Metformin mitigates gas explosion-induced blast lung injuries through AMPK-mediated energy metabolism and NOX2-related oxidation pathway in rats

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Abstract. Gas explosions are a recurrent event in coal mining that cause severe pulmonary damage due to shock waves, and there is currently no effective targeted treatment. To illustrate the mechanism of gas explosion-induced lung injury and to explore strategies for blast lung injury (BLI) treatment, the present study used a BLI rat model and supplementation with metformin (MET), an AMP-activated protein kinase (AMPK) activator, at a dose of 10 mg/kg body weight by intraperitoneal injection. Protein expression levels were detected by western blotting. Significantly decreased expression of phosphorylated (p)-AMPK, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) and metabolic activity were observed in the BLI group compared with those in the control group. However, the mitochondrial stability, metabolic activity and expression of p-AMPK and PGC1α were elevated following MET treatment. These results suggested that MET could attenuate gas explosion-induced BLI by improving mitochondrial homeostasis. Meanwhile, high expression of nicotinamide adenine dinucleotide phosphate oxidase (NOX2) and low expression of catalase (CAT) were observed in the BLI group. The expression levels of NOX2 and CAT were restored in the BLI + MET group relative to changes in the BLI group, and the accumulation of oxidative stress was successfully reversed following MET treatment. Overall, these findings revealed that MET could alleviate BLI by activating the AMPK/PGC1α pathway and inhibiting oxidative stress caused by NOX2 activation.

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Introduction

Gas explosions accidents in coal mining threaten public safety and social economy. The mortality associated with gas accidents reached 54.5% of the total coal industry-associated mortalities from 2010 to 2019 in China (1). Gas explosions are able to cause complex injuries with severe inhalation injury, which puts patients at a high risk of developing systemic infection and respiratory failure (2); however, the mechanism of injury remains unknown.

As a cavity and gas-bearing organ, the lung is affected by in gas explosion-induced blast injury. Thus it can be severely torn and fragmented by gas explosion-induced shockwaves (3). Recently, a study indicated that type I and type II alveolar epithelial cells are the targets in BLI induced by gas explosion, and that the characteristics of the injury are nuclear contraction and mitochondrial structural abnormalities (4). A previous study on canines demonstrated that levels of serum malondialdehyde increased with a blast, suggesting that blast wave injury resulted in oxidative stress in the organism (5). Mitochondria are important organelles involved in adenosine triphosphate (ATP) synthesis that produce ATP and numerous other biosynthetic intermediates. Our previous study revealed that energy is consumed rapidly and that galactose metabolism and the tricarboxylic acid (TCA) cycle are downregulated in

rats exposed to gas explosion (6). This abnormality in energy metabolism is probably closely associated with mitochondrial function. The mitochondrial quality control system plays an important role, not only in maintaining the biological production of energy, but also in controlling oxidant formation by the electron transport chain (ETC) (7). Reactive oxygen species (ROS) produced by the ETC can oxidize lipid molecules and induce DNA damage, especially in mitochondrial DNA (mtDNA) (8). Owing to the lack of a protective histone shield around mtDNA and limited DNA repair mechanisms, mtDNA is more sensitive to oxidative damage compared with nuclear DNA (9,10). This suggests mitochondrial mass, function and stabilization are intimately involved in energy metabolism and oxidative stress (9,10).

AMP-activated protein kinase (AMPK), a major energy sensor at both cellular and whole-body levels (11), maintains energy homeostasis in eukaryotic cells. A previous study observed a correlation between nicotinamide adenine dinucleotide phosphate oxidase (NOX2) expression and energy metabolism (12). The NOX2 heterodimer is composed of p22phox and gp91phox, and contributes to the production of ROS, especially superoxide anions, which are very harmful to cells (13). ROS generated by NOX2 act as the initiator of the pathophysiology of acute lung injury (14). Recent study have proposed that AMPK and NOX2 are mutually regulated (15). Furthermore, AMPK increases mitochondrial biogenesis by activating peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) (16,17). Therefore, AMPK may be a potential target in the treatment of gas explosion-induced blast lung injury.

Metformin, an activator of AMPK, is a key regulator of energy metabolism and balance, and it improves mitochondrial respiration, restores the mitochondrial life cycle by activating the protein kinase AMPK and inhibits oxidative stress through the upregulation of PGC1α and superoxide dismutase 1 in acute lung injury (18,19). The aforementioned studies confirmed that metformin can regulate energy metabolism and oxidative stress via the activation of AMPK.

The present study aimed to explore the role of the AMPK/NOX2 pathway in gas blast-induced lung injury by designing experiments that simulated the effects of a gas explosion on a real roadway with a rat model, which provided evidence to demonstrate the pulmonary-protective effects of metformin and its specific molecular mechanism with respect to targeting and alleviating gas explosion-induced blast lung injury.

Materials and methods

Chemicals and reagents. Metformin (MET) was purchased from Bristol-Myers Squibb Pharmaceuticals Ltd. The total antioxidant capacity (T-AOC) detection kit (cat. no. #A015-3-1) was purchased from the Nanjing Jiancheng Bioengineering Institute. The micro isocitrate dehydrogenase mitochondrial (ICDHm) assay kit (cat. no. #BC2165), micro α-ketoglutarate dehydrogenase (α-KGDH) assay kit (cat. no. #BC0715), phosphofructokinase (PFK) activity assay kit (#BC0535), ATP content assay kit (cat. no. #BC0305) were purchased from Beijing Solarbio Science & Technology Co., Ltd. TRIzol® was purchased from Ambion (Thermo Fisher Scientific, Inc.). The Pierce™ BCA protein assay kit and SuperScript III reverse transcriptase were purchased from Thermo Fisher Scientific, Inc. SDS-PAGE sample loading buffer was purchased from Beyotime Institute of Biotechnology. β-actin (cat. no. #AF7018), AMPK (cat. no. #AF6423) and p-AMPK antibodies (cat. no. #AF3423) were purchased from Affinity Biosciences, Ltd.; NOX2 antibody (cat. no. #A19701) and PGC-1α (cat. no. #A19674) antibody were purchased from ABclonal Biotech Co., Ltd.; and catalase (CAT; cat. no. #12980) antibody was purchased from Cell Signaling Technology, Inc. SYBR® GreenER qPCR SuperMix Universal was purchased from Invitrogen (Thermo Fisher Scientific, Inc.).

