Bioscience Reports (2018) **38** BSR20181042
https://doi.org/10.1042/BSR20181042

PORTLAND PRESS

Research Article

HADC regulates the diabetic vascular endothelial dysfunction by targetting MnSOD

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Vascular dysfunction is a common result of diabetes in humans. However, the mechanism underlying diabetic vascular dysfunction is not fully understood. Here in the present study, we showed that the histone deacetylase 2 (HDAC2) promoted the endothelial dysfunction induced by diabetes. The expression and activity of HDAC2 were up-regulated in vascular endothelial cells (ECs) from diabetic patients and mice. The expression of HDAC2 was also increased by high glucose stress in isolated human ECs. *HDAC2* knockdown repressed the proliferation rate and promoted high glucose-induced apoptosis of ECs, which was associated with the activation of apoptotic pathways (Bcl-2, Caspase 3, and Bax). By contrast, HDAC2 overexpression led to opposing results. Significantly, we observed that HDAC2 regulated the accumulation of reactive oxygen species (ROS) induced by high glucose in ECs, which accounted for the effects of HDAC2 on proliferation and apoptosis because antioxidants, N-acetyl-L-cysteine (NAC) or MnTBAP treatment blocked the effects of HDAC2 on apoptosis of ECs under high glucose condition. Mechanism study revealed that HDAC2 bound to the promoter of *MnSOD* and repressed the expression of *MnSOD* by regulating the level of acetylated H3K9 and H3K27, which led to the promotion of oxidative stress and contributed to the function of HDAC2 in ECs under high glucose condition. Altogether, our evidence demonstrated that HDAC2-MnSOD signaling was critical in oxidative stress and proliferation as well as the survival of ECs under high glucose condition.

Introduction

Diabetes is a serious and increasing global health burden, and estimates of prevalence are essential for appropriate allocation of resources and the monitoring of trends [1]. This disease is a group of metabolic disorders in which there are high blood sugar levels over a prolonged period [2]. Accumulating data show that diabetes is associated with an increased risk of cardiovascular diseases in the presence of an intensive glycemic control. Vascular endothelial cells (ECs) are an important target of hyperglycemic stress [3]. Hyperglycemia-induced increase in the production of reactive oxygen species (ROS) generally leads to cell apoptosis and proliferation arrest or senescence [4]. However, the mechanism underlying diabetic endothelial dysfunction is not fully understood.

Histone acetylation and deacetylation determine the transcription of target genes. Histone acetylation is regulated by histone acetyltransferase and deacetylase (HDAC). Histone deacetylases act via the formation of large multiprotein complexes, and are responsible for the deacetylation of lysine residues on the N-terminal region of the core histones. Amongst the HDACs, the SIRT1 (NAD^+-dependent class III HDAC) has been fully investigated in endothelial dysfunction [5-7]. However, the functions of other HDACs in diabetic endothelial dysfunction remain largely unknown.

Received: 29 June 2018
Revised: 31 August 2018
Accepted: 12 September 2018

Accepted Manuscript Online: 14 September 2018
Version of Record published: 28 September 2018

Bioscience Reports (2018) **38** BSR20181042
https://doi.org/10.1042/BSR20181042

Histone deacetylase 2 (HDAC2) belongs to the class I HDAC and negatively regulates memory formation and synaptic plasticity [8]. HDAC2 blockade by nitric oxide and inhibitors reveals a common target in Duchenne muscular dystrophy treatment [9]. Furthermore, HDAC2 regulates the cardiac hypertrophic response by modulating Gsk3β activity [10]. In addition, a previous report showed that HDAC2 was a key regulator of diabetes- and transforming growth factor-β1-induced renal injury. HDAC2 activity significantly increased in the kidneys of streptozotocin (STZ)-induced diabetic rats and *db/db* mice [11].

Here, we observed that HDAC2 expression and activation were increased in the vascular ECs from diabetic patients and mice as well as ECs treated with high glucose. Gain-of-function and loss-of-function results demonstrated that HDAC2 regulated proliferation and apoptosis under high glucose condition in an ROS-dependent manner. HDAC2 binds the promoter of *MnSOD* and repressed the expression of *MnSOD* under high glucose condition, which partially contributed to the function of HDAC2 in endothelial dysfunction induced by high glucose.

Materials and methods

Patients and blood vessels

Patients' segments of internal mammary arteries and human saphenous veins were obtained from the patients undergoing routine coronary artery bypass surgery at the Xiangya Hospital, Central South University. The Local Research Ethics Committee approved the collection of tissue specimens, and all patients gave written informed consent. The patients with type 2 diabetes mellitus had fasting glucose >5.5 mmol/l and/or current treatment with insulin or oral hypoglycemic agents. An equal number of non-diabetic subjects were matched for other major demographic and clinical risk factors: hypercholesterolemia (total plasma cholesterol >4.8 mmol/l), smoking (current or within last 6 months), and hypertension (current treatment with antihypertensive agents). ECs from human saphenous veins were isolated from non-diabetic donors as described previously [12]. Vessels were collected immediately after surgical harvesting and transported to the laboratory in ice-cold Krebs HEPES buffer for further endothelial isolation. Human vascular ECs were isolated with the CD31 MicroBead Kit (Miltenyi, #130-091-935) for further RNA and protein isolation with the PARIS™ Kit (Sigma, #AM1921). The ECs were used at passage 4 (Supplementary Figure S1A).

