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Histone Deacetylase Inhibitor Trichostatin A Sustains Sodium Pervanadate-induced NF-κB Activation by Delaying IκBα mRNA Resynthesis

*COMPARISON WITH TUMOR NECROSIS FACTOR α**

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NF-κB is a crucial transcription factor tightly regulated by protein interactions and post-translational modifications, like phosphorylation and acetylation. A previous study has shown that trichostatin A (TSA), a histone deacetylase inhibitor, potentiates tumor necrosis factor (TNF) α-elicited NF-κB activation and delays IκBα cytoplasmic reappearance. Here, we demonstrated that TSA also prolongs NF-κB activation when induced by the insulino-mimetic pervanadate (PV), a tyrosine phosphatase inhibitor that initiates an atypical NF-κB signaling. This extension is similarly correlated with delayed IκBα cytoplasmic reappearance. However, whereas TSA causes a prolonged IKK activity when added to TNFα, it does not when added to PV. Instead, quantitative reverse transcriptase-PCR revealed a decrease of *iκbα* mRNA level after TSA addition to PV stimulation. This synthesis deficit of the inhibitor could explain the sustained NF-κB residence in the nucleus. *In vivo* analysis by chromatin immunoprecipitation assays uncovered that, for PV induction but not for TNFα, the presence of TSA provokes several impairments on the *iκbα* promoter: (i) diminution of RNA Pol II recruitment; (ii) reduced acetylation and phosphorylation of histone H3-Lys[14] and -Ser[10], respectively; (iii) decreased presence of phosphorylated p65-Ser[536]; and (iv) reduction of IKKα binding. The recruitment of these proteins on the *icam-1* promoter, another NF-κB-regulated gene, is not equally affected, suggesting a promoter specificity of PV with TSA stimulation. Taken together, these data suggest that TSA acts differently depending on the NF-κB pathway and the targeted promoter in question. This indicates that one overall histone deacetylase role is to inhibit NF-κB activation by molecular mechanisms specific of the stimulus and the promoter.

The ubiquitous nuclear factor (NF)[8]-κB is a critical regulator of the expression of numerous genes implicated in immune and inflammatory responses, cellular proliferation and differentiation, and cell survival (1). This transcription factor is composed of homo- or heterodimers with various combinations of five subunits: p50/p105, p52/p100, p65 (RelA), RelB, and c-Rel. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form through its association with a member of an inhibitory family, of which the most characterized is IκBα (2). Upon cell stimulation by tumor necrosis factor α (TNFα), inducing the classical pathway, IκBα is rapidly phosphorylated on Ser[32] and Ser[36] by the cytoplasmic IκB kinase (IKK) complex, which triggers its polyubiquitination and subsequent degradation. The released NF-κB translocates into the nucleus to regulate the expression of multiple target genes, including those coding for its own inhibitor, IκBα. This negative feedback ensures removing NF-κB from its DNA-binding sites and transporting it back to the cytoplasm, thereby terminating NF-κB-dependent transcription (3).

Tyrosine phosphorylation plays a key role in NF-κB activation. It has been shown that pervanadate (PV), a potent tyrosine phosphatase (protein-tyrosine phosphatase) inhibitor, induces IκBα phosphorylation on Tyr[42] and activates NF-κB (4). Depending on the cell type, this IκBα tyrosine phosphorylation is correlated with either dissociation from NF-κB (5) or IκBα degradation (6). In Jurkat T cells stimulated with PV, the phosphatidylinositol 3-kinase regulatory subunit, p85α, interacts with the Tyr-phosphorylated IκBα, leading to IκBα release from NF-κB without its degradation (7). In HeLa cells, PV as well as hypoxia/reoxygenation, causes Tyr phosphorylation of IκBα through a Src-dependent mechanism (8). Beside this, PV is also considered as an insulino-mimetic compound by its capacity to activate insulin tyrosine kinase receptor and medi-

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[8] The abbreviations used are: NF, nuclear factor; ALLN, *N*-acetylleucylleucylnorleucinal; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; ERK1/2, extracellular signal-regulated kinase 1/2; HDAC, histone deacetylase; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; MSK1, mitogen- and stress-activated protein kinase-1; P, phosphorylated residue; PMA, phorbol myristate acetate; PV, sodium pervanadate; RT, reverse transcriptase; RNA Pol II, RNA polymerase II; SAHA, suberoylanilide hydroxamic acid; TNFα, tumor necrosis factor α; TSA, trichostatin A; GST, glutathione *S*-transferase; CBP, cAMP-response element-binding protein; HIV-1, human immunodeficiency virus type 1.

ate the insulin metabolic actions through activation of extracellular signal-regulated kinase 1/2 (ERK1/2), phosphatidylinositol 3-kinase, and protein kinase B (PKB/Akt) (9).

Acetylation is a pivotal post-translational modification of numerous proteins, such as histones and transcription factors (like NF-κB). Histone acetylation, required for transcriptional activation, is tightly controlled by histone acetyltransferases and histone deacetylases (HDAC). Several cancer-promoting mutations result in repression of transcription through abnormal recruitment and activation of HDAC that may lead to neoplastic transformation. Therefore, HDAC inhibitors have emerged as new agents for cancer treatment by preventing angiogenesis and inducing growth arrest with a remarkable tumor specificity (10, 11).

NF-κB functions are regulated by post-translational modifications including phosphorylation (12, 13) and acetylation (14). These modifications determine both the strength and duration of NF-κB-mediated transcriptional response (15). Among the numerous residues emerging to be phosphorylated on p65, Ser^{276} and Ser^{536} are the best characterized. Phosphorylation of Ser^{276} is mediated by the protein kinase A catalytic subunit (PKAc) or mitogen- and stress-activated kinase 1 (MSK1) (16, 17) and Ser^{536} is targeted by multiple kinases: IKKα, IKKβ, IKKϵ, and TRAF family member-associated (TANK)-binding kinase (TBK1) (18, 19). These phosphorylations allow Lys^{310} acetylation by CBP/p300 and transcriptional activation (20). Importantly, the p65 phosphorylation status determines whether nuclear NF-κB associates with CBP/p300 or HDAC, leading to p65 transcriptional activation or repression, respectively (21–23).

A previous study has shown a potentiation of TNFα-induced NF-κB activation by deacetylase inhibitors (24). The associated cytoplasmic reappearance of IκBα is delayed, which is explained, at least partially, by a prolonged IKK activity. Here we analyzed the influence of the histone deacetylase inhibitor on a NF-κB pathway involving tyrosine phosphorylations induced by PV. We demonstrated that the HDAC inhibitor trichostatin A (TSA) extends the PV-induced NF-κB activation by a distinct mechanism. Indeed, *iκbα* mRNA synthesis appears to be delayed after the co-stimulation with PV and TSA, explaining the NF-κB persistence in the nucleus of treated cells. This delay of *iκbα* mRNA synthesis seems to be due to impairing of the recruitments of IKKα, p65 phosphorylated on Ser^{536} and RNA Pol II, but also acetylation of histone H3 on Lys^{14}, and phosphorylation of histone H3 on Ser^{10}. This extension of NF-κB activation is completely different from the one initiated by TNFα with TSA, which is due to, at least partly, an extension of the IKK activity. Therefore, we show here that the implication of HDAC inhibitors can be quite different depending on the NF-κB-inducing agent.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—HeLa cells were cultured in Eagle's minimal essential medium with 10% fetal calf serum and glutamine (BioWhittaker, Petit Rechain, Belgium). Jurkat T cells were cultured in RPMI 1640 with 10% fetal calf serum and glutamine (BioWhittaker). TSA was used at the concentration of 450 nM (Sigma), TNFα at 200 units/ml (Roche Applied Science), and suberoylanilide hydroxamic acid (SAHA) at 3 μM (Alexis Biochemicals, Zandhoven, Belgium). PV was freshly prepared before each experiment as previously described (5) and it was used at 200 μM. H_2O_2 treatment (250 μM; Sigma) was always preceded by a preincubation with aminotriazole (50 mM for 1 h) (Sigma), a catalase inhibitor (4). The phorbol ester PMA (200 nM) was always combined with the Ca^{2+} ionophore ionomycin (141 nM) (Sigma). *N*-Acetylleucylleucylnorleucinal (ALLN) was used at the concentration of 100 μM (Sigma).

