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2-Methoxyestradiol Attenuates Angiotensin II-Induced Hypertension, Cardiovascular Remodeling and Renal Injury

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Abstract

Estradiol may antagonize the adverse cardiovascular effects of angiotensin II (Ang II). We investigated the effects 2-methoxyestradiol (2-ME), a non-estrogenic estradiol metabolite, on Ang II-induced cardiovascular and renal injury in male rats. First, we determined the effects of 2ME on Ang II-induced acute changes in blood pressure, renal hemodynamics and excretory function. Next, we investigated the effects of 2-ME and 2-hydroxyestardiol (2-HE) on hypertension and cardiovascular and renal injury induced by chronic infusion of Ang II. Further, the effects of 2ME on blood pressure and cardiovascular remodeling in the constricted aorta (CA) rat model and on isoproterenol-induced (ISO) cardiac hypertrophy and fibrosis were examined. 2-ME had no effect on Ang II-induced acute changes in blood pressure, renal hemodynamics or glomerular filtration rate. Both 2-ME and 2-HE reduced hypertension, cardiac hypertrophy, proteinuria, and mesangial expansion induced by chronic Ang II infusions. In CA rats, 2-ME attenuated cardiac hypertrophy and fibrosis and reduced elevated blood pressure above the constriction. Notably, 2ME reduced both pressure-dependent (above constriction) and pressure-independent (below constriction) vascular remodeling. 2-ME had no effects on ISO-induced renin release, yet reduced ISO-induced cardiac hypertrophy and fibrosis. The present study shows that 2-ME protects against cardiovascular and renal injury due to chronic activation of renin-angiotensin system. This study reports for the first time that in vivo 2-ME reduces trophic (pressure-independent) effects of Ang II and related cardiac and vascular remodeling.

INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) has crucial role in pathogenesis of hypertension and cardiovascular and renal disease. Abrogation of the excessive activity of RAAS reduces cardiovascular (CVD) morbidity and mortality, and therefore, RAAS

inhibition has evolved into a cornerstone pharmacotherapy of hypertension, CVD and chronic kidney disease (CKD)¹.

Sex differences in primary hypertension, CVD and CKD are well established. Regardless of race or ethnicity, women have lower blood pressure than men do, and these differences are observed across different species and animal models of hypertension^{2–4}. Furthermore, similar to CVD, the incidence and prevalence of CKD is higher in men than in women⁵ and the rate of progression of CKD is more rapid in men than in women^{5, 6}. Similarly, sex differences are seen in experimental animals in regard to consequences of excessive activity of RAAS; 17- β estradiol, a major female hormone, attenuates angiotensin-II-induced hypertension, and cardiovascular and renal injury in rodents^{3, 7, 8}.

There is a line of evidence that not only estradiol, but also its major metabolites products of 2-hydroxylation pathway, i.e., 2-hydroxyestradiol and 2-methoxyestradiol, may provide cardiovascular and renal protection. Accumulating evidence indicates that, at least in part, the protective effects of estradiol are mediated by these metabolites^{9, 10} and that estradiol metabolism may play significant role in development of vascular disease, including eclampsia and pulmonary hypertension^{11–13}. Finally, most recent studies suggest that estradiol metabolism may modulate angiotensin-II induced hypertension and kidney injury^{14, 15}.

The goal of this study was to investigate the effects of 2-methoxyestradiol (2-ME), a major non-estrogenic metabolite of estradiol, on angiotensin II-induced cardiovascular and renal injury in male rats. This study provides in vivo evidence that in male rats, in three models of angiotensin-II induced cardiovascular and renal injury, 2-ME exerts significant cardiovascular and renal protection and 2-ME modulates trophic (pressure-independent) effects of angiotensin II.

MATERIALS AND METHODS

Animals

Ninety, 12-week-old male Sprague Dawley rats were used in this study. The animals were housed in the University of Pittsburgh Medical Center animal care facility (temperature, 22 °C; light cycle, 12 hours; relative humidity, 55%). The rats were fed Pro Lab RMH 3000 rodent diet (PMI Nutrition Inc., St Louis, MO) and were given water *ad libitum*. Institutional guidelines for animal welfare were followed, and the Institutional Animal Care and Use Committee approved experimental protocols.

