Catechol-O-methyltransferase deficiency leads to hypersensitivity of the pressor response against angiotensin II

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Running title: Angiotensin II Hypersensitivity in COMT Deficiency

Word count of manuscript: 6592

Word count of abstract: 248

Total number of figures: 6

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Abstract

Catechol-O-methyltransferase (COMT) metabolizes 2-hydroxyestradiol into 2-methoxyestradiol (2-ME); COMT deficiency has shown to be associated with hypertension in men and preeclampsia, the disease associated with hypersensitivity of pressor response against angiotensin II (Ang II). Here we found COMT deficiency could explain the hypersensitivity of pressor response against Ang II in mice due to the lack of 2-ME dependent suppression of AT1R. Male C57BL/6 mice were subjected to COMT inhibitor (COMTi: 25 mg/kg/day) or oil (Control) for 4 weeks, with or without low-dose Ang II infusion (ANGII: 70 ng/kg/min) for the last 3 weeks. The Ang II-infused mice were treated with 2-ME (10 ng/day) or vehicle for the last 1 week. We obtained the following experimental groups: Control, ANGII, COMTi, COMTi+ANGII, and COMTi+ANGII+2-ME. We performed similar experiments utilizing the in vivo administration of siRNA of COMT instead of COMTi. Neither ANGII nor COMTi exhibited significant alterations in systolic blood pressure (SBP). Compared with ANGII or COMTi, COMTi+ANGII displayed significantly higher SBP, albuminuria and glomerular endotheliosis; 2-ME normalized such alterations. Similar phenotypes were observed in COMT siRNA-treated mice. In the aorta of COMT-deficient mice,
angiotensin II receptor type 1 (AT1R) expression was increased; 2-ME suppressed
AT1R expression. 2-ME exhibited peroxisome proliferator-activated receptor γ
(PPARγ) agonistic activity in vitro and ex vivo plasma from pregnant female mice as
well. In vitro, 2-ME suppressed both basal and Ang II-induced AT1R levels in a
PPARγ-dependent manner. 2-ME is relevant to combat COMT deficiency-associated
hypertensive disorders via suppressing of AT1R by its PPARγ activity.

Key words: COMT, 2-methoxyestradiol, hypersensitivity, angiotensin II,
preeclampsia

Non-standard Abbreviations and Acronyms

Ang II angiotensin II, COMT catechol-O-methyltransferase, 2-OHE2 2-
hydroxyestradiol, 2-ME 2-methoxyestradiol, sFlt-1 soluble fms-related tyrosine kinase-
1, VEGF vascular endothelial growth factor, PI GF placental growth factor, AT1R
angiotensin II receptor type1, VSMC vascular smooth muscle cell, AoSMC aortic
smooth muscle cell, PPARγ peroxisome proliferator-activated receptor γ
Introduction

Catechol-o-methyltransferase (COMT) is an enzyme known to inactivate catecholamines such as dopamine, adrenaline and noradrenaline. However, any compounds with a catechol structure are candidate substrates of COMT. Estradiol is metabolized to 2-hydroxyestradiol (2-OHE2) by cytochrome P450. 2-OHE2, one of the catechol estrogens, is converted to 2-methoxyestradiol (2-ME) by COMT. The maternal circulatory 2-ME levels increases toward the end of pregnancy; however, the plasma levels of 2-ME and placental COMT activity are significantly lower in women with severe preeclampsia.\textsuperscript{1-4} Functional Val158Met COMT polymorphism has shown to be associated with preeclampsia.\textsuperscript{5-9} In a large Norwegian cohort (HUNT2), a low-COMT activity haplotype was associated with recurrent preeclampsia.\textsuperscript{10} Indeed, we have shown that COMT-deficient pregnant mice exhibited preeclampsia-like phenotypes due to 2-ME defects.\textsuperscript{3} Not only the women’s diseases, COMT deficiency has been shown to associate with hypertension in men\textsuperscript{6,11}, acute coronary heart disease in men\textsuperscript{12}, and metabolic diseases\textsuperscript{7,13}. Therefore, to understand the mechanisms of vascular insult associated with COMT deficiency is quite important for human health.

The renin-angiotensin system (RAS) plays an important role in maintaining circulatory homeostasis, including gestational periods. Gant et al. published a seminal
report regarding the role of the RAS in the pathogenesis of hypertension during pregnancy.\textsuperscript{14} They analyzed the angiotensin II (Ang II) doses required to elicit a pressor response of 20 mmHg in diastolic pressure. Compared to non-pregnant women, normal pregnant women required a high dose of Ang II throughout the gestation periods. The mean required doses of Ang II for a pressor response in women who developed preeclampsia progressively declined compared with those in normotensive pregnant women, suggesting that women who developed preeclampsia have an enhanced pressor response against Ang II. In addition, women with preeclampsia had lower plasma levels of both renin activity and Ang II than normotensive pregnant women.\textsuperscript{14} These findings indicate that hypertension in preeclampsia is associated with hypersensitivity of the pressor response against Ang II.

From the rationale of COMT deficiency in hypertensive disorder and the mechanisms of hypertension in preeclampsia, we got the hypothesis that COMT deficiency is broadly relevant for the vascular insult in human disease via hypersensitivity of pressor response against Ang II.

