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Dual effects of histone deacetylase inhibition by trichostatin A on endothelial nitric oxide synthase expression in endothelial cells

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Abstract

Inhibition of histone deacetylases by trichostatin A (TSA) has pleiotropic effects on gene expression. We demonstrated that at low dose (0.1 μg) TSA increased the eNOS mRNA levels, which was followed by a time- and dose-dependent down-regulation. Cycloheximide, a protein synthesis inhibitor, completely abolished TSA-induced decrease in eNOS expression, indicating that new protein synthesis is required for the inhibiting effect. Mevastatin—an inhibitor HMG-CoA reductase and geranylgeranylation reaction dose-dependently antagonized TSA-induced reduction. This mevastatin-mediated antagonism was completely abolished by geranylgeranylpyrophosphate, suggesting that geranylgeranyl modification is needed to activate the eNOS mRNA destabilizing factor—a mechanism responsible for statin-mediated eNOS upregulation.

Keywords: Histone deacetylase; Sp1; eNOS; Gene expression; TSA

Endothelial nitric oxide synthase (eNOS) gene is constitutively expressed in and relatively confined to endothelium [1,2]. In the vasculature, eNOS plays a major vasodilatory role in the maintenance of vascular integrity through the generation of nitric oxide (NO). A variety of biophysical, biochemical, and hormonal stimuli have been reported to affect eNOS expression [3–5], which in most cases affect eNOS mRNA stabilization. Recently, histone deacetylase (HDAC) specific inhibitor trichostatin A (TSA) has been demonstrated to down-regulate eNOS expression post-transcriptionally; and the down-regulation could not be compensated by the vascular endothelial growth factors (VEGF) [6]. However, mechanisms for TSA-mediated eNOS inhibition are not clear.

Despite the importance of eNOS in vascular diseases, there are virtually no pharmaceutical agents that are specifically used for eNOS regulation. Among few existing agents, HMG-CoA reductase inhibitor statins have been consistently shown to up-regulate eNOS mRNA independent of their designated cholesterol lowering effects [7]. The up-regulation appears to be mediated through eNOS mRNA stabilization [7]. The 3′ untranslated region (3′-UTR) of eNOS mRNA is usually inferred to be a target site for eNOS mRNA destabilizing factor(s) in response to a number of stimuli including statins [8–12]. It is therefore interesting to explore whether statins could antagonize TSA-induced decrease in eNOS expression; and whether eNOS 3′-UTR is the target for TSA-induced reduction. In this study, we investigated the mechanisms of TSA-induced eNOS inhibition. We found time-dependent dual effects of TSA on eNOS expression; and the TSA-induced suppression could be recovered by the statin treatment through the geranylgeranyl modification.

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Materials and methods

Reagents. TSA, cycloheximide (CHX), mevastatin, farnesylpyrophosphate (FPP), and geranylgeranylpyrophosphate (GGPP) were purchased from Sigma (St. Louis, MO). TSA, mevastatin, and CHX were dissolved in dimethyl sulfoxide (Me_2SO) before use. The *Clostridium botulinum* C3 transferase was purchased from List Biological Laboratories (Campbell, CA) and dissolved in PBS. Mevastatin was chemically activated by alkaline hydrolysis prior to use. Briefly, after 5 mg mevastatin was dissolved in 200 μl Me_2SO, we added 0.5 M NaOH 300 μl and incubated at 50 °C for 2 h. The mixture was neutralized with 300 μl HCl. We then added 400 μl PBS to make the stock solution with a final concentration of 10 μM. For cell treatment, TSA, CHX, and mevastatin solutions were added into culture medium; whereas for corresponding controls, the same volume of the corresponding solvent Me_2SO was added into the culture medium.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and cultured at 37 °C with 5% CO_2 in EGM-2 endothelial cell basic medium with Bulletkit (Cambrex). HUVECs were used at passage 4–7 in all experiments.

Real-time quantitative RT-PCR. Total RNA was extracted from the cells with Trizol (Invitrogen) according to the manufacturer's protocol. The RNA solution was treated with 1 U RNase-free DNase I (1 U/μl, Promega) in a final volume of 20 μl at 37 °C for 15 min to remove the trace amount of genomic DNA. The DNase I was inactivated by incubating at 75 °C for 10 min. Reverse-transcription and real-time PCR were performed as described previously [13]. The primers for the human eNOS and housekeeping gene β-actin (human) were as described [13].

