Redox stress regulates cell proliferation and apoptosis of human hepatoma through Akt protein phosphorylation

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Abstract Employing a spin trapping agent combined with electron spin resonance spectroscopy, we were able to capture reactive oxygen species (ROS) in living hepatoma cells and first found that the trapped ROS was superoxide anion ($O_2^{\bullet-}$). $O_2^{\bullet-}$ suppressed by treatment with diphenylene iodonium, a flavoprotein inhibitor, was generated by the flavoprotein-containing NADPH-oxidase complex. Applying endogenous/exogenous pro-oxidant or antioxidant causes different redox states in hepatoma cells. Akt activity and cell growth were significantly stimulated by treating hepatoma cells with low concentration of ROS, which could be abolished by adding antioxidants. The phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (0.15 μM) inhibited Akt phosphorylation induced by ROS. Our results indicate that hepatoma cell growth is ROS-dependent, and fluctuation of the intracellular redox state may regulate hepatoma cell growth through Akt phosphorylation and the PI3K/Akt pathway, resulting in a broad array of responses from cellular proliferation to apoptosis.

Key words: Redox state; Akt phosphorylation; Antioxidant; Apoptosis; Electron spin resonance; Reactive oxygen species

1. Introduction

Cellular redox phenomena are involved in numerous biological and biochemical processes. Reactive oxygen species (ROS), by-products of respiration in aerobic organisms, are highly reactive and can oxidize and modify other intracellular molecules. ROS are formed from both exogenous and endogenous sources, includes superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl anion ($^{\bullet}OH$). Endogenous ROS are generated in cells by reducing antioxidants, or directly induced by oxidants/oxidases. A link between intracellular redox balance and growth control has long been implied [1]. Previous studies have indicated that low concentrations of ROS can compensatorily enhance antioxidative enzyme activity [2] and promote cell proliferation, but high concentrations of ROS will induce apoptosis [3–5].

Recently, ROS were recognized as modulators of receptor-mediated signal transduction in a variety of cell types [6–8]. ROS can be a secondary messenger in cell signaling, and the signal transduction pathway may activate protein kinase. However, there is still no direct proof that ROS can mediate hepatoma cell growth.

The evidence on cell growth indicates that the serine-threonine kinase Akt/protein kinase B (PKB) is a critical enzyme in a cell survival pathway. Akt activated by phosphorylation has been shown to protect cells from apoptosis that is induced by external stimuli in different cell types [9,10]. Oxidative stress, antioxidant treatment and the Akt pathway have been shown to play an important role in the regulation of cell growth; however, the relationship between the phosphatidylinositol 3-kinase (PI3K)/Akt signal pathway and the intracellular redox state is still unclear. It is, therefore, of interest to investigate whether fluctuation of the intracellular redox state regulates cell proliferation and apoptosis via Akt phosphorylation and the PI3K/Akt signal pathway, which might provide new strategies in regulating hepatoma cell growth and triggering the apoptosis process.

In the present study, ROS were used to induce oxidative stress, and the levels of antioxidants further give rise to a reduced interference state in hepatoma cells. In this paper we studied the effects of redox state fluctuation on cell growth, and the relationship between this influence and Akt phosphorylation.

2. Materials and methods

2.1. Materials

TRIzol reagent was obtained from Gibco BRL. TES, penicillin, streptomycin, rabbit phospho-Akt (Thr308), Akt antibody, horseradish peroxide-conjugated goat anti-rabbit antibody, and the PI3K inhibitor wortmannin were from Calbiochem. [^{3}H]Thymidine was purchased from Amersham Life Sciences. β-Carotene, diphenylene iodonium (DPI) and *N*-acetyl L-cysteine (NAC) were obtained from Sigma. *Salvia miltriorrhiza* (SM) was provided by the Pharmacology School of Fudan University.

The 7721 human hepatoma cell line was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The PHβA-SOD(+) and PHβA-SOD(−) constructs containing sense or antisense cDNA of human manganese superoxide dismutase (MnSOD) respectively were generously provided by Prof. Kunitaka Hirose.

