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The F2-isoprostane 8-iso-$PGF_{2\alpha}$ attenuates atherosclerotic lesion formation in Ldlr-deficient mice – Potential role of vascular thromboxane A_2 receptors

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ABSTRACT

The F2-isoprostane 8-iso-$PGF_{2\alpha}$ (also known as 15-F_{2t}-isoprostane, $iPF_{2\alpha}$-III, 8-epi $PGF_{2\alpha}$, 15(S)-8-iso-$PGF_{2\alpha}$, or 8-Isoprostane), a thromboxane A_2 receptor (TP) agonist, stable biomarker of oxidative stress, and risk marker of cardiovascular disease, has been proposed to aggravate atherogenesis in genetic mouse models of atherosclerotic vascular disease. Moreover, the TP plays an eminent role in the pathophysiology of endothelial dysfunction, atherogenesis, and cardiovascular disease. Yet it is unknown, how the TP expressed by vascular cells affects atherogenesis or 8-iso-$PGF_{2\alpha}$-related effects in mouse models of atherosclerosis. We studied Ldlr-deficient vascular endothelial-specific (EC) and vascular smooth muscle cell (VSMC)-specific TP knockout mice ($TP^{EC\ KO}$/Ldlr KO; $TP^{VSMC\ KO}$/Ldlr KO) and corresponding wild-type littermates (TP^{WT}/Ldlr KO). The mice were fed a Western-type diet for eight weeks and received either 8-iso-$PGF_{2\alpha}$ or vehicle infusions via osmotic pumps. Subsequently, arterial blood pressure, atherosclerotic lesion formation, and lipid profiles were analyzed. We found that VSMC-, but not EC-specific TP deletion, attenuated atherogenesis without affecting blood pressure or plasma lipid profiles of the mice. In contrast to a previous report, 8-iso-$PGF_{2\alpha}$ tended to reduce atherogenesis in TP^{WT}/Ldlr KO and $TP^{EC\ KO}$/Ldlr KO mice, again without significantly affecting blood pressure or lipid profiles of these mice. However, no further reduction in atherogenesis was observed in 8-iso-$PGF_{2\alpha}$-treated $TP^{VSMC\ KO}$/Ldlr KO mice.

Our work suggests that the TP expressed in VSMC but not the TP expressed in EC is involved in atherosclerotic lesion formation in Ldlr-deficient mice. Furthermore, we report an inhibitory effect of 8-iso-$PGF_{2\alpha}$ on atherogenesis in this experimental atherosclerosis model, which paradoxically appears to be related to the presence of the TP in VSMC.

1. Introduction

Atherosclerosis is one of the most common causes of vascular disease worldwide, with its major clinical manifestations being coronary heart disease (CHD), ischemic stroke, and peripheral artery disease [1]. The pathogenesis of atherosclerosis is thought to be initiated by endothelial dysfunction and structural alteration of vascular architecture with exposure of proteoglycans that promote intimal storage, retention, and oxidative modification of low-density lipoproteins (LDL) [2,3]. Oxidized LDL (oxLDL) and other factors additionally enhance endothelial expression of adhesion molecules, such as VCAM-1, ICAM-1, as well as chemokines and cytokines, e.g., MCP-1, TNF-α, and, via these, endothelial adhesion and transmigration of inflammatory cells into the subendothelial space [2,3]. Subsequently, these events result in the formation of early atherosclerotic lesions. Growth of lesions and

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Non-standard abbreviations and acronyms

8-iso-$PGF_{2\alpha}$	8-iso-prostaglandin $F_{2\alpha}$
apoE	Apolipoprotein E
AT1 receptor	Angiotensin II type 1 receptor
CD31	Cluster of differentiation 31
CHD	Coronary heart disease
EC	Endothelial cell(s)
FELASA	Federation of European Laboratory Animal Science Association
HDL	High density lipoprotein
ICAM-1	Intercellular Adhesion Molecule 1
LDL	Low-density lipoprotein
Ldlr	Low-density lipoprotein receptor
oxLDL	Oxidized low-density lipoprotein
MCP-1	Monocyte chemoattractant protein 1
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
TNF-α	Tumor necrosis factor α
VCAM-1	Vascular cell adhesion molecule 1
VSMC	Vascular smooth muscle cell(s)

remodeling of the vascular wall, including proliferation and migration of vascular smooth muscle cells (VSMC) into the vascular intima, persistent vascular inflammation, and formation of a necrotic plaque core lead to the formation of complex, unstable fibroatheromatous plaques. This favors the development of cardiovascular disease and the occurrence of acute, mostly thrombotic complications, such as myocardial infarction [2,3].

Isoprostanes are prostaglandin isomers formed *in vivo* primarily by radical-induced peroxidation of arachidonic acid. However, results from human studies indicate that cyclooxygenases are also involved in the formation of isoprostanes, although the evidence from human studies is not yet fully conclusive [4–7]. *In vitro*, however, we have just shown that pharmacological inhibition of cyclooxygenase-2 by celecoxib leads to a reduction of 8-iso-$PGF_{2\alpha}$ formation in human endothelial cells, suggesting an involvement of cyclooxygenase-2 in the process of isoprostane formation [8]. Nonetheless, isoprostanes, in particular F2-isoprostanes, which represent a group of 64 compounds isomeric in structure to cyclooxygenase-derived $PGF_{2\alpha}$, are often used as biomarkers for oxidative stress both in preclinical and clinical studies [9]. For F2-isoprostanes, such as 8-iso-$PGF_{2\alpha}$ (also known as 15-F_{2t}-isoprostane, $iPF_{2\alpha}$-III, 8-epi $PGF_{2\alpha}$, 15(S)-8-iso-$PGF_{2\alpha}$, or 8-Isoprostane), it was shown that they can exert biological activity by activating the thromboxane A_2 receptor (TP) [9–11] and may thus directly affect the pathogenesis of cardiovascular diseases. For instance, 8-iso-$PGF_{2\alpha}$ and other isoprostanes synergistically exert anti-angiogenic and pro-apoptotic effects in human endothelial cells via TP activation and may thereby disturb vascular homeostasis and regeneration of the vascular endothelium [10]. In addition, 8-iso-$PGF_{2\alpha}$ has vasoconstrictor properties, which are particularly pronounced in certain vascular beds, including rat aortic and pulmonary artery rings [12]. At isolated coronary arteries of pigs and cattle, 8-iso-$PGF_{2\alpha}$ also proved to be a potent vasoconstrictor [13]. The vasoconstrictor properties of 8-iso-$PGF_{2\alpha}$ were also observed in preparations of the human internal mammary artery [14]. Moreover, cardiovascular responses to 8-iso-$PGF_{2\alpha}$ were abolished in TP knockout mice, highlighting the important role of the TP in mediating the effects of the isoprostane [11]. Isoprostanes are of clinical interest because they are increasingly formed in patients at cardiovascular risk, e.g., patients with CHD, diabetes, or hypercholesterolemia, but also in mouse models of atherosclerosis [15] raising the question whether isoprostanes play a causal role in the pathogenesis of atherosclerosis and its main complication, CHD [9,16,17]. Consistent with these considerations, the stable F2-isoprostane 8-iso-$PGF_{2\alpha}$ aggravated atherogenesis in two different genetic mouse models of atherosclerosis, namely apolipoprotein E (apoE)- and low-density lipoprotein receptor (Ldlr)-deficient mice, by activating the TP [18]. Thus, these results raise the question of whether an increase in isoprostane bioavailability may drive the pathogenesis of atherosclerosis and whether the vascular TP plays a role in this process.

