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EFFECT OF FLUOXETINE ON RAT LIVER MITOCHONDRIA

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Abstract—The *in vitro* and *in vivo* effects of fluoxetine (and its active metabolite norfluoxetine) on mitochondrial respiration and F_oF_1-ATPase were studied, respectively, in mitochondria and submitochondrial particles isolated from rat liver. Fluoxetine *in vitro* inhibited state 3 mitochondrial respiration for α-ketoglutarate and succinate oxidations (50% of effect at 0.25 and 0.35 mM drug concentrations, respectively); stimulated state 4 for succinate; and induced a decrease in the respiratory control ratio (RCR) for both oxidizable substrates. The F_oF_1-ATPase activity was determined at various pH levels in the absence and presence of Triton X-100. The solubilized form was not affected markedly, but an inhibition, apparently non-competitive, was observed for the membrane-bound enzyme, with 50% of the effect at a 0.06 mM drug concentration in pH 7.4. These results suggest that fluoxetine *in vitro* acts on F_oF_1-ATPase through direct interaction with the membrane F_o component (similar to oligomycin), or first with mitochondrial membrane and then affecting F_o. A very similar behavior concerning the respiratory parameters and F_oF_1-ATPase properties was observed with norfluoxetine. The *in vivo* studies with fluoxetine showed stimulation of mitochondrial respiration in state 4 for α-ketoglutarate or succinate oxidations in acute or prolonged treatments (1 hr after a single i.p. dose of 20 mg of drug/kg of body weight, and 22 hr after 12 days of treatment with a daily dose of 10 mg/kg of body weight, respectively), indicating uncoupling of oxidative phosphorylation. Pronounced changes were not observed in the $K_{0.5}$ values of F_oF_1-ATPase catalytic sites, but the V_{max} decreased during the prolonged treatment. The results show that fluoxetine (as well as norfluoxetine) has multiple effects on the energy metabolism of rat liver mitochondria, being potentially toxic in high doses. The drug effects seem to be a consequence of the drug and/or metabolite solubilization in the inner membrane of the mitochondria.

Key words: antidepressants; fluoxetine; norfluoxetine; mitochondria; F_oF_1-ATPase; rat liver

Fluoxetine is a new atypical antidepressant drug whose therapeutic effect has been attributed to a potent and selective inhibition of presynaptic serotonin reuptake [1]. Its chemical structure differs from that of the tricyclic antidepressants such as imipramine and its analogs, which are tertiary or secondary amines with a three-ring molecular core. Fluoxetine is a secondary amine with one phenyl and one tolyl group in the structure [2]. A high therapeutic index associated with mild transient side-effects has been described for fluoxetine [3, 4], but adverse events during clinical treatment with the drug have also been reported [5–7].

It is known that a variety of drugs may interfere with energy metabolism in liver mitochondria, with loss of respiratory control and uncoupling of oxidative phosphorylation [8]. The psychoactive drugs, including the tricyclic antidepressants, have been investigated widely in this aspect, considering their potential for toxicity when given in high doses. In association with a higher membrane solubility [9, 10], imipramine and its analogs are known to affect mitochondrial function in various tissues [10–14]. In rat liver, they are bound tightly to the mitochondria [13].

The subcellular distribution pattern of fluoxetine and its active metabolite, norfluoxetine, in rat brain, after intravenous or oral administration, shows that 40% of the drug or its metabolite is recovered in synaptosomes and mitochondria [15]. This pattern of fluoxetine distribution and the well-known potential of psychoactive drugs for toxicity [13] were considered in the present study. The aim was to assess the potential of fluoxetine to affect the mitochondrial bioenergetic processes, through investigation of the *in vitro* and *in vivo* effects of the drug or its active metabolite on liver mitochondrial respiration and F_oF_1-ATPase kinetic properties, in Wistar rats. A comparison is made to previously described effects of tricyclic antidepressants.

