Role of Histone Deacetylation in Cell-specific Expression of Endothelial Nitric-oxide Synthase*

Received for publication, November 16, 2004, and in revised form, January 25, 2005
Published, JBC Papers in Press, February 19, 2005, DOI 10.1074/jbc.M412960200

Yehua Gan‡§, Ying H. Shen‡, Jian Wang‡, Xinwen Wang‡, Budi Utama‡, Jing Wang‡, and Xing Li Wang‡¶

From the ‡Division of Cardiothoracic Surgery, Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, Texas 77030 and §Center for TMJ Disorders, Peking University School of Stomatology, Beijing 100081, China

Histone acetylation plays an important role in chromatin remodeling and gene expression. The molecular mechanisms involved in cell-specific expression of endothelial nitric-oxide synthase (eNOS) are not fully understood. In this study we investigated whether histone deacetylation was involved in repression of eNOS expression in non-endothelial cells. Induction of eNOS expression by histone deacetylase (HDAC) inhibitors trichostatin A (TSA) and sodium butyrate was observed in all four different types of non-endothelial cells examined. Chromatin immunoprecipitation assays showed that the induction of eNOS expression by TSA was accompanied by a remarkable increase of acetylation of histone H3 associated with the eNOS 5′-flanking region in the non-endothelial cells. Moreover, DNA methylation-mediated repression of eNOS promoter activity was partially reversed by TSA treatment, and combined treatment of TSA and 5-aza-2′-deoxycytidine (AzadC) synergistically induced eNOS expression in non-endothelial cells. The proximal Sp1 site is critical for basal activity of eNOS promoter. The induction of eNOS by inhibition of HDACs in non-endothelial cells, however, appeared not mediated by the changes in Sp1 DNA binding activity. We further showed that Sp1 bound to the endogenous eNOS promoter and associated with HDAC1 in non-endothelial HeLa cells. Combined TSA and AzadC treatment increased Sp1 binding to the endogenous eNOS promoter but decreased the association between HDAC1 and Sp1 in HeLa cells. Our data suggest that HDAC1 plays a critical role in eNOS repression, and the proximal Sp1 site may serve a key target for HDAC1-mediated eNOS repression in non-endothelial cells.

Nitric oxide (NO) is a free radical with diverse functions in many biological systems. In the vasculature NO is mostly generated by endothelial nitric-oxide synthase (eNOS).1 Endothelial NO plays a crucial role in maintaining vascular homeostasis (1). Murine or human eNOS promoter/LacZ transgenic mouse models and human eNOS whole genome containing introns/green fluorescence protein transgenic mouse model have all demonstrated that the eNOS gene is constitutively expressed in and relatively confined to endothelium (2–4). However, the molecular mechanism involved in endothelium-specific expression of eNOS is not fully understood. A recent study has demonstrated that the human eNOS proximal promoter DNA is heavily methylated in non-endothelial cells, whereas it is hardly methylated in endothelial cells. It is suggested that promoter DNA methylation may play an important role in the cell-specific eNOS expression in the vascular endothelium (5). However, to control cell-specific gene expression, DNA methylation requires cooperation from histone modifications and chromatin remodeling factors (6). It is not clear whether histone deacetylation is involved in the cell-specific eNOS expression, i.e., the repression of eNOS in non-endothelial cells, and whether there is any relationship between DNA methylation and histone deacetylation in cell-specific expression of eNOS.

Modifications of core histones are fundamentally important in alteration of chromatin structure and gene transcription (7). Acetylation of core histone unpacks the condensed chromatin and renders the target DNA accessible to transcriptional machinery, hence contributing to gene expression. In contrast, deacetylation of core histones increases the chromatin condensation and prevents the binding between DNA and transcriptional factors, which lead to transcriptional silence (8, 9). Histone acetyltransferases and histone deacetylases (HDACs) regulate the acetylation of histones and interact with components of the transcription machinery (10). Although histone acetylation is related to gene activation, global inhibition of HDACs does not induce widespread transcription (11, 12). For instance, treatment of human lymphoid cell line with HDACs inhibitor trichostatin A (TSA) revealed a change of expression (up- and down-regulation) in only 8 of 340 genes examined (11). It appears that histone deacetylase inhibitors may only activate some specific genes. Several studies have shown that inhibition of HDACs can selectively induce gene expression in the non-expressing cells (13–16).

In this study we examined the human eNOS mRNA, the eNOS promoter activity, and acetylation of histones associated with the 5′-flanking region of the eNOS in non-endothelial cells treated with HDACs inhibitors. We also investigated the effects of HDACs inhibitors on eNOS promoter DNA methylation status and on the DNA methylation-mediated repression of eNOS promoter activity. Furthermore, we examined whether Sp1 binds to endogenous eNOS promoter and the relationship between Sp1 and HDAC1. We found that eNOS was induced in

* This study was supported by National Institutes of Health Grant R01-HL068053. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ An American Heart Association Established Investigator. To whom correspondence should be addressed: Michael E. DeBakey, Dept. of Surgery, MS NAB 2010, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-5485; Fax: 713-798-1705; E-mail: xlwang@bcm.tmc.edu.

