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Oxidative Stress by Monoamine Oxidase Mediates Receptor-Independent Cardiomyocyte Apoptosis by Serotonin and Postischemic Myocardial Injury

Pascale Bianchi, PhD; Oxana Kunduzova, PhD; Emanuela Masini, MD; Claudie Cambon, PhD; Daniele Bani, MD; Laura Raimondi, PharmD; Marie-Helene Seguelas, BSc; Silvia Nistri, PhD; Wilson Colucci, MD; Nathalie Leducq, PhD; Angelo Parini, MD, PhD

Background—Serotonin (5-hydroxytryptamine [5-HT]), released by activated platelets during cardiac ischemia, is metabolized by the mitochondrial enzyme monoamine oxidase A (MAO-A). Because hydrogen peroxide is one of the byproducts of 5-HT degradation by MAO-A, we investigated the potential role of reactive oxygen species generated by MAOs in 5-HT–dependent cardiomyocyte death and post–ischemia-reperfusion cardiac damage.

Methods and Results—Treatment of isolated adult rat cardiomyocytes with 5-HT induced intracellular oxidative stress and cell apoptosis. The apoptotic cascade triggered by 5-HT involves release of cytochrome c, upregulation of proapoptotic Bax protein, and downregulation of antiapoptotic Bcl-2 protein. These effects were prevented by inhibition of amine transporter or MAO, antioxidants, or iron chelation. In contrast, cardiomyocyte apoptosis was only slightly affected by the 5-HT_{2B} receptor antagonist SB 206553. In vivo, inhibition of MAO-A largely reduced myocardial ultrastructural damage induced by 30 minutes of ischemia followed by 60 minutes of reperfusion in the rat heart. Cardioprotective effects of MAO inhibitors were associated with the prevention of postischemic oxidative stress, neutrophil accumulation, and mitochondrial-dependent cell death and were not reverted by SB 206553. Administration of MAO-A inhibitors during ischemia was still effective in preventing cardiac damage.

Conclusions—Our results supply the first direct evidence that oxidative stress induced by MAO is responsible for receptor-independent apoptotic effects of 5-HT in cardiomyocytes and postischemic myocardial injury. These findings provide new insight into the mechanisms of 5-HT action in the heart and may constitute the basis for novel therapies. **(*Circulation*. 2005;112:3297-3305.)**

Key Words: apoptosis ■ ischemia ■ enzymes ■ myocytes ■ serotonin

Serotonin (5-HT) is a biogenic amine produced in the central nervous system by cells originating in the raphe nuclei of the brainstem. In periphery, serotonin is produced predominantly by intestinal enterochromafin cells and stored in platelets. Serotonin affects a wide variety of physiological functions through the interaction with specific G-coupled membrane receptors. In the heart, 5-HT has been involved in regulation of normal cardiac development[1] and in different diseases, including arrhythmia,[2] ventricular hypertrophy,[3] and cardiac valvular insufficiency associated with carcinoid tumors.[4] During the last years, several studies showed that serotonin accumulates in heart during ischemia-reperfusion (I/R) and contributes to the progression of myocardial injury and dysfunction.[5–7] The deleterious effects of serotonin have been related to indirect mechanisms involving coronary vasoconstriction[8] and reactive oxygen species (ROS)–dependent stimulation of cardiac sympathetic afferents.[5,9] At present, the possibility that serotonin acts directly on cardiac cells to induce apoptosis and necrosis has not been investigated. However, this possibility is suggested by our recent studies showing that the serotonin-degrading mitochondrial enzyme monoamine oxidase A (MAO-A) is an important source of hydrogen peroxide (H_2O_2) in the heart.[10] Monoamine oxidases are outer mitochondrial membrane enzymes that, based on genetic criteria,[11] substrate specificity, and inhibition by synthetic compounds,[12] have been subdivided into 2 major forms, A and B. The degradation of biogenic amines (serotonin and catecholamines) has been considered the major physiological function of these enzymes. However, we have recently shown that in kidney H_2O_2 produced by MAOs during dopamine degradation mediates cell apoptosis.[13] Although heart contains large amount of MAO-A,[14] the role of this enzyme in

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From INSERM U388, Toulouse, France (P.B., O.K., C.C., M.-H.S., A.P.); Department of Preclinical and Clinical Pharmacology, Department of Anatomy, Histology and Forensic Medicine, University of Florence, Florence, Italy (L.M., D.B., L.R., S.N.); Boston University Medical Center, Boston, Mass (W.C.); and Cardiovascular-Thrombosis Research Department, Sanofi-Synthélabo Research, Toulouse, France (N.L.).

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Correspondence to Angelo Parini, MD, PhD, IRF31, BP 84225, INSERM U388, 31432 Toulouse Cedex 4, France. E-mail parini@toulouse.inserm.fr

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TABLE 1. Area at Risk and Infarct Size

	LV WT, g	Infarct WT, g	Infarct % of LV	Risk Region WT, g	Risk Region % of LV
I/R	0.33±0.015	0.1±0.004	29.8±3.6	0.17±0.008	52±2.1
P+I/R	0.35±0.005	0.02±0.0004*	7.13±0.47*	0.17±0.002	47.5±2
C+I/R	0.35±0.004	0.04±0.0004*	10.75±0.25*	0.18±0.002	50±1.9
P+SB+I/R	0.36±0.012	0.04±0.001*	11.36±0.25*	0.18±0.006	50.5±1.5
C+SB+I/R	0.35±0.0012	0.05±0.002*	13.77±1.3*	0.18±0.006	51±1.2

LV indicates left ventricular; WT, weight; P, paragyline; C, clorgyline; and SB, SB 206553. Data are mean±SEM.
*$P<0.001$ vs I/R.

regulation of cardiac functions is still not clearly defined. Some studies indicated that cardiac MAO-A regulates noradrenaline concentrations.[15,16] Our demonstration that MAO-A is also an important source of H_2O_2 in the heart suggests that this enzyme may contribute to ROS-dependent cardiomyocyte apoptosis. To verify this hypothesis, we investigated the potential role of ROS generated by MAO-A during 5-HT degradation in cardiomyocyte death and the role of MAO in post-I/R cardiac damage.

