Melatonin reverses tunicamycin-induced endoplasmic reticulum stress in human hepatocellular carcinoma cells and improves cytotoxic response to doxorubicin by increasing CHOP and decreasing Survivin

Abstract: Chemoresistance in hepatocellular carcinoma (HCC) is associated with multiple cellular responses to environmental stresses, such as nutrient deprivation and hypoxia. Nevertheless, whether ER stress resulting from nutrient deprivation and tumor hypoxia contributes to drug resistance remains unclear. Melatonin increased the efficacy of chemotherapeutic drugs in hepatocellular carcinoma in our previous studies. However, the effects of melatonin on endoplasmic reticulum (ER) stress-induced resistance to chemotherapeutic agents in HCC have not been tested. The effect of the endoplasmic reticulum (ER) stress response during resistance of human hepatocellular carcinoma cells against doxorubicin was investigated in this study. Pretreatment of HepG2 and SMMC-7721 cells (two human hepatocellular carcinoma cell lines) with tunicamycin, an ER stress inducer, drastically decreased the rate of apoptosis generated by doxorubicin. Interestingly, co-pretreatment with tunicamycin and melatonin significantly increased apoptosis induced by doxorubicin. Simultaneously, the expression of phosphorylated AKT (p-AKT) was elevated in HepG2 and SMMC-7721 cells given tunicamycin but reduced in the presence of melatonin. Furthermore, consistent with inhibition of AKT activation by using the PI3K inhibitor LY294002, melatonin elevated the levels of CHOP (C/EBP-homologous protein) and reduced the levels of Survivin (a member of the inhibitor of apoptosis protein family) suggesting that inhibition of the PI3K/AKT pathway by melatonin-reversed ER stress-induced resistance to doxorubicin in human hepatocellular carcinoma cells. These results demonstrate that melatonin attenuates ER stress-induced resistance to doxorubicin in human hepatocellular carcinoma cells by down-regulating the PI3K/AKT pathway, increasing the levels of CHOP and decreasing the levels of Survivin.

Introduction

Hepatocellular carcinoma (HCC) is the most common malignant hepatobiliary disease and one of the leading causes of cancer mortality worldwide [1, 2]. To date, there are few effective chemotherapeutic agents for this highly malignant cancer. A major obstacle in the treatment of hepatocellular carcinoma is the resistance acquired by hepatocellular carcinoma cells against current chemotherapy drugs [3]. Thus, approaches to overcoming drug resistance have been a challenging target for the hepatocellular carcinoma therapy.

A number of cellular stress conditions, such as nutrient deprivation, hypoxia, and alterations in glycosylation status, lead to the accumulation of unfolded and/or misfolded proteins in the lumen of the endoplasmic reticulum (ER) leading to ER stress [4]. ER stress triggers an evolutionarily conserved response termed the unfolded protein response (UPR) [5, 6]. The activation of the UPR is believed to alleviate ER stress and promote cell survival via the activation of survival or proliferative pathways [7]. There is increasing evidence that ER stress is activated in various solid tumors including cervical, brain, pancreatic, head and neck, and prostate cancers [8–10]. During tumor development, hypoxia and low glucose levels are known to induce resistance to chemotherapy [11]; however, it is unclear whether the ER stress response due to tumor hypoxia induces chemoresistance of hepatocellular carcinoma cells [12, 13]. Therefore, the identification of ER stress-associated resistance mechanisms and the development of effective agents to evade the resistant phenotype are important clinical issues.

It is commonly accepted that melatonin (N-acetyl-5-methoxytryptamine), the most relevant pineal secretory...
product, controls important physiological functions including circadian rhythms and seasonal reproduction [14–17]. Interestingly, melatonin displays oncostatic actions [18, 19] and increases the efficacy of chemotherapeutic drugs [20]. However, studies related to the effect of melatonin on ER stress-induced resistance to chemotherapeutic agents in HCC are limited. In this study, we provide evidence that melatonin reverses the effects of ER stress-induced resistance to doxorubicin by inhibiting the PI3K/AKT pathway through increasing the levels of CHOP (GADD153) and decreasing the levels of Survivin.

