AMP-Activated Protein Kinase Suppresses Endothelial Cell Inflammation Through Phosphorylation of Transcriptional Coactivator p300

Yuan Zhang, Jian Qiu, Xiaoming Wang, Yuhua Zhang, Min Xia

Objective—Considerable evidence supports the early involvement of monocyte/macrophage recruitment to activated endothelial cells by leukocyte adhesion molecules during atherogenesis. AMP-activated protein kinase (AMPK) is highly expressed in vascular endothelial cells, but its impact on monocyte adhesion and the related mechanisms are not fully understood. The present study was designed to evaluate the impact of and gain mechanistic insight into the signaling coupling AMPK function to the antiinflammatory response.

Methods and Results—5-Aminoimidazole-4-carboxamide-1-β-D-ribonucleotide (AICAR) treatment or overexpression of constitutively active AMPK markedly reduced human monocyte THP-1 cell adhesion and the expression of vascular cell adhesion molecule-1 in tumor necrosis factor-α-activated human aortic endothelial cells. Furthermore, AICAR or constitutively active AMPK overexpression strongly inhibited the histone acetyltransferase activity of the transcriptional coactivator p300 by phosphorylation of Ser89, which in turn decreased tumor necrosis factor-α-activated p300-mediated acetylation of nuclear factor-κB p65 on Lys221 and reduced the DNA binding activity of nuclear factor-κB by inhibiting its recruitment to its target gene promoters. AMPK phosphorylates the transcriptional coactivator p300 via the atypical protein kinase Cu/A.

Conclusion—Our findings demonstrate that transcriptional coactivator p300 phosphorylation at Ser89 by AMPK is critical for the therapeutic effect of AMPK and may be a potential target for pharmaceutical intervention in inflammatory diseases such as atherosclerosis. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: vascular biology ■ AMPK ■ inflammation ■ p300

Atherosclerosis is a complex pathological process that possesses many features of chronic inflammation and is considered an immunoinflammatory disease.1,2 Interactions between circulating monocytes and the intimal endothelial cell (EC) monolayer, which is regulated by multiple cell adhesion molecules, such as selectins, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 (VCAM-1) (which are expressed on the surface of ECs in response to inflammatory stimuli), may be crucial for the localization and the progression of atherogenesis.3,4 Therefore, modulation of monocyte adhesion to the vascular endothelium is regarded as an important therapeutic target for the prevention and treatment of atherosclerosis.

Increasing evidence indicates that histone modifications, such as phosphorylation, acetylation, and methylation, are important for the transcriptional activity state of genes that may contribute to the regulation of inflammation.5 These posttranslational modifications are altered by histone-modifying enzymes, such as histone deacetylases (HDACs) and histone acetyltransferases (HATs).6 HATs increase histone acetylation, thereby reducing DNA-histone binding and facilitating gene transcription, whereas HDACs act in the opposite way.7 Among the HAT family members, the transcriptional coactivator p300 is a critical component of the transcriptional machinery that modifies chromatin organization and participates in the regulation of gene transcription.8,9

The orchestration of these activities by p300 involves an enzymatic activity through a HAT domain for histone H3 and H4 acetylation and several other substrates, including transcriptional regulators, resulting in enhanced gene transcription along diverse biological pathways, including inflammation.10–13 Because p300 activity is regulated via phosphorylation, it is believed that p300 HAT activity is a central integrator of various signaling pathways in the nucleus.14–16 Thus, epigenetic histone acetylation modifiers have become new targets for therapy in cardiovascular disease.

AMP-activated protein kinase (AMPK) is a member of a metabolite-sensing protein kinase family, contains a catalytic

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From the Department of Cardiovascular Medicine, General Hospital of Guangzhou Military Command (Yuan Zhang, J.Q., X.W., Yuhua Zhang, M.X.); Guangdong Provincial Key Laboratory of Food, Nutrition and Health (X.W., Yuhua Zhang, M.X.); Department of Nutrition, School of Public Health, Sun Yat-sen University, Guangzhou, China (X.W., Yuhua Zhang, M.X.).
Drs Yuan Zhang and Qiu contributed equally to this work.
Correspondence to Min Xia, Department of Nutrition, School of Public Health, Sun Yat-sen University (Northern Campus), Guangzhou, Guangdong Province 510080, People’s Republic of China. E-mail: xiamin@mail.sysu.edu.cn
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Figure 1. AMP-activated protein kinase (AMPK) inhibits monocyte adherence to human aortic endothelial cells (HAECs). A, HAECs were pretreated with 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleotide (AICAR) (0–2 mmol/L) for 1 hour or treated with AICAR (2 mmol/L) in the presence of compound C (10 mmol/L). The cells were then stimulated with 10 ng/mL tumor necrosis factor-α (TNF-α)
α subunit and regulatory β and γ subunits, and functions as a protein serine/threonine kinase. The activation of AMPK attenuates anabolic processes, such as the synthesis of proteins, fatty acids, and cholesterol, but stimulates ATP-generating catabolic pathways. Recent findings, including our own, have demonstrated that AMPK is also present in the endothelium and maintains endothelial homeostasis in response to a number of stimuli (including proinflammatory cytokines and oxidative damage). However, the impact of AMPK on EC activation and the underlying epigenetic mechanism remains unknown.

There are several reports linking AMPK activation with alterations in gene expression via the phosphorylation and modulation of histone acetylases. Given the importance of histone acetylases in regulating the endothelial proinflammatory response, the possible link between AMPK activity and acetylation/deacetylation is warranted. In this study, we evaluated the effect of AMPK on histone acetylases activity and its impact on endothelial activation in cultured human ECs. First, we found that pharmacological and genetic activation of AMPK in cultured human ECs led to the attenuation of the endothelial proinflammatory response, which was characterized by the inhibition of monocyte adhesion and adhesion molecule expression. This was due in part to enhanced nuclear factor-κB (NF-κB) transcriptional activity by acetylation at Lys672, which increased its binding on its target gene promoters. We determined that AMPK is an important inhibitor of p300 activity, inhibiting p300 HAT activity by phosphorylation at Ser89. Specifically, loss of AMPK activity enhanced p300 HAT activity, which in turn increased NF-κB acetylation and potently stimulated NF-κB-induced transcription. Overall, our results demonstrate that hyperactivation of p300 HAT activity is responsible, at least in part, for increased NF-κB transactivation potency and for the development of endothelial inflammation. These findings point to p300 phosphorylation at Ser89 by AMPK as critical for its therapeutic effect and highlight this process as a potential target for pharmaceutical intervention in proinflammatory states.

