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Potential interference of aluminum chlorohydrate with estrogen receptor signaling in breast cancer cells

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Abstract

Aluminum salts are widely used as the active antiperspirant in underarm cosmetic. Experimental observations indicate that its long term application may correlate with breast cancer development and progression. This action is proposed to be attributed, among others, to aluminum possible estrogen-like activities. In this study we showed that aluminum, in the form of aluminum chlorohydrate (ACH), caused increase in estrogen receptor alpha (ERα) protein levels, in ERα-positive MCF-7 cells. This effect was accompanied by moderate activation of Estrogen Response Elements (ERE)-driven reporter gene expression and 20%−50% increase in certain estrogen responsive, ERE-independent genes expression. Genes affected were ERα, p53, cyclin D1, and c-fos, crucial regulators of breast cancer development and progression. ACH-induced genes expression was eliminated in the presence of the estrogen antagonist: ICI 182780, in MCF-7 cells, whereas it was not observed in ERα-negative MDA-MB-231 breast cancer cells, indicating aluminum interference with estrogen signaling. Moreover, ACH caused increase in the perinuclear localization of estrogen receptor alpha in MCF-7 breast cancer cells and increase in the mitochondrial Bcl-2 protein, possibly affecting receptors-mediated mitochondrial actions and mitochondrial-dependent apoptosis. ACH-induced perinuclear localization of estrogen receptor beta was also observed in MDA-MB-231. Our findings indicate that aluminum actions on estrogen receptors protein level and subcellular localization possibly affect receptors-mediated actions and thus, aluminum interference with estrogen signaling.

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Conflicts of Interest

The authors declare no conflicts of interest.

Introduction

Aluminum is the most abundant metal and the third most widely used. Humans are exposed to aluminum compounds through diet, antacids, vaccines, and various house hold products (Corain *et al.* 1990, Darbre *et al.* 2010). Moreover, application of Al-based antiperspirant to the underarm provides a high and continuous exposure to aluminum of an area located to the near proximity to the human breast. Aluminum salts in anti-perspirants are soluble at very low pH. Moreover, aluminum ion absorption and bioavailability varies based on many factors such as the chemical form of aluminum and the integrity of the skin. (Bakir *et al.* 2015, Darbre *et al.* 2013, 2016). According to the opinion of the European Union Scientific Committee on Consumer Safety (SCCS/1525/2014), due to the lack of adequate data on dermal penetration of aluminum, to estimate the internal dose of aluminum following cosmetic uses, risk assessment cannot be performed. However, accumulating evidence indicate that the aluminumion (Al^{+3}) is toxic. Abnormally high levels of aluminum are related to pathological conditions such as dialysis dementa, iron-adequate microcytic anemia, osteomalacia, neurodegerative diseases, and breast cancer (Darbre *et al.* 2016, Dórea *et al.* 2015, Gherardi *et al.* 2016, Maya *et al.* 2016, Mold *et al.* 2016).

As regards aluminum compounds implication to breast cancer development and progression, measurements of aluminum in human breast tissue (Exley *et al.* 2007) and in nipple aspirate fluid (Mannello *et al.* 2001) have shown higher levels of aluminum in tissue from women affected by breast cancer compared to the healthy ones (Darbre *et al.* 2009). Moreover, relative high levels of aluminum have been measured in the fluid collected from breast cysts observed in gross cystic breast disease (Mannello *et al.* 2009), a benign breast disorder believed to facilitate the appearance of breast cancer.

In addition, experimental data show that aluminum chloride promotes anchorage independent growth in human mammary epithelial cells (Sappino *et al.* 2012). Moreover, long-term exposure to aluminum salts of oestrogen-responsive, MCF-7, as well as oestrogen unresponsive, MDA-MB-231, human breast cancer cells caused increase in their migratory properties (Bakir *et al.* 2015, Darbre *et al.* 2013, 2016). The metastatic properties of aluminum are attributed to aluminum-induced alterations to metalloproteinases levels and secretion (Bakir *et al.* 2015).

It is also proposed that aluminum act as a metalloestrogen. Dabre and colleagues (Darbre *et al.* 2006, 2010) have shown that long exposure of MCF-7 cells to aluminum salts, such as aluminum chloride, ($AlCl_3$) and aluminum chlorohydrate, [($Al_2Cl(OH)_5$, ACH) at concentrations of 10^{-4} M, caused approximately two fold activation of estrogen response elements (HRE) driven reporter gene expression. Taking into account the crucial role of estrogens in breast physiology (Jia *et al.* 2015, Pasqualini *et al.* 2004), an involvement of aluminum in genomic and/or non-genomic estrogens actions via their cognate receptors, estrogen receptors (ERs) (Jia *et al.* 2015), may have great consequences in breast tissue pathophysiology.

The pathogenic mechanisms of aluminum undesired harmful effects are still under investigation. In this study the mechanisms of aluminum actions as a metalloestrogen and its

possible implication in estrogen receptor related breast cancer development were further investigated.

Materials and Methods

Chemicals

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), MitoTracker Red CMXRos (CMX), and lipofectamin 2000 were obtained from Invitrogen. Molecular weight protein markers were from Fermentas, complete protease inhibitors cocktail were purchased from Roche. All other chemicals in cluding estradiol (E2) and ICI 182780 (ICI), and Hoechst 33342 were purchased from Sigma-Aldrich.

Antibodies

The ERβ-H150, ERα-G20 affinity purified polyclonal antibodies, and the α-tubulin monoclonal antibodies were provided by Santa Cruz Biotechnology. Monoclonal mouse antibodies against human β-actin (Sigma Aldrich), ERβ (Serotec), p53 (DAKO), and c-myc, Bcl-2 (Cell Signalling) were also applied.

Cell Culture

MCF-7 and MDA-MB-231 breast cancer cells were maintained in DMEM, supplemented with 10 % FBS, 2 mM glutamine, and penicillin/streptomycin. Growth medium for MCF-7 cells was also supplemented with 1 μg/ml insulin and 10^{-10} E2. Cells were grown at 37ºC in a humidified atmosphere with 5% CO_2. 48–72 hrs before treatment, cells were cultured in phenol red free-DMEM medium supplemented with 10% charcoal inactivated FBS, 1 μg/ml insulin, 2 mM glutamine, and penicillin/streptomycin.