Experimental animals and grouping. In total, 90 six-month-old male specific pathogen-free (SPF) Sprague-Dawley rats with an average weight of 200±20 g were purchased from Speifu (Beijing) Biotechnology Co., Ltd. and the animal license number was SCXK 2019-0010. The animals were housed in a SPF animal room and maintained under a controlled environment (24±1˚C temperature, relative humidity of 40-50% and 12 h light/dark cycle) for 1 week with adaptive feeding to ensure rats adaptation to the new environment and free diet before commencement of the experiments. The rats were randomly divided into three groups (n=30/group) as follows: Control group, gas explosion injury (BLI) group and gas explosion injury + metformin (BLI + MET) group. A total of 10 rats, randomly selected from each group, were euthanized on days 1, 3 and 7 after treatment. Rats were dissected and the lungs of rats were isolated and sectioned. The animal experiments were performed in accordance with Guide for the Care and Use of Laboratory Animals (20), and were approved by the Medical Ethics Committee of Xinxiang Medical University (approval no. XYLL-2019001; Jan 13, 2019).

BLI model and intervention. Preliminary experiments confirmed that the explosion flame range was 160 m (data not shown); thus, rats were placed 240 m away from the center of the explosion to ensure that they were only affected by shock waves. The explosion chamber, with a total volume of 100 m3 contained air with 9.3% methane gas, and the ignition energy was 20 J. Rats in the BLI and MET groups were deeply anesthetized with 1% sodium pentobarbital (50 mg/kg) and placed in a custom iron cage. After the explosion, rats of the BLI + MET group were administered metformin daily by intraperitoneal injection (10 mg/kg). A total of 10 rats from each group were randomly selected and euthanized on the 1st, 3rd and 7th day. Before collecting the blood, rats were deeply anesthetized as aforementioned. The rats were cervically dislocated after the blood was collected and the organs were retained after death was confirmed. Lung tissues were isolated and weighed. The right lower lung tissue was fixed in 4% paraformaldehyde at 25˚C for 24 h, and the remaining lung tissue was frozen in liquid nitrogen at -80˚C for further tests.

Lung function detection in rats. Rats were placed in the test room for at least 30 min for habituation. Indicators of lung function, peak inspiratory flow rate (PIFR) and minute ventilation (MV), were measured using a whole-body plethysmography system (Buxco Electronics, Inc.).

Assessment of lung injury based on hematoxylin and eosin (HE) staining. The lower lobe of right lung tissue was fixed with 4% formaldehyde at 25˚C for 24 h, dehydrated, embedded in paraffin, cut into 4-μm sections and kept at 60˚C for 1 h. Tissue was deparaffinized with xylene for twice 20 min and then rehydrated, stained with hematoxylin at 25˚C for 5 min and eosin at 25˚C for 3 min before histopathological observation. Overall, five visual fields were randomly selected for each section under a light microscope and scored based on the following four aspects: Alveolar wall thickness, congestion, hemorrhage and inflammatory cell infiltration. A five-point scale from 0 to 4 was used to generate a systematic scoring system for acute lung injury as follows: 0=No damage; l=mild damage; 2=moderate damage; 3=severe damage; and 4=very severe damage (21).

Detection of energy metabolism-related indicators using a biochemical assay kit. Lung tissues frozen at -80˚C were used for biochemical analyses using the aforementioned ICDHm, α-KGDH, PFK activity and ATP content assay kit according to the manufacturer's instructions. Approximately 30 mg of lung tissue was added to 300 μl of homologous extraction solution for ice-bath grinding and then centrifuged at 8,000-11,000 x g at 4˚C for 10-15 min. The supernatant was collected and placed on ice for further testing. Working reagents and the sample supernatant were added to each well of a 96-well plate and incubated for several minutes at 37˚C according to the manufacturer's instructions. Product formations were detected using a standard microplate reader (PerkinElmer, Inc.; EnSpire Multimode Plate Reader) at a specific absorbance wavelength (340 and 505 nm).

Observation of mitochondrial morphology via transmission electron microscopy (TEM). Fresh rat lung tissue was obtained and cut into cubes of ~1 mm^3 to minimize mechanical damage, such as pulling, contusion and extrusion. The small lung tissue cubes were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer overnight at 4˚C and then washed in ice-cold PBS three times. The fixed samples were post-fixed in 1% osmium tetroxide in 0.1 M PBS overnight at 25±3˚C and rinsed in PBS as previously described. Next the samples were dehydrated, infiltrated, embedded in resin and polymerized overnight in an oven at 60˚C. The embedded tissue was cut into ultrathin sections of 60-80 nm using an ultrathin slicer (Leica UC7; Leica Microsystems GmbH). After staining with 2% uranium acetate saturated alcohol solution and 2% lead citrate solution (25˚C for 15 min each), the ultrastructures of cells were observed under a transmission electron microscope (HT7700; Hitachi, Ltd.) and the captured images were analyzed by Image J 1 (National Institutes of Health).

Detection of the total serum antioxidant capacity. Blood was obtained from the abdominal aorta before the animals were euthanized. The collected blood samples were clotted at 25±3˚C for 30 min and then centrifuged at 4,000 x g at 4˚C for 15 min. The serum was removed and stored in the tube at -80˚C. Freshly prepared $FeSO_4$-$7H_2O$ standard solutions were used; the remaining steps in the experiment were performed according to the instructions of the T-AOC detection kit.

Reverse-transcription-quantitative (RT-qPCR). Total RNA from lung tissues was extracted using TRIzol®. The extracted RNA was reverse-transcribed into cDNA using SuperScript III reverse transcriptase according to the manufacturer's instructions. All mRNA levels were detected using SYBR® GreenER™ qPCR SuperMix Universal. The cycling conditions were as follows: 95˚C Pre-denaturation for 1 min, 40 cycles of 95˚C for 15 sec, 60˚C for 15 sec and 72˚C for 30 sec. Finally, 65˚C for 1 min, 95˚C for 20 sec and 37˚C for 1 min. Fold-changes in mRNA expression levels were calculated using $2^{-\Delta\Delta Cq}$ values (22). The primer sequences of the targeted genes used in this study were as follows: Receptor of advanced glycation end products (*RAGE*), forward, 5'-CAATGGTTC ACTCCTCCTT-3', and reverse 5'-TCTGGTAGACTCGGA CTC-3'; *AMPK*, forward, 5'-AATTCGCAGGGAGATTCA GA-3', and reverse, 5'-ACAGCTCTCCTCCAGAAACG-3'; *PGC1-α*, forward 5'-GCACTGACAGATGGAGACGTGA-3', and reverse, 5'-TCATTGTAGCTGAGCTGAGTGTTGG-3'; Transcription factor A of mitochondrial (*TFAM*), forward 5'-TGAAGCTTGTAAATCAGGCTTGGA-3', and reverse, 5'-GAGATCACTTCGCCCAACTTCAG-3'; Gp91phox (*GP91*), forward 5'-CTGCCAGTGTGTCGGAATCT-3', and reverse, 5'-TGTGAATGGCCGTGTGAAGT-3'; *CAT*, forward, 5'-AAGCTGGTTAATGCGAATGG-3', and reverse, 5'-AAG TTTTTGATGCCCTGGTC-3'; *GADPH* forward, 5'-TCAACG GCACAGTCAAGG-3', and reverse 5'-TCAACGGCACAG TCAAGG-3'.

