Cardiovascular pharmacology

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

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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 Serine1177 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 pinacilid. Increased acetylcholine vasorelaxation was abolished by a calcium-activated K⁺ channel (KCa) blocker, but not by an inhibitor of KATP 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 enhances endothelium-dependent and independent relaxation of mesenteric resistance arteries by mechanisms that involve increased eNOS activity, NO generation, and KCa channels activation. These effects may contribute to the cardiovascular effects associated with chronic fluoxetine treatment.

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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
vasomotor tone regulation, such as 5-HT2C receptor (Ni and Miledi, 1997), 5-HT3 receptor (Fan, 1994) and nicotinic receptors (Maggi et al., 1998); voltage-dependent sodium (Na+) and K+ channels (Panzarico 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 consumption 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 (CO2) 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, KH2PO4 1.18, MgSO4 1.17, NaHCO3 14.9, glucose 5.5, EDTA 0.03, CaCl2 1.6], at a constant temperature of 37 °C, pH 7.4, and gassed with a mixture of 95% O2 and 5% CO2.

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 (10−4 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 KATP and KCa 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 [Trition 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 aprotonin 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, Piscataway, 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).
2.6. Fluorescence detection of nitric oxide production

NO production was determined by the fluorescent NO indicator, 5,6 Diamino fluorescein diacetate (DAF-2 DA). Mesenteric arteries from Wistar rats were embedded in Tissue Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA). Unfixed frozen cross sections (10 μm) were incubated with DAF-2 DA (12.5 μM; Sigma) diluted in phosphate buffer with CaCl2 (0.4 mM); in a light protected and humidified chamber at 37 °C for 1 h. Fluorescence was detected with a 490–515 nm long-pass filter, under a microscope (Olympus, BX50) with a 20 × objective lens coupled to a digital camera. Fluorescent images were analyzed by measuring the mean optical density of the fluorescence in a computer system (Image J software) and normalized by the area.

2.7. NOS activity

The method adapted from Bredt and Snyder (1989), which measures the conversion of [C14] arginine to [C14]-citrulline, was used to determine NOS activity. Mesenteric bed fragments were homogenized with 0.3 ml of Hepes buffer (pH 7.4) containing 1.25 mM CaCl2, 1 mM DTT and 1 mM tetrahydrobiopterin (BH4). After homogenization, 1 mM NADPH, and 200,000 cpm of [C14] arginine (270 Ci/mmol) were added and the homogenates were incubated for 10 min at 37 °C. The resulting supernatants were applied to 2 ml columns of Dowex AG WX-8 (Na+ form) and diluted with double distilled water. The levels of [C14]-citrulline were determined using a beta counter. This method is based on the equimolar production of NO and citrulline from arginine, which is mediated by NOS. The results are expressed as the production of [C14]-citrulline per mg of tissue.

2.8. Detection of superoxide anion generation

Superoxide anion generation was measured by detection of fluorescent Dihydroethidium bromide (DHE) oxidation products. Mesenteric arteries from Wistar rats were embedded in Tissue Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA). Unfixed frozen cross sections (10 μm) were incubated with DHE (5 μM; polycyces) diluted in phosphate buffer with diethylene-triaminepentaacetic acid (DTPA [100 μM]), in a light protected and humidified chamber at 37 °C for 30 min. Fluorescence was detected with a 490–515 nm long-pass filter, under a microscope (Olympus, BX50) with a 20 × objective lens coupled to a digital camera. Fluorescent images were analyzed by measuring the mean optical density of the fluorescence in a computer system (Image J software) and normalized by the area.

2.9. Drugs

Fluoxetine, phenylephrine, acetylcholine, sodium nitroprusside, Bay 41-2272, pinacidil, glibenclamide, and charybdotoxin were from Sigma-Aldrich (St. Louis, MO, U.S.A.), 8-Bromo-cGMP was from Tocris (Ellisville, MO, U.S.A.).