1 The abbreviations used are: eNOS, endothelial nitric-oxide synthase; HDAC, histone deacetylase; TSA, trichostatin A; SB, sodium butyrate; ChIP, chromatin immunoprecipitation; AzadC, 5-aza-2′-deoxycytidine; HSF, human skin fibroblast cell; EMSA, electrophoresis mobility shift assay; ChIP, chromatin immunoprecipitation; DAPA, DNA affinity purification assay; DIG, digoxigenin-11-dUTP; RT, room temperature.
non-endothelial cell types by HDACs inhibitors. Moreover, Sp1 was found to bind to endogenous eNOS promoter and interacted with HDAC1.

**EXPERIMENTAL PROCEDURES**

**Reagents**—TSA, 5-aza-2'-deoxycytidine (AzadC), actinomycin D, and cycloheximide were purchased from Sigma and dissolved in dimethyl sulfoxide (MeSO) except AzadC, which was dissolved in phosphate-buffered saline. Sodium butyrate (SB) solution was purchased from Upstate Group, Inc.

**Cell Culture**—HeLa (human cervical epithelial cancer cell line) and 293 (human embryonic renal epithelial cell line) cells were cultured in Dulbecco's modified Eagle's medium (Cellgro) with 10% fetal bovine serum at 37 °C with 5% CO2. Human umbilical vein endothelial cells were purchased from Cell Applications, Inc (San Diego, CA). Human aortic endothelial cells and human coronary artery smooth muscle cells were purchased from Cell Application, Inc (San Diego, CA). HSFs were cultured in minimum essential medium Eagle's (ATCC) with 10% fetal bovine serum at 37 °C with 5% CO2. Human coronary artery smooth muscle cells were cultured in smooth muscle cell growth medium (Cell Applications) at 37 °C with 5% CO2. Human umbilical vein endothelial cells were cultured at 37 °C with 5% CO2 in EBM-2 endothelial cell basic medium with Bulletkit (Cambrex) containing hydrocortisone, fibroblast growth factor B, vascular endothelial growth factor, R2-insulin-like growth factor-1, ascorbic acid, epidermal growth factor, GA-1000, heparin, and 2% fetal bovine serum. Primary cells were used at passage 4–7 in all experiments. The regents of TSA, SB, actinomycin D, and cycloheximide were added in culture medium for 24 h at concentrations of 0.5 μg/ml, 10 μmol/liter, and 40 μg/ml, respectively, if not specifically mentioned. For corresponding controls, the same volume of MeSO or water, whichever was used as solvent, was added in culture medium.

**AzadC Treatment**—HeLa cells were treated with 7 μmol/liter AzadC for 8 days. Culture medium containing AzadC was replaced every 48 h.

**Real-time Quantitative Reverse Transcription-PCR**—Primers were designed with Primer Express Software for Real Time PCR (Applied Biosystems). Designers 2'-deoxyadenosine (Ado) (Roche Applied Science) and UV cross-linking. After blocked in the blocking buffer twice for 5 min each, the DNA was resolved through a 6% native polyacrylamide gel at 80 V (5 V/cm gel) under the aid of yeast tRNA. The recovered DNA was resuspended in 30 μl of 5 mol/liter NaCl. After proteinase K treatment, DNA was heated at 65 °C for at least 4 h to reverse the cross-link by the addition of 20 μl of a mixture of salmon sperm DNA/protein A/protein G at 4 °C with rotation for 30 min. Immunoprecipitation was carried out with 2 μg of anti-acetylhistone H3 at 4 °C overnight with rotation. After immobilization of salmon sperm DNA/protein A/protein G was added and incubated at 4 °C with rotation for 30 min and followed by brief centrifuge. The precipitates were washed twice (5 min each at 4 °C) with low salt buffer, once with high salt buffer, and once with LiCl buffer. Then the precipitates were washed again with TE buffer twice for 5 min each. The immune complexes were extracted three times with 200 μl of elution buffer. The elutes and the input were analyzed by real-time PCR using the primers covering the 5'-flanking region of the human eNOS gene.

**Transfection**—293 (human embryonic renal epithelial cell line) cells were cultured in DMEM high glucose (Invitrogen). TSA reagent was added into medium 4 h after the transfection, and the transfected cells were lysed 24 h after the transfection with cell lysis buffer (Promega). Luciferase activity was measured with a FB12 luminometer (Berthold, Germany) using luciferin (Promega) as the substrate.