Western blotting. Rat lung tissues were rapidly homogenized using an ultrasonic tissue disruptor (SCIENTZ-48; Ningbo Xinzhi Biotechnology Co., Ltd.) with cold RIPA extraction buffer (Beyotime Institute of Biotechnology) to ensure complete homogenization. Lung tissue remained on ice for 30 min, and then it was centrifuged at 15,000 x g at 4˚C for 15 min to obtain the supernatant. Protein concentration was measured using the Pierce™ BCA protein assay kit and denatured with SDS-PAGE sample loading buffer at 100˚C for 5 min. 30 ng denatured protein samples were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (30 min at 90 V, 90 min at 120 V), and electro-transferred to a 0.45 μm polyvinylidene difluoride membrane for 70 min at 300 mA. The membranes were incubated in blocking solution (5% skim milk) at 25±3˚C for 90 min, and subsequently incubated with the antibodies targeting the following proteins at 4˚C overnight: β-actin (1:3,000), AMPK (1:2,000), p-AMPK (1:2,500), NOX2 (1:2,000), PGC-1α (1:2,000) and CAT (1:2,000). The next day, the membrane was washed three times with 5% tris-buffered saline with tween for 30 min (10 min each) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3,000, cat. no. #S0001, Affinity Biosciences, Ltd.) for 2 h at 25±3˚C. Finally, the membranes were washed three times for 10 min each, and chemiluminescence detection was performed using the Amersham Image Quant800 (Cytiva) system. The images were analyzed by Image J 1 (National Institutes of Health).

Statistical analysis. All data were analyzed using SPSS 20.0 (IBM Corp.). Continuous variables of normally distributed were expressed as the mean ± SD, and significant differences between groups were determined using one-way ANOVA

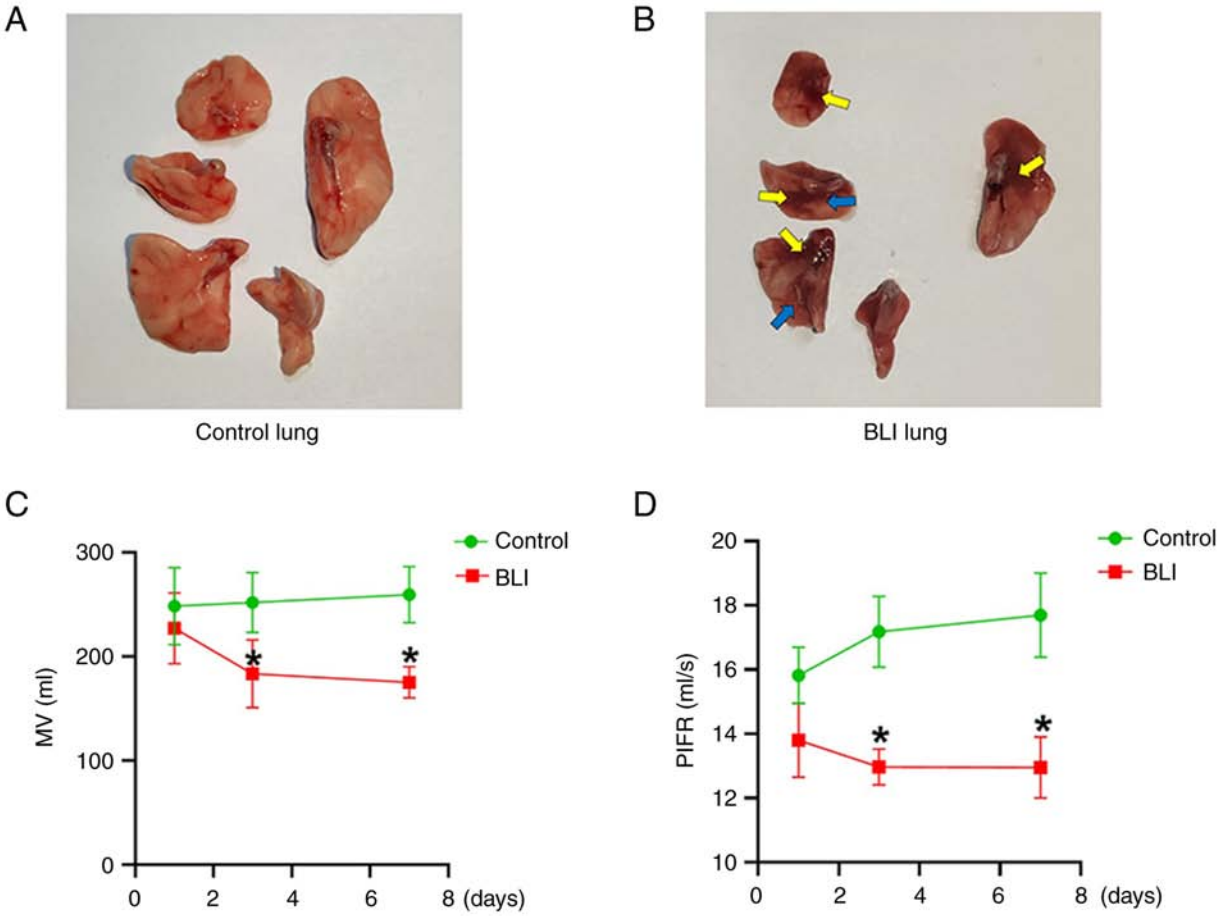

Figure 1. Gas explosions cause lung tissue damage and loss of pulmonary function. (A and B) Changes in lung appearance and pulmonary function measured using (C) MV and (D) PIFR. Yellow arrow, bleeding; blue arrow, alveolar collapse. n=8. *P<0.05 compared with the control group. MV, ventilation per minute rate; PIFR, peak inspiratory flow rate; BLI, blast lung injury.

followed by Tukey's post hoc test. Ordinal variables were expressed as the median with interquartile range and were analyzed using Kruskal-Wallis test and Dunn's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Gas explosions cause lung tissue damage and pulmonary dysfunction. In the present study, lung injuries were clearly observed in rats exposed to gas explosions. Compared with that in the control group, gas explosion exerted marked effects on the lungs of rats in the BLI group, characterized by evident lung bleeding points (yellow arrows) and lung alveolar collapse (the blue arrows) (Fig. 1A and B). Furthermore, gas explosion reduced lung function as follows: MV and PIFR of rats in the BLI group were significantly lower compared with those in the control group (Fig. 1C and D). Compared with those in the control group, the differences in lung function of rats in the BLI group at all time points were statistically significant, and this trend became more pronounced over time. These results indicate the BLI rat model was established successfully.