Protocol I: Effects of 2-Methoxyestradiol on Angiotensin II-induced Acute Changes in Blood Pressure and Renal Hemodynamics and Excretory Function

Male, 12-week old Sprague-Dawley rats (n = 8 per group) were anesthetized (pentobarbital 50mg/kg, i.p.) and instrumented for measurements of blood pressure and renal hemodynamics and excretory function as described previously¹⁶. Next, an infusion of [14C] inulin (0.035 mCi/20 mL saline/min) was initiated. Animals also received an intravenous infusion of either saline (50 μ L/min, control groups), or 2-methoxyestradiol (10 μ g/kg/h; 2-ME group). After 90 minutes, a 30-minute urine collection was conducted and a midpoint

blood samples was taken, and blood and urine [^{14}C]- inulin was measured, and renal clearance of [^{14}C] inulin was calculated as an estimate of glomerular filtration rate (GFR). A midpoint 5-minute average for mean arterial blood pressure (MABP) and renal blood flow were recorded and used to calculate renal vascular resistance (RVR). Three additional 30-minute clearance periods were conducted in the presence of increasing doses of Angiotensin II (3, 30 and 300 pmoles/min).

Protocol II: Effects of 2-Methoxyestradiol and 2-Hydroxyestradiol Angiotensin II-Induced Hypertension and Renal Injury

Thirty-six, male, 12-week old Sprague Dawley rats were used in this protocol. Animals were randomly implanted with osmotic minipumps containing saline (Control; n=10) or angiotensin II (Ang II, 200 ng/min; n=26). A subsets of animals receiving Ang II were also treated with either 2-hydroxyestradiol (10 $\mu\text{g/kg/h}$, via osmotic mini-pumps AngII+2HE group; n=8;), 2-methoxyestradiol (10 $\mu\text{g/kg/h}$, Ang II+ 2ME group; n=8;), or vehicle (PEG-400 2.5 $\mu\text{l/h}$, Ang II group; n=10).

Seven and fourteen days into the treatments, animals were placed in metabolic cages and allowed to acclimatize for two days. Twenty-four hour measurements of urine volume, and food and water intakes were conducted; urine samples were analyzed and 24-hour urinary creatinine, and protein excretion were calculated. A tail vein blood samples were also taken for measurement of plasma creatinine. Plasma and urine samples were analyzed for creatinine concentrations using a creatinine analyzer (Creatinine Analyzer 2, Beckman Instrument, Inc., Fullerton CA, USA), respectively. Total urine proteins were measured by a spectrophotometric assay using bicinchoninic acid reagent (Pierce; Rockford, IL)

After 14 days of treatment, animals were anesthetized with pentobarbital (45 mg/kg i.p) and instrumented for measurements of renal hemodynamics and excretory function as described in Protocol I. Animals were euthanatized by anesthetic overdose and kidneys and hearts were removed and weighed.

One kidney was fixed in 10 % formalin buffer for subsequent light microscopy and immunohistochemistry. The second kidney was quick frozen in liquid nitrogen and stored at -70°C for immunohistochemistry. The kidney tissue sample was sectioned and was processed into paraffin blocks for light microscopy. Five-micron tissue sections from formalin-fixed, paraffin-embedded renal cortices were dewaxed and stained with Periodic Acid Schiff (PAS) stain for histological assessment.

Protocol III: Effects of 2-Methoxyestradiol on Blood Pressure and Cardiac and Vascular Remodeling in Constricted Aorta Rat Model

Experiments were carried out in twenty-eight, male, 12-week old, Sprague Dawley rats. The animal was anesthetized with pentobarbital (50mg/kg/i.p.) and median laparotomy was performed, the abdominal aorta was exposed and a silk ligature (4/0) was placed around the aorta below the right and above the left renal artery. A 1.0 mm blunt steel wire was placed next to the aorta, and the ligature was tied tightly around the wire and the aorta. Then, the wire was removed, allowing the aorta to re-expand inside the ligature and the abdominal wall was closed. Aged-matched control animals underwent a median laparotomy and the

abdominal aorta was isolated but not constricted (shame operated; Control, n=6). A subset of constricted aorta rats (CA group, n=11) and control animals were implanted with osmotic minipumps (2ML1, ALZET, DURECT Corporation, Cupertino, CA) delivering vehicle (PEG-400, 10 µl/h), whereas the remaining CA rats were treated with 2-methoxyestradiol, 10µg/kg/h via osmotic mini pumps (CA+2ME group, n=11). After 8 days, animals were anesthetized, and blood pressure was measured in carotid and femoral artery.