MATERIALS AND METHODS

Materials. Fluoxetine hydrochloride (Lot 490402) and norfluoxetine hydrochloride (Lot U09-6FK-20)

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were donated by Laboratory "Eli Lilly do Brasil" and "Eli Lilly & Co., USA," respectively. The other compounds were purchased from the Sigma Chemical Co. (St. Louis, MO) and Merck (Rahway, NJ).

Management of the animals. The assays analysed the effect of drug or its metabolite on mitochondria and submitochondrial particles isolated from normal rats (*in vitro* studies) or from animals after drug treatment (*in vivo* studies). For the *in vivo* studies, fluoxetine hydrochloride in sterile water was administered i.p. (0.2 mL) either as a single dose of 20 mg/kg of body weight, and the animals were killed 1 hr later (acute treatment); or as a daily dose of 10 mg/kg body weight for 12 days, and the rats were killed 22 hr after the last injection (prolonged treatment). Acute 30-min and prolonged- 22-day treatments were also analysed. In all cases, the control animals were injected with sterile water. The conditions of drug administration used in the acute and prolonged treatments were based on previous reports for fluoxetine [16, 17] and tricyclic antidepressants [14].

Preparation of mitochondria and submitochondrial particles. For mitochondrial preparation, male Wistar strain rats weighing approximately 250 g were anesthetized with ether, followed by removal of the liver. The livers were sliced into small pieces; homogenized with a Potter–Elvehjem-type homogenizer in a medium with 250 mM sucrose, 2 mM EDTA and 1 mg/mL BSA, pH 7.4; and isolated by differential centrifugation in a medium with 250 mM sucrose and 1 mg/mL BSA, pH 7.4 [18]. For respiration, all experiments were carried out with freshly prepared mitochondria. For preparation of submitochondrial particles, the final pellet was frozen and stored at −20°. After 24 hr, the pellet was thawed, and the submitochondrial particles were prepared as previously described for rat heart [19]. Protein was determined according to Murphy and Kies [20].

Oxygen consumption assays. Oxygen consumption was analysed polarographically at 30° in an oxygraph equipped with a Clark oxygen electrode (Gilson Medical Electronics, Middleton, WI, U.S.A.), and the respiratory parameters were determined as described previously [21]. α-Ketoglutarate and succinate, at concentrations of 5 mM, were used as oxidizable substrates in 1.4 mL of medium with 250 mM sucrose, 8.5 mM potassium phosphate, 1 mM EDTA and 0.14 mg/mL BSA in 10 mM Tris–HCl, pH 7.4; 2.5 mg of mitochondrial protein was used, and state 3 respiration was induced with 400 nmol MgADP.

ATPase assays. F_oF_1-ATPase activity was determined at 37° by measurement of inorganic phosphate liberated from MgATP hydrolysis, according to Heinonen and Lahti [22]. Enzymatic reaction was started by the addition of MgATP to the reaction medium (1 mL final volume) containing the submitochondrial particles (20 μg of protein) and fluoxetine in 100 mM sucrose, 80 mM KCl and 50 mM HEPES–KOH; it was stopped at various times by the addition of 0.5 mL of 30% trichloroacetic acid. Kinetic parameters were estimated by using the Hill equation.

Statistics. The Wilcoxon or Mann–Whitney test was used for statistical evaluation of mean values of the respiratory parameters between experimental and control animals.

