SIRT1 regulates oxidant- and cigarette smoke-induced eNOS acetylation in endothelial cells: Role of resveratrol

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Abstract

Endothelial nitric oxide synthase (eNOS) plays a crucial role in endothelial cell functions. SIRT1, a NAD$^+$-dependent deacetylase, is shown to regulate endothelial function and hence any alteration in endothelial SIRT1 will affect normal vascular physiology. Cigarette smoke (CS)-mediated oxidative stress is implicated in endothelial dysfunction. However, the role of SIRT1 in regulation of eNOS by CS and oxidants are not known. We hypothesized that CS-mediated oxidative stress downregulates SIRT1 leading to acetylation of eNOS which results in reduced nitric oxide (NO)-mediated signaling and endothelial dysfunction. Human umbilical vascular endothelial cells (HUVECs) exposed to cigarette smoke extract (CSE) and H_2O_2 showed decreased SIRT1 levels, activity, but increased phosphorylation concomitant with increased eNOS acetylation. Pre-treatment of endothelial cells with resveratrol significantly attenuated the CSE- and oxidant-mediated SIRT1 levels and eNOS acetylation. These findings suggest that oxidant-mediated reduction of SIRT1 is associated with acetylation of eNOS which have implications in endothelial dysfunction.

Keywords

Cigarette smoke; oxidants; SIRT1; eNOS; endothelial dysfunction

Introduction

Reactive oxygen species (ROS) either generated endogenously or by exposure to cigarette smoke (CS) play a major role in the progression of several diseases including cardiovascular and pulmonary diseases [1,2]. CS is known to cause endothelial dysfunction which is characterized by reduced cell migration, angiogenesis vasodilation, proinflammatory and prothrombic properties associated with the development of cardiovascular diseases (CVD) [1,2] Endothelial nitric oxide synthase (eNOS), a vital signaling molecule, plays a crucial role in endothelial cell functions. eNOS regulates vascular tone, blood flow, platelet aggregation, cell adhesion and leukocyte-endothelial cell interactions which are characteristics of endothelial function [3]. It has been shown that accelerated ROS production leads to decreased

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eNOS levels, which play a central role in CS-mediated endothelial dysfunction [4]. We and others have recently shown that CS-mediated oxidative stress downregulate eNOS levels leading to decreased NO production and endothelium-dependent vasodilatation in endothelial cells [5-8]. However, it is not known whether eNOS is subjected to post-translational modifications, in particular acetylation, rendering it inactive in response to CS-mediated oxidative stress in endothelial cells.

SIRT1, which is homologous to yeast (*S. cerevisiae*) silent information regulator protein 2 (Sir 2), plays a critical role in aging, cell cycle regulation, apoptosis and inflammation [9,10]. It is highly expressed in vascular endothelial cells and plays a key role in regulating the endothelial function [11-15]. Although SIRT1 has been shown to play a critical role in endothelial vascular biology, it is not known whether SIRT1 regulates endothelial function via acetylation/deacetylation of eNOS in response to CS and oxidants. We therefore hypothesized that CS-mediated oxidative stress downregulates SIRT1 by phosphorylation and degradation leading to eNOS acetylation in endothelial cells. To test this hypothesis, human umbilical vein endothelial cells (HUVECs) were exposed to cigarette smoke extract (CSE) and H_2O_2, and the role of SIRT1 in regulation of eNOS acetylation/deacetylation was determined. We also studied whether pharmacological activation and inhibition of SIRT1 can regulate eNOS acetylation in these cells.

Materials and Methods

Human umbilical vein endothelial cell culture

HUVECs culture was established as described previously [7,8] by using human umbilical cords collected within 48 hr of delivery and were grown in EGM-2 (Lonza, Walkersville, MD, USA, previously known as Cambrex) media containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown in 75 mm^2 flask coated with 0.1% gelatin, and treatments were performed in 0.1% gelatin-coated 6-well plates [7,8].

CSE preparation

Research grade cigarettes (1R3F) were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Lexington, KY). 10% CSE was prepared by bubbling smoke from one cigarette into 10 ml of culture medium supplemented with 1% FBS at a rate of one cigarette per minute as described previously [7,8].

