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Angiotensin II induces mitochondrial oxidative stress and mtDNA damage in osteoblasts by inhibiting SIRT1–FoxO3a–MnSOD pathway

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ABSTRACT

Previous report showed that angiotensin II accelerates osteoporosis, and recent clinical studies suggest that several antihypertensive drugs, especially angiotensin-converting enzyme inhibitors, reduced bone fractures. However, the underling mechanism by which angiotensin II induces bone dysfunction is largely unknown. Here in this study, we show that angiotensin II induces mitochondrial oxidative stress and mitochondrial DNA (mtDNA) damage. We find that the protein and RNA levels of mitochondrial catalase and manganese superoxide dismutase (MnSOD) are decreased in osteoblasts in the presence of angiotensin II. Further, we show that angiotensin II inhibits the protein level of SIRT1, but not SIRT3, which results in the hyperacetylation of the forkhead box O3a (FoxO3a) and inhibition of the expression of catalase and MnSOD. Finally, we show that SRT3025 (Sirt1 activator) and Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP, a MnSOD mimetics) can markedly reduce mitochondrial oxidative stress and mtDNA damage. In summary, we identify a novel SIRT1–FoxO3a–MnSOD axis in angiotensin II-induced mitochondrial oxidative stress and mtDNA damage in osteoblasts.

1. Introduction

The renin–angiotensin system (RAS) plays a pivotal role in cell function, cardiovascular homeostasis [1]. Angiotensin-(1-7) is one of the most important active peptides of the renin–angiotensin system (RAS) with recognized cardiovascular relevance. Angiotensin II (Ang-II) is cleaved from angiotensin I by angiotensin-converting enzyme, and Ang-II can increase blood pressure and lead to hypertension and subsequent disorders, such as cardiac hypertrophy [2].

Previous reports indicate that Ang-II can regulate the bone metabolism through its angiotensin types 1 and 2 receptors (AT1R and AT2R) [3,4]. Ang-II has been shown to promote the differentiation and activation of osteoclasts by inducing the expression of receptor activator of nuclear factor-κB ligand (RANKL) [5]. In osteoblasts, Ang-II induces modulation of calcium channel currents [6]. Ang-II can also induce other dysfunctional phenotypes in osteoblasts, including inflammation [7], differentiation [8], and oxidative stress [8–10].

Oxidative stress cascade, resulting from persistent generation of reactive oxygen species (ROS), can damage various cellular components of the osteoblasts, and therefore is considered as one of the initiating factors for the impaired osteoblastic bone formation in postmenopausal osteoporosis and senile osteoporosis [11–13]. Mitochondria is one of the main sources of ROS generation. Mitochondria dysfunction essentially participates in osteoblast through regulating processes including mitophagy, apoptosis, and mitochondrial DNA (mtDNA) damage [14–16]. Improvement of mitochondrial function by antioxidative agents or factors could protect osteoblast cells from cytotoxicity and dysfunction [15,17].

Here in this study, we report that Ang-II induces mitochondrial oxidative stress and mtDNA damage in bone MSC-derived osteoblasts by targeting SIRT1, a member of the class III NAD+-dependent deacetylase family. Ang-II reduces the expression of SIRT1, which leads to FoxO3a hyperacetylation and repression of catalase and MnSOD expression. The rescue experiments using SIRT1 activator or MnSOD mimetics could significantly block Ang-II-induced oxidative stress and mtDNA damage in osteoblasts.

2. Materials and methods

2.1. Cell culture and ex vivo differentiation of osteoblasts

Osteoblasts were differentiated from bone marrow mesenchymal stem cells (MSCs) of C57BL/6N mice [18–20]. Briefly, MSC cells

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were isolated and cultured for 3–7 days in MSC medium (Mouse Mesen Cult Proliferation Kit; Stem Cell Technologies), then differentiated in α-MEM (Gibico) containing 10% fetal bovine serum (FBS, Gibco), 5 mM β-glycerophosphate, and 100 μg/mL ascorbic acid (mineralization medium) (GPAA mixture) for 9 days. Mature osteoblasts were confirmed as alkaline phosphatase-positive (ALP+) cells by using fast red violet LB salt.

