Hydrogen Peroxide Induces Muscle Nociception via Transient Receptor Potential Ankyrin 1 Receptors

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ABSTRACT

Background: H2O2 has a variety of actions in skin wounds but has been rarely studied in deep muscle tissue. Based on response to the transient receptor potential ankyrin 1 antagonists after plantar incision, we hypothesized that H2O2 exerts nociceptive effects via the transient receptor potential ankyrin 1 in muscle.

Methods: Nociceptive behaviors in rats (n = 269) and mice (n = 16) were evaluated after various concentrations and volumes of H2O2 were injected into the gastrocnemius muscle or subcutaneous tissue. The effects of H2O2 on in vivo spinal dorsal horn neuronal activity and lumbar dorsal root ganglia neurons in vitro were evaluated from 26 rats and 6 mice.

Results: Intramuscular (mean ± SD: 1,436 ± 513 s) but not subcutaneous (40 ± 58 s) injection of H2O2 (100 mM, 0.6 ml) increased nociceptive time. Conditioned place aversion was evident after intramuscular (–143 ± 813 s) but not subcutaneous (–2 ± 111 s) injection of H2O2. These H2O2-induced behaviors were blocked by transient receptor potential ankyrin 1 antagonists. Intramuscular injection of H2O2 caused sustained in vivo activity of dorsal horn neurons, and H2O2 activated a subset of dorsal root ganglia neurons in vitro. Capsaicin nerve block decreased guarding after plantar incision and reduced nociceptive time after intramuscular H2O2. Nociceptive time after intramuscular H2O2 in transient receptor potential ankyrin 1 knockout mice was shorter (173 ± 156 s) compared with wild-type mice (931 ± 629 s).

Conclusions: The greater response of muscle tissue to H2O2 may help explain why incision that includes deep muscle but not skin incision alone produces spontaneous activity in nociceptive pathways. (Anesthesiology 2017; 127:695-708)
Materials and Methods

All of the procedures in this study were approved by the University of Iowa Animal Care and Committee (approval No. 5011267; Iowa City, Iowa) and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Bethesda, Maryland). Adult male and female Sprague–Dawley rats (200 to 300 g, Harlan, USA) were housed in groups of 2 in clear plastic cages (40 × 60 × 30 cm) on fresh bedding with free access to food and water. The environment was controlled with a 12-h light-dark cycle and a room temperature of 22° ± 2°C. The physical conditions of the animals were carefully monitored every weekday for clinical signs of illness during the experiments. The animals did not show any signs of stress (except nociception-related behavior) or illness throughout the experiment. Based on our previous experience, we used six to nine rats for the behavioral studies and collected six neurons per group for the extracellular Ca2+ imaging. Sixteen mice were assigned for nociceptive behavior after injection of H2O2 or cinnamaldehyde, 58 rats for conditioned place aversion (CPA), 11 rats for DHN recording, 27 rats for nociceptive behavior after capsacain nerve block, and 8 rats for Ca2+ imaging. Sixteen mice were assigned for nociceptive time after H2O2 injection, and six mice were used for Ca2+ imaging.

Measurement of Nociceptive Behaviors

Measurement of Nociceptive Time. Rats were acclimated individually on a small plastic mesh floor covered with a clear plastic cage top for 1 h per day for at least 2 days before testing. Rats were acclimated to being held in a towel. H2O2 was made daily from a 30% solution (Sigma-Aldrich, USA) by diluting into synthetic interstitial fluid (SIF; in mM: 107.8 NaCl, 3.5 KCl, 1.5 CaCl2·2H2O, 0.7 MgSO4·7H2O, 1.7 Na2HPO4·2H2O, 26.2 NaHCO3, 9.6 C6H11NaO7, and 7.6 sucrose; pH 7.4; equilibrated with 95% O2/5% CO2). On testing day, rats were gently held after being wrapped in a towel in the same way as the acclimation session. Various concentrations (10 to 100 mM) and volumes (0.4 to 1.0 ml) of H2O2 were injected into the left gastrocnemius muscle or subcutaneous tissue overlying the gastrocnemius muscle using a 1-ml syringe with a 30-gauge needle. Rats were returned to the testing cage immediately after injection and observed for 60 min. Data were collected in 5-min bins after H2O2 injection. Equivalent volumes of SIF were injected as a control. H2O2-induced nociceptive behavior in rats was recorded as total time spent flinching, lifting, and licking of the hind leg during the 60-min observation period. In our preliminary experiments, Methylene blue dye (Thermo Fisher Scientific, USA) was injected into the gastrocnemius muscle in various volumes (0.4 to 1.0 ml) to trace its distribution. After euthanasia, it was observed that the 0.4 to 0.8 ml of injectate was confined within the muscle, whereas leakage outside of the muscle was observed after injection of 1.0 ml of dye.

