G-Protein Estrogen Receptor as a Regulator of Low-Density Lipoprotein Cholesterol Metabolism

Cellular and Population Genetic Studies

Yasin Hussain, Qingming Ding, Philip W. Connelly, J. Howard Brunt, Matthew R. Ban, Adam D. McIntyre, Murray W. Huff, Robert Gros, Robert A. Hegele, Ross D. Feldman

Objective—Estrogen deficiency is linked with increased low-density lipoprotein (LDL) cholesterol. The hormone receptor mediating this effect is unknown. G-protein estrogen receptor (GPER) is a recently recognized G-protein–coupled receptor that is activated by estrogens. We recently identified a common hypofunctional missense variant of GPER, namely P16L. However, the role of GPER in LDL metabolism is unknown. Therefore, we examined the association of the P16L genotype with plasma LDL cholesterol level. Furthermore, we studied the role of GPER in regulating expression of the LDL receptor and proprotein convertase subtilisin kexin type 9.

Approach and Results—Our discovery cohort was a genetically isolated population of Northern European descent, and our validation cohort consisted of normal, healthy women aged 18 to 56 years from London, Ontario. In addition, we examined the effect of GPER on the regulation of proprotein convertase subtilisin kexin type 9 and LDL receptor expression by the treatment with the GPER agonist, G1. In the discovery cohort, GPER P16L genotype was associated with a significant increase in LDL cholesterol (mean±SEM): 3.18±0.05, 3.25±0.08, and 4.25±0.33 mmol/L, respectively, in subjects with CC (homozygous for P16), CT (heterozygotes), and TT (homozygous for L16) genotypes (P<0.05). In the validation cohort (n=339), the GPER P16L genotype was associated with a similar increase in LDL cholesterol: 2.17±0.05, 2.34±0.06, and 2.42±0.16 mmol/L, respectively, in subjects with CC, CT, and TT genotypes (P<0.05). In the human hepatic carcinoma cell line, the GPER agonist, G1, mediated a concentration-dependent increase in LDL receptor expression, blocked by either pretreatment with the GPER antagonist G15 or by shRNA-mediated GPER downregulation. G1 also mediated a GPER- and concentration-dependent decrease in proprotein convertase subtilisin kexin type 9 expression.

Conclusions—GPER activation upregulates LDL receptor expression, probably at least, in part, via proprotein convertase subtilisin kexin type 9 downregulation. Furthermore, humans carrying the hypofunctional P16L genetic variant of GPER have increased plasma LDL cholesterol. In aggregate, these data suggest an important role of GPER in the regulation of LDL receptor expression and consequently LDL metabolism. (Arterioscler Thromb Vasc Biol. 2015;35:213-221. DOI: 10.1161/ATVBAHA.114.304326.)

Key Words: cholesterol, LDL. GPER protein, human receptors, LDL. PCSK9 protein, human

The importance of estrogen as a regulator of plasma lipid metabolism has been most clearly demonstrated in settings of estrogen deficiency (eg, menopause). Bilateral ovariectomy in premenopausal women causes an increase in plasma levels of low-density lipoprotein (LDL) when compared with premenopausal women with preserved ovaries. Furthermore, LDL cholesterol levels increase after onset of menopause.

Multiple mechanisms have been proposed by which estrogens regulate lipoprotein metabolism in humans, including induction of hepatic LDL receptor expression, reduction of hepatic secretion of acyl-coenzyme A: cholesterol acyltransferase 2–derived cholesteryl esters in plasma lipoproteins, reduction of hepatic lipase, and reduction of levels of proprotein convertase subtilisin kexin type 9 (PCSK9), a key regulator of LDL receptor metabolism.

The receptor(s) mediating estrogen’s effects on LDL metabolism is unclear. Estrogen’s effects are triggered via 2 main receptor types. Classic estrogen receptors (ERs) have been shown to have both nuclear and cytoplasmic/membrane-associated sites of effect. More recently, a G-protein–coupled receptor, G-protein ER (GPER; aka GPR30) has been shown to mediate some of the so-called rapid (nongenomic) effects of estrogen. We have recently shown that GPER is also activated by aldosterone.

