Peripheral NMDA Receptor/NO System Blockage Inhibits Itch Responses Induced by Chloroquine in Mice

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Intradermal administration of chloroquine (CQ) provokes scratching behavior in mice. Chloroquine-induced itch is histamine-independent and we have reported that the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway is involved in CQ-induced scratching behavior in mice. Previous studies have demonstrated that activation of *N*-methyl-d-aspartate receptors (NMDARs) induces NO production. Here we show that NMDAR antagonists significantly decrease CQ-induced scratching in mice while a non-effective dose of an NMDAR agonist potentiates the scratching behavior provoked by sub-effective doses of CQ. In contrast, combined pre-treatment with sub-effective doses of an NMDAR antagonist, MK-801, and the NO synthase inhibitor, L-N-nitro arginine methyl ester (L-NAME), decreases CQ-induced scratching behavior. While intradermal administration of CQ significantly increases the concentration of intradermal nitrite, the end product of NO metabolism, effective doses of intraperitoneal and intradermal MK-801 significantly decrease intradermal nitrite levels. Likewise, administration of an effective dose of L-NAME significantly decreases CQ-induced nitrite production. We conclude that the NMDA/NO pathway in the skin modulates CQ-induced scratching behavior.

Key words: itch; chloroquine; *N*-methyl-D-aspartate receptor (NMDA) antagonists; nitric oxide; mice.

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Chloroquine (CQ) has been used to treat malaria, certain viral infections (1) and a number of autoimmune diseases including rheumatoid arthritis (2) and systemic lupus erythematosus (3). Chloroquine also induces itch in humans and scratching in mice (4, 5) via a histamine-independent pathway linked to activation of Mrgprs (6). Chloroquine activates human MRGPRX1 and the homologous mouse receptor, MrgprA3 (6). As expected, CQ-induced scratching behavior is diminished in Mrgpr cluster knockout mice, which lack MrgprA3 (7). In addition, the kappa-opioid agonist, nalfurafine, has been shown to decrease CQ-induced scratching behavior in mice (8). MrgprA3 coupling to $G_{\beta\gamma}$ modulates TRPA1 to result in itch (6, 9). $G_{\beta\gamma}$ modulates several ion channels via direct binding (10). The *N*-methyl-d-aspartate receptor (NMDAR) is a non-selective cation ion channel (11). NMDAR is implicated in synaptic plasticity, memory (11, 12) and pain (13, 14). The administration of NMDA or glutamate to the rat hind paw evokes nociceptive behaviors that are decreased by the injection of NMDAR antagonists in the periphery (14, 15). Peripherally-acting NMDAR antagonists have been used for inflammatory and visceral pain and seem to be devoid of CNS side effects (16) while oral administration of gabapentin and pregabalin act centrally and are used to treat the neuropathic itch associated with brachioradial and post-herpetic pruritus (17–21).

Intrathecal injection of NMDA induces scratching behavior in mice (22) while NMDAR antagonists suppress such behavior (17, 23). The physiology is analogous in rats, where intrathecal administration of the NMDAR antagonists ketamine and kynurenic acid decrease scratching behavior induced by intradermal injection of the serotonin derivative 5-methoxytryptamine (23). Intrathecal application of the NMDAR antagonists dizocilpine (MK-801) or D (-) -2-amino- 5-phosphonovalerate (APV) prevented intracutaneous histamine-induced expansion of mechanical receptive fields in rats (24). Separately, it has been shown that administration of a nitric oxide (NO) donor to skin enhances substance P-induced itch in mice (25), while serotonin-induced itch was inhibited by systemic nitric oxide synthase (NOS) inhibitors (26).

Taken together, previous reports have revealed that activation of the NMDA/NO pathway is associated with pain (27) while inhibition of this pathway decreases pain and itch (23, 25, 27). Activation of this pathway is also associated with increased NO production (28), which itself is linked to cGMP (29). NO production is associated with itch while inhibition of NOS suppresses itch. We reported recently that the NO/cGMP pathway participates in CQ-induced itch (30). Here we extend these findings with the demonstration that the NMDA/NO pathway participates in CQ-induced scratching behavior.

