Metformin protects against PM$_{2.5}$-induced lung injury and cardiac dysfunction independent of AMP-activated protein kinase α2

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A R T I C L E   I N F O

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A B S T R A C T

Fine particulate matter (PM$_{2.5}$) airborne pollution increases the risk of respiratory and cardiovascular diseases. Although metformin is a well-known antidiabetic drug, it also confers protection against a series of diseases through the activation of AMP-activated protein kinase (AMPK). However, whether metformin affects PM$_{2.5}$-induced adverse health effects has not been investigated. In this study, we exposed wild-type (WT) and AMPKα2$^{-/-}$ mice to PM$_{2.5}$ every other day via intratracheal instillation for 4 weeks. After PM$_{2.5}$ exposure, the AMPKα2$^{-/-}$ mice developed more severe lung injury and cardiac dysfunction than were developed in the WT mice; however, the administration of metformin was effective in attenuating PM$_{2.5}$-induced lung injury and cardiac dysfunction in both the WT and AMPKα2$^{-/-}$ mice. In the PM$_{2.5}$-exposed mice, metformin treatment resulted in reduced systemic and pulmonary inflammation, preserved left ventricular ejection fraction, suppressed induction of pulmonary and myocardial fibrosis and oxidative stress, and increased levels of mitochondrial antioxidant enzymes. Moreover, pretreatment with metformin significantly attenuated PM$_{2.5}$-induced cell death and oxidative stress in control and AMPKα2-depleted BEAS-2B and H9C2 cells, and was associated with preserved expression of mitochondrial antioxidant enzymes. These data support the notion that metformin protects against PM$_{2.5}$-induced adverse health effects through a pathway that appears independent of AMPKα2. Our findings suggest that metformin may also be a novel drug for therapies that treat air pollution associated disease.

1. Introduction

Fine particulate matter (PM$_{2.5}$, aerodynamic diameter ≤ 2.5 μm) ambient pollution has become a major issue that threatens public health in China and other developing countries [1]. PM$_{2.5}$ contains many toxic chemical compositions, including polycyclic aromatic hydrocarbons, transition metals, and endotoxins [2,3]. Because of the small size of its components, PM$_{2.5}$ is easily inhaled into the airway and deposited in lung alveoli [4]. Particles that deposit in the alveolar tissue can translocate from the alveoli into the bloodstream to affect the cardiovascular system [5]. Epidemiological studies have demonstrated that long-term exposure to high concentrations of PM$_{2.5}$ increases the risk of a number of cardiorespiratory illnesses, such as asthma [6], bronchitis [7], chronic obstructive pulmonary disease (COPD) [8], coronary artery disease [9] and heart failure [10]. As revealed by many toxicological studies, the adverse health effects of PM$_{2.5}$ are associated with enhanced inflammation and oxidative stress [11,12]. Considering that ambient PM$_{2.5}$ has maintained high levels for several years and air quality could not be improved in a short period, it is urgent to find effective therapeutic approaches for attenuating PM$_{2.5}$ associated diseases.