**Methods summary**

Eight-week-old male C57BL/6 mice were subjected to COMT inhibitor Ro41-0960
(COMTi: 25 mg/kg/day intraperitoneal administration (i.p.)) or olive oil (Control) for 4 weeks, with or without low-dose Ang II infusion (ANGII: 70 ng/kg/min continuous subcutaneous infusion) for the last 3 weeks. The Ang II-infused mice were treated with 2-ME (10 ng/day i.p.), 2-OHE2 (10 ng/day i.p.), or vehicle for the last week. We performed similar experiments utilizing the in vivo administration of siRNA (siCOMT: 20 nmol/week i.p.) of COMT instead of COMT inhibitor. To obtain the plasma of pregnant mice, six-week-old female C57BL/6 mice were mated with male C57BL/6 mice. Beginning at day 10 of the pregnancy, the mice were injected with Ro41-0960 (25 mg/kg/day) with or without 2-ME (10 ng/day) or placebo subcutaneously. For non-invasive monitoring of blood pressure, mice were trained for at least five days before measurement of blood pressure. Urinary albumin and creatinine measurement were performed as previously described. Plasma Ang II and catecholamines, soluble fms-related tyrosine kinase-1 (sFlt-1), placental growth factor-2 (PIGF-2) concentrations were measured with an enzyme-linked immunosorbent assay system. Western blot analysis for COMT, angiotensin II type 1 receptor (AT1R), and RT-PCR for human AT1R were performed with standard methods. Electron microscopic analysis was performed by Hanaichi Ultrastructure Research Institute (Tokyo, Japan). Peroxisome proliferator-activated receptor (PPAR) luciferase activity was evaluated using the Dual-
Luciferase® Reporter Assay System (Promega). In vitro and ex vivo experiments were performed with human aortic smooth muscle cells (AoSMCs; LONZA), or human embryonic kidney 293-T cells (HEK293Ts) over-expressing hemagglutinin (HA)-tagged mouse AT1a receptor or transfected with pcDNA (kindly gifted by Dr. Junji Ishida and Dr. Akiyoshi Fukamizu, University of Tsukuba, Japan).

Results

COMT deficiency in mice exhibited hypersensitivity to angiotensin II

To evaluate the role of COMT deficiency in the sensitivity to Ang II, we tested the effect of COMT deficiency in mice on SBP and albuminuria with low-dose Ang II infusion (Fig. 1A). Compared to the Control group, neither the ANGII group nor the COMTi group displayed an alteration in SBP. In contrast, the SBP in the COMTi+ANGII group was significantly increased compared with the SBP in either the ANGII or COMTi group. The SBP in COMTi+ANGII was significantly decreased by the treatment with 2-ME but not by the treatment with 2-OHE2 (Fig. 1A). The albuminuria in COMTi+ANGII was significantly increased compared with the albuminuria in both ANGII and COMTi. 2-ME significantly reduced the levels of albuminuria in COMTi+ANGII (Fig. 1B). Ultrastructure analysis revealed that
COMTi+ANGII displayed glomerular endotheliosis characterized by glomerular endothelial swelling and occlusion of the lumen capillary. 2-ME ameliorated the glomerular endothelial morphology (Fig. 1C).

In normal male C57BL/6 mice, membrane-bound (MB)- and soluble (S)-COMT proteins were highly expressed in the liver compared with their levels in the kidney, heart and aorta (Fig. S1A). In the liver, siRNA-mediated gene silencing affected MB-COMT levels. In the kidney, the expression levels of MB- and S-COMT proteins were unaltered (Fig. S1B). The SBP in siCOMT+ANGII group was significantly increased compared with the SBP in both SCRsiRNA+ANGII and siCOMT group. The SBP in siCOMT+ANGII group was significantly decreased when the mice were treated with 2-ME (Fig. 2A). The albuminuria in mice treated with COMT siRNA exhibited similar trends; the magnitude of differences between the groups were less than that observed with COMT inhibitor administration (Fig. 2B).

No association of plasma sFlt-1, PI GF or sFlt-1/PlGF levels in COMT deficiency-associated hypertension induced by Ang II

sFlt-1, which is a soluble form of the vascular endothelial growth factor (VEGF) receptor, acts as an antagonist of VEGF and PI GF. In this regard, an excess level of
plasma sFlt-1 is hypothesized to contribute to the pathomechanism of hypertension in preeclampsia.\textsuperscript{15} However, in the COMT deficient mice treated with low-dose Ang II, the mouse plasma sFlt-1 and PlGF-2 were not correlated with their hypertension (Fig. 3A, B, S1C, D). In our experiment, neither sFlt-1, PlGF-2 nor the sFlt-1/PlGF-2 ratio could explain the hypertension in COMT deficiency.

**Plasma catecholamine levels were not altered in COMT-deficient mice**

COMT is an enzyme that degrades and inactivates catecholamines. COMT deficiency might be assumed to increase the levels of catecholamines. However, COMT deficiency did not significantly influence adrenaline and noradrenaline levels in plasma as reported elsewhere\textsuperscript{16} (Fig. 3C).

**COMT deficiency was associated with the induction of AT1R levels**

Women with preeclampsia displayed lower plasma Ang II levels when compared to normotensive pregnant women.\textsuperscript{14} Similarly, the plasma Ang II levels in both COMTi and siCOMT were significantly decreased compared with those of each control group (Fig. 4A). Both COMTi and siCOMT displayed normal blood pressure, suggesting that Ang II levels of COMT-deficient mice appeared to be suppressed for the homeostasis to
maintain blood pressure at normal levels. In preeclampsia, the vascular AT1R expression was induced to a greater extent than in normal pregnancy.\textsuperscript{17} To evaluate mechanisms for Ang II hypersensitivity, we examined whether AT1R expression in the aorta was induced in COMT deficiency. Several AT1R antibodies are available commercially, but have been reported to display non-specific binding in mouse tissues.\textsuperscript{18} To test the specificity of the antibody used in our experiments, we first performed western blot analysis in AoSMCs transfected with AGTR1 siRNA, and HEK293Ts over-expressing HA-tagged mouse AT1a receptor.\textsuperscript{19} AT1R belongs to the family of G-protein-coupled receptors which are known typical seven transmembrane domain receptors, and encodes 359 amino acids.\textsuperscript{20} The predicted molecular size of human and rodents AT1R form, which represent the non-glycosylated AT1R, is about 41 kDa. Whereas the glycosylated AT1R has a molecular mass of about 65-150 kDa.\textsuperscript{18,20} By western blot analysis, the antibody we used could detect around 41kDa bands in scramble siRNA transfected AoSMCs; the bands were suppressed in the AoSMCs transfected with AGTR1 siRNA (Fig. S2A). Furthermore, in HEK293Ts over-expressing HA-tagged mouse AT1R, the antibody detected higher levels of 41kDa bands when compared to HEK293Ts transfected with pcDNA3. Anti-HA antibody could detect the same band size (Fig. S2B). These data demonstrated that the antibody we used exhibited
highly specific to both human AT1R and mouse AT1Ra.

