Cannabinoid Receptor Type 1 Antagonist, AM251, Attenuates Mechanical Allodynia and Thermal Hyperalgesia after Burn Injury

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ABSTRACT

Background: Burn injury causes nociceptive behaviors, and inflammation-related pathologic pain can lead to glial cell activation. This study tested the hypothesis that burn injury activates glial cells, and cannabinoid receptor 1 (CB1R) antagonist, AM251, will decrease burn pain.

Methods: Anesthetized rats received 0.75-cm² third-degree burn on dorsal hind paw. Vehicle or AM251 30 μg intrathecally (older rats, n = 6 per group) or, either vehicle, 0.1 or 1.0 mg/kg intraperitoneally (younger rats, n = 6 per group), started immediate postburn, was administered for 7 days. Mechanical allodynia and thermal hyperalgesia were tested on ventral paw for 14 days. Microglial and astroglial activity was assessed by immunocytochemistry.

Results: Allodynia, observed on burn side from day 1 to 14, was significantly (P < 0.05) attenuated by intrathecal and intraperitoneal AM251 (1 mg/kg) starting from 3 to 14 days. Hyperalgesia, observed from day 3 to 12, was completely (P < 0.05) reversed by intrathecal and intraperitoneal AM251 (1 mg/kg). AM251 0.1 mg/kg had no effect. Microglial activity (n = 3 per time point) increased (P < 0.05) 18.5 ± 7.5 and 12.3 ± 1.6 (mean ± SD) fold at 7 and 14 days, respectively. Astroglial activity (n = 4 per time point) increased 2.9 ± 0.3 fold at day 7 only. Glial activities were unaltered by AM251.

Conclusions: AM251 inhibited nociceptive behaviors after burn even beyond 7-day period of administration. Although many studies have documented the utility of CB1R agonists, this study indicates that endogenous cannabinoids may have an unexpected pronociceptive effect during development of burn pain, explaining why CB1R antagonist, AM251, improves nociceptive behaviors. The decreased nociception with AM251 without altering glial activity indicates that AM251 acts further downstream of activated glial cells. (Anesthesiology 2014; 121:1311-9)

The endocannabinoid system refers to a group of neuromodulatory lipids and their receptors that are involved in many physiological processes including pain sensation, appetite, and memory.1–4 Pain-related cannabinoid receptors are of two types, cannabinoid receptors 1 and 2 (CB1R and CB2R), and are located in the central and peripheral nervous systems.5–6 Exogenous cannabinoid agonists have been used to attenuate pain responses in several pain models including inflammatory and neuropathic pain.7,8 More specifically, the CB1R together with N-methyl d-aspartate receptor induces analgesia although the mechanism underlying this interaction is unclear.8 Thus, CB1R agonist ligands via CB1R influence neuronal excitability by a variety of mechanisms, and these effects are relevant to pain perception and behavior.6–8 Recent reports, however, showed that global (whole body) CB1R-deficient mice or mice lacking CB1R in dorsal horn inhibitory interneurons were protected from capsaicin-induced mechanical sensitization.9,10 Moreover, preadministration of rimonabant, a CB1R antagonist, resulted in a decrease of hyperalgesic and alldynic areas in a human model; there were no changes, however, in the area of acute pain.10 Therefore, an exogenous CB1R antagonist seems to have beneficial effects in pathologic-state–induced chronic nociceptive behaviors. Based on these latter studies,9,10 it seems that endogenous cannabinoids may induce the disinhibition of nociceptive transmission in the spinal cord and dorsal horn during pathologic-state–induced pain.
Alldynia and hyperalgesia are concomitant features of burn injury in humans and is seen both at the site of burn and at areas in close proximity to the injury.11,12 This pain in humans is exaggerated during procedures (e.g., dressing changes).11,13 Management of burn pain in the perioperative period and in intensive care unit has been challenging.14,15 Burn injury–induced pain probably has inflammatory and neuropathic components. Although previous studies in rodent pain models have documented alterations in the expression of N-methyl D-aspartate, protein kinase C-γ, and/or opioid receptors,16–18 the basic mechanisms underlying burn-induced pain and its treatment have received very little attention. Recent studies on non–burn-induced neuropathic pain conditions suggest that astroglia and microglia are activated in pathologic pain states.3–5 Although approximately 50% of astroglia and approximately 80% of microglia in rat spinal cord have immunoreactivity to CB1R, as to how endogenous cannabinoids and exogenous cannabinoid ligands modulate pain, and the activity of astroglia and microglia in burn injury–induced pain remains unknown. In this study, using rat hind paw burn injury model of thermal hyperalgesia and mechanical allodynia,16–18 we tested the hypothesis that burn injury is associated with increased activity of glial cells in the spinal cord, and that administration of CB1R antagonist, AM251, will beneficially modulate the burn injury–associated hyperalgesia and alldynia.

