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Gβγ Dimers Modulate Kidney Repair Following Ischemia-Reperfusion Injury in Rats

Sarah M. White, Lauren M. North, Emily Haines,
Megan Goldberg, Lydia M. Sullivan, Jeffrey D. Pressly,
David S. Weber, Frank Park, Kevin R. Regner

Department of Medicine, Division of Nephrology (SMW, LMN, EH, MG, KRR),
Cardiovascular Research Center (KRR),
Medical College of Wisconsin, Milwaukee, WI
Department of Pharmaceutical Sciences, University of Tennessee Health Sciences Center, Memphis, TN (JDP, FP)
Department of Physiology, University of South Alabama, Mobile, AL (LMS, DSW)

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RUNNING TITLE PAGE

a) Running title: Role of Gβγ in acute kidney injury

b) Corresponding authors:

Kevin R. Regner, MD, MS, FASN
Medical College of Wisconsin
8701 Watertown Plank Rd.,
HRC 4105
Milwaukee, WI 53226
Ph. 414-456-4755
Fax. 414-456-6312
E-mail: kregner@mcw.edu

Frank Park, PhD
University of Tennessee Health
Sciences Center
Department of Pharmaceutical Sciences
881 Madison Ave., Rm 442
Memphis, TN 38163
Ph. 901-448-1992
E-mail: fpark@uthsc.edu

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d) List of nonstandard abbreviations used in the paper: AGS: Activator of G-protein Signaing; AKI: Acute kidney injury; BrdU: 5'-bromo-2'-deoxyuridine; COMT: catechol-O-methyltransferase; DAPI: 4',6-diamidino-2-phenylindole; GPCR: G-protein coupled receptor; GPR: G-protein Regulatory; GPSM1: G-protein Signaling Modulator 1; GRK2ct: G-protein coupled receptor kinase 2 carboxyl terminus; IRI: ischemia-reperfusion injury; LC-MS/MS: liquid chromatography-tandem mass spectrometry; MAPK: mitogen activated protein kinase; NRK-52E: normal rat kidney epithelial cells; PCNA: proliferating cell nuclear antigen; RGS: Regulator of G-protein Signaling

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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, Gβγ dimer activity was evaluated during the process of renal tubular repair following renal ischemia-reperfusion injury (IRI) in male Sprague Dawley rats. Rats were treated with a small molecule inhibitor of Gβγ activity, gallein (30 or 100 mg/kg), 1 hr after reperfusion and every 24 hours for 3 additional days. Following IRI, renal dysfunction was prolonged following the high-dose gallein treatment in comparison to 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 following treatment with high dose gallein (100 mg/kg) in comparison to low dose gallein (30 mg/kg), or the vehicle and fluorescein control groups. Gallein treatment significantly reduced ($P<0.05$) the number of PCNA-positive tubular epithelial cells after 24 hours following 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 to vehicle-treated cells as determined by 5'-bromo-2'-deoxyuridine incorporation. Taken together, these data suggest that Gβγ 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.

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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 following 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 following AKI. Activation of repair pathways in renal tubules is promoted by intrinsic and extrinsic factors exerted upon the surviving, sub-lethally injured epithelial cells (Humphreys et al., 2011; Humphreys et al., 2008) 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 (Bonventre and Yang, 2011; Kunzendorf et al., 2010). Heterotrimeric G proteins are composed of α, β, and γ subunits and upon GPCR stimulation, the heterotrimeric G protein alpha subunits (Gα) facilitate the exchange of GDP for GTP leading to the dissociation of the heterotrimer into a GTP-bound Gα and free (unbound) Gβγ (Gilman, 1987). Both the active GTP-bound Gα and dissociated Gβγ can subsequently function as independent, active signaling modules that regulate various downstream effectors (Gilman, 1987). After the Gα subunit hydrolyzes the GTP to GDP, a conformation change occurs in which the Gα subunit re-associates with the unbound Gβγ subunits to return to an inactive state. Prior studies have demonstrated activation of Gα in models of AKI. For example, pharmacologic agonists of the

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adenosine A2A GPCR (Yap and Lee, 2012) or genetic modulation of Gα12 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 Gα 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 Gα- or Gβγ subunit activation (Blumer et al., 2005; Cismowski et al., 1999; Sato et al., 2006; Siderovski and Willard, 2005; Takesono et al., 1999) 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 Gα or Gβγ 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 Gαi (Bernard et al., 2001). However, GPSM1/AGS3 exerts its biological effects by modulating the activity unbound Gβγ dimers (Bowers et al., 2008; Kwon et al., 2012; Nadella et al., 2010; Sanada and Tsai, 2005). We have previously shown that GPSM1/AGS3 modulates renal tubular repair through a Gβγ-dependent mechanism in rodent models of renal ischemia-reperfusion injury (IRI) (Regner et al., 2011) and polycystic kidney disease (Kwon et al., 2012; Nadella et al., 2010). More

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specifically, unilateral IRI in mice with a deficiency in full-length expression of GPSM1/AGS3 exhibited decreased number of proliferating outer medullary proximal tubular cells, and increased number of persistently injured renal tubules compared to wild-type mice (Regner et al., 2011). Other accessory proteins have also been shown to regulate effectors through Gβγ function (Takesono et al., 2002), including mitogen-activated protein kinase (MAPK) and phospholipase C signaling, ion channel activity, and mitotic spindle orientation (Kwon et al., 2012; Lin and Smrcka, 2011; Sanada and Tsai, 2005). Although there is a lack of pharmacological inhibitors targeting AGS proteins, Gβγ dimer function can be manipulated *in vivo* through the use of small molecule inhibitors. For example, administration of the small molecule inhibitors of Gβγ, gallein and M119, 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 Gβγ signaling in kidney injury remains poorly characterized.

