Cycloheximide Protects HepG2 Cells from Serum Withdrawal-Induced Apoptosis by Decreasing p53 and Phosphorylated p53 Levels

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ABSTRACT

Cycloheximide (CHX), an inhibitor of protein synthesis, has been reported to prevent cell death in a wide variety of cell types and produced by different apoptotic stimuli. However, the mechanisms by which CHX protects cells from apoptosis are still unclear. In this study, we investigated whether p53 plays a role in the protection by CHX against serum withdrawal-induced apoptosis. Deparivation of serum from the culture medium causes apoptosis in HepG2 cells, and CHX dramatically protects cells from death. p53, p21, and Bax protein levels were elevated, and cell cycle arrest was produced after serum withdrawal. CHX abolished this elevation of p53, p21, and Bax as well as the cell cycle arrest induced by serum deprivation. The p53 inhibitor pifithrin-α protects HepG2 cells against apoptosis induced by serum withdrawal. HepG2 cells expressing a dominant negative form of mutant p53 and Hep3B cells lacking p53 were resistant to serum withdrawal-induced apoptosis. Lowering of p53 by small interfering RNA protects HepG2 cells from serum withdrawal-induced apoptosis. p53 phosphorylation was induced by serum withdrawal and other chemotherapeutic reagents such as actinomycin D, doxorubicin, and etoposide. CHX decreases the levels of phosphorylated p53 (pp53) even in the presence of a proteasome inhibitor, which maintains the total p53 levels, whereas it does not affect the dephosphorylation of pp53. These results suggest the possibility that kinases that phosphorylate p53 might be affected by CHX administration. In summary, CHX protects HepG2 cells from serum withdrawal-induced apoptosis through inhibiting the synthesis of p53 and the phosphorylation of p53.

Cycloheximide (CHX), a protein synthesis inhibitor, has been widely employed in studies of apoptosis. However, its effects on apoptosis vary from inhibition to enhancement depending on the apoptotic stimulators, e.g., CHX sensitizes cells to tumor necrosis factor-α or Fas-induced apoptosis (Fulda et al., 2000), but it prevents rat hepatocytes from apoptosis induced by transformation growth factor β (Sánchez et al., 1997) and protects rat thymocytes and mouse lymphoid cell lines against apoptosis induced by glucocorticoids and a calcium ionophore (Wyllie et al., 1984). The mechanisms by which CHX regulates apoptosis are not clear and still under investigation. The hypotheses whereby CHX sensitizes cells to CD95-induced apoptosis include down-regulating FLICE-inhibitory protein and receptor-interacting protein expression (Fulda et al., 2000) or inhibiting the synthesis of antiapoptotic proteins such as Bcl-2 or Bcl-xL. The standard view of the mechanism whereby CHX prevents cell death is that it inhibits the production of “killer proteins” by blocking protein synthesis. However, until now, such killer proteins have not been identified. Other mechanisms such as preventing cell death by activation of antiapoptotic signaling pathways have also been considered (Mattson and Furukawa, 1997).

The activation of wild-type p53, a major tumor suppressor protein, which is implicated in cell cycle control, DNA repair, and apoptosis (Liebermann et al., 1995; Steller, 1995; Levine, 1997), has been reported to be involved in the process of apoptosis (Fritsche et al., 1993; Steller, 1995). An increase in p53 levels results in large part from stabilization of the p53 protein against degradation, and accumulation of p53 occurs because of this increased protein stability in the presence of ongoing translation (Fritsche et al., 1993). As a transcription factor, p53 protein enhances the transcription rate of several genes including p21WAF1, GADD45, and 14-3-3-α, which are genes involved in cell cycle arrest (Kastan et al., 1992; el-Deiry et al., 1995; Hermeking et al., 1997), and Bax, CD95,
Cell viability was presented as percentage of viable cells. The cell viability data obtained from this assay is not affected by the change of cell proliferation due to serum withdrawal or the treatment of CHX since equivalent total cells are being counted.

