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Upregulation of SIRT1 and FOXO3a Contributes to The Protective Role of Estrogen on HPH in Rats

Li Wang

The Second Hospital of Hebei Medical University

The Second Hospital of Hebei Medical University https://orcid.org/0000-0002-6634-387X

Xiaowei Gong

The Second Hospital of Hebei Medical University

Jianjun Mao

The Second Hospital of Hebei Medical University

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Abstract

Background: SIRT1 has anti-proliferation effects on cells through regulating the expression and activity of FOXOs. Estrogen (E2) has protective effects against hypoxic pulmonary hypertension (HPH), but the involvement of SIRT1 and FOXOs in the proliferation of pulmonary artery smooth muscle cells (PASMCs) and contribution to the effects of E2 on HPH are poorly understood. To use E2 to explore the roles of SIRT1 and FOXO3a in the pathogenesis and progression of HPH and pulmonary vascular remodeling (PVR) in vivo and in vitro.

Methods: Female Sprague-Dawley rats with bilateral ovariectomy were randomized to normoxia, normoxia+E2, hypoxia, and hypoxia+E2. Serum E2 levels, hemodynamic, and pulmonary vascular pathomorphology were assessed. The anti-proliferation effect of E2 was determined in human PASMCs under hypoxia/normoxia. Immunohistochemistry, western blotting, and real-time PCR were used to assess SIRT1, FOXO3a, and PCNA in rat pulmonary artery and hPASMCs. SIRT1 activity was assayed.

Results: Hypoxia increased mean pulmonary artery pressure (mPAP), medial width of pulmonary arterioles, right ventricular hypertrophy index (RVHI), decreased expression SIRT1 and FOXO3a and increased PCNA expression in rats; E2 alleviated these changes. In vitro, E2 significantly inhibited hypoxia-induced hPASMCs proliferation, associated with improvements in SIRT1 and FOXO3a expression, consistent with the in vivo results. SIRT1 inhibition attenuated the effects of E2 on hPASMCs proliferation and the expression of FOXO3a. A SIRT1 activator mimicked the effects of E2 on hPASMCs proliferation and the expression of FOXO3a.

Conclusions: Upregulation of SIRT1 and FOXO3a contributes to the protective role of estrogen on HPH in rats, as supported by in vitro results using hPASMCs.

Background

Hypoxia-induced pulmonary hypertension (HPH) is one of the subtypes of pulmonary hypertension (PH) (1). HPH usually results from chronic hypoxia such as lung diseases-related hypoxia and chronic exposure to high altitude. In addition to hypoxia-induced pulmonary vasoconstriction, proliferation of pulmonary arterial smooth muscle cells (PASMCs) and pulmonary vascular remodeling (PVR) are central to the pathogenesis of HPH (2). Hypoxia-induced PVR can result in progressive increase of pulmonary vascular resistance, increased afterload of right ventricle (RV), and ultimately right heart failure (3).

A previous study by our group showed that ovariectomy exacerbates HPH in rats, whereas E2 replacement in ovariectomized animals attenuates the disease (4). It suggests that E2 exerts protective effects in HPH (5). The protective effects of E2 involve increased prostacyclin release and production of nitric oxide (6, 7), inhibition of endothelin-1 expression (8), and downregulation of angiotensin converting enzyme and angiotensin type 1 receptor (9), all leading to vasodilatation. In addition, E2 markedly alleviates hypoxia-induced PVR and proliferation of PASMCs (10). Moreover, E2 possesses anti-

proliferation effects in other proliferative vascular diseases (11). Nevertheless, the exact mechanisms underlying these effects of E2 are not fully understood.

SIRT1 is a highly conserved nicotinamide adenine dinucleotide (NAD)⁺-dependent deacetylase, and regulates DNA damage repair, oxidative stress, cell senescence, apoptosis, and proliferation via its ability to deacetylate a variety of substrates such as transcription factors, coregulators, and histones (12). SIRT1 is highly expressed in the vasculature and plays a critical role in the development of many vascular diseases including PH (13). Decreased SIRT1 expression may contribute to PVR associated with PH and up-regulated SIRT1 mediates the inhibition on MCT-induced rat PVR and proliferation of PVSMCs (14).

