RGS10 Regulates the Expression of Cyclooxygenase-2 and Tumor Necrosis Factor Alpha through a G Protein–Independent Mechanism

Mohammed Alqinyah, Faris Almutairi, Menbere Y. Wendimu, and Shelley B. Hooks

Hooks Laboratory, Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, Georgia

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ABSTRACT

The small regulator of G protein signaling protein RGS10 is a key regulator of neuroinflammation and ovarian cancer cell survival; however, the mechanism for RGS10 function in these cells is unknown and has not been linked to specific G protein pathways. RGS10 is highly enriched in microglia, and loss of RGS10 expression in microglia amplifies production of the inflammatory cytokine tumor necrosis factor α (TNFα) and enhances microglia-induced neurotoxicity. RGS10 also regulates cell survival and chemoresistance of ovarian cancer cells. Cyclooxygenase-2 (COX-2)–mediated production of prostaglandins such as prostaglandin E2 (PGE2) is a key factor in both neuroinflammation and cancer chemoresistance, suggesting it may be involved in RGS10 function in both cell types, but a connection between RGS10 and COX-2 has not been reported. To address these questions, we completed a mechanistic study to characterize RGS10 regulation of TNFα and COX-2 and to determine if these effects are mediated through a G protein–dependent mechanism. Our data show for the first time that loss of RGS10 expression significantly elevates stimulated COX-2 expression and PGE2 production in microglia. Furthermore, the elevated inflammatory signaling resulting from RGS10 loss was not affected by Gαi inhibition, and a RGS10 mutant that is unable to bind activated G proteins was as effective as wild type in inhibiting TNFα expression. Similarly, suppression of RGS10 in ovarian cancer cells enhanced TNFα and COX-2 expression, and this effect did not require Gi activity. Together, our data strongly indicate that RGS10 inhibits COX-2 expression by a G protein–independent mechanism to regulate inflammatory signaling in microglia and ovarian cancer cells.

Introduction

Regulators of G protein signaling (RGS) are a family of proteins that classically act as activators of the intrinsic GTPase activity of heterotrimeric Gα subunits (Watson et al., 1996). Owing to this GTPase-accelerating protein (GAP) activity and inhibition of signaling initiated by G protein–coupled receptors (GPCRs), RGS proteins play numerous roles in physiologic and pathologic conditions in diverse systems. However, multiple studies have revealed actions of RGS proteins that are independent of GTPase-accelerating activity, recently reviewed in Sethakorn et al. (2010). These noncanonical functions of RGS proteins can affect a variety of targets, including GPCRs, kinases, and transcription factors (Sethakorn et al., 2010). Therefore, to investigate the molecular mechanism of specific RGS protein actions, a critical initial question to answer is whether the RGS protein is acting in a classic GAP-dependent or noncanonical GAP-independent mechanism. The small RGS protein RGS10 regulates inflammatory and survival signaling in multiple cell types (Hooks et al., 2010; Lee et al., 2011, 2013), and has been proposed as a potential drug target for neuroinflammatory disease and ovarian cancer. However, the mechanisms by which RGS10 affects inflammatory and survival signaling are undefined, hampering the development of RGS10–targeted therapeutic strategies.

RGS10 is the smallest member of the R12 RGS subfamily with no functional domains outside of the RGS domain. RGS10 has been shown to selectively target Gαi family G proteins via classic GAP activity (Hunt et al., 1996), and is highly enriched in immune cells, including peripheral macrophages and microglia (Lee et al., 2008, 2013). Loss of RGS10 in microglia amplifies production of inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin 1β, and enhances microglia-induced neurotoxicity triggered by the toll-like receptor (TLR) ligand lipopolysaccharide (LPS) (Lee et al., 2011). Reciprocally, activation of microglia by LPS induces epigenetic silencing of RGS10, which we predict serves to amplify inflammatory signaling (Alqinyah et al., 2017). In addition to its anti-inflammatory role in microglia, RGS10 also regulates survival of ovarian cancer cells, and loss of RGS10 induces chemoresistance in ovarian cancer cells.

