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ORIGINAL RESEARCH

Novel Mechanism of Attenuation of LPS-Induced NF-κB Activation by the Heat Shock Protein 90 Inhibitor, 17-N-allylamino-17-demethoxygeldanamycin, in Human Lung Microvascular Endothelial Cells

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Abstract

Heat shock protein (hsp) 90 inhibition attenuates NF-κB activation and blocks inflammation. However, the precise mechanism of NF-κB regulation by hsp90 in the endothelium is not clear. We investigated the mechanisms of hsp90 inhibition by 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) on NF-κB activation by LPS in primary human lung microvascular endothelial cells. Transcriptional activation of NF-κB was measured by luciferase reporter assay, gene expression by real-time RT-PCR, DNA binding of transcription factors by chromatin immunoprecipitation assay, protein–protein interaction by coimmunoprecipitation/immunoblotting, histone deacetylase (HDAC)/histone acetyltransferase enzyme activity by fluorometry, and nucleosome eviction by partial micrococcal DNase digestion. In human lung microvascular endothelial cells, 17-AAG–induced degradation of IKBα was accomplished regardless of the phosphorylation/ubiquitination state of the protein. Hence, 17-AAG did not block LPS-induced NF-κB nuclear translocation and DNA binding activity. Instead, 17-AAG blocked the recruitment of the coactivator, cAMP response element binding protein binding protein, and prevented the assembly of a transcriptionally competent RNA polymerase II complex at the κB elements of the IKBα (an NF-κB–responsive gene) promoter. The effect of LPS on IKBα mRNA expression was associated with rapid deacetylation of histone-H3(Lys9) and a dramatic down-regulation of core histone H3 binding. Even though treatment with an HDAC inhibitor produced the same effect as hsp90 inhibition, the effect of 17-AAG was independent of HDAC. We conclude that hsp90 inhibition attenuates NF-κB transcriptional activation by preventing coactivator recruitment and nucleosome eviction from the target promoter in human lung endothelial cells.

Keywords: human lung microvascular endothelial cells; heat shock protein 90 inhibitor; LPS; NF-κB; cAMP response element binding protein binding protein

Clinical Relevance

NF-κB is a master regulator of inflammation. We report on a new mechanism regulating NF-κB activity that could reveal new targets for the management of acute lung injury/acute respiratory distress syndrome and other pulmonary inflammatory disease.

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The physical interaction between heat shock protein (hsp) 90 and its "clients," which include transcription factors and protein kinases (1), is essential for their maturation and function (2). Disruption of the hsp90–client interaction results in the degradation of the client protein (3). Thus, hsp90 inhibitors have been tested for their ability to block pathological pathways, especially in cancer (3). As hsps are also involved in immune responses (4), they can be exploited for selectively targeting of pathways in human immune disorders.

Severe gram-negative bacterial infection often leads to uncontrolled immune response as a result of hyperactivated NF-κB signaling (5). The bacterial endotoxin, LPS, stimulates Toll-like receptor-4 receptors on the surface of immune cells and triggers IKKβ activation (6). Activated IKKβ, in turn, phosphorylates the inhibitory IKBα, leading to the degradation of IKBα (7). After IKBα degradation, NF-κB dimers are released from the cytosol and translocated into the nucleus, where they bind to the κB enhancer element, GGGRNNYYCC (R = purine, Y = pyrimidine), present on the promoters of NF-κB–responsive genes and initiate transcription (8). NF-κB hyperactivation in the lung endothelium after severe bacterial septicemia, is a fundamental step in the development of acute inflammatory lung diseases (9, 10). The role of NF-κB activation in endothelial cells is twofold. First, it elicits proinflammatory cytokine production, and second, it accelerates leukocyte infiltration by inducing cell adhesion molecules (6). Several key regulators of Toll-like receptor-4 receptor pathway, such as TAK-1 kinase, RIP kinase, and IκB kinases are clients of hsp90 (1), suggesting that the NF-κB pathway could be highly sensitive to hsp90 inhibitors, such as 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), which has been demonstrated to be highly specific to hsp90 in numerous reports (11).

Inhibition of hsp90 evokes complex NF-κB regulation. In tumor, vascular smooth muscle, and macrophage cells, hsp90 inhibition blocks NF-κB signaling by suppressing its nuclear translocation (12–15). Hsp90 inhibition also blocks mitogen-activated protein kinase pathway activation in mice (16) and NF-κB post-translational modifications in mouse macrophages (17). In yeast, inhibition of the hsp90/hsp70 chaperone complex by genetic manipulation blocks nucleosome eviction from a transcriptionally competent promoter (18). However, the precise mechanism of hsp90 in endothelial NF-κB hyperactivation is still not clearly understood. In this study, we investigated three distinct mechanisms that may be responsible for hsp90 regulation of LPS-induced NF-κB activation in human lung microvascular endothelial cells (HLMVECs): NF-κB nuclear translocation, cAMP response element binding protein binding protein (CBP) coactivator recruitment, and nucleosome reorganization.

Materials and Methods

Reagents and Antibodies

Escherichia coli endotoxin (LPS) L-3137 was purchased from Sigma-Aldrich (St. Louis, MO). 17-AAG was from Selleck Chemicals (Houston, TX). All other inhibitors were purchased from ENZO Life Sciences (Farmingdale, NY). Anti-IKBα and anti–phospho-IKBα (Ser32/36) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–acetyl-H3(Lys9), anti-H3, anti-HA, anti–Poly-(ADP-ribose) polymerase and anti–Lamin-associated protein 2α antibodies were from Cell Signaling Technology (Danvers, MA). Anti-p65, anti-CBP, and anti-ubiquitin antibodies were from Abcam (Cambridge, MA). Anti–phospho-RNA polymerase (Pol) II Ser5 antibody was from Active Motif (Carslbad, CA). Anti–β-actin and horseradish peroxidase–conjugated secondary antibodies (mouse and rabbit IgG) were from Sigma Aldrich. α-Tubulin antibody was from Covance Research Products (Denver, PA).

Cell Culture and Treatment

Primary cultures of HLMVECs were harvested, isolated, and cultured in house, as previously described (19).

Western blotting and coimmunoprecipitation were performed as previously described (20).

Adenoviral Transduction and NF-κB Luciferase Reporter Assay

NF-κB firefly luciferase (Luc) reporter adenovirus was obtained from Vector Biolabs (Philadelphia, PA). Green fluorescent protein (GFP)-expressing adenovirus was generated and characterized as described previously (21). HLMVECs were cotransduced with NF-κB–Luc (10 MOI) and GFP (100 MOI) in 96-well plates for 3 days, then treated with 1 EU/ml LPS for 4 hours in the presence and absence of 17-AAG (5 μg/ml, 16 h). Equal amounts of the lysate were used in duplicates for determining GFP fluorescence (485/528 nm) using a Biotek Synergy HT microplate reader (Winooski, VT). Luminescence was measured using the Bright Glo Luc reagent (Promega, Madison, WI) with GloMax luminometer (Promega) and normalized to GFP fluorescence.

Transfection

HLMVECs were transfected with cytomegalovirus promoter driven mammalian expression plamids–3HA-IKBa or IKBaSer32/36(alanine [Ala]/Ala) double mutant, purchased from Addgene (Cambridge, MA), using Effectene transfection reagent (QIAGEN, Valencia, CA). HLMVECs were grown in 100-mm dishes and transfected with 2.5 μg plasmid mixed with 60 μl of the transfection reagent. After 3 days, the cells were treated with LPS (1 EU/ml) for 1 hour in the presence or absence of 17-AAG (16 h). IKBα expression levels were assessed by Western blotting using anti-HA antibody (Cell Signaling Technology).

Micrococcal DNase Assay

Treated HLMVECs were fixed in 1% formaldehyde for 10 minutes and blocked with 125 mM glycine for 5 minutes at room temperature. The cells were washed 3× with chilled PBS, resuspended in 10 mM Hepes (pH 8) buffer, containing 3 mM $MgCl_2$, 10 mM KCl, 0.5% Nonidet-P40, 1 mM DTT, 1 mM PMSF, and protease inhibitor, and incubated for 10 minutes on ice. The suspension was partially digested with 50 EU micrococcal DNase (New England Biolabs, Ipswich, MA) in 0.1 ml 1× digestion buffer supplemented with 100 μg/ml BSA and 0.1% Triton X-100 for 5 minutes at 37°C. The reaction was stopped by adding 0.1 ml 100 mM Tris-HCl (pH 8), containing 20 mM EDTA, 4% SDS, and 400 mM NaCl. The mixture was incubated for 10 hours at 65°C to reverse cross-linking, and DNA was isolated using PrepEase DNA columns (Affymetrix, Santa Clara, CA). Eluted DNA (20 ng) was used to PCR amplify the IKBα promoter, and was normalized to glyceraldehyde 3-phosphate dehydrogenase.

