Anesthesiology 2005; 102:832–7

Spinal Glucocorticoid Receptors Contribute to the Development of Morphine Tolerance in Rats

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Background: **Opioid analgesic tolerance is a pharmacologic phenomenon involving the mechanisms of cellular adaptation. Central glucocorticoid receptors (GRs) have been implicated in the cellular mechanism of neuronal plasticity that has many cellular steps in common with the mechanism of opioid tolerance. In a rat model of morphine tolerance, the authors examined the hypothesis that spinal GRs would play a significant role in the development of tolerance to the antinociceptive effect of morphine.**

Methods: **In experiment 1, each group of rats received the GR antagonist RU38486 (0.5 or 1 μg), the mineralocorticoid receptor antagonist spironolactone (3 μg), or a vehicle, given intrathecally with morphine (10 μg) twice daily for 6 days. In experiment 2, four groups of rats were used, and each group received intrathecally 10 μg morphine plus 5 μmol GR antisense oligodeoxynucleotide, sense oligodeoxynucleotide, mixed-base oligodeoxynucleotide, or vehicle. Western blotting was used to examine the expression of GRs within the spinal cord dorsal horn. In experiment 3, the GR agonist dexamethasone (4 μg) was given intrathecally twice daily in combination with 10 μg morphine. For all experiments, the development of morphine antinociceptive tolerance was assessed using the tail-flick test.**

Results: **The development of tolerance to the antinociceptive effect of morphine was substantially attenuated when the GR antagonist RU38486 (1 > 0.5 μg > vehicle) but not spironolactone was coadministered with morphine for 6 days. A single treatment with RU38486 did not affect morphine antinociception, nor did it reverse morphine tolerance on day 7. A similar reduction of morphine tolerance was observed in those rats treated with a GR antisense oligodeoxynucleotide but not a sense or mixed-base oligodeoxynucleotide. The administration of the GR antisense oligodeoxynucleotide also prevented GR up-regulation within the spinal cord dorsal horn. Moreover, the GR agonist dexamethasone facilitated the development of morphine tolerance.**

Conclusions: **The results indicate an important role of spinal GRs in the cellular mechanisms of morphine tolerance in rats and may have significant implications in clinical opioid therapy.**

THE development of opioid analgesic tolerance hampers the clinical use of opioids, a class of most effective analgesics in treating many forms of acute and chronic pain. Investigation into the mechanisms of opioid tolerance has been a focus of intense interest for many years.[1–5] Recent studies have implicated the activation of *N*-methyl-D-aspartate (NMDA) receptors and protein kinase C as well as regulation of glutamate transporters in the mechanisms of opioid tolerance,[6–11] suggesting a possible link between neural plasticity and the cellular mechanisms of opioid tolerance.

Endogenous glucocorticoid hormones such as cortisol bind to peripheral glucocorticoid receptors (GRs), which play a crucial role in the regulation of inflammatory responses through both genomic and nongenomic mechanisms. However, neuronal GRs have been located within a number of central regions[12,13] and implicated in neuronal plastic changes induced by neuronal injury[14] and the process of learning and memory.[15–18] Our recent study has indicated that the spinal cord dorsal horn is a prominent site of neuronal GR expression that is enhanced after peripheral nerve injury.[19] Of interest is that activation of GRs may have a modulatory effect on morphine-induce locomotor activity[20] and the related dopamine-dependent responses.[21,22] GRs may also have an effect on morphine antinociception in rodents.[23,24] To date, the role of central GRs in the cellular mechanisms of opioid tolerance has not been directly examined.

Given that central GRs are involved in the mechanism of neuronal plasticity that has many cellular steps in common with the mechanism of opioid tolerance, it is possible that neuronal GRs may play a unique role in the cellular mechanism of opioid tolerance. Using a rat model of antinociceptive tolerance induced by repeated intrathecal morphine administration, we examined the hypothesis that spinal GRs would contribute to the development of morphine tolerance. The results show that morphine tolerance was substantially attenuated through either inhibition of the GR activation with the GR antagonist RU38486 or prevention of morphine-induced GR up-regulation using a GR antisense oligodeoxynucleotide.

