

Predominant Vascular Dilatation with NOS Expression in Lung Lower Lobe of Thioacetamide Induced-Cirrhotic Rat

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Objective: About eighteen percent of cirrhotic patients come along with decreased systemic arterial oxygenation and expansion of pulmonary venous plexus which triggered by nitric oxide. The level of nitrate and iNOS significantly increase in the cirrhotic patients. However, the localization of nNOS and iNOS in the lung tissue has not yet been clarified. The present study, therefore, aimed to demonstrate the sites of expansion of pulmonary blood vessels and to localize nNOS and iNOS in the lung tissue of cirrhotic rat models induced by thioacetamide (TAA).

Material and Method: The rats were divided into 5 groups. The first group was the control. The other four groups were treated with 200 mg/kg body weight of TAA 3 times per week for 1, 2, 3, or 4 month(s), respectively. At the end of each month rats in each treated group were sacrificed. Lung histology and pulmonary NOS expression was studied by light microscope and immunohistochemical technique, respectively.

Results: It was found that diameter of blood vessels were highest increased in the right lower lobe at the 4-months TAA-treated group. At month 4, iNOS and nNOS expression was localized at epithelium of respiratory tract, endothelium of pulmonary vessel and macrophage.

Conclusion: The present study demonstrated that the pulmonary blood vessels at the right lower lobe with cirrhotic background got enormous dilatation. iNOS and nNOS were immunostained at epithelium of respiratory tract, pulmonary endothelium and macrophages. Our observations suggest that enhanced NOS expression is important in the development of systemic hyperdynamic circulatory abnormalities in cirrhosis.

Keywords: Nitric oxide synthase, Hepatopulmonary syndrome, Cirrhosis, Thioacetamide

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Hepatopulmonary syndrome (HPS) is a physiopathology of pulmonary dysfunction seen in 18 percent of patients with cirrhosis or portal hypertension. It is usually accompanied by hypoxemia and intrapulmonary vasodilatation causing from a great difference in the alveolar-arterial oxygen gradient⁽¹⁻³⁾. HPS might involve a low vascular resistance, ventilation-perfusion imbalance, intrapulmonary shunt and change in gas diffusion⁽⁴⁾. While cirrhosis developed, blood from the portal vein is no longer able

to perfuse the liver. The blood then flows retrogradely to, among others, the esophageal venous plexus and possibly induces the formation of varices. This reverse blood flow could predominantly induce dilatation of the blood vessel in right lower lobe of the lung⁽⁵⁾. Patients with chronic cirrhosis have been reported to suffer from problems with breathing. Nitric oxide (NO) is thought to be the main factor that causes dilatation of pulmonary vasculature⁽⁶⁾. Nitric oxide is synthesized by nitric oxide synthase (NOS). Three isoforms of NOS has been characterized: eNOS⁽⁷⁾, nNOS⁽⁸⁾, and iNOS⁽⁹⁾. e-NOS enzyme is found in the endothelium of blood vessel in the abdomen^(10,11,12). n-NOS is expressed in neurons and vascular smooth muscle cells⁽¹³⁾. iNOS is an inducible form of NOS which is proved to be synthesized from macrophage and vascular smooth muscle of blood vessel after cirrhosis is generated⁽¹⁴⁾.

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It is not yet clear which isoform(s) of NOS cause(s) dilatation of pulmonary vessels. The present study aimed to explore the immunoexpression of iNOS and nNOS in the lung tissue as well as the lung histology of the thioacetamide-treated cirrhotic rat.

Material and Method

Animals

Twenty male Wistar rats, 120–180 g, used in the present study were purchased from the National Laboratory Animal Center (NLAC, Salaya, Thailand). The rats were divided into 5 groups. Four rats were assigned to the control and sixteen to the treated group. The treated group was given 200 mg/kg body weight of thioacetamide (TAA), an inducer of liver cirrhosis, 3 times weekly via an intraperitoneal injection. At the end of 1, 2, 3 and 4 month(s), one rat in the control group and four rats in the treated group were sacrificed. All four lobes; right upper lobe (RUL), right lower lobe (RLL), left upper lobe (LUL) and left lower lobe (LLL), were collected. All experiments were performed in accordance with experimental protocols approved by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University (under license No. 3/2550).

