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The Serotonin Transporter Promotes a Pathological Estrogen Metabolic Pathway in Pulmonary Hypertension via Cytochrome P450 1B1

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Running title: Serotonin transporter, estrogen, CYP1B1 and pulmonary hypertension

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Abstract

Pulmonary arterial hypertension (PAH) is a devastating vasculopathy that predominates in women and has been associated with dysregulated estrogen and serotonin signalling. Over-expression of the serotonin transporter ($SERT^+$) in mice results in an estrogen-dependent development of pulmonary hypertension (PH). Estrogen metabolism by cytochrome P450 1B1 (CYP1B1) contributes to the pathogenesis of PAH and serotonin can increase CYP1B1 expression in human pulmonary arterial smooth muscle cells (hPASMCs). We hypothesized that an increase in intracellular serotonin via increased SERT expression may dysregulate estrogen metabolism via CYP1B1 to facilitate PAH. Consistent with this hypothesis, we found elevated lung CYP1B1 protein expression in female $SERT^+$ mice accompanied by PH, which was attenuated by the CYP1B1 inhibitor, 2,3',4,5'-tetramethoxystilbene (TMS). Lungs from female $SERT^+$ mice demonstrated an increase in oxidative stress which was marked by the expression of 8-hydroxyguanosine; however this was unaffected by CYP1B1 inhibition. SERT expression was increased in monocrotaline-induced PH in female rats; however, TMS did not reverse PH in monocrotaline-treated rats, but prolonged survival. Stimulation of hPASMCs with the CYP1B1 metabolite 16α-hydroxyestrone increased cellular proliferation, which was attenuated by an inhibitor of estrogen receptor alpha (ERα; MPP) and a specific ERα antibody. Thus, increased intracellular serotonin caused by increased SERT expression may contribute to PAH pathobiology by dysregulation of estrogen metabolic pathways via increased CYP1B1 activity. This promotes PASMC proliferation by the formation of pathogenic metabolites of estrogen that mediate their effects via ERα. Our studies indicate that targeting this pathway in PAH may provide a promising anti-proliferative therapeutic strategy.

248 words

Introduction

Pulmonary arterial hypertension (PAH) is a fatal cardiovascular disorder with a poorly understood aetiology. It is characterised by obliteration of the small and medium pulmonary arteries resulting in increased pulmonary pressures and subsequent enlargement and failure of the right ventricle. Female sex is possibly the strongest and most established risk factor for PAH, with more than 70% of patients diagnosed being female[1-3]. 17β-estradiol (estrogen) is recognized as a pathogenic mediator in numerous diseases either through aberrant estrogen receptor (ER) mediated signalling and/or metabolism[4]. More recently, increasing evidence suggests that PAH may also be affected by abnormal activity of the estrogen pathway to adversely affect pulmonary vascular cell homeostasis[5-12]. It was initially hypothesized that estrogens and/or their metabolites may promote disease development and progression. However, in the 'classical' animal models of PAH (monocrotaline (MCT) and hypoxia), exogenous administration of estrogen was shown to have a beneficial effect, by in part, promoting cardiac function[13-16]. This is in line with the extensive literature that reports cardio-protective effects of estrogens[17]. More recently, we have provided compelling evidence that the endogenous synthesis of estrogen within the pulmonary arteries by the cytochrome P450 (CYP) enzyme aromatase, promotes the development of pulmonary hypertension (PH) in female rodents[6,12].

One criticism of utilizing the 'classical' animal models of PAH to study the effects of gender is that they do not recreate the gender disparity that is observed in clinic. We have recently described several novel murine models of PH that are specific to female mice. These include $SERT^{+}$ mice[13], mice that over-express the S100A4 calcium binding protein[18] (which requires co-operation between the 5-HT1B receptor and SERT[19]) and mice administered with dexfenfluramine (an indirect serotinergic agonist)[20]. Ovariectomy was shown to reverse PH in these mice confirming a role for sex hormones in these female specific models[13,18,20]. Of

interest, these models all depend upon up-regulation of the serotinergic pathway, suggesting interplay of the serotonin and estrogen pathways in disease development.

The metabolic fate of estrogen is regulated by the activity of CYP enzymes to yield biologically active metabolites[21]. We have previously shown that at least one CYP, CYP1B1, is highly expressed within the pulmonary arterial lesions of patients with PAH, whilst minimal expression is observed in patients without any reported evidence of PAH[5]. CYP1B1 metabolizes estrogen primarily to the 4-hydroxylated estrogens and to a lesser extent to the 2- and the 16α-hydroxylated estrogens (amongst others)[22-24]. CYP1B1 polymorphisms that result in a reduced 2-OHE1/16α-OHE1 estrogen metabolite ratio have been reported in patients with PAH associated with mutations in the bone morphogenic protein receptor type II (BMPR2) [7]– the predominant genetic basis for the development of heritable PAH[25]. Importantly, inhibition of estrogen metabolism by CYP1B1 can attenuate the development of experimental PH induced by hypoxia-, SU5416+hypoxia[5] and dexfenfluramine[20].

Pulmonary arterial smooth muscle cell (PASMC) proliferation is an established and dominant feature associated with PAH pathobiology[26,27]. Estrogen induces proliferation of PASMCs and this is attenuated by CYP1B1 inhibition[5]. CYP1B1 expression is increased in PASMCs isolated from patients with idiopathic PAH and in female mice that overexpress SERT[28] and serotonin can increase the expression of CYP1B1 in healthy PASMCs[28]. We therefore hypothesized that CYP1B1 may play a causative role in the development of PH in female $SERT^+$ mice by promoting pathologic estrogen metabolism. To test this hypothesis, we assessed the therapeutic potential of CYP1B1 inhibition in female $SERT^+$ mice. We also tested this in the MCT model of PAH which has elevated SERT expression[29] and examined the molecular mechanisms that may mediate the pathogenic signalling associated with elevated CYP1B1.

Methods

Ethics

All experimental procedures were carried out in accordance with the United Kingdom Animal Procedures Act (1986) and with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Rodents were housed in a 12-hour light dark cycle with access to food and water ad libitum. Experimental procedures using hPASMCs conform to the principles outlined in the Declaration of Helsinki. All animals were randomly allocated to groups and all measurements and analysis were done in a blinded fashion, where possible.

Animal models and *In Vivo* CYP1B1 inhibition with 2,3',4,5'-Tetramethoxystilbene

5-6 month-old female mice ubiquitously overexpressing the human SERT gene ($SERT^+$ mice) were generated as previously described on a C57Bl/6J CBA background[30]. These were a kind gift from the late Professor Tony Harmar (University of Edinburgh). Age-matched littermates (here-after referred to as wild-type) were studied as controls. Mice were subdivided into groups to receive either the highly potent and selective CYP1B1 inhibitor, 2,3',4,5'-Tetramethoxystilbene (TMS; 1.5mg/kg/day by intra peritoneal (i.p.) injection; Tocris Biosciences, UK) or vehicle (~5% (v/v) ethanol in 0.9% saline) for 14 days prior to assessment of hemodynamic parameters.

Male and female Wistar rats (Harlan Laboratories, Oxon, UK), 10-12 weeks old at termination procedure received either the pneumotoxin MCT pyrrole (Sigma-Aldrich, Dorset, UK) or its vehicle at a dose of 60mg/kg by subcutaneous (s.c.) injection in the flank of the rat at day 0. MCT was dissolved in 1mol/L hydrogen chloride (HCl) at a concentration of 100 mg/mL, neutralized with 1mol/L sodium hydroxide (NaOH) and diluted with sterile distilled water to 6 mg/mL. All animals were weighed every day and monitored for any signs of

discomfort. One single injection of MCT is sufficient to induce a PH phenotype in rats (MCT does not induce PH in mice). MCT mediates damage to the pulmonary endothelium and promotes the extension of smooth muscle cells into normally non-muscular pulmonary arteries promoting PH and right ventricular heart failure [31]. 14 days post MCT injection animals were administered with the CYP1B1 inhibitor TMS at 3mg/kg/day or its vehicle (~5% (v/v) ethanol in 0.9% saline) by i.p. injection for an additional 14 days prior to hemodynamic analysis.

In vivo hemodynamic measurements

Rats and mice were anaesthetized with isoflurane supplemented with a constant flow of oxygen. Right ventricular systolic pressures (RVSP) were obtained by transdiaphragmatic puncture of the right ventricle with a saline-filled 25-gauge needle (in mice) and by jugular vein cannulation of the right ventricle using a fluid-filled catheter (in rats). To monitor off-target effects on the systemic vasculature, mean systemic arterial pressures (SAP) were monitored by micro-cannulation of the left common carotid artery.

Right ventricular hypertrophy

RV hypertrophy was assessed by the Fulton Index[32] by expressing the dry weight of the right ventricle over the dry weight of the left ventricle + septum.

Lung histopathology

The inferior lobe of the lung was fixed in 10% neutral buffered formalin for a minimum of 4 hours with gentle agitation prior to embedding in paraffin. 3µm sections were cut and stained using elastin and counterstained with Picro Sirius Red for identification of vascular thickening in the small arteries with an internal diameter up to 80µm. This was characterized by an increase in the vessel wall diameter in more than 50% of the arterial wall.

