TWEAK/Fn14 promotes oxidative stress through AMPK/PGC-1α/MnSOD signaling pathway in endothelial cells

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Abstract. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) contributes to dysfunction of endothelial cells via its receptor, Fn14. However, its role in the production of reactive oxygen species (ROS), particularly mitochondrial ROS (mtROS) and the subsequent decrease in nitric oxide (NO) in endothelial cells remains unclear. In this study, the effect of TWEAK/Fn14 on generation of ROS, mtROS and NO in endothelial cells and its potential mechanism was investigated. Human umbilical vein endothelial cells (HUVECs) were treated with TWEAK with Fn14 small interfering (si)RNA or negative control RNA. It was demonstrated that TWEAK induced the production of ROS and mtROS in HUVECs, which were detected by fluorescent microscope, and flow cytometry. In addition, TWEAK decreased the generation of NO as indicated using the Nitric Oxide Assay kit. Furthermore, TWEAK aggravated mtDNA damage as measured by quantitative polymerase chain reaction analysis. Inhibition of Fn14 by Fn14 siRNA decreased TWEAK-induced ROS and mtROS production, as well as mtDNA damage, while it increased the production of NO in endothelial cells. In addition, TWEAK inhibited the expression of active AMP-activated protein kinase (AMPK) and its downstream protein peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and manganese superoxide dismutase (MnSOD). Notably, Fn14 siRNA enhanced the expression of the aforementioned proteins. Taken together, TWEAK/Fn14 contributes to endothelial dysfunction through modulation of ROS and mtROS. In addition, the underlying mechanism is implicated in the AMPK/PGC-1α/MnSOD signaling pathway.

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Introduction

Atherosclerosis is a chronic inflammatory disease involving numerous cytokines (1). Tumor necrosis factor-like weak inducer of apoptosis (TWEAK), an inflammatory cytokine of tumor necrosis factor (TNF) superfamily, participates in regulation of multiple cellular responses, including proinflammatory activity, angiogenesis and cell proliferation (2). When binding to its receptor fibroblast growth factor inducible molecule 14 (Fn14), TWEAK exerts adverse biological functions in atherosclerosis, resulting in dysfunction of endothelial cells (2,3) and smooth muscle cells (4) and inducing inflammatory response of monocytes/macrophages (5-7).

Endothelial dysfunction is an early hallmark of the onset of atherosclerosis (8). Excessive production of reactive oxygen species (ROS) and the subsequent decrease in vascular bioavailability of nitric oxide (NO) have long been proposed to be the common pathogenetic mechanism of the endothelial dysfunction (9). The mitochondrial respiratory chain is a major intracellular source of ROS (10) and an abnormal production of ROS in the mitochondria plays a critical role in the development of atherosclerosis, including oxidation of LDL and damage of mitochondria DNA (mtDNA) (11).

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a transcriptional coactivator, recruits transcription factors to regulate mitochondria numbers and functions (12). PGC-1α plays a crucial protective role in the regulation of mitochondrial oxidative stress in endothelial cells (13,14). Its underlying mechanism is to upregulate the mitochondrial antioxidant defense system such as manganese superoxide

dismutase (MnSOD) (13). Furthermore, PGC-1α is activated by AMP-activated protein kinase (AMPK) (14), which is an important metabolic sensor.

In the present study, we demonstrated the role of TWEAK/Fn14 on oxidative stress especially that derived from mitochondrial and NO generation in human umbilical vein endothelial cells (HUVECs). In addition, the underlying mechanism is implicated in the AMPK/PGC-1α/MnSOD signaling pathway.

Materials and methods

Reagents. Recombinant human TWEAK was from Alexis (Läufelfingen, Switzerland). GSK621 (S7898) was purchased from Selleck Chemicals (Houston, TX, USA). Rabbit polyclonal antibody against AMPK (ab131512) was purchased from Abcam (Cambridge, MA, USA), and rabbit monoclonal antibody against GAPDH (2118S), pho-AMPK (Thr^{172}) (2535S), PGC-1α (2178S) and MnSOD (13141S) were purchased from Cell Signaling (Beverly, MA, USA). The Fn14-siRNA duplexes were designed and synthesized by Ribo-Bio (Guangzhou, China).