AMP sensitive protein kinase (AMPK) is an important cellular energy sensor that is activated by an elevated AMP/ATP ratio [13]. Recently, it has been reported that PM$_{2.5}$ exposure activated AMPK to promote autophagy in human lung epithelial A549 and BEAS-2B cells [14,15]. Using a whole-body exposure system, we recently demonstrated that AMPKα2 deficiency exacerbated long-term PM$_{2.5}$-induced lung injury and cardiac dysfunction [16]. In contrast, the activation of AMPK by 5-aminomidazole-4-carboxamide-1-beta-4-ribofuranside (AICAR) ameliorates the ambient PM$_{2.5}$-induced inflammatory response...
Metformin, one of the most widely prescribed antidiabetic drugs, is a well-known AMPK activator. In experimental animal models, metformin was found to attenuate pulmonary and cardiovascular inflammation and/or oxidative stress through activation of AMPK [18–22]. Interestingly, a recent study showed that metformin prevents concentrated ambient PM2.5-induced vascular insulin resistance and changes in endothelial progenitor cell homeostasis [23]. Metformin also reduces the generation of mitochondrial reactive oxygen species (ROS) and interleukin 6 (IL-6) release in PM2.5-exposed alveolar macrophage cells, and prevents PM2.5-induced acceleration of carotid thrombosis [24]. However, whether metformin affects PM2.5-induced pulmonary and cardiovascular disorders remains unclear. Furthermore, we previously demonstrated that metformin was equally effective in attenuating transverse aortic constriction (TAC)-induced left ventricular hypertrophy and heart failure in both wild-type (WT) and AMPKα2−/− mice [25]. Thus, in this study, we treated PM2.5-exposed WT and AMPKα2−/− mice with metformin to determine whether metformin could protect against PM2.5-induced lung injury and cardiac dysfunction through an AMPKα2 dependent pathway.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies against peroxiredoxin 3 (PRDX3), peroxiredoxin 5 (PRDX5), superoxide dismutase 2 (SOD2), thioredoxin reductase 2 (TRXR2), thioredoxin 2 (TRX2), Bcl-2 and (PRDX5), superoxide dismutase 2 (SOD2), thioredoxin reductase 2 (TRXR2), thioredoxin 2 (TRX2), Bcl-2 and peroxiredoxin 3 (PRDX3) were purchased from Cell Signaling Technology (#5831 and #5832, Danvers, MA, USA). Anti-α-tubulin, and ELISA kits for IL-6, tumor necrosis factor alpha (TNFa) and 3'-nitrotyrosine (3'-NT) were purchased from Abcam PLC (#ab73349, #ab180587, #ab13533, #ab180493, #ab185544, #ab194583, #ab6046, #ab100712, #ab108910, and #ab116691, Cambridge, UK). AMPKα1 and AMPKα2 antibodies were obtained from GenTex (#GTX60403 and #GTX103487, Irvine, CA, USA). AMPKα1 and phospho-AMPKαThr172 antibodies were purchased from Cell Signaling Technology (#S831 and #2535, Denvers, MA, USA). Galectin-3 and neutrophil antibodies were obtained from Bios Biotechnology Co. Ltd. (#bs-20700R and #bs-6982R, Beijing, China), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), TRIZol and MTT were purchased from Life Technologies (Gaithersburg, MD), ThermoFisher Scientific (Waltham, MA) or WGA germ agglutinin (WGA, #29022, Bio-Tein Inc, Fremont, CA, USA) or DHE for 30 min to assess myocyte cross-sectional areas and superoxide levels, respectively. At least 4 mice per group were used for these experiments.

2.2. Experimental animals

Animal studies were performed in accordance with the principles of laboratory animal care (NIH publication no. 85–23, revised 1985) and with approval from the University of Chinese Academy of Sciences Animal Care and Use Committee. Male C57BL/6J and AMPKα2−/− mice [27] (C57BL/6J background), at 8–10 weeks of age, were treated with 10 μl phosphate-buffered saline (PBS) or 10 μg/kg PM2.5 in 10 μl PBS every other day via intratracheal instillation and the mice were sacrificed at 4 weeks after the instillation treatment. The PM2.5−exposed mice were treated with metformin in drinking water (300 mg/kg/day) during the experimental period.

2.3. Histopathology staining

After perfusion with PBS, the mouse lung and heart tissues were harvested, washed, fixed with formalin and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E), Masson's trichrome stain kit (Slytek Laboratories, Inc., UT, USA) or a TUNEL stain kit to assess fibrosis and apoptosis, respectively. As previously described [16], lung sections were also stained with monoclonal galectin-3 and neutrophil antibodies to identify macrophages and neutrophils, respectively. Frozen heart sections (4 μm) were stained with 4,6-diamidino-2-phenylindole (DAPI) or DHE for 30 min to assess myocyte cross-sectional areas and superoxide levels, respectively. At least 4 mice per group were used for these experiments.

2.4. Echocardiographic measurement

As previously described [28], the mice were anesthetized with 1.5% isoflurane and echocardiographic images were obtained using a VisualSonics high resolution Vevo 2100 system (VisualSonics, Toronto, ON, Canada).

