INTRODUCTION

Proper functioning of the nociceptive system is essential to protect the body from tissue damage. Inflammation sensitizes the nociceptive system, leading to a lower threshold to painful stimuli (hyperalgesia) (1). This process is thought to serve adaptive purposes, but becomes maladaptive when hyperalgesia persists after resolution of inflammation. The proinflammatory cytokine interleukin (IL)-1β directly sensitizes nociceptors, leading to transient hyperalgesia (2,3). Peripheral injection of other inflammatory mediators such as prostaglandin E2 (PGE2) also increases the sensitivity of nociceptors and the response to painful stimuli (4). Moreover, there is evidence that proinflammatory cytokines such as IL-1β and tumor necrosis factor (TNF)-α contribute to the genesis of neuropathic pain (5,6).

Many of the signals involved in inflammatory hyperalgesia are generated via activation of G protein–coupled receptors (GPCRs) expressed in sensory neurons. The activity of GPCRs is regulated by the family of GPCR kinases (GRK) 1–7. Agonist-activated GPCRs are phosphorylated by GRKs, inducing rapid uncoupling from the G protein, a process.
called homologous receptor desensitization. GRK-mediated GPCR phosphorylation facilitates binding of arrestin proteins, promoting GPCR internalization (7,8). GRKs are also capable of interacting with a variety of downstream signaling molecules, thereby regulating cellular signaling independently of GPCRs (8,9).

GRK6 plays a crucial role in inflammatory pathologies. For example, GRK6 deficiency increases acute inflammatory arthritis as well as colitis in male mice (10,11). Recently, we showed that post-inflammatory visceral hyperalgesia is enhanced in female GRK6−/− mice without affecting inflammation (12). This previous study indicated that GRK6 plays a role in regulating visceral post-inflammatory pain, but did not give insight into the mechanisms involved.

Although most of the substrates of GRK6 are probably still unknown, it has been shown that GRK6 regulates desensitization of the chemokine receptor CXCR4, the BLT1 receptor for the leukotriene B4 (LTB4) and the calcitonin gene-related peptide (CGRP) receptor (13–16). Furthermore, GRK6 binds and phosphorylates PDZ domains in Na+/H+ exchanger regulatory factor (NHERF) and binds to downstream regulatory element antagonistic modulator (DREAM), both regulators of ion channels, indicating that GRK6 can also regulate cellular signaling via mechanisms independent of GPCR desensitization (17,18).

We aimed to determine the contribution of GRK6 to somatic inflammatory hyperalgesia. As a model, we induced local hyperalgesia by a single injection of carrageenan or the proinflammatory cytokines IL-1β or TNF-α into the paw. In search of the mechanism via which GRK6 regulates hyperalgesia, we analyzed the consequences of GRK6 deficiency for cytokine signaling to p38 and PI 3-kinase.

**MATERIAL AND METHODS**

**Animals**

Female GRK6-deficient C57BL/6 and wild-type (WT) control littermates were bred in the Utrecht University Central Animal Facility (12) and genotyped by polymerase chain reaction (PCR) analysis on genomic DNA. All experiments were performed in accordance with international guidelines and approved by the University Medical Center Utrecht experimental animal committee or were approved under the United Kingdom Home Office Animals (Scientific Procedures) Act 1986.

**Induction of Cytokine-Induced Thermal Hyperalgesia**

Mice received an intraplantar injection of 5 μL 1% λ-carrageenan (Sigma-Aldrich, St. Louis, MO, USA), 5 μL recombinant mouse IL-1β (0.2–200 ng/mL; Preprotech, Rocky Hill, NC, USA) or TNF-α (20 ng/mL; Preprotech) in saline or 5 μL saline as a control (19).

Heat withdrawal latency times were determined using the Hargreaves test at three different intensities (n = 8). (B) Thresholds to mechanical stimulation were determined using von Frey hairs (n = 14). (C) WT and GRK6−/− mice (n = 8–12) received an intraplantar injection of IL-1β, and the decrease in heat withdrawal latency was determined 2 h after injection. Two-way ANOVA: genotype: P < 0.001; dose: P < 0.001; interaction: P < 0.001. (D, E) Time course of IL-1β–induced thermal hyperalgesia in WT and GRK6−/− mice (D: 10 pg IL-1β; E: 1,000 pg IL-1β; n = 8–14). (F) Time course of IL-1β–induced mechanical allodynia in WT and GRK6−/− mice (n = 8). Data are expressed as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.
phosphate-buffered saline. Cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) containing 10% fetal bovine serum (Gibco), 2 mM/L glutamine (Gibco), 10,000 IU/mL penicillin-streptomycin (Gibco) and 100 ng/mL nerve growth factor (NGF) (Sigma-Aldrich) and poly-L-lysine– and laminin-coated wells. Cells were stimulated with IL-1β for 5 min 15–25 h after plating.