The total number of remodeled vessels was expressed over the total number of vessels present within a lung section.

CYP1B1 immunolocalization

CYP1B1 immunolocalization was determined in mouse and rat pulmonary arteries by immunohistochemistry, as described previously[5]. Briefly, 3μm sections were deparaffinised and rehydrated through a xylene-ethanol gradient to water. After antigen retrieval with citric acid buffer, endogenous peroxidase and biotin activity was blocked followed by a 1 hour incubation with 2.5% normal horse serum to block non-specific binding. Sections were incubated overnight at 4°C with an antibody against CYP1B1 (0.6μg/mL, ab32649, Abcam, UK), proliferating cell nuclear antigen (PCNA; 0.2μg/mL, ab2426, Abcam), SERT (1:200, whole antiserum, ab44520, Abcam) or α-smooth muscle cell actin (α-SMA; 0.4μg/mL, ab5694, Abcam). Excess primary antibody was removed by 3 consecutive washes in TBS-T and then incubated with a secondary anti-rabbit antibody for 1 hour at room temperature. Cells positive for CYP1B1 were visualized by VIP (which stains pink; Vector Laboratories, UK) in mice and DAB (which stains dark brown; Vector Laboratories) in rats and humans. Cells positive for PCNA and α-SMA were visualized by DAB. Rat sections were counterstained with hematoxylin. Images were captured using a Zeiss Axio Imager M1. For all antibodies, an IgG control was performed at the same concentration to control for non-specific binding.

PCNA Quantification

The percentage of PCNA expressing nuclei was calculated using a free web application (ImmunoRatio) used for automated analysis of immunostained tissue sections. The application segments diaminobenzidine-stained (DAB) and hematoxylin-stained nuclei regions from the user-submitted image and calculates the percentage of DAB-stained nuclear area over total nuclear area. A total of 3-4 pulmonary arteries were analysed per lung section.

Determination of reactive oxygen species in lung sections by immunofluorescence

Immunohistochemistry of the reactive oxygen species (ROS) marker, 8-hydroxyguanosine (8-OHG) was determined in the lungs of wild-type, $SERT^{+}$ and $SERT^{+}$ TMS treated mice. Sections were deparaffinised in a xylene-ethanol to water gradient. Antigen retrieval was performed by boiling slides in EDTA (pH 8.0) for 15 min prior to a 1 hour incubation in a humidified chamber at room temperature with 10% donkey serum/1% BSA in 1xTBS-T to block non-specific binding. Sections were incubated with an antibody against 8-OHG overnight at 4°C (5µg/mL, ab10802, Abcam). For identification of 8-OHG positive cells, sections were incubated with Alexa-fluor-488-conjugated donkey anti-goat secondary antibody (Molecular probes, A-11055, Life Technologies, UK) for 45 mins at room temperature in the dark. Lipofuscin-mediated auto-fluorescence was removed using 0.1% Sudan Black B (Sigma-Aldrich, UK) in methanol w/v for 10 mins. Slides were mounted using ProLong Gold anti-fade mounting media containing DAPI (Molecular probes, P-36931, Life technologies, UK). Fluorescence imaging was measured in an Axiovert 200M microscope with a laser-scanning module LSM 510 (Carl Zeiss AG, Germany). DAPI was excited at 405 nm and Alexa-fluor 488 at 488 nm. Images were captured using the LSM 510 evaluation software physiology (Zeiss, UK). Mean fluorescence intensity of 8-OHG was measured on at least 5 different calibrated images of lung sections of the different groups at x40 magnification by using Image J software. An auto threshold for each image was applied by using the Huang method in Image J.

Protein analysis

Whole lung samples were homogenized and lysed in ice-cold 0.1% lauryl maltoside solution in PBS (v/v) and protein concentrations were measured by a BCA assay (Thermo Scientific, UK). 30µg of protein was loaded for whole lung homogenates and samples were separated by SDS-PAGE. Protein expression of CYP1B1 (2µg/mL; sc-32882, Santa Cruz

Biotechnology, USA) and SERT expression (2µg/mL; AB1594P, Millipore, UK) was determined by overnight incubation at 4°C and normalized to GAPDH (0.2µg/mL; ab8264, Abcam).

Estrogen immunoassay

The levels of estrogen were determined by competitive based immunoassay in whole lung homogenate samples from female wild-type, $SERT^+$ and $SERT^+$ mice treated with TMS. Lung samples were homogenized in ice-cold 1% lauryl maltoside solution in PBS (v/v) and solubilized on ice followed by cellular fractionation. Protein concentrations were determined by a BCA assay. 400µg of protein was loaded for mouse lung samples and assayed in duplicate as per manufacturer's instructions (Cayman Chemical, Michigan, USA). The plate was read at a wavelength of 405nm for end point measurements (SpectraMax M2 plate reader, Molecular Devices, California, USA). Data was normalized to per µg of protein.

Human pulmonary arterial smooth muscle cells

hPASMCs were provided by Professor Nicholas W. Morrell (University of Cambridge). Female hPASMCs were explanted from the distal pulmonary microvasculature from subjects with no reported presence of PAH. Three independent female cell lines were used in this study and characterized by morphological assessment and staining for the smooth muscle cell marker, α-smooth muscle actin. Assays were performed between passages 2 and 7. Cells were seeded in 24-well plates (cell counts) and 96-well plates (BrdU incorporation) at a density of 7500 cells per cm^2. Cells were grown to ~60% confluency and then cell-cycle synchronized by serum-deprivation (0.2% charcoal-stripped FBS) in phenol-red free DMEM (Invitrogen, UK) for 24 hours.

Charcoal-stripped fetal bovine serum

Fetal bovine serum (FBS; Sera Labs, UK) was charcoal stripped twice to remove estrogens. Dextran-coated charcoal (Sigma-Aldrich, UK) was added to FBS at a concentration of 1g/100mL and left overnight at 4ºC under gentle agitation. Samples were centrifuged at 1811g at 4ºC for 30 minutes. The stripped serum was decanted and filtered through a 0.22µm filter. ELISA analysis of 50µl of FBS confirmed successful removal of estrogen (as described in section above).

Cell proliferation assays

hPASMC proliferation was assessed by two approaches; live cell counts (cell viability) using a haemocytometer and by a BrdU incorporation assay, as per manufacturers protocol (Millipore, UK). Cells were treated for 72 hours, in phenol red free 2% charcoal stripped FBS DMEM with 1nM 16α-OHE1, (Steraloids, Inc., USA) and 0.1µM MPP Dihydrochloride (ERα antagonist, Tocris, UK) or 0.002pg/mL ERα antibody (sc-7207, Santa Cruz Biotechnology, USA). Antagonists/antibodies were added 30 minutes prior to the addition of 16α-OHE1. Cell culture media and drugs were replaced after 48 hours and PASMC proliferation was assessed at 72 hours.

Statistics

Data is represented as the group mean ± SEM. Data was analyzed by either a student's two-tailed t-test or a one-way ANOVA followed by a Tukey's Post Hoc test. The statistical test used is indicated within the figure legends.

Results

Female SERT$^+$ mice develop PH via CYP1B1

Female mice overexpressing the human SERT develop a spontaneous PH phenotype that is dependent on estrogen[13]. As serotonin can modulate CYP1B1 expression[28] and CYP1B1 contributes to the development of PH[5], we investigated whether the development of PH in these mice was related to increased CYP1B1 activity. Overexpression of SERT resulted in elevated RVSPs (reflective of PH) compared to wild-type mice and CYP1B1 inhibition with the highly potent and selective inhibitor TMS attenuated this effect (Fig. 1A). As previously reported, female SERT$^+$ mice did not develop RV hypertrophy despite elevated RVSPs. This is consistent with observations in numerous transgenic models of PH in normoxic conditions[12,18,33-35] and no further effects were observed in mice treated with TMS (Fig. 1B). Overexpression of SERT and CYP1B1 inhibition had no effect on mean SAPs (Fig. S1A). SERT$^+$ mice had lower heart rates compared with wild-type mice and this was normalized by CYP1B1 inhibition (Fig. 1C). SERT$^+$ mice had a significant degree of pulmonary vascular remodelling within the distal vasculature (where arteries are normally composed of a thin vascular layer) and this was attenuated by CYP1B1 inhibition (Fig. 1D).

CYP1B1 expression was increased in SERT$^+$ mice and CYP1B1 inhibition increased estrogen levels in the lung

Overexpression of the human SERT gene in mice increased CYP1B1 protein expression in whole lung homogenates compared with wild-type mice. CYP1B1 expression in SERT$^+$ TMS treated mice was not significantly different from wild-type mice, suggesting that TMS reduced CYP1B1 expression. These findings were confirmed by immunohistochemistry staining for CYP1B1 (Fig. 2A-B).

