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Interleukin-8 Expression Is Regulated by Histone Deacetylases through the Nuclear Factor-κB Pathway in Breast Cancer[S]

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ABSTRACT

We have reported recently that the chemokine interleukin 8 (IL-8)/CXCL8 was overexpressed in invasive estrogen receptor (ERα)-negative breast cancer cells compared with ERα-positive breast cancer cells. We now demonstrate that histone deacetylases (HDACs) play an essential role in the regulation of IL-8 gene expression in ERα-positive MCF-7 breast cancer cells. Treatment of MCF-7 cells with the HDAC inhibitor trichostatin A (TSA) led to a strong up-regulation of IL-8 protein and RNA levels in MCF-7 cells. The up-regulation of IL-8 in MCF-7 cells was time- and concentration-dependent. Moreover, run-on and transfection experiments demonstrated that IL-8 induction by HDAC inhibitors was transcriptional and involved mainly the nuclear factor-κB (NF-κB) site of the IL-8 promoter. These observations are corroborated by an up-regulation of NF-κB activity in MCF-7 cells in the presence of TSA. In addition, blocking NF-κB pathway by adenoviral delivery of a dominant-negative IκB or IκB kinase complex 2 (IKK2) mutant abolished IL-8 gene induction by histone deacetylase inhibitors. HDAC inhibitors triggered IKK phosphorylation and up-regulated p65 nuclear translocation, although they decreased the protein levels of IκBα, which accounts for NF-κB activation. TSA increased binding of acetylated histone 3 to the IL-8 gene promoter. In summary, our results demonstrate that NF-κB pathway repression by HDAC is responsible for the low expression of IL-8 in ERα-positive breast cancer cells.

IL-8 (CXCL8), initially known for its function in recruitment and activation of immune and inflammatory cells during inflammation, is a multifunctional chemokine, which is involved in a variety of physiopathological processes (Moser et al., 2004). IL-8 has been shown to be expressed by a number of cancer cells, including breast cancer (Freund et al., 2003, 2004; Ali and Lazennec, 2007; Bièche et al., 2007; Chavey et al., 2007). IL-8 is believed not only to favor the invasion and the metastatic spread of cancer cells but also to increase angiogenesis in growing tumors (Freund et al., 2003; Lin et al., 2004; Bièche et al., 2007; Yao et al., 2007). We have reported previously that IL-8 is overexpressed in highly invasive breast cancer cells lacking the estrogen receptor α (ERα) compared with ERα-positive breast cancer cells (Freund et al., 2003; Ali and Lazennec, 2007).

We hypothesized that the low expression of IL-8 in ERα-positive breast cancer cells could be the result of epigenetic repression. Epigenetic events like DNA methylation and histone acetylation are known to play an important role in regulating gene expression and in cancer (Marks et al., 2000; Margueron et al., 2003; Duong et al., 2006; Yang and Seto, 2007). Unwinding of the closed, repressive chromatin configuration during active transcription of genes permits accessibility to transcription factors and transcriptional control. It is believed that histone acetylation and acetylation of transcription factors, governed by histone acetyltransferase activity, generally promotes transcriptional activation of genes after conformational changes within the chromatin, whereas repression of transcriptional activity is commonly correlated

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ABBREVIATIONS: IL-8, interleukin-8; ER, estrogen receptor; HDAC, histone deacetylase; TSA, trichostatin A; IKK, IκB kinase; NIK, nuclear factor-κB-inducing kinase; NF-κB, nuclear factor-κB; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; bp, base pair; ELISA, enzyme-linked immunosorbent assay; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; AP-1, activator protein 1; dn, dominant negative; TNF-α, tumor necrosis factor α; C/EBP, CCAAT/enhancer-binding protein; rS9, ribosomal protein S9; MG-132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

with histone hypoacetylation due to histone deacetylase (HDAC) activity (Wu, 1997; Kuo and Allis, 1998; Wade, 2001). Differential acetylation of histones and transcription factors plays an important regulatory role in the developmental process, proliferation, and differentiation (Timmermann et al., 2001). Consistent with this is the involvement of aberrant acetylation in cancer. Both histone hyperacetylation and hypoacetylation seem to be implicated in the neoplastic process, depending on the target gene involved. Thus, in cancer, some genes such as tumor suppressor genes might be repressed by the inappropriate recruitment of HDACs (Glozak and Seto, 2007; Yang and Seto, 2007). Inhibitors of histone deacetylase are now being evaluated for their therapeutic effects in cancer (Marks et al., 2000; Rasheed et al., 2007).

In this study, we investigated whether IL-8 expression in breast cancer cells is epigenetic. Our present data show that inhibition of HDAC activity using the HDAC inhibitor trichostatin A (TSA) increases IL-8 expression in ERα-positive MCF-7 breast cancer cells producing low IL-8 levels. Moreover, IL-8 induction by TSA in MCF-7 cells involves the NF-κB pathway. This occurs through an increase of p65 nuclear translocation and IKK phosphorylation, a concomitant decrease of IκBα levels, and an acetylation of histone H3 on the IL-8 promoter. In summary, our results demonstrate that NF-κB pathway repression by HDAC is responsible for the low expression of IL-8 in ERα-positive breast cancer cells.

