Published in final edited form as:

Biochem J. ; 474(14): 2301–2313. doi:10.1042/BCJ20170093.

G-protein coupled receptor kinase-2 is a critical regulator of TNF α signaling in colon epithelial cells

Michael D. Steury¹, Peter C. Lucas², Laura R. McCabe¹, and Narayanan Parameswaran^{1,*}

¹Department of Physiology, Michigan State University, East Lansing, MI 48824

²Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania

Abstract

G protein-coupled receptor kinase-2 (GRK2) belongs to the GRK family of serine/threonine protein kinases critical in the regulation of GPCRs. Apart from this canonical role, GRK2 is also involved in several signaling pathways via distinct intracellular interactomes. In this study, we examined the role of GRK2 in TNF α signaling in colon epithelial cell-biological processes including wound healing, proliferation, apoptosis, and gene expression. Knockdown of GRK2 in the SW480 human colonic cells significantly enhanced TNF α -induced epithelial cell wound healing without any effect on apoptosis/proliferation. Consistent with wound healing effects, GRK2 knockdown augmented TNF α -induced matrix metalloproteinases (MMP) 7 and 9 as well as urokinase plasminogen activator (uPa) (factors involved in cell migration and wound healing). To assess the mechanism by which GRK2 affects these physiological processes, we examined the role of GRK2 in TNF α -induced MAPK and NF κ B pathways. Our results demonstrate that while GRK2 knockdown inhibited TNF α -induced I κ B α phosphorylation, activation of ERK was significantly enhanced in GRK2 knockdown cells. Our results further demonstrate that GRK2 inhibits TNF α -induced ERK activation by inhibiting generation of reactive oxygen species (ROS). Together, these data suggest that GRK2 plays a critical role in TNF α -induced wound healing by modulating MMP7, 9 and uPA levels via ROS-ERK pathway. Consistent with the in vitro findings, GRK2 heterozygous mice exhibited enhanced intestinal wound healing. Together, our results identify a novel role for GRK2 in TNF α signaling in intestinal epithelial cells.

INTRODUCTION

G-protein coupled receptors kinases (GRKs) are serine/threonine kinases that include 7 distinct proteins separated into three subfamilies based on their sequence homology and functional similarities. These include the retinal kinase (GRK1 and GRK7), the GRK2 (GRK2 and GRK3), and the GRK4 (GRK4, 5, and 6) subfamilies [1]. GRKs were initially described for their canonical regulation of G-protein coupled receptor (GPCR) phosphorylation and desensitization. However, it is now clear that their role in cell signaling is not limited to this canonical function. GRK2 can interact with and regulate (or be regulated by) a wide variety of non-GPCRs as well as non-receptor substrates, including

*Corresponding author: Narayanan Parameswaran BVSc., Ph.D., 567 Wilson Road, # 2201 Biomedical Physical Sci Bldg, Department of Physiology, East Lansing, MI 48824, USA.

IGF-1R, insulin-R, EGFR, ERK, MEK, I κ B α , and p38, which demonstrates its capacity to impact a diverse set of cellular functions through both phosphorylation-dependent and -independent mechanisms [2]. These non-canonical roles of GRK2 allow for this kinase to influence basic cellular processes such as inflammatory gene expression, cellular migration, as well as mitochondrial metabolism [3]. The role for GRK2 in these cellular functions has been extensively examined in cardiac, immune and other cell types [2,4,5], but the role of GRK2 in intestinal epithelial cells is not well known.

Mucosal surfaces, including the intestinal tract, are lined by epithelial cells and are critical in defining and maintaining a barrier between the host and external environment. Dysregulation of this barrier integrity leads to inflammatory processes including inflammatory bowel disease (IBD) [6,7][8,9]. Intestinal epithelial cell healing (proliferation, differentiation, migration) and other epithelial dynamics such as permeability become impaired in inflammatory bowel disease (IBD) [10]. In addition, patients with chronic IBD are also at increased risk for colitis-associated colon cancer. Thus, understanding the signaling mechanisms that regulate colonic epithelial cell biology is important for long-term drug development strategies for IBD and colitis-associated colon cancer.

IBD is associated with an increase in inflammatory cytokines, such as TNF α , and these cytokines have been shown to alter the ability of epithelial cells to repair damage to the monolayer. These changes are mediated via intracellular signaling through regulation of NF κ B and MAPK pathways which affect epithelial cell apoptosis, proliferation and migration from the crypts [11]. In previous studies, we and others showed that GRK2 is an important regulator of TNF α signaling in myeloid cells both *in vitro* and *in vivo* [12–17]. However, the function of GRK2 in intestinal epithelial cells in the context of TNF α signaling processes is not known. Therefore, in this study, we examined the role of GRK2 in colonic epithelial cells in terms of various cell biological responses including cell signaling pathways that are modulated by GRK2. Our studies underscore a critical role of GRK2 in intestinal epithelial cell wound healing *in vitro*. Part of this regulation occurs via the role of GRK2 in TNF α -induced ROS-ERK pathway and subsequent MMP expression. Consistent with *in vitro* findings, we also demonstrate that intestinal wound healing is enhanced in GRK2 heterozygous knockout mice *in vivo*.

Materials and Methods

Materials

Cell Culture and knockdown of GRK2—Human colon epithelial cell line SW480 was obtained from ATCC and was maintained in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose and supplemented with 10% (v/v) fetal bovine serum (Life Technologies) along with 5% penicillin/streptomycin (Life Technologies). These cells were grown at 37° C in a humidified 5% CO₂ incubator. One hour prior to stimulation, cells were replenished with culture media without FBS and then stimulated with 20 ng/ml of TNF α (PeproTech) for various time points as indicated. For knockdown of GRK2, 1 million cells were treated with 25 pmoles of "SmartPool siRNA" containing a mixture of four designed siRNAs targeting GRK2 or scrambled siRNA (Dharmacon) and electroporated using an Amaxa V electroporation instrument using the standard protocol provided by the supplier

and solution V (Lonza) and program L-024. The cells were then incubated for 48 hours to facilitate knockdown before being used for experiments.

