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Inhibition of NF-κB nuclear translocation via HO-1 activation underlies α-tocopheryl succinate toxicity☆

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Abstract

α-Tocopheryl succinate (α-TOS) inhibits oxidative phosphorylation at the level of mitochondrial complex I and II, thus promoting cancer cell death through mitochondrial reactive oxygen species (ROS) generation. Redox imbalance activates NF-E2 p45-related factor 2 (Nrf2), a transcription factor involved in cell protection and detoxification responses. Here we examined the involvement of heme oxygenase-1 (HO-1) in the regulation of nuclear factor κB (NF-κB) signaling by short exposure to α-TOS in prostate cancer cells. A short-term (4 h) exposure to α-TOS causes a significant reduction in cell viability (76%±9%) and a moderate rise in ROS production (113%±8%). α-TOS alters glutathione (GSH) homeostasis by inducing a biphasic effect, i.e., an early (1 h) decrease in intracellular GSH content (56%±20%) followed by a threefold rise at 4 h. α-TOS increases nuclear translocation and electrophile-responsive/antioxidant-responsive elements binding activity of Nrf2, resulting in up-regulation of downstream genes cystine-glutamic acid exchange transporter and HO-1, while decreasing NF-κB nuclear translocation. This effect is suppressed by the pharmacological inhibition of HO-1 and mimicked by the end-products of HO activity, i.e., bilirubin and carbon monoxide. Results suggest a little understood mechanism for α-TOS-induced inhibition of NF-κB nuclear translocation due to HO-1 up-regulation.

Keywords: Glutathione; Reactive oxygen species; Heme oxygenase-1; Cystine-glutamic acid exchange transporter; Nrf2; NF-κB

1. Introduction

α-Tocopheryl succinate (α-TOS) is one of the most potent anticancer derivatives of vitamin E [1–3] with *in vitro* efficacy on prostate cancer cells [4]. This derivative is thought to promote cancer cell death through mitochondrial effects that include mitochondrial complex I and II inhibition and reactive oxygen species (ROS) generation [5–8]. Intracellular oxidative stress is sensed by NF-E2 p45-related factor 2 (Nrf2), a transcription factor which, binding to the antioxidant/electrophile response element (EpRE/ARE), induces the expression of phase II/antioxidant genes. Nrf2 protein is regulated by kelch-like ECH-associated protein 1 (Keap1), which binds to and sequesters Nrf2 in the cytoplasm [9]. ROS signaling induces thiol modifications in Keap1 leading to Nrf2 release and nuclear translocation. Nrf2 regulates the expression of several antioxidant enzymes, such as NAD(P)H:quinone oxidoreductase, heme oxygenase-1 (HO-1), thioredoxin reductase 1, L-cystine and L-glutamic acid exchange transporter (XCt), glutamate-cysteine ligase modifier subunit (GCLM) and glutamate-cysteine ligase catalytic subunit (GCLC) [10,11].

HO-1 is a microsomal enzyme catalyzing the first, rate-limiting step in the degradation of heme and playing an important role in iron recycling. By cleaving heme α-meso carbon bridge, HO-1 yields equimolar quantities of carbon monoxide (CO), iron ions (Fe^{2+}) and biliverdin. The enzymatic activity of HO-1 results in decreased oxidative stress [12]. HO-1 also controls cell growth and proliferation and mitigates inflammation. Hence, induction of HO-1 is a key

Abbreviations: α-TOH, α-tocopherol; α-TOS, α-tocopheryl succinate; BSO, L-buthionine-(*S*,*R*)-sulfoximine; CO, carbon monoxide; CORM-2, tricarbonyldichlororuthenium (II) dimer; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; EpRE/ARE, electrophile-responsive/antioxidant-responsive elements; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GSH, reduced glutathione; GSSG, oxidized glutathione; HO-1, heme oxygenase-1; Keap1, kelch-like ECH-associated protein 1; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-dephenyl tetrazolium bromide; NF-κB, nuclear factor κB; Nrf2, NF-E2 p45-related factor 2; ROS, reactive oxygen species; XCt, cystine-glutamic acid exchange transporter; ZnPPIX, zinc protoporphyrin IX.

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event in cellular responses to pro-oxidative and proinflammatory insults [13]. To date, HO-1 expression and nuclear localization define a new subgroup of prostate cancer primary tumors, suggesting that HO-1 may represent a new approach to prostate cancer therapy [14].

Nuclear factor-κB (NF-κB) is an inducible, widely expressed, pleiotropic transcription factor implicated in several physiological and pathological processes, such as infection, inflammation and cancer [15]. The term NF-κB commonly refers to a p50-p65 heterodimer, which is the major Rel/NF-κB complex in most cells. In basal conditions, NF-κB is sequestered in the cytoplasm by inhibitor proteins, usually IκBα. Several NF-κB inducers phosphorylate IκBα that becomes a substrate for ubiquitination and subsequent degradation by the 26S proteasome. The released NF-κB dimer can then translocate to the nucleus and activate target genes by binding with high affinity to κB elements in their promoters [16].

Since anti-inflammatory and anticarcinogenetic agents suppress NF-κB signaling while activating Nrf2-EpRE/ARE pathway [17–19], we decided to investigate the involvement of α-TOS-induced ROS generation in the up-regulation of Nrf2-driven genes and the contribution of these genes to NF-κB inhibition in prostate cancer cell lines.

2. Materials and methods

2.1. Materials

All the reagents, unless otherwise stated, were from Sigma Aldrich (St Louis, MO, USA). All the antibodies, unless otherwise stated, were from Santa Cruz Biotech (Santa Cruz, CA, USA). Cell culture reagents were from Life Technologies (GibcoBRL, Gaithersburg, MD, USA). Tricarbonyldichlororuthenium (II) dimer (CORM-2) was solubilized in dimethyl sulfoxide (DMSO). The inactive form of the compound (negative control) was prepared by solubilizing the compound to DMSO and leaving it for 18 h at 37°C in a 5% CO_2 humidified atmosphere to liberate CO. The inactive CO-releasing molecule (iCORM) solution was bubbled with nitrogen to remove the residual CO present in the solution.

