European Journal of Pharmacology 765 (2015) 375–383

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular pharmacology

Chronic fluoxetine treatment increases NO bioavailability and calcium-sensitive potassium channels activation in rat mesenteric resistance arteries

Camila A. Pereira [a,*], Nathanne S. Ferreira [a], Fabiola L. Mestriner [a], José Antunes-Rodrigues [b], Paulo R.B. Evora [c], Leonardo B.M. Resstel [a], Fernando S. Carneiro [a], Rita C. Tostes [a,*]

[a] Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto 14049-900, SP, Brazil
[b] Department of Physiology, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto 14049-900, SP, Brazil
[c] Department of Surgery and Anatomy, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto 14049-900, SP, Brazil

ARTICLE INFO

Article history:
Received 28 July 2015
Received in revised form
26 August 2015
Accepted 1 September 2015
Available online 8 September 2015

Keywords:
Fluoxetine
Chronic treatment
Vascular reactivity
Nitric oxide
Potassium channel

ABSTRACT

Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), has effects beyond its antidepressant properties, altering, e.g., mechanisms involved in blood pressure and vasomotor tone control. Although many studies have addressed the acute impact of fluoxetine on the cardiovascular system, there is a paucity of information on the chronic vascular effects of this SSRI. We tested the hypothesis that chronic fluoxetine treatment enhances the vascular reactivity to vasodilator stimuli by increasing nitric oxide (NO) signaling and activation of potassium (K^+) channels. *Wistar* rats were divided into two groups: (I) vehicle (water for 21 days) or (II) chronic fluoxetine (10 mg/kg/day in the drinking water for 21 days). Fluoxetine treatment increased endothelium-dependent and independent vasorelaxation (analyzed by mesenteric resistance arteries reactivity) as well as constitutive NO synthase (NOS) activity, phosphorylation of eNOS at $Serine^{1177}$ and NO production, determined by western blot and fluorescence. On the other hand, fluoxetine treatment did not alter vascular expression of neuronal and inducible NOS or guanylyl cyclase (GC). Arteries from fluoxetine-treated rats exhibited increased relaxation to pinacidil. Increased acetylcholine vasorelaxation was abolished by a calcium-activated K^+ channel (K_{Ca}) blocker, but not by an inhibitor of K_{ATP} channels. On the other hand, vascular responses to Bay 41-2272 and 8-bromo-cGMP were similar between the groups. In conclusion, chronic fluoxetine treatment increases endothelium-dependent and independent relaxation of mesenteric resistance arteries by mechanisms that involve increased eNOS activity, NO generation, and K_{Ca} channels activation. These effects may contribute to the cardiovascular effects associated with chronic fluoxetine treatment.

1. Introduction

Among the different classes of commercially available antidepressants, fluoxetine, a selective serotonin reuptake inhibitor (SSRI) approved by the Food and Drug Administration in 1987 (FDA, 2014), was the best-selling drug in the United States in 1996, and accounted for 6 million filled prescriptions in the United Kingdom in 2011 (Verispan, 2012). According to the National System of Management of Controlled Products (SNGPC), 3.5 tons of fluoxetine were used in Brazil in 2006 (ANVISA, 2006), not only for the treatment of depression, but also as an appetite suppressant drug (Wise, 1992).

Side effects associated with the use of fluoxetine include, among others, orthostatic hypotension, sedation, arrhythmia and mental confusion (Pacher et al., 2001), sexual dysfunction (Lee et al., 2010), inappropriate secretion of anti-diuretic hormone (Liu et al., 1996), stroke (Hung et al., 2013), gestational hypertension and preeclampsia (Toh et al., 2009).