**Western Blot Analysis**

Cells were homogenized in lysis buffer (200 mM L NaCl, 50 mM L Tris-HCl, pH 7.5, 10% glycerol, 1% NP-40, 2 mM/L sodium orthovanadate, 2 mM/L phenylmethylsulfonyl fluoride (PMSF), 2 μmol/L leupeptin, protease inhibitor mix [p3840, 1:200; Sigma-Aldrich]). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Blots were stained with mouse key anti-rabbit-peroxidase (Amersham International) and developed by enhanced chemiluminescence plus (Amer sham International). Band density was quantified using a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA, USA).

**mRNA Isolation and Real-Time PCR**

Lumbar DRGs were homogenized in Trizol (Invitrogen, Paisley, UK). Total RNA was isolated with RNasey Mini Kit (Qiagen) and reverse-transcribed using an iScript™ Select cDNA Synthesis Kit (Invitrogen). Real-time quantitative PCR was performed with an iQ™ SYBR® Green Supermix (Invitrogen). Primer pairs used were as follows:

**GRK6:** CTTGG TCTCA TAGGC (forward), CTCGG TCTCA TAGGC (reverse); GRK2: CCGGACTTC TGCTC AACCC ATCTG (forward), CTCGG CTCCGA GCAC (reverse); β-arrestin1: AAGGGA

**ACAGG AGTGT TCAAG A (forward), CCCGC TTTCG CAGGT AGAC (reverse); β-arrestin2:** GGCAA GCGCG (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): TGGCA CTCTCA AACTC (reverse).

**Data Analysis**

Data are expressed as mean ± standard error of the mean (SEM) and analyzed using the Student t test, one-way analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni analysis. A P value <0.05 was considered statistically significant.

All supplementary materials are available online at www.molmed.org.

**RESULTS**

**Increased IL-1β-Induced Thermal and Mechanical Hyperalgesia in GRK6–/– Mice**

Heat-withdrawal latencies of WT and GRK6–/– mice at baseline were compared using the Hargreaves test at increasing intensities. Under baseline conditions, there was no genotype-dependent difference in heat sensitivity (Figure 1A). Similarly, sensitivity to mechanical stimuli, as measured with the Von Frey test, was not different between genotypes (Figure 1B).

Intraplantar injection of IL-1β dose-dependently increased thermal hyperalgesia in WT mice, as determined at 2 h after injection (Figure 1C). In GRK6–/– mice, the dose-response curve was sharply shifted to the left (see Figure 1C). Notably, the lowest dose of IL-1β (100 pg) that induced detectable thermal hyperalgesia in WT mice was 100-fold higher than the lowest dose that induced thermal hyperalgesia in GRK6–/– mice (1 pg).

To determine whether GRK6 also regulates duration of IL-1β–induced thermal hyperalgesia, we followed intraplantar...
IL-1β–induced thermal hyperalgesia over time. Hyperalgesia induced by intraplantar injection of either a low (10 pg/paw) or high (1,000 pg/paw) dose of IL-1β was markedly prolonged in GRK6-deficient mice (3 or 8 d after injection of a low or high dose of IL-1β in GRK6−/− mice versus 6 h or 1 d in WT mice; Figures 1D, E). Mechanical alldynia induced by IL-1β (1,000 pg/paw) was also markedly prolonged in GRK6−/− mice compared with WT mice (4–6 d after injection in GRK6−/− mice versus <1 d in WT mice; Figure 1F). At 24 h after intraplantar IL-1β (1,000 pg/paw), there were no differences in expression of COX2, IL-6 and TNF-α mRNA, indicating that the prolongation of hyperalgesia was independent of inflammatory activity (Supplementary Figure 1).

Carrageenan-induced thermal hyperalgesia was also significantly prolonged in GRK6−/− mice in comparison to WT mice (Figure 2A, recovery within 6–8 d in GRK6−/− mice versus 2–3 d in WT mice). Additionally, carrageenan-induced mechanical alldynia in GRK6−/− mice lasted three times longer than in WT mice (Figure 2B, recovery within 8–10 d in GRK6−/− versus 2–3 d in WT mice).

