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Induction of Apoptosis in Estrogen Receptor-Negative Breast Cancer Cells by Natural and Synthetic Cyclopentenones: Role of the IκB Kinase/Nuclear Factor-κB Pathway

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ABSTRACT

Nuclear factor-κB (NF-κB), a transcription factor with a critical role in promoting inflammation and cell survival, is constitutively activated in estrogen-receptor (ER)-negative breast cancer and is considered a potential therapeutic target for this type of neoplasia. We have previously demonstrated that cyclopentenone prostaglandins are potent inhibitors of NF-κB activation by inflammatory cytokines, mitogens, and viral infection, via direct binding and modification of the β subunit of the IκB kinase complex (IKK). Herein, we describe the NF-κB-dependent anticancer activity of natural and synthetic cyclopentenone IKK inhibitors. We demonstrate that the natural cyclopentenone 15-deoxy-$\Delta^{12,14}$prostaglandin J_2 (15d-PGJ_2) is a potent inhibitor of constitutive IκB-kinase and NF-κB activities in chemotherapy-resistant ER-negative breast cancer cells. 15d-PGJ_2-induced inhibition of NF-κB function is rapidly followed by down-regulation of NF-κB-dependent antiapoptotic proteins cIAPs 1/2, Bcl-X_L, and cellular FLICE-inhibitory protein, leading to caspase activation and induction of apoptosis in breast cancer cells resistant to treatment with paclitaxel and doxorubicin. We then demonstrate that the cyclopentenone ring structure is responsible for these activities, and we identify a new synthetic cyclopentenone derivative, 3-*tert*-butyldimethylsilyloxy-5-(*E*)-iso-propylmethylenecyclopent-2-enone (CTC-35), as a potent NF-κB inhibitor with proapoptotic activity in ER-negative breast cancer cells. The results open new perspectives in the search for novel proapoptotic molecules effective in the treatment of cancers presenting aberrant NF-κB regulation.

Apoptosis is an essential physiological process required for embryonic development, immune system function, and the maintenance of tissue homeostasis in multicellular organisms (Hengartner, 2000). When the decision to undergo apoptosis is made in response to physiological signals, a proteolytic cascade involving different caspases is triggered in the suicidal cell, which ultimately results in activation of nucleases that degrade chromosomal DNA (Hengartner, 2000). One of the major regulators of life or death decisions is the nuclear factor-κB (NF-κB) (Karin and Lin, 2002; Karin et al., 2002).

NF-κB normally exists as an inactive cytoplasmic complex, whose predominant form is a heterodimer composed of p50 and p65 (Rel A) subunits, bound to inhibitory proteins of the IκB family (IκBs), and it is induced in response to a variety of pathogenic stimuli, including UV radiation and exposure to proinflammatory cytokines or mitogens, and to bacterial or viral infection (Karin and Lin, 2002; Li and Verma, 2002; Santoro et al., 2003). In most instances, induction requires the activation of the IκB kinase IKK, a multisubunit complex

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ABBREVIATIONS: NF-κB, nuclear factor-κB; IKK, IκB kinase complex; TNF, tumor necrosis factor; cIAP, cellular inhibitors of apoptosis; cFLIP, cellular FLICE-inhibitory protein; ER, estrogen receptor; cyPG, cyclopentenone prostaglandin; 2-Cy, 2-cyclopenten-1-one; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$prostaglandin J_2; CTC-8, 4-(*S*)-*tert*-butyldimethylsilyloxy cyclopent-2-enone; CTC-35, 3-*tert*-butyldimethylsilyloxy-5-(*E*)-iso-propylmethylenecyclopent-2-enone; AA, arachidonic acid; PG, prostaglandin; Hsp70, 70-kDa heat shock protein; PI, propidium iodide; DAPI, 4,6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; ns, nonspecific; MDP, modular toolkit for data processing; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; FACS, fluorescence-activated cell sorting; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; E2, 17β-estradiol; ERE, estrogen response element.

containing two catalytic subunits (IKK-α and IKK-β) and the IKK-γ regulatory subunit (Rothwarf et al., 1998; Israel, 2000). The IKK complex phosphorylates IκBs triggering their ubiquitination and proteasome-mediated degradation (Karin and Ben-Neriah, 2000). Release of IκBs results in nuclear translocation of NF-κB and its binding to DNA at specific κB sites, rapidly inducing a variety of genes encoding, among others, cell adhesion molecules, matrix metalloproteinases, inflammatory and chemotactic cytokines, cytokine receptors, and enzymes that produce inflammatory mediators, such as cyclooxygenase-2 and the inducible form of nitric-oxide synthase (Karin and Lin, 2002; Li and Verma, 2002). As a consequence, NF-κB is considered a critical regulator of the inflammatory and immune responses (Li and Verma, 2002).

More recently, NF-κB activation has been connected with multiple aspects of oncogenesis, including the control of apoptosis (Baldwin, 2001). NF-κB acts as an antiapoptotic factor and its activation can suppress cell death pathways by switching on genes that dampen proapoptotic signals, including members of the Bcl-2 family (Bcl-X_L and A1/Bfl-1), cellular inhibitors of apoptosis (cIAPs) (cIAP-1, cIAP-2, and X-linked mammalian inhibitor of apoptosis protein), TNF receptor-associated factors 1 and 2, and the caspase-8/Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein cFLIP (Karin and Lin, 2002; Karin et al., 2002). In addition, NF-κB has been recently shown to be constitutively activated in several types of cancer cells, including lymphoid malignancies and some types of breast cancer (Nakshatri et al., 1997; Rayet and Gelinas, 1999). In particular, both the IκB kinase and NF-κB have been shown to be constitutively activated in estrogen receptor (ER)-negative breast cancer cell lines and primary tumors (Nakshatri et al., 1997; Sovak et al., 1997; Biswas et al., 2000; Romieu-Mourez et al., 2001), and this event has been associated with resistance to apoptosis induced by chemotherapeutic drugs (Patel et al., 2000; Weldon et al., 2001).

Cyclopentenone prostaglandins (cyPGs) are potent bioactive molecules that possess antiviral (Santoro, 1997) and anticancer activity (Santoro et al., 1989; Straus and Glass, 2001). The unique characteristic of cyPGs is the presence of an α,β-unsaturated carbonyl group in the cyclopentane ring, which renders this portion of the molecule able to form Michael adducts with cellular nucleophiles and to covalently bind to thiol groups of target cysteine residues of specific proteins (Chen et al., 1999). The cyclopentenone structure has been shown to be essential for the biological activity (Rossi et al., 1996; Santoro, 1997; Straus and Glass, 2001).

We have shown that natural cyPGs are potent inhibitors of NF-κB activation by inflammatory cytokines, mitogens, and viral infection, via direct binding and modification of the β subunit of the IκB kinase (Rossi et al., 1997, 2000; Amici et al., 2001, 2004). Starting from these observations, in the present study we investigated the effect of cyPGs on NF-κB activity and survival of ER-positive (ER+) and ER-negative (ER−) breast cancer cells. We report that the natural prostanoid 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) is able to inhibit constitutive NF-κB activity in different chemoresistant ER− breast cancer cell lines, including MDA-MB-231, SKBR-3, and MDA-MB-468 cells, at concentrations that have no effect in ER+ cells. NF-κB inhibition is rapidly followed by down-regulation of NF-κB-dependent antiapoptotic proteins cIAP1/2, Bcl-X_L, and cFLIP, leading to caspase activation and induction of apoptosis in chemoresistant ER− breast cancer cells. We also show that these effects are mimicked by the 2-cyclopenten-1-one (2-Cy) ring by itself, and two 2-Cy synthetic derivatives, CTC-8 and CTC-35. Finally, we have identified CTC-35 as a potent inducer of apoptosis in chemoresistant ER− breast cancer cells.