Scratch Wound Healing Assay—SW480 cells were cultured in a 6-well plate and the confluent cells were used for *in vitro* scratch-wound assay [18]. Cells were treated with TNF α (20 ng/ml) or vehicle at time 0 hours. To create the wound, a uniform scratch was created vertically in the culture dish using a 200 μ l plastic pipette tip and the plate was then incubated for 48 additional hours in the presence of mitomycin C (1 μ g/ml) to inhibit proliferation. The scratch was assessed and photos of the wound were taken at 0, 16, 24, 32, and 48 hours. Images were taken with cellSens Software (Olympus) and the distance of the wound was calculated by measuring the distance between the leading edges of the wound using ImageJ software. The effect of TNF α on wound closing was calculated by determining the amount of wound closed relative to the initial time point in the respective groups.

Proliferation Assay—After siRNA transfection, SW480 cells were cultured in a 96 well plate at a concentration of 50,000/well for 24 or 48 hours and stimulated with TNF α (20 ng/ml) or vehicle for 24 or 48 hours. The amount of proliferation was determined using the CyQuant Cell Proliferation Assay (Thermo Scientific). In short, after TNF α treatment the media was removed and the plate was incubated at -80° C overnight. The following day the plate was thawed and a DNA binding dye in lysis buffer was added to each well and the fluorescence was read on a Tecan M2000 spectrophotometer at 485 nm excitation and 530 nm emission and compared to a standard curve.

Apoptosis Assay—After siRNA transfection, SW480 cells were cultured in 6-well plates at a concentration of 1 million cells/well for 48 hours and were treated with TNF α (20 ng/ml) for 24 or 48 hours. Apoptosis was quantified using Annexin V Apoptosis Detection Kit (eBioscience) according to the manufacturer's protocol. Cells were quantified using LSR II flow cytometer and data was analyzed using FlowJo software. Data was divided into 4 categories: Live (Annexin V “–”, Propidium Iodide (PI) “–”), Early Apoptotic (Annexin V “+”, PI “–”), Apoptotic (Annexin V “+”, PI “+”), and Necrotic/Dead (Annexin V “–”, PI “+”).

ROS Assay—After siRNA transfection, SW480 cells were cultured in 6-well plates at a concentration of 1 million cells/well for 48 hours and stimulated for 10 or 20 minutes with TNF α (20 ng/ml) or vehicle. ROS was measured using CellRox Green Reagent (Thermo Scientific). Briefly, the cells were pretreated with CellRox Green reagent (5 μ M) for 30 minutes. The cells were then treated with TNF α and after the treatment washed 3 times with PBS. The cells were fixed with 3.7% formaldehyde for 15 minutes, washed 3 times with FACS buffer (0.5% BSA, 0.05% Sodium Azide, in 1x PBS) and quantified using LSR II flow cytometer and analyzed using FlowJo software. Data is expressed as mean fluorescent intensity (MFI).

Invasion Assay—After siRNA transfection, SW480 cells were cultured in a 6-well plate to facilitate the siRNA mediated knockdown of GRK2. After 48 hours, the cells were removed from the plate and 20,000 cells were seeded in serum free media in the upper

chamber of transwell inserts coated with matrigel (Cell BioLabs Inc) and cellular migration was determined per manufacturer's protocol. In brief, serum-containing media was inserted in the lower chamber and the cells were incubated for 48 hours to allow for migration through the membrane in the presence or absence of TNF α (20ng/ml). After 48 hours the migrated cells were detached using the manufacturer's detachment solution and lysed in lysis buffer. A fluorescent dye was added to the lysate and fluorescence was read on a Tecan M2000 spectrophotometer at 485 nm excitation and 530 nm emission.

RT-PCR—RNA was extracted from SW480 cells using the RNEasy Kit (Qiagen) and treated with DNase (LifeTech) per manufacturer's protocol. A total of 1 μ g RNA was used for the template for single-stranded cDNA synthesis. Quantitative real-time PCR was performed for the various genes and the cDNA was amplified using SYBR Green Pro Master Mix and the genes of interest were normalized to the corresponding hypoxanthine-guanine phosphoribosyltransferase (HPRT) controls as described before [19].

Western Blot Analysis—Sample preparation and Western blot analysis were done as described before [20]. Briefly, cells were lysed in lysis buffer (20mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl) containing 1% Triton X-100 and protease inhibitors (protease inhibitor cocktail, Roche Diagnostics) and phosphatase inhibitors. Insoluble material was removed by centrifugation and protein concentration was determined by Bradford assay. Proteins in the cellular lysates were separated 10% SDS-PAGE and the gel was electro-blotted against nitrocellulose membrane (Thermo Fisher Scientific). Following this transfer, the blot was probed with specific primary antibodies for pERK 1/2, total ERK-2, p38, pp38, JNK1/2, pJNK1/2 and tubulin (Santa Cruz) as well as I κ B α and pI κ B α (Cell Signaling) diluted in LiCor blocking buffer (LiCor) and followed by anti-rabbit or anti-mouse IR dye-conjugated secondary antibodies (LiCor). The blot was scanned and analyzed with an Odyssey LiCor instrument along with its software Odyssey.

Mice—GRK2 heterozygous mice were obtained from Jackson Labs (kindly donated by Dr. Robert Lefkowitz, Duke University). Animals were bred at Michigan State University by breeding GRK2 heterozygous mice with wild-type mice. Note that homozygous knockout of GRK2 are embryonically lethal [21]. All animals were housed in a specific-pathogen-free facility maintained at 22–24 C with a 12H light-dark cycle and were given mouse chow and water *ad libitum*. All animals were performed with age- and sex- matched mice (males) between 8 and 10 weeks of age. All animal procedures were approved by the Michigan State University IACUC and conformed to NIH guidelines.

Statistical Analysis—All values are represented as mean $\pm$ SEM. All data were tested for statistical analysis for statistical significance using unpaired Student's T-test (two sample comparisons) and Analysis of Variance (ANOVA) with Tukey post-hoc test (more than 2 sample comparisons). The analysis was done using Prism GraphPad software. A p-value <0.05 was considered significant.

