Histone Deacetylase Inhibitors Suppress TF-κB-dependent Agonist-driven Tissue Factor Expression in Endothelial Cells and Monocytes*

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Histone deacetylase inhibitors (HDACi), such as trichostatin A (TSA), can regulate gene expression by promoting acetylation of histones and transcription factors. Human tissue factor (TF) expression is partly governed by a unique, NF-κB-related "TF-κB" promoter binding site. We find that TSA and four other HDACi (apicidin, MS-275, sodium butyrate, and valproic acid) all inhibit by ∼90% TF activity and protein level induction in human umbilical vein endothelial cells stimulated by the physiologic agonists tumor necrosis factor (TNF)-α, interleukin-1β, lipopolysaccharide, and HOSCN without affecting expression of the NF-κB-regulated adhesion molecules ICAM-1 and E-selectin. TSA and butyrate also blunt TF induction ∼50% *in vitro* in peripheral blood mononuclear cells and *in vivo* in thioglycolate-elicited murine peritoneal macrophages. In human umbilical vein endothelial cells, TSA attenuates by ∼70% TNF-α stimulation of TF mRNA transcription without affecting that of ICAM-1. By electrophoretic mobility shift assay analyses, TNF-α and lipopolysaccharide induce strong p65/p50 and p65/c-Rel heterodimer binding to both NF-κB and TF-κB probes. TSA nearly abolishes TF-κB binding without affecting NF-κB binding. A chromatin immunoprecipitation assay and a promoter-luciferase reporter system confirm that TSA inhibits TF-κB but not NF-κB activation. Chromatin immunoprecipitation and small interfering RNA inhibitor studies demonstrate that HDAC3 plays a significant role in TNF-α-mediated TF induction. Thus, HDACi transcriptionally inhibit agonist-induced TF expression in endothelial cells and monocytes by a TF-κB- and HDAC3-dependent mechanism. We conclude that histone deacetylases, particularly HDAC3, play a hitherto unsuspected role in regulating TF expression and raise the possibility that HDACi might be a novel therapy for thrombotic disorders.

Tissue factor (TF)[2] is the critical initiator of physiologic and pathologic coagulation that binds VIIa to form a TF·VIIa complex that cleaves factors IX and factor X to activate the coagulation protease cascade (1, 2). In addition to its pivotal role in hemostasis and thrombosis, TF-dependent signaling also participates in the processes of inflammation, angiogenesis, metastasis, and cell migration (3). TF gene expression is regulated mainly at the transcriptional level (4). Diverse biologic stimuli, such as bacterial endotoxin (lipopolysaccharide; LPS), inflammatory cytokines (*e.g.* tumor necrosis factor (TNF)-α and interleukin (IL)-1β), growth factors (*e.g.* vascular endothelial growth factor), oxidized and acetylated low density lipoproteins, hypoxia, shear stress, and oxidants (5) induce TF expression in endothelial cells and monocytes. These agonists activate several signal transduction pathways, including mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase C (2). The human TF gene promoter contains binding sites for activator protein-1 (AP-1), epidermal growth response-1 (Egr-1), and specificity protein-1 (SP-1) transcription factors and has an NF-κB-like "TF-κB" sequence that binds members of the NF-κB family, including p65/p50 and p65/c-Rel (4). In monocytes, SP-1 sites contribute to the constitutive expression of base-line TF, whereas the AP-1, Egr-1, and TF-κB sites mediate inducible TF expression (6). In endothelial cells, constitutive AP-1 and inducible TF-κB activation mediate TF induction by LPS, TNF-α, IL-1β (7), and, as we have recently shown, the principal phagocyte peroxidase-derived oxidant, HOSCN (5).

Specific reversible histone deacetylase inhibitors (HDACi), such as trichostatin A (TSA) and suberoylanilide hydroxamic acid, were originally described to mediate acetylation of chromatin-associated histones and thereby open promoter loci to permit interaction with transcription factors to promote gene expression. However, more recent studies show that HDACi can also promote acetylation of transcription factors, including the p50 and p65 subunits of NF-κB (8), and can suppress (9–11) as well as promote (12, 13) gene expression. Recently, HDACi were found to inhibit the expression of proinflammatory cytokines and mediators in some inflammatory disease models (14–19). For example, suberoylanilide hydroxamic acid attenuates the expression of certain NF-κB-regulated cytokines, including TNF-α, IL-1β, IL-6, and interferon-γ, in a mouse endotoxemia model (16). These findings led us to hypothesize

that inhibition of HDAC activity by HDACi would, through inhibition of TF-κB activation, suppress the expression of tissue factor in agonist-stimulated endothelial cells and monocytes.

We tested the effect of five HDACi (TSA, apicidin, MS-275, sodium butyrate, and valproic acid) on TF expression induced by the physiologic agonists LPS, TNF-α, IL-1β, and HOSCN. We find that HDACi potently inhibit agonist-induced TF activity in human endothelial cells and monocytes through specific blockade of TF-κB activation. TSA also suppresses murine macrophage TF activity induction *in vitro* and decreases peritoneal macrophage TF activity in an *in vivo* mouse peritonitis model. Our results suggest that HDAC3 plays an unanticipated and pivotal role in regulating TF expression and suggest that HDACi might be a novel therapy for thrombotic diseases.

EXPERIMENTAL PROCEDURES

Reagents—Trichostatin A (T8552), lipopolysaccharide (*Escherichia coli* 055:B5), sodium butyrate (B5887), PMA (phorbol 12-myristate 13-acetate), and protease inhibitor mixture (P8340) were from Sigma. Apicidin, MS275, and valproic acid were from Calbiochem. Recombinant human TNF-α and IL-1β were from R&D Systems (Minneapolis, MN). Lipofectamine™ 2000 was from Invitrogen (Carlsbad, CA). Rabbit polyclonal antibodies against human ICAM-1, Egr-1, SP-1, c-Rel, p65, p50, IκB-α, Ac-histone H3, Ac-histone H4, goat polyclonal antibody against actin, and alkaline phosphatase-conjugated goat anti-rabbit IgG and donkey anti-goat IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies against human tissue factor (20) were kindly provided by Dr. Ronald Bach (Veterans Affairs Medical Center, Minneapolis, MN). The chromatin immunoprecipitation (ChIP) assay kit was from Upstate Biotechnology, Inc. (Lake Placid, NY; catalog number 17-295). Nuclear extraction kit (AY2002) was from Panomics, Inc. (Redwood City, CA). Reagent HOSCN was synthesized and quantitated as previously described using lactoperoxidase (21, 22).

Human Umbilical Vein Endothelial Cell (HUVEC) Culture—HUVEC were isolated from umbilical cords and cultured as previously described (23). HUVEC were used for experiments at passage 2 or 3.

Peripheral Blood Mononuclear Cell (PBMC) Preparation—Fresh human blood was collected from healthy, adult volunteers after their informed consent into acid-citrate-dextrose (38 mM citric acid, 75 mM trisodium citrate, and 100 mM dextrose; 1:7 acid-citrate-dextrose final concentration). After removal of platelet-rich plasma by centrifugation of the whole blood at $100 \times g$ at 22 °C for 15 min, the cell pellet was diluted with ice-cold phosphate-buffered saline to original blood volume and layered onto the same volume of HISTOPAQUE®-1077 (catalog number 1077-1; Sigma) and centrifuged at $400 \times g$ at 22 °C for 30 min (24). The supernatant and interface were collected and washed twice by phosphate-buffered saline. The PBMC were resuspended in RPMI medium with 10% fetal bovine serum and incubated at 37 °C for 2 h before use.

