Protective Effects of Fluvoxamine against Ischemia/Reperfusion Injury in Isolated, Perfused Guinea-Pig Hearts

Tatsuya Muto,a,b Haruki Usuda,a Aya Yamamura,a Koji Yoshida,a Ai Ohashi,a Kumiko Mitsu-Saitoh,a,c Junichi Sakai,a,c Yumi Sugimoto,d Hideki Mizutani,a Tsunemasa Nonogaki,a and Yoshihiro Hotta*a,c

a College of Pharmacy, Kinjo Gakuin University; Omori, Moriyama, Nagoya 463–8521, Japan; b Meitetsu Hospital Pharmacy; Sakou, Nishi, Nagoya 451–8511, Japan; c Faculty of Sports and Health Science, Nagoya Gakuin University; Kamishinano, Seto 480–1298, Japan; d Laboratory of Pharmacology, Department of Clinical Pharmacy, Yokohama College of Pharmacy; Totsuka, Yokohama 245–0066, Japan; and e Department of Pharmacology, Aichi Medical University School of Medicine; Karimata, Nagakute, Aichi 480–1195, Japan.

Received July 12, 2013; accepted February 2, 2014

Serotonin (5-hydroxytryptamine; 5-HT) is known to be activated during ischemia–reperfusion and triggers contractile dysfunction and pathological apoptosis. Here, the beneficial effects of the selective serotonin reuptake inhibitor (SSRI) fluvoxamine was demonstrated on ischemia–reperfusion injury in guinea-pig hearts perfused using the Langendorff technique. The recovery (%) of left ventricular developed pressure (LVDP) by fluvoxamine (5×10^{-8} mol) was 95.4% (control: 32%), which was consistent with the inhibition of mitochondrial Ca^{2+}(\text{[Ca}^{2+}]_{m}) uptake induced by changes in the Ca^{2+} content and acidification of the perfusate, and similar to reperfusion following global ischemia in Langendorff-perfused hearts. Fluvoxamine inhibited the increase in \text{[Ca}^{2+}]_{m} induced by changes in the Ca^{2+} content of the perfusate in perfused preparations of mitochondria, which was similar to the results obtained with the mitochondrial permeability transition pore (MPTP) opener atractyloside. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL)-positive cells were significantly less in fluvoxamine-treated hearts than in control hearts, with decreases in caspase-3 activity. These results suggest that SSRI inhibits opening of the MPTP by preventing \text{[Ca}^{2+}]_{m} overload-induced apoptosis related to the endogenous accumulation of 5-HT in ischemia–reperfusion hearts.

Key words fluvoxamine; left ventricular developed pressure; \textsuperscript{31}P-NMR; mitochondrial permeability transition pore (MPTP); ischemia–reperfusion injury; apoptosis

Serotonin (5-hydroxytryptamine; 5-HT) is a neurohormone that has been shown to regulate cardiovascular functions.\textsuperscript{1,2} Circulating 5-HT can also be taken up by sympathetic neurons and vascular endothelial cells, from which it can then be co-released.\textsuperscript{3} Previous studies reported that antagonists of 5-HT\textsubscript{2A} may be potential therapeutic agents for hypertension as well as peripheral vascular disease,\textsuperscript{4,5} and also prevented cardiac dysfunction in ischemic–reperfusion injury hearts.\textsuperscript{4,5} Sarpogrelate, a 5-HT\textsubscript{1A} antagonist, was shown to have a beneficial effect on ischemia–reperfusion injury in isolated Langendorff-perfused guinea-pig hearts perfused with no-blood solution.\textsuperscript{6} The cardiac stimulatory effects of 5-HT in the heart\textsuperscript{7} were similarly attributed to the 5-HT\textsubscript{2} receptors present in cardiomyocytes.\textsuperscript{8} However, the role of 5-HT antagonists in myocardial ischemic cellular damage remains unclear.

The identification of factors regulating cardiomyocyte survival and growth is important to more clearly understand the pathogenesis of congenital heart diseases. Interest in the link between the signaling circuitry for external stimuli and mitochondrial apoptotic machinery in cardiac disease is increasing. Mitochondria are also known to play an important role in apoptotic signaling,\textsuperscript{9} especially in the heart.\textsuperscript{10} In relation to our investigation of \text{[Ca}^{2+}]_{m} systems, the effects of an increase in mitochondrial Ca^{2+} ((\text{[Ca}^{2+}]_{m}) uptake on changes in the Ca^{2+} content or acidification of the perfusate, similar to the end of ischemia or reperfusion following global ischemia in hearts, was previously examined after the administration of each optimal isomer of a 5-HT\textsubscript{1A} antagonist pyrapyridolol.\textsuperscript{11} Changes in \text{[Ca}^{2+}]_{m} were measured to assess their contribution to injury using an opener or inhibitor of the mitochondrial permeability transition pore (MPTP).\textsuperscript{12}

