Cell death-inducing DFF45-like effector C gene silencing alleviates pulmonary vascular remodeling in a type 2 diabetic rat model

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Keywords
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ABSTRACT
Aims/Introduction: Cell death-inducing DFF45-like effector C (CIDEC) was proven to be closely associated with the development of insulin resistance and metabolic syndrome. We aimed to investigate whether CIDEC gene silencing could alleviate pulmonary vascular remodeling in a type 2 diabetes rat model.

Materials and Methods: We built a type 2 diabetes rat model. An adenovirus harboring CIDEC small interfering ribonucleic acid was then injected into the jugular vein to silence the CIDEC gene. After hematoxylin–eosin and Sirius red staining, we detected indexes of the pulmonary arterioles remodeling. Immunohistochemical staining of proliferating cell nuclear antigen was used to evaluate the pulmonary arterial smooth muscle cell proliferation. Apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling reaction and western blotting. The levels of signaling pathway proteins expression were measured by western blotting analyses.

Results: Histological analysis of the pulmonary artery showed that the thickness of the adventitia and medial layer increased notably in type 2 diabetes rats. Immunohistochemistry showed that more proliferating cell nuclear antigen positive pulmonary arterial smooth muscle cells could be seen in type 2 diabetes rats; and after CIDEC gene silencing, proliferating cell nuclear antigen positive cells decreased accordingly. Cleaved caspase-3 and cleaved poly (adenosine diphosphate-ribose) polymerase measured by western blotting showed increased apoptosis with overexpressed CIDEC in diabetes. Terminal deoxynucleotidyl transferase dUTP nick end labeling reaction showed that the apoptosis mainly occurred in endothelial cells. Western blotting analysis showed CIDEC overexpression in rats with diabetes, and phosphorylated adenosine 5’ monophosphate-activated protein kinase-α expression was significantly decreased. After CIDEC gene silencing, the expression of phosphorylated adenosine 5’ monophosphate-activated protein kinase-α was upregulated.

Conclusions: The CIDEC/5’ monophosphate-activated protein kinase signaling pathway could be a potential therapeutic candidate against pulmonary vascular diseases in type 2 diabetes patients.

INTRODUCTION
Pulmonary hypertension (PH) is a complex disease with significant morbidity and mortality. Pulmonary vascular remodeling, including endothelial apoptosis and dysfunction, medial layer smooth muscle cell proliferation, adventitial fibroblast activation, differentiation and proliferation, and collagen synthesis, plays a key role in pulmonary hypertension1–3. Recent studies suggested that the metabolic dysregulation might be an...
important factor contributing to the pulmonary vascular remodeling. Diabetes and metabolic syndrome were considered to be strongly associated with PH independent of coronary artery disease, congestive heart failure or smoking. The procedures and mechanisms of pulmonary vascular remodeling associated with metabolic syndrome are complex, including endothelial dysfunction, medial smooth muscle cell proliferation, adventitial fibroblast activation and collagen synthesis. A number of inflammatory cells and cytokines, such as adipocytes, macrophages and adiponectin, are involved in this pathophysiological process. Adenosine 5'-monophosphate activated protein kinase (AMPK), a key molecule in the regulation of energy metabolism, plays an important role in the study of diabetes and other metabolic diseases. Recent studies have shown that the activation of AMPK could inhibit the pulmonary arterial smooth muscle cell proliferation, and reduce collagen production in the lung and kidney.

The cell death-inducing DFF45-like effector (CIDE) family proteins could induce cell apoptosis. CIDE (known as Fsp27 in mice), which can be detected in many tissues, has been found to be closely associated with the development of metabolic diseases, such as obesity, diabetes and liver steatosis. Studies showed that CIDE could directly interact with the AMPKα1 subunit and downregulate AMPKα through an ubiquitin/proteasome pathway. Therefore, the overexpression of CIDE could significantly reduce AMPK activity.