One-stage Clotting TF Activity Assay—Confluent HUVEC monolayers or PBMC were pretreated with TSA (0.01–1 μM) for 4 h and then stimulated by either 1 μg/ml LPS, 1 ng/ml TNF-α, 1 ng/ml IL-1β, or 100 μM reagent HOSCN for another 4 h. Tissue factor activity was detected by a one-stage clotting assay standardized against recombinant human TF as described before (5). Alternatively, HUVEC were treated with increasing doses of apicidin, MS-275, sodium butyrate, or valporic acid and then stimulated by LPS and TNF-α for another 4 h before assaying TF activity. The 50% inhibitory concentration (IC_{50}) and maximal inhibition were calculated from the inhibition curve.

Reverse Transcription-PCR Analysis—Confluent HUVEC monolayers were pretreated with 1 μM TSA for 4 h and then stimulated by 1 ng/ml TNF-α for 1 or 2 h. Total cellular RNA was isolated, and reverse transcription-PCR was performed as previously described (5). Specific primers were purchased from Integrated DNA Technologies (Coralville, IA): long tissue factor, sense (5'-GGCACTACAAATACTGTGGCAGCA-3') and antisense (5'-ACTCTTCCGGTTAACTGTTCGGGA-3'); full-length tissue factor, sense (5'-CGCCGCCAACTGGTAGAC-3') and antisense (5'-TGCAGTAGCTCCAACAGTGC-3'); ICAM-1, sense (5'-CAGTGACCATCTACAGCTTTCCGG-3') and antisense (5'-GCTGCTACCACAGTGATGATGACAA-3'). PCR products were analyzed by electrophoresis loading 10-μl aliquots on 1.2% agarose gels. Gels were stained with 0.5 μg/ml ethidium bromide and imaged with ultraviolet transillumination.

Nuclear Protein Extraction—Monolayers of HUVEC were pretreated 4 h with 0.3 or 1.0 μM TSA and then stimulated by 1 ng/ml TNF-α for 30 min, and nuclear protein was prepared with a nuclear extract kit (Panomics) according to the manufacturer's instructions and stored at −80 °C until use.

Electrophoretic Mobility Shift Assay (EMSA)—NF-κB consensus (5'-AGTTGAGGGGACTTTCCCAGGC-3') and NF-κB mutant (5'-AGTTGAGGCGACTTTCCCAGGC-3') and SP-1 consensus (5'-ATTCGATCGGGGCGGGGCGAGC-3') and SP-1 mutant (5'-ATTCGATCGGTTCGGGGCGAGC-3') oligonucleotides were purchased from Santa Cruz Biotechnology. Oligonucleotides for the TF-κB consensus site (5'-GTCCCG-GAGTTTCCTACCGGG-3') and a TF-κB-like mutant control (5'-GTCCCGGAGTTAGATACCGGG-3') and proximal TF AP-1 consensus site (5'-CTGGGGTGAGTCATCCCTT-3') and AP-1 mutant control (5'-CTGGGGTGAGTTGTCCCTT-3') were from Integrated DNA Technologies, Inc. (Coralville, IA). Oligonucleotides were end-labeled with [γ-^{32}P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (Amersham Biosciences). Five μg of nuclear extract was loaded per lane (10 μg for the TF-κB probe), and EMSA was carried out as described (5).

Western Blot Analysis of TF—HUVEC monolayers were pretreated with TSA and stimulated by agonists as described above (see "One-stage Clotting TF Activity Assay"). Whole cell lysates were prepared and analyzed on 8% SDS-polyacrylamide gels and for nuclear extracts on 12% gels. Western blot analysis was performed as described before (5), using specific primary antibodies against human tissue factor, ICAM-1, c-Rel, p65, p50, Egr-1, SP-1, IκB-α, Ac-histone H3, Ac-histone H4, and actin.

ChIP Assay—HUVEC monolayers were pretreated with Me₂SO or TSA (0.3 or 1 μM) for 4 h and then stimulated with 1 ng/ml TNF-α for 0.5 h. HUVEC monolayers were fixed by adding formaldehyde directly to the medium to a final concentration of 1% and incubated for 10 min at 37 °C. ChIP assay was

performed according to the kit instructions. Immunoprecipitations were carried out at 4 °C overnight with 4 μg of rabbit IgG or rabbit polyclonal antibodies against p65 (sc-372), HDAC1 (sc-7872), HDAC2 (sc-7899), or HDAC3 (sc-11417) (Santa Cruz Biotechnology). About one-third of the immunoprecipitated DNA was used in each PCR (25). Promoter-specific primers for TF and ICAM-1 were as follows: TF forward, 5′-GCCCTCCCTTTCCTGCCATAGA-3′; TF reverse, 5′-CCTCCCGGTAGGAAACTCCG-3′; ICAM-1 forward, 5′-ACCTTAGCGCGGTGTAGACC-3′; ICAM-1 reverse, 5′-CTCCGGAACAAATGCTGC-3′.

HUVEC Transfection and Luciferase Promoter Assay—The human TF wild-type promoter (-227 to $+121$) and the TF-κB mutant (5′-CGGAGTT<u>TCC</u>-3′ $\rightarrow$ 5′-CGGAGTT<u>AGA</u>-3′) reporter plasmid in pGL2 basic were provided by Dr. Nigel Mackman (6) (Scripps Research Institute, La Jolla, CA). The ICAM-1 wild-type luciferase reporter plasmid (26) was a gift from Dr. Asrar B. Malik (University of Illinois College of Medicine, Chicago, IL). HUVEC were plated into 6-well plates 12–18 h before transfection. Transfections of HUVEC were performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendations. Briefly, reporter DNA (1 μg) was mixed with 1.5 μl of Lipofectamine in 200 μl of Opti-MEM reduced serum medium. The DNA-Lipofectamine complexes were incubated with HUVEC for 4 h at 37 °C in the 5% CO_2 incubator and then replaced with growth medium. After 36 h, cells were pretreated with an Me_2SO solvent control or TSA (30, 100, or 300 nM) for 2 h, followed by stimulation with 10 μg/ml LPS, 10 ng/ml TNF-α, or 50 nM PMA for another 6 h. Cell lysates were assayed for luciferase activities using the luciferase assay system (Promega, Madison, WI). The pCMVβ vector (BD Clontech, Palo Alto, CA) was cotransfected so that the transfection efficiencies could be normalized with the β-galactosidase activities determined using a luminescent β-galactosidase detection kit II (BD Clontech) (27).

HUVEC Transfection and RNA Interference—siRNA duplexes for human HDAC2 (sc-29345) and HDAC3 (sc-35538) and a scrambled control (sc-37007, causing no specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology. siRNA transfection was performed following the manufacturer's instructions. Briefly, HUVEC were plated at 2×10^5 cells in a 6-well cell culture plate. HUVEC were incubated with 45 nM siRNA/9 μl of Lipofectamine 2000 (Invitrogen) mixture in Opti-MEM (Invitrogen) medium for 5 h at 37 °C in a humidified CO_2 incubator and then replaced with growth medium (28). After 72 h, cells were stimulated by 1 ng/ml TNF-α for another 4 h. TF activity of HUVEC lysates was assayed by a one-stage clotting assay, and protein levels of HDAC3, HDAC2, TF, and ICAM-1 were detected by Western blot using specific antibodies against human HDAC3, HDAC2, TF, and ICAM-1. Alternatively, cells were stimulated by TNF-α for 0.5 h, and a ChIP assay was conducted as described above using specific antibodies against HDAC2 and HDAC3.