**Electrophoresis Mobility Shift Assay (EMSA)**—HeLa cells were treated with 0.2 μg/ml TSA or MeSO for 24 h, and then nuclear proteins were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's instructions. EMSA was performed with a non-radioisotope method with digoxigenin-11-dUTP (DIG)-labeled probe using DIG gel shift kit (Roche Applied Science) according to the manufacturer's instructions as described below (17). Briefly, the oligonucleotide containing the human eNOS proximal Sp1 site (5'-GGA TAG GGC GGC GAG GAC G-3', 111–93) were annealed with its antisense strand and labeled at 3' end with DIG by terminal transferase. The binding reaction was performed with 30 fmol of DIG-labeled probes incubated with 5 μg of nuclear extraction in the binding buffer containing 1 μl of poly(dI-dC) in a final volume of 20 μl at room temperature (RT) for 15 min. The DNA-protein complex was resolved by gel electrophoresis through a 6% native polyacrylamide gel at 80 V (5 V/cm gel) followed by electroblotting onto a positively charged nylon membrane (Roche Applied Science) and UV cross-linking. After blocked in the blocking buffer, the blot was then incubated with anti-DIG antibody conjugated with alkaline phosphatase at RT for 30 min following washing and chemiluminescent detecting with CSPD (disodium 3-(4-methoxyxypirro1,2-dioxetane-3,2-(5'-chloro/triclo[3.3.1.1']decane)-4-ylphenylphosphate) as the substrate and exposed to x-ray film.
Histone Deacetylation and Cell-specific Expression of eNOS

RESULTS

Induction of eNOS mRNA Expression by HDACs Inhibitors in Non-endothelial Cells—To investigate whether HDACs are involved in the repression of eNOS expression in non-endothelial cells, we treated HeLa, 293, HSFs, and human coronary artery smooth muscle cells with HDACs inhibitor TSA (0.2 μg/ml) for 24 h. We found that eNOS mRNA level was significantly induced up to 8–14-fold by TSA in all the examined non-endothelial cell types (Fig. 1A). The induction of eNOS mRNA by TSA was dose-dependent in HeLa cells and all other examined cell types (Fig. 1B). SB, a HDAC inhibitor differing from TSA in structure, showed similar results in all the examined cell lines (data not shown). The induction of eNOS mRNA in non-endothelial cells by TSA or SB was not blocked by cycloheximide but was completely blocked by actinomycin D (data not shown). The eNOS mRNA level was quantitated by real-time reverse transcription-PCR and normalized to housekeeping gene β-actin. However, in contrast to its effects on eNOS in non-endothelial cell lines, TSA decreased eNOS mRNA in endothelial cells under a totally different mechanism (21).

Basal Activity of eNOS Promoter in Non-endothelial and Endothelial Cells and Activation of eNOS Promoter by HDACs Inhibitors—Previous studies demonstrated that eNOS promoter activity was mainly in the proximal region (−1001 to +109 bp) (22). In the current experiment, we raised two questions. 1) Do non-endothelial cells possess transcriptional machinery comparable with endothelial cells for eNOS promoter activation? 2) Can TSA directly activate eNOS promoter in non-endothelial cells, and if so, what is the responsive element? The epimodal promoter/reporter may help to answer the two questions and is broadly used in TSA activation studies as it can help to identify TSA-responsive elements (13–16), although it may not have the same methylation status that the corresponding endogenous DNA has. Therefore, we made a luciferase-coupled eNOS proximal promoter (−1140 to −22 bp) reporter construct. Human umbilical vein endothelial and HeLa cells were transiently transfected with the promoter/reporter constructs together with the pGL3 control plasmid. As shown in Fig. 2, the basal activity of eNOS promoter normalized as percentage of that of pGL3 control vector containing SV40 promoter and enhancer was comparable in both HeLa and human umbilical vein endothelial cells. The results suggest that non-endothelial cells possess endothelial equivalent transcriptional machinery to activate eNOS promoter. Furthermore, the episomal eNOS promoter was activated by TSA in HeLa cells, indicating that TSA could directly activate eNOS promoter in non-endothelial cells. The activation of the episomal eNOS promoter was also observed in TSA-treated 293 and in SB-treated HeLa and 293 cells (data not shown).

Increase of Acetylation of Histone H3 at eNOS 5′-Flanking Region in Non-endothelial Cells by HDACs Inhibitors—To ex-
amine the effect of HDACs inhibitors on the pattern of acetylation of histones associated with the eNOS gene, we performed the ChIP assay. To examine the chromatin status in a broad 5'-flanking region of the eNOS, we used four pairs of primers complementary to the eNOS 5'-flanking region (4917 to 13 bp before transcription start site) in the ChIP assay, including the eNOS enhancer region (4917 to 4626 bp) (23). Acetylation of histone H3 associated with the eNOS 5'-flanking region (4917 to 13 bp) in HeLa cells was low or undetectable before TSA treatment. However, a remarkable increase in acetylation of histones H3 in the same region of HeLa cells treated with TSA (0.2 μg/ml, 24 h) was observed (Fig. 3). An increase of acetylation of histone H3 was also observed in TSA-treated 293 and HSF cells and in SB-treated HeLa and 293 cells. Interestingly, the status of histone H3 at all these sites in endothelial cells was highly acetylated as comparing to non-endothelial cells, and TSA treatment did not induce a further increase of acetylation (Fig. 3).

