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Stimulation of nitric oxide-sensitive soluble guanylate cyclase in monocrotaline-induced pulmonary hypertensive rats

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ABSTRACT

Aims: In this study, we examined whether a disruption in the balance between nitric oxide (NO)-sensitive and -insensitive soluble guanylate cyclase (sGC) is observed in pulmonary hypertension (PH) and whether treatment with NO-enhancing drugs can halt disease progression.

Main methods: Rats were injected subcutaneously with saline or 60 mg/kg monocrotaline (MCT). At 14 days after injection, the vascular reactivity of isolated extralobar pulmonary arteries was assessed by organ chamber technique. In a separate experiment, isosorbide mononitrate (0.3 or 1 g/L) or sodium nitrite (30 or 300 mg/L) was administered in drinking water for the last 14 days (from day 15 to day 28), and their therapeutic potential was evaluated.

Key findings: The NO-sensitive sGC stimulant BAY 41-2272 and the NO-insensitive sGC stimulant BAY 60-2770 both relaxed the pulmonary arteries, which was comparable between saline- and MCT-injected rats. Treatment with isosorbide mononitrate suppressed the MCT-induced right ventricular systolic pressure (RVSP) elevation and pulmonary arterial medial thickening but not right ventricular hypertrophy. However, the beneficial effects on RVSP and pulmonary vascular remodeling were not observed when a high dose was administered. The same results were obtained following the sodium nitrite treatment. Interestingly, NO-enhancing drugs did not increase plasma nitrite plus nitrate levels at a dose that provided the greatest therapeutic advantage.

Significance: These findings suggest that the balance between NO-sensitive and -insensitive sGC is not disrupted in the early stage of MCT-induced PH. Furthermore, supplementation with an adequate amount of NO may be a useful therapy to prevent the progression of PH.

1. Introduction

Nitric oxide (NO) is an important signaling molecule mediating several physiological functions including vasodilation, platelet anti-aggregation, inhibition of interstitial fibrosis, and smooth muscle anti-proliferation. NO exerts its diverse effects mainly through the activation of soluble guanylate cyclase (sGC) and its subsequent cGMP production [1]. NO activates sGC by binding to its reduced (Fe^{2+}) heme moiety, and is unable to activate sGC if it contains an oxidized (Fe^{3+}) heme moiety or if the heme group is missing altogether [2]. This sGC redox equilibrium is disrupted under stress conditions [3] and could decrease NO bioavailability.

Pulmonary hypertension (PH) is associated with impaired NO bioavailability, and thus, drugs targeting the NO/sGC/cGMP pathway are clinically used to treat PH [4]. Several hypotheses have been made as to underlying mechanisms by which NO bioavailability decreases during PH and they include: (1) the suppression of NO production in the pulmonary endothelium [5,6], (2) the scavenging of NO by the superoxide anion generated at the site of the lesion [7,8], and (3) the stimulation of cGMP degradation in the lung and pulmonary circulation [9,10]. However, there is no concrete evidence that indicates that the sGC redox state, or the balance between NO-sensitive and -insensitive forms, is altered in the pulmonary vessels during the progression of PH.

The therapeutic efficacy of exogenously administered NO-enhancing drugs against PH remains controversial. The inhalation of nebulized nitrite [11] or the daily intraperitoneal administration of

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inorganic nitrite [12] has been demonstrated to reverse established PH induced by a monocrotaline (MCT) injection in rats. Additionally, continuous subcutaneous infusion of molsidomine [13], daily oral administration of isosorbide dinitrate [14], or gavage treatment with pentaerythritol tetranitrate [15] prevented the development of MCT-induced PH. Conversely, chronic NO inhalation did not exert such beneficial effects [16,17]. Furthermore, patients with PH are sometimes refractory to the therapeutic action of NO supplementation [18,19].

This study aimed to investigate whether the sGC redox equilibrium is shifted towards the NO-insensitive state in the pulmonary artery before full establishment of severe PH and whether treatment with NO-enhancing drugs from that point can halt disease progression. To address these issues, MCT-injected rats were used as an experimental model of PH.

2. Materials and methods

2.1. Animals

A total of 60 male Sprague-Dawley rats (8 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were maintained at the Animal Testing Facility, Osaka University of Pharmaceutical Sciences until experiments were performed. The animals were housed in a light-controlled room with a 12-h light/dark cycle and were allowed access to food and water ad libitum. The Experimental Animal Research Committee of Osaka University of Pharmaceutical Sciences approved the use of rat materials along with the experimental protocols in this study.

2.2. Experimental protocols

Experimental protocols are illustrated in Fig. 1. Rats were given a single subcutaneous injection of 0.9% saline (referred to as “control”) or 60 mg/kg MCT. In the first experiment, at 14 days after the saline or MCT injections, each rat was anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg) and a polyethylene catheter was inserted into the right carotid artery to measure central hemodynamics, such as mean arterial pressure (MAP) and heart rate (HR), using a pressure transducer coupled to a polygraph system (RM 6000, Nihon Kohden, Tokyo, Japan). Another polyethylene catheter was inserted into the right ventricle through the right jugular vein to measure right ventricular systolic pressure (RVSP). The rats were then euthanized, and the heart and lungs were excised, weighed, and used for morphometric analysis. At this time, extralobar pulmonary arteries were isolated for measurement of vascular reactivity.

