2-METHOXYESTRADIOL ATTENUATES THE DEVELOPMENT AND RETARDS THE PROGRESSION OF HYPOXIA- AND ALPHA-NAPHTHYLTHIOUREA-INDUCED PULMONARY HYPERTENSION

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ABSTRACT

Pulmonary arterial hypertension (PH), a progressive, incurable, and deadly disease, predominantly develops in women. Growing body of evidence suggest that dysregulated estradiol (E2) metabolism influences the development of PH and that some of the biological effects of E2 are mediated by its major non-estrogenic metabolite, 2-methoxyestradiol (2ME). The objective of this study was to examine effects of 2ME in chronic hypoxia (CH)-induced PH and alpha-naphthylthiourea (ANTU)-induced acute lung injury and PH. In addition, we investigated the effects of exposure to different levels of CH on development of PH. Chronic exposure to 15% or 10% oxygen produced similar increases in right ventricle peak systolic pressure (RVPSP) and pulmonary vascular remodeling, but oxygen concentration-dependent increase in hematocrit. Notably, right ventricle (RV) hypertrophy correlated with level of hypoxia and hematocrit, rather than with magnitude of RVPSP. The latter suggests that, in addition to increased afterload, hypoxia (via increased hematocrit) significantly contributes to RV hypertrophy in CH model of PH. In CH-PH rats, preventive and curative 2ME treatments reduced both elevated RVPSP and pulmonary vascular remodeling. Curative treatment with 2ME was more effective in reducing hematocrit and right ventricular hypertrophy, as compared to preventive treatment. Single ANTU injection produced lung injury, i.e., increased lungs weight and induced pleural effusion. Treatment with 2ME significantly reduced pleural effusion and, more importantly, eliminated acute mortality induced by ANTU (33% vs 0%, ANTU vs. ANTU+2ME group). Chronic treatment with ANTU induced PH and RV hypertrophy and increased lungs weight. 2-ME significantly attenuated severity of disease (i.e., reduced RVPSP, RV hypertrophy and pulmonary vascular injury). This study demonstrates that 2ME has beneficial effects in chronic hypoxia- and acute lung injury-induced PH and provides preclinical justification for clinical evaluation of 2ME in pulmonary hypertension.

Keywords: Pulmonary hypertension, 2-methoxyestradiol, hypoxia, polycythemia, vascular remodeling, right ventricle, alpha-naphthylthiourea

INTRODUCTION

Pulmonary arterial hypertension (PH) is a progressive, incurable, and deadly disease. PAH

is characterized by pulmonary vasoconstriction, severe pulmonary vascular remodeling and elevation in pulmonary arterial pressure. This leads

to development of right ventricle (RV) hypertrophy, heart failure and significant decrease in life expectancy. In the past two decades, numerous effective treatments have evolved. However, despite the significant progress in diagnosis and treatment of PH, mortality remains high and there is unmet medical need for new therapies. Notably, PH predominantly develops in women. Epidemiological studies and various registry worldwide reported female to male ratio ranging from 2:1 to 4:1 [1-4] and up to 9:1 in connective tissue disease [5, 6]. In 2005, we were first to propose that estradiol metabolism may influence the development of this predominantly female disease [7]. Specifically, we demonstrated that 2ME (a major non-estrogenic metabolite of estradiol) exhibits preventive and therapeutic effects in monocrotaline-induced PH [7] and later documented that 2ME mediates the beneficial effects of estradiol in experimental PH [8, 9]. Furthermore, we suggested significant interaction between estradiol metabolism and vascular disease in PH [10] that may be of high clinical relevance. Over the past fifteen years, an expanding body of animal and human data points toward dysregulated estradiol metabolism as a risk factor for PH and involvement of estradiol metabolism in the development, progression, and prognosis of PH [11-19]. Finally, 2ME not only mediates (at least in part) the beneficial effects of estradiol in PH, but also 2ME exhibits opposing effects to estradiol on several processes relevant to the underlying pathophysiology of PH, including angiogenesis, metabolic reprogramming, inflammation, and immunity [12]. Therefore, recently we suggested that 2ME should be viewed as a disease modifier in women with PH [20]. The objective of this study was to examine the preventive and curative effects of 2ME in chronic hypoxia (CH)-induced PH in male rats. In addition, we investigated the effects of chronic exposure to different levels of CH (15 vs. 10% oxygen) on development of pulmonary hypertension. Finally, we studied the effects of 2ME on acute pulmonary vascular injury and development of PH induced by alpha-naphthylthiourea (ANTU).