In both COMTi and COMTi+ANGII groups, AT1R protein levels in the aorta were increased compared to those in Control and ANGII groups; 2-ME suppressed aortic AT1R expression in COMTi+ANGII (Fig. 4B and S3A). In both the siCOMT and siCOMT+ANGII groups, AT1R protein levels in the aorta were significantly increased compared to those in the SCRsiRNA and SCRsiRNA+ANGII groups; 2-ME suppressed aortic AT1R expression in siCOMT+ANGII (Fig. 4C and S3B). These results suggested that mice with COMT deficiency exhibited Ang II hypersensitivity via the induction of AT1R expression in vessels; 2-ME suppressed such COMT deficiency-associated AT1R expression.

2-ME suppressed AT1R levels in cultured human smooth muscle cells

Ang II increases vasoconstriction via AT1R activation in vascular smooth muscle cells (VSMCs). In AoSMCs, compared with the control, Ang II significantly induced both AT1R protein and mRNA levels; 2-ME suppressed this induction of AT1R expression (Fig. 5A, B and S3C). COMT inhibitor significantly induced AT1R mRNA and protein levels in AoSMCs; 2-ME, but not 2-OHE2, significantly suppressed this increase in AT1R mRNA and protein levels (Fig. 5C and S3D).
2-ME suppressed AT1R levels in a PPARγ-dependent manner

2-ME has structural similarity with PPAR ligands\textsuperscript{23}, such as pioglitazone and rosiglitazone. 2-ME has been shown to activate PPARγ in VSMCs\textsuperscript{23,24}. In addition, a PPARγ activator was shown to suppress AT1R expression in VSMCs\textsuperscript{25,26}. In AoSMCs, 2-ME (100 nmol/L and 500 nmol/L) increased PPARγ activities to levels similar to those induced by the PPARγ agonist pioglitazone (30 μmol/L) (Fig. 6A). In AoSMCs, both 2-ME and pioglitazone suppressed both mRNA and protein levels of AT1R. The PPARγ antagonist GW9662 diminished the effects of 2-ME and pioglitazone on AT1R levels (Fig. 6B and S3E). These results suggested that 2-ME suppressed AT1R expression in a PPARγ-dependent manner.

The plasma from COMT-deficient male or pregnant mice exhibited less PPARγ transcriptional activity

PPARγ transcriptional activity was nearly doubled in cells treated with serum from normal pregnant women when compared to those from non-pregnant women; the serum from severe preeclamptic women exhibited reduced levels of PPARγ transcriptional activity compared with the serum from normal pregnant women\textsuperscript{27,28}. Additionally,
dominant negative mutations in human PPARγ were associated with the onset of preeclampsia.\textsuperscript{29} We found that reduced PPARγ transcriptional activity and increased AT1R protein levels in AoSMCs were induced by plasma from COMT inhibitor-treated male mice; these protein levels were diminished in plasma from male mice treated with COMT inhibitor plus 2-ME associated with the restoration of PPARγ transcriptional activity (Fig. 6C and S3F). COMT inhibitor-treated pregnant mice, the mice exhibited preeclampsia phenotype\textsuperscript{3}, exhibited low PPARγ transcriptional activity in plasma. Such low PPARγ transcriptional activity was associated with increased AT1R levels; 2-ME restored these levels (Fig. 6D and S3G).

**Discussion**

COMT deficiency led to the hypersensitivity to Ang II with increased AT1R expression; 2-ME suppressed AT1R expression in a PPARγ-dependent manner. Previously, we showed that COMT-deficient pregnant mice displayed a preeclampsia-like phenotype due to the absence of 2-ME.\textsuperscript{3} However, the pathomechanisms of pregnancy-induced hypertension in pregnant COMT-deficient mice remained unclear. In the present study, we found that COMT deficiency was indeed associated with hypersensitivity of the pressure response against Ang II. Jennings et al. reported that
cytochrome P450 1B1, which convert estradiol into 2-OHE2, protected higher dose (700 ng/kg/min) of Ang II-induced hypertension by 2-ME-inhibiting oxidative stress and the activity of these signaling molecules.\textsuperscript{30} Also, we confirmed that 2-ME ameliorated low-dose (70 ng/kg/min) Ang II-induced hypertension in COMT deficiency. Here, we found that COMT deficiency and the resultant lack of 2-ME lead to the induction of AT1R levels in vitro and in vivo. Furthermore, we found that 2-ME exhibited PPAR\textgreek{y} agonistic activity and that such PPAR\textgreek{y} transcriptional activity of 2-ME was important for the suppression of AT1R levels in both in vitro and ex vivo plasma experiments. 2-ME has shown to be elevated toward the end of pregnancy.\textsuperscript{2,3} These data clearly demonstrate the fundamental role of 2-ME in the homeostasis of blood pressure through the regulation of sensitivity against Ang II.

In normal pregnancy, plasma renin activity and Ang II levels are higher; however, the vascular responsiveness to Ang II appears to be reduced. In contrast, preeclamptic women exhibited lower levels of plasma renin activity and Ang II levels despite a lower plasma volume.\textsuperscript{31,32} Approximately 40 years ago, Gant et al. established a logical explanation for the hypertension mechanism of preeclampsia as hypersensitivity against Ang II.\textsuperscript{14} AbdAlla et al. found that AT1R-bradykinin B2 receptor heterodimers were increased in platelets and omental vessels from preeclamptic women, resulting in Ang II
hypersensitivity. In addition, platelet Ang II binding sites were increased in women with preeclampsia. Wallukat et al. described the presence of agonistic autoantibodies for AT1R (AT1-AAs) in preeclampsia, and such AT1-AAs may facilitate hypersensitivity against Ang II. These reports indicate that the pathogenic mechanisms of hypertension in preeclampsia might be related to elevated AT1R levels that induce hypersensitivity to Ang II.