A recent estrogen metabolic analysis of Cyp1b1 knockout mice reported increased lung concentrations of estrogen in Cyp1b1 knockout mice compared with wild-type mice[36]. CYP enzymes regulate estrogen metabolism and it is therefore plausible that the increase in estrogen concentrations in these mice is attributed to reduced metabolism by CYP1B1. It was therefore of interest to assess estrogen concentrations in the lungs of $SERT^+$ mice to determine the effect of SERT overexpression and CYP1B1 inhibition. Whole lung estrogen concentrations were determined by ELISA and found to be similar in $SERT^+$ mice and wild-type mice. However, CYP1B1 inhibition in $SERT^+$ mice increased estrogen concentrations (Fig. 2C).

Oxidative stress is increased in $SERT^+$ lungs

The formation of ROS by the metabolism of estrogen by CYP1B1 has been previously described[37] and increased oxidative stress may be associated with the pathobiology of PAH[38]. We therefore investigated whether oxidative stress was regulated by CYP1B1 activity in our model by immunohistochemical analysis of 8-OHG (a sensitive measure of ROS. Female $SERT^+$ mice had increased levels of 8-OHG compared to wild-type mice and CYP1B1 inhibition with TMS had no effect on 8-OHG levels (Fig. 3A-B). 8-OHG was expressed within numerous cellular compartments in $SERT^+$ mice treated with vehicle and TMS, including alveoli, bronchi, bronchioles and vessels, whereas it was visually less in control mice (Fig. 3A-B). 8-OHG was observed in both the cytoplasm and the nucleus indicating increased RNA and DNA oxidation.

CYP1B1 inhibition did not improve right ventricular function in MCT-induced PH but prolonged survival

Previous studies have reported increased SERT expression in the MCT model of PH[29]. Because the over-expression of human SERT increased CYP1B1 expression in our mouse model, we rationalised that CYP1B1 expression would also be increased by MCT.

Whole lung expression of SERT protein was increased in female but not in male rats (Fig. 4A). However, CYP1B1 protein expression (Fig. 4B) was unchanged in the lungs from both male and female rats that had been treated with MCT for 28 days (all tissue examined was taken from animals that survived until day 28). MCT induced significant increases in RVSP (Fig. 5A) and RV hypertrophy (Fig. 5B). TMS had no effects on RVSP (Fig. 5A) and only moderate, yet significant effects in reducing RV hypertrophy in male rats, whilst no effects were observed in female rats (Fig. 5B). Male rats developed more severe RV hypertrophy in comparison with female rats in response to MCT, despite developing a similar degree of increased RVSP (Fig. 5A-B). MCT and TMS treatment had no effects on mean SAPs in female and male rats (Fig. S2A). MCT lowered the heart rate in male rats and this was normalized by TMS to pressures similar to vehicle-treated rats. No changes in heart rate were observed in female rats (Fig. 5C). Mortalities were reported 23 and 26 days after MCT administration in male and female rats, respectively. TMS prolonged survival in male rats and no mortalities were reported in female rats treated with TMS two weeks after administration with MCT (Fig. 5D). MCT induced a significant degree of pulmonary vascular remodelling in the distal vasculature and this was not affected by CYP1B1 inhibition (Fig. 6A-B). CYP1B1 immuno-positive cells were predominantly observed in pulmonary arterial endothelial cells (Fig. 6C).

The CYP1B1 metabolite 16α-OHE1 stimulates PASMC proliferation via ERα

PASMC proliferation is an established and dominant feature associated with PAH pathobiology[26,27] and we have previously shown that estrogen induces hPASMC proliferation via CYP1B1[5]. Using immunohistochemistry, we determined that both SERT and CYP1B1 are highly expressed in α-SMA positive cells (PASMCs) in pulmonary arteries from patients with PAH, whereas minimal expression is observed in non-PAH arteries (Fig. 7). SERT overexpression in mice increased the expression of CYP1B1, suggesting that the PH

phenotype observed in these mice may be mediated by increased cellular proliferation. To investigate this, we performed immunohistochemistry analysis of the DNA replication marker PCNA. PCNA expression was increased in $SERT^+$ and this was attenuated by CYP1B1 inhibition (Fig. 8A-B). We next investigated whether the proliferative CYP1B1 estrogen metabolite 16α-OHE1 promoted proliferation via the estrogen receptor ERα by selectively blocking ERα by a specific antagonist and antibody. 16α-OHE1 induced significant increases in PASMC proliferation and this was completely attenuated by blocking ERα signalling using both a specific antagonist and antibody, as assessed by cell number (Fig. 8C, E) and BrdU incorporation (Fig. 8D, F). To prove that these effects were directly related to an inhibition of 16α-OHE1 signalling through ERα and not by indirect effects mediated by the antagonist or antibody, we stimulated hPASMCs with each treatment alone and observed no effects (data not shown).

Discussion

The incidence of PAH is more common in women than in men[1-3]. For example, in the largest PAH registry in the World, the Registry to Evaluate Early and Long-Term PAH Disease Management (REVEAL), more than 80% of patients diagnosed were females[3]. This suggests that estrogens and/or its metabolites may be associated with the pathobiology of PAH. Understanding the biological basis of this gender disparity may offer a new treatment paradigm in this devastating cardiovascular disorder that currently has a high unmet clinical need.

We have previously shown that serotonin can upregulate the expression of CYP1B1 in hPASMCs[13]. Accordingly, female mice that overexpress the human SERT gene have increased CYP1B1 expression and inhibition of CYP1B1 reduces CYP1B1 expression and can attenuate PH in these mice. This is consistent with the literature, where TMS has been shown to inhibit CYP1B1 mRNA and protein expression in a concentration-dependent manner[39]. Of note, female $SERT^{+}$ mice do not develop RV hypertrophy in the face of increased RVSP and muscularization of the pulmonary arteries. This is consistent with numerous transgenic models where mice develop a spontaneous PH phenotype in normoxic conditions[12,18,33-35]. Although the reasons for this observation in the PH field remains undetermined, we speculate that this may be related to an inability of mice to develop compensatory RV hypertrophy in the face of moderate PH.

In the 1980's there was a PAH epidemic in women that had taken the anorexigens, dexfenfluramine and aminorex[40,41]. Both of these drugs are substrates for SERT and act as indirect serotinergic agonists[42]. This outbreak formulated the 'serotonin hypothesis of PAH' and suggested a direct interaction between SERT and the development of PAH. More recently, we have shown that administration of dexfenfluramine in mice results in the development of PH in female mice only and this is dependent on intact ovaries and

CYP1B1[20]. These observations suggest that SERT facilitates the development of estrogen-related PH via CYP1B1. This may be relevant to human PAH as CYP1B1 is both highly up-regulated in disease[5] and polymorphisms of CYP1B1 confer increased susceptibility amongst patients with a BMPR2 mutation[7]. In addition, we recently demonstrated that proliferation of female hPASMCs is increased via a reduction in BMPR2 signalling and that the CYP1B1 metabolite 4-hydroxyestradiol can modulate BMPR2 signalling in a gender specific fashion[12].

Overexpression of SERT increased CYP1B1 expression *in* vivo and this was accompanied by increased expression of the proliferative marker PCNA. Inhibition of CYP1B1 in $SERT^+$ mice reduced CYP1B1 and PCNA expression in the pulmonary arteries and the degree of pulmonary vascular remodelling. Estrogen promotes proliferation of hPASMCs[5,13] and CYP1B1 inhibition can attenuate this effect in a dose-dependent fashion[5]. Proliferative screens of the most prominent CYP1B1 metabolites of estrogen revealed that the 16α-hydroxylated estrogens promote PASMC proliferation[5]. Inhibition of estrogen synthesis and its receptor ERα can attenuate experimental PH in female rodents[6,43]. ERα is predominantly regarded as a nuclear receptor, but its expression may also be observed at the cell membrane. Upon ligand binding, the nuclear receptor dimerises and translocates to the nucleus where it acts as a regulator of gene transcription. Previous studies have shown that BMPR2 mutant pulmonary microvascular endothelial cells signal through non-canonical pathways by reduced trafficking of ERα to the cell nucleus[8]. Furthermore, ERα can suppress the transcriptional activation of the BMPR2 gene by binding to a conserved estrogen response element within its promoter[8]. Here, we show that ERα mediates the proliferative effects of the CYP1B1 metabolite 16α-OHE1. This is of interest as the concentrations of 16α-OHE1 are elevated in the urine of mice exposed to hypoxia[5], BMPR2 mutant mice[9] and importantly also in patients with PAH[7]. We rationalized to focus our studies on ERα given its more prominent role in the pathobiology of PAH[6,8,43,44]. However it should be noted that 16α-

OHE1 has a high binding affinity for ERβ[45] and may therefore also be signalling through this receptor.

