G-Protein $\beta\gamma$ Subunit Dimers Modulate Kidney Repair after Ischemia-Reperfusion Injury in Rats[§]

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ABSTRACT

Heterotrimeric G-proteins play a crucial role in the control of renal epithelial cell function during homeostasis and in response to injury. In this report, G-protein $\beta\gamma$ subunit ($G\beta\gamma$) dimer activity was evaluated during the process of tubular repair after renal ischemia-reperfusion injury (IRI) in male Sprague Dawley rats. Rats were treated with a small molecule inhibitor of $G\beta\gamma$ activity, gallein (30 or 100 mg/kg), 1 hour after reperfusion and every 24 hours for 3 additional days. After IRI, renal dysfunction was prolonged after the high-dose gallein treatment in comparison with vehicle treatment during the 7-day recovery period. Renal tubular repair in the outer medulla 7 days after IRI was significantly ($P < 0.001$) attenuated after treatment with high-dose gallein

(100 mg/kg) in comparison with low-dose gallein (30 mg/kg), or the vehicle and fluorescein control groups. Gallein treatment significantly reduced ($P < 0.05$) the number of proliferating cell nuclear antigen-positive tubular epithelial cells at 24 hours after the ischemia-reperfusion phase in vivo. In vitro application of gallein on normal rat kidney (NRK-52E) proximal tubule cells significantly reduced ($P < 0.05$) S-phase cell cycle entry compared with vehicle-treated cells as determined by 5'-bromo-2'-deoxyuridine incorporation. Taken together, these data suggest that $G\beta\gamma$ signaling contributes to the maintenance and repair of renal tubular epithelium and may be a novel therapeutic target for the development of drugs to treat acute kidney injury.

Introduction

Acute kidney injury (AKI) leads to adverse outcomes in hospitalized patients including prolonged length of stay, higher health care costs, and increased mortality (Chertow et al., 2005; Hsu et al., 2013). In addition to these short-term complications, recovery of renal function after AKI is often impaired resulting in chronic kidney disease or end-stage renal disease (Coca et al., 2009; Triverio et al., 2009). Repair of injured renal tubular epithelium is a critical step required for recovery of renal function after AKI. Activation of repair pathways in renal tubules is

promoted by intrinsic and extrinsic factors exerted upon the surviving, sublethally injured epithelial cells (Humphreys et al., 2008, 2011) or through the actions of stem cells residing within or honed to the kidney (Chen et al., 2008).

Cell surface G-protein coupled receptor (GPCR)-mediated signal transduction pathways may play a critical role in regulating the epithelial cell response during injury and repair (Kunzendorf et al., 2010; Bonventre and Yang, 2011). Heterotrimeric G proteins are composed of α , β , and γ subunits, and upon GPCR stimulation the heterotrimeric G protein α subunits ($G\alpha$) facilitate the exchange of GDP for GTP, leading to the dissociation of the heterotrimer into a GTP-bound $G\alpha$ and free (unbound) $G\beta\gamma$ (Gilman, 1987). Both the active GTP-bound $G\alpha$ and dissociated $G\beta\gamma$ can subsequently function as independent, active signaling modules that regulate various downstream effectors (Gilman, 1987). After the $G\alpha$ subunit hydrolyzes the GTP to GDP, a conformation change occurs in which the $G\alpha$ subunit reassociates with the unbound $G\beta\gamma$ subunits to return to an inactive state. Prior studies have demonstrated activation of $G\alpha$ in models of AKI. For example, pharmacologic agonists of the adenosine A2A GPCR (Yap and Lee,

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ABBREVIATIONS: AGS, activator of G-protein signaling; AKI, acute kidney injury; BrdU, bromodeoxyuridine; COMT, catechol-O-methyltransferase; DAPI, 4',6'-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle medium; ERK1/2, extracellular signal-regulated kinase 1/2; $G\beta\gamma$, G-protein $\beta\gamma$ subunit; GPCR, G-protein coupled receptor; GPR, G-protein regulatory; GPSM1, G-protein signaling modulator 1; GRK2ct, G-protein coupled receptor kinase 2 carboxyl terminus; HRP, horseradish peroxidase; IRI, ischemia-reperfusion injury; M119, 2-(3,4,5-trihydroxy-6-oxoxanthene-9-yl) cyclohexane-1-carboxylic acid; MAPK, mitogen-activated protein kinase; NRK-52E, normal rat kidney epithelial cells; PCNA, proliferating cell nuclear antigen; RGS, regulator of G-protein signaling.

2012) or genetic modulation of $G\alpha_{12}$ subunit function (Yu et al., 2012) have been shown to alter the severity of AKI in animal models. However, these studies primarily focused on signaling via $G\alpha$ subunits through biased signaling cascades activated by upstream GPCRs.