Thromboxane A_2 (TxA_2) is a potent mediator of platelet aggregation as well as vasoconstriction and exerts its numerous effects via the heptahelical TP [9]. 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 [9]. Via an induction of platelet aggregation, the TP plays a major role in the pathogenesis of thromboembolic complications in patients with cardiovascular disease [9]. Besides, the TP contributes to the formation of a dysfunctional proinflammatory endothelium, enhances the interaction between leukocytes and endothelium, and has proapoptotic and antiangiogenic effects in endothelial cells [10,19,20]. In this context, we just recently uncovered a TP-driven and COX-2-dependent auto/paracrine positive feedback loop by which the receptor is able to trigger sustained self-activation independent of exogenous sources of TP ligands, thereby inducing endothelial dysfunction and impairing angiogenesis [21]. Consistent with our aforementioned findings, previous studies indicated that the TP also plays an essential role in the pathogenesis of endothelial dysfunction and atherosclerosis in mouse models *in vivo* [18,22,23]. Moreover, pharmacological TP inhibition has been shown to improve endothelial dysfunction in individuals at high cardiovascular risk, supporting the notion that pharmacological disruption of the TxA_2-TP axis has the potential to improve vascular homeostasis in humans [24]. Nevertheless, it was not known how the vascular TP affects atherogenesis or 8-iso-$PGF_{2\alpha}$-related effects in Ldlr-deficient mice, which we therefore analyzed in the present study. Here we show that VSMC-, but not EC-specific knockout of the TP, attenuated atherogenesis without affecting the arterial blood pressure or plasma lipid profiles of mice. However, in contrast to a previous report [9], the TP agonist 8-iso-$PGF_{2\alpha}$ attenuated atherogenesis in wild-type littermates of EC- and VSMC-specific TP knockout mice on the Ldlr-deficient background as well as in EC-specific TP knockout mice on the Ldlr-deficient background. However, 8-iso-$PGF_{2\alpha}$ infusion did not affect blood pressure or the lipid profile of these mice. Moreover, neither VSMC- or EC-specific knockout nor 8-iso-$PGF_{2\alpha}$ infusion altered body or organ weights or organ-to-body weight ratios in our experimental set-up.

2. Materials and methods

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, USA), unless stated otherwise. 8-iso-$PGF_{2\alpha}$ (#16350, purity >99%) was purchased from Cayman Chemical Europe (Tallin, Estland).

2.1. Animals

Generation of conditional TP knockout mice has been described previously [25]. These mice were obtained from The Jackson Laboratory (Bar Harbor, USA; stock #021985). To generate mice with specific deletion of the TP in the vascular endothelium, floxed homozygous mice were crossed with Tie2-cre mice obtained from The Jackson Laboratory (stock #004128) [26] as reported previously [21]. To generate mice with specific deletion of the TP in vascular smooth muscle cells, floxed

homozygous mice were crossed to SM22α-cre mice obtained from The Jackson Laboratory (stock #004746) [27]. The resultant mice were bred to Ldlr knockout mice to generate homozygous TP-floxed mice on the Ldlr-deficient background. Generation of Ldlr knockout (KO) mice (The Jackson Laboratory, strain #002207) has been published previously [28]. 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 on the Ldlr KO background were analyzed and compared with corresponding sex-matched Ldlr-deficient TP-expressing littermates (TP^{WT}/Ldlr KO; cre-negative). We studied mice that were 12 weeks old when the Western-type diet was started.

All animal experiments were carried out in accordance with the directive 2010/63/EU and the German law (Tierschutzgesetz). The experiments were ethically and legally approved by the governmental, local animal committee (approval number Az 42502-2-1292 MLU, Landesverwaltungsamt Sachsen-Anhalt). According to the Federation of European Laboratory Animal Science Association (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 at 22 ± 2 °C. The mice had free access to water and received a standard rodent diet (Altromin) or as described below a (Western-type) high-fat diet (Altromin Western-type diet; 15% milk fat and 1.25% cholesterol) *ad libitum*. The cages were supplied with some paper towels as nesting material and housing options. Animal well-being was controlled daily. Mice which exceeded a previously determined welfare assessment score were killed immediately. At the end of the experiments, the mice were sacrificed for organ explantation by cervical dislocation.

2.2. Isolation of mouse lung endothelial cells (MLECs)

For MLEC isolation, vascular endothelial TP-deficient mice ($TP^{EC\ KO}$/Ldlr KO; Tie2-cre-positive) and wild-type (TP^{WT}/Ldlr KO; Tie2-cre-negative) mice were sacrificed by cervical dislocation. Lungs were explanted and dispersed using the mouse lung dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. Afterwards, endothelial cells were isolated by magnetic-activated cell separation (MACS). For enrichment of CD31-positive endothelial cells, endothelial cells were magnetically separated with CD31 antibody-coated microbeads from dispersed lung tissue according to the manufacturer's protocol (Miltenyi Biotec). mRNA derived from CD31-positive cell fractions was gained by trizol purification and analyzed by real-time RT-PCR as described below.

2.3. 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). Mice were habituated to the procedure for two weeks. The measurement period started six weeks before 8-iso-$PGF_{2\alpha}$ infusion and lasted for 10 weeks. Each session consisted of 20 inflation and deflation cycles per set, of which the first five cycles were acclimation cycles and were not used for analysis. Measurements took place twice a week between 7 a.m. and 11 a.m. after ending of the dark-cycle. Only measurements taken in the week before to four weeks after the start of infusion of 8-iso-$PGF_{2\alpha}$ or vehicle control were used to evaluate the blood pressure of the mice.