The results of the present study indicate that the selective serotonin reuptake inhibitor (SSRI) fluvoxamine (Fig. 1A) may inhibit opening of the MPTP by preventing \text{[Ca}^{2+}]_{m} overload-induced apoptosis in ischemia–reperfusion hearts, similar to the 5-HT\textsubscript{1A} antagonist pyrapyridolol.\textsuperscript{11} Evidence now suggests that apoptosis, or programmed cell death, is an important response of the myocardium to ischemia, which precedes cell necrosis and appears to contribute to the overall sequelae of cardiac injury.\textsuperscript{13} The molecular mechanisms of cardiac functions triggered by 5-HT have to be elucidated in detail. The role of 5-HT in this response can be delineated with 5-HT inhibitors.\textsuperscript{14} Therefore, we examined which protective action was associated with the beneficial effects of SSRI, with respect to reductions in the number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL)-positive myocytes and caspase-3 activity, in order to compare these effects to those of 5-HT-related drugs; a 5-HT\textsubscript{2A} antagonist\textsuperscript{6} and 5-HT\textsubscript{1A} antagonist.\textsuperscript{11}

![Chemical Structure of the Selective Serotonin Reuptake Inhibitor (SSRI) Fluvoxamine](image)

Fig. 1. Chemical Structure of the Selective Serotonin Reuptake Inhibitor (SSRI) Fluvoxamine

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: yhotta@kinjo-u.ac.jp © 2014 The Pharmaceutical Society of Japan
MATERIALS AND METHODS

Guinea-Pig Hearts Heart Preparation and Examination Procedures: Hartley strain guinea pigs of either sex, weighing 300–350 g, were anesthetized with diethyl ether and heparinized (250 IU, intraperitoneally (i.p.)). The heart was rapidly excised and the aorta was cannulated. The Langendorff-perfused heart preparations were then perfused with Krebs–Henseleit solution (KH solution, pH 7.4, at 37°C) containing in mM: NaCl 115, NaHCO₃ 25, KCl 4.7, CaCl₂ 2.0, MgCl₂ 1.2, KH₂PO₄ 1.2 and glucose 10. The KH solution was presaturated with a gas mixture containing 95% O₂ and 5% CO₂, and the heart was perfused at a constant pressure of 75 cm H₂O in an NMR sample tube (20 mm in diameter) in a superconducting magnet as described previously. Subsequent to an equilibration period of 30 min required to stabilize the mechanical function, the perfused hearts were exposed to 40 min of global ischemia by clamping the perfusion flow line, and were then reperfused for 40 min with the control medium of perfusate. In the pretreatment groups, fluvoxamine (5×10⁻⁸ m) was introduced into the perfusate 4 min before ischemia was initiated and added to the perfusate during reperfusion. To examine the TUNEL and caspase-3 activity, the hearts were subjected to 4 min before ischemia for 1 h of ischemia and 5 h of reperfusion. Drugs (5×10⁻⁸ m) were administered for 4 min before ischemia and for 5 h throughout reperfusion.

Measurement of Myocardial Function Coronary flow rate (FR) in the NMR analysis was measured continuously with an in-line flow probe connected to an ultrasonic flow meter (transonic T101, Advance, Ithaca, NY, U.S.A.). A latex balloon (Hirokawa, Niigata, Japan) was introduced into the left ventricle via the left atrium and connected to a strain-gauge transducer (MIP-5100, Baxter, Tokyo, Japan) for the measurement of isovolumic left ventricular pressure. Left ventricular end-diastolic pressure (LVEDP) was adjusted to 10 mmHg during the equilibration period in each heart, and the volume of the balloon was unchanged during the experiments. Left ventricular developed pressure (LVDP) was calculated by subtracting LVEDP from left ventricular systolic pressure.

³¹P-NMR Measurement and Data Analysis The myocardial temperature was maintained at 37±0.5°C with a water-jacketed perfusion line and a continuous stream of air around the NMR sample tube. The heart connected to the Langendorff perfusion line was placed in a standard 20-mm NMR jacketed perfusion line and a continuous stream of air around the NMR sample tube. The heart connected to the Langendorff-perfused heart preparations were exposed to 40 min of global ischemia by clamping the perfusion flow line, and were then reperfused for 40 min with the control medium of perfusate. In the pretreatment groups, fluvoxamine (5×10⁻⁸ m) was introduced into the perfusate 4 min before ischemia was initiated and added to the perfusate during reperfusion. To examine the TUNEL and caspase-3 activity, the hearts were subjected to 4 min before ischemia for 1 h of ischemia and 5 h of reperfusion. Drugs (5×10⁻⁸ m) were administered for 4 min before ischemia and for 5 h throughout reperfusion.

Measurement of Myocardial Function Coronary flow rate (FR) in the NMR analysis was measured continuously with an in-line flow probe connected to an ultrasonic flow meter (transonic T101, Advance, Ithaca, NY, U.S.A.). A latex balloon (Hirokawa, Niigata, Japan) was introduced into the left ventricle via the left atrium and connected to a strain-gauge transducer (MIP-5100, Baxter, Tokyo, Japan) for the measurement of isovolumic left ventricular pressure. Left ventricular end-diastolic pressure (LVEDP) was adjusted to 10 mmHg during the equilibration period in each heart, and the volume of the balloon was unchanged during the experiments. Left ventricular developed pressure (LVDP) was calculated by subtracting LVEDP from left ventricular systolic pressure.

