Restoration of renal function by a novel prostaglandin EP4 receptor-derived peptide in models of acute renal failure

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Submitted 5 April 2012; accepted in final form 30 October 2012

ACUTE RENAL FAILURE (ARF) is characterized by an abrupt and sustained decline in the glomerular filtration rate (46). Despite significant advances in supportive therapy over the last few decades, ARF remains a serious medical condition associated with high levels of morbidity and mortality (7, 18). Depending on the definition used, ARF has been reported to affect from 1% to 25% of patients admitted to intensive care units and has led to mortality rates from 15% to 60% (17). On the basis of the progress made in understanding the pathophysiology of ARF, many therapeutic agents have been developed and tested in animal models of ARF (24). However, only limited success has been achieved in human clinical trials with pharmacological agents, such as loop diuretics, mannitol, dopamine receptor agonists, atrial natriuretic peptide, adenosine antagonists, N-acetylcysteine, IGF-1, and calcium entry blockers (24, 46). The lack of effective treatments for ARF warrants the identification of new molecular targets and approaches to develop efficient therapeutic agents for the treatment of renal insufficiency.

Prostaglandins, which are derivatives of the cyclooxygenase-catalyzed conversion of arachidonic acid, are potent mediators of renal function and hemodynamics (3, 8). PGE2 is one of the major prostanoids found in the kidney, and it exerts its biological actions through one of the four EP receptors, EP1–EP4. The EP4 receptors are predominantly expressed in the glomerulus and in preglomerular vessels, but they are also expressed in the distal convoluted tubule, cortical collecting duct, and outer medullary vasa recta (4, 16). Activation of EP4 by PGE2 has a vasodilatory effect on renal vascular tone (41), stimulates renin release in juxtaglomerular cells (10), and promotes cell survival of podocytes (1). An EP4-specific orthosteric (natural ligand binding site) agonist was found to reduce nephrotoxic injury and increased the survival rate of rats with mercury chloride-induced ARF (49), and an EP4 agonist prevented the development of glomerulonephritis (34). These studies suggest that modulating EP4 receptor activity could improve renal function in ARF.

In contrast to orthosteric ligands, functionally selective allosteric modulators of G protein-coupled receptors (GPCRs) offer advantages by exerting effects on some (desirable) receptor-mediated pathways without interfering with other pathways that are best left intact (12, 13, 19, 25, 45); hence, orthosteric ligands exhibit undesirable properties by lacking selectivity. There is increasing evidence that peptides derived from various extracellular and juxtamembranous regions of GPCRs can interfere with their respective activities, as demonstrated, for example, by the prostaglandin F2α (13, 38), vasopressin type 2 peptide, THG213.29, was tested for its efficacy to improve renal function (glomerular filtration rate, renal plasma flow, and urine output) and histological changes in a model of ARF induced by either cisplatin or renal artery occlusion in Sprague-Dawley rats. THG213.29 modulated PGE2-binding dissociation kinetics, indicative of an allosteric binding mode. Consistently, THG213.29 antagonized EP4-mediated relaxation of piglet saphenous vein rings, partially inhibited EP4-mediated cAMP production, but did not affect Go, activation or β-arrestin recruitment. In vivo, THG213.29 significantly improved renal function and histological changes in cisplatin- and renal artery occlusion-induced ARF models. THG213.29 increased mRNA expression of heme-oxygenase-1, Bcl2, and FGF-2 in renal cortex; correspondingly, in EP4-transfected HEK293 cells, THG213.29 augmented FGF-2 and abrogated EP4-dependent overexpression of inflammatory IL-6 and of apoptotic death domain-associated protein and BCL2-associated agonist of cell death. Our results demonstrate that THG213.29 represents a novel class of diuretic agent with noncompetitive allosteric modulator effects on EP4 receptor, resulting in improved renal function and integrity following acute renal failure.
(40), and β2 adrenergic (14) receptors. Peptides that target regions of the receptor remote from the (natural ligand) orthosteric binding site often exhibit noncompetitive allosteric properties and modulate only a subset of the receptor-mediated activities, allowing more specific pharmacological intervention targeting some, but not all, receptor signaling pathways. This concept, referred to as functional selectivity, has been described for numerous compounds (12, 45), including noncompetitive antagonists (19, 25).

The aim of the present work is to study the efficacy of a new (D-) peptidomimetic EP4 receptor modulator in tempering ARF; in this process, we investigated its mode of action. We, hereby, describe that the optimized peptidomimetic THG213.29, which possesses functional selectivity toward the EP4 receptor, proved to be efficacious in improving renal function and structural outcome in ischemia- and nephrotoxin-induced rat models of ARF.

