Novel treatment options for bronchopulmonary dysplasia

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Chapter 3

Metformin attenuates hyperoxia-induced lung injury in neonatal rats by reducing the inflammatory response

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Am J Physiol Lung Cell Mol Physiol. 2015;309(3):L262-70.

Abstract

Because therapeutic options are lacking for bronchopulmonary dysplasia (BPD), there is an urgent medical need to discover novel targets/drugs to treat this neonatal chronic lung disease. Metformin, a drug commonly used to lower blood glucose in type 2 diabetes patients, may be a novel therapeutic option for BPD by reducing pulmonary inflammation and fibrosis, and improving vascularization.

We investigated the therapeutic potential of daily treatment with 25 and 100 mg/kg metformin, injected subcutaneously in neonatal Wistar rats with severe experimental BPD, induced by continuous exposure to 100% oxygen for 10 days. Parameters investigated included survival, lung and heart histopathology, pulmonary fibrin and collagen deposition, vascular leakage, right ventricular hypertrophy and differential mRNA expression in the lungs of key genes involved in BPD pathogenesis, including inflammation, coagulation, and alveolar development.

After daily metformin treatment rat pups with experimental BPD had reduced mortality, alveolar septum thickness, lung inflammation and fibrosis, demonstrated by a reduced influx of macrophages and neutrophils, and hyperoxia-induced collagen III and fibrin deposition (25mg/kg), as well as improved vascularization (100 mg/kg) compared to control treatment. However, metformin did not ameliorate alveolar enlargement, small arteriole wall thickening, vascular alveolar leakage and right ventricular hypertrophy.

In conclusion metformin prolongs survival and attenuates pulmonary injury by reducing pulmonary inflammation, coagulation and fibrosis, but does not affect alveolar development or prevent pulmonary arterial hypertension and right ventricular hypertrophy in neonatal rats with severe hyperoxia-induced experimental BPD.

Introduction

Treatment for neonatal respiratory distress syndrome with mechanical ventilation and supplemental oxygen for a prolonged period injures the immature lung in very premature infants and frequently results in neonatal chronic lung disease (bronchopulmonary dysplasia, BPD). BPD leads to permanently enlarged alveoli, caused by lung damage, an arrest in alveolar and vascular development, and a subsequent reduction of the alveolar surface and lung function (5,19). BPD is seriously complicated in the perinatal period by inflammation and oxidative stress, and at later stages associated with pulmonary arterial hypertension (PAH)-induced right ventricular hypertrophy (RVH) and lung fibrosis (1,5,8,34). Similar to premature infants at risk for developing BPD, neonatal rats are born in the saccular stage of lung development. After exposure to hyperoxia, neonatal rats develop chronic lung inflammation, followed by persistent alveolar simplification, fibrosis, PAH and RVH (8,9).

Because inflammation plays an important role in the pathogenesis of BPD, anti-inflammatory agents may have therapeutic potential by reducing inflammation-induced tissue damage in the lung as demonstrated in multiple studies of experimental BPD (10,46). Metformin, an effective therapeutic option for type 2 diabetes by lowering blood glucose levels (39), has potent anti-inflammatory properties as well, both *in vitro* (3,14,26) and *in vivo* (28,38). In addition metformin has protective effects on vascular function by improving capillary blood flow and limb perfusion (35,43), and disease, including atherosclerosis, vascular remodeling, revascularization after ischemia and apoptosis (16,35). These pleiotropic effects of metformin are probably mediated via activation of AMP-activated protein kinase (AMPK; 20,29). AMPK agonists have anti-inflammatory properties *in vitro*, as shown in multiple cell types exposed to lipopolysaccharides (LPS), including macrophages (17,18,45) and airway epithelial cells (26), and *in vivo*, as demonstrated in obese mice with allergic eosinic inflammation (7) and in mice with inflammatory bowel disease (4).

The role of metformin in chronic lung disease, including BPD is unknown. Because inflammation is an important contributor to the pathogenesis of chronic lung disease, treatment with the potent anti-inflammatory AMPK agonist metformin may result in the identification of a novel therapy for BPD and chronic obstructive pulmonary disease (COPD). To advance our knowledge on AMPK activation in neonatal cardiopulmonary disease *in vivo*, we studied the effects of metformin in neonatal rats with experimental BPD, induced by prolonged exposure to hyperoxia, by investigating inflammation, coagulation, alveolarization, and PAH in the lung (9).

Materials and Methods

Animals

The research protocol was approved by the Institutional Animal Care and Use Committee of the Leiden University Medical Center. For each experiment, newborn wild type Wistar rat pups from 2-3 litters were pooled and equally distributed over two experimental groups: an oxygen group (N=12) and two room air (RA) exposed control groups (N=6 each). For the intervention experiments, newborn rat pups were distributed over two groups, i.e. an

oxygen-NaCl (N=6) and an oxygen-metformin group (N=6), and two room air-exposed control groups (N=6 each) were daily injected subcutaneously either with 100 µl 0.9% NaCl or metformin (25 or 100 mg/kg; D150959, Sigma-Aldrich, St. Louis, MO, USA). All pups were fed by Wistar foster dams. Foster dams were rotated daily between the oxygen-exposed pups and 2 groups of RA exposed pups to avoid oxygen toxicity: 24 hrs in 100% oxygen and 48 hours in RA. Oxygen concentration, body weight, evidence of disease, and mortality were monitored daily. Pups were continuously exposed to 100% oxygen for 10 days. Lung and heart tissue was collected on day 10. Separate experiments were performed for (I) lung (N=8) and heart histology (N=10), (II) lung tissue homogenates for fibrin deposition (N=12) and RT-PCR (N=10), and (III) collection of bronchoalveolar lavage fluid (N=10). For all parameters studied two independent experiments were performed.