Statistical Analysis

Data represent more than three separate experiments and are reported as means (± SE). Differences among groups were analyzed using one-way ANOVA and *post hoc* tested with the Tukey-Kramer multiple comparison test. The *t* test for independent samples was used for comparisons between two groups. Significance was accepted at

P less than 0.05. Statistical calculations were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

Results

The hsp90 Inhibitor, 17-AAG, Blocks LPS-Induced IKBα Phosphorylation and Ubiquitination, but Not Degradation, in HLMVECs

Short-term (2-h) 17-AAG treatment had no effect on LPS-induced IKKβ activation, but 16-hour treatment significantly reduced IKKβ activation (Figure 1A). Furthermore, only 16-hour 17-AAG treatment blocked LPS-induced IKBα phosphorylation, but failed to prevent LPS-induced IKBα degradation (Figure 1B). This was not due to suboptimal inhibition of hsp90 by 17-AAG by a short-term treatment, as both 2- and 16-hour treatments blocked extracellular signal–regulated kinase 1/2 mitogen-activated protein kinase activation by LPS to a similar extent (Figure 1B). In addition, there was a dramatic induction of hsp70 mRNA expression by 2-hour 17-AAG treatment (*see* Figures E1 and E2 in the online supplement), and a modest increase in hsp70 protein expression (Figure 1B). The synergistic effect of LPS and 17-AAG on IKBα degradation was partially blocked by the proteasomal inhibitor, PS-341 (Figure 1C). In addition, the degradation of IKBα in the presence of 17-AAG was not due to residual IKK activity, as it was not affected by the IKK

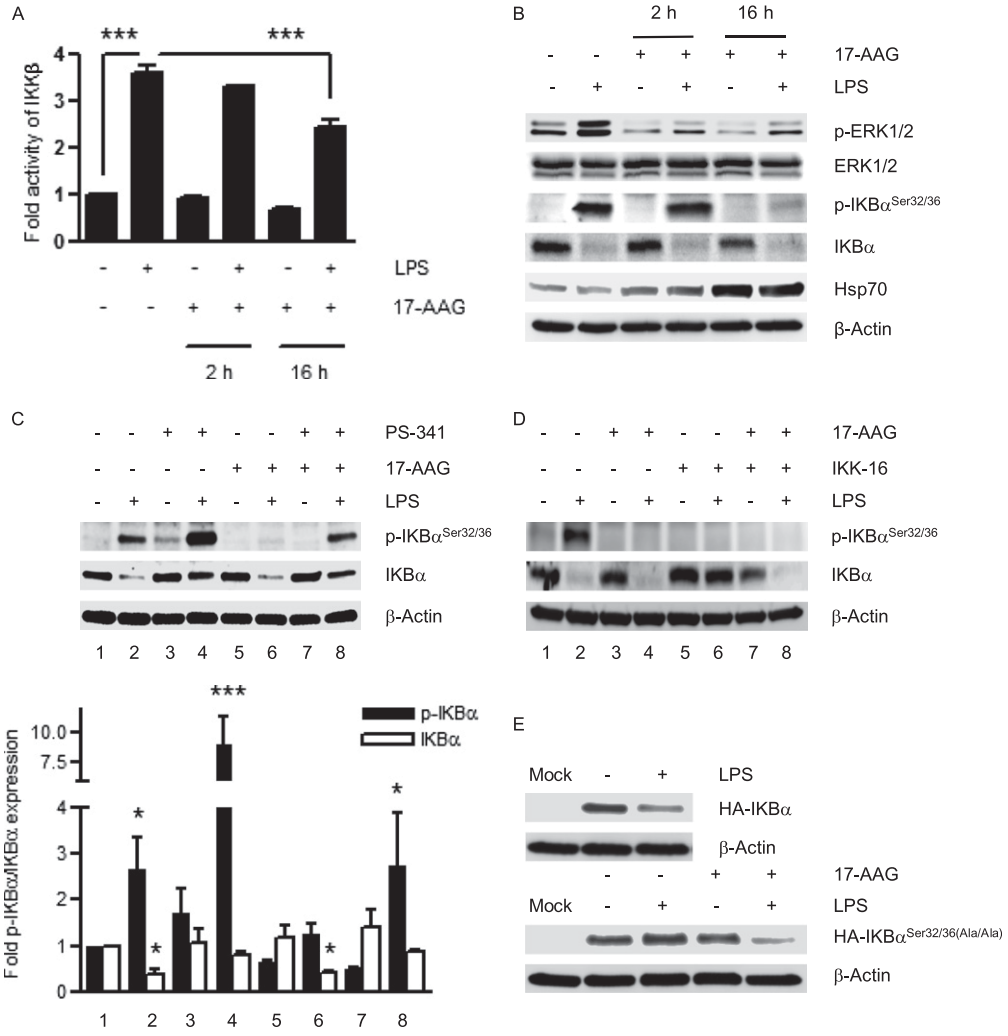

Figure 1. The heat shock protein (hsp) 90 inhibitor, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), abolishes LPS-induced IKBα phosphorylation, but does not prevent IKBα degradation. Human lung microvascular endothelial cells (HLMVECs) were pretreated for 2 or 16 hours with 5 μg/ml 17-AAG followed by LPS (1 EU/ml) for 15 minutes, and IKKβ activation was determined by ELISA. Only the long-term (16-h) 17-AAG pretreatment significantly blocked IKKβ activation (*A*) (***$P < 0.001$). Means ± SE of vehicle fold activated IKKβ from three independent experiments. Both 2- and 16-hour 17-AAG treatment blocked the LPS-mediated extracellular signal–regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK) phosphorylation, and produced a gradual induction of hsp70 expression in HLMVECs. However, only the 16-hour 17-AAG treatment blocked LPS (1 EU/ml, 1 h) -induced IKBα phosphorylation, but did not prevent it's degradation (*B*). The figure is representative of one of the three independent experiments; a histogram of IKBα regulation by 17-AAG is presented in Figure E1A. The synergism between 17-AAG and LPS was attenuated by the 26S proteasomal inhibitor, PS-341 (1 μM for 1 h; compare *lanes 6* and *8* [*C*]; *$P < 0.05$ and ***$P < 0.001$). Residual IKKβ activity was not responsible for the LPS + 17-AAG–induced IKBα degradation, as the IKK inhibitor, IKK16 (5 nM for 1 h), administered after 16-hour exposure of HLMVECs to 17-AAG, could not restore IKBα protein levels after LPS stimulation (compare *lanes 4* and *8* [*D*]). To confirm that 17-AAG induces degradation of nonphosphorylated IKBα, the HA-tagged phosphorylation-deficient IKBα Ser32/36 (Ala/Ala) double mutant was overexpressed in HLMVECs. The cells were then treated with LPS for 1 hour in the presence and absence of 17-AAG (16 h). In the presence of LPS, 17-AAG promoted the degradation of the IKBα double mutant in HLMVECs (*E*).

inhibitor, IKK16, administered *after* 17-AAG (Figure 1D).

NF-κB–associated IKBα has a longer half-life compared with free IKBα (22). Thus, we tested the hypothesis that hsp90 inhibition may destabilize this interaction and promote IKBα degradation by LPS. 17-AAG destabilized the association of IKBα with p65 in LPS-stimulated HLMVECs (Figure E3). Because 17-AAG did not block the LPS-induced degradation of IKBα, we expected that it might not affect the LPS-induced ubiquitination of IKBα, as well. Surprisingly, inhibition of hsp90 suppressed IKBα phosphorylation as well as IKBα poly-ubiquitination (Figure E4). This suggested that, contrary to accepted theory, hsp90 inhibition promotes the degradation of nonphosphorylated IKBα. To confirm this, we overexpressed HA-tagged wild-type and phosphorylation-deficient IKBαSer32/36(Ala/Ala) in HLMVECs and exposed them to LPS in the presence and absence of 17-AAG. The phosphorylation-deficient IKBα was not affected by LPS alone, but was profoundly degraded in the presence of both LPS and 17-AAG (Figures 1E and E5).

