Research Article

MOLECULAR PAIN

Molecular Pain
Volume 13: 1–9

DOI: 10.1177/1744806917713907
journals.sagepub.com/home/mpx
SAGE

Differential involvement of reactive oxygen species in a mouse model of capsaicin-induced secondary mechanical hyperalgesia and allodynia

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Abstract

Intradermally injected capsaicin induces secondary mechanical hyperalgesia and allodynia outside the primary (i.e., capsaicin-injected) site. This secondary mechanical hypersensitivity is attributed to central sensitization in which reactive oxygen species (ROS) play a key role. We examined whether ROS would be differentially involved in secondary mechanical hyperalgesia and allodynia using a mouse intraplantar capsaicin injection model. In mice, capsaicin-induced secondary mechanical hyperalgesia outlasted its allodynia counterpart. Unlike the hyperalgesia, the allodynia was temporarily abolished by an anesthetic given at the capsaicin-injected site. The ROS scavenger phenyl-N-tert-butylnitrone slowed the development of both secondary mechanical hyperalgesia and allodynia when administered before intraplantar capsaicin injection, whereas it inhibited only the allodynia when administered after capsaicin had already induced secondary mechanical hyperalgesia and allodynia. Intrathecal injection of the ROS donor KO_2 induced both mechanical hyperalgesia and allodynia with the former outlasting the latter. Metformin, an activator of redox-sensitive adenosine monophosphate-activated protein kinase, selectively inhibited capsaicin-induced secondary mechanical allodynia and intrathecal KO_2-induced mechanical allodynia. These results suggest that ROS is required for rapid activation of central sensitization mechanisms for both secondary mechanical hyperalgesia and allodynia after intraplantar capsaicin injection. Once activated, the mechanism for the hyperalgesia is long-lasting without being critically dependent on ongoing afferent activities arising from the capsaicin-injected site and the continuous presence of ROS. On the contrary, the ongoing afferent activities, ROS presence and adenosine monophosphate-activated protein kinase inhibition are indispensable for the maintenance mechanism for capsaicin-induced secondary mechanical allodynia.

Keywords

Hyperalgesia, allodynia, reactive oxygen species, central sensitization, capsaicin

Date received: 14 March 2017; revised: 9 May 2017; accepted: 11 May 2017

Introduction

Central sensitization is defined as an increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input (see IASP taxonomy webpage: http://www.iasp-pain.org/). Its role in mechanical hyperalgesia (increased pain from a mechanical stimulus that normally provokes pain) and mechanical allodynia (pain due to a mechanical stimulus that does not normally provoke pain) has been readily demonstrated in experiments where an intradermal capsaicin injection induces mechanical hyperalgesia and allodynia in the ''secondary'' skin area surrounding the primary (i.e., capsaicin-injected) site. The capsaicin-induced secondary mechanical hyperalgesia and allodynia occur with an increase

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in responsiveness to mechanical stimuli of spinal cord dorsal horn projection neurons but without a change in that of mechanosensitive peripheral afferents innervating the secondary skin area.[1–5]

The capsaicin-induced secondary mechanical hyperalgesia and allodynia in humans differ in several ways besides the obvious differences in the stimulus intensity provoking them and the type of sensory fibers involved. While the allodynia is resolved in ~2 h and nearly abolished by blocking ongoing nerve activities at the capsaicin-injected primary site, the hyperalgesia is long-lasting (~20 h) and not strongly inhibited by the nerve block at the primary site.[2] This suggests that capsaicin-activated central sensitization mechanism for secondary mechanical hyperalgesia may be different from that for secondary mechanical allodynia.

Reactive oxygen species (ROS) were shown to play a critical role in central sensitization mechanism(s) activated by intradermal capsaicin. Specifically, ROS scavengers such as phenyl-N-tert-butylnitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl were shown to reverse the capsaicin-induced decrease in foot withdrawal mechanical threshold[6] and attenuate the capsaicin-induced increase in foot withdrawal responses to innocuous punctate mechanical stimulation in the secondary zone.[7,8] Collectively, these studies suggest that ROS are important molecular components in central sensitization mechanism for capsaicin-induced secondary mechanical allodynia.