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The role of GPER in hepatic lipid metabolism is unknown. GPER is expressed in normal hepatocytes.\textsuperscript{13} Notably, mice with genetic deletion of GPER have increased cholesterol levels.\textsuperscript{14} In contrast, total cholesterol levels have not been reported to be elevated in ER\textsubscript{α} knockout mice.\textsuperscript{15}

To examine the relationship between GPER function and lipid metabolism in humans, we have studied the effect of carrying a common hypofunctional GPER genetic variant. The P16L GPER genetic variant is common with an allelic frequency in the general population of \(\approx 20\%\). Recent studies from our laboratory have shown that this proline to leucine change at amino acid residue 16 of GPER (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=11544331) results in a hypofunctional response to the GPER agonist G1.\textsuperscript{16}

In these studies, we evaluate the association of GPER P16L genotype with plasma LDL cholesterol in a previously characterized,\textsuperscript{17} genetically isolated population of Northern European descent from Alberta (n=415). To validate our findings, we examine a London, Ontario-based population of healthy women (n=339).\textsuperscript{18}

We found that in both population samples, the hypofunctional GPER P16L variant was associated with increased LDL concentrations in women, following a recessive model. Furthermore, in cellular studies, we demonstrate that G1 agonist activation of GPER in HepG2 cells, which endogenously express GPER,\textsuperscript{19,20} leads to reduced PCSK9 expression and increased LDL receptor expression. Overall, these studies support an important role of GPER in regulation of plasma lipoprotein metabolism.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Female Carriers of GPER P16L Have Higher Plasma LDL Cholesterol

In the discovery cohort, the GPER P16L allele frequency was 12.3%, with no significant difference between men (13.1%) and women (11.7%). The frequencies of CC, CT, and TT genotypes were 77.1%, 21.2%, and 1.7%, respectively; these did not significantly deviate from the Hardy–Weinberg distribution. The body mass index and waist circumference were not different between genotypes (Table 1). The plasma LDL and total cholesterol concentrations in this sample were significantly higher in homozygotes for the P16L allele, and followed an apparent recessive model (\(P<0.05\); Table 2). After adjusting for covariates, there was no change in the significance of the associations (Table 3).

Subgroup analysis showed that the increase in LDL cholesterol levels in carriers of the GPER P16L variant was sex specific; in particular, women but not men showed the increase in plasma LDL and total cholesterol levels (Table 2). After adjusting for covariates in women, there was no change in the significance of the associations (Table 3).

In the validation cohort of 339 women, the allele frequency of the P16L GPER variant was 25.2%. The frequencies of GPER variant genotype were 56.3%, 36.9%, and 6.8% for wild type (CC), heterozygote (CT), and P16L homozygote (CT) and did not significantly deviate from the Hardy–Weinberg distribution. The plasma LDL cholesterol levels were significantly higher in those carrying the P16L GPER variant (TT>CT>CC; \(P<0.05\); Table 4). The body mass index and waist circumference did not vary significantly between genotypes (Table 5). The increase in plasma LDL cholesterol levels associated with the expression of the P16L GPER variant remained significant after adjusting for covariates (Table 6).

<table>
<thead>
<tr>
<th>Table 1. Subject Demographics: Alberta Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Whole population (n=415)</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>BMI, kg/m\textsuperscript{2}</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
</tr>
<tr>
<td>Women (n=235)</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>BMI, kg/m\textsuperscript{2}</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
</tr>
<tr>
<td>Men (n=180)</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>BMI, kg/m\textsuperscript{2}</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; and NS, not significant.
To determine whether the increase in LDL cholesterol levels in carriers of the GPER P16L variant might indicate a role for GPER in the regulation of lipoprotein metabolism, we studied whether GPER activation regulated LDL receptor protein expression, which is an important determinant of plasma LDL levels. In HepG2 cells, the GPER agonist, G122, induced a concentration-dependent increase in LDL receptor protein expression with a maximal effect of a $36\pm 6\%$ (mean±SE; n=10) increase in LDL receptor protein above baseline at a G1 concentration of 0.1 μmol/L (Figure 1); this was comparable with the increase in LDL receptor protein induced by atorvastatin (33±9%, n=3; Figure 1).

To confirm the GPER dependence of the observed effect of G1, we examined the effect of the GPER antagonist, G15, on G1-mediated stimulation of LDL receptor protein expression. Pretreatment of HepG2 cells with G15 inhibited the G1-mediated increase in LDL receptor protein expression (Figure 2).