Crestani et al. (2011) demonstrated that chronic treatment of rats with fluoxetine increases blood pressure and alters hemodynamic responses induced by the administration of vasoactive agents. Also, Ribback et al. (2012) reported that different antidepressants (amitriptyline, tranylcypromine, and fluoxetine) induce rat aortic relaxation regardless of the pre-contractile agent (e.g. noradrenaline or serotonin). Mechanisms involved in the effects of these antidepressants include the activation of the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP)/potassium (K^+) channels signaling pathway. In addition, fluoxetine has been shown to inhibit several receptors and ion channels directly participating in the

* Corresponding authors.
E-mail addresses: mila_cap@yahoo.com.br (C.A. Pereira), rtostes@usp.br (R.C. Tostes).

http://dx.doi.org/10.1016/j.ejphar.2015.09.002
0014-2999/

vasomotor tone regulation, such as 5-HT_{2C} receptor (Ni and Miledi, 1997), 5-HT_3 receptor (Fan, 1994) and nicotinic receptors (Maggi et al., 1998); voltage-dependent sodium (Na^+) and K^+ channels (Pancrazio et al., 1998; Perchenet et al., 2001; Thomas et al., 2002) and chloride (Cl^-) channels (Maertens et al., 1999).

These reports clearly show that fluoxetine has effects beyond its antidepressant properties, altering mechanisms involved in blood pressure and vasomotor tone control. Otherwise, most of the published investigations have studied acute fluoxetine effects on the cardiovascular system. Therefore, based on this rationale, we tested the hypothesis that chronic fluoxetine treatment enhances vascular responses mediated by NO signaling and K^+ channel activation.

2. Materials and methods

2.1. Animals

All experimental procedures performed in this study were approved by the Ethics Committee on Animal Experiments of the Ribeirão Preto Medical School, University of São Paulo (protocol. 013/2013) and are in accordance with the Guidelines of the Brazilian College of Animal Experimentation (COBEA).

Male *Wistar* rats, weighing 230–250 g were used in the experimental protocols. The animals were housed in high-top-filter cages (3 rats per cage – 48.3 cm × 33.7 × 25.3 cm) in a room with controlled humidity (45 ± 5%) and temperature (21 ± 2 °C), and light/dark cycles of 12 h. Animals had free access to food (commercially available standard rat chow, Purina) and potable tap water.

2.2. Treatment with fluoxetine

Fluoxetine was administered (10 mg/kg/day) in the drinking water, which was changed daily (Alper, 1992; Lino-de-Oliveira et al., 2001). The average consumption of water by the rats was monitored for three days, and the daily concentration required to achieve a dose of 10 mg/kg/day was calculated. The bottles were light protected to prevent degradation or oxidation of the drug. Rats were divided randomly into two groups: (I) vehicle, water for 21 days or (II) chronic fluoxetine (10 mg/kg/day for 21 days).

2.3. Vascular reactivity – isolated mesenteric resistance arteries

The method described by Mulvany and Halpern (1977) was used. The animals were euthanized in a carbon dioxide (CO_2) chamber. Segments of third-branch mesenteric arteries, measuring about 2 mm in length, were mounted in a small vessel myograph (Danish Myo Tech, Model 620M, A/S, Århus, Denmark). Arteries were maintained in Krebs Henseleit solution [(in mmol/L) NaCl 130, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 14.9, glucose 5.5, EDTA 0.03, $CaCl_2$ 1.6], at a constant temperature of 37 °C, pH 7.4, and gassed with a mixture of 95% O_2 and 5% CO_2.

Mesenteric resistance artery preparations were set to reach a tension of 13.3 kPa (kilopascal) and remained at rest for 30 min for stabilization. The arteries were stimulated with Krebs solution containing a high concentration of potassium [K^+ (120 mM)] to evaluate the contractile capacity of the segments. After washing and returning to the basal tension, arteries were pre-contracted with phenylephrine (10^{-6} M) and then stimulated with acetylcholine (10^{-5} M) to determine the presence of a functional endothelium. Arteries exhibiting a vasodilator response to acetylcholine greater than 80% were considered endothelium-intact vessels. The failure of acetylcholine to elicit relaxation of arteries that were subjected to rubbing of the intimal surface with human hair was taken as proof of endothelium removal. After washing and another period of stabilization, concentration–response curves were performed.

