Redox Biology 11 (2017) 286–296

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

Aldehyde dehydrogenase 2 protects against oxidative stress associated with pulmonary arterial hypertension

Tao Xu[a,*,1], Shuangyue Liu[b,1], Tingting Ma[b], Ziyi Jia[c], Zhifei Zhang[d], Aimei Wang[b,*]

[a] Life Science Institute, Jinzhou Medical University, Jinzhou, Liaoning 121000, PR China
[b] Department of Physiology, Jinzhou Medical University, Jinzhou, Liaoning 121000, PR China
[c] College of Economics and Management, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China
[d] Department of Physiology and Pathophysiology, Capital Medical University, School of Basic Medical Sciences, Beijing 100069, PR China

ARTICLE INFO

Keywords:
Pulmonary arterial hypertension
4-hydroxynonenal
Aldehyde dehydrogenase 2
Oxidative stress
NF-κB
Alda-1

ABSTRACT

The cardioprotective benefits of aldehyde dehydrogenase 2 (ALDH2) are well established, although the regulatory role of ALDH2 in vascular remodeling in pulmonary arterial hypertension (PAH) is largely unknown. ALDH2 potently regulates the metabolism of aldehydes such as 4-hydroxynonenal (4-HNE), the endogenous product of lipid peroxidation. Thus, we hypothesized that ALDH2 ameliorates the proliferation and migration of human pulmonary artery smooth muscle cells (HPASMCs) by inhibiting 4-HNE accumulation and regulating downstream signaling pathways, thereby ameliorating pulmonary vascular remodeling. We found that low concentrations of 4-HNE (0.1 and 1 μM) stimulated cell proliferation by enhancing cyclin D1 and c-Myc expression in primary HPASMCs. Low 4-HNE concentrations also enhanced cell migration by activating the nuclear factor kappa B (NF-κB) signaling pathway, thereby regulating matrix metalloprotein (MMP)-9 and MMP2 expression *in vitro*. *In vivo*, Alda-1, an ALDH2 agonist, significantly stimulated ALDH2 activity, reducing elevated 4-HNE and malondialdehyde levels and right ventricular systolic pressure in a monocrotaline-induced PAH animal model to the level of control animals. Our findings indicate that 4-HNE plays an important role in the abnormal proliferation and migration of HPASMCs, and that ALDH2 activation can attenuate 4-HNE-induced PASMC proliferation and migration, possibly by regulating NF-κB activation, in turn ameliorating vascular remodeling in PAH. This mechanism might reflect a new molecular target for treating PAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is a serious and fatal clinical syndrome characterized by pulmonary vascular remodeling, which leads to a mean pulmonary artery pressure above 25 mm Hg, right ventricular failure, and death [1]. PAH is a multi-factorial process with a very complex pathological mechanism. Abnormal proliferation of the pulmonary arterial smooth muscle cells (PASMCs) typically underpins its pathology [2,3]. In recent years, extensive studies in both animal models and patients suggested that oxidative stress plays a key role in pathological remodeling of the pulmonary vasculature [4]. Excessive lipid peroxidation participates in the abnormal proliferation of PASMCs [5].

4-hydroxynonenal (HNE) is a major end product of lipid peroxidation, derived from the oxidation of *n*−6 polyunsaturated fatty acids, such as linoleic, γ-linolenic, or arachidonic acids [6]. 4-HNE is not only a marker of oxidative stress, but can also form protein adducts and dysregulate cell signaling to contribute to multiple diseases, including cancer, atherosclerosis, and hypertension [7–9]. 4-HNE has been reported to stimulate the proliferation of vascular smooth muscle cells [10–12]. The accumulation of 4-HNE in the pulmonary arteries in patients with PAH has been recognized as an important contributor to disease progression [13,14]; however, the role of 4-HNE in the abnormal proliferation of pulmonary vascular smooth muscle cells, and the associated signaling pathways involved remain unknown.

Aldehyde dehydrogenase (ALDH) 2 is a key enzyme mediating the conversion of aldehydes, e.g., 4-HNE, into much less reactive chemical species [15]. Previous data have indicated that ALDH2 activity is closely associated with several cellular functions, including proliferation and responses to oxidative stress [16]. The *ALDH2* gene is linked to susceptibility to cardiovascular diseases [17,18]. Polymorphism of the *ALDH2* gene is implicated in inflammatory processes associated

* Corresponding authors.
E-mail addresses: 5xu5_888@163.com (T. Xu), aimeiwang@lnmu.edu.com (A. Wang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.redox.2016.12.019
Received 30 October 2016; Received in revised form 9 December 2016; Accepted 17 December 2016
Available online 21 December 2016
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with coronary heart disease and hypertension [19,20]. Although the exact role of ALDH2 in pulmonary vascular remodeling is unknown, it is very likely that ALDH2 contributes to the development and progression of PAH. In this study, we investigated whether 4-NHE accumulation was responsible for the abnormal proliferation and migration of pulmonary vascular smooth muscle cells, and whether Alda-1 (an ALDH2 agonist) affects perfusion, accelerating 4-NHE clearance and thereby attenuating PAH.