MATERIALS AND METHODS

Animals and reagents. Animals were used according to a protocol of the Ste-Justine Hospital Animal Care Committee and along the principles of the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. THG213.29 was a kind gift from Theratechnologies (Montreal, QC, Canada); peptides were synthesized using F-moc chemistry and the solid phase Merrifield method, and peptide purity was assessed by HPLC and mass spectrometry. PGE2, U-46619, and GW627368X were purchased from Cayman Chemical (Ann Arbor, MI). L-902688 (51) was a kind gift from Merck Frosst (Kirkland, QC, Canada). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Vascular ring preparation. Newborn piglets (1–4 days old) were anesthetized with halothane (1.5%), and the lower external saphenous vein was cannulated for infusion of saline containing the anesthetics and [3H]PGE2, in this process, we investigated its mode of action. We, hereby, describe that the optimized peptidomimetic THG213.29, which possesses functional selectivity toward the EP4 receptor, proved to be efficacious in improving renal function and structural outcome in ischemia- and nephrotoxin-induced rat models of ARF.

Renal function. All experimental procedures were performed under anesthesia. Male Sprague-Dawley adult rats (250–300 g) were anesthetized using 1.5% isoflurane in oxygen. U-46619 was added. Tension was measured by force-displacement transducers and was recorded using Work Bench software (Kent Scientific, Litchfield, CT).

Renal function. All experimental procedures were performed under anesthesia. Male Sprague-Dawley adult rats (250–300 g) were anesthetized using 1.5% isoflurane in oxygen. U-46619 was added. Tension was measured by force-displacement transducers and was recorded using Work Bench software (Kent Scientific, Litchfield, CT).

Histology. Kidneys were fixed in 10% formalin and paraffin-embedded; then, the specimens were stained with periodic acid-Schiff stain. Histological changes were evaluated by quantitative measurements of glomerular structural changes, as well as obstruction of collecting ducts and tubules—performed in a blinded manner relative to treatment assignment (by X. Hou and D. R. Varma).

Cell culture. Human embryonic kidney (HEK) 293E (Invitrogen) and EP4 stably transfected HEK293 cells (HEK293/EP4) were cultured in DMEM supplemented with 10% FBS (Wisconsin, Saint-Jean Baptiste, QC, Canada), 100 units/ml penicillin/streptomycin, and 2 mM l-glutamine (Invitrogen, Carlsbad, CA) and 200 μg/ml G418. Transient transfections were performed in 6-well or 10-cm dishes using the polyethylenimine (Polysciences, Warrington, PA) method, as described previously (26).

Plasmids. The expression vectors containing human EP4 receptor and Gαq were obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). Plasmids encoding GFP10-Epac-Rluc3, Gaα11-91Rluc, YFP-Gβ1, Epα-YFP, and Rluc-β-arestatin 2 have been described previously (26).

Radioligand binding. HEK293/EP4 cells were incubated for 2 h at 4°C in PBS/0.5% BSA (w/v) buffer with 10 nM of [3H]PGE2, in the presence or absence of 10 μM unlabeled PGE2 to determine specific binding. Cells were washed three times with PBS/0.5% BSA and lysed with 0.2 N NaOH/0.1% Triton X-100. Bound radioactivity was measured on cell lysates with a liquid scintillation counter. For [3H]PGE2 dissociation rate experiments, a time course of displacement was performed by removing [3H]PGE2 (10 nM) after 2 h and replacing it with 1 μM unlabeled PGE2 in the presence or absence of THG213.29 (50 μM); reaction was stopped by removing the buffer and lysing the cells.

Bioluminescence resonance energy transfer measurement. Bioluminescence resonance energy transfer (BRET) measurements were performed as described previously (26). Briefly, transiently transfected HEK293E cells were seeded in 96-well white clear bottom microplates (ViewPlate, PerkinElmer) coated with poly-d-lysine and left in culture for 24 h. Cells were washed once with PBS and the Rluc Samples were then collected every 20 min for an additional period of 2 h. The radioactivity in the blood and urine samples was measured with a liquid scintillation counter. Urine flow rates, GFR, and renal plasma flow (RPF) were determined at different times and averaged for a 60-min period starting 20 min after drug administration. Urinary volume was calculated and expressed as microliters of urine per minute corrected for the weight of the animal. GFR and RPF were calculated as the ratio of urinary [3H]inulin to plasma [3H]inulin or the ratio of urinary [14C]aminohippuric acid to plasma [14C]aminohippuric acid, respectively, corrected for the volume of the urine and the weight of the animal. Intrafemoral arterial blood pressure was also measured. Moreover, blood urea nitrogen and serum creatinine levels were measured using QuantiChrom assay kits (BioAssay Systems, Hayward, CA).

Cisplatin-induced model of acute renal failure. ARF and tubular necrosis were induced by intraperitoneal injection of 17.5 mg/kg of cisplatin to Sprague-Dawley male rats on day 1. Rats were treated (intravenously) with saline or THG213.29, either 1 mg/kg on day 5 or 5 mg/kg three times per day from days 2 to 5. Renal function was then assessed on day 5, as described above. Ischemic-renal artery occlusion model of acute renal failure. Male Sprague-Dawley rats were cannulated, and basal GFR was determined as described above. The left and right renal arteries were then clamped for a period of 60 min to induce acute renal ischemia. Following the ischemic period, clamps were removed, and the animals were immediately treated with THG213.29 (1 mg/kg iv bolus), fenoldopam (0.6 μg/kg bolus dose, followed by 0.6 μg·kg⁻¹·h⁻¹) or saline via the jugular vein. Sham-operated rats underwent the same procedure without out renal artery clamping. At the end of their assigned period, the animals were either killed to obtain tissue samples or anesthetized to measure renal parameters as described above.

