Serotonin receptor antagonist inhibits monocrotaline-induced pulmonary hypertension and prolongs survival in rats

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Abstract

Objectives: It has been reported that serotonin (5-HT) is involved in the development of pulmonary arterial hypertension (PAH) with pulmonary vascular remodeling. The purpose of the present study was to examine the role of a 5-HT2A receptor antagonist, sarpogrelate hydrochloride, in preventing or reversing monocrotaline (MCT)-induced PAH in rats. Methods: Rats were injected with 40 mg/kg of MCT subcutaneously and randomized to either sarpogrelate (50 mg/kg, intraperitoneally) or placebo for 3 weeks. Animals treated with MCT and survived for 3 weeks were assigned to either sarpogrelate (50 mg/kg, intraperitoneally) or placebo for next 3 weeks. The animals had pressure measurement of the pulmonary artery, and then underwent histologic, immunohistochemical, and Western blot analyses of the lung tissue. Survival rate was also assessed after treatment. Results: Sarpogrelate immediately following MCT injection suppressed PAH with severe pulmonary vascular remodeling and right-sided heart failure. The survival rate was significantly increased in the sarpogrelate-treated group compared with the placebo group (71% vs. 44%, p < 0.05). Intense expression of P-selectin was found on the endothelium of the pulmonary arteries in the placebo group, and it was markedly attenuated in the sarpogrelate-treated group. The numbers of the CD45-positive cells and those of the proliferating cell nuclear antigen (PCNA)-positive cells in the lung tissue were significantly increased in the placebo group, and the increases in these cells were prevented by sarpogrelate. Endothelial nitric oxide synthase (eNOS) expression in the lung tissue was markedly decreased in the placebo group, but it was prevented by sarpogrelate (p < 0.001). In contrast, late treatment with sarpogrelate failed to reverse established PAH. Conclusions: Specific 5-HT2A receptor blockade with sarpogrelate immediately after MCT inhibited PAH and prolongs survival in rats. These effects were accompanied by anti-inflammatory and anti-proliferative effects in the lung tissue and marked improvement of pulmonary vascular endothelial dysfunction and activation.

Keywords: Endothelial function; Pulmonary circulation; Serotonin (5-HT)

1. Introduction

Recent advances in our understanding of pathogenesis of pulmonary arterial hypertension (PAH) led us to introduce newer effective therapeutic strategies that target vascular remodeling, including abnormal proliferation of pulmonary vascular cells [1,2]. Large clinical trials suggest prostaglandins [3,4] and endothelin receptor antagonists [5,6] as first line of treatment of PAH. Phosphodiesterase inhibitors might be used to enhance the efficacy of prostaglandins in less advanced disease [7,8]. Serotonin (5-HT) released from blood platelets accelerates platelet coagulation and is one of the most potent vasoconstrictor of pulmonary arteries [9].
has also been reported that 5-HT is involved in the development of PAH with pulmonary vascular remodeling including the migration and proliferation of smooth muscle cells [9]. Several studies revealed that plasma level of 5-HT was markedly elevated in patients with primary pulmonary hypertension [10] and that 5-HT2 receptor antagonist attenuated development of PAH in both the clinical and experimental settings [11–14]. In addition, there is growing evidence that proliferation of vascular smooth muscle cell is inhibited by a 5-HT2 receptor antagonist in vitro [15,16]. However, the precise mechanisms for its beneficial effects on PAH have not been fully clarified in vivo.

Using a rat model of monocrotaline (MCT)-induced PAH, the present study was undertaken to examine the effects of a 5-HT2A receptor antagonist, sarpogrelate hydrochloride, immediately following MCT injection on endothelial dysfunction and activation of the pulmonary vessels and inflammatory processes and infiltration of the proliferative cells in the lung tissue, and to assess treatment effects of sarpogrelate on survival. We also investigated whether or not sarpogrelate reversed the pathologic effects of MCT once severe PAH had been established in this model.

