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Effect of Tamoxifen and Retinoic Acid on Bradykinin Induced Proliferation in MCF-7 Cells

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ABSTRACT

Chemopreventive approaches for the treatment of breast cancer have been validated clinically and with in vitro studies. The combined action of tamoxifen/all-trans retinoic acid was advantageous in MCF-7 cells, reducing cell proliferation, Bcl-2 and c-Myc protein levels and increasing E-Cadherin protein levels and Gap junctional Intercellular Communication. We further investigated their combined effect in the presence of bradykinin, a pro-inflammatory agent, previously reported to contribute to the proliferation of breast cancer cells. Bradykinin increased MCF-7 cell proliferation, c-Myc levels and ERK1/2 activity. The co-incubation of bradykinin-MCF-7 cells with tamoxifen/all-trans retinoic acid reduced cell proliferation, ERK1/2 activity, as well as Bcl-2, c-Myc, and bradykinin receptor-2 levels, without altering the enhanced E-cadherin levels induced by tamoxifen/all-trans retinoic acid. We showed that the anti-tumoral effect of tamoxifen/all-trans retinoic acid is beneficial in MCF-7 breast cancer cells grown in a bradykinin-pro-mitogenic environment, an effect that might be, at least in part, through the MAPK pathway and B2-bradykinin receptor inhibition. J. Cell. Biochem. 106: 473–481, 2009.

KEY WORDS: TAMOXIFEN; RETINOIC ACID; BRADYKININ; BREAST CANCER; CHEMOPREVENTION

Breast cancer is the most common malignancy in American and north-western European women. It is estimated that one in eight American women and one in 12 women in the United Kingdom will develop breast cancer in their lifetime and the incidence rates in industrialized nations are on the rise [Simstein et al., 2003].

Today, breast cancer is considered to be a result of damage to DNA produced by factors that lead to an increased rate of mutation and decreased repair. Although many epidemiological risk factors, biological co-factors and promoters have been identified, the majority of breast cancer incidences remain unexplained, and the primary cause is unknown. Several studies have correlated the development of breast cancer with epigenetic variables, thus rendering targets for early intervention before malignant cells acquire invasive properties [Jacobs, 2000]. Natural or pharmacological agents that are able to inhibit, retard or reverse carcinogenic processes have been the basis for the development of chemopreventive approaches, which began more than 30 years ago. Since then, suitable agents for several specific molecular targets have been identified. Specifically, the selective oestrogen receptor modulator, Tamoxifen (Tx) that targets preferentially breast cancers with oestrogen receptor expressing cells and, compounds of the retinoid family engaged in the modulation of genes involved in cell differentiation. The use of a combination of anti-oestrogens and retinoids arises from the possibility of improving their individual effect on breast cancer cells, due to their different and potentially complementary mechanisms of action, and because active and tolerable agents from both classes are available. We have previously reported the benefits of using the Tx and all-trans retinoic acid (RA) combined treatment on MCF-7 breast cancer cell line [Saez et al., 2003], demonstrating reduced cell proliferation, decreased Bcl-2 and c-Myc protein levels, increased Gap junction intercellular communication and E-cadherin protein levels. Furthermore, a recent report showed that the secretion of the transforming growth factor β (TGF-β) was synergistically stimulated by Tx and RA in MCF-7 cells to induce apoptosis [Wang et al., 2007].

Approximately 30% of breast cancer cells are found to over-express the c-myc oncogene and it is generally considered a bad prognosis marker [Nass and Dickson, 1997]. On the other hand, bcl-2, one of the major genes implicated in the regulation of apoptosis [Reed, 1994], is also over-expressed in many breast cancers and together with c-myc, they confer tumor chemoresistance, resulting

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in an unfavorable prognosis. Down regulation of c-myc gene expression has been reported to occur after Tx exposure in several breast and cervical cancer cell lines, with the exception of MCF-7 cells, in which c-Myc expression was not affected [Kang et al., 1996]. Nevertheless, a significant decrease in c-Myc and Bcl-2 along with up-regulation of E-cadherin was observed in MCF-7 when treated with RA and Tx in concert. Moreover, preliminary results suggest that Tx and RA treatment sensitize MCF-7 cells towards the action of chemotherapeutic drugs regularly utilized for breast cancer treatment [Saez et al., 2003].

Even though evidence for the advantageous action of Tx and RA as a combined treatment towards malignant cell lines seems to be recognized, their anti-tumoral effect under the influence of a pro-mitogenic or pro-tumorigenic agent has not been investigated.

Bradykinin (BK), a vasoactive peptide released during inflammation [Chen et al., 1988; Yu et al., 1998], has been considered a multipotential stimulant of cancer growth for several reasons: increasing blood flow, thus promoting the supply of nutrients and oxygen, reducing the barriers between blood and the tumor by the secretion of metalloproteinases [Robert and Gulick, 1989; Maeda et al., 1999], and directly inducing proliferation [Greco et al., 2005]. In addition, we have previously shown that BK stimulation of VSMC leads to the activation of the mitogen-activated protein kinase (MAPK) cascade [Velarde et al., 1999] which can be a responsible mediator of cellular proliferation [Velarde et al., 2004]. BK performs its action via two known receptors, namely, the B1 and B2 receptors [Regoli and Barabé, 1980; Vavrek and Stewart, 1985; Ma et al., 1994; el-Dahr et al., 1997; Pesquero and Bader, 1998]. In a large survey of cancers, the receptors for BK were among the most commonly found on tumor cells, being expressed either in established cancer cell lines or in fresh surgical lung cancer specimens [Stewart, 2003]. In prostate cancer, both, BK B1 and B2 receptors are linked to cell proliferation and consequently, BK antagonists have been generated for the treatment of this disease [Barki-Harrington and Daaka, 2001]. In MCF-7 cells BK can activate PKC and ERK [Greco et al., 2004, 2005, 2006], confirming the contribution of this peptide to the malignant progression of the disease.

