

Basic Study

Role of ferroptosis in the process of diabetes-induced endothelial dysfunction

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Abstract

BACKGROUND

Endothelial dysfunction, a hallmark of diabetes, is a critical and initiating contributor to the pathogenesis of diabetic cardiovascular complications. However, the underlying mechanisms are still not fully understood. Ferroptosis is a newly defined regulated cell death driven by cellular metabolism and iron-dependent lipid peroxidation. Although the involvement of ferroptosis in disease pathogenesis has been shown in cancers and degenerative diseases, the participation of ferroptosis in the pathogenesis of diabetic endothelial dysfunction remains unclear.

AIM

To examine the role of ferroptosis in diabetes-induced endothelial dysfunction and the underlying mechanisms.

METHODS

Human umbilical vein endothelial cells (HUVECs) were treated with high glucose (HG), interleukin-1 β (IL-1 β), and ferroptosis inhibitor, and then the cell viability, reactive oxygen species (ROS), and ferroptosis-related marker protein were tested. To further determine whether the p53-xCT (the substrate-specific subunit of system Xc⁻)-glutathione (GSH) axis is involved in HG and IL-1 β induced ferroptosis, HUVECs were transiently transfected with p53 small interfering ribonucleic acid or NC small interfering ribonucleic acid and then treated with HG and IL-1 β . Cell viability, ROS, and ferroptosis-related marker protein were then assessed. In addition, we detected the xCT and p53 expression in the aorta of

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db/db mice.

RESULTS

It was found that HG and IL-1 β induced ferroptosis in HUVECs, as evidenced by the protective effect of the ferroptosis inhibitors, Deferoxamine and ferrostatin-1, resulting in increased lipid ROS and decreased cell viability. Mechanistically, activation of the p53-xCT-GSH axis induced by HG and IL-1 β enhanced ferroptosis in HUVECs. In addition, a decrease in xCT and the presence of de-endothelialized areas were observed in the aortic endothelium of db/db mice.

CONCLUSION

Ferroptosis is involved in endothelial dysfunction and p53-xCT-GSH axis activation plays a crucial role in endothelial cell ferroptosis and endothelial dysfunction.

Key Words: Diabetes mellitus; Endothelial dysfunction; Ferroptosis; Reactive oxygen species; p53; Glutathione

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Core Tip: Endothelial dysfunction is a critical and initiating contributor to the pathogenesis of diabetic cardiovascular complications. Ferroptosis is characterized by the accumulation of iron-induced lipid reactive oxygen species and depletion of plasma membrane unsaturated fatty acids. Our findings demonstrated that ferroptosis is involved in endothelial dysfunction and the p53-xCT (the substrate-specific subunit of system Xc⁻)-glutathione axis activation plays a crucial role in endothelial cell ferroptosis and endothelial dysfunction. These results provide important insights as inhibiting activation of the p53-xCT-glutathione axis and ferroptosis could attenuate diabetes-induced endothelial dysfunction and may be a novel strategy for the treatment of vascular complications in diabetes mellitus.

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INTRODUCTION

Diabetes mellitus is a chronic, metabolic disorder characterized by insulin resistance and impaired glucose homeostasis^[1]. A growing number of diabetic patients suffer from cardiovascular complications which can cause mortality and morbidity. Evidence suggests that diabetes-induced endothelial dysfunction is a critical and initiating factor in the genesis of diabetic macrovascular and microvascular complications^[2], although the underlying mechanisms are still not fully understood. Extensive research has shown that endothelial dysfunction in cardiovascular complications is primarily characterized by enhanced oxidative stress, decreased release of nitric oxide (NO), increased production of inflammatory factors, impaired endothelial repair, and abnormal angiogenesis^[3].

Cell death is critical in diverse aspects of mammalian development, homeostasis, and disease, and plays a vital role in diabetes-induced endothelial dysfunction. Our previous studies demonstrated that activation of NF κ B signaling induced by high glucose (HG) and interleukin-1 β (IL-1 β) caused endothelial cell apoptosis and endothelial dysfunction^[4]. Whether and how other forms of regulated cell death (RCD) are involved in diabetic endothelial dysfunction remains less clear. Ferroptosis, a recently defined, non-apoptotic, RCD process with the hallmark of the accumulation of iron-dependent lipid peroxides was named by Dixon *et al*^[5] in 2012. Ferroptosis may occur in various physiological and pathological processes in humans and animals^[6]. For example, excessive ferroptosis has been linked to neurodegeneration, kidney

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failure, liver fibrosis, and ischemia-reperfusion injury, whereas insufficient ferroptosis can lead to cancer^[7-9]. In addition, Alzheimer's disease, ischemic and hemorrhagic stroke, Huntington disease, and Parkinson's disease are among the candidate neurodegenerative diseases that may involve ferroptosis^[10]. Recent evidence suggests that ferroptosis plays a pathophysiological role in cardiomyopathy^[11]. Bai *et al.*^[12] indicated that ferroptosis might occur during the initiation and development of atherosclerosis (AS). Inhibition of ferroptosis could protect against the aggravation of AS in thoracic aorta through attenuating lipid peroxidation and endothelial dysfunction. These studies also raise intriguing questions of whether ferroptosis is involved in diabetes-induced endothelial dysfunction. Ferroptosis is characterized by the accumulation of iron-induced lipid reactive oxygen species (ROS) and depletion of plasma membrane unsaturated fatty acids^[13]. Also enhanced oxidative stress and ROS, which inactivate NO, is a dominant feature of diabetes-induced endothelial dysfunction^[14]. However, very little is known about the role of ferroptosis in diabetes-induced endothelial dysfunction. Therefore, this study aimed to investigate the role and regulatory mechanism of ferroptosis in diabetes-induced endothelial dysfunction.