Materials and methods

Reagents

The PI3K inhibitor LY294002, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), melatonin, RNase A, propidium iodide (PI), and tunicamycin (TM) were from Sigma Chemical (St. Louis, MO, USA). Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) System was purchased from Roche (Indianapolis, IN, USA). Doxorubicin was purchased from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China). The anti-AKT antibody was obtained from Abcam (Cambridge, MA, USA). The phospho (p)-AKT(Ser473) antibody was from Cell Signaling Technology, Inc (Danvers, MA, USA). Anti-GRP78, anti-CHOP, anti-survivin, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DMEM was obtained from Gibco BRL Life Technologies (Grand Island, NY, USA).

Cell culture

Human hepatoma cell lines HepG2 and SMMC-7721 were purchased from Shanghai cell bank (Chinese Academy of Sciences, Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37°C in a humidified 5% CO2 atmosphere.

MTT assay

Cells were seeded in 96-well plates at a density of 1 × 104 cells per well and subsequently exposed to the appropriate treatment at 37°C. After the treatments, MTT was added into each well and then incubated for 4 hr at 37°C. The supernatant was discarded, and the pellet was resuspended in 70% ethanol at −20°C for at least 12 hr. Cells were subsequently treated in PBS with RNase A for 30 min at room temperature and stained with propidium iodide (PI). Flow cytometric analysis was performed using an EPICS XL-MCL model counter (Beckman Coulter, Fullerton, CA, USA). A total of 1 × 106 cells/mL were analyzed for each sample, and the experiment was repeated at least three times.

TUNEL assay

Cells seeded on coverslips were fixed with freshly prepared 4% paraformaldehyde in PBS for 1 hr at room temperature, washed with PBS and permeabilized in 0.1% triton X-100 and 0.1% sodium citrate for 2 min at 4°C. Cells were processed for TUNEL staining according to the manufacturer’s instructions (Roche in Situ Cell Death Detection Kit). TUNEL-positive cells were enumerated using the Nikon ECLIPSE 80i biology microscope and Nikon Digital Camera DXM 1200F, ACT-1 version 2.63 software (Yokohama, Kanagawa, Japan).

![Graph](Image)

**Fig. 1.** Effect of tunicamycin on cell viability induced by doxorubicin in HepG2 and SMMC-7721 cells. HepG2 (A) and SMMC-7721 (B) cells were pretreated with tunicamycin (0.1, 0.5, 1.5, 3, 6 μM) for 8 hr then exposed to different concentrations of doxorubicin (0.63, 1.25, 2.5, and 5 mg/L) for 24 hr. Cell viability of HepG2 and SMMC-7721 cells was determined by the MTT assay. Data are expressed as the mean ± S.D. of three independent experiments. (*P < 0.05, **P < 0.01, compared with HepG2/SMMC-7721 cells treated with doxorubicin alone).
Western blotting

Total protein from cells was extracted with RIPA buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.15 M NaCl, 1% NP-40, 0.5% sodium dodecyl sulfate, 1 mg/mL aprotinin, 1 mM phenyl methyl sulphonyl fluoride) on ice for 30 min. Protein concentration was determined by the Lowry Protein Assay. Each extract containing approximately 40 μg protein was subjected to 10% polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (MilliPore, Bedford, MA, USA). The membranes were blocked in TBST (5 mM Tris-HCl, pH 7.4, 136 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 2 hr at room temperature and hybridized with the primary antibody overnight at 4°C, followed by three washes for 5 min with TBST. Then the membranes were incubated with HRP-conjugated secondary antibody for 2 hr at room temperature and washed three times with TBST. Proteins were detected using the enhanced chemiluminescence western blotting detection kit (ECL, Pierce, Rockford, IL) according to the manufacturer’s instruction. β-actin served as the loading control.