Human umbilical cord veins ECs (HUVECs) were also freshly isolated from human umbilical cord veins as previously described with the CD31 MicroBead Kit (Miltenyi, #130-091-935), and cultured in M200 medium (Cascade Biologics Inc., #M-200-500) containing 5 mM D-glucose and supplemented with low-serum growth supplement (Cascade Biologics, #S-003-10), according to the manufacturer's recommendations. The ECs were used at passage 4–8 (Supplementary Figure S1B).

Diabetic mouse model

Eight-week-old male C57BL/6 mice were randomly divided into two groups. Diabetes was induced in one group by peritoneal injection of STZ (Sigma, #S0130). STZ was freshly dissolved in sterile citrate buffer (0.05 mol/l sodium citrate; pH: 4.5) and peritoneally injected into mice (50 mg/kg body weight) within 60 min of preparation. STZ was administered for five consecutive days. Mice with a random blood glucose level > 16 mmol/l for three consecutive weeks after STZ injection were specifically included in the diabetic group. The *db/db* mice (#000697) were purchased from Jackson Lab. Mouse ECs were isolated from lung arteries of diabetic and control mice with the CD31 MicroBeads kit (Miltenyi, #130-097-418) for further RNA and protein isolation with the PARIS™ Kit (Sigma, #AM1921). The ECs were used at passage 3 (Supplementary Figure S1C,D).

Quantitative real-time PCR

Total RNA (1 μg) was subjected to RNA that was subjected to cDNA synthesis with ProtoScript® II First Strand cDNA Synthesis Kit (New England BioLabs, #6560), and then cDNA was used to analyze the expression of target genes using quantitative real-time PCR (qRT-PCR) with the YBR™ Green PCR Master Mix (Thermo Fisher, #4309155). The primers used were listed as follows:

Human *GAPDH* forward: 5′-TGTGGGCATCAATGGATTTGG-3′,
Human *GAPDH* reverse: 5′-ACACCATGTATTCCGGGTCAAT-3′,
Human *HDAC2* forward:5′-ATGGCGTACAGTCAAGGAGG-3′,
Human *HDAC2* reverse:5′-TGCGGATTCTATGAGGCTTCA-3′,
Human *MnSOD* forward: 5′-GCTCCGGTTTTGGGGTATCTG-3′,
Human *MnSOD* reverse: 5′-GCGTTGATGTGAGGTTCCAG-3′.

Western blot

ECs were lysed with cell lysis buffer (Beyotime, P1003) supplemented with protease inhibitor cocktail (Thermo Fisher, #78429) or subjected to RNA and protein isolation with PARIS™ Kit (Sigma, #AM1921). Twenty micrograms of total proteins were applied to 12% SDS/polyacrylamide gel separation. After electrophoresis, the proteins were transferred to PVDF membranes, followed by blocking in the buffer containing 5% fat-free milk in TBST for 1 h at room temperature. Then, membranes were incubated with indicated antibodies overnight at 4°C, and then washed with TBST and incubated with HRP–conjugated secondary antibodies for 2 h at room temperature. Finally, the proteins were visualized using Chemiluminescent ECL reagent. Anti-HDAC2 (#ab7029), Anti-Ac-H3K9 (#ab10812), Anti-Ac-H3K27 (#ab4729), and antibodies were purchased from Abcam. Anti-MnSOD antibody (#06-984) was obtained from EMD Millipore. Anti-GAPDH (#sc-32233), Anti-Bcl-2 (#sc-7382), and Anti-Bax (#sc-7480) antibodies were purchased from Santa Cruz Biotechnology. Anti-Cleaved Caspase 3 (#9661) antibody was purchased from Cell Signaling Technology.

Retrovirus packaging

For *HDAC2* or *MnSOD* knockdown, we designed retrovirus carrying shRNA targetting these two genes. The shRNAs were synthesized from Invitrogen and cloned into the pSUPERre. For *HDAC2* overexpression, human *HDAC2* (NM_001527.3) was cloned into pQCXIP (Clontech). For retroviral packaging, HEK293T cells were co-transfected with pVPackVSV-G, pVPack-GP (Stratagene) and the knockdown or pSUPERretro control constructs, overexpression or pQCXIP control constructs. The viral supernatant was collected after 48 h. For transduction, cells were incubated with virus-containing supernatant in the presence of 8 mg/ml polybrene. After 48 h, infected cells were selected for 72 h with puromycin (2 mg/ml). The sequences targetting *HDAC2* and *MnSOD* were listed as follows:

sh*HDAC2*-1#: 5′-AATCCGCATGACCCATAACTT-3′,
sh*HDAC2*-2#: 5′-GTATCATCAGAGAGTCTTATT-3′,
sh*MnSOD*: 5′-GGAGCACGCTTACTACCTTCA-3′.