Murine Macrophage TF Activity Measurement—Male mice (C57 Bl/6J, 25–30 g body weight) were injected intraperitoneally with 2 ml of 4% thioglycolate medium. Three days later, peritoneal macrophages were harvested by peritoneal lavage

using 10 ml of sterile Hanks' balanced salt solution buffer supplemented with 5 mM EDTA. Lavage fluids were pooled and centrifuged at $200 \times g$ for 10 min. The cell pellet was washed once in sterile Hanks' balanced salt solution, and red blood cells were lysed in 10 ml of ice-cold sterile water followed by adding 10 ml of $2\times$ Hanks' balanced salt solution after 30 s (29). This suspension was pelleted, resuspended in RPMI (10% fetal bovine serum), and counted using a hemocytometer. Lavage leukocytes were diluted in RPMI medium (1–2×10^6 cells/ml), aliquoted into 6-well plates, and then incubated (37 °C and 5% CO_2) overnight. The next day, nonadherent cells were washed away, and adherent macrophages were overlaid with fresh RPMI medium. Macrophages were pretreated with 0.3 or 1 μM TSA for 4 h before stimulation with 10 μg/ml LPS, 20 ng/ml human TNF-α or IL-1β for 5 h. Macrophage TF activity in whole cell lysates was detected by a one-stage clotting assay, as described before (5). Relative TF activity was normalized using a standard curve generating using recombinant human TF protein.

Mouse Peritonitis Model—Male mice (C57 Bl/6J; 25–30 g body weight) were intraperitoneally injected with either TSA (1 mg/kg, as used in Refs. 18 and 70) ($n = 6$) or Me_2SO vehicle control ($n = 6$) 4 h before intraperitoneal injection of 2 ml of 4% thioglycolate medium. In an additional three control animals, saline rather than thioglycolate was injected intraperitoneal without TSA or Me_2SO treatment. Mice were given a second intraperitoneal injection of TSA or Me_2SO at day 2 and sacrificed at day 3. Peritoneal cavities were lavaged, and total leukocytes were enumerated and identified as described before (24). This model generates leukocytes with a cell population that was 65–75% macrophages, the remainder equally split between lymphocytes and neutrophils. Total TF activity in whole cell lysates was detected by a one-stage clotting assay and normalized to macrophage cell number.

RESULTS

TSA Inhibits Agonist-induced TF Activity and Protein Expression in Human Endothelial Cells—HDACi induces apoptosis in many cancer cells with little or no toxicity for primary untransformed cells (30, 31). We found that ≤ 1 μM TSA has no significant effect on the viability of HUVEC as detected by trypan blue staining after a 24-h treatment (data not shown). For our studies, we chose doses and treatment times similar to those previously used successfully to investigate the influence of TSA on endothelial NO synthase expression and angiogenesis in HUVEC *in vitro* (15, 32) and some inflammatory disease models *in vivo* (18, 32).

We first assessed the effect of the TSA on agonist-stimulated TF expression in HUVEC. HUVEC were pretreated with TSA (10 nM to 1 μM) for 4 h and then stimulated by LPS, TNF-α, IL-1β, or HOSCN for another 4 h. HUVEC lysates were then assayed for TF activity by a one-stage clotting assay (Fig. 1), and whole cell detergent lysates were probed for TF protein by Western blot analysis (Fig. 2). As shown in Fig. 1, 1 μg/ml LPS (Fig. 1*A*), 1 ng/ml TNF-α (Fig. 1*B*), 1 ng/ml IL-1β (Fig. 1*C*), or 100 μM HOSCN (Fig. 1*D*) all induced TF activity 25–1000-fold, as expected. TSA treatment inhibited by up to 90% TF induction by all four agonists in a dose-dependent manner with an
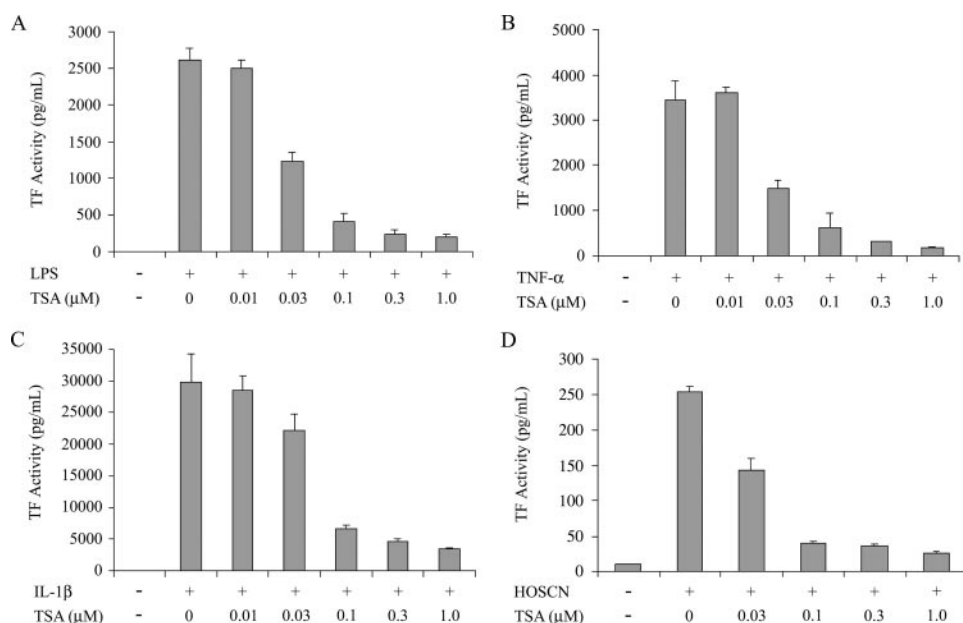
FIGURE 1. **TSA suppresses tissue factor activity induced by physiologic agonists in HUVEC.** HUVEC monolayers were preincubated with 0.01–1 μM TSA for 4 h and then exposed to 1 μg/ml LPS (*A*), 1 ng/ml TNF-α (*B*), 1 ng/ml IL-1β (*C*), or 100 μM HOSCN (*D*) for another 4 h. Scraped, freeze-thawed monolayer lysates were prepared and assayed for TF activity using a one-stage clotting assay. All data are shown $\pm$ S.D. TF activities in TSA-treated HUVEC were all statistically significantly ($p < 0.05$) different from that of agonist alone at TSA concentrations from 0.03 to 1.0 μM.
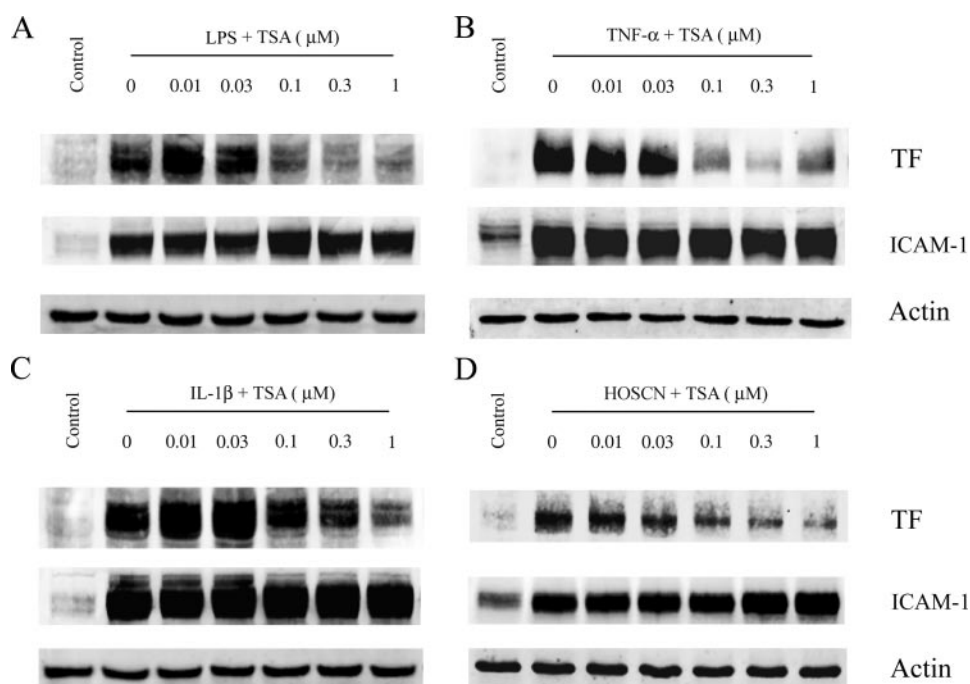
FIGURE 2. **TSA suppresses TF protein expression of agonist-stimulated HUVEC.** HUVEC monolayers were treated with 0.01–1 μM TSA for 4 h and then exposed to 1 μg/ml LPS (*A*), 1 ng/ml TNF-α (*B*), 1 ng/ml IL-1β (*C*), or 100 μM HOSCN (*D*) for another 4 h. Whole cell lysates were prepared and probed for TF and ICAM-1 expression by Western blot analysis. Actin was detected as a loading control.

