Inhibitors of Histone Deacetylation Downregulate the Expression of Endothelial Nitric Oxide Synthase and Compromise Endothelial Cell Function in Vasorelaxation and Angiogenesis

Lothar Rössig, Huige Li, Beate Fisslthaler, Carmen Urbich, Ingrid Fleming, Ulrich Förstermann, Andreas M. Zeiher, Stefanie Dimmeler

Abstract—The histone deacetylase (HDAC) inhibitor trichostatin A (TSA) inhibits hypoxia-stimulated angiogenesis. Endothelial nitric oxide synthase (eNOS)–derived NO is central to angiogenesis signaling in endothelial cells (ECs). We hypothesized that the HDAC-dependent regulation of angiogenesis may involve a modulatory effect on eNOS expression. The HDAC inhibitors TSA, butyric acid (BuA), and MS-275 time- and concentration-dependently suppressed eNOS protein levels to 41+/−2%, 46+/−12%, and 40+/−12% of control, respectively. In parallel, TSA and BuA also downregulated eNOS mRNA expression to 21+/−4% and 37+/−4% of control. TSA also attenuated the NO-dependent relaxation of porcine coronary arteries (P<0.0001, TSA 1 μmol/L) and prevented tube formation in a human angiogenesis assay. Although vascular endothelial growth factor substitution did not compensate for the inhibitory effect of TSA, exogenous NO reversed the inhibition of angiogenesis by TSA. To address the underlying signaling mechanism, we characterized the effect of TSA on eNOS gene transcription and mRNA half-life. Although TSA decreased both eNOS protein and mRNA levels, TSA paradoxically enhanced the activity of the eNOS promoter, and did not alter the eNOS transcription rate in nuclear run-on experiments, suggesting that TSA posttranscriptionally targets eNOS mRNA. These data indicate that HDAC-dependent mechanisms contribute to the regulation of eNOS expression in ECs. (Circ Res. 2002;91:837-844.)

Key Words: endothelial nitric oxide synthase ■ angiogenesis ■ endothelial cell ■ histone deacetylase ■ gene expression

The interplay between histone deacetylases (HDACs) and histone acetyltransferases (HATs) differentially regulates the acetylation status of histone and nonhistone proteins and regulates gene expression in various ways. First, acetylation of nucleosomal core histone proteins determines the structure of chromatin, which regulates the accessibility of certain DNA sequences to the transcription machinery. Dynamic changes in histone acetylation contribute to the so-called epigenetic control of gene expression. A predominance of HDAC activity results in histone hypoacetylation, which is associated with the assembly of repressive heterochromatin structures and subsequent gene silencing, whereas histone hyperacetylation is associated with euchromatin formation and transcriptional activation. In addition to histone modification, acetylation/deacetylation processes may also directly regulate the activity of transcription factors. In contrast with the common paradigm of histone deacetylation as a gene silencing mechanism and histone acetylation leading to the induction of gene expression, the acetylation of transcription factors may result in both inhibition as well as activation of gene expression. Indeed, acetylation of the tumor suppressor protein p53 facilitates promoter activation, whereas acetylation of c-Jun at lysine 271 results in transcriptional repression, and acetylation of the bivalent transcription factor Yin-Yang-1 (YY-1) is required for its maximal repressive effect.

Nitric oxide (NO) generated by the endothelial NO synthase (eNOS) plays a crucial role in the regulation of vascular tone and angiogenesis signaling. Although eNOS is referred to as a constitutively expressed gene, a large variety of physiological and pathological stimuli affect eNOS mRNA expression. For example, lysophosphatidylcholine, TGF-β, and H2O2 enhance eNOS gene transcription. The posttranscriptional modulation of eNOS mRNA half-life is probably the major determinant of eNOS mRNA levels, and eNOS mRNA can be stabilized by exposing endothelial cells...
to 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors,\textsuperscript{13} vascular endothelial growth factor (VEGF),\textsuperscript{14} and fluid shear stress.\textsuperscript{15} TNF-\alpha,\textsuperscript{16} oxLDL,\textsuperscript{17} hypoxia,\textsuperscript{18,19} and cell confluence,\textsuperscript{20} on the other hand, accelerate the degradation of eNOS mRNA. Two of the latter stimuli, TNF\alpha and cell confluence, are associated with the induction of 60 kDa\textsuperscript{21} and 51 kDa\textsuperscript{20} cytosolic proteins, which bind to eNOS mRNA and destabilize it. As yet, however, the detailed molecular mechanisms underlying eNOS mRNA destabilization remain to be fully characterized.

