Gallic Acid Suppresses Lipopolysaccharide-Induced Nuclear Factor-κB Signaling by Preventing RelA Acetylation in A549 Lung Cancer Cells

Kyung-Chul Choi,[1,2] Yoo-Hyun Lee,[4] Myung Gu Jung,[1,2] Seung Hyun Kwon,[3] Mi-Jeong Kim,[1,2] Woo Jin Jun,[5] Jeongmin Lee,[6] Jae Myun Lee,[2,3] and Ho-Geun Yoon[1,2]

[1]Department of Biochemistry and Molecular Biology, Institute of Genetic Science, Center for Chronic Metabolic Disease Research, [2]Brain Korea 21 Project for Medical Sciences, and [3]Department of Microbiology, Yonsei University College of Medicine, Seodaemun-gu, Seoul, South Korea; [4]Department of Food and Nutrition, The University of Suwon, Hwaseong, South Korea; [5]Department of Food and Nutrition, Chonnam National University, Gwangju, South Korea; and [6]Department of Medical Nutrition, Kyung Hee University, Suwon, South Korea

Abstract

Although multiple studies have revealed that gallic acid plays an important role in the inhibition of malignant transformation, cancer development, and inflammation, the molecular mechanism of gallic acid in inflammatory diseases is still unclear. In this study, we identified gallic acid from *Rosa rugosa* as a histone acetyltransferase (HAT) inhibitor with global specificity for the majority of HAT enzymes, but with no activity toward epigenetic enzymes including sirtuin (silent mating type information regulation 2 homologue) 1 (*S. cerevisiae*), histone deacetylase, and histone methyltransferase. Enzyme kinetic studies indicated that gallic acid uncompetitively inhibits p300/CBP-dependent HAT activities. We found that gallic acid inhibits p300-induced p65 acetylation, both *in vitro* and *in vivo*, increases the level of cytosolic IκBα, prevents lipopolysaccharide (LPS)-induced p65 translocation to the nucleus, and suppresses LPS-induced nuclear factor-κB activation in A549 lung cancer cells. We have also shown that gallic acid treatment inhibits the acetylation of p65 and the LPS-induced serum levels of interleukin-6 *in vivo*. Importantly, gallic acid generally inhibited inflammatory responses caused by other stimuli, including LPS, IFN-γ, and interleukin-1β, and further downregulated the expression of nuclear factor-κB–regulated antiapoptotic genes. These results show the crucial role of acetylation in the development of inflammatory diseases. (Mol Cancer Res 2009;7(12):2011–21)

Introduction

Gallic acid (3,4,5-trihydroxybenzoicacid) is a polyhydroxy-phenolic compound which is found in various natural products such as gallnuts, sumac, tea leaves, oak bark, green tea, apple peels, grapes, strawberries, pineapples, bananas, lemons, and in red and white wine (1). Gallic acid is cytotoxic to cancer cells and has anti-inflammatory and antimutagenic properties (2, 3). Gallic acid has been described as an excellent free radical scavenger and as an inducer of differentiation and programmed cell death in a number of tumor cell lines (4, 5). Recently, it was revealed that gallic acid from rose flowers exhibits antioxidative effects even in senescence-accelerated mice and can restore the activities of catalase and gluthatione peroxidase (6). Even though many reports have revealed that gallic acid plays an important role in the prevention of inflammation and cancer development, it is still unclear how gallic acid inhibits cancer-related inflammation.

An imbalance of protein acetylation and deacetylation in cellular signaling can lead to human diseases, including inflammation and cancer. Currently, the functions of more than 70 proteins are known to be regulated through acetylation of their lysine residues (7). Acetylation of specific lysine residues within the amino terminal tails of histones is generally linked to chromatin disruption and the transcriptional activation of genes. Depending on the functional domain that is modified, acetylation can also regulate the functions of non-histone proteins, such as DNA-binding affinity, protein stability, protein-protein interaction, and subcellular localization (8, 9). The RelA (p65) subunit of nuclear factor-κB (NF-κB) is also known to be activated in an acetylation-dependent manner in response to cytokine stimulation (10, 11). Reversible acetylation of RelA thus functions as a molecular switch that both controls the duration of the NF-κB transcriptional response and replenishes the cytoplasmic pool of latent NF-κB/IκBα complexes, thereby readying the cell for the next NF-κB–inducing signal (11). NF-κB is a ubiquitously expressed family

of transcription factors controlling the expression of numerous genes involved in inflammatory and immune responses and cellular proliferation (12, 13). Given the ubiquitous expression of NF-κB and the important number of inducing stimuli and target genes, it is not surprising that NF-κB is involved in numerous and diverse diseases (14-16). Therefore, NF-κB may be a therapeutic target for pharmaceutics that interfere with the establishment and the progression of these pathologic states (17).

Among the small molecules that are capable of modulating epigenetic status, histone deacetylase (HDAC) inhibitors have been extensively studied and several are currently in clinical trials (18). One example is vorinostat, which has been approved by the Food and Drug Administration for the treatment of advanced solid tumors or lymphomas (19). In addition, DNA methyltransferase inhibitors, azacitidine and decitabine, have been developed for the treatment of leukemia. On the other hand, there is little information available on histone acetyltransferase (HAT) inhibitors (HATi). As suggested in recent studies, the development of HATi from dietary compounds is the next therapeutic goal. Recent investigations have suggested that several dietary factors, such as garcinol, curcumin, and anacardic acid, have the ability to inhibit HAT activity (20-22). Garcinol has been found to inhibit p300 and PCAF, *in vitro* and *in vivo*, anacardic acid to inhibit TIP60 as well as p300 and PCAF, and curcumin to inhibit p300 and PCAF (20). These dietary compounds are associated with the prevention of cancer and other diseases. Importantly, anacardic acid has been recently shown to suppress the expression of NF-κB–regulated gene products by inhibiting IκBα kinase and acetylating p65 (22). That study showed that downregulation of the *p300 HAT* gene by RNA interference inhibits the effect of anacardic acid on NF-κB suppression, illustrating a critical role of this enzyme.

In this study, we report that gallic acid from *Rosa rugosa* possesses potent anti-HAT activity and has global specificity for the majority of HAT enzymes. Our studies show that gallic acid generally induces hypoacetylation of p65 by directly inhibiting the activity of HAT enzymes. This hypoacetylation of p65 leads to the downregulation of NF-κB activation in response to diverse inflammatory signals. In addition, gallic acid also blocks the acetylation of p65 and lipopolysaccharide (LPS) and cytokine-induced inflammatory responses *in vivo*. In summation, this study shows that selective modulation of NF-κB acetylation by HATi is a potential mechanism for a new class of anti-inflammatory and chemotherapeutic drugs.

Results

Gallic Acid from R. rugosa Is a Specific Inhibitor of Histone Acetyltransferases

During our ongoing screen for natural compounds with anti–histone acetyltransferase (HAT) activity, we previously found that *R. rugosa* has potent anti-HAT activity against histone acetyltransferase p300/CBP (23). Through mass spectrometry and nuclear magnetic resonance spectroscopy, the component responsible for the anti-HAT activity was identified as gallic acid. As shown in Fig. 1A, gallic acid inhibited 90% of HAT activity in a dose-dependent manner. Because several small molecules have been known to possess anti-HAT activity

(20-22), we next compared the relative anti-HAT activity of gallic acid with other HATi. As shown Fig. 1B, gallic acid exhibited the highest anti-HAT activity among the reported HATi, although the anti-HAT activity of curcumin was similar to that of epigallocatechin gallate (Supplementary Fig. S1A). Next, we examined the enzyme specificity of gallic acid. The HAT activities of immunoprecipitated p300, CBP, PCAF, and Tip60 were measured in the presence and absence of gallic acid. Gallic acid was found to be a highly efficient inhibitor of p300 and CBP acetyltransferase activities with an IC_{50} of ~14 and ~24 μmol/L, respectively (Fig. 1B). Under similar conditions, gallic acid also inhibited Tip60 and PCAF histone acetyltransferase activities with an IC_{50} of ~25 and ~34 μmol/L, respectively, although to a lesser extent, suggesting that gallic acid generally inhibited most HAT enzyme activities (Fig. 1B).

To examine enzyme specificity, we first sought to assess the effect of gallic acid on HDAC activities. When HeLa nuclear extracts were used as a source of HDAC enzymes, deacetylase activity was not affected by the presence of gallic acid, but TSA efficiently blocked nuclear HDAC activity (Fig. 1C, *left*). This result suggests that gallic acid does not have any specificity for HDACs. We next investigated the effect of gallic on histone methyltransferase (HMT) activity. HeLa core histones were methylated with [^{3}H] (*S*)-adenosyl methionine by HeLa nuclear extracts. As shown in Fig. 1C (*right*), histone methylation remained the same in the presence or absence of gallic acid. We further investigated gallic acid's inhibition of p300/CBP histone acetyltransferase activities by studying the kinetics of p300 HAT activity in the presence of gallic acid in reactions with changing histone concentrations. We found that K_m and V_{max} were decreased with gallic acid, demonstrating that gallic acid does not bind to the active sites of histone, but to some other site on the enzyme (Fig. 1D). These results establish that gallic acid generally and uncompetitively inhibits histone acetyltransferase activities, but not other epigenetic enzymes.