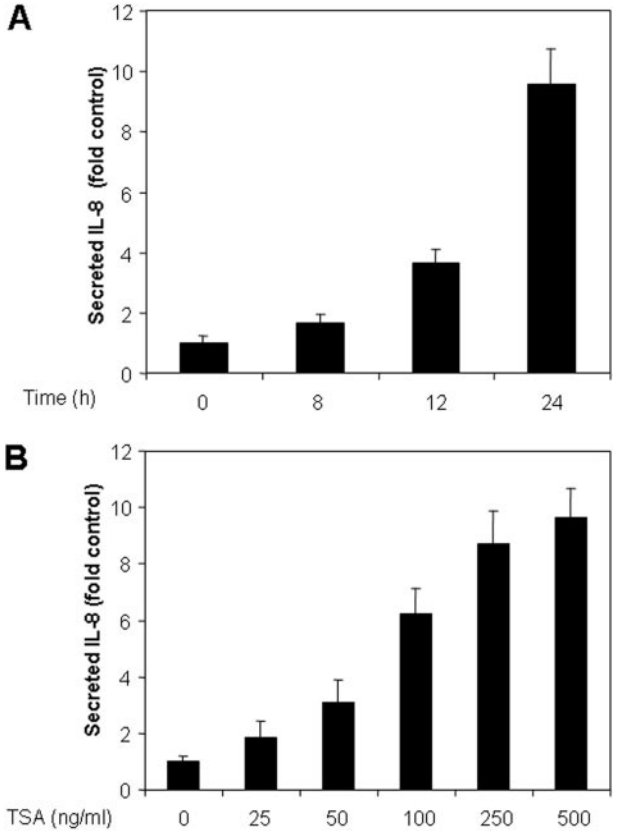

Fig. 1. Induction of IL-8 protein secretion in MCF-7 cells by TSA is time- and concentration dependent. A, MCF-7 cells were treated with ethanol vehicle (control) or TSA (500 ng/ml) for 0, 8, 12, or 24 h. Culture medium was collected, and IL-8 secretion was evaluated by ELISA. Results are expressed as -fold induction compared with control cells and represent the mean ± S.D. of three independent experiments. B, MCF-7 cells were treated with ethanol vehicle (control) or TSA at the designated concentrations for 24 h and evaluated for IL-8 secretion. Results are expressed as -fold induction compared with control cells and represent the mean ± S.D. of three independent experiments.

Materials and Methods

Cell Culture. MCF-7 cells were maintained in DMEM/F-12 medium supplemented with 10% fetal calf serum (FCS) and gentamicin as described previously (Lazennec et al., 1996). CAMA-1 and BT-474 cells were cultured in DMEM/F-12 medium supplemented with 10% FCS.

Plasmids. Wild-type and mutant constructs of IL-8 promoter corresponding to −1481/+44 bp have been described previously (Freund et al., 2004). NF-κB family expression vectors (p50 and p65) and the dominant-negative form of IκBα, IKK2, were a kind gift of Dr. B. B. Aggarwal, Dr. W. C. Greene, Dr. R. De Martin, and Dr. H. Nakshatri. pNF-κB reporter plasmid was from Clontech (Mountain View, CA). CMV-GAL corresponds to the β-galactosidase gene under the control of the cytomegalovirus (CMV) promoter.

Transient Transfection. Cells (3×10^5) were plated in six-well plates in DMEM/F-12 supplemented with 10% FCS 24 h before transfection. Transfections were performed as described previously (Lazennec et al., 2001) with Lipofectamine using 4 μg of luciferase reporter along with 100 ng of each expression vector and 0.8 μg of the internal reference reporter plasmid (CMV-Gal) per well. After overnight lipofection, the medium was removed, and the cells were placed into fresh medium supplemented with control vehicle ethanol or TSA. Eighteen hours later, cells were harvested and assayed for luciferase activity on a Centro LB960 Berthold luminometer (Berthold Technolo-

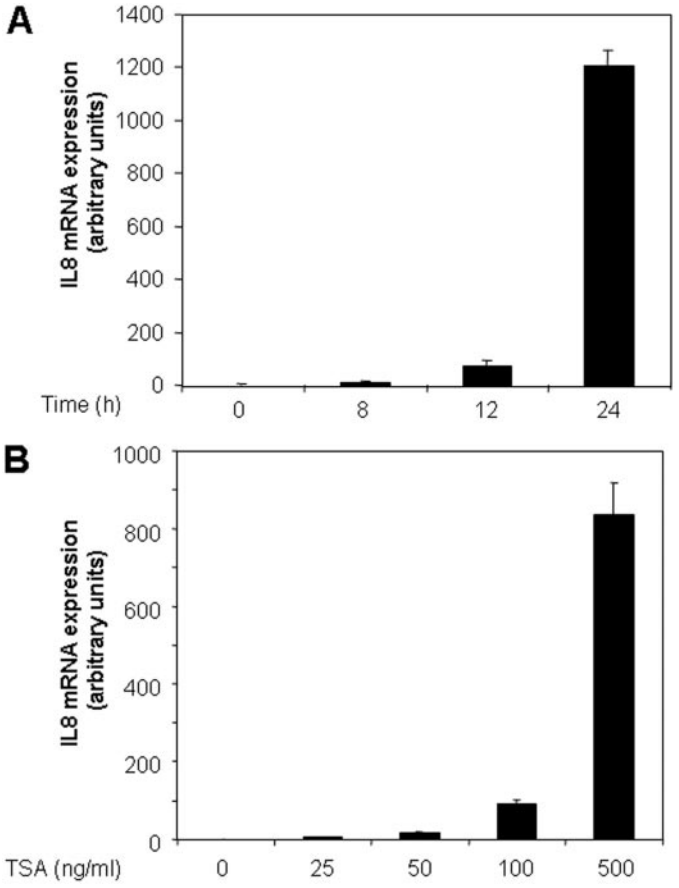

Fig. 2. IL-8 mRNA expression is induced by TSA. RNA was extracted and reverse-transcribed from the same cells as in Fig. 1 and IL-8 RNA levels were determined by quantitative PCR on isolated mRNA. Results are expressed as arbitrary units after normalization to rS9 levels and represent the mean ± S.D. of three independent experiments.

gies, Bad Wildbad, Germany). β-Galactosidase was determined as described previously (Duong et al., 2006).

Recombinant Adenovirus Construction, Propagation, and Infection. The adenoviruses Ad5 and dominant-negative IκB [IκB(SA)2, with S32A and S36A mutations], dominant-negative IKK2, and dominant-negative NIK used in this study and their propagation have been described previously (He et al., 1998; Lazennec et al., 2001; Oitzinger et al., 2001; Haller et al., 2002; Russo et al., 2002; Lucas et al., 2003; Freund et al., 2004). MCF-7 cells were infected for 6 h at a multiplicity of infection of 100 with the different adenoviruses in DMEM/F-12 10% FCS. The next day, the medium was changed, and the cells were treated with ethanol vehicle or TSA for 18 h before collecting culture medium and harvesting cells for ELISA assays and RNA isolation, respectively.