Tissue Preparation

After the lungs were dissected out, they were fixed overnight in 4% formaldehyde. Thereafter, each sample was routinely histological processed prior to embed in paraplast. Serial sections of 5–7 µm in thickness was prepared and mounted on poly L-lysine-coated slides. Lung samples from each group were stained with hematoxylin and eosin for studying the diameter of pulmonary blood vessels, while the remaining slides were stored at 4°C for subsequent immunohistochemical study.

Immunohistochemistry

The sections were deparaffinized in xylene, rehydrated in alcohol and washed in PBS. The sections were then autoclaved in 10 mM sodium citrate pH 6.0 for 10 min to retrieve antigens and to inactivate endogenous alkaline phosphatase, cooled down at room temperature for at least 15 min, wash in PBS, blocked in TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20; pH 8.0), containing either 10 % normal goat, fetal calf, or rabbit serum, for 30 min in a moist incubation chamber, incubated overnight (without prior washing) at room temperature with rabbit polyclonal anti iNOS antibody (Chemical, USA) at 1:50 dilution and rabbit polyclonal anti nNOS antibody

Table 1. Density of immunolocalization of iNOS and nNOS in the right lower lobe of the lung of rats treated for 4 months with thioacetamide

Immunostaining structure	iNOS	nNOS
Bronchial, bronchiolar and alveolar epithelium	+	+
Alveolar endothelial cells	+	++
Pulmonary alveolar macrophage	+	+

(Chemical, USA) at 1:50 dissolved in the blocking solution. Subsequently, the sections were washed in PBS and incubated for 2 hours at room temperature with the alkaline phosphatase-conjugated goat anti-rabbit IgG (Dako Inc., Glostrup, Denmark). After incubation the sections were re-washed once more as described. To reveal antibody binding, the sections were incubated with nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt at room temperature and washed in distilled water. In order to assess nNOS and iNOS antibodies, the tissue sections containing ganglion associated with the rat lower urinary tract were used as positive control.

Statistical analysis

Measurement of the pulmonary blood vessels was accomplished by using version 3.00 of the image tool for Windows ([www.http://ddsdx.uthscsa.edu/dig/download.html](http://ddsdx.uthscsa.edu/dig/download.html).) To compare the diameter of blood vessel in the four lobes of lung at various times of TAA treatment, one-way ANOVA analysis with Tukey's post-hoc test was utilized. A significant difference was set at $p < 0.05$.

Results

Pulmonary histology

With light microscopy, the histological architecture of pulmonary parenchyma of all TAA-treated groups was identical to the control (Figs. 1-4). However, the change in diameter of pulmonary blood vessels was observed. It was shown that the mean diameter of blood vessels of the right lower lobe was significantly increased compared to those of the left lower lobe in 2-, 3- and 4-months TAA-treated groups (Fig. 5). After 2 months of TAA administration, the diameter of the vessels of left upper lobe (LUL) (Fig. 2A), left lower lobe (LLL) (Fig. 2B), right upper lobe (RUL) (Fig. 2C), and right lower lobe (RLL) (Fig. 2D) started to increase compared to the controls of

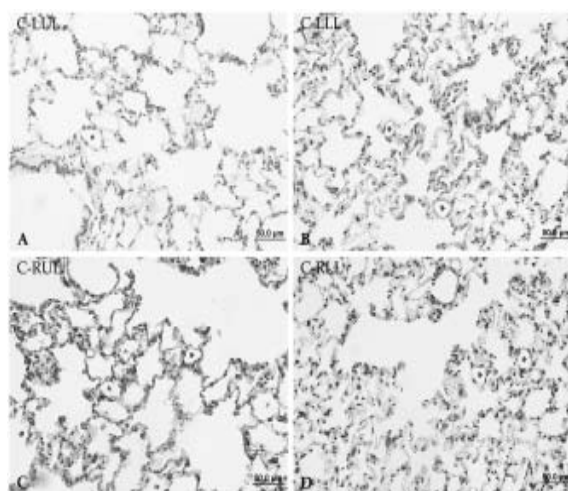


Fig. 1 H&E staining of the left upper lobe (LUL) (A), left lower lobe (LLL) (B), right upper lobe (RUL) (C), and right lower lobe (RLL) (D) of the lung in the control group with the normal vascular diameter (*)

