Polydatin inhibits the oxidative stress-induced proliferation of vascular smooth muscle cells by activating the eNOS/SIRT1 pathway

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Abstract. Oxidative stress-mediated proliferation of vascular smooth muscle cells (VSMCs) contributes to plaque formation and the progression of atherosclerosis. Polydatin is a derivative of resveratrol, and is widely present in certain herbal medications used for the treatment of cardiovascular diseases. In the present study, we examined whether polydatin was capable of attenuating VSMC proliferation induced by oxidative stress as well as the potential involvement of the endothelial nitric oxide synthetase (eNOS)/SIRT1 pathway. Briefly, VSMCs were exposed to H_2O_2 for 24 h in the absence or presence of polydatin (10-100 μM) prior to performing a cell proliferation assay. In mechanistic studies, the cells were incubated with the silent information regulator 1 (SIRT1) inhibitor, EX527, or the eNOS inhibitor, L-NAME, prior to polydatin treatment. The results showed that polydatin inhibited VSMC proliferation and the level of reactive oxygen species, increased the expression of Kip1/p27, SIRT1 and eNOS, whereas the expression of cyclin B1, Cdk1 and c-myc was decreased. The number of cells in the G2/M phase was increased. Pre-treatment with L-NAME attenuated the inhibitory effects of polydatin on cell proliferation, inhibited the expression of SIRT1 and the phosphorylation of eNOS. Pre-treatment with EX527 also attenuated the inhibitory effects of polydatin on cell proliferation, but failed to reduce the activation of eNOS and the production of nitric oxide. Taken together, these findings suggest that, polydatin inhibited the oxidative stress-induced proliferation of VMSCs by activating the eNOS/SIRT1 pathway.

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Introduction

Drinking wine in moderation, in particular red wine, may lower the incidence of cardiovascular diseases, presumably due in part to the benefits of resveratrol in the wine (1). Polydatin is a glycoside derivative of resveratrol, and widely present in various plant sources including red wine, grapes skins and Japanese knotweed (2). Polydatin is an ingredient of many herbal medications used for the treatment of cardiovascular diseases in China. Similar to resveratrol, polydatin has anti-oxidant (3), anti-inflammatory (4) and anti-ageing (5) effects.

It has been shown that oxidative stress promotes the proliferation of vascular smooth muscle cells (VSMCs) and induces blood vessel remodeling (6), thereby contributing to the pathogenesis of atherosclerosis (7,8). Consequently, inhibiting VSMC proliferation may be used as a future therapeutic strategy for the treatment of atherosclerosis. Previous studies have indicated that polydatin may protect cardiac function from acute injury by reducing oxidative stress and inhibiting sarcoplasmic reticulum Ca^{2+} leakage (9), inhibiting platelet aggregation and intercellular cell adhesion molecule-1 (ICAM-1) expression, and weakening white blood cell-endothelial cell adhesion (10). However, the effect of polydatin on the oxidative stress-induced proliferation of VSMCs remains unclear.

Silent information regulator 1 (SIRT1) is a key factor that regulates cell responses to oxidative stress by deacetylating non-histone targets (11). Inhibiting SIRT1 expression has been demonstrated to accelerate cell senescence and promote cell injury (12,13). Knockdown of SIRT1 by small interfering RNA in HUVECs resulted in impaired antioxidant ability (14). A previous study from this laboratory revealed that the expression of SIRT1 was reduced in ageing (15). The improvement of endothelial function in the thoracic artery of ageing rats by atorvastatin was associated with the increased expression of SIRT1 and endothelial nitric oxide synthase (eNOS) (15).

In the present study, we demonstrated that polydatin inhibited the oxidative stress-induced proliferation of VSMCs. We then examined the potential role of the eNOS/SIRT1 pathway with EX527, a SIRT1 inhibitor, and L-NAME, an eNOS inhibitor.

Materials and methods

Cell culture. VSMCs were isolated from the thoracic aorta of 12-week-old male Sprague-Dawley rats (Experimental Animal Center, Southern Medical University, Guangzhou, China) as previously described (16). The present study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (15). Experimental procedures were approved by the Animal Research Committee of Nanfang Hospital. The purity of the isolated VSMCs was verified using immunohistochemical staining of smooth muscle α-actin. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Australia), in a humidified incubator containing 5% CO_2 at 37˚C. VSMCs at 4-6 passages, and at 40% confluence, were used for the experiments.

Treatment. In the preliminary dose-finding experiments, VSMCs were exposed to the vehicle (0.1% DMSO) and 100 μM H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) (14) in the absence or presence of polydatin (purity ≥98%, HPLC) (10, 50 or 100 μM; Baoji Herbest Bio-Tech Co., Ltd., Baoji, China) for 24 h prior to performing a cell proliferation assay using a CCK-8 assay kit (Beyotime Institute of Biotechnology, Nantong, China), as previously described (17). Absorbance was measured with an enzyme-linked microplate assay reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm. In the subsequent set of experiments, the cells were treated with 100 μM eNOS inhibitor L-NAME (18) or 10 μM SIRT1 inhibitor EX527 (both from Sigma-Aldrich) (13) for 2 h prior to treatment with H_2O_2 and polydatin (100 μM).

Measurement of reactive oxygen species (ROS), superoxide dismutase (SOD) and nitric oxide (NO) levels. Re-suspended cells were incubated in pre-warmed DMEM containing 2,7-dichlorofluorescein diacetate (H_2DCFDA; Gibco) fluorescent probe (5 μM) at 37˚C for 30 min. The cells were washed with PBS twice. Intracellular ROS was examined using a spectrofluorometer (Agilent Technologies, Inc., Palo Alto, CA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, as previously described (19). A thiobarbituric acid method was used to detect SOD with WST-1 assay kit (Jiancheng Bioengineering Institute, Nanjing, China) (20). NO in the supernatant was examined using a Total Nitric Oxide Assay kit (Jiancheng Bioengineering Institute), as previously described (21). Since NO is rapidly converted to nitrite (NO_2^-) and further to nitrate (NO_3^-), NO content was reflected by nitrite plus nitrate, as measured with Griess reagent.

