**Background:** The authors have demonstrated a decrease in pH in the incisional wound environment, suggesting a possible contribution of low pH to postsurgical pain. In this study, the authors characterized the acid-responsiveness of nociceptors innervating the plantar aspect of the rat hind paw 1 day after plantar incision and compared this to plantar skin from unincised control rats.

**Methods:** Using the rat glabrous in vitro skin-tibial nerve preparation, afferent nerve activities from single mechanosensitive nociceptors were recorded. Differences in mechanosensitivity, spontaneous activity, and chemosensitivity of units were evaluated. For chemosensitivity, acid-responsiveness of nociceptors to lactic acid (pH 5.5 to 6.5) was studied.

**Results:** C-fibers showed dose-dependent, sustained responses to lactic acid. A greater proportion of C-fibers from 2 mm or less from the incision was activated by pH 6.0 lactic acid (52.9%) compared to control (14.3%). Total evoked potentials during acid exposure were greater in C-fibers innervating 2 mm or less from the incision compared to those in uninjured skin. The prevalence of acid responses and total evoked potentials during acid exposure in C-fibers innervating more than 2 mm from the incision were not different from control. Few A-fibers responded to lactic acid, with a range of pH 5.5 to 6.5 in both incision and control groups. Increased spontaneous activity and mechanosensitivity were also evident.

**Conclusions:** C-fibers in the vicinity of the incision showed qualitatively and quantitatively greater chemosensitivity to pH 6.0 lactic acid compared to control. This change was localized to 2 mm or less from the incision, suggesting increased chemosensitivity of nociceptive C-fibers 1 day after plantar incision.

WE have developed and characterized models of incisional pain and described a variety of pain-related behaviors to better understand mechanisms for postoperative pain. In a previous study, we demonstrated that a decrease in pH occurs immediately after incision and is sustained for several days. The decreased pH is localized at the incision site, and pain-related behaviors are evident during the period of low tissue pH. Because low pH activates and sensitizes nociceptors and acid injection causes pain in human volunteers, decreased pH may contribute to nociception after incision and pain in patients after surgery. Several possible channels or receptors activated by low pH are expressed on nociceptors, suggesting drugs blocking pH responses may be candidate analgesics for patients after surgery.

The pH required to produce sustained activation of nociceptors (pH 5.0 to 6.0) is much lower than the pH of incisions (pH 6.8 to 7.0). Recent studies suggest that mediators such as nerve growth factor (NGF) and lactate may enhance pH response of sensory neurons through various mechanisms. Both NGF and lactate are increased in the incisional wound environment, suggesting that they might contribute to sensitization of nociceptors to low pH.

Nociceptor sensitization is a key finding in hyperalgesic, pathologic pain states. In most studies of nociceptor sensitization, heat responsiveness is examined; more recently mechanosensitivity is generating considerable interest. However, chemosensitivity of nociceptors in pathologic states is rarely evaluated.

In this study using a rat glabrous in vitro skin-nerve preparation, we hypothesized that the acid-responsiveness of nociceptive afferents innervating the plantar aspect of the rat hind paw 1 day after plantar incision would be greater than the responsiveness of afferents in the sham-operated rats. As a low pH stimulus, we used lactic acid on the basis of our previous study showing that tissue lactate concentration is increased at the same time that pH is decreased and pain behaviors are obvious. This chemical stimulus may in part simulate the chemical challenge acting on nociceptors in the incisional wound environment in vivo. Using computer-controlled mechanical stimulator, quantitative mechanosensitivity of these fibers to force-controlled stimuli was also studied.

**Materials and Methods**

**General**

All experimental procedures were approved by The University of Iowa Animal Care and Use Committee, Iowa City, Iowa. Rats were treated in accordance with the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals issued by the International Association for the Study of Pain.

Forty-two adult male Sprague-Dawley rats (250–300 g; Harlan, Indianapolis, IN) were used. Rats were housed in groups of two to three in clear plastic cages, with a 12-h light-dark cycle. Food and water were available ad libitum.

