

Characterization of Monoamine Oxidases in Mesenchymal Stem Cells: Role in Hydrogen Peroxide Generation and Serotonin-Dependent Apoptosis

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Early death of grafted bone marrow mesenchymal stem cells (MSCs) represents a major limit to their use in cell therapy of solid organs. It is well known that oxidative stress plays a major role in cell death. We have recently shown that the serotonin-degrading enzyme monoamine oxidase A (MAO-A) generates large amount of hydrogen peroxide (H₂O₂) responsible for cell apoptosis. Hydrogen peroxide generation requires 5-HT internalization into the cell and its degradation by MAO-A. In the present study, we investigated whether MAO-A is expressed in MSCs and we defined its role in serotonin-dependent MSCs apoptosis. RT-PCR analysis and western blots showed that the serotonin transporter (SERT) and the 2 MAO isoenzymes, A and B, are expressed in MSCs. As shown by enzyme assays using [¹⁴C]serotonin or [¹⁴C]β-phenylethylamine as selective MAO-A or MAO-B substrates, MAO-A is largely predominant in MSCs. Incubation of MSCs with the MAO substrate tyramine led to a time-dependent generation of H₂O₂ that was prevented by the MAO inhibitor pargyline. Finally, exposure of the cells to serotonin promoted an increase in MSCs apoptosis prevented by pargyline and the SERT inhibitor imipramine. The pro-apoptotic effect of serotonin was associated to a decrease in the expression of the anti-apoptotic factor Bcl-2. In conclusion, these results show for the first time that the 5-HT-degrading enzyme MAO-A is an important source of H₂O₂ in MSCs and plays a major role in 5-HT-dependent MSCs apoptosis.

Introduction

MESENCHYMAL STEM CELLS (MSCs) are progenitor cells that can be isolated from different tissues, in particular from bone marrow and adipose tissue [1–3]. Results obtained in our and other laboratories showed that direct injection of MSCs into the tissue is associated with a functional recovery of ischemic kidney [4] and heart [5]. In particular, MSCs protect against both chemical and ischemia–reperfusion damage and promote repair process in models of acute renal injury [6,7]. Although intraparenchymal injection allows concentration of MSCs within the injured organ, its use is limited by the extensive early death of grafted cells [8–10]. Different mechanisms have been involved in this phenomenon including hypoxia, inflammation, and oxidative stress [11–13]. The importance of reactive oxygen species (ROS) in death of grafted cells has been supported by studies showing that prevention of oxidative stress through pharmacological approaches [7] or genetic modifications [14]

allowed increasing MSCs survival after graft. At present, the intracellular sources of ROS and their involvement in MSCs death have not been clearly identified. Recently, we have shown for the first time that, in different cell types [15–17], the mitochondrial enzyme monoamine oxidases (MAOs) generate large amount of hydrogen peroxide (H₂O₂). Based on genetic criteria, substrate specificity, and inhibition by selective compounds, MAOs have been classified in 2 isoenzymes, MAO-A and MAO-B [18,19]. Both catalyze the oxidative deamination of biogenic and dietary amines [20] and are primarily involved in the degradation of neurotransmitters within the central nervous system. There are also implicated in the metabolism of biogenic amines in peripheral tissues and vascular cells [20–22]. MAO-A and MAO-B oxidize dopamine, norepinephrine, and tyramine whereas serotonin (5-HT) is the preferred substrate for MAO-A and phenylethylamine is the preferred substrate for MAO-B. The control of substrate availability has been considered as the major

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feature of MAOs in the central nervous system and in the periphery. Our recent studies allowed demonstrating the existence of an additional function of MAO-A. Indeed, we showed that H₂O₂ produced by MAO-A during 5-HT degradation plays a major role in cardiomyocytes apoptosis *in vitro* and *in vivo* [23]. Based on our and other results, it is now accepted that 5-HT may act through 2 different mechanisms: one, the receptor-dependent, involves stimulation of specific 5-HT receptors; the other, the MAO-A-dependent, requires 5-HT internalization into the cell, its degradation by MAO-A, and H₂O₂ generation.