FOXO3a, an isoform of the Forkhead box "O" (FOXO) transcription factors, plays an important role in regulating diverse cellular processes such as proliferation, differentiation, and cell cycle control (15, 16). FOXO3a is closely related to hypoxia and oxidation (17) and FOXOs are associated with PH (18). SIRT1 can deacetylate FOXO3a, controlling its activity or expression, and regulates FOXO-dependent gene transcription (19, 20).

We hypothesized that E2 inhibits the abnormal proliferation of PASMCs via the SIRT1/FOXO3a pathway and contributes to attenuating PVR and alleviating HPH. Therefore, the aim of the present study was to use E2 to explore the roles of SIRT1 and its target FOXO3a in the pathogenesis and progression of HPH and PVR *in vivo*. Furthermore, we verified the effects of SIRT1 and FOXO3a on hPASMCs *in vitro*.

Materials And Methods

Animals and experimental design

Healthy 6 to 8 week old female Sprague-Dawley rats (170-190 g) were purchased from the animal center of the Hebei Medical University, China. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Hebei Medical University and complied with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

The rats underwent bilateral ovariectomy and were randomized into four groups (n = 8/group): normoxia group, normoxia+E2 group, hypoxia group, and hypoxia+E2 group. Previous studies showed that hypoxia exposure for 2-4 weeks can establish HPH models (21, 22). One week after surgery, the animals received 17β -estradiol (20 µg/kg/d, Sigma, St Louis, MO, USA) or saline (0.1 ml/d) subcutaneously for 8 weeks, as described (23). An additional file shows this in more detail (see Additional file 1).

Hemodynamics, hormone assays, and tissue preparation

Hemodynamics were recorded using a Med lab electrophysiolograph (BL-420S, Thaimeng Technology Co., Ltd., Chengdu, China). A commercially available estradiol ELISA kit (DRG International Inc.,

Springfield Township, NJ, USA) was used to detect the hormone levels. The lungs and heart were harvested. An additional file shows this in more detail (see Additional file 1).

Pathology and immunochemical staining

Immunohistochemistry was routinely performed for SIRT1 (1:400; ab110304, Abcam, Cambridge, MA, USA), FOXO3a (1:400; ab109629, Abcam, Cambridge, MA, USA), and PCNA (1:400; 610664, BD Biosciences, Franklin Lake, NJ, USA). An additional file shows this in more detail (see Additional file 1).

Cell culture and treatment

HPASMCs (3110, Sciencell Research Laboratories, Carlsbad, CA, USA) were routinely cultured at 37°C in smooth muscle cell medium (Sciencell Research Laboratories, Carlsbad, CA, USA) supplemented with 2% fetal bovine serum (Sciencell Research Laboratories, Carlsbad, CA, USA), 0.5% smooth muscle cell growth supplement (Sciencell Research Laboratories, Carlsbad, CA, USA), and 0.5% penicillin-streptomycin solution (Sciencell Research Laboratories, Carlsbad, CA, USA). The cells were divided into six groups: one normoxia group, four hypoxia groups treated with four different concentrations of E2 (10⁻⁶, 10⁻⁷, 10⁻⁸, and 10⁻⁹ mol/L), and one hypoxia alone group. An additional file shows this in more detail (see Additional file 1).

Cell proliferation assay

HPASMC proliferation was determined routinely using the MTT assay (MTT; D0801, Sigma, St Louis, MO, USA). An additional file shows this in more detail (see Additional file 1).

Western blotting

Western blotting was performed routinely for SIRT1 (ab110304, Abcam, Cambridge, MA, USA), FOXO3a (ab109629, Abcam, Cambridge, MA, USA), PCNA (610664, BD Biosciences, Franklin Lake, NJ, USA), and GAPDH (Abcam, Cambridge, MA, USA). An additional file shows this in more detail (see Additional file 1).

RT-PCR analysis

RT-PCR was performed routinely for SIRT1, FOXO3a, and GAPDH (172 bp). The expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (24). An additional file shows this in more detail (see Additional file 1).