2.2. Cell culture and transfection

7721 hepatoma cells were grown in RPMI 1640 medium containing

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Abbreviations: ROS, reactive oxygen species; SM, *Salvia miltriorrhiza*; DPI, diphenylene iodonium; PKB, protein kinase B; ESR, electron spin resonance; NAC, *N*-acetyl L-cysteine; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; SOD, superoxide dismutase; PI3K, phosphatidylinositol 3-kinase; DCF-DA, dichlorodihydrofluorescein diacetate

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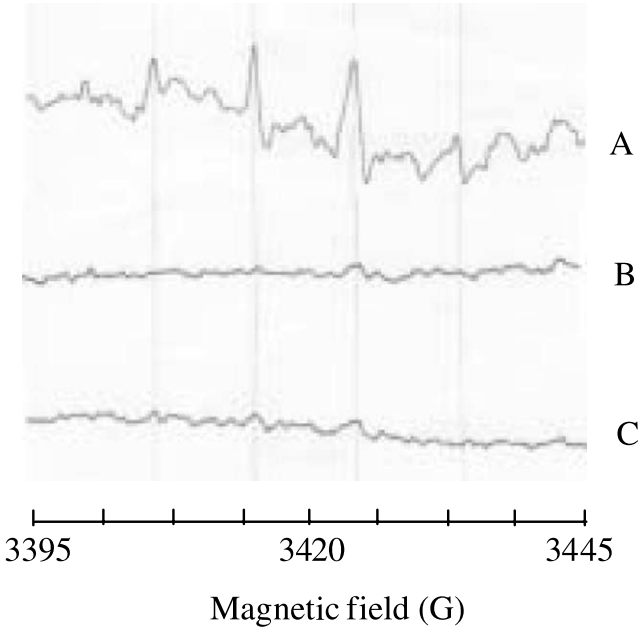

Fig. 1. Spin trapping and ESR spectroscopy. ESR spectra of 7721 hepatoma cells were recorded with the spin trap DMPO on 5×10^6 cells with a Bruker-IBM ER 200D-SRC spectrometer. A: 7721 hepatoma cells displayed a 1:2:2:1 DMPO-OH quartet signal. B: 7721 hepatoma cells treated with SOD (100 U/ml) abolished the free radical signal. C: 7721 hepatoma cells treated with DPI (20 μmol/l) abolished the free radical signal.

10% fetal calf serum, 100 mg/l streptomycin, 100 U/ml penicillin. The sense MnSOD(+) and antisense MnSOD(−) constructs were transfected into hepatoma cells using a standard method [11].

2.3. Cell treatment and incorporation of [^{3}H]thymidine

7721 cells, PHβA-SOD(+) transfected cells (MnSOD-7721) and PHβA-SOD(−) transfected cells (MnSOD-AS7721) were treated with normal saline, Tween-8, oxygen stress (1 or 10 μM H_2O_2) or antioxidants including catalase (5 U/ml), β-carotene (100 μmol/l, dissolved in Tween-8), NAC (20 mmol/l) and SM (40 mg/l). SM can scavenge superoxide and hydroxy radicals [12,13]. Cells were harvested at 2, 4, and 6 days after treatment.

Cell cycle progression was monitored by [^{3}H]thymidine incorporation. Cells were incubated with [^{3}H]thymidine (1 μCi/ml) for 4 h. Total cell DNA was collected on Whatman GF/C filter paper using an automated cell harvester, and incorporated radioactivity was determined by liquid scintillation counting [14].

2.4. Determination of cell apoptosis

The percentage of apoptotic cells was analyzed by flow cytometer. Cells were washed with phosphate-buffered saline (PBS) and fixed with 75% alcohol at −20°C overnight. Then splitting solution was added, and the cells were left stationary for 45 min at room temperature, digested by RNase A (50 μg/ml) for 10 min, and determined with flow cytometer (B.D Company of America). Cells below G1 phase were considered apoptotic cells [5].

