Immunoopharmacology and Inflammation

Anti-inflammatory effect of the spirocyclopiperazinium compound LXM-10 in mice and rats

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ABSTRACT

Anti-inflammatory drugs are clinically limited because of their side effects. The aim of this study was to evaluate the anti-inflammatory activities and mechanisms of the spirocyclopiperazinium compound LXM-10 (2, 4-dimethyl-9-β-phenylethyl-3-oxo-6, 9-diazaspiro[5.5]undecane chloride). We found that LXM-10 produced a significant, dose-dependent decrease in xylene- and carrageenin-induced edema. The anti-inflammatory effect was attenuated by hexamethonium, methyllycaconitine citrate, atropine methylnitrate, and tropicamide. The serum level of TNF-α was reduced by LXM-10 in lipopolysaccharide-challenged mice, and this effect was also inhibited by methyllycaconitine and tropicamide. LXM-10 also reduced the prostaglandin E2 concentration in rat paw tissue. LXM-10 minimised the carrageenin-induced pathological changes and did not affect mice heart rate. LXM-10 did not induce significant changes in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) or alkaline phosphatase (ALP) activity. Median lethal dose (LD_{50}) of LXM-10 was 1573.0 µmol/kg. Our findings suggest that LXM-10 has anti-inflammatory effects by activating α7 nicotinic and M4 muscarinic acetylcholine receptors with limited side effects.

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1. Introduction

Conventional steroidal and nonsteroidal anti-inflammatory drugs are effective (Davies et al., 2000) but have side effects that limit their utility (James and Hawkey, 2003). Steroids can cause immunosuppression, osteoporosis, and metabolic disorders and can retard growth in children (Davis, 1999). Nonsteroidal anti-inflammatory drugs are associated with adverse gastrointestinal, renal, and cardiovascular side effects (Subramanian et al., 2008). Even the selective cyclooxygenase 2 inhibitors have adverse effects (Wallace, 2002). The need is great for anti-inflammatory drugs that are better tolerated and have a more favorable side-effect profile.

Acetylcholine receptor agonists have potential to reduce inflammation (van Westerloo et al., 2006), but as with other anti-inflammatory drugs, their use has been limited by side effects (Decker et al., 1994; Eglen, 2005). The compound LXM-10 (2, 4-dimethyl-9-β-phenylethyl-3-oxo-6, 9-diazaspiro[5.5]undecane chloride), an acetylcholine receptor agonist, is a spirocyclopiperazinium salt compound (Fig. 1) that does not affect motor performance, spontaneous activity, or body temperature in mice, and the minimal lethal dose is 445.0 mg/kg, s.c. (Yue et al., 2007). It may have potential as an anti-inflammatory drug that has fewer side effects. In this study, we evaluated the anti-inflammatory effects of LXM-10 and explored potential mechanisms of these effects.

2. Methods

2.1. Animals

For this study, we used both sexes of adult ICR and BALB/c mice (20–22 g) and Sprague–Dawley rats (180–200 g). The animals were provided by the Department of Laboratory Animal Science of Peking University, and all experiments were approved by the Institutional Animal Care and Use Committee of Peking University. All experiments were performed in awake, unrestrained, age-matched animals and were conducted in a blinded manner. We made efforts to minimise animal suffering, to reduce the number of animals used, and to utilise alternatives to in vivo techniques, if available.

2.2. Drugs and administration

LXM-10 was synthesized by Runtao Li and Qi Sun. Atropine methylnitrate, hexamethonium chloride, methyllycaconitine citrate,
tropicamide, aspirin, carageenin, and lipopolysaccharide were purchased from Sigma (St. Louis, MO, USA). Dexamethasone was purchased from Jinjiao Pharmaceutical Corporation (PR China). Drug doses were selected on the basis of previous reports and our preliminary studies (Beirith et al., 1998; Boccia et al., 2001; Decker et al., 1998). Carageenin and lipopolysaccharide were diluted in a saline solution (0.9% NaCl), CCl₄ was diluted in peanut oil, and all other drugs were dissolved in distilled water immediately before use. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 10 µl/kg by subcutaneous (s.c.), intraperitoneal (i.p.) or intragastric (i.g.) injection.

