Fluoxetine effect on kidney water reabsorption

Zenaide Providello Moyses, Fausto Kigui Nakandakari and Antonio José Magaldi

Laboratório de Pesquisa Básica-LIM 12, Laboratórios de Investigação Médica–Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Brazil

Abstract

Background. The pathogenesis of hyponatraemia caused by fluoxetine (Fx) use in the treatment of depression is not well understood. It has been attributed to a SIADH, although ADH-enhanced plasma level has not yet been demonstrated in all the cases reported in humans. This experiment aimed at investigating the effect of fluoxetine on the kidney and more specifically in the inner medullary collecting duct (IMCD).

Methods. (1) In vivo study: (a) 10 rats were injected daily i.p. with 10 mg/kg fluoxetine doses. After 10 days, rats were sacrificed and blood and kidneys were collected. (b) Immunoblotting studies for AQP2 protein expression in the IMCD from injected rats and in IMCD tubules suspension from 10 normal rats incubated with 10^{-7} M fluoxetine. (2) In vitro microperfusion study: The osmotic water permeability (P_f, \mu m/s) was determined in normal rats IMCD (n = 6), isolated and perfused by the standard methods.

Results. In vivo study: (a) Injected rats with fluoxetine lost about 12% body weight; Na^+ plasma level decreased from 139.3 ± 0.78 mEq/l to 134.9 ± 0.5 mEq/l (p < 0.01) and K^+ and ADH plasma levels remained unchanged. (b) Immunoblotting densitometric analysis of the assays showed an increase in AQP2 protein abundance of about 40%, both in IMCDs from injected rats and in IMCD tubules suspension from 10 normal rats incubated with 10^{-7} M fluoxetine. (2) In vitro microperfusion study: Fluoxetine increased P_f in the IMCD in the absence of ADH from the cont 7.24 ± 2.07 to Fx 15.77 ± 3.25 (p < 0.01).

Conclusion. After fluoxetine use, the weight and plasma Na^+ level decreased, and the K^+ and ADH plasma levels remained unchanged, whereas the AQP2 protein abundance and water absorption in the IMCD increased, leading us to conclude that the direct effect of fluoxetine in the IMCD could explain at least in part, the hyponatraemia found sometime after this drug use in humans.

Keywords: AQP2; fluoxetine; hyponatraemia; water reabsorption

Introduction

Fluoxetine is one of the most widely used antidepressants in the world in the treatment of depression. This drug is a selective serotonin reuptake inhibitor (SSRI) and is one of the first choices for treating depression nowadays. Fluoxetine and its metabolite, norfluoxetine, have a plasma half-life of 3–15 days, providing good conditions for successful treatment [1]. Despite its having been used for a long time, cases of hyponatraemia have been recently reported [1,2], principally in the elderly during the first 2 weeks of treatment. [2–4]. The pathogenesis of this hyponatraemia is not well understood and has been attributed to an inappropriate antidiuretic hormone secretion (SIADH), although the vasopressin enhanced plasma level has not yet been well demonstrated [5–10].

The ingestion of drugs is a very common cause of the SIADH, and drug-induced hyponatraemia has been directly associated with the use of several SSRIs [10–14]. Nevertheless, despite the fact that several clinical cases have already been reported, the SIADH is still only a hypothesis and not a confirmed fact.

Thus, in an attempt to elucidate this hypothesis and considering that no data are available concerning the effect of fluoxetine on water transport in the final segments of the nephron in human beings, the purpose of this study was to directly analyse the effect of this drug on the kidney. For this reason, in vivo and in vitro studies were performed to investigate the water absorption and the Aquaporin 2 (AQP2) expression in rat inner medullary collecting ducts (IMCDs).

Our results showed that fluoxetine enhanced water permeability in the absence of ADH and produced an increase in water absorption in the last segment of the nephron.