Materials and Methods

Reagents. Arachidonic acid (AA), prostaglandin (PG)A_1, PGE_2, $PGF_{2\alpha}$, and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 were obtained from Cayman Chemical (Ann Arbor, MI). 2-Cyclopenten-1-one, doxorubicin-hydrochloride, and paclitaxel were obtained from Sigma-Aldrich (St. Louis, MO). CTC-8 and CTC-35 were synthesized as described previously (Bickley et al., 2004). TNFα and antibodies to human cIAP-1 and cIAP-2 were purchased from R&D Systems (Minneapolis, MN). Monoclonal antibodies to α-tubulin were obtained from Sigma-Aldrich, monoclonal antibodies to Hsp70 were from Stressgen Biotechnologies Corp. (Victoria, BC, Canada), and monoclonal antibodies to Bcl-2 were from Dako Denmark A/S (Glostrup, Denmark). Rabbit polyclonal antibodies to caspase-8, caspase-9, caspase-3, and Bcl-X_L were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), polyclonal antibodies to cFLIP were from Upstate Biotechnology (Charlottesville, VA), and polyclonal antibodies to IKKα were from BD Biosciences PharMingen (San Diego, CA).

Cell Culture and Treatments. Human breast cancer cell lines MCF-7 (ER+) and MDA-MB-231 (ER−) were obtained from American Type Culture Collection (Manassas, VA). SKBR-3 and MDA-MB-468 cell lines were kindly provided by Dr. O. Segatto (Regina Elena Institute, Rome, Italy). T-47D cells were kindly provided by Dr. G. Forni (University of Turin, Turin, Italy). All cell lines were grown at 37°C in 5% CO_2 and 95% humidified air in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, and antibiotics (Invitrogen, Carlsbad, CA) and supplemented with 1 mM sodium pyruvate (MCF-7) or 1× nonessential amino acids (MDA-MB-231). AA, prostaglandins, and cyclopentenones were dissolved in absolute ethanol and diluted in the culture medium immediately before use. Control cells received the same amount of ethanol diluent. Cell viability was determined by vital dye exclusion assay (0.1% trypan blue).

Flow Cytometry. For DNA content determination, cells fixed in 70% ethanol were treated with 0.25 mg/ml RNase and stained with 5 μg/ml propidium iodide. The sub-G_1/G_0 phase fraction was calculated using CellQuest program (BD Biosciences, San Diego, CA). For Annexin-V staining, cells suspended in staining buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl) with Annexin-V-PI (Annexin-V-FITC; BD Biosciences) were analyzed by FACScan, using CellQuest program.

DNA Fragmentation. Cytoplasmic histone-bound DNA fragments (mono- and oligonucleosomes) generated during apoptosis were measured by Cell Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Histone-associated DNA fragments were quantified spectrophotometrically using antibodies against DNA and histones in a colorimetric assay. Enrichment in cytoplasmic nucleosomes was expressed as –fold induction of levels in untreated controls.

4,6-Diamidino-2-phenylindole Staining. Cells grown on coverslips and treated with 20 μM 15d-PGJ_2 for 36 h were fixed with 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with 5 μg/ml DAPI (Sigma-Aldrich). Fluorescence microscopy was performed on a Leica DM-IL microscope equipped with UV excitation filters, and images were captured on a Leica DC-300 camera using Leica Image-Manager500 software (Leica, Wetzlar, Germany).

Electrophoretic Mobility Shift Assay. For nuclear extract preparation, cells (2×10^6 cells/sample) were lysed in hypotonic lysis buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8, 0.5%

Nonidet P-40, and 1 mM dithiothreitol) and then in high-salt extraction buffer (50 mM Tris-HCl, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.5% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 0.2% aprotinin). Equal amounts of protein (6 μg/sample) were incubated with ^{32}P-labeled κB-DNA probe (5′-AGCTTCAGAGGGGACTTTCCGAGAGG-3′ and 3′-AGTCTCCCCTGAAAGGCTCTCCAGCT-5′) (Rossi et al., 1997), followed by analysis of DNA binding activity by EMSA. Binding reactions were performed as described previously (Rossi et al., 1997). Complexes were analyzed by nondenaturing 4% polyacrylamide gel electrophoresis. Specificity of protein-DNA complexes was verified by immunoreactivity with polyclonal antibodies specific for p65/RelA. Quantitative evaluation of NF-κB/κB complex formation was determined by Typhoon-8600 Imager (GE Healthcare, Little Chalfont, Buckinghamshire, UK), using ImageQuant software (MDP analysis). For control of equal loading, NF-κB values were normalized to the level of the nonspecific protein-DNA complex (ns) in the same lane.

Western Blot Analysis. Whole-cell extracts were prepared after lysis in buffer containing 50 mM Tris-Cl, pH 7.4, 0.15 M NaCl, 5 mM EDTA, 0.1% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors. Equal amounts of protein (20 μg/sample) were separated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and incubated with specific antibodies, followed by labeling with peroxidase-labeled anti-mouse or anti-rabbit IgG (enhanced chemiluminescence; GE Healthcare). α-Tubulin (Sigma-Aldrich) Western blot analysis was performed as a control of equal sample loading. Membranes were stripped by washing in glycine stripping buffer [0.2 M glycine, 0.1% SDS, and 1% Tween 20 (Sigma-Aldrich), pH 2.4] for 60 min at room temperature, before exposure to different antibodies. Quantitative evaluation of proteins was determined by Versadoc 1000 analysis using the Quantity One software program (Bio-Rad Laboratories, Hercules, CA). Levels of the different proteins analyzed were normalized to the level of α-tubulin in the same blot.

Kinase Assay. For IKK activity determination, cell lysates were immunoprecipitated with anti-IKKα antibody in the presence of 15 μl of protein A-Sepharose (Sigma-Aldrich) at 4°C for 16 h. After extensive washing, endogenous IKK activity was determined using glutathione transferase-IκB$\alpha_{(1-54)}$ as substrate (Rossi et al., 2000). The reaction mixtures were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, followed by autoradiography. Western blot analysis of IKKα was performed as kinase loading control.

Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted using the TRIzol reagent (Life Technologies, Inc.) RT-PCR was performed (1 μg of RNA) according to the manufacturer's protocols (Invitrogen). The oligonucleotide primer pairs used for RT-PCR were as follows: human cIAP-1 (984 bp), 5′-ATGAACATAGTAGAAAACAGC-3′ and 5′-CCTGTCCTTTAATTCTTATCA-3′; human cIAP-2 (900 bp), 5′-TGACTTTTCCTGTGAACTCT-3′ and 5′-GCCTTTCATTCGTATCAAGA-3′; human c-FLIP (851 bp), 5′-GCTGAAGTCATCCATCAGGT-3 and 5′-CATACTGAGATGCAAGAATT-3′; human Bcl-X_L (601 bp), 5′-TGTGGAAGAGAACAGGACTGAGG-