³¹P-NMR Measurement and Data Analysis The myocardial temperature was maintained at 37±0.5°C with a water-jacketed perfusion line and a continuous stream of air around the NMR sample tube. The heart connected to the Langendorff perfusion line was placed in a standard 20-mm NMR tube with the apex approximately 2.5 cm from the bottom of the tube, and the tube was inserted into the NMR coil. The effluent was removed from a level above the heart with a peristaltic pump, leaving the heart submersed in a fixed volume of the perfusate. In the ischemia–reperfusion experiments, ³¹P-NMR spectra were monitored along with simultaneous recordings of ventricular pressure, as described previously and paced at a rate of 3–4 Hz stimulation via a 3 m KCl agar electrode. ³¹P-NMR spectra were obtained at 161.8 MHz on a GSX 400 spectrometer (JEOL Datum Co., Ltd., Tokyo, Japan) equipped with a 9.4-Tesla vertical-bore magnet. For each spectrum, 90 free-induction decays (4 min) were accumulated after 45° flip-angle pulses (18 µs) using 4096 data points and 15.015 kHz spectral widths with a repetition time of 2 s. Accumulated free-induction decays were exponentially filtered, resulting in a 30-Hz line broadening.

PCr, Pi and β-ATP were quantified by comparison with a capillary tube of standard methylenediphosphonic acid (MDP, 0.25 m) fixed inside the NMR tube. Phosphate peaks, expressed as percentages of the control values, were determined by measuring the area under each resonance peak. The relative intensity of each peak was used for quantitative analysis. Datum Station ALICE software (JEOL Datum) was used to determine the area under each peak using a personal computer. The intracellular pH (pHᵢ) was calculated from the chemical shift between the phosphocreatine (PCr) and inorganic phosphate (Pi) resonances using the following equation: pHᵢ=6.90−log[(δ₀−5.85)/(3.29−δ₀)], where δ₀ is the chemical shift of Pi from PCr expressed as parts per million (ppm).

Intramitochondrial Fluorometric Measurements According to the Change in the Ca²⁺ Concentration or pH of the Perfusate Mitochondria were isolated from guinea-pig hearts according to standard procedures in our laboratory. The final mitochondrial pellet was resuspended with washing medium and the protein was quantified by the Lowry method. The suspensions were incubated at 24°C in a medium with a composition similar to the intracellular ionic composition (100 mM Ca²⁺, 10 mM Na⁺ and 110 mM K⁺) containing respiratory substrates (composition in mM: sucrose 250, MgCl₂ 1, KH₂PO₄ 1, succinate 10, malate 5, and 3-morpholinopropanesulfonic acid (MOPS) 20 adjusted to pH 7.4 with KOH) and containing the Ca²⁺ fluoroprobe fura-2-AM (10 μM) and 0.025% cromepor EL. A 0.5-mL aliquot of mitochondria was allowed to settle on a glass coverslip on the stage of an inverted microscope (CAM230, Japan Spectroscopic Co., Tokyo, Japan) for 30 min, during which it was treated with poly-L-lysine to promote mitochondrial adhesion. After dye loading, the mitochondria on the coverslip were mounted on the stage of an inverted microscope and washed continuously in a dye-free solution for 10 min, followed by continuous circulation in 10 mL of medium solution. Fura-2 Ca²⁺ signals were measured at 500 nm as the ratio of fluorescence strength (R340/380) at 340 nm (F340) and 380 nm (F380). After fura-2 was loaded for 30 min, the amplitude of F340 from the mitochondria increased by 5–7-fold compared with that of unloaded mitochondria. The intramitochondrial Ca²⁺ signal following preloading with high Ca²⁺ concentrations (10 μM) for over 5 min was markedly increased by lowering the Ca²⁺ concentration to a low physiological level (100 nM), by acidifying the perfusate (pH 7.5 → 6.5), or by treatment with the MPTP opener, atracyside. We then determined whether SSR1, fluvoxamine, or a MPTP blocker, cyclosporine A suppressed the Ca²⁺ increase induced by these procedures. The final mitochondrial protein concentration was adjusted to 30–35 mg/mL by dilution.