**In Vivo Role of p38 and PI 3-Kinase in the IL-1β–Induced Hyperalgesia**

To determine the in vivo relevance of the shift in IL-1β–induced activation of the p38 and PI 3-kinase/Akt pathway in DRG neurons of GRK6−/− mice, we compared the effect of intraplantar administration of the p38 inhibitor SB239063 (20 mg/kg, i.p.) or the PI 3-kinase inhibitor LY249002 (10 mg/kg, i.p.) on IL-1β–induced hyperalgesia in WT and GRK6−/− mice. In line with previous reports (2), intraplantar administration of SB239063 significantly attenuated IL-1β–induced hyperalgesia in WT mice. The p38 inhibitor SB239063 (5 mg/kg) also inhibited the magnitude of acute IL-1β–induced hy-
eralgesia in GRK6−/− mice and partially attenuated the duration of hyperalgesia that develops in these mice (Figure 4A).

In contrast, intraplantar administration of the PI 3-kinase inhibitor LY249002 (10 μg/paw) significantly enhanced the magnitude of acute (0.5–6 h) IL-1β-induced hyperalgesia in WT mice but did not have any effect on acute IL-1β hyperalgesia in GRK6−/− mice (Figure 4B). Additionally, IL-1β-induced hyperalgesia in WT mice was significantly prolonged after inhibition of PI 3-kinase. LY249002 increased and prolonged heat withdrawal latencies in WT or GRK6−/− mice. Injection of SB239063 or LY249002 alone did not have any effect on heat withdrawal latencies in WT or GRK6−/− mice (Figure 4C). These findings indicate that a similar mechanism, a switch from activation of both p38 and PI 3-kinase/Akt (23), is operative in GRK6−/− mice leading to the prolongation of p38-sensitive hyperalgesia in GRK6−/− mice (Figure 5A). In addition, the duration of hyperalgesia was limited to mediators signaling via p38, we also analyzed PGE2-induced hyperalgesia that is known to be cAMP-dependent protein kinase A (PKA) dependent (24,25). The data in Figure 6C show that PGE2-induced thermal hyperalgesia was similar in WT and GRK6−/− mice (Figure 5D), indicating that GRK6 deficiency does not affect hyperalgesia induced by an inflammatory mediator that signals via the cAMP/PKA pathway and independently of p38.

**GRK6 mRNA Expression Levels in DRG of Mice with Neuropathic or Inflammatory Pain**

To investigate whether changes in GRK6 do occur in conditions of chronic pain, we investigated GRK6 mRNA expression levels in DRGs of mice with chronic neuropathic or inflammatory pain. Four weeks after unilateral L5 nerve transection (L5 SNT), mice were more sensitive to mechanical stimulation of the ipsilateral paw (Figure 6A). Importantly, at this same time point, GRK6 mRNA levels were significantly reduced in ipsilateral DRGs compared with contralateral DRGs from sham-operated mice (Figure 6B). L5 SNT did not induce changes in mRNA levels for GRK2, β-arrestin1 or β-arrestin2 (Supplementary Figures 2A–C). Chronic inflammatory pain was induced by intraplantar injection of carrageenan. Six days after carrageenan injection, heat-withdrawal latencies were reduced (Figure 6C). At this time, GRK6 mRNA levels were significantly decreased in the DRGs of carrageenan-treated mice compared with vehicle-treated mice (Figure 6D). mRNA levels for GRK2 and β-arrestin2 did not differ between carrageenan- and vehicle-treated mice, whereas β-arrestin1 mRNA levels were slightly reduced (Supplementary Figures 2D–F).
Unfortunately, we were unable to test whether the decrease in GRK6 mRNA was associated with a reduction in GRK6 protein, since no reliable GRK6 antibodies were available.

**DISCUSSION**

In this study, we present the novel concept that the kinase GRK6 plays a pivotal role in regulating the duration and intensity of inflammatory hyperalgesia. GRK6 deficiency strongly enhanced and prolonged thermal hyperalgesia and mechanical allodynia induced by intraplantar injection of either IL-1β or TNF-α. Similarly, hyperalgesia induced by intraplantar injection of carrageenan was markedly prolonged in GRK6−/− mice. We also show that GRK6 deficiency promotes activation of p38 while the activation of PI 3-kinase/Akt is dampened. Thus, the novelty and significance of these results is that GRK6 emerges here as a kinase that constrains neuronal responsiveness to IL-1β and TNF-α and ultimately cytokine-induced hyperalgesia via biased cytokine-induced p38 and PI 3-kinase/Akt activation (Figure 6E). The potential pathophysiological significance of these findings is substantiated by our finding that DRG GRK6 expression levels are reduced in a model of chronic neuropathic pain and inflammatory pain.