Trichostatin A (TSA), an inhibitor of class I and II HDACs, has been reported to inhibit angiogenesis stimulated by either hypoxia or VEGF.\textsuperscript{22,23} The cellular mechanisms underlying these effects involved prevention of the hypoxia-driven induction of HIF-1\alpha and VEGF,\textsuperscript{22} or regulation of HIF-1\alpha transactivation.\textsuperscript{24} Moreover, inhibition of the VEGF-induced VEGF receptor expression and induction of the VEGF competitor, semaphorin III, by TSA have been reported.\textsuperscript{23} In the present study, we investigated the contribution of TSA-sensitive, HDAC-dependent signaling to the regulation of eNOS expression. We demonstrate that HDAC inhibitors downregulate eNOS mRNA expression resulting in both impaired NO-dependent vasorelaxation and angiogenesis and, thus, providing a common pathway by which HDAC inhibitors interfere with endothelial function. Our data suggest that TSA targets eNOS expression posttranscriptionally via induction of a mRNA destabilizing gene, which represents a novel mode of interaction by which HDAC-dependent mechanisms control gene expression.

**Materials and Methods**

**Cell Culture, Materials, and Immuno blotting**

Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Systems/Clonetics, Solingen, Germany, and were cultured as previously described.\textsuperscript{25} Cells were either used at the third passage or as indicated in the text. HUVEC-derived EA.hy 926 cells\textsuperscript{26} were fully characterized.

Reporter Gene Assay

Reporter gene constructs were generated using the vector plasmid pGL3-Basic (Promega), which contains the luciferase gene, as described previously.\textsuperscript{27} pGL-Hu-eNOS-3500 and pGL-Hu-eNOS-1600 contained either a 3.5 kb or 1.6 kb human eNOS promoter fragment. HUVECs (3.5\times10\textsuperscript{5} cells) were transiently transfected with 3 \mu g plasmid DNA using 18 \mu L Superfect (Qiagen) as described.\textsuperscript{28} After incubation in the absence or presence of TSA for 18 hours, cells were lysed with passive lysis Buffer (Promega), and luciferase activity was measured using the Dual-Luciferase System (Promega) with a luminometer (Berthold Luminat LB 9501).

EA.hy 926 cells were transfected with the pGL-Hu-eNOS-3500-neo, then diluted and incubated in selection medium (containing 1 mg/mL G418). Single clones were collected and propagated in selection medium. For analysis of the effects of TSA on eNOS promoter activity, the stably transfected cells were incubated with TSA for 18 hours. Thereafter, cell extracts were prepared using the reporter lysis buffer (Promega), and luciferase activity was determined and normalized to protein content.

Oligonucleotide Transfections

Sequence-specific antisense oligonucleotides with phosphorothioate linkages homologous to the human YY-1 sequence (AS1, 5'-AGGG-TGT/CGCCGGAGGCCAT-3'; AS2, 5'-CTGAACATCTTTGAGCAGC-3') were incubated in 100 \mu L RPMI medium in the presence of 5 \mu L lipofectamine (Gibco) for 30 minutes at room temperature. HUVECs (4.0\times10\textsuperscript{5} cells) were washed with RPMI and incubated with 2 mL RPMI before the lipofectamine/oligonucleotide mixture was added. After incubation for 5 hours, 3 mL complete endothelial basal medium was added, and the cells were incubated in the presence or absence of TSA for 36 hours. Western blot analysis with an antibody directed against YY-1 confirmed that both antisense oligonucleotides completely suppressed YY-1 protein expression.

Northern Blot and Nuclear Run-On Analysis

For detection of eNOS RNA by Northern blotting, RNA was isolated with the TRIzol reagent (Gibco), separated by agarose gel electrophoresis and transferred to nylon membranes. The blots were hybridized with a radioactively labeled full-length human eNOS probe.

