Nitric Oxide Modulates Chromatin Folding in Human Endothelial Cells via Protein Phosphatase 2A Activation and Class II Histone Deacetylases Nuclear Shuttling

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Abstract—Nitric oxide (NO) modulates important endothelial cell (EC) functions and gene expression by a molecular mechanism which is still poorly characterized. Here we show that in human umbilical vein ECs (HUVECs) NO inhibited serum-induced histone acetylation and enhanced histone deacetylase (HDAC) activity. By immunofluorescence and Western blot analyses it was found that NO induced class II HDAC4 and 5 nuclear shuttling and that class II HDACs selective inhibitor MC1568 rescued serum-dependent histone acetylation above control level in NO-treated HUVECs. In contrast, class I HDACs inhibitor MS27–275 had no effect, indicating a specific role for class II HDACs in NO-dependent histone deacetylation. In addition, it was found that NO ability to induce HDAC4 and HDAC5 nuclear shuttling involved the activation of the protein phosphatase 2A (PP2A). In fact, HDAC4 nuclear translocation was impaired in ECs expressing small-t antigen and exposed to NO. Finally, in cells engineered to express a HDAC4-Flag fusion protein, NO induced the formation of a macromolecular complex including HDAC4, HDAC3, HDAC5, and an active PP2A. The present results show that NO-dependent PP2A activation plays a key role in class II HDACs nuclear translocation. **(*Circ Res*. 2008;102:51-58.)**

Key Words: nitric oxide ■ endothelial cells ■ histone deacetylases ■ chromatin

In endothelial cells (ECs) laminar shear stress (SS), the tangential component of hemodynamic forces, enhances NO production via endothelial nitric oxide synthase (eNOS) activation.[1] SS-dependent transcriptional responses are partly attributable to NO production,[2] and NO has been demonstrated to downregulate gene expression in endothelial cells.[3] Nevertheless, the mechanism by which NO regulates gene expression is still unclear.

Changes in chromatin folding are the prerequisite for genes to be turned "on" and "off".[4] Histones acetyltransferases (HATs) and deacetylases (HDACs) are histones modifying enzymes involved in the regulation of chromatin unwinding and wrapping, respectively. HAT activity is mainly linked to transcriptional activation, because acetylated histone tails decrease their affinity for DNA, facilitating the recruitment of other chromatin associated transcriptional complexes. HDACs catalyze the removal of acetyl groups from histone tails, compressing chromatin and promoting the repression of transcription.[5] The reversible nature of acetylation allows chromatin structure to be tightly regulated to permit the fine tuning of gene expression and a disregulated histone deacetylase activity, together with inappropriate epigenetic patterns, have been recently associated with cancer.[6,7] Four classes of histone deacetylases are currently known,[5] being class II HDACs mainly involved in the regulation of differentiation programs.[8] Although the role of histone deacetylases in the biology of the cardiovascular system is still poorly characterized, different HDACs have been involved in the differentiation of stem cells into endothelial cells[9] or in the commitment of endothelial progenitor cells[10] and in the maintenance of vascular integrity.[11]

Our previous work demonstrated that SS regulates gene expression in human ECs and directs embryonic stem cell (ES) differentiation toward the cardiovascular lineage, two processes both associated to the activation of HATs and the opening of chromatin.[12,13] In those experiments, SS-dependent histone acetylation and HATs activation was transient, showing a peak between 1 and 2 hours of SS and

Original received December 22, 2006; resubmission received June 5, 2007; revised resubmission received October 12, 2007; accepted October 22, 2007.

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Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.107.157305**