2.5. Spin trapping and analysis of ROS in living hepatoma cells

ROS released from hepatoma cells were assessed directly using a spin trapping agent combined with electron spin resonance (ESR) spectroscopy. 10 μl 0.1 mol/l spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was added to 90 μl suspension of 7721 hepatoma cells, and it was transferred to a flat quartz ESR aqueous cell, which was fixed in the cavity of the ESR spectrometer. ESR measurements were carried out on an X-band ESR spectrometer (Bruker-IBM ER 200D-SRC, Germany) under the following experimental conditions: TE = 300 K, SP = 20 mV, SF = 9.66 GHz, MA = 2 G, CF = 3420, SW = 100 G. The sample was scanned and accumulated 10 times for 20 min. SOD or DPI was then added and analyzed as described above.

2.6. Effect of redox state on Akt phosphorylation

Cells (including 7721, MnSOD-7721 and MnSOD-AS7721) at 75–85% confluence in 75 mm dishes were made quiescent by incubation with RPMI 1640 containing 0.1% calf serum for 24 h. Part of 7721 hepatoma cells were treated with exogenous H_2O_2 (10 μM) and antioxidant SM (40 mg/l) for 12 h, respectively, while others were pre-incubated with wortmannin (0–0.15 μM) for 1 h before exposure to H_2O_2 (10 μM, 30 min). Total Akt level and Akt phosphorylation were analyzed by Western blotting [15].

3. Results

3.1. $O_2^{\bullet-}$ are trapped in living hepatoma cells

We used DMPO as a spin trap agent to capture the ROS in

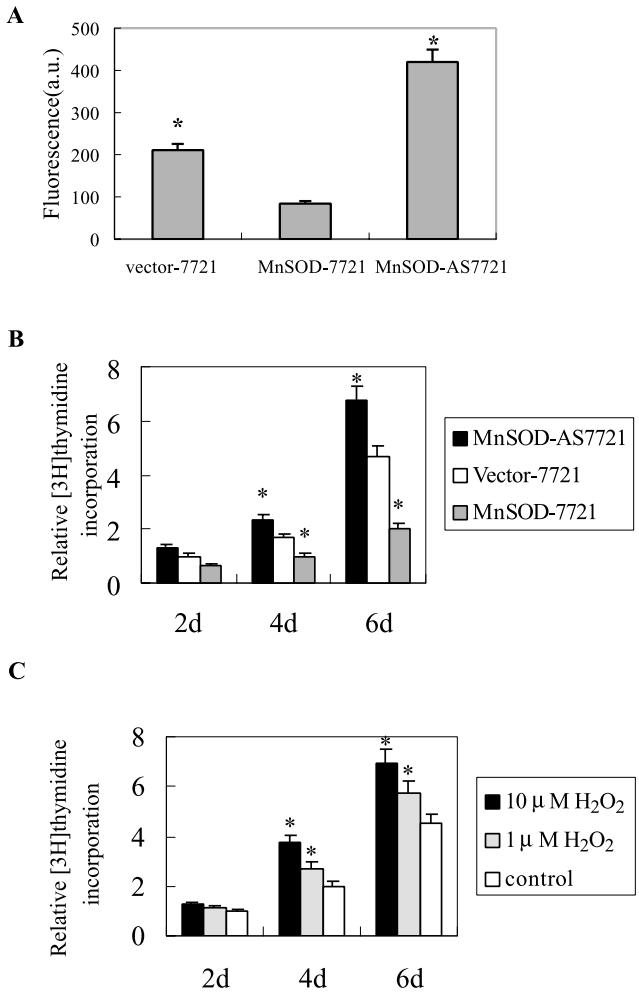

Fig. 2. Effects of redox state on 7721 hepatoma cell proliferation. A: ROS generation in three cell lines. Hepatoma cells were washed three times with PBS, then incubated with DCF-DA (5 μM) at 37°C for 30 min. All cell concentrations were set to 1×10^6 cells/ml. The fluorescence was measured by a fluorescence spectrometer (F-3000, Hitachi) using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Results are expressed as absolute level of fluorescence (arbitrary units). B: Relative [^{3}H]thymidine incorporation in vector-7721, MnSOD-7721 and MnSOD-AS7721 cells. Cells cultured for 2, 4, and 6 days were incubated with [^{3}H]thymidine (1 μCi/ml) for 4 h, [^{3}H]thymidine incorporation was assayed as described in Section 2. C: Relative [^{3}H]thymidine incorporation in 7721 hepatoma cells which were treated with 1 μM or 10 μM H_2O_2; values are the means ± S.D. for three independent experiments. *$P < 0.05$ compared to control cells.