Gallic Acid Inhibits p300-Mediated RelA Acetylation

Based on recent studies showing that gallic acid inhibits inflammatory cytokine production in a broad range of human cancer cells (24, 25), we decided to investigate the molecular basis for gallic acid inhibition of NF-κB–mediated inflammatory cytokine production. As p300 acetyltransferase has been shown to play a major role in the acetylation of p65, we first examined whether gallic acid directly inhibits p300-mediated p65 acetylation *in vitro*. *In vitro* acetylation assays were done in the presence or absence of gallic acid with recombinant GST-p65 as a substrate. As shown in Fig. 2A, the acetylation of GST-p65 was detected in the presence of active GST-p300, but not in the presence of GST. Upon gallic acid treatment, the p300-induced acetylation was inhibited, confirming that gallic acid suppressed the hyperacetylation of p65 *in vitro*. To confirm the prevention of p300-mediated p65 acetylation by gallic acid, we next assessed the effect of gallic acid treatment on LPS-induced acetylation of p65 in A549 lung cancer cells. LPS-treated A549 cells were cultured either in the presence or absence of gallic acid. The cells were collected, and subsequent Western blot analysis was done using an antibody against acetylated p65 (lysine 310). As shown in Fig. 2B, gallic acid treatment reversed the LPS-enhanced acetylation of p65 in A549

cells. In support of this result, gallic acid increased the level of cytosolic IκBα, even in the presence of LPS. We conclude that gallic acid prevents the hyperacetylation of p65 by inhibiting the HAT activity of p300/CBP.

p300-Mediated RelA Acetylation Is Critical for NF-κB Function

Because p300-mediated RelA acetylation is critical for the maintenance of NF-κB function (12, 26), we first examined the effect of gallic acid on the LPS-induced DNA binding activity of NF-κB by performing electrophoretic mobility shift assays (EMSA). As shown in Fig. 3A, LPS treatment enhanced the DNA binding of p65 in A549 cells. However, gallic acid treatment attenuated the LPS-enhanced p65 DNA-binding activity, suggesting that the level of acetylation determines the DNA binding activity of NF-κB. To confirm this result, we carried out chromatin immunoprecipitation (ChIP) assays over the promoter region of the interleukin (IL)-6 gene. The IL-6 gene promoter contains a well-characterized NF-κB–binding site located at −105 to −130 relative to the transcriptional start site. A representative result is presented in Fig. 3B and shows that LPS treatment increased the recruitment of both p65 and acetylated p65 to the promoter of the IL-6 gene, but not the IgG control. The enhanced recruitment of acetylated p65 was abolished by treatment with gallic acid, which is in agreement with the results of the EMSA. In addition, we tested the effect of gallic acid–mediated hypoacetylation on the recruitment of HDAC3 or p300 to the promoter region of the IL-6 gene. As shown in Fig. 3B, LPS treatment led to a significant increase in binding of p300 to the NF-κB binding site of the IL-6 gene. However, addition of gallic acid resulted in the enhanced recruitment of HDAC3 to the NF-κB binding site of the IL-6 gene. Conversely, the recruitment of p300 was abolished by treatment with gallic acid, illustrating the importance of balance between HATs and HDACs to the NF-κB–mediated inflammatory signal.

Because the dissociation of IκBα and the nuclear translocation of NF-κB are critical for NF-κB activation (10), we examined whether gallic acid inhibits LPS-induced NF-κB activation by increasing the association of IκBα with deacetylated p65. As shown in Fig. 3C, gallic acid treatment restored the association of IκBα with p65, which was inhibited by LPS treatment, indicating that deacetylation events lead to a cytoplasmic accumulation of IκBα and subsequent cytosolic sequestration of NF-κB. We next examined whether hypoacetylation by gallic acid inhibits the nuclear translocation of p65 using immunohistochemistry analysis. As shown in Fig. 3D, gallic acid reversed the LPS-induced nuclear translocation of p65 and enhanced the nuclear export of hypoacetylated p65. In addition, leptomycin B treatment inhibited nuclear export of hypoacetylated p65, indicating that the level of acetylation is critical for p65 translocation. Collectively, these results establish that hypoacetylation of p65 by gallic acid decreases NF-κB nuclear function by enhancing the nuclear export of p65, increasing the subsequent association with the inhibitory protein IκBα, and impairing DNA-binding activity.

Gallic Acid Suppresses the LPS-Induced Inflammatory Response, Both In vitro and In vivo

To examine whether gallic acid inhibits LPS-induced NF-κB activation and the inflammatory response, we first measured NF-κB–dependent promoter activity using the luciferase reporter plasmid in A549 cells. To do this, A549 cells were incubated with or without gallic acid, and LPS was subsequently added. As shown in Fig. 4A, gallic acid inhibited LPS-induced NF-κB activation in a dose-dependent manner. Because of the important role for NF-κB in the regulation of a variety of key inflammatory mediators, we next determined whether gallic acid inhibits LPS-induced transcription of NF-κB–regulated genes by real-time PCR analysis. Total RNA was extracted 18 hours after LPS and/or gallic acid treatment. As shown in Fig. 4B, gallic acid treatment greatly reduced LPS-induced expression of IL-6, cyclooxygenase (COX)-2, IL-1β, and NOS2 in A549 cells. Next, we confirmed that gallic acid inhibits the expression of NF-κB–regulated genes in primary peritoneal macrophages. Primary peritoneal macrophages were isolated from 7- to 8-week-old female BALB/c mice injected with thioglycolate. The isolated primary peritoneal macrophages were cultured with or without gallic acid in the presence of LPS. As shown in Fig. 4C, gallic acid strongly inhibited the expression of IL-6, COX-2, IL-1β, and NOS2 stimulated by LPS in the primary mouse macrophages. Because several studies have shown that cytokine treatment results in an enhanced release of IL-6 from epithelial cell cultures, we next assessed the effect of gallic acid on the release of inflammatory markers from A549 cell cultures. The amount of released IL-6 was measured using ELISA kits. Gallic acid treatment of cells resulted in a significant inhibition of the LPS-induced increase in the expression of IL-6, implicating gallic acid as a potent anti-inflammatory molecule (Fig. 4D).

Next, we investigated whether gallic acid inhibits the production of LPS-induced inflammatory molecules in vivo. We pretreated 6-week-old male mice with 40 mg of gallic acid/kg body weight by oral administration for 4 weeks, and then we i.p. injected 0.25 mg of LPS/kg body weight. After 2 hours, serum IL-6 levels were measured. As shown in Fig. 5A, gallic acid dramatically decreased the LPS-induced serum levels of IL-6 compared with levels in the control group. Consistent with this, we also observed reduced levels of p65 acetylation in primary peritoneal macrophages following gallic acid treatment (Fig. 5B). We next examined the effect of gallic acid on the serum levels of multiple cytokines induced by LPS treatment. As shown in Fig. 5C, LPS treatment dramatically increased the serum levels of proinflammatory cytokines and chemokines, such as BLC, I-309, IL-6, IL-7, IP-10, KC, G-CSF, MCP-5, MIP-2, and TREM-1 expressed on myeloid cells, as compared with controls. Conversely, gallic acid treatment efficiently reversed the elevated cytokine and chemokine expression profile in the LPS-treated mice, but not those treated with G-CSF and KC (Supplementary Fig. S2). Taken together, these data show that gallic acid inhibits the LPS-induced p65-dependent inflammatory response, both in vitro and in vivo.