tin (34) (not shown) in agonist-stimulated HUVEC. This phenomenon was reproduced using four other HDACi (apicidin, MS-275, sodium butyrate, and valproic acid) in LPS- and TNF-α-stimulated HUVEC (Table 1). The IC_{50} of TSA is much less than that of the other four HDACi, reflecting their relative potencies as HDAC inhibitors (35). We therefore chose TSA as a prototype to investigate HDACi effects on monocyte/macrophage TF expression and elucidate the molecular mechanisms behind HDACi suppression of agonist-driven TF expression.

TSA Attenuates TF Activity Induction in Human PBMC and Murine Peritoneal Macrophages—Human PBMC were treated with TSA (10 nM to 1 μM) for 4 h and then stimulated by 1 μg/ml LPS (Fig. 3*A*) or 1 ng/ml IL-1β (Fig. 3*B*) for another 5 h before lysates were assayed for TF activity. Both LPS and IL-1β induced the expected 15–50-fold increase in TF activity of PBMC (Fig. 3, *A* and *B*). TSA suppressed by up to 60% this TF activity induction with an IC_{50} of 50–100 nM. To test the influence of TSA upon TF expression function *in vivo*, we utilized a murine thioglycolate peritonitis model to elicit activated murine macrophages. TF activity was assayed both *in vitro*, after incubation for 5 h in the presence of agonists (Fig. 3*C*), and *in vivo*, in freshly isolated peritoneal macrophages (Fig. 3*D*). Both 0.3 and 1 μM TSA treatment decreased TF activity by 60–70% in base-line or LPS-, TNF-α-, or IL-1β-stimulated macrophages *in vitro* (Fig. 3*C*). Thioglycolate induced a 15-fold increase in peritoneal macrophage TF activity over that of resident macrophages (Fig. 3*D*, control group). *In vivo*, TSA treatment significantly decreased (~50%) thiogy-

IC_{50} of 20–50 nM. In data not shown, TSA had no significant effect upon base-line TF expression in nonstimulated HUVEC. Western blot analysis of TF protein (Fig. 2) confirms the pronounced inhibition by TSA of agonist-induced TF expression. In contrast, as previously reported (28), we also found that TSA had no significant impact upon expression of the NF-κB-regulated adhesion molecules ICAM-1 (26, 33) (Fig. 2) and E-selec-

colate-elicited peritoneal macrophage TF activity (Fig. 3*D*) without significantly affecting peritoneal macrophage accumulation (not shown).

TSA Decreases TF mRNA Level in Human Endothelial Cells— To determine whether the inhibition of TF induction by TSA is transcriptionally mediated, we performed reverse transcription-PCR analysis to assess TF mRNA levels. HUVEC
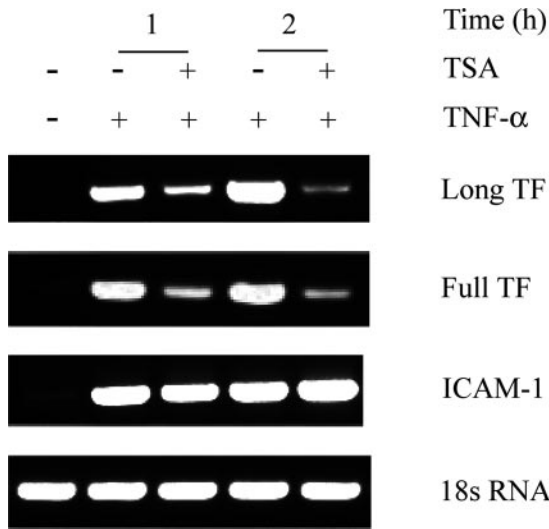
FIGURE 4. **Influence of TSA upon TF mRNA levels in TNF-α-stimulated HUVEC.** HUVEC monolayers were treated with 1 μM TSA for 4 h and then exposed to 1 ng/ml TNF-α for 1 or 2 h. Total cellular RNA was extracted and analyzed by reverse transcription-PCR using Long TF, Full TF, ICAM-1, or 18 S rRNA-specific primers. Gels were stained with ethidium bromide and imaged using ultraviolet transillumination.

LPS-stimulated HUVEC as well. AP-1, Egr-1, and SP-1 were all constitutively activated and unaffected by TNF-α treatment irrespective of whether TSA is present (not shown). Moreover, TSA did not affect SP-1 and Egr-1 levels in the nuclear extracts as detected by Western blot (Fig. 5D). These data suggest that TSA exerts its inhibitory effect on TF transcriptional expression predominantly through suppression of TF-κB, but not NF-κB, promoter site interaction with p65/c-Rel (Fig. 5B) and p65/p50 (not shown).

To confirm the specific blockade of TF-κB activation by TSA, ChIP was performed to analyze p65 binding to the authentic endogenous TF-κB site in the TF promoter and an NF-κB site in the ICAM-1 promoter. As shown in Fig. 6, TSA treatment strongly inhibited p65 binding to the TF-κB site in the TF promoter induced by TNF-α without affecting p65 binding to the ICAM-1 NF-κB site in HUVEC.

