Comparative Effects Between Fucoxanthinol and its Precursor Fucoxanthin on Viability and Apoptosis of Breast Cancer Cell Lines MCF-7 and MDA-MB-231

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Abstract. *Background/Aim: We evaluated whether low doses of the natural carotenoid fucoxanthin and/or of its metabolite fucoxanthinol are effective against proliferation of estrogen-sensitive MCF-7 and estrogen-resistant MDA-MB-231 breast cancer cell lines. Materials and Methods: These cell lines were stimulated with 10 to 20 μM fucoxanthin and/or fucoxanthinol, followed by cell viability assays, Annexin V immunofluorescence to evaluate apoptosis, as well as mRNA and protein extractions for changes in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) members' expressions and nuclear translocations. Results: Fucoxanthin and fucoxanthinol reduced the viability of MCF-7 and MDA-MB-231 cells in a time-dependent manner as a result of increased apoptosis. In both cell lines, modulatory actions of fucoxanthinol on members of the NF-κB pathway were more pronounced than that of fucoxanthin. Conclusion: In MDA-MB-231 cells, fucoxanthinol reduced nuclear levels of NF-κB members' p65, p52 and RelB. Fucoxanthinol and fucoxanthin could be effective for the treatment and/or prevention of breast cancer.*

Breast cancer ranks second among causes of cancer death in North American women. In the United States alone, an estimation of 39,950 women died of breast cancer and over 226,800 new cases have been diagnosed in 2012 (1). With no significant change for trends in cancer incidence and death rates for breast cancer from 2004 to 2008 (1), there is an urgent need for improvements in detection, diagnosis and treatment of this devastating disease for women.

Fucoxanthin (Fx) (Figure 1) is a predominant carotenoid found in edible brown algae, such as wakame (*Undaria pinnatifida*), kombu (*Laminaria japonica*) and arame (*Eisena bicyclis*). Fx represents more than 10% of the estimated total natural carotenoids production (2). Carotenoids and Fx, being hydrophobic, are absorbed at the intestinal level through the same path as dietary fats. Ingested Fx, known to be non-toxic and non-mutagenic (3, 4), is metabolized in the gastrointestinal tract by digestive enzymes to fucoxanthinol (Fxol) (Figure 1), followed by absorption into intestinal cells (5). Then, circulating Fxol is further converted to amarouciaxanthin A in the liver (4, 6), showing that the active forms of Fx in biological systems are Fxol and/or amarouciaxanthin A. Fx and Fxol are known to have many beneficial effects on health, such as anti-mutagenic (7), anti-diabetic (8), anti-obesity (9, 10), anti-inflammatory (11, 12) and show preventive actions on liver, breast, prostate, colon and lung cancers (7, 13-17).

In breast cancer cells, members of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway have been associated to breast cancer development and progression (18, 19). Thus, inhibitory actions on the NF-κB pathway are associated with enhanced sensitivity of breast tumor cells to apoptosis (20, 21). Members of the NF-κB family include NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel (22). These NF-κB proteins bind DNA as dimers and are sequestered in the cytoplasm of most cell types by inhibitory proteins (23, 24). These inhibitors, which belong to a family of proteins, named IκB, mask the NF-κB nuclear localization domain and inhibit its DNA-binding activity. In response to a large variety of stimuli, the IκB inhibitor is rapidly phosphorylated and degraded by the proteasome, thus allowing NF-κB nuclear translocation, dimerization, DNA binding to specific DNA binding elements in promoters and transcription of target genes (25–27). NF-κB is activated by most carcinogens, leading to expression of anti-apoptotic genes, which allow for survival and growth of tumors (28-30). Among NF-κB members, p65 and p50 are constitutively active and over-expressed in breast cancer cells (31) resulting in increased transcription of anti-apoptotic genes (32). Such NF-κB activation seems to be the

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Key Words: Fucoxanthin, fucoxanthinol, MCF-7, MDA-MB-231, NF-κB, SOX9.

Table I. *Sequences of oligonucleotide primers used in quantitative real-time PCR for this study.*

Target gene	Forward primer	Reverse primer	Reference
p65	5'-CTCCGCGGGCAGCATCC-3'	5'-AGCCGCACAGCATTCAGGTCGTAG-3'	
p50	5'-CACCTAGCTGCCAAAGAAGG-3'	5'-AGGCTCAAAGTTCTCCACCA-3'	(84)
p52	5'-GCAAATCTCCGGGGGCATCAAACC-3'	5'-CTCCGCTTCCGCTGCACCTCTTCC-3'	
SOX9	5'-GGAGATGAAATCTGTTCTGGGAATG-3'	5'-TGAAGGTTAACTGCTGGTGTTCTGA-3'	(85)
Rps9	5'-AAGGCCGCCCGGGAACTGCTGAC-3'	5'-ACCACCTGCTTGCGGACCCTGATA-3'	
GAPDH	5'-TGACCACAGTCCATGCCATC-3'	5'-ATGAGGTCCACCACCCTGTT-3'	

result of chronic stimulation of IκB kinases (IKKs) or of defective IκBα (30, 33, 34). Acquired resistance to therapy is linked to constitutive NF-κB in aggressive estrogen receptor (ER) -positive tumors and often leads to estrogen-independent growth (34, 35).

Among NF-κB potential target genes in breast cancer cells, *SOX9* is over-expressed in a wide range of human cancers (36). Under normal conditions, SOX9 is especially known to be expressed in chondrocytes where it regulates the transcription of type II collagen (37, 38) and in the testis where it plays a role in male sex determination and differentiation (39, 40). SOX9 is a member of a large family of transcription factors that interact with DNA through a high mobility group (HMG) box, resulting in DNA bending and target genes' transactivation (41, 42). A role for SOX9-mediated growth inhibition of breast cancer cells by retinoids has been suggested (43). In addition, SOX9 expression has been linked to cells arrest in G_1 phase of the cell cycle and up-regulation of the *p21* gene expression in chondrocytes CFK2 cells (44).

In a previous manuscript (45), we showed that 20 μM Fx and Fxol reduced the viability of MCF-7 and MDA-MB-231 cells in a time-dependent manner as a result of increased apoptosis. Furthermore, Fxol-induced apoptosis was more potent than that of Fx and correlated, for MDA-MB-231 cells, with inhibitory actions on members of the NF-κB pathway p65, p50, RelB and p52. The objective of the current study was to compare the time-dependent inhibitory actions of weak doses of Fx and Fxol on nuclear translocation of NF-κB members and breast cancer cell apoptosis. Since MCF-7 cells express high levels of estrogen receptor alpha (ER-α), whereas MDA-MB-231 are resistant to estrogen, we sought to determine if the presence of a functional ER altered the effects of 10 μM Fx and/or Fxol treatments. Herein we report that 10 μM Fxol moderately reduced breast cancer cells viability and increased apoptosis of MDA-MB-231 cells, possibly through modulation of components of the NF-κB pathway.