Thus, we hypothesized that type 2 diabetes and insulin resistance upregulated the protein expression of CIDE in lung tissue, which reduced the expression of AMPK, resulting in pulmonary vascular remodeling. CIDE gene silencing could contribute to decreasing pulmonary arterioles remodeling and pulmonary hypertension induced by diabetes. We established a type 2 diabetes Sprague-Dawley rat model, and used CIDE gene silencing to determine the relationships among the CIDE/AMPK signaling pathway, type 2 diabetes and pulmonary vascular remodeling.

**EXPERIMENTAL GROUPS**

A total of 40 male Sprague-Dawley rats were randomly assigned to the following four groups: normal control group (group C, 10 rats), type 2 diabetes group (group D, 10 rats), type 2 diabetes + CIDE small interfering ribonucleic acid adenovirus (CIDE gene silencing) group (group A, 10 rats) and type 2 diabetes + empty pAdxsi virus (vehicle) group (group V, 10 rats).

**Animal model**

Group C received normal chow, including 20% protein, 3% fat, 3% dietary fiber and 74% other components (carbohydrates, microelement, etc.). The type 2 diabetes group was fed a high-glucose and high-fat diet (34.5% fat, 17.5% protein and 48% carbohydrates). 4 weeks later, intraperitoneal glucose tolerance test and intraperitoneal insulin tolerance test were carried out again, and diabetes was induced by a single intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO, USA; 27.5 mg/kg i.p. in 0.1 mol/L citrate buffer, pH 4.5) to rats with insulin resistance. Group C received citrate buffer (i.p.) alone. 1 week after streptozotocin administration, fasting blood glucose (FBG) and fasting insulin (FINS) were measured, and the insulin sensitivity index ($\text{insulin sensitivity index} = \ln [\text{FINS} \times \text{FBG}^{\frac{1}{2}}]$) was calculated. Rats with FBG $\geq 11.1$ mmol/L in two consecutive analyses and reduced insulin sensitivity were considered the diabetes rat model. After 12 weeks of diabetes, the type 2 diabetes group rats were randomized to receive CIDE small interfering ribonucleic acid for CIDE gene silencing or vehicle treatment. Animals were then injected in the jugular vein with $5 \times 10^9$ plaque-forming units of an adenovirus harboring CIDE small interfering ribonucleic acid to silence the CIDE gene or control empty pAdxsi virus (vehicle). After 4 weeks, bodyweight, FBG and FINS were measured again, and then all the rats were killed. All experimental procedures were carried out in accordance with animal protocols approved by the Shandong University Animal Care Committee.

**Echocardiography test**

According to recent studies, pulmonary artery acceleration time (PAAT) has been confirmed to be correlated with invasive pulmonary artery pressure, especially in mild-to-moderate pulmonary arterial hypertension. Therefore, we calculated the PAAT by echocardiography to evaluate the mean pulmonary artery pressure. At the 21st week, pulsed-wave Doppler of pulmonary outflow was recorded in the parasternal view at the level of the aortic valve. PAAT was measured from the time of onset of systolic flow to peak pulmonary outflow velocity. The rats were killed after the echocardiography tests. The right ventricle (RV) tissue was cut along the edge of the ventricle and the interventricular septum. The RV and the left ventricle plus the interventricular septum were collected from each rat. They were weighed, and the mass ratio of the RV over the left ventricle plus the interventricular septum was used as an index for RV hypertrophy.

**Histological analysis**

The left lungs of each rat were removed, fixed in 4% paraformaldehyde for 24 h and then imbedded in paraffin. Tissue sections were cut 4 μm in thickness. Pulmonary arterial smooth muscle cell proliferation was observed by hematoxylin–eosin (HE) staining, and the zone that the smooth muscle cells were distributed in was used to evaluate the area and thickness of the medial layer. The adventitial fibrosis was observed by evaluation of Sirius red staining. Green-, yellow- and red-stained collagen fibers were quantified as a measure of the area and thickness of the adventitia. Histological analysis of pulmonary vascular remodeling was carried out using light microscopy. One tissue section was chosen from each rat, and 5–10 pulmonary arterioles with diameters between 50–200 μm were chosen randomly in each section. The pulmonary arteriole remodeling indexes were measured by
Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). The medial layer thickness index = medial layer thickness / vessel wall thickness (HE, magnification: × 400), the medial layer area index = medial layer area/vessel wall area (HE, magnification: × 400), the adventitia thickness index = adventitia thickness / vessel wall thickness (Sirius red staining, magnification: × 200) and the adventitia area index = adventitia area / vessel wall area ( Sirius red staining, magnification: × 200).

