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 deregulated histone deacetylase activity, together with inappropriate epigenetic patterns, have been recently associated with cancer.6,7 Four classes of histone deacetylases are currently known,8 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 cells9 or in the commitment of endothelial progenitor cells10 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...
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. 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. 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.

Materials and Methods

Cell Culture and Treatments

HUVEC were cultured as previously described. 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% CO2 atmosphere in complete Dulbecco’s Modified Eagle Medium (GIBCO) containing 0.11 g/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.

Immunofluorescence

Immunofluorescences were performed as previously described.

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.

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/Thr 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 rTA2-S2 activator and rTS repressor. To express HDAC4, cells were stimulated 24 hours with 1 μg/mL doxycyclin. 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-PP2A 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.
Results

NO Induces Histone Acetylation 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 treatment (Figure 1A). 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.

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 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.

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.
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 phosphodiesterases17 (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, with or without histone deacetylase inhibitors. Western blot analysis was performed on total extracts. Left 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.

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-
transfected cells NTG induced HDAC4 nuclear localization, small-t. Immunofluorescence analysis showed that in mock-infected HUVECs were stably transfected with the viral oncoprotein involved in NO-dependent nuclear translocation of HDAC4, mental Figure V). Therefore, to examine whether PP2A was consistent, NTG also induced an increase in phosphatase okadaic acid (OA) (Figure 4A and supplemental Figure V). Phosphatase activity determined on total cell extracts in infected with an adenovirus encoding for the viral small-t antigen, a well known PP2A inhibitor,24 and after infection 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.

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 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 acetylation transferase activity12 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 enhancement 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.
transduction pathway to open the chromatin in its promoter region. 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. Moreover, although it is well established that NO production rapidly increases after SS exposure, 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. 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.
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. 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 and Erk pathways, 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. 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 and a physical association between HDAC5 and PP2A has also been reported. 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, 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) and a regulatory subunit, which may differ according to the cellular compartment or target. 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 or enhance the trimer overall activity. Our evidence that NO-dependent HDAC4 nuclear translocation involves, at least in part, calcium release (supplemental Figure IV) suggests that a Ca+-dependent PP2A regulatory subunit 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.

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. 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 ineffective, class II HDACs remain in the cytosol, inducing histones hyperacetylation and disregulation of gene expression.

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Disclosures
None.
References


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Supplementary Materials & Methods

Cell culture and treatments.

HUVEC were cultured as previously described. Treatments were performed as follows: cells were starved for an overnight in Endothelial Basal Medium-2 (EBM-2, Clonetics). The day after, culture medium was replaced with EBM-2 plus 1% Fetal Bovine Serum, with or without 100 µM NTG, 500 µM DETA/NO, 1mM 8Br-cGMP, 10 µM A23187. BAPTA-AM, SB203580, okadaic acid, KN92 and KN93 were 5 µM. PD98059 was 30 µM and KT5823 was 1 µM. SMT was 12 µM. 7N was 710 nM.

HEK 293 cells were grown at 37 °C in a 5% CO₂ atmosphere in complete Dulbecco’s Modified Eagle Medium (GIBCO) containing 0.11g/L Pyridoxine, complemented with 2 mM 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 (all the antibiotics were from SIGMA). Small-t antigen adenoviral and retroviral infection of HUVEC were obtained as previously described.

Cell extracts and Western blot

Fractionated cellular extracts were performed as follows. Cells were scraped in ice-cold PBS, centrifuged and the cell pellet was resuspended in 500 µl of ice-cold buffer A, containing 0.1% Triton X-100, 10 mM Hepes pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 1mM PMSF and Proteases and phosphatases inhibitor cocktail. Cells were stored on ice for 30’, passed several times through a 500 µl Hamilton syringe and centrifuged for 10’ at 3500 rpm at 4°C to separate the cytosolic fraction from the nuclear pellet. The latter was washed 3 times with 500 µl of buffer A and resuspended in 300 µl of ice-cold buffer C, containing 20 mM Hepes pH 7.5, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 1mM PMSF and Proteases and
phosphatases inhibitor cocktail. The nuclear pellet was vigorously rocked at 4°C for 45’, centrifuged for 30’ at 12000 rpm and the supernatant, containing the nuclear proteins, was recovered and stored at –80°C.

Western blots were performed as previously described. Anti-HDAC4, -HDAC3, -HDAC5, -Mef2C,-14-3-3,-Grb2,-c-fos, -pCamkIV antibodies were purchased from Santa Cruz Biotechnology. Anti-H3AcK14 and anti-H1 antibodies were purchased from Upstate Biotechnology. Anti PP2Ac antibody was purchased from Transduction Laboratories. Anti-Flag antibody was purchased from SIGMA.