Materials and Methods

Experimental Animals and Drugs

Adults male Sprague–Dawley rats of age 8 to 9 weeks weighing 300 to 350 g or younger male Sprague–Dawley rats of age 4 to 5 weeks weighing 140 to 150 g were used. Adult and younger animals were tested because of our interest in both adult and pediatric burn pain. Rats were housed in individual cages in one of the Massachusetts General Hospital, Boston, Massachusetts, animal care facilities, which were artificially illuminated from 7:00 AM to 7:00 PM, with water and food pellets available ad libitum. The protocol was approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. CB1R antagonist, AM251, was purchased from Sigma-Aldrich (St. Louis, MO). AM251 was dissolved in dimethyl sulphoxide (70%). For all in vivo nociceptive behavior studies, we chose a sample size of n = 6 for each group based on previous experience in this model.16–18

Burn Injury

The model has been previously described by us16–18 and consists of a localized burn injury to the dorsum of the hind paw with demonstration of nociceptive behaviors on the plantar aspect of paw. Before infliction of burn injury, the rats were anesthetized with 50 mg/kg of pentobarbital. A third-degree burn injury was produced by immersion of only the dorsum of hind paw into 85°C water for 12 s. The burn-injured area was limited to an area of approximately 0.75 cm² by pressing the dorsum of hind paw firmly into the hot water through a holed plastic template. This third-degree burn injury most reliably produced postinjury nociceptive behaviors including thermal hyperalgesia and mechanical allodynia in both older and younger rats.16–18 Sham injury was produced by immersing the dorsal part of the right hind paw into a warm water bath (35°C) for 12 s.

Intrathecal Catheter Implantation

One week before burn injury to adult rats, a polyethylene-10 catheter was implanted intrathecally under sevoflurane anesthesia administered via a commercial vaporizer with exit port attached to a hollow tube, which in turn was connected to a funnel that was applied to the snout of the rat. The catheter was inserted into each adult rat at the level of lumbar 1 to 2 as described in the study reported by Yaksh and Rudy.19 Those animals that exhibited neurological deficits after intrathecal catheter implantation were excluded from the experiments. AM251 or vehicle (dimethyl sulphoxide) was delivered via the intrathecal catheter in a total volume of 10 μl followed by a saline flush (10 μl). Although catheters were inserted 1 week before burn injury, AM251 or vehicle administration was started after burn injury (day 0). After recovery from anesthesia, the rats were returned to their cages.

Nociceptive Behavioral Testing

Animals were habituated to the behavioral test environment for 2 consecutive days before burn injury. The adult animals were tested for thermal hyperalgesia and mechanical alldynia preburn and at 1, 3, 5, 7, 10, 12, and 14 days postburn. Nociceptive behaviors in the younger rats were tested on same days except days 10 and 12 after burn. For testing thermal hyperalgesia, each plantar surface of a rat’s hind paw on the ipsilateral and contralateral surface was exposed to a beam of radiant heat through a transparent perplex glass surface as described in the study reported by Hargreaves et al.20 The withdrawal latency was averaged from at least two trials separated by 2-min intervals, and the cutoff was at 20 s to avoid tissue damage.