Materials and Methods

Experimental Animals

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 300–350 g were used. Animals were housed in cages with water and food pellets available *ad libitum*. The animal room was artificially illuminated from 7:00 to 19:00 h. The general experimental protocol was approved through our Institutional Animal Care and Use Committee (Boston, MA).

Intrathecal Catheter Implantation and Drug Delivery

An intrathecal catheter (PE-10) was implanted in each rat according to the previously published method.[25] An-

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Received from the Pain Research Group, MGH Pain Center, Department of Anesthesia and Critical Care, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts. Submitted for publication August 9, 2004. Accepted for publication November 24, 2004. Supported by RO1 grant Nos. DA08835, NS42661, and NS45681 from the National Institutes of Health, Bethesda, Maryland.

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imals that exhibited neurologic deficits such as paralysis after the intrathecal catheter implantation were excluded from the experiments.[9] Drugs were delivered *via* an intrathecal catheter in a total volume of 10 μl followed by a saline flush. Each drug was injected separately *via* the intrathecal catheter during an injection session. The following drugs were purchased from Sigma (St. Louis, MO): mifepristone (RU38486), morphine, spironolactone, and dexamethasone. Morphine was dissolved in normal saline, and other drugs were dissolved in a 10% ethanol solution (vehicle).

For those experiments using GR oligodeoxynucleotides, the sequences for antisense, sense, and mixed-base oligodeoxynucleotides were chosen based on a previous study that has verified the down-regulation of GRs after the administration with the antisense but not sense or mixed-base oligodeoxynucleotide.[26] Therefore, the sequences overlapping the respective initiation codon (Gene Bank No. M14053) were used for targeting GR mRNA: GR antisense (TGG AGT CCA TTG GCA AAT), GR sense (ATT TGC CAA TGG ACT CCA), and the mixed-base (TGA AGT TCA GTG TCA ACT), as described in a previous study.[26] Sequencing was performed by MWG-Biotech (High Point, NC), and the product stability was ensured at the time of delivery. Oligodeoxynucleotides were dissolved in 0.9% saline and injected intrathecally (5 μmol each) twice daily. After the last behavioral testing, spinal cord tissue samples were taken to examine changes in the GR protein concentration.

Induction of Morphine Tolerance and Statistical Analysis

Tolerance to the antinociceptive effect of morphine was induced using an intrathecal treatment regimen of 10 μg morphine given twice daily for 6 consecutive days. Differences in morphine antinociception among treatment groups were assessed using the tail-flick test by testing at 30 min after a probe dose of 10 μg morphine. The dose–response effect of morphine was examined by generating cumulative dose–response curves to reduce the total number of rats used, as described in a previous study.[27] The increment log doses (0.2 log unit) of morphine were given to the same rats, and the morphine antinociceptive effects were examined at 20 min after each morphine injection. This process was repeated until either no additional antinociceptive effect was demonstrated or the cutoff time (10 s) of the tail-flick test was reached in response to a next higher dose, indicative of the maximal antinociceptive effect of morphine under the experimental condition.[27] The routine tail-flick test was used with baseline latencies of 4–5 s and a cutoff time of 10 s. At least two trials were made for each rat, with an intertrial interval of 1 min and with changes of the tail position receiving radiant heat stimulation at each trial.

The percent of maximal possible antinociceptive effect (%MPAE) was determined by comparing the tail-flick latency before (baseline) and after a drug injection using the equation: %MPAE = [(After Drug Injection − Baseline)/(10 − Baseline)] × 100% (the constant 10 refers to the cutoff time). The data were analyzed by using two-way analysis of variance with *post hoc* Newman–Keuls tests. For the dose–response data analysis, AD_{50} values and 95% confidence intervals were generated using a computerized regression model.