Methods

Cell Culture

Calcium-tolerant adult rat ventricular myocytes were obtained from hearts of male Sprague-Dawley rats (250 to 275 g) as previously described.[17] Briefly, hearts were perfused retrogradely with Ca^{2+}-free Krebs-Henseleit bicarbonate buffer and dissociated in the same buffer containing 0.02 mg/mL trypsin and 0.02 mg/mL deoxyribonuclease. The cells were filtered and sedimented through 60 μg/mL BSA (Sigma-Aldrich) to separate ventricular myocytes from nonmyocytes as described by Ellingsen et al.[18] The cell pellet was resuspended and plated in ACCT medium consisting of DMEM containing 2 mg/mL BSA, 2 mmol/L L-carnitine, 5 mmol/L creatine, 5 mmol/L taurine, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

Evaluation of Apoptosis and Necrosis

In vitro, morphological changes in the nucleus were detected as described by Meilhac et al.[19] Necrosis and apoptosis were evaluated concomitantly on intact cultured cells after fluorescent staining using vital fluorescent dyes SYTO-13 (0.6 μmol/L), a permeant DNA intercalating green probe, and propidium iodide (15 μmol/L), a nonpermeant intercalating orange probe (Molecular Probes), and counted with an inverted fluorescence microscope (Fluovert FU, Leitz). Normal nuclei exhibited loose chromatin colored green by SYTO-13; apoptotic nuclei exhibited condensed green chromatin; necrotic cells exhibited orange nuclei with loose chromatin.

In vivo, apoptosis was evaluated with the DeadEnd Fluorometric TUNEL system according to the manufacturer's instructions (Promega). Briefly, the deparaffinized heart sections were incubated in a 20 μg/mL proteinase K solution to permeabilize the tissues, rinsed, and fixed in 4% paraformaldehyde. The sections were then incubated with terminal deoxynucleotidyl transferase (25 U/μL) and fluorescein-12-dUTP for 1 hour at 37°C. After rinsing in 1× PBS, the slides were immersed in propidium iodide solution (1 μg/μL) for 15 minutes.

TABLE 2. Scoring Method of Myocyte and Endothelial Injury

Injury	Score	Degree of Injury	Description
Myocyte	0	Normal myocyte	
	1	Slight	Mild intracellular edema
			Mild mitochondrial swelling
	2	Moderate	Mild intracellular edema
			Contracture of myofibrils
			Marked mitochondrial swelling with matrix clearing
			Occasional foal clumping of mitochondrial cristae
			Mild nuclear chromatin clumping
	3	Severe	Severe mitochondrial swelling with loss of cristae
			Presence of intramitochondrial dense granules
			Disarrangement of myofibrils
			Plasma membrane rupture
			Nuclear degeneration (apoptosis or karyolysis)
Endothelial	0	Normal endothelium	
	1	Slight	Mild to moderate endothelial swelling
	2	Moderate	Marked endothelial swelling
			Decreased pinocytic vesicles
			Mitochondrial swelling
	3	Severe	Severe mitochondrial swelling with loss of cristae
			Plasma membrane rupture
			Nuclear degeneration (apoptosis or karyolysis)
			Neutrophil adhesion and extravasation

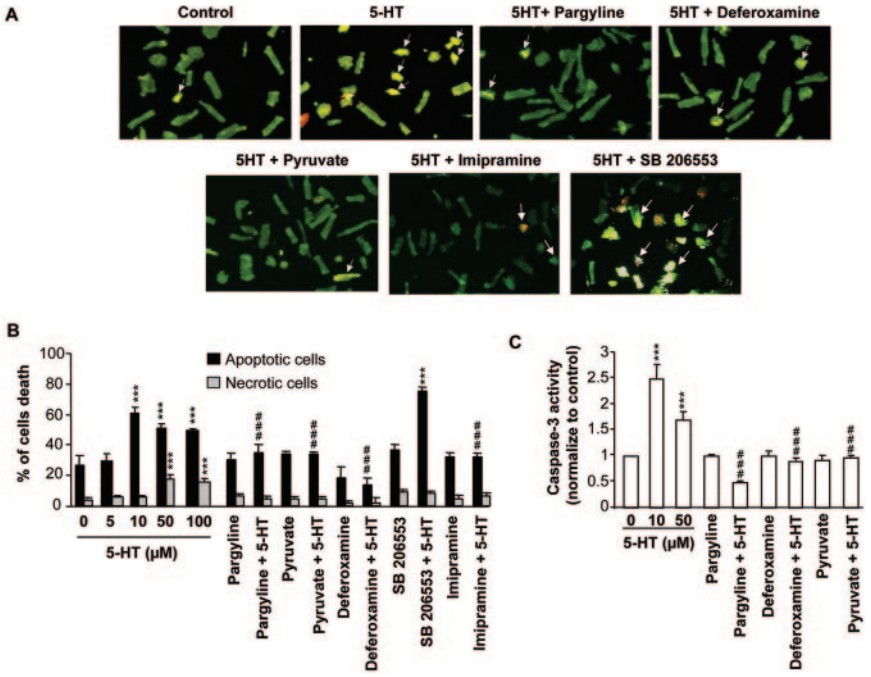

Figure 1. Effect of 5-HT on apoptosis and caspase-3 activation in rat cardiomyocytes. Cell nuclei were double stained with SYTO-13 and propidium iodide, discriminating normal cells from cells undergoing apoptosis (arrow) or necrosis by fluorescence microscopy. A, Photomicrographs of cardiomyocytes pretreated for 30 minutes with or without 1 μmol/L pargyline, 50 μmol/L deferoxamine, 1 μmol/L pyruvate, 25 μmol/L 5-HT transporter blocker imipramine, or 1 μmol/L $5HT_{2B}$ receptor antagonist SB 206553 before addition of 5-HT (10 μmol/L) for 24 hours. B, Quantification of cardiomyocytes stained with SYTO-13 and propidium iodide. Percentage of apoptotic and necrotic cells presented in A. Data result from counting 3 fields of ≈100 cells each per dish. Values are mean±SEM of the percentage of morphologically apoptotic and necrotic cells from three separate experiments. C, Caspase-3 activity was measured fluorometrically with 20 μmol/L Ac-DEVD-AMC as described in Methods. Data are mean±SEM from 3 independent experiments. ***$P<0.001$ vs control, ###$P<0.001$ vs 10 μmol/L 5-HT alone.

Western Blot Analysis

The proteins levels of Bax, Bcl-2, and procaspase-3 were assessed by immunoblotting on cells and heart lysates. The blot was stripped completely of antibodies before reprobing with a polyclonal anti-actin antibody used as a standard. The quantity of proteins loaded from cardiac myocyte extracts was normalized with Ponceau red.

Caspase-3 Activity

Caspase-3 activity was assess with EnzChek Caspase-3 Assay Kit #1 (Molecular Probes) according to the manufacturer's instructions. Briefly, cells were harvested in 1× cell lysis buffer. After centrifugation (5000 rpm for 5 minutes), the supernatant were transferred to a microplate, and 2× substrate working solution (10 mmol/L Z-DEVD-AMC substrate) was added. The microplate was incubated in dark at room temperature for 30 minutes. Substrate cleavage was monitored fluorometrically with a SpectraMax Gemini spectrophotometer (Molecular Devices) with excitation and emission wavelengths of 350 and 450 nm.