Immunofluorescence

Cells grown on coverslips, in DMEM without phenol red supplemented with 10 % charcoal inactivated serum, were incubated for 2 hr at 37 °C with 200 nM CMX, in the presence or absence of 10^{-9} M E2, 10^{-4} M ACH, alone or in combination as indicated, washed with phosphate buffer saline (PBS), fixed for 10 min at −20°C in methanol, transferred to acetone (−20°C) for 2 min, and briefly air-dried. Control cells were treated with vehicles (EtOH and ddH_2O in the same dilution used for E2 and ACH). After three washings in PBST (0.1% Tween 20 in PBS), (5 min each), immunofluoresence was proceeded using primary ERs antibodies (final dilution of 1:50), appropriate secondary antibodies conjugated with Alexa fluor 488, provided by Invitrogen, diluted 1/500 in PBST, and 1 μg/ml Hoechst 33342 (Sigma-Aldrich). Specimens were mounted in polyvinyl alcohol-based anti-fading medium (Psarra *et al.* 2011). Cell specimens were observed with a Leica 2000 DM microscope. Images were obtained with the optiMOS (Qimaging) camera. Quantification of the results was carried out by applying Image J analysis as previously described (McCloy *et al.* 2014). Briefly, an outline of area of interest was drawn; mean fluorescence was measured along with several adjacent background readings. The total corrected fluorescence of area of interest (TCF) = integrated density – (selected area × mean fluorescence of background readings), was calculated. This TCF was then equalized against the mean TCF of vehicle-treated cells. 60 cells were analyzed in each condition. Results presented as fold increase

over the fluorescence density of control cells. Statistical analysis (2-sided *t*-tests) was performed.

Estrogen receptor transcriptional activity

MCF-7 cells growing on 24-well plates, in hormone depleted medium, were co-transfected with an ERE promoter-driven luciferase construct (ER-Luc reporter gene construct) and a β-galactosidase reporter construct using Lipofectamin 2000 according to manufactures instructions. Cells were treated with 10^{-9} M E2 and/or 10^{-4} M ACH for 6 hrs. Subsequently, cells were harvested, lysed in report lysis buffer (Promega), following the manufacturer's protocol, and assayed for the expressed luciferase and β-galactosidase activities (Psarra *et al.* 2009). The light emission was measured using a chemiluminometer (LB 9508, Berthold) and adjusted to the β- galactosidase activity of the sample. Transfection efficiency was expressed as relative luciferase units (RLU).

Crude mitochondrial isolation

Crude mitochondrial fractions were isolated from MCF-7 cells by differential centrifugation (Psarra *et al.* 2005). Approximately 8×10^6 cells were allowed to grow for 48 h in phenol red free DMEM medium supplemented with 10% charcoal inactivated fetal bovine serum and 1 μg/ml insulin, in 15 cm dishes. Cells were incubated for additional 72 hrs with 10^{-9} M E2 and/or 10^{-4} M ACH, washed twice with ice-cold PBS, and harvested in 5 volumes of isotonic buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose) in which protease inhibitors were added (1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and appropriate amount of protease inhibitors cocktail (Roche), according to manufactures instructions). Cells were homogenized at 4°C, with 20 strokes, using a glass Potter-Elvejhem homogenizer, with a Teflon pestle. The homogenate (Total Extract, TE) was centrifuged for 5 min, at 1000 × g. The supernatant was centrifuged at 12,000 × g, for 20 min, to give the crude mitochondrial pellet. The crude mitochondrial pellet was washed twice with buffer B (0.21 M mannitol, 0.07 M sucrose, 5 mM Tris, 2.5 mM EDTA, 2.5 mM EGTA, pH 7.4), and centrifuged at 12,000 × g, for 10 min. The mitochondrial pellets were subsequently lysed in lysis buffer (20 mM Tris pH:7.5, 250 mM NaCl, 0.5 % Triton, 3 mM EDTA) supplemented with cocktail pro-tease inhibitors and protein concentration was determined with the application of Bradford assay (Bradford *et al.* 1976).

Electrophoresis and Western Blotting

Cells grown on 6 well plate, for 48 h in phenol red free medium supplemented with 10 % charcoal inactivated fetal bovine serum, were incubated for additional 72 hrs with 10^{-9} M E2, 10^{-4} M ACH, and 10^{-7} M ICI, a synthetic ER antagonist (Osborne *et al.* 2004), alone or in combination as indicated. Cells were washed in PBSX1, lysed in buffer A (20 mM Tris pH:7.5, 250 mM NaCl, 0.5 % Triton, 3 mM EDTA) supplemented with cocktail protease inhibitors. After Bradford protein determination (Bradford *et al.* 1976), cell or sub-cellular extracts were electrophoresed in discontinuous SDS-PAGE and Western blotted with specific antibodies against ERα, ERβ, β-actin, α tubulin, p53, c myc, Bcl-2 as previously described (Psarra *et al.* 2011). β-actin or α tubulin expression levels were evaluated for the normalization of ERs, p53, c-Myc, Bcl-2, expression levels. SDH was used for the

normalization of the mitochondrial protein levels. Enhanced chemiluminescence was used for the detection of the protein bands.

Real time PCR

Cells grown on 6 well plate, for 48 h in phenol red free medium supplemented with 10% charcoal inactivated fetal bovine serum were further incubated for 2 or 6 hrs in hormone free medium with 10^{-9} M E2 and/or 10^{-4} M ACH and with 10^{-9} M E2 and/or 10^{-4} M ACH in combination with 10^{-7} M ICI. Subsequently, cells were washed with phosphate buffer saline and total RNA was extracted using Trizol followed by DNase treatment (Promega) and reverse transcription into cDNA, using random primers and superscript II reverse transcriptase (Invitrogen). Expressed levels of mRNA were quantified using real-time RCR which was performed after mixing the cDNA with SYBR GreenER qPCR super mix Universal (Invitrogen) and appropriate primers. Products were quantitated with the Mx30005P Real-Time System (Stratagene). Conditions for PCR were: 52 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 55 °C for 20 s, followed by 72 °C for 20 s according to manufacturer suggestions. Primers for ERα p53, c-fos, cyclin D1, c-Myc and β-actin are shown in Table 1.

Statistical analysis

All results are expressed as mean ± SD (n=3–6). Data were analysed by independent t-test or by analysis of variance followed by Tukey's post-hoc test using SPSS software. Differences were considered significant at a two tailed P value < 0.05.

Results

Effect of ACH on estrogen receptor alpha transcriptional activation

In this study, applying luciferase reporter gene assay, we examined whether estrogen receptor transcriptional activation is affected by relative short exposure (6 hrs) of MCF-7 cells to various concentrations of ACH (10^{-6} M, 10^{-5} M, 10^{-4} M). As it is shown in Figure 1, increasing concentrations of ACH, caused only moderate ER transcriptional activation, in ERα-positive MCF-7 cells.

Effect of ACH on the subcellular localization of ERα and ERβ in breast cancer cells

Assessment of the effect of ACH and E2 on the sub-cellular translocation of ERα and ERβ in MCF-7 cells and the subcellular translocation of ERβ in MDA-MB-231 breast cancer cells, cultured in steroid free me dium, was achieved by applying immunofluoresence analysis, using commercially provided affinity purified antibodies against ERα and ERβ. As shown in Figure 2C, 2E, 10^{-4} M ACH similarly to 10^{-9} M E2 caused 2.5 to 3.5 fold increase in the perinuclear localization of ERβ in MDA-MB-231 cells. In ERα-positive MCF-7 cells, the nuclear localization of ERα was not significantly affected either by E2 or ACH (Figure 2A, 2D), whereas 1.5 fold E2-induced increase in the perinu-clear localization of ERβ was also observed (Figure 2B, 2E). ACH did not significantly altered ERα peri-nuclear localization in MCF-7 cells (Figure 2A, 2D).