Mechanical allodynia was determined on ipsilateral and contralateral sides on the same days described above. For examining mechanical allodynia, each rat was placed on a metal mesh floor covered with a plastic box (15 × 15 × 18 cm) and allowed to habituate for 30 min. Mechanical stimulation resulting from the bending force of a von Frey filament was applied to the planter surface of each hind paw. Each trial consisted of five applications of a von Frey filament given every 4 s, and the cutoff force was 10 g. Brisk foot withdrawals (at least three times of five applications) in response to von Frey filament stimulation were considered positive. Depending on the initial response, subsequent filaments were applied in the order of either descending or ascending force to determine the threshold force.21,22
Drug Treatment
The older burn- and sham-burned rats received either CB1R antagonist (AM251 30 μg) or vehicle dimethyl sulphoxide intrathecally daily for 7 days via the previously implanted intrathecal catheter. The dose of intrathecal AM251 was based on previously reported dose of AM251 administered directly into the central venous system.23 The administration was started immediately postburn (day 0). The choice of administration of AM251 versus vehicle to rats was not randomized. The withdrawal threshold to thermal and mechanical stimulation was examined on the ipsilateral and contralateral hind paw for 14 days after burn or sham burn injury. In view of the difficulty in placing intrathecal catheter in the younger rats, one of three therapeutic regimen (1 mg/kg, or 0.1 mg/kg or vehicle once daily, administered intraperitoneally) was started after the burn and continued for 7 days. These doses were based on previous reports of parenteral administration of AM251 to rodents.24,25 To avoid the acute effect of AM251 or vehicle, the injections of AM251 were performed only after behavioral tests. Another set of younger naive animals received AM251 (1.0 mg/kg) or vehicle intraperitoneally, and nociceptive behaviors tested on the hind paw at 0 to 7 days.

Immunohistochemistry
Immunostaining with glial fibrillary acid protein (GFAP) and ionized calcium-binding adapter molecule-1 (Iba1) as markers of astroglial and microglial activation, respectively, was performed in a separate group of young rats at 3, 7, and 14 days after burn. The effect of treatment with AM-250 or vehicle on the expression of astroglia (using GFAP as a marker)26 and of microglia (using Iba1 as a marker)27 was studied on the burn-injured and contralateral sides. For harvesting of spinal cord segments, the rats were anesthetized with pentobarbital, and the chest was opened. A needle was inserted through the heart into the ascending aorta, which was perfused with saline (200 ml via infusion pump), followed by 4% paraformaldehyde (300 ml via infusion pump). Spinal cord lumbar segments were then excised, postfixed overnight, and kept 3 days in 20% sucrose. The spinal cord samples were sectioned with a cryostat (25-μm thick sections). All sections for immunohistochemical procedure were treated under the same conditions on the same day to minimize the between-group variability. Sections were blocked with 3% bovine serum in 0.3% Triton X-100 for 1 h at room temperature and incubated over night at 4°C with primary antibody (GFAP from Abcam, Inc., Cambridge, MA; microglial, Iba1, 1:2,000 dilution, from Chemicon/Millipore, Billerica, MA). The sections were then incubated for 1 h at room temperature with the corresponding fluorotet isothiocyanate or cyanine 3- (CY3-) conjugated secondary antibody (1:800 dilution; Chemicon/Millipore). For analyses of immunostained specimens, the sections were randomly selected and images scanned using a Nikon fluorescence microscope (Nikon Corporation, Tokyo, Japan). Images of dorsal horn regions were then captured with a CCD Spot camera (Diagnostic Instruments, Inc., Sterling Heights, MI) as described previously.22,28 Photoshop program (Adobe, San Jose, CA) was used for quantitation of expression of Iba1 and GFAP.

