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Correspondence and requests for materials should be addressed to M.B. (barton@access.uzh.ch) or E.R.P. (eprossnitz@unm.edu)

* Current address: Department of Cardiology, Bern University Hospital, Bern, Switzerland.

G Protein-coupled Estrogen Receptor Protects from Atherosclerosis

Matthias R. Meyer[1]*, Natalie C. Fredette[1], Tamara A. Howard[1], Chelin Hu[1], Chinnasamy Ramesh[2], Christoph Daniel[3], Kerstin Amann[3], Jeffrey B. Arterburn[2], Matthias Barton[4] & Eric R. Prossnitz[1]

[1]Department of Cell Biology and Physiology, University of New Mexico Health Sciences Center, Albuquerque, NM, USA, [2]Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, NM, USA, [3]Pathologisches Institut, Universität Erlangen-Nürnberg, Erlangen, Germany, [4]Molecular Internal Medicine, University of Zürich, Zürich, Switzerland.

Coronary atherosclerosis and myocardial infarction in postmenopausal women have been linked to inflammation and reduced nitric oxide (NO) formation. Natural estrogen exerts protective effects on both processes, yet also displays uterotrophic activity. Here, we used genetic and pharmacologic approaches to investigate the role of the G protein-coupled estrogen receptor (GPER) in atherosclerosis. In ovary-intact mice, deletion of *gper* increased atherosclerosis progression, total and LDL cholesterol levels and inflammation while reducing vascular NO bioactivity, effects that were in some cases aggravated by surgical menopause. In human endothelial cells, GPER was expressed on intracellular membranes and mediated eNOS activation and NO formation, partially accounting for estrogen-mediated effects. Chronic treatment with G-1, a synthetic, highly selective small molecule agonist of GPER, reduced postmenopausal atherosclerosis and inflammation without uterotrophic effects. In summary, this study reveals an atheroprotective function of GPER and introduces selective GPER activation as a novel therapeutic approach to inhibit postmenopausal atherosclerosis and inflammation in the absence of uterotrophic activity.

Atherosclerosis is a chronic and systemic vascular inflammatory process that forms the pathological basis of coronary artery disease, myocardial infarction, and stroke[1,2]. Coronary artery disease represents the main cause of death in men and women alike, and shows a distinct gender difference with premenopausal women being largely protected[1,2]. Cessation of estrogen production due to natural or surgical menopause increases the risk of developing coronary atherosclerosis[1–3]. Thus, vascular protection in premenopausal[1] but not postmenopausal[4] women has been linked to the ovarian production of estrogens[2,5], of which 17β-estradiol represents the physiologically relevant form[3,5]. Current estimates predict that by the year 2050 one billion women worldwide will be postmenopausal[6], requiring preventive or therapeutic intervention to limit coronary artery disease and its associated health risks[7,8]. Attempts to reduce the increased risk of postmenopausal coronary atherosclerosis and its complications have included the use of estrogens as hormone therapy[1–3]; however, estrogen treatment is associated with adverse effects, such as blood clots and endometrial stimulation, increasing the risk of hyperplasia and carcinoma[2,9].

Natural estrogens, such as 17β-estradiol, exert their vascular effects through soluble nuclear receptors and membrane-bound receptors[5,10]. In human coronary arteries, activation of estrogen receptors exerts both acute and chronic effects, including vasodilation[11], reducing inflammation in atherosclerotic plaques[12], and inhibiting proliferation of vascular smooth muscle cells (VSMC)[13]. Formation of endothelial nitric oxide (NO, a short-lived gas implicated in protection from atherosclerosis and inflammation[14]) and expression of the NO-synthesizing enzyme, eNOS, are also regulated in an estrogen-dependent fashion[15]. *In vitro* studies have shown that the "classical" estrogen receptors, ERα/*esr1*, and ERβ/*esr2* activate eNOS[16], and have identified ERα as one of the mediators of estrogen-dependent inhibition of atherogenesis[17]. However, since the inhibition brought about by estrogen is at least partially maintained in female *esr1*-deficient mice[17,18], estrogen targets distinct from ERα must be involved in its atheroprotective effects.

In humans, exposure of vascular endothelial cells to laminar shear stress inhibits the progression of underlying atheroma[19]. Shear stress represents an important physiological stimulus of endothelial NO production[14], which is centrally involved in protection from cardiovascular disease[5,14,20]. Exposing human endothelial cells to laminar shear stress also led to the detection and cloning of an orphan G protein-coupled receptor (GPR30)[21]. Studies have since established that this receptor binds and signals in response to estrogen[22,23], which led to its designation as G protein-coupled estrogen receptor (GPER)[24]. Utilizing a transgenic *gper*-LacZ reporter mouse, its predominant

expression in endothelial cells and VSMC has been reported[25]. The creation of *gper*-deficient mice[26] and the identification of synthetic ligands that act as selective agonists or antagonists of GPER[27–29] have facilitated studies of the role of GPER in physiology and disease, particularly in the context of ovarian sex steroid function[24].

As inhibition of atherogenesis by estrogen must involve additional mechanisms distinct from ERα[17,18] and because GPER shows a vasculotropic expression profile[21,25,26] with its activation inducing vasodilation[26,30,31] and inhibition of VSMC proliferation[26], we hypothesized that GPER plays a role in atherosclerotic vascular disease[26] and might represent a potential target for estrogen-mediated protection in women[26]. Additional support for this concept comes from the anti-inflammatory activity attributed to GPER[24] as well as its involvement in the PI_3K/Akt signaling pathway[22], which regulates eNOS activation[14]. Thus, we set out to determine whether GPER expression and activity may contribute to the anti-inflammatory[12] and NO-stimulating vascular effects of estrogen[14]. We also assessed whether treatment with a synthetic small molecule, GPER-selective agonist[27] might be suitable as a new pharmacological strategy, distinct from classical hormone therapy, for the treatment of postmenopausal atherosclerosis.

