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External Qi of Yan Xin Qigong Induces Apoptosis and Inhibits Migration and Invasion of Estrogen-Independent Breast Cancer Cells Through Suppression of Akt/NF-κB Signaling

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Key Words

Breast cancer • Akt • NF-κB • External Qi of Yan Xin Qigong • Apoptosis • Metastasis

Abstract

The antitumor effects of external Qi of Yan Xin Qigong (YXQ-EQ) have been widely described over the past three decades. To gain a better understanding of the mechanisms underlying YXQ-EQ's antitumor effects, in the present study we investigated its effects on growth, migration, invasion and apoptosis of breast cancer cells and the underlying molecular mechanisms. We show that YXQ-EQ treatment caused a time-dependent reduction in viability, blocked clonogenic growth and induced apoptosis in estrogen-independent breast cancer MDA-MB-231 cells. Furthermore, YXQ-EQ treatment blocked migration and invasion of MDA-MB-231 cells. Biochemically, YXQ-EQ treatment markedly inhibited constitutive and EGF-induced Akt phosphorylation. YXQ-EQ also substantially repressed NF-κB activity, resulting in decreased expression of anti-apoptotic Bcl-2, Bcl-X_L, XIAP and survivin proteins. These findings suggest that YXQ-EQ may induce apoptosis and inhibition of migration and invasion of MDA-MB-231 cells through the repression of Akt/NF-κB signaling.

Introduction

Breast cancer is the most common cancer among women and the second leading cause of deaths related to cancer [1]. Systemic treatment of breast cancer includes cytotoxic, hormonal, and immunotherapeutic agents [2]. In general, systemic agents are active at the beginning of therapy in most of primary and about half of metastatic tumors. Eventually, however, tumors progress and resistance to therapy occurs, resulting in treatment failure [2].

Studies have shown that the activation of Akt and NF-κB contributes to the resistance of cancer cells to chemotherapeutic agents and treatment failure in cancer chemotherapy [3-7]. Both Akt and NF-κB play important roles in many cellular processes, including cell proliferation, apoptosis, migration, invasion and tumor angiogenesis [8, 9]. Recently, constitutive activation of NF-κB has been found in human breast cancer cells, possibly due to the activation of various signaling pathways, including

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phosphatidylinositol-3 kinase (PI3K)/Akt and mitogen-activated protein kinases [3, 4, 7, 10, 11]. NF-κB has been reported to regulate the expression of many genes that are associated with tumor development, including anti-apoptotic, pro-invasion, and cell cycle-regulatory genes [12-15]. NF-κB inactivation has been reported to induce growth inhibition and apoptosis in breast cancer cells or sensitize breast cancer cells to apoptosis [16-19]. The inhibition of Akt has been shown to repress growth and invasive capacity of breast cancer cells [20]. Down-regulation of Akt/NF-κB signaling could thus be a novel therapeutic approach for achieving optimal results in patients with chemoresistant breast cancer [19].

The concept External Qi (of Qigong) refers to the technology and ability of "Qi deployment" therapy and health preservation of traditional Chinese medicine (TCM) [21, 22]. External Qi therapy of TCM has long been one of the medical practices in China and is managed by the Chinese health authorities [23]. Long-term clinical observations and ongoing studies have shown that the external Qi of Yan Xin Qigong (YXQ-EQ) has positive effects on patients of various diseases including cancer [24-28]. Over the past two decades, a large number of experimental studies have revealed some important properties of YXQ-EQ, including its effects on the structure and properties of biomolecules such as DNA, RNA, proteins and lipids [25, 29-33]. YXQ-EQ has been demonstrated to regulate PI3K and Akt activities and modulate gene expression [21-23, 34]. Recently, the molecular and cellular mechanisms underlying YXQ-EQ's antitumor effects have begun to be addressed [21, 22].

Breast cancer MDA-MB-231 cells are estrogen-independent and highly invasive. Akt and NF-κB are constitutively activated in MDA-MB-231 cells [14, 16]. In the present study we show that YXQ-EQ induced apoptosis and inhibited migration and invasion of MDA-MB-231 cells through suppression of Akt/NF-κB signaling.

