Ga12 Regulates Interleukin-8 Expression after Epithelial Cell Injury

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Received 22 April 2016; accepted 8 July 2016; published 11 July 2016

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Abstract

Acute kidney injury (AKI) is common in hospitalized patients and is strongly correlated with increased morbidity, mortality, and prolonged hospitalization. However, signals that determine whether injured tissues following AKI will repair or fibrose and lead to chronic kidney disease (CKD) are not well defined. Numerous cytokines are activated at various times after injury and recruit inflammatory cells. Interleukin-8 (IL-8) is upregulated following activation of Ga12 by H2O2, a reactive oxygen species (ROS). Herein, we study this occurrence in vitro and in vivo. IL-8 was measured by ELISA in Ga12-silenced (si-Ga12) and inducible QLa12 (constitutively active Ga12) Madin-Darby Canine Kidney (QLa12-MDCK) cell lines after H2O2/catalase cell injury. QLa12- and si-Ga12 MDCK cells showed time-, agonist- and Ga12-dependent increases in IL-8 mRNA and protein. Ga12-silenced MDCK cells demonstrated lower IL-8 expression and blunted IL-8 increases. In transgenic mice (QLa12γGTCre+, proximal tubule Qα12 expression) ischemia reperfusion injury led to significant upregulation of CXCL-1 (IL-8 homologue) at 48 hours that was not observed in Ga12 knockout mice. Macrophages in renal cells from these mice were imaged by immunofluorescent microscopy and QLa12γGTCre+ showed increased macrophage infiltration. We demonstrate that IL-8 is a critical link between H2O2 stimulated Ga12 and renal injury. Ga12 activation led to increased IL-8 expression, a potent mediator of inflammation after injury. Future studies targeting Ga12 for inhibition after injury may blunt the IL-8 response and allow for organ recovery.

Keywords
Ga12, Interleukin-8, Acute Kidney Injury, Inflammation, Fibrosis

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1. Introduction

Acute kidney injury (AKI) is common in hospitalized patients, reporting to affect 1% - 25% of intensive care unit (ICU) patients, and is frequently not recognized in the outpatient setting [1]. AKI in hospitalized patients is strongly correlated with prolonged hospitalization and increased morbidity and mortality, with mortality rates ranging from 15% to 60% in ICU patients [2]-[4]. Etiologies of AKI include ischemic and toxic injury (drugs), obstruction, and delayed graft function after renal transplantation. Clinical features include oliguria, fluid overload, and electrolyte disturbances and often require dialysis for correction of these abnormalities [1] [5].

Importantly, mechanisms initiated with acute kidney injury can lead to renal tubular fibrosis and progressive chronic kidney disease (CKD) [6]-[8]. The amount of interstitial fibrosis is the best predictor of long-term renal outcome, regardless of the etiology [9]. Although significant progress has been made in defining the complexities of AKI (reviewed in [10]), little is known about the molecular switches that determine whether injured epithelia will repair or progress to fibrosis. Reactive oxygen species (ROS) are increased with tissue injury and contribute to progressive fibrosis, diabetic nephropathy and hypertensive nephrosclerosis [11]-[13]. ROS remains elevated for more than 16 days after 30 min of ischemia [14] leading to sustained activation of numerous signaling pathways. Oxidative stress through ROS leads to proximal tubule cell detachment, actin cytoskeleton disruption and TJ disruption [15]; all processes are linked to G protein signaling and specifically, Ga12 [16]-[18].

The heterotrimeric G protein family is comprised of Gαs, Gαi/o, Gαq, and Gα12/13. The α subunits of these signal transduction proteins bind GTP with activation and utilize a conformational switch to promote interactions with downstream effectors. G proteins are essential to cellular function and regulate numerous processes including: proliferation, apoptosis, differentiation, cell attachment and migration and many others. The Ga12 family regulates cell migration and attachment in addition to apoptosis in epithelial cells [16] [19]. We previously demonstrated an essential role for Ga12 in regulating the epithelial cell tight junction and barrier function [17] [20] [21]. Recently, we showed that Ga12 is directly activated by the reactive oxygen species (ROS), H2O2, a major signaling molecule mediating oxidative injury seen in ischemia reperfusion (I/R). We found that silencing Ga12 protected epithelial cells from injury in the H2O2/catalase model of reversible injury. Furthermore, Ga12 knockout mice (Ga12 KO) were highly protected from ischemia reperfusion injury (IRI) and mice with proximal tubule targeted expression of constitutively activated Ga12 (QLα12GTCre+) showed more severe injury [22].