Antibodies—Monoclonal anti-IκBα used for Western blotting was provided by Dr. Ron Hay (University of Dundee, United Kingdom). Polyclonal anti-IκBα used for immunoprecipitation, -p65 used for Western blot, -IKKβ, -RNA Pol II, and -IKKα were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IκBα phosphorylated on Ser^{32} and Ser^{36}, -phospho-p38 and -phospho-ERK, -p65 phosphorylated on Ser^{276} and -p65 phosphorylated on Ser^{536} were from Cell Signaling Technology. Anti-phosphotyrosine, -p65 used for immunoprecipitation, -histone H3 acetylated on Lys^{14}, and -histone H3 phosphorylated on Ser^{10} were from Upstate (Charlottesville, VA). Finally anti-unmodified histone H3 was from Abcam Limited (Cambridge, UK).

Plasmids—Several reporter plasmids containing the Luciferase gene under the control of different promoters were used. The 0.4SK-pGL3 plasmids containing the *iκbα* promoter were kindly provided by J. Hiscott (McGill University, Canada). The plasmid p*icam-1*-Luc was donated by Y. de Launoit (University of Brussels, Belgium).

Transient Transfection and Luciferase Assay—Twenty hours before treatment, HeLa cells were transfected with FuGENE 6™ (Roche Applied Science) according to the manufacturer's recommendations. At 7 h post-treatment, cells were lysed and assayed for luciferase activity. Luciferase activities were normalized with protein concentration. Luciferase assay results are an average of three independent experiments.

Cytoplasmic and Nuclear Protein Extraction—Cells were washed twice with ice-cold phosphate-buffered saline, scraped, and centrifuged. The pellets were resuspended in 100 μl of cold hypotonic buffer (10 mM Hepes-KOH, pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 0.5% IGEPAL, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture Complete (Roche Applied Science)), incubated on ice for 10 min. The lysates were vortexed 5 s, and centrifuged for 30 s at 20,000 × *g* at 4 °C. The supernatants containing cytoplasmic proteins were stored at −80 °C. The pellets were next resuspended in 30 μl of cold hypertonic buffer (50 mM Hepes-KOH, pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 400 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and Complete), incubated on ice for 25 min and centrifuged for 15 min at 20,000 × *g* at 4 °C. Then the supernatants containing nuclear proteins were stored at −80 °C. The protein concentration was determined with a Bio-Rad protein assay.

Western Blotting and Electrophoretic Mobility Shift Assay (EMSA)—Cytoplasmic extracts, obtained as described above, were analyzed by Western blotting as previously described (25). Nuclear extracts, prepared as detailed above, were analyzed by EMSA as previously described (26), using a ^{32}P-labeled oligonucleotide probe (5′-GGTTACAAGGGACTTTCCGCTG-3′;

Eurogentec, Liège, Belgium) corresponding to a HIV-1 long terminal repeat κB site.

IκBα Immunoprecipitation—Whole cell extraction and IκBα immunoprecipitation were previously described by Gloire *et al.* (27). The presence of unmodified IκBα and IκBα phosphorylated on tyrosine was determined by Western blotting.

IKK Complex Immunoprecipitation and in Vitro IKK Kinase Assay—Cytoplasmic extracts were prepared as detailed above in a hypotonic buffer supplemented with phosphatase inhibitors (1 mM Na_3VO_4, 10 mM NaF, 25 mM β-glycerophosphate, 10 mM nitrophenyl phosphate). IKK complex immunoprecipitation and *in vitro* IKK kinase assay were performed as previously described (28) with purified GST-IκBα-(1–54) fusion protein as substrate (a gift from R. Gaynor, University of Texas Southwestern Medical Center, Dallas). This was followed by a Western blotting using an anti-IκBα phosphorylated on Ser^{32} and Ser^{36} antibody.

Total Protein Extraction for Phospho-Western Blotting—Treated cells were washed with ice-cold phosphate-buffered saline and rapidly lysed in SDS-blue lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.03% bromphenol blue powder, and 50 mM dithiothreitol). Total lysates were sonicated for 50 s, boiled for 3 min, and used for Western blotting as previously described (25).

Quantitative Real Time Reverse Transcription-PCR—Total RNA samples were extracted with RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. 1 μg of RNA was submitted to reverse transcription with the Moloney murine leukemia virus reverse transcriptase (Invitrogen). For quantitative real-time RT-PCR, the obtained cDNA was analyzed, in triplicate, with the SYBR Green Master Mix (Applied Biosystems, Foster City, CA) in the ABI Sequence Detection System. The results were normalized with the *β2-microglobulin* transcript. The primers used to analyze the different transcripts were designed with the software Primer Express™ (Applied Biosystems): *iκbα*, FW, 5′-CCAACCAGCCAGAAATTGCT-3′ and RV, 5′-TCTCGGAGCTCAGGATCACA-3′; *icam*-1, FW, 5′-TGCCAAGAGGGGAGGGGTGC-3′ and RV, 5′-GCCCCGGGGAGGCTCCGTGC-3′; *β2-microglobulin*, FW, 5′-GAGTATGCCTGCCGTGTG-3′ and RV, 5′-AATCCAAATGCGGCATCT-3′ (Eurogentec).

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation (ChIP) assays were carried out with solutions prepared in our laboratory following the Upstate Cell Signaling protocol. Chromatin was sheared by sonication for 15 min to lengths between 200 and 1000 base pairs. The sonication was done in a water bath with generation of high power ultrasound (Bioruptor, Diagenode, Belgium): 15 cycles of 30 s ON, 30 s OFF (1 cycle/min) at maximum power. To reduce nonspecific background, protein A-agarose (Pierce), used for immunoprecipitation, was pre-saturated with herring sperm DNA (Sigma). Immunoprecipitations were performed with 2 μg of different antibodies: anti-p65, -RNA Pol II, -histone H3 acetylated on Lys^{14}, -histone H3 phosphorylated on Ser^{10}, -p65 phosphorylated on Ser^{276}, -p65 phosphorylated on Ser^{536} and -IKKα. To test aspecific binding to the beads, an irrelevant antibody was used as control for immunoprecipitation (anti-FLAG antibody, Sigma). A phenol/chloroform DNA extraction was performed and the immunoprecipitated DNA was analyzed by quantitative real time PCR with the SYBR Green Master Mix in the ABI Sequence Detection System. All ChIP assays were performed three times. The primers, corresponding to the promoter region of each gene, were designed using the software Primer Express™: *iκbα*, FW, 5′-CGCTCATCAAAAAGTCCCTG-3′ and RV, 5′-GGAATTTCCAAGCCAGTCAGAC-3′; *icam-1*, FW, 5′-CCCGATTGCTTTAGCTTGGAA-3′ and RV, 5′-CCGGAACAAATGCTGCAGTTAT-3′ (Eurogentec). As control for binding specificity, we amplified a non-coding region next to the albumin gene (29).

RESULTS

It was previously reported by Adam *et al.* (24) that histone deacetylase inhibitors cause the extension of TNFα-induced NF-κB activation when both compounds are added simultaneously. In this work, we compared the influence of TSA, a large spectrum histone deacetylase inhibitor, on two different NF-κB activation pathways: (i) the atypical pathway involving multiple tyrosine kinases mediated either by PV or hydrogen peroxide (H_2O_2), and (ii) the classical pathway induced either by the pro-inflammatory cytokine TNFα or the phorbol ester PMA.

