Crosstalk between endoplasmic reticulum stress and oxidative stress in apoptosis induced by α-tocopheryl succinate in human gastric carcinoma cells

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Abstract
α-Tocopheryl succinate (α-TOS) has been shown to be a potent apoptosis inducer and growth inhibitor in a variety of cancer cells. Our previous studies showed the important role of endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) generation in the apoptosis induced by α-TOS. However, the relationship of oxidative stress with ER stress is still controversial. The objective of the present study was to investigate the interplay between the two stress responses induced by α-TOS in SGC-7901 human gastric cancer cells. In response to α-TOS, cytological changes typical of apoptosis, induction of glucose-regulated protein 78 (GRP78) and C/EBP homologous protein transcription factor (CHOP), and activation of caspase-4 were observed. And the antioxidant N-acetyl-L-cysteine inhibited induction of both GRP78 and CHOP by α-TOS transcriptionally and translationally. Furthermore, knocking down CHOP by RNA interference decreased ROS generation, increased glutathione level and induced glutathione peroxidase mRNA expression in α-TOS-treated cells, whereas catalase and superoxide dismutases mRNA expression were not altered. The results imply that α-TOS induces ER stress response through ROS production, while CHOP perturbs the redox state of SGC-7901 cells treated with α-TOS.

Key words: Apoptosis; Endoplasmic reticulum stress; Oxidative stress; α-Tocopheryl succinate; C/EBP homologous protein transcription factor

Gastric carcinoma is one of the most common malignancies and represents the major cause of death worldwide. New therapeutic strategies are urgently needed for treating gastric carcinoma. The successful eradication of cancer cells through apoptosis is the ultimate aim of chemotherapy and chemoprevention1,2). Recently, it has been suggested that endoplasmic reticulum (ER) stress and oxidative stress can trigger apoptotic signalling, although the interplay between the two is still controversial.

The accumulation of unfolded or misfolded protein in ER can induce ER stress. To cope with accumulated unfolded or misfolded ER proteins, mammalian cells trigger a specific response termed the unfolded protein response, which regulates the expression of ER chaperone genes, such as glucose-regulated protein 78 (GRP78)3. During prolonged ER stress, the C/EBP homologous protein transcription factor (CHOP) is one of the most highly up-regulated genes, which has been implicated in ER stress-induced apoptosis4,5. Caspase-12, localised in the ER, is also cleaved and activated, which results in pro-apoptotic actions of ER stress5,6. Oxidative stress is caused by the imbalance between reactive oxygen species (ROS) production and the elimination of toxic intermediates by antioxidant systems7. The ER is a place where ROS are generated, and provides an environment that is highly optimised for oxidative protein folding8,9. It has been reported that the induction of ER stress response by

Abbreviations: α-TOS, α-tocopheryl succinate; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; CAT, catalase; CHOP, C/EBP homologous protein transcription factor; DAPI, DNA dye 4′,6-diamidine-2′-phenylindole dihydrochloride; ER, endoplasmic reticulum; GPX, glutathione peroxidase; GRP78, glucose-regulated protein 78; GSH, glutathione; NAC, N-acetyl-l-cysteine; PERK, RNA-dependent protein kinase-like endoplasmic reticulum kinase; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; siRNA, small interfering RNA; SOD, superoxide dismutase.

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dehydroxymethylpoxyquinomicin is an event downstream of oxidative stress in hepatoma cells\(^{10}\). Sodium selenite-induced ER stress requires ROS generation in human acute promyelocytic leukaemia NB4 cells\(^{11}\). Moreover, Younce & Kolattukudy\(^{12}\) have reported inhibition of ER stress with the ER-stress-specific inhibitor tauroursodeoxycholate, which results in reduced ROS production in H9c2 cells. Nevertheless, it has been shown that glucose-induced ER stress is independent of oxidative stress, and antioxidants can ameliorate oxidative stress without altering the ER stress induced by hyperglycaemia\(^{13,14}\). Interestingly, a study has also revealed that the suppression of ER stress did not attenuateCd-induced oxidative stress in renal tubular cells\(^{15}\). Therefore, there is still no conclusive demonstration of the precise interplay between ER stress and oxidative stress.