Cell culture. HUVECs were obtained from American Type Culture Collection (Manassas, VA, USA), and grown in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sciencell, Carlsbad, CA, USA). Cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air and grown to 70 to 80% confluence. For the experiments, the HUVECs were treated with 50, 100 and 200 ng/ml TWEAK respectively based on previous studies (15-17). As for Fn14 siRNA, for each well of a 6-well plate, cells were transfected with 5 μl siRNA (20 μM) or negative control (Ncontrol group) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 48 h. To confirm that AMPK activation is required for the expression of PGC-1α and MnSOD, HUVECs were treated by 10 μmol/l GSK621 as previous described (18,19).

Assessment of ROS production. To assess ROS and mitochondrial ROS production, HUVECs were incubated with 2,7-dichlorofluorescein diacetate (DCFH-DA, Beyotime, Shanghai, China) or MitoSOX Red (Thermofisher Scientific, Waltham, MA, USA) in Hank's Buffered Salt Solution (HBSS) at 37˚C for 30 min. After washing two times in HBSS, fluorescent images were captured using an Olympus fluorescent microscope. The fluorescence intensity was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA). For flow cytometry analysis, after 30 min loading of DCFH-DA or MitoSOX, the cells were collected and resuspended in 200 μl of PBS buffer. Then intracellular ROS and mitochondrial ROS levels were performed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Measurement of NO production. Total NO production in culture medium was determined by measuring the concentration of nitrate and nitrite, a stable metabolite of NO, by modified Griess reaction method. The procedure involed use of the Nitric Oxide Assay kit (Nanjing Jiancheng Bioengineering, Nanjing, China).

MtDNA damage quantification. MtDNA damage was determined by quantitative PCR in HUVECs as previously described (20). Total DNA was extracted using the Genomic DNA kit (TransGen Biotech, Beijing, China). Quantitative PCR was performed using the Eppendorf Mastercycler ep realplex PCR System and the sequences of the primers were as follows: mtDNA primers: 5'-CCCCACAAACCCCATTAC TAAACCCA-3'; 5'-TTTCATCATGCGGAGATGTTGGAT GG-3'; β-*globin* primers: 5'-CGAGTAAGAGACCATTGT GGCAG-3'; 5'-GCTGTTCTGTCAATAAATTTCCTTC-3'. PCR was performed under the following conditions: denaturation at 95˚C for 1 min, followed by 40 cycles of 95˚C for 15 sec and 58˚C for 20 sec. The values were determined relative to the control sample after normalizing to β-*globin* gene control values and calculated by the comparative cycle threshold (ΔΔCt) method.

Western blot. HUVECs after treatment were lysed with RIPA lysis buffer (Beyotime, Shanghai, China) containing 10 mM phenylmethylsulfonyl fluoride (PMSF, Beyotime, Shanghai, China). Then the lysates were isolated by centrifugation and the protein concentration was determined using the BCA Protein Assay kit (Beyotime). Western blotting was performed as previously described (21). After quantification, the proteins were separated by 10% SDS-PAGE and proteins transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Billerica, MA, USA). Membranes were blocked with 5% nonfat dried milk, and they were immunoblotted with anti-GAPDH (1:2,000), anti-AMPK (1:1,000), anti-pho-AMPK (Thr^{172}) (1:1,000), anti-PGC-1α (1:1,000), and anti-MnSOD (1:1,000) antibodies at 4˚C overnight. Subsequently, the membranes were incubated with goat anti-rabbit IR-Dye 800cw labeled secondary antisera in 0.1% Tween, 0.01% SDS LiCor blocking buffer for 1 h at room temperature. Membranes were imaged using a LiCor Odyssey scanner.

Statistical analysis. All experiments were performed at least three times. All statistical analysis was conducted with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Data were represented as means ± standard deviation. Statistical significance of the data was performed by unpaired Student test (2-tailed) between two groups or one-way ANOVA followed by the post-hoc Tukey's test, as appropriate. A value of $P<0.05$ was considered significant.