Influence of HDAC Inhibitor on NF-κB Activation, and the Associated IκBα Degradation, Induced by Various Inducers—We investigated the kinetics of NF-κB activation elicited by four inducers (PV, TNFα, PMA, and H_2O_2) in the presence or absence of an HDAC inhibitor, TSA or SAHA, in two cell types, HeLa cells or Jurkat T cells. Time course extractions were carried out after each co-treatment. EMSA, performed with nuclear extracts, revealed that stimulation with PV alone leads to NF-κB activation from 30 min until 2 h (Fig. 1*A*, *upper left panel*), whereas the co-treatment PV plus TSA potentiates this binding until 8 h (Fig. 1*A*, *upper middle panel*). The associated IκBα degradation in the corresponding cytoplasmic extracts was analyzed by Western blotting. A delay in the IκBα cytoplasmic reappearance is clearly detected when TSA is added simultaneously to PV (Fig. 1*A*, *lower left* and *middle panels*). Beside this, we tested what happen to the PV-induced NF-κB activation with either another HDAC inhibitor (SAHA) or another cell type (Jurkat T cells). First, in HeLa cells, we observed by EMSA that the simultaneous addition of SAHA also prolongs the activation of NF-κB induced by PV until 8 h (Fig. 1*A*, *upper right panel*), which is correlated with a delay of IκBα cytoplasmic reappearance shown by Western blotting (Fig. 1*A*, *lower right panel*). Because TSA and SAHA gave similar results, SAHA impacts were not investigated anymore in this report. In Jurkat T cells, Beraud *et al.* (7) have demonstrated that PV induces NF-κB activation via an IκBα phosphorylation on tyrosine associated with its dissociation from NF-κB. Here in this cell type, we detected no significant extension of NF-κB activation when TSA is added (Fig. 1*B*, *upper panels*), but the IκBα phosphorylation, associated with NF-κB activation, is prolonged from 30 min to, at least, 1 h (Fig. 1*B*, *lower panels*). This indicates a sustained activity of IκBα tyrosine kinase. Thus, the use of Jurkat T cells highlights the fact that the intensity of the HDAC inhibitor effects on NF-κB activation could depend on the cell type. The following experiments were performed with

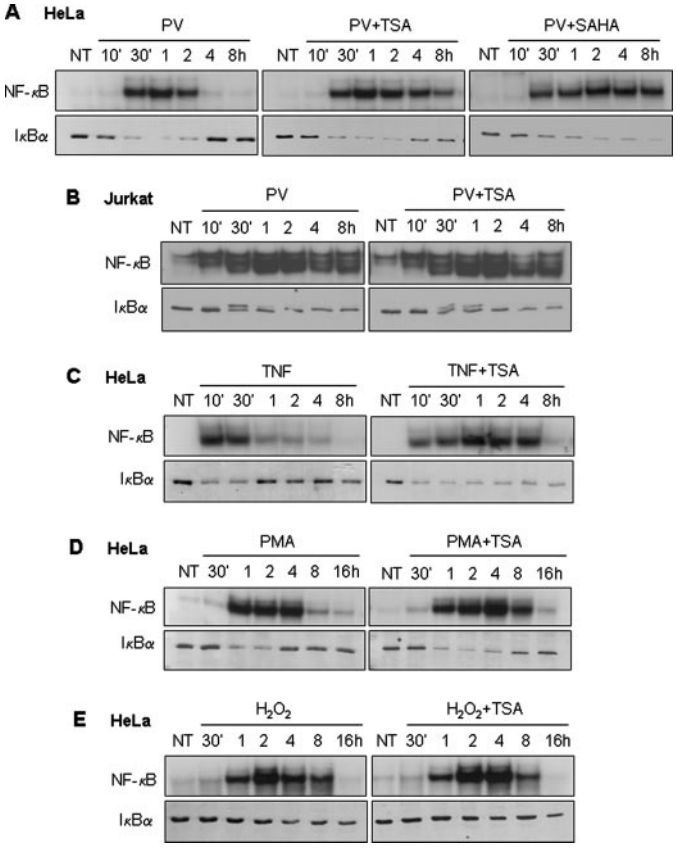

FIGURE 1. **Influence of HDAC inhibitor on NF-κB activation and the associated IκBα degradation induced by PV, TNFα, PMA, or H_2O_2 in HeLa and Jurkat T cells.** *A, C–E*, HeLa cells were not treated (*NT*) or treated with PV (*A*), TNFα (*C*), PMA (*D*), or H_2O_2 (*E*) in the absence/presence of TSA (*A*, *left* and *middle panels*, *C–E*) or SAHA (*A*, *right panels*) for the indicated times. *B*, Jurkat T cells were non-treated or treated with PV in the absence or presence of TSA for the indicated times. Nuclear translocation of NF-κB was measured by EMSA with a probe corresponding to the HIV-1 long terminal repeat κB site (*upper panels*). IκBα degradation was analyzed by Western blotting of cytoplasmic extracts (*lower panels*).

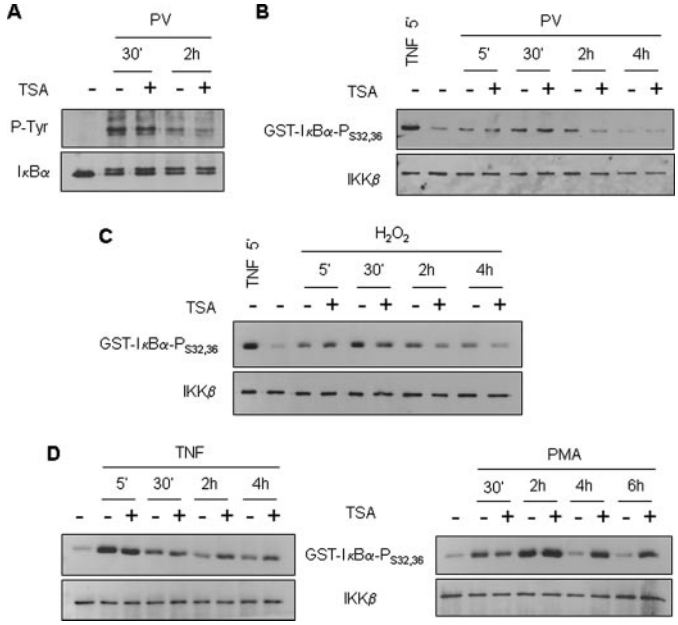

FIGURE 2. **Effect of TSA on IκBα tyrosine phosphorylation or on IKK activity after stimulation by PV, TNFα, PMA, or H_2O_2.** *A*, TSA does not modify IκBα tyrosine phosphorylation after PV. IκBα was immunoprecipitated from total cellular extracts obtained after ALLN pretreatment for 45 min and PV with or without TSA for the specified durations. The presence of IκBα phosphorylated on tyrosine in the immunoprecipitated fraction was detected by Western blotting with a phosphotyrosine antibody. Loading control was carried out with an anti-IκBα antibody. *B–D*, IKK activity is prolonged by TSA after TNFα or PMA treatment, but not after PV or H_2O_2 stimulation. *B*, cells were treated with TNFα for 5 min (positive control) or PV with or without TSA for increasing times. After immunoprecipitation of the complex with an anti-IKKγ antibody, *in vitro* IKK kinase activity was determined by incubation of the immunoprecipitated proteins with purified GST-IκBα-(1–54) fusion protein as substrate. Western blotting was then performed using an antibody specific for Ser^{32-36}-phosphorylated IκBα (*upper panel*). The presence of equal amounts of IKK catalytic subunit (IKKβ) in each sample was confirmed by Western blotting (*lower panel*). *C* and *D*, *in vitro* IKK kinase activity was measured after treatment with H_2O_2, TNFα, or PMA with or without TSA for different times as described in *B*.

the HDAC inhibitor TSA on HeLa cells, for which the effects are the most striking.

The events obtained after treatment of HeLa cells with PV in the absence or presence of TSA seem to be similar to those described by Adam *et al.* (24) and are confirmed here, with HeLa cells treated with TNFα and TSA compared with cells treated with TNFα alone. We observed NF-κB activation from 10 to 30 min with TNFα alone, whereas the addition of TSA extends it until 4 h. The IκBα cytoplasmic reappearance is also delayed for several hours (Fig. 1*C*). The induction by the phorbol ester PMA, leading to NF-κB activation via PKC and IKK complex (30), also shows a prolonged profile when TSA is added; the kinetic is, however, rather different (Fig. 1*D*). In HeLa cells, H_2O_2 was described by Storz and Toker (31) to activate the IKK complex but fail to induce IκBα tyrosine phosphorylation. In this study, H_2O_2 is the only inducer that shows no prolonged NF-κB activation upon simultaneous induction with TSA (Fig. 1*E*). Nevertheless, the binding seems to be stronger at 4 h of co-treatment. IκBα does not appear to be significantly degraded after H_2O_2 or H_2O_2 plus TSA stimulation. Therefore, TSA provokes the extension of NF-κB activation and delays IκBα reappearance in the cytoplasm in PV-, TNFα-, and PMA- induced pathways, whereas NF-κB activation by H_2O_2 is not significantly modified by the presence of TSA.