2.3. Xylene-induced ear edema in mice

The effect of LXM-10 on acute topical inflammation was assessed as previously described (Vogel and Vogel, 1997). Thirty minutes after treatment of mice with LXM-10 (20, 10, or 5 µmol/kg, s.c.), dexamethasone (20 µmol/kg, s.c.), aspirin (2490 µmol/kg, i.g.) or vehicle, edema was induced in each mouse by applying 50 µl of xylene to the inner and posterior surface of the right ear. One hour later, the animals were killed, and both ears were removed. We collected and weighed 6-mm² ear punch biopsy specimens. We calculated ear edema weight by subtracting the weight of the left ear specimen from the weight of the right ear specimen. We determined the degree of inhibition of inflammation by using the following formula: inhibition (%) = [(edema weight of control group − edema weight of experimental group)/edema weight of control group] × 100.

2.4. Carageenin-induced hind paw edema in rats

We injected 100 µl of 1% carageenin solution into the left hind paws of rats (Winter et al., 1962). LXM-10 (24, 12, or 6 µmol/kg, s.c.), dexamethasone (12 µmol/kg, s.c.), aspirin (1660 µmol/kg, i.g.) or vehicle was given 1 h before carageenin injection. The paw edema volume was determined by using a plethysmometer (model YLS-78, Shandong Academy of Medical Sciences, PR China) before and 0.5, 1, 2, 3, and 4 h after carageenin injection. Inflammation was estimated from the difference in paw volume before and after carageenin injection. We calculated edema volume by subtracting the paw volume before carageenin injection from the volume after injection. Degree of inhibition of edema was calculated according to the following formula: inhibition (%) = [(edema volume of control group − edema volume of experimental group)/edema volume of control group] × 100.

2.5. Involvement of nicotinic and muscarinic acetylcholine system

To elucidate the anti-inflammatory mechanisms of LXM-10, mice in the ear edema test were pretreated with one of the following receptor antagonists: hexamethonium, a peripheral neuronal nicotinic acetylcholine receptor antagonist (15, 3, or 0.6 µmol/kg, i.p.); methyllycaconitine citrate, an α7 nicotinic acetylcholine receptor antagonist (3, 1, or 0.3 µmol/kg, i.p.);; methyllycaconitine citrate, an α7 nicotinic acetylcholine receptor antagonist (3, 1, or 0.3 µmol/kg, i.p.); atropine methyl nitrate, a peripheral muscarinic acetylcholine receptor antagonist (15, 3, or 0.6 µmol/kg, i.p.); or tropicamide, an M₄ muscarinic acetylcholine receptor antagonist (3, 1, or 0.3 µmol/kg, i.p.). Fifteen minutes after injection of the antagonist, animals received the injection of LXM-10 (20 µmol/kg, s.c.), dexamethasone (20 µmol/kg, s.c.), aspirin (2490 µmol/kg, i.g.) or vehicle. Other animals were pretreated with vehicle as control, and after 15 min the animals received the injection of LXM-10 (20 µmol/kg, s.c.), dexamethasone (20 µmol/kg, s.c.), aspirin (2490 µmol/kg, i.g.) or vehicle.

2.6. Measurement of serum tumor necrosis factor (TNF)-α level

Mice were pretreated with methyllycaconitine citrate, an α7 nicotinic acetylcholine receptor antagonist (3 µmol/kg, i.p.), tropicamide, an M₄ muscarinic acetylcholine receptor antagonist (3 µmol/kg, i.p.) or vehicle; after 15 min, the animals received the injection of LXM-10 (20 µmol/kg, s.c.). Other animals were pretreated with vehicle, and after 15 min the animals received the second injection of vehicle. Thirty minutes later, experimental-group mice were challenged with lipopolysaccharide (1 mg/kg, i.p.), and control-group mice received an equal volume of saline. Two hours after challenge, blood was collected into plastic tubes and stored on ice before centrifugation at 800 × g at 4°C for 15 min, and then serum was removed and collected (Choi et al., 2007). Serum level of TNF-α was determined by using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA), according to the manufacturer's instruction.

