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# **$\beta$ -Carotene induces apoptosis and up-regulates peroxisome proliferator-activated receptor $\gamma$ expression and reactive oxygen species production in MCF-7 cancer cells**

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## ABSTRACT

Although the pharmacological role of  $\beta$ -carotene in the prevention and treatment of many cancer cells has received increasing attention, the molecular mechanisms underlying such chemopreventive activity are not clear. Since peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ) has been implicated in regulating breast cancer cell differentiation and apoptosis, the effects of  $\beta$ -carotene on the PPAR- $\gamma$ -mediated pathway and its association with reactive oxygen species production in MCF-7 cells were investigated in the present study. The results demonstrated that  $\beta$ -carotene significantly increased PPAR- $\gamma$  mRNA and protein levels in time-dependent manner. In addition,  $\beta$ -carotene increased the cyclin-dependent kinase inhibitor p21<sup>WAF1/CIP1</sup> expression and decreased the prostanoid synthesis rate-limiting enzyme cyclooxygenase-2 expression. 2-chloro-5-nitro-N-phenylbenzamide (GW9662), an irreversible PPAR- $\gamma$  antagonist, partly attenuated the cell death caused by  $\beta$ -carotene. Further, reactive oxygen species (ROS) production was induced by  $\beta$ -carotene, resulting in mitochondrial dysfunction and cytochrome C release. Reduced glutathione (GSH) treatment decreases the intracellular ROS and prevents cytochrome C release and cell apoptosis induced by  $\beta$ -carotene. In total, these observations suggest that the synergistic effect of PPAR- $\gamma$  expression and ROS production may account for  $\beta$ -carotene-mediated anticancer activities.

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## 1. Introduction

Epidemiological studies have shown that consumption of diets with a relatively large amount of fruit and vegetables rich in carotenoids and/or high levels of  $\beta$ -carotene (BC) are associated with a decreased incidence of various cancers.<sup>1,2</sup> This effect has been further verified by several data from experimental studies, animal studies, and observational epi-

demiological studies.<sup>3–5</sup> However, harmful effects have been observed in several randomised human intervention trials. Supplementation of  $\beta$ -carotene alone or in combination with vitamins A or E in heavy smokers and in asbestos workers could actually increase lung cancer incidence and mortality.<sup>6,7</sup> Some recent studies suggest that the effect of  $\beta$ -carotene supplementation on the risk of tobacco-related cancers (lung cancer, digestive cancer et al.) may depend on the individuals'

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habits:  $\beta$ -carotene intake was inversely associated with risk of tobacco-related cancers among nonsmokers but directly associated with risk among smokers.<sup>8,9</sup> These contradictory findings have drawn considerable interest to elucidate the mechanisms by which carotenoids affect cancer cell growth.

Peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) is a ligand-activated transcription factor which has been implicated in many processes related to cellular development, differentiation and physiology.<sup>10,11</sup> It is expressed in a large number of human cancers, including breast, colon, stomach, prostate, pancreas, bladder, placenta, lung, chondrosarcoma, as well as in leukaemia. This suggests that PPAR- $\gamma$  plays an important role in carcinogenesis.<sup>12,13</sup> *In vitro* studies have shown that both PPAR- $\gamma$  natural ligand 15-Deoxy- $\Delta^{12,14}$ -prostaglandin  $J_2$  (15dPG- $J_2$ ) and synthetic PPAR- $\gamma$  agonists such as ciglitazone and troglitazone inhibit cell proliferation in several cancer cell lines including melanoma, prostate, colon, and breast cancer cells.<sup>11,14,15</sup> PPAR- $\gamma$  ligands were also found to inhibit anchorage-independent growth, angiogenesis and induce terminal differentiation of cancer cells.<sup>16,17</sup> Recent mechanistic studies have suggested that the activation of PPAR- $\gamma$  may lead to cell cycle arrest and the initiation of apoptosis in cancer cells.<sup>18</sup>

The effects of carotenoids on PPAR- $\gamma$ -mediated pathways have also been studied. It has been shown that the treatment of some isoprenols such as farnesol and geranylgeraniol cause the transactivation of the PPAR- $\gamma$  reporter gene and increased expression of PPAR-targeted lipid metabolic genes.<sup>19</sup> Takahashi and colleagues reported that various carotenoids at 100  $\mu$ M did not significantly affect PPAR- $\gamma$  transactivation.<sup>20</sup> However, Sharoni and colleagues proposed in their reviews that some carotenoids such as lycopene, phytoene, phytofluene, and  $\beta$ -carotene cause the transactivation of peroxisome proliferator response element in cells co-transfected with PPAR- $\gamma$ .<sup>21</sup> Interestingly, Hosokawa and colleagues reported that combined treatment of fucoxanthin, a carotenoid from the edible seaweed *Undaria pinnatifida*, with the PPAR- $\gamma$  ligand, troglitazone, remarkably induced DNA fragmentation which led to the reduction on survival of human Caco-2 colon cancer cells.<sup>22</sup> However, it is still not clear whether the effect of carotenoids on PPAR- $\gamma$  expression could contribute to the inhibition of cancer cell growth or their other anti-cancer activities.

The ability of  $\beta$ -carotene to act as radical scavengers has been noted, especially in *in vitro* model systems, some of which are relevant to mammalian biochemistry/biology.<sup>23</sup> Also, the prooxidant behaviour of  $\beta$ -carotene has been observed in some situations.<sup>23–25</sup> Apoptosis induced by  $\beta$ -carotene is reported to be associated with reactive oxygen species (ROS) generation in WiDr colon adenocarcinoma and HL-60 human leukaemia cell types.<sup>26</sup> In Molt 4 cells, treatment with  $\beta$ -carotene increased intracellular ROS level, which then triggered caspase 2 activation and feedback amplification loop.<sup>27</sup> However, the detailed role of ROS in  $\beta$ -carotene biological effects, especially those that participate with PPAR- $\gamma$ , is still not clear.

Breast cancer is the most common cancer in women worldwide. The PPAR- $\gamma$  pathway has been reported to play a role in the breast cancer cell differentiation.<sup>28</sup> It was reported that 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), a synthetic triterpenoid, is a PPAR- $\gamma$  ligand, and transactivated

PPAR- $\gamma$  induced growth inhibition, cell cycle arrest in G<sub>1</sub>-S and G<sub>2</sub>-M, and apoptosis in breast cancer cell lines.<sup>29</sup> Human clinical trials have demonstrated that the breast cancer risk is inversely associated with  $\alpha$ -carotene,  $\beta$ -carotene,  $\beta$ -cryptoxanthin, lutein, and total carotenoids.<sup>30</sup> The biological significance of PPAR- $\gamma$  in breast cancer cell proliferation regulated by carotenoids has not been determined. To assess the connection between the PPAR- $\gamma$  pathway and the anti-cancer activity of  $\beta$ -carotene, we examined the effects of  $\beta$ -carotene on the expression of PPAR- $\gamma$  and its target genes such as cyclin-dependent kinase inhibitor p21 and cyclooxygenase-2 (COX-2, a rate-limiting enzyme for prostanoid synthesis) in human breast cancer cell line MCF-7. We also investigated the role of PPAR- $\gamma$  and ROS in cell apoptosis induced by  $\beta$ -carotene.