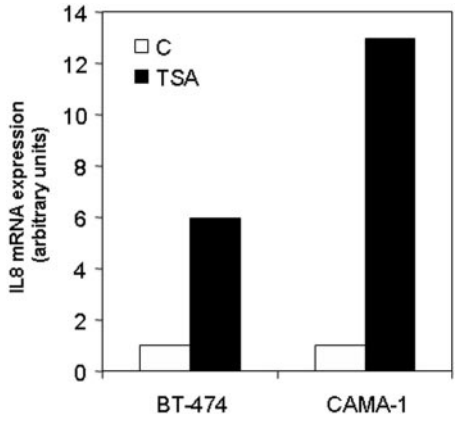

Fig. 3. TSA induces IL-8 mRNA expression in BT-474 and CAMA-1 cells. BT-474 and CAMA-1 cells were treated with ethanol vehicle (C) or TSA (500 ng/ml) for 18 h. RNA was extracted and reverse-transcribed from the same cells as in Fig. 2 and IL-8 mRNA levels were determined by quantitative PCR on isolated mRNA. Results are expressed as arbitrary units after normalization to rS9 levels and represent the mean of two independent experiments.

Cell Lysis and Western Blotting. Cells were harvested, centrifuged (5 min, 800*g*, 4°C), and washed with ice-cold phosphate-buffered saline. For whole-cell extracts, cells were harvested in Tris-glycerol buffer (Tris-HCl 50 mM, EDTA 1.5 mM, and 10% glycerol) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and were then sonicated. For nuclear cell extracts, cells were centrifuged, and pellets were resuspended in buffer A (10 mM HEPES pH 7.2, 60 mM KCl, 1 mM EDTA, and 0.5% Nonidet P-40) supplemented with protease inhibitor cocktail (Roche) and incubated on ice for 15 min and then centrifuged (30 s, 12,000*g*, 4°C). Pelleted nuclei were resuspended in buffer B (10 mM HEPES pH 7.2, 60 mM KCl, and 1 mM EDTA) supplemented with protease inhibitor cocktail, lysed by three freezing/defreezing cycles (liquid nitrogen, 37°C) and then centrifuged (10 min, 13,000 rpm, 4°C). For IKK phosphorylation assessment, cells were scraped in cold phosphate-buffered saline and then lysed in buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 2 mM EDTA) containing phosphatase inhibitors (β-GP 20 mM, sodium fluoride 10 mM, and sodium orthovanadate 1 mM) and protease inhibitors. Protein extracts were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analyses done using p65, p50, IκBα (all Santa Cruz Biotechnology, Santa Cruz, CA), IKKα, phosphor-IKKα (Ser180)/IKKβ (Ser181) (both from Cell Signaling Technology Inc., Danvers, MA), and actin (Sigma-Aldrich, St. Louis, MO). Immunoreactivity was detected with Amersham Enhanced Chemiluminescence system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Actin was used as a loading control. IKK-P phosphorylation was quantified with the Scion Image software (Scion Corporation, Frederick, MD).

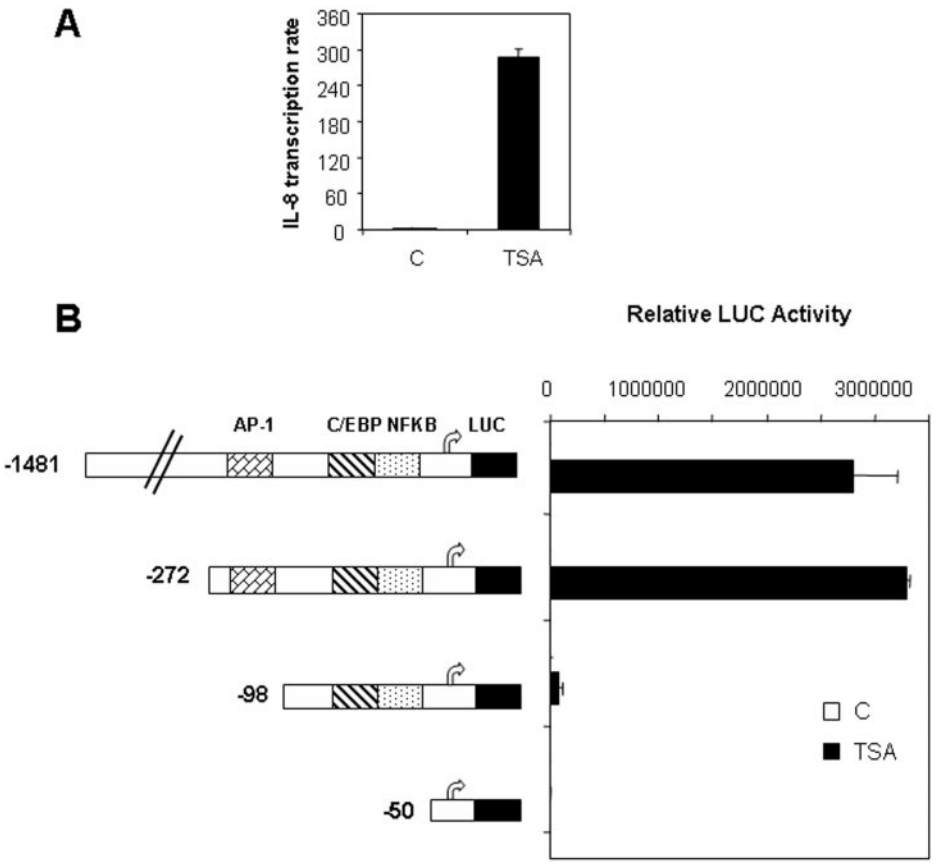

Fig. 4. TSA affects IL-8 gene regulation at a transcriptional level. A, MCF-7 cells were treated with control vehicle ethanol (C) or TSA (500 ng/ml) for 18 h. The transcription rates of IL-8 and rS9 genes were determined using run on assay. The transcription rate, expressed in arbitrary units corresponding to the ratio of IL-8 signal over rS9 signal, represents the mean ± S.D. of three independent experiments. B, MCF-7 cells were transfected with wild-type or deleted constructs corresponding to the first 1481, 272, 98, or 50 bp of the IL-8 promoter. Cells were then treated with control vehicle ethanol (C) or TSA (500 ng/ml) for 18 h before harvesting cells for luciferase assays. Results show relative luciferase activities ($n = 3$) after normalization for β-galactosidase activity.