Results

GPER is an intracellular estrogen receptor in endothelial and vascular smooth muscle cells. Both endothelial cells and VSMCs are essential for atherogenesis, with estrogen exhibiting anti-atherogenic effects in both cell types[2,14]. Thus, we first sought to determine the expression and subcellular localization of GPER in these cells. In endothelial cells, GPER staining revealed an intracellular expression pattern displaying colocalization with both endoplasmic reticulum and Golgi apparatus markers (Fig. 1a–f), similar to previous observations in other cell types[22]. VSMCs also exhibited an intracellular expression pattern. To confirm the predominant intracellular localization of GPER, we employed an anti-GPER antibody targeting the second "extracellular" loop, which would recognize a cell surface receptor in unpermeabilized cells. As this antibody only recognizes GPER under permeabilizing conditions (Fig. 1g–l), we conclude that, under steady state conditions, the great majority of GPER is also localized intracellularly in VSMCs, consistent with studies demonstrating constitutive internalization of surface-expressed GPER[32].

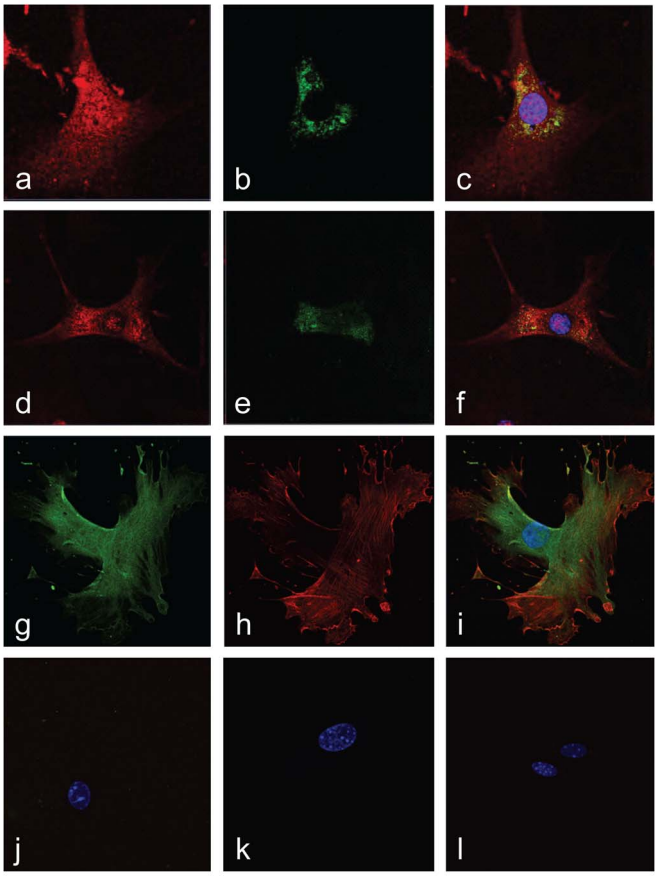

Figure 1 | GPER is an intracellular membrane receptor in vascular endothelial and smooth muscle cells. (a)–(f), Endothelial cells were stained for GPER (red; a, d) and either endoplasmic reticulum (b) or Golgi apparatus (e), demonstrating colocalization of GPER with both markers in the merged images (c), (f), which include DAPI staining of the nucleus (blue). (g)–(j), Vascular smooth muscle cells were stained for GPER (green; g), employing an antibody targeting an "extracellular" epitope, and α-actin (red; h). merged with GPER staining in (i) under both permeabilizing conditions (g)–(i) and non-permeabilizing conditions (j). Pre-immune anti-GPER serum and negative control IgG were used to test the specificity of the GPER anti-serum and α-actin antibodies under permeabilizing (k) and non-permeabilizing (l) conditions. The cell nucleus is stained with DAPI (blue; i–l). The amino terminus of GPER, expected to be extracellular if the receptor is expressed on the cell surface (plasma membrane), is accessible only upon cell permeabilization, indicating that GPER is expressed predominantly on internal membranes.

Increased atherosclerosis in *gper*-deficient mice. Wild type (*gper*+/+) and *gper*-deficient (*gper*−/−) mice were fed an atherogenic diet for 16 weeks and assessed for the development of early atherosclerosis in the aortic root and the aorta. Deletion of *gper* increased atherosclerosis in the aortic root (Fig. 2a) as well as macroscopic lesions in the aorta (Fig. 2b and 2c) and increased total cholesterol and LDL cholesterol plasma levels (Supplementary Table 1). Ovariectomy (surgical menopause[33]) accelerated development of aortic root atherosclerosis (Fig. 2a) as well as gross atherosclerotic lesions (Fig. 2d and e) to a similar extent. In ovariectomized mice, deletion of *gper* further aggravated atherosclerosis progression (Fig. 2a, d and e). In *gper*+/+ mice, the predilection site for atherosclerotic lesions was the proximal segment, with the lesion-covered aortic area decreasing distally, a pattern that was unaffected by *gper* deficiency or by ovariectomy (Fig. 2f–h). However, in mice exhibiting the most severe disease (*gper* deficiency and surgical menopause combined), the atherosclerotic distribution pattern changed, now showing similar levels of atherosclerosis in the proximal (Fig. 2f), middle (Fig. 2g), and distal segment (Fig. 2h) of the aorta. These findings (*i*) confirm atheroprotective effects of endogenous ovarian estrogens in female mice[17,18] and (*ii*) provide evidence for a previously unrecognized atheroprotective function of GPER. The effects mediated by GPER may extend beyond its function as a receptor for ovarian estrogen, since its deletion surprisingly aggravated atherosclerosis even in ovariectomized mice. Thus GPER may exhibit significant basal ligand-independent activity, or being a vasculotropic receptor[21,26], GPER may also be activated by estrogen produced locally in the vascular wall, which is increased in atherosclerosis[34–36].