2.2. Primary prostate cell culture

Murine primary prostate cultures were prepared as described [20]. Briefly, mouse prostates were dissected, spread on the surface of a 25-cm^2 culture flask and allowed to adhere to the plastic substrate. Then, explants were covered with Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% fetal calf serum (FCS), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} amphotericin B and incubated for at least 96 h at 37°C in a humidified 5% CO_2 environment. As soon as cells began to emerge from the explants, the medium was replaced with DMEM, 10% FCS and antibiotics.

2.3. Cell culture

Cell lines, obtained from American Type Culture Collection (Rockville, MD, USA), were grown in RPMI supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 environment. The cultures were split twice a week. Human, mouse and rat cells (Table 1), subcultured before each experiment, were grown to 60% confluence before treatment.

2.4. Cell growth and viability assays

Cells were seeded in 96-well plates and treated with α-TOS for the indicated times. Experimental and control cells were adjusted to the same percentage of vehicle (EtOH/DMSO) to avoid solvent interference with results. At fixed times, cell viability was measured using the conventional 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) reduction assay, and live cells were counted using a hemocytometer. To ensure that results were comparable between the cell counting methods, calibration curves for MTT absorbance vs. cell numbers in parallel cultures were constructed. A linear relationship between absorbance vs. cell number was found. Experiments were performed at least three times with six repeats in each experiment. Results were expressed as the % of reduced MTT, assuming that the absorbance of control cells was 100%.

Table 1
IC50 of α-TOS in several cell lines at 24 h exposure

Cellular lines	Origin	IC50 24 h (μM)
SKBR3	Human breast adenocarcinoma	13
TUBO	BALB-neu T mouse-derived mammary lobular carcinoma	15
A549	Human lung epithelial adenocarcinoma	>100
C6	Rat glioma	0.11
THP1	Human acute monocytic leukemia	>100
PC3	Human androgen-independent prostate adenocarcinoma	58
LNCaP	Human androgen-sensitive prostate adenocarcinoma	100
Prostate primary cultures	C57BL/6 mice	> 1 mM

All cell lines were treated for 24 h with increasing α-TOS concentration, and viability was determined by MTT assay. IC50 values were determined graphically.

2.5. Cell uptake of tocopherol compounds

After incubation with tocopherols, PC3 cells (1×10^6) were harvested, and cell pellets were washed twice with fresh medium and then resuspended in 1 ml of phosphate buffer. Two milliliters of ethanol was added, and after vigorous vortexing (30 s), vitamin E compounds were extracted twice with 2 ml of hexane. Cell vitamin E analysis was carried out without saponification, and tocol was used as the internal standard at the final concentration of 10 μM. Test compounds were extracted by 1 vol of ethanol/methanol (1:1 v/v) and 2 vol of n-hexane. The organic layer was collected and evaporated under a stream of N_2. The dried samples were resuspended in methanol and injected into a high-performance liquid chromatography (HPLC) apparatus consisting of two Pro Star solvent delivery systems with a high-pressure gradient mixer and in-line degasser, a Model 410 autosampler (Varian) and an ESA 5600 CoulArray eight-channel electrochemical detector with potentials of the first two cells set in oxidation mode at 300 and 700 mV (vs. Pd reference) for the analysis of tocopherols and tocol, respectively. To measure α-TOS, a UV/Vis detector was mounted in series before the electrochemical detector and set at 215 nm to obtain sufficient sensitivity. Specificity and linearity of analysis were checked against an authentic standard of α-TOS verified for purity at 287 nm. The chromatographic profile was developed under isocratic conditions (flow rate of 1 ml min^{-1}) using as a mobile phase a mixture of acetonitrile and ultrapure water 98/2% (vol/vol) containing 25 mM sodium perchlorate and a Kromasil C18, 5 μm, 250×4.6-mm column (Eka Nobel) protected with a guard column packed with the same stationary phase. Compounds were identified by spiking the samples with known amounts of individual pure tocopherols also used to calibrate the assay.

2.6. Glutathione determination

In time-course experiments, PC3 cells were treated with α-TOS for the indicated times. When L-buthionine-(*S*,*R*)-sulfoximine (BSO) was used, cells were pretreated for 18 h, and then the inhibitor was maintained until the end of the experiment. The determination of the total and reduced form of cell glutathione was carried out by derivatization with the fluorescent probe SBD-F and subsequent analysis by HPLC with fluorometric detection [21]. Glutathione (GSH) concentrations (μM) were expressed as % of control.

2.7. Western blotting

Cells were treated with α-TOS for the indicated time. When inhibitors were used, cells were pretreated for 18 h (BSO) or 4 h (zinc protoporphyrin IX, ZnPPIX), and the inhibitors were maintained until the end of the experiments. After treatment, cells were harvested by trypsinization, and nuclear and cytosolic extracts were obtained [9,10]. Cytosolic and nuclear extracts (40 μg) were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and incubated with anti-Nrf2 (C-20) and NF-κB p65 (A) antibodies (1:400). β-Actin and lamin B (H-90) antibodies (1:400) were used as marker proteins for cytosolic and nuclear extracts. Immunocomplexes were visualized with an enhanced chemiluminescence kit (ECL, Pierce). Bands were analyzed with the Bandscan software.

2.8. Measurement of DCFH fluorescence

Cells were seeded in 96-well plates and treated with α-TOS for the indicated times. The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) method was used to detect the levels of intracellular ROS [22]. DCFH-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2′,7′-dichlorodihydrofluorescein. This

nonfluorescent fluorescein analogue gets trapped inside the cells and is oxidized by intracellular oxidants to a highly fluorescent 2′,7′-dichlorofluorescein, and fluorescence intensity is proportional to the amount of oxidant species produced by the cells. Cells, treated as described, were exposed to DCFH-DA (10 μM) for 30 min at 37°C. Cell viability was not significantly affected by DCFH-DA treatment. The fluorescence of 2′,7′-dichlorofluorescein was detected at 485 nm excitation and at 535nm emission using a plate reader (Titertek Fluoroscan II; Flow Laboratories, McLean, VA, USA). Results, expressed as % of the control DCF fluorescence, were normalized to cell viability.

