ORIGINAL RESEARCH COMMUNICATION

Oxidant-Induced Activation of cGMP-Dependent Protein Kinase α Mediates Neuropathic Pain After Peripheral Nerve Injury

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Abstract

Aims: Emerging lines of evidence indicate that oxidants such as hydrogen peroxide exert specific signaling functions during the processing of chronic pain. However, the mechanisms by which oxidants regulate pain processing *in vivo* remain poorly understood. Here, we investigated whether cyclic guanosine monophosphate (cGMP)-dependent protein kinase α (cGKI α), which can be activated by oxidants independently of cGMP, serves as a primary redox target during pain processing. **Results:** After peripheral nerve injury, oxidant-induced cGKI α activation is increased in dorsal root ganglia of mice. Knock-in (KI) mice in which cGKI α cannot transduce oxidant signals demonstrated reduced neuropathic pain behaviors after peripheral nerve injury, and reduced pain behaviors after intrathecal delivery of oxidants. In contrast, acute nociceptive, inflammatory, and cGMP-induced pain behaviors were not impaired in these mice. **Innovation:** Studying cGKI α KI mice, we provide the first evidence that oxidants activate cGKI α in sensory neurons after peripheral nerve injury *in vivo*. **Conclusion:** Our results suggest that oxidant-induced activation of cGKI α specifically contributes to neuropathic pain processing, and that prevention of cGKI α redox activation could be a potential novel strategy to manage neuropathic pain. *Antioxid. Redox Signal.* 21, 1504–1515.

Introduction

PAIN USUALLY SERVES as a warning signal to protect the body from tissue injury. However, nerve lesion (neuropathic pain) or tissue damage (inflammatory pain) leads to a state of hypersensitivity that often becomes chronic and refractory to currently available treatments (2, 8). Several lines of evidence indicate that reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), essentially contribute to the development of hypersensitivity during chronic pain (26, 43). For example, persistent neuropathic and inflammatory pain behaviors were reduced in rodents after the administration of various ROS scavengers and antioxidants (30, 33, 49, 57, 58). Moreover, mice lacking the

Innovation

In this article, we demonstrate for the first time that specific redox signaling involving cyclic guanosine monophosphate (cGMP)-dependent protein kinase α (cGKI α) occurs in chronic pain states *in vivo*. Our results indicate that oxidants such as hydrogen peroxide activate cGKI α in sensory neurons after peripheral nerve injury, thereby contributing to the development of neuropathic pain hypersensitivity. The data provide new insights into specific reactive oxygen species-dependent mechanisms of pain sensitization and could offer new therapeutic avenues for the treatment of neuropathic pain in the clinic.

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ROS-producing NADPH oxidases Nox1, Nox2, and Nox4 demonstrated a reduced pain behavior in models of persistent pain (23, 25, 29), whereas inactivation or deficiency of the antioxidant proteins superoxide dismutase and Sestrin 2 was accompanied with increased persistent pain behaviors (4, 24, 48). Nevertheless, to date, the molecular targets of ROS during the processing of persistent neuropathic and/or inflammatory pain remain poorly understood.

An important contributor of persistent pain signaling is cyclic guanosine monophosphate (cGMP)-dependent protein kinase I α (cGKI α , synonym PKG1 α). This enzyme is highly expressed in primary afferent neurons that detect painful stimuli and transmit the nociceptive signal to the central nervous system (35, 42, 45, 46, 54, 55). cGKI α exists as a homodimer that is stabilized *via* the noncovalent interaction of the N-terminal regions of the two subunits, and it is classically activated by cGMP (21). Interestingly, recent data revealed that oxidants can cause interprotein disulfide bond formation between two cysteine residues (Cys⁴²) on adjacent chains in the cGKI α homodimer, rendering the kinase catalytically active, independently of cGMP (9). In the present study, we investigated whether such an oxidant-dependent activation of cGKI α exists in nociceptive neurons and whether this is relevant for pain processing *in vivo*. We demonstrate that oxidant-dependent disulfide activation of cGKI α plays an important role in the maintenance of neuropathic pain after peripheral nerve injury.

Results

H₂O₂ induces cGKI α disulfide dimerization in neurons

We first evaluated whether oxidant-induced dimerization of cGKI α may occur in neurons. We exposed neuroblastoma cells to various concentrations of H₂O₂ *in vitro*, isolated cells in the presence of maleimide to block thiol oxidation (9), and analyzed the amount of cGKI α monomer and dimer by nonreducing western blot analyses. The exposure to H₂O₂ induced a cGKI α disulfide dimerization in a concentration-dependent manner (Fig. 1A, B). In contrast, cGKI α disulfide dimerization did not occur after exposure to 8-(4-chlorophenylthio) guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP), a cGMP analog that activates cGKI α independently from disulfide formation (Fig. 1A). Moreover, the H₂O₂-induced cGKI α disulfide dimerization was prevented by co-incubation with catalase, an enzyme which degrades H₂O₂ (Fig. 1C). These data indicate that oxidants, in contrast to cGMP, may induce a covalent disulfide dimerization of cGKI α in neurons.