Histology. Kidneys were fixed in 10% formalin and paraffin-embedded; then, the specimens were stained with periodic acid-Schiff stain. Histological changes were evaluated by quantitative measurements of glomerular structural changes, as well as obstruction of collecting ducts and tubules—performed in a blinded manner relative to treatment assignment (by X. Hou and D. R. Varma).

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substrates coelenterazine h (for BRET1 experiments; NanoLight Technology, Pinetop, AZ) or coelenterazine 400A (for BRET2 experiments; Biotium, Hayward, CA) added at a final concentration of 5 μM to BRET buffer (PBS, 0.5 mM MgCl₂, 0.1% glucose). BRET readings were collected using a Mithras LB940 plate reader (Berthold) and MicroWin2000 software. BRET1 measurement between Rluc and YFP was obtained by sequential integration of the signals detected in the 460–500 nm (luciferase) and 510–550 nm (YFP) windows, whereas BRET2 readings between Rluc3 and GFP10 were collected by sequential integration of the signals detected in the 365–435 nm (Rluc3) and 505–525 nm (GFP10) windows. The BRET signal was calculated as the ratio of light emitted by acceptor (YFP or GFP10) over the light emitted by donor (Rluc or Rluc3). The values were corrected to net BRET by subtracting the background BRET signal obtained in cells transfected with Rluc (BRET1) or Rluc3 (BRET2) constructs alone. Cells were pretreated or not for 10 min with THG213.29 or GW627368X and treated with agonists at room temperature for 3 (Gα11), 10 (Epac) or 15 (β-arrestin) minutes prior to BRET measurements.

CRE prediction. Prediction of cAMP response element was carried out with CREB target DB of the Salk Institute using the human database (http://natural.salk.edu/CREB/) (52).

Gene expression. HEK293E and HEK293/EP4 cells were stimulated with L-902688 (1 μM) in the presence or absence of THG213.29 (100 μM) for 4 h. Similarly, explants from rat kidney were prepared from young adult rats (~300 g) (Charles River). Tissues were minced in pieces

Fig. 1. A: schematic two-dimensional representation of human EP4 receptor indicating the juxtamembranous region from which peptide THG213 was derived. Structure of THG213 (iftsyecl; B) and THG213.29 [L-(4,4)-biphenyl alanine-tseyal.KK; C] peptides.
of ~25 mg, placed in 96-well plates in DMEM and treated with THG213.29 (100 μM) or PGE2 (1 μM) for 4 h. Cells and explants were collected in TRizol (Invitrogen) and total RNA was isolated. Prior to extraction, explants were homogenized. Five hundred nanograms of RNA was combined to qScript cDNA SuperMix (Quanta Bioscience, Gaithersburg, MD), and cDNA synthesis was performed following the manufacturer’s protocol. Quantitative real-time PCR was performed on MxPro3000 (Stratagene, La Jolla, CA) using iTaq SYBR Green SuperMix with ROX (Bio-Rad, Hercules, CA). Primers were synthesized by AlphaDNA (Montreal, QC, Canada) and sequences were hIL6-F: TCTC-CACAAGGCGCTTCTGGCT; hIL6-R: GTTCTGTGGGGGCGCGC-TACA; hFadd-F: GGGCCTGGGAAAGAAGCCTGT; hFadd-R: CAGGTGGGGCCTGTGTGCT; hDaxx-F: GCCAGGGCTTGG-ACCTGCAC; hBAD-F: ATTCCTGACTACAGGCAAGATCA; hBAD-R: ACAGC-CACGCGGTCCTCATGAT; rbC12-F: GATAACGGAGGCTGGG-AGCC; and rbC12-R: GATACAGGAGGCTGGGATGTC; hBAD-F: ATTCCTGACTACAGGCAAGATCA; hBAD-R: ACAGC-CACGCGGTCCTCATGAT; rbC12-F: GATAACGGAGGCTGGG-AGCC; and rbC12-R: GATACAGGAGGCTGGGATGTC; hBAD-F: ATTCCTGACTACAGGCAAGATCA; hBAD-R: ACAGC-CACGCGGTCCTCATGAT; rbC12-F: GATAACGGAGGCTGGG-AGCC; and rbC12-R: GATACAGGAGGCTGGGATGTC. mRNA expression levels were normalized against 18S rRNA endogenous control levels in each sample and measured using Student’s t-test, and for differences between more than two groups by one-way ANOVA followed by Dunnett’s post hoc test. For comparison between treatments and cell line for gene expression levels, two-way ANOVA followed by Bonferroni’s post hoc test was used. Statistical significance was set at P < 0.05.