Animals were euthanized with an overdose of pentobarbital. The abdominal aorta, heart and kidney were removed and stored in 10% formalin buffer for morphometric and immunohistochemical analysis. Tissues were processed into paraffin blocks for light microscopy. Two histological sections (3–5 microns thick) were cut and stained with hematoxylin-eosin (H&E) and trichrome. Histopathology scores were assessed semi quantitatively as 0 (absent), 0.5 (trace), 1 (mild), 2 (moderate), and 3 (severe) for heart histopathology, including vascular thickening and perivascular fibrosis, and myocardial fibrosis and ischemic and degenerative changes. The Aperio ScanScope XT system (Aperio Inc., Vista, CA, USA) was used to scan entire glass slides at 20x or 40x magnification and Aperio Image Scope software was used to analyze digital slides.

To assess vascular remodeling below and above aortic constriction, aortic 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 vessel wall and media thickening were measured using morphometric methods and digital planimeter on the SAMBA 4000 image analyzer.

Protocol IV: Effects of 2-Methoxyestradiol on Isoproterenol-Induced Cardiac Hypertrophy and Fibrosis

A total of 18 male Sprague Dawley rats (b.w. 314 ± 2 g) were randomized into three groups. Under halothane anesthesia in control animals (n = 6) an osmotic minipump (model 2001, Alzet) containing the vehicle for isoproterenol (1 µl/h of 0.002 N HCl) and another minipump (model 2ML1, Alzet) delivering the vehicle for 2-methoxyestradiol (PEG 400, 10 µl/h) were implanted. The remaining animals were implanted with minipump containing isoproterenol dissolved in 0.002 N HCl (2.0 mg/kg/day; ISO group n=12). A subset of ISO rats were also implanted with second minipump containing 2-methoxyestradiol (10 µg/kg/h, ISO+2ME group). Seven days after osmotic minipumps were implanted, animals were anesthetized (pentobarbital 45 mg/kg. i.p.) and instrumented for blood pressure and heart rate measurement. Briefly, a PE-50 catheter connected to Digi-Med Blood Pressure Analyzer (BPA 200, Micro-Med Inc., Louisville, KY) was inserted into right carotid artery, and blood pressure and heart rate recorded for 20 min. Blood samples were taken, stored at -20°C and later analyzed for plasma renin activity (PRA). Animals were sacrificed by overdose anesthetic. Total heart, left ventricle + septum, and right ventricle weights were recorded and normalized by body weight and brain weight. Measurement of hydroxyproline content in heart was determined by the method of Reddy and Enwemeka¹⁷. Plasma renin activity (PRA) was measured using the Angiotensin II25 RIA kit (Du Pont, NEN Research Products, Boston, MA) with appropriate modifications and validations¹⁸.

Statistical Analyses

All data are presented as mean $\pm$ S.E.M. Statistical analyses were performed using the Number Cruncher Statistical software program (Kaysville, Utah). Group comparison for data from metabolic studies (repeated measurements) were performed by one- (1F) and two (2F) hierarchical analysis of variance (ANOVA) as appropriate, followed by Fisher's LSD test for post-hoc comparison. Comparison of data from acute experiments and from histological analysis (single point data) was performed by 1F-ANOVA (three or four groups) or Student's t-test. The probability value of $p < 0.05$ was considered statistically significant.

RESULTS

The effects of 2-ME on acute changes in MABP, RBF, RVR and GFR induced by increasing doses of Angiotensin II are presented in Figure 1. As expected Ang II in a dose-dependent manner increased MABP and RVR, reduced RBF and had no effects on GFR. Notably, 2-ME had no effects on baseline and Ang-II induced changes in MABP, RVR and RBF and GFR.