Cell treatments

HUVECs were grown (90% confluent) in 6-well culture plates and starved for 6 hr in 0.1% FBS then treated with CSE (0.5% and 1.0%) or H_2O_2 (25, 50 and 100 μM) for 1 hr and 4 hr. For treatments with proteasome inhibitor (N-Acetyl-Leu-Leu-Nle-CHO, ALLN, 5 μM; Calbiochem, San Diego, CA), cells were pretreated for 1 hr followed by exposure to CSE or H_2O_2. To investigate the pharmacological regulation of SIRT1, HUVECs were pretreated for 1 hr with resveratrol, 10 μM (SIRT1 activator) and splitomicin, 10 μM (SIRT1 inhibitor) (Enzo lifesciences, PA, USA) alone or in combination with CSE (1.0%) or H_2O_2 (100 μM) for 4 hr. At the end of the incubations, cells were washed twice with ice-cold PBS and lysed using RIPA buffer containing protease inhibitor cocktail as described previously [8]. Finally, the whole cell lysates were sonicated for 10 seconds and centrifuged at 10,000 g for 15 min, and supernatant was separated and analyzed for protein content using BCA kit (Pierce).

Immunoblotting

Cellular protein (40 μg) was electrophoresed on 7.5% SDS-PAGE gel and transblotted on nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Membranes were

blocked with 5% (w/v) non-fat milk in PBS containing 0.1% (v/v) Tween 20 and incubated with SIRT1 (rabbit- anti-SIRT1, Cell Signaling) and phosphorylated SIRT1 (rabbit anti-phospho-SIRT1 (Ser27 and Ser47), Cell Signaling) antibodies. After washing, bound antibody was detected using anti-rabbit/anti-mouse antibody linked to horseradish peroxidase and bound complexes were detected using enhanced chemiluminescence (Perkin Elmer, Waltham, MA, USA).

SIRT1 activity assay

SIRT1 activity was assayed using a deacetylase colorimetric activity assay kit according to the manufacturer's instructions (Biomol International, Plymouth Meeting, PA).

eNOS acetylation by immunoprecipitation

Whole cell lysate was used for eNOS immunoprecipitation. An antibody against eNOS (1:40 dilution, Cell Signaling) was added to 100 μg of cellular proteins in a final volume of 400 μl, and incubated for 1 hr. Protein-A/G agarose beads (20 μl) (Santa Cruz Biotechnology) were added to each sample and kept overnight at 4°C on a rocker. The beads were washed three times and then resuspended in 40 μl of RIPA buffer. The immunoprecipitated eNOS agarose bead suspension was resolved by 7.5% SDS-PAGE. To assess the acetylation of eNOS, the membranes were first probed against acetyl lysine antibody (Mouse monoclonal acetyl lysine, Cell Signaling) then reprobed for eNOS (rabbit anti-eNOS, Cell signaling).

Statistical analysis

The SigmaStat 3.0 statistical program was used to analyze the data. All pair-wise multiple comparisons were performed using the ANOVA; values of $P < 0.05$ were considered as significant.

Results

CSE and H_2O_2 decreased SIRT1 level in HUVECs

SIRT1 plays important role in vascular inflammation and endothelial dysfunction [15]. However, it is not known that whether SIRT1 level is altered in response to CS-mediated oxidative stress in endothelial cells. Therefore, we determined the effects of CSE on SIRT1 protein levels in HUVECs. Treatment of endothelial cells with CSE (0.5% and 1.0%) for 1 and 4 hr resulted significant dose- and time-dependent decrease in SIRT1 levels when compared to control treatments (P<0.001, Fig. 1A-C). Similarly, the SIRT1 level was reduced by dose- and time-dependent manner in HUVECs treated with H_2O_2 (25, 50 and 100 μM) for 1 and 4 hr (Fig. 1A-C).

CSE and H_2O_2 decreased SIRT1 deacetylase activity in HUVECs

We further investigated whether CSE-mediated oxidative stress which showed decreased SIRT1 levels also show decreased SIRT1 deacetylase activity. Indeed, SIRT1 deacetylase activity was reduced in a time-dependent manner at 1 and 4 hr by CSE (1.0%) and H_2O_2 (100 μM) treatments in endothelial cells (Fig. 1D). Pre-treatment of cells with resveratrol significantly increased the SIRT1 deacetylase activity alone or combination with CSE or H_2O_2 treatments whereas splitomicin alone or in combination with CSE or H_2O_2 treatments further reduced the SIRT1 deacetylase activity (Fig. 1D). These data showed that CSE-mediated reduction in SIRT1 level was associated with decrease in its deacetylase activity in HUVECs.

CSE and H_2O_2 increased SIRT1 phosphorylation in HUVECs

Recent studies suggest that alterations in mRNA level was not associated with reduced SIRT1 protein and activity levels [16,17], suggesting that post-translational modifications are involved in SIRT1 regulation. We therefore investigated the effect of CSE and H_2O_2 treatments on phosphorylation pattern of SIRT1 in HUVECs. Endothelial cells treated with increasing doses of CSE (0.5% and 1.0%) and H_2O_2 (25, 50 and 100 μM) for 1 and 4 hr showed significant phosphorylation of SIRT at Ser27 and Ser47 residues compared to control treatments ($P<0.01$ and $P<0.001$, Fig. 2A-C).

