0026-895X/08/7401-109–121$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 74:109–121, 2008

Vol. 74, No. 1
42838/3347479
Printed in U.S.A.

Histone Deacetylase Inhibitors Exert Time-Dependent Effects on Nuclear Factor-κB but Consistently Suppress the Expression of Proinflammatory Genes in Human Myometrial Cells

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Received November 9, 2007; accepted March 28, 2008

ABSTRACT

Premature activation of the inflammatory processes that mediate human parturition leads to preterm birth, a major clinical problem associated with neonatal morbidity and mortality. Histone deacetylase inhibitors (HDACi) are currently in clinical trials for the treatment of inflammatory disorders. Recent evidence suggests that there may be a therapeutic use for HDACi in the management of preterm birth, with administration of HDACi to pregnant mice shown to delay delivery. Because NF-κB is a key orchestrator of the inflammatory response and plays a pivotal role in parturition, it is important to understand how administration of HDACi might affect NF-κB activity in human uterine tissues. We show here that the effects of HDACi on nuclear factor-κB (NF-κB) in human myometrial cells are time-dependent. Short-term exposure to HDACi enhanced interleukin (IL)-1β-induced NF-κB activity as a result of potentiating IκB kinase (IKK)β activity, thereby leading to persistent turnover of IκBα/ε proteins and prolonging NF-κB phosphorylation, nuclear localization, and DNA binding. Conversely, long-term HDACi treatments resulted in repression of NF-κB DNA binding. Nevertheless, both short- and long-term HDACi treatments inhibited the expression of four labor-associated proinflammatory genes (*COX-2*, *IL-8*, *IL-6*, and *RANTES*), and this was associated with repression of the proinflammatory transcription factor c-Jun. Together, our data indicate that HDACi exert anti-inflammatory effects in human myometrium and may thus be useful in achieving a myometrial gene expression profile that favors uterine quiescence. However, coadministration of an IKKβ inhibitor may be both necessary and sufficient to circumvent potential induction of labor-associated pathways that could result from HDACi-induced augmentation of NF-κB activity.

During parturition, myometrium undergoes a transition from a state of quiescence to one of contractility, resulting in delivery of the neonate. Although the molecular mechanisms that underpin this transition are poorly understood, it is widely accepted that parturition is an inflammatory process, involving the up-regulation of multiple proinflammatory genes (Romero et al., 2006). Premature activation of uterine proinflammatory pathways can lead to preterm birth, a major clinical problem that occurs in 5 to 10% of pregnancies and is associated with 70 to 75% of neonatal morbidity/mortality (Wen et al., 2004).

Histone acetylation plays a major role in gene transcription, enabling the partial unraveling of local chromatin structure that is required to make the DNA accessible for binding by transcription factors and the basal transcription machinery (Li et al., 2004). Acetylation is reversible and reflects a dynamic balance between the activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Aberrant recruitment of HDACs to promoters and/or overexpression of HDACs are observed in cancer cells, and

This work was supported by Tommy's Campaign.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.042838.

ABBREVIATIONS: HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor(s); NF-κB, nuclear factor-κB; SIRT, Sirtuin; IκB, inhibitor of κB; IKK, inhibitor of κB kinase; $SCF^{\beta\text{-}TrCP}$, ligase composed of Skp1, Cdc53/Cu11, and a specificity-conferring F-box protein, in this case β-transducin repeat-containing protein (βTrCP); COX, cyclooxygenase-2; TSA, trichostatin A; IL, interleukin; SAHA, suberoylanilide hydroxamic acid; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; EMSA, electrophoretic mobility shift assay; LUC, luciferase; PCR, polymerase chain reaction; RANTES, regulated on activation normal T cell expressed and secreted; ac-HH4, acetylated histone H4; CRM, chromosome maintenance.

pharmacological HDAC inhibitors (HDACi) are in clinical trials for the treatment of cancers (Bolden et al., 2006). Cancer is an inflammatory disease, and HDACi are beginning to be considered as therapeutic agents in other inflammatory disorders (Lührs et al., 2002). Recent studies suggest that there could also be a clinical use for HDACi in the prevention of preterm delivery (Condon et al., 2003; Phillips et al., 2005). It is noteworthy that human parturition is characterized by a decrease in myometrial levels of HATs, global histone H3 acetylation decreases in both human and murine uterine tissue at term, and administration of HDACi to pregnant mice late in gestation delays delivery (Condon et al., 2003).

Because histone hyperacetylation is associated with transcriptional activation (Li et al., 2004), the inhibition of HDAC activities might be expected to result in a global increase in gene expression. However, microarray studies indicate that HDACi are selective in their effects on gene expression, altering the expression of only 2 to 10% of genes analyzed and down-regulating as many genes as they up-regulate (Kelly and Marks, 2005). The paradoxical inhibitory effects of HDACi on gene expression imply that other components of the transcriptional response, in addition to histones, are regulated by acetylation. Indeed, an increasing number of diverse nonhistone proteins are being identified as targets for HATs and HDACs. Nuclear factor-κB (NF-κB) is a nonhistone protein that both recruits HATs and HDACs to target gene promoters and is itself a substrate for HATs/HDACs (Quivy and Van Lint, 2004). The class I HDACs 1, -2, and -3, and the class III HDAC Sirtuin (SIRT)1 have been implicated in the inhibition of NF-κB-mediated transcription, with HDAC3 and SIRT1 reportedly acting to deacetylate NF-κB itself (Chen et al., 2001; Quivy and Van Lint, 2004; Yeung et al., 2004).

NF-κB is key to the orchestration of the inflammatory response, serving to induce the expression of numerous proinflammatory genes (Lindström and Bennett, 2005b), and the anti-inflammatory effects of HDACi in patients with ulcerative colitis are associated with diminished NF-κB activation (Lührs et al., 2002). NF-κB is a transcription factor family composed of five members, p65, p50, p52, c-rel, and RelB, which form heterogeneous dimers. In resting cells, NF-κB is retained in the cytoplasm in an inactive form through association with the IκB proteins, namely, IκBα, IκBβ, and IκBε. In the classic pathway, proinflammatory signaling converges on the IκB kinase (IKK) complex. Once activated, the IKKβ subunit phosphorylates IκBα on serines 32/36 (Traenckner et al., 1995), and this phosphorylation targets IκBα for ubiquitination by the $SCF^{\beta\text{-TrCP}}$ ubiquitin ligase. Ubiquitinated IκBα is subsequently degraded by the 26S proteasome, thereby releasing p50/p65 NF-κB dimers from the cytoplasmic IκBα/NF-κB complex and allowing them to translocate to the nucleus where they bind to target gene promoters to modulate transcription.

Acetylation/deacetylation can affect NF-κB-mediated transcription on multiple levels. Reports on the effects of HDACi on NF-κB activity are conflicting (Chen et al., 2001; Takada et al., 2006). Likewise, the effects of HDACi on proinflammatory gene expression vary in a promoter-, stimulus-, and cell type-specific manner (Horion et al., 2007). Unsurprisingly given its importance in inflammation, accumulating evidence suggests that NF-κB plays a pivotal role in parturition (Lindström and Bennett, 2005b; Mendelson and Condon, 2005). In the present study, we investigate the effects of HDACi on NF-κB activity and proinflammatory gene expression in primary human myometrial cells to assess the therapeutic potential of HDACi in the maintenance of myometrial quiescence.

Materials and Methods

Reagents. Antibodies against p65, p50, IκBα, IκBε, cyclooxygenase (COX)-2, c-Jun, and the IKKβ antibody used for Western immunoblotting were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against serine 32/36-phosphorylated IκBα, against serine 536-phosphorylated p65, and against the IKKβ antibody used for immunoprecipitation were from Cell Signaling Technology Inc. (Danvers, MA). The anti-acetylated histone H4 antibody was from Millipore (Billerica, MA), and the antibody against lamin B was from Calbiochem (Nottingham, UK). Trichostatin A (TSA) and the α-smooth muscle actin antibody were from Sigma-Aldrich (Exeter, UK). Recombinant human interleukin (IL)-1β was from R&D Systems (Abingdon, UK), and suberoylanilide hydroxamic acid (SAHA) was from BioVision (Mountain View, CA). Sc-514 was purchased from Calbiochem. TSA, SAHA, and sc-514 were dissolved in dimethyl sulfoxide. All experiments were vehicle-controlled and dimethyl sulfoxide, at the concentrations used in this study, did not affect any aspect of the NF-κB signaling pathway.

Primary Human Myometrial Cell Culture. Institutional Ethics Committee approval was obtained for the collection of myometrial tissues from healthy women at 37 to 39 weeks gestation, and all patients gave informed consent. Myometrial biopsies were collected at term from the upper margin of uterine incision at the time of lower segment caesarean section, as indicated by breech presentation or previous caesarean section. Myometrial tissue was dissected, rinsed in phosphate-buffered saline (PBS), and digested in PBS containing 15 mg/ml collagenase 1A (Sigma-Aldrich), 15 mg/ml collagenase X (Sigma-Aldrich), and 50 mg/ml bovine serum albumin (Sigma-Aldrich) for 45 min at 37°C. The cell suspension was filtered through a cell strainer and pelleted at 400*g* for 5 min. Cells were used between passage numbers 1 to 5. Cells were grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS; Sigma-Aldrich), 2 mM L-glutamine (Invitrogen, Paisley, UK), and 100 U of penicillin-streptomycin (Invitrogen), and then they were incubated overnight in DMEM containing 2% FCS, 2 mM L-glutamine, and 100 U of penicillin-streptomycin, and the effects of TSA or SAHA on unstimulated or IL-1β-stimulated cells were examined.

