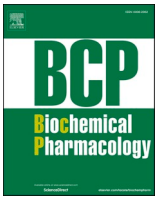
Biochemical Pharmacology 219 (2024) 115916

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm



Deletion of vascular thromboxane A_2 receptors and its impact on angiotensin II-induced hypertension and atherosclerotic lesion formation in the aorta of Ldlr-deficient mice

Heike Braun [a,1], Michael Hauke [a,b,1], Markus Petermann [a,1], Robert Eckenstaler [a], Anne Ripperger [a], Edzard Schwedhelm [c,d], Beatrice Ludwig-Kraus [e], Frank Bernhard Kraus [e], Md Jalal Ahmed Shawon [a], Virginie Dubourg [f], Alma Zernecke [g], Barbara Schreier [f], Michael Gekle [f], Ralf A. Benndorf [a,*]

[a] Department of Clinical Pharmacy and Pharmacotherapy, Institute of Pharmacy, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany
[b] Center for Translational Medicine, Department of Neurology and Pain Therapy, Brandenburg Medical School, Rüdersdorf, Germany
[c] Institute of Clinical Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[d] German Centre for Cardiovascular Research (DZHK), Partner Site Hamburg/Kiel/Lübeck, Hamburg, Germany
[e] Central Laboratory, University Hospital Halle (Saale), Halle (Saale), Germany
[f] Julius-Bernstein-Institute of Physiology, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany
[g] Institute of Experimental Biomedicine, University Hospital Würzburg, Würzburg 97080, Germany

ARTICLE INFO

Keywords:
Angiotensin II
Thromboxane A_2 receptor
Conditional thromboxane A_2 receptor knockout mice
Endothelial cells
Vascular smooth muscle cells
Endothelial dysfunction
Atherosclerosis
Ldlr knockout mice

ABSTRACT

The thromboxane A_2 receptor (TP) has been shown to play a role in angiotensin II (Ang II)-mediated hypertension and pathological vascular remodeling. To assess the impact of vascular TP on Ang II-induced hypertension, atherogenesis, and pathological aortic alterations, i.e. aneurysms, we analysed Western-type diet-fed and Ang II-infused $TP^{VSMC\ KO}$/Ldlr KO, $TP^{EC\ KO}$/Ldlr KO mice and their respective wild-type littermates (TP^{WT}/Ldlr KO). These analyses showed that neither EC- nor VSMC-specific deletion of the TP significantly affected basal or Ang II-induced blood pressure or aortic atherosclerotic lesion area. In contrast, VSMC-specific TP deletion abolished and EC-specific TP deletion surprisingly reduced the *ex vivo* reactivity of aortic rings to the TP agonist U-46619, whereas VSMC-specific TP knockout also diminished the *ex vivo* response of aortic rings to Ang II. Furthermore, despite similar systemic blood pressure, there was a trend towards less atherogenesis in the aortic arch and a trend towards fewer pathological aortic alterations in Ang II-treated female $TP^{VSMC\ KO}$/Ldlr KO mice. Survival was impaired in male mice after Ang II infusion and tended to be higher in $TP^{VSMC\ KO}$/Ldlr KO mice than in TP^{WT}/Ldlr KO littermates. Thus, our data may suggest a deleterious role of the TP expressed in VSMC in the pathogenesis of Ang II-induced aortic atherosclerosis in female mice, and a surprising role of the endothelial TP in TP-mediated aortic contraction. However, future studies are needed to substantiate and further elucidate the role of the vascular TP in the pathogenesis of Ang II-induced hypertension, aortic atherosclerosis and aneurysm formation.

Abbreviations: Ang II, Angiotensin II; apoE, Apolipoprotein E; AT1R, Angiotensin II subtype 1 receptor; CD31, Cluster of differentiation 31; EC, Endothelial cell(s); EGFR, Epidermal growth factor receptor; HDL, High density lipoprotein; LDL, Low-density lipoprotein; KCl, Potassium chloride; KO, knockout; Ldlr, Low-density lipoprotein receptor; SM22α, Smooth Muscle Protein 22-α; $TP_{\alpha/\beta}$, Thromboxane A_2 receptor (α/β isoforms); TxA_2, Thromboxane A_2; TEK/TIE2, TEK tyrosine kinase/tyrosine kinase with Ig and EGF homology domains 2; VSMC, Vascular smooth muscle cell(s).

* Corresponding author at: Department of Clinical Pharmacy and Pharmacotherapy, Institute of Pharmacy, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Str. 3, Halle (Saale) D-06120.
E-mail address: ralf.benndorf@pharmazie.uni-halle.de (R.A. Benndorf).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bcp.2023.115916
Received 28 August 2023; Received in revised form 8 November 2023; Accepted 9 November 2023
Available online 17 November 2023

1. Introduction

Atherosclerosis is a widespread disease in Western industrialized nations [1]. Typical manifestations and complications of atherosclerosis include coronary artery disease and myocardial infarction, cerebrovascular disease and stroke as well as aortic aneurysm formation and aortic dissection [1]. Atherosclerosis arises particularly under the influence of cardiovascular risk factors in response to damage to the vascular endothelium, which becomes dysfunctional as a result [2]. As the disease progresses, a chronic inflammatory response develops in the vessel wall, triggered by the oxidation of lipids (especially LDL) and promoting pathophysiology through the increased expression of adhesion molecules and the release of proinflammatory mediators [2,3]. These detrimental events eventually lead to severe arterial stenosis and compromised downstream blood supply to organs.

Angiotensin II (Ang II) is an important effector peptide of the renin-angiotensin-aldosterone system (RAAS) and exerts its effects predominantly via the angiotensin II subtype 1 receptor (AT1R). Ang II is significantly involved in blood pressure regulation, NaCl homeostasis, and cardiovascular and renal remodeling [4]. Furthermore, Ang II promotes the development and progression of endothelial dysfunction and atherosclerosis via the AT1R [4]. For example, AT1R activation stimulates leukocyte-endothelial interaction, favouring vascular inflammation in the process of atherogenesis [5]. Furthermore, Ang II promotes either hyperplasia or hypertrophy of aortic vascular smooth muscle cells (VSMCs) in a context-specific manner, thereby influencing the structure of the aortic media [4,6]. Indeed, Ang II stimulates the expression of collagen, proteoglycans, adhesion molecules, and chemokines in vascular cells, thereby contributing to pathological changes in vascular architecture [4,7]. Additionally, Ang II is an important trigger of aortic aneurysm formation in Western-type diet-fed low-density lipoprotein (LDL) receptor (Ldlr) knockout or apolipoprotein E (ApoE) knockout mouse models [8,9]. Because of its pathophysiological similarity to atherosclerosis-associated aortic aneurysm formation in humans, the model is of great importance for elucidating the mechanistic principles of disease development [9]. In this model, the pathogenesis of aortic aneurysms is enhanced by, among other factors, advanced age and male sex of the mice as well as elevated LDL plasma concentrations, whereas the extent of Ang II-induced blood pressure elevation most likely has only a minor influence [9]. However, the extent to which the development and progression of Ang II-induced aortic aneurysms is influenced by concomitant pharmacological blockade of AT1R-synergistic receptor systems, such as the thromboxane A_2 (TxA_2) receptor (TP), has remained unclear.

TxA_2 is a potent mediator of platelet aggregation as well as vasoconstriction and exerts its effects via the TP [10]. The murine TP and human TP isoforms (TP_α/TP_β) have been shown to mediate contraction and hyperplasia/hypertrophy of VSMC and to influence vascular remodeling in addition to vasotonus [10]. Via an induction of platelet aggregation, the TP plays a major role in the pathogenesis of thromboembolic complications in patients with cardiovascular disease [10]. In addition, the TP contributes to the formation of a proinflammatory endothelium and exerts antiangiogenic effects in endothelial cells via activation of a $G_{\alpha 13}$-RhoA/C-ROCK-LIMK2-dependent signal transduction pathway [10–15]. In this context, we have recently identified a TP-driven, COX-2-dependent feedback loop and key TP effectors in vascular endothelial cells, through which the receptor controls its own activation as well as induces endothelial dysfunction and inflammation and inhibits blood vessel formation [12]. Furthermore, the TP has been reported to play a role in the pathogenesis of atherosclerosis in mouse models of the disease [16–18]. We recently demonstrated that specific deletion of the TP in VSMCs from Western diet-fed Ldlr knockout mice was associated with a moderate reduction in atherosclerotic plaque formation, whereas endothelium-specific deletion of the TP had no significant effect [18]. In vascular cells, the TP also seems to trigger synergistic effects with other receptor systems, which in principle may contribute to the initiation and progression of vascular disease. For example, with regard to signal transduction and gene expression in vascular cell types, we were able to demonstrate synergism of TP and epidermal growth factor receptor (EGFR), suggesting the importance of other receptors in vascular signal transduction of the TP [19]. In addition, results from other research groups demonstrate that pharmacological blockade or genetic deletion of the TP reduces the vasoconstrictor as well as blood pressure-increasing effect of Ang II and Ang II-mediated vascular remodeling [20–22]. These effects suggested that the TP plays a role in transducing deleterious effects of AT1R in vascular cells and that, in particular, the TP expressed in VSMC may play a role in mediating Ang II-induced arterial hypertension and remodeling. Therefore, the aim of this study was to investigate whether deletion of vascular TP has an effect on Ang II-mediated hypertension, atherosclerosis, aortic aneurysm formation, and mortality in the Western-type diet-fed Ldlr knockout mouse model. In the present study, we were able to demonstrate that vascular deletion of the TP neither significantly affected Ang II-mediated hypertension nor the extent of atherosclerosis, the incidence of aortic aneurysms, or the levels of plasma cholesterol and triglycerides. Moreover, neither VSMC- or EC-specific knockout altered body or organ weights or organ-to-body weight ratios in our experimental set-up. However, sex-specific analysis revealed a trend towards a reduction of Ang II-induced mortality in male $TP^{VSMC\ KO}$/Ldlr KO mice as well as a trend towards a reduction in aortic arch atherosclerotic lesion formation and the incidence of aortic aneurysms in female $TP^{VSMC\ KO}$/Ldlr KO mice. Thus, these data suggest that the TP could represent a gender-specific therapeutic target for reducing vascular complications in patients with activated RAAS.