\(\alpha\)-Tocopherol succinate (\(\alpha\)-TOS), a derivative of natural vitamin E, has been shown to effectively induce apoptosis in a variety of cancer cells as well as suppress tumour growth in animal models\(^{16-20}\). Our previous studies showed that \(\alpha\)-TOS-induced apoptosis is coupled to ER stress and ROS generation in SGC-7901 human gastric cancer cells\(^{21,22}\). In the present study, we sought to determine whether CHOP down-regulation inhibits oxidative stress induced by \(\alpha\)-TOS and whether reduced ROS production by the antioxidant \(N\)-acetyl-l-cysteine (NAC) decreases ER stress in SGC-7901 cells treated with \(\alpha\)-TOS. To our knowledge, this is the first report that the relationship between ER stress and oxidative stress induced by \(\alpha\)-TOS is elucidated in SGC-7901 human gastric cancer cells.

Materials and methods

Cell culture

SGC-7901 human gastric cancer cells were grown as monolayers in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum, 60 \(\mu\)g/ml penicillin, 100 \(\mu\)g/ml streptomycin and 2 \(\mu\)g/ml glutamine in a humidified 5% \(\mathrm{CO}_2\) incubator at 37°C. For experiments, the level of fetal bovine serum was reduced to 2%. \(\alpha\)-TOS (Sigma) was dissolved in sterile absolute ethanol to make 10 mg/ml stock solution, and diluted in RPMI-1640 complete condition medium correspondingly to the indicated concentrations. An equal amount of ethanol was used as a solvent control.

Detection of apoptosis

Apoptosis was assessed as described previously, based on changes in the nuclear morphology by staining the cells with the fluorescent dye 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI). Briefly, the cells were treated with \(\alpha\)-TOS at 20 \(\mu\)g/ml for 24 h. After fixing and permeabilisation, the cells were incubated with 2 \(\mu\)g/ml DAPI in methanol for 30 min at 37°C. Apoptotic nuclei were observed using an Olympus X70 fluorescence microscope (Olympus) with UV excitation at 300–500 nm.

Western blot analysis

SGC-7901 human gastric cancer cells were treated with 5, 10 or 20 \(\mu\)g/ml \(\alpha\)-TOS and 3 \(\mu\)g/ml tunicamycin for 24 h or 20 \(\mu\)g/ml \(\alpha\)-TOS for up to 24 h. Tunicamycin (Sigma) was used as a positive control. The cells were lysed in the lysis buffer (150 \(\mathrm{mM}\) NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 \(\mathrm{mM}\)-Tris, 1 \(\mathrm{mM}\)-dithiothreitol, 5 \(\mathrm{mM}\)-Na3VO4, 1 \(\mathrm{mM}\)-phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml trypsin, 10 \(\mu\)g/ml aprotinin, 5 \(\mu\)g/ml leupeptin; pH 7.4). After incubation for 15 min at 4°C, the sample was centrifuged at 15,000 \(\times\)g for 8 min at 4°C, and the supernatant was collected as whole cell lysate and stored at -80°C until use. Equivalent amounts of protein were separated on 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The following primary antibodies were used: \(\beta\)-actin antibody (Santa Cruz), GRP78, CHOP antibodies (Cell Signaling), caspase-4 antibody (Abcam). After washing with PBS-Tween 20 (PBST), membranes were incubated with the secondary alkaline phosphatase-conjugated IgG and detected with the Western Blue Stabilized Substrate for alkaline phosphatase (Promega). Bands were analysed using the ChemiImager 4000 instrument (Alpha Innotech).

Transient transfections

Predesigned small interfering RNA (siRNA) against human CHOP and control scrambled siRNA were purchased from Santa Cruz Biotechnology. For siRNA transfection, SGC-7901 cells were plated in six-well culture plates at density of 2 \(\times\)10\(^5\) and transfected with siRNA using X-tremeGENE siRNA Transfection Reagent (Roche), according to the manufacturer’s protocol. Briefly, for each well, 10 \(\mu\)l transfection reagent was diluted in 90 \(\mu\)l serum-free Opti-MEM\(^{\text{R}}\)-1 medium (Invitrogen). This mixture was carefully added to a solution containing 2 \(\mu\)g siRNA in 100 \(\mu\)l dilution medium. The solution was incubated for 20 min at room temperature, and then gently dripped into the SGC-7901 cells in 2 ml antibiotic-free medium. Then, 36 h after transfections, the cells were treated with \(\alpha\)-TOS.