MATERIAL AND METHODS

Animals

Ten to twelve weeks old (300-330g) male Sprague Dawley rats were purchased from

Charles River (Wilmington, MA) and were allowed to acclimatize for one week before inclusion into the study. All experimental protocols were approved by the University of Pittsburgh Animal Care and Use Committee, and all experiments were conducted in accordance with the University guidelines for animal welfare.

Protocol 1: The effects of chronic exposure to 15% or 10%-oxygen on PH, hematocrit and pulmonary vascular and right ventricular remodeling

Animals were assigned to exposure to normal air (Control; n=8) or to 21-day exposure to two different levels of hypoxia (15% and 10% O₂) and were placed into normobaric Plexiglas chambers (Bio Spherix, Redfield, NJ, USA). The chambers were continuously flushed with nitrogen to maintain oxygen levels at 15±0.5% or 10±0.5% and CO₂ concentrations at >0.5%. The O₂ and CO₂ concentrations were continuously monitored by PROOX110 BioSpherix oxygen controller and LB-2CO₂ analyzer, (Sensormedics). After 21 day, animals were anesthetized with pentobarbital (45 mg/kg i.p.) and placed on a Deltaphase Isothermal pad (Braintree Scientific, Braintree, MA) to maintain body temperature at 37 ± 0.5°C. A PE-240 polyethylene catheter was inserted into the trachea to facilitate breathing, and the right jugular vein was exposed and a miniature pressure transducer catheter (SPR-513, Millar Instruments, Houston, TX) connected to a digital heart performance analyzer (PowerLab) was advanced into right ventricle (RV). After 20-min stabilization period, 15 minutes recording of RV pressures was conducted. Animals were euthanatized by anesthetic overdose and heart and lungs were dissected and weighed. The ratios of wet weights of heart and lung to body weight (BW) were calculated (H/BW and LV/BW, respectively). The right ventricle (RV) free wall was separated from the left ventricle and the septum (LV+S) to determine the wet weight, the RV to body weight ratio (RV/BW), the LV+S to body weight ratio (LV+S/BW), and the RV to LV+S weight ratio (RV/LV+S, Fulton Index).

The lungs were removed from the chest cavity en block with the trachea and perfused via the trachea with a 10% neutral-buffered formalin at a pressure of 25 cm H₂O. Perfused tissue samples were immersed in the fixative for 24 to 72 hours and samples were embedded in paraffin blocks for light microscopy. Five-micron serial tissue

sections from formalin-fixed, paraffin-embedded lungs were dewaxed and stained with H&E and Masson's trichrome for histological and morphometric assessment. Lung tissues sections were examined by light microscopy and were scored in blinded fashion. To study vascular remodeling of small pulmonary arteries, arterial wall smooth muscle cells were stained using a mouse monoclonal anti-smooth muscle- α actin antibody at a dilution of 1/100 (Lab Vision, Fremont, CA). The measurements of media thickness, and media and adventitia surface were conducted using an Image Analyzing System (Diagnostic Instruments, Inc., Sterling Heights, MI) that included SPOT RT Camera installed on NIKON Eclipse 50 light microscope and specialized computer software program (SPOT Software, Version 4.1). Measurements were done ($\times 20$ magnification) on ten cross sectioned pulmonary artery branches with 50–250-micron diameters. The peripheral lung fields were located at approximately equal distances from the pleural lining. Only vessels with an approximately circular profile (cross-sectional cuts) were studied. For each blood vessel, two rectangular diameters and their four respective media were measured. The media % index was calculated as $2 \times \text{media/diameter} \times 100$ and is presented as average of four media measurements for a single vessel. For each blood vessel, two rectangular diameters and their four respective media were measured, and averages of four individual values of media thickness and media % index were calculated.