Therefore, the present study was designed to evaluate the effect of gallein, a selective inhibitor of Gβγ dimer function during renal tubular repair following experimental AKI.

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MATERIALS AND METHODS

Chemicals and antibodies. Gallein was purchased from Spectrum Chemicals (Philadelphia, PA). Fluorescein sodium (Cat. #46960), 5'-bromo-2'-deoxyuridine (BrdU; Cat. #B5002) and monoclonal β-actin antibody (Cat. #A5441) were purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal BrdU antibody conjugated with Alexa Fluor 488 (Cat. #B35130) and DAPI (Cat. #P36931) were purchased from Life Technologies. Primary antibodies to phospho (Cat. #9107) and total ERK1/2 (Cat. #9102), phospho (Cat. #9215) and total p38 MAPK (Cat. #9212), PCNA (Cat #2586), and secondary goat anti-rabbit (Cat. #7074) and horse anti-mouse IgG (Cat. #7076) 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 min bilateral renal ischemia (or sham) surgeries as previously described (Regner et al., 2011). In brief, the rats were anesthetized with ketamine (50 mg/kg, IM) and sodium pentobarbital (50 mg/kg, IP) 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

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to the kidney, and then the abdominal incision was closed. Approximately 1 hour after the reperfusion of the kidneys, either vehicle (phosphate buffered saline), fluorescein (100 mg/kg) or gallein (30 or 100 mg/kg) was administered into the intraperitoneal (IP) space. Subsequent injections with either 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 Gβγ function (Lehmann et al., 2008), whereas fluorescein is a closely related analog of gallein that does not inhibit Gβγ 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 either 1, 3, or 7 days following 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.

Biological 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. Following 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 LC-MS/MS (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.

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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 μm) for immunostaining with PCNA as previously described by Kwon *et al* (Kwon et al., 2012). In brief, antigen retrieval by heating (90°C) in antigen retrieval solution (IHC World, Woodstock, MD) was performed; 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 DAB precipitation on the sections. Between each of the steps, the slides were rinsed multiple times in Tris-buffered saline (TBS). 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 was counted in five random fields at a magnification of 20X. 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 μm) and stained with hematoxylin and eosin (H&E). Kidney sections were imaged using a Hamamatsu NanoZoomer HT (Hamamatsu Photonics KK) digital scanner. At least 5 random areas from the cortex and 5 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

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kidney at a magnification of 20X (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) for 24 hours. Subsequently, the cells were incubated with DMEM/10% FBS for 10 hours, followed by 4 hours of incubation with 10 μM BrdU. At this point, the media 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 2M 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), 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 RIPA buffer (Millipore, Billerica, MA) with protease and phosphatase Inhibitors (Pierce Biotechnology), and protein lysates were isolated following differential centrifugation. Immunoblot analysis was performed as previous published (Akbulut et al., 2009; Kwon et al., 2012; Nadella

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et al., 2010; Regner et al., 2011). In brief, protein lysates (25 μg) were size-separated on a 4-12% NuPAGE® Bis-Tris Gradient Gel (Life Technologies, Carlsbad, CA), and subsequently transferred to a supported polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked in Odyssey Blocking Buffer (Licor, Lincoln, NE) and blotted with the following primary antibodies overnight at 4ºC: All antibodies were used at a dilution of 1:1000-1:2000. β-actin was used 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 horseradish peroxidase (HRP) at room temperature for 1 hr. The bands were detected on film following incubation in chemiluminescent solution (GE Healthcare, Piscataway, NJ). All films were scanned and band densities were calculated using NIH ImageJ software.

Scratch wound assay. This experiment was performed on normal rat kidney (NRK-52E) renal epithelial cells using a protocol modified from Torres *et al*. (Torres et al.). The NRK-52E cells were cultured to 80 to 90% confluence. Gallein (2 μM) or vehicle solution was applied to the media prior to the experimental period in which artificial wounds were created by scratching the monolayer with a 10 μL pipette tip. At this point, the media was replaced with DMEM containing 10% calf serum and the cells were incubated at 37°C in a 5% CO_2 incubator for up to 18 hours. At the end of the experimental period, the NRK cells were fixed in 5% formaldehyde, stained with Coomassie blue, and images were captured using phase-contrast microscopy. Open area was calculated using Nikon Elements software (Nikon Instruments, Inc., Melville, NY).

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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 previous described in our lab (Nadella et al., 2010; Regner et al., 2011). NRK cells were plated into 6-well dishes and allowed to expand for 48 hours at which the cells were harvested for counting by hemocytometry (Regner et al., 2011). Collecting duct cells were plated into 96-wells and counted after 24 hours using CyQuant cell proliferation assay (Life Technologies, Carlsbad, CA) (Nadella et al., 2010).