Recent studies demonstrated that MSCs are a target of 5-HT. Indeed, it has been shown that 5-HT, through activation of 5-HT₂ receptors, regulate neuroplasticity [24] and proliferation of MSCs [25].

In the present study, we investigated whether MAO-A is expressed in MSCs and we defined its role in serotonin-dependent MSCs apoptosis.

Materials and Methods

Cell culture

Marrow aspirates were obtained from femurs cavity of Lewis rats (Harlan, France) weighing 180–200 g. Bone marrow from femurs cavity was flushed with minimum essential medium (MEM) (ABCYs, France) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and the cell suspension was centrifuged (400g, 5 min). Then, cells were plated in culture flasks (200,000 cell/cm²). Nonadherent cells were removed after 72 h and MSCs were recovered by their capacity to adhere highly to plastic culture dishes. MSCs were then routinely cultured and were used for the experiments after passage 3. Most adherent cells expressed CD90, CD29, CD106 and were negative for CD34, CD45, and CD31.

RT-PCR

Total ribonucleic acid (RNA) was isolated from MSCs, or H9C2 using nucleospin kit (Macherey Nagel, Germany) and cDNA was synthesized from 1 µg total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) was performed in 20 µL reaction buffer containing 17 µL PCR Platinum Mix (Invitrogen), 1 µg cDNA, 1 µg of specific primers. PCR conditions were as follows: 4 min at 94°C, followed by cycles of denaturation (30 s, 94°C), 30 s annealing at 60°C, and 30 s extension at 72°C. PCR products were confirmed on a 1% agarose gel and visualized under UV light after ethidium bromide staining. Followed primers (sense/antisense) were used (Eurogentec, Belgium): SERT-F AGTGCTGTCAGAGTGT AAGGA, SERT-R GCGCCAGGCTATGATGGTGT; MAO-A-R: ATGACGGATCTGGAGAAGCC; MAO-A-F: TGCCTCA CATAACACAGGAAC; MAO-B-R: TTAGATAATTTGTGTG GGTGAGAGAA; MAO-B-F: AAGAAAACAAAAGAACC CAGAAATTATT.

For mRNA expression of SERT, H9C2 cells have been used as a positive control. The H9C2 cardiomyocyte-like cell line (American Type Culture Collection, Molsheim, France) is a rat embryonic myoblast-derived cell line commonly used as an *in vitro* model of cardiomyocyte biology that shows

similar hypertrophic and apoptotic responses as those seen in primary adult and neonatal cardiomyocytes [26].

Apoptosis evaluation

Apoptosis was assessed by double staining cells with Syto 13 (1 µmol/L; Molecular Probes, Eugene, OR) and propidium iodide (6 µg/mL; Molecular Probes) for 15 min at 37°C. Cells were then examined with a fluorescent microscope, and apoptotic cells were characterized by condensed, fragmented nuclear regions. A total of 300 cells were counted for each condition.

Western blot

For western blot (WB), proteins were extracted from MSCs and prepared from rat brain and liver lysates. WB analyses were performed with samples normalized for protein concentration. Membranes were probed with anti-rabbit Bcl-2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse Bax (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-rabbit MAO-A (1:400; Santa Cruz Biotechnology), anti-goat MAO-B (1:400; Santa Cruz Biotechnology), or anti-goat actin (1:1,000; Santa Cruz Biotechnology) antibodies. Following several washes in Tris-buffered saline-Tween (0.2%), membranes were incubated with anti-rabbit or anti-mouse or anti-goat secondary antibody (1:10,000; Santa Cruz Biotechnology). Expression of Bcl-2 and Bax is compared with expression of actin.

H₂O₂ production assay

As previously reported, the luminol-amplified chemiluminescence assay is a sensitive procedure to measure the specific contributions of MAO-A and MAO-B to H₂O₂ production [27].