Chronic 14-day infusion of sub pressor doses of Ang II resulted with development of hypertension; Ang-II significantly increased systolic (SBP), mean (MABP) and diastolic (DBP) blood pressure (Figure 2A) and induced significant cardiac hypertrophy (Figure 2B). Both, 2-ME and its metabolic precursor 2-HE attenuated Ang II-induced hypertension and cardiac hypertrophy (Figures 2A&B). Metabolic and renal excretory function measurements revealed no effect of Ang II on food consumption (table 1), yet Ang II increased fluid intake and urine volume, confirming the well-known (CNS mediated) dipsogenic effect of Ang II. 2HE, but not 2-ME reduced food and water intake and reduced urine volume (Table 1). Angiotensin II reduced GFR (i.e., creatinine clearance), and 2-HE and 2-ME had no effect on Ang II induced changes in renal excretory function (Figure 3A). Notably, Ang II induced proteinuria, and both 2-ME and 2-HE prevented Ang II of inducing proteinuria (Figure 3B). Histopathological analysis of kidney revealed increased mesangial expansion in Ang II treated animals compared to control rats (Figure 4A–C). More importantly, consistent with their ability to reduce proteinuria, 2-ME and 2-HE inhibited Ang II-induced mesangial expansion (Figure 4 D and E, respectively).

Constriction of abdominal aorta led to increase in blood pressure above constriction by ~ 50 mmHg, and had no effect on MABP below constriction (Figure 5). Notably, treatment with 2-ME significantly reduced MABP above constriction and had no effects on blood pressure below constriction. Increased blood pressure was associated with marked cardiac hypertrophy (Figure 6). Furthermore, cardiac hypertrophy in CA rats was associated with perivascular (Figure 7 E, F&J) and interstitial fibrosis (Figure 7 G–J) in the myocardium of the left ventricle. In CA group, in several animals myocardial fibrosis was accompanied by ischemic and degenerative changes (Figure 7 H&I). Notably, 2-ME treatment attenuated constricted aorta-induced cardiac hypertrophy (Figure 6) and perivascular and interstitial fibrosis (Figure 7K&L) and prevented development of ischemic/degenerative injury.

The effects of abdominal aorta constriction on media remodeling above and below constriction are presented in Figure 8. The banding of abdominal aorta and subsequent

increase in blood pressure above constriction resulted with marked cardiac hypertrophy and significant vascular remodeling above constriction, i.e., an increase in media thickness by ~30% (Figure 8 A and C). Although blood pressure was normal below constriction, moderate (~10%), but statistically significant increase in media thickness was detected below constriction. This blood pressure-independent remodeling was most likely due to trophic effects of Ang II. Notably, 2-ME attenuated both pressure-dependent vascular remodeling above constriction and pressure-independent remodeling below constriction (Figure 8A and D).

To further study the effects of 2-ME on Ang II induction of pressure-independent cardiac remodeling (i.e., modulation of trophic effects of Ang II), we examined the effects of 2-ME on isoproterenol-induced cardiac hypertrophy and fibrosis. A seven-day infusion of isoproterenol had no effect on systemic blood pressure, and increased heart rate and plasma renin activity (Figure 9, top panels). Isoproterenol induced cardiac hypertrophy, i.e., increased heart and left and right ventricle weight (Figure 9 mid panels) and induced cardiac fibrosis, as evidenced by increased hydroxyproline content (Figure 9, bottom panels). 2-ME had no effect on blood pressure, heart rate and elevated PRA, yet reduced isoproterenol-induced (pressure independent) cardiac remodeling and fibrosis.

DISCUSSION

In the present study, we examined the therapeutic effects of 2-ME, a major non-estrogenic metabolite of estradiol, on angiotensin II-induced cardiovascular and renal injury in male rats. 2-methoxystardiol was given in daily dose of 240 µg/kg (i.e., infusion rate of 10µg/kg/h) that we have shown to produce high physiological plasma concentrations of 2-ME (~2–3ng/ml) and has no estrogenic effects in male rats¹⁹.