Histology

Formalin-fixed, paraffin-embedded, 4 µm-thick heart and lung sections were stained with hematoxylin and eosin. Furthermore, lung tissue sections were Hart's stained to visualize elastin (31) by incubation for 24 hours in a solution containing 20 ml Resorcin-fuchsin solution (X877.2 Carl Roth GmbH, Karlsruhe, Germany), 200 ml of 70% ethanol, and 4 ml hydrochloric acid. After 4 washes in double distilled water, sections were counterstained with tartrazine solution, containing 0.5 g of tartrazine (86310, Sigma-Aldrich, St. Louis, MO, USA), 200 ml double distilled water, and 0.5 ml acetic acid. Lungs were immunostained additionally with anti-ED-1 (monocytes and macrophages; 1:2), anti-myeloperoxidase (MPO, RB-373-A1, Thermo Fisher Scientific, Fremont, CA, USA; diluted 1:1,500), anti-α smooth muscle actin (ASMA, A2547, Sigma-Aldrich, St. Louis, MO, USA; diluted 1:20,000), anti-collagen III (COL3A1, ab7778, Abcam, Cambridge, United Kingdom; diluted 1:3,200) or anti-von Willebrand factor (vWF, A0082, Dako Cytomation, Glostrup, Denmark; diluted 1:4,000), stained with EnVision-HRP (Dako, Glostrup, Denmark), using the chromogenic substrate NovaRed as recommended by the manufacturer (Vector, Burlingame, CA, USA), and counterstained briefly with hematoxylin using standard methods (9,41). For morphometry of the lung, an eye piece reticle with a coherent system of 21 lines and 42 points (Weibel type II ocular micrometer; Olympus, Zoeterwoude, The Netherlands) was used (41). We used different (immuno)histochemically stained lung sections for each quantification, except for alveolar crest and pulmonary arteriolar wall thickness, which were determined on the same ASMA stained section. To investigate alveolar enlargement in experimental BPD, we studied the number of alveolar crests to exclude potential effects of heterogenous alveolar development. The number of alveolar crests (46), determined on lung sections stained immunohistochemically for ASMA, were assessed in 10 non-overlapping fields at a 400x magnification for each animal and were normalized to field. The density of ED-1 positive monocytes and macrophages or MPO-positive neutrophilic granulocytes was determined in the alveolar compartment by counting the number of cells per field. Results were expressed as cells per mm^2. Per experimental animal 20 fields in one section were studied at 400x magnification. Pulmonary alveolar septal thickness was assessed in HE-stained lung sections at a 400x magnification by averaging 100 measurements per 10 representative fields. Capillary density was assessed in lung sections stained for vWF at a 200x magnification by counting the number of vessels per field. At least 10 representative fields

per experimental animal were investigated. Results were expressed as relative number of vessels per mm^2. Pulmonary arteriolar wall thickness was assessed in lung sections stained for elastin at a 1000x magnification by averaging at least 10 vessels with a diameter of less than 30 µm per animal. Medial wall thickness was calculated from the formula "percent wall thickness = $\frac{2 * wall \cdot thickness}{external \cdot diameter} * 100$" (22). Fields containing large blood vessels or bronchioli were excluded from the analysis. Thickness of the right and left ventricular free walls was assessed in a transversal HE-stained section taken halfway the long axis at a 40x magnification by averaging 6 measurements per structure. RVH was calculated for each heart by dividing average RV free wall thickness and average LV free wall thickness. For morphometric studies in lung and heart, 8 and 10 rat pups per experimental group were studied, respectively. Quantitative morphometry was performed by two independent researchers blinded to the treatment strategy using the NIH Image J program (9,10,46).

Fibrin detection assay

Quantitative fibrin deposition was determined in lung tissue homogenates by Western blotting (41,42; N=12). Lung tissue homogenates for quantitative fibrin deposition by Western blotting were pretreated as described previously (41). Tissue samples, dissolved in reducing sample buffer (10 mM Tris pH 7.5, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, and 0.4 mg/mL of bromophenol blue) were subjected to SDS-PAGE (7.5% gel; 5% stacking gel) and blotted onto PVDF membrane (Immobilon-FL, Millipore, Bredford, MA, USA). The 56-kDa fibrin β-chains were detected with monoclonal 59D8 (Oklahoma Medical Research Foundation, Oklahoma City, OK USA; diluted 1:1000), infrared labeled goat-anti-mouse secondary antibody (IRDye 800CW; Licor Biosciences, Lincoln, NE, USA, diluted 1:5000), and quantified using an infrared detection system (Odyssey infrared imaging system, Licor Biosciences). Fibrin deposition was quantified using rat fibrin as a reference.

Bronchoalveolar lavages and protein assay

Total protein content in lung lavages was determined as an indicator of vascular leakage using a standard protein assay (Dc protein assay, Bio-Rad, Veenendaal, the Netherlands), according to the manufacturer's instructions, using bovine serum albumin (fraction V; Roche Diagnostics, Almere, The Netherlands) as previously described (9; N=10).