Cell proliferation assay

The proliferation of HUVECs was monitored using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher, #V13154) according to the manufacturer's protocol.

Cell apoptosis assay

The apoptosis of HUVEC was analyzed with the Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson, #556547) according to the manufacturer's protocol. The data were analyzed with FACSCalibur flow cytometer.

ChIP assay

ChIP assays were performed in HUVECs with IgG, HDAC2, acetylated H3K9, and H3K27 antibodies as previously described [13], with ChIP assay with the Pierce™ Magnetic ChIP Kit (Thermo, #26157). qRT-PCR was carried out with specific primers to amplify the HDAC2-binding region of the human *MnSOD* promoter (forward: 5′-ACAGGCACGCAGGGCACCCCCGGGGTT-3′, reverse: 5′-TCCTGCGCCGCCCGCGGGCCTTAAGAAA-3′).

Measurement of mitochondrial oxidative stress

HUVECs were treated as indicated in the figure legends. Total ROS Assay Kit (Thermo Fisher, #88-5930-74) was used for detecting ROS generation in HUVECs according to the manufacturer's protocol. The levels of mitochondrial ROS were normalized to the control group.

Statistical analysis

All values are expressed as means ± S.E.M. Student's *t* test was used for analyses between two groups. One-way ANOVAs were used to compare results of data from three or more groups followed by Bonferroni's *post hoc* multiple comparisons tests. *P*-values less than 0.05 were considered significant. Statistical analysis was performed with GraphPad Prism software, version 6.0.

Bioscience Reports (2018) **38** BSR20181042
https://doi.org/10.1042/BSR20181042

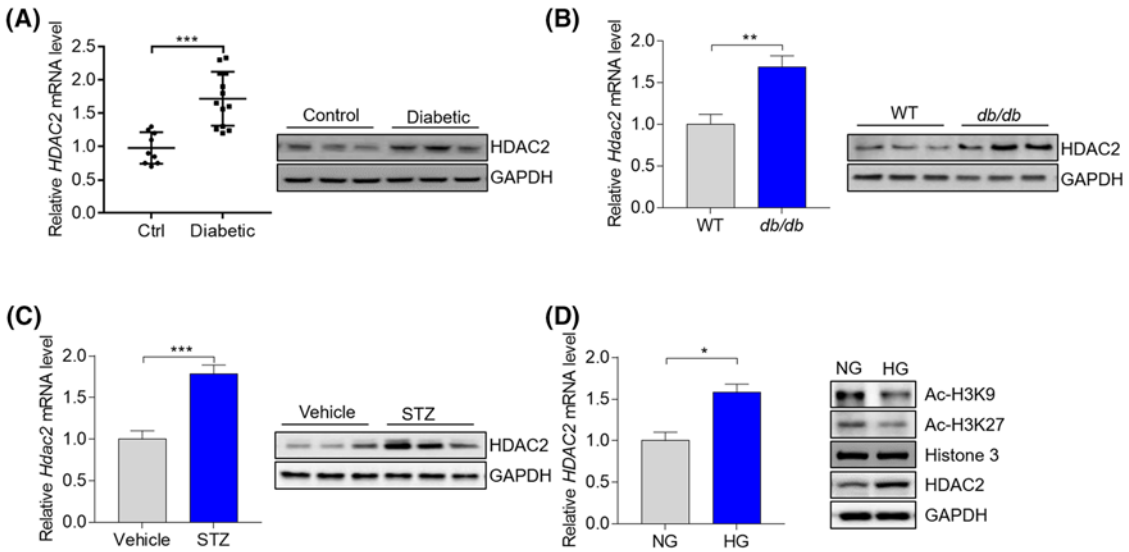

Figure 1. HDAC2 is increased in vascular ECs of diabetic patients and mice

(**A**) *HDAC2* mRNA and protein levels were increased in vascular ECs from diabetic patients. ECs were isolated from internal mammary arteries of diabetic patients (n=12) and non-diabetic donors (n=9) and the RNA and protein were analyzed. (**B**) *HDAC2* mRNA and protein levels were increased in vascular ECs from diabetic (*db*/*db*) mice. ECs were isolated from lung arteries of diabetic mice (n=5) and non-diabetic mice (n=5), and the RNA and protein were analyzed. (**C**) *HDAC2* mRNA and protein levels were increased in vascular ECs from STZ-induced diabetic mice. ECs were isolated from lung arteries of STZ-induced diabetic mice (n=5) and non–diabetic mice (n=5), and the RNA and protein were analyzed. (**D**) HDAC2, H3K9Ac, and H3K27Ac levels in ECs treated with/without high glucose. Human umbilical cord veins ECs (HUVECs) were treated with a normal concentration of glucose (NG) or high glucose (HG, 30 mM) for 24 h and then the cells were analyzed. *P<0.05, **P<0.01, and ***P<0.001 by unpaired Student's t test.

