Estrogen Receptor GPR30 Reduces Oxidative Stress and Proteinuria in the Salt-Sensitive Female mRen2.Lewis Rat

Sarah H. Lindsey, Liliya M. Yamaleyeva, K. Bridget Brosnihan, Patricia E. Gallagher, Mark C. Chappell

Abstract—The current study assessed whether activation of the novel estrogen receptor GPR30 ameliorates salt-dependent renal damage in intact mRen2.Lewis (mRen2) females. Hemizygous mRen2 rats were maintained on either a normal salt (0.5% Na) or high-salt (HS; 4.0% Na) diet for 10 weeks (5 to 15 weeks of age), and HS animals were treated with the GPR30 agonist G-1 or vehicle for 2 weeks. Systolic blood pressure markedly increased with HS diet (149 ± 3 to 219 ± 5 mm Hg; P < 0.01), but G-1 did not influence pressure (P = 0.42). G-1 and estradiol induced relaxation of preconstricted mesenteric vessels from normal salt mRen2 rats, but both responses were attenuated in the HS group. Despite the lack of an effect on blood pressure, G-1 decreased renal hypertrophy, proteinuria, urinary 8-isoprostane excretion, and tubular 4-hydroxynonenal staining. HS diet significantly increased GPR30 mRNA (1.01 ± 0.04 versus 1.59 ± 0.13; P < 0.01) and protein (0.60 ± 0.31 versus 3.99 ± 0.75; P < 0.01) in the renal cortex. GPR30 was highly expressed in the brush border of proximal tubules and colocalized with megalin. Finally, megalin expression was reduced by HS diet and restored with G-1. We conclude that GPR30-mediated beneficial effects in salt-sensitive mRen2 females occurred independent of changes in systolic blood pressure. The failure of G-1 to influence pressure may reflect a salt-induced impairment in GPR30-mediated vasorelaxation. The renoprotective actions of GPR30 may involve attenuation of tubular oxidative stress and activation of megalin-mediated protein reabsorption. (Hypertension. 2011; 58:00-00.)

Online Data Supplement

Key Words: GPR30 ■ estrogen receptors ■ oxidative stress ■ megalin ■ dietary sodium

In contrast to the classic estrogen receptors (ERs) α and β, GPR30 is a membrane-bound G protein–coupled estrogen receptor that induces rapid signaling events.1,2 Synthesis of the selective GPR30 agonist G-1 and antagonist G15 has stimulated studies to elaborate the role of this membrane receptor in the myriad and complex actions of estrogen.3,4 Ongoing studies in our laboratory use the congenic mRen2.Lewis (mRen2) hypertensive rat to elucidate the role of estrogen in cardiovascular disease.5 We showed previously in ovariec tomized mRen2 females that chronic treatment with the GPR30 agonist G-1 markedly reduces blood pressure but does not influence total body or uterine weight, suggesting activation of a receptor distinct from ERα and ERβ.6 We subsequently showed that estradiol and G-1 dilate isolated mesenteric vessels from the female mRen2 to a similar extent, and both responses are blocked by the GPR30 antagonist G15.7 In addition to estrogen sensitivity, mRen2 females exhibit a marked salt-dependent increase in blood pressure.8 In comparison to a normal salt (NS) diet (0.5% sodium), high salt (HS; 4.0% sodium) significantly increases blood pressure, proteinuria, and albuminuria; decreases creatinine clearance; and induces renal and cardiac hypertrophy.5,8 Maintenance of the normotensive Lewis female, the background strain for the mRen2, on an HS diet does not increase blood pressure or proteinuria, suggesting that an activated renin-angiotensin-aldosterone system is essential to the development of salt sensitivity in the mRen2 female. The mRen2 strain is a unique congenic model in that HS profoundly elevates blood pressure in females via an angiotensin II–dependent mechanism, but males do not respond to salt with an increase in pressure.9,10 Estrogen depletion exacerbates the salt-dependent increase in blood pressure, as well as the extent of proteinuria and albuminuria in this model.8,10 Furthermore, we showed previously that G-1 administration in intact HS mRen2 females improves diastolic function and attenuates myocyte hypertrophy without influencing blood pressure.11 Thus, the mRen2 strain presents a unique opportunity to establish the protective effects of GPR30 in a salt-sensitive female. The current study addressed the hypothesis that chronic GPR30 activation ameliorates salt-sensitive renal damage in the absence of blood pressure effects.