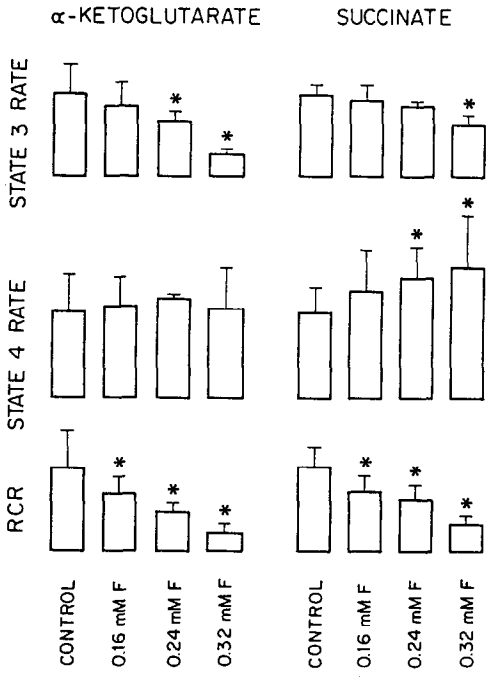

Fig. 1. *In vitro* effect of fluoxetine (F) on respiratory parameters of rat liver mitochondria (2.5 mg of protein) energized with 5 mM α-ketoglutarate and 5 mM succinate/5 μM rotenone, at 30°. The medium (1.4 mL) contained 250 mM sucrose, 8.5 mM potassium phosphate, 1 mM EDTA, 0.14 mg/mL BSA and 10 mM Tris–HCl, pH 7.4. State 3 respiration was induced by the addition of 400 nmol MgADP. Values represent the means ± SD of five assays with different preparations. Control values for state 3 respiration rate for α-ketoglutarate and succinate oxidations: 45 and 49 natoms O/min/mg protein, respectively. Control values for state 4 respiration rate for the same substrates: 8 and 12 natoms O/min/mg protein, respectively. RCR control values: 6.0 and 4.3, respectively. Key: (*) significantly different ($P < 0.05$) vs respective controls.

RESULTS

In vitro *experiments with fluoxetine.* Fluoxetine *in vitro* decreased the state 3 respiration rate for α-ketoglutarate and succinate oxidations, from control rates of 45 and 49 natoms O/min/mg protein, respectively. The effect was greater for the first oxidizable substrate (Fig. 1). The state 4 respiration rate increased for succinate oxidation, although for α-ketoglutarate, only a slight enhancement was observed (control rates of 12 and 8 natoms O/min/mg protein, respectively). Therefore, the decrease in the RCR (respiratory control ratio) for α-ketoglutarate oxidation was due mainly to inhibition of state 3 respiration, whereas that for succinate oxidation was due mainly to stimulation of state 4 (RCR control values of 6.0 and 4.3, respectively).

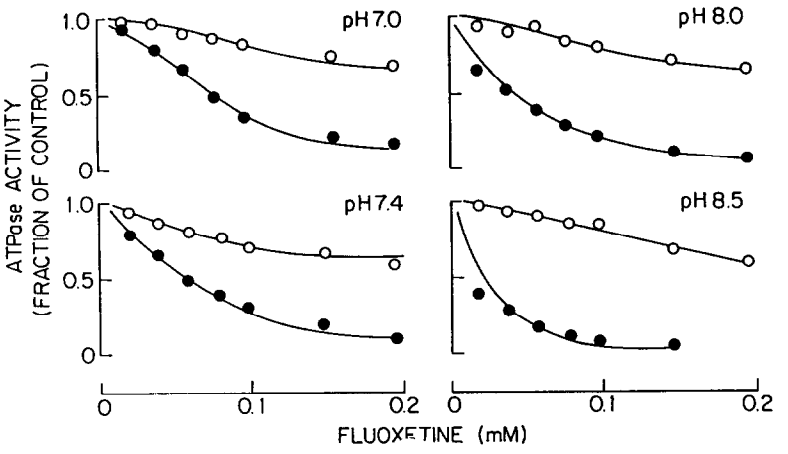

Fig. 2. *In vitro* effect of fluoxetine on MgATP hydrolysis by F_oF_1-ATPase from rat liver submitochondrial particles at various pH levels, in the absence (●) and presence (○) of 2.5 mg/mL Triton X-100. After 5 min of preincubation, enzymatic reaction was started by the addition of 1 mM MgATP in the reaction medium (1 mL final volume) containing submitochondrial particles (20 μg of protein) and fluoxetine in 100 mM sucrose, 80 mM KCl and 50 mM HEPES–KOH, at 37°. The curves are representative of three experiments with different preparations. Control rates for F_oF_1-ATPase activity were 0.82, 0.85, 1.16 and 1.27 μmol P_i/min/mg protein, respectively, in pH 7.0, 7.4, 8.0 and 8.5.