### Table 2. Lipid Profiles by Genotype (Least Squares Means Shown): Alberta Population

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total Cholesterol</th>
<th>LDL Cholesterol</th>
<th>Apo B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>5.28</td>
<td>3.27</td>
<td>1.20</td>
</tr>
<tr>
<td>CT</td>
<td>5.28</td>
<td>3.21</td>
<td>1.19</td>
</tr>
<tr>
<td>TT</td>
<td>6.25</td>
<td>4.30</td>
<td>1.41</td>
</tr>
</tbody>
</table>

### Table 3. Summary of ANOVA for Genotype and Quantitative Traits: Alberta Population

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Total Cholesterol</th>
<th>LDL Cholesterol</th>
<th>Apo B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole population</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPER P16L genotype</td>
<td>4.46</td>
<td>5.72</td>
<td>68.66</td>
</tr>
<tr>
<td>Age, y</td>
<td>24.93</td>
<td>13.88</td>
<td>16.26</td>
</tr>
<tr>
<td>Sex</td>
<td>9.17</td>
<td>14.01</td>
<td>12.56</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>16.26</td>
<td>11.96</td>
<td>7.76</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>71.24</td>
<td>3.51</td>
<td>54.41</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>68.66</td>
<td>3.36</td>
<td>35.87</td>
</tr>
</tbody>
</table>

With the increase in LDL cholesterol levels in carriers of the GPER P16L variant, we investigated whether GPER activation regulated LDL receptor protein expression, which is a key determinant of plasma LDL levels. In HepG2 cells, the GPER agonist, G122, induced a concentration-dependent increase in LDL receptor protein expression with a maximal effect of a $36\pm 6\%$ (mean±SE; n=10) increase in LDL receptor protein above baseline at a G1 concentration of 0.1 μmol/L (Figure 1); this was comparable with the increase in LDL receptor protein induced by atorvastatin (33±9%, n=3; Figure 1).

To confirm the GPER dependence of the observed effect of G1, we examined the effect of the GPER antagonist, G15, on G1-mediated stimulation of LDL receptor protein expression. Pretreatment of HepG2 cells with G15 inhibited the G1-mediated increase in LDL receptor protein expression (Figure 2).

BMI indicates body mass index; GPER, G-protein estrogen receptor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and NS, not significant.
expression. In addition, G15 had no significant effect on atorvastatin-mediated upregulation of LDL receptor protein (Figure 2).

In addition, we confirmed the GPER-dependent effect of G1 on LDL receptor protein expression using a GPER-targeted shRNA to downregulate GPER expression. Infection of HepG2 cells with the adenosGPER construct resulted in ≈50% downregulation of GPER mRNA content when compared with the control construct (adenosGFP; Figure 3A). GPER downregulation, using shGPER, attenuated G1-mediated increase in LDL receptor protein expression (Figure 3B). Atorvastatin-mediated LDL receptor upregulation was not affected by shRNA-induced GPER downregulation.

**Role of GPER on Regulation of LDL Uptake and LDL Receptor mRNA**

To confirm the functional effect of GPER activation on LDL receptor protein expression, we assessed G1-mediated regulation of LDL uptake. G1 induced a concentration-dependent increase in LDL uptake with a maximal response of 27±2% above control at G1 concentrations >0.1 μmol/L (n=4; P<0.05; Figure 4A).

To determine whether the increase in LDL receptor protein expression and LDL uptake was related to an increase in LDL receptor mRNA, we performed quantitative polymerase chain reaction studies. No alterations in LDLR mRNA levels were apparent at G1 concentrations >0.1 μmol/L to 1 μmol/L (n=3). In contrast, atorvastatin increased LDL receptor mRNA levels 57±14% above control (n=4; P<0.05; Figure 4B).