Methods

Please see the online Data Supplement at http://hyper.ahajournals.org for all procedures and methods.
Reduced the response to G-1 (*P<0.05).

G-1–treated animals was not different from NS controls.

Was significantly reduced after G-1, and albuminuria in both proteinuria and albuminuria (Figure 2).8,9 Proteinuria as shown previously, 10 weeks of HS significantly increased systolic blood pressure (219±5 mm Hg) as compared with the NS group (149±3 mm Hg; *P<0.001). Administration of the GPR30 antagonist G-1 for 2 weeks did not significantly alter systolic blood pressure (vehicle: 224±8 versus G-1: 216±4 mm Hg; *P<0.42). In mesenteric vessels, HS blunted vasorelaxation to both G-1 and estradiol by ~60% (P<0.01; Figure 1B); however, the response to acetylcholine (10−6 mol/L) was not altered by salt (NS: 14.0±2.8%; HS: 13.0±3.9%; *P=0.85; data not shown). Pretreatment of HS vessels with the NO synthase inhibitor Nω-nitro-L-arginine methyl ester further reduced the response to G-1 (P<0.05).

Effects of Chronic G-1 on Renal Indices
G-1 significantly decreased renal hypertrophy and increased creatinine clearance (Table). G-1 did not alter urinary excretion of sodium, potassium, norepinephrine, or epinephrine. As shown previously, 10 weeks of HS significantly increased both proteinuria and albuminuria (Figure 2).8,9 Proteinuria was significantly reduced after G-1, and albuminuria in G-1–treated animals was not different from NS controls.

Table. Physiological Parameters in High Salt mRen2.Lewis Females Treated With Vehicle or G-1

<table>
<thead>
<tr>
<th>Measurement</th>
<th>HS + veh (n=9)</th>
<th>HS + G-1 (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>199±4</td>
<td>207±5</td>
</tr>
<tr>
<td>Kidney/body weight ratio, mg/g</td>
<td>5.1±0.1</td>
<td>4.7±0.1*</td>
</tr>
<tr>
<td>Urine volume, mL/d</td>
<td>48±5</td>
<td>50±5</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>1.0±0.1</td>
<td>1.7±0.2*</td>
</tr>
<tr>
<td>Clearance/urine weight ratio, mL/min/mg/g</td>
<td>0.21±0.02</td>
<td>0.36±0.05*</td>
</tr>
<tr>
<td>Urinary sodium, mEq/d</td>
<td>17.1±1.7</td>
<td>18.2±1.8</td>
</tr>
<tr>
<td>Urinary potassium, mEq/d</td>
<td>2.48±0.2</td>
<td>2.67±0.3</td>
</tr>
<tr>
<td>Urinary norepinephrine, ng/d</td>
<td>932±156</td>
<td>1054±156</td>
</tr>
<tr>
<td>Urinary epinephrine, ng/d</td>
<td>102±24</td>
<td>152±27</td>
</tr>
</tbody>
</table>

HS indicates high salt; veh, vehicle.

*P<0.05 vs HS + veh.

HS mRen2 displayed significant staining for the lipid peroxidation product 4-hydroxynonenal in the renal cortex (Figure 3). The majority of staining was localized to the tubulointerstitial area (~50% of pixels) with less staining in glomeruli (~7%). G-1 treatment significantly reduced staining in the whole renal cortex (P<0.05), more specifically in tubules (P<0.05) but not in glomeruli (P=0.94). Medullary 4-hydroxynonenal staining was not altered (data not shown). Finally, urinary excretion of the reactive oxygen species metabolite 8-isoprostane F2α was significantly reduced in the G-1 group and correlated with proteinuria in HS females (Figure 4).