Immunohistochemistry

Paraffin embedded tissue sections were prepared as mentioned before. After blocking, sections were incubated with a mouse monoclonal antibody to proliferating cell nuclear antigen (PCNA; Abcam, Cambridge, UK). Sections were then incubated with secondary horseradish peroxidase labeled anti-rabbit immunoglobulin G polymer (PV-9001; Beijing Zhong Shan Golden Bridge Biotechnology Co. Ltd., Beijing, China). Lung specimens incubated with 0.01 mmol/L phosphate-buffered

Table 1 | Baseline data

|                  | Group C    | Group D    | Group V    | Group A    | n |
|------------------|------------|------------|------------|------------|---|
| Bodyweight (g)   | 168.14 ± 4.34 | 170 ± 3.55 | 169.71 ± 3.56 | 169.29 ± 3.50 | 7 |
| FBG (mmol/L)     | 4.30 ± 0.18  | 4.84 ± 0.21 | 4.93 ± 0.39  | 4.27 ± 0.15  | 7–10 |
| FINS (µg/dL)     | 3.61 ± 0.93  | 2.61 ± 0.35 | 2.61 ± 0.22  | 2.48 ± 0.40  | 6 |
| ISI              | −2.65 ± 0.21 | −2.68 ± 0.18 | −2.46 ± 0.13 | −2.30 ± 0.13 | 6 |

Bodyweight, fasting blood glucose (FBG), fasting insulin and insulin sensitivity index (ISI) had no statistically significant difference among the four groups.

Figure 1 | Body weight, fasting blood glucose (FBG) and fasting insulin (FINS) of the four groups at the 21st week. (a) Bodyweights. (b) FBG. (c) FINS. (d) ISI (**P < 0.01 vs group C; ##P < 0.01 vs group A).
saline in place of the specific primary antibody served as negative controls. The nuclei stained brown or brown yellow can be determined as positive results. The percentage of the PCNA-positive cells in the total arterial medial layer smooth muscle cells, defined as the cell proliferation index, was used to evaluate the pulmonary arterial smooth muscle cell proliferation. The cell proliferation index (magnification: × 200) in each group was statistically analyzed by Image-Pro Plus 6.0 image analysis software.

Analysis of apoptosis
As CIDEC was known to be a potent apoptotic inducer, we also evaluated apoptosis in pulmonary arterial endothelial cells and smooth muscle cells of the four groups. Apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction (FragEL™ DNA Fragmentation Detection Kit, Colorimetric-TdT Enzyme; EMD Millipore, Billerica, MA, USA). The nuclei stained brown could be determined as TUNEL-positive results. The percentage of the TUNEL-positive cells was defined as the cell apoptotic index (AI). The AI (magnification: × 400) in each group was statistically analyzed by Image-Pro Plus 6.0 image analysis software. CIDEC (Abcam), cleaved caspase-3 (Cell Signaling Technology, Beverly, MA, USA) and cleaved poly (adenosine diphosphate-ribose) polymerase (PARP; Anti-Cleaved PARP Antibody; Boster Biological Technology, Wuhan, China) in lung tissue was measured by Western blotting subsequently to detecting the apoptosis.