Recent evidence indicates that hyperalgesia induced by intraplantar administration of IL-1β is mediated via activation of p38 and subsequent modulation of the activity of tetrodoxin-resistant sodium channels (2). TNF-α is also known to signal to p38 and is capable of inducing hyperalgesia via mechanisms that involve increased transient receptor potential channel subfamily V member 1 (TRPV1) expression and modulation of tetrodoxin-resistant sodium channels (26,27). We show here for the first time that GRK6 constrains IL-1β–induced p38 activation in sensory neurons. This conclusion is on the basis of our in vitro finding that IL-1β–induced p38 phosphorylation was enhanced in GRK6−/− DRG cultures. Additionally, our in vivo studies showed that p38 activation is required for IL-1β and TNF-α hyperalgesia both in WT and GRK6−/− mice. Finally, we show that in vivo IL-1β– and TNF-α–induced hyperalgesia is increased in GRK6-deficient mice. Collectively, these findings support a central role of p38 in cytokine-induced hyperalgesia and position GRK6 as a pivotal regulator of cytokine-induced hyperalgesia.

Our findings indicate that GRK6 deficiency enhances IL-1β signaling to p38 in
GRK6 regulates cytokine-induced hyperalgesia

nociceptors. The IL-1 receptor is expressed in virtually all sensory neurons (28). Moreover, IL-1β injection into the rat paw enhances p-p38 in peripherin-positive sensory nerves (2). Finally, in mice in which Nav1.8-positive nociceptors are deleted, peripherin-positive neurons are reduced >85% and inflammatory pain does not develop (29). Therefore, we propose that GRK6 deficiency enhances IL-1β-induced hyperalgesia via promoting p38 signaling in peripherin-positive nociceptors.

Our experiments identified GRK6 as a prerequisite for IL-1β–induced activation of the PI 3-kinase/Akt pathway in primary sensory neurons. This conclusion is on the basis of our finding that IL-1β induced a significant increase in Akt phosphorylation in WT mice, but was unable to do so in GRK6−/− mice. Second, we show here that IL-1β– and TNF-α–induced PI 3-kinase activity is required to constrain cytokine-induced hyperalgesia in magnitude as well as duration. Inhibition of PI 3-kinase enhanced and prolonged IL-1β and TNF-α hyperalgesia in WT mice, whereas inhibition of PI 3-kinase did not affect hyperalgesia in GRK6−/− mice. Thus, our studies in GRK6-deficient mice reveal a completely novel role of the PI 3-kinase/Akt signaling cascade in attenuating the severity and duration of cytokine-induced hyperalgesia. Interestingly, recent evidence shows that PI 3-kinase also mediates a negative feedback loop in preventing neuronal hyperexcitability in the Drosophila neuromuscular junction (30). In mice, it was shown that PI 3-kinase inhibition blocks capsaicin- and NGF-induced increases in pain sensitivity (hyperalgesia) (31), whereas the morphine-induced reduction in pain sensitivity (analgesia) is mediated via PI 3-kinase (32). Our present data demonstrate that PI 3-kinase inhibition increases and prolongs cytokine-induced hyperalgesia in WT mice. The differential effects of PI 3-kinase activity in determining pain sensitivity may depend on the fact that the isoform of PI 3-kinase that is activated in response to NGF is of a different subtype than the PI 3-kinase activated by cytokines or morphine, PI3Kγ (PI 3-kinase γ) (32). Thus, if GRK6 is only required for activation of the PI3Kγ isoform of PI 3-kinase activated by TNF-α and IL-1β, one would expect that NGF-induced hyperalgesia would not be affected by GRK6. Indeed, we show that NGF-induced hyperalgesia was similar in GRK6−/− and WT mice (Supplementary Figures 3A, B).

Opposing effects of the mitogen-activated protein kinase p38 and PI 3-kinases on cellular function have been shown previously. Lipopolysaccharide (LPS)-induced IL-6 release is inhibited when p38 is blocked, whereas inhibition of the PI 3-kinase/Akt pathway enhances IL-6 production (33). Additionally, TNF-α and IL-1β can both induce a PI 3-kinase/Akt-dependent decrease in potassium currents (34), whereas p38 activation enhances potassium currents (35). The exact molecular mechanisms that explain how p38 and PI 3-kinase define the balance in the effects on cytokine-induced hyperalgesia remain to be elucidated (Figure 6E).