**Role of GPER in the Regulation on PCSK9 Expression**

PCSK9 is recognized as a critical regulator of overall LDL receptor functional capacity.1 Furthermore, the regulation of PCSK9 levels has been shown to be cAMP dependent,23 which is the second messenger pathway regulated by GPER activation.24 To determine whether GPER plays a role in PCSK9 protein expression, which in turn could explain the effects of GPER on the regulation of LDL receptor protein, we examined the effect of G1 on PCSK9 levels as assessed in HepG2 conditioned media samples. G1 mediated a concentration-dependent decrease in PCSK9 protein expression in HepG2 cells, with a maximal decrease to 72±8% (n=8) of control with G1 at a concentration of 1.0 μmol/L (Figure 5A). Atorvastatin, in contrast, increased PCSK9 protein content by 40% (P<0.05). Comparable findings were obtained when PCSK9 content was assessed in samples taken from cell lysates (Figure 5B).

To determine whether the decrease in PCSK9 protein expression was related to a decrease in PCSK9 receptor mRNA, we performed quantitative polymerase chain reaction studies. G1 mediated a concentration-dependent decrease in PCSK9 mRNA content (Figure 5C). In contrast (and as expected), atorvastatin treatment increased PCSK9 mRNA content.

To confirm the GPER dependence of the observed effect of G1, we examined the effect of G15 (1 μmol/L). Pretreatment of HepG2 cells with G15 significantly attenuated the decrease in PCSK9 protein induced by G1 (Figure 6). G15 alone had no significant effect on PCSK9 protein expression. In addition, G15 had no significant effect on atorvastatin-mediated upregulation of PCSK9 protein expression. Furthermore, we confirmed the GPER-dependent effect of G1 on PCSK9 levels using the GPER-targeted shRNA. GPER downregulation almost completely attenuated the G1-mediated decrease in PCSK9 expression (Figure 7). The atorvastatin-mediated PCSK9 upregulation was not affected by GPER downregulation.

**Discussion**

The importance of the G-protein–coupled receptor, GPER, in mediating the vascular effects of estrogen (and of aldosterone) has been increasingly appreciated.27–31 However, the importance of GPER in regulation of lipoprotein metabolism in humans has been unexplored to date. Using a combination of human genetics and functional cellular biology, we found that the relatively common missense GPER P16L genotype was associated with higher plasma LDL cholesterol levels in women, but not in men. Furthermore, in HepG2 cells expressing GPER, we demonstrate that GPER activation (1) increased LDL receptor protein expression, without change in mRNA content and (2) decreased PCSK9 mRNA content and protein levels. These independent lines of experimental evidence are directionally consistent and in aggregate support the importance of GPER as a regulator of LDL metabolism, probably via regulation of PCSK9.

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**Table 4. Lipid Profiles by Genotype (Least Squares Means Shown): London, Ontario Female Population**

<table>
<thead>
<tr>
<th></th>
<th>Women (n=339)</th>
<th></th>
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<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CC (n=191)</td>
<td>CT (n=125)</td>
<td>TT (n=23)</td>
<td>CC vs CT</td>
<td>TT</td>
<td>CT vs TT</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.23±0.02</td>
<td>4.39±0.03</td>
<td>4.53±0.03</td>
<td>NS (0.33)</td>
<td></td>
<td></td>
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<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.17±0.03</td>
<td>2.34±0.03</td>
<td>2.42±0.03</td>
<td>NS (0.33)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo B, g/L</td>
<td>0.69±0.03</td>
<td>0.72±0.03</td>
<td>0.76±0.03</td>
<td>NS (0.21)</td>
<td></td>
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</tbody>
</table>

LDL indicates low-density lipoprotein; and NS, not significant.

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**Table 5. Subject Demographics: London, Ontario Female Population**

<table>
<thead>
<tr>
<th></th>
<th>Women (n=339)</th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC (n=191)</td>
<td>CT (n=125)</td>
<td>TT (n=23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>27.51±0.72</td>
<td>28.33±0.93</td>
<td>24.83±1.56</td>
<td>NS</td>
<td></td>
<td></td>
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<tr>
<td>BMI, kg/m²</td>
<td>22.91±0.28</td>
<td>23.35±0.40</td>
<td>22.26±0.59</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>76.26±0.63</td>
<td>77.31±0.89</td>
<td>75.65±1.61</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BMI indicates body mass index; and NS, not significant.
Consistent with a functional effect of the GPER P16L variant, human genetic association studies showed higher LDL cholesterol levels in female carriers. In the northern Alberta sample, heterozygotes and homozygotes for L16 had ≈0.1 and ≈1.0 mmol/L higher plasma LDL cholesterol concentrations, respectively, than homozygotes for P16. This is equivalent to or higher than the effect sizes seen for other relatively common candidate gene variants in lipid metabolism,32–41 (although much smaller than the effect sizes seen in carriers of rare mutations associated with familial hypercholesterolemia).42 Notably, the effect on LDL cholesterol of the GPER P16L allele was less marked (although still clearly evident) in the London, Ontario validation cohort. The reason for the lesser effect size in the Ontario sample in comparison with the Alberta cohort is unclear. Notably, both populations were ethnically similar (ie, predominantly white from Western European origins). However, it should be noted that in the London sample, the LDL determination was performed on a nonfasting sample, which may have minimized the apparent effect of carrying the GPER P16L allele.