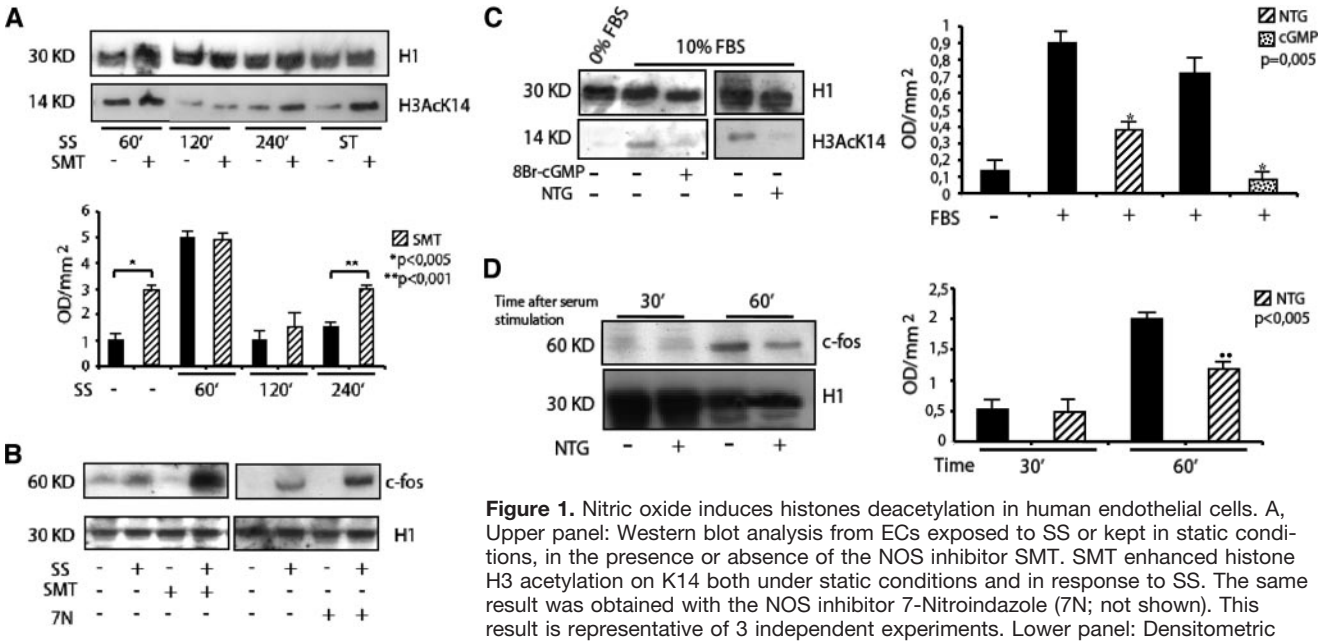

Figure 1. Nitric oxide induces histones deacetylation in human endothelial cells. A, Upper panel: Western blot analysis from ECs exposed to SS or kept in static conditions, in the presence or absence of the NOS inhibitor SMT. SMT enhanced histone H3 acetylation on K14 both under static conditions and in response to SS. The same result was obtained with the NOS inhibitor 7-Nitroindazole (7N; not shown). This result is representative of 3 independent experiments. Lower panel: Densitometric analysis. B, Western blot analysis from ECs exposed to SS (4 hours) or kept in static conditions, in the presence or absence of NOS inhibitors SMT and 7N. NOS inhibitors enhanced SS-induced c-fos expression. The result is representative of 3 independent experiments. C, ECs were starved overnight in culture medium without serum; the day after, were shifted to complete medium supplemented with 10% FBS for 1 hour, either in the presence or absence of NTG or 8Br-cGMP. Left panel: total cell extracts were analyzed by Western blot for acetylated histone H3. The result is representative of at least 4 independent experiments. Right panel: densitometric analysis. D, ECs were starved over night in culture medium without serum; the day after, were shifted to complete medium supplemented with 10% FBS for 1 hour, in the presence or absence of NTG. Left panel: total cell extracts were analyzed by Western blot for c-fos protein. NTG treatment impaired serum-dependent c-fos expression. The result is representative of at least 4 independent experiments. Right panels: densitometric analysis. OD indicates optical density.

declining shortly after. In cells treated with the histone deacetylase inhibitor Trichostatin A (TSA), however, histone acetylation remained elevated beyond the 4 hours time point.[12] Based on this observation, we hypothesized a potential involvement of a HDAC-dependent activity in this process, in a time frame compatible with NO production.[14]

In the present study, we show that NO induces class II HDAC4 and 5 nuclear localization in human endothelial cells. This phenomenon is associated with a decrease in histone acetylation and c-fos expression and may provide a novel molecular mechanism for NO-dependent effect on gene expression.[3]

Materials and Methods

Cell Culture and Treatments

HUVEC were cultured as previously described.[12] Treatments were performed as described in supplemental Materials and Methods (available online at http://circres.ahajournals.org).

HEK 293 cells were grown at 37°C in a 5% CO_2 atmosphere in complete Dulbecco's Modified Eagle Medium (GIBCO) containing 0.11g/L Pyridoxine, complemented with 2 mmol/L L-Glutamine, 0.1 mg/mL Penicillin-Streptomycin, 10% (v/v) Fetal Calf Serum (FCS, GIBCO), 250 μg/mL G418, 0.5 μg/mL Puromycin, and 100 μg/mL Hygromycin. Small-t antigen adenoviral and retroviral infection of HUVECs were performed as previously described.[15]

Immunofluorescence

Immunofluorescences were performed as previously described.[13]

Cell Extracts and Western Blot

For a detailed protocol of cell fractionation procedure see supplemental Materials and Methods. Western blots were performed as previously described.[12,13]

HDAC Assay

HDAC assays were performed by using the HDAC activity assay Kit (Upstate Biotechnology) according to the manufacturer's instructions. For a detailed protocol see supplemental Materials and Methods.

Phosphatase Assay

Phosphatase (PPase) assay were performed by using the Ser/Threo Phosphatase Assay System (Promega) according to the manufacturer's instructions for the detection of PP2A-specific activity. Total cell extracts were performed by using a standard RIPA buffer, without phosphatase inhibitors.

Plasmid and HDAC4-Flag Purification

HDAC4-Flag was cloned in a pTRE2 hyg vector (Clontech) and stably transfected into an HEK 293 EBNA-1 cell line constitutively expressing both Tet rtTA2^s-S2 activator and rTS repressor. To express HDAC4, cells were stimulated 24 hours with 1 μg/mL doxocyclin. After induction, cells were collected and the protein was absorbed onto an anti-Flag resin (Sigma) before competitive elution with a 3× Flag peptide, according to manufacturer instructions.

Confocal Analysis

Confocal analysis, for the detection of HDAC4 and PP2A colocalization, was performed by using an anti-HDAC4 antibody (Santa Cruz Biotechnology) and an anti-PP2Ac antibody (Transduction Laboratory). Nuclei were stained with Topro3 dye. Samples were analyzed using a Zeiss LSM510 Meta Confocal Microscope. Lasers' power, beam splitters, filter settings, pinhole diameters, and scan mode were the same for all examined samples of each sample. Fields reported in the figure are representative of all examined fields.