## 2. Materials and methods

### 2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), foetal calf serum and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Gibco BRL (Grand Island, NY, USA). 2',7'-dichlorofluorescein diacetate (DCF-DA), Hoechst 33258, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT), Glutathione reduced (GSH), 2-Chloro-5-nitro-N-phenylbenzamide (GW9662),  $\beta$ -carotene (purity  $\geq$  97%), trypsin, penicillin, streptomycin and Mitotracker Red CMXRos (MTR) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC)-labelled annexin V was purchased from PharMingen (San Diego, CA, USA). Lipofectamine 2000, TRIZOL reagent and RT-PCR kit were purchased from Invitrogen Co. (San Diego, USA). Cytochrome c Release Apoptosis Assay Kit with mouse monoclonal cytochrome c antibody, which reacts with denatured human, mouse, and rat cytochrome c, was purchased from Oncogene (San Diego, USA). Beta-actin and PPAR- $\gamma$  antibodies were purchased from Santa Cruz Biotechnology (Delaware, USA). Cytochrome c-GFP plasmid<sup>31</sup> was kindly provided by Prof. Chang, D.C (Hong Kong University of Science and Technology). Human breast cancer cell line MCF-7 was purchased from cell centre (Institute of Basic Medical, CAMS, Beijing). Non-small lung cell line H1299 and chronic myelogenous leukemia K562 cell line were gifts from Dr. Guangjun Nie (Lady Davis Institute for Medical Research, Canada).

### 2.2. Cell culture and treatment

Cells were maintained in DMEM supplemented with heat-inactivated foetal calf serum (10%, v/v), glucose (4.5 mg/ml), penicillin (100 IU/ml), and streptomycin (100  $\mu$ g/ml) in humidified 5% CO<sub>2</sub>/95% air at 37 °C.

$\beta$ -carotene was delivered to the cell using tetrahydrofuran (THF) as a solvent. The stock solution of  $\beta$ -carotene was prepared and stored at –20 °C in the dark. From the stock solution, aliquots of  $\beta$ -carotene were injected into culture medium to give the desired concentrations immediately before each use. The concentration of THF was adjusted to be the same in all experiments and final concentration was no more than 0.5% (V/V). Control groups received the same amount of THF. When GW9662 (irreversible PPAR- $\gamma$  antago-

nist) and/or reduced glutathione (GSH, intercellular antioxidant) were used, they were added to the medium 2 h before  $\beta$ -carotene treatment.

### 2.3. Assessment of cell survival

The exponentially growing MCF-7 cells were harvested with 0.25% trypsin–0.02% EDTA treatment and plated at a density of  $10^4$ /well in 96-well plates. After overnight incubation to allow cell attachment, the medium was removed (on day 0) and replaced with fresh medium containing  $\beta$ -carotene at a series of concentrations. The effects of GW9662 and/or GSH on  $\beta$ -carotene-induced cell death were also investigated to evaluate the possible involvement of PPAR- $\gamma$  activation and reactive oxygen species (ROS) in the decrease of the cell survival by  $\beta$ -carotene treatment. Cell survival was determined by adding 500  $\mu$ g/ml MTT (final concentration) to each well and the mixture was incubated for another 4 h at 37 °C.<sup>32</sup> After the medium containing MTT was removed, cells were lysed with DMSO. The absorbance at 595 nm was measured by a Bio-Rad model 3550 microplate reader (Richmond, CA, USA). Samples were measured in eight replicates and each experiment was repeated at least three times. Absorbance values were expressed as a percentage of control.

### 2.4. Detection of apoptosis by Annexin V staining

$\beta$ -carotene-induced apoptosis in MCF-7 cells was measured by using an Annexin V-FITC kit according to the manufacturer's instructions (BioVision, Inc., Palo Alto, CA, USA). MCF-7 cells were plated at a density of  $5 \times 10^5$  in 60-mm cell culture dishes and incubated overnight for cell attachment. The exponentially growing cells were treated as described above with 50  $\mu$ M  $\beta$ -carotene, and/or GW9662 and/or GSH for 3 days. The cells were harvested and washed three times with cold 1 $\times$  PBS, and resuspended in 500  $\mu$ l of binding buffer. The cell suspension was incubated in the dark room for 15 min with 5  $\mu$ l of Annexin V-FITC and 5  $\mu$ l of propidium iodide. Annexin V and PI emissions were detected in the FL1-H and FL2-H channels of a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA), using emission filters of 525 and 575 nm, respectively.

### 2.5. Morphological changes

Changes in nuclear morphology of apoptotic cells were labelled by Hoechst 33258 and examined by fluorescent microscopy.<sup>33</sup> The cells were fixed with Carnoy's fixative buffer containing methanol and glacial acetic acid (3:1, v/v), and incubated with Hoechst 33258 (3  $\mu$ g/ml) for 30 min, then the nuclear morphology was observed under a fluorescence microscopy (Olympus, kx14e). Cells which exhibited reduced nuclear size, chromatin condensation, intense fluorescence and nuclear fragmentation were considered as apoptotic.

### 2.6. Measurement of intracellular ROS

It has been reported that  $\beta$ -carotene is able to significantly modify ROS production in cancer cells and this effect was accompanied by changes in cell growth.<sup>24</sup> The level of intra-

cellular ROS was quantified by fluorescence with 2', 7'-dichlorofluorescein diacetate (DCF-DA).<sup>34</sup> After treatment, the cells were collected and washed three times with 1 $\times$  PBS then incubated with 5  $\mu$ M DCF-DA for 45 min at 37 °C in the dark. Then the cells were washed three times with 1 $\times$  PBS and resuspended in BSS buffer containing 130 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 15 mM Glucose, and 5 mM HEPES, pH 7.4. The relative levels of fluorescence were quantified by a fluorescence spectrophotometer (Hitachi F-4500, Tokyo, Japan, 485-nm excitation and 535-nm emission). The data were expressed as a percentage of the fluorescence relative to the fluorescent value in control cells.

### 2.7. Confocal microscopy and photogrammetry

Cells were plated onto poly-D lysine-coated glass coverslips and grown to about 75% confluence at 37 °C and 5% CO<sub>2</sub>. To image cytochrome c, cells were transfected with the cytochrome c-GFP plasmid using lipofectamine 2000 and cultured for 48–72 h to allow the fusion gene expression. For staining mitochondria, Mitotracker Red CMXRos (MTR) was added to a final concentration of 20 nM. For living cell measurements, a laser scanning confocal microscope (Olympus FV500, Tokyo, Japan) was used to image cytochrome c-GFP distribution and mitochondria morphology simultaneously. Cytochrome c-GFP and Mitotracker were observed at 488 nm and 568 nm, respectively.