2.10. Data analysis

Results are expressed as mean ± standard error of the mean (S.E.M.). Relaxation responses are expressed as the percentage of relaxation about the levels of pre-contraction induced by phenylephrine. Concentration-effect curves were analyzed by non-linear regression using the software GraphPad Prism (GraphPad Prism 5.0, GraphPad Software Inc., San Diego, CA, USA). The potency of agonists is expressed as pD2 (negative logarithm of the molar concentration that produces 50% of the maximal response). Statistical analysis of the pD2 values was performed using non-linear regression analysis followed by Student “t” test. Statistical differences were indicated as P<0.05.

3. Results

3.1. Effects of chronic fluoxetine treatment on endothelium-dependent and -independent vasorelaxation

Endothelium-intact mesenteric resistance arteries from rats chronically treated with fluoxetine exhibited increased acetylcholine-induced relaxation ([pD2]), Veh=7.08 ± 0.07; Fluox=7.4 ± 0.11, P<0.05 (Fig. 1A). Endothelium-denuded arteries also exhibited significantly increased relaxation to sodium nitroprusside ([pD2]), Veh=7.75 ± 0.08; Fluox=8.5 ± 0.11, P<0.05 (Fig. 1B). Phenylephrine-induced contraction was similar between the groups ([mN], Veh=24.1 ± 1.7; Fluox=23.3 ± 0.9).

3.2. Effects of chronic fluoxetine treatment on NO production, bioavailability and NOS activity

Nitric oxide measurement, by the fluorescent NO indicator (DAF-2 DA), showed that fluoxetine treatment increased both basal and acetylcholine-stimulated NO production in mesenteric arteries [arbitrary units (A.U), Veh=4.0 ± 0.5; Fluox=5.5 ± 0.3, and Veh=3.1 ± 0.1; Fluox=5.5 ± 0.6, respectively; P<0.05] when compared to the vehicle group (Fig. 2A and B).

![Fig. 1. Effects of chronic fluoxetine treatment on the relaxation responses of mesenteric resistance arteries to different agonists.](image)
Confirming these results, increased NO metabolite (NOx) levels (μM/mg protein), Veh = 1.2 ± 0.13; Fluox = 2.0 ± 0.13, P < 0.05 (Fig. 2B) were found in mesenteric arterial bed from rats chronically treated with fluoxetine (Fig. 2C). Constitutive NOS activity was also increased in arteries from fluoxetine-treated animals when compared with the vehicle group (Fig. 2D).

3.3. Effects of chronic fluoxetine treatment on eNOS phosphorylation at Ser1177 and eNOS/nNOS/iNOS expression

Mesenteric arteries from animals chronically treated with fluoxetine exhibited increased expression of Ser1177-phosphorylated-eNOS when compared to arteries from the vehicle group (Fig. 3A). Total eNOS, nNOS, and iNOS expressions were similar between mesenteric arteries from the vehicle and fluoxetine groups (Fig. 3A, B, and C, respectively).

3.4. Effects of chronic fluoxetine treatment on the activation and expression of soluble guanylyl cyclase

Bay 41–2272-induced activation of soluble guanylyl cyclase (sGC) produced similar relaxation in arteries from the vehicle and fluoxetine groups [(pD2), Veh = 6.7 ± 0.2; Fluox = 6.8 ± 0.10] (Fig. 4A). Expression of sGC α and β subunits was similar between arteries from vehicle and fluoxetine-treated rats (Fig. 4B).

3.5. Effects of chronic fluoxetine treatment on the relaxation responses to 8-bromo-cGMP and pinacidil

Relaxation responses of the mesenteric resistance arteries to the cGMP analog, 8-bromo-cGMP, were similar between the groups [(pD2), Veh = 4.5 ± 0.4; Fluox = 4.4 ± 0.3] (Fig. 5A). However, pinacidil-induced vasorelaxation was significantly increased in animals treated with fluoxetine when compared to the vehicle group [(pD2), Veh = 5.9 ± 0.12; Fluox = 6.5 ± 0.17, P < 0.05] (Fig. 5B).