The role for estrogen in the pathobiology of PAH has been met with much speculation and controversial data. Indeed numerous pre-clinical studies have reported compelling evidence that estrogen mediates protective effects in the cardiopulmonary unit[13,14,46,47]. Estrogen is a potent vasodilator in the pulmonary vascular bed[48] and physiological increases in circulating estrogens (the estrous cycle) can attenuate pulmonary arterial vasoconstriction under both normoxic and hypoxic conditions[49]. On the contrary, endogenous synthesis of estrogen contributes to the development of PH and associated RV hypertrophy in intact female rodents[6]. Moreover, there is a positive correlation between circulating estrogen levels and the degree of RV hypertrophy suggesting an association between estrogens and disease severity[6]. The estrogen synthesizing enzyme aromatase is expressed within the smooth muscle cell layer of the pulmonary arterial wall[6], suggesting that estrogens can be produced locally. Local estrogens would be predicted to have a much more profound effect than the impact of the relatively low concentrations circulating in the blood stream[17]. Here, we show that CYP1B1 inhibition increases estrogen concentrations in the lung, which is consistent with a previous study in Cyp1b1 knockout mice[36]. This suggests that the pathobiology behind estrogen-associated PAH may be, at least in part, mediated by its downstream metabolism.

Increasing evidence suggests that altered redox signaling may be associated with the pathobiology of PAH and elevated ROS has been observed in numerous animal models and in patients with PAH[50]. Estrogen metabolism by CYP1B1 to the 4-hydroxylated estrogens is associated with the formation of ROS which can generate quinones and semi-quinones that can mediate DNA damage by the formation of DNA adducts[51,52]. We had therefore anticipated that CYP1B1 inhibition might alleviate oxidative stress. Although we observed increased ROS expression in $SERT^+$ mouse lungs, this was unaffected by CYP1B1 inhibition,

suggesting that other pathways unrelated to CYP1B1 activity contribute to the elevated oxidative stress in $SERT^{+}$ mice.

The MCT rat model has been and continues to be used as a model of PH despite having received criticism as a pre-clinical model of PAH[53]. However, previous studies have shown that MCT increases lung expression of SERT and that inhibition of SERT can prevent the development of MCT-induced PH[29]. We therefore questioned whether this was related to elevated CYP1B1 activity and hypothesized that CYP1B1 inhibition may be effective in reversing MCT-induced PH. Although we observed an increase in SERT expression in female rats, this was not accompanied by an increase in CYP1B1 expression. This is likely due to the relatively moderate increase of SERT expression in comparison to the mouse model overexpressing SERT. The lack of increased CYP1B1 expression and the different cellular localization compared with mouse and humans (endothelial vs. smooth muscle cell) may underlie the relatively mild therapeutic effects observed. In male rats, CYP1B1 inhibition reduced RV hypertrophy and improved survival. In female rats, no effect was reported on PH indices, yet a small improvement in survival was observed. Clinically, survival in males with PH is worse than in females and this is thought to be related to a worse RV function[54] and indeed, RV function is a strong predictor of survival[55]. In this study, the degree of RV hypertrophy was more profound in male rats treated with MCT compared with females and this was associated with poorer survival. MCT is bioactivated by CYPs to its active compound MCT pyrrole in the liver. Sex-specific CYPs may regulate MCT metabolism in the liver and underlie the gender difference in response to MCT. Of interest, the exogenous administration of estrogen has been shown to be therapeutic in MCT-treated rats by activation of ERβ[16]. This may explain the reduced degree of RV hypertrophy and the increased survival in female rats compared with male rats. However, we have recently reported pathogenic effects of endogenous estrogens in females in other models of PH[6]. The

influence of estrogens on RV function is still controversial in the literature. In healthy females on hormone replacement therapy, increased estrogen levels correlated with an improved RV function[56]. However, recently it has been show that men with idiopathic PAH have elevated estrogen levels and this is correlated with a poorer RV function and six minute walking test[57].

Serotonin and SERT are mediators in the pathogenesis of PAH. Over-activity of the estrogen and serotonergic pathways may be associated with the development of PAH, particularly in women, by increasing CYP1B1 expression promoting pro-proliferative estrogen metabolism. Targeting CYP1B1 is currently being explored therapeutically in clinical trials in cancer patients, using a vaccination with a DNA plasmid encoding an inactivated form of CYP1B1 (ZYC300). Upon delivery, the immune system elicits a cytotoxic T lymphocyte mediated-response against CYP1B1 resulting in the lysis of CYP1B1 expressing cells[58,59]. This may be a strong therapeutic candidate in female patients that have elevated CYP1B1 expression. The subset of patients that would respond to this therapy could be identified by measuring the urinary 2-OHE1/16α-OHE1 ratio, as previously reported[7]. Current PAH therapies do not encompass this well-defined gender disparity that occurs in PAH, or the potential divergent response to current therapies that may be affected by gender. Future therapies that target dysregulated estrogen metabolism may have a favourable outcome in this highly neglected, devastating cardiovascular disease.

Acknowledgements

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Figure Legends

Figure 1. Inhibition of CYP1B1 with 2,3',4,5'-tetramethoxystilbene (TMS) attenuates PH in female SERT$^+$ mice.

Female SERT$^+$ mice were injected with 1.5mg/kg i.p. TMS for two-weeks and compared with wild-type (C57Bl/6J CBA) and SERT$^+$ vehicle treated mice for its effects on PH indices. (A) RVSP measurement per group as measured by transdiaphragmatic puncture; n=9-10 (B) Right ventricular hypertrophy expressed as the ratio of the weight of the right ventricle (RV) over the left ventricle + septum (LV+S); n=10-11. (C) Heart rate; n=7-9. (D) Pulmonary vascular remodeling; n=5-6. (E) Representative images of pulmonary vascular remodeling in arteries for each group. Scale bar = 20μm. Data was analyzed by a one-way ANOVA followed by a Tukey's post-hoc test. *P<0.05, **P<0.01, ***P<0.001.

Figure 2. CYP1B1 expression is increased in female SERT$^+$ mice and CYP1B1 inhibition increases estrogen concentrations in lung homogenates.

Female SERT$^+$ mice were injected with 1.5mg/kg i.p. 2,3',4,5'-tetramethoxystilbene (TMS) for two-weeks and compared with wild-type (C57Bl/6J CBA) and SERT$^+$ vehicle treated mice. (A) Whole lung homogenates (30μg) were analyzed for CYP1B1 expression by western blotting and normalized to GAPDH, n=5-8. (B) Representative CYP1B1 immunolocalization in pulmonary arteries as determined by VIP positive cells (pink). Scale bar = 20μm. (C) Estrogen concentrations as determined by ELISA in 400μg of whole lung homogenates; n=4-5. Data was analyzed by a one-way ANOVA followed by a Tukey's post-hoc test. ***P<0.001.

Figure 3. SERT$^+$ mice have increased RNA and DNA oxidative damage in the lungs.

(A) Lung sections obtained from wild-type, SERT$^+$ and SERT$^+$ mice treated with the CYP1B1 inhibitor 2,3',4,5'-tetramethoxystilbene (TMS) were immunostained for the

oxidative stress marker 8-hydroxyguanosine (8-OHG). Sections were counterstained with the nuclear stain, DAPI. Scale bar = 50μm. (B) 8-OHG mean fluorescence intensity was measured in whole lung sections using Image J software analysis; n=4-5. Data was analyzed by a one-way ANOVA followed by a Tukey's post-hoc test. *P<0.05, **P<0.01.

Figure 4. SERT and CYP1B1 expression in monocrotaline (MCT) treated rats

Female and male rats were given a single dose of MCT (60mg/kg s.c.) and 28 days later whole lung homogenates were analysed for protein expression by western blotting for (A) SERT, n=6 and (B) CYP1B1, n=8. Data was normalised to GAPDH. Data was analyzed by an unpaired t-test. *P<0.05.

Figure 5. Inhibition of CYP1B1 with 2,3',4,5'-tetramethoxystilbene (TMS) does not reverse monocrotaline (MCT)-induced PH yet improves survival.

Female and male rats were given a single dose of MCT (60mg/kg s.c.) and 14 days later were given TMS (3mg/kg/day i.p.) or vehicle (~5% ethanol in saline) for an additional 14 days and then assessed for indices of PH. (A) Right ventricular systolic pressures (RVSP) as assessed by jugular vein cannulation; n=5-11. (B) Right ventricular hypertrophy as assessed by the weight of the right ventricle (RV) over the left ventricle + septum (LV+S); n=9-14. (C) Heart rate; n=6-12. (D) Kaplan Meier survival analysis in rats treated with MCT (total of 16 animals per group at the start of the experiment). Data was analyzed by a one-way ANOVA followed by a Tukey's post-hoc test. *P<0.05, **P<0.01, ***P<0.001 vehicle vs. MCT; $^{\#}$P<0.05 male vs. female.

Figure 6. Inhibition of CYP1B1 with 2,3',4,5'-tetramethoxystilbene (TMS) does not reverse monocrotaline (MCT)-induced pulmonary arterial remodelling

Female and male rats were given a single dose of MCT (60mg/kg s.c.) and 14 days later were given TMS (3mg/kg/day i.p.) or vehicle (~5% ethanol in saline) for an additional 14 days and

then assessed for indices of PH. (A) Pulmonary vascular remodeling; n=6-7. Data was analyzed by a one-way ANOVA followed by a Tukey's post-hoc test. **P<0.01, ***P<0.001. (B) Representative pulmonary arteries for each group. Scale bar = 20μm. (C) CYP1B1 immunolocalization in representative pulmonary arteries indicates positive cells in the endothelial cell layer and peri-adventitial layer as visualised by DAB (dark brown). Sections were counterstained with haematoxylin. Scale bar = 50μm.