Effect of ACH on ERα protein levels

Based on previous observations showing transcriptional regulation of ERα by 10^{-4}M ACH in MCF-7 cells (Darbre *et al.* 2005) and taking into account our results showing moderate ER transcriptional activation, upon exposure of MCF-7 cells to 10^{-5}M or 10^{-4}M ACH, for a relative short period of time, ACH effect on ERα protein levels was examined by applying Western blot analysis of protein extracts from MCF-7 cells, treated with 10^{-9} M E2, 10^{-4} M ACH, or 10^{-7} ICI alone or in combination with E2 or ACH, for 4 days. As it is shown in Figure 3A,B, ACH caused increase in ERα protein levels, whereas E2 caused moderate reduction in ERα protein levels. In the presence of ICI, alone or in combination with either E2 or ACH, ERα protein levels were decreased.

Effect of ACH on ERα and estrogen responsive genes expression

In order to explore whether the observed increase in ERα protein levels and the moderate increase in ERα transcriptional activation, upon relative short exposure of MCF-7 cells to ACH, is accompanied with alterations in ERα and ERE-independent estrogen responsive genes expression, mRNA levels of ERα, and ERα target genes such as c-myc, c- fos, cyclin D1 and p53 (Björnström *et al.* 2005, Bondesson *et al.* 2015) were examined upon 2h or 6h incubation of MCF-7 cells with E2 (10^{-9} M), ACH (10^{-4} M), or ICI (10^{-7} M) alone or in combination of them as indicated (Figure 4). As shown in Figure 4A, upon 2h incubation of MCF-7 cells, E2 caused approximately 2.5 and 3.5 fold, increase in the mRNA levels of cyclin D1 and, c-fos respectively, compared to control, untreated cells. Similarly, ACH caused approximately 1.5 fold increase in the mRNA levels of c-fos and cyclin D1 compared to control cells. Interestingly, these effects were diminished in cells incubated either with E2 or ACH in combination with ICI, the well-known ERα antagonist (Osborne *et al.* 2004). Moreover, in the presence of E2 in combination with ACH an increase in the mRNA levels of c-fos, ERα and p53, compared to control and E2- or ACH- treated cells, was observed, upon two (Figure 4A) or six hours treatment of the cells (Figure 4B). Evaluation of the effect of ACH and E2 on ERα, p53 and cyclin D1 gene expression, in ERα-negative MDA-MB-231 cells showed no ACH effect on the respective mRNA synthesis (Figure 4C), indicating that the effect of ACH on ERα, p53, and cyclin D1 gene expression is possible, at least in part, ERα-mediated. As regards assessments of c-myc gene expression in MCF-7 cells, we found that E2, upon 2h and 6h incubation, caused 14.6 ± 2.2 and 2.5 ± 0.1 fold increase, respectively, in c-myc gene expression compared to the respective control cells, whereas ACH did not alter c-myc expression (data not shown).

Evaluation of the ACH effect on protein levels of the ACH-affected estrogen targets showed that E2 caused increase in p53 and c-myc protein synthesis compared to control cells, whereas in the presence of ICI, the E2- induced increase in p53 and c-myc protein level was eliminated, verifying the involvement of estrogen receptor in this process (Figure 5A). ACH caused moderate increase in p53 protein synthesis, whereas it had no effect on c-myc protein synthesis, compared to control cells, which is in accordance with the ACH effect on c-myc mRNA levels. The involvement of ERα in ACH-induced increase in p53 protein level was also verified in ERα negative MDA-MB-231 cells, since no remarkable ACH-induced increase in p53 protein level was observed (Figure 5B). In addition no significant ACH-induced effect on ERβ protein level was observed in MCF-7 and MDA-MB-231 breast

cancer cells (Figure 5). Moreover, an antagonistic effect of ACH on E2-induced ERβ protein synthesis was observed in MCF-7 cells treated with ACH in combination with E2 (Figure 5A).

Effect of ACH on mitochondrial related actions

Taking into account the known perinuclear localization of mitochondria in many type of cells (Okatsu *et al.* 2010) and the ACH-induced increase in the perinuclear localization of estrogen receptors, we attempted to evaluate the effect of ACH on mitochondrial localization of certain estrogen responsive regulatory molecules. Thus, evaluation of the ACH-induced mitochondrial localization of ERα, p53 and Bcl2 was performed in total as well as in mitochondrial extracts from MCF-7 cells, treated or not with ACH or E2 (Figure 6). Tubulin was used for the assessment of the mitochondrial purity and the normalization of protein levels from total extracts. Protein levels of succinate dehydrogenase (SDH), a tricarboxylic acids cycle enzyme and a protein component of the mitochondrial inner membrane was analyzed for the normalization of mitochondrial protein levels. As it is shown in Figure 6, the protein levels of Bcl2 were increased by ACH in mitochondrial as well as in total cell extracts. In addition the ACH induced mitochondrial localization of ERα, observed by immunofluoresence and western blot analysis, was also confirmed. The presence of SDH and the absence of α-tubulin from mitochondrial extracts verified the mitochondrial enrichment and the absence of cytosolic contamination from mitochondrial fractions.

Discussion

Estrogens are steroid hormones that regulate, cell growth, differentiation, metabolism and function of many target tissues in the human body. Estrogens mediate their actions through estrogen receptor alpha and beta, which belong to the large superfamily of nuclear receptors and regulate the expression of a large number of genes via genomic and non-genomic mechanisms of actions (Björnström *et al.* 2005, Bondesson *et al.* 2015). Genomic actions involve direct or indirect DNA binding. The classical pathway includes direct binding of estrogen receptors to estrogen response elements, upon ligand activation, and transcriptional regulation of genes carrying ERE at their corresponding promoters, whereas the ERE-independent genomic actions includes protein-protein interactions with other transcription factors and transcriptional regulation of their target genes. Estrogens non-genomic, rapid effect, are proposed to be exerted through ligand dependent activation of membrane receptors or ligand-independent pathways involving cytosolic ERs phosphorylation and activation (Heldring *et al.* 2007).

Estrogens play crucial role in mammary gland morphogenesis and breast cancer progression (Jia *et al.* 2015). It is widely accepted that ERα promotes tumori-genesis and cancer progression in breast tissues. On the contrary, ERβ is proposed to inhibit breast cancer cell proliferation via repression of the activation of MAPK and PI3K signaling pathways, inhibition of ERα activity, and regulation of genes controlling cell cycle progression and apoptosis (Chang *et al.* 2006, Cotrim *et al.* 2013).

Many environmental factors, natural or synthetic chemicals known as xenoestrogen interfere with estrogen receptor signaling and act as endocrine disruptors (Acconcia *et al.* 2016).

