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Tonic Modulation of Nociceptive Behavior and Allodynia by Cannabinoid Receptors in Formalin Test in Rats

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Abstract

Cannabinoids produce anti-nociceptive and anti-hyperalgesic effects in acute, inflammatory and neuropathic pain models. The current study investigated the role of cannabinoid (CB1 and CB2) receptors in modulating formalin-induced nociceptive behavior and mechanical allodynia in the rat. Rats received subcutaneous plantar injections of 5% formalin in the hind paws. Licking, biting and paw flinching nociceptive behaviors were measured 0-60 min after formalin injection. Allodynia was measured at 3 and 6 h, and 1, 3, 5 and 7 days post-injection using the mechanical paw withdrawal threshold. Animals in the experimental group were given i.p. injections of CB1 and CB2 receptor antagonists AM281 and AM630 at a dose of 1 mg/kg concomitant with formalin, and then twice daily for the following 7 days. AM281 and AM630 enhanced nociceptive behaviors, and attenuated the bilateral mechanical paw withdrawal threshold, compared with the vehicle. The results indicate that CB1 and CB2 receptors mediate a tonically inhibitory action on formalin-induced inflammatory pain, especially long-term allodynia, in bilateral hind paws.

Key Words: allodynia, cannabinoid receptor, formalin test, inflammatory pain, rat, tonic modulation

Introduction

Inflammatory pain and hyperalgesia are two of the most common clinical conditions affecting an individual's quality of life. Inflammatory pain and hyperalgesia involve enhanced responses of nociceptive neurons to noxious stimuli (hyperalgesia) or painful responses to innocuous stimuli (allodynia) after injury or inflammation (17). Persistence of inflammatory stimulators from acute inflammatory pain could lead to chronic inflammatory pain. Subcutaneous formalin injection is one well-known model for the study of acute and inflammatory pain mechanisms, and is also used to evaluate the analgesic action of various endogenous and exogenous anti-nociceptive molecules. The response to formalin injections is characterized by two phases of nociceptive responses. The early phase (phase 1) begins immediately after injection and lasts for 5 min. After a short quiescent period of about 10 min, a prolonged tonic response ensues, persisting for over 45 min (phase 2) with a peak response approximately 30 min after injection (30, 32). Recently, it was shown that subcutaneous injection of formalin also produced a delayed and long-term third phase of thermal hyperalgesia and mechanical allodynia (12, 24, 28). However, the mechanism for the chronic inflammatory pain

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that is induced by subcutaneous formalin injection is not clear.

Cannabinoids are currently attracting clinical attention as an alternative or as an adjunct to opioids in treating chronic pain (17). Activation of type 1 and 2 cannabinoid (CB1 and CB2) receptors has been reported to produce anti-nociceptive effects in a neuropathic pain model (2, 13, 23, 31). Anandamide and 2-arachidonoylglycerol, two main endocannabinoids, were shown to potentiate the effects of analgesia induced by stress and fear (7, 14). In addition, anti-nociceptive effects can result from the inhibition of endocannabinoid reuptake or from the inhibition of degradation in neuropathic pain (10, 18). These results imply that endocannabinoids may be involved in modulating neuropathic or injury-induced pain.

Cannabinoids may be involved in depressing the formalin-evoked phase 1 and/or phase 2 nociceptive responses (1, 6, 8, 16, 19). Some studies have reported hyperalgesia in response to systemically administered cannabinoid receptor antagonists (17, 18), whereas others have reported evidences against a role for the endocannabinoid system in the tonic inhibition of pain (5, 6, 19). However, evidence for the role of endocannabinoids in formalin-evoked long-term allodynia is missing. Therefore, it has remained uncertain whether the endocannabinoids could cause tonic activation of the cannabinoid receptors and participate in the intrinsic control of pain initiation, and subsequent allodynia induced by formalin injection. To address this issue, the present study examined the effects of co-administration of CB1 and CB2 receptor antagonists on formalin-evoked nociceptive behavior and delayed long-term mechanical allodynia in the rat.