Statistical analysis. Data are expressed as mean +/- SEM. 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 ANOVA was performed on the *in vivo* biological 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 following ischemia-reperfusion injury (IRI). Prior studies have demonstrated that renal tubular repair following experimental IRI involves de-differentiation, migration, and proliferation of tubular epithelial cells within 2-5 days of injury (Humphreys et al., 2011; Humphreys et al., 2008; Witzgall et al., 1994). To determine the role of Gβγ in tubular repair, we treated rats with low (30 mg/kg IP) or high dose (100 mg/kg IP) of gallein, fluorescein (100 mg/kg IP; a gallein analog with no Gβγ inhibitory effects), or vehicle solution 1 hr following reperfusion, and subsequently

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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 to vehicle treated rats at each time point during the experimental period (**Figure 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 (**Figure 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 to vehicle, fluorescein or low-dose gallein (**Table 1**).

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

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Effect of gallein on renal tubular injury following ischemia-reperfusion injury. In sham operated rats, the extent of tubular injury in the renal cortex and outer medulla (**Figures 2** and **3**) over the 7 day period was not significantly different in the vehicle-, fluorescein-, and gallein-treated rats. However, the renal tubules in the high dose gallein-treated rat kidneys appeared more dilated compared to vehicle- and fluorescein-treated kidneys (**Figure 2**). At day 7 of the experimental period, we observed reduced recovery by the renal tubules in the rat treated with high dose gallein following IRI. High dose gallein significantly increased the proportion of injured tubules in both the cortex (**Figure 3A**) and outer stripe of the outer medulla (**Figure 3B** and **3C**) compared to the other treatment groups ($P<0.001$). In addition, increased expression of neutrophil gelatinase-associated lipocalin (NGAL), a marker of AKI, was detected by Western blot analysis at day 7 in the high dose gallein-treated kidneys, but no bands were detected in vehicle- or fluorescein-treated sham or IRI rat kidney lysates (**Supplemental Figure 2**).

Effect of gallein on renal epithelial cell proliferation in rat kidneys following ischemia-reperfusion injury. 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 cell numbers were significantly ($P\leq0.02$) reduced in two different renal epithelial cell lines over-expressing the carboxy terminus of the G-protein coupled receptor kinase (GRK2ct) compared to control cells (**Supplemental Figure 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 following gallein treatment was secondary to

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inhibition of tubular cell proliferation. To identify cells in late G_1 and early S-phase of the cell cycle, we performed immunostaining for proliferating cell nuclear antigen (PCNA), a cell cycle marker for late G_1 and early S-phase (Witzgall et al., 1994) in kidney sections from gallein- and vehicle-treated rats following IRI or sham surgery. In the high dose gallein-treated rats, the proportion of PCNA-positive tubular epithelial cells was significantly decreased in the outer stripe of the outer medulla (19.1 ± 4.5%; n=6) 1 day after IRI compared to vehicle (39.0 ± 4.2%; n=5; P<0.05) (**Figure 4**). At days 3 and 7, the percentage of PCNA-positive epithelial cells did not differ between the high dose gallein- and vehicle-treated IRI rats. Similarly, there was no difference in the number of PCNA-positive nuclei in rat kidneys treated with fluorescein (14.0 ± 3.7%; n=4) at day 7, compared to the vehicle (15.2 ± 4.9%; n=5) or high-dose gallein (18.2 ± 2.7%; n=6) groups.

In the sham-operated rats, the proportion of PCNA-positive outer medullary tubular epithelial cells in vehicle- and high dose gallein-treated kidneys 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 if the decrease in tubular cell PCNA expression was due to an effect of gallein on cell cycle progression. 5'-bromo-2'-deoxyuridine (BrdU) is 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 μM; 76.7 ± 11.9 BrdU-positive cells) in comparison to vehicle (145.0 ± 11.7 BrdU-positive cells) (**Figure**

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5). To determine whether Gβγ modulates other tubular repair mechanisms, we assessed cellular migration using a scratch wound assay. Incubation of NRK cells with gallein (2 μM; n=3) did not significantly differ in the rate of wound closure in comparison to vehicle treated cells (n=5; **Figure 6**). Taken together, these findings suggest that inhibition of Gβγ function with gallein inhibits tubular repair by modulating tubular epithelial cell proliferation rather than migration.

DISCUSSION

Renal tubular regeneration following experimental IRI involves de-differentiation, migration, and proliferation of tubular epithelial cells within 2-5 days of injury (Humphreys et al., 2011; Humphreys et al., 2008; Witzgall et al., 1994). However, the intracellular mechanisms that regulate this response remain poorly understood. In the present study, we provide evidence that pharmacologic inhibition of Gβγ prolongs renal injury and impairs recovery of kidney function following 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-Gα subunit activation by specific ligands to control signal processing and subsequent regulation of renal epithelial cell recovery (Bajwa et al., 2010; Johnson et al., 2006; Leduc et al., 2013; Yap and Lee, 2012). More recently, the importance of Gα subunits in the recovery of the kidney following ischemic injury was documented in a study by Yu *et al.* (Yu et al., 2012). In their report, constitutively active QLα12 mutant transgenic mice were observed to have attenuated recovery following renal IRI with more severe tubular injury

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compared to the wild-type Gα12 mice. Conversely, the renal tubules in Gα12 knockout mice were protected following 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 pathological situations.