In this study, we demonstrated that HG and IL-1 β induce human umbilical vein endothelial cells (HUVECs) ferroptosis and ferroptosis inhibitor attenuates the ferroptosis of HUVECs induced by HG and IL-1 β . Furthermore, our findings showed that activation of the p53-xCT (the substrate-specific subunit of system Xc⁻)-glutathione (GSH) axis plays a vital role in HG and IL-1 β induced ferroptosis. We also observed iron accumulation, down-regulation of xCT, and the presence of de-endothelialized areas in the aorta of db/db mice. These findings support our hypothesis that activation of the p53-xCT-GSH axis and increased ferroptosis have a pivotal role in diabetes-associated endothelial dysfunction.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice and db/db mice (C57BL/6J-Lepr^{db}/Lepr^{db}) were obtained from Southeast University Animal Center. 10-12 wk old mice were employed. All experiments involving animals were performed under the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (DHWE publication No. 96-01, revised in 2002) and were approved by the Care of Experimental Animals Committee of Southeast University.

Cell culture

Primary HUVECs were purchased from Promocell (C-12200, Heidelberg, Germany) and grown in endothelial culture medium (1001, ScienCell, United States) containing 1% endothelial cell growth supplement (1052, ScienCell), 5% fetal bovine serum (0025, ScienCell) and 1% penicillin/streptomycin solution (0503, ScienCell) at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. All of the experiments were performed using 2-5 passages of HUVECs. HUVECs characterization is shown in [Supplementary Figure 1](#).

Reagents

Recombinant human IL-1 β was purchased from Thermo Scientific (PHC0814, Waltham, MA, United States). The Cell Counting Kit-8 (CCK-8) assay kit was purchased from Dojindo (Kumamoto, Japan). Deferoxamine (DFO) and ferrostatin-1 (Fer-1) were purchased from Med Chem Express (HY-B0988 and HY-100579, Shanghai, China). Necrostatin-1s (Nec-1s) was purchased from BioVision (2263, Milpitas, United States) and Z-VAD-FMK was purchased from ApexBio (A1902, Houston, United States). The following antibodies were used: anti-glutathione peroxidase 4 [(GPX4) (ab125066, Abcam, Cambridge, MA, United States)], anti-FTH1 (ab75972, Abcam, Cambridge, MA, United States), anti-cyclooxygenase (COX)-2 (ab179800, Abcam, Cambridge, MA, United States), anti-p53 (ab1431, Abcam, Cambridge, MA, United States), anti-xCT (ab37185, Abcam, Cambridge, MA, United States), anti-CD31 (ab28364, Abcam, Cambridge, MA, United States) and anti- β -actin (ab8226, Abcam, Cambridge, MA, United States).

Cell viability

Cell viability was typically assessed in 96-well format by CCK-8. CCK-8 solution (10 μ L) was added to HUVECs at various times, and the cells were incubated for 1-4 h at

37°C. The absorbance at 450 nm was measured on a microplate reader (SYNERGY/H4, BioTek, United States).

ROS level measurement

Approximately 1×10^6 cells/well were seeded in 6-well plates overnight followed by treatment for 48 h. Lipid peroxidation was determined by the BODIPYTM 581/591 C11 method (D3861, Invitrogen, United States). The cellular fluorescence was immediately detected using an Accuri C6 flow cytometer (BD Biosciences, United States). A total of 1×10^4 events were acquired for each sample from three independent experiments. Data were analyzed using FlowJo software (ver.10.0, FlowJo LLC, United States).

Small interfering ribonucleic acid transfection in HUVECs

HUVECs were transfected with small interfering ribonucleic acid (siRNA) using Lipofectamine RNA interfere MAX transfection reagent (Thermo Scientific, United States) according to the manufacturer's instructions. siRNA specific for p53 was purchased from Santa Cruz (sc-29436, United States). Control siRNA of p53 was purchased from Santa Cruz (sc-37007, United States).

Real-time quantitative PCR

Total RNA isolation was carried out with the RNAiso plus kit (9109, TaKaRa, Shiga, Japan) according to the manufacturer's instructions. RNA concentration and purity were confirmed with a Nanodrop 2000 (Thermo Scientific, United States). First-strand complementary deoxyribonucleic acid synthesis was then obtained using the PrimeScriptTM RT Reagent Kit with gDNA Eraser (RR047A, TaKaRa, Shiga, Japan). The quantitative real-time reverse transcription-polymerase chain reaction analysis was performed with the TB GreenTM Premix Ex TaqTM II (RR820A, TaKaRa, Shiga, Japan) and Prism 7500 sodium dodecyl sulfate (Applied Biosystems; Thermo Scientific, MA, United States). The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. β -actin was used as the internal control to normalize the data. The sequences of quantitative real-time reverse transcription-polymerase chain reaction primers are presented in [Table 1](#).

Western blot analysis

Total cellular protein was extracted with the Tissue or Cell Total Protein Extraction Kit (C510003, Sangon Biotech, China) to evaluate the levels of GPX4, FTH1, COX-2, p53, and xCT. A BCA kit (KeyGEN, China) was used to determine the protein concentrations according to the manufacturer's instructions. Equal amounts of proteins were electrophoresed in sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were incubated in a blocking solution containing 5% non-fat milk at 37°C for 1 h. The blots were incubated overnight with primary antibodies against GPX4 (ab125066, Abcam), FTH1 (ab75972, Abcam), COX-2 (ab179800, Abcam), p53 (ab1431, Abcam) and xCT (ab37185, Abcam) followed by horseradish peroxidase-labelled secondary immunoglobulin G (sc-516132, Santa Cruz Biotechnology). Signals were detected using an advanced ECL system (GE Healthcare, United Kingdom). β -actin (ab8226, Abcam) was used as an internal control.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described^[4]. HUVECs grown on coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton-X100. After blocking with 10% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature, the coverslips were incubated with primary antibodies against CD31 (ab28364, Abcam) and xCT (ab37185, Abcam) at 4°C overnight. The coverslips were then incubated with secondary antibodies for 1 h at room temperature in the dark, the images were captured using a laser scanning confocal microscope (LSM 510 META, Zeiss, Germany).

Paraffin tissue sections from mice aorta were deparaffinized and rehydrated. After pre-treatment with pepsin for 30 min (1% pepsin in 0.1% HCl) for antigen retrieval, the sections were incubated overnight at 4°C with primary CD31 and xCT antibodies diluted in Tris-buffered saline containing 1% bull serum albumin and 0.1% Tween 20. After pre-treatment, the antigen retrieval sections were washed in Tris-buffered saline and incubated with secondary antibodies. The images were captured using a laser scanning confocal microscope.