Protocol II: Effects of 2-methoxyestradiol on hypoxia induced pulmonary hypertension

A total of 32 male Sprague Dawley rats were randomly assigned to exposure to normal air (Control; $n=8$) or to 21-day exposure to hypoxia (10% O₂; CH rats, $n=24$), as described in Protocol I. On Day 0 and Da-10 of exposure to chronic hypoxia, a subsets of CH rats were implanted ALZET (2ML-4, Durect Corporation, Cupertino, CA) osmotic pumps delivering 2ME (10 $\mu\text{g/kg/h}$; Steraloids, Inc., Newport, RI; 2ME0-21d and CH+2ME10-21d group, respectively). After 21 day, animals were instrumented and RV pressure was recorded and lungs and heart tissue were collected and processed for morphometric and histopathological analysis, as described in Protocol I.

Protocol III: Effects of 2-methoxyestradiol on ANTU-induced lung injury and PH

For acute administration of ANTU and 2ME, animals were anesthetized with pentobarbital (45 mg/kg i.p.) and placed on a Deltaphase Isothermal pad (Braintree Scientific, Braintree, MA) to maintain body temperature at $37 \pm 0.5^\circ\text{C}$. A PE-240 polyethylene catheter was inserted into the trachea to facilitate breathing, and the right jugular vein was exposed and a miniature pressure transducer catheter (SPR-513, Millar Instruments, Houston, TX) connected to a digital heart performance analyzer (PowerLab) was inserted into right ventricle. A left jugular vein was exposed and PE-50 catheters was inserted for infusion of vehicle (0.1% DMSO, 25 $\mu\text{l/min}$ i.v., for 4 hours; control and ANTU groups) or 2-methoxyestradiol (10 $\mu\text{g/kg/h}$; ANTU+2ME group). After 90 minutes, subset of animals (ANTU group) was injected alpha-naphthylthiourea 5 mg/kg. Four hour later, chest cavity was opened and pleural fluid collected and measured. Animals were euthanized by overdose of anesthetic and lungs tissue were collected and processed for histopathological analysis as described in Protocol I.

For chronic experiments, twelve-week old male Sprague Dawley rats were randomly administered saline (Cont, $n=7$), ANTU (5mg/kg i.p.; ANTU group, $n=15$) or ANTU plus 2ME (10 $\mu\text{g/kg/h}$ for 4 weeks via osmotic minipumps; ANTU+2ME group, $n=10$). ANTU injections were repeated after 7, 14 and 21 days. Four weeks into treatments, animals were instrumented for in situ (open chest) measurements of right ventricular (RV) peak systolic pressure (RVSP). Animals were anesthetized and instrumented for measurement of RVPSP and tissue samples were collected and processed for morphometric and histopathological analysis as described in Protocol I.

RESULTS

Before investigating the effects of 2ME on CH- and ANTU-induced induced PH, we examined the effects of two different levels of hypoxia (10% vs 15%) on development of PH. Chronic exposure to 15%- or 10%-oxygen environment produced similar increases in RV peak systolic pressure (RVPSP; Figure 1B), however it produced oxygen concentration-dependent increase in hematocrit (Figure 1C). The Fulton index (RV/LV+S ratio), a measure of isolated RV hypertrophy, correlated with level of hypoxia and hema-

Figure 1. Effects of two different levels (15% and 10% oxygen) of chronic hypoxia (CH) on (A) isolated right ventricle hypertrophy (Fulton index, RV/LV+S), (B) right ventricular peak systolic pressure (RVPSP) and (C) hematocrit. RV hypertrophy correlated with hematocrit, but not with pulmonary artery pressure.

Figure 2. Effects of two different levels (15% and 10% oxygen) of chronic hypoxia (CH) on pulmonary vascular remodeling. Hypoxia-induced adventitia widening, but not media remodeling, correlated with the level of hypoxia

tocrit (Figure 1A), rather than with magnitude of RVPSP. The latter suggests that, in addition to pulmonary artery pressure, hypoxia (via increased hematocrit) significantly contributes to RV hypertrophy in chronic hypoxia model of pulmonary hypertension. Assessment of pulmonary vascular remodeling revealed no effect of O₂ levels on media remodeling (Figure 2 A&B), but O₂ levels correlated with adventitia widening and overall wall thickness Figure (2C&D).