The effects of local injection of TRPA1 antagonist AP-18 (Sigma-Aldrich) on nociceptive behavior elicited by H2O2 injections were also evaluated. Sequential injections of AP-18 (50 mM, 0.3 ml) or vehicle (10% dimethyl sulfoxide; Molecular Probes, USA) in phosphate-buffered saline (PBS; Gibco, USA), followed by H2O2 (100 mM, 0.3 ml), were made into the gastrocnemius muscle. Therefore, the total injection volume was 0.6 ml, and the final concentration of H2O2 was 50 mM. Immediately after injection, nociceptive behavior was recorded for 60 min as described above. The effect of systemic administration of sodium azide (Sigma-Aldrich; dissolved in PBS; 10 mg/kg, intraperitoneal), a catalase inhibitor, on nociceptive behavior after injection of H2O2 (10 or 30 mM, 0.6 ml) into the gastrocnemius muscle or subcutaneous tissue overlying the gastrocnemius muscle was also tested. We used systemic administration of sodium azide (10 mg/kg) to reduce peroxide catalolism and to potentially increase the nociceptive behavior evoked by H2O2 injection.

Nociceptive behavior induced by injection of 30 mM cinnamaldehyde (Sigma-Aldrich; dissolved in PBS; 0.6 ml), a TRPA1 agonist,21 was also evaluated in rats. Cinnamaldehyde was injected into the left gastrocnemius muscle or subcutaneous tissue overlying the gastrocnemius muscle. Nociceptive behavior protocol was similar to that described above for H2O2. For all of the behavioral experiments, a second person randomly assigned and blinded the observer to drug and dose.

Conditioned Place Aversion. For CPA experiments, we used a biased approach.22 All of the animals were handled by a single researcher and housed in a separate area from animals used for other experiments. Bedding and food pellets were changed by the same researcher. One and two days before the preconditioning day, the animals underwent a 5-min handling session each day. After each handling, the animals were placed in a fresh cage and transported to the animal care unit.

The CPA apparatus consisted of three chambers, two larger-end chambers (46.0 × 32.0 × 32.0 cm) and one smaller center-connecting chamber (46.0 × 21.5 × 13.0 cm). The center chamber was lined with white walls. One lateral chamber had gray walls, a rough black floor, and a lemon scent. The other lateral chamber had black- and white-striped walls, a smooth black floor, and a vanilla scent. Baseline data were collected on the preconditioning day. Rats were placed in the center of the CPA apparatus, with free access to all three chambers. The position of the rat was determined by the position of its head; a rat was considered to be in a specific chamber when its head, including both pinnae, were in that chamber. The time spent in each chamber was recorded for 15 min to determine the preconditioning baseline, and the preferred and nonpreferred chambers were identified. Rats spending...
greater than 80% or less than 20% of the time in a chamber were excluded from additional testing. Rats underwent a single conditioning trial the following day as described below.23,24

We conducted two series of CPA experiments. In the first CPA experiment, in the morning of the conditioning day, rats received intragastrocnemius muscle injection of SIF (0.6 ml) as a vehicle. Immediately after SIF injection, rats were placed into the nonpreferred chamber for 45 min, with no access to the other chambers. In the afternoon approximately 4 h later, the rats received one of the following injections: (1) SIF (0.6 ml) intramuscularly; (2) \( \text{H}_2\text{O}_2 \) (100 mM, 0.6 ml) intramuscularly; (3) \( \text{H}_2\text{O}_2 \) (100 mM, 0.6 ml) subcutaneously; or (4) coinjection of \( \text{H}_2\text{O}_2 \) (200 mM, 0.3 ml) and AP-18 (50 mM, 0.3 ml) intramuscularly. Rats were then placed in the preferred chamber for 45 min.

The second series of CPA experiments were performed using 30 mM \( \text{H}_2\text{O}_2 \) instead of 100 mM. Therefore, on the conditioning day, intragastrocnemius muscle injection of SIF (0.6 ml) was paired with the nonpreferred chamber in the morning, and one of the following injections were paired with the preferred chamber in the afternoon: (1) SIF (0.6 ml) intramuscularly; (2) \( \text{H}_2\text{O}_2 \) (30 mM, 0.6 ml) intramuscularly; (3) \( \text{H}_2\text{O}_2 \) (30 mM, 0.6 ml) subcutaneously; or (4) coinjection of \( \text{H}_2\text{O}_2 \) (60 mM, 0.3 ml) and AP-18 (50 mM, 0.3 ml) intramuscularly.

In the morning of the next day, a place aversion test was performed by placing the animals back into the center of the CPA chambers with free access to all chambers for 15 min. The time spent in each chamber was again recorded and analyzed off-line. CPA data were expressed as an absolute measure of preference and aversion, by comparing the total time spent in the initially preferred and nonpreferred chambers during preconditioning baseline versus the total time spent in the same chambers after conditioning. We also used the CPA score, defined as time (seconds) spent in the initially preferred chamber during the test period minus the time spent in that chamber during preconditioning baseline. The person scoring the time spent in each chamber was blinded to the solution injected in the afternoon session.