Table 1 Sequences of the reverse transcription-polymerase chain reaction primers

Sequences of the reverse transcription-polymerase chain reaction primers	
p53 (human)	Forward: 5'- CAGCACATGACGGAGGTTGT - 3'; Reverse: 5'- TACTCCAAATACTCCACACGC - 3'
xCT (human)	Forward: 5'- TCTCCAAAGGAGGTTACTCTGC - 3'; Reverse: 5'- AGACTCCCCTCAGTAAAGTGAC - 3'
β -actin (human)	Forward: 5'- GGCACCCAGCACAATGAAG - 3'; Reverse: 5'- CCGATCCACACGGAGTACTTG - 3'
p53 (mouse)	Forward: 5'-TGGAAGGAAATTTGTATCCGA- 3'; Reverse: 5'-GTGGATGGTGGTATACTCAGAG- 3'
xCT (mouse)	Forward: 5'-GCTGTTTTATCATCACGGGTTT - 3'; Reverse: 5'- AATCCCAAACATCATGCATAAGC- 3'
β -actin (mouse)	Forward: 5'-GTGACGTTGACATCCGTAAGA- 3'; Reverse: 5'-GCCGGACTCATCGTACTCC- 3'

Statistical analysis

The data are shown as the mean \pm SD taken from at least three independent experiments. Analyses were performed using Graphpad Prism 5 Software (Graphpad Software Inc., United States). Significance levels were determined by Student's *t*-test and one-way analysis of variance (ANOVA). Significance was defined as $P < 0.05$.

RESULTS

HG and IL-1 β induces HUVECs ferroptosis

As shown in **Figure 1A**, HG (30 mmol/L) and IL-1 β (10 ng/mL) significantly affected cell viability in HUVECs. To elucidate the involvement of ferroptosis in HG and IL-1 β -mediated cell death, the expression of corresponding proteins was detected by Western blot. HG and IL-1 β induced a significant reduction in GPX4 and an increase in FTH1 and COX-2 (**Figure 1B**). Lipid peroxidation is an essential process for conducting ferroptosis. **Figure 1C** shows a marked right-shift of intracellular ROS release after treatment with HG and IL-1 β for 48 h.

Ferroptosis inhibitor attenuates ferroptosis of HUVECs induced by HG and IL-1 β

To further determine whether ferroptosis inhibitor attenuates HG and IL-1 β -induced ferroptosis, HUVECs were treated with NG, HG (30 mmol/L) and IL-1 β (10 ng/mL), and ferroptosis inhibitor, DFO (100 μ mol/L) or Fer-1 (10 μ mol/L), the necroptosis inhibitor necrostatin-1s (Nec-1s, 2 μ mol/L), and the apoptosis inhibitor Z-VAD-FMK (Z-VAD, 5 μ mol/L). As shown in **Figure 2A**, DFO, Fer-1, Nec-1s, and Z-VAD partially rescued HG and IL-1 β -mediated cell death. It was demonstrated that in addition to necrosis and apoptosis, ferroptosis may play an important role in cell death induced by hyperglycemia and inflammation. Representative phase-contrast microscopy images of cell death rescued by DFO and Fer-1 are shown in **Figure 2B**. In addition, DFO and Fer-1 reversed the decrease in GPX4 and the increase in FTH1 and COX-2 induced by HG and IL-1 β (**Figure 2C**). As shown in **Figure 2D**, after treatment with DFO and Fer-1, the level of ROS in HUVECs reduced significantly.

HG and IL-1 β induce ferroptosis by activating the p53-xCT-GSH axis in HUVECs

GPX4 uses GSH to protect cells from ferroptosis by eliminating phospholipid peroxides^[15]. GSH is one of the major cellular non-protein antioxidants, and is a tripeptide anti-oxidant consisting of glutamate, glycine and cysteine^[16]. Jiang *et al*^[17] found that ferroptosis has p53-mediated activity during tumor suppression and p53 sensitizes cells to ferroptosis by repressing expression of xCT (SLC7A11), a key component of the cystine/glutamate antiporter, and inhibiting cystine uptake. Previous research has established that hyperglycemia promotes the activation of p53^[18,19]. To further determine whether the p53-xCT-GSH axis is involved in HG and IL-1 β -induced ferroptosis, HUVECs were transiently transfected with p53 siRNA or NC siRNA and then treated with NG, HG (30 mmol/L) and IL-1 β (10 ng/mL). As shown in **Figure 3**, HG and IL-1 β induced a significant increase in p53 and a reduction in xCT and GSH in HUVECs. p53 siRNA can attenuate the decrease in xCT and GSH induced by HG and IL-1 β . As presented in **Figure 4A**, p53 siRNA significantly rescued HG and IL-1 β -mediated cell death. **Figure 4B** shows that p53 siRNA attenuated the decrease in GPX4 and the increase in FTH1 and COX-2 induced by HG and IL-1 β . As shown in **Figure 4C**, p53 siRNA attenuated the increase in ROS induced by HG and IL-1 β .