Hydrogen Peroxide Production

H_2O_2 production was determined by the oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) (Molecular Probes) to a fluorescent 2′,7′-dichlorofluorescein (DCF). DCFH was added to a final concentration of 20 μmol/L at 37°C for 30 minutes. After 2 washes, images obtained with an inverted fluorescence microscope (Fluovert FU, Leitz) were used to quantify fluorescence intensity with SigmaScan Pro 5 software.

Measurement of the GSH/GSSG Ratio

The ratio between reduced (GSH) and oxidized (GSSG) glutathione was determined with the Bioxytech GSH/GSSG-412 kit (OxisResearch) adapted for cells. Briefly, cells were washed with 1× PBS and harvested into GSH assay buffer. After homogenization, they were centrifuged at 10 000*g* for 15 minutes. Then, 5% metaphosphoric acid was added to the supernatant. After centrifugation at 10 000*g* for 2 minutes, the supernatant was divided into 2 samples, 1 for GSH and 1 for GSSG measures. The assays were performed at 412-nm OD during 3 minutes with a SpectraMax 340 pc spectrophotometer (Molecular Devices).

Animals

The experimental protocol was designed in compliance with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals and was approved by the animal care committee of the University of Florence (Italy). Male Wistar albino rats weighing 200 to 250 g (Harlan, Milan, Italy) were anesthetized with intraperitoneal injection of sodium thiopenthal (Penthotal, Abbott; 50 mg/kg). A cannula was inserted into the trachea, and the animals were ventilated with air with a Palmer pump (U. Basile). All rats underwent thoracotomy at the fifth left intercostal space, the pericardium was opened, and a loose 5-0 braided silk suture was placed around the left anterior descending coronary artery, together with a small silicon ring to permit an easy removal of the ligature. Ischemia (30 minutes) was induced by tightening the threads of the coronary suture. Reperfusion (60 minutes) was obtained by reopening the chest and cutting the ligature around the coronary artery. In all animals, survival time was recorded.

Experimental Protocol

The protocol included 8 groups of rats treated as follows: Group 1 (n=10), sham-operated rats in which no tightening of the coronary sutures was performed; group 2 (n=10), rats pretreated with saline and subjected to 30 minutes of ischemia followed by 60 minutes of reperfusion; group 3 (n=10), rats pretreated with the MAO inhibitor pargyline (10 mg/kg) 30 minutes before ischemia and subjected to 30 minutes of ischemia followed by 60 minutes of reperfusion; group 4 (n=10), rats treated under the same conditions as group 3 but with the MAO-A inhibitor clorgyline (5 mg/kg); group 5 (n=6), rats pretreated for 3 days with the $5\text{-}HT_{2B}R$ antagonist SB 206553 (1 mg · kg^{-1} · d^{-1}; the first dose injected subcutaneously, the following doses given in the drinking water, as reported previously)[20] and then treated with pargyline (10 mg/kg) 30 minutes before ischemia and subjected to 30 minutes of ischemia followed by 60 minutes of reperfusion; group 6 (n=6), rats treated under the same conditions as group 5 but with the MAO-A inhibitor clorgyline (5 mg/kg) instead of pargyline; group 7 (n=6), rats subjected to 30 minutes of ischemia followed by 60 minutes of reperfusion treated with pargyline (10 mg/kg) 20 minutes after induction of ischemia; and group 8 (n=6), rats treated under the same conditions as group 7 but with the MAO-A inhibitor clorgyline (5 mg/kg) instead of pargyline. Saline, pargyline, and clorgyline injections were carried out through the dorsal penile vein.

Determination of Area at Risk and Infarct Size

After 60 minutes of reperfusion, in some animals of each group, the left anterior descending coronary artery was reoccluded, and 1 mL of 1% Evans blue dye was injected into the carotid artery. Right ventricular and atrial tissues were removed, and heart was sliced