Figure 7. SERT and CYP1B1 are expressed in α-smooth muscle actin positive cells in human pulmonary arteries

Immunolocalization of SERT, CYP1B1 and α-smooth muscle actin (α-SMA) in human pulmonary arteries of non-PAH and PAH lung sections as visualised by DAB (dark brown). Lung samples were from a female age 26 with heritable PAH and a female age 51 with idiopathic PAH. Scale bar = 200μm.

Figure 8. The CYP1B1 metabolite 16α-hydroxyestrone (16α-OHE1) induces cell proliferation via estrogen receptor α (ERα).

(A) Representative images of immunohistochemistry analysis of the DNA replication marker, PCNA in wild-type, $SERT^{+}$ mice treated with vehicle and the CYP1B1 inhibitor, 2,3',4,5'-tetramethoxystilbene TMS. Scale bar = 20μm. (B) Percentage PCNA positive nuclei. n=3-6; 3-4 pulmonary arteries analyzed per lung. Data was analyzed by a one-way ANOVA followed by a Tukey's post hoc test . ***P<0.001. Female human pulmonary arterial smooth muscle cells (PASMCs) were incubated with 16α-OHE1 for 72 hours in 2% charcoal stripped serum in the presence or absence of ERα inhibition and assessed for proliferation by cell counts and BrdU incorporation. 16α-OHE1 induced PASMC proliferation in the presence of a selective ERα antagonist MPP dihydrochloride (0.1μM) as assessed by (C) cell counts; n=15 replicates per group (D) and BrdU incorporation; n=11 replicates per group. 16α-OHE1 induced PASMC proliferation in the presence of a specific ERα antibody (0.002pg/mL) as

assessed by (E) cell counts; n=6-9 replicates per group and (F) BrdU incorporation; n=16 replicates per group. Data was analyzed by a one-way ANOVA followed by a Tukey's post hoc test. **P<0.01, ***P<0.001.

Figure S1. Inhibition of CYP1B1 with 2,3',4,5'-tetramethoxystilbene (TMS) has no effects on mean systemic arterial pressures (SAP).

Female $SERT^+$ mice were injected with 1.5mg/kg i.p. TMS for two-weeks and compared with wild-type (C57Bl/6J CBA) and $SERT^+$ vehicle treated mice. Mean SAPs were measured by cannulation of the carotid artery; n=7-10. Data was analyzed by a one-way ANOVA followed by a Tukey's post-hoc test.

Figure S2. Inhibition of CYP1B1 with 2,3',4,5'-tetramethoxystilbene (TMS) and monocrotaline (MCT) has no effects on mean systemic arterial pressures (SAP).

Female and male rats were given a single dose of MCT (60mg/kg s.c.) and 14 days later were given TMS (3mg/kg/day i.p.) or vehicle (~5% ethanol in saline) for an additional 14 days. Mean SAPs were measured by cannulation of the carotid artery; n=5-11. Data was analyzed by a one-way ANOVA followed by a Tukey's post-hoc test.

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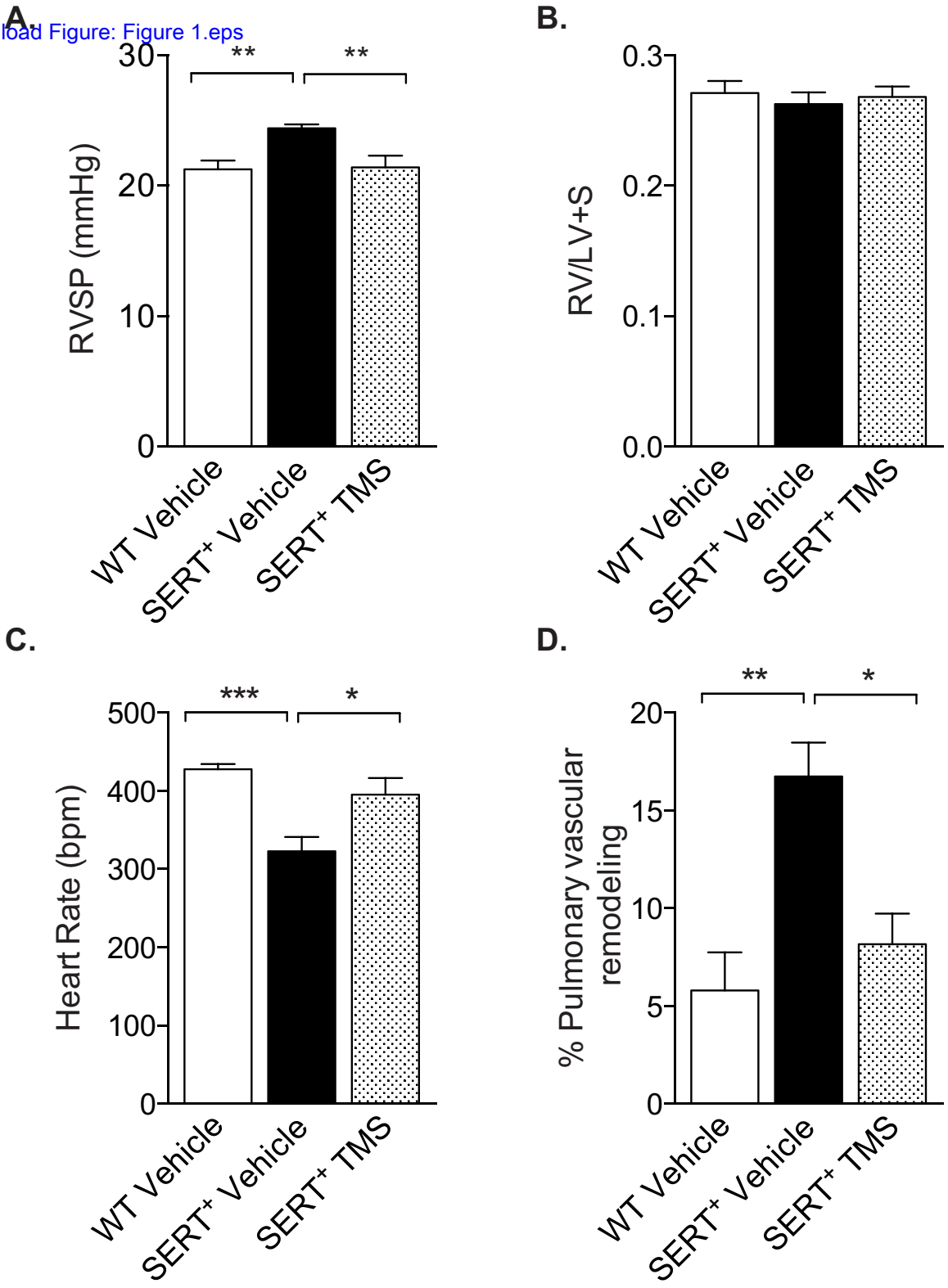