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ABBREVIATIONS: AlF4, aluminum fluoride; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; GAP, GTPase-accelerating protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein–coupled receptor; HRP, horseradish peroxidase; LPA, lysophosphatidic acid; LPS, lipopolysaccharide; PGE2, prostaglandin E2; PTX, pertussis toxin; RGS, regulator of G protein signaling; siRNA, small interfering RNA; TLR, toll-like receptor; TNFα, tumor necrosis factor α; WT, wild type.
Ha-tagged RGS10-2 DNA plasmids were purchased from the cDNA Resource Center (Brooklyn University, Brooklyn, PA). The E52K mutation was generated with the QuickChange site-directed mutagenesis kit (Stratagene, San Diego, CA) using the following primer sequences: forward: 5'-TA AAA AAG GAA TTC AGT GAA AAA AAT-3' and reverse: 5'-GG TAG CCA AAA CAA ACC ATT TT TTT TCA-3'. Mutagenesis resulted in a single nucleotide change from G → A, corresponding to codon GAA → AAA, and glutamic acid → lysine at position 52 of human RGS10-2.

For transfection, 0.5 μg of plasmid DNA was used per well of a 24-well plate, or scaled up or down appropriately for different sized wells or plates. DNA plasmids were added to cells with lipofectamine reagent according to the manufacturer’s instructions (Thermo Fisher Scientific), and cells were cultured an additional 48 hours in antibiotic-free medium prior to assessing function.

Western Blot Analysis. Cells were lysed in SDS-PAGE sample buffer (0.5 M Tris pH 6.8, 10% SDS, glycerol, β-mercaptoethanol, bromophenol blue), and the samples were subjected to SDS-PAGE using standard protocols followed by transfer to nitrocellulose membranes. Primary antibodies for RGS10, COX-2, P65, and GNA13 were purchased from Santa Cruz Biotechnology. Phosphorylated extracellular signal-regulated kinase (ERK), ERK, phospho-P65, P-AKT, and AKT were obtained from Cell Signaling Technology (Danvers, MA), and gliceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Millipore Technologies (Temecula, CA). Following primary antibody incubation, the suitable secondary horseradish peroxidase (HRP)–conjugated antibodies were used to incubate the membranes: donkey anti-goat IgG-HRP (Santa Cruz Biotechnology), goat anti-rabbit IgG-HRP (Millipore Technologies), and goat anti-mouse IgG-HRP (Bethyl, Montgomery, TX). The membranes were visualized utilizing an enhanced chemiluminescent substrate for detection of HRP (ThermoScientific, Waltham, MA), and quantified using FluorChem HD2 software (Proteinsimple, San Jose, CA). The values were normalized to endogenous control GAPDH.

Quantitative Real-Time Polymerase Chain Reaction. Isolation of mRNA was performed using TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA) and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Actin and/or GAPDH were used as housekeeping genes for normalization. Calculating the fold difference was performed using the 2–ΔΔCT method. Mouse actin and COX-2 mouse and human primers were purchased from Sigma-Aldrich. Mouse TNFα primers and human GAPDH and TNFα primers were obtained from Integrated DNA Technologies (Coralville, IA). The mouse primers used were the following: actin forward 5'-GGCTGATTCCCTCATCCG-3', actin reverse 5'-CCATGGTTGAAACGACCTT-3'; COX-2 forward 5'-TGGCAAGATCCAGGCTACTTCC-3', COX-2 reverse 5'-GCCGTTGTAGGCGTTTTG-3'; and TNFα forward 5'-CCTGGATCCGAGCGCTTG-3', TNFα reverse 5'-GGATGCTAACGAGTTTGG-3', and GAPDH reverse 5'-GCCGCTGGTTTACGCTT-3'. The human primers used were the following: GAPDH forward, 5'-GGACTACTGGATCCATT-3', GAPDH reverse, 5'-AAAGGGATGACTTCTCT-3'; TNFα forward 5'-CCATGGTTGAAACGACCTT-3'; and COX-2 forward 5'-CCTGGATCCGAGCGCTTG-3'. The human primers used were the following: GAPDH forward, 5'-GGACTACTGGATCCATT-3', GAPDH reverse, 5'-AAAGGGATGACTTCTCT-3'; TNFα forward 5'-CCATGGTTGAAACGACCTT-3'; and COX-2 forward 5'-CCTGGATCCGAGCGCTTG-3'.