Materials and Methods

Chemicals. Fucoxanthin (Fx) and fucoxanthinol (Fxol) were purchased from Wako Chemicals (Richmond, VA, USA).

Cell culture. Human breast cancer cell lines MCF-7 (hormone sensitive) and MDA-MB-231 (hormone resistant) were obtained from the American Type Culture Collection (ATCC; Bethesda, MD, USA). MCF-7 and MDA-MB-231 cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum (Canadian origin) and 100 U/ml penicillin/streptomycin (Corning, Tewksbury, MA, USA). Cells were cultured at 37˚C and 5% CO_2.

Cell viability. The viability of Fx or Fxol treated cells were measured using the Cell-Titer blue cell viability assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Following treatments of breast cancer cell lines with 10 and 20 μM of Fx or Fxol in a time-dependent (6, 12, 24 or 48 h) manner, cell viability was estimated by measuring the amount of reduced resorufin by its fluorescence at $560_{Ex}/590_{Em}$ using a multimode microplate reader (Varioskan, Thermo Scientific, Waltham, MA, USA).

Apoptotic, necrotic and healthy cell assays. The breast cancer cell lines were treated with 10 μM Fx or Fxol for 24 h, followed by staining with FITC-Annexin V, ethidium homodimer III and Hoechst 33342 according to the manufacturer's protocol (Biotium, Inc., Hayward, CA, USA). Fluorescence was assessed using an Axio Observer A1 inverted fluorescence microscope (Carl Zeiss, Gottingen, Germany) with FITC, rhodamine and DAPI filters. Images were merged and analysed for signal densitometry using the ImageJ software (http://rsbweb.nih.gov/ij/).

Quantitative real-time polymerase chain reaction (qPCR). RNA was isolated from cultured breast cancer cell lines using the E.Z.N.A.® Total RNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA). The purity and concentration of the isolated RNA was assessed using a Nanodrop 1000; 260/280 ratios were approximately 2:1. The High Capacity cDNA Reverse Transcription Kit (Life Technologies, Burlington, ON, Canada) was used to synthesize cDNA, using 1μg of RNA. qPCR was performed using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The primers (Integrated DNA Technologies, Coralville, IA, USA) used are described in Table I. Primers' specificities were assessed by melt curve analysis. Gene expression levels were evaluated by the ΔΔ threshold cycle (Ct) method after confirmation that amplification efficiency was between 90% and 110% for all primer pairs. The geometric average of reference genes *RPS9* and *GAPDH* mRNAs was used as control for total mRNA recovery. Experiments were repeated four times and quantified as technical duplicates.

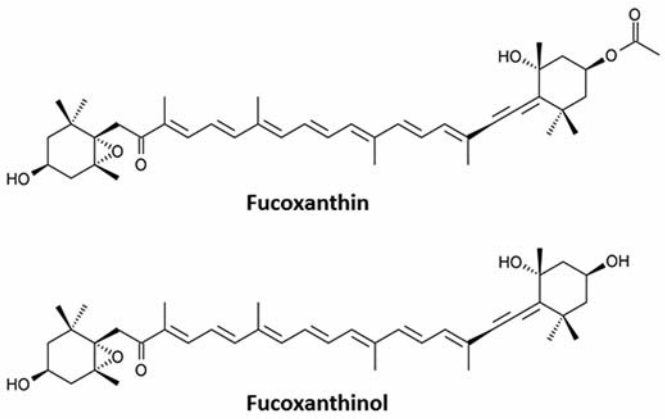

Figure 1. *Chemical structures of Fucoxanthin (Fx) and Fucoxanthinol (Fxol).*

Western blot analysis. The breast cancer cell lines were treated with 10 μM Fx or Fxol for 12 or 24 h, followed by protein extractions for nuclear and cytoplasmic proteins using hypertonic buffer with phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4 and 20 mM glycerol2-phosphate) and protease inhibitors (30 μg/ml aprotinin, 2.5 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mM phenylmethyl fluoride) as described previously (46). Protein concentrations were evaluated using the Bradford method (47). Ten μg (for nuclear extracts) or 30 μg (for cytoplasmic extracts) of protein from each sample was separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated in blocking solution containing 5% non-fat dry milk in TBST buffer (TBS buffer containing 0.1% Tween 20) for 1 h at room temperature, followed by incubation with a specific primary antibody overnight at 4˚C. Specific proteins were detected using the following primary antibodies: NF-κB family member antibody sampler kit (1:1000, Cat.: 4766, Cell Signaling, Danvers, MA, USA), monoclonal anti-Phospho-NF-κB p65 (Ser536) (1:1,000, Cat.: 3033, Cell Signaling), polyclonal anti-SOX9 (1:500, Cat.: AB5535, Millipore, Billerica, MA, USA), monoclonal anti-α-TUBULIN (1:5,000, Cat.: 05-829, Millipore) and polyclonal anti-NCL (1:1,000, Cat.: 12247S, Cell Signaling). The membranes were washed three times in TBST buffer for 5 min, followed by incubation for 1h with horseradish peroxidase-conjugated secondary antibody. The membranes were washed again and developed using an enhanced chemiluminescent detection system (Luminata Forte, Millipore) according to the manufacturer's instructions. Images were taken using the ChemiDoc MP imaging system (Bio-Rad Laboratories) and analysed for signal densitometry using the ImageJ software (http://rsbweb.nih.gov/ij/). The Western blotting results were referred to NCL (for nuclear extracts) and α-TUBULIN (for cytoplasmic extracts) levels as loading controls.

Statistics. Experiments were repeated at least three times and the data are presented as means±standard error of the mean (S.E.M). Statistical analysis of the data was performed using one-way and two-way analysis of variance (ANOVAs) followed by multiple comparisons tests with GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). $P<0.05$ was considered significant.

Results

Fxol is more antiproliferative than Fx against human breast cancer cells. We first examined the effects of 10 and 20 μM Fx and Fxol on cell viability of human breast cancer cell lines MCF-7 and MDA-MB-231 (Figure 2). Fxol-induced suppression of cell viability was more rapid and more pronounced, compared to Fx. Indeed, viability of MCF-7 cells was reduced by 58% in response to a 48-h treatment with 20 μM Fxol, whereas Fx had no inhibitory effect on proliferation of these breast cancer cells (Figures 2A and 2C). For MDA-MB-231 cells, viability was reduced by 13% and 47% in response to 48-h treatments with 10 μM and 20 μM Fxol, respectively (Figure 2D). At 10 μM, only MDA-MB-231 cells showed a reduction of viability in response to a 48-h treatment with Fxol. Thus, our results suggest that 10 μM Fx/Fxol treatments have minor effects on breast cancer cells viability and such dose can be used to study the preliminary changes of signaling pathways related to apoptosis. In addition, these results also demonstrate a more efficient inhibitory action of Fxol on viability of hormone-independent MDA-MB-231, compared to hormone-dependent MCF-7 cells.

