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Impact of dietary iron restriction on the development of monocrotaline-induced pulmonary vascular remodeling and right ventricular failure in rats

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ABSTRACT

Pulmonary hypertension (PH) is characterized by pulmonary vascular remodeling leading to right ventricular (RV) failure. Recently, iron deficiency is reported to be prevalent in patients with PH. However, the mechanism by which iron deficiency occurs in patients with PH remains unknown. Here, we investigated the effects of dietary iron restriction on the development of monocrotaline-induced pulmonary vascular remodeling and the involved mechanisms. Male Sprague–Dawley rats were subcutaneously injected with monocrotaline (60 mg/kg). Afterwards, monocrotaline-injected rats were randomly divided into two groups and were given a normal diet ($n = 6$) or an iron-restricted diet ($n = 6$) for 4 weeks. Saline-injected rats given a normal diet were served as controls ($n = 6$). Monocrotaline-injected rats showed pulmonary vascular remodeling, increased RV pressure, RV hypertrophy, and decreased RV ejection fraction, followed by RV failure after 4 weeks. In contrast, iron restriction attenuated the development of pulmonary vascular remodeling and RV failure. Of interest, expression of cellular iron transport protein, transferrin receptor 1 was increased in the pulmonary remodeled artery and the failing right ventricle of monocrotaline-injected rats, as compared with the controls. Moreover, a key regulator of iron homeostasis, hepcidin gene expression was increased in the failing right ventricle of monocrotaline-injected rats. Iron restriction attenuated the development of monocrotaline-induced pulmonary vascular remodeling and RV failure. Cellular iron transport might be involved in the pathophysiology of PH and PH induced RV failure.

1. Introduction

Pulmonary hypertension (PH) is characterized by progressive pulmonary vascular remodeling resulting from significant proliferation of pulmonary artery smooth muscle cells and endothelial cells [1]. PH is a public health problem leading to right ventricular (RV) failure and sudden death. RV failure is the main cause of death in patients with PH [2]. Thus, understanding of the pathophysiology of PH and PH induced RV failure is important for the improvement of the morbidity and mortality.

Iron is a necessary element for maintaining physiological homeostasis in the body, whereas excess iron leads to toxicity and free radical damage by the Fenton reaction, thereby resulting in tissue damage. Hence, iron is considered to be implicated in the pathogenesis of several cardiovascular diseases [3–5]. Of interest, several reports suggest that iron availability affects acute pulmonary vascular responses to hypoxia [6,7]. Additionally, recent reports indicate that iron deficiency is prevalent without overt anemia in patients with idiopathic and heritable PH [8–10]. However, the mechanism by which iron deficiency occurs in patients with PH remains unknown.

Most cells regulate iron uptake by modulating the amount of transferrin receptor 1 (TfR1), which plays a key role in cellular iron transport. Low iron conditions normally lead to upregulate TfR1 expression. Conversely, high iron conditions induce to downregulate TfR1 expression [11]. We have recently shown that TfR1 is involved in the development of vascular remodeling in Dahl

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salt-sensitive rats. In addition, dietary iron restriction has preventive effects on salt-induced cardiovascular remodeling in Dahl salt-sensitive rats. [12]. To the best of our knowledge, there is no report to investigate whether TfR1 is involved in the development of PH and PH associated RV failure. Besides, it is completely unknown whether dietary iron restriction impacts the development of pulmonary vascular remodeling. We therefore hypothesized that TfR1 participates in the development of pulmonary vascular remodeling and dietary iron restriction might affect the development of pulmonary vascular remodeling. In the present study, we investigated the effects of dietary iron restriction on monocrotaline (MCT)-induced pulmonary vascular remodeling and the involved mechanisms.

