MEKK1-MKK4-JNK-AP1 Pathway Negatively Regulates Rgs4 Expression in Colonic Smooth Muscle Cells

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Abstract

Background: Regulator of G-protein Signaling 4 (RGS4) plays an important role in regulating smooth muscle contraction, cardiac development, neural plasticity and psychiatric disorder. However, the underlying regulatory mechanisms remain elusive. Our recent studies have shown that upregulation of Rgs4 by interleukin (IL)-1β is mediated by the activation of NFκB signaling and modulated by extracellular signal-regulated kinases, p38 mitogen-activated protein kinase, and phosphoinositol-3 kinase. Here we investigate the effect of the c-Jun N-terminal kinase (JNK) pathway on Rgs4 expression in rabbit colonic smooth muscle cells.

Methodology/Principal Findings: Cultured cells at first passage were treated with or without IL-1β (10 ng/ml) in the presence or absence of the selective JNK inhibitor (SP600125) or JNK small hairpin RNA (shRNA). The expression levels of Rgs4 mRNA and protein were determined by real-time RT-PCR and Western blot respectively. SP600125 or JNK shRNA increased Rgs4 expression in the absence or presence of IL-1β stimulation. Overexpression of MEKK1, the key upstream kinase of JNK, inhibited Rgs4 expression, which was reversed by co-expression of JNK shRNA or dominant-negative mutants for MKK4 or JNK. Both constitutive and inducible upregulation of Rgs4 expression by SP600125 was significantly inhibited by pretreatment with the transcription inhibitor, actinomycin D. Dual reporter assay showed that pretreatment with SP600125 sensitized the promoter activity of Rgs4 in response to IL-1β. Mutation of the AP1-binding site within Rgs4 promoter increased Rgs4 expression in the absence or presence of IL-1β stimulation. Mutation of the AP1-binding site within Rgs4 promoter increased the promoter activity. Western blot analysis confirmed that IL-1β treatment increased the phosphorylation of JNK, ATF-2 and c-Jun. Gel shift and chromatin immunoprecipitation assays validated that IL-1β increased the in vitro and ex vivo binding activities of AP1 within rabbit Rgs4 promoter.

Conclusion/Significance: Activation of MEKK1-MKK4-JNK-AP1 signal pathway plays a tonic inhibitory role in regulating Rgs4 transcription in rabbit colonic smooth muscle cells. This negative regulation may aid in maintaining the transient level of RGS4 expression.

Introduction

Signal transduction is a key process of converting one signal to another, leading to a series of signaling reactions. One critical class of signal-transduction pathways is the signaling controlled by the guanine–nucleotide-binding heterotrimeric proteins (G proteins). G protein-coupled receptors (GPCRs), also known as seven-transmembrane domain receptors, comprise a large protein family of transmembrane receptors. GPCRs are involved in a vast array of physiological and pathological processes and are also the targets of approximately 40% of all modern medicinal drugs [1,2]. The ligand binding to GPCRs, such as the acetylcholine (ACh) receptor, catalyzes GDP-GTP exchange on the α-subunit of a heterotrimeric G-protein complex. The dissociation of GTP-bound α-subunit from βγ subunits leads to the regulation of downstream effectors. GPCR signaling is terminated by the intrinsic GTPase activity of the Gα-subunit, which is accelerated by the regulator of G-protein signaling (RGS) proteins as GTPase-activating proteins. RGS proteins regulate the strength and duration of Gα signaling [2]. Each RGS protein regulates the function of multiple GPCRs, while some RGS proteins have a clear preference for particular receptor-G protein complexes. RGS4 is one of seven members of a classic R4 RGS protein family that accelerates the intrinsic GTPase activity of the Gα1/α and Gαq/11 family members [3]. RGS4 plays an important role in regulating smooth muscle contraction, cardiomyocyte development, neural plasticity and psychiatric disorders [4–7]. In particular, RGS4 has been widely shown to be an underlying risk factor for schizophrenia, even though it is not true in some human populations [4,8–12].

In neuronal cell lines, expression of Rgs4 is reduced after treatment with nerve growth factor [13], cAMP [14] or camptothecin [15], whereas opioid receptor agonists lead to an
increase in the expression levels of Rgs4 mRNA [16] and RGS4 protein [17]. Administration of corticosterone to adult rats decreases the level of Rgs4 mRNA in the paraventricular nucleus of the hypothalamus and increases the levels in locus coeruleus [18], but has no effect in the hippocampus [19,20]. Long-term opiate administration is associated with an increase in RGS4 immunoreactivity in the rat and human brain [21,22]. Rapid kindling leads to an increase of Rgs4 mRNA in hippocampus and forebrain, but not in brainstem or cerebellum [23]. Rgs4 expression is downregulated in prefrontal cortex and striatum by neonatal status epilepticus [24]. In rat adrenal glands, Rgs4 is upregulated by aldosterone secretagogues, both in vivo and in vitro [25]. Rgs4 mRNA is expressed only in glial cell line-derived neurotrophic factor-responsive neurons [26]. In cardiomyocyte, Rgs4 expression is induced by endotoxin and interleukin (IL)-1β [27,28] and may contribute to the loss of Gαq-mediated phospholipase C activation by endothelin-1 [29]. In human aortic smooth muscle cells (SMC), RGS4 is highly expressed at the mRNA level and inhibits S1P3 receptor-mediated signaling [30]. In gastrointestinal smooth muscle, Rgs4 negatively regulates Gαq signaling activated by M3 or motilin receptors [31,32] and thus inhibits agonist-induced initial contraction [6,7,33]. In our previous studies, we demonstrated for the first time that Rgs4 expression is increased in both dispersed and cultured rabbit SMC after IL-1β treatment [7]. These findings suggest that Rgs4 expression is regulated dynamically by inflammatory mediators such as cytokines and growth factors.