The hsp90 Inhibitor, 17-AAG, Does Not Prevent the LPS-Induced Nuclear Translocation of NF-κB (p65) in HLMVECs

Once released from IKBα, NF-κB rapidly translocates into the nucleus and binds κB enhancer elements in target promoters. We tested whether 17-AAG blocks the nuclear translocation of NF-κB and modulates the DNA binding affinity of NF-κB proteins. 17-AAG did not affect LPS-induced p65 or p50 nuclear translocation (Figure 2A). Also the DNA-binding affinity of p65 (as measured by ELISA) was similar between LPS and LPS plus 17-AAG–treated

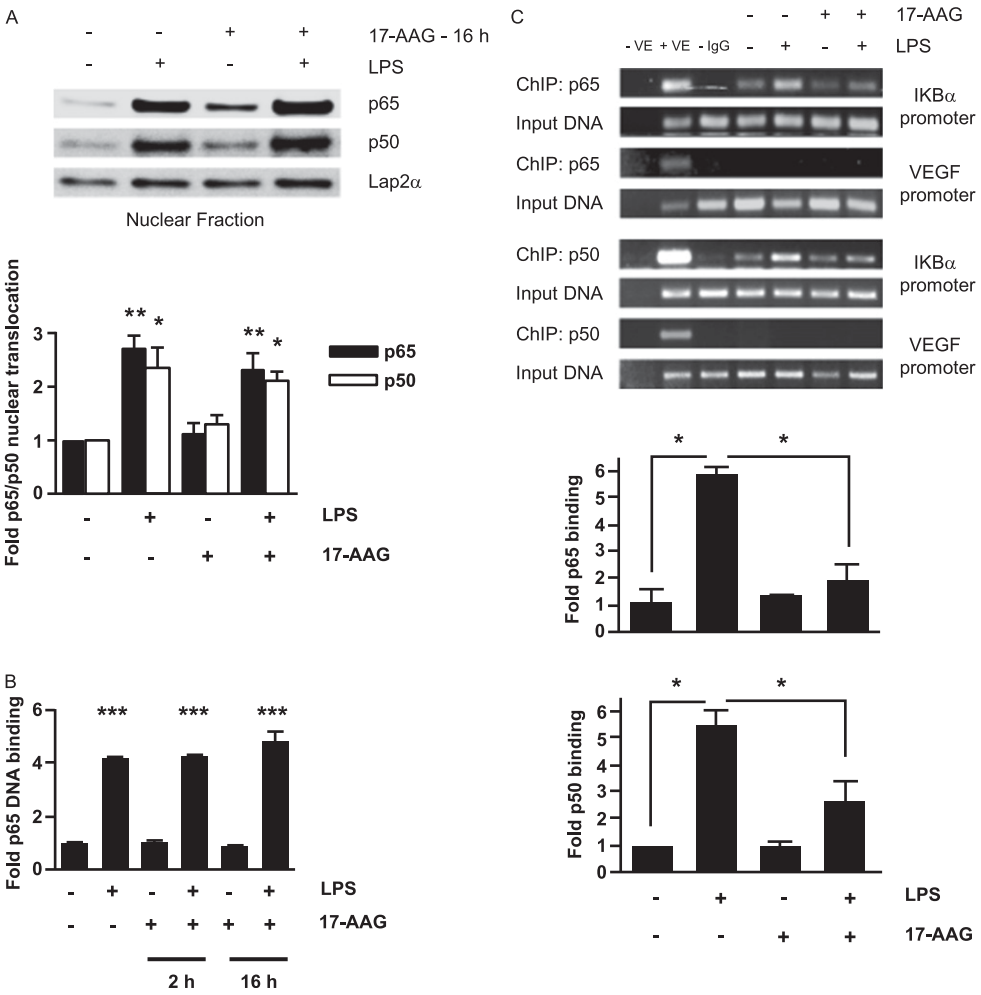

Figure 2. The hsp90 inhibitor, 17-AAG, prevents NF-κB transcriptional activation without interfering with NF-κB nuclear translocation and intrinsic DNA binding. HLMVECs were treated with LPS for 1 hour in the presence or absence of 17-AAG (16 h). NF-κB nuclear translocation was assessed by p65 and p50 immunoblotting. The LPS-induced nuclear translocation of NF-κB was not prevented by 17-AAG (*A*); $^{*}P < 0.05$ and $^{**}P < 0.01$. The LPS-induced DNA binding efficiency of p65 was also assessed in the presence or absence of 17-AAG by the TransAM NF-κB p65 kit (Active Motif). 17-AAG did not reduce the LPS-induced p65 DNA binding in HLMVECs (*B*); means ± SE of vehicle-fold p65 DNA binding from three independent experiments, each done in duplicate; $^{***}P < 0.001$. However, chromatin immunoprecipitation (ChIP) assay of p65/RelA and p50 showed that LPS (10 EU/ml, 1 h) induced binding to the IKBα promoter that was abolished by 17-AAG ($P < 0.01$) (*C*). The HIF-1α enhancer site in the human VEGF promoter served as the negative control for the assay. Means ± SE of three independent experiments.

HLMVECs (Figure 2B). To confirm that this observation was unique to HLMVECs, these experiments were repeated in bovine pulmonary arterial endothelial cells. As previously reported in other tissues, hsp90 inhibition prevented both the LPS-induced nuclear translocation of p65 (Figure E6) and p65 binding to DNA (Figure E7). We then studied the effect of 17-AAG on NF-κB binding to the IKBα (an NF-κB target gene) promoter by chromatin immunoprecipitation (ChIP) assay. We found that 17-AAG attenuated the LPS-stimulated p65 and p50 binding to the IKBα promoter (Figure 2C). Real-time quantitative RT-PCR confirmed that 17-AAG blocked the induction of three NF-κB target genes (IKBα, IL-8, and matrix metalloproteinase-9) by LPS (Figure E8). This suggested that the inhibition of hsp90 might regulate the transcriptional activation of NF-κB by regulating the accessibility of NF-κB to the target promoter.

To confirm the effect of 17-AAG on LPS-induced NF-κB transcriptional activation, an NF-κB Luc reporter and GFP were overexpressed in HLMVECs. NF-κB reporter assay was performed in triplicate and normalized to the corresponding GFP fluorescence. 17-AAG abolished the LPS-induced Luc activity (Figure 3A). In addition, short hairpin RNA–mediated knockdown of hsp90 similarly blocked LPS-induced NF-κB reporter activation (Figure 3B), and concomitantly increased hsp70 expression, a hallmark of hsp90 inhibition. Furthermore, the overexpression of hsp90α overcame the inhibitory effect of 17-AAG on LPS-induced NF-κB reporter activation (Figure 3C). These results suggested that the effect of 17-AAG was due to specific inhibition of hsp90.

LPS Suppresses Histone-H3(Lys9) Acetylation and Down-Regulates Core Histone H3 Binding to the IKBα Promoter

Nucleosome reorganization during transcription initiation determines the accessibility of transcription factors to their cognate enhancer elements (23). To study whether the observed effects of hsp90 inhibitors on NF-κB target gene activation involve regulation of nucleosome

