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ORIGINAL ARTICLE

Novel choline analog 2-(4-((1-phenyl-1*H*-pyrazol-4-yl)methyl) piperazin-1-yl)ethan-1-ol produces sympathoinhibition, hypotension, and antihypertensive effects

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Abstract

The search for new drugs remains an important focus for the safe and effective treatment of cardiovascular diseases. Previous evidence has shown that choline analogs can offer therapeutic benefit for cardiovascular complications. The current study investigates the effects of 2-(4-((1-phenyl-1*H*-pyrazol-4-yl)methyl)piperazin-1-yl)ethan-1-ol (LQFM032) on cardiovascular function and cholinergic-nitric oxide signaling. Synthesized LQFM032 (0.3, 0.6, or 1.2 mg/kg) was administered by intravenous and intracerebroventricular routes to evaluate the potential alteration of mean arterial pressure, heart rate, and renal sympathetic nerve activity of normotensive and hypertensive rats. Vascular function was further evaluated in isolated vessels, while pharmacological antagonists and computational studies of nitric oxide synthase and muscarinic receptors were performed to assess possible mechanisms of LQFM032 activity. The intravenous and intracerebroventricular administration of LQFM032 elicited a temporal reduction in mean arterial pressure, heart rate, and renal sympathetic nerve activity of rats. The cumulative addition of LQFM032 to isolated endothelium-intact aortic rings reduced vascular tension and elicited a concentration-dependent relaxation. Intravenous pretreatment with L-NAME (nitric oxide synthase inhibitor), atropine (nonselective muscarinic receptor antagonist), pirenzepine, and 4-DAMP (muscarinic M1 and M3 subtype receptor antagonist, respectively) attenuated the cardiovascular effects of LQFM032. These changes may be due to a direct regulation of muscarinic signaling as docking data shows an interaction of choline analog with M1 and M3 but not nitric oxide synthase. Together, these findings demonstrate sympathoinhibitory, hypotensive, and antihypertensive effects of LQFM032 and suggest the involvement of muscarinic receptors.

Keywords Choline analog · Muscarinic receptor · Nitric oxide synthase · Sympathoinhibition

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Introduction

Cardiovascular disease is a leading cause of mortality and is associated with a higher annual death rate than any other disease category (Stern and Lebowitz 2010). The search for new drugs that target the cardiovascular system is recognized as a necessary strategy for effective treatment (Fajemiroye et al. 2014). It was suggested that newer drugs with fewer side effects and with novel mechanisms of action could provide therapeutic options (Magoon et al. 2017). Targeting the autonomic nervous system, specifically by reducing sympathetic signaling, is an avenue of particular interest, as studies have demonstrated significantly enhanced sympathetic activity in hypertensive patients and spontaneously hypertensive rats (SHRs) (Li et al. 2013; Ye et al. 2013).

The autonomic nervous system controls heart rate, inotropy, and blood pressure, and regulates the cardiovascular system. Chronic autonomic sympathetic and parasympathetic imbalance plays a crucial role in chronic kidney disease, hypertension, renal disease progression, cardiovascular morbidity, and mortality (Penne et al. 2009; Grassi et al. 2011). Autonomic signals such as epinephrine, norepinephrine, and acetylcholine contribute to normal physiological responses within the cardiovascular system, and drive many of the abnormal pathophysiological responses (Roy et al. 2015). In hypertensive rats, renal norepinephrine spillover (an index of renal sympathetic nerve activity) is increased (Schlaich et al. 2004). The firing frequency of renal sympathetic nerve activity (RSNA) has been reported to be doubled in SHRs compared to normotensive Wistar–Kyoto rats (Lundin et al. 1984). Thus, data from human studies and animal models indicate that the elevation of sympathetic nerve activity plays a causal role in the hypertensive process.

Although many therapeutic strategies have targeted the adrenergic signaling induced by aberrant sympathetic activity, studies have also suggested the potential benefit of targeting cholinergic signaling. Choline analogs, including acetylcholine, are known to induce hypotensive effects, and several pharmacological approaches have been used to increase the responsiveness of blood vessels to acetylcholine-induced vasodilation (Tominaga et al. 1994; Hye Khan et al. 2014). A previous report also demonstrated that pharmacological inhibition of acetylcholinesterase activity led to the attenuation of hypertension in SHRs (Lataro et al. 2015). The reduction of heart rate and blood pressure by choline analogs and agonists of muscarinic acetylcholine receptor (mAChR) suggests that targeting muscarinic signaling may be beneficial for the treatment of hypertension (Yalcin et al. 2005; Deng et al. 2018). A better understanding of muscarinic signaling pathways and the potential roles they play in regulating the heart and vasculature may provide new therapeutic strategies for treating cardiovascular disease (Harvey 2012).

Considering these evidences of cholinergic-mediated cardiovascular changes, researchers are now working to develop novel compounds targeting mAChR. With the overall goal of developing a novel cholinergic signaling molecule that could decrease incidents of cardiovascular dysfunction, the synthesis of a new choline analog 2-(4-((1-phenyl-1*H*-pyrazol-4-yl)methyl)piperazin-1-yl)ethan-1-ol (LQFM032) was proposed and designed from molecular hybridization of LQFM008 (piperazine derivative) and JWB-1-84-1 (analog of acetylcholine) (Brito et al. 2017).

In this study, we investigate 2-(4-(1-phenyl-1*H*-pyrazol-4-yl)methyl) piperazin-1-yl)ethan-1-ol (LQFM032)-induced cardiovascular alterations and the participation of mAChR mechanisms in regulating cardiovascular responsiveness in normotensive and hypertensive rats treated with LQFM032. Pharmacological tools and computational studies were employed to unravel the molecular mechanism of action and to investigate non-acetylation of this choline analog by acetyl coenzyme A and choline acetyltransferase.

Material and methods

Drugs and treatments

Halothane (Cristália, Itapira, SP, Brazil), urethane (Sigma-Aldrich, St. Louis, MO, USA), N^G-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich, St. Louis, MO, USA), indomethacin (Sigma-Aldrich, St. Louis, MO, USA), atropine sulphate (Cristália, Itapira, SP, Brazil), 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP; Sigma-Aldrich, St. Louis, MO, USA), phenylephrine (PHE; Sigma-Aldrich, St. Louis, MO, USA) were used in this study. Saline (0.3 mL/kg, 0.9% NaCl) was administered as vehicle in control animals. A 0.3-mL drug solution or vehicle per kilogram body weight (0.3 mL/kg) was administered intravenously (IV), while 5 μg of LQFM032 was administered by intracerebroventricular (ICV) injection at a final volume of 5 μL. All reagents were of analytical purity (≥ 95%).