RESULTS

GRK2 suppresses TNF α induced epithelial wound closure

The present studies were designed to investigate the role of GRK2 in TNF α signaling in intestinal epithelial cells. In other studies, TNF α has been shown to significantly modulate the intestinal wound healing response and protect from intestinal epithelial apoptosis [11]. To this end, we performed a scratch wound-healing assay with SW480 cells (human colonic epithelial cell line) to identify the role of GRK2 in TNF α -induced wound healing. For this, cells were transfected with either control siRNA or GRK2 siRNA and plated as described in the methods. GRK2 knockdown was ~80–90% compared to controls (Fig 1a–b). The wound was induced by scratch assay and wound closure was monitored up to 48 hours.

Proliferation was inhibited using mitomycin C and therefore any cells in the wound space are the result of cell migration and not due to cell proliferation. The scratch-induced wound size was similar in each experimental group at time 0. The effect of TNF α on wound closing was calculated by determining the amount of wound closed through quantification of the gap distance using Image-J software and comparing the amount of wound closed to the initial time point in the respective groups. We observed that the amount of wound closure was similar between untreated and TNF α treated groups in the control siRNA transfected cells, at all the time points tested. However, in the GRK2 knockdown cells, wound size was markedly reduced in response to TNF α (Figure 1c–d). This suggests that GRK2 normally inhibits TNF α -induced wound healing and therefore, its knockdown unmasks the healing potential of TNF α . Importantly, the concentration of TNF α used in these experiments had no effect on SW480 proliferation or apoptosis between the control and the GRK2 knockdown cells (Figure 2a–b). To further understand whether this phenomenon was kinase activity-dependent we performed the wound healing experiments in the presence of a GRK2 kinase inhibitor, paroxetine. Paroxetine binds to the active site of GRK2 inhibiting its kinase function [22]. Pretreatment of SW480 cells with paroxetine (10 μ M) did not affect wound healing in the absence or presence of TNF α , suggesting that our observations with GRK2 knockdown are likely kinase independent (data not shown). Together, our results indicate that knockdown of GRK2 in intestinal epithelial cells accelerates TNF α -induced wound healing process.

GRK2 differentially modulates TNF α signaling in SW480 cells

Previous studies have shown that GRK2 can influence TNF α signaling in macrophages [12]. Our results here suggest that GRK2 can significantly impact TNF α -induced wound healing in epithelial cells. To examine the signaling mechanisms by which this occurs, we treated control and GRK2 siRNA transfected SW480 cells with TNF α for various time points (0 to 90 min) and examined the cell signaling pathways. TNF α stimulation led to increases in phosphorylation of I κ B α and ERK1/2 in the control siRNA treated cells (Figure 3a–b). Surprisingly, these effects were differentially affected in the GRK2 knockdown cells. TNF α -induced I κ B α phosphorylation was significantly inhibited in the GRK2 knockdown cells whereas ERK1/2 phosphorylation was markedly enhanced (Figure 3a–b). These results demonstrate opposing roles of GRK2 in MAPK and NF κ B pathways, with GRK2 augmenting the NF κ B pathway and simultaneously inhibiting the ERK pathway. Other signaling pathways including JNK and p38 were not consistently affected by GRK2

knockdown (data not shown). Overall, these results suggest that GRK2 is an important regulator of ERK1/2 and I κ B α signaling pathways in intestinal epithelial cells.

GRK2 alters TNF α induced gene expression

Both ERK1/2 and NF κ B pathways are potent regulators of gene transcription; therefore we sought to identify which inflammatory and matrix modifying genes are altered by GRK2 knockdown at 6 and 24 hours post-TNF α treatment. TNF α -induced expression of genes that encode extracellular matrix degradation, including matrix metalloproteinase 7 (*MMP7*), matrix metalloproteinase 9 (*MMP9*), as well as urokinase plasminogen activator (*uPA*), were all markedly enhanced in the GRK2 knockdown cells as compared to control cells at 24 hours (Figure 4a). Whereas after 6 hours of stimulation we observed a mixed response in inflammatory gene expression where TNF α -induced expression of *CXCL8* and *TNF α* was markedly enhanced in the GRK2 knockdown cells (compared to control siRNA cells) and *IL6* expression was significantly decreased in GRK2 knockdown compared to control cells (Figure 4a). After 24 hours of TNF α treatment, there essentially was no difference in the expression of these 3 genes between control and GRK2 knockdown groups.

Because MMPs are important in cell migration and MMP9 has been shown to be regulated via the ERK pathway, we investigated whether the TNF α -induced MMP9 in SW480 cells is ERK dependent. To this end, we treated control and GRK2 knockdown cells with MEK1/2 inhibitor U0126 (10 μ M, 40-minute pretreatment) prior to TNF α treatment. Inhibition of MEK-ERK pathway abolished TNF α -induced *MMP9* expression, confirming that *MMP9* expression is driven via the ERK pathway in both control and GRK2 knockdown groups (Figure 4b). We also examined the transcripts of *MMP7* and *uPA* but observed that their regulation is not ERK dependent. We attempted to inhibit the I κ B α pathway using the IKK inhibitor BMS-345541 but the combination of the inhibition of the pro-survival NF κ B and the stimulation with TNF α was detrimental to the cell survival in culture at both 6 and 24 hours (data not shown). To further confirm that an increase in MMP9 activity is important in migration through an extracellular matrix we subjected the SW480 cells to a matrigel invasion assay. Control and GRK2 knockdown SW480 cells were seeded in the upper chamber of a transwell insert coated with matrigel and allowed to migrate towards serum for 48 hours in the presence or absence of TNF α (20ng/ml). As shown, GRK2 knockdown cells treated with TNF α showed enhanced degradation of the matrigel layer and subsequent increase in migration towards the serum stimulus (Figure 4c). Recently, groups have demonstrated that, in other epithelial cell models, MMP9 is critical for the onset and activation of epithelial migration and wound repair [23] suggesting that the effect of GRK2 on ERK1/2 activation and MMP9 expression may be responsible for the increase in TNF α induced wound healing in these cells.