It is notable that the GPER P16L genotype is associated with higher LDL cholesterol in women only. GPER was initially characterized as mediating the effects of estrogen,11 hence an effect of expression of a hypofunctional GPER leading to increased LDL cholesterol solely in women would not be unexpected. However, our recent studies demonstrated that aldosterone is also a potent GPER agonist.12 If so, it might have been expected that the effect on LDL cholesterol of expressing a hypofunctional GPER might have been comparably apparent in men and women. The reason for this apparent inconsistency is unclear.

### Table 6. Summary of ANOVA for Genotype and Quantitative Traits: London, Ontario Female Population

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Total Cholesterol</th>
<th></th>
<th>LDL Cholesterol</th>
<th></th>
<th>Apo B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F Value</td>
<td>P Value</td>
<td>F Value</td>
<td>P Value</td>
<td>F Value</td>
</tr>
<tr>
<td>Women (n=339)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPER P16L genotype</td>
<td>3.91</td>
<td>0.02</td>
<td>3.91</td>
<td>0.02</td>
<td>3.07</td>
</tr>
<tr>
<td>Age, y</td>
<td>30.81</td>
<td>&lt;0.0001</td>
<td>30.81</td>
<td>&lt;0.0001</td>
<td>12.58</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>1.61</td>
<td>NS (0.2)</td>
<td>1.61</td>
<td>NS (0.2)</td>
<td>1.52</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>47.01</td>
<td>&lt;0.0001</td>
<td>10.16</td>
<td>0.0016</td>
<td>3.71</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>108.72</td>
<td>&lt;0.0001</td>
<td>10.33</td>
<td>0.0014</td>
<td>86.76</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; GPER, G-protein estrogen receptor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and NS, not significant.

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**Figure 1.** G1-mediated effects on low-density lipoprotein (LDL) receptor expression. Concentration-dependant G1-mediated increase in LDL receptor expression. Hep G2 cells were incubated for 24 hours with multiple G1 concentrations (0.01–1.0 μmol/L) or vehicle control only. Atorvastatin (10 μmol/L) is used as a positive control. Significant increases are evident at concentrations of 0.01 and 0.1 μmol/L G1 (data represent the means±SE of 3–10 independent experiments performed under identical conditions). Inset, Representative immunoblot demonstrating the G1- and atorvastatin-mediated increase in LDL receptor. *P<0.05 vs control (cells treated with vehicle only).

**Figure 2.** G15 attenuates G1-mediated increase in low-density lipoprotein (LDL) receptor expression. Pretreatment of Hep G2 cells with the G-protein estrogen receptor antagonist G15 (1 μmol/L) attenuated effects of G1 (0.1 μmol/L) on LDL receptor expression. G15 pretreatment did not attenuate the increase in LDL receptor expression seen with atorvastatin (10 μmol/L). The data represent means±SE of 3 to 8 independent experiments performed for each condition. Inset, Representative immunoblot demonstrating the effects of pretreating cells with the antagonist G15, on G1- and atorvastatin-mediated regulation of LDL receptor expression. *P<0.05 vs control (cells treated with vehicle only).
We considered why this genetic variant has not been previously identified as a significant determinant on prior genome-wide association studies of plasma LDL cholesterol levels. However, as far as we have been able to determine, none of the commonly used microarrays in the catalogue of published genome-wide association studies carried the single nucleotide polymorphism of interest (rs11544331) (https://www.genome.gov/page.cfm?p=ageid=26525384#searchForm). In this context, the findings in the present study emphasize that the previous genome-wide association studies may be incomplete about potential candidate genes in dyslipidemia because the associations are limited to the content on the microarray. A recent genome-wide association study did identify a variant of GPR146 (viz., rs1997243) as being associated with cholesterol levels (Table II in the online-only Data Supplement). Notably, this genetic variant was also reported to be in linkage disequilibrium with P16L GPER ($r^2=0.554$), supporting our present findings.

