Behavioral/Systems/Cognitive

Reactive Oxygen Species Derived from NOX1/NADPH Oxidase Enhance Inflammatory Pain

Masakazu Ibi,[1] Kuniharu Matsuno,[1] Dai Shiba,[2] Masato Katsuyama,[1] Kazumi Iwata,[1] Tomoko Kakehi,[1] Takayuki Nakagawa,[3] Kazunori Sango,[4] Yasuhito Shirai,[5] Takahiko Yokoyama,[2] Shuji Kaneko,[3] Naoaki Saito,[5] and Chihiro Yabe-Nishimura[1]

Departments of [1]Pharmacology and [2]Anatomy and Developmental Biology, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan, [3]Department of Molecular Pharmacology, Graduate School of Pharmaceutical Science, Kyoto University, Kyoto 606-8501, Japan, [4]Department of Developmental Morphology, Tokyo Metropolitan Institute for Neuroscience, Tokyo 183-8526, Japan, and [5]Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, Kobe 657-8501, Japan

The involvement of reactive oxygen species (ROS) in an augmented sensitivity to painful stimuli (hyperalgesia) during inflammation has been suggested, yet how and where ROS affect the pain signaling remain unknown. Here we report a novel role for the superoxide-generating NADPH oxidase in the development of hyperalgesia. In mice lacking *Nox1* (*Nox1*$^{-/Y}$), a catalytic subunit of NADPH oxidase, thermal and mechanical hyperalgesia was significantly attenuated, whereas no change in nociceptive responses to heat or mechanical stimuli was observed. In dorsal root ganglia (DRG) neurons of *Nox1*$^{+/Y}$, pretreatment with chemical mediators bradykinin, serotonin, or phorbol 12-myristate 13-acetate (PMA) augmented the capsaicin-induced calcium increase, whereas this increase was significantly attenuated in DRG neurons of *Nox1*$^{-/Y}$. Concomitantly, PMA-induced translocation of PKCε was markedly perturbed in *Nox1*$^{-/Y}$ or *Nox1*$^{+/Y}$ DRG neurons treated with ROS-scavenging agents. In cells transfected with tagged PKCε, hydrogen peroxide induced translocation and a reduction in free sulfhydryls of full-length PKCε but not of the deletion mutant lacking the C1A domain. These findings indicate that NOX1/NADPH oxidase accelerates the translocation of PKCε in DRG neurons, thereby enhancing the TRPV1 activity and the sensitivity to painful stimuli.

Key words: **NOX1; NADPH oxidase; PKCε; TRPV1; hyperalgesia; inflammation**

Introduction

An augmented sensitivity to painful stimuli (hyperalgesia) is characteristic of inflammatory pain and persists until the tissue heals. Chemical mediators released from damaged tissue and inflammatory cells enhance the perception of pain by decreasing the activation threshold of transient receptor potential vanilloid 1 (TRPV1) through a variety of posttranslational mechanisms (Cesare and McNaughton, 1996; Khasar et al., 1999; Hu et al., 2002; Chuang et al., 2001; Ohta et al., 2006). Among these mechanisms, protein kinase A (PKA)- and PKC-mediated phosphorylation of serine residues is known to modulate the sensitivity of TRPV1 (Bhave et al., 2002; Numazaki et al., 2002). TRPV1 is a nonselective cation channel with six transmembrane domains expressed predominantly in Aδ-fibers and unmyelinated C-fibers. It is activated by several stimuli, including heat (>43°C), capsaicin, and protons. Mice with a disruption in the *trpv1* gene were incapable of sensing capsaicin and showed little thermal hypersensitivity during inflammation (Caterina et al., 2000).

The involvement of reactive oxygen species (ROS) in nociceptive signaling has been reported in recent studies. The application of a compound that mimics the enzymatic activity of superoxide dismutase (SOD) or a radical scavenger TEMPOL attenuated hyperalgesia elicited by injection of an inflammatory agent, carrageenan (Wang et al., 2004; Khattab, 2006). Nitration of endogenous manganese (Mn) SOD in the spinal cord during carrageenan-induced hyperalgesia deactivates the enzyme, allowing superoxide levels to remain elevated and sustain nociceptive signaling (Wang et al., 2004). Although these results suggest that ROS mediate pain that accompanies inflammation, important issues left unanswered include the source of ROS and how ROS are involved in pain signaling.

NADPH oxidase is a superoxide-generating flavoenzyme comprising a membrane-bound catalytic subunit NOX and several cytosolic regulatory subunits. Among several homologs of NOX identified so far, NOX2 has been implicated in various neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (Wu et al., 2002, 2006; Abramov et al., 2004). In contrast, a relative paucity of data exists on the role of NOX1. NOX1 is primarily expressed in colon epithelial cells and to a lesser extent in vascular smooth muscle cells (Lambeth, 2004). In a preceding work using cultured neu-

Received April 28, 2008; revised Aug. 4, 2008; accepted Aug. 4, 2008.

This work was partly supported by Grant-in-Aid for Young Scientists (B) 17790172 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (M.I.). We thank Dr. D. Lambeth at Emory University and Dr. K. Rokutan at Tokushima University for providing anti-NOX1 antibodies, and Dr. M. Tominaga at Okazaki Institute for Integrative Bioscience for insightful discussions and critical reading of this manuscript.

Correspondence should be addressed to Dr. Chihiro Yabe-Nishimura, Department of Pharmacology, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan. E-mail address: nchihiro@koto.kpu-m.ac.jp.

DOI:10.1523/JNEUROSCI.1857-08.2008

Copyright © 2008 Society for Neuroscience 0270-6474/08/289486-09$15.00/0

ronal cells, we found an intriguing role for NOX1 in nerve growth factor (NGF)-induced neurite outgrowth (Ibi et al., 2006). Increased superoxide generation after upregulation of NOX1 induced by NGF negatively regulated neuronal differentiation by suppressing excessive neurite outgrowth. As an extension of this study, we investigated whether NOX1/NADPH oxidase is involved in neuronal development and signaling using mice deficient in the *Nox1* gene (*Nox1*$^{-/Y}$) (Matsuno et al., 2005). Based on the findings obtained in these mice and cultured cells, we report here a novel role for this superoxide-generating enzyme in the development of inflammatory pain.

Materials and Methods

Reagents. Ham's F-12 medium, DMEM/F-12, horse serum, and SuperScript III Platinum SYBR Green One-Step qRT-PCR were obtained from Invitrogen. Fura-2 AM was purchased from Dojindo. Neuronal-specific nuclear protein (NeuN) was purchased from Millipore Bioscience Research Reagents. Anti-TRPV1 antibody was obtained from Calbiochem. Bradykinin, phorbol 12-myristate 13-acetate (PMA), SOD–polyethylene glycol (PEG), catalase–PEG, cholera toxin B–FITC, isolectin B4 (IB4)–FITC, diphenyleneiodonium (DPI) chloride, NADPH, *N*-acetyl-L-cysteine, dithiothreitol, and streptavidin–agarose were obtained from Sigma. Carrageenan, Formalin, and acetic acid were purchased from Wako Pure Chemicals. Anti-PKCε antibody, anti-PKCδ antibody, and anti-NOX1 antibody were obtained from Santa Cruz Biotechnology. EZ-Link PEO-iodoacetyl Biotin was obtained from Pierce. Anti-PKCα antibody was obtained from BD Biosciences. TransIT-LT1 reagent was purchased from Mirus. Anti-NOX1 antibodies were also provided by Dr. D. Lambeth (Emory University, Atlanta, GA) and Dr. K. Rokutan (Tokushima University, Tokushima, Japan).

Animals. Mice deficient in *Nox1* (*Nox1*$^{-/Y}$) and control littermates (*Nox1*$^{+/Y}$) were housed in a temperature-controlled room (21–23°C) with a 12 h light/dark cycle. For behavioral and neural culture studies, 11- to 12-week-old animals were used. All procedures were done in accordance with the policies and recommendations of the International Association for the Study of Pain and were approved by the Committee for Animal Research at Kyoto Prefectural University of Medicine.

Morphological analysis. Mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. L4 and L5 DRG were removed, soaked in 20% sucrose overnight, and stored at −80°C. Sections (10 μm) were stained with 0.1% toluidine blue for 10 min. NIH ImageJ software was used for the measurement of neuron area.