2.9. Immunocytochemistry

PC3 cells, seeded on chamber slides, were treated for 1 h with 100 μmol L^{-1} α-TOS and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature (RT) for 20 min. After three washes with PBS, the slides were treated with 0.2% Triton X-100 in PBS for 10 min, rinsed and incubated for 60 min at RT with 10% bovine serum albumin (BSA) in PBS. The slides were incubated overnight at 4°C with rabbit polyclonal antibody anti-Nrf2 (1:50) diluted in PBS containing 1% BSA and 0.01% Triton X-100. Control samples were incubated with nonimmune serum. After rinsing, the slides were incubated in the dark at RT with Alexa 488-anti-rabbit IgG (1:1,000) (Molecular Probes-Invitrogen SRL, Milano, Italy) for 1 h and extensively washed, and the cell nuclei were stained with 0.1 mg ml^{-1} diamino-phenylindole (Molecular Probes). Slides were washed and covered with coverslips in Vectashield mounting medium (Vector Lab, Inc., Burlingame, CA, USA), and cells were analyzed with a Nikon Eclipse Te2000-S fluorescence microscope (Nikon Instruments, Spa Calenzano, Italy) equipped with Olympus analysis cell software.

2.10. Electrophoretic mobility shift assay (EMSA)

PC3 cells were treated with α-TOS for 1 or 4 h and then harvested and used for the experiments. Synthetic double-stranded biotin 5′-oligonucleotides containing the Nrf2-binding domain (EpRE/ARE) (5′-TTT TCT GCT GAG TCA AGG TCC G-3′ and 3′-AAA AGA CGA CTC AGT TCC AGG C-5′) and κB responsive element (κBRE) (5′-AGTTGAGGGGACTTTCCCAGGC-3′and 3′-TCAACTCCCCTGAAAGGGTCCG-5′) were used. Nuclear lysates, obtained with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA), were incubated with the labeled probe for 20 min at RT, resolved on a 6% native polyacrylamide gel and transferred to Hybond-N^+ (Amersham Pharmacia). Signals were visualized with peroxidase-conjugated streptavidin (Pierce Biotechnology, Rockford, IL, USA). Specific binding was confirmed with scramble probes, anti-Nrf2 and anti-NF-κB antibodies and nonimmune IgG.

2.11. Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

PC3 cells were treated with α-TOS for 4 h and then harvested and used for the experiments. Total RNA was isolated with TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was performed using the iCycler iQ detection system (Bio-Rad) and SYBR Green chemistry. Human oligonucleotides were obtained from Invitrogen: XCt F: CAACTAGAAGCGTGACAGGT, R: GATGCATGTGCTTTTGTATG; HO-1 F: TGTGGCAGCTGTCTCAAACCTCCA, R: TTGAGGCTGAGCCAGGAACAGAGT; GCLC F: TGCTAAGTCACCATCCACTTCAA, R: ATACTACGAAAGAGCCCCCAAAA; GCLM F: CTGTAAATGGTGTCATTGAGGC, R: CAATACAGAGGTTCATGAGAATGG; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F: TGGTATCGTGGAAGGACTCATGAC, R: ATGCCAGTGAGCTTCCCGTTCAGC.

The SYBR Green RT-PCR amplifications were carried out in a 96-well plate in a 25-μl reaction volume that contained 12.5 μl of 2× iQ SYBR Green SuperMix (Bio-Rad), 400 nM forward and reverse primers, and 5 to 40 ng of cDNA. In each assay, no-template controls were included, and each sample was run in triplicates. The thermal profile consisted of incubation at 95°C 3 min, followed by 40 cycles of denaturation for 10 s at 95°C and an annealing/extension step of 30 s at 58°C.

The mean of Ct values of the stimulated sample was compared to the unstimulated control sample using the Ct value of GAPDH as an internal control.

2.12. Animals

Two/three-month-old male C57BL/6 mice (25–30 g) were housed at the Laboratory Animal Research Centre of Perugia University. The animals were maintained at a constant temperature of 24°C and a 12-h light/dark cycle and were fed *ad libitum*.

2.13. Ethics statement

All experimental procedures were carried out in accordance with European directives, approved by the Institutional Animal Care and Use Committee of Perugia University (N32/2009). Efforts were made to minimize animal stress/discomfort.

2.14. Statistical analysis

All results were confirmed in at least three separate experiments and expressed as mean±S.D. Data were analyzed for statistical significance by two-tailed Student's *t* test using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). *P* values<.05 were considered significant.

3. Results

3.1. α-TOS increases ROS generation and decreases cell viability in prostate cancer cell lines

We analyzed the α-TOS cytotoxic effect in a wide range of cells and showed that α-TOS is selectively cytotoxic in cancer cells since it does not affect viability of normal cell (Table 1). The mechanism underlying α-TOS cytotoxicity is based on generation of ROS [5–8] that, depending on their intracellular levels, can induce redox signaling or oxidative stress [17,23,24]. We started this study by determining the effects of a prolonged exposure to α-TOS on ROS generation and cell viability in two prostate cancer cell lines, i.e., PC3 and LNCaP, and in murine prostate primary cultures. We found that, in prostate cancer cells, α-TOS decreases cell viability and increases ROS generation in a concentration-dependent manner after a 24-h exposure. One hundred micromolars of α-TOS induces a threefold increase in ROS levels and reduces the percentage of viable cells (Fig. 1A). Results show that α-TOS induces oxidative stress. Next, we investigated the early mechanism underlying the α-TOS action and examined the cellular response of PC3 cells to shorter α-TOS exposure (Fig. 1B). Time-course experiments showed that a 4-h exposure to 100 μM α-TOS reduces cell viability and significantly increases ROS levels. Results show that a 4-h exposure is sufficient to trigger cell death.

LNCaP, less sensitive to the toxic effects of α-TOS, showed a concentration-dependent decrease in cell viability and an increase in ROS generation. ROS levels peak at 6-h exposure, while cell viability is not significantly modified (Fig. 1C and D).

Murine prostate primary cultures were not affected by α-TOS treatment at any tested concentration up to 24 h (Fig. 1E).

Data confirm that α-TOS is selectively cytotoxic in cancerous cell lines.