Since capsaicin-induced secondary mechanical hyperalgesia is distinct from its allodynia counterpart, a question is raised as to whether ROS would be differentially involved in the two. We addressed this question by first reverse-translating the human capsaicin studies to animal studies in order to examine secondary mechanical hyperalgesia and allodynia separately in mice. Then, we tested the effect of an ROS scavenger on mouse behaviors resembling the capsaicin-induced secondary mechanical hyperalgesia and allodynia in humans. Portions of data in this study have been reported in abstract form.[9]

Materials and methods

Animals

Male C57BL/6 N mice (9–12 weeks, Charles River, Wilmington, MA) were used throughout this study. The mice were housed on a 12–12 h light–dark cycle in AAALAC (Association for the Assessment and Accreditation of Laboratory and Care International)-accredited animal facility with standard bedding and free access to food and water. All experimental procedures using animals were done according to the guidelines of the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

Behavioral tests

Mice were placed on an elevated metal grid and mechanically stimulated by applying punctate stimulation on the hind paw plantar skin between the third and fourth toe bases with von Frey filaments (VFFs, Figure 1(a)). Since hyperalgesia reflects an increased response at a normal pain threshold and allodynia indicates pain evoked by a stimulus that is normally not painful, mechanical stimulation at and below normal nociceptive threshold intensity can be used after intradermal capsaicin injection to examine secondary mechanical hyperalgesia and allodynia, respectively. In rodents, a nociceptive response threshold to punctate mechanical stimulation applied to a paw is commonly measured as a 50% foot withdrawal threshold.[10,11] Adopting this concept, we first identified a VFF that reliably evoked 40% to 60% foot withdrawals in individual mice at baseline (VFF-high); 9.8 mN (VFF #4.08) was chosen in most cases with some exceptions where 13.7 mN (VFF #4.17) had to be used to produce 40% to 60% responses at baseline. Next, we chose another VFF (VFF-low) to deliver mechanical stimulation at "below threshold (~1/10 force of VFF-high; 1.0 mN and 1.6 mN)"

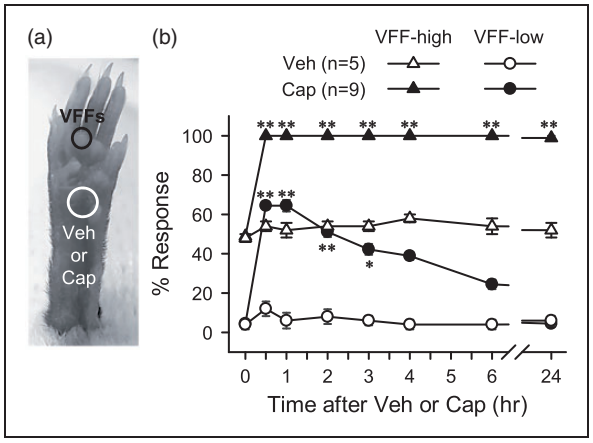

Figure 1. Capsaicin-induced secondary mechanical hyperalgesia and allodynia in the mouse. (a) Three μl of 0.1% capsaicin (Cap) or its vehicle (Veh) was injected at the center of the hind paw (white circle). Mechanical stimulations with two von Frey filaments (VFFs; VFF-high or -low) were applied outside the Cap-injected site, between the third and fourth toe bases (black circle). (b) Before the Cap injection (0 h), VFF-high evoked 49 ± 1% (n = 9) foot withdrawal responses, and VFF-low (delivering ~ 1/10 force of VFF-high), 4 ± 2% (n = 9) responses. Intraplantar Cap injection increased the foot withdrawal responses to VFF-high and VFF-low, indicating the development of secondary mechanical hyperalgesia and allodynia, respectively. At 24 h post-Cap injection, the hyperalgesia persisted, while the allodynia was completely resolved. *Adjusted $p < 0.05$ and ** adjusted $p < 0.01$ versus baseline (0 h) in each group by Friedman repeated measure test followed by post hoc Dunn's test.

intensity. VFF-low evoked no apparent foot withdrawals at baseline. The stimulation intensities of VFF-high are above the highest median value (6.8 mN) of mechanical thresholds (range: 2.0–13.9 mN) in Aδ/C-fibers innervating the hindlimb skin of C57BL/6 J[12] and C57BL/6 N[13] mice; in these mice, the highest median value of Aβ-fiber mechanical thresholds is 1.6 mN. The number of foot withdrawals in response to 10 stimuli with either VFF-high or VFF-low was measured before and after intraplantar capsaicin or intrathecal KO_2 injection and expressed as % response.

Intraplantar capsaicin injection

From 0.5% (wt/vol) capsaicin stock solution (dissolved in an absolute ethanol), 0.1% capsaicin solution was made fresh before each use by mixing the stock solution with Tween-80 and saline at 2:1:7 ratio. Three µl of 0.1% capsaicin solution was injected into the center of the hind paw (Figure 1(a)) under isoflurane (1.5%) anesthesia.