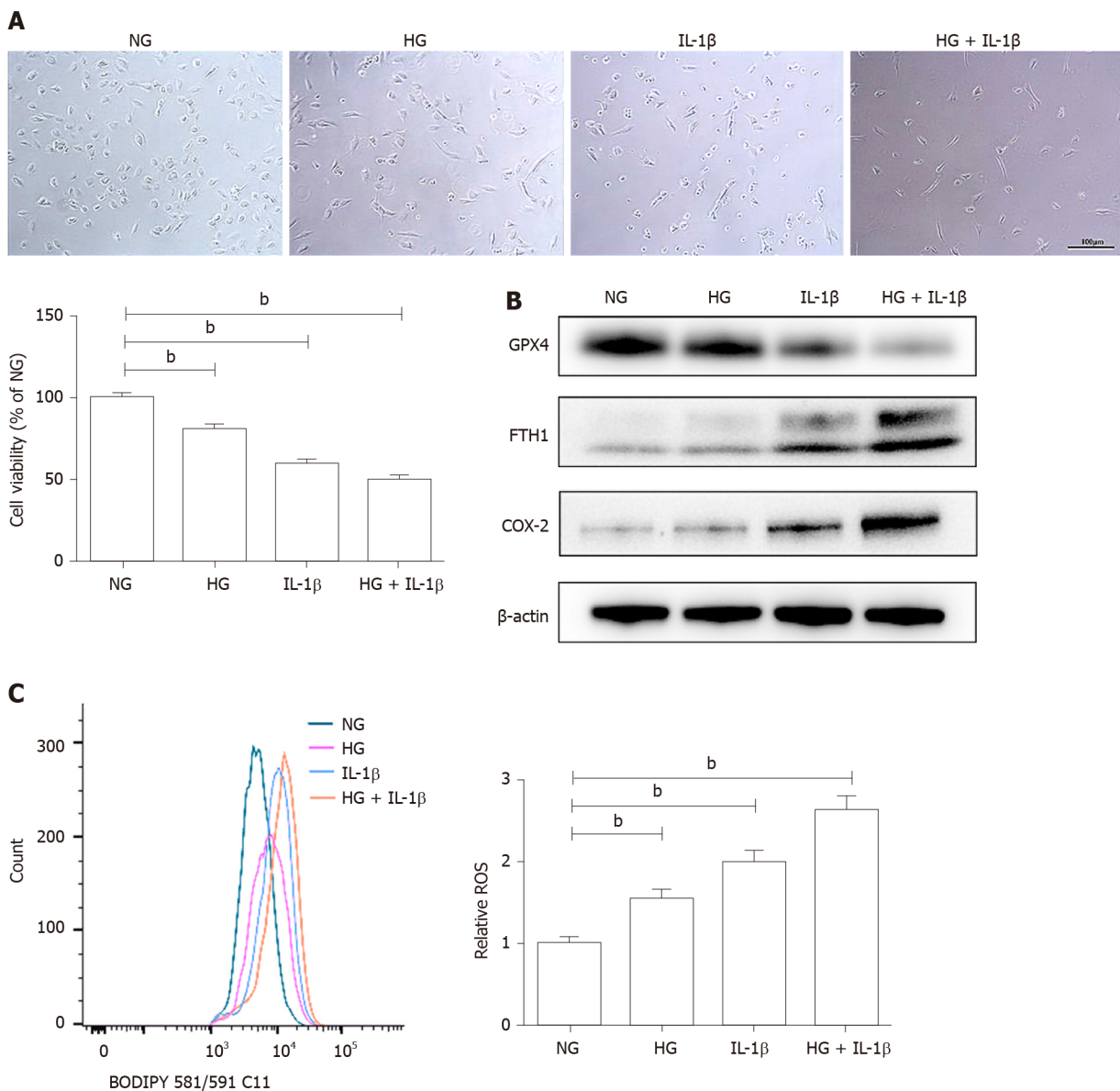


Figure 1 Effects of high glucose and interleukin-1 β on ferroptosis in human umbilical vein endothelial cells. A: Human umbilical vein endothelial cells were treated with normal glucose (5 mmol/L) or high glucose (30 mmol/L) and interleukin-1 β (10 ng/mL). After treatment for 48 h, representative phase-contrast microscopy images of human umbilical vein endothelial cells were obtained and cell viability was determined using Cell Counting Kit-8. Scale bar: 100 μ m; B: The protein levels of glutathione peroxidase 4, FTH1, and cyclooxygenase-2 were determined using Western blot assays. β -actin was used for normalization; C: The generation of reactive oxygen species was determined by the BODIPY[™] 581/591 C11. To maintain constant isotonicity or osmolality, normal glucose (5 mmol/L) medium was supplemented with D-mannitol (25 mmol/L, final concentration) in the current study. ^a $P < 0.05$ vs the control group. ^b $P < 0.01$ vs the control group.

Detection of xCT and p53 expression in the aorta of db/db mice

The localization of CD31, a biomarker of endothelial cells, and xCT were detected by immunofluorescence. **Figure 5A** shows the decrease in xCT and the presence of de-endothelialized areas in the aortic endothelium of db/db mice. As presented in **Figure 5B** and **C**, p53 mRNA was up-regulated, and xCT mRNA was down-regulated in the aorta of db/db mice.

DISCUSSION

Previous research has established that endothelial dysfunction is among the most important factors for vascular complications in diabetic patients^[20]. The endothelium secretes various factors, regulating blood vessel development, cell adhesion, inflammation, metabolism, angiogenesis, vascular permeability, and vessel integrity^[21]. Endothelial dysfunction is an imbalance in the production of vaso-

