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Glucocorticoid triggers endothelial cell ferroptosis via NOX4-mediated reactive oxygen species and lipid peroxidation

Lijun Fang[a], Jiazheng Chen[b], Wenqiang Li[c], Linmao Lyu[d,e,f,g,*]

Abstract

Background: Glucocorticoids (GCs) are widely used in acute and critical illnesses, but long-term and high-dose use of GCs can cause several vascular side effects. However, the underlying mechanisms are not well-understood. Ferroptosis, a novel form of reactive oxygen species (ROS)-dependent cell death, is characterized by intracellular iron accumulation and lipid peroxidation. NADPH oxidase 4 (NOX4) is a major source of ROS. The roles of ferroptosis and NOX4 in GC-induced endothelial injury remain unknown.

Methods: Human umbilical vein endothelial cells (HUVECs) were exposed to varying concentrations of dexamethasone (DEX) to evaluate ferroptosis and NOX4 expression. Further mechanistic studies were conducted using NOX4-overexpressing adenovirus (Ad-NOX4), NOX4 small interfering RNA (siRNA), ferrostatin-1 (FER-1), and erastin.

Results: Our findings demonstrate that DEX induces ferroptosis in HUVECs. Inhibition of ferroptosis with FER-1 prevents DEX-induced reduction in HUVEC viability. Furthermore, DEX treatment increases NOX4 expression in HUVECs, and NOX4 overexpression with Ad-NOX4 promotes ferroptosis. NOX4 knockdown with siRNA suppresses DEX-induced ROS production, lipid peroxidation, and ferroptosis, thereby improving the viability, angiogenesis, and migration capacity of DEX-treated HUVECs. However, the protective effect of NOX4 knockdown is negated by the reactivation of ferroptosis with erastin.

Conclusion: GC-induced endothelial cell ferroptosis occurs through NOX4-mediated ROS production and lipid peroxidation, leading to cell death, impaired angiogenesis, and migration dysfunction. Inhibition of ferroptosis and NOX4 knockdown ameliorate GC-induced endothelial damage and dysfunction.

Keywords: Endothelial cell, Ferroptosis, Glucocorticoid, Lipid peroxidation, NADPH oxidase 4, Reactive oxygen species

Introduction

Glucocorticoids (GCs) are steroid hormones secreted by the adrenal cortex that regulate the biosynthesis and metabolism of sugars, fats and proteins [1]. They exhibit various pharmacological effects, including inhibition of the immune response and anti-inflammatory, antitoxic, and anti-shock properties.[1] Consequently, GCs are widely used to treat acute and critical illnesses, such as hypersensitivity reactions, organ transplant rejection, acute and severe infections, acute respiratory distress syndrome, sepsis, and shock.[2] However, long-term and high-dose use of GCs can lead to vascular-related side effects such as hypertension, atherosclerosis, myocardial infarction, stroke, femoral head necrosis, osteoporosis, pulmonary embolism, and deep vein thrombosis [3,4]. Understanding the mechanisms underlying GC-induced vascular damage is essential for developing interventions to mitigate the adverse effects.

Ferroptosis, first described by Dixon in 2012, is a novel form of cell death that is reactive oxygen species (ROS)-dependent and characterized by intracellular iron accumulation and lipid peroxidation [5,6]. Ferroptosis primarily depends on solute carrier family 7 member 11 (SLC7A11) and glutathione peroxidase 4 (GPX4) expression, ROS and glutathione (GSH) levels, iron and malondialdehyde (MDA) contents, and mitochondrial morphology. The identification of ferroptosis

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

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has provided new insights into strategies for treating vascular diseases [7]. However, its role in GC-induced endothelial injury remains unclear. NADPH oxidase 4 (NOX4), a key member of the NOX superfamily, is a major source of ROS and is closely associated with lipid peroxidation [8]. This study aimed to investigate the roles of ferroptosis and NOX4 in regulating GC-induced vascular injury and dysfunction.