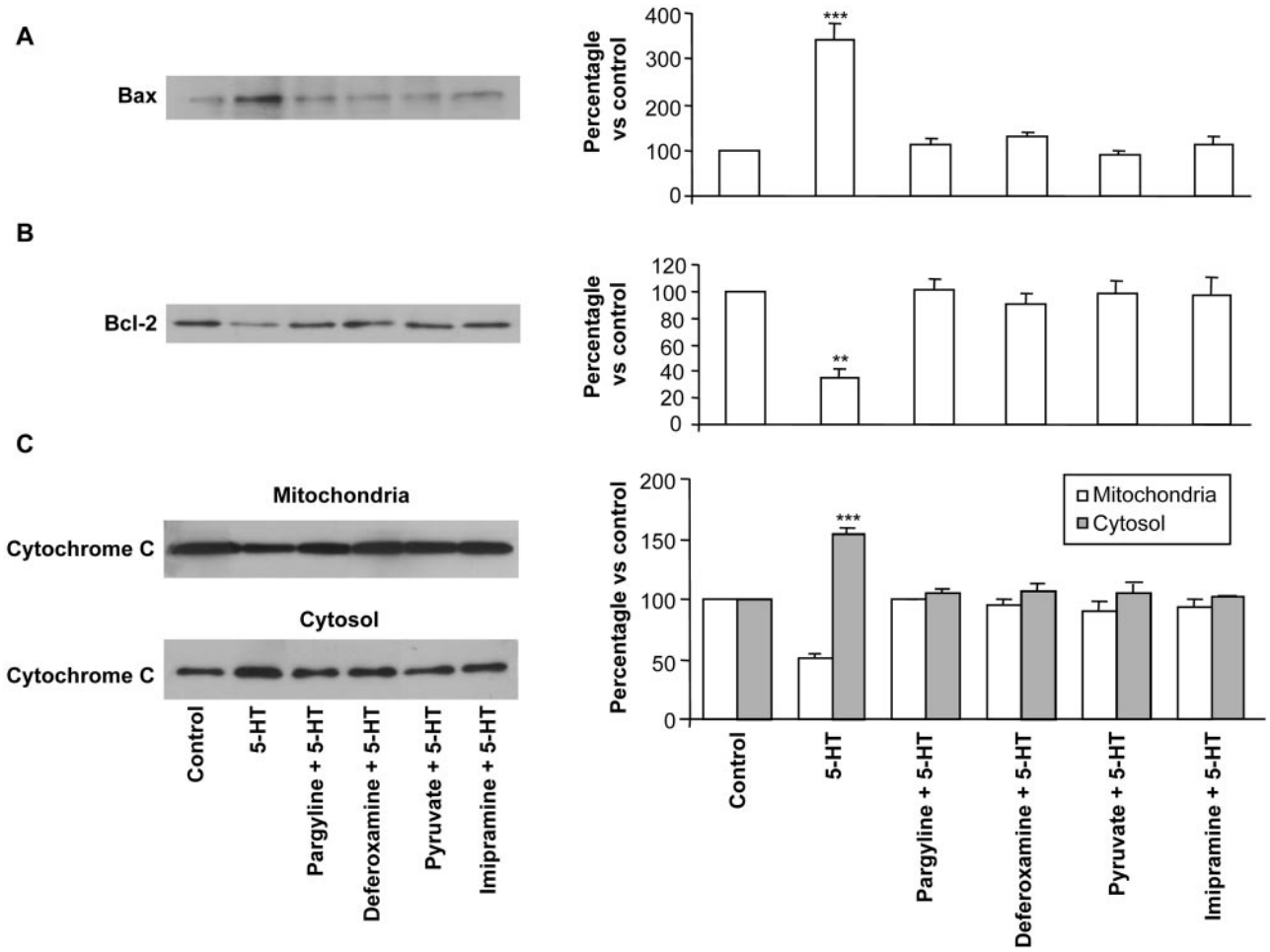

Figure 2. Effect of 5-HT on Bax/Bcl-2 expression and cytochrome c release in cardiomyocytes. Cardiac myocytes were pretreated for 30 minutes with or without 1 μmol/L pargyline, 50 μmol/L deferoxamine, 1 μmol/L pyruvate, or 25 μmol/L imipramine before the addition of 5-HT (10 μmol/L) for 16 hours. The expression of Bax (A) and Bcl-2 (B) proteins and the intracellular distribution of cytochrome c (C) were determined by Western blot in cell lysates (left). Ponceau red was used to confirm equal loading of the extracts. Right, Quantification of Western blot data. Results are mean±SEM from 3 independent experiments. $^{**}P<0.01$, $^{***}P<0.001$ vs control.

transversely (12 to 13 slices) and immersed in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) for 20 minutes. The left ventricular sections were fixed in 10% formalin for 24 hours, weighed, and visualized under an Olympus microscope equipped with a closed-circuit television camera (Sony). In each photograph, the area at risk (unstained by Evans blue dye) and the infarcted area (unstained by TTC) were outlined and measured by planimetry. The mass of tissue in each region was calculated using the areas measured in each slice and the wet weight of each slice. Infarct size was expressed as a percentage of the area at risk (Table 1).

Morphology

Cardiac tissues were fixed in cold 4% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for 3 hours at room temperature and were postfixed in 1% osmium tetroxide in 0.1 mol/L phosphate buffer, pH 7.4, for 1 hour at 4°C. Semithin sections (2 μm) were cut, stained with toluidine blue sodium tetraborate, and studied by light microscopy. Electron microscopic examination was carried out on ultrathin sections stained with uranyl acetate and alkaline bismuth subnitrate and viewed under a Jeol 1010 electron microscope at 80 kV. In each fragment, 2 series of 6 to 8 ultrathin sections cut at 2 different levels were examined and photographed (Table 2). Myocyte and microvascular endothelium injury was quantified from electron micrographs (magnifications ranging from ×3000 to ×20 000).

Evaluation of Myeloperoxidase Activity

Myeloperoxidase (MPO) activity, a marker for neutrophil accumulation, was evaluated according to the method of Bradley et al.[21] Frozen samples of left ventricular tissue (100 mg) were homogenized in 5 mmol/L potassium phosphate buffer and then centrifuged at 30 000*g* for 30 minutes at 4°C before extraction. The resulting pellets were used to measure MPO activity after suspension in 50 mmol/L phosphate buffer containing 0.5% hexadecyltrimethyl ammonium bromide. The pellets were centrifuged at 40 000*g* for 15 minutes; 0.2 mL supernatant specimen was added to 0.8 mL 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% H_2O_2. Absorbance was measured spectrophotometrically at 460-nm wavelength for 2 minutes. MPO activity, normalized to the protein content of the supernatant, was expressed as milliunit per milligram of protein.

Determination of Malondialdehyde Production

Malondialdehyde (MDA), an end product of lipid peroxidation, was determined by measurement of the chromogen generated from the reaction of MDA with 2-thiobarbituric acid as described previously.[22] The cardiac tissues were placed in 50 mmol/L Tris-HCl (pH 7.4) containing 180 mmol/L KCl and 10 mmol/L EDTA in a total volume of 2 mL and homogenized. Briefly, 0.02 mol/L HCl and 0.4% thiobarbituric acid were added to the homogenates and heated to 95°C for 20 minutes. After the addition of 2 mL of 1-butanol, the mixture was centrifuged at 2000 rpm for 10 minutes at 4°C. Absorbance of the upper layer was read spectrophotometrically at 548-nm wavelength. Results are expressed as nanomole of MDA per milligram of protein.

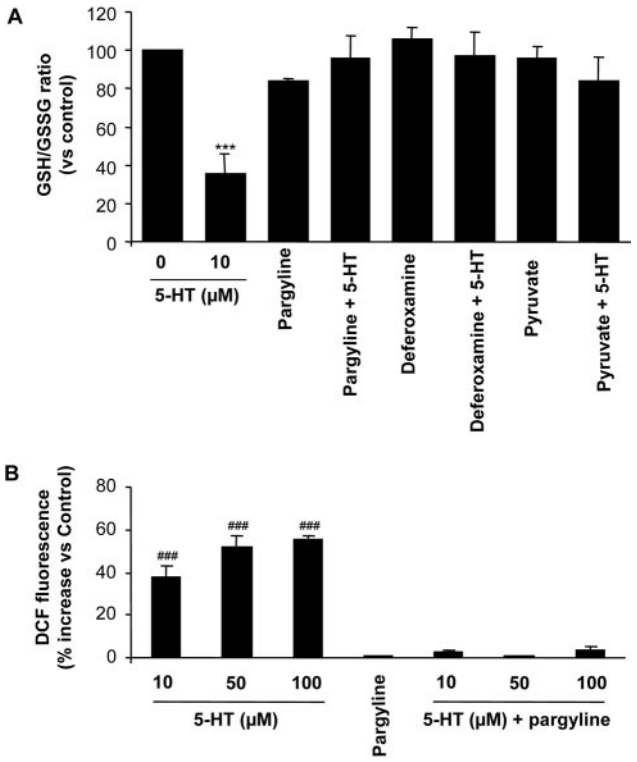

Figure 3. Effect of 5-HT on GSH/GSSG content and H_2O_2 production in rat cardiomyocytes. A, Oxidative stress was assessed by measuring the GSH/GSSG ratio after cardiomyocyte treatment with 10 μmol/L 5-HT for 16 hours in the absence or presence of 1 μmol/L pargyline, 1 μmol/L pyruvate, or 50 μmol/L deferoxamine. B, Effect of 5-HT on H_2O_2 production (DCF fluorescence) in the presence or absence of pargyline (1 μmol/L). Data are mean±SEM from 3 independent experiments. ***$P<0.001$ vs control; ###$P<0.001$ vs pargyline treatment.