RESULTS

THG213.29 is derived from an EP4 extracellular juxtamembranous region and acts via EP4. Initially, the all-D peptide THG213 (itfysc; Fig. 1B) was derived from the first extracellular loop and transmembrane-3 junction of the human EP4 receptor (Fig. 1A), as reported for other GPCRs (13, 38, 40). In the process of optimizing THG213 for efficacy (and solubility), derivatives were generated and tested for their ability to modulate PGE2-induced vasodilation of piglet saphenous veins; this complex multisignal-dependent physiological functional assay was deliberately used to avoid a screening bias toward classical but potentially nonrelevant signaling pathways and because PGE2-induced vasorelaxation of porcine saphenous vein is foremost EP4-dependent (50), whereas stimulation of EP2 and to an even lesser extent of EP3 hardly relaxes saphenous veins (27, 33). Relevantly in the present context, we also tested THG213 derivatives on GFR. The peptidomimetic THG213.29 [L-(4,4)-biphenyl alanine-(all D) tsyea-(all L) LKK; Fig. 1C] induced the most robust increase in rat GFR; THG213.29 (1 μM) also significantly suppressed PGE2- and EP4-selective agonist L-902688 (51) (10 nM)-mediated relaxation of saphenous vein (by nearly 50%; Fig. 2), suggesting that THG213.29 acts via EP4.

Effect of THG213.29 on PGE2 binding and dissociation kinetics. To ascertain the interaction of THG213.29 on the EP4 receptor and determine its receptor-coupled mode of action, we performed competitive binding experiments and investigated major EP4-mediated signaling pathways, including ones relevant to cytotoxicity and cytoprotection as in ARF. Unlabeled PGE2 dose-dependently displaced bound [3H]PGE2 in HEK293 cells specifically expressing only EP4, but not the other EP2 receptors (Fig. 2B). As expected, on the basis of the EP4 receptor region (juxtamembranous) from where the original peptide was derived, THG213.29 did not displace bound [3H]PGE2, indicating that it did not compete for the EP4 orthosteric natural ligand binding site, in line with an allosteric modulator (see below). THG213.29 was able to modulate the [3H]PGE2 dissociation rate in radioligand dissociation kinetics experiments (Fig. 2C). Indeed, the addition of excess unlabeled PGE2 resulted in bound [3H]PGE2 dissociation with a longer half-life (τ1/2) and a corresponding smaller koff in the presence of THG213.29. These results are in line with the properties of an allosteric ligand, as a key manifestation of an allosteric interaction is an alteration of the affinity of an orthosteric ligand for its binding site, and thus of its association and/or dissociation rates (31).

Functional selectivity of THG213.29. As EP4 is classically described to couple to Goi/adenylate cyclase activation, we measured cAMP production in EP4-transfected HEK293E cells coexpressing the previously characterized BRET-based Epac biosensor (26) that allows measurement of changes in intracellular cAMP levels in living cells. Stimulation with PGE2 resulted in a dose-dependent increase in cAMP production (pEC50 of 9.63 ± 0.16), while pretreatment of cells with THG213.29 (100 μM) reduced the Emax of PGE2 by 23% without affecting PGE2 potency (pEC50 of 9.74 ± 0.19) (Fig. 2D). The potency of THG213.29 to inhibit EP4-mediated cAMP production was low (pIC50 of 5.52 ± 0.68; Fig. 2E) but specific, as neither inhibition of PGE2-mediated cAMP production was observed in HEK293E cells transfected with the closely related prostaglandin EP2 receptor, nor of (unrelated) AVP-mediated cAMP production by the vasopressin 2 receptor (Fig. 2F). Moreover, THG213.29 did not exhibit any intrinsic effects on cAMP production in EP4-transfected cells when administered alone (Fig. 2F). As EP4 signaling has also been shown to involve coupling to pertussis toxin-sensitive Goi proteins (11, 26) and β-arrestin-mediated effects (5, 26), we verified the effect of THG213.29 on these two other EP4 signaling outcomes in living cells, using previously characterized BRET assays (26). THG213.29 had no intrinsic effect or no modulatory effect of PGE2-induced structural rearrangements within the Goi1β1γ2 heterotrimeric G protein (reflecting activation of Goi1i; Fig. 2G) or β-arrestin recruitment to the receptor (Fig. 2H) in EP4-expressing HEK293E cells; conversely, the orthosteric EP4-selective antagonist GW627368X completely abrogated the PGE2-induced response in these assays. The results reveal the pharmacological selectivity of THG213.29, as it only partially inhibited specifically EP4-mediated cAMP production without affecting two other signaling outcomes of the receptor.

Effect of THG213.29 on normal dog, rat, and pig renal function. Because EP4 exhibits a role on kidney function (41), we tested whether THG213.29 affects renal function of different species, notably Beagle dogs, juvenile pigs, and Sprague-Dawley rats. GFR essentially doubled with a maximal effect found at a dose of 2 mg/kg in rats and pigs and at 5 mg/kg in dogs (Fig. 3A). Renal plasma flow and urine output were also augmented by THG213.29 administration in normal dogs and pigs, with a borderline increase for renal plasma flow in rats, (Fig. 3, B and C); mean arterial blood pressure remained stable
after injection of THG213.29, as shown on representative averaged tracing of blood pressure in dogs (Fig. 3D) and on compiled data in rats, dogs, and pigs (Fig. 3, E–G). Thus, effects of THG213.29 on renal functional parameters tested were species-independent.