CSE- and H_2O_2- mediated SIRT1 degradation in HUVECs is proteasome dependent

Since SIRT1 degradation is associated with its phosphorylation in response to CSE and oxidative stress. We therefore determined whether CSE- and H_2O_2- mediated reduction of SIRT1 levels was associated with its degradation in proteasomes. Pretreatment of HUVECs with proteasome inhibitor N-acetyl-Leu-Leu-Nle-CHO (ALLN; 5 μM) for 1 hr and followed by CSE (1.0%) or H_2O_2 (100 μM) treatment for 1 and 4 hr resulted in time-dependent attenuation of SIRT1 levels when compared to CSE and H_2O_2 treatments alone (Fig. 3A-C).

CSE and H_2O_2 caused eNOS acetylation in HUVECs

eNOS plays an important role in endothelial function and is inactivated by oxidants [5-8]. We therefore determined whether CSE- and H_2O_2- mediated oxidative stress modifies eNOS, thereby rendering it inactive in HUVECs. We found that acetylation of eNOS (at lysine residues) was significantly increased in response to CSE (0.5% and 1.0%) and H_2O_2 treatments (50 and 100 μM) both at 1 and 4 hr ($P<0.001$, Fig. 4A-C). These data suggest that CS-mediated eNOS acetylation may lead to decreased NO bioavailability in endothelial cells.

Pharmacological activation of SIRT1 regulates CSE-induced eNOS acetylation in HUVECs

We further studied whether CS-mediated eNOS acetylation was associated with decreased SIRT1 levels and pharmacological activation of SIRT1 can modulate CSE-induced eNOS acetylation. Pretreatment of HUVECs with resveratrol (10 μM) for 4 hr alone or in combination with CSE (1.0%) resulted in reduction in eNOS acetylation when compared to CSE alone ($P<0.001$, Fig. 4 D-F). Furthermore, pretreatment of endothelial cells with splitomicin alone or in combination with CSE increased eNOS acetylation. We also showed phosphorylation of eNOS at ser1177 in response to CSE [8], which was reversed by resveratrol (Arunachalam *et al.* unpublished data). These data support the notion that activation of SIRT1 attenuates eNOS post-translational modifications (phosphoacetylation of eNOS) which may regulate endothelial function during oxidative stress.

Discussion

Oxidative stress-mediated chronic inflammation in smokers renders damage to endothelial cells thus increases response to vascular injury [6]. Previously, we have shown that CS-induced oxidative stress impaired the angiogenic function of endothelial cells by downregulating phosphorylation of Akt and eNOS in endothelial cells [7,8]. Recently, it has been shown that activation of SIRT1 protects endothelial cells against CS-induced oxidative stress [18]. However, the downstream target of SIRT1 in protection against oxidative stress-mediated endothelial dysfunction is not fully known. We hypothesized that CS and oxidative stress downregulate SIRT1 and increase eNOS acetylation leading to endothelial dysfunction. Indeed, CSE and H_2O_2 treatments caused dose- and time-dependent reduction of SIRT1 levels and its deacetylase activity in HUVECs. Our data are in agreement with the recent reports showing that CS-mediated oxidative stress decreased SIRT1 levels and its deacetylase activity

in vitro in macrophages and epithelial cells as well as in lungs of patients with chronic obstructive pulmonary disease (COPD) [19-22].

Oxidative stress cause extensive post-translational modifications to signaling proteins leading to loss of their functions [23]. Recent studies have identified several phosphorylation sites on SIRT1 [16,17] and the phosphorylation on these residues alters the SIRT1 protein levels [22, 24]. We proposed that CS-mediated reduction of SIRT1 level is associated with its phosphorylation in endothelial cells. CSE treatments dose-dependently increased phosphorylation of SIRT1 on Ser27 and Ser47 residues. Ubiquitin-dependent proteasomal degradation plays a critical role in degradation of oxidatively modified proteins [25]. Hence phosphorylated SIRT1 may undergo proteasomal degradation. Indeed, we showed that proteasomal inhibition by a potent proteasome inhibitor attenuated the SIRT1 levels suggesting a possible role of ubiquitin-dependent degradation of SIRT1. This observation is supported by a recent study demonstrating that ionizing radiation induces proteasomal degradation of SIRT1 [26].