Materials and methods

Reagents and antibodies

Dexamethasone (HY-14648), ferrostatin-1 (FER-1, HY-100579), and erastin (HY-15763) were purchased from MCE (Shanghai, China). The CCK8 assay kit (CK04) was obtained from Dojindo (Shanghai, China), and the Cytotoxicity Detection Kit (11644793001) was acquired from Roche (Hoffmann, Germany). Matrigel (354230), used for angiogenesis, was purchased from Corning (Rochester, NY, USA). A ferrous ion content assay kit (BC5415) was purchased from Solarbio (Beijing, China). The ROS (S0053), MDA (S0131), and GSH (S0053) assay kits were obtained from Beyotime (Shanghai, China). Human small interfering RNA (siRNA)-control and siRNA-NOX4 were purchased from Gene Pharma (Shanghai, China). Human NOX4-overexpressing adenovirus (Ad-NOX4) and Adenovirus-control (Ad-Ctrl) were acquired from Weizhen BIO (Jinan, China). The antibodies against SLC7A11 (26864-1-AP), GPX4 (67763-1-Ig), NOX4 (14347-1-AP), and GAPDH (60004-1-Ig) were purchased from Proteintech (Wuhan, China).

Cell culture and cell viability assay

Human umbilical vein endothelial cells (HUVECs, Catalog #8000, Sciencell Research Laboratories, Carlsbad, CA, USA) were cultured in endothelial cell medium (ECM) at 37°C with 5% CO_2. Cells were passaged upon reaching approximately 70%–80% confluence, and experiments were performed using cells between passages 3 and 6.

Cell viability was assessed using the CCK-8 assay. HUVECs were seeded at an initial seeding density of 1×10^4 cells per 100-μL medium in each well. After treatment for 72 hours, 10 μL CCK-8 solution was added to each well and the cells were incubated for 2 hours at 37°C. The optical density (OD) values at a wavelength of 450 nm were measured using a microplate reader (BioTek Synergy Neo2 Hybrid).

Release of lactate dehydrogenase

Cell death *in vitro* was measured based on the release of lactate dehydrogenase (LDH) using a Cytotoxicity Detection Kit. HUVECs were cultured with the stimulations in 24-well plates for 48 hours.