Paraformaldehyde-perfused cryosections were used for immunohistochemical analyses for NeuN and TRPV1. Sections were first incubated with NeuN (1:200) for 24 h at 4°C in a blocking buffer followed by blocking with 1% goat serum in 0.1% Triton X-100/PBS. After being washed with PBS, sections were reacted with anti-mouse IgG conjugated with Alexa488 (1:400) for 1 h at room temperature. After another wash and further blocking, incubation of anti-TRPV1 antibody (1:200) in blocking buffer was performed for 2 h at room temperature. Sections were washed with PBS and further incubated with Alexa568-conjugated IgG (1:400) for 1 h. After a final wash with PBS, fluorescence was detected with FV1000 (Olympus).

Analysis of motor function. Initially, mice deficient in *Nox1* were tested for locomotor activity and coordinated movement. Spontaneous locomotor activity was determined in a 25 × 40 cm field for 5 min using ACTMONITOR II (Medical Agent). A rotarod test was performed to examine coordinated movement according to a method described previously with minor modifications (Kuribara et al., 1977). The rod rotated with a stepwise increase in speed from 8 to 16 rpm every 5 min, and coordinated movement was determined as the time taken to fall from the rod at each speed.

Thermal, mechanical, and chemical nociception. The tail-flick test was performed using a Tail Flick Analgesia Meter (Muromachi). The tail at 3.5 cm from the tip was exposed to radiant heat, and the latency of escape from stimuli was measured. The cutoff time was 15 s. The hotplate test was performed at three different temperatures (50, 52, and 55°C). The latency of licking the hindpaw was recorded. The cutoff time was 60 s for 50°C, 40 s for 52°C, and 20 s for 55°C. Tail pressure was added using Randall Selitto (Ugo Basile). Pressure was gradually applied to the tail of mice, and nociceptive thresholds were determined as the pressure (grams) required to elicit tail withdrawal. The cutoff weight was 200 g. To evaluate chemical nociception, acetic acid (0.6%) was intraperitoneally injected (0.1 ml/10 g), and the number of writhes was counted for 20 min. Formalin (10 μl of 0.5% Formalin in saline) was intraplantarly injected under brief ether anesthesia. Mice were subsequently transferred to observation cages, and the time of each lick or bite of the hindpaw during 0–10 min (phase 1) and 20–50 min (phase 2) after injection was measured and integrated.

Carrageenan-induced hyperalgesia. Carrageenan (2% in 30 μl of saline) was injected subcutaneously into the plantar area of the right hindpaw of mice anesthetized with ether. The paw volume was measured with a plethysmometer (Ugo Basile) before the injection of carrageenan and every hour after the injection up to 5 h (Salvemini et al., 1996). The magnitude of edema was expressed by $(V_{post} - V_{pre})/V_{pre} \times 100$, where V_{pre} and V_{post} are the volume of the paw measured before and after carrageenan injection, respectively.

To evaluate thermal hyperalgesia, the hindpaw withdrawal latency was measured by the method of Hargreaves et al. (1988) using a Paw Stimulator Analgesia Meter (IITC). After habituation, a hindpaw was exposed to radiant heat, and the latency of escape from the stimulus was measured. Mechanical hyperalgesia was evaluated using a 0.6 g von Frey hair (Muromachi) as described previously (Takasaki et al., 2000). A mouse was placed in a cage with a wire mesh bottom. After acclimation for at least 30 min, the 0.6 g von Frey hair (Muromachi) was pressed perpendicularly against the plantar skin and held for 3–5 s slightly buckled. Stimulation of the same intensity was applied six times to each hindpaw at intervals of several seconds. Responses to stimuli were ranked as follows: 0, no response; 1, movement away from the von Frey hair; and 2, immediate flinching or licking of the hindpaw. The pain score was calculated as follows: pain score (%) = Σ(average score of each animal)/2 × (number of animals tested) × 100.

Real-time PCR. Total RNA was isolated from DRG by the acid guanidinium thiocyanate/phenol/chloroform method. Real-time PCR was performed by use of the GeneAmp 5700 Sequence Detection System (Applied Biosystems) with the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). The primer sequences used were as follows: Nox1, 5′-ctgacaagtactattacacgagag-3′ and 5′-catatatgccaccagcttatggaag-3′; Nox2, 5′-aactgtatgctgatcctgctgc-3′ and 5′-gttctcattgtcaccgatgtcag-3′; Nox4, 5′-tgaggagtcactgaactatgaagttaatc-3′ and 5′-tgactgaggtacagctggatgttcaca-3′. Copy number was calculated on the basis of standard curves generated using mouse Nox1, Nox2, and Nox4 cDNA templates. Data were expressed as copies per microgram RNA.

Cell culture and transfections. The isolation of DRG neurons was performed as described with minor modifications (Sango et al., 2004). Briefly, DRG at thoracolumbar and lumber levels were isolated from 12-week-old mice, incubated with collagenase at 37°C for 90 min, and digested with 0.05% trypsin for 10 min. Dispersed cells were resuspended in F-12/Ham's medium containing 10% FBS and then plated on poly-L-lysine-coated dishes. Experiments were performed 1–2 d after the preparation of neuronal cultures.

HEK293 and CHO cells were maintained in 10% FBS-containing Eagle's medium essential medium and Ham's F-12, respectively. These cells were transfected with the green fluorescent protein (GFP)-tagged PKCε expression vectors (Kashiwagi et al., 2002) using TransIT-LT1 reagent according to the instructions of the manufacturer. After 24–48 h, transfected cells were exposed to hydrogen peroxide.

Measurement of superoxide by lucigenin luminescence. Extracellular superoxide production by DRG neurons was evaluated by measuring lucigenin luminescence using a Luminescensor (Atto). DRG neurons (5×10^3 cells) at day 1 *in vitro* were harvested with trypsin and washed twice with HBSS. Cells were suspended in 1% BSA/HBSS containing 100 μM NADPH and 1 μM lucigenin. Chemiluminescence was measured at 37°C for 10 min and expressed as relative light counts per 5 min.

Immunoblot for TRPV1. The supernatant fraction of DRG neurons homogenized in lysis buffer (25 mM β-glycerophosphate, 2 mM EGTA, 1% Triton X-100, 1 mM PMSF, and 2 mM DTT in 20 mM TBS) was

separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. An anti-TRPV1 monoclonal antibody and anti-mouse IgG antibody were used for detection with ECL-Plus (GE Healthcare). The density of the band was analyzed using NIH Image J software.

Measurement of intracellular calcium. DRG neurons were loaded with 5 μM fura-2 AM at 37°C for 30 min in DMEM/F-12 medium containing 1% BSA. Ratiometric fluorescence images were captured at 3 s intervals by alternate excitation at 340 and 380 nm, with emission at 510 nm. The fluorescence of fura-2 associated with individual cells was determined by Metafluor imaging software. Results were expressed as the 340 nm/380 nm emission ratio, which is proportional to the intracellular calcium concentration ($[Ca^{2+}]_i$) (Zhang et al., 2005). Forskolin (10 μM), PMA (100 nM), bradykinin (10 μM), or serotonin (30 μM) was applied to the medium before the addition of 3 nM capsaicin.

Localization of PKCε. DRG neurons, starved in a serum-free medium for 3 h before the analysis, were incubated with 100 nM PMA for 5 min. ROS-scavenging agents (300 mU/ml SOD–PEG, 300 mU/ml catalase–PEG, 10 mM *N*-acetyl cysteine, and 1 mM dithiothreitol) were included in the medium 3 h before the addition of 100 nM PMA. After PMA treatment, DRG neurons were fixed and treated as described previously (Rathee et al., 2002). Anti-PKCε antibody (1:400 in 3% BSA) and a secondary anti-rabbit IgG conjugated to Alexa548 (1:400) were used to detect the localization of PKCε. The fluorescence intensity of the cells was analyzed by selecting a straight line across the neural soma and using the plot profile function of NIH Image J software. Membrane regions were initially identified by IB4–FITC binding, which gave more than threefold higher fluorescence intensity than the background. Average fluorescence intensity of the membrane region (ΔF) against the total fluorescence intensity across a line bisecting the neural soma (F) was demonstrated. Small neurons of $r < 10$ μm were selected to evaluate the translocation of PKCε, because most small neurons express TRPV1 (Tominaga et al., 1998).