For determination of the eNOS RNA transcription rate, EA.hy 926 cells and HUVECs were detached with trypsin, lysed with Nonidet P-40, and 2\times10\textsuperscript{5} nuclei were separated by a 20.5% sucrose gradient. Nuclei were incubated in the presence of ATP, GTP, CTP, and [\textsuperscript{32}P]UTP for 30 minutes at 30\degree C to transcribe \textsuperscript{32}P-labeled mRNA. To prepare hybridization membranes, human full-length eNOS cDNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as control were blotted onto nitrocellulose membranes (Amer sham). Blots were cross-linked, hybridized with the radioactively labeled transcripts in ExpressHyb hybridization solution (Clontech) for 36 hours at 65\degree C, washed (0.1% SDS, 2\times SSC), and exposed to x-ray films.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using Tri Reagent (Sigma) from porcine coronary arteries ground to a powder in liquid nitrogen and equal amounts of RNA were used for the reverse transcriptase reaction (AMV polymerase, Promega) with random hexanucleotide primers. An aliquot (2 \mu L) of the RT reaction was used for eNOS PCR using the following conditions and primers: eNOS forward primer, 5'-AACCTGACAAAAAACAGGCTTACCTGCGTCTCCAG-3' eNOS reverse primer, 5'-TTCCGCGTCTCACTATTTCAGTCCC-3' (55\degree C annealing temperature, 30 cycles). PCR products were separated on a 1.2% TAE agarose gel and visualized by staining with ethidium bromide.

To assess the half-life of eNOS, we performed one-step quantitative real-time RT-PCR of 100 ng RNA using the Light Cycler Instrument (Roche): eNOS forward primer, 5'-ACCTGACAAAAAACAGGCTTACCTGCGTCTCCAG-3'; eNOS reverse primer, 5'-AAAAGCTCTGGTGCTGGTATG-3'.

**DAF-2 DA Staining**

To measure NO production in HUVECs, cells were stained with the membrane-permeable NO-sensitive fluorescent indicator dye DAF-2 DA (4,5-diaminofluorescein diacetate, 13.3 \mu g/well, Calbiochem). Subsequently, NO levels of the cells were determined by flow cytometric analysis.
RNase Protection Assay

Total RNA was isolated from EA.hy 926 cells by guanidinium thiocyanate-phenol-chloroform extraction. Radiolabeled antisense RNA probes for human eNOS and β-actin mRNA were generated by in vitro transcription using pCR-eNOS-Hu and pCR-β-actin-Hu plasmids. Total RNA (20 μg) were hybridized with labeled RNA probes. Thereafter, the mixture was digested with a mixture of RNase A and RNase T1 (Roche Diagnostics). The reaction was stopped by proteinase K digestion and purified by phenol extraction. After ethanol precipitation, the reaction products were analyzed by densitometry. Densitometry analyses were performed using a Phospho-Imager (BioRad). The density of each eNOS band was normalized with the corresponding β-actin band.

Coronary Artery Reactivity and Angiogenesis Assay

Hearts were obtained from freshly slaughtered pigs at a local abattoir. The epicardial arteries were excised and cleaned of adventitial material. Arterial rings (2 mm) were then incubated in minimal essential medium (MEM, 0.1% BSA, 50 U/mL penicillin, 50 μL/G streptomycin, and 1 μL/g polymyxin B) in the absence and presence of TSA (0.1 to 1 μL/g, 18 hours). To assess the relaxation response, the rings were mounted in organ chambers and precontracted with U46619 (0.1 to 1 μL/g) and the relaxation to bradykinin was determined in the presence of diclofenac (10 μL/g), as described.28

The human angiogenesis assay (Cell Systems/Clonetics) was performed as previously reported.30 Briefly, HUVECs were cocultured with human fibroblasts in a culture matrix. After changing the medium, cells were repeatedly stimulated at day 1, 4, 7, and 9 with TSA (0.1 μL/mL) and/or SNAP (20 μL/mL) as indicated. On day 11, cells were fixed and labeled with an anti-CD31-antibody and a secondary antibody conjugated with alkaline phosphatase and BCIP/NBT as substrate. Tube length was quantified by measuring the stained tubes using a computer-assisted microscope system (KS300 3.0 Software, Zeiss, Germany).

Statistics

Data are expressed as mean±SEM. Two treatment groups were compared by the independent samples t test, and three or more groups by 1-way ANOVA followed by post hoc analysis adjusted with an LSD correction for multiple comparisons (SPSS). Results were considered statistically significant with a value of P<0.05.