### 2.8. Western blot

To detect mitochondrial cytochrome C release, approximately  $5 \times 10^7$  control or treated cells were harvested and washed twice with cold 1 $\times$  PBS. The cytosolic and mitochondrial fractions were separated according to the manual provided in the cytochrome c release apoptosis assay kit. As for protein expression experiments, harvested cells were lysed in lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.02% (w/v) NaN<sub>3</sub>, 100  $\mu$ g/ml PMSE, 1  $\mu$ g/ml Aprotinin 1  $\mu$ g/ml Pepstatin A, 2  $\mu$ g/ml leupeptin and 1% (v/v) Triton-X 100) on ice for 30 min. Protein content was measured by BCA kit (Pierce Inc) and the protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Forty  $\mu$ g protein was loaded in SDS-PAGE experiments, separated on gels (12% for cytochrome C and p21, 10% for PPAR- $\gamma$  and COX-2), and then transferred to nitrocellulose membranes. Blots were blocked in 0.1 M TBS containing 5% no-fat milk, 0.1% Tween 20 and then incubated with primary antibody (1:1000) overnight with constant agitation at 4 °C. After washing four times the membrane were incubated with the second antibody for 1 h at room temperature with constant agitation, then washed and reacted with the chemiluminescent substrate (Pierce Biotechnology, IL), and exposed to Kodak-XAR film. The image was digitised and analysed by a NIH imaging software.

### 2.9. RT-PCR assay

Total RNAs were extracted from cells using Trizol reagent as described by the manufacturer (Invitrogen, USA). Two  $\mu$ g of total RNA was reverse transcribed into cDNA using a SuperScript. First-Strand Synthesis System (Invitrogen, USA). A

standard 25  $\mu$ l polymerase chain reaction (PCR) containing 2  $\mu$ l of the reverse transcriptase reaction mixture was performed following: 95 °C 45 sec, 30 °C 60 sec, 72 °C 45 sec, 30 cycles with TaKaRa Ex Taq™ Hot Start Version. (Takara Japan) in MJ Research PTC-200 Peltier Thermocycler. The primers used for PCR reaction were as follows: PPAR- $\gamma$  sense, 5'-TCTGGCCACCAACTTTGGG-3'; antisense, 5'-CTTACAAGCATGAACTCCA-3' (360 bp); COX-2 sense, 5'-GGTCTGGTGC CTGGTCTGATGATG-3'; antisense, 5'-GTCCTTCAAGGAATGGTGC-3' (724bp); p21<sup>WAF1/CIP1</sup> sense, 5'-GCCGAAGTCAGTTCTTGTGGA-3'; antisense, 5'-GTGGGCGGATTAGGGCTT-3' (579 bp). The PCR products were electrophoresed on a 2% agarose gel.

## 2.10. Statistical analysis

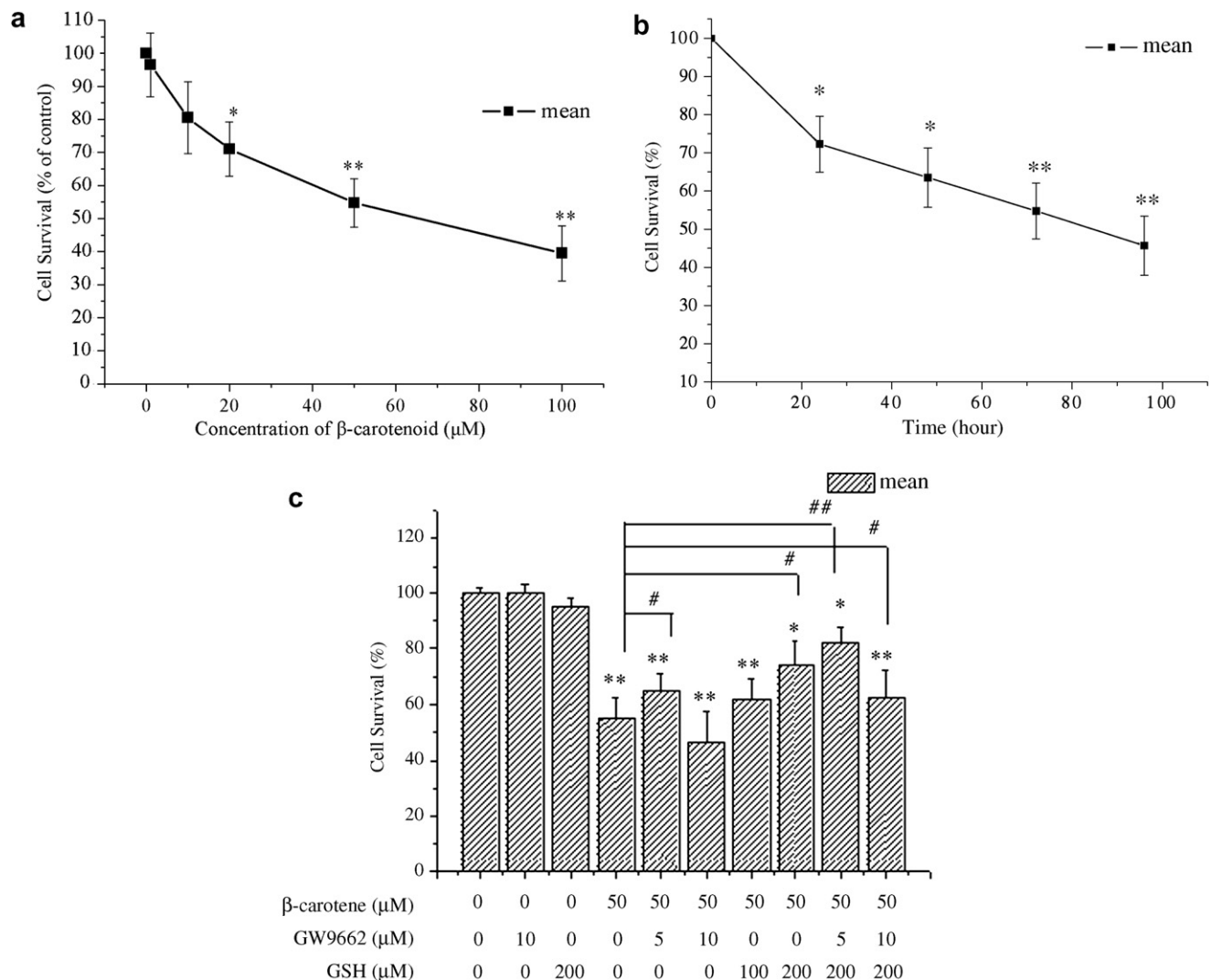
All experiments were performed at least in triplicate. One-way ANOVAs were used to estimate overall significance followed by post hoc Tukey's tests corrected for multiple

comparisons. Data are presented as mean  $\pm$  SEM. A probability level of 5% ( $p < 0.05$ ) was considered significant.