2. Materials and methods

2.1. Animals

9-Week-old male Sprague–Dawley rats (body weight; 350–400 g) were fed on a normal diet for 1 week. Afterwards, Male Sprague–Dawley rats were subcutaneously injected with MCT (60 mg/kg, Sigma). MCT was dissolved in 1 N HCl, the pH was adjusted to 7.4. After 1 day injection, MCT-treated rats were randomly divided into two groups and were given a normal diet ([PH] $n = 6$) or an iron-restricted diet ([PH + iron restriction (IR)] n = 6) for 4 weeks. Saline-injected rats given a normal diet were served as controls ([CTRL] $n = 6$). The nutrients of a normal diet consist of cornstarch 33%, casein 22%, cellulose 5%, sucrose 30%, corn oil 5%, mineral mixture 4%, and vitamin mix 1%. Mineral mixture contains dicalcium phosphate dihydrate 0.43%, potassium dihydrogen phosphate 34.31%, sodium chloride 25.06%, ferric citrate 0.623%, magnesium sulfate 4.8764%, zinc chloride 0.02%, manganese (II) sulfate pentahydrate 0.121%, copper (II) sulfate pentahydrate 0.156%, potassium iodide 0.0005%, calcium carbonate 29.29%, ammonium molybdate tetrahydrate 0.0025%, and microcrystalline cellulose 5.11%. An iron-restricted diet was based on a normal diet, but with a mineral mixture free of ferric citrate [13]. After 4 weeks diet, rats were sacrificed. The tissues were resected and quickly snap-frozen in liquid nitrogen and stored at −80 °C. In a separate study, 7-week-old male Sprague–Dawley rats (body weight; 250–300 g) were treated by a single subcutaneous injection of MCT (60 mg/kg) and were randomly given a normal diet (PH) or an iron-restricted diet (PH + IR) for 8 weeks. The survival rate was compared among the CTRL ($n = 6$), PH ($n = 15$), and PH + IR ($n = 14$) groups for 8 weeks diet. Rats were maintained on a 12 hr light/dark cycle and had free access to food and water. All of our experimental procedures were approved by the Animal Research Committee of Hyogo College of Medicine.

2.2. Cardiac and pulmonary hemodynamics

Rats were anesthetized with ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg). B-mode, M-mode, and pulmonary pulsed-wave Doppler echocardiography were performed by transthoracic echocardiography (Aplio, Toshiba Medical Systems Co.). RV end-diastolic and end-systolic dimension were determined from epigastrium views. RV pressure was also measured directly by inserting a catheter connected to a pressure transducer into the RV before sacrifice (ADVantage, Scisense).

2.3. Assessments of hematologic parameters

Peripheral blood cell count, and serum iron and erythropoietin levels were determined as previously reported [14].

2.4. Histomorphometric analysis

The lung and RV tissues were fixed with buffered 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. Elastica van Gieson stain (EVG) staining was performed using serial lung sections. Pulmonary arteriolar wall thickness was quantified using ImageJ software by measuring the maximum thickness of arteriolar walls. The values are normalized to CTRL (100%). There were at least 5–10 arterioles measured per slide and 2 slides per animal ($n = 3$–4 rats per group). Hematoxilin-eosin (HE) staining and Masson's trichrome staining were performed using serial RV sections. Cross-sectional area and fibrosis area of the RV were evaluated by semiquantitative score using the method as previously described [14].

2.5. Immunohistochemical analysis

The pulmonary and RV sections were immunohistochemically stained with a primary mouse anti-TfR1 antibody (Zymed Laboratories; dilution 1:200). Immunostains were visualized with the use of an avidin–biotin-peroxidase conjugate and 3,3′-diaminobenzidine substrate. Every section was counterstained with hematoxylin. Quantification of TfR1 positive area was performed by counting the number of TfR1 positive cells in 50 pulmonary arteries and 80 cardiomyocytes in 20 randamly selected fields.

2.6. Gene expression analysis

Total RNA was extracted from the tissues using TRIzol reagent (Invitrogen). DNase-treated RNA was reverse-transcribed into cDNA using random primers (Applied Biosystems). Real-time PCR reactions were performed using the ABI PRISM 7900 with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems) [12]. TaqMan Gene Expression Assays used as primers and probes for each gene were as follows: hepcidin (assay ID Rn00584987_m1), interleukin-6 (IL-6) (assay ID Rn01410330_m1), bone morphogenetic protein receptor type II (BMPR2) (assay ID Rn01437214_m1), and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (assay ID Rn99999916_s1). GAPDH was used as an internal control.

2.7. Statistical analysis

Values are reported as the means ± SEM. Statistical analysis was performed using one way analysis of variance. Analysis of variance (Kruskal–Wallis test, followed by Mann–Whitney U test) was used for statistical comparisons. The probability value <0.05 were considered to be significant. Survival rate was assessed by the Kaplan–Meier survival curves.

3. Results

3.1. Effects of iron restriction on the development of pulmonary vascular remodeling in MCT-injected rats

First, we evaluated the effects of iron restriction on the development of pulmonary vascular remodeling. EVG staining showed a single injection of MCT in rats induced massive pulmonary vascular remodeling; however, dietary iron restriction prevented this change (Fig. 1A and B). In addition, Doppler echocardiography demonstrated the appearance of midsystolic notching on pulmonary artery flow and the increase in pulmonary artery acceleration time in MCT-injected rats, whereas iron restriction suppressed these changes (Fig. 1C). Moreover, MCT-injected rats showed a significant increase in RV systolic pressure, while iron restriction