Statistical Analyses
Each group consisted of unique treatment strata (vehicle or AM21) and age strata (older or younger rats). Each rat served as its own control when ipsilateral and contralateral sides were tested at the different time points of testing. All data were analyzed by two-way ANOVA using the method of generalized linear model with repeated measures. Statistical significance was declared at P value of less than 0.05, repeated measure was accounted for the fitted dataset. The effect of treatment with AM251 or no treatment was examined on the contralateral side using pain response as the outcome. If no statistical significant result was observed, the untreated contralateral side was selected as the baseline (comparator) for further assessment over time. Subsequent analyses were focused on assessing the treatment effect, where the burned (ipsilateral) side was examined and the untreated ipsilateral side was used as comparator. Post hoc analyses were performed when significant main effect was observed. Subsequent analysis by study day on treatment effect was further examined. These were analyzed in the same manner as the primary analyses. Statistical significance was declared with corresponding Bonferroni-adjusted α value by the number of pairs (two-sided test). In addition, comparisons on pain responses between older and younger rats were made. All the analyses were performed using SAS® version 9.3 (Cary, NC). The fold change in glial activities is reported as mean ± SD. The person performing the animal or immunohistochemistry experiments were not blinded to the experimental condition of the preparation.

Results
Noicceptive Behaviors
Older Rats. After the placement of intrathecal catheters, six rats demonstrated some form of neurological deficits. These rats were excluded from study and not given burn or sham injury. No rats were lost after initiation of burn or sham burn injury. There were no differences in nociceptive responses among vehicle-treated contralateral and AM251-treated contralateral sides of burned-injured mice (two measurements per rat, six rats per group at each measured day). The adult burned rats receiving vehicle treatment showed significant (P < 0.05) mechanical allodynia (Von Frey testing) on the ipsilateral side of burn compared with contralateral side at all experimental days (starting at day 1 and persisting up to day 14; fig 1). In the adult burned rats receiving AM251 intrathecally, mechanical allodynia that was seen at day 1 postburn was significantly attenuated by day 3. This beneficial effect of AM251 on allodynia was maintained until day 14 after burn even after termination of AM251 on day 7 (fig 1).
When thermal hyperalgesia (thermal withdrawal response) was tested in adult rats, statistically significant ($P < 0.05$) nociceptive behavior was observed in the vehicle group on the ipsilateral (ipsi) compared with contralateral (contra) side. Intrathecal AM251 treatment, started immediately after burn and continued daily for 7 days, attenuated the allodynia starting at 3 days. The beneficial effect of AM251 lasted up to 14 days beyond the infusion period of AM251.

**Younger Rats.** A group of younger naive animals (two measurements per rat, four rats per group) that received 1 mg/kg AM251 showed no differences in pain responses or gait compared with vehicle-treated animals. In other words, AM251 had no beneficial or harmful effects on mechanical allodynia.
nonpathologic states. Statistical difference was not observed in the nociceptive responses in the vehicle-treated contralateral side and AM251-treated (1 or 0.1 mg/kg) contralateral sides. In the rats with burn receiving intraperitoneal vehicle treatment (two measurements per rat, six rats per group for each time period), significant ($P < 0.05$) mechanical alldynia was observed on the ipsilateral side throughout the experimental period (day 1 to 14; fig. 3). In the group receiving 1 mg/kg AM251 intraperitoneally (two measurements per rat, six rats per time period), mechanical alldynia was attenuated at days 1, 3, 5, 7, and 14 days after burn compared with contralateral side (fig. 3). Thus, the beneficial effects of AM251 (1 mg/kg intraperitoneally) persisted up to day 14 postburn, even though the drug was stopped at day 7 after burn. The younger rats receiving 0.1 mg/kg AM251 (two measurements per rat, six rats for each time period) demonstrated alldynia on the ipsilateral side, comparable with that of the burned-injured rats in the vehicle group, throughout the observation period of 1 to 14 days (fig. 3).

When thermal hyperalgesia was tested in the younger rats, the ipsilateral burn side showed exaggerated nociceptive responses at 3, 5, and 7 days (fig. 4). The thermal hyperalgesia exhibited at days 3, 5, and 7 had spontaneously reversed to normal by day 14 (fig. 4). The hyperalgesic responses observed previously at days 3, 5, and 7 were completely reversed by AM251 (1 mg/kg) treatment (fig. 4). The rats receiving 0.1 mg/kg AM251 continued to demonstrate thermal hyperalgesia on the ipsilateral side at days 3, 5, and 7 (fig. 4).