Histone Deacetylation Involved in DNA Methylation-mediated Repression of eNOS Promoter—A recent study has demonstrated that DNA methylation strongly repressed the eNOS promoter activity (5). DNA methylation is usually linked with histone deacetylation (6). Methylated DNA can provide a dock for transcription repressors, such as MeCP2, a methylated CpG dinucleotide-binding protein. MeCP2 can recruit HDACs-mSin3A complex to the specific methylated DNA region (24). We wanted to know if this mechanism is also involved in eNOS repression in non-endothelial cells. To investigate whether histone deacetylation is involved in DNA methylation-mediated repression of the eNOS promoter, we methylated the luciferase-
coupled eNOS promoter/reporter constructs in vitro using methylase M. Sss I, which specifically methylates cytosine of CpG dinucleotides. The methylated the eNOS promoter/reporter constructs, which were confirmed by MspI and HpaII isoschizomer digestion, were then transiently transfected into HeLa cells. Methylation of the eNOS promoter DNA dramatically decreased its promoter activity to 13% of the mock-methylated promoter activity (Fig. 4). Treatment with HDAC inhibitor TSA partially reversed the repression that was induced by the DNA methylation (Fig. 4), indicating that HDAC is involved in DNA methylation-mediated repression of eNOS promoter.

Synergistic Effect of TSA and AzadC on the Induction of the eNOS Expression in Non-endothelial Cells—AzadC is an analog of cytokine, which can be incorporated into DNA and irreversibly binds to DNA methyltransferase, thus inhibiting DNA methyltransferase and reversing epigenetic silencing of methylated genes (25). To further investigate the relationship between histone deacetylation and DNA methylation in the repression of the eNOS expression in non-endothelial cells, we treated HeLa cells with DNA methyltransferase inhibitor AzadC (7 μM) alone for 8 days, 1 μg/ml alone for 24 h, or AzadC for 7 days combined with TSA for another 24 h. TSA and AzadC synergistically induced eNOS mRNA expression in HeLa cells (Fig. 5). However, AzadC alone only mildly induced the eNOS expression compared with the effect of TSA (1 μg/ml) alone (Fig. 5).

DNA Methylation Status of the eNOS Proximal Promoter of HeLa Cells after Treatment with AzadC or TSA—To investigate the DNA methylation status of the eNOS proximal promoter in HeLa cells after treatment with AzadC or TSA, we performed sodium bisulfite genomic sequencing for this region. The DNA methylation status of eNOS proximal promoter of non-treated and treated HeLa cells was summarized in Fig. 6. There were two CpG dinucleotides within the high affinity proximal Sp1 site (−102 and −97), and they were 36.3 and 54.5% methylated, respectively. However, 40% of the two CpG dinucleotides were unmethylated. Treatment with AzadC resulted in a statistically significant decrease of methylation in 8 of 12 (66.6%) CpG dinucleotides in the eNOS proximal promoter region (Fig. 6A). Moreover, the two CpG dinucleotides (−102 and −97) within the proximal Sp1 site totally lost their methylation after the AzadC treatment (Fig. 6A). Treatment with TSA only resulted in the decrease of methylation in 2 of 12 (16.6%) CpG dinucleotides in the eNOS proximal promoter (Fig. 6B).

Roles of Sp1 Site in Induction of eNOS by TSA in Non-endothelial Cells—The proximal Sp1 site is crucial for eNOS promoter activity (22). Sp1 site is suggested to be a TSA-responsive element in several studies (26–28). It would be necessary to know whether HDAC inhibitors can enhance DNA binding activity of Sp1 transcription factor itself. We performed EMSA with a DIG-labeled probe containing the high affinity proximal Sp1 site of eNOS promoter. Incubation of nuclear extracts from HeLa cells treated with or without TSA with the Sp1 probe resulted in one major retarded band, which was competed by adding a 100-fold molar excess of the unlabeled probe (Fig. 7A). The intensity patterns of the retarded band between the nuclear extracts from TSA-treated and non-treated cells were the same, indicating that DNA binding activity of the Sp1 factor was not activated by TSA. Similar results were also observed in TSA-treated HSF and 293 cells
Histone Deacetylation and Cell-specific Expression of eNOS