2.2. Measurement of mitochondrial oxidative stress

Osteoblasts were treated with Ang-II (Sigma), SRT3025 (Sirtris) or MnTBAP (Santa Cruz) as indicated in the figure legends. MitoSox (Invitrogen) was used for detecting mitochondrial ROS generation in osteoblasts as per the manufacturer's protocol. The levels of mitochondrial ROS were normalized to the control group.

2.3. Mitochondrial DNA (mtDNA) damage analysis

mtDNA was assessed by q-PCR as described elsewhere [21]. Briefly, genomic DNA was extracted using the Qiagen Genomic-tip 20/G and Qiagen DNA Buffer Set per the manufacturer's protocol. The cells were incubated in a lysis buffer containing RNase A (Life Technologies) and proteinase K (Qiagen) for 2 h. Total DNA contained in the extracts was bound to Qiagen Genomic-tip 20/G columns, washed and eluted. Eluted DNA was incubated with isopropanol at −80 °C overnight, and centrifuged 12,000*g* for 60 min. To wash DNA, 0.7 mL (70%) ethanol was evenly added to each aliquot and centrifuged at 9500*g* for 30 min. After removing supernatant, TE buffer (pH 7.5) was added the each tube and measured the DNA concentration. PCR was performed using Ex-taq (Clonetech) with specific primers (Table 1) to amplify a fragment of the mitochondrial genome both a short and long fragment as described [21]. Each DNA was quantified by Pico-green (Life Technologies) using the FL600 Microplate Fluorescence Reader following parameters: excitation and emission wavelengths 485s nm and 530. And then, the data were obtained from the small fragment were subsequently used to normalize the results of the mitochondrial long fragment. The number of mitochondrial lesions was calculated by following equation: $D = (1 - 2^{-(\Delta long - \Delta short)})$ 10,000(bp)/size of long fragment (bp).

2.4. Quantitative real-time PCR (q-PCR)

Total RNAs were extracted from osteoblasts with TRIzol (Invitrogen). 1 μg RNA was subjected for synthesis of cDNA with One Step RT-PCR Kit (TaKaRa). q-PCR was performed with the SYBR Green (TaKaRa) detecting method on an ABI-7500 RT-PCR system (Applied Biosystems). GAPDH was used as housekeeping gene. The primers used in this study were listed in Table 1.

2.5. Immunoprecipitation and Western blot

For immunoprecipitation analysis, osteoblast cell lysates (3 mg protein) were mixed with the indicated antibody (2 μg) at 4 °C overnight followed by the addition of 80 μl of protein G-Sepharose (Amersham Biosciences) for 1 h at 4 °C. Immune complexes were washed five times with lysis buffer (Beyotime), supplemented with complete mini-protease inhibitor cocktail (Roche Applied Science).

For Western blot, samples were subjected to SDS/PAGE, transferred to nitrocellulose using the iBlot Dry Blotting System (Invitrogen), and then immunoblotted with the indicated primary antibodies, including anti-GAPDH (Santa Cruz), anti-catalase (Cell Signaling Technology), FoxO3a (Santa Cruz Biotechnology), SIRT1 (Santa Cruz Biotechnology), SIRT3 (Cell Signaling Technology) or acetyl-lysine (Cell Signaling Technology), followed by the appropriate horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Bands were visualized by enhanced chemiluminescence (Pierce).

2.6. Adenovirus infection

Adenovirus carrying human Sirt1 or control GFP were purchased from Applied Biological Materials. For infection, osteoblasts were infected with adenovirus carrying Sirt1 or GFP for 24 h before Ang-II treatment.

2.7. Enzyme activity assay

Catalase and MnSOD activity were analyzed with kits from Abcam. Briefly, the enzymes were immunoprecipitated with 2 μg antibodies from mitochondria lysis (isolated with kit from Thermo) and subjected to enzyme activity assay as per the manufacturer's protocol.

2.8. Statistical analysis

All values are expressed as the means ± SEM. Statistical differences between two groups were determined using non-paired, two-tail Student's *t* test. Two way ANOVA was used to analyze differences among groups more than two. Statistical analysis was performed using GraphPad Prism 6. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Angiotensin II induces mitochondrial oxidative stress and mtDNA damage in osteoblasts

Angiotensin II has long been known to induce osteoblast dysfunction. Mitochondria dysfunction contributes essentially to

Table 1
Primers used for quantitative or real-time PCR.