3.6. Effects of KATP and KCa channels blockers on the increased acetylcholine vasorelaxation induced by chronic fluoxetine treatment

Relaxation responses to acetylcholine were not modified in the presence of KATP channel blocker, glibenclamide. In other words, acetylcholine vasorelaxation was significantly increased in animals treated with fluoxetine when compared to the vehicle group [(pD2), Veh = 7.6 ± 0.11; Fluox = 8.0 ± 0.12, P < 0.05] (Fig. 6A).
However, the K\textsubscript{Ca} blocker, charybdotoxin, abolished the differences in acetylcholine vasorelaxation between the groups \([\text{pD}_2], \text{Veh} = 7.3 \pm 0.15; \text{Fluox} = 7.4 \pm 0.12\) (Fig. 6B).

3.7. Effects of chronic fluoxetine treatment on superoxide anion generation

Chronic fluoxetine treatment did not change superoxide anion generation in mesenteric arteries when compared with the vehicle group \([\text{A.U.}, \text{Veh} = 30.8 \pm 3.0; \text{Fluox} = 31.7 \pm 1.5\] (Fig. 7).

4. Discussion

The major findings of the present investigation about the chronic fluoxetine effects on vascular reactivity are: (1) Fluoxetine treatment increases endothelium-dependent and -independent vasorelaxation as well as the constitutive NO synthases (eNOS and nNOS) activity, the phosphorylation of eNOS at Serine\textsuperscript{1177} and, the NO production; (2) Fluoxetine does not change vascular expression of neuronal and iNOS or guanylyl cyclase (GC); (3) Vasorelaxation induced by pinacidil, an activator of ATP-sensitive K\textsuperscript{+} channels, is increased in arteries from fluoxetine-treated rats; (4) Vasorelaxations induced by a cGMP analog or a sGC activator were not affected; and (5) blockade of K\textsubscript{Ca} channels abolishes increased vasorelaxation. These data indicate that chronic fluoxetine treatment increases the relaxation of resistance arteries by mechanisms that involve increased constitutive NO activity, NO generation and activation of K\textsubscript{Ca} channels. The following discussion intends to cover all these data.

To determine whether chronic fluoxetine treatment changes mechanisms involved in the vasomotor tone control, we initially assessed vascular reactivity to acetylcholine, an endothelium-dependent agonist, and to sodium nitroprusside, an endothelium-independent agent. Mesenteric arteries from rats chronically treated with fluoxetine exhibited increased vasodilator responses to both agents. One of the mechanisms that could facilitate acetylcholine and sodium nitroprusside-induced vasorelaxation is an increased activation of the NO-GC-cGMP pathway. The enzyme sGC contains a ferrous heme heterodimer composed of \(\alpha\) and \(\beta\) subunits, which is activated in response to NO binding to the heme moiety (Meuer et al., 2009). NO-induced activation of sGC enzyme in vascular smooth muscle cells increases intracellular cGMP levels, leading to activation of cGMP-dependent kinases (PKG), reduction of intracellular Ca\textsuperscript{2+} levels, activation of K\textsuperscript{+} channels and relaxation (Vanhoutte, 1989).

To evaluate whether fluoxetine changes the NO-GC-cGMP signaling pathway, we determined the vascular effects of a cGMP analog and a GC activator, but no differences in the vascular responses to these agents were observed. Similarly, there were no differences in the vascular expression of sGC \(\alpha\) and \(\beta\) subunits between the groups. Together, these data suggest that chronic fluoxetine treatment does not increase NO-GC-cGMP signaling.