MET attenuates pathological and endothelial factor damage to lungs caused by gas explosion. The lungs of the rats in the control group had intact alveolar structures, thin, clear alveolar walls and no edema (Fig. 2). Compared with those in the normal control group, alveolar structure disorder, alveolar wall collapse, bleeding, and numerous inflammatory cells were observed in the BLI group (Fig. 2). Histological assessment revealed that the degree of inflammation was most severe on the third day after the gas explosion. To determine the effect of MET on the established BLI rat model, MET (10 mg/kg) was injected intraperitoneally into the rats. As expected, the lungs of the MET group had a significantly reduced amount of inflammatory cells and thinner alveolar walls compared with those of the BLI group. The pathological scores of acute lung injury are presented in Fig. 2B. In addition, the expression of an endothelial factor, RAGE, was detected, which was associated with acute lung injury in the control and BLI group (23) using RT-qPCR. The results (Fig. 2C) revealed that activation of the RAGE damage pathway by the gas explosion was rescued by MET on day 1. The expression of RAGE in the BLI group was not significantly increased at days 3 and 7 compared with the control; however, treatment with MET did markedly decrease the expression of RAGE in the BLI + MET group compared with the BLI group, and this decrease was significant on day 3.

MET regulates the energy metabolic disturbances caused by gas explosions. Pulmonary ATP levels were significantly lower in the BLI group compared with in the control group, and MET treatment significantly increased pulmonary ATP levels in gas explosion-induced rats lung injury (Fig. 3A). Therefore, the activities of several metabolic enzymes in the

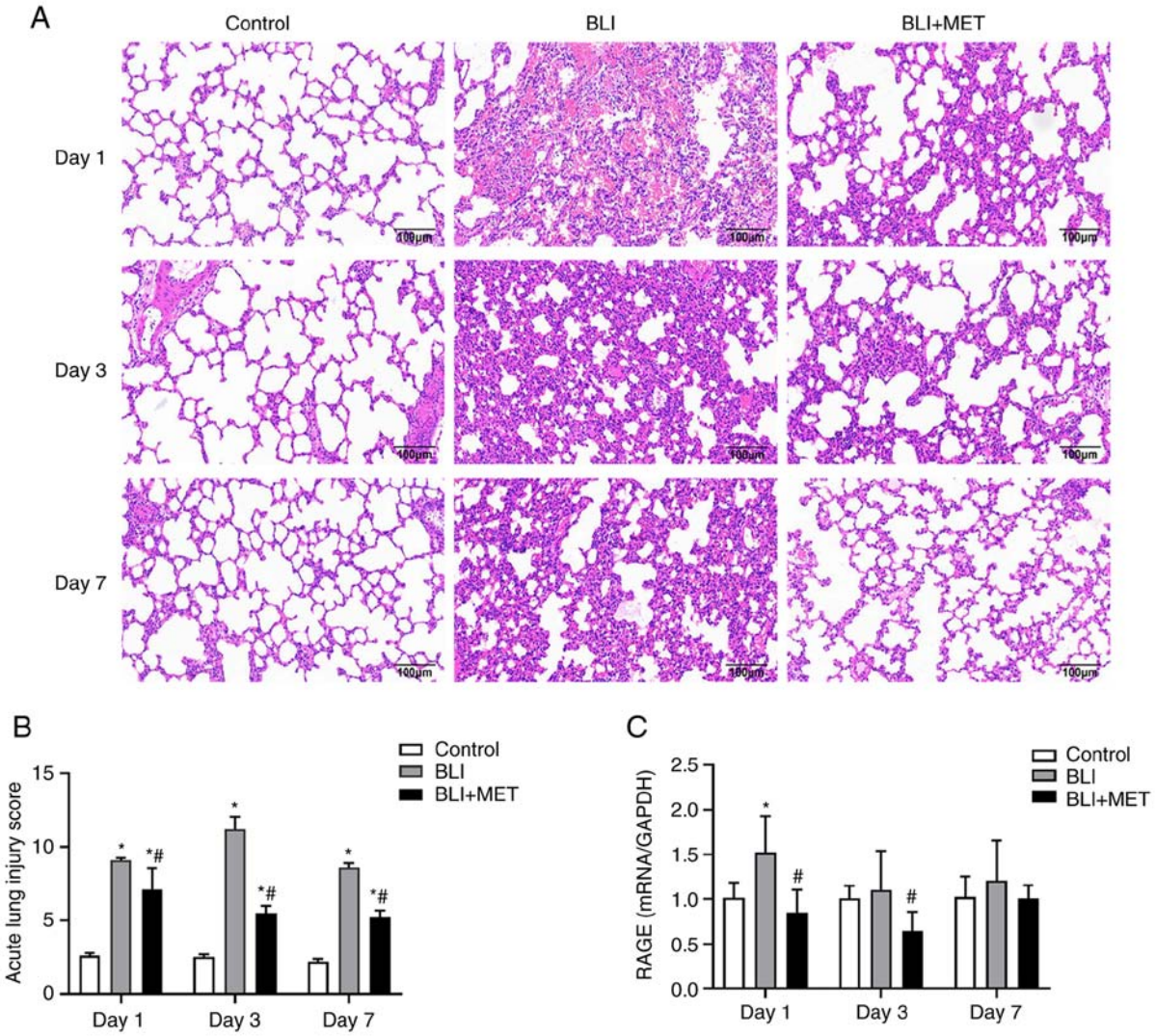

Figure 2. MET reduces pathological and endothelial factor damage to lungs caused by gas explosions. (A) Hematoxylin and eosin staining of lung tissue in each group at 1, 3 and 7 days after gas explosion (magnification, x200; scale bar, 100 μm). (B) Acute lung injury score (from 0 to 4). n=6. (C) Expression of RAGE in lung tissue of rats, measured using RT-qPCR. The relative quantitative expression of *RAGE* mRNA was normalized to that of GAPDH. n=6. *P<0.05 compared with control group; $^{\#}$P<0.05 compared with the BLI group. RT-qPCR, reverse-transcription-quantitative PCR; RAGE, renal tumor antigen 1; BLI, blast lung injury; MET, metformin.