Materials and Methods

Cell culture and Treatment

MDA-MB-231 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS). To examine the effect of YXQ-EQ on the epidermal growth factor (EGF)-mediated Akt activation, MDA-MB-231 cells were serum-starved over night, treated by YXQ-EQ for 5 min, and then 10 min later were stimulated with EGF for 20 min. Whole cell lysates were prepared from the cells and used in Western blot analysis of Akt phosphorylation as described previously [21].

Cytotoxicity assay

Cytotoxicity assessment of YXQ-EQ was performed as previously described [21]. Briefly, cells grown in 96-well plates were exposed to YXQ-EQ for 5 min. Cell viability was determined using trypan blue exclusion assay at 0, 1, 6 and 24 h after the treatment. In some experiments, cells were exposed 3 times to YXQ-EQ for 5 min each time with a 25 min interval [21]. A cell viability assay was performed 10 min after the third exposure to YXQ-EQ, using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay with a CellTiter 96Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI).

Clonogenic assay

Clonogenic assay was performed based on a protocol previously described [35]. Briefly, cells were suspended in RPMI-1640 containing 10% FBS, treated with YXQ-EQ for 5 min, and plated in 6-well plates (1×10^3 cells in each well). Medium was changed every 3-4 days. After incubation for 2 weeks, colonies were stained with crystal violet and counted.

Migration and invasion assay

Migration assay was carried out in Transwell Boyden migration chambers with polycarbonate filters (8-μm pores) (Costar Inc., Bethesda, MD). MDA-MB-231 cells were starved in RPMI 1640 containing 0.1% BSA for 24 h. Cells were trypsinized and resuspended in starvation medium at 5×10^5 cells/ml. After 5 min of YXQ-EQ treatment, cells were added to the filters (1.5×10^5 cells/filter) and 500 μl of RPMI 1640 with 5% FBS (as chemotactic stimulus) was added to the lower chamber. After incubation at 37 °C for 4 h, cells that did not transmigrate were carefully wiped off with cotton-tipped swabs. Migrated cells were fixed with methanol and stained with crystal violet and counted in 5 random fields. The invasive capacity of breast cancer cells was analyzed in Transwell Boyden chambers coated with Matrigel (BD Biosciences, Bedford, MA). Briefly, MDA-MB-231 cell suspension (5×10^4 cells/filter) was added to the filters. Invasion was induced by addition of RPMI 1640 containing 10% FBS to the lower chamber followed by incubation for 24 h. The cells remaining on the top surface of the filters were removed with cotton swabs. The cells that invaded through the Matrigel to the bottom of the filters were fixed, stained and counted as described above.

Flow cytometry analysis of apoptosis and cell cycle distribution

Cells were treated by YXQ-EQ for 5 min and collected 16 h after the treatment by trypsinization. For quantitative evaluation of apoptosis, cells were stained with Annexin V-FITC and propidium (PI) using an apoptosis detection kit (BD Biosciences, San Jose, CA), and subjected to flow cytometry analysis. The apoptotic population was defined as the sum of early (Annexin V-FITC positive, PI negative) and late (Annexin V-FITC positive, PI positive) apoptotic cells. For cell cycle analysis, cells were fixed with ethanol, stained with PI after RNase treatment, and subjected to flow cytometry analysis [21].