Elevated levels of BK can be found in chronic pathologies such as diabetes and inflammation, among others. These conditions might render a favorable progression of an initiated malignancy, and would most probably complicate the disease. Indeed, cancer and chronic inflammation have been correlated since 1863 [Balkwill and Mantovani, 2001]. The possible participation of inflammation in carcinogenesis may include the induction of genomic instability and epigenetic alterations which subsequently results in abnormal gene expression that will promote enhanced cell proliferation and resistance to apoptosis among the many properties that the malignant cells acquired to ensure their growth.

In an inflammatory environment, the presence of BK may potentially induce epigenetic changes in a tumor initiated cell, thus contributing to malignancy. This condition renders the opportunity to evaluate the intervention of chemopreventive agents that aim at retarding or partially reversing the ongoing carcinogenesis.

Consequently, our aim was to investigate whether the advantageous chemopreventive action of the combined treatment with Tx and RA persists when breast cancer cells are immersed in a pro-mitogenic environment as a result of a pro-inflammatory mediator such as BK.

METHODS

CHEMICALS

All-trans-RA and Tx (Sigma) were dissolved in absolute ethanol (Merck) at a concentration of 10^{-2} M and then diluted in culture media to the required final concentration. Stock solution of bradykinin (Sigma) was prepared in phosphate buffer saline (PBS) at 10^{-3} M and diluted in culture media at a final concentration of 10^{-7} M. All stock solutions were kept at -80°C. Procedures involving RA were performed in subdued yellow light.

CELL CULTURE

Human mammary-carcinoma MCF-7 (ERβ) cell lines were cultured in RPMI 1640 media supplemented with 10% fetal calf serum (Gibco) and antibiotics in a humidified incubator (5% CO_2, 95% air) at 37°C. Cells were synchronized by serum deprivation 24 h before the addition of complete media containing RA, Tx, and/or bradykinin. For growth experiments, cells were photographed on an inverted microscope, a Nikon Eclipse TS100.

IMMUNOCYTOCHEMISTRY

Cells grown on glass cover slips were fixed and permeabilized in 70% ethanol at -20°C for 20 min. After blocking non-specific binding sites with normal goat serum (1% in PBS), cells were incubated with polyclonal anti BK-B2 receptor antibody (Transduction). Subcellular localization of the receptor was visualized after incubating the cells with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma) and mounted on Gelvatol Airvol 205 plus 100 mg/ml DABCO (Sigma) with an Olympus BX60 microscope and photographed using a T-max 400 film (Kodak).

WESTERN BLOTTING

Relative protein levels for BK-B2 receptor (Transduction) E-cadherin (Zymed), c-Myc, or Bcl-2 (Santa Cruz) were analyzed by SDS–PAGE and immunoblot analysis. Cells were scrape-collected in the presence of protease inhibitors (phenylmethylsulfonyl fluoride 3×10^{-3} M, soybean trypsin inhibitor 200 mg/ml, EDTA 2×10^{-8} M, pH 10) and phosphatase inhibitors (pyrophosphate 2×10^{-8} M, NaF 10^{-1} M, $NaVO_3$ 2×10^{-1} M), and lysed by sonication with an ultrasonic cell disrupter (Microson, Heat system). Total protein content in cell lysates was determined by the Bradford method (Bradford, Bio-Rad labs, Hercules, CA). Samples containing up to 200 mg of total protein were separated in a SDS–PAGE and electro-transferred to a nitrocellulose membrane. After blocking non-specific binding sites with 5% non-fat milk, the membranes were incubated with the specific antibody at dilutions suggested in accordance with the manufacture's protocol. The membranes were then incubated with a secondary antibody conjugated to horse raddish peroxidase. Antigen-antibody complexes were detected by chemiluminiscense. Equal sample loadings were detected by staining the membranes with Ponceau S. Immunostained protein bands were scanned at a resolution of 200 dpi and quantified.

BrdU NUCLEI INCORPORATION

Cells were grown to early-log phase at approximately 7×10^4 cells/plate in 24-well plates containing a cover glass. Cells were pulsed with 10^{-6} M bromodeoxyuridine (5-bromo-2′-deoxyuridine, Sigma Chemical) for 60 min at 37°C. After incubation, the cells were washed 3 times in PBS and incubated in denaturing DNA solution (HCl 2 M) for 15 min. Cells were washed 3 times with PBS buffer and fixed in 3 ml cold 70% ethanol for 20 min at −20°C. Cells were then incubated for an hour with a monoclonal antibody against-BrdU at a dilution 1:20. Cells were washed and incubated for 45 min at room temperature with a second antibody conjugated to FITC (1:20). Covers were mounted and visualized under a fluorescence microscope Olympus B.

In another experiment, cells were grown in 96 well plates at approximately 1.5×10^3 cells/plate. Cells were pulsed with 10^{-6} M bromodeoxyuridine (5-bromo-2′-deoxyuridine, Sigma Chemical) for 120 min at 37°C, followed by incubation in fixation/denaturation solution. Cells were then incubated for an hour with a monoclonal antibody against-BrdU conjugated to peroxidase at a dilution 1:100. Cells were washed and incubated for 20 min at room temperature with the substrate. The reaction was stopped by adding H_2SO_4 1M. Absorbance of samples was measured at 405 and 490 nm and the relative amount of nuclei in synthesis were expressed by the difference between both absorbances.