2.7. Measurement of tissue prostaglandin E₂ level

LXM-10 (24 µmol/kg, s.c.), aspirin (1660 µmol/kg, i.g.), or vehicle were given 1 h before carageenin injection. Rats were killed 2 h after carageenin injection to determine paw prostaglandin E₂ level (Nantel et al., 1999). The paws were separated at the calcaneus bone, degloved, frozen in liquid nitrogen, and stored at −80°C until use. Ethanol was added to the sample and homogenized. Specimens were incubated at 4°C for 5 min, and then centrifuged at 3000 × g for 10 min to remove precipitated proteins. We adjusted the pH of the resulting supernatant to 4.0 by using 1.0 M citrate buffer. The adjusted supernatant was applied to 6 ml Sep-Pak C18 cartridges (Waters Associate) and eluted with 5 ml ethyl acetate containing 1% methanol. The eluent was dried with nitrogen, and prostaglandin E₂ concentration was measured with an ELISA kit (Cayman Chem, USA), according to the manufacturer's instruction.

2.8. Histological examination

In the carageenin-induced hind paw edema test, biopsies of paws from LXM-10 (24 µmol/kg, s.c.) or vehicle treated rats were taken 2 h after the injection of carageenin. The tissue slices were fixed in 10% formalin, embedded in paraffin, and sectioned. The sections were stained with haematoxylin and eosin.

2.9. Assessment of heart rate

In a separate analysis of the effect of LXM-10 on heart rate, we treated mice with LXM-10 (40 or 20 µmol/kg, s.c.) or vehicle. We measured heart rate with a Cardiofax monitor (model ECG-6511, Shanghai, PR China) 0.5 h, 2 h, and 3.5 h after treatment.

2.10. Determination activities of ALT, AST and ALP

We measured the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) spectrophotometrically in serum of rats. Rats were treated with LXM-10 (48, 24 µmol/kg, s.c.), CCl₄ (10%, i.p.) or vehicle. We collected blood 24 or 48 h later and stored it in ice before centrifugation at 1000 × g at 4°C for 10 min. Then we removed and collected the serum. The activities of ALT, AST and ALP were determined by using assay kits (Nanjing Jiancheng Bioengineering Institute, PR China). Absorbance of
the reaction mixture was measured by a spectrophotometer at 505 nm for ALT and AST and 520 nm for ALP.

2.11. Acute toxicity study

Mice were divided into five groups and were administered with LXM-10 at doses of 1751.2, 1646.1, 1547.2, 1454.4, and 1367.1 µmol/kg, s.c. respectively. All animals were observed for toxic symptoms and death for 24 h.

2.12. Statistical analysis

There were 8 animals per group in the determination activities of ALT, AST and ALP, all other groups had 10 animals each. We used one-way analysis of variance followed by Bonferroni or repeated-measures analysis of variance to test the difference between groups. Differences were considered significant at $P<0.05$.

3. Results

3.1. Anti-inflammatory activity of LXM-10

LXM-10 significantly reduced ear edema induced by xylene in a dose-dependent fashion (Fig. 2A). At doses of 20, 10, and 5 µmol/kg, inhibition ratios were 64%, 58%, and 46%, respectively. Consistent with its effects on ear edema, LXM-10 also decreased paw edema induced by carrageenin in a dose- and time-dependent fashion (Fig. 2B). The difference from vehicle was greatest 2 h after injection; the inhibition ratios of LXM-10 were 52%, 44%, and 36%, respectively, at doses of 24, 12, and 6 µmol/kg. We chose dexamethasone which is a typical steroid or aspirin a nonsteroidal anti-inflammatory drug as controls in the two models.