Results

TWEAK induces production of ROS and mtROS and decreases NO generation in HUVECs. After treated with TWEAK for 24 h, HUVECs were incubated with DCF-DA or MitoSOX probe for 30 min. And then cell images were captured by a fluorescence microscope or fluorescence was detected by flow cytometry. At the same time, NO production in culture medium was determined by the Nitric Oxide Assay kit. Compared to control group, TWEAK significantly increased the production of ROS (Fig. 1) and mtROS (Fig. 2), while it significantly decreased the NO generation (Fig. 1). Furthermore, the effects were dose-dependent within 50-200 ng/ml.

Fn14 mediates TWEAK-induced production of ROS and mtROS and reduction of NO in HUVECs. To determine

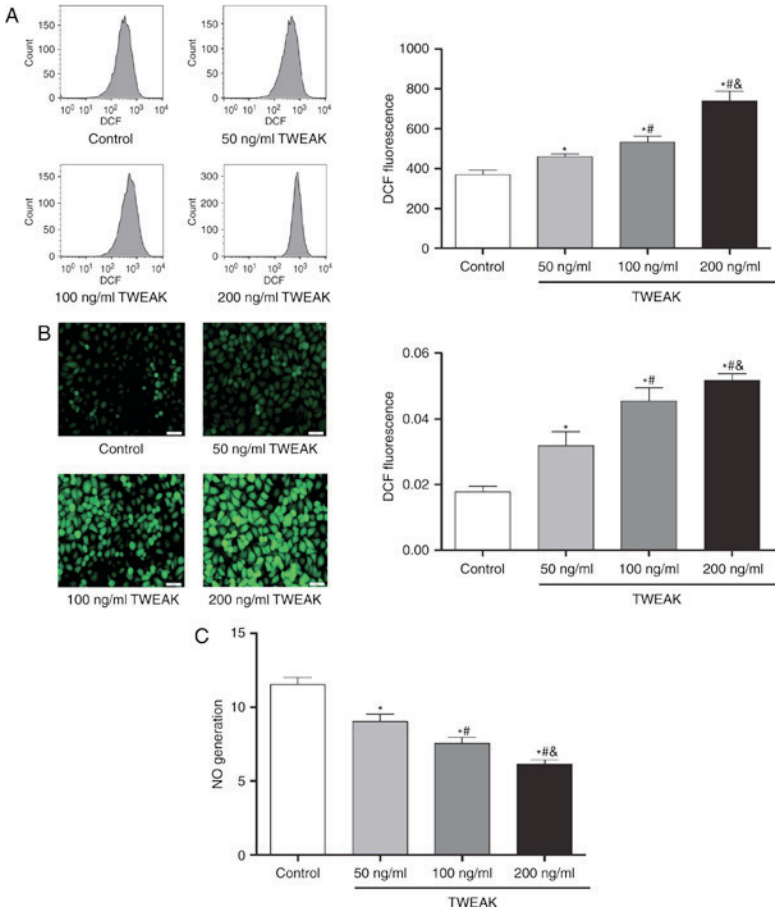

Figure 1. TWEAK induces ROS production and decreases NO generation in HUVECs. After treated with TWEAK for 24 h, HUVECs were incubated with DCF-DA probe for 30 min, and culture medium was used for NO determination. (A) Data are expressed as mean fluorescence intensity which was quantified by flow cytometry. (B) Quantification of fluorescence intensity for ROS levels was analyzed using fluorescence microscopy. (C) NO production in culture medium was determined by the Nitric Oxide Assay kit (μmol/l). Magnification, x200. n=3 in each group. *P<0.05 vs. control group, $^{\#}$P<0.05 vs. 50 ng/ml TWEAK treatment group, $^{\&}$P<0.05 vs. 100 ng/ml TWEAK treatment group.