STATISTICAL ANALYSIS

Data were expressed as mean ± SEM and analyzed by ANOVA for unpaired samples with the post hoc test of Bonferroni. Values were considered significant if $P < 0.05$.

RESULTS

RATx REVERT THE MCF-7 CELL PROLIFERATION ENHANCEMENT INDUCED BY BK

The induction of cell proliferation by BK has been reported in normal and cancerous cells [Velarde et al., 2004; Greco et al., 2004]. Figure 1a shows a representative photograph of MCF-7 cultured for 48 h in the absence (control) or in the presence of BK, showing a greater number of cells in the latter condition. Indeed, cell count showed a 62 ± 13% increase ($P < 0.05$) in MCF-7 cell number when supplemented with BK for 48 h (Fig. 1b). To further confirm this result, BrdU incorporation into the nuclei was measured. As shown in Figure 1c, incubation with BK resulted in 56 ± 4% increase in nuclei that incorporated BrdU as compared with control cells ($P < 0.05$).

In accordance to our previous reported data [Saez et al., 2003] and as observed in Figure 1a,b, the incubation for 48 h in RATx reduced the cell number in 57 ± 7% as compared with control conditions. Accordingly, a 44.7 ± 0.7% decrease in BrdU incorporation compared to control MCF-7 cells ($P < 0.05$) was observed when grown in the presence of the combination of RA and Tx. The decrease in cell number under these conditions did not correlate with an increase of cell death as reported before [Saez et al., 2003].

Interestingly, the cell proliferation enhancement observed in MCF-7 grown in the presence of BK was greatly diminished when BK-MCF-7 cells were co-incubated with 10^{-6} M RATx (Fig. 1a). A

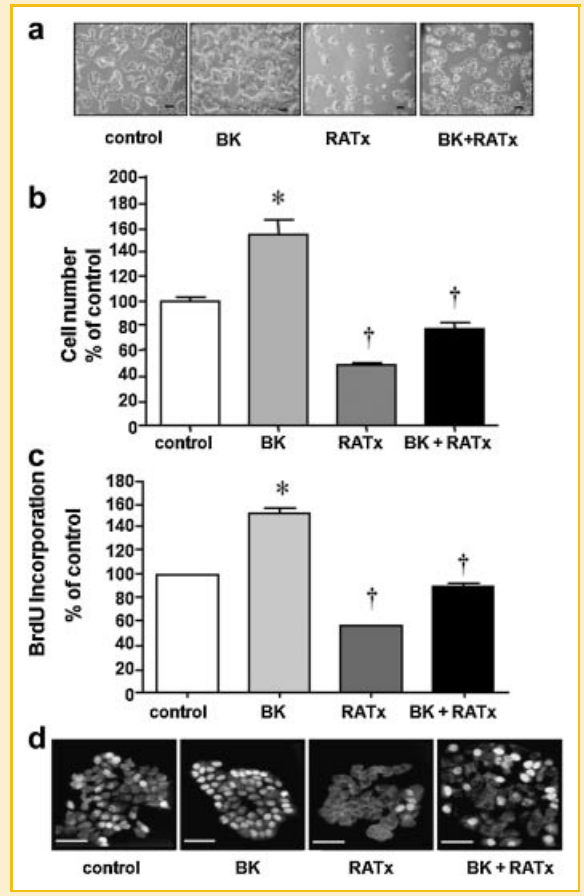

Fig. 1. RATx reverts the MCF-7 cells proliferation enhancement observed with BK. MCF-7 cells were cultured in the absence or presence of BK (100 nM), RATx (RA 1 μM + Tx 1 μM) or the combination of BK + RATx during 48 h, as described in Materials and Methods Section. a: A representative photograph of cell cultures grown under the specified conditions (n = 4). Bars: 50 μm. b: The quantification of MCF-7 cell count under the different conditions. Results are expressed as percentage of control cells. Bars represent the mean ± SEM of four independent experiments. $^*P < 0.05$ versus control; $^\dagger P < 0.05$ versus BK. c: The graph illustrates the quantification of proliferating nuclei for each culture condition, using DNA labeled with BrdU measured by an ELISA method. Bars represent the mean ± SEM of three independent experiments. $^*P < 0.05$ versus control; $^\dagger P < 0.05$ versus BK. d: The photographs are representative pictures of an immunofluorescence aimed to detect BrdU labeled nuclei of MCF-7 cells cultured under the conditions specified. Bar: 50 μm.

23 ± 3% decrease in cell number as compared with control conditions was observed in BK-MCF-7 when they were co-cultured with RATx and a 53 ± 5% decrease when compared with a culture treated only with BK (Fig. 1b). BrdU incorporation further corroborated these observations showing a 42.3 ± 0.3% ($P < 0.05$) less proliferating nuclei in BK- RATx co-cultured cells than in cells solely treated with BK.

The bottom photographs of Figure 1d are representative illustrations of the BrdU labeled nuclei on MCF-7 cells grown under the different specified conditions. As compared with control cells, it is possible to visualize the enhancement in the number of BrdU positive nuclei induced by BK and the significant reduction

due to RATx. Furthermore, it is possible to observe that the enhancement in proliferating cells due to BK is significantly reduced when co incubated with RATx, showing that the combination of chemopreventive agents are able to greatly evade the mitogenic effect of BK in MCF-7 cells.