Synthesis of LQFM032

The procedural details and steps for the synthesis of LQFM032 (molecular weight: 286.18 mg/mmol), as shown in Fig. 1, involve reductive amination of 1-(phenyl)-1*H*pyrazole-4-carbaldehyde and 1-(2-hydroxyethyl)piperazine. This has been described previously in detail (Brito et al. 2017).

Experimental animals

Adult male normotensive Wistar rats (NWRs) or spontaneously hypertensive rats (SHRs) with an average weight of 290 ± 25 g and age of 14 ± 2 weeks were provided by the Central

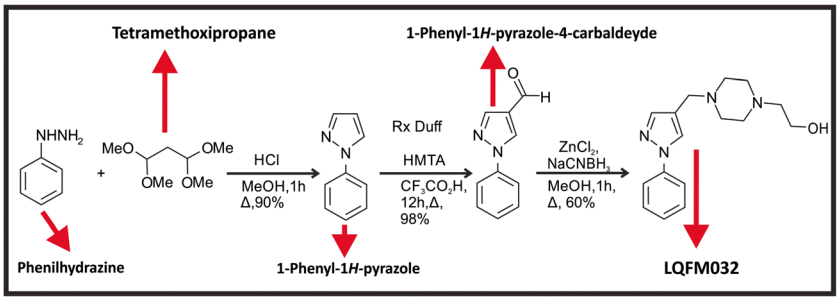


Fig. 1 Synthesis of 2-(4-((1-phenyl-1H-pyrazol-4-yl)methyl)piperazin-1-yl)ethan-1-ol (LQFM032). HCl, hydrochloric acid; MeOH, methanol; Rx Duff, Duff condition; HTMA, hexamethylenetetramine; CF_3CO_2H, trifluoroacetic acid; $NaCNBH_3$, sodium cyanoborohydride; $ZnCl_3$, zinc chloride

Animal House the of Federal University of Goiás. The experimental protocol was approved by the ethics committee of the Federal University of Goiás (protocol #172/09). Rats with blood pressure above 150 mmHg were considered SHRs. The animals were kept under controlled conditions (temperature 25 ± 1 °C, light/dark cycle of 12 h), and provided free access to food and water. All procedures were carried out in strict compliance with the Guiding Principles for Research Involving Animals and Human Beings (Kilkenny et al. 2010).

Surgery and intracerebroventricular (ICV) administration

The skulls of anesthetized rats (with urethane 800 mg/kg i.p.) were surgically exposed prior to implantation of a stainless-steel guide cannula (0.6 mm o.d., 23G) 1 mm above the injection site, using a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Stereotaxic coordinates selected for the implantation of the cerebral cannula (1.0 mm posterior to the bregma, 1.5 mm lateral, and 4.5 mm ventral) were based on the Paxinos and Watson (1998) rat atlas. Cannulas were fixed to the skulls with dental cement and metal screws. A tight-fitting mandrel was kept inside the guide cannula to avoid its occlusion. After a 7-day recovery period, the femoral artery and vein were catheterized, and ICV injection was performed using a 10-μL syringe (Hamilton, Reno, NV, USA). LQFM032 5 μg was injected at a volume of 5 μL (1 μg/μL) over a period of 1 min (Kawasaki et al. 1992; Peng et al. 2009). Incisions were sutured with surgical line following the intramuscular injection of flunixin (0.02 mL/kg), an analgesic drug at prophylactic dose.

Preparation of aortic rings and studies of vascular reactivity to LQFM032

Rats were euthanized using a guillotine, and thoracic aortas were isolated, removed, and cleaned of fat and adherent connective tissues, as described previously (Fajemiroye et al. 2014). An isometric transducer connected to a data acquisition system (World Precision Instruments, Sarasota, FL, USA) was used to record alterations in tension baseline. In order to evaluate the vascular effects of LQFM032, steady vessel tension of isolated endothelium-denuded and endothelium-intact aortic rings of NWRs and SHRs was induced by phenylephrine (PHE) 1 μM prior to the cumulative addition of LQFM032 (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M) and 30 min post-treatment observation. In the experiment, eight concentration of LQFM032 were added at 8-min intervals in a cumulative manner. Each concentration was added in a 10-μL volume (a 10× concentration) after having previously removed 10 μL of the medium in order to maintain the medium volume constant. LQFM032-induced relaxation in the aortic rings was calculated as a percentage of the relaxation in response to PHE. To test the possibility that the vascular function of LQFM032 involves NO and prostaglandin release, the rat aortic rings were pre-incubated with 100 μM L-NAME or indomethacin 100 μM for 1 h prior to the addition of 1 μM PHE to induce contraction.

Evaluation of cardiovascular alterations and mechanism of LQFM032 activity

Cardiovascular effects of peripheral (IV) administration of saline at 0.3 mL/kg (0.9% NaCl) or LQFM032 at 0.3, 0.6, and 1.2 mg/kg on RSNA, heart rate (HR) and blood pressure (BP) were recorded as described in the previous study (Togashi et al. 1990). Baselines of these parameters were recorded for 30 min. In separate experiments, animals received an IV injection of atropine 0.2 mg/kg, L-NAME (infused at 3 μg/kg/min into the femoral vein with a pump; Insight LTDA, Monte Alto, SP, Brazil), pirenzepine 0.2 mg/kg or 4-DAMP 0.4 mg/kg prior to the administration of LQFM032 (at an interval of 60 s) and observed for a duration of 240 s. The abbreviation "a.u.", which means arbitrary units, was adopted for representative tracings. In the present study, the RSNA was recorded and the signal was quantified by integrating the absolute value into 1-s intervals. We used a percentage value of the RSNA for analysis and graphics confection according to previous studies (Shi et al. 2007; De Oliveira-Sales et al. 2010; Pedrino et al. 2016; Pinto et al. 2016; Mourão et al. 2018).

Bioinformatics analysis

Crystallographic structures for the nitric oxide synthase [NOS1, PDB ID: 1lzx (Li et al. 2002)] and the murine muscarinic acetylcholine receptor 3 (mAchR3) complexed with the inhibitor tiotropium [PDB ID: 4u14 (Thorsen et al. 2014)] were obtained from the Protein Data Bank (Berman et al. 2000). We modeled the murine muscarinic acetylcholine receptor 1 (mAchR1, UniProtKB P08482) using its human form [PDB ID: 5cxv (Thal et al. 2016)] as a template with Modeler (Šali and Blundell 1993). LQFM032 structure was constructed with Avogadro (Hanwell et al. 2012). The protonation of all receptors was obtained with PROPKA (Dolinsky et al. 2004). Molecular docking was performed with LQFM032 and each protein using both DockThor (Custódio et al. 2014; De Magalhães et al. 2014) and Vina (Trott and Olson 2010), using the redocking of co-crystallized ligands as control in order to validate the method. Intermolecular interactions were obtained with PoseView (Stierand et al. 2006), and images were produced with PyMOL (DeLano 2002) and Inkscape™.