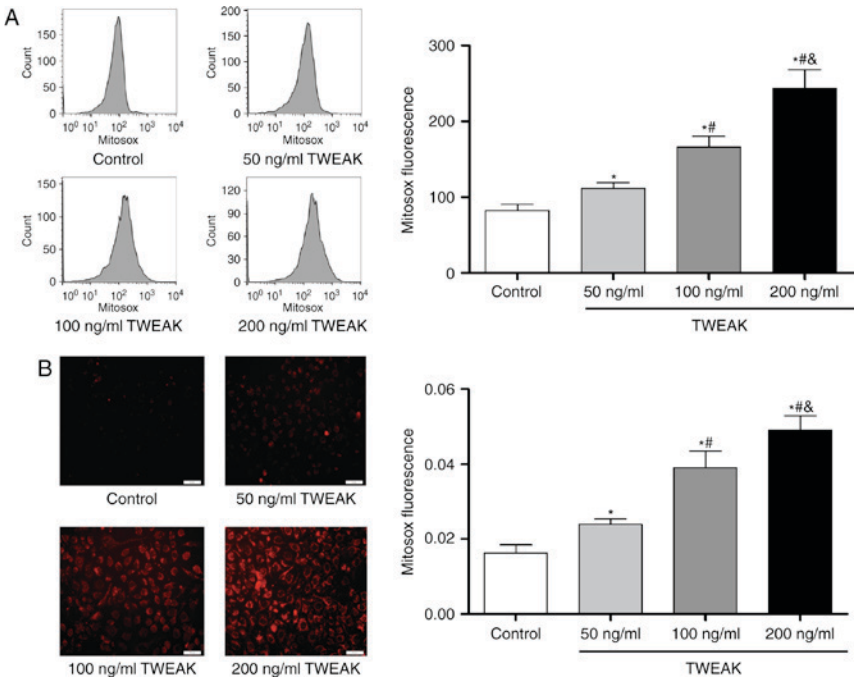

Figure 2. TWEAK increases mtROS production in HUVECs. After treated with TWEAK for 24 h, HUVECs were incubated with MitoSOX probe for 30 min. (A) Data are expressed as mean fluorescence intensity which was quantified by flow cytometry. (B) Quantification of fluorescence intensity for mtROS levels was analyzed using fluorescence microscopy. Magnification, x200. n=3 in each group. *P<0.05 vs. control group, $^{\#}$P<0.05 vs. 50 ng/ml TWEAK treatment group, $^{\&}$P<0.05 vs. 100 ng/ml TWEAK treatment group.