Renal Distribution and Expression of GPR30
Although we previously demonstrated GPR30 expression in aortic and mesenteric vessels of the peripheral vasculature, distribution of the receptor in the mRen2 kidney is not known.6,7 We used the GPR30 antibody LS4272, which was shown previously to have high specificity in immunofluorescence and immunoblot studies.6,12 GPR30 immunostaining was predominantly in the renal cortex of HS mRen2 females with less staining in the medulla (Figure 5). GPR30 immunofluorescence was detected specifically in proximal tubules, with no staining evident in distal tubules or the glomerulus (Figure 5B). A higher magnification image using confocal micro-
copy showed GPR30 localization specifically on the apical surface of proximal tubules (Figure 5C).

Immunoblot analysis using the same antibody as in immunohistochemistry revealed a single band in cortical (Figure 6C) and medullary (Figure 6F) membrane fractions at a molecular weight of 50 kDa, which is identical to that reported in aortic tissue and brain.6 In the renal cortex, GPR30 mRNA (Figure 6A) and protein (Figure 6B) were both significantly increased with HS. Although medullary GPR30 mRNA was increased in the HS group (Figure 6D), protein analysis did not reveal a significant change (Figure 6E).

We also assessed ERα-36, another purported membrane-bound estrogen receptor, using an antibody directed against amino acid residues 247 to 261 of the DNA binding domain, which is conserved in the various molecular forms of ERα.13,14 In the renal cortex, ERα-66 was the predominant isoform of the receptor, whereas ERα-36 was barely detectable by immunoblot (Figure S1A, available in the online Data Supplement). Quantification revealed that ERα-36 expression was 50-fold less than ERα-66. The expression of all 3 ERα isoforms was localized primarily in the soluble fraction of the renal cortex, contrasting with the presence of GPR30 protein exclusively in the 100 000g membrane fraction (Figure S1B). Moreover, maintenance on an HS diet did not alter the expression or localization of ERα isoforms in the renal cortex (P>0.20).

**GPR30 and Megalin**

Because GPR30 immunostaining localized predominantly to the apical surface of proximal tubules, we assessed its relationship with megalin, an endocytic receptor expressed primarily in the brush border that plays a key role in the reabsorption of filtered protein.15 As shown in Figure 7, GPR30 and megalin colocalized in the brush border of proximal tubules. In addition, megalin was significantly reduced with HS and restored with G-1 (Figure 8). Tubular megalin immunofluorescence negatively correlated with proteinuria (Figure 8B). Real-time PCR analysis showed that HS also decreased megalin mRNA (Figure 8C).

**Discussion**

Emerging data suggest that GPR30 is widely expressed in various tissues including the brain, heart, vasculature, pancreas, ovary, and kidney.16 To our knowledge, the current study is the first to characterize receptor distribution within the kidney of female hypertensive rats. GPR30 immunostaining was most prominent in the proximal tubules of the renal cortex, with minimal staining throughout the glomerulus and medulla. The immunoreactive signal for the receptor was predominantly localized to the apical aspect of the epithelium, suggesting that estrogenic signaling may arise from tubular fluid. Although circulating estrogen is predominantly bound to a steroid-binding protein, significant concentrations of estradiol and various metabolites are filtered and present in the urine.17 We further find cortical GPR30 upregulation in the HS mRen2 female. It is not known whether salt directly affects GPR30 expression within the renal cortex or whether the increase in GPR30 reflects a compensatory mechanism of enhanced transcription or mRNA stabilization to oppose the damaging effects of salt within the kidney. Indeed, the
salt-induced increase in cortical GPR30 may be protective under estrogen-intact conditions but result in an exacerbated response to estrogen loss in the mRen2 female and other animal models.8,9,18,19 In contrast, renal medullary GPR30 did not reflect the increase in mRNA induced by HS. Wang et al20 also reported divergent regulation of GPR30 in the hamster ovary in response follicle stimulating hormone and luteinizing hormone stimulation. The underlying mechanisms for these discrepancies remain to be resolved and may involve translational regulation of GPR30.