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METHODS

Animals and material

Male NMRI mice, an outbred strain, (Pasteur Institute, Tehran, Iran) of 5–6 weeks of age and weight range of 23–30 g were used.

Animals were maintained in appropriate facilities with respect to temperature (23–25ºC) and light (lights on from 08:00 AM to 08:00 PM) and had free access to food or water (31).

All of the operational guidelines in the housing, routine husbandry, handling, and experimental procedures were approved by the committee for animal ethics and experiments at Tehran University of Medical Sciences, Tehran, Iran.

Chloroquine bisphosphate (Pubchem CID 64927) was a gift from Pars Darou Pharmaceutical Company (Tehran, Iran).

Loratadine (Pubchem CID 3957), magnesium sulfate (Pubchem CID 24083), ketamine hydrochloride solution (Pubchem CID 15851 – CAS Number: 1867-66-9), MK-801 (Pubchem CID 1207), N-methyl-D-aspartic acid (NMDA) (Pubchem CID 22880), N-nitro-L-arginine methyl ester (L-NAME) (Pubchem CID 39836) and vanadium chloride (Pubchem CID 62647) were purchased from Sigma, St. Louis, MO, USA. Lysis buffer was purchased from Abcam, Cambridge, MA, USA.

All drugs except loratadine were prepared freshly for use by dissolving in physiological saline. Loratadine was dissolved in phosphate-buffered saline (PBS).

Drug administration

Loratadine, a second-generation histamine type 1 (H1) receptor antagonist, was injected intraperitoneally (IP) at a dose of 10 mg/kg 30 min before intradermal (ID) injection of 400 µg CQ.

Magnesium sulfate ($MgSO_4$) was administered IP at doses of 5 and 20 mg/kg 30 min before ID injection of 400 µg CQ in volume of 50 µl per site. Magnesium ions block the NMDA type of ionotropic glutamate receptors (ion GluRs) (32).

We targeted the glutamate site of NMDA receptors by the glutamate channel blockers ketamine and MK-801, at doses of 2, 5 mg/kg and 0.1, 0.25 mg/kg, respectively injected IP 45 min beforehand (32, 33). NMDA was injected intraperitoneally (IP) at a dose of 75 mg/kg, 30 min before ID administration of CQ at a sub-effective dose of 200 µg/site (32, 33). MK-801 was administered ID at a dose of 10 nmol simultaneously with CQ 400 µg/site (34).

We recently reported that IP injection of 3 and 10 mg/kg of L-NAME significantly ($p<0.001$) reduces CQ 400 µg-induced scratching behavior. We also demonstrated that L-NAME at a dose of 1 mg/kg is considered sub-effective and does not significantly inhibit CQ 400 µg-induced scratching behavior (30). For the current study, sub-effective doses of intraperitoneal MK-801 (0.1 mg/kg) and L-NMAE (1 mg/kg) were injected 45 and 30 min before CQ 400 µg, respectively.

Behavioral experiments

All animals were habituated in an acrylic box (10 × 10 × 13 cm) at 23º C ± 1 for 1 h before behavioral experiments. A small amount of bedding was placed in the box. Hair was removed from the rostral back of the mice by depilatory cream. After two days, ID injections were delivered to the shaved area in a volume of 50 µl per site and each mouse was used once. The mice were removed briefly from the box for injections, returned to the same box after injections; and the behavior was recorded using a video camera in unmanned conditions to avoid distraction. The video was played back to quantify the scratching bouts directed at the site of injection. Each scratching bout is initiated by lifting of the hind paw to the area of injection, and ended by returning of the hind paw to the floor or to the mouth.

Open-field locomotor activity

NMDAR antagonists are associated with dissociative symptoms. To rule out the possibility of such effects on the ambulatory behavior of mice, an open-field test (35) was performed to evaluate the effect of NMDAR antagonists on the motor activity of mice. The apparatus consisted of a wooden box measuring 40 × 60 × 50 cm. The floor of the arena was divided into 12 equal squares. The animals were gently placed in the center of the field, and the number of squares crossed with all paws (crossing) was counted in a 6-min session. Although loratadine is a non-sedative H1 receptor antagonist, we performed an open-field test with loratadine to rule out its possible effects on locomotor activity of the mice.