**Plantar Incision**

A plantar incision similar to that described previously was made under 1.5–2% isoflurane anesthesia delivered...
Electrophysiological Studies

Preparation. The rat glabrous in vitro skin-nerve preparation, modeled as saphenous nerve-skin preparation,\(^1\) has been described elsewhere.\(^1\) In brief, rats were euthanized in a carbon dioxide chamber; the medial and lateral planter nerves and their innervated territory on the glabrous hind paw skin were subcutaneously dissected until the nerve and skin could be removed. The skin was placed epidermal side down in the in vitro perfusion chamber and superfused with modified Krebs-Henseleit solution (in mM: 110.9 NaCl, 4.8 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)SO\(_4\), 24.4 NaHCO\(_3\), and 20.0 glucose, pH 7.4), which was saturated with a gas mixture of 95% oxygen and 5% carbon dioxide. The temperature of the bath solution was maintained at 32 ± 0.5°C. The plantar nerves were drawn through a small hole into the recording chamber containing a superficial layer of mineral oil and a bottom layer of modified Krebs-Henseleit solution. The nerve was desheathed on a mirror stage, and small filaments were repeatedly split with sharpened forceps to allow single fiber recording to be made using extracellular gold-wire recording electrodes. Neural activity was amplified (DAM50; Harvard Apparatus, Holliston, MA), filtered, and displayed using standard techniques. Amplified signals were led to a digital oscilloscope and an audiomonitor and fed into a personal computer via a data acquisition system (spike2/CED1401 program; Cambridge Electronic Design Ltd., Cambridge, United Kingdom).

Identification of Afferents. The receptive fields of afferent units were identified by probing the dermis side of the skin with a blunt glass rod; thus, mechanosensitive afferents were recorded. Only units with a clearly distinguishable signal to noise ratio (greater than 2:1) were further studied. Once the receptive field was identified, ongoing spontaneous activity was recorded over a 5-min period for each fiber before any modality testing. After recording of spontaneous activity, a standard protocol of mechanical stimulation followed by lactic acid application was performed.

Feedback-controlled Mechanical Stimulation. To determine quantitative mechanosensitivity, a servo force-controlled mechanical stimulator (Series 300B Dual Mode Servo System; Aurora Scientific, Aurora, Ontario, Canada)\(^1\) was used. A flat-ended cylindrical metal probe (tip diameter, 0.7 mm) attached to the tip of the stimulator arm was placed just close to the most sensitive spot of the receptive field so that no force was generated. First, computer-controlled ramp-shaped force stimuli were applied at 60-s interval to measure the mechanical threshold of the nociceptors. Each force ramp started from 0 to 40 and 80 mN, respectively, in 5 s. Then the ascending series of compressive loads (5–120 mN range of force) were applied to evaluate the suprathreshold mechanosensitivity. Since the neural responses of cutaneous mechanosensitive nociceptors to mechanical stimuli are more highly correlated with compressive stress (force) than compressive strain (displacement),\(^1\)\(^2\)\(^0\) sustained force-controlled stimuli (rise time, 100 ms; duration of sustained force plateau, 1.9 s) were applied at 60-s intervals (see Discussion).

Chemical Stimulation. After mechanical stimulation, chemosensitivity was assessed using lactic acid. To restrict the chemical stimuli to the isolated receptive field, a small metal ring (internal diameter, 5 mm), which could seal by its own weight, was used. In some cases, inert silicone grease was added to ensure a waterproof seal.

After recording baseline for 5 min, the metal ring was placed and the modified Krebs-Henseleit solution inside the ring was removed with a syringe. Then, either pH 6.0 lactic acid (15 mM; 32°C) or control solution (Krebs-Henseleit solution equilibrated with room air; pH 7.4; 32°C) was applied to the receptive field for 5 min and followed by 5-min washout (fig. 1). Thirty-one units (16 C-fibers and 15 A-fibers) from incised rats and 19 units (11 C-fibers and 8 A-fibers) from control rats were tested with pH 6.0 lactic acid. Sixteen units from incised rats and 14 units from control rats were tested with control solution. In the next 20 units (14 C-fibers and 6 A-fibers) from incised rats and 17 units (10 C-fibers and 7 A-fibers) from control rats, 15 mM lactic acid with increasing acidity (pH 6.5, 6.0, and 5.5; 32°C) was sequentially tested for 5 min in each unit, to further characterize the acid-responsiveness and evaluate pH-dependencies in the response. The interval between each lactic acid application was 15 min. In a separate group of five acid-responsive C-fibers 2 mm or less from the incision, pH 6.0 lactic acid (15 mM; 32°C) was repeatedly applied (three times) for 5 min at 5-min intervals in each unit, to evaluate the reproducibility of pH response and the potential for tachyphylaxis. To avoid sensitization/desensitization of nociceptors, fibers having receptive fields in the previously studied area were avoided for subsequent recording.