Gallic Acid Suppresses the Expression of NF-κB–Regulated Antiapoptotic and Cell Survival Genes

Because multiple stimuli could induce inflammatory responses, we next examined whether gallic acid generally inhibits
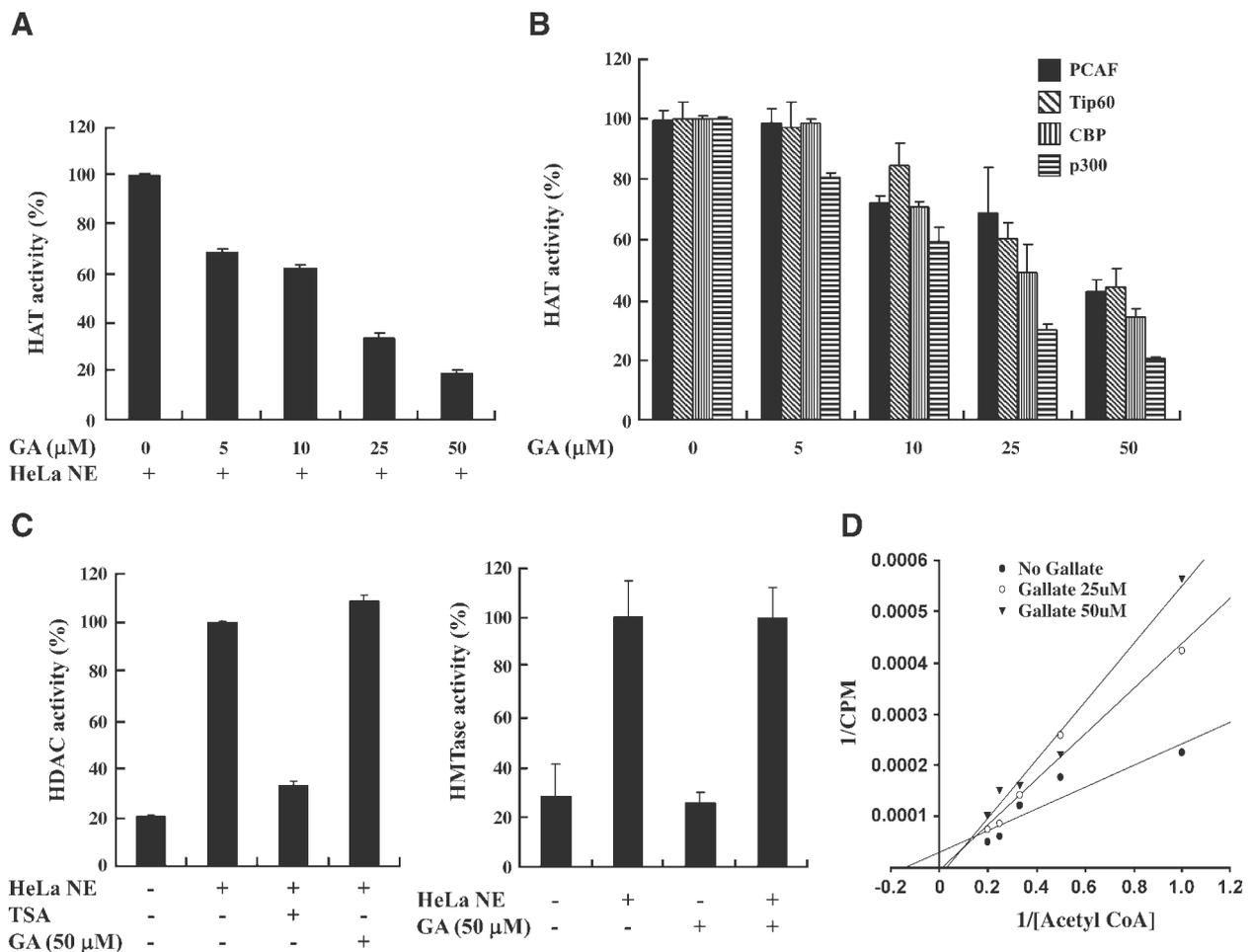
FIGURE 1. Gallic acid from *R. rugosa* is a specific inhibitor of HATs. **A.** Gallic acid has potent anti-HAT activity. HAT activity was assayed with a HAT activity colorimetric assay kit and HeLa cell nuclear extract as a source of HAT enzymes. Columns, mean of three independent experiments; bars, SD. **B.** Gallic acid generally inhibited most HAT enzyme activities. Gallic acid inhibits HAT activity in a dose-dependent manner. HAT activity colorimetric assays were done with the indicated concentrations of gallic acid. **C.** Gallic acid treatment had no effect on both HDAC and HMT activity. HDAC activity was assayed with an HDAC activity colorimetric assay kit. TSA was used as a control inhibitor for HDAC activity. HMT assays were done in 30 μL reactions in the presence or absence of gallic acid using HeLa cell nuclear extract as the enzyme source and then processed using a filter binding assay. Columns, mean of three independent experiments; bars, SD. **D.** Gallic acid uncompetitively inhibits the p300/CBP HAT activity. The Lineweaver-Burk plot shows the effect of gallic acid on p300-mediated acetylation of core histones. HAT assays were carried out with a fixed concentration of histones (8 pmol/L) and an increasing concentration of [³H]acetyl CoA in the presence (25 and 50 μmol/L) or absence of gallic acid (*left*). The results were plotted using Sigma Plot Ver. 10.0 (SYSTAT Software).

inflammatory responses by other stimuli including LPS, IFN-γ, and IL-1β. We first tested the effect of gallic acid on the acetylation of p65 induced by various stimuli. As shown in Fig. 6A, gallic acid efficiently blocked the acetylation of p65 stimulated by LPS, IFN-γ, and IL-1β. Consistently, we also observed that gallic acid treatment led to the cytoplasmic accumulation of IκBα, suggesting that gallic acid generally inhibits inflammatory responses.

Because the antiproliferative or antigrowth activities of gallic acid against lung cancer cell lines have been reported (27, 28), we next wished to decipher the molecular basis of gallic acid's inhibition of the growth and proliferation of cancer cells. We first asked whether the antiproliferative effect of gallic acid was mediated through the downregulation of cell survival or proliferative gene products mainly regulated by NF-κB. For the assessment of cell survival gene products, LPS-treated A549 cells were cultured either in the presence or absence of gallic acid. The cells were collected, and subsequent Western blot analysis was done using antibodies against XIAP, Bcl-xL, and Bcl-2. As shown in Fig. 6B, gallic acid reversed the expression of LPS-induced antiapoptotic proteins. We also assessed the expression of the NF-κB–regulated genes cyclin D1, c-Myc, and COX-2, which are involved in tumor cell proliferation. Western blot analysis revealed that gallic acid significantly decreased the expression of these proteins compared with the control. We conclude that the antiproliferative activity of gallic acid against lung cancer cells may be due to the downregulation of antiapoptotic gene products.

Discussion

Although epigenetic modulators including HDACi and DNMTi have been extensively studied, and several are currently in clinical trials (18), there is little information available on

inhibitors of HATs. There are several HATi now available, which may be useful for clinical development. These HATi compounds may be associated with the prevention of several diseases, such as cancer, diabetes, and neurologic disorders (20-22). Gallic acid is already known to have an antiproliferative and antitumor activity in lung cancer (25, 29), prostate cancer, and leukemia (24, 30). However, the molecular basis for gallic acid's inhibition of the growth and proliferation of cancer cells is still unclear. Recently, we reported the potent anti-HAT activity of *R. rugosa* methanol extract containing polyphenol compounds. In this study, we identified gallic acid as being responsible for the anti-HAT activity of these extracts by employing structural analysis. Gallic acid showed global specificity for the majority of HAT enzymes, but not other epigenetic enzymes, including HDAC, SIRT, and HMT, illustrating that gallic acid is a specific HATi.