eNOS activation leads to NO release which exerts vasoprotective and cardioprotective effects in endothelial cells by regulating blood flow, inhibition of platelet aggregation and inflammatory cell adhesion [27]. Moreover, ROS-mediated downregulation of eNOS plays a critical role in CS-mediated endothelial dysfunction [4,5,7,28]. SIRT1 is recently identified as a critical regulator of endothelial function [29]. In the light of our data showing the reduction of SIRT1 levels by CS, we hypothesized that CS would cause increased eNOS acetylation in endothelial cells (acetylation leads to inactivation of eNOS activity). Indeed, we show increased eNOS acetylation in endothelial cells in response to CSE treatments. Furthermore, SIRT1 activation by pre-treatment with a non-specific SIRT1 activator resveratrol attenuated CS-induced eNOS acetylation whereas SIRT1 inhibition by splitomicin further increased eNOS acetylation in HUVECs. We further showed phosphorylation of eNOS at Ser1177 in response to CSE [8], which was reversed by resveratrol suggesting that CSE induces phosphoacetylation of eNOS. This is supported by other observations that SIRT1 activation promotes endothelial-dependent vasodilatation by targeting eNOS for deacetylation to enhance NO production [30] whereas SIRT1 knock-down showed decreased NO production and impaired endothelial-dependent vasodilatation [31]. Our data support the concept that activation of SIRT1 pharmacologically or by dietary polyphenol resveratrol may protect against deleterious effects of CS/oxidants on endothelial dysfunction [18,31]. Hence it is hypothesized that the impairment of eNOS due to acetylation under oxidative stress would lead to endothelial dysfunction, and resveratrol or a specific SIRT1 activator would deacetylate eNOS and maintain the NO bioavailability/endothelial function. This may be one of the possible mechanisms of resveratrol in maintaining or improving endothelial function. However, further studies are required to investigate whether eNOS acetylation alters its activity and NO bioavailability in response to CS in endothelial cells leading to endothelial dysfunction and alterations in proliferation, migration and angiogenesis. It is also worthwhile to determine whether the acetylation of eNOS has any impact on caveolin-1 acetylation as these molecules play a synergistic role in NO bioavailability and hence endothelial function/angiogenesis [32]. Preliminary data from our lab also indicated acetylation of caveolin-1 in response to CS exposure in endothelial cells.

In summary, we show that CS and oxidative stress downregulate SIRT1 levels and increase its phosphorylation on Ser27 and Ser47 residues concomitant with increased eNOS acetylation in endothelial cells. Pre-treatment of endothelial cells with resveratrol attenuated the CSE-mediated SIRT1 levels and eNOS acetylation. Identification of eNOS as a substrate of SIRT1 under the conditions of oxidative stress is important in understanding the beneficial effect of SIRT1 on endothelial vascular biology in terms of preventing stress-induced endothelial dysfunction, an early step in the pathogenesis of cardiopulmonary diseases.