However, the molecular mechanisms and signaling pathways for RGS4 regulation remain elusive. At the protein level, Rgs4 is regulated by the N-end rule pathway [34,35] and proteasome degradation [6,36]. At the mRNA level, Rgs4 is regulated by a transcription factor Phox2b [37]. Our recent studies demonstrate that IL-1β-induced upregulation of Rgs4 is transcription-dependent [6,39] and mediated by the canonical IKK2/IκBα pathway of NFκB activation [6]. Further studies suggest that IL-1β-induced activation of either extracellular signal-regulated kinase 1/2 (ERK1/2) or p38 mitogen-activated protein (MAP) kinase (MAPK) enhances the upregulation of Rgs4 expression, whereas the PI3K/Akt/GSK3β pathway attenuates IL-1β-induced upregulation of Rgs4 expression [39].

The pathway of c-Jun NH2-terminal kinase (JNK), also known as stress-activated protein kinase, is another key member of MAPK superfamily, and is activated primarily by inflammatory cytokines and environmental stresses [40–42]. The JNK family includes JNK1 (four isoforms), JNK2 (four isoforms), and JNK3 (two isoforms). JNKs are activated by MAP2kinases such as MAPK kinase (MKK)4 and MKK7, which are in turn activated by the MAP3kinases, such as MAP-ERK kinase kinase (MEKK)1, MEKK4, TAK1, ASK1 and MLKs [43]. The JNK MAP3kinase pathways are activated by MAP4kinases that link to a variety of cell receptors [40,44]. The diversity and selection of upstream kinases for JNK activation depend upon the cell types and stimulators [40]. After activation, JNK regulates target gene expression through an array of transcription factors such as AP1, ATF-2, SMAD4, NFAT, etc. [45–47]. In the present study, we

Figure 1. The JNK inhibitor SP600125 dose-dependently increases Rgs4 expression in rabbit colonic smooth muscle cells. Cultured and serum-starved muscle cells were treated with indicated concentration of SP600125 1 h before treatment with IL-1β (10 ng/ml) for 3 h, followed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) (A, B) and Western blot analysis (C, D). The relative level of Rgs4 mRNA expression (fold induction) was presented as compared with the control without SP600125 pretreatment after GAPDH normalization (A, B). Levels of β-actin and GAPDH were used as a loading control (C). The relative optical density (fold change) was presented as compared with the vehicle control (DMSO) after β-actin or GAPDH normalization (D). Values are means ± SE of 3 experiments. * (p <0.05) indicates significant increase after IL-1β treatment as compared with the control. + (p<0.05) indicate significant increase by ANOVA and Newman-Keuls comparison of SP600125 treatment with the vehicle control. doi:10.1371/journal.pone.0035646.g001
investigated the role of MEKK1-MKK4-JNK-AP1 pathway in regulating Rgs4 expression in rabbit colonic SMC and showed that JNK inhibition increased while MEKK1/MKK1 over-expression attenuated both constitutive and IL-1β-induced expression of Rgs4. IL-1β induced transient phosphorylation of JNK and sustained phosphorylation of c-Jun and ATF-2. IL-1β increased the binding activity of c-Fos and c-Jun to Rgs4 promoter. JNK inhibition and mutation of the AP1-binding site within the Rgs4 promoter sensitized the promoter activity of Rgs4 in response to IL-1β. This work provides new insights into how stress-induced signaling pathways regulate G protein signaling and smooth muscle contraction.

Results

Pharmacological inhibition of JNK by SP600125 significantly increased Rgs4 expression in colonic SMC

IL-1β is well known to activate NFκB and MAPK pathways [48–50]. We have shown that the NFκB pathway, as well as the ERK1/2 and p38 MAPK pathways enhance while the PI3K/Akt/GSK3β pathway inhibits the upregulation of Rgs4 expression by IL-1β in colonic SMC [6,39]. To explore the potential role of JNK pathway on Rgs4 expression in colonic SMC, we examined the effect of JNK pathway inhibitor on Rgs4 expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis, the established techniques for detecting Rgs4 mRNA and protein expression [7]. SP600125, a well-established specific inhibitor for the JNK pathway [48,50,51], were selected to pretreat the serum-starved SMC for 1 h at different concentrations before IL-1β (10 ng/ml) stimulation for 3 h. Total RNA extraction and whole cell lysate were prepared. In the preliminary studies, a long range concentration (10 nM to 100 μM) of SP600125 was tested, showing the maximal effect at 10 μM (Fig. 1A). Thus, 1–20 μM was used for the present study. SP600125 treatment alone between 1–10 μM induced a dose-dependent upregulation of Rgs4 mRNA (Fig. 1B) and protein (Fig. 1C). However, a higher concentration of SP600125 (20 μM) did not induce further upregulation but reversed the expression of Rgs4 mRNA and protein (Fig. 1B–D), which may result from the non-specific effects on other kinases. IL-1β treatment alone increased Rgs4 mRNA expression as previously reported [6,7]. Pretreatment with SP600125 enhanced IL-1β-induced upregulation of Rgs4 mRNA in a dose-dependent manner similar to SP600125 alone (Fig. 1B). However, SP600125 at 20 μM did not induce additive or an increased effect over IL-1β, perhaps due to additional toxic effect. These data suggest that inhibition of the JNK pathway increases constitutive and IL-1β-induced expression of Rgs4 in colonic SMC. Therefore, 10 μM of SP600125, consistent with previous reports [48,50–52], was used for further functional and mechanistic studies.