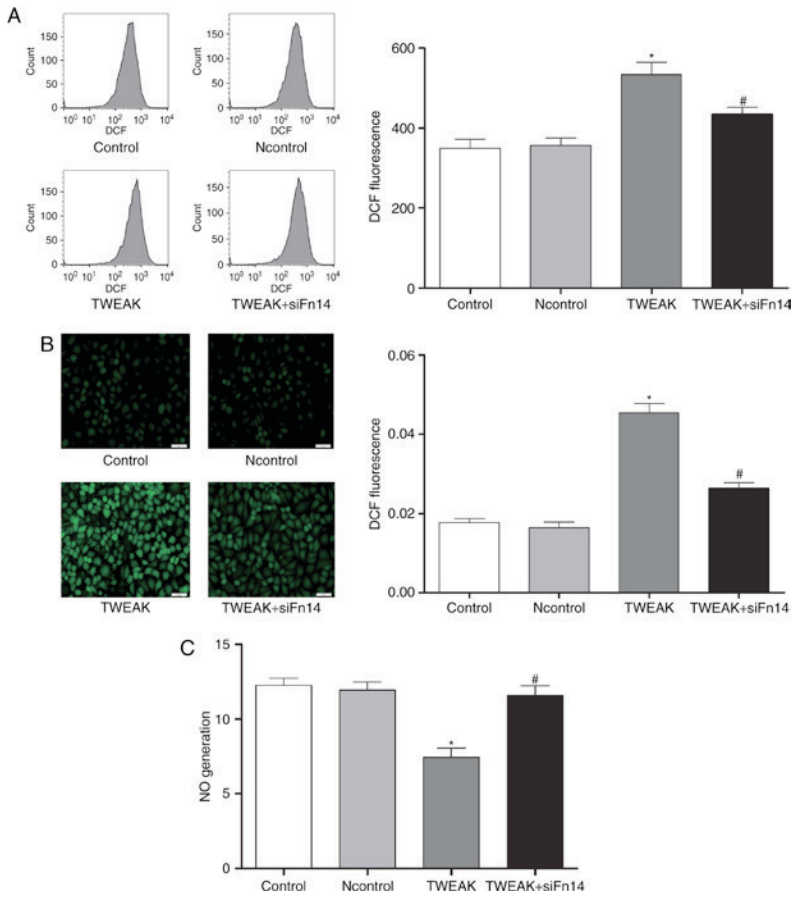

Figure 3. Fn14 mediates TWEAK-induced production of ROS and reduction of NO in HUVECs. After pretreatment with Fn14 siRNA or negative control RNA for 48 h, HUVECs were treated with 100 ng/ml TWEAK for 24 h, and they were incubated with DCF-DA probe for 30 min, and culture medium was used for NO determination. (A) Data are expressed as mean fluorescence intensity which was quantified by flow cytometry. (B) Quantification of fluorescence intensity for ROS levels was analyzed using fluorescence microscopy. (C) NO production in culture medium was determined by the Nitric Oxide Assay kit (μmol/l). Magnification, x200. n=3 in each group. *P<0.05 vs. control group, $^{\#}$P<0.05 vs. TWEAK treatment group.

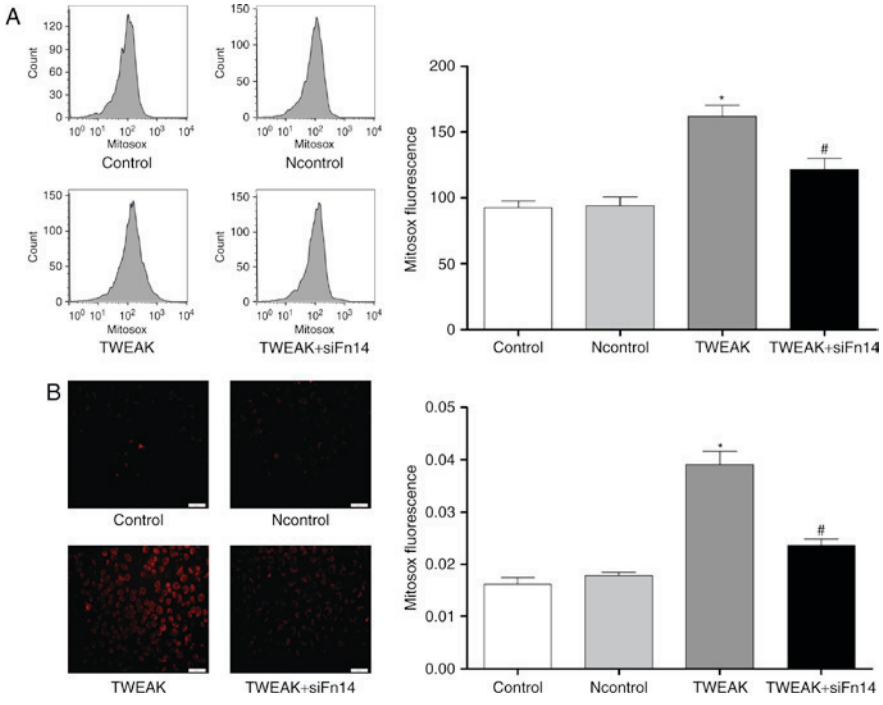

Figure 4. Fn14 mediates TWEAK-induced mtROS production in HUVECs. After pretreatment with Fn14 siRNA or negative control RNA for 48 h, HUVECs were treated with 100 ng/ml TWEAK for 24 h, and they were incubated with MitoSOX probe for 30 min. (A) Data are expressed as mean fluorescence intensity which was quantified by flow cytometry. (B) Quantification of fluorescence intensity for mtROS levels was analyzed using fluorescence microscopy. Magnification, x200. n=3 in each group. *P<0.05 vs. control group, $^{\#}$P<0.05 vs. TWEAK treatment group.

whether Fn14 mediate the effect of TWEAK on production of ROS and mtROS and reduction of NO, HUVECs were treated with 100 ng/ml TWEAK for 24 h after Fn14 siRNA or negative control siRNA pretreatment. Compared to control group, the production of ROS and mtROS was increased significantly while NO generation decreased markedly in TWEAK treatment group. Furthermore, after Fn14 siRNA pretreatment, the generation of ROS and mtROS decreased significantly while NO generation increased markedly compared to TWEAK treatment group (Figs. 3 and 4).

