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Research article

THE EXPRESSION OF PROTEIN KINASE B IN GASTRIC CANCER CELL APOPTOSIS INDUCED BY 12-O-TETRADECANOYLPHORBOL-1,3-ACETATE

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Abstract: Protein kinase B (PKB/Akt) is a serine-threonine kinase functioning downstream of phosphatidylinositol 3-kinase (PI-3 kinase) in response to mitogen or growth factor stimulation. In several cell types, it plays an important anti-apoptotic role. TPA is a potent regulator of the growth of many different cell types. Here, we detected that TPA could induce cell apoptosis in the gastric cancer cell line, BGC-823. We also found that TPA inhibited the expression of PKB/Akt in a TPA concentration- and time-dependent manner. Furthermore, TPA inhibited the phosphorylation of PKB at Ser473, but did not affect the phosphorylation of Thr308. It only attenuated the expression of PKB/Akt and the phosphorylation of Ser473 in the cell nucleus, whereas it did not change the PKB/Akt distribution in BGC-823 cells. These results suggest that PKB/Akt inhibition by TPA may be the important factor in the mechanism of effect of TPA on gastric cell lines.

Key words: TPA, Cell apoptosis, PKB/Akt, Gastric cancer BGC-823 cell line

INTRODUCTION

The phorbol ester, TPA (12-O-tetradecanoylphorbol-1, 3-acetate), can be found in many Chinese medicinal herbs and food types. A number of cultured cell lines are responsive to treatment with TPA. There is considerable data supporting

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Abbreviations used: BrdU – 5-bromo-2'-deoxyuridine; OA – okadaic acid; PDK1/PDK2 – 3-phosphoinositide-dependent kinase; PI – propidium iodide; PI3K – phosphoinositide-3-OH kinase; PKB/Akt – protein kinase B; TPA – 12-O-tetradecanoylphorbol-1,3-acetate

TPA as a tumor promoter agent. For example, it has been shown to synergize with the Epstein-Bar virus to induce carcinoma in live animals [1]. On the other hand, TPA has been shown to induce apoptosis in some cell lines, such as nasopharyngeal carcinoma cells (CNE2) and cerebellar granule cell cancer cells [2, 3]. Thus, it appears that the effect of TPA differs depending on the cell type.

Protein kinase B (PKB) is the cellular homologue of the viral ongene *v-akt*. It is referred to as c-Akt, and is a 60-kDa serine-threonine kinase functioning downstream of phosphatidylinositol 3-kinase (PI-3 kinase) in response to mitogen or growth factor stimulation. PKB/Akt is activated by insulin, serum, insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) through a phosphorylation mechanism [4-6]. The phosphorylation of threonine 308 of PKB and serine 473 of Akt are prerequisites to PKB/Akt activation [4, 7]. After stimulation via the PI-3 kinase pathway, PKB/Akt is recruited to the plasma membrane through its PH domain, followed by its phosphorylation at Thr308 in the activation loop of the kinase domain and at Ser473 in the C-terminus regulatory region domain. The phosphorylation of Thr308 is catalyzed by 3'-phosphoinositide-regulated kinase-1 (PDK-1), whereas the phosphorylation of Ser-473 appears to be catalyzed by the thusfar uncharacterized 3'-phosphoinositide-regulated kinase-2 (PDK-2) [8]. Some studies indicate that single phosphorylation of either site leads to partial activation *in vitro*, whereas dual phosphorylation of the two is synergistic, and is required for maximal activation. In addition, *in vivo*, each phosphorylation event is independent of the other [5].

Numerous studies indicate that PKB/Akt plays key roles in cell cycle control, apoptosis inhibition and glucose metabolism [7, 9]. The goal of this study is to investigate whether TPA induces cell apoptosis of gastric cancer cells, specifically BGC-823 cells, and to investigate the involvement of PKB/Akt in mediating the apoptotic properties of TPA in BGC-823 cells. Our hypothesis was that if PKB/Akt activation is important in the prevention of apoptosis, then the inverse is true: PKB/Akt inactivation/inhibition is behind the apoptotic properties of TPA.