GRK2 localizes in the mitochondria and inhibits ROS production

In previous studies, we have shown that GRK2 can directly regulate TNF α -induced I κ B α phosphorylation [12]. Moreover, because the cellular phenotype (MMP expression and wound healing [23–25]) appears to be regulated via the ERK pathway, we focused on determining the mechanisms by which GRK2 regulates the TNF α -induced ERK pathway. To define how GRK2 knockdown enhanced ERK1/2 phosphorylation, we initially focused

on known GRK2 binding partners with respect to the ERK pathway (mainly MEK1/2). However, our immunoprecipitation approach did not identify MEK1/2 as a binding partner for GRK2 in these cells (data not shown). Therefore, we pursued an unbiased mass-spectrometry approach to identify the various partners of GRK2 in the SW480 cells. Our results indicated that GRK2 may interact with some mitochondrial proteins in this cell line (data not shown).

To further determine if GRK2 can localize to the mitochondria, we isolated mitochondria from untreated and TNF α treated SW480 cells and subjected them to SDS-PAGE followed by Western blotting for GRK2. We found that GRK2 is detectable in the mitochondria even under basal conditions as well as at 10 minutes and 3 hours after TNF α stimulation, indicating that GRK2 is not only present in the mitochondria but remains there even under inflammatory conditions (Figure 5a). To further assess the functional consequences of GRK2 being present in the mitochondria we measured generation of reactive oxygen species (ROS) in control and GRK2 knockdown cells. We focused on ROS because previous studies have shown that ROS generation can lead to ERK activation [26]. Therefore, we treated control and GRK2 siRNA transfected SW480 cells with TNF α and examined ROS production using flow cytometry. There was a modest increase in TNF α -induced ROS production in control cells over the time points tested; however, in the GRK2 knockdown cells, ROS production was significantly enhanced and this was more evident at 10 min following TNF α treatment (Figure 5b–c).

Increase in ROS is responsible for increase in ERK1/2 phosphorylation

ROS is produced by a variety of cell types but is normally viewed as harmful to both cells and tissues. Over the last few decades, the functional capabilities of ROS have been expanding and ROS has been shown to play alternative roles other than causing harmful effects. ROS generation in non-phagocytotic cells (at low concentrations) is recognized as ubiquitous intracellular messengers that can activate mitogen-associated signal transduction pathways, including ERK1/2 [27,28]. To determine if the observed increase in ROS generation was causative for the increases in phosphorylated ERK1/2 in SW480 cells, we treated these cells with a superoxide inhibitor, N-acetylcysteine (NAC), to inhibit the production of ROS and demonstrate the link between ROS and ERK1/2 activation. Control and GRK2 knockdown SW480 cells were pretreated with NAC (100 μ M) for 30 minutes and then stimulated with TNF α for 30 minutes. In the groups untreated with NAC we observed that knocking down GRK2 caused a significant increase in the pERK1/2 (as expected), but in the presence of NAC, ERK activation was suppressed especially in the GRK2 knockdown cells (Figure 6a). This data demonstrates that the enhanced ERK activation observed in the GRK2 knock down cells following TNF α stimulation is due to increase in ROS generation. To further link ROS generation to wound healing response, we repeated the wound healing assay in control and GRK2 knockdown cells pretreated with NAC and untreated or treated with TNF α for 48 hours. As shown NAC significantly inhibited the effect of GRK2 knockdown on TNF α -induced wound closure (Figure 6b). Together this data supports the role that GRK2 inhibits TNF α induced wound healing in these cells via the ROS pathway.

GRK2 heterozygous mice are protected in intestinal wound healing model

Our *in vitro* wound-healing assay indicated that knocking down GRK2 in intestinal epithelial SW480 cells improved their ability to close the wound in the presence of TNF α . A common characteristic of dextran sodium sulfate (DSS) induced colitis in mice is gross epithelial damage and high levels of TNF α in the colon [29,30]. To determine if our *in vitro* results would correlate to an *in vivo* model we used mice that were heterozygous for GRK2 and subjected them to a 2.5% DSS colitis. Both wild-type (WT) and heterozygous GRK2 mice (GRK2^{+/-}) were administered 2.5% DSS in drinking water. Mice were scored daily for weight loss and external signs of disease measured as Disease Activity Score (DAI, indicated by weight loss, loose and bloody stools, hunched posture, crusty eyes, and ruffled hair coat) [31]. After 7 days of DSS, the mice were given clean water and allowed to heal from intestinal inflammation for an additional 5 days. After DSS treatment, DAI increased after day 7 but recovered after day 12 and were back to normal by day 13 in the wild type mice (Figure 7a). Interestingly, the DAI was significantly decreased in the GRK2^{+/-} group. This was also evident in the histopathology where GRK2 heterozygous knockout mice showed marked protection (Figure 7b). GRK2, therefore, is protective in *in vivo* wound healing, perhaps through alterations of the epithelial response to TNF α and its influence on wound healing.

DISCUSSION

Since the discovery of GRK2, it has been shown as a key regulator of G-protein coupled receptor phosphorylation and desensitization. As it continued to be studied, its role has expanded and it has been shown to have many different functions both dependent and independent of its catalytic activity [3]. Furthermore, GRK2 has been shown to regulate non-GPCRs as well as a variety of intracellular targets leading to a number of cellular roles for this kinase. In this study, we investigated these non-receptor regulatory capabilities of GRK2 in intestinal epithelial cells in response to TNF α stimulation.

In previous studies, GRK2 has been demonstrated to play a negative role in wound healing that is also associated with increased levels of TNF α . Measuring levels of inflammatory cytokines in the hearts of mice lacking β 1-adrenergic receptor (B1KO) and expressing β ARKct (a GRK2 inhibitor) there was an increase in TNF α , IL-6 and IL-1 β 24 hours after undergoing myocardial infarction which is indicative of increased healing associated inflammation [32]. Alternatively GRK2 was shown to positively mediate sphingosine-1 phosphate (S1P)-induced ERK1/2 activation and wound closing in HEK293 cells as well as mouse embryonic fibroblasts [33]. In our studies with colonic epithelial cells, GRK2 inhibited TNF α -induced ERK1/2 phosphorylation as well as wound closure, and thus the role of GRK2 in wound healing per se is different between the two studies. In the above study GRK2 regulates ERK signaling through modifications of the scaffolding ability of GIT1 through direct interactions. This modification alters the Rac/PAK/MEK/ERK pathway leading to eventual changes in migration. While these differences in how GRK2 regulates the ERK pathway could be attributed to different cell types and ligands, the role of GRK2 in the SW480 cells offers an alternative pathway of ERK regulation through modification of TNF α -induced ROS production that acts a mitogenic signaling factor to stimulate ERK.