H₂O₂ production was measured by chemiluminescence assay on MSCs scrapped in Hank's balanced salt sodium (HBSS; 5 µg) in the presence of luminol (30 µM) and horse radish peroxidase (0.1 U/µL) by using a thermostatically (37°C) controlled luminometer (Mithras, Berthold, France). The generation of chemiluminescence triggered with tyramine (30 µM), a common MAO substrate, was monitored every 20 s during 60 min.

Assays of MAO activity

Crude extracts of proteins (50 µg) were incubated at 37°C for 20 min, in the presence of 3.125 to 400 µM of [¹⁴C]serotonin or 200 µM of [¹⁴C]β-phenylethylamine to measure MAO-A and MAO-B activities, respectively [28]. To define the nonspecific activities, the MAO-A inhibitor clorgyline and the MAO-B inhibitor deprenyl were used (0.1 µM). The reaction was ended by the addition of 0.1 mL of HCl 4 N at 4°C. The reaction product was extracted (efficiency 92%) with 1 mL of ethyl acetate/toluene (v/v), and the radioactivity contained in the organic phase was counted in a liquid scintillation spectrometer.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical comparison of the data was performed using the *t*-test for comparison between 2 groups or

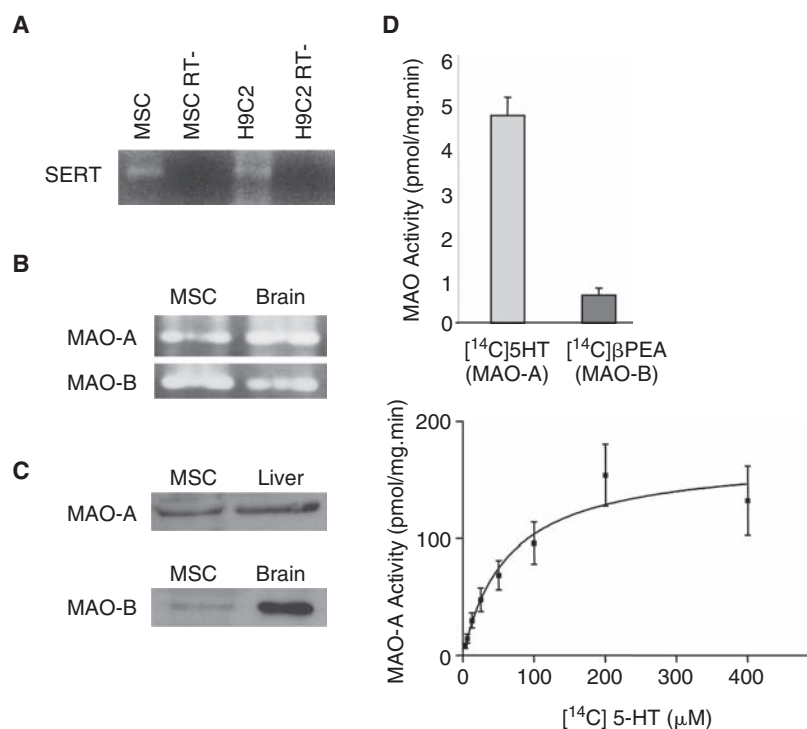


FIG. 1. Serotonin transporter (SERT) expression and monoamine oxidases (MAOs) expression/activities in mesenchymal stem cells (MSCs). (A) SERT mRNA expression in mesenchymal stem cells was defined by RT-PCR. H9C2 cells have been used as positive control. MSCs RT- are relative to negative control in which reverse transcriptase SuperScript II was omitted. (B) MAO-A and MAO-B mRNA expression in mesenchymal stem cells. mRNA from brain is used as positive control. (C) Protein expression of MAO-A and MAO-B was determined by western blot in MSCs lysates. Homogenates of rat liver and brain were used as positive control of MAO-A and MAO-B, respectively. (D) MAO activities were determined using [¹⁴C]5-HT (400 μM) and [¹⁴C]β-PEA (200 μM) as specific substrates for MAO-A and MAO-B, respectively (upper panel). A dose response for MAO-A activity was realized with [¹⁴C]5-HT (3.125–400 μM) in the lower panel. Results are expressed as the difference between total and nonspecific activities, the latter being defined in the presence of selective inhibitors clorgyline (10⁻⁷ M) and deprenyl (10⁻⁷ M) for MAO-A and MAO-B, respectively. These experiments have been done on 3 different MSCs preparation isolated from bone marrow of Lewis rat.