These studies suggest a mechanism whereby GPER activation has beneficial effects on LDL cholesterol metabolism—and thus specify a mechanism by which carrying a hypofunctional GPER variant may lead to increased LDL cholesterol levels. LDL receptor expression is significantly increased by GPER activation with effects comparable with those of HMG (3-hydroxy-3-methyl-glutaryl) CoA reductase inhibition.

In summary, our studies demonstrate that the expression of a common GPER genetic variant parallels increased LDL levels and that GPER activation increases LDL receptor protein expression, probably via downregulation of PCSK9 levels. LDL receptor expression is significantly increased by GPER activation with effects comparable with those of HMG (3-hydroxy-3-methyl-glutaryl) CoA reductase inhibition.

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which are also well known to be associated with estrogen status. Overall, these findings support an important role of GPER in the regulation of LDL metabolism in humans.

Figure 5. G1-mediated effects on proprotein convertase subtilisin kexin type 9 (PCSK9) expression. Concentration-dependent G1-mediated decrease in PCSK9 expression assessed in supernatants (A) and in cell lysates (B). Hep G2 cells were incubated for 24 hours with multiple G1 concentrations (0.01–1.0 μmol/L). Atorvastatin (10 μmol/L) is used as a positive control. A statistically significant decrease in PCSK9 expression is evident at concentration of G1>0.1 μmol/L. Data represent the mean±SE of 3 to 10 independent experiments performed under identical conditions in supernatants (A) and cell lysates (B). Note: The control condition in A was medium from cells treated with vehicle only. The control condition in B was lysates from cells treated with vehicle only. Insets, Representative immunoblots demonstrating the G1-mediated decrease and atorvastatin-mediated increase in PCSK9 expression. *P<0.05 vs control.

Figure 6. G15 attenuates G1-mediated decrease in proprotein convertase subtilisin kexin type 9 (PCSK9) expression. Pretreating Hep G2 cells with the G-protein estrogen receptor antagonist G15 (1 μmol/L) attenuated the effects on PCSK9 expression of G1 (0.1 μmol/L). Atorvastatin-mediated increase in PCSK9 expression was not altered with G15 pretreatment. Data represent mean±SE of 3 to 5 independent experiments performed for each condition. Inset, Representative immunoblot demonstrating the effects of pretreating cells with the antagonist G15, on G-1 and atorvastatin-mediated regulation of PCSK9 expression. *P<0.05 vs control (cells treated with vehicle only).

Figure 7. G-protein estrogen receptor (GPER) downregulation and its effect on G1-mediated decrease in proprotein convertase subtilisin kexin type 9 (PCSK9) expression. shGPER-mediated downregulation of GPER expression caused an attenuation in the G1-mediated decrease (0.1 μmol/L) in PCSK9 expression (data represent the mean±SE of 3 independent experiments performed for each condition) Inset, Representative immunoblot demonstrating the G1-mediated decrease and atorvastatin-mediated increase in PCSK9 expression vs control in both shGFP- and shGPER-infected cells. *P<0.05 vs its respective control (ie, cells infected with the same adenovirus construct, treated with vehicle only).

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Disclosures

None.

References

24. Zhu H, Tucker HM, Greer KE, Simpson JF, Manning AK, Cupples LA, Estus S. A common polymorphism decreases low-density lipoprotein


**Significance**

G-protein estrogen receptor (GPER) is a newly recognized G-protein–coupled receptor linked to the actions of estrogen (and to a lesser extent aldosterone). We have identified that women carrying a common hypofunctional missense genetic variant of GPER, P16L GPER, have increased low-density lipoprotein cholesterol. Furthermore, GPER activation in vitro leads to increased low-density lipoprotein receptor expression mediated at least, in part, by inhibition of PCSK9 expression, an important regulator of low-density lipoprotein receptor degradation. In total, these studies support an important role of GPER in low-density lipoprotein cholesterol regulation.
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Materials and Methods

Cell culture. HepG2 cells (obtained from ATCC, Manassas, Virginia, USA) were cultured in DMEM (Invitrogen, Mississauga, ON) containing penicillin, streptomycin and 10% fetal calf serum (FBS) in humidified atmosphere (37°C, 5% CO2). Previous studies have shown that these cells express GPER\textsuperscript{1,2} as well as ER\textalpha and ER\textbeta\textsuperscript{3}.