A drug concentration of 0.08 mM did not change the respiratory parameters, and the ADP:O ratio (not shown) decreased only with 0.32 mM drug. When fluoxetine was added to mitochondria during state 3 respiration, 50% inhibition was observed at 0.25 and 0.35 mM drug, respectively, for α-ketoglutarate and succinate oxidations. In conformity with the results in Fig. 1, addition of fluoxetine during state 4 respiration led to an expressive rate enhancement only for succinate oxidation (not shown).

The effect of increasing concentrations of fluoxetine on F_oF_1-ATPase activity was investigated at various pH levels, using membrane-bound enzyme (85% inhibited by 1 μg/mL oligomycin) and its Triton X-100 solubilized form (15% inhibited by 1 μg/mL oligomycin). The results in Fig. 2 show that fluoxetine inhibited F_oF_1-ATPase activity (control rates of 0.82, 0.85, 1.16 and 1.27 μmol P_i/min/mg protein, respectively, in pH 7.0, 7.4, 8.0 and 8.5); an expressive effect was seen for the membrane-bound enzyme, but was not observed for its solubilized form. The drug concentrations giving 50% inhibition on the membrane enzyme were 0.08, 0.06, 0.04 and 0.02 mM, respectively, in pH 7.0, 7.4, 8.0 and 8.5.

The curves of MgATP saturation of F_oF_1-ATPase (Fig. 3) show two kinetic phases corresponding to two catalytic sites [19]. Fluoxetine affected V_{max}, but not the $K_{0.5}$ values of these sites or the contribution of each site to the total activity of enzyme. An Arrhenius plot of temperature–effect data showed a two-phase curve with activation energies of 27 and 38 kcal/mol, respectively, above and below a phase transition point at 15°, for enzyme in both the absence and presence of 0.06 mM fluoxetine, in pH 7.4 (not shown).

In vitro *experiments with norfluoxetine.* The *in*

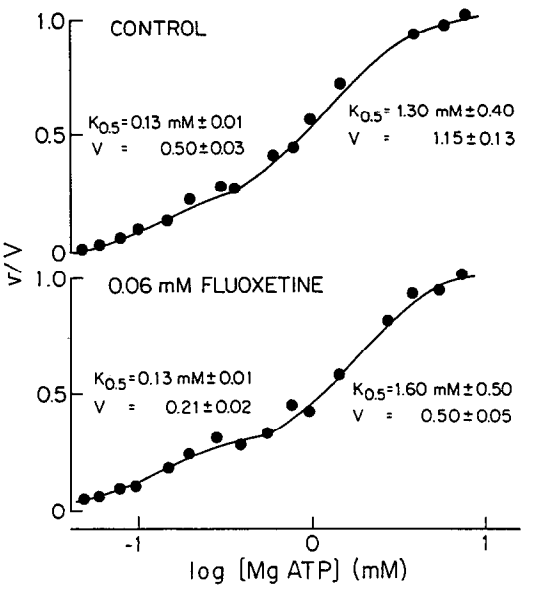

Fig. 3. *In vitro* effect of 0.06 mM fluoxetine on MgATP saturation of F_oF_1-ATPase from rat liver submitochondrial particles, in pH 7.4, and respective kinetic parameters. The experimental conditions are described in Fig. 2, using various substrate concentrations. *V* values (maximal velocity) are expressed as μmol of MgATP hydrolyzed/min/mg of protein. The curves are representative of five experiments with different preparations, and the values of the kinetic parameters are expressed as means ± SD.