2. Methods

2.1. Experimental protocol

2.1.1. Protocol 1

Male Sprague–Dawley rats (aged 4 weeks, weighing 100 to 120 g) were injected with 40 mg/kg of MCT subcutaneously and randomized to either sarpogrelate (50 mg/kg/day, intraperitoneally) (sarpogrelate group) or placebo (placebo group) for 3 weeks. The dose of sarpogrelate in the present study was nearly identical to that in the previous studies [13,14]. The normal male Sprague–Dawley rats (aged 4 weeks, weighing 100 to 120 g) served as controls (normal control group). Fifty-five rats subjected to MCT (28 in the sarpgrelate group and 27 in the placebo group) and 25 normal control animals were followed for 3 weeks to assess survival rate. A total of 11 rats in the sarpogrelate group, 12 in the placebo group, and 11 normal control animals had pressure measurement of the pulmonary artery, and then underwent histologic, biochemical, immunohistochemical, and Western blot analyses of the lung tissue.

2.1.2. Protocol 2

Eighty male Sprague–Dawley rats (aged 4 weeks, weighing 100 to 120 g) injected with 40 mg/kg of MCT subcutaneously and survived for 3 weeks were randomly assigned to either sarpogrelate (50 mg/kg/day, intraperitoneally) (n=40) or placebo (n=40) for the next 3 weeks. Animals survived for 6 weeks also had pulmonary artery pressure measurement, histologic, biochemical, immuno-

2.2. Physiologic and biochemical analyses

After treatment, rats were anesthetized with 60 mg/kg sodium pentobarbital given intraperitoneally, and pulmonary arterial pressure was measured with a fluid-filled catheter inserted into the pulmonary artery via the right jugular vein. The rats were euthanized and wet weights of the individual cardiac chambers and lungs were measured. Plasma level of 5-HT concentration was determined by high-performance liquid chromatography after platelet-rich plasma was separated by centrifugation at 2000 g for 30 min at 4 °C. Plasma level of rat brain natriuretic peptide (BNP) was measured by using sensitive and specific radioimmunoassay kits (Peninsula Laboratories, San Carlos, CA). Serum level of NOx (NO2 + NO3) was then determined by gas chromatography.

2.3. Histological and immunohistochemical analyses

Lung tissue sections were prepared as previously described elsewhere [17,18] and stained with elastic hematoxylin–eosin. Morphometric analysis was performed on muscular arteries with external diameter in the ranges of 50–99 and 100–200 μm, using a color digital camera mounted on a computer-interfaced light microscope. Medial wall thickness was measured as the distance between the external and internal elastic laminae of each artery, and external diameter was measured as the diameter of the external lamina, using a calibrated eyepiece micrometer. The percent medial wall thickness of each artery was calculated by using the following formula as previously described [12,19,20]: %medial wall thickness=(2×medial wall thickness)/(external diameter)×100 (%). For each rat, 8–10 vessels were counted and average was calculated. The remaining tissue sections of the lung were subjected to immunostaining with antibodies against P-selectin (Santa Cruz Biotechnology, Santa Cruz, CA), proliferating cell nuclear antigen (PCNA) (Dako, Osaka, Japan), and the investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Sarpogrelate hydrochloride was kindly provided by Mitsubishi Welpharma (Tokyo, Japan).

2.4. Western blot analyses of the lung tissue

To confirm protein expression in lung tissue, the present experiment was designed to examine the mechanisms for its beneficial effects on PAH. Using a rat model of monocrotaline (MCT)-induced PAH, the present study was undertaken to examine the effects of a 5-HT2A receptor antagonist, sarpogrelate hydrochloride, immediately following MCT injection on endothelial dysfunction and activation of the pulmonary vessels and inflammatory processes and infiltration of the proliferative cells in the lung tissue, and to assess treatment effects of sarpogrelate on survival. We also investigated whether or not sarpogrelate reversed the pathologic effects of MCT once severe PAH had been established in this model.

The experimental protocol was approved by the Animal Subjects Committee of Shinshu University School of Medicine and the investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Sarpogrelate hydrochloride was kindly provided by Mitsubishi Welpharma (Tokyo, Japan).