Data analysis

All values are expressed as mean ± standard error of the mean (SEM). The average value of MAP, HR, or RSNA was measured every 30 s after the injection of LQFM032 or vehicle. Multiple comparisons of groups were carried out through one-way and two-way analysis of variance (ANOVA) prior to Bonferroni post hoc test. All analyses were performed using GraphPad Prism software (San Diego, CA, USA). A value of $p < 0.05$ was considered to be significant.

Results

Effect of intravenous injection of LQFM032 on MAP, HR, and RSNA of NWRs

The line graph in Fig. 2 shows (a) representative tracings of the changes in pulsatile arterial pressure (PAP), heart rate (HR), renal sympathetic nerve activity (RSNA), and integrated RSNA (a.u.) induced by vehicle (0.3 mL/kg) and LQFM032 (0.3, 0.6 and 1.2 mg/kg) in NWRs. The temporal changes in baseline values of (b) MAP, (c) HR, and (d) RSNA were observed following increasing doses of LQFM032. The peak effect in MAP (−4.8 ± 0.8, −8.5 ± 2.1, and −16.6 ± 2.8 mmHg; Fig. 2e), HR (−2.0 ± 1.1, −3.6 ± 1.1, or −6.8 ± 1.2 bpm; Fig. 2f), and RSNA (−11.1 ± 2.2, −14.1 ± 2.1, or −22.7 ± 2.8%; Fig. 2g) were reduced in a dose-dependent manner (0.3, 0.6, and 1.2 mg/kg, respectively). Data were analyzed with two-way ANOVA (Fig. 3b–d) and one-way ANOVA (Fig. 3e–g) followed by Bonferroni post hoc test.

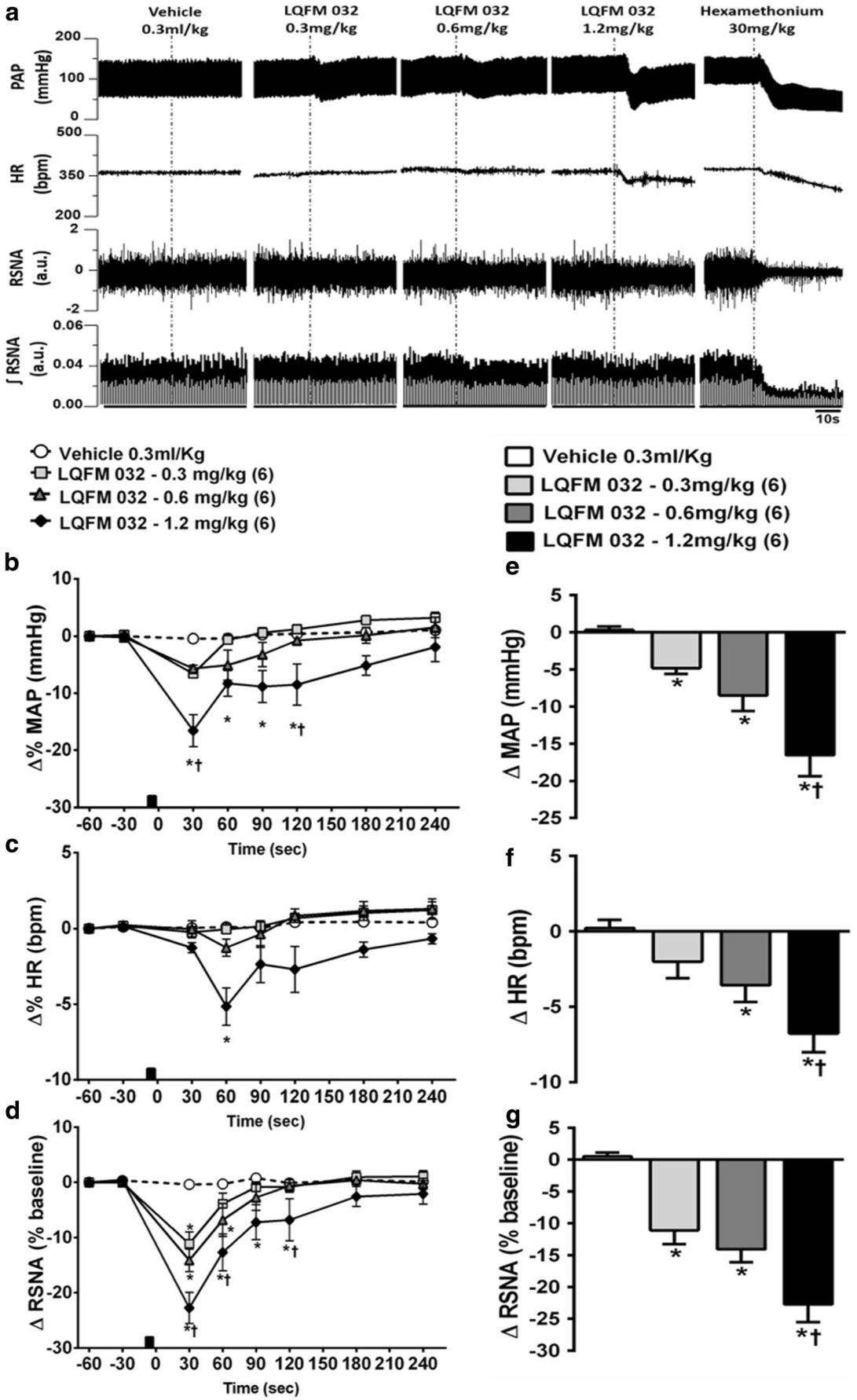
Effect of intravenous injection of LQFM032 on MAP, HR, and RSNA of SHRs

Figure 3a shows representative tracings of the changes in pulsatile arterial pressure (PAP), heart rate (HR), renal sympathetic nerve activity (RSNA), and integrated RSNA (a.u.) induced by vehicle (0.3 mL/kg) and LQFM032 (0.3, 0.6 and 1.2 mg/kg) in SHRs. Within 30 s of LQFM032 administration, there were significant changes in MAP, HR, and RSNA of SHRs (Fig. 3b, c, and d, respectively). The bar chart shows the peak effect in MAP (−10.0 ± 2.5, −6.0 ± 2.0, and −12.3 ± 2.2 mmHg; Fig. 3e); HR (−4.6 ± 1.4, −1.7 ± 0.8, or −3.5 ± 1.4 bpm; Fig. 3f), and RSNA (−9.8 ± 2.3, −12.7 ± 1.8, or −21.3 ± 2.9%; Fig. 3g) following intravenous injection of LQFM032 at 0.3, 0.6, and 1.2 mg/kg, respectively. While a clear dose-dependent reduction in RSNA was observed in response to treatment, HR was significantly reduced only in animals that received the lowest dose (0.3 mg/kg). Data were analyzed with two-way ANOVA (Fig. 3b–d) and one-way ANOVA (Fig. 3e–g) followed by Bonferroni post hoc test.