3.2. α-TOS alters intracellular antioxidant defences

Regulation of redox metabolism is mainly provided by GSH systems responsible for maintaining low redox potential and high free thiol levels in the cell. GSH, a tripeptide (γ-Glu-Cys-Gly) present in all mammalian cells, has cytoprotective functions. To explain the low ROS levels observed after a 4-h α-TOS exposure, we performed a time-course experiment of GSH content in PC3 cells incubated with 100 μM α-TOS. α-TOS halves GSH content after a 1-h incubation, whereas it induces a threefold increase in GSH levels after a 4-h incubation, indicating that the low ROS levels can be ascribed to the GSH-ROS-buffering ability. A 4-h exposure to 100 μM α-TOS does not affect intracellular oxidized glutathione (GSSG) levels (Fig. 2A).

To assess the contribution of GSH synthesis system to the intracellular glutathione increase, we treated PC3 cells with 100 μM BSO 18 h prior to the exposure to 100 μM α-TOS for 4 h. The treatment with the inhibitor suppresses GSH accumulation, suggesting that GSH *de novo* synthesis is responsible for the increase in GSH intracellular levels. The presence of 100 μM BSO, the irreversible inhibitor of γ-glutamylcysteine synthetase, 18 h prior

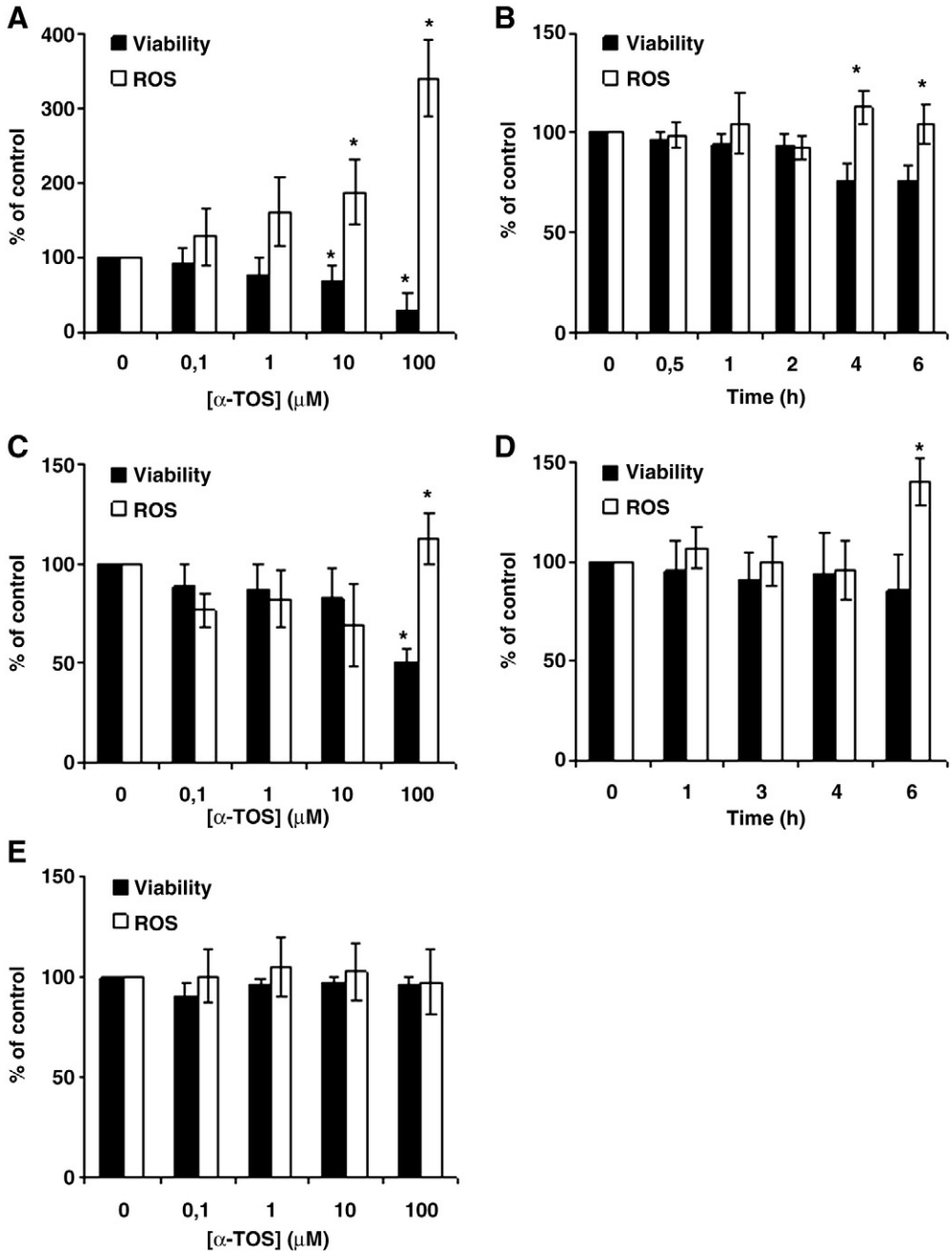


Fig. 1. α-TOS increases ROS generation and decreases cell viability in prostate cancer cell lines. Concentration-dependent response of viability and ROS levels. Prostate cancer PC3 (A) and LNCaP (C) cells and murine prostate primary culture (E) were treated with increasing concentration of (A, C, E) α-TOS (0.1–100 μM) for 24 h and used to determine cell viability and ROS levels. Control values (mean±S.D., n=6) are given as 100% (MTT absorbance 100%=1.12±0.31; DCFH fluorescence 100%=0.95±0.22 for PC3; MTT absorbance 100%=1.32±0.38; DCFH fluorescence 100%=0.82±0.22 for LNCaP; MTT absorbance 100%=0.25±0.004; DCFH fluorescence 100%=0.53±0.08 for murine prostate primary culture). (B and D) PC3 (B) and LNCaP (D) cells were treated with 100 μM α-TOS for the indicated times and used to determine cell viability and ROS levels. Control values (mean±S.D., n=6) are given as 100% (MTT absorbance 100%=0.99±0.27; DCFH fluorescence 100%=0.92±0.18 for PC3; MTT absorbance 100%=1.02±0.28; DCFH fluorescence 100%=0.77±0.12 for LNCaP). *P<.05 vs. control cells.

to the exposure to 100 μM α-TOS for 4 h results in increased ROS generation (Fig. 2B).

Data confirm that GSH is used by the cells in an attempt to counteract α-TOS-induced ROS generation.