Vascular inflammation is enhanced in *gper*-deficient mice. Macrophages and T cells play a central role in atherogenesis and are critical for disease initiation and progression[37]. We next determined whether endogenous GPER expression or surgical menopause affects macrophage and T cell accumulation in the aortic root using quantitative immunohistochemistry. Cell quantitation indicated ~10-fold fewer CD3+ cells (a T cell marker) than CD68+ cells (a macrophage marker) in all groups investigated (Fig. 3a–d). In ovary-intact mice, deletion of *gper* resulted in a striking increase in accumulation of CD68+ cells (Fig. 3a–c). Ovariectomy resulted in comparable effects to *gper*

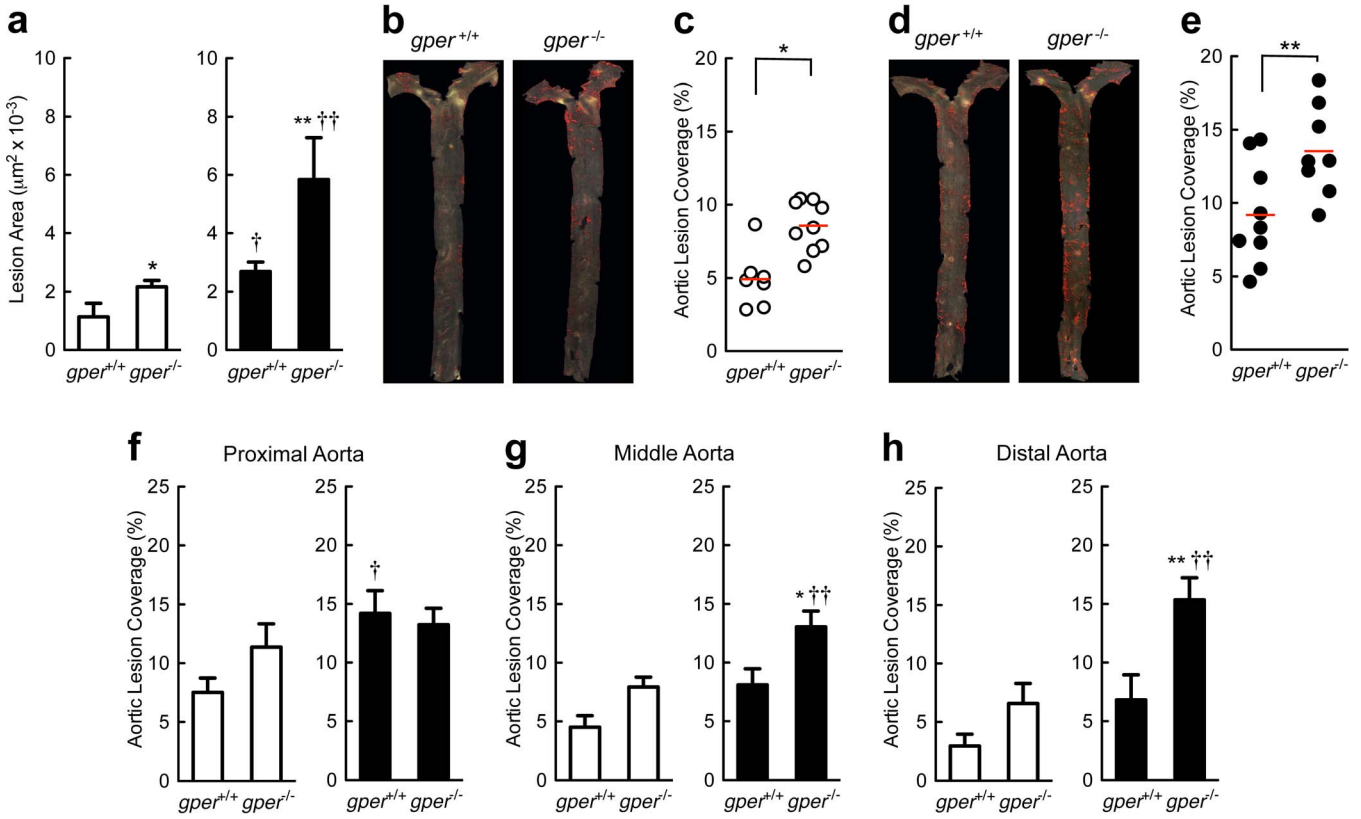

Figure 2 | Increased atherosclerosis in mice lacking GPER. Quantification of atherosclerosis in the aortic root (a) and macroscopic atherosclerosis on the aortic surface (b)–(h). Data are shown for ovary intact (open bars/circles) and ovariectomized (filled bars/circles) mice treated with an atherogenic diet. Deletion of *gper* increased both aortic root atherosclerosis (a) as well as macroscopic atherosclerosis (b)–(e). Both aortic root as well as macroscopic atherosclerosis development were also accelerated after ovariectomy (a), (d), (e). The effect of ovariectomy was further aggravated by deletion of *gper* (a), (d), (e). The predilection site for atherosclerotic lesions in *gper*+/+ animals was the proximal aortic segment (f), with lesions intensity decreasing from the middle (g) to the distal (h) segment. This distribution pattern was unaffected by *gper* deficiency or by ovariectomy alone (f)–(h); however, in ovariectomized *gper*−/− mice, the distribution pattern changed markedly, revealing equally extensive atherosclerosis in all three aortic segments (f)–(h). *P < 0.05 and **P < 0.01 compared with *gper*+/+ mice, †P < 0.05 and ††P < 0.01 compared with ovary intact genotype matched mice (ANOVA with Bonferroni post-hoc test). All data (n = 4–9 per group) represent mean ± s.e.m.

deficiency alone on CD68+ cell staining, which was not further aggravated by *gper* deficiency (Fig. 3c), suggesting that GPER was entirely responsible for the effect observed upon estrogen removal. With regard to CD3+ cell immunostaining, changes were less pronounced. In ovary-intact mice, *gper* deletion alone increased CD3+ cell staining (Fig. 3a, b and d), while ovariectomy had no significant effect (Fig. 3d). Deletion of *gper* also increased CD3+ cell staining in ovariectomized mice (Fig. 3d). These data are compatible with an important inhibitory role of endogenous GPER on vascular accumulation of macrophages and T cells and suggest that GPER-mediated atheroprotective effects likely involve inhibition of vascular inflammation.