DISCUSSION

In the present study, we have provided evidence for the first time that HDACs are involved in the repression of the human eNOS expression in non-endothelial cells. The repression of the eNOS could be partially relieved in several non-endothelial cell types by the inhibition of HDACs (Fig. 1). The induction of eNOS in non-endothelial cells by HDACs inhibitors is more likely at the transcriptional level and less likely due to the enhancement of eNOS mRNA stabilization, since actinomycin D (an inhibitor of DNA-dependent RNA synthesis) completely blocked the induction of eNOS by TSA. Moreover, this eNOS induction in non-endothelial cells appears independent of new protein synthesis, since blocking protein synthesis by cycloheximide did not alter the TSA-induced eNOS mRNA expression. Given that the episomal eNOS promoter/reporter was directly activated by TSA, it is implicated that HDACs may be directly involved in the repression of eNOS promoter in non-endothelial cells. Our experiments have further shown that activities of episomal eNOS promoter/reporter were comparable in endothelial and non-endothelial cells (Fig. 2), indicating that endothelial cells and non-endothelial cells (at least among the cell types we have tested) may have equivalent transcriptional machinery necessary for eNOS transcription. Our study also suggests that the induction of eNOS in non-endothelial cells by HDAC inhibitors could be mediated by direct utilization of the existing transcriptional machinery. The reason that non-endothelial cells do not constitutively express eNOS would be more likely due to repressive chromatin structure.

Histone acetylation is a critical component of chromatin remodeling and transcriptional regulation (6). The acetylation level of core histone results from the balance between the activities of HDACs and histone acetyltransferases. Inhibition of HDACs by the TSA leads to activation of only specific target genes through increased histone acetylation (11, 12). Our experiments showed that induction of eNOS expression by TSA in non-endothelial cells was accompanied by a remarkable increase in acetylation of histone H3 associated with the eNOS 5'-flanking region (−4917 to −13) (Fig. 3). The increase of the core histone acetylation at the broad 5'-flanking region of the eNOS gene after the TSA treatment indicates that the chromatin structure of eNOS promoter area may become a loose and non-condensed structure, which is usually necessary for the start of transcription (8, 9). In contrast, histone H3 of the

binding with the endogenous eNOS promoter or how HDACs were involved in the suppression. The association of Sp1 with HDAC1 was first reported by Doetzlhofer et al. (20), and later the association was shown to be involved in repress genes in several cell lines, including human telomerase reverse transcriptase (29), human luteinizing hormone receptor (30), and transforming growth factor β type II receptor (15). Using a co-immunoprecipitation assay, we tested whether Sp1 was associated with HDAC1 in HeLa cells. As shown in Fig. 8, HDAC1 was detected in anti-Sp1 antibody-precipitated complex, and reversely, Sp1 was also detected in anti-HDAC1 antibody-precipitated complex. But neither Sp1 nor HDAC1 was revealed in negative control anti-FLAG antibody-precipitated complex. Sp1 was shown as a 105-kDa abundant isofrom and a 95-kDa less abundant isofrom, which may be difficult to be detected by Western blot unless a large amount of proteins are loaded. Notably, anti-HDAC1 antibody pulled down an almost equal amount of the two isofroms, implicating that HDAC1 may preferably interact with the 95-kDa isofrom of Sp1. Treatment with TSA or AzadC alone did not alter the association of HDAC1 with Sp1 (Fig. 8C). However, treatment with AzadC and TSA together decreased the association of HDAC1 with Sp1 (Fig. 8).

((data not shown). To further examine the role of the proximal Sp1 site in induction of eNOS by TSA in non-endothelial cells, we mutated five nucleotides within the proximal Sp1 site in the luciferase-coupled eNOS promoter/reporter construct and transfected the construct into HeLa cells. A luciferase assay showed that the basal activity of Sp1 site-mutated eNOS promoter was only 4% that of the wild type (Fig. 7B). To examine whether the binding ability of mutant Sp1 site to Sp1 factor was affected, we did a DAPA assay with the biotinylated probes containing the wild type or the mutant Sp1 site as in the promoter/reporter construct. Corresponding to the results of luciferase assay, DAPA assay showed that the mutant Sp1 site signficantly decreased its binding ability to only 13% of the wild type according to the density measurement (Fig. 7C). Unexpectedly, the mutation of the proximal Sp1 site did not cause a loss of response to TSA treatment, as compared to the wild type (Fig. 7D).

To further investigate the roles of the proximal Sp1 site in TSA-induced eNOS expression in non-endothelial cells, it is important to know whether Sp1 binds to eNOS promoter in vivo in non-endothelial cells. To answer this question, we performed a ChIP assay with Sp1 antibody in HeLa cells. The Sp1 antibody-precipitated chromatin was subjected to DNA extraction and PCR with the primers covering the high affinity proximal Sp1 site (−111/−39). We did four separated experiments and showed that Sp1 bound to the endogenous eNOS promoter in HeLa cells in every assay. As shown in Fig. 8, the occupancy of the endogenous eNOS promoter by Sp1 did not change after treatment with TSA, consistent with our EMSA results. Treatment with AzadC alone also did not alter Sp1 occupancy of the endogenous eNOS promoter (Fig. 8A). However, the combined treatment of AzadC and TSA increased Sp1 occupancy of the endogenous eNOS promoter (Fig. 8A).