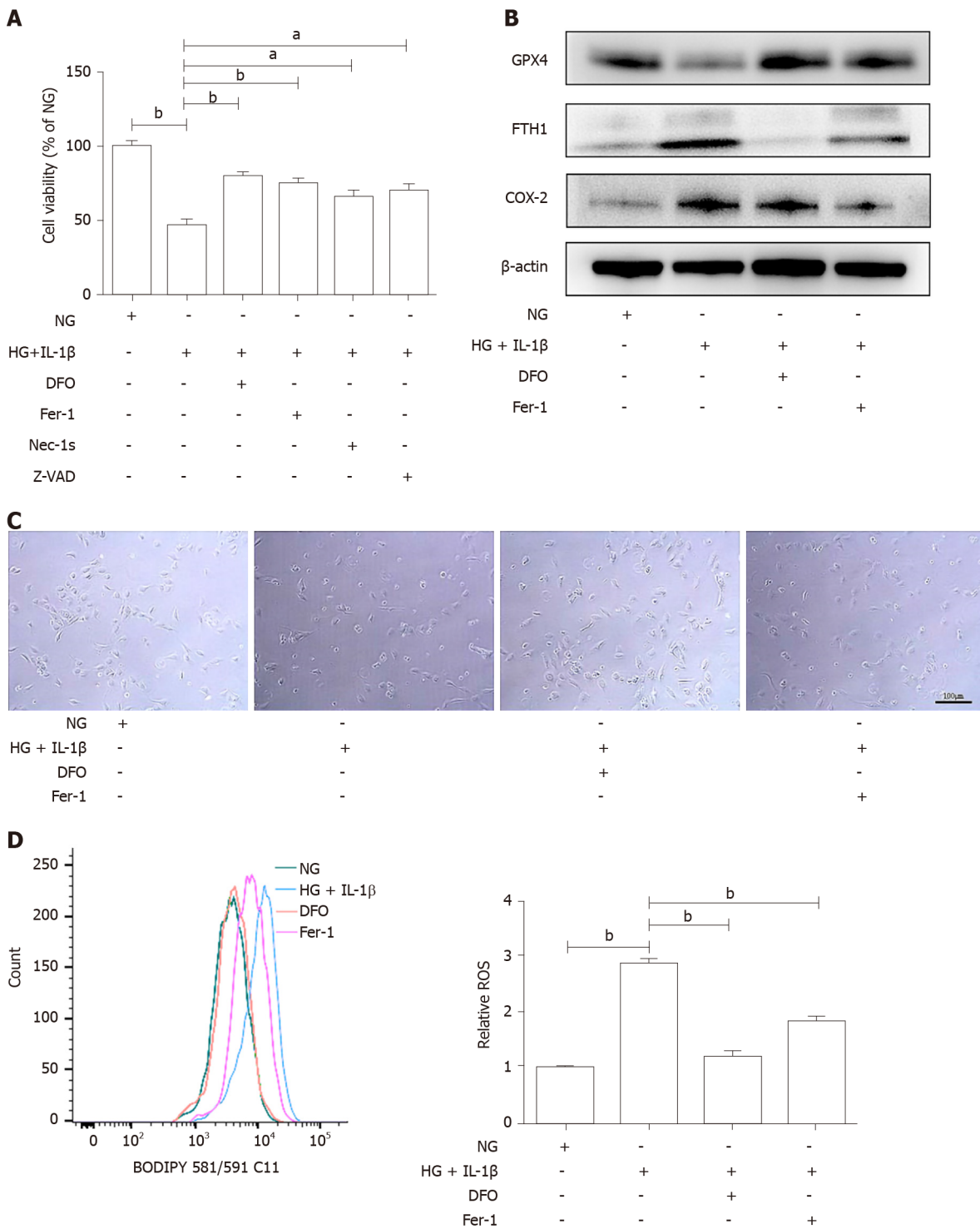


Figure 2 Ferroptosis inhibitor attenuates the ferroptosis of human umbilical vein endothelial cells induced by high glucose and interleukin-1 β . A: Human umbilical vein endothelial cells (HUVECs) were treated with NG, high glucose (30 mmol/L) and interleukin-1 β (10 ng/mL), the ferroptosis inhibitor, Deferoxamine (100 μ mol/L) or Ferrostatin-1 (10 μ mol/L), the necroptosis inhibitor necrostatin-1s (2 μ mol/L), and the apoptosis inhibitor Z-VAD-FMK (Z-VAD, 5 μ mol/L). After treatment for 48 h, cell viability was determined using Cell Counting Kit-8; B: HUVECs were treated with NG, high glucose (30 mmol/L) and interleukin-1 β (10 ng/mL), Deferoxamine (100 μ mol/L), and ferrostatin-1 (10 μ mol/L), after treatment for 48 h, representative phase-contrast microscopy images of HUVECs were obtained. Scale bar: 100 μ m; C: The protein levels of glutathione peroxidase 4, FTH1, and cyclooxygenase-2 were determined using Western blot assays. β -actin was used for normalization; and D: The generation of reactive oxygen species was determined by the BODIPY™ 581/591 C11. ^a $P < 0.05$ vs the control group. ^b $P < 0.01$ vs the control group.

constricting factors, *e.g.*, prostaglandin, angiotensin II, endothelin-1, and vasodilator factors, *e.g.*, prostacyclin, NO, and endothelial-derived hyperpolarizing factor. Disruption of this balance results in vasoconstriction, vascular inflammation, leukocyte adherence, platelet activation, impaired coagulation, thrombosis, and AS^[22].

Existing research recognizes the critical role played by hyperglycemia-induced oxidative stress and increased production of ROS in the development of endothelial dysfunction^[23]. Ferroptosis, a newly defined, iron-dependent form of the RCD process that is induced by excessive lipid peroxidation, is morphologically and mechanistically distinct from apoptosis^[5,7,24]. And the current study found that HG and IL-1 β induced ferroptosis in HUVECs, as evidenced by the protective effect of DFO and Fer-1, resulting in an increase in lipid ROS and a decrease in cell viability. In addition, activation of the p53-xCT-GSH axis enhanced ferroptosis in HUVECs. Interestingly, p53 siRNA attenuated the decrease in xCT and GSH, the increase in ROS and ferroptosis induced by HG and IL-1 β . Our findings were in accordance with the study by Yokoyama *et al*^[25], which showed that, in the diabetic state, endothelial p53 expression was markedly up-regulated and endothelium-dependent vasodilatation was significantly impaired. Wu *et al*^[18] verified the relationship between HG-induced p53 signaling activation and suppression of antioxidant systems in the vascular endothelium, which was consistent with our findings. Moreover, Ortega-Camarillo *et al*^[26] reported that p53 is part of a death signaling pathway in rat insulin-producing cells under hyperglycemic conditions. Gu *et al*^[27] indicated that the inhibition of p53 attenuated diabetes-induced cell senescence, and improved both glycolytic and angiogenic defects. These results showed that endothelial p53 plays a significant role in the development of diabetic vascular complications and inhibition of endothelial p53 could be a novel therapeutic target in diabetics. xCT, a key component of the cystine-glutamate antiporter, is important for the exchange of glutamate and cystine at a ratio of 1:1 across the plasma membrane^[28]. Our findings showed that, in diabetes, p53 could transcriptionally suppress xCT and the downregulation of xCT inhibited cystine uptake and reduced GSH synthesis. These results were also supported by Jiang, who found that retinal cells treated with HG for four days showed a decrease in xCT. Furthermore, a decrease of xCT in the retina of diabetic rats was also found^[29]. Our results were further supported by recent studies which indicated that xCT expression and glutathione levels were reduced whereas ROS were increased in diabetic rats^[30]. Lutchmansingh *et al*^[31] showed that patients with type 2 diabetes have GSH deficiency, especially if they have microvascular complications.

In short, the results of this study indicated that the p53-xCT-GSH axis induced by HG and IL-1 β can repress the expression of xCT and inhibit cystine uptake, ultimately causing increased endothelial cell ferroptosis and endothelial dysfunction. From the perspective of ferroptosis and GSH synthesis disorders, we describe a new mechanism of endothelial vascular injury in diabetes.