Coimmunoprecipitation. For the coimmunoprecipitation experiments in BMV-2 microglia, cells were plated in 15 cm dishes and then lysed with 1.5 ml of a modified lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 6 mM MgCl2, 1% NP40) containing protease/phosphatase inhibitor cocktails (Cell Signaling Technology). Cell lysate was left for 30 minutes on ice and subsequently centrifuged at 27,216 × g for 10 minutes at 4°C. Cells lysates were incubated with either GDP or GTPγS at 20°C with gentle shaking for 30 minutes. Next, 2 μg of Gαi3 antibody (Santa Cruz Biotechnology) or normal rabbit IgG (Santa Cruz Biotechnology) was added. In the coimmunoprecipitation experiments HEK-kiTLR4293 cells were transfected prior to the coimmunoprecipitation with 0.25 μg
of RGS10 WT, RGS10 E52K, and Goα3. Following transfection, the
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Fig. 1. Loss of RGS10 enhances LPS-stimulated COX-2 expression and PGE2 production in BV-2 microglia. (A) BV-2 microglia cells were plated in six-well plates and simultaneously transfected with either control or RGS10 siRNA. Cells were cultured for 24 hours and then incubated with vehicle or LPS (10 ng/ml) for an additional 24 hours. RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction were performed as described in Materials and Methods. Expression of COX-2 mRNA was normalized to the control actin, and relative expression levels were calculated by the \(2^{-\Delta\Delta Ct}\) method. Data were analyzed from two independent experiments. The difference between groups was analyzed by analysis of variance (ANOVA), followed by Tukey’s test. Data are presented as mean ± S.E.M., where * \(P < 0.05\), ** \(P < 0.01\), and *** \(P < 0.001\). (B and C) BV-2 microglia were plated in 24-well plates and transfected with control or RGS10 siRNA for 24 hours and then treated with vehicle or LPS for an additional 24 hours. Cells were lysed, and SDS-PAGE was performed followed by immunoblotting using specific antibodies against COX-2, RGS10, and the loading control GAPDH. (B) The image is representative of two independent experiments. (C) Images from two independent western experiments were quantified with densitometry. (D) BV-2 microglia cells were plated in six-well plates and simultaneously transfected with either control or RGS10 siRNA. Cells were cultured for 24 hours and then incubated with vehicle or LPS (10 ng/ml) for an additional 24 hours. Culture medium was collected and PGE2 levels were measured using enzyme-linked immunosorbent assay. Conversion of raw absorbance values to picograms per milliliter concentration was conducted using a standard curve following the manufacturer’s protocol. Data were analyzed from four independent experiments and the difference between groups was analyzed by ANOVA, followed by Tukey’s test. Data are presented as mean ± S.E.M., where * \(P < 0.05\). (E–G) BV-2 cells were stably infected with control or RGS10-targeted CRISPR/Cas9 lentivirus as described in Materials and Methods. Control and RGS10 knockout cells were treated with 10 ng/ml
receptor classes and downstream inflammatory signaling (Ye, 2001; Fan et al., 2004; Dauphinee et al., 2011). Given the ability of RGS10 to deactivate G\textsubscript{i} and the ability of G\textsubscript{i} pathways to activate ERK, AKT, and NF\textkappa B, we predicted that RGS10 regulation of TLR4-induced COX-2 and PGE\textsubscript{2} production may be mediated by RGS10 deactivation of G\textsubscript{i} proteins that impact the ability of TLR4 to initiate these pathways. To explore this hypothesis further, we tested LPS-induced phosphorylation of p42/44 ERK with or without RGS10 knockdown. RGS10 knockdown had no effect on ERK phosphorylation following 20 minutes of LPS treatment (Fig. 2C). Similarly, acute LPS treatment stimulated phosphorylation of AKT and p38 in BV-2 microglia, but RGS10 knockdown had no effect on these responses (data not shown). Phosphorylation of NF\textkappa B subunit p65, an early event in the NF\textkappa B activation cascade, was also significantly enhanced by LPS treatment, but this response was not affected by RGS10 knockdown (Fig. 2D). Therefore, endogenous RGS10 appears to function as a G\textsubscript{i} GAP, but does not regulate acute LPS-stimulated signaling pathways that are coregulated by G\textsubscript{i}.