2.5. Cell culture and exposure of cells to PM2.5

The human bronchial epithelial BEAS-2B cell line and the rat cardiac myoblast H9C2 cell line were obtained from the China Infrastructure of Cell Line Resource (Beijing, China) and maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin at 37 °C with 5% CO2. After being cultured for 24 h, the cells were pretreated with PBS or 1 mM metformin for 2 h. Then the culture medium was replaced with serum-free (for BEAS-2B) or 1% FBS (for H9C2) medium and the

| Abbreviation | Description |
|----------------|-------------|
| 3'-NT         | 3'-nitrotyrosine |
| 4-HNE         | 4-hydroxynonenal |
| AMPK          | AMP sensitive protein kinase |
| ANP           | atrial natriuretic peptide |
| ATF-3         | transcription factor-3 |
| BCA           | bicinchoninic acid |
| DCFH-DA 2, 7'-dichlorodihydrofluorescein diacetate | |
| DHE           | dihydroethidium |
| DMEM          | Dulbecco's modified eagle medium |
| EF            | ejection fraction |
| FBS           | fetal bovine serum |
| H&E           | hematoxylin and eosin |
| IL            | interleukin |
| LV            | left ventricular |
| mTOR          | mammalian target of rapamycin |
| PRDX          | peroxiredoxin |
| qPCR          | quantitative real-time polymerase chain reaction |
| ROS           | reactive oxygen species |
| SOD2          | superoxide dismutase 2 |
| TGF-β         | transforming growth factor beta |
| TNFa          | tumor necrosis factor alpha |
| TRX2          | thioredoxin 2 |
| TRXR2         | thioredoxin reductase 2 |
| WGA           | germ agglutinin |
cells were exposed to freshly dispersed PM2.5 preparations for 24 h. Cell viability was measured with a MTT assay and intracellular ROS levels were determined by a Synergy H1 Hybrid Multi-Mode microplate reader (Biotek Instruments, Inc., Winooski, VT, USA) using DCFH-DA.

To generate a stable AMPKα2-knockdown cell line, 5 × 10^5 exponentially growing cells were transfected with AMPKα2 shRNA lentivirus for 24 h and then with puromycin (1 μg/mL) for 3 weeks for selection.

2.6. Quantitative real-time PCR and western blotting

Total RNA was extracted with TRIzol reagent and cDNA was synthesized using a PrimeScript RT reagent kit (#RR036B, TaKaRa, Otsu, Japan). A quantitative real-time polymerase chain reaction (qPCR) assay was performed using the SYBR Premix Ex Taq™ II Kit (#RR820DS, TaKaRa) and the results were normalized to 18S ribosomal RNA. The primers used in the qPCR assay are listed in Table S1.

Proteins were extracted from the lung, heart or cultured cells using buffer (50 mM Tris-Cl, 150 mM NaCl, 100 μg/ml phenylmethylsulfonyl fluoride, protease and phosphatase inhibitor cocktail (#046931124001 and #4906837001, Roche, Basel, Switzerland), and 1% Triton X-100) on ice for 30 min. After 12000 g centrifugation at 4 °C for 20 min, the supernatant was used for Western blot analysis.

2.7. Data and statistical analysis

All values are expressed as the mean ± standard error of means (SEM). One- or two-way analysis of variance (ANOVA) was used to test each variable for differences among the treatment groups with StatView (SAS Institute Inc, Cary, NC, USA). If ANOVA demonstrated a significant effect, pairwise post hoc comparisons were made with the Fisher’s least significant difference test. Statistical significance was defined as p < 0.05.

3. Results

Metformin attenuates PM2.5-induced inflammation and fibrosis in both WT and AMPKα2−/− mice. Compared with the control mice, PM2.5-exposed mice exhibited significantly greater lung weight and higher lung weight to body weight ratio (Table S2). To investigate the protective effects of metformin on PM2.5-induced lung injury and cardiac dysfunction, we treated the PM2.5-exposed mice with metformin or
a vehicle (untreated drinking water) for 4 weeks. PM$_{2.5}$ exposure caused significant increases in serum TNFα and IL-6 levels in the WT and AMPKα2$^{-/-}$ mice, while the AMPKα2$^{-/-}$ mice exhibited higher serum TNFα and IL-6 levels than exhibited in the WT mice. However, metformin significantly attenuated the increase in serum TNFα and IL-6 levels in the PM$_{2.5}$-exposed WT and AMPKα2$^{-/-}$ mice (Fig. 1A–B). Next, we performed qPCR to examine the effects of metformin on the mRNA levels of genes related to inflammation and fibrosis. In the WT mice, we found that PM$_{2.5}$ exposure significantly increased the mRNA levels of TNFα, IL-1, transforming growth factor beta (TGF-β) and collagen I and III, and these increases were significantly attenuated by metformin treatment (Fig. 1C). Moreover, metformin also significantly attenuated the upregulation of TGF-β, and collagen I and III in the PM$_{2.5}$-exposed AMPKα2$^{-/-}$ mice (Fig. 1D).