Vascular basal NO bioactivity is reduced in *gper*-deficient mice with atherosclerosis. Vascular NO bioactivity regulates vascular tone and inhibits atherogenesis, and its production is critically dependent on intact ovarian steroid production[14,38]. Accordingly, a decrease in NO bioactivity has been implicated in the increased risk of cardiovascular disease in postmenopausal women[14,38]. Similar to surgically induced menopause in humans, ovariectomy in mice resulted in a significant reduction in vascular NO bioactivity measured *ex vivo* (Fig. 4a). Deletion of *gper* in ovary-intact animals reduced vascular NO bioactivity to a similar extent (Fig. 4a). However, in ovariectomized *gper*-deficient mice, vascular NO bioactivity was further reduced (Fig. 4a), indicating that endogenous GPER expression maintains NO bioactivity in both pre- and postmenopausal females *in vivo* in a milieu of vascular inflammation and atherosclerosis.

GPER activation stimulates human endothelial nitric oxide synthase. Following the observation that GPER is critical to maintain vascular NO bioactivity *ex vivo* (Fig. 4a), and given that GPER was originally cloned from endothelial cells[21], with vascular expression[25] and function[26], we next set out to determine whether and how GPER activation affects signaling of the L-arginine/NO pathway in human endothelial cells. For these experiments, acetylcholine was used as a known GPCR agonist to stimulate endothelial NO formation[39], G-1 as selective GPER agonist[27], 17β-estradiol (estrogen) as a non-selective agonist of ERα[16], ERβ[16], and GPER[22], and G36 as a GPER-selective antagonist[28,29]. NO formation was determined by measuring the release of the stable NO metabolites NO_2/NO_3[40]. Stimulation of human endothelial cells with G-1 resulted in a robust increase in eNOS protein phosphorylation at the Akt-mediated serine1177 activation site[41,42] (Fig. 4b), and all three agonists induced robust, rapid NO formation (Fig. 4c). G36 completely abrogated G-1-stimulated NO production, and partly inhibited NO formation in response to estrogen, but had not effect on responses to M_3 muscarinic receptor stimulation with acetylcholine (Fig. 4c), demonstrating the selectivity of G-1 and G36 in the modulation of GPER activity. In addition, stimulation of endothelial NO formation in response to G-1 was sensitive to PI_3 kinase inhibition (data not shown).

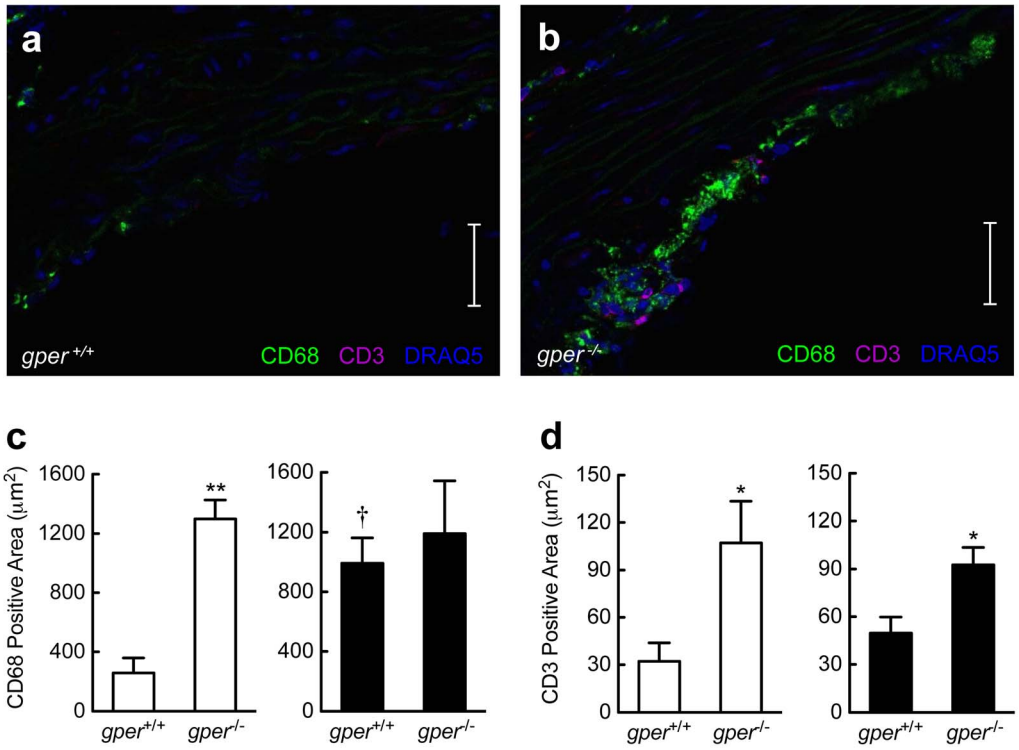

Figure 3 | *Gper* deficiency results in vascular accumulation of inflammatory cells. Quantification of CD68+ cells (macrophages) and CD3+ cells (T cells) in the aortic root using quantitative immunohistochemistry. Data are shown for ovary intact (open bars) and ovariectomized mice (filled bars) treated with an atherogenic diet. Compared with ovary intact *gper*+/+ mice (a), (c), deletion of *gper* yielded a pronounced increase in CD68+ cells (b), (c). Ovariectomy alone also increased staining for CD68+ cells in *gper*+/+ mice, whereas deletion of *gper* had no further effect on this increase (c). Compared to either ovary intact or ovariectomized *gper*+/+ mice (a), (d), respectively, deletion of *gper* yielded a pronounced increase in CD3+ cells (b), (d). Ovariectomy did not further increase CD3+ staining in either *gper*+/+ or *gper*−/− mice (d). *P < 0.05 and **P < 0.01 compared with *gper*+/+ mice, †P < 0.05 compared to ovary intact genotype-matched mice (ANOVA with Bonferroni post-hoc test). All data (n = 3–6 per group) are mean ± s.e.m. Scale bar, 100 μm.