We further investigated how eNOS was transcriptionally suppressed in non-endothelial cells when Sp1 factor was in

histone Deacetylation and Cell-specific Expression of eNOS

DISCUSSION

In the present study, we have provided evidence for the first time that HDACs are involved in the repression of the human eNOS expression in non-endothelial cells. The repression of the eNOS could be partially relieved in several non-endothelial cell types by the inhibition of HDACs (Fig. 1). The induction of eNOS in non-endothelial cells by HDACs inhibitors is more likely at the transcriptional level and less likely due to the enhancement of eNOS mRNA stabilization, since actinomycin D (an inhibitor of DNA-dependent RNA synthesis) completely blocked the induction of eNOS by TSA. Moreover, this eNOS induction in non-endothelial cells appears independent of new protein synthesis, since blocking protein synthesis by cycloheximide did not alter the TSA-induced eNOS mRNA expression. Given that the episomal eNOS promoter/reporter was directly activated by TSA, it is implicated that HDACs may be directly involved in the repression of eNOS promoter in non-endothelial cells. Our experiments have further shown that activities of episomal eNOS promoter/reporter were comparable in endothelial and non-endothelial cells (Fig. 2), indicating that endothelial cells and non-endothelial cells (at least among the cell types we have tested) may have equivalent transcriptional machinery necessary for eNOS transcription. Our study also suggests that the induction of eNOS in non-endothelial cells by HDAC inhibitors could be mediated by direct utilization of the existing transcriptional machinery. The reason that non-endothelial cells do not constitutively express eNOS would be more likely due to repressive chromatin structure.

Histone acetylation is a critical component of chromatin remodeling and transcriptional regulation (6). The acetylation level of core histone results from the balance between the activities of HDACs and histone acetyltransferases. Inhibition of HDACs by the TSA leads to activation of only specific target genes through increased histone acetylation (11, 12). Our experiments showed that induction of eNOS expression by TSA in non-endothelial cells was accompanied by a remarkable increase in acetylation of histone H3 associated with the eNOS 5'-flanking region (−4917 to −13) (Fig. 3). The increase of the core histone acetylation at the broad 5'-flanking region of the eNOS gene after the TSA treatment indicates that the chromatin structure of eNOS promoter area may become a loose and non-condensed structure, which is usually necessary for the start of transcription (8, 9). In contrast, histone H3 of the
proximal promoter and the putative enhancer of eNOS gene in endothelial cells are already in a hyperacetylated status before the TSA treatment (Fig. 3), a typical phenomenon for activated chromatin (8, 9). These data provide further evidence that histone deacetylation plays an important role in the repression of eNOS in non-endothelial cells.

DNA methylation is another important epigenetic process involved in regulating chromatin structure and gene transcription (6). DNA methylation is linked with histone deacetylation through a transcriptional repressor MeCP2 (a methylated CpG-binding protein), which recruits HDACs to specific promoter region, resulting in repressive chromatin structure and suppress gene expression (24, 31). A recent study has demonstrated that eNOS proximal promoter DNA is heavily methylated in non-endothelial cells and that DNA methylation plays an important role in the repression of eNOS in non-endothelial cells.

DNA methylation is another important epigenetic process involved in regulating chromatin structure and gene transcription (6). DNA methylation is linked with histone deacetylation through a transcriptional repressor MeCP2 (a methylated CpG-binding protein), which recruits HDACs to specific promoter region, resulting in repressive chromatin structure and suppress gene expression (24, 31). A recent study has demonstrated that eNOS proximal promoter DNA is heavily methylated in non-endothelial cells and that DNA methylation plays an important role in the repression of eNOS in non-endothelial cells.

DNA methylation is another important epigenetic process involved in regulating chromatin structure and gene transcription (6). DNA methylation is linked with histone deacetylation through a transcriptional repressor MeCP2 (a methylated CpG-binding protein), which recruits HDACs to specific promoter region, resulting in repressive chromatin structure and suppress gene expression (24, 31). A recent study has demonstrated that eNOS proximal promoter DNA is heavily methylated in non-endothelial cells and that DNA methylation plays an important role in the repression of eNOS in non-endothelial cells.