Exposure to chronic hypoxia induced PH, i.e., increased RVPSP and produced isolated RV hypertrophy (Figure 3) and also increased hematocrit from 45% (Control group) to 65% (CH group; Figure 3, top right panel). Chronic hypoxia also induced significant vascular remodeling in pulmonary circulation (Figure 4 & 5), as evidenced by increased media thickness and surface area (Figure 4 A&B), adventitia widening and wall surface area (Figure 4 C&D). Preventive and curative treatment

Figure 3. Preventive (0-21d) and curative (10-21d) treatment with 2-methoxyestradiol (2ME) attenuated chronic hypoxia (CH)-induced pulmonary hypertension, right ventricle (RV) hypertrophy and polycythemia (hematocrit)

Figure 4. Preventive (0-21d) and curative (10-21d) treatment with 2-methoxyestradiol (2ME) attenuated chronic hypoxia (CH)-induced media remodeling (A&B), adventitia widening (C) and wall thickness (D)

Figure 5. The effects of 2-methoxyestradiol on chronic hypoxia-induced pulmonary vascular remodeling (A-D Trichrome and E-H Movat's-pentachrome stain). Representative images of control (A&F), chronic hypoxia (CH group: B&F), preventive (CH+2ME0-21d: C&G) and curative (CH+2ME 10-21: D&H) treatments with 2-methoxyestradiol. Both 2ME treatments reduced CH-induced media remodeling (B&F vs. C,D and G,H, respectively), adventitia widening (F back arrow), perivascular fibrosis (B, white arrow) and perivascular inflammatory cells infiltration (F, black arrow; (B&F vs. C,D and G,H, respectively)).

Figure 6. Acute administration of ANTU induced pleural effusion (A) increased lungs weight (B), and perivascular edema and inflammation in both small size arteries (D-F) and veins (G); D-G, Movat's-pentachrome stain. Pre-treatment with 2ME reduced pleural effusion (A), abolished acute mortality (C) and has no effects on lungs weight (B) and histopathological changes (data not shown)

Figure 7. 2-Methoxyestradiol attenuated PH (i.e., right ventricular peak systolic pressure (RVPSP) and RV hypertrophy induced by chronic administration of ANTU (bottom panels)

Figure 8. Representative images of control (top images) and ANTU-treated animals (bottom images). ANTU induced significant congestion, perivascular inflammation and pulmonary vasoconstriction, with moderate media remodeling (all images H&E stain)

with 2ME similarly reduced elevated RVPSP (Figure 3, top middle panel) and pulmonary vascular remodeling (Figure 4, 5). Yet, only curative treatment (CH+2ME10-21d group), but not preventive treatment (CH+2ME group), reduced elevated hematocrit (Figure 3, top right panel), isolated RV hypertrophy (Figure 3, top left panel) and RV mass (Figure 3, bottom panels). These data further support the notion that hematocrit has significant effect of development of RV hypertrophy.

Single ANTU injection acutely increased lung weight, caused pleural effusion (PE), and resulted in 33% acute mortality rate (Figure 6 top panels). Histopathological analysis of lungs tissue revealed perivascular edema and inflammation in both small size arteries (Figure 6D-6F) and veins (Figure 6G). 2ME had no effect of lungs weight (Figure 6B), however significantly reduced PE volume (Figure 6A) and more importantly eliminated the acute mortality induced by ANTU (Figure 6C). Repeated weekly injections of ANTU produced moderate PH, i.e., increased RVPSP, induced isolated RV hypertrophy and increased lung weight (Figure 7D, E and A, respectively). 2ME significantly attenuated severity of disease i.e., reduced RVPSP and RV hypertrophy (Figure 7D-7F). Histopathological evaluation of lung tissue revealed significant congestion, perivascular inflammation and pulmonary vasoconstriction, with moderate media remodeling (Figure 8, bottom images and Figure 9, top images). Chronic treatment with 2ME attenuated ANTU-induced histopathological changes (Figure 9, bottom images).

DISCUSSION

In this study, we further evaluated the therapeutic efficacy of 2ME in two distinctly different models of PH, i.e., chronic hypoxia- and pneumotoxin ANTU-induced PH. There are four major findings related to the effects of polycythemia, 2-methoxyestradiol, and pneumotoxin alpha-naphthylthiourea on PH and RV remodeling, that may have significant pre-clinical and clinical ramifications.