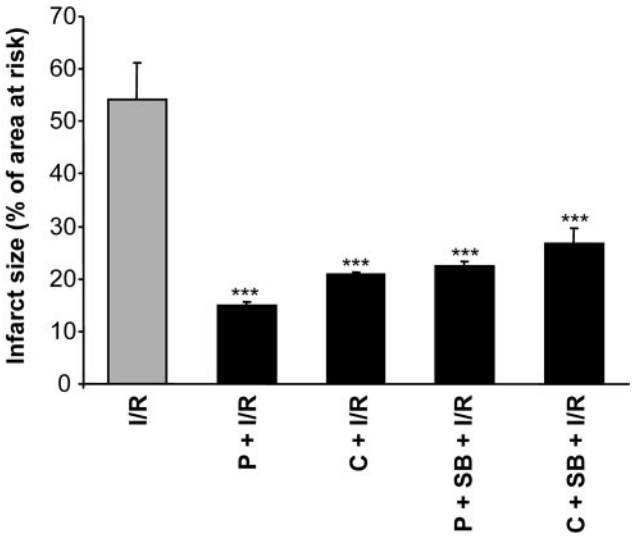

Figure 4. Effects of MAO inhibitors on postreperfusion myocardial injury. Infarct size is expressed as percentage of area at risk. Animal groups are described in Methods. Data are expressed as mean±SEM from 8 separate experiments. Sham indicates sham-operated rats treated with saline; I/R, rats subjected to 30 minutes of ischemia followed by 60 minutes of reperfusion; P+I/R and C+I/R, rats treated before ischemia with pargyline or clorgyline, respectively; and P+SB+I/R and C+SB+I/R, rats treated before ischemia with pargyline and SB 206553 or clorgyline and SB 206553, respectively. ***$P<0.001$ vs I/R group.

Statistical Analysis

Data are given as mean±SEM. The ANOVA procedure, followed by Student-Newman-Keuls multiple comparison test or Student's *t* test, was used for statistical comparison as appropriate (GraphPad Software). A value of $P<0.05$ was considered statistically significant.

Results

Serotonin-Dependent Cardiac Cell Death

Treatment of cardiac myocytes with 5-HT for 24 hours induced a significant increase in SYTO-13 cell labeling (35% versus control; $P<0.001$) and caspase-3 activity (2.5-fold versus control; $P<0.01$) with a maximal effect at 10 μmol/L (Figure 1B and 1C). Higher 5-HT concentrations (50 to 100 μmol/L) resulted in both apoptotic and necrotic cell death (Figure 1B). The apoptotic effect of 5-HT was associated with mitochondrial dysfunction. Indeed, as shown in Figure 2, cardiomyocytes incubated with 5-HT displayed a significant increase in the expression of the proapoptotic protein Bax. The increase in Bax was concomitant with a decrease in the expression of the antiapoptotic protein Bcl-2. Analysis of cytochrome c distribution showed that the alteration of Bax and Bcl-2 expression was followed by release of cytochrome c from mitochondria and its accumulation in the cytosol (Figure 2). Cardiomyocyte apoptosis by 5-HT was concomitant with intracellular oxidative stress and H_2O_2 generation (Figure 3). Indeed, we showed that 5-HT induced a decrease in the intracellular GSH/GSSG ratio (Figure 3A). In addition, 5-HT (10 to 100 μmol/L) also induced dependent intracellular H_2O_2 formation (Figure 3B), as measured with the fluorescent probe DCF.

Serotonin effects on SYTO-13 labeling, caspase-3 activity, Bax increase, Bcl-2 decrease, cytochrome c redistribution, and oxidative stress production were fully prevented by cardiomyocyte treatment with the MAO inhibitor pargyline, the amine transporter inhibitor imipramine, the iron chelator deferoxamine, or the antioxidant pyruvate. In contrast, the increase in 5-HT–dependent cardiomyocyte apoptosis was unaffected by cell pretreatment with the 5-HT_{2B} receptor antagonist SB 206553. Taken together, these data show that 5-HT induces cardiomyocyte apoptosis by a mechanism involving H_2O_2 generation by MAO-A.

Involvement of MAOs in the Postreperfusion Cardiac Damage

To determine the relevance of H_2O_2 production by MAOs in vivo, we investigated the effects of MAO inhibition in cardiac damage after I/R. Figure 4 shows the effect of MAO inhibition on myocardial infarct size of rats subjected to 30 minutes of myocardial ischemia and 60 minutes of reperfusion with or without pretreatment with MAO inhibitors (pargyline and clorgyline). The area at risk is not modified by the different treatments (Table 1). Infarct size is significantly reduced in animals treated with pargyline or clorgyline compared with saline-treated ischemic rats. Rat pretreatment with the 5-HT_{2B} receptor antagonist SB 206553 for 3 days did not reverse the effect of the MAO inhibitors (Figure 4 and Table 1), indicating that the cardioprotective properties of pargyline and clorgyline were not due to 5-HT receptor activation. On the other hand, MAO inhibitors also induced cardioprotection when given 20 minutes after the onset of ischemia (infarct size, 57.3±5.9% in the

