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Chloroquine-induced scratching is mediated by NO/cGMP pathway in mice

Arash Foroutan [a,b,c], Nazgol Sadat Haddadi [a,b], Sattar Ostadhadi [a,b], Narges Sistany [a,b], Ahmad Reza Dehpour [a,b,c,*]

[a] Experimental Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran
[b] Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[c] Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Chloroquine (CQ), a 4-aminoquinoline drug, has long been used in the treatment and prevention of malaria. However its side effect generalized pruritus contributes to treatment failures, and consequently results in the development of chloroquine resistant strains of *Plasmodium falciparum*. It was proposed that the administration of CQ correlated with increase in nitric oxide (NO) production. Nitric oxide is involved in some pruritic disorders such as atopic dermatitis, psoriasis and scratching behavior evoked by pruritogens like substance P. Therefore, the aim of this study was to investigate the involvement of NO/cGMP pathway in CQ-induced scratching in mice. Scratching behaviors were recorded by a camera after intradermal (ID) injection of CQ in the shaved rostral back of the mice. The results obtained show that CQ elicited scratching in a dose-dependent manner with a peak effective dose of 400 μg/site. Injection of non-specific NOS inhibitor, N-nitro-L-arginine methyl ester or neuronal NOS selective inhibitor and 7-nitroindazole, reduced CQ-induced scratching significantly. On the other hand, administration of aminoguanidine as inducible NOS inhibitor has no inhibitory effect on this behavior. Also, injection of L-arginine as a precursor of NO significantly increased this response. Conversely, accumulation of cGMP by sildenafil as a selective phosphodiesterase type 5 inhibitor, potentiated the scratching behavior by CQ. This study therefore shows that CQ-induced scratching behavior is mediated by the NO/cGMP pathway.

1. Introduction

Chloroquine is a drug that has long been used in the treatment of rheumatoid arthritis, systemic lupus and malaria (Wellems, 1992). The most common side effect associated with the use of CQ is pruritus; as observed in up to 70% of Black Africans, during the treatment of malaria fever (Ajayi et al., 1989). Pruritus or scratching is an unpleasant cutaneous sensation that results in scratching (Ikoma et al., 2006). It works as a protective mechanism to help preserve the skin against harmful agents (Ständer et al., 2003). The pruritogenic side-effect of CQ can cause reduced acceptability and compliance of patients during the treatment course, which may result to the development and spread of diseases such as CQ-resistant *Plasmodium falciparum* (Mnyika and Kihamia, 1991) malaria. Therefore, it is important to learn how this drug-related dermatologic disorder can be managed.

The molecular and cellular pathways mediating histamine-independent pruritus by chloroquine in primary sensory neurons are not largely clarified. Chloroquine has been displayed to act on the MrgprA3 receptor on sensory nerves to produce scratching, which is mediated by TRPA1 (Wilson et al., 2011). Previous studies and trials show that CQ-induced scratching can be mediated by endogenous opioid system, (Ajayi et al., 2004; Cowan and Inan, 2009; Inan and Cowan, 2004), in which mu (μ) receptor antagonists and kappa receptor agonists can reverse the scratching response (Ajayi et al., 2004; Olatunde, 1977). On the other hand, studies have also shown that some actions of opioids are mediated by nitric oxide (NO) (Nahavandi et al., 2001). however, the involvement of nitric oxide in CQ-induced scratching is still unknown. Moreover, it has been claimed that CQ-induced scratching is not an allergic reaction, because it happens even during the first exposure to CQ (Abila et al., 1994; Ezeamuzie et al., 1990);

In addition to the experimental model of pruritus (Andoh et al., 1998), further clinical evaluations of some skin diseases such as atopic dermatitis and psoriasis, have clarified the role of other mediators such as nitric oxide (NO) in pruritic disorders (Ormerod et al., 1998; Taniuchi et al., 2001) and acute inflammation (Ademowo et al., 1998).

Thus, the present study aimed to investigate whether the NO/cGMP pathway is involved in CQ-induced scratching.

Abbreviations: CQ, chloroquine; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; L-NAME, L-N-nitro arginine methyl ester; NOS, nitric oxide synthase; nNOS, neuronal NOS; 7-NI, 7-nitroindazole; AG, aminoguanidine.