To determine whether α-TOS is the active form of the vitamin E derivative, we analyzed α-TOS uptake by PC3 cells after a 4-h exposure, corresponding to the increase in GSH levels. We found high intracellular levels of the derivative, while α-tocopherol (α-TOH) concentration in α-TOS-treated cells was below detection. Results show that the derivative does not undergo de-esterification within PC3 cells (Fig. 2D).

3.3. α-TOS induces Nrf2 activation

One of the major cellular antioxidant responses is mediated by the transcription factor Nrf2 that binds to the EpRE/ARE sequences,

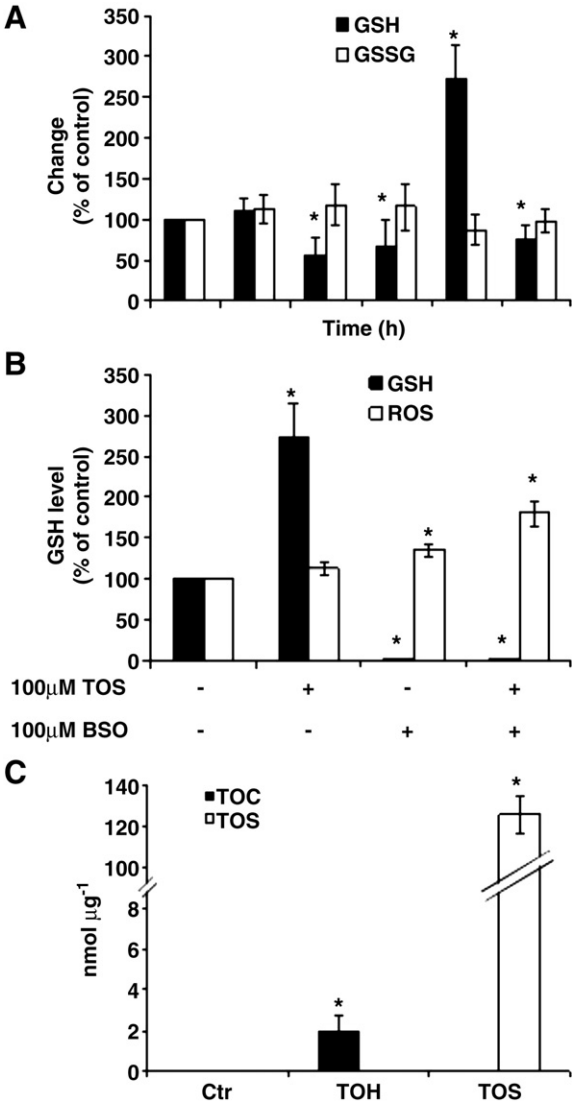


Fig. 2. α-TOS alters intracellular antioxidant defences. (A) Time course of intracellular GSH and GSSG levels. PC3 cells were incubated with 100 μM α-TOS for the indicated times, harvested and used to determine intracellular glutathione levels. Results are given as mean±S.D., $n=6$ independent experiments. Control values are given as 100% (GSH 100%=3.8±1.0 nmol/10^6 cells; GSSG 100%=1.2±0.4 nmol/10^6 cells). (B) Effect of BSO on intracellular GSH and ROS levels. PC3 cells were pretreated for 18 h with 100 μM BSO and then exposed for 4 h to 100 μM α-TOS and used to measure intracellular GSH content and ROS levels. Results are given as mean±S.D., $n=4$ independent experiments. (C) α-TOS uptake. PC3 cells were treated with either 100 μM α-TOH or 100 μM α-TOS for 4 h and used to determine the levels of different tocopherols by HPLC. *$P<.05$ vs. control cells.

thus activating the transcription of antioxidant and phase II detoxifying genes [10,11,17–19].

PC3 cells treated with 100 μM α-TOS display, after 1-h incubation, a transient nuclear accumulation of Nrf2 and an increase in its EpRE/ARE binding capacity, as shown by Western blotting (Fig. 3A), immunocytochemical analyses (Fig. 3B) and EMSA (Fig. 3C). Data show that α-TOS-induced decrease in GSH levels stimulates an antioxidant response through Nrf2 activation.

To determine whether Nrf2 activation is responsible for the increase in GSH intracellular content, we evaluated the expression of GCLC (EC:6.3.2.2) and GCLM by real-time RT-PCR (Fig. 3E). A 4-h exposure to α-TOS affects the mRNA levels of the glutathione-synthesizing enzymes. We then analyzed the expression of XCt since cysteine is the rate-limiting precursor for glutathione *de novo* synthesis. The incubation with 100 μM α-TOS for 4 h induces a sixfold increase in XCt expression (Fig. 3E); thus, the increase in cysteine uptake may be responsible for the rise in intracellular GSH levels.

HO-1 (EC 1.14.99.3) is an EpRE/ARE-regulated gene that participates in maintaining cellular homeostasis and plays an important protective role in the tissues by reducing oxidative injury [12]. A 4-h α-TOS exposure induces a 3.5-fold increase in HO-1 expression (Fig. 3E) in PC3 cells, showing the involvement of this protein in α-TOS action.

3.4. α-TOS inhibits basal NF-κB activation in PC3

Depending on intracellular ROS levels, different redox-sensitive transcription factors are activated and coordinate several biological responses [19,25]. Preincubation with α-TOS significantly inhibits DNA-binding capacity of NF-κB induced by TNF-α in PC3 cells [26]. Since DNA-binding capacity of NF-κB is constitutively high in PC3 cells [27,28], we investigated the ability of α-TOS to reduce basal nuclear levels of NF-κB by Western blotting analysis (Fig. 4A). Time course of NF-κB nuclear accumulation shows that exposure to α-TOS almost halves nuclear NF-κB levels at 4 h as well as decreases κBRE binding capacity (Fig. 4B). Results demonstrated that the vitamin E derivative is capable of inhibiting the survival machinery even in basal conditions.