Statistical analysis

The data represent mean ± S.D. from three independent experiments. Statistical analysis was performed by a Students’ t-test or ANOVA. Significance was noted at \( P < 0.05 \).
Melatonin reverses endoplasmic reticulum stress-induced chemotherapy resistance

Fig. 4. Effect of melatonin on doxorubicin-induced apoptosis in HepG2 cells. Cells were treated with doxorubicin (2.5 mg/L) and/or melatonin (10^{-3} M) for 24 hr. Apoptosis was analyzed as the sub-G1 fraction by fluorescence-activated cell sorting (FACS). a: Untreated HepG2 cells; b: HepG2 cells treated with melatonin alone; c: HepG2 cells treated with doxorubicin alone; d: HepG2 cells cotreated with doxorubicin and melatonin. (B) and (C) Cell morphology and percentage of apoptotic cells were examined by TUNEL staining. a: Untreated HepG2 cells; b: HepG2 cells treated with melatonin alone; c: HepG2 cells treated with doxorubicin alone; d: HepG2 cells cotreated with doxorubicin and melatonin. Data are presented as mean ± S.D. for the three independent experiments. (*P < 0.05, **P < 0.01 compared with untreated HepG2 cells, #P < 0.05, ##P < 0.01 compared with HepG2 cells treated with doxorubicin).

Fig. 5. Effect of melatonin on doxorubicin-induced apoptosis in SMMC-7721 cells. Cells were treated with doxorubicin (2.5 mg/L) and/or melatonin (10^{-3} M) for 24 hr. Apoptosis was analyzed as the sub-G1 fraction by fluorescence-activated cell sorting (FACS). a: Untreated SMMC-7721 cells; b: SMMC-7721 cells treated with melatonin alone; c: SMMC-7721 cells treated with doxorubicin alone; d: SMMC-7721 cells cotreated with doxorubicin and melatonin. (B) and (C) Cell morphology and percentage of apoptotic cells were examined by TUNEL staining. a: Untreated SMMC-7721 cells; b: SMMC-7721 cells treated with melatonin alone; c: SMMC-7721 cells treated with doxorubicin alone; d: SMMC-7721 cells cotreated with doxorubicin and melatonin. Data are presented as mean ± S.D. for the three independent experiments. (*P < 0.05, **P < 0.01 compared with untreated SMMC-7721 cells, #P < 0.05, ##P < 0.01 compared with SMMC-7721 cells treated with doxorubicin).
pared with doxorubicin alone; #P < 0.05, ##P < 0.01, compared with doxorubicin alone; **P < 0.001, compared with values treated with HepG2/SMMC-7721 cells pretreated with doxorubicin (2.5 mg/L) and/or melatonin (10^{-3} \text{m}) for 24 hr. Treatment with melatonin alone slightly increased the number of cells at Sub-G1 phase, while doxorubicin alone significantly increased the sub-G1 cell population undergoing apoptosis. Furthermore, melatonin treatment further increased the doxorubicin-induced apoptotic cells compared with doxorubicin control. Consistently, combined treatment of doxorubicin and melatonin significantly increased the number of TUNEL-positive cells compared with doxorubicin control in HepG2 and SMMC-7721 cells.

To further determine whether melatonin affects ER stress-induced resistance to doxorubicin, HepG2 and SMMC-7721 cells were first treated with different concentrations of melatonin for 8 hr in the presence of tunicamycin. Similar results were observed through TUNEL staining (Figs 2B,C and 3B,C). These results suggest that ER stress can reduce the sensitivity of hepatocellular carcinoma cell lines to doxorubicin treatment, indicating that ER stress may result in chemotherapeutics resistance.

To investigate whether melatonin influences doxorubicin-induced apoptosis, FACS analysis (Figs 4A and 5A) and TUNEL assay (Figs 4B,C and 5B,C) were performed in HepG2 and SMMC-7721 cells. Cells were treated with doxorubicin (2.5 mg/L) and/or melatonin (10^{-3} \text{m}) for 24 hr. Treatment with melatonin alone significantly increased the number of TUNEL-positive cells compared with doxorubicin control. Consistently, combined treatment of doxorubicin and melatonin significantly increased the number of TUNEL-positive cells compared with doxorubicin control in HepG2 and SMMC-7721 cells.

To further determine whether melatonin affects ER stress-induced resistance to doxorubicin, HepG2 and SMMC-7721 cells were first treated with different concentrations of melatonin for 8 hr in the presence of tunicamycin, and then subjected to doxorubicin treatment for 24 hr. Cell viability was determined by the MTT assay. Data are expressed as the mean ± S.D. of three independent experiments. (*P < 0.05, **P < 0.01, compared with doxorubicin alone; #P < 0.05, ##P < 0.01, compared with values treated with HepG2/SMMC-7721 cells pretreated with tunicamycin and then exposed to doxorubicin for 24 hr).