More recently, the diversity of G-protein regulation has been highlighted by the discovery of accessory proteins in the regulator of G-protein signaling (RGS) and activator of G-protein signaling (AGS) families, which can selectively control either $G\alpha$ - or $G\beta\gamma$ subunit activation (Cismowski et al., 1999; Takesono et al., 1999; Blumer et al., 2005; Siderovski and Willard, 2005; Sato et al., 2006) independent of GPCR stimulation (Blumer et al., 2005). Accessory proteins regulate heterotrimeric G-protein function through a number of distinct mechanisms that include the facilitation of guanine nucleotide transfer by the α -subunit, selective binding to $G\alpha$ or $G\beta\gamma$ subunits, or accelerating the catalysis of guanine triphosphate nucleotides by the α -subunit (Blumer et al., 2005).

G-protein signaling modulator 1 (GPSM1)/AGS3 is an AGS protein that functions as a guanine dissociation inhibitor with multiple G-protein regulatory (GPR) motifs that bind selectively to $G\alpha_i$ (Bernard et al., 2001). However, GPSM1/AGS3 exerts its biologic effects by modulating the activity of unbound $G\beta\gamma$ dimers (Sanada and Tsai, 2005; Bowers et al., 2008; Nadella et al., 2010; Kwon et al., 2012). We have previously shown that GPSM1/AGS3 modulates renal tubular repair through a $G\beta\gamma$ -dependent mechanism in rodent models of renal ischemia-reperfusion injury (IRI) (Regner et al., 2011) and polycystic kidney disease (Nadella et al., 2010; Kwon et al., 2012). More specifically, unilateral IRI in mice with a deficiency in full-length expression of GPSM1/AGS3 exhibited a decreased number of proliferating outer medullary proximal tubular cells and an increased number of persistently injured renal tubules compared with wild-type mice (Regner et al., 2011). Other accessory proteins have also been shown to regulate effectors through $G\beta\gamma$ function (Takesono et al., 2002), including mitogen-activated protein kinase (MAPK) and phospholipase C signaling, ion channel activity, and mitotic spindle orientation (Sanada and Tsai, 2005; Lin and Smrcka, 2011; Kwon et al., 2012). Although there is a lack of pharmacologic inhibitors targeting AGS proteins, $G\beta\gamma$ dimer function can be manipulated in vivo through the use of small molecule inhibitors. For example, administration of gallein and M119 [2-(3,4,5-trihydroxy-6-oxoxanthen-9-yl)cyclohexane-1-carboxylic acid], the small molecule inhibitors of $G\beta\gamma$, has been shown to improve cardiac function and slow disease progression in mouse models of heart failure (Casey et al., 2010; Kamal et al., 2014). In contrast to the heart, the role of $G\beta\gamma$ signaling in kidney injury remains poorly characterized.

Therefore, our study evaluated the effect of gallein, a selective inhibitor of $G\beta\gamma$ dimer function during renal tubular repair after experimental AKI.

Materials and Methods

Chemicals and Antibodies. Gallein was purchased from Spectrum Chemicals (Philadelphia, PA). Fluorescein sodium, bromodeoxyuridine and monoclonal β -actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal BrdU antibody conjugated with Alexa Fluor 488 and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Life Technologies (Carlsbad, CA). Primary antibodies to phospho and total extracellular signal-regulated kinase 1/2 (ERK1/2),

phospho and total p38 MAPK, proliferating cell nuclear antigen (PCNA), and secondary goat anti-rabbit and horse anti-mouse IgG conjugated to horseradish peroxidase (HRP) were purchased from Cell Signaling Technology (Danvers, MA).

Animals and Surgical Procedures. Male Sprague Dawley rats (250–300 g) were obtained from Taconic Farms (Oxnard, CA). All rats were allowed ad libitum access to food and water during the course of this experiment. Rats underwent 30-minute bilateral renal ischemia (or sham) surgeries as previously described elsewhere (Regner et al., 2011). In brief, the rats were anesthetized with ketamine (50 mg/kg, i.m.) and sodium pentobarbital (50 mg/kg, i.p.) and placed on a heated surgical table to maintain body temperature at 37°C. A midline abdominal incision exposed the kidneys to isolate the renal arteries and veins, at which point microvascular clamps were placed across the renal pedicles to arrest blood flow for 30 minutes. Time-control sham surgeries were performed in which the renal pedicles were not clamped. Subsequently, the clamps were removed to allow reperfusion of the kidneys, which was verified by observation of the restoration of color to the kidney, and then the abdominal incision was closed. Approximately 1 hour after the reperfusion of the kidneys, vehicle (phosphate-buffered saline), fluorescein (100 mg/kg), or gallein (30 or 100 mg/kg) was administered into the intraperitoneal space. Subsequent injections with vehicle, fluorescein, or gallein were administered every 24 hours for 3 consecutive days. Gallein has been shown to act as a small molecule inhibitor of $G\beta\gamma$ function (Lehmann et al., 2008) whereas fluorescein is a closely related analog of gallein that does not inhibit $G\beta\gamma$ activity due to the absence of hydroxyl groups at positions 4 and 5 of the tricyclic ring structure (Lehmann et al., 2008). Rats were allowed to recover for 1, 3, or 7 days after reperfusion, at which point the rats were euthanized for organ collection. All protocols used in this study were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Biologic Measurements and Tissue Morphometry. During the course of the experimental period, blood was serially collected from some of the rats by submandibular bleeding or intracardiac puncture on the day of sacrifice. After the surgical procedures, the rats were observed on a daily basis to determine whether gallein administration would adversely impact the health of the rats. On each day of sacrifice, rats were weighed and blood was collected to measure plasma creatinine by liquid chromatography with tandem mass spectrometry (UAB Biochemical Core, Birmingham, AL). Upon sacrifice, both kidneys were harvested and cut into two pieces; one-half was used for immunoblot analysis and the other half was fixed in 10% neutral buffered formalin and paraffin embedded. Kidney sections were imaged using a Hamamatsu NanoZoomer HT (Hamamatsu Photonics KK, Hamamatsu City, Japan) digital scanner.