RT-PCR

Total RNA was extracted from SGC-7901 human gastric cancer cells with the TRizol reagent (Invitrogen). RT-PCR was performed according to the manufacturer’s protocol (RNA PCR kit, TaKaRa Shuzo Company, Limited). For each reaction, approximately 1-0 \(\mu\)g of total RNA was used to synthesise the complementary DNA using a thermal program of 25°C for 10 min, 42°C for 30 min and 95°C for 5 min. Forward and reverse sets of RT-PCR primers were designed as follows: \(\beta\)-actin, forward primer 5'-GGGGGCCTGCTCA GGCAACCA-3', reverse primer 5'-GTCTACTCCAA-GCATCATCAATT-3'; \(\alpha\)-TOS, forward primer 5'-GGTGGCCGCTCTAGGCACCAA-3', reverse primer 5'-TTGTTGATGCAGCGGTCG-3'; caspase-4, forward primer 5'-CTCTTTAGTGTCAACGCAG ATTTCT-3' (generates a 540 bp PCR product); CHOP, forward primer 5'-GCACCTCCC-AGAC CGCTACTCC-3', reverse primer 5'-GTCCTATTCCAA-GCTTCCCTCCGGC-3' (generates a 422 bp PCR product); superoxide dismutases (SOD), forward primer 5'-CATCATTAGT TCGAGCAG-3', reverse primer 5'-CTTCTCTCATTTCCACCTT-3'
Endoplasmic reticulum stress and oxidative stress

Our previous studies demonstrated that α-TOS induced a concentration-dependent generation of ROS in SGC-7901 cells.

The antioxidant N-acetyl-L-cysteine abrogates α-tocopheryl succinate-induced endoplasmic reticulum stress response in SGC-7901 cells

To determine whether α-TOS-induced apoptosis is mediated by ER stress in SGC-7901 cells, several ER-stress-associated molecules were examined using Western blot analysis. In these experiments, the glycosylation inhibitor tunicamycin, a conventional inducer of ER stress, served as a positive control. First, the expression of GRP78/Bip, which serves as a gatekeeper to the activation of ER stress transducers, was assessed. Our data showed that treatment of SGC-7901 cells with 20 μg/ml α-TOS led to an increase in the protein levels of GRP78 in a time-dependent manner (Fig. 2(a) and (b)). The ER-related apoptotic proteins CHOP/growth arrest and DNA damage induced gene-153 (GADD153), caspase-4 were also evaluated. We found that α-TOS significantly increased the expression of CHOP, and induced cleavage of caspase-4 in a dose- and time-dependent manner (Fig. 2(a) and (b)).

Endoplasmic reticulum stress is associated with α-tocopheryl succinate-induced apoptosis

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To determine whether CHOP perturbs the cellular redox state, SGC-7901 cells were first transiently transfected with siRNA specific for CHOP for 36 h, and then the levels of ROS, GSH and antioxidant enzyme genes were examined after 12 or 18 h of treatment with α-TOS. When the cells were transfected with CHOP siRNA, the CHOP mRNA was down-regulated, which was not observed in the negative control using a non-silencing siRNA, indicating high efficacy of this approach (Fig. 5(a)). We then found that down-regulation of CHOP significantly reversed α-TOS-induced decrease of GPX mRNA expression; on the other hand, the alterations of CAT and SOD mRNA expression were not reversed by CHOP down-regulation (Fig. 5(a)). Knockdown of CHOP also decreased ROS generation and increased GSH level in α-TOS-treated cells (Fig. 5(b) and (c)). These results suggest that CHOP has an important role in α-TOS-induced oxidative stress in SGC-7901 cells.

Discussion

α-TOS has spurred much recent interest, which can selectively induce apoptosis. Apoptosis induction is arguably the most potent defence against cancer. Recently, the role of redox mechanisms in apoptotic signalling and control attracts great attention, as it is well known that ROS plays a central role in cell signalling. It has been demonstrated that ROS can induce death receptor and mitochondrial apoptotic signalling. ER stress has just been identified as a third apoptotic pathway. However, the relationship of oxidative stress with ER stress is still controversial, which is perhaps as a result of the differences in cell types, treatments and treatment conditions. The present results demonstrated that: (1) after exposure to α-TOS, the main ER-stress-associated molecules – GRP78, CHOP and caspase-4 – were found to be induced and activated, suggesting that ER stress was involved in α-TOS-induced apoptosis of SGC-7901 cells. (2) The antioxidant, NAC, significantly inhibited the induction of GRP78 and CHOP by α-TOS, which indicated that α-TOS induced ER stress response through ROS production in SGC-7901 cells. (3) CHOP down-regulation reversed the alteration of ROS, GSH and GPX levels induced by α-TOS; thus CHOP perturbed the redox state of SGC-7901 cells treated with α-TOS.