2. Materials and methods

U-46619 was obtained from Cayman Chemical (Ann Arbor, USA). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, USA), unless stated otherwise.

2.1. Animals

We recently described the generation of conditional TP knockout mice on the Ldlr knockout background [18]. For this purpose, conditional TP knockout mice, whose generation has been published previously [23], were obtained from The Jackson Laboratory (Bar Harbor, USA; stock #021985) and crossed on the one hand with Tie2-cre mice (The Jackson Laboratory; stock #004128) [24] or with SM22α-cre mice (The Jackson Laboratory; stock #004746) [25] to allow the specific deletion of the TP in the vascular endothelium or VSMC, respectively. The resulting mice were subsequently crossed with Ldlr knockout mice (The Jackson Laboratory, stock #002207) [26] to generate homozygous TP-floxed mice on the Ldlr-deficient background. In this study, male and female endothelial-specific ($TP^{EC\ KO}$/Ldlr KO; Tie2-cre-positive) and vascular smooth muscle cell-specific ($TP^{VSMC\ KO}$/Ldlr KO; SM22α-cre-positive) TP-deficient mice were analyzed on the Ldlr KO background and compared with corresponding Ldlr-deficient TP-expressing littermates (TP^{WT}/Ldlr KO; cre-negative). We examined mice that were 12 weeks of age when the Western-type diet was initiated.

All animal experiments were performed in accordance with the German Animal Welfare Act and the European Directive 2010/63/EU. The local animal welfare commission approved the experiments (approval number Az 42502-2-1292 MLU, H2-1/T1-17 and H2-1/T1-22; Landesverwaltungsamt Sachsen-Anhalt). According to FELASA guidelines, mice were housed in groups of up to 5 animals in a specific pathogen-free environment on a 12-h light/12-h dark cycle and 22±2 °C ambient temperature. Mice had free access to water and were fed a standard rodent diet (Altromin, Lage, Germany) ad libitum or a high-fat diet (Altromin Western-type diet; 15 % milk fat and 1.25 % cholesterol) as specified below. At the end of the experiments, mice were sacrificed for organ explantation by cervical dislocation.

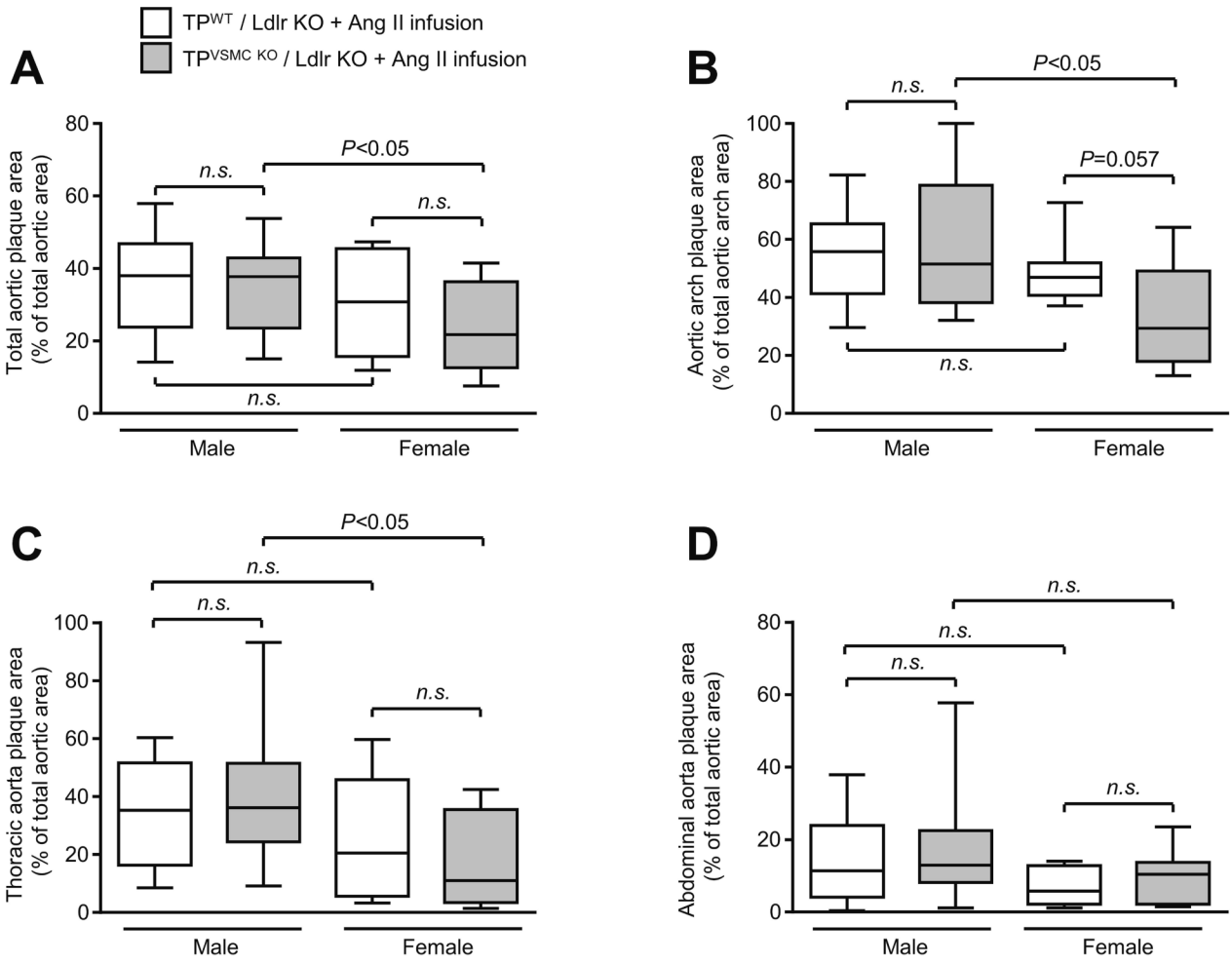


Fig. 1. Deletion of vascular smooth muscle (VSMC) TP does not significantly reduce angiotensin II (Ang II)-induced aortic atherosclerosis in Ldlr knockout mice fed a Western-type diet for 9 weeks but, as a trend, reduces atherosclerosis development in the aortic arch of female mice. (A-D) Statistical analysis of aortic atherosclerotic lesion formation in aortas derived from TP^{VSMC} KO/Ldlr KO or TP^{WT}/Ldlr KO mice. Data for male and female mice are shown separately as min-to-max box-and-whisker plots (including median with 25 % and 75 % percentiles (IQR)). Total aortic lesion area (A), atherosclerotic lesion area in the aortic arch (B), and atherosclerotic lesion area in the thoracic (C) or abdominal aorta (D) are shown separately for male and female mice. Male TP^{WT}/Ldlr KO (n = 13); male $TP^{VSMC\ KO}$/Ldlr KO (n = 13); female TP^{WT}/Ldlr KO (n = 9); female $TP^{VSMC\ KO}$/Ldlr KO (n = 9). n.s. = non-significant.

2.2. Blood pressure measurements

Blood pressure was measured noninvasively via the tail-cuff method using the BP-2000 Blood Pressure Analysis System (Visitech Systems, Inc. North Carolina, USA) as previously described [18] starting five weeks before Ang II infusion.

2.3. Intervention with Western-type diet and implantation of osmotic pumps

12-week-old mice were fed a high-fat (Western-type) diet (Altromin; 15 % milk fat and 1.25 % cholesterol) for nine weeks, starting 5 weeks before treatment with Ang II. Both male and female mice of the different genetic backgrounds ($TP^{VSMC\ KO}$/Ldlr KO, $TP^{EC\ KO}$/Ldlr KO as well as corresponding wild-type littermates (TP^{WT}/Ldlr KO) were studied. For subcutaneous infusion of Ang II (1,4 mg per kg body weight per day, dissolved in sterile 0.9 % NaCl solution), Alzet osmotic pumps (model 1004, Alzet, Cupertino, CA, USA) were implanted under isoflurane anesthesia on the back of the mice, slightly posterior to the scapulae. Carprofen (Zoetis, Berlin, Germany) at a dose of 5 mg per kg body weight was provided as pain relief. After 28 days of Ang II infusion, mice were sacrificed and tissue and plasma were subsequently analyzed.