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SIRT1 deacetylase activity assay

SIRT1 deacetylase activity was assayed using the Universal SIRT Activity Assay Kit (Abcam, Cambridge, MA, USA), according to the manufacturer's instructions.

Statistical analysis

All data were expressed as means ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA, followed by the least significant difference (LSD) test for post hoc multiple comparisons. Significant difference was accepted at P<0.05. Analyses were performed using SPSS 21.0 (IBM, Armonk, NY, USA).

Results

E2 attenuates chronic HPH and pulmonary artery remodeling in rats

Compared with the normoxia group, the E2 levels in the normoxia+E2 group were significantly elevated. Compared with the hypoxia group, the E2 levels in the hypoxia+E2 group were significantly elevated (Figure 1A).

After 8 weeks of hypoxia, the mPAP of the hypoxia group was significantly elevated compared with the normoxia group (Figure 1B). In the hypoxia+E2 group, the mPAP was significantly lower than in the hypoxia group (Figure 1B), but it was still higher than in the normoxia group. There was no significant difference in mPAP between the normoxia and normoxia+E2 groups (Figure 1B).

As shown in Figure 2A, wall-thickened pulmonary arterioles with medial smooth muscle cell proliferation were observed in the hypoxia rats compared with those in the normoxia group. The vessel changes were alleviated in the hypoxia+E2 group compared with the hypoxia group (Figure 2A). Compared with the normoxia group, the MT% and RVHI in the hypoxia group were significantly elevated (Figures 1C and 2B). The rats treated with E2 showed relatively less severity of both indexes (Figures 1C and 2B). There was no significant difference of MT% and RVHI between the normoxia and normoxia+E2 groups (Figures 1C and 2B).

E2 decreases hPASMCs proliferation under hypoxia

Hypoxia significantly increased cell proliferation compared with the normoxia group (Figure 3). The hypoxia-induced proliferation of PASMCs was obviously inhibited by the four different concentrations of E2 (Figure 3). To determine whether SIRT1-FOXO3a mediates the effects of E2 on hPASMCs proliferation, the SIRT1 inhibitor nicotinamide (NAM) was used. Interestingly, hPASMCs cotreated with E2 and NAM exhibited significant attenuation of E2-induced improvements in hPASMCs proliferation under hypoxia (Figure 3B).

We next confirmed whether SIRT1 overexpression could duplicate the effects of E2 on hPASMCs proliferation *in vitro*. Resveratrol (RE) was used as an activator of the SIRT1 protein. Cell proliferation of the RE-treated group was lower than in the hypoxia group (Figure 3B). HPASMCs proliferation was significantly reduced by 40 µM resveratrol.

E2 increases the expression of SIRT1 in HPH rats

To explore whether SIRT1 was involved in hypoxia-induced pulmonary hypertension and artery remodeling in rats, SIRT1 expression and deacetylase activity were detected. Immunochemistry showed that SIRT1 was highly expressed in pulmonary arterioles and image analysis showed that SIRT1 expression in the hypoxia group was decreased compared with the normoxia group (Figure 4A, D, E). Compared with the hypoxia group, SIRT1 expression in the E2 treatment group was significantly increased, but it was still lower than that of normoxia group (Figure 4A, D, E). There was no notable difference in SIRT1 expression between the normoxia and normoxia+E2 group (Figure 4A, D, E).

According to immunohistochemistry, compared with the normoxia group, hypoxia exposure significantly decreased the expression of the transcription factor FOXO3a (Figure 4B, D, E), accompanied with significant increase of PCNA expression (Figure 4C, D, E). Compared with the hypoxia group, FOXO3a expression in the E2 group was significantly increased (Figure 4B, D, E). PCNA expression in the E2 treatment group was significantly decreased (Figure 4C, D, E).

SIRT1 activity in lung specimens

There was no significant difference in SIRT1 activity among the groups (Figure 5).