PCNA Immunohistochemistry. Kidneys from sham and IRI operated rats were formalin fixed, paraffin embedded, and sectioned (4 μ m) for immunostaining with PCNA as previously described by Kwon et al. (2012). In brief, antigen retrieval was performed by heating (90°C) in antigen-retrieval solution (IHC World, Woodstock, MD); the slides were blocked then incubated with a primary antibody against proliferating cell nuclear antigen (PCNA; 1:1000 dilution). The appropriate secondary antibody conjugated with HRP was used to visualize the binding by detecting the *N,N*-dimethyl-4-aminoazobenzene precipitation on the sections. Between each of the steps, the slides were rinsed multiple times in Tris-buffered saline. All kidney sections were subsequently counterstained with hematoxylin, cover-slipped, and digitized using a Hamamatsu NanoZoomer HT digital scanner. The number of PCNA-positive renal tubular epithelial cells in the outer stripe of the outer medulla were counted in five random fields at a magnification of 20 $\times$. A total of 750 tubular epithelial cells were counted for each rat kidney, and a percentage of PCNA-to-total epithelial cells was calculated.

Histopathologic Analysis of Renal Injury. Kidneys from sham-operated and IRI-treated rats were formalin fixed, paraffin embedded, sectioned (4 μ m), and stained with H&E. Kidney sections were imaged using a Hamamatsu NanoZoomer HT (Hamamatsu Photonics KK) digital scanner. At least five random areas from the

cortex and five random areas of the outer stripe of the outer medulla were visualized, imaged, and counted for each kidney. Tubular injury was determined by counting the proportion of injured-to-total tubules using cross-sectional images of the kidney at a magnification of 20 $\times$ (Regner et al., 2011). Tubular injury was defined as the presence of tubular casts, loss of brush border, flattened epithelium, and/or sloughing of tubular epithelial cells.

BrdU Incorporation Assay. NRK-52E cells were plated onto poly-L-lysine coverslips and subjected to low serum (1% fetal bovine serum in Dulbecco's modified Eagle medium [DMEM]) for 24 hours. Subsequently, the cells were incubated with DMEM/10% fetal bovine serum for 10 hours, followed by 4 hours of incubation with 10 μ M BrdU. At this point, the medium was replaced in the absence of BrdU, and the cells were collected 12 hours later. At the end of the experimental period, the cells were fixed in 4% paraformaldehyde, treated with 2 M HCl, and permeabilized with 0.2% Triton X-100. Immunostaining was performed using a mouse monoclonal anti-BrdU antibody conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA), and counter-stained with DAPI (Invitrogen) to mark the DNA in the nuclei. Sixteen-bit monochrome pictures were imaged for green fluorescence (GFP light cube) and blue fluorescence (DAPI light cube) under uniform exposure and brightness setting to ensure standardization across all cell culture groups using an EVOS fluorescent microscope (Advanced Microscope Group, Bothell, WA). The images were analyzed to manually count all blue (total nuclei) and green (BrdU positive). A total of 200 DAPI-stained nuclei were counted per group. Experiments were performed on three different occasions in duplicate.

Immunoblot Analysis. Kidney tissue was homogenized in 1X radioimmunoprecipitation assay buffer (Millipore, Billerica, MA) with protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL), and protein lysates were isolated after differential centrifugation. Immunoblot analysis was performed as previous published (Akbulut et al., 2009; Nadella et al., 2010; Regner et al., 2011; Kwon et al., 2012). In brief, protein lysates (25 μ g) were size separated on a 4–12% NuPAGE Bis-Tris Gradient Gel (Life Technologies) and were subsequently transferred to a supported polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in Odyssey Blocking Buffer (Licor, Lincoln, NE) and blotted with the following primary antibodies overnight at 4°C; all antibodies were used at a dilution of 1:1000–1:2000. We used β -actin as a loading control at a dilution of 1:8000. After subsequent washing steps, membranes were incubated with the secondary anti-rabbit or anti-mouse IgG conjugated with HRP at room temperature for 1 hour. The bands were detected on film after incubation in chemiluminescent solution (GE Healthcare, Piscataway, NJ). All films were scanned, and the band densities were calculated using the National Institutes of Health ImageJ software (<http://imagej.nih.gov/ij/>).

