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Asymmetric dimethyl arginine induces pulmonary vascular dysfunction via activation of signal transducer and activator of transcription 3 and stabilization of hypoxia-inducible factor 1-alpha

Michaela Pekarova [a,*], Adolf Koudelka [a], Hana Kolarova [a], Gabriela Ambrozova [a], Anna Klinke [b], Anna Cerna [a], Jaroslav Kadlec [c], Maria Trundova [d], Lenka Sindlerova Svihalkova [a], Radek Kuchta [c], Zdenka Kuchtova [c], Antonin Lojek [a], Lukas Kubala [a,e]

[a] Institute of Biophysics, Academy of Sciences of the Czech Republic, v.v.i., Kralovopolska 135, 612 65 Brno, Czech Republic
[b] Department of Experimental Cardiology, University Hospital of Cologne, Kerpener Str. 62, 50924 Cologne, Germany
[c] Faculty of Electrical Engineering and Communication, Brno University of Technology, Technicka 3058/10, 616 00 Brno, Czech Republic
[d] Institute of Biotechnology, Academy of Sciences of the Czech Republic, v.v.i., BIOCEV, Videnska 1083, 142 20 Prague 4, Czech Republic
[e] International Clinical Research Center — Center of Biomolecular and Cellular Engineering, St. Anne's University Hospital, Brno, Czech Republic

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ABSTRACT

Pulmonary hypertension (PH), associated with imbalance in vasoactive mediators and massive remodeling of pulmonary vasculature, represents a serious health complication. Despite the progress in treatment, PH patients typically have poor prognoses with severely affected quality of life. Asymmetric dimethyl arginine (ADMA), endogenous inhibitor of endothelial nitric oxide synthase (eNOS), also represents one of the critical regulators of pulmonary vascular functions. The present study describes a novel mechanism of ADMA-induced dysfunction in human pulmonary endothelial and smooth muscle cells. The effect of ADMA was compared with well-established model of hypoxia-induced pulmonary vascular dysfunction. It was discovered for the first time that ADMA induced the activation of signal transducer and activator of transcription 3 (STAT3) and stabilization of hypoxia inducible factor 1α (HIF-1α) in both types of cells, associated with drastic alternations in normal cellular functions (e.g., nitric oxide production, cell proliferation/Ca^{2+} concentration, production of pro-inflammatory mediators, and expression of eNOS, DDAH1, and ICAM-1). Additionally, ADMA significantly enhanced the hypoxia-mediated increase in the signaling cascades. In summary, increased ADMA may lead to manifestation of PH phenotype in human endothelial and smooth muscle cells via the STAT3/HIF-1α cascade. Therefore this signaling pathway represents the potential pathway for future clinical interventions in PH.

1. Introduction

Pulmonary hypertension (PH) is considered as a progressive vasculopathy with poor prognosis, which is associated with imbalance in vasoactive mediators (e.g., nitric oxide, NO; and prostaglandin I_2), massive remodeling of pulmonary vasculature, and right ventricular hypertrophy (reviewed in [1]). Although PH is a rare disease, its prevalence in certain at-risk groups (e.g., patients with HIV, systemic sclerosis, and sickle cell disease) is substantially higher and was shown to range between 0.5–12% [2–7]. Independently of pathogenic origins, PH is characterized by inflammatory processes associated with activation of various metabolic and pro-proliferative signaling pathways within different types of cells (mainly endothelial cells, smooth muscle cells, and fibroblasts) and increased production of inflammatory-derived mediators (e.g., reactive oxygen species, ROS; interleukin-6, IL-6 and tumor growth factor-β). These factors contribute to development of endothelial dysfunction and progression of vascular remodeling by instigating the proliferation and migration of vascular SMC. This vascular pathology can be triggered by genetic and environmental stimuli, including hypoxia [1,8,9].

As it was mentioned above, the reduced bioavailability of NO in patients with PH represents one of the most important functional abnormalities in this disease [1–9]. Under physiological circumstances, NO is released by endothelium in response to shear stress and regulates flow-mediated vasodilation [10]. This reaction is catalyzed by endothelial NO synthase (eNOS), which requires a semi-essential amino acid L-

Abbreviations: ADMA, asymmetric dimethyl arginine; DDAH, dimethyl arginine dimethylaminohydrolase; ET-1, endothelin-1; eNOS, endothelium nitric oxide synthase; HIF-1α, hypoxia inducible factor 1a; ICAM-1, intracellular adhesion molecule 1; IL-6, interleukin-6; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor kappa-B; NO, nitric oxide; HPAECs, human pulmonary artery endothelial cells; PASMCs, pulmonary artery smooth muscle cells; PH, pulmonary hypertension; PDGF, platelet-derived growth factor; PRMT, protein arginine methyltransferase; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; TRPC, transient receptor potential channel.

* Corresponding author.
E-mail address: pekarovam@ibp.cz (M. Pekarova).

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arginine as a substrate [11,12]. Besides regulating the vasodilation, NO is able to suppress the proliferation of vascular smooth muscle cells and inhibit the interaction of circulating blood elements with the endothelial cells [10,12]. Importantly, the loss of NO production in PH patients is further associated with increased plasma levels of asymmetric dimethyl arginine (N^G, N^G-dimethyl-L-arginine, ADMA), an endogenous competitive inhibitor of eNOS enzyme [13–17].