E2 increases the expression of SIRT1 in hPASMCs

In line with the *in vivo* findings, compared with the normoxia control, hypoxia significantly decreased the protein and mRNA expression SIRT1 in hPASMCs (Figure 6A, C). On the other hand, treatment with E2 (10⁻⁶ M) significantly increased the expression of SIRT1 (Figure 6A, C).

In accordance with the *in vivo* findings, hypoxia resulted in reduction of FOXO3a in hPASMCs (Figure 6A, C) and elevation of PCNA (Figure 6B, D) compared with the normoxia group. In the hypoxia+E2 group, the protein and mRNA levels of FOXO3a were significantly increased (Figure 6A, C) and the protein and mRNA levels of PCNA were significantly reduced (Figure 6B, D) compared with the hypoxia group.

Compared with the E2 group, cotreatment with E2 and NAM decreased the expression of SIRT1 (Figure 6A, C) and repressed the expression of FOXO3a (Figure 6A, C), whereas the expression of PCNA was increased in this condition (Figure 6B, D).

We next confirmed whether SIRT1 overexpression could duplicate the effects of E2 on expression of FOXO3a and PCNA *in vitro*. RE significantly increased the expression (Figure 6A, C) and increased the expression of FOXO3a (Figure 6A, C), but decreased the expression of PCNA in hypoxia-induced hPAMSCs (Figure 6B, D).

Hypoxia reduces SIRT1 activity in hPASMCs, without effect of E2

Not exactly consistent with the *in vivo* findings, compared with the normoxia control, hypoxia significantly reduced SIRT1 activity (Figure 7). Whereas, treatment with E2 (10⁻⁶ M) had no effect on SIRT1 activity (Figure 7). Compared with the normoxia group, cotreatment with E2 and NAM decreased the activity of SIRT1 (Figure 7). Compared with the hypoxia group, RE significantly increased the activity of SIRT1 (Figure 7).

Discussion

SIRT1 has anti-proliferation effects on cells through FOXO deacetylation and regulating the expression of FOXOs. E2 has protective effects against HPH, but the involvement of SIRT1 and FOXOs in the proliferation of PASMCs and their contribution to the effects of E2 on HPH are poorly understood. Therefore, this study aimed to use E2 to explore the roles of SIRT1 and FOXO3a in the pathogenesis and progression of HPH and PVR *in vivo* and *in vitro*. The results suggest that upregulation of SIRT1 and FOXO3a contributes to the protective role of estrogen on HPH in rats, as supported by *in vitro* results using hPASMCs.

PH is a progressive and sexually dimorphic disease with high morbidity and mortality. HPH is a severe complication and important cause of death in patients with hypoxia-related lung diseases. HPH has a multi-factorial etiology that often leads to significant hemodynamic changes, severe pulmonary vascular remodeling, and right ventricular failure and death (25). Despite the development of novel therapies over the last 20 years, the clinical prognosis of PH is still poor.

Although females are much more susceptible than males to idiopathic, familial, and sclerodermaassociated PH (26), there are discrepancies in mortality (27) and in the prevalence of HPH due to high altitude (28). In addition, female PH patients exhibit higher cardiac index and lower mPAP and PVR (29), and better RV function (30). Under hypoxia, animal models (rats, chicken, sheep, and swine) develop less severe PH than their male counterparts (31–34). Previous studies by our group and others' showed that hypoxic ovariectomized rats develop more severe PH, PVR, and RV hypertrophy than chronically hypoxic rats with intact ovaries (4, 5, 9). Furthermore, administration of estrogen attenuates the severity of PH in the ovariectomized animals (4, 5, 9). Along these lines, estrogen may influence the development of hypoxia-induced PH. In the present study, hypoxia induced pathologic changes that were typical of HPH, such as elevated mPAP, increased RV/LV + S and MT%. In addition, E2 alleviated these changes. PVR plays a critical role in the progression of HPH in animal models as well as in patients with HPH, and abnormal proliferation of PASMCs leads to media hyperplasia and vessel lumen stenosis, resulting in increased pulmonary vascular resistance and elevated mPAP (3). Although many studies on estrogen revealed that it could effectively alleviate various types of vascular diseases, there are still many controversies over the beneficial effects of estrogen, especially over its anti-proliferation effect (10, 26, 35). To date, the effects of estrogen on PVR are still not clear. In the present study, we found that E2 significantly attenuated hypoxia-induced pulmonary artery remodeling *in vivo* and inhibited hPASMCs proliferation under hypoxia *in vitro*. The results are consistent with those reported by Xu et al. (10). In our opinion, the controversies about estrogen's anti-proliferation effect may due to different animal models, different target cells, different experimental conditions, and different treatments. Nevertheless, the molecular mechanisms responsible for the anti-proliferation effect are complex and have not yet been fully elucidated.