Western blot analysis. Total protein was measured using a bicinchoninic acid (BCA) method. Samples containing 50 μg protein were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 1.5 h, and then incubated overnight at 4˚C with anti-Kip1/p27 antibody (1:500), anti-cyclin B1 antibody (1:500), anti-cyclin dependent kinase (Cdk)1 antibody (1:500), anti-c-myc antibody (1:1,000), phosphorylated (p-) (Ser1177)-eNOS antibody (1:250), anti-eNOS antibody (1:250) or anti-SIRT antibody (1:500). Polyclonal primary antibodies against SIRT1, p-(Ser1177)-eNOS and eNOS were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA) and Kip1/p27, cyclin B1, Cdk1 and c-myc were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Following incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) for 1 h, the blots were visualized using an enhanced chemiluminescence (ECL) method. Bands of interest were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA). β-actin (1:1,000; ZSGB-BIO, Beijing, China) was used as the loading control.

Cell cycle analysis. The cells were fixed with ice-cold 70% ethanol for 4 h, washed with PBS twice and stained with propidium iodide (PI). A minimum of $1x10^3$ cells/sample were counted. Cells in distinct cell cycles were characterized by DNA amount (G0/G1 cells, diploid; S cells, DNA synthesis between diploid and tetraploid; and G2/M cells, tetraploid) using flow cytometry (FCM; Beckman Coulter, Inc., Brea, CA, USA), as previously reported (22).

Reverse transcription quantitative-polymerase chain reaction (RT-qPCR). Total RNA was extracted with RNAiso Plus (Takara Bio, Dalian, China) using a phenol-chloroform method. Reverse transcription was carried out in 20 μl reaction mixture containing 1 μg total RNA. The primers were designed using GenBank sequences and Primer-BLAST (Table I). The resulting cDNA was amplified using a SYBR-Green PCR method. Initial denaturation was performed at 94˚C for 5 min, followed by 40 cycles of denaturation at 94˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 45 sec. SIRT1 and eNOS mRNA were calculated using a comparative threshold cycle (Ct) method (ΔΔCt method) with β-actin as a reference.

Statistical methods. Data were presented as the means ± standard deviation (SD) for at least three sets of independent experiments and analyzed using one-way analysis of variance (ANOVA), followed by post-hoc analysis for pairwise comparison: LSD method upon homogeneity of variance, Dunnett's t-test. $P<0.05$ (two-sided test) was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

Effect of polydatin on proliferation. Polydatin attenuated H_2O_2-induced VSMC proliferation in a concentration-dependent manner ($P<0.05$ at 50 and 100 μM vs. H_2O_2-treated group alone; Fig. 1A). Polydatin concentrations of 50 and 100 μM were selected for subsequent experiments.

Effects of polydatin on ROS, SOD, NO, eNOS and SIRT1. At 50 and 100 μM, polydatin significantly decreased the level of ROS, and increased the activity of SOD and the level of NO ($P<0.05$ vs. H_2O_2-treated group alone; Fig. 1B-D).

Table I. Primers used for RT-qPCR.

Target mRNA	Forward primer (5' to 3')	Reverse primer (5' to 3')
SIRT1	ACAACCTCCTGTTGGCTGATGAGA	AGAATTGTTCGAGGATCGGTGCCA
eNOS	GGATCCAGTGGGGGAAACTG	TGGCTGAACGAAGATTGCCT
β-actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC

SIRT1, silent information regulator 1; eNOS, endothelial nitric oxide synthase.