Knockdown of JNK expression by shRNA increased Rgs4 expression in colonic SMC

To validate the stimulatory effect of JNK pharmacologic inhibition, we tested the effect of JNK specific shRNA silencing on constitutive and IL-1β-induced Rgs4 expression. The efficacy of JNK1 and JNK2 shRNA was validated by Western blot analysis (Fig. 2A) with anti-JNK(FL) antibody, which recognized p46 and p54 isoforms of JNK1, JNK2 and JNK3 (manufacturer’s data sheet). The p46 isoforms contain JNK1a1, JNK1b1, JNK2a1, JNK2b1, and JNK3a1, while the p54 isoforms contain JNK1a2, JNK1b2, JNK2a2, JNK2b2, and JNK3b2 [53]. As shown in Fig. 2B, both JNK1 and JNK2 shRNA dramatically increased the constitutive and IL-1β-induced expression of Rgs4 protein, and the effect of JNK2 shRNA was stronger than that of JNK1 shRNA (Fig. 2A). Consistent with SP600125 (Fig. 1C), both JNK1 and JNK2 shRNA increased the number of bands detected by Rgs4 antibody, implying that JNK may regulate the protein stability of Rgs4 [6,34–36].

The effects of JNK inhibition on the constitutive and IL-1β-induced expression of Rgs4 mRNA were transcription-dependent

To investigate whether the transcriptional mechanism is involved in the enhancing effect of JNK inhibition on Rgs4 mRNA expression, cultured SMC were pretreated with the transcriptional inhibitor, actinomycin D (10 μM) 1 h before SP600125 (10 μM) was applied for 4 h and IL-1β for 3 h. The level of Rgs4 mRNA expression was determined by RT-qPCR and normalized to the house-keeping gene GAPDH. Consistent with previous studies [6], pretreatment with actinomycin D blocked IL-1β-induced upregulation of Rgs4 mRNA expression (Fig. 3A). Actinomycin D pretreatment completely blocked the upregulation of Rgs4 mRNA induced by either SP600125 alone or a combination of SP600125 and IL-1β (Fig. 3A). These data suggest that inhibition of the JNK pathway stimulates the transcription of Rgs4 in colonic SMC.
To further understand the transcriptional mechanism underlying the induction of Rgs4 mRNA expression by JNK inhibition, we performed a luciferase reporter assay for Rgs4 promoter activity by transfecting SMC with rabbit Rgs4 promoter-luciferase reporter plasmid [30]. As shown in Fig. 3B, inhibition of JNK with SP600125 alone significantly increased the promoter activity of Rgs4 in a similar manner to the effect of IL-1β stimulation. Pretreatment with SP600125 before IL-1β exposure sensitized the promoter activity of Rgs4 in response to IL-1β (Fig. 3B). These data suggest that activation of endogenous JNK pathway plays a tonic inhibitory effect on the constitutive and IL-1β-inducible promoter (transcription) activity of Rgs4.

JNK-AP1 pathway maintained a tonic inhibition of Rgs4 transcription

The family of AP1 transcription factor consists of several subfamilies of bZIP-domain (bZIP = basic region leucine zipper) proteins: the Jun (c-Jun, JunB, and JunD), the Fos (c-Fos, FosB, Fra-1 and Fra-2), and the ATF-2 (ATF-2 and ATF-a) [54]. Since AP1 is a major target of the JNK signaling pathway, and an AP1 binding site within the proximal region of rabbit Rgs4 promoter was identified by bioinformatics analysis using MatInspector [38], we hypothesize that the JNK pathway inhibits Rgs4 transcription predominantly via AP1 transcription factor. To test this hypothesis, we first examined the function of AP1 binding site within Rgs4 promoter using Rgs4 promoter luciferase reporter assay and site-directed mutagenesis analysis. As shown in Fig. 4A, mutation of the AP1-binding site within rabbit Rgs4 promoter increased the promoter activity and sensitized IL-1β-induced promoter activity. These data imply that the AP1 binding site is required for the tonic inhibitory effect of the JNK pathway activation on Rgs4 transcription and the transcription factor AP1 functions as a repressor for Rgs4 regulation.

We then determined if IL-1β treatment affects the binding activity of AP1 transcription factor within the Rgs4 promoter both in vitro and ex vivo. Electrophoretic mobility shift assay (EMSA) measuring the in vitro interactions between an oligonucleotide probe containing rabbit Rgs4 attgagtcact sequence and SMC nuclear protein showed that IL-1β induced the formation of an AP1 DNA-binding complex, which was completely blocked by the specific inhibitor of either JNK pathway or NFKβ pathway (Fig. 4B). The in vivo chromatin immunoprecipitation (CHIP) assay on the chromatin of cultured rabbit colonic SMC identified a specific enrichment of AP1 transcription factor within proximal Rgs4 promoter containing the AP1 binding site by CHIP assay with antibodies against c-Fos, c-Jun and ATF-2, the key components of AP1 transcription factor (Fig. 4C). The epitope-matching control IgG was used as a negative control for CHIP and the input chromatin DNA was used as a positive control for PCR. In non-stimulated cells, both c-Fos and ATF-2 were found to bind to Rgs4 promoter but c-Jun was absent (Fig. 4C). IL-1β treatment for 3 h promoted the DNA-binding activity of endogenous c-Fos and c-Jun proteins but removed ATF-2 from the Rgs4 promoter (Fig. 4C). These data suggest that IL-1β promoted DNA-binding activity of Fos/Jun-containing AP1 factors within proximal Rgs4 promoter that ultimately suppressed the transcription of Rgs4.

