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Journal of the Chinese Medical Association 80 (2017) 683–689

www.jcma-online.com

Original Article

The effects of pioglitazone in cirrhotic rats with hepatopulmonary syndrome

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Received April 22, 2017; accepted June 12, 2017

Abstract

Background: Hepatopulmonary syndrome (HPS) is characterized by oxygen desaturation and increased intrapulmonary shunting formation in cirrhosis. Due to an unclarified mechanism, there is still no effective therapy except liver transplantation. Recent studies revealed that pulmonary angiogenesis may participate in pathogenesis, in which nitric oxide (NO) and vascular endothelial growth factor (VEGF) play roles. Pioglitazone, a peroxisome proliferator-activated receptor gamma agonist, exerts anti-angiogenesis effect. However, whether pioglitazone influences pulmonary angiogenesis, shunting and HPS remains unexplored.

Methods: Cirrhosis with HPS was induced in Spraque-Dawley rats with common bile duct ligation (CBDL). Pioglitazone (10 mg/kg/day, oral gavage) or vehicle was administered from 8th to 28th day post CBDL. On the 28th day, the mortality rate, hemodynamic parameters, concentrations of plasma glucose and liver biochemistry parameters, and arterial blood gas data were evaluated. Lungs were dissected for protein expression analyses. In another series, intrapulmonary shunting degree was determined by color microsphere method in paralleled groups.

Results: The survival rates were similar in HPS rats with or without pioglitazone administration. Pioglitazone did not influence the hemodynamic parameters, glucose and liver biochemistry levels, oxygen saturation and alveolar arterial gradient, but significantly down-regulated pulmonary VEGF protein expression, endothelial NO synthase (eNOS) activation, and decreased intrapulmonary shunts. Pioglitazone significantly decreased intrapulmonary shunts as compared with the vehicle (18.1 ± 4.5 vs. 9.8 ± 3.6, $p = 0.015$).

Conclusion: Pioglitazone down-regulated VEGF, eNOS and decreased intrapulmonary shunts without improving oxygenation. The current finding suggests a multifactorial mechanism of HPS that could not be successfully overcome merely by pioglitazone-induced anti-angiogenesis and shunting reduction.

Keywords: Angiogenesis; Hepatopulmonary syndrome; Liver cirrhosis; Pioglitazone; Portal hypertension; Shunting

1. Introduction

Hepatopulmonary syndrome (HPS) is characterized by arterial oxygen desaturation in patients with chronic liver disease, which poses a dismal outcome.[1] Three main components of HPS include hypoxia with increased alveolar arterial oxygen gradient ($AaPO_2$), intrapulmonary vasodilatation and increased shunting vessels, and chronic liver disease (mostly liver cirrhosis with portal hypertension). The intrapulmonary

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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http://dx.doi.org/10.1016/j.jcma.2017.08.010

vasodilatation and increased shunting are responsible for abnormal gas exchange and hypoxia in HPS.[2,3] No effective therapy has been documented except liver transplantation.[4] However, emerging evidence on the pathogenesis of HPS has suggested the possibility of medical treatment.[5,6] One piece of that evidence, angiogenesis has just recently been identified.[5] Zhang et al. demonstrated that HPS was associated with pulmonary angiogenesis and vascular endothelial growth factor (VEGF) production.[5] In animals with CBDL-induced HPS, vascular VEGF synthesis and activation of VEGF-dependent signaling pathway increased pulmonary shunting degree. Furthermore, inhibition of monocyte accumulation by pentoxifylline decreased VEGF generation and was associated with reduced angiogenesis and ameliorated HPS.[5] We found that sorafenib, a multikinase inhibitor with anti-angiogenesis effect, reduced the pulmonary shunting and improved hypoxia in CBDL rats. This action might be mediated through VEGF/VEGF receptor 2 (VEGFR2) pathway inhibition.[7]