TCA cycle and glycolysis were investigated to confirm that MET regulated energy metabolism.

Similarly, the enzymatic activities of rate-limiting enzymes α-KGDH and ICDHm in the TCA cycle (24,25) were significantly lower in the BLI group compared with the control group (Fig. 3B and C), and MET treatment markedly improved these enzymatic activities in rats exposed to a gas explosion at each time point. The same effect was observed in terms of the activity of PFK, which is the rate-limiting enzyme in glycolysis. However, the changes between the MET and the BLI group varied significantly only on days 3 and 7 (Fig. 3D). Overall, these data indicated that MET ameliorated the deregulated energy metabolism attributed to gas explosion-induced injury.

MET activates the AMPK/PGC1 mitochondrial protection pathway. The aforementioned results showed that MET alleviated pathological damage and regulated energy metabolism in the blast-injured lung of rats. Therefore, the present study detected the expression of AMPK and PGC-1α using western blotting and RT-qPCR to confirm the effect of MET on the mitochondrial protective pathway (Fig. 4). The ratio of p-AMPK/t-AMPK levels were significantly lower in the BLI group compared with the control group on the 3rd day, and PGC-1α levels were significantly lower in the BLI group compared with the control group at each time point (Fig. 4A-D); whereas the ratio of p-AMPK/t-AMPK levels and PGC-1α levels were significantly higher in the MET compared with the BLI group on day 3 and 7 (Fig. 4C and D). Similarly, results of RT-qPCR revealed that the mRNA expression levels of *AMPK*, *PGC-1α* and *TFAM* in the rats exposed to gas were lower compared with those in the control group, while MET treatment significantly increased the expression of these mRNA markers in gas blast-injured rats compared with BLI group (Fig. 4E-G).

MET maintains mitochondrial morphological stability. Ultrastructural changes in the lung tissue were observed using TEM (Fig. 5). The results indicated that the mitochondria of rats in the control group had the same normal shape, size and

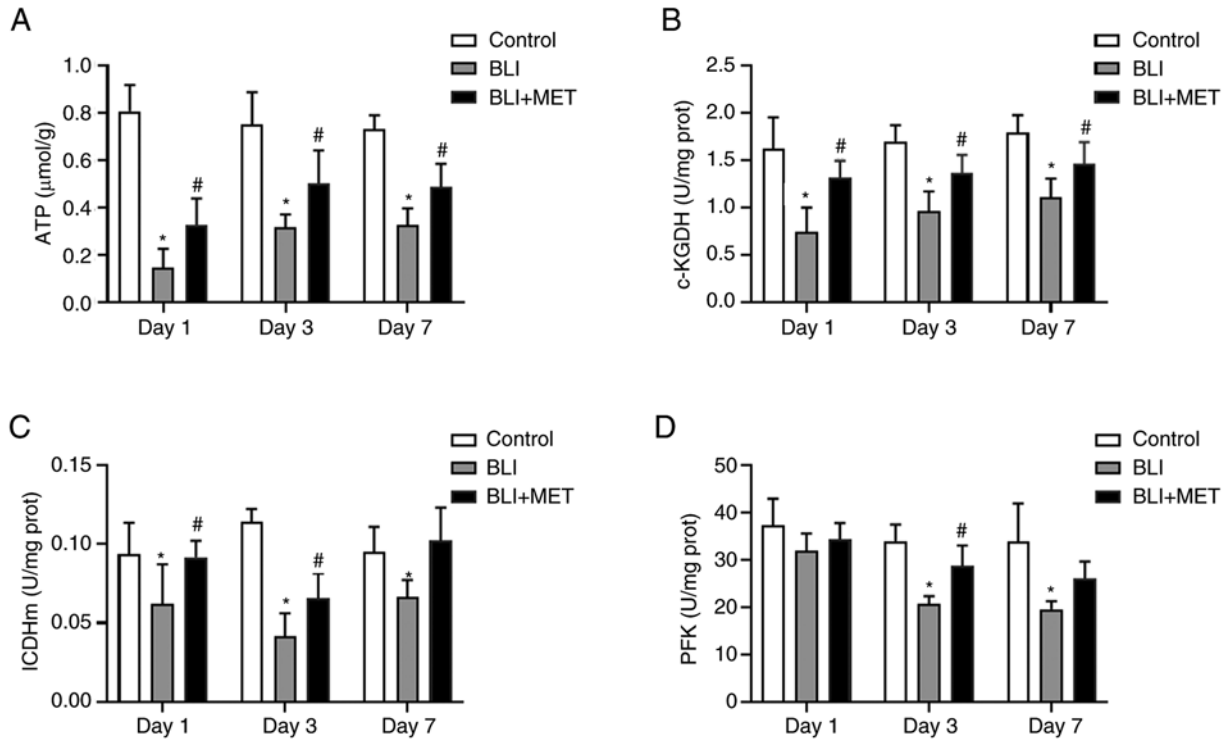

Figure 3. MET regulates the energy metabolic disturbances caused by gas explosions. (A) Changes in ATP content in lung tissue. Changes in (B) α-KGDH, (C) ICDHm and (D) PFK activity in lung tissue. n=8. *P<0.05 compared with the control group; #P<0.05 compared with the BLI group. ICDHm, micro isocitrate dehydrogenase mitochondrial; α-KGDH, micro α-ketoglutarate dehydrogenase; PFK, phosphofructokinase; BLI, blast lung injury; MET, metformin.

arrangement, and that mitochondrial cristae were arranged in layers. However, the rats with gas explosion-related swelling demonstrated a decrease or disappearance of the mitochondria crista and vacuolization. Moreover, loss of the lamellar body structure of alveolar type II cells was observed. As expected, the number of mitochondrial cristae in rats treated with MET increased, and the structures of mitochondria and lamellar bodies were more stable compared with those in the rats exposed to a gas explosion.