Basic characteristics of cGKI α knock-in mice

We used Cys42Ser cGKI α knock-in (KI) mice to analyze whether oxidant-induced cGKI α disulfide dimerization might contribute to pain processing. In these mice, cGKI α cannot be activated by oxidants due to the lack of the redox-sensing thiol (Cys⁴²); whereas the cGMP-induced cGKI α activation remains intact (41). We first compared the cGKI α expression in lumbar dorsal root ganglia (DRGs) of naïve wild-type (WT) and KI mice by western blot analyses. Both monomer and dimer of cGKI α were detected in WT mice (Fig. 2A, B). In contrast, only cGKI α monomer was detected in KI mice (Fig. 2A), confirming that Cys⁴²-mediated disulfide forma-

FIG. 1. Oxidant-induced disulfide dimerization of cyclic guanosine monophosphate (cGMP)-dependent protein kinase I α (cGKI α) in neurons. (A) SH-SY5Y neuroblastoma cells were exposed to different concentrations of the oxidant hydrogen peroxide (H₂O₂) and the cGMP analog 8-(4-chlorophenylthio)guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP). The amounts of cGKI α dimer (150 kDa) and monomer (75 kDa) were analyzed using nonreducing western blot analyses. It should be noted that H₂O₂ evoked a cGKI α disulfide dimerization, whereas 8-pCPT-cGMP had no effect. (B) Ratio of cGKI α monomer and dimer after H₂O₂ stimulation. Monomer was significantly decreased, and dimer was significantly increased as compared with nonstimulated cells ($n=4$). (C) H₂O₂-induced cGKI α disulfide dimerization was prevented by coincubation with catalase, confirming a redox-dependent mechanism. Data are presented as mean $\pm$ standard error of the mean (SEM). * $p < 0.05$.

tion is impaired. We then analyzed the cellular distribution of cGKI α in DRGs of naïve WT and KI mice by immunofluorescence experiments. Consistent with earlier studies in rats and mice (35, 42), cGKI α immunoreactivity was detected in the vast majority of DRG neurons. Double labeling experiments revealed that virtually all substance P (SP)-expressing peptidergic nociceptors and isolectin B4 (IB4)-binding non-peptidergic nociceptors contained cGKI α (Fig. 2C). This distribution pattern was similar in WT and KI mice (Fig. 2C), as was the proportion of DRG neurons positive for IB4, SP, transient receptor potential cation channel V1 (TRPV1), or neurofilament 200 (NF200; Fig. 2D). Moreover, the termination of primary afferent neurons in the dorsal horn (Fig. 2E) and the size and structure of the spinal cord (Fig. 2F) were comparable between WT and KI mice. The normal spinal

cord structure in KI mice essentially differs from that in cGKI knockout (cGKI^{-/-}) mice, which exhibit a reduced ventral-to-dorsal diameter of the spinal cord (55). Together, these data indicate that the lack of cGKIα disulfide activation in the KI mice does not impair the general characteristics of DRG neurons and the spinal cord.

To assess the function of oxidant-induced cGKIα disulfide activation in pain processing *in vivo*, we compared the nociceptive behavior of littermate Cys42Ser cGKIα KI and WT mice in various animal models of pain. The KI mice are viable and fertile, normal in size and weight, and do not show any obvious behavioral abnormalities. We tested both male and female mice (17, 37), but no significant main effects of gender were detected in any assay. Having established that the motor coordination is not impaired in KI mice (median rota-rod fall-off latencies: 120 s in KI and WT mice; $p=0.436$; $n=13-17$), we examined the immediate behavioral responses of KI and WT mice to thermal and mechanical stimulation. No significant differences were found between KI and WT mice after thermal stimulation with different intensities using a Hargreaves apparatus, a 54°C hot plate,

and a 0°C cold plate, and after mechanical stimulation with a Dynamic Plantar Aesthesiometer (Fig. 3). These results indicate that an oxidant-induced cGKIα disulfide activation does not contribute to the immediate response to acute noxious stimulation.