Reporter constructs. We amplified human eNOS full-length cDNA (+4 to +3611, from transcription start site) and 3′-UTR (+3622 to +4029, from transcription start site) with standard PCR techniques from HUVECs cDNA pool; and we cloned the PCR products into pGL3-control vector (Promega) at the *Xba*I site. The eNOS full-length and 3′-UTR were inserted just after the stop codon (1934 bp) of the luciferase coding sequence, with the *Xba*I restriction site on the pGL3 vector to form the pGL3-full-length and pGL3-3′-UTR constructs, respectively. Primers with restriction enzyme *Xba*I site (underlined) for cloning eNOS full-length and 3′-UTR are: sense, 5′-TCT AGA AGT GGA CGC ACA GTA ACA TG-3′; antisense, 5′-TCT AGA CTC TCA GGG GCT GTT GGT GT-3′ and sense, 5′-TCT AGA GAG CCG CCT GGC TTT CCC TT-3′; antisense, 5′-TCT AGA GGG CTG GGG TAG GCA CTT TAG-3′, respectively. The eNOS full-length and 3′-UTR in pGL3 vectors were confirmed by the direct DNA sequencing.

Luciferase reporter assay. Plasmid DNA was prepared using Plasmid Extraction Kit (Qiagen). One microgram of DNA was transfected into the cells cultured in 12-well plates with Lipofectamine 2000 (Invitrogen). Reagents (TSA 0.4 μg/ml or mevastatin 40 μM) were added into the culture medium 4 h after the transfection; and the transfected cells were lysed 24 h after the TSA treatment with cell lysis buffer (Promega). Luciferase activity was measured with FB12 luminometer (Berthold, Germany) using luciferin (Promega) as the substrate.

Co-immunoprecipitation. Co-immunoprecipitation of Sp1 with HDAC1 was performed as described previously [13]. Briefly, HUVECs were washed twice with ice-cold PBS and lysed in mild lysis buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 2 mM sodium orthovanadate, and complete protease inhibitor cocktail (Sigma) on ice for 10 min. The cells were then harvested by centrifugation at 15,000*g* for 15 min at 4 °C and the supernatant was collected as the whole cell extracts. Equal amount (2.5 mg) of extracts was incubated in 500 μl of the extraction buffer with 4 μg Sp1 antibody (Upstate) for 16 h with rotation at 4 °C. After addition of 40 μl of protein A/G–agarose beads (Santa Cruz Biotechnology), the mixture was further incubated with rotation for 1 h at 4 °C. After five washes with 1 ml of the extraction buffer, the bound proteins were released by boiling in 30 μl SDS loading buffer and then subjected to 10% SDS–PAGE and Western blot analysis. Sp1 was immunoprecipitated with an affinity- purified polyclonal rabbit anti-Sp1 antibody (Upstate). HDAC1 was detected with an affinity-purified polyclonal rabbit anti-HDAC1 antibody (eBioscience, San Diego, CA). Anti-FLAG antibody (Sigma) was used as a non-relevant antibody for negative control in the immunoprecipitation of endogenous proteins.

Western blot. Proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. The membrane was blocked in 5% nonfat milk in TBS-T (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. After incubation with primary antibody in TBS-T containing 1% milk for 16 h at 4 °C, the membrane was washed extensively with TBS-T and incubated with secondary anti-rabbit horseradish peroxidase-conjugated antibody for 1 h at room temperature. After extensive washes with TBS-T, the membrane was visualized with ECL plus reagents (Amersham Biosciences).

Statistic analyses. All quantitative data were presented as means ± SD of three separate experiments and one-way ANOVA was used for comparisons among three or more groups (SPSS 12.0). The independent Student's *t* test was used for between-group analysis. The $p < 0.05$ was regarded as statistically significant.

Results

Dual effects of TSA on eNOS mRNA expression

TSA has been demonstrated to down-regulate eNOS expression in endothelial cells after 24 h treatment [6]. While our experiments confirmed this TSA-induced eNOS down-regulation, the true effects of the TSA on eNOS expression appeared to be time- and dose-dependent. Contrary to late inhibition (by 24 h), we observed an induction of eNOS in HUVECs (>2-fold increase) during early stage (as early as 15 min) of the TSA treatment (Fig. 1A). For the dose-dependent effects, we observed that at the low dose (0.1 μg/ml), TSA-induced eNOS expression (Fig. 1B). This was then followed by a dose-dependent inhibition on eNOS expression (Fig. 1B). The eNOS mRNA level was quantified by the real-time RT-PCR and normalized to the housekeeping gene β-actin [13].