BRADYKININ DOES NOT MODIFY Bcl-2 LEVELS IN MCF-7 CELLS

It has previously been reported that when rats are transduced with components of the kallikrein–kinin system, an increase of Bcl-2 protein levels in brain tissue is observed [Xia et al., 2004]. On the other hand, elevated Bcl-2 protein levels are generally associated with bad prognosis in cancer [Piché et al., 1998; Simstein et al., 2003] and we have previously observed that the combined treatment of RATx reduces Bcl-2 protein levels in MCF-7 cells [Saez et al., 2003]. Therefore, we examined whether the mitogenic effect of BK is associated with elevated levels of this tumoral marker as well.

Neither at 24 h nor at 48 h of culture, BK (10^{-7} M) modified the levels of Bcl-2 protein compared to control levels (103.3 ± 1.5% vs. 100% of control for 48 h). In contrast, RATx reduced approximately 30% of control Bcl-2 protein levels (76.0 ± 3.5% vs. 100% of control) after 48 h of treatment.

In addition, the co-stimulation with BK and RATx reduced Bcl-2 protein to levels comparable to the ones observed with RATx alone (70.0 ± 2.5% vs. 76.0 ± 3.5%, respectively), indicating that BK, during the time frame studied does not influence Bcl-2 pathway (Fig. 2).

BRADYKININ AND RATx INDUCED CHANGES IN c-Myc LEVELS THROUGH DIFFERENT PATHWAYS

The proto-oncogene, c-Myc is essential for cell cycling and is over-expressed in approximately one-third of breast cancers, and commonly associated to an unfavorable prognosis [Deming et al., 2000]. BK, on the other hand, increases mRNA levels of c-Myc in cardiomyocytes [Lei and Guo, 1998].

In MCF-7 cells, BK (10^{-7} M) transitorily induced an increase in c-Myc protein levels 24 h after stimulation (150.7 ± 8.3% vs. 100% of control, $P < 0.01$) (Fig. 3a), returning to control levels by 48 h of treatment (94.0 ± 5.0% vs. 100% of control) (Fig. 3b). Conversely, RATx did not affect c-Myc protein levels by 24 h of treatment (88.7 ± 3.5% vs. 100% of control, $P > 0.05$) (Fig. 3a), but significantly reduced them by 48 h of incubation (53.3 ± 2.4% vs. 100% of control, $P < 0.05$) (Fig. 3b).

When MCF-7 cells were incubated in the presence of BK plus the chemopreventive drugs, c-Myc protein levels were equivalent to the observed in cultures treated only with BK at 24 h (142.0 ± 4.5% vs. 150.7 ± 8.3% of BK) (Fig. 3a), but a significant reduction in c-Myc protein levels occurred at 48 h as compared to control or BK-stimulated levels (68.9 ± 2.9% vs. 53.3 ± 2.4% of RATx, $P > 0.05$). The down-regulation achieved with RATx in BK-MCF-7 cells at 48 h

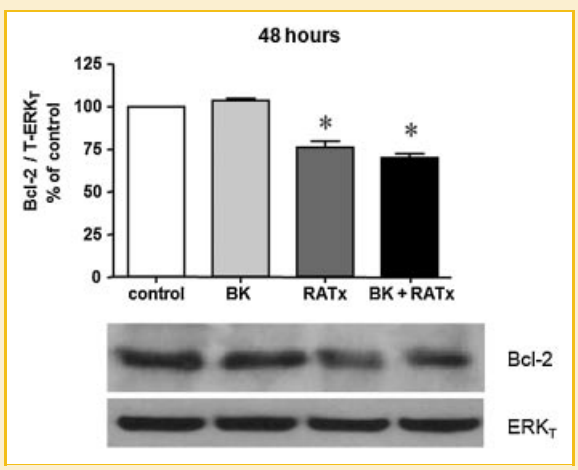

Fig. 2. BK and RATx effect on Bcl-2 protein levels in MCF-7 cells. The graph shows the quantification of Bcl-2 relative protein levels in MCF-7 cells cultured for 48 h in the presence or absence of BK 100 nM, RATx (RA 1 μM and Tx 1 μM) or the combination BK + RATx as described in Materials and Methods Section. Total ERK protein levels were used as loading control. Graph bars represent average ± SEM for four independent experiments. *$P < 0.05$ from control. ANOVA followed by the post hoc test of Bonferroni.