Oxidant-induced pain behavior is reduced in cGKIα KI mice

To evaluate the nociceptive behavioral response of oxidant-induced cGKIα disulfide activation, we induced mechanical hypersensitivity by an intrathecal (i.t.) injection of the oxidant *tert*-butyl hydroperoxide (TBHP; 1.1 μmol) in Cys42Ser cGKIα KI and WT mice. As shown in Figure 4A, an i.t. injection of TBHP evoked mechanical hypersensitivity of the hindpaws in KI and WT mice. However, the hypersensitivity was considerably decreased in KI mice compared with WT littermates, and significant differences between groups occurred between 30 and 45 min after the i.t. injection (Fig. 4A). In contrast, the hypersensitivity induced by an i.t. injection of the cGMP analog 8-pCPT-cGMP (10 nmol) was

FIG. 3. Acute nociceptive pain behavior is normal in Cys42Ser cGKI α KI mice. Immediate responses to noxious thermal stimuli (A) in a Hargreaves apparatus at 9%, 11%, and 13% intensity ($n=8-9$), (B) on a 54°C hot plate ($n=13-17$), and (C) on a 0°C cold plate ($n=13-17$), and (D) mechanical thresholds measured using a Dynamic Plantar Aesthesiometer ($n=18-19$) were similar in KI and WT mice. Data are presented as mean $\pm$ SEM.

indistinguishable between KI and WT mice (Fig. 4B). These data demonstrate that cGKI α disulfide activation essentially contributes to oxidant-induced, but not cGMP-induced, pain hypersensitivity *in vivo*.

Neuropathic pain behavior is reduced in cGKI α KI mice

We then explored whether cGKI α disulfide activation is relevant for disease-induced pain states *in vivo*. For that purpose, we first assessed the behavior of Cys42Ser cGKI α KI mice after spared nerve injury (SNI), a model of neuropathic pain that is associated with increased H_2O_2 production in nociceptive tissues (25). During the first days after SNI surgery, mechanical hypersensitivity of the operated hindpaw developed similarly in KI and WT mice (Fig. 5A). Interestingly, however, at later time points, the extent of mechanical hypersensitivity remained constant in KI mice, while it gradually increased in WT mice. Significant differences between groups occurred between 13 days after nerve injury and the end of the 35-day observation period (Fig. 5A), indicating that cGKI α disulfide activation is required for the maintenance of SNI-induced pain hypersensitivity. Of note, a reduced neuropathic pain behavior in KI mice was also observed in another model of peripheral nerve injury, the chronic constriction injury (CCI) model (Fig. 5B). Significant differences in mechanical hypersensitivity between KI and WT mice again occurred at later stages (Fig. 5B). Moreover, an i.t. administration of a cGKI inhibitor (8-bromoguanosine-3',5'-cyclic monophosphorothioate, Rp- isomer; Rp-8-Br-cGMPs; 50 nmol) (55) at 3 weeks after peripheral nerve

FIG. 4. Oxidant-induced pain behavior is reduced in Cys42Ser cGKI α KI mice. (A) Mechanical hypersensitivity of the hindpaws evoked by intrathecal (i.t.) injection of the reactive oxygen species donor *tert*-butyl hydroperoxide (TBHP; 1.1 μ mol) was considerably reduced in KI mice compared with WT littermates ($n=6$). (B) In contrast, mechanical hypersensitivity induced by i.t. injection of the cGMP analog 8-pCPT-cGMP (10 nmol) did not differ between groups ($n=6$). Data are presented as mean $\pm$ SEM. * $p < 0.05$.

injury ameliorated the mechanical hypersensitivity in WT mice (Fig. 6), further confirming a role of cGKI α in the maintenance of neuropathic pain hypersensitivity. These data demonstrate that oxidant-induced cGKI α disulfide activation contributes to the processing of neuropathic pain after peripheral nerve injury.

To monitor cGKI α expression and disulfide bond formation after peripheral nerve injury, we performed maleimide-protected, nonreducing western blot analyses of DRGs from naïve mice and SNI-operated mice. Unexpectedly, total cGKI α protein expression (sum of cGKI α monomer and dimer) was decreased at 3, 7, and 17 days after SNI in WT mice (Fig. 7A). This injury-induced decrease of cGKI α expression was not observed in KI mice (Fig. 7A). Most importantly, peripheral nerve injury evoked cGKI α disulfide formation in DRGs of WT mice, as indicated by a significant increase of cGKI α dimer ratio at 3, 7, and 17 days after SNI surgery (Fig. 7A, B). A comparable increase in cGKI dimer ratio was also observed in WT DRGs exposed to 100 μ M H_2O_2 *ex vivo* (Fig. 7C). Moreover, quantitative real-time RT-PCR experiments revealed that the injury-induced drop in total cGKI α protein in WT mice (Fig. 7A) was paralleled by a significant decrease

FIG. 5. Neuropathic pain behavior is reduced in Cys42Ser cGKI α KI mice. Paw withdrawal latency times of KI and WT mice after mechanical stimulation in the (A) spared nerve injury (SNI) and (B) chronic constriction injury (CCI) model of peripheral nerve injury ($n=10-12$). It should be noted that KI mice demonstrated reduced mechanical hypersensitivity compared with WT littermates. Data are presented as mean $\pm$ SEM. * $p<0.05$.

in cGKI mRNA levels (Fig. 7D). These findings are in line with earlier observations that oxidants can modulate cGKI expression (39). We conclude that peripheral nerve injury induces cGKI α disulfide activation in DRGs, and that this process affects neuropathic pain hypersensitivity and cGKI α expression.