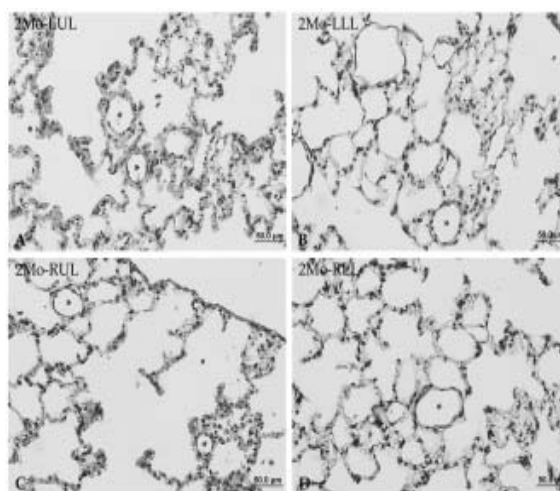


Fig. 2 H&E staining of lung tissues after administration of TAA for 2 months showing many pulmonary blood vessels with large diameter (*) in the left and right lungs but with largest at right lower lobes (RLL) (D)

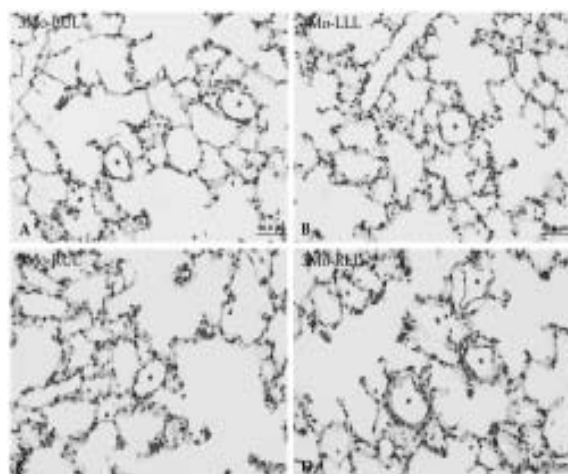


Fig. 3 H&E staining in the right and left lungs of cirrhotic rats treated with thioacetamide for 3 months revealing the largest diameter of blood vessels with irregular shape in right lower lobe (RLL) comparing to the 2- months TAA- treated group and control

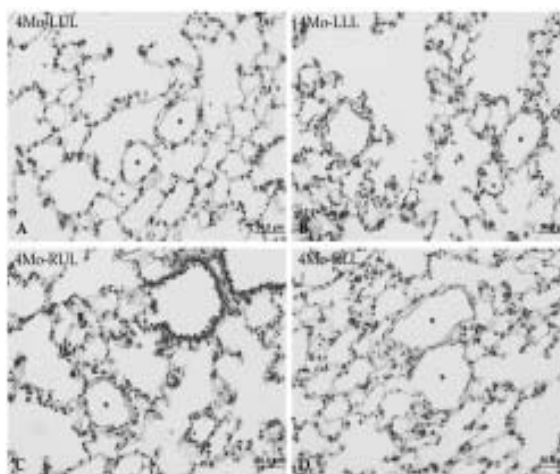


Fig. 4 H&E staining in the lungs of the rats treated with TAA for 4 months showing the largest diameter of blood vessels in the right lower lobe (RLL) compared to that 2 and 3 months treated rat and control group

corresponding months (Figs. 1A,1B,1C,1D, 5). A more pronounced increase in diameter of the blood vessels in RLL was observed after 3 months of TAA treatment (Fig. 3D), while the largest diameter of vessels in RLL were seen after 4 months of TAA treatment (Fig. 4D).

Compared the diameter of pulmonary vasculature at RLL between all three groups of TAA treatment and the control, the largest one was significantly observed at 4 months-TAA treated group ($p < 0.05$) (Fig. 5).

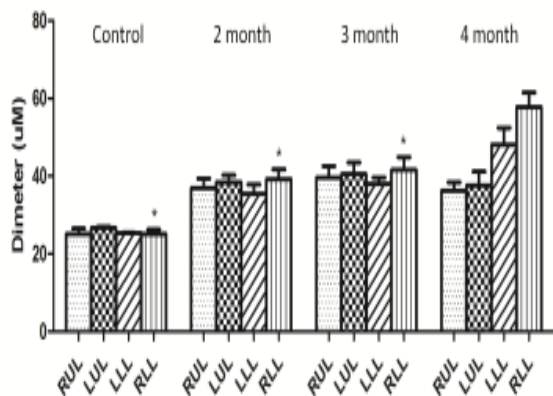


Fig. 5 Comparison the pulmonary vascular diameters in the right lower lobes of the control and thioacetamide-treated rats at different times of exposure. Note the significant increase in diameter of pulmonary vessels in 4- months TAA-treated group ($p < 0.05$)