Assessment of LDL receptor and PCSK9 protein. The effect of the GPER agonist G1\textsuperscript{4} on PCSK9 and LDL receptor protein was assessed by immunoblotting. Cultured HepG2 cells were serum starved for 24 hours in phenol red-free DMEM and then incubated with increasing concentration of G1 for an additional 24 hours. In experiments using the GPER antagonist, G15\textsuperscript{5}, cells were pretreated with G15 for 15 min prior to addition of the agonist. Cells were sequentially washed with phosphate buffered saline (PBS) and lysed in ice-cold buffer containing 20mM Tris, pH 8.0, 1%NP-40.0, 0.1% SDS, 140mM NaCl and 1mM phenylmethylsulfonyl fluoride. Conditioned media (for secreted PCSK9\textsuperscript{24,25}) or cell lysates (for LDL receptor) were resolved by 8%SDS-PAGE and transferred electrophoretically onto Immun-Blot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and blocked for 1 hour at room temperature in a buffer containing 20 mM Tris (pH 7.4), 0.5 M NaCl, and 0.1% Tween 20 and 5% skim milk. The membranes were then incubated with anti-LDL receptor antibody or anti-PCSK9 (Abcam, Toronto ON; 1:1000 dilutions for both). Blots were washed in Tris-buffered saline for 1 h and then incubated in HRP conjugated secondary anti-rabbit (1:5,000 dilution) antibody for 1 h at room temperature. GAPDH was used as a loading control for immunoblots of cell lysates. In immunoblots of supernatant samples for PCSK9 expression, samples were normalized for protein content.

Proteins were detected by chemiluminescence, as described by the manufacturer's protocol (DuPont NEN, Boston, MA). Bands corresponding to PCSK9 or LDL receptor were quantified by densitometry.

Assessment of LDL uptake. LDL uptake was determined by using LDL labeled with the fluorescent probe 1,1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate (DiI-LDL, Alfa Aesar, Ward Hill, MA) as previously described\textsuperscript{6}. Briefly, HepG2 cells in 6-well (35mm) plates were serum-starved for 24 hours in phenol red-free DMEM and then were incubated with increasing concentration of GPER agonist G1(0.001-1μM) for 24 hour. Subsequently, cells were incubated in fresh media in the presence of 10 μg/mL DiI-LDL for 5 h. Cells were trypsinized and resuspended in phosphate-buffered saline for FACS analysis with BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ) Data were collected and analyzed with BD Cell Quest Pro Software.

Real time PCR analysis of the level of LDL receptor and PCSK9 mRNA expression. HepG2 cells were serum-starved in phenol red-free DMEM for 24 hours, and then incubated with increasing concentration of G1 (0.001-1μM) for 24 hours. Total RNA was isolated by using RNeasy Mini Kit (Qiagen, Toronto, ON). RNA (2 μg) was then reverse-transcribed using high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) as per manufacturer instruction, and mRNA abundances were determined by a 2-step quantitative real-time reverse transcription PCR method on an ABI Prism (model 7900HT) Sequence Detection
System (Applied Biosystems, Foster City, CA) as previously described\(^6\). Briefly, cDNA (20 ng) generated by reverse transcription as described above was analyzed in triplicate for LDLR or PCSK9 mRNA expression which was normalized to the abundance of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) utilizing standard curve method. Quantities of mRNA were interpolated from a standard curve plotted as cycle threshold versus the log of the quantity of serial dilutions of known GAPDH and LDLR or PCSK9 cDNA standards. Primers for LDLR, PCSK9 and GAPDH were obtained from Taqman Assays-on-Demand (Applied Biosystems, Foster City, CA).