Transfected CHO cells were starved in serum-free medium for 3 h and exposed to 100 μM H_2O_2 for 2 min. After fixation, H_2O_2-induced translocation of the GFP-tagged full-length PKCε or deletion mutant lacking the C1A domain was visualized by confocal microscopy.

Identification of free sulfhydryl groups in PKCε. The detection of free sulfhydryl groups in PKCε was performed as described previously with modifications (Kim et al., 2001). HEK293 cells were exposed to 100 μM H_2O_2 for 5 min and harvested with lysis buffer without a reducing agent. Cell lysate (250 μg) was incubated with 500 μg of EZ-Link PEO-iodoacetyl Biotin for 16 h at 4°C and then incubated with 40 μl of streptavidin–agarose for 4 h at 4°C. The beads were washed twice with lysis buffer, and bound protein was eluted with sample buffer. To detect free sulfhydryls in PKCε, the eluted sample was subjected to SDS-PAGE and immunoblotted with anti-PKCε antibody.

Statistics. Statistical analyses were performed with one-way ANOVA. For multiple datasets, a *post hoc* multiple comparison (Dunnett's test) was applied. Values are expressed as means ± SEM.

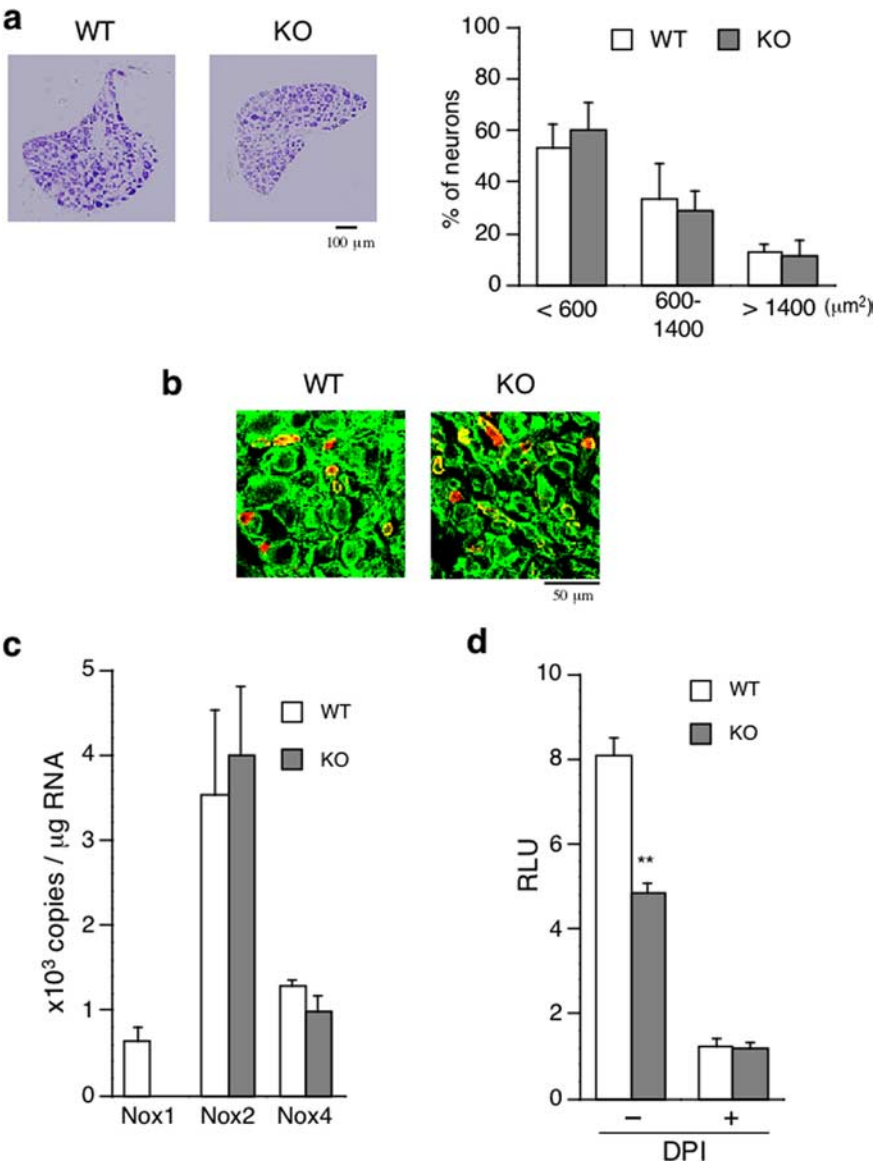

Figure 1. Morphology, expression of Nox isoforms, and superoxide production in DRG of $Nox1^{+/Y}$ and $Nox1^{-/Y}$. ***a***, DRG sections stained with *o*-toluidine blue; ***b***, immunohistochemistry with NeuN (green) and anti-TRPV1 antibody (red); ***c***, expression of Nox isoforms in DRG. The level of each Nox mRNA was determined by real-time PCR. ***d***, Extracellular superoxide production in DRG neurons measured with lucigenin in the absence or presence of DPI (10 μM). WT (wild-type) and KO (knock-out) represent control littermate ($Nox1^{+/Y}$) and Nox1-deficient ($Nox1^{-/Y}$) mice, respectively. Values were obtained from four samples. RLU, Relative light units.

Results

Morphology, expression of Nox isoforms, and superoxide production in DRG

We first examined whether the deletion of *Nox1* altered the morphology and expression of other *Nox* isoforms in DRG. Measurement of cell area stained with toluidine blue by the NIH Image software depicted no difference between the two genotypes in the size distribution of DRG neurons (Fig. 1*a*). In immunohistochemical analyses (Fig. 1*b*), the population of TRPV1-positive neurons, which reacted with both anti-TRPV1 antibody and NeuN antibody, was also equivalent in both genotypes (26.31 ± 4.28% in $Nox1^{+/Y}$ vs 24.76 ± 1.96% in $Nox1^{-/Y}$; $n = 4$).

In quantitative reverse transcription-PCR analyses, the level of *Nox2* mRNA was highest, whereas levels of *Nox1* and *Nox4* mRNAs were comparable in $Nox1^{+/Y}$ DRG. Between the two genotypes, no marked difference was observed in the levels of

Table 1. Motor function of *Nox1*$^{+/Y}$ and *Nox1*$^{-/Y}$

	Locomotor activity	Coordinated movement		
	Counts	Latency to fall (s)		
		8 rpm	12 rpm	16 rpm
Wild types	1504.0 ± 35.0	286.7 ± 13.2	193.5 ± 30.4	182.1 ± 30.5
Knock-outs	1504.5 ± 43.7	291.0 ± 9.0	222.6 ± 30.5	206.6 ± 30.3

Values were obtained from 11–12 animals per group.

Nox2 and *Nox4* mRNAs (Fig. 1*c*). When the level of *Nox1* mRNA expressed in the peripheral nerve was investigated, the level expressed in the spinal cord was much less than that expressed in DRG (data not shown). We further examined the levels of *Nox* mRNAs expressed in the paw. *Nox1* mRNA ($1.34 \pm 0.14 \times 10^4$ copies/μg RNA) was abundantly expressed in the paw compared with DRG ($0.65 \pm 0.14 \times 10^3$ copies/μg RNA). Similarly, Nox2 ($1.14 \pm 0.13 \times 10^6$ in paw vs $3.53 \pm 1.01 \times 10^3$ in DRG) and Nox4 ($1.26 \pm 0.17 \times 10^5$ in paw vs $1.28 \pm 0.07 \times 10^3$ in DRG) mRNAs are highly expressed in the paw tissue. The localization and distribution of NOX1 protein could not be identified by immunohistochemical means using three anti-NOX1 antibodies obtained from different sources. Conversely, the DPI-sensitive extracellular production of superoxide was significantly attenuated in *Nox1*$^{-/Y}$ DRG (Fig. 1*d*).

Motor function tests

Because motor function of each animal affects the results obtained in behavioral studies, we first examined the integrity of motor function in both genotypes. As shown in Table 1, no difference in locomotor activity and coordinated movement was found between *Nox1*$^{+/Y}$ and *Nox1*$^{-/Y}$. In addition, the number of mice that remained on the rod at speeds up to 16 rpm in coordinated movement test was equivalent in both genotypes (data not shown).