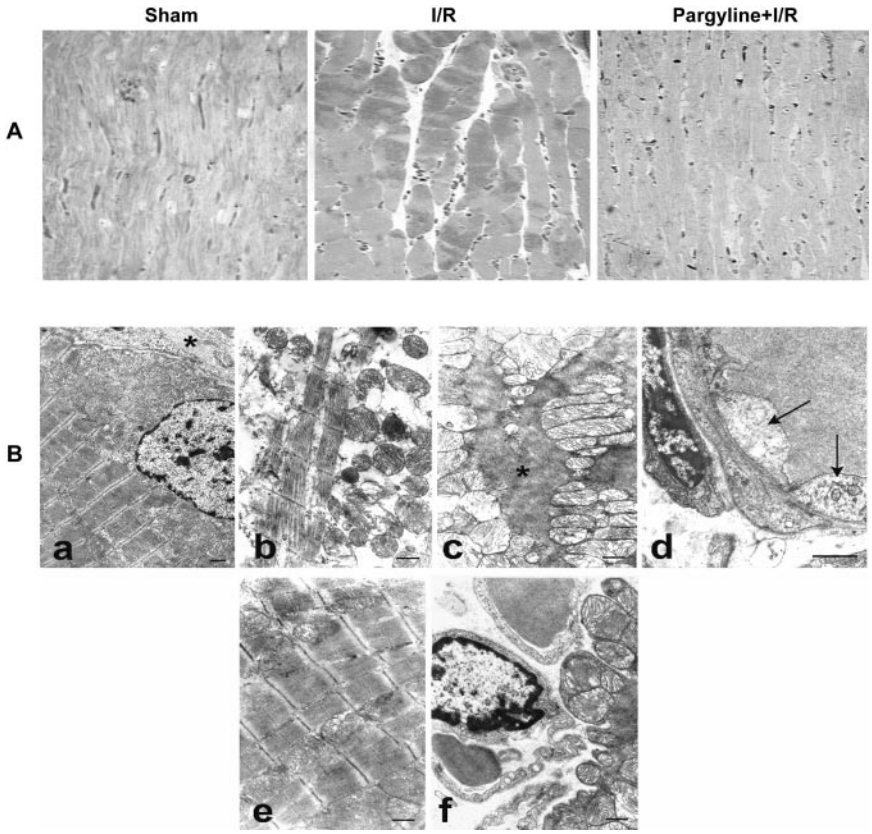

Figure 5. Representative light (A) and electron (B) micrographs of the left ventricle from sham-operated and I/R rats treated with saline or pargyline. A, Toluidine blue–stained sections showing myofibril hypercontraction and interstitial edema in rats subjected to 30 minutes of ischemia followed by 60 minutes of reperfusion (I/R) vs sham-operated (Sham) or pargyline treated (Pargyline+I/R) rats. Bar=100 μm. B, Cardiomyocytes from sham-operated rats (a) contain myofibrils with prominent cross banding and mitochondria rich in cristae; an endothelial cell from a blood capillary (asterisk) shows thin cytoplasm rich in pinocytosis vesicles. Myocardial tissue from rats subjected to I/R showed myofibril hypercontraction and interstitial edema (b), myofibril disarrangement (*) and mitochondrial swelling within a severely injured cardiomyocyte (c), swollen cytoplasm (arrows), and reduction of pinocytotic vesicles in endothelial cell (d). Myocardial tissue from pargyline-treated rats displays cardiomyocytes with nondamaged myofibrils and mitochondria (e) and blood capillaries with normal endothelial cells (f). Bar=1 μm.

I/R animals and 26.2±3.6% in the pargyline-treated I/R animals, $P<0.01$; 31.7±4.3% in clorgyline-treated I/R animals, $P<0.01$).

Light microscopic analysis of postischemic semithin sections demonstrated attenuated myofibril hypercontraction and interstitial edema in pargyline-treated rats (Figure 5A). In addition, ultrastructural cell lesions induced by myocardial ischemia were detected by electron microscopy. Indeed, quantification of ultrastructural tissue damage (Table 2) revealed that myocyte injury was significantly increased in the I/R hearts (sham-operated, 0.3±0.2; I/R, 2.8±0.1; $P<0.001$) and was largely prevented in pargyline-treated rats (I/R, 2.8±0.1; pargyline+I/R, 1.3±0.3; $P<0.01$). Similarly, endothelial cell damage was significantly increased in the I/R hearts (sham-operated, 0.2±0.1; ischemic-reperfused, 2.9±0.1; $P<0.001$) and was strongly reduced by pargyline treatment (I/R, 2.9±0.1; pargyline+I/R, 1±0.2; $P<0.001$).

Effect of MAO Inhibition on Lipid Peroxidation and Phagocyte Infiltration

Oxidative stress and phagocyte infiltration have been proposed a key mediators triggering postreperfusion cardiac damage.[23] As shown in Figure 6, MDA levels (Figure 6A) and MPO activity (Figure 6B), markers of lipid peroxidation and infiltration of ROS-generating inflammatory cells (ie, neutrophils and macrophages), respectively, were increased after 1 hour of reperfusion in ventricules from saline-treated rats compared with sham-operated animals. Treatment with the MAO inhibitor pargyline or the MAO-A inhibitor clorgyline resulted in a significant reduction in myocardial MDA levels and MPO activity compared with untreated I/R animals.

Cardiac Cell Apoptosis, Bax and Bcl-2 Expression, and Procaspase-3 Cleavage

Finally, to determine whether MAO-dependent ROS production may contribute to postreperfusion cardiomyocyte apoptosis, we examined the effects of pargyline on TUNEL staining, Bax/Bcl-2 balance, and procaspase-3 cleavage. As shown in Figure 7A and 7B, I/R induced a significant increase in the number of TUNEL-positive cells compared with sham-operated animals. This effect was strongly prevented by rat treatment with pargyline or clorgyline. I/R also increases the expression of the proapoptotic protein Bax, decreases the expression of the antiapoptotic protein Bcl-2, and induces procaspase-3 cleavage (Figure 7C and 7D). In rats treated with pargyline, the amounts of Bax, Bcl-2, and procaspase-3 were similar to those found in sham-operated animals.

Discussion

The major finding of this study is the demonstration that 5-HT behaves as a proapoptotic factor in cardiomyocytes and that its effect occurs independently of receptor stimulation. The novel mechanism of action of 5-HT that we describe requires 5-HT uptake into cardiac cells, its degradation by MAO-A, and H_2O_2 production. In addition, we demonstrate that H_2O_2 production by MAO-A plays a critical role in post-I/R events leading to cardiac damage.

The demonstration that 5-HT is able to regulate directly cardiomyocyte functions is relatively recent. As reported in a variety of cell types,[24–26] 5-HT displays trophic and protective properties in cardiac cells. Via 5-HT_{2B} receptors, 5-HT induces cardiac hypertrophy[27] and prevents cardiomyocytes from apoptosis.[3] The 5-HT_{2B} receptor–mediated antiapoptotic effect of 5-HT involves inhibition