2-ME had no effects on Ang-II induced acute changes in blood pressure and renal hemodynamics. This is consistent with our previous studies in normotensive and spontaneously hypertensive rats in which short-term (90–150 min) infusion of the same dose of 2-ME (10µg/kg/h) had no effect on systemic blood pressure and renal hemodynamics and excretory function¹⁶. The fact that 2-ME does not inhibit the acute hemodynamic and renal effects of Ang II suggests that 2-ME does not have antagonistic properties for Ang II receptors. Yet, 2-ME reduced elevated blood pressure induced by infusion of sub pressor dose of Ang II or by banding of abdominal aorta. This is consistent with previous reports of the effects of 2-ME on elevated blood pressure in rodents. In this regard, in our previous study in another rat model of high RAAS activity, i.e., hypertension and cardiovascular and renal injury induced by chronic treatment with nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine (LNNA)¹⁶, 2-ME not only reduced severely elevated blood pressure and cardiac and renal injury, but also reduced mortality by 83%. Furthermore, 2-ME was shown to prevent development and attenuates progression of hypertension in DOCA salt rats²⁰, aged and obese ZSF1 rats²¹, and in spontaneously hypertensive rats²².

In the current study, 2-ME prevented Ang II of inducing proteinuria and attenuated Ang-II induced mesangial expansion, an early sing of renal injury. These findings are consistent with previous report showing marked renoprotective effects of 2-ME in male rats treated

with NOS inhibitor LNNA. In this model of high RAAS activity, 2-ME prevented development of proteinuria and abolished LNNA-induced interstitial and glomerular inflammation, attenuated glomerular collagen IV synthesis, and inhibited glomerular and tubular cell proliferation¹⁶. Similarly, in aged (44 weeks old) male ZSF1 rats, a model of obesity, metabolic syndrome and severe kidney disease, 2-ME attenuates proteinuria, increases renal blood flow and glomerular filtration, and reduces renal expression of markers of proliferation, inflammation, and angiogenesis²¹. Finally, most recent study indicates that 2-ME reduces Ang II-induced hypertension and kidney injury in both intact and OXV female and male mice²³.

The infusion of sub pressor dose of Ang II for 14 days increased fluid intake, i.e., induced dipsogenic effect, well-established central effect of systemically administered angiotensin II. Proper control of fluid intake is an essential component of blood pressure regulation²⁴. Systemic infusion of ANG II in male rats increases phosphorylation of MAPK and expression of the Ang II type-1 receptor (AT1R) in both paraventricular nucleus and subfornical organ that are involved in the complex central regulation of blood pressure. Further, the increased expression of AT1R is blocked by p44/42 MAPK inhibition, suggesting that the central RAAS effects on blood pressure require MAPK activation²⁵. Although 2-ME inhibits p44/42MAPK activation²⁶ and downregulates the expression of AT1R^{27–29}, in the current study 2-ME did not affect Ang II-induced dipsogenic effect. However, in recent study in male mice, 2-ME not only reduced Ang II-induced increase in blood pressure, but also attenuated Ang II-induced increase in water intake and urine output in male mice²³. Opposite to 2-ME, its metabolic precursor 2HE attenuated Ang II-induced increase in water intake, which is consistent with inhibitory effects of estradiol on Ang II-induced increase in fluid intake in ovariectomized female rats³⁰. Whether 2-ME inhibits the central effects Ang II on thirst and how is this related to the effects of 2-ME on Ang II-induced hypertension need further clarification.

Chronic pressure overload induces cardiac fibrosis and apoptosis that exacerbates left ventricular remodeling and dysfunction. In the heart of constricted aorta rats, 2-ME markedly reduced cardiac hypertrophy and perivascular and interstitial fibrosis and prevented development of degenerative/ischemic changes that were seen in some CA rats. This is consistent with previously reported effects of 2-ME on perivascular and interstitial inflammation and reduced collagen synthesis in myocardium of NOS deficient male rats¹⁶. Notably, 2-ME or its metabolic precursor 2HE, inhibits collagen synthesis and exerts anti-fibrotic effects not only in vitro⁹, but also in models of renal³¹, systemic^{32, 33} or pulmonary fibrosis³⁴. Some of the injurious cardiovascular and renal effects of Ang II may be induced by aldosterone and not directly by AngII. It should be noted that in OVX rats, estradiol reduces adrenal expression of AT1 receptors and Ang II-induced secretion of aldosterone³⁵. Aortic constriction- and isoproterenol-induced cardiac hypertrophy are associated with increased aldosterone secretion^{36–38}. Furthermore, aldosterone receptor antagonists inhibit isoproterenol-induced cardiac hypertrophy, fibrosis (including collagen-1 accumulation) and related inflammation, oxidative stress and diastolic dysfunction^{37, 38}. Because it appears that 2-ME attenuates the expression of AT₁ receptor, it is plausible that marked anti-fibrotic effects of 2-ME seen in all three models in the current study were due, at least in part, to reduced aldosterone secretion. Currently, the effects of 2-ME on aldosterone secretion in

high RAAS activity states are unknown and future studies will be needed to clarify this issue.