We next assessed the induction of activating transcription factor 3 (ATF3), a sensitive marker of nerve injury (6, 56), in DRG neurons of WT and KI mice at 17 days after SNI. ATF3 expression was induced in DRG neurons of both genotypes (Fig. 8A, B). However, the ATF3 induction was significantly decreased in KI mice as compared with WT mice (Fig. 8C). Considering that ATF3 is induced in an oxidant-dependent manner (20, 24), this observation suggests that oxidant-induced cGKI α activation contributes to ATF3 induction in sensory neurons after peripheral nerve injury. Altogether, these data demonstrate that the disulfide bond formation of cGKI α caused by oxidants is important for the processing of neuropathic pain.

Inflammatory pain behavior is normal in cGKI α KI mice

Finally, we monitored the behavior of Cys42Ser cGKI α KI mice in models of inflammatory pain. In the formalin test, an injection of 0.5% formalin into a hindpaw resulted in the

FIG. 6. Neuropathic pain behavior is reduced after inhibition of cGKI. Paw withdrawal latency times of WT mice after mechanical stimulation in the SNI model. The cGKI inhibitor Rp-8-Br-cGMPS (50 nmol) or vehicle (0.9% saline) was i.t. administered at 3 weeks after SNI ($n=6$). Data are presented as mean $\pm$ SEM. * $p<0.05$, comparing Rp-8-Br-cGMPS- and vehicle-treated animals.

typical biphasic nociceptive response consisting of licking the injected paw. However, the licking behavior was similar between KI and WT mice (Fig. 9A). In a second model of inflammatory pain, we analyzed the hyperalgesia evoked by a zymosan injection into a hindpaw. As shown in Figure 9B, KI mice developed mechanical hypersensitivity in the injected hindpaw that was indistinguishable from that of WT mice. Similarly, no significant differences between genotypes were observed when measuring thermal hypersensitivity after a zymosan injection (Fig. 9C). Moreover, cGKI mRNA expression in DRGs was not altered at 4, 8, and 24 h after a zymosan injection (data not shown). The normal inflammatory pain behavior of cGKI α KI mice is in marked contrast to the reduced inflammatory pain behavior that was observed in cGKI knockout mice (35, 55), suggesting that cGMP rather than oxidants mediate the cGKI activation during inflammatory pain. Hence, our data suggest that an oxidant-induced cGKI α disulfide activation is dispensable for the processing of inflammatory pain after paw inflammation.

Discussion

Oxidants can function as specific regulators of intracellular signaling pathways, in large part through covalent and reversible modifications of redox-sensitive cysteine residues within target proteins. The data in this study provide evidence that cGKI α in sensory neurons serves as a specific redox target after peripheral nerve injury, and that this signaling mechanism is relevant for neuropathic pain processing *in vivo*. Acute nociceptive and inflammatory pain processing, however, seems not to depend on oxidant-induced cGKI α activation.

Several recent studies revealed that the endogenous production of oxidants is increased after peripheral nerve injury. Important pain-relevant sources are the ROS-producing NADPH oxidases Nox4 and Nox2, which in response to peripheral nerve injury generate ROS in neuronal and non-neuronal cells of the nociceptive system, respectively (25, 29, 32). In addition, mitochondria in primary afferent neurons and spinal cord dorsal horn neurons increase their ROS

FIG. 7. cGKI α expression and disulfide dimerization after peripheral nerve injury. (A) Nonreducing western blot with DRGs from naïve mice and after SNI. The nerve injury reduced the total cGKI α protein expression at 3, 7, and 17 days after SNI surgery (sum of cGKI α monomer and dimer) in WT mice (left), but not in KI mice (right). $n=4-7$ animals per time point. cGKI α disulfide dimerization was increased in DRGs of WT mice at 3, 7, and 17 days after SNI (B; $n=4-7$) and in DRGs of naïve WT mice exposed to 100 μ M H₂O₂ *ex vivo* (C; $n=3$). (D) Expression of cGKI mRNA in DRGs of WT mice after SNI assessed by quantitative real-time RT-PCR. cGKI mRNA was significantly decreased at 7 and 14 days after SNI ($n=6$). Data are presented as mean $\pm$ SEM. * $p < 0.05$.