Endocrine disruptors interact with the synthesis, secretion, transport, metabolism, binding, action, and elimination of natural hormones (Marino *et al.* 2012). The measured Kd of many xenoestrogens toward ERα and ERβ is in the micromolar range. Despite the 10,000-fold lower affinity xenoestrogens have for ERs, compared to E2 (Kd= 1 nM), xenoestrogens interfere with E2 signaling through ERα and ERβ receptor (Acconcia *et al.* 2016). Some metals, known as metalloestrogens, are also known to elicit estrogen-like activity (Byrne *et al.* 2013). Among them, aluminum, at concentration of 10^{-5}M - 10^{-4}M, is proposed to act as a metalloestrogen and its implication in breast cancer formation, development and progression is explored (Darbre *et al.* 2005, 2006, 2013, 2016). Although aluminum salts solubility is low at neutral pH, and its dermal absorption varies between 0.1%−2%, the aluminum concentration detected in breast cyst fluids and nipple aspirate fluid from breast cancer patients varies between 1 μM–10 μM (Manello *et al.* 2009, 2011).

In an effort to further investigate the possible estrogenic activity of the aluminum ion (Al^{+3}) and to delineate the molecular mechanisms underlining this action, the effect of aluminum, in the form of aluminum chlorohydrate (ACH) on the subcellular translocation of estrogen receptor alpha and beta in ERα positive MCF-7 and the subcellular translocation of estrogen receptor beta in ERα negative MDA-MB-231 cells, was examined. In addition the estrogenic activity of ACH as regards ERs protein levels regulation, and transcriptional regulation of -ERE-driven reporter gene and -ERE-independent target genes expression was evaluated.

Our results show that relative short exposure (4 hrs) of ERα positive MCF-7 breast cancer cells to ACH at concentration range from 10^{-6}M to 10^{-4}M caused only moderate activation of an ERE driven reporter gene transcription. The discrepancy between our results and those from earlier studies, showing approximately two fold transcriptional activation of estrogen receptor, upon 8 days, exposure of MCF-7 cells to aluminum salts (Darbre *et al.* 2005, 2006), may be attributed to the different incubation period applied.

Similar to the moderate effect of ACH on ER transcriptional activation, and in accordance with previous findings (Darbre *et al.* 2005), evaluation of the estrogenic activity of ACH in MCF-7 cells proliferation, upon 5 days exposure of MCF-7 cells to aluminum chlorohydrate, revealed no statistically significant ACH-induced activation of cell proliferation (data not shown).

Since no remarkable ACH effect on estrogen direct genomic actions was observed, we focused on the elucidation of the possible ACH involvement in ERE-independent, estrogen-indirect genomic actions and on ERα protein level regulation. Interestingly, upon 4 days incubation of MCF-7 cells, with 10^{-4} M ACH, ERα protein levels were increased by 2–3 fold compared to control cells. The molecular mechanism of the ACH-induced increase in ERα protein level could be attributed, at least in part, to aluminum estrogenic activity since this effect is abolished in the presence of the ER antagonist ICI 182780, which is also known to cause ERα protein degradation and reduction (Jaber *et al.* 2006, Johnston *et al.* 2010, Osborne *et al.* 2004). In accordance with previous findings (Pinzone *et al.* 2004, Saceda *et al.* 1988) we showed that 10^{-9}M E2 induced approximately 20% reduction in ERα protein levels in MCF-7 cells. This action is proposed to be attributed either to the direct binding of

the ERα-E2 complex to the estrogen response elements (ERE) of the promoter region of ERα (Pinzone *et al.* 2004) or to the induction of ERα protein degradation (reviewed by Acconcia *et al.* 2016). Interestingly in the presence of ACH, the E2 effect on ERα protein level was attenuated, indicating a possible interference of aluminum with estrogen signaling via an antagonistic effect of ACH on the E2-induced ERα protein level regulation. In order to examine whether the ACH action on ERα protein level is relied on the activation of estrogen receptor gene expression, the effect of E2 and ACH on ERα mRNA synthesis was evaluated. In accordance with previous findings (reviewed by Pinzone *et al.* 2004), 6 hours exposure of MCF-7 cells to 10^{-9}M E2 caused two fold induction in the ERα mRNA synthesis. As regards ACH effect on ERα mRNA synthesis, almost 10% induction was observed. Differential effect on ERα mRNA and protein levels has also been observed in the case of other xenoestrogens such as the Nar xenoestrogen (La Rosa *et al.* 2014). Nevertheless, this effect may also indicate that aluminum has a strong influence on estrogen receptor protein stability. The biochemical mechanism(s) of the regulation of ERα protein stability by ACH needs to be further explored, but it possibly involves ACH-induced conformational changes in ERα molecule, or ACH-induced ERα post-translational modifications rendering ERα less susceptible to proteolysis (Kawahara *et al.* 2011, Tecalco-Cruz *et al.* 2017). ER posttranslational modification affected by ACH could be phosphorylation, palmytoylation, acetylation, sumoylation and or lysine or arginine methylation, events modifying estrogen receptor expression and stability, subcellular localization, and sensitivity to hormonal response (Le Romancer *et al.* 2011).

The ACH-induced increase in ERα protein level may have consequences in the regulation of EREs-dependent or EREs-independent estrogen-responsive genes expression, among them, genes that encode the synthesis of crucial regulators of breast cancer development and progression. Accordingly, our results indicate that ACH may affects the expression of p53, p21, cyclin D1, and c-fos genes.

More specifically, expression of p53, which is an ERα target gene (Berger *et al.* 2012), via association of ERα with Sp1 at the GC-rich motif in the proximal *p53* promoter (Gu *et al.* 2012), was increased in MCF-7 cells, treated for 2 or 6 hrs with ACH in combination with E2, compared to control cells. The additive effect of ACH and E2 on p53 mRNA synthesis possibly indicates estrogen-like activity of ACH. This action could be attributed either to a direct ACH effect on ERα genomic actions or to an indirect effect, resulting from the ACH-induced increase in ER protein level. Increase in estrogen receptor protein level may lead to increase in p53 protein level but also suppression of its anti-tumor activity. This assumption is based on observations showing direct interaction of ERα with p53 (Liu *et al.* 2006, 2009, Sayeed *et al.* 2007), which leads to ERα dependent inhibition of p53 transcriptional activity, and thus suppression of p53-responsive anti-proliferative and apoptotic genes expression (Berger *et al.* 2012, Gu *et al.* 2012, Liu *et al.* 2009, 2016, Sayeed *et al.* 2007). The ERα involvement in the ACH-induced increase in p53 level is also supported by the inability of ACH to affect p53 gene expression in ERα negative MDA-MB-231 breast cancer cells. Increase in p53 protein level is also proposed to be associated with induction of cellular senescence (Qian *et al.* 2013). Recently, induction of cellular senescence and up regulation of the $p53/p21^{Waf1}$ pathway by aluminum, in the form of $AlCl_3$, possibly via an ERα independent manner, has also been demonstrated by Sappino et al (Sappino *et al.* 2012).