Gβγ is the natural binding partner to Gα subunits and is known as the canonical guanine dissociation inhibitor. Gβγ subunits have been shown to have diverse biological 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 biological 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 due to 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 has been performed in earlier studies using rodents by over-expression of the G-protein coupled receptor kinase (GRK2ct) carboxy terminus, and these studies demonstrated that Gβγ dimers can play a central role in modulating drug addiction (Daaka, 2004; Yao et al., 2002; Yao et al., 2003), pain (Xie et al., 1999)

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and cardiovascular disease (Akhter et al., 1997; Eckhart et al., 2002; Iaccarino and Koch, 2003). Subsequently, small molecule inhibitors of Gβγ have been identified and have demonstrated similar effects (Casey et al., 2010; Mathews et al., 2008).

In the present study, we used gallein, a small molecule antagonist of Gβγ 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), and interfere with downstream signaling targets that modulate biological processes known to be involved in tubular repair, including cell proliferation and migration (Lehmann et al., 2008; Regner et al., 2011). Specifically, the Gβγ inhibitors, gallein and M119, were shown to block fMet-Leu-Phe (fMLP) activation of superoxide production and inhibited chemotaxis in HL60 cells (Lehmann et al., 2008). The binding of gallein to Gβγ subunits is dependent on the presence of hydroxyl groups at carbons 4 and 5 of the tricyclic ring structure, since fluorescein and M119B, which are deficient of these hydroxyl groups, were unable to bind to $G\beta_1\gamma_2$ by surface plasmon resonance or unable to 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 (Casey et al., 2010; Lehmann et al., 2008). 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 Gβγ 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 Gβγ inhibition were through mechanisms that

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were directly controlling renal epithelial cell function, or indirectly through hormonal release from the central nervous system. Because of these possibilities, we speculate that Gβγ inhibition has the potential to either block vasopressin-mediated water transport in the collecting ducts of the kidneys, or 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 was a key contributor to the exacerbated epithelial cell damage following IRI in the presence of high-dose gallein, since urine tonicity and urine output was similar to values obtained in the fluorescein-treated IRI group. The dose of gallein needed toexert changes in biological function in the kidney may be higher than 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 biological 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 5-8 fold higher in the kidney compared to the heart or brain (Myohanen et al., 2010). Second, the diversity of Gβγ subunit interactions as described earlier in this section may vary in the kidney compared to other cells and/or organs (Fong et al., 1987; Ruiz-Velasco et al., 2002), which could affect the activation status of Gβγ-dependent mechanisms (McIntire, 2009; Yan et al., 1996) and require increased amounts of gallein to regulate cellular function. Regardless, the present study confirms that gallein can be safely used at lower doses

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similar to 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 ADME studies focusing on gallein metabolism and renal excretion in the context of renal injury are warranted.

The results of the present study indicate that Gβγ may act either alone or in concert with Gα subunit activity to control signaling mechanisms involving epithelial cell proliferation during the repair process following injury. Previous studies by our group have shown that genetic over-expression of GRK2ct or pharmacologic inhibition of Gβγ using low concentrations of gallein decreased renal tubular epithelial cell number *in vitro* (Regner et al., 2011). In the current study, we further interpret these findings by revealing that the effect of Gβγ 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 Gβγ inhibition as determined by wound closure assay. These findings suggest that the predominant role of Gβγ activity in renal epithelial cells *in vitro* is to control cell proliferation, and not cell migratory pathways.

The mechanism by which Gβγ regulates cellular proliferation remains unknown. Immunoblot analysis of cell lysates treated with or without gallein was performed to assess Gβγ-mediated MAPK signaling in renal epithelial cells, but no significant difference in the phosphorylation state of either ERK1/2 or p38 MAPK was detected by

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immunoblot analysis from rat kidney lysates harvested after 1, 3 or 7 days following IRI and treated with either vehicle or gallein (data not shown). These findings are similar to previous findings by Bonacci *et al.* (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 following ischemia resulted in a detectable increase in ERK1/2 activation only after 30 minutes, but not at any later time points. Since free Gβγ 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 Gβγ signaling in the kidney.

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

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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, and Regner.

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

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FOOTNOTES

a) The work in this manuscript was funded by the National Institutes of Health [DK090123] and institutional funds provided by The University of Tennessee Health Sciences Center.

b) Portions of this work were presented at the American Society of Nephrology Kidney Week, November 1-4, 2012, San Diego, CA.

c) The name and full address (with street address or P.O. box and postal code) and e-mail address of person to receive reprint requests: Frank Park, PhD, The University of Tennessee Health Sciences Center, College of Pharmacy, Department of Pharmaceutical Sciences, 881 Madison Ave., Rm 442, Memphis, TN 38163, e-mail: fpark@uthsc.edu

d) Sarah M. White and Lauren M. North contributed equally in the completion of these studies.

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LEGENDS

Figure 1. Effect of Gβγ inhibition on renal function in sham and IRI-treated rats. (A) Plasma creatinine was measured by LC-MS/MS using blood collected from high dose gallein and vehicle treated rats 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. * $P < 0.05$, high dose gallein- versus vehicle-treated IRI rats at day 3; ¶ $P < 0.01$, high dose gallein- versus vehicle-treated IRI rats 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 IP) versus all other sham- and IRI-treated groups.

Figure 2. Histology of sham and IRI rat kidneys in the presence and absence of Gβγ inhibition. Representative hematoxylin and eosin (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 20X.