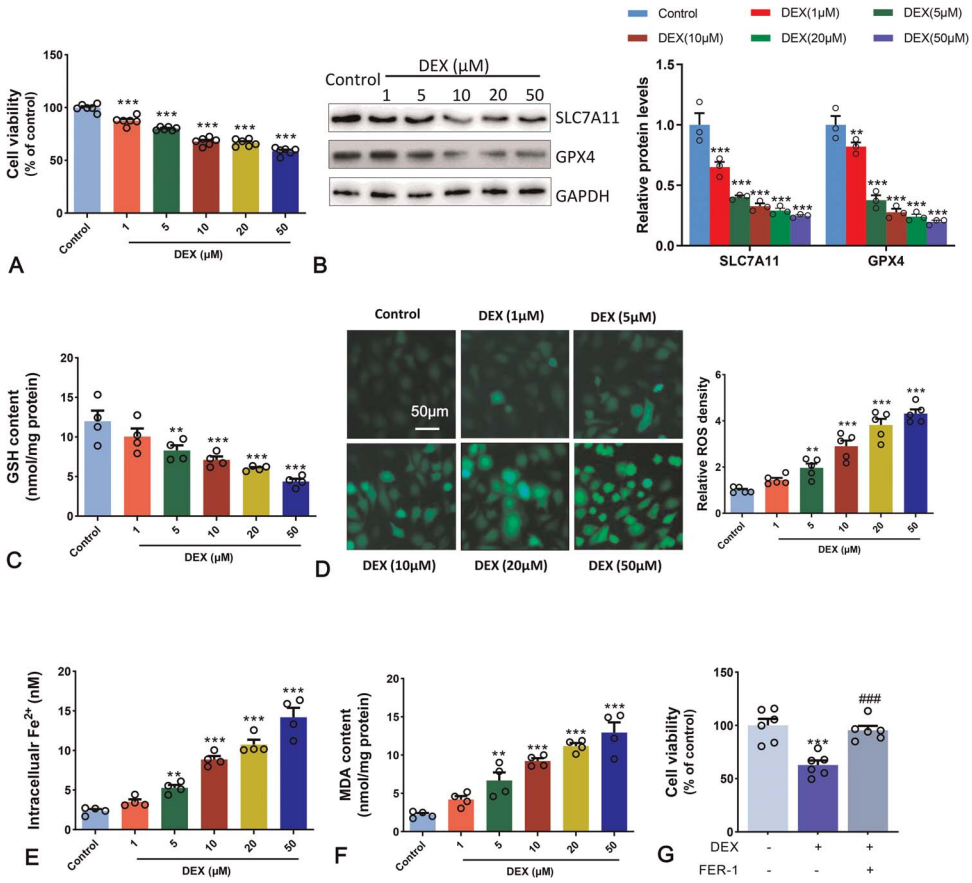

Figure 1. DEX induces ferroptosis in ECs. (A) Viability of HUVECs treated with DEX (1–50 μM, n = 6) was measured with CCK8 assay. (B) The expression of SLC7A11 and GPX4 (n = 3). (C) GSH content (n = 4). (D) Intracellular ROS level (n = 5). (E and F) Iron and MDA contents (n = 4). (G) Effect of FER-1 (1 μM) on the viability of DEX (10 μM)-treated HUVECs (n = 6). ** $P < 0.01$ and *** $P < 0.001$ vs. control group ### $P < 0.001$ vs. DEX group. DEX, dexamethasone; ECs, endothelial cells; GSH, glutathione; ROS, reactive oxygen species.

Subsequently, 100 μL of cell culture medium was mixed with 100 μL of the kit's reaction mixture and incubated at 20–25°C for 15 minutes in the dark. OD was measured at a wavelength of 488 nm.

Real-time polymerase chain reaction

Total RNA was extracted from the HUVECs using a Total RNA Extraction Kit. A UV spectrophotometer was used to measure total RNA concentration. cDNA was synthesized and amplified using 2 × Taq PCR Master Mix and SYBR Green. The primers used were as follows: NOX4 forward 5′-GCCAGAGTATCACTACCTCCAC-3′ and reverse 5′-CTCGGAGGTAAGCCAAGAGTGT-3′. GAPDH forward 5′-GTCTCCTCTGACTTCAACAGCG-3′, and reverse 5′-ACCACCCTGTTGCTGTAGCCAA-3′. GAPDH was used as an internal reference, and the relative mRNA expression level of NOX4 was calculated using the $2^{-\Delta\Delta}$ CT method and normalized to GAPDH.

Western blot

Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk and incubated sequentially with primary and secondary antibodies. The expression levels of specific proteins were normalized to that of GAPDH in the total cell lysate.

Immunofluorescence staining

HUVECs were treated with 4% PFA for 10 minutes, followed by 0.1% Triton X-100 in phosphate buffered saline (PBS) for 10 minutes at 4°C. After blocking with 1% BSA in PBS for 1 hour at 20–25°C, cells were incubated overnight at 4°C with the primary antibody. After 3 washes with PBS, cells were incubated for 5 minutes. Cells were then exposed to specific secondary antibodies for 1 hour and incubated with DAPI for 5 minutes. Finally, the cells were mounted and examined under a fluorescence microscope (Nikon ECLIPSE Ti2).

Measurements of ROS

Intracellular ROS were detected using the dichlorodihydrofluorescein diacetate (DCFH-DA) assay. HUVECs were treated with different stimuli and then incubated with 10 μmol/L DCFH-DA in serum-free medium for 30 minutes at 37°C in the dark, and after washing with PBS, the cells in the medium were observed under a fluorescence microscope. Image-Pro Plus software (version 6.0) was used to quantify the integrated optical density (IOD) of the images.