Results

HDAC2 expression is increased in vascular ECs of diabetic patients and mice

To investigate the potential function of HDAC2 in diabetic vascular diseases, we analyzed the expression of HDAC2 in vascular ECs from diabetic patients and mice. ECs were isolated from vessels from type 2 diabetic patients (n=12) and age-matched donors (n=9), and qRT-PCR and Western blot were performed to analyze the expression of HDAC2. The results showed that the mRNA and protein levels of HDAC2 were remarkedly up-regulated in ECs from diabetic patients compared with that in the control donors (Figure 1A). Next, we analyzed the expression profile of HDAC2 in vascular ECs in *db*/*db* mice, which is a type 2 diabetic mouse model. The results demonstrated that the mRNA and protein levels of HDAC2 were increased in the vascular ECs from *db*/*db* mice compared with wild-type littermates (Figure 1B). We also induced type 1 diabetes in mice by injection of STZ. Similarly, the results showed that the expression of HDAC2 was up-regulated in the vascular ECs from STZ-induced type 1 diabetes (Figure 1C). To explore whether the effects of diabetes on HDAC2 expression in ECs relied on high glucose, we isolated HUVECs and treated the cells with high glucose. High glucose increased the mRNA and protein levels of HDAC2 in HUVECs, which was consistent with the decreased levels of acetylated H3K9 and H3K27 (Figure 1D). Taken together, HDAC2 expression was increased in vascular ECs in diabetic patients and mice.

HDAC2 regulates the proliferation and apoptosis of ECs

Diabetes could induce endothelial dysfunction. To study the effects of HDAC2 in high glucose-induced dysfunction of ECs, we prepared retrovirus-mediated shRNA to knock down the expression of *HDAC2* in HUVECs (Figure 2A and Supplementary Figure S2A). We observed that HDAC2 knockdown promoted the proliferation rate of HUVECs (Figure 2B). High glucose treatment induced apoptosis of ECs, whereas *HDAC2* knockdown repressed high glucose-induced apoptosis of ECs (Figure 2C). In addition, we also analyzed the apoptotic pathway with Western blot. *HDAC2* knockdown repressed high glucose-induced increase in cleaved Caspase 3 and BAX as well as a decrease in

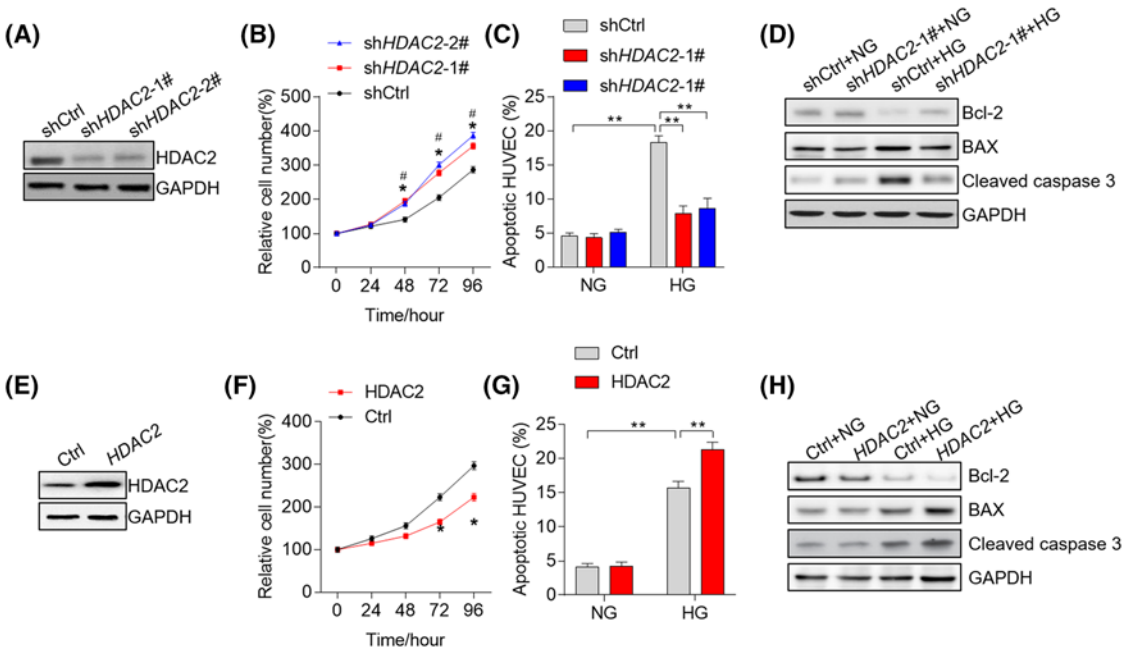

Figure 2. HDAC2 regulates the proliferation and apoptosis of ECs treated with high glucose