The acetylation of p65 is critical to NF-κB–dependent activation (10). Although gallic acid has been shown to suppress the inflammatory response, there was no evidence that gallic acid suppresses p300/CBP-mediated p65 acetylation and subsequent NF-κB activation. In the present study, *in vitro* and *in vivo* acetylation assays with p65 as a substrate clearly showed that gallic acid inhibits p300-induced p65 acetylation. In addition, gallic acid increased the level of cytosolic IκBα, even in the presence of LPS. Thus, our study provides firm evidence to support the idea that gallic acid suppresses p300/CBP-mediated p65 acetylation. The acetylation of p65 is known to be enhanced by prior phosphorylation of p65 at serine 276 or serine 536 (26). By immunoprecipitation and Western blot analysis, we observed that gallic acid treatment leads to the inhibition of p65 phosphorylation (data not shown). This result is somewhat surprising because gallic acid does not
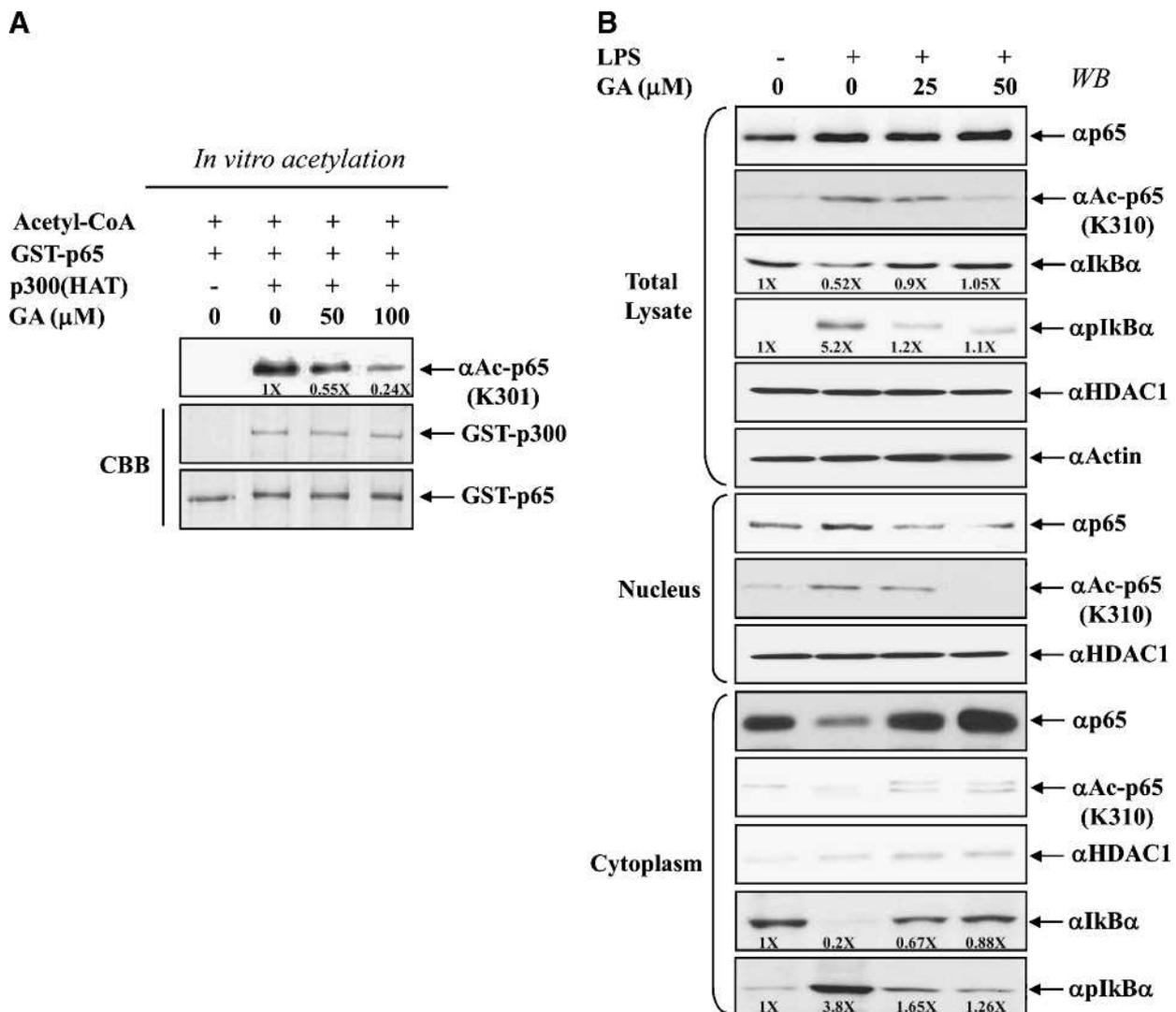

FIGURE 2. Gallic acid inhibits p300-mediated RelA acetylation. **A.** Gallic acid prevents p300-mediated p65 acetylation *in vitro*. Recombinant GST-p300 was incubated with both GST-p65 and acetyl-CoA in the presence or absence of gallic acid, and subsequently processed for Western blot analysis using antibody against acetylated lysine (310) p65. CBB, Coomasie blue staining. The data for quantification was derived using the Quantity One image program (Bio-Rad). **B.** Gallic acid treatment suppressed LPS-induced p65 acetylation *in vivo*. A549 cells were treated with LPS and/or the indicated amounts of gallic acid. The level of p65 acetylation was assayed by Western blot analysis using antibody against acetylated lysine (310) p65. The data for quantification was derived using the Quantity One image program (Bio-Rad).
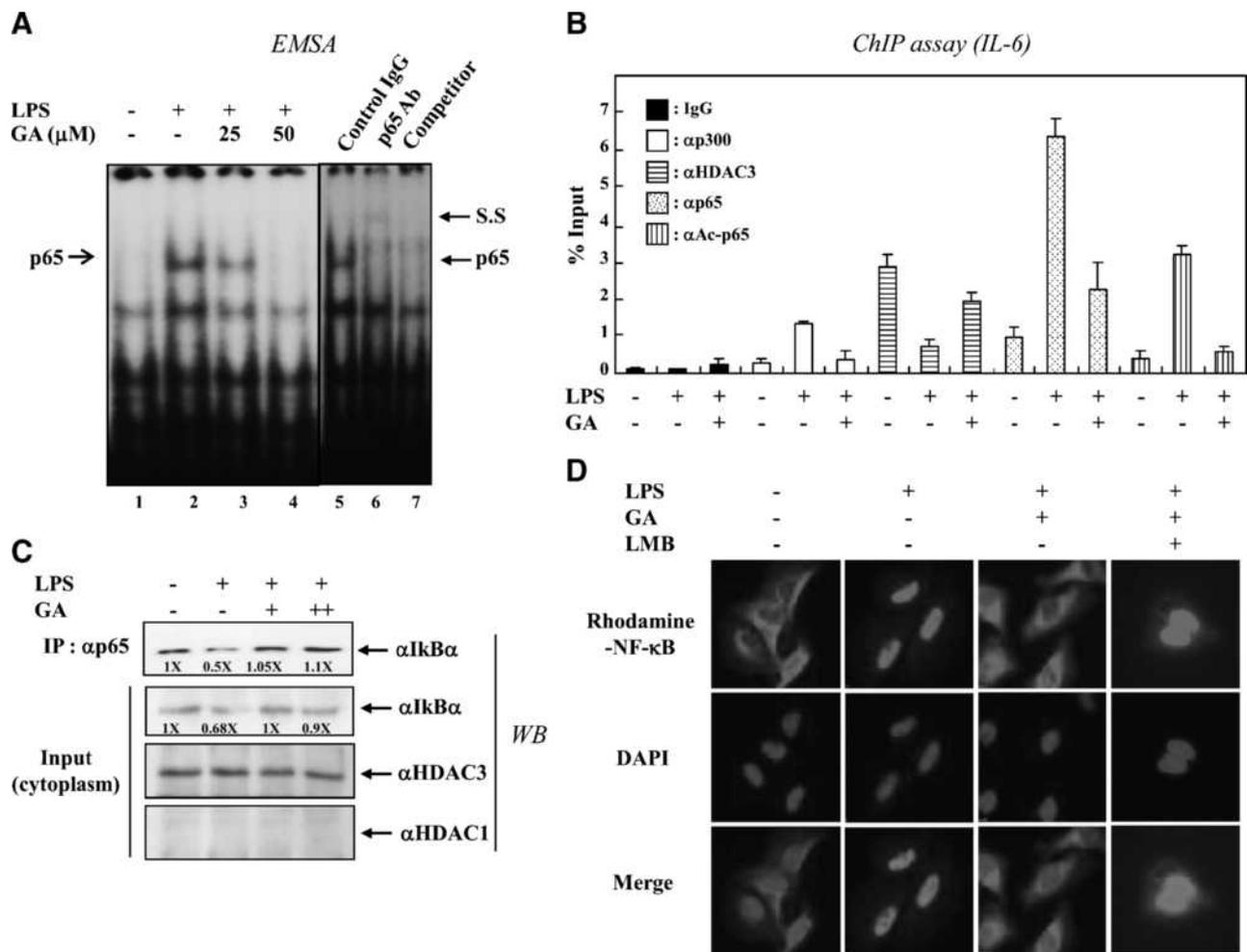
FIGURE 3. Gallic acid represses NF-κB function. **A.** An electromobility assay was done in the presence of LPS and/or gallic acid, as indicated. Gallic acid treatment resulted in a dose-dependent reduction in DNA binding of NF-κB in cells treated with LPS. **B.** Gallic acid–induced p65 hypoacetylation resulted in the association of HDA3 and the dissociation of p300 at the IL-6 promoter region. A549 cells were treated with LPS and/or gallic acid for 1 h. ChIP assays were then carried out to determine the binding of HDAC3, p300, p65, and acetylated p65. **C.** Gallic acid treatment led to a cytoplasmic accumulation of IκBα. A549 cells were treated with LPS and/or gallic acid. Cytoplasmic extracts were prepared and an immunoprecipitation assay was processed for Western blot analysis. **D.** Gallic acid induced the hypoacetylation of p65 and enhanced the nuclear export of p65, as assessed by immunostaining.

inhibit the multiple kinases responsible for phosphorylation of p65, including protein kinase A, glycogen synthase kinase-3β, casein kinase 2, and IkB kinase (Supplementary Fig. S1B). A recent study showed that HDAC3 specifically interacts with both the signal transducer and activator of transcription 3 (STAT3) and protein phosphatase PP2A and enhances the complex formation between STAT3 and PP2A to dephosphorylate STAT3 (31). Because HDAC3 also interacts with p65 and negatively regulates the inflammatory signaling, one likely explanation is that the hypoacetylated form of phosphorylated p65 by either overexpression of HDAC3 or HATi treatment could be dephosphorylated by protein phosphatases similar to STAT3.

