ONLINE SUPPLEMENT

DELETION OF G PROTEIN-COUPLED ESTROGEN RECEPTOR INCREASES ENDOTHELIAL VASOCONSTRICTION

Matthias R. Meyer [a], Kerstin Amann [b], Angela S. Field [a], Chelin Hu [a], Helen J. Hathaway [a], Nancy L. Kanagy [a], Mary K. Walker [c], Matthias Barton [d], Eric R. Prossnitz [a,*]

[a] Department of Cell Biology and Physiology, University of New Mexico Health Sciences Center, Albuquerque, NM, United States
[b] Pathologisches Institut, Universität Erlangen-Nürnberg, Erlangen, Germany
[c] College of Pharmacy, University of New Mexico Health Sciences Center, Albuquerque, NM, United States
[d] Molecular Internal Medicine, University of Zurich, Zürich, Switzerland

***Corresponding Author:**
Eric R. Prossnitz, Ph.D.
Department of Cell Biology and Physiology
University of New Mexico Health Sciences Center
Albuquerque, NM 87131
United States
Tel: +1 505 272 5647
FAX: +1 505 272 1421
eprossnitz@salud.unm.edu

Expanded Materials and Methods

Animals

Male C57Bl6 (3 months of age, The Jackson Laboratory, Bar Harbor, ME) and GPER-deficient ($GPER^0$) mice (Proctor & Gamble, Cincinnati, OH, provided by Jan S. Rosenbaum) were housed at the animal research facility of the University of New Mexico Health Sciences Center. $GPER^0$ mice were bred and genotyped as described,[1] and backcrossed 10 generations onto C57Bl6 mice. Animals were maintained under controlled temperature of 22-23 °C on a 12h light, 12h dark cycle and fed normal chow ad libitum. All procedures were approved by and carried out in accordance with institutional policies and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Isolated Vessel Preparation and Experimental Setup

Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (2.2 mg/g BW) and exsanguinated by cardiac puncture. The aorta was immediately excised and placed in cold (4 °C) physiological Krebs-Ringer bicarbonate solution (composition in mmol/L: NaCl 118.6, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.1, $EDTA_{CaNa2}$ 0.026, Glucose 10.1; pH 7.4). Vessels were carefully dissected free from adherent connective and adipose tissue, and the lower thoracic and upper abdominal aorta was cut into 2mm long rings of equal diameter. Particular care was taken not to damage the endothelium. Isolated rings were mounted onto two 120 μm tungsten wire hooks, transferred to water-jacketed tissue baths (Radnoti, Monrovia, CA, USA) containing Krebs-Ringer bicarbonate solution (37 °C; pH 7.4; oxygenated with 21% O_2, 5% CO_2, and balanced N_2), and connected to force transducers (Grass Technologies, West Warwick, RI, USA) as described.[2-4] After equilibrating for 30 min, rings were progressively stretched to the optimal passive tension for generating force during isometric contraction, which was determined as 1.9-2.0 g in precedent studies. Rings were allowed to equilibrate for another 60 min, and repeatedly exposed to high potassium Krebs-Ringer bicarbonate solution (equimolar substitution of 100 mmol/L potassium for sodium) until a stable response was achieved.

Vascular Function Experiments

When indicated, rings were pretreated with the GPER-selective antagonist G15 (3 μmol/L)[5] or vehicle (final concentration in organ bath: ethanol 0.27%, DMSO 0.03% vol/vol) for 20 min. Concentration-response curves to acetylcholine (0.1 nmol/L – 100 μmol/L) or sodium nitroprusside (SNP, 0.01 nmol/L – 10 μmol/L) were generated in rings precontracted with phenylephrine. Selected rings were pretreated with the non-selective COX inhibitor indomethacin (10 μmol/L) or the TP receptor antagonist SQ 29,548 (1 μmol/L) for 30 min. In addition, concentration-response curves to U46619 (0.1 nmol/L – 10 μmol/L) were obtained. To exclude interference with NO-dependent effects of GPER,[4,6,7] responses to SNP and U46619 were recorded after incubation with the NO synthase inhibitor L-NAME (300 μmol/L) for 30 min. Rings that showed less than 70% relaxation to acetylcholine were considered to have a damaged endothelium and were discarded.

Determination of Basal NO Bioactivity

Two adjacent vessel rings, one of them pretreated with L-NAME (300 μmol/L) for 30 min, were exposed to phenylephrine (300 nmol/L). Basal NO bioactivity was calculated as the increase in contraction to phenylephrine in rings treated with L-NAME compared to untreated rings.[8]

Histological Analyses and Immunohistochemistry
Aortas of WT and GPER^0 mice were carefully harvested, fixed in formalin, and embedded in paraffin. Sections (2 μm) were cut and stained with hematoxylin eosin using a standard protocol. For immunohistochemical analyses, aorta cross sections embedded in paraffin were stained as previously described[9] using the following antibodies: α-smooth muscle actin (1:200), proliferating cell nuclear antigen (PCNA, 1:500), and Ki-67 (1:100).

Quantitative Real-time Polymerase Chain Reaction (qPCR)
The thoracic aorta of WT and GPER^0 mice was snap-frozen in liquid nitrogen, disrupted and homogenized using a rotor-stator homogenizer. Total RNA was extracted using the silica-based RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA). RNA (200 ng) was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). PCR was performed using TaqMan gene expression assays on a 7500 FAST real-time PCR System (Applied Biosystems, Carlsbad, CA, USA). The sets of primers used for amplification of gene-specific cDNA fragments are given in Table S1. Gene expression was calculated using the $2^{-\Delta\Delta C(T)}$ method.[10] GAPDH served as house-keeping control.