Materials and Methods

An expanded Methods section is available in the supplemental materials, available online at http://atvb.ahajournals.org.

Results

AMPK Represses the Adhesive Response of Monocytes to Vascular Endothelial Cells

We first used a calcein-AM fluorescence-based adhesion assay to explore whether AMPK affects cell-cell adhesion between monocytes and ECs under proadhesive conditions. Exposure of HAECs to 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleotide (AICAR) for 24 hours suppressed THP-1 cell adhesion in a dose-dependent manner (Figure 1A and 1B). Coincubation with compound C, a potent AMPK inhibitor, completely reversed the effects of AICAR, suggesting AMPK dependent. AICAR also time-dependently inhibited THP-1 cell binding to HAECs, which plateaued after 24 hours of incubation (Figure 1C). Similar inhibitory effects were observed for unrelated AMPK agonists, such as metformin and A-769662 through the binding of fluorescence-labeled THP-1 cells to HAECs (Supplemental Figure IA). To avoid the potential nonspecific effects of these drugs, we further transected the ECs with plasmids encoding a constitutively active form of AMPKα (CA-AMPKα) and a dominant-negative form of AMPKα (DN-AMPKα). CA-AMPKα transfection caused a robust decrease in THP-1 adhesion. In contrast, overexpression of DN-AMPKα was unable to inhibit THP-1 adhesive response (Supplemental Figure IB). The catalytic subunit of AMPK has 2 isoforms: α1 and α2. It was important to determine the relative contribution of each isoform. Selective depletion of AMPKα1 by small interfering RNA (siRNA) dramatically abrogated the monocyte adherence, whereas AMPKα2 depletion slightly abrogated it (Supplemental Figure IC), showing that the AMPKα1 subunit plays the dominant role. Taken together, these results suggest that AMPK functions as a negative regulator of the cytokine-induced adhesive responses in vascular ECs.

AMPK Decreases the Expression of VCAM-1 in Vascular ECs

VCAM-1 is a well-characterized mediator of monocyte adhesion to the endothelium, leading to the infiltration of monocytes into the subendothelial area and the development of atherosclerosis. To investigate the underlying mechanism of AMPK-mediated inhibition of monocyte adhesion, we next evaluated the effect of AMPK on VCAM-1 expression in HAECs. AICAR treatment broadly and markedly repressed the ability of tumor necrosis factor-α (TNF-α) to stimulate the expression of VCAM-1 mRNA (Figure 1D) and protein (Figure 1E) in HAECs. The effects of AICAR were almost completely abrogated by siRNA-mediated knockdown of AMPK, demonstrating that these antiinflammatory effects
were specifically exerted through AMPK activation (Supplemental Figure IIA and IIB). VCAM-1 is mainly regulated at the transcriptional level. Thus, the effects of AMPK on VCAM-1 promoter activity were explored. TNF-α significantly increased VCAM-1 promoter activity in HAECs. Overexpression of CA-AMPKα strongly inhibited the induction of VCAM-1 luciferase activity. Conversely, DN-AMPK expression had no influence (Supplemental Figure IIC). Furthermore, the function-blocking anti-VCAM-1 antibody essentially abolished TNF-α-induced THP-1 cell adhesion to HAECs, validating the contribution of VCAM-1 to this process (Supplemental Figure IID).

**AMPK Suppresses p300-HAT Activity**

Given these potent cellular selective antiinflammatory effects, we were interested in understanding the specific mechanisms whereby signals from AMPK inhibit inflammatory pathways. To further evaluate whether AMPK can suppress the acetylation of histones during endothelial inflammation, protein extracts from ECs treated with TNF-α alone or in the presence of AMPK activators were subjected to analysis of HAT activity as described in Methods. A, *P < 0.05 or **P < 0.01 compared with TNF-α. B, *P < 0.05 or **P < 0.01 compared with 0 hours. C, Human aortic endothelial cells (HAECs) were infected with adenoviruses encoding constitutively active (CA) or dominant-negative (DN) forms of AMPK in the absence or presence of TNF-α. The degree of phosphorylation (phos) of ACC at serine 79 (the target site for AMPK) was detected by Western blot (top). The HAT activity was determined as indicated and expressed as fold of control (bottom). *P < 0.05. D, HAECs were pretreated with AICAR (0–2 mmol/L) for 1 hour. Next, the cells were washed and stimulated with 10 ng/mL TNF-α for 8 hours. Histone deacetylase (HDAC) activity in the cell pellet was assayed and reported as arbitrary units. Each assay was performed at least 3 times.