Our results indicated that TPA could induce apoptosis in the BGC-823 cell line, and inhibit the expression of PKB/Akt in a TPA concentration- and time-dependent manner. The activity of PKB/Akt was also partly inhibited by TPA in a time-dependent manner. PKB/Akt inhibition by TPA was associated with the inhibition of its Ser473 phosphorylation, but not that of Thr308. Furthermore, TPA attenuated the expression of PKB/Akt and its phosphorylation of Ser473 in the cell nucleus, whereas it did not change the PKB/Akt distribution in the cell. These results suggest that PKB/Akt inhibition by TPA may play an important role in mediating the effect of TPA on gastric cell lines. Apoptosis induced by TPA is partly due to PKB/Akt inhibition and its phosphorylation of Ser473 in the cell nucleus.

MATERIALS AND METHODS

Cell culture conditions

The human gastric cancer cell line BGC-823 was obtained from the Institute of Cell Biology in Shanghai, China. The cells were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 1 mM glutamine and 100 u/ml penicillin, at 37ºC in a humidified 5% CO_2 atmosphere. After the cells were seeded at a density of 1×10^6 per 100-mm culture dish for 24 h, they were treated with the indicated concentrations of TPA (Sigma) or okadaic acid (Sigma) and harvested at different times as required to be subjected to different experimental procedures.

The BrdU (5-Bromo-2'-deoxyuridine assay) assay

Cells were seeded as required and treated with or without TPA for 24 h, and then incubated with BrdU (20 mol/l; Sigma) for 2 h. After collection by centrifugation, the cells were fixed with 4% paraformaldehyde for 30 min at 4ºC and then incubated with saponin (0.1%; Sigma) for another 10 min. The cells were washed twice with PBS containing 0.1% saponin, and resuspended in PBS containing 30 μg DNase I. After incubation with anti-BrdU antibody for 1 h, the cells were given two PBS washes and then incubated with phycoerythrin-linked anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) [10]. Finally, the cells were washed with PBS and analyzed with a flow cytometer (Beckman Coulter, Fullerton, CA).

The immunofluorescence assay of cell apoptosis

Cells were seeded in 100-mm plates with RPMI-1640 medium with 10% fetal bovine serum at 37ºC in a 5% CO_2 humidified atmosphere for 24 h. The cells were treated with or without TPA for 24 h. Then the cells were trypsinized, washed in PBS, and collected by centrifugation. The harvested cells were fixed in 4% paraformaldehyde on ice, washed in PBS, and stained in the dark for 30 min with 50 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) containing 100 μg/ml DNase-free RNase per ml. The cells were observed under an immunofluorescence microscope. Apoptosis was assessed histologically according to specific morphological features, namely nuclear condensation or fragmentation [11].

Cell cycle analysis using PI (Propidium iodide)

Cells were seeded as required and treated with or without TPA for 24 h, then harvested. The cells were fixed in 80% ethanol for 30 min at 4ºC, and dyed with PI (Sigma) for 20 min in the dark. Finally, the cells were washed with PBS and analyzed using a flow cytometer (Beckman Coulter, Fullerton, CA).

Protein Extraction and Western blotting analysis

The cells treated with TPA were harvested by scraping the cells from the culture dishes, and were then collected by centrifugation. After that, the cells were lysed in RIPA buffer (10 mmol/l Tris, pH 7.4; 150 mmol/l NaCl; 1% Trion X-100; 1%

deoxycholic acid; 0.1% SDS; 5 mmol/l EDTA, pH 8.8; 1 mmol/l PMSF; 10% protease inhibitor cocktail, Roche; 1% Dithiothreitol) for 30 min at 4ºC. The lysates were centrifuged at 12000×g for 30 min at 4ºC. The protein concentration was determined using the Bio-Rad protein assay system according to the manufacturer's instructions (Bio-Rad Hercules, CA).