(**A**) Representative Western blot showing HDAC2 knockdown in HUVECs. HUVECs were infected with a retrovirus carrying shRNA targetting *HDAC2* for 48 h, then the protein was subjected to Western blot analysis. (**B**) Knockdown of *HDAC2* promoted HUVEC proliferation. Transduced HUVECs with/without *HDAC2* knockdown were subjected to cell proliferation assay. $^{*}P<0.05$ (shCtrl compared with sh*HDAC2*-1#), $^{\#}P<0.05$ (shCtrl compared with sh*HDAC2*-2#) by two-way ANOVA followed by Bonferroni's *post hoc* multiple comparisons tests. (**C**) Knockdown of *HDAC2* reduced high glucose-induced apoptosis in HUVECs. Transduced HUVECs with/without *HDAC2* knockdown were treated with high glucose (HG, 30 mM) for 48 h, then cells were subjected to apoptosis analysis. $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni's *post hoc* multiple comparisons tests. (**D**) Representative Western blot showing the effects of *HDAC2* knockdown on the apoptotic pathway. Transduced HUVECs with/without HDAC2 knockdown were treated with high glucose (HG, 30 mM) for 24 h, then cells were subjected to Western blot analysis. (**E**) Representative Western blot showing *HDAC2* overexpression in HUVECs. HUVECs were infected with a retrovirus carrying *HDAC2* for 48 h, then the protein was subjected to Western blot analysis. (**F**) Overexpression of *HDAC2* repressed HUVEC proliferation. Transduced HUVECs with/without *HDAC2* overexpression were subjected to cell proliferation assay. $^{*}P<0.05$ by two-way ANOVA followed by Bonferroni's *post hoc* multiple comparisons tests. (**G**) Overexpression of *HDAC2* promoted high glucose-induced apoptosis of HUVECs. Transduced HUVECs with/without *HDAC2* overexpression were treated with high glucose (HG, 30 mM) for 48 h, then cells were subjected to apoptosis analysis. $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni's *post hoc* multiple comparisons tests. (**H**) Representative Western blot showing the effects of *HDAC2* knockdown on the apoptotic pathway. Transduced HUVECs with/without *HDAC2* overexpression were treated with high glucose (HG, 30 mM) for 24 h, then cells were subjected to Western blot analysis.

Bcl-2 in HUVECs (Figure 2D). We next overexpressed *HDAC2* in HUVECs with a retrovirus (Figure 2E and Supplementary Figure S2B). *HDAC2* overexpression repressed the proliferation of HUVECs (Figure 2F) and promoted endothelial apoptosis induced by high glucose (Figure 2G,H). Therefore, HDAC2 repressed proliferation of ECs and promoted high glucose-induced endothelial apoptosis.

HDAC2 regulates oxidative stress to affect the proliferation and apoptosis of ECs

Oxidative stress is a common phenomenon in diabetic vascular disease. We next tested whether HDAC2 regulated oxidative stress in the ECs. We found that high glucose treatment increased the accumulation of ROS in HUVECs. *HDAC2* knockdown repressed whereas *HDAC2* overexpression promoted high glucose-induced accumulation of ROS in ECs (Figure 3A,B). Then, we studied whether ROS contributed to the effects of HDAC2 on apoptosis of ECs under high glucose condition. We treated the ECs with a pan-ROS inhibitor (N-acetyl-L-cysteine (NAC)) and a mitochondrial ROS inhibitor (MnTBAP, an MnSOD mimetic). Either NAC or MnTBAP treatment repressed

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https://doi.org/10.1042/BSR20181042

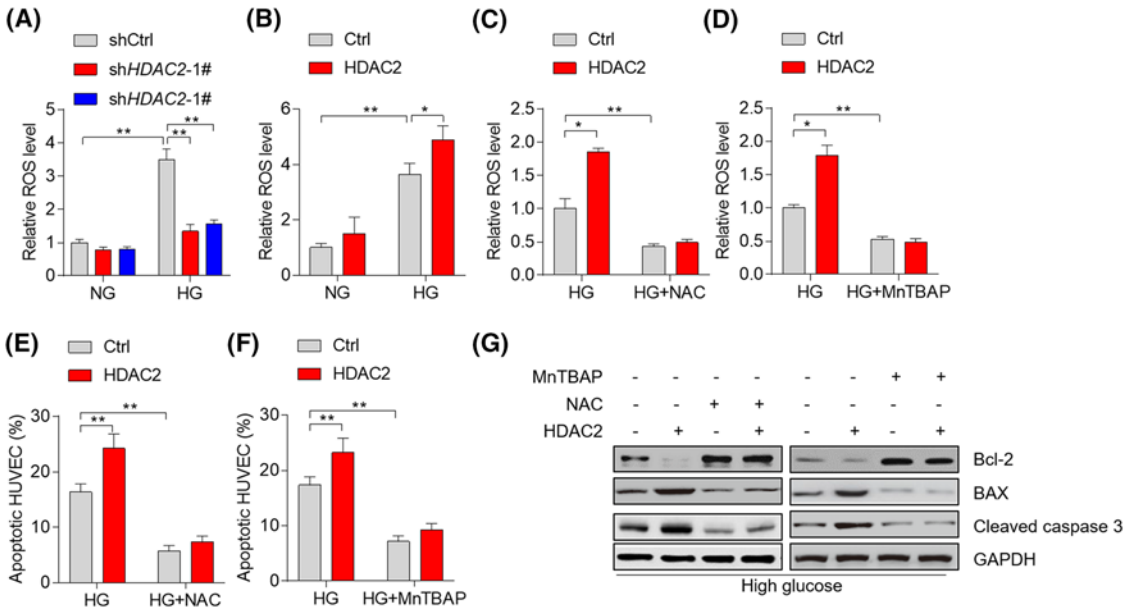

Figure 3. HDAC2 regulates oxidative stress to participate in proliferation and apoptosis