TWEAK/Fn14 promotes mtDNA damage in HUVECs. Given mitochondrial oxidative stress leading to mitochondrial DNA damage, we tested the impact of TWEAK/Fn14 on the damage of mtDNA in HUVECs. MtDNA damage was assessed by the relative expression quantity of DNA amplification. The lower relative expression quantity of DNA would suggest more serious DNA damage. We observed that mtDNA relative amplification was about 56.4% lower in the group of TWEAK treatment, suggesting mtDNA damage increased significantly compared to control group. After Fn14 siRNA pretreatment, mtDNA damage was improved compared to TWEAK treatment group (Fig. 5).

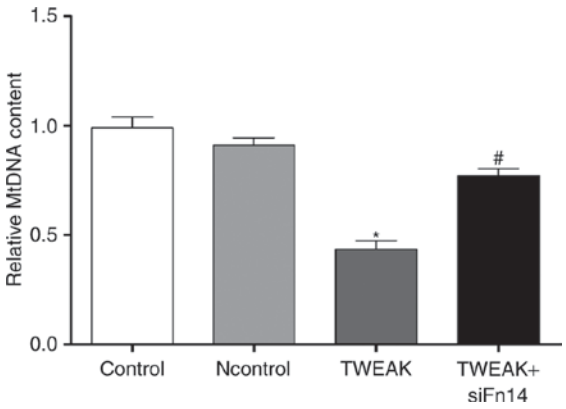

Figure 5. TWEAK/Fn14 induces mitochondrial DNA damage in HUVECs. After pretreatment with Fn14 siRNA or negative control RNA for 48 h, HUVECs were treated with 100 ng/ml TWEAK for 24 h, total DNA was extracted and mitochondrial DNA damage was determined by quantitative PCR in HUVECs. n=3 in each group. *P<0.05 vs. control group, #P<0.05 vs. TWEAK treatment group.

Essential role of TWEAK/Fn14 in the expression of AMPK/PGC-1α/MnSOD in HUVECs. To understand the mechanism of TWEAK/Fn14 inducing mtROS to increase the generation of ROS, we tested the expression of PGC-1α and its downstream protein MnSOD. After 100 ng/ml TWEAK treatment of HUVECs for 24 h, PGC-1α and MnSOD expressions were significantly lower, suggesting PGC-1α/MnSOD may participate in the process above. It also stated that the activation of PGC-1α depended on AMPK activation (21). Therefore, we further tested the pho-AMPK (Thr172) and the expression of AMPK, and TWEAK treatment decreased the expression of pho-AMPK in HUVECs. Furthermore, we also found that, compared with the TWEAK treatment group, the expression of pho-AMPK, PGC-1α and MnSOD were significantly increased in Fn14 siRNA pretreatment group (Fig. 6). In addition, we further tested whether AMPK activation was required for the expression of PGC-1α and MnSOD. As shown in Fig. 7, the pho-AMPK (Thr172) increased significantly in HUVECs when treated with the AMPK activator GSK621 (10 μmol/l), which resulted in increased expression of PGC-1α and MnSOD.

Discussion

In present study, we first determined the effect of one inflammatory factor, TWEAK, and its receptor Fn14 on inducing ROS especially mtROS production and decreasing NO generation in HUVECs. Furthermore, the underlying mechanism associated with the AMPK/PGC-1α/MnSOD pathway was tried to demonstrate clearly.