IL-1β induced rapid activation of the JNK-AP1 pathway in rabbit colonic SMC

The data from pharmacological inhibition, gene reporter assay, mutagenic analysis, EMSA and CHIP assay suggest that JNK-AP1 pathway is activated when rabbit colonic SMC were exposed to IL-1β. To provide further experimental evidence, we performed Western blot analysis using phosphor-specific antibodies against the key members of JNK pathway. IL-1β treatment induced a rapid and transient increase in the phosphorylation of JNK at Thr-183/Tyr-185 (Fig. 5). ATF-2 and c-Jun are the major downstream substrates of JNK kinase and both bind to AP1 response elements in many other types of cells [40,47]. Therefore, we determined the level of JNK-specific phosphorylation of ATF-2 at Thr-71 and c-Jun at Ser-73 in rabbit colonic SMC. As shown in Fig. 5, IL-1β stimulation induced rapid and sustained phosphorylation of both
ATF-2(Thr-71) and c-Jun(Ser-73), implying the activation of ATF-2 and c-Jun by IL-1β-stimulated JNK pathway.

MEKK1-MKK4 overexpression inhibited the constitutive and IL-1β-induced expression of Rgs4 protein

MEKK1 is the key upstream kinase of JNK and induces dual phosphorylation of Thr/Tyr residues within a Thr-Pro-Tyr motif of JNK via the dual specific kinases MKK4 (also known as SEK1 or MEK4) and MKK7 (SEK2) [40,53,55-57]. To address whether MEKK1 regulates Rgs4 expression, MEKK1 was overexpressed in SMC. MEKK1 overexpression inhibited the constitutive and IL-1β-induced expression of Rgs4 protein, which was reversed by coexpressing dominant-negative JNK1 and JNK2 mutants (Fig. 6A, B) as well as JNK1 and JNK2 shRNA (Fig. 6C). Consistently, overexpression of MKK4 inhibited the constitutive Rgs4 expression, while overexpression of MKK4 dominant-negative mutant (MKK4-DN) blocked MEKK1-induced inhibition of Rgs4 expression (Fig. 6B), implying that MKK4 acts downstream of MEKK1 [53,55,57] and negatively regulates Rgs4 expression. In contrast, overexpression of MEK1, the key upstream kinase of ERK pathway, increased the constitutive expression of Rgs4 (Fig. 6B), which is consistent with our previous report showing that MEK1/ERK inhibition blocked IL-1β-induced upregulation of Rgs4 expression [39]. These data suggest that MEKK1-MKK4-JNK pathway harnesses inhibitory effect on Rgs4 expression in colonic SMC.

JNK pathway interacts with p38 MAPK and NFκB pathways

Our previous studies have shown that the canonical IKK2/IKBβ pathway of NFκB activation mediates IL-1β-induced upregulation of Rgs4 [6] and such upregulation is enhanced by the activation of the ERK1/2 pathway [39]. However, the stimulatory effect of p38 MAPK pathway on Rgs4 expression is independent of NFκB signaling [39]. To determine if NFκB, p38 MAPK and ERK1/2 pathways are involved in the JNK-AP1 pathway, we performed Western blot analysis in rabbit colonic SMC treated with selected MAPK inhibitors. The treatment with the JNK specific inhibitor (SP600025, 10 μM) alone induced a constitutive activation of NFκB signaling as determined by the phosphorylation of IκBα (Fig. 7A). Treatment with the p38 MAPK inhibitor (SB203580, 1 μM) increased the constitutive and IL-1β-induced phosphorylation of JNK at Thr-183/Tyr-185 (Fig. 7B). However, the MEK inhibitor (PD98059, 20 μM) had no effect on the constitutive and IL-1β-stimulated phosphorylation of JNK at Thr-183/Tyr-185. The specificity of IL-1β-induced JNK phosphorylation was validated by the complete blockade with the JNK specific inhibitor SP600025 (10 μM). These data suggest that JNK activation inhibits NFκB signaling at the level of IKK2, which may also contribute to the tonic inhibition of JNK pathway on Rgs4 expression, and p38 MAPK negatively regulates JNK activity (Fig. 8).
We have identified the important role of NFκB in regulating Rgs4 expression in rabbit colonic smooth muscle cells. However, the regulatory mechanism of Rgs4 expression has been identified in human [65–67], rat [64] and mouse [65]. Our previous studies showed that IL-1β treatment in rabbit colonic SMC increased the basal level of Rgs4 expression and potentiated IL-1β-induced upregulation of Rgs4 expression; (3) Overexpression of MEKK1/MKK4 inhibited Rgs4 expression while overexpression of MKK4/JNK mutants and JNK shRNA reversed MEKK1-mediated Rgs4 inhibition. The family of MAPks (all members) is activated upon dual phosphorylation at threonine and tyrosine by upstream kinases in response to diverse extracellular stimuli. However, the role and outcome of the activation of MAPK pathways rely on the stimuli, target genes and cell resources. The selective involvement of an individual MAPK pathway can be identified generally by specific manipulation of each pathway. In most cases, the MAPK pathways mediate the upregulation of many target genes including inflammatory mediators, contractile proteins and signaling components/regulators. In airway SMC, IL-1β-induced upregulation of COX-2 and eotaxin is inhibited by either MEK1 inhibitors or p38 MAPK inhibitors [68–70], whereas IL-1β-induced RANTES release is sensitive to inhibition of MEK1 [71] or JNK [72] but not inhibition of p38 MAPK [71]. IL-1β-induced upregulation of MPP-9 [48] and tumor necrosis factor α-induced expression of VCAM-1 [73] are sensitive to the inhibition of all three MAPK pathways. In vascular SMC, IL-1β-stimulated iNOS expression is prevented by MEK1 inhibition but potentiated by p38 MAPK inhibition [74,75]. Inhibition of MEK1 or p38 MAPK, but not PI3K, reduced IL-1β-stimulated expression of LIMK2 and cofilin [76]. However, in human vascular SMC, IL-1β activates only p38 MAPK, which mediates IL-1β-induced IL-8 and VEGF expression [77,78]. In human colonic SMC, IL-1β-induced H2O2 production is inhibited by MEK inhibitor but not p38 MAPK inhibitor [79], while IL-1β-induced upregulation of IL-6, IL-8, and COX-2 is reduced by p38 MAPK inhibitor but not MEK-1 inhibitor [80]. In rabbit colonic SMC, IL-1β-induced upregulation of Rgs4 is attenuated by MEK and p38 MAPK inhibitors but is potentiated by PI3K inhibitors [81]. The present studies demonstrate for the first time that JNK inhibitor and shRNA potentiate the constitutive and inducible expression of Rgs4 in rabbit colonic SMC.

