Fluoxetine (Prozac) interaction with the mitochondrial voltage-dependent anion channel and protection against apoptotic cell death

Edna Nahon, Adrian Israelson, Salah Abu-Hamad, Varda Shoshan-Barmatz*

Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

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Abstract Fluoxetine (Prozac) is a potent antidepressant compound inhibiting serotonin reuptake, but also Na^+, K^+ and Ca^{2+} channels and reported to both trigger and prevent apoptosis. Recently, fluoxetine was found to increase the voltage sensitivity of the mitochondrial voltage-dependent anion channel (VDAC). VDAC which functions in transporting metabolites across the mitochondria also plays a crucial role in apoptosis.

Here, we demonstrate that fluoxetine interacted with VDAC and decreased its conductance. Fluoxetine inhibited the opening of the mitochondrial permeability transition pore, the release of cytochrome *c*, and protected against staurosporine-induced apoptotic cell death. These findings may explain some of the reported fluoxetine side effects.

Keywords: Voltage-dependent anion channel; Fluoxetine; Prozac; Permeability transition pore; Apoptosis; Cytochrome *c*

1. Introduction

Fluoxetine, known also as Prozac, is a clinically used potent antidepressant compound [1]. Fluoxetine is a selective serotonin reuptake inhibitor with a high selectivity for the 5-hydroxytryptamine (5-HT) transporter, and thus, in the brain, modulates synaptic serotonin concentration [2]. However, fluoxetine produces undesired side effects including anxiety, sleep disturbances, sexual dysfunction and gastrointestinal disturbances [3].

Besides the well-known action as serotonin reuptake inhibitor, fluoxetine exerts other effects, such as blockade of muscular and neuronal nicotinic receptors [4] and inhibition of monoamine oxidase A and B [5]. Fluoxetine has also been reported to inhibit the activity of the voltage-dependent Na^+ and K^+ and Ca^{2+} channels [6,7]. In addition, fluoxetine inhibits the multi-drug resistance extrusion pump and thus enhances the response to chemotherapy. Indeed, fluoxetine enhances doxorubicin accumulation within tumors [8].

Several studies have linked fluoxetine with cell proliferation and an increased risk of developing cancer [9–11]. Fluoxetine has been shown to enhance cell proliferation and to prevent apoptosis in dentate gyrus [10], to stimulate DNA synthesis [9] and inhibit UV-induced DNA fragmentation in U937 cells [12]. Contradicting results showing enhancement of programmed cell death in various cell lines have also been reported [13]. Fluoxetine was found to trigger rapid and extensive apoptosis in Burkitt lymphoma cells that is prevented by over-expression of the anti-apoptotic Bcl-2 [13].

Fluoxetine was shown to penetrate the cell membrane and to be distributed in several intracellular compartments. ^{18}F-Fluoxetine was found to bind mostly to mitochondria (60–70%), but also to synaptosomes and other cellular organelles [14].

Recently, Thinnes [15] demonstrated that fluoxetine increases the voltage-dependence of the voltage-dependent anion channel (VDAC1) incorporated into a planar lipid bilayer and proposed that fluoxetine blocks the mitochondrial permeability transition pore (PTP). VDAC, also known as a mitochondrial porin, is a large channel that transports anions, cations, [16,17] adenine nucleotides [18], Ca^{2+} [19] and other metabolites [20] into and out of the mitochondrial intermembrane space. VDAC also plays an important role in apoptosis by participating in the release of intermembrane space proteins, including cytochrome *c* [21].

In this study, we demonstrate that fluoxetine-modified VDAC conductance and prevented the opening of the mitochondrial PTP, release of cytochrome *c* and apoptotic cell death induced by staurosporine (STS). The interaction of fluoxetine with the mitochondrial protein VDAC, inhibiting its activity, may explain some of the clinically reported side effects.

2. Materials and methods

2.1. Materials

Tris, HEPES, asolactin, fluoxetine, and Triton X-100 were purchased from Sigma Chemicals Co., anti-VDAC antibody (Cal Biochem) and HRP conjugated anti-mouse from Protos Immunoresearch (San Francisco, CA). Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad and Celite from the British Drug Houses.

2.2. Mitochondrial preparation

Mitochondria were isolated from rat liver as described previously [19] and used immediately.

2.3. Ca^{2+} accumulation

Freshly prepared mitochondria (1 mg/ml) were incubated at 30 °C for 2 min with fluoxetine in the presence of 225 mM mannitol, 75 mM sucrose, 5 mM HEPES/KOH, pH 7.0, 5 mM succinate and 200 μM Pi. The reaction was initiated by the addition of 120 μM $CaCl_2$ (containing 3×10^4 cpm/nmol $^{45}Ca^{2+}$) and was terminated by rapid Millipore filtration followed by a wash with 5 ml of 0.15 M KCl.

*Corresponding author. Fax: +972 8 6472992.
E-mail address: vardasb@bgu.ac.il (V. Shoshan-Barmatz).

Abbreviations: PTP, permeability transition pore; STS, staurosporine; VDAC, voltage-dependent anion channel

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2.4. Mitochondrial swelling

Ca^{2+}-induced mitochondrial swelling was assayed at 24 °C and under the same conditions as for Ca^{2+} accumulation. Swelling was initiated by Ca^{2+} (200 μM) addition, and absorbance changes at 540 nm were monitored with an Ultraspec 2100 spectrophotometer.