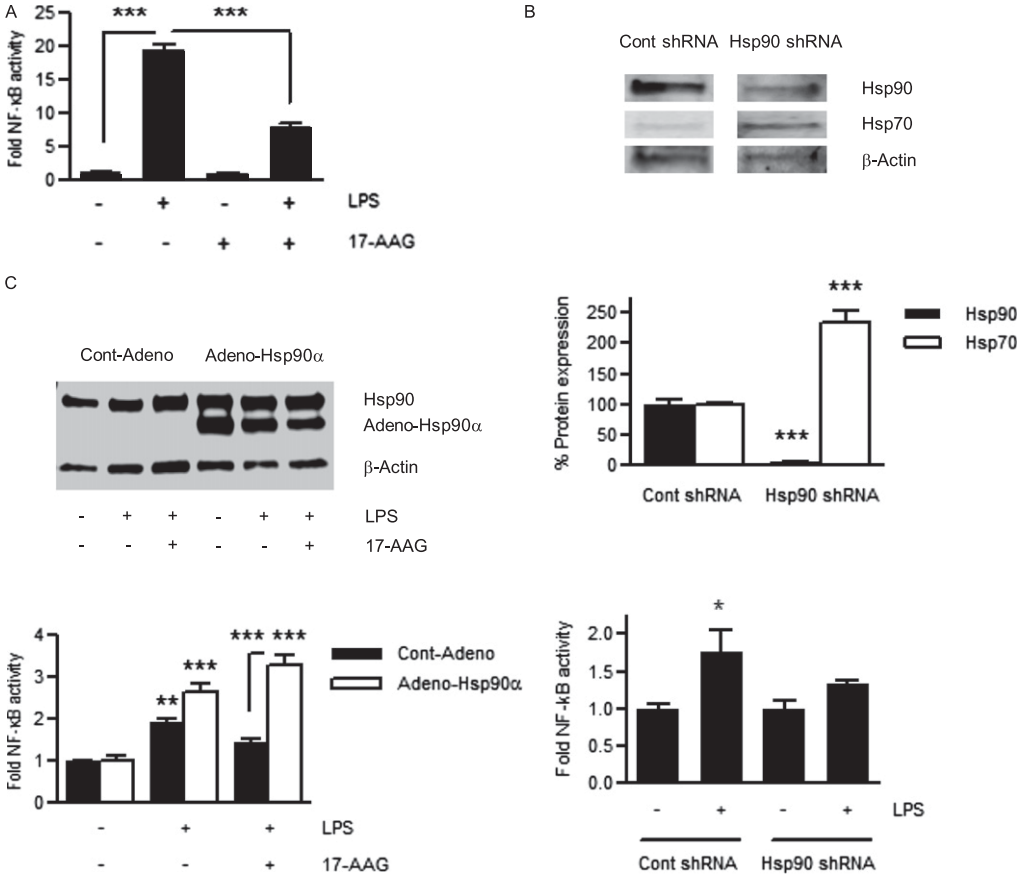

Figure 3. The hsp90 inhibitor, 17-AAG, blocks NF-κB transcriptional activity. HLMVECs were infected with an adenoviral NF-κB luciferase (Luc) reporter together with a green fluorescent protein (GFP) adenovirus (Adeno) 72 hours before a 4-hour exposure to LPS (10 EU/ml) in the presence and absence of 17-AAG (5 μg/ml, 16 h). 17-AAG blocked the LPS-induced NF-κB reporter activation (*A*); ***$P < 0.0001$. To confirm the role of hsp90 in the regulation of NF-κB transcriptional activation, endogenous hsp90 was depleted by hsp90 short hairpin RNA (shRNA) lentivirus in HLMVECs, as described in the MATERIALS AND METHODS in the online supplement. Hsp90 protein down-regulation by shRNA compared with the control shRNA and the associated hsp70 up-regulation is shown in the *upper panels* of (*B*) (the images are cropped from the same gel for better representation). The *middle panels* shows the histogram of means ± SE of fold protein from triplicate experiments (***$P < 0.001$). The *lower panels* shows that hsp90 down-regulation blocked NF-κB Luc reporter activation by LPS (*$P < 0.05$). On the other hand, the overexpression of Adeno–hsp90α reversed the inhibition of NF-κB Luc reporter activity by 17-AAG (*C*). The *upper panel* shows the overexpression of Adeno-hsp90α and the *lower panel* shows the histogram of fold NF-κB activation (means ± SE of triplicates; **$P < 0.01$, ***$P < 0.001$). The experiments were repeated three times.

reorganization, we studied the proximal roughly 0.5-Kb 5′-untranslated region of the IKBα promoter by ChIP assay. The IKBα promoter was associated with histone H3 and was constitutively acetylated at (Lys9) in HLMVECs (Figure 4A). LPS induced a concentration-dependent deacetylation of histone H3 (Lys9) and H3 displacement from the promoter region (Figure 4A). The H3 (Lys9) deacetylation and H3 displacement were specific to the IKBα promoter and was observed neither in the β-actin promoter, which is not regulated by LPS (Figure 4B), nor in the SOCS3 promoter, which is not a target of NF-κB, but is regulated by LPS (Figure 4C). Furthermore, LPS induced H3 Lys9 deacetylation in the total histone extract without affecting acetylation at Lys14 (Figure 4D).

Either Histone Deacetylase or hsp90 Inhibitor Prevent the LPS-Induced Displacement of Nucleosome from the IKBα Promoter in HLMVECs

Histone acetyltransferases and histone deacetylases (HDACs) regulate the acetylation of both histones and nonhistone proteins (24). Because LPS-mediated NF-κB activation involves histone deacetylation, we tested the role of HDAC using the inhibitor, trichostatin A (TSA). TSA blocked the LPS-induced IKBα and IL-8 mRNA expression in HLMVECs (Figure E9). TSA also attenuated p65 binding to the IKBα promoter (Figure 5A). We also tested the effect of TSA on histone H3 (Lys9) deacetylation and core H3 displacement. Somehow, TSA did not restore H3 (Lys9) acetylation at the IKBα promoter; however, TSA effectively blocked the LPS-induced core H3 displacement from the IKBα promoter (Figure 5B).

To study whether the effect of hsp90 inhibition was via an epigenetic mechanism, we tested the effect of 17-AAG on nucleosome reorganization and core histone modification at the IKBα promoter by ChIP assay. 17-AAG significantly reduced the LPS-mediated H3 (Lys9) deacetylation and H3 displacement in HLMVECs (Figure 5C). Because both TSA and 17-AAG affected NF-κB binding and core histone displacement, we investigated