Methods

Normal male Wistar rats, weighing 170–180 g, were obtained from the animal facilities of the University of São Paulo School of Medicine. They were maintained under standard laboratory conditions and given a normal pellet diet and tap water ad libitum.
**In vivo studies**

For i.p. injection, fluoxetine was freshly dissolved in sterilized water to have a final concentration of 10 mg/kg. The injected volume was 1 µl for each grams of body weight.

A group of 10 animals were injected daily with fluoxetine i.p for 10 days [15]. Before the fluoxetine injection and after 10 days, when the animals were killed, blood was collected and placed in tubes immersed in ice for measurements of sodium (Na⁺) and potassium (K⁺) plasma levels (flamephotometer CELM São Paulo, Brazil). ADH was measured only after the use of fluoxetine.

At the same time, the renal medulla was separated and strips were dissected from these medullas to determine the expression of AQP2. Rat weights were measured before fluoxetine injection and at the end of 10 days. In other experiments, renal medullas from 10 normal rats free of fluoxetine were isolated for the normal test tube and the other for the fluoxetine group. From each rat, one kidney was designated for the normal test tube and the other for the fluoxetine test tube [16]. The tubule strips were dissected from the medullas in the same cold solution used in the microperfusion experiments (see below)

**Preparation of membrane fractions**

Medulla samples were homogenized in cold isolation solution (200 mM Mannitol, 80 mM Heps, 41 mM KOH, pH 7.5) containing protease inhibitors (cocktail protease inhibitor, Sigma Chemical, St. Louis, MO, USA) using a Teflon pestle glass homogenizer (Schmidt and Co., Frankfurt/M, Germany) [17]. The homogenates were centrifuged at low speed (2000 g) for 15 min at 4°C in order to remove the nuclei and cell debris. Subsequently, the supernatants were centrifuged at 100 000 g for 1 h at 4°C (rotor 50Ti; Beckman Instruments, Palo Alto, CA, USA) to produce a pellet containing membrane fractions enriched with both plasma membranes and intracellular vesicles. Protein concentration was determined for each sample using the Bradford method (Bio-Rad Laboratories, Richmond, CA).

**Electrophoresis and immunoblotting (western blotting)**

The proteins were separated on denaturing SDS/12% polyacrylamide gels by electrophoresis [17]. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Sweden) by wet electrophoretting for 90 min. Blots were blocked for 60 min at 4°C with 5% nonfat dry milk in PBS-T, pH 7.5 (phosphate-buffered saline, in mM: 100 NaCl, 80 Na₂HPO₄, 20 NaH₂PO₄, 0.1% Tween 20). Blots were incubated overnight with AQP2 antibody (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with control actin antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then washed and incubated with the second antibody (anti-goat secondary antibody HRP-conjugated, diluted at 1:10 000, Sigma, St. Louis, MO, USA) for 1 h. Subsequent detection of the specific proteins was carried out by enhanced chemiluminescence (ECL-Amersham), according to the manufacturer’s instructions. Prestained protein markers (Sigma Chemical Co., St. Louis, MO, USA) were used for molecular weight determinations. Equal protein loading was checked by Coomassie blue staining of the membranes.

**Quantification of AQP2 kidney levels**

Enhanced chemiluminescence films presenting bands within the linear range were scanned using an Image Master VDS (Pharmacia Biotech, Upplands Väsby, Sweden). For AQP2, both the 29 kDa and the 35–50 kDa bands (corresponding to two different states of glycosylation) were quantified by densitometric analysis. Densitometry results were reported as integrated values (area x density of the band) and expressed in percentages when compared to control actin protein abundance (100%).

**In vitro studies—microperfusion**

Six isolated IMCDs, from six different rats, were perfused by previously described techniques [18] in the absence of ADH and in the presence of fluoxetine in order to determine the osmotic water permeability. Tubules were isolated from a small kidney slice that was immersed in a dish of chilled Ringer-HCO₃ buffer (see composition below), oxygenated and kept at pH 7.4 by bubbling the solution with 5% CO₂ and 95% O₂. After isolation, the segment was transferred to a temperature-regulated chamber (37°C) mounted on the stage of an inverted microscope.