Western Blotting

For Western blot, rats were rapidly (< 1 min) killed through decapitation after being anesthetized with pentobarbital (100 mg/kg intraperitoneal). Lumbar spinal cord segments (L3–L5) were removed through a lumbar laminectomy. The spinal segments were divided into dorsal and ventral horns and homogenized in sodium dodecyl sulfate sample buffer containing a cocktail of proteinase inhibitors (Sigma: 2 mM AEBSF, 1 mM EDTA, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin). The lumbar segments were harvested because the intrathecal drug delivery was aimed at this site. Protein samples were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–15% gradient gel; Bio-Rad, Hercules, CA) and transferred to polyvinyl difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% milk and incubated overnight at 4°C with a primary antibody (GR: 1:1,000; rabbit polyclonal from Santa Cruz, Santa Cruz, CA) and 1 h at room temperature with horseradish peroxidase–conjugated secondary antibody (1:7,000; Amersham Biosciences, Arlington Heights, IL). The blots were visualized in existing chemiluminescent solution (NEN, Boston, MA) for 1 min and exposed to hyperfilms (Amersham) for 1–10 min. The blots were then incubated in a stripping buffer (67.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, and 0.7% β-mercaptoethanol) for 30 min at 50°C and reprobed with a polyclonal rabbit anti–β-actin antibody (1:20,000; Alpha Diagnostic International, San Antonio, TX) as a loading control. The Western analysis was made in triplicates. The band density was measured with a computer-assisted imaging analysis system and normalized against the corresponding loading control. Differences were compared using analysis of variance followed by *post hoc* Newman–Keuls tests.

Results

Effect of GR Antagonist RU38486

The role of GRs in the development of morphine tolerance was examined in three groups of rats each receiving the GR antagonist RU38486 (0.5 or 1 μg intrathecal) or a vehicle with morphine (10 μg intrathecal) for 6 days (n = 5 or 6). The RU38486 doses were chosen based on the literature[28] and our pilot experiments.

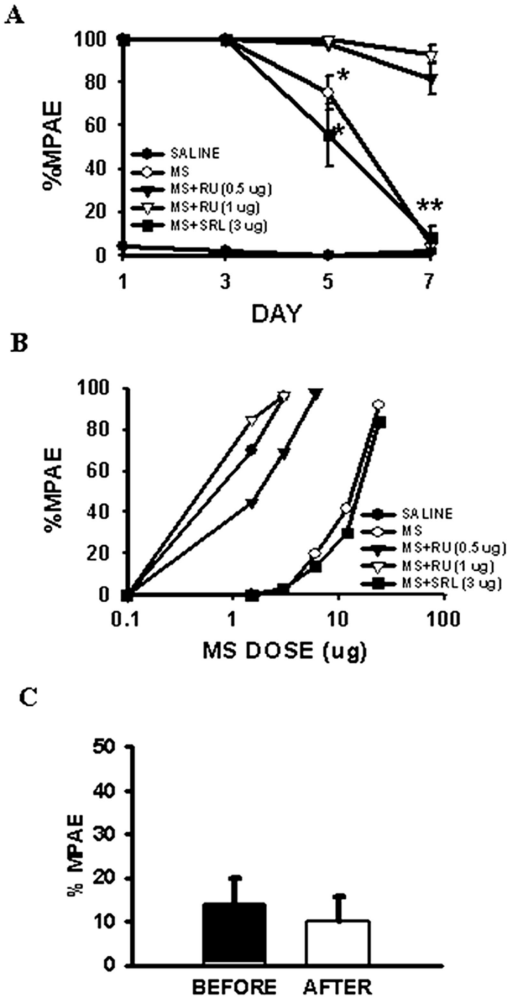

Fig. 1. (*A* and *B*) The development of morphine tolerance was prevented in rats treated with morphine (MS) and RU38486 (RU) but not spironolactone (SRL). Note the differences in the onset of complete tolerance (*A*) and the rightward shift of dose–response curves (*B*) among groups. * $P < 0.05$ and ** $P < 0.01$ as compared with baseline. (*C*) A single injection of 1 μg RU38486 (intrathecal, 30 min AFTER) did not reverse tolerance after a probe morphine dose (10 μg) on day 7 (BEFORE) in those rats made tolerant to morphine (10 μg intrathecal) given twice daily for 6 days. %MPAE = percent of maximal possible antinociceptive effect.