Statistical Analysis

Statistical analysis was performed as previously described.[12,13]

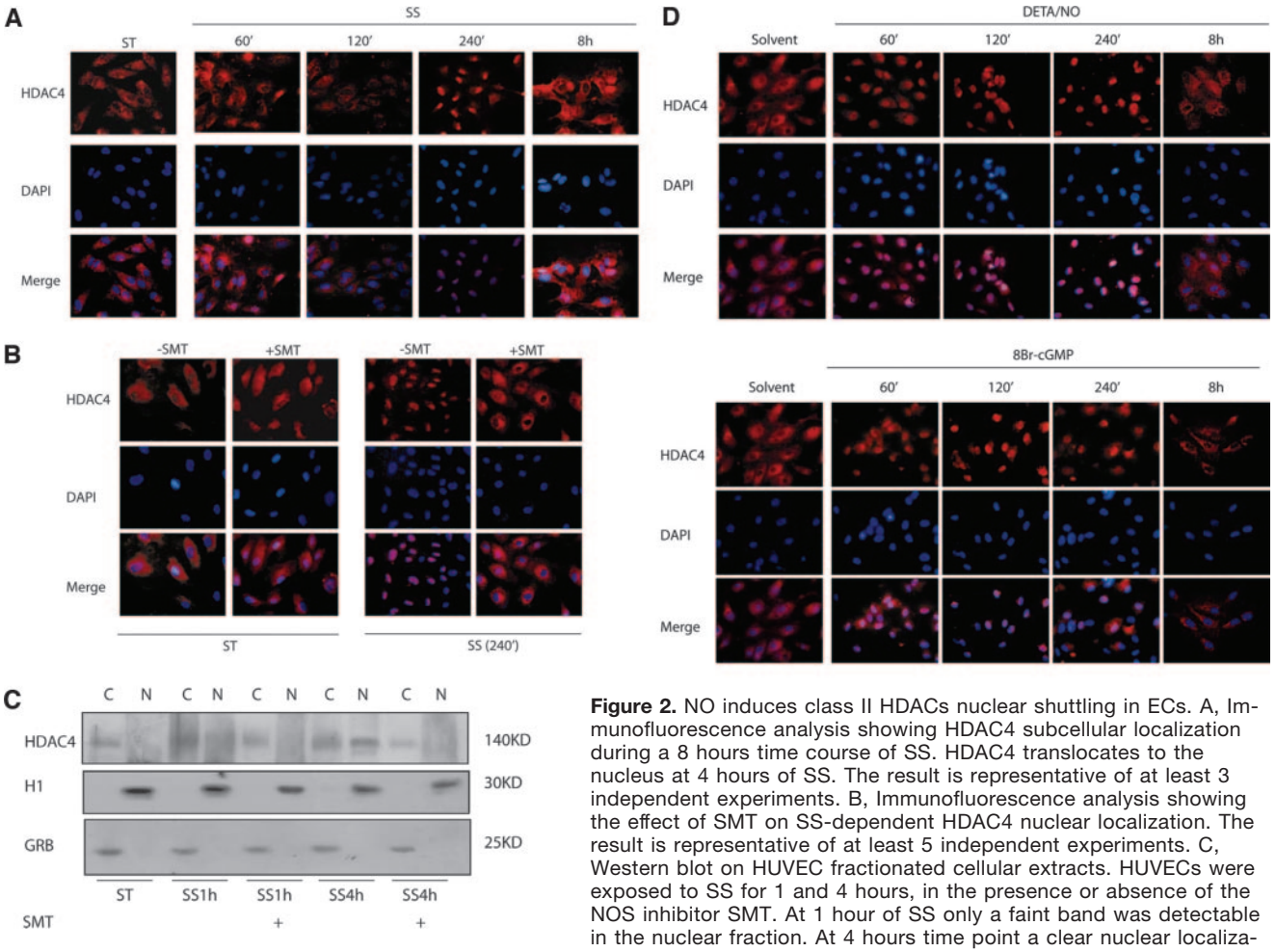

Figure 2. NO induces class II HDACs nuclear shuttling in ECs. A, Immunofluorescence analysis showing HDAC4 subcellular localization during a 8 hours time course of SS. HDAC4 translocates to the nucleus at 4 hours of SS. The result is representative of at least 3 independent experiments. B, Immunofluorescence analysis showing the effect of SMT on SS-dependent HDAC4 nuclear localization. The result is representative of at least 5 independent experiments. C, Western blot on HUVEC fractionated cellular extracts. HUVECs were exposed to SS for 1 and 4 hours, in the presence or absence of the NOS inhibitor SMT. At 1 hour of SS only a faint band was detectable in the nuclear fraction. At 4 hours time point a clear nuclear localization of HDAC4 was observed. SMT treatment completely abolished this phenomenon either at 1 hour or at 4 hours time point. The result is representative of 3 independent experiments. D, ECs were treated for 1 to 8 hours with either DETA/NO or 8Br-cGMP. Immunofluorescence shows NO and cGMP-dependent HDAC4 nuclear translocation at 1 hour of treatment. The phenomenon was transient as the enzyme completely return to the cytoplasm at 8 hours time point. The result is representative of at least 8 independent experiments.

Results

NO Induces Histones Deacetylation in Human Endothelial Cells

SS rapidly induced histone H3 acetylation in human ECs and mouse ES.[12,13] The phenomenon was transient, reaching a peak between 1 and 2 hours and decreasing at the 4 hours time point. To investigate whether a link between NO production and histone deacetylation may exist, HUVECs were exposed to SS for 1 to 4 hours in the presence or absence of the nitric oxide synthase inhibitor S-methylthiosourea (SMT). Western blot analysis was performed to detect acetylated histones. SS-dependent histone H3 acetylation on lysine 14 (K14) was detectable at the 1 hour time point, as expected. Surprisingly, SMT enhanced histone H3 acetylation both under static and at 4 hours time point of SS treamtent (Figure 1A). The same result was obtained with an another NOS inhibitor, the 7-Nitroindazole (7N; not shown). This response was associated with an enhanced SS-dependent c-fos expression (Figure 1B). To further investigate the effect of NO on histone acetylation levels, ECs were exposed to a pulse of serum after an overnight starvation, a condition which induces acetylation of histone H3,[16] and treated either with the potent NO donor nitroglycerin (NTG) or with the NO second messenger 8Bromide-Cyclic Guanosine Monophospate (8Br-cGMP). Western blot analysis on total histones revealed that NTG and cGMP decreased serum-dependent histone H3 acetylation (Figure 1C) and this was paralleled by the inhibition of serum-induced c-fos expression (Figure 1D). Consistently, treatment of ECs with NTG produced an enhancement in nuclear histone deacetylase activity, as indicated by a HDAC activity assay performed on nuclear extracts (supplemental Figure I).