3.5. α-TOS-induced HO-1 expression is responsible for NF-κB inhibition

NF-κB and Nrf2 are redox-regulated transcription factors characterized by a tightly regulated cross talk [17,19]. To verify the existence of this interplay in PC3 cells, i.e., the activation of Nrf2-EpRE/ARE pathway responsible for the observed NF-κB inhibition, we investigated whether the α-TOS-induced GSH increase is responsible for the observed inhibition of NF-κB nuclear translocation. In the presence of 100 μM BSO, α-TOS-induced effect on NF-κB inhibition is not reverted (Fig. 5A). α-TOS induces the expression of HO-1, and pretreatment with 500 nM ZnPPIX, a selective inhibitor of HO-1, inhibits the decrease in NF-κB nuclear levels (Fig. 5A), confirming the involvement of HO-1 enzymatic activity in α-TOS-induced effect. To validate the involvement of HO-1 in the observed NF-κB inhibition, we treated PC3 cells with products of HO-1 activity, i.e., 100 μM bilirubin and 50 μM CORM-2 for 4 h (Fig. 5B). NF-κB nuclear translocation is significantly reduced (Fig. 5B), while the iCORM has no effect on NF-κB nuclear translocation (data not shown).

These are the first results showing that, in PC3 cells, the basal nuclear accumulation/activation of NF-κB can be inhibited by HO-1 activity.

4. Discussion

Here we showed for the first time that α-TOS, promptly taken up by PC3 cells, is capable of inhibiting neoplastic basal activity of NF-κB by inducing the Nrf2-mediated increase of HO-1 expression. An important cellular protective system that prevents ROS-mediated damage is α-TOH, a lipophilic compound that scavenges oxygen radicals and protects membranes against lipid peroxidation. The anionic vitamin E ester α-TOS is more effective than unesterified α-TOH in protecting isolated hepatocytes against many different toxic oxidative challenges [29]. These anionic properties are likely responsible for the ability of α-TOS to selectively kill tumor cells but not normal cells [1,5,30].

α-TOS, preferentially accumulating in the mitochondria [31], is known to act as a mitocan that induces apoptosis in cancer cells

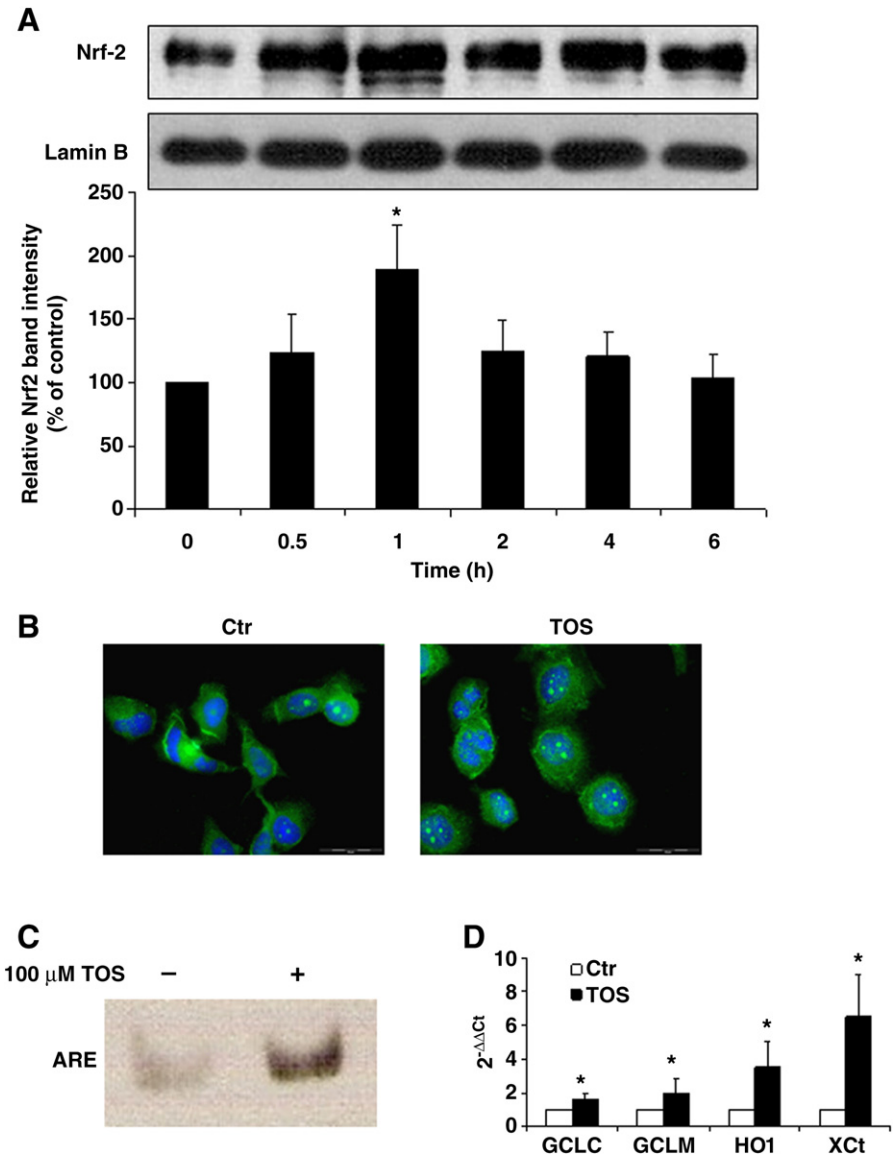


Fig. 3. α-TOS induces Nrf2 activation. (A) Time course of Nrf2 nuclear translocation. PC3 cells were incubated with 100 μM α-TOS for the indicated times and then harvested. The nuclear extracts (40 μg of proteins) were subjected to Western blotting analysis with the indicated antibodies. Anti-lamin B antibody was used as marker for nuclear extracts. Blots of representative experiments are shown. Results of densitometric analyses are given as mean±S.D., $n=3$ independent experiments. (B) Immunofluorescence staining of PC3 cells by anti-Nrf2 antibody (1:50) and DAPI after 1-h exposure to 100 μM α-TOS. (C) EMSA of PC3 cells after 1-h exposure to 100 μM α-TOS with EpRE/ARE biotinylated probe. One out of three representative experiment is shown. (D) Gene expression analysis by real-time PCR in PC3 cells after 4-h exposure to 100 μM α-TOS. Expression of each gene was normalized to GAPDH and reported as $2^{-\Delta\Delta Ct}$. Relative mRNA level of each gene in untreated cells was assumed as 1 and reported as control. Results are given as mean±S.D., $n=3$ independent experiments. *$P<.05$ vs. control.

by mitochondrial destabilization [5] following the formation of mitochondrial outer membrane pores [32]. α-TOS-induced apoptosis is related to the increase in ROS via inhibition of mitochondrial complex I and II activity [6,8], and cells that lack mitochondrial respiratory chain activity are insensitive to α-TOS toxicity [33]. α-TOS-induced apoptosis may also be related to the interaction with the BH3 domain of the Bcl-2 family proteins that disrupts the interaction between Bak, Bcl-x_L and Bcl-2 in prostate cancer cells [34] as well as to the induction of Bax translocation into mitochondria in breast cancer cells [35]. Moreover, Bax dimerization and mitochondrial translocation are related to oxidative stimuli [36].