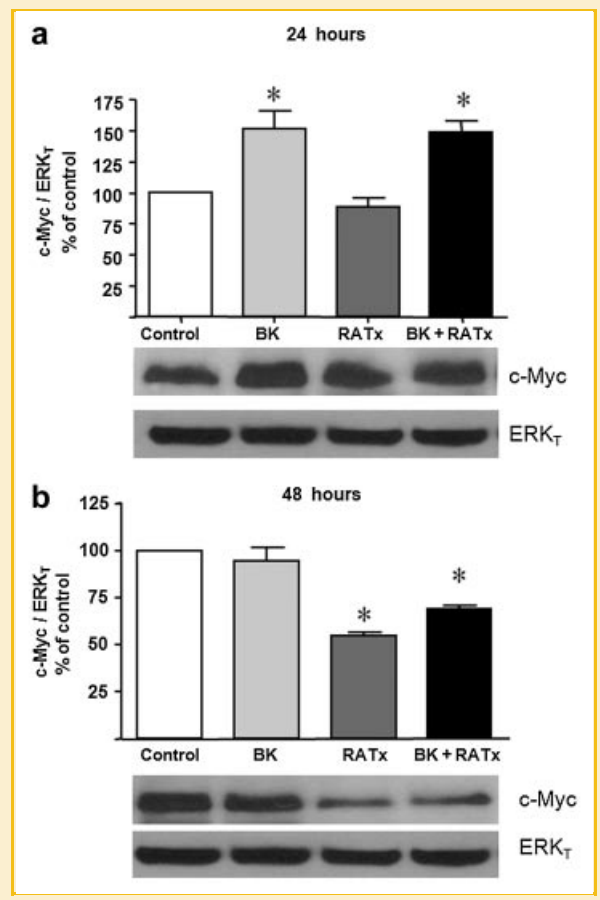

Fig. 3. Effect of BK and RATx on c-Myc protein levels in MCF-7 cells. The graphs show the quantification of c-Myc protein levels of MCF-7 cells incubated for 24 h (a) or 48 h (b) in the presence or absence of BK 100 nM, RATx (RA 1 μM and Tx 1 μM) or the combination BK + RATx. Total ERK protein levels were used as loading control. Graph bars represent the average ± SEM for four independent experiments. *$P < 0.05$ from control. ANOVA followed by the post hoc test of Bonferroni.

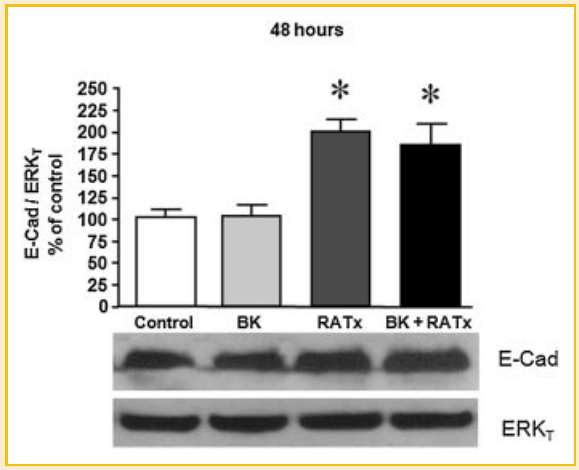

Fig. 4. BK and RATx effect on E-cadherin protein levels in MCF-7 cells. Cells were serum starved for 24 h and incubated for the next 48 h in the presence or absence of BK 100 nM, RATx (RA 1 μM and Tx 1 μM) or the combination BK + RATx. Each bar of the graph represents the average ± SEM of E-cadherin protein levels obtained from the culture conditions specified in three independent experiments. Total ERK protein levels were used as loading control. *$P < 0.05$ from control. ANOVA followed by the post hoc test of Bonferroni.

of treatment was not different to the one observed in cultures with RATx alone. These results suggested that BK and the chemopreventive agents targeted c-Myc through different pathways.

BRADYKININ DOES NOT MODIFY E-CADHERIN LEVELS IN MCF-7 CELLS

The increase in E-cadherin in epithelial cells has been correlated to a more differentiated stage and to a decrease in cell proliferation [González-Mariscal et al., 2007]. In general, cancerous cells, after treatment with RA (10^{-6} M) express higher protein levels of E-cadherin, denoting a reversal of their un-differentiated state [Saez et al., 2003].

E-cadherin protein levels were not modified by BK (10^{-7} M) in MCF-7 cells, neither at 24 h (data not shown) nor at 48 h of culture (105.2 ± 19.7% vs. 100% of control) (Fig. 4). In agreement to our previous report, RATx almost doubled E-cadherin protein levels at 48 h of treatment (198.0 ± 5.3% vs. 100% of control) and in accordance with these results, the co-culture of BK-MCF-7 cells with RATx increased E-cadherin protein levels to a similar extent to that of RATx alone (182.7 ± 22.6% vs. 198.0 ± 5.3%) (Fig. 4).

THE ACTIVATION OF THE MAPK PATHWAY INDUCED BY BK IS BLOCKED BY RATx

Cell proliferation is regulated by several pathways among which, one of the most studied is the ERK 1/2 pathway. Previous results in VSMC showed that BK can increase the activation of the ERK 1/2 pathway [Velarde et al., 1999]. In MCF-7 cells, BK induced a time dependent increase in ERK 1/2 phosphorylation that was maximal at 3 min and returned to control levels at 10 min of culture (Fig. 5a). Interestingly, RA or Tx added to MCF-7 cells separately or in combination, did not modify p-ERK levels as compared to the control cultures (Fig. 5).

The increase in p-ERK induced by BK was not significantly modified when cells were pre-incubated with RA for 30 min (200 ± 95% in RA + BK vs. 265 ± 5% in BK) (Fig. 5b). Similarly, the pre-incubated with Tx for 30 min did not alter the effects of BK on ERK phosphorylation (159 ± 53% in Tx + BK vs. 265 ± 5% in BK) (Fig. 5c).

Remarkably, when cells were incubated with BK in the presence of RATx, the p-ERK enhancement induced by BK was completely inhibited, returning to values found in control cultures (105 ± 9% in RATx + BK vs. 265 ± 5% in BK) (Fig. 5d). These results showed that the combination of RATx is greatly advantageous towards p-ERK levels reduction in MCF-7 cells in response to BK, effect that is non-proportional to the added action of Tx or RA when used independently.

RATx DECREASE THE B2 BRADYKININ RECEPTOR PROTEIN LEVELS

B2 receptors for BK are commonly involved in cell signaling related to cell proliferation and ERK-pathway stimulation [Velarde et al., 1999]. However, it is not known if they are modulated by chemopreventive agents such as RATx. Therefore, we determined BK-B2 receptor protein levels and visualized its sub-cellular localization in MCF-7 cells under the different culture conditions described.