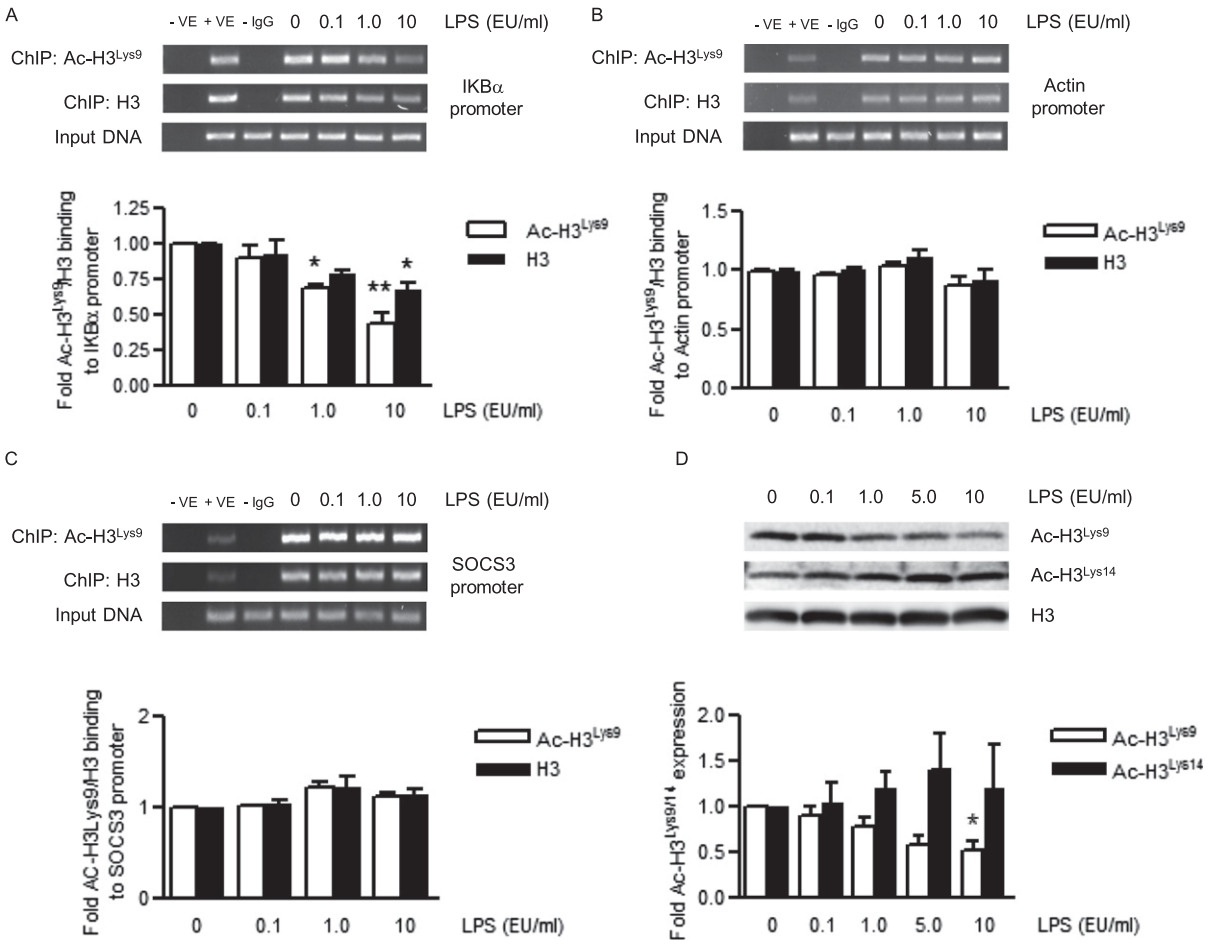

Figure 4. LPS suppresses histone-H3(Lys9) acetylation and down-regulates core histone H3 binding to the IKBα promoter. HLMVECs were treated for 1 hour with increasing concentrations of LPS (0.1–10 EU/ml). The regulation of core histone H3 at the IKBα promoter was assessed by ChIP assay. LPS induced a concentration-dependent deacetylation of H3 and binding of core H3 to the IKBα promoter (*A*). Mean ± SE of three experiments (*$P < 0.05$, **$P < 0.01$). This regulation of core histone H3 by LPS was not observed in non–NF-κB target gene promoters, β-actin (*B*) and SOCS3 (*C*). Furthermore, immunoblotting of total histone extract demonstrated that Lys9 deacetylation was specific to H3 (Lys9), as deacetylation of H3 (Lys 14) was not observed in LPS-treated HLMVECs (*D*).

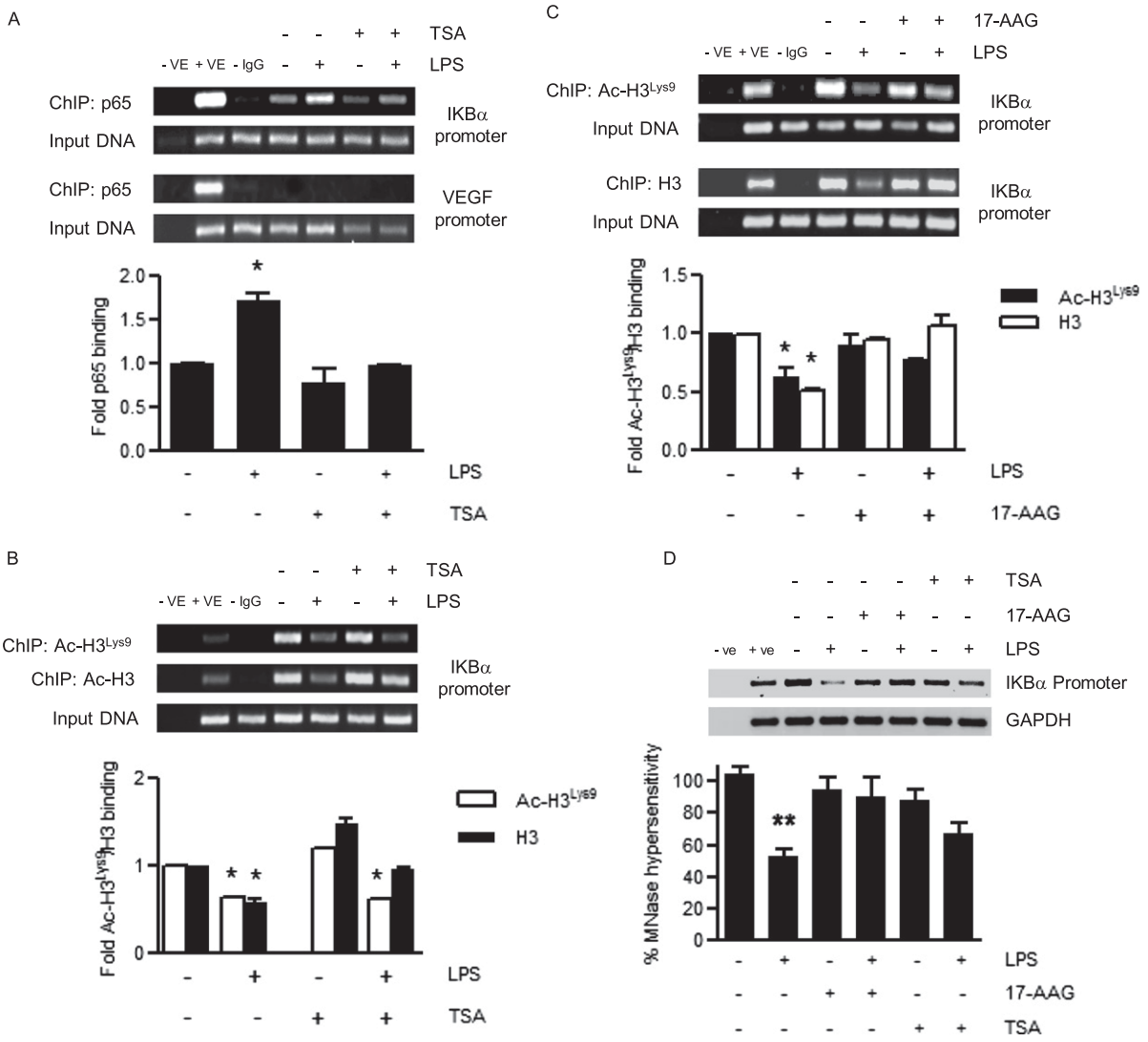

Figure 5. Both histone deacetylase (HDAC) and hsp90 inhibition down-regulate LPS-mediated NF-κB activation in HLMVECs. HLMVECs were treated with LPS (4 h, 10 EU/ml) in the presence or absence of the HDAC inhibitor, trichostatin A (TSA; 1 μM for 1 h) or 17-AAG (5 μg/ml for 16 h). TSA also blocked the LPS-induced p65 binding to the IKBα promoter (*$P < 0.5$) (*A*), and prevented the LPS-induced core histone H3 displacement from the IKBα promoter (*$P < 0.01$). However, TSA did not prevent the LPS-induced deacetylation of promoter-associated H3 (Lys9) ($P < 0.05$) (*B*). ChIP assay demonstrated that, unlike TSA, 17-AAG blocked both the deacetylation of H3 (Lys9), as well as the displacement of core H3 from the IKBα promoter (*C*). However, both HDAC and hsp90 inhibitors block the LPS-induced hypersensitivity of IKBα promoter to micrococcal DNase (MNase) (*D*). The *upper panel* in (*B*) shows the inverted image of a representative PCR-amplified IKBα promoter after partial MNase digestion. The *lower panel* in (*B*) depicts means ± SE of three experiments. 17-AAG completely prevented the effect of LPS (**$P < 0.01$), whereas TSA only partially blocked MNase hypersensitivity.

whether hsp90 inhibition and HDAC inhibition shared a common mechanism. The partial micrococcal DNase assay demonstrated that both 17-AAG and TSA attenuated LPS-induced DNase hypersensitivity, albeit to different extents (Figure 5D). 17-AAG blocked the LPS effect, whereas TSA produced a partial attenuation. To determine whether hsp90 inhibition acted via HDAC inhibition, we sudied the effect of 17-AAG on LPS-induced HDAC activation. 17-AAG did not affect LPS-induced HDAC activation (Figure E10). This suggests that hsp90 inhibitors work through an alternate pathway of H3 deacetylation, and possibly regulate nucleosomal reorganization via mechanisms other than HDAC inhibition.