Previous studies have shown that site-specific acetylation of p65 regulates discrete biological action of the NF-κB complex, including transcriptional activation, DNA binding activity (11), and IκBα assembly. Indeed, mutation of all of these lysine residues leads to the loss of NF-κB function. By EMSA, gallic acid treatment was shown to inhibit the DNA binding of NF-κB in cells treated with LPS. As a consequence, gallic acid–induced

hypoacetylation of p65 also suppressed LPS-induced NF-κB activation by decreasing the dissociation of IκBα from p65 and causing a delay in the cytoplasmic reappearance of IκBα. We also found that gallic acid suppresses LPS-induced p65 translocation to the nucleus, confirming that hyperacetylation is critical for NF-κB translocation. Similar to the case of EGCG, a recently identified HATi, the hypoacetylation of p65 and histone by gallic acid resulted in an exchange between HDAC3 and p300 in the promoter region. ChIP analysis showed that the addition of gallic acid resulted in the association of HDAC3 and the dissociation of p300 to the NF-κB binding site of the IL-6 gene, supporting the importance of balance between HATs and HDACs in NF-κB–mediated inflammatory signaling. The importance of balance between acetylation and deacetylation has been emphasized in the functional control of several proteins. The acetylation of β-catenin is a key posttranslational modification, enhancing the affinity of β-catenin for Tcf4 and the coactivator function of β-catenin (32, 33). Like p65, β-catenin is acetylated by p300, and acetylation of β-catenin regulates the recruitment of

coregulators to the basal transcription machinery at Wnt target genes. Thus, our study uncovers a feasible mechanism in which HAT coactivators compete with HDAC corepressors for binding to promoter regions and/or protein substrates, and consequently modulate the level of NF-κB–dependent transcription.

Chronic low-grade inflammation is linked to carcinogenesis in several organ systems (34). Our findings from this study provide some insight into the gallic acid–mediated host defense system against environmental pathogens. First, gallic acid treatment inhibited LPS-enhanced acetylation of p65 and NF-κB activation in human non–small cell lung cancer cells. In addition, the gallic acid–mediated hypoacetylation of p65 resulted in a reduction in the protein levels of NF-κB target genes and inflammatory markers in cultured lung cancer cells. Second, in *in vivo* experiments using BALB/c mice, gallic acid was shown to inhibit LPS-induced NF-κB activation and the serum levels of multiple proinflammatory cytokines and chemokines, in a dose-dependent manner. Importantly, the *in vivo* levels of p65 acetylation were greatly suppressed by gallic acid treatment, providing firm evidence for the relevance of p65 acetylation

in cytokine-induced inflammatory signaling. Thus, we confirmed that gallic acid–mediated hypoacetylation of p65 leads to the suppression of inflammatory responses. By examining the selective effects of gallic acid–mediated hypoacetylation on the inflammatory responses caused by various stimuli, we observed that gallic acid efficiently blocks p65 acetylation and the release of inflammatory markers by LPS, TGF-β, and IL-6. Importantly, another HATi, anacardic acid, has been shown to suppress the expression of NF-κB–regulated genes related to antiapoptotic signaling by inhibiting p300-dependent p65 acetylation, providing new insight into the critical role of HATi in cancer cell survival.

In sum, we have provided evidence that gallic acid, a novel anti-HATi, inhibits the p65 acetylation-dependent NF-κB activation and production of inflammatory markers. Gallic acid–mediated hypoacetylation of p65 led to a complete loss of NF-κB function, indicating that the acetylation of p65 is also necessary for NF-κB–mediated inflammatory signaling. We have also provided a feasible mechanism by which the relative occupancy of HDAC versus HAT in the promoter region of NF-κB–regulated
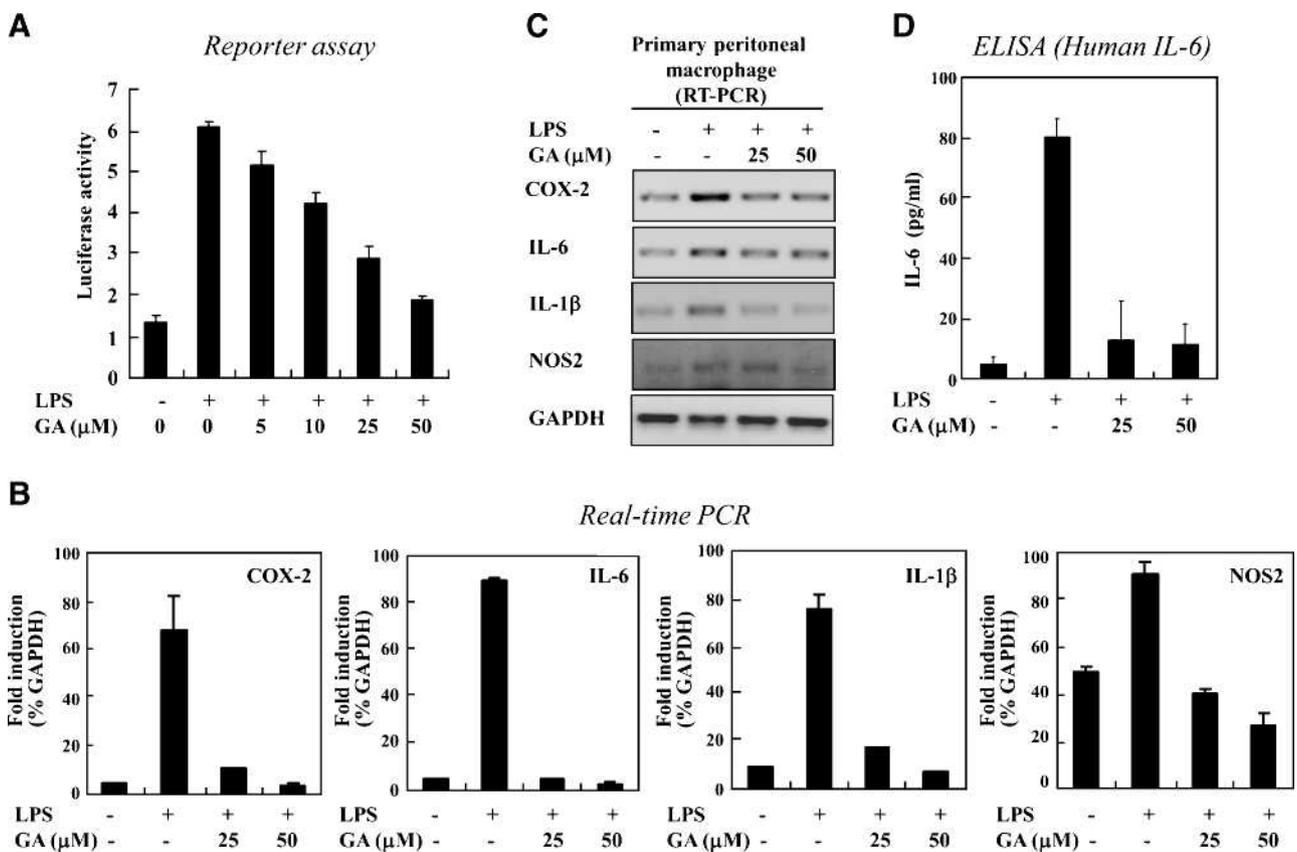