Western blotting
After the rats were killed, the right lung tissue was divided into several 100-mg segments, and then was kept in the −80°C liquid nitrogen for protein analysis. The primary antibodies included a rabbit polyclonal CIDEC antibody (Abcam), a rabbit polyclonal AMPKα antibody (Cell Signaling Technology), a rabbit polyclonal phosphorylated AMPKα antibody (Cell Signaling Technology) and a mouse monoclonal β-actin antibody (Abcam). The secondary antibodies included a goat anti-rabbit immunoglobulin G/horseradish peroxidase (ZDR-5306;
Figure 5 | (a) Pulmonary arterial smooth muscle cell proliferation observed by hematoxylin–eosin staining. (b) The medial layer thickness index (**P < 0.01, ##P < 0.01 vs group C, ▲▲P < 0.01 vs group C, ★★P < 0.01 vs group D, ★★★P < 0.01 vs group V). (c) The medial layer area index (**P < 0.01, ★★P < 0.01 vs group C, ▲▲P < 0.01 vs group C, ★P < 0.05 vs group D, *P < 0.05 vs group V).
Beijing Zhong Shan Golden Bridge Biotechnology Co. Ltd.) and a goat anti-mouse immunoglobulin G/horseradish peroxidase (ZDR-5307; Beijing Zhong Shan Golden Bridge Biotechnology Co. Ltd.). The relative optical density of electrophoretic bands was quantitatively calculated by Image-Pro Plus 6.0 image analysis software.

Statistical analysis
All data are expressed as the mean ± standard error. SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA) was used to carry out all the calculations. ANOVA followed by a Tukey–Kramer test was used for statistical analyses of differences between groups. A level of \( P < 0.05 \) was considered statistically significant.

RESULTS
Bodyweight, FBG and FINS of the four groups
As expected, at the 21st week, bodyweights and insulin sensitivity indexes were significantly lower in group D and group V than in group C and group A, and FBGs and FINSs were significantly higher in group D and group V than in group C and group A (Table 1; Figure 1).

![Image of PASMC proliferation](http://onlinelibrary.wiley.com/journal/jdi)

**Figure 6** | (a) Pulmonary arterial smooth muscle cell (PASMC) proliferation evaluated by immunohistochemical staining of proliferating cell nuclear antigen. (b) The cell proliferation indexes (\(^*\)\(^*\) \(< 0.01, \ast \) \(< 0.05 \) vs Group C, \(^\#\) \(< 0.05 \) vs group D, \(\mathcal{O} \) \(< 0.05 \) vs group V).
Figure 7 | (a) The adventitial fibrosis observed by Sirius red staining. (b) The adventitia thickness index (**P < 0.01, ##P < 0.01 vs group C, *P < 0.05 vs group C, †P < 0.05 vs group D, ‡P < 0.05 vs group V). (c) The adventitia area index (**P < 0.01, ##P < 0.01 vs group C, *P < 0.05 vs group C, †P < 0.05 vs group D, ‡P < 0.05 vs group V).
Effects of diabetes and CIDEC gene silencing on PAAT and RV hypertrophy

At the 21st week, pulsed-wave Doppler of pulmonary outflow was recorded to measure the PAAT (Figure 2). PAAT was significantly shortened in group D and group V compared with group C. After CIDEC gene silencing, PAAT was lengthened correspondingly (Figure 3). The index of RV hypertrophy had no statistically significant difference among the four groups (Figure 4).

Histological analysis of pulmonary vascular remodeling

Pulmonary arterial smooth muscle cell proliferation was observed by HE staining. In group D and group V rats, the pulmonary arterioles medial layer had thickened, as a result of the smooth muscle cell proliferation. However, in group A rats, the smooth muscle cell proliferation and the medial layer thickening in the pulmonary arterioles were reduced (Figure 5a). The medial layer thickness index of group D and group V were significantly increased compared with those of group C (group D vs group C: 0.4972 ± 0.0265 vs 0.2461 ± 0.0130, \( P < 0.01 \); group V vs group C: 0.4537 ± 0.0204 vs 0.2461 ± 0.0130, \( P < 0.01 \)) After CIDEC gene silencing, the medial layer thickness indexes were significantly decreased compared with those of group D and group V (Figure 5b). Similar results were obtained when compared with the medial layer area index among the four groups (Figure 5c). Immunohistochemical