In our previous studies, we showed that IL-1β consistently induced a 10–20 fold increase in mRNA expression of endogenous Rgs4 in colonic SMC [6,7}. However, reporter gene assay using Rgs4 promoter detected only a 1–2 fold induction by IL-1β in rabbit colonic SMC [38]. Weak induction in the reporter gene assay also occurred as to the stimulatory effect of SP600125 (Fig. 2). Such discrepancy may be interpreted as the following: (i) IL-1β-induced upregulation of endogenous Rgs4 mRNA level involves not only the transcriptional mechanism but also other mechanisms such as HuR-mediated mRNA stability [81]; (ii) The constitutive promoter activity without IL-1β treatment is already high, which may limit further induction; (iii) The promoter used contains only the proximal region, not reflecting the true full-length functional promoter of Rgs4 [38]; and (iv) JNK pathway may regulate the endogenous Rgs4 through other signaling pathways not related to the promoter region. The JNK-AP1 pathway has been shown to regulate mRNA stability of many genes through down-regulating the expression of HuR [82,83] or upregulating tristetraprolin [84]. AP1-DNA binding activity was significantly increased by IL-1β treatment in rabbit colonic SMC. Western blot analysis demonstrated a rapid activation of the JNK-AP1 pathway by IL-1β. The activation of the JNK-AP1 pathway induced a tonic repression of Rgs4 transcription. The following evidence supports our findings: (1) Either specific inhibition of JNK with SP600125 or mutation of the proximal AP1 binding site within rabbit Rgs4 promoter significantly increased the basal and IL-1β-inducible promoter activity; (2) Specific inhibition of JNK with SP600125 and shRNA increased the basal level of Rgs4 expression and potentiated IL-1β-induced upregulation of Rgs4 expression; (3) Overexpression of MEKK1/MKK4 inhibited Rgs4 expression while overexpression of MKK4/JNK mutants and JNK shRNA reversed MEKK1-mediated Rgs4 inhibition.

**Discussion**

The salient finding of this study is the identification of the tonic inhibitory regulation of Rgs4 transcription by the activation of MEKK1-MKK4-JNK-AP1 signaling pathway. In a series of previous studies, we demonstrated that pro-inflammatory cytokine IL-1β upregulates Rgs4 expression in rabbit colonic SMC [7] through the canonical IKK2/IκBκ pathway of NFκB activation [6] as well as ERK1/2 and p38 MAPK pathways [39]. This upregulation of Rgs4 is negatively regulated by the activation of PI3K/Akt/GSK3β pathway [39]. Here, we demonstrate an additional signaling pathway MEKK1-MKK4-JNK-AP1 that maintains a tonic inhibitory regulation on Rgs4 transcription. The positive and negative regulatory mechanisms of Rgs4 expression reflect an intricate and delicate system for gene regulation (Fig. 8).

Rgs4 is implicated in intestinal inflammation [6,7,59,60], cardiovascular diseases [61–63] and psychiatric disorders [4,8–12]. However, the regulatory mechanism of Rgs4 expression has not been well understood. We and others have demonstrated that Rgs4 expression is transcriptionally regulated [6,38,64–67]. We have cloned and characterized the promoter region of rabbit Rgs4 [38]. This promoter contains a canonical TATA box, and predicted binding sites for several transcription factors such as NFκB, AP1, GATA, MyoD, etc. Similar promoter regions have been identified in human [63–67], rat [64] and mouse [65] Rgs4. Within human RGS4 promoter, the inverted CCAAT box element (ICE) and the cAMP response element (CRE) mediate activation while the B-cell lymphoma 6 (Bcl6)-binding site mediates repression of RGS4 transcription [67]. Within rat Rgs4 promoter, a variant AP1-related site mediates transcriptional repression [64]. For mouse Rgs4 promoter, no experimental evidence for the functional regulation has been reported [65]. For rabbit Rgs4 promoter, we have identified the important role of NFκB binding site in mediating IL-1β-induced upregulation of Rgs4 mRNA expression [6]. In the present study, we validated the AP1 binding site within the proximal region of rabbit Rgs4 promoter using *in vivo* CHIP, *in vitro* EMSA and site-directed mutagenic analysis. The

![Figure 5. IL-1β induces a rapid and transient phosphorylation of JNK and sustained phosphorylation of ATF-2 and c-Jun in rabbit colonic smooth muscle cells. Cultured and serum-starved muscle cells were treated with IL-1β (10 ng/ml) for the indicated time period, followed by Western blot analysis with indicated anti-phospho antibodies.](image)