Acknowledgments

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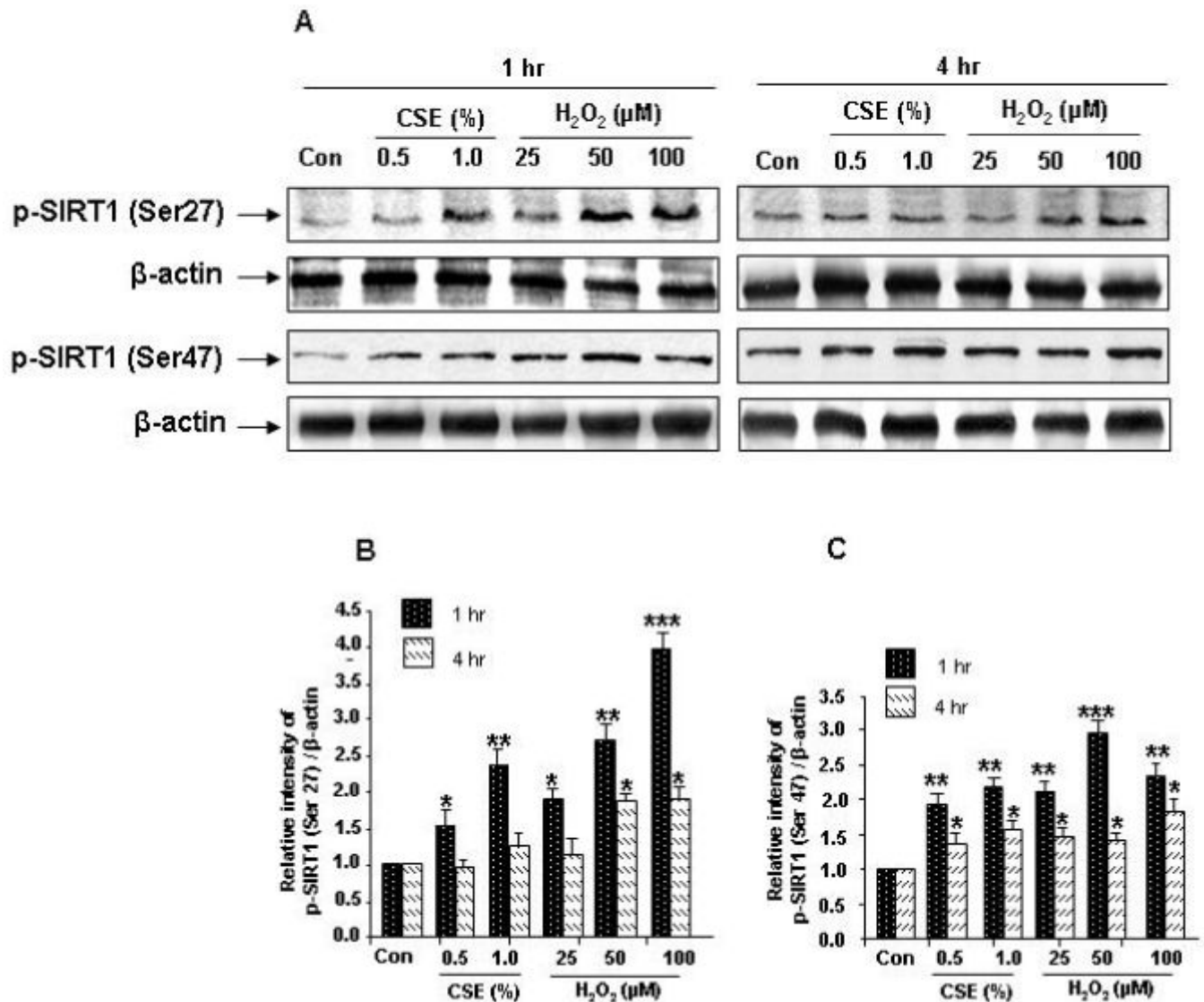
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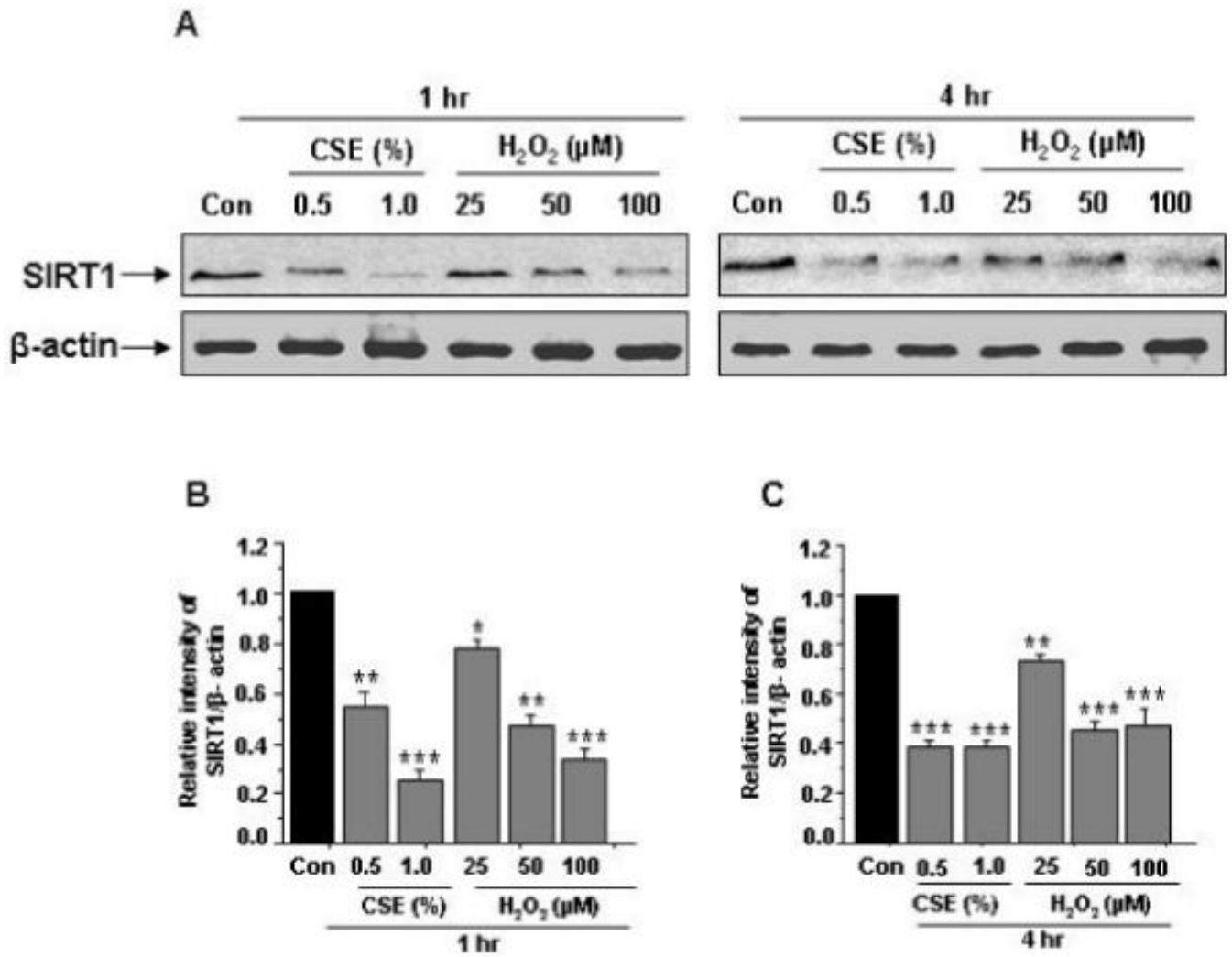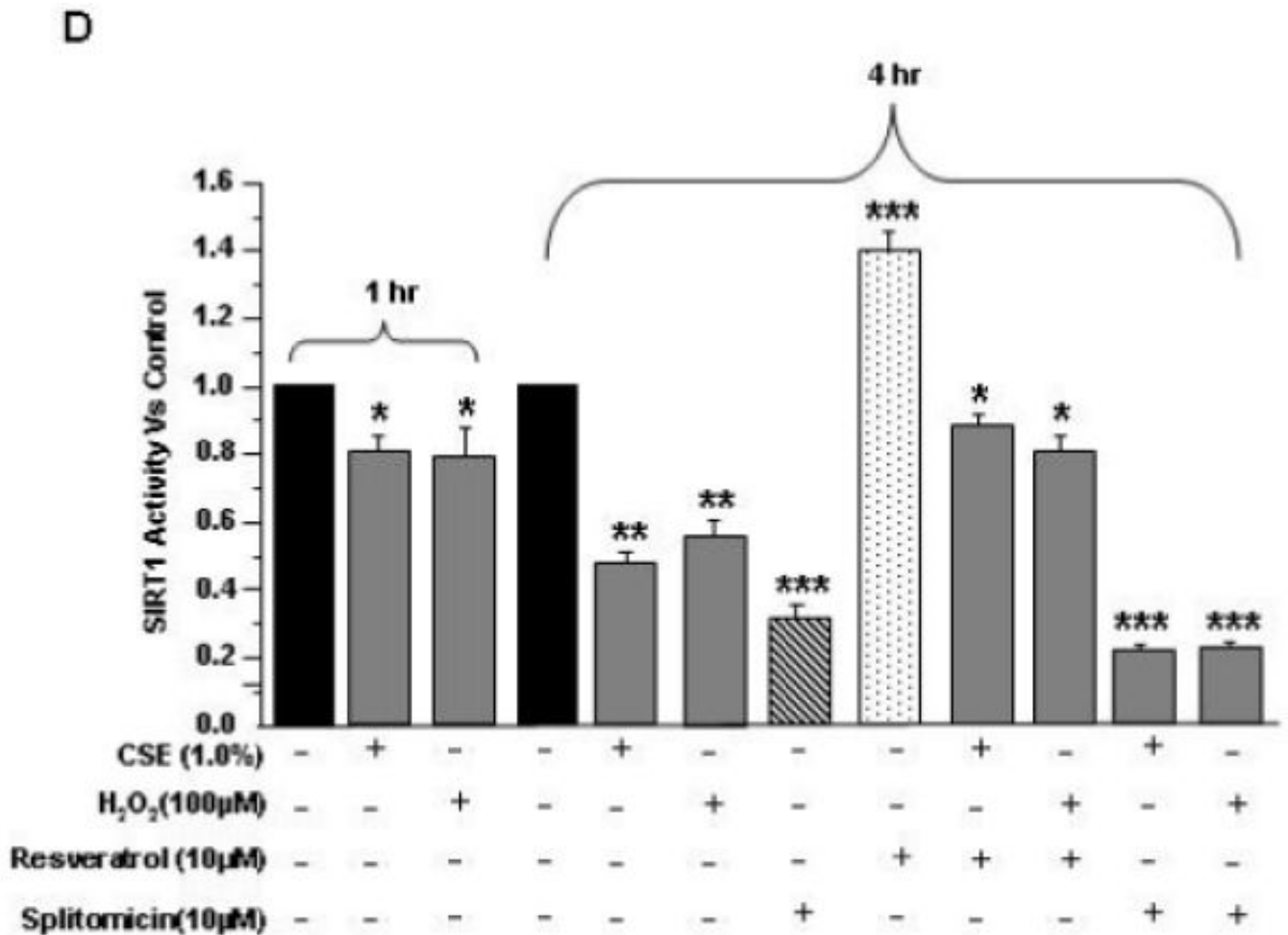
Figure 1. Effect of CSE and H_2O_2 on SIRT1 levels and activity in HUVECs
SIRT1 levels: A) HUVECs were treated with CSE (0.5% and 1.0%) and H_2O_2 (25, 50 and 100 μM) for indicated time points. B) and C) Histograms represent the relative intensity of SIRT1 levels (bands) compared to respective controls (n=3). SIRT1 activity: D) HUVECs were treated with CSE (1.0%) and H_2O_2 (100 μM) for 1 hr, and at 4 hr alone or in combination with pretreatment of resveratrol (10 μM) and splitomicin (10 