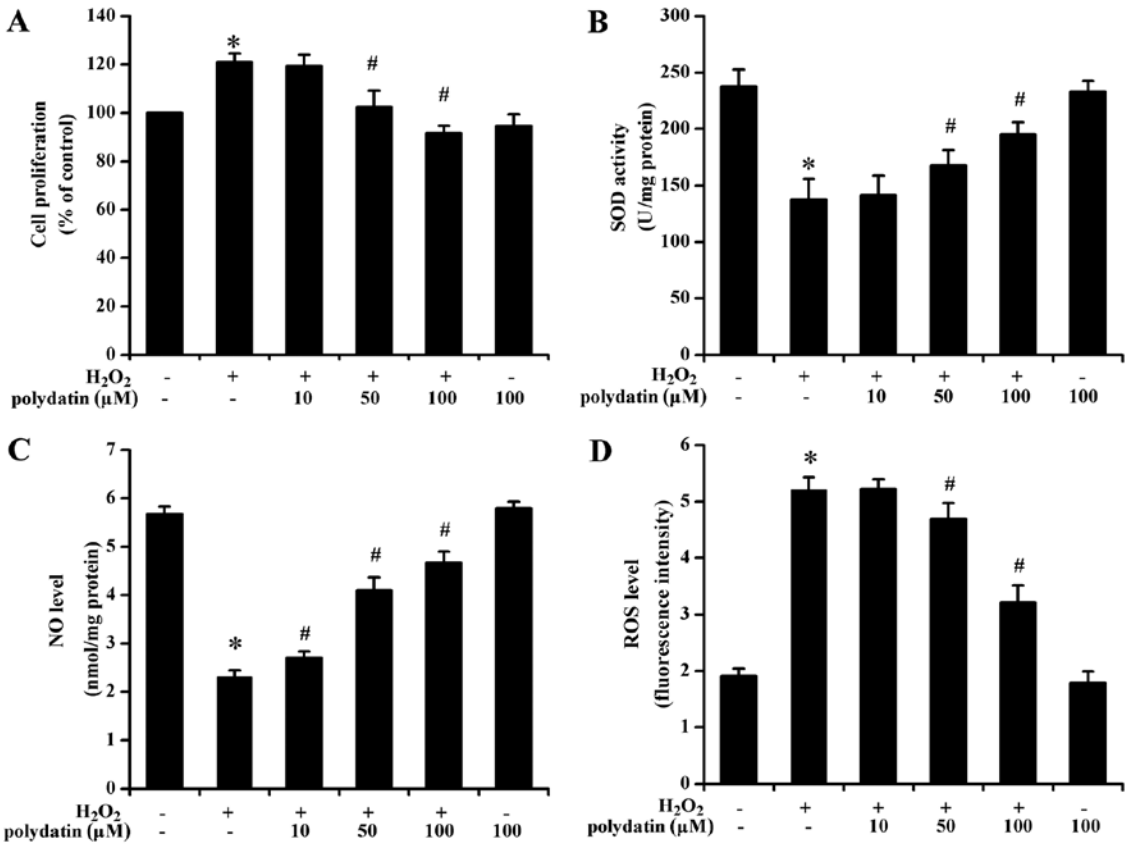

Figure 1. Effects of polydatin on vascular smooth muscle cells (VSMCs) under H_2O_2-induced oxidative stress. VSMCs were incubated with either vehicle (DMSO) or 10-100 μM polydatin in the presence of H_2O_2 (100 μM) for 24 h. (A) Cell proliferation in different groups. (B) Superoxide dismutase (SOD) activity of VSMCs in different groups. (C) Nitric oxide (NO) level of VSMCs in different groups. (D) ROS level of VSMCs in different groups. Data are presented as the means ± SD; n=5 per group. *P<0.05 vs. control group; #P<0.05 vs. H_2O_2-treated group alone.

Polydatin (100 μM) increased the mRNA level of SIRT1 and eNOS in cells exposed to H_2O_2 (P<0.05 vs. 50 μM polydatin-treated group; Fig. 2A and B). The results obtained with western blot analysis were generally consistent with the mRNA findings (Fig. 2C).

Effects of polydatin on the cell cycle. Treatment with polydatin increased the number of cells in the G2/M phase (from 3.78±0.26 to 15.62±0.22% and 23.31±0.34% at 50 and 100 μM, respectively) and decreased the number of cells in the G0/G1 phase (from 92.15±0.19 to 77.02±0.31% and 66.30±0.18% at 50 and 100 μM, respectively) (P<0.05 at 50 and 100 μM vs. H_2O_2-treated group alone; Fig. 3).

Effects of polydatin on the cell cycle proteins. H_2O_2 treatment alone increased cyclin B1 and Cdk1 and decreased Kip1/p27 (P<0.05 vs. the control group; Fig. 4A). Polydatin decreased cyclin B1 and Cdk1 expression and increased Kip1/p27 expression in cells exposed to H_2O_2 (P<0.05 vs. the H_2O_2-treated group alone). H_2O_2 increased the protein level of c-myc. Polydatin inhibited such a response (P<0.05 vs. the H_2O_2-treated group alone; Fig. 4B).

Effects of L-NAME and EX527 pre-treatment. The CCK-8 assay revealed that polydatin inhibited VSMC proliferation (Fig. 5A). L-NAME pre-treatment reversed the effects of polydatin on SOD activity (Fig. 5B) and ROS levels (Fig. 5D). Additionally, L-NAME pre-treatment decreased NO levels (Fig. 5C). Polydatin (100 μM) increased the phosphorylation of eNOS protein (p-eNOS) at Ser1177 of its catalytic subunit, and L-NAME treatment attenuated such a response (P<0.05; Fig. 5E).