Results

Downregulation of eNOS Protein and mRNA Expression in HDAC Inhibitor–Treated ECs

Incubation of HUVECs for 36 hours with either TSA, butyric acid (BuA), or MS-275 concentration– (Figure 1A) and time-dependently (Figure 1B) decreased eNOS protein levels. In contrast to its effects on eNOS, TSA treatment induced the expression of the cell cycle regulatory protein p21<sup>CRIP</sup> (Figure 1B), whereas the cytoskeletal proteins actin and tubulin were unaffected (Figures 1A and 1B). In parallel with the observed decrease in eNOS protein, both TSA and BuA also downregulated the expression of eNOS mRNA (Figure 1C). TSA incubation also significantly reduced the synthesis of NO assessed by flow cytometric analysis after fluorescent DAF-2 DA staining (Figure 1D and 1E).

TSA Inhibits NO–Mediated Relaxation

To assess the functional relevance of the observed downregulation of eNOS mRNA and protein, we determined the effect of TSA on the bradykinin-induced, NO-mediated relaxation of the porcine coronary artery. TSA decreased eNOS expression in native porcine coronary endothelial cells (eNOS levels were reduced to 69.3±26.5% and 39.3±15.4% of control levels after treatment with 0.3 and 1 μL/g TSA for 18 hours, respectively; n=3), and concentration-dependently inhibited bradykinin-induced relaxation (Figure 2A).

NO Reverses Angiogenesis Inhibition by TSA

TSA is a potent inhibitor of angiogenesis under basal conditions23 as well as in an animal model of hypoxia.22 Because NO is a key mediator of proangiogenic signaling, we examined whether or not the downregulation of eNOS contributes to the antiangiogenic effect of TSA. Incubation with TSA for 11 days inhibited tube formation in an angiogenesis assay (Figure 2B). However, addition of the NO donor SNAP reversed the antiangiogenic effect of 0.1 μL/mL TSA (Figure 2B) as well as that observed using 0.3 μL/mL TSA (data not shown). These observations suggest that exogenous NO can compensate for the loss of endogenous NO. TSA (0.3 μL/mL) also inhibited control and VEGF-stimulated angiogenesis to a similar extent (tube length in TSA-treated cocultures was 48±2% versus 50±20% of control values in the absence and presence of VEGF; NS). These findings indicate that, in contrast to hypoxia-driven angiogenesis,22 the antiangiogenic effect of TSA observed under normoxic conditions is not primarily due to a decrease in VEGF expression.

TSA Downregulates eNOS mRNA Despite an Increased Transcription Rate

In order to investigate whether the downregulation of eNOS by TSA is due to the repression of eNOS–specific promoter activity, we used a series of luciferase-coupled eNOS reporter gene constructs. HUVECs were transiently transfected with two luciferase reporter gene constructs that contained either 1600 or 3500 base pairs of the 5<sup>'</sup> flanking region of the eNOS gene promoter. In an apparent contradiction to the inhibitory effect of TSA on mRNA expression, the HDAC inhibitor increased the activity of both eNOS promoter constructs. A similar, concentration-dependent activation of eNOS promoter constructs containing 633, 326, and 263 base pairs was observed in transiently transfected EA.hy 926 cells after TSA treatment (data not shown). Because epigenetic mechanisms might differentially regulate chromatin-embedded and single plasmid DNA, we generated an EA.hy 926 cell line that stably expressed the 3500 base pair eNOS promoter construct. In these cells, TSA treatment concentration-dependently decreased endogenous eNOS mRNA levels, assessed using an RNase protection assay, although at the same time TSA elicited a pronounced activation of the eNOS promoter (Figure 3B), suggesting a transcription-independent downregulatory mechanism.