Thus, the critical factor between both of these studies is the activation of the ERK pathway. The role of the ERK pathway in wound healing is becoming more and more apparent through studies in other epithelial cells types and several studies have now shown ERK to be a critical regulator of wound healing and cellular migration [23,24]. Importantly, activation of the ERK pathway has been linked to improved wound healing responses in a variety of different cell types, including colonic epithelial cells [34]. Thus, understanding the mechanisms by which the ERK pathway is regulated in colonic epithelial cells could lead to identification of better therapeutic targets for intestinal inflammation. In this context, our results using GRK2 suggest that this may be one additional mechanism by which ERK pathway and its consequent effect on wound healing is regulated. Thus, we show for the first time that GRK2 inhibits the TNF α induced wound healing response in colonic epithelial cells.

The ERK1/2 pathway has a diverse set of roles within the cell including regulation of cell growth, survival, as well as the regulation of cell motility [35]. A necessary mechanism for cellular migration and wound healing is the capacity for matrix degradation through the localized expression of matrix metalloproteinases. One suggested mechanism for the ability of ERK1/2 to alter migration and wound healing is the upregulation of MMPs for extracellular matrix remodeling [36]. We provide evidence that MMP7, MMP9, and urokinase plasminogen activator (uPA) (another ECM remodeler) are all elevated in GRK2 knockdown cells and that the increase in MMP9 expression especially is ablated by inhibition of the ERK pathway. Other studies demonstrate that MMP9 is critical to the onset and activation of epithelial migration and wound repair in both SW480 cells as well as alternative cell types such as HEK293 cells [23–25]. Consistent with those studies, our data has shown that GRK2 knockdown cells have increased invasive capabilities through a matrigel layer. Taken together, our studies suggest that GRK2 regulation of the ERK pathway in SW480 cells modulates MMP9 and consequent epithelial cell migration.

It is possible that MMP7 and uPA (as well as the inflammatory cytokines, CXCL8, IL6, and TNF α) are regulated through the changes observed in NF κ B signaling or other unidentified pathways. Our lab has previously confirmed that GRK2 regulates the expression of CXCL8 and IL6 through alterations of NF κ B in murine macrophages [17]. In this study, we focused on mechanisms and consequences by which GRK2 regulates the ERK pathway. Thus, regulation of MMP9 through pERK1/2 and the importance of this MMP in published studies indicate that it may be sufficient to drive the phenotype we observe in our system.

Previously we showed that GRK2 could directly influence TNF α signaling through binding to I κ B α [12]. Additionally, GRK2 has been shown to interact with other signaling proteins such as PI3K [37], AKT [38], GIT [39], and MEK1 [40]. Based on these previous studies we hypothesized that GRK2 would interact with an unknown component of the TNF α signaling pathway to influence regulation of ERK1/2 in SW480 cells. Interestingly, our preliminary data suggests that GRK2 may be interacting with different mitochondrial proteins. Indeed, when we isolated the mitochondrial fraction from these cells we observed that GRK2 is present in the mitochondria both basally as well as after TNF α stimulation. This ability for GRK2 to be present in the mitochondria has been shown by other groups in both fibroblasts [41] as well as macrophages [42] and cardiac myocytes [43] but has never been shown in

colonic epithelial cells. Previous work investigating the role of GRK2 in the mitochondria showed that knocking down GRK2 increases the mitochondrial generation of ROS in response to lipopolysaccharide in macrophages [42]. Our results on ROS generation in SW480 cells, following TNF α stimulation, are consistent with these findings. Our results on ROS generation and ERK activation are consistent with previous studies demonstrating a strong link between ROS and ERK pathway [26,46]. In line with this, treatment with superoxide inhibitor completely abolished the enhanced ERK activation observed in GRK2 knockdown cells. Together these results indicate that GRK2 is present both in the cytoplasm as well as in the mitochondria under normal and inflammatory conditions in the colonic epithelial cells and this presence regulates ROS generation and subsequent modulation of ERK-mediated wound healing response.

Inflammatory bowel disease is a chronic inflammatory state characterized by persistent damage to the epithelial barrier in the colon. A common characteristic of dextran sodium sulfate (DSS) induced colitis model in mice is gross epithelial damage and high levels of TNF α in the colon. To determine if our *in vitro* results with SW480 cells can be correlated to *in vivo* pathophysiology, we used mice that were heterozygous for GRK2 and subjected them to a wound healing DSS model. Our data indicates that knocking down GRK2 does, in fact, protect against intestinal inflammation, consistent with the *in vitro* data. It should however, be noted that *in vivo* intestinal inflammation involves several different cell types. Even though the injury is initiated by DSS at the level of the intestinal epithelial cells, pathogenesis of inflammation involves participation of many different cell types including immune cells. Since GRK2 is expressed in many different cell types, it is possible that the effect observed in the heterozygous mice is not restricted to its role in intestinal epithelial cells. These certainly are important questions for future studies.

Although very little is known about the role of GRK2 in the colon or in colitis, GRK2 has been implicated in several diseases including multiple sclerosis [14], Alzheimer's disease [47], and rheumatoid arthritis as well as sepsis and endotoxemia [15]. In addition, others have examined the role of other GRKs, including GRK6, in DSS-induced colitis model and shown that mice deficient in GRK6 have enhanced immune cell infiltration and enhanced severity of colitis [48]. These results suggest that the role of the various GRKs in colitis models could be different and warrant further detailed investigation. Altogether these studies show a regulatory capacity for GRKs in the colon and a vital role of GRK2 in inflammation, this merits future work on the mechanism of protection and the role of GRK2 in colitis.