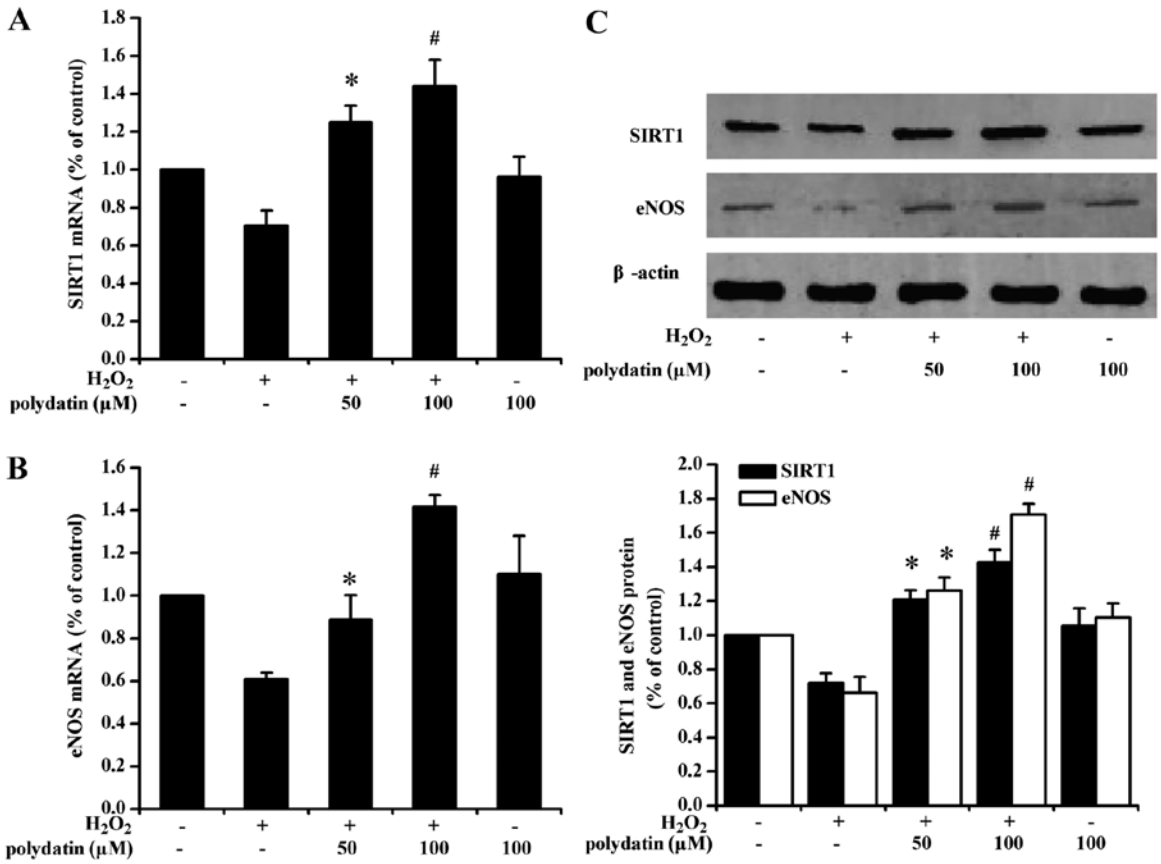

Figure 2. Effects of polydatin on the mRNA level and protein expression of silent information regulator 1 (SIRT1) and eNOS. (A) Measurement of SIRT1 mRNA level in different groups. (B) Measurement of eNOS mRNA level in different groups. (C) Measurement of SIRT1 and eNOS protein expression in different groups. Data are presented as the means ± SD; n=3 per group. *P<0.05 vs. H_2O_2-treated group alone; #P<0.05 vs. H_2O_2 combined with 50 μM polydatin treatment group.

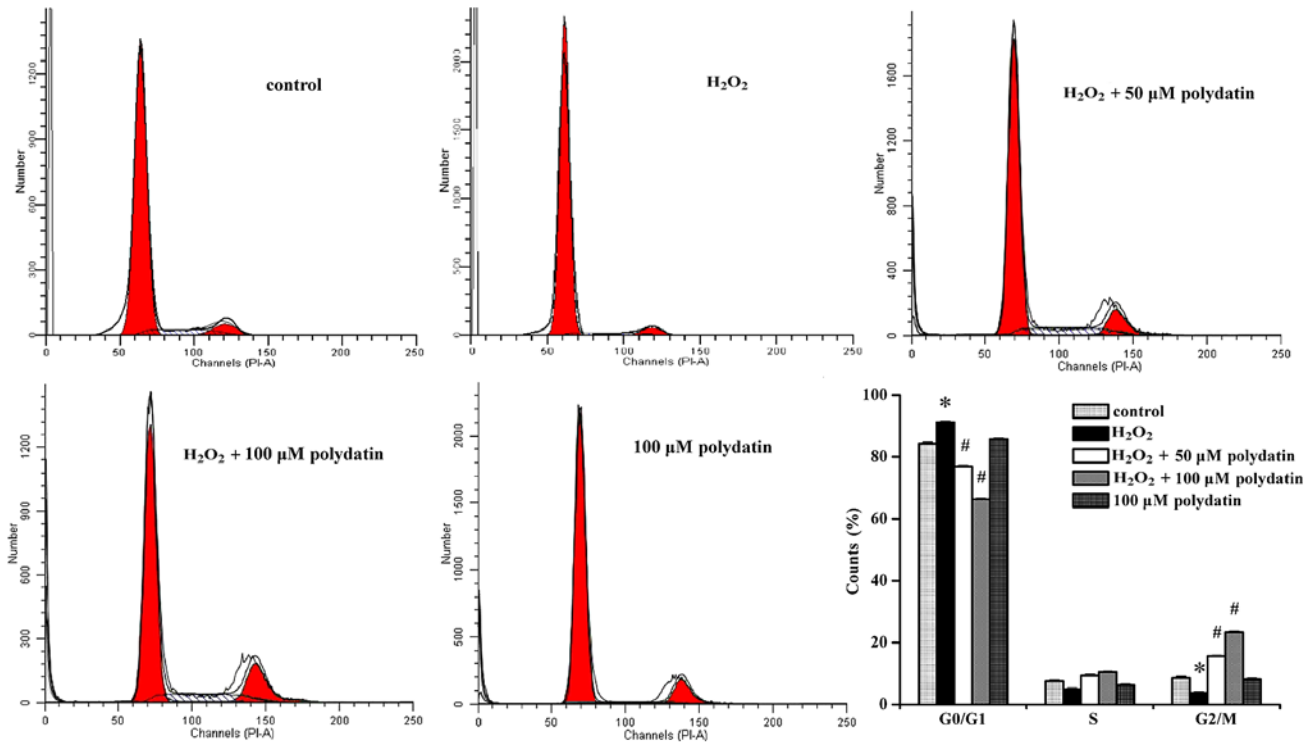

Figure 3. Effects of polydatin on the cell cycle of vascular smooth muscle cells (VSMCs) in the presence of H_2O_2. Cells were co-treated with different concentrations of polydatin (50 and 100 μM) and H_2O_2 or vehicle-treated control for 24 h. Cell cycle analysis was then performed by measuring the cellular DNA content. Data are presented as the means ± SD; n=3 per group. *P<0.05 vs. control group; #P<0.05 vs. H_2O_2-treated group alone.