Materials and Methods

Animals and Drug Treatments

Twenty-four adult male Sprague-Dawley rats (200-250 g) were used for the experiment, n = 6 per study group. The rats were sourced from the Experimental Animal Center, Gansu College of Traditional Chinese Medicine, P. R. China. Experiments protocols were approved by the Institutional Animal Care Committee of Lanzhou University, and were in accordance with the guidelines of the International Association for the Study of Pain (33) and all efforts were made to minimize the number of animals used and their suffering. All rats were housed at room temperature (22°C) on a 12-h light/dark cycle, and given food and water *ad libitum*.

The rats received a 50-μl subcutaneous injection of 5% formalin into the plantar surface of the right hind paw (30, 32). Nociceptive behaviors were measured from 0 (immediate) to 60 min after formalin injection, while allodynia was measured at 3 and 6 h, and 1, 3, 5 and 7 days post-formalin injection (27). Animals in the experimental group were treated with i.p. injections of CB1 and CB2 cannabinoid receptor antagonists, AM281 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide; Tocris, Biosci., Bristol, UK) and AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1H-indol-3-yl](4-methoxyphenyl) methanone; Tocris), at a dose of 1 mg/kg each in 500 μl. The dose of 1 mg/kg for AM281 and AM630 was chosen on the basis of our preliminary experiments and previous literature data (2). The mixture of AM281 and AM630 or their vehicle (12% DMSO, i.p.) was given 20 min prior to the formalin injection and then twice daily (at 10:00 h and 16:00 h) for the following 7 days.

Acute Nociceptive Behavior

Animals were placed in a clear plastic box (16 × 10 × 8 cm) with a mirror below the floor surface to allow an unobstructed view of the paws. To allow familiarization with surroundings, individual rats were habituated in the test chambers for 20 min daily for 3 days. On the day of testing, following 5% formalin injections, rats were immediately placed in the observation chamber. The response to formalin injection was monitored by two experimenters as reported previously (30, 32). Measurements included total duration of licking/biting and the incidence of flinching of the injected hind paw per 5 min in the 60-min observation period.

Secondary Mechanical Allodynia

The experimental treatment protocol used for allodynia was the same as for nociception behaviors (above). Animals were acclimated to their testing boxes (transparent Plexiglass boxes of 46 × 30 × 16 cm) placed on a raised metal mesh grid for 30-60 min. The mechanical paw withdrawal threshold (PWT) in the bilateral hind paws was measured using the up-down method (9). A series of eight von Frey monofilaments (Stoelting, Inc., Wood Dale, IL, USA) were applied to the lateral aspect of the plantar surface of the hind paw. First, an intermediate monofilament (number 4.31, exerting ~2.0 *g* of force) was applied with enough force to cause the monofilament to bend. In the case of a positive response (rapid withdrawal of the paw within 6-8 s), a smaller filament was tested. If there was no withdrawal response (negative), the next larger force was delivered. Positive and negative responses were recorded and a 50% threshold was determined using the formula according to Chaplan *et al.* (1994). If the PWT measured by von Frey fila-