To separate the cytoplasmic and nuclear fractions, the cells were suspended in 2 ml MS buffer (210 mmol/l mannitol; 70 mmol/l sucrose; 5 mmol/l Tris-HCl, pH 7.5; and 1 mmol/l EDTA, pH 7.5), containing a 1% protease inhibitor cocktail, and then homogenized using a Dounce homogenizer. The homogenate was spun at 12000×g for 30 sec at 4ºC to pellate the nuclei and unbroken cells. The supernatant was the cytoplasmic fraction. The pellets were washed with ice-cold PBS and resuspended in MS buffer. The nuclei were purified in a second centrifugation for 30 min at 12000×g [12].

50 μg protein was subjected to SDS-PAGE (8-10%) and transferred to nitrocellulose membrane for western blotting analysis [12]. Each membrane was subsequently blocked with 5% dry no-fat milk in TBS-T (Tris bufferred saline and 2.5% tween20) and then incubated with primary antibodies: PKB/Akt (Santa Cruz Biotechnology, Santa Cruz, CA), P-PKB/Akt-Ser473 and Thr308 (Cell Signaling Technology, Inc. Beverly, MA), and β-actin, Tubulin and Lamin B (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed with TBS-T and incubated with secondary antibodies conjugated with peroxidase, and the signal was detected using the chemiluminescent detection system (Pierce) according to the manufacturer's instructions.

Confocal laser scanning microscopy

The cells were cultured on a cover glass overnight and treated at different times with TPA. The cells were then fixed in 4% paraformaldehyde, followed by a PBS wash. To display the expression of PKB/Akt, the cells were incubated first with anti-PKB/Akt antibody (as above), and then reacted with FITC-conjugated anti-IgG (Pharmingen) as a secondary antibody. To visualize the nuclei, the cells were stained with propidium iodine (PI, 50 μg/ml) containing 100 μg of DNase-free RNase per ml [13]. The fluorescent images were observed under a laser-scanning Confocal microscope (Bio-Rad MRC-1024ES). This analysis allowed the determination of the subcellular distribution of PKB/Akt.

Immunoprecipitation and the PKB/Akt activity assay

After being harvested, the cells were lysed for 30 min in an ELB lysis buffer (140 mM NaCl; 0.5% NP-40; 100 mM NaF; 50 mM Tris/HCl, pH 8.0; 1 mmol/l PMSF; 10% protease inhibitor cocktail; 1% dithiothreitol) at 4ºC, and centrifuged at 12000×g for 30 min at 4ºC. The protein concentration was determined using the Bio-Rad protein assay system (Bio-Rad Hercules, CA).

For the PKB/Akt activity assay [14], 500 μg protein were incubated for 1 h under constant agitation with 5 μl of the monoclonal antibody of PKB/Akt at 4ºC, then 20 μl Protein A-agarose was added and incubation was continued for 2 h under constant agitation. The agrose beads were washed twice in lysis buffer

and twice in the PKB/Akt kinase buffer (50 mM HEPES, pH 7.5; 1 mM EDTA; 10 mM $MgCl_2$; 0.1 mM Na_3VO_4; 1 mM NaF; 0.3 mM PMSF; 20 μg/ml leupetin; 20 μg/ml aprotinin; 1 mM dithiothreitol; and 15 mM β-glycerophosphate). The assay mixturess (20 μl) contained 50 mM HEPES, pH 7.5; 1 mM EDTA; 10 mM $MgCl_2$; 0.1 mM Na_3VO_4; 1 mM NaF; 0.3 mM PMSF; 20 μg/ml leupetin; 20 μg/ml aprotinin; 1 mM dithiothreitol; 15 mM β-glycerophosphate; 10 μg MBP as the kinase substrate; 200 μM ATP and 10 μCi[γ-^{32}P] ATP. The samples were incubated in assays for 30 min at 30ºC, and the reaction was then stopped by adding 2×sample buffer and boiling for 5 min. The reaction mixtures were subsequently resolved by 12% SDS-PAGE, and the results were analyzed by quantitative autoradiography.