Renin-angiotensin system plays critical role in development of pressure-overload LV hypertrophy and vascular remodeling in rats with abdominal aortic constriction³⁹. Both in vitro and in vivo studies suggest that trophic responses to increased afterload (i.e., increased stretch and diastolic wall stress) involve Ang II^{40, 41}. In constricted aorta rats, 2-ME attenuated pressure overload-induced left ventricular hypertrophy. The effects of 2-ME on elevated blood pressure above constriction could potentially contributed to its antihypertrophic effects and inhibition of vascular remodeling above the constriction. More importantly, 2-ME also reduced pressure-independent vascular remodeling below constriction. The later indicates that 2-ME may modulate trophic effects of Ang II. To study this possibility, we examined the effects of 2-ME in male rates with isoproterenol-induced cardiac hypertrophy, yet another model of increase RAAS activity⁴². 2-ME had no effect on isoproterenol-induced increase in plasma renin activity, heart rate and normal systemic blood pressure, but reduce isoproterenol-induced cardiac hypertrophy and fibrosis. Altogether, this data provide evidence that 2-ME may modulate the trophic (pressure-independent) effects of Ang II and thereby offer vasoprotection. In this regard, 2-ME reduces atherosclerotic lesions in female Apo lipoprotein E deficient mice⁴³, induces vasodilation by stimulating NO release⁴⁴ and inhibits neointima formation²⁶.

Although in the present study 2-ME did not block acute hemodynamic and renal effects of Ang II, the cumulating data and present study suggest that 2-ME could be viewed as biological antagonist of Ang II. In this regard, 2-ME downregulated Ang II type I (AT1) receptor^{27–29, 45}. Furthermore, deficiency in COMT, a key enzyme for conversion of catechol estrogen 2-HE into 2-ME, leads to hypersensitivity to pressor effects of Ang II and exacerbates Ang-II induced renal injury. Notably, 2-ME abolishes hypersensitivity to Ang II in COMT deficient mice²⁸

The mechanism by which 2-ME attenuates adverse effects of Ang II in the kidney, heart and vascular and isoproterenol-induced cardiac hypertrophy and fibrosis remains unknown. In vitro, 2-ME is a potent anti-mitogen and inhibits migration, proliferation, and collagen synthesis of human and rat VSMCs, proliferation and collagen synthesis in rat cardiac fibroblasts and mesangial cell proliferation, migration, and extra-cellular matrix production⁹. In vivo, 2-ME inhibits injury-induced neointima formation and blocks SMC proliferation by inhibiting key pathways involved in cell growth²⁶. The effects of 2-ME on cardiovascular and renal cells are independent of classical nuclear estradiol receptors. The parent hormone, 17-beta estradiol produces its actions mainly via estrogen receptors. Recently G protein-coupled estrogen receptor (GPER), located on the cell membrane and endoplasmic reticulum, was discovered in the hearts and vasculature and activation of this receptor has been linked to cardiac and vascular protection in male and female rats⁴⁶. Furthermore, activation of GPER attenuates Ang II-induced hypertrophy of cardiomyocytes and left ventricular remodeling caused by ovariectomy in female mRen.Lewis rats⁴⁷, inhibits cardiac fibroblasts proliferation⁴⁸ and is involved in vascular remodeling⁴⁹. GPER has also been implicated in cardiovascular effects of 2-ME. In this regard, recently it has been reported that 2-ME binds the membrane GPER and, via GPER mediated transactivation of EGFR and

ERK1/2 phosphorylation, it downregulates AT₁ receptor^{27, 29}. Yet, the ability of 2-ME to inhibit the proliferation of BV2 microglia cells and VSMCs was shown to be independent of GPER or nuclear estrogen receptors⁵⁰. Whether protective effects of 2-ME against Ang II-induced injury are mediated by GPER remains to be determined.