μM), and SIRT1 deacetylase activity was determined. Data are Mean ± SE (n=3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* controls.
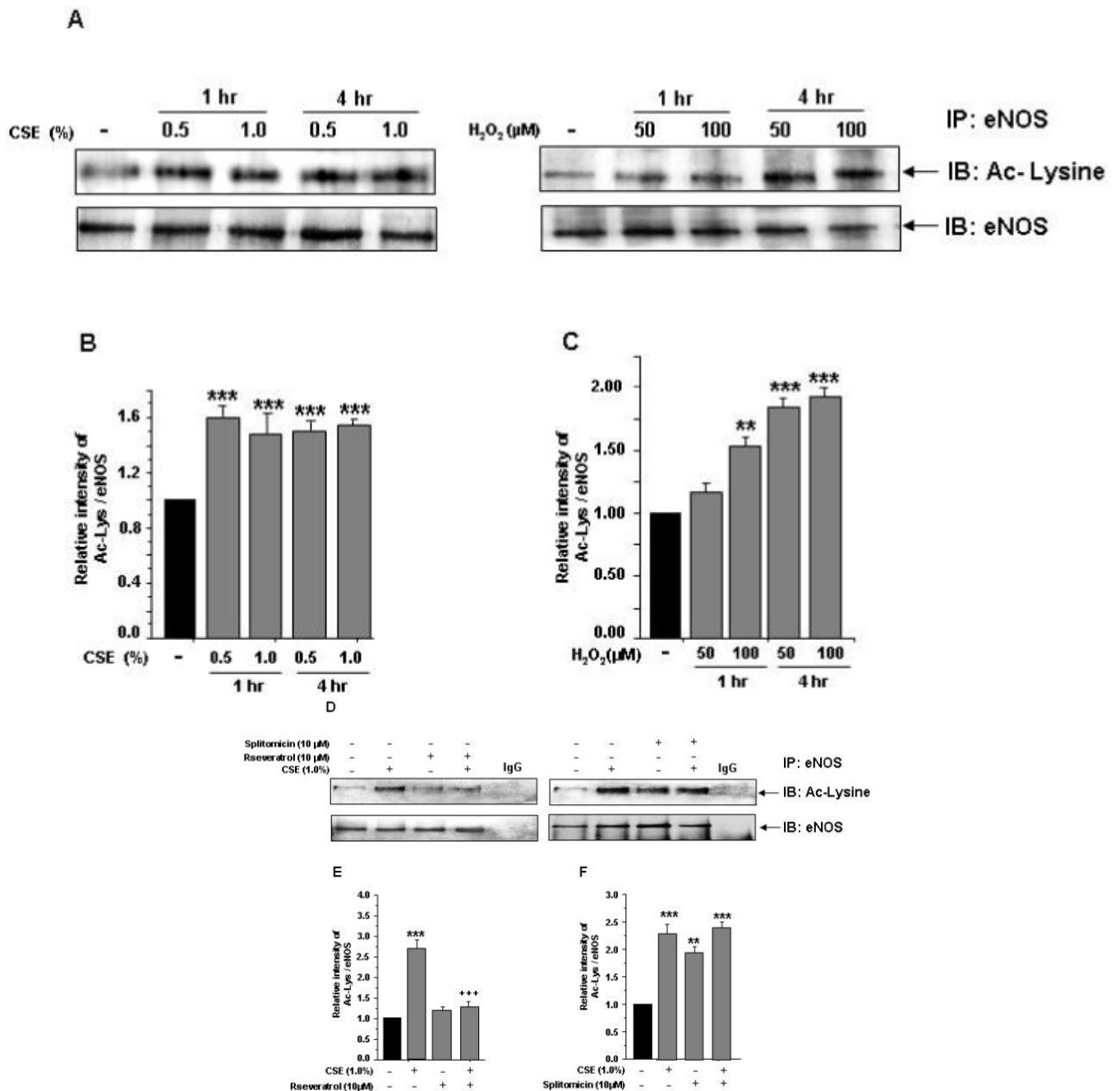
Figure 2. Effect of CSE and H$_2$O$_2$ on SIRT1 phosphorylation in HUVECs
HUVECs were treated with CSE (0.5% and 1.0%) and H$_2$O$_2$ (25, 50 and 100 μM) for indicated time points. A) CSE and H$_2$O$_2$ increased the phosphorylation of SIRT1 (at Ser27 and Ser47). B) and C) Histograms represent relative intensity of phosphorylated SIRT1 levels compared to respective control treatments (n=3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs*. controls. p-SIRT1 (Ser27), phosphorylation of SIRT1 at Ser27; p- SIRT1 (Ser47), phosphorylation of SIRT1 at Ser 47.