In another group of ten C-fibers from sham control skin, the incision was made during recording to evaluate whether the tissue disruption caused by incision affects acid sensitivity by providing better access of lactic acid to nociceptive nerve terminals. First, after recording baseline activity for 5 min, pH 6.0 lactic acid was applied

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to the receptive field for 5 min and the response was measured. This was followed by 5-min washout. Then an incision was made approximately 1 mm from the receptive field. Ten minutes later, the unit was tested with pH 6.0 lactic acid again, and the responses before and after the incision were compared.

Lactic acid for chemical stimulation was made by replacing NaHCO₃ (24.4 mM) normally contained in modified Krebs-Henseleit solution with L-lactic acid (Sigma, St. Louis, MO; 85% to a final concentration of 15 mM). The pH of lactic acid was measured and adjusted to pH 6.0 with a few drops of 1 N NaOH before application. To further increase or decrease pH, additional 1 N NaOH or 1N HCl was added. The final osmolarity of the lactic acid solution was 312 mOsm; the sodium concentration was 125 mM.

**Conduction Velocity and Fiber Categorization.** The conduction velocity was always measured at the end of the experiment. The conduction velocity of each unit was determined by monopolar electrical stimulation (5–20 V, 0.5–2.0 ms duration, 0.2–1.0 Hz) into the most mechanosensitive site in the receptive field. Then the distance between the receptive field and the recording electrode (conduction distance) was divided by the latency of the action potential. Afferent fibers conducting slower than 2.5 m/s were classified as C-fibers, those conducting between 2.5 and 24 m/s as Aδ-fibers, and those conducting faster than 24 m/s as Aβ-fibers. Units were classified as mechanosensitive nociceptors on the basis of their graded response throughout the innocuous and noxious range of mechanical force stimuli. Rapidly adapting fibers were not studied.

**Data Analyses.** Action potentials collected on a computer were analyzed offline with a template-matching function of Spike 2 software (Cambridge Electronic Design Ltd.). If more than one fiber was present in a recording, data were analyzed only if the spike shapes and amplitudes were different and could be easily discriminated. If a unit discharged at a rate of 0.1 imp/s or more without any intentional stimuli, it was categorized as spontaneously active. For chemical responses, unit discharges were counted in 10-s bins, and total responses were averaged during baseline, during acid application and after washout. A unit was considered activated (responsive) when it discharged greater than 0.1 imp/s during chemical stimulation. If background activity was present, the unit was regarded as responsive if the activity was increased at least two standard deviations greater than the background activity during the chemical stimulation period. To count impulses generated by acid in a unit with spontaneous activity, background activity was subtracted from the evoked responses during stimulation. Responses to pH 6.0 lactic acid and control Krebs–Henseleit solution were compared in 50 units. Acid dose-response curves were generated in 37 units. To analyze responses to pH 6.0 lactic acid, units from both groups were combined. For mechanical responses, activity was counted in 1-s bins. Mechanical threshold was determined as the lowest force that elicited the first action potential in responses to ramp-shaped force. If background activity was present, threshold was determined by the lowest force that increased background activity by at least two standard deviations greater than the background average for 10 s (1-s bins). For the suprathreshold mechanosensitivity, total spikes during the 1.9-s sustained force were analyzed. Background activity was subtracted from any evoked responses, thus assuming background activity was sustained during the stimulus period.

