Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2010;25:761-766

Accepted: March 10, 2010

CHOP-dependent Regulation of p21/waf1 During ER Stress

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Key Words
p21/waf1 • ER stress • CHOP/GADD153 • Tunicamycin

Abstract
The transcription factor CHOP/GADD153 is induced during the unfolded protein response (UPR) and is associated to the induction of ER stress-related apoptosis. However, how the transition between the pro-survival and the pro-apoptotic role of ER stress is being orchestrated remains poorly understood. Here we show that tunicamycin, an antibiotic promoting ER stress, suppresses the expression of p21, a tumor suppressor that induces cell cycle arrest and inhibits apoptosis. This suppression of p21 levels was independent of p53 that is the major transcriptional regulator of p21, but could be reproduced by forced expression of CHOP. Consistently with these findings, siRNA-mediated inhibition of p21 levels restored the sensitivity of CHOP-deficient cells to tunicamycin. Our findings are consistent with a CHOP-dependent role for p21 in the shift from the pro-survival to the pro-apoptotic function of UPR.

Introduction

The accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (ER) results in cellular stress that initiates a specialized response designated as the unfolded protein response (UPR) [1, 2]. The transduction of UPR is mediated by the binding of the chaperone BiP (GRP78) to the misfolded proteins and its concomitant dissociation from each of the three major transducers of ER stress, the proteins IRE1, ATF6 and PERK [1, 2]. This dissociation results in their activation and subsequent initiation of a cascade of downstream signals that ultimately aim to the preservation of cellular homeostasis facilitating cell survival. Prolonged ER stress however, beyond levels at which cellular homeostasis can be maintained, becomes proapoptotic triggering programmed cell death [3-6]. The pro-apoptotic branch of the UPR is represented by PERK, a protein kinase that upon activation, besides attenuating protein translation by inhibiting eIF2 activity, stimulates the expression of the pro-apoptotic transcription factor CHOP [4, 7-9]. While the molecular cues governing ER stress sensing and transduction have been studied adequately, the shift in the balance between pro-survival and pro-apoptotic UPR-related signaling remains poorly understood [10].

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p21/waf1 is an inhibitor of cyclin dependent kinases and is regulated transcriptionally by the p53 tumor suppressor [11-13]. p21 expression is a potent inhibitor of cell cycle progression and is considered as a major mediator of p53-dependent cell cycle arrest [14, 15]. During genotoxic stress p53 activation stimulates p21 expression inhibiting cell cycle progression. Besides however this well documented anti-proliferative function, p21 also operates as an inhibitor of apoptosis exhibiting a pro-survival mode of action as it protects from p53-dependent and p53-independent apoptosis [15]. This dual ability of p21 to regulate negatively cell cycle progression and to inhibit apoptosis prompted us to explore whether it plays a role in the pro-survival towards the pro-apoptotic shift during ER stress. Our results show that p21 expression levels are suppressed during the UPR and by forced CHOP suppression, while its transient suppression reduces the sensitivity of CHOP-deficient cells to apoptosis. Importantly, the effects of ER stress to p21 appear to be p53 independent pointing to a mechanism that bypasses the transcriptional regulation of p21 by p53. Our findings implicate p21 in ER stress-related signaling and provide hints regarding the regulation of UPR-associated apoptosis.

Materials and Methods

Cell culture and transfections

Mouse Embryonic Fibroblasts (MEFs, E12.5) were isolated from CHOP-deficient [9], p53-deficient [16, 17] or isogenic wild type (wt) littermates using standard methods and maintained in DMEM containing 10% FBS and antibiotics/antimycotics at 37°C in a humidified atmosphere of 5% CO_2-95% air. Experiments were performed before cells reached passage 10. Suppression of p21 in the fibroblasts was achieved by transfecting wild-type mouse embryonic fibroblasts (MEFs) with siRNA specific for mouse (clone ID 160142, Ambion) according to the manufacturer's instructions. Scrambled RNA was used as control. A549 lung cancer cells were maintained as described above were transfected with a pcDNA3 plamid containing mouse CHOP cDNA construct using the Lipofectamine 2000 (Invitrogen) reagent following the manufacturer's instructions. Cell proliferation assays were performed by counting the cells under light microscope by using trypan blue exclusion. Data were obtained by assessing cell numbers in at least 8 optic fields. The experiments were performed at least three times and similar results were obtained. Results of a representative experiment are shown here. Treatment conditions for the tunicamycin, nutlin and MG-132 are described in the results section and the corresponding figure legends. All chemicals were obtained from Sigma unless otherwise specified.