ER stress results from the alteration of ER homeostasis brought about by various pathological conditions and treatments with a variety of agents. There are three distinct signalling pathways that are triggered in response to ER stress, mediated by RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). Under non-stress conditions, IRE1 binds to the three components and renders them inactive. During ER stress, IRE1 preferentially associates with the unfolded proteins instead of PERK, ATF6 and inositol-requiring enzyme 1, leading to activation of their downstream signalling. A number of molecules, including CHOP, caspase-12 and c-Jun N-terminal kinase, are involved in ER stress-induced cell death. However, the human caspase-12 gene contains several mutations in the recent adaptation process of humans, which makes it enzymatically inactive. But, it has recently been shown that caspase-4 performs the function of caspase-12 and plays an important role...
in ER-stress-induced apoptosis in humans. In this study, our results showed that α-TOS enhanced the cleavage of caspase-4 and induction of CHOP, GRP78 in a time-dependent manner. Furthermore, in our previous studies, we demonstrated that PERK/activating transcription factor 4 (ATF4), ATF6 and inositol-requiring enzyme 1/X-box binding protein 1 pathways were activated by α-TOS treatment, and phosphorylation of c-Jun N-terminal kinase were responsible for the induction of apoptosis in SGC-7901 cells. Taken together, our data have confirmed that α-TOS-induced apoptosis of human gastric cancer cells was coupled to ER stress and unfolded protein response.

Oxidative stress, a cytopathic consequence of excessive ROS production and the inhibition of ROS scavenging by antioxidant defence system, is known to induce apoptosis. This antioxidative system includes antioxidant enzymes such as SOD, CAT, GPX and low-molecular antioxidants such as GSH. Our previous studies showed that production of ROS and depletion of GSH were induced in a dose-dependent manner in α-TOS-treated cells. Mitochondrial complex II, an important driver of ROS production, has been identified as a molecular target for α-TOS in vitro and in vivo. Therefore, oxidative stress has the potential to induce apoptosis in SGC-7901 cells exposed to α-TOS. In the present investigation, we disclosed that the induction of GRP78 and CHOP mRNA

![Figure 3](https://journals.cambridge.org/abstract富有/10.1017/S0007114512001882/Published_online_by_Cambridge_University_Press)

**Fig. 3.** N-acetyl-L-cysteine (NAC) significantly decreased α-tocopheryl succinate (α-TOS)-induced reactive oxygen species production in SGC-7901 cells. (a) SGC-7901 cells were pretreated or not with the antioxidant NAC (20 mM) for 2 h, and then with 20 μg/ml α-TOS for 12 h. The cells were incubated with the fluorescent probe 2,7'-dichlorofluorescin diacetate (DCFH-DA) and visualised by a confocal microscope. (b) Cells were pretreated or not with the antioxidant NAC at 20 mM for 2 h, and then with 20 μg/ml α-TOS for 12 h. The cells were harvested and incubated with DCFH-DA, and the fluorescence was measured by flow cytometry. Values are means of three separate experiments, with standard deviations represented by vertical bars. * Mean values were significantly different compared with control (P<0.05). † Mean values were significantly different compared with α-TOS-treated cells (P<0.05). (A colour version of this figure can be found online at journals.cambridge.org/bjn)

![Figure 4](https://journals.cambridge.org/abstract富有/10.1017/S0007114512001882/Published_online_by_Cambridge_University_Press)