Measurement of nitric oxide levels in skin tissue

Mice were sacrificed by cervical dislocation and the rostral skin (the site of injection) was removed 5, 15, 25 and 35 min after ID injection of CQ and saline. Next, we evaluated the changes in nitrite concentration after injection of an effective dose of L-NAME (10 mg/kg, IP) and 15 min after injection of CQ (400 µg, ID) (the time of maximum scratching behavior and nitrite concentration). We also evaluated the effects of MK-801 (0.25 mg/kg, IP and 10 nmol/site, ID) on the nitrite level 15 min after CQ injection (400 µg, ID).

NO metabolite, nitrite, was measured in the homogenized supernatant samples using the Griess reaction (36). One tenth ml of washout samples were pipetted into a 96-well micro titer plate, then 0.1 ml of Griess reagents containing 2.5% w/v sulphanilamide and 2.5% N-(1-naphthyl) ethylenediamine hydrochloride were added and incubated at room temperature for 15 min to allow color development. One tenth ml of 5% w/v vanadium chloride was added and incubated at 37°C for 45 min. Nitrite concentration was calculated using ELISA and the results were expressed as pmol/mg.

Data analysis

Data were processed (GraphPad Prism 6.0 graphing and statistics software) by one-way or two-way analysis of variance (ANOVA) along with Dunnett's test or Tukey's multiple comparisons tests. The *t*-test was performed for some data analyses. In all the experiments, $p<0.05$ was considered significant. Data are presented as mean ± standard error of the mean (SEM).

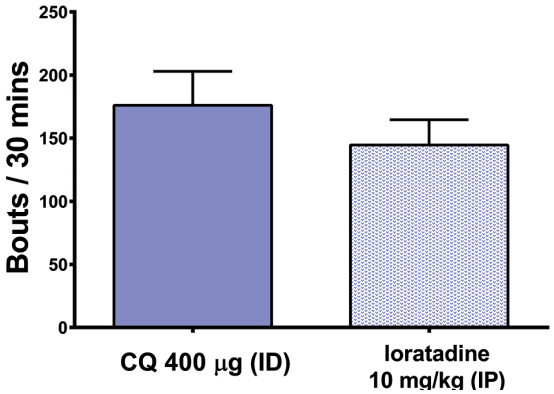

Fig. 1. The effect of loratadine on chloroquine (CQ)-induced scratching behavior. Administration of a non-sedative H1 antagonist, loratadine, (10 mg/kg, intraperitoneally (IP)) 30 min before 400 µg CQ (intradermally (ID)) does not significantly reduce CQ-induced scratching behavior ($p=0.3625$). Values are expressed as mean ± SEM ($n=8$) and were analyzed using a *t*-test. $p>0.05$.

RESULTS

Chloroquine induces histamine-independent scratching behavior in NMRI mice

Previous studies with C57BL/6 mice have demonstrated that CQ-induced itch is histamine-independent (6). To confirm these findings, we evaluated the effect of loratadine, a non-sedating H1 receptor antagonist, on CQ-induced scratching behavior in NMRI mice. The administration of loratadine (10 mg/kg, IP) before CQ (400 µg/site) did not significantly decrease the pruritic behavior ($p > 0.05$) (**Fig. 1**). Consistent with its non-sedating activity, loratadine (10 mg/kg, IP) had no significant effect on the locomotor activity of mice in the open-field test ($p > 0.05$; data not shown) (37).

NMDAR antagonists decrease chloroquine-induced scratching behavior

Previous studies have revealed that NMDA induces itch (22) while NMDAR antagonists suppress itch (17, 23). We thus asked if NMDAR antagonists would decrease scratching behavior from CQ. Mice were pretreated with $MgSO_4$ (5 and 20 mg/kg), ketamine (2 and 5 mg/kg), and MK-801 (0.1 and 0.25 mg/kg) before intradermal CQ injection (400 µg/site). Chloroquine-induced scratching behavior was significantly decreased when effective doses of $MgSO_4$ (20 mg/kg), ketamine (5 mg/kg) or MK-801 (0.25 mg/kg) were administered intraperitoneally ($F(7, 56) = 11.75$, $p < 0.0001$) (**Fig. 2**). MK-801, ketamine and magnesium sulphate had no significant effect on the locomotor activity of mice in the open field test ($F(3, 28) = 0.83$, $p > 0.05$; data not shown) at these concentrations. **Fig. 3** shows the effect of MK-801 (10 nmol/site) on scratching induced by CQ 400 µg/site. A two-way ANOVA type III showed the effect of intradermal MK-801 ($F(1, 28) = 0.0218$, $p > 0.05$), the effect of CQ 400 µg/site ($F(1, 28) = 53.9007$, $p < 0.0001$) and the effect of intradermal MK801 × CQ 400 µg/site interaction ($F(1, 28) = 27.4496$, $p < 0.0001$). Intradermal MK-801 (10 nmol/site) decreased CQ-induced scratching levels to those of mice treated with saline alone.

