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Effect of intermedin/adrenomedullin$_2$ on the pulmonary vascular bed in hypoxia-induced pulmonary hypertensive rats

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ABSTRACT

Aims: This study aimed to investigate the effect and mechanism of action of intermedin/adrenomedullin$_2$ (IMD/AM$_2$) on the pulmonary vascular bed in pulmonary hypertensive rats.

Materials and methods: Male Sprague-Dawley rats were exposed to hypobaric hypoxia for 3 weeks to induce pulmonary hypertension (PHT). The development of PHT was confirmed by histopathological analyses and measurement of hematocrit, basal perfusion pressure, and right ventricle hypertrophy. Subsequently, the effect of IMD/AM$_2$ in pulmonary hypertensive rats was assessed with both, isolated organ bath and isolated lung perfusion studies.

Key findings: In the PHT group, the basal perfusion pressure and % hematocrit were increased, and right ventricle hypertrophy occurred after 3 weeks of hypoxia exposure. Increased medial wall thickness was also observed in the pulmonary artery with histopathological analysis. In the PHT, the nitric oxide-mediated vasodilation caused by IMD/AM$_2$ in the pulmonary vascular bed and this was as potent as the control group. Acetylcholine responses were also protected in pulmonary hypertensive rats.

Significance: Our results showed for the first time in *in vitro* studies that IMD/AM$_2$ administration causes potent, concentration-dependent vasodilation in the main and resistance pulmonary arteries of rats with PHT. Based on these results, IMD/AM$_2$ might be considered as a future therapeutic target for PHT treatment.

1. Introduction

Pulmonary hypertension (PHT) is a rare and severe disease, characterized by elevated pulmonary arterial pressure. It progresses rapidly, causing serious consequences, such as right ventricular failure and death. Though PHT pathobiology is still not well-understood, vasoconstriction, pulmonary vessel wall remodeling, and thrombosis are known to result in elevated resistance to the pulmonary vascular system. Many endogenous mediators, growth factors, and exogenous stimuli play important roles in these pathological changes. Adjunctive and symptomatic therapies targeting these factors are applied for PHT treatment. Although new drugs such as prostanoids, endothelin receptor antagonists, and phosphodiesterase-5 inhibitors have recently provided significant improvements in hemodynamic parameters, there is still no definitive treatment for the disease [1–5].

The members of the calcitonin gene-related peptide (CGRP) family, especially CGRP and adrenomedullin, demonstrate potent activity in cardiovascular systems. They are both cardioprotective and effective in cardiac diseases such as congestive heart failure and myocardial ischemia [6]. Based on the cardioprotective and potent vasorelaxant action of these peptides, their effect on PHT has been tested in many studies. Endogenous CGRP levels were reduced in hypoxia-induced PHT; hence, CGRP infusions prevented hypoxia-induced PHT [7,8]. Adrenomedullin reduced the pulmonary arterial pressure in pulmonary hypertensive rats [9–11]. Additionally, intravenous administration or inhalation of adrenomedullin decreased the pulmonary arterial pressure and vascular resistance in PHT patients [12].

Intermedin/adrenomedullin$_2$ (IMD/AM$_2$) is the latest member of the CGRP family; similar to other members of the family, IMD/AM$_2$ has a significant effect on the cardiovascular system [13,14]. Intraperitoneal IMD/AM$_2$ injections attenuate myocardial injury and protect the heart against ischemia-reperfusion injury [15]. Another study showed that low dose administration of IMD/AM$_2$ reduces pulmonary ischemia/reperfusion injury in lungs of mice [16]. IMD/AM$_2$ is also a potent vasorelaxant in several vascular beds such as rat aorta, mesenteric and porcine coronary arteries, and reduces arterial blood pressure [14,17–21]. IMD/AM$_2$ was suggested as a putative drug candidate for cardiometabolic diseases based on its positive effect on hemodynamic

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and cardiac functions [22]. Our previous studies reported that IMD/AM_2 provides a marked vasorelaxation in the isolated main pulmonary artery (PA); additionally, intra-arterial (i.a.) bolus injections of IMD/AM_2 decreased the perfusion pressure of the pulmonary vascular bed in isolated rat lungs [23]. In recent years, several studies have been reported the effect of CGRP and adrenomedullin on PHT. However, no study investigated the effect of IMD/AM_2 on pulmonary vascular bed with PHT in vitro. In this study, we determined the role and mechanism of action of IMD/AM_2 on the isolated main PA and perfused lungs of rats with hypoxia-induced PHT.

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats (300–350 g) were used in all the experiments. The rats were housed in a room with a 12 h/12 h light/dark cycle at a constant temperature (22 ± 1 °C), and were provided access to food and water ad libitum. All experiments were approved by the Hacettepe University Animal Experimentations Local Ethics Board (2015/2–3). All procedures involving animals were conducted in accordance with the EU Directive 2010/63/EU.

2.2. Chemicals

Rat intermedin (IMD/$AM_{2\ (17–47)}$) was purchased from Bachem Labs (Torrance, CA, USA). All other chemicals were purchased from Sigma Aldrich (St Louis, Missouri, USA). All chemicals were dissolved in distilled water except for U46619 (Sigma Aldrich, St Louis, Missouri, USA), which was dissolved in ethanol.