**Immunoreactivity**

The immunoreactivity studies were performed only in the younger rats at days 3, 7, and 14 days after burn. The Iba1 immunoreactivities between sham burn and contralateral side of burn were not different ($n = 3$ for burn, $n = 3$ for sham burn).
for sham burn for each time point). After burn injury, the dorsal horn immunoreactivity of Iba1 (microglial activity) on the ipsilateral side significantly ($P < 0.05$) increased 18.5 ± 7.5 and 12.3 ± 1.6 fold compared with contralateral dorsal horn at 7 and 14 days, respectively. The expression of Iba1 between 7 and 14 days did not differ. Representative fluorescence images for each time period demonstrating the activation of Iba1 is shown (fig. 5). The immunoreactivity of GFAP reflecting astroglial activity ($n = 4$ for burn, $n = 4$ for sham burn at each time point of 3, 7, and 14 days) showed no differences in activities between sham burn and contralateral side of burn. The astroglial activity (GFAP expression) on the ipsilateral side relative to contralateral side showed a 2.9 ± 0.3 fold increase ($P < 0.05$) at day 7 only (fig. 6).

In view of the increased microglial and astroglial activities at day 7 after burn and improvement of nociceptive behaviors when treated with AM251 at day 7, we examined whether treatment with AM251 would alter glial activities. The administration of AM251 to the burned-injured animals did not change the activities (intensities) of Iba1 on the ipsilateral and contralateral side when examined at day 7 after burn (fig. 7). Similarly, although the (astroglial) activity was increased at day 7 after burn, the administration of AM251 (1.0 mg/kg for 7 days) did not change the expression of GFAP on burned, contralateral, and also in naïve mice) when compared with vehicle treatment (fig. 6).

**Discussion**

The current study demonstrates that (1) burn injury to adult rats leads to hyperalgesic and allodynic responses on the ipsilateral side of burn compared with the contralateral side; (2) postburn intrathecal treatment of adult rats with the CB1R antagonist AM251 results in partial or complete reversal of the altered nociceptive behaviors during and beyond the period of infusion; (3) younger rats with burn injury showed nociceptive responses similar to that of adult animals; (4) interaperitoneal treatment of young rats with AM251 started immediately after burn also attenuated or reversed the nociceptive (hyperalgesia and allodynia) behaviors induced by burn injury; (5) burn injury results in an increase in activity of microglia at days 7 and 14 after burn evidenced by increased Iba1 immunoreactivity; (6) the astroglial activity, reflected by GFAP expression, was up-regulated only at day 7 after burn; (7) reversal of the nociceptive behaviors was not associated with quantitative changes in the activities of microglia or astroglia; and (8) in all rats, the contralateral nociceptive responses were not affected by the administration of AM251, when compared with preburn and untreated contralateral side nociceptive responses. Although many studies have documented the utility of cannabinoids agonists to attenuate pain, the results of this study indicate that endogenous cannabinoids may have an unexpected pro-nociceptive effect during the development of burn-induced pain.

**Fig. 6.** Increased glial fibrillary acidic protein expression at 7 days after burn is unaltered by treatment with AM251. Astroglial activity on the spinal cord dorsal horn was determined using fluorescent glial fibrillary acidic protein antibody at day 7. Groups studied ($n = 4$ per group) included sham burn treated with vehicle (Sham + Veh), ipsilateral side of burn treated with vehicle (Burn + Veh), contralateral side to burn treated with vehicle (Contra + Veh), ipsilateral to burn treated with AM251 (Burn + AM251), and contralateral to burn treated with AM251 (Contra + AM251). Scale bars for A and B indicate 100 μm and for Ca, Cb, Da, and Db indicate 25 μm. Burn injury significantly increases astroglia activity 2.9 ± 0.3 fold ($P < 0.05$) on the ipsilateral side compared with contralateral side at day 7. Treatment with AM251 does not change the activity-expression of astroglia on the ipsilateral side compared with vehicle treatment of ipsilateral side ($n = 4$ per group).
pain. This may explain why inhibition of endogenous CB1R activity by an antagonist, AM251, results in improvement of nociceptive behaviors.