Requirement of new protein synthesis for the TSA-induced decrease in eNOS mRNA

To determine whether a new protein synthesis is required for the TSA-induced eNOS reduction, we investigate the effects of a protein synthesis inhibitor on TSA-induced decrease in eNOS mRNA. As shown in Fig. 2, the protein synthesis inhibitor cycloheximide (40 μg/ml) completely blocked TSA-induced down-regulation in eNOS mRNA, whereas cycloheximide alone induced a less pronounced decrease in eNOS mRNA (Fig. 2). Those data indicate that new protein synthesis is necessary for the TSA-induced eNOS down-regulation.

Effects of HMG-CoA reductase inhibitor on TSA-induced eNOS reduction

As vascular endothelial growth factor (VEGF) could not compensate for the TSA-induced eNOS reduction [6], we explored whether the HMG-CoA reductase inhibitor mevastatin, which is reported to prolong eNOS mRNA half-life [7,14], could antagonize the effect of TSA. As

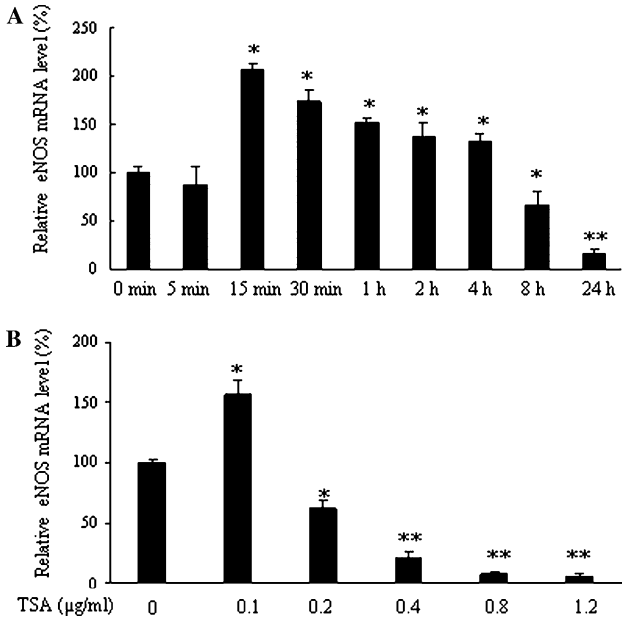

Fig. 1. Dual effects of HDAC inhibition by TSA on eNOS mRNA expression in HUVECs. (A) Induction of eNOS mRNA by TSA was observed at 15 min. This was followed by a dramatic decrease and was below the baseline level by 24 h. The cells were treated with 0.4 μg/ml TSA (Me_2SO for control cells). (B) Induction of eNOS mRNA by TSA at a lower dose (0.1 μg/ml) for 24 h, which was followed by a dose-dependent decrease. The eNOS mRNA level was quantified by the real-time quantitative RT-PCR. All data are presented as means + SD of three separate experiments. $*P < 0.05$ and $**P < 0.01$ vs control.

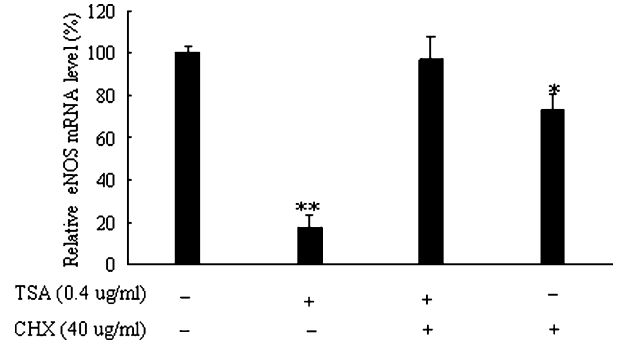

Fig. 2. Requirement of new protein synthesis for TSA-induced decrease in eNOS mRNA levels in HUVECs. The cells were treated with TSA or CHX or both for 24 h before the cells were harvested for the eNOS mRNA measurements by the real-time quantitative RT-PCR. All data are presented as means + SD of three separate experiments. $*P < 0.05$ and $**P < 0.01$ vs control.

shown in Fig. 3, mevastatin dose-dependently reversed the TSA-induced reduction in eNOS mRNA after added into the culture medium at the same time with TSA for 24 h. However, the effect of mevastatin, which depletes cellular isoprenoid lipid and thereby inhibits prenylation (geranylgeranylation and farnesylation), was completely abolished by the GGPP, but not by FPP. Our findings indicate that geranylgeranylation but not farnesylation is required for the mevastatin to reverse the TSA-induced reduction in eNOS mRNA.