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2.4. Western blot analysis

Proteins were prepared from the lung tissue. Immunoblotting was performed by means of monoclonal antibodies to endothelial nitric oxide synthase (eNOS) (Transduction Laboratories, Lexington, KY). The eNOS protein was detected using the enhanced chemiluminescence immunoblotting detection kit (Amersham Life Science, Piscataway, NJ). Densitometric analysis was performed and the protein levels in each specimen were expressed relative to those of the normal control animals.

2.5. Statistical analysis

Data are presented as mean ± S.E.M. Analysis of variance with Bonferroni’s multiple comparison test was used to analyze the differences among the groups. Survival data were analyzed by Kaplan–Meier method with log-rank test and $\chi^2$ analysis. A probability value of $< 0.05$ was considered statistically significant.

3. Results

3.1. Protocol 1

3.1.1. Treatment effects on survival

During the follow-up of 3 weeks, 23 of the 80 animals died, including 8 in the sarpogrelate group and the remaining 15 in the placebo group. Thus, the survival rate at 3 weeks after treatment was significantly increased in the sarpogrelate-treated group compared with the placebo group.

![Survival curves. Survival rate at 3 weeks after treatment is significantly greater in the sarpogrelate-treated group than in the placebo group (71% vs. 44%, $p<0.05$).](image-url)

Fig. 1. Survival curves. Survival rate at 3 weeks after treatment is significantly greater in the sarpogrelate-treated group than in the placebo group (71% vs. 44%, $p<0.05$).

Table 1

Physiologic, morphologic, and biochemical data after treatment with sarpogrelate immediately following monocrotaline injection

<table>
<thead>
<tr>
<th></th>
<th>Normal control</th>
<th>Placebo</th>
<th>Sarpogrelate (50 mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>11</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Mean PAP (mm Hg)</td>
<td>14.5 ± 0.6</td>
<td>35.5 ± 2.1</td>
<td>14.9 ± 1.6**</td>
</tr>
<tr>
<td>RVW/(LV + S)W</td>
<td>0.30 ± 0.01</td>
<td>0.58 ± 0.02</td>
<td>0.35 ± 0.02**</td>
</tr>
<tr>
<td>%Medial wall thickness (%)&lt;100 μm</td>
<td>8.8 ± 0.5</td>
<td>31.3 ± 1.4**</td>
<td>13.7 ± 1.3**</td>
</tr>
<tr>
<td>100–200 μm</td>
<td>4.2 ± 0.2</td>
<td>32.5 ± 1.4**</td>
<td>7.1 ± 0.6**</td>
</tr>
<tr>
<td>Plasma 5-HT level (ng/ml)</td>
<td>1.08 ± 0.08</td>
<td>2.84 ± 0.17**</td>
<td>2.09 ± 0.11*</td>
</tr>
<tr>
<td>Plasma BNP level (pg/ml)</td>
<td>31.9 ± 1.8</td>
<td>445.0 ± 38.7**</td>
<td>74.1 ± 4.6**</td>
</tr>
<tr>
<td>Serum NOx level (nmol/ml)</td>
<td>41.2 ± 2.2</td>
<td>12.6 ± 1.6**</td>
<td>29.3 ± 2.6**</td>
</tr>
</tbody>
</table>

BNP, brain natriuretic peptide; 5-HT, serotonin; LV, left ventricle; NO, nitric oxide; PAP, pulmonary arterial pressure; RV, right ventricle; S, interventricular septum; W, weight.

Values are mean ± S.E.M.

* $p<0.05$ vs. placebo group.

** $p<0.01$ vs. placebo group.

$^a$ $p<0.05$ vs. normal control group.

$^{ab}$ $p<0.01$ vs. normal control group.
(71% vs. 44%, \( p < 0.05 \)) (Fig. 1). All of the 23 animals died of right heart failure with massive pericardial and pleural effusions and ascites.

3.1.2. Physiologic and morphologic data

Mean pulmonary arterial pressure was significantly elevated in the placebo group compared with normal control group, and it was improved to the normal control level in the sarpogrelate-treated group (Table 1). The ratio of the right ventricular weight to the weight of left ventricle plus septum was significantly increased in the placebo group compared with the normal control animals. The sarpogrelate-treated group showed an intermediate value for the ratio between the other two groups (Table 1).