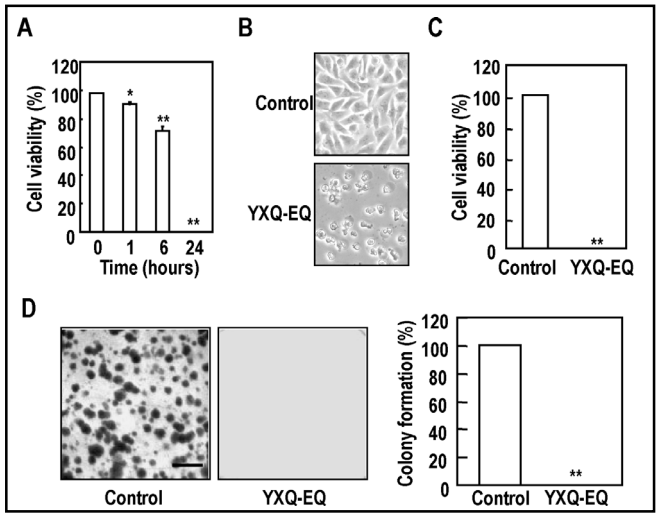

Fig. 1. Cytotoxic effect of YXQ-EQ on MDA-MB-231 cells. A and B. Cells were treated with YXQ-EQ for 5 min. Cell viability (A) was determined with trypan blue exclusion analysis at time points as indicated. Results are presented as the mean ± SD of percent viability from 3 independent experiments. Morphological changes (B) were examined 24 h after the treatment using phase-contrast microscopy (x100). C. Cells were sequentially treated with YXQ-EQ for 3 times as described in Materials and Methods. Cell viability was evaluated 10 min after the third treatment. Results are presented as the mean ± SD of percent viability from 4 independent experiments. D. Colony formation. Colonies were stained with crystal violet and counted. Results are presented as the mean ± SD of percent colony formation from 3 independent experiments. * $P < 0.05$ and ** $P < 0.01$, versus control cells. Scale bar, 5 mm.

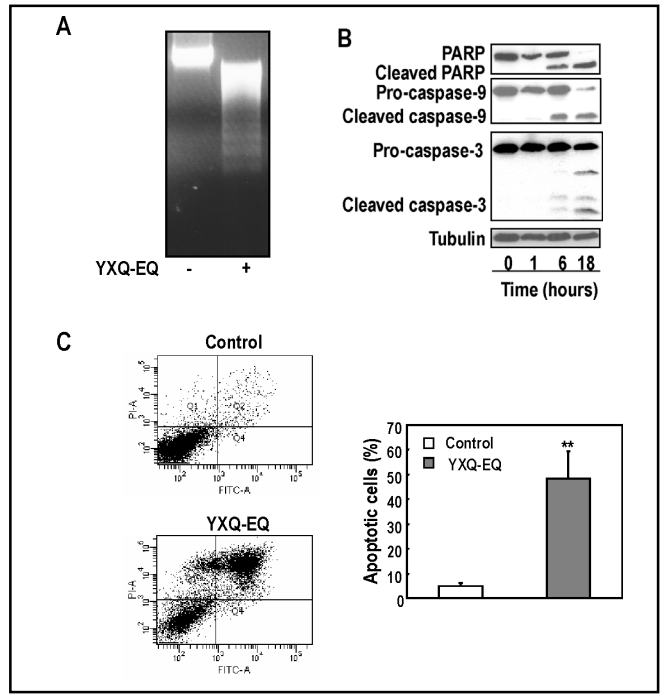

Fig. 2. YXQ-EQ induced apoptosis in MDA-MB-231 cells. Cells were treated with 5 min of YXQ-EQ and assessed for apoptosis. A. DNA fragmentation assay 16 h after YXQ-EQ treatment. B. Western blot analysis of PARP, caspase-3 and caspase-9 at time points as indicated. C. Flow cytometry analysis at 16 h after the treatment. Apoptotic cells were defined as Annexin V-FITC positive/PI negative (early apoptosis) and Annexin V-FITC positive/PI positive (late apoptosis). Results are presented as the mean ± SD of percent apoptotic cells from 3 independent experiments. ** $P < 0.01$, versus control cells.

Western blot analysis

For Western blot analysis, cells were grown on 60 mm culture dishes and treated with YXQ-EQ for 5 min. Cells (including attached and detached cells) were harvested at time points as indicated and whole cell lysates were prepared in lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with protease inhibitors (Roche, Minneapolis, MN). Equal amount of the lysates were subjected to electrophoresis on SDS polyacrylamide gels. The levels of phospho-Akt, phospho-Rb (Ser^{780}), caspase-3 and -9, poly(ADP-ribose) polymerase (PARP), cyclins D1 and E, Bcl-2, Bcl-X_L, XIAP, survivin, β-actin and tubulin were detected using specific antibodies, respectively. Antibodies against β-actin and tubulin were purchased from Sigma (St Louis, MO). Antibody against cyclin E was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rest of antibodies were purchased from Cell Signaling Technology.