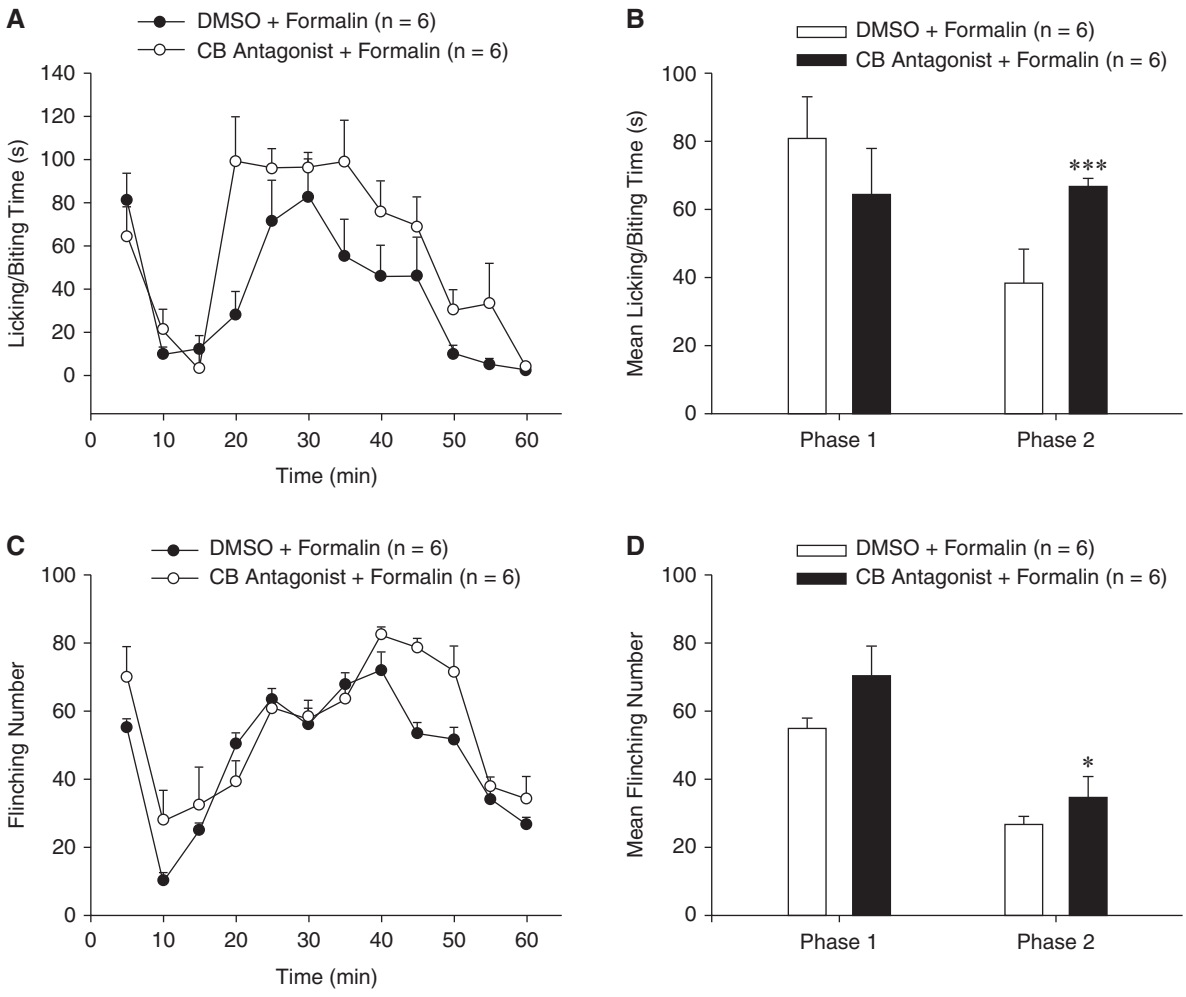

Fig. 1. CB receptor blockade facilitated formalin-induced nociceptive behaviors during the acute stage. Time-course plots showing the facilitated effects of i.p. administration of CB receptor antagonists (AM281 and AM630) on formalin-evoked licking/biting (A) and flinching behaviors (C). Mean licking/biting time (B) and flinching number (D) in phase 1 and phase 2 are shown. *$P < 0.05$ and ***$P < 0.001$ compared with DMSO vehicle group.

ments after formalin injection was significantly smaller than the baseline value before injection, mechanical allodynia was judged as having occurred.

Statistical Analysis

All data are presented as mean ± SEM Statistical differences in total observation time and at each time point were tested between treatment groups using a two-way analysis of variance (two-way ANOVA), followed by a *post-hoc* multiple comparison (Bonferroni *t*-test). $P < 0.05$ was considered to be statistically significant. SigmaStat software v.3.5 (Systat Software Inc., San Jose, CA, USA) was used to perform all statistical analyses.