**HDAC Assay**

HDAC assays were performed by using the HDAC activity assay Kit (Upstate Biotechnology) according to the manufacturer’s instructions. Nuclear extracts were performed as previously described. Briefly, the nuclear extracts (200 µg) were added directly in a 96 multiwell provided by the kit, in the presence of HDAC assay buffer and HDAC substrate (acetylated histone H4). Control wells were represented by the same mixture plus TSA. The samples were assayed in duplicate. The mixture was incubated for 1h at 30°C. Thereafter, an activator solution was added and the plate was incubated for 15 minutes at room temperature. The plate was red by fluorometric detection at an excitation wavelenght of 380 nm and at an emission wavelenght of 460 nm, by using a LS55 luminescence spectrometer (Perkin Elmer).
SUPPLEMENTARY RESULTS

Erk and p38 kinase inhibition do not interfere with NO-dependent HDAC4 nuclear translocation.

Since SS and NO activate multiple signal transduction pathways,\[1,2,3\] to discriminate between SS and nitric oxide-dependent signals in mediating class II HDACs nuclear translocation, human endothelial cells were treated for 2 hrs with DETA/NO, in the presence or absence of the Erk inhibitor PD98059, p38 inhibitor SB203580 and the PKG inhibitor KT5823. We found that inhibiting Erk or p38 did not interfere with NO-dependent HDAC4 nuclear shuttling, which, remarkably, was completely abolished by blocking PKG activity (fig. S2). The same result was obtained for HDAC5 (not shown). This data further confirmed a specific role for NO signalling pathway accounting for class II HDACs nuclear localization in HUVEC.

HDAC4 nuclear translocation is calcium dependent.

Since a crosstalk between NO and calcium (Ca\[^{2+}\]) it has been well established,\[4,5\] it was examined whether NO effect on HDAC4 sub-cellular localization was, at least in part, dependent on Ca\[^{2+}\] release. HUVEC were pre-treated with the calcium chelator BAPTA-AM and then exposed to DETA/NO. Immunofluorescence analysis showed that BAPTA-AM partially prevented NO-dependent HDAC4 nuclear translocation, while the direct exposure of ECs to the calcium ionophore A23187 dramatically induced HDAC4 nuclear shuttling (fig. S3B). This data was validated by western blot analysis, which showed HDAC4 translocation from the cytoplasm to the nucleus of ECs upon A23187 treatment (fig. S3C). These results are suggestive of a role of calcium in mediating NO effects on HDAC4 cellular localization.
SUPPLEMENTARY REFERENCES


SUPPLEMENTARY FIGURE LEGENDS

Fig. S1 HDAC activity assay. HUVEC were treated for 1h with NTG or control solvent. Nuclear extracts were performed as previously described \(^1\) and an \textit{in vitro} HDAC activity assay was performed (see Supplementary materials and Methods). The graph show the enhancement in HDAC activity in nuclear extracts of NTG-treated ECs. As expected, the addition of TSA in the reaction mixture inhibited HDAC activity.

Fig. S2 Immunofluorescence showing HDAC5 nuclear enrichment in the presence DETA/NO (panel A) and cGMP (panel B). The result is representative of at least 6 independent experiments.

Fig. S3 Immunofluorescence analysis showing the effect of the Erk inhibitor PD98059 (30 \(\mu\)M), of the p38 inhibitor SB203580 (5 \(\mu\)M) and of the PKG inhibitor KT5823 (1 \(\mu\)M) on NO-dependent nuclear shuttling of HDAC4 in HUVEC. The result is representative of 3 independent experiments.

Fig. S4 Panel A: CamK inhibitor KN93 alone is sufficient to induce HDAC4 nuclear shuttling in HUVEC. Immunofluorescence shows a positive nuclear HDAC4 staining in HUVEC treated with KN93. The result is representative of 3 independent experiments. Panel B: Immunofluorescence analysis showing the effect of the calcium chelator BAPTA-AM and of the calcium ionophore A23187 on HDAC4 cellular localization. In the presence of DETA/NO and BAPTA-AM, HDAC4 is partially retained in the cytoplasm, while A23187 strongly induced its nuclear translocation. Panel C: Western blot analysis showing the nuclear translocation of HDAC4 in the presence of the calcium ionophore A23187.

Fig. S5 Time-dependent regulation of total PPase activity in response to DETA/NO, NTG and 8Br-cGMP in non-infected and in small-t antigen-infected ECs. PPase activity increased at 1 h time point to decline in 4 hrs in cells treated with DETA/NO and 8Br-cGMP. In cells treated with NTG, the PPase activity remained elevated.