2.3. Chronic hypoxia-induced pulmonary hypertension model

PHT was developed in rats by hypobaric hypoxia exposure for 3 weeks. First, the rats were acclimatized for one day in plexiglass chambers, in which the atmospheric pressure was reduced at a rate of ¼th of the atmospheric pressure. Subsequently, the atmospheric pressure in chambers was reduced of ½ of the atmospheric pressure for the remaining 3 weeks. The development of PHT was assessed by right ventricle hypertrophy, increased baseline perfusion pressure, and polycythemia. The hypertrophy in the right ventricle (RV) was evaluated as a weight ratio of the right ventricle (RV) to the sum of left ventricle (LV) and septum (S). To determine hematocrit levels, 5 ml of blood was centrifuged at 10.000 rpm (17.700 g) for 5 min and the percentage of the length ratio of red cells to total blood in the tube was measured. Vascular remodeling was also evaluated by measuring medial wall thickness after hematoxylin and eosin (HE) staining. The vessels were fixed by immersion in 10% neutral buffered formalin at room temperature and were embedded in paraffin using an automated vacuum tissue processor. Cross sections with a thickness of 5 μm were stained with HE. Photomicrographs of each cross section was generated using a light microscope (Leica DMR 6000B, Westlar, Germany) attached to a digital camera (Model DFC 490, Leica Westlar, Germany), so that the entire thickness of vessel wall was visible at the lowest magnification. Bright-field images were captured and measured quantitatively using an image-processing program (LAS, Leica Inc. Westlar Germany). The tunica media, consisting of circularly arranged smooth muscle fibers and elastic lamellae in each image, was measured at 10 different points. The tunica media layer was characterized based on its morphology in HE- stained sections.

2.4. Isolated main pulmonary artery

The rats were euthanized by exsanguination from the carotid artery. The rat lungs were isolated and immersed in cold Krebs-Henseleit solution (KHS, comprised of the following: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM Mg_2SO_4, and 10 mM glucose). Main PAs were isolated and cleaned to remove connective tissues and excess fat. The vessels were cut into approximately 3 mm-long rings. Two stainless steel triangles were inserted into each ring; one triangle was fixed and the other was connected to a transducer in an organ bath containing 5 ml of KHS. The solution was continuously gassed with 95% oxygen and 5% CO_2 at 37 °C. The isometric changes in tension were recorded with a force transducer (MP36, Commat, Ankara, Turkey). The vessels were stretched under a tension of 1.5 g and were equilibrated for 60 min by washing with KHS every 10 min. Subsequently, PA rings were precontracted with thromboxane A_2 agonist U46619 and cumulatively treated with ACh (10^{-8}-3×10^{-6} M) for assessing endothelial integrity. The endothelium was accepted intact when vasorelaxation responses were over 70%. To test the vasorelaxation responses of IMD/AM_2, PA rings were again precontracted with U46619 at approximately 1 g. After they had reached a steady state, IMD/AM_2 (10^{-9}-3×10^{-7} M) was applied cumulatively. L-NAME (10^{-4} M) was used for blocking nitric oxide-mediated responses. It was added to organ baths 30 min before precontraction and afterwards, the administration of IMD/AM_2 were repeated.

2.5. Isolated lung perfusion

Rats were anesthetized with ketamine/xylazine (90/10 mg/kg) and the tracheas were cannulated. The thoracic cavity was opened and heparin (200 IU) was injected into right ventricle. PA was cannulated via the right ventricle and animals were exsanguinated from the abdominal aorta. A small incision was made in the left atrium and ventricles were removed for enabling the free efflux of perfusate. The lungs were isolated and perfused at a constant flow rate (6 ml/min) by using a peristaltic pump (Gilson Model M312, Middleton, USA), while KHS was gassed with 95% oxygen and 5% CO_2 continuously at 37 °C. The modified Langendorff system was used for measuring isolated lung perfusion. After 15 min of equilibrium, the perfusion pressure was elevated by approximately 10 mmHg using U46619, and i.a. IMD/AM_2 bolus injection (2 μg/0.1 ml) was administered. After the perfusion pressure had reached a steady state, an i.a. bolus injection of ACh (2 μg/0.1 ml) was also applied, the lungs were perfused with L-NAME (10^{-4} M) for 30 min, to investigate the role of the endothelium. Then, the vasodilation responses of IMD/AM_2 was replicated in the presence of L-NAME (10^{-4} M).

2.6. Statistical analysis

All data were expressed as mean ± SEM values. Statistical analysis was performed with the Student's *t*-test. The normality of distribution for histomorphometric measurements was assessed by the Mann Whitney *U* test. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Development of chronic hypoxia-induced pulmonary hypertension

Secondary PHT was developed in rats after 3 weeks of hypobaric hypoxia exposure. Basal perfusion pressure of these rats were increased by approximately two-fold compared to that in the control group (*$p < 0.05$; Fig. 1A). Right ventricular hypertrophy occurred in the PHT group. (*$p < 0.05$; Fig. 1B). The increased hematocrit level was found to be statistically significant compared to that of the control group (*$p < 0.05$; Fig. 1C).

A statistically significant increase in muscle content was detected in the tunica media of PAs from PHT group (136.93 ± 14.62 μm) when compared to that from the control group (62.19 ± 5.32 μm). The measured values exhibited normal distribution. The thickness of the tunica media of PAs from the PHT and control groups were shown in