During the past decade, it was discovered that ADMA is a very powerful molecule and is believed to be associated with development of endothelial dysfunction triggered mainly by ADMA-mediated decrease in NO production and increase in ROS formation [18–26]. Moreover, it serves as a predictor of mortality not only in PH patients, but also in people suffering from other serious diseases (such as renal failure, hypercholesterolemia, hypertension and coronary artery disease) [13–17,27,28]. ADMA is endogenously synthesized during methylation of protein arginine residues by protein arginine methyltransferases (PRMTs). Although it can be eliminated by renal excretion, more than 90% of endogenous ADMA is metabolized by specific enzyme — dimethyl arginine dimethylaminohydrolase (DDAH) [29]. DDAH exists in two isoforms and DDAH1, which is predominantly expressed in the vascular endothelium, was shown to be associated with endothelial dysfunction in PH [13,14,29,30].

Based on the above-mentioned facts, decreased expression/activity of DDAH is believed to represent a key mechanism responsible for increased accumulation of ADMA, as well as for subsequent impairment of NO-dependent regulation of vascular homeostasis [18,24,29–31]. The suppression of DDAH1 was already partially explained by ADMA-dependent activation of microRNA in hypoxia induced PH [30]. Nevertheless, there were also published interesting results, showing the evidence that ADMA is able to regulate the basic cellular functions in NO/DDAH-independent manner [19–22,32,33]. Although it was discovered that ADMA activates the signaling pathways associated with stress response in different types of cells (e.g., mitogen activated protein kinases, MAPKs and nuclear factor-kappa B, NF-κB) [19–22,32,33], there exists only incomplete information about the molecular mechanism by which ADMA contributes to development of pulmonary endothelial dysfunction.

Therefore, our study was focused on the effect of ADMA on physiological functions of human pulmonary artery endothelial cells (HPAECs) and pulmonary smooth muscle cells (PASMCs), which are relevant for progression of PH. We determined the expression of eNOS (phospho eNOS), intracellular adhesion molecule 1 (ICAM-1), DDAH1, and PRMT1, as well as production of NO, ROS, and other inflammatory-derived mediators (e.g., ICAM-1, IL-6 and platelet-derived growth factor, PDGF) in HPAEC. We were also interested in proliferation of PASMC, which is associated with changes in intracellular Ca^{2+} concentration and expression of transient receptor potential channels (TRPCs). TRPC1, 3/6 were selected based on the previously published literature, which demonstrated that these channels play a particularly crucial role in regulating SMC contraction and proliferation in the PH [34–36].

Additionally we elucidated the role of ADMA in specific signaling cascades. Although the activation or stabilization of transcription factors STAT3 (signal transducer and activator of transcription 3) and HIF-1α (hypoxia-inducible factor 1-alpha) has been demonstrated to play a crucial role in PH pathology [1,37–39], as far as we know there exists no information concerning the effect of ADMA. Interestingly, STAT3 and HIF-1α were shown to be responsible for regulation of cell survival and proliferation, angiogenesis, as well as for inflammatory processes, associated with production of different types of inflammatory-derived mediators described above [37–46].

2. Material and methods

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Cell cultures

HPAEC (Lonza, Switzerland) and PASMC (Lonza) were cultivated in complete EGM™-2 medium (Lonza) or complete MCDB medium (Gibco®, Life Technologies), respectively. Confluent cells at passages 2–5 were used for the experiments which were performed in basic medium supplemented only with 2% of fetal bovine serum (FBS). During the experiments, the concentration of amino acid L-arginine was reduced to 100 μM, which is considered as physiological concentration in human plasma [47].

2.2. Detection of cell viability and proliferation

Cell viability was measured based on total cellular mass of adherent cells using the detergent-compatible protein assay reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard [33]. Cell viability was not significantly decreased in any of the tested groups (data not shown).

Additionally, the proliferation of cells was determined using the ATP test (BioThema, Handen, Sweden) which was performed according to the supplier's instructions. Briefly, cells were incubated for 72 h and then lysed with Somatic cell ATP releasing reagent. The ATP substrate was added to each cell lysate and the luminescence signal was recorded for 5 min. The ATP standard was used for calculation of ATP levels in individual samples [48].

The activity of caspase-3 and expression of Bax protein were determined in both types of cells and the results are summarized in Suppl 1.

2.3. Determination of NO production

The production of NO was measured using the Griess colorimetric assay, which is based on determination of nitrites, the end product of NO metabolism in cultivation medium [49]. Briefly, supernatants (150 μl) were mixed with Griess reagent at a ratio of 1:1 and incubated at room temperature for 15 min. The absorbance was measured at 540 nm using a SPECTRA Sunrise microplate reader (Tecan, Mannedorf, Switzerland).

2.4. Detection of protein expression using a Western blot technique

The expression of different proteins was detected in cell samples which were processed using a standard SDS-lysing buffer. An equal amount of proteins (30 μg) from each sample was subjected to SDS-polyacrylamide gel electrophoresis on a 7.5% or 10% acrylamide gel. The proteins were transferred to immobilon polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Billerica, MA, USA) and then incubated with primary antibodies against total eNOS, phospho-eNOS (p-eNOS, Ser1177), total STAT3 and phospho-STAT3 (pSTAT3, Tyr705) (all purchased from Cell Signaling Technology, Danvers, MA, USA) or against HIF-1α, ICAM-1, PRMT1, DDAH1, and TRPC1, 3/6 (all purchased from Santa Cruz Biotechnology, Santa Cruz, California, USA). The membranes were further incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Cell Signaling Technology) for 1 h. The blots were visualized using a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL, USA) and exposed to CP-B X-ray films (Agfa, Brno, Czech Republic). Relative levels of proteins were quantified by scanning densitometry using the ImageJ™ program (National Institutes of Health, Bethesda, MD, USA), with the individual band density value expressed in arbitrary units (optical density, OD). An equal protein loading was verified by β-actin immunoblotting (Santa Cruz Biotechnology) [50]. The data in graphs represent the ratio between the individual values for OD of bands determined for phosphorylated, total form of protein or β-actin. The results are displayed as % of negative control.