living hepatoma cells. It showed that cells displayed a 1:2:2:1 quartet signal (with $\alpha H = \alpha N = 14.9$ G) indicative of DMPO-OH adduct, indicating that the free radical signal in hepatoma cells was successfully captured (Fig. 1A). The DMPO-OH adduct can be performed either by direct trapping of $^{\bullet}OH$ or by rapid breakdown of DMPO-OOH, the adduct formed by $O_2^{\bullet-}$. SOD, a specific scavenger of $O_2^{\bullet-}$, quenched the captured signal of free radicals (Fig. 1B), which suggested the ESR signal was made from $O_2^{\bullet-}$; meanwhile SOD suppressed DNA synthesis and hepatoma cell growth, whereas catalase did not have these effects. The result further suggested that the free radical signal was attributable to $O_2^{\bullet-}$ trapping rather than to $^{\bullet}OH$ derived from H_2O_2 in hepatoma cells, and was capable of mediating DNA synthesis and cell growth.

To determine the source of $O_2^{\bullet-}$, we treated hepatoma cells with DPI, a potent and highly selective flavoprotein inhibitor. The DMPO-OH signal was diminished in DPI-treated hepatoma cells (Fig. 1C), which indicates that $O_2^{\bullet-}$ was generated from the flavoprotein-containing NADPH-oxidase complex.

3.2. Oxidative state stimulates hepatoma cell proliferation

Intracellular ROS levels were measured using the peroxide-sensitive fluorophore dichlorodihydrofluorescein diacetate (DCF-DA) as described by Jakubowski et al. [16]. The oxidation of DCF-DA was significantly augmented in MnSOD-AS7721 cells, there was a significant (about two-fold) enhancement of DCF compared with the control (vector-7721 cells); in contrast, the oxidation of DCF in MnSOD-7721 cells was reduced about 60% compared to the control (Fig. 2A). Upregulation of MnSOD expression in MnSOD-7721 cells decreased the intracellular oxidative levels, and downregulation of MnSOD expression in MnSOD-AS7721 cells increased the intracellular oxidative levels [17]. The DNA synthesis in these cells showed that overexpression of the MnSOD gene suppressed cell proliferation and downregulation of MnSOD expression stimulated cell proliferation (Fig. 2B). This finding demonstrates that oxidative state could affect cell growth, and cell proliferation was drastically suppressed by reducing endogenous ROS level. In addition, exogenous H_2O_2 (1 or 10 μM) also stimulated cell growth (Fig.