Next, we looked at possible early modulations of an important marker of apoptosis, Annexin V, in response to 10 μM Fx or Fxol treatments of breast cancer cells for 24 h. Increased apoptosis of breast cancer cell lines in response to these treatments was confirmed using fluorescence microscopy (Figure 3A). For both cell lines, Fx and Fxol significantly increased apoptotic levels as shown with FITC-Annexin V green fluorescence (Figure 3B). However, there was no difference in the apoptotic levels between treatments with Fx and Fxol for both cell lines. While apoptotic levels tended to be higher in MDA-MB-231 cells treated with 10 μM Fx (by 45%) or Fxol (by 24%), compared to MCF-7 cells, these results were non-significant. In addition, although non-

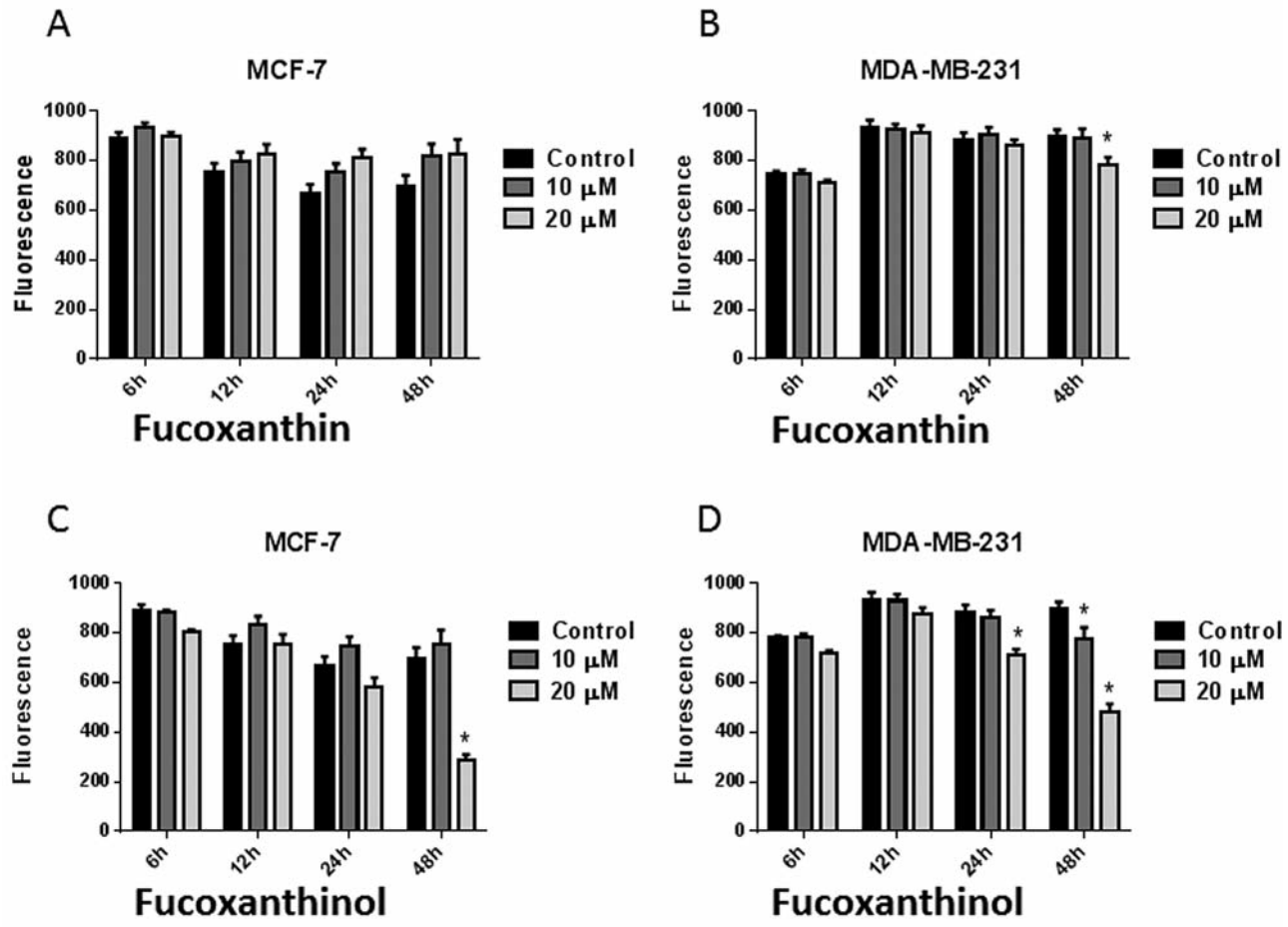

Figure 2. *Influences of Fx and Fxol on cell proliferation of MCF-7 and MDA-MB-231 cell lines. Exponentially-growing cells were incubated in the absence or presence of 10 or 20 μM of Fx or Fxol, for 6, 12, 24 and 48 h, followed by determination of cell viability, as described in Material and Methods. The results are presented as absolute fluorescence signal and are expressed as means±standard error of mean deviation of four independent experiments in which each treatment was performed in triplicate. Comparisons to control were done using a two-way ANOVA followed by Dunnett multiple comparison test (*=significant, p<0.05).*

significant for MCF-7 cells, Fx and Fxol treatments also resulted in decreased levels of necrosis as shown with ethidium homodimer III red fluorescence (Figure 3B). Thus, these results suggest that treatments with 10 μM Fx or Fxol may initiate the apoptotic pathway in breast cancer cells.

Modulation of NF-κB protein synthesis and nuclear accumulation in response to Fx and Fxol. Since members of the NF-κB family play a central role in the inhibition of apoptosis in breast cancer cells (48-50), we examined whether Fx and/or Fxol could inhibit their mRNA and/or protein expressions. After a 12- to 24-h exposure to 10 μM Fx or Fxol, no changes in *p65, p50* and *p52* mRNA expressions could be observed in MCF-7 and MDA-MB-231 cells (Figure 4A-F). In addition, *SOX9*, known to be a NF-κB target gene in other cancer cells (51, 52), was not influenced by Fx/Fxol treatments in MCF-7 cells (Figure 4G). However, Fxol treatment of MDA-MB-231 cells for 24h caused a significant reduction of *SOX9* expression by 25% (Figure 4H).