FIGURE 4. Gallic acid suppresses the LPS-induced NF-κB–dependent inflammatory response. **A.** Gallic acid inhibited LPS-induced NF-κB–dependent promoter activity in A549 cells in a dose-dependent manner. A549 cells were transfected with a NF-κB binding site–driven luciferase reporter plasmid and was treated with LPS and/or various concentrations of gallic acid. Whole cell extracts were used in luciferase assays. Columns, means of two independent experiments done in triplicate. **B.** Gallic acid reduced mRNA levels of NF-κB target genes in A549 cells. Total RNA was prepared from each sample and used for quantitative RT-PCR to measure the expression of the NF-κB target genes. As controls, the level of GAPDH mRNA was also measured by quantitative RT-PCR using the same batch of RNA samples. **C.** Gallic acid reduced mRNA levels of NF-κB target genes in primary mouse macrophages. Peritoneal cavities from BALB/c female mice were lavaged 3 d after injection of 3% thioglycolate. Isolated primary peritoneal cells were treated with gallic acid and cultured for 2 h with LPS (20 ng/mL) treatment, and then cells were harvested. The protein levels were assayed by Western blot analysis using antibodies, as indicated. **D.** Gallic acid treatment of cells resulted in a significant inhibition of the LPS-induced IL-6 production. The release of IL-6 into the A549 cell culture medium was analyzed with a human IL-6 ELISA kit.
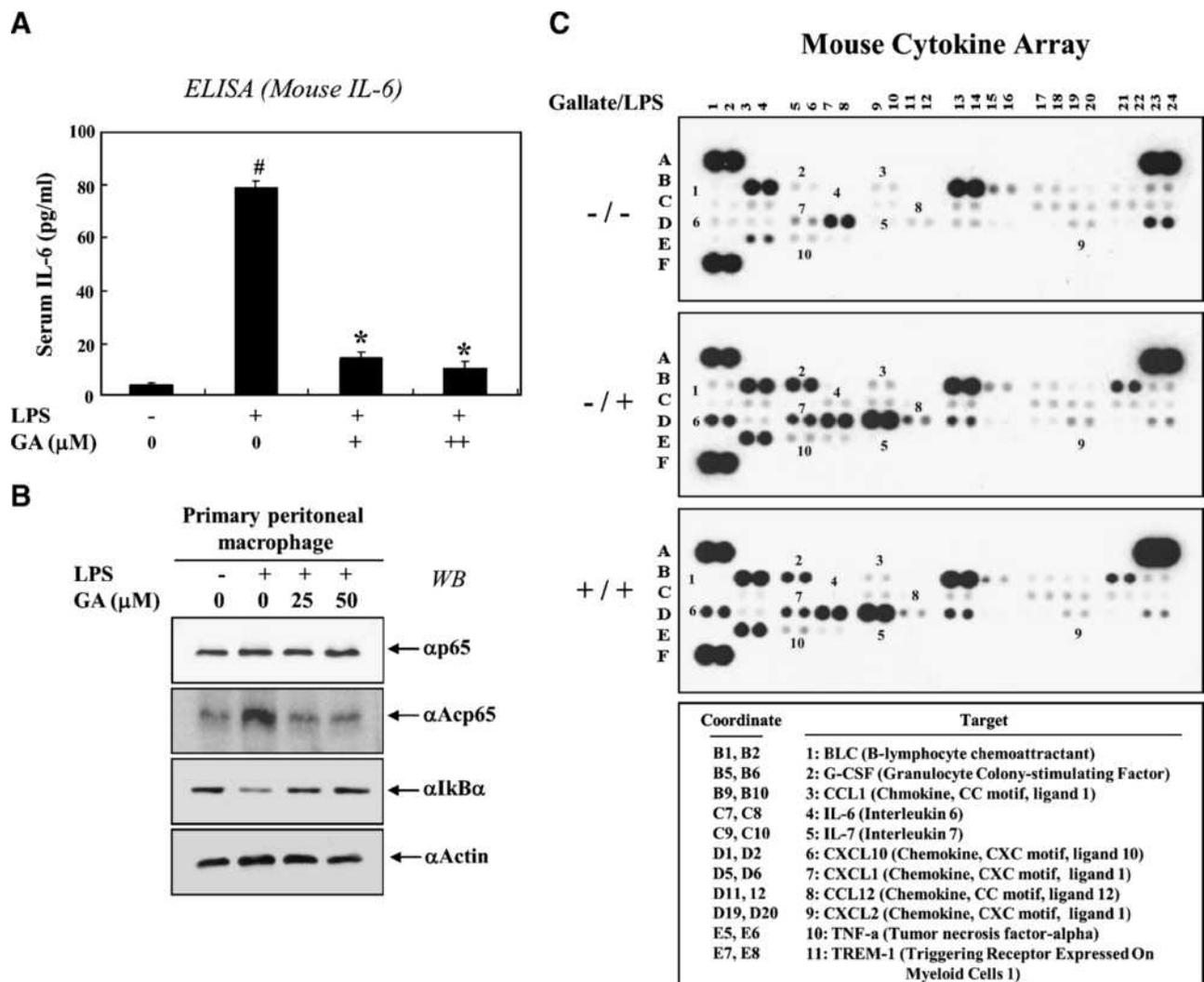
FIGURE 5. Gallic acid inhibits LPS-mediated inflammatory signals *in vivo*. **A.** Gallic acid inhibits the production of LPS-induced inflammatory molecules *in vivo*. Male BALB/c mice were pretreated with the indicated doses of gallic acid by oral administration for 4 wk, and then all were treated with LPS, as indicated. After 2 h, mice were killed, and serum was collected for serum IL-6 levels. A one-way ANOVA test was used to determine the effects of the treatment. Columns, mean; bars, SD ($n = 8$; *, $P < 0.05$ vs. control; #, $P < 0.05$ vs. LPS-treated). **B.** Gallic acid efficiently suppressed the *in vivo* acetylation of p65. Primary peritoneal cells from gallic acid–treated or untreated mice were washed in PBS and were cultured for 2 d. The level of p65 acetylation was assessed by Western blot analysis with antibody against acetyl-p65. As a control, cytosolic accumulation of IκBα was assessed by Western blot analysis. **C.** Gallic acid suppresses cytokine production *in vivo*. Mouse cytokine array analysis was done using the Mouse Cytokine Antibody Array 3.0, and membranes were incubated with serum from mice untreated or treated with gallic acid. Images were visualized using a LAS 3000 image analyzer (Fuji).

genes determines the overall cytokine-mediated transcription, suggesting that reversible acetylation of RelA serves as an important intranuclear regulatory mechanism. Taken together, the results of this study suggest that selective interference with p65 acetylation by small molecules, such as gallic acid, will lead to a new class of anti-inflammatory or immune suppression drugs.

Materials and Methods

Cell Culture and Reagents

All cell lines were obtained from the American Type Culture Collection. A549 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone), 1% antibiotics, and antimycotics. Cells were treated with various amounts of gallic acid (Sigma) and were stimulated with LPS. All cell lines were grown at 37°C in 5% CO_2. DMEM, antibiotics, and antimycotics were purchased from Hyclone. The HAT activity colorimetric assay kit and HDAC activity colorimetric assay kit were from BioVision Biotechnology. An Easyspin total RNA extraction kit and a Maxime RT premix kit were from Intron, Korea. The antibodies were purchased from Santa Cruz Biotechnology, Upstate Biotechnology, and Cell Signaling Technology. Protein A/G PLUS agarose beads were purchased from Santa Cruz Biotechnology. The Effectene transfection reagent was purchased from Qiagen.

Mouse Experiments

We maintained male BALB/c mice (6 wk old) ~20 to 25 g in body weight in accordance with the guidelines and under the

approval of the Animal Care Committee of Yonsei University, Korea. Mouse peritoneal macrophages were isolated according to a previously described method (30). Peritoneal cells were washed in PBS and were cultured at 37°C under 5% CO_2 in DMEM supplemented with L-glutamine, antibiotics, and 10% fetal bovine serum. After incubation for 2 d, cells treated with gallic acid were cultured for 2 h with LPS (20 ng/mL) treatment and then harvested.

HAT, HDAC, and HMT Activity Assay

HeLa cell nuclear extracts were prepared as previously described (35). HAT activity and HDAC activity assays were done using nuclear extracts following the protocols of the manufacturer (BioVision Biotechnology). For HAT activity assays, immunoprecipitations were done using anti-p300, anti-Tip60, anti-CBP, and anti-pCAF (Santa Cruz Biotechnology) with HeLa nuclear extracts. Precleared nuclear extract was incubated with antibodies overnight with Protein A/G PLUS agarose beads at 4°C. Immunoprecipitations were collected and washed with HAT assay buffer [50 mmol/L Tris (pH 8.0), 10% glycerol, 0.1 mmol/L EDTA]. For recombinant p300 HAT activity assays, the p300 active domain (amino acids 1066-1707) was cloned and purified as a GST fusion protein. HAT activity assays were done using purified p300 following the protocols of the manufacturer (BioVision Biotechnology). All samples were counted with a multipurpose scintillation counter, LS 6500 (Beckman). For Fig. 1B, immunoprecipitation samples and purified GST-p300 were incubated with HAT assay buffer [50 mmol/L HEPES (pH 8.0), 10% glycerol, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L sodium butyrate, 1 µL [³H]acetyl-CoA], 5 µg of H4-peptides, and 5 µg each of gallic acid at 30°C for 1 h. Samples were separated on a 15% SDS-PAGE gel and analyzed by autoradiography. For HMT activity assays, assay buffer and core histones were from the HMT Assay Reagent kit (Upstate Biotechnology). HMT activity assays were done following the protocols of the manufacturer. Core histones were incubated in methyltransferase buffer [final concentration, 50 mmol/L Tris (pH 8.0), 1 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L DTT], 1 µL (0.55 µCi) of S-adenosyl-L-[methyl-³H]methionine (³H-AdoMet; Amersham Pharmacia Biotech), and 1 µg/µL of SET7/9 methyltransferase (ATgen, Korea) for 1 h at 30°C in a total volume of 20 µL. The reaction was spotted on P-81 paper for scintillation counting. P-81 paper was washed thrice with 10% trichloroacetic acid for 15 min (P-81; Upstate Biotechnology). P-81 papers were then washed with 95% ethanol for 5 min at room temperature and allowed to dry. Dry P-81 papers were counted with a multipurpose LS 6500 scintillation counter (Beckman).