First, in hypoxic PH, we show that hypoxia-induced polycythemia have significant effect on PH and related RV hypertrophy. Our data in CH-PH rats are in accordance with fifty five years old findings that, in absence of hypoxia, polycythemia (induced by cobalt chloride or by administration of packed red blood cells) per se induces RV hypertrophy [21, 22]. Chronic exposure to hypoxia, in addition to hypoxic pulmonary vasoconstriction, is also associated with an increase in red blood cell mass which, by an increase in blood viscosity, further aggravates PH. Notably, hypoxia-induced polycythemia also increases RV afterload and should be expected to increase RV hypertrophy [23]. Therefore, any experimental study of hypoxic PH (rats and mice CH-PH model and CH+SU5416 murine model of angioproliferative PH) should report hematocrit levels and potential effects of any interven-

Figure 9. Chronic treatment with 2ME attenuated ANTU-induced histopathological changes (bottom vs. top images; all images H&E stain)

tion on red blood cells count. Similarly, clinical studies, at least in patients with group III (hypoxia related) PH, should report red blood cells related parameters.

Second, our data confirm previously reported [24-26] beneficial effects of 2ME in hypoxic PH and suggest that these beneficial effects of 2ME (both on pulmonary pressure and RV hypertrophy) are, at least in part, due to decrease in polycythemia. In this regard, in the current study, the preventive treatment with 2ME reduced PH, yet had no effect on RV hypertrophy and hematocrit. In contrast, the rescue treatment with 2ME further reduced the PH and reduced both hematocrit and RV hypertrophy. These data provide further evidence that polycythemia have great impact on hypoxia-induced PH and RV hypertrophy, and eventually on disease prognosis (development of RV failure, not addressed in the current study). What is precise mechanism of the effects of 2ME on hypoxic polycythemia is unknown. Yet, hypoxia via HIF1 α -VEGF axis is a major controlling factor of erythropoietin synthesis and erythropoiesis. 2ME is an VEGF antagonist and downregulates HIF1 α [12, 20] and thereby, should be expected to decrease hypoxia-induced erythropoiesis. 2ME also inhibited hypoxia-induced pulmonary vascular remodeling, i.e., reduced the media thickness and adventitia widening of small pulmonary arteries. This is consistent with our previous reports of inhibition of vascular remodeling by 2ME in monocrotaline- and bleomycin-induced PH [7-9, 27, 28].

Third, pretreatment with 2ME attenuated ANTU-induced lung injury and associated with single injection of this pneumotoxin. ANTU is an organosulfur compound and rodenticide specifically toxic in the endothelium of pulmonary capillaries and venules, which leads to increased permeability micro-leakage, pleural effusion and edema, increased vascular reactivity and pulmonary hypertension [29-31]. Pretreatment with 2ME had no effect of lungs weigh, yet significantly reduced pleural effusion and more importantly eliminated the 33% acute mortality rate produced by ANTU. This data are in accordance with reported vasoprotective effects of 2ME in systemic circu-

lation, as well as in altered precapillary vessels in PH [10].

Finally, 2ME treatment attenuated hemodynamic and pathological PH induced by weekly injections of ANTU. This study suggests that 2ME, a major non-estrogenic metabolite of estradiol, may have protective effects in PH induced by acute lung injury.

Altogether, the presented data and above discussion provide strong impetus for further evaluation of potential therapeutic effects of 2ME in PH. The efficacy and safety of 2ME have not been tested in PH patients. Nonetheless, in numerous phase I and phase II clinical trials conducted in patients with various solid malignancies 2ME was well tolerated and safe in doses up to 3 g/day [32]. Unfortunately, due to its low solubility and high pre-systemic metabolism (glucuronidation), to achieve effective concentrations large doses 2ME were used. In clinical studies, low bioavailability (1-2%) was reflected with very high inter- and intra-patient variability [32].