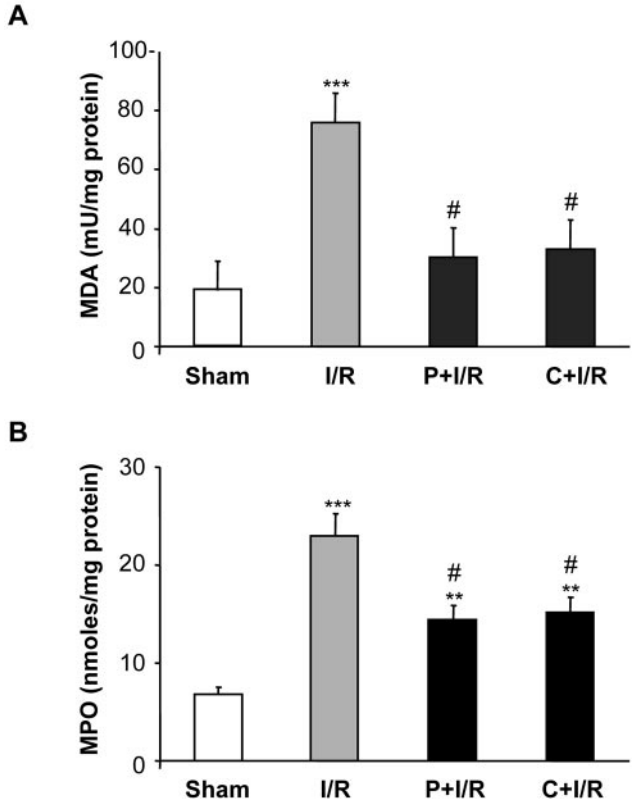

Figure 6. Effect of MAO inhibition on myocardial postreperfusion lipid peroxidation MDA (A) and MPO (B) activity. Sham indicates sham-operated rats treated with saline; I/R, rats subjected to ischemia followed by reperfusion; and P+I/R and C+I/R, rats treated before ischemia with pargyline or clorgyline, respectively. Data are expressed as mean±SEM from 8 separate experiments. $^{**}P<0.01$ and $^{***}P<0.001$ vs sham-operated group; $\#P<0.05$ vs I/R group.

of the mitochondrial apoptotic machinery via cross-talks between the ERK and Akt pathways. Our results show for the first time that 5-HT may also promote cardiomyocyte apoptosis. This effect occurs at concentrations (10 μmol/L) higher than those observed for the antiapoptotic activity (1 μmol/L). In contrast to that observed for the antiapoptotic properties of 5-HT, cell apoptosis by 5-HT does not require receptor stimulation. Indeed, we showed that the increase in cardiomyocyte apoptosis by 5-HT was unaffected by cell treatment with the 5-HT_{2B} receptor antagonist SB 206553 and was completely prevented by the amine transporter inhibitor imipramine. The effect of 5-HT involves the activation of the mitochondrial proapoptotic pathway as shown by the release of cytochrome c, upregulation of the proapoptotic Bax protein, and downregulation of the antiapoptotic Bcl-2 protein. Taken together, our results and those previously reported by Nebigil and coworkers[3] suggest that, depending on the concentrations and the mechanisms of action, 5-HT may have opposite effects on cardiomyocyte survival. It is conceivable that, in vivo, these dual concentration-dependent effects of 5-HT may contribute in different ways to the initiation and progression of ventricular dysfunction.

We showed that the receptor-independent apoptotic effects of 5-HT in cardiac cells requires ROS production. Various sources of ROS, including mitochondrial electron-transport chain, arachidonic acid metabolism, and nonphagocyte NADPH oxidase, have been proposed to mediate cell apoptosis in the heart in response to different agents.[28] Concerning serotonin, we have identified the mitochondrial enzyme MAO-A as the intracellular source of ROS involved in cardiomyocyte apoptosis. During the oxidative deamination of serotonin, this enzyme generates H_2O_2, which can be inactivated by antioxidants or, in the presence of iron, converted to hydroxyl radical by the Fenton reaction. At the concentrations leading to cardiomyocyte apoptosis, we showed that serotonin induces H_2O_2 generation and intracellular oxidative stress, which were prevented by MAO inhibition, antioxidant, or iron chelation. The localization of MAO-A within the outer mitochondrial membrane and ROS production in mitochondrial environment may be particularly relevant for triggering mitochondrial dysfunction associated with apoptotic cell death. This possibility is supported by previous findings showing that treatment of isolated mitochondria from brain with the MAO substrate tyramine induced a decrease in mitochondrial respiration concomitant with glutathion oxidation.[29] According to these results, we showed that MAO-dependent ROS generation induced an increase in the Bax/Bcl-2 ratio and mitochondrial cytochrome c release, leading to cardiac cell apoptosis. These findings point out the critical role of ROS produced by MAO-A during serotonin degradation in triggering the proapoptotic mitochondrial cascade.

Oxidative stress and mitochondrial dysfunction are considered key mediators of cardiomyocyte apoptosis associated with post-I/R cardiac damage.[28] In addition, large amounts of serotonin are released by activated platelets during vascular injury[30] and I/R.[5] It is noteworthy that the 5-HT concentration inducing cardiomyocyte apoptosis in vitro (10 μmol/L) is compatible with those potentially observed in vivo. Indeed, it has been shown that, in cats[5] and mice,[1] platelets can release up to 27-μmol/L concentrations of 5-HT. In addition, Fu and Longhurts[5] have recently shown that large amounts of 5-HT (5 μmol/L) can be released by activated platelets in cardiac venous blood after I/R in cats. From these observations, we investigated the potential role of MAO-A in oxidative stress and cardiac damage in a rat model of cardiac I/R.

In our in vitro studies, we showed that the 5-HT–degrading enzyme MAO-A participates in the induction of the cardiomyocyte damage after I/R. Indeed, we demonstrated that rat pretreatment with the irreversible MAO-A/B inhibitor pargyline or the selective MAO-A inhibitor clorgyline significantly reduced infarct size. This effect was not reversed by the 5-HT_{2B} receptor antagonist SB 206553, indicating that the increase in cardiac 5-HT that may follow MAO inhibition is not responsible for cardiac protection. These findings are in agreement with previous results indicating that stimulation of 5-HT receptors is involved in postreperfusion cardiac damage rather than cardioprotection.[6,31,32] Interestingly, MAO inhibitors also decreased cardiac damage after I/R when administered 10 minutes before reperfusion. These results suggest that MAO inhibitors may have clinical relevance for the prevention of postreperfusion cardiac injury by treating patients in the period occurring between the ischemic event and reperfusion.