**Statistics**
Conduction velocity of afferent units was compared by unpaired t test. A Fisher exact test was used to compare
the percentage of acid-responsive fibers, the percentage of spontaneously discharging fibers, and the percentage of fibers responding to each force level. Total evoked potentials or average discharge rates during acid application and the mechanical thresholds were compared by Kruskal-Willis followed by Dunn’s test. The pH-dependent responses to lactic acid were analyzed using linear association test and two-way ANOVA with repeated measures on one factor. The responses of C-fibers to repeated application of pH 6.0 lactic acid were analyzed using Friedman’s test. Mechanical thresholds were compared by one-way ANOVA followed by Scheffe post hoc test, and the stimulus-response relationship for the mechanical responses was compared using two-way ANOVA with repeated measures on one factor; significant main effects of incision group or interactions were followed by separate one-way ANOVAs and Scheffe post hoc test at each force level. Data are presented as mean ± SE or median [range]. Statistical analysis was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL).

Results

General Properties of Afferents

A total of 132 mechanosensitive nociceptors were studied. Seventy-two fibers (47 C-fibers, 24 Aδ-fibers, and 1 Aβ-fibers) were studied from 25 incised rats; 60 fibers (39 C-fibers and 21 Aδ-fibers) were studied from 22 unincised, sham-control rats. There was no difference in the conduction velocity of afferents between the incised and the unincised groups (C-fibers, 1.15 ± 0.08 m/s vs. 1.11 ± 0.11 m/s; Aδ-fibers, 5.16 ± 0.61 m/s vs. 5.66 ± 0.81 m/s). The conduction velocity of one Aβ-nociceptor identified from an incised rat was 25.6 m/s.

The receptive fields of all fibers were located in glabrous hind paw skin. For analyses, we subdivided those having receptive fields 2 mm or less from the incision and those having receptive fields greater than 2 mm from the incision. This was based on our previous study, which showed that most C-fibers sensitized by heat were localized to 2 mm or less from the incision, in vitro, 1 day after plantar incision.14 Of 47 C-fibers and 25 A-fibers (Aδ-fibers and Aβ-fibers) recorded from incised rats, 30 C-fibers and 13 A-fibers had receptive fields 2 mm or less from the incision.

Response to Chemical Stimulation

Chemical responses to pH 6.0 lactic acid were evaluated in 17 C-fibers no more than 2 mm from the incision, 13 C-fibers more than 2 mm from the incision, and 21 sham control C-fibers. An example of an acid-responsive C-fiber with the receptive field 2 mm or less from the incision is shown in figure 1A. A greater proportion of C-fibers 2 mm or less from the incision was responsive (52.9%, 9 of 17; P < 0.05 by Fisher exact test; fig. 2A and B) compared to sham control (14.3%, 3 of 21). The prevalence of responsive C-fibers innervating more than 2 mm from the incision was not different from sham control (23.1%, 3 of 13; fig. 2B). For A-fibers, 12 fibers no more than 2 mm from the incision, 9 fibers greater than 2 mm from the incision, and 15 sham control fibers were tested for their responsiveness to pH 6.0 lactic acid. Few A-fibers responded to pH 6.0 lactic acid: 2 of 12 fibers (16.7%) innervating 2 mm or less from the incision were activated; no unit innervating greater than 2 mm from the incision or sham control paw was responsive (fig. 2 A and C). In a separate group of 16 units (12 C-fibers and 4 A-fibers) from incised skin and 14 units (8 C-fibers and 6 A-fibers) from sham control skin, responses to pH 7.4 Krebs–Hensleit solution were tested, and none were excited. (fig. 1B and fig. 2B and C). Among 12 C-fibers from incised skin tested with pH 7.4 control solution, 8 fibers were from no more than 2 mm from the incision.

The majority of units activated by lactic acid were C-fibers; therefore, responses of these units to pH 6.0 lactic acid were further quantified by counting activity in 10-s bins and by averaging responses during baseline, acid application, and washout period (fig. 3). In figure 3, panels A–C show the sample recording trace of a responsive unit during lactic acid application for incision of 2
mm or less, incision greater than 2 mm, and sham control. The mean spike density histograms during 5-min acid exposure are shown in panels D–F of figure 3. Units discharged in a sustained manner throughout the acid application period. Total evoked potentials during acid exposure were greater in C-fibers innervating 2 mm or less from the incision (fig. 3D; median 179 [61–514] imp; \( P < 0.05 \) by the Kruskal-Wallis followed by Dunn’s test) compared to those of sham control (fig. 3F; median 50 [34–78] imp). Total evoked potentials of C-fibers having receptive fields greater than 2 mm from the incision (fig. 3E; median 57 [35–174] imp) were not different from sham control. The individual magnitude of C-fiber responses is shown in panels G–I of figure 3. In C-fibers having receptive fields 2 mm or less from the incision (fig. 3G), the median rate during acid application (median 1.34 [0.20–2.78] imp/s) was greater than that of sham control C-fibers (fig. 3I; median 0.17 [0.11–0.26] imp/s; \( P < 0.05 \) by the Kruskal-Wallis followed by Dunn’s test).