Our initial studies in several rats models of cardiovascular and renal disease, indicated that estradiol metabolites (product of 2-hydroxylatoxin pathway) have cardiovascular and renal protective effects^{16, 21, 31, 51} and that 2-ME mediate protective effects of E2 in experimental pulmonary hypertension^{10, 34}. In the past eight years, growing body of evidence supports the notion that estradiol metabolism not only influence the development of pulmonary hypertension¹², but may also play role in cardiovascular and renal protection. Several recent study from Dr. Malik lab clearly showed that estradiol metabolism and Cyp1B1 activity influence the effects of Ang II to produce cardiovascular and renal injury^{14, 15, 52, 53}. Since the inception of the current study, two reports highly relevant to cardiovascular protection of 2-ME against Ang II-induced injury have been published. First, Dr. Malik's group report suggests that Cyp1B1 may play critical role in production of 2-ME and cardioprotective effects of this non-estrogenic estradiol metabolite against Ang-II induced injury. In this regard, Ang II induces oxidative stress, endothelial dysfunction and cardiovascular and renal injury in Cyp1B1^{-/-}, but not in Cyp1B1^{+/+} female mice, these changes coincide with reduced 2-ME production in Cyp1B1^{-/-} mice and 2-ME treatment of these animals abolishes Ang-II-induced oxidative, vascular and renal injury²³. Furthermore, 2-ME and its metabolic precursor 2-HE protects against Ang II-induced hypertension in CYP1B1^{-/-}, but not in CYP1B1^{+/+} female mice¹⁴, suggesting that Cyp1B1 protects against Ang II-induced cardiovascular injury. However, CYP1B1 effects on Ang II-induced injury are sex specific, as CYP1B1 gene disruption minimizes Ang-II induced injury⁵². Second recent study suggests that antihypertrophic effects of 2-ME in male rats with pressure overload-induced LV hypertrophy are associated with a significant inhibition of CYP 1B1 activity⁵⁴.

Whether CYP1B1 is an important regulator of 2-ME production in healthy people and patients with cardiovascular disease need further investigation. Initial data indicated that CYP1A1 predominantly control the conversion of E2 to catechol estrogen 2-HE that is converted to 2-ME by COMT, whereas human CYP1B1 forms predominantly 4-hydroxyestradiol (4-HE), a metabolite that is carcinogenic in animal models⁵⁵. In contrast to human CYP1B1 that favors four-fold greater production of 4-HE versus 2-HE, the rat CYP1B1 favors two-fold production of 2-HE compared to 4-HE⁵⁶. In vitro, 2-ME exerts feedback inhibition of CYP1B1 and CYP1A1 activity⁵⁷. Furthermore, in MCF-7 breast tumor epithelial cells that do not constitutively express CYP450 enzymes that metabolize E2 to catechol estrogens, 2-ME inhibits induction of CYP1B1 by 2,3,7,8-tetrachloro dibenzo-p-dioxin and production of downstream reactive metabolites and related oxidative stress. Noteworthy, in addition playing crucial role in metabolism of catechol estrogens, CYP1B1 also metabolizes arachidonic acid into mid-chain hydroxyeicosatetraenoic acids (HETEs) and to a lesser degree into terminal HETEs and epoxyeicosatrienoic acids (EETs). Cytochrome P450 1B1, expressed in vascular smooth muscle cells, can metabolize arachidonic acid in vitro into several products including 12- and 20-hydroxyeicosatetraenoic acids that stimulate vascular smooth muscle cell growth⁵⁸. Noteworthy, CYP1B1 inhibition attenuates doxorubicin-induced heart failure and cardiac fibrosis, as well as formation of

mid-chain HETE formation⁵⁹. It should be mentioned that in vitro feedback inhibition on cytochrome P450 1A1 and 1B1 by 2-ME occurs at micromolar concentrations (10–30 μ mol)⁵⁷. More importantly, anti-hypertrophic effects of 2-ME in pressure overload-induced cardiac remodeling is associated with significant inhibition of CYP1B1 and reduced formation of mid-chain HETE⁵⁴. Whether 2-ME inhibition of CYP1B1 leads to elevated E2 and 2-HE levels and ER activation, as well as reduced production of cardioprotective EETs is unknown.