Lack of transcriptional regulation of NF-κB members by Fx/Fxol suggests that post-transcriptional regulation of these transcription factors may be involved. Although Fx and Fxol had no inhibitory effect on cytoplasmic accumulations of p65 and p50 in MCF-7 cells (Figure 5A), Fxol reduced the nuclear accumulation of phosphorylated p65 by 50% following a 24-h treatment of these cells (corrected with NCL as a loading control) (Figure 5B). In MDA-MB-231 cells, treatments with 10 μM Fx/Fxol resulted in decreased cytoplasmic levels of p65 phosphorylation, p50 and its precursor p105, whereas the cytoplasmic levels of p65 were increased in response to Fx treatment for 24 h (Figure 5C). In addition, nuclear levels of total and phosphorylated p65 were decreased by more than

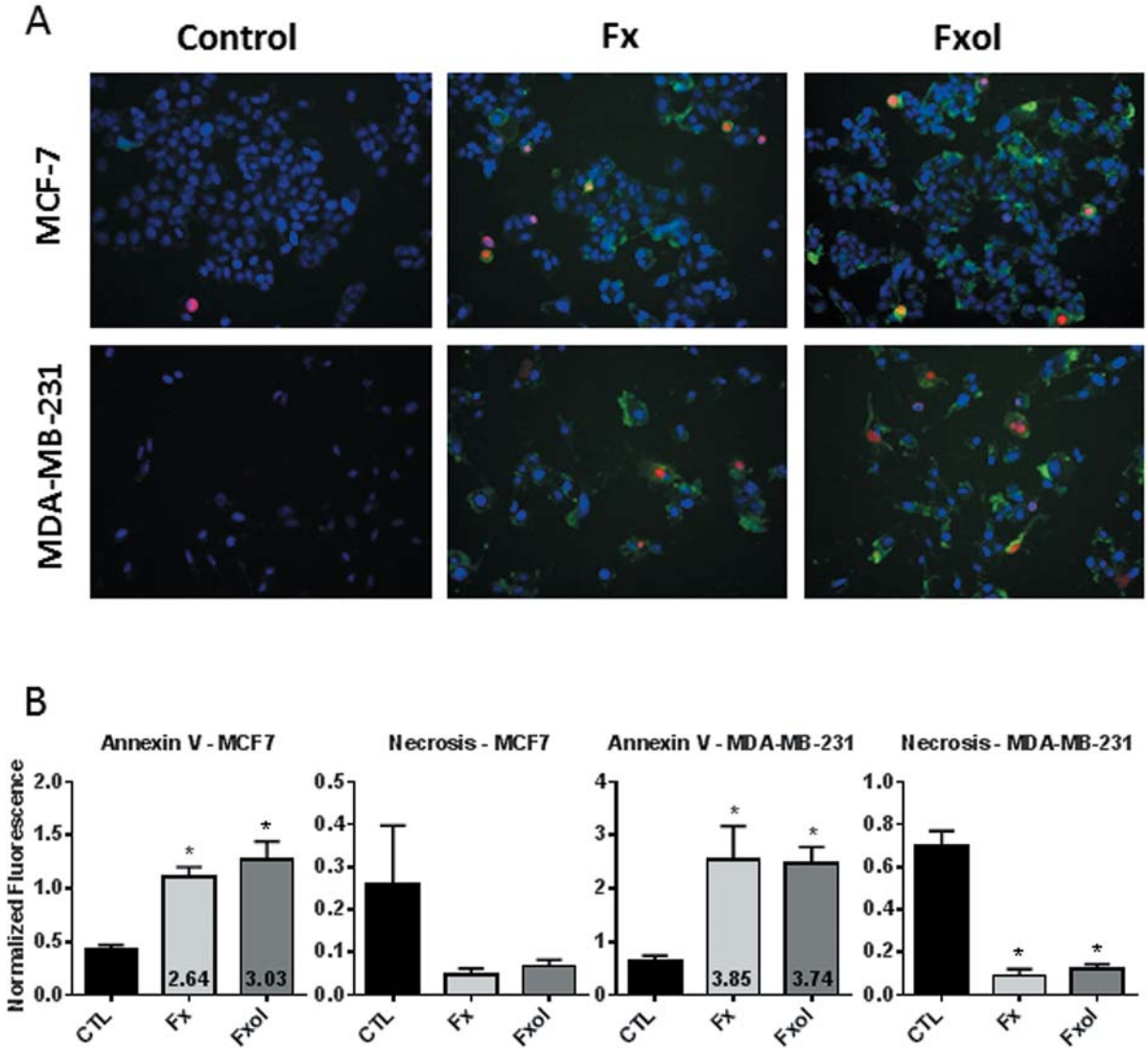

Figure 3. *Effects of Fx and Fxol on apoptotic markers in breast cancer cells. (A) Following treatment with 10 μM Fx or Fxol for 24 h, MCF-7 cells (upper panel) and MDA-MB-231 cells (lower panel) were stained with Hoechst 33342 (for viable cells in blue), FITC-Annexin V (for apoptotic cells in green) and ethidium homodimer III (for necrotic cells in red), followed by imaging with an inverted fluorescence microscope with total magnifications of ×200. In (B), densitometry assays of signals obtained in fluorescence microscopy were assessed using the ImageJ software. Fluorescence signals for FITC-Annexin V and ethidium homodimer III were normalized to Hoechst 33342. Fold increases in fluorescence for FITC-Annexin V of treated samples compared to control (CTL) are presented inside columns. The experiments where repeated three times. Statistics were performed using a one-way ANOVA followed by Holm-Sidak's multiple comparisons test (*=significant, $p<0.05$).*

47% in response to Fxol treatments (when compared to NCL) in these cells (Figure 5D) suggesting that protein syntheses of p65 and p105/50 are decreased in response to 10 μM Fxol in MDA-MB-231 cells. Fx treatments of MDA-MB-231 cells resulted in inconsistent results at the nuclear level, as shown by a 32% decrease in p65 after 12-h and no effect after 24-h stimulations. Thus, these results suggest that inhibitory actions of low doses of Fxol involve down-regulation of cell nuclear localization of the NF-κB member p65 in MDA-MB-231 cells.