We also assessed the subcellular localization of ERα, the predominant steroid receptor for estrogen in the kidney, using an antibody directed against the DNA binding domain, which recognized all of the molecular forms of the receptor. G-1 does not bind ERα-66 or ERα-46; however, Kang et al13 reported that G-1 activates the membrane-associated ERα-36 in the breast cancer cell line SK-BR-3. In cortical homogenates, ERα-36 was only detectable in the soluble fraction, was >50-fold lower than ERα-66, and was not altered with HS. In contrast, GPR30 protein was only detected in the membrane fraction, consistent with the protein motif of a G protein–coupled receptor. Although we cannot completely exclude ERα-36 from contributing to the renal actions of G-1, the low level of expression suggests that the renoprotective actions of G-1 in the salt-sensitive mRen2 female are primarily mediated by GPR30.

In the current model of salt sensitivity, placement of the female mRen2 on a 4% sodium diet for 10 weeks induced a moderate degree of proteinuria and albuminuria despite a high systolic blood pressure (>200 mm Hg). Nevertheless, 2-week G-1 treatment significantly reduced proteinuria without influencing systolic blood pressure, contrasting with our previous studies in the ovariectomized mRen2 on an NS diet in which the same dose of G-1 significantly lowers systolic blood pressure.6 Although telemetry can detect more discrete changes in blood pressure, the tail-cuff method has provided consistent detection of changes in pressure to salt or estrogen depletion in the mRen2, as well as after renin-angiotensin-al-
dosterone system blockade.5,6,8,9 We demonstrated recently that G-1 dilates mesenteric vessels from intact and ovariec-
tomized mRen2 to a similar extent as estradiol and is composed of both an NO-dependent and -independent com-
ponent.7 In the current study, G-1 and estradiol relaxation was blunted by >60% in HS vessels, which may contribute to the salt-sensitive increase in blood pressure. Pretreatment of HS

Figure 4. A, G-1 reduced urinary 8-isoprostane in high salt (HS) mRen2.Lewis (mRen2) females (**P<0.01; n=9). B, Excretion of 8-isoprostane positively associated with proteinuria.

A

B

Figure 5. A, In the kidneys of high salt (HS) fed mRen2.Lewis (mRen2) females, immunofluorescent GPR30 staining (green) in the medulla (right) was lower than the cortex (left). 4',6-Diamidino-2-
phenylindole (blue) was used to stain nuclei. B, The renal cortex showed a prominent signal primarily on the luminal membrane of proximal tubules with minimal staining in glomeruli and distal tubules. C, Higher magnification using confocal microscopy identifying proximal (P) and distal (D) tubules according to presence of a brush border. D, Preabsorption of primary antibody with the anti-
genic peptide attenuated immunostaining.
antioxidant effects of GPR30 within the kidney of salt-sensitive mRen2 females remains to be established, estrogenic effects are generally associated with attenuation of oxidative stress. Ji et al\(^22\) reported that NADPH oxidase activity in renal wrap hypertension is exacerbated by ovariec- tomy and reversed with estradiol replacement. In addition, estrogen activates several reactive oxygen species scavenging pathways, including thioredoxin, thiol/disulfide oxidoreduc- tase, and glutathione peroxidase.\(^23\)–\(^25\) In this regard, Broughton et al\(^26\) showed recently that G-1 reduces NADPH-dependent oxidase activity in isolated carotid and intracranial arteries of normotensive Sprague-Dawley rats. Elucidation of the underlying mechanisms of GPR30 to attenuate reactive oxygen species within the proximal tubules, as well as the tissue levels of G-1 that were achieved after chronic admin- istration of the agonist, awaits future studies.