Figure 3. Quantitative analysis of renal tubular injury in the renal cortex and outer medulla following 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

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treated with high dose gallein (▼) or vehicle (▽). n=3-7 kidneys/group. ***P < 0.001, high dose gallein- versus vehicle-treated IRI rats at day 7. As a control, fluorescein (100 mg/kg IP) was administered over a 3 day period following IRI as an additional control for high dose gallein. (**C**) The data for the outer medulla in (**B**) was re-graphed to compare the effects of vehicle (white), low (hatched) and high dose (black) gallein, and fluorescein (grey) 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 to sham-operated rats; *** P<0.001 significant difference between high dose gallein- versus all other sham- and IRI-treated rat groups.

Figure 4. Gβγ inhibition delays renal epithelial cell proliferation in rat kidneys following IRI. Kidney sections from each rat group were immunostained for proliferating cell nuclear antigen (PCNA) 1 day (**A**,**D**,**G**,**J**), 3 days (**B**,**E**,**H**,**K**) and 7 days (**C**, **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, high dose gallein versus vehicle treated IRI rats.

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Figure 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 μM; **E**-**H**). Nuclei were labeled with DAPI (**B**, **D**, **F**, **H**). White boxes in **A, B, E, F** indicate the corresponding region magnified in **C, D, G, H**, respectively. Green fluorescence within nuclei indicates BrdU incorporation (white arrows; **C**,**D**,**G**,**H**). Red arrows in **C**,**D**,**G**,**H** indicate nuclei without BrdU incorporation. Scale bar in (**A**) = 200 μm and scale bar in (**C**) = 50 μm. (**I**) Number of BrdU-positive nuclei in vehicle and gallein treated cells. * $P<0.05$ vs vehicle. Data from three independent experiments are depicted.

Figure 6. Effect of Gβγ inhibition on renal epithelial cell migration. (**A**) Representative light micrographs of vehicle- and gallein (2 μM)-treated NRK cells 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 following the initial scratch regardless of the experimental conditions. n=3-6 different experiments for each time point/group.

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Table 1: Body weight, water intake, and urine output 7 days following sham or IRI surgery

	Sham				IRI			
	Veh	Fl	Gal		Veh	Fl	Gal	
			30	100			30	100
Body weight (g)	263 ± 13	275 ± 8	294 ± 7	212 ± 8 **	290 ± 7	285 ± 7	256 ± 7	188 ± 23 ***
Water intake (mL)	32.4 ± 1.0	38.5 ± 1.9	37.2 ± 2	56.7 ± 4.0 *	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 **	16.8 ± 1.5	25.8 ± 3.9	25.8 ± 3.5	24.8 ± 5.2
Urine osmolality (mOsm/kg H_20)	1814 ± 89	1559 ± 184	1532 ± 102	689 ± 52 ****	1450 ± 28	1005 ± 99 #	998 ± 86 #	765 ± 102 ##

Table 1. Physiologic parameters in sham and IRI-treated rats 7 days following renal IRI. Urine osmolality, urine output, water intake and body weight were measured at day 7 following 24 hour collection of urine and other parameters using rats housed in individual metabolic cages. * P<0.05 significant difference between high dose gallein versus other sham-operated groups; ** P<0.01 significant difference between high dose gallein versus other sham-operated groups; *** P<0.001 significant difference between high dose gallein versus other IRI-treated groups; **** P<0.0001 significant difference between high dose gallein versus other sham-operated groups; # P<0.01 significant difference compared to vehicle-treated IRI rats; ## P<0.001 significant difference between high dose gallein versus vehicle-treated IRI rats