NO Induces HDAC4 and HDAC5 Nuclear Shuttling in HUVECs

To clarify NO-dependent mechanism involved in the regulation of gene expression under SS conditions, the subcellular localization of class I and II HDACs was investigated by immunofluorescence. In response to 4 hours of SS class II HDAC4 translocated to the nucleus of ECs, to return in the cytoplasm at 8 hours time point (Figure 2A). Interestingly, this phenomenon was inhibited by SMT treatment (Figure 2B

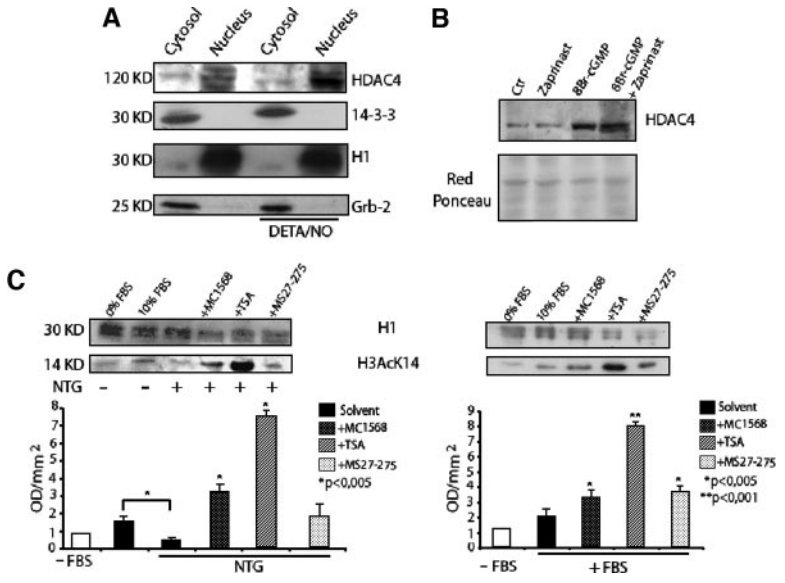

Figure 3. NO stimulates class II HDACs activity. A, Western blot analysis on HUVEC fractionated cellular extracts. In the presence of DETA/NO, the ECs cytosolic fraction was completely deprived of HDAC4, which was highly enriched in the nuclear fraction. Histone H1 and Grb-2 proteins were used to verify the purity of the cell compartments and the loading of proteins. The result is representative of 3 independent experiments. B, HUVECs were treated for 4 hours with 8Br-cGMP, in the presence or absence of the PDEs' inhibitor Zaprinast. Upper panel: Western blot analysis on HUVEC nuclear extracts shows the presence of HDAC4 in ECs nuclei in the presence of cGMP. A further enrichment is observed in Zaprinast-treated cells. Lower panel: red ponceau staining was used to normalize protein loading. The result is representative of 3 independent experiments. C, HUVECs were starved for an overnight and the day after were shifted for 1 hour to complete medium supplemented with 10% FBS, with or without NTG, with or without histone deacetylase inhibitors. Western blot analysis was performed on total extracts. Left upper panel: in the presence of NTG, serum-induced histone H3 acetylation is reduced. The class II HDACs selective inhibitor MC1568 prevented NTG-dependent histone deacetylation, whereas the class I specific inhibitor MS27–275 had a minor effect. TSA induced H3 hyperacetylation, as expected. Histone H1 was used to normalize protein loading. Right upper panel: control Western blot experiment showing the effect of the different HDACs inhibitors used on serum-induced histone H3 acetylation. HUVECs were starved for an overnight and the day after were shifted for 1 hour to complete medium supplemented with 10% FBS, with or without histone deacetylase inhibitors. TSA greatly enhanced serum-induced histone acetylation. MS27–275 and MC1568 also produced an increase in serum-dependent histone H3 acetylation, being MC1568 less effective. Histone H1 was used to normalize protein loading. The result is representative of 3 independent experiments. Lower panels: densitometric analysis. OD indicates optical density.

and 2C). The same results were obtained for HDAC5 (not shown), whereas other HDACs tested (1, 2, 3, 6, 7, 8, 9) did not show any change in their cellular localization (not shown). To further verify NO effect on the subcellular localization of class II HDAC4 and 5, ECs were treated either with diethylenetriamine/nitric oxide adduct (DETA/NO), to allow a constant NO release, or with 8BR-cGMP for 1 to 8 hours, and immunofluorescence analysis was performed. As shown in Figure 2D, HDAC4 shuttled to the nucleus at 1 hour of treatment, to return in the cytoplasm at 8 hours time point. HDAC5, which had a more diffuse localization under basal conditions, showed a nuclear enrichment on DETA/NO or 8Bromide-cGMP exposure (supplemental Figure II). Interestingly, in cells treated with 8Br-cGMP, HDAC4 and 5 begin to exit from the nucleus at 4 hours time point. cGMP is metabolized by phosphodiesterases[17] (PDEs), and PDE5 has a major role in the pathophysiology of the cardiovascular system.[18,19] Treatment of ECs with the PDE5 inhibitor Zaprinast allowed a nuclear enrichment in HDAC4 protein levels at 4 hours of cGMP treatment, as demonstrated by Western blot on ECs nuclear extracts (Figure 3B). In addition, blocking either Erk or p38 kinase, the latter being involved in SS-induced histone acetylation,[12] did not interfere with NO-dependent HDAC4 nuclear shuttling; indeed, inhibiting cGMP-activated protein kinase G (PKG) completely abolished HDAC4 nuclear translocation (see supplemental Results and Figure III), addressing a specific role to the NO / cGMP / PKG axis in modulating class II HDACs subcellular localization. Similar results were obtained with HDAC5 (not shown).