2.4. Atherosclerotic lesion quantification and detection of pathological changes of the aorta

Atherosclerotic lesion quantification was performed as described previously [18]. Briefly, mice were sacrificed and subsequently perfused very gently via syringe by injection of 2×10 mL PBS and 1x 10 mL 4 % PFA in PBS after cannulation of the left ventricle. After collecting the organs, the entire aorta was dissected and stored for fixation overnight in 4 % PFA/PBS at 4 °C. Directly after preparation of the aorta, an analysis of macroscopically visible pathological changes of the aorta (including aortic aneurysms, aortic dissections or other visible impairment of the integrity of the aorta, adventitial hemorrhage), which were associated with a recognizable change in structure, shape or diameter of the aorta, was performed. All pathological aortic changes mentioned were evaluated qualitatively (as pathological changes visibly present or not). The next day, the loose adipose tissue and adventitia were carefully removed. Aortas opened by longitudinal sectioning were stained with Sudan IV staining solution as described previously [27] Subsequently, the aortas were embedded on slides in Kaisers glycerol jelly. Images were acquired with a Keyence microscope (Keyence, Osaka, Japan) and single pictures were assembled with the associated software. The image analysis software ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) was used to determine the atherosclerotic lesion area

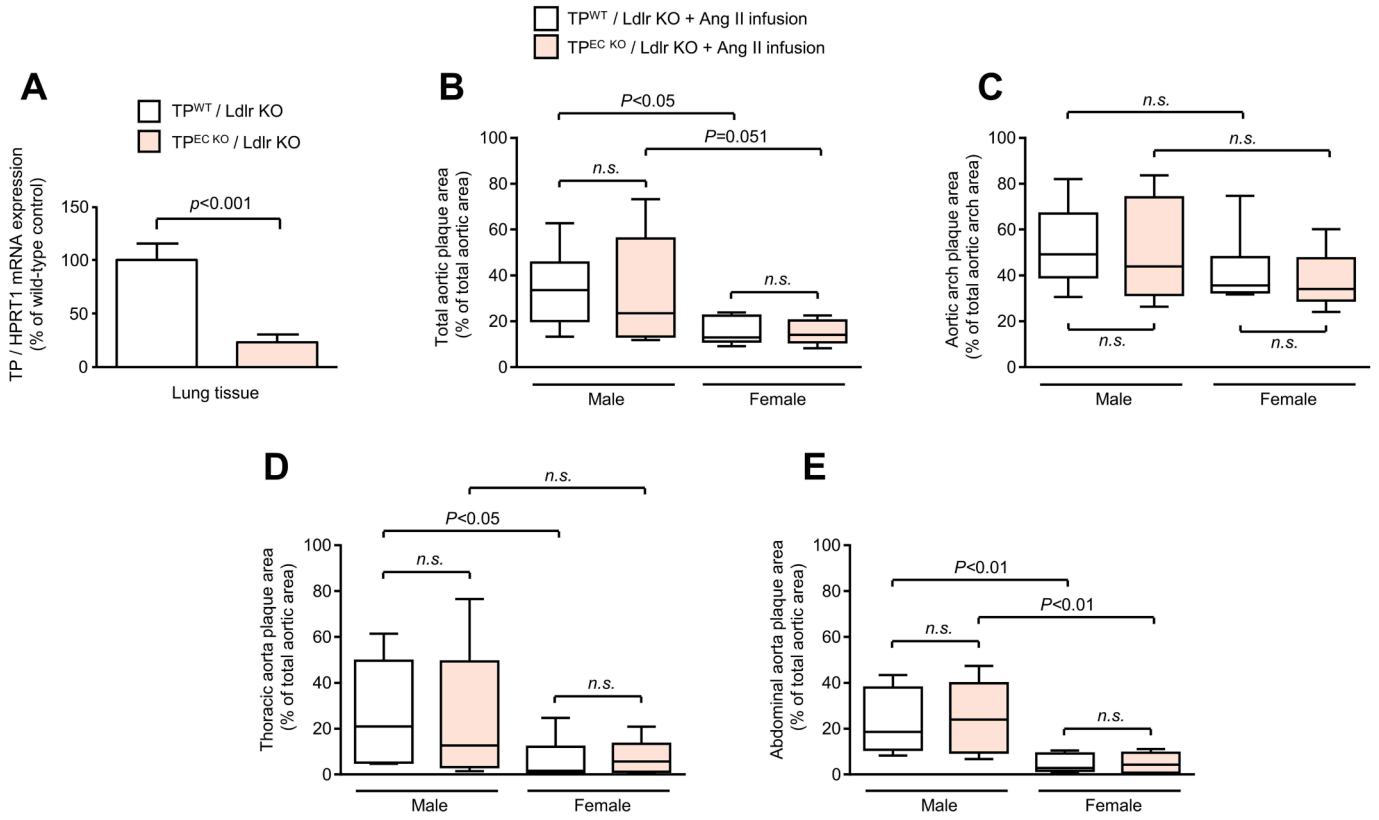


Fig. 2. Endothelial (EC)-specific deletion of the TP does not affect angiotensin II (Ang II)-induced aortic atherosclerosis of Ldlr knockout mice fed a Western-type diet for nine weeks. TP expression in lung tissue (A, n = 4–6) derived from $TP^{EC\ KO}$/Ldlr KO mice or TP^{WT} (wild-type, cre-negative)/Ldlr KO littermates. Data are shown as mean ± SD. Statistical analysis of aortic atherosclerotic lesion formation in aortas derived from male and female TP^{EC} KO/Ldlr KO or TP^{WT}/Ldlr KO mice. Data are shown as min-to-max box-and-whisker plots (including median with 25 % and 75 % percentiles (IQR)). Total aortic lesion area (B), atherosclerotic lesion area in the aortic arch (C) and atherosclerotic lesion area in the thoracic (D) or the abdominal aorta (E) are shown for male and female mice, respectively. Male TP^{WT}/Ldlr KO (n = 11); male $TP^{EC\ KO}$/Ldlr KO (n = 8); female TP^{WT}/Ldlr KO (n = 7); female $TP^{EC\ KO}$/Ldlr KO (n = 8). n.s. = non-significant.

and the total area of the aortic intima. Male and female mice were studied, and both pooled as well as sex-specific analyses of atherosclerotic lesion formation were performed. In these analyses, the total atherosclerotic lesion area of the aorta was determined, but also the atherosclerotic lesion areas separately for different aortic sections: the aortic arch, the thoracic aorta, and the abdominal aorta. The height of the 7th rib and the diaphragm were used as anatomic landmarks to subdivide the aortic sections.

2.5. Measurement of aortic ring force

Male and female mice of minimum 15 weeks, of endothelial-specific ($TP^{EC\ KO}$/Ldlr KO; Tie2-cre-positive) and vascular smooth muscle cell-specific ($TP^{VSMC\ KO}$/Ldlr KO; SM22α-cre-positive) background and their respective WT littermates (Cre-negative) were sacrificed. From each mouse, two aortic rings of the thoracic segment (between the pairs of branches of the intercostal arteries 2–4 and 8–9) and the abdominal segment (anterior and posterior to the pair of ilio-lumbal arteries) were isolated in ice-cold Krebs-Ringer solution. The four isolated rings were transferred to the myograph chambers (DMT Wire Graph 620, DMT, Saeby, Denmark) using wires (24 μm diameter). Measurement of aortic ring force was performed as described previously [7]. In brief, aortic rings were equilibrated in modified aerated Krebs-Ringer solution (20 % O_2, 5 % CO_2, NaCl 119.90 mM, KCl 5.40 mM, $MgCl_2x6H_20$ 1.10 mM, $NaHCO_3$ 22.60 mM, glucose 5.05 mM, $NaH_2PO_4x1H_20$ 0.42 mM, $CaCl_2x2H_20$ 2.5 mM, ascorbic acid 0.28 mM, EDTA 0.05 mM) at 37 °C for 30 min. At the beginning and the end of the equilibration, the solution was changed once, followed by the application of a strain resulting in a force of approximately 12mN. This strain resulted in a similar change in vessel circumference and similar effective pressure values in all genotypes and was applied for 2×5 min prior to the addition of the first test substance. After each measurement, the chambers were rinsed five times with Krebs-Ringer solution, until 12mN baseline force was reached, resulting in an approximately 100,000-fold dilution of the previously tested substance before a new substance was analyzed. However, this was not the case for the vasodilator carbamoylcholine chloride (carbachol). The muscarinic receptor agonist was applied at the point of stable force development of the previously administered vasoconstrictor serotonin (10 μmol/L). The sequence of applied substances and washing for each ring was: Potassium chloride (25 mM) – 5x washing – Ang II (0.1 nM – 1 μM) – 5x washing – U-46619 (0.1 nM – 1 μM) – 5x washing – serotonin (10 μM) – carbachol (1 nM – 100 μM). Substances of the next higher concentration were applied at the point of stable force development of the previous one. Values were Δ mean force in mN, taken from highest stable peak of vasoconstriction/vasorelaxation minus the respective baseline value before substances were applied, recorded with the LabChart 8 Software (ADInstruments, Sydney, Australia). Since the agonists were tested sequentially in these experiments, only a limited number of agonist concentrations were measured (with the exception of the final carbachol analyses) in order to minimize the stress on the aortic tissue caused by this sequential stimulation. Therefore, the calculation of EC_{50} values was only performed using the carbachol datasets. The EC_{50} was calculated by fitting the data with the sigmoid model using the computer program GraphPad Prism (Version 6, GraphPad Software Inc., Boston, MA, USA). Significant differences between EC_{50} values were determined by Student's paired *t*-test.