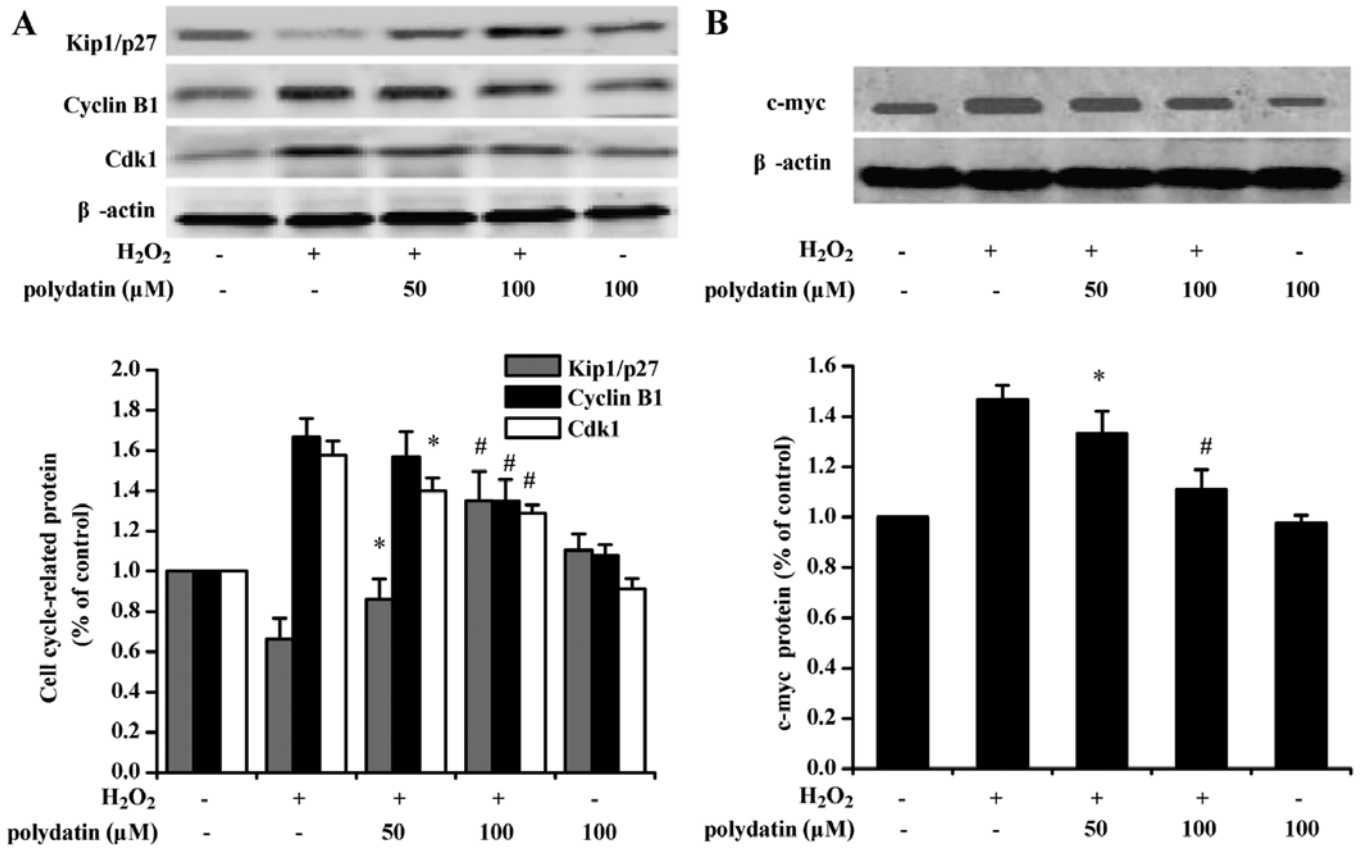

Figure 4. Effects of polydatin on cell cycle-related protein levels in the presence of H_2O_2. (A) Kip1/p27, cyclin B1 and Cdk1. (B) c-myc. The total cellular lysis products for protein extraction were prepared and adjusted for western blot analysis with proper dilutions of specific antibodies for Kip1/p27, cyclin B1, Cdk1, c-myc and β-actin. Data are presented as the means ± SD; n=3 per group. *P<0.05 vs. H_2O_2-treated group alone; #P<0.05 vs. H_2O_2 combined with 50 μM polydatin treatment group.

EX527 pre-treatment attenuated the anti-proliferative effects of polydatin (Fig. 6A). EX527 pre-treatment also decreased SOD activity (Fig. 6B) and increased ROS levels (Fig. 6D). EX527 pre-treatment significantly decreased SIRT1 expression at the protein level (P<0.05). There was no decrease in NO levels (Fig. 6C), and eNOS and p-eNOS expression at the protein level (Fig. 6E). L-NAME and EX527 pre-treatment decreased the expression of Kip1/p27 at the protein level, and increased cyclin B1 (Fig. 7).

Discussion

The results from the present study have shown that polydatin, an analog of resveratrol, attenuated the proliferation of VSMCs under oxidative stress. Such an effect may be attributed to G2/M-phase arrest, an increased antioxidant capacity, and activation of the eNOS-SIRT1 signaling pathway in the VSMCs.

Resveratrol has been previously reported to have anti-proliferative (23), antioxidant (24) and anti-aging (25,26) properties *in vitro* and *in vivo*. Polydatin, also known as piceid, is a derivative of resveratrol. Polydatin and resveratrol have been demonstrated to produce their effects via the stilbene synthesis pathway (27). Previous studies have indicated that stilbene derivatives with more phenolic moieties and an increased number of OH groups on the phenol ring have improved biological effects compared with resveratrol (28), including lower toxicity, better anticancer activities and sirtuin activation (29). Hydroxystilbene analogues with more hydroxyl groups on the phenol rings of the stilbene structure tend to have higher anti-radical activity than resveratrol (29). Polydatin has more potent anti-proliferative effects on intestinal epithelial cells than resveratrol (30), possibly due to an increased number of hydroxyl groups on the ring-structure.