RESULTS

TPA induces cell apoptosis in the BGC-823 gastric cancer cell line

We first tested whether cell apoptosis of BGC-823 cells could be induced by TPA. The apoptotic nuclear morphology in BGC-823 cells was observed by DAPI staining under an immunofluorescence microscope. The results indicated that treating BGC-823 cells with TPA for 24 h caused the classical morphological characteristics of apoptosis, including nuclear condensation and fragmentation (Fig. 1A). Meanwhile, the BrdU assay, used to identify DNA-synthesizing cells [15], showed that treatment with TPA for 24 h strongly inhibited BrdU incorporation in the BGC-823 cells (Fig. 1B). The cell cycle analysis also indicated that the sub-G_1 was observed in BGC-823 cells treated with TPA (Fig. 1C). Thus, this data demonstrated that TPA indeed induced apoptosis in gastric cancer cells of the BGC-823 line.

TPA inhibits the expression of PKB/Akt in the BGC-823 gastric cancer cell line

Previous studies showed that PKB/Akt is activated through the PI3K pathway by several growth factors (such as EGF and insulin [4-6]) in a number of tumor cells, including a primary gastric adenocarcinoma [16], and ovarian and pancreatic cancers [17, 18]. PKB/Akt plays a major role in the cell survival pathway by regulating cell survival and apoptotic processes [4-6, 16-18]. Since TPA could induce cell apoptosis of BGC-823 cells, we assessed the expression of PKB/Akt in BGC-823 cells with or without TPA treatment. The western blotting results showed that the expression of PKB/Akt was inhibited when the cells were exposed to TPA for different periods. The expression of PKB/Akt began to reduce at 12 h, and appeared to be increasingly inhibited after 48 h (Fig. 2A). A similar repressive tendency was also observed in a TPA concentration-dependent manner. Fig. 2B shows the results for BGC-823 cells treated with different concentrations of TPA (0 ng/ml, 50 ng/ml, 100 ng/ml and 150 ng/ml) for 24 h. 50 ng/ml TPA is sufficient to reduce the expression of PKB/Akt (Fig. 2B), so TPA can inhibit the expression of PKB/Akt in BGC-823 cells in both a time- and concentration-dependent manner.