In a separate group of five acid-responsive C-fibers 2 mm or less from the incision, pH 6.0 lactic acid was repeatedly applied (three times) for 5 min at 5-min intervals (fig. 4). The magnitude of responses of C-fibers to repeated pH 6.0 lactic acid was reproducible, and no tachyphylaxis was noted (fig. 4B); the mean discharge rates during repeated application of pH 6.0 lactic acid were not significantly different.

Chemical responses of nociceptors to lactic acid of three different pH levels are summarized in figure 5. Sample recordings of an acid-responsive C-afferent from an incised rat during the application of 15 mM lactic acid with different pH (pH 6.5, 6.0, and 5.5) are shown in figure 5A. C-fibers showed pH-dependent responses to lactic acid; greater acidity activated more C-fibers (\( P < 0.05 \), linear by linear association test; fig. 5B) and generated greater discharge rates during acid application (\( P < 0.05 \), two-way ANOVA with repeated measures on one factor; fig. 5D). Few A-fibers responded to lactic acid, with a pH range of 6.5 to 5.5 in both incision and sham-control groups (fig. 5C).
In another group of ten C-fibers from sham-control skin, nine were not activated by pH 6.0 lactic acid before the incision, and these nine units remained unresponsive after the incision. The prevalences of responsive units both before and after the incision (10.0%, 1 of 10) were not different from sham control (23.1%, 3 of 13). One of ten fibers was activated by pH 6.0 lactic acid before the incision, and the discharge rates during acid application before and after the incision were the same (0.13 imp/s and 0.11 imp/s, respectively). Therefore, acid responsiveness did not change immediately after incision.

Spontaneous Discharge

The distribution of receptive fields of C- and A-nociceptors with or without spontaneous activity is shown in figure 6A. The prevalence of spontaneously discharging C-fibers (48.0%, 12 of 25) and A-fibers (46.2%, 6 of 13) innervating 2 mm or less from the incision was greater...
Controlled mechanical stimuli. A ramp-shaped force stimulus was used to determine mechanical threshold (fig. 7A), and then the ascending series of sustained force stimuli was applied to evaluate the suprathreshold mechanosensitivity (fig. 7B). A summary of mechanical responses of C- and A-fibers 1 day after incision is shown in panels C-F of figure 7. Mean response threshold of C-fibers innervating 2 mm or less and greater than 2 mm from the incision was $14.2 \pm 2.4$ mN and $20.7 \pm 4.7$ mN, respectively. The mean mechanical threshold of sham-control C-fibers was $23.1 \pm 3.8$ mN. There was no difference in mechanical threshold among the groups. The force-response curve of C-fibers showed increases in responses to greater mechanical forces ($P < 0.001$ by two-way ANOVA with repeated measures on one factor; fig. 7D). When compared with sham-operated control, C-fibers that had receptive fields 2 mm or less from the incision showed greater responses to 20 and 40 mN stimuli ($P < 0.05$ by one-way ANOVA followed by Scheffé post hoc test; fig. 7D).

The mean threshold of A-fibers innervating 2 mm or less and greater than 2 mm from the incision was $9.6 \pm 3.4$ mN and $15.4 \pm 6.5$ mN, respectively. There was no difference in the mean mechanical thresholds of A-fibers among the groups. When the percentage of mechanosensitive fibers was compared at each force stimulus level, a greater percentage of A-fibers innervating 2 mm or less from the incision responded to 10-mN stimulus ($83.3\%$, $10$ of $12$; $P < 0.05$ by Fisher exact test; fig. 7E) compared to control ($40\%$, $8$ of $20$). A-fibers showed increases in responses to greater mechanical forces ($P < 0.001$ by two-way ANOVA with repeated measures on one factor), but there was no difference in force-response curves among three groups (fig. 7F).