Western Immunoblotting. Nuclear and cytoplasmic extracts were prepared as described previously (Lindström and Bennett, 2005a). For whole-cell lysates, cells were lysed for 20 min on ice in radioimmunoprecipitation assay buffer (1% NP-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris, pH 8.0, and 2 mM NaF). Protein concentrations of cell lysates were determined using detergent-compatible protein assay reagents (Bio-Rad Laboratories, Hertfordshire, UK). Western blotting was performed as described previously (Lindström and Bennett, 2005a). The levels of cellular α-smooth muscle actin were used as a loading control, and membranes were reprobed with antibodies against lamin B to confirm the integrity of cell fractionation.

Electrophoretic Mobility Shift Assay. Nuclear lysates were prepared, and electrophoretic mobility shift assays (EMSAs) were performed as described previously (Lindström and Bennett, 2005a). Consensus double-stranded oligonucleotides were obtained from Promega (Southampton, UK), NF-κB: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (specific binding site sequences underlined).

Transient Transfections. Cells at ~70% confluence in 24-well plates were transfected using the liposome reagent FuGENE-6 (Roche, Welwyn Garden City, UK) according to the manufacturer's instructions. Cells were transfected with 0.4 μg/well of a NF-κB-

dependent firefly luciferase reporter construct (NF-κB-LUC) at a 3:1 ratio of FuGENE-6 to DNA, in DMEM and 10% FCS, together with 0.04 μg/well cytomegalovirus-*Renilla reniformis* as an internal control for transfection efficiencies. NF-κB-LUC contains two tandem repeats of the sequence 5′-GGG GAC TTT C CC TGG GGA CTT TCC CTG GGG ACT TTC CC-3′, which contains three copies of the decameric κB site (underlined) upstream of a minimal β-globin promoter driving a luciferase gene (in a pGL3 vector). NF-κBmut-LUC, in which the κB sites are mutated, was used as a control to confirm NF-κB-mediated transactivation. The medium was replaced with DMEM containing 2% FCS 16 h after transfection, and the cells incubated for a further 24 h before treatment with TSA, SAHA, and/or IL-1β. Reporter activity was analyzed in a firefly luciferase assay (Promega), and firefly values were normalized according to the internal control *R. reniformis* luciferase values. The *R. reniformis* luciferase assay was carried out by adding coelenterazine substrate (Calbiochem) in 0.5 M HEPES, pH 7.8, and 40 mM EDTA to the firefly luciferase reaction.

In Vitro IKK Kinase Assay. After treatment, cells were washed two times in PBS, scraped into 10 ml of PBS, and centrifuged at 400*g* for 5 min. The PBS was removed, and the cell pellet resuspended in 400 μl of nondenaturing cell lysis buffer (New England Biolabs, Hitchin, Hertfordshire, UK). Cell lysates were incubated on ice for 20 min, and then they were centrifuged for 20 min at 13,000 rpm at 4°C. The supernatants were transferred to a fresh Eppendorf tube, and endogenous IKKβ was immunoprecipitated from 400 to 500 μg of protein lysate using a rabbit anti-IKKβ antibody (L570; Cell Signaling Technology Inc.) at a 1/100 dilution. The cell lysate/antibody mix was incubated at 4°C on a rotator overnight. Twenty microliters of protein Sepharose A beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was added to the cell lysate/antibody mix and replaced on the rotator for a further 2 h. The beads were then centrifuged at 13,000 rpm at 4°C for 30 s, the supernatant was discarded, and the beads were washed two times in lysis buffer and two times in kinase buffer (New England Biolabs, Ipswich, MA). Fifty microliters of kinase buffer supplemented with 100 μM ATP (New England Biolabs) and 2 μg of glutathione transferase (GST)-IκBα fusion protein (Europa Bioproducts Ltd., Cambridge, UK) was added to the beads. The kinase reaction was vortexed and incubated in a 30°C shaking water bath for 30 min. Then, 10 μl of 3× Laemmli sample buffer was added to stop the reaction, and the samples were boiled for 5 min at 100°C. Samples were centrifuged at 13,000 rpm for 30 s, and the supernatants resolved on a 10% SDS-polyacrylamide gel electrophoresis gel. Whole-cell lysate was loaded in parallel as a positive control, and samples immunoprecipitated with preimmune rabbit IgG were included as a negative control. Western immunoblot analysis was next performed using antibodies against IκBα phosphorylated on serines 32/36 to assess kinase activity. To confirm equal immunoprecipitation of the kinase, membranes were also probed with an antibody against IKKβ (Santa Cruz Biotechnology, Inc.). Membranes were then stripped and reprobed for total IκBα (Santa Cruz Biotechnology, Inc.), to confirm the presence of equal amounts of substrate.

Quantitative Real-Time PCR. Total RNA was extracted using RNA STAT-60 reagent (AMS Biotechnology, Abingdon, Oxon, UK) according to the manufacturer's specifications. To remove any contaminating DNA, 1 μg of total RNA was digested with 0.5 μl of DNaseI in 1× DNaseI reaction buffer (Invitrogen) in a total volume of 5 μl at room temperature for 15 min. The reaction was terminated by the addition of 0.5 μl of 25 mM EDTA and incubation at 65°C for 15 min. The whole of this reaction was subsequently used for first-strand cDNA synthesis with SuperScript first-strand synthesis system for RT-PCR (Invitrogen), according to the manufacturer's instructions. For quantitative real-time PCR, the resulting cDNA was analyzed in triplicate using SYBR Green Master Mix (Applied Biosystems, Warrington, Cheshire, UK) in the ABI Prism 7700 sequence detection system (Applied Biosystems). The values obtained were normalized according to transcript levels of the ribosomal protein *L19*, whose expression has been used as a control to normalize gene expression in many contexts, including in human endometrium in a study of recurrent pregnancy loss (Francis et al., 2006), and in a proinflammatory setting (Wan et al., 2007). The primers used to analyze the different transcripts were designed with the software Primer Express (Applied Biosystems): *IKBA*: forward, 5′-CCTGGCCCAAAACGTCTTATT-3′; reverse, 5′-TGATCTTTCTCGTCCCCT ACAAA-3′, *COX-2*: forward,5′-GCTCAAACATGATGTTTGCATTC-3′; reverse, 5′-GCTGGCCCTCGCTTATGA-3′, *IL-8*: forward, 5′-AAGGAACCATCTCACTGTGTGTAAAC-3′; reverse, 5′-ATCAGGAAGGCTGCCAAGAG-3′, *IL-6*: forward, 5′-CACCGGGAACGAAAGAGAAG-3′; reverse, 5′-AGGCGCTTGTGGAGAAGGA-3′, regulated on activation normal T cell expressed and secreted (*RANTES*): forward, 5′-GCATCTGCCTCCCCATATTC-3′; reverse, 5′-AGTGGGCGGGCAATGTAG-3′, and *L19*: forward, 5′-GCGGAAGGGTACAGCCAAT-3′; reverse, 5′-GCAGCCGGCGCAAA-3′.

Results

HDACi Exert Time-Dependent Effects on NF-κB Activation. Data on the effect of pharmacological HDAC inhibition on NF-κB seem contradictory, with some studies showing a potentiation (Chen et al., 2001; Adam et al., 2003; Yeung et al., 2004) and others reporting an inhibition (Lührs et al., 2002; Takada et al., 2006) of NF-κB activity. Several studies using the same HDACi compound used very different treatment protocols, with exposure of cells to HDACi ranging from 1 to 24 h. IL-1β activates NF-κB, it is considered a master regulator of inflammation in diverse disease processes, its expression increases in the uterus as term approaches and in association with labor onset (Elliott et al., 2001; Mendelson and Condon, 2005), and administration of IL-1β can induce labor in the rhesus monkey (Vadillo-Ortega et al., 2002). We therefore tested both long- and short-term effects of the HDACi TSA on IL-1β-induced NF-κB activity in human myometrial cells.

Cells were preincubated with 50, 100, or 500 nM TSA for 1 h to establish inhibition of HDAC activity, and then they were stimulated with IL-1β, to activate NF-κB, for 2 or 24 h. Treatment of cells with TSA did not lead to any overt morphological changes at any time point, nor did it induce the cleavage of caspase-3 or poly(ADP-ribose) polymerase (data not shown), in agreement with previous studies showing that HDACi induce apoptosis in cancer cells but not in normal cells (Kelly and Marks, 2005). Nuclear extracts were prepared and binding of proteins to a consensus κB probe was assessed by EMSA (Fig. 1, A and B). TSA alone had no effect on NF-κB DNA binding at any time point. However, TSA enhanced IL-1β-stimulated NF-κB DNA binding at 2 h (lanes 3–6), whereas, conversely, it repressed IL-1β-induced NF-κB DNA binding at 24 h (lanes 12–15). Nuclear extracts used in the EMSA experiments were subjected to Western immunoblot analysis using an antibody that specifically recognizes acetylated histone H4 (ac-HH4) to confirm the efficacy of TSA. IL-1β alone had no effect on HH4 acetylation (Fig. 1D). Although TSA alone had no effect on NF-κB DNA binding (Fig. 1A), TSA treatment resulted in the appearance of ac-HH4 by 2 h, presumably as a consequence of preventing HH4 deacetylation (Fig. 1D). However, ac-HH4 could not be detected at 24 h. To exclude nonspecific effects of the TSA compound, SAHA, a structurally similar HDACi that also inhibits class I and II HDACs and is currently in clinical trials for cancer treatment (Kelly and Marks, 2005), was also