When injected into pregnant mice, affinity-purified AT1-AA from preeclamptic women lead to hypertension, proteinuria, glomerular endotheliosis, and elevated sFlt-1 levels. Whereas, nonpregnant mice injected with AT1-AA induced hypertension without proteinuria and glomerular endotheliosis, elevated sFlt-1 levels. In our study, however, COMTi+ANGII group displayed glomerular endotheliosis without sFlt-1 induction; 2-ME ameliorated the glomerular endothelial morphology. 2-ME has been shown to inhibit hypoxia-inducible factor-1α (HIF-1α), the molecule induced by Ang II. Pregnant mice overexpressing HIF-1α had hypertension and proteinuria with glomerular endotheliosis. Silencing of HIF-1α gene significantly improved the glomerular sclerotic damages (as indicated by glomerular mesangial expansion with hypercellularity, capillary collapse, and fibrous deposition in glomeruli) induced by Ang II infusion (200ng/kg/min) in Sprague-Dawley rats. It is possible that albuminuria and
glomerular endotheliosis are mediated by increased HIF-1α levels. Therefore, we assume that both absence of 2-ME and infusion of low-dose Ang II, not by each of them alone, may lead to the accumulation of HIF-1α resulting in glomerular injury.

Both 2-OHE2 and 2-ME have little affinity for estrogen receptors (ERs) and inhibit the growth of VSMCs\textsuperscript{40,41} and cardiac fibroblasts\textsuperscript{42}, and renal mesangial cells\textsuperscript{43,44}. COMT deficiency eliminates many of the vasoprotective effects of estradiol and 2-OHE2.\textsuperscript{40} These anti-growth effects via ERs-independent mechanisms are mediated through 2-ME. 2-ME protects cardiovascular and renal system, and reduces mortality in severe disease models.\textsuperscript{45} Furthermore, COMT is efficient with regard to converting 2-OHE2 to 2-ME\textsuperscript{46} and rapidly methylate 2-OHE2 in heart and kidneys\textsuperscript{47}. These reports suggest COMT-mediated conversion of 2-OHE2 to 2-ME play vital protective role in cardiovascular and renal system.

Our study has several limitations. First, we used indirect methods for measuring BP in mice because 1) we repeated measurements of SBP in large numbers of conscious mice during studies of long duration\textsuperscript{48}, 2) pulse-based tail-cuff measurements have been proven accurate by comparison to simultaneous telemetry measurements\textsuperscript{49,50}. Secondly, blood was collected into heparin-coated tube without inhibitors as reported elsewhere (such as protease inhibitor and renin inhibitor, angiotensin converting inhibitor)\textsuperscript{51,52}. 
Indeed, the measurement of plasma Ang II levels was not planned at the beginning of our experiments. Nussberger et al. reported that blood samples were collected in the presence of inhibitor solution for prevention of generation and degradation of Ang II.\textsuperscript{53} Therefore, it is possible that the Ang II levels in our study may under-/over-estimate “true“ values. Both COMT deficient mice (inhibitor or siRNA) displayed suppressed levels of Ang II when compared to each control mouse. Even though such technical limitations, the levels of Ang II in our experiments could be relevant to demonstrate the Ang II hypersensitivity in COMT deficiency. Next, we could not measure the plasma 2-ME concentration by ELISA using a commercially available system in this study. 2-ME is present at approximately 3 ng/mL (approximately 10 nmol/L) in the blood stream of normal pregnant women during the third trimester by HPLC with MS/MS detection.\textsuperscript{3} In previous study, we measured the plasma levels of 2-ME in pregnant mice, the condition associated with high 2-ME levels, and found that COMT knockout pregnant mice displayed depleted levels of 2-ME\textsuperscript{3}; however, even in the pregnant mice, the levels of 2-ME were very low, and we did not observe any statistical significant difference between the groups, probably due to low estrogen levels in mice when compared to pregnant women.\textsuperscript{54-57} Despite these limitations, we successfully demonstrated that PPAR\textgamma transcriptional activities were upregulated by 2-ME in vitro and ex vivo. These data
strongly suggest that 2-ME plays a vital role in blood pressure regulation via the suppression of AT1R through PPARγ transcriptional activation. In addition, we utilized male C57BL/6 wild-type mice for our study. Primary purpose of our study was to investigate whether COMT deficiency could induce vascular injury in the presence of low Ang II levels; we considered analyzing female mice for our study since most strong evidence between COMT deficiency and hypertensive disorder was indeed preeclampsia. However, female mice have a very short estrous cycle (approximately 4-5 days), which significantly alters estrogen and 2-ME levels. Despite these limitations, we successfully demonstrated that COMT-deficient pregnant female mice, as a model of preeclampsia, exhibited the suppression of PPARγ transcriptional activity in plasma. This PPARγ suppression in COMT-deficient pregnant female mice was restored by 2-ME. Thus, COMT/2-ME deficiency is relevant in both male and female mice.

Perspectives

This present study showed that COMT deficiency leads to Ang II hypersensitivity with increased vascular AT1R expression; 2-ME suppresses AT1R expression via PPARγ activation. In both men and women, low-COMT activity polymorphisms and haplotypes are associated with hypertension, CVD, metabolic disease, recurrent preeclampsia. Preeclamptic women display a significant depletion of plasma 2-ME
levels\textsuperscript{1-3} and have a well-established association with the incident of hypertension and CVD in later life. 2-ME is nonfeminizing because of a low affinity for ERs. Therefore, these data could provide the mechanistic rationale for the 2-ME replacement therapy to combat COMT deficiency-associated hypertensive disorders irrespective of age or gender.

Acknowledgments

The authors declare no conflicts of interest related to this work. Boehringer Ingelheim, MitsubishiTanabe Pharma, and Ono Pharmaceutical contributed to establishing the Division of Anticipatory Molecular Food Science and Technology. We thank Dr. Junji Ishida and Dr. Akiyoshi Fukamizu (University of Tsukuba, Japan) for providing over-expressing HA-tagged mouse AT1aR or pcDNA3 HEK293T cells.