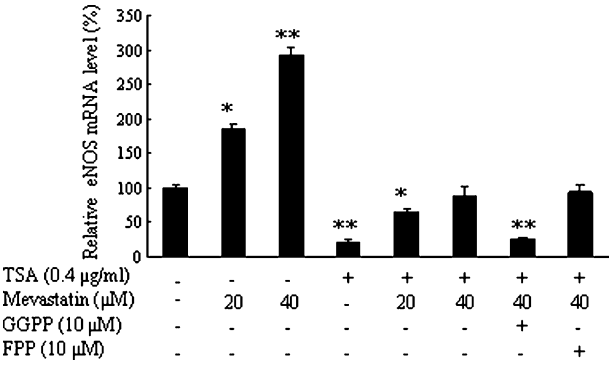

Fig. 3. Involvement of geranylgeranylation in TSA-induced decrease in eNOS mRNA. Inhibition of HMG-CoA reductase by mevastatin dose-dependently antagonized TSA-induced eNOS mRNA reduction in HUVECs, and the mevastatin-mediated antagonism was completely abolished by GGPP, but not by FPP, treatment. The cells were treated with the reagents for 24 h. All data are presented as means + SD of three separate experiments. $*P < 0.05$ and $**P < 0.01$ vs control.

Roles of ROCK and Rho GTPase in TSA-induced eNOS reduction

To understand the signaling pathway underlying the rescue effect by the mevastatin on TSA-induced eNOS mRNA reduction, we investigated the effects of inhibitors of Rho kinase (ROCK, the only known down-stream effector of Rho GTPase) and Rho GTPase on TSA-induced decrease in eNOS mRNA. ROCK inhibitor Y-27632 (10 and 20 μM for 24 h, added at the same time with the TSA treatment) and Rho GTPase inhibitor *C. botulinum* C3 transferase (25 μg/ml for 24 h, added at the same time with the TSA treatment), an exoenzyme which inactivates Rho by ADP-ribosylation [14], did not show the effects of antagonizing TSA-induced decrease in eNOS mRNA. While TSA (0.4 μg/ml) treatment reduced eNOS mRNA to $11.7 \pm 3.4\%$ of the control cells, cells simultaneously treated with Y-27632 or C3 had similar eNOS mRNA levels ($15.4 \pm 5.7\%$ and $14.4 \pm 3.2\%$, respectively) as those treated by TSA alone ($p = \text{NS}$). The results suggest that the ROCK kinase or Rho GTPase may not contribute to the rescuing effect by the mevastatin.

Association of Sp1 with HDAC1 in endothelial cells

The constitutive expression of eNOS gene is proximal Sp1 site dependent [13,15]. To understand the up-regulation of eNOS at the early stage of TSA treatment and down-regulation at the late stage, we investigated the association of Sp1 with HDAC1 in endothelial cells, which was reported in several other cell lines [13,16–18]. Consistently, HDAC1 was detected in anti-Sp1 antibody-precipitated complex from HUVECs, but not in negative control anti-FLAG antibody-precipitated complex (Fig. 4), indicating that Sp1 associates with HDAC1 in endothelial cells.

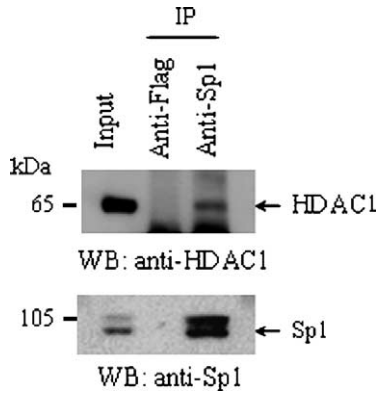

Fig. 4. Association of HDAC1 with Sp1 in HUVECs. Whole cell lysates were immunoprecipitated with anti-Sp1 antibody and anti-FLAG antibody (negative control). The immunocomplexes were subjected to SDS–PAGE and analyzed by Western blot with anti-HDAC1 antibody. Input (40 μg whole cell lysate) was served for the electrophoretic position of HDAC1. The membrane was stripped for the detection of immunoprecipitated Sp1 protein using anti-Sp1 antibody (lower panels).