Scratch Wound Assay. The scratch wound experiment was performed on normal rat kidney (NRK-52E) renal epithelial cells using a protocol modified from Torres et al. (2011). The NRK-52E cells were cultured to 80 to 90% confluence. Gallein (2 μ M) or vehicle solution was applied to the medium before the experimental period in which artificial wounds were created by scratching the monolayer with a 10- μ l pipette tip. At this point, the medium was replaced with DMEM containing 10% calf serum, and the cells were incubated at 37°C in a 5% CO₂ incubator for up to 18 hours. At the end of the experimental period, the NRK cells were fixed in 5% formaldehyde and stained with Coomassie blue, and images were captured using phase-contrast microscopy. Open area was calculated using Nikon Elements software (Nikon Instruments, Melville, NY).

Cell Counting Analysis. NRK-52E (Regner et al., 2011) and primary immortalized collecting duct epithelial cells (Nadella et al., 2010) were transduced with lentiviral vectors expressing the carboxy terminus from the G-protein coupled receptor kinase 2 as previously described in our laboratory (Nadella et al., 2010; Regner et al., 2011). NRK cells were plated into six-well dishes and allowed to expand for 48 hours, at which point the cells were harvested for counting by hemocytometry (Regner et al., 2011). Collected duct cells were plated

into 96-well plates and counted after 24 hours using a CyQuant cell proliferation assay (Life Technologies) (Nadella et al., 2010).

Statistical Analysis. Data are expressed as mean $\pm$ S.E.M. All statistical analyses were performed using Prism 5.0 software (SAS Institute Inc., Cary, NC). Unpaired *t* tests were performed on the in vitro experiments. One-way analysis of variance was performed on the in vivo biologic measurements where appropriate, and Newman-Keuls post-hoc test was performed when *P* < 0.05 was calculated for significance.

Results

Effect of Gallein on Kidney Function after IRI. Prior studies have demonstrated that renal tubular repair after experimental IRI involves dedifferentiation, migration, and proliferation of tubular epithelial cells within 2 to 5 days of injury (Witzgall et al., 1994; Humphreys et al., 2008, 2011). To determine the role of G β γ in tubular repair, we treated rats with low-dose (30 mg/kg i.p.) or high-dose (100 mg/kg i.p.) gallein, fluorescein (100 mg/kg i.p., a gallein analog with no G β γ inhibitory effects), or vehicle solution 1 hour after reperfusion, and subsequently every 24 hours for 3 consecutive days. In sham-operated rats, administration of high-dose gallein did not significantly alter renal function as assessed by plasma creatinine in comparison with vehicle-treated rats at each time point during the experimental period (Fig. 1A). At day 7, the plasma creatinine in the low- and high-dose gallein sham-operated groups did not significantly differ from the time-control vehicle- and fluorescein-treated rats (Fig. 1B). In sham-operated rats, there were significant changes in body weight (*P* < 0.01), urine output (*P* < 0.01), water intake (*P* < 0.05), and urine osmolality (*P* < 0.0001) after high-dose gallein treatment compared with vehicle, fluorescein, or low-dose gallein (Table 1).

After IRI, high-dose gallein treatment did not significantly increase the severity of renal dysfunction (Fig. 1) or tubular injury (Fig. 2, A and B) in comparison with vehicle treatment at day 1. However, high-dose gallein delayed the recovery of the kidney function compared with vehicle as indicated by significantly elevated plasma creatinine levels 3 days (Fig. 1A; *P* < 0.05) and 7 days (Fig. 1, A and B; *P* < 0.01) after IRI. Similar to the sham groups, the effect of gallein on renal function as measured by plasma creatinine (Fig. 1B; *P* < 0.01) and creatinine clearance (Supplemental Fig. 1; *P* < 0.001) was significantly impaired only at the high dose of gallein at day 7 compared with the other control (vehicle and fluorescein) and low-dose gallein groups. Water intake did not significantly differ between groups at day 7 after IRI, but urine osmolality was significantly decreased (*P* < 0.001) in the rats treated with high-dose gallein in comparison with the rats treated with vehicle, fluorescein, or low-dose gallein (Table 1).