Table 1. *In vitro* effect of 0.16 mM norfluoxetine (NF) on respiratory parameters of rat liver mitochondria

Oxidizable substrate		State 3 rate	State 4 rate	RCR
α-Ketoglutarate	Control	1.00 ± 0.12	1.00 ± 0.47	1.00 ± 0.31
	NF	0.65 ± 0.18*	1.30 ± 0.40	0.54 ± 0.17*
Succinate	Control	1.00 ± 0.12	1.00 ± 0.14	1.00 ± 0.13
	NF	0.87 ± 0.17*	2.03 ± 0.64*	0.41 ± 0.09*

Absolute control values are the same as those described in Results for fluoxetine effect *in vitro*.

The values (±SD) in the presence of NF represent fractions of controls (1.00 ± SD). They are means of five experiments with different preparations.

* Significantly different ($P < 0.05$) vs respective controls.

vitro effect of norfluoxetine on respiratory parameters of rat liver mitochondria is shown in Table 1. Similar to fluoxetine, inhibition of the state 3 respiration rate was greater for α-ketoglutarate, and stimulation of state 4 was greater for succinate oxidation. The RCR decreased to approximately half of the control values, and the ADP:O ratio (not shown) decreased approximately 25%. When norfluoxetine was added to mitochondria during state 3 respiration (not shown), 50% inhibition was observed at metabolite concentrations of 0.18 and 0.26 mM, respectively, for α-ketoglutarate and succinate oxidations.

The effect of norfluoxetine on F_oF_1-ATPase activity was assayed in pH 7.4, in the absence and presence of Triton X-100 (not shown). Behavior very similar to that of fluoxetine (Fig. 2) was observed, with an IC_{50} of 0.075 mM for membrane-bound enzyme.

In vivo *experiments with fluoxetine.* Prolonged treatment of rats with fluoxetine led to a 38% decrease in body weight gain compared with control animals. This effect of fluoxetine is in accord with previous reports in rats, and has been associated with one possible anorectic activity of drug [16]. However, the liver weight and the hepatic glycogen concentration were not changed significantly.

Our *in vivo* experiments with fluoxetine (Fig. 4) showed that the state 4 respiration rate increased approximately 50% for α-ketoglutarate and succinate oxidations, in both acute and prolonged treatments, but the state 3 respiration rate, RCR values, and ADP:O ratio (not shown) were not modified significantly. Acute treatment with fluoxetine did not affect the kinetic parameters of F_oF_1-ATPase, although V_{max} values decreased markedly in the prolonged treatment (Table 2).

Simultaneous studies after acute 30-min or prolonged 22-day treatment (not shown) gave results similar to those of 60-min and 12-day treatments, respectively.

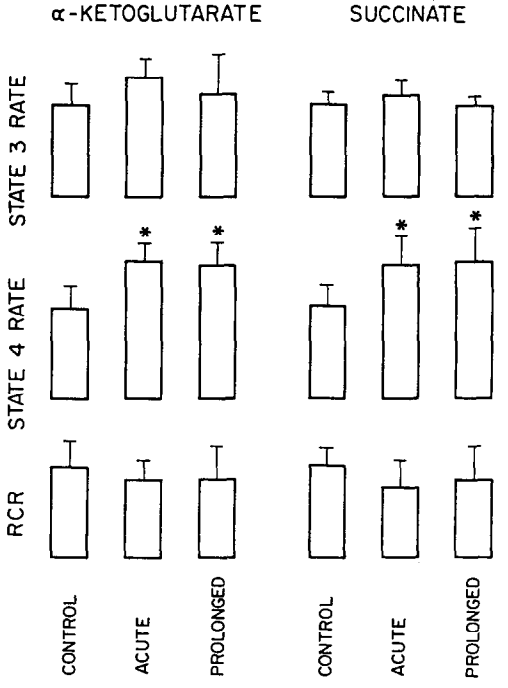

Fig. 4. Effect of acute and prolonged *in vivo* fluoxetine treatment on respiratory parameters of rat liver mitochondria (2.5 mg of protein) energized with 5 mM α-ketoglutarate and 5 mM succinate/5 μM rotenone. The assay conditions are described in Fig. 1. The values represent means ± SD of eight assays with different preparations. Key: (*) significantly different ($P < 0.05$) vs respective controls.