SIRT1, as an important class III histone deacetylase, participates in many cell functions such as proliferation, differentiation, senescence, and apoptosis (36–39). SIRT1 plays critical roles in vascular homeostasis and remodeling (39, 40). Bae et al. (41) demonstrated that SIRT1 attenuates neointima formation by inhibiting HIF-1a expression in neointimal lesions of a murine model of wire-injured femoral artery. Gao et al. (42) reported that SIRT1 expression is decreased in the aortas of angiotensin II infusion-induced hypertension mouse models. Overexpression of SIRT1 in VSMCs prevents the increase in systolic blood pressure caused by AngII infusion and alleviates vascular remodeling in thoracic and renal aortas. With respect to VSMC proliferation and arterial remodeling within the pulmonary vasculature, studies also showed that decreased SIRT1 expression may contribute to PVR in monocrotaline (MCT)-induced PH models, and up-regulated SIRT1 revealed the inhibition effect on MCT-induced rat PVR and PVSMCs proliferation by modulating different molecules (14, 43).

In this study, data showed that hypoxia decreased the expression of SIRT1 along with increase of PCNA expression. E2 reversed these changes of SIRT1 and PCNA, accompanied with the attenuation of PH and PVR induced by hypoxia. Interestingly, there was a positive association between SIRT1 and FOXO3a. Moreover, similar phenomena were also observed at the cellular level. SIRT1 activity was not changed after E2 treatment both *in vitro* and *in vivo*, but it was reduced after hypoxia in cultured hPASMCs. These results suggest that the protective effects of E2 on the HPH and hPASMCs proliferation were related to the improvement of SIRT1 expression levels and elevation of FOXO3a expression levels.

FOXO transcription factors play critical roles in cell proliferation, apoptosis, and cell cycle arrest and are becoming the focus of research in a variety of vascular diseases and cancers (15, 16, 44). As an important isoform of the FOXO family, FOXO3a has proved to be an important regulator that modulates vascular homeostasis, VSMC proliferation, and neointimal hyperplasia (45). In addition, FOXO3a is regulated by SIRT1 (46). Huang et al. (47) revealed that physiological cyclic stretch increased SIRT1 expression, which subsequently up-regulated the expression of FOXO3a and maintained the normal contractile differentiation of VSMCs. In contrast, Zhang et al. (48) showed that in MSCs, inhibition of miR-34a led to an increase in SIRT1 expression, which down-regulated the protein expression of FOXO3a, resulting in decreased cell apoptosis and increased survival. In the present study, there was a positive association between SIRT1 and FOXO3a expression. Further studies in cultured hPASMCs showed that cells cotreated with E2 and NAM exhibited significant reduction of expression and activity of SIRT1 and marked attenuation of E2-induced improvements in hPASMCs proliferation and FOXO3a expression under hypoxia. Resveratrol (RE), an activator of the SIRT1 protein, mimicked the effects of E2 on hPASMCs proliferation and expression of FOXO3a. These results revealed that E2 increased the expression of SIRT1, which subsequently modulated the expression of FOXO3a and then the proliferation of PASMCs. The expression of FOXOs are regulated by SIRT1 in several types of cells (49, 50). Recent research revealed that the host cell factor 1 (HCF-1) participates in the SIRT1-regulated expression of FOXOs in worms (38). In addition, there is also evidence that SIRT1 deacetylates FOXOs, thus attenuating cell apoptosis and cell-cycle arrest (46). Accordingly, we could not exclude that SIRT1-mediated deacetylation of FOXO3a or other molecules may also contribute to the anti-remodeling effect of E2 and further investigation is needed.