NO Induces Class II HDACs-Dependent Histone Deacetylase Activity in ECs

A series of experiment were performed to investigate whether NO-stimulated histone deacetylation relied on class II HDACs activity. HUVECs were starved for an overnight and the day after were shifted for 1 hour to complete medium supplemented with 10% FBS with or without NTG, in the presence or absence of histone deacetylase inhibitors. As shown in Figure 3C, left panel, NTG significantly decreased serum-dependent histone H3 acetylation. Treatment of ECs with the selective class II HDACs inhibitor MC1568[20] rescued NTG-dependent histone H3 deacetylation to above control level, whereas the inhibition of class I HDAC1, 2, and 3 activity by the specific inhibitor MS27–275[21] had a minor effect. As expected, TSA, which inhibits either class I or class II HDACs, induced a hyperacetylation of histone H3. Altogether, these data suggest an important role for NO in the regulation of histone acetylation by modulating the activity of class II HDACs complexes in ECs.

PP2A Mediates NO-Dependent HDAC4 Nuclear Shuttling

When phosphorylated by calcium-calmodulin dependent kinases (CaMKs) class II HDACs 4 and 5 localize in the cytoplasm bound to the 14-3-3 chaperonins and shuttle to the nucleus in their unphosphorylated form.[22] To investigate whether CaMKs may have a role in retaining class II HDACs in the cytoplasm of ECs, HUVECs were exposed to the CaMK inhibitor KN93, in the absence of NO donors. Immunofluorescence analysis showed that KN93 treatment was sufficient to induce HDAC4 nuclear translocation (see sup-

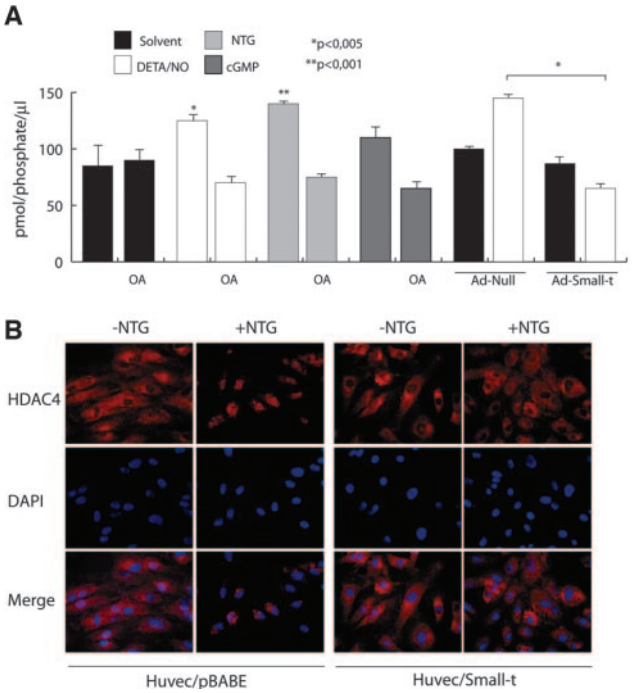

Figure 4. NO activates a PP2A-related activity. A, Phosphatase (PPase) activity assay. ECs were infected with Ad-small-t or Ad-Null adenoviruses. 72 hours later cells were treated for 1 hour with DETA/NO. NO-dependent increase in the overall cellular phosphatase activity was observed in total cell extracts from either noninfected or Ad-Null expressing cells. The phenomenon was abrogated in small-t expressing cells and by the incubation of the cellular extract with the PP2A inhibitor okadaic acid (OA). NTG also increased total PPase activity in HUVECs. 8Br-cGMP enhanced total PPase activity to a lesser extent. B, ECs transfected with the empty vector pBABE or with the vector encoding SV40 small-t antigen were treated for 2 hours with NTG or control solvent, and HDAC4 localization was analyzed by immunofluorescence analysis. Cells transfected with small-t retained HDAC4 in the cytoplasm after NTG treatment. The result is representative of 3 independent experiments.

plemental Figure IV), indicating that a CaMkinase activity is required to allow HDAC4 cytosolic retention.

It has been recently demonstrated that CaMKIV and the protein phosphatase PP2A play a role in regulating HDAC5 subcellular localization,[23] hence we hypothesized that a PP2A-related activity was involved in NO-dependent HDAC4 nuclear shuttling. To this aim, HUVECs were infected with an adenovirus encoding for the viral small-t antigen oncoprotein, a well known PP2A inhibitor,[24] and after 72 hours were treated for 1 to 4 hours with DETA/NO. Phosphatase activity determined on total cell extracts increased in control cells after 1 hour of exposure to DETA/NO, however it was abrogated either in cells expressing small-t antigen or in the presence of the PP2A inhibitor okadaic acid (OA) (Figure 4A and supplemental Figure V). Consistently, NTG also induced an increase in phosphatase activity, which was abolished by OA (Figure 4A and supplemental Figure V). Therefore, to examine whether PP2A was involved in NO-dependent nuclear translocation of HDAC4, HUVECs were stably transfected with the viral oncoprotein small-t.[15] Immunofluorescence analysis showed that in mock-transfected cells NTG induced HDAC4 nuclear localization, whereas cells expressing small-t antigen retained HDAC4 in the cytoplasm, suggesting an involvement of PP2A in the regulation of the nuclear shuttling of this enzyme (Figure 4B).