one-way analysis of variance (ANOVA) and post hoc Tukey's test for comparison of >2 groups. A value of $P < 0.05$ was considered significant.

Results

Expression of 5-HT transporter and monoamine oxidases in MSCs

Serotonin transport into the cell and its degradation by MAO-A is necessary for H₂O₂ generation. RT-PCR performed on mRNA obtained from rat MSCs showed the expression of mRNA encoding for the 5-HT transporter SERT (serotonin transporter), and for the 2 isoforms of MAOs, MAO-A and MAO-B (Fig. 1, panels A and B, respectively). As shown in Figure 1C, western blot analysis performed on MSCs lysates revealed 2 proteins with the apparent molecular weight expected for MAO-A and MAO-B (~61 and 60 kDa, respectively). The expression of both MAO-A and MAO-B was confirmed by radioenzyme assay using [¹⁴C]5-HT and [¹⁴C]β-PEA as substrates for MAO-A and MAO-B, respectively (Fig. 1D, upper panel) and by dose response using [¹⁴C]5-HT (Fig. 1D, lower panel). Results indicated that the 2 isoforms were found in MSCs with a higher expression of the MAO-A.

H₂O₂ production by MAOs in MSC

In order to determine whether MAOs are a source of ROS in MSCs, H₂O₂ production was assessed using a continuous luminol-amplified chemiluminescence assay. We have previously shown that this technique allows the measurement of H₂O₂ generation by MAOs in intact cells using the MAO-A/MAO-B substrate tyramine [27]. As shown in Figure 2, incubation of MSCs with tyramine (30 μM) led to a time-dependent increase in chemiluminescence. This effect was prevented by preincubation of MSCs with the irreversible MAO inhibitor pargyline (5 μM). We observed that ROS generation by tyramine was prevented by pargyline by 78% (94.8–21). Tyramine is a MAO substrate but it has also been shown that it can be metabolized by other amino oxidases, in particular, the semicarbazide-sensitive amine oxidase (SSAO). Consequently, it is possible that residual H₂O₂ generation may be related to the SSAO activity. H₂O₂ generation was also observed using higher tyramine concentrations (300 μM and 2 mM). However, this effect was observed only within 5 min after tyramine addition and rapidly declined with time probably because of MSCs death. This hypothesis is supported by our previous results showing that tyramine (50 μM, 24 h) induced MSCs apoptosis measured by MTT

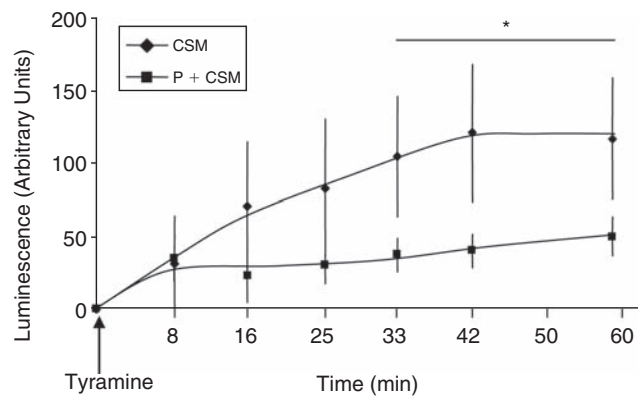


FIG. 2. Monoamine oxidase (MAO)-dependent H_2O_2 production in mesenchymal stem cells (MSCs) lysates. Generation of chemiluminescence was monitored for 60 min after the addition of tyramine (30 μ M) to MSCs as described in "Materials and Methods" section. MSCs were preincubated with pargyline (5 μ M, 20 min) before tyramine addition. The plot is representative of 4 separate experiments. * $P < 0.05$ versus pargyline-treated MSCs.