## 3. Results

### 3.1. Effect of $\beta$ -carotene on the survival of MFC-7 cancer cells

MCF-7 cancer cells were treated with increasing concentrations (1–100  $\mu$ M) of  $\beta$ -carotene, and the cell survival was examined.  $\beta$ -carotene remarkably decreased the survival of MCF-7 cells in a dose-dependent manner (Fig. 1a). After 72 h of incubation with 20 and 50  $\mu$ M of  $\beta$ -carotene, the numbers of viable cells decreased to 70% and 50%, respectively, in comparison to controls. The effect of  $\beta$ -carotene on the cell survival was also time-dependent (Fig. 1b). When the treatment time was prolonged from 24 h to 96 h, the cell survival rate was decreased from 71% to 42% in the presence of 50  $\mu$ M  $\beta$ -



**Fig. 1 –  $\beta$ -carotene causes cell survival loss in time- and dose-dependent manners.** (a) MCF-7 cells were exposed to different concentrations of  $\beta$ -carotene (1–100  $\mu$ M) for 72 h; (b) MCF-7 cells were exposed to 50  $\mu$ M  $\beta$ -carotene for different time points (24–96 h); (c) The effects of GW9662 and GSH on cell survival loss induced by  $\beta$ -carotene (72 h). Data are presented as the means  $\pm$  SEM,  $n = 8$ , \* $p < 0.05$ , \*\* $p < 0.01$  versus control group; # $p < 0.05$ , ## $p < 0.01$  versus  $\beta$ -carotene-treated group.



carotene. Similar effects on cell survival loss induced by  $\beta$ -carotene were also found in H1299 and K562 cells (Fig. S1 in supplement data).

As shown in Fig. 1c, pre-incubation of 5  $\mu$ M GW9662 partly increased cell survival and such effect disappeared when concentration of GW9662 increased to 10  $\mu$ M. GSH prevented  $\beta$ -carotene-induced cell death in a dose-dependent manner. Pre-incubation of 200  $\mu$ M GSH increases cell survival from 50% to 70% and this effect is reinforced by co-application of 5  $\mu$ M GW9662.

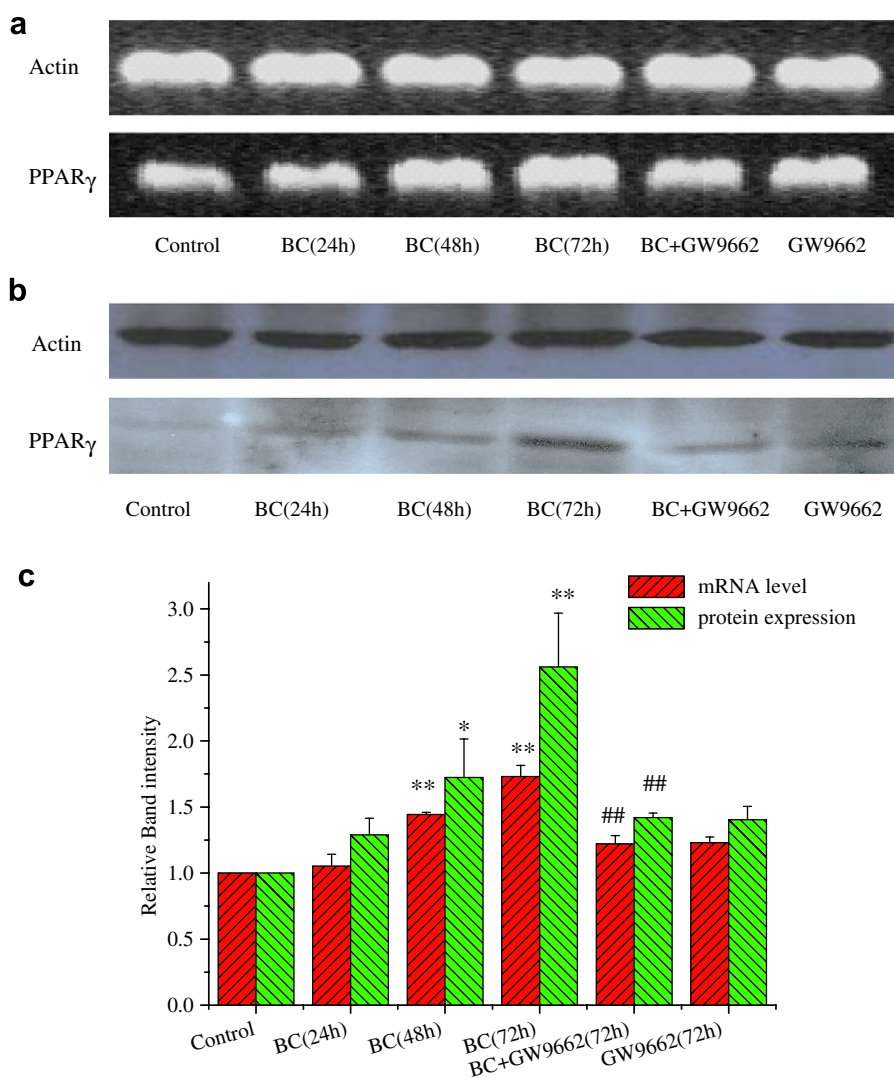
### 3.2. $\beta$ -carotene treatment up-regulates the PPAR- $\gamma$ mRNA and protein expression levels

To study the effect of  $\beta$ -carotene on PPAR- $\gamma$  gene expression, RT-PCR and western blot were performed to examine changes in the PPAR- $\gamma$  mRNA and protein expression following  $\beta$ -carotene treatment. As shown in Fig. 2,  $\beta$ -carotene exposure

significantly enhanced the expression levels of PPAR- $\gamma$  mRNA and protein in a time-dependent manner. Seventy two hour treatment increased the expression levels of PPAR- $\gamma$  mRNA and protein about 1.7 and 2.5 fold, respectively. GW9662 is an irreversible antagonist of PPAR $\gamma$  with high affinity and selectivity and can fully abrogate PPAR $\gamma$ -dependent mechanism.<sup>35</sup> Pre-incubation with GW9662 significantly attenuated the increase of PPAR- $\gamma$  gene expression induced by  $\beta$ -carotene. The effects of  $\beta$ -carotene up-regulation on PPAR- $\gamma$  expression were also observed in K562 cells (Fig. S2 in supplement data) and H1299 cells (data not shown).

### 3.3. $\beta$ -carotene down-regulates COX-2 but up-regulates p21 gene expression

P21<sup>WAF1/CIP1</sup> is cyclin-dependent kinase inhibitor which is activated by PPAR- $\gamma$  and inhibits cell cycle progression leading