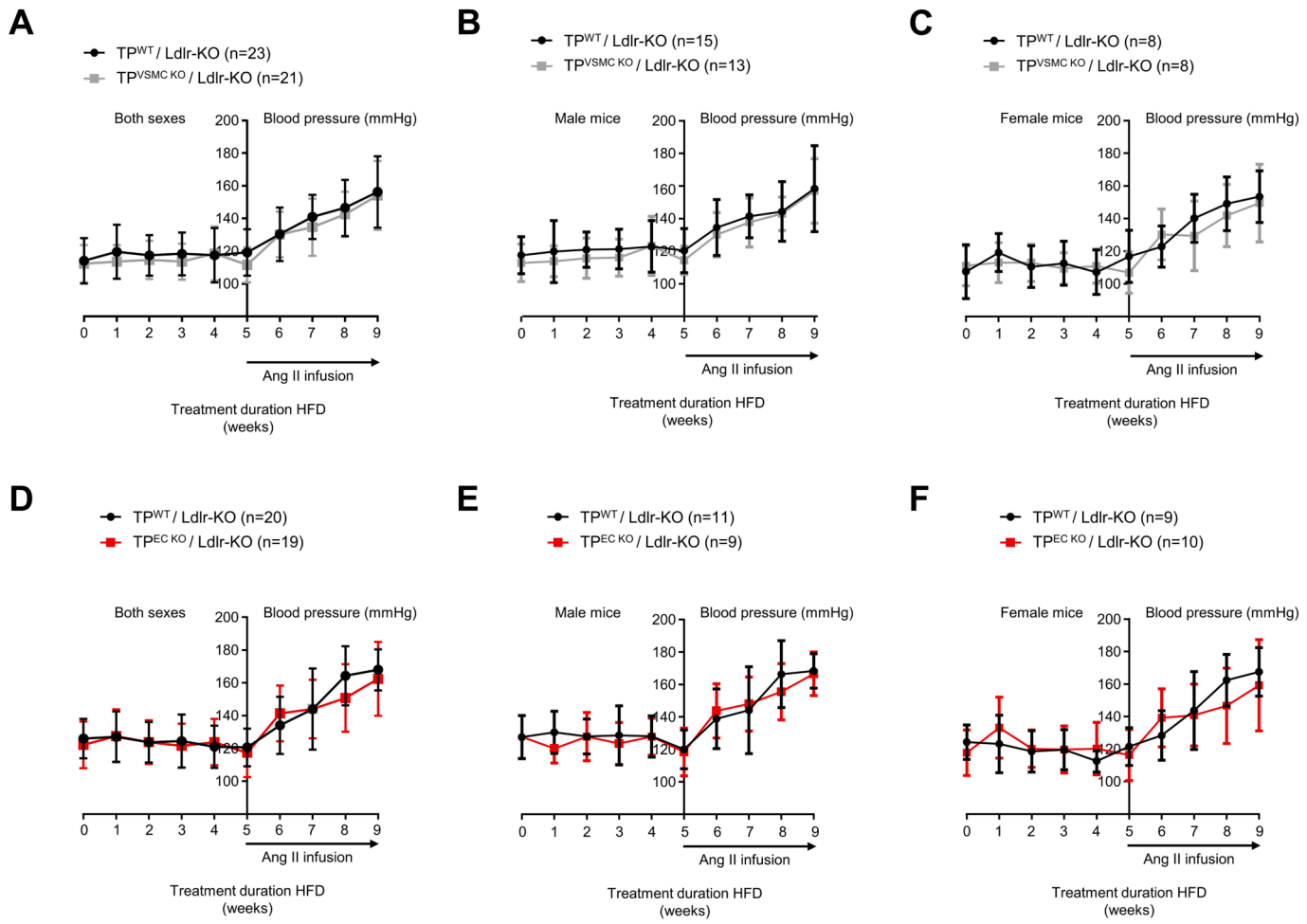


Fig. 3. Infusion of angiotensin II (Ang II) significantly increases systemic blood pressure to a similar extent in VSMC-specific TP knockout mice on the Ldlr-deficient background ($TP^{VSMC\ KO}$/Ldlr KO; A-C) and in TP wild-type littermates ($TP^{WT\ KO}$/Ldlr KO). Furthermore, sex has no significant effect on Ang II-induced hypertension in these mice (data are shown for male (B) or female mice (C), respectively). Data are shown as mean ± SD. Also, infusion of Ang II increases systemic blood pressure to a similar extent in EC-specific TP knockout mice on the Ldlr-deficient background (TP^{EC} KO/Ldlr KO; D-F) and in TP wild-type littermates (TP^{WT} KO/Ldlr KO). Again, sex has no significant effect on Ang II-induced hypertension in these mice (data are shown for male (B) or female mice (C), respectively). Data are shown as mean ± SD.

2.6. Lipid measurements

For the analysis of cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides in mouse plasma, colorimetric assays (CHOL2, cholesterol; Gen.2, HDL cholesterol; Gen.4, LDL cholesterol; Gen.3, Triglycerides; all Roche Diagnostics, Rotkreuz, Switzerland) were used, which were analyzed on a Roche cobas c701 or a c502 analyzer integrated with a fully automated Roche Cobas 8000 platform. All analyses on the Roche cobas analyzers were performed according to the manufacturer's instructions and manuals and in compliance with routine maintenance and quality control procedures. Due to the high analyte concentrations, all samples were diluted fivefold prior to measurement to match the analytical ranges of the assays.

2.7. Real time RT-PCR

Total RNA was isolated, reverse transcribed and analyzed as described previously [28,29] and mRNA expression was quantified using the ABI 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, USA). TaqMan reactions were carried out in 96-well plates according to the manufacturer's instructions using pre-made TaqMan™ Gene Expression Assays (probes) for the TP (Mm00436917_m1). Hypoxanthine-guanine phosphoribosyl transferase 1 (HPRT1) was used as an endogenous control (Mm01545399_m1). All TaqMan™ Gene Expression Assays used in this study had been previously validated by the manufacturer, Thermo Fisher Scientific, and all of these probes span exons. We performed relative quantification of gene expression as previously described using the delta-delta Ct method [30,31].

2.8. Statistical analyses

Statistical analyses were performed using one-way analysis of variance followed by Sidak's multiple comparisons post hoc test or the unpaired student s *t*-test. For statistical analyses the Graph Pad Prism 6 software package was used (Graph Pad Software, Inc., La Jolla, USA). Data were expressed as min-to-max box-and-whisker plots (including median with 25 % and 75 % percentiles (IQR)) or as mean ± standard deviation (SD) or standard error of the mean (SEM) as indicated. Probability values were considered significant at a $P < 0.05$.

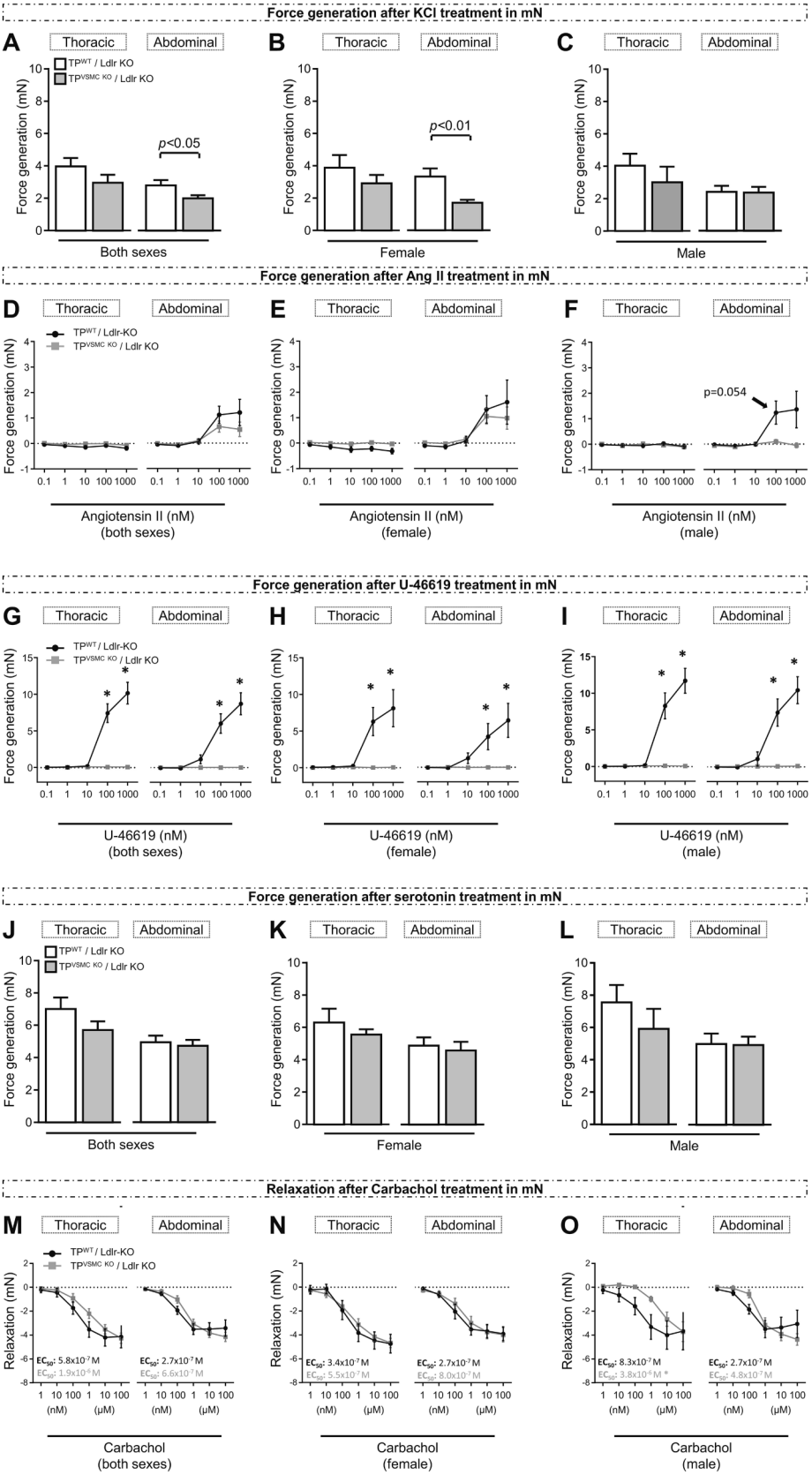


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Fig. 4. Effect of VSMC-specific TP knockout ($TP^{VSMC\ KO}$/Ldlr KO) on vascular reactivity in *ex vivo* aortic ring force measurements after successive treatment with 25 mM KCl (A-C), 0.1 nM − 1 μM Ang II (D-F), 0.1 nM − 1 μM U46619 (G-I) as well as treatment with 10 μM serotonin (J-L) immediately followed by 1 nM – 100 μM carbachol (M−O). Aortic rings were obtained from untreated $TP^{VSMC\ KO}$/Ldlr KO mice and their respective wild-type littermates. Data are shown as delta mean in mN ± SEM as compared to baseline. Aortic rings from female (female) and male (male) animals harvested from the thoracic (thoracic) and abdominal portions (abdominal) of the aorta, respectively, were analyzed either separately or collectively (both sexes): both sexes TP^{WT}/Ldlr KO (white bars/lines each n = 14); both sexes $TP^{VSMC\ KO}$/Ldlr KO (grey bars/lines each n = 14); male TP^{WT}/Ldlr KO (white bars/lines each n = 8); male $TP^{VSMC\ KO}$/Ldlr KO (grey bars/lines each n = 6); female TP^{WT}/Ldlr KO (white bars/lines each n = 6); female $TP^{VSMC\ KO}$/Ldlr KO (grey bars/lines each n = 8). EC_{50} values for carbachol treated groups are plotted as mean in mol/L. * = $P < 0.05$.