It has also been noted that endothelin-1 (ET-1) produced by the injured liver activates pulmonary ET_B receptors, resulting in nitric oxide (NO)-mediated vasodilation via endothelial NO synthase (eNOS) upregulation.[8] In agreement with this notion, ET_B receptor knockout inhibited pulmonary eNOS activation and improved HPS in CBDL rats.[9] Also, increased tumor necrosis factor-α (TNF-α) related to bacterial translocation in cirrhosis led to intravascular macrophage accumulation/activation and enhanced inducible NOS (iNOS) expression in animals with experimental HPS, which was improved by TNF-α inhibition.[10]

Thiazolidinediones (TZDs) are anti-diabetic agents that improve insulin sensitivity. Due to the concern of hepatic and cardiovascular adverse effects, nowadays, only pioglitazone is approved for clinical use based upon the following findings: first, the incidence of hepatotoxicity of pioglitazone is not different from other anti-diabetic drugs[11,12]; second, meta-analysis and a clinical trial reveal that pioglitazone use is associated with a significantly lower risk of myocardial infarction and stroke.[13,14] Pioglitazone is a nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) activator.[15,16] Using a high-cholesterol fructose diet-feeding rat model, Collino et al. demonstrated that pioglitazone significantly reduced hepatic expression of TNF-α.[17] Furthermore, pioglitazone improved hepatic ischemia/reperfusion injury in rats via TNF-α suppression.[18]

It is worth noting that pioglitazone also inhibits carcinogenesis,[19] which may be due to its anti-angiogenesis and anti-inflammation properties.[20] Pioglitazone elicited partial remission in patients with advanced sarcoma and malignant vascular tumors via anti-angiogenesis.[21,22] It also inhibited corneal neovascularization, rendering it promising for the treatment of diabetic retinopathy.[23] In addition, pioglitazone appears beneficial in patients with active psoriatic arthritis due to its anti-angiogenesis and anti-inflammation effects.[24] With regard to its influences on NO, it has been reported that in patients with type 2 diabetes, pioglitazone significantly reduced the level of eNOS.[25] Pioglitazone ameliorated eNOS activity in diabetic mice as well.[26] It is noteworthy that pioglitazone decreased portosystemic shunting via modulation of splanchnic inflammation and neoangiogenesis in cirrhotic rats, which was related to splanchnic eNOS and VEGF down-regulation.[27]

Taking the aforementioned factors into consideration, this study aimed to explore if pioglitazone suppressed pulmonary angiogenesis, shunting and improved HPS in rats with CBDL-induced liver cirrhosis and HPS. The underlying mechanism was also evaluated.

2. Methods

2.1. Animal model

Male Sprague-Dawley rats weighing 240–270 g at the time of surgery were used. The rats were allowed free access to food and water then fasted for 12 h before the operation. Secondary biliary cirrhosis and HPS[5] were induced in the rats by CBDL. Under ketamine anesthesia (100 mg/kg, intramuscularly), the common bile duct was exposed through a midline abdominal incision, catheterized by a PE-10 catheter and doubly ligated with 3-0 silk. The first ligature was made below the junction of the hepatic ducts and the second ligature above the entrance of the pancreatic duct. The PE-10 catheter was then removed and the ligatures tightened, followed by section of the common bile duct between the ligatures. The incision was then closed and the animal allowed to recover. A high yield of secondary biliary cirrhosis was noted four weeks after the ligation.[28,29] To avoid coagulation defects, CBDL rats received weekly vitamin K injection (50 μg/kg intramuscularly). The procedures adhered to the Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985). This study was approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital (IACUC2013-090).

2.2. Study protocol

Pioglitazone (10 mg/kg/day, oral gavage) or vehicle (2 ml/day, distilled water) was administered beginning on the 8th day post CBDL for 3 weeks. Four weeks after CBDL, hemodynamic parameters were measured and blood was collected to determine the plasma levels of glucose, liver biochemistry parameters, and TNF-α. Arterial blood was withdrawn for blood gas analysis. Arterial gas exchange, represented by $AaPO_2$, was calculated as $150-(PCO_2/0.8)-PO_2$.[30] In the parallel groups of CBDL rats with or without pioglitazone treatment, color microsphere technique was applied to determine the intrapulmonary shunting degree.