Electrophoresis mobility shift assay

Nuclear extract preparation and electrophoresis mobility shift assay (EMSA) were performed as previously described [21]. Briefly, Cells were treated with YXQ-EQ for 5 min and harvested 10 min later by trypsinization. Cells were incubated in hypotonic buffer for 20 min on ice. Nuclei were collected by centrifugation at 10,000 rpm, 4 ºC for 5 min. After extraction of the nuclei with high-salt buffer for 15 min on ice, a nuclear fraction was prepared by centrifugation at 10,000 rpm for 15 min. EMSA was performed with ^{32}P-labeled NF-κB oligonucleotide according to the protocol provided by the manufacturer (Promega, Madison, WI).

DNA fragmentation analysis

For DNA fragmentation analysis cells were treated with YXQ-EQ for 5 min. DNA was isolated from the cells 16 h after the treatment and analyzed as previously described [21].

Statistic analysis

Results are presented as mean ± SD. The significance of differences in means was determined using the two-tailed Student's *t*-test. $P < 0.05$ was considered significant.

Results

Cytotoxic effect of YXQ-EQ

We first examined the cytotoxic effect of YXQ-EQ on MDA-MB-231 cells. YXQ-EQ treatment of MDA-

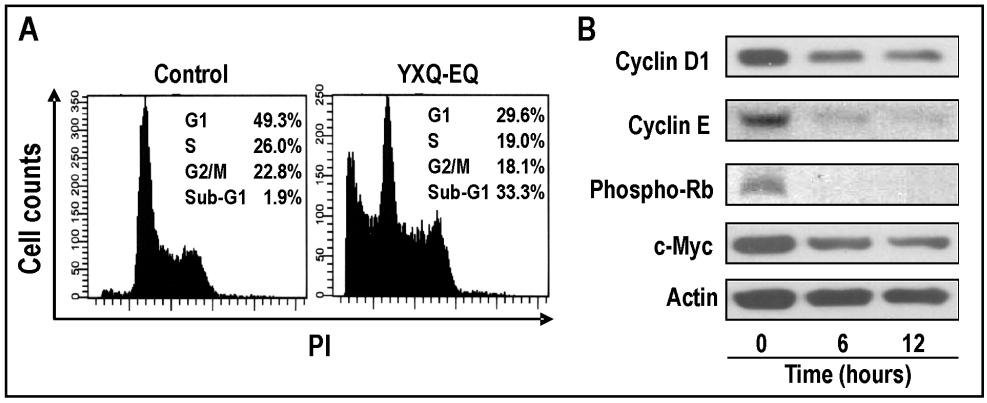

Fig. 3. Effect of YXQ-EQ on MDA-MB-231 cell cycle. Cells were treated with YXQ-EQ for 5 min. A. Flow cytometry analysis of cell cycle distribution at 16 h after the treatment. Percentages of cells at G1, S, G2/M, and sub-G1 phases are also shown. B. Western analysis of cyclins D1 and E, phospho-Rb, and c-Myc at time points as indicated.

