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

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Received 20 June 2005; revised 5 August 2005; accepted 10 August 2005
Available online 24 August 2005
Edited by Vladimir Skulachev

Abstract Fluoxetine (Prozac) is a potent antidepressant compound inhibiting serotonin reuptake, but also Na⁺, K⁺ and Ca²⁺ 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 Ca²⁺ 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. 18F-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], Ca²⁺ [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). Hydroxypatite (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. Ca²⁺ 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 CaCl₂ (containing 3 × 10⁶ cpm/nmol ⁴⁰Ca²⁺) and was terminated by rapid Millipore filtration followed by a wash with 5 ml of 0.15 M KCl.
2.4. Mitochondrial swelling

Ca\textsuperscript{2+}-induced mitochondrial swelling was assayed at 24 °C and under the same conditions as for Ca\textsuperscript{2+} accumulation. Swelling was initiated by Ca\textsuperscript{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\textsuperscript{2+} (200 µM) in a solution containing 150 mM KCl, 25 mM NaHCO\textsubscript{3}, 5 mM succinate, 1 mM MgCl\textsubscript{2}, 3 mM KH\textsubscript{2}PO\textsubscript{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 \textit{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 \(\beta\)-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 \(\beta\)-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\textsuperscript{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\textsuperscript{2+} accumulation and swelling of energized mitochondria, was examined.

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

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 (\(G_\text{m}\)). (C) Coomassie (CBB) staining and immunoblot (Ab) of purified VDAC used in these experiments.
This transient Ca\(^{2+}\) retention requires the presence of Pi and suggests that mitochondria undergo a permeability transition, losing the accumulated Ca\(^{2+}\) via the PTP. The concentration dependence of fluoxetine for preventing PTP opening was examined at two time points – 1 and 4 min after initiating Ca\(^{2+}\) accumulation (Fig. 2B). As expected, according to Fig. 2A, after 1 min, Ca\(^{2+}\) content of the mitochondria was the same in the absence or the presence of up to 50 \(\mu\)M fluoxetine. On the other hand, 4 min after initiation of Ca\(^{2+}\) accumulation, in the absence of fluoxetine, all accumulated Ca\(^{2+}\) was released. This, however, was inhibited in the presence of fluoxetine in a concentration-dependent manner, with 50% and 100% inhibition of PTP opening obtained at about 18 and 50 \(\mu\)M, respectively (Fig. 2B). The Ca\(^{2+}\) efflux mediated via activated PTP and inhibited with fluoxetine is also sensitive to the well-known inhibitor of PTP, cyclosporin A (CsA) (Fig. 2C).

As shown in Fig. 3, in the presence of fluoxetine, mitochondrial swelling, as monitored by the absorbance change following Ca\(^{2+}\) addition, was inhibited by fluoxetine (20 or 50 \(\mu\)M). Fluoxetine inhibited PTP opening when added either prior to PTP induction (Fig. 3A) or 1 min after initiation of Ca\(^{2+}\) accumulation, but before PTP opening (Fig. 3B). As shown previously [23], this mitochondrial swelling is prevented by CsA (Fig. 3A).

Thus, both swelling and Ca\(^{2+}\) accumulation, which reflect PTP assembly or opening, are prevented by fluoxetine.

Next, the effect of fluoxetine on the release of cytochrome \(c\) from mitochondria was tested. Mitochondria were incubated under conditions shown previously to activate cytochrome \(c\) release [22]. In the presence of Ca\(^{2+}\) and succinate, cytochrome \(c\) was released from mitochondria (as revealed by Western blot analysis), where 20 and 50 \(\mu\)M of fluoxetine prevented 50% and 100%, respectively, of this release (Fig. 3C).

### 3.3. Fluoxetine prevents STS-induced apoptotic cell death

Next we tested whether fluoxetine, which prevented the release of cytochrome \(c\) (Fig. 3C), would suppress apoptosis induced by STS in U-937 cells. Apoptotic cell death was induced by exposure of control cells and cells incubated with fluoxetine (10, 20 or 50 \(\mu\)M) to STS (Fig. 4). Upon exposure of control cells to STS for 5 h, about 70% of the cells underwent apoptosis, whereas in cells not exposed to STS, 5% of the cells died (Fig. 4A and B). When U-937 cells were incubated with 50 \(\mu\)M fluoxetine, a decrease of up to 80% in the degree of STS-induced apoptotic cell death was observed after 4–8 h incubation, and lower levels of protection were obtained with 10 and 20 \(\mu\)M fluoxetine (Fig. 4C).
Fluoxetine produced 50% protection against STS-induced apoptosis when incubated with cells for 8 h at 20 μM or for 1 h at 50 μM (Fig. 4D).