Name	Forward (5′–3′)	Reverse (5′–3′)
Catalase	GGAGGCGGGAACCCAATAG	GTGTGCCATCTCGTCAGTGAA
MnSOD	ACAGGCCTTATTCCACTGCT	CAGCATAACGATCGTGGTTT
Prx-3	GGTTGCTCGTCATGCAAGTG	CCACAGTATGTCTGTCAAACAGG
GPX-1	CCACCGTGTATGCCTTCTCC	AGAGAGACGCGACATTCTCAAT
Trx-1	CTTTTGCCCGTCTCTCAATCA	AGGGTATTTCACACTTAGGTCCT
FoxO3a	TCACTGCATAGTCGATTCA	CATATGTGCGTACCTAGCA
SIRT1	TGATTGGCACCGATCCTCG	CCACAGCGTCATATCATCCAG
SIRT3	GAGCGGCCTCTACAGCAAC	GGAAGTAGTGAGTGACATTGGG
GAPDH	AATGGATTTGGACGCATTGGT	TTTGCACTGGTACGTGTTGAT
Mitochondrial short (117 bp)	CCCAGCTACTACCATCATTCA AGT	GATGGTTTGGGAGATTGGTTGATGT
Mitochondrial long (10 kb)	GCCAGCCTGACCCATAGCCATAATAT	GAGAGATTTTATGGGTGTAATGCGG

the dysfunction and apoptosis of osteoblasts. Here we tried to explore the effects of Ang-II on mitochondrial dysfunction in osteoblasts. We first induced osteoblasts from bone MSCs, and the osteoblasts were confirmed by ALP staining. Further, we treated those osteoblasts with Ang-II, and analyzed mitochondrial oxidative stress by testing ROS level with MitoSox staining. We found that Ang-II significantly induced mitochondria oxidative stress in a dose-dependent and time-dependent manner (Fig. 1A and B). Mitochondrial oxidative stress could induce various aspects of mitochondrial dysfunction, including mtDNA damage. Therefore, we analyzed mtDNA damage in osteoblasts treated with Ang-II. In consistent with the above results, Ang-II induced a significant increase in mtDNA damage in osteoblasts in a dose-dependent and time-dependent manner (Fig. 1C and D). Taken together, the findings indicate that Ang-II induces mitochondrial oxidative stress and mtDNA in mouse osteoblasts differentiated from bone MSCs.

3.2. Angiotensin II inhibits the expression of mitochondrial catalase and MnSOD in osteoblasts

Both increase in ROS production and decline in ROS clear can contribute to the increased mitochondrial oxidative stress and subsequent mtDNA damage. In the present study, we focused on the antioxidative enzymes in mitochondria. We performed q-PCR assay to analyze the mRNA levels of mitochondrial antioxidative enzymes, including catalase, MnSOD, peroxiredoxin 3 (Prx3), glutathione peroxidase 1 (GPX-1), and thioredoxin 1 (Trx-1). The results showed that Ang-II could significantly reduce the mRNA levels of catalase and MnSOD (Fig. 2A). The Western blot results also indicated that catalase and MnSOD levels were down-regulated in osteoblasts treated with Ang-II (Fig. 2B). Furthermore, we analyzed the enzyme activity of catalase and MnSOD. We found that Ang-II reduced the enzyme activity of catalase and MnSOD (Fig. 2C). In summary, Ang-II inhibits catalase and MnSOD expression and reduces their enzyme activity in osteoblasts.