Thermal, mechanical, and chemical nociception

No change was observed in *Nox1*$^{-/Y}$ compared with responses in *Nox1*$^{+/Y}$ when responses to acute thermal stimuli were examined by tail flick (5.96 ± 0.47 s latency in *Nox1*$^{+/Y}$ vs 5.93 ± 0.38 s in *Nox1*$^{-/Y}$; $n = 8$–10) and hotplate tests (Fig. 2*a*). Furthermore, no significant difference in the threshold for mechanical stimuli was observed between the two genotypes (106.12 ± 8.35 g in *Nox1*$^{+/Y}$ vs 98.63 ± 6.25 g in *Nox1*$^{-/Y}$; $n = 8$–10). We subsequently analyzed the response to chemical stimuli by injecting Formalin into the hindpaw. In this model, a biphasic response is observed. In phase 1, Formalin directly stimulates nociceptors to induce the pain response, and, in phase 2, the subsequently induced inflammation elicits the pain response (Tjolsen and Hole, 1992). As determined by the licking/biting response time, the phase 1 responses were similar between the genotypes. In phase 2, however, a significant reduction in licking time was observed in *Nox1*$^{-/Y}$ (Fig. 2*b*). Intraperitoneal injection of acetic acid induces a typical behavior (writhing) in mice, which is used for quantifying visceral pain with inflammation (Tjolsen and Hole, 1992). When mice were injected with acetic acid, the number of writhes in *Nox1*$^{-/Y}$ was significantly diminished compared with that in *Nox1*$^{+/Y}$ (31.7 ± 2.45 in *Nox1*$^{+/Y}$ vs 23.2 ± 1.87 in *Nox1*$^{-/Y}$; $p < 0.01$; $n = 8$–10). Accordingly, the pain response after direct stimulation of nociceptors by Formalin was not affected, whereas the response during the chemical-induced inflammation appeared to be significantly attenuated in *Nox1*$^{-/Y}$.

Inflammatory pain is attenuated in *Nox1*$^{-/Y}$

To further verify the findings obtained by chemical stimuli that elicit inflammatory responses, we next studied another model of inflammatory pain. Intraplantar injection of carrageenan into the hindpaw induced a time-dependent development of edema and hyperalgesia. In *Nox1*$^{-/Y}$, carrageenan similarly evoked marked edema at 1 h after administration, which persisted until 5 h (Fig. 3*a*). Conversely, the development of thermal and mechanical hyperalgesia was significantly attenuated in *Nox1*$^{-/Y}$ compared with *Nox1*$^{+/Y}$ (Fig. 3*b*,*c*). These findings suggest that ROS derived from NOX1/NADPH oxidase affect the response properties of nociceptor afferents under inflammatory conditions.

Augmentation in the capsaicin-induced calcium increase by chemical mediators is attenuated in *Nox1*$^{-/Y}$

Posttranslational modifications and transcriptional changes of nociceptors alter their threshold and excitability, leading to pain hypersensitivity. Nociceptor afferent terminals transduce noxious stimuli into electrical activity, which is conducted to the dorsal horn of the spinal cord. To further delineate the molecular mechanisms underlying the reduced hyperalgesia observed in *Nox1*$^{-/Y}$, we isolated DRG containing cell bodies of afferent neurons. Because TRPV1, which works as an inflammatory sensor, has been implicated in the development of thermal hyperalgesia, we focused on TRPV1 activity in DRG neurons. Previously it was reported that NOX2/NADPH oxidase mediates NGF-induced increase in TRPV1 protein expression in rat PC12 cells (Puntambekar et al., 2005). We therefore examined the level of TRPV1 protein expressed in DRG neurons. Densitometric analyses of immunoblots indicated that the level of TRPV1 protein in DRG neurons of *Nox1*$^{-/Y}$ was comparable with that in the neurons of *Nox1*$^{+/Y}$ (data not shown).

Capsaicin, an agonist of TRPV1, dose dependently increased intracellular calcium levels in DRG neurons of both genotypes in a similar manner (Fig. 4*a*). The expression level and function of TRPV1 in DRG neurons are therefore unaffected in *Nox1*$^{-/Y}$. In the development of hyperalgesia, PKA and PKC are known to participate in the activation of TRPV1 (Cesare et al., 1999; Bhave et al., 2002). As shown in Figure 4*b*, forskolin and a phorbol ester, PMA, known to activate PKA and PKC, respectively, markedly augmented the increase in calcium induced by capsaicin. Although no difference in the effect of forskolin was observed between the genotypes, the effect of PMA was significantly attenuated in *Nox1*$^{-/Y}$ DRG neurons. Bradykinin and serotonin are inflammatory mediators that increase the heat response via activation of the PKC-dependent signaling pathway (Cesare et al., 1999; Numazaki et al., 2002). The finding obtained with PMA was thus verified by applying these inflammatory mediators to DRG neurons. As shown in Figure 4*c*, bradykinin or serotonin alone moderately increased the level of intracellular calcium in both genotypes to the same extent. In *Nox1*$^{+/Y}$ neurons, the capsaicin-induced calcium increase was markedly augmented in the presence of bradykinin or serotonin, whereas such an increase was abolished in *Nox1*$^{-/Y}$ DRG neurons. Accordingly, the absence of NOX1/NADPH oxidase affects the PKC-dependent activation of TRPV1, downstream of the receptor for inflammatory mediators.

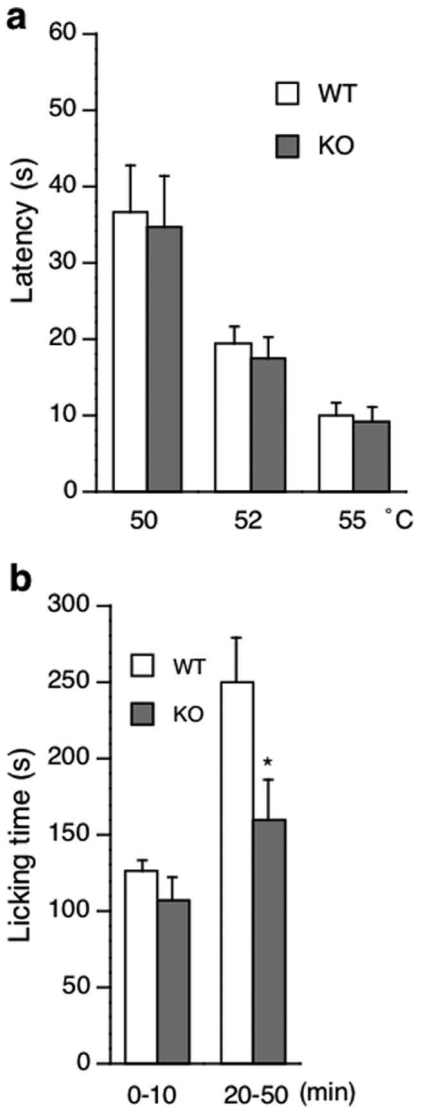

Figure 2. Thermal and chemical nociception in *Nox1*$^{+/Y}$ and *Nox1*$^{-/Y}$. ***a***, Latency for hindpaw licking and/or jumping in hotplate tests; ***b***, the licking and/or biting time 0–10 and 20–50 min after Formalin was injected to the hindpaw. WT (wild-type) and KO (knock-out) represent control littermate (*Nox1*$^{+/Y}$) and *Nox*1-deficient (*Nox1*$^{-/Y}$) mice, respectively. Values were obtained from 8 to 10 animals per group. *$p < 0.05$, compared with WT.