Sources of Funding

This work was partially supported by grants from the Japan Society for the Promotion of Science awarded to M Kanasaki (24790329), KK (23790381, 23790381), and DK (25282028, 25670414) and several research awards for KK (the Daiichi-Sankyo Foundation of Life Science, the Ono Medical Research Foundation, the Takeda Science
Foundation, Novo Nordisk Insulin Research Foundation and the Banyu Life Science Foundation International). This work was partially supported by a Grant for Collaborative Research awarded to DK (C2011-4, C2012-1), a Grant for Promoted Research awarded to KK (S2013-13, S2014-4, S2015-3) and a Grant for Specially Promoted Research from Kanazawa Medical University (head of team: DK, SR2012-06) from Kanazawa Medical University.

**Disclosures**

KK is under a consultancy agreement with Boehringer Ingelheim (Germany).
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Novelty and Significance

1) What Is New?

- Catechol-O-methyltransferase (COMT) deficiency leads to hypersensitivity of the pressor response against angiotensin II (Ang II) with increased expression of angiotensin II type 1 receptor (AT1R) in aorta.

- 2-methoxyestradiol (2-ME), a natural metabolite of estradiol synthesized by COMT, suppressed AT1R expression in vivo and in vitro.

- 2-ME suppressed AT1R expression via peroxisome proliferator-activated receptor γ (PPARγ) activation in vitro.

2) What Is Relevant?

- COMT knockout pregnant mice exhibit preeclampsia like phenotypes via absence of 2-ME. It is assumed that the pathomechanism of hypertension in preeclampsia is hypersensitivity of the pressor response against Ang II. Notably, COMT deficiency and/or absence of 2-ME may exacerbate hypertension in pregnancy.

3) Summary

COMT deficient (caused by COMT inhibitor or COMT siRNA) male mice, not wild-type male mice, exhibited hypertension and albuminuria, glomerular endotheliosis with
low-dose Ang II; 2-ME, not 2-hydroxyestradiol which is precursor of 2-ME, ameliorated phenotypes in COMT deficient mice with low-dose Ang II. COMT deficient mice increased AT1R expression in aorta; 2-ME suppressed such COMT deficiency-associated AT1R expression. In human aortic smooth muscle cells (AoSMCs), compared with control, Ang II significantly induced both AT1R protein and mRNA levels; 2-ME suppressed this induction of AT1R expression. In AoSMCs, 2-ME (100 nmol/L and 500 nmol/L) increased PPARγ activities to levels similar to those induced by the PPARγ agonist pioglitazone (30 μmol/L). In AoSMCs, both 2-ME and pioglitazone suppressed both mRNA and protein expression of AT1R. The PPARγ antagonist GW9662 diminished the effects of 2-ME and pioglitazone on AT1R levels. These results suggested COMT deficiency and/or absence of 2-ME increased AT1R expression, and 2-ME suppressed AT1R expression in a PPARγ-dependent manner.
**Figure 1.** COMT inhibitor-treated mice exhibit Ang II hypersensitivity. (A) The experimental protocol of the study is presented. Blood pressure measurements in Oil (n=10), Oil+ANGII (n=10), COMTi (n=10), COMTi+ANGII (n=10), COMTi+ANGII+2-ME (n=10), COMTi+ANGII+2-OHE2 (n=10) groups. Mean systolic blood pressure (SBP) at day 21 and 28 were compared across all groups. (B) Urinary albumin/creatinine ratio (μg/mg) for Oil (n=5), Oil+ANGII (n=6), COMTi (n=5), COMTi+ANGII (n=7), and COMTi+ANGII+2-ME (n=5) groups. (C) Electron microscopy was performed for Oil, Oil+ANGII, COMTi, COMTi+ANGII, and COMTi+ANGII+2-ME groups. Electron micrographs of glomeruli show glomerular endotheliosis, including glomerular endothelial swelling and occlusion of the capillary lumen, as indicated by the white dotted lines. Scale bar, 1 μm. Data are expressed as the mean ± SEM, *p values analyzed by 1-way ANOVA with Tukey’s test; *p<0.05, **p<0.01. Ang II: angiotensin II; COMTi: COMT inhibitor; 2-OHE2: 2-hydroxyestradiol; 2-ME: 2-methoxyestradiol.
Figure 2. COMT siRNA-treated mice exhibit Ang II hypersensitivity. (A) The experimental protocol of the study is presented. Blood pressure measurements in SCRsiRNA (n=10), SCRsiRNA+ANGII (n=10), siCOMT (n=10), siCOMT+ANGII (n=11), siCOMT+ANGII+2-ME (n=11) groups. Mean SBP at day 21 and 28 were compared across all groups. (B) Urinary albumin/creatinine ratio (μg/mg) for SCRsiRNA (n=5), SCRsiRNA+ANGII (n=5), siCOMT (n=5), siCOMT+ANGII (n=6), and siCOMT+ANGII+2-ME (n=5) groups. Data are expressed as the mean ± SEM, *p values analyzed by 1-way ANOVA with Tukey’s test; *p<0.05, **p<0.01.
**Figure 3.** Plasma sFlt-1 levels and sFlt-1/PlGF-2 ratio, catecholamine levels are unlikely associated with the hypertension in COMT-deficient mice. (A and B) ELISA was performed for sFlt-1 and PlGF-2 on plasma from mice in Oil (n=5), Oil+ANGII (n=5), COMTi (n=5), COMTi+ANGII (n=5), and COMTi+ANGII+2-ME (n=5) groups (A) and from mice in SCRsiRNA (n=5), SCRsiRNA+ANGII (n=5), siCOMT (n=5), siCOMT+ANGII (n=5), and siCOMT+ANGII+2-ME (n=5) groups (B). sFlt-1: soluble fms-like tyrosine kinase-1; PlGF-2: placental growth factor-2. (C) ELISA of plasma adrenaline and noradrenaline from Oil (n=5), Oil+ANGII (n=5), COMTi (n=5), COMTi+ANGII (n=5), and COMTi+ANGII+2-ME (n=5) groups. Data are expressed as the mean ± SEM, *P* values analyzed by 1-way ANOVA with Tukey’s test; *P*<0.05, **P*<0.01; n.s.: non-significance.
**Figure 4.** COMT-deficient mice exhibit the elevated levels of AT1R protein expression in aorta. (A) ELISA of plasma angiotensin II from Oil (n=4) and COMTi (n=4), SCRsiRNA (n=4) and siCOMT (n=4). (B) The panel shows a densitometric analysis of mouse aorta AT1R protein expression normalized to GAPDH (n=3) in Oil, Oil+ANGII, COMTi, COMTi+ANGII, and COMTi+ANGII+2-ME groups. GAPDH levels are shown as a loading control. (C) The panel shows a densitometric analysis of mouse aorta AT1R protein expression normalized to GAPDH (n=3) in SCRsiRNA, SCRsiRNA+ANGII, siCOMT, siCOMT+ANGII, and siCOMT+ANGII+2-ME groups. Data are expressed as the mean ± SEM, *P* values analyzed by 1-way ANOVA with Tukey’s test; *P*<0.05, **P**<0.01. AT1R; angiotensin II type 1 receptor.
**Figure 5.** AT1R expression in AoSMCs is downregulated by 2-ME. (A) The panel shows a densitometric analysis of AT1R protein expression normalized to GAPDH (n=3) in human aortic smooth muscle cells (AoSMCs). (B) AT1R mRNA expression in AoSMCs determined by quantitative polymerase chain reaction (qPCR) with 18S as an internal control (n=4). (C) AT1R mRNA expression in AoSMCs determined by qPCR with 18S as an internal control (n=3). Data are expressed as the mean ± SEM, $P$ values analyzed by 1-way ANOVA with Tukey’s test; *$P$<0.05, **$P$<0.01.
**Figure 6.** PPARγ transcriptional activity is decreased by plasma from COMT-deficient mice. (A) PPARγ luciferase activity in AoSMCs. The relative luciferase activities are presented as fold induction with respect to that of 2-ME 0 nmol/L. Pio: pioglitazone (30 μmol/L). (B) AT1R mRNA expression in AoSMCs determined by qPCR with 18S as an internal control (n=5). The next panel shows a densitometric analysis of AT1R protein expression normalized to GAPDH (n=4) in AoSMCs. GW9662: a PPARγ inhibitor. (C) The data including PPARγ luciferase activity; a densitometric analysis of AT1R protein expression normalized to GAPDH shows the results in AoSMCs treated with 5% plasma from male mice in Oil (control), COMTi, and COMTi+2-ME groups (n=4 each group). PPARγ relative luciferase activities are presented as fold induction with respect to those of the Oil group. (D) The data including PPARγ luciferase activity (n=4); a densitometric analysis of AT1R protein expression normalized to GAPDH (n=3) shows the results in AoSMCs treated with 5% plasma from pregnant female mice in Oil (control), COMTi, and COMTi+2-ME groups. PPARγ relative luciferase activities are presented as fold induction with respect to that of the Oil group. Data are expressed as the mean ± SEM, $P$ values analyzed by 1-way ANOVA with Tukey’s test; *$P<0.05$, **$P<0.01$. 
Figure 5
A.