Figure 8 | (a) The apoptosis of the pulmonary arterial endothelial cells (ECs) and smooth muscle cells (SMCs) detected by terminal deoxynucleotidyl transferase dUTP nick end labeling reaction. (b) The apoptotic indexes (**\( P < 0.01 \), *\( P < 0.05 \) vs group C, ##\( P < 0.01 \) vs group D, @@\( P < 0.01 \) vs group V).
staining of PCNA was used to evaluate the pulmonary arterial smooth muscle cell proliferation. More PCNA-positive pulmonary arterial medial layer smooth muscle cells could be seen in group D and group V, and after CIDEC gene silencing, PCNA-positive cells decreased accordingly (Figure 6a). The cell proliferation indexes were significantly higher in group D and group V (group D vs group C: 0.187 ± 0.022 vs 0.077 ± 0.014, P < 0.01; group V vs group C: 0.188 ± 0.021 vs 0.077 ± 0.014, P < 0.01), and after CIDEC gene silencing, the proliferation index became lower accordingly (group A vs group C: 0.134 ± 0.019 vs 0.077 ± 0.014, P < 0.05; group A vs group D: 0.134 ± 0.019 ± 0.187 ± 0.022, P < 0.05; group A vs group V: 0.134 ± 0.019 ± 0.188 ± 0.021, P < 0.05; Figure 6b).

The adventitial fibrosis was observed and evaluated by Sirius red staining. Green-, yellow- and red-stained collagen fibers were quantified as a measure of the area and thickness of the adventitia. In group D and group V rats, the pulmonary arterioles adventitia had thickened as a result of the collagen deposition. After CIDEC gene silencing, the collagen deposition and the adventitia thickening in the pulmonary arterioles were reduced (Figure 7a). The adventitia thickness index of group D and group V were significantly increased compared with those of group C (group D vs group C: 0.3899 ± 0.0176 vs 0.2642 ± 0.0186, P < 0.01; group V vs group C: 0.3921 ± 0.0175 vs 0.2642 ± 0.0186, P < 0.01). After CIDEC gene silencing, the adventitia thickness indexes were significantly decreased compared with those of group D and group V (Figure 7b). Similar results were obtained when compared with the adventitia area index among the four groups (Figure 7c).

**Analysis of apoptosis**

TUNEL was applied to detect the apoptosis of the pulmonary arterial endothelial cells and smooth muscle cells. More TUNEL-positive cells could be detected in group D and group V. After CIDEC gene silencing, TUNEL-positive cells decreased accordingly, and as observed, the differences in apoptosis mainly occurred in endothelial cells (Figure 8a). The AIs were significantly higher in group D and group V (group D vs group C: 0.0791 ± 0.0056 vs 0.0292 ± 0.0047, P < 0.01; group V vs group C: 0.0837 ± 0.0047 vs 0.0292 ± 0.0047, P < 0.01), and after CIDEC gene silencing, AI became lower accordingly (group A vs group C: 0.0530 ± 0.008 ± 0.0292 ± 0.0047, P < 0.05; group A vs group D: 0.0530 ± 0.008 vs 0.0791 ± 0.0056, P < 0.01; group A vs group V: 0.0530 ± 0.008 vs 0.0837 ± 0.0047, P < 0.01; Figure 8b). Cleaved caspase-3 and cleaved PARP in lung tissue of rats were measured by western blotting (Figure 9a). Both cleaved caspase-3 and cleaved PARP were significantly overexpressed in group D and group V compared with group C. After CIDEC gene silencing, the expression of cleaved caspase-3 and cleaved PARP were relatively downregulated (Figure 9b,c).