2.4. 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 eight weeks, starting 4 weeks before treatment with 8-iso-$PGF_{2\alpha}$. A similar number of males and females were studied in each treatment group. For subcutaneous infusion of 8-iso-$PGF_{2\alpha}$ (5 μg/kg body weight/hour, dissolved in sterile 0.9% NaCl solution containing 10% ethanol) or vehicle control (sterile 0.9% NaCl solution containing 10% ethanol), Alzet osmotic pumps (model 1004) were implanted under isoflurane anesthesia on the back of the mice, slightly posterior to the scapulae. Control mice received only the ethanol-containing solvent, without 8-iso-$PGF_{2\alpha}$, for four weeks via osmotic pumps. After 28 days of 8-iso-$PGF_{2\alpha}$ infusion, mice were sacrificed and tissue and plasma were subsequently analyzed.

2.5. Atherosclerotic lesion quantification

For fixation and before analysis of aortic tissue, mice (after cervical dislocation) were perfused very gently via syringe by injection of 2x 10 mL PBS and 1x 10 mL 4% paraformaldehyde (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. 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 [29]. 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 was used to determine the atherosclerotic lesion area and the total area of the aortic intima. A similar number of males and females were studied in each treatment group, and a pooled analysis of atherosclerotic lesion formation was performed. Analysis of the aortas was performed separately for different 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. In addition, the total atherosclerotic lesion area of the aorta was determined.

2.6. Lipid measurements

Cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides were analyzed by colorimetric assays (CHOL2, cholesterol; Gen.2, HDL cholesterol; Gen.4, LDL cholesterol; Gen.3, triglycerides; all Roche Diagnostics, Rotkreuz, Switzerland) on either a Roche cobas c701 or c502 analyzer integrated with a fully automated Roche Cobas 8000 platform. All analyses on Roche cobas analyzers were performed with routine maintenance and quality control procedures and according to the manufacturer's instructions and manuals. 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 [30,31]. mRNA expression was quantified using the ABI 7500 Real-Time PCR System (Thermo Fisher Scientific). TaqMan reactions were carried out in 96-well plates according to the manufacturer's instructions using premade 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 (probes) 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 [32].

2.8. Statistical analyses

Statistical analyses were performed using one-way analysis of variance followed by Sidak's multiple comparisons 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 mean ± standard deviation (SD) or as indicated otherwise.

Probability values were considered significant at a $p < 0.05$.

3. Results

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

We here studied Ldlr KO mice with a VSMC-specific deletion of the TP ($TP^{VSMC\ KO}$/Ldlr KO) fed a Western-type diet for eight weeks (Fig. 1). To verify successful knockout of the TP in aortic vascular smooth muscle cells derived from $TP^{VSMC\ KO}$/Ldlr KO mice, we analyzed TP mRNA expression in denuded aortas in which the adventitia had been removed. In these analyses, we observed a reduction of TP mRNA expression by approximately 95% in aortic tissue obtained from $TP^{VSMC\ KO}$/Ldlr KO mice as compared with aortic tissue obtained from TP^{WT}/Ldlr KO littermates (Fig. 2A). The selective deletion of the TP in VSMC was associated with a significant reduction in Western-type diet-induced atherosclerotic lesion formation in the aorta (Fig. 2B, F). Regarding the different aortic segments, we observed a significant reduction in atherosclerotic lesion formation in the aortic arch (Fig. 2C, F) and a non-significant trend towards a decrease in atherosclerotic lesion formation in the thoracic and abdominal aorta (Fig. 2D–F).

To validate the knockout of the TP in vascular endothelial cells derived from $TP^{EC\ KO}$/Ldlr KO mice, we analyzed TP mRNA expression in lung tissue and in MACS-isolated CD31-positive lung endothelial cells. In these experiments, $TP^{EC\ KO}$/Ldlr KO mice showed a reduction of TP mRNA expression in lung tissue by approximately 75% and in CD31-positive lung endothelial cells by approximately 88% (Fig. 3A). However, $TP^{EC\ KO}$/Ldlr KO mice showed no significant change in Western diet-induced atherosclerotic lesion formation in the aorta after 8 weeks of diet (Fig. 3B–F).

3.2. Effect of 8-iso-$PGF_{2\alpha}$ infusion on Western-type diet-induced atherosclerotic lesion formation in Ldlr-deficient vascular TP knockout mice and wild-type littermates

We also examined the effect of 8-iso-$PGF_{2\alpha}$ infusion on atherosclerotic plaque formation in both vascular (EC- and VSMC-specific) TP KO mice and their respective wild-type littermates. The rationale for these studies was that a TP-mediated pro-atherosclerotic effect of 8-iso-$PGF_{2\alpha}$ had been previously described in Ldlr KO mice [18]. Therefore, by using VSMC-specific or EC-specific KO mice, we aimed to clarify the role of the vascular TP in mediating this proatherogenic effect of 8-iso-$PGF_{2\alpha}$. Surprisingly, however, our experiments showed that infusion of 8-iso--$PGF_{2\alpha}$ in wild-type littermates of VSMC-specific TP KO mice caused not an increase but a moderate decrease in atherosclerotic lesion formation (Fig. 2B–F). Furthermore, this anti-atherosclerotic effect of 8-iso-$PGF_{2\alpha}$ infusion was also independently replicated in the wild-type littermates of EC-specific TP KO mice (Fig. 3B–F) and primarily resulted in a decrease in aortic arch atherosclerotic lesion formation in both TP wild-type mouse strains (TP^{WT}/Ldlr KO) analyzed (Figs. 2C and 3C). Interestingly, a trend towards a reduction in atherosclerotic plaque area by 8-iso-$PGF_{2\alpha}$ infusion was also evident in EC-specific TP KO mice, although this effect became statistically significant only in the abdominal aorta (Fig. 3E). In contrast, infusion of 8-iso-$PGF_{2\alpha}$ in VSMC-specific TP KO mice on the Ldlr-deficient background did not reduce atherogenesis further in our experimental set-up (Fig. 2B–F). These results may suggest that the TP expressed in VSMC is responsible for mediating anti-atherosclerotic effects induced by 8-iso-$PGF_{2\alpha}$ in Ldlr-deficient mice.

3.3. Effects of vascular-specific TP deletion and 8-iso-$PGF_{2\alpha}$ infusion on blood pressure, plasma lipid profiles, and organ weights of Ldlr-deficient mice on Western-type diet

To analyze a potential blood pressure modulating effect of the vascular-specific TP KO and/or 8-iso-$PGF_{2\alpha}$ infusion, we performed blood pressure measurements by the tail-cuff method in mice. Here, we first observed that neither EC-specific nor VSMC-specific TP KO led to a significant change in systolic blood pressure compared with their respective wild-type littermates (Fig. 4), although systolic blood pressure tended to be lower in VSMC-specific TP KO mice before 8-iso-$PGF_{2\alpha}$ treatment (Fig. 4A). Furthermore, four-week infusion of 8-iso-$PGF_{2\alpha}$ did not result in a significant change in systolic blood pressure in either vascular TP KO mice or their respective TP wild-type littermates (Fig. 4). These results therefore suggest that the inhibitory effect of VSMC-specific TP knockout or 8-iso-$PGF_{2\alpha}$ infusion on atherogenesis most likely cannot be explained by the effect of the respective intervention on systemic blood pressure.