**Efficacy of THG213.29 in a cisplatin-induced nephrotoxic acute renal failure rat model.** Nephrotoxic effects resulting from the use of antineoplastic, radiocontrast, and antimicrobial agents can result in ARF (35). Cisplatin is a common and effective chemotherapeutic agent used for the treatment of various malignancies with unfortunate dose-limiting nephrotoxicity observed in 25–35% of treated patients (39). We tested the efficacy of THG213.29 in a cisplatin-induced nephrotoxicity rat model of acute tubular necrosis and renal failure. Five days after administration of cisplatin to Sprague-Dawley rats, there was a dramatic decline in GFR, renal plasma flow, and urine output (Fig. 4A), and, consequently, a progressive increase of serum blood urea nitrogen (BUN) and creatinine levels (Fig. 4B); levels of plasma proteins, bicarbonate, sodium, and chloride did not change (results not shown). Late administration of a single dose of THG213.29 (1 mg/kg) on day 5 (after cisplatin) improved renal function, while three times per day (5 mg/kg) treatment with THG213.29 from days 2 to 5 (Fig. 4A) further restored measured renal function parameters; BUN and serum creatinine levels measured on day 5 also decreased in animals treated from day 2 to 5 after cisplatin (Fig. 4C). Histological assessment of kidneys of cisplatin-injected rats 72 h after treatment with saline revealed hypertrophy and/or erythrocyte extravasation in the majority of glomeruli, as well as occlusion of a small portion of collecting ducts; treatment with THG213.29 (5 mg/kg, three times a day) dramatically reduced glomerular hypertrophy and glomerular erythrocyte extravasation; collecting duct obstruction (percent) was unaffected by THG213.29 (Fig. 4, D and E). Together, these results reveal that THG213.29 improves renal function and glomerular structure in a model of nephrotoxin-induced ARF.

**Efficacy of THG213.29 in a renal artery occlusion rat model of acute renal failure.** A variety of clinical conditions, including hemorrhage, heart failure, volume depletion, and shock can lead to compromised renal blood flow and ischemia, which accounts for the largest number of cases of ARF (44). We assessed the efficacy of THG213.29 in the widely used ischemic renal artery occlusion (RAO) rat model of ARF. In this model, the effect of THG213.29 was compared with fenoldopam, a selective dopamine D1 receptor agonist that has been shown to improve renal perfusion and decrease serum creatinine in limited clinical studies (46). Bilateral renal artery clamping was carried out for 60 min to induce acute renal ischemia, leading subsequently to a marked decrease in renal function. As illustrated in Fig. 5A, administration of either THG213.29 (1 mg/kg) or fenoldopam (0.6 μg/kg) increased urine output, but only THG213.29 was effective in significantly raising GFR and renal plasma flow 2 h after renal artery clamping; THG213.29 and fenoldopam were comparably effective in decreasing BUN and serum creatinine 72 h after RAO (Fig. 5B). Histological examination of kidneys collected 24 and 72 h after renal ischemia revealed structural damage compared with sham-operated rats, showing glomerular erythrocyte extravasation and tubular obstruction with cell debris (Fig. 5, C–G). These histological anomalies were considerably attenuated in rats treated with THG213.29. Results indicate that THG213.29 improves renal function and protects against tissue damage in a model of ischemia-induced ARF.

**Effects of THG213.29 on EP4-dependent gene expression.** In an attempt to understand the effects of THG213.29 in renal function and structure in ARF models, we tested in renal cortex whether THG213.29 can specifically induce genes consonant with its protective actions. Interestingly, THG213.29 stimulation of renal cortex induced mRNA expression (including on renal tissue) of the anti-inflammatory heme-oxygenase-1 (9), the anti-apoptotic Bcl2, and the cytoprotective FGF-2, whereas another gene of potential relevance, namely, endothelial nitric oxide synthase (eNOS), was unaffected (Fig. 6A). To further illustrate that the effects of THG213.29 in altering expression of cytoprotective, inflammatory, and proapoptotic genes are specifically mediated via EP4, we treated HEK293 cells stably transfected with EP4, with the EP4 orthosteric agonist L-902688 in the absence or presence of THG213.29. L-902688 slightly increased mRNA expression of FGF-2, and this was further augmented by cotreatment with THG213.29 (Fig. 6), consistent with findings on the renal cortex. Also, treatment with THG213.29 reduced L-902688-induced expression of proinflammatory cytokine IL-6, and of apoptotic factors BCL2-associated agonist of cell death (BAD) and death do-