Intracerebroventricular (ICV) microinjection of LQFM032

To determine whether the effects of LQFM032 in NWRs and SHRs were mediated within the central nervous system (CNS), we next administered LQFM032 (1 μg/μL) directly to the brain with ICV injection. Figure 4 shows the MAP (a), HR (b), and RSNA (c) responses to ICV microinjection of LQFM032 (1 μg/μL). In NWRs and SHRs, the LQFM032 elicited (i) a decrease in the values of MAP (−10.7 ± 0.3 and −13.8 ± 0.2, respectively) and RSNA (−11.3 ± 0.3 and −16.6 ± 0.4, respectively), as well as (ii) a slight increase in HR (1.2 ± 0.09 and 1.8 ± 0.2), when compared to the vehicle-treated group. Data were analyzed with one-way ANOVA followed by Bonferroni post hoc test.

Fig. 2 Cardiovascular and sympathetic effects of LQFM032 acute administration in normotensive Wistar rats (NWRs). Representative tracings showing the changes in pulsatile arterial pressure (PAP), heart rate (HR), renal sympathetic nerve activity (RSNA), and integrated RSNA (a.u.) induced by intravenous administration vehicle (0.3 mL/kg) and LQFM032 (0.3, 0.6, and 1.2 mg/kg) in NWRs (panel **a**). Panels **b**, **c**, and **d** show the temporal line graphs of mean arterial pressure (Δ% MAP), Δ% HR, and Δ% RSNA after intravenous LQFM032 in NWRs. Panels **e**, **f**, and **g** show the summary data for peak changes in Δ MAP, Δ HR, and Δ RSNA caused by the dose-dependent effects of LQFM032 (0.3, 0.6, and 1.2 mg/kg). Data are shown as mean ± SEM. $P < 0.05$ [two-way ANOVA (panels **b**, **c**, and **d**) and one-way ANOVA (panels **e**, **f** and **g**) followed by Bonferroni post hoc test]. *Different from the vehicle; †different from the 0.3-mg/kg and 0.6-mg/kg doses of LQFM032


a
Vehicle 0.3ml/kg
LQFM 032 0.3mg/kg
LQFM 032 0.6mg/kg
LQFM 032 1.2mg/kg
Hexamethonium 30mg/kg
PAP (mmHg)
HR (bpm)
RSNA (a.u.)
∫ RSNA (a.u.)
10s
Vehicle 0.3ml/Kg
LQFM 032 - 0.3 mg/kg (6)
LQFM 032 - 0.6 mg/kg (6)
LQFM 032 - 1.2 mg/kg (6)
Vehicle 0.3ml/Kg
LQFM 032 - 0.3mg/kg (6)
LQFM 032 - 0.6mg/kg (6)
LQFM 032 - 1.2mg/kg (6)
b
Δ% MAP (mmHg)
Time (sec)
c
Δ% HR (bpm)
Time (sec)
d
Δ RSNA (% baseline)
Time (sec)
e
Δ MAP (mmHg)
f
Δ HR (bpm)
g
Δ RSNA (% baseline)

Vascular reactivity to LQFM032

The line graph in Fig. 5 depicts concentration–response curves of cumulative addition of vehicle and LQFM032 to endothelium-intact and endothelium-denuded aortic rings. LQFM032 caused a concentration-dependent relaxation of (a) NWR-isolated aortic rings with endothelium and (b) SHR-isolated aortic rings with endothelium when compared with their respective group treated with vehicle (control). The maximum relaxant effect of LQFM032 on PHE-induced contraction was 50.3% ± 6.3% (Fig. 5a) and 65.6% ± 8.4% (Fig. 5b) for NWR- and SHR-endothelium-intact aortic rings, respectively. Unlike indomethacin 100 μM, the aortic rings pre-incubated with L-NAME 100 μM blocked relaxation induced by the cumulative addition of LQFM032 (10^{-12} to 10^{-5} M) at the plateau phase of PHE-induced contraction. No significant reduction was observed in vessel tension of NWR- or SHR-isolated aortic rings without endothelium. Data were analyzed with two-way ANOVA followed by the Bonferroni post hoc test.

Effect of muscarinic receptors antagonists on cardiovascular activity of LQFM032

To verify that the effects of LQFM032 1.2 mg/kg were mediated by muscarinic receptors (the more prevalent acetylcholine receptors in autonomic effector organs), LQFM032 and the prototypical muscarinic antagonist atropine were administered intravenously. The effect of LQFM032 on (a) MAP, (b) HR, and (c) RSNA was significantly attenuated by atropine pretreatment in NWRs (Fig. 6a–c) and SHRs (Fig. 7a–c). One of the vasomodulating pathways downstream of muscarinic acetylcholine receptors is nitric oxide synthase (NOS). Thus, we also assessed whether pretreatment with L-NAME, an inhibitor of NOS, could also reduce the effects of LQFM032. Co-administration of L-NAME with LQFM032 also significantly attenuated the effects of this compound on MAP, HR, and RSNA in NWRs (Fig. 6a–c) and SHRs (Fig. 7a–c). Because atropine is a nonselective mAChR antagonist, we assessed the effects of an M1-specific antagonist, pirenzepine, and an M3-specific antagonist, 4-DAMP. Co-administration with either the M1 or M3 antagonists significantly attenuated the hypotensive effects of LQFM032 in SHRs (Fig. 8a–c), suggesting nonselective agonism of mAChR by LQFM032.