Immunoreactivities of astroglia (by fluorescent GFAP) and microglia (by fluorescent Ibal) were examined at 3, 7, and 14 days after burn. Increased immunoreactivity of microglia was observed at days 7 and 14 after burn. The increased immunoreactivities of microglia after burn injury were consistent with the observations of others that immunoreactivity of microglia is increased in neuropathic syndromes and/or inflammation of central nervous system.28–30 The activity of astroglia was up-regulated at day 7 after burn. Nociceptive behaviors, however, were seen at day 3 although astroglial activity was not increased at this time. Although allodynia was observed at day 14, the hyperalgesia had reverted to normal by this time in association with normalization of the increased astroglial activity observed at day 7. This disparate relation between astroglial expression and pain behaviors has been observed previously in other pain syndromes.31,32 There can be a dissociation between pain and microglial activation also.33,34 Once increased glial activity occurs, the altered activity can persist even after recovery from nociceptive pain behaviors, as observed for microglia and hyperalgesia at day 14.31–34 Thus, altered glial activities may reflect the presence or persistence of a pathologic state rather than reflection of a type of nociceptive behavior.

CB1R antagonist, AM251, has been shown to directly inhibit the increase in glial calcium levels, which can lead to glutamate release and activation of N-methyl d-aspartate receptors.35 Thus, inhibition of endogenous C1BR by AM251 may decrease the activation of calcium-induced activation of N-methyl d-aspartate receptors. There is usually, but not always, a close relation between glial cell activation and cytokine levels in pathologic pain conditions.36,37 In fact, localized burn injury, similar to the model described in this study, resulted in the activation of cytokines.38 Spinal intrathecal injection of antisense oligonucleotides for gp130, a receptor subunit shared by members of the interleukin-6 family of cytokines, attenuated burn-induced pain.38 In view of the close relation between glial cell expression and cytokine levels,32,33 and the lack of changes in glial expression after administration of AM251, cytokine expression with and without AM251 treatment was not examined in the current study. In our study, the reversal or attenuation of the nociceptive behaviors in the absence of changes in glial cell activities suggests that the actions of AM251 may not be at the glial level, but further downstream. Pharmacological examples of reversal of deleterious effects of activated signaling proteins without altering their expression has been described previously after burn injury and other pathologic states.39–42 For example, inhibitors of glycogen synthase kinase-3β and inducible nitric oxide synthase reversed the...
deleterious actions of these signaling proteins without altering their expression.\textsuperscript{39–42} In other words, the deleterious effects of activated proteins can be modulated without altering their expression but by altering their downstream signaling effects.

AM251 was effective during intrathecal and intraperitoneal routes. These beneficial effects on nociception had a latent period of onset (2 days), but persisted after termination of AM251 administration at 7 days. From a clinical perspective, the ability to reverse nociceptive behaviors when administered by interperitoneal route is practically more relevant to the burned patient where intrathecal administration may be more restricted because of the infection potential when drugs are administered into the central nervous system of immunocompromised burned patients. The utility of agonists of CBIR for pain treatment has been documented in many previous studies. As observed in this study, if the use of CBIR antagonists proves to be useful in additional studies, this could be a new strategy to modulate the development of acute or chronic burn pain or even burn-induced hyperalgesia. There are several ongoing clinical trials on the use of oral CB1R antagonists to treat various pathologic states (NCT00603109, NCT00588731). These clinical trials are testing the utility of CBIR antagonist for nonpain conditions such as appetite, weight control, and cognitive dysfunction. Based on efficacy and safety reports from these trials, the potential use of endocannabinoid antagonists to treat burn-induced pain in humans would be of great interest to burn care physicians and related medical personnel. Thus CBIR antagonists, would be a novel, previously unreported, application to treat pain, specifically burn pain. This bench observation in rats possibly may have almost immediate application to the bedside, provided those clinical trials enumerated above provide proof of safety in humans.

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Competing Interests

The authors declare no competing interests.

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