**Figure 5.** IL-1β induces a rapid and transient phosphorylation of JNK and sustained phosphorylation of ATF-2 and c-Jun in rabbit colonic smooth muscle cells. Cultured and serum-starved muscle cells were treated with IL-1β (10 ng/ml) for the indicated time period, followed by Western blot analysis with indicated anti-phospho antibodies. The β-actin was used for the loading control. doi:10.1371/journal.pone.0035646.g005
The mechanism underlying the inhibition of JNK-AP1 pathway on Rgs4 transcription remains to be determined. In rat Rgs4 promoter, FRA-2-dependent dismissal of the transcriptional co-activator, CRE-binding protein is involved in AP1-mediated transcriptional repression [64]. In the present study, we demonstrated that IL-1β treatment induced the recruitment of both c-Fos and c-Jun but dismissed ATF-2 from the AP1-binding site of rabbit Rgs4 promoter. Thus, different dimers of AP transcription factor may function in different ways. IL-1β induction may promote preferentially the binding of Fos/Jun heterodimer and/or Jun/Jun homodimer to the heptamer consensus sequence of AP1 site [TGA/C/G/TCA]. Such binding represses rabbit Rgs4 transcription. In contrast, ATF-2-containing dimers may normally bind to the AP1 site and activate Rgs4 transcription. Upon JNK activation by IL-1β induction, the ATF-2-containing activator was removed and Jun-containing repressor was strengthened, leading to tonic inhibition of Rgs4 transcription. JNK1 and JNK2 have mostly overlapping functions due to their concurrent and ubiquitous expression, although recent evidence identified different dimers of AP transcription factors are involved in the knockdown of JNK1 and JNK2, but not p38 pathway, are responsible for IL-1β-induced inhibition in the contractile response to endothelin receptor agonist [97]. JNK pathway also mediates Toll-like receptor-mediated airway hyper-responsiveness to bradykinin [98]. In vascular SMC, all three MAPK pathways are involved in the contractile signaling [93,99]. In ileal SMC, sphingosyl phosphorylcholine-induced contraction is blocked by MEK-1 inhibitor but not p38 MAPK inhibitor [100]. In esophageal SMC, ERK1/2 but not p38 and JNK contributes to sphingosine 1-phosphate-induced contraction [15,101] andbone...
besin-induced contraction [102]. However, all three MAPK pathways (p38, ERK1/2 and JNK) mediate LPS-induced inhibition on acetylcholine-stimulated contraction in rabbit duodenum containing SMC and enteric nervous system [51,103,104]. In animal colitis induced by 2,4,6-trinitrobenzene sulfonic acid, ERK1/2 mediates the restoration of the reduced muscle contractility by meloxacin, a COX2 inhibitor [105]. In dextran sulfate sodium-induced colitis, both ERK and p38 MAPK pathways contribute to hypercontractility but JNK was not studied [106]. The present study provides the first evidence that the JNK pathway maintains the low level of Rgs4 expression in colonic SMC and subsequently leads to the promotion of SMC contraction. The tonic inhibition of Rgs4 expression by JNK pathway provides a new mechanism for the contribution of JNK pathway in regulating smooth muscle contraction [93,98].

The cross-talk between JNK pathway and other MAPK and NFκB pathways is not well understood. The ERK1/2 pathway has been widely shown to affect IL-1β-induced NFκB activation and regulate Rgs4 expression [39]. The p38 MAPK pathway stimulates Rgs4 expression independently of NFκB signaling [39]. In the present study, we showed that p38 MAPK negatively regulates JNK activity but ERK1/2 pathway does not affect JNK pathway. However, the JNK and NFκB pathways regulate each other during IL-1β-induced upregulation of Rgs4 expression in rabbit colonic SMC. JNK activation inhibits NFκB signaling at the level of IKK2. To the contrary, IKK2-mediated NFκB signaling promotes IL-1β-induced activation of the JNK-AP1 pathway because IKK2 inhibitor abolished IL-1β-stimulated AP1-binding activity within Rgs4 promoter. Our conclusion is supported by several previous studies showing a positive regulation of JNK pathway by IKK [107–109]. The mechanism underlying IKK2-mediated activation of JNK pathway remains to be determined.

In conclusion, activation of MEKK1-MKK4-JNK-AP1 signaling pathway plays a tonic inhibitory role in regulating Rgs4 transcription in rabbit colonic SMC. Rgs4 expression is dynamically and strictly regulated by both the positive signaling pathways of NFκB, ERK1/2 and p38 MAPK and the negative pathways of P38-Akt-ASKJβ and MEKK1-MKK4-JNK-AP1. This intricate and orchestral regulation may aid in maintaining the transient function of Rgs4 for smooth muscle contraction/relaxation as well as cardiovascular and neuronal functions.

Materials and Methods

Reagents and antibodies

IL-1β was obtained from Alexis Biochemicals (San Diego, CA). SP600125 (Anthra[1,9-cd]pyrazol-6(2H)-one, 1,9-pyrazoloanthrone), PD98059 (2′-Amino-3′-methoxystyline), SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)1H-imidazole), and IKK2-IV (IKK2 inhibitor IV, 5-[p-Fluorophenyl]-2-ureido)thiophene-3-carboxamide) were obtained from EMD Chemicals (San Diego, CA) and dissolved in dimethyl sulfoxide (DMSO). Antibodies against c-Fos, c-Jun, ATF-2, JNK FL, IκBα, GAPDH and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified anti-Rgs4 antibody was kindly provided by Dr. Susanne M. Mumby (University of Texas Southwest Medical Center). Antibodies against phospho-JNK(Thr183/Tyr185), phospho-ATF-2(Thr71), phospho-c-Jun (Ser73), phospho-IKK2 (Ser177/181), phospho-IκBα (Ser32/36) and phospho-p65 (Ser546) were from Cell Signal Technology (Davers, MA). All the other reagents were from Sigma (St. Louis, MO).