IL-8 ELISA. IL-8 concentration in culture supernatants was determined by ELISA with Duoset kit (R&D Systems, Minneapolis, MN) as recommended by the manufacturer.

RNA Extraction, Reverse Transcription, and Quantitative PCR. Total RNA was isolated with TriReagent reagent (Euromedex, Souffelweyersheim, France) as described by the manufacturer. Reverse transcription was performed using 5 μg of total RNA, random primers, and Superscript II enzyme (Invitrogen, Carlsbad, CA). Quantitative PCR was performed with FastStart DNA Master SYBR Green I kit (Roche) on a Light Cycler instrument (Roche) as specified by the manufacturer. Ribosomal protein S9 (rS9) was used as an internal control. Primers used were as follows: IL-8: sense, CACCGGAAGGAACCATCTCACT; antisense, TCAGCCCTCTTCAAAAACTTCTCC; IκBα: sense, CGTTATGAGCGCAAAGG; antisense, AGTCACTCGAAGCACA; P50: sense, GCAAATCTCCGGGGGCATCAAACC; antisense, CTCCGCTTCCGCTGCACCTCTTCC; p65: sense, CTCCGCGGGCAGCATCC; antisense, AGCCGCACAGCATTCAGGTCGTAG; and rS9: sense, AAGGCCGCCCGGGAACTGCTGAC; antisense, ACCACCTGCTTGCGGACCCTGATA.

Run-On Assay. IL-8 and rS9 transcription was measured by run-on assays described previously (Freund et al., 2004). In brief, equal amounts (10^7 cpm/ml) of labeled nuclear RNA were hybridized at 65°C for 24 h to ζ-probe membranes (Bio-Rad, Hercules, CA) bound previously with 5 μg of linearized plasmid DNAs. The immobilized plasmids used were pCR2-IL8 and pCR2-rS9. After washing, the filters were subjected to autoradiography. Radioactive transcripts were quantified on a Fuji Bas Reader (Fuji Film, Tokyo, Japan). Data were normalized to transcription of the rS9 gene.

Chromatin Immunoprecipitation Analysis. MCF-7 cells were stimulated with TSA (500 ng/ml) for 0, 1, 6, and 18 h, and chromatin immunoprecipitation (ChIP) assays were performed an Upstate-Cell Signaling kit (Millipore Bioscience Research Reagents, Temecula, CA) as described previously (Mühlbauer et al., 2008). Immunoprecipitation was carried out using 5 μg of anti-acetyl-histone H3 antibody (acetylated lysines in position 9 and 14 of the protein; Upstate-Cell Signaling Solutions). IL-8 promoter-specific primers (−121 to +61 of the IL-8 promoter) were as follows: IL-8 sense, GGGCCATCAGTTGCAAATC; and ChIP IL-8 antisense, TCCTTCCGGTGGTT-