Western Blotting. Cell lysates were prepared by sonicating cells followed by centrifugation. The protein concentration of the supernatant was measured (DC protein assay reagent; Bio-Rad, Hercules, CA), and 30 μg of denatured protein was resolved on 10% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (Bio-Rad). The membrane was incubated with primary antibody followed by incubation with horseradish peroxidase-conjugated secondary antibody. Detection by the chemiluminescence reaction was carried out for 1 min using the ECL kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) followed by exposure to Kodak X-OMAT X-ray film (Eastman Kodak, Rochester, NY).

Intracellular Measurement of Reactive Oxygen Species. Fluorescence spectrophotometry was used to measure production of intracellular reactive oxygen species (ROS) with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) as the probe as described previously (Bai et al., 1999). In brief, HepG2 cells were incubated in serum-free medium with or without 10 μM CHX for 0, 4, 8, 24, 48, and 72 h followed by incubation with 5 μM DCFDA in MEM for 30 min at 37°C in the dark. The cells were washed in PBS, trypsinized, and resuspended in 3 ml of PBS, and the intensity of fluorescence was immediately read in a fluorescence spectrophotometer (650-108; PerkinElmer Life and Analytical Sciences, Boston, MA) at 503 nm for excitation and at 529 nm for emission.

DNA Fragmentation Assay. The DNA fragmentation pattern (DNA ladder) was carried out by agarose gel electrophoresis. Cells (5 × 10⁶) treated with various reagents were scraped and centrifuged at 1200 rpm for 10 min. The cell pellet was resuspended in 0.5 ml of lysis buffer consisting of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA, 100 μg/ml protease K, and 0.5% SDS and incubated for 2 h at 50°C. DNA was extracted with 0.5 ml of phenol, pH 8.0, followed by extraction with 0.5 ml of phenol/chloroform (1:1) and chloroform. The aqueous phase was precipitated with 2.0 volumes of ice-cold ethanol and 0.1 volumes of 3 M sodium acetate, pH 5.2, at 20°C overnight. The precipitates were collected by centrifugation at 13,000g for 10 min. The pellets were air-dried and resuspended in 50 μl of Tris/EDTA (10 mM Tris, 1 mM EDTA, pH 8.0) buffer supplemented with 100 μg/ml RNase A. DNA was loaded onto a 1.5% agarose gel containing ethidium bromide, electrophoresed in Tris/acetate/EDTA buffer for 2 h at 50 V, and photographed under UV illumination.

Flow Cytometry for Cell Cycle Analysis. Cell cycle was analyzed by flow cytometry. Cells (5 × 10⁶) were seeded onto six-well plates and incubated with medium containing 10% serum or medium without serum in the presence or absence of 10 μM CHX for 16 h. Cells were harvested by trypsinization and washed with PBS, followed by centrifugation at 2000 rpm for 10 min. The pellet of cells was resuspended in 80% ethanol and stored at 4°C for overnight. Cells were washed twice with PBS. The pellet was resuspended in PBS containing 100 μg/ml RNase A, incubated at 37°C for 30 min, stained with propidium iodide (50 μg/ml), and analyzed by flow cytometry for DNA analysis.

Statistics. Results are expressed as mean ± S.D. One-way analysis of variance with subsequent post hoc comparisons by Scheffe was used for the DCFDA fluorescence data analysis. p < 0.05 was considered as statistically significant. Student’s t test was used for analysis for all other data with p < 0.05 considered as the level of significance.