Cell proliferation is accompanied by the activation of cell cycle proteins (31). The cell cycle is controlled by many factors, including cyclins/CDK complexes and CDK inhibitors (CDKI) (32). High activity of the cyclin B1/CDK complex allows the progression of cells through the G2/M phase, and thus, is critical for mitosis (33). Previous findings have shown that resveratrol inhibited VSMC proliferation by inducing cell cycle arrest and increasing DNA synthesis (22). The results from the present study indicated that the promotion of VSMC proliferation by oxidative stress was accompanied by an increased expression of cyclin B1 and Cdk1, and a decreased expression of Kip1/p27 (Fig. 4). A previous study indicated that the downregulation of CDKI and Kip1/p27, interfered with G2/M arrest in response to DNA injury stress, thereby increasing genetic instability (34). Consistent with those findings, our data indicated that polydatin

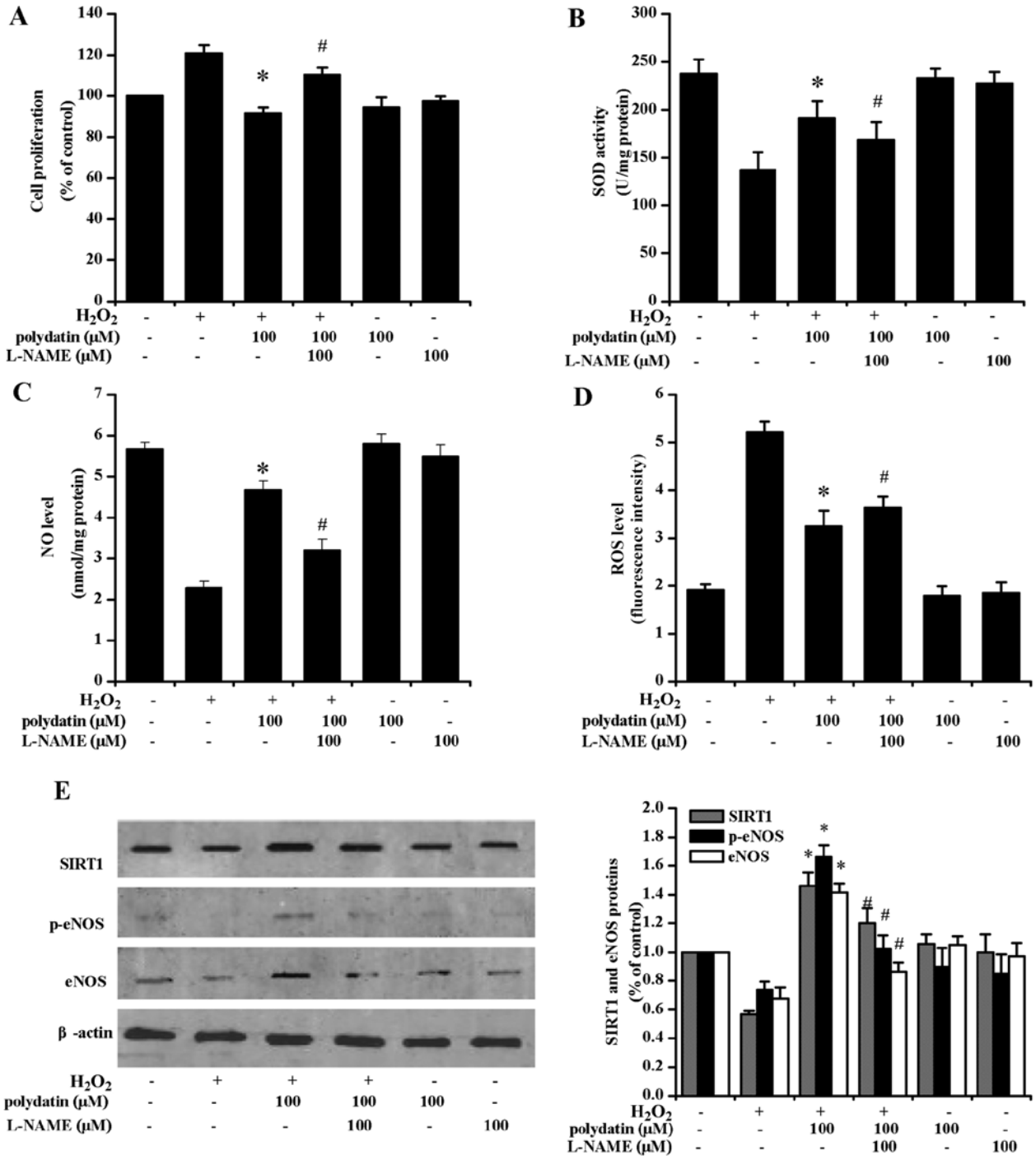

Figure 5. Effects of L-NAME in vascular smooth muscle cells (VSMCs). (A) Measurement of cell proliferation in VSMCs treated with L-NAME. (B) Measurement of SOD activity in different groups. (C) Measurement of NO levels in different groups. (D) Measurement of ROS levels in different groups. (E) Quantification of silent information regulator 1 (SIRT1), p-eNOS and eNOS protein expression in different groups detected by western blot analysis. Data are presented as the means ± SD of three independent experiments. *P<0.05 vs. H_2O_2-treated group alone; #P<0.05 vs. H_2O_2 combined with 100 μM polydatin treatment group.

inhibited oxidative stress-induced VSMC proliferation, in part by arresting the cell cycle at the G2/M phase (Fig. 3). This finding is associated with the upregulation of CDKI and Kip1/p27, and the downregulation of cyclin B1 and Cdk1. Pre-treatment with the eNOS inhibitor, L-NAME, or the SIRT1 inhibitor, EX527, attenuated Kip1/p27 expression, and increased cyclin B1 levels (Fig. 7). These results indicated that the effect of polydatin on VSMC proliferation is mediated by eNOS and SIRT1.