3.1.3. Histologic, immunohistochemical, and Western blot analyses

Medial hypertrophic and hyperplastic changes in the medium- to small-sized pulmonary arteries were prominent in the placebo group compared with normal control animals 3 weeks after MCT injection. These features were markedly inhibited in the sarpogrelate-treated group. Thus, 3 weeks of sarpogrelate prevented the increase in the %medial wall thickness (Table 1).

Intense expression of P-selectin was found on the endothelium of the pulmonary arteries in the placebo group, and it was markedly attenuated in the sarpogrelate-treated group (Fig. 2). The numbers of the CD45-positive cells and those of the PCNA-positive cells in the lung tissue were significantly increased in the placebo group compared with the normal control group, and they were significantly decreased after sarpogrelate treatment (Fig. 2). However, the numbers of these cells were still significantly different between the normal control and sarpogrelate-treated groups (Fig. 3). In the placebo group, majority of the CD45-positive cells were found to be mononuclear leukocytes and they infiltrated throughout the lung tissue. Nuclear staining with PCNA antibody was observed in mononuclear cells and spindle-shaped nuclear cells around the alveolar walls and small pulmonary vessels (Fig. 2).

eNOS protein level in the lung tissue was markedly decreased in the placebo group compared with the normal control animals, but 3 weeks of sarpogrelate prevented its downregulation by MCT (\( p < 0.001 \)) (Fig. 4). Thus, the

![Fig. 2. Immunohistochemical analysis showing P-selectin expression and CD45-positive and PCNA-positive leukocytes in the lung tissue and pulmonary vessels. P-selectin is intensely expressed on the endothelium of the pulmonary arteries in the placebo group, and its expression is markedly suppressed in the sarpogrelate-treated group (original magnification × 250). The numbers of the CD45-positive and PCNA-positive cells in the lung tissues are significantly increased in the placebo group compared with the normal control group, and these are significantly decreased after sarpogrelate treatment (original magnification × 160).](image-url)
magnitude of eNOS expression was restored to the 71% of the normal control level after sarpogrelate.

3.1.4. Biochemical data

Plasma levels of 5-HT and BNP were markedly elevated in the placebo group compared with the normal control group, and they were significantly decreased after sarpogrelate treatment (Table 1). Serum NOx concentration was significantly reduced in the placebo group compared with the normal control animals, and 3 weeks of sarpogrelate improved the NOx level (Table 1).

3.2. Protocol 2

Of the 80 rats, 15 (8 with sarpogrelate-treated and 7 with placebo-treated animals) survived for 6 weeks. Mean pulmonary arterial pressure, cardiac weights, and the extent of pulmonary vascular remodeling did not differ significantly between the two groups (Table 2). There were also no significant differences in the biochemical data and the magnitude of P-selectin expression on the endothelium of the pulmonary arteries, accumulation of the CD45-positive leukocytes and PCNA-positive leukocytes, and the extent of eNOS expression in the lung tissue between the two groups.

Table 2
Effects of treatment on established pulmonary arterial hypertension induced by monocrotaline

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Sarpogrelate (50 mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Mean PAP (mm Hg)</td>
<td>36.5 ± 2.4</td>
<td>36.7 ± 2.7</td>
</tr>
<tr>
<td>RVW/(LV + S)W</td>
<td>0.59 ± 0.07</td>
<td>0.60 ± 0.07</td>
</tr>
<tr>
<td>%Medial wall thickness (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100 µm</td>
<td>31.5 ± 2.2</td>
<td>32.2 ± 1.8</td>
</tr>
<tr>
<td>100–200 µm</td>
<td>32.6 ± 1.9</td>
<td>32.9 ± 2.1</td>
</tr>
<tr>
<td>Plasma 5-HT level (ng/ml)</td>
<td>3.74 ± 0.34</td>
<td>3.65 ± 0.35</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.
Abbreviations as in Table 1.
4. Discussion

This study demonstrated that (1) specific 5-HT2A receptor blockade with sarpgrelate hydrochloride immediately following MCT injection inhibited PAH and prolongs survival in rats, (2) these effects were accompanied by marked improvement of pulmonary vascular endothelial activation/injury, anti-inflammatory and anti-proliferative effects and upregulation of eNOS in the lung tissue, and (3) late treatment with intraperitoneal administration of sarpgrelate failed to reverse established PAH and pulmonary vascular remodeling in this model. Miyata et al. [13] reported that sarpgrelate suppressed PAH and attenuated recruitment of proliferative cells in the lung tissue of MCT-treated rats. Our study was the first to demonstrate that extremely diminished expression of eNOS protein in the lung tissue was markedly improved after sarpgrelate in the model.