production after peripheral nerve injury (15, 40). It is likely that oxidants derived from additional sources contribute to pain processing, because various enzymes, including cyclooxygenases, lipoxygenases, cytochrome P450 monooxygenases, xanthine oxidase, and uncoupled nitric oxide synthase, may produce ROS in certain pathophysiological conditions (26). Although the elucidation of ROS sources in

FIG. 8. Activating transcription factor 3 (ATF3) induction in DRGs in response to peripheral nerve injury is decreased in Cys42Ser cGKI α KI mice. (A, B) Representative immunofluorescence of ATF3 in DRGs of WT (A) and KI (B) mice at 17 days after SNI (scale bar, 100 μ m). (C) Quantification of relative ATF3 expression ($n=5$ animals per genotype). Data are presented as mean $\pm$ SEM. * $p < 0.05$.

the nociceptive system awaits further analyses, the available data strongly suggest specific functions of ROS during neuropathic pain processing. For example, Nox2-derived ROS mediate microglia activation in the spinal cord, whereas Nox4-derived ROS induce dysmyelination of peripheral nerves (25, 29). The signaling specificity is most likely achieved by the colocalization of the ROS source near the intended target as well as to antioxidant molecules (16).

So far, various redox targets have been identified in the nociceptive system. These targets include transient receptor potential (TRP) channels, NMDA receptors, glutamate transporter, and transcription factors [for a review, see Kallenborn-Gerhardt *et al.* (26)]. For example, electrophysiological experiments revealed that transient receptor potential cation channel A1 (TRPA1) is activated by several classes of endogenous thiol-reactive molecules (1), and that TRPV1 is sensitized by H₂O₂-induced covalent cysteine modifications (12, 53). Furthermore, hyperalgesia in response

FIG. 9. Inflammatory pain behavior is not impaired in Cys42Ser cGKI α KI mice. (A) Formalin test. Both genotypes showed a similar biphasic licking response to 0.5% formalin injected into a hindpaw ($n=5-8$). (B) Mechanical and (C) thermal hypersensitivity induced by zymosan injection in a hindpaw. Paw-withdrawal latency times after mechanical stimulation did not differ between KI and WT mice at all times tested ($n=7-8$). Data are presented as mean $\pm$ SEM.

to a paw injection with H_2O_2 was ameliorated in mice lacking TRPA1 or TRPV1 (5, 28). However, since these studies were performed *in vitro* or in animals administered with exogenous oxidants, it remained elusive at which specific pain states these and other redox targets contribute to pain processing *in vivo*. Here, we show that the cysteine residue 42 of cGKI α is a redox target during the processing of neuropathic pain. In the absence of cysteine 42-mediated cGKI α dimerization, the neuropathic pain hypersensitivity was considerably de-

creased in KI mice. Similar effects occurred in both the SNI and the CCI model of peripheral nerve injury, confirming that the pain phenotype is consistent across models. Moreover, the proportion of DRG neurons expressing ATF3 was decreased in KI mice after nerve injury. ATF3 is a member of the activating transcription factor/cAMP-responsive element binding protein family (ATF/CREB family) that activates or represses transcription by binding to other proteins, such as c-Jun and gadd153 (11). The detailed functions of ATF3 induction in DRG neurons are still not clear, but it seems to be associated with regeneration processes of peripheral nerves in response to injury of axons (38, 50). ATF3 is a widely used marker for damaged DRG neurons after peripheral nerve injury, because its induction correlates with the degree of injury (6, 27, 56). Hence, our data suggest that differential degrees of neuronal injury may underlie the differing evoked behavioral phenotypes in KI and WT mice.

We also observed that cGKI α mRNA and protein expression was suppressed after peripheral nerve injury in WT mice but not in KI mice. In general, cGKI expression can be transcriptionally controlled by several factors, including Sp1, upstream stimulatory factor 1/2, Krüppel-like transcriptional factor 4, RhoA, Rac1, FoxO1a, and the tumor suppressor p53 (51). Conversely, cGKI has been shown to regulate the activity of many transcription factors, including cAMP-response element binding protein, transcription factor II-I, activator protein 1, early growth response protein 1, and nuclear factor-kappa B. However, it is not known whether these transcription factors are also involved in modulating cGKI expression (51). Considering that many of these transcription factors are regulated by redox events (7) and that the cGKI α down-regulation did not occur in KI mice, it is conceivable that oxidant-induced cGKI α activation regulates not only neuropathic pain processing but also the cGKI α expression in sensory neurons. This idea is supported by earlier observations that oxidants may regulate cGKI expression in pulmonary vessels (39). On the other hand, since many transcription factors are regulated by cGKI α , it is possible that the decreased cGKI α expression affects the expression of other pain-relevant proteins and signaling pathways, which, in turn, might compensate for the altered cGKI α function. The exact molecular mechanisms governing the cGKI α decrease and its consequences after peripheral nerve injury remain to be elucidated.