RNA isolation and RT-PCR

RNA was isolated using Trizol RNA isolation protocol (Invitrogen), according to the manufacturer's instructions. cDNA was obtained after a two step reaction using AMV-RT (Promega) using 2μl from the extracted RNA for each reaction. Cells were approximately 90% confluent when RNA was isolated. PCRs were performed using Go-Taq polymerase (Promega) according to manufacturer's instruction. PCR reactions were performed at conditions according to which preliminary experiments have determined the exponential phase of the reaction. Each experiment has been performed at least 3 times independently, and similar results were obtained.

The oligonucleotide primers and cycling conditions were as follows: GAPDH (223bp) 5'AAC TTT GGC ATT GTG GAA GG3'(left) and ACA CAT TGG GGG TAG GAA CA3' (right), at 95°C for 30 sec, 55°C at 30 sec and 72°C at 30 sec for 30 cycles. p21 (359 bp): 5'GTC CAA TCC TGG TGA TGT CC3'(left) and 5'GCT CAG ACA CCA GAG TGC AA3' (right), at 95°C for 30 sec, 60°C at 30 sec and 72°C for 70 sec for 30 cycles. PCR products were electrophoresed into 2% agarose gel and visualized by ethidium bromide.

Western Blot Analysis

Proteins were extracted from cultured A549 cells or MEFs as previously described before [17, 18]. Briefly, The cells were lysed at 4°C for 20 min using a lysis buffer (Ripa, Invitrogen and proteinase inhibitor). Whole cells lysates were subsequently centrifuged at 13.000 rpm for 10 min at 4°C and the supernatants were collected. Protein content in the supernatants was determined by the Bradford assay. Each 100 μg aliquot of the proteins extracted from cells, was electrophoresed on a 10% SDS-PAGE under reducing conditions. The proteins were electrophoretically transferred from gels to nitrocellulose membranes. The blots were exposed overnight to primary antibodies, followed by 1h incubation with secondary antibodies. The antigen- antibody complexes were detected with the ECL chemiluminescence detection system (Thermo Scientific). The experiments were repeated with at least three different cultured specimens with similar results and the reported results are representative. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Results

Suppression of p21 expression during ER stress

In order to test if ER stress modulates p21 expression levels, MEFs from wild type mice were exposed to tunicamycin for 24h at 5 μg/ml and p21 levels were evaluated by western blot. Tunicamycin is an antibiotic that is an inhibitor of N-linked glycosylation and formation of N-glycosidic protein-carbohydrate linkages that induces stress in the ER [19]. As shown in Fig. 1 tunicamycin treatment inhibited potently the expression of p21 in MEFs. At these conditions ER stress was potently induced as evidenced by the stimulation of the BiP/GRP78 expression

that is diagnostic for the initiation of the UPR [20] showing that ER stress induction coincides with p21 suppression. Considering that p53 is the major regulator of p21 expression we asked if the effect of ER stress in the levels of p21 depends on p53. Therefore, MEFs were treated with nutlin at 25 μM for 24h and the effect of tunicamycin on p21 expression was evaluated. Nutlin is a small molecule inhibitor of p53-Mdm2 interaction and potent activator of p53 activity [21]. The latter has been confirmed by the stimulation of p21 levels after exposure of cells with nutlin (Fig. 1a) As shown in Fig. 1a, nutlin, while activated p21 expression, was incapable of inhibiting the suppressive effect of tunicamycin in the levels of p21 pointing to an effect that is p53-independent. However, in p53 deficient cells some suppression of p21 expression was also apparent following tunicamycin treatment, - notwithstanding the overall inhibition of p21 levels due to the absence of p53-, suggesting that in the presence of p53 the effect of tunicamycin in p21 levels is potentiated. We also note that BiP levels, in the presence of nutlin, were less potently induced by tunicamycin implying an involvement of p53 in the induction of UPR that is also supported by the minimal effect of tunicamycin in BiP levels in p53-null cells (Fig. 1). This effect was not likely to be due to the proteasome-mediated degradation of p21 because exposure of cells to MG132 at 5 μM, a potent proteasome inhibitor, during tunicamycin treatment had no effect in the reduction of p21 expression levels (Fig. 1b).