Redox signaling is a reversible phase of physiological regulatory reactions occurring over short periods in the presence of moderate ROS levels, whereas oxidative stress, occurring in the presence of high ROS levels, causes a persistent and often irreversible oxidative shift that characterizes pathological states [24]. We confirmed that a prolonged α-TOS exposure induces oxidative stress and markedly decreases cell viability, and showed for the first time that a short α-TOS exposure induces redox signaling via Nrf2 activation. Low ROS levels induce Nrf2, a transcription factor implicated in the transactivation of genes encoding for antioxidant/detoxifying enzymes [37,38]. We showed that α-TOS induces early glutathione depletion in PC3 cells. This finding is compatible with glutathione depletion by α-TOS, already reported for leukemia and glioblastoma cells [39,40]. The early decrease in intracellular GSH occurs in the presence of a moderate increase in ROS levels, suggesting that, in the first hour, GSH might be utilized to buffer ROS generation, and its decrease is sensed by the cell that reacts by increasing intracellular antioxidant defences. The decrease in GSH content is

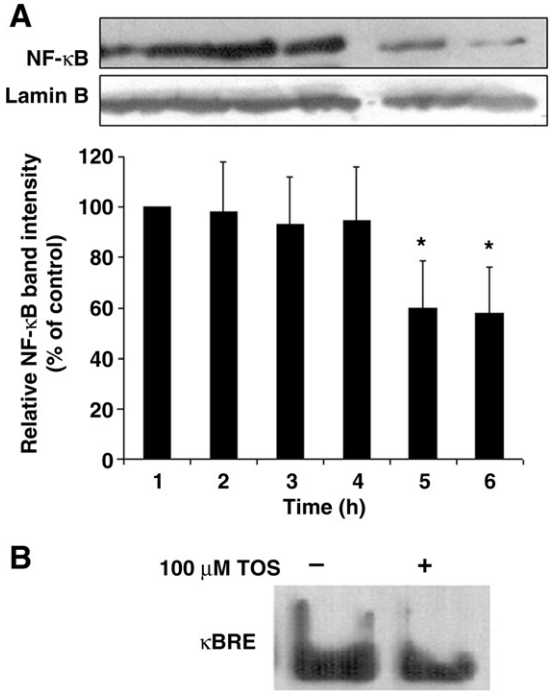


Fig. 4. α-TOS inhibits basal NF-κB activation in PC3. (A) Time course of NF-κB nuclear translocation. PC3 cells were incubated with 100 μM α-TOS for the indicated times and then harvested. The nuclear extracts (40 μg of proteins) were subjected to Western blotting analysis with the indicated antibodies. Anti-lamin B antibody was used as marker for nuclear extracts. Blots of representative experiments are shown. Results of densitometric analyses are given as mean±S.D., $n=3$ independent experiments, *$P<.05$ vs. control. (B) EMSA of PC3 cells after 4-h exposure to 100 μM α-TOS with κBRE biotinylated probe. One out of three representative experiment is shown.

linked to the increase in Nrf2 nuclear translocation and DNA binding capacity, indicating that GSH levels are finely sensed by Nrf2 that, upon activation, increases the expression of antioxidant genes. These results are compatible with reports showing that, in astrocytic cell line and mouse embryonic fibroblasts, the reduction in GSH levels by BSO treatment effectively activates the Nrf2 pathway as a cellular adaptive mechanism [41,42].

Nrf2, by translocating into the nucleus and binding to EpRE/ARE, is capable of inducing the expression of enzymes involved in glutathione *de novo* synthesis, i.e., GCLM, GCLC and XCt [10,11]. α-TOS slightly increases the expression of both GCLC and GCLM and markedly increases the expression of XCt that carries out an essential step in the synthesis of glutathione, thus explaining the observed GSH accumulation.

α-TOS is known to suppress either TNF-related apoptosis-inducing ligand or lipopolysaccharide-stimulated NF-κB activation [43,44]. Here we showed that the vitamin E derivative is capable of reducing the basal nuclear levels of NF-κB and increasing intracellular GSH. Although *S*-glutathionylation of p65 NF-κB is known to be responsible for NF-κB inactivation [45], we showed that GSH accumulation is not involved in the α-TOS-induced NF-κB inhibition, indicating the existence of an alternative inhibitory mechanism.

In endothelial cells, cinnamaldehyde activated Nrf2 and up-regulated HO-1 while inhibiting TNFα-induced NF-κB activation, thus indicating the existence of a cross talk between Nrf2 and NF-κB systems [19,46,47]. Furthermore, biliverdin, a product of the HO-1 catalysis [11], inhibits NF-κB activation [48]. In our experimental conditions, exposure to α-TOS is responsible for a marked increase in HO-1 expression. Compatible with data from endothelial cells [46,47], we showed that pharmacological inhibition of HO-1 eliminates the α-TOS-induced effects on NF-κB. The NF-κB inhibition by HO-1 end-products, i.e., bilirubin and CO, confirms the crucial role of this enzyme in NF-κB signaling suppression by α-TOS.

Collectively, our results describe the mechanism by which α-TOS inhibits NF-κB nuclear translocation and causes cytotoxicity. α-TOS alters intracellular redox homeostasis that, in turn, activates Nrf2 and induces HO-1 expression/activity. The end-products of HO-1 activity cause the inhibition of NF-κB nuclear translocation (Scheme 1).

In conclusion, α-TOS killing of prostate cancer cells is a multilevel process that might be exploited for anticancer therapy.