TF-κB-dependent Modulation of Gene Expression by TSA in a Promoter-Luciferase Reporter System—To test the apparently critical role of TF-κB in TSA suppression of TF expression, we also assessed its effect in a TF promoter-luciferase reporter system. Luciferase plasmids containing a TF promoter (either a wild-type TF-κB- containing promoter or one containing a TF-κB non-binding mutant) or an ICAM-1 NF-κB-containing
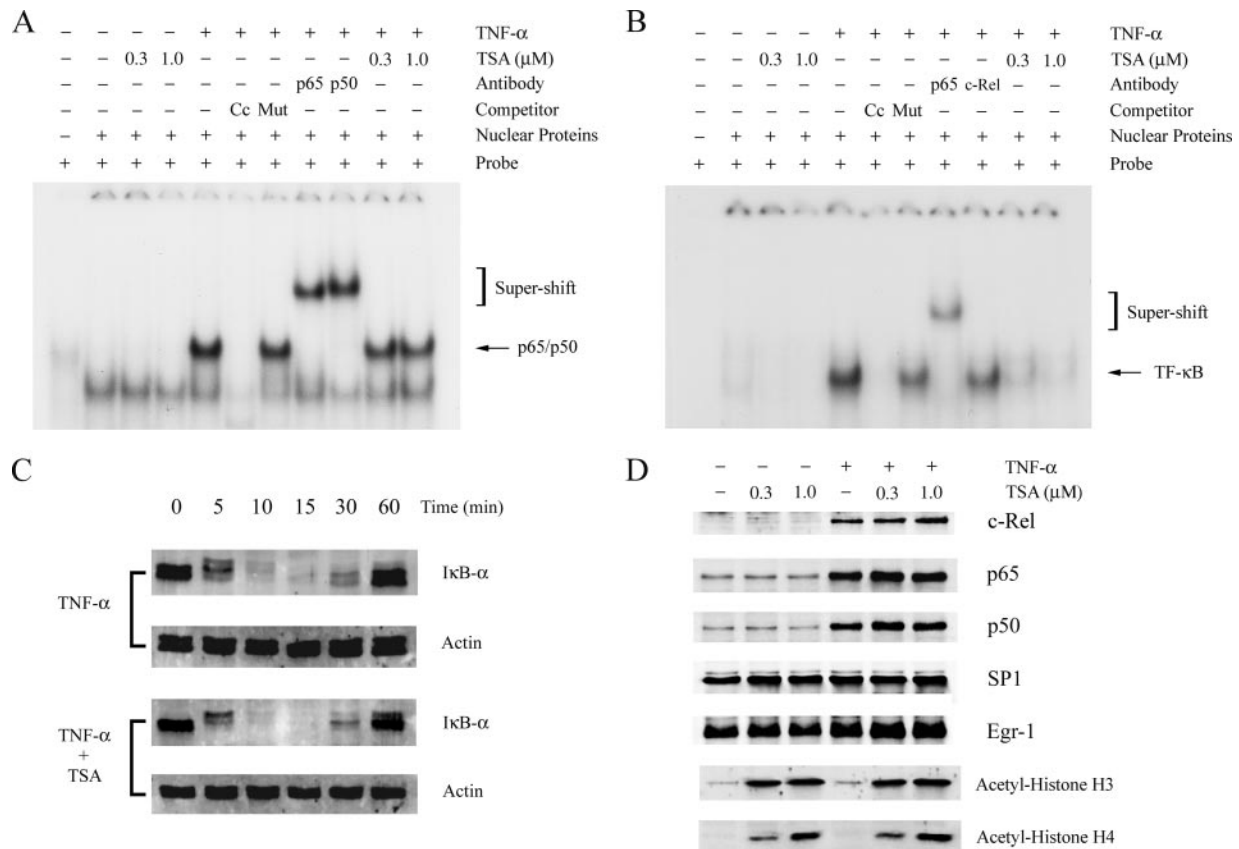
FIGURE 5. **TSA effects on NF-κB signaling pathway in stimulated HUVEC.** *A*, EMSA analysis of NF-κB activation. HUVEC were pretreated with 0.3 or 1 μM TSA for 4 h and then exposed to 1 ng/ml TNF-α for 0.5 h prior to extraction of nuclear proteins. Five-μg aliquots of nuclear extracts were incubated with a radiolabeled NF-κB consensus binding sequence oligonucleotide and separated on 5% nondenaturing polyacrylamide gels. The mobility of the shifted NF-κB consensus probe is shown by the *arrow* on the *right*; the slower migration of supershifted bands is shown in *brackets*. The *lanes* designated *Cc* show the effect of adding a 50-fold excess of unlabeled consensus probe; *Mut*, the effect of adding a 50-fold excess of unlabeled mutant NF-κB oligonucleotide; *p65* and *p50*, the effects of adding antibodies to the designated proteins. *B*, EMSA analysis of TF-κB activation. The same nuclear extracts in *A* and a probe comprising the authentic TF-κB sequence were used. *C*, kinetic analysis of IκB-α degradation. HUVEC were pretreated with 1 μM TSA for 4 h and then exposed to 1 ng/ml TNF (for 0–60 min prior to extraction of whole cell lysates). The kinetics of IκB-α degradation was followed by Western blot analysis. Actin was a loading control. *D*, Western blot of nuclear extracts. The same nuclear extracts used in *A* and *B* were probed using polyclonal antibodies specific for c-Rel, p65, p50, SP-1, Egr-1, acetylated histone H3, and acetylated histone H4.
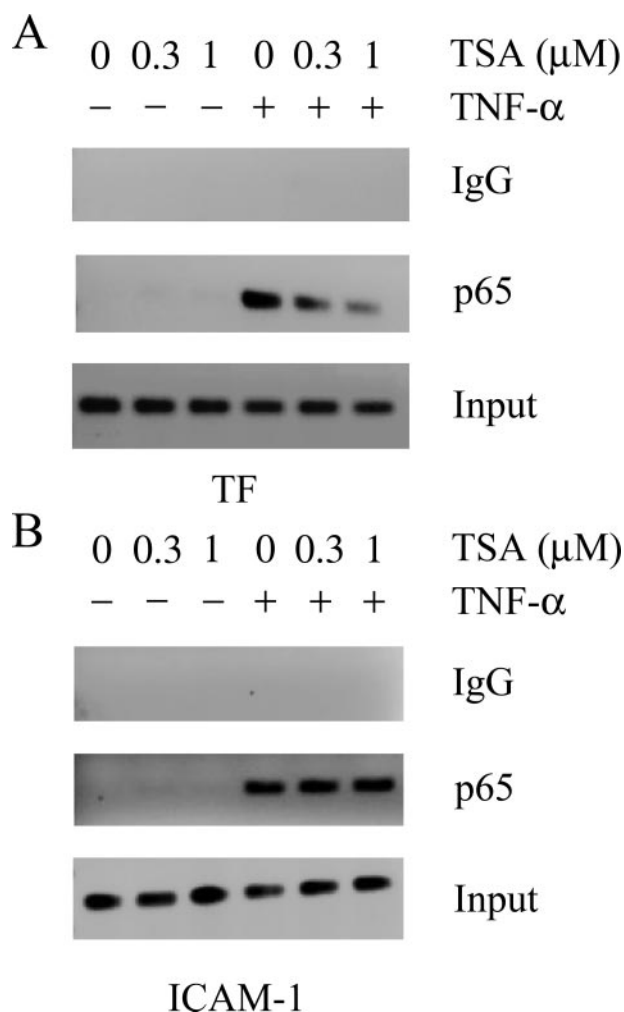
FIGURE 6. **Effect of TSA on p65 binding to the TF-κB site in the TF pro-moter.** HUVEC monolayers were pretreated by Me₂SO or TSA (0.3 or 1 μM) for 4 h and then stimulated with 1 ng/ml TNF-α for 0.5 h. HUVEC monolayers were fixed by 1% formaldehyde. HUVEC were lysed and sonicated to shear the DNA. Chromatin immunoprecipitations were performed using either rabbit IgG control or rabbit anti-human p65 antibody. The immunoprecipitated DNA was detected by PCR amplification using promoter-specific primers for TF (*A*) and ICAM-1 (*B*).