**Results**

In order to determine the effects of the ER stress inducer, tunicamycin, on doxorubicin-induced cytotoxicity in HepG2 and SMMC-7721 cells (two hepatocellular carcinoma cell lines), cells were first pretreated with tunicamycin for 8 hr and then exposed to different concentrations of doxorubicin for 24 hr. Cell viability was assessed using the MTT assay (Fig. 1). Pretreatment with different concentrations of tunicamycin significantly decreased doxorubicin-induced cytotoxicity in both HepG2 and SMMC-7721 cells between a doxorubicin dosage range of 0.31–5 mg/L. Sub-G1 analysis was then conducted by fluorescence-activated cell sorting analysis (FACS). The morphological changes indicative of apoptosis were also assessed by TUNEL staining. As shown in Figs 2(A) and 3(A), treatment of HepG2 and SMMC-7721 cells with doxorubicin resulted in a dramatic increase in the sub-G1 cell population, which was significantly reduced in the presence of tunicamycin. Similar results were observed through TUNEL staining (Figs 2B,C and 3B,C). These results suggest that ER stress can reduce the sensitivity of hepatocellular carcinoma cell lines to doxorubicin treatment, indicating that ER stress may result in chemotherapeutics resistance.

To investigate whether melatonin influences doxorubicin-induced apoptosis, FACS analysis (Figs 4A and 5A) and TUNEL assay (Figs 4B,C and 5B,C) were performed in HepG2 and SMMC-7721 cells. Cells were treated with doxorubicin (2.5 mg/L) and/or melatonin (10^{-3} \text{m}) for 24 hr. Treatment with melatonin alone significantly increased the number of cells at Sub-G1 phase, while doxorubicin alone significantly increased the sub-G1 cell population undergoing apoptosis. Furthermore, melatonin treatment increased the doxorubicin-induced apoptotic cells compared with doxorubicin control. Consistently, combined treatment of doxorubicin and melatonin significantly increased the number of TUNEL-positive cells compared with doxorubicin control in HepG2 and SMMC-7721 cells.

To further determine whether melatonin affects ER stress-induced resistance to doxorubicin, HepG2 and SMMC-7721 cells were first treated with different concentrations of melatonin for 8 hr in the presence of tunicamycin, and then subjected to doxorubicin treatment for 24 hr. Cell viability was determined by the MTT assay. Melatonin (10^{-5} and 10^{-3} \text{m}) significantly increased doxorubicin-induced cytotoxicity when co-pretreated with tunicamycin in HepG2 (Fig. 6A) and SMMC-7721 cells (Fig. 6B), whereas melatonin at 10^{-7} \text{m} did not induce changes in doxorubicin-induced cytotoxicity. Apoptosis was assessed by FACS analysis (Figs 7A and 8A) and TUNEL staining (Figs 7B,C and 8B,C). Apoptosis induced by doxorubicin was increased by co-pretreatment of tunicamycin and melatonin. In this group, the sub-G1 percentage and the number of TUNEL-positive HCC cells indicative of apoptosis increased compared to cells pretreated with tunicamycin alone. Together, these results support the reversing effect of melatonin on ER stress resistance to doxorubicin.

To investigate the underlying mechanisms involved in reversing effect of melatonin on ER stress resistance to doxorubicin, alterations in the protein expression of AKT and GRP78 (a hallmark of ER stress) in HepG2 and SMMC-7721 cells were determined through western blotting. As shown in Fig. 9A–D administration of tunicamycin to HepG2 and SMMC-7721 cells induced an early increase in GRP78 expression in both cells, indicative of ER stress. Simultaneously, the expression of phospho (p)-AKT in both HepG2 and SMMC-7721 cells rapidly increased after treatment with tunicamycin. These data suggest that the PI3K/AKT pathway may be directly involved in ER stress-induced resistance to doxorubicin.
However, the expression of phospho (p)-AKT was decreased in both cells simultaneously pretreated with both melatonin and tunicamycin (Fig. 10). These data implied that the PI3K/AKT pathway induced by tunicamycin could be altered by melatonin treatment.