Notably, in the present study and in all our previous experimental studies (reviewed in references 10-12 and 18) therapeutic effects of 2ME were achieved by much lower parenteral doses (240 μ g/kg/day via osmotic minipump) and in the absence of any estrogenic effect. Noteworthy, this parenteral dose produces high physiological concentrations of 2ME (~3 ng/ml) similar to levels observed during the last trimester of pregnancy [33]. Our dose-escalating study in monocrotaline-induced PH suggests that parenteral administration substantially improves bioavailability and disposition of 2ME and further increase of the dose (although do not additionally reduce PH and RV hypertrophy) does further inhibit media remodeling and inflammation [27]. Similarly, in contrast to oral administration in healthy volunteers and cancer patients, we have shown that in healthy male volunteers, subcutaneous administration of a long-acting formulation of 2ME in doses up to 10 mg produced blood levels of 2ME >1 ng/ml over a 3-week period, with no estrogenic or other adverse effects reported [34]. As several parenteral formulations of 2ME with supposedly high bioavailability are currently under investigation [35, 36], clinical evaluation of 2ME in PH could be expected to take place in the near future.

CONCLUSIONS

This study demonstrates that 2ME has beneficial effects in chronic hypoxia- and acute lung injury-induced PH and further provides preclinical justification for clinical evaluation of 2ME in pulmonary hypertension, a deadly disease that predominantly develops in women. Based on its biological and pharmacological properties, 2-methoxyestradiol could be viewed as a disease modifier in pulmonary arterial hypertension.

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Резиме**2-МЕТОКСИЕСТАРДИОЛ ГО НАМАЛУВА РАЗВОЈОТ И ЈА ЗАБАВУВА ПРОГРЕСИЈАТА НА ХИПОКСИЈА-И АЛФА-НАФТИЛТИОУРЕА-ИНДУЦИРАНА БЕЛОДРОБНА ХИПЕРТЕНЗИЈА**

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Белодробната артеријска хипертензија (БХ) е прогресивно, неизлечиво и смртоносно заболување, кое преобладава кај жените. Растечки број на докази сугерира дека нарушувањето во метаболизмот на естрогените хормони влијае врз развојот на БХ и дека дел од биолошките ефекти на естардиол се должат на 2-метоксиестардиол (2МЕ), главен неестроген метаболит на естрадиолот. Целта на овој труд беше да ги испитае ефектите на 2МЕ кај хроничната хипоксија (ХХ) индуцирана БХ и алфа-нафтилтиуреа (АНТУ)-индуцирано акутно белодробно оштетување и БХ. Покрај тоа, ние ги испитавме ефектите на две нивоа на ХХ врз развивањето на БХ. Хроничната изложеност на 15 % или 10 % на кислород доведе до слично покачување на максималниот притисок во десната комора (МПДК), ремоделирање на белодробните крвни садови и до покачување на хематокритот, зависно од концентracијата на кислород. Забележително, хипертрофијата на десната комора (ДК) корелираше со концентracијата на кислородот и хематокритот, но не со големината на МПДК. Тоа сугерира дека, покрај притисочното оптоварување (afterload), хипоксијата (преку покачен хематокрит) значително придонесува за хипертрофија на ДК кај модел на хипоксична БХ. Кај белодробнохипертензивните стаорци, превентивниот и куративниот третман со 2МЕ го намали зголемениот МПДК и ремоделирањето на белодробните крвни садови. Споредено со превентивниот третман, куративниот третман со 2МЕ беше поефикасен во однос на намалувањето на хематокритот и хипертрофијата на ДК. Единечна инјекција на АНТУ доведе до белодробно оштетување, т.е., ја зголеми тезината на белите дробови и предизвика плеурален излив. Третманот со 2МЕ го намали плеуралниот излив и, што е уште позначајно, ја отстрани акутната смртност предизвикана од АНТУ (33 % наспроти 0 %, АНТУ наспроти АНТУ+2МЕ група). Хроничниот третман со АНТУ доведе до зголемување на тежината на белите дробови, БХ, хипертрофија на десната комора и белодробни васкуларни оштетувања. 2МЕ значително ја намали сериозноста на оштетувањата (т.е. ги намали МПДК, ДК хипертрофија и ремоделирање на белодробните крвни садови). Оваа студија покажува дека 2МЕ има полезни ефекти кај хроничната хипоксија- и акутно белодробно оштетување-предизвикана БХ, и изнесува додатни предклинички податоци за потреба од клиничка евалуација на 2МЕ во белодробната хипертензија.

Клучни зборови: белодробна хипертензија, хематокрит, 2-метоксиестрадиол, белодробно оштетување