The hsp90 Inhibitor, 17-AAG, Attenuates CBP/HAT Activity and Prevents Its Recruitment to the IKBα Promoter

Because 17-AAG did not alter HDAC activity, we considered that it could prevent the LPS-induced activation of histone acetyltransferases. LPS alone induced nuclear HAT activation and 17-AAG blocked this activation (Figure 6A). NF-κB employs several cofactors for transcriptional activation of target genes, including CBP/p300 and PCAF (25). CBP belongs to a family of HAT, and regulates NF-κB–responsive gene expression (26). Thus, we used immunoprecipitated CBP from nuclear lysates to test the hypothesis that 17-AAG prevents LPS-stimulated CBP/HAT activation. LPS induced CBP activation and 17-AAG attenuated the

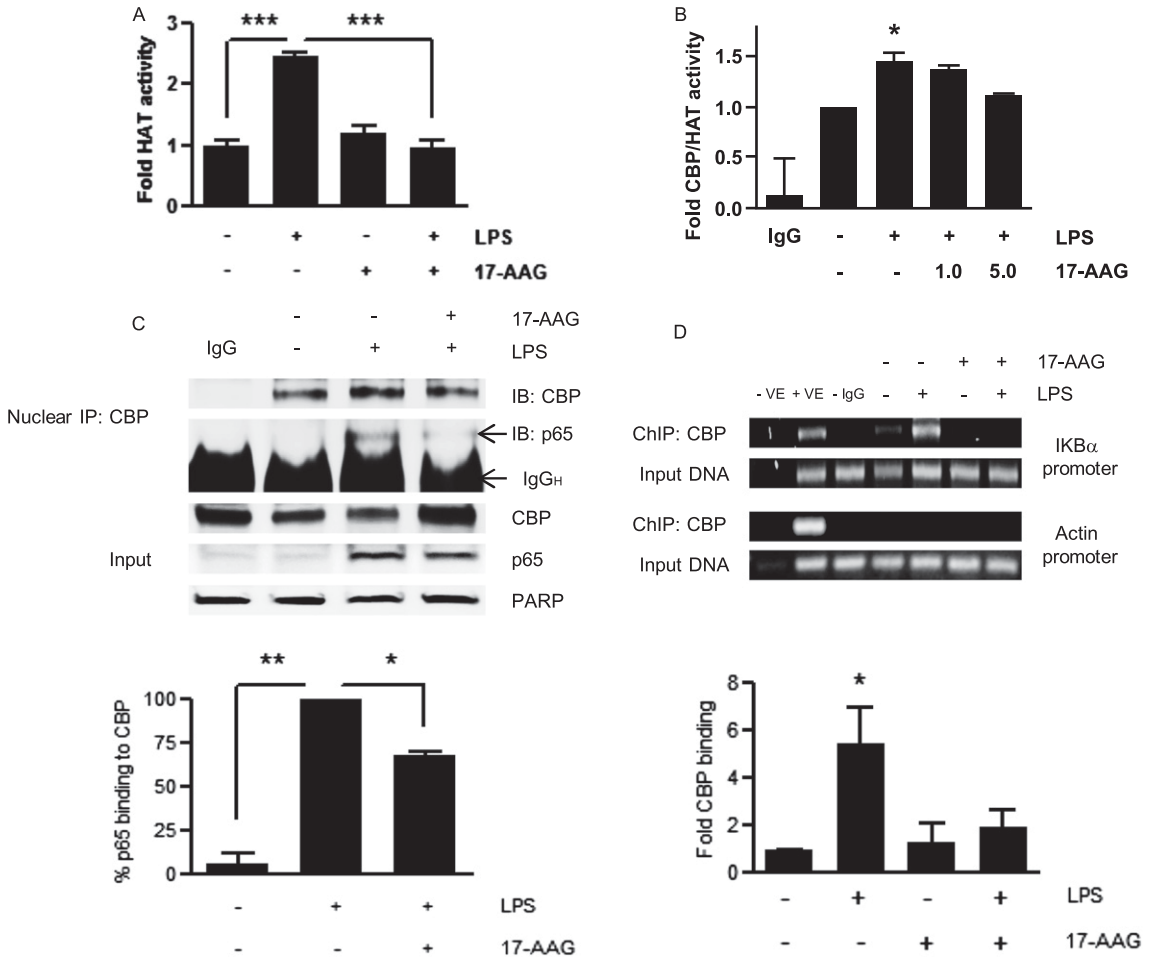

Figure 6. The hsp90 inhibitor, 17-AAG, blocks LPS-induced CBP/histone acetyltransferase (HAT) activation and recruitment to the IKBα promoter. HLMVECs were treated with LPS (10 EU/ml, 1 h) in the presence and absence of 17-AAG (16 h). Nuclear extracts were used to estimate total nuclear HAT activity and CBP activity using the HAT activity assay kit. 17-AAG attenuated the LPS-induced total HAT activation (*A*) (***$P < 0.0001$) and blocked the LPS-induced nuclear CBP activation in a concentration-dependent manner (*B*) (**$P < 0.01$). Means ± SE of three independent experiments, each performed in duplicate. In addition, CBP protein was immunoprecipitated from HLMVEC nuclear extracts, and its association with p65 was investigated by Western blotting. LPS induced an association between CBP and p65 that was prevented by 17-AAG (*C*). We also checked the recruitment of CBP to the IKBα promoter by ChIP assay. LPS (10 EU/ml, 15 min) promoted CBP binding to the IKBα promoter, and 17-AAG (5 μg/ml, 16 h) blocked this binding (*D*) (**$P < 0.01$). Means ± SE of three experiments (*$P < 0.05$).

activation in a concentration-dependent manner (Figure 6B). Furthermore, the physical interaction between p65 and CBP is considered to be important for NF-κB activity and gene induction (26). Thus, in coimmunoprecipitation studies, we examined the interaction of p65 and CBP. LPS induced CBP/p65 association in HLMVEC nuclear extracts, and 17-AAG suppressed this interaction (Figure 6C). We also tested the recruitment of CBP to the IKBα promoter, and observed that 17-AAG blocked the LPS-induced recruitment of CBP (Figure 6D).

Ser276 phosphorylation of p65 by mitogen and stress activated kinase-1 and cAMP-dependent protein kinase promotes the interaction and recruitment of CBP to target promoters (27–29). Thus, we investigated the effect of 17-AAG on the LPS-induced p65 (Ser276) phosphorylation in HLMVECs. LPS induced p65 Ser276 phosphorylation, and this was attenuated by hsp90 inhibition (Figure E11). We then overexpressed adenoviral p65 wild type and Ser276→Asp mutant in HLMVECs and examined the LPS-mediated activation of NF-κB Luc reporter. The phosphomimic did not prevent the 17-AAG–mediated attenuation of the LPS-stimulated NF-κB reporter activity (Figure E12). This suggests that p65Ser276 phosphorylation does not play a significant role in NF-κB regulation by 17-AAG, and is unlikely to be involved in the recruitment of CBP by p65.

The recruitment of CBP by p65 promotes cooperative binding of NF-κB to multiple elements (26). This, in turn, induces an optimal DNA conformation for subsequent downstream events, such as binding of nucleosome remodeling enzymes and other factors that regulate the transcription of inducible genes (30). Ser5-phosphorylated RNA Pol II is important for initiation of transcription, and Ser5 phosphorylation increases enzyme activity (31). We thus examined, by ChIP assay, whether hsp90 inhibition prevents the assembly of a functional RNA Pol II

complex at the IKBα promoter of HLMVECs. LPS induced the phosphorylation of RNA Pol II Ser5 at the IKBα promoter; this phosphorylation event was attenuated by 17-AAG (Figure 7A). As a control, we also studied the β-actin promoter. RNA Pol II was hyperactivated in the constitutively active β-actin promoter, and the level of Ser5 phosphorylated RNA Pol II was high. No additional stimulation/phosphorylation by LPS was observed, and no attenuation by 17-AAG. Similarly, no change in RNA Pol II Ser5 phosphorylation was observed in the SOCS3 promoter under these conditions. These findings suggest that 17-AAG attenuated LPS-mediated RNA Pol II complex assembly specifically at the NF-κB–responsive IKBα promoter.

Discussion

NF-κB activation is regulated at multiple steps: nuclear translocation, post-translational modification, and chromatin remodeling at the promoter region (32, 33). Our data suggest that, in HLMVECs, hsp90 inhibition blocks IKKβ activation and IKBα phosphorylation and polyubiquitination by LPS (canonical pathway), but cannot prevent IKBα degradation. Crystal structure (22) and thermodynamic studies have revealed an interaction between NF-κB and IKBα (34) that is responsible for the difference in the turnover rate between the bound and free IKBα (34, 35). Any modification that destabilizes this interaction promotes IKBα degradation. The destabilization of IKBα by 17-AAG in LPS-stimulated HLMVECs could be due to reduced binding of IKBα to p65.