The fact that cGKI α contributes to neuropathic pain processing has already been observed in earlier studies. For example, neuropathic pain produced by DRG compression in rats was ameliorated after treatment with a cGKI inhibitor (14, 52). Interestingly, cGMP analogs failed to enhance the hyperalgesic behavior after DRG compression (52), pointing to a cGMP-independent cGKI activation. In addition to neuropathic pain processing, cGKI α essentially contributes to inflammatory pain processing, as revealed by the ameliorated pain behavior in mice and rats after treatment with cGKI inhibitors (45, 54) and in mice with a global or nociceptor-specific deletion of cGKI (35, 55). Our data implicate that the cGKI α activation during inflammatory pain is mediated by cGMP but not by oxidants, because the inflammatory pain behavior was normal in cGKI α KI mice. Unlike in many other tissues, cGKI α in DRG neurons is not colocalized with nitric oxide-sensitive guanylyl cyclase (synonym "soluble" guanylyl cyclase) (44). Instead, cGKI in DRG neurons is

colocalized with the membrane-bound, “particulate” guanylyl cyclases, natriuretic peptide receptor A and B (NPR-A and NPR-B, respectively) (31, 44, 59). The expression of NPR-A and its endogenous ligand, B-type natriuretic peptide, is up-regulated in small DRG neurons on noxious hindpaw stimulation by complete Freund’s adjuvant, an agent that induces tissue inflammation and pain sensitization. Notably, an increased cGMP formation has recently been shown to prevent disulfide formation of cGKI α due to an allosteric structural change that reorients the redox-sensitive cysteines (10). Based on these data, it is tempting to speculate that cGKI α is primarily activated by cGMP produced by up-regulated natriuretic peptides and guanylyl cyclases during inflammatory pain processing; whereas its function in neuropathic pain processing mainly depends on oxidant-induced disulfide activation due to increased oxidant production.

Altogether, here, we provide evidence that specific redox signaling involving cGKI α occurs in persistent neuropathic pain states after nerve injury. Targeting oxidant-induced cGKI α activation might be considered a novel option in attenuating neuropathic pain.

Materials and Methods

Animals

The generation of “redox-dead” Cys42Ser cGKI α KI mice on a pure C57BL/6 background has been described in detail elsewhere (41). In these KI mice, the cysteinyl thiol 42 has been constitutively replaced by a hydroxyl group, which prevents oxidative disulfide activation of cGKI α while maintaining classic cGMP-dependent stimulation. Heterozygous mice on a C57BL/6 background were intercrossed with one another to obtain homozygous WT and KI mice. Behavioral experiments were performed in animals of either sex at the age of 6 to 14 weeks. Male C57BL/6N mice (Harlan) were additionally used for RT-PCR and western blot analyses. Animals were housed on a 12/12 h light/dark cycle with water and standard rodent chow available *ad libitum*. All experiments were performed in accordance with the International Association for the Study of Pain and approved by the local Ethics Committee for Animal Research.

Behavioral testing

All behavioral investigations were performed with littermate mice and done by a blinded observer.

Rotarod test. Motor coordination was assessed using a Rotarod treadmill for mice (Ugo Basile) at a constant rotating speed of 32 rpm. All mice had approximately five training sessions before the experiment. The cut-off time was 120 s, and every mouse was tested thrice.

Hargreaves test. Paw withdrawal latency after thermal stimulation was measured using a Plantar Analgesia Meter (model 390G; IITC). Mice were placed in plexiglas cages on top of a heated surface (32°C). The radiant heat source (9%, 11%, and 13% intensity) was aimed at the plantar side of the hindpaw, and the paw withdrawal latency was calculated as the mean of three to four trials with at least 30 s in between (18).

Hot- and cold-plate test. Mice were placed on a heated or cooled metal plate that was surrounded by a plexiglas cylinder (Hot/Cold Plate; Ugo Basile). In the hot-plate test, the time between placement and licking or shaking of one hindpaw was recorded. The plate was heated on 54°C and the cut-off time was 20 s, to prevent tissue damage. In the cold-plate test, the number of reactions (shaking and licking of a hindpaw, or jumping) after placement on a 0°C plate was counted during 5 min. In both tests, only one test per mouse was performed.

Formalin test. A 0.5% formaldehyde solution (20 μ l; formalin) was injected subcutaneously in the dorsal side of one hindpaw (22). The time spent licking the injected paw was measured at 5 min intervals during 60 min after the injection.