Because the reporter gene constructs carry the 5<sup>'</sup>-flanking region of the eNOS gene, even when integrated into the genome, could be regulated differently from the 5<sup>'</sup>-flanking region of the native eNOS gene on chromosome 7, we performed nuclear run-on experiments to establish whether or not TSA affects the rate of eNOS transcription. We observed no change in eNOS transcription in EA.hy 926 cells and in HUVECs in the presence of TSA (Figure 3C and data not shown).
To characterize the molecular mechanism underlying the observed decrease in eNOS protein, we used antibodies against acetylated lysine residues to analyze protein acetylation levels in ECs after HDAC inhibition. TSA treatment increased the lysine acetylation of histone H3 and histone H4. This effect was similar in HUVECs (Figure 3D), COS-7, and HEK 293 cells (data not shown). These observations indicate that the effect of TSA on eNOS mRNA levels may be associated with histone hyperacetylation. Furthermore, TSA induces the hyperacetylation of an ~50-kDa nonhistone protein (Figure 3D), indicating that in HUVEC TSA treatment affects the posttranslational modulation of proteins other than histones. Histone hyperacetylation is usually associated with gene induction rather than suppression; however, acetylation of nonhistone proteins, particularly transcription factors, may also lead to gene repression. For example, acetylation of the Krüppel-like transcription factor YY-1, which regulates eNOS gene transcription, is required for its maximal repressive effect. To determine the possible role of YY-1 in regulating eNOS levels in TSA-treated cells, the effect of the HDAC inhibitor was assessed in normal HUVECs and in HUVECs treated with YY-1 antisense oligonucleotides. Consistent with our finding that TSA transcription independently regulates eNOS expression, TSA suppressed eNOS levels to a similar extent in both cell groups (Figure 3E). Antisense-mediated downregulation of another eNOS promoter repressor, the zincfinger transcription factor MAZ, also failed to affect the TSA-induced decrease in eNOS levels (data not shown). In summary, these data demonstrate that acetylation of YY1 or MAZ does not mechanistically underlie the suppression of eNOS by TSA.
TSA-Induced Downregulation of eNOS mRNA Involves the Transcriptional Induction of a Gene

The half-life of eNOS mRNA was assessed by mRNA chase experiments using the transcription inhibitor actinomycin D. As reported previously, in ECs the half-life of eNOS mRNA was approximately 24 hours when cells were maintained in the presence of serum (Figure 4A). In the combined presence of TSA and actinomycin D, eNOS mRNA half-life was unchanged (Figure 4A). However, TSA could act via induction of an eNOS mRNA destabilizing factor, which cannot be detected when transcription is blocked by actinomycin D. Indeed, after 18-hour treatment with TSA in the presence of actinomycin D, eNOS mRNA expression (assessed by RT-PCR analysis) still amounted to 58% of control, which is similar to the effect of actinomycin D alone (53% of control), whereas in the absence of actinomycin D, TSA reduced eNOS mRNA to 30±5% of control levels. Accordingly, incubation with TSA for 36 hours in the presence of actinomycin D reduced eNOS protein levels to a significantly lower degree when compared with TSA on its own (Figure 4B), indicating that transcriptional activity is necessary for the regulation of eNOS expression by TSA.

Experiments performed in parallel using 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) as an inhibitor of transcription yielded identical results (data not shown), indicating that the sensitivity of the TSA effect to actinomycin D is not due to an unspecific action of actinomycin D.

VEGF Substitution Does Not Compensate for eNOS Reduction in Response to TSA

Because VEGF has previously been reported to increase eNOS mRNA half-life, and TSA prevents the induction of VEGF by hypoxia by increasing the expression of p53, we determined whether or not a similar mechanism could also be involved in the TSA-induced decrease in eNOS expression in HUVECs under normoxic conditions. Low levels of p53 were expressed in HUVECs under basal conditions, as reported previously. However, TSA treatment reduced p53 levels (Figure 4C). This effect contrasted with the effect of the HDAC inhibitor on p21 levels (see Figure 1B). Application of VEGF, which reduces the rate of eNOS mRNA degradation, did not neutralize the dose-dependent downregulatory effect of TSA on eNOS protein levels (Figure 4D and 4E), excluding that a suppression of VEGF could account for the downregulation of eNOS by TSA under normoxic conditions.