2.3. Systemic and portal hemodynamic measurement

The right internal carotid artery 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 (MAP) and heart rate (HR) were performed on a multi-channel recorder (model RS 3400, Gould, Inc., Cupertino, CA, USA). The external zero reference was placed at the level of the mid-portion of the rat. The

abdomen was then opened with a mid-line incision, and a mesenteric vein was cannulated with a PE-50 catheter connected to a Spectramed DTX transducer. The abdominal cavity was closed and the PP was recorded on a Gould model RS 3400 recorder.

2.4. Determination of plasma TNF-α level

Plasma samples were centrifuged at 3000 rpm for 10 min at 4 °C and stored at −80 °C until tested. The plasma TNF-α levels were measured with a commercially available solid-phase sandwich enzyme-linked immunosorbent assay (rat TNF-α kits; R&D Systems, MN, USA) according to the protocol supplied by the manufacturer. The lower limit of detection is 5 pg/ml.

2.5. Western blot analysis for protein expressions

Rats were sacrificed, and their lungs were dissected, frozen in liquid nitrogen immediately, and stored at −80 °C until required. The protein extracts were incubated with the primary antibody [anti-VEGF rabbit polyclonal antibody (Gene Tex, Irvine, CA, USA); anti-VEGFR-2 (1:500; Millipore Corporation, Billerica, MA, USA); anti-p-VEGFR2 rabbit polyclonal antibody (OriGene, Rockville, USA); anti-eNOS, -iNOS (1:1000; Millipore Corporation, Billerica, MA, USA); anti-p-eNOS (1:1000; Cell Signaling Technology, Danvers, MA, USA)]. Then, the blots were incubated with the corresponding secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG antibody, Sigma Chemical Co., St. Louis, MO, USA). β-actin expression served as loading control. With a computer-assisted video densitometer and digitalized software (Kodak Digital ScienceTM ID Image Analysis Software, Eastman Kodak Co., Rochester, NY, USA), the blots were scanned, and photographed, then the signal intensity (integral volume) of the appropriate band was analyzed.

2.6. Intrapulmonary shunting analysis

Intrapulmonary shunting degree was determined using the technique described by Fallon and Zhang[8,30] with minor modification. Before microsphere injection, animals underwent the placement of indwelling PE-50 femoral arterial and venous catheters. On the day of measurement, 2.5×10^6 custom mixed and counted cross linked polystyrene-divinylbenzene microspheres labeled red (size range 6.5−10 μm; Interactive Medical Technologies, Los Angeles, CA, USA) in 0.20 ml of sterile PBS were injected over 2−4 s through the femoral vein catheter, which was immediately flushed with 0.2 ml of sterile PBS over 2−4 s. An aliquot of microspheres was removed from the injection syringe immediately before injection and counted to verify the numbers of microspheres injected. A reference blood sample was withdrawn from the femoral arterial catheter, starting at the time of femoral vein injection for a total of 90 s at a constant rate of 1.0 ml/min. The volume removed was replaced with an equal volume of sterile PBS. Samples of beads before venous injection and reference blood samples were coded. The absorbance of the solution was read at 448-nm wavelength by a spectrophotometer (Shimadzu, Columbia, MD, USA), and the number of microspheres was calculated by comparison with standards. Spillover between wavelengths was corrected with the matrix inversion technique. Total numbers of microspheres passing through the pulmonary microcirculation were calculated as reference blood sample microspheres per milliliter times estimated blood volume. Blood volume of each animal was derived from the following formula: blood volume (ml) = 0.06 × body wt (g) + 0.77.[31] Intrapulmonary shunting degree (%) was calculated as: (total number of microspheres passing through the pulmonary microcirculation/total beads injected into the venous circulation) × 100.