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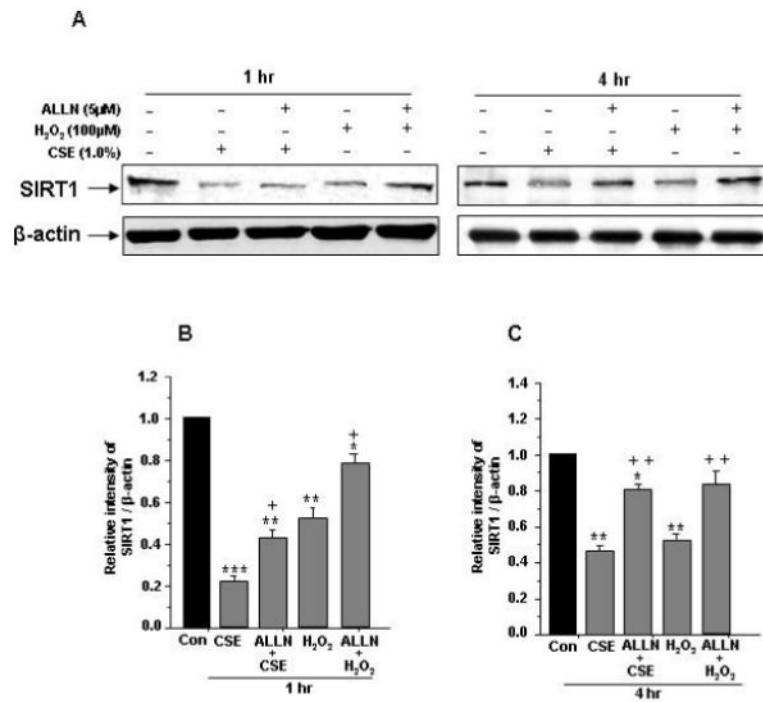
Figure 3. Effect of proteasome inhibitor on CSE- and H_2O_2-induced degradation of SIRT1 degradation in HUVECs