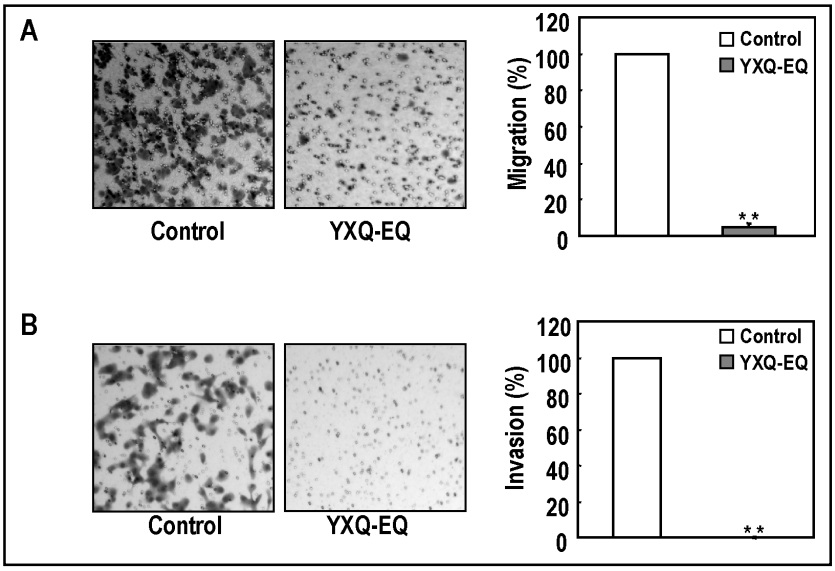

Fig. 4. Effect of YXQ-EQ on migration and invasion of MDA-MB-231 cells. Cells were treated with YXQ-EQ for 5 min. A. Cell migration was determined in Boyden Chambers after 4 h of stimulation with 5% FBS. B. Invasion was determined in Boyden Chambers coated with Matrigel after stimulation with 10% FBS for 24 h. Cells that migrated or invaded through Matrigel were fixed, stained and counted as described in Materials and Methods. Results are presented as the mean ± SD of percent migration or invasion from 3 independent experiments. ** $P < 0.01$ vs. control cells.

MB-231 cells for 5 min caused a time-dependent reduction in cell viability (Fig. 1A). A substantial viability reduction was observed at 6 h post treatment and all cells appeared to be dead 24 h after the treatment. In agreement, YXQ-EQ-treated cells underwent dramatic cell morphological changes (Fig. 1B). Furthermore, prolonged YXQ-EQ treatments caused a rapid and drastic reduction in cell viability (Fig. 1C). To determine long-term effect of YXQ-EQ, a clonogenic assay was performed. As shown in Figure 1D, colony formation of MDA-MB-231 cells was fully blocked by YXQ-EQ treatment. These results demonstrate potent cytotoxic effects of YXQ-EQ on MDA-MB-231 cells.

Induction of apoptosis by YXQ-EQ

We assessed the effect of YXQ-EQ on apoptosis in MDA-MB-231 cells to investigate the cellular and molecular mechanisms underlying YXQ-EQ's cytotoxic effect. YXQ-EQ treatment caused DNA fragmentation (Fig. 2A) and cleavage of pro-caspase-3, pro-caspase-9 and PARP (Fig. 2B). These results indicate that YXQ-EQ treatment induced apoptosis in MDA-MB-231 cells and are in agreement with the apoptotic morphological changes seen after YXQ-EQ treatment including cell shrinkage and cytoplasmic condensation (Fig. 1B). The percentage of apoptotic cells was evaluated using Annexin V-FITC/PI staining assay 16 h after YXQ-EQ treatment. The number of apoptotic cells increased from 5.1 ± 1.1% in the control to 48.5 ± 10.7% in the treated cells (Fig. 2C).

Effect of YXQ-EQ on cell cycle

We next examined the effect of YXQ-EQ on MDA-MB-231 cell cycle progression. The populations of cells at G1, S, and G2/M phases were reduced by 19.7%, 7.0% and 4.7%, respectively, accompanied by a 31.4% increase in the sub-G1 population 16 h after YXQ-EQ treatment (Fig. 3A). Furthermore, YXQ-EQ treatment repressed the expression of cyclins D1 and E and Rb phosphorylation (Fig. 3B), which are known to promote the G1 to S phase transition [36, 37]. These findings suggest that G1 cell cycle arrest followed by apoptosis might have been induced by YXQ-EQ treatment.