* Corresponding author at: Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, P.O. Box 13145-784, Tehran, Iran. Tel.: +98 21 88973652; fax: +98 21 66402569.
E-mail address: dehpoura@sina.tums.ac.ir (A.R. Dehpour).

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2. Methods

2.1. Animals

Nuclear Magnetic Resonance Imaging (NMRI) was conducted on male mice (Pasteur Institute, Tehran, IRAN) of 5–6 weeks of age and weight range of 23–30 g was used. They were maintained in the facilities appropriate for the housing and care of mice in terms of environmental variables such as temperature (23–25 °C) and light (lights on from 08:00 AM to 08:00 PM) and had free access to food and water (Fawcett, 2012). All 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.

2.2. Materials

Chloroquine bisphosphate (Pubchem CID 64927) was received as a gift from Pars Darou Pharmaceutical Company (Tehran, Iran) and sildenafil citrate (Pubchem CID 62853) was purchased from Poursina Pharmaceuticals (Tehran, Iran). 7-Nitroindazole (Pubchem CID 1893), N-nitro-L-arginine methyl ester (L-NAME) (Pubchem CID 39836), aminoguanidine (Pubchem CID 2146) and L-arginine (Pubchem CID 6322), were purchased from Sigma Chemicals (St. Louis, MO, USA).

7-Nitroindazole was suspended in saline after adding a few drops of Tween-80 (Pubchem CID 5281955) purchased from Merck Chemicals (Darmstadt, Germany). Other agents were dissolved in the physiological saline.

The hair was removed from the rostral part of the back using hair removal creams. After two days, CQ was intradermally (ID) injected in a volume of 50 μl per site.

2.3. Behavioral experiments

The experimental groups and administration protocol conducted in the present study are shown in Table 1.

Before the experiments, all animals were separately placed in an acrylic cage (10 * 10 * 13 cm) at 23 ± 1 °C for 1 h for acclimation. A small amount of bedding was placed within the cages in order to absorb any urine voided during the experiment. After CQ injection, they were returned to the same cage and behaviors were recorded using a video camera in unmanned conditions. The video was played back by the expert observer to count the scratching of the injected site by the mice using the hind paws (Andoh et al., 1998). The mice generally demonstrated scratching for about 1 s and a series of movements were counted as one bout of scratching (Kuraishi et al., 1995).

2.4. Data analysis

Data were processed (GraphPad Prism 5.0 software) using one-way analysis of variance (ANOVA) along with Dunnett's Test for multiple comparisons and t-test analysis for two experiments. In all the experiments, $p < 0.05$ was regarded as significant and data were presented as mean ± S.E.M.

3. Result

Experiment (1) showed a significant effect of ID administration of different doses of CQ (200, 400, and 800 μg/site) on scratching behavior. One-way ANOVA analysis represented a significant effect ($F_{(3, 28)} = 117.9$, $p < 0.0001$). Post hoc Dunnett's multiple comparison test showed a significant pruritogenic effect for CQ at the doses of 400 and 800 μg/site compared with the saline-treated control animals. Four hundred micrograms per site of CQ, which induced a significant

Table 1
Injection procedures for experiment.