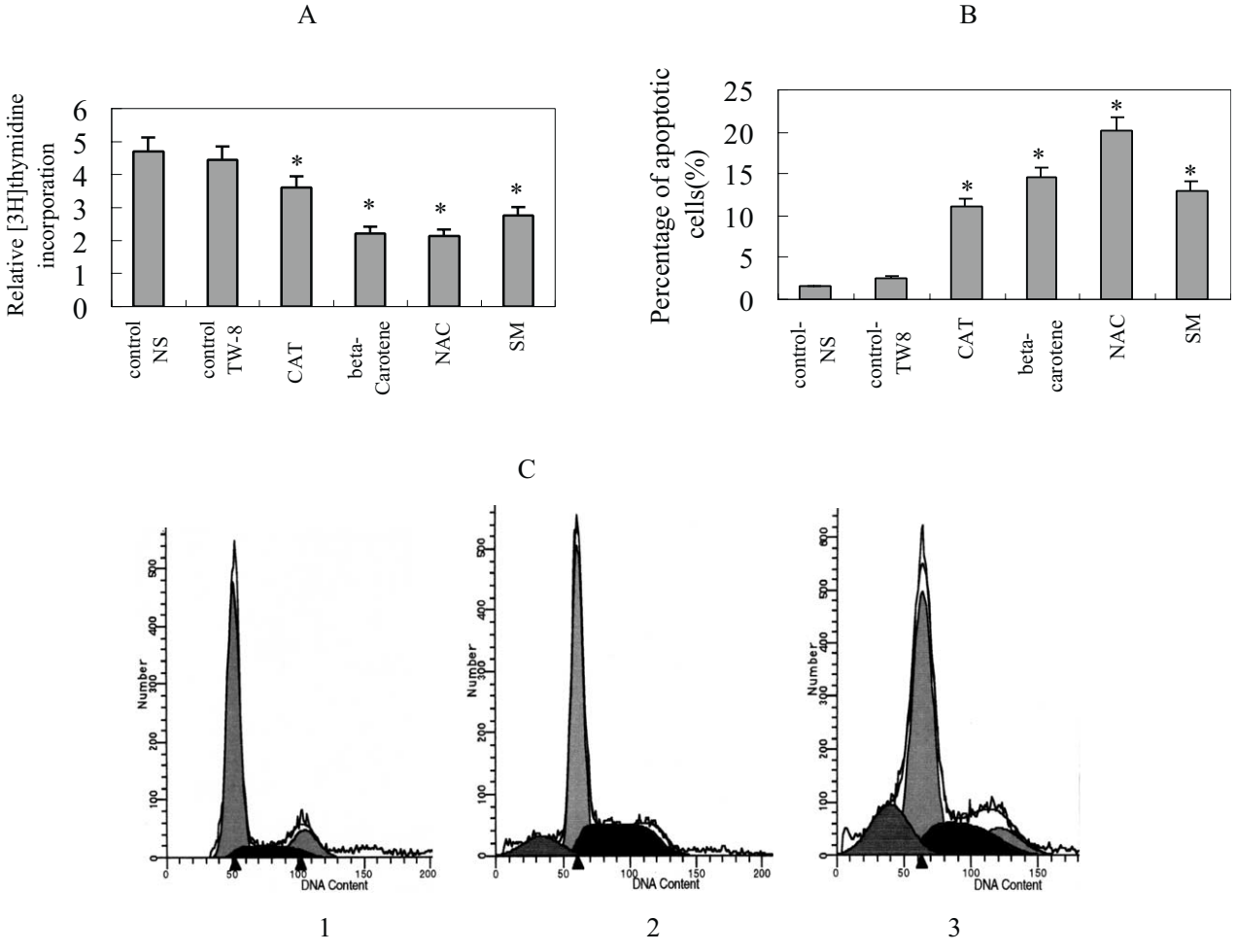

Fig. 3. Effects of antioxidants on hepatoma cells. A: The inhibitory effect of antioxidants on hepatoma cell proliferation. 7721 hepatoma cells were treated with antioxidants for 6 days, then incubated with [^{3}H]thymidine (1 μCi/ml) for 4 h, [^{3}H]thymidine incorporation was assayed as described in Section 2. B: The effect of antioxidants on hepatoma cell apoptosis. 7721 hepatoma cells were treated with antioxidants for 6 days, then apoptosis was detected by flow cytometry (FCM, B.D Company of America). C: Dose response of apoptosis by antioxidant SM in hepatoma cells. 7721 hepatoma cells were treated with (1) 0 mM, (2) 40 mM SM, or (3) 60 mM SM for 6 days, then related nuclear DNA contents were measured by flow cytometry. The percentage of apoptotic cells in (1)–(3) was 0%, 10.96%, 18.7%, respectively. Values are the means ± S.D. for three independent experiments. $^*P < 0.05$ compared to control cells.

2C), but the exogenous antioxidant SM (40 mg/l) inhibited cell proliferation (data not shown). This finding indicates that the intracellular oxidative state could regulate hepatoma cell proliferation.

3.3. Antioxidants inhibit hepatoma cell growth and induce apoptosis

To further study the role of antioxidants in hepatoma cell growth, various antioxidants were applied to 7721 hepatoma cells. The results indicate that antioxidants inhibited DNA synthesis in hepatoma cells to different extents (Fig. 3A); meanwhile, the percentage of apoptotic cells in the antioxidant interference groups increased significantly (Fig. 3B) depending on the dose of SM (Fig. 3C). Therefore, antioxidant interference could not only inhibit hepatoma cell proliferation but also induce apoptosis. It suggests that upregulation of the reduction level may regulate cell growth and suppress the effect of ROS on hepatoma cell growth and apoptosis.