Drug administration

Bupivacaine (0.5%, 3 µl), the local anesthetic, was injected at the capsaicin injection site. PBN (50 mg/kg body weight), an ROS scavenger, and metformin (Met, 200 mg/kg body weight), an activator of adenosine monophosphate-activated protein kinase (AMPK), were given intraperitoneally (i.p.). KO_2 (100 or 200 mM), an ROS donor, was dissolved in artificial cerebrospinal fluid containing (in mM): 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 Glucose. Five µl of KO_2 was injected intrathecally by lumbar puncture between L5 and L6 vertebrae. All chemicals were purchased from Sigma-Aldrich (St. Louis, MA) unless noted otherwise.

Data analyses

Data are expressed as mean ± SEM with n, the number of mice. We performed both a priori and post hoc statistical tests. When % responses at multiple time points were compared with those at baseline in each group, Friedman repeated measures test followed by post hoc Dunn's multiple comparison test (vs. single control) was used. In case that paired % responses between two time points (before and after treatments) were compared, Wilcoxon signed rank test was used. Mann–Whitney U test was used when % responses to either VFF-high or VFF-low between vehicle- and drug-treated groups were compared at a given time point. For Dunn's multiple comparison test, adjusted p-values were used to determine statistical significance. Results were considered significant when $p < 0.05$.

Results

As in Figure 1(b), 0.5 h after intraplantar capsaicin injection, the foot withdrawal response to VFF-high increased from 49 ± 1% to 100% ($n = 9$, adjusted $p < 0.01$ by Dunn's test following Friedman test) and that to VFF-low, from 4 ± 2% to 64 ± 2% ($n = 9$, adjusted $p < 0.01$ by Dunn's test following Friedman test), showing the development of hyperalgesia- and allodynia-like mechanical hypersensitivity, respectively. While the increased response to VFF-low gradually decreased over the next 6 h and completely disappeared at 24 h post-capsaicin, the increased response to VFF-high was maintained throughout the 24 h testing period; the response to VFF-high found to be back to normal at 48 h post-capsaicin (51 ± 2%, $n = 9$). Injection of the vehicle of capsaicin (a mixture of ethanol, Tween-80 and saline at 2:1:7 ratio) did not significantly change the response to either VFF-high ($p = 0.49$ by Friedman test) or VFF-low ($p = 0.51$ by Friedman test) throughout the testing period.

We then tested whether the capsaicin-induced secondary mechanical hypersensitivity in mice would depend on ongoing nerve activities at the primary site. For this test, we first validated the duration and site-specificity of local bupivacaine (0.5%, 3 µl) effect in normal mice using their withdrawals from VFF-high stimulation as an indicator of intact sensation. A half-hour after the injection of bupivacaine at the center of hind paw, local anesthesia (absence of withdrawals from VFF-high) was apparent ($n = 4$, adjusted $p < 0.01$ by Dunn's test following Friedman test) only at the site, not at the toe bases. This local anesthesia faded out within 2 h (Figure 2(a)). Next, we injected bupivacaine (0.5%) at the primary zone (i.e., capsaicin-injected paw center) after capsaicin-induced secondary mechanical hypersensitivity had been established. The increased response to VFF-high in the secondary zone (toe bases) was not affected by the injection of bupivacaine at the primary zone (Figure 2(b)). In stark contrast, however, the increased response to VFF-low was completely abolished 0.5 h after the injection of local anesthetic. As the effect of bupivacaine was wearing off, the increased response to VFF-low gradually reappeared (Figure 2(b)). These data collectively indicate that, in response to intraplantar capsaicin injection, mice developed long-lasting secondary mechanical hyperalgesia whose maintenance is independent of ongoing afferent activity arising from the primary site, and secondary mechanical allodynia, relatively short-lasting and critically dependent on such afferent activity.

We next investigated whether interfering with ROS accumulation would hamper the induction of secondary mechanical hyperalgesia and allodynia. PBN, an ROS scavenger, did not alter the foot withdrawal response to either VFF-high (−0.5 h vs. 0 h in Figure 3; $p = 0.31$