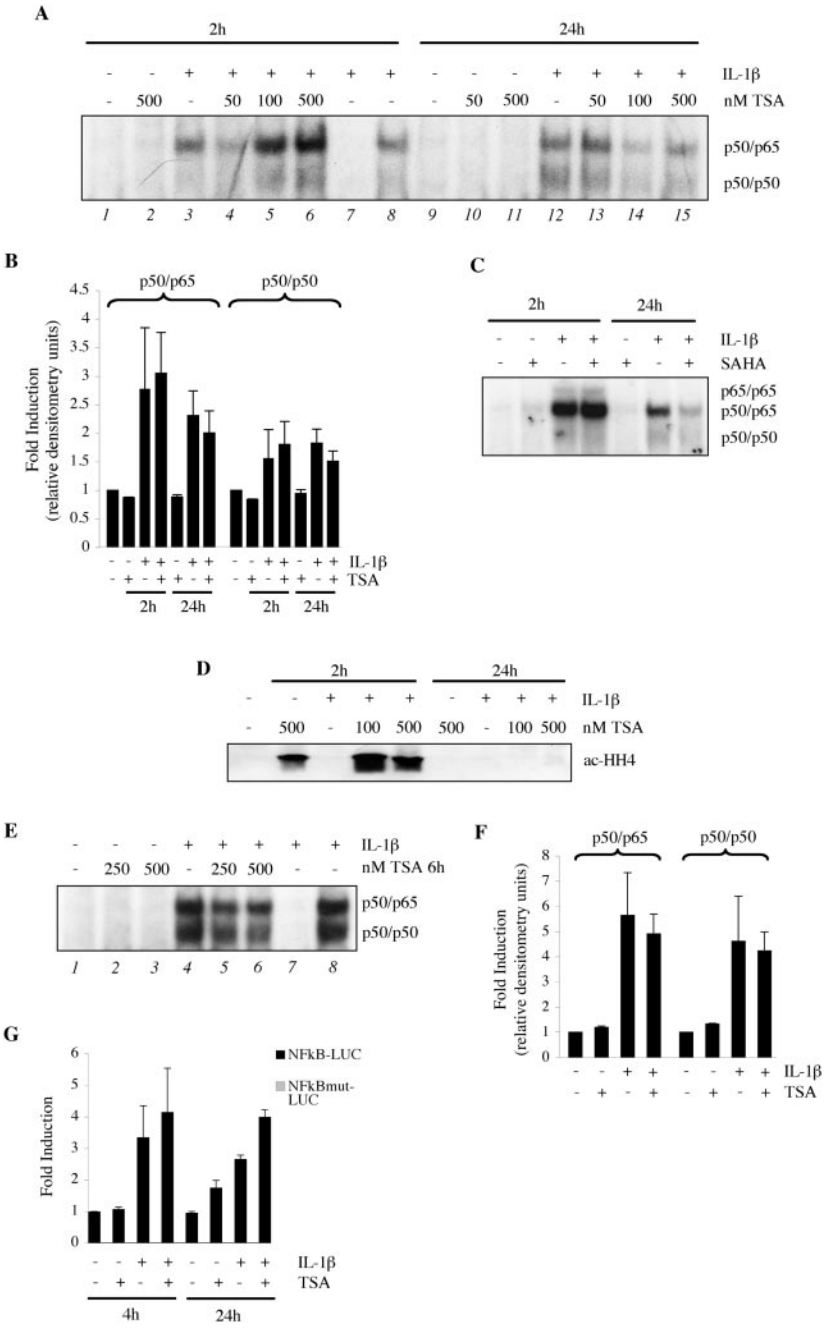

Fig. 1. Time-dependent effects of HDACi on NF-κB activity. A, myometrial cells were preincubated with the indicated concentrations of TSA for 1 h, and then they were stimulated with 1 ng/ml IL-1β for 2 or 24 h, and nuclear lysates were assessed by EMSA for NF-κB DNA binding using a consensus κB probe. A 100-fold excess of unlabeled κB (xs spec) or irrelevant (xs nonspec) oligonucleotide was added to lanes 7 and 8, respectively, before the addition of ^{32}P-labeled probe as DNA binding competitor to verify specificity of binding. B, densitometric quantitation of p50/p65 and p50/p50 DNA-binding complexes normalized against actin protein levels. Data are presented as -fold induction relative to nonstimulated cells at the start of the experiment, and they are the mean ± S.E.M. from two independent experiments. The bars representing TSA or TSA + IL1β bands reflect data from cells incubated with 500 nM TSA. C, myometrial cells were preincubated with 5 μM SAHA for 1 h, and then they were stimulated with 1 ng/ml IL-1β for 2 or 24 h, and nuclear lysates were assessed by EMSA for NF-κB DNA binding using a consensus κB probe. We have previously identified the NF-κB complexes binding to DNA in response to IL-1β in myometrial cells as p65/p65, p50/p50, and p50/p65 at early time points, and p50/p50 and p50/p65 at late time points (Lindstrom and Bennett, 2005a; unpublished data), as indicated. D, nuclear lysates from A were assessed for expression of acetylated HH4 (ac-HH4) by Western immunoblotting. E, myometrial cells were preincubated with the indicated concentrations of TSA for 1 h, and then they were stimulated with 1 ng/ml IL-1β for 6 h, and nuclear lysates were assessed by EMSA for NF-κB DNA binding using a consensus κB probe. A 100-fold excess of unlabeled κB (xs spec) or irrelevant (xs nonspec) oligonucleotide was added to lanes 7 and 8, respectively. F, densitometric quantitation of p50/p65 and p50/p50 DNA-binding complexes normalized against actin protein levels. Data are presented as -fold induction relative to nonstimulated cells at the start of the experiment, and they are the mean ± S.E.M. from two independent experiments. The bars representing TSA or TSA + IL1β bands reflect data from cells incubated with 500 nM TSA. G, myometrial cells were transiently transfected with 0.4 μg/well of a NF-κB-dependent luciferase reporter (NF-κB-LUC), pretreated for 1 h with 500 nM TSA, followed by stimulation with 1 ng/ml IL-1β for 4 or 24 h. NF-κBmut-LUC, in which the κB sites are mutated, was used as a control to confirm NF-κB-mediated transactivation. Cells were cotransfected with a cytomegalovirus-*R. reniformis* reporter plasmid as an internal control, and luciferase activity normalized for *R. reniformis* reporter readout. Results are the mean ± S.E.M. from three independent experiments performed in triplicate, and they are presented as the -fold induction relative to reporter activity in cells treated with vehicle only at 4 h.

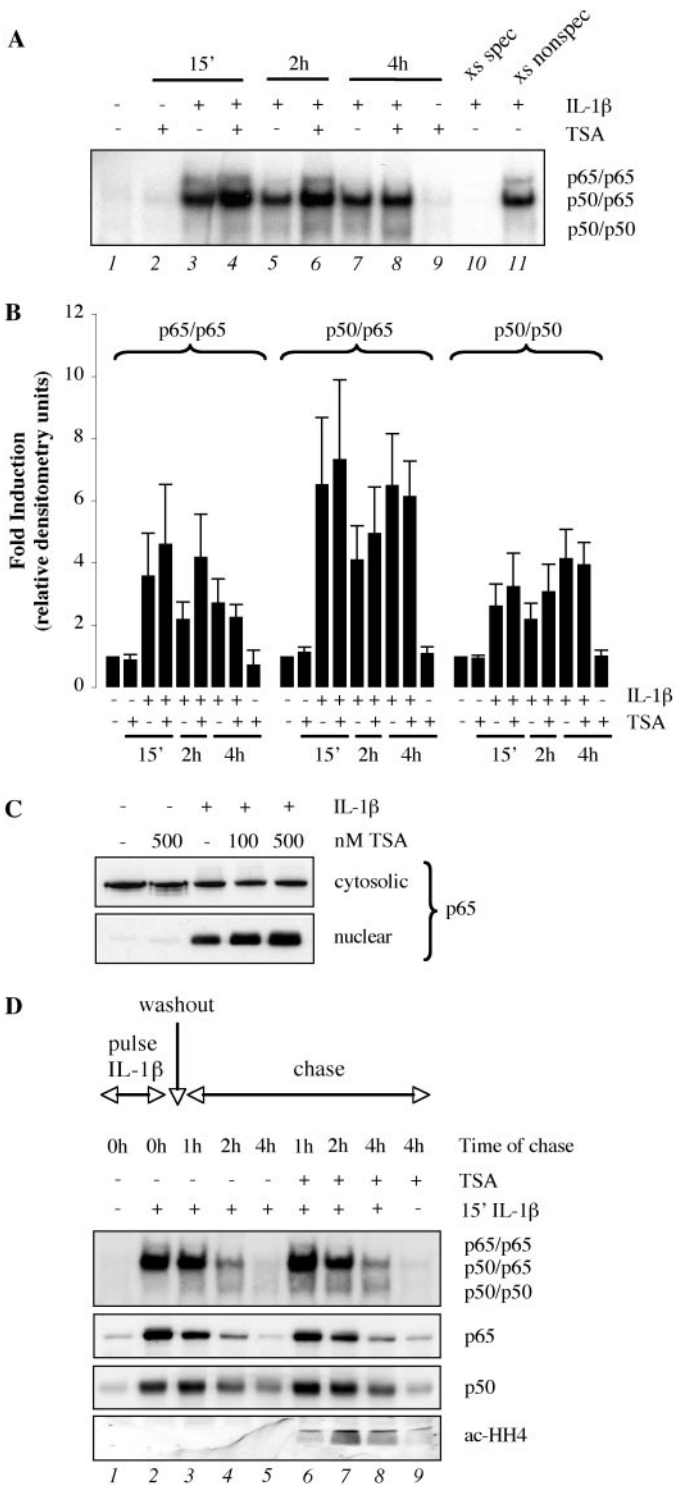

Fig. 2. Short-term exposure to HDACi prolongs NF-κB nuclear localization and DNA binding in response to IL-1β. A, myometrial cells were preincubated with 500 nM TSA for 1 h, and then stimulated with 1 ng/ml IL-1β for 15 min, 2 h, or 4 h, and nuclear lysates were assessed by EMSA for NF-κB DNA binding using a consensus κB probe. A 100-fold excess of unlabeled κB (xs spec) or irrelevant (xs nonspec) oligonucleotide was added to lanes 10 and 11, respectively, before the addition of ^{32}P-labeled probe as DNA binding competitor to verify specificity of binding. B, densitometric quantitation of p65/p65, p50/p65 and p50/p50 DNA binding complexes normalized against actin protein levels. Data are presented as -fold induction relative to nonstimulated cells at the start of the experiment, and they are the mean ± S.E.M. from three independent experiments. C, myometrial cells were preincubated with 100 or 500 nM TSA for 1 h, and then stimulated with 1 ng/ml IL-1β for 2 h. Cytosolic and nuclear extracts were prepared and assessed for levels of p65 by Western immunoblotting. D, myometrial cells were pulse-stimulated with 1 ng/ml IL-1β for 15 min, the IL-1β was then washed out and incubation continued for 1, 2, or 4 h in medium alone or in the presence of 500 nM TSA. Nuclear extracts were prepared and assessed by EMSA for NF-κB DNA binding using a consensus κB probe, and for nuclear levels of p65, p50, and ac-HH4 proteins by Western immunoblotting.