Results

CHX Protects HepG2 Cells from Apoptosis Induced by Serum Deprivation. HepG2 cells were cultured in MEM with 10% FBS or without the 10% FBS (serum deprivation conditions)
Serum withdrawal caused severe cell death at 48 and 72 h. Morphology change of cells after 72-h serum withdrawal was visualized under the light microscope as shown in Fig. 1A. Cells cultured in serum withdrawal medium (–serum, –CHX) displayed shrinkage and nuclei condensation; however, cells cultured in serum withdrawal medium plus 10 μM CHX (–serum, +CHX) showed normal morphology. CHX at different concentrations (0.5, 1, 5, 10, and 40 μM) protected HepG2 cells from cell death induced by serum deprivation (cell viability from 46–89% compared with ~20% viability in the absence of CHX). At a concentration of 10 μM, CHX provided maximal protection and did not cause significant toxicity to HepG2 cells (Fig. 1Ba); therefore, 10 μM CHX was used in the following experiments. Cell viabilities at different time points (0, 24, 48, and 72 h) of treatment with or without 10 μM CHX in serum-free medium were evaluated by trypan blue exclusion as shown in Fig. 1Bb. Cell viability decreased in cells cultured in serum withdrawal medium for 48 and 72 h (approximately 40 and 20% 0-h control values, respectively). CHX effectively blocked serum withdrawal-induced cell death (90% survival at 48 and 72 h) (Fig. 1Bb). However, if CHX was administrated at 4, 8, or 24 h after serum deprivation, the cell viability at 72 h after serum withdrawal (<20% in the absence of CHX) decreased to 80, 60, or 40%, respectively, and no protective effect was observed if adding CHX at 36 h after serum deprivation. To evaluate whether serum deprivation induces apoptosis, the DNA was extracted from cells cultured in medium with 10% FBS or serum deprivation medium and treated with or without 10 μM CHX for 48 and 56 h. Formation of a DNA ladder was observed in cells cultured in serum deprivation medium for 48 and 56 h, whereas no DNA ladder was detected in cells cultured in serum withdrawal medium plus CHX for 56 h. No DNA ladder was found in cells cultured in 10% FBS medium plus CHX for 56 h (Fig. 1C).

**CHX Does Not Decrease Intracellular ROS Production Caused by Serum Deprivation.** Serum withdrawal

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**Fig. 1.** CHX protects HepG2 cells from apoptosis induced by serum deprivation. HepG2 cells were cultured in MEM with 10% FBS or without FBS and treated with or without CHX. A, morphology change of cells at 72 h after culture in medium with or without serum in the presence or absence 10 μM CHX was visualized and photographed under the light microscope. B, cell viability evaluated by trypan blue exclusion assay. a, cells were cultured in serum-free medium in the absence or presence of different concentrations CHX (0.5, 1, 5, 10, and 40 μM) for 72 h. b, Cells were cultured in serum-free medium in the absence or presence of 10 μM CHX for different times (0, 24, 48, and 72 h). Cell viability is presented as percentage of viable cells. Data are the mean of three independent experiments. C, DNA was extracted from cells cultured in MEM with 10% FBS or without FBS and treated with or without 10 μM CHX for 48 and 56 h, electrophoresed on agarose gels, and stained with ethidium bromide and photographed under UV illumination.
can induce reactive oxidative stress, which is believed to play an important role in cell death (Satoh et al., 1996; King et al., 2003). Fluorescence spectrophotometry was used to measure intracellular ROS production with DCFDA as the probe. Intracellular levels of ROS in cells increased approximately 4-fold after 24 h of serum deprivation compared with cells incubated in medium containing 10% FBS (control). However, production of ROS in HepG2 cells cultured in serum-free medium plus CHX was even greater than that in cells cultured in serum-free medium without CHX at 8 and 24 h (Fig. 2A). At 48 and 72 h of serum withdrawal, the DCF fluorescence was still maintained at a high level compared with control 0-h values, but there were no significant differences between cells treated with and without CHX (Fig. 2A). Trolox, an antioxidant chemical reagent, could protect against serum deprivation-induced cell death, validating that ROS plays an important role in the toxicity produced by serum withdrawal. Morphology changes of HepG2 cells after 48-h serum withdrawal with or without 50 µM trolox treatment were visualized under the light microscope as shown in Fig. 2B.