Oxidative stress is linked to tubular damage within the kidney and may contribute to microvilli remodeling and subsequent proteinuria.\(^27\) In this study, urinary excretion of the lipid oxidation marker 8-isoprostane highly correlated with proteinuria, and chronic G-1 treatment significantly attenuated oxidative stress and protein excretion. Proteinuria results from glomerular damage and/or a decrease in tubular protein reabsorption mediated by megalin, a large 600-kDa receptor localized primarily to the luminal surface of epithelial cells.\(^28\) Megalin is responsible for the endocytosis of multiple ligands, and the marked proteinuria in megalin knockout mice emphasizes its vital role in the reabsorption of filtered proteins.\(^15\) We demonstrate colocalization of GPR30 and megalin on the apical surface of proximal tubules, suggesting that GPR30 may influence megalin-mediated protein reabsorption. HS decreased tubular megalin protein and gene expression, indicating regulation at the transcriptional level. Although others have shown megalin downregu- lation in diabetic nephropathy, albumin overload, and in the hypertensive male mRen2(27) fed an NS diet, to our knowl- edge this is the first report of megalin regulation in response to salt loading.\(^27,29,30\) G-1 increased megalin protein but not mRNA, potentially indicating a posttranscriptional mecha- nism. The protective effects of GPR30 may result from activation of the receptor-associated protein (RAP), a chap- erone for megalin that promotes its localization to the brush border.\(^31\) Alternatively, GPR30 may decrease luminal mega- lin shedding and promote its retention in the tubule, perhaps through the regulation of various secretases or sheddases along the nephron.\(^32,33\)

**Perspectives**

In conclusion, we show that the novel estrogen receptor GPR30 was upregulated within the renal cortex of salt-sensitive mRen2 females after HS intake and that chronic administration of the GPR30 agonist reduced renal damage independent of changes in systolic blood pressure. Recent clinical trials reveal that salt sensitivity increases after meno- pause, and estradiol treatment reverses this effect.\(^34,35\) The HS-induced upregulation of renal GPR30 and the ability of G-1 to ameliorate renal damage further support a protective role for estrogen within the kidney.
Figure 7. A, GPR30 immunofluorescence in the renal cortex of a HS mRen2.Lewis (mRen2) female was localized to the luminal surface of proximal tubules. B, Megalin immunofluorescence was also primarily in the brush border. C, GPR30 and megalin were highly colocalized. D, Bright field image identifying proximal (P) and distal (D) tubules according to presence of a brush border.

Figure 8. A, Quantification of tubular megalin immunofluorescence showed a significant decrease in high-salt (HS) versus normal salt (NS) mRen2.Lewis (mRen2) females. G-1 treatment reversed the effect of salt on megalin expression (*P<0.05, **P<0.01; n=4 to 5). B, Tubular megalin negatively associated with proteinuria. C, HS also decreased megalin mRNA, but G-1 treatment did not significantly alter megalin gene expression (*P<0.05, **P<0.01; n=5 to 8). D through F, Representative images of tubular megalin staining.
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Disclosures

None.

References


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METHODS

Animals.
Hemizygous mRen2 congenic rats were obtained from the Wake Forest School of Medicine Hypertension Center transgenic breeding colony. Rats were given free access to food and water in a temperature-controlled room (22 ± 2°C) with a 12 hour light to dark cycle. The normal salt diet (NS; 0.5% sodium) was switched to a high salt diet (HS; 4% sodium) beginning at five weeks of age. The GPR30 agonist G-1 (EMD Chemicals, Gibbstown, NJ) was dissolved in 50% DMSO and delivered via osmotic minipump implanted subcutaneously at the dorsum of the neck at a dose of 400 µg/kg/day (Model 2ML2; Alza Corporation, Palo Alto, CA) as previously described.1 G-1 was administered for two weeks beginning at 13 weeks of age. Animals were randomly assigned to three experimental groups: intact NS, intact HS + vehicle and intact HS + G-1 (n = 9). Urine was collected in metabolic cages over a 24 hour period. All procedures were approved by the university Animal Care and Use Committee.

Urinary Markers.
Urinary albumin, protein, and creatinine were measured as described previously.2,3 The oxidative stress marker 8-isoprostane F2α was determined with an enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI). Norepinephrine and epinephrine were measured using the Bi-CAT kit (ALPCO Diagnostics, Salem, NH). Urinary electrolytes were measured in an automated system (NOVA Biomedical Electrolyte Analyzer).

Blood pressure
Systolic blood pressure was monitored using an automated tail-cuff system (Narco Bio-systems, Houston, TX) while warming at 35°C under slight restraint.1 Multiple measurements were taken for each animal, and the last five readings were averaged for mean systolic blood pressure.