As already mentioned, Ribback and colleagues (2012) demonstrated that NO-cGMP signaling is an important mechanism of fluoxetine and other SSRIs to promote acute vascular relaxation in vitro since NO and cGMP inhibitors decreased relaxation to these drugs. Also, K\textsuperscript{+} channel activation contributes to fluoxetine- and SSRIs-induced vasorelaxation. NO-induced activation of K\textsuperscript{+} channels and the consequent hyperpolarization and vasorelaxation may occur indirectly, via cGMP (Jackson, 2005), but also directly and independently of cGMP, as demonstrated by Bolotina et al., (1994). Accordingly, NO has been shown to activate directly K\textsubscript{Ca} and K\textsubscript{ATP} channels (Kawano et al., 2009).

Our results indicate that fluoxetine does not alter mechanisms associated with GC activation and cGMP-induced responses, but it changes responses mediated by activation of K\textsuperscript{+} channels. Also, arteries of rats chronically treated with fluoxetine exhibited increased relaxant responses to pinacidil suggesting that increased relaxation to acetylcholine and sodium nitroprusside are due to increased K\textsuperscript{+} channels activation. To confirm this hypothesis we determined the inhibitors of K\textsubscript{ATP} and K\textsubscript{Ca} channels effects on acetylcholine vascular reactivity. Increased dilation to acetylcholine in arteries from fluoxetine-treated rats was only abolished by charybdotoxin, a K\textsubscript{Ca} blocker. This result was initially unexpected.
considering that arteries from fluoxetine-treated rats exhibited increased relaxation to pinacidil, an activator of KATP channels. However, this result would suggest the possibility of nonspecific activation of K⁺ channels by pinacidil. Accordingly, Gelband and McCullough (1993) demonstrated that pinacidil increases the opening probability of single KCa channels in rabbit aortic smooth muscle microsomes.

By using activators and inhibitors of K⁺ channels to identify mechanisms associated with fluoxetine-induced dilatation in skeletal muscle arteries, Pacher et al. (1999a, 1999b) concluded that K⁺ channels activation is not involved in fluoxetine-induced vasorelaxation. Also, fluoxetine has also been reported to block K⁺ channels (Park et al., 1999; Perchenet et al., 2001). It is important to mention that the studies by Pacher (1999a, 1999b), Perchenet (2001), Park (1999) and colleagues tested acute effects of fluoxetine. It is possible that whereas K⁺ channels are not activated (or are blocked) by acute administration of fluoxetine, chronic treatment with this SSRI leads to increased K⁺ channels function.

Fluoxetine and other SSRIs, such as paroxetine, increase NOx (NO metabolites) levels, an additional mechanism that may contribute to the increased vasodilator responses in the fluoxetine-treated group. In the present study, increased NO production in the mesenteric bed of rats chronically treated with fluoxetine was determined by an NO-sensitive fluorescent probe and NOx levels. Supporting our data, Chrapko and colleagues (2006) demonstrated that treatment for eight weeks with paroxetine, an SSRI, increased NOx plasma levels in healthy subjects and patients with the major depressive disorder treated for 4 or 8 weeks with milnacipran, a serotonin-norepinephrine reuptake inhibitor (SNRI). Increased NOx levels after fluoxetine treatment seem to be a time-dependent event, since as demonstrated by Krass and colleagues (2011) a single dose of fluoxetine is unable to promote changes in the brain NOx levels.

To investigate mechanisms involved with the increased NO generation, we determined the expression of total and phosphorylated eNOS. The western blot analysis showed increased eNOS phosphorylation at Ser1177 in mesenteric resistance arteries of rats treated with fluoxetine. Ofek et al. (2012) reported that endothelial cells incubated with fluoxetine exhibit increased eNOS phosphorylation and greater interaction with heat shock protein 90 (HSP90), a protein known to regulate trafficking and signaling of several other proteins. Garcia-Cardena et al. (1998) demonstrated that HSP90 association with eNOS increases NO production. More importantly, HSP90/eNOS interaction is increased after chronic fluoxetine treatment. NOS activity was also determined to confirm these results. Our findings show that constitutive NOS activity is increased in the mesenteric bed of fluoxetine-treated rats when compared with the control group. Contrary to our findings, Chrapko et al. (2006) did not observe changes in platelets NOS activity after paroxetine treatment, indicating that tissue-specific differences may account for the apparent discrepancy.