Hypertensive pulmonary vascular disease is characterized by abnormal proliferation of vascular endothelial and smooth muscle cells [21,22], eventually leading to occlusion of small pulmonary arteries. Recent advances of understanding of the pathogenesis of PAH led us to the development and clinical application of novel treatment such as prostaglandins and endothelin receptor antagonists, which exerted primarily through anti-proliferative actions [1,2]. However, several potential limitations still exist in these therapeutic strategies, and the development of additional effective therapeutic approaches which target pulmonary vascular remodeling is required. Recently several investigators demonstrated that statins were beneficial in the treatment of a rat model of MCT-induced PAH and pulmonary vascular remodeling in this model. Miyata et al. [13] reported that sarpgrelate suppressed PAH and attenuated recruitment of proliferative cells in the lung tissue of MCT-treated rats. Our study was the first to demonstrate that extremely diminished expression of eNOS protein in the lung tissue was markedly improved after sarpgrelate in the model.

Accumulating evidence suggests that inflammatory process contributes to the development and progression of severe PAH [31]. We used CD45 immunohistochemistry in order to further characterize the involvement of inflammatory leukocytes [32] and to examine the treatment effects of sarpgrelate in the lung tissue. Our results clearly demonstrated a marked reduction in the accumulation of CD45-positive leukocytes after treatment and confirmed the finding by the previous study that the 5-HT2A receptor blocker exerts anti-proliferative effects in the lung tissue [13].

It has been reported that enhanced expression of eNOS inhibits vascular smooth muscle cell proliferation [33] and that underexpression of vasodilators such as NO along with overexpression of vasoconstrictors such as endothelin influences pulmonary vascular tone and thus contributes to the vascular remodeling [1]. In the present study, there was extremely diminished expression of eNOS protein in the lung tissue which was associated with decreased serum NOx level in the placebo group, and it was dramatically upregulated after 3 weeks of sarpgrelate.

Although the precise reason for the anti-inflammatory action and upregulation of eNOS protein after sarpgrelate treatment remains uncertain, based on the results of our study, anti-inflammatory and anti-proliferative actions and marked improvement of pulmonary vascular endothelial dysfunction and activation are suggested as possible mechanisms for the beneficial effects in the setting of MCT-induced PAH in rats.
4.2. Effects of sarpogrelate on established PAH and vascular remodeling

It would be of great interest to assess whether sarpogrelate could exert regressive effects on the pathobiology of PAH once it had been induced by MCT, since majority of the patients have already developed relatively advanced pathophysiologic processes when PAH is found in the clinical setting. Our results indicate that sarpogrelate failed to reverse or palliate established PAH and pulmonary vascular remodeling in this model.

4.3. Limitations and implications

There were several limitations in the present study. First, the precise mechanism responsible for the reduction of plasma 5-HT concentration after sarpogrelate was not clear. We assumed that it was due to a diminished release and/or increased uptake by platelets, by inhibiting disease progression. Second, sarpogrelate was beneficial to suppress PAH and pulmonary vascular remodeling and prolong survival in a rat model of MCT-induced PH, but this agent could not rescue the advanced disease condition nor completely inhibit pathobiology of MCT-induced PAH. Thus, sarpogrelate may be beneficial in treating patients with less advanced PAH, in which inflammatory mechanisms with pulmonary vascular endothelial dysfunction may contribute to the development and progression of disease conditions. These include primary pulmonary hypertension, PAH associated with collagen vascular diseases, and chronic pulmonary thromboembolism. Well-designed, carefully performed, large clinical trials will be necessary to convince the evidence of efficacy and safety of sarpogrelate in the treatment of PAH.

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References