Figure 2. AMP-activated protein kinase (AMPK) blocks p300-histone acetyltransferase (HAT) activity. A and B, The dose and time course for the effect of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) on the HAT activity of p300. Cells were treated with AICAR at different dosages (A) or for the indicated times (B) in the presence of tumor necrosis factor-α (TNF-α) and then harvested and subjected to analysis of HAT activity as described in Methods. A, *P < 0.05 or **P < 0.01 compared with TNF-α. B, *P < 0.05 or **P < 0.01 compared with 0 hours. C, Human aortic endothelial cells (HAECs) were infected with adenoviruses encoding constitutively active (CA) or dominant-negative (DN) forms of AMPK in the absence or presence of TNF-α. The degree of phosphorylation (phos) of ACC at serine 79 (the target site for AMPK) was detected by Western blot (top). The HAT activity was determined as indicated and expressed as fold of control (bottom). *P < 0.05. D, HAECs were pretreated with AICAR (0–2 mmol/L) for 1 hour. Next, the cells were washed and stimulated with 10 ng/mL TNF-α for 8 hours. Histone deacetylase (HDAC) activity in the cell pellet was assayed and reported as arbitrary units. Each assay was performed at least 3 times.
Figure 3. Acetylation of nuclear factor-κB (NF-κB) p65 at lysine 221 by p300 is essential for tumor necrosis factor-α (TNF-α)-mediated transcriptional activity. A, Human aortic endothelial cells (HAECs) were infected with Ad-p300 and Ad–dominant-negative (DN)–p300 for 24 hours and then treated with TNF-α (10 ng/mL) for 8 hours. The NF-κB p65 acetylation at lysine 221 was determined...
presence of AICAR or their vehicles were subjected to immunoblot with the acetylated antibody against histone-3 or histone-4. Incubation with AICAR strongly inhibited the TNF-α-induced acetylation of histone-3 and histone-4 in ECs (Supplemental Figure IIIA). Then, we analyzed the presence of nuclear hyperacetylation in HAECs by immunostaining with an anti-acetylated lysine antibody. Stimulation of HAECs with TNF-α markedly induced nuclear staining, indicating hyperacetylation. This phenomenon was almost completely abrogated in the presence of AICAR (Supplemental Figure IIIB). Blocking p300-HAT activity inhibited histone acetylation induced by TNF-α, whereas increased p300-HAT activity promoted it, as demonstrated by Western blot (Supplemental Figure IIIIC) and the global acetylation of H3 and H4 (Supplemental Figure IIID and IIIE). Furthermore, overexpression of p300 potently ablated the effects of AICAR to reduce monocyte adhesion (Supplemental Figure IIIf) and VCAM-1 expression (Supplemental Figure IIIG). AICAR to reduce monocyte adhesion (Supplemental Figure IIID and IIIE). Furthermore, overexpression of p300 strongly induced NF-κB p65 luciferase activity (Figure 3C) and VCAM-1 expression (Figure 3D) in HAECs, and this effect was abolished by co-incubation with Lys-CoA-TAT, a cell-permeable chemical inhibitor of p300 HAT.16 This result was also confirmed by a siRNA specific to p300 (Figure 3E and 3F). Because of the important role of lysines 218, 221, and 310 as major acetylation sites in p65, we next determined which individual lysine residue is essential for TNF-α-induced NF-κB activation. As shown in Figure 3G, the mutation of lysine 221, but not 218 or 310, markedly decreased p65 acetylation in response to TNF-α compared with wild-type (WT) p65. Consistent with this result, TNF-α enhanced NF-κB activation in cells transfected with WT p65, p65-Lys218Arg, and p65-Lys310Arg but not with the p65-Lys221Arg mutant (Figure 3H). Thus, it is evident that the lysine 221 residue is critical for mediating enhancement of p65 acetylation by TNF-α and the subsequent DNA-binding and transcriptional activity of NF-κB.

Notably, AICAR treatment repressed the TNF-α-induced increase in the acetylated form at lysine 221 of NF-κB p65 (Figure 4A). Consistently, overexpression of CA-AMPK suppressed NF-κB p65 Lys221 levels, whereas overexpression of DN-AMPK aggravated NF-κB p65 acetylation in TNF-α-treated ECs (Figure 4B). AICAR also diminished the TNF-α-mediated increase in the binding of NF-κB p65 with p300 (Supplemental Figure IVA and IVB). The amounts of total NF-κB in the lysates after immunoprecipitation were similar among the 3 groups, indicating equal protein loading. For the reverse experiments, the same extracts were then immunoprecipitated with anti-p300 antibody, followed by immunoblotting with anti-NF-κB antibody. AICAR still diminished the TNF-α-mediated induction of p300 binding with NF-κB (Supplemental Figure IVC and IVD). Transfection with CA-AMPK abolished the TNF-α-mediated induction of NF-κB p65 luciferase activity, whereas DN-AMPK had no effect (Supplemental Figure IVE). We also assessed NF-κB DNA binding activity by using an ELISA-based assay. Notably, CA-AMPK, but not DN-AMPK, markedly suppressed the TNF-α-mediated induction of NF-κB p65 DNA binding activity (Supplemental Figure IVF). In addition, the inhibitory effects of AMPK on NF-κB transcriptional activity and DNA binding activity were reversed by infection with Ad-p300 (Supplemental Figure IVE and IVF). These findings demonstrate that AMPK abolishes TNF-α-mediated NF-κB activation by inhibiting p300-induced acetylation of NF-κB p65.

AMPK Blocks p300-Mediated Acetylation of NF-κB p65 at Lysine 221

NF-κB had been implicated in the transcriptional regulation of adhesion molecule genes.26 To achieve maximum activation, NF-κB must undergo a variety of posttranslational modifications, including acetylation. We found that the stimulation of HAECs with TNF-α strongly induced NF-κB p65 acetylation at lysine 221 (Figure 3A). A previous report describing the regulation of NF-κB activation by direct protein-protein interaction with p300 prompted us to investigate the functional significance of p300 in NF-κB signaling.27 Overexpression of p300 promoted TNF-α-induced acetylation of NF-κB p65, whereas DN-p300 expression prevented it (Figure 3A and 3B). Furthermore, incubation with TNF-α significantly enhanced NF-κB p65 luciferase activity (Figure 3C) and VCAM-1 expression (Figure 3D) in HAECs, and this effect was abolished by co-incubation with Lys-CoA-TAT, a cell-permeable chemical inhibitor of p300 HAT.16 This result was also confirmed by a siRNA specific to p300 (Figure 3E and 3F). Because of the important role of lysines 218, 221, and 310 as major acetylation sites in p65, we next determined which individual lysine residue is essential for TNF-α-induced NF-κB activation. As shown in Figure 3G, the mutation of lysine 221, but not 218 or 310, markedly decreased p65 acetylation in response to TNF-α compared with wild-type (WT) p65. Consistent with this result, TNF-α enhanced NF-κB activation in cells transfected with WT p65, p65-Lys218Arg, and p65-Lys310Arg but not with the p65-Lys221Arg mutant (Figure 3H). Thus, it is evident that the lysine 221 residue is critical for mediating enhancement of p65 acetylation by TNF-α and the subsequent DNA-binding and transcriptional activity of NF-κB.

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AMPK Prevents the Occupancy of VCAM-1 Promoter by p300 and NF-κB p65

To further address the role of p300, we next examined whether p300 directly interacted with the VCAM-1 promoter.