Effect of TSA on IκBα Tyrosine Phosphorylation or on IKK Activity after Stimulation by PV, TNFα, PMA, or H_2O_2—To understand the origin of this prolonged NF-κB activation, we focused on the events responsible for IκBα phosphorylation and its subsequent degradation. PV is known to induce global tyrosine phosphorylation of proteins by its ability to inhibit tyrosine phosphatases. IκBα, one of the targets of tyrosine kinases, is phosphorylated on Tyr^{42} upon PV stimulation (5). Thus, we wanted to explore whether this tyrosine phosphorylation is influenced by the addition of TSA. HeLa cells were pretreated for 45 min with a proteasome inhibitor, ALLN, to protect phosphorylated IκBα from degradation, and then treated with PV with or without TSA for 30 min or 2 h. Total extracts were used to immunoprecipitate IκBα and its phosphorylation status on tyrosine was determined by Western blotting with an anti-phospho-Tyr antibody (Fig. 2*A*, *upper panel*). When we compare the level of tyrosine phosphorylation 30 min after PV stimulation without and with TSA, there is no obvious difference. Identical results are obtained after 2 h of treatment. The amount of immunoprecipitated IκBα, visualized using an anti-IκBα antibody, is quite similar in the samples

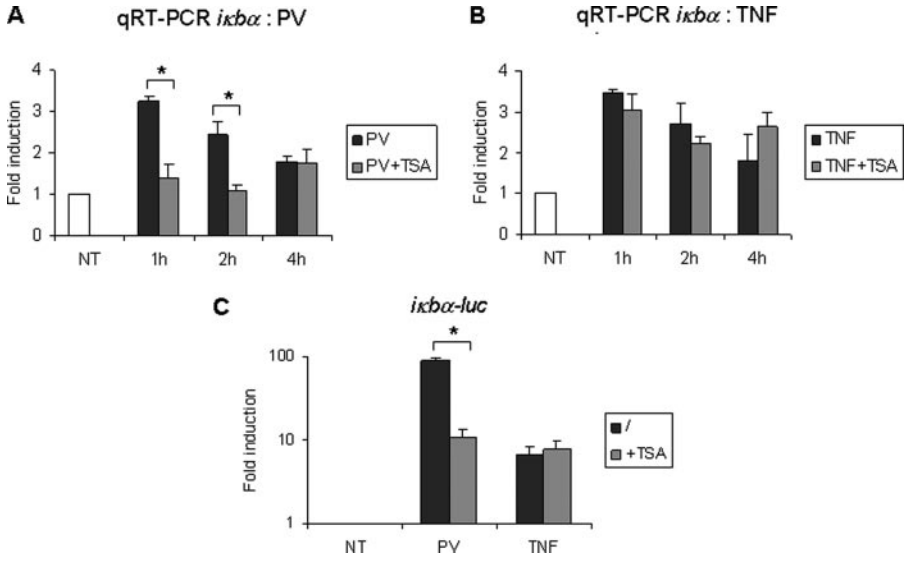

FIGURE 3. **TSA reduces PV- but not TNFα-induced *iκbα* mRNA synthesis and NF-κB transcriptional activity.** Total RNAs were isolated at various times of treatment with either PV or PV + TSA (*A*), and TNFα or TNFα + TSA (*B*). The *iκbα* mRNA expression level was analyzed by quantitative (*q*) real time RT-PCR with specific primers. The results were normalized with the *β2-microglobulin* transcript. *C*, transient transfections were performed with a *luciferase reporter* gene under the control of the *iκbα* promoter. Twenty hours after transfection, cells were treated with PV or TNFα with or without TSA for 8 h before cell lysis and detection of luciferase activity, normalized by the protein amount. *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments. *NT*, not treated.

without and with TSA at each time point, even if the total level of IκBα is slightly decreased after 2 h (Fig. 2*A*, *lower panel*).

IKK activity, which is responsible for IκBα phosphorylation on Ser^{32} and Ser^{36}, was then tested after exposure to PV with or without TSA for periods ranging from 5 min to 4 h. A treatment with TNFα for 5 min was used as a positive control. The IKK complex was immunoprecipitated from cytoplasmic extracts and submitted to an *in vitro* kinase assay with purified GST-IκBα-(1–54) fusion protein as substrate. A Western blot was then performed with an antibody recognizing IκBα phosphorylated on Ser^{32} and Ser^{36}. We observed that the PV- and H_2O_2-induced IKK activity, already shown in HeLa cells by Storz and Toker (31), is weaker than the one observed with TNFα and is not considerably affected, or even slightly decreased, by the presence of TSA (Fig. 2, *B* and *C*, respectively, *upper panels*). On the opposite, when TSA is added to TNFα or PMA, the IKK activity is prolonged, respectively, up to 2 or 4 h of treatment (Fig. 2*D*, *upper panels*). The quality of the immunoprecipitation is visualized with an anti-IKKβ antibody in each IKK kinase assay (Fig. 2, *B–D*, *lower panels*). Taken together, these results indicate that TSA has no effect upstream of PV- and H_2O_2-elicited IκBα degradation, whereas there is an extension of TNFα- and PMA-induced IKK activity by the addition of TSA.

For the following results, we focused on the comparison of the two inducers, PV and TNFα. PMA stimulation was not further considered as it displays the same profile as TNFα when TSA is added. Likewise, because TSA has only a slight influence on H_2O_2 induction in these experiments, we decided to not study this rather modest effect further.

TSA Decreases PV, but Not TNFα, -induced iκbα mRNA Expression—Because the delayed IκBα cytoplasmic reappearance after PV plus TSA treatment is not due to a prolonged phosphorylation and subsequent degradation, we investigated the *iκbα* mRNA synthesis. HeLa cells were treated with either PV or TNFα with or without TSA and total RNA was isolated. *iκbα* specific primers were used to examine RNA by quantitative real time RT-PCR. The results were normalized with the *β2-microglobulin* transcript. When cells are treated for 1 h with PV alone, a 3-fold stimulation of the *iκbα* mRNA synthesis was observed and was gradually decreased at 2 and 4 h of treatment (Fig. 3*A*, *black bars*). When TSA was added to PV, there is a strong reduction of *iκbα* mRNA synthesis at 1 and 2 h of treatment, bringing it to levels quite comparable with the nontreated signal (Fig. 3*A*, *gray bars*). This effect of TSA on PV-induced *iκbα* mRNA expression seems to be stabilized at 4 h, where we detected a slightly delayed resynthesis. This experiment was confirmed by the ribonuclease protection assay (data not shown). In contrast, no significant difference appears between TNFα or TNFα plus TSA (Fig. 3*B*), as it was previously described by the ribonuclease protection assay (24). Therefore, these results demonstrate that *iκbα* mRNA synthesis is delayed after the addition of TSA to PV, but not after the addition of TSA to TNFα. This reduction might explain why the nuclear presence of NF-κB is prolonged, as in the absence of newly synthesized inhibitor, NF-κB cannot be brought back to the cytoplasm.

PV-induced NF-κB Transcriptional Activity on the iκbα Promoter Is Reduced in the Presence of TSA—To clarify the functional role of the NF-κB-binding site on *iκbα* promoter inducibility by PV, transient transfection assays were performed with a *luciferase reporter* gene under the control of the *iκbα* promoter (32). The addition of TSA decreases nearly 10-fold the PV-induced NF-κB transcriptional activity, whereas there is no significant difference of TNFα-elicited NF-κB transcriptional activity in the presence of TSA (Fig. 3*C*, logarithmic scale). Thus, NF-κB transcriptional activity on the *iκbα* promoter is clearly modified when TSA is combined with PV.