H&E and Masson’s staining of the lung sections showed that PM$_{2.5}$ exposure caused significant lung injury and fibrosis in the WT and AMPKα2$^{-/-}$ mice, as showed by alveoli collapse, airway epithelial thickening and collagen deposition. Immunohistochemical staining using antibodies against neutrophils and galectin-3 (a macrophage-specific marker) showed that PM$_{2.5}$ exposure resulted in significant infiltration of neutrophils and macrophages in the lungs of WT and AMPKα2$^{-/-}$ mice. After PM$_{2.5}$ exposure, the lungs of AMPKα2$^{-/-}$ mice exhibited more severe injury, fibrosis and infiltration of macrophages and neutrophils than the lungs of the WT mice. In both the WT and AMPKα2$^{-/-}$ mice, PM$_{2.5}$-induced lung injury, fibrosis and inflammatory cell infiltration were significantly attenuated by metformin administration (Fig. 2). Taken together, these results indicate that metformin protects against lung fibrosis and inflammation in PM$_{2.5}$-exposed mice in an AMPKα2 independent manner.

Metformin prevents PM$_{2.5}$-induced oxidative stress and cell death in the lungs of WT and AMPKα2$^{-/-}$ mice. To determine whether metformin affects PM$_{2.5}$-induced pulmonary oxidative stress, we measured the levels of two oxidative stress markers, 3′-NT and 4-HNE. PM$_{2.5}$ exposure for 4 weeks significantly increased 3′-NT and 4-HNE levels in the lungs of the WT and AMPKα2$^{-/-}$ mice. Compared with the lungs of WT mice, those of the AMPKα2$^{-/-}$ mice exhibited significantly higher levels of 3′-NT and 4-HNE. Metformin administration significantly decreased pulmonary 3′-NT and 4-HNE levels in PM$_{2.5}$-exposed WT and AMPKα2$^{-/-}$ mice (Fig. 3A–B). TUNEL staining revealed that PM$_{2.5}$ exposure caused more TUNEL-positive cells in the lungs of AMPKα2$^{-/-}$ mice than in the lungs of the WT mice, while metformin treatment significantly decreased the apoptotic cells in the lungs of the PM$_{2.5}$-exposed WT and AMPKα2$^{-/-}$ mice (Fig. 3C–D). Thus, these results indicated that the protective effect of metformin against PM$_{2.5}$-induced oxidative stress and cell apoptosis is AMPKα2 independent.

In the lungs of the WT mice, the phosphorylation of AMPK was reduced in response to PM$_{2.5}$ exposure, and this reduction in p-AMPK levels was diminished by metformin administration (Fig. 3E). PM$_{2.5}$ exposure with or without metformin treatment did not affect the expression of AMPKα1 and AMPKα2 (Fig. S1). Because AMPKα2 deletion affects mitochondrial swelling and PRDX5 expression in PM$_{2.5}$-exposed lungs [16], we performed Western blot analysis to determine the protein expression of pulmonary mitochondrial antioxidant enzymes. As shown in Fig. 3E, PM$_{2.5}$ exposure had no obvious effect on SOD2 and PRDX3 expression, but significantly decreased the protein expression of

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**Fig. 2.** Metformin ameliorates PM$_{2.5}$-induced pulmonary fibrosis and inflammation. (A) Representative lung sections from the PBS- and PM$_{2.5}$-exposed WT and AMPKα2$^{-/-}$ mice with or without metformin (Met) treatment were stained with hematoxylin and eosin (H&E), Masson’s trichrome, and antibodies specific for neutrophils and macrophages (galectin-3, Gal-3) (brown staining). Scale bar = 100 μm. The relative collagenous fiber area (B), and the number of Gal-3 positive cells (C) and neutrophils (D) were quantified. N = 4; ** indicates 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.)
PRDX5, TRXR2 and the anti-apoptotic protein Bcl-2. Metformin treatment significantly attenuated the PM2.5-induced reduction in PRDX5, TRXR2 and Bcl-2 expression. In the lungs of AMPKα2−/− mice, PM2.5 exposure resulted in significant decreases in p-AMPK, PRDX5 and Bcl-2 expression, and increases in AMPKα1 expression. The decreases in p-AMPK and Bcl-2 levels, as well as the induction of AMPKα1, were attenuated by metformin treatment. Moreover, metformin significantly increased the protein expression levels of SOD2, PRDX3 and TRXR2 in the lungs of the PM2.5-exposed AMPKα2−/− mice (Fig. 3F, Fig. S1).