3.3. Sirt1–FoxO3a axis inhibition is involved in angiotensin II function in osteoblasts

Factors belonging to the Foxo subfamily have been shown to inhibit ROS generation by enhancing the activities of MnSOD and catalase [22,23]. We analyzed the role of FoxO3a in Ang-II-induced inhibition of MnSOD and catalase expression. However, we did not observe any change in FoxO3a mRNA and protein levels before and after Ang-II treatment in osteoblasts (Fig. 3A and B). Interesting, the acetylation of FoxO3a was significantly upregulated by Ang-II (Fig. 3C), which could reduce the activity of FoxO3a as a transcription factor [24]. Because SIRT3 and SIRT1 have been found to have redundant effects of protecting cells during stress, and SIRT1 and SIRT3 have the ability to control the activity of Foxo3a by deacetylation [24,25]. We postulated that Ang-II may regulate SIRT1 and/or SIRT3 expression in osteoblasts. The results showed that SIRT1 protein but not mRNA level was reduced by Ang-II treatment (Fig. 3D and E), which may be consistent with previous report [26]. Indeed, SIRT1 overexpression inhibited hyperacetylation of FoxO3a induced by Ang-II (Fig. 3F). SIRT1 overexpression also promoted the expression of MnSOD and catalase (Fig. 3G). Finally, we showed that SRT3025, a new activator of SIRT1, markedly up-regulated the mRNA levels of MnSOD and catalase in osteoblasts, and the effects of Ang-II was blocked (Fig. 3H). Therefore, inhibited SIRT1–FoxO3a axis contributes to MnSOD and catalase down-regulation induced by Ang-II.

3.4. Sirt1 activator and MnSOD mimetics reduce angiotensin II function in osteoblasts

As SIRT1–FoxO3a–MnSOD pathway was repressed by Ang-II in osteoblasts, we finally wanted to know whether activation of this pathway could block Ang-II-induced mitochondrial oxidative stress and mtDNA damage. We treated osteoblasts with Ang-II in combination with SRT3025. The results showed that SRT3025

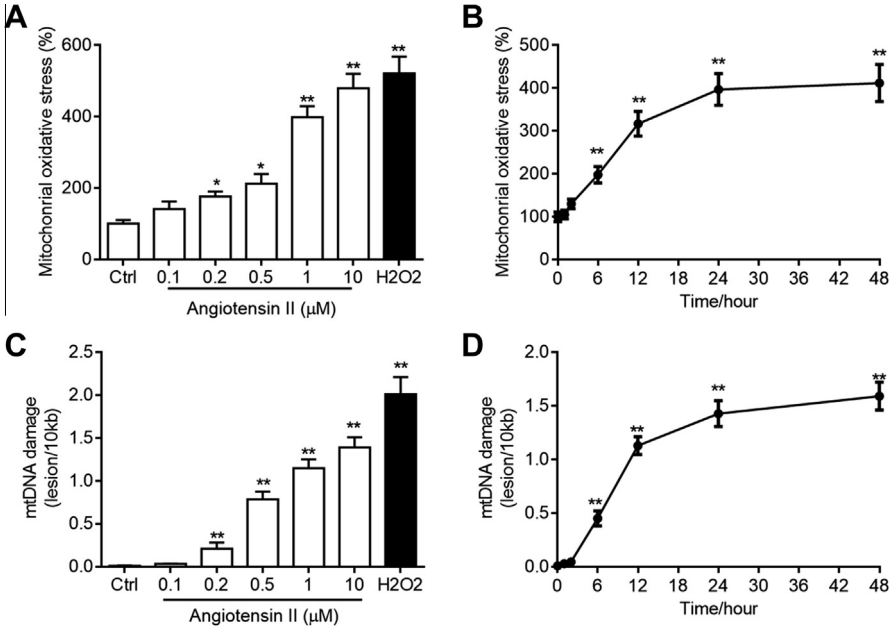

Fig. 1. Angiotensin II (Ang-II) induces mitochondrial oxidative stress and mtDNA damage in osteoblasts. (A and B) Ang-II induces mitochondrial oxidative stress in osteoblasts. (A) Osteoblasts were treated with Ang-II with the indicated concentration for 24 h. MitoSox probe staining was used to evaluate the ROS level in diverse groups. H_2O_2 (50 μM) was used as a positive control. (B) Osteoblasts were treated with 1 μM Ang-II for the indicated time. MitoSox probe staining was used to evaluate the ROS level in diverse groups. (C and D) Ang-II induces mtDNA damage in osteoblasts. (C) Osteoblasts were treated with Ang-II with the indicated concentration for 24 h. Q-PCR was utilized to quantify the damage in mtDNA as described in the method section. H_2O_2 (50 μM) was used as a positive control. (D) Osteoblasts were treated with 1 μM Ang-II for the indicated times. Q-PCR was utilized to quantify the damage of mtDNA.