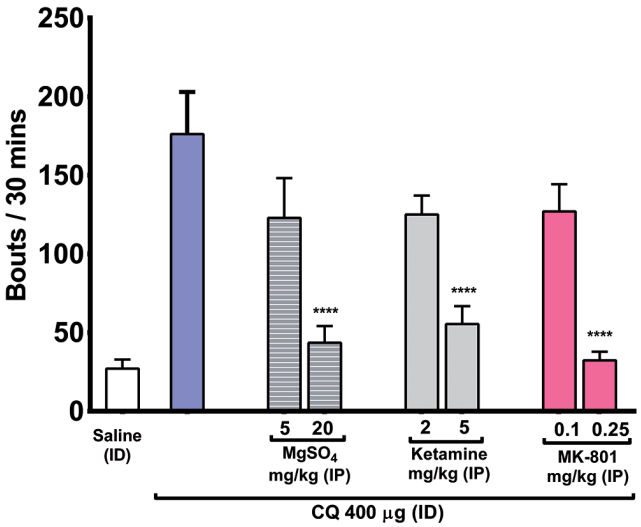

Fig. 2. Systemic administration of NMDA receptor antagonists decreases chloroquine (CQ)-induced scratching behavior. $MgSO_4$ (5 and 20 mg/kg, intraperitoneally (IP)) was administered 30 min before 400 µg CQ (intradermally (ID)) in volume of 50 µl per site. MK-801 (0.1 and 0.25 mg/kg, IP) and ketamine (2 and 5 mg/kg, IP) were administered 45 min before CQ 400 µg (ID). Values are expressed as mean ± SEM ($n = 8$) and were analyzed using a one-way ANOVA followed by Dunnett's test. ****$p < 0.0001$.

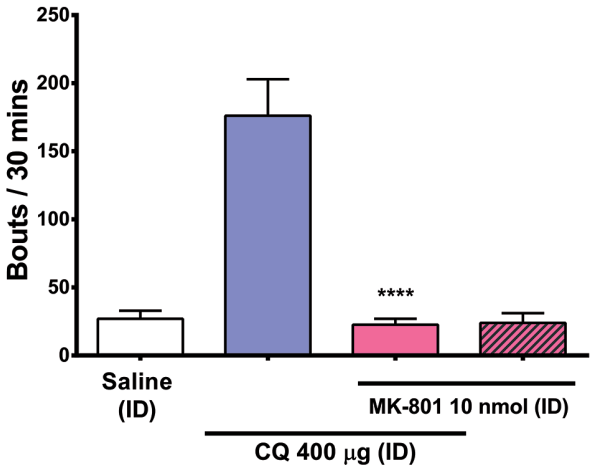

Fig. 3. Intradermal MK-801 (10 nmol/site) significantly decreases chloroquine (CQ)-induced scratching behavior. MK-801 (10 nmol/site, intradermally (ID)) *per se* did not have any significant pruritic effects. Values are expressed as mean ± SEM ($n = 8$) and were analyzed using a two-way ANOVA type III followed by Tukey's multiple comparisons test. ****$p < 0.0001$.