Metformin attenuates PM2.5-induced cardiac dysfunction and fibrosis. PM2.5 exposure had no obvious effect on body weight, heart weight or the ratio of heart weight to body weight in the WT mice, but significantly increased the heart weight, ratio of heart weight to body weight and cardiac myocyte cross-sectional area in the AMPKα2−/− mice. Metformin treatment significantly attenuated the increase in the ratio of heart weight to body weight and cardiac myocyte cross-sectional area in the PM2.5-exposed AMPKα2−/− mice (Tables S2–S3, Fig. 4A, and Fig. S2). Echocardiographic examination showed that PM2.5 exposure resulted in cardiac dysfunction in the WT and AMPKα2−/− mice, as indicated by the significant reduction in left ventricular (LV) ejection fraction (EF). In response to PM2.5 exposure, the AMPKα2−/− mice developed more severe LV dysfunction than the WT mice. Metformin significantly increased the LV EF values in the PM2.5-exposed WT and AMPKα2−/− mice (Fig. 4B). Furthermore,
Metformin also significantly attenuated PM$_{2.5}$-induced upregulation of myocardial atrial natriuretic peptide (ANP; a marker for cardiac stress) (Fig. 4C). The results from Masson's staining and TUNEL assay demonstrated that the hearts from the WT and AMPKα$_{2}^{-/-}$ mice developed obvious fibrosis and cardiomyocyte apoptosis after PM$_{2.5}$ exposure, while metformin significantly decreased the collagenous fibrotic area and apoptotic cell numbers in the hearts of the PM$_{2.5}$-exposed mice (Fig. 4D–F). As shown in Fig. 4G–H, metformin also attenuated PM$_{2.5}$-induced increases in myocardial collagen I and III mRNA levels in WT and AMPKα$_{2}^{-/-}$ mice. Together, these results indicated that metformin effectively attenuates PM$_{2.5}$-induced cardiac dysfunction in both wild type and AMPKα$_{2}^{-/-}$ mice.
Metformin attenuates PM$_{2.5}$-induced myocardial oxidative stress. PM$_{2.5}$ exposure significantly increased myocardial 3′-NT and 4-HNE levels in the WT and AMPKα2$^{-/-}$ mice, while these increases were significantly attenuated by metformin treatment (Fig. 5A–B). To investigate the effects of PM$_{2.5}$/metformin on mitochondrial superoxide generation, the heart sections were stained with Mitosox to evaluate the mitochondrial superoxide levels. As shown in Fig. 5C–D, PM$_{2.5}$ exposure caused significant increases in the mitochondrial superoxide levels in the hearts of the WT and AMPKα2$^{-/-}$ mice. In both WT and AMPKα2$^{-/-}$ mice, PM$_{2.5}$-induced myocardial superoxide overproduction was significantly attenuated by metformin administration (Fig. 5C–D). Similar results were also obtained from DHE staining (Fig. S3). In the hearts of the WT mice, PM$_{2.5}$ exposure significantly decreased the protein expression of SOD2, PRDX3, PRDX5 and TRXR2, and this reduction was significantly attenuated by metformin treatment. PM$_{2.5}$ exposure decreased AMPKα1 expression but had no significant effect on AMPKα2 or p-AMPKα expression levels. Metformin significantly increased the phosphorylation levels of AMPKα and TRX2 expression in the PM$_{2.5}$-exposed hearts but did not affect AMPKα1 or AMPKα2 expression (Fig. 5E, Fig. S4). In hearts of AMPKα2$^{-/-}$ mice, PM$_{2.5}$ exposure had no obvious effect on SOD2, PRDX3 or AMPKα1 expression, but significantly decreased p-AMPK levels and the protein expression of PRDX5, TRXR2 and TRX2, and these decreases were significantly attenuated by metformin (Fig. 5F, Fig. S4).