Antinociceptive tolerance developed in the morphine plus vehicle group, whereas RU38486 significantly attenuated the development of morphine tolerance when examined on days 5 and 7 ($P < 0.05$; fig. 1A). Consistently, the rightward shift of the morphine dose–response curve on day 7 in the morphine-plus-vehicle group was prevented in the morphine-plus-RU38486 groups (fig. 1B and table 1). In contrast, intrathecal coadministration of 10 μg morphine with the mineralocorticoid receptor antagonist spironolactone (3 μg) for 6 days did not prevent the development of morphine tolerance ($P > 0.05$, n = 5; figs. 1A and B), nor did spironolactone alone change baseline nociceptive threshold, indicating a selective role of GRs in this process. The selected spironolactone dose has been shown to be effective for blocking the mineralocorticoid receptor.[22]

Table 1. Effects of RU38486 on Morphine Tolerance

Group	AD_{50}	95% CI
MS–VEH (D1)	1.9	1.5–3.2
MS–VEH (D7)	6.3	4.9–6.8
MS–0.5 μg RU (D7)	2.3	1.0–3.7
MS–1 μg RU (D7)	1.5	1.0–2.7
MS–3 μg SPL (D7)	6.7	5.6–7.1

Only the baseline AD_{50} value on day 1 from the morphine-plus-vehicle group is shown in the table because there were no significant differences in baseline morphine dose–response among all groups.

AD_{50} = morphine antinociceptive dose with a 50% maximum possible antinociceptive effect; CI = confidence interval; D1 = day 1 of the experimental period; D7 = day 7 of the experimental period; MS = morphine; RU = RU38486; SPL = spironolactone; VEH = vehicle.

Two additional groups of rats (n = 5) each received RU38486 (1 μg intrathecal) or saline alone for 6 days. Such treatment did not change the baseline tail-flick latency, nor did the RU38486 treatment change the motor activity, indicating a specific effect of RU38486. The effect of RU38486 on morphine tolerance was unlikely due to a direct effect on morphine antinociception, because a single dose of RU38486 (1 μg) did not reverse morphine tolerance on day 7 in those rats made tolerant to morphine ($P > 0.05$, n = 5; fig. 1C), nor did it change the antinociception induced by a single morphine (10 μg intrathecal) treatment in a separate group of rats (%MPAE, morphine alone: 91 ± 2.3%; morphine plus RU38486: 89 ± 3.1%). Therefore, the data indicate that the GR antagonist RU38486 effectively attenuated the development of morphine tolerance.

Effect of GR Antisense Oligodeoxynucleotide

In addition to using RU38486, we examined the functional role of GRs in morphine tolerance by interfering with the GR expression within the spinal cord dorsal horn. Although there are reports on mice with genetically altered GRs, they are either lethal or unavailable.[29–31] As an alternative, an antisense oligodeoxynucleotide against GRs was given as described previously.[26] In this experiment, a total of four groups of rats were used, and each group (n = 5–7) received 10 μg morphine in combination with 5 μmol GR antisense oligodeoxynucleotide, sense oligodeoxynucleotide, mixed-base oligodeoxynucleotide, or vehicle. The oligodeoxynucleotide treatment (twice daily, intrathecal) was first given alone for 7 days before the morphine regimen. These rats then received the intrathecal morphine treatment along with an oligodeoxynucleotide for 6 days. The spinal cord dorsal horn samples were collected from these groups at the end of behavioral testing.

The intrathecal morphine regimen induced a substantial up-regulation of GRs within the spinal cord dorsal horn when examined on day 7, whereas the same morphine treatment did not up-regulate GRs in the morphine–