The Effect of G\textsubscript{i} Inhibition on RGS10 Knockdown–Induced Enhancement of TLR4 Signaling. To further explore the role of G\textsubscript{i} in RGS10’s effects on inflammatory signaling, we next sought to determine whether enhanced COX-2 expression resulting from RGS10 knockdown is the result of amplified G\textsubscript{i} signaling. If the primary mechanism by which RGS10 regulates LPS-stimulated inflammatory signaling is through regulation of G\textsubscript{i} GAP activity, then the effects of RGS10 knockdown should be reversed by inhibition of G\textsubscript{i}. To test this prediction, we measured the expression of COX-2 mRNA after LPS treatment and RGS10 knockdown with or without pretreatment with the G\textsubscript{i} family inhibitor PTX. Surprisingly, the RGS10 siRNA-mediated increase in LPS-stimulated COX-2 mRNA production (Fig. 3A) or protein levels (Fig. 3B) was completely resistant to PTX pretreatment. Since RGS10 loss has also been shown to enhance the production of the inflammatory cytokine TNF\textalpha, we further tested whether RGS10 knockdown–induced increase in TNF\textalpha expression was sensitive to G\textsubscript{i} inhibition. Like COX-2, the increase in LPS-stimulated TNF\textalpha expression mediated by RGS10 knockdown was not affected by PTX (Fig. 3C). To ensure that the dose of PTX used is fully efficacious in BV-2 cells, we confirmed that PTX completely blocked CXCL12-stimulated AKT phosphorylation, which has been shown to be mediated by G\textsubscript{i} signaling (Kumar et al., 2012) (Fig. 3D). Collectively, these data suggest that RGS10 knockdown–mediated amplification of COX-2 and TNF\textalpha expression is not mediated by enhanced G\textsubscript{i} signaling.
Evaluating HEK293-HourTLR4 Cells as a Model to Study the Effect of RGS10 on TLR4-Induced Inflammatory Signaling. To more directly test the possibility that RGS10 regulates LPS-stimulated inflammatory signaling through a GAP-independent mechanism, we sought to assess whether RGS10 G protein binding activity is required for this effect by comparing the effects of exogenous WT and GAP-deficient RGS10. BV-2 microglia cells are not an appropriate model for this experiment due to their low transfection efficiency and high levels of endogenous RGS10. As an alternative, we used HEK293-hourTLR4 cells because they are easy to transfect, express low endogenous RGS10 levels, and stably overexpress TLR4 receptors. To confirm that this cell line is responsive to LPS treatment, we treated HEK293-hourTLR4 cells with LPS (10 ng/ml), a dose that potently activates TLR4 signaling in BV-2 microglia. LPS caused a dramatic increase in TNFα mRNA levels (Fig. 4A), and phosphorylation of p65-NFκB and p44/42 mitogen-activated protein kinase (data not shown) in HEK293-hourTLR4 cells. However, COX-2 expression was not affected by LPS treatment in these cells, demonstrating that not all downstream TLR4 signaling pathways observed in BV-2 microglia will be recapitulated in HEK293-hourTLR4 cells. Nonetheless, the cells provided a suitable platform to assess the effect of exogenous RGS10 on LPS-induced production of TNFα. To address this question, we overexpressed WT RGS10 via transient plasmid transfection, inducing robust expression of exogenous RGS10 mRNA and protein. RGS10 overexpression suppressed LPS-induced production of TNFα after 24 hours of LPS treatment (Fig. 4A). In contrast, RGS10 overexpression activates TLR4 signaling in BV-2 microglia.
had no effect on acute LPS-induced activation of ERK and NFκB (data not shown). Finally, to determine if this effect is shared among closely related RGS domains, we overexpressed the RGS domain of RGS14 and compared the effect on LPS-stimulated TNFα expression. The exogenous RGS14 RGS domain, when expressed at similar levels to exogenous RGS10, did not have any effect on LPS-stimulated TNFα expression (Fig. 4B). These results suggest that RGS10 regulates TLR4-stimulated gene expression to a greater extent than homologous RGS domains.