(**A**) *HDAC2* knockdown repressed high glucose-induced ROS accumulation in HUVECs. Transduced HUVECs with/without *HDAC2* knockdown were treated with high glucose (HG, 30 mM) for 12 h, then the cells were subjected to analysis of total cellular ROS level. $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons tests. (**B**) *HDAC2* overexpression promoted high glucose-induced ROS accumulation in HUVECs. Transduced HUVECs with/without *HDAC2* overexpression were treated with high glucose (HG, 30 mM) for 12 h, then the cells were subjected to analysis of total cellular ROS level. $^{*}P<0.05$, $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons tests. (**C**) NAC reduced the *HDAC2*-mediated increase in ROS level in high glucose-treated HUVECs. Transduced HUVECs with/without *HDAC2* overexpression were treated with high glucose (HG, 30 mM) and/or NAC (1 mM) for 12 h, then the cells were subjected to analysis of total cellular ROS level. $^{*}P<0.05$, $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons tests. (**D**) MnTBAP reduced the *HDAC2*-mediated increase in ROS level in high glucose-treated HUVECs. Transduced HUVECs with/without *HDAC2* overexpression were treated with high glucose (HG, 30 mM) and/or MnTBAP (100 μM) for 12 h, then the cells were subjected to analysis of total cellular ROS level. $^{*}P<0.05$, $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons tests. (**E**) NAC reduced the *HDAC2*-mediated increase in apoptosis in high glucose-treated HUVECs. Transduced HUVECs with/without *HDAC2* overexpression were treated with high glucose (HG, 30 mM) and/or NAC (1 mM) for 48 h, then the cells were subjected to analysis of apoptosis. $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons tests. (**F**) MnTBAP reduced the *HDAC2*-mediated increase in apoptosis in high glucose-treated HUVECs. Transduced HUVECs with/without *HDAC2* overexpression were treated with high glucose (HG, 30 mM) and/or MnTBAP (100 μM) for 48 h, then the cells were subjected to analysis of apoptosis. $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons tests. (**G**) NAC and MnTBAP blocked the effects of *HDAC2* on apoptotic signaling pathways in high-glucose-treated HUVECs. Transduced HUVECs with/without *HDAC2* overexpression were treated with high glucose (HG, 30 mM) and/or NAC (1 mM) or MnTBAP (100 μM) for 24 h, then the cells were subjected to Western blot analysis. $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons tests.

HDAC2-mediated increase in ROS accumulation in HUVECs under high glucose condition (Figure 3C,D). Importantly, NAC, and MnTABP treatment could repress HDAC2-induced increase in endothelial apoptosis under high glucose condition (Figure 3E,F). Consistently, the effects of HDAC2 on apoptotic signalings were blocked by NAC and MnTBAP (Figure 3G). Collectively, ROS was critically involved in HDAC2 function in endothelial apoptosis.

MnSOD is a target for HDAC2 in regulating ROS and apoptosis of ECs

To further investigate the molecular mechanism underlying the HDAC2 function in ROS and apoptosis regulation, we tested the expression of MnSOD, which is a mitochondrial antioxidant. We found that MnSOD expression was increased by *HDAC2* knockdown and decreased by *HDAC2* overexpression in HUVECs (Figure 4A,B). However, *HDAC2* knockdown did not change the expression of catalase (Supplementary Figure S3). Our ChIP assay revealed