The Ringer-HCO₃ perfusion solution had 295 ± 5 mOsmol/kgH₂O (osmometer, Advanced Instruments) and the following composition (in mM): 115.0 NaCl, 25.0 NaHCO₃, 10.0 CH₃COONa, 5.0 KCl, 1.0 CaCl₂, 1.2 MgSO₄, 1.2 NaH₂PO₄ and 5.0 D-glucose. The bath solution was made hypertonic (510 ± 5 mOsmol/kgH₂O) by the addition of NaCl. FD&C green dye was added to the perfusate to be used as a visual marker. The net water absorption (Jᵥ) was measured with [¹⁴C]inulin, which had been dialyzed immediately before the experiments. The isotope was added to the perfusion solution at a final concentration of 25–100 cpm/nl. Jᵥ was calculated as Vᵢ/Vₒ/L, in which Vᵢ is the perfusion rate, Vₒ the collecting rate and L the length of the tubule studied (tubule length and inner diameter (ID) can be measured with a precalibrated micrometer in the eyepiece of an inverted microscope to observe the perfusion, with 0.05 mm of precision). Vₒ was directly measured on the basis of collection time, whereas
$V_i$ was calculated by the rate of appearance of the impermeant marker [14C]ulin in the collection pipette according to the equation $V_i = V_o (I_n/I_n)$, in which $I_n$ is the counts per minute of the collected fluid and $I_n$ is the counts per minute of the perfusate. Timed tubular samples were collected for analysis under mineral oil by aspiration into a calibrated pipette. The ID of the perfused segments ranged from 25 to 35 µm and the tubular length ($L$) from 1.5 to 2.0 mm. The area ($A$) was calculated as $\pi L$ and expressed as $\times 10^4$ cm$^2$. The osmotic water permeability ($P_f$) was determined by measuring net fluid movement in response to an imposed osmolar gradient. The net fluid reabsorption was induced by perfusing with a 295 mOsmol/kgH$_2$O isotonic perfusion solution and bathing with a 510 mOsmol/kgH$_2$O hypertonic solution. The $P_f$ ($\mu$m/s) was calculated in each experiment using the following equation [19]:

$$P_f = \frac{RT \cdot L_p}{V_w},$$

in which $L_p$ is the hydraulic conductivity and determined by the equation

$$L_p = \frac{1}{RTA} C_b^2 [C_b (V_i - V_o) + C_c V_i \ln (C_b - C_c) V_i - \ln (C_b V_o - C_c V_i)],$$

in which $C_b$ and $C_c$ are the osmolalities of the bath in the initial perfusion fluid, respectively; $R$ is the gas constant; $T$ is the absolute temperature; $V_w$ is the partial molar volume of water and $A$ the luminal surface area. The solutions were checked for osmolality and pH, with an osmometer (Advanced Instruments) and a pH meter (Iris 7; Tecnow, São Paulo, Brazil), respectively. The bath fluid was changed every 10 min to reduce the effect of evaporation and, consequently, to avoid an increase in the osmotic gradient. The bath osmolality did not change significantly during the 10-min period (from 295 ± 1 mOsmol/kgH$_2$O to 297 ± 2 mOsmol/kgH$_2$O). Timed fluid collections were made with a constant-volume constriction pipette and rinsed four times in a vial with 0.5 ml of water. A 5 ml aliquot of scintillation liquid was then added. The isotopic concentration was determined with a liquid scintillator spectrometer (Tri-Carb 1600TR; Packard, Downers Grove, IL). The data for each period are represented by the average of three to four collections.

The isotopic materials used were from Amersham International and New England Nuclear. Fluoxetine was kindly supplied by the Pharmacy Division of the Clinical Hospital, Medical School of São Paulo University. All other drugs were purchased from Sigma Chemical (St. Louis, MO, USA). Peptide-derived polyclonal antibodies specific to the AQP2 water channel were kindly supplied by Dr. Mark Knepper (NHLBI, NIH, Bethesda, MD, USA). ADH was tested by the RIA $^{115}$I, AVP DSL-1800.