A synthetic small molecule GPER agonist inhibits postmenopausal atherosclerosis and inflammation *in vivo*. Treatment of postmenopausal women and experimental animals with natural estrogen is generally associated with a reduction in atherosclerosis and inflammation, yet increases the risks of endometrial hyperplasia and cancer due to uterotrophic stimulation[9]. This has been one of the key concerns of postmenopausal hormone therapy and requires additional preventive measures[9]. In the present study, using a model of postmenopausal atherosclerosis, we identified an endogenous inhibitory function of GPER on atherosclerosis progression (Fig. 2), the vascular inflammation associated with it (Fig. 3), and its protective effect on vascular NO bioactivity (Fig. 4a). We next set out to test whether treatment of postmenopausal mice with the synthetic small molecule GPER-selective agonist G-1[27] translates into inhibition of atherosclerosis progression and vascular inflammation. Treatment with G-1 for 16 weeks resulted in a marked reduction in aortic root atherosclerosis (Fig. 5a), yet was devoid of feminizing effects on the uterus (Fig. 5b). The reduction in atherosclerosis was accompanied by an inhibition of CD68+ cell infiltration, compatible with the notion that G-1 reduces vascular macrophage accumulation *in vivo* (Fig. 5c, e and f). Immunostaining for CD3+ cells (which was an order of magnitude lower than that for CD68+ cells) was however unaffected by G-1 treatment (Fig. 5d–f). These findings indicate that selective GPER activation by the synthetic small molecule G-1 may serve as a novel therapeutic approach to inhibit postmenopausal atherosclerosis and its associated vascular inflammation without undesirable estrogenic effects on the uterus.

Discussion

In the present study, we demonstrated that the *in vivo* activity of the intracellular, transmembrane G protein-coupled estrogen receptor (GPER) plays an essential, previously unrecognized role in atherogenesis, dyslipidemia and the associated inflammation. Treatment with G-1, a highly selective small molecule agonist of GPER[27], was effective in reducing postmenopausal atherosclerosis and vascular inflammation without uterotrophic activity. Consistent with the vasculoprotective effect of its activation, genetic loss of *gper* worsened atherogenesis and inflammation.

Clinical studies have demonstrated that atherosclerotic vascular disease in women, the most prevalent cause of death, is strongly dependent on ovarian estrogen production[2,33], and that natural estrogen(s) may effectively interfere with atherogenesis with treatment early but not late after menopause[3,43]. We thus chose to employ a therapeutically relevant model that resembles incipient atherogenesis in females, characterized by fatty streaks and early atheroma formation[18]. Our study revealed three principal new findings with regard to the role of GPER in atherosclerosis. First, *gper* deletion increases atherogenesis and plasma cholesterol, whereas pharmacological GPER activation following menopause attenuates disease progression. Second, the effect of *gper* deletion is further aggravated following surgical menopause, which itself accelerates atherogenesis to a similar extent as *gper* deficiency. Third, with regard to the topography of lesion development, the typical distribution pattern known for this model is dramatically altered in animals that are both postmenopausal and lack *gper*, wherein atherosclerosis increases in the