used. After a 1-h preincubation period, 5 μM SAHA enhanced IL-1β-stimulated NF-κB DNA binding at 2 h, but repressed IL-1β-stimulated DNA binding at 24 h (Fig. 1C), confirming the results obtained with TSA. Inhibition of IL-1β NF-κB DNA binding was already detectable by 6 h of incubation in the presence of TSA and IL-1β (Fig. 1, E and F).

To determine whether the effects of HDACi on NF-κB DNA binding translated to an effect on NF-κB transcriptional activity, myometrial cells were transiently transfected with a NF-κB-dependent luciferase reporter. When cells were preincubated with TSA for 1 h and stimulated with IL-1β for 4 h, TSA alone had no effect on reporter activity, but it resulted in a modest increase in IL-1β-induced reporter activity (Fig. 1G), consistent with the effects of HDACi on NF-κB DNA binding. We were surprised to find that when cells were preincubated with TSA for 1 h and then stimulated with IL-1β for 24 h, TSA did not diminish NF-κB transcriptional activity; rather, it exhibited a modest augmentation of IL-1β-induced reporter activity (Fig. 1G). At this time, TSA alone was able to increase activity of the NF-κB reporter. Mutation of the κB sites abolished basal reporter activity, as well as the effects of IL-1β, TSA, or both, confirming that the luciferase readout was NF-κB-specific under all treatment conditions (Fig. 1G). The transfection data from the long-term incubation experiments are in contrast to the observations on NF-κB DNA binding at these time points. A possible explanation is that TSA is exerting a positive effect on the transcriptional activity of NF-κB independently of effects on NF-κB DNA binding activity (see *Discussion*).

Thus, caution must be exercised when interpreting and comparing results based on experiments using different lengths of exposure to HDACi. Short-term effects of HDACi most likely reflect direct effects of prolongation of acetylation on the NF-κB pathway, whereas long-term treatments with HDACi reveal secondary, indirect effects on NF-κB. Because enhancement of NF-κB activity would be undesirable in the management of preterm labor, it was important to next elucidate the mechanisms by which HDACi potentiate NF-κB activation during short-term treatments.

HDACi Prolong IL-1β-Induced Nuclear Localization and DNA Binding of NF-κB. In initial experiments aimed at characterizing the primary, potentiating effects of HDACi on NF-κB activity, myometrial cells were preincubated with TSA for 1 h and then they were stimulated with IL-1β for 15 min, 2 h, and 4 h; finally, the nuclear lysates were prepared for analysis of NF-κB DNA binding. TSA alone had no effect on NF-κB DNA binding at any time, but TSA treatment enhanced IL-1β-induced NF-κB DNA binding at 15 min and 2 h after stimulation, but not at 4 h (Fig. 2, A and B). Western immunoblot analysis demonstrated that TSA increased nuclear levels of p65 in a dose-dependent manner after IL-1β stimulation, but not in the absence of IL-1β (Fig. 2C). This was not due to a TSA-induced increase in p65 expression, because cytosolic levels of p65 remained unchanged by TSA treatment. Although IL-1β stimulation resulted in a readily

detectable increase in nuclear p65 protein levels, a concomitant decrease in cytosolic p65 levels was not detected. This is commonly observed in a number of cell types and is a function of the proportionally high levels of cytosolic p65, such that the loss of a small fraction of total cytosolic p65 is not readily detectable (Mattioli et al., 2004). Likewise, treatment of cells with SAHA plus IL-1β also resulted in increased p65 nuclear levels compared with IL-1β treatment alone (data not shown).

A chase experiment was next performed to determine whether the potentiating effect of TSA on IL-1β-induced NF-κB requires the continuous presence of IL-1β. Myometrial cells were stimulated with IL-1β for 15 min. IL-1β was subsequently withdrawn from the cells, and incubation continued for the indicated times in medium alone or in medium containing TSA. Cells incubated with TSA exhibited more sustained NF-κB DNA binding after the transient stimulation with IL-1β, compared with cells incubated in medium alone (Fig. 2D, compare lanes 3–5 with lanes 6–8). This was associated with prolonged IL-1β-induced nuclear expression of p65 and p50 proteins in TSA-treated cells.

Taken together, these findings suggest that the augmenting effect of TSA on IL-1β-induced NF-κB DNA binding occurs as a result of IL-1β inducing the activation, and possibly acetylation, of components of the NF-κB pathway, and TSA blocking the deacetylation of such components, thus delaying the postinduction down-regulation of the NF-κB response.

HDACi Suppress IκB Protein Reappearance. NF-κB activation is governed by an autoregulatory "postinduction repression" mechanism. Because of the presence of κB sites in the promoter of the *IKBA* gene, activation of NF-κB leads to the rapid resynthesis of IκBα, which accumulates in the nucleus and dissociates NF-κB from DNA-bound complexes. These newly formed IκBα/NF-κB complexes are then exported out to the cytoplasm, a process dependent on a nuclear export signal present in IκBα (Huang and Miyamoto, 2001). We have found that the levels of resynthesized IκBα after stimulation influence the duration of NF-κB activation in myometrial cells (T. M. Lindström and P. R. Bennett, unpublished data). It therefore seemed possible that modulation of IκBα by HDACi underlies the prolonged nuclear localization and DNA binding of NF-κB in response to these compounds. The effect of HDACi on IκBα protein expression in myometrial cells was therefore examined. Cells were preincubated with 500 nM TSA for 1 h and subsequently stimulated with IL-1β for 15 min, 2 h, 4 h, 8 h, and 24 h. In cells treated with IL-1β alone, IκBα was completely degraded by 15 min after stimulation and resynthesized by 2 h (Fig. 3A). In cells pretreated with TSA, IL-1β-induced IκBα degradation occurred as normal, but resynthesized IκBα protein levels were markedly reduced at every time point after stimulation. The reduction in IκBα levels was associated with a concomitant increase in nuclear levels of p65 at early time points (Fig. 3A). In cells treated for 8 or 24 h with TSA and IL-1β, nuclear p65 expression did not differ from cells treated with IL-1β alone, whereas IκBα levels were still comparatively lower.

Like IκBα, IκBε can shuttle between the nucleus and cytoplasm, and it may participate in postinduction repression of NF-κB (Lee and Hannink, 2002). Therefore, the question of whether TSA-induced changes in IκBε protein levels might contribute to the potentiation of NF-κB at early time points and/or the inhibition of NF-κB DNA binding at later times was also addressed. In cells treated with IL-1β alone, IκBε was degraded 15 min after stimulation and resynthesized by 4 h (Fig. 3A). In cells pretreated with TSA, IL-1β-induced IκBε degradation occurred as normal, but resynthesized IκBε protein levels were reduced at 4 h after stimulation. However, unlike IκBα, the repressive effect of TSA on IκBε did not persist, with IκBε protein levels returning to normal postinduction levels by 8 h (Fig. 3A).

IκBβ proteins also sequester a substantial proportion of NF-κB dimers. Unlike IκBα and IκBε, however, IκBβ does not exhibit nucleocytoplasmic shuttling, and it does not contribute to postinduction termination of the NF-κB response, because it lacks a nuclear export signal (Huang and Miyamoto, 2001; Malek et al., 2001). Conversely, some studies have described a role for IκBβ in persistence of the NF-κB response, either as a result of continual IκBβ turnover creating an imbalance in the relative levels of NF-κB and IκB proteins (Xu et al., 2006), or as a result of resynthesized nuclear IκBβ acting in a chaperone-like manner to prevent the nuclear export of NF-κB by IκBα (DeLuca et al., 1999). However, we found that in myometrial cells, IκBβ is resynthesized with delayed kinetics in the presence of IL-β alone, and it does not contribute to the persistence of NF-κB acti-