Other genes that contain GC-rich promoter sequences and constitute ERs target via ERs interaction with the Sp1 transcription factor (Porter *et al.* 1997) are those of c-fos (Duan *et al.* 1998) and cyclin D1 (Castro-Rivera *et al.* 2001). In this study the E2-induced expression of c-fos and cyclin D1 gene expression was verified. Although to a lower compared to E2 extent, ACH caused increase in both genes expression. In addition, the additive effect of E2 and ACH on c-fos and cyclin D1 gene expression, upon two and six hours incubation of MCF-7 cells, respectively, and the elimination of the ACH effect on both genes expression in the presence of ICI, further support the notion of a possible estrogenic activity of ACH. The ACH-induced effect on cyclin D1 gene expression may be, at least in part, ERα-mediated since this action was abolished in ERα negative MDA-MB-231 cells. Taking into account that p53, cyclin D1 and c-fos are important regulators in carcinogenesis and breast cancer progression (Sana *et al.* 2015), their induction by ACH could possibly contribute to cancer development.

In contrast to the effect of ACH on ERα, p53, c-fos and cyclin D1 expression, no ACH effect on cmyc gene expression was observed. On the other hand, consistent with previous observations, E2 induced cmyc gene expression (Chen *et al.* 2015, Wang *et al.* 2011). It is possibly indicated that the ERα conformational changes and/or post-translational modifications, such us phosphorylation, palmitoylation, sumoylation and/or ER methylation, potentially affected by ACH, may differentially favor binding and requirement of certain components of the multi-protein dynamic transcription complex leading to differential regulation of ER target genes expression and ERs subcellular targeting.

ERs subcellular localization was also affected by aluminum chlorohydrate. ACH similarly to E2, caused remarkable increase in the perinuclear translocation of ERβ, in both types of breast cancer cells. Based on previous findings showing mitochondrial localization of ERβ, (Chen *et al.* 2004, Liao *et al.* 2015, Psarra *et al.* 2008) as well as perinuclear accumulation of mitochondria (Al-Mehdi *et al.* 2012, Lonergan *et al.* 2007, Okatsu *et al.* 2010), our observations may indicate an increase in the mitochondrial localization of ERβ by ACH. Mitochondrial estrogen receptor beta is proposed to favor mitochondrial biogenesis, bioenergetics and anti-apoptotic signaling (Chen *et al.* 2004, 2009, Levin *et al.* 2009). In addition, increased oxidative mitochondrial metabolism is associated with increased tumor growth in human epithelial breast cancer cells, providing them with the necessary energy fuels and precursors molecules to cover their energy and proliferative demands (Al-Mehdi *et al.* 2012, Martinez-Outschoorn *et al.* 2011). Thus, increase in the mitochondrial localization of ERβ by ACH may favor breast cancer formation. In the same context, ACH-induced increase in the mitochondrial antiapoptotic Bcl2 molecule, that constitute an estrogen target gene (Perillo *et al.* 2000), may support mitochondrial viability and anti-apoptotic actions, leading to the observed ACH-induced cellular senescence (Childs *et al.* 2014, Raffetto *et al.* 2001, Sappino *et al.* 2012).

To conclude our results show that aluminum salts induce a remarkable increase in estrogen receptor protein level possibly via interference with estrogen receptor gene expression or estrogen receptor protein stability. This effect may have consequences in breast physiology, affecting estrogen receptor mediated gene expression via direct or indirect estrogen receptor DNA binding. Genes affected were genes of p53, cyclin D1, cfos, crucial regulators of breast

cancer development and progression and/or cellular senescence. Although the ACH – induced increase in the above mentioned ERα target genes took place to a moderate degree, the ACH effect on ERα protein level or stability could cause sustainable increase in ERα target gene expression and attenuation of the regulatory feedback mechanism of estrogen on ERα-mediated estrogen response. In addition, aluminum via induction of perinu-clear localization of estrogen receptors might affect mitochondrial physiology and function to provide cancer cells with the necessary energy production and supply of precursor molecules. Our findings do not provide conclusive evidence that aluminum is a breast carcinogen. However, the daily exposure to aluminum in connection with its possible role as an endocrine disruptor raises concerns about the safety of its use.

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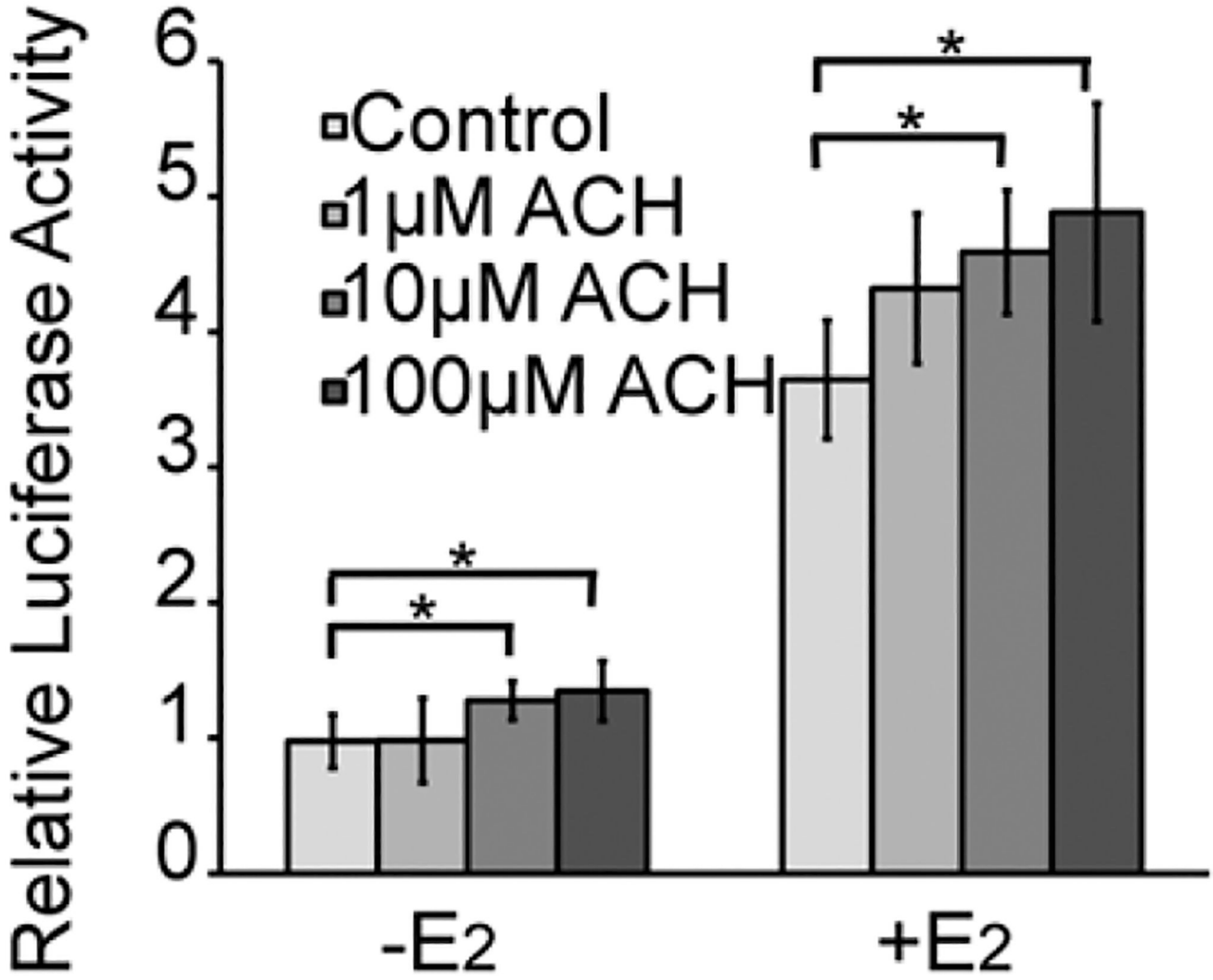