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Figure 1

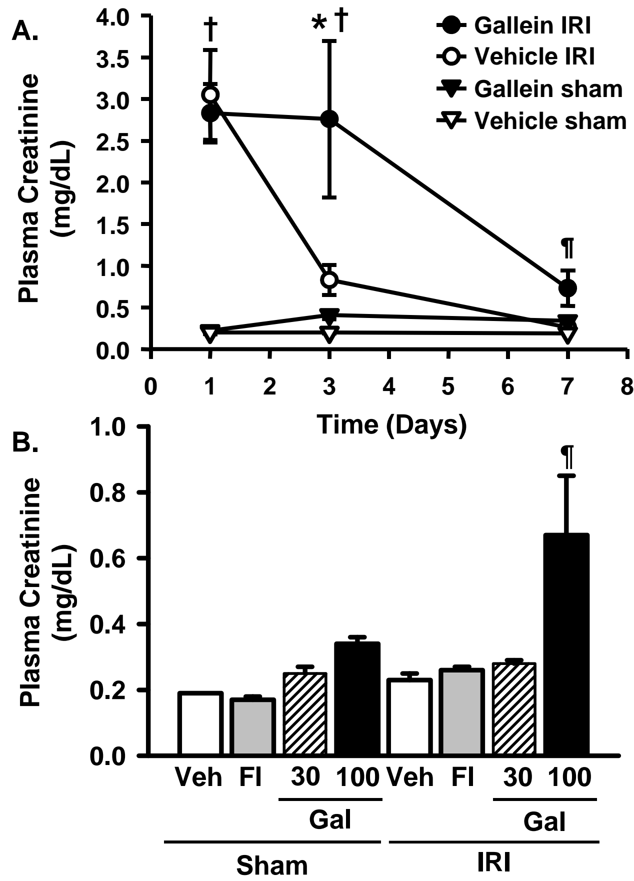

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Figure 2

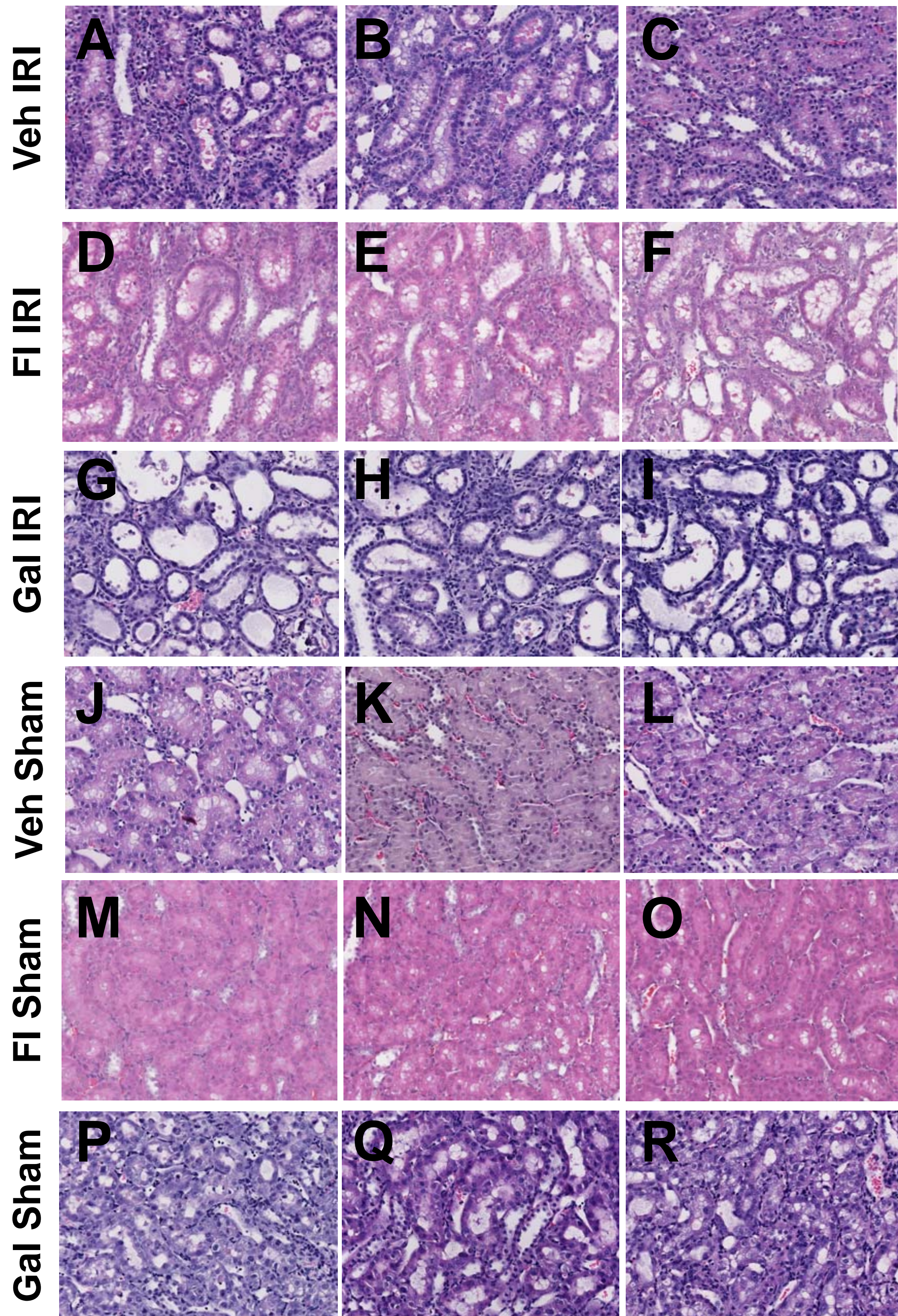