NMDA potentiates CQ-induced scratching behavior

As NMDA can itch (22), we asked if NMDAR agonists could allow for itch to be induced by a sub-itch-inducing

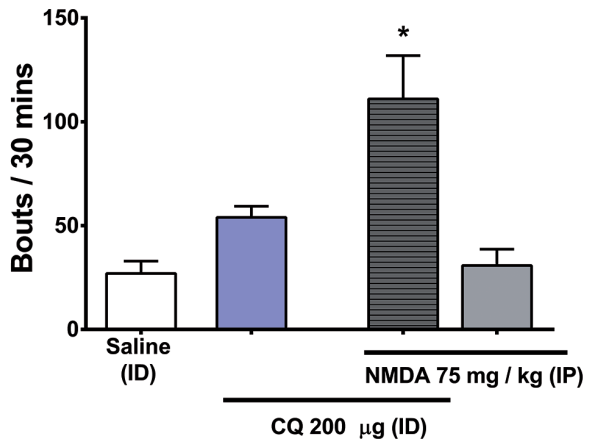

Fig. 4. NMDA potentiates chloroquine (CQ)-induced scratching behavior. Pretreatment with the NMDA agonist (NMDA, 75 mg/kg, intraperitoneally (IP)) 30 min before administration of CQ 200 µg (intradermally (ID)) potentiated the scratching responses ($p = 0.0103$). NMDA (75 mg/kg, IP) per se does not have any significant pruritic effects. Values are expressed as mean ± SEM ($n = 8$) and were analyzed using two-way ANOVA type III followed by Tukey's multiple comparisons test. *$p < 0.05$.

dose of CQ. **Fig. 4** demonstrates the effect of a NMDAR agonist on CQ-induced scratching behavior. Mice were pretreated with NMDA (75 mg/kg, IP) in advance of sub-pruritic intradermal CQ (200 µg/site) injection. A two-way ANOVA type III showed that CQ (200 µg/site) × NMDA (75 mg/kg, IP) injection induces significant scratching behavior ($F(1, 28) = 5.0501$, $p < 0.05$). No significant pruritic effect was observed with NMDA (75 mg/kg, IP) injection ($F(1, 28) = 0.0501$, $p > 0.05$). We conclude that NMDA per se does not induce considerable scratching behavior in mice but CQ (200 µg/site) induced scratching behavior, is significantly potentiated in the presence of NMDA (75 mg/kg, IP).

Combined sub-effective doses of MK-801 and L-NAME decrease chloroquine-induced scratching behavior

As NMDAR antagonists and NOS antagonists can suppress itch (17, 23, 25), we next asked if low, thus individually sub-effective doses of such compounds, would have an additive effect and decrease CQ-induced scratching. Sub-effective doses of L-NAME and MK-801 alone had no significant effect on CQ-induced scratching behavior, the combination of sub-effective doses of L-NAME (1 mg/kg) and MK-801 (0.1 mg/kg), significantly decreased CQ-induced scratching behavior ($F(3, 28) = 19.50$, $p < 0.0001$) (**Fig. 5**).

Intradermal administration of chloroquine increases the concentration of nitrite

Given that NO is associated with itch (38), we next asked if administration of CQ would increase the level of nitrite, the end product of NO metabolism in the skin. Intradermal CQ (400 µg/site) induced scratching behavior with maximum scratching behavior at 10 to 20 min after injection (**Fig. 6**A). A two-way ANOVA showed the effect of CQ 400 µg/site ($F(1, 56) = 32.28$, $p < 0.0001$), the effect of time ($F(3, 56) = 37.57$, $p < 0.0001$) and the effect of CQ 400 µg/site × time interaction ($F(3, 56) = 8.212$, $p = 0.0001$). Intradermal CQ significantly increased the concentration of nitrite compared to the saline group in 5 and 15 min in the site of injection with maximum concentration at 15 min after CQ administration (Fig. 6B). L-NAME (10 mg/kg, IP) and MK-801 (0.25 mg/kg, IP and 10 nm/site, ID) significantly decreased the nitrite levels 15 min after CQ 400 µg/site injection ($F(4, 35) = 27.72$, $p < 0.0001$) (**Fig. 7**).

