Peroxidasin promotes diabetic vascular endothelial dysfunction induced by advanced glycation end products via NOX2/HOCl/Akt/eNOS pathway

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A B S T R A C T

Reactive oxygen species (ROS) derived from NADPH oxidases (NOX) plays an essential role in advanced glycation end products (AGEs)-induced diabetic vascular endothelial dysfunction. Peroxidasin (PXDN, VPO1) is one member of peroxidases family that catalyzes hydrogen peroxide (H2O2) to hypochlorous acid (HOCI). This present study aimed to elucidate the role of PXDN in promoting vascular endothelial dysfunction induced by AGEs in diabetes mellitus. We found that, compared to non-diabetic (db/m) mice, PXDN expression was notably increased in db/db mice with impaired endothelium-dependent relaxation. Knockdown of PXDN in vivo through tail vein injection of siRNA restored the impaired endothelium-dependent relaxation function of db/db mice which is accompanied with up-regulation of eNOS Ser1177 phosphorylation and NO production. AGEs significantly elevated expression of PXDN and 3-Cl-Tyr, but decreased phosphorylation of Akt and eNOS and NO release in HUVECs. All these effects induced by AGEs were remarkable alleviated by silencing PXDN with small interfering RNAs. In addition, HOCI treatment alone as well as HOCI added with Akt inhibitor MK2206 inhibited phosphorylation of Akt and eNOS, reducing NO production. More importantly, AGEs-induced up-regulation of PXDN and 3-Cl-Tyr with endothelial dysfunction were transformed by NOX2 silencing and H2O2 scavengers. Thus, these results support the conclusion that PXDN promotes AGEs-induced diabetic vascular endothelial dysfunction by attenuating eNOS phosphorylation at Ser1177 via NOX2/HOCI/Akt pathway.

1. Introduction

Vascular complications as a result of diabetes mellitus (DM) currently poses a significant public health risk in the world, as nearly 75% of diabetic patients die of vascular disease [1,2]. The intima of blood vessels is comprised of an endothelial barrier that maintains physiological homeostasis by releasing molecules. Vasoconstriction stimulus such as noradrenaline, thrombin and hypoxia, triggers endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO), and promotes vascular tone relaxation [3,4]. Many studies reported hyperglycemia, insulin resistance, advanced glycation end products (AGEs) and its receptor RAGE inhibit eNOS activity, reduce NO production, promote endothelial dysfunction and eventually lead to the occurrence of diabetic vascular disease [5–7].

Under a prolonged state of hyperglycemia, non-enzymatic glycation or glycoxidation of proteins, lipids, and nucleic acids induced AGEs and reactive oxygen species (ROS) accumulation in endothelium, which contributes to diabetic endothelial dysfunction [8]. ROS that generated from mitochondrial and especially NADPH oxidase 2 (NOX2) in endothelial cells inhibits eNOS activity by promoting eNOS uncoupling and decreasing phosphorylation of eNOS [9–11]. However, the exact mechanism of how AGEs/NOX2/ROS regulate eNOS activity remains unknown.

Peroxidasin (PXDN) was firstly reported in Drosophila by Nelson et al. [12] and plays an important role in ocular development [13,14]. In
2008, PXDN was renamed vascular peroxidase1 (VPO1) for the reason of detecting highly expressed in the cardiovascular system including endothelial cells, vascular smooth muscle cells, cardiomyocytes as well as extracellular matrix [15–19]. PXDN generates hypothalamic acids (HOCI) by catalyzing hydrogen peroxide (H2O2), which aggravates oxidative stress in vasculature [20,21]. We have previously reported that PXDN decreases eNOS expression in response to angiotensin II [17]. However, the role of PXDN in diabetic endothelial dysfunction was unclear.

Here we reported for the first time that PXDN promotes diabetic vascular endothelial dysfunction through the NOX2/HOCl/Akt/eNOS pathway induced by AGES.

2. Materials and methods

Refer to supplementary materials for detailed methods.

2.1. Animal

Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all experimental protocols were approved by the Medicine Animal Welfare Committee of the Third Xiangya Hospital of Central South University (Approval No: 201703121; Changsha, China). 11-week-old male db/db mice with a C57BLKS/Jnu background (db/db, Lepr<sup>−/−</sup>) and control db/m mice with the same background were obtained from Model Animal Research Center of Nanjing University (Nanjing, China).

2.2. Metabolic parameters test

Body weights of mice were measured using an electronic scale (PL6001E, Mettler Toledo, Zurich, Switzerland). Blood glucose was assessed in random-fed status mice using a glucometer (SAFE-ACCU, Sinocare Inc, Changsha, China) to analyze blood samples collected from tail veins. Glycosylated hemoglobin (HbAlc) and other serum biochemical markers were evaluated by assay kit according to the manufacturer’s protocol.