Figure 1.

Effect of ACH on ERE-driven luciferase gene expression in MCF-7 cells. ERα positive MCF-7 cells cultured in hormone depleted medium were transiently cotransfected with an ER-Luc reporter gene construct, and a β-galactosidase reporter construct. Cells were further incubated for 48 hrs and subsequently treated with the indicated concentrations of ACH, and/or 10^{-9}M E2 (diluted in EtOH) for 6 hrs. Control cells were treated with an equal volume of ddH_2O and EtOH (1:1000). Cells were lysed and activity of luciferase and β-galactosidase was measured in cell extracts. Luciferase activity was normalized against β-galactosidase activity and expressed as relative luciferase unit. Data are expressed as mean ± S.D. (n=6), *P<0.05.

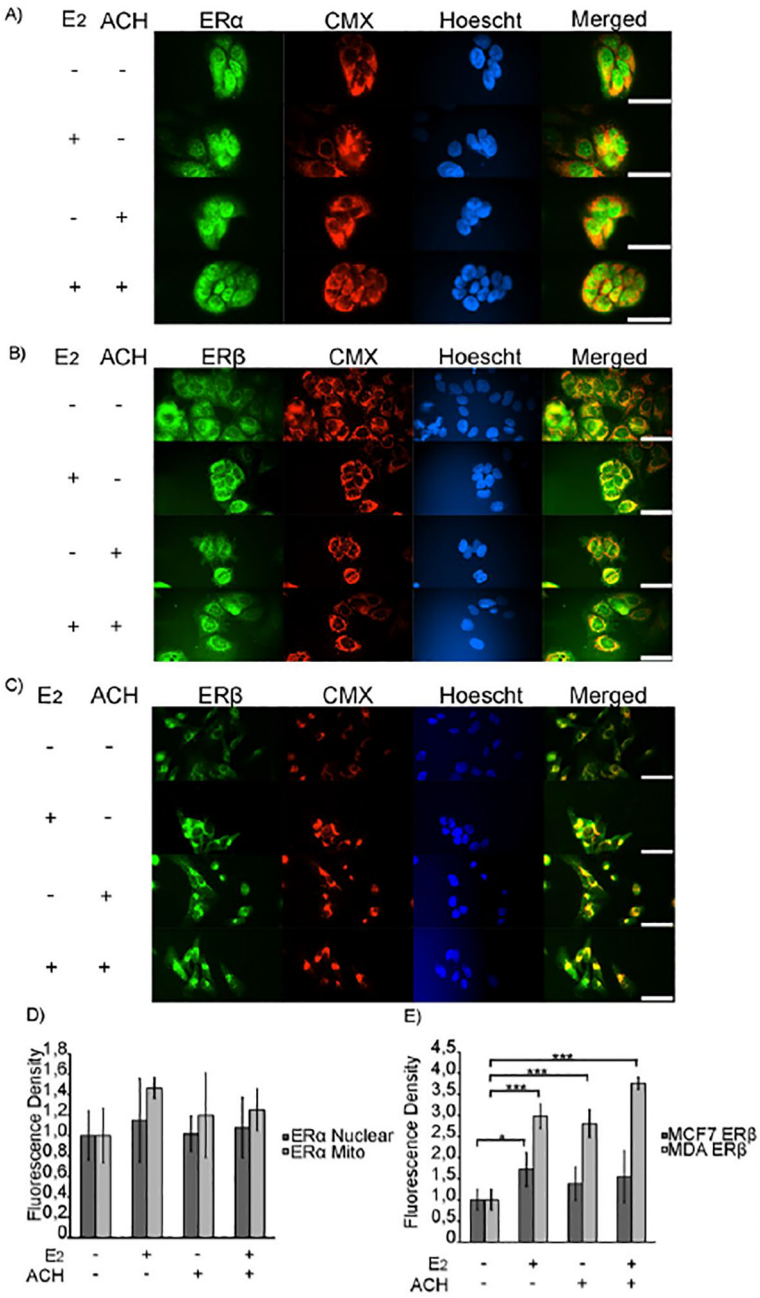

Figure 2.

ACH induced perinuclear localization of ERα and ERβ in breast cancer cells. MCF-7 (A,B) and MDAMB-231 (C) cells cultured in hormone depleted medium for 48 hrs were incubated with 10^{-4}M ACH or 10^{-9}M E2 in DMEM hormone free medium supplemented with 200 nM CMX. Control cells were treated with ddH_20 and EtOH at the same dilution as treated cells. Following 2 hrs incubation, cells were washed in PBS and fixed in methanol-acetone. For immunochemistry analysis antibodies against ERβ (ERβ-H150) (B,C), ERα (ERα-G20) (A) and anti-rabbit secondary antibodies *Alexa Fluor® 488 c*onjugated (Green) were applied. Hoechst 33342 (Blue) dye for nuclear staining was also used. Representative

images were presented. Bars indicate 50 μm. ERα nuclear and perinuclear-mitochondrial localization in MCF-7 cells (D) and ERβ perinuclear-mitochondrial localization (E) in E2- or ACH- treated cells is expressed as relative fluorescence density compared to control cells. Data are expressed as mean ± S.D. (n= 60), *P<0.05, ***P<0.001, compared to controls.