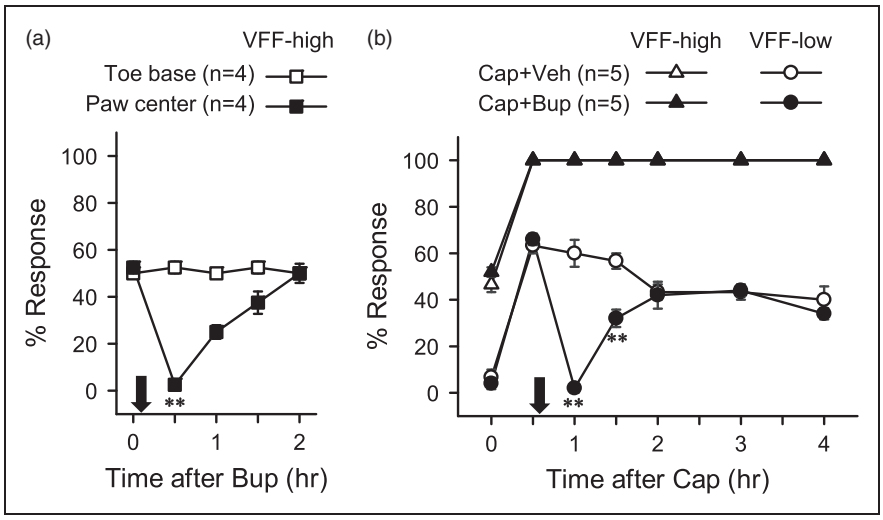

Figure 2. Effects of local anesthetic given at the capsaicin-injected site on capsaicin-induced secondary mechanical hyperalgesia and allodynia. (a) Three μl of 0.5% bupivacaine (Bup) was injected at the center of hind paw (marked by black arrow). The Bup injection produced a site-specific local anesthesia wearing off within 2 h. (b) Bup or its vehicle (Veh) was injected (marked by black arrow) at the capsaicin (Cap)-injected site (paw center) when Cap-induced secondary mechanical hyperalgesia (increased response to VFF-high) and allodynia (increased response to VFF-low) developed at toe bases. Bup abolished the allodynia but had no effect on the hyperalgesia. Responses at 0 h indicate baseline withdrawal responses to the two VFFs before the Cap injection. **Adjusted $p < 0.01$ versus baseline (0 h) in each group by Friedman repeated measure test followed by post hoc Dunn's test in (a); **$p < 0.01$ versus corresponding Veh by Mann–Whitney U test in (b).

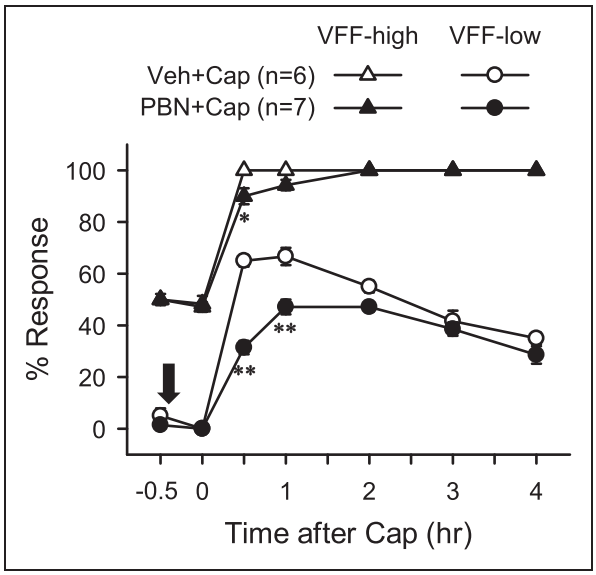

Figure 3. Effects of scavenging reactive oxygen species (ROS) on the development of capsaicin-induced secondary mechanical hyperalgesia and allodynia. When phenyl-N-tert-butylnitrone (PBN, 50 mg/kg body weight, i.p.), an ROS scavenger, was administered (marked by black arrow) before intraplantar capsaicin (Cap) injection, the rapid onset of capsaicin-induced secondary mechanical hyperalgesia (increased response to VFF-high) and allodynia (increased response to VFF-low) was inhibited. Responses at −0.5 h indicate withdrawal responses to the two VFFs before the injection of PBN or its vehicle (Veh). Those at 0 h indicate baseline responses before the Cap injection. *$p < 0.05$, **$p < 0.01$ versus corresponding Veh by Mann–Whitney U test.

by Wilcoxon test) or VFF-low ($p = 0.25$ by Wilcoxon test) prior to capsaicin injection. Immediately after measuring responses to VFF-high and VFF-low 0.5 h after PBN treatment (0 h in Figure 3), we injected capsaicin into the hind paw. Unlike vehicle-pretreated mice that showed rapid onset of capsaicin-induced secondary mechanical hyperalgesia and allodynia, PBN-pretreated mice showed a delayed peak of the hyperalgesia and allodynia (Figure 3), suggesting that ROS are necessary for the rapid development of capsaicin-induced secondary mechanical hyperalgesia and allodynia. In another set of experiments, PBN was administered after secondary mechanical hyperalgesia and allodynia had been already induced by intraplantar capsaicin. PBN significantly inhibited the allodynia but had no effect on the hyperalgesia (Figure 4). Administered at 24 h post-capsaicin when only secondary mechanical hyperalgesia was present, PBN still had no effect on it (data not shown).