In addition, the plasma lipid profiles of the mice were examined. For this purpose, we first examined the lipid profiles of TP^{WT}/Ldlr KO mice fed a standard diet and compared the results with those of Ldlr wild-type mice fed the same standard diet (Fig. 5A–D). Ldlr KO mice showed the expected increase in total plasma cholesterol (Fig. 5A), LDL cholesterol (Fig. 5B), HDL cholesterol (Fig. 5C), and triglyceride levels (Fig. 5C) compared with wild-type mice. Moreover, the Western-type diet greatly increased total cholesterol (Fig. 5E,I), LDL cholesterol (Fig. 5F,J), and triglyceride levels (Fig. 5H,L) in VSMC-specific or EC-specific TP KO mice and similarly in their corresponding wild-type littermates on the Ldlr KO background. However, neither VSMC-specific nor EC-specific

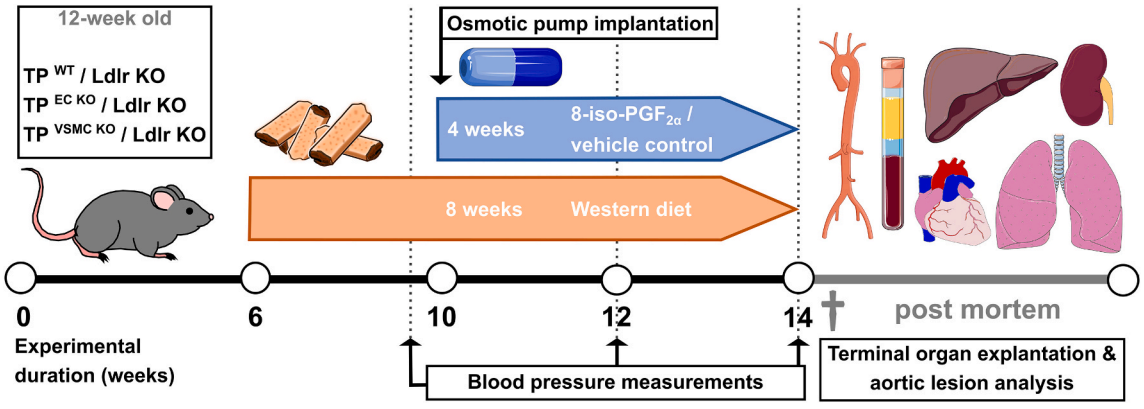

Fig. 1. Schematic representation of the experimental setup of the animal studies used to analyze atherogenesis in Ldlr-deficient mice. Shown is the period during which mice were fed a Western diet and also infused with 8-iso-$PGF_{2\alpha}$ or vehicle control via implanted osmotic pumps. Time points of blood pressure measurements are indicated by arrows. After the animals were sacrificed, body and organ weights were determined, plasma was collected, and aortic tissue was prepared and utilized for atherosclerotic lesion analysis.