Numerous cytokines are activated at various times after injury and these molecules play critical roles in recruitment of inflammatory cells and activation of other pro-inflammatory/pro-fibrotic factors. Now we demonstrate that IL-8 is a critical link between H2O2 stimulated Ga12 and renal injury. We show that Ga12 stimulates IL-8 production in cell culture and activated Ga12 (QLα12) enhances IL-8 expression and macrophage infiltration after injury. IL-8 is significantly upregulated in human acute kidney injury [23] and these results suggest that Ga12 is an important proximal mediator of IL-8 production. Taken together, these studies extend previous findings indicating an important role of Ga12 in propagating injury signals and link Ga12 activation to increased IL-8 expression, a potent mediator of inflammation after injury. Targeting Ga12 for inhibition after injury may blunt the IL-8 response and contribute to more rapid and complete organ recovery.  

2. Methods

2.1. MDCK Cell Lines, Cell Culture and H2O2/Catalase Injury Model

Tet-off inducible Ga12- and QLα12-MDCK cell lines (as previously described in [17]) were cultured at 37°C in 5% (vol/vol) CO2 and maintained in DMEM (Cellgro) containing 5% (vol/vol) FBS (Clontech) (DMEM) and 100 μg/mL G418 and 40 ng/mL doxycycline. Ga12 expression was induced by dox removal. Si-Ga12- and Si-GFP-MDCK cells were previously described [16]. Monolayers were serum starved for 24 h and then incubated with 10 U/mL thrombin or 2.5 mM H2O2 as described in [24]. Recovery at T = 0 was induced by the addition of the ROS scavenger catalase (5000 U/mL) and cells removed at various times for analysis of IL-8 concentration (T = 0, 16, 20, 24 hours).

2.2. IL-8 ELISA

Canine IL-8 from MDCK cells was measured by ELISA in 96-well plates (Linbro/Titertek; ICN Biochemicals,
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Costa Mesa, CA) coated overnight with 1 g/mL anti-rabbit IL-8 monoclonal antibody and detected with rabbit anti-canine IL-8 polyclonal antibody. Concentrations of IL-8 in si-Ga12, si-GFP, and QLa12 cells at t = 0, 16, 20, and 24 hours after exposure to thrombin or H2O2 were determined by a curve of optical density vs. concentration.

2.3. Real-Time PCR

Kidneys were obtained from QLa12GTCRe+ mice, Ga12 knockout mice, and C57/B6 control mice 48 h following ischemia reperfusion (murine models previously described in [22]). cDNA was isolated from whole kidneys and TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA, USA) were performed on the cDNA using an ABI 7300 (Applied Biosystems) with the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The CXCL-1 5′ sense primer was ACCCGCTCGCTTCTCTGT and the 3′ antisense primer was AAGGGAGCTTCAGGGTCAAG. Data analysis used the ΔΔCt method where the Ct was normalized to the CXCL-1 expression in the C57/B6 mice.

2.4. Immunofluorescence Microscopy

Macrophages in kidney sections from transgenic mice obtained 48 h after I/R were stained with F4/80 antibody and co-stained with DAPI. Coverslips were mounted in Fluoromount (Southern Biotechnology Associates) and viewed by using a Nikon Labophot-2 microscope with digital camera. Images were processed by using Adobe Photoshop and assembled in Adobe Illustrator (Adobe Systems Incorporated).

2.5. Statistics

Data are expressed as medians or means ± SEM as indicated. Statistical analysis was performed by using Excel using the two-tailed t test. Statistical significance was identified at P < 0.05.

3. Results

To identify potential targets of Ga12 activation, inducible QLa12 MDCK cells (previously described in [17]) were utilized in a microarray analysis comparing baseline (no QLa12 expression (+dox)) to induced QLa12 expression (~dox for 3 days). In addition to changes in integrin expression [25], it was noted that within the cytokine family, IL-8 was highly induced. Relative message levels for the IL-6, IL-10, and others showed no significant change (defined as <2-fold with p < 0.05; not shown), but IL-8 was significantly upregulated from ~30 to 120 fold. Based on the importance of IL-8 in inflammation and our recent findings showing that Ga12 knockout mice were protected from injury, we further examined a possible link between Ga12 activation and IL-8 expression.