Real-time RT-PCR

Total RNA isolation from lung tissue homogenates (RNA-Bee, Tel-Test Inc, Bio-Connect BV, Huissen, the Netherlands), first-strand cDNA synthesis (SuperScript Choice System, Life Technologies, Breda, the Netherlands), and *real-time* quantitative PCR, using β-actin as a housekeeping gene reference, were performed on a Light Cycler 480 (Roche, Almere, The Netherlands) of the Leiden Genome Technology Center (Leiden, The Netherlands) as described previously (9; N=10). Primers are listed in Table 1.

Gene Product	Forward Primer	Reverse Primer
AMPK	5'-TGAAGCAGCTGGACTTTGAA-3'	5'-TTTTCACGTAATTGCCAGTCA-3'
CINC1	5'-GCACCCAAACCGAAGTCATA-3'	5'-GGGGACACCCTTTAGCATCT-3'
FGFR4	5'-GTTGGCACGCAGCTCCTT-3'	5'-GCAGGACCTTGTCCAGAGCTT-3'
MCP1	5'-ATGCAGTTAATGCCCCAGTCA-3'	5'-TTCTCCAGCCGACTCATTGG-3'
PAI-1	5'-AGCTGGGCATGACTGACATCT-3'	5'-GCTGCTCTTGGTCGGAAAGA-3'
TF	5'-CCCAGAAAGCATCACCAAGTG-3'	5'-TGCTCCACAATGATGAGTGTT-3'
β-actin	5'-TTCAACACCCCAGCCATGT-3'	5'-AGTGGTACGACCAGAGGCATACA-3'

Table 1. Sequences of oligonucleotides for forward and reverse primers for real-time RT-PCR

Statistical analysis

Values are expressed as mean ± SEM. Differences between groups were analyzed by one-way ANOVA for independent samples, followed by Tukey's multiple comparison test, using the GraphPad Prism version 6 software package (San Diego, CA, USA). Differences at *p* values < 0.05 were considered statistically significant.

Results

Dose finding for metformin in experimental BPD

To determine the optimal dosing of metformin, we performed a pilot experiment in which hyperoxia-exposed rat pups were treated daily with 10-250 mg/kg metformin or NaCl. Because metformin has anti-inflammatory and –coagulant properties (3,29), we used fibrin deposition in the lung as a read-out. We found that metformin reduced fibrin deposition in a broad concentration range of 10-250 mg/kg/day with 25 mg/kg/day of metformin as the most optimal dose to reduce fibrin deposition in the lung in hyperoxia-induced BPD (Figure 1, N=6). Because 100 mg/kg/day of metformin, administered intraperitoneally, is effective in attenuating experimental pulmonary hypertension in adult rats (2) we used 25 and 100 mg/kg/day of metformin in our experiments.

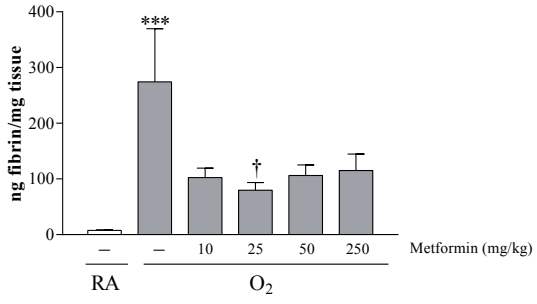

Figure 1

Pilot experiment to find the optimal dose of metformin for treatment of experimental BPD by determining fibrin deposition in lung tissue homogenates (N=6) in room air (RA) pups injected daily with NaCl (open bar) and O2-exposed pups (shaded bars) injected daily with NaCl or metformin: 10, 25, 50 and 250 mg/kg/day). Data are expressed as mean ± SEM. ***p < 0.001 versus RA controls. †p < 0.05 versus age-matched NaCl-treated O2-exposed controls.

Effects of metformin on growth and survival

Body weight on day 10 was comparable in NaCl treated rat pups kept in room air (19.5 g; Figure 2A) and in 100% oxygen (13.5 g). Exposure to hyperoxia resulted in a 40% survival on day 10 in NaCl-treated rat pups (Figure 2B). Treatment of experimental BPD with 25 mg/kg/day of metformin increased survival to 61% ($p < 0.05$; compared to hyperoxia-exposed controls), whereas 100 mg/kg/day had no beneficial effects on hyperoxia-induced mortality. All room air (RA)-exposed pups showed no morbidity or mortality during the experimental period of 10 days.

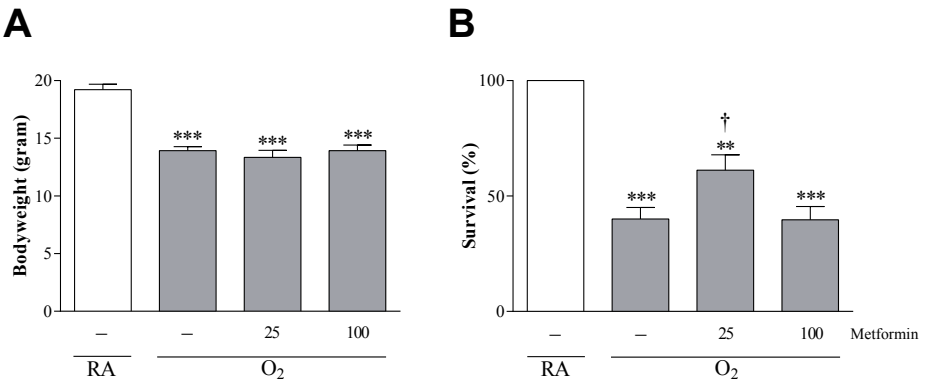

Figure 2

Growth (A) and survival (B) on day 10 (N=5 experiments) in room air (RA) and age-matched O2-exposed rat pups (O2). RA pups were injected daily with NaCl (open bar) and O2 pups (shaded bars) were injected daily with NaCl or metformin (25 or 100 mg/kg/day) until 10 days of age. Data are expressed as mean ± SEM. **p < 0.01 and ***p < 0.001 versus RA controls. †p < 0.05 versus age-matched NaCl-treated O2-exposed controls.