The question arises through which mechanisms GRK6 is required for the activation of PI 3-kinase and how GRK6 inhibits p38 activation. IL-1β–induced activation of PI 3-kinase requires recruitment of PI 3-kinase to the interleukin-1 receptor (IL-1R) (36). Another member of the GRK family, GRK2, was implicated in facilitating agonist-induced PI3Kγ recruitment to the β2-adrenergic receptor (37). Similarly, it is possible that GRK6 regulates recruitment of PI3Kγ to the IL-1β receptor, enabling IL-1β–induced activation of PI 3-kinase. In addition, GRK2 inhibits p38 activation by phosphorylation of p38 at Thr-123, a residue located at its docking groove (38). Importantly, the central serine/threonine kinase catalytic domain as well as the N-terminal regulator of G protein signaling (RGS)-like domain are highly conserved in all GRKs (39). Thus, it is possible that GRK6 interferes with p38 activation via binding and phosphorylation of p38 similar to what has been described for GRK2. Future studies will have to unravel the precise mechanisms.

We recently described that a partial reduction in GRK2 increases hyperalgesia induced by carrageenan and the GPCR ligands CCL3 and PGE2 (24,40). However, reduced GRK2 did not affect the magnitude of IL-1β–induced acute hyperalgesia (40,41). Vice versa, we show here that GRK6 deficiency does not affect PGE2-induced hyperalgesia. These findings indicate that GRK6 and GRK2 regulate hyperalgesia induced by inflammatory mediators via separate mechanisms. These distinct effects of GRK6 and GRK2 on inflammatory hyperalgesia conform to the notion that the different GRKs have specific and often contrasting effects on signaling pathways (42).

GRK6 and other members of the GRK family have been originally identified because of their capacity to phosphorylate agonist-occupied GPCRs (43). However, to the best of our knowledge, there is no evidence that IL-1β and TNF-α signal directly via a GPCR. One possible explanation for our findings could be that IL-1β induces the local production of GPCR ligands (for example, PGE2) and that GRK6 regulates signaling via this receptor with consequences for hyperalgesia. However, inhibition of COX2 did not affect IL-1β–induced hyperalgesia in WT or GRK6−/− mice (Supplementary Figure 4). Additionally, we showed that the magnitude and duration of PGE2 hyperalgesia was similar in WT and GRK6−/− mice. We cannot exclude that other GPCR ligands are produced at the site of injection of the cytokines. However, this explanation seems unlikely in view of our finding that the change in phosphorylation of p38 and Akt in sensory neurons was already observed 5 min after stimulation with IL-1β in vitro. Overall, these data point to a role of GRK6 in regulating cytokine-induced events that are independent of GPCR signaling.

The data presented herein indicate that GRK6 deficiency enhances the sensitivity to IL-1β–induced hyperalgesia. It may therefore be that GRK6 deficiency also facilitates other inflammatory processes involving IL-1β. Indeed,
this hypothesis is supported by our earlier studies. For example, we have shown that increased inflammatory visceral hyperalgesia in GRK6–/– mice is characterized by increased neuronal and behavioral responses to noxious colonic stimulation with capsaicin. We also showed that overexpression of GRK6 attenuated IL-1β-induced TRPV1 sensitization (12). Interestingly, blocking TRPV1 with capsazepine partially inhibited the ongoing IL-1β hyperalgesia 24 h after IL-1β administration (Supplementary Figure 5), suggesting enhanced TRPV1 sensitization may underlie the enhanced intraplantar IL-1β–induced heat hyperalgesia as well. Finally, we demonstrated that in vivo, GRK6 deficiency leads to increased severity as well as duration of colitis (11). In these models of visceral hyperalgesia and colon inflammation, IL-1β is produced in the colon, and thus increased sensitivity to IL-1β–induced events may contribute to the increased and prolonged pain and colitis. In addition, Tarrant et al. (10) showed that GRK6–/– mice develop more severe joint inflammation in the K/BxN model of acute inflammatory arthritis (10). These authors showed that serum IL-1 levels are normal, but IL-6 levels are elevated in GRK6–/– mice. This study was supported in part by National Institutes of Health grants RO1-NS073939 to R Dantzer, KW Kelley and A Kavelaars and RO1-NS074999 to A Kavelaars. Part of the work of N Eijkelkamp was supported by a Rubicon fellowship of the Netherlands Organization for Scientific Research.

**CONCLUSION**

In summary, here we have identified for the first time GRK6 as a crucial kinase that is required to constrain cytokine signaling and cytokine-induced hyperalgesia. This contribution of GRK6 to hyperalgesia is likely to be mediated by regulating the balance of cytokine-induced p38 and PI 3-kinase/Akt activation in normal mice. This important relevant function of GRK6 in modulating cytokine-induced signaling events may also play a role in chronic inflammatory conditions.

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**REFERENCES**