Besides members of the canonical pathway, Fx/Fxol may also influence transcriptional activity of non-canonical NF-κB members in breast cancer cells. In MCF-7 cells, Fx and Fxol stimulations for 24 h resulted in decreased cytoplasmic levels, accompanied by increased nuclear accumulation of p52, whereas its precursor, p100, was decreased by at least 40% at the nuclear level in response to Fx/Fxol treatments (Figure 6A-B). However, this trend was not observed in MDA-MB-231 cells, where only p100 was decreased at the nuclear level in

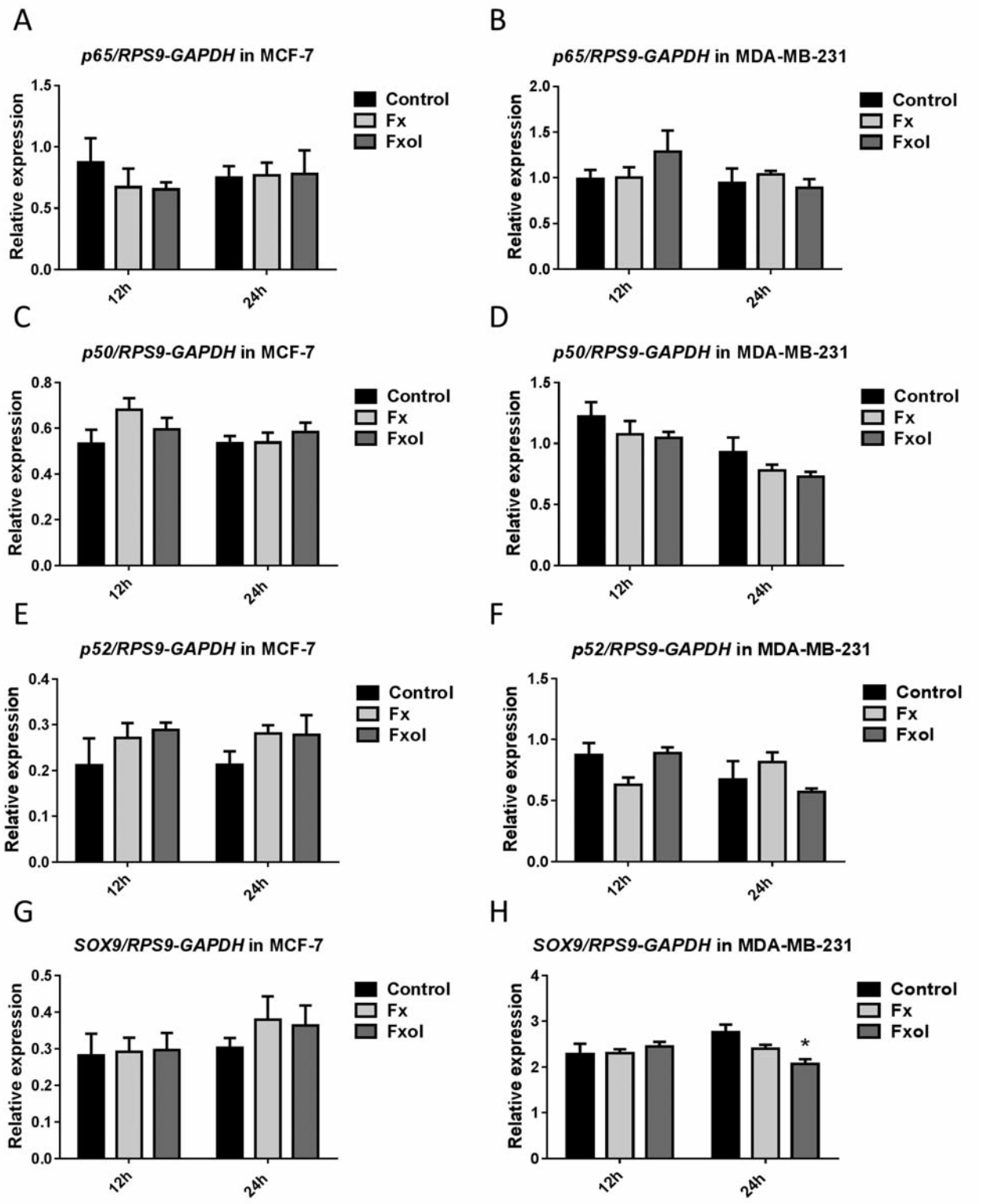

Figure 4. *Influences of Fx and Fxol on transcriptional regulation of members of the NF-κB pathway, p65, p50 and p52, as well as their target gene SOX9. MCF-7 and MDA-MB-231 cell lines were treated with 10 μM Fx or Fxol for 12 and 24 h, followed by total RNA extraction. The levels of mRNA expressions for target genes were determined using quantitative real-time PCR analysis, as described in Material and Methods. The geometric average of reference genes RPS9 and GAPDH mRNAs was used as control for total RNA recovery. Experiments were repeated four times and quantified as technical duplicates. Statistics were performed using a two-way ANOVA followed by Dunnett's multiple comparisons test (*=significant, p<0.05).*