ELISA and Mouse Cytokine Array

HEK 293 cells and mouse primary peritoneal macrophages treated with gallic acid were cultured for 2 h with LPS (20 ng/mL), and then cell culture supernatants were collected. IL-6 was measured with a human (or mouse) IL-6 TiterZyme Enzyme Immunometric Assay Kit (Assay Designs) according to the instructions of the manufacturer. Values were calculated on the basis of a standard curve constructed for each assay. For the analysis of mouse cytokine expression profiles, 6-wk-old male mice were pretreated with 50 mg of gallic acid/kg body weight by oral administration for 4 wk, and then i.p. injected with 0.5 mg of LPS/kg body weight. At 2 h after injection, the mice were sacrificed and blood was collected via cardiac puncture. TransSignal Mouse Cytokine Antibody Array membranes (Panomics) were
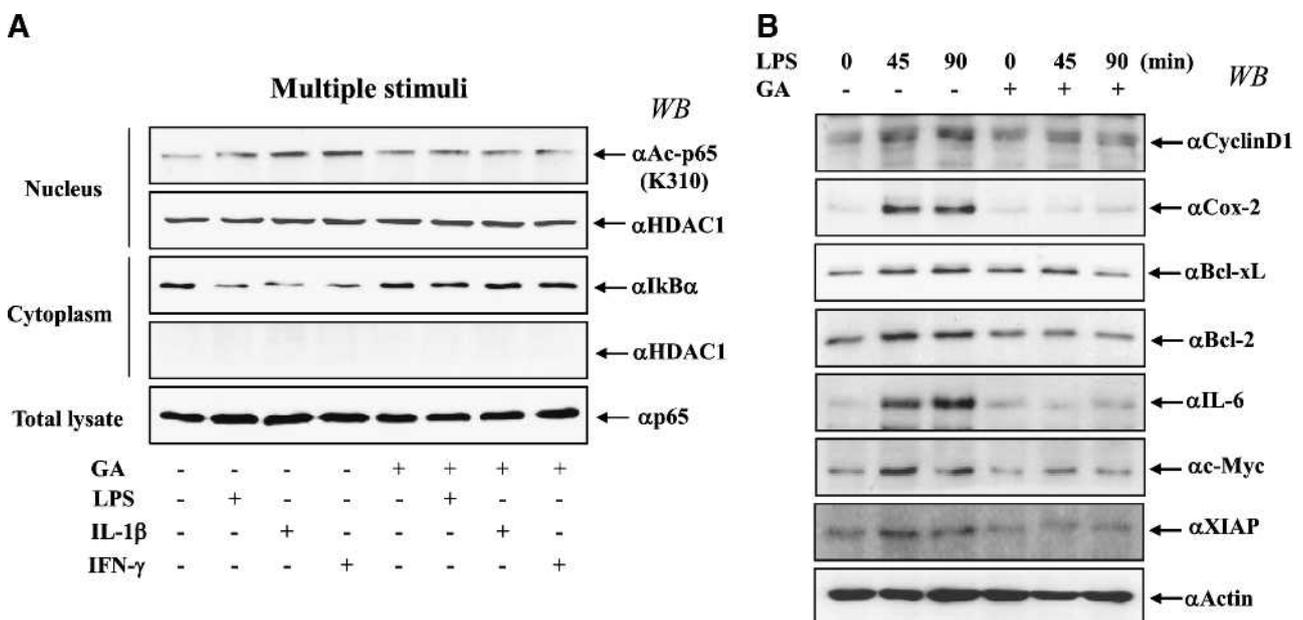

FIGURE 6. Gallic acid suppresses the expression of NF-κB–regulated antiapoptotic genes. **A.** Gallic acid generally inhibits inflammatory responses. The effect of gallic acid treatment on the level of p65 acetylation in the A549 cells was assessed by Western blot analysis. **B.** Gallic acid inhibits the expression of LPS-induced antiapoptotic proteins. A549 cells were incubated with 50 µmol/L of gallic acid and then were treated with LPS for the indicated times. Whole cell extracts were prepared and analyzed by Western blotting using the indicated antibodies.

incubated with blood serum. Images were visualized using a LAS 3000 image analyzer (Fuji).

EMSA

A549 cells incubated with and without gallic acid were cultured in DMEM with 10% fetal bovine serum and subsequently treated with LPS. The preparation of nuclear extracts and EMSA were done as previously described. For EMSA, a double-stranded oligonucleotide containing the consensus sequence for NF-κB binding (Supplementary Table S1) was labeled with [γ-^{32}P]ATP and polynucleotide kinase, and then purified on a 4% nondenaturing polyacrylamide gel. Double-stranded oligonucleotide sequences for EMSA assay were 5′-AGTTGAGGGGACTTTCCCAGGC (sense) and 5′-GCCTGGGAAAGTCCCCTCAACT (antisense).

Immunoprecipitation and Western Blot Analysis

Cell extracts were prepared using lysis buffer, incubated with the NF-κB p65 (Santa Cruz Biotechnology) and p300 (Upstate), and then incubated with 20 μL of protein A/G agarose overnight at 4°C. Coimmunoprecipitated proteins were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes, subjected to immunoblot analysis, and visualized by autoradiography. After treatment with LPS and/or gallic acid, cells were lysed in a lysis buffer [50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 10 mmol/L NaF, 10 mmol/L sodium pyrophosphate, and protease inhibitors]. Each sample was separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and visualized by autoradiography.

RNA Extraction, Reverse Transcription-PCR, and Real-time PCR Analysis

Total RNA was isolated with the RNA Easyspin kit according to the instructions of the manufacturer (Intron, Korea). Total RNA from each sample was reverse transcribed with random primers using a StrataScript reverse transcriptase kit (Stratagene) according to the protocols of the manufacturer. Primers for amplification of the human IL-6 were 5′-CCCCCAGGAGAA-GATTCCAA and 5′-GCTGCTTTCACACATGTTACTCTTG. Primers for amplification of the mouse IL-6 were TGGGACT-GATGCTGGTGACA and TTTCCACGATTTCCCAGAGAA. Primers for amplification of the human COX-2 were 5′-GTCCCTGAGCATCTACGGTTT and 5′-CAACTGCTCAT-CACCCCATT. Primers for amplification of the mouse COX-2 were 5′-TGGAGGCGAAGTGGGGTTTTA and 5′-GAGTGG-GAGGCACTTGCATT. Primers for amplification of the human NOS2 were 5′-ACATTCAGATCCCCAAGCTCTA and 5′-GCCGAGATTTGAGCCTCATG. Primers for amplification of the mouse NOS2 were 5′-TCTTGGAGCGAGTTGTGGAT and 5′-GGGTCGTAATGTCCAGGAAGT. Primers for amplification of the human IL-1β were 5′-ACCTGAGCTCGCCAGT-GAA and 5′-TCGGAGATTCGTAGCTGGAT. Primers for amplification of the mouse IL-1β were 5′-GGCTG-CTTCCAAACCTTTGA and 5′-CCACGGGAAAGACA-CAGGTA. Real-time PCR analysis was done with SYBR Green PCR Master Mix reagents on an ABI Prism 7300 Sequence Detection System. The singularity and specificity of amplification were verified by Dissociation Analysis Software. All samples were normalized to human GAPDH. All reactions were done

in triplicate. Relative expression levels and SDs were calculated using the comparative method.

ChIP Assays

Chromatin was isolated as previously described (36, 37). Briefly, ~2 × 10^9 A549 cells in a T75 flask were treated with PBS containing 1% formaldehyde for 10 min, washed twice with PBS, and incubated with 100 mmol/L Tris (pH 9.4) and 10 mmol/L DTT at 30°C for 15 min. The cells were then rinsed twice with PBS and resuspended in 600 μL of Sol A buffer [10 mmol/L HEPES (pH 7.9), 0.5% NP40, 1.5 mmol/L MgCl$_2$, 10 mmol/L KCl, and 0.5 mmol/L DTT]. After the sample was centrifuged for 5 min at 500 × g at 4°C, the pellets were resuspended in Sol B [20 mmol/L HEPES (pH 7.9), 25% glycerol, 0.5% NP40, 0.42 mol/L NaCl, 1.5 mmol/L MgCl$_2$, and 0.2 mmol/L EDTA] containing protease inhibitors, followed by vigorous pipetting in order to extract the nuclear proteins. After centrifugation at 6,000× g for 30 min at 4°C, the nuclear pellets were resuspended in immunoprecipitation buffer [1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris/HCl (pH 8.0), 150 mmol/L NaCl, and protease inhibitors] and sonicated to break chromatin into fragments with an average length of 0.5 to 1 kb. ChIP assays were then done with the indicated antibodies essentially as previously described but with SDS omitted from all buffers. The antibodies against acetylated p65, p65, and HDAC3 were purchased from Santa Cruz Biotechnology, and RNA Polymerase II and p300 antibodies were purchased from Upstate Biotechnology. Primers used for ChIP assays included the following: hIL-6, F, 5′-TGCTC-AAGTGCTGAGTCACT-3′, R, 5′-AGACTCATGGGAAAATCC-CA-3′; GAPDH, F, 5′-CCCAGCCCGGAGAGA-3′, R, 5′-GGTTTCTGCACGGAAGGT-3′.