Discussion

The results of this study demonstrate that inhibition of HDAC activity is associated with histone hyperacetylation, reduced expression of eNOS in native and cultured endothelial cells, as well as an impaired relaxant and angiogenic capacity. The reduction in eNOS expression was achieved by three structurally unrelated HDAC inhibitors including the hydroxamic acid trichostatin A, the short-chain fatty acid sodium butyrate, and the synthetic benzamide derivative MS-275. Although eNOS mRNA levels were decreased, HDAC inhibition paradoxically enhanced the eNOS promoter activity, indicating that HDAC inhibition posttranscriptionally downregulates eNOS mRNA. However, when assessed by mRNA chase experiments, TSA did not alter eNOS mRNA stability. These data suggest that TSA may induce the de novo expression of an eNOS mRNA destabilizing factor, which cannot be detected in mRNA decay studies performed in the presence of actinomycin D and DRB. Indeed, such a factor/protein has previously been suggested to account for the downregulation of eNOS mRNA in confluent endothelial cells, as well as in cells treated with either TNFα or LPS. In the latter studies, UV cross-linking experiments revealed that the binding of 51 kDa and ~60 kDa proteins to the 3′ untrans-
lated region (3'-UTR) of eNOS-mRNA was associated with mRNA degradation. As yet, however, these proteins have not been further characterized or identified.

In addition to the specific regulation of eNOS mRNA, the induction of an mRNA destabilizing factor represents a novel mechanism, by which HDAC inhibition may also downregulate the expression of genes other than eNOS. In accordance with the relevance of acetylation processes in the regulation of gene expression, HDAC inhibitors have been shown to induce bidirectional changes in the expression of a number of genes in yeast and mammalian cells. Indeed, differential display analysis of a TSA-treated human lymphoid cell line revealed a change in only 8 out of 340 genes tested, with the majority being upregulated, whereas a smaller number was suppressed. The upregulation of mRNA expression in response to HDAC inhibitor-treatment is generally believed to reflect an increased accessibility of DNA associated with hyperacetylated histones (ie, euchromatin) to transcription factors. A variety of mechanisms have been proposed to mediate the downregulation of certain genes by HDAC inhibitors, including inhibition of the transcriptional elongation of c-myc, acetylation of the transcription factor TCF by CREB-binding protein (CBP), and acetylation of the integrative transcription factor HMG I(Y). Of particular importance, a recent study reported that HDAC inhibition regulates HIF-1-dependent transcriptional activity, which could also have implications for the regulation of eNOS expression.

Our finding that HDAC-dependent mechanisms regulate eNOS expression has a number of implications for cardiovascular homeostasis. For example, because HDAC activity may be affected by various physiological and pathophysiological situations, the subsequent changes in histone acetylation levels may effectively regulate eNOS expression. Replicative senescence is a condition that potentially contributes to the initiation and/or development of atherosclerosis.
is associated with a reduction in eNOS expression. Indeed, HDAC inhibitors induce a senescent cell phenotype in human fibroblasts and in endothelial cells, which can be reversed by exogenous addition of the NO donor SNAP (L. Rössig and S. Dimmeler, unpublished data, 2002). In addition, eNOS deletion results in impaired angiogenesis in eNOS−/− animals. In accordance, our results demonstrate that the inhibitory effect of TSA on angiogenesis in vitro can at least partially be rescued by exogenous NO, indicating that the regulation of eNOS expression levels in ECs contributes to the effects of HDAC inhibition on angiogenesis signaling.

In conclusion, the data presented here provide evidence for a novel regulatory mechanism involved in the absence or presence of actinomycin D (2.5 μg/mL). A, RT-PCR analysis of eNOS mRNA half-life in HUVECs treated with actinomycin D (Act D, 2.5 μg/mL) in the absence (●) or presence (▪) of TSA (1 μmol/L). B, Effect of TSA (1 μmol/L, 36 hours) on eNOS protein in the absence or presence of VEGF (30 ng/mL). Top, Representative Western blots; Bottom, Densitometric analysis of n=3 individual experiments corrected for tubulin expression. *P<0.05. C, Effect of TSA (1 μmol/L, 36 hours) on p53 protein levels in non-HUVECs and HEK 293 cells. Top, Western blot stained against p53; Bottom, Tubulin reprobe; both representative of n=3 experiments. D, Effect of incubation with TSA for 36 hours on eNOS protein levels in the absence or presence of VEGF (30 ng/mL); representative Western blot. E, Concentration-dependent effect of TSA in the absence or in the presence of 30 ng/mL VEGF; densitometric analysis of n=3 to 6 individual experiments, corrected for tubulin expression. *P<0.05.

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References


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