B.

C.

Ang II  -  -  +  +
2-ME    -  +  -  +

Ang II  -  -  +  +
2-ME    -  +  -  +

COMT i  -  +  -  +  +
2-HE    -  -  +  +  -
2-ME    -  -  -  -  +

Relative density (AT1R/GAPDH)
Relative mRNA Expression AT1R/18S (Fold)
Figure 6

A. PPAR transcriptional activity

B. Relative mRNA Expression (AT1R/18S, Fold)

C. Relative density (AT1R/GAPDH)

D. PPAR transcriptional activity

- 2-ME (nmol/L)
- Pioglitazone
- GW9662

C. COMT i
- 2-ME
Catechol-O-methyltransferase deficiency leads to hypersensitivity of the pressor response against angiotensin II

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Running title: Angiotensin II Hypersensitivity in COMT Deficiency

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Supplemental Methods

Animal experiments

We utilized eight-week-old male C57BL/6 mice (Sankyo Lobo Service, Tokyo, Japan). Mice were subjected to COMT inhibitor Ro41-0960 (COMTi: 25 mg/kg/day intraperitoneal administration (i.p.); SIGMA-ALDRICH, St. Louis, MO) or olive oil (Control) for 4 weeks, with or without low-dose Ang II infusion (ANGII: 70 ng/kg/min continuous subcutaneous infusion (CSI); BACHEM, Bubendorf, Switzerland) for the last 3 weeks. The Ang II-infused mice were treated with 2-methoxyestradiol (2-ME: 10 ng/day i.p.; SIGMA-ALDRICH), 2-hydroxyestradiol (2-OHE2: 10 ng/day i.p.; SIGMA-ALDRICH), or vehicle for the last week. The levels and regulation of 2-ME is largely unknown. 2-ME present at approximately 3 ng/mL in the plasma of pregnant women during third trimester. In our previously we thought to keep 2-ME levels as similar to the level as human pregnancy\(^1\). Therefore, we injected 10 ng/day (since circulatory volume of adult mice would be 1.2-2.4 mL and pregnant mice were estimated to be 1.4-2.9)\(^2\). However, the 2-ME levels estimation in previous study revealed that 2-ME levels in pregnant wild type mice was approximately 250 pg/mL\(^1\). COMT deficient pregnant mice displayed notable 2-ME defect; 2-ME injection (10 ng/day) in COMT deficient pregnant mice reached the levels of 2-ME similar to that of pregnant wild type mice. Therefore, in our study, we used 10 ng of 2-ME with knowledge of previous our pregnant mice study. Dose of COMT inhibitor was followed by Emilia Rivas et al.\(^3\) and our previous study\(^1\). 2-OHE2 is the precursor of 2-ME, therefore same 10 ng/day was used.