Figure 3 (Continued). by immunoblotting using an antiacetylated antibody. B, Quantification of Western blot data. *P<0.05, **P<0.01. C and D, Cultured HAECs were pretreated with the specific inhibitor of p300, Lys-CoA-TAT (10 μmol/L) for 2 hours. Then, the cells were left alone or stimulated with TNF-α (10 ng/mL) for 8 hours. *P<0.05. E and F, HAECs were treated with TNF-α (10 ng/mL) in the presence of control small interfering RNA (siRNA) or p300 siRNA. *P<0.05. G, Mutation of lysine 221, but not 218 or 310, markedly decreased p65 acetylation in response to TNF-α compared with wild-type (WT) p65. H, TNF-α-mediated NF-κB-dependent transcriptional activity in cells transfected with p65-Lys218Arg and p65-Lys310Arg, but not with the p65-Lys221Arg mutant compared with cells transfected with WT p65. *P<0.05.
Figure 4. AMP-activated protein kinase (AMPK) suppresses the acetylation of nuclear factor-κB (NF-κB) p65 at Lys221. A and B, Human aortic endothelial cells (HAECs) were pretreated with 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleotide (AICAR) (1.0 mmol/L) for 1 hour (A) or transfected with constitutively active AMPK (CA-AMPK) or dominant-negative AMPK (DN-AMPK) (B). After that, the cells were stimulated with 10 ng/mL tumor necrosis factor-α (TNF-α) for 8 hours. The cellular extracts were assayed for NF-κB p65 acetylation.
in cultured ECs. Chromatin immunoprecipitation assays revealed that the recruitment of p300 and NF-κB p65, as well as the acetylation levels of histones H3K9 and H4K8, at the VCAM-1 promoter were markedly increased after TNF-α stimulation (Figure 4C–4F). These results suggest that p300 and NF-κB are associated with and co-occupy the VCAM-1 promoter on TNF-α stimulation. All these bindings were almost completely prevented by transfecting the ECs with CA-AMPK. In contrast, overexpression of DN-AMPK had no impact on the promoter occupancy. Furthermore, inhibition of p300 expression decreased the TNF-α-stimulated association of NF-κB p65 with the VCAM-1 promoter, demonstrating that p300 is necessary for the binding of NF-κB to DNA (Figure 4G).

AMPK Induces the Ser89 Phosphorylation of p300

To understand the molecular mechanism underlying p300 inhibition by AMPK, we evaluated in the sites where p300 is phosphorylated by AMPK. We initially tested for phosphorylation at Ser89, which was shown to be a universal mechanism for regulating p300 transcriptional activity. Both AICAR and metformin stimulated p300 phosphorylation at the Ser89 site in HAECs (Figure 5A). We further cotransfected increasing amounts of an AMPK expression vector in HAECs, and this caused an increase in Ser89 phosphorylation of p300 (Figure 5B). Based on these effects, we next investigated whether phosphorylation at Ser89 of p300 by AMPK affected its physiological function. We transfected expression constructs containing a Gal4 DNA-binding domain with an antiacetylated antibody. C and D, Chromatin immunoprecipitation (ChIP) assay showing p300 (C) and NF-κB (D) recruitment to the vascular cell adhesion molecule-1 (VCAM-1) promoter following treatment with TNF-α. Data are mean ± SEM from 3 independent experiments. *P < 0.05. E and F, H3K9 (E) and H4K8 (F) recruitment to the promoter of VCAM-1 was measured by ChIP studies. The immunoprecipitated VCAM-1 promoter sequence was analyzed by quantitative polymerase chain reaction. The amount of immunoprecipitated H3 and H4 at the DNA was unchanged on p300 overexpression or silencing and was used as a control to normalize H3K9 and H4K8 acetylation levels to the VCAM-1 promoter. Data are means ± SEM from the 3 independent experiments. **P < 0.01.

Figure 5. AMP-activated protein kinase (AMPK) induces p300 phosphorylation in human aortic endothelial cells (HAECs). A, Western blot analysis of p300 with specific anti–phospho-Ser89 antibody in response to 5-aminoimidazole-4-carboxamide-1-β-D-ribo nucleotide (AICAR) and metformin (met). Data are representative of 3 independent experiments. B, AMPK overexpression stimulated p300 phosphorylation after AMPK infection in the absence or presence of compound C in HAECs. Data are representative of 3 independent experiments. C, Measurement of Gal4-wild-type (WT) and Ser89Ala p300 activity in HAECs overexpressing constitutively active AMPKα (CA-AMPKα). Data are the average of 3 independent experiments. *P < 0.05. D, Western blot analysis of p300 Ser89 phosphorylation after AMPK infection in the absence or presence of compound C in HAECs. Data are representative of 3 independent experiments. E, Measurement of luciferase activity in HAECs overexpressing Gal4-WT-p300 with AMPK after compound C treatment. Data are the average of 3 independent experiments. **P < 0.01.
main fused to either WT p300 (Gal4-WT-p300) or p300 containing a point mutation at Ser89 to alanine (Gal4-Ser89Ala-p300) into HAECs along with the AMPK constructs. Coexpression of CA-AMPK suppressed Gal4-WT-p300 activity by 3-fold compared with control, whereas CA-AMPK had no effect on Gal4-Ser89Ala-p300 activity (Figure 5C). Interestingly, Gal4-Ser89Ala-p300 was more active than Gal4-WT-p300, suggesting that phosphorylation of p300 by AMPK inhibits its transcriptional ability. This explanation was supported by the observation that compound C, an inhibitor of AMPK activity, markedly reduced amounts of Ser89 p300 phosphorylation in cells overexpressing CA-AMPK (Figure 5D), and as a result, its transcriptional activity was markedly increased (Figure 5E).

AMPK Induces p300 Phosphorylation via Atypical Protein Kinase C (aPKC)/\(\alpha\) Activation

Protein kinase C (PKC) was previously reported to mediate the phosphorylation of p300, and analysis of the amino acid sequence of p300 led to the identification of Ser89 as a consensus recognition phosphorylation motif. We further determined that the class of PKC is responsible for p300 phosphorylation at Ser89, and we treated vascular ECs with 2 PKC inhibitors. The nonisoform-selective PKC-specific inhibitor Ro31-8220 blocked AICAR-induced p300 phosphorylation; however, an inhibitor of classical and novel PKC isoforms, Go6976, had no effect (Figure 6A). These data suggest that AICAR mediates p300 phosphorylation through atypical PKCs (aPKCs).

PKC\(\zeta\) and PKC\(\alpha\) constitute the aPKCs that are conserved across species and are ubiquitously expressed in most tissues. We then sought to identify the responsible PKC isof orm using specific siRNA. RAI-mediated knockdown of aPKC\(\zeta\) in ECs blocked the induction of p300 phosphorylation by AMPK (Figure 6B). Furthermore, silencing of aPKC\(\alpha\) also markedly diminished AICAR-mediated reduction of monocyte adhesion (Supplemental Figure VA) and VCAM-1 expression (Supplemental Figure VB). Transfection of an increasing amount of aPKC\(\alpha\) expression vector caused the Ser89 phosphorylation of p300 (Figure 6C), supporting the notion that aPKC\(\alpha\) directly phosphorylates p300.