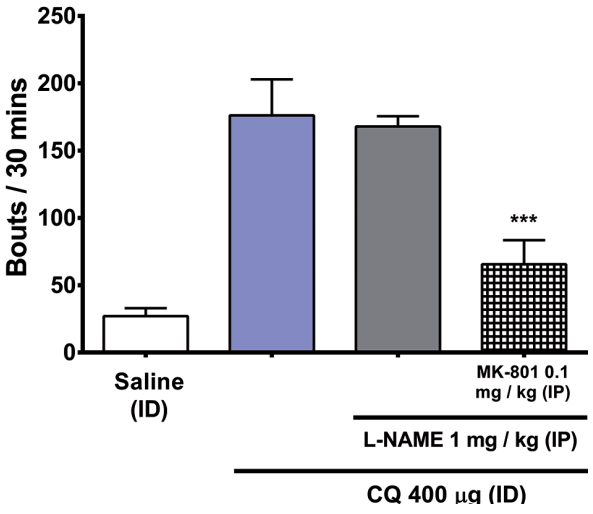

Fig. 5. The effect of MK-801 and L-NAME on chloroquine (CQ)-induced scratching behavior. Combined treatment with sub-effective doses of MK-801 (0.1 mg/kg, intraperitoneally (IP)) 45 min before and L-NAME (1 mg/kg, IP) 30 min before administration of CQ 400 µg intradermally (ID) significantly reduced CQ-induced scratching responses ($p = 0.0002$). Values are expressed as the mean ± SEM ($n = 8$). ***$p < 0.001$.

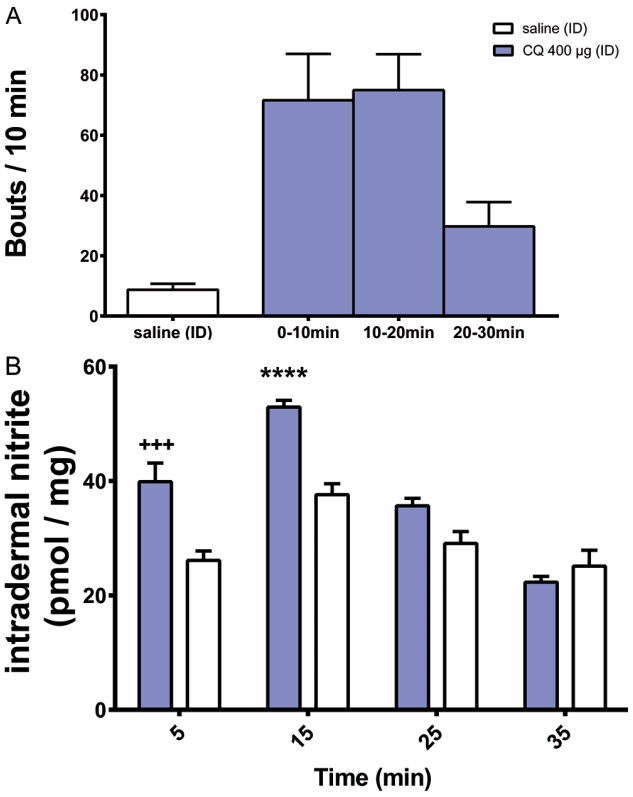

Fig. 6. Chloroquine (CQ)-induced scratching behavior and production of nitrite in mouse skin. (A) The time-course of scratching activity following intradermal injection of CQ 400 µg/site ($n = 8$). (B) The time-course of nitrite production following intradermal injection of CQ 400 µg/site. Skin injected with chloroquine was isolated 5, 15, 25 and 35 min after CQ injection. The concentration of nitrite is increased compared to the saline group in 5 ($p = 0.0004$) and 15 min ($p < 0.0001$) in the site of injection. Each group consisted of 8 animals. Values are expressed as mean ± SEM ($n = 8$) and were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test. +++$p < 0.001$, ****$p < 0.0001$.

DISCUSSION

The NMDA/NO pathway has been associated with pain (27) while inhibition of this pathway decreases pain and itch (23, 25–27). Activation of this pathway is also associated with increased production of NO (28). NO is associated with itch while inhibition of NOS suppresses itch (25, 26, 30). We reported recently that the NO/cGMP