As these results suggested that increased ROS level would be required for rapid development of capsaicin-induced secondary mechanical hyperalgesia and allodynia but differentially contributory to their maintenance, we hypothesized that a direct elevation of ROS level would result in behavioral consequences similar to those of intraplantar capsaicin injection. To test this hypothesis, we intrathecally administered a freshly made KO_2, an ROS donor, and measured foot withdrawal responses to VFF-high and VFF-low. KO_2 (100 mM) induced mechanical hyperalgesia (increased response to VFF-high) and allodynia (increased response

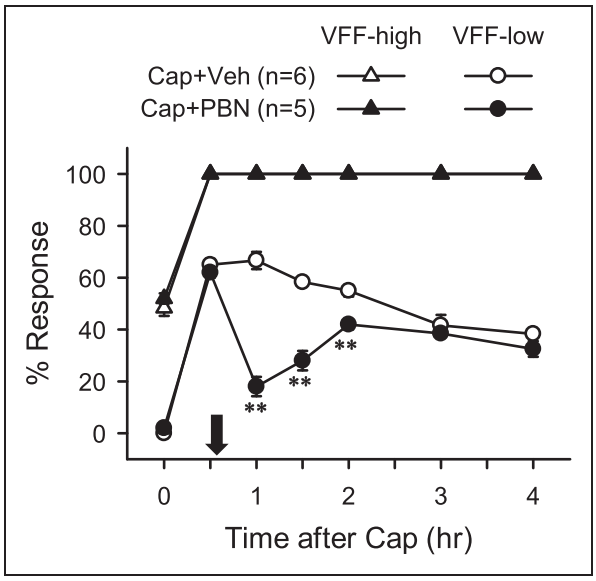

Figure 4. Effects of scavenging ROS on the maintenance of capsaicin-induced secondary mechanical hyperalgesia and allodynia. When phenyl-N-tert-butylnitrone (PBN, 50 mg/kg body weight, i.p.) was administered (marked by black arrow) after intraplantar capsaicin (Cap) had already induced secondary mechanical hyperalgesia (increased response to VFF-high) and allodynia (increased response to VFF-low), it only inhibited the allodynia, not the hyperalgesia. Responses at 0 h indicate baseline withdrawal responses to the two VFFs before the Cap injection. $^{**}p < 0.01$ versus corresponding vehicle (Veh) by Mann–Whitney U test.

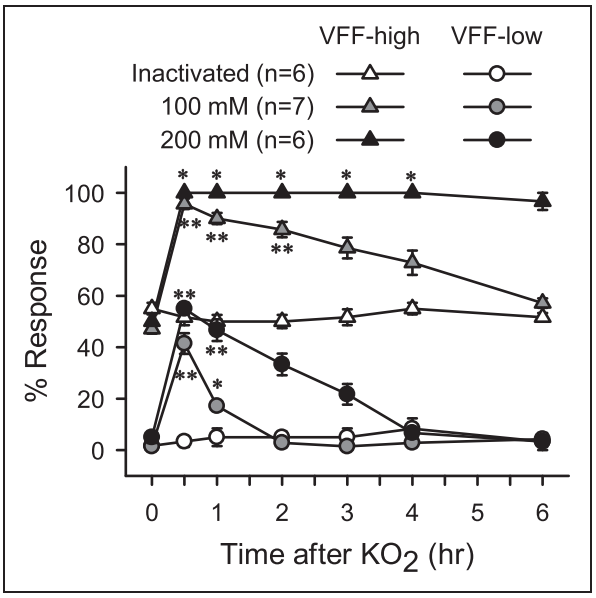

Figure 5. Mechanical hyperalgesia and allodynia induced by ROS donor. When 5 μl of freshly made KO_2 (100 or 200 mM), an ROS donor, was intrathecally injected, mice developed both mechanical hyperalgesia (increased response to VFF-high) and allodynia (increased response to VFF-low) with the former outlasting the latter. In contrast, inactivated KO_2 (incubated for 2 h at room temperature) had no effect on the foot withdrawal response to either VFF-high or VFF-low. Responses at 0 h indicate baseline responses before the intrathecal KO_2 injection. *Adjusted $p < 0.05$, ** adjusted $p < 0.01$ versus baseline in each group by Friedman repeated measure test followed by post hoc Dunn's test.

to VFF-low) with the former outlasting the latter. Mice that received a higher dose of KO_2 (200 mM) manifested a clearer separation between the time courses of mechanical hyperalgesia and allodynia (Figure 5). This mechanical hypersensitivity is unlikely to be due to the increased K^+ in the intrathecal space after KO_2 injection because inactivated KO_2 (200 mM, incubated for 2 h at room temperature to exhaust its ROS-liberating ability) did not alter mechanosensitivity ($p = 0.56$ by Friedman test for VFF-high; $p = 0.87$ for VFF-low in Figure 5).