Phosphorylation plays an important role in activating PKC activity. For this reason, we examined the phosphorylation status of aPKC\(\alpha\). AICAR and AMPK overexpression stimulated phosphorylation of aPKC\(\alpha\) at Thr403 and Thr555 (Figure 6D and 6E), which are located in the activation loop of the kinase domain and have been shown to be critical for the kinase activity of aPKC\(\alpha\). Moreover, inhibition of PKC\(\zeta\) (Supplemental Figure VIA) or PKC\(\epsilon\) (Supplemental Figure VIB) by the TAT-conjugated peptide inhibitor did not interfere with the effects of AICAR on p300 phosphorylation.
Thus, AMPK mediates p300 phosphorylation via αPKCs/A activation.

Discussion

In this study, we show that AMPK functions as a p300-HAT inhibitor in proinflammatory ECs. By signaling through the phosphorylation of p300, AMPK activation through AICAR treatment and CA-AMPK overexpression mediates potent anti-inflammatory effects to inhibit TNF-α inflammatory signaling pathways. The mechanism of AMPK-mediated anti-inflammation involves the induction of p300 Ser89 phosphorylation and subsequent inactivation of p300 HAT activity, resulting in the attenuation of NF-κB acetylation and its dependent monocyte adhesion to human ECs in response to TNF-α (Figure 6F). Because chronic tissue inflammation is an important mechanism that causes the generation of atherosclerotic lesions, the anti-inflammatory actions of AMPK exert potent antiatherogenic effects.

AMPK is a crucial regulator of energy metabolic homeostasis and thus a major survival factor in a variety of metabolic stresses. Recently, there have been several reports describing how the activation of AMPK can inhibit inflammatory response induced by different stimulating insults, whereas a decrease in AMPK activity is associated with increased inflammation. In this study, we demonstrated that pharmacological and genetic activation of AMPK decreases expression of adhesion molecules VCAM-1 in TNF-α-activated ECs, thus preventing the adhesion of THP-1 cells to vascular ECs. The concentration of AMPK agonist AICAR used in this study seems high; however, this dosage of AMPK seems comparable to the concentration of AICAR used in this study. To exclude the off-target effects of AICAR, AICAR was commonly used and is comparable to the concentration of a recent report showing that AICAR prevents postischemic inflammation through the inhibition of p300 HAT activity. The p300-associated HAT activity can be regulated by multiple mechanisms of AMPK activation and found a consistent influence of these agents on monocyte adhesion. This contention is supported by our further observations that genetic activation of AMPK by CA-AMPKα mimics the protective effects of AICAR. All these data indicate that AMPK activation indeed suppressed endothelial inflammation. There are 2 isoforms of AMPKα, each of which localizes to different subcellular compartments and downstream enzymes. Using RNAi technology, we demonstrated the major contribution of the α1 isoform in AMPK-mediated regulation of TNF-α-induced endothelial inflammation. This notion is inconsistent with a recent report showing that AICAR prevents postschismic inflammation by a mechanism involved with both isoforms. This discrepancy was mainly due to the predominant isoform expressed in ECs being the α1 catalytic subunit.

NF-κB is a key transcriptional factor involved in regulating the expression of proinflammatory mediators, including adhesion molecules, thereby playing a critical role in mediating inflammatory responses. To achieve its biological functions, NF-κB must undergo a variety of posttranscriptional modifications, including acetylation. Of particular interest, we provide direct evidence that acetylation of NF-κB p65 by p300 constitutes a regulatory mechanism for TNF-α-dependent VCAM-1 transcription and monocyte adhesion to ECs. First, suppression of p300-HAT activity results in impaired NF-κB transcriptional activity, whereas upregulation of p300-HAT activity promotes NF-κB activation, suggesting that p300-HAT plays a critical role in the activation of NF-κB signaling. Actually, p300 was shown to recruit to the VCAM-1 promoter in a TNF-α-dependent manner, promoting NF-κB p65 binding, as well as histone H3K9 and H4K8 acetylation. Furthermore, the major acetylated site of NF-κB p65 by p300 is the Lys221 residue located within its DNA-binding domain. Mutation of NF-κB p65 Lys221 resulted in decreased DNA binding and transcription activity, indicating that the acetylation of NF-κB p65 is required for its capacity to interact with its target gene promoters. This observation correlates with previous reports showing that increasing acetylation within the DNA-binding domain of transcription factors, such as p53, Nrf2, or HNF4, enhances their binding to the DNA, allowing an increase in their transcriptional activity. Although Lys221 was identified as the major acetylation site for NF-κB p65, mutation of Lys218 appears to partially diminish the acetylation and transactivation of p65. Given that lysine 218 is located adjacent to lysine 221, it is possible that mutation of lysine 218 may interfere with the function of lysine 221 as a result of a possible conformational change. Taken together, our results demonstrate that p300 coactivates TNF-α-mediated induction of proinflammatory gene expression by acetylating both histones and NF-κB itself, which may contribute to increased association of NF-κB p65 with the promoter.

Histone acetylation is regulated by p300-HAT and plays pivotal role in vascular disease. The results of the present study indicate that inhibition of histone acetylation is a key mechanism for the anti-inflammatory action of AMPK and that p300-HAT serves as its molecular target. By blocking p300-HAT activity, AMPK may inhibit the acetylation levels of both histone and NF-κB itself, thus largely attenuating the increased occupancy of p300 and NF-κB at the VCAM-1 promoter after treatment with TNF-α. As a result, AMPK activation by AICAR treatment or CA-AMPK overexpression diminishes the amplification of the proinflammatory signaling cascades in the ECs. Together, these findings further indicate that AMPK protects from inflammation through the inhibition of p300 HAT activity. The p300-associated HAT activity can be regulated by multiple mechanisms in addition to changes in p300 abundance, such as phosphorylation and acetylation of p300. We observed that AICAR treatment promoted the phosphorylation levels of p300 at Ser89 without affecting the overall cellular level of p300. Mutation of Ser89 to alanine abrogates AMPK-mediated reduction of p300 transcriptional activity, confirming that the Ser89 is the major target of AMPK in regulation of HAT activity. aPKCα represents a subgroup of the PKC family comprising the λ/ι and ζ isoforms and is involved in several signal transduction pathways. In the current study, we also provide a molecular link that aPKCα mediates p300 phosphorylation by AMPK.

