellular senescence, proliferation, apoptosis, and DNA damage and repair [31–35]. p21 is a critical regulator with negative and positive functions of cell cycle and also acts as a positive mediator of cell survival upon terminal differentiation or genotoxic damage [35]. The role of p21 in oxidative stress remains controversial. Although most studies have provided evidence that it functions as a protective factor during stress, due to its growth inhibitory properties, there is evidence that p21 is proapoptotic in certain situations [36]. Thus, the accumulation of $p21^{WAF1/CIP1}$ by H_2O_2 may activate a cell cycle checkpoint to rescue the cells from DNA damage.

In summary, our study demonstrated that JNK stabilized $p21^{WAF1/CIP1}$ protein in K562 cells through proteasome-mediated p21 ubiquitination decrease. These finding provided the new insight for understanding the regulatory effects of JNK on cellular p21 accumulation in stress signal pathway.

Acknowledgments

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