Differences among the means of multiple parameters were analysed by ANOVA followed by the Student–Newman–Keuls test. Differences between two parameters were analysed by either unpaired t-test or nonparametric methods (Mann–Whitney test). Values of $P < 0.05$ were considered significant.

**Results**

**In vivo Studies**

The data obtained before the i.p. fluoxetine dose were considered as control data. The animals were injected daily with 10 mg/kg diluted in sterilized water.

**Body weight.** The results, shown in Table 1, revealed that with the use of the SSRI the body weight decreased from 179 ± 1.28 g, in the control period (cont), to 158 ± 1.15 g ($p < 0.0001$) at the end of the 10th day. This weight loss was about 12%. The food ingestion was not measured during this period.

**Na$^+$, K$^+$ and ADH plasma levels.** The Na$^+$ plasma level had a significant decrease after the treatment, from 139.3 ± 0.7 mEq/l to 134.9 ± 0.5 mEq/l ($p < 0.0001$), the later levels being considered as hyponatraemic. On the other hand, the K$^+$ plasma level, from 5.37 ± 0.1 mEq/l to 5.04 ± 0.2 mEq/l, remained statistically unchanged (Table 1). The ADH plasma level for the control period was not measured and for the animals treated with fluoxetine it was normal, 13 ± 2 pg/ml, since the normal value for the kit test used is 14 pg/ml.

**Table 1.** Plasma Na$^+$ and K$^+$ levels and weight of the rats before and after fluoxetine injection

<table>
<thead>
<tr>
<th>Rats</th>
<th>Na$^+$ Control</th>
<th>Na$^+$ Flux</th>
<th>K$^+$ Control</th>
<th>K$^+$ Flux</th>
<th>Weight Control</th>
<th>Weight Flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>143</td>
<td>135</td>
<td>4.8</td>
<td>4.3</td>
<td>182</td>
<td>160</td>
</tr>
<tr>
<td>2</td>
<td>137</td>
<td>134</td>
<td>5.6</td>
<td>5.7</td>
<td>187</td>
<td>166</td>
</tr>
<tr>
<td>3</td>
<td>138</td>
<td>135</td>
<td>5.0</td>
<td>4.8</td>
<td>172</td>
<td>154</td>
</tr>
<tr>
<td>4</td>
<td>141</td>
<td>136</td>
<td>5.8</td>
<td>5.4</td>
<td>175</td>
<td>153</td>
</tr>
<tr>
<td>5</td>
<td>138</td>
<td>136</td>
<td>5.0</td>
<td>4.8</td>
<td>181</td>
<td>159</td>
</tr>
<tr>
<td>6</td>
<td>137</td>
<td>137</td>
<td>5.4</td>
<td>5.6</td>
<td>179</td>
<td>157</td>
</tr>
<tr>
<td>7</td>
<td>138</td>
<td>135</td>
<td>5.1</td>
<td>5.0</td>
<td>177</td>
<td>158</td>
</tr>
<tr>
<td>8</td>
<td>140</td>
<td>132</td>
<td>6.0</td>
<td>5.4</td>
<td>180</td>
<td>160</td>
</tr>
<tr>
<td>9</td>
<td>142</td>
<td>134</td>
<td>5.6</td>
<td>4.4</td>
<td>178</td>
<td>156</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>179</td>
<td>158</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>$139 ± 0.7$</td>
<td>$134.9 ± 0.5^*$</td>
<td>$5.37 ± 0.1$</td>
<td>$5.04 ± 0.2$</td>
<td>$179 ± 1.28$</td>
<td>$158.1 ± 1.15^*$</td>
</tr>
</tbody>
</table>

Flux: fluoxetine; Na$^+$ and K$^+$: mEq/l; weight: g.