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**Fig. 2.** A: inhibition of EP4-mediated vasoconstriction in isolated piglet saphenous vein rings by THG213.29. Saphenous vein rings pre-exposed or not to THG213.29 (1 μM) were contracted with the TP receptor agonist U-46619 (0.2 μM) and were then exposed to 1 μM PGE2 (i) or to 10 nM of the EP4-specific agonist L-902688 (ii). Results are expressed as means ± SE of 22 (PGE2-treated) or 9 (L-902688-treated) rings. **P < 0.01.***P < 0.0001 compared with saline. B and C: effects of THG213.29 on the specific binding and dissociation kinetics of [3H]PGE2 to EP4 receptors. B: displacement of bound [3H]PGE2 (10 nM) by increasing concentrations of unlabeled PGE2 or THG213.29. Values are expressed as means ± SE of three experiments, each performed in duplicate. C: dissociation kinetics of [3H]PGE2 (10 nM) binding by excess unlabeled PGE2 (1 μM) in the presence or absence of THG213.29 (213.29; 50 μM) in HEK293/EP4 cells. Data from one representative experiment are shown. Inset: means ± SE of dissociation half-life and kobs from 4 experiments, each performed in duplicate. **P < 0.01 compared with PGE2. D: functional selectivity of THG213.29. PGE2 concentration-response curves of EP4–receptor-mediated cAMP production assessed by BRET in living HEK293 cells cotransfected with Epac biosensor and EP4. Data from one representative experiment of four (each performed in triplicate) are shown (to avoid visual cluttering). F: specificity of THG213.29 for EP4. Effect of THG213.29 on cAMP production assessed by Epac-based BRET assay. HEK293E cells cotransfected with Epac sensor and either EP4, or vasoresspin type 2 (V2R) receptors were pretreated or not with THG213.29 (100 μM) and stimulated with PGE2 or AVP (0.1 μM). Values are expressed as means ± SE of 3–6 experiments, each performed in triplicate; ***P < 0.001. G: BRET assay to monitor EP4–mediated Gαo activation was performed in living HEK293E cells cotransfected with Gαo–Fluc, YFP–GpI, G2Y, and EP4. Variation of the BRET signal compared with basal conditions was measured in cells pretreated or not with THG213.29 (100 μM) or EP4 antagonist GW627368X (1 μM) and stimulated or not with PGE2 (0.1 μM). Values are expressed as means ± SE of five experiments, each performed in triplicate. H: β-arrestin recruitment to EP4 receptor was monitored by BRET in living HEK293E cells coexpressing EP4–YFP and Fluc–β-arrestin 2. Ligand-promoted BRET variation was measured in cells pretreated or not with THG213.29 (100 μM) or EP4 antagonist GW627368X (1 μM) and stimulated or not with PGE2 (0.1 μM). Values are expressed as means ± SE of four experiments, each performed in triplicate.
main-associated protein (DAXX) (Fig. 6B); importantly, no response to L-902688 and/or THG213.29 was observed in HEK293 cells devoid of EP4, confirming that the effects of THG213.29 are EP4 dependent. The data also reveal that THG213.29 exhibits biased functional selectivity, depending as illustrated on the type of gene affected, resulting in inhibition of some and stimulation of others, in line with allosteric modulators (13, 20, 25, 45).

Fig. 3. Effects of THG213.29 on kidney function in normal rats, dogs, and pigs. THG213.29 administration (intravenous bolus dose; rats and pigs: 2 mg/kg, dogs: 5 mg/kg) significantly increased glomerular filtration rate (GFR; A), renal plasma flow (B), and urine output (C) in normal Sprague-Dawley rats, Beagle dogs, and pigs. Values are expressed as means ± SE from 4 (rats) or 6 (dogs, pigs) animals; *P < 0.05, ***P < 0.001 compared with saline. D: averaged representative tracing of mean arterial blood pressure in dogs, before and after intravenous injection of THG213.29 (at dose indicated above). Data are expressed as means ± SE; n = 4. E–G: mean arterial blood pressure at baseline, and at 30 and 60 min after intravenous THG213.29 in rats, dogs, and pigs (at doses indicated above). Data are expressed as means ± SE; n = 4 per species.

Fig. 4. Efficacy of THG213.29 in a cisplatin-induced nephrotoxic model of acute renal failure. Sprague-Dawley rats were injected with cisplatin (17.5 mg/kg ip on day 1) to induce acute tubular necrosis and renal failure. A: renal function (GFR, renal plasma flow, and urine output) were measured in normal untreated rats or 5 days after administration of cisplatin. A single administration of THG213.29 on day 5 (1 mg/kg iv) improved kidney function compared with saline-treated rats; these parameters were normalized in rats treated with THG213.29 three times a day (5 mg/kg iv) from days 2 to 5. Data are expressed as means ± SE from 106 normal untreated rats or 4–6 rats per treatment group, *P < 0.05, **P < 0.01 compared with cisplatin protocol rats treated with saline. B: BUN and serum creatinine were measured in saline-treated rats daily for 5 days after administration of cisplatin. C: animals were injected with cisplatin on day 1, treated with saline or THG213.29 three times a day (5 mg/kg iv) from days 2 to 5 after cisplatin, and BUN, and serum creatinine were measured on day 6 (n = 4). Data are expressed as means ± SE. *P < 0.05 compared with saline-treated rats (ANOVA followed by trend analysis). D: hypertrophic glomeruli and/or erythrocyte extravasation and collecting duct obstruction were quantified from histology kidney sections of cisplatin-injected rats collected 72 h after treatment with saline or THG213.29 (5 mg/kg ip, three times a day from days 2 to 5 after cisplatin). **P < 0.01 compared with saline-treated rats.

AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00138.2012 • www.ajpregu.org
DISCUSSION

Despite improved supportive care, ARF remains a serious clinical condition associated with high mortality and morbidity, which have not significantly decreased over the past few decades; accordingly, new therapeutic modalities are needed for this medical condition. Approximately 85% of ARF is of ischemic or nephrotoxic origin (44). PGE$_2$ and its receptors, including EP$_4$, are widely expressed in the kidney and regulate renal function (3, 16, 24). Orthosteric EP$_4$ agonists have been
shown to improve renal function in models of nephrotoxicity and glomerulonephritis (34, 49). However, functionally selective allosteric modulators of GPCRs offer numerous advantages over orthosteric ligands by exerting effects on some (desirable) receptor-mediated functions but not others best left intact. We, hereby, describe the effects of a first functionally selective EP4 modulator, THG213.29, which is effective in preserving renal function and structural integrity in distinct models of ARF.

Extracellular loops and the extracellular portions of transmembrane helices play an important role in the overall pharmacology of GPCRs (6), and there is a conformational coupling between the extracellular surface and the orthosteric binding site (2). THG213.29 was derived from a juxtamembranous region of the EP4 receptor that is distinct from the presumed orthosteric binding site, which is located in the case of the closely related prostacyclin receptor, mostly within the transmembrane core (42). Consequently, THG213.29 exhibited noncompetitive properties toward EP4 as it did not displace bound PGE2 but modulated PGE2 binding dissociation kinetics (Fig. 2, B and C). The noncompetitive nature of THG213.29 is corroborated by the inability of high concentrations of PGE2 to overcome the partial inhibitory effects of THG213.29 on PGE2-induced cAMP production (insurmountable antagonism) (Fig. 2, D and E); in contrast, competitive orthosteric antagonism by definition can be surmounted by increasing concentrations of the natural ligand (30). THG213.29 exerted specificity for the EP4 receptor, as it inhibited EP4-specific agonist L-902688-mediated piglet saphenous vein vasodilation (Fig. 2A, ii); 2) attenuated cAMP response in EP4, but not EP2 or V2R-expressing HEK293 cells (Fig. 2F); 3) modulated PGE2 binding dissociation kinetics to the EP4 receptor (Fig. 2C); and 4) modulated the L-902688-induced increased expression of various mRNAs in EP4-expressing cells only (Fig. 6B).

In the past several years, it has become increasingly clear that G protein-coupled receptors can activate independently a variety of signaling effectors and that the ability of a compound to modulate a given signaling pathway cannot be extrapolated to another signaling pathway triggered by the same receptor. The conceptual basis for this is that GPCRs do not have merely “inactive” and “active” conformations but that ligands can stabilize distinct receptor conformations, which are more or less potent and efficient in activating a given readout (21). This infers that a drug can affect some, but not all, functions evoked by a receptor, a concept referred to as functional selectivity (45). The difficulty of accommodating functional selectivity in screening campaigns has been pointed out (22); hence, a complex isolated-tissue physiological functional assay (piglet saphenous vein vasorelaxation) was initially used to determine the efficacy of peptides to avoid overseeing signaling responses based on reductionist in vitro recombinant systems. THG213.29 inhibited both PGE2 and L-902688-mediated vasorelaxation in this system. In an effort to decipher the effect of THG213.29 on EP4 activities, we investigated more specific signaling pathways using BRET-based assays in living HEK293 cells, EP4-mediated cAMP production was partially, but specifically, inhibited by THG213.29, while the Goxi activation and β-arrestin recruitment pathways activated by PGE2 were unaffected by THG213.29 (Fig. 2, F–H). The observed functional selectivity is likely made possible by THG213.29-induced stabilization of a unique receptor conformation that, in essence, creates a “modified” receptor conformation with distinct functional properties, characteristic of allosteric modulators. The modulation of specific receptor activities may confer greater selectivity and possibly reduce undesirable side effects, compared with orthosteric antagonists that block all (beneficial and pathological) responses of the receptor to stimuli (19).

A significant feature of this study relates to the in vivo effects of THG213.29 on renal integrity following insults that lead to ARF. THG213.29 attenuated renal dysfunction associated with two separate models of ARF. Using the EP4-selective agonist CP-044,519–02, Vukicevic and colleagues (49) have previously shown that EP4 agonism reduces serum creatinine levels, proximal tubular necrosis, and abundance of apoptotic cells in a nephrotoxic mercury chloride rat model of ARF; fenoldopam has also been shown to be tubulo-protective in nephrotoxic and ischemic ARF (29, 32), which may explain its significant improvement in urine output, BUN, and creatinine with marginal effects on GFR (Fig. 5). We have shown that THG213.29 is a partial negative modulator of EP4-mediated cAMP production and saphenous vein vasorelaxation but has no effect on EP4-mediated Goxi activation or β-arrestin recruitment; whether these cell signaling pathways operate in renal function is unclear. On the other hand, data at the transcriptional level are consistent with beneficial effects of THG213.29. In the renal cortex, THG213.29 induced expression of cytoprotective and anti-inflammatory genes (Fig. 6). In cultured cells specifically expressing EP4, THG213.29 correspondingly abrogated the expression of cytotoxic (proapoptotic) and proinflammatory genes, potentially regulated by cAMP and known to be involved in tissue degeneration following kidney injury (28, 43) (47, 48), and again increased expression of cytoprotective FGF-2 (Fig. 6). It has been shown that members of the extrinsic death receptor-dependent (DAXX) and the intrinsic mitochondrial (BAD) apoptosis pathways are rapidly induced following ischemic ARF in animal models and may be involved in tubule cell loss following ischemia-reperfusion injury (28, 43). Recovery from tubular damage requires regenerative mechanisms implicating the paracrine or autocrine action of growth factors, including FGF-2, which has been shown to induce a reexpression of
morphogenic proteins and accelerates the recovery process after renal damage in a model of ischemic ARF (47, 48). It has also been reported that increased IL-6 production by infiltrating macrophages exacerbates ischemic murine acute renal failure (23). Hence, the EP4-dependent modulation of inflammatory and cytotoxic factors by THG213.29 can explain the relative preservation of renal structural integrity observed after acute renal insults.