Ethics Statement

All procedures involving rabbit were approved by the IACUC committee at Temple University (approval protocol # 3164) or Virginia Commonwealth University (approval protocol # 0510-5402).

Isolation and culture of SMC

Rabbit colonic circular muscle cells were isolated and cultured as previously described [7]. Briefly, distal colon from euthanized New Zealand White rabbits (2–2.5 kg) was placed in HEPES-buffered smooth muscle media. The circular smooth muscle layer was dissected from the mucosa and longitudinal muscle layer using stereo microscope and treated with 0.1% collagenase (type II) and 0.1% soybean trypsin inhibitor for 30 min at 31°C. The isolated single muscle cells were harvested after several rounds of spontaneous dispersion by filtration through 500-μm Nitex and centrifuged twice at 350 g for 10 min. The isolated SMC were cultured in 100 mm dish with DMEM containing 10% fetal bovine serum and 1% antibiotics and antimycotics. After 10–14 days, the SMC attained confluence and were then passaged once.
for use in various experiments. Full confluent muscle cells were deprived of serum for 24 h before experiments.

Promoter cloning, site-directed mutagenesis and vector construction

The rabbit Rgs4 promoter containing a fragment of −962/+50 (from the putative transcription start site) was cloned into pMlu3 AccepTor vector as described previously. The potential binding site for AP1 transcriptional factor was identified by MatInspector (http://www.genomatix.de) and TFSEARCH (http://www.cbrc.jp) and located at −213/−203 of rabbit Rgs4 promoter as previously described [38]. Mutation of the AP1 binding site (ATTGAGTCACT) in the pMlu3-Rgs4-P2 reporter vector construct was performed using site-directed mutagenesis using the QuickChange kit (Stratagene). Mutagenic primers (sense, 5'-GAACATCAGTCGTTTTTCATGTAT-3', and anti-sense 5'-GCAGCCTGGAGAAGCTGCTAA-3') were used to introduce a nucleotide change in the entire binding site for AP1 transcriptional factor. Mutation was confirmed by nucleotide sequencing.

Mammalian expression vectors encoding MEK1 and MKK1 were obtained from Clontech. Mammalian expression vectors encoding MKK4, MKK4-DN, JNK1-APF and JNK2-APF were generously provided by Dr. Riches J. David (National Jewish Center, Denver, CO) [53,55].

The JNK shRNA expression vectors were generated as previously described [110]. JNKs originate from three genes that yield 10 isoforms through alternative mRNA splicing. Since colonic SMCs express JNK1 and JNK2, we designed two shRNA-encoding sequences for JNK1 and JNK2. The JNK1A and JNK1B shRNA targeted the nucleotides 124–149 and 339–360 of rabbit JNK1 (XM_002722871). The JNK2A and JNK2B shRNA targeted the nucleotides 647–699 and 747–771 of rabbit JNK2 (XM_002721308.1). The shRNA expression cassette was generated through consequent, two rounds of PCR, and cloned into pLL3.7 lentiviral vector which contains CMV-promoted EGFP (enhanced green fluorescent protein) marker as an internal control [110]. The sequence of each shRNA expression cassette in the vector was confirmed by restriction enzyme digestion and DNA sequencing.

Cell transfection and reporter Assays

All the mammalian expression vectors were prepared using EndoFree Plasmid Maxi kit (Qiagen). All transfections in rabbit colonic SMCs were performed utilizing a Lipofectamine-2000 kit (Invitrogen) as previously validated [6,39,110]. The transfection efficiency of rabbit SMC (~60%) was determined by the expression of internal EGFP in the pLL3.7 shRNA expression vector. For Western blot analysis, cells (5 × 10^4/well) cultured in a 6-well plate were cotransfected with indicated vectors for 24–48 h followed by serum starvation and treatment. For reporter assays, cells (2–4 × 10^4/well) cultured on a 96-well plate were cotransfected with the renilla luciferase reporter constructs and the 1:10 normalization firefly luciferase vector pGL4-CMV (Promega).

After incubation with IL-1β for 24 h in the absence or presence of JNK inhibitor SP600125, the media were harvested for measurement of renilla luciferase activity and the cell lysate was used for measurement of firefly luciferase activity. The renilla luciferase was determined with a renilla luciferase vector pGL4-CMV (Promega). The firefly luciferase was determined using a ONE-Glo luciferase assay system (Promega). The luminescence was measured using EnVision multilabel plate reader (Perkin Elmer). Data are normalized by dividing renilla luciferase activity with that of the corresponding firefly luciferase activity. Four to six separate experiments were conducted and data was calculated in each experiment as the average of 4–6 samples.

Reverse transcription (RT) quantitative PCR (RT-qPCR)