In conclusion, our studies unravel a critical role for GRK2 in TNF α signaling in colonic epithelial cells and potential role for GRK2 in intestinal inflammation. Through regulation of ROS-generated ERK1/2 phosphorylation GRK2 knockdown enhances the ability of SW480 cells to close a wound possibly through increased MMP9 expression. These studies suggest GRK2 as a possible therapeutic target in intestinal inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the support from NIH (grants HL095637, AI099404 and AR056680 to N.P.). We thank the University lab animal resources for taking excellent care of our animals, and the Histopathology laboratory for their excellent service.

Bibliography

1. Gurevich EV, Tesmer JJG, Mushegian A, Gurevich VV. G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. *Pharmacol Ther*. Elsevier Inc. 2012; 133:40–69.
2. Packiriswamy N, Parameswaran N. G-protein-coupled receptor kinases in inflammation and disease. *Genes Immun*. 2015; 16:367–77. [PubMed: 26226012]
3. Evron T, Daigle TL, Caron MG. GRK2: Multiple roles beyond G protein-coupled receptor desensitization. *Trends Pharmacol Sci*. Elsevier Ltd. 2012; 33:154–164.
4. Penela P, Murga C, Ribas C, Lafarga V, Mayor F. The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets. *Br J Pharmacol*. 2010; 160:821–832. [PubMed: 20590581]
5. Woodall MC, Ciccarelli M, Woodall BP, Koch WJ. G protein-coupled receptor kinase 2: A link between myocardial contractile function and cardiac metabolism. *Circ Res*. 2014
6. Sansonetti PJ. War and Peace at Mucosal Surfaces. *Nat Rev Immunol*. 2004; 4:953–964. [PubMed: 15573130]
7. Kagnoff MF, Eckmann L. Epithelial cells as Sensors for Microbial Infection. *J Clin Invest*. 1997; 100:6–10. [PubMed: 9202050]
8. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*. 2007; 448:427–434. [PubMed: 17653185]
9. Gribar SC, Richardson WM, Sodhi CP, Hackam DJ. No Longer an Innocent Bystander: Epithelial Toll-Like Receptor Signaling in the Development of Mucosal Inflammation. *Mol Med*. 2008; 14:645–659. [PubMed: 18584047]
10. Okamoto R, Watanabe M. Cellular and Molecular Mechanisms of the Epithelial Repair in IBD. *Dig Dis Sci*. 2005; 50:34–38.
11. Leppkes M, Roulis M, Neurath MF, Kollias G, Becker C. Pleiotropic functions of TNF- α in the regulation of the intestinal epithelial response to inflammation. *Int Immunol*. 2014; 26:509–515. [PubMed: 24821262]
12. Patial S, Luo J, Porter KJ, Benovic JL, Parameswaran N. G-protein coupled receptor kinases mediate TNF α induced NF κ B signaling via direct interaction with and phosphorylation of I κ B α . *Biochem J*. 2011; 425:169–178.
13. Patial S, Parameswaran N. Tumor Necrosis Factor α Signaling in Macrophages. *Crit Rev Eukaryot Gene Expr*. 2010; 20:87–103. [PubMed: 21133840]
14. Vroon A, Kavelaars A, Limmroth V, Lombardi MS, Goebel MU, Van Dam AM, Caron MG, Schedlowski M, Heijnen CJ. G protein-coupled receptor kinase 2 in multiple sclerosis and experimental autoimmune encephalomyelitis. *J Immunol*. 2005; 174:4400–6. [PubMed: 15778405]
15. Parvataneni S, Gonipeta B, Packiriswamy N, Lee T, Durairaj H, Parameswaran N. Role of myeloid-specific G-protein coupled receptor kinase-2 in sepsis. *Int J Clin Exp Med*. 2011; 4:320–30. [PubMed: 22140603]
16. Durairaj H, Steury MD, Parameswaran N. Paroxetine differentially modulates LPS-induced TNF α and IL-6 production in mouse macrophages. *Int Immunopharmacol*. Elsevier BV. 2015; 25:485–492.
17. Patial S, Saini Y, Parvataneni S, Appledorn DM, Dorn GW, Lapres JJ, Amalfitano A, Senagore P, Parameswaran N. Myeloid-specific GPCR kinase-2 negatively regulates NF κ B1p105-ERK pathway and limits endotoxemic shock in mice. *J cell Physiol*. 2011; 226:627–637. [PubMed: 20717897]
18. Gao C, Negash S, Guo HT, Ledee D, Wang H, Zelenka P. CDK5 Regulates Cell Adhesion and Migration in Corneal Epithelial Cells. *Mol Cancer Res*. 2002; 1:12–24. [PubMed: 12496365]