Effect of Gallein on Renal Tubular Injury after IRI. In sham-operated rats, the extent of tubular injury in the renal cortex and outer medulla (Figs. 2 and 3) over the 7-day period was not significantly different in the rats treated with vehicle, fluorescein, or gallein. However, the renal tubules in the kidneys of rats treated with high-dose gallein appeared more dilated compared with vehicle- and fluorescein-treated kidneys (Fig. 3). At day 7 of the experimental period, we observed reduced recovery by the renal tubules in the rat treated with high-dose gallein after IRI. High-dose gallein significantly increased the proportion of injured tubules in

Fig. 1. Effect of $G\beta\gamma$ inhibition on renal function in sham and IRI-treated rats. (A) Plasma creatinine was measured by liquid chromatography with tandem mass spectrometry using blood collected from rats treated with high-dose gallein or vehicle at days 1, 3, and 7 after IRI or sham surgery. IRI rats treated with high-dose gallein (●) or vehicle (○); sham-operated rats treated with high-dose gallein (▼) or vehicle (▽); $n = 5-11$ rats/group. * $P < 0.05$, IRI rats treated with high-dose gallein versus vehicle at day 3; † $P < 0.01$, IRI rats treated with high-dose gallein versus vehicle at day 7. ‡ $P < 0.01$, IRI versus sham-treated rats at days 1 and 3. (B) Plasma creatinine at day 7 in sham and IRI-treated rats administered vehicle, fluorescein, or gallein. ¶ $P < 0.01$ difference between gallein-treated IRI rats (100 mg/kg i.p.) versus all other sham and IRI-treated groups.

both the cortex (Fig. 2A) and outer stripe of the outer medulla (Fig. 2, B and C) compared with the other treatment groups ($P < 0.001$). In addition, increased expression of neutrophil

gelatinase-associated lipocalin, a marker of AKI, was detected by Western blot analysis at day 7 in the kidneys treated with high-dose gallein, but no bands were detected in vehicle- or fluorescein-treated sham or IRI rat kidney lysates (Supplemental Fig. 2).

Effect of Gallein on Renal Epithelial Cell Proliferation in Rat Kidneys after IRI. We previously reported that genetic or pharmacologic inhibition of $G\beta\gamma$ decreased renal tubular epithelial cell number in vitro (Regner et al., 2011). Consistent with those findings, we found that the cell numbers were significantly ($P \leq 0.02$) reduced in two different renal epithelial cell lines overexpressing the carboxy terminus of the G-protein coupled receptor kinase (GRK2ct) compared with control cells (Supplemental Fig. 3). The GRK2ct sequesters free $G\beta\gamma$ subunits to block its function (Koch et al., 1994). Because of these confirmatory results, we speculated that the impairment of renal repair observed after gallein treatment was secondary to the inhibition of tubular cell proliferation. To identify cells in the late G_1 and early S-phase of the cell cycle, we performed immunostaining for PCNA, a cell cycle marker for the late G_1 and early S-phase (Witzgall et al., 1994) in the kidney sections from the rats treated with gallein and vehicle after IRI or sham surgery. In the rats treated with high-dose gallein, the proportion of PCNA-positive tubular epithelial cells was significantly decreased in the outer stripe of the outer medulla ($19.1 \pm 4.5\%$; $n = 6$) 1 day after IRI compared with vehicle ($39.0 \pm 4.2\%$; $n = 5$; $P < 0.05$) (Fig. 4). At days 3 and 7, the percentage of PCNA-positive epithelial cells did not differ between the IRI rats treated with high-dose gallein or vehicle. Similarly, there was no difference in the number of PCNA-positive nuclei in rat kidneys treated with fluorescein ($14.0 \pm 3.7\%$; $n = 4$) at day 7, compared with the vehicle ($15.2 \pm 4.9\%$; $n = 5$) or high-dose gallein ($18.2 \pm 2.7\%$; $n = 6$) groups.

In the sham-operated rats, the proportion of PCNA-positive outer medullary tubular epithelial cells in the kidneys treated with vehicle or high-dose gallein did not significantly differ, which is consistent with the relatively low mitotic activity of renal epithelial cells from mature rodent kidneys (data not shown).

We performed additional in vitro experiments to determine whether the decrease in tubular cell PCNA expression was due to an effect of gallein on cell cycle progression. BrdU is

TABLE 1

Body weight, water intake, and urine output 7 days after sham or IRI surgery

Physiologic parameters in sham and IRI-treated rats 7 days after renal IRI. Urine osmolality, urine output, water intake, and body weight were measured at day 7 after using rats housed in individual metabolic cages for 24 hours to collect urine.

	Sham				IRI			
	Vehicle	Fluorescein	Gallein		Vehicle	Fluorescein	Gallein	
			30	100			30	100
mg/kg				mg/kg				
Body weight (g)	236 ± 13	275 ± 8	294 ± 7	212 ± 8 ^a	290 ± 7	285 ± 7	256 ± 7	188 ± 23 ^b
Water intake (ml)	32.4 ± 1.0	38.5 ± 1.9	37.2 ± 2	56.7 ± 4.0 ^c	46.3 ± 1.8	59.3 ± 5.8	43.1 ± 7.1	40.5 ± 3.8
Urine output (ml)	11.6 ± 0.8	14.5 ± 1.8	16.3 ± 1	32.6 ± 3.7 ^a	16.8 ± 1.5	25.8 ± 3.9	25.8 ± 3.5	24.8 ± 5.2
Urine osmolality (mOsm/kg H ₂ O)	1814 ± 89	1559 ± 184	1532 ± 102	689 ± 52 ^d	1450 ± 28	1005 ± 99 ^e	998 ± 86 ^e	765 ± 102 ^f

^a $P < 0.01$, significant difference between high-dose gallein versus other sham-operated groups.