Drugs and Antibodies
L-NAME (L-N^G-Nitroarginine methyl ester), SQ 29,548, and U46619 were from Cayman Chemical (Ann Arbor, MI, USA). SNP was from MP Biomedicals (Solon, OH, USA). All other drugs were from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared according to the manufacturer's instructions. G15 was synthesized as described (provided by Jeffrey Arterburn, New Mexico State University, Las Cruces, NM, USA),[5] dissolved in DMSO, and subsequently diluted in ethanol 99% to get a stock solution of 3 mmol/L. If needed, stock solutions were diluted in Krebs-Ringer bicarbonate solution to the required concentration before use. Concentrations are expressed as final molar concentration in the organ chamber. α-Smooth muscle actin and PCNA antibodies were from DAKO Diagnostika (Hamburg, Germany), and Ki-67 antibody from LabVision (Wedel, Germany).

Statistical Analyses
Contraction is given as the percentage of contraction to high potassium Krebs-Ringer bicarbonate solution, and relaxation is expressed as the percentage of precontraction. Area under the curve (AUC) and EC_{50} values (as negative logarithm: pD_2) were calculated by non-linear regression analysis.[11] Data were analyzed using two-way repeated-measures ANOVA followed by Bonferroni post hoc analysis, the unpaired Student's *t*-test, or the Mann-Whitney *U* test where appropriate. Values are shown as means±SEM of independent experiments. A $P<0.05$ value was considered statistically significant.

References

1. Wang C, Dehghani B, Magrisso IJ, Rick EA, Bonhomme E, Cody DB, Elenich LA, Subramanian S, Murphy SJ, Kelly MJ, Rosenbaum JS, Vandenbark AA, Offner H. GPR30 contributes to estrogen-induced thymic atrophy. *Mol Endocrinol.* 2008;22:636-648.

2. Barton M, Haudenschild CC, d'Uscio LV, Shaw S, Munter K, Luscher TF. Endothelin ET_A receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. *Proc Natl Acad Sci U S A.* 1998;95:14367-14372.

3. Haas E, Bhattacharya I, Brailoiu E, Damjanovic M, Brailoiu GC, Gao X, Mueller-Guerre L, Marjon NA, Gut A, Minotti R, Meyer MR, Amann K, Ammann E, Perez-Dominguez A, Genoni M, Clegg DJ, Dun NJ, Resta TC, Prossnitz ER, Barton M. Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. *Circ Res.* 2009;104:288-291.

4. Meyer MR, Baretella O, Prossnitz ER, Barton M. Dilation of epicardial coronary arteries by the G protein-coupled estrogen receptor agonists G-1 and ICI 182,780. *Pharmacology.* 2010;86:58-64.

5. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, Bologa CG, Leitao A, Brailoiu E, Deliu E, Dun NJ, Sklar LA, Hathaway HJ, Arterburn JB, Oprea TI, Prossnitz ER. In vivo effects of a GPR30 antagonist. *Nat Chem Biol.* 2009;5:421-427.

6. Broughton BR, Miller AA, Sobey CG. Endothelium-dependent relaxation by G protein-coupled receptor 30 agonists in rat carotid arteries. *Am J Physiol Heart Circ Physiol.* 2010;298:H1055-1061.

7. Lindsey SH, Carver KA, Prossnitz ER, Chappell MC. Vasodilation in response to the GPR30 agonist G-1 is not different from estradiol in the mRen2.Lewis female rat. *J Cardiovasc Pharmacol.* 2011;57:598-603.

8. Hamilton CA, Brosnan MJ, McIntyre M, Graham D, Dominiczak AF. Superoxide excess in hypertension and aging: a common cause of endothelial dysfunction. *Hypertension.* 2001;37:529-534.

9. Haas CS, Amann K, Schittny J, Blaser B, Muller U, Hartner A. Glomerular and renal vascular structural changes in alpha8 integrin-deficient mice. *J Am Soc Nephrol.* 2003;14:2288-2296.

10. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25:402-408.

11. DeLean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol.* 1978;235:E97-102.

Gene (Accession Number)	Forward Primer	Reverse Primer
COX-1 (NM_008969)	5'-ACT CAG CGC ATG ACT ACA TC-3'	5'-CTT CTC AGC AGC AGC TGT TG-3'
PGI_2 Synthase (NM_008968)	5'-ACA GCA TCA AAC AAT TTG TCG TC-3'	5'-GCA TCA GAC CGA AGC CAT ATC T-3'
Thromboxane A_2 Synthase (L18868)	5'-CAG AAG ATC CCT TTG TGC AAC-3'	5'-CCT CTC TTC TGC TGC TTG CTG-3'
TP Receptor (D10849)	5'-GCC TTG TTC TCA CCG ACT TC-3'	5'-CAG CCC GAA GAA CAC CAT AG-3'
eNOS (NM_008713)	5'-AGA GCC TGC AAT TAC TAC CA-3'	5'-GTG GAT TTG CTG CTC TGT AG-3'
Soluble GCα NM_021896)	5'-TTT GTC ATC CGG GTG AGG AG-3'	5'-CCT TGA CGA TTT CTT CAC CGA G-3'
Soluble GCβ (NM_017469)	5'-TTG CGT GTC CTG GGA TCT AAT-3'	5'-GGC ATC GAG GTT CTG CAA AA-3'
GAPDH (NM_008084)	5'-TTC ACC ACC ATG GAG AAG GC-3'	5'-GGC ATG GAC TGT GGT CAT GA-3'

Table S1. Sets of primers used for amplification of gene-specific cDNA fragments by qPCR. COX, cyclooxygenase; eNOS, endothelial NO synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCα, guanylate cyclase, α-subunit; GCβ, guanylate cyclase, β-subunit; PGI_2, prostacyclin; TP receptor, thromboxane prostanoid receptor.