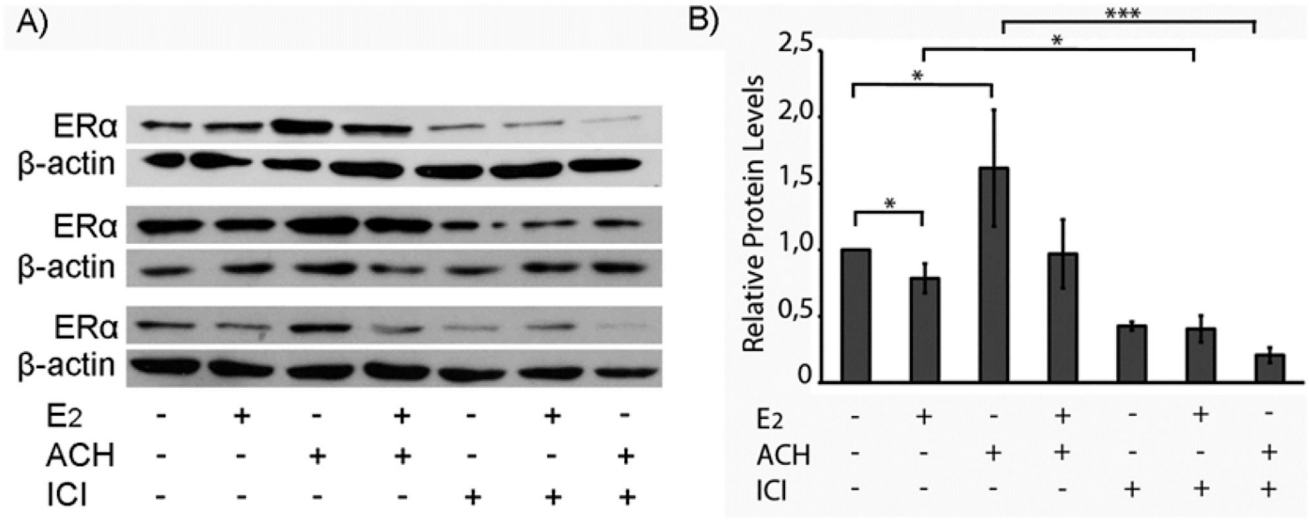

Figure 3.

ACH induced increase in ERα protein levels in MCF-7 cells. Evaluation of ACH effect on ERα protein levels was achieved by Western blot analysis of ERα, and β-actin protein levels, in total extracts from ERα positive MCF-7 breast cancer cells, treated with 10^{-4}M ACH, 10^{-9}M E2, 10^{-7}M ICI and in combination of them, as indicated, in hormone depleted medium, for 4 days. Commercially provided antibodies were used. B. Normalization of protein levels against β-actin protein levels. Results are expressed as mean ± SD (n=3), *P <0.05, ***P<0.001.

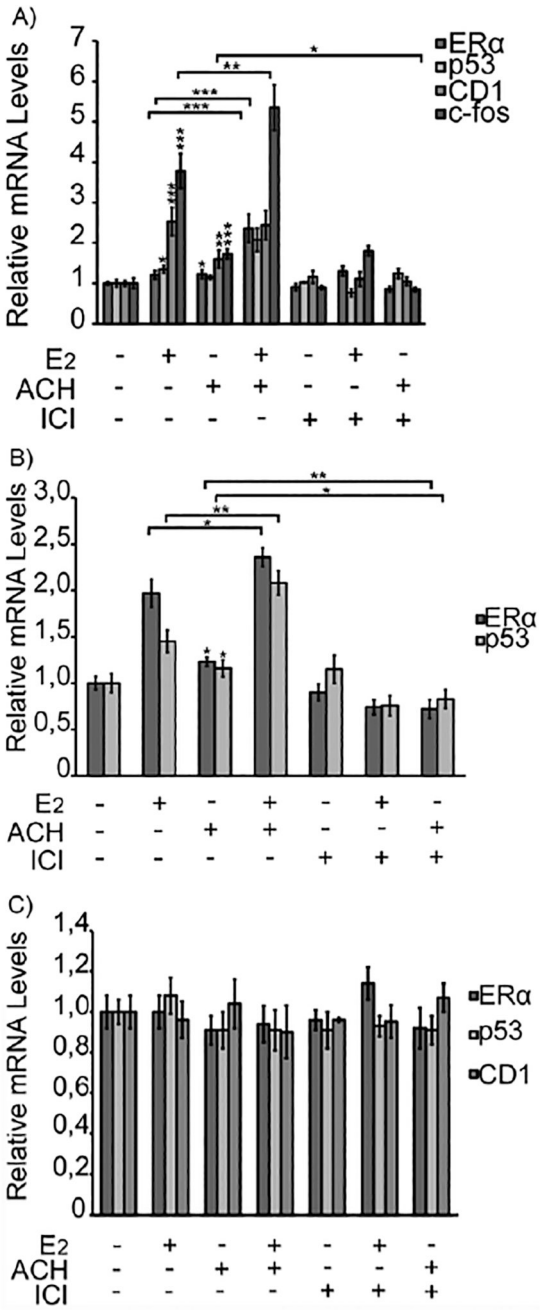

Figure 4.

Effect of ACH on mRNA synthesis of estrogen responsive molecules. Real-time PCR was applied to determine the mRNA levels of ERα, p53, Cyclin D1 (CD1), c-Fos, and β-actin in MCF-7 (A,B) and MDA-MB-231 cells (C), upon 2h (A) and 6 hr (B,C) incubation of the cells with 10^{-9}M E2, 10^{-4}M ACH, or 10^{-7}M ICI alone or in combination of them, as indicated, in hormone depleted medium. β-actin was used as a reference gene. The results of three independent experiments as means ± S.D. are shown. Vertical aligned asterisks indicate statistical significance differences compared to control vehicle treated cells. *P <0.05, **P<0.01, ***P<0.001, when compared with the respective cells, as indicated.