The above-mentioned data, along with the absence of significant differences in nNOS and iNOS expression between arteries from vehicle- and fluoxetine-treated rats, indicate that increased eNOS phosphorylation and increased eNOS-derived NO generation contributes to the vascular changes observed with chronic fluoxetine treatment.
fluoxetine treatment. Corroborating our findings, Yoshino et al. (2015) did not observe changes in nNOS or iNOS expression after fluoxetine treatment.

From a clinical point of view, many patients with depression also exhibit cardiovascular diseases (Mast et al., 2004; Rabkin et al., 1983). Reduced NO biosynthesis or bioavailability has been considered a potential mechanism to explain this correlation (Joynt et al., 2003). Reduction of NO bioavailability and eNOS activity or decreased responsiveness of vascular smooth muscle cells to NO are common mechanisms described in cardiovascular diseases, such as arterial hypertension (Hayakawa and Raji, 1998; Luscher et al., 1987). The increased NO generation induced by fluoxetine may be a potential advantage in the depression treatment this disorder is associated with cardiovascular diseases (Lara et al., 2003). Some antidepressants, such as imipramine or venlafaxine, decrease NOx levels in the brain (Krass et al., 2011). It is possible that beneficial effects of fluoxetine may be related to opposite effects on NOx levels.

Its antioxidant properties represent another potential mechanism involved in fluoxetine-induced increased NO bioavailability. To test this evidence, DHE fluorescence, a technique that detects superoxide anion generation, was determined in mesenteric arteries sections. No differences in DHE fluorescence, or superoxide anion generation, were observed between the groups.

Galecki et al. (2009) demonstrated that fluoxetine therapy attenuates oxidative stress, i.e. it increases the activity of antioxidative enzymes, in patients with major depressive disorder. It is important to note that these patients already presented a pro-oxidative status before the treatment was started. Similarly, Chung et al. (2010) observed antioxidant effects with fluoxetine treatment in the presence of inflammatory conditions, suggesting that fluoxetine effectively has antioxidant properties in oxidative stress-associated conditions. Since no differences in superoxide anion generation were observed after chronic treatment of healthy animals with fluoxetine, one may suspect that fluoxetine does not have significant antioxidative effects when reactive oxygen species production is normal/balanced.

In summary, chronic fluoxetine treatment increases endothelium-dependent and -independent relaxant responses in mesenteric resistance arteries by mechanisms that involve increased eNOS activity, NO generation, and KCa channels activation. Whether these effects contribute to the side effects of the cardio-vascular system or whether this may represent a beneficial effect associated with chronic fluoxetine treatment needs to be further investigated. It is important to emphasize, once again, that the aim of the investigation was to study the effects of chronic fluoxetine use in vascular reactivity. Since these effects were determined only in arteries from the mesenteric circulation, further studies in other vascular beds are necessary.

Authors’ contributions

CAP, NSF and FLACM performed wet laboratory experiments. CAP, RCT, LBR, and FSC designed the study. CAP wrote the manuscript and JAR, PRE, FSC, RCT revised its scientific content.

Conflict of interest

The results presented in this paper have not been published previously in whole or part, except in abstract format.
Acknowledgments

The work was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Brazil.

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Fig. 7. Effects of chronic fluoxetine treatment on superoxide anion generation. (A) Representative image and (B) bar graph showing dihydroethidium (DHE; 5 μM)-derived fluorescence in sections of mesenteric resistance arteries from Wistar rats chronically treated with vehicle (Veh) or fluoxetine (Fluox). Each point represents the mean ± S.E. M. N= 5. Scale bar: 100 μm.