The Effect of TSA on PV Induction Is Promoter-dependent—To determine whether this TSA effect on the activity of the IκBα was observed with other NF-κB-dependent promoters, several promoters (*il-8*, *il-6*, and *icam-1*) were tested and the *icam-1* promoter (33) interestingly showed different results. HeLa cells were treated with either PV or TNFα with or without TSA and total RNA was isolated. *icam-1* gene-specific primers were used to analyze RNA by quantitative real time RT-PCR. The results were normalized with the *β2-microglobulin* transcript. The comparison between PV and PV plus TSA stimulation revealed no significant difference of *icam-1* mRNA synthesis (Fig. 4*A*). Unexpectedly, the addition of TSA to TNFα initiates a 2-fold decrease of *icam-1* mRNA expression after 1 h, which was sta-

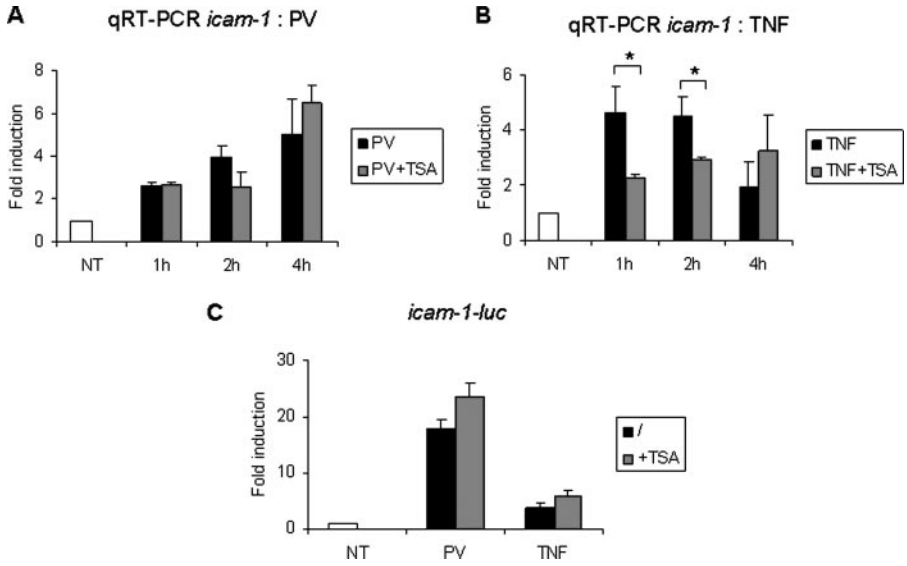

FIGURE 4. **TSA decreases TNFα- but not PV-induced *icam-1* mRNA synthesis.** Total RNAs were isolated at various times of treatment with either PV or PV + TSA (*A*), TNFα or TNFα + TSA (*B*). *icam-1* mRNA expression was analyzed by quantitative real time RT-PCR with specific primers. The results were normalized with the *β2-microglobulin* transcript. *C*, transient transfection with a *luciferase reporter* gene under control of the *icam-1* promoter. Twenty hours after transfection, cells were treated with PV or TNFα with or without TSA for 8 h before cell lysis and determination of luciferase activity, normalized by the protein amount. *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments. *NT*, not treated.

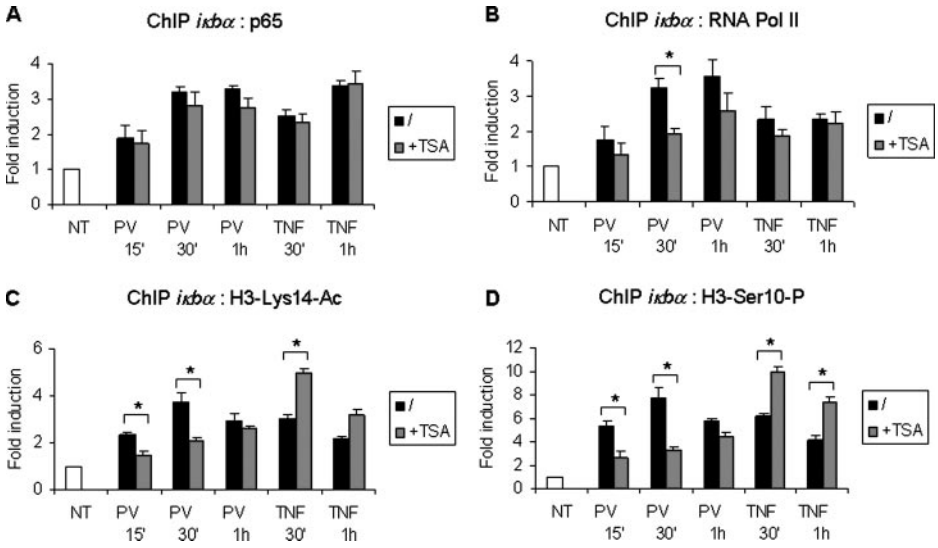

FIGURE 5. **Impact of TSA on p65 and RNA Pol II recruitment and histone H3 modifications on the *iκbα* promoter after PV or TNFα induction.** ChIP assays were performed on HeLa cells treated with either PV or TNFα with or without TSA using different antibodies for the immunoprecipitation directed against: (*A*) p65, (*B*) RNA Pol II, (*C*) histone H3 acetylated on Lys^{14}, and (*D*) histone H3 phosphorylated on Ser^{10}. The immunoprecipitated chromatin was submitted to a quantitative real time PCR analysis using primers amplifying the promoter region of *iκbα*. *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments. *NT*, not treated.

bilized and even slightly up-regulated at 4 h (Fig. 4*B*). This effect was quite similar to the one obtained after PV with or without TSA on the *iκbα* promoter.

We also examined the functional role of the NF-κB binding site in the inducibility of the *icam-1* promoter. Transient transfection assays were performed with a *luciferase reporter* gene under the control of the *icam-1* promoter (Fig. 4*C*). For both PV and TNFα inductions, the presence of TSA does not lead to a significant change of NF-κB driven transcription. These results indicate that the *icam-1* promoter seems to react differently than the *iκbα* promoter after a co-stimulation of PV plus TSA. In summary, the effect of TSA on PV or TNFα induction is clearly promoter-dependent.

Impact of TSA on p65 and RNA Pol II Recruitments and Histone H3 Modifications on the iκbα Promoter after PV or TNFα Induction—In the previous paragraph, we showed that NF-κB, present in the nucleus of the co-treated (PV with TSA) cells, was able to bind longer to an *in vitro* probe by EMSA (Fig. 1*A*), whereas *iκbα* mRNA expression was impaired (Fig. 3*A*). We thus investigated, by ChIP, *in vivo* p65 and RNA Pol II recruitments, as well as histone H3 modifications, on the *iκbα* promoter. Whatever the stimulus, PV or TNFα, the approximate 3-fold increase in p65 binding was not significantly modified by the presence of TSA (Fig. 5*A*). As p65 is correctly recruited to the *iκbα* promoter but mRNA synthesis is impaired, we next decided to analyze the RNA Pol II recruitment on the *iκbα* promoter. At 15 min post-treatment, the slight PV-induced RNA Pol II binding was not significantly modified by TSA, whereas, after 30 min, the addition of TSA diminishes the 3-fold increased recruitment by about 40%. At longer times, after 1 h, the difference was not significant. When we compared TNFα alone and TNFα plus TSA, there was no change of RNA Pol II binding (Fig. 5*B*). It is commonly accepted that histone acetylation is a prerequisite to basal transcriptional machinery recruitment (34). Beside this, Yamamoto *et al.* (35) have demonstrated that histone H3 must be phosphorylated on Ser^{10} prior to being acetylated on Lys^{14} on the *iκbα* promoter. Therefore, *in vivo* histone H3 acetylation and phosphorylation were tested by ChIP assays and normalized with unmodified histone H3 (Fig. 5, *C* and *D*). We noticed that histone H3 acetylation on Lys^{14} and phosphorylation on Ser^{10} are significantly down-regulated after 15 and 30 min of PV plus TSA treatment compared with PV alone. After 1 h, the levels were approximately similar. However, when cells were treated with TNFα, TSA addition leads to an increase of both post-translational modifications on the *iκbα* promoter. For