Vascular Reactivity
Second order mesenteric vessels were isolated after sacrifice and mounted on a myograph as previously described.4 Vessels were pre-constricted with 10-5 M phenylephrine and the responses to increasing concentrations of G-1 or estradiol were measured. All vessels were assessed for response to acetylcholine (10-6 M). Some vessels were pretreated with the nitric oxide synthase inhibitor L-NAME (10-4 M).

Immunoblots
Kidneys were separated into cortical and medullary fractions and frozen until analysis. Tissue was lysed in buffer containing protease inhibitors (Sigma, St. Louis, MO) and separated into soluble and membrane fractions by 100,000 x g centrifugation (Optima Ultracentrifuge, Beckmann, Fullerton CA). Lysates (50 to 100 µg) were separated by electrophoresis, transferred to a PVDF membrane, and probed with primary antibodies for GPR30 (1:500; LS-A4272, Lot #8969/70; MBL International, Woburn, MA) or ERα (1:500; ab2746-50, Lot #823870; Abcam, Cambridge, MA). Membranes were treated with HRP-labeled secondary antibodies and detected with chemiluminescent substrates (Pierce Biotechnology, Rockford, IL). Membranes were reprobed with mouse monoclonal anti-β-actin (1:5000; Sigma, St. Louis, MO) as a loading control, and bands were quantified using MCID densitometry software (InterFocus Imaging, Linton, England).

Immunohistochemistry
Kidney sections mounted on slides were blocked with 0.1% Tween, 1% bovine serum albumin, and 5% normal donkey serum. Anti-GPR30 (1:250; LS-A4272, Lot #8969/70, MBL International), anti-4-hydroxy-2-nonenal (4-HNE; 1:15,000; Calbiochem, La Jolla, CA), or anti-
megalin (1:150, ab21849, Abcam, Cambridge, MA) were incubated overnight at 4°C. For a negative control, some sections were pre-absorbed with the antigenic peptide. For fluorescent staining, Alexa Fluor 488 or 562 secondary antibodies (Invitrogen, Carlsbad, CA) were applied and coverslips were mounted using ProLong mounting media with DAPI (Invitrogen). For colorimetric staining, biotinylated goat anti-rabbit (VectorLabs, Burlingame, CA) was applied and antibody binding was detected using Vectastain Elite avidin-biotin complex kit (VectorLabs) and 0.1% diaminobenzene (Sigma). Slides were counterstained with hematoxylin (Sigma). Images were acquired with Simple PCI (Hamamatsu, Sewickley, PA), and Adobe Photoshop was used to analyze images. All images were background subtracted before analysis. For fluorescent quantification, ten cross-sectioned tubules were selected from a 20X image and data was expressed as percent of pixels stained x intensity and normalized to controls. For colorimetric images, data was expressed as percent of pixels stained.

**RNA Isolation and Real-time PCR**
RNA from renal cortical tissue was analyzed for GPR30 and megalin gene expression as previously described. Results were quantified as C_t values, where C_t is the threshold cycle at which the amplified product is initially detected, and data were expressed as relative gene expression (ratio of target/18S rRNA control).

**Statistics**
All measurements were expressed as the mean ± standard error of the mean (SEM). Data were analyzed with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Unpaired t-test was used to analyze data with P<0.05 considered significant. One-way ANOVA with Newman Keuls post test was used for multiple comparisons with a confidence limit of 95% considered significant.
REFERENCES


Figure S1. A, A full length immunoblot of soluble (S) and membrane (M) fractions following 100,000 x g centrifugation of the kidney cortex probed for ERα expression. The ERα antibody, which was directed against amino acid residues 247-261 in the DNA binding domain, revealed predominant expression of ERα-66 and ERα-46 forms, but minimal signal for ERα-36. ERα expression was primarily in the soluble fraction and was not influenced by the high salt diet. B, A full length immunoblot of kidney cortex homogenates probed for GPR30 (50 kDa) reveals expression exclusively in the 100,000 x g membrane fraction.