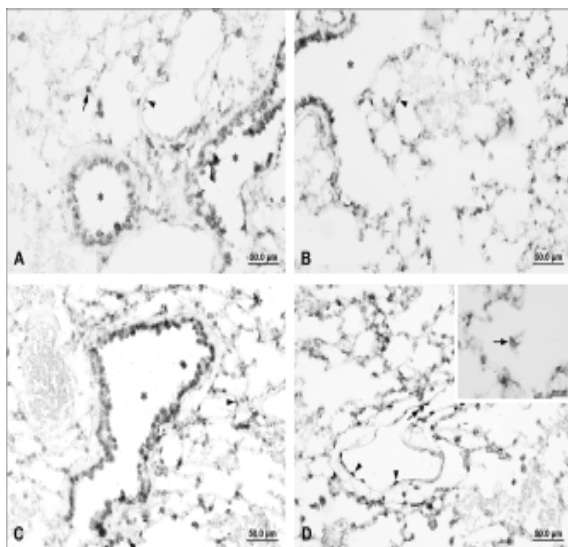


Fig. 6 Micrographs demonstrating the immunolocalization of iNOS (A, B) and nNOS (C, D) in the right lower lobes of the lung after 4 months of treatment with TAA in the epithelium of bronchioles (*), endothelium (%), and macrophages (!) in the blood vessels and airspaces of the respiratory tract. Note the macrophage immunostained with nNOS (!) at high magnification (see inset in Figure D)

Immunohistochemistry

Since the dilatation of pulmonary vessels was enormously observed in RLL of 4-months TAA-treated lung, the immunostaining of nNOS and iNOS was, therefore, performed in this group only.

Upon immunostaining of the RLL tissue in 4-months TAA-treated rats, expression of iNOS and nNOS was found to be localized in the cytoplasm of bronchial, bronchiolar and alveolar epithelium and endothelium, and the macrophages (Fig. 6). There was no expression of iNOS and nNOS in the lung tissue of the control group.

Discussion

The present study demonstrated that the blood vessels in the rat lungs with cirrhosis due to long-term treatment with TAA start to dilate from 2 months onward. The most pronounced effects were seen after the longest treatment period (4 months) and in the RLL (relative to the RUL) of the lung. The underlying mechanism might involve the up-regulation of pulmonary NOS expression⁽¹⁵⁾. In previous studies of the common bile duct-ligation model, it was found that endothelin-1 (ET-1) that was released from the bile duct epithelium, triggers the expression of eNOS in the lung which in turn caused pulmonary vasodilatation^(2,16).

In liver fibrosis, shear stress originating from the obstruction of blood flow to the liver could up-regulate the expression of endothelin B (ET_B) receptor in the pulmonary capillaries^(2,16). Coupling of ET-1 with the ET_B receptor mediated an increase of eNOS expression in the pulmonary endothelium and a high production of nitric oxide (NO) in the lung⁽¹⁷⁾, which in turn caused the relaxation of pulmonary vascular smooth muscles⁽¹⁸⁾. The pulmonary expression of eNOS and the ET_B receptor was significantly decreased after administration of an ET_B receptor antagonist⁽¹⁹⁾. This mechanism induced intrapulmonary vascular dilatation and eventually led to the so-called “hepatopulmonary syndrome”⁽²⁰⁾.

It has been reported that macrophages accumulated in the wall of the pulmonary blood vessels during the development of the hepatopulmonary syndrome. Macrophages expressed iNOS and heme oxygenase 1 (HO-1), which could, in turn, produce NO and CO, respectively^(21,22). In agreement, the authors showed that iNOS and nNOS expressed in the macrophages and endothelium of lung vessels of cirrhotic rats might mediate NO- and CO-dependent vascular dilatation.

The present study demonstrated that the

pulmonary blood vessels of RLL of rats with cirrhosis were profoundly dilated. This finding corresponded to the clinical observation of Anand et al⁽²³⁾, who reported that only the pulmonary venous plexus of the lower lobes, but not the upper lobes of the lung were increased in diameter in 18% of their cirrhotic patients with hepatopulmonary syndrome. The initiating factor that caused dilatation of the pulmonary vessels at lower lobes may due to the steal shunts in the coronary-azygos shunt⁽²⁴⁻²⁶⁾. From this regard, it could be one evidence confirming that there was a real connection between inferior pulmonary vein and azygos vein at the embryonic period but obliterated later. This connection could get re-canalized due to the backflow circulation, e.g. consequence of cirrhosis.