**Guarding Behavior.** Detailed methods for plantar incision-induced guarding were described previously.4,25 Briefly, rats were first acclimated to the testing environment for three days. Then a baseline test was performed one day before incision. The next day rats underwent plantar hind paw incision as described in the “Plantar Hind Paw Incision” section. Guarding nociceptive behaviors were measured up to 10 days after incision using the guarding scores as follows.

To measure guarding behavior, rats were placed individually on a small plastic mesh floor (grid 8 × 8 mm) covered with a clear plastic cage top (21 × 27 × 15 cm). Both incised and nonincised hind paws were closely observed during a 1-min scoring period, and a score of zero, one, or two was given. Zero was scored when the incised area was touching the mesh and the area was blanched or distorted by the mesh; one was scored when the incised area touched the mesh without blanching or distortion; two was scored for the position when the incised area was completely off of the mesh. We scored once every 5 min for 1 h after incision. Therefore, a cumulative score was obtained by adding the 12 scores during the 1-h testing period (0 to 24) for each hind paw. The guarding score was then obtained by subtracting the score of the incised hind paw from that of the nonincised hind paw. The person scoring the guarding score was blinded to treatment.

**In Vivo Extracellular Recording of DHNs**

The surgical preparation of rats for DHN recording was performed according to previously described methods.26 Briefly, anesthesia was initially induced with 5% isoflurane in air in a plastic box followed by 2% isoflurane via a nose cone. Then a tracheotomy was performed and the tracheal cannula was connected to a ventilator (Harvard Apparatus, Inc., USA). End-tidal carbon dioxide was measured in the first two rats to ensure that ventilator parameters maintained end-tidal carbon dioxide between 36 and 40 mmHg. The animal was artificially ventilated with 100% oxygen, and anesthesia was maintained with 2% isoflurane. The rat was then positioned in a stereotactic frame. The head and vertebral column of the rat were stabilized with ear bars and vertebral clamps, respectively. Limited laminectomies were performed to expose the dorsal spinal cord at the lumbar enlargement between thirteenth thoracic and third lumbar vertebra. The underlying dura was removed and the spinal cord was covered with mineral oil. During the DHN recording, the body temperature was maintained at an approximate range of 35° to 37°C with a servo-controlled electric heating lamp and an underbody heating pad.

After these surgical preparations, isoflurane was decreased to 1.2% during the subsequent recording period. For DHN recording, small holes were made with fine forceps on the pia mater of the lumbar enlargement between the lumbar three and five segments. A tungsten parylene-coated electrode (1.0 to 1.5 mΩ impedance, Microprobe Inc., USA) was then driven through the pia mater hole, and the depth was set 0 μm once the electrode entered the surface of the dorsal spinal cord. Then the electrode was advanced into the spinal cord slowly at 10 μm per step via a micropositioner (David Kopf Instruments, USA) until a neuron was encountered or a depth of 1,000 μm was reached. Innocuous mechanical search stimuli (tapping and touching) were applied to the gastrocnemius region; gently squeezing of the gastrocnemius was also tested. Neurons were tested for response to pinch of the skin of the gastrocnemius region using a small blunt, curved forceps, and squeezing the gastrocnemius muscle using a large forceps; Neurons were accepted for additional study if they responded to both stimuli. The depth of the neuron...
toward the proximal femur. Stimulation frequency was at 2 Hz, and the pulse duration was 200 microseconds. As the needle approached the sciatic nerve, dorsiflexion and/or plantar flexion of the ankle was observed. Before injection of the drug, the stimulating needle was positioned to maximize the response at the ankle to a current of 0.50 to 0.55 mA. Using this combined landmark and stimulation technique for the sciatic nerve block, we were able to observe reversible loss of knee flexion and ankle/hind paw motor function in rats injected with bupivacaine. Nerve stimulator–guided sciatic nerve block was performed using either 0.05% capsaicin mixed in 0.5% bupivacaine or 0.5% bupivacaine-containing vehicle. Capsaicin was first dissolved in ethanol and Tween 80 (Sigma-Aldrich) to yield 2.5% stock solution and then diluted 50 times with 0.5% bupivacaine to a final concentration of 0.05% capsaicin. For the control group, the injectate consisted of 1% ethanol and 1% Tween 80 diluted in 0.5% bupivacaine. One day after nerve block with the above-mentioned drugs, rats underwent plantar incision. The guarding score was recorded through postoperative day 10.