Hsp90 inhibition did not affect DNA binding affinity of NF-κB/p65. However, it blocked its binding to the IKBα promoter. Free NF-κB binds cognate elements inside the nucleus. However, in most cases, multiple κB elements need to be occupied before transcriptional induction. The human IKBα promoter has three critical NF-κB consensus sites in the proximal

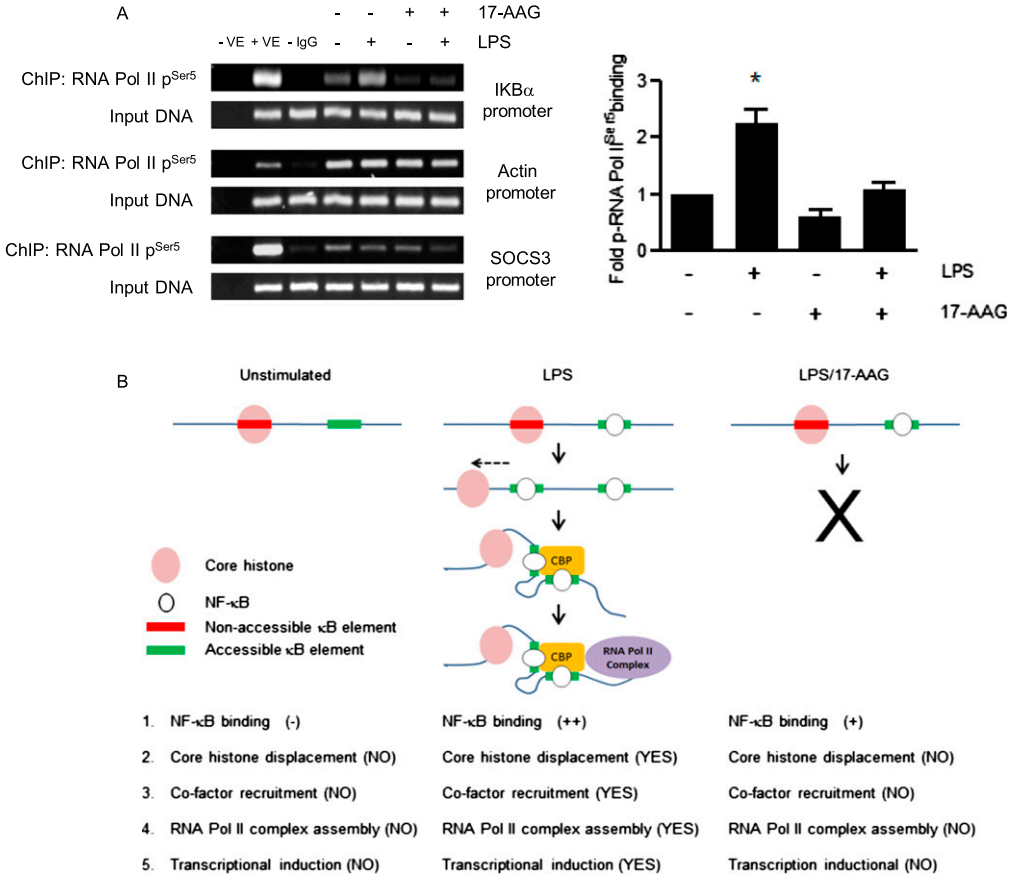

Figure 7. The hsp90 inhibitor, 17-AAG, prevents the assembly of RNA polymerase (Pol) II complex at the IKBα promoter. To study whether 17-AAG affected the assembly of an active RNA Pol II complex at the IKBα promoter, ChIP assay of phosphor-RNA Pol II Ser5 was performed in HLMVECs. LPS induced binding of phosphor-RNA Pol II Ser5 on the IKBα promoter, and this was prevented by 17-AAG. The effects of LPS and 17-AAG were unique for the IKBα promoter and not observed at the actin and SOCS3 promoters, which are insensitive to LPS and NF-κB, respectively. Means ± SE of three experimental repeats (*A*); $^*P < 0.05$). (*B*) The proposed model of hsp90 regulation of LPS-stimulated NF-κB transcriptional activation in HLMVECs. Under physiologic conditions, nucleosome-associated κB enhancer in the promoter is not accessible for NF-κB binding. LPS displaces core histones that unmask κB enhancer elements. Recruitment of CBP by p65 stabilizes the NF-κB/enhancer complex and, at the same time, promotes its interaction with RNA Pol II. The inhibition of hsp90 by 17-AAG blocks core histone displacement and prevents CBP recruitment, thereby terminating transcriptional initiation of NF-κB target genes by LPS.

500 bp of the 5′-untranslated region (36). The accessibility of NF-κB to these sites is determined by DNA conformation and the presence or absence of core histones. Eukaryotic DNA is packed into chromatins composed of repeated units of 147 bp DNA tightly wound around the histone octamer. These nucleosomes render most elements inaccessible to transcription factors. During gene transcription, nucleosomes are believed to slide along the DNA by an ATP-dependent remodeling process (37). Although certain NF-κB–mediated transcription does not require nucleosomal remodeling (33), we now demonstrate that nucleosome remodeling and displacement of core histone H3 from the IKBα promoter is a key mechanism of LPS-induced NF-κB transcriptional activation in HLMVECs. Furthermore, the displacement of histone H3 was associated with specific deacetylation of H3 (Lys9), suggesting the involvement of HDAC. Our data indicate that hsp90 inhibition and HDAC inhibition regulate NF-κB activation differently. For example, the inhibition of HDAC by TSA did not restore promoter-associated H3 (Lys9) acetylation, but blocked core histone H3 displacement by LPS. On the other hand, 17-AAG blocked both H3 (Lys9) deacetylation and H3 displacement from the IKBα promoter.

The role of hsp90 in chromatin remodeling was demonstrated in yeast, where deletion of either hsp70 or hsp90 chaperone suppressed nucleosome eviction from the actively transcribing galactose inducible promoter 1 (GAL1) promoter. The yeast orthologs of hsp70 and hsp90 are recruited to GAL1 promoter on induction, and their deletion prevents transcription, in the absence of any defects in galactose signaling and GAL4 DNA binding (18). Our data also suggest that hsp90 inhibition by 17-AAG prevented LPS-mediated NF-κB target gene expression without affecting NF-κB nuclear translocation and DNA binding. We then demonstrated that the suppression of transcription is due to failure of nucleosome eviction and core histone displacement from the IKBα promoter. Nuclear actions of Hsp90 have been reported before. Hsp90 nuclear shuttling has been observed in yeast; also, hsp90 complexes with the immature steroid receptor and shuttles between the cytoplasm and the nucleus (38). Thus, hsp90 is likely to play a key role in nuclear events, such as nucleosome remodeling during transcription initiation.

The binding of NF-κB/p65 to the coactivator, CBP, and its recruitment to the target promoter has a profound effect on the transcriptional activation of NF-κB–responsive genes. CBP is a HAT, and modifies both histone and nonhistone proteins (39). LPS promoted p65 interaction with CBP and recruitment to the IKBα promoter. On the other hand, 17-AAG abolished CBP recruitment, and this is likely due to the prevention of p65 binding to the IKBα promoter. As p65 binding to promoter is obligatory for CBP recruitment (25, 27, 29), the down-regulation of CBP binding by 17-AAG may not appear to explain the attenuation of NF-κB binding to IKBα promoter (Figures 2D and 2E). On the other hand, cofactor binding is known to promote the cooperative binding of transcription factors to multiple enhancer elements (33). Thus, we propose that the lack of CBP recruitment to the IKBα promoter on hsp90 inhibition most likely precludes NF-κB binding to all enhancer elements in the IKBα promoter, and thereby effectively lowers the promoter binding efficiency of NF-κB.

Coactivator recruitment by NF-κB is also important for assembly of active transcriptional machinery on the RNA Poll II preinitiation complex. CBP directly interacts with p65 as well as several members of the basal transcriptional complex, such as TATA binding protein, transcription factor IIB, and RNA Poll II (30). Thus, CBP acts as a bridge between the activator complex and the basic transcription machinery. We now demonstrate that hsp90 inhibition also prevents the LPS-induced formation of an active RNA Pol II complex at the IKBα promoter. Thus, the attenuation of CBP recruitment in the presence of 17-AAG could be one of the mechanisms for suppression of LPS-mediated NF-κB transcriptional activation in HLMVECs. The overall scheme of NF-κB transcriptional regulation by hsp90 inhibition in HLMVECs is depicted in Figure 7B.