DISCUSSION

The function of the liver mitochondria *in vitro* was altered by the presence of fluoxetine, as demonstrated by the large decrease in RCR values. The state 3 respiration rate was affected more by the drug during NAD^+-dependent substrate oxidation, and state 4 during succinate oxidation. The ability of the drug to uncouple oxidative phosphorylation, an effect also described for tricyclic antidepressants and other psychotropic drugs [10–13], is hypothesized here, considering the stimulation observed for the state 4 respiration. The inhibition detected in the state 3 respiration, which could be a result of reduction in the electron transport of the respiratory chain, led us to assay the activity of F_oF_1-ATPase in submitochondrial particles in the presence of increasing concentrations of the drug. One

Table 2. Effects of acute and prolonged *in vivo* fluoxetine treatment on kinetic parameters of F_oF_1-ATPase from rat liver submitochondrial particles

	$K_{0.5}$ (1)	$K_{0.5}$ (2)	V_{max} (1)	V_{max} (2)	Total V_{max}
	(mM)			(μmol P_i/mg/min)	
Control	0.13 ± 0.01	1.30 ± 0.40	0.50 ± 0.03	1.15 ± 0.13	1.65 ± 0.15
Acute treatment	0.12 ± 0.02	1.00 ± 0.42	0.48 ± 0.03	1.07 ± 0.10	1.55 ± 0.13
Prolonged treatment	0.16 ± 0.04	1.60 ± 0.42	0.35 ± 0.02	0.75 ± 0.07	1.10 ± 0.09

The values (±SD) were obtained from five saturation curves using different preparations.

classical inhibitor of the mitochondrial respiration in state 3 is the antibiotic oligomycin, which blocks the proton translocation across the mitochondrial membrane by interacting with the membrane F_o component of the F_oF_1-ATPase ([23, 24]; for reviews on enzyme, see Refs. 25 and 26]). The membrane-bound enzyme, but not its solubilized form, is inhibited by oligomycin [27]. In our assays, the inhibiting effect of fluoxetine on F_oF_1-ATPase, similar to oligomycin, was marked on the activity of membrane-bound enzyme, but not on its solubilized form. This behavior suggests that fluoxetine *in vitro* acts on F_oF_1-ATPase, either through direct interaction with the membrane F_o component (similar to oligomycin), or first with the mitochondrial membrane and then affecting F_o. However, in both situations, the membrane structure should be necessarily involved. Here, it should be considered that the concentration of oligomycin needed to inhibit enzyme activity is around 1.4 μM (1 μg/mL), and, therefore, fluoxetine is a less effective inhibitor than oligomycin. The type of inhibition seems to be non-competitive, since the V_{max} values of the catalytic sites, but not the $K_{0.5}$ values, were altered in the presence of drug.

Antidepressant and other psychoactive drugs are known to be highly membrane soluble and, therefore, able to affect biological events associated with the membrane [9, 10]. The pH dependence of ATPase inhibition reinforces that fluoxetine *in vitro* may act inside the membrane. Drug concentrations giving 50% inhibition indicate that the free base form of drug (higher pH) is more effective than its ionized form (lower pH). The greater solubility of the free base form of fluoxetine in the membrane phase would then enhance its inhibitory effect on the enzyme. However, the presence of fluoxetine in the mitochondrial membrane apparently does not affect the physical state of the membrane lipids. This hypothesis, based on previous reports of temperature effect on F_oF_1-ATPase [28], arises from the fact that the drug had no effect on the activation energies and phase transition point of the membrane enzyme.

This effect of fluoxetine, through direct or indirect interaction with F_o, has not been described for antidepressant drugs. Some reports for tricyclic antidepressants show inhibition of state 3 respiration of rat brain and rat liver mitochondria [11], and inhibition of ATPase activity of submitochondrial particles and soluble F_1-ATPase from rat liver [13]. On the other hand, state 3 respiration and mitochondrial ATPase activity of rat heart mitochondria are not affected by imipramine [12]. The results of our *in vitro* studies demonstrate that the effect of fluoxetine on mitochondria is, in some aspects, similar to that described for tricyclic antidepressants, showing, in addition, an action on F_o in association with mitochondrial membrane.

