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Original Article

Metformin reduces intrahepatic fibrosis and intrapulmonary shunts in biliary cirrhotic rats

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Abstract

Background: Liver fibrosis causes portal hypertension which dilates collateral vasculature and enhances extra-hepatic angiogenesis including intrapulmonary shunts, which subsequently complicates with hepatopulmonary syndrome. Metformin is an anti-diabetic agent which possesses anti-inflammation and anti-angiogenesis properties. This study evaluated the effect of metformin treatment on liver and lung in a non-diabetic rat model with biliary cirrhosis induced via common bile duct ligation (CBDL).

Methods: CBDL rats were fed with metformin 150 mg/kg/day during the 8th−28th day post operation. The hemodynamic and biochemistry parameters were tested, and blood gas analysis was performed. The liver and lung were dissected for protein analysis and immuno-histochemical stains. Intrapulmonary shunting degree was determined using color microsphere method.

Results: Metformin treatment neither induced obvious hypoglycemic event nor altered hemodynamics in cirrhotic rats. The plasma levels of alanine aminotransferase were significantly reduced by metformin (control vs. metformin: 269 ± 56 vs. 199 ± 21 IU/L, P = 0.02). Sirius Red stains and CD-68 stains showed that metformin reduced intrahepatic fibrosis and CD-68-positive macrophages. Metformin did not influence hypoxia and intrapulmonary angiogenesis; however, it significantly reduced intrapulmonary shunts (31.7 ± 10.1 vs. 15.0 ± 6.6%, $P = 0.006$.). Furthermore, metformin reduced the protein expressions of COX-2 and PI3K in liver and COX-1 in lung.

Conclusion: Metformin reduced liver injury and improved hepatic fibrosis in cirrhotic rats. It also attenuated the intrapulmonary shunts. However, the effects of metformin on pulmonary angiogenesis and hypoxia were insignificant.

Keywords: Angiogenesis; Hepatopulmonary syndrome; Liver cirrhosis; Metformin

1. Introduction

Metformin is generally accepted as the first-line treatment in type 2 diabetes mellitus patients.[1] It improves peripheral and hepatic sensitivity to insulin, reduces liver gluconeogenesis and enhances the utilization of glucose by peripheral tissues. Apart from hypoglycemic effects, a large body of evidence reveal that metformin has anti-inflammation and anti-angiogenesis properties. The administration of metformin

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prevented and reversed hepatic inflammation in a non-diabetic mouse model of non-alcoholic steatohepatitis.[2] Also, metformin has been noticed in anti-cancer treatment. A nationwide case-control study indicated that metformin reduced the risk of hepatocellular carcinoma in diabetic patients in a dose-dependent manner.[3] Another case-control study showed that diabetes was an independent risk factor for intrahepatic cholangiocarcinoma, and that metformin treatment was associated with a 60% reduction of cancer risk in diabetic patients.[4] The effect of metformin on the prevention and treatment of hepatic cancer comes from, at least in part, inhibition of abnormal angiogenesis.

Hepatopulmonary syndrome (HPS) is a severe complication of liver cirrhosis which is characterized by deoxygenation in cirrhotic patients. Three important components of HPS are: (1) hypoxia with increased alveolar-arterial oxygen gradient ($AaPO_2$); (2) increased intrapulmonary shunts; and (3) chronic liver disease.[5] Recent animal studies show that anti-inflammation and anti-angiogenesis therapies alleviate HPS.[6,7] Using common bile duct ligation (CBDL)-induced biliary cirrhotic rats, researchers have built up a reliable animal model mimicking the clinical presentation of HPS.[8]