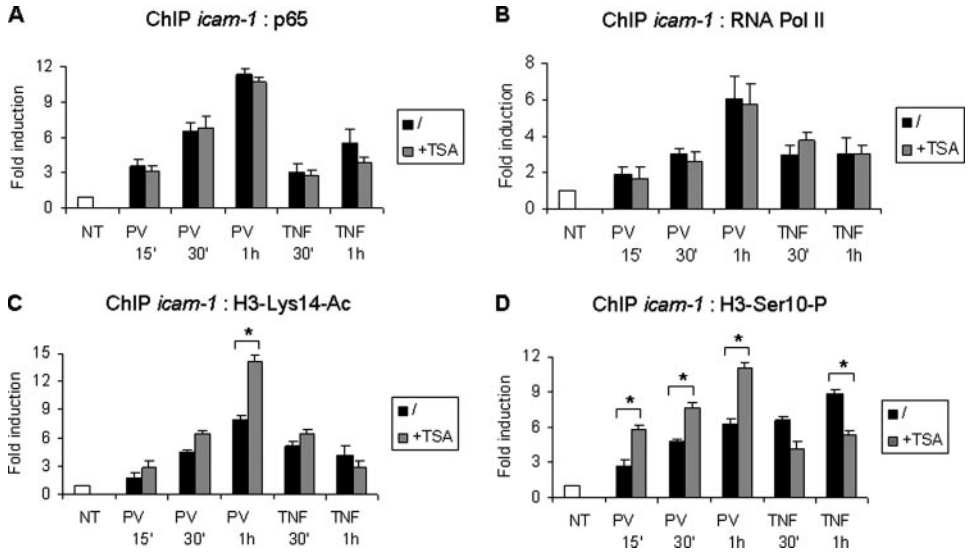

FIGURE 6. **Impact of TSA on p65 and RNA Pol II recruitment and histone H3 modifications on the *icam-1* promoter after PV or TNFα induction.** ChIP assays were performed on HeLa cells treated with either PV or TNFα with or without TSA with different antibodies for the immunoprecipitation directed against: (*A*) p65, (*B*) RNA Pol II, (*C*) histone H3 acetylated on Lys[14], and (*D*) histone H3 phosphorylated on Ser[10]. The immunoprecipitated chromatin was submitted to a quantitative real time PCR analysis using primers amplifying the promoter region of *icam-1*. *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments. *NT*, not treated.

each experiment, negative controls were performed with irrelevant immunoglobulins (FLAG antibody) for immunoprecipitation and quantitative PCR on the *albumin* promoter (data not shown). In conclusion, the impairment of *iκbα* mRNA expression induced by co-stimulation of PV and TSA is likely due to, at least in part, a delay of RNA Pol II recruitment and histone H3 acetylation on Lys[14] and phosphorylation on Ser[10]. The results are obviously different in the presence of another inducer, TNFα.

Impact of TSA on p65 and RNA Pol II Recruitment and Histone H3 Modifications on the icam-1 Promoter after PV or TNFα Induction—Because the influence of TSA on NF-κB-responsive genes appears to depend on the considered promoter, we next explored the *icam-1* promoter and its *in vivo* p65/RNA Pol II recruitments and histone H3 modifications by ChIP assay. Whatever the inducer, PV or TNFα, neither p65 nor RNA Pol II recruitment were affected when TSA was added simultaneously (Fig. 6, *A* and *B*). For histone H3 acetylation on Lys[14], TSA causes an up-regulation of the PV induction, particularly at 1 h, but has no significant effect on TNFα stimulation (Fig. 6*C*). Interestingly, histone H3 phosphorylation on Ser[10] was increased after TSA addition on PV stimulation as early as 15 min and that up-regulation was maintained up to 1 h. The opposite was observed with TNFα as a diminution of histone H3 phosphorylation was induced by the presence of TSA (Fig. 6*D*). This indicates that TSA differentially affects *iκbα* and *icam-1* promoter regulation in a stimulus-specific mode.

The MAPK Activation Pathway Remains Unchanged after Adding TSA to PV Induction—MSK1, activated by ERK and p38 MAPK (36), is responsible for the phosphorylation of p65 on Ser[276] (17) and H3 histone on Ser[10] (37). Because H3 histone phosphorylation is reduced on the *iκbα* promoter when TSA is added to PV stimulation (15 and 30 min) (Fig. 5*D*), the MAPK activation pathway was analyzed by Western blotting on total protein extracts. No change in ERK and p38 activation was detected, using phosphorylated antibodies, when cells were treated with PV in the absence or presence of TSA (Fig. 7*A*). The global phosphorylation status of two MSK1 targets, histone H3 and p65, were also checked after PV treatment and demonstrated that TSA addition has no impact on it (Fig. 7*B*). The elevated level of histone H3 phosphorylation in the untreated cells can be explained by the fact that this phosphorylation occurs after cellular stress or mitogenic stimulation, as well as during mitosis. For the latter, MSK1 is not involved (37). As histone H3 phosphorylation on Ser[10] is reduced on the *iκbα* promoter when PV was used in the presence of TSA (Fig. 5*D*), we checked the recruitment of p65 phosphorylated on Ser[276] by ChIP assay (Fig. 7*C*). Whatever the promoter (*iκbα* or *icam-1*) and stimulus (PV or TNFα with or without TSA), the binding of p65-Ser[276]-P was not significantly modified. The *icam-1* promoter results, shown in Fig. 7*C*, are representative of the one obtained on the *iκbα* promoter. In summary, TSA addition on PV induction does not influence global ERK and p38 MAPK activation and global phosphorylation of MSK1 targets. This strengthens the idea of a promoter dependence after co-treatment of PV with TSA.

TSA Could Interfere with p65 Transactivation Potential Depending on the Stimulus and Promoter—We then investigated whether the p65 transactivation potential induced by PV or TNFα was affected by the presence of TSA. The p65-Ser[536], included in the transactivation domain, is targeted by multiple kinases (18, 19). Its phosphorylation allows Lys[310] acetylation by CBP/p300 and is, at least partially, responsible for the p65 transactivation capacity (20). The p65 global phosphorylation status on Ser[536] was tested by Western blotting on total protein extracts. We observed that the co-stimulation of PV plus TSA does not modify the p65 global phosphorylation compared with PV alone (Fig. 7*D*). We then performed the ChIP assay to check if the promoter-specific recruitment of p65 phosphorylated on Ser[536] could be modified by TSA, even if we observed no change of the unmodified p65 binding whatever the stimulus or promoter (Figs. 5*A* and 6*A*). We first examined binding on the *iκbα* promoter (Fig. 8*A*). The p65-Ser[536]-P begins to be recruited after 15 min of PV stimulation and a significant reduction of the binding at 30 min was detected in the presence of TSA. For TNFα treatment, the addition of TSA leads to up-regulation of the recruited p65-Ser[536]-P. The analysis of the *icam-1* promoter revealed that TSA does not modify the PV-induced p65-Ser[536]-P recruitment at 15 and 30 min of stimulation, but there was a sustained recruitment at 1 h when TSA was added (Fig. 8*B*). The TNFα stimulus induces a 3-fold increased binding of