Region of eNOS mRNA involved in TSA-induced reduction in eNOS mRNA

It is hypothesized that the down-regulation of eNOS by TSA could be due to a destabilizing factor of the eNOS mRNA; and the eNOS 3′-UTR is inferred to be a target for the destabilizing factor [6]. To identify which region of the eNOS mRNA is involved in the TSA-induced eNOS mRNA destabilization, we investigated the effects of eNOS 3′-UTR and full-length region on the activities of luciferase using the pGL3 reporter. pGL3 vector (Promega) uses SV40 promoter to drive luciferase gene. As expected, TSA strongly activated luciferase expression in pGL3 control vector (Fig. 5). TSA also equally activated the construct pGL3-3′-UTR (Fig. 5). Yet, TSA exerted little up-regulating effects on pGL3-full-length construct. Our experiments indicate that insertion of the eNOS 3′-UTR to the 3′ end of the luciferase gene did not introduce destabilizing effects on luciferase mRNA upon TSA treatment. Instead, the destabilizing effects could be mediated by a certain region in the eNOS coding sequence since TSA had an attenuated promoting effect on pGL3-full-length construct. This speculation is further supported by the finding that mevastatin partially recovered the effects of TSA (Fig. 5), which also implicates that the eNOS coding region may be a target for the eNOS destabilizing factor.

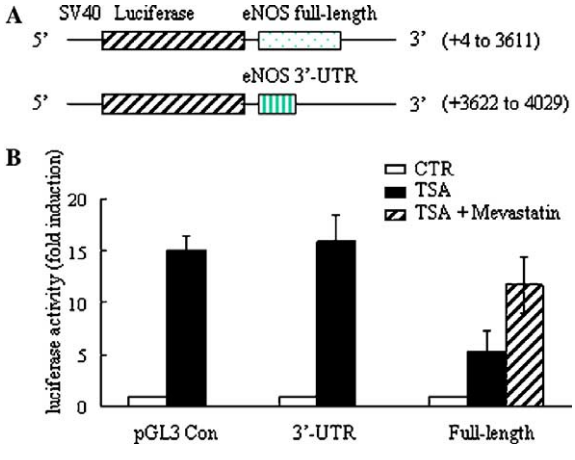

Fig. 5. Involvement of eNOS coding region in TSA-induced eNOS mRNA reduction in HUVECs. (A) Scheme of constructs. The eNOS 3′-UTR and full-length were inserted at the end of luciferase coding sequence in the pGL3-control vector, respectively. (B) Luciferase activity was measured 24 h after the TSA treatment. The HUVECs were transiently transfected with the respective vector constructs. The data shown are means + SD of three separate experiments.

Discussion

In the present study, we demonstrated that inhibition of HDACs by TSA had a dual effect on the eNOS expression in a time- and dose-dependent fashion. Although previous studies demonstrated that TSA down-regulated eNOS by 24 h [6], our study showed that TSA could activate eNOS at the early stage of TSA treatment (by 15 min), and at the low doses (0.1 μg/ml). This up-regulating effect is followed by a time- and dose-dependent repression in the eNOS mRNA expression. We suggest that HDAC inhibition may have complex effects on the eNOS expression as recently described by Fish et al. [19]. Indeed, TSA has been previously demonstrated to directly activate the eNOS promoter; but paradoxically the eNOS is down-regulated by the TSA in endothelial cells [6,13]. The findings imply that the positive effects of TSA on eNOS could be overwhelmed by the negative effects of TSA on eNOS mRNA time- and dose-dependently.

Currently, the mechanisms for TSA-mediated eNOS regulation are not clear. Association of Sp1 with HDAC1 has been previously demonstrated to repress Sp1-mediated transcriptional activation in several cell lines; and the repression of Sp1 will be released through inhibition of HDAC1 enzymatic activities by TSA [13,16,17,20]. Given that the association of Sp1 with HDAC1 inhibits Sp1 activity and that the constitutive expression of eNOS gene is proximal Sp1 site dependent [13,15], our results of Sp1-HDAC1 association in endothelial cells may, to some extent, explain the up-regulatory effect on the eNOS expression at the early stage of TSA treatment.