Effects of metformin on lung airway development and inflammation

Oxygen exposure for 10 days resulted in lung edema, a heterogeneous distribution of enlarged air-spaces with a decreased number of alveolar crests (2.4-fold, $p < 0.001$; Figures 3B and 4A), surrounded by septa with increased thickness (1.7-fold, $p < 0.001$; Figures 3B and 4D), reduced pulmonary vessel density (2.4-fold, $p < 0.001$; Figures 3B and 4B), and increased pulmonary arterial wall thickness (2.5-fold, $p < 0.001$; Figures 3F and 4C). Exposure to hyperoxia also induced an inflammatory response, characterized by an influx of macrophages (10.3-fold, $p < 0.001$ Figures 3J and 4E) and neutrophils (13.1-fold, $p < 0.01$, Figures 3N and 4F). Administration of metformin to RA-exposed controls did not have an effect on the parameters investigated (not shown). Compared to oxygen exposed controls, administration of 25 mg/kg/day of metformin during oxygen exposure reduced alveolar septal thickness (1.3-fold, $p < 0.05$; Figures 3C and 4D) and the influx of macrophages (1.8-fold, $p < 0.01$; Figures 3K and 4E) and of neutrophils (2.1-fold, $p < 0.05$; Figures 3O and 4F). Administration of 100 mg/kg/day of metformin during oxygen exposure increased the number of blood vessels compared to hyperoxia-exposed controls (1.4-fold, $p < 0.001$, Figure 4B). However, metformin did not affect hyperoxia-induced inhibition of alveolarization and increase of arterial medial wall thickness.

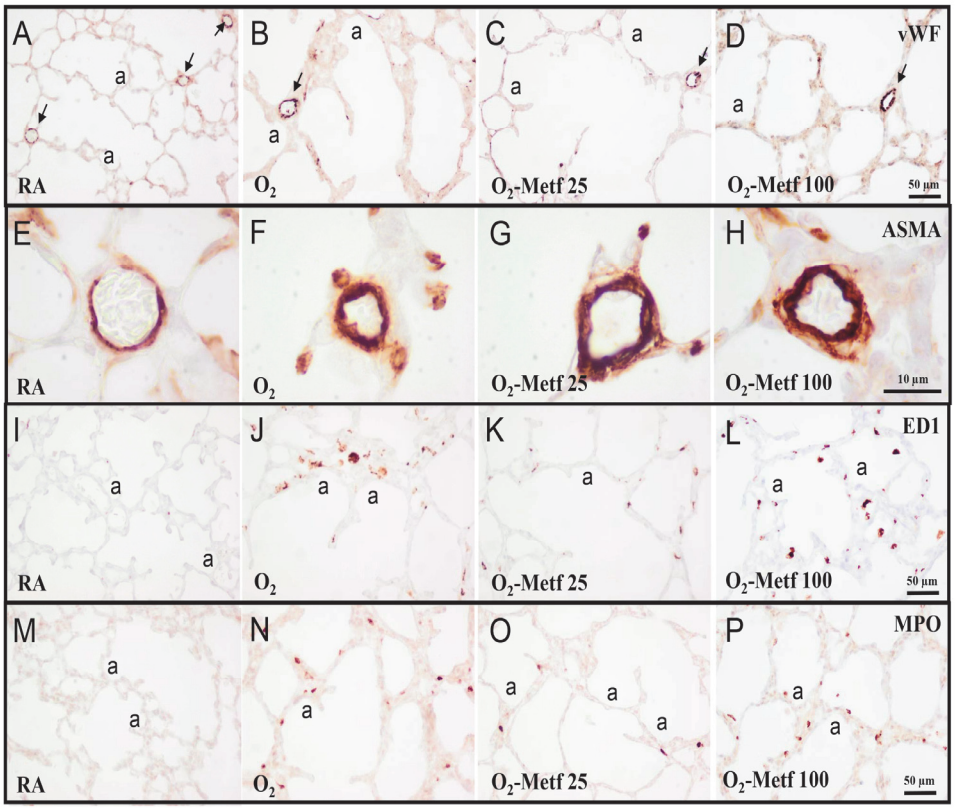

Figure 3

Representative lung sections stained for von Willebrand Factor (vWF; A-D), α smooth muscle actin (ASMA; E-H), the monocyte and macrophage marker ED1 (I-L) or myeloperoxidase (MPO) as a marker for neutrophilic granulocytes (M-P) of rat pups kept in room-air (RA; A, E, I and M) or 100% O2 (B-D, F-H, J-L and N-P). Pups were injected daily with 0.9% NaCl (A, B, E, F, I, J, M and N) or metformin (25 mg/kg/day: C, G, K and O) or 100 mg/kg/day: D, H, L and P) until 10 days of age. a = alveolus. Arrows in panels A-D indicate vWF-positive blood vessels.