DNA methylation is another important epigenetic process involved in regulating chromatin structure and gene transcription (6). DNA methylation is linked with histone deacetylation through a transcriptional repressor MeCP2 (a methylated CpG-binding protein), which recruits HDACs to specific promoter region, resulting in repressive chromatin structure and suppress gene expression (24, 31). A recent study has demonstrated that eNOS proximal promoter DNA is heavily methylated in non-endothelial cells and that DNA methylation plays an important role in the repression of eNOS in non-endothelial cells.
mediated repression of eNOS in non-endothelial cells. Consensus Sp1 site has been shown to be a responsive element in several HDAC-regulated genes (26–28). In the case of eNOS, the proximal Sp1 site also appeared as a key element in TSA induction of eNOS expression in non-endothelial cells. Consistent with the published data (22), the proximal Sp1 site was responsible for most (96%) of the eNOS proximal promoter basal activity since the mutation within the site resulted in only 13% of Sp1 binding ability and 4% of basal activity (Fig. 7). Furthermore, we demonstrated with the Sp1 ChIP assay that Sp1 still bound to the endogenous eNOS proximal promoter (containing the proximal Sp1 site) in vivo (Fig. 8), although eNOS is repressed in HeLa cells. Treatment with AzadC resulted in unmethylation status of both CpG dinucleotides within the proximal Sp1 site (Fig. 6A); however, the occupancy of the endogenous eNOS promoter by Sp1 showed no difference between the AzadC-treated and -non-treated HeLa cells (Fig. 8A), indicating that the binding to the endogenous eNOS promoter is not affected by the Sp1 site methylation.

Based on these results it would be logical to ask how eNOS is repressed in non-endothelial cells by HDACs. Our results indicate that the induction of eNOS in non-endothelial cells by HDACs inhibitor is mediated not through increased Sp1 DNA binding activity or its occupancy of endogenous eNOS proximal promoter (Figs. 7A and 8A). Doetzlhofer et al. (20) suggest that Sp1 can serve as a target for HDAC1-mediated transcriptional repression, although Sp1 is usually known as a positive transcription factor. Studies have further shown that the association of HDAC1 or 2 with Sp1 and Sp3 is involved in repressing gene expression in several cell lines (15, 29, 30), which could be a universal mechanism for cell-specific repression of the Sp1 dependent expression of genes. Indeed, our study also has demonstrated the interaction between HDAC1 and Sp1 in HeLa cells (Fig. 8). Given that the proximal Sp1 site is crucial for eNOS promoter activity, Sp1 associates with HDAC1, and Sp1 binds to the endogenous eNOS proximal promoter, it is likely that the proximal Sp1 site may serve as a target for HDAC1-involved eNOS suppression in non-endothelial cells. Based on these findings, we propose that repression of eNOS in non-endothelial cells is mediated by the recruitment of HDACs to the proximal promoter region by the complex of methylated DNA, MeCP2, and other co-repressors and, more importantly, the recruitment of HDAC1 by Sp1 to the proximal Sp1 site. We further speculate that the association of HDAC1 with Sp1 somehow limits the Sp1 transcriptional activating function. The HDAC-specific inhibitor TSA abrogates HDAC1 enzymatic activities of deacetylation and, hence, shifts acetylation status of core histones to hyperacetylation and restores Sp1 transcriptional function. The synergistic effect on eNOS activation in HeLa cells by the combined treatment of AzadC and TSA could
be the results of increased Sp1 binding to the endogenous eNOS promoter and decreased association of Sp1 with HDAC1 (Fig. 8). Further studies will be needed to understand how the association of HDAC1 with Sp1 inhibits the Sp1 function and how TSA restores the function of Sp1.

Our experiments have also shown that episomal eNOS promoter was not repressed in non-endothelial cells and was responsive to TSA activation. After the episomal eNOS promoter, which is methylation-free, is transfected into the non-endothelial cells, it will form mini-chromatin but not in a repressive chromatin structure as the endogenous eNOS promoter in these cells. Therefore, the balance between HDAC-related inhibition and histone acetyltransferase-related activation determines the basal activity of the episomal eNOS promoter. Inhibition of HDACs by TSA will shift the balance and lead to the episomal promoter activation.

In conclusion, our study has demonstrated that HDACs are involved in the eNOS repression in non-endothelial cells. The proximal Sp1 site may be the key target for HDAC1-mediated inhibition of eNOS in non-endothelial cells. The findings that constitutively repressed eNOS gene in non-endothelial cells can be re-activated by the inhibition of HDACs will not only enhance our understanding in tissue-specific eNOS expression but will also have much wider implications in several clinically relevant processes including developmental differentiation, stem cell transformation, and functional restoration after vascular injury. Further investigations in dissecting exact mechanisms about how the association of HDAC1 with Sp1 prevents Sp1 transcriptional activation will be needed to understand tissue-specific expression of genes in endothelium.