Groups	Pretreatment injection	Intradermal injection in 50 μl per site
Experiment 1 (Green et al., 2006; Inagaki et al., 2001)[a]		
Group 1	–	Saline
Group 2	–	CQ 200 μg
Group 3	–	CQ 400 μg
Group 4	–	CQ 800 μg
Experiment 2 (Andoh and Kuraishi, 2003)	(30 min before)	
Group 1	L-NAME 1 mg/kg IP	Saline
Group 2	L-NAME 3 mg/kg IP	Saline
Group 3	L-NAME 10 mg/kg IP	Saline
Group 4	L-NAME 1 mg/kg IP	CQ 400 μg
Group 5	L-NAME 3 mg/kg IP	CQ 400 μg
Group 6	L-NAME 10 mg/kg IP	CQ 400 μg
Experiment 3 (Andoh and Kuraishi, 2003)		
Group 1	–	L-NAME 100 nMol + Saline
Group 2	–	L-NAME 100 nMol + CQ 400 μg
Experiment 4 (Andoh and Kuraishi, 2003)		
Group 1	–	7-Nitroindazole 10 nMol + CQ 400 μg
Experiment 5 (Shahsavarian et al., 2014)	(45 min before)	
Group 1	Aminoguanidine 200 mg/kg IP	CQ 400 μg
Experiment 6 (Andoh and Kuraishi, 2003)	(30 min before)	
Group 1	L-Arginine 100 mg/kg IP	Saline
Group 2	L-Arginine 10 mg/kg IP	CQ 200 μg
Group 3	L-Arginine 100 mg/kg IP	CQ 200 μg
Experiment 7 (Andoh and Kuraishi, 2003)		
Group 1	–	L-Arginine 300 nMol + Saline
Group 2	–	L-Arginine 300 nMol + CQ 200 μg
Experiment 8 (Riazi et al., 2006)	(30 min before)	
Group 1	Sildenafil 20 mg/kg IP	Saline
Group 2	Sildenafil 5 mg/kg IP	CQ 200 μg
Group 3	Sildenafil 10 mg/kg IP	CQ 200 μg
Group 4	Sildenafil 20 mg/kg IP	CQ 200 μg

[a] References for injected doses.

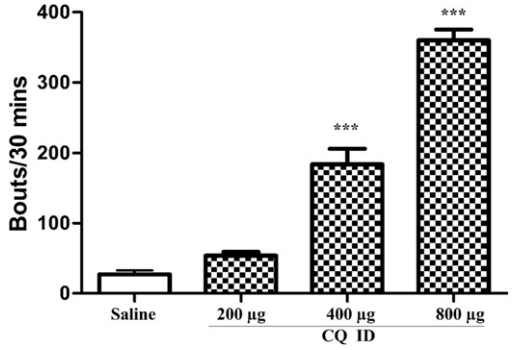

Fig. 1. CQ-induced scratching dose response. Different doses of CQ injected intradermally. Scratching behavior was analyzed by counting bouts per 30 min, and comparing it with the saline injection. As shown in the figure ***$p < 0.001$ compared with saline-treated control group. Each group consisted of eight mice (Experiment 1).

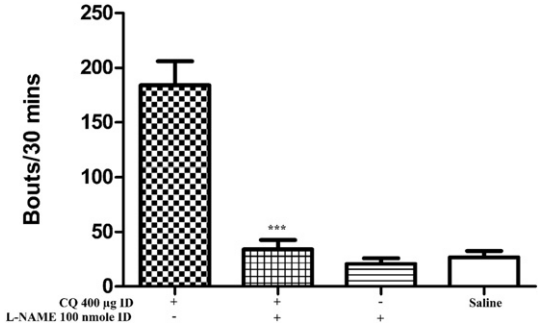

Fig. 3. Effect of co-administration 400 µg/site CQ and L-NAME ID compared to the ID injection of 400 µg CQ alone and 100 nmol/site L-NAME. Reversed scratching behavior was analyzed by counting bouts per 30 min and L-NAME injected groups. As it is shown above (***$p < 0.001$) compared with 400 µg/site CQ-treated group. Each group consisted of eight mice (Experiment 3).

pruritogenic effect on NMRI mice ($p < 0.001$), was selected for further experiments in order to allow better detection of possible pruritogenic effects (Fig. 1). In other studies, scratching was evoked by CQ at a dose of 200 µg/site; but in the present study, 400 µg/site was selected as an effective dose of CQ to elicit the response in NMRI mice. This may be attributed to the fact that different genotypes showed different responses to CQ (Green et al., 2006).

Experiment (2) was performed to show the effect of acute intraperitoneal (IP) administration of different doses of L-NAME (1, 3, and 10 mg/kg) for 30 min before the intradermal injection of CQ at the dose of 400 µg/site. Two control ($n = 8$) groups only received pretreatment injection at doses of 1, 3 and 10 mg/kg L-NAME, which represented lack of pruritogenic effect of L-NAME.

One-way ANOVA analysis showed a significant suppression of scratching activity on administration of L-NAME, before ID CQ injection ($F_{(7, 56)} = 23.67$, $p < 0.0001$). Dunnett's post hoc multiple comparison test showed that IP injection of 3 and 10 mg/kg of L-NAME significantly ($p < 0.001$) reduced CQ-induced scratching (Fig. 2).