Cells were treated with the Trizol reagent (Invitrogen, Carlsbad, CA) for total RNA extraction. The potentially-contaminated genomic DNA was removed by treating 10 µg of the RNA sample at 37°C for 30 min with 1 µl of TURBO DNase (Ambion, Austin, TX) followed by extraction with phenol:chloroform:isoamylalcohol (25:24:1). Real time PCR analysis was carried out on the ABI Prism® 7300 Sequence Detection System (Applied Biosystems, Foster, CA). Expression of Rgs4 was analyzed using the TaqMan® PCR Master Mix Reagents Kit (Applied Biosystems). The TaqMan probe and primers for rabbit Rgs4 designed using the Primer Express® 2.0 version were as follows: (forward, nucleotides 232–252, exon 2) 5’-tccacagaaagacccaaag-3’, (reverse, nucleotides 303–324, exon 3) 5’-tggccacagacacctg-3’ and (probe, nucleotides 254–279, across exon 2 and 3 with 321 bp of intron 2) 5’-tgaatgacaccttggacacaa-3’. The cDNA was synthesized from 500 ng of RNA using the TaqMan® RT Reagents Kit (Applied Biosystems). The optimized concentrations for real-time PCR were 0.4 µM for both primers and 0.2 µM for the probe, and 5 ng cDNA in a 20 µl reaction volume. Rabbit GAPDH primers (forward 5’-ccttgagagagcttgctgtagt-3’, reverse 5’-cagctgtcctgctcggtag-3’) were used as an internal control. Each sample was tested in
triplicate. Cycle threshold (Ct) values were obtained graphically for Rgs4 and GAPDH. The difference in Ct values between GAPDH and Rgs4 were represented as ∆Ct values. The ∆∆Ct values were obtained by subtracting the ∆Ct value of the control samples from that of the treated samples. Relative fold change in gene expression was calculated as 2−∆∆Ct.

Western blot analysis
Cells were solubilized for 30 min in Triton X-100-based lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 100 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 30 mM sodium fluoride and 5 mM sodium vanadate. After centrifugation of the lysates at 20,000 g for 10 min at 4°C, the protein concentrations of the supernatant were determined with a De Ducre Protein Assay kit from BioRad (Hercules, CA). Equal amounts of protein were fractionated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane (BioRad). Blots were blocked in 5% nonfat dry milk/tris-buffered saline (pH 7.6) plus 0.1% Tween-20 (TBS-T) for 1 h and then incubated overnight at 4°C with various primary antibody in TBS-T plus 1% milk. The dilution of 1:1000 was used for most primary antibodies except for anti-Rgs4 (1:10,000) and b-actin (1:100,000). After incubation for 1 h with horseradish peroxidase-conjugated corresponding secondary antibody (1/2,000; 10 µg/ml, Pierce) in TBS-T plus 1% milk, immunoreactive proteins were visualized using SuperSignal Femto maximum sensitivity substrate kit (Pierce, Rockford, IL). All washing steps were performed with TBS-T.

Electrophoretic Mobility Shift Assay (EMSA)
Rabbit colonic SMC were cultured into full confluence and starved with serum-free culture media for 24 h. Cells were pretreated with vehicle (DMSO), JNK inhibitor SP600125 or IKK2 inhibitor IKK2-IV for 1 h before treatment with IL-1β for 1 h. Nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce, Rockford, IL). The oligonucleotide probe covering the predicted AP1 binding site within the promoter of rabbit Rgs4 was used. Synthesized sense (5'-tgcgATTTTGTAGAGATTGAGTGCATTCT-3') and antisense (5'-tgcgAAAGTGAATCCTACTCTGAAATG-3') oligonucleotides were annealed to generate a double-strand DNA probe with an overhang TCGA for end-labeling. The probe was labeled with γ32P-ATP and T4 polynucleotide kinase (Promega), and added to the binding reactions in the presence of poly(dI-dC):poly(dI-dC) (Sigma), herring sperm DNA (Invitrogen, Carlsbad, CA), and nuclear extracts. Equal amounts of extracts (10 µg) were loaded for each binding reaction. After 30 min incubation at room temperature, samples were loaded onto a pre-electrophoresed 0.5 x tris-borated EDTA buffer (TBE), 6% polyacrylamide gel and run at 150 V for approximately 1.5 h. The gels were then fixed and dried, and autoradiographs obtained.

Chromatin immunoprecipitation (CHIP) assay
CHIP assay was performed according to the manufacturer’s protocol (Upstate Biotechnology Inc., Lake Placid, NY). Cells were cultured in 10-cm dishes until full confluence was established and then serum-starved overnight. Cells were treated with IL-1β (10 ng/ml) for 3 h. The DNA-chromatin of cells were cross-linked by the addition of 280 μl of 37% formaldehyde to 10 ml of culture medium for 30 min at room temperature and stopped with 0.125 M glycine. Cells were washed twice with PBS and harvested with 1 ml of SDS lysis buffer (20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 1% SDS, 1% deoxycholic acid, 2 mM EDTA, and freshly added protease inhibitors). After sonication and centrifugation, the supernatants were used for standard immunoprecipitation with anti-c-Fos antibody or control IgG and protein A/G agarose bead (Santa Cruz). The immune complexes were eluted, reverse cross-linked using 5 M NaCl, and purified by phenol/chloroform extraction. Ethanol-precipitated DNA pellets were dissolved in Tris-EDTA buffer. The supernatant of an immunoprecipitation reaction carried out in the absence of antibody was purified and diluted 1:100 as total input DNA control. PCR was carried out on 1 µl of each sample using sense and anti-sense primers against the cloned promoter region of rabbit Rgs4. PCR products were analyzed in 1% agarose gels and images were analyzed with NIH ImageJ densitometric measurements. Relative changes were calculated using the mean density after background subtraction.

Statistical analysis
The images from Western blot, EMSA and CHIP assays were scanned and analyzed with NIH ImageJ (1.46a version) densitometric measurements. The data were expressed as integrated density and presented as relative fold in comparison with the corresponding control. Quantitative data were expressed as means ± SE of n experiments and statistical significance was determined using Student’s t-test for unpaired values or ANOVA and Newman-Keuls comparison.

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Author Contributions
Conceived and designed the experiments: WH KM. Performed the experiments: FL YZ HW SL SM WH. Analyzed the data: WH FL YZ. Contributed reagents/materials/analysis tools: KM KK. Wrote the paper: WH.

References