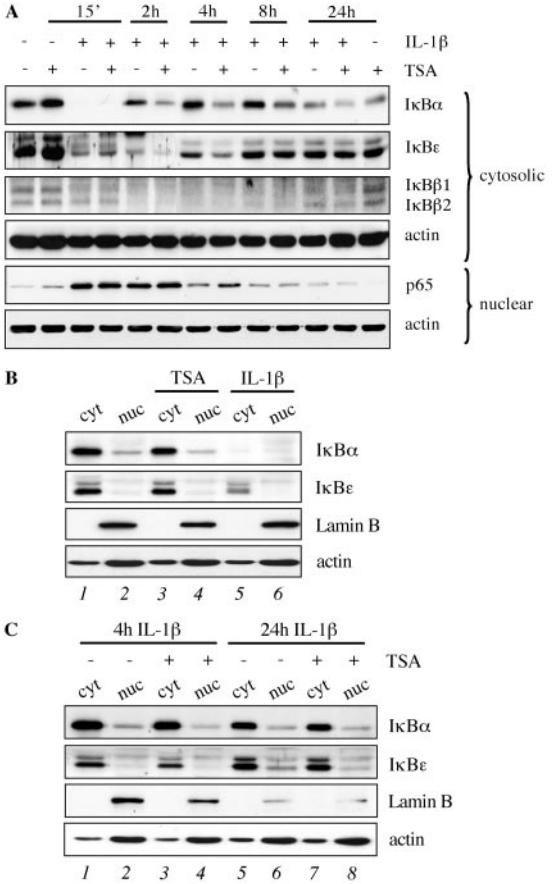

Fig. 3. HDACi reduce levels of resynthesized IκBα and IκBε proteins after IL-1β stimulation, but they do not influence the IκB cytosolic/nuclear ratio. Myometrial cells were preincubated with 500 nM TSA for 1 h, and then they were stimulated with 1 ng/ml IL-1β for the indicated times, and cytosolic/nuclear extracts were prepared. A, cytosolic IκBα, IκBε, and IκBβ protein levels, and nuclear p65 protein levels were assessed by Western immunoblotting. B and C, cytosolic and nuclear levels of IκBα and IκBε were compared by Western immunoblotting. Blots were reprobed for lamin B expression to confirm the integrity of cell fractionation.

vation (Fig. 3A; T. M. Lindström and P. R. Bennett, unpublished data). Furthermore, TSA had no effect on the IL-1β-induced degradation or resynthesis of IκBβ (Fig. 3A).

The heterodimeric importin α/β complex is responsible for the nuclear import of proteins containing nuclear localization signals, and importin α is subject to dynamic acetylation (Bannister et al., 2000). The second ankyrin repeat of IκBα serves as a substitute for a classic nuclear localization signal, and nuclear import of IκBα requires both importin α and β (Turpin et al., 1999). IκBε is likely to use a similar mechanism to IκBα to enter the cell nucleus (Lee and Hannink, 2002). We therefore asked whether HDACi prolong the nuclear localization of NF-κB at early time points by disrupting the nuclear import of resynthesized IκBα or IκBε. Cytosolic and nuclear fractions of myometrial cells treated with TSA ± IL-1β were assessed for levels of IκBα and IκBε proteins. IκBα and IκBε proteins were detected in both the cytoplasmic and the nuclear fractions under basal conditions, although nuclear IκBε levels were low (Fig. 3B), consistent with previous work showing that nuclear import of IκBε is less efficient than that of IκBα (Lee and Hannink, 2002). Stimulation of myometrial cells with IL-1β for 15 min resulted in the degradation of both cytoplasmic and nuclear IκBα and IκBε proteins (Fig. 3B). Treatment of cells with TSA alone did not result in accumulation of IκB proteins in the cytosol (Fig. 3B). Likewise, the ratio of cytosolic/nuclear levels of resynthesized IκB proteins was not greater in cells treated with IL-1β plus TSA compared with cells treated with IL-1β alone (Fig. 3C). This suggests that the HDACi-induced prolongation of NF-κB nuclear localization at early time points is not due to disrupted import of inhibitory IκB proteins.

Both IκBα and IκBε require the chromosome maintenance (CRM)1-dependent nuclear export pathway to exit the nucleus and restore cytosolic pools of NF-κB (Huang and Miyamoto, 2001; Lee and Hannink, 2002). Because there was also no difference in the ratio of nuclear/cytosolic levels of resynthesized IκB proteins between cells treated with IL-1β plus TSA and cells treated with IL-1β alone at early time points (Fig. 3C), it seems that the HDACi-induced prolongation of NF-κB nuclear localization at early time points is not due to a failure to export IκB-bound NF-κB from the nucleus. As seen above (Fig. 1, A–E), long-term exposure (>6 h) of myometrial cells to TSA results in inhibition of IL-1β-induced NF-κB DNA binding independently of effects on nuclear levels of NF-κB. Such inhibition could potentially reflect a scenario in which NF-κB is dissociated from DNA by IκBα/ε but cannot be exported to the cytoplasm because of delayed disruption of the CRM1 export pathway. However, we also failed to detect any difference in the ratio of nuclear/cytosolic levels of resynthesized IκB proteins between cells treated with IL-1β plus TSA and cells treated with IL-1β alone at late time points (Fig. 3C), indicating that CRM1-mediated export is not targeted by TSA.

Taken together, these findings suggest that HDACi prolong NF-κB nuclear localization and DNA binding after IL-1β stimulation by decreasing the levels of resynthesized IκBα and IκBε proteins, but not by disrupting the nuclear import/export of NF-κB/IκB complexes. It seems that the secondary, inhibitory effects of HDACi on NF-κB DNA binding are not related to changes in IκBα or IκBε protein levels; rather, they target NF-κB downstream of its release from IκB proteins.

HDACi Do Not Inhibit IκBα mRNA Expression. We next sought to examine the effect of HDACi on IκBα expression in more detail. As with TSA, pretreatment of myometrial cells for 1 h with SAHA also resulted in decreased IκBα protein levels 2 h after IL-1β stimulation (Fig. 4A). The cytosolic lysates corresponding to the nuclear lysates used in the chase experiment for NF-κB were next probed for levels of IκBα protein. Converse to the findings for NF-κB, IκBα

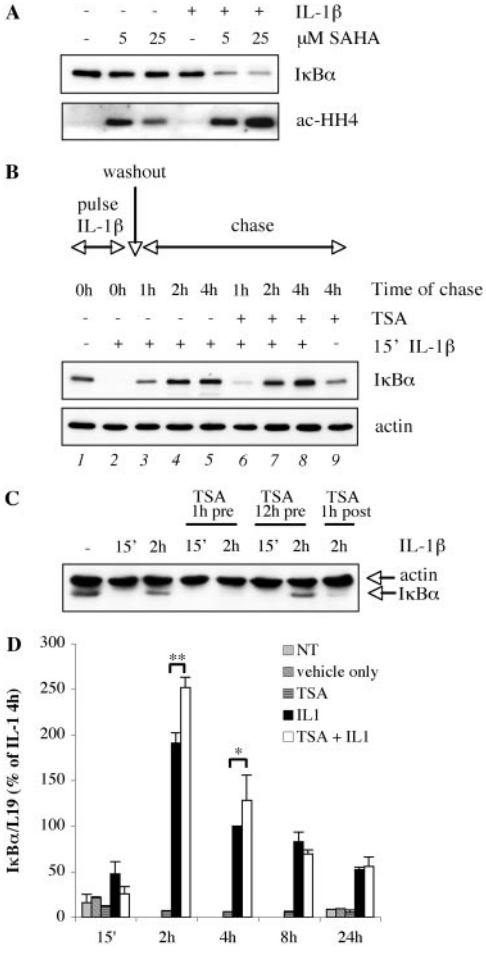

Fig. 4. HDACi reduce resynthesized IκBα protein levels, independently of effects on IκBα mRNA expression. A, myometrial cells were preincubated with 5 or 25 μM SAHA for 1 h, and then they were stimulated with 1 ng/ml IL-1β for 2 h. Cytosolic and nuclear extracts were prepared and assessed by Western immunoblotting for protein levels of cytosolic IκBα and nuclear ac-HH4. B, myometrial cells were pulse-stimulated with 1 ng/ml IL-1β for 15 min, and the IL-1β was then washed out and incubation was continued for 1, 2, or 4 h in medium alone or in the presence of 500 nM TSA. Cytosolic extracts were prepared and assessed for IκBα protein levels by Western immunoblotting. C, myometrial cells were either stimulated with 1 ng/ml IL-1β alone for 15 min or 2 h, or they were incubated with 500 nM TSA for 12 or 1 h before the addition of IL-1β (12h pre or 1h pre), or they had TSA added to them 1 h after the start of stimulation with IL-1β (1h post). Cytosolic extracts were prepared and assessed for expression of IκBα protein by Western immunoblotting. D, myometrial cells were preincubated with 500 nM TSA for 1 h and stimulated with 1 ng/ml IL-1β for the indicated times. Total RNA was isolated and mRNA expression of IκBα was examined by real-time PCR. IκBα mRNA values were normalized against expression levels of the *L19* house-keeping gene. Data are presented as a percentage of the expression level at 4-h IL-1β (the median time point, to allow inhibition by HDACi of IL-1β-induced expression to be assessed in a background of patient-to-patient variation in degree of responsiveness to IL-1β), and they are the mean ± S.D. from three independent experiments. *, $p < 0.005$; **, $p < 0.0001$ (by unpaired t test, comparing IL-1β versus TSA + IL1-β at each time point). NT, nontreated.

protein levels were decreased in cells incubated with TSA after a transient stimulation with IL-1β compared with cells incubated only with IL-1β (Fig. 4B, compare lanes 3–5 with lanes 6–8). To determine whether administration of HDACi after the initial degradation of IκBα would have the same effect as preincubation of cells with HDACi, TSA was added to myometrial cells 1 h after the addition of IL-1β, and this also resulted in marked reduction of IκBα protein expression at 2 h after IL-1β stimulation (Fig. 4C). At 1 h after IL-1β stimulation, IκBα protein has already been resynthesized in these cells, but continual IκBα protein synthesis is needed to sustain the concentration of IκBα throughout the course of the stimulation (T. M. Lindström and P. R. Bennett, unpublished data). We therefore investigated whether the inhibition of IκBα protein expression in IL-1β-stimulated myometrial cells by TSA occurred at the level of transcription/mRNA stability, as recently reported for pervanadate-stimulated HeLa cells (Horion et al., 2007). To assess the effects of HDACi on transcription of the *IKBA* gene and/or IκBα mRNA stability, IκBα mRNA expression was analyzed by real-time PCR in myometrial cells preincubated for 1 h with 500 nM TSA and stimulated with IL-1β for the indicated times. IL-1β induction of IκBα mRNA expression was detected as early as 15 min and peaked at 2 h (Fig. 4D). TSA alone had no effect on IκBα mRNA expression. However, TSA treatment resulted in a modest increase in IL-1β-stimulated *IKBA* mRNA expression at 2 and 4 h, which was lost at later time points (Fig. 4D). Similar results were obtained with SAHA (data not shown). These findings indicate that HDACi do not decrease IκBα protein levels by impairing the transcriptional activation of the *IKBA* promoter or decreasing IκBα mRNA stability and that they likely reduce IκBα expression by targeting the stability of the protein. In addition, the HDACi-induced augmentation of IL-1β-induced IκBα mRNA expression is consistent with the observed potentiation of NF-κB by HDACi, because *IKBA* transcription is driven by NF-κB.