Histological Examination and Assessment of Internucleosomal DNA Cleavage by the TUNEL Method and Measurement of Caspase-3 Activity The Langendorff-perfused heart preparations were perfused with normal Krebs–Henseleit solution (KH solution, pH 7.4, at 37°C) presaturated with a gas mixture containing 95% O₂ and 5% CO₂. Subsequent to an equilibration period of 30 min required to stabilize mechanical function, the preparations were perfused with a modified KH solution, in which glucose 10 mM was replaced by 2-deoxy-d-glucose 5 mM and glucose 5 mM 4 min before ischemia for 1 h. After reperfusion for 5 h, the left ventricle was cut into several pieces. Fluvoxamine (5×10⁻⁸ m) was introduced into the modified perfusate for 4 min before the start of ischemia and was...
added to the drug-containing medium used for reperfusion. The changes in the cellular levels of high-energy phosphates in the heart, together with simultaneous recordings of LVDP, LVEDP and FR, were monitored using $^{31}$P-NMR. The proximal portions of the isolated Langendorff-perfused hearts were fixed for 2 h at room temperature with a phosphate-buffered solution containing 2% paraformaldehyde and embedded in paraffin for subsequent routine histological examination and the TUNEL method.24–25

Other sections of each heart were stored in liquid nitrogen for the measurement of caspase-3 activity using a CPP32/Caspase-3 fluorometric protease assay kit.26 The assay is based on the detection of the cleavage of the substrate DEVD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). DEVD-AFC emits blue light ($\lambda_{\text{max}}=400$ nm), but upon cleavage with CPP32 or related caspases, free AFC emits a yellow-green fluorescence ($\lambda_{\text{max}}=505$ nm), which can be quantified using a fluorometer. Comparison of the fluorescence of AFC from an apoptotic sample with an uninduced control allows determination of the fold-increase in CPP32 activity. The uninduced control was a heart preparation that had been perfused with normal solution (control-1).

**Drugs** The following reagents were obtained from the sources indicated: 5-hydroxytryptamine creatinine sulphate (5-HT), atractyloside (Atr), FK506, and cyclosporin A (CsA) were obtained from Sigma (St. Louis, MO, U.S.A.). Fura-2 AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Fluvoxamine was generously donated by Meiji Seika Co., Ltd. (Tokyo, Japan). The CPP32/Caspase-3 fluorometric protease assay kit was purchased from NBL (Nagoya, Japan). All other chemicals used in this study were of analytical grade and obtained from commercial sources.

**Animals** Throughout the experiments, all animals were handled in accordance with the guidelines for animal experimentation set by the Japanese Association for Laboratory Animal Science.

**Statistical Analysis** All values are presented as the mean±S.E.M. unless otherwise specified. The unpaired t-test or ANOVA followed by Dunnett’s method was used for the comparison of means between the groups. Statistical significance was defined as $p<0.05$.

**RESULTS**

**Beneficial Effects of Fluvoxamine during Ischemia–Reperfusion in Langendorff-Perfused Heart Preparations** The following reagents were obtained from the sources indicated: 5-hydroxytryptamine creatinine sulphate (5-HT), atractyloside (Atr), FK506, and cyclosporin A (CsA) were obtained from Sigma (St. Louis, MO, U.S.A.). Fura-2 AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Fluvoxamine was generously donated by Meiji Seika Co., Ltd. (Tokyo, Japan). The CPP32/Caspase-3 fluorometric protease assay kit was purchased from NBL (Nagoya, Japan). All other chemicals used in this study were of analytical grade and obtained from commercial sources.

**Postischemic End-Diastolic Pressure** The values of LVEDP obtained at the end of 40 min of ischemia (ischemic contracture) in hearts pretreated with fluvoxamine during ischemia and reperfusion were lower than those in the untreated or posttreated control hearts. The main feature of the recovery of postischemic function was the difference in end-diastolic pressure between the control and drug-treated hearts. In the control group, LVEDP tended to increase during the first 5 min of reperfusion, but thereafter decreased and remained low. In the hearts pretreated with fluvoxamine, there was no further increase in diastolic pressure, which gradually returned to the preischemic value (end of reperfusion, 16.9±4.5 mmHg, $p<0.05$, in fluvoxamine-pretreated hearts versus 42.0±2.0 mmHg in the control hearts). The LVEDP values obtained at the end of reperfusion were significantly lower than those in the control group and improved the recovery of LVDP during the first 30 min of reperfusion. Actual values (mmHg) of...

![Fig. 2](image-url)  
**Fig. 2.** Beneficial Effects of Fluvoxamine (FLV) ($5 \times 10^{-8}$s) during Ischemia–Reperfusion in Langendorff-Perfused Heart Preparations  
(A) Pretreatment with fluvoxamine (FLV) enhanced the recovery of LVDP after reperfusion compared to the control. Control: no drug was given during ischemia–reperfusion. **$p<0.01$, significantly different from the control (drug-free) value. (B) Relation of high-energy phosphates (ATP) estimated from $^{31}$P-NMR spectra in the presence and absence of fluvoxamine (FLV) ($5 \times 10^{-8}$s) during ischemia–reperfusion. **$p<0.01$, significantly different from the control (drug-free) value. Data are expressed as the mean of 5 determinations; Vertical lines represent S.E.M.
LVDP and LVEDP are shown in Table 1.

**Postischemic Coronary Flow Rate** At the end of reperfusion in the control hearts, coronary flow (FR) was $4.6 \pm 0.6$ mL/min, significantly lower than the preischemic level, $7.9 \pm 0.8$ mL/min. However, compared to the controls, the fluvoxamine-pretreated hearts showed significant differences ($p < 0.05$) at the end of reperfusion (Table 1).