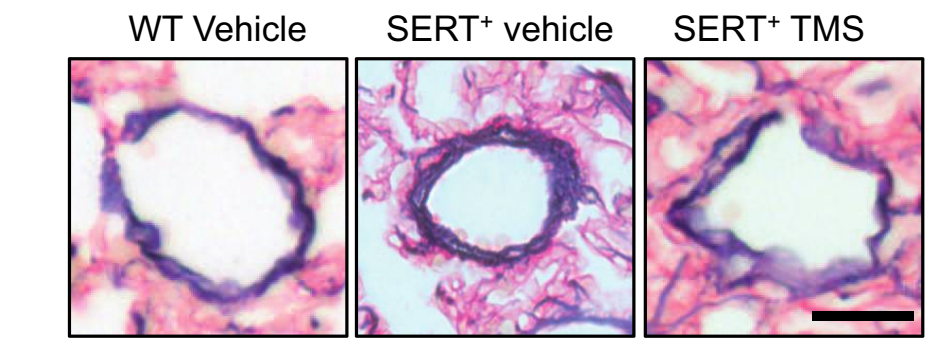

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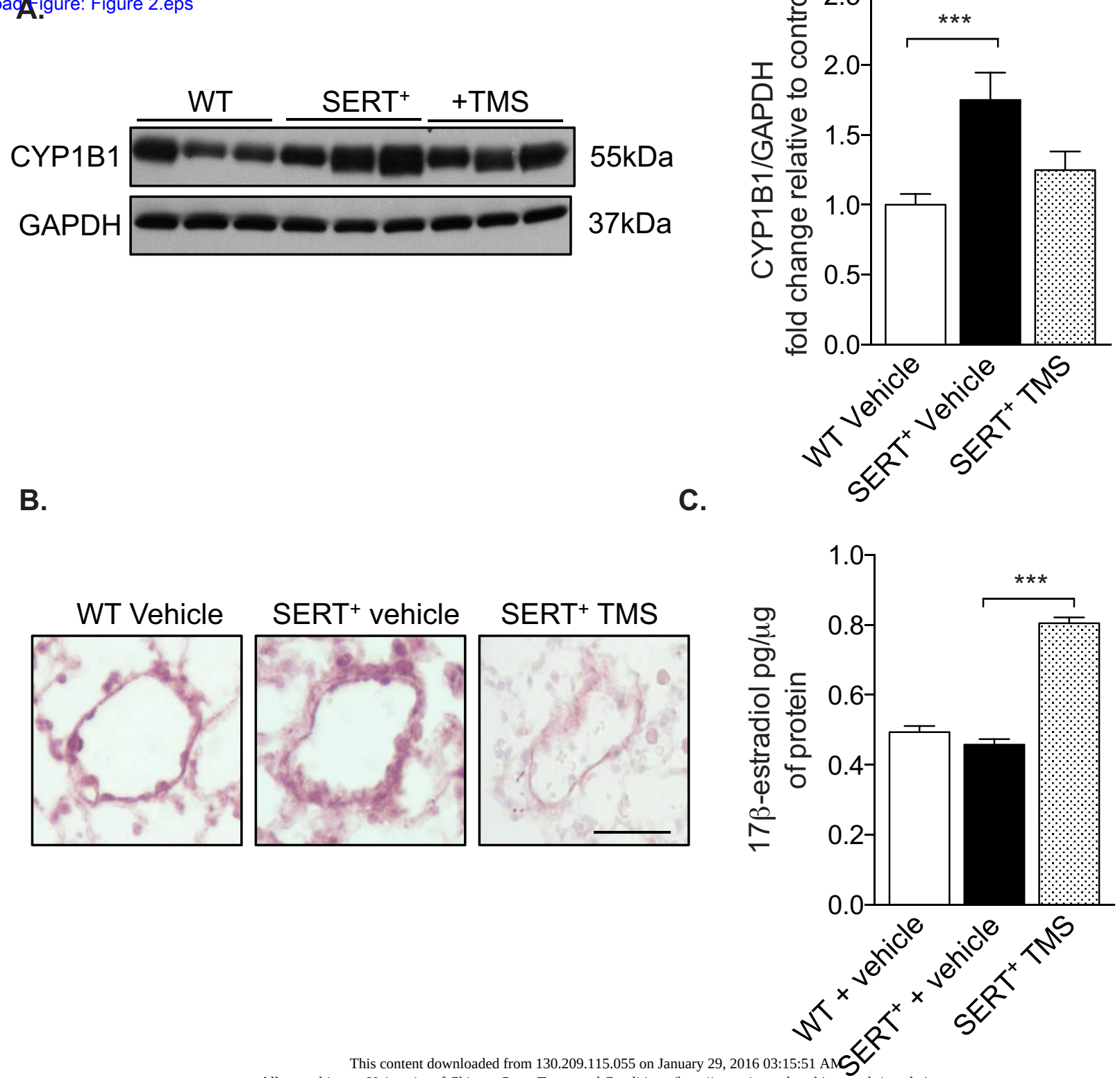

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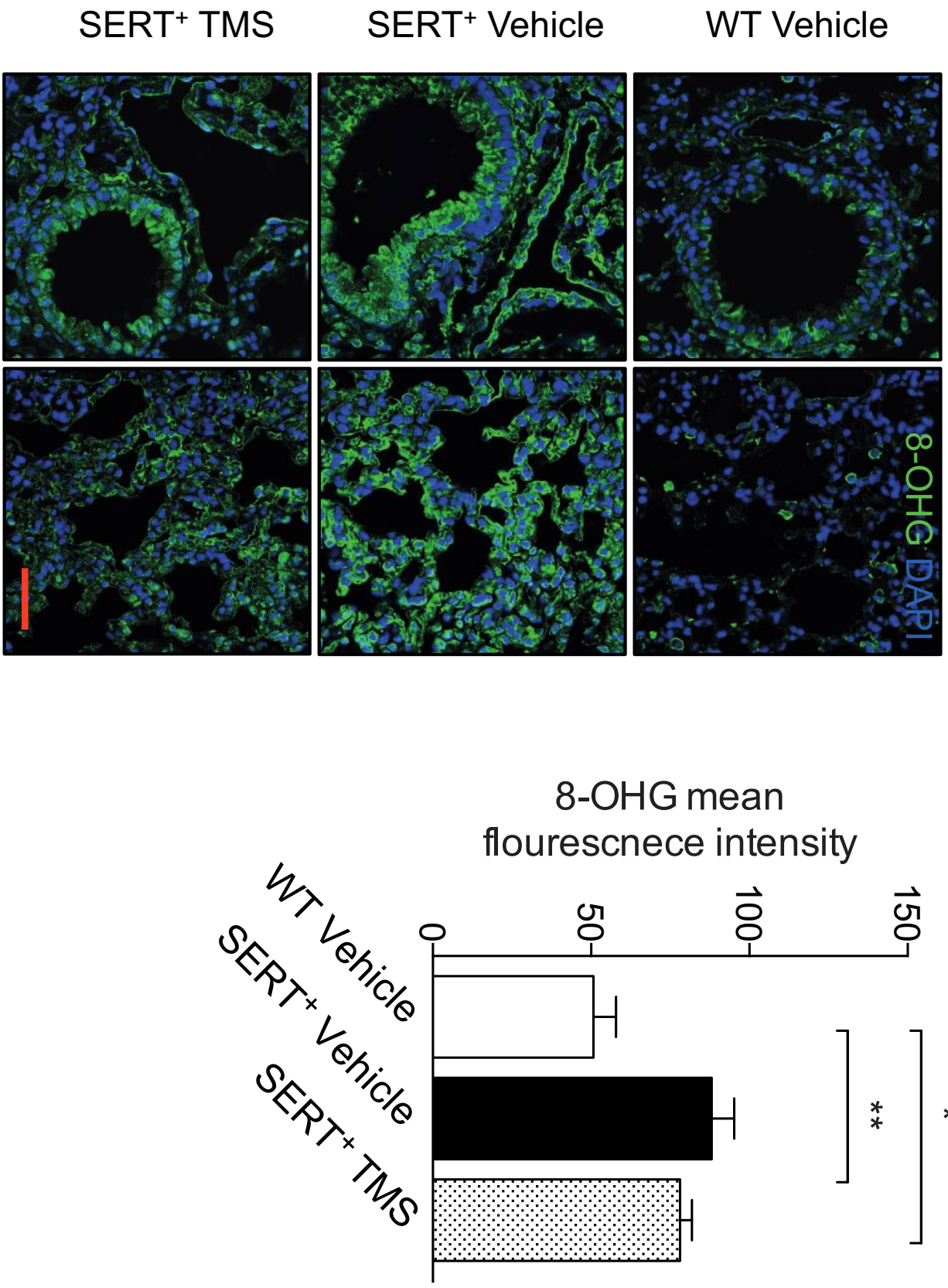

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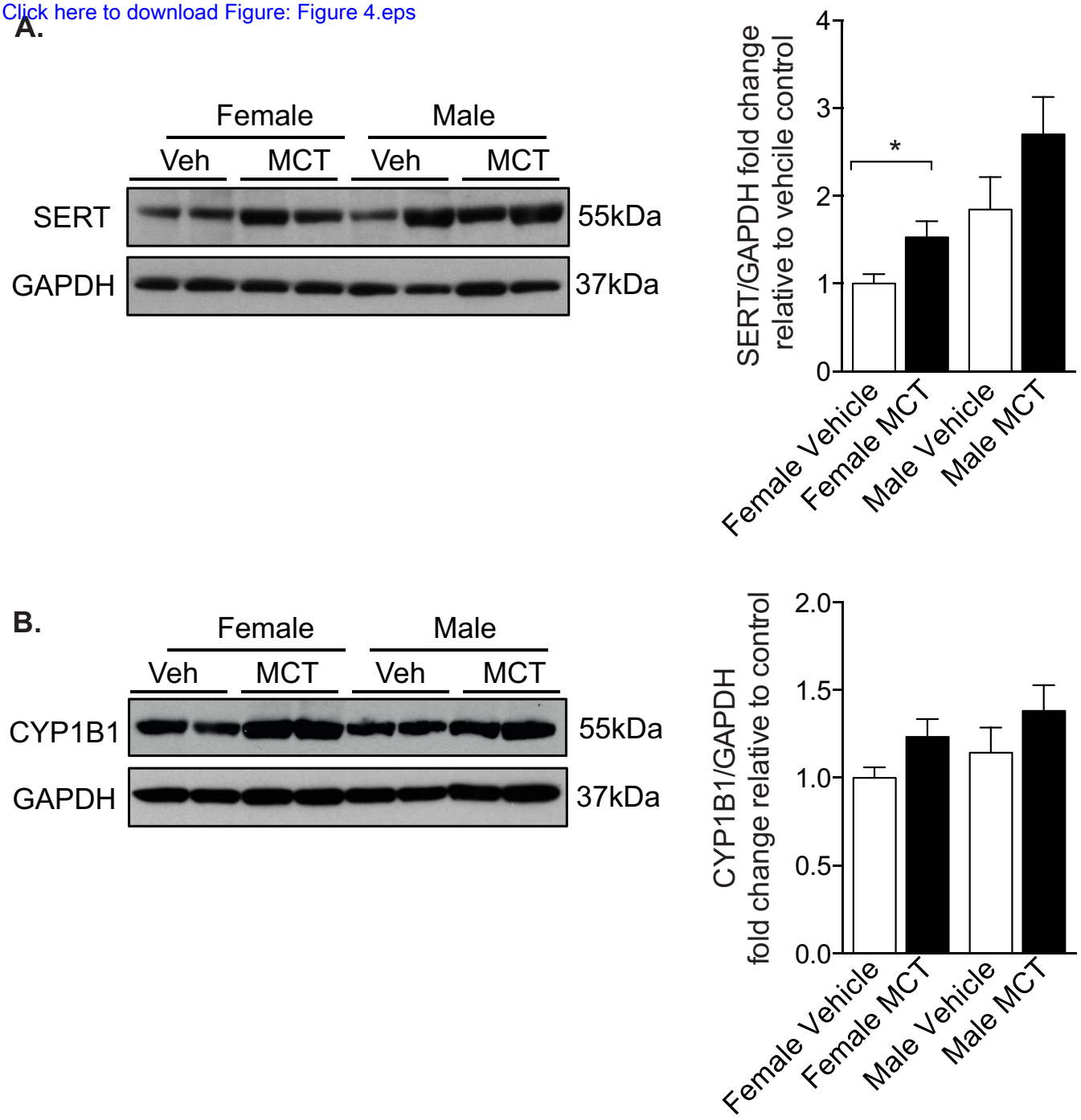