The Effect of RGS10 WT and E52K Mutant on TLR4-Induced Signaling in HEK293-HourTLR4 Cells. We generated a single amino acid mutation E52K in RGS10 corresponding to a previously characterized GAP-dead mutation in the RGS domain of RGS12 (Sambi et al., 2006). To confirm that the mutation did not affect expression or protein stability, we compared RGS10 protein levels 48 hours after transient transfection with plasmid encoding RGS10 WT or RGS10 E52K in HEK293-hourTLR4 cells. Our results indicate that the RGS10E52K mutant was expressed equally to the WT (Fig. 5A). To confirm that the RGS10 E52K mutant is indeed unable to bind the active form of G proteins, we transfected HEK293-hourTLR4 cells with WT and E52K RGS10 and performed communoprecipitation to compare their G protein binding abilities. Only RGS10WT, and not the E52K mutant, was able to bind active Go;i3-GDP-AlF4 (Fig. 5B), which confirms that the RGS10 E52K mutant has deficient GAP activity. Next, we aimed to confirm that the RGS10 GAP-deficient mutant lost the ability to suppress Gi-mediated signaling pathways. Unfortunately, comparing the effect of overexpressed WT and mutant RGS10 on GPCR-evoked Gi signaling in HEK293-hourTLR4 cells was not possible because we have not observed any effect of exogenous WT RGS10 overexpression on signaling stimulated by specific Gi-coupled GPCRs, including the multiple purinergic, lysosphospholipid, and chemokine receptor agonists that we tested (data not shown). It is possible that RGS10 regulates Gi-mediated signaling downstream from select GPCRs that we have not yet tested. However, it is also possible that, although RGS10 can interact with activated Gi in cell lysates, it does not function to regulate acute signaling following activation of cell-surface GPCRs. To indirectly address the effect of the EK mutant on signaling, we turned to the SKOV-3 cells, in which we have shown that exogenous RGS10 overexpression regulates basal ERK mitogen-activated protein kinase activity in the presence of serum. In these cells, overexpression of WT RGS10, but not EK mutant RGS10, suppressed ERK phosphorylation (Fig. 5C), suggesting that this mutant is defective in regulating G protein signaling pathways.

Finally, we compared the ability of WT and GAP-dead E52K RGS10 to suppress LPS-induced TNFα mRNA in HEK293-hourTLR4 cells. In sharp contrast to the lack of Go;i interaction activity of RGS10 E52K, we show that overexpression of both RGS10 WT and E52K mutant resulted in similar inhibition of LPS-induced production of TNFα (Fig. 5D). RGS10 mRNA was measured in parallel with TNFα in each experiment to ensure equivalent transfection efficiency of RGS10WT and E52K. These data provide further compelling evidence that RGS10 inhibits TLR4 signaling in a mechanism independent of its G protein interaction and GTPase accelerating activity.