assay (data not shown). According to our findings, Marcocci et al. demonstrated that tyramine at concentrations from 100 to 500 μ M altered the mitochondrial membrane permeability transition [29], an event generally associated with cell apoptosis [30].

These results indicate that MAOs are able to produce H_2O_2 during substrate degradation.

5-HT treatment induced apoptosis of MSC

In these experiments, we investigated whether MAOs are involved in 5-HT-dependent MSCs apoptosis. Cell apoptosis was evaluated by analysis of DNA fragmentation and the measure of cell incorporation of 2 fluorescence dyes SYTO-13, a permeant DNA intercalating green-colored probe, and propidium iodide, a nonpermeant intercalating orange probe. Apoptotic and necrotic cells were discriminated by green (SYTO-13) and orange (propidium iodide) staining, respectively. Cell treatment with increasing concentrations of 5-HT for 24 h induced a significant increase (25% to 40% for 5-HT 10 μ M) in the number of SYTO-13-stained cells as compared to untreated cells (Fig. 3A). In contrast, 5-HT treatment did not increase cell staining by propidium iodide, the marker of necrotic cells. Exposure of MSCs to 5-HT (10 μ M) at different times (3, 6, 12, and 24 h) induced a time-dependent increase in apoptosis (Fig. 3B).

As we and others have demonstrated that 5-HT could induce apoptosis through a receptor-independent pathway, we have decided to clarify the involvement of MAOs in 5-HT-induced apoptosis. MSCs have been pretreated with the pargyline (5 μ M, 24 h) before 5-HT treatment and results indicated that inhibition of MAO activity prevented apoptosis (Fig. 3C). Furthermore, use of the imipramine (10^{-7} M), a serotonin uptake inhibitor, and SB206553 (0.1 μ M, 1 h), a 5-HT_{2B} antagonist, also prevented the pro-apoptotic effect of 5-HT on MSCs.

These results show that, in order to induce MSCs apoptosis, 5-HT needs to be internalized into the cells and metabolized by MAO-A and that 5-HT_{2B} receptor could play a role in this mechanism.

Effect of serotonin on Bcl-2 and Bax expression and mitochondrial cytochrome c release in MSCs

Apoptotic processes could be associated to mitochondrial dysfunction involving members on the Bcl-2 protein family such as the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax. Exposure of MSCs to 5-HT (10 μ M, 24 h) promoted an increase in cell apoptosis (Fig. 3B) that was associated to a decrease in Bcl-2 expression (Fig. 4A). This effect was partially prevented when MSCs were pretreated with pargyline (5 μ M, 24 h). We next examined the effect of serotonin on Bax expression (Fig. 4B). Western blot analysis showed that incubation of MSCs with 5-HT did not modify the level of Bax protein.

Release of cytochrome *c* from mitochondria to cytosol is a critical step of the mitochondrial-dependent cell apoptosis. As shown in Figure 4C, MSCs treatment with 5-HT (10 μ M, 24 h) induced a slight increase in cytosolic cytochrome *c* as defined by western blotting.

Altogether, these results indicate that 5-HT induces MSCs apoptosis through a MAO-A-dependent mechanism. This effect could involve ROS generation concomitant to a decrease in the amount of the anti-apoptotic factor Bcl-2 that could contribute to cell apoptosis.

Discussion

In the present work, we showed the expression of the 5-HT-degrading enzyme MAO-A in bone marrow MSCs and its role in H_2O_2 generation. In addition, we demonstrated that MAO-A is involved in 5-HT-dependent MSC apoptosis.