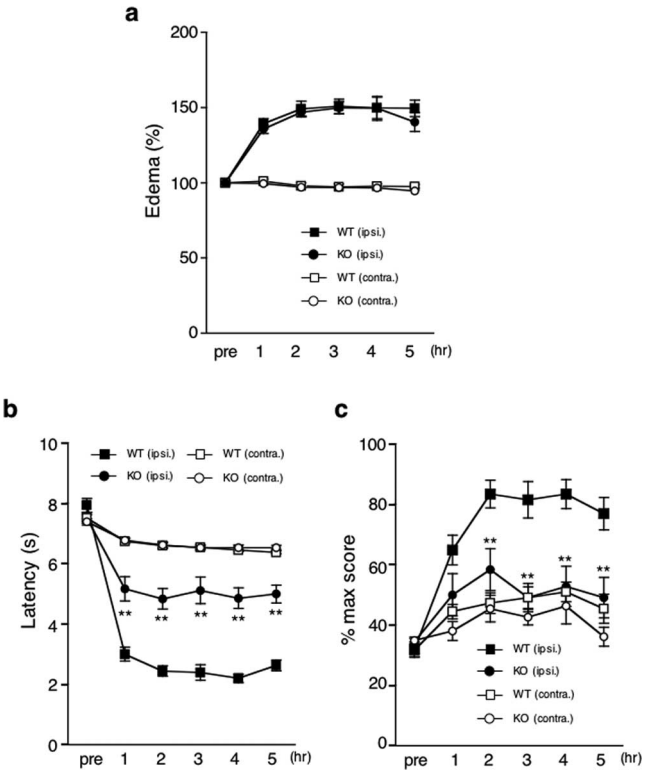

Figure 3. Carrageenan-induced inflammatory pain is attenuated in *Nox1*$^{-/Y}$. ***a***, Development of edema after intraplantar injection of carrageenan; ***b***, the hindpaw withdrawal latency for thermal stimuli; ***c***, the pain score determined with the von Frey test. Thermal and mechanical hyperalgesia were significantly attenuated in *Nox1*$^{-/Y}$. Filled and open squares indicate ipsilateral and contralateral hindpaws of *Nox1*$^{+/Y}$, and filled and open circles indicate ipsilateral and contralateral hindpaws of *Nox1*$^{-/Y}$ (KO, for knock-out), respectively. Values were obtained from eight to nine animals per group. **$p < 0.01$, compared with the ipsilateral hindpaw of *Nox1*$^{+/Y}$ (WT, for wild-type).

PMA-induced translocation of PKCε is attenuated in *Nox1*$^{-/Y}$

To delineate the role of NOX1/NADPH oxidase in the PKC-dependent activation of TRPV1, we next examined the translocation of PKCε in DRG neurons. The bradykinin-induced TRPV1 activation is dependent on the PKCε pathway (Cesare et al., 1999; Puntambekar et al., 2005), and direct phosphorylation of the serine residues of TRPV1 by PKCε increases the channel activity (Numazaki et al., 2002). Exposure of DRG neurons isolated from *Nox1*$^{+/Y}$ to PMA markedly increased the translocation of PKCε from the cytosolic to membrane fractions. In contrast, PMA-induced PKCε translocation was significantly perturbed in DRG neurons of *Nox1*$^{-/Y}$ (Fig. 5*a*). Because ROS derived from NOX1/NADPH oxidase may affect the membrane translocation of other PKC isoforms implicated in hyperalgesia (Olah et al., 2002; Obreja et al., 2005), we examined the translocation of PKCα and PKCδ in DRG neurons. Interestingly, PMA-induced translocations of PKCα and PKCδ were similarly reduced in *Nox1*$^{-/Y}$ DRG neurons. When *Nox1*$^{+/Y}$ neurons were pretreated with a compound that mimics the activity of SOD or catalase, the translocation of PKCε induced by PMA was significantly attenuated. Similar results were obtained when *Nox1*$^{+/Y}$ neurons were pretreated with a sulfhydryl protective agent, *N*-acetyl cysteine or dithiothreitol (Fig. 5*b*). These findings suggest that ROS derived from NOX1/NADPH oxidase modulate the translocation and activation of PKCε in DRG neurons.

Oxidation of sulfhydryl residues of the C1A domain enhances the translocation of PKCε

We then investigated the molecular mechanisms underlying the reduced translocation of PKCε in *Nox1*$^{-/Y}$ DRG neurons. It has been suggested that ROS activate PKC isoforms through post-translational modifications. During treatment of cells with hydrogen peroxide, PKC isoforms including PKCε are phosphorylated at the tyrosine residues conserved in the catalytic domain of the PKC family and enzymatically activated (Konishi et al., 1997). We first examined whether PKCε is tyrosine phosphorylated during stimulation and translocation to plasma membranes using a specific antiphosphorylated tyrosine antibody. However, no difference was observed in the phosphorylation of tyrosine residues of PKCε in HEK293 cells exposed to hydrogen peroxide (data not shown). Another mechanism underlying the ROS-induced activation of PKC is reported for the conventional PKC isoform containing both the C1 and C2 motifs (Knapp and Klann, 2000; Lin and Takemoto, 2005). Because the C1 motif containing two lipid-binding sites, C1A and C1B, is conserved in

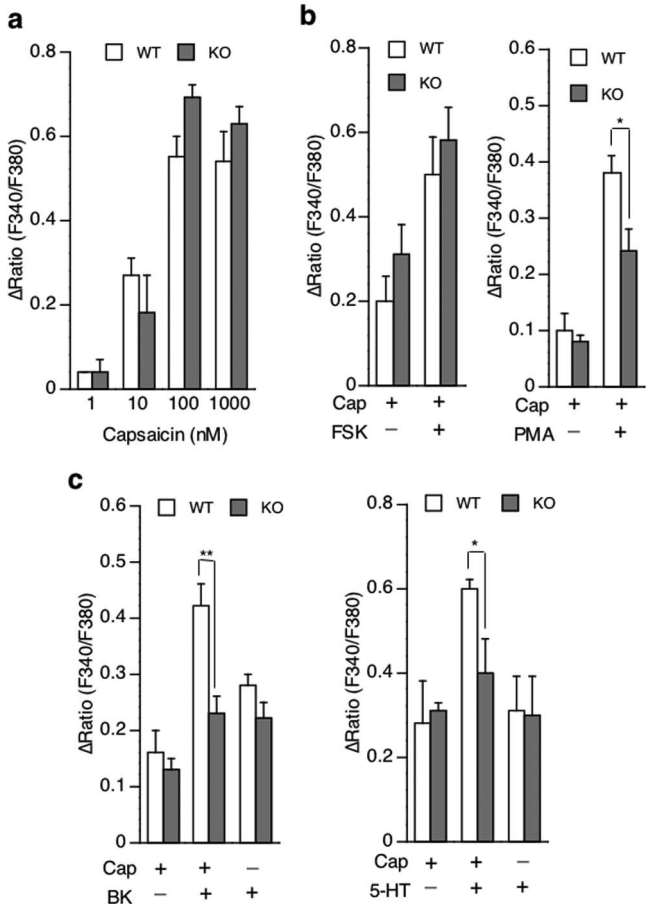

Figure 4. Augmentation of the capsaicin-induced calcium increase by chemical mediators is blunted in *Nox1*$^{-/Y}$ DRG neurons. ***a***, Functional characterization of TRPV1. Capsaicin, an agonist of TRPV1, dose dependently increased intracellular calcium levels in DRG neurons of both genotypes. Values were obtained from four to six experiments. ***b***, Effects of forskolin (10 μM) or PMA (100 nM) on capsaicin-induced increase in intracellular calcium. ***c***, Effects of bradykinin (10 μM) or serotonin (30 μM) on capsaicin-induced increase in intracellular calcium. Forskolin (FSK), PMA, bradykinin (BK), or serotonin (5-HT) was applied before the addition of capsaicin (Cap). Values were obtained from four to eight experiments. *$p < 0.05$; **$p < 0.01$.

PKCε, direct oxidation of PKCε and possibly other PKC isoforms in DRG neurons at the C1 domain may accelerate translocation of the domain to plasma membranes. We therefore transfected HEK293 cells with a GFP-fused full-length PKCε or deletion mutant lacking the C1A (ΔC1A) or C1B (ΔC1B) domain and exposed them to hydrogen peroxide. Treatment with hydrogen peroxide reduced free sulfhydryl groups of full-length PKCε, as well as of the deletion mutant lacking the C1B site (ΔC1B). Conversely, the level of free sulfhydryl groups was unaffected in the C1A deletion mutant (ΔC1A) treated with hydrogen peroxide (Fig. 6*a*). We then transfected CHO cells with full-length PKCε–GFP or ΔC1A–GFP and stimulated with hydrogen peroxide or with PMA. Hydrogen peroxide induced translocation of full-length PKCε to plasma membranes, whereas translocation of ΔC1A was significantly reduced (Fig. 6*b*). In contrast to these findings, PMA-induced translocation of ΔC1A was retained as observed previously (Kashiwagi et al., 2002). These findings suggest that ROS derived from NOX1/NADPH oxidase enhance the translocation of PKCε via modulation of the redox state of cysteine residues located at the C1A site.