FIGURE 7. **TSA influence upon TF-κB- or NF-κB-dependent gene expression in a promoter-luciferase reporter system.** *A*, luciferase plasmids containing a wild-type TF promoter (*TF*) or mutant TF-κB promoter (*TFₘ*) were transfected into HUVEC and treated with TSA (or Me₂SO) for 4 h followed by stimulation with 10 μg/ml LPS (*gray bars*) or 10 ng/ml TNF-α (*white bars*) for 6 h prior to assay of luciferase activity. *B*, HUVEC were transfected with wild-type ICAM-1 promoter luciferase plasmid and treated as in *A*. *C*, HUVEC were transfected with plasmids containing a wild-type TF promoter (*gray bars*), wild-type ICAM-1 (*diagonal bars*) or a mutant TF-κB (*white bars*) promoter and treated with TSA (or Me₂SO) for 4 h followed by stimulation with 50 nM PMA for 6 h. The relative luciferase activity is shown as -fold induction normalized to the signal of nonstimulated HUVEC. All data are shown ± S.D. (*, $p < 0.05$ *versus* agonist alone).

promoter (wild-type) were transfected into HUVEC. Trans-fected HUVEC were pretreated with TSA and then stimulated by LPS (10 μg/ml), TNF-α (10 ng/ml), or PMA (50 nM) prior to assay of luciferase activity. As shown in Fig. 7, both LPS and TNF-α induced a significant increase of luciferase activity in cells transfected with wild-type TF (Fig. 7*A*) and wild-type ICAM-1 (Fig. 7*B*) constructs. TSA treatment inhibited the luciferase activity induction of wild-type TF (Fig. 7*A*) without affecting that of wild-type ICAM-1 (Fig. 7*B*) in a dose-dependent manner. No agonist induction was observed for the mutant TF-κB construct (Fig. 7*A*, *far right bars*). PMA strongly induced TF expression by activating both TF-κB and Egr-1 transcription factors in endothelial cells (5, 7, 37). We find that 100 nM TSA attenuates by 75% TF activity induced by 50 nM PMA in HUVEC (data not shown). In luciferase-reporter-transfected HUVEC, PMA induced luciferase activity 4.5-fold for wild-type TF, 2-fold for mutant TF-κB, and 6-fold for wild-type ICAM-1 (Fig. 7*C*). TSA (30–300 nM) treatment decreased the induction of TF

wild type from 4.5- to 2-fold, the same as that of the mutant TF-κB construct, without altering the induction of the wild-type ICAM-1 and mutant TF-κB constructs.

FIGURE 8. **Role of HDAC3 in HUVEC TF gene expression.** *A*, ChIP assay. HUVEC monolayers were stimulated with phosphate-buffered saline or 1 ng/ml TNF-α for 0.5 h. ChIP was performed as described for Fig. 6 using either rabbit IgG control or rabbit anti-human HD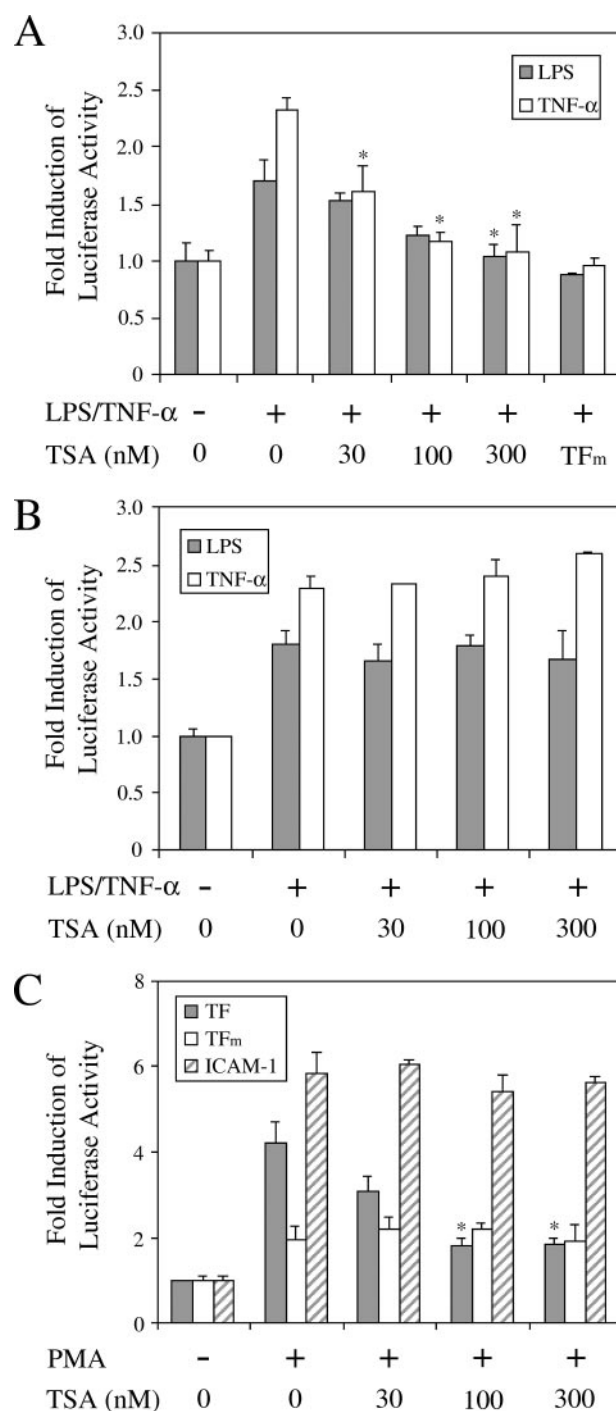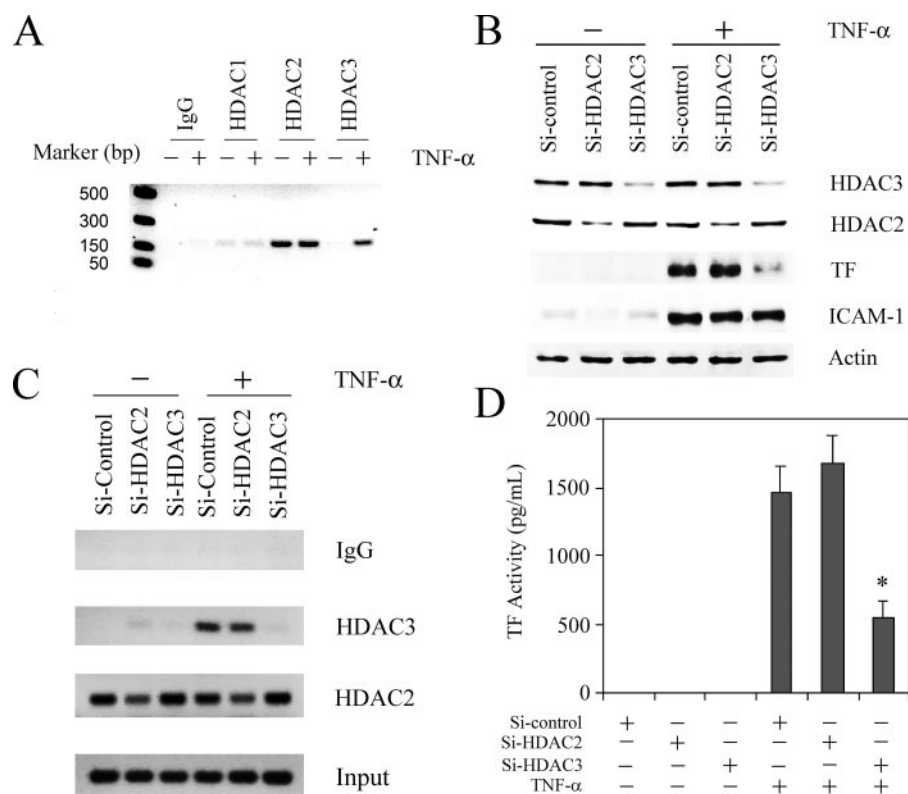AC1, HDAC2, or HDAC3 antibodies. The immunoprecipitated DNA was detected by PCR amplification using promoter-specific primers for TF. *B–D*, siRNA analysis. HUVEC were transfected with control (*Si-control*), HDAC2 (*Si-HDAC2*), or HDAC3 (*Si-HDAC3*) siRNA duplex. 72 h after transfection, HUVEC were stimulated by TNF-α for another 0.5 h (*C*) or 4 h (*B* and *D*). *B*, protein levels of HDAC2, HDAC3, TF, and ICAM-1 were detected by Western blot using actin as a loading control. *C*, binding of HDAC2 and HDAC3 to the TF promoter was assayed by a ChIP assay. *D*, TF activity of transfected HUVEC lysates was assayed by a one-stage clotting assay. All data shown ± S.D. (*, $p < 0.05$ *versus* Si-HDAC2 or Si-control).