3. Results

3.1. Impact of VSMC- or EC-specific TP deletion on Ang II- and Western-type diet-induced atherosclerotic lesion and aneurysm formation in Ldlr-deficient mice

In the first set of experiments we studied Ldlr KO mice with a VSMC-specific deletion of the TP ($TP^{VSMC\ KO}$/Ldlr KO) and the respective TP wild-type littermates (TP^{WT}/Ldlr KO). In these experiments, mice were fed a Western-type diet for nine weeks and treated with Ang II during the last four weeks of the diet (Graphical abstract). Successful knockout of the TP in aortic vascular smooth muscle cells derived from $TP^{VSMC\ KO}$/Ldlr KO mice had been verified previously in denuded, adventitia-free aortas [18]. In these analyses, a reduction in TP mRNA expression of approximately 95 % was observed in aortic tissue obtained from $TP^{VSMC\ KO}$/Ldlr KO mice compared with aortic tissue collected from TP^{WT}/Ldlr KO littermates [18].

In pooled analyses of male and female mice, deletion of TP in VSMC was not associated with a reduction in Ang II- and Western-type diet-induced aortic atherosclerotic lesion formation, regardless of whether the whole aorta or specific aortic segments (aortic arch, thoracic aorta, or abdominal aorta) were studied. In sex-specific analyses (Fig. 1A – D), there was a trend towards plaque area reduction in female $TP^{VSMC\ KO}$/Ldlr KO mice in the aortic arch (34.4±17.7 % ($TP^{VSMC\ KO}$/Ldlr KO) vs 48.4±10.5 % (TP^{WT}/Ldlr KO), $P = 0.057$, n = 9, Fig. 1B). In addition, there was a trend towards fewer Ang II-induced aortic alterations (including abdominal or thoracic aneurysms and aortic dissections) in $TP^{VSMC\ KO}$/Ldlr KO mice (48 %, 12 of 25 mice affected) compared with TP^{WT}/Ldlr KO littermates (59 %, 16/27 mice), which was attributable to a non-significant reduction in the incidence in females (11 % (1/9 $TP^{VSMC\ KO}$/Ldlr KO mice)) vs. 44 % (4/9 TP^{WT}/Ldlr KO mice). Ang II-induced mortality almost exclusively affected males in all genotypes examined and tended to be lower in $TP^{VSMC\ KO}$/Ldlr KO mice 13 % (2/15) than in TP^{WT}/Ldlr KO mice 28 % (5/18).

Endothelial-specific knockout of the TP had been verified previously in MACS-isolated CD31-positive lung endothelial cells and lung tissue derived from $TP^{EC\ KO}$/Ldlr KO mice [18]. In the present study, we reanalysed TP mRNA expression in lung tissue in a subset of $TP^{EC\ KO}$/Ldlr KO mice and wild-type littermates (Fig. 2A). In these experiments, $TP^{EC\ KO}$/Ldlr KO mice showed a reduction of TP mRNA expression in lung tissue by more than 75 % (Fig. 2A). However, $TP^{EC\ KO}$/Ldlr KO mice showed no significant change in Ang II- and Western-type diet-induced atherosclerotic lesion formation in the aorta. Sex-specific analyses showed a trend towards lower, and in some experimental groups significantly lower, atherosclerotic plaque burden in female compared with male mice (Fig. 2B – E). However, sex-specific analyses did not reveal any differences in atherosclerotic plaque burden between the genotypes (Fig. 2B – E). Similarly, no significant differences were observed in the incidence of pathological aortic alterations ($TP^{EC\ KO}$/Ldlr KO mice (25 %, 4/16 mice affected) versus TP^{WT}/Ldlr KO littermates (39 %, 7/18 mice affected)) or mortality ($TP^{EC\ KO}$/Ldlr KO mice (16 %, 3/19 mice affected) versus TP^{WT}/Ldlr KO littermates (10 %, 2/20 mice affected)).

3.2. Effect of vascular TP knockout on Ang II infusion-induced hypertension in Western-type diet-fed Ldlr-deficient mice

We also examined the effect of Ang II infusion on systemic blood pressure in both vascular TP KO mice (EC- and VSMC-specific) and their respective wild-type littermates. The rationale for these studies was that VSMC-specific deletion of the TP had been demonstrated to reduce Ang II-induced hypertension [22]. Therefore, by using VSMC-specific or EC-specific KO mice, we aimed to clarify the role of the vascular TP in the blood pressure response to Ang II. Surprisingly, however, our experiments showed that infusion of Ang II caused an almost identical increase in blood pressure in $TP^{VSMC\ KO}$/Ldlr KO mice and TP^{WT}/Ldlr KO littermates (Fig. 3A). In addition, both female and male mice responded similarly to Ang II (Fig. 3B and C). Also, no significant differences in basal blood pressure could be detected between the genotypes (Fig. 3). Endothelium-specific knockout of the TP did not alter the Ang II-induced blood pressure response and resulted in a similar increase in systemic blood pressure as observed in wild-type littermates (Fig. 3D). As with VSMC-specific knockout, sex had no effect on basal blood pressure or Ang II-induced blood pressure response in endothelial-specific TP knockout mice (Fig. 3E and F).

3.3. Impact of vascular-specific TP deletion on aortic ring reactivity ex vivo

To analyze a potential role of the TP in Ang II-induced vasoconstriction, we tested the vasoreactivity of the abdominal and thoracic aorta of VSMC- (Fig. 4) and EC-specific (Fig. 5) TP knockout mice on the Ldlr-deficient background. The ring sections were subjected to vasoactive substances such as potassium chloride (KCl, Fig. 4A – C, Fig. 5A – C), Ang II (Fig. 4D – F, Fig. 5D – F), the TP agonist U46619 (Fig. 4G – I, Fig. 5G – I) and serotonin (Fig. 4J – L, Fig. 5J – L). Finally, the rings were subjected to the muscarinic agonist carbachol to assess endothelial-dependent aortic relaxation from plateaued serotonin-induced preconstriction (Fig. 4M – O, Fig. 5M – O).

When aortic sections were treated with KCl, aortic rings from both vascular TP knockout mouse strains showed significantly attenuated vasoconstriction compared with aortic rings from their respective wild-type littermates, particularly in abdominal sections derived from female mice (Fig. 4A – C, Fig. 5A – C). However, aortic rings from male mice of both vascular TP knockout strains showed comparable vasoconstriction to wild-type male aortas, suggesting an influence of vascular TP on the KCl-induced vasoconstriction pathway exclusively in female mice (Fig. 4A – C, Fig. 5A – C).

Interestingly, addition of Ang II at concentrations ranging from 0.1 nM to 1 μM (Fig. 4D – F, Fig. 5D – F) did not result in Ang II-dependent vasoconstriction of aortic rings of the thoracic segment, regardless of genotype or sex of the mice studied (Fig. 4E, Fig. 5E). In contrast, abdominal sections of the aorta showed the expected Ang II-induced vasoconstriction *ex vivo* with a maximum force generated at 100 nM (Fig. 4D – F, Fig. 5D – F). Interestingly, endothelial-specific knockout had no significant effect on Ang II-mediated constriction of the abdominal aorta (Fig. 5D – F), whereas we observed a trend towards weaker Ang II-mediated constriction of the abdominal aorta of male VSMC-specific TP knockout mice compared with male wild-type littermates (Fig. 4F).