REFERENCES
1. Li, H., Wallerath, T., Munzel, T., and Forstermann, U. (2002) *Nitric Oxide* 7, 149–164
2. Teichert, A. M., Miller, T. L., Tai, S. C., Wang, Y., Bei, X., Robb, G. B., Phillips, M. J., and Mavrden, P. A. (2000) *Am. J. Physiol. Heart Circ. Physiol.* 278, 1352–1361
3. Guillot, P. V., Liu, L., Kuivenhoven, J. A., Guan, J., Rosenberg, R. D., and Aird, W. C. (2000) *Physiol. Genomics* 2, 77–83
4. van Haperen, R., Cheng, C., Mees, B. M., van Deel, E., de Waard, M., van Damme, L. C., van Gent, T., van Aken, T., Krams, R., Duncker, D. J., and de Crom, R. (2003) *Am. J. Pathol.* 163, 1677–1686
5. Chan, Y., Fish, J. E., D’Abreo, C., Lin, S., Robb, G. B., Teichert, A. M., Karantoniou-Fegaras, F., Keightley, A., Steer, B. M., and Marsden, P. A. (2004) *J. Biol. Chem.* 279, 35087–35100
6. Geiman, T. M., and Robertson, K. D. (2002) *J. Biol. Chem.* 87, 117–125
7. Iinuki, M., and Smith, M. M. (2003) *Curr. Opin. Genet. Dev.* 13, 154–160
8. Davie, J. R., and Spencer, V. A. (1999) *J. Cell. Biochem. Suppl.* 32–33, 141–148
9. Pazin, M. J., and Kadonaga, J. T. (1997) *Cell* 89, 325–328
10. Legube, G., and Trouche, D. (2000) *EMBO Rep.* 1, 944–947
11. Van Lint, C., Emiliani, S., and Verdin, E. (1996) *Gene Expr.* 5, 245–253
12. Della Rapione, F., Crimi, V., Della Pietra, V., Borril, O., Oliva, A., Indaco, S., Yamamoto, T., and Zappia, V. (2001) *FEBS Lett.* 499, 199–204
13. Cong, Y. S., and Bacchetti, S. (2000) *J. Biol. Chem.* 275, 35665–35668
14. Zhao, S., Venkatsubbarao, K., Li, S., and Freeman, J. W. (2003) *Cancer Res.* 63, 2624–2630
15. Zhang, X., Wharton, W., Yuan, Z., Tsai, S. C., Ohashi, N., and Seto, E. (2004) *Mol. Cell. Biol.* 24, 5106–5118
16. Wang, J., Dudley, D., and Wang, X. L. (2002) *Arterioscler. Thromb. Vasc. Biol.* 22, e1–e4
17. Warnecke, P. M., Stirzaker, C., Song, J., Grunau, C., Melki, J. R., and Clark, S. J. (2002) *Methods* 27, 101–107
18. Li, H., and Dahiya, R. (2002) *Bioinformatics* 18, 1427–1431
19. Doetschhofer, A., Rotheneder, H., Lagger, G., Kuranda, M., Kurtev, V., Bresch, G., Wintersberger, E., and Seiser, C. (1999) *Mol. Cell. Biol.* 19, 5504–5511
20. Rossig, L., Li, H., Fisslthaler, B., Urbich, C., Fleming, I., Forstermann, U., Zeiher, A. M., and Dimmeler, S. (2002) *Circ. Res.* 91, 837–844
21. Karantoniou-Fegaras, F., Antoniou, H., Lai, S. L., Kulkarni, G., D’Abreo, C., Wong, G. K., Miller, T. L., Chan, Y., Atkins, J., Wang, Y., and Marsden, P. A. (1999) *J. Biol. Chem.* 274, 3076–3083
22. Laumonnier, Y., Nadaud, S., Agrapart, M., and Soubrier, F. (2000) *J. Biol. Chem.* 275, 40732–40741
23. Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolfe, A. P. (1998) *Nat. Genet.* 19, 187–191
24. Brown, R., and Plumb, J. A. (2004) *Expert Rev. Anticancer Ther.* 4, 501–510
25. Sowa, Y., Orita, T., Minamikawa, S., Nakano, K., Mizuno, T., Nomura, H., and Sakai, T. (1997) *Biochem. Biophys. Res. Commun.* 241, 142–150
26. Kim, H. S., Park, J. S., Hoon, S. J., Woo, M. S., Kim, S. Y., and Kim, K. S. (2003) *Biochem. Biophys. Res. Commun.* 312, 960–967
27. Camarero, N., Nadal, A., Barrero, M. J., Haro, D., and Marreño, P. F. (2003) *Nucleic Acids Res.* 31, 1693–1703
28. Won, J., Yim, J., and Kim, T. K. (2002) *J. Biol. Chem.* 277, 38239–38228
29. Zhang, Y., and Dulfu, M. L. (2002) *J. Biol. Chem.* 277, 33431–33438
30. Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, R. M., Eisenman, R. N., and Bird, A. (1998) *Nature* 393, 386–389