HDACi Potentiate IKKβ Activity. Degradation of IκBα protein is carried out by the 26S proteasome, for which phosphorylation by IKKβ on serines 32/36 is a prerequisite (Traenckner et al., 1995). We therefore examined whether enhanced IKKβ activity could account for the reduction in IκBα protein levels in the presence of TSA plus IL-1β. Endogenous IKKβ was immunoprecipitated from myometrial cells treated with IL-1β, TSA, or IL-1β plus TSA, and IKKβ kinase activity was assayed by using a GST-IκBα fusion protein as substrate and then visualizing its phosphorylation by Western immunoblotting with an antibody that recognizes IκBα phosphorylated on serines 32/36. As expected, IL-1β caused an induction of IKKβ activity, which peaked at 15 min and was still detectable but much reduced at 2 h, at which time IκBα protein was resynthesized (Fig. 5A). Cells preincubated with TSA for 1 h exhibited increased IKKβ activity at 2 h of IL-1β stimulation compared with cells treated with IL-1β alone. The increase in IKKβ activity was associated with a decrease in levels of resynthesized IκBα. Similar findings were reported in tumor necrosis factor-α-stimulated HeLa cells (Adam et al., 2003). Thus, enhanced/prolonged IKKβ activity leading to persistent degradation of resynthesized IκBα (and IκBε) probably underlies the prolonged nuclear localization of NF-κB in the presence of IL-1β and HDACi.

Several distinct kinases have been reported to phosphorylate p65 at serine 536, a modification that plays an important role in the subsequent acetylation of this protein and in NF-κB transcriptional activity (Buss et al., 2004; Hoberg et al., 2006). Although pharmacological inhibition of RSK1 or p38 had no effect on IL-1β-induced phosphorylation of p65 at serine 536 in myometrial cells (data not shown), such phosphorylation was abrogated in both the cytoplasm and the nucleus by the selective IKKβ inhibitor sc-514 (Fig. 5B). Thus, because IKKβ mediates the serine 536-phosphorylation of p65 in response to IL-1β stimulation, the augmentation of IKKβ activity by TSA could result in the prolonged phosphorylation of p65 at this residue. On examination of whole-cell lysates, levels of serine 536-phosphorylated p65 were found to be greater in cells treated with TSA plus IL-1β compared with cells treated with IL-1β alone at the 4- and

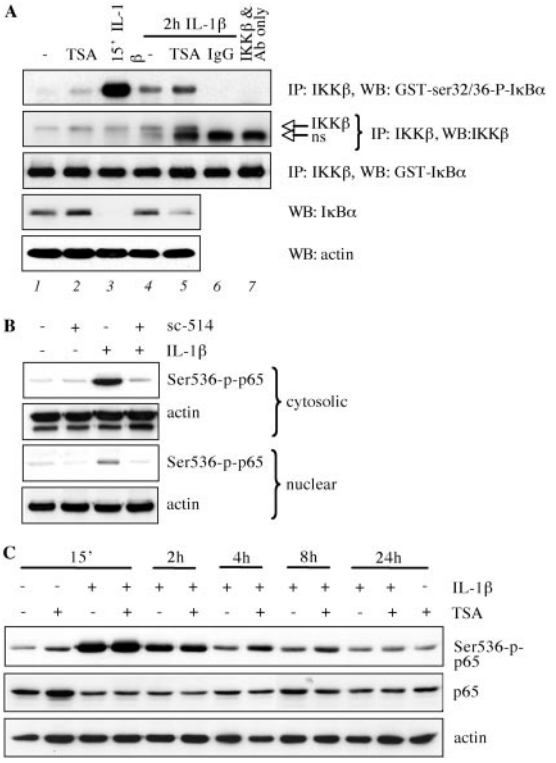

Fig. 5. TSA augments IL-1β-induced IKKβ activity. A, myometrial cells were preincubated with 500 nM TSA for 1 h, and then they were stimulated with 1 ng/ml IL-1β for 15 min or 2 h, and whole-cell extracts were prepared. Endogenous IKKβ was immunoprecipitated, and IKKβ kinase activity was assayed using a GST-IκBα fusion protein as substrate and visualization of its phosphorylation by Western immunoblotting with an antibody that recognizes IκBα phosphorylated on serines 32/36 (Ser32/36-P-IκBα). As negative controls, lysates were immunoprecipitated with preimmune rabbit IgG instead of IKKβ antibody (lane 6) or IKKβ antibody was incubated with protein Sepharose A beads in the absence of cell lysate (lane 7). Membranes were reprobed with an antibody against IKKβ to confirm the presence of equal immunoprecipitated IKKβ, and the presence of equal GST-IκBα substrate was verified by Western immunoblotting with an antibody against total IκBα. Whole-cell extracts were also assessed for expression of endogenous IκBα protein by Western immunoblotting. ns, nonspecific. B, myometrial cells were preincubated for 1 h with 100 μM sc-514, and then they were stimulated with 1 ng/ml IL-1β for 2 h. Cytosolic and nuclear extracts were prepared and assessed by Western immunoblotting for levels of serine 536-phosphorylated p65 (Ser536-p-p65). C, myometrial cells were preincubated with 500 nM TSA for 1 h and stimulated with 1 ng/ml IL-1β for the indicated times. Whole-cell extracts were assessed by Western immunoblotting for levels of Ser536-p-p65 and total p65.

8-h time points, whereas total p65 protein expression was not affected by either treatment (Fig. 5C).

Therefore, HDACi-induced potentiation of IKKβ activity can enhance NF-κB activity via the prolongation of NF-κB nuclear localization and via the post-translational modification of the p65 NF-κB subunit.

Both Short- and Long-Term Exposure to HDACi Represses COX-2, IL-6, IL-8, and RANTES Expression, and Inhibits the c-Jun Transcription Factor. The proinflammatory genes encoding IL-6, IL-8, RANTES, and COX-2 have all been implicated in human parturition and contain NF-κB recognition elements in their promoters (Lindström and Bennett, 2005b). The outcome of HDACi administration on the expression of endogenous NF-κB target genes cannot be predicted from the in vitro data on NF-κB. The transient reporter assays described should reflect primary and secondary effects of HDACi on the NF-κB signaling pathway, but they do not reflect potential effects on histone modifications since transiently transfected plasmids are not efficiently packaged into chromatin (Hebbar and Archer, 2008). Furthermore, because multiple signaling pathways may be targeted by HDACi to affect transcription of NF-κB target genes in a promoter-specific manner, the effect of HDACi on specific NF-κB-regulated genes must be empirically determined.

The effect of exposure of myometrial cells to HDACi on the expression the four labor-associated genes, *COX-2*, *IL-6*, *IL-8*, and *RANTES*, was therefore examined. Because HDACi may produce differential primary and secondary effects on inflammatory gene expression (Suuronen et al., 2006), mRNA expression of these genes was examined after both short- and long-term treatments with HDACi plus IL-1β. Real-time PCR analysis was performed on RNA isolated from myometrial cells preincubated with TSA for 1 h, and stimulated with IL-1β for 15 min, 2 h, 4 h, 8 h, and 24 h. IL-1β induced COX-2 mRNA expression by 2 h, which declined progressively from 4 to 24 h (Fig. 6). IL-1β-stimulated IL-6 mRNA was detected at 2 h, and it remained at maximal levels from 2 to 8 h, exhibiting a slight decrease at 24 h. IL-8 expression was induced by 2 h, remaining elevated up to 24 h. IL-1β induced RANTES expression by 2 h, which was maximal at 8 and 24 h. TSA alone had no effect on expression of any of the genes at any time point, indicating that the *COX-2*, *IL-6*, *IL-8*, and *RANTES* genes are not repressed by class I/II HDACs under basal conditions. However, TSA inhibited IL-1β-induced expression of COX-2, IL-6, IL-8, and RANTES mRNA at all time points (Fig. 6). Similar results were obtained with SAHA (data not shown). Western immunoblot analysis of lysates from myometrial cells pretreated with TSA for 1 h followed by IL-1β stimulation over a 24-h period confirmed inhibition of COX-2 expression by TSA at the protein level, which was observed at both early and late time points (Fig. 7A). SAHA was also found to inhibit COX-2 protein expression after IL-1β stimulation (Fig. 7B). Thus, these findings demonstrate that both short-term and long-term inhibition of class I and II HDAC activities represses the expression of key proinflammatory genes involved in human parturition.