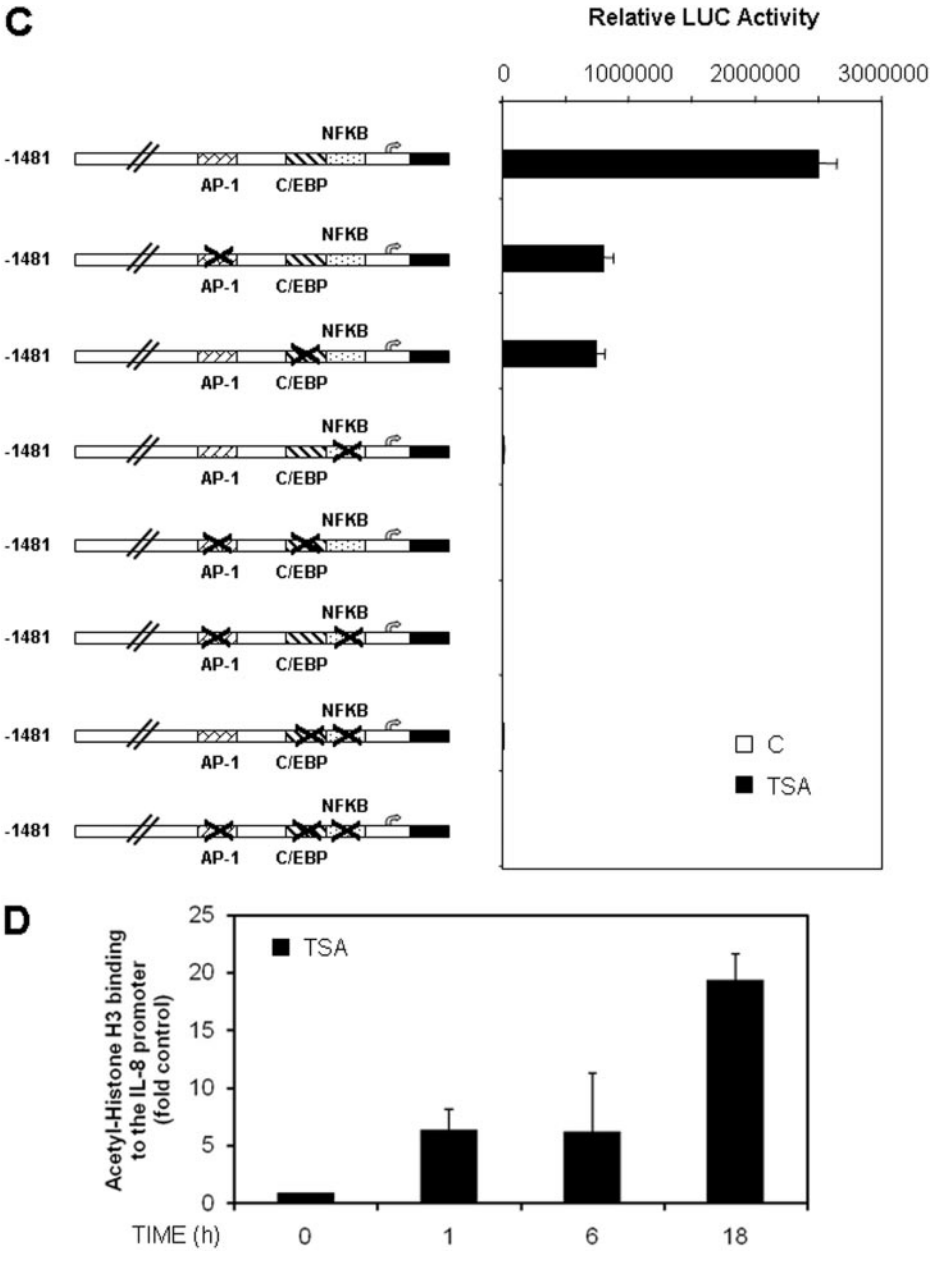

Fig. 4. C, IL-8 promoter constructs harboring single, double, or triple mutations of AP-1, C/EBP, or NF-κB sites were transfected in MCF-7 cells in the same conditions as in B. Results show relative luciferase activities (n = 3) after normalization for β-galactosidase activity. D, MCF-7 cells were treated with control vehicle ethanol (control) or TSA (500 ng/ml) for 0, 1, 6, and 18 h and ChIP assays performed on the IL-8 promoter using an anti-histone H3 antibody as described under *Materials and Methods*. Quantitative PCR was performed, and relative -fold changes were determined by a semiquantitative assay using the ABI 7700 sequence detection system. The results are representative of three independent experiments.

TCTTC. Relative -fold changes in H3 were determined by a semi-quantitative assay using the ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA) as described previously (Mühlbauer et al., 2008).

Results

Deacetylase Inhibition Results in Increase of IL-8 Expression in MCF-7 Breast Cancer Cells. ERα-positive MCF-7 breast cancer cells express low levels of IL-8 (Freund et al., 2003), which could be the result of an epigenic repression. To determine whether IL-8 gene expression is modified by acetylation regulation, we treated MCF-7 breast cancer cells with the deacetylase inhibitor TSA. As shown in Fig. 1A, such treatment resulted in a 10-fold increase of secreted IL-8 by MCF-7 cells compared with nontreated control cells. The basal level of IL-8 secretion of MCF-7 was low and between 25 and 50 pg/ml, according to these experiments. The TSA-mediated induction of IL-8 secretion in MCF-7 cells was time- and concentration-dependent with maximal induction at 24 h and 500 ng/ml, respectively (Fig. 1B). As shown by quantitative PCR, treatment of MCF-7 cells with TSA also increased IL-8 mRNA expression above control levels in a time- and dose-dependent manner. Consistent with IL-8 secretion levels, maximal induction of IL-8 mRNA was observed at 24 h and 500 ng/ml, respectively (Fig. 2). It is remarkable that maximal induction of IL-8 mRNA expression in MCF-7 cells exceeded control levels up to 300-fold. Higher doses of TSA did not lead to a further induction of IL-8 gene (Supplemental Fig. 1). The induction of the IL-8 gene expression by TSA was not restricted to MCF-7 cells but could be also observed in BT-474 and CAMA-1 breast cancer cells (Fig. 3).

IL-8 Regulation by TSA Is Transcriptional and Involves the NF-κB Pathway. To determine whether the effects of HDAC inhibitors observed in MCF-7 cells were transcriptional, we performed run-on experiments (Fig. 4A). We observed a strong up-regulation of IL-8 gene activity by approximately 100-fold in the presence of TSA. This led us to the question of which sequences of the IL-8 promoter could account for this regulation by TSA. MCF-7 cells were transfected with various 5′-truncated IL-8 promoter-constructs and assayed for their responsiveness to TSA (Fig. 4B). Elimination of sequences located between −1481 and −272 bp of the promoter did not affect significantly the transcriptional response to TSA. On the contrary, a drastically decreased induction by TSA was observed with the −98-bp promoter construct lacking the AP-1 site, compared with the full-size IL-8 promoter. Moreover, the inducibility by TSA was completely lost with the −50-bp IL-8 promoter construct, in which the three upstream sequence elements for AP-1, NF-κB, and C/EBP are lacking (Fig. 4B). To further characterize the contribution of defined sequence elements, we tested different point-mutated IL-8 promoter constructs (Fig. 4C). Mutation of the AP-1 or C/EBP site inhibited IL-8 transcriptional activation by TSA by approximately 3-fold, whereas mutation of NF-κB completely abolished the responsiveness to TSA, demonstrating that NF-κB was the predominant element in TSA-mediated transcriptional activation. It is interesting that the mutation of both C/EBP and AP-1 sites led also to a complete shutdown of TSA induction of IL-8 promoter, suggesting that these sites are also contributing to IL-8 regulation by HDAC inhibitors. We next investigated whether TSA could enhance histone H3 acetylation of the endogenous IL-8 gene promotor. ChIP experiments showed that the levels of acetylated histone H3 present on the IL-8 gene promoter were strongly enhanced by HDAC inhibitor treatment (Fig. 4D).