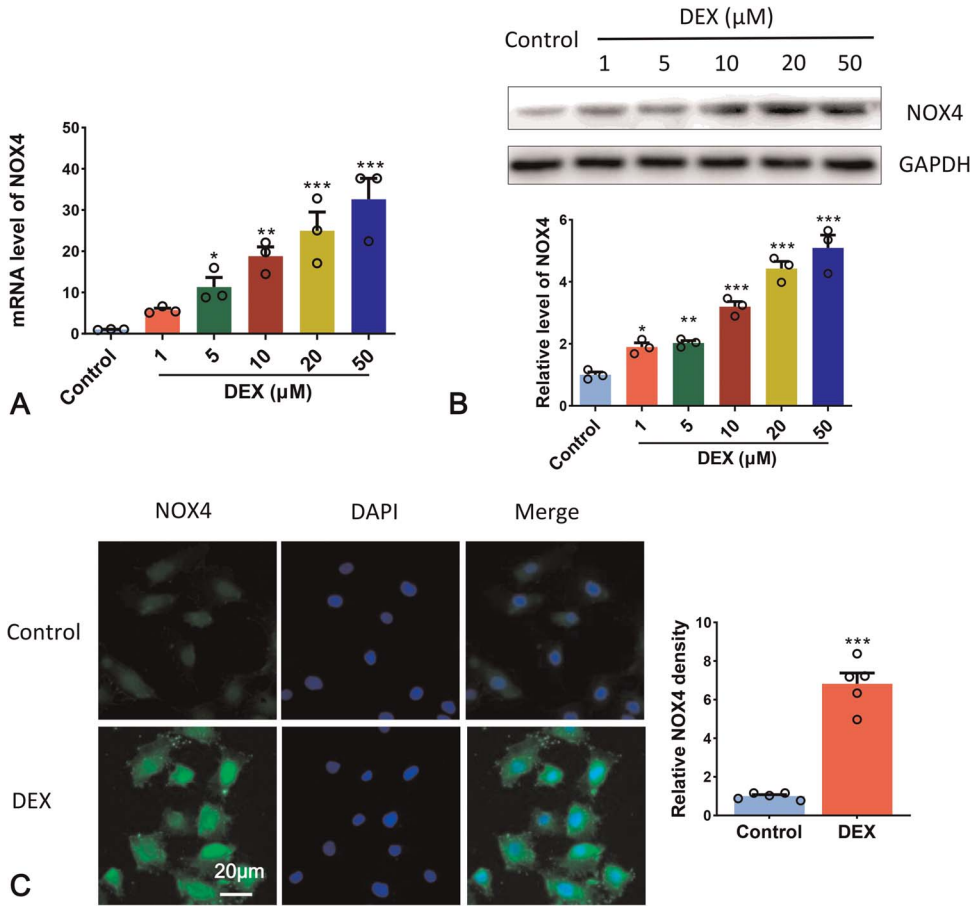

Figure 2. DEX upregulates NOX4 in ECs. (A) NOX4 mRNA levels of HUVECs treated with DEX (1–50 μM, n = 3). (B) NOX4 protein levels of HUVECs treated with DEX (1–50 μM, n = 3). (C) Determination of NOX4 levels in HUVECs treated with DEX (10 μM) using immunofluorescence staining (n = 5). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control group. DEX, dexamethasone; ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells.

Contents of Fe^{2+}, MDA, and GSH

Cells (1×10^6 per sample) were collected and lysed for the detection of Fe^{2+}, MDA, and GSH levels, following the manufacturer's instructions for each respective assay kit.

Transmission electron microscope

Fresh HUVECs were fixed in 2% glutaraldehyde in PBS for 1 hour at 20–25°C. The cells were then postfixed with 1% osmium tetroxide (OsO_4) in PBS for 1 hour, followed by embedding in Epon resin. The sections were analyzed using a TEM (JEM1200 EX2; JEOL Ltd.) to assess the mitochondria of the different groups.

Tube formation assay

Cold Matrigel (80 µL) was added to each well of a 96-well plate and incubated at 37°C for 30 minutes. HUVECs were seeded at a density of 5×10^4 cells per well and cultured in ECM supplemented with 2.5% FBS and 0.25% ECGS, with or without stimulation, for 6 hours. The formation of capillary-like structures was assessed by observing the polygonal patterns under a light microscope. Image analysis was performed using the Angiogenesis Analyze plug-in in the ImageJ software (NIH, Bethesda, MD, USA).

Wound-healing Assay

HUVECs were seeded in a 6-well plate and cultured with complete ECM. When the cells reached confluence, the monolayer was scratched using a sterile plastic tip to create parallel lines. After 2 washes with the culture medium, the medium was replaced with ECM containing 1% FBS and 0.1% ECGS, with or without stimulation. After 24 hours, the cells were examined under an inverted microscope, and images were captured.