MET downregulates oxidative stress caused by NOX2 due to gas explosions. Western blotting revealed that NOX2 levels in lung tissues were significantly increased in the BLI group compared with those in the control group, and MET treatment decreased NOX2 levels, the main source of ROS in acute lung injury (Fig. 6A and B). In addition, CAT levels were significantly decreased in the lung tissues of gas explosion-exposed rats compared with the control group; however, MET supplementation increased the antioxidant capacity of the lung (Fig. 6A and C). Similarly, RT-qPCR revealed that the levels of *GP91*, which is the main subunit of the superoxide anion-generating NADPH oxidase complex, and *CAT* were consistent with the western blotting results (Fig. 6D and E). Finally, the serum T-AOC of rats exposed to gas explosions was significantly lower compared with that in the rats of the control group, whereas MET treatment increased this capacity in gas explosion-exposed rats (Fig. 6F).

Discussion

The shock wave pressure caused by a gas explosion increases following multiple reflections, resulting in extensive damage and serious harm due to the complex structure of the mine. The high-temperature flame from a gas explosion can cause serious damage to the upper and lower respiratory tracts and alveolae (26). Previous studies have indicated that shock waves from a gas explosion contribute damage to the cavity viscera, such as the lungs and brain (27,28). The mechanism of gas explosion-induced lung injury is unclear, and our previous metabolomic study identified nine different metabolites that differ significantly between the control rats and explosion-induced acute lung injury rats (6). The identified biomarkers reflect well-known adverse health effects of a gas explosion, such as inflammation and impairment of glucose homeostasis (29). In addition, NOX2 is an important source of ROS in acute lung injury, and NOX2-induced ROS causes oxidative damage to macromolecules, such as proteins, DNA and lipids (13). AMPK, an important energy sensor for the maintenance of cellular energy homeostasis, can be activated by MET (11). Recent studies have indicated that in addition to the treatment of type 2 diabetes, MET helps alleviate the clinical symptoms of cancer, aging (30), cardiovascular disease (31) and kidney disease (32). Therefore, the present study explored the effect of MET on energy metabolism during gas explosion-exposed pulmonary injury in rats. The results indicated that MET could attenuate gas explosion-induced lung injury via AMPK-mediated energy metabolism and the NOX2-related oxidase pathway.

The present study used an actual roadway to imitate a real gas explosion situation in a tunnel. There were bleeding points in the lungs and a large number of free red blood cells were observed under the microscope; this indicated that shock waves caused by a gas explosion could induce pulmonary

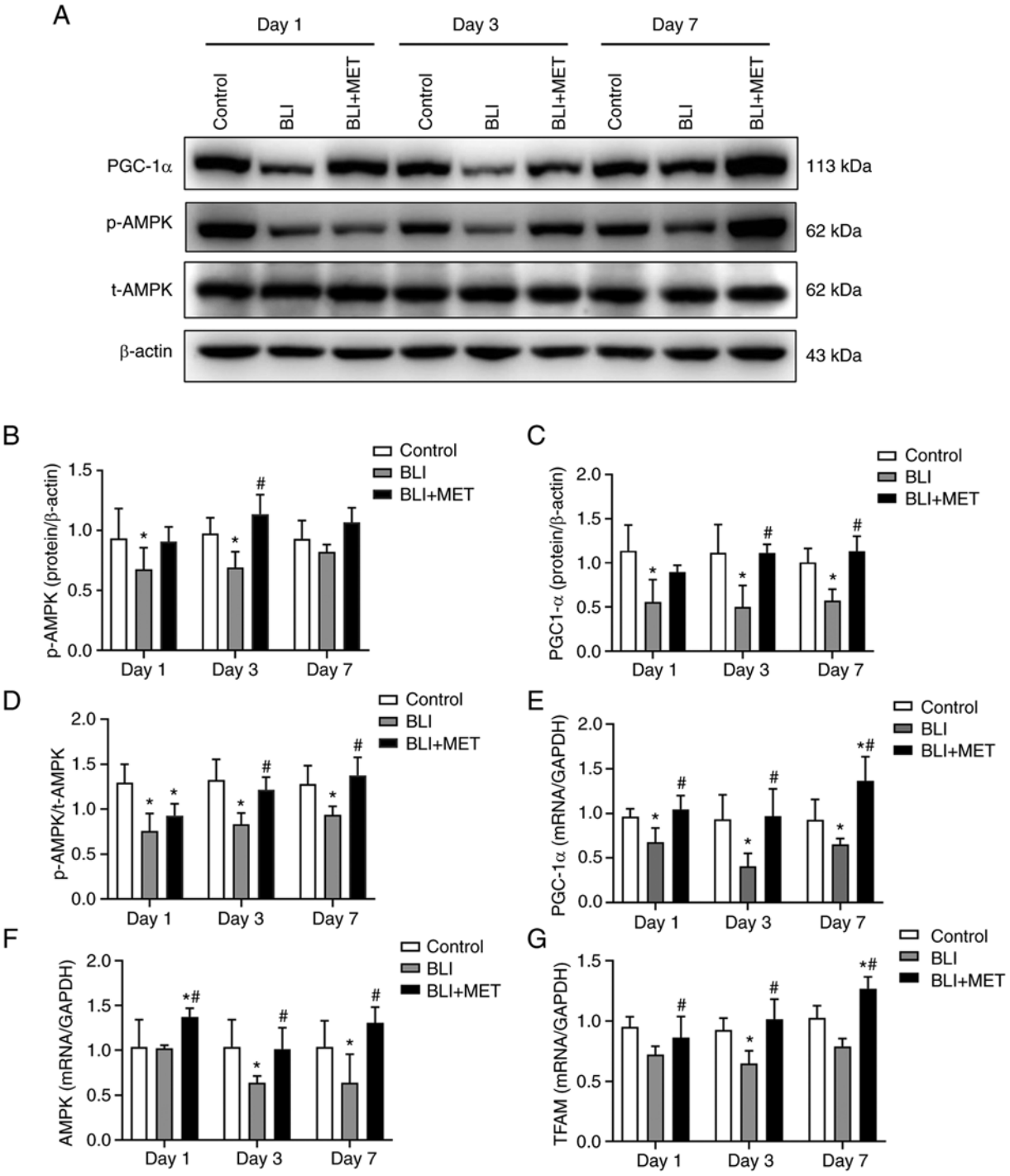

Figure 4. MET activates the AMPK/PGC1α mitochondrial protection pathway. (A) Expression of p-AMPK, t-AMPK and PGC-1α. Protein levels of (B) p-AMPK, (C) PGC-1α and (D) p-AMPK/t-AMPK were quantified using Image J Software. n=8. RT-qPCR analysis of the relative quantitative expression levels of (E) *PGC-1α* and (F) *AMPK* and (G) *TFAM* in lung tissues of rats, and the relative quantitative expression of each gene was normalized to that of *GAPDH*. n=6. *P<0.05 compared with the control group; #P<0.05 compared with the BLI group. RT-qPCR, reverse-transcription-quantitative; BLI, blast lung injury; MET, metformin; p-, phosphorylated; t-, total; AMPK, AMP-activated protein kinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; TFAM, transcription factor A of mitochondrial.

hemorrhage and decreased MV and PIFR, indicating that a gas explosion in a real roadway environment could lead to a decrease in respiratory function and lung tissue damage in a rat model. The peak of inflammatory cell influx was related to the impairment of lung function. Pulmonary edema induced by inflammation often shows an enhanced respiration pause (33), and the reduction of MV and PIFR indicates the occurrence of airway stenosis. These results prove the applicability of the present gas explosion blast lung injury-induced rat model.