It could be summarized that in the cirrhotic rat treated by TAA, the dilatation of pulmonary blood vessels was demonstrated especially at the right lower lobe. This finding corresponded to the observation by clinicians^(20,23,26).

Potential conflicts of interest

The present study was granted by the National Research Council of Thailand through Srinakharinwirot University under the contract no. 008/2550 and 019/2551.

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การขยายตัวของหลอดเลือดและการแสดงออกของไนตริกออกไซด์ซินเทสในปอดกลีบล่างของหนูที่ถูกชัก นำให้เกิดภาวะตับแข็งด้วยสารไฮโออะเซตาไมด์

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ภูมิหลัง: จากการศึกษาในผู้ป่วยที่เป็นโรคตับแข็ง พบว่าประมาณ 18 เปอร์เซ็นต์ของผู้ป่วยมีการลดลงของระดับออกซิเจนในเลือดร่วมกับมีการขยายตัวของหลอดเลือดในปอด ทั้งนี้อาจเนื่องมาจากผลของไนตริกออกไซด์ซึ่งระดับของไนเตรท และ iNOS มีปริมาณสูงขึ้นอย่างมีนัยสำคัญ อย่างไรก็ตามการศึกษากการกระจายและปริมาณของไนตริกออกไซด์ซินเทสชนิดต่างๆ ในเนื้อเยื่อนั้นยังไม่เป็นที่กระจ่างชัด

วัตถุประสงค์: เพื่อศึกษาตำแหน่งการขยายตัวของหลอดเลือดในปอดและการแสดงออกของ iNOS และ nNOS ในเนื้อเยื่อปอดของหนูที่ถูกกระตุ้นให้เกิดภาวะตับแข็งด้วยไฮโออะเซตาไมด์

วัสดุและวิธีการ: หนูทดลองถูกแบ่งเป็น 5 กลุ่ม กลุ่มที่ 1 เป็นกลุ่มควบคุม กลุ่มที่ 2, 3, 4 และ 5 เป็นกลุ่มที่ถูกชักนำให้เกิดภาวะตับแข็งด้วยการฉีดสารไฮโออะเซตาไมด์ ขนาด 200 มิลลิกรัมตอกลิโลกรัม เป็นเวลา 3 ครั้งต่อสัปดาห์ต่อเนื่องเป็นเวลา 1, 2, 3 และ 4 เดือนตามลำดับ จากนั้นนำเนื้อเยื่อปอดของหนูมาศึกษาทางด้านจุลพยาธิวิทยาและการแสดงออกของโปรตีน iNOS และ nNOS ด้วยวิธีการทางอิมมูโนฮิสโตเคมีสตรี้

ผลการศึกษา: การศึกษานี้พบว่าเส้นผ่านศูนย์กลางของหลอดเลือดในเนื้อเยื่อปอดขวา กลีบล่างของหนูที่ถูกกระตุ้นจนเกิดภาวะตับแข็งเป็นเวลา 4 เดือน มีการเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับปอดขวา กลีบล่างของหนูที่ถูกกระตุ้นเป็นเวลา 2, 3 เดือนและกลุ่มควบคุม นอกจากนี้ยังพบการแสดงออกของ iNOS และ nNOS บริเวณเซลล์เยื่อปอดผิวของท่อทางเดินหายใจ บริเวณเซลล์บุหลอดเลือดในปอดและเซลล์ macrophage

สรุป: การศึกษานี้สรุปได้ว่า ในสภาวะที่หนูเกิดภาวะตับแข็งจากการถูกกระตุ้นด้วยสารไฮโออะเซตาไมด์เป็นเวลา 4 เดือน จะส่งผลให้หลอดเลือดในกลีบล่างของปอดขวาขยายตัวมากขึ้นอย่างมีนัยสำคัญ ร่วมกับการพบการแสดงออกของ iNOS และ nNOS บริเวณเซลล์บุหลอดเลือดในปอดด้วย การขยายตัวของหลอดเลือดในปอดที่พบนี้อาจแสดงให้เห็นว่าเป็นหลักฐานว่ามีการเชื่อมต่อระหว่าง inferior pulmonary vein กับ azygos vein จริงในช่วงของการพัฒนาการในระยะเอ็มบริโอ จึงทำให้เลือดสามารถไหลย้อนขึ้นไปยังปอดได้