Experiment (3) was carried out to determine the ID co-administration of 100 nmol/site of L-NAME and 400 µg/site of CQ. One-way ANOVA analysis showed that co-administration of these two agents reduced CQ-induced scratching significantly ($F_{(3, 28)} = 38.76$, $p < 0.0001$). Moreover, co-injection of 100 nmol/site L-NAME with 400 µg/site of CQ significantly reduced scratching activity compared with CQ alone ($p < 0.001$) (Fig. 3).

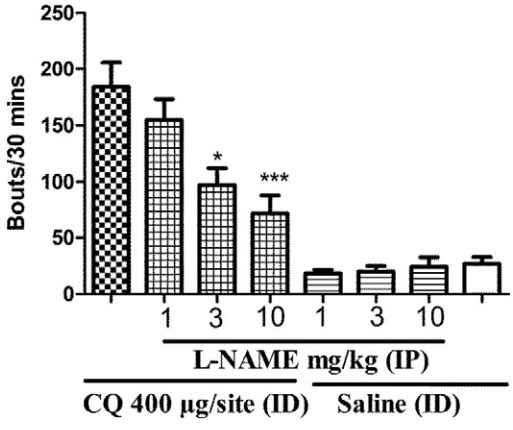

Fig. 2. The effect of pretreatment of the animals with the IP injection of L-NAME at 1, 3, and 10 mg/kg on the scratching response induced by the ID injection of 400 µg/site CQ. Analysis of the responses as a scratching behavior was achieved by counting the bouts per 30 min and comparing it with the CQ injected group. As shown in the figure *$p < 0.05$ and ***$p < 0.001$ compared with 400 µg/site CQ-treated group. IP injection of L-NAME has no effect on response in saline-treated animals (ID). Each group consisted of eight mice (Experiment 2).

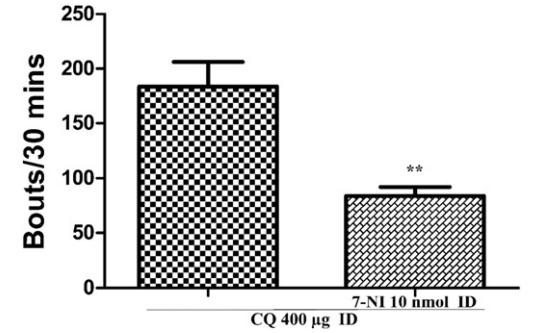

Fig. 4. Comparison of the effect of ID co-injection of 10 nmol of 7-nitroindazole (7-NI) and 400 µg/site CQ with response of the condition in which only 400 µg/site CQ is injected. 7-NI significantly reversed the scratching response induced by 400 µg/site CQ and **$p < 0.01$ compared with 400 µg/site CQ ID injection alone. Each group consisted of eight mice (Experiment 4).

In Experiment (4), in order to evaluate the peripheral role of nNOS on CQ-induced scratching, 7-nitroindazole at 10 nmol/site was intradermally injected simultaneously with 400 µg/site of CQ. This injection significantly reduced scratching activity of CQ (independent t-test, $p = 0.0086$) (Fig. 4).

Experiment (5) shows that aminoguanidine is an iNOS inhibitor, IP injection at 200 mg/kg for 45 min before the ID injection of 400 µg/site with CQ. Independent t-test showed that aminoguanidine could not significantly reverse the scratching response ($p = 0.4972$) (Fig. 5).

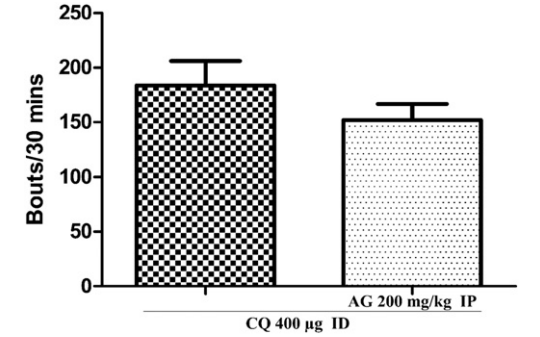

Fig. 5. Reversing pruritic response in the pretreated animals by IP injection of aminoguanidine (AG) as a specific iNOS inhibitor at dose of 200 mg/kg 45 min before the ID injection of 400 µg/site CQ was assessed. The number of bouts per 30 min indicated that the difference was non-significant ($p > 0.05$) compared with another group whose animals had ID injection of 400 µg/site CQ. Each group consisted of eight mice (Experiment 5).