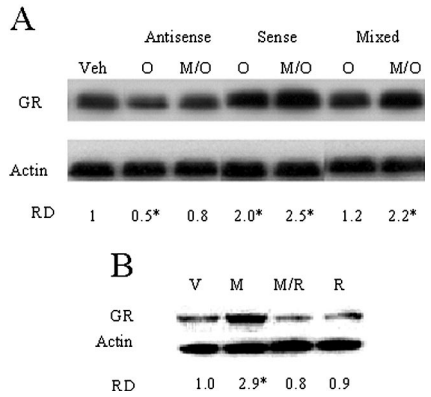

Fig. 2. (*A*) Changes in glucocorticoid receptor (GR) protein (Western blot) level within the spinal cord dorsal horn after treatment (twice daily × 6 days) with either a vehicle or morphine (10 μg intrathecal) and one of the following GR oligodeoxynucleotides (5 μmol each, intrathecal): sense, antisense, or mixed base. Lane assignment: Veh = vehicle alone; O = an oligodeoxynucleotide alone; M/O = morphine plus an oligodeoxynucleotide. * $P < 0.05$ as compared with the vehicle group. (*B*) RU38486 (1 μg intrathecal) attenuated GR up-regulation within the spinal cord dorsal horn induced by morphine (10 μg intrathecal) administration. Both agents were given twice daily for 6 days. * $P < 0.05$ as compared with the vehicle group. V = vehicle; M = morphine; R = RU38486. In *A* and *B*, RD refers to relative density (an average of triplicates) of each GR band as normalized against the corresponding loading band.

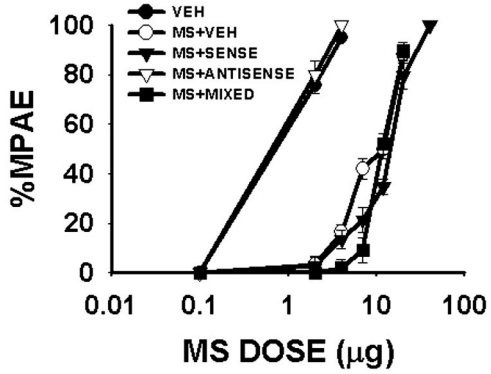

Fig. 3. The development of morphine tolerance was prevented in rats treated with morphine (MS) and the glucocorticoid receptor antisense oligodeoxynucleotide but not a sense or mixed-base oligodeoxynucleotide. The cumulative dose–response curves were generated on day 7. %MPAE = percent of maximal possible antinociceptive effect; VEH = vehicle.

plus–antisense oligodeoxynucleotide group, as compared with each of the groups receiving morphine plus vehicle, a sense oligodeoxynucleotide, or mixed-based oligodeoxynucleotide ($P < 0.05$; fig. 2A). No motor changes such as paralysis were observed after oligodeoxynucleotide treatment. Of note is that the RU38486 treatment that prevented the development of morphine tolerance (see below) also reduced the GR up-regulation induced by morphine administration ($P < 0.05$, n = 5; fig. 2B), whereas RU38486 alone did not change the GR expression as compared with the vehicle group ($P > 0.05$; fig. 2B). Consistent with the Western blot results, the development of morphine tolerance was substantially diminished in the morphine–plus–GR antisense group as compared to each of the remaining groups ($P < 0.05$, n = 6; fig. 3). These results indicate that morphine tolerance was significantly attenuated when the morphine-induced GR up-regulation within the spinal cord dorsal horn was diminished by the antisense oligodeoxynucleotide treatment.

Effect of Dexamethasone

Coadministration of morphine (10 μg intrathecal) and the GR agonist dexamethasone (4 μg intrathecal),[23,32] given twice daily for 4 days, significantly shortened the onset of morphine tolerance such that antinociceptive tolerance to 10 μg morphine developed on day 5 of the morphine cycle (%MPAE: 46.5 ± 7.1%) as compared with the morphine alone group (%MPAE: 79.2 ± 2.3%) ($P < 0.05$, n = 6; fig. 4). Dexamethasone alone did not affect the baseline nociceptive threshold.

Discussion

The current findings demonstrate that (1) the development of morphine antinociceptive tolerance was substantially attenuated by the GR antagonist RU38486, (2) a single treatment with RU38486 did not affect morphine antinociception, (3) a GR antisense oligodeoxynucleotide but not a sense or mixed-base oligodeoxynucleotide also attenuated the development of morphine tolerance as well as the up-regulation of GRs within the spinal cord dorsal horn, and (4) the GR agonist dexamethasone significantly shortened the onset of morphine tolerance. Collectively, these data indicate that central GRs may play a significant role in the development of morphine tolerance in rats.