2.3.1. Cumulative concentration–response curves

Mesenteric resistance arteries were stimulated with phenylephrine (10^{-6}–3×10^{-6} M) to produce contraction (measured as an increase in baseline tension). After 15 min, concentration–response curves to acetylcholine (10^{-10}–3×10^{-5} M), sodium nitroprusside (10^{-12}–10^{-5} M), Bay 41-2272 (10^{-10}–10^{-6} M), 8-bromo-cGMP (10^{-10}–10^{-4} M) or pinacidil (10^{-10}–10^{-4} M) were carried out. Concentration–response curves to acetylcholine were also performed in the presence of glibenclamide (10^{-5} M) and charybdotoxin (10^{-7} M), inhibitors of K_{ATP} and K_{Ca} channels, respectively.

2.4. Western blot

The mesenteric bed was isolated in ice-cold Krebs solution, cleaned from adventitial tissue and immediately frozen in liquid nitrogen. The tissue was pulverized and homogenized in ice-cold lysis buffer [Triton X-100 1%, 100 mM tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM ethylene diamine tetraacetic acid (EDTA), 10 mM sodium orthovanadate, 2 mM phenyl methyl sulfonyl fluoride (PMSF) and aprotinin 0.01 mg/ml]. The tissue extracts were centrifuged, and total protein content was quantified using the Bradford method (Bio-Rad) (Bradford, 1976).

Proteins (40–100 μg) were then separated by electrophoresis in polyacrylamide (SDS-PAGE) gel and transferred to nitrocellulose membranes. Nonspecific binding sites on the membrane were blocked with 1% bovine serum albumin (BSA) in tris-buffered saline solution with 1% of Tween (TBS-T) for 1 h at room temperature. Membranes were then incubated at 4 °C with specific antibodies, overnight. Membranes were washed 3 times with TBS-T and incubated with specific secondary antibodies for 1 h at room temperature. Signals were revealed after reaction with ECL (Amersham ECL Prime Western Blotting Detection Reagent) and the images captured in ImageQuant 350 (GE Healthcare, Piscata Way, NJ, USA). Results were normalized by β-actin or the total protein and expressed relatively to the vehicle (100%) in the experimental protocols. Antibodies dilutions were used as follows: phospho-eNOS (1:100, Cell Signaling, #9571), eNOS (1:500, Cell Signaling, #9572), anti-guanylyl cyclase α-1 (1:1000, Abcam, Ab50358), anti-guanylyl cyclase β (1:2000, Sigma, SAB4501344), anti-nNOS (1:1000, Cell Signaling, #4234S), anti-iNOS (1:5000, Sigma, N7782) and β-actin (1:3000, Cell Signaling, #2148S).

2.5. Chemiluminescence

The mesenteric bed, free from adipose tissue, was immediately frozen in liquid nitrogen, pulverized and homogenized in 20 mM Tris–HCl (pH 7.4). The samples were centrifuged (5000g, 10 min, 4 °C) and the total protein content was quantified using the Bradford method (Bio-Rad) (Bradford, 1976). The samples were analyzed in duplicate for nitrite and nitrate (NOx) using chemiluminescence-based assay ozone. Briefly, mesenteric bed samples were treated with cold ethanol (1:2 mesenteric bed to ethanol, for 30 min at −20 °C) and centrifuged (4000g, 10 min). NOx levels were measured by injecting 25 μL of supernatant in a container vent glass containing 0.8% of Vanadium (III) in HCl (1 N) at 90 °C, which reduces NOx into NO gas. A stream of nitrogen was bubbled through the purge vessel containing vanadium (III) with sodium hydroxide [NaOH (1 N)], and then through an analyzer (Sievers Nitric Oxide Analyzer® 280, GE Analytical Instruments, Boulder, CO, USA).