3.2. Potential anti-inflammatory mechanisms of LXM-10

3.2.1. Involvement of nicotinic acetylcholine system

When mice were predosed with hexamethonium (15, 3, 0.6 µmol/kg, i.p.), the inhibition ratio of LXM-10 was reduced to 6%, 16%, and 36% at doses of 20, 10, and 5 µmol/kg, respectively. When mice were predosed with methyllycaconitine (MLA, 3, 1, 0.3 µmol/kg, i.p.), the inhibition ratio of LXM-10 was reduced to 6%, 16%, and 36% at doses of 20, 10, and 5 µmol/kg, respectively.

Fig. 2. Inhibitory effect of LXM-10 in the inflammation model. (A) LXM-10 reduced the ear edema induced by xylene in mice. The mice were administered LXM-10 (20, 10, 5 µmol/kg, s.c.), dexamethasone (Dex, 20 µmol/kg, s.c.), aspirin (2400 µmol/kg, i.g.) or vehicle (Vel). LXM-10 produced a significant and dose-dependent decrease in xylene-induced edema. (B) LXM-10 attenuated the paw edema induced by carrageenin in rats. LXM-10 (24, 12, 6 µmol/kg, s.c.) decreased the carrageenin-induced paw edema at each time period in a dose-dependent fashion. Dexamethasone (12 µmol/kg, s.c.) and aspirin (1660 µmol/kg, i.g.) also significantly inhibited the edema induced by carrageenin. All data are mean ± S.E.M. of 10 animals per group. Significant differences from the vehicle group at the same time period are indicated by *$P<0.05$ and **$P<0.01$.

Fig. 3. Anti-inflammatory activity of LXM-10 was reversed by nicotinic receptor antagonists in xylene-induced mice ear edema. (A) Hexamethonium (Hex, 15, 3, 0.6 µmol/kg, i.p.) administered before LXM-10 (20 µmol/kg, s.c.) blocked the reduction of ear edema caused by LXM-10 in mice. (B) Methyllycaconitine (MLA, 3, 1, 0.3 µmol/kg, i.p.) also inhibited the anti-inflammatory effect of LXM-10 (20 µmol/kg, s.c.) in a dose-dependent fashion. Each bar represents mean ± S.E.M. of 10 mice per group. *$P<0.05$ and **$P<0.01$ vs Vel/Vel group; †$P<0.05$ and ††$P<0.01$ vs Vel/LXM-10 group at the same time point.
31% (Fig. 3A). Hexamethonium blocked the anti-inflammatory effect of LXM-10 at the higher doses (15, 3 µmol/kg, i.p.). Hexamethonium (15 µmol/kg, i.p.) did not affect the anti-inflammatory effect of dexamethasone and aspirin. In the presence of methyllycaconitine citrate (3, 1, 0.3 µmol/kg, i.p.), the anti-inflammatory effect of LXM-10 was reduced to 3%, 25%, and 30% (Fig. 3B). Methyllycaconitine citrate blocked the effect at the highest dose (3 µmol/kg, i.p.).

3.2.2. Involvement of muscarinic acetylcholine system

Atropine methylnitrate (15, 3, 0.6 µmol/kg, i.p.) also significantly inhibited the anti-inflammatory effect of LXM-10 to inhibition ratios of 17%, 27%, and 35% (Fig. 4A). Atropine methylnitrate blocked the effect of LXM-10 at the highest doses (15 µmol/kg, i.p.). Atropine methylnitrate (15 µmol/kg, i.p.) did not affect the anti-inflammatory effect of dexamethasone and aspirin. Tropicamide (3, 1, 0.3 µmol/kg, i.p.) reduced the effect of LXM-10 to inhibition ratios of 15%, 26%, and 30% and blocked the effect at the highest dose (3 µmol/kg, i.p.) (Fig. 4B).

3.2.3. Effect on serum TNF-α

Lipopolysaccharide increased serum TNF-α level from 28 pg/ml to 225 pg/ml. LXM-10 (20 µmol/kg) inhibited the lipopolysaccharide-induced increase of TNF-α by 38%. Methyllycaconitine citrate and tropicamide attenuated this effect to 10% and 15%, respectively (Fig. 5).