**Expression of signaling pathway proteins in lung tissue**

Protein expression of CIDEC, AMPKα and phosphorylated AMPKα in lung tissue of rats were measured by western blotting (Figure 10a). CIDEC was significantly overexpressed in group D and group V compared with group C. After CIDEC gene silencing, the expression of CIDEC was significantly downregulated (Figure 10b). The expression of phosphorylated AMPKα was significantly decreased in group D and group V compared with group C. After CIDEC gene silencing, the expression of phosphorylated AMPKα in group A was significantly increased, and had no statistically significant difference compared with group C (Figure 10c). The expression of AMPKα had no statistically significant difference among the four groups (P > 0.05; Figure 10d).
DISCUSSION

The salient finding of the present study was that the CIDEC/AMPK signaling pathway plays a major role in the pulmonary vascular remodeling in a type 2 diabetes rat model. More importantly, silence of CIDEC partially reversed diabetic pulmonary vascular remodeling. Thus, the CIDEC/AMPK signaling pathway could be a potential therapeutic candidate against pulmonary vascular diseases in type 2 diabetes.

We established a type 2 diabetes Sprague–Dawley rat model to investigate the relationships among the CIDEC/AMPK signaling pathway, type 2 diabetes and the pulmonary vascular remodeling. The CIDEC gene was silenced in order to study the effects of CIDEC. In the present study, the results showed that diabetes induced mildly elevated mean pulmonary artery pressure, and this process was partially reversed after the CIDEC gene silencing. The RV hypertrophy index of the four groups showed that diabetes had not induced notable RV hypertrophy yet. We detected the pulmonary arterioles remodeling by histological analysis. The pulmonary arterioles medial layer and the adventitia had thickened with diabetes as a result of the smooth muscle cell proliferation and the collagen deposition. Cleaved caspase-3 and cleaved PARP in lung tissue measured by western blotting showed increased apoptosis with overexpressed CIDEC in diabetes. TUNEL reaction showed that the differences in apoptosis mainly occurred in endothelial cells. Therefore, the present study showed that diabetes could cause pulmonary vascular remodeling at least by endothelial apoptosis, medial layer smooth muscle cell proliferation, adventitial fibroblast activation and collagen synthesis. These findings were consistent with the reported characteristics of pulmonary vascular remodeling19,20. As observed in the present study, silence of CIDEC reversed the pathological pulmonary arterioles remodeling. Protein expression of CIDEC has been detected by western blotting in the lung tissue of each group. In rats with diabetes, CIDEC was significantly overexpressed, which induced the downregulation of the expression of phosphorylated AMPKα. After CIDEC gene silencing, the expression of phosphorylated AMPKα was significantly increased.

Pulmonary vascular remodeling plays a key role in pulmonary hypertension. Many systemic diseases, including chronic
obstructive pulmonary disease, chronic pulmonary thromboembolism and connective tissue disease, were considered to contribute to pulmonary vascular remodeling and PH. Recent animal and human studies have highlighted that metabolic dysregulation and chronic inflammation were linked to the development of pulmonary vascular remodeling. As the most common and important metabolic disorder, diabetes was considered to be strongly associated with PH. In diabetes, vascular remodeling extends to capillaries, microvascular beds and arteries of different caliber. As an important part of the circulatory system, pulmonary vessels are also affected by diabetes, and more attention has been paid to the mechanisms of pulmonary vascular remodeling associated with diabetes. AMPK is a key molecule in the regulation of biological energy metabolism. It plays an important role in regulating cell growth and proliferation, establishing and stabilizing cell polarity, and regulating physiological rhythm. The effect of AMPK on pulmonary hypertension induced by chronic hypoxia has been reported. The role of AMPK in diabetic pulmonary vascular remodeling is yet to be confirmed. CIDEA (known as Fsp27 in mice) was proven to be closely associated with the development of insulin resistance and metabolic syndrome. Studies showed that CIDEA and CIDEC could directly interact with the AMPK alpha 1 (AMPKα1) subunit and downregulate AMPKβ through an ubiquitin/proteasome pathway, and CIDEC could also influence the activity of AMPK by phosphorylation. Therefore the overexpression of CIDEC could significantly reduce AMPK activity. The present research confirmed that diabetes and insulin resistance upregulated the protein expression of CIDEC in lung tissue, which inhibited the AMPK signaling pathway and induced pulmonary arterioles remodeling, and the protective effects with CIDEC silencing suggested a potential role in treating diabetic pulmonary vascular diseases.

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DISCLOSURE

The authors declare no conflict of interest.

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