Discussion

We demonstrated a new function of NOX1/NADPH oxidase in the development of inflammatory pain. The principal findings obtained include the following. (1) Thermal and mechanical hyperalgesia was significantly attenuated in *Nox1*$^{-/Y}$, whereas no difference in nociceptive responses to heat or mechanical stimuli was observed between *Nox1*$^{+/Y}$ and *Nox1*$^{-/Y}$. (2) In DRG neurons derived from *Nox1*$^{+/Y}$, pretreatment of bradykinin, serotonin, or PMA augmented the capsaicin-induced increase in calcium, whereas this increase was significantly attenuated in *Nox1*$^{-/Y}$. (3) PMA-induced translocation of PKCε was significantly reduced in *Nox1*$^{-/Y}$ or in *Nox1*$^{+/Y}$ neurons treated with ROS-scavenging agents. (4) In cells transfected with tagged PKCε, hydrogen peroxide induced translocation and a reduction in free sulfhydryls of full-length PKCε but not of the deletion mutant lacking the C1A domain. These results suggest that ROS derived from NOX1/NADPH oxidase oxidize the C1A domain of PKCε and accelerate the translocation to plasma membranes of DRG neurons, thereby enhancing the TRPV1 activity and the sensitivity to painful stimuli.

Between *Nox1*$^{+/Y}$ and *Nox1*$^{-/Y}$, no significant difference was demonstrated in the size distribution of DRG neurons or the population of TRPV1-positive neurons. These histological findings did not reflect our previous study, indicating a regulatory role for NOX1 in NGF-induced neurite outgrowth in cultured PC12 cells (Ibi et al., 2006). Quantitative mRNA analyses of *Nox1*$^{+/Y}$ DRG verified expression of *Nox1* together with expression of other isoforms of NADPH oxidase, *Nox2* and *Nox4*. We could not, however, confirm the localization and distribution of NOX1 protein in DRG using immunohistochemical techniques. This could be attributed to the low level of protein expressed in DRG compared with colon epithelial cells in which abundant expression of NOX1 is reported (Szanto et al., 2005). Conversely, the reduced production of superoxide demonstrated in *Nox1*$^{-/Y}$ clearly indicated the functional contribution of NOX1/NADPH oxidase to ROS generated in DRG neurons. The level of *Nox1* mRNA expressed in the paws was much higher than the level in DRG. Because abundant expression of NOX1 is reported in colon epithelial cells and also observed in pulmonary tissue (K. Iwata and K. Matsuno, unpublished data), NOX1 might be preferentially localized in the tissue exposed to environmental irritants.

We presently showed that ROS derived from NOX1/NADPH oxidase in DRG neurons play a crucial role in the development of hyperalgesia using *Nox1*-deficient mice. In contrast, it was reported that ROS generated in mitochondria of the dorsal horn neurons mediate the development of inflammatory hyperalgesia evoked by carrageenan (Wang et al., 2004). Inactivation of mitochondrial SOD induced by superoxide was a key event in this hyperalgesic response, by allowing the levels of superoxide to remain elevated and maintain nociceptive signaling. In fact, intrathecal administration of an SOD mimetic completely reversed hyperalgesia (Wang et al., 2004). Inactivation of spinal MnSOD was demonstrated in NMDA-mediated hyperalgesia as well (Muscoli et al., 2004), and ROS generated in mitochondria were further implicated in the development of capsaicin-induced hyperalgesia (Schwartz et al., 2008). It is well known that, during tissue injury and inflammation, hyperalgesia results from a persistent state of peripheral afferent sensitization that subsequently initiates spinal sensitization. Accordingly, oxidative stress in the spinal cord might be also reduced as a result of decreased peripheral sensitization in *Nox1*$^{-/Y}$. The data presented here, together with those of preceding studies, suggest that ROS formed at mul-

tiple levels of the nociceptive signaling cascade may contribute to the development of hyperalgesia.

In our study, both *Nox1*$^{+/Y}$ and *Nox1*$^{-/Y}$ developed edema to a similar extent, whereas ROS scavengers are documented to ameliorate edema as well as hyperalgesia (Wang et al., 2004; Khattab, 2006). Because inflammatory cells such as neutrophil and macrophage predominantly express NOX2 and produce a large amount of ROS under inflammatory conditions, it is reasonable to assume that ROS derived from NOX2/NADPH oxidase may be involved in the development of edema. Conversely, our present findings indicate that ROS derived from NOX1/NADPH oxidase take part in the development of hyperalgesia.

Several lines of evidence indicate that glial cells are important in the development of hyperalgesia. Indeed, intrathecal administration of agents that disrupt glial function or disrupt the action of glial products blocked Formalin-induced hyperalgesia in rats (Watkins et al., 1997). Recent investigation of hyperalgesia at the spinal transcriptome level indicated that carrageenan-induced inflammation of rat hindpaws leads to a rapid but sustained increase in S100A8 and S100A9 expression, two members of the S100 family implicated in the pathology of numerous inflammatory diseases (Mitchell et al., 2008). These proinflammatory genes derived from a population of neutrophils that mobilized to the spinal cord. Because NOX2 is predominantly expressed in glial cells and neutrophils, it is surprising that NOX1 was shown to be the major isoform involved in the development of Formalin- and carrageenan-induced hyperalgesia. Our findings might be attributed to the close proximity between NOX1/NADPH oxidase and the target molecule identified in this study, which enables direct interaction and modulation of TRPV1 activity in DRG neurons.