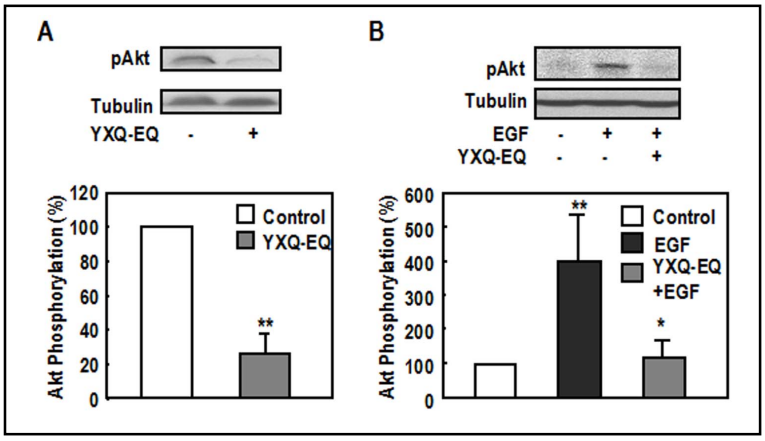

Fig. 5. YXQ-EQ inhibits Akt phosphorylation in MDA-MB-231 cells. A. Constitutive Akt phosphorylation. Cells were treated with YXQ-EQ for 5 min and harvested for analysis 1 h later. B. EGF-induced Akt phosphorylation. Serum-starved cells were treated with YXQ-EQ for 5 min and 10 min later were stimulated with EGF for 20 min. Akt phosphorylation was determined by Western blot analysis of whole cell extracts. Results are presented as the mean ± SD of percent Akt phosphorylation from 3 independent experiments. * $P < 0.05$ vs. cells treated with EGF alone; ** $P < 0.01$ vs. control cells.

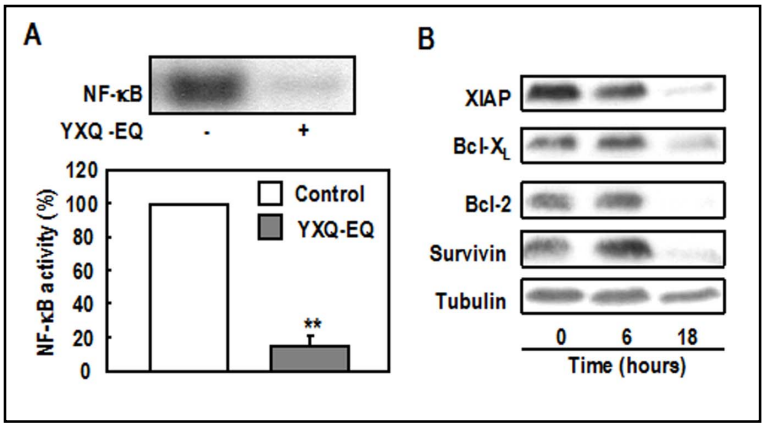

Fig. 6. Effect of YXQ-EQ on NF-κB signaling in MDA-MB-231 cells. A. Inhibition of nuclear NF-κB DNA binding activity by YXQ-EQ. Cells were treated with YXQ-EQ for 5 min and then 10 min later nuclear extracts were prepared. NF-κB DNA binding activity was determined by EMSA using ^{32}P-labeled consensus NF-κB oligo probe. Results are presented as the mean ± SD of the percent NF-κB DNA binding activity from 3 independent experiments. ** $P < 0.01$ vs. control cells. B. Downregulation of NF-κB target gene expression by YXQ-EQ. The expression of NF-κB target genes was determined by Western blot analysis of whole cell extracts.

Effect of YXQ-EQ on migration and invasion

MDA-MB-231 breast cancer cells are highly metastatic. The ability of cancer cells to metastasize is associated with migration and invasion behavior. We therefore investigated if YXQ-EQ affects MDA-MB-231 cell migration and invasion capacity. MDA-MB-231 cells were exposed to YXQ-EQ for 5 min and cell migration was determined after 4 h of FBS stimulation. As shown in Fig. 4A, YXQ-EQ markedly inhibited MDA-MB-231 cell migration (inhibition by 95.2 ± 2.1%). Invasion was determined with Matrigel-coated filters after 24 h of FBS stimulation. As seen in Fig. 4B, YXQ-EQ treatment fully blocked MDA-MB-231 cell invasion.