In addition, THG213.29 was found to augment renal hemodynamics (renal plasma flow) and GFR resulting in increased urine output. A dilation of the afferent arterioles with or without relaxation of the efferent arterioles would be required to induce these functional changes. Although, EP4 stimulation generally induces relaxation of vascular tissue [including saphenous vein (Fig. 2A)], in kidneys, EP4-induced renin release may counter these effects (41), but effects of renin are foremost only on efferent arterioles. In addition to the complex actions of EP4 stimulation on vasomotoricity, THG213.29 caused a modest decrease in cAMP, which cannot explain enhanced in vivo renal hemodynamics. However, THG213.29 triggered expression of the inducible heme-oxygenase-1 (9), a primary generator of the potent vasorelaxant carbon monoxide; in the renal cortex, this could explain augmented renal hemodynamics in vivo (Fig. 6A).

Other than the mechanisms described above, additional EP4 receptor activities not yet characterized may also be modulated (positively or negatively) by THG213.29 and may be implicated in the efficacy of the peptide. The EP4 receptor data in the kidney have a corollary in the control of patent ductus arteriosus (DA) (15). The use of an EP4 agonist maintains the DA patent even when dosed with indomethacin. The quandary in this case is that the EP4 receptor-null mice also show a patent DA. PGE2 has been shown to have both constrictor and dilatory functions acting through the EP4 receptor. PKA activation after EP4 stimulation can directly promote vasodilation by inhibiting myosin light chain kinase and maintains the DA patent during fetal development. PKA activation can also result in stimulation of hyaluronic acid formation, which forms a basis for extracellular matrix and DA closure. Signal pathway downstream of the EP4 receptor can clearly be divergent dependent on timing and the environment of the challenge, adding to the complexity of THG213.29 actions. Altogether, the complex actions of the biased-signaling modulator THG213.29 on EP4 likely provide it with its intricate properties.

In summary, we have documented the characterization of a small peptidomimetic, THG213.29, which is derived from a juxtamembranous region of the prostaglandin EP4 receptor and is effective in models of ischemia- and nephrotoxic-induced ARF. THG213.29 exhibited properties consistent with those of a noncompetitive, functionally selective allosteric modulator. The use of therapeutic peptides [particularly D-peptides (as is the case herein) and/or peptidomimetics] has become increasingly attractive in recent years, mainly due to improvements in half-life and bioavailability (37), which may be of lesser concern in seriously ill (hospital-bound) patients with ARF; in addition, peptides can be used as scaffolds for subsequent conversion into small molecules. Moreover, peptides that tar-
get regions of the receptor remote from the orthosteric natural ligand-binding site might exhibit noncompetitive allosteric properties and modulate only a subset of the receptor-mediated activities, allowing more specific pharmacological intervention targeting some, but not all, receptor signaling pathways (37). Hence, THG213.29, a novel class allosteric modulator of the EP<sub>4</sub> receptor effective in increasing renal function and diuresis in different species, may in the future yield promise in the clinical setting.

**Perspectives and Significance**

Drug treatments for ARF have so far not been successful. Historically, drug discovery efforts have been optimized for the discovery of standard orthosteric agonists and antagonists. However, one drawback often encountered with orthosteric ligands is undesirable side effects. Allosteric ligands, which bind to a receptor region remote from the natural ligand orthosteric site, have the potential to provide more versatility in receptor signaling modulation through functional selectivity and separate control of efficacy and affinity. We have exploited these properties with the characterization of a small peptido-mimetic, THG213.29, which is derived from a juxtamembrane region of the prostaglandin EP<sub>4</sub> receptor. The peptide exhibited pharmacological properties consistent with those of a noncompetitive, functionally selective allosteric modulator, and most relevantly improved renal function in two different models of ARF. THG213.29 is a novel class of allosteric modulator of the EP<sub>4</sub> receptor with diuretic properties, which may have potential as therapy for acute kidney injury.

**ACKNOWLEDGMENTS**

The authors thank Hendrika Fernandez and Mélissa Paumet for excellent technical assistance.

**GRANTS**


**DISCLOSURES**

K.G. Peri is an employee of Theratechnologies, Incorporated; S. Chemtob has received funding from Theratechnologies to complete part of the research described within this article.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


