Supplementary Information

G Protein-Coupled Estrogen Receptor Protects from Atherosclerosis

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Supplementary Methods

Mice. For generation of *gper-/-* mice, the *gper* gene was targeted in 129 SvEvTac embryonic stem (ES) cells by a specific vector with Neo[r] insertion[1]. ES cells were microinjected into C57BL/6J blastocysts and implanted into recipient pseudo-pregnant CD1 female mice, which were backcrossed 10 generations onto the C57BL/6J background. Successful gene targeting was verified by PCR genotyping and Southern blot[1]. Before sacrifice, systolic and diastolic blood pressure was measured in conscious mice using a non-invasive volume-pressure recording monitoring system (CODA-6, Kent Scientific, Torrington, CT, USA), which produces blood pressure readings with similar sensitivity and specificity as invasive measurements[2]. Following a training period of 5-7 days, measurements recorded on 5-8 consecutive days were averaged for each mouse.

Quantification of atherosclerosis. Following an overnight fast, mice were euthanized by intraperitoneal injection of sodium pentobarbital (2.2 mg/g body weight). The heart and the aorta were excised and washed in physiological saline solution (PSS, composition in mmol/L: 129.8 NaCl, 5.4 KCl, 0.83 MgSO4, 0.43 NaH2PO4, 19 NaHCO3, 1.8 CaCl2, and 5.5 glucose; pH 7.4). For quantification of atherosclerosis in histologic aortic sections, the aortic sinus was embedded in optimal cutting temperature (O.C.T.) compound, and stored at -80 °C until processing. Embedded aortic roots were cryosectioned with a microtome cryostat (Microm HM 550, Thermo Fisher Scientific, Waltham, MA, USA). After sixteen week of an atherogenic, high-fat diet containing high cholesterol and cholate[3], atherosclerosis in the aortic root was quantified as previously described and validated by Paigen *et al*.[3] Eighteen alternate sections per aortic root covering a distance of 360 μm were examined by light microscopy (Nikon Eclipse E400 connected to a Nikon DS-Fi1 camera and NIS Elements F 3.0 software; Melville, NY, USA)**.** To quantify macroscopic atherosclerosis and lesion distribution throughout the aortic tree in *en face* stained preparations[4], atherosclerosis was quantified separately in the proximal segment (ascending aorta and aortic arch), middle segment (descending and suprarenal aorta) and distal segment of the aorta (infrarenal aorta to the iliac bifurcation).

Vascular NO bioactivity in WT and *gper-/-* mice *ex vivo*. The mouse common carotid artery was chosen for these studies due to its particularly high NO bioactivity[5]. Immediately after sacrifice, common carotid arteries were excised, transferred into cold (4 °C) PSS, and cleaned of fat and connective tissue to exclude perivascular adipose-dependent contractile effects[6]. Arteries

were cut into rings 3 mm in length and mounted on a Mulvany-Halpern myograph (620M Multi Wire Myograph System, Danish Myo Technology, Aarhus, Denmark) to record isometric tension using a PowerLab 8/35 data acquisition system and LabChart Pro software (AD Instruments, Colorado Springs, CO, USA)[7]. Vascular function experiments were performed as recently described[7]. Briefly, functional integrity of the vascular smooth muscle was assessed by repeatedly exposing vessels to KCl (PSS with equimolar substitution of 60 mmol/L potassium for sodium). The presence of an intact endothelium was confirmed by precontracting arterial rings with phenylephrine (~60% of KCl-induced contractions), and subsequently exposing them to cumulative concentrations of acetylcholine (0.1 nmol/L – 10 μmol/L) inducing endothelium-dependent, NO-mediated relaxation. Experiments were conducted in the presence of meclofenamate (1 μmol/L) to exclude effects of cyclooxygenase-derived endothelial vasoconstrictor prostanoids. A relaxation response of >90% was considered to represent an intact and functional endothelium. Functional integrity of vascular smooth muscle was confirmed by relaxations to the NO donor sodium nitroprusside (10 pmol/L – 10 μmol/L) in rings precontracted with phenylephrine. Basal NO bioactivity was determined as described[8, 9].

GPCR-stimulated NO formation in human endothelial cells. Human endothelial cells of a h*TERT*-immortalized umbilical vein endothelial (TIVE) cell line were kindly provided by Rolf Renne (University of Florida, FL, USA) and generated as described[10]. These cells express the endothelial-cell-specific markers CD31, Flt, UEA, CD105, and factor VIII/vWF; the observed expression patterns of TIVE cells at passage 12 remain identical to those derived from primary HUVEC at passage 2, cells from which the *gper* gene was originally cloned[11]. Endothelial cells were cultured in M199 basal medium supplemented with 20% FBS bovine endothelial cell growth factor (6ug/mL) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL gentamycin). Cells up to passage 12 were used for experiments. Cells were serum starved overnight prior to experiments. Serum-free M199 was removed and replaced with HEPES-PSS for 60 min prior to experiments. Cells were lysed and prepared for colorimetric detection of the stable NO metabolites NO_2/NO_3 using the Griess reaction[12] according to the manufacturer's instructions (Abcam, Cambridge, MA, USA).