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Figure 3

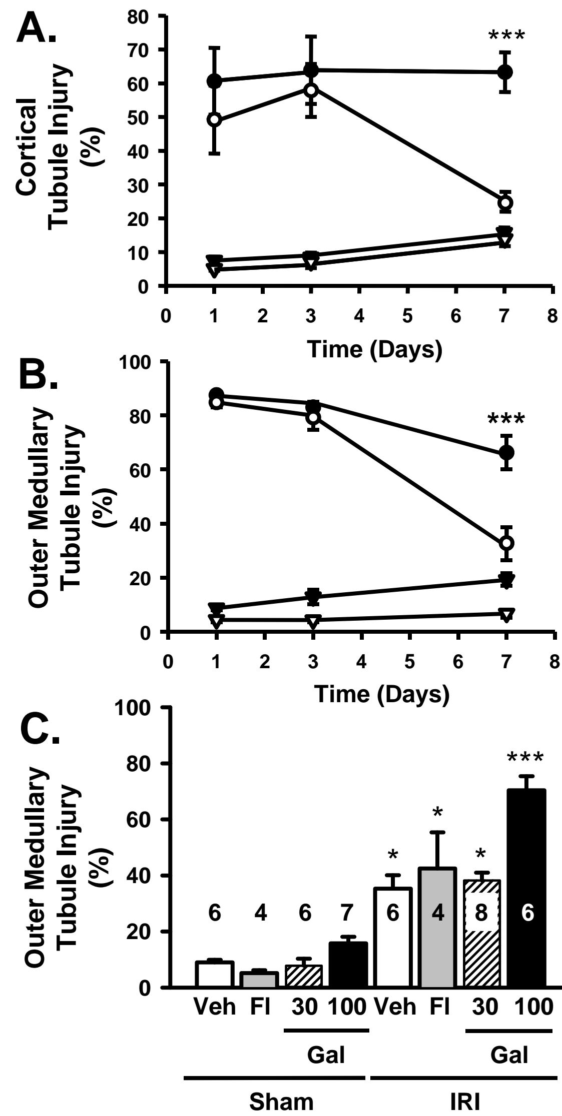

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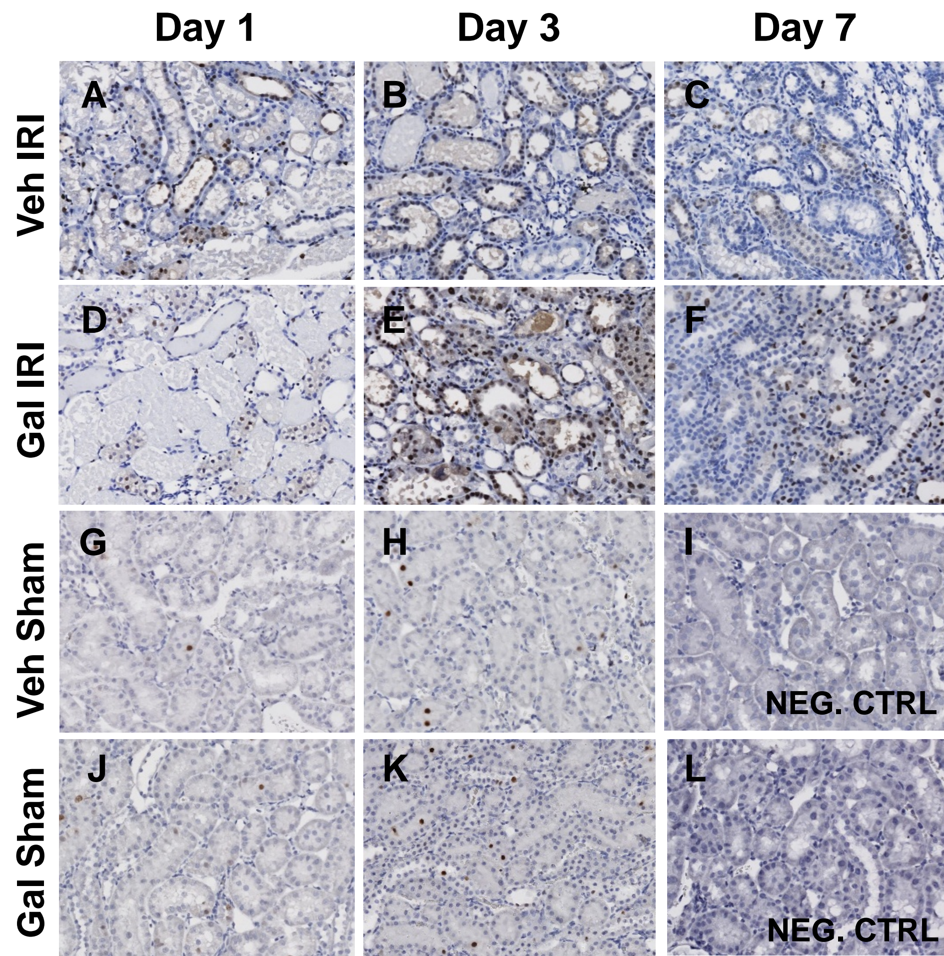
Figure 4