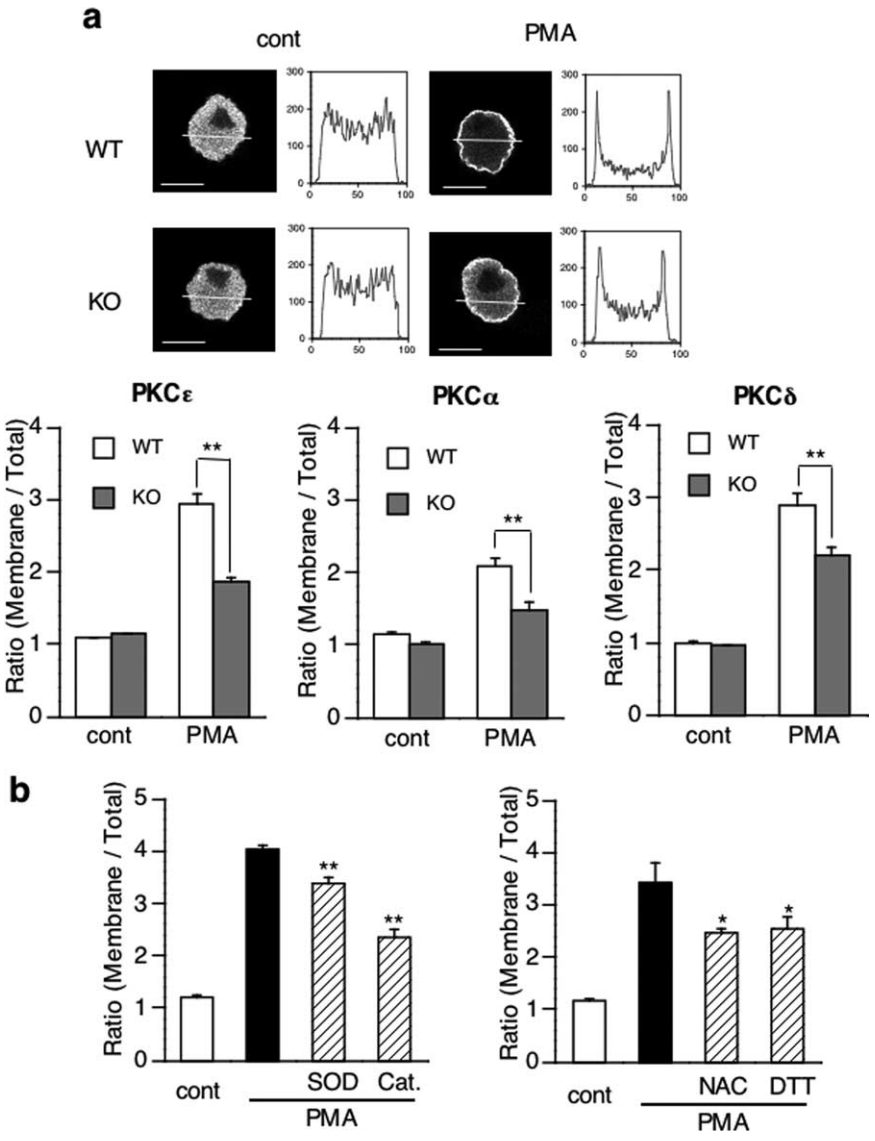

Figure 5. PMA-induced translocation of PKC is attenuated in *Nox1*$^{-/Y}$ DRG neurons. ***a***, Representative photographs and fluorescence intensity histograms of PKC. Top row demonstrates localization of PKCε in DRG neurons. The histogram is taken at a line bisecting the neural soma. Scale bars, 10 μm. Bottom row demonstrates quantitative measurements of the fluorescence intensity of PKCε, PKCα, and PKCδ. $^{**}p < 0.01$. Values were obtained from 18 to 30 neurons. ***b***, Effects of ROS-scavenging agents on PMA-induced PKCε translocation. Three hours before the addition of 100 nM PMA, 300 mU/ml SOD–PEG (SOD), 300 mU/ml catalase–PEG (Cat.), 10 mM *N*-acetyl cysteine (NAC), or 1 mM DTT was applied. Values were obtained from 15 to 25 neurons. $^{*}p, < 0.05$, $^{**}p < 0.01$, compared with PMA alone. WT (wild-type) and KO (knock-out) represent control littermate (*Nox1*$^{+/Y}$) and *Nox*1-deficient (*Nox1*$^{-/Y}$) mice, respectively.

Chemical mediators released from damaged tissue and inflammatory cells decrease the activation threshold of TRPV1 via phosphorylation of serine residues in a PKA- or PKC-dependent manner (Bhave et al., 2002; Numazaki et al., 2002). In this study, no difference in the effect of forskolin, an activator of PKA, on capsaicin-induced TRPV1 activation was observed between the genotypes, whereas the effect of PMA was significantly attenuated in *Nox1*$^{-/Y}$ DRG neurons. Moreover, PMA-induced translocation of PKC was significantly reduced in *Nox1*$^{-/Y}$ neurons. ROS derived from NOX1/NADPH oxidase were thus suggested to modulate TRPV1 activity in a PKC-dependent manner. In cells exposed to ROS, PKC isoforms including PKCε are phosphorylated at the tyrosine residues conserved in the catalytic domain of the PKC family and enzymatically activated (Konishi et al., 1997). Although we initially examined whether PKCε is tyrosine phosphorylated during stimulation and translocation to plasma membranes, no difference was observed in the phosphorylation of tyrosine residues of PKCε in cells exposed to hydrogen peroxide.

Hydrogen peroxide also elicits direct oxidation of PKCγ in the cysteine-rich C1 domain and ensuing translocation of the domain to plasma membranes (Knapp and Klann, 2000; Lin and Takemoto, 2005). Because the C1 motif containing two lipid-binding sites, C1A and C1B, is conserved in PKCε, direct oxidation of PKCε at the C1 domain may accelerate translocation of the domain to plasma membranes. In our study, the level of free sulfhydryl groups was unaffected in the C1A deletion mutant treated with hydrogen peroxide. Although PMA-induced translocation of ΔC1A was retained, hydrogen peroxide-induced translocation of ΔC1A was significantly reduced in cells trans-

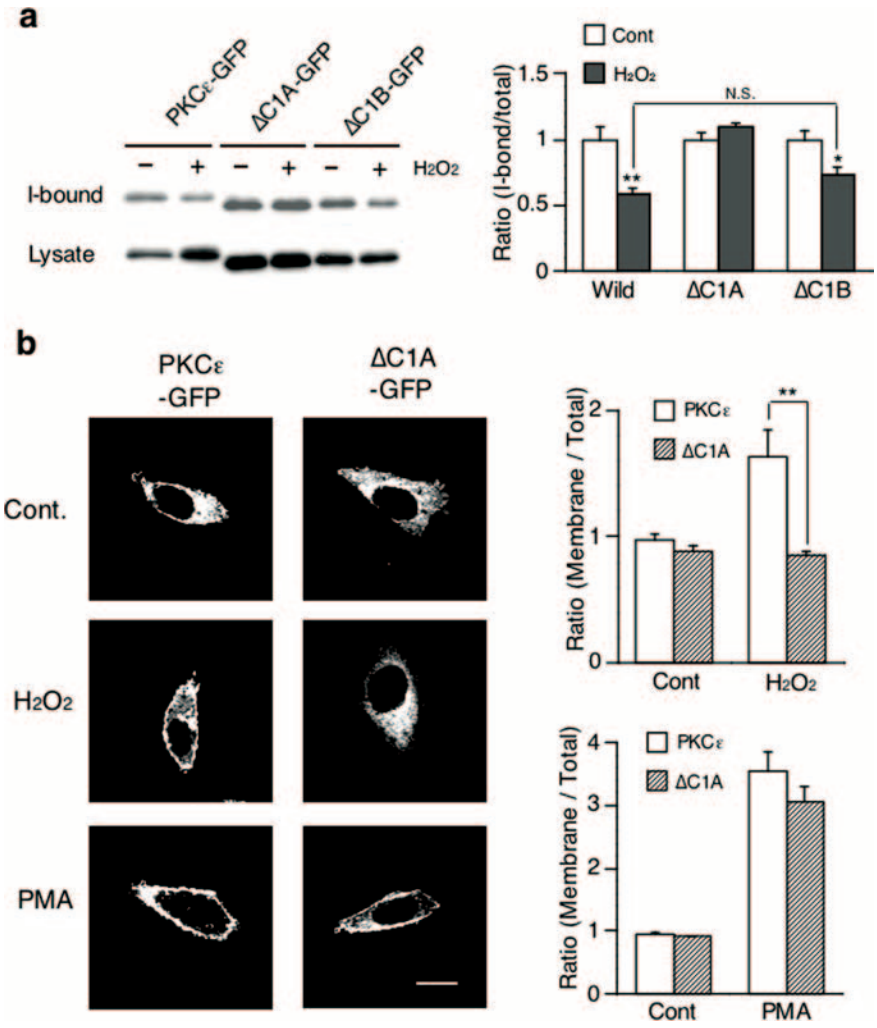

Figure 6. Involvement of the redox state of sulfhydryl residues in the C1A domain of PKCε. ***a***, Determination of free sulfhydryl residues in PKCε and deletion mutants after H_2O_2 treatment. HEK293 cells transfected with a GFP-fused full-length PKCε or deletion mutant lacking the C1A (ΔC1A) or C1B (ΔC1B) domain were exposed to 100 μM H_2O_2 for 5 min. Top row demonstrates iodoacetyl-PEO_2 biotin-bound PKCε (I-bound), which corresponds to the enzyme containing reduced cysteine residues. Bottom row indicates the total PKCε level in the lysate. Right demonstrates quantitative densitometric analysis of the ratio of I-bound to total PKCε. Results are representative of five independent experiments. $*p < 0.05$, $**p < 0.01$, compared with control. N.S., Not significant. ***b***, Translocation of PKCε induced by H_2O_2 or PMA. CHO cells transfected with full-length PKCε–GFP or ΔC1A–GFP were stimulated with 100 μM H_2O_2 for 2 min or with 1 μM PMA for 10 min. Scale bar, 10 μm. Representative photographs of three experiments are shown (left). Right demonstrates quantitative measurements of the fluorescence intensity of PKCε–GFP or ΔC1A–GFP. $**p < 0.01$. Values were obtained from 9 to 18 neurons.

fected with the tagged PKCε construct. These findings suggest that ROS derived from NOX1/NADPH oxidase enhance the translocation of PKCε via modulation of the redox state of cysteine residues located at the C1A site.

It has been reported that various inflammatory factors are involved in hyperalgesia via a sequential activation of PKCε and TRPV1 (Cesare and McNaughton, 1996; Khasar et al., 1999; Chuang et al., 2001; Zhang et al., 2005; Amadesi et al., 2006; Ohta et al., 2006). Meanwhile, the modification of reactive cysteine residues of TRP channels is documented to cause channel activation. Given that oxidative modifications (*S*-oxidation) of cysteine residues by hydrogen peroxide were shown to activate TRPV1 (Yoshida et al., 2006), ROS derived from NOX1/NADPH oxidase might also interact with TRPV1 to regulate the channel activity. The possibility of direct oxidative modifications of TRPV1 by NOX1/NADPH oxidase remains under consideration.