Loss of RGS10 Enhances TNFα and COX-2 Expression in Ovarian Cancer Cells, and This Effect Is Mediated via a Go;i-Independent Mechanism. The previous data demonstrate RGS10 suppression of the inflammatory cytokine TNFα and the inflammatory enzyme COX-2 in microglia. However, RGS10 has also been shown to play important roles in other physiologic systems, most notably in ovarian cancer cells (Hooks et al., 2010; Ali et al., 2013). RGS10 suppresses survival signaling and maintains sensitivity to chemotherapeutic-induced cell death in these cells, but the mechanism is unknown and has not been directly linked to a Go;i-mediated pathway. Inflammatory signaling including COX-2- and TNFα-mediated pathways are directly linked to ovarian cancer chemoresistance (Symowicz et al., 2005; Gu et al., 2008). Based on our previous results demonstrating that
RGS10 regulates the expression of both COX-2 and TNF-α in microglia, we aimed to test whether RGS10 also regulates inflammatory mediators in ovarian cancer, and if so whether RGS10 also functions in a G protein–independent mechanism in ovarian cancer cells. We transfected SKOV-3 ovarian cancer cells with RGS10 siRNA or control siRNA in the presence or absence of PTX, and assessed the mRNA levels of COX-2 and TNF-α. SKOV-3 cells transfected with RGS10 siRNA produced significantly higher levels of COX-2 (Fig. 6A) and TNF-α (Fig. 6B) mRNA compared with cells transfected with control siRNA. SKOV-3 cells did not require receptor stimulation to observe an effect of RGS10 knockdown on COX-2 or TNF-α expression, and these cells did not respond to LPS treatment with enhanced COX-2 or TNF-α expression. As observed in microglia, PTX treatment had no effect on RGS10 siRNA-mediated upregulation of COX-2 and TNF-α expression. Successful knockdown of RGS10 following siRNA transfection in SKOV-3 cells was confirmed by real-time polymerase chain reaction (Fig. 6C), and we confirmed that the dose of PTX used in the experiment was sufficient to fully inhibit LPA-mediated ERK phosphorylation in SKOV-3, an established Gαi-mediated event (Fig. 6D) (Hurst et al., 2008). These results demonstrate for the first time that RGS10 regulates inflammatory signaling pathways in ovarian cancer cells and that RGS10 anti-inflammatory actions and mechanisms are not exclusive to immune cells but extend to other models. These findings provide new insight into understanding the mechanisms by which RGS10 suppresses chemoresistance and survival of ovarian cancer cells.

Discussion

RGS10 regulates cellular physiology and fundamental signaling pathways in microglia (Lee et al., 2008, 2011), macrophages (Lee et al., 2013), T-lymphocytes (Lee et al., 2016), neurons (Lee et al., 2012), osteoclasts (Yang and Li, 2007; Yang et al., 2007, 2013), cardiomyocytes (Miao et al., 2016), platelets (Hensch et al., 2016, 2017), and cancer cells (Hooks et al., 2010; Ali et al., 2013; Cacan et al., 2014; Hooks and Murph, 2015). However, despite its small size and seemingly simple function as a G protein GAP, the molecular mechanisms accounting for RGS10 effects have not been defined. The results presented here significantly expand our understanding of the scope and mechanism of RGS10 regulation of inflammatory signaling. First, we show for the first
time that RGS10 regulates COX-2 expression and subsequent prostaglandin production. Given the essential role of COX-2 and PGE2 in the physiology of diverse systems, this finding expands the potential relevance of RGS10 in physiology and disease. Furthermore, our results provide the first evidence of regulation of inflammatory signaling pathways in cancer cells by RGS10, establishing a potential explanation for the effect of RGS10 on ovarian cancer cell survival. Finally, and most importantly, our results demonstrate that the effect of RGS10 on inflammatory signaling in both microglia and ovarian cancer cells cannot be explained by the ability of RGS10 to suppress Gaia signaling. We further show that exogenously expressed GAP-deficient RGS10 suppresses LPS-stimulated TNFα to the same extent as WT RGS10. This surprising result suggests novel RGS10 mechanisms and potentially novel binding partners linking RGS10 to inflammatory signaling mediators TNFα and COX-2.

RGS10 is highly expressed in microglia and RGS10 has profound effects on microglial function. Microglia cells are resident macrophages of the central nervous system and normally function to eliminate pathogens and debris (Kettenmann et al., 2011). However, chronic activation of these cells leads to amplified production of inflammatory cytokines, prostaglandins, and other neurotoxic molecules, ultimately resulting in significant neuronal death and neurodegenerative diseases (Lull and Block, 2010). Microglial RGS10 suppresses the release of TNFα and other inflammatory cytokines following inflammatory triggers (Lee et al., 2008) and plays a protective role against microglia-induced neurodegeneration of dopaminergic neurons (Lee et al., 2011). Reciprocally, microglial activation by either LPS or TNFα suppresses the expression of RGS10 and reduces RGS10 protein levels by approximately 50%–70% for approximately 48 hours (Alqinyah et al., 2017). Thus, endogenous RGS10 silencing mechanisms likely serve to amplify and enhance inflammation in a feed-forward mechanism that sustains a continuous cycle of RGS10 suppression and enhanced production of inflammatory cytokines (Lee et al., 2008; Alqinyah et al., 2017). Importantly, the magnitude and duration of RGS10 suppression induced by transient siRNA transfection recapitulates that observed during suppression of RGS10 expression induced by endogenous activation of microglia (Alqinyah et al., 2017), suggesting that the effects reported here following siRNA knockdown will likely serve to amplify the magnitude of COX-2 and PGE2 signaling during endogenous microglial activation.