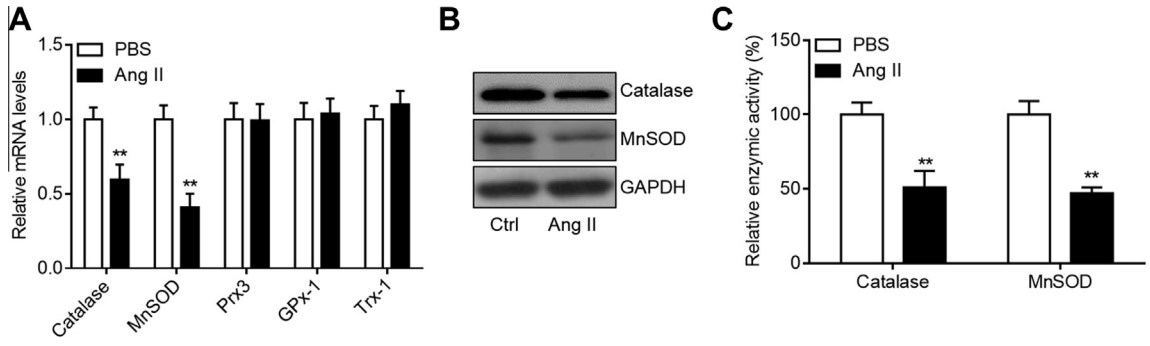

Fig. 2. Ang-II inhibits the expression of mitochondrial catalase and MnSOD in osteoblasts. Osteoblasts were treated with 1 μM Ang-II for 24 h. (A) Ang-II reduces catalase and MnSOD mRNA levels in osteoblasts. (B) Ang-II reduces catalase and MnSOD protein levels in osteoblasts. (C) Ang-II reduces catalase and MnSOD activity in osteoblasts. The activity of catalase and MnSOD was analyzed with kits.