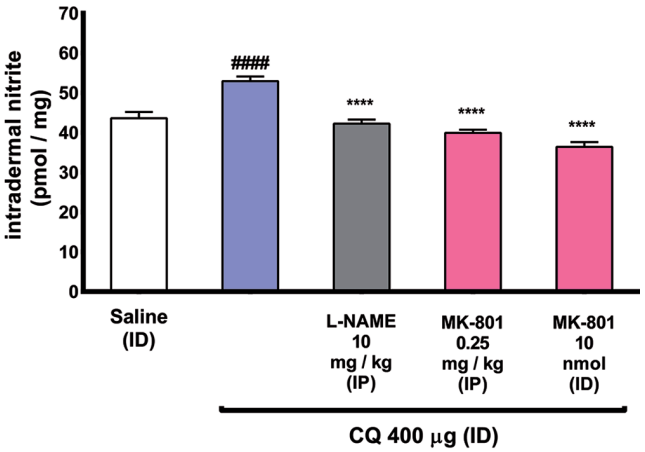

Fig. 7. L-NAME and MK-801 significantly decrease chloroquine-(CQ) induced nitrite levels in the skin. Mice were treated with L-NAME and MK-801 and nitrite levels were measured 15 min after CQ (400 μg/site) injection. Values are expressed as mean±SEM ($n=8$). ####$p<0.0001$ and ****$p<0.0001$ compared with saline and CQ alone, respectively.

pathway participates in CQ-induced itch (30). The data here extend these findings. These data demonstrate that the NMDA/NO pathway participates in CQ-induced scratching behavior. These data also reveal that CQ-induced scratching behavior is significantly decreased by the NMDA receptor antagonists MK-801, ketamine and $MgSO_4$. Conversely, CQ-induced scratching is potentiated in the presence of NMDA. Consistent with these findings, CQ was found to promote the generation of nitrite, a proxy for NO production, the production of which was decreased after intradermal and intraperitoneal injection of the NMDAR antagonist MK-801. The data thus support a role for peripheral NMDA receptors together with the NO pathway in CQ-induced itch.

CQ activates MrgprA3 and coupling of this receptor to TRPA1 via $G_{\beta\gamma}$ is necessary for CQ-induced itch (39). Chloroquine does not induce itch in TRPA1 knockout mice (9). Previous investigations have described interactions between glutamatergic signaling and TRPA1 channels (27). Intraplantar injection of formalin produces TRPA1-dependent biphasic nociceptive responses in rodents. Formalin directly activates TRPA1 channels on primary afferents followed by the release of inflammatory mediators (40). Glutamate, an endogenous NMDAR ligand (41), is also increased in the glabrous skin of the rat hind paw after formalin administration (42). TRPA1 channels may mediate CQ-induced itch by activating the glutamatergic system and NMDA receptors. Previous studies have demonstrated that more than 80% of the mouse dorsal root ganglion neurons responding to chloroquine, are immunopositive for vesicular glutamate transporter type 2 (VGLUT2) proposing a role for glutamate in CQ-induced itch (43). Glutamate binds the NMDAR, leading to ion channel opening and release of Mg^{2+} (44) which allows for an influx of Ca^{2+}, stimulation of nNOS activity and subsequent NO production (28). In addition to pain (27, 29, 45), the NMDA/NO synthase pathway has been implicated in modulation of many other behavioral processes including depression, learning, anxiety, aggression and locomotion (46–50). Our findings in the periphery, that activation of NMDA receptors following CQ injection stimulates NO production in skin, complement what has been found centrally.

NO functions as a neurotransmitter in itch (25, 26, 38, 51). Similar to our findings with CQ, previous studies have shown that NO enhances substance P-induced itch in mice (25). In addition, NO production is increased in the skin of patients with pruritic skin diseases such as atopic dermatitis and psoriasis (52, 53). Although, the exact cite of action of CQ-induced NO is not clear, it has been suggested that NO may directly activate primary sensory neurons (54). In addition, NO can also bind to TRPA1 and modulate its function by cysteine S-nitrosylation (55, 56). Keratinocytes and primary sensory neurons express NMDAR, nNOS and TRPA1 (34, 57–63) while CQ activates MrgprA3 (6). Sensory neurons may thus not be the only source of NO following intradermal CQ injection. CQ also induces mast cell degranulation, (64, 65) releasing mediators that interact with afferent nerve fibers in the skin.

In conclusion, our findings reveal that peripheral NMDA receptors and the production of NO in skin may be part of the pathway that underlies CQ-induced itch. Targeting peripheral NMDA receptors may be of benefit in the treatment of histamine-independent itch.

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The authors declare no conflicts of interest.

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