**Fig. 4.** The antioxidant N-acetyl-L-cysteine (NAC) reverts α-tocopheryl succinate (α-TOS)-induced endoplasmic reticulum stress response. (a) SGC-7901 cells were pretreated or not with 20 mM-NAC for 2 h, and then treated with 20 μg/ml α-TOS for 24 h. Immunoblotting was used to analyse glucose-regulated protein 78 (GRP78) and C/EBP homologous protein transcription factor (CHOP) protein expression in the cell lysate. (b) Cells were pretreated or not with the antioxidant NAC at 20 mM for 2 h, and then treated with 20 μg/ml α-TOS for 15 h. GRP78 and CHOP mRNA were detected by RT-PCR. β-Actin was used as a loading control. The data are representative of at least three independent experiments.
and proteins were inhibited in the presence of NAC, indicating that α-TOS induced ER stress in a ROS-dependent manner in SGC-7901 cells. Consistent with the present results, some research also reported that ROS generation may be an early event that triggers ER stress. Kadara et al. (43) have showed that enhanced ROS formation in human head and neck cancer cells treated with N-(4-hydroxyphenyl)retinamide induces ER stress response. The antioxidants such as ascorbic acid or NAC blocked bovine serum albumin-induced increases in GRP78 activation and eukaryotic initiation factor 2 (eIF2α) phosphorylation in renal proximal tubule cells (44). TNFα-induced activation of unfolded protein response was inhibited by the antioxidant, butylated hydroxyanisole in L929 cells (45). In contrast, high ambient dextrose concentration induces ER stress in human umbilical endothelial cells, and cannot be reversed by treatment with the antioxidants, ascorbic acid and α-tocopherol (14). Mooradian & Haas have also reported that ER stress in endothelial cells can be uncoupled from oxidative stress (13). Currently, the mechanisms underlying ROS-induced ER stress remain elusive. One possibility is that ROS might cause ER stress through inhibition of Ca^{2+}-ATPase, leading to disturbances in Ca homeostasis (15). Our previous data showed that exposure to α-TOS resulted in an increase in the cytosolic Ca^{2+} concentration in a dose-dependent manner (21).

![Fig. 5.](https://doi.org/10.1017/S0007114512001882)
CHOP is a transcription factor involved in ER-stress-induced apoptosis through various mechanisms such as down-regulation of B-cell lymphoma 2 (Bcl-2) (4). Brains from Bcl-2-deficient mice had a higher content of oxidised proteins and higher activities of the antioxidant enzymes (46). It has been reported that the PERK-eIF2α-ATF4 pathway, the main inducer of CHOP transcription, has a special role in protecting cells against oxidative stress (47). Namba et al. (48) have showed that an increase in ROS production was suppressed in CHOP-null mice. These reports indicate that CHOP probably plays a certain role in the regulation of oxidative stress. We report here that a decrease of ROS formation and increase of GSH level were observed in α-TOS-exposed cells with down-regulated CHOP using the RNA interference technology. Data presented here also show that the decrease of GPX mRNA expression by α-TOS, but not the decrease of SOD and CAT, was selectively counteracted by the knockdown of CHOP. All these data provide strong evidence for the first time that CHOP may perturb the cellular redox state via regulating ROS production, GSH level and GPX expression in α-TOS-treated SGC-7901 cells. An interesting question raised here is how CHOP leads to redox imbalance. The possible explanation is that CHOP down-regulates Bcl-2 in α-TOS-exposed cells. Bcl-2 over-expressing has been shown to increase the levels of antioxidant enzymes and GSH, whereas the effects of Bcl-2 on the expression or activity of antioxidant enzymes are cell type-specific and context-dependent (49–51). In the present study, only the antioxidant enzyme GPX is regulated by CHOP in SGC-7901 cells.

Taken together, we conclude that ER stress response and oxidative stress are all involved in α-TOS-induced apoptosis in human gastric cancer cells, and that there is a crosstalk between the two pathways, as depicted in Fig. 6. However, the detailed molecular mechanisms that couple these processes are still unclear. Further investigation will be required to elucidate how and what kinds of ROS induce ER stress.

![Fig. 6. A scheme of proposed mechanisms of α-tocopheryl succinate (α-TOS)-induced apoptosis in the SGC-7901 cells depicting the interplay between oxidative stress and endoplasmic reticulum (ER) stress responses. α-TOS induced reactive oxygen species (ROS) production, and decreased glutathione (GSH) level and the enzyme activities of glutathione peroxidase (GPX), superoxide dismutases (SOD) and catalase (CAT), leading to oxidative stress in SGC-7901 cells. And ROS generation was an early event that triggered ER stress, activating branches of unfolded protein response (UPR), including RNA-dependent protein kinase-like ER kinase (PERK)/activating transcription factor 4 (ATF4), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1)/X-box binding protein 1 (XBP1) pathways. The apoptotic molecules, C/EBP homologous protein transcription factor (CHOP), caspase-4 and c-Jun N-terminal kinase (JNK), were involved in α-TOS-induced apoptosis, while CHOP perturbs the cellular redox state via regulating ROS production, GSH level and GPX expression in α-TOS-treated SGC-7901 cells. NAC, N-acetyl-L-cysteine; siCHOP, small interfering CHOP. (A colour version of this figure can be found online at journals.cambridge.org/bjn)](https://doi.org/10.1017/S0007114512001882)
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References