^b $P < 0.001$, significant difference between high-dose gallein versus other IRI-treated groups.

^c $P < 0.05$, significant difference between high-dose gallein versus other sham-operated groups.

^d $P < 0.0001$, significant difference between high-dose gallein versus other sham-operated groups.

^e $P < 0.01$, significant difference compared with vehicle-treated IRI rats.

^f $P < 0.001$, significant difference between IRI rats treated with high-dose gallein versus vehicle.

Fig. 2. Quantitative analysis of renal tubular injury in the renal cortex and outer medulla after IRI. Kidneys were analyzed to determine the percentage of injured cortical (A) and outer medullary (B) tubules during the 7-day experimental period. IRI rats treated with high-dose gallein (●) or vehicle (○); sham-operated rats treated with high-dose gallein (▼) or vehicle (▽); $n = 3-7$ kidneys/group. *** $P < 0.001$, IRI rats treated with high-dose gallein versus vehicle at day 7. Fluorescein (100 mg/kg i.p.) was administered over a 3-day period after IRI as an additional control for high-dose gallein. (C) The data for the outer medulla in (B) was regraphed to compare the effects of vehicle (white), low-dose gallein (hatched), and high-dose (black) gallein, and fluorescein (gray) as shown in (C) for kidney sections at day 7. Number of kidneys per group analyzed at day 7 is shown in the graph. * $P < 0.01$, significant difference between IRI-treated rats compared with sham-operated rats; *** $P < 0.001$, significant difference between rat groups treated with high-dose gallein versus all other sham and IRI groups.

a standard halogenated nucleoside used to assess the progression through the S-phase of the cell cycle (Gratzner, 1982). In cultured normal rat kidney (NRK-52E) cells, incorporation of BrdU was significantly decreased ($P < 0.05$) by approximately 50% in cells incubated with gallein ($0.5 \mu\text{M}$; 76.7 ± 11.9 BrdU-positive cells) in comparison with vehicle (145.0 ± 11.7 BrdU-positive cells)

Fig. 3. Histology of sham and IRI rat kidneys in the presence and absence of $G\beta\gamma$ inhibition. Representative H&E-stained kidney sections from the outer stripe of the outer medulla at day 7 after IRI or sham operation. Three different rat kidneys are shown from sham (J–R) and IRI rats (A–I) treated with vehicle (A–C, J–L), fluorescein (D–F, M–O), or high-dose gallein (G–I, P–R). Original magnification $20\times$.

(Fig. 5). To determine whether $G\beta\gamma$ modulates other tubular repair mechanisms, we assessed cellular migration using a scratch wound assay. Incubation of NRK cells with gallein ($2 \mu\text{M}$; $n = 3$) did not significantly differ in the rate of wound closure in comparison with vehicle-treated cells ($n = 5$; Fig. 6). Taken together, these findings suggest that inhibition of $G\beta\gamma$ function with gallein inhibits tubular repair by modulating tubular epithelial cell proliferation rather than migration.

Discussion

Renal tubular regeneration after experimental IRI involves dedifferentiation, migration, and proliferation of tubular epithelial cells within 2 to 5 days of injury (Witzgall et al., 1994; Humphreys et al., 2008, 2011). However, the intracellular mechanisms that regulate this response remain poorly understood. With our present study, we provide evidence that pharmacologic inhibition of $G\beta\gamma$ prolongs renal injury and impairs recovery of kidney function after renal IRI in rats. These findings provide further evidence to suggest that G-proteins are involved in renal tubular repair.

Prior studies have evaluated the effects of selective GPCR- $G\alpha$ subunit activation by specific ligands to control signal processing and subsequent regulation of renal epithelial cell recovery (Johnson et al., 2006; Bajwa et al., 2010; Yap and

Fig. 4. *Gβγ* inhibition delays renal epithelial cell proliferation in rat kidneys after IRI. Kidney sections from each rat group were immunostained for PCNA 1 day (A, D, G, J), 3 days (B, E, H, K), and 7 days (C and F) after IRI or sham procedures. Brown-stained nuclei in each panel indicate PCNA-positive cells. Representative photomicrographs of negative controls in vehicle and high-dose gallein-treated sham-operated rats at 7 days are presented in (I) and (L), respectively. (M) The number of total and PCNA-positive renal tubular epithelial cells was determined in IRI-treated rats with vehicle (black) or high-dose gallein (white). The mean percentage of PCNA-positive nuclei in each group is presented. The number of animals in each group is shown in the bars. **P* < 0.05, IRI rats treated with high-dose gallein versus vehicle.