p65 and IκB Are the Main Regulators of IL-8 Promoter Induction in Response to TSA. Because the NF-κB site was the main regulator of IL-8 gene regulation by TSA, we analyzed in more detail the contribution of the NF-κB pathway to this phenomenon. We further tested the functional involvement of the p50 and p65 subunits of NF-κB in TSA-mediated IL-8 promoter induction in MCF-7 cells. Although cotransfection with p65 alone resulted in approximately 3-fold potentiation of IL-8 promoter activity in response to TSA in MCF-7 cells, cotransfection with p50 did not further modulate TSA induction of IL-8 promoter (Fig. 5A). Cotransfection of both p50 and p65 increased IL-8 promoter activity by approximately 2-fold in the presence of TSA. Next, we took advantage of an NF-κB super-repressor, IκBα IκB(SA)2 (Haller et al., 2002), which expresses an IκB protein that is not degraded by the proteasome and thus prevents both p65 nuclear translocation and DNA binding. It is interesting that IκB(SA)2 reduced TSA induction of IL-8

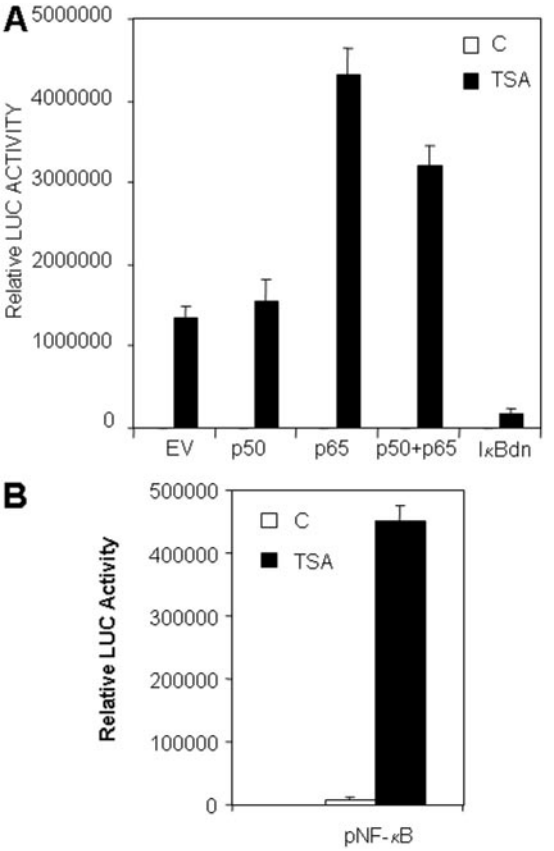

Fig. 5. p65 potentiates induction of IL-8 transcriptional activity in response to TSA. A, 100 ng of expression vectors for p50 and p65, IκBdn (SA)2, or empty vector (EV) were cotransfected along with xp2-IL8 reporter in MCF-7 cells. Cells were treated with ethanol vehicle (C) or with 500 ng/ml TSA for 18 h before harvesting cells for luciferase assays. Results show relative luciferase activities ($n = 3$) after normalization for β-galactosidase activity. B, MCF-7 cells were transfected with pNF-κB reporter construct and treated as in A. Results show relative luciferase activities ($n = 3$) after normalization for β-galactosidase activity.

promoter by approximately 80% (Fig. 5A). Based on these results, we analyzed whether NF-κB activity was differentially regulated by TSA in MCF-7 cells, by transfecting a NF-κB reporter (Fig. 5B). When TSA was added, the reporter activity was enhanced approximately 60-fold in MCF-7 cells, demonstrating that NF-κB signaling was activated (Fig. 5B).

To analyze more precisely the NF-κB signaling pathway, we infected the cells with dominant-negative forms of IκB, IKK2, and NIK (Fig. 6A). Dominant-negative NIK reduced by half IL-8 RNA induction by TSA, whereas IKK2dn and IκBdn decreased by more than 90% IL-8 RNA levels (Fig. 6A). Measurement of secreted IL-8 levels showed that Ad-IκBdn virus could reduce by approximately 80% IL-8 secretion in the presence of TSA (Fig. 6B). We next assessed whether IKK was subjected to phosphorylation upon TSA treatment. Our results showed that IKKα total levels were not affected by TSA treatment, whereas IKKα and IKKβ phosphorylation were increased by TSA addition (Fig. 6C).

NF-κB Signaling Is Modulated by HDAC Inhibitors. Because NF-κB signaling was strongly enhanced by TSA, we evaluated whether p50 and p65 levels were modulated by HDAC inhibition. At the RNA level, p65 levels remained unchanged, whereas p50 RNA was increased by 2-fold (Fig. 7A). In terms of proteins, total p65 protein levels remained unchanged (Fig. 7B, right), whereas p65 nuclear translocation increased (Fig. 7B, left). In contrast, nuclear p50 protein levels were not affected (Fig. 7C). In addition, IκB expression was reduced both at the RNA and protein levels by TSA (Fig. 7, D and E). IκB down-regulation by TSA could be reduced by the use of MG-132 proteasome inhibitor, demonstrating that IκB protein was also subject to degradation by HDAC inhibitor treatment (data not shown).

Discussion

In the present work, we explored the hypothesis that the low expression of IL-8 gene in ERα-positive breast cancer cells could be the result of an inhibition by HDACs. We show that in MCF-7 cells, in which constitutive IL-8 gene expression was low, histone deacetylase inhibition with TSA increased dramatically IL-8 production. This result could be obtained with other ERα-positive breast cancer cells such as BT-474 and CAMA-1 cells. The increase in IL-8 secretion was also consistent with an increase in IL-8 RNA, gene transcription, histone H3 acetylation on the IL-8 promoter, and increased activity of the IL-8 promoter. This regulation seems relatively early, because IL-8 RNA levels are already increased after 8 h of TSA treatment. However, to obtain a full induction, a treatment for 24 h with HDAC inhibitors was required. Such strong regulation of cytokine genes by HDAC inhibitors has been reported for IL-6 when cells were cotreated with TNF-α and TSA (Zhong et al., 2002; Adam et al.,