2.3. Measurement of vascular function

Endothelium-dependent and endothelium-independent relaxation of the mice thoracic aortas in response to acetylcholine (ACH) and sodium nitroprusside (SNP), respectively, were assessed as previously described [22–24].

2.4. Cell culture and experiments

Human umbilical vein endothelial cells (HUVECs) were obtained from ScienCell Research Laboratories (San Diego, CA, USA). HUVECs were cultured in endothelial cell medium (ScienCell Research Laboratories, CA, USA) with 5% fetal bovine serum (FBS) and 1% endothelial cell growth supplement (ECGS) at 37 °C and 5% CO2. For experiments, cells were used between passages 3 and 8.

2.5. Western blot analysis

Isolated thoracic aortic tissues or cultured HUVECs were lysed in commercially purchased RIPA lysis buffer (P0013B, Beyotime, Shanghai, China) spiked with 1 mmol/L phenylmethanesulfonyl fluoride (ST506, Beyotime, Shanghai, China) to obtain protein products. The antibodies against the following proteins were used: PXDN (1 µg/mL, ABS1675, Merck, Frankfurt, Germany), RAGE (0.3 µg/mL, ab37647, Abcam, Cambridge, UK), NOX2 (0.25 µg/mL, ab80508, Abcam, Cambridge, UK), NOX1 (0.5 µg/mL, ab131088 and ab121009, Abcam, Cambridge, UK), NOX4 (0.33 µg/mL, ab154244, Abcam, Cambridge, UK), 3-chlorotyrosine (0.1 µg/mL, 3-Cl-Tyr, HP5002, Hycult biotech, Uden, Netherlands), 4-hydroxyxynonenal (0.43 µg/mL, 4-HNE, ab46545, Abcam, Cambridge, UK), Akt (0.5 µg/mL, SAB4500797, Sigma-Aldrich, USA), p-Akt (0.143 µg/mL, ab18206, Abcam, Cambridge, UK), eNOS (0.25 µg/mL, 61029, BD, Biosciences, NJ, USA) and p-eNOS (0.056 µg/mL dilution, Ser1177, MA5-14957, Invitrogen, CA, USA). Expression of the protein GAPDH (0.5 µg/mL, SAB1405848, Sigma-Aldrich, MO, USA) was used for data normalization. PVDF membranes were incubated with a horseradish peroxidase linked secondary antibody and bands were visualized using gel documentation system (Bio-Rad).

2.6. Immunofluorescence staining

Cryosection of thoracic aortas and mesenteric arteries were blocked in 5% bovine serum albumin (BSA, A1933, Sigma-Aldrich, MO, USA) diluted in phosphate buffered saline (PBS) for 1 h at room temperature and subsequently incubated with goat anti-PXDN (10 µg/mL, sc-168598, Santa Cruz, TX, USA) and rabbit anti-CD31 (1:20 dilution, ab28364, Abcam, Cambridge, UK), anti-RAGE (1 µg/mL, ab37647, Abcam, Cambridge, UK) and anti-3-chlorotyrosine (2 µg/mL, HP5002, Hycult biotech, Uden, Netherlands) antibodies overnight at 4 °C in a humidity box. Samples were then incubated with secondary antibody for 1 h at room temperature. Finally, DAPI (5 µg/mL, D9542, Sigma-Aldrich, MO, USA) was used for nuclear staining.

2.7. Detection of superoxide

Intracellular superoxide levels were evaluated by Dihydroethidium (DHE) fluorescence probe. For thoracic aortas and mesenteric arteries, cryosection were incubated with DHE (Ex518/Em606 nm, 5 µmol/L; D23107, Invitrogen)-containing PBS at 37 °C for 15 min. For HUVECs, cells were seeded in the six-well plates and incubated with DHE in PBS at 37 °C for 30 min. Fluorescence image were obtained with a fluorescence microscopy (Eclipse, Nikon, Japan) and analyzed by measuring the fluorescence intensity.

2.8. Assessment of NO production

The level of NO in plasma and cell supernatants was indirectly
3.1. PXDN expression is elevated in db/db mice with impaired endothelium-dependent relaxation

The db/db mice were used as diabetic animal model and the db/m were used as the control in this study. Body weight, fasting blood glucose, Hba1c and other serum biochemical markers were measured and described in supplementary table 1. Vascular functions of mice were evaluated by measuring dilatory responses of pressurized thoracic aortas to vasoactive agents. Endothelium-dependent relaxations in response to Ach were impaired by 62.50% (p < 0.01) in db/db mice compared with db/m mice (Fig. 1A), while no significant difference in SNP-induced endothelium-independent relaxation was observed (Fig. 1B). Moreover, the NO production in plasma was significantly decreased in db/db mice compared to db/m mice (29.03 ± 7.91 μmol/L vs 61.68 ± 12.09 μmol/L, p < 0.01, Fig. 1C). The expression of RAGE, PXDN, 3-Cl-Tyr (a surrogate measure of HOCl production), p-eNOS, p-Akt, NOX1, 2, 4, and the levels of H2O2, superoxide, oxidative stress products (4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA)) were measured by Western blot, fluorescence probe or kits. We found that RAGE protein expression was elevated (Fig. 1D and Figure S1A) in aortas of db/db mice compared to db/m mice. The protein expression of NOX family (NOX1, 2 and 4) was up-regulated in thoracic aortas from db/db mice compared with the db/m group, and the increase of NOX2 was more obvious than NOX1 and NOX4 (Fig. 1D, Figure S1D-F). The ROS level including H2O2, superoxide, 4-HNE and MDA were up-regulated concurrently in diabetic mice (Fig. 1H, G, K and E). ROS levels detected using DHE probe were inhibited by non-specific NOX inhibitor (VAS2870), specific NOX2 inhibitor (GSK2795039), and mitochondrial superoxide scavenger (Mito-TEMPO), while the effect of ROS attenuation treated with NOX2-specific inhibitor was more significant than that of non-specific NOX inhibitor and mitochondrial superoxide scavenger (Figure S1L-N). Furthermore, expression of PXDN and 3-Cl-Tyr in db/db mice was also obviously increased compared with the db/m (2.9-fold and 3.3-fold, respectively, Fig. 1F-J). Alternatively, the phosphorylation level of eNOS on Ser1177 in db/db mice was significantly decreased (Fig. 1D, Figure S1B). Although the protein expression of total eNOS was also decreased, while was no statistical difference (p = 0.1238, Figure S1C).

In the endothelium of both mesenteric arteries and thoracic aortas, the expression of PXDN in db/db mice was significantly increased and co-localized with CD31 (marker of endothelium) compared to db/m mice (Fig. 1G, Figure S1H and Figure S1I). Moreover, expression level of RAGE, 3-Cl-Tyr was also increased in the mesenteric artery and thoracic aortas endothelium of db/db animals (Fig. 1G, Figure S1J and K) compared to db/m mice.

3.2. Knockdown of PXDN attenuates AGEs induced endothelial dysfunction in db/db mice

To clarify the role of PXDN in AGEs induced diabetic endothelial dysfunction, PXDN siRNA and AGEs breaker (Alagebrium, ALT-711) were used in diabetic mice. After tail vein siRNA injection three times a week, mice were injected intraperitoneally with ALT-711 simultaneously until sacrificed at 12 weeks (Fig. 2A). Body weight, fasting blood glucose, Hba1c and other serum biochemical markers of mice were measured and summarized in Supplementary table 2. The knockdown efficiency of PXDN was detected by Western blot. As shown in Fig. 2G, PXDN expression was significantly inhibited after injection of PXDN siRNA in aortas of mice (78.6% reduction, p < 0.01). We further found that si-PXDN treatment significantly improved the Ach-induced aortic endothelium-dependent relaxation (64.67% vs 34.20%, Fig. 2B) and plasma NO level (44.35 ± 8.46 μmol/L vs 24.17 ± 6.85 μmol/L, Fig. 2F) compared to db/db mice, while without affecting the endothelium-independent relaxation responses to SNP (Fig. 2D). In addition, treatment with ALT-711 combined with si-PXDN in diabetic mice improved vascular function (Ach-induced relaxation: 66.97%; NO level: 50.11 ± 10.59 μmol/L), which was similar to the effect of si-PXDN but more significant than ALT-711 alone (Ach-induced relaxation: 47.45%; NO level: 38.36 ± 6.98 μmol/L) (Fig. 3–D). Whereas all of these treatments did not alter vascular function in non-diabetic mice. ALT-711 treatment remarkably inhibited RAGE, NOX2 expression and H2O2 production in the aortas of db/db mice (Fig. 2G and H and Figure S2A) while si-PXDN did not affect this change. PXDN and 3-Cl-Tyr level were declined significantly in db/db mice treated with ALT-711, si-PXDN or both, which accompanied with the reduce of superoxide and oxidative stress products 4-HNE and MDA (Fig. 2G–I, 3A–C and Figs. 3A and S3C). Moreover, the level of eNOS phosphorylation on Ser1177 in aortas of db/db mice was rescued after treated with ALT-711, si-PXDN or both. (Fig. 2G).

Researches show that P38/Akt pathway is involved in regulating endothelial ENOS phosphorylation and vasodilation [25–27]. In addition, we found that phosphorylation level of Akt on Ser473 in aortas of db/db mice was significantly decreased (Fig. 1D, Figure S1G). This reduction was recovered after PXDN knockdown (Figure S2B), which suggests that Akt may be involved in PXDN-mediated diabetic vascular endothelial dysfunction.

3.3. Knockdown of PXDN reduces HOCl and ROS levels in both microvascular and macrovascular endothelium of db/db mice

Diabetic endothelial dysfunction occurs more than in the macrovascular and also in microvessels such as the mesenteric artery. To further determine the effect of PXDN knockdown on different arteries, DHE and immunofluorescent staining was used to detect arterial superoxide and HOCl production after PXDN knockdown. As shown in Figure S4, superoxide levels were declined in mesenteric artery after db/db were treated with ALT-711, si-PXDN or both. Consistent with the results of Western blot, 3-Cl-Tyr protein expression was significantly decreased in the thoracic aortas of db/db mice treated with si-PXDN (Fig. 3B–D). In addition, si-PXDN combined with ALT-711 has more significant effect on reducing 3-Cl-Tyr protein expression than treated with ALT-711 alone in db/db mice (Figure S3B and S3D). Interestingly, these changes also appeared in the mesenteric artery, which was even more obvious (Fig. 3B–E, Figure S3B and S3E).
Fig. 1. PXDN expression is elevated in db/db mice with impaired endothelium-dependent relaxation. A and B, endothelium-dependent and endothelium-independent relaxation of thoracic aortic rings were assessed by vascular functional experiments; C, nitrite concentration in plasma was measured by Total Nitric Oxide Assay Kit; D, expression of RAGE, NOX2, p-eNOS<sub>ser1177</sub>/total eNOS and p-Akt<sub>ser473</sub>/total Akt in thoracic aortic tissues of db/db and db/m mice was measured by Western blot; E, expression 4-HNE in thoracic aortic tissues of db/db and db/m mice was measured by Western blot; F and J, expression of PXDN and 3-Cl-Tyr in thoracic aortic tissues of db/db and db/m mice was measured by Western blot and quantified by Image Lab software; G, H<sub>2</sub>O<sub>2</sub> concentration of thoracic aortas was assessed by Hydrogen Peroxide Assay Kit; I, MDA concentration of thoracic aortas was measured by MDA kit; n = 6 mice for each group. Tissue superoxide level was measured by DHE staining, and expression of PXDN, 3-Cl-Tyr and RAGE in thoracic aortas and mesenteric arteries was measured by immunofluorescence staining. G, representative cross-sections of mesenteric arteries and thoracic aortas stained with DHE (red), PXDN (red), CD31 (green), RAGE (green), 3-Cl-Tyr (green) and DAPI (blue) in db/m and db/db mice; K, quantification of DHE staining fluorescence intensity within the thoracic aortic and mesenteric arterial endothelial layer; n = 6 mice for DHE staining; n = 3 mice for immunofluorescence staining. Data are expressed as the mean ± SEM. Bar = 500 μm for DHE staining of thoracic aortas and 50 μm for other fluorescence staining. Two-way ANOVA and Student–Newman–Keuls post-hoc testing was used for concentration–response curves. Unpaired Student’s t-test was used for other comparison of compared groups. **p < 0.01, ***p < 0.001.
Fig. 2. Knockdown of PXDN attenuates AGEs induced endothelial dysfunction in db/db mice. A, experimental procedure for injecting siRNA, ALT-711 or both in mice; B-E, endothelium-dependent and endothelium-independent relaxation of thoracic aortic rings after mice received injections of siRNA, ALT-711 or both; F, Plasma nitrite concentration of db/m and db/db mice after received siRNA, ALT-711 or both injections was measured by Total Nitric Oxide Assay Kit; G, expression of RAGE, PXDN, 3-Cl-Tyr, 4-HNE and p-eNOSSer1177/total eNOS in thoracic aortas was assessed by Western blot, and quantification of protein expression by Image Lab. H, H$_2$O$_2$ concentration of thoracic aortas was assessed by Hydrogen Peroxide Assay Kit; I: MDA concentration of thoracic aortas was measured by MDA kit. Data are expressed as the mean ± SEM (n = 6 for each group). Two-way ANOVA and Student–Newman–Keuls post-hoc testing were used for concentration-response curves. One-way ANOVA test followed by Student–Newman–Keuls or Kruskal-Wallis H followed by Dunnett’s Test were used for other comparison of compared groups. **p < 0.01 versus db/m mice; #p < 0.05 versus db/db mice, ##p < 0.01 versus db/db mice.
Fig. 3. Knockdown of PXDN reduces microvascular and macrovascular endothelial HOCl and ROS levels. A, representative cross-sections of thoracic aortas stained with DHE probe in db/m and db/db mice after injected with PXDN siRNA; B, representative cross-sections of thoracic aortas and mesenteric arteries stained with 3-Cl-Tyr (green) and DAPI (blue) in db/m and db/db mice after injected with PXDN siRNA. C, quantification of DHE staining fluorescence intensity within the thoracic aortic endothelial layer using Image J; D and E, quantification of microvascular and macrovascular 3-Cl-Tyr staining fluorescence intensity within the endothelial layer using Image J. Bar = 500 μm for DHE staining of thoracic aortas and 50 μm for other fluorescence staining. Data are expressed as the mean ± SEM (n = 6 mice for each group). One way-ANOVA followed by Student–Newman–Keuls test was used for compared groups. **p < 0.01; ***p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
3.4. AGE-BSA attenuates phosphorylation of eNOS and activates PXDN/HOCl pathways in cultured HUVECs

AGEs were used as a stimulus to mimic the pathologic state of diabetic endothelial dysfunction in vitro. As shown in Figure S5A and D, HUVECs were treated with AGE-BSA, protein expression of RAGE was up-regulated in a concentration-dependent and time-dependent manner. Inversely, phosphorylation level of eNOS on Ser1177 and Akt on Ser473 was reduced (Figure S5B, C and F). In addition, the expression level of PXDN and 3-CI-Tyr was increased (2.5-fold, Figure S5G; 2.3-fold, Figure S5I; 2.2-fold, S2H; 2.2-fold, Figure S5J). Moreover, the induction of PXDN expression peaked at 48 h after treatment with 200 μg/mL AGE-BSA.

3.5. PXDN silencing attenuates the effect of AGE-BSA on eNOS phosphorylation

To further explore the mechanism of PXDN regulating AGEs induced endothelial dysfunction, HUVECs were transfected with siRNA against PXDN. PXDN protein expression was successfully knocked down by PXDN-siRNA in HUVECs (75.0% reduction, p < 0.01, Figure S6A). The production of superoxide, oxidative stress products (MDA and 4-HNE) were significantly reduced in PXDN-siRNA transfection compared to the negative control siRNA transfected cells after treatment with AGEs, while the level of H$_2$O$_2$ was not affected (Fig. 4A–C and Figure S7A-C). In addition, expression of 3-CI-Tyr was suppressed by si-PXDN after AGEs stimulation (Fig. 4D and E). Consistent with results of mice, PXDN silencing improved the reduction of Akt and eNOS phosphorylation (Fig. 4F and G) and NO level (20.72 ± 1.97 μmol/L vs 9.46 ± 2.23 μmol/L, Fig. 4H) upon AGE-BSA treatment.

3.6. HOCl reduces eNOS phosphorylation via phosphorylation of Akt

Previous studies have demonstrated that PXDN-derived HOCl exacerbates vascular oxidative stress [17,28]. To clarify this effect on endothelial dysfunction, HUVECs were incubated with different concentrations of HOCl (10 μmol/L and 100 μmol/L). HOCl treatment remarkably reduced phosphorylation of Akt on Ser473 and eNOS on Ser1177 (Fig. 5A and B). NO level was also reduced (10.78 ± 2.80 μmol/L vs 23.25 ± 2.48 μmol/L, Fig. 5E) in HOCl treated cells. Moreover, the effects of HOCl on eNOS phosphorylation and NO production (5.85 ± 1.87 μmol/L vs 11.69 ± 2.11 μmol/L) were aggravated by Akt specific inhibitor MK2206 (Fig. 5C, D and F).

3.7. H$_2$O$_2$, ROS scavengers and NOX inhibitors attenuate PXDN-mediated endothelial dysfunction induced by AGEs

PXDN is reported to aggravate NOX-mediated oxidative stress by utilizing chlorine and H$_2$O$_2$ to generate HOCl in endothelial cell apoptosis and smooth muscle cell proliferation [16,29]. To further explore the role of this mechanism in AGEs-induced endothelial dysfunction, H$_2$O$_2$ scavengers (PEG-catalase and ebsehol), NOX inhibitors (VAS2870 and GSK2795039) and ROS scavenger (tempol) were used. We found that, H$_2$O$_2$ scavengers, NOX inhibitors and ROS scavenger decreased intracellular oxidative stress (Fig. 6A–C and Figure S7G-J). Moreover, these scavengers attenuated the induction of PXDN and 3-CI-Tyr expression by AGEs (Fig. 6D and E), accompanied by the recovery of phosphorylation of Akt and eNOS (Fig. 6F and G) and NO production (20.14 ± 2.36 μmol/L, 18.93 ± 2.03 μmol/L, 20.54 ± 2.14 μmol/L, 21.85 ± 2.74 μmol/L and 18.31 ± 1.83 μmol/L vs 12.28 ± 2.15 μmol/L, Fig. 6H) in HUVECs.

3.8. NOX2 silencing attenuates the effect of AGE-BSA on PXDN and its downstream pathway, restoring endothelial function

Studies show that NOX2 play a critical role in AGEs-induced ROS production and endothelial dysfunction [30,31]. In the present study, we also observed a significant increase of NOX2 expression in the aortas of db/db mice (Fig. 1D). And NOX2-specific inhibitor had more significant effect on ROS attenuation than non-specific NOX inhibitor and mitochondrial ROS scavenger (Figure S1-L-N), which suggested NOX2 may be a major source of ROS in diabetic mice. In order to elucidate whether NOX2 mediates PXDN in AGEs-induced endothelial dysfunction, NOX2 siRNA was used. NOX2 protein expression was successfully knocked down by NOX2-siRNA in HUVECs (80.4% reduction, p < 0.01, Figure S5B). Surprisingly, NOX2-siRNA transfection did not affect the expression of its family member NOX4 while decreased the level of NOX1 in HUVECs (Figure S5A and S5B). The results show that intra-cellular H$_2$O$_2$, superoxide, 4-HNE and MDA were declined with NOX2-siRNA transfection, compared to the control siRNA transfected cells treated with AGEs (Fig. 7A–C and Figure S7D-F). We also found the expression of PXDN and 3-CI-Tyr was significantly down-regulated in the si-NOX2 transfected group after treated with AGEs compared with the control group (Fig. 7D and E). Additionally, NOX2 silencing improved phosphorylation reduction of Akt and eNOS (Fig. 7F and G) and NO level in cell supernatants (18.79 ± 2.42 μmol/L vs 10.59 ± 2.68 μmol/L, Fig. 7H) upon AGE-BSA treatment.

4. Discussion

Various clinical trials (ACCORD, ADVANCE and VADT Trials) have shown that diabetes-related vascular complications exist and even develop despite strict and long-term glycemic control [32–34]. AGEs are recognized as a key inductor for contributing to this phenomenon, while the mechanism remains unknown [35]. In the present study, we found that: (i) In db/db mice with impaired endothelial function, PXDN expression was increased. Knockdown of PXDN restored the impaired endothelium-dependent relaxation function of db/db mice, increased eNOS phosphorylation and NO production. (ii) In cultured HUVECs cells, PXDN silencing significantly inhibited AGEs-induced endothelial dysfunction, including improved eNOS phosphorylation on Ser1177 and NO production. (iii) The effect of PXDN on AGEs-induced endothelial dysfunction is mediated by NOX2/HOCl/Akt/eNOS pathway.

In diabetes with a prolonged state of hyperglycemia, AGEs were accumulated from the non-enzymatic glycation or glycoxidation of proteins, lipids, and nucleic acids. It is well-known that AGEs increase ROS by binding to RAGE and promote diabetic vascular endothelial dysfunction [36]. Myeloperoxidase (MPO), the heme-containing peroxidase highly expressed in leukocytes, is previously reported to leads to endothelial dysfunction by reducing the bioavailability of NO under inflammatory conditions of diabetic vascular disease, [37,38]. Unlike MPO, PXDN is highly expressed in the cardiovascular system including endothelial cell, vascular smooth muscle cell and extracellular matrix [18,29,39,40]. Moreover, as a secreted protein, the concentration of PXDN in plasma is 1000-fold higher than MPO [41]. It has been previously reported that PXDN catalyzes the oxidation of chloride in the presence of H$_2$O$_2$ to generate HOCl. The enzymatic function of PXDN plays a physiological role in participating in host immune against gram-negative bacterial pathogens [42,43], and was reported to aggravate NOX-mediated oxidative stress under pathological progress [16,29]. Multiple studies reported that PXDN contributes to pathological procedure of cardiovascular diseases such as vascular calcification, hypertension and cardiac hypertrophy [18,44,45]. Especially, our former researches have demonstrated that PXDN plays a key role in the endothelial cell senescence, apoptosis and endothelial progenitor cell dysfunction [46-50]. What’s more, the preliminary research found that PXDN showed a significant function in regulating eNOS expression and activity [31]. These findings suggests that PXDN may be an important molecule in mediating AGEs-induced diabetic endothelial dysfunction. Here, we observed that the expression of PXDN and 3-CI-Tyr was increased in the thoracic aortic and mesenteric arterial endothelium of db/db mice and AGEs-induced HUVECs cells. Knocking down of PXDN...
Fig. 4. PXDN silencing attenuates the effect of AGE-BSA on eNOS phosphorylation through HOCl/Akt pathway. Cells were transfected with either a negative control siRNA (si-NC) or PXDN siRNAs (si-PXDN) for 24 h. The medium was then replaced by endothelial cell medium containing 200 μg/mL AGE-BSA for 48 h. A, intracellular superoxide was measured using DHE staining; B, quantification of DHE staining fluorescence intensity by Image J; C, intracellular H$_2$O$_2$ concentration was assessed by Hydrogen Peroxide Assay Kit; D and E, cell expression level of 3-Cl-Tyr was measured by Western blot and quantified by Image Lab; F and G, phosphorylation level of p-Akt$_{Ser473}$/total Akt and p-eNOS$_{Ser1177}$/total eNOS was measured by Western blot and quantified by Image Lab; H, nitrite concentration in cell supernatants was measured by Total Nitric Oxide Assay Kit. Bar = 200 μm for DHE staining of HUVECs. Data are expressed as the mean ± SEM (n = 6 for each group). An ANOVA and a subsequent Student–Newman–Keuls test were conducted for this figure. **P < 0.01 versus control, ***P < 0.001; ##p < 0.01, ###p < 0.001; NS: not significant.
restored endothelium-dependent relaxations, increased NO production in db/db mice. Consistent with the findings of mice, PXDN knockdown also inhibited AGEs-induced endothelial dysfunction in cultured HUVECs. Therefore, we speculate that PXDN may mediates AGEs-induced diabetic vascular endothelial dysfunction.

Previous studies have confirmed that alteration of eNOS activity in the endothelium is one important mechanism of endothelial dysfunction in diabetes [52,53]. The activity of eNOS is mainly regulated by the formation of homodimer, and the post-modification of multisite phosphorylations [54,55]. Among these mechanisms, decreased phosphorylation level in serine 1177 site is believed to have a pivotal role in diabetic endothelial dysfunction [56,57]. In this study, we found that phosphorylation of eNOS on ser1177 was reduced both in thoracic aortas of db/db mice and AGEs-induced HUVECs cells. Moreover, PXDN knockdown significantly improved the reduced eNOS phosphorylation on ser1177, which suggest that PXDN may promote AGEs-mediated diabetic endothelial dysfunction by inhibiting the phosphorylation of eNOS on Ser1177. Akt/protein kinase B (PKB) phosphorylates eNOS at serine 1177, which directly increases eNOS activity [27,58]. Previous studies have found that Akt/eNOS signaling pathways are extensively damaged in diabetes models induced by different pathogenic factors such as insulin resistance and hyperglycemia [59–61]. We further found Akt phosphorylation were impaired by measuring it in thoracic aortas of diabetic mice and HUVECs induced by AGEs. More critically, these injuries were recovered after PXDN knockdown.

We have previously reported that PXDN catalyzes HOCl formation and participates in many pathophysiology of cardiovascular diseases [16,20,62]. In order to further elucidate the specific mechanism of PXDN in endothelial dysfunction, HUVECs were treated with exogenous HOCl. We found that HOCl treatment significantly reduced eNOS
Fig. 6. H$_2$O$_2$, ROS scavengers and NOX inhibitors attenuate PXDN-mediated vascular endothelial dysfunction induced by AGEs. Cells were incubated with 400 units PEG-catalase, 40 μM ebselen, 10 μM VAS2870, 25 μM GSK2795039 or 10 μM Tempol for 1 h and then exposed to AGE-BSA (200 μg/mL) for 24 h. A and B, DHE staining and quantification of DHE fluorescence intensity by Image J; C, intracellular H$_2$O$_2$ concentration was assessed by Hydrogen Peroxide Assay Kit; D and E, cell expression level of PXDN and 3-Cl-Tyr was measured by Western blot and quantified by Image Lab; D and E, phosphorylation level of p-Akt$^{Ser473}$/total Akt and p-eNOS$^{Ser1177}$/total eNOS was measured by Western blot and quantified by Image Lab; H, nitrite concentration in cell supernatants was measured by Total Nitric Oxide Assay Kit. Bar = 200 μm for DHE staining of HUVECs. Data are expressed as the mean ± SEM (n = 6 for each group). An ANOVA and a subsequent Student–Newman–Keuls test were conducted for this figure. **p < 0.01 versus control, ##p < 0.01 versus AGE-BSA treatment.
phosphorylation on Ser1177 by attenuating Akt phosphorylation, which finally reduced the production of cellular NO. And the Akt inhibitor (MK2206) reduced eNOS phosphorylation and NO level induced by HOCl. These results suggest that HOCl mediated eNOS phosphorylation via activating Akt, leading to NO consumption and AGEs-induced endothelial dysfunction.

Our previously studies have identified PXDN is response to NOX-activated redox signaling [16,18,29]. NOX2 that highly expressed in...
endothelial cells is the main source of ROS and plays a critical role in AGEs-induced endothelial dysfunction [11,31,63–65]. In addition to NOX2, other isoforms of NOX (NOX1 and NOX4) are also expressed in vascular tissues, of which NOX1 is mainly expressed in smooth muscle cells and NOX4 is mainly expressed in endothelial cells [66,67]. Excess superoxide production derived from NOX2 and NOX1 in response to diabetic stimulation results in impaired endothelial function, while defining the role of NOX4 has been elusive [68,69]. Some studies suggest that NOX4 may exhibit protective function for decreasing insulin resistance via inhibition of PTP1B [70]. Therefore, NOX2 is considered to be a key enzyme in the NOX family that promotes diabetic endothelial dysfunction. In agreement of these results, we found that expression of NOX2 in aortas of db/db mice was significantly higher than that of NOX1 and NOX4. Moreover, NOX2-specific inhibitor significantly reduced ROS production in the aortas and mesenteric arteries of db/db mice compared to non-specific NOX inhibitor and mitochondrial ROS scavenger. Previous studies have found that the NOX2/PXDN pathway is involved in a series of pathophysiological processes including hypoxia-induced vascular remodeling and angiogenesis B-induced myocardial hypertrophy [45,71]. Based on the high expression and cellular localization of NOX2 in db/db mice, we hypothesized NOX2 may participate in PXDN-mediated endothelial dysfunction induced by AGEs. We found NOX2 significantly increased in thoracic aortas of db/db mice, which could be suppressed by AGEs breaker. After NOX2 silencing, H2O2, ROS scavenger and NOX inhibitors were used to HUVECs, we found that both NOX2 silencing, H2O2, ROS scavengers and NOX inhibitors attenuated the AGEs-induced damage on phosphorylation of Akt and eNOS, up-regulating NO level by inhibiting PXDN expression. These results suggest PXDN is a downstream regulator of NOX2 in AGES-induced endothelial dysfunction.

Here, we firstly showed that PXDN is a mediator in AGES-induced diabetic vascular endothelial dysfunction via HOCl/Akt/eNOS pathway. We found both PXDN and HOCl could regulate phosphorylation of Akt in diabetic mice and endothelial cells. Admittedly, the mechanism that PXDN regulates Akt phosphorylation remains to be further elucidate. Previous study reported Akt Ser 473 phosphorylation is related to several factors such as mTORC2 Ack1, Src, PTK6, TBK1 and IKBK [72,73]. Thus, more studies need to be performed to explore the pathway of PXDN-mediated Akt phosphorylation. What is more, to clarify the role of PXDN in AGES in induced vascular endothelial dysfunction in vivo, endothelium-specific genetically modified animals are needed in the future.

5. Conclusion

To summarize, our results suggested that PXDN may promote AGES-induced diabetic vascular endothelial dysfunction by attenuating eNOS phosphorylation on Ser1177 via NOX2/HOCl/Akt pathway (Fig. 8). These novel findings suggest a potential target for the early intervention of diabetic vascular disease.

Data availability

All the raw data are available on request from the corresponding authors.

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Fig. 8. The proposed pathway by which PXDN mediates AGES-induced diabetic endothelial dysfunction. AGES bind their receptor RAGE and subsequently upregulates the expression of NOX2, which is an important source of ROS. This increase in ROS, increases PXDN expression and HOCl production. PXDN-derived HOCl decreases phosphorylation of eNOS on serine 1177 by blocking Akt phosphorylation on serine 473. The decrease of eNOS phosphorylation were found to correlate intimately with endothelial dysfunction.

Contribution statement

Jing Cao, Guogang Zhang, and Ruizheng Shi conceived and designed the study; Jing Cao, Zhaoya Liu, and Qian Xu contributed to data acquisition; Guangjie Cheng and Chan Li analyzed the data; all authors contributed to the writing of this manuscript. Ruizheng Shi is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Declaration of competing interest

None.

Authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102031.

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