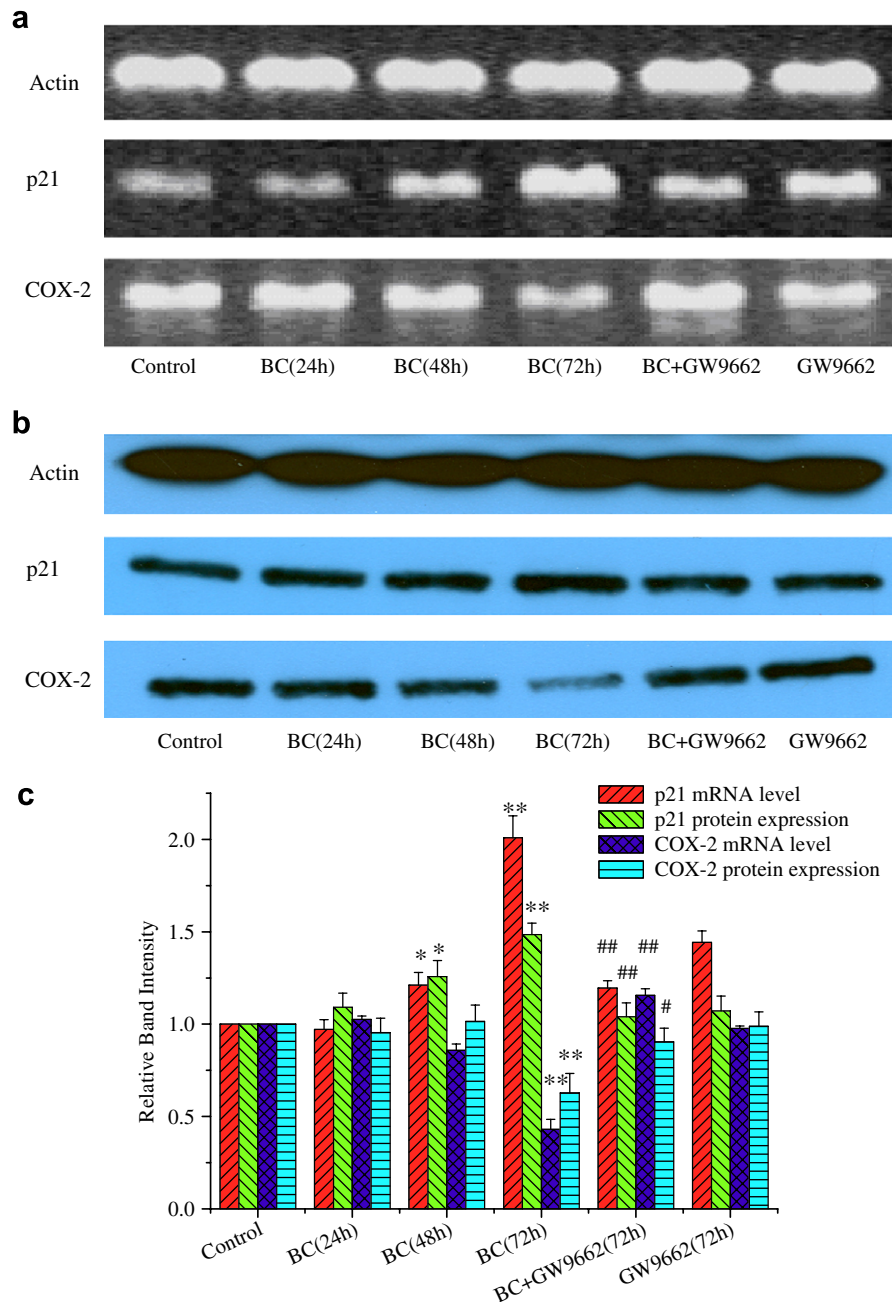
**Fig. 2 –  $\beta$ -carotene increases PPAR- $\gamma$  mRNA and protein expression levels in a time dependent manner.** MCF-7 cells were exposed to 50  $\mu$ M  $\beta$ -carotene (BC) and/or 5  $\mu$ M GW9662 for 24 h, 48 h and 72 h. (a) RT-PCR results of PPAR- $\gamma$  and  $\beta$ -Actin; (b) Western blot results of PPAR- $\gamma$  and  $\beta$ -Actin; (c) Relative band intensity analysis. The expression levels of samples are normalised to the corresponding levels of  $\beta$ -actin. Data are presented as percent of control and the bars are the means  $\pm$  SEM values for three independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01 versus control group; # $p$  < 0.05, ## $p$  < 0.01 versus  $\beta$ -carotene-treated group.

to G<sub>1</sub> arrest. Cyclooxygenase enzyme is overexpressed in a number of tumour types and it is also activated by PPAR- $\gamma$  and may be a target for chemoprevention. The effect of  $\beta$ -carotene on mRNA and protein levels of p21<sup>WAF1/CIP1</sup> and COX-2 were studied by RT-PCR and western blot analysis respectively. Fig. 3 shows that  $\beta$ -carotene down-regulated the COX-2 but up-regulated the p21 mRNA level and protein expression in a time dependent manner (Fig. 3a,b). The regulation effect of  $\beta$ -carotene on p21 and COX-2 was inhibited by the PPAR- $\gamma$

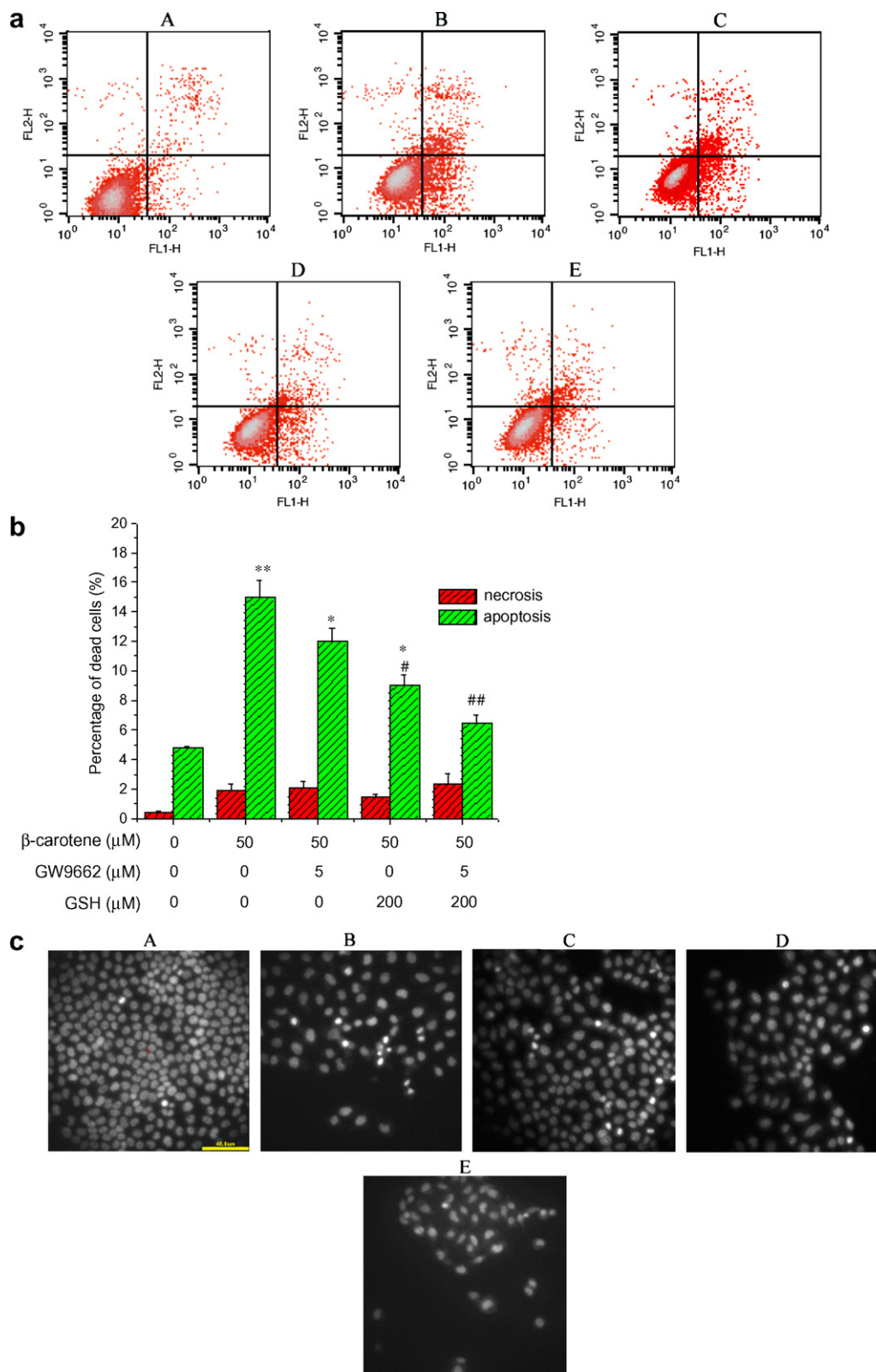
antagonist GW9662 (Fig. 3), indicating that  $\beta$ -carotene modulate p21 and COX-2 through PPAR- $\gamma$  dependent manner.