We then attempted to study molecular components of ROS-mediated central sensitization underlying mechanical hyperalgesia and allodynia. AMPK is a redox-sensitive protein kinase[14–16] shown to be involved in various types of pathological pains.[17–20] We administered metformin (200 mg/kg body weight, i.p.), an AMPK activator, either before (Figure 6(a) and (b)) or after (Figure 6(c) and (d)) intraplantar capsaicin or intrathecal KO_2 injection. The metformin dose was chosen based on the literature demonstrating its analgesic action without an effect on normal nociception.[18,19] Regardless of administration timing, metformin selectively inhibited mechanical allodynia in mice that received intraplantar capsaicin or intrathecal KO_2 injection.

Discussion

This study demonstrates that capsaicin-induced secondary mechanical hyperalgesia and allodynia differ in ROS-related mechanisms. The findings in this study are: (1) capsaicin-induced secondary mechanical hyperalgesia outlasts its allodynia counterpart; (2) only the allodynia is abolished by blocking ongoing nerve activities at the capsaicin-injected primary site; (3) an ROS scavenger slows the development of both the hyperalgesia and allodynia; (4) an ROS scavenger inhibits already induced secondary mechanical allodynia but not the hyperalgesia; (5) an ROS donor itself induces long-lasting mechanical hyperalgesia and short-lasting mechanical allodynia; and (6) an AMPK activator alleviates ROS-mediated mechanical allodynia.

We acknowledge the limitations of translating animal withdrawal behaviors to pain/nociception and designating increases in such behavioral responses to high- and low-intensity stimulations as hyperalgesia and allodynia in the strict sense. In other words, the increased paw withdrawals from VFF-high and -low stimulations in the mouse could be regarded as two different types of mechanical hypersensitivity rather than mechanical hyperalgesia and allodynia. It being considered,