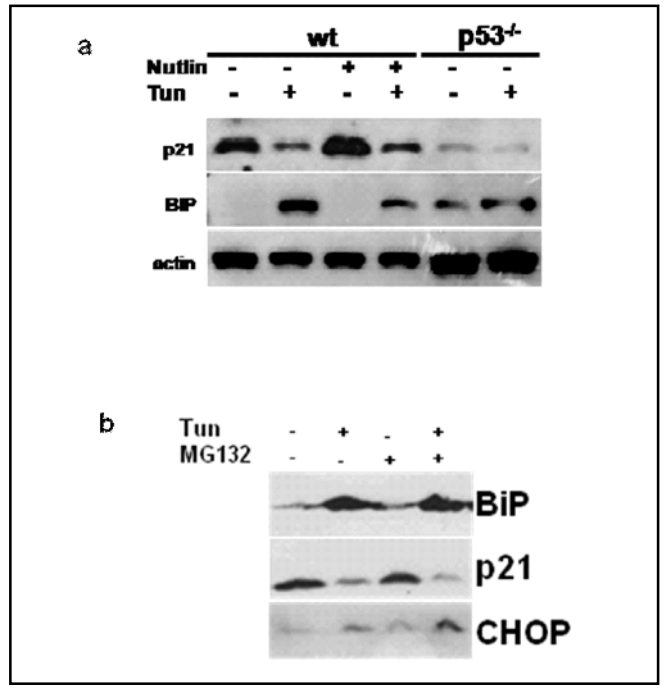

Fig. 1. (a). Immunoblot analysis for p21 and BiP in wt and p53-null fibroblasts after exposure of cells to tunicamycin for 24h at 5 μg/ml that induces stress in the ER. p53 stabilization by nutlin at 25 μM for 24h was also performed in wt MEFs alone, or in combination with tunicamycin treatment. Actin levels are shown as a loading control. (b). Immunoblot analysis for CHOP, p21 and BiP in wt MEFs following exposure of cells to tunicamycin alone or in combination with proteasome inhibitor MG132 at 5 μM for 24h.

CHOP suppresses p21 expression

As shown in Fig. 1b we noticed that p21 suppression during treatment of cells with tunicamycin, coincides with the activation of the expression of CHOP transcription factor. We also noted that CHOP induction by tunicamycin was potentiated by MG132, which is consistent with a dynamic role for proteasome-related regulation of CHOP expression levels [22]. The latter also provides an internal control for the effective treatment of the cells with the proteasome inhibitor. CHOP is activated by the ER stress transducer PERK and induces apoptosis under prolonged ER stress [7-9]. This observation, in association with the anti-apoptotic effect of p21 prompted us to reason that CHOP might regulate p21 expression. Thus, wt MEFs and A549 human lung cancer cells were transfected with a CHOP expression plasmid and p21 levels were evaluated. Western blot analysis showed that increasing amounts of CHOP caused a dose-dependent reduction in the levels of p21 in both cell types that is consistent with a regulatory role for PERK in the levels of p21

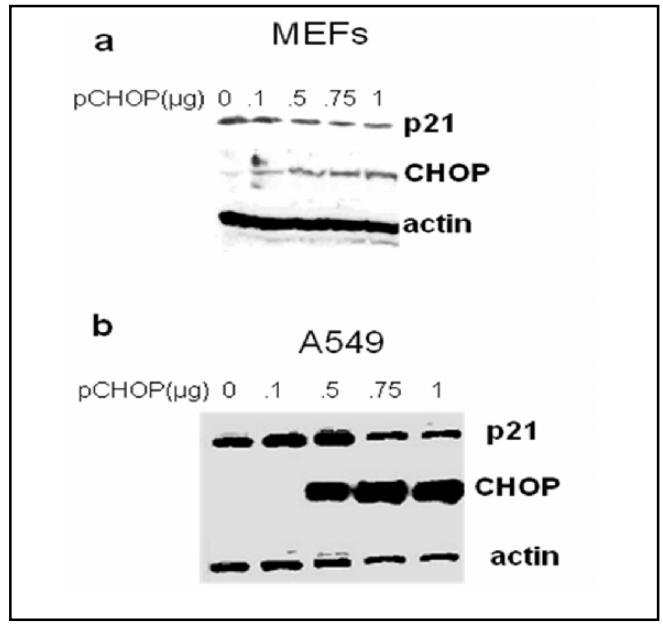

Fig. 2. Immunoblot analysis for CHOP and p21 in MEFs (a) and A549 (b) human lung cancer cells following transfection with a CHOP-expressing plasmid. Plasmid amounts are also indicated. Actin levels are shown as a loading control.