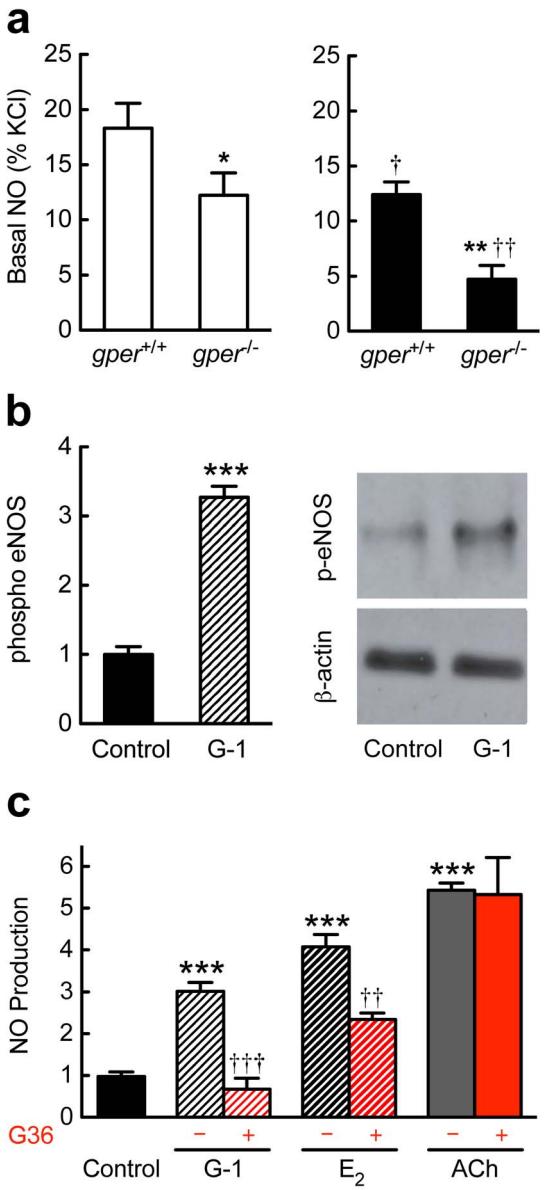

Figure 4 | GPER regulates NO synthase function *ex vivo* and *in vitro*. Basal vascular nitric oxide (NO) bioactivity was measured *ex vivo* (a) in mice treated with an atherogenic diet, with eNOS phosphorylation (b) and NO formation (c) determined in human endothelial cells. (a), Basal vascular NO bioactivity in ovary intact (open bars) and ovariectomized (filled bars) mice. Deletion of *gper* reduced vascular NO bioactivity to a similar extent (about 30%) as did ovariectomy in *gper*+/+ mice. However, in ovariectomized *gper*−/− mice, NO bioactivity was further reduced by more than 70%. *P < 0.05 and **P < 0.01 compared with *gper*+/+ mice, †P < 0.05 and ††P < 0.01 compared to ovary intact genotype-matched mice (ANOVA with Bonferroni post-hoc test). All data are mean ± s.e.m. (n = 5–7). (b), Stimulation of human endothelial cells with the GPER-selective agonist G-1 increased levels of activated eNOS-phosphorylated on serine1177. ***P < 0.001 compared with control (vehicle only, Student's *t*-test). Data are mean ± s.e.m. (n = 4). (c), Endothelial NO production was determined through the detection of stable NO metabolites NO_2^-/NO_3^-. Stimulation of GPER in human endothelial cells with the selective (G-1) or non-selective (17β-estradiol, E_2) GPER agonist increased NO formation, as did the M_3 muscarinic receptor agonist, acetylcholine (ACh). A selective GPER antagonist, G36, completely blocked G-1-stimulated endothelial NO formation, while E_2-stimulated NO formation was only partly reduced. G36 had no effect on ACh-stimulated NO formation. ***P < 0.001 compared with control, ††P < 0.01 and †††P < 0.001 compared with no antagonist (Student's *t*-test). All data (n = 3–9 per group) are mean ± s.e.m.

otherwise largely lesion-resistant middle and abdominal portions of the aorta[18]. The findings reported here suggest that GPER plays an important role in mediating part of the atheroprotective effects of estrogen in premenopausal (ovary-intact) mice. Our results further indicate that even after menopause GPER retains a beneficial function and that its expression determines atherosclerotic lesion topography.

Currently known natural ligands of GPER include 17β-estradiol[22] and 2-methoxy-estradiol[44]. Both steroids are produced within the arterial vascular wall[36,45], and production has been shown to increase

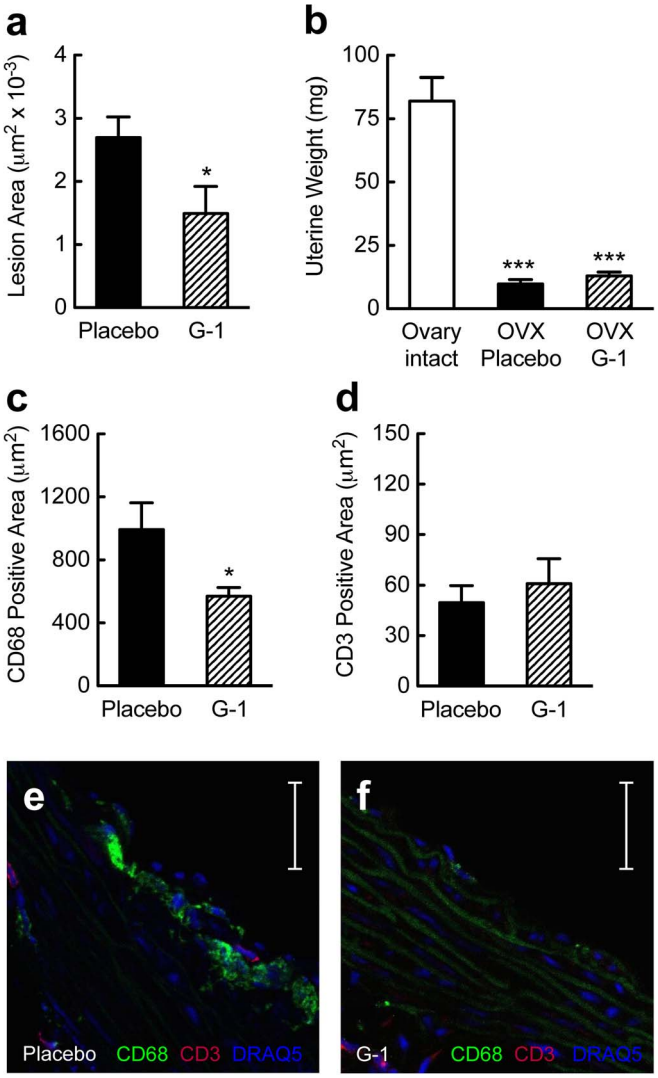

Figure 5 | A small molecule GPER-selective agonist inhibits atherosclerosis and vascular inflammation. Treatment effects on atherosclerosis (a), uterine wet weight (b) or quantification of CD68+ (c), (e), (f) and CD3+ (d)–(f) staining of macrophages and T cells, respectively, in the aortic root. Data were obtained in ovariectomized (surgically postmenopausal) *gper*+/+ mice, which display accelerated atherogenesis (*cf.* Fig 1a–d). Effects are shown in response to treatment with placebo (filled bars) or G-1 (hatched bars), a selective small molecule agonist of GPER. G-1 treatment reduced atherosclerosis by 45% (a). Ovariectomy reduced uterine weight by about 90% compared with ovary-intact animals (b), in which estrogen levels are high. G-1 treatment had no feminizing effect on the uterus similar to placebo treatment (b). G-1 treatment reduced staining for CD68+ cells by 43% (c), (e), (f), but had no effect on CD3+ immunostaining (d)–(f). *P < 0.05 compared with placebo, ***P < 0.001 compared with ovary intact (Student's *t*-test). All data (n = 5–11 per group) are mean ± s.e.m. Scale bar, 100 μm.

in human atherosclerosis[35,36], suggesting an alternative and localized source of estrogen following menopause, particularly under disease conditions. Moreover, adrenal hormones such as dehydroepiandrosterone are converted to estrogen in the vascular wall[46], and inhibit VSMC proliferation via a mechanism involving a yet unidentified membrane G protein-coupled receptor[47]. In addition, numerous GPCRs exhibit limited constitutive activity, thus contributing to signal transduction even in the absence of ligand activation[48]. Thus, an increased local production of ligands in atherosclerotic tissue and/or constitutive activity of GPER could well explain the effect of *gper* deletion that we observed in postmenopausal mice. Interestingly, a similar aggravation of atherosclerosis following ovariectomy has been shown in *esr1*-deficient mice[18].