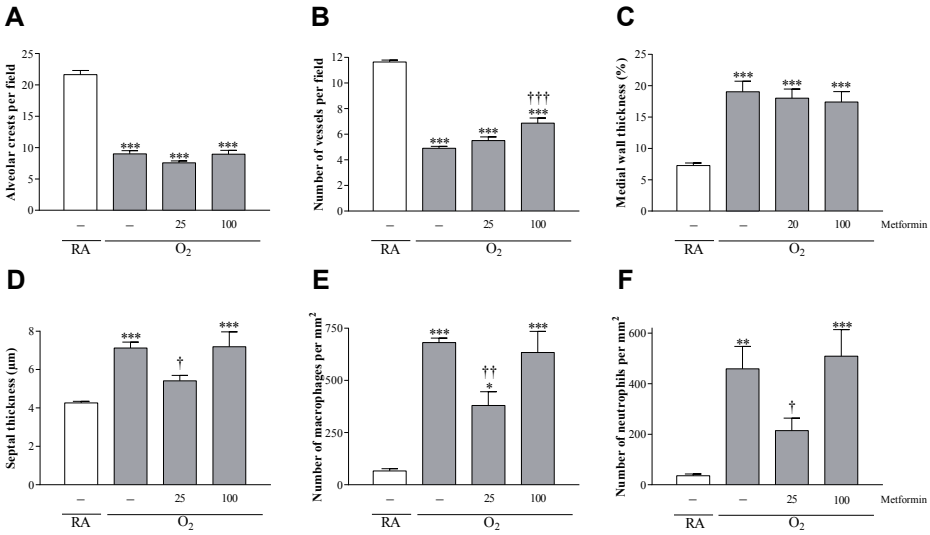

Figure 4

Lung morphometry, including the quantifications of alveolar crests (A), number of pulmonary vessels (B), arterial medial wall thickness (C), septal thickness (D) and influx of macrophages (E) and neutrophilic granulocytes (F) was determined on paraffin sections in rat pups on day 10. RA pups were injected daily with NaCl (open bar) and O2 pups (shaded bars) were injected daily with NaCl or metformin (25 or 100 mg/kg/day) until 10 days of age. Values are expressed as mean ± SEM (N=8). *p < 0.05, **p < 0.01 and ***p < 0.001 versus RA controls. †p < 0.05, ††p < 0.01 and †††p < 0.001 versus age-matched NaCl-treated O2-exposed controls.

Effects of metformin on pulmonary deposition of collagen, elastin and fibrin, and vascular leakage

In rat pups kept in normoxia, collagen III was only present at high levels in the perivasculature of large and small blood vessels (Figure 5A). Expression was low or absent in alveolar septa. In lungs of pups exposed to hyperoxia for 10 days, collagen III deposition increased 9.4-fold ($p < 0.001$; Figure 5I), and was present in the perivasculature of blood vessels and in thick alveolar septa (Figure 5B). Treatment with 25 mg/kg/day of metformin for 10 days reduced collagen III expression by 29% ($p < 0.05$; Figure 5I) in alveolar septa (Figures 5C). In rat pups kept in RA elastin was predominantly present on the septal tips and in the wall of blood vessels (Figure 5E). Elastin expression decreased 1.5-fold under hyperoxia ($p < 0.001$; Figure 5J) and was predominantly present in the alveolar walls rather than on septal tips and in the walls of small blood vessels (Figure 5F). Treatment with 25 mg/kg/day of metformin decreased elastin expression further: 2.2-fold ($p < 0.001$ compared to RA controls) and 1.5-fold ($p < 0.01$ compared to oxygen-exposed controls) in blood vessels and alveolar walls (Figures 5G and J).

Pulmonary fibrin deposition was studied in homogenates as a read-out for lung damage (Figure 5K). Fibrin deposition was at reference levels during normal neonatal pulmonary development on day 10 (< 10 ng fibrin/mg tissue), and increased 44-fold ($p < 0.01$) in lungs of pups exposed to 100% oxygen for 10 days. Administration of 25 mg/kg/day of metformin reduced hyperoxia-induced fibrin deposition by 73% ($p < 0.05$), whereas

administration of 100 mg/kg/day of metformin showed a tendency towards lower levels compared to hyperoxia-exposed controls. Total protein concentration in bronchoalveolar lavage fluid (BALF) was determined to establish the effect of pulmonary edema by capillary-alveolar leakage (Figure 5L). Protein concentration on postnatal day 10 showed a tendency to increase after hyperoxia, but was not affected by metformin. Administration of 100 mg/kg/day of metformin did not have beneficial effects on hyperoxia-induced pulmonary deposition of collagen, elastin and fibrin, and vascular leakage compared to hyperoxia-exposed controls (Figure 5, panels D and H-L).