Results

Blockage of CB Receptors Facilitated Acute Stage Nociceptive Behavior

Intraplantar injection of 5% formalin induced typical biphasic nociceptive behaviors (flinching and licking/biting of the injected paw). Administration of the CB antagonist vehicle (12% DMSO, 500 μl, i.p.) did not alter the formalin-induced nociceptive behaviors from that of formalin only (data not shown). Concomitant injection of AM281 and AM630 (1 mg/kg each, 500 μl) 20 min prior to the formalin injection significantly increased the formalin-induced nociceptive behaviors compared with the vehicle ($F_{(1, 120)} = 14.709$, $P < 0.001$ for licking/biting response; $F_{(1, 120)} = 20.336$, $P < 0.001$ for flinching response) and across time ($F_{(11, 120)} = 12.490$, $P < 0.001$; $F_{(11, 120)} = 14.355$, $P < 0.001$), but not for their interaction ($F_{(11,120)} = 1.658$, $P = 0.091$; $F_{(11, 120)} = 1.292$, $P = 0.237$) (Fig. 1). Further analyses of this acute response revealed that the increased nociceptive behaviors induced by

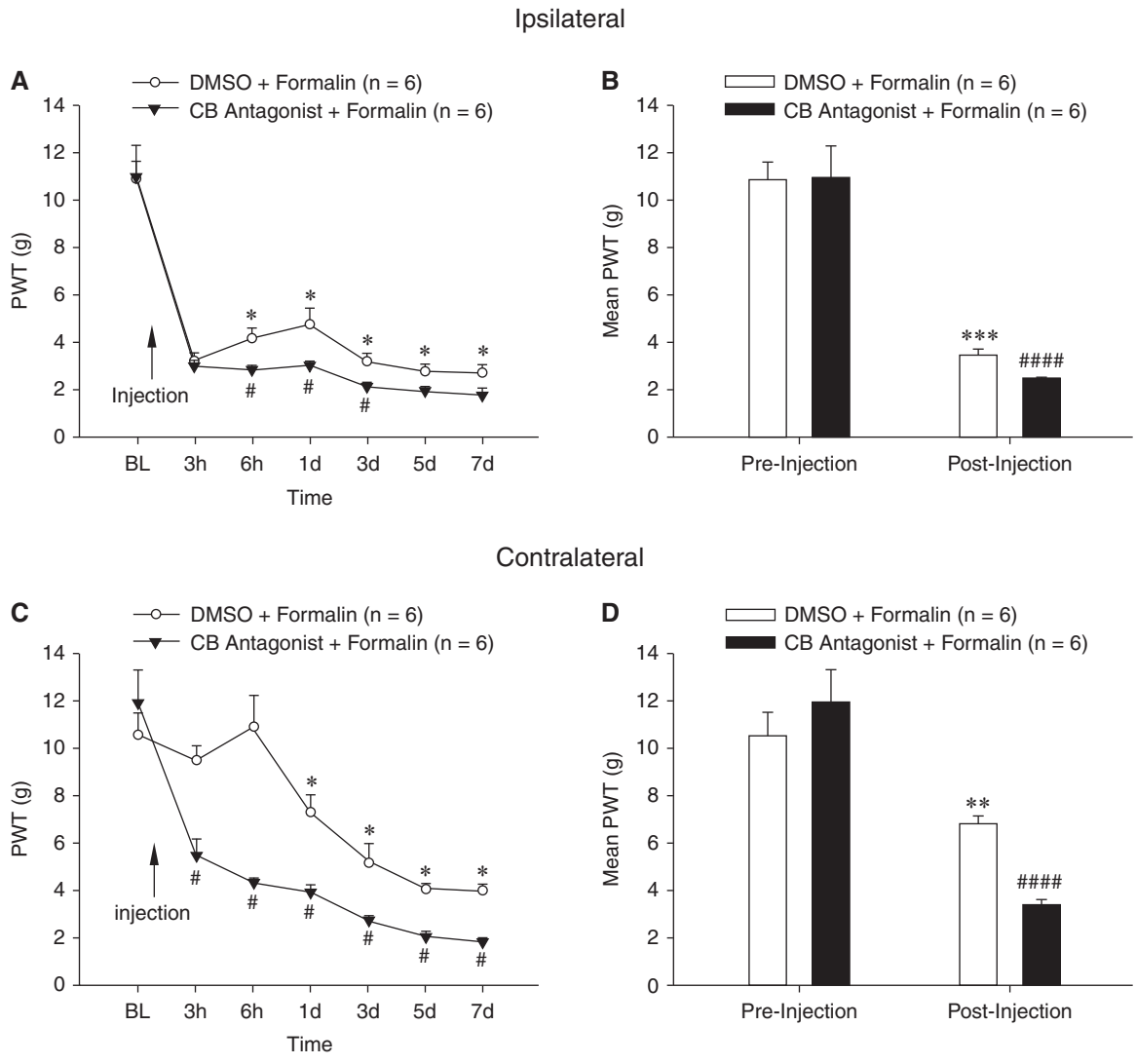

Fig. 2. CB receptor blockade facilitated formalin-induced mechanical allodynia during the late stage. Time-course plots showing i.p. administration of CB receptor antagonists (AM281 and AM630) further reduced the decreased paw withdrawal threshold (PWT) evoked by formalin injection in ipsilateral (A) and contralateral hind paws (C). Mean PWT in ipsilateral (B) and contralateral hind paws (D) are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with baseline (BL) of pre-injection of formalin; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with DMSO vehicle group.

AM281/AM630 injection occurred during phase 2, but not during phase 1 (Fig. 1).

Blockage of CB Receptors Facilitated Chronic Stage Mechanical Allodynia

Animals were tested for mechanical allodynia at 3 and 6 h, and 1, 3, 5 and 7 days following formalin injection. The PWT of the injured paw (ipsilateral to the formalin injected paw) was significantly reduced at 3 and 6 h, and 1, 3, 5 and 7 days post-injection compared with the baseline value ($P < 0.05$) (Fig. 2A). The PWT of the uninjured paw (contralateral to the formalin injected paw) was significantly smaller than the baseline value at 1, 3, 5 and 7 days post-injection ($P < 0.05$) (Fig. 2C).