**CHX Could Block p53 Activation and Cell Cycle Arrest Induced by Serum Deprivation.** Serum withdrawal induces p53 activation. Total p53, p21, Bax, and especially phosphorylated (pSer-15) p53 levels were elevated at 8, 16, and 24 h after serum withdrawal compared with control cells (Fig. 3A). In cells treated with serum withdrawal plus 10 µM CHX, total p53 levels were strongly decreased, phosphorylated p53 was not detected, and levels of p21 and Bax declined to control levels (Fig. 3A). Cell cycle analysis showed that serum withdrawal caused G1-S cell cycle arrest. The cell population in the G0-G1 phase increased in cells cultured with serum deprivation medium for 16 h (74%) compared with cells cultured in 10% serum medium (58%). CHX abolished this increase induced by serum withdrawal (57% cells in G0-G1) (Fig. 3B, a and b). Similar results of the cell population in the G0-G1 phase were observed at 24 h after serum deprivation because the percentage cells in the G0-G1 phase was 76% in the absence of CHX and 56% in the presence of CHX (data not shown).

**HepG2 Cells Treated with a p53 Inhibitor or HepG2 Cells Expressing Mutant p53 or Hep3B Cells Lacking p53 Are More Resistant to Apoptosis Induced by Serum Deprivation.** Pifithrin-α, an effective p53 inhibitor, has been widely used in studies with p53. It inhibits p53-mediated apoptosis and p53-dependent gene transcription such as p21, bax, and mdm2 expression (Komarov et al., 1999; Liu et al., 2004; Murphy et al., 2004). To confirm the inhibitory effect of pifithrin-α on p53-dependent gene transcription, p21 and Bax and total p53 protein levels in HepG2 cells treated with 0, 30, or 60 µM pifithrin-α for 24 h were evaluated. The p21 and Bax protein level in HepG2 cells treated with 30 µM pifithrin-α was 50% lower compared with cells treated without pifithrin-α and was further lowered in cells treated with 60 µM pifithrin-α, although the total p53 levels were not significantly different in the absence or presence of pifithrin-α (Fig. 4Aa). Cells treated with 30 and 60 µM pifithrin-α were more resistant to serum withdrawal-induced apoptosis. Cell viabilities were 60 and 80%, respectively, in cells cultured in serum-free medium plus 30 and 60 µM pifithrin-α for 48 h compared with 36% viable cells after treatment with serum-free medium plus DMSO (control) (Fig. 4Ba). The morphological changes as shown in Fig. 4Ca confirm the protection by pifithrin-α against the serum withdrawal-induced apoptosis. To further evaluate the role of p53 in the toxicity induced by serum withdrawal, a HepG2 cell line overexpressing a dominant negative mutant p53 (hepg2-mtp53) was established by transfecting HepG2 cells with pCMV-p53mt135 plasmid. pCMV-p53mt135 expresses the p53mt135 mutant, which because of a conformational change, blocks normal p53 function. When p53mt135 and p53 are coexpressed, they form a mixed tetramer that is unable to interact with p53-binding sites; therefore, the downstream effects of p53 are blocked (Vogelstein and Kinzler, 1992). HepG2-mtp53 cells showed higher levels of p53 protein due to the expression of mutant p53 compared with control cells transfected with empty vector plasmid (HepG2-neo); however, their p21 and Bax levels were lower than HepG2-neo cells because of the decrease in functional p53 as a result of the interaction of mutant p53 with normal p53 (Fig. 4Ab, lanes 1 and 2). Hep3B cells, another hepatoma cell line that lacks normal p53 expression, showed a very low p21 protein level. The Bax level in Hep3B cells, although not as dramatically low as the level of p21, is approximately 30% lower than the Bax level in HepG2-neo cells (Fig. 4Ab, lane 3). HepG2-mtp53 cells or Hep3B cells were resistant to serum withdrawal-induced apoptosis compared with HepG2-