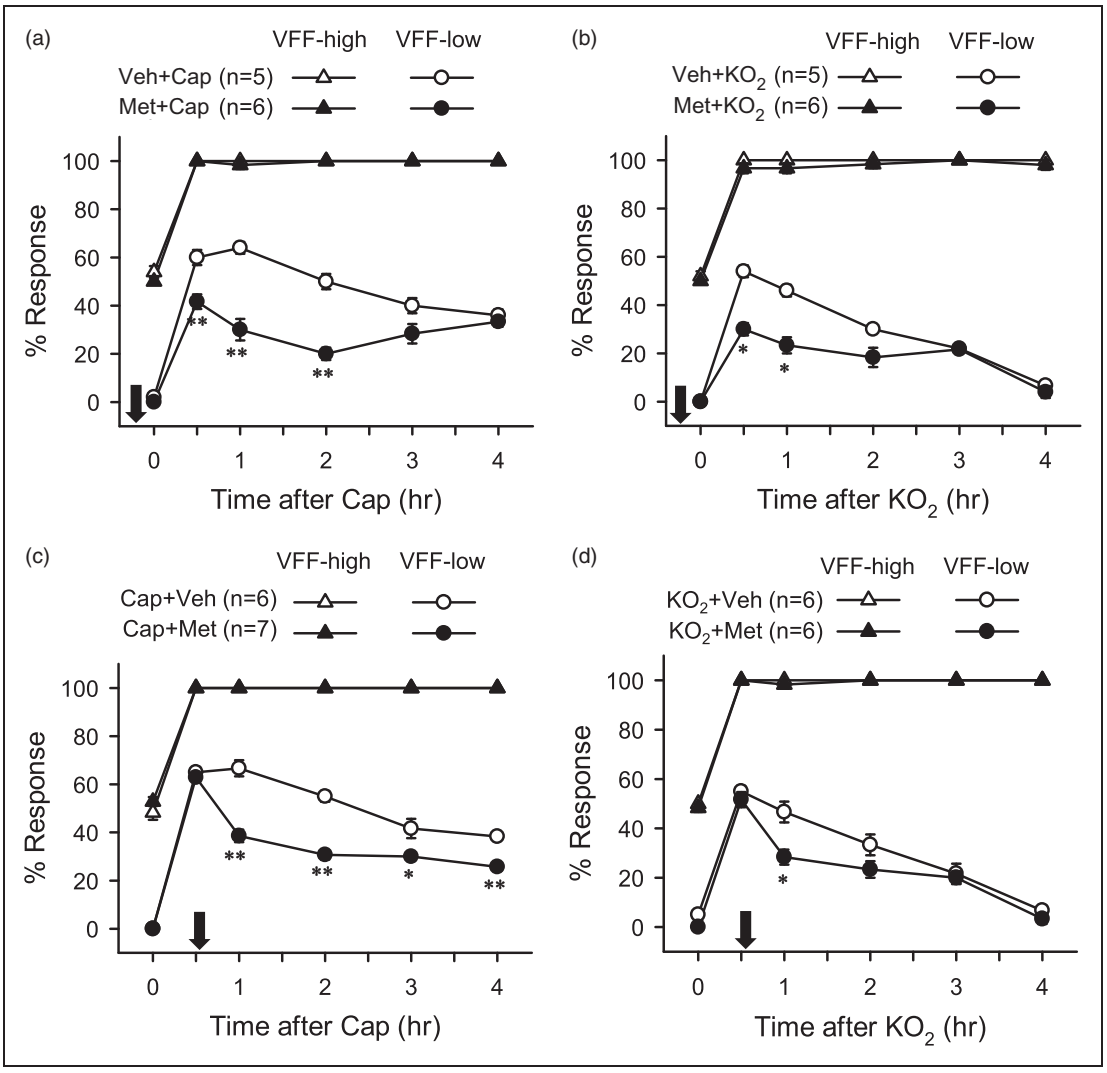

Figure 6. Effects of adenosine monophosphate protein kinase (AMPK) activator on mechanical hyperalgesia and allodynia. Administered (marked by black arrows) immediately before either (a) intraplantar capsaicin (Cap) or (b) intrathecal KO_2 (200 mM) injection, the AMPK activator metformin (Met, 200 mg/kg body weight, i.p.) selectively hampered the development of mechanical allodynia (increased response to VFF-low). When given 0.5 h after (c) Cap or (d) KO_2 injection, metformin alleviated mechanical allodynia only. Responses at 0 h indicate baseline responses before the injection of Cap or KO_2. *$p < 0.05$, **$p < 0.01$ versus corresponding vehicle (Veh) by Mann–Whitney U test.

however, that the VFF-high stimulation intensities (9.8–13.7 mN) are above the highest median value of mechanosensitive Aδ/C-fiber thresholds (6.8 mN) and the VFF-low, at that of Aβ-fiber thresholds (1.6 mN) in the skin of mouse hindlimb,[12,13] it seems that increased withdrawals to VFF-high and VFF-low in this study do reflect the mouse version of mechanical hyperalgesia (increased nociception from mechanical stimulus around the normal mechanical nociceptor threshold intensity) and allodynia (nociception from a stimulus below the normal mechanical nociceptor threshold intensity).

Previously we reported that intradermal capsaicin generates excess ROS in the spinal cord and the high level of ROS activates central sensitization mechanisms for the capsaicin-induced secondary mechanical allodynia.[7,8] Together with the previous reports, our current findings suggest that, once activated by increased ROS in response to intradermal capsaicin, the central sensitization mechanism for secondary mechanical hyperalgesia is long-lasting and independent of ongoing aberrant afferent activity arising from the primary site. In contrast, the ROS-activated central sensitization mechanism for secondary mechanical allodynia lasts for a short period of time, and thus it can be only sustained by continuous ROS generation by the ongoing aberrant afferent activity. The critical role of such afferent activity in the maintenance of capsaicin-induced secondary mechanical allodynia is further supported by the gradual

reappearance of the allodynia after the effect of the local anesthetic or the ROS scavenger had faded out. The reappeared allodynia later declined over the same time-course of secondary mechanical allodynia in vehicle-treated counterparts. These findings indicate that the degree of capsaicin-induced secondary mechanical allodynia, after local anesthesia or ROS scavenging wears off, is determined by the level of remaining afferent activities arising from the capsaicin-injected primary site at that time. Indeed, the dependency of mechanical allodynia on ongoing aberrant afferent activity was reported in other acute chemogenic pain and chronic neuropathic pain conditions. Gracely et al.[21] described four cases of complex regional pain syndrome in which peripheral lidocaine infiltration at the most painful foci completely abolished spontaneous pain and mechanical allodynia remote from the infiltration sites only for the duration of peripheral nerve blockade. Likewise, Koltzenburg et al.[22] found a strong correlation between the magnitude of ongoing pain and the intensity of mechanical allodynia in healthy human subjects that received topical application of mustard oil and in chronic neuralgia patients. In rats with L5 spinal nerve ligation (SNL), Sheen and Chung[23] and Yoon et al.[24] were able to abolish the SNL-induced mechanical allodynia by surgically or pharmacologically blocking ongoing aberrant afferent inputs coming through L5 dorsal root to the spinal cord.