We next inquired whether AKT activity inhibited by melatonin influenced intracellular levels of CHOP and Survivin. HepG2 and SMMC-7721 cells were first pretreated with tunicamycin in the presence of LY294002 for 8 hr, followed by doxorubicin treatment for 24 hr. CHOP (CCAAT/enhancer-binding protein homologous protein), also called GADD153, is one of the primary effectors of ER stress-mediated cell apoptosis. Survivin is a member of the inhibitor of apoptosis protein family. Western blotting analysis was performed to detect the expression of CHOP and Survivin. As shown in Figs 11(A,B) and 12(A,B), the expression of CHOP protein was markedly increased and the expression of Survivin was decreased in the presence of LY294002 and tunicamycin. Of interest, consistent with the effect of AKT inactivation by using LY294002, the levels of CHOP were elevated, and the levels of Survivin were reduced by the combined pretreatment of melatonin and tunicamycin (Figs 11C,D and 12C,D). These results suggest that melatonin may have a reversing effect on ER stress resistance to doxorubicin by targeting the PI3K/AKT pathway increasing the levels of CHOP and decreasing the levels of Survivin.

Discussion

Hepatocellular carcinoma (HCC) is one of the most common solid malignancies, which is characterized unfortu-
nately by a high degree of drug resistance [21]. The underlying mechanisms of chemotherapeutic resistance in HCC have not been well addressed. Recently, chemoresistance generated by environmental stresses has been widely elucidated. Resistance can be produced by long-term exposure of cancer cells to various stresses, such as anoxia and lack of nutrition [22, 23], but how these conditions contribute to chemoresistance remains largely unknown. A recent study suggested that ER malfunctions are an important effect that is closely correlated with resistance [24], implying that ER stress might be a novel event connecting the pathophysiologic conditions associated with tumor resistance to chemotherapeutic agents at the molecular level. Therefore, ER stress-mediated resistance to anticancer drugs, in particular in HCC, needs a clearer cellular identification.

The endoplasmic reticulum (ER) stress response constitutes a cellular process that can be triggered by a great variety of conditions that cause imbalances in intracellular homeostasis and threaten proper cell functioning. In response, the ER stress response activates an adaptive effort aimed at neutralizing these threats and re-establishing homeostasis. However, if these countermeasures are unsuccessful and severe imbalances persist, the ER stress response may abandon its pro-survival efforts and instead may initiate a pro-apoptotic program to eliminate the faulty cell for the benefit of the organism as a whole [4–7]. As the tumor grows, cancer cells undergo increasing nutrient starvation and hypoxia, which are strong inducers leading to ER stress and the adaptive, pro-survival components of the ER stress response system that are frequently

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Fig. 8. Effect of copretreatment with melatonin and tunicamycin on cell apoptosis induced by doxorubicin in SMMC-7721 cells. (A) SMMC-7721 cells were treated with 3 μM tunicamycin for 8 hr, in either the absence or the presence of 10−3 μM melatonin and then exposed to doxorubicin (2.5 mg/L) for 24 hr. Apoptosis was analyzed as the sub-G1 fraction by fluorescence-activated cell sorting (FACS). a: Untreated SMMC-7721 cells; b: SMMC-7721 cells treated with doxorubicin alone; c: SMMC-7721 cells copretreated with tunicamycin and then exposed to doxorubicin for 24 hr; d: SMMC-7721 cells copretreated with tunicamycin and melatonin and then exposed to doxorubicin for 24 hr. (B) and (C) Cell morphology and percentage of apoptotic cells were examined by TUNEL staining. a: Untreated SMMC-7721 cells; b: SMMC-7721 cells treated with doxorubicin alone; c: SMMC-7721 cells pretreated with tunicamycin and then exposed to doxorubicin for 24 hr; d: SMMC-7721 cells copretreated with tunicamycin and melatonin and then exposed to doxorubicin for 24 hr. Data are presented as mean ± S.D. for the three independent experiments. (*P < 0.05, **P < 0.01 compared with SMMC-7721 cells treated with doxorubicin alone, #P < 0.05, ##P < 0.01 compared with SMMC-7721 cells pretreated with tunicamycin and then exposed to doxorubicin for 24 hr).
found chronically activated in tumor cells [25]. In order to establish a link between ER stress (preconditioned by tunicamycin) and doxorubicin-induced cell apoptosis in HepG2 and SMMC-7721 cells, HepG2 and SMMC-7721 cells were pretreated with tunicamycin for 8 hr and then exposed to various concentrations of doxorubicin and incubated for 24 hr. The repression of doxorubicin-induced cytotoxicity and apoptosis by tunicamycin were confirmed by the MTT assay, FACS analysis, and TUNEL staining. Our results indicate that pretreatment with tunicamycin significantly decreased doxorubicin-induced cytotoxicity. Moreover, although treatment of HepG2 and SMMC-7721 cells with doxorubicin led to both a significant increase in the sub-G1 cell population and the numbers of apoptotic cells stained with TUNEL, pretreatment of these cells with tunicamycin-repressed apoptosis initiated by doxorubicin. In this study, we show that the induction of ER stress renders HepG2 and SMMC-7721 cells even more resistant to chemotherapeutic drugs doxorubicin. Our results suggest that ER stress may be a possible mechanism by which human hepatocellular carcinoma cells become resistant to available chemotherapeutic drugs. There is, therefore, an urgent need for exploring novel treatment strategies especially targeting ER stress for HCC.