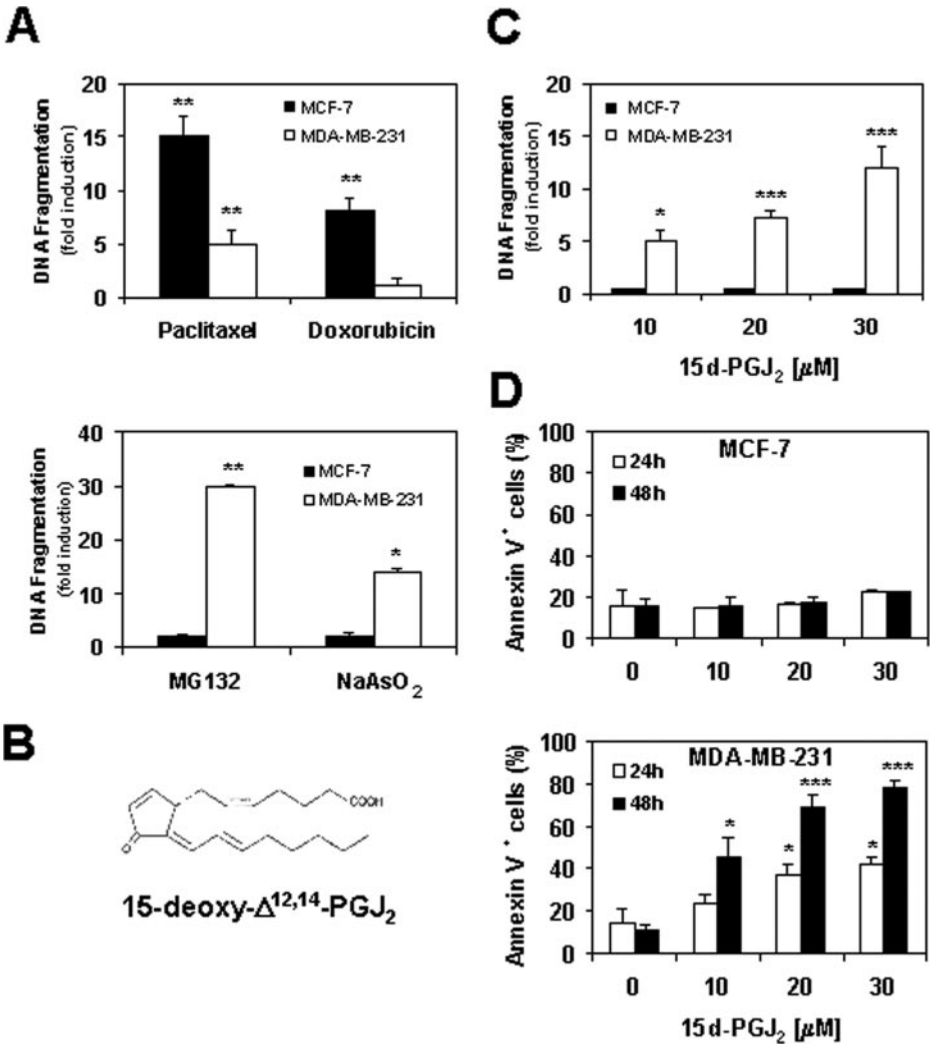

Fig. 1. Differential response of ER+ and ER− breast cancer cells to conventional chemotherapeutic drugs and to NF-κB inhibitors. A, ER+ MCF-7 (filled bars) and ER− MDA-MB-231 (empty bars) cells were treated with 0.1 μM paclitaxel and 5 μM doxorubicin (top) or with 1 μM MG132 and 50 μM $NaAsO_2$ (bottom). After 48 h, apoptosis was evaluated by measuring DNA fragmentation, as described under *Materials and Methods*. The enrichment of nucleosomes in the cytoplasm of treated cells is expressed as -fold induction of levels in untreated controls. B, structure of 15d-PGJ_2. C and D, MCF-7 and MDA-MB-231 cells were treated with different concentrations of 15d-PGJ_2 or with control diluent. Apoptosis was evaluated at 48 h after treatment by measuring DNA fragmentation (C). In a parallel experiment, the percentage of Annexin-V^+ cells was determined by flow cytometry after Annexin-V-FITC/PI staining at 24 h (empty bars) and 48 h (filled bars) after treatment with 15d-PGJ_2 (D). Data represent the mean ± S.E.M. of three independent experiments, each in duplicate. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

C-3′ and 5′-GAAAGAGTGAGCCCAGCAGAACCA-3′; human β-actin (210 bp) 5′-GCGCTCAGGAGGAGCAAT-3′ and 5′-GCACTCTTCCA-GCCTTCC-3′. PCR was performed using the following thermocycler parameters: denaturing temperatures of 95°C for 1 min, annealing temperatures of 58°C for 1 min, and elongation temperatures of 72°C for 2 min for 30 cycles. The cycles for human β-actin were 28. PCR products were electrophoresed alongside DNA Molecular Weight Marker-IX (Roche Diagnostics, Basel, Switzerland) in 2% agarose gels and then stained with ethidium bromide.

Statistical Analysis. Statistical analysis was performed using Student's *t* test for unpaired data. Data are expressed as the mean ± S.E. of duplicate or triplicate samples. $p < 0.05$ was considered significant.

Results

Differential Sensitivity of ER-Positive and ER-Negative Breast Cancer Cells to Conventional Chemotherapeutic Drugs and to NF-κB Inhibitors. Antiestrogen-resistant breast cancers are generally invasive and respond poorly to chemotherapy compared with ER-positive tumors (Clarke et al., 1993). To compare the effect of conventional chemotherapeutic drugs on apoptosis induction in ER+ and ER− breast cancer cells, the human cell lines MCF-7, ER+ and hormone-dependent, and MDA-MB-231, ER− and hormone-independent, were used. Cells were plated at the density of 2×10^5 cells/ml and treated with 0.1 μM paclitaxel, 1 μM doxorubicin, or control diluent for 48 h. Apoptosis was determined by analysis of DNA fragmentation. As shown in Fig. 1A (top), treatment with paclitaxel or doxorubicin was very effective in inducing apoptosis in ER+ MCF-7 cells, whereas MDA-MB-231 cells were found to be resistant to doxorubicin and to respond poorly to paclitaxel. Similar results were obtained when cells were treated with higher concentrations of both drugs (data not shown).

Because constitutive NF-κB DNA binding activity has been associated with resistance to chemotherapy in breast cancer, in a parallel experiment the effect of two NF-κB inhibitors, sodium arsenite ($NaAsO_2$) (Mathas et al., 2003) and the proteasome inhibitor MG132 (Dai et al., 2003), on the induction of apoptosis was examined in MCF-7 and MDA-MB-231 cells. MG132 (1 μM) and 50 μM $NaAsO_2$ were found to be ineffective in inducing DNA fragmentation in MCF-7 cells up to 48 h after treatment (Fig. 1A, bottom). It is noteworthy that both molecules had a potent proapoptotic effect in MDA-MB-231 cells.

15d-PGJ_2 Induces Apoptosis in ER− Breast Cancer Cells, Which Are Resistant to Conventional Chemotherapeutic Drugs. We have previously shown that cyclopentenone prostanoids, which are known to induce cell death in many tumor cell lines (Straus and Glass, 2001), are potent inhibitors of NF-κB activation (Rossi et al., 1997, 2000; Amici et al., 2001, 2004). We then investigated the effect of the natural cyPG 15d-PGJ_2 on cell death of ER+ and ER− breast cancer cells. MCF-7 and MDA-MB-231 cells were plated at the density of 2×10^5 cells/ml and treated with different concentrations of 15d-PGJ_2 or with control diluent. Apoptosis was evaluated at 48 h after treatment by measuring DNA fragmentation. In a parallel experiment, the percentage of Annexin-V^+ cells was determined by flow-cytometry after Annexin-V-FITC/PI staining at 24 and 48 h after treatment with 15d-PGJ_2. As shown in Fig. 1C, 15d-PGJ_2 caused a dose-dependent increase in cytoplasmic mono- and oligonucleosomes in MDA-MB-231 cells, whereas it had no effect on ER+ MCF-7 cells at the same concentrations. The proapoptotic activity of 15d-PGJ_2 on ER− breast cancer cells was confirmed by FACS analysis of Annexin-V^+ cells. After 24 h of exposure, 15d-PGJ_2 induced apoptosis in MDA-MB-231 cells in a dose-dependent manner, reaching 40% of positive cells at the concentration of 30 μM (Fig. 1D, bottom). At 48 h, the percentage of Annexin-V^+ MDA-MB-231 cells was increased to 70% at 20 μM and to 80% at 30 μM 15d-PGJ_2. In contrast, no significant change in Annexin-V binding was observed in MCF-7 cells up to 48 h of treatment (Fig. 1D, top).