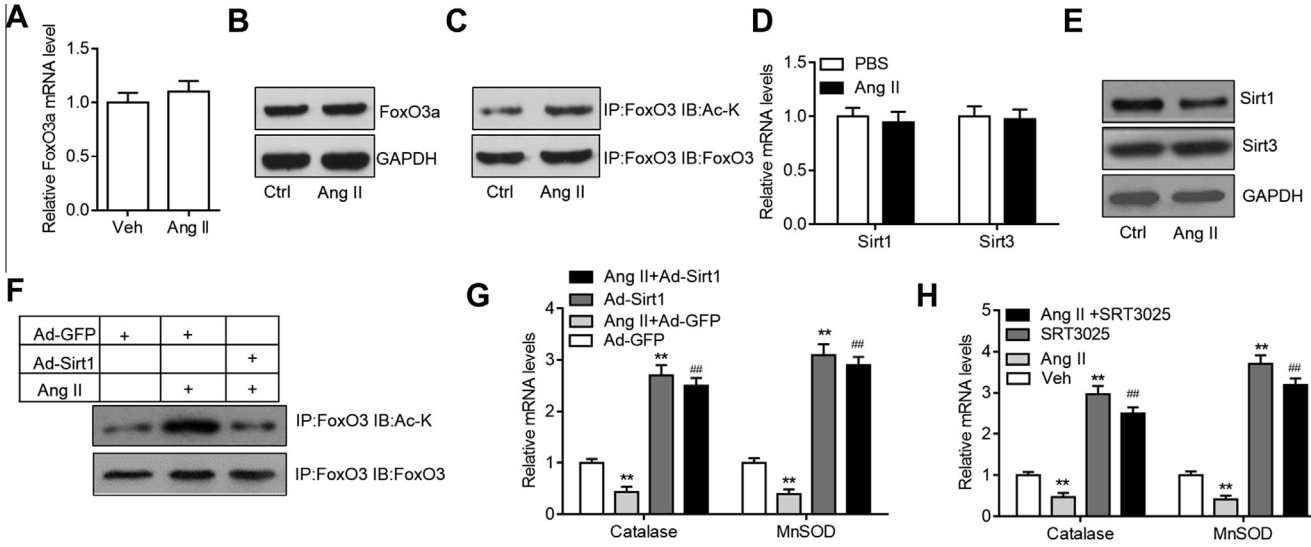

Fig. 3. Sirt1–FoxO3a axis inhibition is involved in Ang-II function in osteoblasts. (A–C) Ang-II promotes FoxO3a acetylation level in osteoblasts. Osteoblasts were treated with 1 μM Ang-II for 24 h. (A) FoxO3a mRNA level in osteoblasts treated with Ang-II. (B) FoxO3a protein level in osteoblasts treated with Ang-II. (C) FoxO3a protein acetylation level in osteoblasts treated with Ang-II. (D and E) Ang-II inhibits SIRT1 but not SIRT3 level in osteoblasts. Osteoblasts were treated with 1 μM Ang-II for 24 h. (D) mRNA levels of SIRT1 and SIRT3. (E) Protein levels of SIRT1 and SIRT3. (F) SIRT1 overexpression reduces FoxO3a acetylation in osteoblasts. Osteoblasts were infected with adenovirus carrying human SIRT1 or control GFP for 24 h, then were treated with 1 μM Ang-II for additional 24 h. (G) SIRT1 overexpression promotes catalase and MnSOD expression in osteoblasts. Osteoblasts were infected with adenovirus carrying human SIRT1 or control GFP for 24 h, then were treated with 1 μM Ang-II for additional 24 h. (H) SIRT1 activator SRT3025 promotes catalase and MnSOD expression in osteoblasts. Osteoblasts were treated with 1 μM Ang-II with/without 10 μM SRT3025 for 24 h.

significantly increased the activity of MnSOD and catalase, and the effects of Ang-II was blocked (Fig. 4A). Furthermore, we also found that SRT3025 treatment reduced angiotensin II-induced mitochondrial oxidative stress and mtDNA damage (Fig. 4B and C). Similarly, we found that MnTBAP, a mimetics of MnSOD, could up-regulate MnSOD activity and inhibit Ang-II-induced mitochondrial oxidative stress and mtDNA damage in osteoblasts (Fig. 4D–F). Taken together, pharmacological activation of SIRT1–FoxO3a–MnSOD pathway suppresses Ang-II induced mitochondrial oxidative stress and mtDNA in osteoblasts.

4. Discussion

In the present work, we identify a novel mechanism by which Ang-II induces mitochondrial oxidative stress and mtDNA damage in osteoblasts. We show that Ang-II reduces the protein level of SIRT1, which leads to hyperacetylation of FoxO3a. FoxO3a hyperacetylation reduces MnSOD and catalase expression and enzyme activity. Finally, we provide data that SIRT1 activator and MnSOD mimetics could largely repress Ang-II-induced mitochondrial oxidative stress and mtDNA damage.

Angiotensin II was previously reported to up-regulate global ROS in mouse osteoblastic MC3T3-E1 cells [9], and human osteoblast-like cell [8]. In the present work, we used a distinctive model of osteoblasts differentiated from mouse bone MSCs. We found that Ang-II induced mitochondrial ROS production, which is consistent with the finding of Li et al. in primary mouse calvaria osteoblast [27]. We also showed that Ang-II induced mtDNA damage, which is a downstream damage of persistent ROS production. Li et al. [27] showed that Ang-II induces mitochondrial dysfunction and promotes apoptosis. However, they did not uncover the underlying mechanism by which Ang-II function.

Previous work has indicated that Ang-II selectively induces mitochondrial oxidative stress in cardiomyocytes [28], and Ang-II reduces the expression of catalase and MnSOD [24], two pivotal antioxidative enzymes that could locate in mitochondria [29]. Factors belonging to the Foxo subfamily have been shown to inhibit ROS generation by enhancing the activities of MnSOD and catalase [22,23]. We found that Ang-II could induce the hyperacetylation of FoxO3a, but not affect its expression level. SIRT1 and