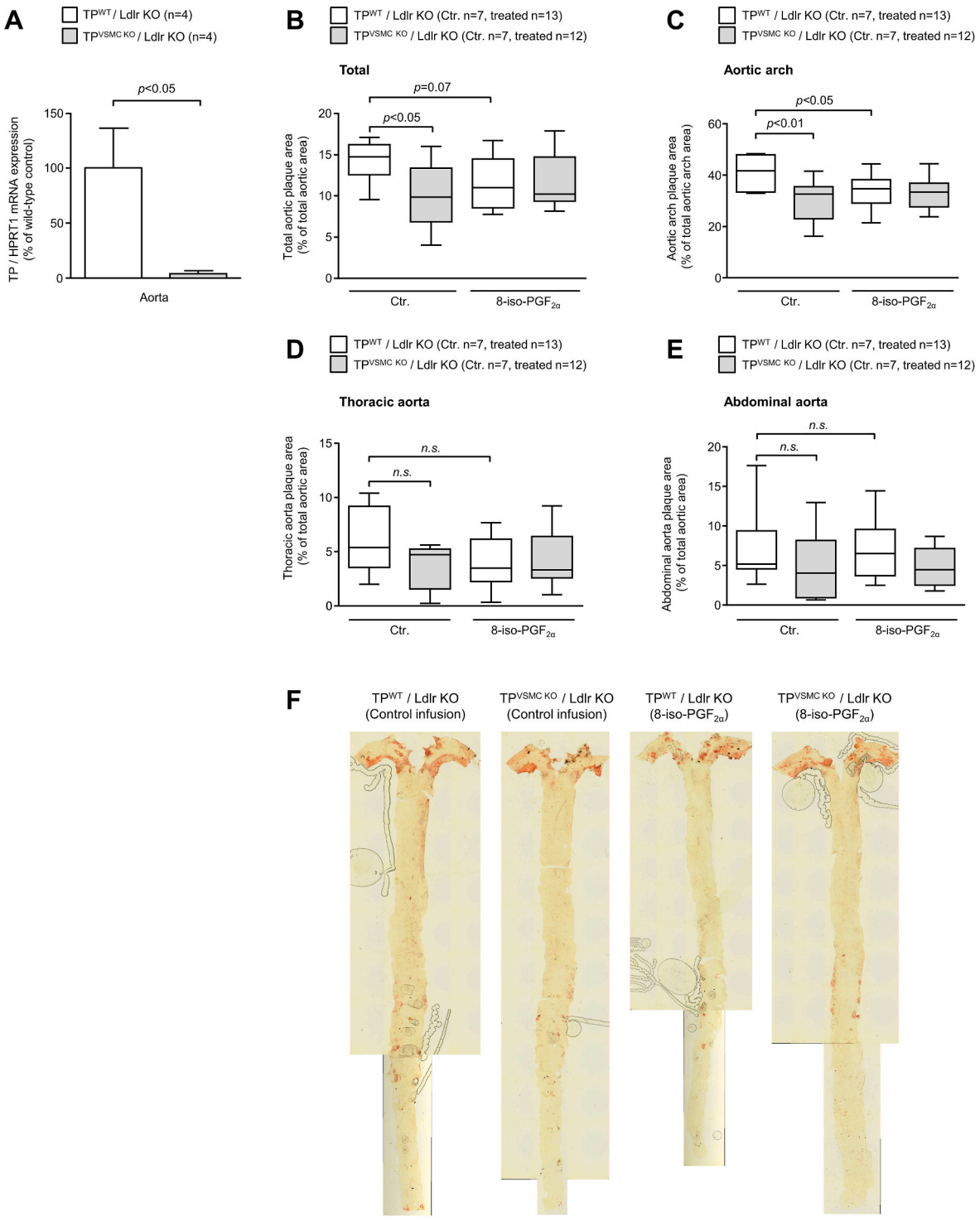

Fig. 2. Vascular smooth muscle (VSMC)-specific deletion of the TP reduces aortic atherosclerosis of Ldlr knockout mice fed a Western-type diet for eight weeks. Moreover, infusion of 8-iso-$PGF_{2\alpha}$ moderately reduces atherogenesis in thromboxane A_2 receptor (TP)-expressing Ldlr knockout mice but not in VSMC-specific TP knockout littermates. (**A**) TP mRNA expression in aortic tissue derived from $TP^{VSMC\ KO}$/Ldlr KO mice or TP^{WT} (wild-type, cre-negative)/Ldlr KO littermates. Data are shown as mean ± SD. (n = 4). Analysis of aortic atherosclerotic lesion formation in aortas derived from TP^{VSMC} 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 abdominal aorta (**E**) are shown. (n = 7, Ctr. TP^{WT}/Ldlr KO); (n = 7, Ctr. $TP^{VSMC\ KO}$/Ldlr KO); (n = 13, 8-iso-$PGF_{2\alpha}$-treated TP^{WT}/Ldlr KO); (n = 12, 8-iso-$PGF_{2\alpha}$-treated $TP^{VSMC\ KO}$/Ldlr KO). Two-group statistical analysis was performed using the unpaired two-tailed Student's t-test, while for multiple-group statistical analysis, one-way analysis of variance followed by the Sidak multiple comparison test was used. (**F**) Representative pictures of aortas derived from these mice.

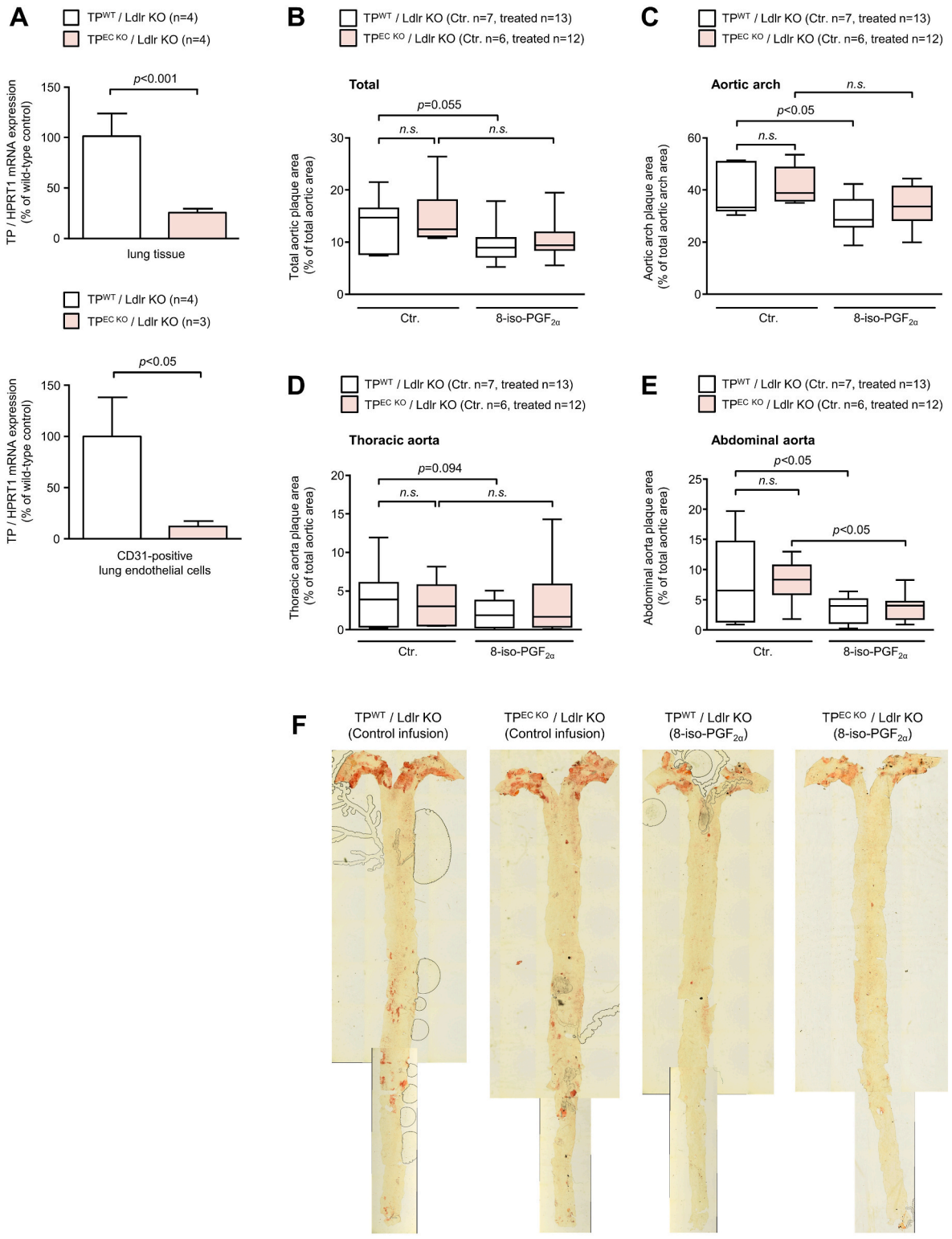

Fig. 3. Endothelial (EC)-specific deletion of the TP does not affect aortic atherosclerosis of Ldlr knockout mice fed a Western-type diet for eight weeks. However, infusion of 8-iso-$PGF_{2\alpha}$ moderately reduces atherogenesis in thromboxane A_2 receptor (TP)-expressing Ldlr knockout mice as well as in EC-specific TP knockout littermates, indicating that the endothelial TP is not relevant for 8-iso-$PGF_{2\alpha}$-mediated suppression of atherosclerotic lesion formation. TP expression in lung tissue (**A**, upper panel, n = 4) or MACS-isolated CD31-positive lung endothelial cells (**A**, lower panel, n = 3–4) derived from $TP^{EC\ KO}$/Ldlr KO mice or TP^{WT} (wild-type, cre-negative)/Ldlr KO littermates. Data are shown as mean ± SD. Analysis of aortic atherosclerotic lesion formation in aortas derived from 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. (n = 7, Ctr. TP^{WT}/Ldlr KO); (n = 6, Ctr. $TP^{EC\ KO}$/Ldlr KO); (n = 13, 8-iso-$PGF_{2\alpha}$-treated TP^{WT}/Ldlr KO); (n = 12, 8-iso-$PGF_{2\alpha}$-treated $TP^{EC\ KO}$/Ldlr KO). Two-group statistical analysis was performed using the unpaired two-tailed Student's t-test, whereas for multiple-group statistical analysis, one-way analysis of variance followed by the Sidak multiple comparison test was used. (**F**) Representative pictures of aortas derived from these mice.