3.2.4. Effect on prostaglandin E2 level

The anti-inflammatory effect of LXM-10 was also observed in the tissue level of prostaglandin E2. Two hours after carrageenin injection, the prostaglandin E2 level in the paw was higher than in paws of unchallenged animals (Fig. 6). Treatment with LXM-10 (24 µmol/kg, s.c.) reduced the paw prostaglandin E2 level by 23% in carrageenin-treated animals. In comparison, aspirin lowered the prostaglandin E2 level by 40%.

3.3. Histological analysis

Two hours after carrageenin injection, histological examination of biopsy specimens from rat paws revealed marked inflammatory changes. We observed edema, a spongy-like appearance and bulla in...
epidermis (Fig. 7B) and neutrophil infiltrates in the dermis (Fig. 7C). Treatment with LXM-10 (24 µmol/kg, s.c.) minimised the pathological changes—the epidermis looked normal (Fig. 7D)—and reduced the neutrophil infiltration in the dermis (Fig. 7E).

3.4. Effect on heart rate

Heart rate values in each group are presented in Table 1. Compared with control, LXM-10 (40 or 20 µmol/kg, i.p.) had no significant effect on heart rate in mice 0.5, 2.0, or 3.5 h after administration.

3.5. Effect on ALT, AST and ALP activities

The effects of LXM-10 on serum ALT, AST, and ALP activities are shown in Table 2. Rats treated with CCl₄ developed significant hepatotoxicity at 24 and 48 h compared with the normal group, and serum ALT, AST and ALP were elevated markedly. LXM-10 (48 µmol/kg and 24 µmol/kg) slightly increased the level of AST at 24 and 48 h, but the values were still in normal ranges. Administration of LXM-10 (48 µmol/kg and 24 µmol/kg) had no significant effects on the levels of serum ALT and ALP.

3.6. Acute toxicity study

The death rates were 90%, 60%, 50%, 20%, and 10%, when mice were administered LXM-10 at doses of 1751.2, 1646.1, 1547.2, 1454.4, and

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Mice pretreated with LXM-10 (40 or 20 µmol/kg, i.p.) had no significant effect on heart rate at 0.5, 2.0, or 3.5 h. The values represent mean ± S.E.M. of 10 mice per group. Differences were considered significant at $P<0.05$.
1367.1 µmol/kg, s.c. respectively. Median lethal dose (LD₅₀) was analyzed by SPSS software as 1573.0 µmol/kg (95% confidence interval 1509.0–1641.8 µmol/kg).

4. Discussion

We found that LXM-10 significantly reduced ear edema induced by xylene in mice and paw edema induced by carrageenin in rats. The effect was dose-dependent, and the highest dose of LXM-10 reduced ear edema by 64% and paw edema by 52%. Both the acute toxicity study and assays for serum hepatic enzymes showed that LXM-10 possessed limited side effects.

Recent studies have shown the vagus nerve to be a target for reducing inflammation. Stimulating the vagus nerve can control systemic inflammation in rodents (Bernik et al., 2002). Acetylcholine, the principal neurotransmitter of the vagus nerve, can limit the production of pro-inflammatory cytokines from human macrophages (Miyazawa et al., 2003). Selective nicotinic agonists are a potential pharmacologic strategy to control inflammation.

Previous studies show that LXM-10 produces an antinociceptive effect by activating peripheral neuronal nicotinic acetylcholine receptors (Yue et al., 2007). Therefore, we tested whether nicotinic acetylcholine receptors are also involved in the anti-inflammatory mechanism. We found that the effect was blocked by hexamethonium, which shows that the anti-inflammatory effect of LXM-10 is related to peripheral nicotinic acetylcholine receptors.

Research has identified the critical importance of the α7 nicotinic acetylcholine receptor in mediating vagus nerve anti-inflammatory signaling (Gallowitsch-Puerta and Pavlov, 2007; Wang et al., 2003). The α7 receptor is expressed on non-neuronal cells, including macrophages, lymphocytes, endothelial cells, dermicit cells, and keratinocytes. α7 receptor agonists inhibit inflammatory cytokine release in a variety of experimental inflammatory models (Gallowitsch-Puerta and Pavlov, 2007). To explore whether the α7 receptor is involved in the anti-inflammatory effect of LXM-10, we tested the effect of methyllycaconitine citrate. We found that methyllycaconitine citrate blocks the anti-inflammatory effect of LXM-10, indicating that the effect of LXM-10 is related to activation of peripheral α7 receptors.