![Fig. 2. Serum deprivation induces intracellular ROS production and an antioxidant protects against cell death induced by serum deprivation. A, HepG2 cells were incubated in serum-free medium with or without 10 µM CHX for 0, 4, 8, 24, 48, and 72 h followed by incubation with 5 µM DCFDA in MEM for 30 min at 37°C in the dark. The cells were washed in PBS, trypsinized, and resuspended in PBS, and the intensity of fluorescence was immediately read in a fluorescence spectrophotometer at 503 nm for excitation and at 529 nm for emission. The results are expressed as arbitrary units of the fluorescence intensity. Data are the mean ± S.D. of three independent experiments conducted in duplicate. **, p < 0.01 versus CHX, 8 h; ***, p < 0.0001 versus CHX, 24 h. B, HepG2 cells were incubated in serum-free medium with or without 50 µM trolox for 48 h. Morphology change of HepG2 cells was visualized under the light microscope.](https://jpet.aspetjournals.org/content/1438-1447/10/8SHIP4-1278802/1 figure)
neo cells. Cell viabilities were around 80% in HepG2-mtp53 cells and Hep3B cells compared with 38% in HepG2-neo control cells (Fig. 4Bb), and morphological changes as shown in Fig. 4Cb confirm the lack of toxicity in the HepG2-mtp53 and Hep3B cells subjected to serum withdrawal.

siRNA Lowering of p53 Protects HepG2 Cells from Serum Withdrawal-Induced Cell Death. To further evaluate the role of p53 in the toxicity induced by serum deprivation, siRNA-p53 and siRNA-control (a nontargeting siRNA) were transfected into HepG2 cells. Twenty-four hours after transfection, cells were cultured in medium with or without serum for an additional 24 h. p53 and pp53 as well as Bax levels were evaluated by Western blot analysis as shown in Fig. 5A. The total p53 and Bax levels in HepG2 cells transfected with p53 siRNA dramatically decreased compared with cells transfected with control siRNA. Serum deprivation increased pp53 and Bax levels in HepG2 cells transfected with control siRNA but not in cells transfected with p53 siRNA (Fig. 5A). Cell viability evaluated after serum deprivation for 48 h was higher in cells transfected with siRNA-p53 compared with cells transfected with siRNA-control (62 versus 39%, p < 0.05) (Fig. 5B).

CHX Decreases p53 Accumulation and p53 Phosphorylation Induced by ActD, Doxorubicin, and Etoposide. To further study how CHX inhibits p53 activation and levels of p53, HepG2 cells were treated with therapeutic reagents 2.5 μM ActD, 2.5 μM doxorubicin, and 25 μM etoposide with or without 10 μM CHX for 16 h. Total p53 and pp53 (Ser-15) levels were evaluated by Western blot. These three reagents induce total p53 and pp53 levels compared with control. CHX blocked this elevation of total p53 and pp53 (Fig. 6), showing that lowering of p53 levels and activation of p53 to pp53 by CHX is not limited to only the serum withdrawal model.

The CHX Decrease of p53 Accumulation Is Related to p53 Phosphorylation but Not to pp53 Dephosphorylation. p53 accumulation caused by DNA-damaging reagents is mainly due to posttranslational modifications such as p53 phosphorylation.
phosphorylation as catalyzed by DNA kinases. Phosphorylated p53 is much more stable against proteasome-mediated degradation than nonphosphorylated p53. Once phosphorylated, pp53 can be dephosphorylated by phosphatases. CHX inhibition of p53 protein synthesis could be a major explanation for the decrease in total p53 levels by CHX. However, CHX might also regulate p53 and pp53 levels by affecting the phosphorylation or dephosphorylation of p53 via acting on the DNA kinases or phosphatases responsible for these post-translational modifications. To evaluate this, the proteasome inhibitor 10 \( \mu \)M MG115 was used to prevent degradation of p53 and thus maintain the total p53 level (unlike results in Fig. 6, where total p53 levels declined in the presence of CHX). Two hours after treatment with MG115, the HepG2 cells were treated with 2.5 \( \mu \)M ActD for 4 h to induce pp53 accumulation, followed by treatment with 10 \( \mu \)M CHX for an additional 6 h. This allows evaluation of the effect of CHX on already accumulated pp53 and p53. Figure 7A shows that MG115 alone, as expected, increased p53 levels but did not cause an increase in pp53 levels. ActD in the presence of MG115 increased both total p53 and pp53 levels. However, 6-h additional treatment with CHX did not lower pp53 levels. This suggests that CHX did not promote/activate p53 dephosphorylation (e.g., activation of pp53 phosphatases) so that once formed, pp53 levels are resistant to the CHX-mediated decline; and once total p53 is stabilized against proteasome-dependent degradation, CHX no longer lowers this stabilized p53. In a second experiment, the HepG2 cells were again pretreated with 10 \( \mu \)M MG115 for 2 h and then treated with either 2.5 \( \mu \)M ActD alone, or 10 \( \mu \)M CHX alone, or both ActD...
CHX for 6 h. In the presence of MG115, total p53 was maintained at the same level in the absence or presence of CHX or CHX plus ActD (Fig. 7B). Thus CHX does not lower p53 as long as the p53 cannot be degraded by the proteasome. ActD treatment induces pp53 formation. CHX plus ActD produced a decrease in the pp53 level compared with ActD alone, although total p53 levels were similar in these two groups (Fig. 7B). This suggests the possibility that CHX lowers the activity of DNA kinases, thereby decreasing the phosphorylation of p53, resulting in lowering of both total p53 and pp53 levels.