2.7. Drugs

Pioglitazone was purchased from Takeda Pharmaceuticals (Takeda Pharmaceuticals Taiwan, Ltd., Taipei, Taiwan).

2.8. Data analysis

The results are expressed as mean ± S.D. Statistical analyses were performed using the unpaired Student's *t*-test. Results were considered statistically significant at a two-tailed *p* value less than 0.05.

3. Results

3.1. Mortality rate of pioglitazone- and vehicle-treated cirrhotic rats with HPS

The mortality rates of vehicle- and pioglitazone-treated rats with CBDL-induced HPS are similar [2 out of 13 (15.4%) vehicle-treated rats died; 3 out of 12 (12.7%) pioglitazone-treated rats died].

3.2. Body weight, hemodynamic and liver biochemistry parameters

Table 1 depicts the body weight, hemodynamics, liver biochemistry and glucose levels of vehicle- and pioglitazone-

Table 1
Body weight, hemodynamics, liver biochemistry parameters and glucose level in vehicle- and pioglitazone-treated rats with CBDL-induced HPS.

	Vehicle (n = 7)	Pioglitazone (n = 6)
BW (g)	335 ± 15	350 ± 29
MAP (mmHg)	112 ± 14	114 ± 17
HR (beat/min)	347 ± 37	371 ± 52
PP (mmHg)	20 ± 4.2	16 ± 4.4
AST (IU/L)	1401 ± 581	1190 ± 441
ALT (IU/L)	258 ± 112	222 ± 95
TB (mg/dL)	8.57 ± 1.3	8.76 ± 1.6
Glucose (mg/dL)	88 ± 16	96 ± 16

BW: body weight, MAP: mean arterial pressure, HR: heart rate, PP: portal pressure, AST: aspartate aminotransferase, ALT: alanine aminotransferase, TB: total bilirubin. All $p > 0.05$ between both groups.

treated HPS rats. There was no significant difference between the two groups ($p > 0.05$).

3.3. Arterial oxygenation

The partial pressure of oxygen (PaO_2) and $AaPO_2$ were not significantly different between vehicle- (n = 7) and pioglitazone-treated (n = 6) HPS rats [vehicle vs. pioglitazone: PaO_2 (mmHg): 89.4 ± 3.9 vs. 91.8 ± 3.12, Fig. 1; $AaPO_2$ (mmHg): 13.3 ± 3.6 vs. 13.5 ± 2.7, Fig. 2, both $p > 0.05$].

3.4. Intrapulmonary shunts

The intrapulmonary shunting ratio was significantly decreased by pioglitazone [vehicle (n = 4) vs. pioglitazone (n = 4) (%): 18.1 ± 4.5 vs. 9.8 ± 3.6, $p = 0.015$, Fig. 3].

3.5. Plasma level of TNF-α

Pioglitazone significantly decreased the plasma level of TNF-α in HPS rats [vehicle vs. pioglitazone (ng/ml): 22.1 ± 2.1 vs. 15.9 ± 1.1, $p = 0.045$, Fig. 4].

3.6. Western blot

Pioglitazone significantly down-regulated pulmonary VEGF expression [vehicle vs. pioglitazone (/β-actin): 0.9271 ± 0.0798 vs. 0.6153 ± 0.0696, $p = 0.026$, Fig. 5] and eNOS activation (p-eNOS/eNOS: 1.2653 ± 0.1135 vs. 0.6729 ± 0.0724, $p = 0.002$, Fig. 5).