Electron microscopic study of postischemic rat hearts showed that MAO inhibition prevented ultrastructural abnormalities, including myofibril hypercontraction, interstitial edema, and mitochondrial swelling. The cardioprotective effects of MAO inhibition were associated with a significant reduction of postreperfusion oxidative stress. We found that MAO inhibition also prevented cardiac accumulation of ROS-generating phagocytes. These results are in

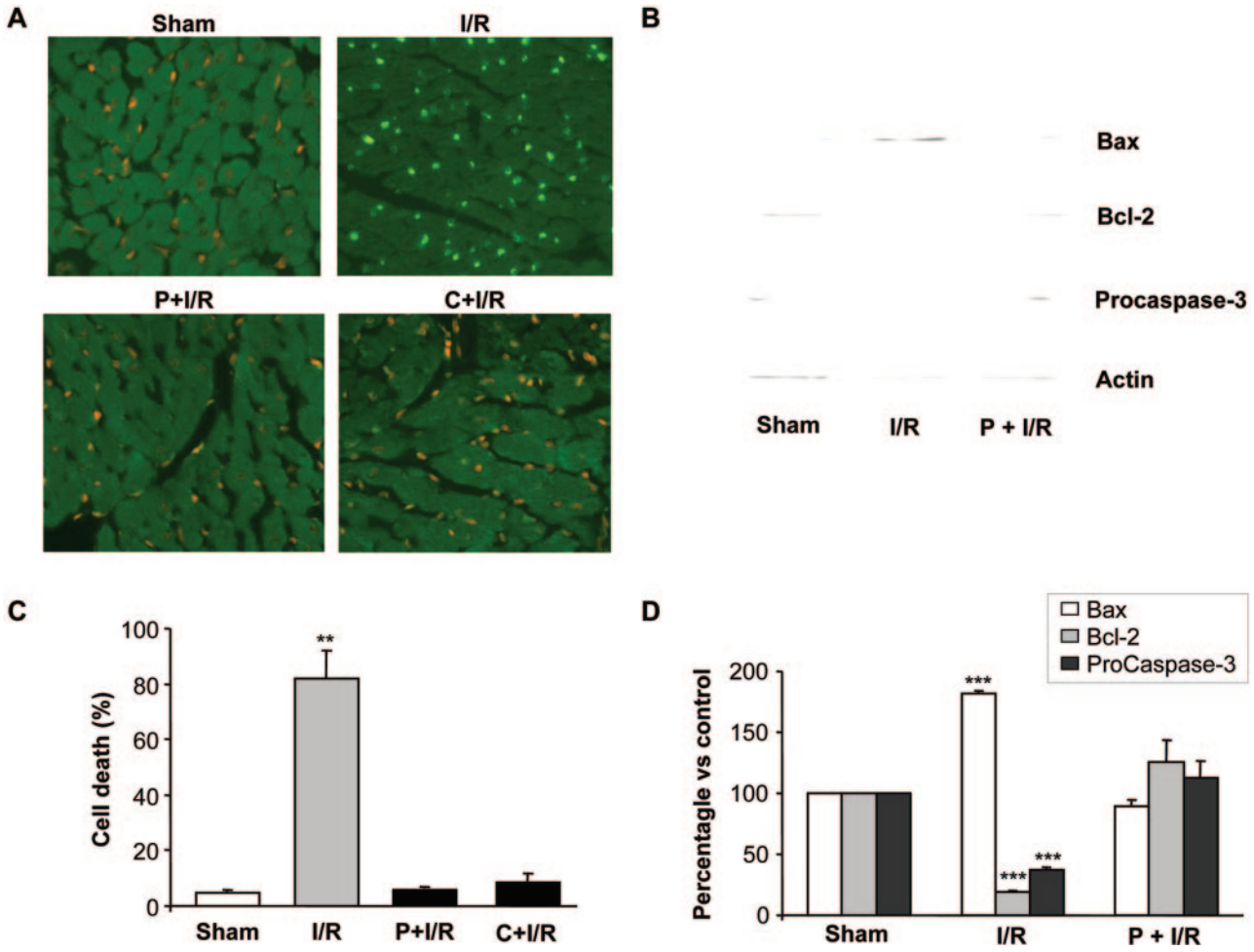

Figure 7. Effect of MAO inhibition on myocardial cell death, Bax/Bcl-2 expression, and procaspase-3 cleavage. A, B, Apoptosis was evaluated by TUNEL staining heart sections as describe in Methods. A, Light photomicrographs (magnification ×40) of heart sections. Bright green dots correspond to TUNEL-positive nucleus. B, Percentage of TUNEL-positive cells in heart sections of A. Data are expressed as mean±SEM from 4 separate experiments quantified in triplicate. C, Western blot analysis of Bax, Bcl-2, and procaspase-3 expression. D, Quantification of Western blot analysis of Bax, Bcl-2, and procaspase-3 expression. Data are expressed as mean±SEM from 4 separate experiments. Sham indicates sham-operated rats treated with saline; I/R, rats subjected to ischemia followed by reperfusion; P+I/R, rats treated with pargyline before ischemia; and C+I/R, rats treated with clorgyline before ischemia. $^{**}P<0.01$ vs sham-operated group; $^{***}P<0.001$ vs sham-operated group.

agreement with previous reports suggesting that an initial burst of ROS at the onset of reperfusion may participate in delayed ROS generation by phagocytes.[23] Prevention of postreperfusion cardiac damage and oxidative stress by MAO inhibition was associated with a decrease in caspase activation, upregulation of Bax, and downregulation of Bcl-2. Taken together, these results show that MAO-A is a major source of ROS-triggered myocardial apoptosis after reperfusion.

In conclusion, the identification of a new MAO-dependent mechanism involved in the 5-HT–triggered cardiomyocyte apoptosis opens new perspectives for the comprehension of the role of this biogenic amine in the heart. It is conceivable that the serotonin-MAO pathway may play a role not only in post-I/R syndrome but also in other heart diseases associated with cardiomyocyte apoptosis. Additional studies are necessary to verify this possibility and to define the potentiality of MAO-A as a pharmacological target for the prevention and therapy of cardiac diseases.

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CLINICAL PERSPECTIVE

In the heart, serotonin has been involved in the regulation of normal cardiac development and in different diseases, including arrhythmias, ventricular hypertrophy, and cardiac valvular insufficiency associated with carcinoid tumors. Recently, several studies showed that serotonin, released by activated platelets, accumulates in the heart during I/R and contributes to the progression of myocardial injury and dysfunction. The availability of cardiac serotonin depends on the activity of the MAO-A. During serotonin degradation, MAO-A produces large amounts of H_2O_2. The major findings of this study are that serotonin behaves as a proapoptotic factor in cardiomyocytes and that its effect occurs independently of serotonin receptor stimulation. The novel mechanism of action of serotonin that we describe requires serotonin uptake into cardiac cells, its degradation by MAO-A, and H_2O_2 production. In addition, we demonstrate that H_2O_2 production by MAO-A plays a critical role in post-I/R apoptosis, leading to cardiac damage. The identification of a new MAO-dependent mechanism involved in the serotonin-triggered cardiomyocyte apoptosis offers new perspectives for the role of this biogenic amine in the heart. It is conceivable that the serotonin-MAO pathway may represent a potential pharmacological target for the prevention and therapy not only of cardiac I/R but also of other heart diseases associated with cardiomyocyte apoptosis.