19. Loniewski K, Shi Y, Pestka J, Parameswaran N. Toll-like receptors differentially regulate GPCR kinases and arrestins in primary macrophages. *Mol Immunol*. 2008; 45:2312–2322. [PubMed: 18180038]
20. Parameswaran N, Pao CS, Leonhard KS, Kang DS, Kratz M, Ley SC, Benovic JL. Arrestin-2 and G protein-coupled receptor kinase 5 interact with NFkappaB1 p105 and negatively regulate lipopolysaccharide-stimulated ERK1/2 activation in macrophages. *J Biol Chem*. 2006; 281:34159–70. [PubMed: 16980301]
21. Jaber M, Koch WJ, Rockman H, Smith B, Bond RA, Sulik KK, Ross J Jr, Lefkowitz RJ, Caron MG, Giros B. Essential role of β -adrenergic receptor kinase 1 in cardiac development and function. *Proc Natl Acad Sci U S A*. 1996; 93
22. Thal DM, Homan KT, Chen J, Wu EK, Hinkle PM, Huang ZM, Chuprun JK, Song J, Gao E, Cheung JY, et al. Paroxetine is a direct inhibitor of g protein-coupled receptor kinase 2 and increases myocardial contractility. *ACS Chem Biol*. 2012; 7:1830–9. [PubMed: 22882301]
23. Bove PF, Hristova M, Wesley UV, Olson N, Lounsbury KM, van der Vliet A. Inflammatory levels of nitric oxide inhibit airway epithelial cell migration by inhibition of the kinase ERK1/2 and activation of hypoxia-inducible factor-1 alpha. *J Biol Chem*. 2008; 283:17919–28. [PubMed: 18424783]
24. Kim HC, Kim YS, Oh H, Kim K, Oh SS, Kim J, Kim BY, Lee SJ, Choe Y, Kim DH, et al. Collagen Triple Helix Repeat Containing 1 (CTHRC1) acts via ERK-dependent induction of MMP9 to promote invasion of colorectal cancer cells. *Oncotarget*. 2014; 5:519–529. [PubMed: 24504172]
25. Luo Y, Liang F, Zhang ZY. PRL1 Promotes Cell Migration and Invasion by Increasing MMP2 and MMP9 Expression through Src and ERK1/2 Pathways. *Biochemistry*. 2009; 48:1838–1846. [PubMed: 19199380]
26. Iovine B, Iannella ML, Nocella F, Pricolo MR, Bevilacqua MA. Carnosine inhibits KRAS-mediated HCT116 proliferation by affecting ATP and ROS production. *Cancer Lett*. 2012; 315:122–128. [PubMed: 22137144]
27. Zhang J, Jin N, Liu Y, Rhoades RA. Hydrogen Peroxide Stimulates Extracellular Signal-regulated Protein Kinases in Pulmonary Arterial Smooth Muscle Cells. *Am J Respir Cell Mol Biol*. 1998; 19:324–332. [PubMed: 9698606]
28. Chen KC, Zhou Y, Xing K, Krysan K, Lou MF. Platelet derived growth factor (PDGF)-induced reactive oxygen species in the lens epithelial cells: the redox signaling. *Exp Eye Res*. 2004; 78:1057–1067. [PubMed: 15109912]
29. Neurath MF. New targets for mucosal healing and therapy in inflammatory bowel diseases. *Mucosal Immunol*. 2014; 7:6–19. [PubMed: 24084775]
30. Iizuka M, Konno S. Wound healing of intestinal epithelial cells. *World J Gastroenterol*. 2011
31. Sharma D, Malik A, Steury MD, Lucas PC, Parameswaran N. Protective Role of β -arrestin2 in Colitis Through Modulation of T-cell Activation. *Inflamm Bowel Dis*. 2015; 12:2766–2777.
32. Salazar NC, Vallejos X, Siryk A, Rengo G, Cannavo A, Liccardo D, De Lucia C, Gao E, Leosco D, Koch WJ, et al. GRK2 blockade with β ARKct is essential for cardiac β 2-adrenergic receptor signaling towards increased contractility. *Cell Commun Signal*. 2013; 11:64. [PubMed: 23984976]
33. Penela P, Ribas C, Aymerich I, Eijkelkamp N, Barreiro O, Heijnen CJ, Kavelaars A, Sánchez-Madrid F, Mayor F. G protein-coupled receptor kinase 2 positively regulates epithelial cell migration. *EMBO J*. 2008; 27:1206–18. [PubMed: 18369319]
34. Murray D, Morrin M, McDonnell S. Increased Invasion and Expression of MMP-9 in Human Colorectal Cell Lines by a CD44-dependent Mechanism. *Anticancer Res*. 2004; 24:489–494. [PubMed: 15152948]
35. Vial E, Pouyssegur J. Regulation of Tumor Cell Motility by ERK Mitogen-Activated Protein Kinases. *Ann NY Acad Sci*. 2004; 1030:208–218. [PubMed: 15659800]
36. Mccawley LJ, Li S, Wattenberg EV, Hudson LG. Sustained Activation of the Mitogen-activated Protein Kinase Pathway. *J Biol Chem*. 1999; 274:4347–4353. [PubMed: 9933637]
37. Naga Prasad SV, Barak LS, Rapacciuolo A, Caron MG, Rockman HA. Agonist-dependent Recruitment of Phosphoinositide 3-Kinase to the Membrane by Beta-Adrenergic Receptor Kinase 1. *J Biol Chem*. 2001; 276:18953–18959. [PubMed: 11259422]

38. Liu S, Premont RT, Kontos CD, Zhu S, Rockey DC. A crucial role for GRK2 in regulation of endothelial cell nitric oxide synthase function in portal hypertension. *Nat Med.* 2005; 11:952–958. [PubMed: 16142243]
39. Premont RT, Claing A, Vitale N, Freeman JLR, Pitcher JA, Patton W, Moss J, Vaughan M, Lefkowitz RJ. Beta2-Adrenergic receptor regulation by GIT1, a G protein-coupled receptor kinase-associated ADP ribosylation factor GTPase-activating protein. *Proc Natl Acad Sci.* 1998; 95:14082–14087. [PubMed: 9826657]
40. Jiménez-Sainz MC, Murga C, Kavelaars A, Jurado-Pueyo M, Krakstad BF, Heijnen CJ, Mayor F, Aragay AM. G protein-coupled receptor kinase 2 negatively regulates chemokine signaling at a level downstream from G protein subunits. *Mol Biol Cell.* 2006; 17:25–31. [PubMed: 16221891]
41. Fusco A, Santulli G, Sorriento D, Cipolletta E, Garbi C, Dorn GW, Trimarco B, Feliciello A, Iaccarino G. Mitochondrial localization unveils a novel role for GRK2 in organelle biogenesis. *Cell Signal.* 2012; 24:468–475. [PubMed: 21983013]
42. Sorriento D, Fusco A, Ciccarelli M, Rungi A, Anastasio A, Carillo A, Dorn GW, Trimarco B, Iaccarino G. Mitochondrial G protein coupled receptor kinase 2 regulates proinflammatory responses in macrophages. *FEBS Lett, Federation of European Biochemical Societies.* 2013; 587:3487–94.
43. Chen M, Sato PY, Chuprun JK, Peroutka RJ, Otis NJ, Ibeti J, Pan S, Sheu S, Gao E, Koch WJ. Prodeath Signaling of G Protein–Coupled Receptor Kinase 2 in Cardiac Myocytes After Ischemic Stress Occurs Via Extracellular Signal–Regulated Kinase-Dependent Heat Shock Protein 90-Mediated Mitochondrial Targeting. *Circ Res.* 2013; 112:1121–1134. [PubMed: 23467820]
44. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, Tourlomousis P, Däbritz JHM, Gottlieb E, Latorre I, et al. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell.* 2016; 167:1–14. [PubMed: 27662081]
45. Yankovskaya V, Horsefield R, Tornroth S, Luna-Chavez C, Miyoshi H, Leger C, Byrne B, Cecchini G, Iwata S. Architecture of Succinate Dehydrogenase and Reactive Oxygen Species Generation. *Science* (80–). 2003; 299:700–704.
46. Samavati L, Monick MM, Sanlioglu S, Buettner GR, Oberley LW, Hunninghake GW. Mitochondrial K ATP channel openers activate the ERK kinase by an oxidant-dependent mechanism. *Am J Physiol Cell Physiol.* 2002; 283:273–281.
47. Obrenovich ME, Palacios HH, Gasimov E, Leszek J, Aliev G. The GRK2 Overexpression Is a Primary Hallmark of Mitochondrial Lesions during Early Alzheimer Disease. *Cardiovasc Psychiatry Neurol.* 2009; 2009:327360. [PubMed: 20204079]
48. Eijkelkamp N, Heijnen CJ, Lucas A, Premont RT, Elsenbruch S, Schedlowski M, Kavelaars A. G protein-coupled receptor kinase 6 controls chronicity and severity of dextran sodium sulphate-induced colitis in mice. *Gut.* 2007; 56:847–54. [PubMed: 17229795]