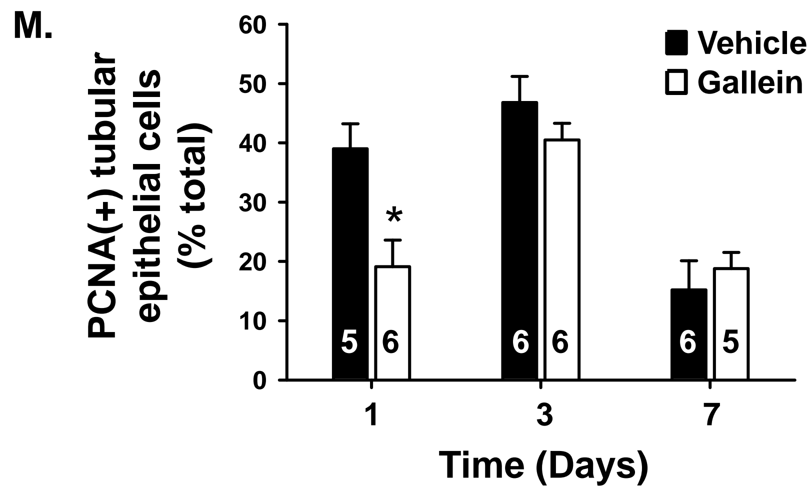


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Figure 5

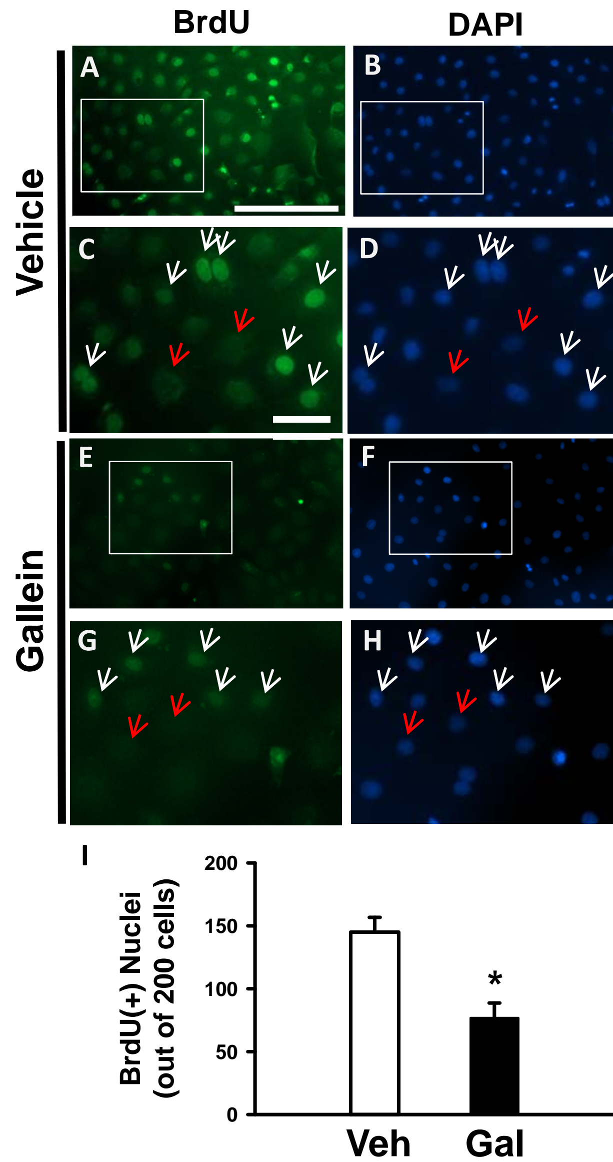

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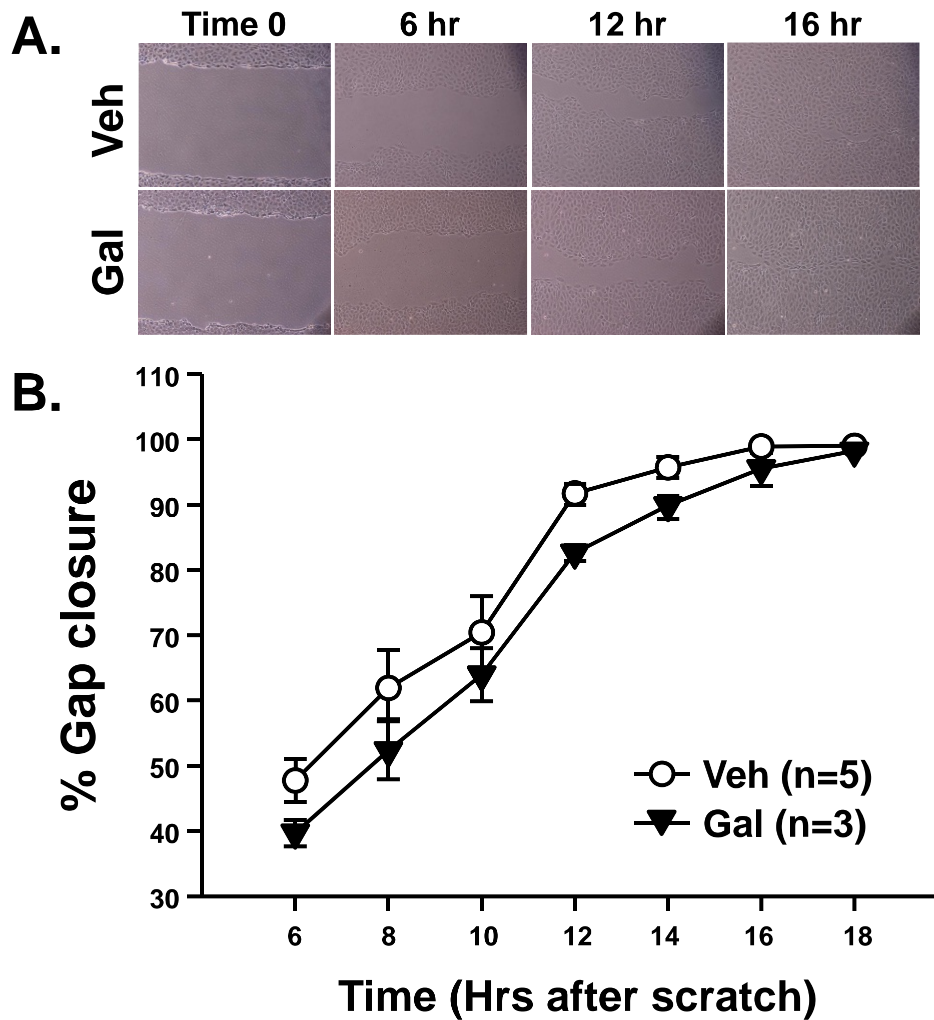
Figure 6



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