**Effect of Fluvoxamine on High Phosphorous Energy during Ischemia and Reperfusion Determined with $^{31}$P-NMR Spectroscopy** As mentioned, these experiments were carried out in Krebs–Henseleit (KH)-perfused hearts; ischemia and reperfusion were measured for 40 min and 40 min. The effects of fluvoxamine were investigated, and the drug was introduced into the perfusate 4 min before global ischemia was induced by stopping the perfusate (pre-treated groups). High phosphorous energy, $\beta$-ATP, values are presented graphically as percentages relative to the pre-ischemic baseline with the post-ischemia recovery rate of LVDP in Fig. 2B. During 20 min of ischemia in the pre-treated group, $\beta$-ATP and PCr did not change significantly. At the end of 40 min of ischemia, significant differences in the preservation of ATP were obtained only in the hearts pretreated with fluvoxamine ($49.0 \pm 5.6\%$ of pre-ischemic value, $p < 0.01$) compared with the control ($27.0 \pm 3.1\%$). During reperfusion, there was a resynthesis of ATP in pretreated hearts. Differences between drug-treated and control hearts were significant at 20 min of reperfusion and thereafter. The level of ATP at the end of 40 min of reperfusion was significantly higher in the fluvoxamine-treated groups ($p < 0.01$) than the control group ($68.2 \pm 9.2\%$, control: $39.2 \pm 4.2\%$, with pre-ischemia as 100%). A rapid decrease in PCr was observed during ischemia in all groups.

Changes in pH during ischemia and reperfusion were calculated using the equation described in Materials and Methods. The initial (pre-ischemia) level of pH was almost identical in each group ($7.46 \pm 0.03$). At the end of 36–40 min ischemia, the pH did not differ significantly between the con-

---

**Table 1. Left Ventricular Developed Pressure (LVDP), Ventricular End Diastolic Pressure (LVEDP), Coronary Flow Rate (FR), and Intracellular pH (pHi) Changes during Ischemia–Reperfusion of Langendorff-Perfused Hearts**

<table>
<thead>
<tr>
<th></th>
<th>LVDP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>FR (mL/min)</th>
<th>pHi</th>
<th>HR (times/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Isch Rep</td>
<td>Pre Isch Rep</td>
<td>Pre Isch Rep</td>
<td>Pre Isch Rep</td>
<td>Pre Isch Rep</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>64.3 ± 7.6</td>
<td>0</td>
<td>20.6 ± 5.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40.2 ± 5.2</td>
<td>20.0 ± 2.0</td>
<td>10.5 ± 0.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.6 ± 0.6</td>
<td></td>
<td>7.46 ± 0.03</td>
<td>6.02 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.35 ± 0.06</td>
<td></td>
<td>7.35 ± 0.06</td>
<td>7.35 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>182 ± 10.4</td>
<td></td>
<td>65.0 ± 10.1</td>
<td>0</td>
</tr>
<tr>
<td>FLV $5 \times 10^{-3}$ M</td>
<td>6</td>
<td>64.9 ± 5.2</td>
<td>0</td>
<td>61.9 ± 6.9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>15.2 ± 3.2</td>
<td>16.9 ± 4.5</td>
<td>11.5 ± 1.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.9 ± 0.8</td>
<td></td>
<td>7.46 ± 0.03</td>
<td>6.15 ± 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.43 ± 0.07</td>
<td></td>
<td>7.43 ± 0.05</td>
<td>189 ± 11.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>175 ± 10.2</td>
<td></td>
<td>175 ± 10.2</td>
<td>0</td>
</tr>
</tbody>
</table>

Changes during 40 min of ischemia and a subsequent 40 min of reperfusion. Values are the mean ± S.E.M. $a) p < 0.01$, $b) p < 0.05$; significantly different from the control value. Pre, preischemia; Isch, ischemia; Rep, reperfusion.

---

**Fig. 3. Recordings of Intramitochondrial Ca$^{2+}$ from Fura-2-Loaded Mitochondrial Preparations Affected by Changing the Ca$^{2+}$ Concentration (A) or pH of the Perfusate (B)**

The mitochondrial Ca$^{2+}$ signal ([Ca$^{2+}$]$_{m}$) gradually increased with extremely high Ca$^{2+}$ ($10 \mu$M), and perfusion with a low-Ca$^{2+}$ solution ($100 \mathrm{nm}$) rapidly increased the intramitochondrial Ca$^{2+}$ level (A, top). Acidification (pH 6.5) in the low-Ca$^{2+}$ solution ($100 \mathrm{nm}$) produced a more rapid rise in Ca$^{2+}$ than that seen with changes in Ca$^{2+}$ content (B, top). Fluvoxamine (FLV) ($10^{-7}$M) reduced the increase in intramitochondrial Ca$^{2+}$ signals induced by a change of perfusate (A and B, bottom). The rise in [Ca$^{2+}$]$_{m}$ brought about by the perfusate acidification decreased on the addition of fluvoxamine (FLV) ($10^{-7}$–$10^{-4}$M). Subsequent treatment with a new solution (pH 6.5) restored the [Ca$^{2+}$]$_{m}$ (B, bottom).
control, and pre-treated hearts (control: 6.02±0.05, fluvoxamine: 6.15±0.07). During reperfusion, the recovery from intracellular acidification observed in pre-treated hearts was faster than that of the control. However, pH values in these hearts at 30 min of reperfusion were not significantly different from the control value (Table 1).