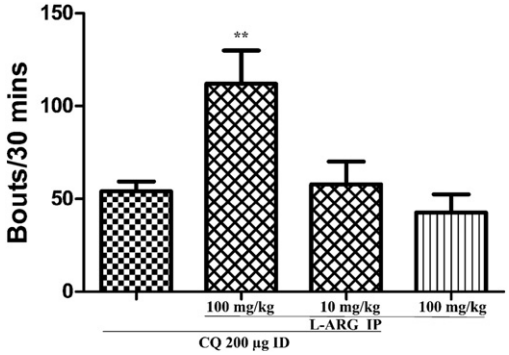

Fig. 6. The effect of pretreatment with NO precursor, L-arginine (L-ARG), at doses of 10 and 100 mg/kg IP on the pruritogenic effect of sub-effective 200 µg/site ID dose of CQ. **p < 0.01 compared with the group merely got 200 µg/site CQ. Each group consisted of eight mice (Experiment 6).

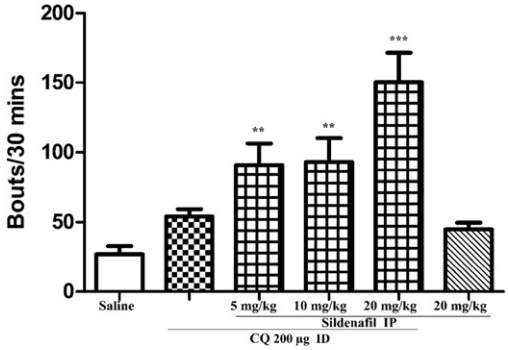

Fig. 8. The effect of pretreatment with sildenafil at different doses of 5, 10, and 20 mg/kg and potentiating response of scratching. IP injection of sildenafil 30 min before injection of sub-effective dose of CQ (200 µg/site) showed a significant increase vs saline group. (**p < 0.01) at doses of 5 and 10 mg/kg and (***p < 0.001) 20 mg/kg in response (Experiment 8).

Experiment (6) was carried out to show the effect of pretreatment with different doses of L-arginine, as a precursor of NO at 10 and 100 mg/kg for 30 min before the injection of a sub-effective dose of CQ (200 µg/site). One-way ANOVA analysis showed a significant increase in the scratching response ($F_{(3, 28)} = 6.35$, $p < 0.01$). Dunnett's multiple comparison test shows a significant difference between the L-arginine pretreated group and the group that only received the sub-effective 200 µg/site dose of CQ ($p < 0.01$) (Fig. 6).

Experiment (7) was undertaken to potentiate scratching response by injecting 300 nmol/site L-arginine as the precursor of NO at the time of injecting the sub-effective dose of CQ (200 µg/site) ($p < 0.05$). Data analysis by one-way ANOVA showed a significant increase in response to scratching ($F_{(2, 21)} = 5.86$, $p < 0.01$) (Fig. 7).

Experiment (8) indicated the involvement of cGMP in scratching and sildenafil (Riazi et al., 2006). Thus IP injection as a selective phosphodiesterase type 5 inhibitor at doses of 5, 10, and 20 mg/kg for 30 min before the injection of the sub-effective dose of CQ (200 µg/site). One-way ANOVA analysis showed a significant increase in response to scratching ($F_{(5, 42)} = 10.94$, $p < 0.0001$) and Dunnett's Test showed a significant difference when the sildenafil treated group at dose of 20 mg/kg was compared with the experimental group that received a sub-effective dose of CQ ($p < 0.001$) (Fig. 8).