### 3.4. $\beta$ -carotene induces MCF-7 cell apoptosis

The early apoptosis and necrosis/late apoptosis induction by  $\beta$ -carotene was studied by flow cytometry analysis using Annexin V and PI double staining to assess the cause of the decrease of the cell survival by  $\beta$ -carotene treatment. Fig. 4



**Fig. 3 –  $\beta$ -carotene up-regulates p21 and down-regulates the COX-2 mRNA level and protein expression in time and PPAR- $\gamma$  dependent manners.** MCF-7 cells were exposed to 50  $\mu$ M  $\beta$ -carotene (BC) and/or 5  $\mu$ M GW9662 for 24 h, 48 h and 72 h. (a) RT-PCR results of p21, COX-2 and  $\beta$ -Actin; (b) Western blot results of p21 and COX-2; (c) Relative band intensity analysis. The expression levels of samples are normalised to the corresponding levels of  $\beta$ -actin. Data are presented as percent of control and the bars are the means  $\pm$  SEM values for three independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01 versus control group. # $p$  < 0.05, ## $p$  < 0.01 versus  $\beta$ -carotene treated 72 h group.



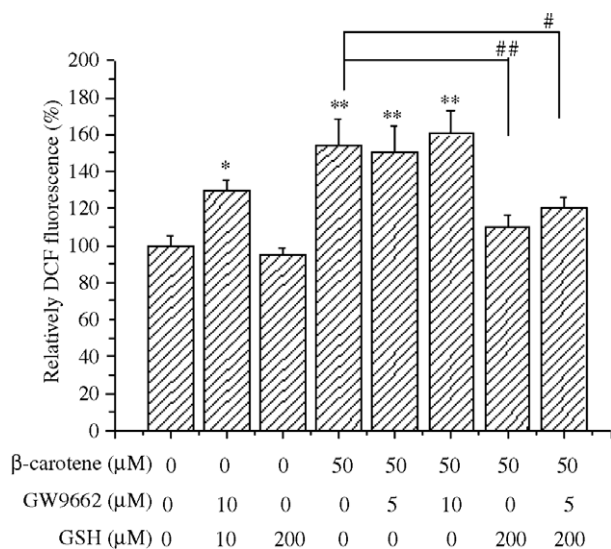
**Fig. 4** – Cell apoptosis and necrosis induced by  $\beta$ -carotene (72 h). (a) Flow cytometry analysis: A, control group; B,  $\beta$ -carotene treated group; C, GW9662 plus  $\beta$ -carotene treated group; D, GSH plus  $\beta$ -carotene treated group; E, GW9662 plus GSH and  $\beta$ -carotene treated groups. (b) Percentage of dead cell. Data are presented as the means  $\pm$  SEM,  $n = 3$ , \* $p < 0.05$ , \*\* $p < 0.01$  versus control group; # $p < 0.05$ , ## $p < 0.01$  versus  $\beta$ -carotene-treated group. (c) Fluorescence micrographs of MCF-7 cell nuclei: (A) Control; (B) Cells exposed to 50  $\mu$ M  $\beta$ -carotene for 72 h; (C) Pre-treatment with 5  $\mu$ M GW9662 (D) Pre-treatment with 200  $\mu$ M GSH; (E) Pre-treatment with GW9662 and GSH. The cells were stained with the DNA-binding fluorochrome Hoechst 33258. Scale bar = 40  $\mu$ m.

shows representative cytograms relative to apoptosis of MCF-7 cells incubated in the absence (A in Fig. 4a) or presence of 50  $\mu$ M  $\beta$ -carotene (B in Fig. 4a) for 72 h, as measured by this method.  $\beta$ -carotene treatment significantly increased the percentage of early apoptosis and the effect was partly attenuated by pre-incubation with GW9662 or GSH (C or D in Fig. 4a). A synergistic effect was found when both were used (E in Fig. 4a).  $\beta$ -carotene treatment had little effect on the percentage of necrotic cells (Fig. 4b).

In addition,  $\beta$ -carotene also induced morphological changes associated with apoptosis. Apoptotic nuclei, as indicated by condensed nuclei and nuclear fragmentation, were apparent after  $\beta$ -carotene treatment (B in Fig. 4c). The apoptotic nuclear changes were significantly reduced in the cells pre-treated with 200  $\mu$ M GSH (D in Fig. 4c) or 200  $\mu$ M GSH + 5  $\mu$ M GW9662 (E in Fig. 4c). The corresponding effect of GW9662 was smaller compared to GSH (C in Fig. 4c).

### 3.5. $\beta$ -carotene increases intracellular ROS

As shown in Fig. 1c, GSH prevented the decrease in cell survival induced by  $\beta$ -carotene treatment, indicating that ROS induced by  $\beta$ -carotene treatment may contribute to the loss of cell survival. Therefore, intracellular ROS levels were examined by using DCF-DA (Fig. 5). MCF-7 cells treated with 50  $\mu$ M  $\beta$ -carotene for 72 h exhibit a significant increase in the DCF signal relative to the control. This increase was significantly attenuated by pre-treatment with 200  $\mu$ M GSH. Pre-incubation with GW9662 (5 or 10  $\mu$ M) alone did not reduce the  $\beta$ -carotene-mediated effect on the intercellular ROS level. Moreover, the value of DCF signal in the presence of 10  $\mu$ M GW9662 was 120% ( $p < 0.05$ ) relative to the control.



**Fig. 5 –  $\beta$ -carotene treatment increases intracellular ROS level.** MCF-7 cells were exposed to  $\beta$ -carotene and/or GW9662/GSH for 72 h. Data are presented as percent of control and the bars are the means  $\pm$  SEM,  $n = 3$ . \* $p < 0.05$ , \*\* $p < 0.01$  compared with control group, # $p < 0.05$ , ## $p < 0.01$  compared with  $\beta$ -carotene treated group.

### 3.6. $\beta$ -carotene induced cytochrome C release

$\beta$ -carotene-induced apoptosis was further examined by cytochrome C release. Co-localisation of cytochrome c-GFP (Fig. 6a, grid 1) and mitochondrial marker MTR (Fig. 6a, grid 2) confirmed that cytochrome c-GFP was located in mitochondria; the overlap of GFP and MTR images shows thin filaments (Fig. 6a, grid 3).<sup>36</sup>  $\beta$ -carotene (50  $\mu$ M) exposed for 72 h resulted in changes in distribution of green and red fluorescence (Fig. 6a). The red fluorescence (mitochondria) became convergent and spherical, while green fluorescence (cytochrome c-GFP protein) was dispersed and separated from mitochondria. The cytochrome C release triggered by  $\beta$ -carotene exposure was further confirmed by western blot (Fig. 6b). The process of cytochrome C release was significantly inhibited by pre-treated with 200  $\mu$ M GSH or 200  $\mu$ M GSH plus 5  $\mu$ M GW9662 (Fig. 6b,c).