Lee, 2012; Leduc et al., 2013). More recently, the importance of *Gα* subunits in the recovery of the kidney after ischemic injury was documented in a study by Yu et al. (2012). In their report, constitutively active *QLα12* mutant transgenic mice were observed to have attenuated recovery after renal IRI with more severe tubular injury compared with the wild-type *Gα12* mice. Conversely, the renal tubules in *Gα12* knockout mice were protected after IRI. Mechanistically, *Gα12* subunits were found to disrupt epithelial cell tight junctions in the presence of hydrogen peroxide in vitro. These studies demonstrate that the *Gα* subunits from heterotrimeric G-proteins play a crucial role in renal epithelial cell function during normal and pathologic situations.

Gβγ is the natural binding partner to *Gα* subunits and is known as the canonical guanine dissociation inhibitor. *Gβγ*

Fig. 5. *Gβγ* inhibition decreases S-phase progression in renal epithelial cells in vitro. (A–H) Representative images of normal rat kidney (NRK-52E) epithelial cells incubated with vehicle (A–D) or gallein (0.5 μ M; E–H). Nuclei were labeled with DAPI (B, D, F, H). White boxes in (A), (B), (E), and (F) indicate the corresponding region magnified in (C), (D), (G), and (H), respectively. Green fluorescence within nuclei indicates BrdU incorporation (white arrows; C, D, G, H). Red arrows in (C), (D), (G), and (H) indicate nuclei without BrdU incorporation. Scale bar in A = 200 μ m; scale bar in C = 50 μ m. (I) Number of BrdU-positive nuclei in vehicle- and gallein-treated cells. **P* < 0.05 versus vehicle. Data from three independent experiments are depicted.

subunits have been shown to have diverse biologic effects due, in large part, to the potential multitude of interactions that can exist between the 5 β and 12 γ subtypes identified in the human genome (Downes and Gautam, 1999; Hurowitz et al., 2000; Lander et al., 2001; Venter et al., 2001). There is evidence to suggest that there are preferred *Gβ* and γ subtype interactions that dictate the biologic function of *Gβγ* within specific cells (McIntire, 2009). Localization studies have demonstrated *Gβγ* expression throughout the kidney (Brunskill et al., 1991; Yanagisawa et al., 1994). However, it remains a major challenge to determine the specificity of *Gβγ* subunit associations in the kidney because of the numerous cell types that express various isoforms of *Gβ* and γ subunits.

At the present time, selective blockade of specific *Gβγ* subunits in vivo is not readily achievable without performing complex genetic manipulation studies. However, global blockade of *Gβγ* function was performed in earlier studies using rodents by overexpression of the G-protein coupled receptor kinase

Fig. 6. Effect of G $\beta\gamma$ inhibition on renal epithelial cell migration. (A) Representative light micrographs of NRK cells treated with vehicle or gallein (2 μ M) at 0, 6, 12, and 16 hours after the scratch wound of the confluent cells. (B) No significant difference in the closure of the scratch wound after the initial scratch regardless of the experimental conditions. $n = 3$ –6 different experiments for each time point/group.

(GRK2ct) carboxy terminus, and these studies demonstrated that G $\beta\gamma$ dimers can play a central role in modulating drug addiction (Daaka, 2004; Yao et al., 2002, 2003), pain (Xie et al., 1999), and cardiovascular disease (Akhter et al., 1997; Eckhart et al., 2002; Iaccarino and Koch, 2003). Subsequently, small molecule inhibitors of G $\beta\gamma$ have been identified and have demonstrated similar effects (Mathews et al., 2008; Casey et al., 2010).

In our present study, we used gallein, a small molecule antagonist of G $\beta\gamma$ activity in the M119 class, which has been shown to bind to G $\beta_1\gamma_2$, as determined by surface plasmon resonance (Lehmann et al., 2008). In addition, gallein can interfere with downstream signaling targets that modulate biologic processes known to be involved in tubular repair, including cell proliferation and migration (Lehmann et al., 2008; Regner et al., 2011). Specifically, the G $\beta\gamma$ inhibitors gallein and M119 were shown to block fMet-Leu-Phe (fMLP) activation of superoxide production and inhibit chemotaxis in HL60 cells (Lehmann et al., 2008). The binding of gallein to G $\beta\gamma$ subunits is dependent on the presence of hydroxyl groups at carbons 4 and 5 of the tricyclic ring structure because fluorescein and M119B, which are deficient of these hydroxyl groups, cannot bind to G $\beta_1\gamma_2$ by surface plasmon resonance or block superoxide production (Lehmann et al., 2008).