AM281 and AM630 significantly enhanced the formalin-induced mechanical allodynia ($F_{(1,60)} = 24.418$, $P < 0.001$ for ipsilateral paw; $F_{(1, 60)} = 92.477$, $P < 0.001$ for contralateral paw) and across time ($F_{(5, 60)} = 6.685$, $P < 0.001$; $F_{(5,60)} = 23.957$, $P < 0.001$), and their interaction was significant in the contralateral paw ($F_{(5,60)} = 3.919$, $P = 0.04$), but not for their interaction in the ipsilateral paw ($F_{(5,60)} = 1.027$, $P = 0.410$) (Fig. 2). Further analyses indicated that the decreased PWT induced by AM281 and AM630 was significantly smaller than that in the DMSO-treated group at three time points (6 h, and 1 and 3 days) in the ipsilateral paw, and at all time points (3 and 6 h, and 1, 3, 5, 7 days) in the contralateral paw ($P < 0.05$) (Figs. 2,

A and C). The comparison between the two groups across the entire observation period is shown in Figs. 2, B and D.

Discussion

The results from the present study demonstrate that dual blockade of CB1 and CB2 receptors significantly increased the formalin-evoked second-phase nociceptive behavioral response and the late, long-term mechanical allodynia. These results suggest that CB1 and CB2 receptors have a tonic activity capable of depressing formalin-induced persistent pain and long-lasting inflammatory hypersensitivity.

The formalin test used here is an inflammatory pain model that has been extensively used in studies investigating mechanisms of inflammatory pain and the action of analgesic agents (4, 29). Subcutaneous injection of dilute formalin into the hind paw of the rat or mouse evokes characteristic biphasic nociceptive behaviors for about 1 h. The first phase (phase 1) is elicited immediately after formalin injection by peripheral unmyelinated and myelinated nociceptive afferents, and the second phase (phase 2) is closely related to local ongoing inflammatory afferents and central sensitization (21, 26). The present study demonstrated the formalin-evoked typical biphasic nociceptive behaviors (licking/biting and flinching the injected paw), and indicated that i.p. co-administration of the CB1 and CB2 receptor antagonists, AM281 and AM630, enhanced phase 2, but not phase 1, nociceptive behaviors in the rat. These results suggest that under the inflammatory pain state the cannabinoid receptors may exert a tonic inhibitory action on the phase 2 nociceptive responses. Calignano *et al.* (1998) reported that intravenous administration of a CB1 antagonist SR141716A to mice potentiated formalin-evoked phase 1 and phase 2 nociceptive behaviors, while a CB2 antagonist SR144528 only potentiated phase 1, but not phase 2, nociceptive behavior by removing an endogenous cannabinoid tone (8).