COX-2 enhances neuroinflammation and is implicated in microglia-induced neurotoxicity. To further assess inflammatory pathways regulated by RGS10 in microglia, we tested the effect of RGS10 loss on the expression of COX-2 and production of PGE2. RGS10 knockdown enhanced the LPS-induced production of COX-2 and the subsequent release of PGE2 from BV-2 microglia. These findings confirm previous reports demonstrating the anti-inflammatory roles of RGS10 in microglia, and further expand the scope of this effect. COX-2 and PGE2 have been shown to enhance neurodegeneration of dopaminergic neurons.
(Teismann et al., 2003; Sánchez-Pernaute et al., 2004). Therefore, it is possible that the effect of RGS10 on COX-2 and PGE2 production accounts, fully or partially, for the neuroprotective properties of RGS10 on dopaminergic neurons (Lee et al., 2008). Furthermore, since COX-2 and PGE2 play diverse roles in the central nervous system, it is likely that RGS10 serves additional functions in the central nervous system related to COX-2 regulation.

Remarkably, the mechanisms by which RGS10 exerts its anti-inflammatory and neuroprotective actions have not been defined. RGS proteins classically function to accelerate the inactivation of heterotrimeric G proteins, thereby inhibiting G protein–mediated signaling pathways (Watson et al., 1996). Indeed, RGS10 has been shown to function as a bona fide GAP with specificity to Gαi subunits (Hunt et al., 1996), and our data confirm that endogenous RGS10 in microglia does bind transition state Gαi, consistent with its classic GAP function. Gαi has been shown to enhance LPS-induced activation of multiple acute signaling pathways, including Akt and ERK kinase cascades (Fan et al., 2004; Dauphinee et al., 2011), as well as NFκB signaling, which is an essential pathway regulating the production of inflammatory cytokines (Ye, 2001). We also show that LPS stimulation of BV-2 cells enhances RGS10 interaction with Gαi. Therefore, our initial hypothesis was that RGS10, via its classic GTPase-accelerating activity on Gαi, would suppress these acute signaling pathways following LPS treatment. However, RGS10 knockdown had no effect on LPS-induced acute activation of ERK, Akt, or NFκB. This suggested the possibility that RGS10 may function in a mechanism that is independent of its effect on Gαi.

Our data also revealed that the amplified TNFα and COX-2 production observed with RGS10 knockdown was not altered by Gαi inhibition with PTX. While PTX is an established and commonly used inhibitor of Gαi, it can produce G protein–independent effects (Mangmool and Kurose, 2011). To more rigorously assess the role of Gαi in RGS10 function, we created a RGS10 mutant E52K construct based on a previously characterized GAP-dead RGS12 construct (Sambil et al., 2006). Overexpression studies comparing RGS10 E52K and WT RGS10 were performed in HEK293-hourTLR4 cells, which stably express TLR4, MD-2, and CD14 co-receptor genes to recapitulate functional TLR4 signaling. Indeed, we confirmed that HEK293-hourTLR4 cells show dramatically enhanced TNFα expression and activation of NFκB and ERK phosphorylation in response to LPS treatment with similar dose response as BV-2 cells. We demonstrated that overexpression of GAP-deficient mutant RGS10 suppressed LPS-induced TNFα to the same degree as WT, suggesting that Gαi interaction is not required for the ability of RGS10 to inhibit TLR4 signaling. These data, combined with the observation that RGS10 regulation of LPS-stimulated TNFα and COX-2 is PTX insensitive in BV-2 microglia cells, strongly suggest that RGS10 anti-inflammatory effects are mediated by G protein–independent mechanisms. Furthermore, we have validated the RGS10E52K mutant as a valuable tool to assess the role of RGS10 GAP activity in mediating the overall functions of RGS10.