While our experimental data on secondary mechanical allodynia and the above-mentioned literature emphasize the importance of unceasing peripheral sensory activity in the maintenance of pathological pain, the data on secondary mechanical hyperalgesia supports the existence of central long-term changes that could also underlie the ''chronic'' aspect of pathological pain. In this regard, it is noteworthy that after a brief experimental conditioning stimulation or natural noxious stimulation, spinal cord dorsal horn neurons show long-term potentiation (LTP) of excitatory synaptic strength.[25,26] Considering that such long-term synaptic plasticity mostly occurs at Aδ- and C-fiber synapses that are implicated in ''nociceptor'' inputs, one would expect a long-lasting hyperalgesia (increased pain from a normally pain-evoking stimulus) after a conditioning event. This expectation is in accordance with our observation that mechanical hyperalgesia, not tactile allodynia, lasted long after triggering events (intraplantar capsaicin or intrathecal KO_2 injection).

ROS have been found to be key molecules in long-term synaptic plasticity in the brain and spinal cord. For instance, ROS scavengers block the induction of LTP, while ROS-generating manipulations induce LTP by itself, in the hippocampus and spinal cord dorsal horn.[27–30] Because excess ROS were shown to be required for the rapid induction of capsaicin-induced secondary mechanical hyperalgesia and allodynia in our study, it could be that potentiation of excitatory synaptic strength by ROS is a common central sensitization mechanism for the secondary mechanical hyperalgesia and allodynia. However, only the synapses for nociceptive inputs, not the ones for touch sensory inputs, could become long-term potentiated, resulting in the differential contribution of ROS to the maintenance of mechanical hyperalgesia and allodynia. It is our future research interest to investigate whether the duration of ROS-induced synaptic potentiation differs between Aδ/C-high threshold fiber (nociceptive) and Aβ/Aδ-low threshold fiber (touch sensory) synapses on the spinal cord dorsal horn neurons. Comparison of the response profiles of the two synapse types to ROS might lead to identification of cellular machinery that converts LTP into short-lasting potentiation or vice versa.

It remains to be elucidated what signaling molecules are downstream of excess ROS accountable for pathological pains. In this regard, AMPK deserves a notion because it is a redox-sensitive protein kinase whose activity is either increased or decreased by high levels of ROS.[14–16] In pathological pain conditions, AMPK activators show a potent analgesic effect on neuropathic and post-surgical mechanical allodynia[17–19] in which ROS play a significant role.[31–33] Conversely, conditional knockout of AMPKα1 gene per se increases excitatory synaptic activities in the spinal cord superficial dorsal horn neurons and reduces mechanical threshold in an ROS-dependent manner.[34] Aligned with these reports, the AMPK activator metformin inhibited mechanical allodynia induced by intraplantar capsaicin or intrathecal KO_2 injection in the present study. That only mechanical allodynia development was significantly affected by metformin pretreatment suggests that metformin-sensitive cellular machinery including AMPK is downstream of ROS signaling involved only in central sensitization mechanism for mechanical allodynia, further demonstrating the difference between ROS-induced mechanical hyperalgesia and allodynia in molecular mechanisms. We are aware that caution is needed in interpreting the metformin data as it could affect cellular energy state independently of AMPK activation.[35,36] Future studies are warranted to look into multiple cellular pathways regulated by ROS in the context of pathological pain.

In conclusion, the results of present study suggest that central sensitization mechanisms for capsaicin-induced secondary mechanical hyperalgesia and allodynia are different. Although both require a high level of ROS to be rapidly activated, the mechanism for secondary mechanical hyperalgesia is maintained long-term without being critically dependent on ROS-generating ongoing afferent activities arising from the capsaicin-injected primary site. On the other hand, ongoing aberrant afferent activities and the concomitant ROS generation are indispensable

for the maintenance mechanism for secondary mechanical allodynia. Thus, this study supports the existence of two chronic pain-underlying central sensitization mechanisms involving excess ROS, one critically dependent on ongoing aberrant afferent activity and the other not.

Author contributions

J-HL: study design, data analysis/interpretation, and manuscript drafting. JW: data acquisition, data analysis/interpretation, and manuscript revision. AB: assisting experiments, data interpretation, and manuscript revision. HSS and CB: data interpretation and manuscript revision. JMC: research funding acquisition, data interpretation, and manuscript revision. J-HL and JW contributed equally to this work.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by a NIH grant R01 NS031680.

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