4. Discussion

In this study, we have characterized fluoxetine modulation of the mitochondrial phase of apoptosis using purified VDAC, isolated mitochondria and tumor-derived cells in culture. The results show that fluoxetine: (a) interacted directly with purified VDAC reconstituted into a planar lipid bilayer to decrease channel conductance; (b) prevented PTP opening as demonstrated by preventing the release of accumulated Ca²⁺ and by swelling of energized mitochondria; (c) inhibited release of cytochrome c from mitochondria and (d) protected against STS-induced apoptotic cell death. These results suggest that fluoxetine interacts with a mitochondrial component and prevents mitochondria-mediated cell death. The specific protein(s) interacting with fluoxetine has not yet been identified. Accumulating evidence suggests VDAC, located at the OMM is a key player in apoptosis [19,26–29]. VDAC is a component of the PTP, is involved in release of cytochrome c and regulates apoptotic cell death [26–29]. Fluoxetine interacting with VDAC and modifying its conductance (Fig. 1), as well as inhibiting mitochondrial PTP opening and release of cytochrome c (Figs. 2 and 3) make VDAC an appropriate candidate for a fluoxetine target. Thus, the actions of fluoxetine preventing PTP opening and release of cytochrome c may result from its interacting with VDAC and modifying its activity/conformation. The effect of fluoxetine in delaying the onset of PTP opening is similar to that of other PTP inhibitors such as L-glutamate [30]. However, its effect differs from those of CsA [23], RuR or Ru360 [19], hexokinase-I [22] or R68-3400 [24] which prevent PTP opening rather than delaying it.

Recently [31], it has been proposed that plasma membrane VDAC is involved in apoptotic cell death. Thus, the effect of fluoxetine on cell death might result not only from its interaction with mitochondrial VDAC, but also with plasma membrane VDAC. Fluoxetine known as selective serotonin reuptake inhibitor but it was found to possess a wide range of biological activities such as interaction with Na⁺ and K⁺ and Ca²⁺ channels [6,7]. In isolated mitochondria, fluoxetine indirectly affects electron transport and (F1Fo)ATPase activity, and thus inhibits oxidative phosphorylation, as a result of interfering with the physical state of the lipid bilayer of the inner mitochondrial membrane [32]. However, these effects of fluoxetine on mitochondrial activities may result from interacting with VDAC and decreasing its conductance as a channel providing passage for Ca²⁺ [19], adenine nucleotides [18] and other metabolites [20] into and out of mitochondria [16]. The modification of VDAC conductance, and thereby of mitochondrial activities, may explain some of the clinically reported side effects for this drug [3].

Recent epidemiological and laboratory studies have raised questions regarding the association of antidepressant use with cancer risk [33]. However, conflicting results of non-association have been reported in a number of other studies [34]. In vitro studies with tumor cells exposed to fluoxetine have shown inhibition [13] or stimulation [9,12] of cell growth or DNA synthesis. Since alterations in cell proliferation, cell death or both lead to cancer development, the possible relation between inhibition of apoptotic cell death by fluoxetine and cancer requires further study. This includes elucidating the mechanism by which fluoxetine prevents PTP opening and the release of the pro-apoptotic cytochrome c, as well as clarifying the relationship between the
observed cancer-related effects and fluoxetine interaction with mitochondria.

Acknowledgments: This research was supported by grants from the Israel Science Foundation.

References

Fig. 4. Fluoxetine protects against apoptotic cell death induced by STS. (A) Human U-937 cells were incubated without (a,c) or with fluoxetine (20 μM) for 18 h (b,d) and were exposed to STS (1.25 μM) (c,d). After five hours cells were stained with acridine orange and ethidium bromide. Arrows and arrowheads indicate cells in early and late apoptotic state, respectively. Scale bar, 20 μm. B–D present quantitative analysis of apoptotic cell death. In B, quantitative analysis of apoptotic cell death was assessed 5 h after their exposure to STS (1.25 μM) by ANOVA and t-test. P < 0.001 was considered statistically significant (***). Data are the means ± S.E.M, n = 3. In each independent experiment, approximately 200 cells were counted. In C, U-937 cells were incubated with 10 μM (●), 20 μM (○) or 50 μM (▲) of fluoxetine for the indicated time and the degree of apoptotic cells was analyzed. The results are presented as % of protection against STS-induced cell death. In D, cells were pre-incubated for 1 h (●) or 8 h (○) with the indicated concentration of fluoxetine and then apoptotic cell death was induced by 5 h incubation with STS.