Statistical analysis

Statistical analyses were conducted using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). The data are expressed as the mean ± standard deviation from a minimum of 3 independent experiments. Student's *t* test was used to compare the differences between 2 groups, whereas one-way ANOVA was used to compare multiple groups. A *P* value of less than 0.05 was considered statistically significant.

Results

DEX induces ferroptosis in endothelial cells

DEX is a commonly used, long-acting GC. In our study, HUVECs were treated with various concentrations of DEX (1–50 µM) and subjected to subsequent experiments. The CCK8 assay reflects cell viability, and MDA is a marker of lipid peroxidation. Our results showed that DEX decreased the viability of HUVECs in a concentration-dependent manner (Fig. 1A). DEX also reduced the protein expression levels of SLC7A11 and GPX4, decreased the GSH content, and increased the cellular ROS, iron (Fe^{2+}), and MDA (Fig. 1B–F). Thus, DEX causes significant ferroptosis in endothelial cells (ECs).

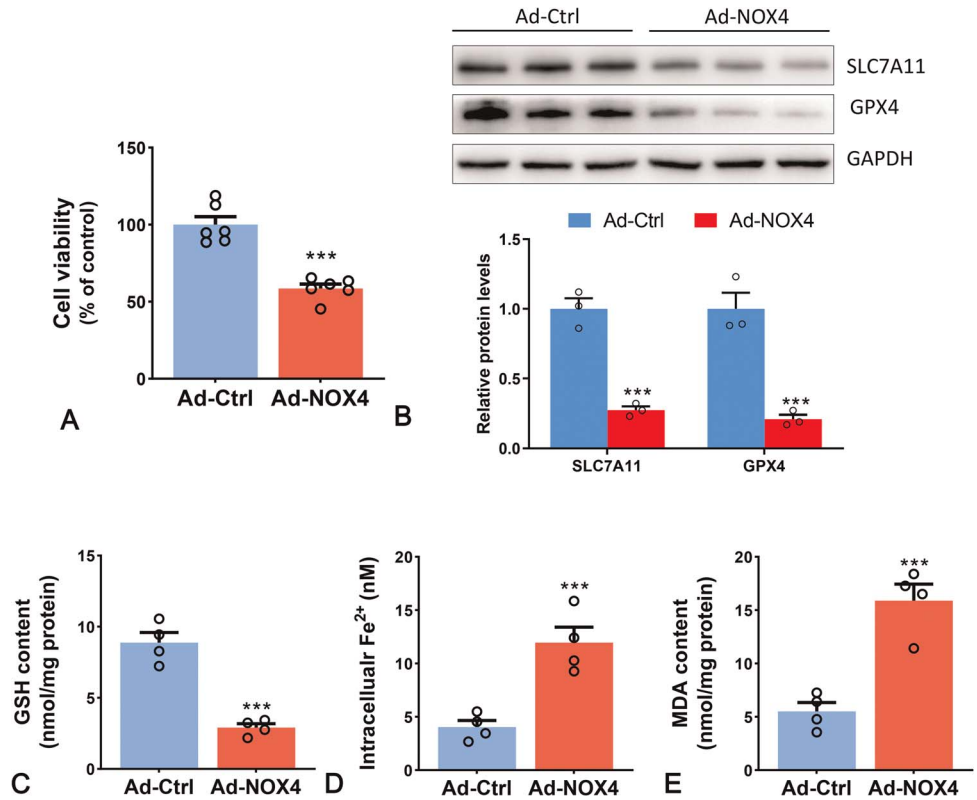

Figure 3. Over expression of NOX4 induces ferroptosis in ECs. (A) Viability of HUVECs treated with Ad-Ctrl or Ad-NOX4 (n = 6). (B) The expression of SLC7A11 and GPX4 (n = 3). (C–E) GSH, iron and MDA contents (n = 4). *** $P < 0.001$ vs. Ad-Ctrl group. ECs, endothelial cells; GSH, glutathione; HUVECs, human umbilical vein endothelial cells; MDA, malondialdehyde.

Ferrostatin-1 (FER-1) is a potent and selective ferroptosis inhibitor that prevents membrane lipid damage through a reducing mechanism [5]. Our data showed that FER-1 could inhibit DEX-induced EC damage (Fig. 1G). These results suggested that a high dose of DEX may induce EC damage by promoting ferroptosis.