To investigate whether MET could mitigate gas explosion-induced blast injury, 10 mg/kg of MET was administered to rats in the present study. The results revealed that MET alleviated the pathological injury induced by exposure to a gas

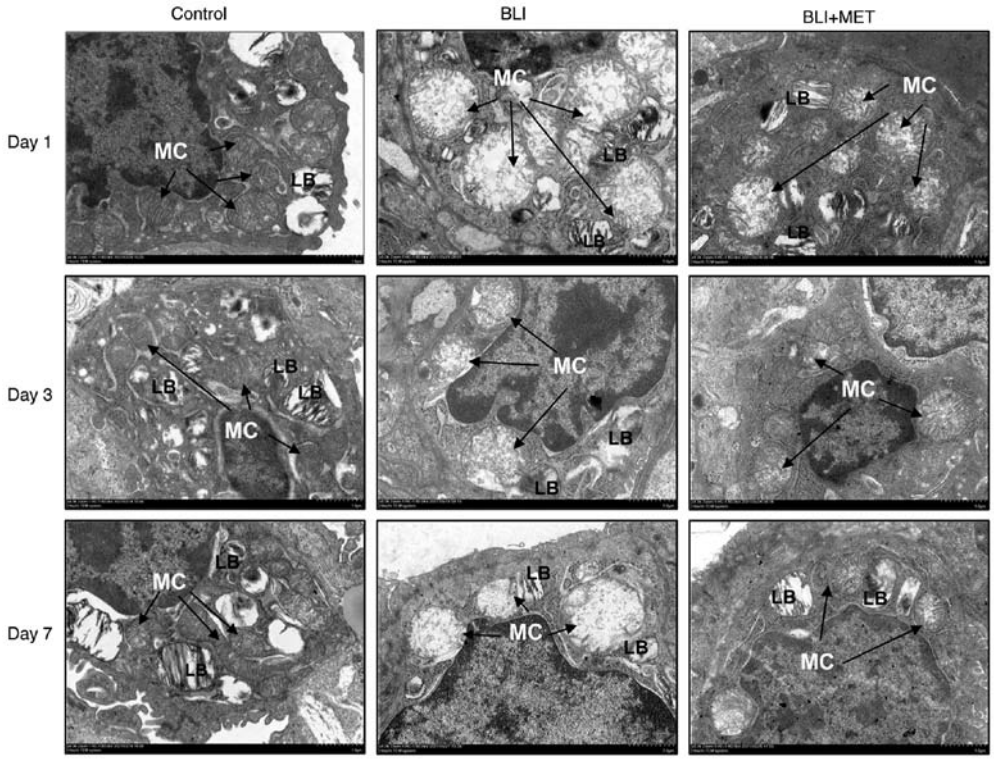

Figure 5. MET maintains mitochondrial morphological stability. Representative images of lung tissue as observed using transmission electron microscopy on 1, 3 and 7 days after the gas explosion. Images were obtained with TEM (magnification, x8,000; scale bar, 1.0 μm). MC, mitochondria; LB, lamellate bodies; BLI, blast lung injury; MET, metformin.