To link increased IL-8 mRNA levels to increased secreted IL-8 protein, Ga12- and QLa12-MDCK cells (+/−dox) were assayed for IL-8 expression by ELISA. Figure 1 shows a time course of induced IL-8 protein levels in Ga12- and QLa12-MDCK cells (+/−dox). Within 24 h of dox removal (~dox), Ga12 and QLa12 proteins are induced and plateau by days 2 - 3 (see [17]). IL-8 protein levels increased in parallel with induced Ga12 expression in both Ga12- and QLa12-MDCK cell lines. However, QLa12 expression (~dox) led to >20 fold higher IL-8 levels at day 1 when compared to Ga12 +/-dox or QLa12-MDCK maintained in +dox (Figure 1). Levels of IL-8 further increased at day 2 and plateaued by day 3 of ~dox exposure correlating with the known time course of QLa12 protein expression in these cells. At day 3, IL-8 levels were >5 fold higher in QLa12 expressing cells when compared with the +dox control. Similar findings were seen with Ga12-MDCK cells but lower levels of IL-8 were induced consistent with the previously reported low level activation of Ga12 effectors seen with higher Ga12 expression. Figure 1(a) (inset) shows IL-8 protein expression in QLa12+dox and Ga12-MDCK cells (+/−dox) on an expanded scale. There is a small increase in IL-8 protein levels with induced Ga12 expression (~dox vs +dox). QLa12-MDCK+dox reveal subtle phenotypes due to leaky expression and increased IL-8 was observed.

Next, Ga12-MDCK cells +/-dox were stimulated with thrombin (a Ga12 agonist) for 24 h. Prior to inducing Ga12 expression with ~dox, baseline IL-8 levels were 400 ± 300 pg/mL (n = 4) (Figure 1(b)). Ga12-MDCK cells (~dox) show a 4-fold increase in IL-8 production when stimulated with thrombin (Figure 1(b)). To extend these findings, baseline IL-8 was measured in previously characterized Ga12-silenced MDCK cells (si-Ga12) and controls (si-GFP) [26]. Figure 1(c) shows significantly lower IL-8 expression in the si-Ga12 cells in comparison to the controls.
Figure 1. IL-8 is upregulated by Ga12 activation. (a) Time course of increased IL-8 release with expression of both wild-type and constitutively active (QL) Ga12. Ga12 and QLa12-MDCK cells were incubated +/−dox (−dox = Ga12 expression; +dox = control) for 3 days (n = 8 for each set). Supernatants were collected and assayed by ELISA for IL-8 levels; (b) Ga12-MDCK cells −dox showed greater IL-8 production than +dox cells (n = 4) 1 day after thrombin stimulation (P < 0.0118); (c) Baseline IL-8 levels in the si-Ga12 in comparison to the si-GFP MDCK cells (P < 0.0001).

H$_2$O$_2$ is a key ROS mediator of injury and directly activates Ga12 [22]. To determine if H$_2$O$_2$ stimulated Ga12 regulates IL-8 expression, we utilized the well-established ROS model of reversible epithelial injury with H$_2$O$_2$/catalase [22] [24] in si-Ga12 and si-GFP MDCK cells (Figure 2). si-Ga12 and si-GFP MDCK cells were compared +/− catalase at baseline, and at multiple times up to 24 h after exposure to 2.5 mM H$_2$O$_2$ (Figure 2). Control cells (si-GFP) have significantly higher baseline IL-8 expression levels than si-Ga12-cells (0 h in Figure 2) and similar to what is shown in Figure 1(c). At T = 0, cells are exposed to H$_2$O$_2$ +/- catalase and si-GFP cells were more prone to barrier disruption (see [22]). With H$_2$O$_2$ injury, si-Ga12 MDCK are protected and secrete significantly lower amounts of IL-8 in comparison with controls.

The link between Ga12 activation and IL-8 expression was further investigated in in vivo using ischemia reperfusion injury in two transgenic models; QLa12$^{GTCre}$ mice and Ga12 KO mice [22]. The functional homologue of IL-8 in mice is CXCL-1/KC and CXCL-1/KC was quantified utilizing real-time PCR in previously reported Ga12 KO mice (protected from injury)and QLa12$^{GTCre}$ mice (show accelerated injury) [22]. Figure 3(a) shows that CXCL-1 gene expression was significantly upregulated in the QLa12$^{GTCre}$ mice 2 days following ischemia reperfusion injury, corresponding to a ~4 fold increase (Figure 3(a)). The CXCL-1 relative gene expression in the Ga12 KO mice was indistinguishable from the controls. Thus, the more severe injury seen in QLa12$^{GTCre}$ mice may reflect enhanced CXCL-1 expression and increased inflammation. IL-8 is released from inflammatory macrophages following injury [27]. To determine whether increased IL-8 also demonstrated increased macrophage (M1) infiltration, Ga12 KO and QLa12$^{GTCre}$ mice were stained at 48 hours following IRI. Analysis of macrophage staining demonstrated that the infiltration of macrophages was enhanced in the QLa12$^{GTCre}$ mice (Figure 3(b)). This suggests that activated Ga12 enhances macrophage infiltration 2 days following ischemia reperfusion injury and similar findings were seen in 3 mice.