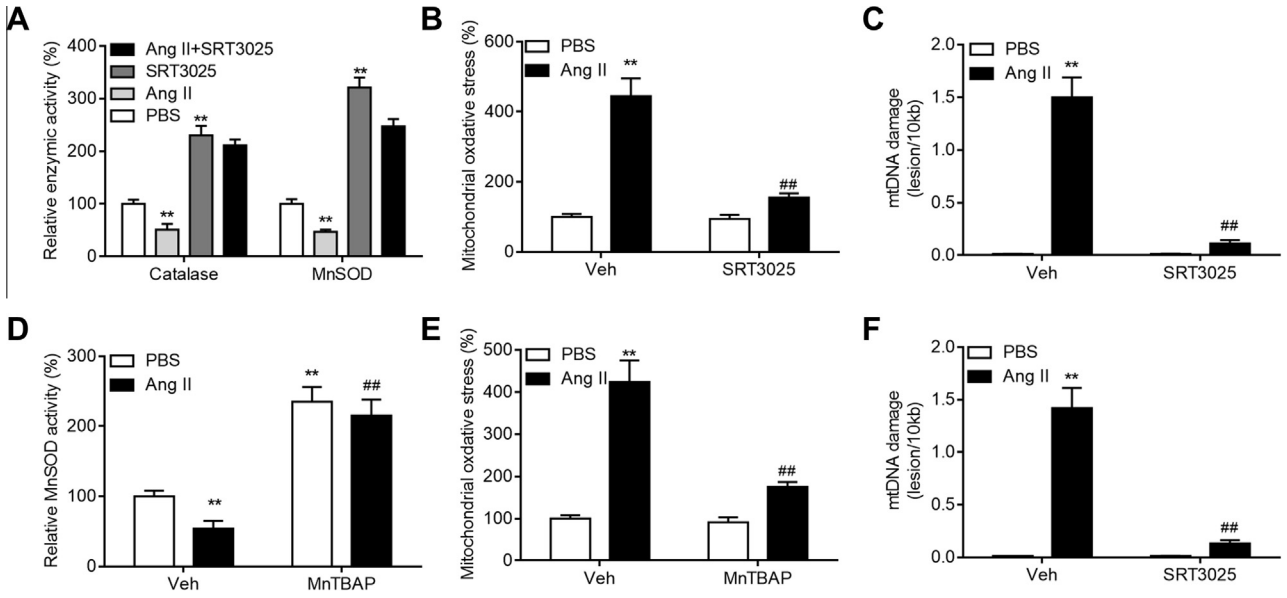

Fig. 4. Sirt1 activator and MnSOD mimetics reduce angiotensin II induced mitochondrial oxidative stress and mtDNA damage in osteoblasts. (A) SRT3025 promotes catalase and MnSOD activity. (B) SRT3025 reduces Ang-II-induced mitochondrial oxidative stress. (C) SRT3025 reduces Ang-II-induced mtDNA damage. (D) MnTBAP promotes MnSOD activity. (E) MnTBAP reduces Ang-II-induced mitochondrial oxidative stress. (F) MnTBAP reduces Ang-II-induced mtDNA damage.

SIRT3 have been found to have redundant effects of protecting cells during stress, and SIRT1 and SIRT3 have the ability to control the activity of Foxo3a by deacetylation [24,25]. Interesting, Ang-II reduces the expression of SIRT1 but not SIRT3. A recent work by Huang et al. [26] indicated that Ang-II promoted insulin-like growth factor receptor II (IGF-IIR) expression and cardiomyocyte apoptosis by inhibiting heat-shock transcription factor 1 (HSF1) *via* JNK activation and SIRT1 degradation. In osteoblasts, we also found that Ang-II reduced SIRT1 protein but not mRNA level, indicating that the mechanism exists widely in diverse cell types.

Finally, we also attempt to block Ang-II-induced mitochondrial oxidative stress and therefore may rescue osteoblast function. We used two drugs, SRT3025 and MnTBAP. SRT3025 is a new developed SIRT1 activator, which is reported to provide atheroprotection in ApoE−/− mice by reducing hepatic Pcsk9 secretion and enhancing Ldlr expression [30]. More importantly, SRT3025 down-regulates sclerostin and rescues ovariectomy-induced bone loss and biomechanical deterioration in female mice [31]. Repression mitochondrial oxidative stress may be a novel mechanism for SRT3025-induced bone protection. Therefore, SRT3025 may be promising candidate drug for osteoporosis treatment via improving osteoblast function. In addition, we also analyzed MnTBAP, a mimetics of MnSOD. Similar results were obtained as SRT3025 treatment. This is the first time for demonstrating the protective role of MnTBAP in osteoblasts and bone homeostasis.

In summary, we find a novel mechanism that Ang-II induces mitochondrial oxidative stress and mtDNA damage by inhibiting SIRT1–FoxO3a–MnSOD axis. SRT3025 and MnTBAP may be potential drugs for osteoporosis therapy by improving osteoblast function.

5. Conflict of interest

None.

Acknowledgments

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