DEX upregulates NOX4 in ECs

Given the established correlations among NOX4, ferroptosis, oxidative stress, and lipid peroxidation, the present study investigated the effects of DEX on NOX4 expression in HUVECs. RT-PCR results demonstrated that 5–50 μM DEX could upregulate NOX4 mRNA expression in ECs (Fig. 2A). Western blot analysis further showed that 1–50 μM DEX significantly increased the protein level of NOX4 in ECs (Fig. 2B). In addition, the cell immunofluorescence assay confirmed the upregulation of endothelial NOX4 by DEX (Fig. 2C).

Overexpression of NOX4 leads to ferroptosis in ECs

To the best of our knowledge, the modulation of ferroptosis by NOX4 in ECs has not been previously reported. Therefore, Ad-NOX4 was constructed to study the potential effect. The results demonstrated that NOX4 overexpression induced a decline in cell viability, protein expression levels of SLC7A11 and GPX4, and GSH content in HUVECs (Fig. 3A–C). Conversely, Ad-NOX4 increased iron and MDA levels (Fig. 3D and E). Collectively, these findings suggest that elevated NOX4 expression may contribute to ferroptosis in ECs.

NOX4 knockdown alleviates DEX-induced ferroptosis in ECs

Our results showed that knockdowning NOX4 with siRNA improved cell viability, SLC7A11 and GPX4 expressions, and GSH level, all of which were suppressed by DEX treatment (Fig. 4A–C). Furthermore, the suppression of NOX4 protected HUVECs from DEX-induced ROS generation, mitochondrial damage, iron accumulation, and lipid peroxidation (Fig. 4D–G).

Mitochondria function as initiators and amplifiers of ferroptosis.[9,10] In our study, the TEM results showed that DEX treatment caused swelling, breakage, and defects in the cristae of mitochondria in HUVECs (Fig. 4E). However, the inhibition of NOX4 prevented HUVECs from experiencing mitochondrial damage (Fig. 4E). Consequently, it has been suggested that NOX4 mediates DEX-induced ferroptosis.

Activation of ferroptosis eliminates the protective effects of NOX4 knockdown in ECs

To further clarify the role of NOX4-mediated ferroptosis in DEX-induced EC death and dysfunction, the ferroptosis agonist erastin was used in subsequent experiments. In this part of the study, the LDH release assay was used to assess cell death, and the angiogenic potential of HUVECs was quantified using a tube formation assay.