*p < 0.001 versus control.
Fig. 1. (A) Western blot analysis of the AQP2 protein from normal control rats and from fluoxetine-injected rat tubule suspension showing an increase in the AQP2 expression. The bands shown are representative for 5 experiments of 10 performed. (B) Densitometry quantification showing an increase of about 43% in the AQP2 expression in fluoxetine-injected rats \( (n = 10) \) when compared with the control rats \( (n = 5) \). The densitometric graphs were done using all the fluoxetine bands compared with all bands from normal rats. Values are means ± SE; Fx: fluoxetine \( *p < 0.05 \) versus control.

Analysis of expression of AQP2 by western blot. The AQP2 antibody revealed that both the 29 kDa and the 35–50 kDa bands were increased, corresponding to the nonglycosilated and glycosilated AQP2, respectively. The densitometric analysis of the immunobloting assays showed an increase in AQP2 protein abundance of about 43%, both in Group a fluoxetine-injected rats \( (\text{cont} 99.6 ± 5.17 \text{ versus Fx} 145.6 ± 16.93, p < 0.05) \) (Figure 1) and in Group b IMCD fluoxetine-incubated tubule suspension \( (\text{cont} 100.0 ± 3.5. \text{ versus} 143.0 ± 2.0, \text{p} < 0.01) \) (Figure 2). These data revealed that fluoxetine treatment is correlated with the increased AQP2 expression.

In vitro Microperfusion Studies

Microperfusion. The osmotic water permeability \( (P_t, \mu \text{m/s}) \) was measured in tubules directly dissected from normal rat medullas in three periods: control, \( 10^{-7} \text{ M fluoxetine} \) and recovery. The fluoxetine was added to the bath solution in the absence of ADH. Drug addition to the perfusion solution had no effect. In the presence of fluoxetine, the water permeability increased from cont 7.24 ± 2.07 to Fx 15.77 ± 3.25 \( (p < 0.01) \) and recovered to 6.21 ± 1.13 \( (n = 6) \), confirming the above data, i.e. the data obtained in \textit{in vivo} experiments and with molecular biology experiments.

Discussion

The purpose of this study was to investigate the origin of the decrease in the plasma sodium level observed in patients who use SSRI drugs, principally fluoxetine, since its side effect has not yet been well characterized [20,21]. Our data provided evidence that this decrease in the plasmatic sodium level can be attributed, at least in part, to the intrinsic capacity of fluoxetine to increase the water permeability in the IMCD, allowing an increase in water absorption.

It is well known that one cause of hyponatraemia may be the increase in renal water absorption [22] and, as a consequence, water intoxication can occur. Several pharmacologic agents have been shown to have water-retaining properties, and SSRIs are some of them [23,24]. Indeed, these drugs are thought to produce a SIADH, but this inappropriate secretion has not been well characterized [11,12].

Body weight

It has been reported that this drug produces anorexia and is used as an adjuvant therapy to treat obesity [25,26]. The anorexigenic effect of fluoxetine was not the object of this study, and because of that, food ingestion was not measured. Nevertheless, our data confirmed the literature and showed that the animals injected with fluoxetine lost weight at the end of the 10th day (about 12%), probably as a result of a decrease in food ingestion stimulated by the drug, as had been already reported by Marar and Amico in experiments with rats [15].

\( \text{Na}^+, \text{K}^+ \) and ADH

In the \textit{in vivo} study, the \( \text{Na}^+ \) and \( \text{K}^+ \) urinary levels, the urinary volume and the fluid ingestion were not measured,
nor was the ADH plasma level before treatment with fluoxetine. After 10 days of fluoxetine use, the rats presented a decrease in sodium plasma levels, whereas the potassium plasma levels remained unchanged. This experimental data showed levels considered to be hyponatraemic (below 135 mEq/l) and exhibited a hyponatraemia similar to clinical data demonstrated in several case reports [2–4,7,20]. This hyponatraemia is usually attributed to abnormal water reabsorption [22]. In articles found in the literature presenting cases of hyponatraemia associated with the use of fluoxetine, the main hypothesis considered to explain this side effect, in all the articles, was an unexpected release of ADH (SIADH). Some of these articles proposed that in the presence of SSRI drugs, renal responsiveness to ADH could be increased [8]. Despite the control plasma ADH levels not having been measured in our work, our data showed that these levels remained unchanged after fluoxetine injection, showing that a SIADH did not occur, reinforcing the idea that our data can illustrate the importance of knowing the physiological effects of this SSRI drug, which may contribute to the understanding of its pharmacological significance and provide information for its correct use to avoid iatrogenic diseases, even having been carried out in animals.