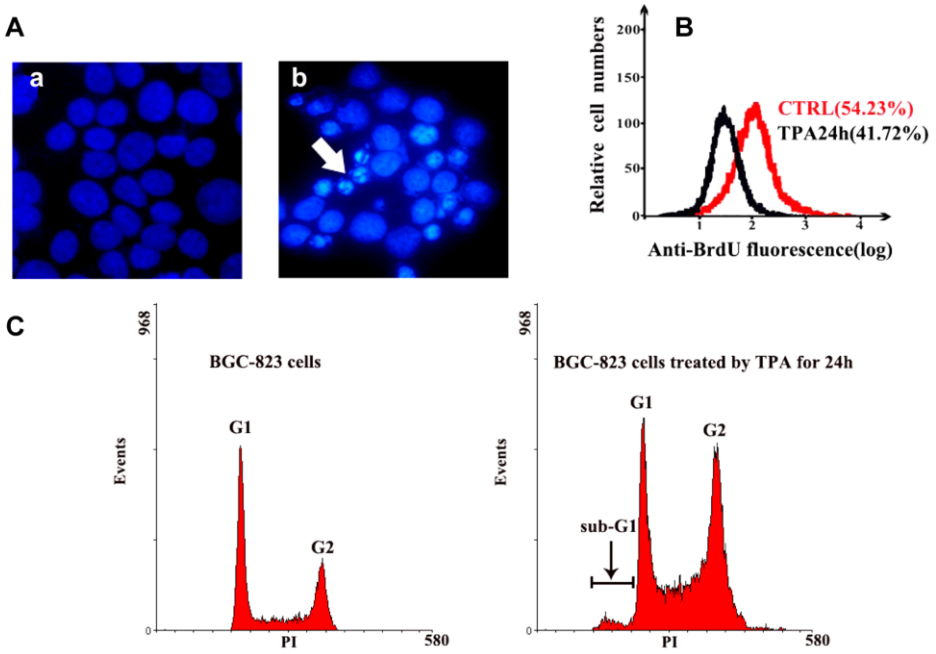

Fig. 1. TPA induced cell apoptosis in BGC-823 gastric cancer cells. A – BGC-823 cells were treated with TPA (100 ng/ml) for 24 h, and then harvested. After staining with DAPI, the cells were observed under a fluorescent microscope (x 200); a – control group, b – experimental group. The arrow indicates the apoptotic bodies of the apoptotic cells. B – BGC-823 cells were treated with TPA (100 ng/ml) for 24 h, and then the cells were maintained in BrdU medium for 2 h before harvesting. The cells were prepared as described in the Materials and Methods section, and assayed via FACS. C – BGC-823 cells were treated with TPA (100 ng/ml) for 24 h, and then the cells were harvested. The cells were prepared as described in the Materials and Methods section, and assayed via FACS. The data is representative of three independent experiments, each yielding similar results.

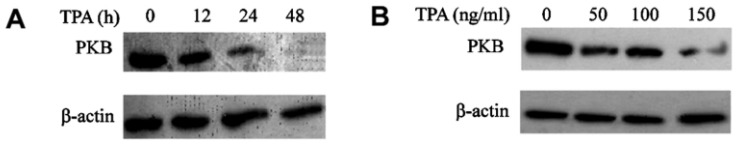

Fig. 2. TPA inhibited the expression of PKB/Akt in BGC-823 gastric cancer cells. A – The effect of TPA on PKB/Akt expression at different times. Cells were treated with TPA (100 ng/ml) for the indicated time, and Western blotting analysis was used to show the expression of PKB/Akt. The blots were normalized to an endogenous protein (β-actin). B – The effect of different concentrations of TPA on PKB/Akt expression. The cells were treated with different concentrations of TPA (0 ng/ml, 50 ng/ml, 100 ng/ml, and 150 ng/ml) for 24 h. Western blotting analysis was used to show the expression of PKB/Akt, and the blots were normalized to an endogenous protein (β-actin). The data is representative of three independent experiments, each yielding similar results.

TPA represses PKB/Akt kinase activity and its phosphorylation of Ser473 in the BGC-823 gastric cancer cell line

The inhibition of protein expression is always followed by a decline in its activity [19]. PKB/Akt kinase activity was measured in immunocomplex kinase assays using MBP as a substrate *in vitro* (Fig. 3A). TPA treatment resulted in a decline in PKB/Akt activity in the BGC-823 cells, even though a slow and progressive decrease in the enzymatic activity was observed (Fig. 3A). Therefore, we investigated if TPA could repress PKB/Akt kinase activity following the inhibition of its protein expression.