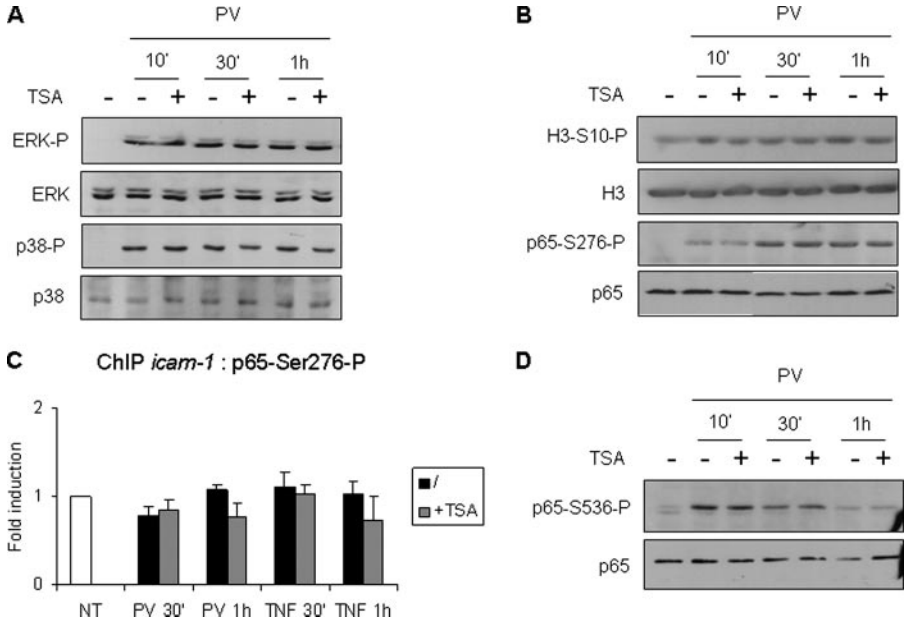

FIGURE 7. **Phosphorylation status of ERK and p38 MAPK, histone H3, and p65 after adding TSA to PV induction.** *A*, ERK and p38 MAPK: HeLa cells were treated with PV or PV with TSA for increasing times. Total proteins were rapidly extracted in SDS-blue lysis buffer and Western blotting was carried out using different antibodies to evaluate ERK and p38 phosphorylation; *B*, MSK1 targets: histone H3-Ser^{10} and p65-Ser^{276}. *C*, p65-Ser^{276}-P: ChIP assay was performed on HeLa cells treated with either PV or TNFα in the presence or absence of TSA with an antibody for the immunoprecipitation directed against p65-Ser^{276}-P. The immunoprecipitated chromatin was submitted to a quantitative real-time PCR analysis using primers amplifying the promoter region of *icam-1*. *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments. *D*, p65-Ser^{536}-P: after PV or PV + TSA treatment, total proteins were rapidly extracted in SDS-blue lysis buffer and Western blotting was carried out using the p65-Ser^{536}-P antibody. For each Western blot (*A*, *B*, and *D*), the corresponding loading control was obtained with the antibody directed against the unmodified form of the protein.

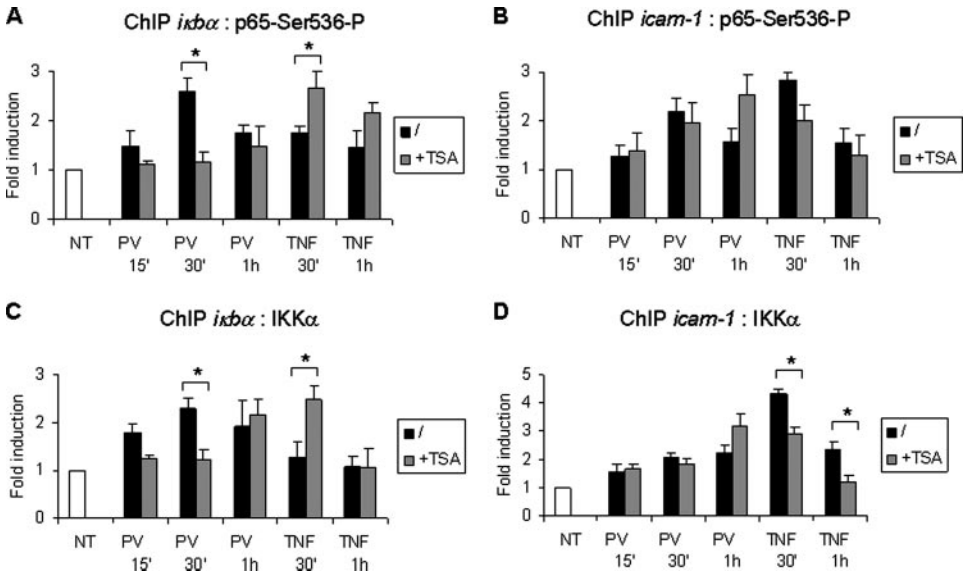

FIGURE 8. **TSA influence on the p65 transactivation potential depends on the stimulus and the promoter.** ChIP assays were performed on HeLa cells treated with either PV or TNFα with or without TSA using different antibodies for the immunoprecipitation directed against: p65 phosphorylated on Ser^{536} (*A* and *B*) and IKKα (*C* and *D*). The immunoprecipitated chromatin was submitted to quantitative real time PCR analysis using primers amplifying the promoter region of *iκbα* (*A* and *C*) or *icam-1* (*B* and *D*). *, significantly different (p value < 0.05). The results presented on the graphs are an average of three independent experiments.

p65-Ser^{536}-P at 30 min, which is slightly reduced in the presence of TSA. Thus, we can conclude that the p65 transactivation potential on the *iκbα* promoter could be affected by the addition of TSA on PV stimulation, partially explaining the delay of *iκbα* mRNA expression. This effect is clearly promoter-specific because it was not observed with *icam-1*, another NF-κB-dependent gene.

IKKα Recruitment on a Defined Promoter Could be a Target for TSA Effect Depending on the Stimulus—Several studies have recently described an important nuclear role for IKKα in transcriptional activation. On the one hand, IKKα was shown to phosphorylate histone H3 on Ser^{10} on the *iκbα* promoter, which allows subsequent histone H3 acetylation on Lys^{14} by CBP/p300 (35, 38). On the other hand, Mayo and co-workers (39, 40) have highlighted an IKKα-mediated phosphorylation of p65 on Ser^{536} and SMRT on Ser^{2410} leading to derepression of SMRT-HDAC3 complexes and p65 acetylation on Lys^{310} by CBP/p300. Because the influence of the co-treatment of PV with TSA on the delayed *iκbα* mRNA expression appears to result from decreased phosphorylations of both histone H3 on Ser^{10} and p65 on Ser^{536}, we then wanted to determine whether IKKα recruitment on the *iκbα* promoter could be affected by the addition of TSA on PV stimulation. Indeed, this co-treatment leads to a transient down-regulation of IKKα binding on the *iκbα* promoter (15 and 30 min), whereas for stimulation with TNFα plus TSA, IKKα recruitment was increased (Fig. 8*C*). We next analyzed the *icam-1* promoter and observed that the presence of TSA induces no significant modification after PV induction, whereas for TNFα, a decrease of IKKα binding was detected (Fig. 8*D*). Taken together, these results indicate that the delayed *iκbα* mRNA resynthesis, observed when HeLa cells are treated with PV plus TSA, is due to, at least partly, a decreased presence of IKKα. Once again, the nature of the NF-κB-inducing agent can lead to different response that is promoter specific.

DISCUSSION

Acetylation of histones and transcription factors is tightly regulated by histone acetyltransferases and HDAC. Imbalances of these modifications frequently occur in tumor cells.

For instance, abnormal recruitment and activation of HDAC could lead to transcriptional repression of tumor suppressor genes. Therefore, long-time exposure to cells (longer than 24 h) with HDAC inhibitors, inducing hyperacetylation, appears to have anti-tumoral effects by reactivating gene expression and altering growth of tumor cells (11, 41). Nevertheless, TSA, which is a broad HDAC inhibitor, was previously demonstrated to modify the expression of only 2% of the genes (42). Moreover, among these 2%, genes are up-regulated as well as down-regulated. This highlights the complexity of the acetylation phenomenon and the need to further study the regulation mechanisms. Thus, we decided to examine the effect of TSA on the activation of the anti-apoptotic transcription factor, NF-κB. Adam *et al.* (24) have shown that short-time exposure to simultaneous addition of TSA can potentiate TNFα-induced NF-κB activation by, at least partially, extending IKK activity and delaying IκBα cytoplasmic reappearance. A complementary explanation can come from the fact that HDAC3 is responsible for p65 deacetylation leading to an increased affinity for IκBα thereby promoting its export to the cytoplasm. Therefore, HDAC inhibition by TSA could impair both IκBα interaction and nuclear export leading to sustained p65 nuclear residence (43).