Metformin pretreatment attenuates PM$_{2.5}$-induced cell death and oxidative stress. To determine whether metformin affects PM$_{2.5}$-induced cell death in an AMPKα2-dependent manner, BEAS-2B and H9C2 cells were stably transfected with a shRNA lentiviral vector targeting scramble sequence (shScr) or AMPKα2 (shAMPKα2). The efficiency of knockdown was confirmed by Western blot analysis (Fig. S5). The cells were pretreated with 1 mM metformin for 2 h and were then replaced with fresh medium and exposed to 50 μg/ml PM$_{2.5}$ for 24 h. After PM$_{2.5}$ exposure, the shAMPKα2-BEAS-2B cells exhibited significantly lower cell viability and higher intracellular ROS levels than the shScr-BEAS-2B cells. Pretreatment with metformin significantly attenuated PM$_{2.5}$-induced cell viability loss and increases in intracellular ROS levels in both shScr- and shAMPKα2-BEAS-2B cells (Fig. 6A–B). In the shScr-BEAS-2B cells, PM$_{2.5}$ exposure had no obvious

![Fig. 5. Metformin attenuates myocardial oxidative stress in PM$_{2.5}$-exposed mice.](image-url) After PM$_{2.5}$ exposure, heart tissue was collected, and the myocardial 3′-nitrotyrosine (3′-NT) (A) and 4-HNE levels were measured. The heart sections were stained with Mitosox (scale bar = 20 μm), and the relative Mitosox fluorescence intensity was quantified (C, D). Lysates of the heart tissue were examined by western blotting for the expression of p-AMPKα, SOD2, PRDX3, PRDX5 and TRXR2. β-Tubulin was used as a loading control (E, F). N = 4; data are presented as the mean ± SEM; * indicates p < 0.05, ** indicates p < 0.01.
effect on the phosphorylation levels of AMPK or the expression of PRDX3 and TRXR2 but resulted in significant decreases in SOD2 expression and increases in PRDX5 expression. Metformin pretreatment increased p-AMPK and SOD2 expression in the PM2.5-exposed shScr-BEAS-2B cells (Fig. 6C). In the shAMPKα2-BEAS-2B cells, PM2.5 exposure significantly increased p-AMPK levels, whereas SOD2, PRDX3, PRDX5 and TRXR2 expression was decreased. Although metformin pretreatment did not significantly increase p-AMPK levels in PM2.5-exposed shAMPKα2-BEAS-2B cells, it significantly attenuated the reduction in SOD2, PRDX5 and TRXR2.

In the H9C2 cells, PM2.5 exposure significantly decreased cell viability, whereas it increased intracellular ROS levels in both the shScr and shAMPKα2 cells. However, the shAMPKα2-H9C2 cells exhibited significantly lower cell viability and higher intracellular ROS levels than the shScr-H9C2 cells. Pretreatment with 1 mM metformin significantly attenuated PM2.5-induced cell viability loss and oxidative stress in both the shScr and shAMPKα2 cells (Fig. 7A–B). In the shScr-H9C2 cells, PM2.5 exposure decreased PRDX3 and TRXR2 expression, whereas it increased PRDX5 expression. In shAMPKα2-H9C2 cells, PM2.5 exposure significantly decreased the expression of SOD2, PRDX3, PRDX5 and TRXR2. In these PM2.5-exposed cells, metformin pretreatment significantly increased p-AMPK, SOD2, PRDX3 and PRDX5 expression (Fig. 7C–D). We also stably transfected BEAS-2B and H9C2 cells with a shRNA lentiviral vector targeting AMPKα1 (shAMPKα1). In these shAMPKα1 stably transfected BEAS-2B and H9C2 cells, PM2.5-induced cell viability loss and oxidative stress could also be attenuated by pretreatment with metformin (Fig. 5G). Taken together, these results indicate that metformin protects against PM2.5-induced cell death and oxidative stress in an AMPKα1 or AMPKα2 independent manner.

4. Discussion

The present study has two major new findings. First, we demonstrated that metformin was effective in attenuating PM2.5-induced lung injury and cardiac dysfunction in both WT and AMPKα2−/− mice, indicating that metformin protects mice from PM2.5 exposure in part, through an AMPKα2-independent pathway. Second, the protective effect of metformin in PM2.5-exposed mice was associated with the upregulation of mitochondrial antioxidant enzymes.