MCF-7 cells incubated for 48 h with BK alone did not alter total protein levels for BK-B2 receptors (96 ± 7% vs. 100% of control, $P > 0.05$) as compared with the control conditions). In contrast, cells treated with RATx for 48 h significantly decreased the BK-B2 receptor protein levels (70.3 ± 0.6% vs. 100% of control, $P < 0.05$) (Fig. 6A).

As it could be anticipated, BK-B2 receptor protein levels were significantly reduced in MCF-cells co-incubated with BK and the chemopreventive agents reaching levels similar to that of cells incubated with only RATx (69 ± 3% in BK + RATx vs. 70.3 ± 0.6% in RATx, $P > 0.05$).

MCF-7 cells were immunostained to visualize the BK-B2 receptor localization within the cell (Fig. 6B), showing the receptor, under control culture conditions, abundant in the cell cytoplasm (white arrows) and at the cell membrane apposition of many cells (dark arrows) as well.

In spite of having a similar total protein level to that found in control cultures, cells stimulated with BK showed a re-distribution of the BK-B2 receptor sub-cellular localization. Some of the receptor was still found at the cell membrane apposition (dark arrows) instead some cells showed abundant intracellular immunoreactivity, clustering in the peri-nuclear area (white arrows) (Fig. 6B).

After 48 h of RATx treatment, in agreement with the total protein levels, the immunostaining for B2 receptor was clearly diminished but still localized at the apposition membrane of a few cells (dark arrows) and peri-nuclear in the cytoplasm as well (white arrows). Consistent with the total protein levels, at 48 h of MCF-7 cells co-incubated with BK and the chemopreventive agents, the relative immunoreactive intensity for the B2 receptor was equivalent to the cells treated only with RATx and with a similar sub-cellular distribution (Fig. 6B). These results suggest that the combination of RA and Tx is effective in reducing the receptor availability in MCF-7

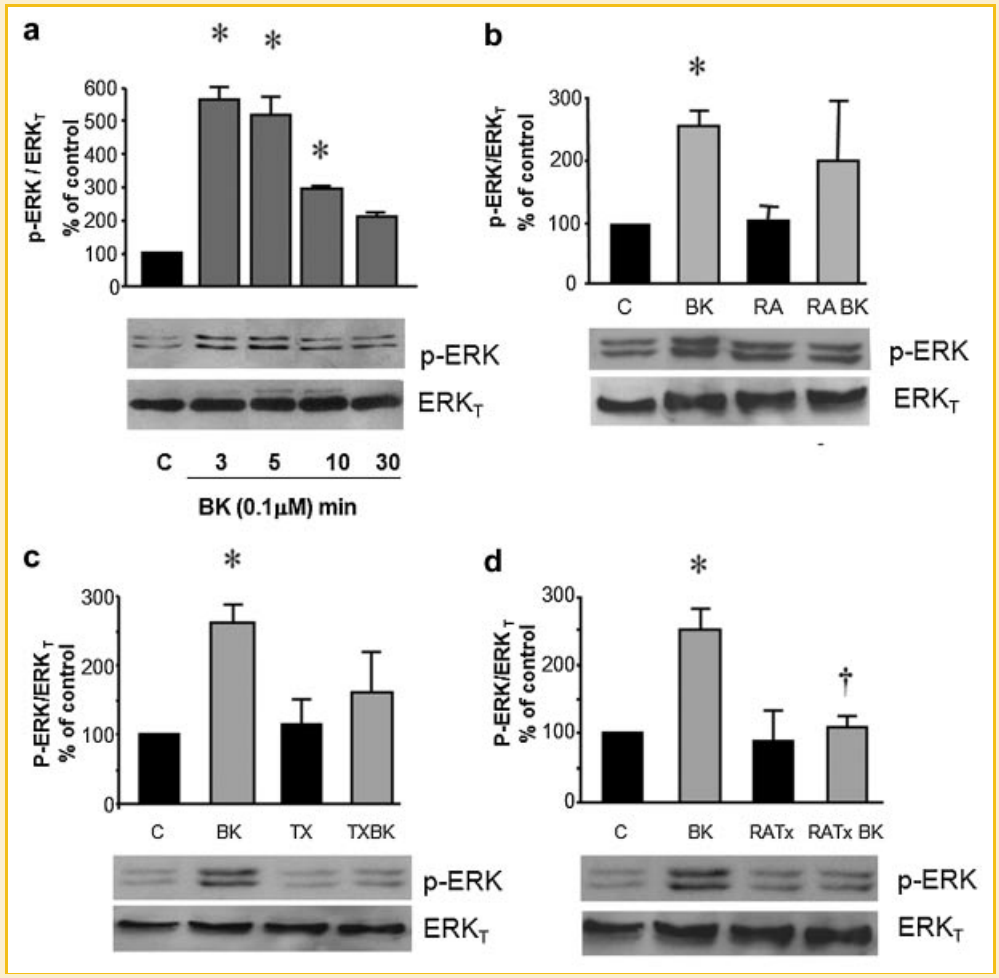

Fig. 5. The activation of the MAPK pathway induced by BK is abolished by RATx. a: The quantification of p-ERK protein levels in MCF-7 cells stimulated with BK 100 nM for different periods. b–d: Each column of the graphs represents the average of the quantification of p-ERK on MCF-7 cells pre-incubated for 30 min with (b) RA (1 μM), (c) Tx (1 μM), or (d) the combination RATx, and further stimulated with BK 100 nM for 5 min. Results are expressed as the ratio between the optical densities for pERK/ERK and illustrated as percentage of p-ERK activity in MCF-7 control cells. Bars represent mean ± SEM for three independent experiments; $^*P < 0.05$ versus control. $^\dagger P < 0.05$ versus BK. ANOVA followed by the post hoc test of Bonferroni.

cells, possibly denoting one step in the mechanism of action for the combination of these chemopreventive agents.