In this work, we examined TSA effects on the PV-induced NF-κB activation compared with the TNFα-induced effect. The main conclusions are the following. (i) The presence of TSA sustains NF-κB activation after each stimulus. This highlights an inhibitory role of HDAC on NF-κB activation in both pathways, but by distinct mechanisms. (ii) For PV stimulation, the extension of NF-κB activation by TSA could be explained by a delay of *iκbα* mRNA synthesis, whereas, for TNFα, a prolonged IKK activity is expected to be implicated. (iii) The *iκbα* promoter is affected by co-treatment PV with TSA, leading to reduction of RNA Pol II recruitment, histone H3 modifications, p65-Ser^{536} phosphorylation, and IKKα binding. (iv) According to previous works (35, 38, 40), we hypothesize, for PV induction, a role of HDAC in IKKα recruitment on the *iκbα* promoter. This influences the subsequent histone H3 modifications, RNA Pol II binding but also p65-Ser^{536} phosphorylation and transactivation via CBP/p300 involvement. (v) TSA effects are clearly promoter-specific as the *icam-1* promoter displays different results.

More precisely, we investigated the impact of TSA on various NF-κB signaling pathways such as the classical pathway induced by TNFα or PMA and the atypical pathways elicited by PV or H_2O_2. At first, we demonstrated that TSA acts on NF-κB activation in a way depending on the stimulus and the cell type. Indeed, in HeLa cells, TSA prolongs NF-κB activation after TNFα, PMA, and PV but not after H_2O_2 treatments. As TSA combined with PMA extends NF-κB activation by sustaining the IKK activity, such as previously demonstrated for TNFα, we did not further study that inductor. The prolonged IKK activity more pronounced with PMA than with TNFα suggests differences in the implicated pathways that leads to activation/deactivation of the IKK complex. The IKK activation by H_2O_2 was slightly decreased by the presence of TSA, but we decided not to investigate further, in this study, this rather modest effect, and only compare TSA effects after TNFα and PV stimulation. The prolonged NF-κB activation seen with TSA appears to be reproducible to other HDAC inhibitors as well, as it can also be observed with SAHA. Nevertheless, the effect of TSA on PV induction is not general for all cell types. It is very weak in Jurkat T cells indicating that the effect is cell type-dependent. For both studied stimuli, PV and TNFα, TSA leads to an extension of NF-κB activation, but the mechanisms seem to be quite different, highlighting a stimulus dependence. The TNFα-induced expression of *iκbα* mRNA is not significantly modified by the presence of TSA and the prolonged IKK activity is postulated to be a cause for the delayed IκBα cytoplasmic reappearance (24). For PV stimulation, the addition of TSA does not affect either the IKK or tyrosine kinase activities but appears to delay *iκbα* mRNA synthesis, thereby explaining the prolonged NF-κB residence in the nucleus. Thus, we further analyzed the molecular mechanism of epigenetic modifications responsible for the delayed expression of *iκbα* mRNA. We observed a decrease of histone H3 phosphorylation on Ser^{10}, histone H3 acetylation on Lys^{14}, and RNA Pol II recruitment when TSA was added to PV, but not to TNFα. These reductions clarify the delay of *iκbα* mRNA synthesis. Recently a nuclear role for IKKα was discovered in histone H3 phosphorylation on Ser^{10} with the *iκbα* promoter, and this phosphorylation appears to be required for subsequent histone H3 acetylation on Lys^{14} by CBP/p300 (35, 38, 44). Furthermore, histone H3 must be acetylated to allow accessibility for components of the basal transcriptional machinery, especially RNA Pol II. Thus, if histone H3 phosphorylation is impaired on the *iκbα* promoter after co-stimulation of PV with TSA, it is likely that CBP/p300 is temporarily impeded and cannot acetylate histone H3 on Lys^{14} explaining why the RNA Pol II recruitment and *iκbα* mRNA synthesis are delayed. Referring to the work of Yamamoto and collaborators (35), we hypothesize that TSA affects the IKKα activity or its recruitment on the *iκbα* promoter leading to a decrease of histone H3 phosphorylation on Ser^{10}. Indeed, an additional ChIP assay (Fig. 8*C*) showed a reduced IKKα recruitment on the *iκbα* promoter, which could be the cause of the decreased phosphorylation and acetylation of histone H3 and the delay of RNA Pol II recruitment and *iκbα* mRNA synthesis.

MSK1 is another histone H3 kinase situated downstream of ERK and p38 in the MAPK pathway (36). The analysis of PV-induced activation of the upstream kinases, ERK and p38 MAPK, and the downstream targets, histone H3 (Ser^{10}) and p65 (Ser^{276}), revealed no modification in the presence of TSA, indicating that the global MSK1 activity was not affected. In addition, the binding of p65-Ser^{276}-P on *iκbα* and *icam-1* promoters does not appear to be significantly modified after PV or TNFα treatment with or without TSA.

The p65 transactivation potential was also studied through the recruitment of p65 phosphorylated on Ser^{536} on the *iκbα* promoter. We observed that TSA clearly impairs this PV-, but not TNFα-, induced binding, even if a similar amount of p65 is present in the nucleus in these two experimental conditions. This raises the possibility that either phosphorylated

p65-Ser^{536} could bind to the promoter and be immediately dephosphorylated, or p65 could be phosphorylated on Ser^{536} after its promoter recruitment. It has been recently demonstrated that chromatin-bound IKKα coordinates simultaneous phosphorylation of p65 on Ser^{536} and SMRT on Ser^{2410} on *ciap-2* and *il-8* promoters (40). These phosphorylations deactivate SMRT-HDAC3 repressor complexes and allow p65 to become acetylated on Lys^{310} by CBP/p300, increasing p65 transactivation. Therefore, we can postulate that the decreased *iκbα* mRNA expression after co-treatment of PV with TSA is a result of a transient impairment of IKKα recruitment on the *iκbα* promoter, as it was demonstrated in this study by ChIP assay (Fig. 8*C*). Another argument in favor of the implication of IKKα in our model is that p65 binding on the *iκbα* promoter is unaffected by TSA. It was already described that *IKKα*$^{-/-}$ cells display correct p65 recruitment on the *iκbα* promoter but impair phosphorylation on Ser^{10} and acetylation on Lys^{14} of histone H3 (35). Whereas, in *IKKβ*$^{-/-}$ cells, Hoberg and co-workers (39) have observed a loss of p65 binding on *ciap-2* and *il-8* promoters. Of course, we cannot exclude the role of another kinase phosphorylating p65 on Ser^{536}.

Therefore, by using the HDAC inhibitor TSA in a PV stimulation context, we postulate that HDAC could have a role in recruiting IKKα on the *iκbα* promoter. Indeed, the presence of TSA to PV treatment induces an impairment of IKKα binding on the *iκbα* promoter, which influences the two following events important for transcription. On the one hand, it reduces histone H3 phosphorylation on Ser^{10}, a prerequisite for histone H3 acetylation on Lys^{14} and RNA Pol II recruitment. And on the other hand, it decreases p65 phosphorylation on Ser^{536} needed for increasing transactivation via CBP/p300.

Another interesting observation from our study is that the influence of TSA on NF-κB activation seems to be clearly specific of the promoters. We demonstrated that *iκbα* and *icam-1* promoters display distinct responses after co-stimulation by PV or TNFα and TSA with respect to protein recruitments or histone H3 modifications. This is in good agreement with the work of Saccani *et al.* (45) who described the importance of histone H3 phosphorylation for transcription depending on the nature of each promoter. In this report, we showed that, for the *icam-1* promoter, the presence of TSA increases the PV-induced histone H3 phosphorylation and IKKα binding, whereas it reduces the TNFα one. This situation is opposite to the one observed on the *iκbα* promoter. Nevertheless, RNA Pol II recruitment on the *icam-1* promoter does not appear to be modified by TSA addition on PV or TNFα induction. The mechanism on the *icam-1* promoter needs to be better understood.

In conclusion, our results suggest that a large range HDAC inhibitor such as TSA is able to influence NF-κB activation in multiple ways. Moreover, an overall role of the HDAC is to inhibit NF-κB activation by different mechanisms that depend on the inducer and the considered promoter. This high specificity of NF-κB activation/repression represents an efficient regulation strategy.

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