## 4. Discussion

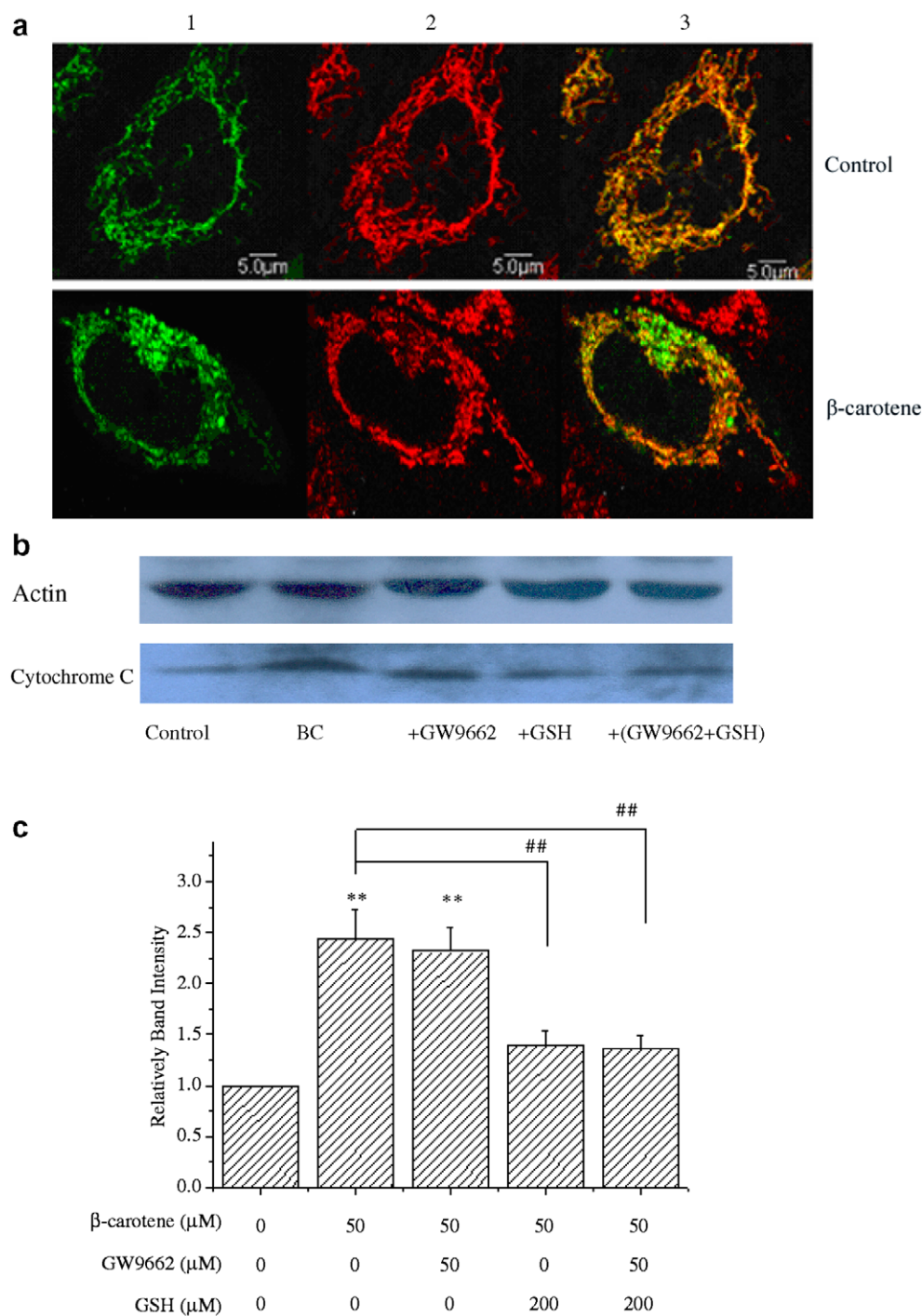
Numerous studies have shown that the risk of cancer is inversely correlated with dietary  $\beta$ -carotene. But the mechanism for the anti-cancer effect of  $\beta$ -carotene remains poorly understood. In this study, we attempted to elucidate a possible mechanism by which  $\beta$ -carotene regulates cell apoptosis. In human breast cancer cell line MCF-7, our study demonstrated that  $\beta$ -carotene modulated the expression of PPAR- $\gamma$  and its related target genes. This effect may cooperate with ROS induced by  $\beta$ -carotene to induce apoptosis.

### 4.1. Effects of $\beta$ -carotene on the expression of PPAR- $\gamma$ , p21 and COX-2

It has been reported that human breast cancer cell lines, as well as primary and metastatic breast adenocarcinomas express PPAR- $\gamma$  and ligand activation of PPAR- $\gamma$  can cause inhibition of proliferation in cultured breast cancer cell lines.<sup>37</sup> In the present study the results demonstrated that  $\beta$ -carotene significantly increased the mRNA and protein level of PPAR- $\gamma$  in a time-dependent manner (Fig. 2). This effect was blocked by addition of the PPAR- $\gamma$  antagonist GW9662 at 5  $\mu$ M. Kawada and colleagues demonstrated that carotenoids and retinoids regulated adipogenesis by changing PPAR- $\gamma$  activity and that isoprenols including carotenoids were also activators of PPAR- $\alpha$  and PPAR- $\gamma$  by using GAL4 ligand-binding domain chimera assay system.<sup>38</sup> It has been presumed that some phytochemicals including carotenoids are possible activators of PPARs.<sup>20,38</sup> Our results are in agreement with these previous observations, suggesting that  $\beta$ -carotene somehow causes an increase in the PPAR- $\gamma$  transactivation.<sup>21,39</sup>

It has been shown that the cyclin-dependent kinase inhibitor p21<sup>WAF1/CIP1</sup> inhibits cell cycle progression leading to G<sub>1</sub> arrest. P21<sup>WAF1/CIP1</sup> plays an important role in the antiproliferative effect of  $\beta$ -carotene<sup>40</sup> and the expression of p21 is regulated by several signalling pathways including the PPAR- $\gamma$ -mediated pathway.<sup>41</sup> The present results demonstrated clearly that  $\beta$ -carotene caused an increase of p21 expression, and that effect is inhibited by an irreversible PPAR- $\gamma$  antagonist (Fig. 3). Our results also showed that  $\beta$ -carotene acts as a prooxidant to increase intracellular ROS (Fig. 5)