Metformin, a miracle drug, is well known for controlling hyperglycemia via regulation of hepatic glucose production. However, growing epidemiological and experimental studies have suggested that metformin has multiple additional health benefits in diabetic patients [29–31]. For example, metformin was found to decrease the risk of mortality from all causes and cardiovascular death in patients with type-2 diabetes mellitus [30]. Moreover, metformin is also known for its anti-aging [32] and anti-cancer effects [33]. Interestingly, two recent studies demonstrated that metformin prevents PM2.5-inhaled vascular inflammation. Our results were consistent with those findings. First, we demonstrated that metformin decreases serum TNFα and IL-6 levels and pulmonary TNFα and IL-1β mRNA levels in PM2.5-exposed mice suggested that metformin protects PM2.5-induced lung injury, at least partially, by suppressing inflammatory responses [36–38]. The finding that metformin decreased serum TNFα and IL-6 levels and pulmonary TNFα and IL-1β mRNA levels in PM2.5-exposed mice suggested that metformin protects PM2.5-induced lung injury, at least partially, by suppressing inflammation. Our results were consistent with those findings.

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Fig. 6. Metformin attenuates PM2.5-induced cell death and oxidative stress in BEAS-2B cells. Scramble shRNA (shScr)- and AMPKα2-specific shRNA (shAMPKα2)-stably transfected BEAS-2B cells were pretreated with PBS or 1 mM metformin for 2 h and then exposed to 50 μg/ml PM2.5 for 24 h. Cell viability (A) and intracellular ROS levels (B) were then determined. Lysates of the control and PM2.5-exposed shScr (C) or shAMPKα2 (D) with or without metformin pretreatment were examined by western blotting to determine the expression of phosphorylated AMPKα, SOD2, PRDX3, PRDX5 and TRXR2. β-Tubulin was used as a loading control. N = 3–4; data are presented as the mean ± SEM; * indicates p < 0.05, ** indicates p < 0.01, NS, not significant.
consistent with previous reports that showed that metformin alleviated systemic and pulmonary inflammation in lipopolysaccharide (LPS)-induced endotoxic mice [18,34]. The anti-inflammatory actions of metformin have also been found in different models, and the involved molecular mechanisms include suppression of mammalian target of rapamycin (mTOR) or toll-like receptor 4 signaling [18,34], induction of transcription factor-3 (ATF-3) [39], phosphorylation of histone deacetylase 5 (HDAC5) and restoration of Krüppel-like factor 2 (KLF2) [40]. Since AMPK can directly or indirectly affect the inflammatory response, these anti-inflammatory mechanisms of metformin were dependent on AMPK activation. Although AMPKα2 deletion exacerbated the PM2.5-induced pulmonary inflammatory response, in this study we found that metformin was effective in decreasing the systemic and pulmonary inflammatory responses in PM2.5-exposed AMPKα2−/− mice, indicating that metformin can also suppress inflammation through an AMPKα2-independent pathway.

PM2.5 has the ability to induce high levels of ROS [41], and the overproduction of intracellular ROS causes inflammatory cytokine release and DNA damage, which leads to lung injury [42]. PM2.5 exposure may also promote oxidative stress by decreasing the expression of antioxidant enzymes [16,37]. In the present study, we found that PM2.5 exposure significantly increased pulmonary 3′-NT and 4-HNE levels, which were associated with the reduction in mitochondrial antioxidant enzymes (PRDX5 and TRXR2). The finding that metformin attenuates PM2.5-induced pulmonary oxidative stress and the reduction in PRDX5 and TRXR2 expression suggested that metformin may act as a kind of antioxidant. Moreover, a similar antioxidative effect of metformin has also been described in other experimental models [43–45]. For example, metformin attenuates ischemia reperfusion-induced oxidative stress in fatty livers by increasing antioxidant enzyme activity [44]. Metformin also prevents PM2.5-induced mitochondrial ROS generation in alveolar macrophages, and mitochondrially targeted antioxidants mimic the effects of metformin in vitro and in vivo [24]. As AMPKα2 deletion exacerbated PM2.5-induced oxidative stress and the reduction in PRDX5 [16], it is possible that metformin may suppress ROS production through activating AMPKα. However, we did find that metformin decreased the 3′-NT or 4-HNE levels in the lungs of PM2.5-exposed AMPKα2−/− mice. Moreover, metformin also attenuated PM2.5-induced cell death and oxidative stress in AMPKα2-depleted BEAS-2B cells. Thus, it is likely that metformin inhibits PM2.5-induced pulmonary oxidative stress independent of AMPKα2.