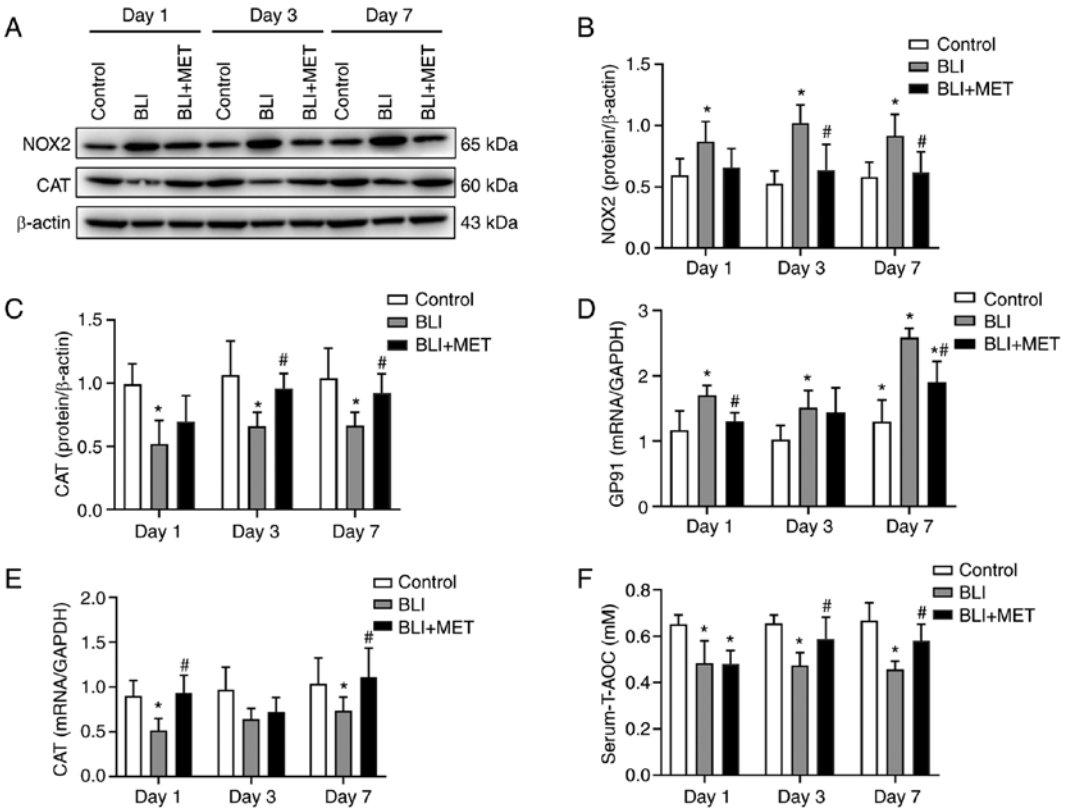

Figure 6. MET downregulates NOX2-induced oxidative stress caused by gas explosions. (A) Protein expression levels of NOX2 and CAT. Protein expression levels of (B) NOX2 and (C) CAT were quantified. n=8. RT-qPCR analysis for the relative quantitative expression of (D) GP91 and (E) CAT in lung tissue of rats, and the relative quantitative expression was normalized to that of GAPDH. n=6. (F) Changes in the total antioxidant capacity (T-AOC) in the serum of rats with gas explosion-induced injury. n=6. *P<0.05 compared with the control group; $^{\#}$P<0.05 compared with the BLI group. RT-qPCR, reverse-transcription-quantitative; MET, metformin; NOX2, nicotinamide adenine dinucleotide phosphate oxidase; CAT, catalase; GP91, gp91 phox; T-AOC, total antioxidant capacity; BLI, blast lung injury.

explosion. To demonstrate that MET could positively regulate the energy metabolism associated with injured alveolar cells in gas explosion, the changes in enzyme activity were measured. Under normal circumstances, >80% of ATP required by the human body is provided by oxidative phosphorylation (34). However, when oxidative phosphorylation is inhibited, glycolysis can rapidly produce ATP to provide energy to the human body by converting glucose into lactic acid (34). Jin *et al* (35) revealed that ATP production and metabolic reorganization from the TCA cycle to glycolysis are reduced in the lungs of rats exposed to different dosages of seasonal particulate matter less than 2.5 μm in diameter. By investigating the activities of the rate-limiting enzymes in the TCA cycle, specifically α-KGDH and ICDHm, the present study revealed that ATP content was decreased, and the TCA cycle was inhibited after the gas explosion. Similarly, a previous study demonstrated that the rapid consumption of energy by rats with gas explosion-induced injury downregulates galactose metabolism and the TCA cycle, both of which are consistent with the Warburg effect (6). In addition, the present study revealed that gas explosions not only inhibited oxidative phosphorylation but also affected glycolysis. PFK is the source rate-limiting enzyme of glycolysis, and its enzymatic activity was inhibited by gas explosion injuries. Therefore, ATP production is directly inhibited, but MET can improve metabolic abnormalities to some extent.

In the present study, MET promoted the phosphorylation of AMPKα and increased the expression of PGC1α. In addition, *AMPK*, *PGC1α* and *TFAM* mRNA levels were increased in the BLI + MET group. The transcription of mtDNA requires the participation of nucleus-encoded proteins, such as additional factors necessary for TFAM (36). In turn, factors are activated directly or indirectly by the PGC-1 family (37). PGC-1α increases the number of mitochondria to enhance oxidative phosphorylation in lung epithelial cells (38). Similarly, the AMPK/PGC1α pathway is activated to regulate mitochondrial homeostasis and then attenuate gas explosion-induced BLI (17).

The results of the present study are consistent with the results aforementioned. Ultrastructural changes in lung tissue demonstrated the improvement in the structural stability of the mitochondria after MET treatment. In previous studies, considerable damage to the right lower lobe, mitochondrial abnormalities and the loss of a lamellar body structure have been observed after acute primary blast injury by an electron microscope. Moreover, the lesions noted in the lung may be progressive in the first 24 h after injury (39). However, the present study revealed that the most severe lung injury was observed after 3 days using TEM. In summary, MET activated the AMPK/PGC1α mitochondrial protective pathway and maintained the mitochondrial structural stability.

Finally, the present study demonstrated that MET downregulated oxidative stress caused by NOX2, which was activated in gas explosion-induced BLI. Oxidative stress is known to induce mitochondrial functional protein destruction or mtDNA damage (9). A previous study indicated that the activation of NOX2 increases cardiac superoxide production, causing mitochondrial dysfunction and decreased contractility (40). The present results confirmed that NOX2 mediated oxidative stress in lung injury caused by a gas explosion. By contrast, AMPK agonists can improve mitochondrial function, increase energy levels and thereby reduce oxidative stress to improve overall cell viability and tissue health (41). In a study by Schuhmacher *et al* (42), alveolar type II intervention in AMPKα1-knockout mice resulted in a significant upregulation of NOX2 mRNA and protein in the vascular endothelium of mice, while AMPK downregulates NOX2 expression, reduces oxidative stress, decreases mitochondrial inner membrane ROS production and protects mitochondrial structures from being compromised (42). Thus, the inhibition of NOX2 and promotion of antioxidation may be another mechanism by which MET protects mitochondria from gas explosion-induced acute lung injury.

On a temporal level, the damage to the lung function due to gas explosions is continuously aggravated. The results from the present experiment indicated that the situation was the most severe on day 3 after the injury. This is consistent with the pre-experimental results of our previous studies. However, between days 3 and 7 after the gas explosion, there was a weak tendency of abnormal molecular indicators to return to their normal levels, but this self-recovery was particularly limited. The present results provided evidence that if MET was administered as early as possible after a gas explosion-induced injury, it could reduce the persistent aggravation of intrapulmonary injury caused by a gas explosion. In summary, MET mitigated gas explosion-induced blast lung injuries through AMPK-mediated energy metabolism and NOX2-related oxidase pathway in rats.

In the present study, only one dose intervention group was used. Future experiments should use multiple dose groups of metformin to determine the best dose of treatment.

In conclusion, gas explosions can cause the energy metabolism abnormalities associated with acute lung injury and lead to the onset of oxidative stress in the lungs. MET activates AMPK and downregulates NOX2 expression. This maintains the mitochondrial structure, enhances intrapulmonary antioxidant capacity, promotes intrapulmonary ATP production and finally alleviates the extent of BLI induced by a gas explosion.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MZ, WJR and SQY conceived the study and designed experiments. CJD and SH performed animal experiments. MZ, YZS and XWD performed the main experiments. SQY and

WJR confirm the authenticity of all the raw data. NL, YG and LZ were responsible for data acquisition and analysis. WY and JC analyzed and interpreted data. MZ and SQY wrote and revised the manuscript. WY, JC, WJR and SQY were accountable for all aspects of the work in ensuring that questions are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal study protocol was approved by the Medical Ethics Committee of Xinxiang Medical University (approval no. XYLL-2019001; Jan 13, 2019).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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