4. Discussion

In this study, the results show that CQ induces scratching in a dose-dependent manner and was suppressed by L-NAME (a non-selective NOS inhibitor). This demonstrates that NO has a potential role in CQ-induced scratching. Meanwhile, aminoguanidine as an inducible NOS inhibitor (Griffiths et al., 1993) had no effect on scratching, represents

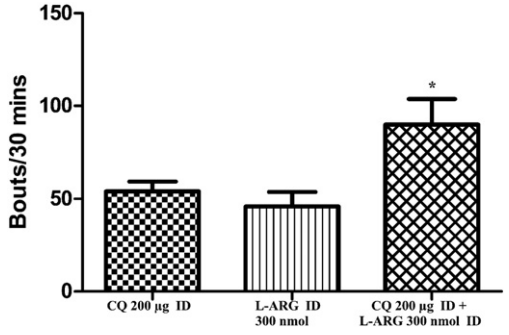

Fig. 7. ID co-injection of L-arginine (L-ARG) as a NO precursor at dose of 300 nmol/site and sub-effective 200 µg/site dose of CQ showed a significant increase in the number of bouts per 30 min in comparison with the group which only received the sub-effective dose of CQ (*p < 0.05). Each group consisted of eight mice (Experiment 7).

that inducible NOS is not involved in the induction of this response. Regarding the inhibitory effects of 7-nitroindazole as a selective neuronal NOS inhibitor (Ayajiki et al., 2001) on scratching behavior, it seems that nNOS plays a crucial role in this phenomenon. On the other hand, based on our findings, sildenafil, a selective phosphodiesterase 5 inhibitor (Boolell et al., 1996; Jackson et al., 1999; Riazi et al., 2006), can potentiate CQ-induced scratching and increase the scratching activity of CQ through the accumulation of cGMP.

Fundamentally, malaria accounts for approximately 1–2 million deaths per year, and African children below the age of 5 years are principally susceptible (Woster, 2003). In the past 40 years, CQ has been an important anti-malaria drug with a quinolone-ring chemical structure (Fitch, 2004) and is used in the treatment of systemic lupus (Borba et al., 2004), rheumatoid arthritis (Romanelli et al., 2004) and some viral infections (Aghahowa et al., 2010; Savarino et al., 2003; Wellems, 1992). Pruritus is one of the most common side effects of the administration of chloroquine, especially in dark skinned Africans, Caucasians, and African albinos, and can lead to reduced acceptability and compliance of patients (Aghahowa et al., 2010). Other side effects of chloroquine include psychosis, delirium and behavioral changes (Good and Shader, 1982).

Furthermore, pruritus is an unpleasant sensory and emotional experience, which causes the desire to scratch the skin and may occur after a stimulus (Paus et al., 2006) and develops by various mediators (Hägermark, 1992). Investigating CQ-induced scratching, which is mainly suggested as a histamine-independent response for CQ, results in further understandings about its exact mechanisms (Abila et al., 1994; Ezeamuzie et al., 1990). Previous studies have demonstrated the role of endogenous opioids (Ajayi et al., 2004; Cowan and Inan, 2009; Inan and Cowan, 2004; Onigbogi et al., 2000), gastrin-releasing peptide receptors (Liu et al., 2009), and toll-like receptor 7 in CQ-induced scratching (Liu et al., 2010).

Opioids are among the mediators for CQ-induced scratching (Ajayi et al., 2004); as such, mu (μ) antagonists and kappa agonists can reverse it (Ajayi et al., 2004; Inan and Cowan, 2004). On the other hand, NO and opioids are involved in various processes such as analgesia (Brignola et al., 1994; Ferreira et al., 1991), tolerance and dependency (Dambisya and Lee, 1996; Kolesnikov et al., 1993), thermal regulation (Benamar et al., 2001) and gastric protection (Gyires, 1994). Pharmacological data have also shown that opioids can release NO in the neural system (Homayoun et al., 2002; Nahavandi et al., 2001). However, correlation of NO with different opioid activities led to the investigation of the crosstalk between scratching and NO. Several kinds of cells including endothelial cells, keratinocytes, macrophages, and primary sensory neurons can release NO in the skin (Moncada et al., 1991).