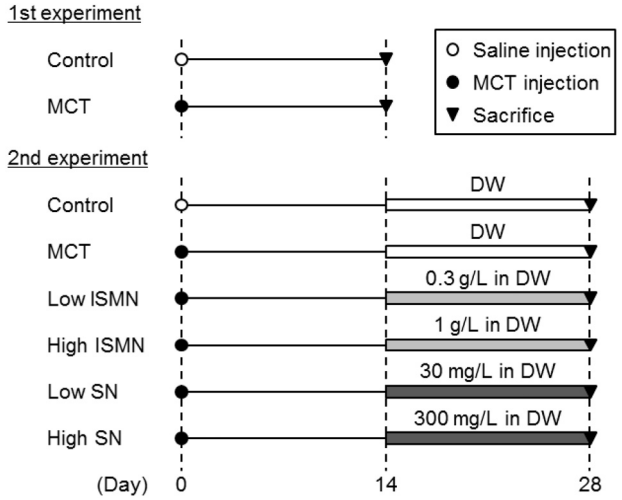

Fig. 1. Schematic diagram depicting the experimental protocols. MCT, monocrotaline; ISMN, isosorbide mononitrate; SN, sodium nitrite; DW, drinking water.

In the second experiment, rats were injected with saline or MCT as described above, and 14 days later, MCT-injected rats were assigned to receive either water, isosorbide mononitrate (0.3 or 1 g/L), or sodium nitrite (30 or 300 mg/L). Saline-injected rats were given water. All interventions were administered via their drinking water and the solutions were changed every 2–3 days for 14 days. The average water intake per day was as follows: control 47 mL, MCT 35 mL, low-dose isosorbide mononitrate 39 mL, high-dose isosorbide mononitrate 44 mL, low-dose sodium nitrite 41 mL, and high-dose sodium nitrite 41 mL. At 14 days after the initiation of treatment, hemodynamic measurements were performed, and then blood samples were collected from the inferior vena cava to determine plasma nitrite plus nitrate (NOx) concentration, and finally organs were harvested for analyses.

2.3. Histological analysis

Pulmonary arterial medial thickening was assessed as previously reported [20]. Briefly, left lungs were fixed in 10% phosphate-buffered formalin and routinely embedded in paraffin. Tissue sections were cut at 4-μm thickness and stained with Elastica van Gieson for examination by light microscopy. Vessels with two well-defined elastic lamellae and with a layer of smooth muscle between the two lamellae were defined as resistance pulmonary arteries. The external diameter and medial wall thickness were measured for 10–15 muscular arteries (50–150 μm in external diameter) per lung section using an image analyzer (AE-6905C, ATTO, Tokyo, Japan). For each artery, the percent wall thickness was calculated and expressed as follows: medial thickness (%) = [(medial thickness × 2) / external diameter] × 100. The values obtained were averaged per animal and then per group.

2.4. Vascular reactivity

Extralobar pulmonary arteries were cut into 2-3-mm rings. To exclude the influence of endothelium-derived NO, the endothelium of the rings was intentionally removed by infusing the tissue with sodium deoxycholate. This step is necessary because the presence of NO makes it difficult to assess the sGC redox state [3]. The rings were then suspended on stainless steel wires in a muscle bath (10 mL capacity) containing Krebs-Ringer bicarbonate solution with the following composition: 118.5 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, and 10.0 mM glucose. The solution was bubbled with a gas mixture of 95% O_2 and 5% CO_2 (pH 7.4), and the temperature was maintained at 37 ± 0.3 °C. The upper wire was connected to the lever of a force-displacement transducer (TB-612 T, Nihon Kohden) and isometric contractions and relaxations were displayed on an ink-writing oscillograph. The resting tension was adjusted to 1.0 g, which is optimal for inducing maximum contraction. Before starting the experiments, all rings were allowed to equilibrate in the bathing medium for 90 min, during which time the solution was replaced every 15 min.

The rings were partially contracted with 1 μM phenylephrine. After the contraction reached a plateau, concentration-response curves for the reduced sGC stimulant BAY 41-2272 and the oxidized/heme-free sGC stimulant BAY 60-2770 were obtained by adding the drug directly into the bathing medium in cumulative concentrations. At the end of each experiment, 100 μM papaverine was added to induce maximal relaxation, which was taken as 100% for the relaxations induced by sGC stimulants. As an aside, endothelial denudation was confirmed by the lack of acetylcholine (1 μM)-induced relaxation of precontracted pulmonary arterial rings.

2.5. NOx measurement

Plasma NOx concentration was measured using an ENO-20 NOx

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