The initiation of HPS comes from abnormal vasodilatation, inflammation and angiogenesis in the lung. A recent animal study demonstrated that enhanced chemokine fractalkine expression and signaling contributed to pulmonary monocyte accumulation, angiogenesis and development of HPS.[6] Additionally, Thenappan et al. found that the pulmonary accumulation of CD68-positive macrophages played an important role in development of HPS and speculated depletion of macrophages might be targeted to control HPS.[7] These activated monocytes or macrophages are important modulators to initiate intrapulmonary inflammation and abnormal angiogenesis. The activation of vascular endothelial growth factor (VEGF)-dependent signaling pathways would subsequently increase intrapulmonary shunts then aggravate HPS. We have shown that sorafenib, a tyrosin kinase inhibitor, improves HPS via the attenuation of pulmonary angiogenesis through VEGF/VEGF-R2/Akt pathway inhibition.[9] In addition, we also documented that rosuvastatin alleviated experimental HPS through pulmonary inflammatory angiogenesis blockade, which was related to the down-regulation of tumor necrosis factor-α/nuclear factor kappa B and VEGF/Rho-associated A kinase pathways.[10] Taking the evidence into consideration, the relevant impacts of metformin on intrapulmonary inflammation, angiogenesis, shunting and liver fibrosis is worthy of investigation. In the present study, we evaluated the effect of a 3-week metformin treatment on rats with CBDL-induced experimental HPS.

2. Methods

2.1. Animal model

Male Sprague–Dawley rats weighing 240–270 g at the time of surgery were used for experiments. The rats were housed in plastic cages and allowed free access to food and water. All rats were fasted for 12 h before the operation. Rats with secondary biliary cirrhosis were induced by CBDL. The operation was performed under ketamine anesthesia (100 mg/kg, intramuscularly). A high yield of secondary biliary cirrhosis was noted four weeks after the ligation.[11] To avoid the coagulation defects, CBDL rats received weekly vitamin K injection (50 μg/kg intramuscularly). In all experiments, the principle of laboratory animal care (NIH publication no. 86-23, revised 1985) was followed. This study was approved by the Taipei Veterans General Hospital Animal Committee (approval number: IACUC 2013-161).

2.2. Study protocols

CBDL-induced cirrhotic rats were orally gargled with 150 mg/kg/day metformin or vehicle (0.9% sodium chloride, 1 ml/day, control group) from the 8th to 28th day after CBDL. On the 28th day, the body weight, mortality rate and hemodynamic data were measured (n = 6:6). The blood was collected and protein expressions were examined. Also, liver and lung were examined with hematoxylin and eosin (H&E), Sirius Red, and immunohistochemical stains. Another two parallel groups (n = 7:6) were tested for intrapulmonary shunts using color microsphere method.

2.3. Systemic and portal hemodynamic measurements

The right internal carotid artery of rats was cannulated with a PE-50 catheter that was connected to a Spectramed DTX transducer (Spectramed Inc., Oxnard, CA, USA). Continuous recordings of mean arterial pressure and heart rate were performed on a multi-channel recorder (model RS 3400, Gould, Inc., Cupertino, CA, USA). The abdomen was opened with a mid-line incision, and a mesenteric vein was cannulated with a PE-50 catheter to measure portal pressure.

2.4. Biochemistry and blood gas analysis

The femoral artery and vein of CBDL rats were cannulated with PE-50 catheters one day before experiments. Both catheters were fixed over the back and flushed with a solution contained heparin. The blood was withdrawn from the femoral vein for determining plasma concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, creatinine and glucose levels on the day of experiment. Then, arterial blood was withdrawn from the femoral artery for arterial blood gas analysis. Arterial gas exchange including partial pressure of oxygen (PaO_2) and carbon dioxide ($PaCO_2$) was evaluated, and the $AaPO_2$ was calculated as $150-(PaCO_2/0.8)-PaO_2$.

2.5. Intrapulmonary shunting analysis

Intrapulmonary shunts were determined using the color-microsphere technique.[9,10] Cross-linked (2.5×10^6) colored microspheres (size range 6.5–10 μm; Interactive Medical Technologies, Los Angeles, CA, USA) were injected through the femoral vein catheter. A reference blood sample was