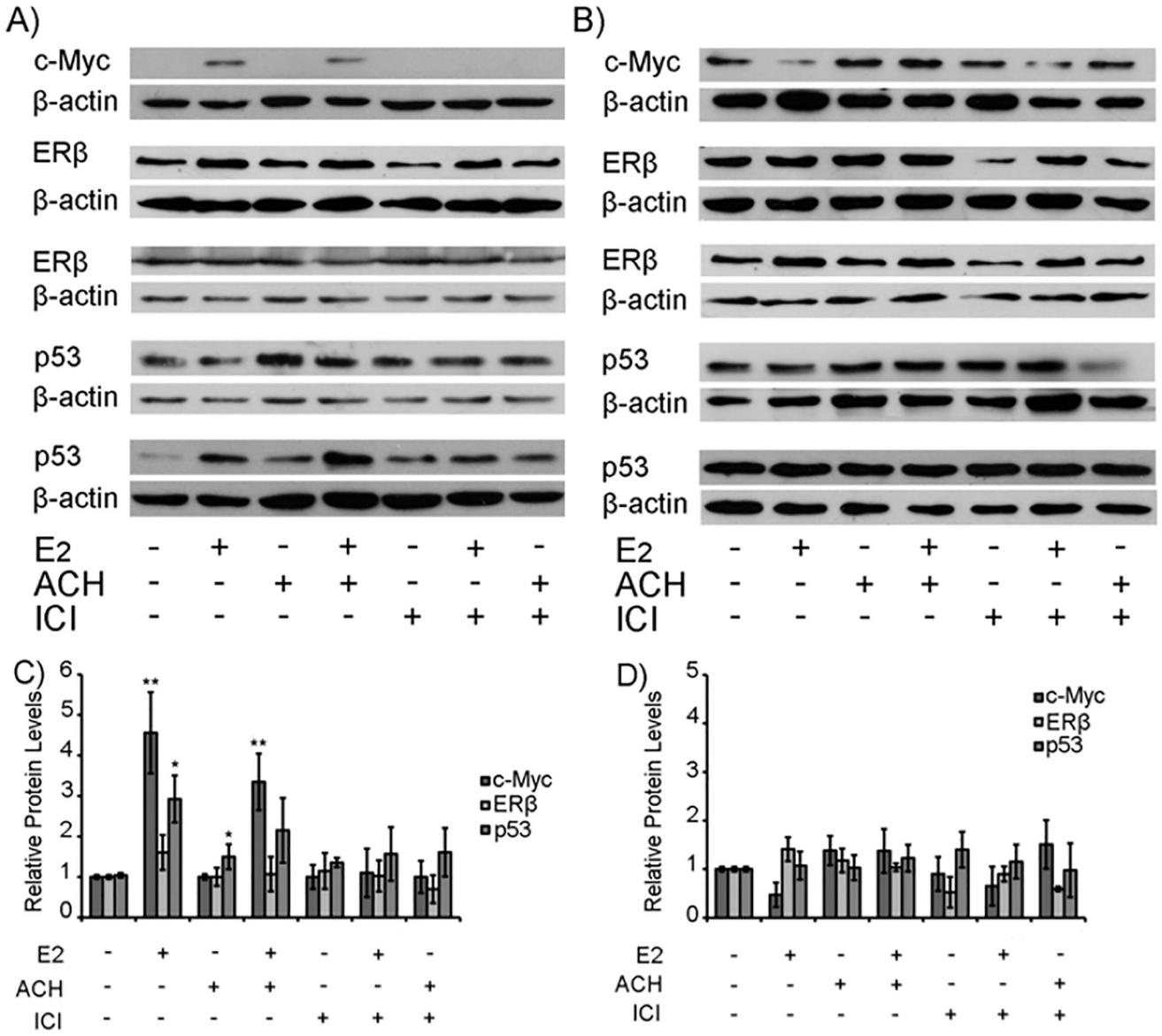

Figure 5.

ACH effect on protein synthesis of estrogen responsive molecules in MCF-7 and MDA-MB-231 cells. Western blot analysis of ERβ, p53, c-myc, and β-actin protein levels was performed in total extracts from MCF-7 (A) and MDA-MB-231 (B) human breast cancer cells treated with 10^{-4}M ACH, 10^{-9}M E2, 10^{-7}M ICI and in combination of them, in hormone depleted medium, for 4 days. Commercially provided antibodies were used. β-actin protein levels was used for the normalization of the results. Quantification of the results in MCF-7 (C) and MDA-MB-231 (D) are expressed as mean ± S.D. (n=3), *P <0.05, **P<0.01, when compared with the respective control cells, as indicated.

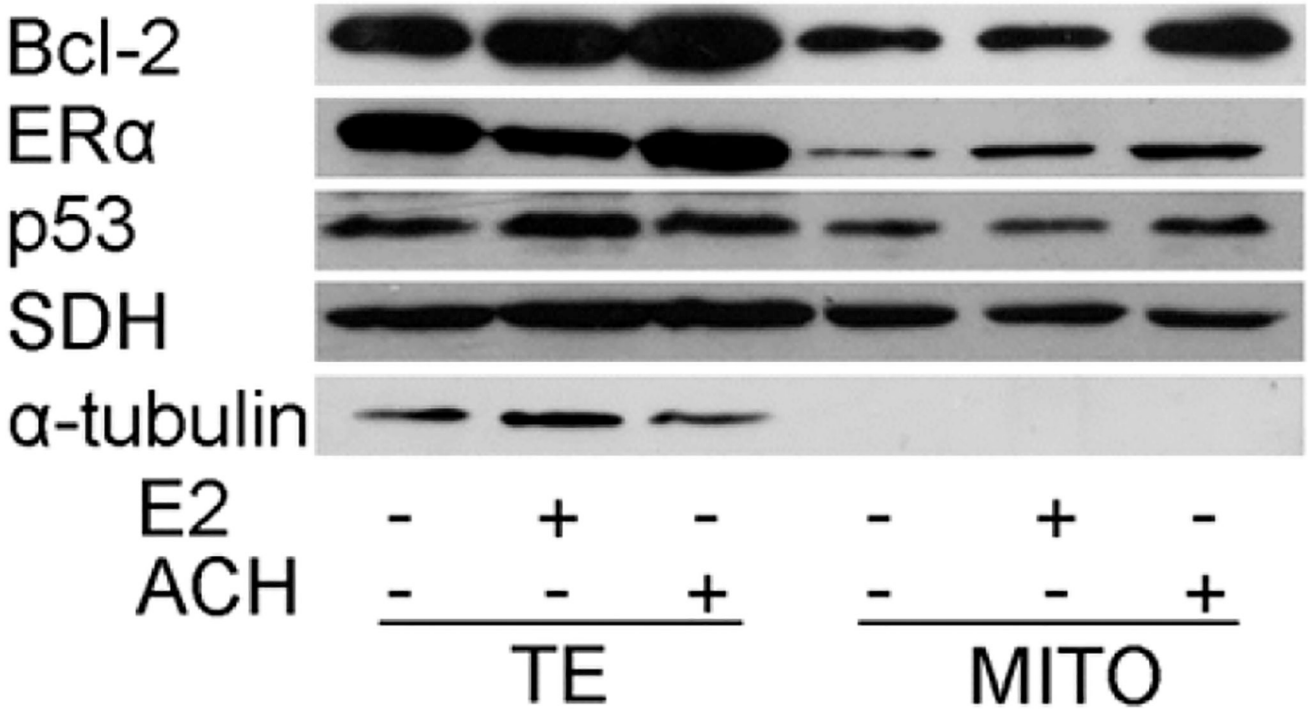

Figure 6.

ACH-induced increase in mitochondrial related molecules. Western blot analysis of Bcl-2, ERα, p53, SDH and α-tubulin, in total and crude mitochondrial extracts from ACH- or E2-treated MCF-7 cells, using commercially provided antibodies, is presented. SDH and α-tubulin were used, for the normalization of mitochondrial and total extract protein levels, respectively.

Table 1.

Sequences of primers for RT-PCR.

	Forward Primer	Reverse Primer
ERa	TGATCCTACCAGACCCTTCAGT	CCAATCATCAGGATCTCTAGCC
p53	AGGAAATTTGCGT GTGGAGTAT	TCCGTCCCAGTAGATTACCACT
CD1	GTGGCCTCTAAGATGAAGGAGA	GCATTTTGGAGAGGAAGTGTTC
c-Fos	GCTTCAACGCAGACTACGAG	AGTGACCGTGGGAATGAAGT
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTTCCACGATACCAAAGT