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Protectin DX prevents H_2O_2-mediated oxidative stress in vascular endothelial cells via an AMPK-dependent mechanism

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ABSTRACT

Protectin DX (PDX), which is a novel regulator of 5′ adenosine monophosphate-activated protein kinase (AMPK), has recently gained attention for its ability to improve several metabolic diseases. However, the function of PDX in vascular endothelial cells remains unclear. To confirm the protective effects of PDX on endothelial oxidative stress, human umbilical vein endothelial cells (HUVECs) were treated with hydroperoxide (H_2O_2) and PDX. PDX treatment significantly increased the level of AMPK phosphorylation, and this elevation was attenuated after treatment with G-protein coupled receptor 120 (GPR120) antagonist or GPR120 knockdown. Expressions and activities of antioxidant proteins, including catalase and superoxide dismutase 2 (SOD2), were elevated by PDX and were inhibited by treatment with AMPK inhibitor or with GPR120 antagonist. Production of H_2O_2-induced reactive oxygen species (ROS), the Bax/Bcl-2 ratio, and the loss of mitochondrial membrane potential were all reversed by PDX, leading to improved cell viability and reduced release of lactate dehydrogenase (LDH). Using flow cytometry, we also found that PDX significantly reduced the H_2O_2-induced apoptotic population of cells. These protective effects of PDX were all reversed after treatment with AMPK inhibitor or GRP120 antagonist. These results show that the PDX-AMPK axis has a protective role against H_2O_2-induced oxidative stress in vascular endothelial cells.

1. Introduction

Vascular endothelial dysfunction is a major physiologic feature observed in patients with cardiovascular disease (CVD) and can initiate atherosclerotic processes, leading to plaque formation and rupture [1]. Generally, endothelial dysfunction is accelerated by excessive oxidative stress [2]. Oxidative stress leads to oxidation of low-density lipoprotein, whose uptake by macrophages is easier compared to that of non-oxidized lipoprotein [3]. Although the imbalance of reactive oxygen species (ROS) production results in inflammation, loss of vasodilation, and disruption of endothelial homeostasis [4], there are no effective therapies for the reduction of oxidative stress to prevent the progression of atherosclerosis.

Protectin DX (PDX), a known isomer of protectin D1, is produced through double lipoxygenation of docosahexaenoic acid (DHA), an unsaturated fatty acid that belongs to the omega-3 family [5]. Omega-3 fatty acids have potential anti-inflammatory activity in a variety of human diseases [6]. Yan et al. [7] reported that stimulation of macrophages with omega-3 fatty acids, including DHA, abolished activation of nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome and inhibited subsequent caspase-1 activation. Additionally, Liu et al. [8] demonstrated for the first time that PDX decreases ROS production, inhibits NADPH oxidase (NOX) activation, and reduces myeloperoxidase (MPO) release from neutrophils, suggesting that PDX could be a protective agent against neutrophil invasion in chronic inflammatory diseases. Recently, PDX has received considerable attention as a novel regulator of several metabolic disorders. White et al. [9] found that PDX treatment stimulated the release of interleukin-6 from skeletal muscle in obese diabetic *db/db* mice, thereby blunting hepatic glucose production and improving insulin sensitivity through a myokine-liver glucoregulatory axis. Furthermore, Jung et al. [10,11] reported that PDX ameliorates palmitate-induced

Abbreviations: AMPK, 5′ adenosine monophosphate-activated protein kinase; ANOVA, analysis of variance; Bax, Bcl2-associated X protein; Bcl2, B-cell lymphoma-2; CVD, cardiovascular disease; DHA, docosahexaenoic acid; GPR120, G-protein coupled receptor 120; H_2O_2, hydroperoxide; HUVECs, human umbilical vein endothelial cells; LDH, lactate dehydrogenase; PDX, Protectin DX; PI, propidium iodide; ROS, reactive oxygen species; SOD2, superoxide dismutase 2

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hepatic endoplasmic reticulum (ER)-stress and muscular inflammatory response via an AMP-activated protein kinase (AMPK)-dependent mechanism. In those studies, gluconeogenesis induced by a high-fat diet (HFD) was significantly decreased after PDX treatment [10], and impaired insulin signaling was recovered by PDX in palmitate-treated C2C12 cells and in soleus skeletal muscle of HFD-fed mice [11]. Although all these results strongly suggest the potential of PDX to maintain metabolic homeostasis under oxidative stress, there has been no study examining the protective role of PDX on vascular endothelial dysfunction in response to oxidative stress.

To determine the anti-oxidative effects of PDX against hydroperoxide (H_2O_2) in endothelial cells, we examined the following: i) whether PDX treatment enhances AMPK-mediated signaling pathways; ii) whether PDX treatment reduces ROS production by increasing the expression of antioxidant genes; and iii) whether PDX treatment regulates the expression of apoptosis-related molecules, mitochondrial membrane potential, and vascular endothelial cell-death under H_2O_2 treatment. More specifically, we examined whether the effects of PDX treatment are modulated by G-protein-coupled receptor 40 (GPR40) or 120, which are activated by medium or long-chain fatty acids [12].

2. Materials and methods

2.1. Cell culture and compounds

HUVECs (Invitrogen, Carlsbad, CA, USA) were cultured on gelatin-coated dish with 0.2% M200PRF medium (Invitrogen) containing low serum growth supplement (LSGS) (Invitrogen). The cells were incubated at 37 °C in a 5% CO_2 atmosphere. When reaching > 90% confluence, the cells were stimulated with several compounds, including H_2O_2 (Biosesang, Korea), PDX (Cayman Chemical, MI, USA), AMPK inhibitor (Compound C; Sigma Aldrich, MO, USA), GPR40 antagonist (GW1100, Cayman Chemical), and GPR120 antagonist (AH7614, Cayman Chemical).

2.2. GPR120 knockdown

When HUVECs reached > 90% confluence, scramble or GPR120 siRNAs (Santa Cruz Biotechnology, CA, USA) were inserted into the cells using Lipofectamine™ transfection reagent (Invitrogen) according to the manufacturer's instructions. After one day, the efficiency of GPR120-knockdown was verified by Western blotting.

2.3. Western blotting

PRO-PREP™ (iNtRON, Sungnam, Korea) was used to extract total protein from HUVECs. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose (NC) membranes (Amersham Bioscience, Westborough, MA, USA). The NC membranes were sequentially incubated with blocking solution (0.05% TBST containing 5% non-fat dry milk or 5% bovine serum albumin), blocking solution containing primary antibodies, and blocking solution containing horseradish peroxidase (HRP)-conjugated secondary antibodies (Vector Laboratories, CA, USA). The HRP-bound NC membranes were washed using 0.05% TBST and then incubated with chemiluminescence solution (Bio-Rad) to detect immunoreactive bands in the dark. The bands were analyzed using the ImageJ program (National Institutes of Health, MD, USA).

Antibody sources were as follows: anti-beta actin mouse monoclonal IgG (1:5000 dilution), anti-total-AMPK rabbit polyclonal IgG (1:1000 dilution), anti-GPR120 mouse monoclonal antibody (1:1000 dilution), anti-catalase mouse monoclonal IgG (1:1000 dilution), anti-superoxide dismutase 2 (SOD2) mouse monoclonal IgG (1:500 dilution; Santa Cruz Biotechnology), anti-p-AMPK rabbit monoclonal antibody (1:2000 dilution), anti-Bcl2-associated X protein (Bax) rabbit polyclonal antibody (1:1000 dilution), anti-B-cell lymphoma-2 (Bcl-2) rabbit polyclonal antibody (1:500 dilution; Cell Signaling Technology, Boston, MA, USA).

2.4. ROS detection

To measure the production of ROS, HUVECs were incubated with M200PRF medium containing 10 μM dihydroethidium (DHE, red; Invitrogen) for 30 min at 37 °C. The cells were washed with phosphate buffered saline (PBS, Biosesang) and fixed in 3.6% formaldehyde (Junsei Chemical, Japan) for 10 min. The fixed cells were observed under a fluorescence microscope (Olympus, Japan) to obtain fluorescence images, and then ROS levels were quantified using the ImageJ program (National Institutes of Health).

2.5. The removal rate of ROS

Total protein from HUVECs was quantified using Bradford methods. The removal rate of superoxide and H_2O_2 was determined from the equal amounts of protein using EZ-Catalase Assay (DG-CAT400) and EZ-SOD Assay (DG-SOD200) kits (DoGenBio CO., Ltd. Seoul, Korea) according to the manufacturer's instructions.

2.6. JC-1 staining

To identify the role of PDX in mitochondrial membrane potential, HUVECs were stained with M200 medium containing 5 μg/mL cyanine dye JC-1 (BioVision, CA, USA) for 30 min. The cells were washed and immediately observed under a fluorescence microscope (Olympus). Intracellular JC-1 dye enters in energized mitochondria because of its higher membrane potential and then converted into red fluorescence aggregates from green fluorescence monomers. Loss of mitochondrial membrane potential was determined by the ratio of green fluorescence to red fluorescence [13].

2.7. Measurement of cell toxicity

Cell viabilities and lactate dehydrogenase (LDH) release levels were measured using an EZ-CYTOX kit (DAEILAP, Seoul, Korea) and EZ-LDH kit (DAEILAP) according to the manufacturer's instructions. The value of optical density (OD) was measured on a microplate reader (Bio-rad).

2.8. Flow cytometry

To measure the apoptotic rate, HUVECs were stained with FITC-conjugated Annexin V antibody and propidium iodide (PI) solution (Cell Signaling Technology). The stained cells were washed and immediately analyzed using a Cytomic FC500 (Beckman Coulter, CA, USA).

2.9. Statistical analysis

The significance of differences between groups was determined by analysis of variance (ANOVA). All graphs are described as the mean ± SD and were obtained from more than three experiments. Results were considered to be significant at P-value < 0.05.

3. Results

3.1. PDX induced AMPK phosphorylation via GPR120 in HUVECs

PDX increased phosphorylated AMPK level through a time- and dose-dependent mechanism (Fig. 1A and B). These elevations were inhibited by treatment with AH7614, a GPR120 antagonist, but not with GW1100, a GPR40 antagonist (Fig. 1C). To assess the role of GPR120 in PDX-induced AMPK phosphorylation, siRNA oligomers for GPR120 or scramble control siRNAs were inserted into HUVECs, and PDX-induced AMPK phosphorylation was significantly inhibited in GPR120-

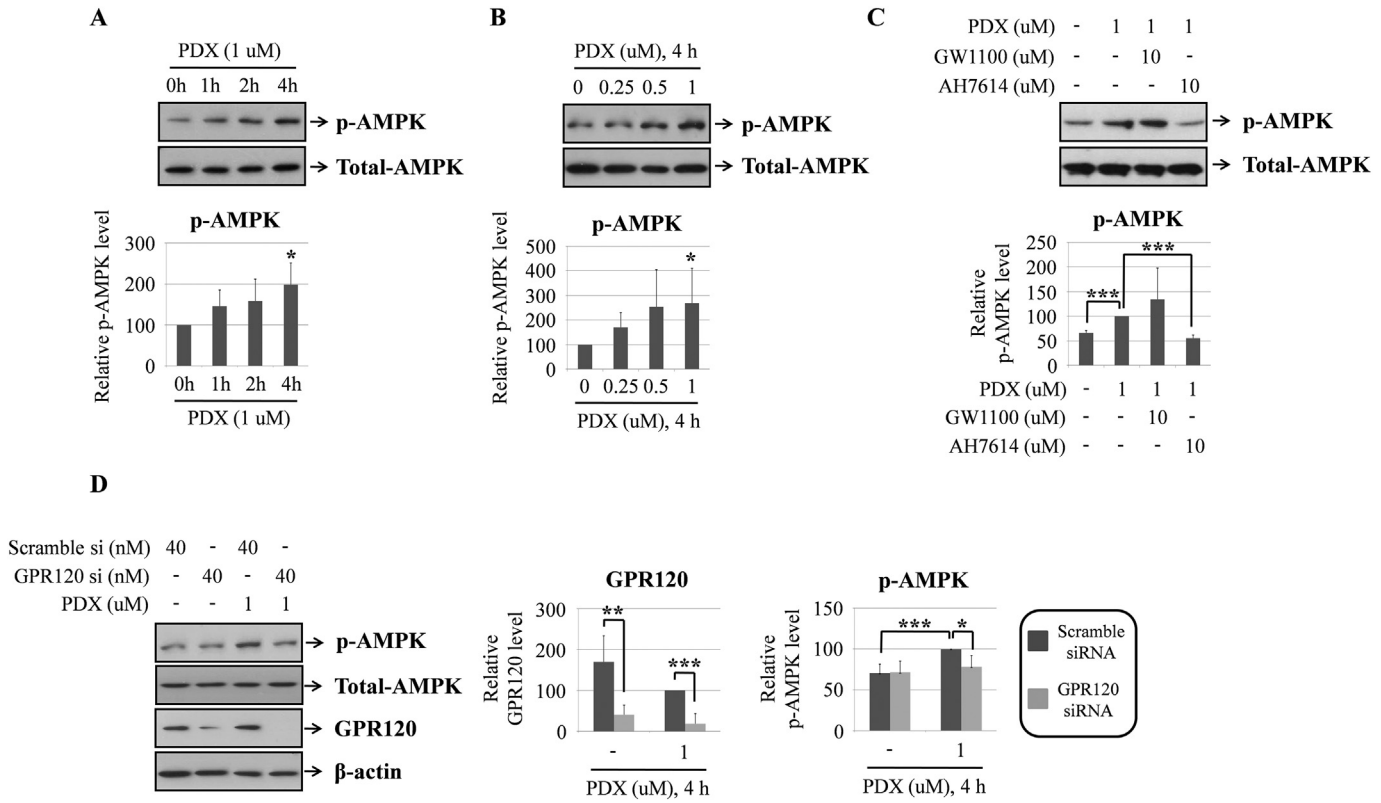

Fig. 1. Treatment with PDX increases AMPK phosphorylation via GPR120 in HUVECs.
A, HUVECs were incubated with PDX (1 μM) for 0, 1, 2, or 4 h. B, Cells were stimulated with the indicated dose of PDX for 4 h. C, HUVECs were treated with PDX, PDX plus GW1100 (10 μM), or PDX plus AH7614 (10 μM) for 4 h. D, Scramble or GPR120 siRNAs (si) were inserted into cells and incubated with PDX for 4 h. Phosphorylated AMPK level was detected by Western blotting. All data were obtained from three separate experiments. Error bars indicate the mean ± SD (*, $P < 0.05$; ***, $P < 0.0005$; ANOVA).

knockdown HUVECs (Fig. 1D).

3.2. PDX reduced ROS production by inducing the expression of antioxidant genes in HUVECs

PDX regulated the expression levels of antioxidant proteins such as catalase and SOD2. Both proteins were significantly upregulated after PDX treatment in a time- and dose-dependent manner (Fig. 2A and B). Treatment with AMPK inhibitor, compound C, or AH7614 reduced PDX-mediated expression of catalase and SOD2 in HUVECs (Fig. 2C). Likewise, the PDX-induced increase in the removal rate of superoxide and H_2O_2 was reversed by treatment with compound C or AH7614 (Fig. 2D). Furthermore, the increased ROS production by H_2O_2 treatment, measured by DHE staining, was significantly decreased after PDX treatment, which was also significantly attenuated by AMPK inhibitor or GPR120 antagonist treatment (Fig. 2E). This suggests that PDX-induced recovery of oxidative stress in endothelial cells is dependent on the AMPK pathway and GPR120 receptor.

3.3. The PDX-AMPK axis inhibits H_2O_2-mediated cytotoxicity in HUVECs

To determine the cytoprotective effects of PDX against H_2O_2-mediated oxidative stress, we first assessed the Bax/Bcl2 ratio and mitochondrial membrane potential in HUVECs. The Bax/Bcl2 ratio is associated with loss of mitochondrial membrane potential and increase in mitochondria-mediated apoptosis [14]. The cyanine dye JC-1 is observed as red fluorescence in mitochondria with high membrane potential, while it fluoresces green in the cytoplasm [13]. The H_2O_2-induced increase in Bax/Bcl-2 ratio was reduced by PDX; however, this protective effect of PDX disappeared after treatment with compound C or AH7614 (Fig. 3A and B). The H_2O_2-mediated decrease in the level of red fluorescence, as indicated by JC-1 staining, was recovered after the treatment of PDX, meaning that the number of functional mitochondria was increased by PDX (Fig. 3C); however, this elevation was significantly attenuated by treatment with compound C or AH7614. The H_2O_2-mediated reduction of cell viability and the elevation of LDH release were blocked after PDX treatment (Fig. 4A and B). Both effects were also reversed after treatment with compound C or AH7614 (Fig. 4C and D). Furthermore, we calculated the rate of apoptosis using flow cytometry. H_2O_2-induced annexin V-positive (total dead cells) and annexin V-positive/PI-negative (early apoptotic cells) populations were all decreased after treatment with PDX, which was significantly reversed by AMPK inhibitor or GPR120 antagonist (Fig. 4E), suggesting that PDX inhibits H_2O_2-mediated cytotoxicity in endothelial cells through AMPK and GPR120.

4. Discussion

We made the following discoveries in endothelial cells: i) PDX treatment increased AMPK phosphorylation; ii) PDX upregulated the expression of antioxidant genes, such as catalase and SOD2; iii) PDX attenuated H_2O_2-mediated ROS production and loss of mitochondrial membrane potential; iv) PDX ameliorated H_2O_2-induced cell death; and v) all PDX-mediated protective effects under oxidative stress were attenuated by treatment with AMPK inhibitor or GPR120 antagonist.

PDX is a newly identified di-oxygenated derivative from DHA, which can be produced by human neutrophils incubated with DHA and can be found in inflammatory exudates [15]. Xia et al. [16] reported that PDX increases survival in a mouse model of sepsis by enhancing phagocytosis of macrophages, decreasing the inflammatory response, and facilitating M2 polarization of peritoneal macrophages. Recently, PDX has been considered a novel AMPK regulator as well as an

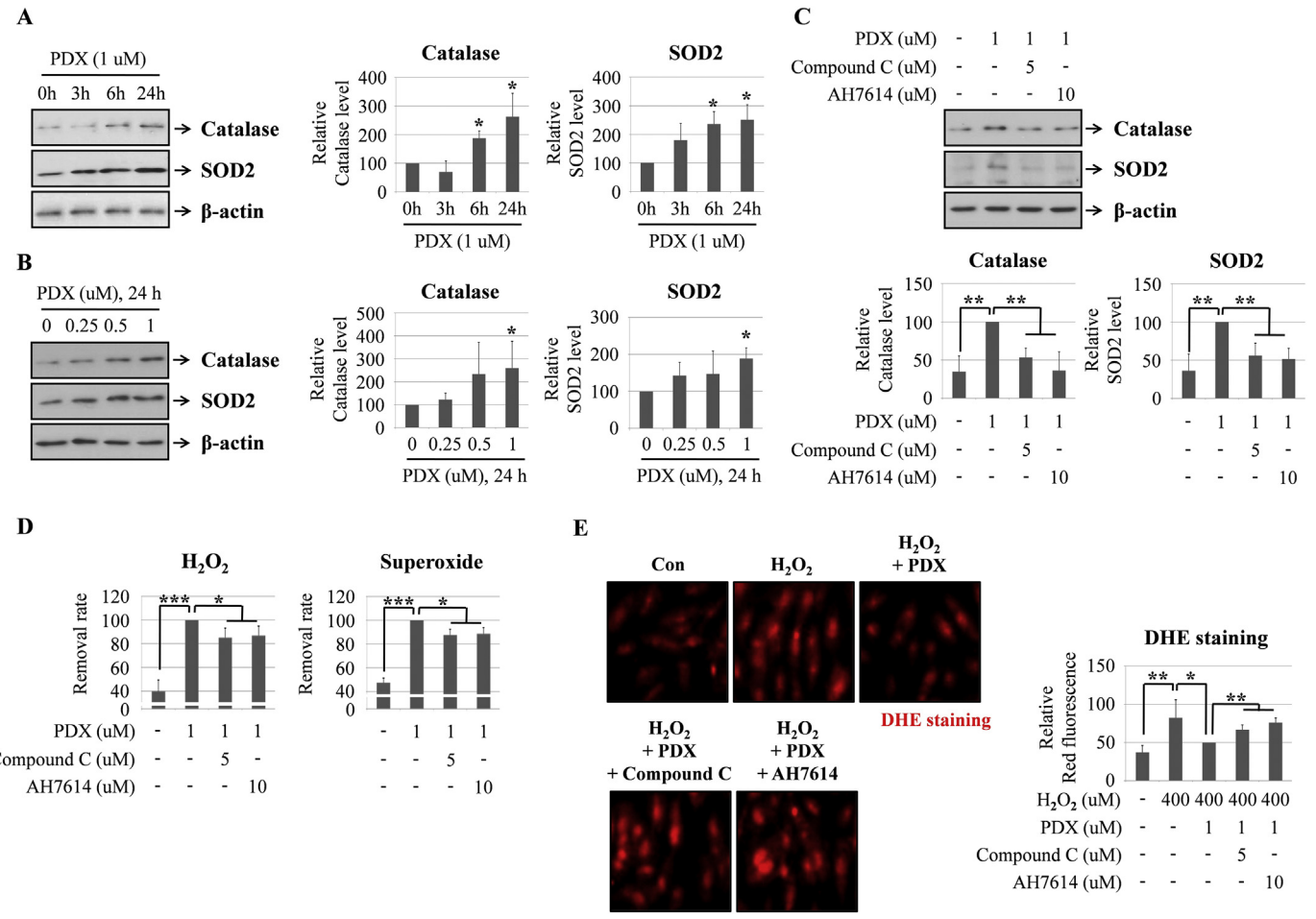

Fig. 2. PDX induces antioxidative effects in HUVECs.
A, HUVECs were stimulated with PDX (1 μM) for 0, 3, 6, or 24 h. B, Cells were incubated with various doses of PDX (0, 0.25, 0.5, or 1 μM) for 24 h. C, Cells were treated with PDX, PDX plus compound C (5 μM), or PDX plus AH7614 (10 μM) for 24 h. Catalase and SOD2 expressions were determined by Western blotting. D. The removal rate of superoxide and H_2O_2 was analyzed using EZ-Catalase Assay and EZ-SOD Assay kits. E, Cells were stained by DHE (red fluorescence; 10 μM) to quantify ROS production. All data were obtained from three separate experiments. Error bars indicate the mean ± SD (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ANOVA).

inflammatory modulator. AMPK signaling is strongly linked to antioxidative, anti-inflammatory, and anti-diabetic effects [17]. White et al. [9] found that PDX treatment activated AMPK in mouse skeletal muscle and alleviated insulin resistance. Furthermore, in palmitate-treated C2C12 cells, AMPK phosphorylation induced by PDX treatment blocked phosphorylation of inhibitor of kappa-B (IκB) and translocation of nuclear factor kappa-B (NF-κB) from the cytosol to the nucleus [11]. In an obese mouse model, decreased hepatic AMPK phosphorylation recovered after PDX administration, which improved hepatic steatosis and endoplasmic reticulum stress [18]. These previous studies demonstrate the critical roles of PDX in maintaining metabolic homeostasis via activation of the AMPK pathway. AMPK also functions as an important molecule that bridges the immune system with atherosclerotic reactions. In macrophages, AMPK activation attenuates NF-κB activity through Sirtuin1 (SIRT1)-mediated deacetylation [19], and it ameliorates oxidized LDL-induced foam cell formation [20]. Based on these studies, we sought to clarify the beneficial effects of PDX on the inhibition of atherosclerotic reactions via the AMPK pathway in endothelial cells. As a result, we found that PDX reduced oxidative stress in endothelial cells, leading to decreased endothelial cell death by AMPK phosphorylation. Next, we determined whether nuclear factor erythroid 2-related factor 2 (Nrf2) is involved in PDX-mediated antioxidative effects via AMPK pathway. Nrf2 is one of the AMPK-downstream molecules and a transcription factor for various antioxidant genes [21]. Recently, Jung et al. reported that the PDX-mediated cellular protective effects were regulated by AMPK-Nrf2 pathway in HepG2 cell line [10]. However, in our experimental condition, the expression of heme oxygenase 1 (HO-1), which is a typical marker of Nrf2 activation, was not changed after the treatment with PDX (data not shown). Besides the Nfr2 gene, there are several other AMPK-downstream molecules to prevent oxidative stress such as peroxisome proliferator activated receptor-gamma coactivator 1alpha (PGC1α) and forkhead Box O3 (Foxo3). Overexpression of PGC1α induced the expression of catalase and SOD2 which resulted in the decrease of ROS production and cellular death [22]. Foxo3-knockdown reduced the expression of various antioxidant genes containing PGC1α [23]. Therefore, these molecules might be the targets to explain the antioxidant effects by the PDX-AMPK axis.

Intracellular ROS levels have been strongly linked to endothelial dysfunction and atherosclerotic processes. Increased endogenous ROS sources, such as NOX, MPO, and nitric oxide synthase (NOS), are the main inducers of oxidative stress in vascular walls and can cause vasoconstriction, platelet aggregation, and attachment of immune cells to the endothelium [3]. In apolipoprotein (ApoE) knockout mice, the overexpression of antioxidant genes, like catalase and SOD1, significantly reduced aortic lipid peroxidation and lesion area [24]. Therefore, inhibiting excessive ROS production in the vascular endothelium is necessary to overcome or prevent atherosclerosis. In the present study, we

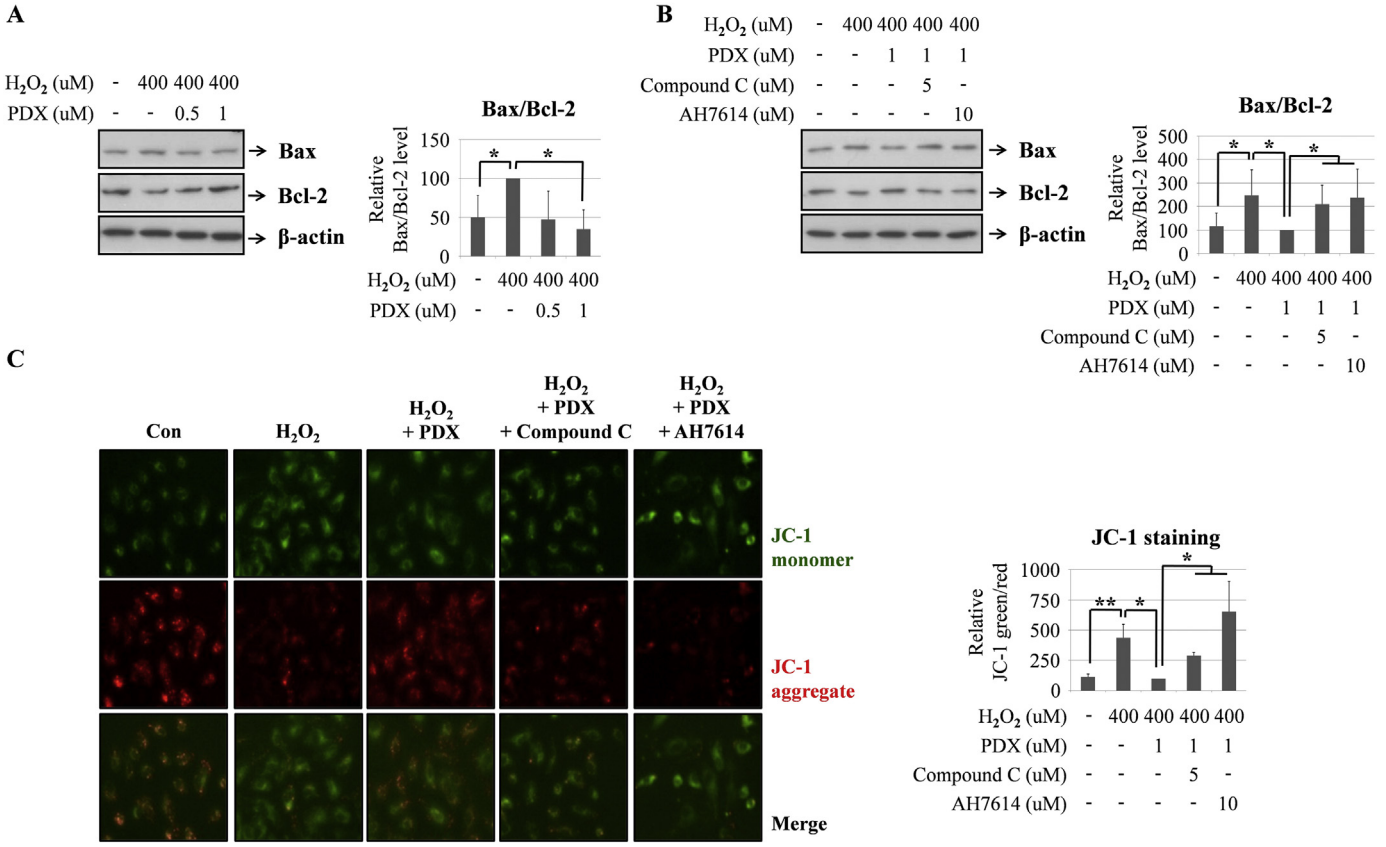

Fig. 3. PDX inhibits H_2O_2-mediated mitochondrial damage in HUVECs.
Cells were pre-incubated with PDX (0.5 or 1 μM), PDX plus compound C (5 μM), or PDX plus AH7614 (10 μM) for 24 h and then stimulated with H_2O_2 (400 μM) plus PDX for 3 h. A and B, The levels of Bax and Bcl-2 were detected by Western blotting. C, HUVECs were treated with JC-1 dye (5 μg/mL) to detect mitochondria with high membrane potential (red fluorescence). Fluorescence images were obtained from each sample using fluorescence microscopy. All data were obtained from three separate experiments. Error bars indicate the mean ± SD (*, $P < 0.05$; **, $P < 0.005$; ANOVA).

identified antioxidative effects of PDX in H_2O_2-treated HUVECs. H_2O_2 treatment can induce intracellular ROS production thought NADPH oxidase, an enzymatic source of superoxide, in endothelial cells [25]. Antioxidant proteins, including catalase and SOD2, were more highly expressed after PDX treatment, and H_2O_2-induced ROS production was significantly decreased in PDX-treated HUVECs. Similar to our data, Liu et al. [8] reported that increased intracellular ROS levels after treatment with phorbol myristate acetate or formyl-methionyl-leucyl-phenylalanine were significantly reduced by PDX in human neutrophils. Oxidative neutrophils exist in atherosclerotic plaques and accelerate pro-inflammatory cytokine release and immune cell recruitment [26]. Therefore, we thought that PDX functioned as an antioxidant molecule to improve vascular endothelial function and atherosclerotic processes. Furthermore, PDX inhibited H_2O_2-induced loss of mitochondrial membrane potential and cell death, which are important features of atherosclerosis. Mitochondrial dysfunction increases ROS generation and promotes cell apoptosis [27]. Endothelial cell apoptosis is an important initiating step in atherosclerosis, with LDL uptake and leukocyte adhesion occurring at damaged vascular sites [28]. In the present study, we demonstrated that the increased Bax/Bcl-2 ratios by H_2O_2, which is a crucial molecular event associated with mitochondria-mediated cell death, were significantly decreased after PDX treatments. In addition, H_2O_2-mediated loss of mitochondrial membrane potential was also recovered after PDX administration. All these results suggest the protective role of PDX on the progression of atherosclerotic lesions by relieving oxidative stress and endothelial cell death.

In the present study, the beneficial effects of PDX on endothelial dysfunction disappeared after treatment with AMPK inhibitor or GPR120 antagonist. GPR40 and GPR120, which are medium- and long-chain fatty acid receptors, are considered novel therapeutic targets in treating metabolic disorders because activation of either receptor is related to anti-inflammatory and anti-diabetic effects [7,29]. Oh et al. [30] showed that GPR120 functions as an omega-3 fatty acid sensor and enhances insulin sensitivity in mice. Furthermore, a selective, high-affinity, orally available, small-molecule GPR120 agonist exerts potent anti-inflammatory effects on macrophages in vitro and in obese mice in vivo [31]. Some studies revealed that these GPR-mediated protective effects are associated with AMPK phosphorylation [32–34]. Treatment with the GPR40 agonist, GW9508 significantly increased AMPK and acyl-CoA carboxylase (ACC) phosphorylation, which led to the blockade of hepatic fatty acid synthesis in HepG2 cell line [33]. DHA-mediated GPR120 activation improved muscular glucose uptake [34] and inhibited hepatic lipid accumulation [32] via AMPK-dependent signaling. Therefore, we hypothesized that PDX-mediated AMPK phosphorylation might be a downstream event of the activation of GPR40 and/or GPR120 because PDX is a long-chain fatty acid composed of 22 carbons. In HUVECs, PDX-induced AMPK phosphorylation was decreased after treatment with AH7614, a GPR120 antagonist, and after the attenuation of GPR120 expression by siRNA transfection. However, AMPK phosphorylation was not attenuated by a GPR40 antagonist, suggesting that PDX-induced AMPK phosphorylation is regulated by GPR120 in endothelial cells. Although it has not been clarified whether or not PDX is a direct ligand of GPR120, we assumed that GPR120 is one of the molecules associated with the mechanism of PDX.

In conclusion, we report for the first time that PDX has protective effects against H_2O_2-induced oxidative stress in HUVECs (Fig. 5). PDX significantly reduced H_2O_2-mediated ROS production by enhancing the expression of antioxidant proteins, such as catalase and SOD2. In