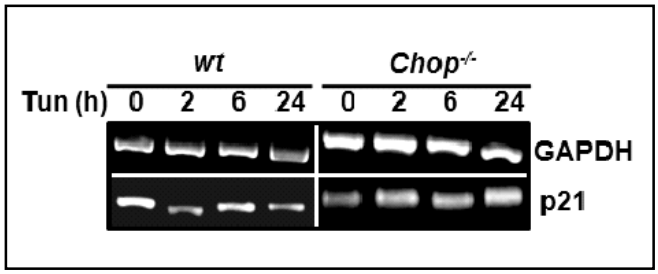

Fig. 3. Semiquantitative RT-PCR analysis for p21 mRNA levels in wt and CHOP-deficient fibroblasts in the presence or absence of tunicamycin at 5 μg/ml. The treatment periods are indicated. GAPDH levels are shown as a loading control.

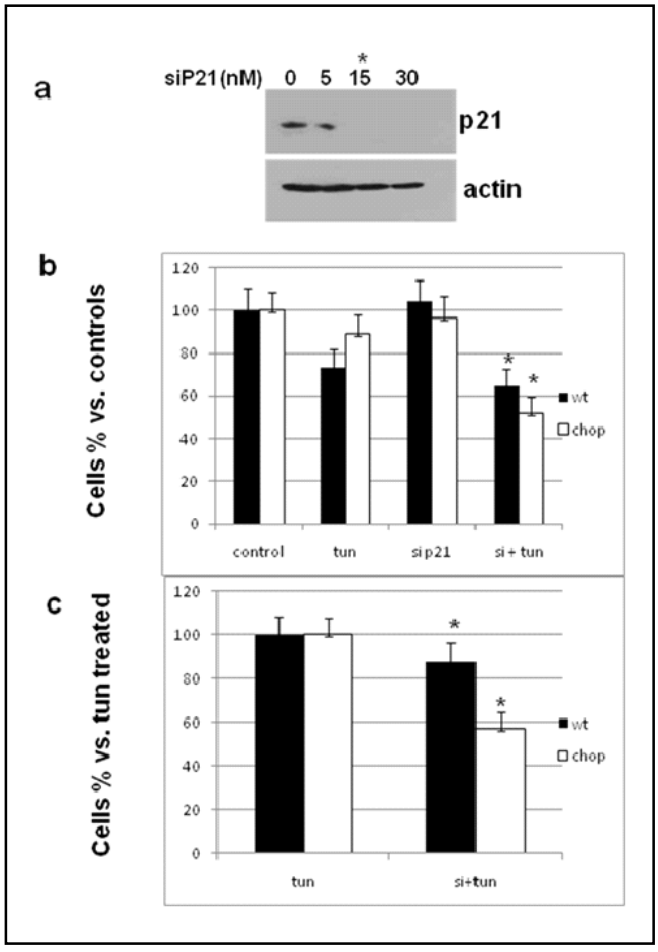

Fig. 4. Effect of knocking down p21 in cell survival during tunicamycin treatment. (a). Immunoblot analysis for p21 in wt MEFs following siRNA-mediated suppression of p21. Transfection of cells with siRNA at 15nM was selected as the optimal concentration for subsequent experiments. p21 was knocked-down in wt and CHOP-null cells and cell proliferation was evaluated following exposure of cells to tunicamycin at 0,5 μg/ml, after 24h. Transfection of scrambled RNA at 15 nM was performed in the controls Cell number % vs. controls (b) and % vs. tunicamycin treated (c) is shown. *, $p<0.05$ vs. corresponding controls (Students' t-test).