A separate group of rats underwent nerve block with either 0.05% capsaicin/0.50% bupivacaine mixture or 0.5% bupivacaine with vehicle as described in the previous paragraph. Rats were tested for nociception induced by injection of \( \text{H}_2\text{O}_2 \) into the gastrocnemius muscle at several time points (one, three, five, and seven days) after the nerve block. On each day of testing, \( \text{H}_2\text{O}_2 \) (100 mM, 0.6 ml) was injected into the gastrocnemius muscle, and the total time spent flinching, lifting, and licking as nociceptive behavior was recorded for 60 min, as described in the “Measurement of Nociceptive Time” section. The person performing the behavioral experiments was blinded to drug used for the nerve block.

**In Vitro Responses of Lumbar DRG Neurons to \( \text{H}_2\text{O}_2 \)**

Rats were anesthetized with isoflurane and then euthanized using increasing concentrations of carbon dioxide. Lumbar DRGs (L2 to L5) were removed bilaterally, placed into Hank’s Balanced Salt Solution (HBSS; Gibco), and minced. DRGs were enzymatically treated by 40 to 60 units of activated papain ( Worthington Biochemical, USA) for 20 min and then by collagenase type 4 (2 mg/ml, Worthington Biochemical) and dispase (2.5 mg/ml, Gibco) for 20 min in a 37°C water bath. The dissociated lumbar DRG neurons were placed on polylysine-coated glass coverslips (Sigma-Aldrich) inside 35-mm culture dishes and incubated at 37°C in 5% \( \text{CO}_2 \) and 90% humidity overnight before \( \text{Ca}^{2+} \) imaging.

The DRG neurons were incubated in 3 \( \text{mM} \) Fura-2 \( \text{AM} \) (acetoxyethyl ester of Fura-2; Molecular Probes) in HBSS containing 0.2% pluronic acid F-127 at room temperature for 1 h, washed with HBSS, and left in this solution at room temperature in the dark for 1 h so the cells could stabilize. Ratiometric \( \text{Ca}^{2+} \) imaging was performed using an inverted fluorescent microscope (Olympus IX71, Olympus, USA). Coverslips were placed on epifluorescence microscope and...
continuously perfused (5 ml/min) with HBSS. Fura-2 was excited alternately with ultraviolet light at 340 and 380 nm and the fluorescence emission was detected at 510 nm using a computer-controlled monochromator. Fluorescent images and ratios were acquired every 1 s. Timing of excitation, and the acquisition of images were controlled using the program Slidebook 5.0 (Intelligent Imaging Innovation, USA) running on a personal computer. Digital images were stored for off-line analysis. One coverslip usually contained 20 to 30 DRG neurons/microscopic field at ×40 magnification. Drugs were diluted in HBSS and delivered via bath application using a gravity-driven system (infusion rate of 5 ml/min). The drugs used were H$_2$O$_2$ (1 mM, 20 s), capsaicin (0.5 μM, 20 s), and allyl isothiocyanate (AITC; Sigma-Aldrich, 100 μM, 20 s). Cells were considered responsive if their F340/F380 ratio increased by greater than 20% during the drug application. All of the experiments were performed at room temperature. At the end of each protocol, 50 mM KCl extracellular solution was used to depolarize neurons, thereby allowing for identification of viable neurons from nonneuronal cells or nonfunctioning neurons.

**TRPA1 Knockout Mice**

Experiments were conducted on four- to six-month-old male and female TRPA1 knockout (TRPA1−/−) mice (B6.129P-Tspa1tm1Kykw/J; Jackson Laboratories, USA) in which the exons essential for the TRPA1 gene function were deleted (see Kwan et al.27). The TRPA1−/− mouse line was created on a C57BL/6J background. Nociceptive behavior after injection of H$_2$O$_2$ (30 mM, 0.05 ml) into the gastrocnemius muscle was measured for 60 min in both TRPA1−/− and TRPA1 wild-type (TRPA1+/+) mice, in a similar manner as described above for rats.

Intracellular Ca$^{2+}$ transient evoked by H$_2$O$_2$ (1 mM, 20 s) was examined in the lumbar DRG neurons from TRPA1−/− and +/+ mice using Ca$^{2+}$ imaging. The Ca$^{2+}$ imaging protocols were similar to those described above for rats.

**Statistical Analysis**

Statistics were conducted using GraphPad Prism (version 5.04, GraphPad Software, Inc., USA). Kolmogorov–Smirnov tests were applied to all continuous data sets to test for normality. Parametric tests were used for normally distributed, continuous data, which were expressed as mean and SD. Nonparametric analyses were used for nonnormally distributed and categorical data. Nonnormally distributed data were presented as median and interquartile range. All of the analyses were two tailed. Nociceptive behavioral data in rats were compared using two-way ANOVA followed by post hoc t test with Bonferroni correction, one-way ANOVA with Tukey post hoc test, or unpaired t test. CPA experimental data were analyzed using paired t test or one-way ANOVA with Tukey post hoc test. In vivo extracellular recording data were compared using Kruskal–Wallis test. The data for behavioral tests after nerve blockade with capsaicin were compared using two-way ANOVA with repeated measures on one factor followed by Bonferroni post hoc test. The data from the TRPA1−/− mice experiments were compared using unpaired t test and chi-square test. Values of $P < 0.05$ were considered significant.