2.5. Release of cytochrome c

Mitochondria (1 mg/ml) were incubated with fluoxetine for 2 min and additional 15 min with Ca^{2+} (200 μM) in a solution containing 150 mM KCl, 25 mM $NaHCO_3$, 5 mM succinate, 1 mM $MgCl_2$, 3 mM KH_2PO_4, 20 mM HEPES, pH 7.4 [22]. Samples were centrifuged and the supernatants (40 μl) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis using monoclonal anti-cytochrome *c* antibody (1:500) and HRP-conjugated anti-mouse IgG as a secondary antibody (1:10000).

2.6. Purification of VDAC and single channel recording and analysis

VDAC was purified from rat liver mitochondria using LDAO and hydroxyapatite followed by carboxymethyl (CM)-cellulose in which LDAO was replaced by β-octylglucoside as previously described [16]. Reconstitution of purified VDAC into a planar lipid bilayer, single channel current recording, and data analysis were carried out as previously described [19].

3. Results

3.1. Fluoxetine interacts with VDAC to modify channel conductance and voltage dependence

Purified VDAC (Fig. 1C), isolated from rat liver mitochondria using CM-cellulose and β-octylglucoside, was reconstituted into a planar lipid bilayer and its channel activity was studied under voltage-clamp conditions. Current passing through VDAC in response to voltages stepped from a holding potential of 0 mV to −10 or −40 mV was recorded before and after the addition of fluoxetine (Fig. 1). At relatively small membrane potentials (−10 mV), the channel remained stable in the full conducting state for over 30 min of recording. However, upon addition of fluoxetine, the channel was stabilized in its low-conducting state (Fig. 1A). At −40 mV, transitions between the main conductance state and the sub-conductance state occurred and fluoxetine stabilized the channel in the low-conducting state (Fig. 1A). Fluoxetine-promoted VDAC closure was observed at all voltages tested (Fig. 1B).

3.2. Fluoxetine prevents PTP opening and release of cytochrome c

It has been suggested that activation of the Ca^{2+}-dependent mitochondrial PTP is a key event committing the cell to an apoptotic fate [23]. PTP is a large channel proposed to be formed by a direct association between VDAC in the OMM, adenine nucleotide translocator, located in the IMM and cyclophilin D in the matrix [16,23–26]. Since VDAC is a proposed component of the PTP, the effect of fluoxetine on PTP opening, as monitored by Ca^{2+} accumulation and swelling of energized mitochondria, was examined.

When freshly isolated mitochondria were allowed to generate a membrane potential, a transient Ca^{2+} accumulation was observed; it reached a maximal level, and then rapidly released

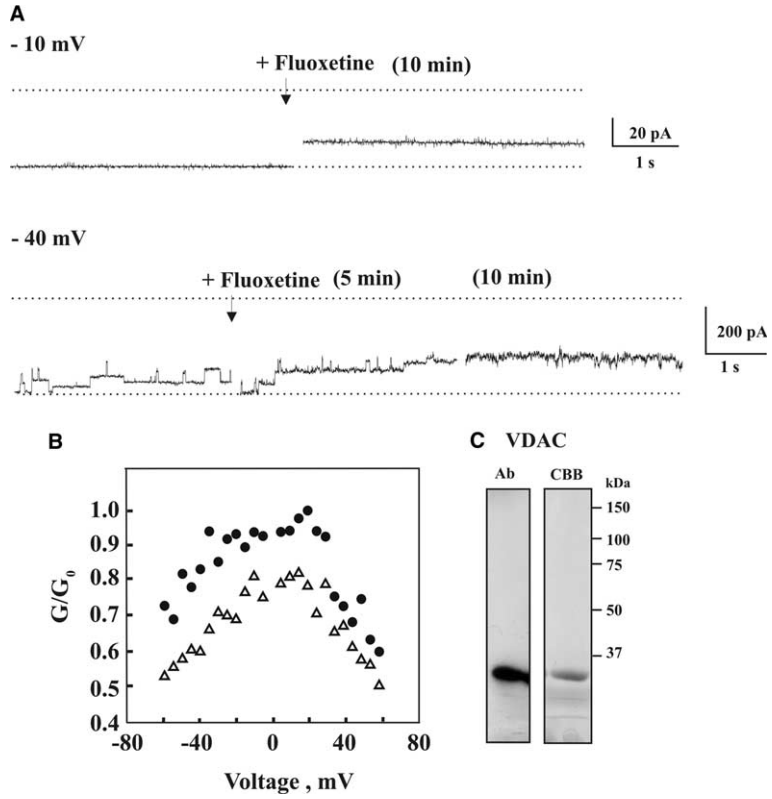

Fig. 1. Fluoxetine decreases VDAC channel conductance. (A) Purified VDAC was reconstituted into a planar lipid bilayer, and channel currents through VDAC, in response to a voltage step from 0 to −10 mV or 0 to −40 mV, were recorded before and 5 or 10 min after the addition of fluoxetine (50 μM). The dashed lines indicate the zero-current and the maximal current levels. (B) Multi-channel recordings of the average steady-state conductance of VDAC before (●) and 10 min after the addition of 50 μM fluoxetine (△) as a function of voltage are shown. Relative conductance was determined as the ratio of conductance at a given voltage (G) and the maximal conductance (G_0). (C) Coomassie (CBB) staining and immunoblot (Ab) of purified VDAC used in these experiments.