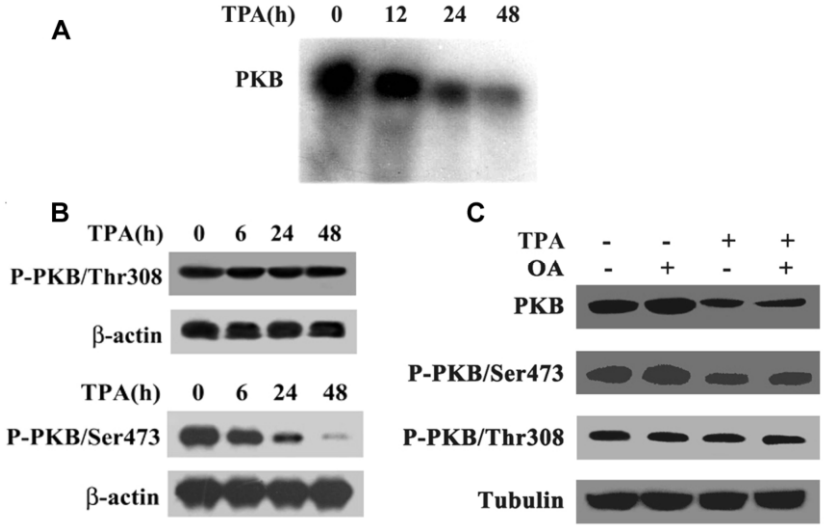

Fig. 3. TPA inhibited PKB/Akt kinase activity and the phosphorylation of Ser473 in BGC-823 gastric cancer cells. A – TPA inhibited kinase activity of PKB/Akt in a time-dependent manner. Cells were treated with TPA (100 ng/ml) for different times as indicated. Cell lysates were prepared as described in the Materials and Methods section. PKB kinase activity was measured following immunoprecipitation using MBP as a substrate. B – TPA affected the phosphorylation of PKB/Akt at Thr 308 and Ser473. Cells were treated with TPA (100 ng/ml) for different times as indicated. Cell lysates were prepared as described in the Materials and Methods section, and then analyzed by Western blotting with an antibody against P-PKB/Akt-Thr308 or P-PKB/Akt-Ser473. β-actin expression served as control for indicating similar proteins in each lane. C – The effect of okadaic acid (OA) on the expression of PKB/Akt, and the phosphorylation of Ser473 and Thr308. The cells were pretreated with or without okadaic acid (100 nm/l) for 2 h, followed by TPA (100 ng/ml) for 24 h. Western blotting analysis was used to show the PKB/Akt expression levels and the blots were normalized to an endogenous protein (Tubulin). The data is representative of three independent experiments, each yielding similar results.

PKB/Akt activation is a multistep process mainly requiring the phosphorylation of the Ser473 and Thr308 of PKB/Akt [8, 20-22]. We determined the phosphorylation status of PKB/Akt Ser473 and Thr308 after different periods of

treatment of TPA. The determination was done by Western blotting with a Ser473 and Thr308 site-specific antiphospho-PKB/Akt antibody (Fig. 3B). The results shown in the lower panel in Fig. 3B showed that the phosphorylation status of PKB/Akt at Ser473 was not reduced at 6 h. A distinct decrease in its expression was observed when it was exposed to TPA for 24 and 48 h (Fig. 3B, lower panel). The phosphorylation status of PKB/Akt at Thr308 was assessed too, but the effect of TPA on it was not as distinct as that at Ser473 (Fig. 3B, upper panel). These results suggest that the apoptosis effect of TPA on BGC-823 cells is partly mediated via the inhibition of the phosphorylation of PKB/Akt Ser473 and the concomitant repression of PKB/Akt activity.

Previous studies have shown that PKB/Akt kinase is primarily activated through phosphorylation [19, 20] and inactivated through dephosphorylation by a proteinphosphatase 2A (PP2A) [23, 24]. In order to eliminate the possibility that the PKB/Akt inhibition is related to the dephosphorylation of PKB/Akt caused by PP2A in BGC-823 cells while exposed to TPA, we assessed the expression of PKB/Akt. The expression of PKB/Akt still degraded to some extent under TPA treatment for 24 h following a 2-h pretreatment with okadaic acid (OA, an inhibitor of PP2A; Fig. 3C).

The effects of TPA on the subcellular distribution of PKB/Akt in the BGC-823 gastric cancer cell line

Protein function may be influenced by its subcellular distribution. The stimulation of some cells with insulin or IGF-1 is associated with subcellular redistribution of PKB/Akt, and the cellular distribution of PKB/Akt seems to be context dependent with respect to the various cell lines studied [25-29]. Several studies have demonstrated that PKB/Akt undergoes nuclear translocation upon stimulus, and that the intranuclear translocation of PKB/Akt is an important step in the signaling pathways that mediate cell proliferation [25, 26]. Here, we found that PKB/Akt could be observed simultaneously in the cytoplasm and the nucleus in intact BGC-823 cells using a confocal laser scanning microscope via the immunofluorescence method (Fig. 4A). When BGC-823 cells were treated with TPA for 6, 24 and 48 h, the PKB/Akt in the cytoplasm was also visually similar to that of intact BGC-823 cells (Fig. 4A). However, the level of nuclear PKB/Akt was largely reduced after the cells were treated with TPA for 24 h, and had completely disappeared when the treatment was extended to 48 h (Fig. 4A). Cellular fractional analysis via Western blotting further supported this finding. As shown in Fig. 4B, after BGC-823 cells were treated with TPA for 48 h, PKB was barely detected in the nucleus but had remained at almost the same levels in the cytoplasm as that of intact BGC-823 cells (Fig. 4B).