**Results**

**Nociceptive Behavior after Injection of H$_2$O$_2$**

Injection of 100 mM H$_2$O$_2$ into the gastrocnemius muscle produced significantly greater nociceptive behavior at all of the volumes tested compared with intramuscular injection of SIF (fig. 1A, A and B). Nociceptive behavior was the greatest immediately after injection of H$_2$O$_2$ and subsided by the end of the 60-min period in the 0.4- and 0.6-ml groups but not in the 0.8- and 1.0-ml groups (fig. 1A). Total time of nociceptive behavior (summarized data from fig. 1A) was 1393 ± 750 ($P = 0.0035$ vs. SIF), 1675 ± 926 ($P = 0.0004$ vs. SIF), 1698 ± 1353 ($P = 0.0005$ vs. SIF), and 2658 ± 430 s ($P < 0.0001$ vs. SIF), respectively, after injection of 0.4, 0.6, 0.8, and 1.0 ml of 100 mM H$_2$O$_2$ (fig. 1B). Nociceptive time after injection of 0.6 ml of H$_2$O$_2$ into the gastrocnemius muscle was 7 ± 7, 64 ± 60, 255 ± 245, and 1436 ± 513 s for SIF and 10, 30, and 100 mM of H$_2$O$_2$, respectively (fig. 1C). Intramuscular injection of 100 mM H$_2$O$_2$ produced significantly greater nociceptive behavior compared with the lower concentrations of H$_2$O$_2$ ($P < 0.0001$ vs. 30 mM group and $P < 0.0001$ vs. 10 mM group) or the SIF group ($P < 0.0001$). Nociceptive time after injection of H$_2$O$_2$ into the subcutaneous tissue was 21 ± 23, 24 ± 32, 32 ± 34, and 40 ± 58 s, respectively, for SIF and 10, 30, and 100 mM of H$_2$O$_2$ (fig. 1C). Nociceptive time after subcutaneous injection of H$_2$O$_2$ (10, 30, and 100 mM) was not significantly different from that after SIF injection ($P > 0.9999$ vs. subcutaneous SIF for all of the concentration groups). Intramuscular injection of H$_2$O$_2$ produced greater nociceptive behavior compared with subcutaneous injection in the 100 mM group ($P < 0.0001$) but not in the 10 ($P > 0.9999$) or 30 mM groups ($P > 0.9999$). Pretreatment with locally injected TRPA1 antagonist AP-18 significantly reduced nociceptive behavior induced by intramuscular injection of H$_2$O$_2$ compared with vehicle ($P = 0.0001$; fig. 1D). Nociceptive time after intramuscular injection of 10 and 30 mM of H$_2$O$_2$ was significantly greater in sodium azide-treated (10 mg/kg) rats compared with that in the vehicle-treated rats ($P = 0.0286$ vs. vehicle group for 10 mM H$_2$O$_2$ and $P = 0.0286$ vs. vehicle group for 30 mM H$_2$O$_2$; fig. 1E). Nociceptive time after subcutaneous injection of 10 and 30 mM of H$_2$O$_2$ was not different between the sodium azide group and the vehicle group (fig. 1E). Nociceptive time after intramuscular injection of 30 mM of cinnamaldehyde was significantly greater compared with the subcutaneous injection of cinnamaldehyde or intramuscular injection of vehicle (fig. 1F).