The observed inhibitory effect of HDACi on the expression of *COX-2*, *IL-6*, *IL-8*, and *RANTES* genes is in contrast to the positive effects of HDACi on NF-κB activity and *IKBA* transcription at early time points. Transcriptional activation of these genes requires the coordinated action of multiple transcription factors, and the activity of proinflammatory activators other than NF-κB may be negatively regulated by acetylation. Apart from NF-κB, the proinflammatory transcription factor c-Jun is also required for the transcription of *COX-2*, *IL-6*, *IL-8*, and *RANTES* in many cell types (Saccani et al., 2001). We therefore exam-

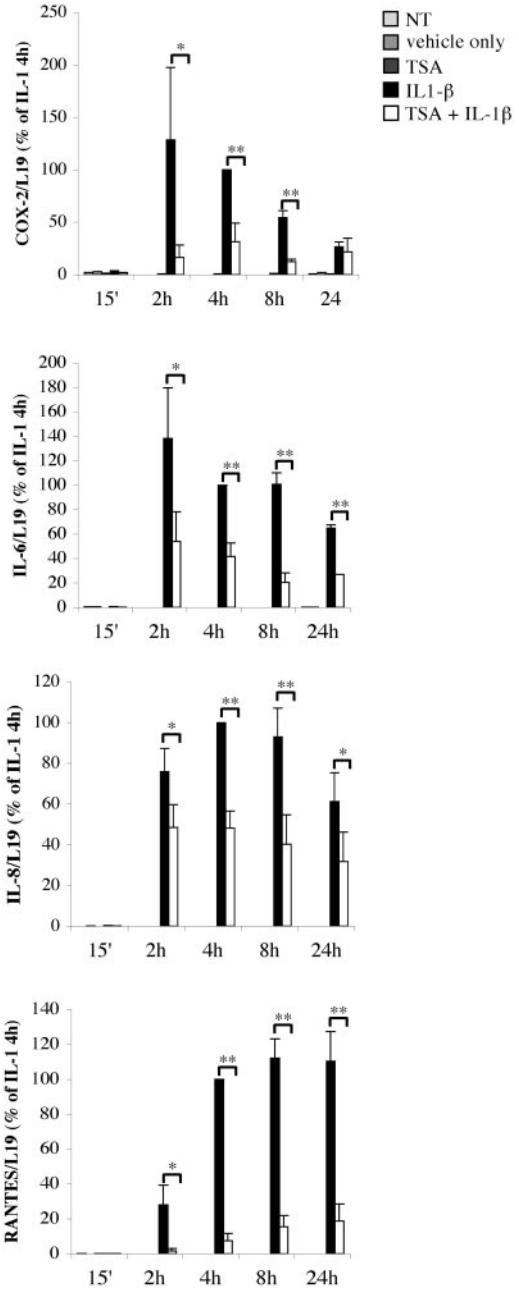

Fig. 6. Exposure of myometrial cells to TSA inhibits IL-1β-induced COX-2, IL-6, IL-8, and RANTES mRNA expression. Myometrial cells were preincubated with 500 nM TSA for 1 h, and then they were stimulated with 1 ng/ml IL-1β for the indicated times. Total RNA was isolated and mRNA expression of COX-2, IL-6, IL-8, and RANTES was examined by real-time PCR. Values were normalized against expression levels of the *L19* house-keeping gene. Data are presented as a percentage of the expression level at 4-h IL-1β (the median time point, to allow inhibition by HDACi of IL-1β-induced expression to be assessed in a background of patient-to-patient variation in degree of responsiveness to IL-1β), and they are the mean $\pm$ S.D. from three independent experiments. *, $p < 0.005$; **, $p < 0.0001$ (by unpaired t test, comparing IL-1β versus TSA + IL1-β at each time point). NT, nontreated.

ined the effects of HDACi on c-Jun in myometrial cells. Western immunoblot analysis of lysates from myometrial cells pretreated with TSA for 1 h followed by IL-1β stimulation over a 24-h period showed that TSA decreased IL-1β-induced nuclear levels of c-Jun at both early and late time points. SAHA was also shown to reduce nuclear c-Jun levels after IL-1β stimulation (Fig. 7B). The HDACi-induced reduction in nuclear levels of c-Jun was found to be due, at least in part, to decreased expression of c-Jun protein (Fig. 7C).

Thus, both short- and long-term exposure of myometrial cells to HDACi inhibits the proinflammatory transcription factor c-Jun and suppresses the expression of key labor-associated genes (Table 1).

Discussion

HDACi are in clinical trials for the treatment of inflammatory disorders (Lührs et al., 2002; Bolden et al., 2006), and recent evidence suggests that they may also have therapeutic potential in the management of preterm labor (Condon et al., 2003; Phillips et al., 2005; Mitchell, 2006). Because NF-κB is a key orchestrator of the inflammatory response and plays a pivotal role in parturition, it is important to understand how administration of HDACi might affect NF-κB activity in human uterine tissues. In the present study, we show that HDACi exert time-dependent effects on NF-κB activity in primary human myometrial cells. Whereas short-term treatment with HDACi resulted in the prolongation of IL-1β-stimulated NF-κB DNA binding and the augmentation of NF-κB transcriptional activity, long-term treatment reduced IL-1β-stimulated NF-κB DNA binding. Thus, although some of the discrepancies between studies regarding the effects of HDACi on NF-κB may be due to cell type-specific differences, it seems that a certain proportion might be attributable to differences in experimental protocol.

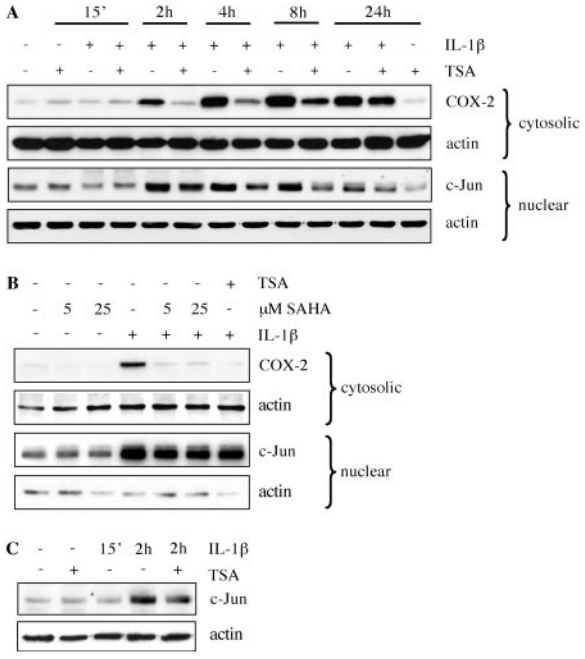

Fig. 7. Inhibition of COX-2 expression by HDACi is associated with reduced c-Jun expression. Myometrial cells were preincubated with 500 nM TSA for 1 h, and then they were stimulated with 1 ng/ml IL-1β for the indicated times. Cytosolic and nuclear extracts were prepared and assessed by Western immunoblotting for levels of cytosolic COX-2 protein and nuclear c-Jun. B, myometrial cells were preincubated with 5 or 25 μM SAHA or 500 nM TSA for 1 h, and then they were stimulated with 1 ng/ml IL-1β for 2 h. Cytosolic and nuclear extracts were prepared and assessed by Western immunoblotting for levels of cytosolic COX-2 and nuclear c-Jun proteins. C, myometrial cells were preincubated with 500 nM TSA for 1 h, and then they were stimulated with 1 ng/ml IL-1β for 15 min or 2 h. Whole-cell extracts were prepared and assessed by Western immunoblotting for total c-Jun protein expression.

HDACi alone did not induce NF-κB activity in myometrial cells but acted to enhance the stimulatory effects of IL-1β, even after IL-1β was withdrawn. This implies that a stimulus is required for the initial activation and acetylation of NF-κB pathway components, whereas HDACi prolong such acetylation, and thereby prolong NF-κB activity. The primary, positive effects of HDACi on NF-κB activity were shown to be due, at least in part, to the suppression of IκBα and IκBε protein reappearance after stimulus-induced degradation. This was a consequence of the augmentation of IL-1β-induced IKKβ activity by HDACi, leading to the persistent turnover of IκBα/ε. Because IκBα (Huang and Miyamoto, 2001), and possibly IκBε (Lee and Hannink, 2002), mediate the postinduction shut-off on the NF-κB response, the absence of these critical inhibitors of NF-κB resulted in sustained NF-κB nuclear localization and DNA binding, which translated to an increase in NF-κB transcriptional activity (Fig. 8).

The observed effects of HDACi may also reflect changes in post-translational modification of NF-κB itself. IKKβ was found to mediate IL-1β-induced phosphorylation of p65 at serine 536, an important determinant of subsequent p65 acetylation and NF-κB transcriptional activity (Buss et al., 2004; Hoberg et al., 2006), and such phosphorylation was augmented by TSA. Thus, the HDACi-induced potentiation of IKKβ activity might additionally enhance NF-κB activity independently of effects on NF-κB DNA binding, via the prolongation of p65 Ser536 phosphorylation and associated acetylation. HDACi may also modulate NF-κB signaling via direct effects on the acetylation status of NF-κB subunits (i.e., by blocking their deacetylation). Acetylation of p65 at lysine 221 has been shown to enhance its binding to DNA and impair its assembly with IκBα, whereas acetylation at lysine 310 promotes p65 transcriptional activity (Chen et al., 2001; Chen and Greene, 2003). DNA binding of p50 may also be enhanced by acetylation (Furia et al., 2002).

The inhibitory effects of HDACi on NF-κB observed at later time points are likely to be indirect, involving broader changes in cell physiology and possibly resulting from changes in protein synthesis, because this would require more time than changes in the activity of a latent transcription factor. Studies in other cell types have shown that many of the effects of HDACi on gene transcription are lost if translation is inhibited (Reid et al., 2005). In cancer cells, inhibition of tumor necrosis factor-α-induced NF-κB by HDACi occurs as a function of decreased IκBα degradation, due either to repression of proteasome activity (Place et al., 2005) or repression of IKK activation (Takada et al., 2006). These mechanisms cannot account for the delayed repression of NF-κB in myometrial cells, however, because the inhibitory effects of HDACi on NF-κB DNA binding were not related to an increase in IκB protein levels, indicating that the inhibitory actions of HDACi must target NF-κB downstream of its release from IκBα.