4. Discussion

An appropriate animal model provides a convincing basis for the survey of a disease entity. Several experimental rat models have been evaluated for the feasibility of HPS study, including partial portal vein ligation (PPVL), thioacetamide

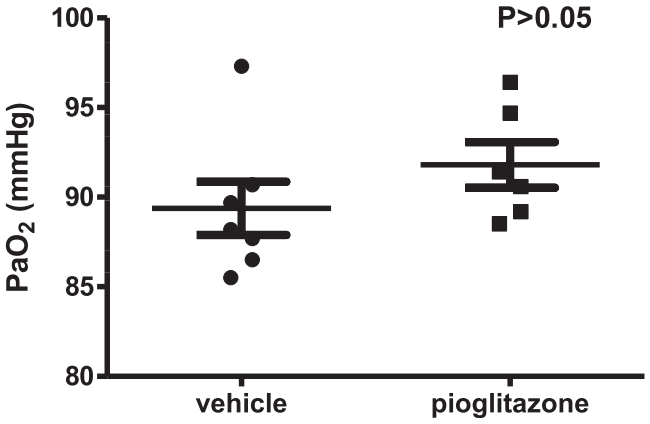

Fig. 1. The partial pressure of arterial oxygen (PaO_2) of vehicle- and pioglitazone-treated rats with CBDL-induced HPS. There was no significant difference between the two groups ($p > 0.05$).

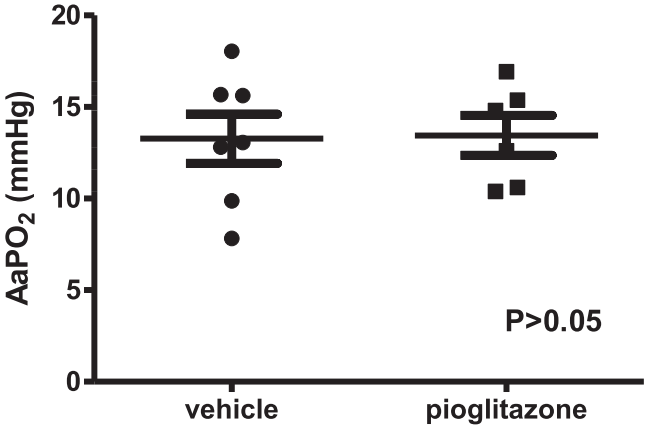

Fig. 2. The alveolar-arterial oxygen gradient ($AaPO_2$) of vehicle- and pioglitazone-treated rats with CBDL-induced HPS. There was no significant difference between the two groups ($p > 0.05$).

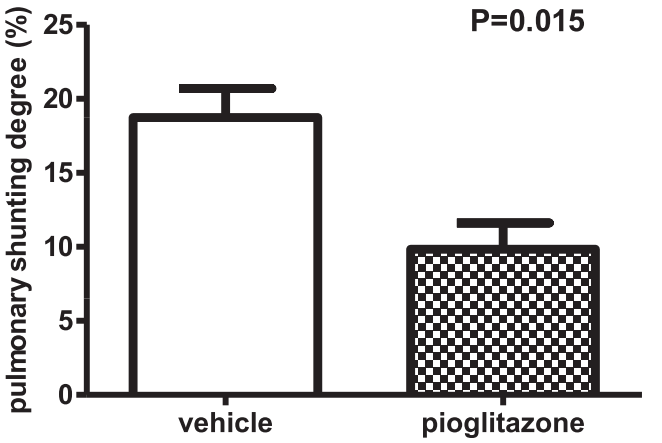

Fig. 3. The intrapulmonary shunting degrees of vehicle- and pioglitazone-treated rats with CBDL-induced HPS. Pioglitazone significantly decreased intrapulmonary shunts ($p < 0.05$).

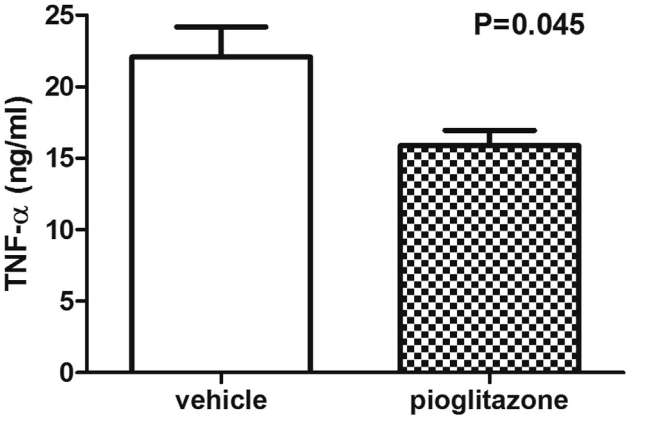

Fig. 4. The plasma levels of TNF-α in HPS rats with vehicle or pioglitazone treatment. Pioglitazone significantly reduced the TNF-α level ($p < 0.05$).