To investigate the effect of selective endothelial or VSMC-specific TP

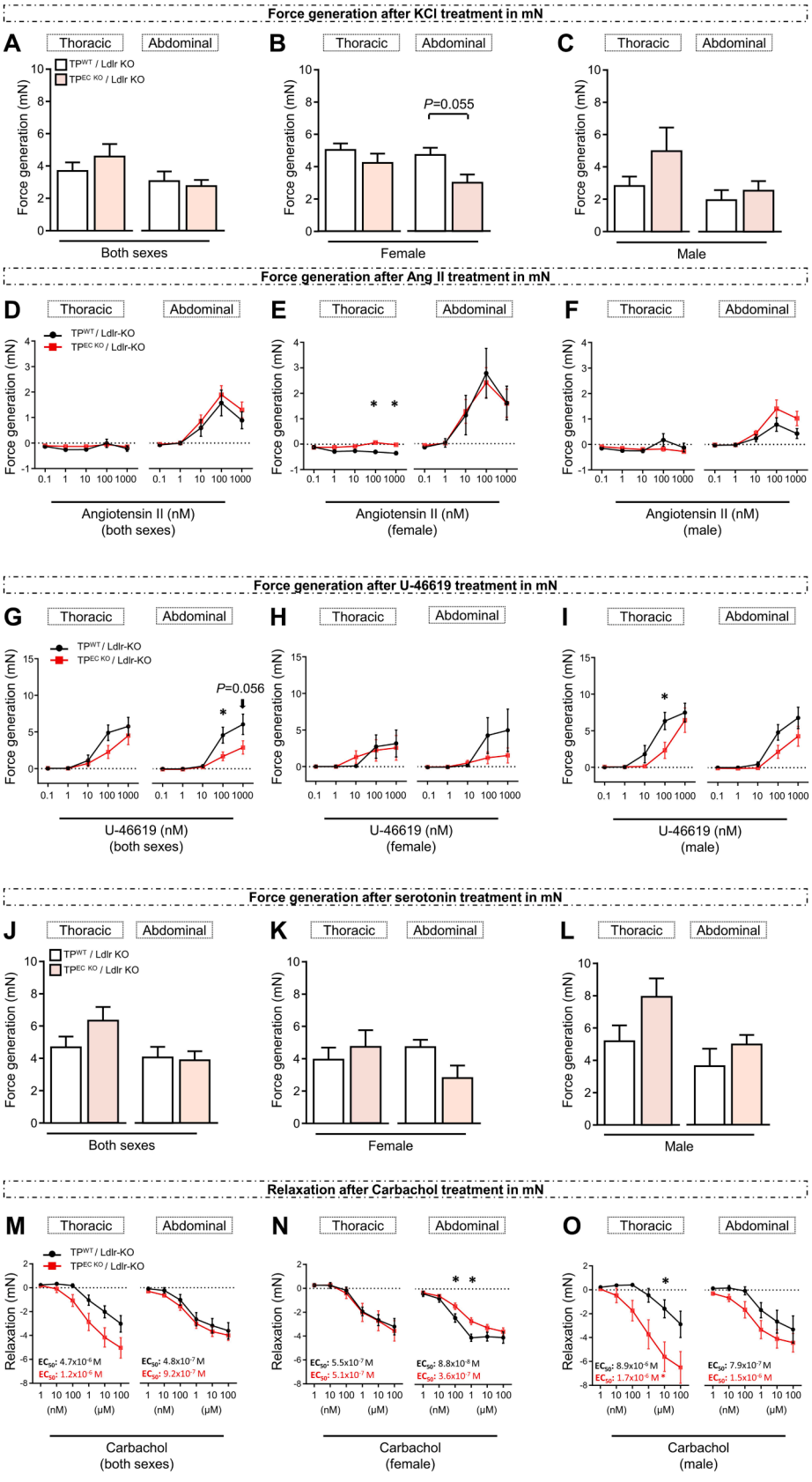

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Fig. 5. Effect of vascular endothelial-specific TP knockout ($TP^{EC\ KO}$/Ldlr KO) on vascular reactivity in ex vivo aortic ring force measurements after successive treatment with 25 mM KCl (A-C), 0.1 nM − 1 μM Ang II (D-F), 0.1 nM − 1 μM U46619 (G-I) as well as treatment with 10 μM serotonin (J-L) immediately followed by 1 nM – 100 μM carbachol (M−O). Aortic rings were obtained from untreated $TP^{EC\ KO}$/Ldlr KO mice and their respective wild-type littermates. Data are shown as delta mean in mN ± SEM as compared to baseline. Aortic rings from female (female) and male (male) animals harvested from the thoracic (thoracic) and abdominal portions (abdominal) of the aorta, respectively, were analyzed either separately or collectively (both sexes): both sexes TP^{WT}/Ldlr KO (white bars/lines each n = 10); both sexes $TP^{EC\ KO}$/Ldlr KO (pink bars/red lines each n = 16); male TP^{WT}/Ldlr KO (white bars/lines each n = 6); male $TP^{EC\ KO}$/Ldlr KO (pink bars/red lines each n = 8); female TP^{WT}/Ldlr KO (white bars/lines each n = 4); female $TP^{EC\ KO}$/Ldlr KO (pink bars/red lines each n = 8). EC_{50} values for carbachol treated groups are plotted as mean in mol/L. * = $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

knockout on TP-mediated aortic vasoconstriction, the effect of the TP agonist U46619 was examined at a concentration range of 0.1 nM to 1 μM in aortas from mice with vascular TP knockout compared with aortas from the corresponding TP wild-type littermates (Fig. 4/Fig. 5G – I). In these experiments, even the highest U-46619 concentration of 1 μM did not cause vasoconstriction in either thoracic or abdominal sections of $TP^{VSMC\ KO}$/Ldlr KO of each sex, whereas their TP wild-type littermates showed gender-independent vasoconstriction at concentrations of 10 nM U46619 or above (Fig. 4G – I). These results suggest that VSMC-specific knockout of the TP not only affects TP expression in the aortic wall but also that, as expected, the TP expressed in VSMC plays an essential role in U-46619-mediated aortic constriction. Interestingly, the aorta of $TP^{EC\ KO}$/Ldlr KO mice showed a trend toward reduced U46619-dependent vasoconstriction, particularly in male mice in thoracic ring sections (Fig. 5I), indicating a previously unrecognized role of endothelial TP in TP-induced vasoconstriction. This finding indicates that the endothelial TP plays a role in TP-mediated contraction of the aorta, possibly by releasing secondary mediators of vasoconstriction, which subsequently then increase VSMC tone.

Serotonin was used to preconstrict the aortic rings before the addition of the vasorelaxant muscarinic agonist carbachol, which resulted in similar vasoconstriction of the aortic rings in all genotypes and both sexes (Fig. 4/Fig. 5J – L). On the basis of serotonin-induced preconstriction, we subsequently investigated endothelium-dependent vasorelaxation induced by carbachol. Interestingly, thoracic sections of the aorta from male $TP^{VSMC\ KO}$/Ldlr KO mice showed a reduced endothelium-dependent vasodilation compared with thoracic aortic rings from male wild-type littermates (Fig. 4O; TP^{WT}/Ldlr KO: $EC_{50} = 8.3\times10^{-7} \pm 4.6\times10^{-7}$ mol/L vs. $TP^{VSMC\ KO}$/Ldlr KO: $EC_{50} = 3.8\times10^{-6} \pm 7.1\times10^{-7}$ mol/L, $P < 0.05$). In contrast, endothelium-dependent vasodilation was significantly increased in male $TP^{EC\ KO}$/Ldlr KO mice compared with male wild-type littermates (Fig. 5O; TP^{WT}/Ldlr KO: $EC_{50} = 8.9\times10^{-6} \pm 1.5\times10^{-6}$ mol/L vs. $TP^{EC\ KO}$/Ldlr KO: $EC_{50} = 1.7\times10^{-6} \pm 7.0\times10^{-7}$ mol/L, $P < 0.05$), indicating an opposing influence of the TP expressed in the endothelium and VSMC, respectively, on endothelium-dependent vasodilation of the aorta *ex vivo* in male mice (Fig. 5O).

3.4. Effects of vascular-specific TP deletion on plasma lipid profiles and organ weights of Ang II-treated Ldlr-deficient mice on Western-type diet

To analyze a possible effect of vascular-specific TP knockout on plasmatic cholesterol and triglyceride concentrations in mice, we measured the plasma lipid profiles of Western-type diet-fed and Ang II-treated endothelial- and VSMC-specific TP knockout mice and their wild-type littermates (Fig. 6). However, neither VSMC-specific nor EC-specific TP knockout had a significant effect on plasmatic cholesterol or triglyceride levels in these mice. Moreover, sex-specific analyses did not reveal any significant differences in plasmatic cholesterol or triglyceride levels between the genotypes investigated (Fig. 6A – H). However, female mice of all genotypes tended to have lower HDL plasma levels than male mice under the given treatment (Fig. 6C and G).

Furthermore, we analysed the effects of vascular-specific TP deletion on heart, lung, liver, kidney, and body weights of Ldlr KO mice fed a Western-type diet and treated with Ang II (Fig. 7). Again, vascular-specific TP knockout had no significant effect on organ or body weight or organ-to-body weight ratios of mice (Fig. 7). In contrast, body weights were lower in female mice than in male mice, regardless of the genotype studied (Fig. 7A and G). In addition, lung-to-body weight ratios and liver-to-body weight ratios tended to be higher in female than in male mice (Fig. 7D and J).

4. Discussion

4.1. Role of the TP in cardiovascular disease

The TP is an important therapeutic target in the treatment of cardiovascular disease [10]. Indeed, TP has been shown to contribute to platelet aggregation and thromboembolic complications in cardiovascular disease [10], to promote the pathogenesis of atherosclerosis in mouse models [16–18], and to foster endothelial dysfunction in humans [32]. In this context, we were able to show recently that VSMC-specific deletion but not vascular endothelial cell-specific knockout of the TP moderately reduced spontaneous atherosclerotic lesion formation in Western-type diet-fed Ldlr-deficient mice, thereby pointing to a relevant role of the TP expressed in VSMC in atherogenesis [18]. Therefore, it is of considerable scientific interest that an increase in vascular TP expression has been observed in patients at increased cardiovascular risk and in atherosclerotic lesions in mice, suggesting an amplification of TP-mediated signal transduction in the pathogenesis of cardiovascular disease [33,34]. Of note, we recently demonstrated that upregulation of TP in vascular endothelium can be triggered by stimuli such as constitutive RhoA and increased myosin II activity, cellular effectors that may be dysregulated in cardiovascular disease [12,14]. Moreover, several studies suggested that the TP is a major contributor to the development of hypertension and vascular remodeling in pathophysiological situations involving activation of the renin-angiotensin system [21,22,35,36]. However, data were lacking on the potential influence of vascular TP on Ang II-induced atherogenesis and pathological aortic changes, such as aneurysm development. Therefore, an important goal of the present work was to elucidate in which way the vascular TP affects Ang II-induced hypertension, mortality, atherogenesis and aortic aneurysm formation in the Ldlr-deficient mouse model.