Mechanical paw sensitivity. Paw withdrawal latency after mechanical stimulation was measured using a Dynamic Plantar Aesthesiometer (Ugo Basile). In this test, a thin rod (diameter 0.5 mm) is pushed with increasing force against the plantar side of the hindpaw. The device stops automatically when the mouse withdraws the paw and records the latency time. The force increased from 0 to 5 g in 10 s (ramp 0.5 g/s) and was then held at 5 g for a further 10 s (19, 44). Every measurement was repeated four to five times with at least 20 s in between.

Zymosan-induced hypersensitivity. Twenty microliter of a zymosan A suspension (5 mg/ml in 0.1 M phosphate-buffered saline [PBS], pH 7.4; Sigma-Aldrich) was subcutaneously injected into the plantar side of one hindpaw (36). Mechanical and thermal hypersensitivities were determined using the Dynamic Plantar Aesthesiometer and Plantar Analgesia Meter, respectively, as described earlier.

Neuropathic pain. To investigate neuropathic pain behavior, we used the SNI model (13) and a modified version of the CCI model (3). Surgeries were performed under isoflurane anesthesia. For SNI, two branches of the sciatic nerve were ligated and cut distally, leaving the sural nerve intact. For CCI, the common sciatic nerve was exposed unilaterally at the mid-thigh level by blunt dissection. Proximal to the trifurcation of the sciatic nerve, ~5 mm of nerve was freed of adhering tissue, and three silk ligatures (6-0) that constricted the nerve by 30%–50% were tied around it with ~1 mm spacing (25). The Dynamic Plantar Aesthesiometer was used to determine mechanical paw sensitivity.

i.t. injections. TBHP [1.1 μ mol (24); Sigma-Aldrich], 8-pCPT-cGMP [10 nmol (19); Biolog] or Rp-8-Br-cGMPS [50 nmol (55); Biolog] were diluted in 5 μ l of sterile 0.9% saline and intrathecally administered by direct lumbar puncture in awake mice as described elsewhere (33, 34). Mechanical paw sensitivity was determined using the Dynamic Plantar Aesthesiometer.

Cell culture

Human neuroblastoma cells (SH-SY5Y) were maintained in a 1:1 mix of minimal essential medium (ATCC) and F-12

Nutrient Mix (Gibco) containing 15% fetal calf serum, 1% glutamine, 1% nonessential amino acid-mix, and 1% penicillin-streptomycin at 5% CO₂ and 37°C, and passaged once a week. They were cultured for 3 days before stimulation with 10, 100, and 1000 μ M H₂O₂ for 5 min or 10, 100, and 1000 μ M 8-pCPT-cGMP for 15 min. Incubation with catalase (312.5 U/ml; Sigma-Aldrich) was done at 15 min before and during H₂O₂ stimulation.

Western blot

Mice were killed by CO₂, and DRG (L4-L5) were taken out, snap frozen in liquid nitrogen, and stored at -80°C until use. For *ex vivo* stimulation with H₂O₂, 20–30 DRGs of naïve mice were pooled in Hanks' balanced salt solution at 4°C, incubated with Accutase solution (Sigma-Aldrich) for 30 min, stimulated with 100 μ M H₂O₂ for 10 min, and washed with PBS. Neuroblastoma cells were detached and washed with PBS. DRGs or cells were homogenized in Phosphosafe buffer (Novagen) combined with a protease inhibitor cocktail (Complete Mini; Roche Diagnostics) containing 100 mM maleimide (Sigma-Aldrich) and centrifuged at 20,000 g for 30 min at 4°C. Extracted proteins (30 μ g per lane) were separated by nonreducing SDS-polyacrylamide gel electrophoresis and blotted on a nitrocellulose membrane. The membranes were blocked in blocking buffer (Odyssey blocking buffer, LI-COR Bioscience; diluted 1:1 in PBS) for 1 h and incubated with rabbit anti-PKG (1:500; Stressgen; diluted in blocking buffer containing 0.1% Tween-20) over two nights at 4°C. After incubation with the secondary antibody conjugated with Alexa Fluor 680 or 800 (1:10,000; Invitrogen) for 90 min, proteins were detected using an Odyssey Infrared Imaging System (LI-COR Bioscience) and quantified by densitometry using NIH ImageJ software.