Melatonin is an indoleamine synthesized in the pineal gland. Major areas of melatonin research include its effects on seasonal reproduction, circadian rhythms, and free radical scavenging [26–28]. Other new properties of melatonin also have been reported. An emerging field of research on melatonin is its oncostatic and antiproliferative effects. Recent studies have shown that melatonin is a potent therapeutic agent in combination with other drugs to support the efficacy of conventional anticancer agents and also to reduce their side effects [29–32]. In this study, cell cycle analysis and TUNEL assays also showed that the synergism is correlated with apoptosis induction in HepG2 and SMMC-7721 cells. Combined treatment with melatonin and doxorubicin further augmented doxorubicin-induced apoptosis in HepG2 and SMMC-7721 cells. Nevertheless, the effect of melatonin on ER stress-induced resistance to doxorubicin has not been reported. MTT assays, FACS analysis, and TUNEL quantification demonstrated that copretreatment with tunicamycin and melatonin significantly restored doxorubicin-induced cytotoxicity as well as the number of apoptotic cells stained through FACS and TUNEL. The data reveal that melatonin abrogated the protection of hepatocellular carcinoma cells undergoing ER stress against doxorubicin-induced apoptosis.

Currently, a dearth of information exists concerning how melatonin reverses the protection of hepatocellular carcinoma cells under ER stress against doxorubicin-induced apoptosis. What is currently known is that ER stress generates a cytoprotective response via the activation of survival and proliferation pathways. For example,

![Graphs showing the effect of melatonin on ER stress](image_url)

Fig. 9. Effect of tunicamycin treatment on the expression of phosphor (p)-Akt and GRP78 in HepG2 and SMMC-7721 cells. HepG2(A, B) and SMMC-7721(C, D) cells were treated with 3 µM tunicamycin (TM) for 0 (control), 4 and 8 hr. Equal protein amounts of cell lysates were subjected to western blot assay using specific antiphospho (p)-Akt, anti-Akt, and anti-GRP78 antibody. β-actin in the same HepG2/SMMC-7721 cells extract was used as an internal reference. Optical density reading values of the specific protein versus the loading control protein β-actin are represented as fold of the control values. (*P < 0.05, **P < 0.01 compared with untreated HepG2/SMMC-7721 cells).
activation of the PI3K/AKT pathway is associated with drug resistance in many human malignances [33–35]. In view of this evidence, we examined the status of phosphophorylated AKT (p-AKT) in hepatocellular carcinoma cells during ER stress. Our results indicate that p-AKT was strongly increased upon ER stress induced by tunicamycin. Activation of the PI3K/AKT pathway may be an important protective mechanism against doxorubicin in hepatocellular carcinoma cells undergoing ER stress. Notably, melatonin remarkably impaired phosphophorylated AKT accumulation in HepG2 and SMMC-7721 cells as well as attenuating the protective effects of ER stress on doxorubicin-induced apoptosis. Taken together, these results indicate that the inhibition of PI3K/AKT by melatonin sensitizes human hepatocellular carcinoma cells to doxorubicin-induced apoptosis under ER stress induced by tunicamycin.