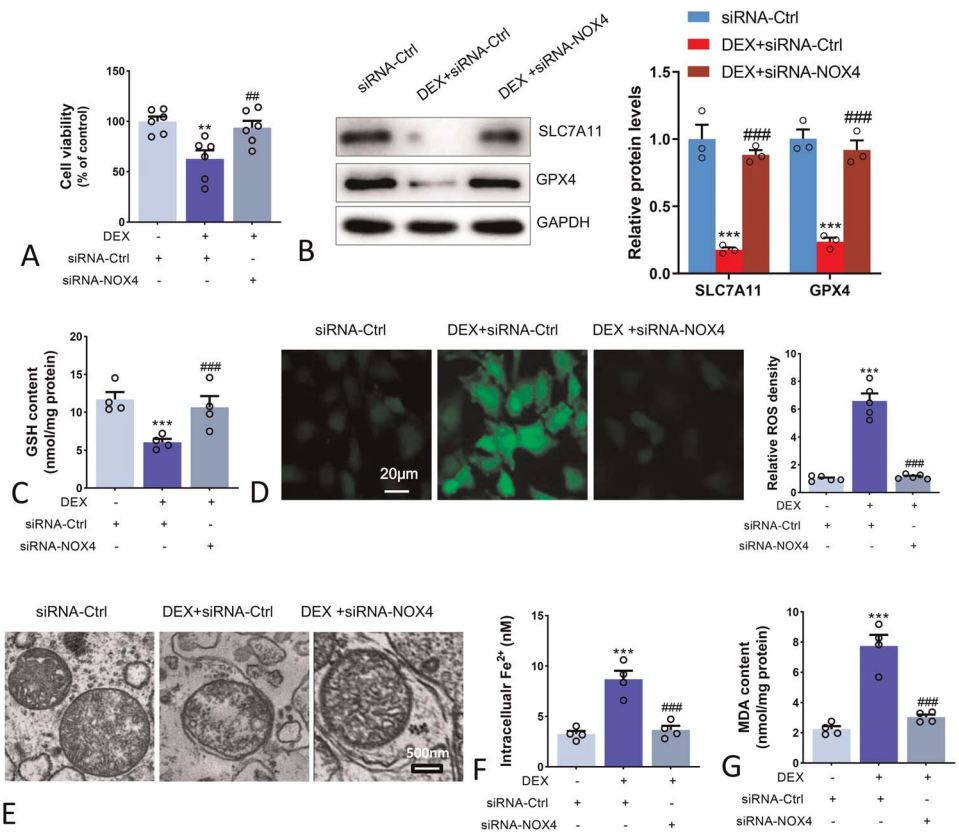

Figure 4. Knockdown of NOX4 alleviates DEX-induced ferroptosis in ECs. (A) Viability of HUVECs treated with DEX (10 μM), siRNA-Ctrl, or siRNA-NOX4 (n = 6). (C) The expression of SLC7A11 and GPX4 (n = 3). (C) GSH content (n = 4). (D) ROS assay (n = 5). (E) Mitochondrial structure was observed by TEM. Scale bar: 500 nm. (F and G) Iron and MDA contents (n = 4). ** $P < 0.01$, *** $P < 0.001$ vs. control group; ## $P < 0.01$, ### $P < 0.001$ vs. DEX group. DEX, dexamethasone; ECs, endothelial cells; GSH, glutathione; MDA, malondialdehyde; ROS, reactive oxygen species; TEM, transmission electron microscope.

A wound-healing assay was used to study cell migration and proliferation. The results showed that NOX4 knockdown inhibited DEX-induced cell death, defects of angiogenesis, and migration in HUVECs; however, reactivation of ferroptosis with erastin abolished the endothelial protective effects of NOX4 knockdown (Fig. 5). These results suggested that NOX4-mediated ferroptosis is involved in DEX-induced endothelial damage and dysfunction.

Discussion

GCs are among the most popular anti-inflammatory and immunomodulatory drugs and have been widely used in acute and critical illness for over 70 years.[4] In the last 30 years, significant progress has been made in elucidating the underlying molecular mechanisms.[4] In addition to their therapeutic effects, GCs have significant side effects on the vascular system. At the cellular level, high doses of GCs induce decreased viability, cell death, angiogenesis, and migration dysfunction in ECs.[11] The molecular mechanisms underlying GC-induced endothelial and vascular lesions are complex. Glucocorticoid receptor activity, ROS production, autophagy, and endoplasmic reticulum stress are involved in GC-induced endothelial and vascular dysfunction.[4,12]

Ferroptosis, a novel type of cell death characterized by excessive lipid peroxidation due to the downregulation of SLC7A11, GPX4, and system X c–.[5] Now, the role of ferroptosis in regulating vascular pathology has received increasing attention. Ferroptosis is involved in GC-induced osteonecrosis of the femoral head [13]. However, the role of ferroptosis in the DEX-induced endothelial injury remains unclear. Our findings demonstrate that DEX causes ferroptosis in HUVECs and that inhibiting ferroptosis with FER-1 can rescue the DEX-induced decline in cell viability. Thus, it is suggested that DEX may induce EC damage by promoting ferroptosis.

NOX4 induces ferroptosis by oxidative stress-induced lipid peroxidation via the impairment of mitochondrial metabolism [14,15]. In our study, DEX treatment increases the expression of NOX4 and overexpression of NOX4 leads to ferroptosis in HUVECs. Our results also demonstrate that NOX4 knockdown upregulates SLC7A11 and GPX4, suppresses oxidative stress, lipid peroxidation, and ferroptosis, thereby improving cell viability, angiogenesis, and migration capacity in DEX-treated ECs. However, reactivation of ferroptosis with erastin has been shown to abolish the protective effect of NOX4 knockdown in ECs. Evidently, suppression of NOX4 and ferroptosis may serve as therapeutic strategies to alleviate GC-induced vascular lesions.

Limitations

Nevertheless, this study has some limitations. Further *in vivo* experiments are needed to explore the implications of these effects and their underlying mechanisms.