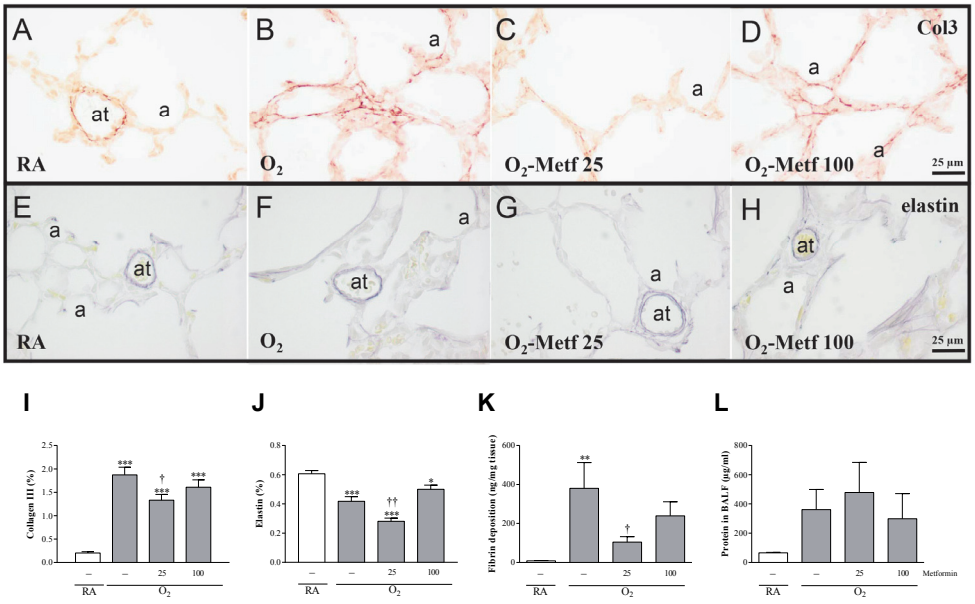

Figure 5

Representative lung sections stained for collagen III (A-D) or elastin (E-H) of rat pups kept in room-air (RA; A and E) or 100% O2 (B-D and F-H) injected daily with NaCl (A, B, E and F), 25 mg/kg/day of metformin (C and G) or 100 mg/kg/day of metformin (D and H) until 10 days of age. Quantification of collagen III deposition (I) and elastin (J) on lung tissue paraffin sections (N=8), extravascular fibrin deposition in lung homogenates (N=12, K) and total protein concentration in bronchoalveolar lavage fluid (BALF; N=10, L) as a marker for vascular leakage on day 10. RA pups were injected daily with NaCl (open bar) and O2 pups (shaded bars) were injected daily with NaCl or metformin: 25 or 100 mg/kg/day until 10 days of age. Values are expressed as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 versus RA controls. †p < 0.05, ††p < 0.01 versus age-matched O2-exposed controls.

Effects of metformin treatment on mRNA expression in lung tissue

Administration of metformin for 10 days during normal neonatal development in room air increased AMP-activated kinase (AMPK) mRNA expression 1.6-fold ($p < 0.05$; not shown), but did not affect expression of the pro-inflammatory factors monocyte chemoattractant protein (MCP)-1 (Figure 6A) and the chemokine-induced neutrophilic chemoattractant-1 (CINC1; Figure 6B), the pro-coagulant factor tissue factor (TF; Figure 6C), anti-fibrinolytic protein plasminogen activator inhibitor 1 (PAI-1; Figure 6D), and fibroblast growth factor receptor type 4 (FGFR4; Figure 6E). Ten days of oxygen exposure resulted in an increase in mRNA expression of MCP-1 (17-fold, p < 0.001), CINC-1 (10-fold, p < 0.001), TF (4.2-fold, p

< 0.001 in) and PAI-1 (62-fold p < 0.001), FGFR4 mRNA expression was reduced in lungs of oxygen-exposed pups (10-fold p < 0.001), whereas AMPK expression was not different from room air controls (Figure 6F). Treatment of oxygen-exposed pups with metformin for 10 days did not affect mRNA expression compared to NaCl treated oxygen-exposed pups.

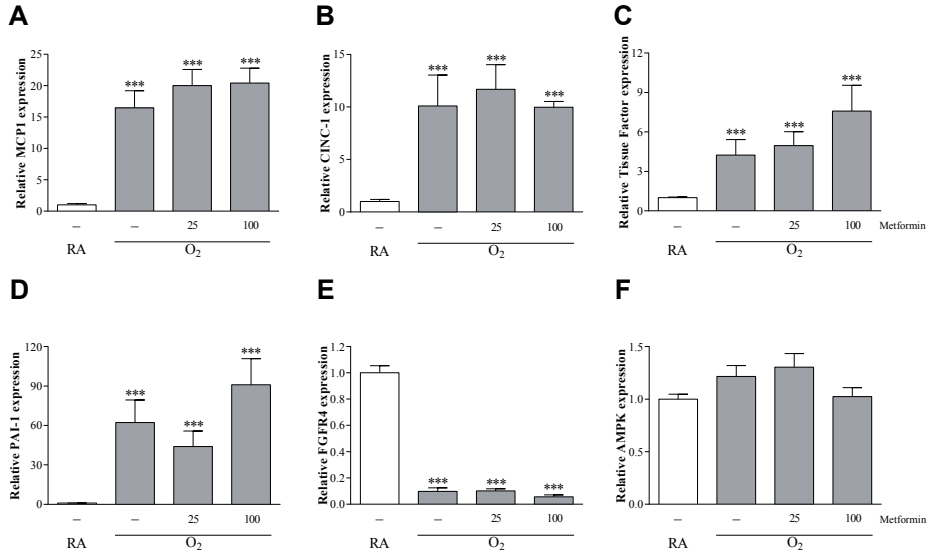

Figure 6

Relative mRNA expression in lung homogenates (N=10) of monocyte chemoattractant protein 1 (MCP1; A), chemokine-induced neutrophilic chemoattractant-1 (CINC1; B), tissue factor (TF; C), plasminogen activator inhibitor 1 (PAI-1; D), fibroblast growth factor receptor type 4 (FGFR4; E), and AMP-activated kinase (AMPK; F) on day 10 in rat pups. RA pups were injected daily with NaCl (open bar) and O2 pups (shaded bars) were injected daily with NaCl or metformin : 25 or 100 mg/kg/day until 10 days of age. Values are expressed as mean ± SEM. ***p < 0.001 versus RA controls.