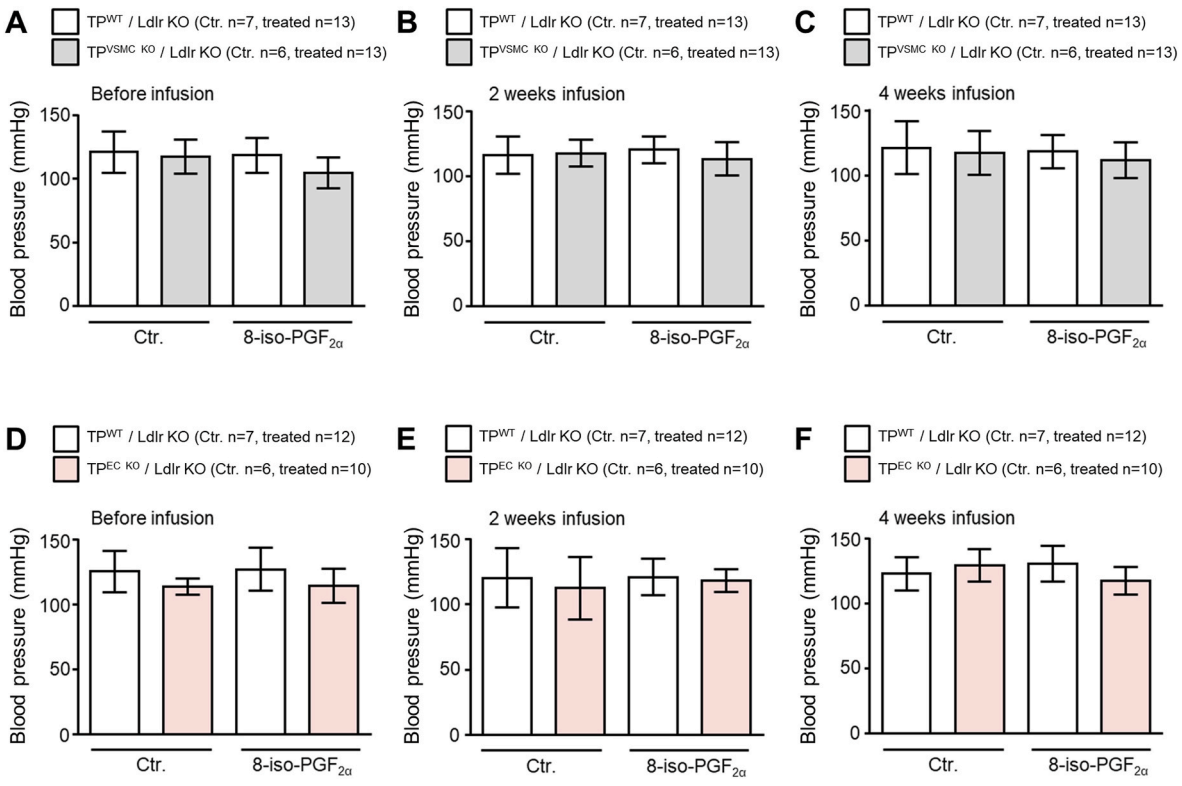

Fig. 4. Neither TP knockout nor 8-iso-PGF$_{2\alpha}$ infusion have a significant effect on systemic blood pressure in Ldlr-deficient mice. Infusion of 8-iso-PGF$_{2\alpha}$ does not significantly affect systemic blood pressure in VSMC-specific TP knockout mice on the Ldlr-deficient background (TP$^{VSMC\ KO}$/Ldlr KO; **A-C**) or in TP wild-type littermates (TP$^{WT\ KO}$/Ldlr KO). Blood pressure tended to be lower in TP $^{VSMC\ KO}$/Ldlr KO mice before infusion of 8-iso-PGF$_{2\alpha}$ (**A**). Data are shown as mean ± SD. (n = 7, Ctr. TP^{WT}/Ldlr KO); (n = 7, Ctr. TP$^{VSMC\ KO}$/Ldlr KO); (n = 13, 8-iso-PGF$_{2\alpha}$-treated TP^{WT}/Ldlr KO); (n = 13, 8-iso-PGF$_{2\alpha}$-treated TP$^{VSMC\ KO}$/Ldlr KO). Also, infusion of 8-iso-PGF$_{2\alpha}$ does not affect systemic blood pressure in EC-specific TP knockout mice on the Ldlr-deficient background (TP^{EC} KO/Ldlr KO; **D-F**) or in TP wild-type littermates (TP^{WT} KO/Ldlr KO). Data are shown as mean ± SD. (n = 7, Ctr. TP^{WT}/Ldlr-deficient); (n = 6, Ctr. TP$^{EC\ KO}$/Ldlr KO); (n = 12, 8-iso-PGF$_{2\alpha}$-treated TP^{WT}/Ldlr KO); (n = 10, 8-iso-PGF$_{2\alpha}$-treated TP$^{EC\ KO}$/Ldlr KO). In addition, VSMC-specific or endothelial-specific TP knockout in Ldlr-deficient mice does not significantly affect systemic blood pressure (**A-F**). All statistical analyses were performed using one-way ANOVA followed by the Sidak multiple comparisons test.

TP knockout had a significant effect on plasmatic cholesterol or triglyceride levels in Ldlr KO mice (Fig. 5E-L). Moreover, plasmatic cholesterol or triglyceride levels were not significantly affected by infusion of 8-iso-PGF$_{2\alpha}$ (Fig. 5E-L).

Furthermore, we analyzed the effects of vascular-specific TP deletion and 8-iso-PGF$_{2\alpha}$ infusion on heart, lung, liver and kidney weight, and body weights of Ldlr KO mice fed a Western-type diet (Supplemental Fig. 1). Again, neither vascular-specific TP knockout nor 8-iso-PGF$_{2\alpha}$ infusion had a significant effect on organ or body weights or the organ-to-body weight ratios of the mice (Supplemental Fig. 1).