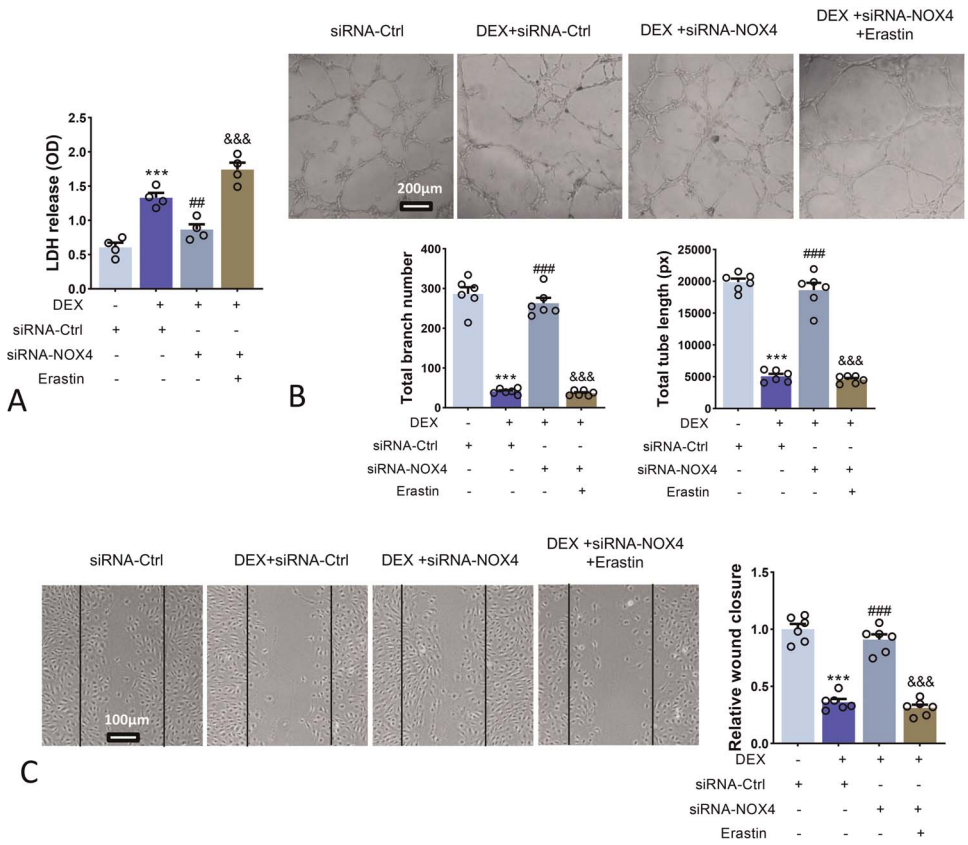

Figure 5. Effects of NOX4 knockdown and erastin on DEX-induced EC injury, angiogenesis and migration dysfunction. (A) HUVECs were treated with DEX, siRNA-Ctrl, siRNA-NOX4, or erastin (5 μM), and cell death was measured with LDH release assay (n = 4). (B) Angiogenesis was detected with tube formation assay (n = 6). (C) Cell migration was measured with wound healing assay, the lines indicate the former scratch area (n = 6). *** $P < 0.001$ vs. control group; ## $P < 0.01$ and ### $P < 0.001$ vs. DEX group; &&& $P < 0.001$ vs. DEX + siRNA-NOX4 group. DEX, dexamethasone; HUVECs, human umbilical vein endothelial cells; LDH, lactate dehydrogenase.

Conclusion

This study demonstrates that GC induces endothelial cell ferroptosis through NOX4-mediated ROS and lipid peroxidation, which in turn leads to endothelial cell death, angiogenesis, and migration dysfunction. These findings provide a new theoretical basis and intervention strategy for improving GC-induced vascular and endothelial damage.

Conflict of interest statement

The authors declare no conflict of interest.

Author contributions

Lyu L and Fang L participated in the research design and wrote the article. Fang L, Chen J, and Li W performed the experiments and data analysis.

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Ethical approval of studies and informed consent

Approval of all experiments was granted by the Ethics Committee of Qilu Hospital of Shandong University (NO. KYLL-202011-222).

Acknowledgments

None.

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