Effect of YXQ-EQ on Akt phosphorylation

It has been reported that Akt is constitutively activated in breast tumors and plays a crucial role in cancer cell survival, migration and invasion [3, 8, 20]. Thus we investigated the effect of YXQ-EQ on Akt phophsorylation in MDA-MB-231 cells. As seen in Fig. 5A, Akt phosphorylation levels of YXQ-EQ-treated cells were only 25.9 ± 14.4% of those of control cells. In cells treated with EGF alone Akt phosphorylation levels were 402 ± 139% of those of control cells, while in cells treated with YXQ-EQ followed by EGF Akt phosphorylation levels were only 121 ± 70% of those of control cells (Fig. 5B). Thus, YXQ-EQ treatment significantly reduced basal Akt phosphorylation as well as EGF-stimulated Akt phosphorylation. This is in agreement with the findings that YXQ-EQ treatment decreased the expression of Akt downstream target genes, including c-myc, cyclins D1 and E, and Rb phosphorylation (Fig. 3B).

Effect of YXQ-EQ on NF-κB signaling

We examined the effect of YXQ-EQ on NF-κB signaling in MDA-MB-231 cells as NF-κB has been reported to be constitutively activated in breast cancer cells and play an important role in cell invasion and growth [10, 14]. The DNA binding activity of NF-κB was determined by EMSA. As seen in Fig. 6A, NF-κB activity in YXQ-EQ-treated cells was 16.1 ± 6.3% of that of control cells, indicating that constitutive NF-κB activity was substantially repressed by YXQ-EQ treatment. We further examined the effect of YXQ-EQ on NF-κB-regulated expression of Bcl-2, Bcl-X_L, XIAP, and survivin, which are known important anti-apoptotic proteins [5, 13, 38]. As shown in Fig. 6B, YXQ-EQ treatment significantly downregulated the expression of

Bcl-2, Bcl-X_L, XIAP, and survivin proteins.

Discussion

Resistance to chemotherapeutic agents is the major cause for treatment failure in cancer chemotherapy. Studies have found constitutive activation of Akt/NF-κB signaling in many tumors that contributes to resistance to apoptosis-inducing chemotherapeutic agents of cancer cells resulting in treatment failure [3-7, 38]. Both Akt and NF-κB regulate a variety of cellular processes including proliferation, apoptosis, invasion and angiogenesis [4, 5, 8, 9, 14, 39]. Akt and NF-κB are being extensively explored as molecular targets for cancer therapeutics [40], as studies have shown that the repression of Akt and/or NF-κB activity inhibits breast cancer cell growth, migration and invasion, and sensitizes breast cancer cells to apoptosis [11, 13, 16-18, 20, 37, 41, 42]. In agreement with these previous studies, we showed here that YXQ-EQ treatment caused apoptosis and inhibition of migration and invasion of MDA-MB-231 cells through the suppression of Akt/NF-κB signaling.