Our results have not strictly observed the expected roles of the CB1 and CB2 antagonists on formalin-evoked nociceptive behavior. An obvious difference between our result and that reported by Calignano *et al.* (1998) is that in the present study co-administration of both the antagonists (AM281 and AM630) did not enhance the phase 1 nociceptive behavior in rats (see Fig. 1) (8). From previous formalin test studies, it appears that administration of the CB1 and CB2 antagonists do not enhance formalin-evoked pain behavior in rats or mice, suggesting that endogenous cannabinoids do not tonically attenuate inflammatory hyperalgesia (5, 6, 19). We suggest that the contradictory results may be due to the differences in experimental protocols, especially with regard to the species, dose and route of drug (5). Importantly, in the present study, the CB1 and CB2 antagonists were concomitantly administrated, whereas in the above-mentioned studies, CB1 and CB2 antagonists were individually administrated (5, 6, 19). Because the intrinsic ability of endocannabinoids to tonically depress the nociceptive transmission may simultaneously activate both the CB1 and CB2 receptors (8), blocking only the CB1 receptor or the CB2 receptor, hence, cannot completely eliminate the intrinsic activity of endocannabinoids, leading to an ineffective response to nociception.

Recent studies have indicated that formalin injection into the plantar or dorsal surface of the hind paw of rats produces long-lasting thermal hyperalgesia and mechanical allodynia 2 h to 4 weeks after formalin injection (12, 24, 28). The present study characterized the formalin-evoked long-lasting mechanical allodynia, demonstrating that the PWT in the bilateral hind paw was significantly attenuated during the entire observation period after formalin injection. Furthermore, co-administration of the CB1 and CB2 receptor antagonists further attenuated the formalin-evoked decrease in PWT in the bilateral hind paw. These results suggest that subcutaneous injection of formalin evokes a secondary and long-lasting inflammatory hypersensitivity (allodynia). Further, endocannabinoid receptors may mediate a tonic inhibitory action on the long-lasting inflammatory hypersensitivity, and, thus, by removing the endocannabinoid tone, the hypersensitivity was enhanced. In a rat model of paw incision-induced mechanical allodynia, concomitant administration CB1 and CB2 receptor antagonists AM281 and AM630 during the acute phase prevented the resolution of post-operative allodynia (2). In CB1 or CB2 receptor knock-out mice, there is an exaggerated basal pain and a significantly more hypersensitivity (hyperalgesia and allodynia) after complete Freund's adjuvant inflammation and after spared nerve injury than their wild-type littermates (1, 22). These results provide support for the suggestion that under neuropathic and inflammatory states, CB1 and CB2 receptors exert a tonic inhibitory action on painful hypersensitivity.

Mirror-image pain is another pathological response to chronic pain in contralateral areas after injury and it has recently been studied in neuropathic pain models (20, 25). However, mirror-image hyperalgesia induced by formalin injection is poorly described in the literature. Our study indicated that dual blockade of CB1 and CB2 receptors significantly facilitated the mechanical allodynia of the uninjured paw, suggesting that CB1 and CB2 receptors may be involved in modulating mirror-image pain during long-term inflammation. The mechanism may be due to persistent activation of peripheral nociceptive afferents (3) and may involve gap junctions (25), which play a

role in the endocannabinoid modulation of neuronal activity (15), and which can be activated by cannabinoids during inflammatory situations (11).

In conclusion, the present study indicated that dual blockade of CB1 and CB2 receptors enhanced acute nociceptive behaviors and chronic allodynia evoked by formalin. These results suggest that, under the persistent inflammatory pain state, endocannabinoids have an intrinsic activity *via* CB1 and CB2 receptors that tonically depresses the nociceptive transmission. Thus, therapeutic strategies designed to enhance endocannabinoid signaling may reduce the incidence of persistent inflammatory pain.

Acknowledgments

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