In addition to its anti-inflammatory effects in microglia and macrophages, RGS10 has also been shown to regulate ovarian cancer cell survival and chemosensitivity. We have previously postulated that the ability of RGS10 to regulate cell survival in ovarian cancer cells was based on its ability to regulate signaling through Gαi-coupled receptors for growth factors such as LPA, a well-established autocrine survival factor that is upregulated in ovarian cancer (Hurst et al., 2008). However, while RGS10 suppression enhanced cell survival and basal AKT signaling, it did not enhance LPA-stimulated AKT or ERK signaling in our previous studies (Hooks et al., 2010). Our current observations demonstrate that RGS10 suppression strongly enhances basal TNFα and COX-2 expression in SKOV-3 ovarian cancer cells, indicating that RGS10 suppresses the production of inflammatory mediators in both immune and nonimmune cells. As observed in BV-2 microglia, Gαi inhibition had no effect on RGS10 knockdown–induced upregulation of COX-2 and TNFα in SKOV-3. TNFα and COX-2 are strongly implicated in the development of chemoresistance and control of cell survival of many cancers including ovarian cancer, suggesting that these pathways may mediate the effect of RGS10 on chemoresistance (Kulbe et al., 2007; Greenhough et al., 2009). Further studies are warranted to determine if the ability of RGS10 to regulate inflammatory signaling in cancer cells fully accounts for its effects on chemoresistance, and if this mechanism extends to other cancer types.

The notion of GAP-independent mechanisms for RGS proteins is not new, and has been described in multiple RGS family members (see the review in Sethakorn et al., 2010). However, many of these RGS proteins possess, in addition to RGS domains, additional domains that mediate some of these GAP-independent functions. For example, RGS12 and RGS14, the RGS family members most closely related to RGS10, interact with RAS and MEK2 proteins via RBD and PDZ domains, respectively, to perform multiple GAP-independent functions (Willard et al., 2007). In contrast, RGS10 contains only the conserved RGS domain and short, disordered N- and C-terminal extensions containing sites for regulatory modifications, but no defined functions. This suggests that the RGS domain itself may mediate the GAP-independent function of RGS10. Indeed, G protein–independent interactions and associations have been mapped to the RGS domains of multiple RGS proteins (Popov et al., 2000; Nguyen et al., 2009; Sethakorn et al., 2010), supporting the notion that the RGS domain is much more than just a GAP for G proteins. Structural studies have identified modest differences in the structure and flexibility of the RGS10 RGS domain, compared with other family members, which may allow distinct binding interactions (Soundararajan et al., 2008). Therefore, we predict that G protein–independent mechanisms and binding partners facilitate a significant subset of RGS10 functions. Identification and delineation of these novel molecular mechanisms is a critical next step in understanding RGS10 function, and will facilitate strategic targeting of RGS10 in the diverse pathologies in which its function is implicated.

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Authorship Contributions

Participated in research design: Alqinyah, Hooks.

Conducted experiments: Alqinyah, Almutairi, Wendimu.

Performed data analysis: Alqinyah, Almutairi, Wendimu, Hooks.

Wrote or contributed to the writing of the manuscript: Alqinyah, Hooks.
References


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Mohammed Alqinyah, Faris Almutairi, Menbere Y. Wendimu, Shelley B. Hooks.

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Supplemental data Figure 1.

Specificity of transient transfection of RGS10-targeted siRNA duplexes.

BV-2 microglia cells were plated in a 6 well plate and transiently transfected with control or RGS10-targeted siRNA constructs for 48 hours. Cells were harvested in TRIzol, and RNA was isolated. RGS10 (A), RGS2 (B), RGS12 (C), and RGS14 (D) transcript levels were quantified using quantitative RT-PCR and normalized to the housekeeping gene Actin. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method. Data in (A-D) are compiled from two independent experimental repeats. Data were analyzed for statistical differences using an unpaired $t$ test. Data are presented as Mean ± SEM where *: $P<0.005$ indicates the levels of significance.