**Fig. 6 –  $\beta$ -carotene induced cytochrome C releases.** (a) Representative examples show MCF-7 cell expressing the cytochrome c-GFP plasmid (grid 1, green), with MTR staining (grid 2, red), the overlap of GFP and MTR fluorescence (grid 3, yellow) in a single cell. White scale bar = 5  $\mu$ m. (b) MCF-7 cells were subjected to  $\beta$ -carotene and/or GW9662 and/or GSH treatments. Cytoplasmic cytochrome c and  $\beta$ -actin were detected by western blot. (c) Relative band intensity analysis. The expression levels are normalised against the corresponding  $\beta$ -actin levels. Data are presented as percent of control and the bars are the means  $\pm$  SEM for three independent experiments. \*\* $p$  < 0.01 versus control group, ## $p$  < 0.01 versus  $\beta$ -carotene treated 72 h group. (For interpretation of the references to colour in figure, the reader is referred to the web version of this article.)

which is consistent with previous data suggesting that the up-regulation of p21 expression is partly attributed to the pro-oxidant property of  $\beta$ -carotene.<sup>42</sup> However, it is notable that the PPAR- $\gamma$  antagonist did not affect intracellular ROS in our

study (Fig. 5). This suggests that the up-regulation of p21 expression is specifically mediated by the activation of PPAR- $\gamma$  and that PPAR- $\gamma$  activation may be associated with the antiproliferative effect of  $\beta$ -carotene.

The activation of PPAR- $\gamma$  causes the decrease of COX-2 expression at the transcriptional level.<sup>43</sup> For example,  $\beta$ -carotene down-regulates heregulin- $\alpha$ -induced COX-2 expression in colon cancer cells.<sup>44</sup> Our data showed that  $\beta$ -carotene down-regulates the COX-2 gene expression in a time-dependent manner, and that 5  $\mu$ M of PPAR- $\gamma$  antagonist GW9662 significantly attenuated this effect (Fig. 3), suggesting that the decrease of COX-2 by  $\beta$ -carotene may be mediated via the PPAR- $\gamma$  pathway. Collectively, it appears that  $\beta$ -carotene causes the up-regulation of PPAR- $\gamma$  expression and modulates the expression of its downstream components and these effects may contribute, at least in part, to the  $\beta$ -carotene-mediated anticancer activities.

#### 4.2. PPAR- $\gamma$ , ROS and cell apoptosis

*In vitro* studies have demonstrated that PPAR- $\gamma$  ligands inhibit the cell growth and induce differentiation in a variety of transformed cells. However, these PPAR- $\gamma$  ligands could also induce cell apoptosis via PPAR- $\gamma$  independent mechanisms, such as inhibition of Bcl-xL/Bcl-2 functions,<sup>45</sup> mitochondrial dysfunction and ROS production.<sup>46</sup> Inhibition of E2F/DP DNA binding,<sup>47</sup> induction of p21,<sup>48</sup> down-regulation of cyclin D1 expression<sup>49</sup> or PI3-kinase signalling<sup>50</sup> have all been suggested as potential molecular mechanisms implicated in the control of cell cycle progression and apoptotic pathways. In this study the expression of PPAR- $\gamma$  was significantly enhanced by  $\beta$ -carotene and it was associated with reduced cell survival. 5  $\mu$ M of GW9662 partly attenuated the cell survival loss, nuclear morphology changes and cell apoptosis induced by  $\beta$ -carotene suggesting that the up-regulation of PPAR- $\gamma$  expression has some contribution to the inhibition of the cell growth by  $\beta$ -carotene. Moreover, GW9662 did not decrease the intracellular ROS level and inhibit cytochrome C release, indicating that  $\beta$ -carotene induced ROS production is not through PPAR- $\gamma$  receptor. The fact that 10  $\mu$ M rather than 5  $\mu$ M of GW9662 is not capable of attenuating  $\beta$ -carotene-mediated cell survival loss is more likely due to the different effects of the antagonist doses.<sup>51</sup> The dose response effects of GW9662 on cell survival and/or apoptosis need to be further studied.

Ligands of PPAR- $\gamma$  suppress carcinogenesis and induce the differentiation of human tumourigenic cells. Activated PPAR- $\gamma$  forms a heterodimer with retinoid X receptor  $\alpha$  and binds to specific PPAR response elements in the promoter region of its target genes, affecting cell survival, growth, differentiation, and apoptosis.<sup>52</sup> Excentral conversion of  $\beta$ -carotene to retinoids and apocarotenoids occurs *in vitro* and in animal models.<sup>53</sup> However, it is still not clear whether retinoids produced from the oxidation of  $\beta$ -carotene or from intact  $\beta$ -carotene modulated PPAR- $\gamma$  expression and contributed to apoptosis in this study. It would appear that intact carotenoid molecules, not retinoids, may up-regulate PPAR- $\gamma$  expression. Elucidating the mechanism involved in the conversion of  $\beta$ -carotene to retinoids in MCF-7 cells would contribute to the understanding of  $\beta$ -carotene anticancer ability.

$\beta$ -carotene can serve as antioxidant or prooxidant, depending on its intrinsic properties as well as on the redox potential of the biological environment in which it acts.<sup>24,54,55</sup>  $\beta$ -Carotene also interacts with various regulatory factors, including Ref-1, nuclear factor-kappa B, and activating pro-

tein-1 via different mechanisms.<sup>56</sup> A series of reports from Palozza's Lab show that  $\beta$ -carotene regulates intracellular redox status and affects redox-sensitive molecular pathways involved in the regulation of cell cycle progression and apoptosis.<sup>5,24,25,44,57</sup> At low concentrations (less than 5  $\mu$ M), the carotenoid may serve as an antioxidant, inhibiting free radical production, while at relatively high concentrations and/or in the presence of a chronic oxidative stress (i.e. smoke), it may behave as a prooxidant, propagating free radical-induced reactions, consuming endogenous antioxidants and inducing DNA oxidative damage.<sup>24</sup> It has been reported that breast cancer cells are more susceptible to oxidation than normal cells.<sup>58</sup> The present results (as observed in Fig. 5) confirmed that  $\beta$ -carotene increases the intracellular ROS production and induces cell apoptosis. In addition,  $\beta$ -carotene caused the release of cytochrome C (Fig. 6b). Collectively, our data as well as data from other research suggest that both PPAR- $\gamma$  activation and ROS production play important roles in the regulation of  $\beta$ -carotene-induced cancer cell apoptosis. The two pathways may be synergistically converged in the regulation of  $\beta$ -carotene-induced apoptosis since the co-treatment of GW9662 and GSH show an improved protective effect.

In summary, our results demonstrated that  $\beta$ -carotene can regulate cell apoptosis through pleiotropic mechanisms. Both PPAR- $\gamma$  activation and ROS production are involved in the apoptosis pathway.  $\beta$ -carotene increases the PPAR- $\gamma$  mRNA and protein level in a time-dependent manner. The expression of p21 and COX-2 are regulated by  $\beta$ -carotene in the PPAR- $\gamma$ -dependent manner. In addition,  $\beta$ -carotene acts as prooxidant to increase intracellular ROS, which may be responsible for mitochondrial dysfunction and cytochrome C release. The loss of cell survival and apoptosis induced by  $\beta$ -carotene were significantly attenuated by co-application with GW9662 and GSH.

#### Conflict of interest statement

None declared.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ejca.2007.08.015](https://doi.org/10.1016/j.ejca.2007.08.015).

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