Numerous epidemiological studies have demonstrated that PM2.5 exposure is strongly tied to cardiovascular diseases, including arrhythmias, ischemic heart disease, heart failure, and cardiac death [4,10,46]. In this regard, the finding that metformin reduced cardiac dysfunction in PM2.5-exposed mice is of considerable interest. Metformin has been shown to be effective in improving cardiac function in diabetic patients and experimental animal models, including rapid ventricular pacing-, TAC-, or doxorubicin-induced heart failure [25,35,47,48]. Mechanistically, the cardioprotective effect of metformin is associated with AMPK-mediated enhancement of the myocardial metabolic energy status, reducing oxidative stress and inhibiting...
cardiomyocyte apoptosis in failing hearts [47–50]. The finding that metformin treatment decreases myocardial 3′-NT, 4-HNE and superoxide levels in PM$_{2.5}$-exposed mice suggested that metformin attenuates PM$_{2.5}$-induced cardiotoxicity by decreasing oxidative stress. Moreover, metformin also attenuates PM$_{2.5}$-induced cardiac dysfunction and myocardial oxidative stress in AMPKα2⁻/⁻ mice. These data were consistent with those of a previous study showing that metformin attenuates TAC-induced hypertrophy, LV dysfunction and myocardial oxidative stress in AMPKα2⁻/⁻ mice [25]. Therefore, metformin may exert cardiac protective effects through an AMPKα2-independent pathway.

It is noticeable that PM$_{2.5}$ exposure had no obvious effect on pulmonary PRDX3 and SOD2 expressions, but significant decreased myocardial PRDX3 and SOD2 expressions. Furthermore, there were also significant differences between pulmonary and myocardial PRDX3 and SOD2 expressions in the PM$_{2.5}$-exposed AMPKα2⁻/⁻ mice after metformin treatment. We speculated that these discrepancies were relative to with PM$_{2.5}$ exposure mode, sensitivity to oxidative stress, and activity of redox-related transcription factors or kinases. However, more careful studies are needed to address this speculation.

Growing evidence has indicated that metformin exerts biological and physiological functions through both AMPK-dependent and AMPK-independent molecular pathways [51]. In the liver, metformin inhibits hepatic gluconeogenesis in an AMPK-independent manner [52,53]. Metformin also alleviates unilateral ureteral obstruction-induced renal fibrosis through both AMPKα2-dependent and AMPKα2-independent pathways [54]. Here, we demonstrated that metformin could attenuate PM$_{2.5}$-induced inflammation and oxidative stress in an AMPKα2-independent manner, and the underlying mechanism is related to the regulation of mitochondrial antioxidant enzymes. There is evidence that some redox-related transcription factors or kinases, including signal transducer and activator of transcription 3 (STAT3) [55], hypoxia-inducible factor 1α (HIF-1α) [56], mTOR [57,58] and SIRT1 [59], are regulated by metformin through an AMPKα2-independent pathway. However, the extent to which these actions of metformin regulate mitochondrial antioxidant enzymes and attenuate PM$_{2.5}$-induced lung injury and cardiac dysfunction independent of AMPKα2 is not clear.

In summary, our study indicates that metformin, in dependent of AMPKα2, protects against PM$_{2.5}$ exposure-induced lung injury and cardiac dysfunction by maintaining the expression of mitochondrial antioxidant enzymes, thereby decreasing oxidative stress and inflammation. These results suggest that metformin administration is a potential strategy to treat air pollution-associated diseases.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2019.101345.
Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent and independent manners, in vivo experiments. The effects of metformin on liver injury include amelioration of hepatic steatosis, improvement of liver function, and reduction of liver inflammatory markers. In vitro studies have also shown that metformin inhibits AMPK-dependent and independent signaling pathways. In vivo studies have demonstrated that metformin reduces liver fat content and improves liver function in models of steatosis and obesity. The mechanisms by which metformin reduces liver injury include suppression of liver inflammation and modulation of the metabolic and autophagic pathways.