4.2. Role of the vascular TP in angiotensin II-related hypertension and aortic reactivity

To clarify this issue, we used previously generated VSMC- and EC-specific TP knockout mice on the Ldlr-deficient background [18], fed these mice a Western-type diet for nine weeks, infused Ang II for the last four weeks of Western-type diet and analysed systemic blood pressure, mortality and atherosclerotic lesion as well as aneurysm formation in the aorta. In addition, we investigated TP-related and Ang II-mediated contraction of aortic rings derived from (otherwise untreated) mice of these genetic backgrounds and their wild-type littermates *ex vivo*. The aim was to collect initial data on whether knockout of the vascular TP alters aortic tissue force generation after stimulation with U-46619 or angiotensin II or endothelium-dependent vasodilation, although we are of course aware of the fact that Ang II treatment in combination with an additional high-fat diet can significantly alter aortic vasoreactivity compared with the untreated state. However, considering the stress that said intervention induces in the mice, we decided to first analyse vasoreactivity in untreated mice of the same genotype. The results of these experiments demonstrate that neither VSMC-specific nor endothelial-specific TP knockout had a significant effect on basal blood pressure

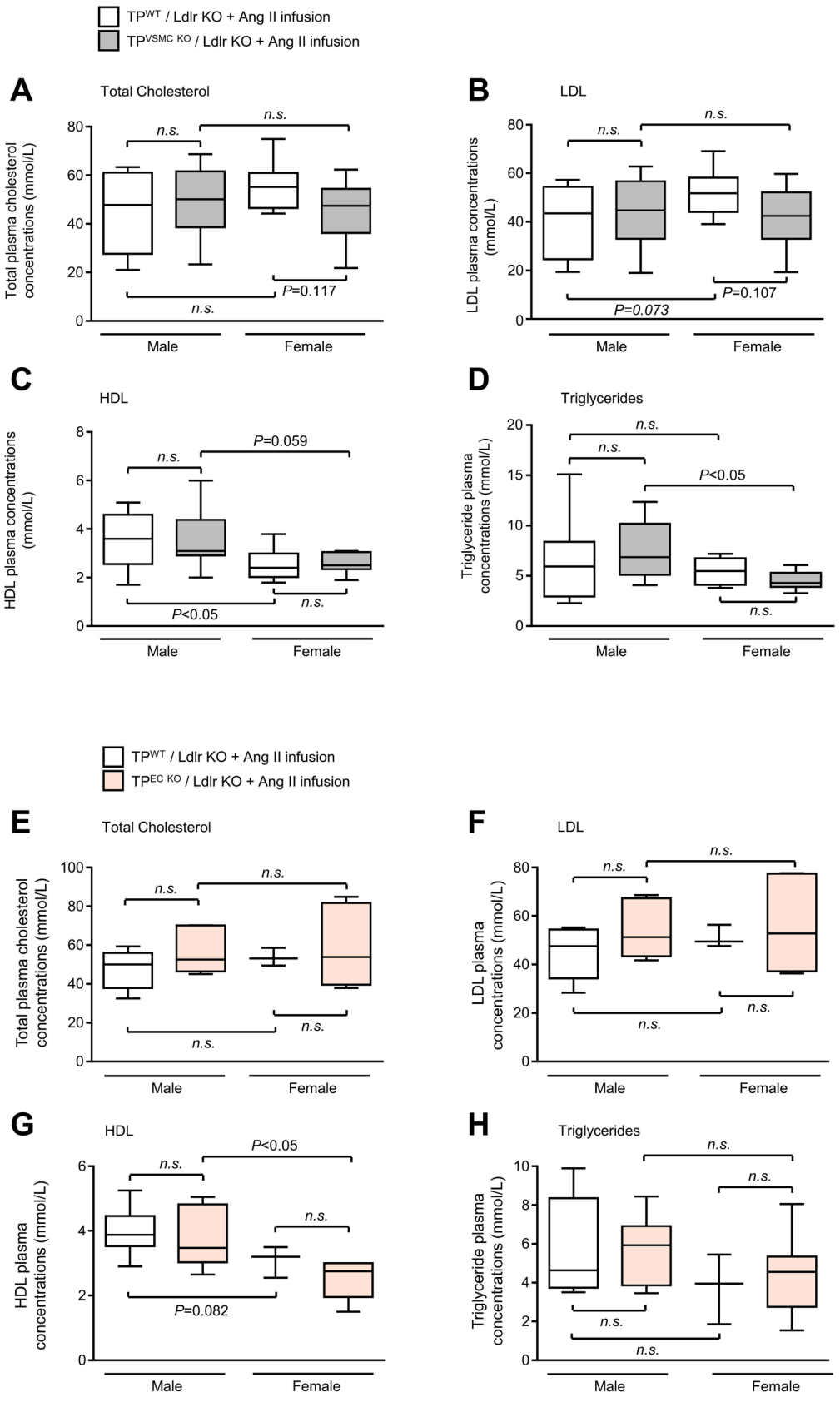

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Fig. 6. (A-D) Impact of vascular smooth muscle cell-specific knockout of the TP on plasma cholesterol and triglyceride levels in Ldlr knockout mice fed a western-type diet and treated with Ang II for four weeks. Data for male and female mice are shown separately as min-to-max box-and-whisker plots (including median with 25 % and 75 % percentiles (IQR)). (n = 11, male TP^{WT}/Ldlr KO); (n = 12, male $TP^{VSMC\ KO}$/Ldlr KO); (n = 8, female TP^{WT}/Ldlr KO); (n = 7, female $TP^{VSMC\ KO}$/Ldlr KO). (E-H) Impact of vascular endothelial cell-specific knockout of the TP on plasma cholesterol and triglyceride levels in Ldlr knockout mice fed a western-type diet and treated with Ang II for four weeks. Data for male and female mice are shown separately as min-to-max box-and-whisker plots (including median with 25 % and 75 % percentiles (IQR)). (n = 8, male TP^{WT}/Ldlr KO); (n = 6, male $TP^{EC\ KO}$/Ldlr KO); (n = 3, female TP^{WT}/Ldlr KO); (n = 7, female $TP^{EC\ KO}$/Ldlr KO). n.s. = non-significant.

or Ang II-induced hypertension in Ldlr-deficient mice, although VSMC-specific TP knockout abolished the contraction induced by the stable prostaglandin H_2 analogue and TP agonist U-46619 and also reduced Ang II-induced vasoconstriction of abdominal aortic rings derived from male mice *ex vivo*. Furthermore, and surprisingly, endothelial-specific knockout of the TP reduced U-46619-induced aortic constriction *ex vivo*, suggesting that TP stimulation in endothelial cells could also affect VSMC tone in the aorta via mechanisms such as paracrine mediator release or other types of cell-cell communication or interaction. In line with these findings, the TP was recently found to drive an autocrine COX2-dependent activation loop in the vascular endothelium, through which it inhibits angiogenesis processes in vitro and in vivo and also induces a pro-inflammatory, dysfunctional endothelial cell phenotype that includes a reduction in endothelial cell protective factors such as eNOS and prostacyclin synthase [12]. However, in the present work endothelial-specific knockout of TP did not alter systemic blood pressure or Ang II-induced mortality or vascular pathology, and thus the in vivo relevance of this finding remains unclear. Indeed, despite the pronounced vasoconstrictor effect of TP agonists on arterial blood vessels, the TP does not appear to play a critical role in the regulation of systemic blood pressure. For instance, basal systemic blood pressure has been demonstrated to be normal in mice completely lacking TP receptors [37]. This is consistent with our findings that VSMC-specific TP knockout mice do not show a significant change in basal systemic blood pressure. These data are also in line with findings from other studies showing that administration of pharmacological TP blockers appears to have negligible effects on systemic blood pressure [38]. However, it should be noted here that in our study, systemic blood pressure was measured by the less accurate (non-invasive) tail-cuff technique, so that subtle differences between genotypes may not have been detected.

Similarly, we were able to demonstrate that VSMC-specific deletion of the TP tended to reduce Ang II-induced contraction of aortic rings *ex vivo*. How VSMC-specific deletion of the TP affects Ang II-related vasoreactivity of the aorta is presently unclear. Nonetheless, it can be speculated that Ang II induces TP agonist formation in the aortic vessel wall via AT1 receptor activation, thereby causing autocrine and paracrine TP activation, which in turn leads to vascular contraction. Indeed, an AT1 receptor-triggered release of TP ligands, such as TxA_2 or 8-*iso*-$PGF_{2\alpha}$, in vascular tissue has been described suggesting that both AT1 receptor and TP function as an autocrine and paracrine signal transduction unit, with effects on VSMC tone [20,39]. Also, in Ang II-induced hypertension, urinary levels of the renal TxA_2 metabolite TxB_2 increased significantly, which may correspondingly indicate an Ang II-mediated induction of TxA_2 biosynthesis [22].

In contrast, VSMC-specific and endothelial-specific TP knockout mice showed an Ang II-induced increase in blood pressure comparable to their wild-type littermates which was unaffected by the sex of the animals. This was partly surprising, as global or VSMC-specific TP knockout (in mice with a functional Ldlr system) has been described to reduce Ang II-mediated hypertension in mice [21,22]. For instance, Sparks and colleagues demonstrated that deletion of the TP in VSMC significantly attenuated Ang II-induced hypertension and related vascular remodelling and that VSMC-specific TP knockout was associated with a reduction in urinary TxB_2 excretion. The reason for this discrepancy is unclear but could be due to differences in the genetic background of the mice (Ldlr knockout versus functional Ldlr system), diet (normal chow versus Western-type diet), or other unknown factors. Another possibility is the type of blood pressure assessment we chose (plethysmographic tail cuff blood pressure measurement), which, in contrast to telemetric invasive blood pressure measurements, may have overlooked smaller differences or time-of-day dependent differences. Nevertheless, further studies are needed to clarify the role of the TP, particularly the TP expressed in VSMC, in Ang II-induced hypertension.