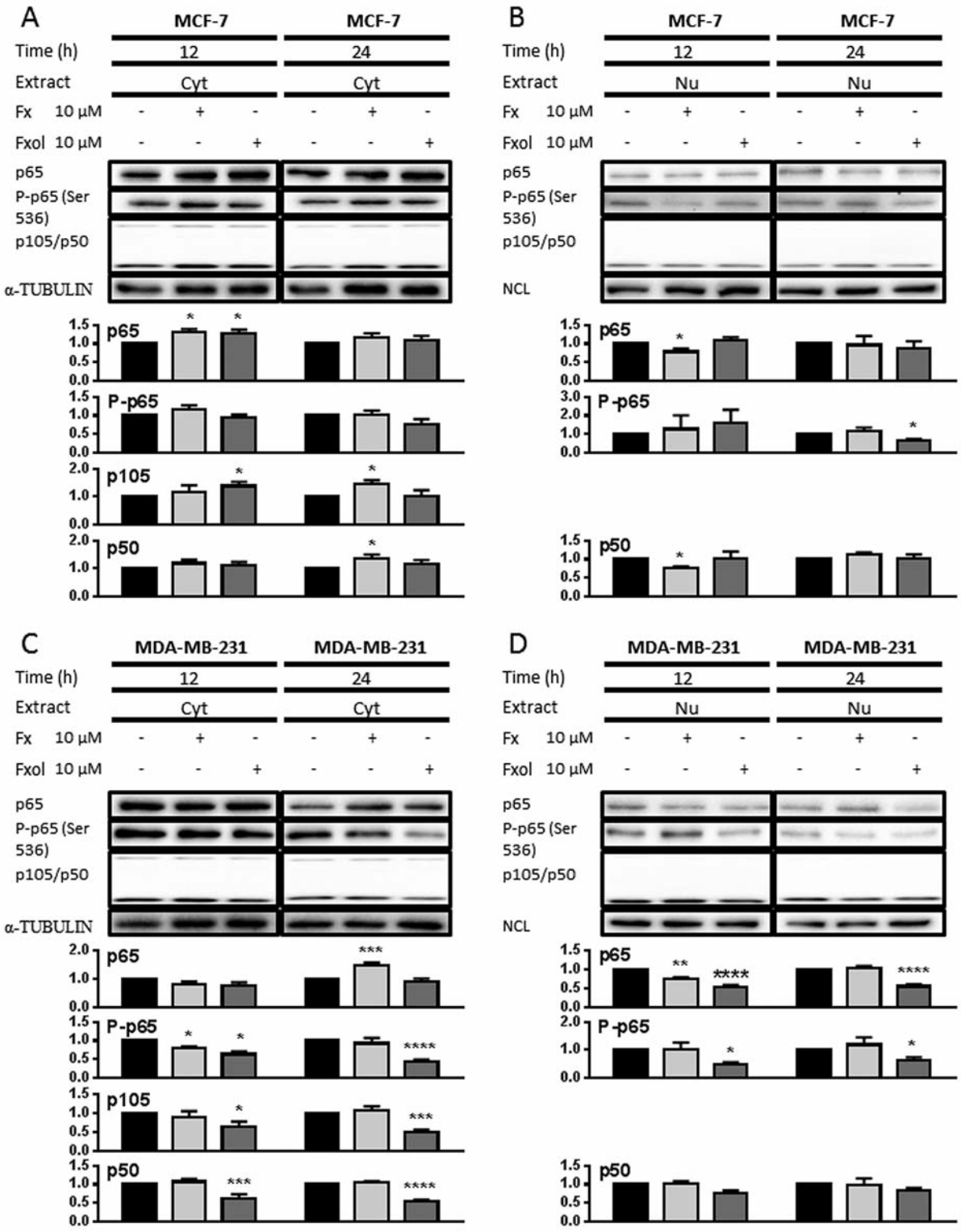

Figure 5. *Effects of Fx and Fxol on canonical members p65/p50 of the NF-κB pathway. MCF-7 and MDA-MB-231 cell lines were treated with 10 µM Fx or Fxol for 12 and 24 h, followed by cytoplasmic and nuclear protein extraction. The levels of target proteins were determined using western blot analysis as described in Material and Methods. α-TUBULIN and Nucleolin (NCL) were used as loading controls for cytoplasmic and nuclear extracts, respectively. Every experiment was repeated three times. A compilation of densitometry assays for protein levels is presented at the bottom portion of each figure. Comparisons to control were done using a one-way ANOVA followed by Dunnett's multiple comparison tests. Asterisks (*: p<0.05, **: p<0.005, ***: p<0.0005, ****: p<0.0001) indicate a statistically significant difference from control.*

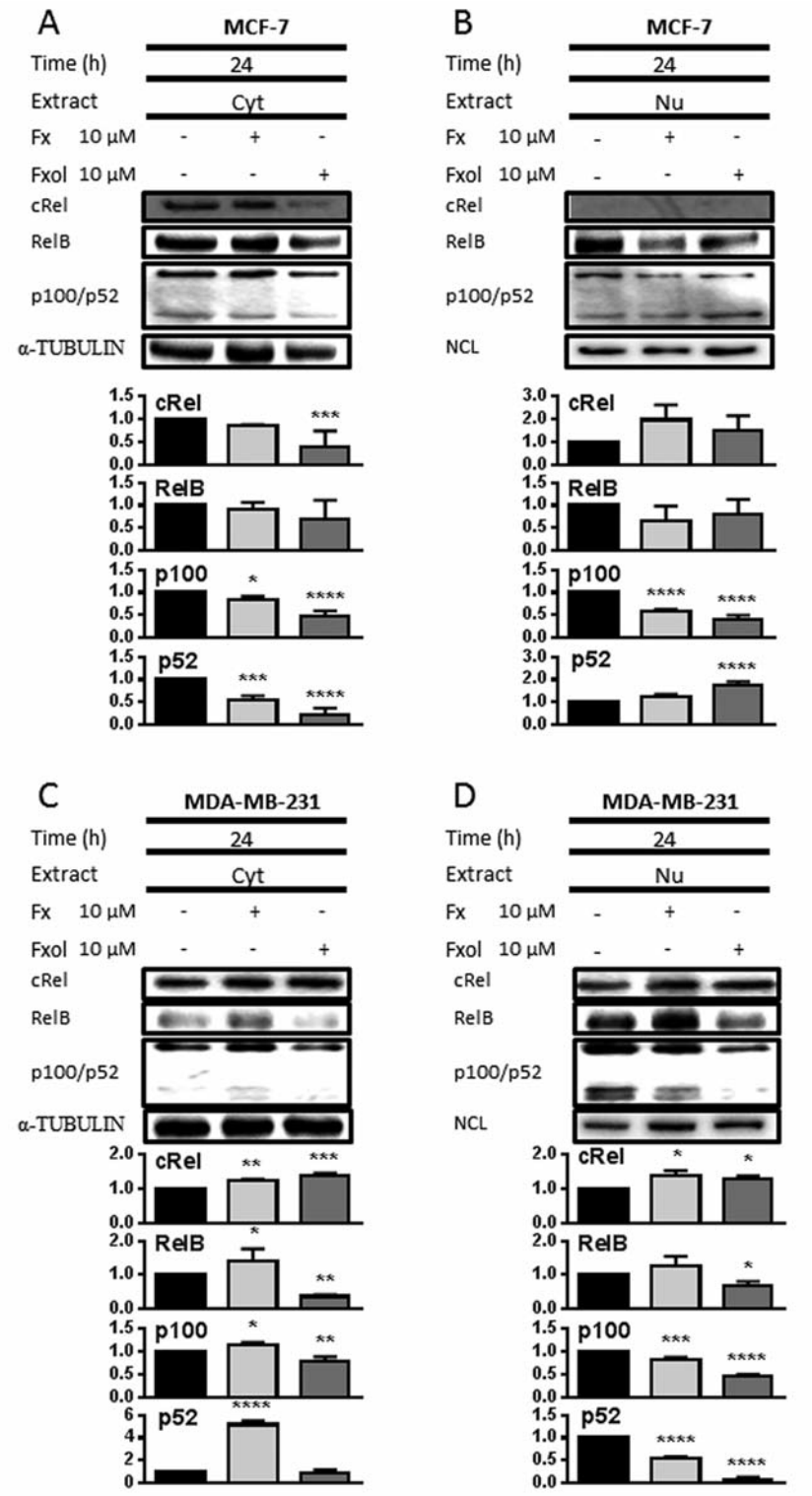

Figure 6. *Influences of Fx and Fxol on non-canonical members cRel, RelB and p52 of the NF-κB pathway. MCF-7 and MDA-MB-231 cell lines were treated with 10 µM for 24 h, followed by cytoplasmic and nuclear protein extraction. The levels of target proteins were determined using western blot analysis as described in Material and Methods. α-TUBULIN and Nucleolin (NCL) were used as loading controls for cytoplasmic and nuclear extracts, respectively. Every experiment was repeated three times. A compilation of densitometry assays for protein levels is presented at the bottom portion of each figure. Comparisons to control were done using a one-way ANOVA followed by Dunnett's multiple comparison tests. Asterisks (*: p<0.05, **: p<0.005, ***: p<0.0005, ****: p<0.0001) indicate a statistically significant difference from control.*