Several technical considerations are worthwhile mentioning. First, although RU38486 (mifepristone, RU486) has been extensively used as a conventional GR antagonist and is the only commercially available GR antagonist, it does have the antiprogesterone effect.[28] The antiprogesterone effect is unlikely to be involved in this study because we only included male rats and the GR

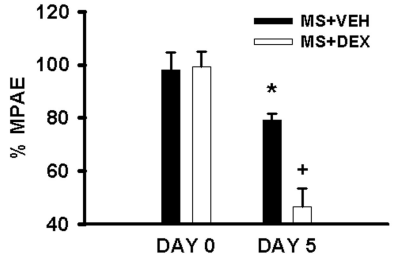

Fig. 4. The glucocorticoid receptor agonist dexamethasone (DEX; 4 μg intrathecal), with coadministration of morphine (MS; 10 μg intrathecal) given twice daily for 4 days, significantly diminished morphine antinociception when examined on day 5. * $P < 0.05$ as compared with the morphine-plus-vehicle group; + $P < 0.05$ as compared with morphine-alone and vehicle groups. %MPAE = percent of maximal possible antinociceptive effect; VEH = vehicle.

knockdown experiment using an antisense oligodeoxynucleotide produced a result comparable to that from using RU38486. Second, mineralocorticoid receptors are unlikely to play a significant role in this process because the mineralocorticoid receptor antagonist spironolactone within the dose range effective for blocking the mineralocorticoid receptor[22] did not influence the development of morphine tolerance. Third, although the current data indicate a spinal locus of GR actions because morphine and RU38486 or the GR antisense oligodeoxynucleotide were delivered using an intrathecal treatment regimen, it does not exclude the possibility that a similar GR-mediated mechanism of morphine tolerance may be involved in other central nervous system regions.

Although GRs have been shown to act through nongenomic mechanisms,[33,34] the role of GRs in morphine tolerance probably is not due to a direct interaction between GRs and μ-opioid receptors, because a single injection with the GR antagonist RU38486 did not reverse the established morphine tolerance and the same RU38486 treatment did not affect the morphine antinociceptive effect in nontolerant rats. At the cellular level, GRs are activated through the formation of a GR homodimer after the dissociation from a cytosolic complex consisting of heat shock proteins.[35] A GR homodimer binds to specific nuclear DNA–responsive elements to activate gene transcription and translation for a variety of cellular elements.[35] Given that the development of morphine tolerance was attenuated in this model after either repeated administration of RU38496 or prevention of GR up-regulation with an antisense oligodeoxynucleotide, it is plausible that GRs may have a modulatory role in one or more intracellular elements contributory to the cellular mechanisms of morphine tolerance.[1–5,36]

It may be considered that an interaction between GRs and NMDA receptors might play a role in this process because NMDA receptors have been known to contribute to the cellular mechanism of neural plasticity related to learning and memory as well as opioid tolerance.[6–10,37–39] For example, GRs have a direct modulatory effect on the NMDA receptor function[40] and potentiate NMDA-induced responses in dopamine-sensitive neurons in the ventral tegmental area.[41] In addition, a recent study suggested the involvement of proinflammatory cytokines in opioid tolerance,[42] raising the possibility that the glucocorticoid effects may be mediated in part though the actions of spinal cytokines. Future studies would examine these possibilities.

The relation among stress, corticosteroids, and morphine analgesia has been suggested in several previous studies.[43,44] For example, Takahashi *et al.*[43] have demonstrated that the antinociceptive effect of morphine was potentiated by adrenalectomy in mice, suggesting that endogenous corticosteroids might have a negative effect on morphine antinociception. However, in the same study, adrenalectomy seemed to be ineffective in affecting the development of morphine tolerance in mice, although that study did not specifically examine the effect of GR activation. The current data support a potential role of endogenous glucocorticoids in the mechanisms of opioid tolerance *via* activation of neuronal GRs, suggesting that those factors (*e.g.*, stress, emotional disturbance, Addison or Cushing disease) capable of changing the endogenous corticosteroid level could be linked to the cellular mechanism of opioid tolerance through neuronal GRs. Our results also suggest that a GR antagonist such as RU38486 could be a useful pharmacologic tool, alone or in combination with other agents (*e.g.*, an NMDA receptor antagonist), to prevent the development of opioid tolerance, an issue of clinical relevance.[45,46]

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