In conclusion, we propose that hsp90 inhibition not only affects cytosolic signaling pathways, but also influences the reorganization of nucleosomes during transcriptional activation of NF-κB. Thus, the versatile property of hsp90 inhibitors, such as 17-AAG, could be exploited to effectively target the NF-κB signaling pathway and develop novel, potent anti-inflammatory drugs for acute inflammatory diseases. ■

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References

1. Picard D. Hsp90 interactors: University of Geneva, Department of Cell Biology (updated Dec 2012; assessed May 2013). Available from: http://www.picard.ch/downloads/hsp90interactors.pdf
2. Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G. The 90-kDa molecular chaperone family: structure, function, and clinical applications: a comprehensive review. *Pharmacol Ther* 1998;79: 129–168.
3. Zhang H, Burrows F. Targeting multiple signal transduction pathways through inhibition of hsp90. *J Mol Med* 2004;82:488–499.
4. Robert J. Evolution of heat shock protein and immunity. *Dev Comp Immunol* 2003;27:449–464.
5. Abraham E. Nuclear factor-kappaB and its role in sepsis-associated organ failure. *J Infect Dis* 2003;187:S364–S369.
6. Dauphinee SM, Karsan A. Lipopolysaccharide signaling in endothelial cells. *Lab Invest* 2006;86:9–22.
7. Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* 1997;91:243–252.
8. Hayden MS, Ghosh S. Signaling to NF-kappaB. *Genes Dev* 2004;18: 2195–2224.
9. Bohrer H, Qiu F, Zimmermann T, Zhang Y, Jllmer T, Mannel D, Bottiger BW, Stern DM, Waldherr R, Saeger HD, *et al*. Role of NFkappaB in the mortality of sepsis. *J Clin Invest* 1997;100: 972–985.
10. Orfanos SE, Mavrommati I, Korovesi I, Roussos C. Pulmonary endothelium in acute lung injury: from basic science to the critically ill. *Intensive Care Med* 2004;30:1702–1714.

11. Dimopoulos MA, Mitsiades CS, Anderson KC, Richardson PG. Tanespimycin as antitumor therapy. *Clin Lymphoma Myeloma Leuk* 2011;11:17–22.
12. Wang X, Ju W, Renouard J, Aden J, Belinsky SA, Lin Y. 17-Allylamino-17-demethoxygeldanamycin synergistically potentiates tumor necrosis factor–induced lung cancer cell death by blocking the nuclear factor-kappaB pathway. *Cancer Res* 2006;66:1089–1095.
13. Yeo M, Park HK, Lee KM, Lee KJ, Kim JH, Cho SW, Hahm KB. Blockage of hsp 90 modulates *Helicobacter pylori*–induced IL-8 productions through the inactivation of transcriptional factors of AP-1 and NF-kappaB. *Biochem Biophys Res Commun* 2004;320: 816–824.
14. Lewis J, Devin A, Miller A, Lin Y, Rodriguez Y, Neckers L, Liu ZG. Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor-kappaB activation. *J Biol Chem* 2000;275:10519–10526.
15. Madrigal-Matute J, Fernandez-Garcia CE, Gomez-Guerrero C, Lopez-Franco O, Munoz-Garcia B, Egido J, Blanco-Colio LM, Martin-Ventura JL. HSP90 inhibition by 17-DMAG attenuates oxidative stress in experimental atherosclerosis. *Cardiovasc Res* 2012;95: 116–123.
16. Yun TJ, Harning EK, Giza K, Rabah D, Li P, Arndt JW, Luchetti D, Biamonte MA, Shi J, Lundgren K, *et al*. EC144, a synthetic inhibitor of heat shock protein 90, blocks innate and adaptive immune responses in models of inflammation and autoimmunity. *J Immunol* 2011;186:563–575.
17. Luo S, Wang T, Qin H, Lei H, Xia Y. Obligatory role of heat shock protein 90 in iNOS induction. *Am J Physiol Cell Physiol* 2011;301: C227–C233.
18. Floer M, Bryant GO, Ptashne M. HSP90/70 chaperones are required for rapid nucleosome removal upon induction of the Gal genes of yeast. *Proc Natl Acad Sci USA* 2008;105:2975–2980.
19. Catravas JD, Snead C, Dimitropoulou C, Chang AS, Lucas R, Verin AD, Black SM. Harvesting, identification and barrier function of human lung microvascular endothelial cells. *Vascul Pharmacol* 2010;52: 175–181.
20. Antonov A, Snead C, Gorshkov B, Antonova GN, Verin AD, Catravas JD. Heat shock protein 90 inhibitors protect and restore pulmonary endothelial barrier function. *Am J Respir Cell Mol Biol* 2008;39: 551–559.
21. Zhang Q, Malik P, Pandey D, Gupta S, Jagnandan D, Belin de Chantemele E, Banfi B, Marrero MB, Rudic RD, Stepp DW, *et al*. Paradoxical activation of endothelial nitric oxide synthase by NADPH oxidase. *Arterioscler Thromb Vasc Biol* 2008;28:1627–1633.
22. Bergqvist S, Ghosh G, Komives EA. The IkappaBalpha/NF-kappaB complex has two hot spots, one at either end of the interface. *Protein Sci* 2008;17:2051–2058.
23. Cockerill PN. Structure and function of active chromatin and DNase I hypersensitive sites. *FEBS J* 2011;278:2182–2210.
24. Shakespear MR, Halili MA, Irvine KM, Fairlie DP, Sweet MJ. Histone deacetylases as regulators of inflammation and immunity. *Trends Immunol* 2011;32:335–343.
25. Sheppard KA, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, Thanos D, Rosenfeld MG, Glass CK, Collins T. Transcriptional activation by NF-kappaB requires multiple coactivators. *Mol Cell Biol* 1999;19:6367–6378.
26. Gerritsen ME, Williams AJ, Neish AS, Moore S, Shi Y, Collins T. CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc Natl Acad Sci USA* 1997;94:2927–2932.
27. Reber L, Vermeulen L, Haegeman G, Frossard N. Ser276 phosphorylation of NK-kB p65 by MSK1 controls SCF expression in inflammation. *PLoS ONE* 2009;4:e4393.
28. Zhong H, Voll RE, Ghosh S. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol Cell* 1998;1:661–671.
29. Kim MO, Lee YJ, Park JH, Ryu JM, Yun SP, Han HJ. PKA and cAMP stimulate proliferation of mouse embryonic stem cells by elevating GLUT1 expression mediated by the NF-kappaB and CREB/CBP signaling pathways. *Biochim Biophy Acta* 2012;1820:1636–1646.
30. Chan HM, La Thangue NB. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J Cell Sci* 2001;114:2363–2373.
31. Phatnani HP, Greenleaf AL. Phosphorylation and functions of the RNA polymerase II CTD. *Genes Dev* 2006;20:2922–2936.
32. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. *Cell* 2008;132:344–362.
33. Natoli G. Control of NF-kappaB–dependent transcriptional responses by chromatin organization. *Cold Spring Harb Perspect Biol* 2009;1: a000224.
34. Bergqvist S, Croy CH, Kjaergaard M, Huxford T, Ghosh G, Komives EA. Thermodynamics reveal that helix four in the NLS of NF-kappaB p65 anchors IkappaBalpha, forming a very stable complex. *J Mol Biol* 2006;360:421–434.
35. O'Dea EL, Barken D, Peralta RQ, Tran KT, Werner SL, Kearns JD, Levchenko A, Hoffmann A. A homeostatic model of IkappaB metabolism to control constitutive NF-kappaB activity. *Mol Syst Biol* 2007;3:111.
36. Ito CY, Kazantsev AG, Baldwin AS Jr. Three NF-kappa B sites in the I kappa B-alpha promoter are required for induction of gene expression by TNF alpha. *Nucleic Acids Res* 1994;22:3787–3792.
37. Cosgrove MS, Boeke JD, Wolberger C. Regulated nucleosome mobility and the histone code. *Nat Struct Mol Biol* 2004;11:1037–1043.
38. Tapia H, Morano KA. HSP90 nuclear accumulation in quiescence is linked to chaperone function and spore development in yeast. *Mol Biol Cell* 2010;21:63–72.
39. Goodman RH, Smolik S. CBP/p300 in cell growth, transformation, and development. *Genes Dev* 2000;14:1553–1577.