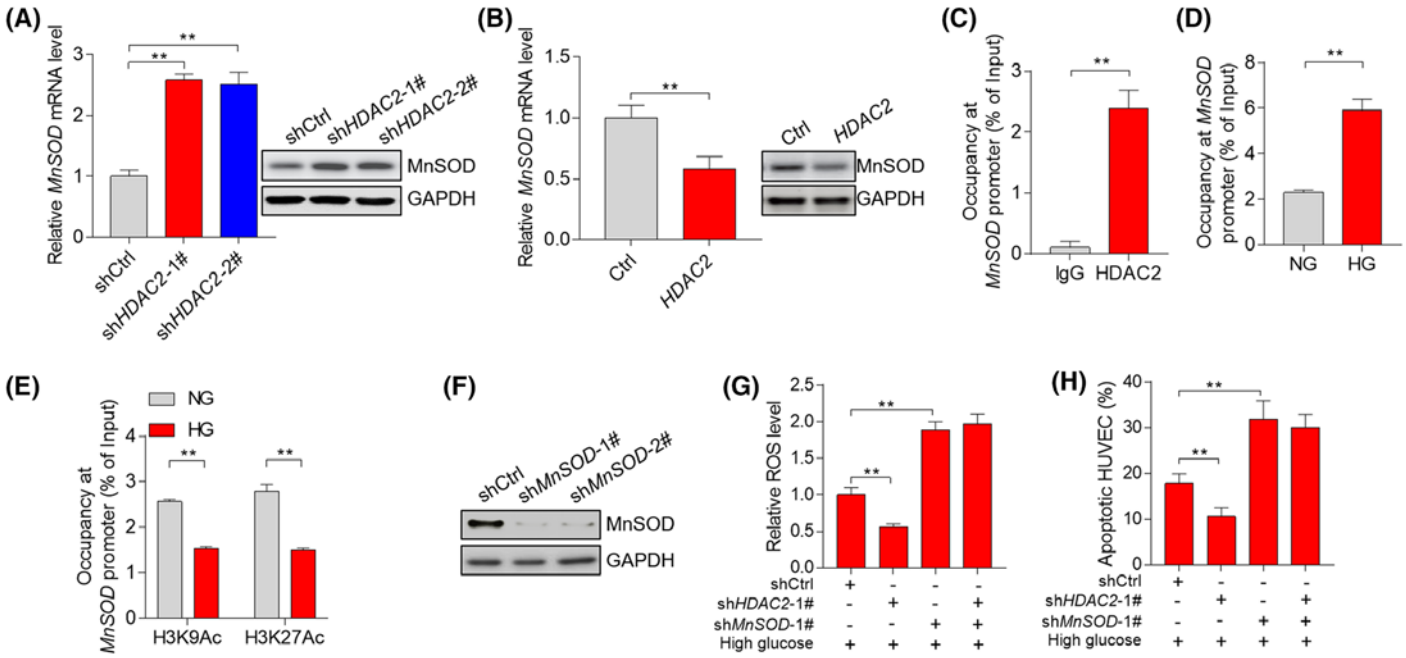

Figure 4. HDAC2 regulates the expression of MnSOD in ECs

(**A**) Knockdown of *HDAC2* increases the expression of *MnSOD*. HUVECs were infected with a retrovirus carrying shRNA targetting *HDAC2* for 48 h, then the mRNA and protein levels were analyzed. $^{**}P<0.01$ by one-way ANOVA followed by Bonferroni *post hoc* multiple comparisons tests. (**B**) Overexpression of *HDAC2* reduces the expression of *MnSOD*. HUVECs were infected with a retrovirus carrying *HDAC2* for 48 h, then the mRNA and protein levels were analyzed. $^{**}P<0.01$ by unpaired Student's *t* test. (**C**) Representative ChIP results showing HDAC2 binds the promoter of *MnSOD.* HUVECs were subjected to ChIP assay with anti-IgG or anti-HDAC2 antibodies, followed by qRT-PCR experiments to detect the binding of HDAC2 to *MnSOD* promoter. $^{**}P<0.01$ by unpaired Student's *t* test. (**D**) High glucose promotes the binding of HDAC2 to MnSOD promoter. HUVECs were treated with/without high glucose (HG, 30 mM) for 24 h and the cells were subjected to ChIP assay with anti-IgG or anti-HDAC2 antibodies, followed by qRT-PCR experiment to detect the enrichment of HDAC2 at *MnSOD* promoter. $^{**}P<0.01$ by unpaired Student's *t* test. (**E**) High glucose reduces H3K9Ac and H3K27Ac at MnSOD promoter. HUVECs were treated with/without high glucose (HG, 30 mM) for 24 h and the cells were subjected to ChIP assay with anti-H3K9Ac or anti-H3K27Ac antibodies, followed by qRT-PCR experiment to detect the enrichment of H3K9Ac and H3K27Ac at *MnSOD* promoter. $^{**}P<0.01$ by unpaired Student's *t* test. (**F**) Representative Western blot showing *MnSOD* knockdown in HUVECs. HUVECs were infected with a retrovirus carrying shRNA targetting *MnSOD* for 48 h. (**G**) *MnSOD* knockdown blocks the effects of *HDAC2* knockdown on the high glucose-induced accumulation of ROS in HUVECs. Transduced HUVECs with/without HDAC2 and/or MnSOD knockdown were treated with high glucose (HG, 30 mM) for 12 h, then the cells were subjected to analysis of total cellular ROS level. $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons tests. (**H**) MnSOD knockdown blocks the effects of *HDAC2* knockdown on high glucose-induced apoptosis in HUVECs. Transduced HUVECs with/without *HDAC2* and/or *MnSOD* knockdown were treated with high glucose (HG, 30 mM) for 48 h, then the cells were subjected to analysis of apoptosis. $^{**}P<0.01$ by two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons tests.

that HDAC2 bound the promoter of *MnSOD*, which was enhanced by high glucose treatment in HUVECs (Figure 4C,D). Consistently, high glucose decreased the levels of acetylated H3K9 and H3K27 (Figure 4E), which might lead to the down-regulation of *MnSOD* expression. To further explore whether *MnSOD* was critically involved in HDAC2 function on ROS and apoptosis of HUVECs, we knocked down the expression of *MnSOD* with retrovirus-mediated shRNA in HUVECs (Figure 4F and Supplementary Figure S4). *MnSOD* knockdown blocked the effects of *HDAC2* knockdown on ROS accumulation and cell apoptosis under high glucose condition (Figure 4G,H). Collectively, these findings demonstrated that MnSOD was a target for HDAC and was critically involved in the function of HDAC2 in ECs.