3.4. Redox state regulates Akt phosphorylation via the PI3K-dependent pathway

To understand the mechanism of how antioxidants induce apoptosis in hepatoma cells, we examined the effects of exogenous and endogenous ROS on phosphorylation of Akt. As shown in Fig. 4A (lanes 2, 3), H_2O_2 (10 μM) could stimulate Akt phosphorylation, but significantly decreased the phosphorylation when the antioxidant SM (40 mg/l) added. In addition, we found that upregulation of MnSOD expression inhibited Akt phosphorylation, while downregulation of MnSOD expression induced Akt phosphorylation (Fig. 4A, lanes 5, 6). Proliferation of hepatoma cells increased upon Akt phosphorylation, but was suppressed upon Akt dephosphorylation. Exogenous and endogenous ROS could thus regulate cell growth by regulating Akt phosphorylation; however

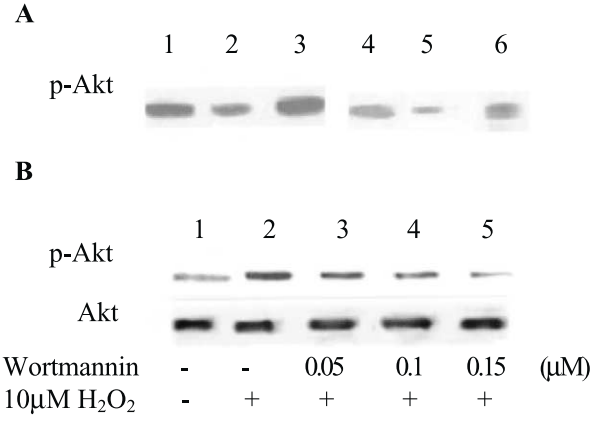

Fig. 4. Effects of redox state on Akt phosphorylation. A: Akt phosphorylation in response to redox stress. Western blotting analysis of cell extracts (50 μg/lane) probed for Thr308 phosphorylated Akt, the protein bands were detected by enhanced chemiluminescence (ECL). Lanes 1–3: Akt phosphorylation in response to control, antioxidant SM (40 mg/l) and H_2O_2 (10 μM) induction for 12 h; lanes 4–6: Akt phosphorylation in vector-7721, MnSOD-7721 and MnSOD-AS7721 cells. B: Dose response of Akt phosphorylation by the PI3K inhibitor wortmannin in H_2O_2-induced hepatoma cells. 7721 hepatoma cells were preincubated with wortmannin (0–0.15 μM) for 1 h before exposure to H_2O_2 (10 μM, 30 min), and cell extracts (50 μg/lane) were analyzed by probing for Thr308 phosphorylated Akt (top) and total Akt levels (bottom). Total Akt levels were similar in various lanes.

the mechanism of Akt phosphorylation induced by ROS is still unclear. In this work, Akt phosphorylation was enhanced when 7721 cells were treated with 10 μM H_2O_2. When 7721 cells were pretreated with wortmannin (a PI3K inhibitor) for 1 h before H_2O_2 stimulation, wortmannin dose-dependently blocked Akt phosphorylation which was induced by ROS (Fig. 4B, top); however, the total levels of Akt were steady in various redox states (Fig. 4B, bottom). This suggests that fluctuation of redox state regulates Akt phosphorylation through a PI3K-dependent pathway.

4. Discussion

Previous studies have indicated that redox state plays a crucial role in carcinogenesis of hepatoma and other cell types [18–20]. It was found that patients with liver cancer or other neoplastic disease have a chaotic antioxidative system. When antioxidants such as vitamin E, vitamin C, vitamin A or some natural antioxidants were used to assist the treatment, effects were significant such as lowering the level of lipid peroxides and decreasing the infection rate of the liver after operation [21,22]. It was reported that β-carotene possesses potent antitumor activity. In the model of chemically induced rat liver cancer, the animals fed β-carotene for a long period of time before the treatment with the chemical toxin had a significantly lower incidence rate of cancer [23,24]. Our previous study also indicated that antioxidants were protective against tumors inoculated at the mouse's axilla [3,5,25]. However, it is still unclear why antioxidant interference can inhibit cancer initiation and progression.