4. Discussion

The TP has been shown to drive atherogenesis in mouse models of atherosclerosis [18,22,23], to promote endothelial dysfunction in humans [24], and to contribute to platelet aggregation and thromboembolic complications in cardiovascular disease [9]. Interestingly, in this context, previous expression analyses suggested elevated TP levels in the intimal layer of blood vessels of cardiovascular risk patients and murine atherosclerotic lesions [33,34]. The role of the vascular TP in this process, however, had not been elucidated before. An important goal of this work was therefore to clarify in which way the vascular TP affects atherogenesis in the Ldlr-deficient mouse model of atherosclerosis. For this purpose, we generated VSMC- and EC-specific TP knockout mice on the Ldlr-deficient background, fed these mice a Western-type diet for eight weeks and subsequently analyzed atherosclerotic lesion formation in the aorta. The results of these experiments indicate that VSMC-specific knockout moderately reduces atherosclerotic lesion formation in Ldlr-deficient mice, whereas EC-specific deletion of the TP has no considerable impact on this process. To the best of our knowledge, our study is the first to investigate the role of the vascular TP in the process of atherosclerosis using a conditional TP knockout mouse model on the Ldlr-deficient background. Nonetheless, VSMC-specific TP knockout mice (with a functional Ldlr system) have been previously studied in the context of angiotensin II-mediated hypertension and vascular remodeling by Sparks and colleagues [35]. In this study, the TP in VSMC was shown to play a role in the transduction of angiotensin II-mediated signals in the vascular system. Indeed, deletion of the TP in VSMC significantly attenuated Ang II-induced vascular remodeling and hypertension *in vivo*. These results together with our findings therefore suggest that VSMC-specific deletion of the TP may attenuate pathological vascular remodeling, such as angiotensin II-triggered vascular remodeling or atherosclerotic lesion formation of the aorta. The mechanisms by which the TP expressed in VSMC triggers these effects are as yet unclear but may be related to a reduction in arterial blood pressure. In this regard, pharmacological blockade or genetic deletion of the TP have been shown to reduce the vasoconstrictor as well as blood pressure-increasing effect of angiotensin II [35–38]. These effects might be triggered by AT1 receptor-mediated release of TP ligands, such as TxA_2 or 8-iso-PGF$_{2\alpha}$, suggesting that both AT1 receptor and TP function as an autocrine and paracrine active signal transduction unit, with effects on VSMC [35–40]. Indeed, VSMC-specific deletion of TP was also associated with a nonsignificant trend toward a reduction in arterial blood pressure in our analyses, so blood pressure must also be considered as an influencing factor in our experiments. In addition, these findings also raised the question of