In conclusion, our present work demonstrates that AMPK prevents the development of the endothelial inflammatory response by blocking p300-HAT–dependent NF-κB acetyla-
tion. AMPK thus becomes an attractive therapeutic candidate for treating inflammatory diseases.

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Disclosures
None.

References


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Supplement Figure I

**Figure A**

- Vertical axis: THP-1 cell adhesion (%)
- Horizontal axis: Control, TNFα, A769662, Met
- Data points:
  - Control: 2.5 ± 0.5
  - TNFα: 20 ± 2.0
  - A769662: 5.0 ± 0.5
  - Met: 10.0 ± 1.0
- Significance: **p < 0.01

**Figure B**

- Vertical axis: THP-1 cell adhesion (%)
- Horizontal axis: Control, TNFα
- Data points:
  - Control:
    - Ad-Lac Z: 2.0 ± 0.5
    - Ad-CA-AMPKα: 5.0 ± 0.5
    - Ad-DN-AMPKα: 10.0 ± 1.0
  - TNFα:
    - Ad-Lac Z: 15.0 ± 2.0
    - Ad-CA-AMPKα: 18.0 ± 2.0
    - Ad-DN-AMPKα: 22.0 ± 2.0
- Significance: *p < 0.05

**Figure C**

- Vertical axis: THP-1 cell adhesion (%)
- Horizontal axis: Control, TNFα, CA-AMPKα, AMPKα1 si, AMPKα2 si
- Data points:
  - Control: 2.0 ± 0.5
  - TNFα: 15.0 ± 2.0
  - CA-AMPKα: 5.0 ± 0.5
  - AMPKα1 si: 10.0 ± 1.0
  - AMPKα2 si: 12.0 ± 1.0
- Significance: *p < 0.05
Supplement Figure I. AMPK agonists inhibits mpnocyte adhesion to vascular ECs. (A) HAECs were pretreated with metformin (1 mmol/L) or A769662 (100 μ mol/L) for 1 hour. Then cells were stimulated with 10 ng/mL TNF α for 8 hours and incubated with calcein-labeled THP-1 cells for another 1 hour. **P<0.01 compared with TNF α. (B) HAECs were transduced with Ad-LacZ, Ad-CA-AMPK α, or Ad-DN-AMPK α. Forty-eight hours after adenovirus transduction, HAECs were incubated in the absence or presence of 10 ng/mL TNF α and the adhesion assays were performed as indicated. Results are represented as the means ± SEM of three independent experiments. *P<0.05. (C) HAECs were transfected with CA-AMPK α alone or in the presence of either AMPK α 1 siRNA or AMPK α 2 siRNA. Thereafter, cells were stimulated with TNF α and THP-1 adhesion was assessed as indicated. *P<0.05.
Supplement Figure II

A. VCAM-1 expression (Fold of Control)

B. Western blot analysis of VCAM-1 and β-actin

C. VCAM-1 luciferase activity (Fold of Control)

D. THP-1 cell adhesion (%)

VCAM-1 expression and luciferase activity were analyzed in THP-1 cells treated with TNFα, AICAR, AMPK α1 siRNA, or control siRNA. THP-1 cell adhesion was measured in the presence of TNFα and anti-VCAM-1 antibodies.
Supplement Figure II. AMPK inhibits VCAM-1 expression

(A-B) HAECs were treated with AICAR (1 mmol/L) in the presence of control siRNA or AMPK α 1 siRNA for 24 hours. Then, the cells were stimulated with 10 ng/mL TNF α  for 8 hours. (A) VCAM-1 mRNA and (B) protein expression were assessed by quantitative RT-PCR and Western blot, respectively. *P<0.05. (C) HAECs infected with CA-AMPK α  or DN-AMPK α  plus VCAM-1 reporter were stimulated with 10 ng/mL of TNF α  for 8 hours. Next, the luciferase activity was performed using β-gal as a control. Results of three independent experiments are expressed as fold of control. *P<0.05. (D) HAECs were pretreated with antibodies (50 µg/mL) for 30 minutes and then incubated in the presence of PBS (control) or TNF α  (10 ng/mL) for 8 hours, and static adhesion assays were performed. *P<0.05.
Supplement Figure III

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Ad-p300 and Ad-DN-p300 treatments are also included in the experiment.
Supplement Figure III. AICAR inhibits TNF α -induced acetylation

HAECs were treated alone or with AICAR (1 mmol/L) for 24 hours. Then ECs were stimulated with 10 ng/mL TNF α for 8 hours. (A) Proteins isolated by acid extraction from these cells were subjected to Western blot for acetylated histone-3/4 or total histone-3/4 as indicated. (B) These cells were subjected to indirect immunofluorescence analysis with an antibody against acetylated lysine. Magnification, ×20. (C-E) Cells were infected with Ad-p300, Ad-DN-p300, or Ad-GFP for 24 hours and then treated with 10 ng/mL TNF α for 8 hours. (C) The global acetylation of histones (D) the acetylation of histone H3, and (E) the acetylation of histone H4 were determined. *P < 0.05. (F-G) HAECs were treated with AICAR (1 mmol/L) in the absence or presence of Ad-p300. Then ECs were stimulated with 10 ng/mL TNF α for 8 hours. (F) THP-1 cells adhesion and (G) VCAM-1 protein expression were assayed as indicated. *P < 0.05. The data shown are means ± SEM from three independent experiments.
Supplement Figure IV

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p300-associated p65/total p65

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Supplement Figure IV. AMPK inhibits the binding of NF-κB p65 with p300

(A) HAECs were left alone or stimulated with 10 ng/mL TNF-α in the presence of AICAR (1 mmol/L) for 24 hours. The protein extracts (100 μg of protein) were immunoprecipitated with anti-p65 antibody or (B) anti-p300 antibody and then subjected to Western blot with anti–acetylated lysine antibody, anti-p300 antibody, and anti-p65 antibody. (C) The amounts of p65-associated p300/total p65 binding and (D) p300-associated p65/total p300 binding were quantified, and the data shown are means ± SEM from three independent experiments.

(E) HAECs were infected with NF-κB reporter, plus CA-AMPK or DN-AMPK, or CA-AMPK alone in the presence of Ad-p300 for 24 hours and then incubated with 10 ng/mL TNF-α for another 8 hours. The luciferase activity was determined using β-gal as the control. Results of three independent experiments are expressed as fold of control.