**CPA after H$_2$O$_2$ Injection**

In the first series of CPA experiments, one rat that spent more than 80% time in one chamber during the preconditioning.
Fig. 1. Nociceptive behavior in rats as total time spent flinching, lifting, and licking of the hind leg during a 60-min period. (A) Time course of the nociceptive behavior after various volumes of 100 mM H2O2 were injected into the gastrocnemius muscle. Data were collected in 5-min bins from six animals in each group. Data points show the average nociceptive time in 5-min bins, and error bars were omitted for clarity. (B) Total time of nociceptive behavior more than 60 min after various volumes of 100 mM H2O2 were injected into the gastrocnemius muscle (summarized data from Fig. 1A). Each group contained six rats. *P = 0.0035, †P = 0.0004, ‡P = 0.0005, #P < 0.0001 compared with the synthetic interstitial fluid (SIF) injection group by two-way ANOVA (interaction factor: F3,40 = 2.227, P = 0.0999; injection volume factor: F3,40 = 2.022, P = 0.1263; group factor: F1,40 = 91.61, P < 0.0001) followed by post hoc t test with Bonferroni correction. (C) Spontaneous nociceptive behavior after various concentrations of 0.6 ml H2O2 were injected subcutaneously (SQ) or intramuscularly (IM). Each group contained six rats. *P < 0.0001 compared with the IM SIF injection group, †P < 0.0001 compared with IM 10 mM H2O2 injection group, ‡P < 0.0001 compared with IM 30 mM H2O2 injection group, #P < 0.0001 compared with SQ 100 mM H2O2 injection group by one-way ANOVA (F7,40 = 34.92, P < 0.0001) followed by post hoc Tukey test. (D) Effects of local preinjection of a transient receptor potential ankyrin 1 (TRPA1) antagonist AP-18 (50 mM, 0.3 ml) on nociceptive behavior caused by intramuscular injection of H2O2 (100 mM, 0.3 ml). Vehicle + SIF group, n = 7; vehicle + H2O2 group, n = 7; AP-18 + H2O2 group, n = 6. *P < 0.0001 compared with vehicle + SIF injection group, †P = 0.0001 compared with AP-18 + H2O2 injection group by one-way ANOVA (F2,17 = 25.65, P < 0.0001) followed by (Continued)
baseline session was excluded. Rats spent significantly less time in their initially preferred chamber after conditioning with intra-muscular injection of 0.6 ml of 100 mM H₂O₂ ($P = 0.0075$) but not after conditioning with intramuscular injection of SIF, subcutaneous injection of H₂O₂, or intramuscular coinjection of AP-18 and H₂O₂ (fig. 2A). The CPA score of the intramuscular H₂O₂ group ($–143 ± 81$ s) was significantly greater than those of the intramuscular SIF group ($–3 ± 57$ s; $P = 0.0199$), the subcutaneous H₂O₂ group ($–2 ± 111$ s; $P = 0.0194$), and the coinjection of AP-18 and H₂O₂ group ($–21 ± 22$ s; $P = 0.0479$; fig. 2B).

The second series of CPA experiments used a lower concentration of H₂O₂ (30 mM) that did not cause significant

**Fig. 2.** The effects of intramuscular (IM) or subcutaneous (SQ) injection of H₂O₂ (100 mM, 0.6 ml) on conditioned place aversion (CPA) in rats. Each column represents the time spent in the preferred and nonpreferred chambers during the preconditioning and postconditioning sessions (A) and the CPA scores (B). For the intramuscular coinjection of H₂O₂ and AP-18, sequential injections of AP-18 (50 mM, 0.3 ml), followed by H₂O₂ (200 mM, 0.3 ml), were made into the gastrocnemius muscle. Therefore, the total injection volume was 0.6 ml, and the final concentration of H₂O₂ was 100 mM. Each group contained six rats. All of the data are expressed as mean ± SD. $*P = 0.0075$ by paired t test; †$P = 0.0199$ compared with the synthetic interstitial fluid (SIF) group; ‡$P = 0.0194$ compared with the subcutaneous H₂O₂ group; # $P = 0.0479$ compared with the intramuscular coinjection of H₂O₂ and AP-18 group by one-way ANOVA ($F_{2,18} = 6.923, P = 0.0059$) followed by post hoc Tukey test.
flinching or lifting. Compared with the baseline, rats spent less time in their initially preferred chamber after conditioning with intramuscular injection of H$_2$O$_2$ ($P=0.0011$) but not after conditioning with intramuscular injection of SIF, subcutaneous injection of H$_2$O$_2$, or intramuscular coinjection of AP-18 and H$_2$O$_2$ (fig. 3A). The CPA score of the intramuscular H$_2$O$_2$ group ($-189 \pm 68$ s) was significantly greater compared with the intramuscular SIF group ($-15 \pm 121$ s; $P=0.0036$), the subcutaneous H$_2$O$_2$ group ($-9 \pm 108$ s; $P=0.0026$), and the coinjection of AP-18 and H$_2$O$_2$ group ($-25 \pm 57$ s; $P=0.0048$; fig. 3B).