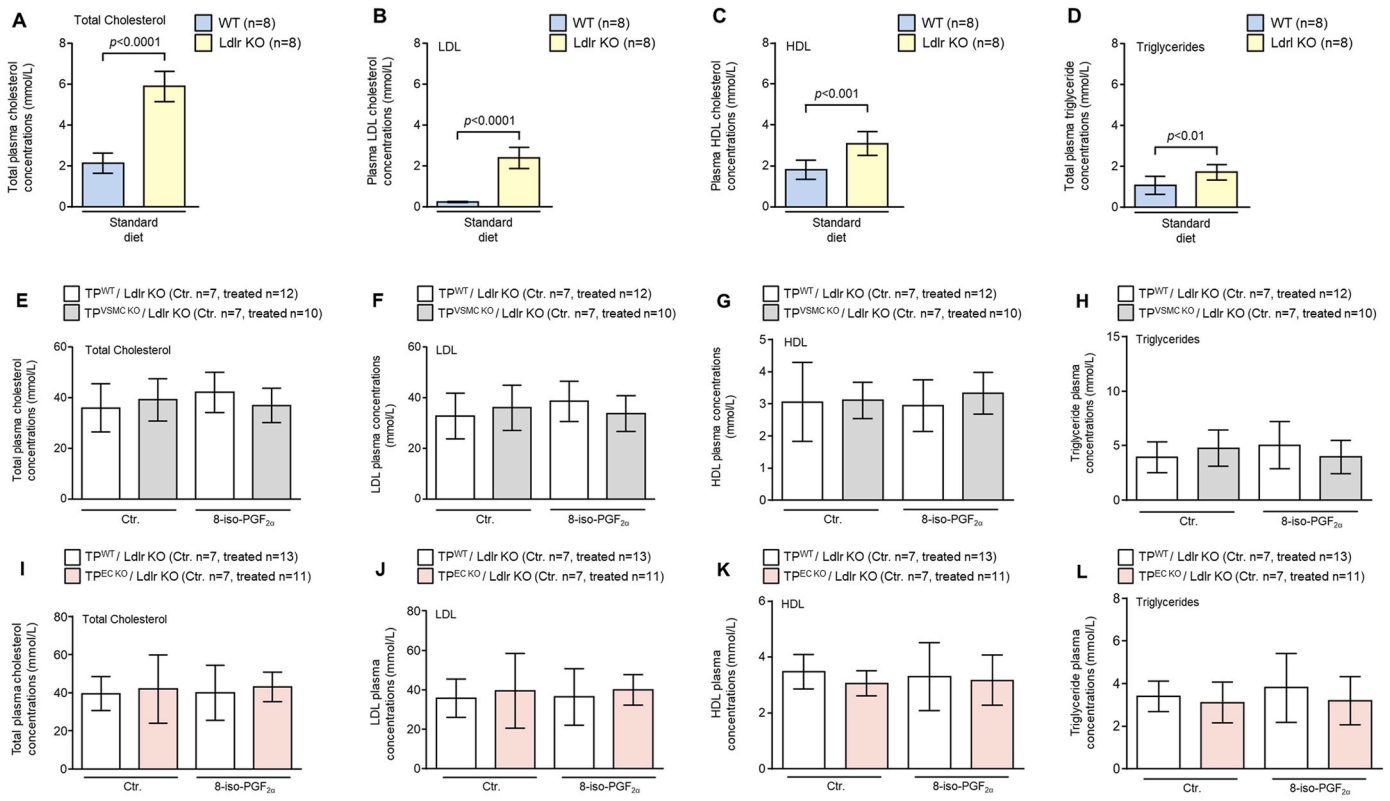

Fig. 5. Neither TP knockout nor 8-iso-PGF$_{2\alpha}$ infusion affect plasma lipid levels in Ldlr knockout mice. A-D) Ldlr knockout mice that are additionally homozygous for the floxed TP allele (Ldlr KO) show profound changes in plasma cholesterol and triglyceride levels compared with wild-type (WT) mice, even when fed a standard diet. In Ldlr KO mice highly significant increases in total cholesterol (**A**), LDL (**B**), HDL (**C**), and triglycerides (**D**) were observed. Data are shown as mean ± SD. (n = 8). Eight weeks of Western-type diet strongly increases total cholesterol, LDL cholesterol, and triglycerides levels in TP$^{VSMC\ KO}$/Ldlr KO and in corresponding wild-type littermates (TP^{WT}/Ldlr KO, **E-H**). Data are shown as mean ± SD. (n = 7, Ctr. TP^{WT}/Ldlr KO); (n = 7, Ctr. TP$^{VSMC\ KO}$/Ldlr KO); (n = 12, 8-iso-PGF$_{2\alpha}$-treated TP^{WT}/Ldlr KO); (n = 10, 8-iso-PGF$_{2\alpha}$-treated TP$^{VSMC\ KO}$/Ldlr KO). Western-type diet also considerably increases total cholesterol, LDL cholesterol, and triglycerides levels in TP$^{EC\ KO}$/Ldlr KO and corresponding wild-type littermates (**I-L**). Data are shown as mean ± SD. (n = 7, Ctr. TP^{WT}/Ldlr KO); (n = 6, Ctr. TP$^{EC\ KO}$/Ldlr KO); (n = 13, 8-iso-PGF$_{2\alpha}$-treated TP^{WT}/Ldlr KO); (n = 11, 8-iso-PGF$_{2\alpha}$-treated TP$^{EC\ KO}$/Ldlr KO). However, neither VSMC-specific nor EC-specific TP knockout significantly affect plasmatic total cholesterol (**E,I**), LDL cholesterol (**F,J**), HDL cholesterol (**G,K**) or triglyceride levels (**H,L**). In addition, plasmatic cholesterol or triglyceride levels were not significantly affected by infusion of 8-iso-PGF$_{2\alpha}$ (**E-L**). Two-group statistical analysis was performed using the unpaired two-tailed Student's t-test, while for multiple-group statistical analysis, one-way analysis of variance followed by the Sidak multiple comparison test was used.

whether an increase in isoprostane bioavailability may affect atherogenesis via pronounced TP activation.

Surprisingly, however, 8-iso-PGF$_{2\alpha}$ infusion did not aggravate atherosclerotic lesion formation in our experimental system. In contrast to a previous report [18], we observed that it reduced atherogenesis in two different TP-expressing Ldlr-deficient mouse strains, namely wild-type littermates of VSMC-specific and EC-specific TP-knockout mice on the Ldlr-deficient background. Interestingly, in these Ldlr-deficient mouse strains, 8-iso-PGF$_{2\alpha}$ significantly reduced atherosclerotic lesion formation in the aortic arch. In addition, 8-iso-PGF$_{2\alpha}$ also reduced atherogenesis in EC-specific TP knockout mice on the Ldlr-deficient background. In contrast, 8-iso-PGF$_{2\alpha}$ infusion did not further reduce atherogenesis in VSMC-specific TP knockout mice. Although the differential effect of 8-iso-PGF$_{2\alpha}$ in EC-specific and VSMC-specific TP knockout mice is difficult to interpret, it may indicate that the anti-atherogenic effect of 8-iso-PGF$_{2\alpha}$ depends on the presence of the TP in VSMC, whereas the presence of the endothelial TP is not required. On a speculative basis and as previously described by other research groups [41], 8-iso-PGF$_{2\alpha}$ could act as a partial antagonist at the TP expressed by platelets under conditions of strong thromboxane A$_2$ formation, thereby suppressing TP-induced platelet-related vascular effects and paracrine thromboxane A$_2$ release in atherogenesis. Moreover, certain isoprostanes, including 8-iso-PGF$_{2\alpha}$, have been shown to exert anti-inflammatory effects on monocytes/macrophages *in vitro*, effects that may also have an impact on the differentiation to macrophages to foam cells and the process of atherosclerotic plaque formation *in vivo* [42,43]. In this context, for example, addition of 8-iso-PGF$_{2\alpha}$ to RAW 264.7 macrophages during endotoxin challenge decreased gene expression of Nos2 and Il1β and reduced cytokine production of IL6, while increasing production of IL10, G-CSF, and IL17 [43]. However, the latter very interesting point of potential immunological effects of isoprostanes remains to be demonstrated in appropriate future studies in animal models of atherosclerosis. However, it should be noted that 8-iso-PGF$_{2\alpha}$ appears to exert its moderate anti-atherogenic effects without significantly altering the blood pressure or lipid profile of the mice, and it had no effect on the body weight or organ weight-to-body weight ratio of the heart, lungs, liver, or kidneys of the treated mice.

The reasons for the apparent discrepancy between our findings and those published by Tang and colleagues are somewhat unclear. As in our study, in the study by Tang and Colleagues the Ldlr knockout mouse model of atherosclerosis was used. Also, the aortic arch was analyzed together with the descending thoracic aorta by the *en face* method and atherosclerotic lesions were visualized with Sudan IV. Nevertheless, relevant experimental differences may lie in the modification of TP activity, the mode of application of 8-iso-PGF$_{2\alpha}$, and the applied dose of 8-iso-PGF$_{2\alpha}$. In this regard, we applied a tissue-selective TP knockout approach to affect 8-iso-PGF$_{2\alpha}$ activity *in vivo*, whereas Tang and colleagues antagonized the effects of 8-iso-PGF$_{2\alpha}$ by concomitant pharmacological TP inhibition. Moreover, we continuously applied 8-iso-PGF$_{2\alpha}$ to the mice at a dose of 5 μg/kg body weight per hour via osmotic

pumps, whereas Tang and colleagues delivered 8-iso-$PGF_{2\alpha}$ to the mice via intraperitoneal injection at a dose of 1 μg/kg body weight every three days. Thus, our approach using the osmotic pumps translates into an approximately 350-fold higher dose of 8-iso-$PGF_{2\alpha}$. However, even when these experimental differences are considered, it remains difficult to explain why continuous delivery of higher doses of 8-iso-$PGF_{2\alpha}$ should lead to opposite anti-atherosclerotic effects in the same Ldlr-deficient mouse model. Nevertheless, in light of the results we have obtained, we believe that further independent studies are needed to elucidate the role of isoprostanes such as 8-iso-$PGF_{2\alpha}$ in the process of atherosclerotic lesion formation and to clarify a potential mechanistic role of the TP expressed by VSMC in this process.

5. Conclusion

In conclusion, our work with conditional TP knockout mice suggests that VSMC-specific but not EC-specific deletion of TP moderately reduces atherosclerotic lesion formation in the well-characterized Ldlr-deficient mouse model of atherosclerosis. We also report an unexpected inhibitory effect of 8-iso-$PGF_{2\alpha}$ on atherogenesis in this experimental model, which contradicts previously published findings on the impact of isoprostanes on atherosclerotic lesion formation. Although our analyses need to be confirmed by further independent studies, they may indicate that the increased formation of isoprostanes in cardiovascular risk patients could serve to slow the progression of atherosclerotic vascular disease.

Data availability

The data that support the findings of this study are available within the article or from the corresponding author upon reasonable request.

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Disclosure of conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2022.04.010.

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