The differential sensitivity of ER− and ER+ breast cancer cells to 15d-PGJ_2-induced apoptosis was also shown by de-

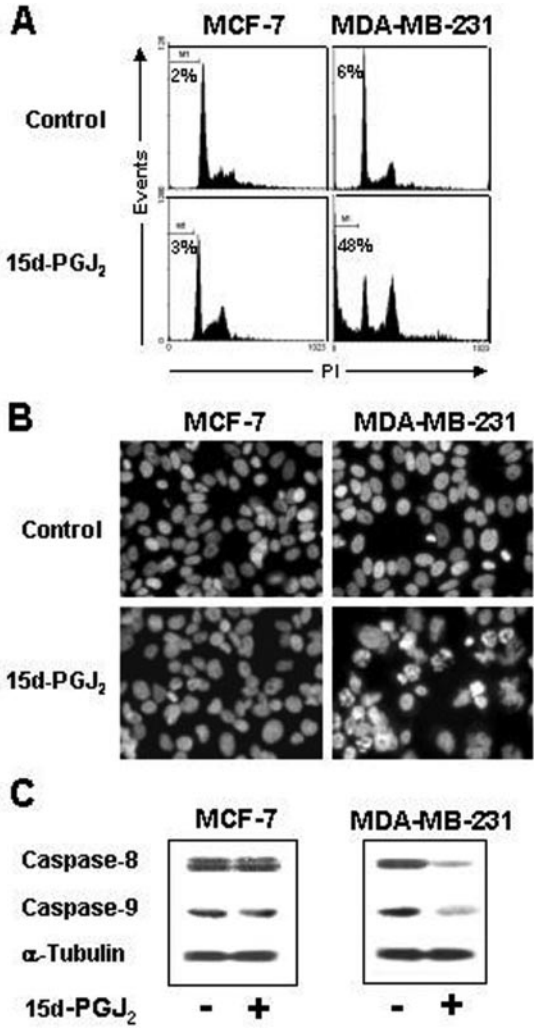

Fig. 2. 15d-PGJ_2 induces apoptosis through multiple caspase activation in ER− MDA-MB-231 cells, but not in ER+ MCF-7 cells. Unsynchronized MCF-7 and MDA-MB-231 cells were treated with 20 μM 15d-PGJ_2 or control diluent (control). A, cell cycle profile was evaluated at 48 h after treatment by PI staining using flow cytometry. The percentage of sub-G_0/G_1 cells is indicated on each panel. Alternatively, cells were fixed, permeabilized, and stained with DAPI at 36 h after treatment (B). Nuclear morphology was analyzed by fluorescent microscopy (original magnification 400×). Apoptotic cells containing condensed and fragmented fluorescent nuclei are visible in 15d-PGJ_2-treated MDA-MB-231 cells, but not in MCF-7 cells. C, MCF-7 and MDA-MB-231 cells were treated with 20 μM 15d-PGJ_2 or control diluent (control) for 24 h. Whole-cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. The levels of α-tubulin are shown as control. Data are representative of three separate experiments with similar results.

termination of DNA content through FACS analysis of hypodiploid cells (sub-G_0/G_1 phase). As shown in Fig. 2A, untreated MCF-7 and MDA-MB-231 cells showed the expected pattern of cell cycle distribution. After 48 h of treatment, a sub-G_0/G_1 peak, indicative of a 48% increase of apoptotic cells, was evident in 15d-PGJ_2-treated MDA-MB-231 cells (Fig. 2A, left). In contrast, no change in the percentage of the sub-G_0/G_1 population was observed in MCF-7 cells (Fig. 2A, right). This observation was further confirmed by analysis of nuclear morphology. In MDA-MB-231 cells, treatment with 20 μM 15d-PGJ_2 for 36 h caused a marked increase in nuclear fragmentation and condensation, as indicated by the pattern of DAPI staining, whereas no nuclear morphology changes were observed in MCF-7 cells (Fig. 2B).

To investigate whether 15d-PGJ_2 treatment induced caspase activation, MCF-7 and MDA-MB-231 cells were treated with 20 μM 15d-PGJ_2 or control diluent and, after 24 h, whole-cell lysates were processed for procaspase-8, -9, and -3 detection by immunoblotting. As shown in Fig. 2C, treatment with 15d-PGJ_2 caused a reduction in both procaspase-8 and -9 levels in MDA-MB-231 cells. In addition, the level procaspase-3 was decreased in the ER− cell line (data not shown). No changes in the level of caspase-8 and -9 (MCF-7 cells lack caspase-3) were instead detected in ER+ cells treated with 15d-PGJ_2 (Fig. 2C).

15d-PGJ_2 Inhibits Constitutive IKK and NF-κB Activities in ER− Breast Cancer Cells. NF-κB has been shown to be constitutively activated in ER− breast cancer cells, and this event has been associated with resistance to apoptosis induced by chemotherapeutic drugs (Sovak et al., 1997; Patel et al., 2000; Weldon et al., 2001). cyPGs inhibit TNFα- and 12-*O*-tetradecanoylphorbol-13-acetate-induced NF-κB activation by blocking IKK activity (Rossi et al., 2000). To determine the levels of constitutive IKK and NF-κB activities in MCF-7 and MDA-MB-231 cells and to investigate whether these would be affected by 15d-PGJ_2, ER+ and ER− breast cancer cells were treated with different concentrations of 15d-PGJ_2, and, after 3 h, whole-cell and nuclear extracts were analyzed for IKK activity by kinase assay and NF-κB DNA binding activity by EMSA, respectively. The levels of IKK and NF-κB activities were then quantified by MDP analysis. As shown in Fig. 3A, IKK activity was found to be 3 times higher in MDA-MB-231 cells than in MCF-7 cells. Furthermore, the levels of IKK activity correlated strictly with the levels of NF-κB DNA binding activity (Fig. 3B), confirming an increased constitutive DNA binding activity of the nuclear factor in more malignant tumors.

A 3-h treatment with 15d-PGJ_2 was found to inhibit constitutive IKK activity in both cell lines; however, the prostanoid was more effective in MDA-MB-231 cells, where IKK activity was decreased by more than 35 and 80% after treatment with 10 or 20 μM 15d-PGJ_2, respectively. Inhibition of IKK was associated with a dramatic inhibition of NF-κB DNA binding activity in ER− breast cancer cells. Quantitative evaluation by MDP analysis revealed that 15d-PGJ_2, at the concentration of 10 and 20 μM, inhibited NF-κB DNA binding activity by more than 65 and 95%, respectively, in