Monoamine oxidase A is widely distributed in the body. While the function of MAO-A has been extensively investigated in the central nervous system, less is known concerning its role in the periphery. As in neuronal cells, MAO-A in the liver [31] and in the lung [32] is involved in the clearance of 5-HT. In other cell types, like cardiomyocytes, we showed that MAO-A also participates in the cell effects of 5-HT [15]. The MAO-A-dependent effects of 5-HT requires 5-HT transport into the cell and its metabolism by MAO-A. Our results show for the first time that this mechanism of action of 5-HT exists in MSCs. Indeed, we showed that 2 partners, necessary for the receptor-independent effects of 5-HT, SERT, and MAO-A, are expressed in MSCs. As previously reported for cardiomyocytes, 5-HT induced MSC apoptosis and this effect was prevented by the SERT inhibitor imipramine, by the MAO inhibitor pargyline, and more surprisingly by SB206553. We may explain these very intriguing results by interactions between 5-HT receptors and SERT. Indeed, studies have showed that activation of 5-HT receptors could trigger the phosphorylation of SERT by PKC, PKG, or p38. These kinases modulate the SERT membrane expression and, consequently, promote a reduction or an acceleration of the kinetics of 5-HT uptake [33–35]. Furthermore, in platelets, SERT-mediated accumulation of 5-HT results in "transamidation" to small GTPases such as Rho-A and Rho-4 [36]. In this case, 5-HT_{2A} receptors synergizes with SERT. Consistent with these results, we also showed that 5-HT decreased the expression of the anti-apoptotic protein Bcl-2 through a MAO-A-dependent mechanism. These results show that 5-HT and MAO-A may behave as pro-apoptotic factors in MSCs. In our experiments, we observed only a slight increase of cytochrome *c* release after 5-HT treatment.