The c-myc is an important oncogene that regulates cell growth. Activation of c-myc promotes the proliferation of many cells, including VSMCs (35). In our study, c-myc expression was significantly increased by H_2O_2 and this response was inhibited by polydatin.

Resveratrol is a trihydroxy stilbene that is effective against oxidative stress and in the treatment of cardiovascular diseases (36). Numerous stilbene analogues have been developed based on the structure of resveratrol. A previous study

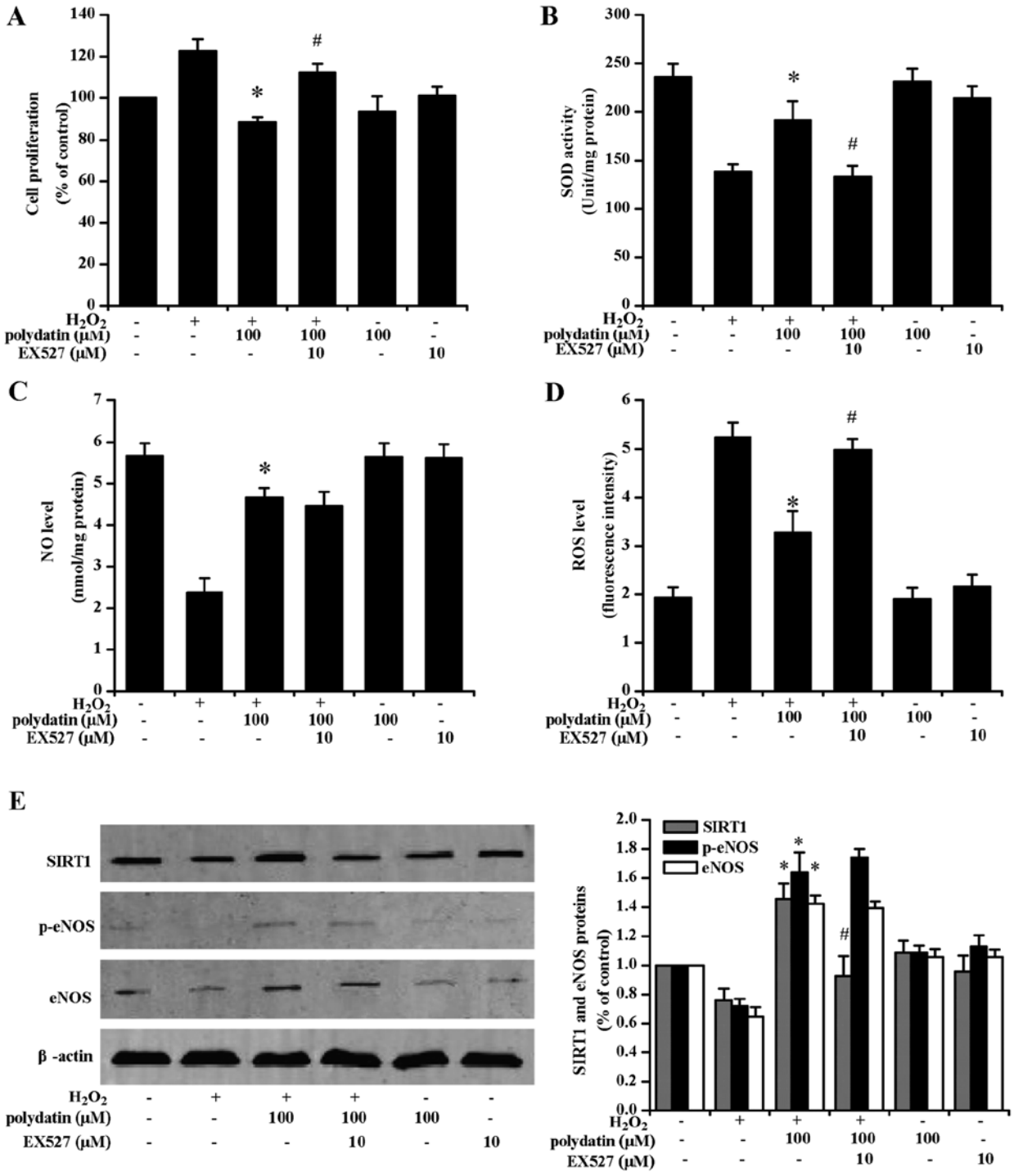

Figure 6. Effects of EX527 in vascular smooth muscle cells (VSMCs). (A) Meaurement of cell proliferation in VSMCs treated with EX527. (B) Measurement of SOD activity in different groups. (C) Measurement of NO levels in different groups. (D) Measurement of ROS levels in different groups. (E) Quantification of silent information regulator 1 (SIRT1), p-eNOS and eNOS protein expression in different groups detected by western blot analysis. Data are presented as the means ± SD of three independent experiments. *P<0.05 vs. H_2O_2-treated group alone; #P<0.05 vs. H_2O_2 combined with 100 μM polydatin treatment group.

indicated that polydatin and resveratrol possess scavenging activity against hydroxyl radicals *in vitro* (36). To evaluate the effect of polydatin on H_2O_2-treated VSMCs, we assessed NO, SOD, and ROS levels. In our experiments, the levels of NO and SOD were significantly increased following treatment with 50 and 100 μM polydatin, with a concomitant decrease in ROS levels. The results suggested that polydatin plays an anti-oxidant role by increasing the cellular oxidative tolerance to extracellular oxidative environmental change, as well as by eliminating ROS caused by oxidative stress in VSMCs.