DISCUSSION

The inhibition or reversal of the carcinogenesis process by pharmacological agents of low cytotoxicity, termed chemoprevention, has increasingly become the focus of cancer prevention efforts.

Using proteomic analysis, Wang et al. [2007] demonstrated the synergistic effect of the combination of RA and Tx in breast cancer cells providing new insights into proteins that may mediate RATx mechanism in this cell type. We have previously observed the benefits of using the combination of RA and Tx in reversing the tumorigenic phenotype of MCF-7 breast cancer cells. This involves an important decrease in cell proliferation and a significant decline in c-Myc and Bcl-2 protein levels, two malignant markers shown to correlate with bad prognosis when over expressed [Reed, 1994; Nass and Dickson, 1997]. In addition, we have found that the combined action of these chemopreventive agents induced an increase of intercellular E-cadherin protein levels, considered to be an epithelial marker for cell differentiation, and an improvement of Gap Junction Intercellular communication, shown to be related with a decrease in tumor cell proliferation and differentiation [Saez et al., 2003].

Recently, the search for mechanisms that may explain the existing relationship between inflammation and cancer has received great interest. Genetic or epigenetic changes during malignant growth may be favored by the existence of inflammatory mediators present in the tumor cell environment. Bradykinin, a pro-inflammatory nonapeptide is shown to be elevated not only during inflammatory states but also in other pathological conditions such as sepsis, diabetes [Tschöpe et al., 1999], and certain malignancies [Costa-Neto et al., 2008], such as lung and prostate cancer.

The ability of RA and Tx to act synergistically when combined to reverse MCF-7 breast cancer cells malignancy represents an important means to challenge their action in a BK pro-inflammatory environment.

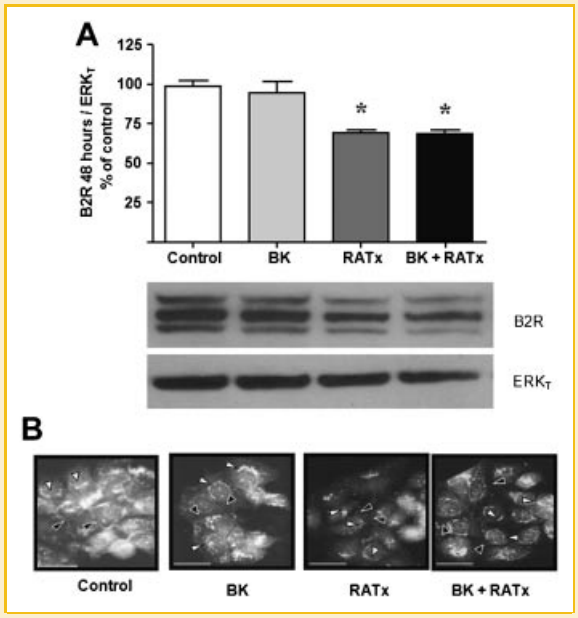

Fig. 6. RATx reduces B2 kinin receptor protein levels in MCF-7 cells. A: The graph shows the quantification of BK-B2 total protein levels in MCF-7 cells grown under control conditions, stimulated with BK (100 nM) or RATx (1 μM each) or pre-incubated for 30 min with RATx (RA 1 μM and Tx 1 μM) and further stimulated with BK 100 nM for 48 h. Results are expressed as the ratio between the optical densities for BK-B2/ERK-T. Bars represent mean ± SEM for three independent experiments; $^{*}P < 0.05$ versus control. ANOVA followed by the post hoc test of Bonferroni. B: Illustrates representative photographs of the immune-BK-B2 localization in MCF-7 cells grown under the specified conditions. Black arrowheads indicate the BK-B2 receptor at the cell membrane apposition and white arrowheads show the cytoplasmic and perinuclear location of the receptor in MCF-7 cells. Bar: 20 μm.

In this study we showed that in MCF-7 breast cancer cells, RATx was able to reduce the cell proliferation increased by BK, and was also capable of retaining its effect by lowering the levels of tumoral markers even in the presence of BK action. Most importantly, RATx was able to inhibit p-ERK enhancement due to BK in a larger magnitude than when the agents were tested individually. In addition, we showed for the first time that RATx down-modulated BK-B2 receptor in MCF-7 cells possibly indicating a new mechanism of action.

The effect of BK on cell proliferation has been addressed in several malignant and normal cells. In EFM-192A mammary carcinoma cell line, BK induced a significant increase in cell proliferation [Drube and Liebmann, 2000] and, in normal breast epithelial cell lines BK doubled the number of cells, an effect mediated through BK-B2 receptors [Greco et al., 2004]. Indeed, BK antagonists have been proposed as anti-cancer agents showing important tumor cell growth inhibition in animal models for several cancers [Stewart, 2003]. In some of the cell models studied, BK exerted its proliferative action through MAPK signaling pathway. Greco et al. [2004] have demonstrated that BK induced MCF-7 cell proliferation through a pathway that involves the participation of PKC, Akt, and ERK1/2. To our knowledge, our study is the first in which the chemopreventive agents, RATx are challenged towards breast cancer cells in a pro-mitogenic environment, such as BK. Moreover, the effect of these compounds on MAPK pathway has been mostly studied for RA or Tx separately. In VSMC for example, Tx inhibits ERK1/2 activation induced by ET1 [Park et al., 2003] and RA seems to interfere with ERK signaling by blocking the interaction of this active enzyme with proteins that associate with the DNA [Benkoussa et al., 2002].