CONCLUSION

As illustrated in **Figure 6**, in diabetes, p53 signaling is activated, and p53 can transcriptionally suppress xCT, in endothelial cells. The downregulation of xCT inhibits cystine uptake and reduces glutathione synthesis, which is involved in triggering ferroptosis in endothelial cells and ultimately leads to endothelial dysfunction. In conclusion, the current study demonstrated that ferroptosis is involved in endothelial dysfunction for the first time, and p53-xCT-GSH axis activation plays a crucial role in endothelial cell ferroptosis and endothelial dysfunction. These facts support the hypothesis that inhibiting activation of the p53-xCT-GSH axis and ferroptosis could attenuate diabetes-induced endothelial dysfunction.

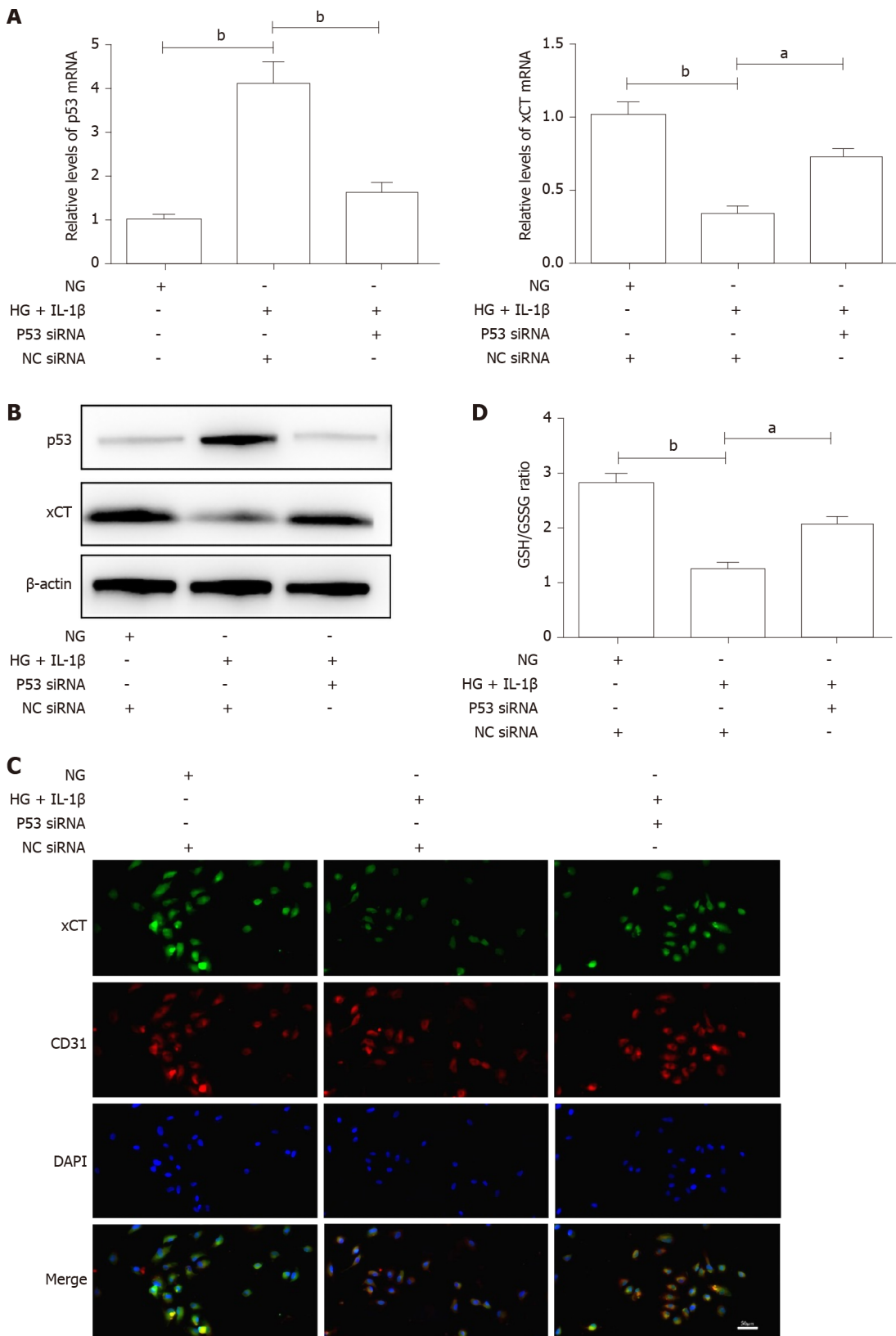


Figure 3 High glucose and interleukin-1 β activate the p53-xCT (the substrate-specific subunit of system Xc⁻)-glutathione axis in human umbilical vein endothelial cells. A: Human umbilical vein endothelial cells were transiently transfected with p53 small interfering ribonucleic acid or NC small interfering ribonucleic acid and then treated with NG, high glucose (30 mmol/L) and interleukin-1 β (10 ng/mL). After treatment for 48 h, the messenger ribonucleic acid levels of p53 and xCT in human umbilical vein endothelial cells with different treatments were determined using real-time polymerase chain reaction analyses. β -actin was used for normalization; B: The protein levels of p53 and xCT were determined using Western blot assays. β -actin was used for normalization; C: The localization of xCT was detected by immunofluorescence. xCT is labeled green, and CD31 is labeled red. The nucleus is labeled blue. Scale bar: 50 μ m; and D: The intracellular glutathione/oxidized glutathione (glutathione/oxidized glutathione) ratio was measured with the glutathione and oxidized glutathione assay Kit. ^a $P < 0.05$ vs the control group. ^b $P < 0.01$ vs the control group.