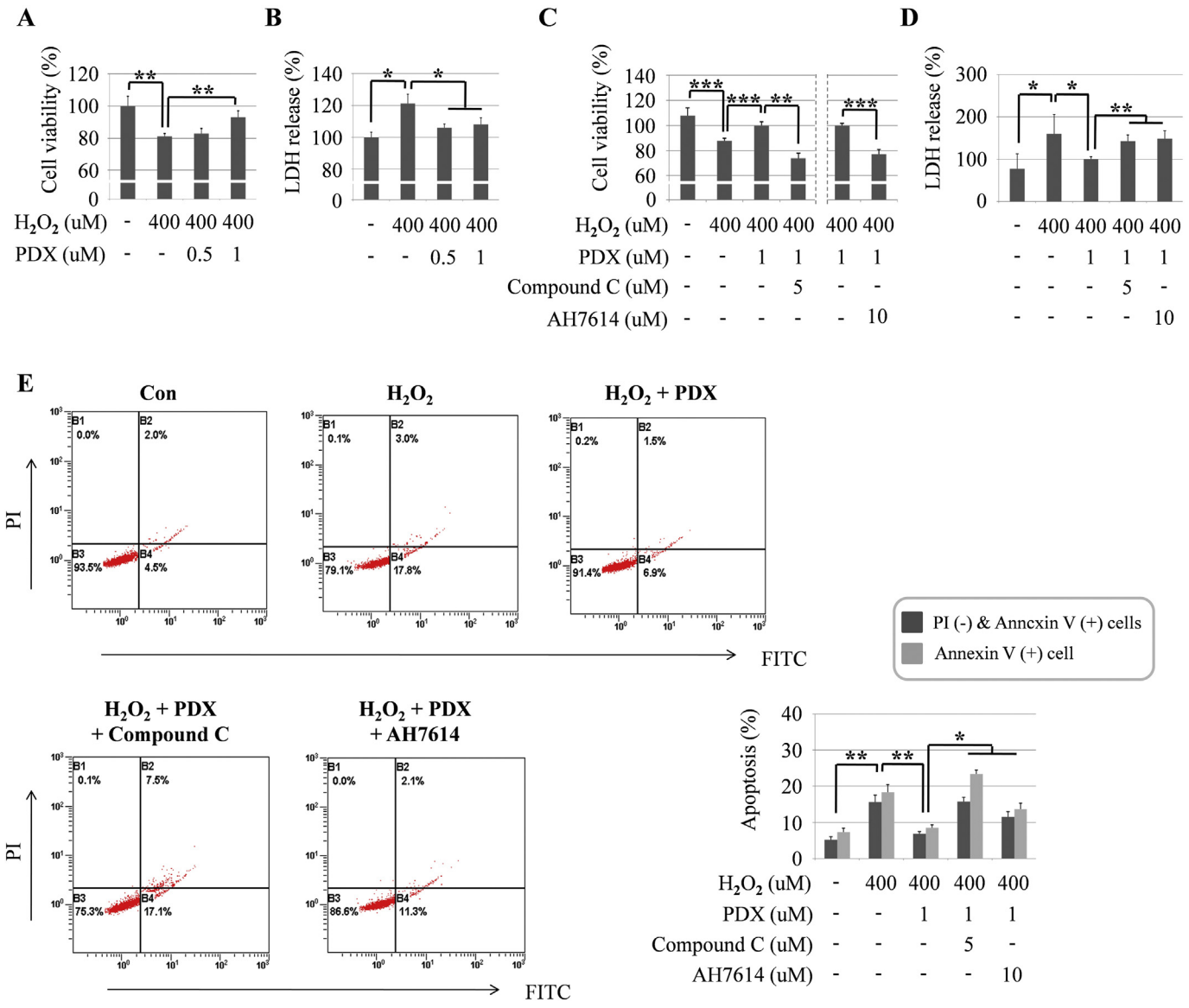

Fig. 4. PDX blocks H_2O_2-induced cytotoxicity in HUVECs.
Cells were pre-incubated with PDX (0.5 or 1 μM) plus compound C (5 μM) or AH7614 (10 μM) for 24 h and then stimulated with H_2O_2 (400 μM) plus the indicated reagents for 3 h. A and C, Cell viabilities were measured using the EZ-CYTOX kit. B and D, LDH release was detected using the EZ-LDH kit. E, The percentage of apoptotic cells was calculated by identifying annexin V/PI double staining cells by flow cytometry. All data were obtained from three separate experiments. Error bars indicate the mean ± SD (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ANOVA).

addition, increased cell death signaling and loss of mitochondrial membrane potential by H_2O_2 were inhibited after PDX treatment, thereby improving cell viability. These effects were attenuated by treatment with AMPK inhibitor or GPR120 antagonist. In conclusion, PDX inhibited H_2O_2-induced oxidative stress and cell death in HUVECs, suggesting that PDX is a potential novel therapeutic target for preventing endothelial dysfunction. Further in vivo studies using a model of atherosclerosis should be warranted to reinforce the role of PDX on vascular health.

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Conflict of interest

The authors have declared that they have no conflicts of interest.

Author contributions

H.J.H. and H.J.Y participated in the design of the study. H.J.H. performed the experiments. H.J.H. and H.J.Y. drafted the manuscript, conceived the study, participated in the study design, coordinated the study, and helped draft the manuscript. All authors read and approved the final manuscript.

Disclosure

The authors have nothing to disclose.

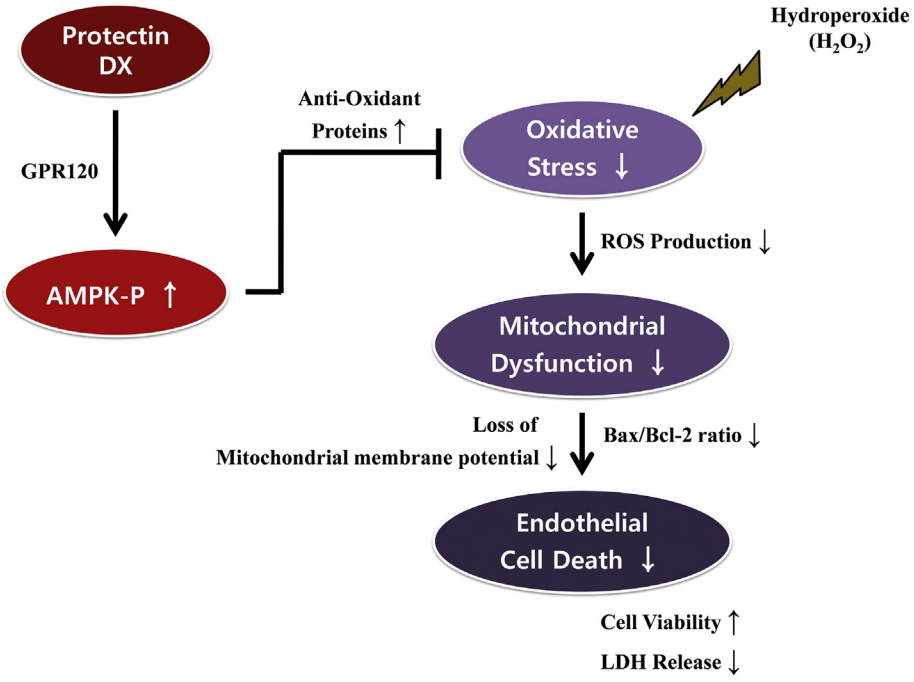

Fig. 5. Schematic diagram of endothelial PDX functions.
PDX reduced H_2O_2-mediated ROS production, loss of mitochondrial membrane potential, and cell death in HUVECs through AMPK phosphorylation (AMPK-P).

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