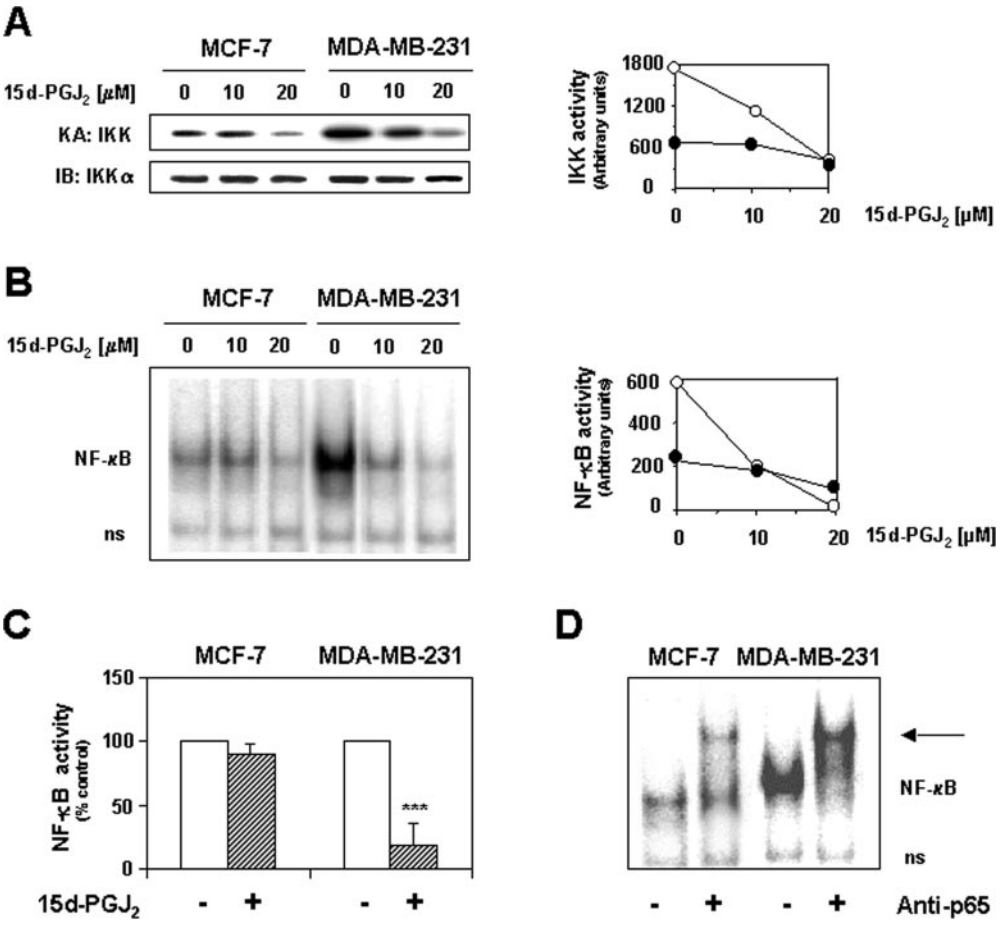

Fig. 3. 15d-PGJ_2 inhibits constitutive IKK and NF-κB activities in ER− breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with the indicated concentrations of 15d-PGJ_2 or with control diluent. After 3 h, protein extracts were analyzed for IKK activity by kinase assay (KA: IKK) (A) and NF-κB activation by EMSA (B). Endogenous IKK recovery was determined in the same samples by immunoblot analysis for IKKα (IB: IKKα). A section of the fluorograms is shown (right). Positions of NF-κB-DNA (NF-κB) and nonspecific protein-DNA (ns) complexes are indicated. The levels of IKK (A) and NF-κB (B) activities in MCF-7 (●) and MDA-MB-231 (○) cells were quantified by MDP analysis and expressed as arbitrary units (left). Data are representative of three separate experiments with similar results. C, NF-κB-DNA binding activity in MCF-7 and MDA-MB-231 cells treated with 10 μM 15d-PGJ_2 or control diluent is expressed as the mean ± S.E.M. of three independent experiments. ***, $p < 0.001$. D, specificity of NF-κB-DNA complexes in MCF-7 and MDA-MB-231 cells, as determined by supershift analysis using anti-p65 antibodies (1:100 dilution). The shifted NF-κB-DNA complexes are indicated by the arrow.

MDA-MB-231 cells, whereas it was inactive in the ER+ cells (Fig. 3, B and C).

To investigate whether the proapoptotic effect of 15d-PGJ_2 was specific for MDA-MB-231 cells or could be a general property of the prostanoid in ER− breast cancer cells, two human ER− (SKBR-3 and MDA-MB-468) and one ER+ (T-47D) breast cancer cell line were examined for their sensitivity to 15d-PGJ_2 treatment. Breast cancer cells were treated with different concentrations of 15d-PGJ_2, and after 3 h, nuclear extracts were analyzed for NF-κB DNA binding activity by EMSA. Levels of NF-κB activity were quantified by MDP analysis. The results shown in Fig. 4A indicate that ER− SKBR-3 and MDA-MB-468 cells expressed constitutively high levels of NF-κB DNA binding activity, whereas NF-κB activity was barely detected in ER+ T-47D cells. Quantitative evaluation by MDP analysis revealed that 15d-PGJ_2, at the concentration of 20 μM, inhibited NF-κB DNA binding activity by 64 and >90% in SKBR-3 and MDA-MB-468 cells, respectively, whereas it was inactive in ER+ T-47D cells. In a parallel experiment, the effect of 15d-PGJ_2 on induction of apoptosis was determined in the same cell lines after 48 h of treatment. As shown in Fig. 4B, inhibition of NF-κB was associated with a dose-dependent increase in the number of Annexin-V^+ cells in both ER− cell lines. In contrast, no significant change in the number of apoptotic cells was observed in the ER+ T-47D cell line, which presented low levels of NF-κB DNA binding activity not altered by 15d-PGJ_2 treatment.

15d-PGJ_2 Down-Regulates the Expression of Antiapoptotic Proteins in ER− Breast Cancer Cells. Because induction of apoptosis by 15d-PGJ_2 was found to be associated with NF-κB inhibition in ER− breast cancer cells, we evaluated the effect of this prostanoid on the level of several survival-regulating proteins, whose expression is modulated by the transcriptional activity of NF-κB, using as a model MDA-MB-231 cells. At different times after treatment with 20 μM 15d-PGJ_2, MDA-MB-231 nuclear extracts were analyzed for NF-κB DNA binding activity by EMSA, whereas the expression of NF-κB-dependent gene products was determined by Western blot analysis in whole-cell extracts. As a control of 15d-PGJ_2 activity, we determined the level of Hsp70, whose expression is known to be induced by cyclopentenone prostanoids via the activation of the heat shock transcription factor HSF-1 (Rossi et al., 1996). The high level of NF-κB DNA binding activity in MDA-MB-231 cells was found to be dramatically decreased by 15d-PGJ_2 also at 24 and 48 h after treatment (Fig. 5A, top). At the same times, 15d-PGJ_2 was found to decrease the expression of antiapoptotic proteins, including cIAP-1 and cIAP-2, cFLIP, and Bcl-X_L (Fig. 5A, bottom, and B). In contrast, Bcl-2 protein levels were not reduced, whereas the level of Hsp70 was greatly increased as expected. In the same experiment, the number of Annexin-V^+ cells in 15d-PGJ_2-treated samples was increased to 70%, compared with 9% of control, at 48 h after treatment.

To determine whether the decrease in the antiapoptotic proteins was a consequence of reduced mRNA levels, the accumulation of the survival protein mRNAs was evaluated in MDA-MB-231 cells treated with 15d-PGJ_2 at different concentrations. In the same experiment, NF-κB activity was analyzed by EMSA 3 h after treatment, whereas mRNA levels were determined by RT-PCR 24 h after 15d-PGJ_2 administration. As expected, MDA-MB-231 NF-κB constitutive activity was dramatically decreased already 3 h after treatment (Fig. 5C, top). The analysis of NF-κB-modulated genes showed that treatment with 15d-PGJ_2 caused a dose-dependent reduction in the levels of cIAP-1, cIAP-2, cFLIP, and Bcl-X_L mRNAs (Fig. 5C, bottom), indicating that the decrease in the antiapoptotic proteins described above is due to inhibition of NF-κB-dependent transcriptional activity.

The Cyclopentenone Ring Structure of Natural Prostaglandin Is Responsible for NF-κB Inhibition and Cell Death Induction in ER− Breast Cancer Cells. We have previously shown that, among the different AA metabolites, only the cyclopentenone prostaglandins are able to inhibit TNFα- or mitogen-induced NF-κB activation and that, in fact, the presence of a cyclopentenone structure is essential for NF-κB inhibition (Rossi et al., 1997, 2000; Amici et al., 2001). We then evaluated the effect of AA and different AA