Furthermore, the phosphorylation of Ser473 was found to be principally degraded in the nucleus, compared with intact BGC-823 cells as assessed by Western blotting. After 48 h treatment with TPA, the phosphorylation of Ser473 was almost unobserved in the nucleus (Fig. 4C). These results determined that TPA did not change the distribution of PKB/Akt in the cytoplasm and nucleus,

but that it might inhibit the expression of PKB/Akt and its phosphorylation of Ser473 in the nucleus.

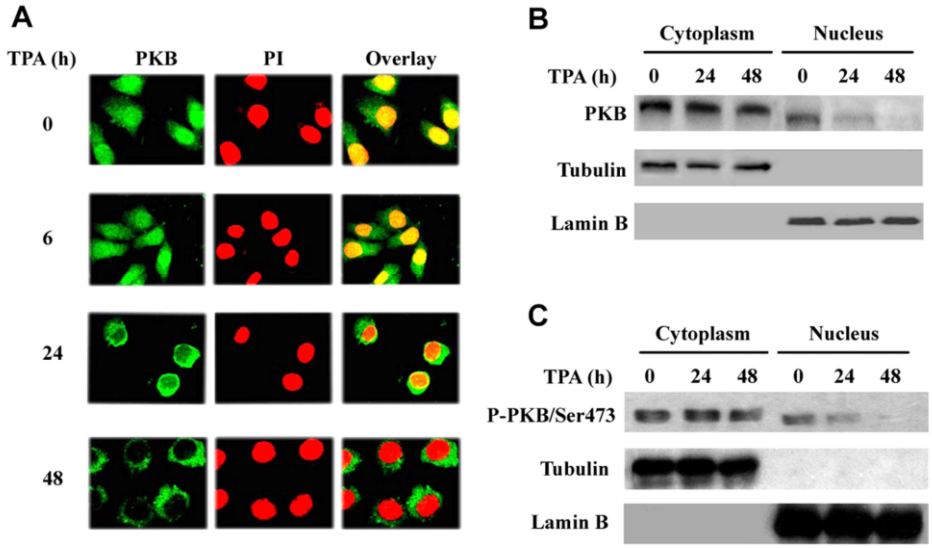

Fig. 4. TPA inhibited the expression of PKB/Akt and the phosphorylation of Ser473 in the nucleus of BGC-823 gastric cancer cells. A – TPA inhibited the expression of PKB/Akt in the nucleus. Cells were treated with TPA (100 ng/ml) for different times as indicated, then immunostained with the corresponding antibody against PKB/Akt or dye (Propidium Iodide, PI) as described in the Materials and Methods section. The fluorescent images were observed under a laser-scanning confocal microscope. B – TPA affected PKB expression in the cytoplasm and nucleus. Cells were treated with TPA (100 ng/ml) for different times as indicated, and then cytoplasmic and nuclear fractions were prepared as described in the Materials and Methods section. Cytoplasmic and nuclear proteins were subjected to Western blotting with an antibody against PKB/Akt. The purities of the cytoplasmic and nuclear fractions were determined by Western blotting with antibodies, respectively against Tubulin and Lamin B. C – TPA affected the expression of PKB/Akt-Ser473 in the cytoplasm and nucleus. Cells were treated with TPA (100ng/ml) for different times as indicated, and then cytoplasmic and nuclear fractions were prepared as described in the Materials and Methods section. Cytoplasmic and nuclear proteins were subjected to Western blotting with an antibody against P-PKB/Akt-Ser473. The purities of the cytoplasmic and nuclear fractions were determined by Western blotting with antibodies, respectively against Tubulin and Lamin B. The data is representative of three independent experiments, each yielding similar results.

DISCUSSION

In this study, we put forward our hypothesis that TPA could induce cell apoptosis in BGC-823 cells, and PKB/Akt inactivation/inhibition is behind the apoptotic properties of TPA.

We showed that TPA could induce cell apoptosis in BGC-823 cells (Fig. 1), and that the expression of PKB/Akt was inhibited in a time- and TPA concentration-dependent manner in these cells (Fig. 2). This data is in agreement with that from previous studies that showed that the expression of PKB/Akt could be inhibited in the cell apoptosis of different cancer cell lines induced by certain factors, such as 17-allylaminogeldanamycin (17-AAG) and H_2O_2 [19, 30].