Discussion

In the current study, we observed that CHX protects HepG2 cells from apoptosis induced by serum withdrawal. This is in general agreement with previous reports in various cell culture models, e.g., CHX prevents apoptosis of cultured sympathetic neurons induced by withdrawal of nerve growth factor (Martin et al., 1988); prevents trophic factor deprivation-induced and glutamate-induced death of PC12 cells (Pittman et al., 1993; Serghini et al., 1994); and protects cultured retinal ganglion cells against excitotoxicity (Dreyer et al., 1995). All of these studies provided evidence in favor of a protein synthesis inhibition-based mechanism for the antiapoptotic action of CHX. In a study of antiapoptotic action of CHX and its inhibition of protein synthesis, Wyllie et al. (1984) reported that concentrations of CHX greater than 1 μM protected rat thymocytes and mouse lymphoid cell lines against apoptosis induced by glucocorticoids and a calcium ionophore and were effective in inhibiting protein synthesis by more than 50%. Martin et al. (1988) also reported that 3 to 10 μM CHX prevented apoptosis of cultured sympathetic neurons following withdrawal of nerve growth factor and caused greater than 90% inhibition in overall protein synthesis. In our study, CHX concentrations from 5 to 40 μM were found to effectively prevent HepG2 cells from apoptosis induced by serum withdrawal (Fig. 1Ba). These reports suggest that the cell death process requires synthesis of killer proteins and CHX prevent cell death by inhibiting the synthesis of these killer proteins, although other mechanisms such as
preventing cell death by activation of antiapoptotic signaling pathways need to be considered (Mattson and Furukawa, 1997; Yu et al., 2003).

Serum deprivation induces oxidative stress in cells as demonstrated by an excess production of ROS, such as superoxide anion, hydrogen peroxide, hydroxyl radical, and organic peroxides that can be inhibited by intracellular antioxidants (Satoh et al., 1996; King et al., 2003). We also found that trolox protects HepG2 cells from serum withdrawal-induced cell death (Fig. 2B). CHX might increase resistance to oxidative insults as suggested by the work of Ratan et al. (1994), who showed that CHX can enhance cellular antioxidant pathways by shunting cysteine from global protein synthesis into the formation of the antioxidant glutathione. However, we did not detect any evidence that the administration of CHX decreases intracellular ROS (Fig. 2A). Conversely, we found that the ROS levels significantly increased in cells treated with CHX compared with cells not treated with CHX at the time points of 8 and 24 h after serum withdrawal, which might be explained by CHX decreasing the synthesis of antioxidant enzymes, e.g., catalase and glutathione synthase. Sánchez et al. (1997) also reported that CHX prevents apoptosis, ROS production, and reduced glutathione depletion induced by TGF-β in fetal rat hepatocytes. However, they concluded that the CHX protection against oxidative stress induced by TGF-β in fetal hepatocytes is not related to an enhancement in the production of cellular antioxidants, such as glutathione, as it appeared, the relationship between TGF-β induced apoptosis and endogenous production of ROS was not causal but rather the consequence of cell lysis. Our results indicate that the protection by CHX is not due to a decrease in ROS production after serum withdrawal.