It is hypothesized that a putative destabilizing factor for the eNOS mRNA may be responsible for the TSA-induced eNOS down-regulation in endothelial cells; and the 3′-UTR of eNOS mRNA could be the target for the factor [6]. Consistently, we showed that inhibition of protein synthesis completely abolished TSA-induced eNOS mRNA reduction (Fig. 2), indicating that new protein synthesis is required for the TSA effect. The data also support the hypothesis that a putative destabilizing factor of eNOS mRNA is induced by the TSA treatment. Our findings rule out the possibility that the inhibitory effect of TSA on eNOS expression is due to the reduction of a transcriptional repressor. However, our results cannot exclude the possibility that the TSA-inducible sONE, an overlapping

antisense transcript to eNOS, may be responsible for the decrease in eNOS RNA levels following the long-term TSA treatment [21]. According to our data, however, it is more likely that the effects of the induced putative destabilizing factor on the eNOS mRNA expression overwhelmed TSA activation on eNOS, resulting in a lower eNOS mRNA. Since it usually takes about 4 h for a new protein to be synthesized, this finding explains why TSA treatment increased the eNOS mRNA levels before 4 h, which was then followed by a reduction.

We have further considered that geranylgeranyl modification may be involved in TSA-induced putative eNOS mRNA destabilizing factor. Geranlygeranyl modification is an important process for some proteins to be translocated to membrane and activated [22,23]. Blocking Rho geranylgeranylation is reported as an important mechanism for statins to stabilize eNOS mRNA [14]. In the present study, we found that mevastatin could antagonize the TSA-induced eNOS down-regulation. However, the effect of mevastatin is completely abolished by GGPP, implying that geranylgeranylation may be an important modification for the putative eNOS mRNA destabilizing factor. Considering membrane has an important role in RNA transportation and localization, the functional significance of geranylgeranyl modification of the putative destabilizing factor may facilitate the bindings between the destabilizing factor and the eNOS mRNA. The results may also explain why VEGF could not compensate for the TSA-induced decrease in eNOS mRNA [6] since VEGF does not block geranylgeranyl modification, but statins do [14]. In addition, although ROCK and Rho GTPase are the downstream signals of geranylgeranyl modification [23] and are involved in eNOS expression [14], their specific inhibitors could not antagonize TSA-mediated eNOS down-regulation. Our results indicate that the two kinases may not be involved in the TSA-induced eNOS downregulation.

The 3′-UTR is frequently considered to be the target for mRNA degradation in many cases [24]. Likewise, the eNOS 3′-UTR is also thought to be the target influencing eNOS mRNA stability [25]. Using luciferase reporter assay we tested whether eNOS 3′-UTR is indeed a target for TSA-induced decrease in eNOS. Surprisingly, our results do not support the involvement of the 3′UTR in the TSA-induced eNOS suppression (Fig. 5). Instead, the eNOS coding region may contain the target for TSA-induced putative destabilizing factor. While more work is clearly needed to identify the target region in the eNOS coding sequence, there are cases showing that coding regions are involved in mRNA degradation, e.g., the coding region of c-fos and c-myc [26–28].

Currently, TSA-induced gene expression in most cases is Sp1-dependent genes [13,16,17,20]. Accordingly, we propose that HDAC1 inhibition by TSA releases the repression of Sp1, which results in an immediate eNOS promoter activation. At the same time, TSA may also activate the putative destabilizing factor of eNOS mRNA. When there is a sufficient expression by the destabilizing factor, which degrades eNOS mRNA, the net outcome may show as reduced eNOS mRNA levels. It is also intriguing that eNOS is constitutively expressed in endothelial cells, yet the Sp1 is associated with the HDAC1. Previous studies have demonstrated that eNOS promoter is almost DNA methylation free in endothelial cells [3]; and that the histone H3 of the eNOS promoter is in a status of hyperacetylation and TSA treatment did not induce a further increase of acetylation [13]. Therefore, the effects of inhibition of Sp1 by HDAC1 on eNOS expression in normal endothelial cells seem limited. Indeed, our results showed the activation of eNOS promoter in the early stage of TSA treatment was moderate in endothelial cells, reflecting that HDAC1 has a limited inhibition on the Sp1 in eNOS expression.

In conclusion, we have shown that inhibition of HDACs by TSA has complex effects on the eNOS expression. The up-regulation of the eNOS at the early stage of TSA treatment is gradually overwhelmed by the induced putative eNOS mRNA destabilizing factor. Geranylgeranyl modification is necessary for the factor to degrade eNOS mRNA. The coding region of eNOS mRNA, but not the 3′-UTR, is involved in the TSA-induced eNOS mRNA degradation. Further studies are needed to identify the putative destabilizing factor and *cis*-acting element in eNOS coding region.

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