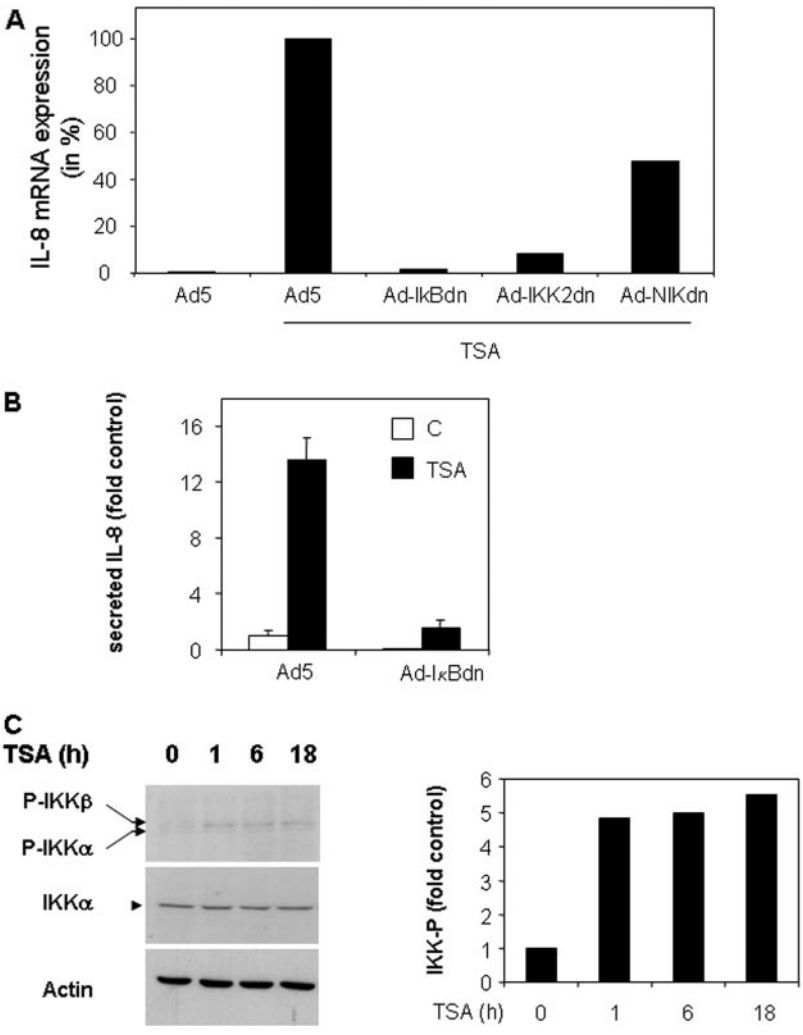

Fig. 6. IκB abolishes induction of IL-8 secretion and RNA levels in response to TSA. A, MCF-7 cells were infected with empty Ad5, Ad-IκBdn, Ad-IKK2dn, or Ad-NIKdn adenoviral vectors. Twenty-four hours after infection, cells were treated with ethanol vehicle or TSA (500 ng/ml) for 18 h before harvesting cells. RNA from the cells was harvested and IL-8 contents were determined by quantitative PCR. IL-8 mRNA levels of Ad5-infected cells in the presence of TSA were set to 100%. Results represent the mean of two independent experiments. B, MCF-7 cells were infected with Ad5 or Ad-IκBdn adenoviral vectors. Twenty-four hours after infection, cells were treated with ethanol vehicle or TSA (500 ng/ml) for 18 h before harvesting cells. Culture medium was collected, and IL-8 secretion was evaluated by ELISA. Results are expressed as -fold induction compared with control cells and represent the mean ± S.D. of three independent experiments. C, MCF-7 cells were treated with TSA (500 ng/ml) for 1, 6, or 18 h. Total IKKα, and P-IKKα and P-IKKβ levels were measured by Western blot. Left, a representative experiment is shown. Right, quantification of P-IKKα and P-IKKβ levels together with the basal phosphorylation (0 h) of both IKK set to 1.