**Gene transfer studies.** The AdMax\(^\text{TM}\) adenovirus creation kit was used to generate the adenoviral vectors used in these studies as per manufacturer’s instructions (Microbix BioSystems Inc., Toronto, Canada), as we have previously described\(^7\). Hep G2 cells were cultured for 24 hours and then infected with adenovirus expressing GPER shRNA (shGPER) or shGFP (control) for 16 hours, followed by incubation in phenol red-free DMEM for 24 hours. Finally, cells were treated with receptor agonists for 24 hours.

**Human genetic studies**

**Discovery cohort: Alberta population of Northern European descent:** 415 healthy subjects were studied from 21 adjacent Hutterite colonies in Alberta\(^8\). Both females and males were included. Subjects were 17-73 years of age, with the following demographic characteristics: 56.6% were women; age, body mass index (BMI) and waist circumference were (mean± standard deviation) 36.9±14.9 years, 28.2±5.3 kg/m\(^2\) and 86.6±13.7 cm, respectively. Fasting blood samples (12 hours) were drawn to determine the biochemical profile and genotype of the subjects. Exclusion criteria were restricted to blood samples inadequate for genetic and/or biochemical profiling. Informed consent was obtained for all analyses, with approval from the Western University Research Ethics Board.

**Validation cohort: London Ontario based population:** We studied 339 normal, healthy females, 18-56 years of age. Recruitment was based on local advertising and email invitations for volunteers within the Robarts Research Institute and the University of Western Ontario. Informed consent was obtained for all analyses, with approval from the Western University Research Ethics Board. This included 251 subjects from a previously reported cohort study\(^9\), which comprises all of the female subjects in whom non-fasting plasma lipids/lipoproteins were determined. The cohort is predominantly white (87%) of predominantly Anglo-Saxon origin with small subsets of East Asians (9%), south Asians (3%) and blacks (1%). Exclusion criteria included: history of cardiovascular events, average alcohol intake over 2 units per day, pregnancy and use of anti-hypertensive drugs or anticoagulants. Age, BMI and waist circumference were 27.6±0.5 years; 23.0±0.2 kg/m\(^2\) and 76.6±0.5 cm, respectively.

**Biochemical analysis:** Plasma concentrations of total cholesterol, high density lipoprotein (HDL) cholesterol, triglyceride and apolipoprotein (apo) B were determined in the J. Alick Little Lipid Research Laboratory at St. Michael’s Hospital (for the discovery cohort)\(^\text{8,10}\) and in the laboratory of Dr. Murray Huff, Robarts Institute (for the validation cohort)\(^11\). The low density lipoprotein (LDL) value was calculated.

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**Genotyping:** Genomic DNA was extracted from whole blood and genotyped for GPER by London Regional Genomic Centre (www.lrgc.ca) as previously described\(^1\).  

**Data analysis:** *Cellular studies:* Initial analysis by one-way ANOVA was followed by Dunnett’s multiple comparison tests for multiple group comparisons. The significance of difference between paired groups was determined by Students test for paired data. \( P < 0.05 \) on a two-sided test was taken as a minimum level of significance.  

**Human genetic studies:** Statistical significance of difference in quantitative variables between wild type P16/P16 homozygotes (CC) and carriers of P16L GPER variant groups (CT plus TT) was determined by non-parametric one way analysis of variance (Prism 4.0, GraphPad Software, San Diego, CA). \( P<0.05 \) was taken as the nominal significance level. The chi-square test was used to compare the allele frequencies in the males vs. females of each population. As previously reported, we used a general linear model ANOVA to test for the association of GPER genotype with plasma levels of LDL and total cholesterol after adjusting for covariates\(^13\). The GPER genotype was introduced into the ANOVA under an additive (co-dominant) model (CC, wild type; CT, heterozygote carrier; and TT, homozygote for the variant). The dependent variable introduced was either LDL cholesterol, total cholesterol or apo B and the independent variables were GPER genotype, age, BMI, sex, HDL cholesterol and triglyceride. Genotype frequencies deviation from the Hardy-Weinberg equilibrium predicted frequencies were evaluated using the conditional chi-square goodness of fit test using one degree of freedom for both populations\(^14\). This analysis was done for the Alberta and London Ontario based samples using SAS statistical software, version 9.1 (SAS institute, Inc., Cary, NC).
References