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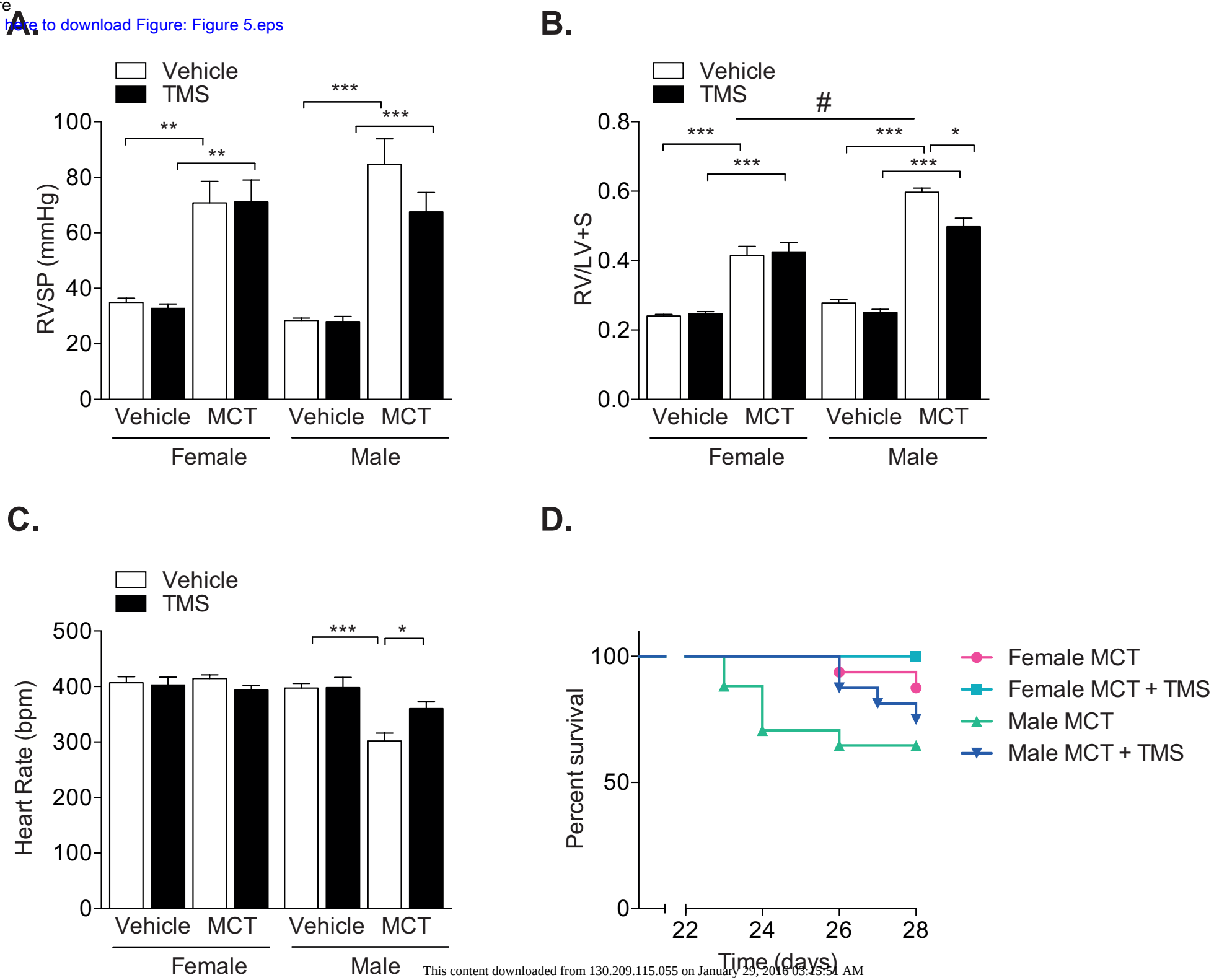

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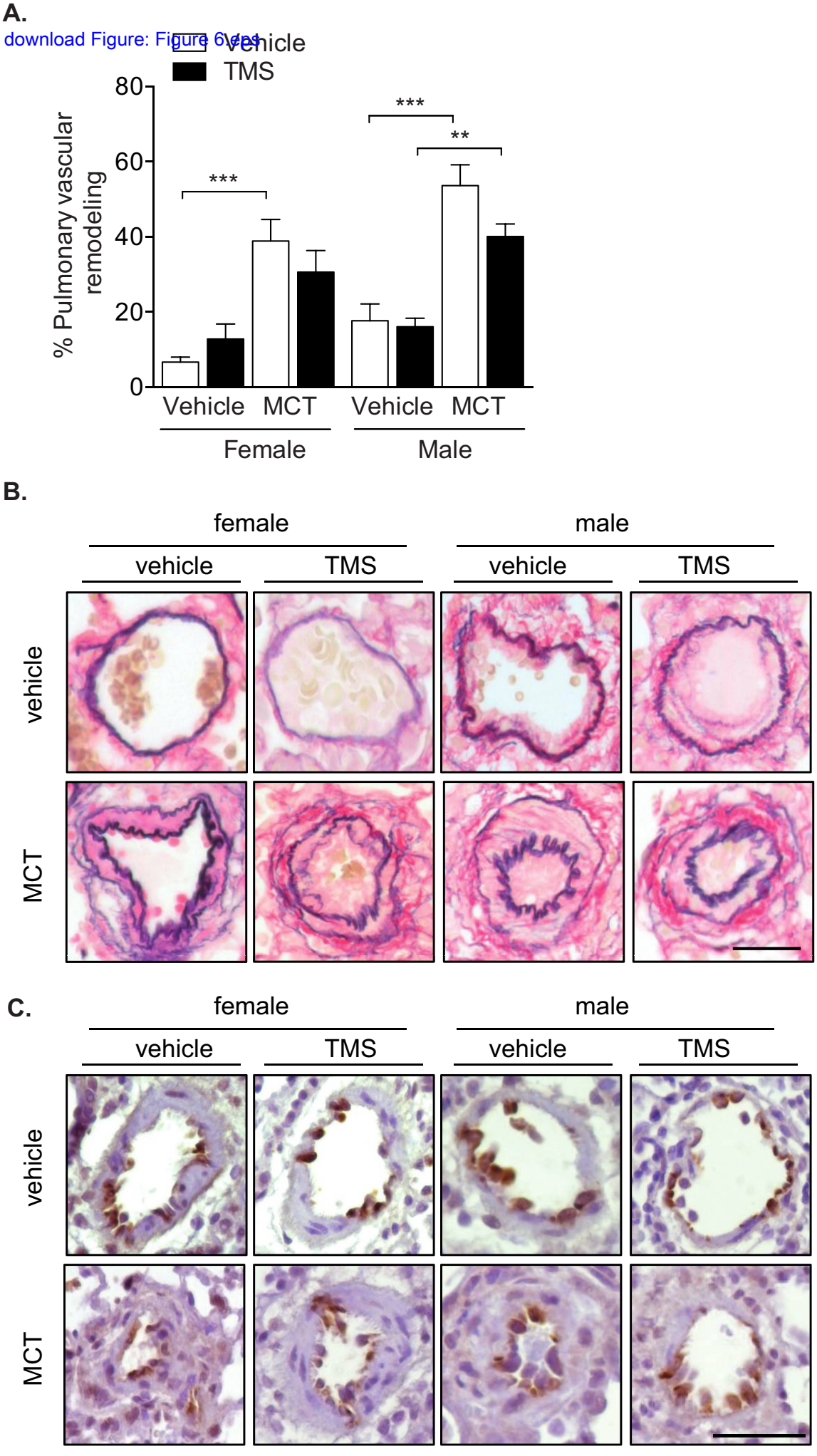