Data on bioinformatics studies

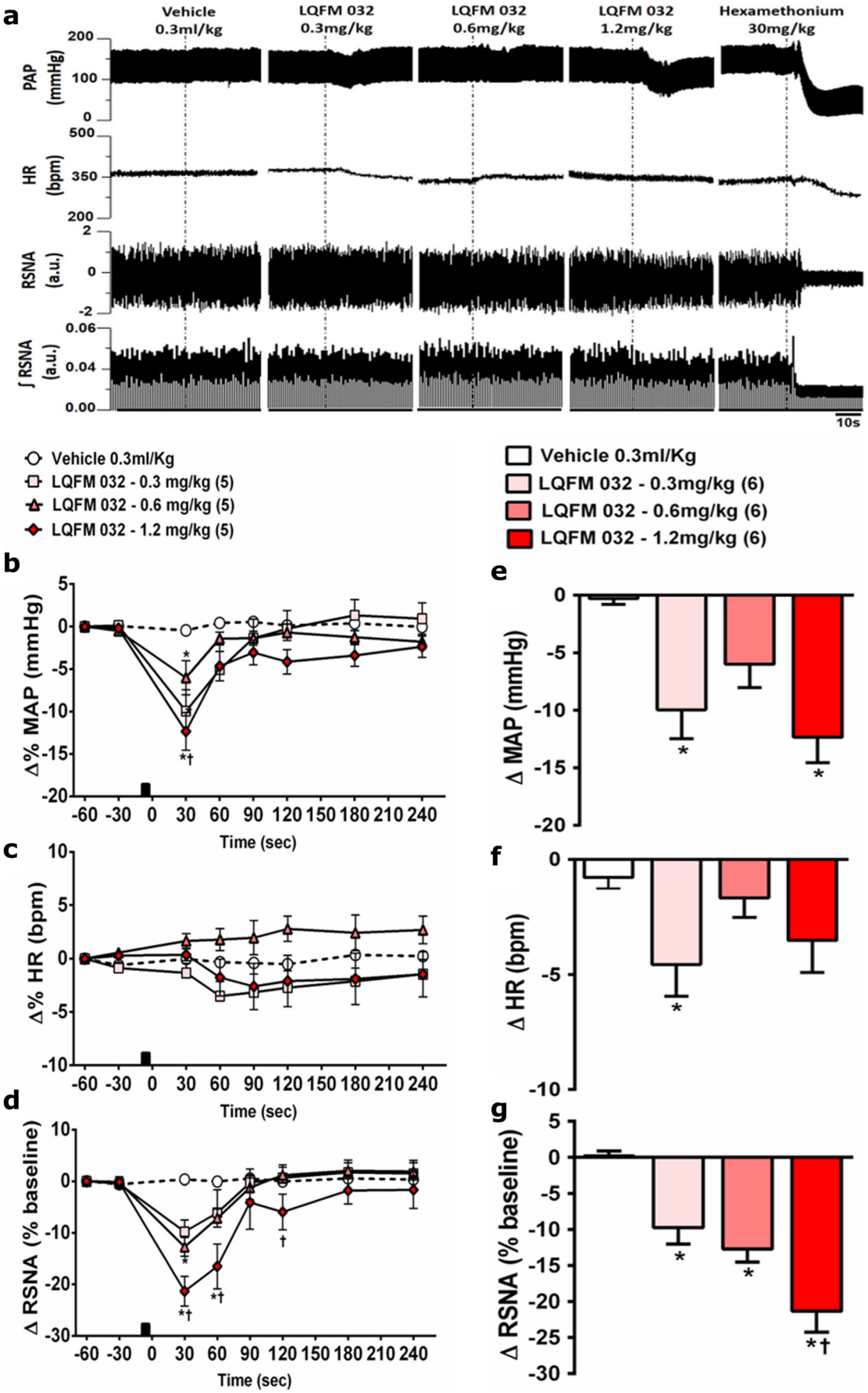
Having established that LQFM032 modulated muscarinic and/or downstream nitrous oxide signaling in the intact cardiovascular system, we next assessed the possible direct interaction between LQFM032 and NOS or the M1 and M3 cholinergic receptors using molecular docking studies. For each, a co-crystallized ligand was used as reference for LQFM032 docking with the programs DockThor and Vina. While redocking of NOS and the ligand N-omega-hydroxy-L-arginine using both docking programs was successful, no interactions with LQFM032 were detected, suggesting that it does not interact with NOS. Regarding mAchR1 and mAchR3 (Fig. 9), LQFM032 was shown to interact with the key asparagine for inhibition by the inverse agonist tiotropium (Asn382). Furthermore, LQFM032 also interacted with all three tyrosine residues that form the lid that covers the orthosteric binding site (Tyr106-Tyr381-Tyr404 and Tyr148-Tyr506-Tyr529 for mAchR1 and mAchR3, respectively) (Kruse et al. 2012). Between the two murine acetylcholine receptors, LQFM032 made contact with key residues in mAchR1 with both docking programs, indicating that it potentially interacts with the same residues as the inhibitor tiotropium.

Discussion

Chemical synthesis and biological screening of compounds are important strategies for new drug discovery (Fajemiroye et al. 2017). Recently, a novel choline analog was developed as a hybrid of piperazine and choline scaffold [Brito et al, 2019]. This compound is of a particular interest, as the six-membered nitrogen containing a heterocycle is of great significance for cardioprotection (Rathi et al. 2016). Unlike LQFM008—one of the parent compounds—the presence of choline substituents with a piperazine ring greatly enhanced solubility and increased the possibility of cholinergic-mediated cardiovascular effects of LQFM032. According to Rathi et al. (2016), a significant increase in research covering the activity of the piperazine ring suggests a successful emergence of the pharmacophore. In the present study, LQFM032—a choline analog—was synthesized in order to evaluate its effect on cardiovascular parameters.

In order to achieve this objective, the cardiovascular effects of LQFM032 were determined with respect to the in vivo physiological readouts in non-hypertensive and

Fig. 3 Cardiovascular and sympathetic effects of LQFM032 acute administration in spontaneously hypertensive rats (SHRs). Representative tracings showing the changes in pulsatile arterial pressure (PAP), heart rate (HR), renal sympathetic nerve activity (RSNA), and integrated RSNA (a.u.) induced by intravenous administration of vehicle (0.3 mL/kg) and LQFM032 (0.3, 0.6, and 1.2 mg/kg) in SHRs (panel **a**). Panels **b**, **c**, and **d** show the temporal line graphs of mean arterial pressure (Δ% MAP), Δ% HR, and Δ% RSNA after intravenous LQFM032 in NWRs. Summary data of peak changes in Δ MAP, Δ HR, and Δ RSNA caused by a dose-dependent effect of LQFM032 (0.3, 0.6 and 1.2 mg/kg) are shown in panels **e**, **f** and **g**, respectively. Data are shown as mean ± SEM. $P < 0.05$ [two-way ANOVA (panels **b**, **c**, and **d**) and one-way ANOVA (panels **e**, **f**, and **g**) followed by Bonferroni post hoc test]. *Different from the vehicle; †different from the 0.3-mg/kg and 0.6-mg/kg doses of LQFM032


a
Vehicle 0.3ml/kg
LQFM 032 0.3mg/kg
LQFM 032 0.6mg/kg
LQFM 032 1.2mg/kg
Hexamethonium 30mg/kg
PAP (mmHg)
HR (bpm)
RSNA (a.u.)
∫ RSNA (a.u.)
10s
Vehicle 0.3ml/Kg
LQFM 032 - 0.3 mg/kg (5)
LQFM 032 - 0.6 mg/kg (5)
LQFM 032 - 1.2 mg/kg (5)
Vehicle 0.3ml/Kg
LQFM 032 - 0.3mg/kg (6)
LQFM 032 - 0.6mg/kg (6)
LQFM 032 - 1.2mg/kg (6)
b
Δ% MAP (mmHg)
Time (sec)
c
Δ% HR (bpm)
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d
Δ RSNA (% baseline)
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Δ HR (bpm)
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Δ RSNA (% baseline)