Isolation and subsequent culture of primary murine VSMC. VSMC were isolated from aortas of *gper+/+* mice (n=6). The aorta was excised, transferred into cold (4 °C) HEPES-PSS, and cleaned of perivascular adipose tissue[6]. The adventitia was carefully removed following a 20 min incubation with collagenase (1 mg/mL) in HEPES-PSS at 37 °C. The aorta was then cut open

longitudinally and the endothelium mechanically denuded. After a 3 h incubation in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamate in a humidified incubator at 37 °C and 5% CO_2, VSMC were dissociated by incubating the vessel with a mixture of collagenase (2 mg/mL) and elastase (0.5 mg/mL) in HEPES-PSS for 20 min at 37 °C. After incubation, the tissue was titurated by a fire polished pipette, and enzyme activity was quenched with 10 mL DMEM with 20% FBS. Cells were centrifuged at 600x g for 10 min, resuspended and seeded in DMEM with 10% FBS, which was replaced every 3 d. Subconfluent cells (80-90%) were passaged at a 1:2 ratio and identified by their characteristic morphology and positive staining for α-actin. Experiments were performed using cells from passages 2 to 5.

Subcellular localization of GPER. TIVE cells were stained with a rabbit anti-mouse GPER antibody targeting a sequence within the second extracellular loop (acetyl-FADVREVQWLEVTLGFIC, 1:10,000)[13], with the endoplasmic reticulum marker calnexin (1:1000) or the Golgi marker golgin97 (1:1000). Permeabilized (0.01% Triton-X100) or non-permeabilized VSMC were stained with GPER antibody, pre-immune GPER rabbit serum, anti-α-actin antibody (1:500), or negative control IgG overnight at 4 °C. Cells were washed with PBS (with or without Triton-X100) and incubated for 1 hour at room temperature with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:500) for GPER, and goat anti-mouse IgG conjugated to Alexa Fluor 568 (1:500) for α-actin.

Clinical biochemistry. A blood sample was taken at sacrifice after an overnight fast, plasma was separated and stored at -80 °C until measurement. Cholesterol and triglycerides were determined at IDEXX Laboratories (Westbrook, ME, USA).

Reagents. Meclofenamate and L-NAME were from Cayman Chemical (Ann Arbor, MI, USA). Sodium nitroprusside was from MP Biomedicals (Solon, OH, USA), and O.C.T. Tissue-Tek compound from Sakura Finetek (Torrance, CA, USA). The GPER-selective agonist G-1 and the GPER-selective antagonist G36 were synthetized as previously described[14]. All other drugs were from Sigma-Aldrich (St. Louis, MO, USA).

Supplementary Results

Role of endogenous *gper* on physiological parameters. Body weight, conscious arterial blood pressure, plasma cholesterol levels and uterine wet weight were measured in *gper+/+* and *gper-/-* mice. Animals were normotensive, and there were no significant differences between groups with regard to body weight (Supplementary Table 1). Deletion of the *gper* gene and surgical menopause (ovariectomy) of wild type mice both increased plasma cholesterol levels, suggesting a regulatory role of *gper* (Supplementary Table 1). Successful ovariectomy was confirmed by markedly reduced uterine wet weight compared to animals that underwent sham surgery (Supplementary Table 1).

Effect of GPER-selective agonist G-1 on physiological parameters. In ovariectomized *gper+/+* mice treated with placebo or the small molecule GPER-selective agonist G-1, body weight, arterial blood pressure and plasma cholesterol levels were measured. All animals were normotensive, and there were no significant differences between groups with regard to body weight or plasma cholesterol levels (Supplementary Table 2).

Confirmation of intact carotid artery endothelial cell and vascular smooth muscle cell function. To ensure that basal NO measurements were conducted in vascular rings with intact endothelium, arteries from *gper+/+* and *gper-/-* mice were assessed for endothelial and vascular smooth muscle cell function. In all rings tested, endothelium-dependent relaxation to acetylcholine was >90%, with no difference between groups (Supplementary Figure 1a). The integrity of vascular smooth muscle was confirmed by relaxation to the NO donor sodium nitroprusside, which was >100% with no difference between groups (Supplementary Figure 1b).