Norfluoxetine is the active metabolite of fluoxetine, which may contribute to the pharmacological and clinical effects of drug [29]. As in human tissues, fluoxetine and norfluoxetine are extensively distributed in rat tissues [15, 30]. When fluoxetine is administered intraperitoneally, the drug and its metabolite rapidly reach higher concentrations in organs such as liver, lung and brain [31]. In rat brain, fluoxetine concentration rises from 12.8 nmol/g at 5 min to 48.7 nmol/g at 90 min; norfluoxetine is detected within 30 min, reaching concentrations of 27.2 nmol/g after 90 min [16]. The *in vitro* effects of fluoxetine and norfluoxetine on mitochondrial respiration and F_oF_1-ATPase were very similar. Therefore, in the *in vivo* condition, the effects of both the drug and its metabolite on mitochondria are probably also similar. This is an important point considering that, after administration, fluoxetine is demethylated rapidly to norfluoxetine, and the half-life of this metabolite is 2–3 times that of the parent drug [15]. Then, in acute or prolonged treatment *in vivo*, the effects observed should be the sum of both drug and metabolite activities.

Enhancement of state 4 respiration, observed in acute and prolonged treatments, reinforces the hypothesis that fluoxetine (or norfluoxetine) *in vivo* may uncouple the oxidative phosphorylation in mitochondria. Similar effects for antidepressants, such as imipramine, have been discussed in terms of the accumulation of these highly membrane-soluble drugs in the lipid bilayer. This would render the membrane less flexible, disturbing the function of the membrane-associated enzymes or inhibiting the transfer of ATP through the inner mitochondrial membrane [10]. Solubilization on mitochondrial membrane seems likely for fluoxetine, considering the *in vitro* drug effect on F_oF_1-ATPase activity. In spite of the anorectic action described for fluoxetine and norfluoxetine in rats [16], the decrease observed in the body weight gain of rats given the prolonged treatment is consistent with the *in vivo* uncoupling of the oxidative phosphorylation.

Absence of *in vivo* inhibition on state 3 respiration, or even a slight stimulation, was described also for

the effect of imipramine on rat liver mitochondria [14]. One possible explanation for this fact is that the presence of the drug or metabolite is required to inhibit state 3 respiration (as *in vitro* conditions); and that, despite their lipophilicity, they are largely removed during isolation of the mitochondria. In this regard, the inhibition observed *in vitro* on state 3 respiration may not be directly associated with that on F_oF_1-ATPase activity, since enzyme was affected *in vivo*, as demonstrated by the decrease in V_{max} after prolonged treatment. Therefore, it seems that, while an effect on state 3 respiration requires the concomitant presence of drug, an effect on F_oF_1-ATPase, and also state 4 respiration, remains for some time after removal of drug from the membrane. Another possibility to be considered is that the drug concentration reaching the mitochondrial membrane after administration is not sufficient to alter coupled respiration.

Finally, two points should be considered in the analysis of the present results: (1) effects of chemical agents on the function of isolated mitochondria are not necessarily associated with action mechanisms, in spite of the sensitivity of the mitochondria to a myriad of chemical agents being the basis of assays to detect potential environmental toxins; and (2) in our experiments, the dose of drug administered was near to the maximum tolerated, and therefore higher than the therapeutic doses used in humans. Taking into account these points, we can interpret the multiple effects of fluoxetine (and norfluoxetine) on the energy metabolism of liver mitochondria as an indication that the drug is potentially toxic when administered in high doses. The effects seem to be a consequence of the drug and/or metabolite solubilization in the inner membrane of mitochondria, which occurs instantaneously *in vitro*, or *in vivo* as early as 30 min after drug administration, remaining for at least 22 days of continued treatment.

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