In conclusion, we demonstrated a novel role for NOX1/NADPH oxidase in the development of thermal and mechanical hyperalgesia. ROS derived from NOX1/NADPH oxidase accelerate the translocation of PKC and augment TRPV1 activity in DRG neurons. The present study provides the first evidence that NOX1/NADPH oxidase is a source of ROS implicated in inflammatory pain, thus making it a novel target for future therapeutic strategies.

References

Abramov AY, Canevari L, Duchen MR (2004) β-Amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase. J Neurosci 24:565–575.

Amadesi S, Cottrell GS, Divino L, Chapman K, Grady EF, Bautista F, Karanjia R, Barajas-Lopez C, Vanner S, Vergnolle N, Bunnett NW (2006) Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cepsilon- and A-dependent mechanisms in rats and mice. J Physiol 575:555–571.

Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW 4th (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. Neuron 35:721–731.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306–313.

Cesare P, McNaughton P (1996) A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. Proc Natl Acad Sci U S A 93:15435–15439.

Cesare P, Dekker LV, Sardini A, Parker PJ, McNaughton PA (1999) Specific involvement of PKC-epsilon in sensitization of the neuronal response to painful heat. Neuron 23:617–624.

Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. Nature 411:957–962.

Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32:77–88.

Hu HJ, Bhave G, Gereau RW 4th (2002) Prostaglandin and protein kinase A-dependent modulation of vanilloid receptor function by metabotropic glutamate receptor 5: potential mechanism for thermal hyperalgesia. J Neurosci 22:7444–7452.

Ibi M, Katsuyama M, Fan C, Iwata K, Nishinaka T, Yokoyama T, Yabe-Nishimura C (2006) NOX1/NADPH oxidase negatively regulates nerve growth factor-induced neurite outgrowth. Free Radic Biol Med 40:1785–1795.

Kashiwagi K, Shirai Y, Kuriyama M, Sakai N, Saito N (2002) Importance of C1B domain for lipid messenger-induced targeting of protein kinase C. J Biol Chem 277:18037–18045.

Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, Green PG, Hodge CW, Levine JD, Messing RO (1999) A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. Neuron 24:253–260.

Khattab MM (2006) TEMPOL, a membrane-permeable radical scavenger, attenuates peroxynitrite- and superoxide anion-enhanced carrageenan-induced paw edema and hyperalgesia: a key role for superoxide anion. Eur J Pharmacol 548:167–173.

Kim KY, Rhim T, Choi I, Kim SS (2001) *N*-acetylcysteine induces cell cycle

arrest in hepatic stellate cells through its reducing activity. J Biol Chem 276:40591–40598.

Knapp LT, Klann E (2000) Superoxide-induced stimulation of protein kinase C via thiol modification and modulation of zinc content. J Biol Chem 275:24136–24145.

Konishi H, Tanaka M, Takemura Y, Matsuzaki H, Ono Y, Kikkawa U, Nishizuka Y (1997) Activation of protein kinase C by tyrosine phosphorylation in response to H_2O_2. Proc Natl Acad Sci U S A 94:11233–11237.

Kuribara H, Higuchi Y, Tadokoro S (1977) Effects of central depressants on rota-rod and traction performances in mice. Jpn J Pharmacol 27:117–126.

Lambeth JD (2004) NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 4:181–189.

Lin D, Takemoto DJ (2005) Oxidative activation of protein kinase Cgamma through the C1 domain. Effects on gap junctions. J Biol Chem 280:13682–13693.

Matsuno K, Yamada H, Iwata K, Jin D, Katsuyama M, Matsuki M, Takai S, Yamanishi K, Miyazaki M, Matsubara H, Yabe-Nishimura C (2005) Nox1 is involved in angiotensin II-mediated hypertension: a study in Nox1-deficient mice. Circulation 112:2677–2685.

Mitchell K, Yang HY, Tessier PA, Muhly WT, Swaim WD, Szalayova I, Keller JM, Mezey E, Iadarola MJ (2008) Localization of S100A8 and S100A9 expressing neutrophils to spinal cord during peripheral tissue inflammation. Pain 134:216–231.

Muscoli C, Mollace V, Wheatley J, Masini E, Ndengele M, Wang ZQ, Salvemini D (2004) Superoxide-mediated nitration of spinal manganese superoxide dismutase: a novel pathway in *N*-methyl-D-aspartate-mediated hyperalgesia. Pain 111:96–103.

Numazaki M, Tominaga T, Toyooka H, Tominaga M (2002) Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues. J Biol Chem 277:13375–13378.

Obreja O, Biasio W, Andratsch M, Lips KS, Rathee PK, Ludwig A, Rose-John S, Kress M (2005) Fast modulation of heat-activated ionic current by proinflammatory interleukin 6 in rat sensory neurons. Brain 128:1634–1641.

Ohta T, Ikemi Y, Murakami M, Imagawa T, Otsuguro K, Ito S (2006) Potentiation of transient receptor potential V1 functions by the activation of metabotropic 5-HT receptors in rat primary sensory neurons. J Physiol 576:809–822.

Olah Z, Karai L, Iadarola MJ (2002) Protein kinase Ca is required for vanilloid receptor 1 activation. J Biol Chem 277:35752–35759.

Puntambekar P, Mukherjea D, Jajoo S, Ramkumar V (2005) Essential role of Rac1/NADPH oxidase in nerve growth factor induction of TRPV1 expression. J Neurochem 95:1689–1703.

Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, Nau C, Kress M (2002) PKA/AKAP/VR-1 module: a common link of Gs-mediated signaling to thermal hyperalgesia. J Neurosci 22:4740–4745.

Salvemini D, Wang ZQ, Bourdon DM, Stern MK, Currie MG, Manning PT (1996) Evidence of peroxynitrite involvement in the carrageenan-induced rat paw edema. Eur J Pharmacol 303:217–220.

Sango K, Tokashiki A, Ajiki K, Horie M, Kawano H, Watabe K, Horie H, Kadoya T (2004) Synthesis, localization and externalization of galectin-1 in mature dorsal root ganglion neurons and Schwann cells. Eur J Neurosci 19:55–64.

Schwartz ES, Lee I, Chung K, Chung JM (2008) Oxidative stress in the spinal cord is an important contributor in capsaicin-induced mechanical secondary hyperalgesia in mice. Pain, in press.

Szanto I, Rubbia-Brandt L, Kiss P, Steger K, Banfi B, Kovari E, Herrmann F, Hadengue A, Krause KH (2005) Expression of NOX1, a superoxide-generating NADPH oxidase, in colon cancer and inflammatory bowel disease. J Pathol 207:164–176.

Takasaki I, Andoh T, Shiraki K, Kuraishi Y (2000) Allodynia and hyperalgesia induced by herpes simplex virus type-1 infection in mice. Pain 86:95–101.

Tjolsen T, Hole K (1992) Animal models of analgesia. In: The pharmacology of pain (Dickenson A, Besson JM, eds), pp 1–20. Berlin: Springer.

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21:531–543.

Wang ZQ, Porreca F, Cuzzocrea S, Galen K, Lightfoot R, Masini E, Muscoli C, Mollace V, Ndengele M, Ischiropoulos H, Salvemini D (2004) A newly identified role for superoxide in inflammatory pain. J Pharmacol Exp Ther 309:869–878.

Watkins LR, Martin D, Ulrich P, Tracey KJ, Maier SF (1997) Evidence for the involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat. Pain 71:225–235.

Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, Choi DK, Ischiropoulos H, Przedborski S (2002) Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. J Neurosci 22:1763–1771.

Wu DC, Ré DB, Nagai M, Ischiropoulos H, Przedborski S (2006) The inflammatory NADPH oxidase enzyme modulates motor neuron degeneration in amyotrophic lateral sclerosis mice. Proc Natl Acad Sci U S A 103:12132–12137.

Yoshida T, Inoue R, Morii T, Takahashi N, Yamamoto S, Hara Y, Tominaga M, Shimizu S, Sato Y, Mori Y (2006) Nitric oxide activates TRP channels by cysteine S-nitrosylation. Nat Chem Biol 2:596–607.

Zhang N, Inan S, Cowan A, Sun R, Wang JM, Rogers TJ, Caterina M, Oppenheim JJ (2005) A proinflammatory chemokine, CCL3, sensitizes the heat- and capsaicin-gated ion channel TRPV1. Proc Natl Acad Sci U S A 102:4536–4541.