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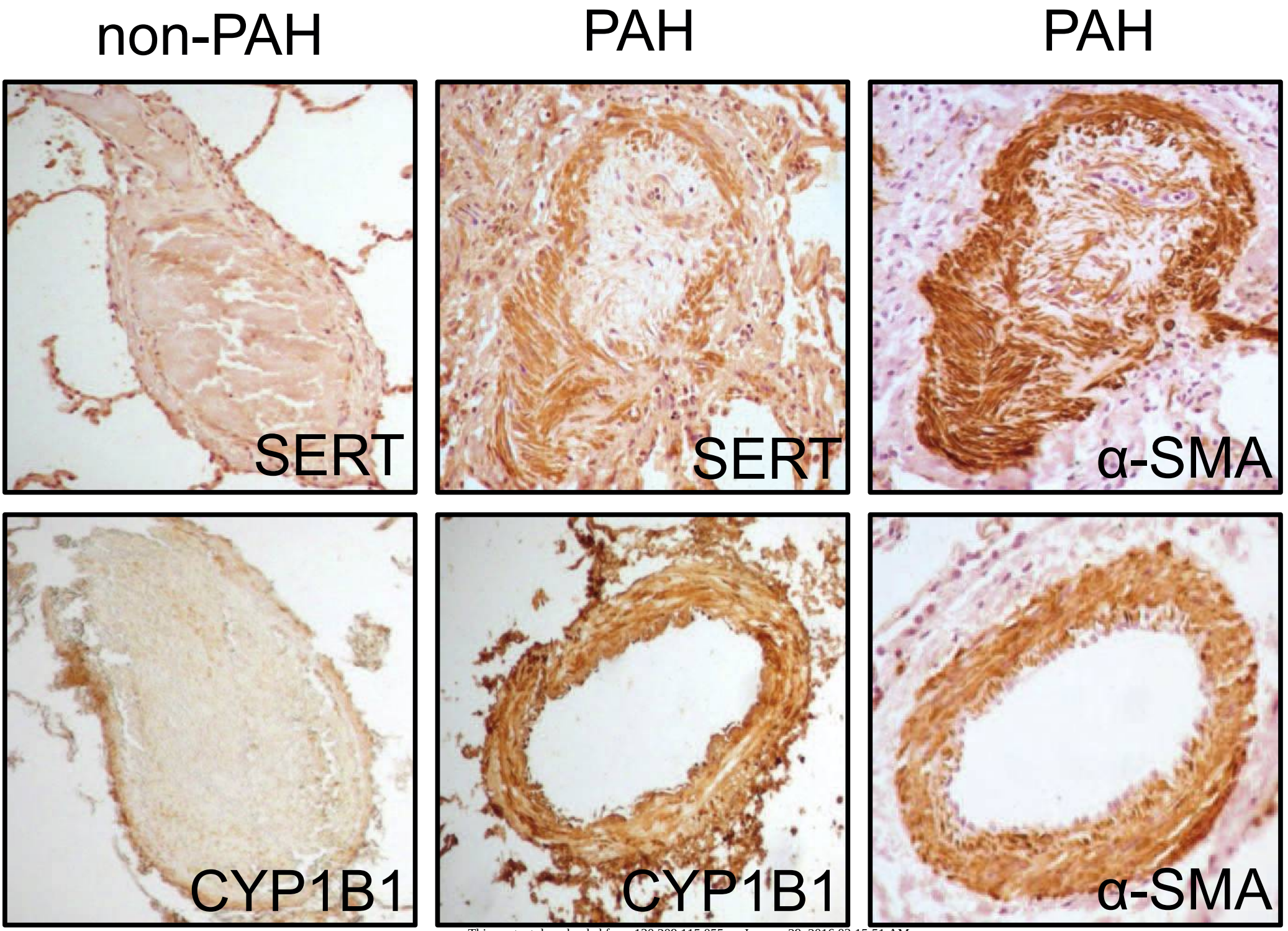

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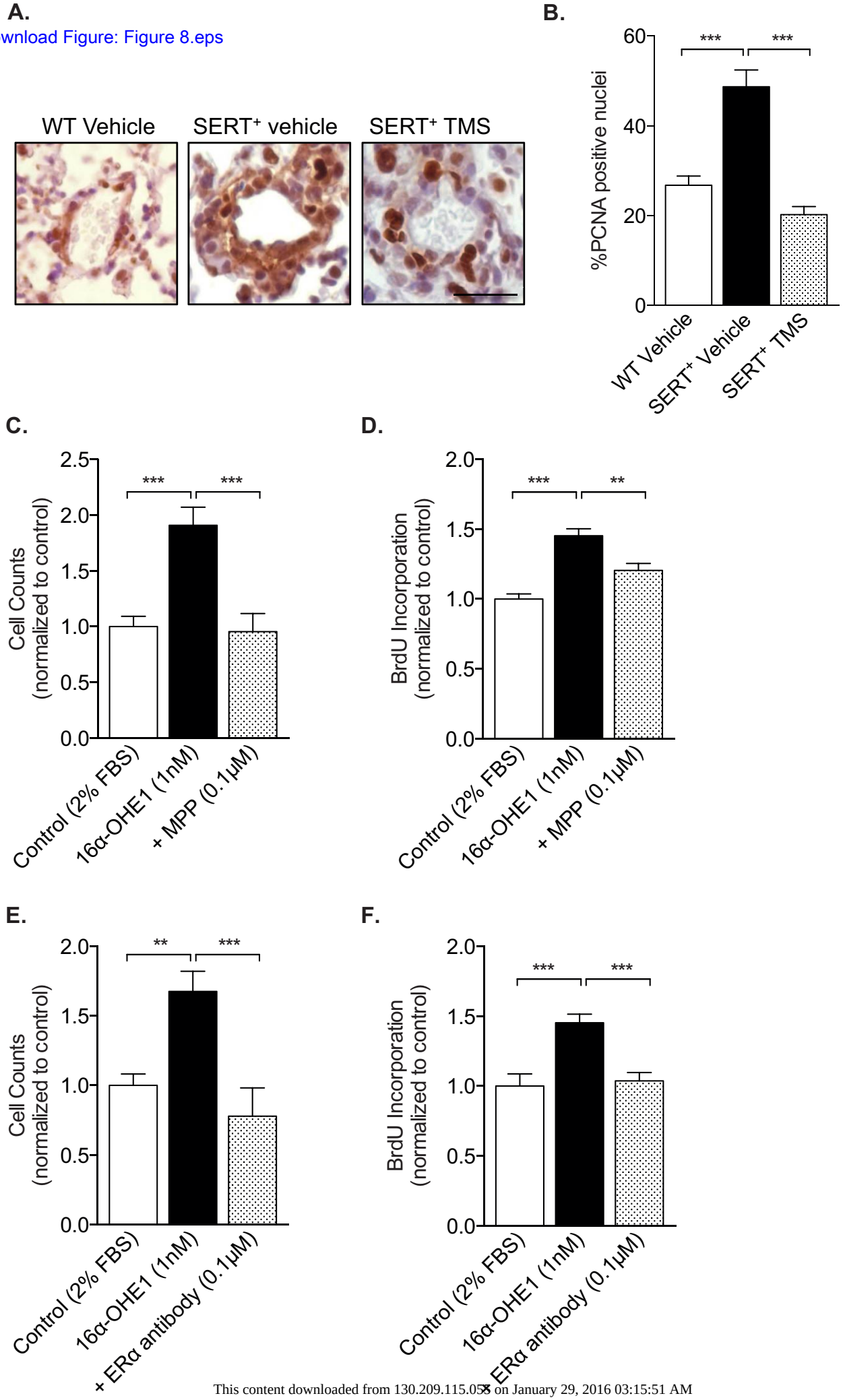

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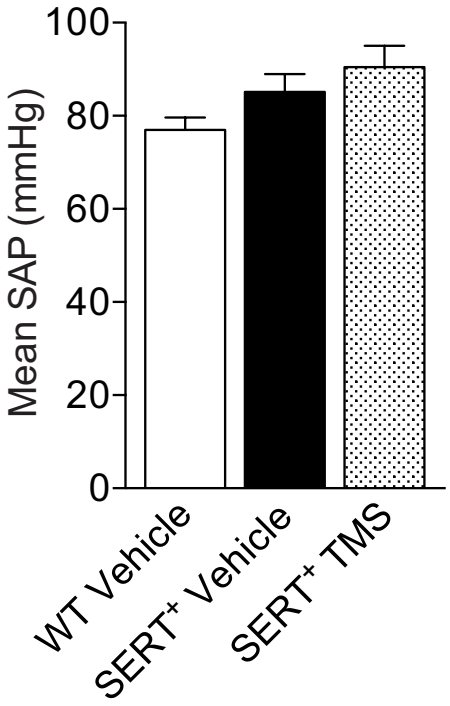
A.



Figure

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