A) HUVECs cells were pre-treated with or without proteasomal inhibitor N-Acetyl-Leu- Leu-Nle-CHO (ALLN, 5 μM) for 1 hr and then treated with CSE (1.0%) or H_2O_2 (100 μM) for 1 and 4 hr. B) and C) Histograms represent Mean ± SE of relative intensity of SIRT1 levels compared to respective controls (n=3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs*. controls. [+]$P < 0.05$, [++]$P < 0.01$ *vs*. CSE and H_2O_2 treatments alone.

Figure 4. Effect of CSE and H_2O_2 on eNOS acetylation and its regulation by SIRT1 in HUVECs
eNOS acetylation: A) HUVECs were treated with CSE (0.5% and 1.0%) and H_2O_2 (50 and 100 μM) for indicated time points. B) and C) Histograms represent the relative intensity of acetylated lysine levels of eNOS compared to respective controls (n=3). SIRT1 regulation of eNOS acetylation: D) HUVECs cells were pre-treated with or without SIRT1 activator (resveratrol, 10 μM) and inhibitor (splitomicin, 10 μM) for 1 hr and then treated with CSE (1.0%) for 4 hr. E) and F) Histograms represent the relative intensity of acetylated lysine levels of eNOS compared to respective controls (n=3). IgG was used as an isotype control. Data shown are Mean ± SE. **$P < 0.01$, ***$P < 0.001$ *vs*. control groups. +++$P < 0.001$ *vs*. CSE treatment alone.