administration, and CBDL.[5,32,33] Among these models, CBDL rats mimic pathophysiologic findings of HPS patients the most. A unique finding in the CBDL rat model is that cholangiocytes, a major source of ET-1, proliferate after CBDL and then

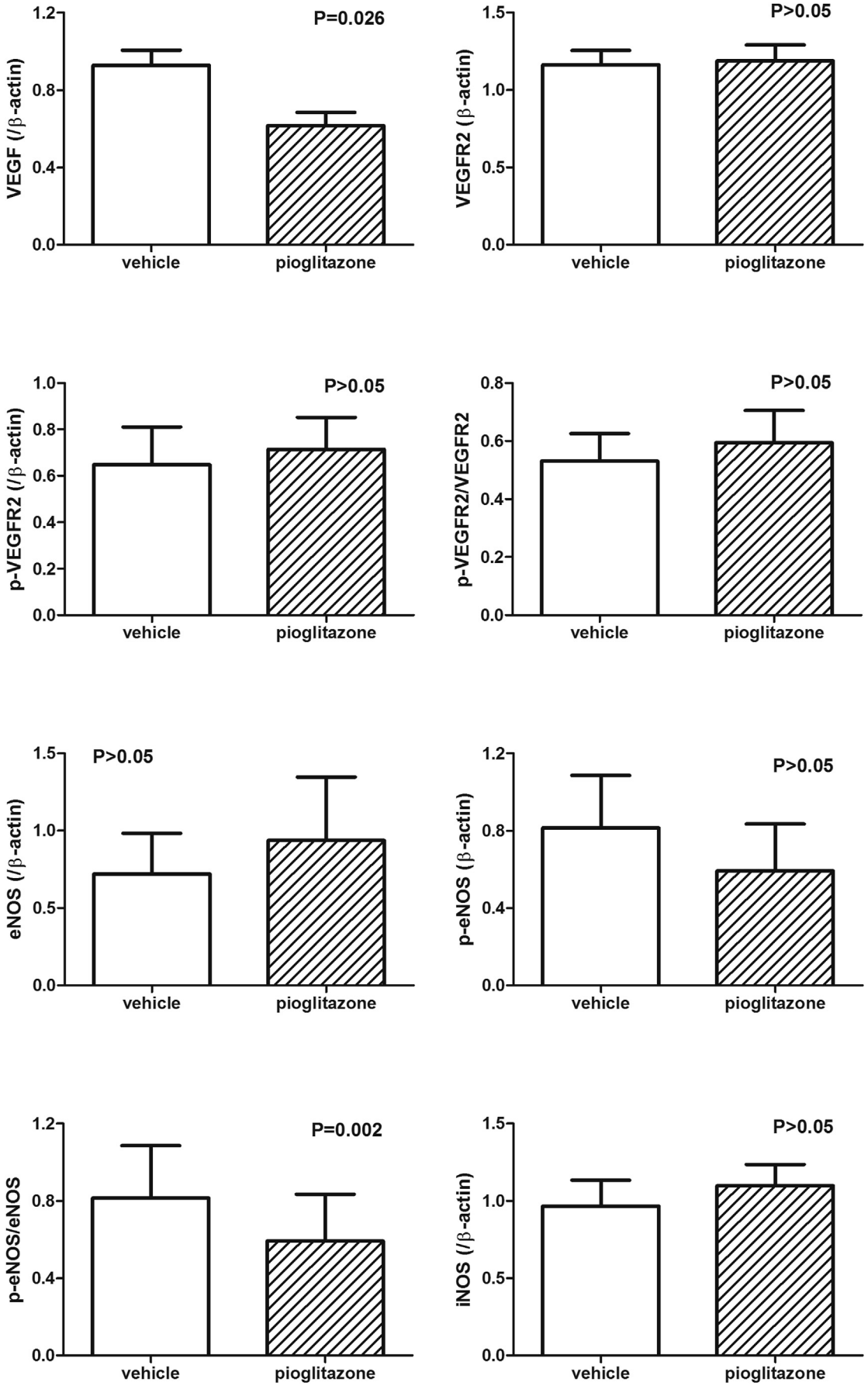

Fig. 5. Pulmonary protein expressions of VEGF, VEGFR2, p-VEGFR2, p-VEGFR2/VEGFR2, eNOS, p-eNOS, p-eNOS/eNOS, iNOS in vehicle- and pioglitazone-treated rats with CBDL-induced HPS. Pioglitazone significantly down-regulated VEGF expression and p-eNOS/eNOS as compared with vehicle ($p < 0.05$).

significantly increase circulating ET-1 levels,[34] which has been identified as a crucial factor to induce HPS.[35] In contrast, prehepatic portal hypertension induced by PPVL does not elevate ET-1 level or develop HPS.[36] Therefore, in this study, CBDL was used to induce liver cirrhosis and HPS.

The present study revealed that pioglitazone reduced intrapulmonary shunting. In liver cirrhosis and portal hypertension, shunting formation can be ascribed to vasodilatation[37] and angiogenesis,[38] in which VEGF and NO play pivotal roles. The modulation of VEGF and NO had been demonstrated to improve the excessive angiogenesis and shunting in cirrhotic and portal hypertensive rats.[37,39] The current study identified that pioglitazone down-regulated pulmonary VEGF expression and eNOS activation, suggesting that pioglitazone may reduce the severity of intrapulmonary shunting via VEGF and NO modulation.

The circulating TNF-α level in cirrhotic rats with HPS was reduced by pioglitazone, compatible with findings of previous studies that pioglitazone suppressed TNF-α generation in animal models with different liver injuries.[17,18] Although TNF-α has been known to induce iNOS expression and subsequent signaling pathways,[40] the current study found that pulmonary iNOS expression was not significantly influenced. The different results may be ascribed to different study designs, organs, disease entities, and rat species. Nevertheless, our findings suggest that pioglitazone reduces the intrapulmonary shunting via eNOS downregulation rather than iNOS.