In summary, this study confirms cardiovascular and renal protective effects previously reported by others and us and provide the in vivo evidence that in addition to pressure-dependent adverse effects of Ang II, 2-ME inhibits trophic (pressure-independent) effects of Ang II.

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Figure 1.

The effects of 2-methoxyestradiol (2-ME) on angiotensin-II-induced acute changes in mean arterial blood pressure (MABP), renal blood flow (RBF) and vascular resistance (RVR), and glomerular filtration rate (GFR).

Figure 3.
The effects of 2-hydroxyestradiol (2HE) and 2-methoxyestradiol (2ME) on creatinine clearance (A) and urinary protein excretion (B) in rats with angiotensin II-induced renal injury.

Figure 4. The effects of 2-hydroxyestradiol (2HE) and 2-methoxyestradiol (2ME) on mesangial expansion in rats with angiotensin II-induced renal injury (A). Control animal showing very low dense matrix in the mesangium (B), angiotensin II treated animal showing very high dense matrix in the mesangium (C) and Ang II+2ME (D) and AngII+2HE (E) rats showing very low to moderate dense matrix in the mesangium, respectively; (PAS, x400 magnification).

Figure 5.
The effects of 2-methoxyestradiol (2ME) on blood pressure in rats with constricted aorta (CA) (Fisher's LSD test $p<0.05$: * - vs Control, a - vs CA+2ME)

Figure 6.
The effects of 2-methoxyestradiol (2ME) on constricted aorta (CA)-induced cardiac hypertrophy (Fisher LSD test p<0.05: * - vs Control, a – vs. CA+2ME)

Figure 7.

The effects of 2-methoxyestradiol (2ME) on cardiac and vascular remodeling in rats with isoproterenol-induced cardiac hypertrophy. Control animal showing very low perivascular and myocardial fibrosis (**A-D**); Isoproterenol treated animal showing perivascular (**E,F,J**) and myocardial fibrosis (**G&I**) and ischemic and degenerative changes (**H&I**); 2ME treatment attenuated perivascular (**K**) and interstitial fibrosis and prevented development of ischemic/degenerative injury (**L**).

Figure 8.

The effects of 2-methoxyestradiol (2ME) on media hypertrophy in aorta above and below constriction (A). Representative photos of abdominal aorta media thickness above constriction from control, sham-operated animal (B), constricted aorta [CA] rat (C) and CA rat treated with 2-methoxyestradiol (D).

Figure 9.

The effects of 2-methoxyestradiol on isoproterenol (ISO)-induced changes in heart rate, blood pressure and plasma renin activity (top panels) and cardiac hypertrophy (mid panels) and fibrosis (bottom panels); □-Control, ■-ISO and ▨-ISO+2ME group.

Table 1:

Metabolic and renal excretory function measurements

Treatment	N	Study period	Body Weight	Food intake	Fluid Intake	Urine volume
			g	gr/kg/24h	ml/kg/24h	ml/kg/24h
65+Control	10	7 days	419	65.2	128.7	58.6
			8	1.9	8.2	5.0
Ang II	10	7 days	370 ^a	56.9	178.3 ^a	134.1 ^a
			15	5.1	30.0	25.3
Ang II+2ME	8	7 days	351 ^{a,b}	48.6 ^a	171.0 ^a	119.2 ^a
			8	5.9	21.1	13.8
Ang II+2OH	8	7 days	350	42.4 ^{a,b}	132.3	101.2
			13	4.4	11.8	11.7
Control	10	14 days	435	57.9	119.5	56.5
			7	1.8	10.1	6.0
Ang II	10	14 days	392 ^a	65.7	180.4 ^a	109.5 ^a
			10	4.6	16.8	12.7
Ang II+2ME	8	14 days	368 ^{a,b}	67.1	214.7 ^a	122.0 ^a
			8	3.7	22.2	19.7
Ang II+2OH	8	14 days	358 ^{a,b}	50.2 ^c	132.5	90.1 ^a
			16	4.3	16.4	13.0
2F-ANOVA						
(A) Treatment		p<	0.001	0.018	0.024	0.008
(B) Time		p<	0.001	0.028	0.315	0.192
A x B Interaction		p<	0.069	0.035	0.206	0.459

Fisher's LSD test: p<0.05,

^a - vs Control;^b - vs Ang II;^c - vs Ang II & Ang+2ME