4. Discussion

IL-8 is an important mediator of the inflammation process. IL-8 is highly specific to CXCR1, a canonical seven-helical transmembrane G-protein receptor. When oxidative stress disturbs the permeability barrier of epithelial cells, IL-8 is secreted and binds to CXCR1 and CXCR2 expressed on neutrophils [28]. This results in rapid changes in cell morphology, activation of integrins, and the release the granule contents of neutrophils. IL-8 acts as a leukocyte chemotactic activating cytokine recruiting T lymphocytes and basophils to induce inflammation to the site of injury [29]. In renal injury, urinary IL-8 levels are associated with sustained renal allograft dysfunction due to ischemia-reperfusion injury [30]. Serial plasma IL-8 levels have been shown to predict
Figure 2. H2O2/catalase reversible injury model shows blunted IL-8 stimulation in si-Gα12 MDCK cells. Time course of IL-8 levels in si-GFP and si-Gα12 MDCK cells after exposure to 2.5 mM H2O2 +/- catalase at T = 0 hours. The effect of IL-8 increase was blunted with respect to exposure to 2.5 mM H2O2 without catalase.

Figure 3. Ga12 stimulates the IL-8 mouse homologue, CXCL1. (a) Relative expression of CXCL1 in QLa12GTCre+ mice and Ga12 KO mice shown relative to the control mice 48 hours following ischemia reperfusion. CXCL1 was increased in QLa12GTCre+ mice in comparison to the Ga12 KO mice (P < 0.0001). Data is based on 5 wildtype mice, 8 QLa12GTCre+, and 2 Ga12 KO mice; (b) Macrophages were imaged by immunofluorescent microscopy after staining with F4/80 antibody and co-stained with DAPI. Kidneys were obtained at sacrifice 48 h after IRI. There was less macrophage staining in the Ga12 KO mice and increased staining in the QLa12GTCre+ compared to the WT controls. Similar findings were seen in 3 mice.

the development in AKI in patients with sepsis [31]. Plasma IL-8 levels have also been shown to be elevated among critically ill patients with AKI that did not survive during hospitalization [27].

We have demonstrated that activation of Ga12 by H2O2, a reactive oxygen species (ROS), promotes increased IL-8 expression in vivo and in vitro. As Ga12 activation is critical in the pro-inflammatory pathway, this study links Ga12 activation to IL-8 expression. However, numerous cytokines and pathways are stimulated with I/R injury and the definitive experiment of blocking IL-8 activity in QLa12GTCre+ mice remains to be performed. Despite this, our findings further establishes Ga12 as a potential therapeutic target for ameliorating ROS mediated injury and add to the narrative of Ga12’s role in IL-8 activation and in injury responses. It has been found that Ga12/13 regulates NF-κB activation [30] and NF-κB bound to IL-8 acts as a transcriptional activator at the IL-8 promoter in all cell types [32]. Furthermore, Ga12 specifically regulates NF-κB-mediated Cyclooxyge-
nase-2 (COX-2), a critical gene in the inflammatory responses during platelet aggregation and thrombosis [33]. Ga12 has also been suggested to stimulate IL-6 and IL-8 activation in the oral squamous carcinoma cell, which in turn promotes the oral squamous carcinoma cell’s invasive behavior characteristic of cancerous cells [34]. Thus we believe targeting Ga12 for inhibition after injury may blunt the IL-8 response and permit engagement of recovery pathways through a mechanism that reduces inflammation after injury and thus prevent AKI evolving into CKD. Although exposure to H2O2 activates Ga12 and leads to barrier disruption, silencing Ga12 protected cells from tight junction disassembly despite H2O2 exposure [22]. Additionally, it has been previously found that Ga12 knockout mice are protected in bilateral ischemia reperfusion [22]. Since Ga12 knockout mice are phenotypically normal (indicating that the absence of Ga12 signaling is tolerated), we believe future studies investigating inhibitors of Ga12 as a potential drug treatment to prevent progressive injury following acute injury will be fruitful and well-tolerated. Although there are many downstream signaling pathways linked to Ga12, targeting the activated conformation of Ga12 would limit drug effects only to cells at the site of injury. Future studies include investigating the pathway(s) involved in the activation of IL-8, as well as studies pursuing the identification of molecules that inhibit activated Ga12 to promote repair processes following AKI.

5. Conclusion
Signals that determine whether injured tissues following AKI will repair or fibrose and lead to chronic kidney disease (CKD) are not well defined. We demonstrate that Ga12 activation by H2O2, a reactive oxygen species (ROS), leads to increased IL-8 expression in vivo and in vitro. Future studies inhibiting Ga12 after injury may reduce the IL-8 response and pro-fibrotic pathway, and permit more complete organ recovery.

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