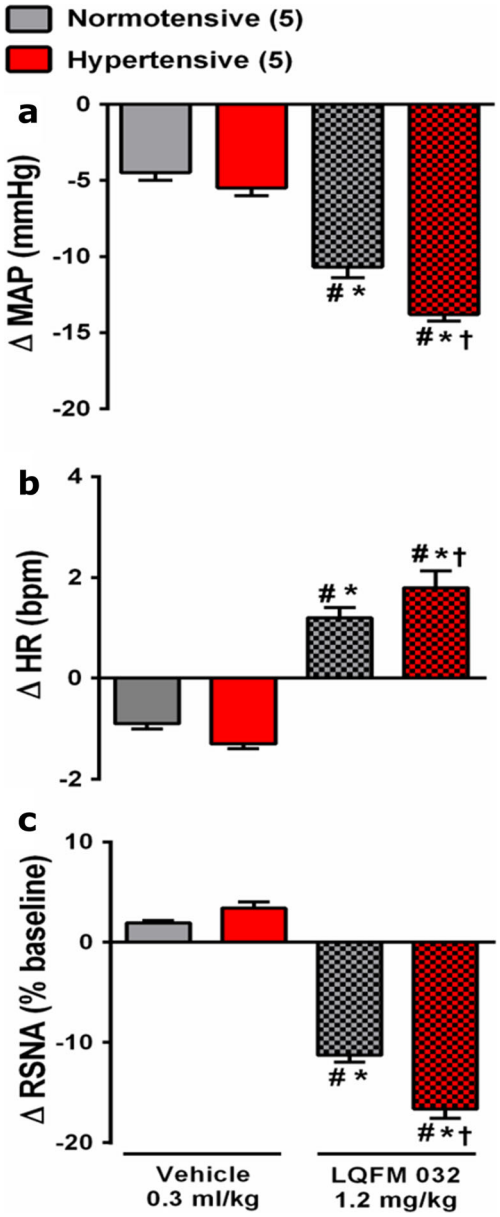

Fig. 4 Effects of intracerebroventricular (ICV) administration of LQFM032 in normotensive Wistar rats (NWRs) and spontaneously hypertensive rats (SHRs). The bar graph shows the effect of ICV microinjection of vehicle or LQFM032 (5 μg was injected at a volume of 5 μL over a period of 1 min) on the (**a**) mean arterial pressure (Δ MAP), (**b**) heart rate (Δ HR), and (**c**) renal sympathetic nerve activity (RSNA) of normotensive and hypertensive rats. Data are shown as mean ± SEM. $P < 0.05$ (one-way ANOVA followed by Bonferroni post hoc test). *Different from the vehicle NWRs; #different from the vehicle SHRs; †different from the LQFM032 in NWRs

spontaneously hypertensive rats (SHRs). The SHR model is an important experimental protocol being used for the screening of antihypertensive drugs (Leong et al. 2015). In summary, LQFM032 elicited temporal reduction of mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) of normotensive rats (NWRs) and SHRs. An increase in RSNA is known to impair sodium excretion by promoting sodium reabsorption and enhancing preglomerular renal vascular resistance, which in turn decrease the glomerular filtration rate (DiBona and Kopp 1997). Thus, the LQFM032-mediated reduction in RSNA may be beneficial for renal function in hypertensive patients.

Similar to some centrally acting antihypertensive drugs such as clonidine and moxonidine with nitric oxide mechanisms (Dobrucki et al. 2001; Moreira et al. 2006), ICV administration of LQFM032 elicited significant antihypertensive activity in SHRs. Considering this compound as an agonist of muscarinic receptors, our result seems to be inconsistent with the established role of centrally mediated effects of cholinergic neurons. Blood pressure regulation has been associated with cholinergic neuronal activity in numerous brain regions (Buccafusco 1996). Cholinergic agonists, antagonists, and acetylcholinesterase inhibitors enhance or attenuate centrally mediated pressor response (Sundaram et al. 1988; Lazartigues et al. 1999; Kubo 1998).

The lateral parabrachial nucleus comprises cholinergic neurons that play an important role in the pathogenesis of

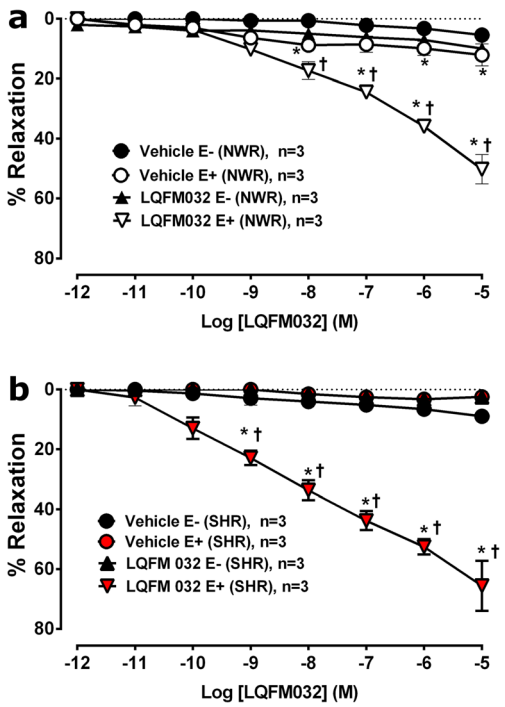

Fig. 5 Vascular effects of LQFM032. Vasorelaxant effect of LQFM032 (10^{-12} to 10^{-5} M) on phenylephrine (PHE 1 μM)-induced pre-contraction of endothelium-denuded (E−) or endothelium-intact (E+) aortic rings in normotensive Wistar rats (NWRs, **a**) and spontaneously hypertensive rats (SHRs, **b**). The vascular function of LQFM032 (10^{-12} to 10^{-5} M) involves NO and prostaglandin release, and the rat aortic rings were pre-incubated with 100 μM L-NAME or indomethacin 100 μM for 1 h prior to the addition of 1 μM PHE to induce contraction (**c**). Data are shown as mean ± SEM. $P < 0.05$ (two-way ANOVA followed by Bonferroni post hoc test). *Different from the vehicle; †different from the LQFM032 E−

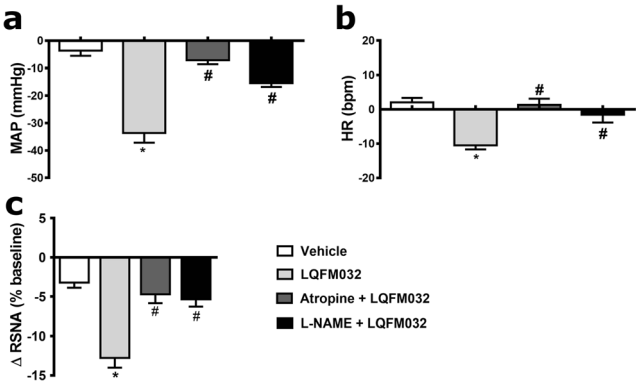

Fig. 6 Cardiovascular and sympathetic responses evoked by atropine and L-NAME pretreatments on LQFM032 effects in normotensive Wistar rats (NWRs). Effects of intravenous pretreatment with atropine (0.2 mg/kg) or L-NAME (3 μg/kg/min) on LQFM032 1.2 mg/kg-induced changes (Δ) in MAP (**a**), HR (**b**), and RSNA (**c**). Data are expressed as mean ± SEM (one-way ANOVA followed by Bonferroni post hoc test). $P < 0.05$. *Different from the vehicle; #different from the LQFM032