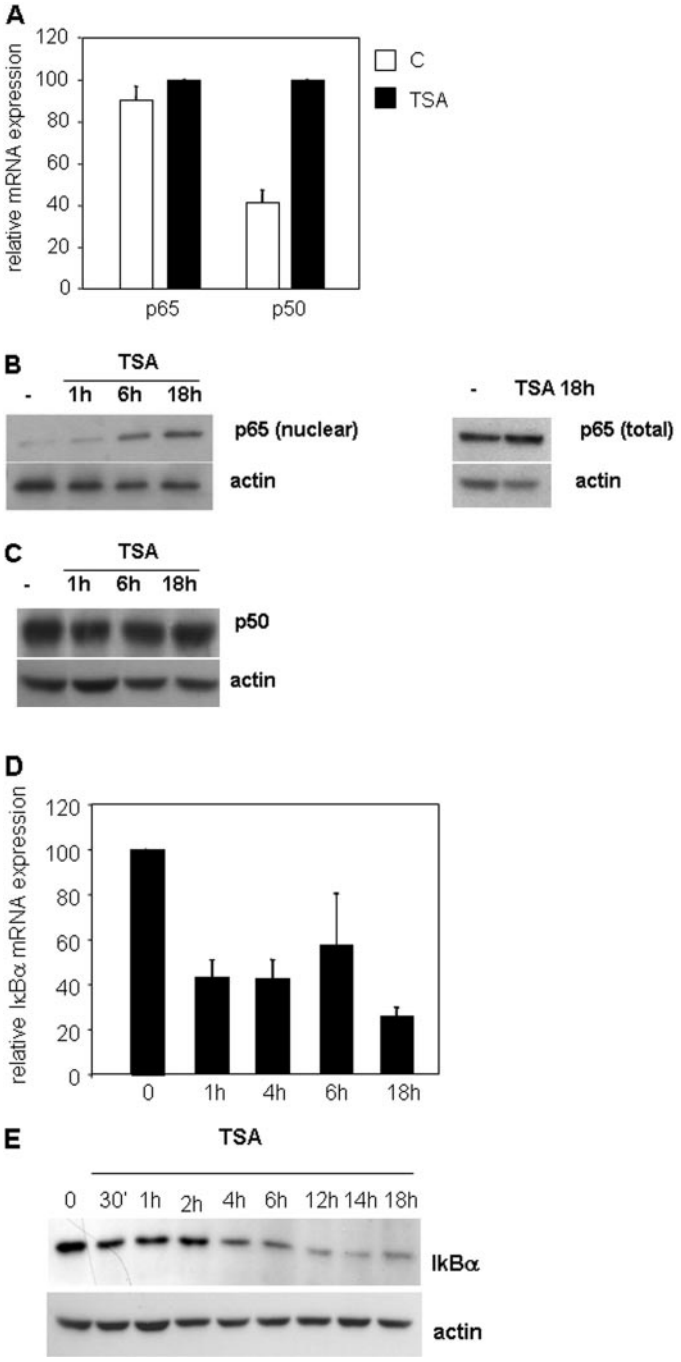

Fig. 7. TSA regulates the expression of different NFκB members. A, MCF-7 cells were treated with ethanol vehicle or TSA (500 ng/ml) for 18 h before harvesting cells. RNA was isolated, and p50 and p65 mRNA expression was determined by quantitative PCR. p50 and p65 RNA levels in the presence of TSA were set to 100%. Results represent the mean ± S.E.M. of three independent experiments. B, left, nuclear extract from MCF-7 cells treated with ethanol vehicle or TSA (500 ng/ml) for 1, 6, or 18 h were prepared for Western blot analysis using antibodies against p65 and actin. Right, whole-cell extracts of MCF-7 cells prepared in the same conditions. Data shown are representative of three independent experiments. C, nuclear levels of p50 protein were measured by Western blot as shown in B. D, MCF-7 cells were treated with ethanol vehicle or TSA (500 ng/ml) for 1, 4, 6, or 18 h before harvesting cells. RNA was isolated, and IκBα mRNA expression was determined by quantitative PCR. Results are expressed as percentage of control cells after normalization by RS9 level and represent the mean ± S.E.M. of three independent experiments. E, whole protein extract from MCF-7 stimulated with ethanol vehicle or TSA (500 ng/ml) for the indicated time was prepared for IκBα protein levels and measured by Western blot analysis.

2003), whereas HDAC inhibitors are also capable of repressing the lipopolysaccharide-induced expression of cytokines such as TNF-α, IL-1β, or interferon-γ (Leoni et al., 2002). HDAC inhibitors have been shown to induce IL-8 gene expression in most studies investigating cell types from different origins, but this occurs generally in the presence of inducers such as TNF-α but not with HDAC inhibitors alone (Ashburner et al., 2001; Rahman et al., 2002; Gilmour et al., 2003; Tomita et al., 2003). However, it is worth mentioning that, in most instances, HDAC inhibitors were acting mainly in cooperation with other inducers of IL-8 gene expression. This is the case for the synergy between HDAC inhibitors and IL-1β (Ito et al., 2001; Wen and Wu, 2001; Böcker et al., 2003) or TNF-α (Ashburner et al., 2001; Iwata et al., 2002; Adam et al., 2003). On the other hand, in some cell types, IL-8 expression is even down-regulated by HDAC inhibitors (Hoshimoto et al., 2002; Iwata et al., 2002).

In breast cancer cells, HDAC inhibitors were sufficient to induce IL-8 transcription. Using serial deletions and mutations of the IL-8 promoter, we show that NF-κB is the major pathway involved in TSA-induced IL-8 gene expression in MCF-7 breast cancer cells, although AP-1 and C/EBP sites also contribute to achieve maximal induction by HDAC inhibitors. The importance of NF-κB in TSA-induced IL-8 regulation was further demonstrated by using the NF-κB inhibitors capsaicin (data not shown), IκB super-repressor and dominant-negative IKK2, and the fact that TSA strongly up-regulates the activity of a synthetic NF-κB responsive gene, which suggests that both upstream and downstream mediators of NF-κB pathway are affected. In addition, we demonstrate that nuclear translocation of p65 was increased with TSA treatment, whereas IκBα protein levels decreased. This is in contrast to the results obtained by Mayo et al. (2003) in non–small-cell lung cancer cells, who did not observe any change in IκBα levels after TSA treatment, suggesting that IκB regulation might be cell-specific (Mayo et al., 2003). Our findings are also in line with recent reports in which TNF-α was shown to increase nuclear localization of IKKα and p65 (Anest et al., 2003). IKKα and IKKβ are required for histone H4 acetylation by TNF-α but not by TSA, whereas histone H3 phosphorylation by TNF-α or TSA involves IKKα and IKKβ (Yamamoto et al., 2003). We observed that IκBα down-regulation by TSA could be antagonized by using proteasome inhibitors such as MG-132 (data not shown). Our results suggest that the hyperactivation of NF-κB pathway and concomitant increased IL-8 expression by TSA is the result of an enhancement of p65 translocation, a reduced sequestration of p65 by IκBα, and an enhanced H3 acetylation on the gene promoter. In addition, we observed an increased phosphorylation of IKKα and IKKβ with TSA treatment. It has been reported that the p65 subunit of NF-κB is subject to reversible deacetylation by HDAC3, which promotes effective binding to IκBα and leads in turn to nuclear export of NF-κB, thus ensuring control of the duration of the NF-κB transcriptional response (Chen et al., 2001). Deacetylase inhibition has been shown to potentiate TNF-induced DNA binding activity and nuclear localization of NF-κB (Gilmour et al., 2003). This is in correlation with a delayed restoration of cytoplasmic inhibitor IκBα, probably because of persistent degradation by the proteasome (Adam et al., 2003). On the other hand, other studies have not

observed modifications on the DNA binding capacity of NF-κB proteins (Mayo et al., 2003).

Taken together, our results indicate that activation of the IL-8 gene expression in breast cancer cells is acetylation-dependent and requires NF-κB activation. Based on our previous results showing that IL-8 can account for the higher aggressiveness of breast cancer cells, the use of HDAC inhibitors in anticancer strategies could also have adverse effects by increasing the expression of proinflammatory molecules.

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