Although the intrapulmonary shunting was alleviated by pioglitazone, $AaPO_2$ and PaO_2 were not significantly improved. This finding suggests that poor oxygenation in HPS could be multifactorial and may not be reversed merely by shunting reduction. For instance, rapid pulmonary capillary blood transit due to hyperdynamic circulation in cirrhosis may play another role, since it may adversely affect the alveolar−capillary equilibration for oxygen.[41] Actually, pioglitazone did not influence the hemodynamic parameters in cirrhosis, both in the current study and in a previous study published by Schwabl et al.,[27] so rapid blood transit is not likely to be improved. Indeed, the mechanism of HPS is quite complicated and not totally elucidated. Further study is required.

Our data also indicate that pioglitazone does not exert a significantly detrimental effect in rats with CBDL-induced HPS, since the mortality rate and liver biochemistry parameters were not adversely affected. The use of pioglitazone did not induce significant hypoglycemia in cirrhotic rats, either.

In conclusion, pioglitazone reduced intrapulmonary shunting in cirrhotic rats with HPS, which was due to reduce pulmonary VEGF expression and eNOS activation. There was no significant influence on survival, liver biochemistry or blood glucose level, and the circulating TNF-α concentration was lowered, suggesting an acceptable safety profile of pioglitazone use in cirrhosis. Nevertheless, further clinical investigation might be required.

Acknowledgments

This study was supported from a grant of Taipei Veterans General Hospital, Taipei, Taiwan (V102A-024).

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