Although muscarinic acetylcholine receptors are expressed on macrophages and other cytokine-producing cells, studies do not support a role for peripheral muscarinic receptors in transmitting the anti-inflammatory effects exerted by the vagus nerve (Pavlov et al., 2005). Most tissues or cell types express multiple muscarinic receptors in a complex, overlapping pattern. These complicating factors have led to many seemingly conflicting results regarding the physiologic roles of the individual receptor subtypes (Wess et al., 2007).

Although the cholinergic anti-inflammatory pathway appears to depend on peripheral α7 nicotinic receptors, we cannot rule out the involvement of muscarinic receptors in mediating cholinergic anti-inflammatory output. Muscarine, which agonizes muscarinic acetylcholine receptors, inhibits the release of TNF in endotoxin-stimulated primary human macrophages (Borovikova et al., 2000). Our studies show that the anti-inflammatory effect of LXM-10 is attenuated by atropine methylnitrate, which shows that the anti-inflammatory effect of LXM-10 is related to peripheral muscarinic acetylcholine receptors.

However, the clinical use of these agonists is often limited by side effects caused by the non-selective activation of multiple acetylcholine receptors (Yoon et al., 2007). Classical vagus nerve cholinergic functions, including the control of heart rate and respiration, are mediated through different subtypes of muscarinic receptors, such as M₂R-mediated decreases in heart rate and M₃R-mediated increases in smooth-muscle contractility and glandular secretion (Caulfield, 1993). M₄ receptors do not seem to regulate critical peripheral functions (Caulfield, 1993; Duttaroy et al., 2002). LXM-10 did not affect heart rate in mice, nor was glandular secretion affected (data not shown), so we propose that the anti-inflammatory mechanism of LXM-10 is related to the M₆ receptor. After administering tropicamide, the anti-inflammatory effect of LXM-10 was reversed, which indicates that peripheral M₆ receptors were involved. Results showed that antagonizing either the α7 nicotinic receptor or M₆ receptor was fully effective in blocking the anti-inflammation of LXM-10, which implies that activation of both receptors was required.

TNF-α is a pro-inflammatory cytokine that plays a critical role in both acute and chronic inflammation (Beutler, 1995). Several inflammatory agents can induce the synthesis of TNF-α (Nishikiori et al., 2002; Pavlov and Tracey, 2004). We found that LXM-10, at the highest dose, suppressed the release of TNF-α induced by lipopolysaccharide, which shows that LXM-10 may inhibit inflammation because it reduces TNF-α release. Further, the suppression of TNF-α by LXM-10 is blocked by both methyllycaconitine citrate and tropicamide, which shows that LXM-10 acts on the nicotinic α7 and M₆ receptors.

More importantly, the effects of TNF-α on prostaglandin E₂ production are due to the enhanced expression of the COX-2 gene (Park et al., 2004). TNF-α induces the COX-2 enzyme which in turn catalyzes the synthesis of prostaglandin E₂ (Habashe et al., 2005; Gray et al., 2004). We thought that LXM-10 may suppress prostaglandin E₂ by inhibiting TNF-α release, because LXM-10 suppressed the increase in either TNF-α or prostaglandin E₂ levels in different tests.

In conclusion, we have shown that the spirocyclopiperazinium compound LXM-10 is a potential anti-inflammatory agent. Simultaneously, our findings show that the anti-inflammatory pathway is through peripheral α7 and M₆ receptors, which decreased the release of TNF-α and prostaglandin E₂. Our results suggest that activation of both α7 and M₆ receptors was required to produce the anti-inflammatory effect. We plan to test whether cholinergic receptors are continuously activated by prolonged administration of LXM-10 and explore the effect on the number and sensitivity of cholinergic receptors. These findings may lead to the development of anti-inflammatory drugs with reduced side effects.

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