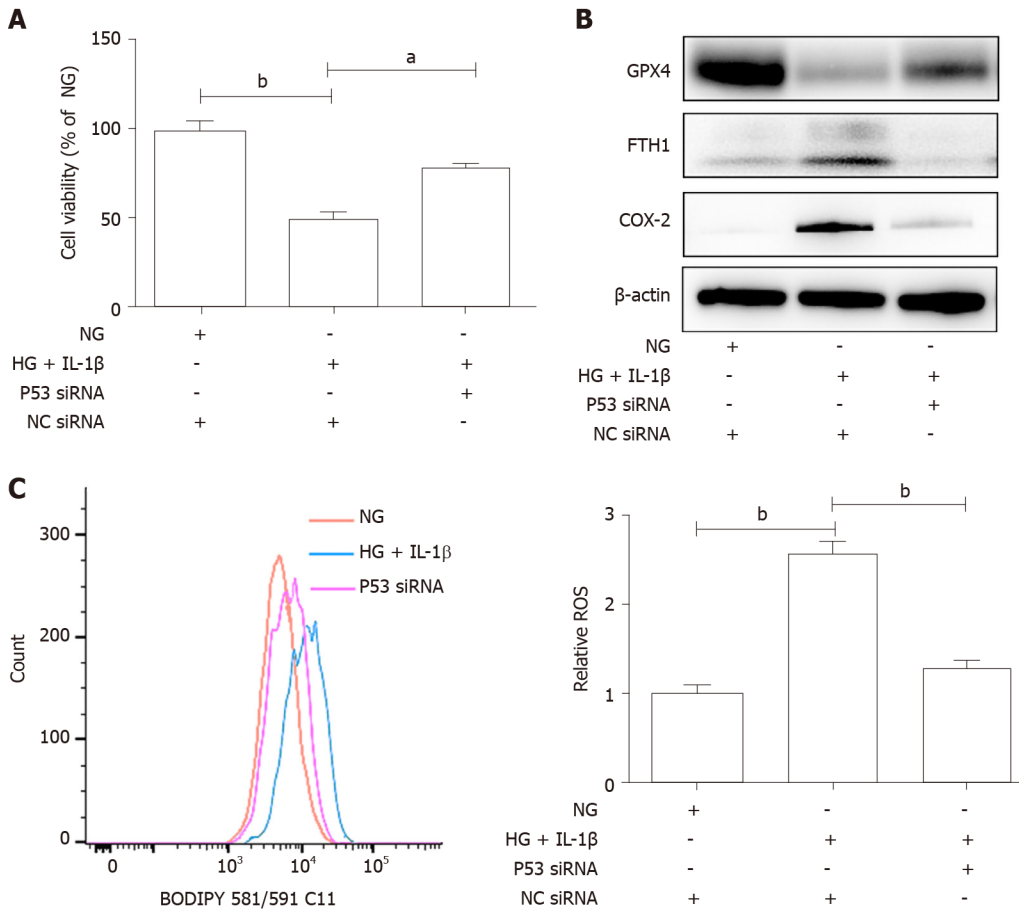


Figure 4 The p53-xCT (the substrate-specific subunit of system Xc-) glutathione axis functions as a pivotal master in high glucose and interleukin-1 β -induced ferroptosis in human umbilical vein endothelial cells. A: Human umbilical vein endothelial cells were transiently transfected with p53 small interfering ribonucleic acid or NC small interfering ribonucleic acid and then treated with NG, high glucose (30 mmol/L) and interleukin-1 β (10 ng/mL). After treatment for 48 h, representative phase-contrast microscopy images of human umbilical vein endothelial cells were obtained and cell viability was determined using Cell Counting Kit-8; B: The protein levels of glutathione peroxidase 4, FTH1, and cyclooxygenase-2 were determined using Western blot assays. β -actin was used for normalization; and C: The generation of reactive oxygen species was determined by the BODIPY™ 581/591 C11. ^a $P < 0.05$ vs the control group. ^b $P < 0.01$ vs the control group.

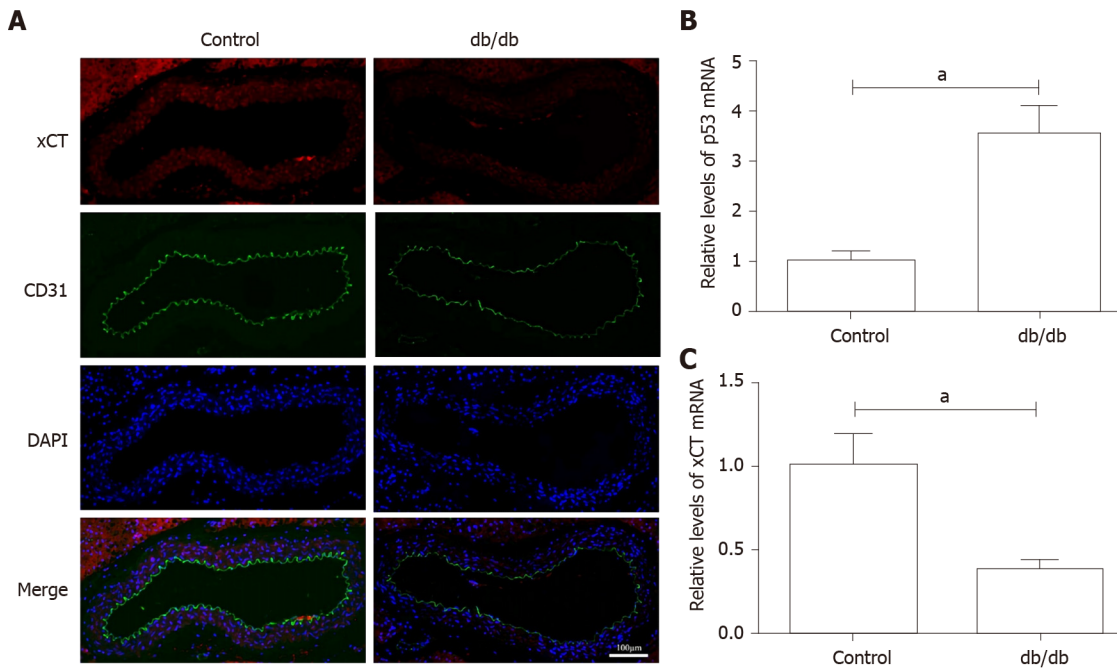


Figure 5 Detection of xCT (the substrate-specific subunit of system Xc⁻) and p53 expression in the aorta of db/db mice. A: The localization of xCT was detected by immunofluorescence. xCT is labeled red, and CD31 is labeled green. The nucleus is labeled blue. Scale bar: 100 μm; B and C: The messenger ribonucleic acid levels of p53 and xCT in mice aorta were determined using real-time polymerase chain reaction analyses. β-actin was used for normalization. ^aP < 0.05 vs the control group. ^bP < 0.01 vs the control group.