In postmenopausal women with an intact uterus, hormone therapy with estrogens requires additional pharmacological interventions to limit endometrial stimulation and the resulting risk of hyperplasia and malignancies. With this in mind, we set out to test the potential therapeutic efficacy of selective GPER activation with the synthetic, small molecule compound G-1[27] in postmenopausal atherosclerosis. We observed a robust inhibition of atherosclerosis, and importantly, G-1 treatment was without any uterotrophic effect[28]. This indicates that treatment with G-1, unlike currently used hormone formulations, affords vascular protection in postmenopausal female mice but is devoid of classical uterotrophic effects, which may have important therapeutic implications. Moreover, neither *gper* deficiency nor G-1 treatment had any effects on arterial blood pressure, further supporting a specific effect of this receptor on the vascular disease process.

Estrogens exert immunomodulatory functions that play numerous roles in physiology and disease[49]. The present study identifies GPER and ovarian function (i.e. estrogen production) as suppressors of vascular macrophage and T cell accumulation in atherogenesis and demonstrates that treatment with the GPER-selective agonist G-1 reduces macrophage accumulation in mice with postmenopausal atherosclerosis. These findings recapitulate observations in women with coronary artery disease, where inflammation and the severity of atherosclerotic lesions are much lower in premenopausal than in postmenopausal women[12], and can be reduced in postmenopausal women by estrogen treatment[12]. Similarly, ovariectomy has been shown to exert proinflammatory effects in experimental atherosclerosis[18], including macrophage infiltration and the recruitment of T cells[50]. The targets previously thought to be solely involved in these responses are the "classical" estrogen receptors, ERα and ERβ[2], which are expressed in macrophages[50]. Recently, GPER expression has been demonstrated in leukocytes, and its activation exerts anti-inflammatory effects by inhibiting IL-1β and increasing IL-10 production[51]. In addition, GPER activation reduces TLR-4 expression[52] and the CRP-induced upregulation of IL-8, ICAM-1, P-selectin, and several chemokines in murine macrophages[50]. The present study now demonstrates that GPER exerts inhibitory effects on vascular macrophage and T cell recruitment *in vivo*. Although we cannot exclude changes in the inflammatory environment secondary to altered atheroma formation, the extent of atherosclerosis did not entirely correlate with immune cell accumulation, which was disproportionally high in premenopausal *gper*-deficient mice. This argues in favor of direct GPER-mediated inhibition of vascular inflammation by endogenous estrogens, which was recapitulated with G-1 treatment in postmenopausal animals. As we have recently shown that GPER regulates Foxp3 expression and induces IL-10 expression under TH17-polarizing conditions[53,54] it is intriguing to speculate that the TH17/IL-17A pathway, which plays an important role in atherogenesis and the associated inflammation[55], is also involved in the anti-inflammatory and atheroprotective effects of GPER.

In women with coronary atherosclerosis, reduced NO bioactivity increases the risk of adverse cardiovascular events and death[20]. Endothelium-derived NO, which has anti-inflammatory properties[14,50], is produced in response to laminar shear stress[14,18] and in an estrogen-dependent fashion[16]. Previously, estrogen-induced NO release from human endothelial cells was thought to be mediated solely by ERα and ERβ[14,16]. Of note, GPER was cloned from human endothelial cells exposed to laminar shear stress[21], and was shown here to be expressed as an intracellular membrane receptor in human endothelial cells. Furthermore, since we previously observed NO- and endothelium-dependent relaxation of epicardial coronary arteries in response to GPER agonists *ex vivo*[31], we speculated that deletion of *gper* and/or menopause might affect NO bioactivity in the setting of atherosclerosis and vascular inflammation. In the present study, we identified GPER as an important determinant that contributes to vascular NO bioactivity in ovary-intact animals. Of note, much of the NO bioactivity in wild type mice is unaffected by ovariectomy, suggesting additional regulatory mechanisms. However, in surgically postmenopausal mice lacking *gper*, which also showed the most severe progression of atherosclerosis, NO bioactivity was dramatically reduced. These findings prompted us to further examine whether and how GPER might be involved in the regulation of endothelial NO synthase activity and the formation of NO. In human endothelial cells, we observed G-1-mediated phosphorylation of eNOS at serine1177, an Akt-mediated event that results in increased eNOS activity and NO production[41,42]. To recapitulate our *ex vivo* observations, the role of GPER was determined in the absence and presence of G36[29], a GPER-selective antagonist, by measuring stimulated cellular NO release. The results demonstrate not only that NO is released from human endothelial cells in response to GPER activation, but also that a considerable portion of 17β-estradiol-mediated NO formation is GPER-dependent, whereas M_3 muscarinic receptor-stimulated NO release (using the prototypic agonist acetylcholine[39]) is completely insensitive to GPER inhibition. Thus, both the *ex vivo* as well as the *in vitro* experiments identify GPER as a novel and important regulator of NO bioactivity, which likely contributes to GPER-mediated inhibitory effects on vascular inflammation and ultimately atherogenesis.

In summary, using both loss and gain of function approaches in female mice with atherosclerosis, dyslipidemia and vascular inflammation, we have identified a previously unrecognized role for GPER in regulating vascular disease progression. We further found that activating this receptor with a synthetic small molecule GPER-selective agonist is effective in reducing atherosclerosis in the absence of uterotrophic effects. In view of the limitations of current hormone therapies[3,9] and the reported vasoprotective effects of G-1 in isolated human arteries[26,30], the concept of selective GPER activation introduced in the present study could be considered as a new therapeutic strategy for the treatment and secondary prevention of coronary artery disease in postmenopausal women.

Methods

Expression and subcellular localization of GPER. Expression and localization of GPER were determined in telomerase-immortalized human umbilical vein endothelial cells and primary aortic vascular smooth muscle cells (VSMC) isolated from *gper*+/+ mice. VSMC and endothelial cells were seeded on coverslips and fixed in PBS containing 4% paraformaldehyde for 12 min at room temperature. For staining, cells were treated with either permeabilizing (PBS containing 3% BSA and 0.1% Triton X-100) or non-permeabilizing (PBS containing 3% BSA) blocking buffer for 1 h at room temperature, and incubated with the corresponding antibodies overnight at 4°C. Slides were then washed, incubated with secondary antibody for 1 h at room temperature, washed, mounted in Vectashield supplemented with DAPI, and visualized using a Zeiss LSM510 Meta confocal fluorescent microscope (Zeiss, Oberkochen, Germany). See Supplementary Information for details.