(F) Cells were infected with CA-AMPK, DN-AMPK, or CA-AMPK plus Ad-p300 for 24 hours, and the protein extracts were assayed for p65 DNA-binding activity. The results were reproducible in three independent experiments.
Supplement Figure V. Inhibition of PKC \( \iota / \lambda \) prevents AICAR-mediated monocyte adhesion and VCAM-1 expression

HAECs were treated with AICAR (1 mmol/L) in the presence of control siRNA or PKC \( \iota / \lambda \) siRNA for 24 hours. Then, the cells were stimulated with 10 ng/mL TNF \( \alpha \) for 8 hours. (A) THP-1 monocytes adhesion and (B) VCAM-1 protein expression was determined as indicated. Results are shown as means ± SEM or representative blots from three independent experiments. *\( P < 0.05 \).
Supplement Figure VI. Inhibition of PKC ζ and PKC ε did not affect the p300 phosphorylation induced by AICAR. Cultured HAECs were treated with medium or AICAR (1 mmol/L) or in the presence of the TAT carrier peptide, (A) TAT-PKC ζ, (B) TAT-PKC ε inhibitor (both at 1 mmol/L) for 1 hour. The phosphorylation levels of p300 was determined by western blot. Blots representative of three independent experiments are shown.
Supplemental Materials for ATVB/2011/237453

AMP-Activated Protein Kinase Suppresses Endothelial Cell Inflammation through Phosphorylation of Transcriptional Coactivator p300

Yuan Zhang1,4, Jian Qiu1,4, Xiaoming Wang1,2,3, Yuhua Zhang1,2,3, Min Xia1,2,3,*

1 Department of Cardiovascular Medicine, General Hospital of Guangzhou Military Command
2 Guangdong Provincial Key Laboratory of Food, Nutrition and Health
3 Department of Nutrition, School of Public Health, Sun Yat-sen University
4 These authors contributed equally to this work
*To whom correspondence should be addressed:
Department of Nutrition, School of Public Health, Sun Yat-sen University (Northern Campus), Guangzhou, Guangdong Province, 510080, P.R.China.
E-mail: xiamin@mail.sysu.edu.cn

Materials and Methods

Materials

AMPK agonist AICAR (5-aminoimidazole-4-carboxamide ribonucleoside), anti-phospho AMPK Thr-172, anti-AMPK and anti-β-actin were purchased from Cell Signaling Technology. Metformin was purchased from Sigma (Sigma-Aldrich). Compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine) was obtained from Calbiochem (San Diego, Calif). A-769662, a cell permeable drug that potently activates AMPK in cells, was provided by TOCRIS bioscience (Cat No. 3336, Genetimes Technology, Inc). The peptide inhibitors of PKCε, Glu-Ala-Val-Ser-Leu-Lys-Pro-Thr was provided by Santa Cluz (Catalog sc-3095) Each peptide was reversibly conjugated to Tat using a disulphide conjugation through N-terminal cysteine residues to produce peptides that are released in the reducing intracellular environment. The siRNA targeting AMPKα1 and AMPKα2 catalytic-subunits were purchased from QIAGEN (Shanghai, China).

Cell Culture
Human aortic endothelial cells (HAECs) were purchased from Cell Applications Inc. (San Diego, CA) and cultured in M199 medium supplemented with FBS (20% vol/vol), penicillin (100 U/mL), streptomycin (100 μg/mL), heparin (90 μg/mL), and endothelial cell growth supplement (20 μg/mL). The cells were grown at 37°C in humidified 5% CO2 and used for experiments between passages 3 and 5 (1).

**Recombinant Adenoviruses**

The plasmids encoding c-Myc-tagged forms of dominant-negative AMPKα mutant (DN-AMPK) and a constitutively active form of AMPK (CA-AMPK) were kindly donated as a gift by Dr J. Ha (Department of Molecular Biology, Kyung Hee University, College of Medicine, Seoul, Korea). The cDNA encoding DN-AMPK or Ca-AMPK was inserted into the EcoRI/XhoI sites of the pAdTrack-CMV shuttle vector. The resulting vector was then electroporated into BJ5138 cells containing the AdEasy adenoviral vector to produce the recombinant adenoviral plasmid. The recombinant viruses were amplified in HEK293 cells and isolated by cesium chloride density centrifugation. The viruses were collected and desalted, and the titers were measured using Adeno-XTM Rapid titer (BD Bioscience, San Jose, CA) according to the manufacturer’s instructions (2).

**Transfection with p300 expression plasmids**

To examine the effects of increased p300 levels on basal and TNFα-induced inflammation, HAECs were transfected with plasmids expressing Myc-tagged wild-type p300 (p300WT) or Myc-tagged p300 mutated in its HAT activity domain and lacking HAT activity, p300(HAT−;both provided by Dr. W. C. Greene, Gladstone Institute of Virology and Immunology, San Francisco, CA) or an empty vector (pcDNA3). Using plasmids expressing the fusion protein p300/Myc facilitated the detection of p300 by employing an anti-Myc antibody for Western blot analysis (3). Transfection efficiency based on p300 expression was 60% to 75%.

**Quantitative Real-Time Polymerase Chain Reaction**

Total mRNA was isolated with TRIZOL Reagent (Invitrogen) according to manufacturer’s instructions. Real-time PCR was performed with the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, Calif) on the ABI 7500 DNA Sequence
Detection System with standard fluorescent chemistries by using
5'-GATAAAGCCTTGGTCAGCCC-3' (sense) and
5'-CGCATCCTTCAACTGGCCTT-3' (antisense) for the VCAM-1. The correlation
between the amounts of RNA used and of PCR products obtained with target gene
and with the internal standard (β-actin) was examined.

Small Interfering RNA (siRNA) Transfection
Control siRNA and siRNA against p300 were obtained from Santa Cruz
Biotechnology, and HAECs were transfected according to the manufacturer's
instruction. Briefly, 2×10^6 cells were seeded on 100-mm plates the day before
transfection. The medium was switched to Opti-MEM and either control siRNA or
p300 siRNA in Oligofectamine was added to the culture medium for 4 hours (final
concentration 50 nmol/L), after which the medium was replaced with normal HAECs
medium. Fluorescein-labeled dsRNA oligomer, used to assess transfection efficiency,
had the same length, charge, and configuration as the siRNAi.