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https://doi.org/10.1042/BSR20181042

Discussion

Diabetic vascular disease is a common complication in patients with diabetes. ECs are the first target of hyperglycemia. Prevention of hyperglycemia-induced endothelial dysfunction is a major goal for the treatment of diabetic vascular diseases [4]. In the past decades, accumulating mechanism was provided to understand the diabetic endothelial dysfunction. Histone acetylation and deacetylation were observed to be an imbalance at the promoter of diverse genes associated with diabetic endothelial dysfunction, and HDACs have been reported to play considerable roles in the endothelial function in diabetes. For instance, repression of $p66^{Shc}$ expression by SIRT1 contributes to the prevention of hyperglycemia-induced endothelial dysfunction [14]. Long-term administration of the HDAC inhibitor Vorinostat attenuates renal injury in experimental diabetes through an endothelial nitric oxide synthase dependent mechanism [15]. Here, we identified the roles of HDAC2 in diabetic endothelial dysfunction. We have provided several lines of evidence that *HDAC2* mRNA and protein level have been up-regulated in ECs from diabetic patients and mice *in vivo*. Our *in vitro* findings revealed that the mRNA and protein levels of HDAC2 were up-regulated in HUVECs treated with high glucose, which was associated with decreased acetylation of H3K9 and H3K27.

Hyperglycemia-induced oxidative stress, apoptosis, and growth arrest are core hallmarks of diabetic endothelial dysfunction [2]. We observed that HDAC2 overexpression reduced the proliferation rate and promoted high glucose-induced apoptosis of ECs. This interesting finding was inconsistent with the observations in cancer cells. HDAC2 attenuates TRAIL-induced apoptosis of pancreatic cancer cells [16]. HDAC2 overexpression confers oncogenic potential to human lung cancer cells by deregulating the expression of apoptosis and cell cycle proteins [17]. Therefore, HDAC2 may play opposing roles in the regulation of cell cycle and apoptosis, which may largely depend on the specific targets of HDAC2 under a given condition.

Oxidative stress is a common result induced by high glucose. Uncontrolled oxidative stress could lead to proliferation arrest and apoptosis of cells. We found that HDAC2 promoted the accumulation of total cellular ROS levels under the condition of high glucose in ECs. Indeed, high level of oxidative stress partially contributed to the function of HDAC2 because HDAC2 effects on apoptosis and apoptotic signaling pathways could be reduced by either inhibition of total ROS with NAC or mitochondrial ROS with MnTBAP. MnTBAP is a mimetic of MnSOD, which is located in the mitochondria and plays a role as a core antioxidant in the mitochondria [18]. We observed that HDAC2 could bind the promoter and repress the expression of *MnSOD* under high glucose condition, which was associated with the acetylation level of H3K9 and H3K27. Importantly, our loss-of-function experiments demonstrated that knockdown of *MnSOD* blocked the function of HDAC2 knockdown on ROS accumulation and apoptosis induced by high glucose. Therefore, repression of MnSOD expression and up-regulation of ROS level were critically involved in the function of HDAC2 in diabetic endothelial dysfunction. Interestingly, oxidative stress may also be one of the mechanisms underlying HDAC2 up-regulation induced by high glucose. A previous report showed that hydrogen peroxide increased HDAC2 activity, and the treatment with an antioxidant NAC almost completely reduced TGF-β1-induced activation of HDAC2 [11]. However, further studies are needed to confirm this hypothesis.

In conclusion, in the present work, we demonstrated that HDAC2 was up-regulated in vascular ECs from diabetic patients and mice. HDAC2 up-regulation was associated with endothelial apoptosis, oxidative stress via an MnSOD-dependent mechanism. Therefore, HDAC2 may be a potential target for the treatment of diabetic vascular diseases.

Funding

This work was supported by the Natural Science Foundation of China for the Youth [grant number 81000337 (to Z.L.)].

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Author contribution

Q.H. and K.H. performed most of the experiments and collected data. X.L. participated in isolation of human and mouse ECs. J.Q. generated lentivirus expressing shRNAs and overexpressing HDAC2. Z.L. designed the study and wrote the paper.

Abbreviations

EC, endothelial cell; HDAC2, histone deacetylase 2; HUVEC, human umbilical cord vein EC; NAC, N-acetyl-L-cysteine; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; STZ, streptozotocin.

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