Mice. Female wild-type C57BL/6J mice (*gper*+/+, Harlan Laboratories, Indianapolis, IN, USA) and GPER-deficient (*gper*−/−) mice (Proctor & Gamble, Cincinnati, OH, USA, provided by Jan S. Rosenbaum) were housed at the Animal Resource Facility of the University of New Mexico Health Sciences Center. Animals were maintained under controlled temperature of 22–23°C on a 12 h light, 12 h dark cycle and had access to chow and water *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. See Supplementary Information for details.

Treatment protocols. At six weeks of age, female *gper*+/+ and *gper*−/− mice were changed from normal chow to an atherogenic, phytoestrogen-free, high-fat, high-cholesterol diet (15.8% fat, 1.25% cholesterol, 0.5% sodium cholate; Teklad TD.90221, Harlan Laboratories, Madison, WI, USA). Prior to changing to the atherogenic diet, all animals had undergone either sham surgery or ovariectomy (to induce surgical menopause). In a subset of menopausal mice, pellets releasing the GPER-selective agonist G-1[27,56] (33 μg/day) or placebo (Innovative Research, Sarasota, FL, USA) were implanted subcutaneously prior to changing to the atherogenic diet. Successful ovariectomy was confirmed post mortem by uterine atrophy (wet weight).

Quantification of atherosclerosis. Quantification of atherosclerosis was performed microscopically (in aortic root sections) and macroscopically (utilizing *en face* staining of the aorta). Briefly, alternate 10 μm-thick sections of the aortic root were stained with Oil Red O (ORO) for neutral lipid, followed by hematoxylin and light green for counter-staining. Atherosclerotic lesion area was quantified using a computer-assisted imaging system (Image *J*, version 1.46r, National Institutes of Health, USA) by an investigator blinded to treatment and genotype, and values are expressed as average per section. For the *en face* quantification of atherosclerosis, the aorta from the ascending part to the iliac bifurcation was carefully cleaned from adherent fat and connective tissue, cut open longitudinally, and fixed in paraformaldehyde (4%). Aortas were mounted *en face* and stained with ORO, and lesion area relative to total aortic surface was quantified. See Supplementary Information for details.

Quantification of CD68+ and CD3+ cells. Aortic root sections were stained for macrophages using rat anti-mouse CD68 antibody (clone FA11, AbD Serotec, Raleigh, NC, USA), and T cells using Armenian hamster anti-mouse CD3ε antibody (clone 145-2C11, BioLegend, San Diego, CA, USA), detected by Alexa Fluor 488 goat anti-rat (Life Technologies, Grand Island, NY, USA) and Cy3 goat anti-Armenian hamster (Jackson ImmunoResearch, West Grove, PA, USA) secondary antibodies. Nuclei were stained with DRAQ5 (Cell Signaling Technology, Danvers, MA, USA). Slides were analyzed utilizing a Leica SP5 confocal microscope (Wetzlar, Germany). Immunostained cells were quantified by computer-assisted histomorphometry (ImageJ).

Vascular NO bioactivity. Basal NO bioactivity was determined *ex vivo* in isolated carotid arteries. Briefly, contraction to phenylephrine (30 nmol/L) was recorded in the absence and presence of the NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME, 300 μmol/L for 30 min), and NO bioactivity was calculated as the difference between both contractions. See Supplementary Information for details.

eNOS activation and NO formation in human endothelial cells. To determine whether GPER affects eNOS ser1177 phosphorylation, endothelial cells were treated with the GPER-selective agonist G-1[27] (100 nmol/L) or solvent (DMSO 0.01%) for 20 min, lysed, electrophoresed by SDS PAGE and Western blotted for phosphorylation of eNOS residue ser1177 (antibody 9571, Cell Signaling, Danvers, MA, USA). Receptor-stimulated NO formation was determined by colorimetric detection of the stable NO metabolites NO_2/NO_3 (Abcam, Boston, MA, USA) in cells starved overnight in M199 medium, incubated with HEPES-PSS (composition in mmol/L: 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 0.026 EDTA, 10 glucose, and 10 HEPES; pH 7.4), and exposed either to the GPER-selective agonist G-1 (1–100 nmol/L)[27], 17β-estradiol (1–100 nmol/L), acetylcholine (100 nmol/L), or solvent (DMSO 0.01%) for 10 min. A subset of cells was exposed to the GPER-selective antagonist G36[29] (100 nmol/L) for 30 min prior to stimulation. NO metabolite concentrations were normalized to total protein (Bradford protein assay, BioRad, Hercules, CA, USA). See Supplementary Information for details.

Statistical analyses. Data were tested for distribution normality and analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, the unpaired Student's *t*-test (two-tailed), or the non-parametric Mann-Whitney *U* test, as appropriate (Prism version 5.0 for Macintosh, GraphPad Software, San Diego, CA, USA). Values are expressed as the mean ± s.e.m. of independent experiments; *n* equals the number of animals or number of independent experiments. Statistical significance was accepted at p values of <0.05.

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Author contributions

M.R.M., N.C.F., T.A.H., C.H. and C.D. performed experiments; R.C. and J.B.A. synthesized G-1 and G36; M.R.M., N.C.F., C.D., M.B. and E.R.P. analyzed data; M.R.M., K.A., M.B. and E.R.P. interpreted results of experiments; M.R.M., M.B. and E.R.P. prepared figures and wrote the manuscript; M.R.M., N.C.F., T.A.H., C.H., C.D., K.A., M.B. and E.R.P. approved the final version of manuscript; M.R.M., M.B. and E.R.P. were involved in conception and design of research.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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