**Intramitochondrial Ca\(^{2+}\) Measurements with Fura-2 AM**

The intramitochondrial fura-2 Ca\(^{2+}\) signal ([Ca\(^{2+}\)]\(_m\)) increased steadily and linearly but was only 10% higher at extremely high concentrations (1 µM–1 mM) of Ca\(^{2+}\) in the perfusate (Figs. 3A, 4E). The displacement of the normal matrix Ca\(^{2+}\) concentration (100 nM) with perfusate produced a rapid and intense increase of up to 8.9-fold (1.42±0.2), while the change in [Ca\(^{2+}\)]\(_m\) caused by displacement of the perfusate to a high Ca\(^{2+}\) concentration was regarded as 100%. Pretreatment of mitochondria with fluvoxamine (10\(^{-7}\) m) markedly suppressed the increase in [Ca\(^{2+}\)]\(_m\), which in the drug-free perfusate rose by 8.5% (fluvoxamine: 0.12±0.06, p < 0.01).

Changing the pH from 7.4 to 6.5 in the low-Ca\(^{2+}\) perfusate (100 nM) elevated [Ca\(^{2+}\)]\(_m\) to the maximal extent up to about ten times as much relative to the pH 7.4 perfusate, which was regarded as 100% (Figs. 3B, 4E). Furthermore, the increase was greater and more rapid than that by perfusion with a low-Ca\(^{2+}\) perfusate. The appreciable increase in [Ca\(^{2+}\)]\(_m\) induced by acidification (1.50±0.15) was reduced by the pretreatment with fluvoxamine (10\(^{-7}\) m), by 6.0% (0.10±0.05, p < 0.01).

**Fig. 4.** The Increase in [Ca\(^{2+}\)]\(_m\) Brought about by Acidification Decreased on the Addition of Fluvoxamine (FLV) (10\(^{-5}\)–10\(^{-4}\) m).

(A) Subsequent treatment with a new solution (pH 6.5) restored the [Ca\(^{2+}\)]\(_m\). (B) The rise in [Ca\(^{2+}\)]\(_m\) brought about by an external Ca\(^{2+}\) concentration change was decreased on the addition of the MPTP inhibitor, cyclosporin A (CsA, 10\(^{-5}\) m), but FK506 (10\(^{-4}\) m), which inhibits the phosphatase calcineurin without inhibiting MPTP, had no effect. (C) The increase in [Ca\(^{2+}\)]\(_m\) brought about by the perfusate acidification was also decreased by cyclosporin A (CsA, 10\(^{-5}\) m) and FK506 (10\(^{-4}\) m). (D) Atractylloside (Atr, 3×10\(^{-5}\) m), a MPTP opener, increased the [Ca\(^{2+}\)]\(_m\) the same as the acidification. The rise in [Ca\(^{2+}\)]\(_m\) decreased on addition of cyclosporin A (CsA, 2×10\(^{-4}\) m). Comparison of inhibition of Ca\(^{2+}\)-uptake into mitochondria in the control and in the presence of fluvoxamine (FLV) (10\(^{-5}\)–10\(^{-4}\) m). (E) The mitochondrial Ca\(^{2+}\) level was elevated by an external Ca\(^{2+}\) concentration change (left) or external acidification (right). The [Ca\(^{2+}\)]\(_m\) elevation was reduced by fluvoxamine (FLV). **p < 0.01, significantly different from the control (drug-free) values. Data are expressed as the mean of 5 determinations; Vertical lines represent S.E.M. (F) The rise in [Ca\(^{2+}\)]\(_m\) brought about by acidification decreased on the addition of fluvoxamine (FLV) (10\(^{-5}\) m) or the MPTP inhibitor, cyclosporin A (CsA, 10\(^{-5}\) m). *p < 0.05; significantly different from the control (drug-free) values. Data are expressed as the mean of 5 determinations; Vertical lines represent S.E.M.
more, it was greater and more rapid than that caused by perfusion with the low-Ca\(^{2+}\) perfusate (Figs. 3A, B).

Changing the pH from 7.4 to 6.5 in the low-Ca\(^{2+}\) perfusate (100\text{nM}) elevated \([\text{Ca}^{2+}]_m\) to the maximal extent up to about ten times as much relative to the pH 7.4 perfusate, which was regarded as 100% (Fig. 4A). When the pH was changed to 7.4, the appreciable increase in \([\text{Ca}^{2+}]_m\) induced by acidification (0.14±1.50±0.15) was reduced by the pretreatment with fluvoxamine (10\text{−8}M) by 74.5% (0.14±1.12±0.18) (Fig. 4F). The \([\text{Ca}^{2+}]_m\) increase was reduced by the addition of the MPTP inhibitor cyclosporin A (CsA, 10\text{−4}M) by 41.3% (0.14±0.62±0.2) (Fig. 4F).