Endothelial dysfunction resulting in disturbance in endothelial homeostasis is considered as a characteristic feature of atherosclerosis (22). The hallmark of endothelial dysfunction is impaired endothelium-dependent vasodilation, which is mediated by NO (23). As an inflammatory factor in the TNF family, TWEAK has been demonstrated to participate in dysfunction of endothelial cells by inducing generation of adhesion molecules (16), monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) (3). In present study, we first demonstrated that TWEAK reduced the generation of NO obviously. Besides, a growing data suggests that increased production of ROS have a pivotal role in reduction of NO (9). Excessive production of ROS induced by TWEAK was measured in our study, and unsurprisingly, TWEAK increased production of ROS in HUVECs and similar results were found in monocytes/macrophages (24). In that article, the authors demonstrated that TWEAK induces ROS through NADPH oxidase in monocytes. As another major intracellular source of ROS (25), however, mitochondrial ROS was also examined in cells for the first time in our study, and it is not unexpected that TWEAK promoted production of mitochondrial ROS significantly in HUVECs. Moreover, the increase of ROS especially mitochondrial ROS and decrease of NO were curbed when treated with Fn14 siRNA, suggesting that Fn14 may mediate these effects.

Given that human mtDNA lacks protective histones (26) and is located proximal to ROS generation, it is vulnerable to damage by ROS, which also induced oxidative damage (26). MtDNA damage was assessed by the relative expression quantity of DNA amplification as previous described (20,27). As for our study, TWEAK/Fn14 axis was capable of inducing mtDNA damage, resulting from excessive production of ROS in the cells, which was not reported before. Meanwhile, as a vicious cycle, mtDNA damage further leads to an increase in oxidative stress, and both of two effects promote atherosclerosis by contributing to endothelial dysfunction (27-29). However, previous studies shown that TWEAK/Fn14-repressed mitochondrial biogenesis might lead to decrease of mtDNA content (30,31). Therefore, it is needed to determine that this is due to the inhibition of DNA synthesis or induction of DNA damage, as well as whether there is interaction between them.

As a positive regulator of oxidative metabolism, PGC-1α upregulates the induction of a set of antioxidant proteins response to mitochondrial oxidative stress, which increases the cellular capacity to detoxify mitochondrial ROS in turn,