In agreement with the literature, our results showed that MCF-7 cells responded to BK, resulting in an increase in cell proliferation through the induction of ERK phosphorylation. Unexpectedly, neither RA nor Tx independently or together, altered the levels of p-ERK seen in control MCF-7 cultures during the time frame analyzed. Similarly, pre-treatment with RA or Tx separately did not significantly prevent the increase of p-ERK induced by BK. However, pre-treatment with RATx together, greatly prevented ERK phosphorylation enhancement induced by BK. These results strongly suggest that the ability of RATx to reduce the increase of MCF-7 cell proliferation, induced by the pro-mitogenic inflammatory peptide BK, is targeting a common signaling pathway. Importantly, these results also confirmed the benefits of the use of RATx in combination instead separately, thus unveiling a new mechanism of action.

The BK-B2 receptor belongs to the G protein-coupled receptors superfamily (GPCRs) and it is known to mediate diverse pathophysiological effects upon interaction with its major agonist, BK [McEachern et al., 1991]. The deduced molecular weight for the receptor is 42 kDa but several post-translational modifications, such as phosphorylations, *N*-glycosilations or palmitoylations that are related to modifications of its trafficking, ligand binding, half-life time and functionality can be revealed by different electrophoretical mobility [Blaukat et al., 1996; Pizard et al., 2001; Michineau et al., 2004]. MCF-7 cells showed three distinctive immunoreactive proteins for the receptor, implying specific post-translational modifications. Since the kind of protein modifications were not investigated in this study, we quantified the three immunoreactive bands as total BK-B2 protein levels to show that even though BK induced a sub-cellular re-distribution of the receptor, its total protein levels did not significantly change. However, it is possible to speculate that the sub-cellular BK-B2 changes induced by BK are related to a slight protein re-distribution among the three immunoreactive bands observed in the Western blot. It has been described that the BK-B2 receptor is rapidly desensitized upon agonist binding, as a mechanism to protect the cell from an excessive signaling [Bachvarov et al., 2001]. Thus, it is possibly to speculate that in MCF-7 cells, BK induces a post-translational modification of the BK-B2 receptor aimed at reducing receptor availability in the cell membrane that does not involve changes of the total receptor protein level, but possibly related to a protein re-distribution within the specific posttranslational modifications.

The addition of RATx induced a decrease in BK-B2 total protein levels, clearly observed as a reduction of the immunoreactive staining in MCF-7 cells. As in the BK-induced B2 receptor changes, in addition to a total protein reduction, it is possible to observe differences of the receptor protein levels within the three immunoreactive bands. Therefore, it can be suggested that part of the RATx mechanism on BK-B2 may be related to altering post-

translational modifications of the BK-B2 receptor. Importantly, the RATx effect on MCF-7 breast cancer cells prevailed in the presence of BK, stressing the advantage of using this combination of chemopreventive agents in this cell model. At this time, we have not elucidated whether RATx action causing BK-B2 receptor reduction is due to a diminished expression or to an accelerated degradation of the receptor.

Despite the recognition of BK as an inducer of cell proliferation and p-ERK enhancement in several cell lines, its relationship with tumoral markers such as c-Myc or Bcl-2 has been less studied. The oncogene, c-Myc, commonly over-expressed in cancer, is crucial for cell cycling and when found along with elevated Bcl-2 protein levels, it results in bad prognosis and confers chemoresistance [Fanidi et al., 1992]. Treatment of MCF-7 cells with RATx produced a significant decrease of both, c-Myc and Bcl-2 protein levels which is related to the cell proliferation inhibition observed [Saez et al., 2003] but not to a significant increase of cell apoptosis. As it may be expected, the pro-mitogenic action of BK came along with an increase of c-Myc levels at 24 h of culture that was not altered by RATx. However, at 48 h of culture, MCF-7 control levels of c-Myc remained unchanged with BK but a significant down-regulation was observed with the co-treatment with RATx, which was equivalent to that obtained with RATx alone. These results suggest that BK increases c-Myc levels in MCF-7 cells possibly as part of the mechanism for its pro-mitogenic action. On the other hand, BK did not modify Bcl-2 protein levels in the time frame studied, nor induced alteration of the cell differentiating marker, E-cadherin. However, the beneficial action of RATx in down regulating Bcl-2 and c-Myc protein levels in MCF-7 cells and enhancing the epithelial marker E-cadherin remained despite the presence of BK.

Overall, our results showed that the enhancement of MCF-7 cell proliferation induced by BK was mediated, at least in part, through an increase of ERK phosphorylation and c-Myc protein levels and that the action of the chemopreventive agents, RATx, hampered the pro-mitogenic effect of BK, impeding p-ERK enhancement, lowering the availability of BK-B2 receptor at the cell membrane, diminishing c-Myc and Bcl-2 protein levels and maintaining the enhancement of E-cadherin protein levels. Our results also highlight the beneficial action of the combination of RATx over their separated use in MCF-7 breast cancer cells, even when immersed in a pro-inflammatory pro-mitogenic environment provided by BK.

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