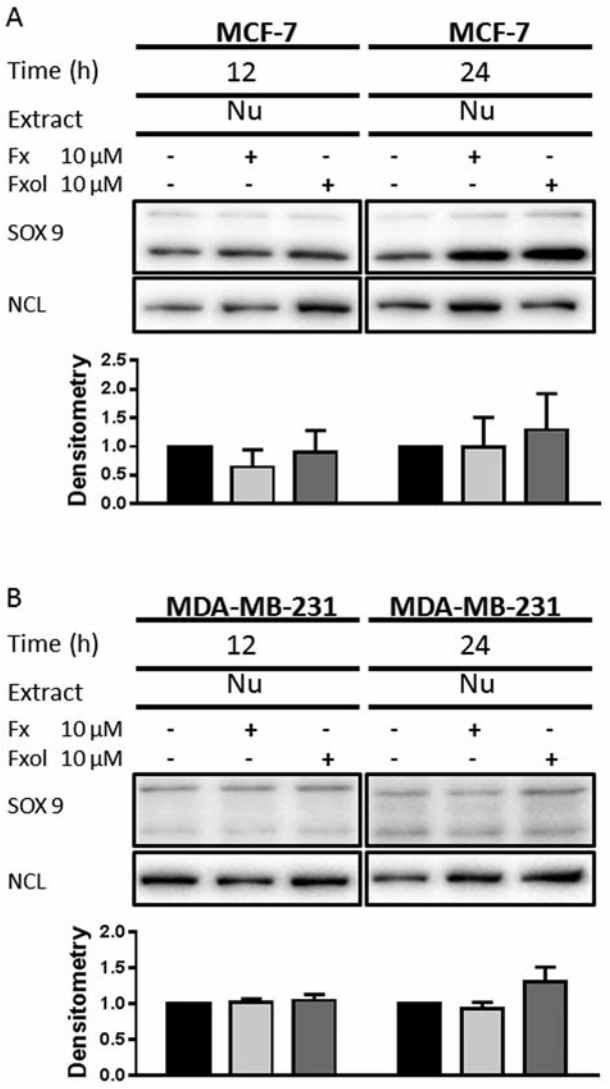

Figure 7. *Effects of Fx and Fxol on nuclear levels of SOX9 protein. MCF-7 (A) and MDA-MB-231 (B) cell lines were treated with 10 µM for 12 and 24 h, followed by nuclear protein extraction. The levels of target proteins were determined using western blot analysis, as described in Material and Methods. Nucleolin (NCL) was used as loading control for nuclear extracts. Every experiment was repeated three times. A compilation of densitometry assays of SOX9 protein levels is presented at the bottom portion of each figure. Comparisons to control were done using a one-way ANOVA followed by Dunnett's multiple comparison test (non-significant results were obtained, $p<0.05$).*

response to Fx/Fxol and p52 was rather down-regulated by 95% in nuclear extracts following Fxol treatments (Figure 6D). In addition, Fx up-regulated p52 cytoplasmic synthesis, while RelB protein synthesis was inhibited by 62% in response to Fxol in MDA-MB-231 cells (Figure 6C). Interestingly, Fx/Fxol treatments of MCF-7 cells did not result in changes of RelB at the cytoplasmic and nuclear levels (Figure 6A-B). Thus, inhibitory actions of Fxol on estrogen-independent breast cancer cells may involve decreased p52 and, to a minor extent, RelB nuclear translocations, dimerizations, DNA binding to specific DNA binding elements in promoters and decreased transcription of target genes.

Influences of Fx and Fxol on SOX9 nuclear levels. Next, we investigated potential changes in nuclear accumulation of SOX9, which is regulated by NF-κB in other cancer cells (51, 52) in response to 10 µM Fx and/or Fxol treatments of breast cancer cell lines in a time-dependent manner. However, no change in nuclear levels of SOX9 was observed in response to Fx and Fxol stimulations of MCF-7 cells (Figure 7A) and of MDA-MB-231 cells (Figure 7B). SOX9 Western blots from nuclear extracts showed the presence of two forms of the protein. Such result might be attributed to SOX9 phosphorylation, as previously shown by protein kinase A in Sertoli-like cells (53). No cytoplasmic accumulation of SOX9 could be observed in response to Fx/Fxol treatments (data not shown), which may be attributed to the rapid nuclear translocation of SOX9 following its translation (54).

Discussion

In the present study, we demonstrated that 10 µM Fx or Fxol show minor antiproliferative and/or apoptosis-inducing effects on two human breast cancer cell lines, MCF-7 and MDA-MB-231. These effects may involve modulatory actions of Fx and Fxol on components of the NF-κB pathway and seem to be independent of nuclear accumulation of SOX9.

As shown previously in 3T3-L1 cells (55), 10 µM Fx did not affect viability or cause cytotoxicity of breast cancer cells. However, others have shown that Fx significantly inhibited proliferation of human hepatoma HepG2 cells by 17% and 28% after incubation with a 10-µM dose for 24 h and 48 h, respectively (56). As in primary effusion lymphoma cell lines (57), we showed that Fxol-induced suppression of cell viability was more pronounced than that of Fx for breast cancer cell lines MCF-7 and MDA-MB-231. Compared to Fx, Fxol also had a stronger inhibitory effect on the viability of human prostate (PC-3) (6), breast (MCF-7) (58), colon (Caco-2) (58) and osteosarcoma (59) cancer cell lines. Thus in agreement with our current results, Fx and Fxol can induce growth inhibition and apoptosis in different cell lines (4, 60); however, the effective concentrations differ among cell lines and the detailed molecular mechanisms remain to be elucidated.