Effects of metformin on right ventricular hypertrophy

Right ventricular hypertrophy (RVH) was investigated using haematoxylin and eosin stained heart sections. Exposure to hyperoxia for 10 days resulted in an 1.4-fold increase in the RV/LV free wall thickness ratio in Wistar control pups compared to room air controls (Figure 7; $p < 0.01$). Metformin had no beneficial effects on RVH compared to hyperoxia-exposed controls.

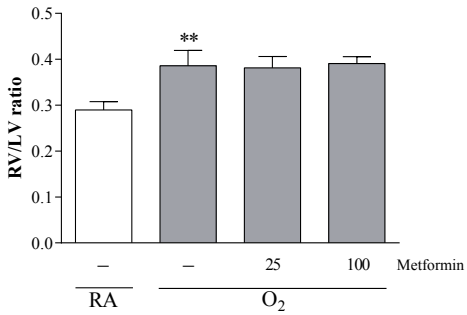

Figure 7

Right ventricular hypertrophy (RVH) was determined on day 10 in rat pups by morphometry in paraffin sections stained with haematoxylin and eosin and depicted as RV/LV free wall thickness ratio in room air (RA) and age-matched O2-exposed pups (O2). RA pups were injected daily with NaCl (open bar) and O2 pups (shaded bars) were injected daily with NaCl or metformin: 25 or 100 mg/kg/day until 10 days of age. Values are expressed as mean ± SEM (N=10). **p < 0.01 versus RA controls.

Discussion

Treatment of rat pups with hyperoxia-induced lung injury, an *in vivo* model for experimental bronchopulmonary dysplasia or BPD (41), with metformin prolongs survival, reduces lung injury by attenuating lung inflammation, coagulation, septal thickness and collagen III expression, and stimulates vascularization. Metformin had no beneficial effects on alveolarization, capillary alveolar leakage, arterial medial wall thickness (pulmonary arterial hypertension; PAH) and right ventricular hypertrophy (RVH), and no adverse effects on normal lung and heart development. These data demonstrate that metformin may be a suitable candidate to reduce lung inflammation, coagulation and fibrosis in preterm infants with severe BPD.

Metformin is a potent anti-diabetic drug that is commonly used in type 2 diabetic patients to lower glucose levels in the circulation. In comparison to other treatment modalities for type 2 diabetes patients treated with metformin were protected against mortality in cardiac disease and had less cancer, suggesting that metformin has various biological functions other than its blood glucose lowering effect in diabetic patients (12,13,33). Many of the pleiotropic effects of metformin are related to reduced inflammation, cancer and cardiovascular disease, and improved vascular function. Evidence is accumulating that these beneficial effects of metformin on inflammation and the cardiovasculature are mediated via activation of AMPK-dependent signaling (29,35) and subsequent inhibition of mammalian target of rapamycin (mTOR) and NF-κB signaling (27,30,32). Inflammation is an important player in the pathogenesis of BPD, because it may contribute to severe tissue damage and fibrosis, and because treatment with anti-inflammatory agents provides protection against hyperoxia-induced neonatal lung disease or experimental BPD (10,46). Metformin protected against hyperoxia-induced neonatal lung injury in rat pups by reducing mortality, fibrosis, coagulation and inflammation, as demonstrated by a reduced pulmonary influx of inflammatory cells, including macrophages and neutrophils. This anti-inflammatory effect of metformin in neonatal rats with experimental BPD is supported by observations *in vivo* in adult mice, in which AMPK agonist treatment, including metformin, suppressed ovalbumin-induced allergic eosinophilic lung inflammation (7,29) and inflammatory bowel disease (4). In addition, anti-inflammatory properties of metformin and other AMPK agonists were observed *in vitro*, in pro-inflammatory monocytes and macrophages, vascular smooth muscle cells, umbilical vein endothelial cells and primary bronchial epithelial cells (3,14,17,20,26,45). BPD is a multifactorial disease in which lung immaturity, aberrant alveolar development, inflammation, coagulation, vascular leakage and pulmonary hypertension contribute to injury, disease severity and mortality (1,5,19). Mortality associated with experimental BPD may also be related to outcomes metformin does not influence, including pulmonary arterial hypertension (PAH) and right ventricular hypertrophy (RVH). Indeed we demonstrated in a previous study that treatment of experimental BPD with the endothelin receptor antagonist ambrisentan had no beneficial effects on alveolarization, vascularization, inflammation and coagulation, but attenuated PAH, RVH and fibrosis, and also improved survival (42).

The anti-coagulant effect of metformin, demonstrated by reduced pulmonary fibrin deposition in neonatal rats with experimental BPD, may, at least in part, be explained by the