Because HH4 acetylation is observed in myometrial cells after short-term but not long-term exposure to HDACi, as is also observed in other cell types (Waterborg and Kapros, 2002; Ajamian et al., 2004), it is possible that TSA is intracellularly metabolized to an inactive form within a few hours. However, TSA is still able to inhibit the mRNA expression of IL-6, IL-8, and RANTES at 24 h. This could be because the downstream effects of TSA have not yet been reversed. Alternatively, the loss of detectable HH4 acetylation, as well as the secondary effects of HDACi on NF-κB, could be due to a shift in the cellular acetylation/deacetylation balance, resulting from autoregulatory feedback loops that affect HDAC (Waterborg and Kapros, 2002; Ajamian et al., 2004) or HAT (Covault et al., 1982) activity. Differential regulation by HDACi of distinct HDAC isoforms that affect different activities of NF-κB (Chen and Greene, 2003; Kyrylenko et al., 2003; Yeung et al., 2004) could explain the observation that NF-κB transcriptional activity is modestly potentiated in the face of decreased NF-κB DNA binding after long-term exposure to HDACi plus IL-1β.

The observed effects of HDACi on NF-κB activity per se represent global effects on NF-κB in myometrial cells and indicate changes in the potential of NF-κB to drive inflammation. Effects of HDACi on gene expression, in contrast, are

TABLE 1
Summary of the short-term and long-term effects of HDAC inhibitors on proinflammatory transcription factors and genes in IL-1β-stimulated myometrial cells

	Short-Term Effects	Long-Term Effects
Transcription factors		
NF-κB	Increased nuclear localization, DNA binding, and transcriptional activity	Decreased DNA binding
c-Jun	Decreased expression and nuclear levels	Decreased expression and nuclear levels
IKBA gene	Increased expression	No effect
Proinflammatory genes		
COX-2	Decreased expression	Decreased expression
IL-8	Decreased expression	Decreased expression
IL-6	Decreased expression	Decreased expression
RANTES	Decreased expression	Decreased expression

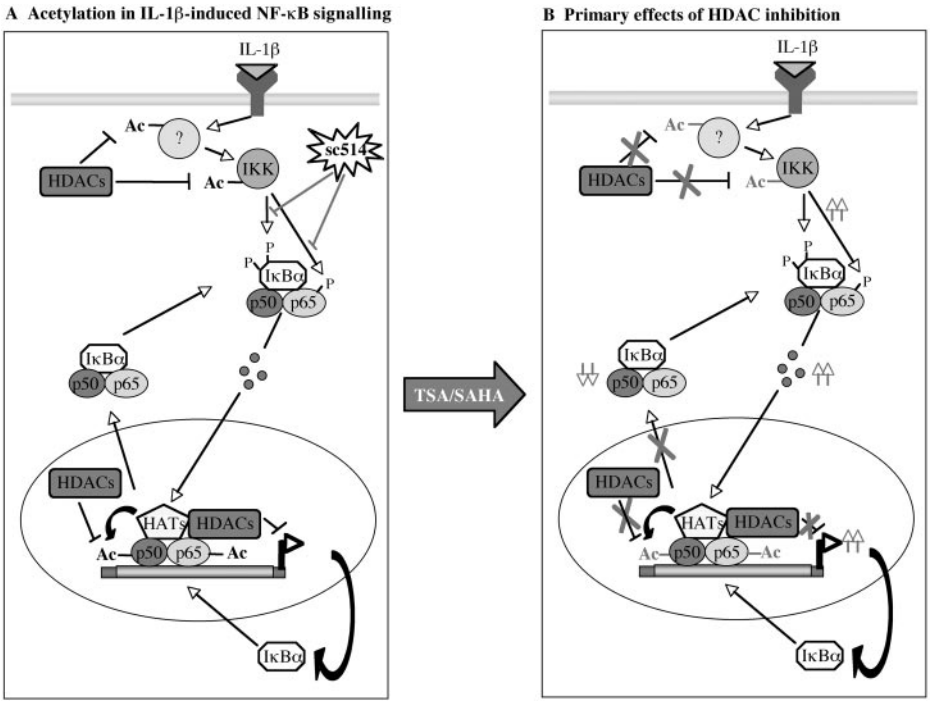

Fig. 8. Schematic diagram of the proposed primary, potentiating effects of HDACi on the NF-κB signaling pathway. A, acetylation/deacetylation modifications can modulate multiple steps in the NF-κB pathway. Targets of HAT-mediated acetylation include IKK and the NF-κB subunits themselves. Acetylation of IKK, or of an activating factor(s) upstream of IKK, promotes IKK activity after IL-1β stimulation, thereby promoting IκBα degradation and NF-κB nuclear localization/DNA binding. IKK-mediated phosphorylation of p65 also facilitates interaction of NF-κB with the basal transcription machinery, as well as promoting p65 acetylation. Acetylation of p50 may enhance NF-κB DNA binding, whereas acetylation of p65 may decrease association of NF-κB with IκBα and enhance NF-κB DNA binding and transcriptional activity. HDACs act to oppose the activity of HATs by removing acetyl groups from proteins. Aside from being a target of acetylation/deacetylation, NF-κB may also recruit HATs and HDACs to the promoters of target genes to regulate transcription by modulating local chromatin structure and recruitment of bromodomain-containing regulators of transcription. B, inhibition of HDAC activities by TSA or SAHA may thus augment NF-κB signaling by prolonging and enhancing these acetylation modifications, leading to increased IKK activity, which results in increased IκB degradation, thereby prolonging nuclear localization and DNA binding of NF-κB. Enhanced acetylation of p50 and p65 may also contribute to an increase in DNA binding and transcriptional activity of NF-κB per se, whereas inhibition of the activities of HDACs at the promoters of NF-κB target genes may enhance transcription independently of effects on NF-κB. As shown in A, an IKKβ inhibitor (e.g., sc514) can inhibit both the degradation of IκBα and the phosphorylation/acetylation of p65, thus counteracting the potentiating effects of HDACi on NF-κB activity. P, phosphorylation; AC, acetylation.

promoter-specific; hence, they must be empirically determined. In addition to effects on the activity of NF-κB, HDAC inhibition can exert effects on chromatin structure surrounding an NF-κB target promoter (Fig. 8), and it may also differentially modulate the expression or activity of distinct transcription factors that cooperate in the transcriptional induction of a given gene. Thus, it is not surprising that the effects of HDACi on NF-κB do not always correlate with their effects on the expression of NF-κB target genes. Indeed, we found that, in myometrial cells, both TSA and SAHA inhibited the expression of COX-2, IL-8, IL-6, and RANTES mRNA under conditions in which they enhanced NF-κB activity, suggesting that effects on this transcription factor are unlikely to underlie the inhibition of *COX-2*, *IL-6*, *IL-8*, and *RANTES* expression by HDACi (Table 1). In addition, because HDACi were shown to induce HH4 acetylation, and histone acetylation is known to facilitate transcriptional induction, effects of HDACi on local chromatin conformation are not likely to account for the inhibition of these genes. Rather, the anti-inflammatory effects of HDACi may be due to the inhibition of proinflammatory pathways other than the NF-κB pathway. Indeed, we show here that HDACi reduced the expression of c-Jun, in agreement with previous studies showing that TSA inhibits c-Jun expression by blocking the recruitment of RNA polymerase II to the *c-jun* promoter (Yamaguchi et al., 2005). c-Jun is essential for the transcriptional induction of *IL-6*, COX-2, and *RANTES* genes in many cell types (Saccani et al., 2001; Wu, 2005). Thus, inhibition of c-Jun could potentially account for some of the observed anti-inflammatory effects of HDACi in myometrial cells.

Condon and colleagues have suggested that a global decrease in HAT activity within the myometrium leads to the initiation of labor by reducing progesterone receptor activity at the promoters of genes that maintain uterine relaxation (Condon et al., 2003), and exposure of myometrial cells to TSA has been shown to up-regulate the expression of the chorionic gonadotrophin/luteinizing hormone receptor (Phillips et al., 2005), a receptor that signals to increase levels of the smooth muscle relaxant cAMP. Alternatively, we show here that HDACi inhibit the expression, in human myometrium, of several key proinflammatory genes implicated in parturition, suggesting that at least a proportion of the impact of decreased HAT and/or increased HDAC activity on uterine contractility at term may result from the stimulation or derepression of proinflammatory gene expression. Taken together, these findings indicate that HDACi administration may lead to a myometrial gene expression profile that favors uterine quiescence, both by inhibiting proinflammatory pathways and by derepressing pathways that promote uterine relaxation, and they suggest that these compounds could have therapeutic potential in the management of preterm labor.

Although HDACi did not increase the expression of the endogenous NF-κB-regulated genes examined in the present study, it remains possible that the expression of NF-κB-dependent genes, whose induction does not involve activators that are negatively regulated by acetylation (e.g., c-Jun), will be up-regulated by HDACi. Combining HDACi with an NF-κB inhibitor, as has been done in clinical trials for cancer treatment (Bolden et al., 2006), should negate any undesirable effects resulting from potentiation of NF-κB activity at early time points, and data presented here suggest that an IKKβ inhibitor should be efficacious in this regard. HDACi-induced inhibition of c-Jun, in contrast, might be expected to additionally suppress the expression of other proinflammatory or labor-associated genes that are not NF-κB-regulated. The ideal tocolytic HDACi compound should target the specific HDAC isoforms that are expressed in uterine tissues in association with labor onset, and efforts to develop more selective HDACi compounds are currently a major focus of research into the therapeutic use of HDACi (Kelly and Marks, 2005).

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