**In Vivo Extracellular DHN Recording**

Extracellular single-unit activities were recorded from 18 DHNs; an example of the responses of three neurons are shown in figure 4A. All of the neurons had receptive fields in both the skin overlying the gastrocnemius region and in the gastrocnemius muscle based on response to pinch. There was no difference in depth of DHNs among groups (fig. 4B). The time-course trend of changes in activity of the DHNs after injection of H$_2$O$_2$ are shown in figure 4C. Activities of the DHNs were greatly increased in the intramuscular H$_2$O$_2$ injection group (100 mM, 0.6 ml) during the 60-min period after injection (fig. 4C). Activity in the intramuscular SIF and the subcutaneous H$_2$O$_2$ injection groups was transiently increased early after injection and then returned to the preinjection level (fig. 4C). There were no differences in total activity of DHNs among groups before injection (fig. 4D). Total activity of DHNs...
Fig. 4. Effects of intramuscular injection of H$_2$O$_2$ on activity of dorsal horn neurons (DHNs). (A) Example recordings of neurons after subcutaneous (SQ) injection of H$_2$O$_2$ (upper panel), intramuscular (IM) injection of synthetic interstitial fluid (SIF; middle panel), and IM injection of H$_2$O$_2$ (lower panel). Arrows represent pinching the skin (SP), squeezing gastrocnemius muscle (MS), and the time of injections. Bin width = 1 s. Unit represents each single action potential. (B) The average depth from the surface of spinal cord in which DHNs were recorded in each groups. Data are expressed as median with interquartile range. (C) Time–course trend of changes in activity of the DHNs after injection of H$_2$O$_2$ or vehicle. Data points show the median impulse/second (imp/s) for six neurons in each group in 5-min bins. (D) Total activity in 5 min of the DHNs before injection. Data are expressed as median with interquartile range. Each group contained six neurons. (E) Total activity of the DHNs during 60 min after injection. Data are expressed as median with interquartile range. Each group contained six neurons. *$P = 0.0482$, #$P = 0.0261$ by Kruskal–Wallis test.
for 60 min after injection in the intramuscular H₂O₂ group was greater compared with the intramuscular SIF group (P = 0.0482) and the subcutaneous H₂O₂ group (P = 0.0261; fig. 4E). Similar to the nociceptive behaviors, DHN activities were greatest early after injection and abated toward the end of the 60-min period.

**Sciatic Nerve Block with Capsaicin**

Capsaicin 0.05% in 0.5% bupivacaine applied to the sciatic nerve the day before incision decreased the guarding score on postoperative day zero (P = 0.0202), day one (P < 0.0001), and day two (P < 0.0001) compare with vehicle plus 0.5% bupivacaine group (fig. 5A), in agreement with previous studies. In the vehicle plus 0.5% bupivacaine group, H₂O₂ injection evoked nociceptive behaviors when injected one, three, five and seven days after nerve block (fig. 5B). Injections of H₂O₂ on alternating days elicited the same response, thus not producing tachyphylaxis. Nerve block with 0.05% capsaicin in 0.5% bupivacaine significantly reduced nociceptive behaviors induced by H₂O₂ injection into the gastrocnemius muscle through five days (fig. 5B). Recovery of responses to injections of H₂O₂ was evident by day seven after capsaicin nerve block.

**Ca²⁺ Imaging**

After evaluating the hind-limb responses to H₂O₂ injection, Ca²⁺ transients in 128 lumbar DRG neurons were recorded during the sequential application of 1 mM H₂O₂ and 0.5 μM capsaicin. The example traces are shown in figure 6A. Twenty of 128 total neurons (16%) responded to 1 mM H₂O₂, and most of these H₂O₂-responsive neurons (95%, 19 of 20) also responded to 0.5 μM capsaicin (fig. 6, B and C). Sixty-one of 128 total neurons (48%) responded to 0.5 μM capsaicin but not to 1 mM H₂O₂ (fig. 6, B and C).

Ca²⁺ transients were recorded during the sequential application of 1 mM H₂O₂ and 100 μM of the TRPA1 activator, AITC, in 190 neurons from four rats. The example traces are shown in figure 6D. Twenty-one (11%) of 190 DRG neurons responded to both 1 mM H₂O₂ and 100 μM AITC, and all of the H₂O₂-responsive lumbar DRG neurons also responded to AITC (100%, 21 of 21; fig. 6E). Thirty-two (17%) of 190 neurons responded to AITC but not to H₂O₂ (fig. 6, E and F).

**TRPA1−/− Mice**

Total time of nociceptive behavior after intramuscular injection of H₂O₂ was less in TRPA1−/− mice compared with TRPA1+/+ mice (P = 0.0051; fig. 7A). Ca²⁺ transients were recorded from 76 lumbar DRG neurons from three TRPA1+/+ mice and 79 lumbar DRG neurons from three TRPA1−/− mice. The example traces of Ca²⁺ transients of DRG neurons from TRPA1−/− and +/+ mice during the application of 1 mM H₂O₂ are shown in figure 7, B and C, respectively. Although 14 (18%) of 79 DRG neurons from TRPA1+/+ mice were activated by 1 mM H₂O₂, none of the 76 neurons from TRPA −/− mice were activated by 1 mM H₂O₂ (P < 0.0001; fig. 7D).
In the present study, we have demonstrated that intramuscular injection of H$_2$O$_2$, but not subcutaneous injection of H$_2$O$_2$, produced nociceptive and aversive behaviors via the TRPA1 receptor. Intramuscular injection of H$_2$O$_2$ caused a sustained increase in activity of DHNs that had a similar time course as the behavioral responses. Using Ca$^{2+}$ imaging, H$_2$O$_2$ activated a subset of lumbar DRG neurons that also responded to AITC, a TRPA1 agonist, and to capsaicin, a TRPV1 agonist. Capsaicin nerve block inhibited guarding behavior after plantar incision and the nociceptive response.
after intramuscular injection of \( \text{H}_2\text{O}_2 \). The actions of intramuscular \( \text{H}_2\text{O}_2 \) through the TRPA1 receptor on behavior and on \( \text{Ca}^{2+} \) imaging in lumbar DRG neurons were confirmed in TRPA1–/– mice.