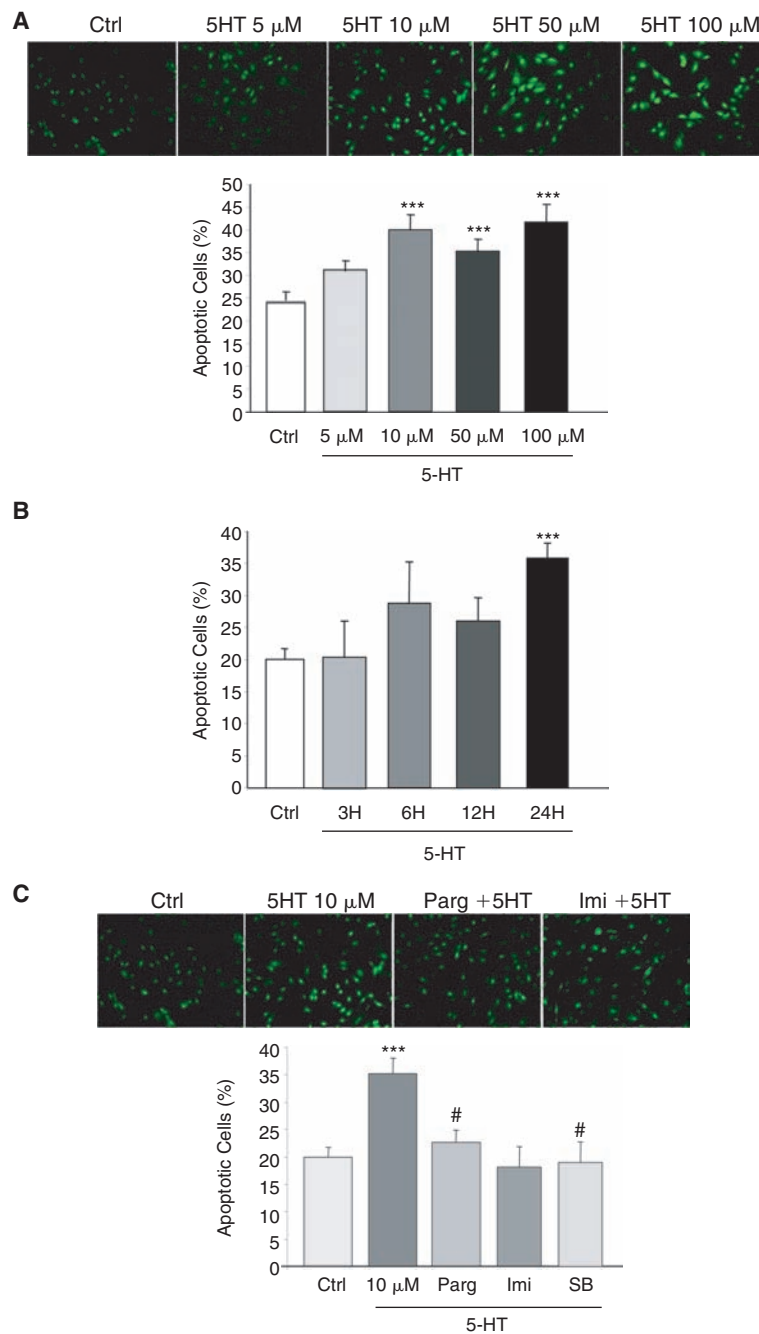


FIG. 3. Time- and dose-dependent effects of 5-HT on apoptosis. Cell nuclei were double-stained with SYTO-13 and propidium iodide, allowing discriminating normal cells from cells undergoing apoptosis or necrosis by fluorescence microscopy. (A) Photomicrographs of mesenchymal stem cells (MSCs) treated with increasing concentration of 5-HT (5, 10, 50, or 100 μM) for 24 h. (B) Photomicrographs of MSCs treated with 5-HT (10 μM) for different times (3, 6, 12, and 24 h). (C) Photomicrographs of MSCs pretreated or not with pargyline (5 μM, 24 h), imipramine (0.1 μM, 1 h), or SB206553 (0.1 μM, 1 h) before addition of 5-HT (10 μM, 24 h). Lower panels showed the quantification of apoptotic cells illustrated in upper panels. Results are mean ± standard error of the mean (SEM) from 3 independent MSCs preparations isolated from bone marrow of Lewis rat. ****P* < 0.001 versus control, #*P* < 0.05 versus 5-HT (10 μM).

This could be explained by the kinetics of the experiments. Indeed, cytochrome *c* release from mitochondria occurs in the early step of the apoptosis. After its release, cytochrome *c* is associated with Apaf-1 to form the apoptosome that will induce the caspase activation. Our experiments were done 24 h after 5-HT treatment. It is conceivable that, at this time, cytochrome *c* may be partially cleaved in apoptotic cells.

Nevertheless, the slight increase in cytosolic cytochrome *c* supports the involvement of mitochondrial dysfunction in apoptosis of MSCs.

The relevance of 5-HT/MAO-A-mediated apoptosis of MSCs *in vivo* is supported by different observations. In the periphery, 5-HT is mainly stored in platelet and released by activated platelets. Platelet activation occurs in different