iments precludes a definitive quantitative comparison of their functional roles in TF expression, these results nonetheless show strikingly disparate roles for HDAC2 and HDAC3.

DISCUSSION

Histone acetyltransferases and HDACs were originally described as enzymes that catalyze, respectively, either acetylation or deacetylation of ε-amino groups of lysine residues in the N-terminal tails of core histones (recently reviewed by Huang and Pardee (40)). Acetylation of histones, in turn, diminishes their positive charge and reduces their affinity for DNA, thereby altering nucleosomal structure to facilitate the binding of transcription factors and enhancing transcription (41, 42). Conversely, HDACs, by catalyzing the removal of histone acetyl groups, increase histone affinity for DNA, hinder the access of transcription factors, and suppress transcription. However, since the initial description of this histone acetylation paradigm, fully 18 members of the HDAC family have now been identified and categorized into three classes based on their sequence homology to yeast homologs (43). Recent work has shown that HDAC, in addition to regulating histone acetylation, can associate with and acetylate transcription factors, including E2F, NF-κB, p300, Stat3, p53, and the retinoblastoma protein (41, 43, 44). Moreover, HDAC can also modify other nonhistone proteins, such as α-tubulin (44) and Hsp90 (45, 46), as well as influence the ubiquitination pathway of protein degradation (43, 47).

Given the numerous facets of cellular biochemistry that are affected by acetylation, it is not surprising that HDACi have been found to exert protean effects on cellular function. The HDACi TSA modifies transcription in a limited (2%) set of genes and can both stimulate and suppress gene transcription (48). Currently, a major focus of investigations with HDACi is on their potential efficacy as an anti-cancer treatment (41, 43, 44). The mechanism for HDACi anti-cancer activity is complex and may reflect transcriptional regulation of genes involved in proliferation, differentiation, and apoptosis (40, 49–52). HDACi have also been found to have potent activity as a treatment for inflammatory diseases, including inflammatory bowel disease (53), systemic lupus erythematosus (14, 17), and rheumatoid arthritis (54). Thus, although the multiple aspects of cellular function influenced by HDACi-induced changes in acetylation have yet to be fully defined, HDACi seem to have promise as potential therapy for important human diseases.

Role of HDAC3 in TNF-α-mediated TF Expression—HDAC1, HDAC2, and HDAC3 have been shown to participate in the regulation of NF-κB activation (12, 13, 38, 39). We tested whether any of these HDACs binds to the TF-κB region in the TF promoter by ChIP analysis. As shown in Fig. 8*A*, in unstimulated HUVEC, HDAC2, but not HDAC1 or HDAC3, bound to the TF promoter (TF-κB region). TNF-α treatment induced a significant increase in HDAC3 binding without affecting that of HDAC1 and HDAC2 (Fig. 8*A*), and these interactions were unaffected by TSA treatment (data not shown), suggesting a possible functional role for HDAC3 in TF gene transcription. We tested this possibility using HDAC3 siRNA knockdown analysis (Fig. 8, *B–D*). An siRNA duplex specific for human HDAC2, HDAC3, or a scrambled control was transfected into HUVEC. After 72 h, Western blots showed a specific 85% knockdown of HDAC3 and 65% knockdown of HDAC2 protein (Fig. 8*B*). ChIP analysis showed a complete blockage of HDAC3 and 40% decrease of HDAC2 binding to the TF promoter (Fig. 8*C*). Compared with HUVEC transfected with siRNA control, HDAC2 siRNA-treated cells have a slight (15%) increase of TF activity induced by TNF-α, but HDAC3 siRNA suppresses TF protein (Fig. 8*B*) and activity (Fig. 8*D*) induction by 65% without affecting ICAM-1 expression (Fig. 8*B*). Although the difference in degree of HDAC2 *versus* HDAC3 knockdown in these exper-
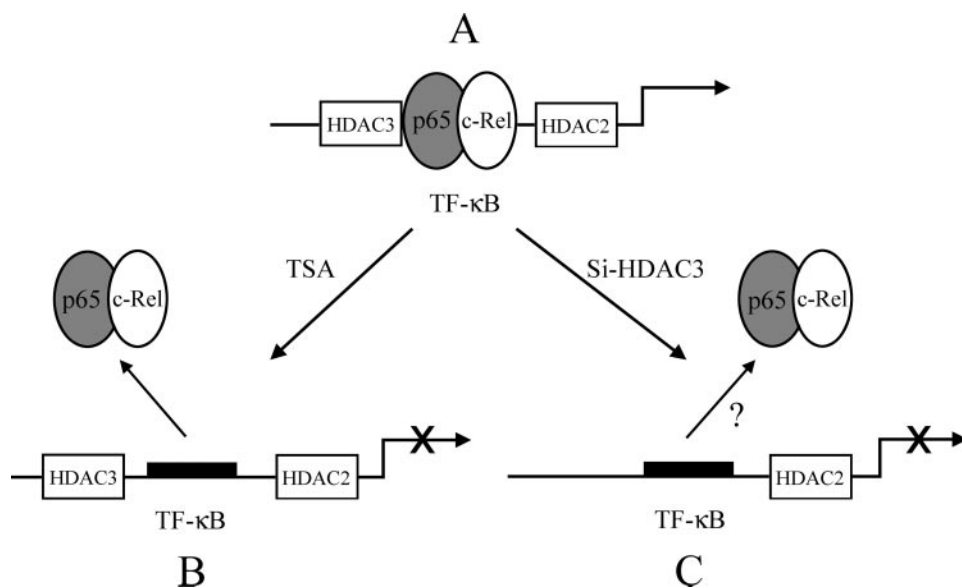
FIGURE 9. **Schema of possible HDAC3 interactions with the TF gene promoter TF-κB site in TNF-α- stimulated HUVEC.** *A*, TNF-α stimulation induces p65/c-Rel (and P65/p50) binding to, and HDAC3 binding to or near, the TF-κB site and promotes TF transcription. HDAC2 binds constitutively to the TF promoter with or without TNF-α treatment. *B*, HDACi (TSA) treatment decreases the binding of p65/c-Rel to the TF-κB site without significantly affecting HDAC2 or HDAC3 binding and impairs TF gene transcription. *C*, Si-HDAC3 treatment blocks HDAC3 binding to the TF-κB site TF promoter and impairs TF transcription, possibly by suppressing binding of p65/c-Rel to the TF-κB site.