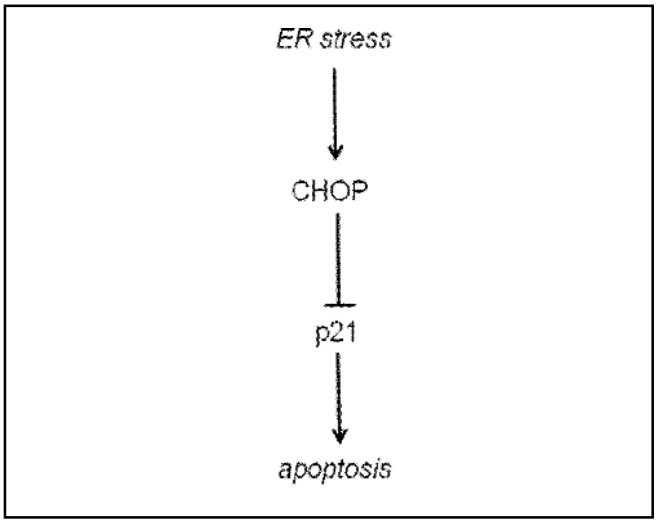

Fig. 5. Diagrammatic presentation of the proposed role of p21 during ER stress.

(Fig. 2). Furthermore we found that in CHOP-deficient MEFs, tunicamycin not only was incapable of suppressing p21 mRNA levels as in wt MEFs within 2h of exposure, but also led to some minor stimulation of p21 levels (Fig. 3). During prolonged treatment periods and contrary to the strong suppression of p21 protein levels, the suppressive effect of tunicamycin to the mRNA levels of p21 was partially compromised which implies a regulation of p21 at a post-transcriptional level. Thus, we concluded that CHOP regulates p21 during ER stress.

p21 inhibition restores sensitivity of CHOP-deficiency to tunicamycin

The notion that the CHOP-mediated modulation of p21 expression is associated with the commitment of cells into a pro-apoptotic fate renders a testable hypothesis, that the manipulation of p21 activity will affect the sensitivity of cells to tunicamycin. Therefore, the viability of wt and CHOP deficient cells was assessed during exposure to tunicamycin, following suppression of p21 expression by siRNA (Fig. 4a). Consistently with previous findings [9] we found that CHOP-deficiency offered resistance against tunicamycin. However, when p21 expression was compromised by siRNA at 15 nM, CHOP-deficient cells were more sensitive to tunicamycin than their wt counterparts (Fig. 4b,c). Thus, it appears that p21 expression increases the resistance of cells against tunicamycin and that its suppression is a perquisite for the effective antiproliferative action of tunicamycin.

Discussion

The commitment of cells to certain pro-apoptotic cues during ER stress remains poorly understood particularly in view of the fact that the latter has to follow a pro-survival molecular program dictated by the execution of the early steps of UPR. Recently, the suppression of CHOP by toll-like receptor signaling was shown to promote survival in TLR expressing cells during ER stress [23]. In the present study we reported that pharmacological induction of ER stress by tunicamycin suppresses p21 levels, an effect that co-insides with the upregulation of CHOP. We have also provided evidence that CHOP transcription factor, a major regulator of ER stress-related apoptosis, may be involved in the regulation of the cell cycle regulator p21/waf1 during ER stress facilitating the commitment of cells into a pro-apoptotic program. Our findings are consistent with a mode of action at which high levels of p21 induce cell cycle arrest and inhibit apoptosis that facilitates the pro-survival role of the UPR during the initial stages of ER stress (Fig. 5). The subsequent however, CHOP-dependent inhibition of p21 expression levels is consistent with the pro-apoptotic effects of ER stress. Thus, it appears that CHOP, besides inducing apoptosis *per se*, also relieves the anti-apoptotic activity of p21. Collectively our results implicate p21 in the regulation of the UPR and provide hints regarding the transition from the pro-survival to the pro-apoptotic role of ER stress.

Abbreviations

UPR (unfolded protein response); ER (endoplasmic reticulum); RT-PCR (reverse transcription PCR); MEFs (mouse embryonic fibroblasts); wt (wild type).

Acknowledgements

We thanks Dr Walz (Freiburg University) for providing us with the CHOP plasmid. This work was supported by grants from the University of Athens (Kapodistrias), the European Social Fund (EPEAEK II) and the KESY Oncology Program.

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