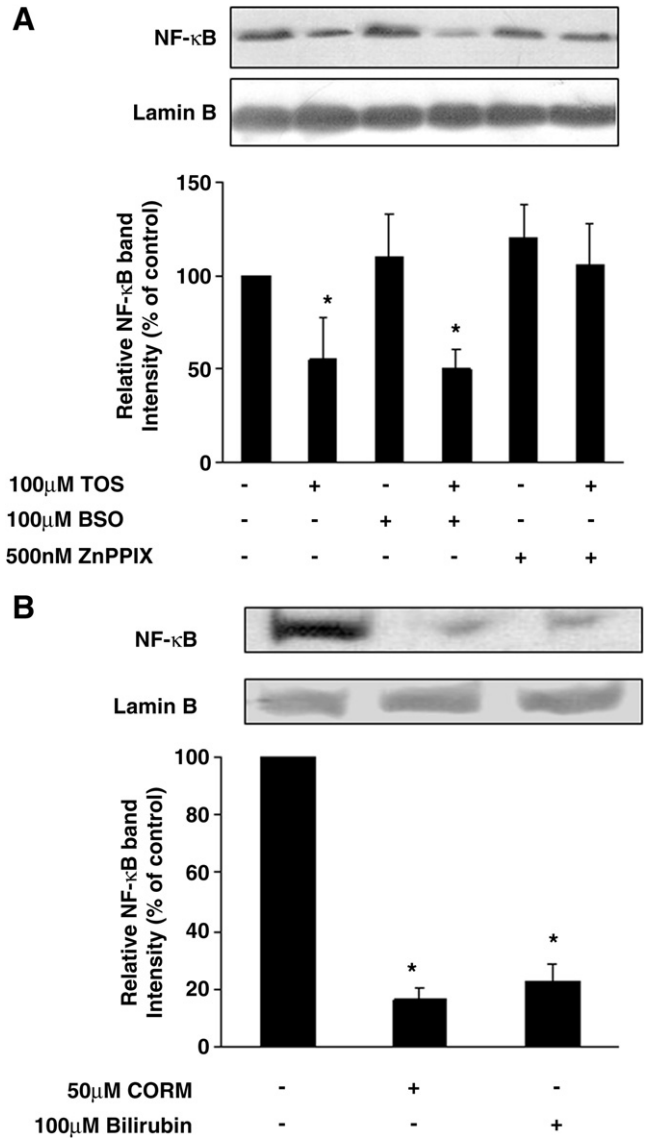
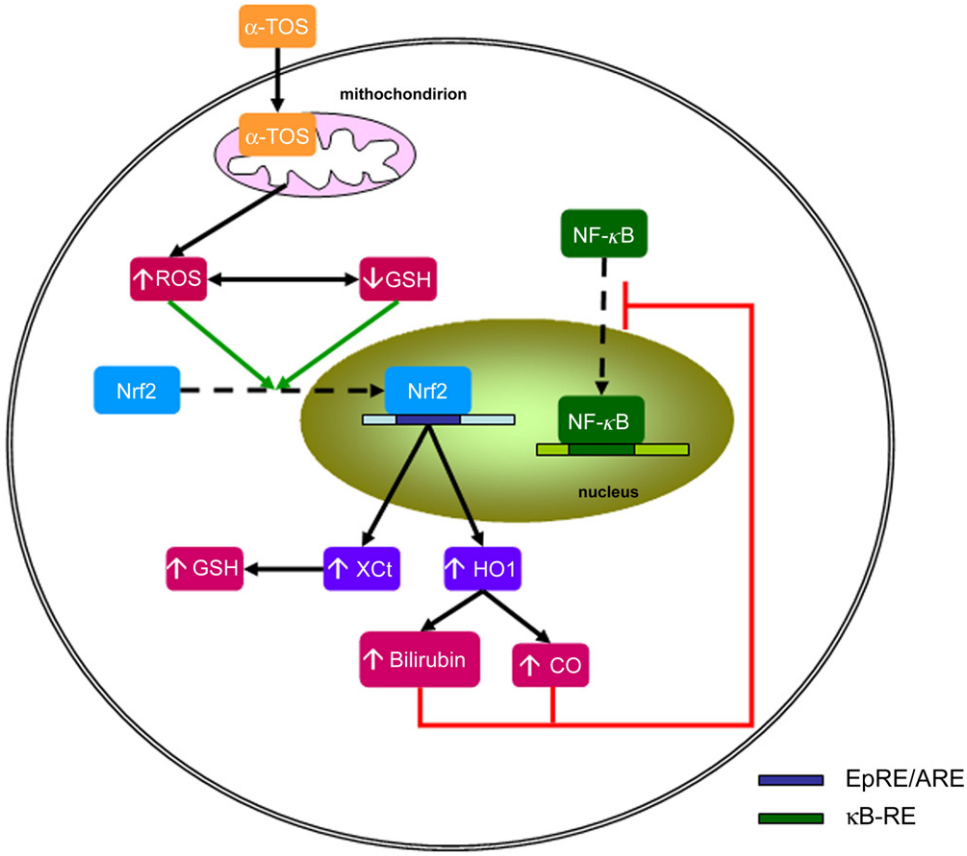


Fig. 5. α-TOS-induced HO-1 expression is responsible for NF-κB inhibition. NF-κB nuclear translocation. (A) PC3 cells were pretreated either with 100 μM BSO for 18 h or with 500 nM ZnPPIX for 4 h and then incubated with 100 μM α-TOS for 4 h. (B) PC3 cells were treated for 4 h with either 100 μM Bilirubin or 50 μM CORM-2. The nuclear extracts (40 μg of proteins) were subjected to Western blotting analysis with the indicated antibodies. Anti-lamin B antibody was used as marker for the nuclear extracts. Blots of representative experiments are shown. Results of densitometric analyses are given as mean±S.D., $n=3$ independent experiments, *$P<.05$ vs. control.



Scheme 1. Proposed mechanism for the early α-TOS effects. α-TOS induces an early GSH depletion to buffer ROS generation, thus activating Nrf2 which, in turn, up-regulates the expression of either XCt or HO-1. While the increased expression of XCt is responsible for the rise in GSH levels, HO-1 activity leads to the inhibition of NF-κB nuclear translocation.

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