anti-inflammatory properties of metformin. Inflammation and coagulation are two closely related processes. Activated inflammatory cells, including macrophages, may activate coagulation directly by upregulating endogenous tissue factor (TF) expression or triggering the release of TF-bearing microparticles (6), resulting in alveolar thrombin generation and fibrin deposition (15, 25). In addition, pro-inflammatory cytokines, including tumor necrosis factor (TNF) alpha and interleukin (IL)-1ß, may have procoagulant effects. In addition, metformin decreases the activity of the potent fibrinolytic inhibitor PAI-1 thereby promoting fibrin degradation and reducing fibrin deposition (24). Moreover, neutrophils may worsen pulmonary fibrin deposition by increased degradation of the important anti-coagulant factor activated protein C by neutrophil elastase (11). These anti-inflammatory and –coagulant effects were not confirmed by the expression of the mRNAs of the inflammatory markers monocyte chemoattractant protein-1 (MCP1) and chemokine-induced neutrophilic chemoattractant 1 (CINC1), the pro-coagulant factor TF and the regulator of fibrinolysis plasminogen activator inhibitor 1 (PAI-1), suggesting the involvement of other chemokines and cytokines, other mechanisms involved in inflammation, including cell adhesion, secretion and migration or regulation of gene expression at a post-transcriptional level. Although neonatal exposure to hyperoxia does not affect AMPK transcription in the lung, protein activity may be reduced by pro-inflammatory mediators, including TNF alpha and IL6, and activated by the anti-inflammatory mediator IL10 (30). Stimulation of NF-κB signalling by oxidative stress plays an important role in inflammatory responses, including hyperoxia-induced neonatal lung injury. The anti-inflammatory and –coagulant effect of metformin may be explained by activation of AMPK and its downstream targets, including sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1alpha), p53 and forkhead box O (FoxO) factors, thereby inhibiting NF-κB signaling (30). Metformin reduced hyperoxia-induced fibrosis in rats with severe experimental BPD by attenuating extravascular collagen III deposition. This anti-fibrotic effect of metformin-induced AMPK activation is supported by the beneficial effects of metformin in mice with bleomycin-induced lung fibrosis (29) and an aggravated pulmonary fibrotic response towards bleomycin in heterozygous AMPKα1-deficient mice (29).

Metformin had a small, but significant beneficial effect on hyperoxia-induced reduced vascularization, which may be explained by a pro-angiogenic effect observed previously *in vitro* in pulmonary arterial endothelial cells from sheep with pulmonary hypertension after AMPK activation (36) and *in vivo* in mice in which metformin stimulated angiogenesis and recovery after experimental stroke (40) and revascularization after hindlimb ischemia via an eNOS dependent pathway (35). This observation is supported by the beneficial effects of stimulation of the NO-eNOS-cGMP pathway with inhaled NO, apelin and sildenafil on experimental lung and heart disease in animal models of experimental BPD by us and others (8,9,21,37). Because metformin had a beneficial effect on reduced vascularization and pro-angiogenic factors have beneficial effects on aberrant alveolar development in (experimental) BPD (5,9,19,21) we expected to find reduced alveolar enlargement as well after metformin treatment. We speculate that the pro-angiogenic effect of metformin is probably too small to trigger alveolarization of the simplified lung.

The absence of a beneficial effect of metformin on PAH-induced RVH in neonatal rats with hyperoxia-induced lung disease is in contrast to adult rats in which metformin protected

against monocrotalin or hypoxia-induced PAH (2) and *in vitro* studies, in which metformin inhibited endothelin-1- or hypoxia-induced proliferation of pulmonary arterial vascular smooth muscle cells (23,44). This discrepancy in response of metformin towards PAH in neonates and adults may be explained by differences in age, development of disease in different animal models or concentration of metformin used in the experimental rat models of PAH.

Daily treatment of neonatal rats with 25 mg/kg of metformin was the most optimal dose to attenuate hyperoxia-induced neonatal lung injury and is a similar dose used for prolonged oral daily treatment of diabetic type 2 patients (2-3 g/day in adolescents and adults). In adult rats and mice, in which metformin was administered orally via gavage or gastric tube, or intraperitoneally, the dose was higher and ranged from 100-350 mg/kg/day to treat ovalbumin- and fungal-associated allergenic protease-induced asthma (29), bleomycin-induced fibrosis (29), allergic eosinophilic inflammation in obese mice (7), hypoxia- or monocrotalin-induced pulmonary hypertension (2), reduced vascularization in hindlimb ischemia (35) and pancreatic cancer (27). The efficacy of the relatively low dose of metformin, administered subcutaneously, in neonatal rats with BPD compared to adult rats and mice may be explained by age, route of drug administration, species or development of disease in an immature lung. A relatively low effective dose of metformin in neonatal rats will help to prevent or reduce potential adverse effects including abdominal discomfort and diarrhea and potential bronchial edema development due to inhibition of the epithelial Na^+ channel ENaC (26).

Because therapeutic options are lacking for BPD, there is an urgent medical need to discover novel targets/drugs to treat this neonatal chronic lung disease. If the absence of adverse effects of treatment with metformin in rats can be confirmed in newborn infants, extrapolation of the beneficial effects of metformin and other AMPK activators in rat pups with experimental BPD to premature infants with respiratory failure, may provide in a new treatment option to attenuate lung inflammation and fibrosis which are major reasons for mortality or morbidity in preterm infants with severe BPD.

Acknowledgements

The authors gratefully acknowledge Dr. E. de Heer (Department of Pathology, LUMC, Leiden, the Netherlands) for providing the ED-1 antibody and Dr. J.J. Baelde (Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands) for providing the COL3A1 antibody.

List of abbreviations

AMPK	AMP-activated protein kinase
ASMA	α smooth muscle actin
BALF	bronchoalveolar lavage fluid
BPD	bronchopulmonary dysplasia
CINC1	chemokine-induced neutrophilic chemoattractant-1
CLD	chronic lung disease
FGFR4	fibroblast growth factor receptor-4
IVS	interventricular septum
LV	left ventricle
MCP1	monocyte chemoattractant protein 1
MPO	myeloperoxidase
O_2	oxygen
PAH	pulmonary arterial hypertension
PAI1	plasminogen activator inhibitor-1
RA	room air
RT-PCR	reverse transcriptase polymerase chain reaction
RV	right ventricle
RVH	right ventricular hypertrophy
TF	tissue factor
vWF	von Willebrand factor

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