We here find that TSA and four other HDACi suppress TF induction induced by TNF-α, LPS, IL-1β, and HOSCN up to 90% in HUVEC (Figs. 1 and 2), and TSA inhibits LPS- and IL-1β-stimulated TF activity by 50–60% in human PBMC and murine macrophages (Fig. 3). TSA exhibits a remarkably low IC_{50} (20–50 nM) for inhibition of agonist-induced TF induction, raising the possibility that TSA or related HDACi might have utility as an *in vivo* antithrombotic therapeutic agent. This IC_{50} is up to orders of magnitude less than that of other known TF inhibitors, such as salicylates (55), curcumin (56), anisodamine (57), and andrographolide (5, 27).

Investigating the mechanism underlying TSA inhibition of TF expression in HUVEC, we find that TSA suppresses the TF mRNA increase induced by TNF-α (Fig. 4). In other experiments not shown, we found that TSA does not significantly affect TF mRNA translational efficiency as assessed in a polyribosomal mRNA distribution assay (58); nor does it affect ubiquitination-dependent TF degradation assessed in the presence of the ubiquitination-dependent protease pathway inhibitor MG-132. Further investigation of potential transcription factors involved in TF expression showed no impact of TSA upon SP-1, Egr-1, or AP-1 activation by EMSA analysis (not shown), and nuclear protein extract levels of SP-1 and Egr-1 were unaffected by TNF-α stimulation regardless of whether TSA was present (Fig. 5D). However, TSA nearly completely blocks binding of p65 and c-Rel (Fig. 5B) and p50 (not shown) to the TF-specific TF-κB promoter binding sequence. These data together with the findings using the ChIP assay (Fig. 6) and luciferase reporter system (Fig. 7) clearly confirm that TSA suppression of TF expression in TNF-α-stimulated HUVEC is strictly TF-κB-dependent.

In striking contrast, TSA has no effect on TNF-α stimulated expression of ICAM-1 (Fig. 2) and E-selectin (not shown). Two

NF-κB sites in ICAM-1 promoter, which bind the p65/p65 homodimer and p65/p50 heterodimer, respectively, play an important role in ICAM-1 gene expression (33). For E-selectin expression, the p65/p50 heterodimer is the most abundant form, binding to three NF-κB sites (34). Our EMSA data show that TSA has no significant effect on p65/p50 binding to a consensus NF-κB binding probe (Fig. 5A), a finding compatible with its lack of effect on ICAM-1 and E-selectin expression. However, TSA significantly suppresses TNF-α-mediated VCAM-1 expression in endothelial cells (28), a finding we confirmed in HUVEC (not shown). The *VCAM-1* gene promoter contains two NF-κB binding sites with distinct sequences that both bind the p65/p50 heterodimer (59). It is possible that TSA, irrespective of its effect on NF-κB, inhibits the activity of other transcription factors, such as endothelial interferon-regulatory factor 1 (60) and GATA (61), that are critically involved in regulating VCAM-1 expression in cytokine-activated HUVEC. We acknowledge, however, that our findings were made in HUVEC, and it is possible, perhaps even likely, that TSA might down-regulate expression of NF-κB-regulated genes in other cell types.

Our studies do not define the mechanism whereby TSA down-regulates TF-κB-dependent TF expression without affecting NF-κB activation in HUVEC, a focus of our ongoing investigations. The effects of HDACi on NF-κB activation are highly variable, with activation (12, 13), inhibition (9–11), and lack of effect (62) described, depending on the HDACi and cell type. The NF-κB family has five members: p105/p50 (NF-κB1), p100/p52 (NF-κB2), p65 (RelA), RelB, and c-Rel. The p65/p50 heterodimer is the most abundant form that regulates expression of a wide spectrum of genes, including cytokines, chemokines, and cell adhesion molecules, including ICAM-1, VCAM-1, and E-selectin. Various coactivator and corepressor proteins, including p300/CBP, HDAC1, HDAC2, and HDAC3, participate in the regulation of NF-κB activation (8). Both p50 and p65 can be acetylated at multiple lysine residues by p300/CBP acetyltransferases, and acetylated p65 or p50 can be deacetylated by HDAC1 (38, 39), HDAC2 (38), or HDAC3 (11, 12). Human TF has a TF-κB (κB-like) upstream site that binds the p65/c-Rel heterodimer. TF-κB contains a cytosine at position 1 (5′-$\underline{C}$GGAGTTTCC-3′) and does not fit the consensus κB binding site sequence (5′-$\underline{G}$GGRNNYYCC-3′, where R represents a purine, N represents any nucleotide, and Y represents a pyrimidine) (63).

One possible explanation for the relative specificity of TSA for inhibition of TF-κB over NF-κB activation is a differential effect of p65 acetylation upon its interaction with these two

sites. Acetylated p65 recombinant protein has a decreased binding affinity for an NF-κB probe *in vitro* (11). It is possible, for example, that the binding affinity of hyperacetylated p65/c-Rel or hyperacetylated p65/p50 is decreased much more for TF-κB than for NF-κB, and therefore HDACi selectively suppresses activation of TF-κB. Alternatively, different acetylation-modulated coactivators or corepressors may localize on a TF-κB site than on NF-κB sites, a possibility supported by the demonstration that a single nucleotide change in a κB site can determine cofactor specificity for NF-κB dimers (64). Myriad other possibilities exist for this complex system, so significant further experimentation will be necessary to define the basis for the selective effect of TSA on TF-κB.

Although we performed our experiments predominantly *in vitro* with primary culture endothelium, PBMC, and macrophages, the demonstration that TSA suppresses macrophage TF induction in response to thioglycolate-evoked inflammation in the murine peritonitis model (Fig. 3*D*) shows that TSA can inhibit TF induction *in vivo* as well. This, in turn, suggests the possibility that HDACi, particularly TSA, could decrease aberrant TF expression in thrombotic diseases as well as in inflammatory/thrombotic states such as sepsis. Both vessel wall-derived TF and the circulating leukocyte-associated pool of TF participate in thrombus formation (65, 66). The circulating pool of TF, derived almost exclusively from monocytes and monocyte-derived microparticles, plays an important role in thrombus propagation by sustaining thrombin production on the clot surface (65). Vessel wall-derived TF plays roles in thrombus initiation immediately after vessel wall damage, especially in the macrovascular system (66). Of note, previous studies found that the HDACi TSA (67) and butyrate (67–69) increase expression of tissue-type plasminogen activator, a key activator of fibrinolysis, in HUVEC (67, 69) and human peritoneal mesothelial cells (68). Thus, HDACi, by simultaneously decreasing agonist-induced expression of TF in vessels and monocytes as well as increasing endothelial tissue-type plasminogen activator expression, might have utility as a therapy for thrombosis. We are testing this hypothesis in a murine deep venous thrombosis model.

Finally, our HDAC-specific ChIP and siRNA experiments show that HDAC3 plays a hitherto unsuspected and influential role in regulating TF expression in TNF-α-stimulated endothelial cells (Fig. 8). Fig. 9 is a schema summarizing our results and depicting one possible mechanistic scenario for the role of HDAC3 in regulation of TF expression. Finally, we note that TSA is a broadly active HDACi affecting both class I (HDAC1, -2, -3, -8, and -11) and class II (HDAC4, -5, -6, -7, -9, and -10) HDACs. A more targeted HDAC3 inhibitor might exhibit more specificity for suppressing aberrant TF expression in thrombotic states than a nonselective HDACi such as TSA.

In summary, we find that HDACi transcriptionally inhibit TF expression in agonist-stimulated endothelial cells, monocytes, and macrophages by specific blockade of TF-κB activation, and HDAC3 plays an important role in regulating TF expression.

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