p53 is implicated in cell cycle control, DNA repair, and apoptosis (Liebermann et al., 1995; Steller, 1995; Levine, 1997). It has been reported that CHX prevented apoptosis of HeLa cells induced by p53 (Yonish-Rouach et al., 1995). In the current study, we found that serum withdrawal induces phosphorylation and accumulation of p53. CHX decreases total p53, pp53, p21, and Bax levels and blocks cell death (Fig. 3A). CHX also blocks the G1-S cell cycle arrest induced by serum withdrawal, most probably due to the decrease in p53 and p21 (Fig. 3B). The p53 inhibitor pifithrin-α protects HepG2 cells from serum withdrawal-induced cell death. Hep3B cells lacking p53 and HepG2 cells expressing dominant negative p53 are more resistant to apoptosis induced by serum withdrawal than control cells (Fig. 4). Decreasing p53 levels by treatment with siRNA of p53 protects HepG2 cells from serum withdrawal-induced apoptosis (Fig. 5). These data indicate that p53 plays an important role in the apoptosis caused by serum withdrawal in HepG2 cells and that an important mechanism by which CHX prevents cell death is linked to the CHX-mediated decline in p53 and pp53 levels.

p53 accumulation caused by DNA damaging reagents is mainly due to posttranslational modifications such as p53 phosphorylation caused by DNA kinases. Phosphorylated p53 has a long half-life compared with nonphosphorylated p53 due to its slower degradation, which then causes p53 accumulation. The phosphorylated p53 can also be dephosphorylated by phosphatases. CHX inhibition of p53 protein synthesis could be a major explanation for its decreasing total p53. However, it might also regulate p53 and pp53 levels by affecting the phosphorylation or dephosphorylation of p53 via acting on DNA kinases or phosphatases. To study the effects of CHX on p53, ActD and the cancer therapeutic reagents doxorubicin and etoposide were used to induce p53. These reagents activate p53 and cause increases of both p53 and pp53. CHX effectively decreases p53 and pp53 levels induced by these reagents (Fig. 6). To determine the effects of CHX on phosphorylation or dephosphorylation of p53, the proteasome inhibitor MG115 was used to maintain the total p53 level at a constant level. MG115 increases the total p53 level, but it does not increase the pp53 level. In the presence of MG115, ActD does not cause any further increase in total p53 levels over the levels found with MG115 alone, but it did increase the pp53 level. Four hours after treatment with ActD to induce pp53, administration of CHX for 6 h does not affect the pp53 level compared with the no CHX-treated levels (Fig. 7A). This suggests that CHX does not stimulate the dephosphorylation of pp53. However, in the presence of MG115 to maintain the total p53 levels, CHX, when added together with ActD, prevents the ActD mediated increase in pp53 levels (Fig. 7B). This suggests that CHX inhibits the phosphorylation of p53 and raises the possibility that kinases that phosphorylate p53 might be inhibited by CHX administration. ATM, a DNA kinase that catalyzes p53 phosphorylation, was therefore evaluated. However, there was no difference in content or activity of ATM in the presence and absence of CHX (data not shown). Other kinases that phosphorylate p53 will require future investigation. These data demonstrate that the CHX decrease of p53 and pp53 levels might be mediated through inhibiting p53 protein synthesis and inhibiting the phosphorylation of p53. The latter will also decrease the total p53 levels via proteasome catalyzed degradation due to the longer half-life of pp53 than p53.

In summary, these experiments indicate that CHX protects HepG2 cells from serum withdrawal-induced apoptosis. Killer protein p53 is involved in the apoptosis induced by serum withdrawal. CHX decreases the p53 and pp53 level via inhibiting p53 protein synthesis and its phosphorylation, thereby preventing cell death.

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References


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