Conclusions

In conclusion, the present study strongly suggests that E2 upregulates the expression of SIRT1 and consequently increases the expression of FOXO3a in hypoxia-induced hPASMCs, which contributed to the attenuation of pulmonary arterial remodeling and the alleviation of PH in hypoxia-treated rats. It suggests that E2 exerts a protective role in HPH, at least in part through the regulation of the SIRT1-FOXO3a signaling axis in vascular smooth muscle cells.

Abbreviations

HPH: hypoxic pulmonary hypertension

NAM: nicotinamide

PH: pulmonary hypertension

RE: resveratrol

PASMCs: pulmonary artery smooth muscle cells

mPAP: mean pulmonary arterial pressure

E2: estrogen

MT: medial wall thickness

- SIRT1: silent mating type information regulation 2 homolog-1
- PCNA: proliferated cell nuclear antigen

Declarations

Ethics approval and consent to participate

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Hebei Medical University and complied with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LW conceived and designed the experiments, performed experiments and drafted the manuscript. YY contributed to conception and critically revised the manuscript. XG and JM performed experiments and participated in analysis. All authors read and approved the final manuscript.

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- 51. Additional file 1.
- 52. DOC.
- 53. Supplemental. materials and methods.

Figures



Effects of E2 on chronic hypoxia-induced pulmonary hypertension and right ventricular hypertrophy of rats. (A) 17 β -estradiol levels in serum. (B) Mean pulmonary artery pressure (mPAP). (C) Right ventricular hypertrophy, showing data as the mass of the right ventricle divided by the masses of the left ventricles and septum [RV/(LV+S)]. **P<0.01,*P<0.05 vs. the normoxia group. ##P<0.01, #P<0.05 vs. the hypoxia group.



Figure 2

Effects of E2 on chronic hypoxia-induced pulmonary vascular remodeling of rats. (A) Hematoxylin and eosin staining of pulmonary arterioles (magnification of 20× and scale bars of 50 μ m). (B) Medial wall thickness (MT%) of pulmonary arterioles. **P<0.01 vs. the normoxia group. ##P<0.01 vs. the hypoxia group.



Effects of E2 (A) and SIRT1 activator/inhibitor (B) on hPASMCs proliferation under hypoxia (MTT assay). **P<0.01, *P<0.05 vs. the normoxia group. ##P<0.01, #P<0.05 vs. the hypoxia group.



Effects of E2 on SIRT1, FOXO3a, and PCNA expression in rats. (A) Immunohistochemistry of SIRT1. (B) Immunohistochemistry of FOXO3a. (C) Immunohistochemistry of PCNA. (D) Densitometry analysis of the immunohistochemistry images. (E) Positive cell percentage based on the immunohistochemistry images. Magnification of 40× and scale bars of 25 μ m. **P<0.01, *P<0.05 vs. the normoxia group. ##P<0.01, #P<0.05 vs. the hypoxia group.



Effects of E2 on SIRT1 activity in rats. Quantitative analysis of SIRT1 activity in lung specimens



Effects of E2 and SIRT1 activator/inhibitor on SIRT1, FOXO3a, and PCNA expression in hPASMCs. (A) Representative western blotting analysis of SIRT1 and FOXO3a protein levels in hPASMCs. (B) Representative western blotting analysis of PCNA protein levels in hPASMCs. (C) Representative RT-PCR analysis of SIRT1 and FOXO3a mRNA levels in hPASMCs. (D) Representative RT-PCR analysis of PCNA mRNA levels in hPASMCs. **P<0.01 vs. the normoxia group. ##P<0.01, #P<0.05 vs. the hypoxia group. &&P<0.01 vs. the hypoxia+E2 group.



Effects of E2 and SIRT1 activator/inhibitor on SIRT1 activity in hPASMCs. Quantitative analysis of SIRT1 activity in hPASMCs. **P<0.01, *P<0.05 vs. the normoxia group. ##P<0.01 vs. the hypoxia group.

Supplementary Files

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