NO Increases PP2A Binding to HDAC4

To investigate whether NO stimulated the physical association between HDAC4 and PP2A, 293 cells were stably transfected with a doxycyclin inducible HDAC4-Flag fusion protein. 293 cells transfected with the empty vector and induced with doxycyclin were used as control. Western blot analysis on fractionated cellular extracts confirmed the effect of NO on HDAC4 nuclear shuttling also in this cellular model (Figure 5A). Purification of the recombinant protein onto an anti-Flag antibody column and Western blot analysis revealed that in absence of NO, HDAC4-Flag was predominantly associated with the phosphorylated form of CaMKIV[25] (p-CaMKIV) and 14-3-3, whereas it was only slightly bound to PP2A, MEF-2,[22] HDAC3,[26] and HDAC5[27] (Figure 5B).

NO treatment caused the dephosphorylation of CaMKIV, coincidently to 14-3-3 detachment, PP2A binding to HDAC4 (see also Figure 6), and the recruitment of MEF-2, HDAC3, and HDAC5 (Figure 5B). PP1 phosphatase was undetectable either in presence or in absence of DETA/NO (not shown). In this condition, HDAC4-associated phosphatase activity was increased on NO exposure, as assessed by an in vitro assay performed by using the recombinant HDAC4-Flag purified from 293 cells untreated or treated with DETA/NO; this activity was inhibited by okadaic acid, either in the presence or absence of NO (Figure 5D).

Discussion

SS exerts a pivotal role in the control of vascular cell structure and function, including the regulation of vascular tone, vessel wall remodeling, and homeostasis, and it is well established that NO is a crucial mediator of some SS properties, including the inhibition of apoptosis and the prevention of atherogenesis.[1] NO transcriptional effects have been extensively investigated in different cell types and tissues, including the nervous system,[28] the myoblasts,[29] and the heart.[30,31] In endothelial cells, NO has been described to globally downmodulate gene expression,[3] and one of the postulated mechanisms evokes the involvement of DNA methylation, which represses gene expression by favoring a close chromatin conformation.[32] The present report describes that the inhibition of NOS prolongs either SS-dependent histone H3 acetylation and c-fos expression (Figure 1A and 1B). The failure of nitric oxide inhibition to produce an increase in H3 acetylation on K14 at earlier time points may be attributable either to the high histone acetyltransferase activity[12] or to the low HDAC activity present in endothelial cells at 1 hour of SS exposure. Therefore, the inhibition of histone deacetylase activity is likely to be undetectable at this time point. When histone acetylation begins to decrease, because of both a decrease in HAT activity and an increase in histone deacetylase activity, the inhibition of the latter, by means of nitric oxide inhibitors, becomes visible as an enhacement in histone H3 acetylation. Consistently, it is highly probable that c-fos expression failed to be increased by NOS inhibitors in static conditions because of the requirement of SS-dependent signal