Thus, we obtained the following 6 experimental groups: Control, ANGII, COMTi, COMTi+ANGII, COMTi+ANGII+2-ME and COMTi+ANGII+2-OHE2. We performed similar experiments utilizing the in vivo administration of siRNA (siCOMT: 20 nmol/week i.p.; BIONEER) of COMT instead of COMT inhibitor. Mice were subjected to COMT siRNA-atelocollagen (AteloGene®, KOKEN, Tokyo, Japan) complex or scramble siRNA (SCRsiRNA as control)-atelocollagen complex for 4 weeks, with or without low-dose Ang II infusion (70 ng/kg/min CSI) for the last 3 weeks. The Ang II-infused mice were treated with 2-ME (10 ng/day i.p.) or vehicle for the last week. Thus, we obtained the following 5 experimental groups: SCRsiRNA, SCRsiRNA+ANGII, siCOMT, siCOMT+ANGII, and siCOMT+ANGII+2-ME. Mice were anesthetized with pentobarbital (40-50 mg/kg i.p.), and osmotic pumps (Alzet®, MODEL 2004, Cupertino, CA) were implanted subcutaneously to infuse Ang II. All mice were euthanized with pentobarbital (100-150 mg/kg i.p.), and whole aorta, kidney, liver,
heart, and plasma were removed. To obtain the plasma of pregnant mice, six-week-old female C57BL/6 mice were mated with male C57BL/6 mice. Successful mating was evaluated by the appearance of a vaginal plug, and the midday of the day when the vaginal plug was observed was considered to be 12 h after fertilization, embryonic day 0.5 (E0.5). The mice with a vaginal plug were placed in a different cage (maximum three pregnant mice to a cage) until being sacrificed at day 17 of gestation. Beginning at day 10 of the pregnancy, the mice were injected with Ro41-0960 (25 mg/kg/day) with or without 2-ME (10 ng/day) or placebo (olive oil) subcutaneously. The mouse studies were approved by the Kanazawa Medical University Institutional Animal Care and Use Guidelines (2013-109, 2014-90, 2015-100, 2016-70).

**Blood pressure measurement**

For non-invasive monitoring of blood pressure, measurements were taken using a tail-cuff system from Softron™ (Tokyo, Japan). Mice were trained for at least five days before systolic blood pressure (SBP) evaluation. The SBP data were obtained by the average of 10 measurements.

**Blood Collection**

Mouse blood were collected into heparin-coated tube via cardiac puncture under anesthesia. Plasma is the supernatant fluid obtained when heparinized blood has been centrifuged at 3,000 rpm for 20 min at 4°C. Plasma was kept at −80 °C until assay.

**Urine albumin evaluation**

24-hour urine samples collected from mice placed in metabolic cages on day 27. Urine albumin levels were analyzed using the Murine Microalbuminuria ELISA kit (Albuwell M; Exocell, Philadelphia, PA), and creatinine levels were analyzed using the Creatinine Assay kit (DICT-500; BioAssay systems, Hayward, CA). Urine albumin excretion was estimated as the quotient of urine albumin and urine creatinine.

**Electron microscopy**

Electron microscopic analysis was performed by Hanaichi Ultrastructure Research Institute (Aichi, Japan). In the following steps for analysis of kidney samples. Immersion fixation with glutaraldehyde 2.5% in sodium cacodylate buffer (pH 7.4) at 4°C for overnight and post-fixation with 2% osmium tetroxide in 0.1 mol/L phosphate buffered saline at 4°C for 2 hours. Samples were washed in 0.1 mol/L cacodylic acid buffer at 4°C for 2×20min. Dehydration at room temperature with 50% ethanol for
15min, 70% ethanol for 15min, 95% ethanol for 15min, 100% ethanol for 15min, 100%
propylene oxide for 2×20min, 1:1 propylene oxide: resin for 2 hours. Embedding with
EPON812 at 60°C for 48 hours.

**Cell culture**

Human aortic smooth muscle cells (AoSMCs; LONZA) were grown at 37°C in
growth medium (Clonetics™ SmGM™-2 Smooth Muscle Growth Medium-2;
LONZA). Human embryonic kidney 293-T cells (HEK293Ts) over-expressing
hemagglutinin (HA)-tagged mouse AT1a receptor or transfected with pcDNA (kindly
gifted by Dr. Junji Ishida and Dr. Akiyoshi Fukamizu, University of Tsukuba, Japan)
were grown at 37°C in Dulbeco’s modified Eagle’s medium with 1 g/L glucose (low
glucose DMEM; NACALAI) supplemented with 10% fetal bovine serum.

**AGTR1 siRNA design and delivery**

The following sequences were used; human AGTR1 siRNA (sense: 5′-
AAUAUCUGCAAUUCUACAG-3′, antisense: 5′-CUGUAGAAUUGCAGAUAUU-3′)

AoSMCs grown in 6-well plates to 70-90% confluence at 37°C were transfected
with AGTR1 siRNA or scramble siRNA using lipofectamine 2000 (Life Technologies)
in Opti-MEM I for 6 hours, and then the medium was changed back to growth medium
for 48 hours incubation.

**Western blot analysis**

The whole aortas were removed from the mice and were homogenized with
TRIzol® Reagent (Thermo Fisher Scientific, Waltham, MA) following the
manufacturer’s instructions. Protein pellets were solubilized in 8 mol/L urea solution.
The concentration of protein lysates was evaluated using the Pierce BCA Protein Assay
(Thermo Fisher Scientific). Protein lysates were denatured by boiling in sodium dodecyl
sulfate (SDS) sample buffer at 94°C for 5 minutes. Protein lysates were separated on a
5-20% ePAGEL® (ATTO Corporation, Tokyo, Japan) with Running Buffer Solution
(WAKO, Tokyo, Japan) and transferred onto PVDF Membrane (Clear Trans®, WAKO).
Blots were probed with anti-COMT antibody (1:5000, AB5873, CHEMICON,
Millipore, Temecula, CA), anti-Angiotensin II receptor Type 1 antibody (1:1000,
SAB3500209, SIGMA-ALDRICH), anti-GAPDH antibody (1:20000, G8795, SIGMA-
ALDRICH), and anti-β-actin antibody (1:4000, A2228, SIGMA-ALDRICH), followed
by horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, Anti-rabbit
IgG, #7074, Anti-mouse IgG, #7076, Cell Signaling, Danvers, MA). After blocking with
TBST (Tris-buffered saline containing 0.05% Tween 20) containing 5% bovine serum albumin, the membranes were incubated with the primary antibodies of the target molecules in TBST containing 5% bovine serum albumin at 4°C overnight. The membranes were washed three times and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive bands were visualized with an enhanced chemiluminescence detection system (Pierce Biotechnology, Rockford, IL) using the ImageQuant LAS 400 camera system (GE Healthcare Life Sciences, Uppsala, Sweden).