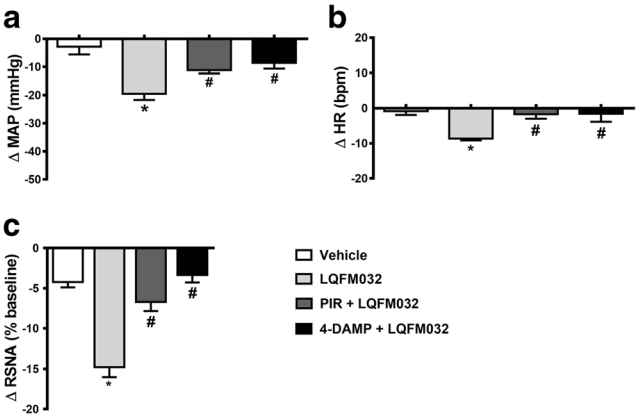

Fig. 8 Pharmacological blockade of the effect of LQFM032 in SHRs with pirenzepine and 4-DAMP. The effects of intravenous pretreatment with pirenzepine (PIR 0.2 mg/kg) or 4-DAMP (0.4 mg/kg) on LQFM032 1.2 mg/kg-induced changes in (**a**) MAP, (**b**) HR, and (**c**) RSNA. Data are expressed as mean ± SEM (one-way ANOVA followed by Bonferroni post hoc test). *$p < 0.05$ compared to the vehicle-treated group; #$p < 0.05$ compared to the LQFM032-treated group

hypertension (Sundaram et al. 1988). The muscarinic receptors on the rostral ventrolateral medulla (RVLM; an essential source of efferent sympathetic activity) are potential pharmacological targets in a hypertensive state. In addition, the inhibition of acetylcholinesterase by physostigmine and release of acetylcholine by 3,4-diaminopyridine increased the firing rate of RVLM neurons (Kubo et al. 1997). Previous pharmacological and biochemical studies of the RVLM revealed an enhancement in the release of acetylcholine and an increase in experimental hypertension (Kubo et al. 1997; Kubo 1998).

Being a choline analog, LQFM032 could compete as a substrate for acetyl coenzyme A and choline acetyltransferase (i.e. enzymatic acetylating processes), choline high-affinity transporter, or vesicular acetylcholine transporter.

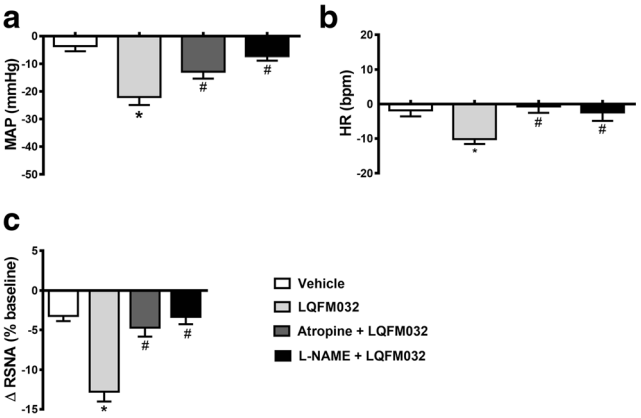

Fig. 7 Cardiovascular and sympathetic responses evoked by atropine and L-NAME pretreatments on LQFM032 effects in spontaneously hypertensive rats (SHRs). Effects of intravenous pretreatment with atropine (0.2 mg/kg) or L-NAME (3 μg/kg/min) on LQFM032 1.2 mg/kg-induced changes (Δ) in MAP (**a**), HR (**b**), and RSNA (**c**). Data are expressed as mean ± SEM (one-way ANOVA followed by Bonferroni post hoc test). $P < 0.05$. *Different from the vehicle; #different from the LQFM032

Hypothetically, the reversible or irreversible competition for these cellular processes could interfere in the synthesis and release of endogenous acetylcholine. A decrease in the level of acetylcholine could trigger positive feedback to enhance synthesis and release as well as an increase in the hydrolysis of this neurotransmitter. The previous unpublished data which showed unaltered activity of acetylcholinesterase following its incubation with LQFM032 suggests noninterference of this compound in the rate of acetylcholine hydrolysis. Together, a disruption in the synthesis and release of acetylcholine without an inhibition of its hydrolysis could lead to overall decrease in its synaptic level in the brain. Additionally, Kubo (1998) demonstrated the involvement of the hypothalamic defense area (an area believed to be involved in the hypertension induced by chronic stress) in the release of acetylcholine in the RVLM and the existence of direct projections from the hypothalamic structures to the lateral parabrachial nucleus. This study seems to be consistent with the demonstration of the anti-anxiety properties of this cholinergic analog (Brito et al. 2017), and reinforces the possible link between stress pathway and antihypertensive properties of LQFM032. In the future, additional studies of specific brain regions will provide a more comprehensive understanding of the central mechanisms of LQFM032-induced cardiovascular alterations and the role of endogenous acetylcholine.

Despite the central effects of LQFM032, its oral administration makes the participation of peripheral mAChRs in the cardiovascular outcome plausible. An isolated organ in a physiological solution remains an important research tool for testing localized or peripheral effects of drugs. Thus, aortic rings were isolated from