Figure 1. GRK2 inhibits wound healing in SW480 cells

A) SW480 cells were transfected with either scrambled siRNA (siCtrl) or GRK2 siRNA (siGRK2) and incubated for 48 hours to facilitate knockdown of GRK2. Representative western blot and their quantification showing levels of GRK2 after siCtrl or siGRK2 treatment. Tubulin is shown as loading control. **B)** Gene expression levels of GRK2 in cells treated with either siCtrl or siGRK2 (n=5). **C)** Confluent SW480 cells were wounded through the generation of a linear scratch and the cells were photographed at 0, 16, 24, 40 and 48 hours. At the time of wounding mitomycin C was added to prevent proliferation. The cells were treated with TNF α (20 ng/ml) at the onset of the linear scratch. Differences between amount of wound closed at each time point between untreated and treated cells for both siCtrl and siGRK2 cells was calculated through ImageJ Software and by identifying leading edge of the wound. Data expressed as fold increase from 16 hours for the respective groups. (n=9). **D)** Representative image from each group shows the area of wound closure at 0, and 48 hours at 4 $\times$ magnification. Mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2. GRK2 does not affect TNF α -induced apoptosis or proliferation in SW480 cells

A) After siRNA transfection, SW480 cells were cultured in 6-well plates at a concentration of 1 million cells/well and were treated +/- TNF α (20 ng/ml) for 24 and 48 hours in order to induce apoptosis. Data is expressed as % total cells quantified by flow cytometry. (n=3)

B) Proliferation was assessed by measuring total DNA from either siCtrl or siGRK2 treated cells +/- TNF α (20 ng/ml) for 24 and 48 hours. Equal numbers of cells were plated and DNA concentration was measured after TNF α treatment. (n=6) Mean +/- SEM, * p < 0.05.

A

■ siCtrl □ siGRK2

Figure 4. GRK2 differentially regulates TNF α -induced gene expression in SW480 cells

A) GRK2 knockdown alters TNF α -induced gene expression in SW480 colon epithelial cells transfected with siCtrl or siGRK2. Gene expression levels were assessed by analyzing mRNA levels using real-time PCR after TNF α stimulation (20ng/ml) for 0 (untreated), 6, and 24 hours and normalized to HPRT then expressed as % maximal expression. (n=5) **B)** Cells were either treated with siCtrl or siGRK2 and stimulated with TNF α (24 hours) in the presence or absence of MEK inhibitor U0126. Gene expression levels of the indicated genes were assessed and normalized to HPRT then expressed as % maximal expression. (n=3) **C)** Following siRNA transfection cells were seeded in the upper chamber of a transwell insert coated with matrigel. Following 48 hours incubation with TNF α , migrated cells were detached and lysed and quantified using a fluorescent dye and expressed as fold control (n=3). Means $\pm$ SEM, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5. GRK2 is present in the mitochondria in SW480 cells and regulates TNF α -induced ROS generation

Isolated Mitochondria shows GRK2 localization in mitochondria in the absence and presence of TNF α (20 ng/ml). **A)** Representative western blot identifies location of GRK2 in different cellular fractions. To see specificity of fractionation, tubulin is used for cytoplasmic fraction control and VDAC is used for mitochondrial fraction. (n=3) **B)** GRK2 knockdown enhances ROS Expression. SW480 cells were treated with either siCtrl or siGRK2 and after 0, 10, or 20 minutes of TNF α stimulation; ROS production was measured

and analyzed using flow cytometry. Data expressed as % maximal expression (n=3). **C)** Representative histogram of ROS expression after 10 minutes of TNF α expression. Lighter color is siCtrl and darker is siGRK2. (n=3). Means $\pm$ SEM, * $p < 0.05$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. ROS scavenging prevents effect of GRK2 on ERK activation and wound closure
A) Treatment with N-acetylcysteine (NAC) prevents TNF α -induced increase in pERK in siGRK2 cells. Cells were untreated or treated with TNF α and NAC as shown. TNF α treatment was performed for 30 minutes. pERK/ERK levels were assayed as described in the methods. Representative blot is shown in the top and quantitation in the bottom. Expressed as fold siCtrl for either no treatment or NAC treatment. (n=3). Means $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$. **B)** Treatment with NAC prevents TNF α -induced wound closure in siGRK2 cells. Wound healing experiments were done as described in the methods. siControl and siGRK2 cells were untreated or pretreated with NAC followed by treatment (or not with TNF α) for 48 hours as shown. (N=3). *** $p < 0.01$.

Figure 7. Intestinal wound healing is enhanced in GRK2 heterozygous mice

Wild type and GRK2 heterozygous mice were subjected to 2.5% DSS for the indicated times. **A)** Disease activity index was determined as described in the text (n=6–8). **B)** Histology of the colon from mice subjected to intestinal inflammation at day 13 (n=6–8). Means $\pm$ SEM, * $p < 0.05$.