**Discussion**

The major finding of the current study is that, 1 day after plantar incision, a greater proportion of C-fibers are activated by pH 6.0 lactic acid **in vitro**, and total evoked potentials during lactic acid exposure are greater in C-fibers 2 mm or less from the incision compared to sham control. These data are the first to demonstrate the chemical sensitization of C-nociceptors after incision. Consistent with previous study,14 more nociceptors have spontaneous activity in incised skin. Our data also suggest evidence for mechanical sensitization of C- and A-nociceptors 2 mm or less from the incision when tested with force-controlled mechanical stimuli.

In the current study, 15 ms lactic acid with pH 6.5, 6.0, and 5.5 was used to assess the chemosensitivity of skin nociceptors **in vitro**. We have previously demonstrated that incision of the plantar hind paw, the gastrocnemius muscle, and the paraspinal region increases the
tissue lactate concentration at the same time that pH decreases and pain behaviors are increased. These results suggested that decreases in pH and increases in lactate together could contribute to pain caused by incisions. Our current data showing increased chemosensitivity of C-nociceptors to lactic acid after incision further supports the possibility that cofactors such as lactate or others might facilitate nociceptor activation by low pH and contribute to postsurgical pain.

Lactate was shown to enhance the response of acid-sensing ion channel 3 (ASIC-3) to low pH in vitro by the mechanism of decreasing of divalent ions in the extracellular media. ASIC-3 is expressed on nociceptors and is a candidate to mediate acid-induced nociception, in addition to transient receptor potential vanilloid receptor 1 (TRPV1). In isolated sensory neurons and ASIC-3 expressing cells, 15 mM lactate produced more than a 70% increase in current evoked by a reduction in pH to 7.0. In the same system, when applied at pH 8.0 or 7.4, lactate produced no depolarization and no current. Likewise, although we did not test the response of nociceptors to 15 mM lactate at neutral pH separately in the current study, it is unlikely that the activation of nociceptors during lactic acid stimulation is through direct activation by lactate itself. The relationship between lactate and pH response in the in vitro skin-nerve preparation warrants future study; the contributions of proton and lactate to the activation of nociceptors by lactic acid will be further evaluated.

Fig. 7. Mechanical responses of afferent units 1 day after plantar incision. (A) Sample recordings of the mechanical responses of a control C-fiber to the ramp-shaped force stimulus. The upper and lower panels show the digitized oscilloscope tracings and the force stimulus applied, respectively. The mechanical threshold of this unit was 16.4 mN. Inset displays the action potential of this unit. CV = conduction velocity. (B) Responses of the same unit to the ascending series of sustained force stimuli, showing greater discharge response to higher force stimuli. The upper, middle, and lower panels show the spike density histograms (bin width = 1 s), raw spike discharges, and the force stimuli applied, respectively. (C, D) The percentage mechanosensitivity (C) and stimulus-response function (D) of C-fibers in sham control (n = 27), 2 mm or less from the incision (n = 24) and greater than 2 mm from the incision (n = 17) (* P < 0.05 vs. sham control, one-way ANOVA followed by Scheffe post hoc test). (E, F) The percentage of mechanosensitivity (E) and stimulus-response function (* P < 0.05 vs. control, Fisher exact test) of A-fibers in control (n = 20), 2 mm or less from the incision (n = 12) and greater than 2 mm from the incision (n = 11).
ACID SENSITIVITY OF NOCICEPTORS AFTER INCISION
modification of mechanical stimulus pattern, neuronal responses during constant, sustained compressive stress were able to be characterized by applying constant force.

In conclusion, this study indicates chemical sensitization of C-fibers in vitro 1 day after plantar incision. C-fibers in the vicinity of the incision showed qualitatively and quantitatively greater responses to pH 6.0 lactic acid compared to control. We have previously demonstrated increased lactate and decreased pH in the incisional wound environment, and increased chemosensitivity of nociceptors to lactic acid after incision supports the possibility that lactate as a cofactor may facilitate nociceptor activation by low pH and contribute to postsurgical pain. C-nociceptors and A-nociceptors close to the incision also showed spontaneous discharge and mechanical sensitization, in vitro.

References

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