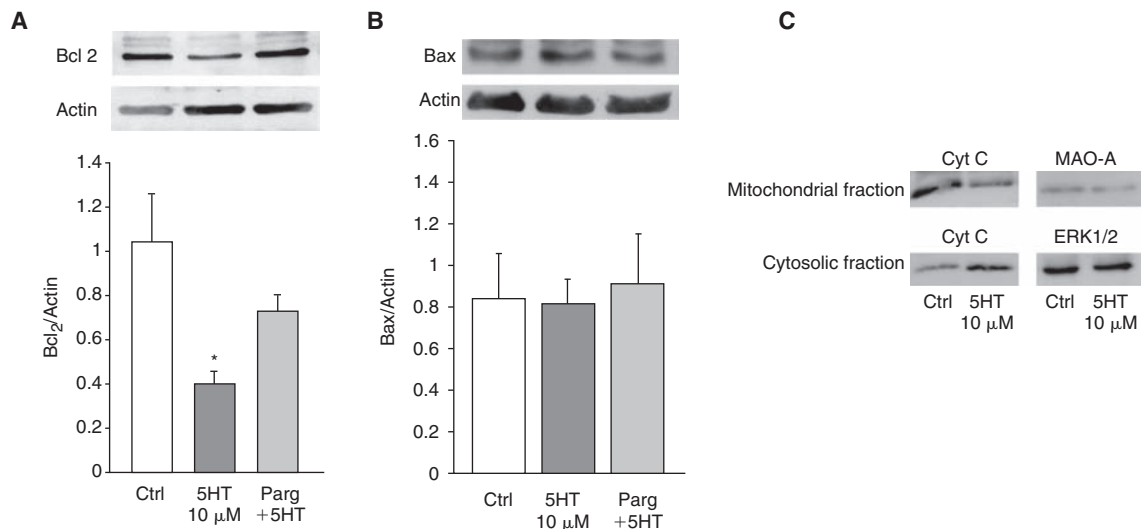


FIG. 4. Effect of 5-HT on Bcl-2/Bax expression and cytochrome *c* release in mesenchymal stem cells (MSCs). (A and B) MSCs were pretreated or not with pargyline (5 μM, 24 h) before the addition of 5-HT (10 μM, 24 h). The expression of Bcl-2 (panel A) and Bax (panel B) proteins were determined by western blot in cell lysates (upper panel). Actin expression was used to confirm equal loading of the extracts. Lower panel showed a quantification of western blot data. Results are mean ± standard error of the mean (SEM) from 3 independent experiments. (C) Western blot analysis of the cytochrome *c* (Cyt *c*) in mitochondria and the cytosol of MSCs. MAO-A and ERK1/2 were used as markers of mitochondria and cytosol, respectively. **P* < 0.05 versus control.

pathological situations manageable by cell therapy and characterized by the increase in circulating or local 5-HT. In the heart, several studies showed that 5-HT accumulates during ischemia–reperfusion, and contributes to the progression of myocardial injury and dysfunction [37–39]. In addition, circulating 5-HT concentrations also increase in chronic diseases such as diabetes [40,41] and atherosclerosis [42,43] and could be elevated because of blood platelets involvement in immune inflammatory processes [44–46]. Based on our results, it is conceivable that the 5-HT/MAO-A pathway and the consequent H₂O₂ generation may contribute to the death of MSCs after intraparenchymal injection. Further studies will be necessary to confirm this possibility.

The demonstration of the expression of MAO-A and of the apoptotic effects of 5-HT in MSCs may exceed the field of cell therapy. Indeed, during the last years, several studies showed that 5-HT plays a critical role in proliferation of MSCs [25] and in the function of MSC-derived cells such as osteoblasts [47] and fibroblasts [48]. In addition, recent studies indicate that 5-HT also plays an important role in differentiation and survival of bone marrow hematopoietic cells [49]. Recent studies reported that ROS are implicated in the reduction of MSCs numbers and differentiation capacity associated with aging [50,51]. Interestingly, it has been also shown that the tissue MAO-A expression and activity increases with age [52,53]. Therefore, MAO-A appears to be a potential candidate to the increase in H₂O₂ generation during MSCs aging. Based on these and our results, it is conceivable that 5-HT and MAO may play an important role in the regulation of bone marrow cells in physiological and pathological situations.

In conclusion, we have shown for the first time the expression of the 5-HT-degrading pathway SERT/MAO-A in MSCs and its involvement in 5-HT-dependent MSCs apoptosis. These results open new perspective in the comprehension of the role

of MAO-A in the regulation of 5-HT effects in MSCs and its involvement in physiological and pathological processes.

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Author Disclosure Statement

No competing financial interests exist.

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