Previous studies have shown that gallein is relatively safe for mice and rats with no reports of nephrotoxicity (Lehmann et al., 2008; Casey et al., 2010). Similarly, there was no apparent nephrotoxicity associated with multiple low and high doses of gallein in our study, but there was increased urine output and decreased urine osmolality observed with

the higher dose of gallein. These data suggest that G $\beta\gamma$ dimer activity may be involved in the regulation of water and electrolyte balance, but this mechanism of action is poorly understood. Our results cannot discern whether the changes in the fluid and electrolyte balance mediated by G $\beta\gamma$ inhibition were through mechanisms that were directly controlling renal epithelial cell function, or indirectly through hormonal release from the central nervous system. Because of these possibilities, we speculate that G $\beta\gamma$ inhibition has the potential either to block vasopressin-mediated water transport in the collecting ducts of the kidneys or to inhibit the release of vasopressin into the systemic circulation at the level of the hypothalamic-pituitary axis, but further studies are needed to elucidate the mechanism of action.

However, it is unlikely that disruptions in fluid and electrolyte balance were a key contributor to the exacerbated epithelial cell damage after IRI in the presence of high-dose gallein because the urine tonicity and urine output were similar to the values obtained in the fluorescein-treated IRI group. The dosage of gallein needed to exert changes in biologic function in the kidney may be higher than for other organs for a number of reasons. First, the metabolism of gallein may be higher in the kidney, leading to lower intracellular concentrations available for biologic activity. Gallein has a polycyclic catechol structure, which may be a prime target for chemical modification by enzymes such as catechol-*O*-methyltransferase (COMT) (Zhang et al., 2011). COMT is known to be highly expressed in the kidney, including the S3 segment of the proximal tubule (Meister et al., 1993), and the activity level of COMT is 5- to 8-fold higher in the kidney compared with the heart or brain (Myohanen et al., 2010). Second, the diversity of G $\beta\gamma$ subunit interactions as described earlier in this section may vary in the kidney compared with other cells and/or organs (Fong et al., 1987; Ruiz-Velasco et al., 2002), which could affect the activation status of G $\beta\gamma$ -dependent mechanisms (Yan et al., 1996; McIntire, 2009) and require increased amounts of gallein to regulate cellular function. Regardless, our study confirms that gallein can be safely used at lower doses, similar to the other published studies, but that some caution needs to be considered at higher doses, particularly in cases of renal injury, to avoid any additional adverse effects. At this time, we cannot definitively exclude that off-target effects may have occurred at the high-dose range of gallein, and that further absorption, distribution, metabolism, and excretion studies focusing on gallein metabolism and renal excretion in the context of renal injury are warranted.

The results of our study indicate that G $\beta\gamma$ may act either alone or in concert with G α subunit activity to control signaling mechanisms involving epithelial cell proliferation during the repair process after injury. Previous studies by our group have shown that genetic overexpression of GRK2ct or pharmacologic inhibition of G $\beta\gamma$ using low concentrations of gallein decreased renal tubular epithelial cell number in vitro (Regner et al., 2011). In our current study, we further interpret these findings by revealing that the effect of G $\beta\gamma$ inhibition on cell number is due in part to impaired cellular proliferation. In this regard, progression through the S-phase of the cell cycle, as measured by BrdU incorporation (Gratzner, 1982), was markedly reduced in renal epithelial cells incubated with gallein. However, renal epithelial cell migration was not affected by G $\beta\gamma$ inhibition as determined by wound closure assay. These findings suggest that the predominant role of G $\beta\gamma$

activity in renal epithelial cells in vitro is to control cell proliferation and not cell migratory pathways.

The mechanism by which $G\beta\gamma$ regulates cellular proliferation remains unknown. No significant difference in the phosphorylation state of either ERK1/2 or p38 MAPK was detected by immunoblot analysis from rat kidney lysates harvested after 1, 3, or 7 days after IRI and treated with either vehicle or gallein (data not shown). These findings are similar to previous findings by Bonacci et al. (2006) using HL60 cells and with previous studies in mice (Park et al., 2001; Park et al., 2002) and rats (Pombo et al., 1994) where short-term reperfusion after ischemia resulted in a detectable increase in ERK1/2 activation only after 30 minutes, but not at any later time points. Because free $G\beta\gamma$ dimers can interact with other effector molecules, such as phospholipases, adenylyl cyclases, and ion channels (Smrcka, 2008), further studies will be necessary to elucidate the mechanistic pathways that are involved in $G\beta\gamma$ signaling in the kidney.

In conclusion, we report for the first time that inhibition of $G\beta\gamma$ dimer function prolongs the duration of renal dysfunction and tubular injury after experimental AKI. This effect was partly attributed to $G\beta\gamma$ -mediated regulation of tubular epithelial cell proliferation. Because $G\beta\gamma$ contributes to the repair process of renal tubular epithelium after injury, the development of inhibitors targeting $G\beta\gamma$ subunits may be a novel strategy to treat AKI.

Authorship Contributions

Participated in research design: Weber, Park, Regner.

Conducted experiments: White, North, Haines, Sullivan, Goldberg, Pressly, Park, Regner.

Performed data analysis: White, North, Park, Regner.

Wrote or contributed to the writing of the manuscript: White, North, Weber, Park, Regner.

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