4.3. Role of vascular TP in angiotensin II-related atherosclerosis and aortic pathology development

Furthermore, we investigated the influence of vascular TP deletion on Western-type diet and Ang II-induced mortality, atherogenesis, and aortic aneurysm development. Interestingly, mortality in this model was observed almost exclusively in male mice. Indeed, regardless of genotype, only just under 3 % of females died, whereas 21 % of all males were affected. This is in agreement with the observations of other research groups that have also identified male sex as an important risk factor for Ang II-mediated mortality in the Western-type diet-fed Ldlr or ApoE knockout mouse model of atherosclerosis [9]. With respect to vascular TP deletion, we did not observe significant differences between the genotypes studied. However, mortality tended to be lower in male VSMC-specific TP knockout mice than in male wild-type littermates, possibly indicating a protective effect of VSMC-specific TP knockout in this context. The reasons for this phenomenon remain unclear, but other groups have observed a reduction in Ang II-mediated hypertensive end-organ damage in mice lacking the TP in VSMC, which may also contribute to a potential survival benefit in our model [22].

In the Western-type diet-fed Ldlr knockout mouse model of atherosclerosis used, these Ang II-mediated end-organ damages (in addition to the increase in atherosclerotic plaque area) include mainly aortic aneurysms, which is why this model is also used to elucidate the pathophysiological mechanisms involved in the development of atherosclerosis-associated aortic aneurysms [9]. With regard to the incidence of aortic pathologies, however, again no significant differences were observed between the genotypes studied. Interestingly, sex-specific analyses also showed a more frequent occurrence of Ang II-induced aortic pathologies, such as aortic aneurysms or dissections, in male than in female mice, independent of genotype. Moreover, it was noticeable that the incidence of aortic pathologies tended to be lower in female VSMC-specific TP knockout mice than in female wild-type littermates. In agreement with this finding, also a trend towards a reduced atherosclerotic lesion area in the aortic arch was observed in female VSMC-specific TP knockout mice. The reasons for potential sex-specific effects of VSMC-specific TP deletion in the Ldlr-deficient mouse model remain unclear. However, it is tempting to speculate that sex-specific effects in VSMC promote AT1R-TP synergism by positively affecting receptor expression or ligand synthesis and release. Nevertheless, further studies are needed to substantiate and further elucidate the role of the vascular TP in the pathogenesis of angiotensin II-induced hypertension, aortic atherosclerosis, and aneurysm formation.

In conclusion, in the present study, we demonstrated that neither VSMC-specific nor endothelium-specific knockout of TP significantly affected Ang II-mediated mortality, hypertension, aortic atherosclerosis, or the occurrence of aortic aneurysms. However, sex-specific analysis revealed a trend towards a reduction in Ang II-induced mortality in male VSMC-specific TP knockout mice and a trend towards a reduction in aortic arch atherosclerotic lesion formation and the aortic aneurysm occurrence in female VSMC-specific TP knockout mice. These data could suggest that the TP may represent a sex-specific therapeutic target to

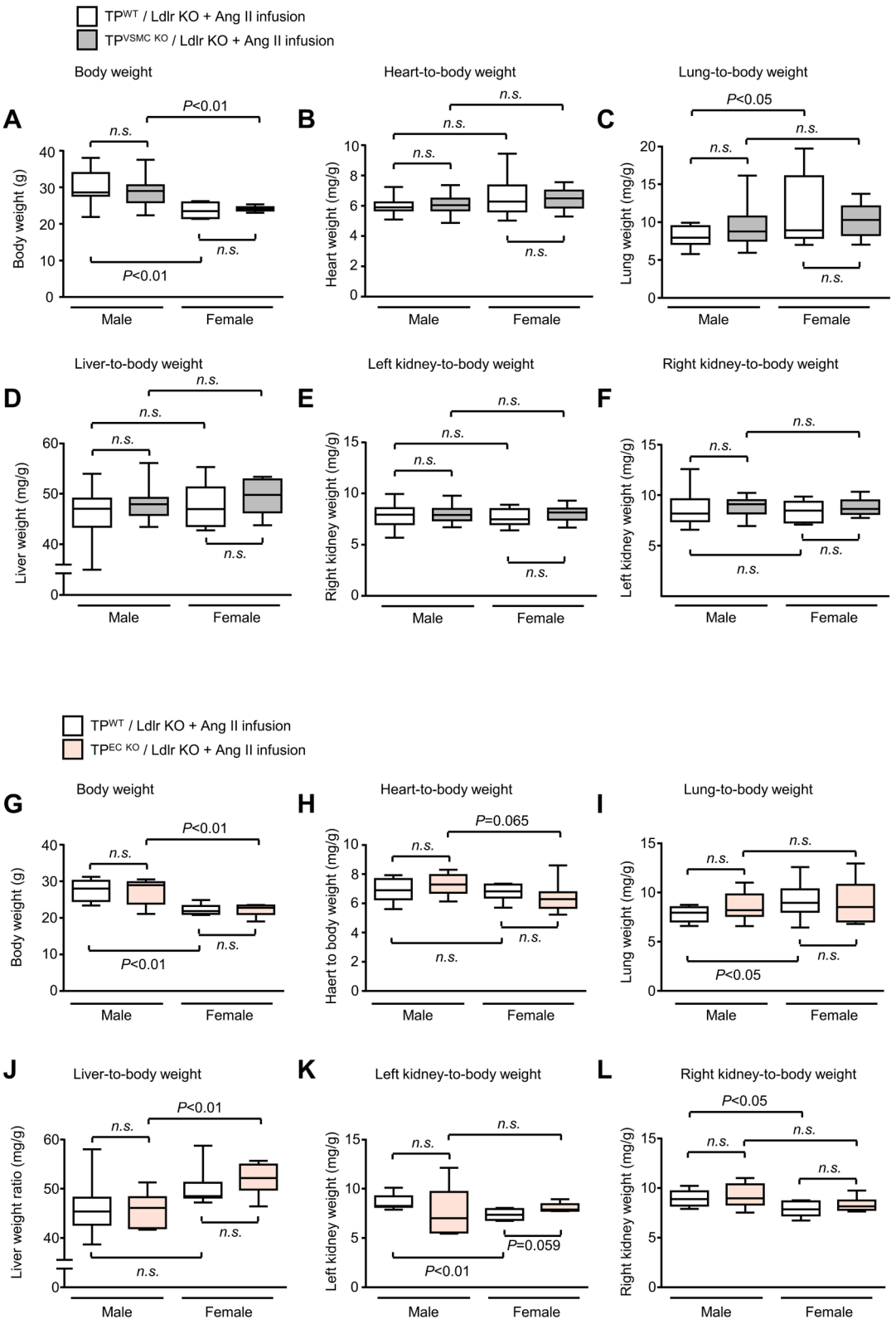

Fig. 7. Effect of VSMC-specific (A-F) or endothelial-specific (G-L) TP knockout on body weight (A,G), the heart-to-body weight ratio (B,H), the lung-to-body weight ratio (C,I), the liver-to-body weight ratio (D,J), or the kidney-to-body weight ratios (E-F,K-L) in Ldlr KO mice fed a cholesterol-rich Western-type diet for nine weeks and additionally treated with Ang II in the last four weeks of the diet. Data for male and female mice are shown separately as min-to-max box-and-whisker plots (including median with 25 % and 75 % percentiles (IQR)). Male TP^{WT}/Ldlr KO (n = 13); male $TP^{VSMC\ KO}$/Ldlr KO (n = 13); female TP^{WT}/Ldlr KO (n = 9); female $TP^{VSMC\ KO}$/Ldlr KO (n = 9). Male TP^{WT}/Ldlr KO (n = 11); male $TP^{EC\ KO}$/Ldlr KO (n = 8); female TP^{WT}/Ldlr KO (n = 7); female $TP^{EC\ KO}$/Ldlr KO (n = 8). n.s. = non-significant.

reduce vascular complications in patients with activated RAAS and thus warrants further research in this area.

CRediT authorship contribution statement

Heike Braun: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Michael Hauke:** Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Markus Petermann:** Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Robert Eckenstaler:** Writing – original draft, Writing – review & editing. **Anne Ripperger:** Investigation, Writing – review & editing. **Edzard Schwedhelm:** Formal analysis, Writing – review & editing. **Beatrice Ludwig-Kraus:** Formal analysis, Writing – review & editing. **Frank Bernhard Kraus:** Formal analysis, Writing – review & editing. **Md Jalal Ahmed Shawon:** Formal analysis, Writing – review & editing. **Virginie Dubourg:** Writing – review & editing. **Alma Zernecke:** Methodology, Writing – review & editing. **Barbara Schreier:** Conceptualization, Funding acquisition, Writing – review & editing. **Michael Gekle:** Conceptualization, Funding acquisition, Writing – review & editing. **Ralf A. Benndorf:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors gratefully acknowledge the expert technical assistance of Manuela Thiemecke and Dorothea Frenzel. This work was supported by the DFG (BE 3246/6-1) and by the European Regional Development Fund of the European Commission (W21029490) to R.A.B., by the DFG (374031971 – TRR 240) to A.Z., and by the DFG (GE 905/24-1) to M.G.

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