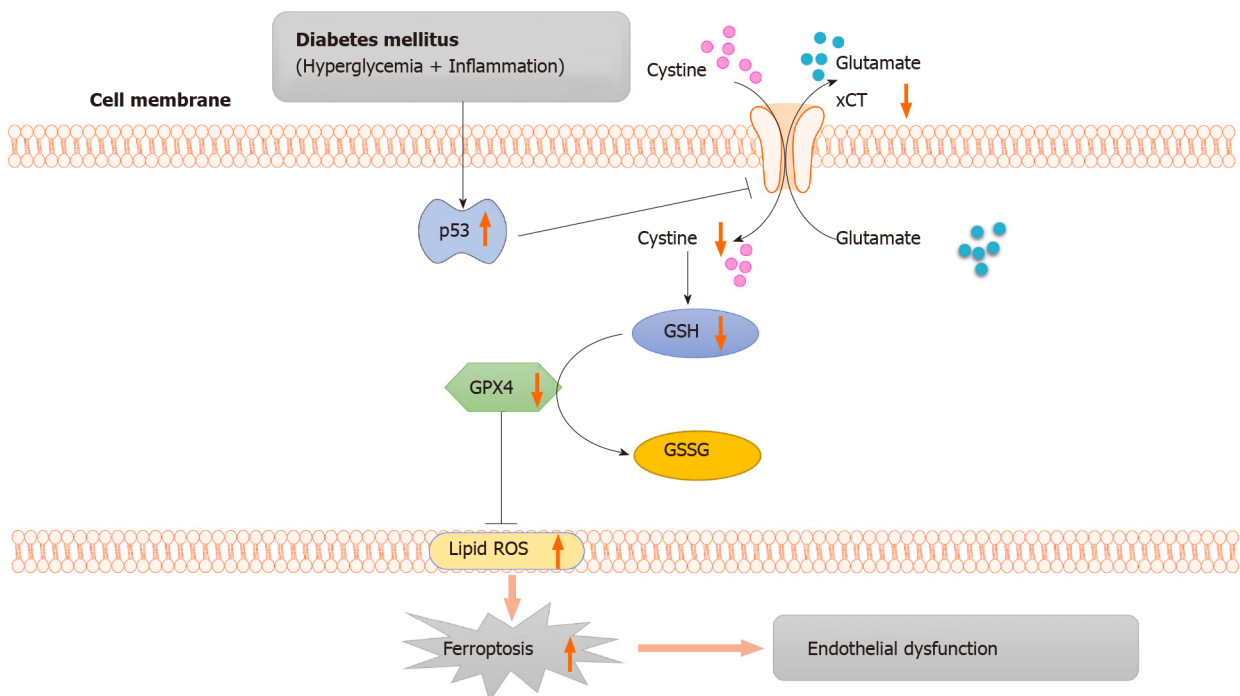


Figure 6 Working model: activation of the p53-xCT (the substrate-specific subunit of system Xc⁻)-glutathione axis and ferroptosis play a vital role in diabetes-induced endothelial dysfunction. In diabetes, p53 signaling is activated, and p53 can transcriptionally suppress xCT, in endothelial cells. The downregulation of xCT inhibits cystine uptake and reduces glutathione synthesis, which is involved in triggering ferroptosis in endothelial cells and ultimately leads to endothelial dysfunction. GSH: Glutathione; GSSG: Oxidized glutathione; GPX4: Glutathione peroxidase 4; ROS: Reactive oxygen species; ECs: Endothelial cells.

ARTICLE HIGHLIGHTS

Research background

Endothelial dysfunction, a hallmark of diabetes, is a critical and initiating contributor to the pathogenesis of diabetic cardiovascular complications. However, the underlying mechanisms are still not fully understood. Ferroptosis is a newly defined regulated cell death driven by cellular metabolism and iron-dependent lipid peroxidation. Although the involvement of ferroptosis in disease pathogenesis has been shown in cancers and degenerative diseases, the participation of ferroptosis in the pathogenesis of diabetic endothelial dysfunction remains unclear.

Research motivation

We tried to provide new insight into the mechanism of diabetic endothelial dysfunction.

Research objectives

The objective of this study is to investigate the role and regulatory mechanism of ferroptosis in diabetes-induced endothelial dysfunction.

Research methods

Human umbilical vein endothelial cells (HUVECs) were treated with high glucose (HG), interleukin-1 β (IL-1 β) and ferroptosis inhibitor, and then cell viability, reactive oxygen species (ROS) and ferroptosis-related marker protein were assessed. To further determine whether the p53-xCT (the substrate-specific subunit of system Xc)-glutathione (GSH) axis is involved in HG and IL-1 β -induced ferroptosis, HUVECs were transiently transfected with p53 small interfering ribonucleic acid or NC small interfering ribonucleic acid and then treated with HG and IL-1 β . Cell viability, ROS and ferroptosis-related marker protein were then tested. In addition, we detected xCT and p53 expression in the aorta of db/db mice.

Research results

It was found that HG and IL-1 β induced ferroptosis in HUVECs, as evidenced by the protective effect of the ferroptosis inhibitors, Deferoxamine and Ferrostatin-1, resulting in increased lipid ROS and decreased cell viability. Mechanistically, activation of the p53-xCT-GSH axis induced by HG and IL-1 β enhanced ferroptosis in HUVECs. In addition, the decrease in xCT and de-endothelialized areas were observed in the aortic endothelium of db/db mice.

Research conclusions

The current study demonstrated that ferroptosis is involved in endothelial dysfunction and p53-xCT-GSH axis activation plays a crucial role in endothelial cell ferroptosis and endothelial dysfunction.

Research perspectives

These results provide important insights as inhibiting activation of the p53-xCT-GSH axis and ferroptosis could attenuate diabetes-induced endothelial dysfunction and may be a novel strategy for the treatment of vascular complications in diabetes mellitus.

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