The rise in \([\text{Ca}^{2+}]_m\) decreased on the addition of the MPTP inhibitor cyclosporin A (CsA, 10\text{−4}M), but FK506 (10\text{−5}M), which inhibits phosphatase calcineurin without inhibiting MPTP, had no effect (Figs. 4B, C). Atractyloside (Atr, 3\times10\text{−3}M), an opener of MPTP, also increased the \([\text{Ca}^{2+}]_m\) the same as acidification (Fig. 4D). The increase brought about by atractyloside decreased on the addition of cyclosporin A. There was a great possibility that MPTP participated in the ischemia–reperfusion injury.

**Detection of Apoptosis**

Examples of apoptotic tissues obtained after the various treatments are shown in Figs. 5A and 5B. Apoptotic cells were first observed in the ventricle after 5h of reperfusion after 1h of ischemia with KH solution containing 2-deoxy-D-glucose. As shown in Figs. 5C and 5D, 5h of reperfusion resulted in an average of 28.0±2.6 cells per 100 microscopic fields, but 4h of reperfusion did not produce apoptotic cells. Fluvoxamine (5\times10\text{−8}M) reduced the incidence of apoptosis in the ischemic and reperfused myocardium to an average of 4.8±1.3 (p<0.01) cells per 100 microscopic fields, respectively, compared with the controls (Fig. 5C).

The caspase-3 activity in the control and apoptotic samples was measured using the same frozen tissues with the TUNEL method. Control-1 was a heart preparation perfused for 30min with normal KH solution (considered to be 2.0), while control-2 was a heart subjected to 1h of ischemia and 5h of reperfusion with a drug-free solution (TUNEL: control). The decrease in caspase-3 activity in the heart pretreated with fluvoxamine [control-1: 2.7, control-2: 11.3±1.4, fluvoxamine: 4.8±0.8 (p<0.01 for control-2)], was similar to the result of the TUNEL assay (Fig. 5D).

**Mechanical Function and High-Energy Phosphates in Ischemia–Reperfusion Hearts for Detection of Apoptosis**

Changes in mechanical function and high-energy phosphates in hearts treated with fluvoxamine at 5\times10\text{−8}M after 5h of re-

---

**Fig. 5.** Representative Light Micrographs (A, B: ×240) Illustrating Apoptotic Cells

TUNEL-positive cardiomyocytes were detected in the outer two-thirds of the left ventricular wall. No stained cells were observed in control hearts not subjected to ischemia. A: control, B: fluvoxamine (5\times10\text{−8}M). Scale=100\text{µm} (C) The incidence of apoptosis observed with the TUNEL technique described in Materials and Methods. Values are expressed as the means for five preparations±S.E.M. and indicate the number of positively stained cells identified per 100 microscopic fields. **p<0.01, significantly different between the fluvoxamine (FLV)-treated groups (n=5) and control groups (n=3). (D) Control-1: The uninduced control was a heart preparation perfused at 30min with normal KH solution. Control-2 (n=3): fluvoxamine (FLV) and the same heart tissue were used with the TUNEL method. Values are expressed as the means for 5 preparations±S.E.M. and indicate the caspase-3 activity. *p<0.05; significantly different from the control (drug-free) values.
perfusion were significantly different from those in the control hearts. No significant differences were observed in the LVDP, but the LVEDP values obtained at the end of reperfusion were significantly lower in hearts pretreated with fluvoxamine (22.0±3.1 mmHg, p <0.05, versus 43.2±3.1 mmHg in control hearts). Coronary flow (FR) was significantly increased (7.9±0.8 mL/min, p <0.05 versus 4.8±0.7 mL/min in control hearts).

ATP and PCR levels at the end of 40 min of reperfusion were significantly higher in the fluvoxamine-treated groups (p <0.05) (ATP: 68.2±9.2%, control: 39.2±4.2%, PCR: 32.3±4.2%, control: 12.3±7.9% with pre-ischemia as 100%). Based on these results, the preventive effects of fluvoxamine on tissue levels of high-energy phosphate were greater than those of control-2.