**Angiotensin II enzyme immunoassay**

Mouse plasma angiotensin II levels were measured with an Angiotensin II EIA kit (RAB0010, SIGMA-ALDRICH) following the manufacturer's instructions.

**Enzyme-linked immunosorbent assays**

sFlt-1(MVR100, R&D Systems, Minneapolis, MN), PlGF2 (MP200, R&D Systems), adrenaline (BA E-5400, LDN®, Nordhorn, Germany), and noradrenaline (BA E-5400, LDN®) levels in mouse plasma were evaluated using commercially available kits following the manufacturers' instructions.

**Quantitative polymerase chain reaction**

The total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific) following the manufacturer’s instructions and was quantified with a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific). The complementary DNA was generated by a PrimeScript™ RT-PCR Kit (TaKaRa, Shiga, Japan). RT-PCR was performed with the following primers: Human AGTR1 (forward: 5’-TCCAGATTGTTCTGTCCAGTTTCC-3’; reverse: 5’-TCTGCAACTTGACGACTACTGCTTA-3’), Human 18S (forward: 5’-ACTCAACACGGAAACCTCA-3’; reverse: 5’-AACCAGACAAATCGCTCCAC-3’). Conditions for the PCR were as follows: 95°C for 20 seconds; 95°C for 1 second, 60°C for 20 seconds (40 cycles). PCR product was monitored using Sybr green (THUNDERBIRD® SYBR®; TOYOBO, Osaka, Japan).

**PPAR reporter assay**

AoSMCs grown in 12-well plates to 70-90% confluence were transfected with peroxisome proliferator-activated receptor (PPAR) reporter vectors (Cignal PPAR Reporter Luciferase Kit; QIAGEN, Hilden, Germany), the mixture of a PPAR-
responsive luciferase construct and a constitutively expressing Renilla element (40:1). In each transfection, 100 ng of PPAR reporter vectors was transfected in the serum-free RPMI 1640 medium. Six h after transfection, the serum-free RPMI 1640 medium was changed and cells were treated with 2-ME (0 nmol/L, 1 nmol/L, 10 nmol/L, 100 nmol/L, 500 nmol/L) and pioglitazone (30 μmol/L) for 24 h in RPMI 1640 containing 0.2% BSA or treated with 5% mouse plasma in the serum-free RPMI 1640 medium for 24 h. The activity of PPAR signaling was evaluated using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega, Fitchburg, WI).

References
S1. (A) Western blot analysis for MB-COMT (30 kDa) and S-COMT (25 kDa) protein of heart, aorta, liver and kidney in normal male mice. Actin and GAPDH levels are shown to indicate loading control. MB: membrane-bound; S: soluble; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. (B) Western blot analysis for COMT protein of liver and kidney in scrambled siRNA- or COMT siRNA-treated mice. Actin protein levels are shown as a loading control. (C and D) ELISA was performed for PlGF-2 on plasma from mice in Oil (n=5), Oil+ANGII (n=5), COMTi (n=5), COMTi+ANGII (n=5), and COMTi+ANGII+2-ME (n=5) groups (C) and from mice in SCRsiRNA (n=5), SCRsiRNA+ANGII (n=5), siCOMT (n=5), siCOMT+ANGII (n=5), and siCOMT+ANGII+2-ME (n=5) groups (D). Data are expressed as the mean ± SEM, P values analyzed by 1-way ANOVA with Tukey’s test; *P<0.05, **P<0.01.
**S2.** The specific reactivity of the anti-AGTR1 antibody (SAB3500209, 1:1000, SIGMA-ALDRICH). (A) Representative immunoblots showing human AT1R protein in human aortic smooth muscle cells. The predicted molecular size of human AT1R is 41kDa. SCRsiRNA: scramble siRNA (B) Representative immunoblots showing mouse AT1aR protein in human embryonic kidney 293-T cells over-expressing hemagglutinin (HA)-tagged mouse AT1aR. The predicted molecular size of mouse AT1aR is 41kDa. Anti-HA antibody (HA-Tag (C29F4) Rabbit mAb #3724, 1:1000, Cell Signaling)
**S3.** Western blot analysis for AT1R protein (41 kDa) in mouse aorta or in human aortic smooth muscle cells (AoSMCs). GAPDH levels are shown as a loading control. (A) AT1R protein of mouse aorta in Oil (control), Oil+ANGII, COMTi, COMTi+ANGII, and COMTi+ANGII+2-ME groups. (B) AT1R protein of mouse aorta in SCRsiRNA (control), SCRsiRNA+ANGII, siCOMT, siCOMT+ANGII, and siCOMT+ANGII+2-ME groups. (C) AT1R protein in AoSMCs treated with 2-ME or/and Ang II. (D) AT1R protein in AoSMCs treated with COMTi or/and 2-OHE2 or 2-ME. (E) AT1R protein in AoSMCs treated with 2-ME or/and GW9662. GW9662: a PPARγ inhibitor. (F) AT1R protein in AoSMCs treated with 5% plasma from male mice in Oil, COMTi, and COMTi+2-ME groups. (G) AT1R protein in AoSMCs treated with 5% plasma from pregnant female mice in Oil, COMTi, and COMTi+2-ME groups. Data are expressed as the mean ± SEM, *P* values analyzed by 1-way ANOVA with Tukey’s test; **P**<0.05, ***P***<0.01. Ang II: angiotensin II; COMTi: COMT inhibitor; 2-OHE2: 2-hydroxyestradiol; 2-ME: 2-methoxyestradiol.