**Nociceptive Behaviors after Intramuscular Injection of \( \text{H}_2\text{O}_2 \)**

Several studies have shown that \( \text{H}_2\text{O}_2 \) activates sensory pathways. \( \text{H}_2\text{O}_2 \) induces asthma when inhaled\(^{20}\) and emesis when ingested by activating vagal afferent sensory fibers.\(^{28}\) \( \text{H}_2\text{O}_2 \) may also contribute to nociceptive behaviors in animal models of acute joint pain\(^{29,30}\) and ischemia-reperfusion injury.\(^{31}\) In previous studies by others, hind paw injections of \( \text{H}_2\text{O}_2 \) produced only brief nociceptive responses, and specific subcutaneous *versus* deep muscle tissue injections were not characterized.\(^{32}\) When applied to the rat skin-saphenous nerve preparation *in vitro*, \( \text{H}_2\text{O}_2 \) had only brief and limited excitatory effects on C-fiber terminals.\(^{33}\) In contrast, *in vivo* injection of \( \text{H}_2\text{O}_2 \) markedly activated all of the recorded group IV chemosensitive afferents innervating the rat tibialis anterior muscle.\(^{34}\) Intramuscular injection of \( \text{H}_2\text{O}_2 \) induced place aversion, which was blocked by a TRPA1 antagonist (figs. 2 and 3). Recent studies have shown the CPA test to be an effective method to measure spontaneous nociception.\(^{8,22}\) CPA produced aversion in doses that did not produce overt nociceptive behaviors (fig. 1C vs. fig. 3); thus, CPA appears to be more sensitive toward detecting nociception by intramuscular \( \text{H}_2\text{O}_2 \) injection in rats.
TRPA1 Responses and TRPV1-expressing Neurons

We have reported previously that plantar incision-induced guarding nociception is inhibited by pretreatment with capsaicin infiltration9,10 and with capsaicin application to the branches of the sciatic nerve.10 We also showed that guarding behavior requires deep muscle tissue injury.5 In the present study, similar to our previous studies, guarding behavior was inhibited by sciatic nerve block using a mixture of bupivacaine and capsaicin. The sciatic nerve block using the mixture of bupivacaine and capsaicin also inhibits the nociceptive response to H2O2 injection into the gastrocnemius muscle. The dilute capsaicin solution that we have used does not cause sensory fiber degenerative loss; rather, it depletes neuropeptides and impairs heat and chemosensitivity but not mechanosensitivity.9 These data suggest that the analgesic effect of capsaicin-induced sensory fiber desensitization is associated with temporary loss of responses to chemical stimuli, to both lactic acid and to H2O2.

Our results from Ca2+ imaging of lumbar DRG neurons demonstrating chemosensitivity of TRPV1-expressing DRG neurons to H2O2 via TRPA1 are consistent with a previous study.35 First, only AITC-responsive neurons responded to H2O2, and responses to H2O2 were absent in TRPA1−/− mice.19,36 Fewer neurons responded to 1 mM H2O2 than to 100 μM AITC in the present study, but H2O2 concentrations higher than 1 mM will likely produce greater responses.37 Second, all of the H2O2-responsive lumbar DRG neurons were capsaicin sensitive.36 Thus, the capsaicin treatment that inhibits guarding nociception is associated with loss of responses to H2O2 and lactic acid, both of which are acutely increased in incised muscle.12,38,39 H2O2 is one of the reactive oxygen species with diverse biologic actions including wound healing.16,18,41 The greater responses to H2O2 injection into muscle compared with subcutaneous injection may, in part, explain why an incision that includes deep muscle produces spontaneous activity in nociceptive pathways but skin incision alone does not.

Conclusions

This study demonstrates that in vivo intramuscular injection of H2O2 into the hind limb evoked nociceptive behavior and activated lumbar spinal DHNs. H2O2 also increased intracellular Ca2+ in lumbar DRG neurons in vitro via the TRPA1 receptor. Guarding after deep tissue incision and nociceptive behavior after intramuscular injection of H2O2 were reduced by nerve blockade with capsaicin. Together, these findings indicate that reduced guarding after incision of deep plantar tissue by a TRPA1 antagonist like HC-03003112 and capsaicin nerve block may in part be related to reduced responses to reactive oxygen species like H2O2 in incised deep tissue. Incision that includes muscle may be more painful than skin incision because of greater responses to reactive oxygen species like H2O2 in muscle.

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Competing Interests

The authors declare no competing interests.

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