Preparation of Mouse Primary Peritoneal Macrophages

Mouse primary peritoneal macrophages were prepared as described (38). BALB/c female mice between 8 and 9 wk of age were injected with 2 mL of 3% thioglycolate (weight / volume) (Sigma). Peritoneal cavities were lavaged 3 d after injection. Primary peritoneal cells were washed in PBS and were cultured at 37°C under 5% CO$_2$ in DMEM supplemented with L-glutamine, antibiotics, and 10% fetal bovine serum.

Immunofluorescence

A549 cells were plated at a density of 2 × 10^5 cells per 22-mm glass coverslip for 24 h before gallic acid treatment. After incubation with gallic acid for 24 h, cells were treated with LPS (20 ng/mL) for 2 h, fixed for 30 min in cold 4% (weight / volume) paraformaldehyde, and permeabilized for 2 min at 25°C in 0.25% (volume / volume) Triton X-100. Expressed NF-κB p65 was detected by incubation at 4°C with anti–NF-κB p65 rabbit polyclonal antibody (Santa Cruz Biotechnology). Anti–NF-κB p65 was used to detect nuclear translocation of NF-κB. After three washes in PBS, coverslips were incubated for 1 h at 37°C with the secondary antibody Alexa-aFluor 555 goat anti-rabbit (Invitrogen). Coverslips were then mounted in medium containing 4′,6-diamino-2-phenylindole (Vector Laboratories). Cells were examined with a DeltaVision RT Imaging Microscope System (Applied Precision).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Ng TB, He JS, Niu SM, et al. A gallic acid derivative and polysaccharides with antioxidative activity from rose (*Rosa rugosa*) flowers. J Pharm Pharmacol 2004;56:537–45.

2. Kroes BH, van den Berg AJ, Quarles van Ufford HC, van Dijk H, Labadie RP. Anti-inflammatory activity of gallic acid. Planta Med 1992;58:499–504.

3. Pellegrina CD, Padovani G, Mainente F, et al. Anti-tumour potential of a gallic acid-containing phenolic fraction from *Oenothera biennis*. Cancer Lett 2005;226:17–25.

4. Hsu CL, Lo WH, Yen GC. Gallic acid induces apoptosis in 3T3-1 pre-adipocytes via a Fas- and mitochondrial-mediated pathway. J Agric Food Chem 2007;55:7359–65.

5. Inoue M, Suzuki R, Sakaguchi N, et al. Selective induction of cell death in cancer cells by gallic acid. Biol Pharm Bull 1995;18:1526–30.

6. Li L, Ng TB, Gao W, et al. Antioxidant activity of gallic acid from rose flowers in senescence accelerated mice. Life Sci 2005;77:230–40.

7. Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non-histone proteins. Gene 2005;363:15–23.

8. Asano Y, Czuwara J, Trojanowska M. Transforming growth factor-β regulates DNA binding activity of transcription factor Fli1 by p300/CREB-binding protein-associated factor-dependent acetylation. J Biol Chem 2007;282:34672–83.

9. Zeng L, Zhang Q, Gerona-Navarro G, Moshkina N, Zhou MM. Structural basis of site-specific histone recognition by the bromodomains of human coactivators PCAF and CBP/p300. Structure 2008;16:643–52.

10. Chen LF, Mu Y, Greene WC. Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-κB. EMBO J 2002;21:6539–48.

11. Kiernan R, Bres V, Ng RW, et al. Post-activation turn-off of NF-κB-dependent transcription is regulated by acetylation of p65. J Biol Chem 2003;278:2758–66.

12. Hayden MS, Ghosh S. Signaling to NF-κB. Genes Dev 2004;18:2195–224.

13. Shishodia S, Aggarwal BB. Nuclear factor-κB activation: a question of life or death. J Biochem Mol Biol 2002;35:28–40.

14. Cao Y, Karin M. NF-κB in mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia 2003;8:215–23.

15. Lin A, Karin M. NF-κB in cancer: a marked target. Semin Cancer Biol 2003;13:107–14.

16. Perkins ND. The Rel/NF-κB family: friend and foe. Trends Biochem Sci 2000;25:434–40.

17. Pande V, Ramos MJ. NF-κB in human disease: current inhibitors and prospects for *de novo* structure based design of inhibitors. Curr Med Chem 2005;12:357–74.

18. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 2006;5:769–84.

19. Duvic M, Vu J. Vorinostat: a new oral histone deacetylase inhibitor approved for cutaneous T-cell lymphoma. Expert Opin Investig Drugs 2007;16:1111–20.

20. Balasubramanyam K, Altaf M, Varier RA, et al. Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. J Biol Chem 2004;279:33716–26.

21. Balasubramanyam K, Varier RA, Altaf M, et al. Curcumin, a novel p300/CREB-binding protein-specific inhibitor of acetyltransferase, represses the acetylation of histone/nonhistone proteins and histone acetyltransferase-dependent chromatin transcription. J Biol Chem 2004;279:51163–71.

22. Sun Y, Jiang X, Chen S, Price BD. Inhibition of histone acetyltransferase activity by anacardic acid sensitizes tumor cells to ionizing radiation. FEBS Lett 2006;580:4353–6.

23. Lee YH, Jung MG, Kang HB, et al. Effect of anti-histone acetyltransferase activity from *Rosa rugosa* Thunb. (Rosaceae) extracts on androgen receptor-mediated transcriptional regulation. J Ethnopharmacol 2008;118:412–7.

24. Madlener S, Illmer C, Horvath Z, et al. Gallic acid inhibits ribonucleotide reductase and cyclooxygenases in human HL-60 promyelocytic leukemia cells. Cancer Lett 2007;245:156–62.

25. Jagan S, Ramakrishnan G, Anandakumar P, Kamaraj S, Devaki T. Antiproliferative potential of gallic acid against diethylnitrosamine-induced rat hepatocellular carcinoma. Mol Cell Biochem 2008;319:51–9.

26. Chen LF, Williams SA, Mu Y, et al. NF-κB RelA phosphorylation regulates RelA acetylation. Mol Cell Biol 2005;25:7966–75.

27. Serrano A, Palacios C, Roy G, et al. Derivatives of gallic acid induce apoptosis in tumoral cell lines and inhibit lymphocyte proliferation. Arch Biochem Biophys 1998;350:49–54.

28. Ohno Y, Fukuda K, Takemura G, et al. Induction of apoptosis by gallic acid in lung cancer cells. Anticancer Drugs 1999;10:845–51.

29. Faried A, Kurnia D, Faried LS, et al. Anticancer effects of gallic acid isolated from Indonesian herbal medicine, *Phaleria macrocarpa* (Scheff.) Boerl, on human cancer cell lines. Int J Oncol 2007;30:605–13.

30. Raina K, Rajamanickam S, Deep G, Singh M, Agarwal R, Agarwal C. Chemopreventive effects of oral gallic acid feeding on tumor growth and progression in TRAMP mice. Mol Cancer Ther 2008;7:1258–67.

31. Togi S, Kamitani S, Kawakami S, et al. HDAD3 influences phosphorylation of STAT3 at serine 727 by interacting with PP2A. Biochem Biophys Res Commun 2009;379:616–20.

32. Levy L, Wei Y, Labalette C, et al. Acetylation of β-catenin by p300 regulates β-catenin-Tcf4 interaction. Mol Cell Biol 2004;24:3404–14.

33. Wolf D, Rodova M, Miska EA, Calvet JP, Kouzarides T. Acetylation of β-catenin by CREB-binding protein (CBP). J Biol Chem 2002;277:25562–7.

34. Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. Cell 2006;124:823–35.

35. Yoon HG, Chan DW, Reynolds AB, Qin J, Wong J. N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso. Mol Cell 2003;12:723–34.

36. Yoon HG, Chan DW, Huang ZQ, et al. Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1. EMBO J 2003;22:1336–46.

37. Yoon HG, Choi Y, Cole PA, Wong J. Reading and function of a histone code involved in targeting corepressor complexes for repression. Mol Cell Biol 2005;25:324–35.

38. Choi KC, Lee YS, Lim S, et al. Smad6 negatively regulates interleukin 1-receptor-Toll-like receptor signaling through direct interaction with the adaptor Pellino-1. Nat Immunol 2006;7:1057–65.

Molecular Cancer Research

Gallic Acid Suppresses Lipopolysaccharide-Induced Nuclear Factor- κB Signaling by Preventing RelA Acetylation in A549 Lung Cancer Cells

Kyung-Chul Choi, Yoo-Hyun Lee, Myung Gu Jung, et al.

Updated version	Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-09-0239
Supplementary Material	Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2009/12/31/1541-7786.MCR-09-0239.DC1

Cited articles	This article cites 38 articles, 12 of which you can access for free at: http://mcr.aacrjournals.org/content/7/12/2011.full#ref-list-1
Citing articles	This article has been cited by 2 HighWire-hosted articles. Access the articles at: http://mcr.aacrjournals.org/content/7/12/2011.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/7/12/2011. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.