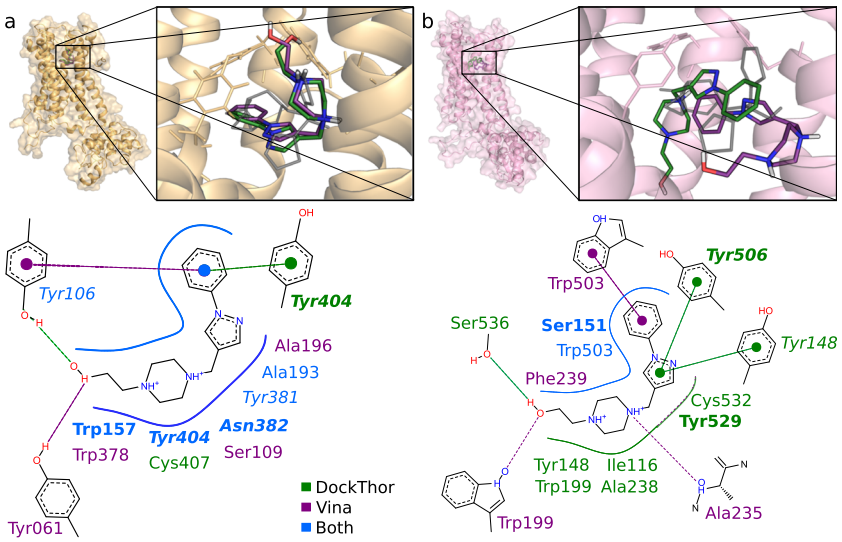

Fig. 9 Interaction of LQFM032 and the inhibitor tiotropium with mAchR1 and mAchR3. **a** Top: Overview of mAchR1, zooming on LQFM032 docked by DockThor (purple) and Vina (green) compared to tiotropium (black). Sticks represent the lid-forming Tyr residues and the interacting Asn382. Bottom: Predicted interactions between mAchR1 and LQFM032 according to DockThor (green), Vina (purple), or both (blue). **b** Top: Overview of mAchR3 and close-up of LQFM032 docked by DockThor (purple) and Vina (green) compared to tiotropium (black). The sticks represent the lid-forming Tyr residues and the interacting Asn. Bottom: Predicted interactions between mAchR3 and LQFM032 according to DockThor (green), Vina (purple), or both (blue). For both receptors, interactions with tiotropium are shown in bold, and the lid-forming Tyr residues and the interacting Asn are shown in italics

normotensive and spontaneously hypertensive rats to test vascular response to LQFM032. Unlike isolated vessels without endothelium, this choline analog reduced vascular tone and elicited a relaxation of isolated aortic rings with endothelium from normotensive and spontaneously hypertensive rats. This result suggests the release and involvement of vasoactives such as nitric oxide (NO), prostaglandins, and endothelium-derived hyperpolarization factors. Unlike indomethacin 100 μM (nonsteroid anti-inflammatory drug/prostaglandin inhibitor), when the rat aortic rings were pre-incubated with L-NAME 100 μM prior to the PHE-induced contraction, cumulative addition of LQFM032 did not elicit relaxation. Hence, this result supports the participation of NO in the vascular function of this compound. According to Stankevicius et al. (2002), these endogenous vasoactives play important roles in vascular relaxation.

Moreover, the use of pharmacological tools such as antagonists and computational studies were explored to unravel the research hypothesis and the potential mechanism of the cardiovascular activity of this choline analog. The hypothesis of the muscarinic action of this compound and its cardiovascular properties was postulated; however, it was not tested until this current study. The attenuation of cardiovascular effects of LQFM032 by L-NAME (a nonselective NOS inhibitor), atropine (muscarinic receptor antagonist), pirenzepine (M1 receptor antagonist), and 4-DAMP (M3 receptor antagonist) pretreatments seems to be consistent with our cholinergic hypothesis and suggest the specific involvement of muscarinic-nitric oxide pathways.

The attenuation of the LQFM032-induced cardiovascular activity could be associated with central and/or peripheral blockade of muscarinic receptors. According to Pediani et al. (2016), pirenzepine does not effectively cross the blood–brain barrier effectively, and as such does not inhibit M1-mediated cholinergic function in the CNS. In this manner, the blockade of the effect of intravenously administered LQFM032 by pirenzepine supports the involvement of peripheral components.

Although pirenzepine and 4-DAMP have been characterized pharmacologically by some authors as selective antagonists of M1 and M3 receptors, respectively (Höglund and Baghdoyan 1997; Pediani et al. 2016; Jaiswal et al. 1989; Borella et al. 2008), the administration of a single dose of pirenzepine (0.2 mg/kg) or 4-DAMP (0.4 mg/kg) in the present study does not necessarily confirm a selective blockade of M1 and M3 in the attenuation of the cardiovascular effects of LQFM032. The close values of K_D and K_B of these antagonists on muscarinic receptors subtypes seem to weaken the possibility of their selectivity with a single dose in vivo. The previous study which revealed lack of correlation in the affinities and abilities of the 4-DAMP to discriminate muscarinic receptor subtypes (Waelbroeck et al. 1992) supports the need for future evaluation of specific roles of all muscarinic receptor subtypes in the activity of LQFM032.

The compounds mimicking acetylcholine actions are dilators of vascular beds and are often used as an investigative tool for the evaluation of endothelial function (Vita et al. 1990;

Faraci and Sigmund 1999; Klonizakis et al. 2009). The endothelial muscarinic receptors participate in triggering the release of the vasorelaxing agents, such as NO (Leung et al. 2006). Since L-NAME blocked the effect of LQFM032, it was assumed that NOS plays an important role in the current cardiovascular effects.

The NOS has a sympathoinhibitory effect under physiological and disease conditions by acting on the paraventricular nucleus, the nucleus of the solitary tract, the rostral ventrolateral medulla, the carotid body, and nerves in the kidney (Wang and Golledge 2013). According to Sander et al. (1995), hypertensive responses to L-NAME in rats with versus without sympathectomy could be related to inhibition of nitric oxide synthesis. Since renal nerves transmit signals from the periphery (kidneys) to the CNS and vice versa (Nishi et al. 2015), the blockade of the inhibitory effects of LQFM032 on RSNA by L-NAME pretreatment suggests crosstalk between the sympathetic nervous system and nitric oxide signaling.

Since NOS catalyzes the synthesis of NO from L-arginine (Ricciardolo et al. 2004), the hypotensive effects of LQFM032 suggests an increase in (i) the activity of this enzyme, and (ii) the synthesis and (iii) release of NO. The overall effect could trigger a reduction in vascular resistance in normotensive Wistar rats. However, these effects could involve direct activation of endothelial NOS or indirect muscarinic action through the activation of the upstream M1 and M3 receptors. Because interactions between NOS and LQFM032 were not established with our molecular docking approaches while LQFM032 interacted with both muscarinic receptors (M1 and M3), our findings support the aforementioned cholinergic hypothesis.

Conclusions

In summary, experimental data suggest muscarinic-mediated sympathoinhibitory, hypotensive, and antihypertensive effects of LQFM032. Future research will be focused on extensive investigation of the pharmacokinetic and toxicological profiles of this new choline analog. Additional modification of LQFM032 will be carried out in the future to study the structural activity relationship and to develop analogs that bind a specific muscarinic receptor subtype.

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Author's contribution JOF, NMA and GRP: synthesis and chemical characterization of LQFM032. BV and HV: in silico assays. JOF, CHX, CHC, ONS, OLF, IOS: devising the research method/methodology used. AAM, EAC, CHX, CHC, GRP: conducting research, statistical analysis and calculations. RM, FSC, LML: devising the concept and assumptions of the article. All authors wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the Federal University of Goiás (protocol #172/09) as compliant with Brazil law.

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