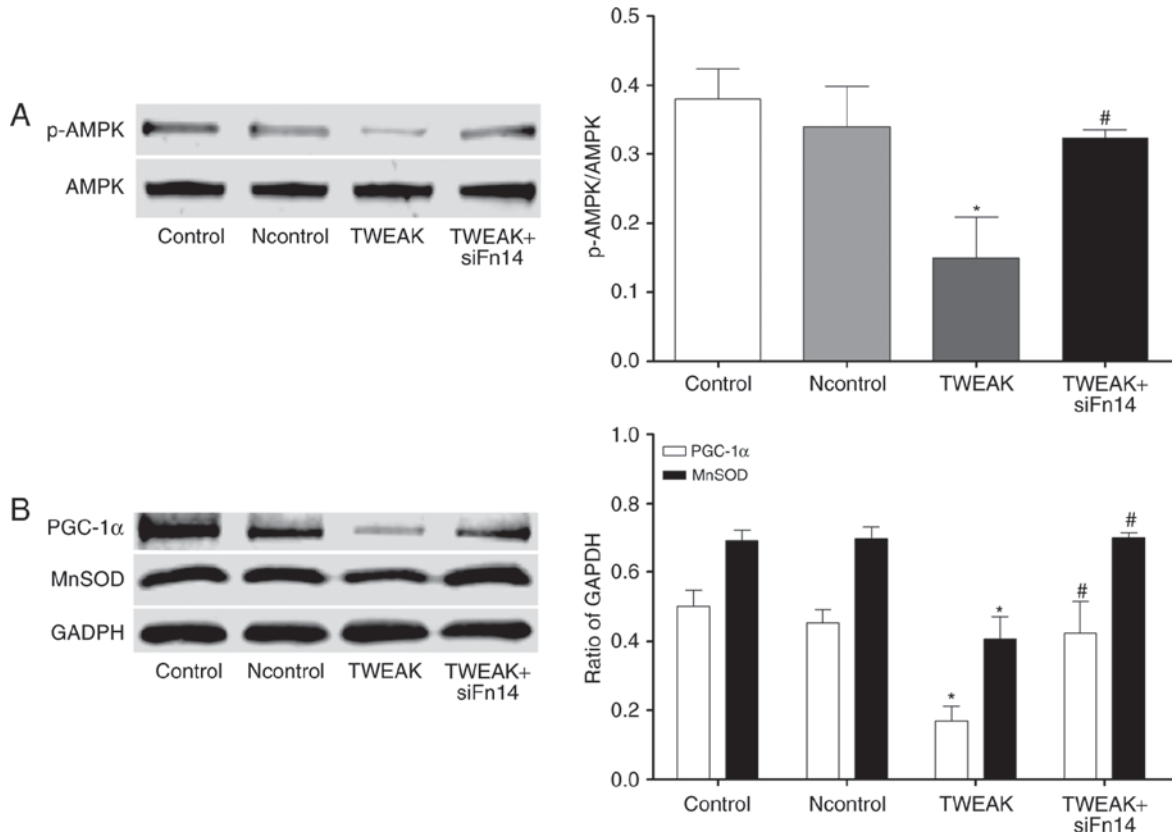

Figure 6. TWEAK/Fn14 down-regulates the expression of AMPK/PGC-1α/MnSOD in HUVECs. After pretreatment with Fn14 siRNA or negative control RNA for 48 h, HUVECs were treated with 100 ng/ml TWEAK for 24 h. (A) TWEAK/Fn14 inhibited AMPK activation in HUVECs. (B) TWEAK/Fn14 decreased relative expression of PGC-1α and MnSOD in HUVECs. n=3 in each group. *P<0.05 vs. control group, #P<0.05 vs. TWEAK treatment group.

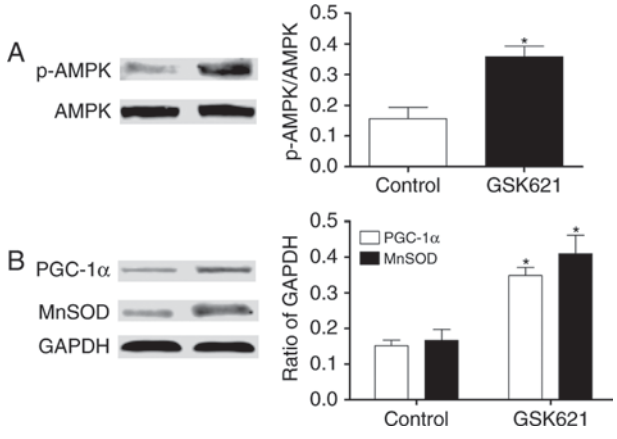

Figure 7. AMPK activation is required for the expression of PGC-1α and MnSOD. After treated with 10 μmol/l GSK621, the expression of AMPK/PGC-1α/MnSOD in HUVECs was performed by western blot. (A) GSK621 induced AMPK activation in HUVECs. (B) GSK621 induced the expression of PGC-1α and MnSOD in HUVECs. n=3 in each group. *P<0.05 vs. control group.

preventing endothelial dysfunction in response to oxidative stress conditions (13). Until recently, the only well-known and primary antioxidant mitochondrial protein is MnSOD (32), which has been strongly implicated in endothelial function via regulation of ROS within mitochondria (33). In our study, we found that the decreased expression of PGC-1α and subsequently that of MnSOD were induced by TWEAK, which was mediated by binding to Fn14. Besides, the induction of PGC-1α has been reported to depend on the activation of AMPK via phosphorylating the enzyme at Thr[172] (21). In this study, we also demonstrated that TWEAK/Fn14 axis decreased the relative expression of phosphorylation levels of AMPK. Furthermore, we reconfirmed that AMPK activation increased the expression of PGC-1α and its downstream protein MnSOD. All these results suggest that TWEAK/Fn14 induces mitochondrial oxidative stress through regulation of the AMPK/PGC-1α/MnSOD pathway. However, previous studies reported that TWEAK/Fn14 repressed PGC-1αand mitochondrial biogenesis (30,31). It is not clear that whether TWEAK/Fn14-repressed mitochondrial biogenesis results in the production of ROS and mtROS. Therefore, further research is needed to confirm.

In conclusion, this study first described the role of TWEAK/Fn14 in upregulation of ROS and mtROS generation in HUVECs, which provide novel evidence that TWEAK/Fn14 may become a key target of interference in atherosclerosis development. Furthermore, the AMPK/PGC-1α/MnSOD pathway may be involved in the potential mechanism, providing a new treatment strategy.

Acknowledgements

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