The results of this study show that, although, CQ-induced scratching response decreased after intradermal injection of L-NAME and 7-NI,

aminoguanidine as iNOS inhibitor could not reduce it. Nitric oxide is generated when L-arginine is transformed to L-citrulline via catalysis by NOS. Intracellular calcium is essential for the stimulation of neuronal NOS and endothelial NOS, but not inducible NOS. nNOS, a soluble enzyme, is constitutively expressed in the brain and peripheral nerves (Förstermann and Sessa, 2012). As previously reported, CQ increased the intracellular level of calcium in neurons (Wilson et al., 2011). Therefore, nNOS and eNOS, but not iNOS, may be involved in the CQ-induced NO production. It was also previously reported that increased epidermal level of NO by nNOS after intradermal injection of substance P, is necessary for scratching behavior (Andoh and Kuraishi, 2003). An intradermal administration of 7-nitroindazole significantly reduced the CQ-induced scratching. Since 7-nitroindazole is a selective inhibitor of nNOS (Ayajiki et al., 2001), the findings of this study proposed that nNOS plays an essential role in the CQ-induced NO production in the skin. Though, since 7-nitroindazole also suppresses eNOS at higher concentrations (Ayajiki et al., 2001), the involvement of eNOS cannot be ruled out. Endothelial cells in the skin express eNOS (Shimizu et al., 1997), and CQ stimulates the release of NO from murine, porcine, and human endothelial cells (Ghigo et al., 1998). Thus, it is also possible for intradermal CQ to induce the release of NO from the endothelial cells in the skin.

This study reveals that CQ can stimulate NOS activity in the skin to produce itch sensation; however, the exact target cells, which release NO and the mechanisms of CQ in the skin, could not be completely evaluated in this study. More experiments should be conducted to understand the mechanism of this response. The other possible mechanism is the involvement of NO in TRPA1 activation after chloroquine induced-MrgprA3 stimulation. The Mas-related G protein-coupled receptor (Mrgpr) family has appeared as a new class of histamine-independent itch receptors (Liu et al., 2009). MrgprA3 is a receptor for chloroquine-induced scratching (Bautista et al., 2014). Chloroquine induced itch also needs the TRPA1 receptor, which acts as the primary transduction channel downstream of MrgprA3 (Wilson et al., 2011). Increased intracellular calcium level after CQ administration (Wilson et al., 2011) can increase the calcium-dependent nNOS activity and NO level. TRPV1 and TRPA1 mediate peripheral nitric oxide-induced nociception (Miyamoto et al., 2009). Nitric oxide activates TRP channels by cysteine S-nitrosylation (Yoshida et al., 2006) and this activation may lead to neuronal depolarization and itch transmission. The other possible mechanism is the activation of adenylyl cyclase by Gβγ subunit of MrgprA3. It was shown that although PLCB is not involved in Mrgpra3 signaling, Gβγ subunit is necessary for this (Wilson et al., 2011). Moreover, Gβγ subunit has distinct effects on some classical second messenger enzymes such as adenylyl cyclase (Sunahara et al., 1996). Adenylyl cyclase catalyzes the conversion of adenosine triphosphate (ATP) to 3′, 5′-cyclic AMP (cAMP). The activation of cAMP-dependent protein kinase A (PKA) results in nNOS phosphorylation and finally increased the level of NO (Yen et al., 2011). Finally, as described above, nitric oxide activates TRP channels (Yoshida et al., 2006), but further studies are required in order to evaluate the role of MrgpA3/AC/PKA/NO/TRPA1 pathway on chloroquine-induced pruritus, as well as establishing the role of NO in this pathway.

Although NO-mediated physiological signaling by S-nitrosylation (Jaffrey et al., 2001), one of the well-established effects of NO is the activation of soluble guanylyl cyclase (Arnold et al., 1977). As such, in the next step, NO/cGMP's involvement in CQ-induced scratching was investigated. Sildenafil is a selective phosphodiesterase type 5 inhibitor (Boolell et al., 1996), which promotes the effect of nitric oxide (NO) in target tissues by inhibiting cGMP degradation (Corbin and Francis, 1999). The result of this study shows that sildenafil increased CQ-induced scratching significantly when administrated before sub-effective dose of CQ. Thus, it is understood that the NO/cGMP pathway is involved in the CQ-induced scratching behavior.

Finally, it can be concluded that CQ elicits scratching at least in part via the NO/cGMP pathway and the role of nNOS is more prominent than iNOS and eNOS, therefore, it could be a new therapeutic target for treating pruritus. Indeed, more experiments are required to develop the current knowledge about this mechanism and treatment.

Acknowledgments

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