It has been shown that protein expression is always associated with its activity [19]. Phosphorylation appears to be critical for PKB/Akt kinase activity since its dephosphorylation causes its inactivation [31, 32]. Some extracellular stimuli could decrease the expression of PKB/Akt by inhibiting its phosphorylation, e.g. there was an observed decline in the expression of PKB/Akt followed by the inhibition of PKB/Akt phosphorylation in gemcitabine-treated pancreatic cancer cells, topotecan-treated lung cancer cells, and cells treated with several kinds of stress-inducing agents, including hyperosmosis, γ-irradiation, and UV-irradiation [23, 24, 33]. It is also known that the phosphorylation of Thr308 and Ser473 occurs in response to growth factors and that extracellular stimuli are essential for PKB/Akt activation [5]. To date, several papers have demonstrated that PDK1 phosphorylates PKB/Akt at the Thr308 and this phosphorylation of Thr308 is an absolute requirement for PKB/Akt activation [5, 21, 22, 34]. The phosphorylation of Ser473 alone was insufficient to generate activity, but was necessary for maximal activation [35]. In this study, TPA-mediated PKB/Akt inhibition and Ser473 phosphorylation inhibition were observed in a time-dependent manner in BGC-823 cells, but similar results were not obtained for Thr308. Similar results also proved that TPA only down-regulates the phosphorylation of Ser473 of PKB/Akt in mouse keratinocytes [36, 37]. Tumor necrosis factor-α activation in WEHI-164 cells is accompanied by increased phosphorylation of Ser473, but not of Thr308 [35], and ceramide inhibits PKB/Akt by promoting the dephosphorylation of Ser473 in TF-1 cells [35-37]. It is suggested that PKB/Akt inhibition may be partially due to the inhibition of PKB/Akt phosphorylation of Ser473, but not of Thr308, in TPA-treated BGC-823 cells.

The redistribution of proteins between nucleus and cytoplasm is an essential event for the regulation of their activities and the execution of their functions [12]. Ahmed *et al.* [38] demonstrated the myristoylation of v-PKB/Akt: subcellular distribution shown a dispersed presence (approximately 40% on the plasma memebrane, 30% in the nucleus, and 30% in the cytosol). Once PKB/Akt is phosphorylated at both regulatory sites, the active PKB/Akt disengages from the plasma membrane and translocates through the cytosol to distinct subcellular compartments including the nucleus [39, 40]. For example, Borgatti *et al.* [41] reported that in the osteoblast-like clonal cell line MC3T3-E1, treatment with IGF-1 and PDGF led to nuclear translocation of active PKB. Gupta *et al.* [42] also found that predominant nuclear distribution of phosphorylated PKB-α was observed in HepG2 cells stably transfected with PKB/Akt. These papers indicated that the nuclear distribution of the phosphorylated pattern of PKB/Akt is significant, considering that active PKB/Akt can interact with some nuclear

factors, such as Tcl1 (involved in the development of human mature T-cell leukemia) and FOXO1 after nuclear translocation [43, 44]. The nuclear distribution of active PKB-α in HepG2-PKB-CA cells is associated with the activation of the p65 subunit of transcription factor NF-κB with plausible constitutive activation of the PI-3kinase pathway inside the nucleus [42]. However, we found that TPA did not change the distribution of PKB/Akt, but only attenuated the expression of total PKB/Akt and the phosphorylation of PKB/Akt at Ser473 in the nucleus in BGC-823 cells. It is believed that PKB/Akt inhibition may be associated with the attenuation of PKB/Akt and the phosphorylation of Ser473 in nucleus. It will be interesting to further delineate the interaction of PKB/Akt with other nuclear targets.

In conclusion, the results of our studies are mechanistic evidence that PKB/Akt inhibition might play an important role in mediating the effect of TPA on this gastric cancer cell line. Apoptosis induced by TPA is partly due to PKB/Akt inhibition and the phosphorylation of Ser473 in the nucleus. Our study is helpful in understanding the mechanism of TPA action and possibility of its clinical application in gastric cancer treatment.

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