2. Materials and methods

2.1. Animal models

All animal care and experimental procedures were approved and conducted in accordance with the Institutional Animal Care and Use Committee of Jinzhou Medical University and conformed to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health. Male Sprague–Dawley rats (n=48; weighing 220–250 g) were purchased from Vital River Laboratories Animal Company (Beijing, China). The animals were intraperitoneally (i.p.) injected with a single dose of monocrotaline (MCT; 60 mg/kg; Sigma-Aldrich, St. Louis, MO) to induce severe PAH within 2 or 4 weeks (n=8 each group). For experiments involving pre-treatment with Alda-1 (Sigma-Aldrich Co., St. Louis, MO), MCT-injected rats were randomly divided into 3 groups, including the MCT group (n=12), the vehicle-alone group (n=6) administered 50% polyethylene glycol (PEG) and 50% dimethyl sulfoxide (DMSO) by volume, and the Alda-1 group (n=6). Control rats (n=8) were injected with an equal volume of 0.9% phosphate-buffered saline (PBS). The MCT-treated rats were subcutaneously implanted with mini-osmotic pumps (model 2004; ALZET, Cupertino, CA) and continuously infused with Alda-1 (10 mg kg^{-1} d^{-1}) for 4 weeks.

2.2. 4-HNE-His adduct and malondialdehyde (MDA) assays

4-HNE and MDA levels were determined using the OxiSelect™ HNE-His Adduct ELISA Kit (Cell Biolabs, San Diego, CA) and the Lipid Peroxidation (MDA) Assay Kit (MAK085; Sigma-Aldrich), respectively. The enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions, and the activity was measured using a Varioskan Flash microplate reader (Thermo Scientific, Waltham, MA).

2.3. ALDH2 activity assay

ALDH2 activity was measured using a ALDH2 Activity Assay Kit (GMS50131; GenMed, Pfizer, CA), according to the manufacturer's instructions. Enzyme activities were measured using a microplate reader by monitoring the production of NADPH at 340 nm.

2.4. Cell culture

Human pulmonary artery smooth muscle cells (HPASMCs) were purchased from ScienCell Research Laboratories (San Diego, CA) and were cultured in smooth muscle cell-growth medium (SMCM) at 37 °C, in a humidified atmosphere containing 5% CO_2. HPASMCs were used within 3–5 passages of the primary culture.

2.5. Cell-proliferation assay

Cell proliferation was quantified using the methyl thiazolyl tetrazolium (MTT) assay (Sigma-Aldrich). Briefly, cells were initially grown in 96-well microplates in complete SMCM for 24 h and, after washing with PBS, were incubated in serum-free SMCM medium for 24 h. The cells were then treated with different concentrations of 4-HNE for 24, 48, or 72 h. Absorbance of the cultures was measured at 570 nm using a microplate reader.

2.6. BrdU-incorporation assay and cell cycle analysis

BrdU-incorporation assays were performed to measure HPASMC proliferation, using BrdU Flow kits (BD Pharmingen, Franklin Lakes, New Jersey). Briefly, HPASMCs were plated in 35 mm plates at a density of 1×10^6 cells/well and were synchronized over 24 h under serum starvation. HPASMCs were then incubated with PBS or 0.1 μM 4-HNE for 48 h. When required, 20 μM Alda-1 was added 30 min before the addition of 4-HNE. The cells were labeled with BrdU, according to the manufacturer's instructions. The results were acquired using a BD LSRFortessa Cell Analyzer (Becton Dickinson, Franklin Lakes, New Jersey).

2.7. Analysis of cell migration and invasion

HPASMC migration was evaluated in scratch-induced, wound-healing assays [21]. HPASMCs were seeded into 6-well plates. Near-confluent HPASMCs were wounded by scraping with a standard 1-mL pipette tip to create a gap along the diameter of the well. HPASMC invasion was assessed by performing a Boyden chamber assay [22]. HPASMCs were seeded into the upper surface of an 8-μm pore size chamber, with serum-free SMCM medium, with or without 0.1 μM 4-HNE in the lower chamber. When required, 20 μM Alda-1 was added 30 min before the addition of 4-HNE.

2.8. Immunohistofluorescence

For double immunofluorescence staining, 5 μm-thick lung sections were incubated at 4 °C overnight with a mixture of mouse anti-4-HNE or anti-ALDH2 monoclonal antibodies (1: 200 dilution; Abcam, Cambridge, UK), and an anti-α-smooth muscle actin (α-SMA) mouse antibody (1:200 dilution; Abcam); alternatively, the primary antibody was substituted with an isotype control (1:200 dilution; Abcam). Immunohistofluorescence images were obtained using confocal microscopy (TCS-SP5, Leica Microsystems, Wetzlar, Germany).

2.9. Western blot analysis

Lung tissues (20 mg) or cells (1×10^6) were sonicated in 100 μL radioimmunoprecipitation assay buffer (Aidlab, Beijing, China) and homogenized. HPASMC nuclear proteins were extracted using a Nuclear Protein Extraction Kit (Beyotime, Jiangsu, China), according to the manufacturer's instructions. Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Billerica, MA). The membranes were then incubated overnight with the indicated primary antibodies at 4 °C. The following primary antibodies were used for western blotting: monoclonal antibodies against rabbit anti-cyclin D1, anti-c-Myc, anti-matrix metalloprotein (MMP)-9 and anti-MMP-2 (1:1000 dilution; Cell Signaling Technology, USA); polyclonal antibodies against rabbit anti-nuclear factor-kappa B (NF-κB) p65, anti-phospho-NF-κB p65, anti-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), and anti-phospho-IκBα (1:1000 dilution; Cell Signaling Technology, USA); monoclonal antibodies against mouse anti-4-HNE monoclonal antibody and mouse anti-ALDH2 (1: 500 dilution; Abcam, Cambridge, UK). Protein levels were analyzed using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

2.10. Real-time polymerase chain reaction (PCR)

Total RNA was extracted from HPASMCs using the RNAprep Pure Kit (Tiangen Biotech, Beijing, China), according to the manufacturer's instructions. The first-strand cDNA was reverse-transcribed from the

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