Supplementary Tables

Supplementary Table 1.

|	gper+/+		gper-/-	
	Ovary intact	OVX	Ovary intact	OVX
Body weight (g)	22±1	22±0	23±1	24±1
BP systolic (mmHg)	127±4	128±2	124±4	125±5
BP diastolic (mmHg)	89±1	88±3	85±2	87±3
Cholesterol (mg/dL)	71±7	123±18*	132±16†	114±17†
Uterine weight (mg)	82±9	10±1***	87±15	12±2***

Supplementary Table 1. Effects of sham surgery (ovary intact) or ovariectomy (OVX) on physiological parameters in *gper +/+* and *gper -/- mice.* Values are given for body weight, systolic and diastolic arterial blood pressure (BP), plasma cholesterol levels, and uterine wet weight at time of sacrifice after 16 weeks of treatment with an atherogenic diet. *P<0.05, ***P<0.001 compared with ovary intact genotype-matched mice; †P<0.05 compared with ovary intact *gper+/+* mice (Bonferroni post test). All data (n=4-15) are mean±s.e.m.

Supplementary Table 2.

	Placebo	G-1
Body weight (g)	22±1	24±1
BP systolic (mmHg)	123±4	124±1
BP diastolic (mmHg)	86±1	86±1
Cholesterol (mg/dL)	124±16	123±15

Supplementary Table 2. Effects of treatment with placebo or the GPER-selective agonist G-1 on physiological parameters in ovariectomized (surgically postmenopausal) *gper+/+* mice. Values are shown for body weight, systolic and diastolic arterial blood pressure (BP) and plasma cholesterol levels at time of sacrifice following 16 weeks of treatment with an atherogenic diet. All data (n=5-12) are mean±s.e.m.

Supplementary Figure
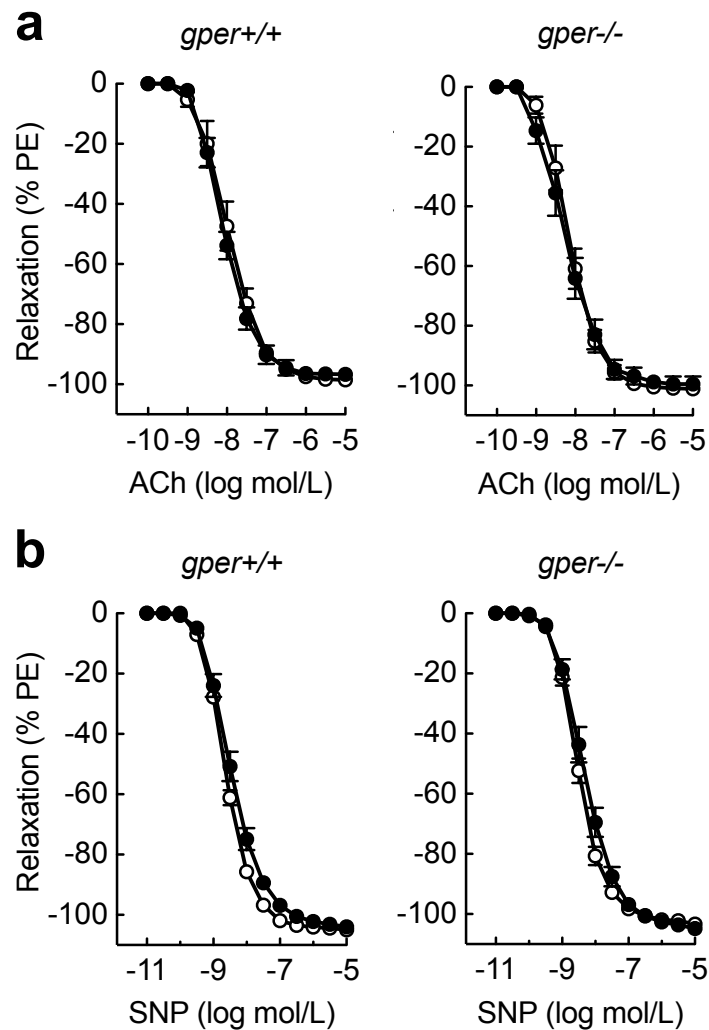
Supplementary Figure 1. Confirmation of functional integrity of endothelial cells (a) and vascular smooth muscle cells (b) in the common carotid artery of the study animals. Experiments were performed following 16 weeks of atherogenic diet in ovary intact (open circles) and ovariectomized (closed circles) *gper+/+* and *gper-/-* mice. Endothelium-dependent responses to acetylcholine (ACh) and endothelium-independent responses to sodium nitroprusside (SNP) confirmed functional integrity of endothelial and vascular smooth muscle cells in all groups and were unaffected by genotype or ovariectomy. All data (n=5-8) are mean±s.e.m.

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