To pursue the potential relationship between inhibition of AKT activation by melatonin and other ER stress-related molecules in hepatocellular carcinoma cells, CHOP and Survivin were examined. CHOP (C/EBP-homologous protein also known as GADD153), is a critical initiator of ER stress-induced apoptosis [36]. Survivin, a member of the inhibitor of apoptosis protein family, has been shown to play an important role in ER stress-mediated apoptosis [37]. In light of these events, we evaluated the effect of pretreatment with melatonin on the CHOP and Survivin expression. Surprisingly, the levels of CHOP were dramatically increased, and the levels of Survivin were decreased by the combined treatment of melatonin and tunicamycin. This effect was similar using an inhibition of AKT activation by the PI3K inhibitor, LY294002. Because melatonin also had a similar effect on the same cells suggesting that the inactivation of PI3K/AKT by melatonin in ER stress-mediated resistance to doxorubicin was associated with the expression of CHOP and Survivin. Our results appear to provide several new insights into the effect of melatonin on cancer therapy. Melatonin was shown to attenuate ER stress-induced resistance to doxorubicin, possibly by regulating the PI3K/AKT pathway increasing the levels of CHOP and decreasing the levels of Survivin.

In conclusion, the results of this study, for the first time, provide mechanistic evidence that melatonin reverses the cellular effects of ER stress-induced resistance to doxorubicin in hepatocellular carcinoma cells. Our results provide evidence that melatonin may be a promising approach in abrogating ER stress-induced resistance to chemotherapeutic agents as a therapeutic strategy for the treatment of HCC. However, it is also important to confirm whether melatonin attenuates ER stress-induced resistance against doxorubicin in vivo.

Acknowledgements

This work was supported in part by the Natural Science Foundation of China (No.81071986 and NO.81272739). The authors would like to acknowledge Dr. Dake Huang, the Central Laboratory of morphology of Anhui Medical University for image analysis and Dr. Qingtong Wang, the institute of clinical pharmacology of Anhui Medical University for technical assistance for FACS analysis.

Author contributions

Lulu Fan performed the experiments and wrote the manuscript. Guoping Sun designed the study and revised the manuscript. Dr. Wei Wei revised the manuscript. Tai Ma, Fei Zhong, Yu Lei, Xiaoqiu Li provided the vital reagents and a technical assistance.

Conflicts of interest

The authors declare that there is no conflict of interest.
Fig. 11. Effect of cotreatment with tunicamycin and LY294002/melatonin on the expression of CHOP and Survivin in HepG2 cells. HepG2 cells were treated with 3 μM tunicamycin in either the absence or the presence of LY294002 (30 μM) (A,B) or melatonin (10−3 M) (C,D) for 8 hr. Equal amounts of cell lysates were subjected to western blot analysis using specific anti-p-AKT, anti-AKT, anti-CHOP antibody, and Survivin. β-actin in the same HepG2 cell extract was used as an internal used as an internal reference. Optical density reading values of the specific protein versus the loading control protein β-actin are represented as fold of the control values. (*P < 0.05, **P < 0.01, compared with untreated HepG2 cells, #P < 0.05, ##P < 0.01, compared with HepG2 cells treated with tunicamycin alone).

Fig. 12. Effect of cotreatment with tunicamycin and LY294002/melatonin on the expression of CHOP and Survivin in SMMC-7721 cells. SMMC-7721 cells were treated with 3 μM tunicamycin in either the absence or the presence of LY294002 (30 μM) (A,B) or melatonin (10−3 M) (C,D) for 8 hr. Equal amounts of cell lysates were subjected to western blot analysis using specific anti-p-AKT, anti-AKT, anti-CHOP antibody, and Survivin. β-actin in the same SMMC-7721 cell extract was used as an internal used as an internal reference. Optical density reading values of the specific protein versus the loading control protein β-actin are represented as fold of the control values. (*P < 0.05, **P < 0.01, compared with untreated SMMC-7721 cells, #P < 0.05, ##P < 0.01, compared with SMMC-7721 cells treated with tunicamycin alone).

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