Immunostaining

Mice were intracardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in PBS (pH 7.4) under deep anesthesia. The lumbar (L4-L5) spinal cords and DRGs were dissected, postfixed in 4% PFA for 10 min, cryoprotected into 20% sucrose overnight, and frozen in tissue-freezing medium on dry ice. Cryostat sections were cut at a thickness of 16 μ m and stored at -80°C . Tissue sections were permeabilized for 5 min in PBST (0.1% Triton-X100 in PBS), blocked for 1 h using 3% bovine serum albumin (BSA) and 10% normal goat or donkey serum, and incubated with primary antibodies that were diluted in 3% BSA in PBS overnight at 4°C or for 2 h at room temperature. The following antibodies were used: rabbit anti-PKG (1:100; Stressgen), rat anti-SP (1:200; BD Biosciences), mouse anti-NF200 (clone N52, 1:1000; Sigma-Aldrich), rabbit anti-TRPV1 (1:800; Alomone), and rabbit anti-ATF3 (1:1500; Santa Cruz). Then, sections were washed with PBS and stained with secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen) or Cy3 (Sigma-Aldrich) for 2 h at room temperature. For staining with *Griffonia simplicifolia* IB4, sections were incubated with Alexa Fluor 488-conjugated IB4 (10 μ g/ml in PBS; Invitrogen) for 2 h at room temperature. To reduce the lipofuscin-like autofluorescence, slides were immersed in 0.06% Sudan black B (in 70% ethanol) for 5 min (47), washed with PBS, and

coverslipped. In experiments with more than one primary antibody, incubation was performed consecutively. Images were taken using an Axio Observer Z1 microscope (Zeiss) with a monochrome CCD camera (AxioCam Mrm; Zeiss). Images of different filters were pseudocolored and superimposed using the Zeiss Axio Vision 4.7.2 software. Brightness and contrast were adjusted using Adobe Photoshop CS software (Adobe Systems). Control experiments were performed without primary and/or secondary antibodies.

Determination of spinal cord size

Lumbar (L4-L5) spinal cords of WT and cGKI α KI mice (seven per genotype) were cut (16 μ m) and one to two sections, at least 100 μ m apart, per animal were analyzed. Size was determined using NIH ImageJ.

Cell counting

DRGs (L4-L5) of WT and cGKI α KI mice were cut (14–16 μ m), and randomly chosen sections with at least 70 μ m apart were counted. Only DRG neurons showing staining clearly above background and containing nuclei were included. The percentage of cells positive for markers (IB4, SP, TRPV1, NF200) was calculated from three to five animals per genotype and two to four sections per animal (>950 cells per genotype). ATF3 induction was calculated from five animals per genotype and from four to six sections per animal (>3500 cells per genotype). Data are expressed as a percent of the total number of DRG neurons counted.

Real-time RT-PCR

DRGs (L4-L5) were rapidly dissected, snap frozen in liquid nitrogen, and stored at -80°C . Total RNA was extracted under RNase-free conditions using an RNA isolation kit (RNAqueous Micro Kit; Ambion) according to the manufacturer's instructions. DNase was treated for 15 min to minimize genomic DNA contamination and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). cDNA was synthesized from 200 ng of RNA using random hexamers of the Verso cDNA kit (Thermo Scientific). Real-time RT-PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using TaqMan gene expression assays for murine cGKI (Mm00440954_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm99999915_g1), purchased from Applied Biosystems. Reactions (total volume, 10 μ l) were performed in duplicate by incubation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and of 1 min at 60°C. Water controls were included to ensure specificity. Relative expression of target gene levels was determined using the comparative $2^{-\Delta\Delta C_t}$ method, with C_t indicating the cycle number at which the signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR. The amount of sample RNA was normalized to GAPDH.

Statistics

Statistical analysis was performed with SPSS software. Student's *t*-test was used for paired comparisons, and one-way ANOVA followed by Fisher *post-hoc* test was used for

multiple comparisons. Rotarod fall-off latencies were analyzed using Mann–Whitney *U*-test. All data are presented as the mean $\pm$ standard error of the mean. For all tests, a probability value $p < 0.05$ was considered statistically significant.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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Abbreviations Used

8-pCPT-cGMP = 8-(4-chlorophenylthio) guanosine-3',5'-cyclic monophosphate
 ATF3 = activating transcription factor 3

BSA = bovine serum albumin
 CCI = chronic constriction injury
 cGKI α = cGMP-dependent protein kinase I α
 Cys42Ser = cysteine substituted by serine at position 42 of cGKI α
 DRG = dorsal root ganglion
 GAPDH = glyceraldehyde 3-phosphate dehydrogenase
 H₂O₂ = hydrogen peroxide
 i.t. = intrathecal
 IB4 = isolectin B4
 KI = knock-in
 NF200 = neurofilament 200
 Nox = NADPH oxidase
 NPR = natriuretic peptide receptor
 O₂⁻ = superoxide
 PBS = phosphate-buffered saline
 PFA = paraformaldehyde
 ROS = reactive oxygen species
 Rp-8-Br-cGMPs = 8-Bromoguanosine-3',5'-cyclic monophosphorothioate, Rp- isomer
 SEM = standard error of the mean
 SNI = spared nerve injury
 SP = substance P
 TBHP = *tert*-butyl hydroperoxide
 TRP = transient receptor potential
 TRPA1 = transient receptor potential cation channel A1
 TRPV1 = transient receptor potential cation channel V1
 WT = wild-type