eNOS regulates VSMC proliferation (37). Several protein kinases, including Akt/PI3K and AMPK, activated eNOS by phosphorylating Ser1177 in response to various stimuli (38,39). A previous study showed that resveratrol increased eNOS mRNA and protein expression levels and promoted NO production in endothelial cells (40). Resveratrol has been

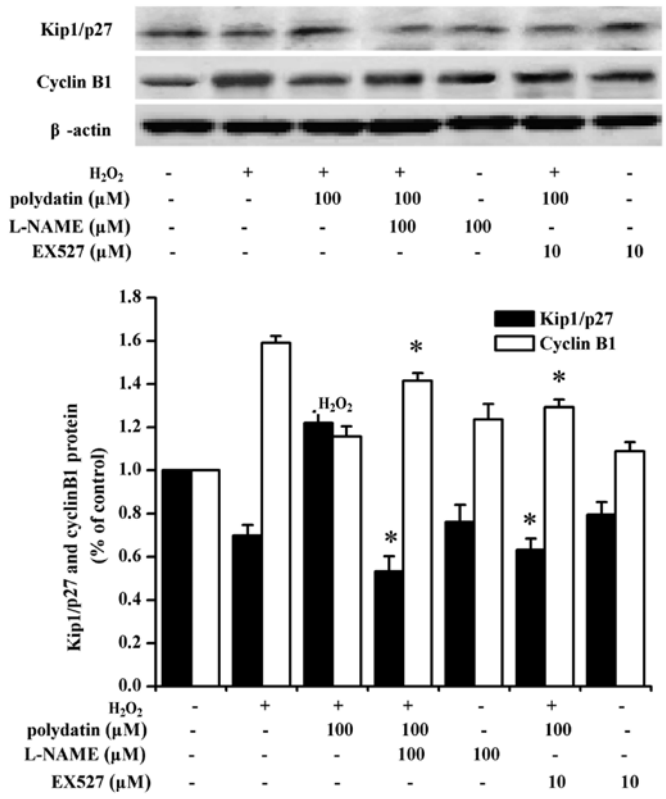

Figure 7. Effects of L-NAME and EX527 on polydatin-mediated cyclin B1 and Kip1/p27 protein expression in vascular smooth muscle cells (VSMCs). The cells were treated with L-NAME (100 μM) or EX527 (10 μM) for 2 h, and then treated with polydatin and H_2O_2 for 24 h. The total cellular protein extracts were prepared and adjusted for western blot analysis with appropriate dilutions of specific antibodies for cyclin B1, Kip1/p27 and β-actin. Data are presented as the means ± SD (n=3). *P<0.05 vs. H_2O_2 combined with 100 μM polydatin treatment group.

shown to inhibit rat aortic VSMC proliferation through estrogen receptor-dependent NO production (41). The present study demonstrated that the inhibition of eNOS by H_2O_2 was accompanied by a decreasing NO expression level. Although eNOS/NO expression by polydatin was not equivalent to the restoration of VSMC function in the normal control group in the present study. Thus, polydatin may attenuate VSMC proliferation. In our experiments, L-NAME, a reversible eNOS inhibitor, downregulated the expression of eNOS, SIRT1 and Kip1/p27, with the concomitant upregulation of cyclin B1 protein expression. We hypothesized that L-NAME reversed the inhibitory effects of polydatin on cell proliferation by inhibiting eNOS expression.

SIRT1 regulates many pathways for nutrient bioavailability, cellular energy status and various receptor signaling pathways. SIRT1 has been proven to inhibit oxidative stress (42), interact with eNOS to improve vascular function and retard endothelial senescence (43). Resveratrol induced VSMC differentiation by stimulating SIRT1 and AMPK (44). SIRT1 was regulated by NO when shuttled between the nucleus and cytosol, upregulated ROS scavengers, Mn-SOD and catalase, and catabolized toxic H_2O_2, which in turn was detoxified to water (45). SIRT1 is a crucial regulator of radical scavengers and transcriptional activation, and inhibits inflammatory expression via induction of eNOS (46,47). Thus, the SIRT1-eNOS/NO signaling pathway may be regarded as a potential target against atherosclerosis (48). In the present study, the expression of SIRT1 protein in VSMCs was significantly induced by polydatin. The peak effect on SIRT1 expression at 100 μM polydatin positively correlated with eNOS expression, suggesting that SIRT1 maybe involved in NO synthesis.

EX527, a specific inhibitor of SIRT1, did not affect the expression of eNOS in VSMCs. We hypothesize that SIRT1 may act as antioxidant regulator by activating upstream eNOS/NO in VSMCs. Taken together with the findings regarding cell cycle-related proteins, results of the present study suggest that SIRT1 functions as a vital regulator of the anti-proliferative effect of polydatin in VSMCs.

There are some limitations to our study. Firstly, it remains undetermined whether polydatin and resveratrol are capable of enhancing the activation of the eNOS/SIRT1 pathway in VSMCs. Secondly, the H_2O_2 model does not faithfully represent the *in vivo* oxidative damage microenvironment. Thirdly, the present study did not include the AMPK/eNOS pathway, which is apparently important for cell proliferation.

In conclusion, our study has demonstrated that polydatin improves oxidative stress-related changes by increasing SOD levels, ameliorating the level of ROS, inhibiting cell proliferation, upregulating eNOS/NO-SIRT1 expression during H_2O_2-induced oxidative damage in VSMCs. The present findings may contribute to the development of strategies for the prevention and treatment of oxidative lesions in the vasculature by targeting the eNOS/NO-SIRT1 pathway.

Acknowledgements

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