Reporter Gene Constructs and Luciferase Assays
To construct the VCAM-1 luciferase plasmids, the full legendth of regions spanning
–1716 to +119 bp of the human VCAM-1 promoter was fused into yT&A cloning
vector (ECOS Inc) and digested with KpnI and BglII restriction enzymes. The
resulting fragments (1835 bp) were cloned with T4 DNA ligase (Quick Ligation Kit,
New England BioLabs Inc., Beverly, MA) into the corresponding sites of the
luciferase reporter gene of vector pGL3-basic (Promega). The orientation of the insert
was determined by DNA sequencing. HAECs were transfected with 1 µg of the
plasmids and 1 µg of the control pCMV-β-gal plasmid using LipofectAMINE Plus
reagents (Invitrogen). Cell extracts were prepared 24 hours after transfection,
and luciferase assays were performed using the Dual-Luciferase® Reporter
(DLR™) Assay System as described in the manufacturer’s protocol
(Promega). Renilla luciferase activity was used to monitor the efficiency of
transfection (4,5)

Chromatin Immunoprecipitation.
Antibodies used for immunoprecipitation of p300 and p65 were from Santa Cruz
biotechnology. After removing crosslinks, DNA was extracted using phenol–chloroform and ethanol precipitated. NF-κB p65 occupancy over the VCAM-1 promoter was quantified using SYBR green real-time PCR with the primers flanking the p65 site of human VCAM-1 promoter. All signals were normalized to input chromatin signals. ChIP experiments were repeated at least three times with reproducible results.

**Chromatin Immunoprecipitation (ChIP)**

To detect the association of p300 and NF-κB p65 with human VCAM-1 promoter, chromatin immunoprecipitation (ChIP) analysis by using the ChIP Assay Kit (Upstate Biotechnology) following manufacturer’s instructions. HAECs in 100-nm dishes were grown to confluence and treated with 10 ng/mL TNFα in the absence or presence of CA-AMPKα or DN-AMPKα. The protein–DNA complexes were fixed by 1% formaldehyde in medium. The fixed cells were lysed and sonicated at 4 °C until the DNA size became 200–1000 base pairs. The samples were centrifuged, and the soluble chromatin was precleared by incubation with sheared salmon sperm DNA-protein agarose A slurry (Upstate) for 30 minutes at 4 °C with rotation. After precleared, samples were centrifuged at 6000 rpm for 2 minutes and the supernatant was transferred to a new tube. The concentration of samples was quantified and balanced. One portion of samples was used as DNA input control, and the remains were subdivided into several portions and then incubated with anti-p300 or anti-p65 antibodies overnight at 4 °C. To precipitate chromatin, 2.5 volumes of ethanol were added to the samples and incubated at -20°C overnight. Chromatin samples were then pelleted and pre-cleared. Pre-cleared samples were used for immunoprecipitation with specific antibodies and subjected to PCR amplification with the forward primer 5′-AAATCAATTCACATGGCATA-3′ and the reverse primer 5′-AAGGGTCTTGTTGCAGAGG-3′, which were specifically designed from the VCAM-1 promoter region (−403 to −30) (6).

**NF-κB p65 DNA-binding activity**

Five microgram of nuclear extracts was used to determine p65 DNA-binding activity by using an ELISA-based assay, according to the manufacturer’s instructions.
Briefly, κB oligonucleotide-coated plates (in a 96-well format) were incubated for 1 hour with the nuclear extracts. Specificity was achieved through incubation with anti-p65 primary antibodies for 1 hour. HRP-conjugated secondary antibodies were used for the detection of p65 bound to the κB sequences (7).

**Histone acetylation**

Acid extraction of histones was performed by using a commercial kit according to the manufacturer's protocol (Upstate). Western blot for acetylated histone-3/4 and for total histone-3/4 was performed using goat anti–acetylated histone-3/4 polyclonal antibodies (Upstate) and rabbit anti–histone-3/4 polyclonal antibodies (Upstate), respectively. Blots were washed again, and immunoreactive bands were visualized by enhanced chemoluminescence (8).

**NF-κB Acetylation State**

Immunoprecipitation and Western blot for acetylated lysine were performed as previously described (9,10). We used goat anti-NF-κB polyclonal antibody (Santa Cruz Biotechnology Inc.) for immunoprecipitation and rabbit polyclonal antibody against acetylated lysine (Cell Signaling), rabbit anti-NF-κB polyclonal antibody, rabbit anti-p300 polyclonal antibody (Santa Cruz Biotechnology Inc.), mouse anti–β-actin monoclonal antibody (Sigma-Aldrich) for Western blot.

**p300-HAT activity**

HAECs were cotransfected with expression vector for p300. 24h after transfection cells were collected by scraping in 1ml of ice-cold phosphate buffered saline, and pelleted by centrifugation. The cells were resuspended in 300 μl of cell lysis buffer (50 mM tris-HCl, pH8.0, 120 mM NaCl, 0.5% nonidet P-40, 2 mg/ml leupetin, 2 mg/ml aprotinin, 100 mM PMSF, 2 mM DTT) and incubated on ice for 30 min to extract the whole cells proteins. The p300 HAT activity was directly quantified with a colorimetric diagnostic kit according to the manufacturer’s instructions (HAT activity colorimetric assay; bioVision).

**HDAC Activity Assay**

HDAC activity was measured by colorimetric HDAC assay kit (ab1438; Abcam) as
described previously. The absorbance of samples is expressed as arbitrary units equivalent to the absorbance obtained with specific concentrations of deacetylated standard (11).

**Monocyte Adhesion Assay**

THP-1 monocytes were prestained with 5 µmol/L calcein-AM (Invitrogen) at 37°C for 30 minutes. After washing in PBS, fluorescently labeled THP-1 monocytes were added onto the HAEC monolayers at the density of 10⁶ cells/mL. To block VCAM-1 function, HAEC monolayers were incubated with blocking antibodies against VCAM-1 (25 µg/mL, BBA5, R&D Systems) for 1 hour before the addition of THP-1 monocytes. Nonadherent monocytes were removed by gently washing with complete medium after 30 minutes. Fluorescence intensity (FI) was measured using the Infinite F200 Fluorescent ELISA Reader (TECAN) set at excitation and emission wavelengths of 485 and 530 nm (12).

**Statistical Analyses**

Results are expressed as mean ± SEM. Comparison between groups was analyzed via one-way analysis of variance followed by Student-Newman-Keuls test. *P*<0.05 was considered significant. Nonquantitative results were representative of at least three independent experiments.

**Reference**