The accumulation of ROS in the body could increase the intracellular oxidative level, leading to a state of oxidative stress, which might further result in the chaotic cellular metabolism involved in various diseases processes. Using antioxidant interference or enhancing the level of antioxidase to scavenge intracellular ROS can enhance the intracellular reductive level and regulate the intracellular redox state to a new balance, and the metabolism in the body will come to order accordingly, which contributes to the prevention of various diseases initiated by cellular injury. Based on this evidence, how to employ interference rationally to change the redox state has attracted much attention in protective strategies, modulating the function of tissue and cells so as to prevent the initiation and progression of cancer.

It is known that some tumor cells produce ROS, but the exact source of these products is unclear. To explore the role of cellular redox state, we further determined the variety and source of ROS generated in hepatoma cells. Employing a spin trapping agent combined with ESR spectroscopy, we successfully captured ROS in living hepatoma cells. We first detected $O_2^{\bullet-}$ in 7721 human hepatoma cells and found it disappeared by treatment with SOD or DPI. MnSOD-7721 hepatoma cells showed that DNA synthesis was not active when the level of free radicals was low. This suggested that $O_2^{\bullet-}$ generated by the flavoprotein-containing NADPH-oxidase complex may directly mediated DNA synthesis and cell proliferation in hepatoma.

Tumors can be initiated by many kinds of factors. ROS, an important factor of initiating carcinogenesis, can modify DNA structure, further leading to the mutation of oncogene/anti-oncogene, and finally cause carcinogenesis. However, ROS can also act as essential intracellular secondary

messengers in regulation of protein kinase activity, relative gene expression, and in modulating cell growth, apoptosis, differentiation and transformation [26]. The activity of protein kinase is associated with its reversible phosphorylation. Cancer cells usually have a lower reductive level and a higher oxidative level, which causes an imbalance in the intracellular redox state. Therefore, fluctuation of the redox state may regulate specific protein phosphorylation and further modulate hepatoma cell growth.

Recent evidence indicates that the serine-threonine kinase Akt/PKB mediates many of the downstream events controlled by PI3K. Following activation of PI3K, Akt isoforms are recruited from the cytosol to the plasma membrane through their interaction with secondary messengers such as phosphatidylinositol 3,4,5-triphosphate and/or phosphatidylinositol 3,4-bisphosphate, where they are thought to undergo a conformational change and the Thr308 residue is exposed, which becomes easily activated by phosphorylation [27]. Our study demonstrated that exogenous and endogenous ROS could improve Akt phosphorylation leading to hepatoma cell proliferation, while PI3K inhibitor or antioxidants could suppress Akt phosphorylation resulting in hepatoma cell apoptosis. Therefore, intracellular redox state fluctuation may regulate hepatoma cell growth through reversible Akt phosphorylation and the PI3K/Akt pathway.

Employing endogenous and exogenous redox interference, we have observed the effects of redox state on Akt phosphorylation and the expression of the related genes. Our experiments suggested that upregulation of intracellular oxidative level by a low concentration of H_2O_2 or antisense MnSOD transfection resulted in an increase of cellular DNA synthesis, Akt phosphorylation and AP-1 transcript factor subunit c-Fos/c-Jun expression (data not shown). In contrast, downregulation of the oxidative level by antioxidant interference or sense MnSOD transfection resulted in a decrease of cellular DNA synthesis, AP-1 transcript factor expression and Akt phosphorylation, but an increase of hepatoma cell apoptosis. Employing various doses of SM, we found the antioxidant-induced apoptosis of hepatoma cells was dose-dependent. The data suggest that the PI3K/Akt pathway may be involved in the regulation of redox state which plays an important role in the growth of hepatoma cells and apoptosis.

In conclusion, our study showed that hepatoma cell growth was ROS-dependent, and endogenous and exogenous ROS or antioxidant interference were capable of regulating Akt phosphorylation through inhibiting Akt phosphorylation and decreasing c-Fos/c-Jun expression so as to suppress hepatoma cell proliferation, or induce apoptosis. This finding further stresses the role of redox state in regulating cancer initiation and progression, and exhibits a broad perspective of antioxidant interference in cancer prevention and therapy.

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