DISCUSSION

Several lines of experimental data have indicated that 5-HT may be such a mediator. 5-HT is released from blood platelets upon their aggregation, which it further amplifies. 27 It also causes vascular contraction via 5-HT2A and 5-HT1B receptors. 28 Thus, 5-HT may exacerbate thrombosis and coronary spasms during myocardial ischemia–reperfusion injury. 29 5-HT levels in the atrial and ventricular walls of Langendorff-perfused rabbit and rat hearts have been clearly established by HPLC. 30–33 Transcardiac plasma 5-HT concentrations were shown to be higher in some patients with angina pectoris. 34 An antagonist of 5-HT2A, ketanserin, may have potential as a therapeutic agent for hypertension as well as peripheral vascular disease, and was shown to prevent cardiac dysfunction in ischemic rat hearts. 35 Furthermore, the blockade of 5-HT receptors by cinanserin had a protective effect on pacing-induced ischemia in an in vivo model using dogs. 36 Sarpogrelate, a 5-HT2A antagonist, had a beneficial effect against ischemia–reperfusion injury in isolated Langendorff-perfused guinea-pig hearts perfused with no-blood solution. 37 It also prevented the downregulation of the anti-apoptosis protein Bel-2 and protected the rat heart against ischemia–reperfusion injury. 38

In contrast, (S)-(−)-pyrapyridolol, a 5-HT1A receptor antagonist, suppressed the increase in myocardial nitric oxide (NO) levels caused by the administration of 5-HT in normal Langendorff-perfused hearts, 39 which confirmed the existence of 5-HT2A, 40–42 5-HT1A, 37 and 5-HT1B receptors in the Langendorff-perfused heart. The increase in 5-HT levels during ischemia has been implicated in the endothelial release of NO from another receptor (5-HT1B or 5-HT2B receptors) in the heart. 43 NO exogenously supplied by a specific NO donor, such as FK409, 44 or the antioxidative 5-HT derivatives isolated from safflower 45 were shown to be responsible for the cardioprotective action observed, presumably by acting directly as oxygen radical scavengers during reperfusion. These NO donors may have potential therapeutic use as NO-mediated vasorelaxers and afford additional protection to reperfusion-injury hearts. The existence of endothelial NOS (eNOS) in myocardial mitochondria was demonstrated in our previous study using immunoblotting and electron microscopic analysis. 22 NO may play an important protective role in reperfusion cardiac injury following ischemia by inhibiting Ca2+ influx into mitochondria, which are otherwise damaged by O2. 29 Further studies are needed to more adequately resolve the relationship between apoptotic caspase activity and the 5-HT1A antagonist effect.

Mitochondrial fura-2 Ca2+ uptake ([Ca2+]m) was previously shown to rapidly increase due to changes in Ca2+ concentrations and acidification of the perfusate, similar to reperfusion following global ischemia in hearts perfused using the Langendorff technique. 31 The marked elevation in [Ca2+]m due to the fluvoxamine treatment was significantly suppressed relative to that in control hearts. The elevations in [Ca2+]m due to a perfuse change in the mitochondrial preparation were similar to those obtained with the MPTP opener, atractyloside. 19 Meanwhile, a depression in the [Ca2+]m elevation due to the fluvoxamine or (S)-(−)-pyrapyridolol treatment was similar to that obtained with cyclosporin A, pH 8.5, or Mg2+, a well-known inhibitor of the MPTP. 11 Therefore, these elevations observed in [Ca2+]m with perfuse changes in the mitochondrial preparation may make it possible to anticipate the mitochondrial function associated with the regulation of the MPTP. Irrespective of its exact composition, the MPTP complex contains multiple targets for pharmacological investigations on different apoptotic pathways as sensors for stress and damage, as well as for certain signals connected to the receptors. 46–48 Evidence now suggests that apoptosis is an important response of the myocardium to ischemia and reperfusion, and appears to contribute to the overall sequelae of cardiac injury. Substances induced by cardiac injury, which prevent opening of the MPTP, may preserve mitochondrial function and, thus, have potential beneficial effects. Fluvoxamine affected the number of TUNEL-positive cardiomyocytes and caspase-3 activity related to the endogenous accumulation of 5-HT in ischemia–reperfusion hearts, similar to the 5-HT1A antagonist pyrapyridolol. 11 Therefore, the effects of fluvoxamine on ischemia–reperfusion-injury was investigated under the same experimental conditions in the present study in order to compare these effects to those of 5-HT-related drugs; a 5-HT2A antagonist 6 and 5-HT1A antagonist. 11 The level of 5-HT, a trigger of cardiac dysfunction, was shown to increase during ischemia–reperfusion; therefore, SSRIs may decrease the amount of 5-HT in hearts. These findings suggest that SSRI inhibits opening of the MPTP by preventing [Ca2+]m overload-induced apoptosis related to the endogenous accumulation of 5-HT in ischemia–reperfusion hearts.

The identification of factors regulating cardiomyocyte cell death is important to more clearly understand the pathogenesis of congenital heart diseases. Future studies should examine the effects of a 5-HT4 antagonist 43 on Langendorff–reperfusion injury and establish a system for 5-HT ischemia–reperfusion injury. Further investigations of the different cardiac effects of the 5-HT antagonist and SSRIs may provide valuable information on the normal physiology of cell death, and be useful in the development of cardioprotective drugs.

Acknowledgments The authors wish to thank Mr. Makoto Naruse and Mr. Minoru Fukayama (Aichi Medical University) for skilful NMR measurements and Miss Kaoru Mimura for technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (No. 16590443) and Kinjo Gakuin University-Parent Teacher Association Special Research Subsidy.