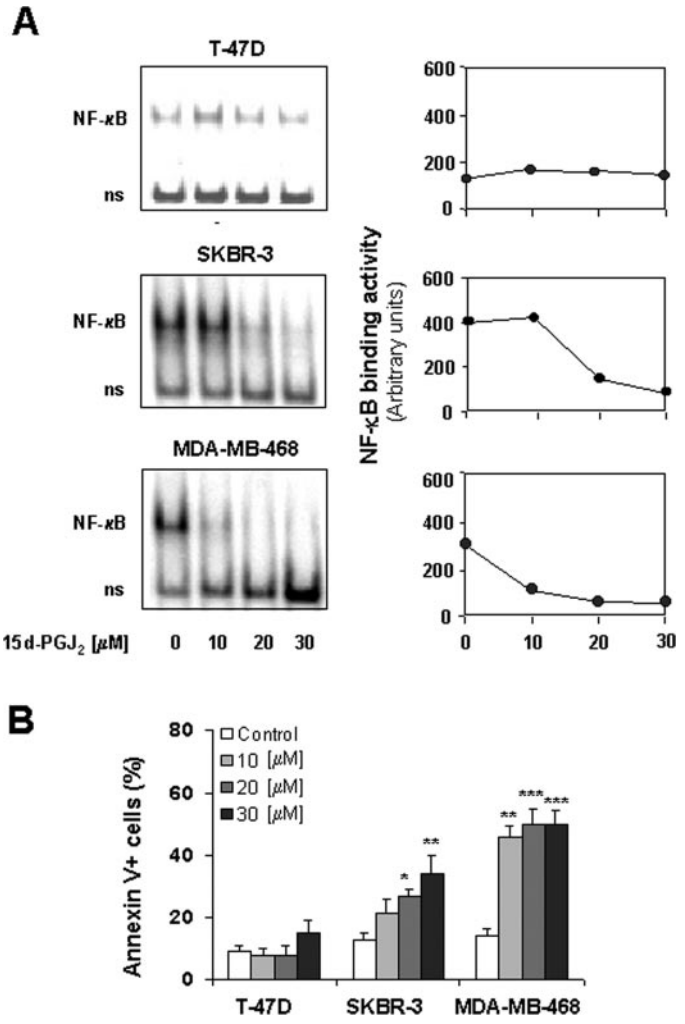

Fig. 4. 15d-PGJ_2 inhibits constitutive NF-κB activity and induces apoptosis in ER− breast cancer cells. ER+ T-47D cells and ER− SKBR-3 and MDA-MB-468 cells were treated with the indicated concentrations of 15d-PGJ_2 or with control diluent. A, after 3 h, nuclear extracts were analyzed for NF-κB activation by EMSA (right). Levels of NF-κB DNA binding activity quantified by MDP analysis are expressed as arbitrary units (left). B, in a parallel experiment, the percentage of Annexin-V^+ cells was determined by flow cytometry after Annexin-V-FITC/PI staining at 48 h after treatment with 15d-PGJ_2 at the indicated concentrations. Data represent the mean ± S.E.M. of two independent experiments, each in duplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

metabolites on NF-κB inhibition and induction of apoptosis in ER− breast cancer cells. MDA-MB-231 cells were treated with 30 μM AA, the noncyclopentenone AA-derivatives PGE_2 and $PGF_{2\alpha}$, the reactive cyclopentenone prostanoids PGA_1 and 15d-PGJ_2, or control diluent. After 3 h, nuclear extracts were assayed for NF-κB activity by EMSA, whereas apoptosis was evaluated by FACS analysis of Annexin-V^+ cells 48 h after treatment. As shown in Fig. 6, among the different AA derivatives, only the cyclopentenone prostanoids were found to reduce NF-κB DNA binding activity and to induce apoptosis, with 15d-PGJ_2 being the most active compound. In contrast, the noncyclopentenone prostaglandins PGE_2 and $PGF_{2\alpha}$ and AA itself had no effect on either NF-κB activity or apoptosis.

As indicated in the Introduction, the unique characteristic of cyPG is the presence of an α,β-unsaturated carbonyl group in the cyclopentane ring, 2-Cy, which renders this portion of the molecule able to bind to specific proteins (Chen et al., 1999). To investigate whether the cyclopentenone ring structure itself, in the absence of the lateral aliphatic side chains, could be effective in inhibiting NF-κB and inducing apoptosis in ER− breast cancer cells, MDA-MB-231 cells were treated with different concentrations of 2-cyclopenten-1-one, and NF-κB activity and the number of Annexin-V^+ cells were determined at 3 or 48 h after treatment, respectively. In a parallel experiment, the activity of 2-Cy was compared with the activity of the 2-Cy derivatives CTC-8 and CTC-35, described in Fig. 7A, which were found to inhibit NF-κB much more potently than the parent compound in a luciferase reporter assay (Bickley et al., 2004). As shown in Fig. 7, B and C (right), 2-cyclopenten-1-one was found to inhibit NF-κB and induce apoptosis, although at concentrations much higher than the natural prostaglandins (>500 μM). Concentrations as high as 1 mM were necessary to induce apoptosis in 60% of MDA-MB-231. The 2-Cy derivatives CTC-8 and CTC-35 were found to be much more active than 2-Cy, inhibiting NF-κB and inducing apoptosis in ER− breast cancer cells at concentrations similar to those of the natural prostanoids. Data shown in Fig. 7 indicate that those of the chemical modification of the cyclopentenone ring (Fig. 7A) caused an increase in the proapoptotic activity of CTC-8 and CTC-35 between 30- and 150-fold compared with 2-Cy. In particular, CTC-35 was found to be more active than 15d-PGJ_2, being able to induce apoptosis in approximately 70% of the cells at the concentration of 5 μM (Fig. 7C).

Discussion

Breast cancer represents one of the leading causes of death among women between the ages of 40 and 55 (Sovak et al., 1997). Estrogen is known to play a major role in the progression of the disease (McGuire et al., 1991). The estrogen receptor, which is required for estrogen-dependent growth, is expressed in more than 60% of breast cancers. Interaction of 17β-estradiol (E2) with ER initiates a sequence of events leading to ER activation and binding to its response element (ERE), which results in the modulation of the expression of hormone-responsive genes responsible for enhanced proliferation of mammary epithelial cells (Biswas et al., 2000). Antihormones such as tamoxifen bind to ER without conferring on it an active configuration, thus blocking consequent downstream events (Jordan, 1992). As the breast cancer progresses, tumor cells can acquire growth autonomy and no longer require estrogen, becoming resistant to antihormones