Previously, Fx was shown to induce apoptosis in human prostate (PC-3, DU 145 and LNCaP) (61), human leukemia (HL-60) (62) and human colon (Caco-2, HT-29 and DLD-1) (63) cancer cell lines. Pro-apoptotic actions of Fx involve the caspase pathway and regulation of Bcl expression (Bcl-2 and Bcl-xL) in HL-60 and PC-3 cells (17, 63, 64). The current study demonstrates that 10 µM Fx or Fxol also induces

apoptosis in MCF-7 and MDA-MB-231 breast cancer cells, as shown with increased Annexin V signal in fluorescence microscopy. Among pathways possibly involved, Fx and Fxol also induced cell-cycle arrest in G_1 phase, inhibited the activation of NF-κB and down-regulated anti-apoptotic proteins in primary effusion lymphoma cells (57). Also, Fx has been shown to inhibit cancer cells proliferation by increasing gap junction intercellular communication (65, 66). Thus, activation of apoptotic pathways associated to decreased cell viability of breast cancer cells in response to Fx/Fxol may also involve cell-cycle arrest and/or increased cell to cell communication. Moreover, our previous study clearly demonstrated that higher doses of Fxol (20 μM) induced apoptosis of breast cancer cells MDA-MB-231 and MCF-7 by cleavages of procaspase-3 and PARP (45). Decreased levels of necrosis, which we observed in response to Fx/Fxol treatments of breast cancer cells, may be attributed to their antioxidant activities, as previously reported with β-carotene in rat hepatocytes (67).

Expression of estrogen receptors in breast cancer is highly correlated with prognosis. Indeed, ER-positive breast tumors are related to a better outcome. However, in up to 25 % of cases, ER-positive breast tumors become non-responsive to therapy, possibly through constitutive expression and activation of NF-κB members, leading to estrogen-independent growth (30, 34, 35). Indeed, constitutive nuclear localization of p50, p52, c-Rel and over-expression of p100/p52 in breast cancer have been reported (18, 19). In addition, p65 is activated in most human breast cancer cell lines and correlated with the conversion of breast cancer cells to hormonal independent growth, a characteristic of more aggressive and metastatic tumors (31). In this study, we looked at possible differences in the inhibitory actions of 10 μM Fx and/or Fxol on components of the NF-κB pathway between estrogen-sensitive MCF-7 and estrogen-resistant MDA-MB-231 breast cancer cell lines. An inhibitory action of Fxol on p65, p52 and Rel-B nuclear accumulations, as we showed in MDA-MB-231 cells, supports a possible anti-cancer effect of this agent on breast cancer cells. Indeed, use of NF-κB antagonists caused apoptotic death in ER-breast cancer cells (68) and mammary epithelial tumors (69). In addition, we showed that stimulations with 10 μM Fxol resulted in a consistent decrease in phosphorylated p65 in the nucleus of MCF-7 and MDA-MB-231 cells. Phosphorylation of p65 at Ser536 enhances nuclear localization, protein-protein interactions and transcriptional activity (70). Thus, decrease in p65 phosphorylation in response to Fxol may lead to increased apoptosis of breast cancer cells, as previously reported (70). Overall, major differences were observed in the inhibitory mechanisms of Fx and Fxol on the activation of components of both canonical (p65) and non-canonical (p52, Rel-B) NF-κB pathways in breast cancer cell lines. In addition, others have shown that Fx inhibited p50 and p65 nuclear translocations in lipopolysaccharide-stimulated RAW 264.7 macrophages cells (11, 71), thus supporting our current results.

We have shown previously that Fxol treatment decreased nuclear levels of p65, p50, RelB and p52/p100 in hormone independent MDA-MB-231 cells (45). Using treatments with 10 μM of Fxol, we showed that RelB protein levels were consistently reduced at the cytoplasmic and nuclear levels in MDA-MB-231 cells. Reductions in RelB protein expression and transcriptional activity have been correlated previously with increased apoptosis in prostate cancer cells (72). Thus, Fxol treatment may contribute to reduce viability of aggressive estrogen-independent tumor growth and may involve inhibitions of nuclear translocation and transcriptional activity of members of the NF-κB pathway. Indeed, other NF-κB inhibitors, such as celastrol and triptolide, have been shown to reduce MDA-MB-231 cells viability (73-75). Consistent with others, showing that inhibition of NF-κB activation in MCF-7 cells results in reduced cell viability (50), our results suggest that decreased MCF-7 cells viability in response to Fx/Fxol may involve NF-κB inhibition. Indeed, treatments of MCF-7 cells with Fx/Fxol resulted in increased apoptosis and correlated with nuclear decreases of p65 and p100 NF-κB transcription factors. However, these results do not exclude that other signaling pathways may be involved. Among them, AP-1 and Akt have been shown to be silenced by Fx/Fxol in primary effusion lymphomas (57).

SOX9, being regulated by NF-κB in other cell types (51, 52), is normally undetectable in normal breast tissue. However, SOX9 expression is highly correlated to invasiveness and poor clinical outcome of breast cancer. Indeed, cytoplasmic accumulation of SOX9 has been shown to increase in invasive ductal carcinoma and metastatic breast cancer (76). SOX9 has been identified as a downstream target of different signaling pathways contributing to breast cancer aggressiveness (77–82). However, it was also suggested that SOX9 expression and nuclear accumulation was rather correlated to breast cancer cells growth inhibition in the presence of retinoic acid (43, 83). Discrepancies between studies on the role of SOX9 in breast cancer cells may be attributed to the escape of SOX9-mediated growth inhibition in certain types of breast cancers due to the fact that: the *SOX9* gene may contain certain inactivating mutations; certain critical downstream growth controlling genes may be unresponsive to SOX9; the oncogenic pathways responsible for the malignant phenotype bypass putative SOX9-regulated genes; or SOX9 may accumulate in the cytoplasmic compartment, as suggested elsewhere (43, 83). Here, we report an absence of SOX9 nuclear increase (correlated to mRNA expression) in response to Fx/Fxol treatments of breast cancer cells, suggesting that SOX9 is not involved in cell growth inhibition through accumulation in the nucleus. Moreover, treatments with higher doses of Fx or Fxol (20 μM) resulted in decreased nuclear levels of SOX9 (45). According to these results, SOX9 could be involved in MDA-MB-231 cells, proliferation and down-regulation of its phosphorylation may contribute to the inhibitory effects of Fx

and Fxol on viability of estrogen-resistant breast cancers. However, 10 μM treatments of breast cancer cells with Fx/Fxol did not result in significant changes in SOX9 phosphorylation status (data not shown).

In conclusion, weak doses of 10 μM Fxol effectively reduced cell viability and increased apoptosis of MDA-MB-231 cells where these effects are associated with functional regulation of components of the NF-κB pathway. These results suggest that dietary Fx or Fxol could be potentially helpful for the treatment of certain types of breast cancers. Nonetheless, further investigations using animal models are needed to better define the molecular mechanisms involved in Fx and Fxol actions against breast cancer. Indeed, although apoptosis is correlated to decreased nuclear accumulations of p65, p52 and RelB in MDA-MB-231 cells, more research will be required to clearly establish a regulatory action of Fxol on members of NF-κB.

Conflicts of Interest

The Authors declare that there is no conflict of interest that would prejudice their impartiality.

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