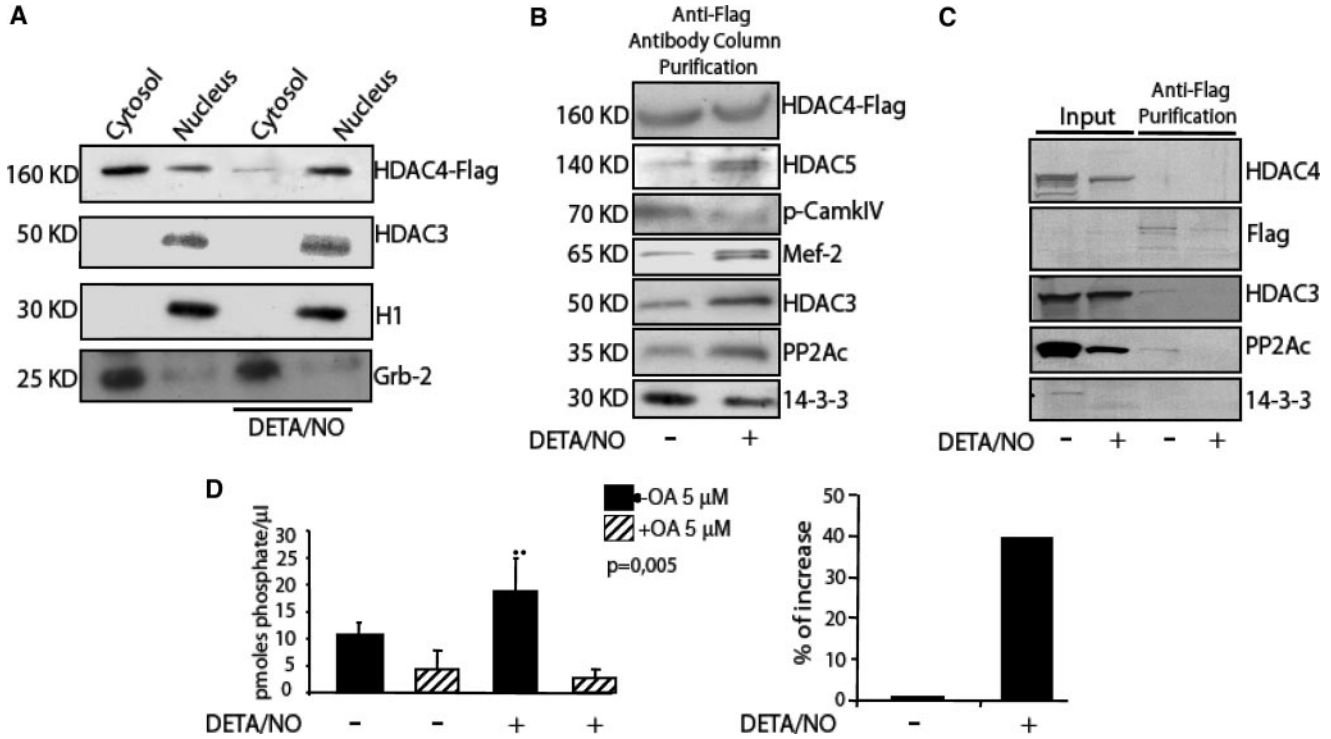

Figure 5. NO induces the formation of a macromolecular complex containing an active PP2A phosphatase. A, Western blot analysis showing the nuclear translocation of an HDAC4-Flag recombinant protein in 293 stably transfected cells on 2 hours of exposure to DETA/NO. In the presence of DETA/NO, a slightly increase of the endogenous HDAC3 in the nuclear fraction was also observed. Anti-Grb2 and anti-H1 antibodies were used to normalize the protein loading and to assess the purity of the cellular compartments. The result is representative of 4 independent experiments. B, Western blot was performed by using 600 ng of purified HDAC4-Flag fusion protein. In absence of NO, HDAC4 associated p-CaMKIV and 14-3-3 chaperonins, as expected. PP2A, MEF-2, HDAC3, and HDAC5 were slightly bound to the complex. NO enhanced PP2A, MEF-2, HDAC3, and HDAC5 binding to HDAC4; in contrast, NO decreased both 14-3-3 and p-CaMKIV binding to the complex. The result is representative of 3 independent experiments. C, Control Western blot of anti-Flag antibody affinity column purification. 293 cells were transfected with a pTRE2 hyg empty vector (Clontech) and induced with doxycylin for 24 hours. The day after cells were treated for 2 hours with DETA/NO or control solvent and purification was performed as described in supplemental Material and Methods. No detectable signals for HDAC4, HDAC3, $PP2A_c$, 14-3-3, and Flag protein was obtained either in the presence or absence of DETA/NO. D, In vitro PPase assay showing a 40% increase in HDAC4-Flag associated PPase activity in presence of NO. OA indicates okadaic acid.

transduction pathway to open the chromatin in its promoter region.[12] Thus, the genome wide effect of NOS inhibition on histone acetylation may not be paralleled by an effect at gene-specific level, at least in certain conditions. The direct exposure of ECs to the NO donors or to the NO metabolite cGMP abolished the serum induced histone H3 acetylation and c-fos expression (Figure 1C and 1D), indicating a possible direct effect of NO in modulating chromatin structure. In our experimental conditions, ECs nuclear histone deacetylase activity increased after 1 hour of treatment with NTG (supplemental Figure I). These experiments suggested that NO may directly regulate HDACs function. Indeed, we found that SS induced class II HDAC4 (and HDAC5, not shown) nuclear shuttling at 4 hours of treatment and this phenomenon was completely abolished in the presence of the NOS inhibitor SMT (Figure 2A and 2B). Consistently, HDAC4 and 5 shuttled from the cytoplasm to the nucleus of ECs either in the presence of DETA/NO or in the presence of cGMP. This phenomenon was time-dependent, being evident at 1 hour of treatment to decline at 8 hours (Figure 2D). The discrepancy in the timing of class II HDACs nuclear translocation in ECs treated directly with NO donors or exposed to SS (1 hour versus 4 hour) is likely attributable to the requirement of the multiple pathways activated by SS to exert its chromatin remodelling function.[12] Moreover, although it is well established that NO production rapidly increase after SS exposure,[33] its effects on ECs epigenome may be evident at later time points. Consistent with this hypothesis, it has been described that exposure to shear stress increases NO production in ECs in a biphasic manner.[34] An initial rapid NO release occurs at the onset of flow, followed by a less rapid, but sustained production. This constant NO release may allow the NO-dependent class II HDACs nuclear translocation

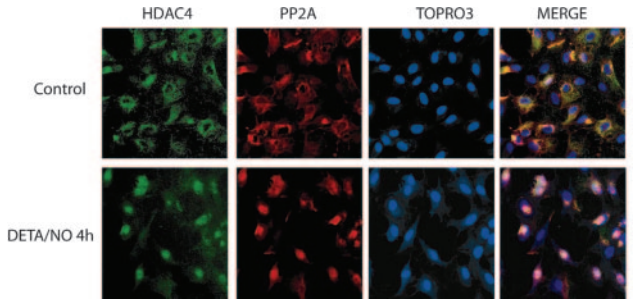

Figure 6. HDAC4 and PP2A associates in vivo in ECs. Confocal analysis showing the nuclear colocalization of HDAC4 and PP2A in response to NO.

which, in turn, accounts for the regulation of long-term SS-dependent gene expression. In light of this consideration, we reasoned that the transient effect of NO and cGMP relied on the activation of PDEs, which are known to hydrolyze cGMP.[17] Indeed, exposure of ECs to Zaprinast, prolonged HDAC4 retention in ECs nuclei, thus supporting our hypothesis of an involvement of PDEs in the nuclear export of class II HDACs (Figure 3B).