AQP2 expression

In order to investigate the hypothesis of renal responsiveness to fluoxetine, AQP2 expression in the IMCD tubule suspension from rats injected with fluoxetine was determined. In the injected group, the AQP2 expression increased by around 45%, both in the 29 and in the 35–50 kD bands, confirming our hypothesis and revealing that this finding can be attributed only by a direct proper action of the fluoxetine in stimulating water channel insertion in the IMCD luminal membrane. The data from the IMCD tubule suspension from normal rats incubated with fluoxetine showed an increase in the AQP2 expression of about 43% in the two bands, emphasizing the injected-rat results.

Microperfusion in vitro

The osmotic water permeability in the IMCD from normal rats in presence of fluoxetine demonstrated that this drug can increase water permeability when AQP2 is inserted into the tubular luminal membrane, as shown above. This result demonstrated that this SSRI drug must be included in the group of drugs that are able to stimulate water absorption by a non-ADH modulation of AQP2 in the kidney. It is well known that some compounds can stimulate water absorption, producing an ADH-like effect, such as hydrochlorothiazide [27], chlorpropamide [28], angiotensin 1–7 [29], rosiglitazone [30], antiepileptic drugs [31] and others.

Recently, Rouch et al. [32] showed that fluoxetine inhibited the IMCD water transport in the presence of ADH and that this effect is mediated by alpha-2 adrenoceptors. This result did not explain the hyponatraemic effect observed in the literature and did not confirm our results. Thus, further studies will be necessary to elucidate the intrinsic fluoxetine mechanisms.

In summary, the data presented in this study confirm, at least in part, the hypothesis that the decrease in the sodium plasma level, associated with the use of SSRI drugs, specifically fluoxetine, could be the result of an increase in water permeability in the IMCD, as was observed in the microperfusion experiments. This increase in water permeability, is in turn, the consequence of the increase in AQP2 expression in the IMCD cells. All these data taken together, specifically the fact that the ADH level remained unchanged, showed that a direct effect of the fluoxetine in the IMCD could explain the hyponatraemia and not an inappropriate secretion of ADH, as the articles in the literature suggest. However, we cannot exclude the possibility that there might be other coexisting mechanisms that contribute to inducing this hyponatraemia.

Finally, this study was performed in animals because no studies in humans have been conducted to date to elucidate the real origin of this hyponatraemia. Thus, we believe that our data can illustrate the importance of knowing the physiological effects of this SSRI drug, which may contribute to the understanding of its pharmacological significance and provide information for its correct use to avoid iatrogenic diseases, even having been carried out in animals.

To the best of our knowledge, there is no other investigation in which the effect of fluoxetine on water metabolism was studied in normal rats to date focusing on the molecular basis of water transport.

Acknowledgments. This study received financial support from the Conselho Nacional de Pesquisa (CNPq, National Counsel for Scientific and Technological Development), the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Foundation for the Support of Research in the State of São Paulo), the Fundação Faculdade de Medicina (FFM, School of Medicine Foundation) and the Laboratórios de Investigação Médica do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (LMHICFMUSP, University of São Paulo Hospital das Clínicas Medical Investigation Laboratories). Z.P. and A.J.M. were recipients of a CNPq fellowship. Parts of this study were presented at the ERA-EDTA Congress of Nephrology in 2005 and at the FASEB Congress 2006. The authors are grateful to Miriam Pellegrini and Brian Weymouth for providing editorial assistance.

Conflict of interest statement. None declared.

References

7. Liu BA, Mittmann N, Knowles SR et al. Hyponatremia and the syndrome of inappropriate secretion of antidiuretic hormone associated

Received for publication: 9.12.06
Accepted in revised form: 12.9.07