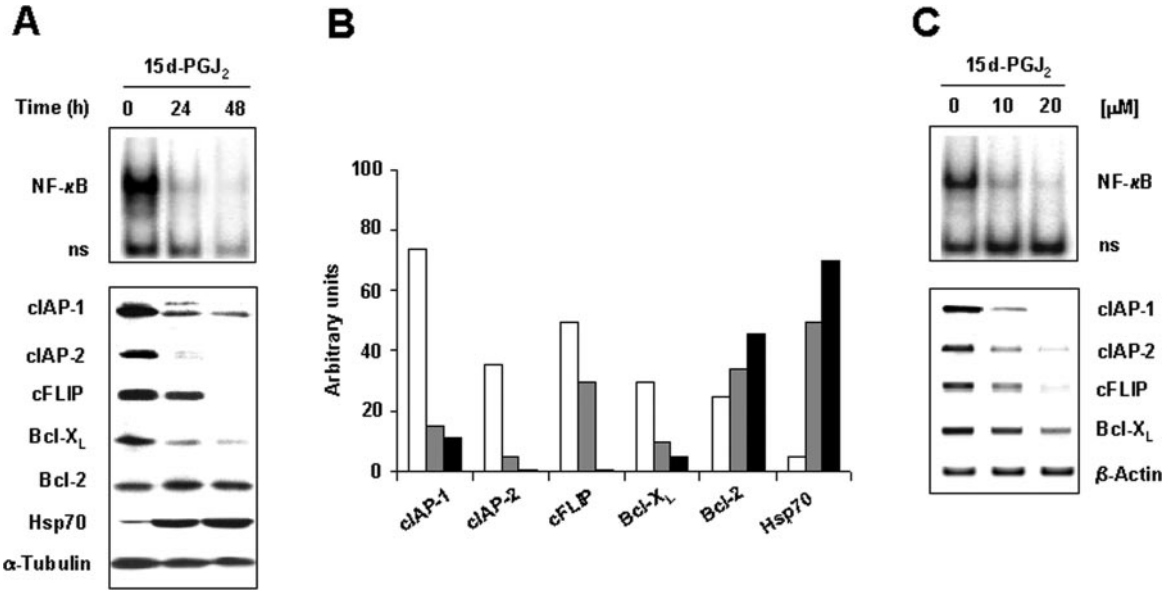

Fig. 5. 15d-PGJ_2 down-regulates the expression of cIAPs, cFLIP, and Bcl-X_L in ER− breast cancer cells. A, MDA-MB-231 cells were treated with 20 μM 15d-PGJ_2 or control diluent. At the indicated times, nuclear extracts were analyzed for NF-κB DNA binding activity by EMSA (top). A section of the fluorograms is shown. Positions of NF-κB-DNA (NF-κB) and nonspecific protein-DNA (ns) complexes are indicated. In the same experiment, whole-cell lysates were immunoblotted with the indicated antibodies. The different proteins were detected on the same blot after stripping (bottom). The levels of Hsp70 and α-tubulin are shown as positive control and loading control, respectively. Inhibition of NF-κB by 15d-PGJ_2 is associated with a decrease in the expression of cIAP-1, cIAP-2, cFLIP, and Bcl-X_L antiapoptotic proteins. B, levels of proteins shown in A, quantified by densitometric analysis, are expressed as arbitrary units after normalization to α-tubulin levels in the same sample. Empty, gray, and filled bars represent protein levels at 0, 24, and 48 h after 15d-PGJ_2 treatment, respectively. C, MDA-MB-231 cells were treated with different concentrations of 15d-PGJ_2. At 3 h after treatment, nuclear extracts were analyzed for NF-κB activity by EMSA (top). Levels of cIAP-1, cIAP-2, XIAP, cFLIP, and Bcl-X_L mRNA in whole-cell extracts were analyzed by RT-PCR 24 h after treatment (bottom). β-Actin mRNA levels were determined and used for normalization.

(Clarke et al., 1993). These antiestrogen-resistant tumors are usually highly invasive and metastatic, and they respond poorly to chemotherapy and radiotherapy. Mutation of ER, down-regulation of ER expression, disregulation of ER-responsive genes, and clonal selection of ER− cells are among the factors believed to be responsible for antiestrogen-resistant growth of breast cancer. ER− breast cancers, which lack the E2-ER-ERE-mediated hormone-dependent cell proliferation pathway, have been shown to contain constitutively elevated levels of NF-κB (Nakshatri et al., 1997; Biswas et al., 2000), and this event has been associated with resistance to apoptosis induced by chemotherapeutic drugs (Patel et al., 2000; Weldon et al., 2001).

In ER− breast cancer, constitutive NF-κB activation is considered to be a downstream consequence of the abnormal epidermal growth factor receptor signaling (Biswas et al., 2000) and to be linked to elevated expression and activity of IKK (Romieu-Mourez et al., 2001). Inhibition of NF-κB by stable expression of IKKβ dominant-negative mutants or by treatment with Gö6976, which blocks NF-κB activation via protein kinase C inhibition, was found to inhibit the growth and to cause regression of ER− mammary tumors in vitro and in vivo (Biswas et al., 2003). The IKK inhibitor curcumin was also shown to possess anti-invasive activity in ER− breast cancer cells by down-regulating the expression of NF-κB-dependent genes that promote tumor invasion, including MMP-2 and VEGF (Bharti et al., 2003). Together, these findings identify IKK and NF-κB as potential therapeutic targets for estrogen receptor negative breast cancer.

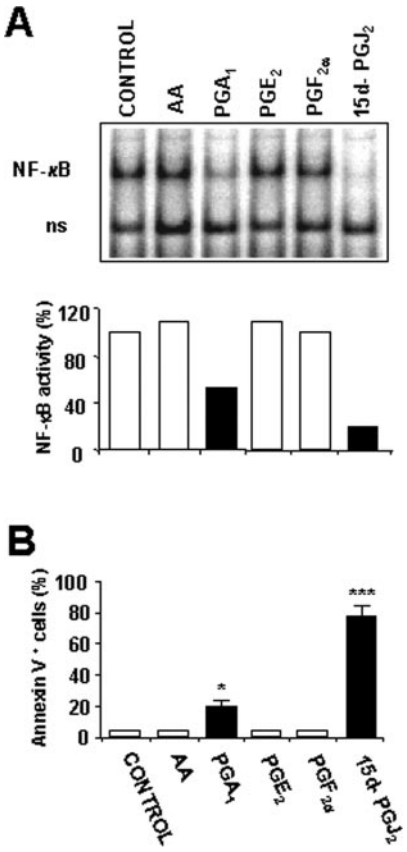

Fig. 6. Effects of arachidonic acid metabolites on cell death and NF-κB activity in MDA-MB-231 cells. MDA-MB-231 cells were treated with 30 μM AA, PGA_1, PGE_2, $PGF_{2\alpha}$, and 15d-PGJ_2 or control diluent. A, after 3 h, nuclear extracts were assayed for NF-κB DNA binding activity by EMSA (top). The levels of NF-κB DNA binding activity were quantified by MDP analysis and expressed as percentage of control (bottom). B, at 48 h after treatment, apoptosis was evaluated by FACS analysis of Annexin-V^+ cells. Data represent the mean ± S.E.M. of duplicate samples. *, $p < 0.05$; ***, $p < 0.001$.

On the basis of our previous observation that cyclopentenone prostanoids prevent virus- and inflammatory cytokine-induced NF-κB activation by direct modification of the IKK complex via binding to the IKK β subunit (Rossi et al., 1997, 2000; Amici et al., 2001, 2004), in the present study we investigated the effect of natural and synthetic cyclopentenones on constitutive NF-κB activity and survival of ER+ and ER− breast cancer cells. In particular, we focused our attention on the natural prostanoid 15d-PGJ_2, which has been shown to possess potent antineoplastic activity in several in vitro and in vivo tumor models, including breast cancer (Clay et al., 2001; Straus and Glass, 2001; Qin et al., 2003).

Because antiestrogen-resistant breast cancers are generally invasive and respond poorly to chemotherapy compared with ER+ tumors (Clarke et al., 1993), we first compared the effect of the conventional chemotherapeutic drugs paclitaxel and doxorubicin on apoptosis induction in ER+ and hormone-dependent MCF-7 cells and in ER− and hormone-independent MDA-MB-231 cells. The results confirmed that the anticancer treatment was very effective in inducing apoptosis in ER+ cells, whereas MDA-MB-231 cells were found to be resistant to doxorubicin and to respond poorly to paclitaxel. Conversely, two well known NF-κB inhibitors, sodium arsenite (Mathas et al., 2003) and the proteasome inhibitor MG132 (Dai et al., 2003), were found to have a potent pro-apoptotic activity in antiestrogen-resistant MDA-MB-231 cells, whereas they had no effect in ER+ MCF7 cells. 15d-PGJ_2 behaved similarly to the second group of compounds, being extremely effective in inducing apoptosis selectively in ER− breast cancer cells. At 48 h after treatment, the percentage of apoptotic MDA-MB-231 cells was increased to 70% at 20 μM and 80% at 30 μM 15d-PGJ_2, whereas no significant change was observed in MCF-7 cells. The differential sensitivity of ER− and ER+ breast cancer cells to 15d-PGJ_2-induced apoptosis was also confirmed by analysis of nuclear condensation and DNA fragmentation. In agreement with previous observations (Clay et al., 2001), apoptosis of MDA-MB-231 cells was mediated by simultaneous activation of multiple caspases demonstrated by the cleavage of initiator caspase-8 and -9 and executioner caspase-3.

When levels of constitutive IKK and NF-κB activities were analyzed in MCF-7 and MDA-MB-231 cells, both activities were found to be 3 times higher in ER− MDA-MB-231 cells than in MCF-7 cells, confirming an increased constitutive DNA binding activity of the nuclear factor in more malignant tumors. A short treatment with 15d-PGJ_2 was found to be effective in inhibiting constitutive IKK activity in MDA-MB-231 cells, where the kinase activity was decreased by more than 35 and 80% already 3 h after treatment with 10 or 20 μM 15d-PGJ_2, respectively. At the higher concentration, 15d-PGJ_2 inhibited the low level of IKK activity also in MCF-7 cells. In ER− breast cancer cells, IKK inhibition was associated with a dramatic inhibition of NF-κB DNA binding activity. Quantitative determination by MDP analysis revealed that 15d-PGJ_2, at the concentration of 10 μM, markedly (>65%) inhibited constitutive NF-κB DNA binding activity

already 3 h after treatment in MDA-MB-231 cells (whereas it was inactive in ER+ cells), and almost completely suppressed it at higher concentrations up to 48 h after treatment. Similar results were obtained in two other human ER− breast cancer cell lines (SKBR-3 and MDA-MB-468), indicating a general effect of 15d-PGJ_2 in this type of cancer.