SS and NO may activate multiple signals, like p38[35] and Erk pathways,[36] which are known to be involved in the NO-dependent regulation of cell proliferation and apoptosis. Exposure of ECs to DETA/NO in the presence or absence of Erk and p38 inhibitors had no effect on HDAC4 nuclear translocation, while inhibiting PKG caused HDAC4 cytosolic retention (supplemental Figure III), suggesting an important role for the NO/cGMP/PKG signal transduction pathway in mediating class II HDACs nuclear shuttling in endothelial cells.

A large body of literature assesses that members of the class I and class II HDAC associate with corepressors in a macromolecular complex that mediates deacetylation of histones and repression of transcription.[26] Specifically, class II HDACs may function as a structural platform in the context of this chromatin remodeling machinery, where class I histone deacetylases retains the major functional role. In our experiments, however, we found that NO stimulates the histone deacetylase activity of class II HDACs (Figure 3C), addressing a specific role to these enzymes in NO-dependent gene expression and modulation of chromatin structure. Class II HDACs are nuclear in their unphosphorylated form[22] and a physical association between HDAC5 and PP2A has also been reported.[23] According to these evidences, we demonstrated that HDAC4 (and HDAC5, not shown) failed to localize to the nucleus of ECs in the presence of small-t antigen (Figure 4B), a well known inhibitor of PP2A,[24] which also counteracted NO-induced increase of cellular phosphatase activity (Figure 4A). Moreover, we show that PP2A was bound to HDAC4 and HDAC5 in a multiprotein complex, by using 293 cells stably expressing an HDAC4-Flag fusion protein (Figure 5).

The molecular mechanism by which NO may activate PP2A remains to be clarified. The members of PP2A family of phosphatases have a trimeric structure, constituted by a scaffold subunit (the A subunit or PR65) a catalytic subunit ($PP2A_c$) and a regulatory subunit, which may differ according to the cellular compartment or target.[37] Hypothetically, NO may cause a posttranslational modification of PP2A, which accounts for its association with HDAC4. In this regard, it has been demonstrated that phosphorylation of the PP2A regulatory subunit PR61δ may either change PP2A substrate affinity[38] or enhance the trimer overall activity.[39] Our evidence that NO-dependent HDAC4 nuclear translocation involves, at least in part, calcium release (supplemental Figure IV) suggests that a Ca^{2+}-dependent PP2A regulatory subunit[40] may associate to and dephosphorylate HDAC4 in response to NO. Mass spectrometric analysis of HDAC4-Flag bound complex will help to identify which regulatory partner enters the PP2A trimer in response to NO.

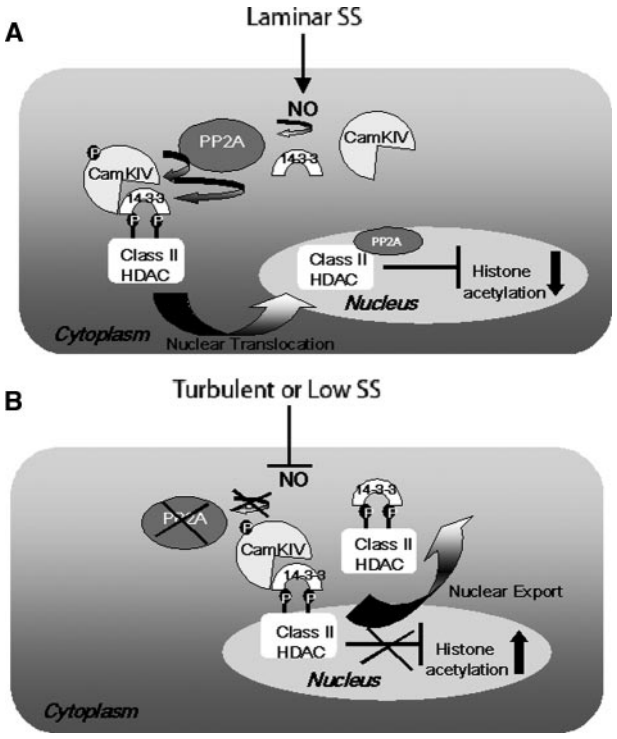

Figure 7. A model for NO-dependent class II HDACs nuclear translocation in endothelial cells. In physiological conditions, NO may activate PP2A, which in turn associates to pCamkIV/HDACs complex, dephosphorylating both proteins. Thus, class II HDACs shuttle to the nucleus of ECs and deacetylate histones. When NO production is impaired, this pathway is uneffective, class II HDACs are retained in the cytosol, inducing histones hyperacetylation and disregulation of gene expression.

In conclusion, here we show, for the first time, that nitric oxide induces class II HDAC4 and HDAC5 nuclear shuttling via PP2A activation and provide mechanistic insights into the NO-dependent regulation of gene expression through the regulation of chromatin folding. Although further experiments have to be performed to dissect the molecular mechanisms underlying the NO-induced activation of this chromatin modifier complex, our work may be relevant for a better understanding of the pathogenesis of NO-deficient diseases, like atherosclerosis or inherited pathologies such as duchenne muscular dystrophy.[21] According to our results, in fact, a model for NO-dependent class II HDACs nuclear shuttling may be hypothesized (Figure 7). In physiological conditions, NO activates a specific PP2A-related activity which dephosphorylates both CamkIV and class II HDACs, allowing HDACs nuclear translocation and chromatin remodeling. When NO production is impaired, this pathway becomes uneffective, class II HDACs remain in the cytosol contributing to the hyperacetylation of histones or other HDACs target proteins, leading to the disregulation of NO-dependent gene expression.

Sources of Funding

This work was supported in part by grant FIRB # RBLA035A4X-1-FIRB to M.C.C., AIRC regional grant to C.G., UE FP6 grant # UE-LHSB-CT-04-502988 to M.C.C., AFM grant # 12042 to C.G.

Disclosures

None.

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