In the present study we showed that brief YXQ-EQ treatment resulted in DNA fragmentation, cleavage of pro-caspase-3, pro-caspase-9 and PARP, indicating that YXQ-EQ induced apoptosis in breast cancer cells. The cleavage of pro-caspase-9 suggests that the intrinsic mitochondrial apoptosis pathway was activated [43]. In many cancer cells including breast tumor cells, both PI3K/Akt and NF-κB pathways are constitutively active and play an important prosurvival and anti-apoptotic role [3-7, 38]. It has been reported that NF-κB enhances the expression of several anti-apoptotic proteins, including Bcl-2, Bcl-X_L, survivin, and XIAP [12-16, 44, 45]. Bcl-2 and Bcl-X_L exert anti-apoptotic function through heterodimerization with several proapoptotic proteins thereby blocking release of cytochrome C from mitochondria and preventing the activation of pro-caspase- 9 and other downstream caspases [46-48]. The inactivation of Akt and/or NF-κB leads to downregulation of Bcl-2 and/or Bcl-X_L resulting in cytochrome C release from the mitochondria and initiation of apoptosis through activating caspase 9 [49]. Our findings that YXQ-EQ repressed Akt and NF-κB activity and downregulated the expression of Bcl-2, Bcl-X_L, survivin and XIAP proteins thus suggest that YXQ-EQ might induce MDA-MB-231 cell apoptosis through downregulating the expression of these anti-apoptotic proteins. YXQ-EQ might also induce apoptosis through disruption of mitochondrial membrane integrity and release of cytochrome C as it has been shown that YXQ-EQ had a profound impact on the structure of liposomes, a model for studies of biomembranes [31]. The exact mechanism(s) by which YXQ-EQ induces cancer cell apoptosis remains to be further investigated. It is also to be investigated if YXQ-EQ activates the extrinsic apoptotic pathway in MDA-MB-231 cells. Nevertheless, our previous studies have shown that YXQ-EQ activated both the intrinsic and extrinsic apoptotic pathways in prostate and pancreatic cancer cells [21, 22]. It has been suggested that the activation of Bcl-2, Bcl-X_L, survivin and/or XIAP might be a mechanism for tumor resistance to drug-induced apoptosis [41, 44, 45, 50, 51]. The inhibition of expression of these anti-apoptotic genes by YXQ-EQ thus also suggests that YXQ-EQ may sensitize cancer cells to apoptosis-based therapeutic agents.

In our study, we found that YXQ-EQ treatment completely blocked colony formation of MDA-MB-231 cells. This is most likely attributed to the potent cytotoxic effect of YXQ-EQ on MDA-MB-231 cells. Nevertheless, YXQ-EQ could have anti-proliferative effects and inhibit the growth of MDA-MB-231 cells, as YXQ-EQ repressed the expression of cyclins D1 and E and Rb phosphorylation that have been reported to play central roles in the G1 to S transition [36, 37].

In addition to induction of apoptosis, our studies also show that YXQ-EQ inhibited migration and invasion of highly metastatic MDA-MB-231 cells. Migration and invasion are essential events in cancer metastasis. It is well documented that PI3K/Akt and NF-κB promote cancer cell migration and invasion, and that the repression of Akt and/or NF-κB signaling by various agents inhibited migration and invasion of MDA-MB-231 cells [14, 20, 41, 50, 51]. Similarly, YXQ-EQ may inhibit MDA-MB-231 cell migration and invasion through the repression of Akt/NF-κB signaling. This could be achieved through repression of the expression of Akt/NF-κB target genes that are involved in migration and invasive behavior and/or through induction of apoptosis. More studies are needed to further elucidate the molecular mechanisms for the inhibitory effects of YXQ-EQ on breast cancer cell migration and invasion.

EGF receptor (EGFR) plays a central role in the development and progression of a number of common malignancies including breast, colon, pancreatic, and lung cancers [52-55]. EGF and its signaling through EGFR promote cell proliferation and survival by suppressing apoptosis [56]. Stimulation of MDA-MB-231 cells with EGF protects them from apoptosis through EGFR-

mediated Akt activation and blockage of mitochondrial cytochrome C release [57]. EGFR has emerged as an attractive molecular target for cancer therapy. Monoclonal antibodies and small-molecule tyrosine kinase inhibitors that specifically block EGFR signaling and induce apoptosis in cancer cells have been developed [52, 53]. Our findings that YXQ-EQ inhibited the activation of Akt mediated by EGF/EGFR signaling in MDA-MB-231 cells suggest that YXQ-EQ might inhibit growth and induce apoptosis of cancer cells by targeting the EGF/EGFR signaling pathway.

In addition to breast cancer cells, we have previously reported potent cytotoxic effects of YXQ-EQ on pancreatic and prostate cancer cells [21, 22]. In contrast, YXQ-EQ has no cytotoxic effect on normal cells including fibroblasts, human umbilical vein endothelial cells, and peripheral blood mononuclear cells [21, 22]. YXQ-EQ even protects neurons from apoptosis induced by oxidative stress [23, 58]. These findings suggest that YXQ-EQ may exert cytotoxic effects selectively on cancer cells and could be a novel approach for cancer therapy including breast cancer therapy.

Acknowledgements

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