Inhibition of NF-κB by 15d-PGJ_2 in ER− breast cancer cells was found to lead to down-regulation of the expression of several NF-κB-dependent antiapoptotic gene products, including Bcl-X_L, cIAP-1/2, and cFLIP, followed by activation of multiple caspases. All together, these findings indicate that 15d-PGJ_2 is able to control IKK and NF-κB aberrant regulation in malignant antihormone-resistant breast cancer cells.

As indicated above, cyclopentenone prostaglandins have been shown to possess potent antitumor activity in different in vitro and in vivo cancer models (Straus and Glass, 2001; Piva et al., 2005). Multiple mechanisms of proapoptotic activity have been suggested, including binding to peroxisome proliferator-activated receptor-γ, activation of oxidative stress-mediated mitochondrial apoptosis pathway, and stimulation of mitogen-activated protein kinase kinases (Padilla et al., 2000; Straus and Glass, 2001; Liu et al., 2003; Nencioni et al., 2003). We now suggest that inhibition of NF-κB activity and NF-κB-dependent expression of cell survival proteins may play a major role in the proapoptotic activity of 15d-PGJ_2 in ER− breast cancer. However, we cannot exclude that different mechanisms, other than inhibition of NF-κB activity, may participate in the proapoptotic activity of cyclopentenone prostanoids.

Among several prostaglandins tested, only the cyclopentenone prostanoids PGA_1 and 15d-PGJ_2 were found to reduce NF-κB DNA binding activity and to induce apoptosis in ER− breast cancer cells, with 15d-PGJ_2 being the most active compound, whereas the noncyclopentenone prostaglandins PGE_2 and $PGF_{2\alpha}$ and the prostaglandin precursor arachidonic acid had no effect on either NF-κB activity or apoptosis. As indicated above, the unique characteristic of cyPG is the presence of an α,β-unsaturated carbonyl group in the cyclopentane ring, 2-cyclopenten-1-one, which renders this portion of the molecule able to bind to cysteine-179 in the IKKβ activation loop (Rossi et al., 2000). We then investigated whether the cyclopentenone ring structure itself, in the absence of the lateral aliphatic side chains, could be effective in inhibiting NF-κB and inducing apoptosis in ER− MDA-MB-231 cells. In addition, the activity of 2-cyclopenten-1-one was compared with the activity of two 2-Cy derivatives, CTC-8 and CTC-35, whose synthesis has been recently described (Bickley et al., 2004). 2-Cyclopenten-1-one was found to be able to inhibit NF-κB and induce apoptosis in MDA-MB-231 cells, but at concentrations much higher than the natural prostaglandins (>500 μM). It is noteworthy that 2-Cy derivatives CTC8 and CTC35 were much more effective than the parent compound, with an activity comparable or greater than the natural prostanoids, both in inhibiting NF-κB and inducing apoptosis in ER− breast cancer cells. These results indicate that a simple substitution of a hydrophobic group into a cyclopentenone molecule enhances the biological activ-

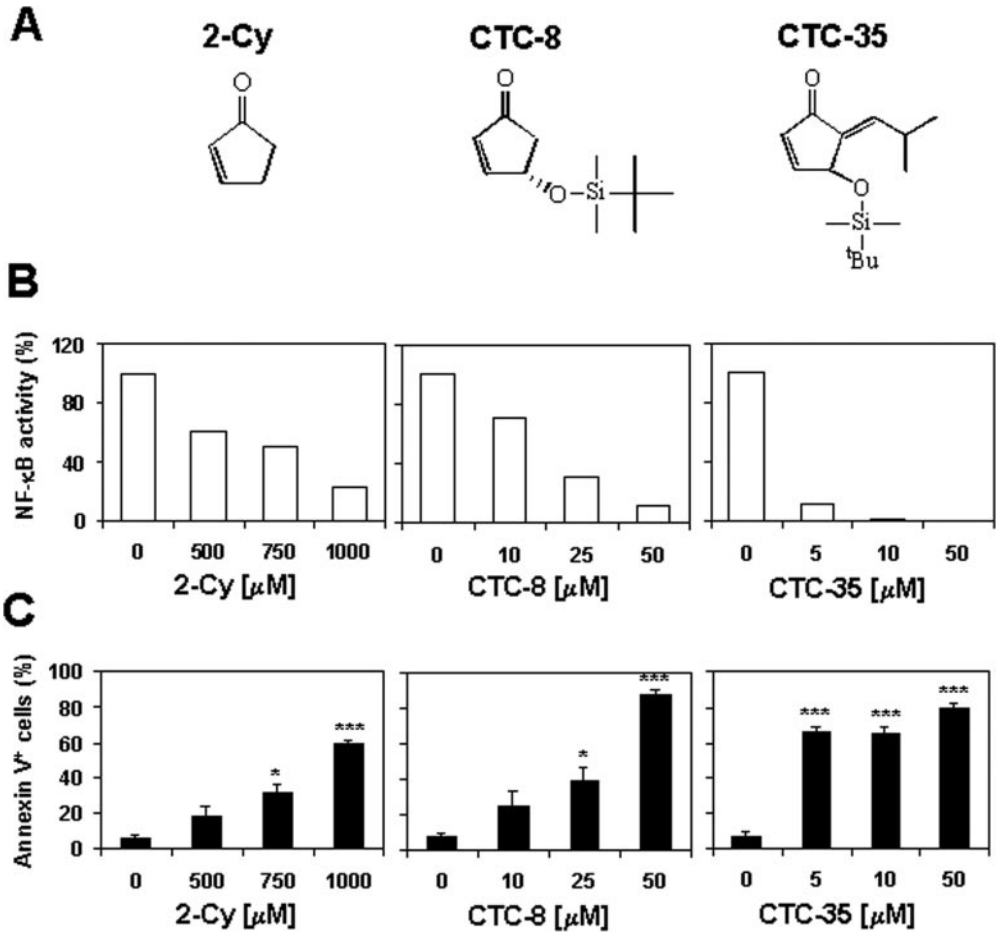

Fig. 7. 2-Cy and 2-Cy derivatives CTC-8 and CTC-35 inhibit NF-κB activity and induce apoptosis in ER− breast cancer cells. A, structures of 2-cyclopenten-1-one, CTC-8, and CTC-35. B and C, MDA-MB-231 cells were treated with different concentrations of 2-Cy (right), CTC-8 (middle), and CTC-35 (left). After 3 h, nuclear extracts were assayed for NF-κB DNA binding activity by EMSA. The levels of NF-κB DNA binding activity were quantified by MDP analysis and expressed as percentage of control (B). In the same experiment, apoptosis was measured 48 h after treatment by FACS analysis of Annexin-V^+ cells (C). Data represent the mean ± S.E.M. of duplicate samples. *, $p < 0.05$; ***, $p < 0.001$.

ity of the five-membered ring system and identifies CTC35 as a new potent anticancer agent.

Prostaglandins are used clinically in the treatment of gastroduodenal ulcers, congenital heart disease, and erectile dysfunction, and to facilitate labor, and they are generally effective and well tolerated (Vane and O'Grady, 1993). The results described in the present report indicate that cyclopentenone prostanoids are potent inducers of apoptosis in aggressive ER− breast cancer that is often resistant to chemotherapy. We also show that the cyclopentenone ring structure itself is essential for the anticancer activity. The fact that 2-cyclopenten-1-one and the 2-Cy derivatives CTC-8 and CTC-35 were able to inhibit constitutive NF-κB activity and induce apoptosis in ER− breast cancer cells suggests that a new class of novel NF-κB inhibitors could be designed, which could be of therapeutic value in the treatment of breast cancers presenting aberrant NF-κB regulation.

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